Analysis of *rev* Gene Function on Human Immunodeficiency Virus Type 1 Replication in Lymphoid Cells by Using a Quantitative Polymerase Chain Reaction Method

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Most detailed analyses of the human immunodeficiency virus type 1 (HIV-1) *rev* gene product have relied on transfection of subgenomic *env* constructs into cells in which amplification of the transfected DNA occurs. This was necessitated by difficulties in quantitating low-abundance HIV-1 mRNA species and in distinguishing different RNAs of similar sizes. We have modified the conventional polymerase chain reaction method for general use as an extremely sensitive procedure for quantitative analysis of RNA species. Using this method, we assessed the role of the HIV-1 *rev* gene in viral replication following mutagenesis of an infectious molecular clone, HIV-1_{JR-CSF}. Following transfection of wild-type and mutant proviral constructs, we can specifically detect unspliced RNA and distinguish between the spliced *tat-rev* and *nef* mRNAs, which are not resolved by standard RNA analyses. Our results show that the *rev* protein of HIV-1_{JR-CSF} simultaneously down regulates the expression of *tat-rev* and *nef* RNAs and up regulates the level of unspliced full-length HIV-1 RNA. A *cis*-acting element(s), located exclusively within the *env* sequences, is essential to exhibit this regulation. Fractionation of cells shows that the ultimate effect of Rev is to direct the appearance of unspliced or singly spliced RNAs in the cytoplasm. Models are discussed for possible mechanisms of Rev action.

In addition to the three genes expressed by all replication-competent retroviruses (*gag*, *pol*, and *env*), human immunodeficiency virus type 1 (HIV-1) contains the additional genes *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* (11). Three of these genes have been shown to affect the expression of HIV-1 RNA and protein. The *tat* gene product increases the level of both HIV-1-specific RNA and protein through a *trans*-acting responsive element located in the long terminal repeat (LTR) and at the 5' termini of all HIV-1 RNA species (6, 7, 17, 22, 23, 25, 26, 31, 34). The *nef* gene product appears to down regulate expression from the LTR through a negative regulatory element in the LTR (1, 19).

The *rev* gene product appears to be necessary for high-level expression of *gag* and *env* mRNAs and protein acting at a posttranscriptional level. Rev function has been proposed as an inhibitor of splicing, as an activator of nuclear transport, and as a source of relief to a translational block (3, 8, 9, 12-14, 21, 27, 28, 30). Previous studies of the mechanism of Rev action have relied on various replicating vector systems to generate assayable levels of virus-specific RNA. A system commonly used to assay Rev function involves transfection of simian virus 40-transformed monkey kidney cell lines (COS) with replicating vectors (13, 14, 20, 21, 27, 28). These studies generally used *env* vectors in which *env* is expressed as an unspliced RNA. Proviral constructs have also been used to examine the effect of Rev on the expression of all HIV-1-specific RNAs (3, 8, 9, 12, 28, 30). However, these studies did not discriminate among HIV-1 RNAs of similar sizes. It has recently been demonstrated that Rev is necessary for the accumulation of high levels of cytoplasmic unspliced RNA in transfected HeLa and COS cells; this was attributed to an effect on both transport and stability of the unspliced RNA (9, 21). A *rev*-responsive element (RRE) found within the HIV-1 *env* gene has been

shown to be involved in the regulation of HIV-1 RNA expression by Rev (9, 12, 13, 21, 27).

To more precisely define the role of Rev in the regulation of HIV-1 replication, we have constructed mutants in an infectious molecular clone, pYKJRCSE, of a primary isolate of HIV-1_{JR-CSF}. This isolate replicates to high titers in primary human lymphoid cells (16). To detect RNA from proviral constructs in lymphoid cells, we developed a quantitative assay for RNA by using a modified polymerase chain reaction (PCR) method. Because of the increased sensitivity and specificity of this technique over those of conventional techniques, we can independently examine the regulation of full-length, *tat-rev*, and *nef* RNAs (3, 22, 24). Our results demonstrate that in lymphoid cells, Rev regulates the distribution of HIV-1 RNAs within the cell.

MATERIALS AND METHODS

Cell lines and electroporation. 729 and Jurkat cell lines were maintained in Iscove medium supplemented with 5 and 10% fetal calf serum, respectively. Before electroporation, cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum at a concentration of 2×10^7 /ml. Cells (10^7) were electroporated with a total of 50 μ g of plasmid DNA as described previously (5). For RNA and p24 analysis, cells were harvested at 48 h postelectroporation.

Plasmid constructions. The proviral clone pYKJRCSE was described previously (16). Mutant Δ Sal was made by filling in a *Sal*I site at nucleotide (nt) 8483 of pYKJRCSE with the Klenow fragment of DNA polymerase. This resulted in a frameshift mutation in the *rev* gene and the creation of a new *Pvu*I site verified by restriction enzyme digestion. Mutant Δ BX was generated by adding a *Bam*HI linker to an *Ssp*I site at nt 8262 and replacing the wild-type *Bam*HI (nt 7324)-to-*Xho*I (nt 8911) fragment with the *Ssp*I-to-*Xho*I fragment. This resulted in a deletion of nt 7328 to 8261 from the proviral construct. Mutant Δ Pst was made in proviral clone

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FIG. 1. Oligonucleotide primer pairs for PCR on HIV-1 RNAs. (A) Schematic representation of the HIV-1_{JR-CSF} genome showing the location and orientation of the oligonucleotide primers used for PCR. The splice donors (SD) and splice acceptors (SA) for *env*, *tat/rev*, and *nef* RNAs are shown. The spliced forms of these RNAs are depicted below the schematic diagram. The locations of the *gag*, *RRE*⁻, and *rev* mutations in the genome are shown (not drawn to scale). (B) Sizes of amplified products generated by RNA PCR with pairs of oligonucleotide primers specific for HIV-1 RNAs. ND, RNA species for which specific products were not detected because of their large theoretical sizes.

pN/BJRCSF, which is identical to pYKJRCFSF except for an additional 2 kilobases of cellular flanking sequence upstream of the 5' LTR. Mutant ΔPst was generated by restriction enzyme digestion with *Pst*I and religation, resulting in the deletion of nt 1205 to 1418 within the *gag* gene.

RNA preparation and p24 antigen detection. Total cellular RNA was prepared by lysing the cells in 4.7 M urea–1.3% sodium dodecyl sulfate–0.23 M NaCl–6.7 mM Tris (pH 8.0)–0.67 mM EDTA. This was followed by three phenol-chloroform (1:1) extractions, one chloroform-isoamyl alcohol (24:1) extraction, and ethanol precipitation. The total recovered nucleic acid was treated twice at 37°C for 1 h with RNase-free DNase (Worthington Diagnostics) to remove the DNA. Nuclear and cytoplasmic RNAs were fractionated by lysing the cells in 0.65% Nonidet P-40–150 mM NaCl–10 mM Tris (pH 7.8)–1.5 mM MgCl₂ and by pelleting the nuclei by low-speed centrifugation. The nuclear and cytoplasmic fractions were then treated as described above. Cell-free supernatants from electroporated cells were assayed for *gag* p24 antigen by using a specific enzyme-linked immunosorbent assay kit (Abbott Laboratories).

RNA PCR analysis. RNA (≤5 μg) was subjected to a primer extension hybridization reaction in a volume of 10 μl containing 10 ng of primer (antisense), 250 mM KCl, 10 mM Tris (pH 8.3), and 1 mM EDTA at 65°C for 1 h. A portion (5

μl) of this reaction was added to each of two tubes containing 10 μl of 1.5× reverse transcriptase buffer (1.5 mM deoxynucleoside triphosphates, 7.5 mM dithiothreitol, 15 mM MgCl₂, 0.09M KCl, 0.63 mM Tris [pH 8.3]). Reaction mixtures were incubated in the presence or absence of 5 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) at 37°C for 1 h. Portions (2 μl each) of these primer extension reactions were subsequently subjected to analysis by PCR. The PCR amplifications each contained 0.25 mM deoxynucleoside triphosphates, 50 mM NaCl, 5 mM Tris (pH 8.0), 5 mM MgCl₂, 100 ng of the 3' (antisense) oligonucleotide, 0.67 to 1.2 U of Taq polymerase (Promega Biotec or New England BioLabs, Inc.), and 20 to 30 ng of the 5' (sense) oligonucleotide end labeled with T4 DNA kinase to a specific activity of 1 × 10⁸ to 6 × 10⁸ cpm/μg, in a total volume of 25 μl. PCR was performed in a DNA thermal cycler (Cetus or Ericomp) for 25 cycles of denaturation at 91°C for 1 min and polymerization at 65°C for 2 min. PCR-amplified products were separated on a 6% polyacrylamide gel and visualized by autoradiography.

The nucleotide sequences of the oligonucleotide primers used for PCR (Fig. 1) are as follows: for M667, 5'-GGC TAACTAGGGAACCCACTG-3'; for M668, 5'-CGCGTCCC TGTTCCGGGCGCC-3'; for LA8, 5'-GCGCGCACAGCAAG AGGCGA-3'; for LA9, 5'-GACGCTCTCGACCCATCTC-

TABLE 1. Production of *gag* p24 from electroporated lymphoid cells^a

Construct	Amt of <i>gag</i> p24 (pg/ml) ^b produced in:				
	729		Jurkat		
	Expt 1 ^c	Expt 2 ^c	Expt 1	Expt 2 ^c	Expt 3
pYKJRCSF (wild type)	>2,700/>2,700	>2,700/>2,700	1,500	>2,700/>2,700	>2,700
ΔPst (<i>gag</i>)	NT	<30/<30	NT	NT	<30
ΔSal (<i>rev</i>)	<30/<30	<30/<30	<30	<30/<30	<30
ΔBX (RRE ⁻)	<30/<30	<30/<30	<30	NT	NT
ΔSal + ΔBX	>2,700/>2,700	1,732/2,139	NT	1,312/>2,700	NT
ΔSal + ΔPst	625/561	877/1,175	426	215/435	1,229
ΔBX + ΔPst	35/79	NT	NT	<30/<30	NT

^a Culture supernatants from 10⁷ cells transfected with a total of 50 μg of plasmid DNA were assayed at 48 h posttransfection.

^b Detected by enzyme-linked immunosorbent assay (Abbott). Values of <30 are below the sensitivity of this assay. NT, Not tested.

^c Results of duplicate electroporations are shown.

3'; for LA45, 5'-GGCTTAGGCATCTCCTATGGC-3'; and for LA41, 5'-TGTCGGGTCCCCTCGTTGCTGG-3'.

RESULTS

Rev is necessary for production of *gag* p24 antigen. To determine the function of Rev in the expression of *gag* proteins in lymphoid cells, mutants were generated in an infectious clone of HIV-1_{JR-CSF}, pYKJRCSF (Fig. 1A). A *rev* mutant (ΔSal) contains a 4-base-pair (bp) insertion at the *Sal*I site (nt 8483), resulting in a frameshift in the *rev* coding sequences; an RRE⁻ mutant (ΔBX) contains a deletion of nt 7328 to 8261 which encompasses the RRE (9, 12, 21, 27) but does not interrupt the *rev* coding sequences; and a *gag* mutant (ΔPst) contains an out-of-frame deletion of almost the entire *gag* p24 coding sequences and is thus incapable of expressing *gag* p24 antigen. This mutant was designed to conserve expression of all the *trans*-acting regulatory proteins so that it could complement a defect in p24 *gag* production due to loss of *rev* function.

These constructs were electroporated either separately or in combination with each other into a human B-cell (729) or T-cell (Jurkat) line. At 48 h postelectroporation, culture supernatants were analyzed for the presence of *gag* p24 antigen by using a specific enzyme-linked immunosorbent assay kit (Abbott). The results are summarized in Table 1. The wild-type proviral construct gave rise to consistently high values of *gag* p24 in both cell types tested. The *gag* mutant was used as a negative control, since it was incapable of producing any *gag* p24 protein. The *rev* and RRE⁻ mutants were also incapable of producing any p24 antigen when electroporated alone. Thus, both the *rev* protein and the RRE are necessary for the production of any detectable *gag* proteins.

Complementation experiments were performed to determine whether it was possible to rescue p24 production in these mutants. High levels of p24 were produced when the *rev* mutant was coelectroporated with either the RRE⁻ or *gag* mutant, suggesting that the *gag* and RRE⁻ mutants produced a functional *rev* protein able to rescue the *rev* mutant in *trans*. In contrast, coelectroporation of *gag* and RRE⁻ mutants did not rescue p24 production or gave rise only to very low levels of measurable p24 production, indicating that the RRE⁻ mutant contained a *cis* defect. This low level was most likely due to recombination between the two constructs, since coelectroporation of either mutant with a vector producing only Rev did not give rise to any detectable p24 antigen (data not shown). The *rev* mutant could produce wild-type levels of p24 antigen when coelec-

toporated with the vector producing only Rev (data not shown). These results suggest that both Rev and the RRE must be present in order to obtain expression of *gag* p24 antigen in lymphoid cells and that both are therefore essential to the replication of HIV-1. As indicated by results in other systems, Rev functions in *trans* through a *cis*-acting element to allow expression of *gag* protein.

Quantitative PCR analysis of HIV-1-specific RNAs. To detect low-abundance HIV-1 RNA transcripts in lymphoid cells, we employed the PCR method (18, 29) with RNA isolated from HIV-1-infected cells by first converting the RNA to cDNA with reverse transcriptase. This method offers a large increase in sensitivity over that available with conventional Northern (RNA), nuclease protection, or primer extension analyses. The oligonucleotide pairs were designed to direct synthesis of PCR products which would span known splice donors and acceptors. The location of the oligonucleotide pairs in the HIV-1 genome with respect to known splice donors and acceptors and the predicted sizes of the PCR products generated for each HIV-1 RNA with these oligonucleotide pairs are shown in Fig. 1. Since large PCR products (>250 bp) are not generated under the conditions used with these oligonucleotide pairs, only the sizes of detectable products are shown. Oligonucleotide primers M667 and M668 will direct synthesis of a 161-bp amplified product corresponding to all HIV-1 RNAs; primers LA8 and LA9 should generate a 95-bp PCR product specific for HIV-1 RNA which has not utilized the 5' splice donor at nt 743 (full length). Primers LA45 and LA41 will generate a 123-bp PCR product specific for HIV-1 RNA which has joined the splice donor at nt 6055 with the splice acceptor at nt 8365 (*tat* and *rev*); LA8 and LA41 should generate a PCR product of 133 bp specific for HIV-1 RNA which has joined the splice donor at nt 743 with the splice acceptor at nt 5788 as well as the splice donor at nt 6055 with the splice acceptor at nt 8365 (*nef*).

Total RNA prepared from peripheral blood lymphocytes infected with HIV-1_{JR-CSF} was treated with RNase-free DNase to remove all contaminating DNA and then converted to cDNA with reverse transcriptase by using the appropriate 3' (antisense) oligonucleotide primer. The products of this reaction were then subjected to 25 cycles of PCR amplification with the addition of the 5' (sense) oligonucleotide primer end labeled with ³²P. We have modified the standard PCR protocol to allow quantitative analysis of samples. By using an end-labeled oligonucleotide primer, we can omit the transfer and hybridization steps used in other protocols and decrease the number of cycles of denaturation

FIG. 2. Quantitative detection of specific HIV-1 RNAs by PCR. (A) Detection of HIV-specific RNAs by PCR on RNA from infected peripheral blood lymphocytes. RNA was extracted from infected (inf.) and uninfected (uninf.) peripheral blood lymphocytes. RNA from 10^5 cells was subjected to primer extension reactions in the presence (+) or absence (–) of reverse transcriptase. Portions of these reactions were subjected to 25 cycles of PCR. The 3' (antisense) oligonucleotide was used for primer extension, and the 5' (sense) oligonucleotide primer was end labeled with [γ - 32 P]ATP and included in the PCR reaction. Products were separated on a 6% polyacrylamide gel and visualized by autoradiography. Numbers on the right indicate sizes in base pairs. Molecular weight marker (M) is pGEM-2 (Promega Biotec) digested with *Hpa*II. (B) PCR on twofold dilutions of RNA from HIV-1-infected peripheral blood lymphocytes. RNA was prepared from infected cells, diluted, and then converted to cDNA by primer extension. cDNA was subjected to PCR as described above.

and polymerization. Decreasing the number of cycles of PCR from 40 to 25 and eliminating the transfer and hybridization steps result in a PCR method which is both extremely sensitive and quantitative. In applying this method, it is also critical that the annealing step in each PCR cycle be omitted and that different primer pairs be tested for appropriately low backgrounds. PCR with HIV-1-specific oligonucleotide pairs can generate a major specific amplified product of the predicted size from HIV-1-infected-cell RNA (Fig. 2A). These specific bands are not present in uninfected, total-cell RNA. In some cases, minor bands other than those predicted are generated. Nucleotide sequence analysis indicates that most minor bands generated during PCR are HIV-1-specific products but result from nonspecific priming of the antisense oligonucleotide primer and specific priming of the sense primer. This nonspecific priming most likely occurs at the reverse transcriptase step, and these aberrant DNA products are then amplified during PCR. Since the oligonucleotide pairs M667-M668 and LA8-LA9 can also detect HIV-1-specific DNA, mock primer extensions in which reverse transcriptase was omitted from the reactions to

control for any contamination of the RNA with DNA were performed; no HIV-1 DNA was detected in these samples.

To determine whether HIV-1-specific RNAs could be detected quantitatively by this RNA PCR procedure, sequential twofold dilutions of total cellular RNA from HIV-1-infected cells were subjected to PCR analysis (Fig. 2B). The signals produced by RNA PCR were quantitative across a 2,000-fold range of RNA concentrations with all oligonucleotide pairs tested. In all cases, increasing signal intensities were detected from increasing amounts of RNA. However, for a given number of PCR amplification cycles, the magnitude of the increase in signal intensities was not linear and reached a plateau as higher signal intensities were achieved, probably because concentrations of nucleotides or oligonucleotides were limiting. Thus, it is important to recognize that differences in signal strength intensities demonstrated by PCR may be underestimates of relative differences. Absolute quantitation requires the use of appropriate RNA standards subjected to PCR in parallel. We estimate that the sensitivity of this method is more than 10^4 -fold that of standard RNA detection systems, making detection of low-level RNA species and the use of far fewer cells possible.

Effect of Rev on levels of *gag-pol*, *tat-rev*, and *nef* RNAs. To examine the block to *gag* p24 production exhibited by the proviral mutants, the expression of HIV-1-specific RNAs in electroporated lymphoid cells was examined by PCR. Using specific oligonucleotide primer pairs, we can discriminate between *tat-rev* and *nef* RNAs, unlike other researchers, who have grouped these RNAs into a same-size class of double-spliced RNAs.

Both the *rev* mutant (Δ Sal) and the *RRE*[–] mutant (Δ BX) produced RNA profiles very different from that of the wild type (Fig. 3). Both mutants produced reduced levels of unspliced full-length RNA and a concomitant large increase in both *tat-rev* and *nef* RNAs relative to the wild type. A comparison of the PCR signals produced by wild-type and mutant constructs with standard curves generated by dilutions of infected-cell DNA has allowed us to determine the magnitude of these differences. The levels of *tat-rev* and *nef* RNAs in both the *rev* and *RRE*[–] mutants reproducibly increase to more than 10-fold the levels in the wild type, and the level of unspliced full-length HIV-1 RNA with these mutants decreases more than 5-fold relative to the wild type. The levels of total HIV-1-specific RNA with these mutants were not significantly different from those of the wild type, suggesting that Rev does not affect overall RNA levels of HIV-1. Coelectroporation of the *rev* and *RRE*[–] mutants resulted in restoration of the high level of full-length RNA seen with the wild type. The levels of both *tat-rev* and *nef* RNAs remained higher than that seen with the wild type. This can be explained by an inability to *trans*-complement the *cis* mutation of the *RRE* in the Δ BX mutant.

Similar results were obtained with another construct which expresses Rev. The *gag* mutant (Δ Pst) can complement the *rev* mutant, as measured by p24 production (Table 1). Coelectroporation of both mutants resulted in a high level of full-length RNA and low levels of both *tat-rev* and *nef* RNAs (Fig. 3B), similar to those seen in the wild type. RNA from cells electroporated with the *gag* mutant exhibited wild-type levels of *tat-rev* and *nef* RNAs. However, the level of unspliced full-length HIV-1 RNA was severely reduced, with an overall reduction in the level of total HIV-1-specific RNA. This could be due to a decrease in RNA stability, similar to that seen in *gag* mutants of Rous sarcoma virus (2).

FIG. 3. Effect of Rev on HIV-1 RNA accumulation. (A) Analysis of RNA from 729 cells electroporated with the indicated plasmid DNAs. In each pair of oligonucleotide primers, equivalent amounts of RNA from these cells were subjected to primer extension and subsequent PCR. (B) Analysis of RNA from electroporated 729 cells. Experiments were performed as described above.

These results indicate that Rev alters the distribution of HIV-1 RNAs by up regulating expression of full-length HIV-1 RNA and down regulating expression of both *tat-rev* and *nef* RNAs. The levels of *tat-rev* and *nef* RNAs appear to be coregulated by Rev, since equivalent increases in these RNAs are seen with both *rev* and *RRE*⁻ mutants. The effect of Rev on the regulation of HIV-1 RNA distribution requires a *cis*-acting element, the RRE.

Effect of Rev on cellular distribution of HIV-1 unspliced full-length RNA. To determine whether Rev was affecting the cellular distribution of HIV-1 RNAs, nuclear and cytoplasmic RNA fractions were prepared from lymphoid cells electroporated with wild-type and mutant proviral constructs. Cell equivalents of RNA were then subjected to analysis by PCR with oligonucleotide pairs specific for unspliced full-length HIV-1 RNA (LA8-LA9) and *tat-rev* RNA (LA45-LA41) (Fig. 4). Although nuclear levels of full-length RNA were similar to those of wild-type and mutant constructs, only the wild type accumulated high

FIG. 4. Effect of Rev on nuclear and cytoplasmic RNA distribution. RNA from electroporated 729 cells was separated into nuclear and cytoplasmic fractions. Nuclear or cytoplasmic RNA from 8×10^5 electroporated cells was subjected to RNA PCR analysis by using oligonucleotides specific for full-length and *tat-rev* RNAs.

levels of unspliced RNA in the cytoplasmic fraction. In contrast, the *rev* and *RRE*⁻ mutants exhibited higher levels of *tat-rev* RNA in the cytoplasmic fraction than did the wild type. The decreased levels of cytoplasmic full-length HIV-1 RNA with the *rev* and *RRE*⁻ mutants were inversely correlated with the large increases in *tat-rev* RNA in the cytoplasm of cells electroporated with these mutants. The majority of spliced RNA of all the constructs was found in the cytoplasmic fractions, indicating that *tat-rev* RNA is transported rapidly to the cytoplasm and does not accumulate in the nucleus.

Expression of *rev* and the presence of the RRE appear to be necessary for the accumulation of unspliced full-length HIV-1 RNA in the cytoplasm. If Rev could exert an effect through a *cis*-acting element other than RRE, this effect should be observed with the *RRE*⁻ mutant, which can still produce Rev. Since both the *rev* mutant, ΔSal, and the *RRE*⁻ mutant, ΔBX, exhibit identical levels of each of the HIV-1 RNAs and identical distributions of the HIV-1 full-length RNA, it seems likely that Rev exerts its effect on RNA expression solely through the RRE and not through any other element within the viral genome.

DISCUSSION

Using PCR, we have developed a novel method for the quantitative detection of specific low-abundance HIV-1 RNAs. Oligonucleotide pairs which detect specific HIV-1 RNA species through *in vitro* synthesis of cDNA and subsequent detection by PCR amplification have been generated. This method offers significant advantages over conventional methods used to measure RNA accumulation, such as Northern, nuclease protection, or primer extension analyses. By using an end-labeled oligonucleotide primer, performing fewer cycles of PCR, and eliminating the transfer and hybridization steps of the conventional PCR method, quantitative detection of HIV-1 RNAs in lymphoid cells is possible. Because of the large increase in sensitivity provided by this method, we can easily detect low-abundance HIV-1 RNAs in electroporated lymphoid cells and perform hundreds of assays with RNA from 10^7 cells. Because of the specificity of the oligonucleotide primers, we can discrimi-

nate between RNAs such as *tat-rev* and *nef*, which are difficult to distinguish by conventional techniques. The non-linear nature of this detection method does not affect quantitation; over a large range of input RNAs and in a large variety of oligonucleotide pairs, we have seen a direct and reproducible correlation between the amount of specific-input RNA and the intensity of the signal produced. Furthermore, if linearity of the dose response is desired for a given range of sample values, the number of PCR cycles can be empirically determined to maintain them within the linear part of the curve. Accurate quantitation by PCR necessitates the use of standard curves on RNA dilutions done in parallel.

Using this RNA PCR procedure, we can discriminate between the *tat-rev* and *nef* RNAs produced in lymphoid cells infected with HIV-1 or transfected with HIV-1 proviral constructs. This has allowed us to analyze the effect of Rev on each of these RNAs independently rather than on doubly spliced RNAs as a single size class. Consequently, we have determined that Rev down regulates the expression of both *tat-rev* and *nef* RNAs concordantly in lymphoid cells while increasing the level of cytoplasmic full-length HIV-1 RNA. Little or no effect on the levels of nuclear unspliced HIV-1 RNA is seen in the presence of Rev. Rev exerts its effect solely through the RRE located in the *env* gene, which is present in both unspliced and singly spliced RNAs. Removal of this element results in levels and distributions of HIV-1-specific RNAs indistinguishable from those of a *rev* mutant. These data demonstrate that this target region, which is removed during splicing to the *tat-rev* and *nef* RNAs, is critical for Rev to exhibit any effect on HIV-1 RNA expression.

It is interesting that although the *rev* and RRE⁻ mutants produce more than 10 times as much *tat-rev* RNA as the wild-type construct does, an increase in total RNA levels was not observed. In these mutants, an increase in total RNA levels due to increased levels of *trans*-activation by Tat might be expected. It is possible that there is no increase in *tat* protein with these mutants. Alternatively, since *nef* RNA is also increased, a concomitant increase in *nef* protein might counteract any increase in *tat trans*-activation. Deletions within *nef* have previously been shown to enhance viral replication (1, 19), and it has been further demonstrated that the *nef* protein down regulates Tat-dependent transcription from the HIV-1 LTR. This repression is apparently exerted through the negative regulatory element located in the LTR (1). Additionally, the RRE⁻ mutant might be expected to overexpress *rev* protein in comparison with the wild type. Since little or no effect on the level of total HIV-1 RNA was seen with the RRE⁻ or *rev* mutants, we cannot demonstrate any effect of Rev on HIV-1 RNA stability, as described by Felber et al. (9), although small effects on the stability of total RNA or larger effects on the stability of minor RNA species would not be seen.

Our parallel analysis of different RNA species is consistent with several models for Rev action. Rev might be necessary for transport to the cytoplasm of unspliced and singly spliced RNAs containing the RRE, resulting in less substrate for splicing to *tat-rev* and *nef* RNAs. This theory has been previously proposed by other investigators (9, 13, 21). A defect in *rev* would lead to an increase in the nuclear pool of unspliced RNA available for subsequent splicing to *tat-rev* and *nef* RNAs. The fates of singly spliced RNAs are less clear. The *env* RNA could potentially be further spliced to *tat-rev* RNA, or it could be transported to the cytoplasm by the action of Rev. The putative *vif* and *vpr* RNAs could be generated by a splice from the splice donor at nt 743 to splice

acceptors at nt 4924 and 5401, respectively (22, 24). Like *env* RNA, these RNAs (which would contain the RRE) might be further transported to the cytoplasm by the action of Rev, or they could be spliced to aberrant doubly spliced products; candidates for such aberrant products have been detected in HIV-1-infected cells (22). Thus, the transport theory would require that Rev act on unspliced and singly spliced RNAs to transport before and after splicing of the first intron, respectively. The effect of Rev on the cytoplasmic level of singly spliced RNAs has been addressed only indirectly by using constructs which express *env* RNA as an unspliced RNA (13, 21). An alternate hypothesis is that Rev might interfere with the splicing machinery by allowing the unspliced and singly spliced RNAs, both containing the RRE, to bypass complete splicing to doubly spliced *tat-rev*, *nef*, and other aberrant RNAs. This would result in an accumulation of unspliced and singly spliced RNAs in the cytoplasm only in the presence of Rev. This theory would explain why the singly spliced RNAs are not further spliced to completion, as occurs with most cellular RNAs. Further regulation of the ratio of unspliced to singly spliced RNAs might be accomplished by *cis*-acting sequences such as those found in Rous sarcoma virus (2). Although the effect of Rev has been demonstrated with vectors containing no known splice donors, implying that splicing is not involved (9, 20, 21, 27), mutations of the 5' splice donor can frequently lead to activation of cryptic splice donors (32, 33). Additionally, the presence of a 5' splice donor does not appear to be necessary for spliceosome formation (4, 10, 15). Rev may prevent a permanent association of RRE-containing RNAs with the spliceosome, which might indirectly block the transport of these RNAs to the cytoplasm. Further splicing would remove the RRE, releasing the doubly spliced RNA and allowing its transport to the cytoplasm.

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