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Absolute Quantitation of Viremia in Human Immunodeficiency Virus Infection by Competitive Reverse Transcription and Polymerase Chain Reaction

STEFANO MENZO,[1] PATRIZIA BAGNARELLI,[1] MAURO GIACCA,[2] ALDO MANZIN,[1] PIETRO E. VARALDO,[1] AND MASSIMO CLEMENTI[1]*

Institute of Microbiology, University of Ancona, I-60100 Ancona,[1] and International Centre for Genetic Engineering and Biotechnology, Area Science Park, 1-34100 Trieste,[2] Italy

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A competitive polymerase chain reaction (PCR)-based assay for the quantitative detection of human immunodeficiency virus type 1 (HIV-1) viremia was developed and optimized. This method consists of the reverse transcription and subsequent amplification in the same tube of two similar RNA templates, the wild-type template to be quantified and a known amount of the internally deleted synthetic template, both with identical primer recognition sites. The same strategy also proved to be useful in the quantitative assay of HIV-1-specific cellular transcripts and proviral DNA sequences from peripheral blood mononuclear cells by using competitor DNA. The method might be of interest in the study of the precise level of HIV-1 activity during the different clinical phases of the infection and in the simple, fast, and methodologically correct molecular investigation of patients treated with specific antiviral compounds.

Similar to other lentivirus infections (16), the time course of human immunodeficiency virus type 1 (HIV-1) infection is slow (10), and it is commonly believed that the initial viremia present in patients with primary HIV-1 infection is followed by antibody production and a decrease in virus replication in target cells (1, 2, 9, 11, 14, 18). Subsequently, depletion of circulating T lymphocytes that express the CD4 surface molecule and progression of disease are correlated with increased viral activity, as demonstrated in the past few years by (i) detection of HIV-1 p24 antigen in sera from most patients with AIDS (14, 25), (ii) virus isolation from plasma and peripheral blood mononuclear cells (PBMCs) in almost all patients with AIDS (1, 9, 19), and (iii) the association of clinical progression with increased 50% tissue culture infective dose titers in plasma (8, 18).

More recently, reverse transcription (RT)-polymerase chain reaction (PCR) amplification has provided unequivocal evidence of efficient HIV-1 transcription and replication in both patients with AIDS and symptomless infected patients (3, 4, 17, 22, 23, 27, 32). These data have confirmed the hypothesis (5) that, even during the course of clinically latent virus infection, a basal level of viral activity is present in almost all of the infected subjects. Thorough investigation of this aspect seems to be of major importance in gaining a more precise understanding of the pathogenetic events relevant in the progression from the asymptomatic phase of HIV-1 infection to AIDS. In addition, from a clinical point of view, the results cited above indicate that highly sensitive and, at the same time, quantitative laboratory procedures are needed for a more correct diagnosis of and prognosis for all HIV-1-infected subjects and for virological monitoring during the treatment with specific anti-HIV-1 compounds.

A competitive approach for quantitative PCR has recently been proposed (13). The general concept of competitive PCR (cPCR) consists of coamplifying, in the same tube, two different templates with similar lengths bearing the same primer recognition sequences, thus ensuring identical thermodynamics and amplification efficiency for both template species. The quantity of one of the templates introduced must be known, and after PCR, amplification products must be clearly distinguishable by gel electrophoresis analysis, in order to allow densitometric comparison of the relative intensities of the bands for both species. The ratio of PCR products may be related to the initial template concentration.

Following these concepts, we planned, developed, and optimized a competitive RT (cRT)-PCR assay in which the RT step is also internally controlled by means of a synthetic competitor RNA which is reverse transcribed, as is the case with the wild-type RNA. The assay proved to be useful in the quantitative detection of HIV-1 viremia, virus-specific cellular transcripts, and using a competitor DNA, proviral DNA sequences in PBMCs.

MATERIALS AND METHODS

Positive controls. The plasmid pHXB2 containing a single copy of proviral HIV-1 DNA (29) and the HIV-1-infected H9 cell line (26) (kindly donated by R. C. Gallo) were used as positive cPCR and cRT-PCR controls (sources of wild-type HIV-1 proviral DNA and extracellular viral genomic RNA, respectively).

Primers and probes. The oligonucleotides used as primers and probes in this study are listed in Table 1. They were synthesized in our laboratory by using the phosphoramidite chemistry and with a DNA-SM synthesizer (Beckman Instruments, Inc., Fullerton, Calif.).

Competitor DNA: synthesis and cloning. For the competitive analysis, we chose the *gag* fragment of the HIV-1 genome (positions 1551 to 1665); the fragment is both highly conserved and amplified by the sensitive and widely used SK38-SK39 (24) primer set. A modified primer, named SK38Δ, was synthesized with the following sequence pattern on the HIV-1 genome: positions 1559 to 1620, including the 18-base deletion from positions 1579 to 1596, thus resulting

* Corresponding author.

TABLE 1. Oligonucleotides used as primers and probes in this study, with the respective lengths and positions on the ARV2/SF2 proviral genome

Oligonucleotide	No. of bases (positions)	Sequence
SK38	28 (1551–1578)	5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′
SK39	28 (1665–1638)	5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′
SK38Δ	44 (1559–1620)	5′-CCTATCCCAGTAGGAGAAATCCTGGGATTAAATAAAATAGTAAG-3′
SK19	41 (1595–1635)	5′-ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC-3′
SK38-*Hin*dIII	36	5′-GGAAGCTTATAATCCACCTATCCCAGTAGGAGAAAT-3′
SK39-*Bam*HI	35	5′-GGGATCCTTTGGTCCTTGTCTTATGTCCAGAATGC-3′

in a 44-base-long oligonucleotide (Table 1 and Fig. 1). A clinical sample was amplified with the SK38Δ-SK39 primer pair, thus yielding a deleted version of the original *gag* sequence (positions 1559 to 1665). Subsequently, this product was amplified with the modified SK38-*Hin*dIII and SK39-*Bam*HI primers bearing the respective restriction sites in addition to the original sequence at the 5′ end. This final PCR product was cleaved with both restriction endonucleases and ligated in the pBS plasmid vector (formerly Bluescribe; Stratagene, La Jolla, Calif.) cloning box, which

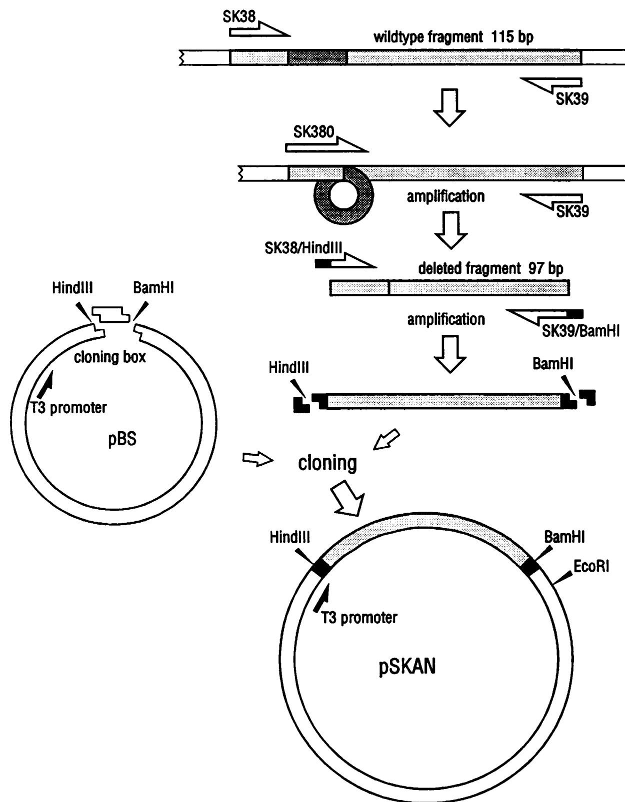

FIG. 1. Synthesis of the deleted competitor and cloning strategy for plasmid pSKAN. Wild-type proviral DNA from a clinical sample (PBMCs) was amplified with the deletion containing the primer SK38Δ paired with the primer SK39. The deleted amplified product was reamplified with primers SK38-*Hin*dIII and SK39-*Bam*HI bearing the respective restriction sites. The deleted amplified products and the plasmid vector pBS were digested with these enzymes, recovered from the agarose gel, and ligated together. Upon transformation of *Escherichia coli* dH5α, bacteria from one colony were grown and large amounts of recombinant plasmid pSKAN were recovered on a CsCl gradient.

lies 3′ of the T3 RNA polymerase promoter sequence. Large amounts of this plasmid (called pSKAN) were purified on a cesium chloride gradient (Centrikon T2060 centrifuge; Kontron Instruments, Zurich, Switzerland) and were carefully quantitated by spectrophotometric analysis and gel electrophoresis to determine the copy number. This estimation was also confirmed by end-point dilution amplification and the Poisson distribution of positive samples, the sensitivity being down to 1 molecule (data not shown). The 18-bp deletion (positions 1579 to 1596) was confirmed by sequencing the *gag* fragment (positions 1551 to 1665) of pSKAN (the competitor DNA) by the chain termination method (Sequenase; Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Synthesis of the competitor RNA. One microgram of the pSKAN plasmid was cleaved with *Eco*RI (22 bp downstream of the insert) in order to synthesize a 136-nucleotide-long transcript. The linearized plasmid was precipitated and resuspended in 100 μl of transcription buffer (Bethesda Research Laboratories, Bethesda, Md.) containing 10 mM dithiothreitol, 0.5 mM (each) ribonucleotide triphosphates, and 80 U of RNasin (Promega, Madison, Wis.). T3 RNA polymerase (50 U; Bethesda Research Laboratories) was added to start the transcription, which was carried out at 37°C for a total of 4 h. Aliquots of the reaction mixture were collected after 1, 2, 3, and 4 h and were run on a 6% denaturing polyacrylamide gel for kinetic analysis; after 4 h the reaction was in the linear phase, and the molar yield was 1,300 RNA molecules per DNA molecule. Briefly, 2.7 U of RNase-free DNase I (Boehringer Mannheim, Penzberg, Germany) was added to reduce the pSKAN load, after which RNA was extracted twice by using phenol-chloroform-isoamyl alcohol, ethanol precipitated and vacuum dried to eliminate enzymes, buffers, and ribonucleotide triphosphates. The RNA pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated water, and aliquots were immediately frozen in dry ice. Spectrophotometric analysis and gel electrophoresis were carried out to determine the quality and copy number of the competitor RNA; a total yield of 26.4 μg of RNA was obtained (roughly 1,300 RNA molecules for each DNA template molecule). After DNase treatment, the residual DNA (calculated by end-point dilution PCR amplification of RNA suspensions with no RT) was calculated to be approximately 1 molecule per 300,000 RNA molecules; thus, it did not interfere in the competitive reactions. End-point dilutions of the competitor RNA and the Poisson distribution of positive samples enabled a final cRT-PCR sensitivity of roughly two target molecules to be established.

Nucleic acid purification. Fresh plasma from HIV-1-infected patients was centrifuged at 3,000 × *g* for 20 min in order to remove platelets and cell debris. One milliliter of this supernatant was mixed with 9 ml of RPMI 1640 medium

(Whittaker, Walkersville, Md.) and ultracentrifuged in a swing-out rotor at 150,000 × *g* for 2 h, and the RNA was extracted from the pelleted virus by the guanidinium-thiocyanate method as described previously (3).

PBMCs were isolated on a Ficoll density gradient; DNA and RNA transcripts were purified from 10^6 PBMCs as reported previously (4).

cPCR and cRT-PCR. In our cPCR assay, each amplification was carried out in a total volume of 100 μl of standard PCR buffer (1.5 mM $MgCl_2$) containing 0.2 mM (each) deoxynucleoside triphosphate, 500 nM (each) primer (SK38 and SK39), a variable and known copy number of DNA competitor (pSKAN.D18), and finally, a constant amount of wild-type DNA. Clinical samples were subdivided into four parallel reactions, each of which contained increasing copy numbers of DNA competitor molecules (10, 20, 100, and 500). We adopted this series after we tested wider ones (data not shown), since it conveniently covers the clinical range of proviral DNA concentrations in the plasma of infected subjects at a reasonable dilution factor. After 10 min of denaturation at 94°C and the addition of 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 50 thermal cycles (automatic cycler from Violet, Rome, Italy) were carried out as follows: 93°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The final incubation was at 72°C for 10 min.

The cRT-PCR was performed in two steps by a method optimized in our laboratory for RT-PCR (3, 4). cDNA was synthesized in the presence of either HIV-1 genomic sequences purified from plasma samples or HIV-1-specific transcripts present in PBMCs and the RNA competitor. The copy numbers of RNA competitor molecules were adjusted to 6,250, 1,250, 250, and 50 in the respective tubes against a constant amount of wild-type viral RNA extract. After RT, the amplification profile was the same as that described above for DNA. Following PCR amplification of both DNA and cDNA, 5 μl of the reaction mixtures was run on a 10% polyacrylamide minigel at 180 V for 50 min in order to obtain complete separation of the 115-bp wild-type fragments from the 97-bp deleted fragments. Liquid-phase hybridization was carried out by using the 5'-end-labeled (^{32}P) SK19 internal probe to confirm the specificity of cPCR and cRT-PCR products as described previously (3).

Competition analysis and mathematical aspects. To make the final step easier, faster, and more direct, gel electrophoresis and analysis of cPCR products after ethidium bromide staining were preferable to radioactive assays. Gels were scanned by using a video densitometer (Ultra Violet Products Ltd., Cambridge, United Kingdom) by positive fluorescent emission on the transilluminator. Peak areas (WA, wild-type area; DA, deleted area) of both amplified products were calculated by the machine software (Gel Analysis Program; Ultra Violet Products) in each lane. DA was corrected (DAc) for its lower molar ethidium bromide incorporation as follows: DAc = DA × (wild-type length/deleted length) = DA × 1.1855. The DAc/WA ratio was calculated for each sample and plotted on the *y* axis against the copy number of the deleted competitor (*D*). A simple regression curve was fitted for positive controls and for each sample. The copy number of the wild-type template (*W*) could be calculated from the curve expression for DAc/WA = 1.

RESULTS

Plasmid pHXB2 containing a single copy of proviral HIV-1 DNA and plasmid pSKAN were carefully quantified by spectrophotometric analysis and end-point dilution amplification. Subsequently, in order, first, to confirm the pSKAN plasmid copy number by cPCR, second, to study the competition kinetics between pSKAN and wild-type HIV-1 DNA, and, third, to elaborate a mathematical model suitable for the application to clinical samples, the following cPCR was performed. In this experiment, a constant copy number (12,500) of prototype HIV-1 pHXB2 DNA was challenged with an increasing pSKAN copy number (from 500 to 200,000). The results (Fig. 2A) show how the lower band corresponding to an increase in the copy number of the deleted HIV-1 DNA *gag* sequence (pSKAN) progressively competes with the upper band corresponding to wild-type HIV-1 DNA sequence (pHXB2) in a constant copy number. The graph at the bottom of Fig. 2A shows the simple regression curve obtained by comparing the deleted/wild-type ratio (measured by densitometric analysis of ethidium bromide-stained bands) with the copy number of the deleted sequence for each amplification tube. The equivalence point, calculated by this regression curve, fully confirmed the spectrophotometric results.

In order to optimize the cRT-PCR assay for the quantitative detection of free, extracellular HIV-1 genomic RNA, cell-free tissue culture medium of the HIV-1-infected H9 cell line was used as the source of extracellular virions. The competitor RNA was synthesized and quantified; wild-type RNA (from fresh culture medium of actively growing, HIV-1-infected H9 cells collected on day 5 after passage) was extracted and treated for 10 min with 2.7 U of RNase-free DNaseI; this was followed by a 10-min denaturation step at 94°C. The extracted RNA was stored in aliquots at −80°C. A fixed, unknown amount of wild-type HIV-1 genomic RNA (equivalent to 2 μl of supernatant) was challenged against a twofold increasing copy number of pSKAN-derived, deleted HIV-1 RNA sequence in a series of microtubes. Both templates were reverse transcribed and amplified. Figure 2B (top) shows the results of a series of such reactions ranging across the equivalence point. This equivalence point was calculated according to the curve equation (see legend to Fig. 2B), thus assigning the value of 3,028 to the unknown wild-type RNA copy number. This experiment was performed in triplicate, starting with different vials of the stored RNA, and the two other resulting copy numbers, 3,105 and 2,849 respectively, confirmed the reliability of cRT-PCR (2,980 ± 120 [mean ± standard deviation]; coefficient of variation, 4%).

Additionally, in order to evaluate the effects of RNA purification on template recovery and to further control the reliability of quantitative results, 1,780 *gag* HIV-1 RNA copies, calculated by the quantitation method reported above, were reextracted and challenged against a competitor series in four repetitions. A mean loss of 36% was observed. In our opinion, this indicates that all absolute quantitative data regarding RNA should be corrected for a percentage loss determined specifically for the extraction method used. However, the absolute quantitative data presented in this report were not corrected to allow a direct approach to the cRT-PCR results.

Since this method was planned for application to a vast number of clinical samples, we had to define a standard set of competitor dilutions which could adequately meet the needs of extensive use; ideally, the maximum information yield with the minimum number of amplification reactions per sample. Preliminary experiments with clinical material (data not shown) indicated that four fivefold dilutions of competitor RNA or DNA could cover a convenient range of nucleic acid copy numbers in most samples. Figure 3 shows

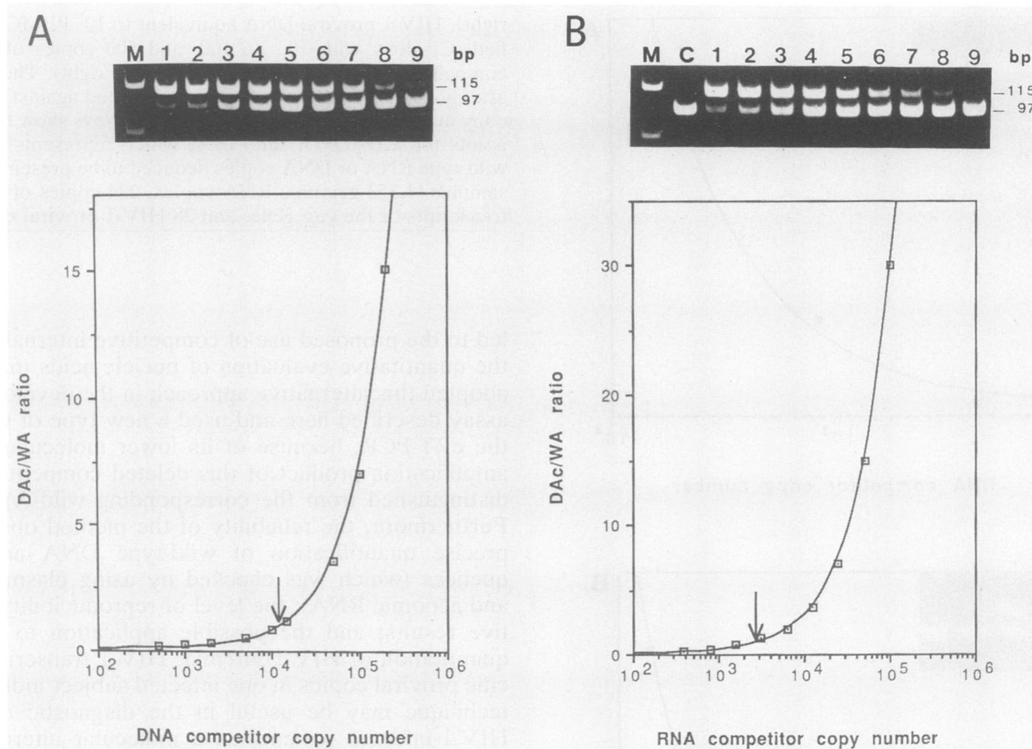

FIG. 2. Optimization of cPCR and cRT-PCR. (A) Coamplification of 12.5 × 10^3 pHXB3 DNA copies with increasing copy number of pSKAN DNA (lanes 1 to 9, 0.5 × 10^3, 1 × 10^3, 2 × 10^3, 5 × 10^3, 10 × 10^3, 15 × 10^3, 50 × 10^3, 100 × 10^3, and 200 × 10^3, respectively; lane M, pGEM molecular weight marker). The regression curve ($y = a + bx$) was calculated by plotting the DAc/WA ratio against deleted competitor copy number, as follows: $y = 8.6035 \times 10^{-2} + 7.3339 \times 10^{-5} x$ ($r^2 = 0.999$). (B) cRT-PCR. A constant amount of HIV-1 genomic RNA from H9 cell supernatant was reverse transcribed along with increasing copy numbers of pSKAN RNA (lanes 1 to 9, 391, 781, 1,563, 3,125, 6,250, 12.5 × 10^3, 25 × 10^3, 50 × 10^3, and 100 × 10^3, respectively; C, deleted RNA control). The regression curve was calculated as described above, as follows: $y = 9.8705 \times 10^{-2} + 2.9764 \times 10^{-4} x$ ($r^2 = 0.999$). Arrows show theoretical equivalence points ($y = 1$) corresponding to 12,469 copies of DNA (A) and 3,028 copies of RNA (B).

an example of how a single clinical sample (from an asymptomatic HIV-1-infected patient, 640 $CD4^+$ T lymphocytes per mm^3) can be studied to obtain quantitative information on three different virological parameters. Viral genomic RNA in plasma, intracellular virus-specific transcripts, and proviral DNA from PBMCs were extracted and challenged against the standard set of competitor dilutions (see legend to Fig. 3 for data).

DISCUSSION

We described here an RT-PCR-based technique (the cRT-PCR) for the quantitative analysis of HIV-1 viremia, HIV-1-specific cellular transcripts, and as cPCR, proviral sequences in PBMCs from clinical specimens from HIV-1-infected subjects.

It has been estimated that about 50% of HIV-1-infected adults develop AIDS within 10 years following infection (21). However, although the major viral reservoir in peripheral blood has been identified (28), our understanding about the molecular history of this infection and about the precise role of other events (including other viral infections) that are capable of modulating HIV-1 activity in vivo (for a recent review, see reference 15) is still incomplete. Since it may partially depend on the lack of sensitive molecular methods that allow the quantitative evaluation of HIV-1 activity in vivo, there is a growing need for sensitive and quantitative assays for use in pathogenetic investigations and the clinical management of HIV-1-infected patients.

The procedure described here is an improvement on other quantitative or semiquantitative RT-PCR-based methods that have been proposed in the past few years. In fact, these latter methods are mainly based on the use of end-point dilution analysis (7, 27, 30, 32) or on the coamplification of single-copy cellular gene sequences together with the viral sequences (20). The first approach is expensive (since it requires a high number of amplification reactions) and time-consuming and does not allow precise control of the RT step (operators must assume a given theoretical reaction efficiency); under these conditions, minimal changes in terms of RT efficiency might strongly influence the subsequent PCR amplification. For these main reasons, this strategy cannot be used for the quantitation of RNA sequences and, since it points to relative rather than absolute quantitation, is open to criticism when it is used to quantify DNA templates. The second strategy (coamplification of a single-copy cellular gene) appears to be in contrast to the PCR theory, since different PCR templates may have different thermodynamics and amplification efficiencies, thus greatly influencing the results (the relative amounts of both PCR products). Furthermore, this method cannot be applied to extracellular templates.

The exponential features of PCR and the possible sample-to-sample variability in amplification efficiency have recently

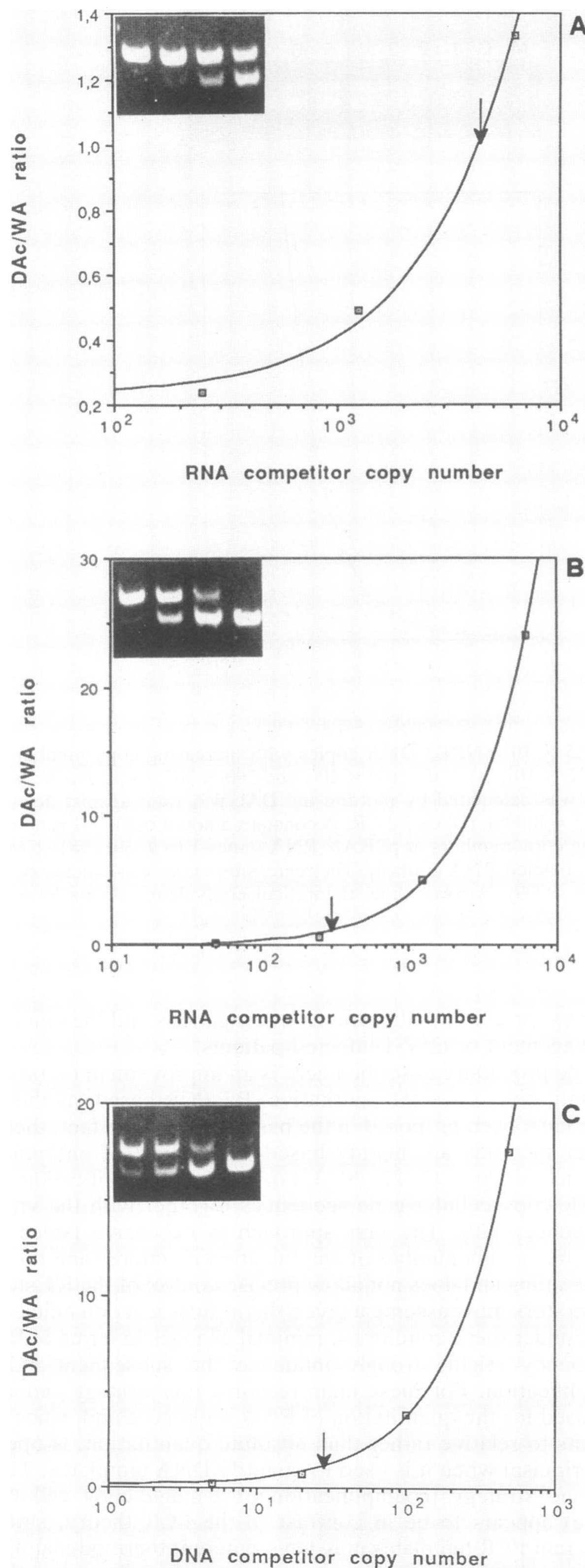

FIG. 3. Quantitation of HIV-1 nucleic acid copy number from clinical samples of an infected subject by cPCR and cRT-PCR. HIV-1 genomic RNA equivalent to 100 μl of plasma (A) and HIV-1 transcripts equivalent to 10^5 PBMCs (B) were challenged against 50, 250, 1,250, and 6,250 copies of deleted RNA competitor in RT and subsequent amplification (insets of panels A and B; from left to right). HIV-1 proviral DNA equivalent to 10^5 PBMCs was coamplified (C) along with 10, 20, 100, and 500 copies of deleted DNA competitor (inset of panel C; from left to right). The data obtained after gel densitometric analysis were plotted against the competitor copy number in the respective graphs. Arrows show the equivalence points for a DAc/WA ratio of 1, which represents the number of wild-type RNA or DNA copies deduced to be present in the original samples (4,353 genomic RNA copies, 224 copies of HIV-1-specific transcripts of the *gag* gene, and 28 HIV-1 proviral copies).

led to the proposed use of competitive internal standards in the quantitative evaluation of nucleic acids (6, 12, 13). We adopted this alternative approach in the development of the assay described here and used a new type of competitor in the cRT-PCR; because of its lower molecular weight, the amplification product of this deleted competitor is directly distinguished from the corresponding wild-type sequence. Furthermore, the reliability of the method observed in the precise quantification of wild-type DNA and RNA sequences (which was checked by using plasmid molecules and genomic RNA); the level of reproducibility of quantitative results; and the possible application to the absolute quantitation of HIV-1 viremia, HIV-1 transcripts, and specific proviral copies in one infected subject indicate that this technique may be useful in the diagnostic evaluation of HIV-1-infected patients as a molecular alternative to the time-consuming and probably less precise biological quantitative methods (8, 18). In addition, the method described here bypasses the need for additional steps, such as the enzymatic restriction and hybridization (the efficiencies of which may affect quantitative results) used to distinguish an amplified wild-type from a competitor of similar length and bearing a new restriction site (31). Theoretically, it is conceivable that, after specific optimization, the same strategy could be used in the quantitative laboratory evaluation of the transcriptional activity of almost all viral, eukaryotic, and prokaryotic genes, thus also offering an alternative to the traditional semiquantitative Northern blot analysis.

Overall, the data presented here indicate that the cRT-PCR assay is a suitable method for the molecular characterization of HIV-1-infected patients regardless of their clinical stage and highlight the reliability of this technical approach in the unequivocal virological monitoring of patients treated with specific anti-HIV-1 compounds.

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