JOURNAL OF VIROLOGY, Mar. 1992, p. 1809–1813
0022-538X/92/031809-05$02.00/0
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Vol. 66, No. 3

Efficient Replication of Human Immunodeficiency Virus Type 1 Requires a Threshold Level of Rev: Potential Implications for Latency

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Received 1 August 1991/Accepted 12 December 1991

The Rev protein of human immunodeficiency virus type 1 (HIV-1) is essential for the expression of the structural genes of HIV-1. To determine whether a functional threshold level of Rev is required to allow efficient HIV-1 replication, CD4-positive HeLa cells, constitutively expressing a Rev-deficient provirus, were transfected with various quantities of a Rev-expressing plasmid. Compared with the quantity of the Rev-producing plasmid transfected, HIV-1 replication was distinctly nonlinear as measured by HIV-1 p24 antigen and HIV-1-specific RNA production. A quantitative RNA polymerase chain reaction (PCR) demonstrated that Rev mRNA expression was linearly correlated with the quantity of Rev-expressing plasmid which was transfected into these cells. These data suggest that a critical threshold of Rev is required for a highly productive HIV-1 infection. This threshold level of Rev may be involved in the generation and maintenance of HIV-1 proviral latency.

Various cellular and virally encoded factors are involved in the control of human immunodeficiency virus type 1 (HIV-1) replication (10). Of the cellular factors which are important in regulating HIV-1 replication, the DNA-binding protein nuclear factor kappa B (NF-κB), which binds to two 11-bp motifs in the HIV-1 long terminal repeat (LTR), is the most thoroughly characterized (26, 27, 38). A number of HIV-1-encoded proteins have also been identified as *trans*-acting factors involved in HIV-1 replication (10). The Tat protein binds to a section of RNA transcribed from the R region of the 5′ LTR, Tar, and dramatically stimulates HIV-1 LTR-directed transcription and possibly also acts on post-transcriptional events (10, 13, 37, 45). The virally encoded Rev protein has been demonstrated to rescue unspliced (9.2-kb) HIV-1 RNA from the nuclei of HIV-1-infected cells (2, 14, 47). This occurs through the binding of Rev to a target sequence, located within the envelope portion of the viral RNA of HIV-1, called the Rev response element (RRE). Whether Rev functions via an alteration in nucleus-to-cytoplasm transport, by protecting the unspliced HIV-1 RNA from the splicing complex, and/or by allowing the translation of some HIV-1-specific mRNAs is controversial (1, 6, 11, 15, 20, 21, 25, 31, 32).

The HIV-1 RNAs may be separated into three classes, according to their sizes. The multiply spliced (2-kb) species encode the regulatory proteins Tat, Rev, and Nef. The singly spliced (4.3-kb) HIV-1-specific RNA species encode at least the envelope proteins, while the unspliced (9.2-kb) HIV-1-specific RNA species function as both the viral genomic RNA and as mRNA for the Gag-Pol and Gag proteins (44).

We have recently described a molecular model of HIV-1 proviral latency in cell culture systems characterized by an aberrant pattern of HIV-1-specific RNA (42). In this model of latent infection, cells are maintained in a state analogous to the early nonproductive stage of an acute HIV-1 infection (23). In latently infected subclones of monocytoid and T-lymphocytic cell lines, U1 (17) and ACH-2 (9) cell lines, respectively, the baseline pattern of HIV-1-specific RNA consists of multiply spliced and singly spliced RNA species but little or no unspliced HIV-1 RNA (42). This HIV-1 RNA pattern mimics, as well, the pattern detected with cells containing a Rev-negative mutant virus (14, 49). Upon stimulation of these latently infected cells to significantly increase viral production by using a variety of factors which activate NF-κB (9, 16, 17, 41, 42), such as phorbol esters, tumor necrosis factor alpha (TNF-α), and lipopolysaccharide, there is a dramatic rise in the level of multiply spliced HIV-1 RNA species prior to an increase in the level of unspliced RNA (42). On the basis of this time course and of the pattern of HIV-1 RNA in latently infected cells that mimics the RNA pattern of a *rev*-negative HIV-1 mutant (14, 49), we have suggested that a threshold level of Rev protein may be necessary for efficient HIV-1 replication (42). In these latently infected cells, a low level of transcriptional activity may generate a baseline level of Rev below a functional threshold necessary to rescue most unspliced HIV-1 RNA from the nucleus.

To test this hypothesis, we initially attempted to transfect a Rev-producing plasmid into the latently infected U1 and ACH-2 cell lines. Unfortunately, all transfection techniques that were evaluated stimulated HIV-1 replication in these cells, even though no Rev-expressing plasmid was added (data not shown). Therefore, as an alternative approach, we analyzed the effect of introducing increasing amounts of Rev into cells containing a *rev*-defective HIV-1 provirus to see whether there was a threshold for Rev function. The previously described HT4 (V-dhfr) cell line is a HeLa cell derivative that constitutively expresses a modified HIV-1 provirus, called V-dhfr, and carries CD4 on its surface (50). V-dhfr was derived from the HIV-1 HXB-Rip7 provirus (33) and contains a four-amino-acid insertion in the proximal fifth of Rev (Fig. 1). This mutation inactivates Rev function, as shown by Northern (RNA) blot analysis of cytoplasmic RNA from HT4 (V-dhfr) (49). The HT4 (V-dhfr) cells were transfected with increasing quantities of a Rev-expressing

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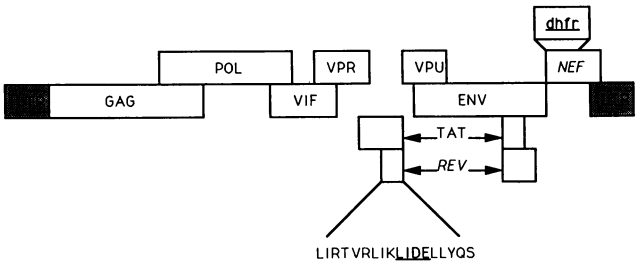

FIG. 1. Provirus expressed by HT4 (V-dhfr) cells. V-dhfr was derived from the W13 version (23) of HXB-Rip 7, a well-characterized clone of HIV-1 (33), by first inserting the coding sequence for a mutant dihydrofolate reductase gene (46) between the initiation codon for Nef and the proximal border of the 3′ LTR and then inactivating the *rev* gene by partial digestion with *Hin*dIII, treatment with the Klenow fragment of DNA polymerase I, and 8-mer *Cla*I linker insertion. This mutation yielded a four-amino-acid insertion in the proximal fifth of Rev. The HT4 (V-dhfr) cells were obtained by transfection of HT4-6C (7), a CD4-positive HeLa cell derivative, with V-dhfr and selection in 2 μM methotrexate (50). Even though the mutation also introduces a stop codon at the 3′ end of the first exon of Tat, Northern blot analysis of cytoplasmic RNA obtained from HT4 (V-dhfr) cells revealed wild-type levels of the 2-kb HIV-1 RNA, confirming that the *rev* and not the *tat* mutation accounts for the replication defect in this mutant provirus (49).

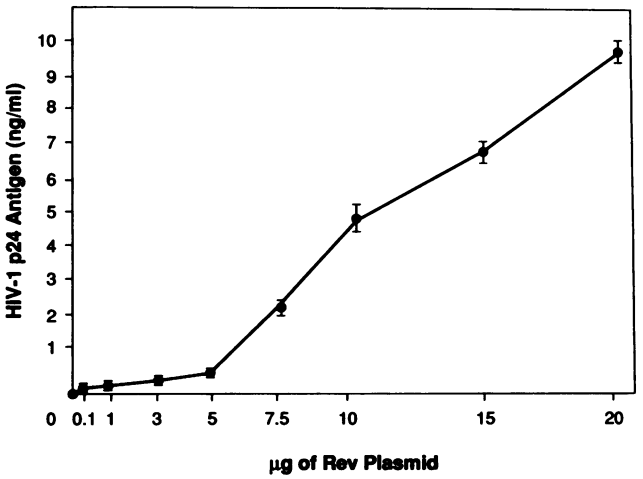

FIG. 2. Transfection of various quantities of pSV-Rev into HT4 (V-dhfr) cells. HT4 (V-dhfr) cells were cultured overnight in 100-mm-diameter petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A total of 1.5×10^6 cells in 10 ml of medium were added to each plate, yielding approximately 30% cellular confluence at the time of transfection. Various quantities of the pSV-Rev plasmid were utilized in transient transfections, using a previously described DEAE-dextran-chloroquine method (3). The total amount of DNA per transfection (22 μg) was normalized by using a plasmid containing only the SV40 promoter in a pSP65 backbone (Promega). Of note, when there was more than 22 μg of total transfected plasmid DNA, cell viability decreased (data not shown). Transfection efficiency was standardized by cotransfection of 2 μg of a CMV-CAT plasmid (i.e., a *Bam*HI-*Hin*dIII CAT fragment cloned downstream of the CMV immediate-early promoter in a pSP65 backbone). CAT activity in the cellular extracts was measured, as previously described (19, 40), after the protein contents of the cell extracts were measured and adjusted (5). The HIV-1 p24 antigen levels in the supernatant were measured 72 h posttransfection, as shown. HIV-1 p24 antigen levels measured at 48 h posttransfection yielded a curve of similar contours (data not shown). The datum points shown here represent the arithmetic means of three independent transfections, performed in duplicate, plus and minus the standard deviations.

plasmid (pSV-Rev). This plasmid consists of an *Mst*II-*Rsa*I fragment of a *rev* cDNA, cloned downstream of a simian virus 40 (SV40) promoter (37). The total amount of transfected DNA was normalized, in all cases, by the addition of a plasmid containing an SV40 promoter but without a *rev* gene. The transfection efficiencies were standardized by cotransfection of a construct containing the cytomegalovirus (CMV) immediate-early promoter linked to the chloramphenicol acetyltransferase gene (CMV-CAT) and subsequent determination of CAT activity in cellular extracts (19) (see legend to Fig. 2). After 72 h, supernatants from each transfection were harvested and assayed for HIV-1 p24 antigen levels by an enzyme-linked immunosorbent assay (ELISA) (Dupont Inc., Wilmington, Del.). As illustrated in Fig. 2, the levels of HIV-1 p24 antigen detected in the supernatants of the transfections revealed a distinctly nonlinear relationship to the quantity of the Rev-expressing plasmid utilized in the transfections. A marked change in the response of viral replication, as assessed by the level of HIV-1 p24 antigen, appeared to occur at levels of pSV-Rev above 5 μg (Fig. 2). Of note, the slope of this curve appears to increase relatively abruptly within a small range of pSV-Rev added to the transient transfections (Fig. 2). This is consistent with a low-efficiency mechanism of HIV-1 replication at low levels of Rev, while above a critical Rev threshold level, a high-efficiency mechanism of HIV-1 replication occurs. As a negative control, a plasmid expressing a mutated *rev* gene, created via a frameshift mutation at the *Bam*HI site in the cDNA clone of *rev* (37) downstream of the SV40 promoter in the same plasmid backbone as pSV-Rev, was utilized. Transfections of this plasmid (1 to 20 μg) into HT4 (V-dhfr) cells led to no change in HIV-1 production (data not shown). These data support a model in which HIV-1 replication is inefficient at low levels of Rev, since mostly the viral regulatory proteins are synthesized, while above a critical threshold level of Rev the viral structural proteins are made, allowing the infection to become productive.

To confirm these findings and to demonstrate that the increase in HIV-1 p24 antigen production was due to the appearance of unspliced HIV-1 RNA in the cytoplasm of these cells, HIV-1-specific RNA patterns in these transfections were evaluated by Northern blot hybridization. As illustrated in Fig. 3, no change in the unspliced (9.2-kb) HIV-1-specific RNA was noted with transfections until 20 μg of pSV-Rev was used. Of note, a *rev*-negative RNA pattern (i.e., mainly 2-kb species) was observed with these cells at baseline (Fig. 3, lane 1), consistent with expression from the Rev-negative provirus integrated in each cell. Transfections of 5 to 15 μg of pSV-Rev induced a small but significant increase in the level of singly spliced HIV-1 RNAs (Fig. 3, lanes 2 to 5). An abrupt switch to a near wild-type pattern of RNA expression (i.e., all three HIV-1-specific RNA species) was demonstrated when 20 μg of pSV-Rev was utilized in these transient transfections (Fig. 3, lane 6). The abrupt change in HIV-1 RNA pattern noted with 20 μg of plasmid, compared with the dramatic increase in HIV-1 p24 antigen levels when transfections included between 5 and 10 μg of Rev-expressing plasmid, appears to be due to the exquisite sensitivity of the HIV-1 p24 antigen ELISA compared with that of the Northern blot hybridization for HIV-1-specific

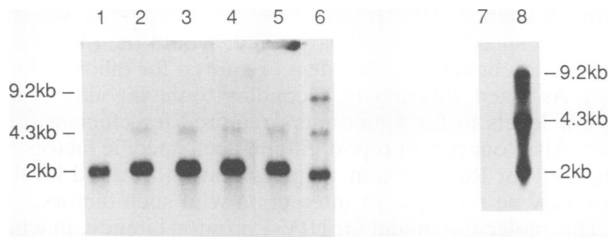

FIG. 3. Northern blot hybridization analysis of HT4 (V-dhfr) cells transfected with various quantities of pSV-Rev. Lanes 1 to 6 show Northern blot hybridizations of RNA isolated from HT4 (V-dhfr) cells transfected with the following amounts of pSV-Rev: 0 μg (lane 1), 5 μg (lane 2), 7.5 μg (lane 3), 10 μg (lane 4), 15 μg (lane 5), and 20 μg (lane 6). Lane 7, RNA isolated from HT4-6C cells (negative control); lane 8, RNA isolated from a T-lymphocytic cell line (H9) productively infected with HIV-1 (positive control). Total cellular RNA was isolated from cells 48 h after transfection by utilizing a guanidinium isothiocyanate-cesium chloride method (8). As previously described (42, 48), equal quantities of RNA (20 μg) were electrophoresed on a 1.1% agarose plus formaldehyde gel, transferred to nitrocellulose, and hybridized at 60°C to a ^{32}P-labelled RNA probe. Filters were washed three times in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C prior to autoradiography. The RNA probe was complementary to nucleotides −120 to +80 of the HIV-1 genome, within the 5′ LTR (13). The probe was generated by using an in vitro transcription system with T7 RNA polymerase (Boehringer-Mannheim). This RNA probe was utilized because it would not bind to the transfected Rev-expressing plasmid.

RNA. A difference in technique sensitivity may be especially important in evaluating transient transfections in which only a small percentage of cells in a culture actually receive and express the transfected plasmid (3).

These results strongly reinforced the hypothesis that a threshold level of Rev is necessary for HIV-1 structural gene expression. However, it could still be argued that the levels of Rev expression obtained with the HT4 (V-dhfr) cells might not have linearly correlated with the amount of pSV-Rev used for transfection. Precise quantitation of Rev protein levels is technically quite difficult at extremely low levels. In addition, assessment by Western blot or radioimmunoprecipitation analyses would be greatly hampered by the modified HIV-1 provirus in HT4 (V-dhfr) cells, which expresses a nonfunctional mutant Rev protein that differs from wild-type Rev only by four additional amino acids (50). Therefore, we measured levels of Rev mRNA expression in these transfections by using a quantitative reverse transcriptase (or RNA) polymerase chain reaction (PCR). The quantitative HIV-1 RNA-PCR methodology which was utilized (Fig. 4) was based on a technique previously described (2), with important modifications. After the treatment of total cellular RNA with RNase-free DNase (3), reverse transcription of the RNA to cDNA was performed by using an oligonucleotide located in the second exon of *rev* as a primer. The sense and antisense primers used to amplify multiply spliced RNAs are located in the first and second exons of *rev* and are 5′-GAAGAAGCGGAGACAGCGA CG-3′ (nucleotides 5977 to 5996) and 5′-GGCCTGTCGGGT CCCCTCG-3′ (nucleotides 8412 to 8393), respectively. These primers amplify a 102-bp fragment in nonmutant HIV-1.

The antisense primer (10 ng) was hybridized to 1 μg of total cellular RNA in a hybridization buffer (0.25 M KCl, 0.01 M Tris [pH 8.0], 1 mM EDTA) at 65°C for 1 h. Primer extensions were accomplished by adding portions of the above reaction mixture to a buffer containing 0.07 M KCl; 0.01 M Tris (pH

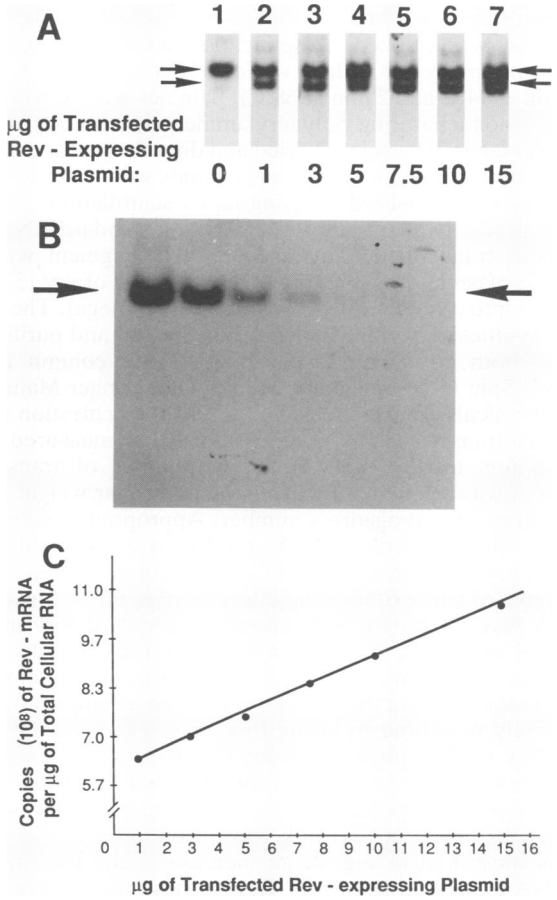

FIG. 4. Quantitation of Rev mRNA expressed from pSV-Rev by utilizing an RNA PCR. (A) Autoradiograph of RNA PCR analyses of pSV-Rev transfections into HT4 (V-dhfr) cells. The upper arrows point to the specific band obtained from Rev mRNA expressed from V-dhfr. The lower arrows point to the band obtained from Rev mRNA expressed from pSV-Rev. This autoradiograph is a representative example from three separate experiments. (B) Autoradiograph of a standard dilution curve (fourfold consecutive dilutions) of in vitro-transcribed HIV-1 Rev mRNA after reverse transcriptase PCR. (C) Graphical representation of copies of Rev mRNA produced from pSV-Rev per microgram of total cellular RNA, plotted against numbers of micrograms of transfected pSV-Rev DNA.

8.3); 5 mM dithiothreitol; 1.0 μM (each) dATP, dTTP, dGTP, and dCTP; and 10 mM $MgCl_2$ either with or without 5 U of reverse transcriptase (Life Sciences, Inc.) at 37°C for 1 h. A tube without reverse transcriptase was used as a negative control to test for DNA contamination. A PCR amplification procedure, with parameters modified to minimize nonspecific hybridization and efficiently amplify only small fragments (<250 nucleotides), was then performed (2).

The 5′ oligonucleotide primer was radioactively labelled by using [γ-^{32}P]ATP and T4 polynucleotide kinase (3). In this way, a hybridization step using a labelled probe was not necessary. A mixture containing 10 ng of radiolabelled oligonucleotide (10^7 cpm) and 50 ng of the 3′ unlabelled oligonucleotide was added to a mixture containing a PCR buffer (25 mM Tris [pH 8.0]; 5 mM $MgCl_2$; 50 mM NaCl; 100 μM (each) dATP, dTTP, dCTP, and dGTP; 20 mg of bovine serum albumin per ml) and 0.5 μl of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). In the PCR amplifi-

cation steps, one-half of the above reverse transcription reaction was utilized. Amplification was conducted in a DNA thermocycler with 25 cycles of two temperature steps (1 min at 94°C and 2 min at 68°C). Samples were resolved on an 8% nondenaturing polyacrylamide gel, with appropriate marker lanes. The gel was dried and directly exposed to film. After appropriate exposure times, bands were cut and radioactivity was measured by using liquid scintillation.

To precisely quantitate Rev mRNA, a standard RNA was in vitro transcribed. An *Eco*RI-*Bam*HI fragment was cut from a plasmid encoding the cDNA version of *rev* (37) and cloned into a pSP64-Poly-A backbone (Promega). The RNA was synthesized with SP6 RNA polymerase and purified by using both an oligo(dT) chromatographic column and a Quick Spin Q-50 Sephadex column (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.). The concentration of the in vitro-transcribed RNA standard was then measured spectrophotometrically, and the copy number of transcripts present was calculated by using the molecular weight of the transcript and Avogadro's number. Appropriate dilutions of the in vitro-transcribed RNA standard were then subjected to the reverse transcriptase PCR protocol described above. A standard curve of fourfold dilutions of in vitro-transcribed RNA was generated, with copies of standard Rev mRNA plotted against the counts per minute of radioactivity of the amplified fragment. Therefore, within the linear amplification range, the quantity of Rev mRNA in a sample could be precisely measured by using this standard curve. Of note, the Rev mRNA produced from V-dhfr could be differentiated on a polyacrylamide gel from the Rev mRNA expressed by pSV-Rev, as the Rev mRNA expressed by V-dhfr is 12 bp larger because of a *Cla*I linker insertion (50).

As illustrated in Fig. 4, an increase in the level of Rev mRNA expressed from pSV-Rev in these transfections was demonstrated, and it was linearly correlated with the quantity of pSV-Rev in each transfection. This linear correlation was demonstrated throughout the entire range of pSV-Rev transfections. Therefore, these experimental results support the hypothesis that the nonlinearity of HIV-1 production in this system is based on a threshold level of Rev and is not due to poor Rev expression at low levels of transfected plasmid.

These data suggest that a functional threshold for Rev is necessary to allow efficient HIV-1 replication. The specific threshold level, however, may vary in different cell types and under different conditions. These findings support recent studies which documented multiple binding sites for Rev on the RRE (24), multimerization of Rev in vitro (39), and the requirement that Rev be multimerized in order for it to function efficiently after it is bound to the RRE, thus allowing the cytoplasmic expression of unspliced HIV-1 RNA (30). Whether Rev multimer formation is necessary for binding to the RRE or is required for efficient function after binding to the RRE is controversial (30, 39, 52). Still, a critical level of Rev protein in the nucleus may be necessary to allow Rev multimers to form. Also, these experiments and our previous work (42) suggest that the expression of singly spliced HIV-1 mRNAs, which encode at least the viral envelope, requires lower levels of Rev than that of unspliced RNA, which codes for Gag and Pol.

This system further supports, in a different context, our model of HIV-1 proviral latency based on a subthreshold level of Rev. In certain cells, a low level of baseline HIV-1 LTR-directed transcription, possibly based on specific cellular transcription factors, may occur. With the U1 cell line, we have suggested that an exquisitely tight control of NF-κB activation may be an important factor involved in HIV-1 proviral latency (41). This low level of transcription, which is due to a specific intracellular milieu, would then keep Rev production below a critical level required for efficient function. As noted, this may be secondary to the inability of Rev at low levels to form multimers required for efficient function. Also, our recent report (50) that cell-specific factors are required for Rev function suggests that a threshold level of Rev may be required for interaction with such factors.

This molecular model for HIV-1 proviral latency, in which an aberrant pattern of HIV-1-specific RNA is detected in latently infected cells and may be based on a subthreshold level of Rev, has recently been confirmed (34) and also documented for other cell lines (35). Preliminary data suggest that such an aberrant pattern of HIV-1 RNA, consistent with this model of HIV-1 proviral latency, may exist in vivo in specific cells from certain HIV-1-infected individuals (reference 43 and unpublished data). Still, a variety of mechanisms may account for the prolonged asymptomatic period in certain HIV-1-infected individuals (12, 22, 28, 36), and HIV-1 latency may involve other molecular mechanisms which affect preintegration stages in the viral life cycle (18, 51).

The findings presented in this report suggest that a critical level of Rev may be necessary for efficient HIV-1 replication. This lends support to the prospects of utilizing "intracellular immunization" techniques to introduce *trans*-dominant negative Rev mutations into HIV-1-infected cells as a therapeutic modality for individuals with clinical HIV-1 infection (4, 29).

We thank Mark Feinberg and Mark Muesing for providing the pSV-Rev plasmid, Bryan Cullen for helpful discussions, David Baltimore for support and insight, and Brenda Gordon and Rita Victor for secretarial assistance.

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