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Viral DNA and mRNA Expression Correlate with the Stage of Human Immunodeficiency Virus (HIV) Type 1 Infection in Humans: Evidence for Viral Replication in All Stages of HIV Disease

NELSON L. MICHAEL,* MARYANNE VAHEY, DONALD S. BURKE, AND ROBERT R. REDFIELD

Department of Retroviral Research, Division of Retrovirology, Walter Reed Army Institute of Research, 13 Taft Court, Suite 200, Rockville, Maryland 20850

Received 1 August 1991/Accepted 8 October 1991

Studies of cultivatable human immunodeficiency virus type 1 (HIV-1) from plasma samples from infected patients have shown a correspondence between increasing viral burden and disease progression, but these measurements are selective and thus nonrepresentative of the in vivo viral load. Quantitation of proviral DNA sequences by the polymerase chain reaction in purified $CD4^+$ T cells has shown a similar relationship but does not provide a measure of viral gene expression. We have studied viral DNA, genomic RNA, and spliced mRNA expression of HIV-1 in infected patients with a quantitative polymerase chain reaction assay. Viral RNA expression is detected in all stages of infection. These data show that the natural history of HIV infection is associated with a shift in the balance of viral expression favoring the production of genomic RNA without a preceding period of true viral latency.

The determination of host viral burden is a fundamental issue in human immunodeficiency virus type 1 (HIV-1) disease. The accurate determination of the expression of the HIV-1 genome in the human host is essential for understanding the natural history of HIV-1 disease, predicting disease progression in individuals, and assessing the efficacy of antiviral intervention strategies. The paucity of expressed viral components in natural infection has made the assessment of viral burden technically difficult. Viral antigen detection is a poor measure of disease progression (2). Quantitative viral culture studies have shown a direct correlation with disease progression (1, 4, 7), but these studies are influenced by the strain-selective nature of in vitro coculti-vation systems (9). The quantitative detection of HIV-1 DNA in peripheral blood mononuclear cells (PBMCs) by the polymerase chain reaction (PCR) has been used as a measure of host viral burden (6, 16, 17). However, since this approach measures viral DNA and not RNA, it does not take into account that portion of the viral load which represents active expression.

We previously studied viral burden in terms of HIV-1 RNA expression and its relationship to the generation of progeny virions in persistently infected T-cell and promonocytic cell lines (10). The amount of specific HIV-1 transcripts, especially genomic RNA, was found to be more reflective of virion production than the level of total HIV-1 RNA. We demonstrated that productive infection is accompanied by a shift away from the generation of spliced RNA species and toward the production of unspliced, genomic RNA in these cell lines. We further showed in these lines that there is expression of HIV RNA and virions in the resting state as well as in all subsequent stages of induction. This demonstrated the absence of true viral latency in these persistently infected cell culture models.

We now report the extension of our studies of HIV-1 gene expression and its relationship to viral burden to the natural infection of the human host. A panel of cryopreserved PBMCs from 31 infected patients representing all stages of HIV-1 infection were chosen for analysis. None of these patients had received antiretroviral agents prior to sample collection. Viral *gag* and *nef* DNA sequences were analyzed by quantitative PCR. Primer pairs were also chosen to study total, genomic, and *vpr* mRNAs in linked reverse transcriptase (RT)-PCR assays.

These data show that the natural history of disease progression in infected patients is associated at the HIV-1 viral RNA level by a shift toward the production of genomic RNA. Furthermore, there is a significant expression of genomic and subgenomic HIV-1 RNA species in all stages of natural infection. These data refute the hypothesis that HIV-1 disease is associated with a period of true viral latency. This quantitative assessment of viral burden in natural infection has implications for the mechanism of HIV-1 pathogenesis in the human host, approach to diagnosis of HIV-1 disease, feasibility of retroviral vector therapies, and assessment of antiretroviral efficacy.

MATERIALS AND METHODS

Preparation of nucleic acids from PBMCs. Blood samples were collected in tubes containing EDTA. Mononuclear cells were then purified through Ficoll-Hypaque and cryopreserved. $CD4^+$ cell counts were determined on separate mononuclear cell samples by flow cytometry. Aliquots of PBMCs (approximately 5×10^6 cells) were thawed on ice, pelleted at $1,000 \times g$ at 4°C for 5 min, and washed once with cold phosphate-buffered saline (PBS). Cells were suspended in 5 ml of cold PBS, and 1 ml was removed for DNA preparation. All samples were monitored for cell number and viability by the trypan blue dye exclusion technique. For the preparation of DNA, 1 ml of washed cells was pelleted and resuspended at 10^4 cells per μl in 100 μl of a solution containing 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.45% Triton X-100, and 0.45% Tween 20. The lysate was digested with freshly reconstituted proteinase K (Bethesda Research Laboratories) at a final concentration of 60 μg/ml for 1 h at 60°C. The protease was inactivated by treatment at 100°C for

* Corresponding author.

15 min. The resulting material was used directly as the PCR template or stored at −80°C.

For the preparation of RNA, 4-ml samples of washed cells were pelleted and resuspended in 3.0 ml of a solution containing 4 M guanidine isothiocyanate, 0.3 M sodium acetate (pH 6.0), and 0.3 M β-mercaptoethanol. Samples were homogenized for 30 to 60 s in an Omnimixer (Sorvall), overlaid on 1.5-ml 5.7 M cesium chloride cushions, and centrifuged for 16 to 20 h at 35,000 rpm with a SW55Ti rotor (Beckman). RNA pellets were resuspended in 0.3 M sodium acetate, ethanol precipitated, and resuspended in 100 μl of diethylpyrocarbonate-treated water. The RNA was then heated to 100°C for 15 min and then quickly chilled on ice. While held on ice, the following were added: 135 U of RNase-free DNase I (Boehringer-Mannheim), 100 U of RNasin (Promega), and a solution which adjusted the sample to 2.5 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, and 10 mM dithiothreitol (DTT). The RNA was incubated for 60 min at 37°C and then for 15 min at 95°C in order to inactivate the DNase. The RNA sample was subsequently cooled on ice prior to the addition of another 100 U of RNasin. The RNA was then either used immediately as the RT-PCR template or stored at −80°C for future analysis.

Plasmid constructs. DNA fragments of HIV-1 target sequences were generated by RT-PCR from ACH-2 cell (A3.01 clone persistently infected with the LAI strain of HIV-1) RNA and ligated into pGEM-3Z (Promega) or pBluescript II KS- (Stratagene) plasmids. All constructions were confirmed by DNA sequencing using the Sequenase 2.0 system (U.S. Biochemical Corp.) and [^{35}S]dATP. HIV-1 sequence positions are given according to the HIVMN sequence in the Los Alamos HIV Sequence Database. pIC-001 was constructed by cloning a 1,273-bp fragment of the R-U5-*gag* region (positions 495 to 1,767) into the *Sma*I site of pGEM-3Z in the forward orientation. This plasmid was linearized with *Xba*I prior to amplification with the *gag* primer set in DNA PCR assays. *Xba*I-linearized pIC-001 was transcribed with T7 RNA polymerase for use in quantitative *gag* (genomic RNA) RT-PCR assays. pIC-004 contains a 305-bp fragment of the U3-*nef* region (positions 9165 to 9469) cloned into the *Eco*RI site of pBluescript II KS− in the reverse orientation. This plasmid was linearized with *Hin*dIII prior to amplification with the *nef* primer set in DNA PCR assays. *Hin*dIII-linearized pIC-004 was transcribed with T7 RNA polymerase for use in quantitative *nef* (total HIV-1 RNA) RT-PCR assays, pIC-006 contains a 211-bp *vpr* cDNA cloned into the *Sma*I site of pGEM-3Z in the forward orientation. This cDNA begins at position 710 in the tRNA primer binding site and continues to the major splice donor at position 739. The next nucleotide, position 5405, represents the first position of the *vpr* splice acceptor. The fragment then continues to position 5585 inside the *vpr* coding sequence. *Xba*I-linearized pIC-006 was transcribed with T7 RNA polymerase for use in quantitative *vpr* RT-PCR assays. The pβ-globin plasmid contains a 268-bp fragment representing sequence positions −195 to +73 (GenBank accession number HUMHBB5E) of β-globin cloned into the *Sma*I site of pBluescript II KS− in the forward orientation. The *Bam*HI-linearized pβ-globin plasmid was used in quantitative β-globin DNA PCR assays. The pβ-actin plasmid was constructed by cloning a 317-bp cDNA representing sequence positions 2029 to 2346 (GenBank accession number HUMACCYBB) of β-actin into the *Sma*I site of pBluescript II KS− in the forward orientation. The *Bam*HI-digested pβ-actin plasmid was transcribed with T3 RNA polymerase for use in quantitative β-actin RT-PCR assays.

Quantitation of PCR and RT-PCR using cloned templates. For PCR, linearized plasmid DNA was quantified spectrophotometrically, diluted in the presence of 4 μg of carrier tRNA per ml, and used directly in the PCR assay. For RT-PCR assays, plasmid DNA was linearized with an appropriate restriction enzyme, and in vitro transcription was carried out for 2 h at 40°C using 2 μg of linearized DNA in the presence of 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 U of RNasin (Promega) per μl, 500 μM nucleoside triphosphates, and 0.2 U of the appropriate bacteriophage RNA polymerase per μl. RNase-free DNase I (2.5 μl) (Boehringer-Mannheim) was added to the reaction mixtures for 15 min at 37°C prior to extraction with phenol, chloroform, and isoamyl alcohol (25:24:1) and ChromoSpin C100 (Clontech) spin column purification. RNA integrity was assessed by electrophoresis through denaturing polyacrylamide gels and subsequent visualization with ethidium bromide staining. Only in vitro-transcribed RNA preparations containing greater than 95% full-length material were used as standards. Dilutions of RNA were made in the presence of 4 μg of carrier tRNA per ml, 5 mM DTT, and 1 U of RNasin (Promega) per μl. RNA was converted to cDNA by annealing 8 μl of each RNA dilution with 10 pmol of 3′ primer at 70°C for 10 min, cooling on ice, and then incubating with 200 U of cloned RNase H-minus Moloney murine leukemia virus RT (Bethesda Research Laboratories) for 1 h at 45°C in the presence of a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT, 0.5 mM deoxynucleoside triphosphates, and 1 U of RNasin per μl in a final reaction mixture volume of 20 μl. The entire RT reaction was then used as the template for a 100-μl PCR assay. PCR was performed in the presence of a solution containing 18 mM Tris-HCl (pH 8.3), 55 mM KCl, 1.64 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM deoxynucleoside triphosphates, 1 μM (each) of the specific 5′ and 3′ primers, and 2.5 U of *Taq* DNA polymerase. The PCR scheme was 95°C for 30 s, 55°C for 30 s, and 72°C for 3 min in Perkin-Elmer-Cetus thermocyclers. All reactions were cycled through a final incubation at 72°C for 10 min prior to completion. For each PCR assay, three aliquots, each representing 20% of any given PCR assay, were analyzed on horizontal 1.5% agarose gels, pressure blotted (Stratagene) to nylon filters (HyBond-N; Amersham), cross-linked with 12 μJ of UV irradiation (Stratagene), and probed with ^{32}P-end-labeled oligonucleotides for sequences internal to the PCR-generated fragment. Hybridization was performed with a standardized radioactive probe concentration of 1.5×10^5 cpm per ml of hybridization solution. All reactions were set up in class II biological containment hoods. Amplification was performed in the same manner and at the same time as experimental samples. Amplified standards were cohybridized with experimental samples. Signal quantitation was performed with a storage phosphor imaging system (Molecular Dynamics). Copy number signals were quantitated on the same phosphor screen as that of the experimental samples. A standard curve was determined from the mean and standard error of an iteration of three points at each concentration. Experimental samples were assigned copy number values by interpolation of the standard curve.

Amplification of experimental samples. The annealing temperatures and the number of cycles necessary for optimal PCR results were determined for each primer pair with cloned DNA templates. For DNA PCR, 3×10^5 cell equivalents were used in a 100-μl reaction mixture. For *nef* and *gag* DNA sequences, 25 cycles of amplification with an

annealing temperature of 55°C were used. DNA signals were normalized by comparison with the results of the amplification of cloned β-globin DNA carried out with 2×10^4 cell equivalents, an annealing temperature of 50°C, and 22 cycles of amplification.

For RT-PCR analysis, an aliquot containing 1.5×10^5 cell equivalents of RNA was used in each of two separate RT reactions. Reverse transcription was performed precisely as described for RNA standards. Parallel reactions were carried out in the absence of RT as a control for RNA specificity. Control amplifications were also performed in the absence of template to monitor for contamination. The entire 20-μl RT reaction was then used as the template in a 100-μl PCR assay. For total RNA and genomic RNA, 28 cycles with an annealing temperature of 55°C were used. For *vpr* RNA, an annealing temperature of 55°C was used for 27 cycles. Three 32-μl aliquots of each PCR assay were electrophoresed through 1.5% agarose gels, pressure blotted (Stratagene) to nylon filters, UV cross-linked, and probed with ^{32}P-end-labeled oligonucleotides specific for sequences internal to the amplified product. Quantitative image analysis was carried out using storage phosphor technology (Molecular Dynamics). RNA signals were normalized to the results of the amplification of a primer pair specific for β-actin with 1.5×10^5 cell equivalents and an annealing temperature of 45°C for 24 cycles. The nucleic acid values were further normalized to 1,000 CD4$^+$ cells, and the data are expressed as relative copy numbers of nucleic acid species per 1,000 CD4$^+$ cells.

Oligodeoxynucleotide synthesis and sequences. The sequences of the primer pairs and their cognate probes (HIVMN sequence positions are given in parentheses) are as follows: (i) *gag* DNA and genomic RNA, GAG-3 (CAATGA GGAAGCTGCAGAATGGGATAG [1412 to 1438]), GAG-6 (CATCCATCCTATTTGTTCCTGAAGG [1547 to 1523]), probe GPR-5 ATGAGAGAACCAAGGGGAAGTGACATA GCA [1479 to 1506]), (ii) *nef* DNA and total RNA, NEF-7 (ACACAAGGCTACTTCCCTGATT [9167 to 9188]), NEF-6 (CCCAGCGGAAAGTCCCTTGTAG [9470 to 9450]), probe NEFP-3 (TTGGATGGTGCTTCAAGCTAGTACCAGTT [9232 to 9260]); and (iii) *vpr* RNA, SD1-1 (CGCACGGCAA GAGGCGAGGG [710 to 729[), VPR-2 (GGCTTGTTCCA TCTATCCTCTGTCA [5585 to 5561]), probe VPRP-1 (AA GATAAAGCCACCTTTGCCTAGTGTTA [5527 to 5554]).

RESULTS

Quantitative detection of HIV-1 nucleic acids. We developed an assay to measure small amounts of HIV-1 nucleic acids directly and quantitatively from patient PBMCs using PCR. This approach avoids the potential influence on viral load introduced by cocultivation. This technique is described in detail (see Materials and Methods). Briefly, cloned templates of HIV-1 sequences were prepared to allow for the quantitation of nucleic acid copy number in experimental samples relative to known amounts of defined template. These templates were used directly as DNA standards or transcribed in vitro prior to use as RNA standards in parallel amplification reactions. All experimental DNA amplifications were normalized to signals obtained from a β-globin primer pair, and all experimental RNA amplifications were normalized to signals obtained from a β-actin primer pair. Amplified DNA fragments were quantitated by hybridization of radioactive probes to internal fragment sequences using storage phosphor technology. The results of this analysis were further normalized to 1,000 CD4$^+$ cells. All data are expressed as copy numbers per 1,000 CD4$^+$ cells relative to parallel amplifications with cloned template standards (pIC plasmids).

The primers used for quantitative PCR in these experiments are shown in Fig. 1. The genomic organization of HIV-1 is shown aligned with genomic and *vpr* transcripts. Relevant splice donor and acceptor sites are indicated, and primers are indicated by half arrows. NEF-7 and NEF-6 amplify a 305-bp fragment in the common 3′ exon of HIV-1 downstream of splice acceptor 5 (SA5). This primer pair represents a measure of total HIV-1 RNA as well as total viral DNA. GAG-3 and GAG-6 amplify a 136-bp fragment contained completely within the *gag* region. This primer pair

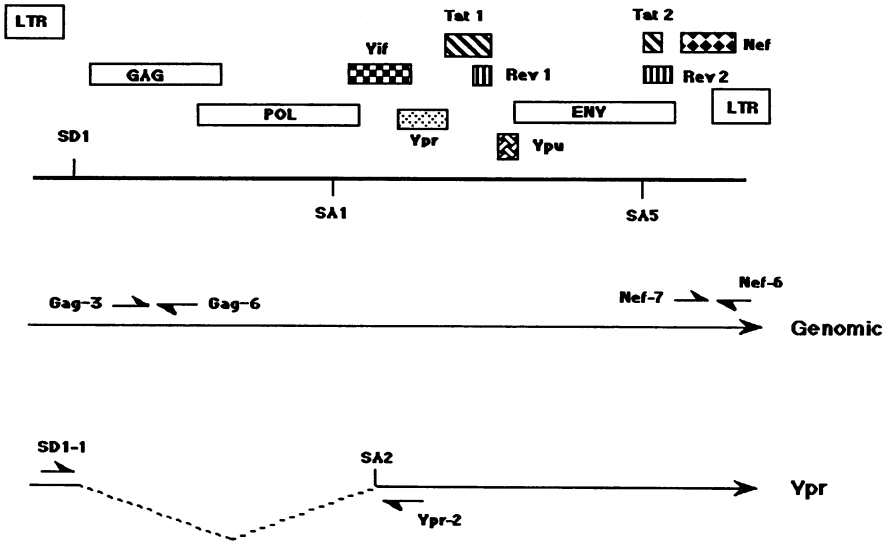

FIG. 1. Strategy for detection of HIV-1 nucleic acids by PCR. The positions of primer pairs are shown aligned with the genomic organization and partial transcription map of HIV-1. Relevant splice donor (SD) and splice acceptor (SA) sites are indicated. Primers are indicated by half arrows. Probe positions are not indicated. The nucleotide sequences and HIVMN positions of the primer pairs and their cognate probes are given in Materials and Methods. LTR, long terminal repeat.

amplifies unspliced, genomic HIV-1 RNA as well as total viral DNA. SD1-1 and VPR-2 amplify a 195-bp fragment specific for *vpr* mRNA which is spliced at splice donor 1 (SD1) and joined at splice acceptor 2 (SA2). It should be noted that this primer pair amplifies only one member, *vpr* mRNA, of a class of singly spliced HIV-1 RNA molecules that also includes *env/vpu1*, *env/vpu2*, and *vif* mRNAs.

A representative experiment using serial dilutions of cloned template is shown in Fig. 2. A series of copies of the U5-*gag* clone pIC-001 were used as templates for 25 cycles of PCR amplification with the *gag* primers GAG-3 and GAG-6. Three iterations of the indicated initial copy number were resolved on agarose gels, blotted to nylon filters, and hybridized with a ^{32}P-end-labeled probe, GPR-5, that maps between but does not overlap with the amplifying primers. A logarithmic-logarithmic plot between DNA copy number and signal intensity (relative phosphor units) with a standard error of ≤10% between iterations was obtained. The coefficient of correlation for the data shown in Fig. 2 was 0.87. The signals from the iterations performed in the absence of template (H_2O lanes) were equivalent in intensity to background. Coamplified experimental samples were assigned a relative copy number from such a curve by interpolation.

Viral RNA and DNA load increase with disease progression. The results of quantitative PCR and RT-PCR analyses from the PBMCs of 31 patients are summarized in Table 1 in descending order of CD4$^+$ cell count. These data are graphically presented in Fig. 3. Relative copy numbers of both *nef* DNA and *gag* DNA increased approximately 100-fold with declining CD4$^+$ number and the progression of disease (Table 1; Fig. 3A and B). The increase in *gag* DNA sequences was more pronounced than that of *nef* DNA sequences, which appeared to level off, temporarily, in the range of 200 to 500 CD4$^+$ cells. There was an approximately fourfold excess of *nef* DNA to *gag* DNA at all times during infection, although this number varied greatly between individual patient samples. The relative copy numbers of total and genomic (unspliced) HIV-1 RNA showed a similar 100-fold rise with declining CD4$^+$ count (Table 1; Figure 3C and D). This observation is indicative of the increased rate of viral expression in a reduced population of circulating CD4$^+$ cells late in disease. Three samples with 557, 319, and 180 CD4$^+$ cells paradoxically showed a higher relative copy number of genomic RNA (*gag* primer set) than total HIV-1 (*nef* primer set). DNA amplifications from the latter two samples with the same primer sets did not show this discrepancy. This could be explained by a differential sequence heterogeneity in the expressed component of viral load (RNA content) that was not reflected in the potential component of viral load (DNA content). The patient sample with 713 CD4$^+$ cells showed an atypically large amount of total HIV-1 RNA with the same *nef* primer set, which indicated a small amount of *nef* DNA sequences. Thus, these data likely represent a true outlier and not an artifact of sequence heterogeneity.

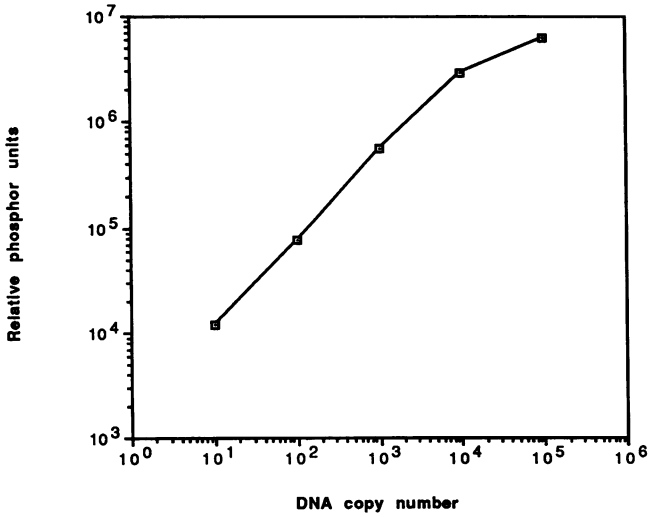

FIG. 2. Quantitation of relative copy number of amplified sequences using cloned templates. A representative standard curve for the determination of copy number for quantitative RT-PCR is shown. DNA fragments of HIV-1 target sequences were generated by RT-PCR from ACH-2 cells, cloned into a plasmid (pBluescript II KS−; Stratagene), linearized with *Hin*dIII, and quantified by spectrophotometry. Known template inputs of these standards were amplified for 25 cycles with the NEF-7 and NEF-6 primer set, blotted to nylon filters, and probed with a ^{32}P-end-labeled oligonucleotide homologous to internal fragment sequences. Signal quantitation was performed with a storage phosphor imaging system (Molecular Dynamics). Signal intensity is expressed in arbitrary units known as relative phosphor units. A standard curve was determined from the mean and standard error of an iteration of three points at each template concentration. Experimental samples were assigned a value for relative copy number by interpolation of the standard curve.

Viral RNA is detected in all stages of HIV-1 disease. To assess the dynamics of the actively expressing component of the viral load in HIV-1-infected hosts, we designed a series of primers that allowed us to determine levels of HIV-1 RNA sequences including total RNA, genomic RNA, and *vpr* mRNA (a singly spliced RNA). Viral RNA was detected in the PBMCs of 21 of the 31 patients studied. The remaining 10 patient samples all were positive for viral DNA sequences. These same 10 samples uniformly exhibited a cell viability of ≤10% on trypan blue dye exclusion analysis prior to the preparation of total cellular RNA. β-Actin amplifications revealed virtually no amplifiable RNA template in these samples, although β-globin DNA could be amplified from the samples. Thus, viral RNA was detected in all 21 samples from which intact template RNA was initially present. These patients represented a spectrum of CD4$^+$ cell counts ranging from 713 to 50 cells per μl and an associated Walter Reed stage from WR 1 (early asymptomatic disease) to WR 6 (AIDS-defining disease) (13).

An excess of genomic HIV-1 RNA to its cognate *gag* HIV-1 DNA sequences was found in all stages of disease (Table 1; Fig. 3F). Since the presence of genomic RNA represents a measure of infectious RNA either packaged or capable of being packaged into virions, these data demonstrate that active expression of HIV-1 RNA is an inherent feature of all stages of natural infection in humans. This observation suggests that there is no absolute virologic latent state in HIV-1 infection.

Disease progression is associated with an increasing proportion of unspliced (genomic) HIV-1 RNA. We and others have previously shown a shift from the production of spliced viral RNA to predominantly unspliced (genomic) RNA following induction of the persistently infected cell lines ACH-2 and U1 (10, 12). This pattern of RNA expression is also seen in acute infection cell culture models (8). The in vivo data suggest an increase in a singly spliced HIV-1 transcript with declining CD4$^+$ cell number (Table 1; Fig. 3F). The ratio of genomic RNA to *gag* DNA was close to unity in early infection. However, as the disease progressed and CD4$^+$ cell numbers declined, infected PBMCs exhibited a preponderance of genomic RNA and lesser amounts of spliced RNA

TABLE 1. Relative copy numbers of HIV-1 nucleic acids in PBMCs from infected patients[a]

CD4 cell count	Relative copy number of nucleic acids in:				*vpr* mRNA/ genomic RNA	Genomic RNA/*gag* DNA
	nef DNA	*gag* DNA	Total RNA	Genomic RNA		
713	10	–	223	4	6	–
669	30	1	33	1	–	2
665	45	3	32	2	26	1
655	21	1	12	1	3	2
640	–	4	–	2	3	1
627	48	2	32	2	0	1
557	–	10	–	–	–	–
557	–	12	14	26	2	4
530	–	10	–	–	–	–
530	–	4	19	16	2	7
520	–	14	–	–	–	–
520	188	3	36	8	4	4
500	–	17	–	–	–	–
473	–	6	–	–	–	–
473	633	6	25	11	1	4
330	–	8	–	20	2	8
328	–	40	–	–	–	–
323	–	4	–	9	3	6
319	113	22	329	939	–	135
317	–	59	–	–	–	–
315	–	7	–	25	0	12
314	–	33	–	–	–	–
286	576	6	202	29	0	18
263	247	7	182	–	–	–
211	–	145	–	103	2	3
180	380	38	776	1,628	–	236
164	525	104	–	–	–	–
54	1,489	16	2,460	513	1	578
50	72	15	8,840	3,060	2	4,091
30	1,066	266	–	–	–	–
18	832	333	–	–	–	–

[a] Values are expressed as the relative numbers of copies of viral sequence per 1,000 $CD4^+$ cells. The patient data is presented in descending order of $CD4^+$ cell count (defined as the number of $CD4^+$ cells per microliter of whole blood). Each data point is the result of two PCR assays and the mean of six iterations from subsequent Southern blot analysis. The standard error of the mean is less than or equal to 15%. Details of quantitative PCR and RT-PCR assays are given in Materials and Methods. –, not determined.

(Table 1; Fig. 3E). The ratio of genomic RNA to *gag* DNA increased dramatically with disease progression (Fig. 3F). This may reflect an increased rate of viral replication from available proviral sequences.

DISCUSSION

HIV-1 viral RNA detection defines the actively expressing component of viral burden. The results presented here indicate that HIV-1 expression at the level of viral DNA and RNA increases with the decline in $CD4^+$ T-cell count and the progression of disease in vivo. We have obtained these results free of the biases that culture amplification and previous antiretroviral therapy introduce. Thus, these data represent a portrayal of the natural history of HIV-1 nucleic acid expression in humans. Precedent exists for a correlation between increasing viral burden and disease progression in HIV-1 disease from both semiquantitative viral culture data and PCR studies from infected PBMCs (1, 4, 6, 7, 16, 17). We extend these findings to the actively expressing component of viral burden by demonstrating an increasing load of viral RNA in the PBMCs of infected patients as the disease progresses. Since HIV-1 is a positive-stranded RNA virus that both enters and leaves the cell in an RNA form, we feel that an RNA-based measure of viral burden is critical to understanding HIV-1 pathogenesis. Measurements of HIV-1 DNA do not reflect the portion of viral burden that represents the immediately infectious viral quantum. Culture-based measurements of viral burden indirectly assay for infectious virus, but they are cumbersome, inherently slow, and subject to selection of viral strains that are suited for in vitro recovery (9). Cocultivation is further removed from a direct measurement of viral load in that viral recovery is assayed by indirect measurements such as supernatant RT activity and p24 antigen detection. A previous report of viral RNA detection using PCR in PBMCs from infected patients showed a correlation between HIV-1 genomic RNA and disease progression, but this study was both nonquantitative and included many patients on active antiretroviral therapy (15). Despite their specific limitations, all of these studies, combined with the data presented in this report, provide a substantial body of evidence for a direct pathogenic role of HIV-1 in the manifestations of HIV-1 disease.

***nef* DNA sequence copy number consistently exceeds that for *gag* DNA sequences.** Throughout the spectrum of HIV-1 infection, the data demonstrated an excess of *nef* DNA to *gag* DNA sequences. This disparity was not uniformly noted when the same primer sets were used to quantify total RNA (*nef* primer set) and genomic RNA (*gag* primer set). This suggests that the differences noted at the viral DNA level were not strictly a consequence of increased sequence diversity in the *gag* and *nef* regions of the HIV-1 genome, although this cannot be ruled out. An alternative interpretation of these data is that the process of reverse transcription of HIV-1 proviral sequences in vivo results in a proportion of arrested or incomplete replication complexes that contain the *nef* region but not the *gag* region. The *gag*-containing portion of total HIV-1 DNA load may represent a complete, functional subset of a process that is subject to premature termination. There is evidence for this hypothesis of arrested viral reverse transcription complexes from in vitro studies of the role of T-lymphocyte activation in HIV-1 replication (18). The formation of such partial replication products are of uncertain relevance to productive infection but may play a role in the cumulative pathogenesis of HIV-1 infection. It is intriguing that previous work has shown an accumulation of unintegrated viral DNA in monocytoid cells in the central nervous systems of patients with marked neurologic manifestations of disease (11). We will pursue this question in subsequent studies by the use of additional, nonoverlapping primer sets in the *gag* and *nef* regions to address the question of sequence diversity affecting the quantitation of these sequences as well as dissecting the proportions of these DNAs that are integrated into the host genome or are epichromosomal in nature.

HIV-1 is expressed in all stages of natural infection. The data presented here show that HIV-1 RNA can be detected throughout the spectrum of HIV-1 disease. We detected viral RNA in all 21 patient PBMC samples from which amplifiable RNA template survived cryopreservation and storage. The expression of genomic RNA in all stages of disease is particularly significant, as these transcripts repre-

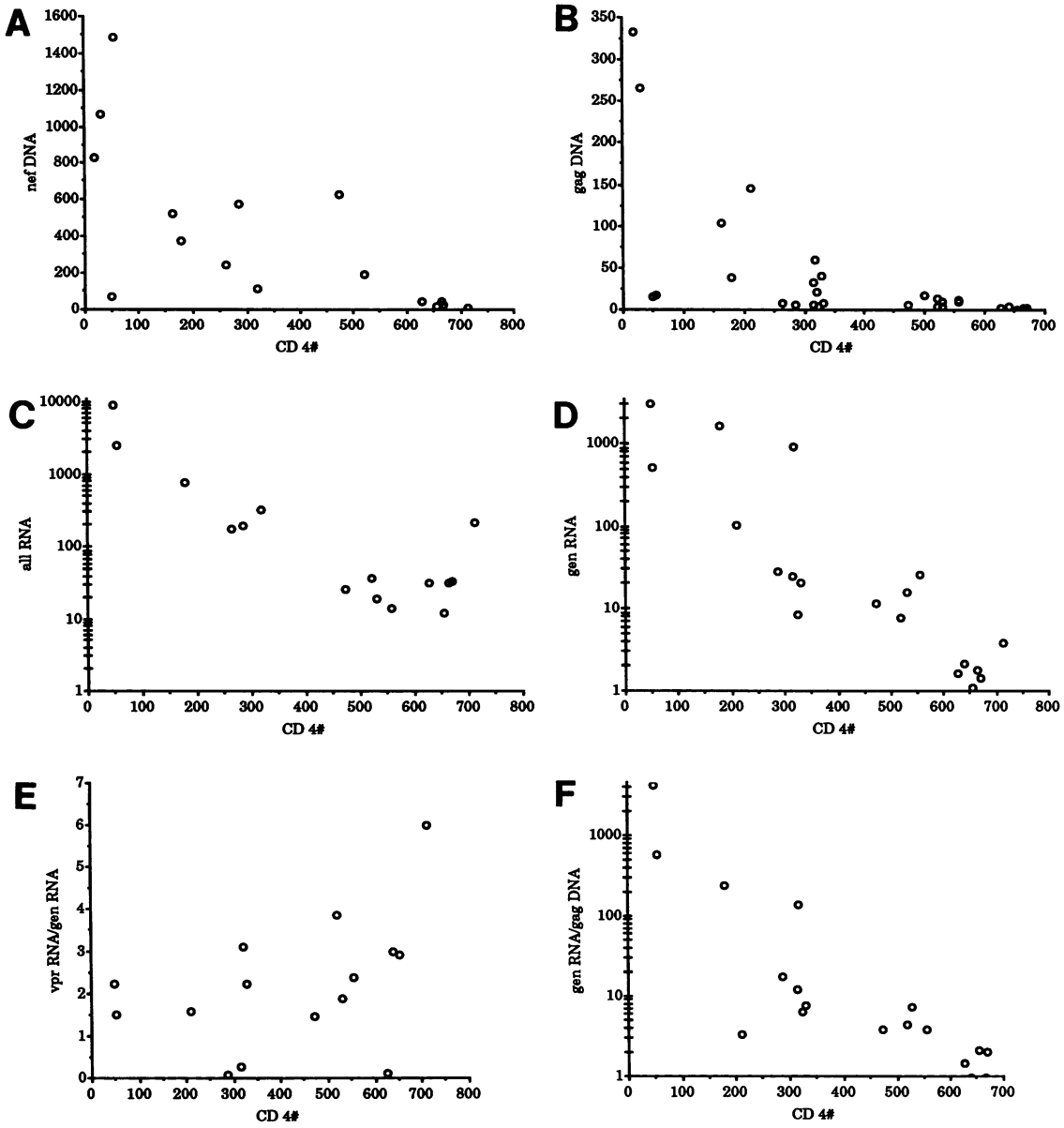

FIG. 3. The relationship of HIV-1 DNA and the expression of viral RNA is indicative of viral replication and correlates with disease stage. Values on the ordinate are the relative copy numbers of viral nucleic acid per 1,000 $CD4^+$ cells. Values on the abscissa are the numbers of $CD4^+$ cells for each isolate. Data are expressed as the mean of three (PCR) or six (RT-PCR) iterations and exhibit a standard error of less than or equal to 15%. (A) *nef* DNA; (B) *gag* DNA; (C) total RNA; (D) genomic RNA; (E) *vpr* RNA/genomic RNA; (F) genomic RNA/*gag* DNA.

sent infectious RNA that is capable of packaging into virions and continuing the virus life cycle. These data support the concept of low-level expression of HIV-1 in early infection that increases with disease progression. These data refute the concept of viral latency in the natural history of HIV-1 infection and suggest instead that at least some cells express viral RNA at all stages of disease. Recent data showing high levels of viral replication in acute HIV-1 infection add further weight to this assertion (3, 5).

The data further show that the state of HIV-1 RNA expression, as measured by the expression ratio of genomic RNA to viral DNA sequences, correlates directly with HIV disease state. Early disease is associated with one to seven copies of genomic RNA per copy of viral DNA. Late in disease, this ratio increases to over 1,000. This suggests that the expression of viral RNA from available DNA templates is much more efficient in late stages than in early stages of disease. Whether this is due to a virus- or host-driven mechanism is unclear. However, these data show that the rate of viral replication in natural infection increases over time. The HIV-1 expression ratio provides a measurement of active viral expression that is relatively insensitive to sequence heterogeneity, since it is experimentally derived with the same primer sequences for both the numerator and the denominator. This dynamic measurement of viral load may provide a powerful tool for the assessment of therapeutic modalities, because it provides insight into the pace of expression of the etiologic agent.

HIV-1 disease progression is characterized by an enrichment for genomic RNA. The increasing fraction of genomic RNA in the viral RNA pool contrasts with a decline in the fraction of HIV-1 RNA which is singly spliced. This is consistent with observations of HIV-1 expression in acutely and persistently infected cell lines (8, 10, 12, 14). These data suggest that early in the natural history of HIV disease, infected cells contain more singly spliced RNA than genomic RNA and that the level of total HIV-1 RNA exceeds that of HIV-1 DNA. The ratio of genomic RNA to *gag* DNA is close to one in early infection. However, as disease progresses and $CD4^+$ cell numbers decline, infected PBMCs contain a preponderance of genomic RNA and lesser amounts of spliced RNA. This quantitative analysis of the viral burden in the PBMCs of infected patients refines the interpretation of the role of functional RNA classes in HIV disease progression. The measurement of specific HIV-1 transcripts in vivo raises the question of the mechanism that mediates the progressive shift in HIV-1 expression toward unspliced viral RNA. An understanding of this mechanism may provide another pathway for therapeutic intervention in HIV-1 disease.

Implications of HIV-1 RNA expression as a measure of viral burden. These studies have major implications for the understanding of the pathogenesis of HIV-1 infection, the approach to prognosis of HIV-1 disease, and the evaluation of specific therapeutic interventions. We have shown that the actively expressing component of viral burden increases with disease progression in HIV-1 infection. The data further show that there is no viral latency state in natural HIV-1 infection. This substantiates the medical rationale for early diagnosis and treatment in HIV-1 disease, because there is no period in which viral replication is truly quiescent. The progressive increase in the viral expression ratio during HIV-1 disease suggests that viral gene expression is a dynamic feature of HIV-1 pathogenesis. This provides a framework for the feasibility of gene therapy approaches that rely on the expression of HIV-1 gene products (e.g., Tat or Rev) for the activation of payloads lethal to the HIV-1-infected cell. Further, the progressive shift toward unspliced viral RNA with advanced disease partially substantiates data from cell culture models of infection. This validation of in vitro models of natural infection is important in the interpretation of future studies employing such model systems.

It is our intent to adapt viral transcript analysis to the assessment of clinical interventions in HIV-1 disease. We have now begun to evaluate quantitative HIV-1 expression analysis as a rapid marker of therapeutic efficacy in multicenter immunotherapy and chemotherapy trials.

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