Rev and the Fate of Pre-mRNA in the Nucleus: Implications for the Regulation of RNA Processing in Eukaryotes

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Although a great deal is known about the regulation of gene expression in terms of transcription, relatively little is known about the modulation of pre-mRNA processing. In this study, we exploited a genetically regulated system, human immunodeficiency virus type 1 (HIV-1) and its *trans*-activator Rev, to examine events that occur between the synthesis of pre-mRNA in the nucleus and the translation of mRNA in the cytoplasm. Unlike the majority of eukaryotic pre-mRNAs whose introns are efficiently recognized and spliced prior to nucleocytoplasmic transport, HIV-1 mRNAs containing functional introns must be exported to the cytoplasm for the expression of many viral proteins. Using human T cells containing stably integrated proviruses, we demonstrate that such incompletely spliced viral mRNAs are exported to the cytoplasm only in the presence of the Rev *trans*-activator. In the absence of Rev, these intron-containing RNAs are sequestered in the T-cell nucleus and either spliced or, more commonly, degraded. Because Rev does not inhibit the expression of fully spliced viral mRNA species in T cells, we propose that Rev, rather than inhibiting viral pre-mRNA splicing, is acting here both to prevent the nuclear degradation of HIV-1 pre-mRNAs and to induce their translocation to the cytoplasm. Taken together, these findings indicate that the cellular factors responsible for the nuclear retention of unspliced pre-mRNAs, although most probably splicing factors, do not invariably commit these RNAs to productive splicing and can, instead, program such transcripts for degradation.

Human immunodeficiency virus type 1 (HIV-1) is generally regarded as the prototype of the lentivirus subfamily of pathogenic retroviruses. All lentiviruses possess complex genetic organizations that feature not only the three structural genes (*gag*, *pol*, and *env*) common to all replication-competent retroviruses but also several additional regulatory and auxiliary genes not found in the simpler avian and murine retroviruses (7, 44). HIV-1, like the other lentiviruses, employs an intricate scheme of alternative splicing of its primary (~9-kb) transcript to generate the array of singly spliced (~4-kb) mRNAs and fully, or multiply, spliced (~2-kb) mRNAs necessary for the synthesis of all viral proteins (14, 42, 49, 51). The 9-kb transcript encodes Gag and Pol, the 4-kb mRNAs encode Env as well as the Vif, Vpr, and Vpu gene products (17, 52), and the 2-kb mRNAs encode the regulatory proteins Tat, Nef, and Rev (13, 14, 27).

Following the onset of viral transcription, the proteins encoded by the fully spliced 2-kb transcripts are the first to be expressed (27). Tat is a potent transcriptional *trans*-activator essential for the efficient expression of all viral genes, whereas Nef has been shown to be dispensable for viral replication in culture (21, 28). Rev, meanwhile, is absolutely essential for viral replication and acts selectively to induce the expression of viral proteins encoded by the intron containing 9- and 4-kb transcripts (11, 13, 15, 20, 31, 39, 56). However, because the concentration of Rev must reach a critical point before full activity is displayed (46), expression of these incompletely spliced transcripts is delayed relative to that of the 2-kb transcripts. Notably, this markedly temporal pattern of viral mRNA expression has

been observed subsequent to either virus infection (27) or activation of a latent provirus (47). Although Rev was first identified in HIV-1, analogous gene products have now been identified in other lentiviruses, including HIV-2, simian immunodeficiency virus (36), visna virus (59), equine infectious anemia virus (57), and feline immunodeficiency virus (29).

The HIV-1 Rev *trans*-activator is a 116-amino-acid phosphoprotein localized to the nucleus and nucleoli of expressing cells (4, 8, 15, 22). Rev binds specifically and with high affinity to an elaborate stem-loop structure, the Rev response element (RRE), present in all intron-containing viral transcripts (10, 19, 39, 50, 62). The precise mechanism by which the Rev-RRE interaction culminates in the expression of viral proteins from these pre-mRNAs remains controversial. Nevertheless, a clear distinction can be made between models proposing that Rev induces the translocation of intron-containing mRNAs from the nucleus to the cytoplasm (3, 11, 15, 20, 25, 39) and alternative models suggesting a critical cytoplasmic role in the activation of viral mRNA translation (1, 9). The essential difference between these two categories of model is that, in the absence of Rev, the former predicts that unspliced viral transcripts are confined to the nucleus, whereas the latter requires that these mRNAs are constitutively expressed in the cytoplasm but are not translated. Although data in favor of both models have been presented, they were invariably derived following the transient transfection of either human or nonhuman cell lines with, generally, subgenomic HIV-1 expression plasmids.

Here we describe a reexamination of Rev function by using an assay system closely akin to the natural environment of HIV-1. Because the primary viral reservoir for HIV-1 in the peripheral bloodstream is the CD4⁺ lymphocyte (12), we have used the human CD4⁺ lymphoblastoid

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line CEM-SS for the production of cell lines stably transduced with HIV-1 proviruses that do or do not express a functional *rev* gene. Analysis of these human T-cell lines demonstrates that the presence of intron-containing HIV-1 transcripts in the cytoplasm, and hence structural protein synthesis, is critically dependent on the viral Rev *trans*-activator. In addition, we show these pre-mRNAs to be highly unstable in the absence of Rev in the nuclei of T cells yet remarkably stable when Rev is present. These findings are in disagreement with data suggesting a cytoplasmic role for Rev in the activation of viral mRNA translation and, instead, strongly support the hypothesis that Rev acts in the nuclei of human T lymphocytes to induce the stabilization and nucleocytoplasmic export of RRE-containing HIV-1 transcripts.

MATERIALS AND METHODS

Construction of molecular clones. The pIIIB expression vector contains a full-length replication-competent HIV-1 provirus in which the *vpr* gene of the HXB-3 molecular clone has been repaired (17, 24). The derivative of pIIIB that confers resistance to G418, pIIIB/N, is analogous to vectors previously described by us and others (37, 58, 60) and was constructed as follows. Initially, an *Nco*I site was introduced at the initiation codon of the *nef* gene in pIIIB by targeted mutagenesis (AGATGG→ACCATGG). Next, a 799-bp *Bsp*HI-*Asp* 718 fragment encoding the neomycin phosphotransferase (*neo*) gene was prepared by digestion of the products of a polymerase chain reaction (PCR) and inserted between the introduced *Nco*I site and the *Asp* 718 site located 70 bp upstream of the 5' boundary of the 3' long terminal repeat U3 region. Finally, to partially compensate for the increased length of the provirus, a 300-bp *Eco*RV fragment known to be dispensable for efficient viral replication in T cells (34) was deleted from the U3 region of the 3' long terminal repeat. The net increase in genome length is therefore only 281 nucleotides. In pIIIB/N, because *neo* has replaced the *nef* gene, it is, like Nef, expressed from a fully spliced 2-kb viral transcript independent of Rev. The Rev-defective (Δ Rev) derivatives of the pIIIB/N expression vector, pIIIB/ Δ 2Rev/N and pIIIB/ Δ 12Rev/N, were generated by site-directed mutagenesis utilizing the PCR. pIIIB/ Δ 2Rev/N has a nonsense mutation in the codon for serine at position 54 in the second coding exon of Rev; this TCG→TAG change is silent for the overlapping *env* gene. pIIIB/ Δ 12Rev/N has a second nonsense mutation in the codon for lysine at position 14 in the first coding exon of Rev in addition to the mutation in the second exon described above. This AAG→TAG change is a missense mutation for the overlapping *tat* gene, as it changes the glutamine at position 60 to leucine; however, this failed to perturb Tat function in transfected cells (data not shown). The expression vector for the *rex* gene of human T-cell leukemia virus type I (HTLV-I), pcREX, has been described previously (48).

Cell culture, DNA transfection, construction of G418-resistant cell lines, and virus infection. The monkey cell line COS was maintained in Iscove's modified Dulbecco's medium and transfected with DEAE-dextran and chloroquine as previously described (6). The human CD4⁺ lymphoblastoid cell line CEM-SS (43) was maintained at 0.5×10^6 to 2.5×10^6 cells per ml in RPMI 1640 medium with 10% fetal bovine serum and 50 μ g of gentamicin sulfate per ml.

CEM-SS cells were rendered G418 resistant by transduction with the *neo*-modified viruses as follows. Dishes (35 mm) of COS were transfected with either 660 ng of the

replication-competent pIIIB/N vector or 660 ng of a Δ Rev vector (pIIIB/ Δ 2Rev/N or pIIIB/ Δ 12Rev/N) and 330 ng of the *trans*-complementing expression vector pcREX (refer to text for details). At 48 h, the presence of virus in the culture supernatant was confirmed by using an enzyme-linked immunosorbent assay (ELISA) system for soluble p24^{gag} expression (Dupont, NEN, Inc., Billerica, Mass.) and the culture medium was exchanged for 2 ml of RPMI 1640. On the next day, the culture volume was increased to 4 ml, and 2×10^6 CEM-SS cells were added for cocultivation. After 48 h of cocultivation, the culture medium, containing predominantly CEM-SS cells, was removed. The cells were harvested and maintained in fresh RPMI 1640 for a further 48 h. At the end of this period (7 days posttransfection), the cells were transferred into RPMI 1640 supplemented with 1 mg of G418 (GIBCO-BRL Inc., Gaithersburg, Md.) per ml at 2×10^6 cells per ml. It took approximately 2 weeks for transduced G418-resistant cultures to emerge when the Δ Rev viruses were used and approximately 4 weeks when the replication-competent virus was used. We attribute the delay observed with the IIIB/N virus to the cytotoxic nature of HIV-1 replication in CEM-SS cells. The mechanism by which survivor cells refractory to these cytotoxic effects emerge is unknown at this time. Cell lines derived with a particular virus are named in accordance with its expression vector and are prefixed with C; hence, the cell line harboring proviruses with a nonsense mutation in the second exon of the *rev* gene is termed C/IIIB/ Δ 2Rev/N.

Acute infections of CEM-SS cells with the IIIB virus were initiated by adding 0.5 ml of filtered high-titer virus stock to 10^6 cells in 1 ml of RPMI 1640. The cultures were expanded gradually and maintained at 1.0×10^6 to 2.5×10^6 cells per ml. Typically, the cells were used for analysis 8 days after infection when the level of soluble p24 in the medium was on the order of 200 ng/ml.

Immunoprecipitation. CEM-SS cells (parental, acutely infected, or stably transduced; 6×10^6) were starved in RPMI 1640 lacking methionine and cysteine (ICN Biomedicals, Costa Mesa, Calif.) for 1 h prior to being metabolically labeled for 16 h with [³⁵S]methionine and [³⁵S]cysteine (Tran³⁵S-label; ICN Biomedicals). The cells were harvested, washed once with phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay buffer as previously described (38). Labeled HIV-1 proteins were immunoprecipitated with either a 1:200 dilution of a rabbit anti-p24^{gag} serum (kindly provided by Carol Carter and Hans-Georg Kräusslich) or a 1:1,000 dilution of a sheep anti-gp120^{env} serum (22a) followed by addition of protein A- or protein G-agarose (GIBCO-BRL Inc.), respectively. Precipitated proteins were resolved on sodium dodecyl sulfate-polyacrylamide gels and visualized by autoradiography.

RNA isolation and S1 nuclease protection assays. Total, nuclear, and cytoplasmic RNAs were isolated either from 100-mm dishes of COS cells 48 to 60 h after transfection or from 12×10^6 CEM-SS cells (acutely infected or stably transduced) as previously described (39) except that the fractionation buffer used for the CEM-SS cells comprised 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.6), 50 mM KCl, 5 mM MgCl₂, and 0.05% Nonidet P-40. In the experiments that addressed mRNA stability, all new RNA polymerase II-dependent transcription was inhibited by adding either actinomycin D (Boehringer Mannheim Corp., Indianapolis, Ind.) to a concentration of 5 μ g/ml or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (Calbiochem Corp., La Jolla, Calif.) to 200

μ M to the medium; RNAs were then isolated from fractionated cells 1, 3, or 6 h later.

S1 nuclease protection assays were performed as previously described (38) with either of two HIV-1 specific end-labeled DNA probes and, in some experiments, with a second end-labeled probe specific for endogenous *c-myc* transcripts. The probe used to distinguish between viral mRNAs that are unspliced (9 kb) and those that are either singly or fully spliced (4- and 2-kb transcripts, respectively) spans the 5' splice site upstream of the *gag* gene. This 462-nucleotide probe was end labeled with Klenow enzyme at a *Hind*III site and extends beyond the above-mentioned 5' splice site at nucleotide 211 to an introduced *Sph*I site at nucleotide 260. A tag of non-HIV-1 sequences at the *Sph*I site permits the resolution of the input probe from the rescued fragments. A previously described 798-nucleotide *tat* gene probe (38) that spans the 5' splice site at the 3' end of the first coding exon of *tat* was used to discriminate between fully spliced mRNA (202-nucleotide rescued fragment) and unspliced and singly spliced mRNA (506-nucleotide rescued fragment). A 548-nucleotide probe derived from a human *c-myc* cDNA clone was labeled with Klenow enzyme at a *Bst*YI site within the second exon and is rescued as a protected fragment of 319 nucleotides by fully spliced endogenous *c-myc* mRNA. Protected fragments were resolved on 5% denaturing polyacrylamide gels and visualized by autoradiography. In some experiments, the relative levels of different transcripts were determined by densitometry with an LKB soft laser scanner.

RESULTS

The purpose of this study was to examine Rev function in human CD4⁺ lymphoid cells by using proviruses established via authentic retroviral infection, reverse transcription, and integration. To do this we constructed Rev-containing and Rev-deficient (Δ Rev) HIV-1 proviral expression vectors in which *neo*, the bacterial gene that confers resistance to the antibiotic G418, had replaced the viral *nef* gene. It had previously been established that the ability of HIV-1 to replicate efficiently in culture was not compromised by the loss of *nef* either by mutation or by substitution with a nonviral gene (21, 28, 37, 58, 60). Because Nef is expressed from a subset of the 2-kb viral mRNAs (49, 51), it was anticipated that the expression of *neo*, and therefore the acquisition of G418 resistance, would not require functional expression of the Rev protein. The experimental scheme for the generation of the cell lines is represented in Fig. 1. Infectious virions were initially generated by transfecting COS cells with *neo*-containing proviral expression vectors. In the case of pIIIB/N, transfection of this plasmid alone yielded infectious virus. However, because the Δ Rev vectors are unable to express structural proteins, cotransfection of these plasmids with a *trans*-complementing vector was necessary for the recovery of virus. In an attempt to minimize the likelihood of regeneration, by recombination, of proviruses encoding functional Rev, we elected to cotransfect these vectors with an expression vector for the functionally analogous Rex protein of human HTLV-I, pcREX (48). Rex has been shown to functionally substitute for Rev and, because of the minimal sequence similarity between the two genes, was the *trans*-activator of choice for these experiments. CEM-SS cells (43) were infected by cocultivation with the transfected COS cultures, and stable cell lines harboring the modified proviruses were selected in G418. The cell line termed C/IIIB/N contains the Rev-producing,

FIG. 1. The derivation of CEM-SS cell lines harboring Rev-deficient HIV-1 proviruses. The specific details of the approach are described in the text. The locations of the *gag*, *pol*, *env*, *rev*, and *neo* (replaces *nef*) genes within the provirus are indicated.

replication-competent proviruses whereas the C/IIIB/ Δ 2Rev/N and C/IIIB/ Δ 12Rev/N lines contain two different mutant proviruses lacking functional *rev* genes.

Rev is essential for HIV-1 structural protein synthesis. The HIV-1 Rev protein has been shown by many laboratories to be absolutely required for expression of viral structural proteins, including Gag and Env (11, 13, 15, 20, 31, 39, 56). The patterns of viral protein synthesis in the three cell lines carrying modified proviruses as well as in the parental CEM-SS cells (negative control) and in CEM-SS cells acutely infected with the IIIB virus (positive control) were therefore analyzed by immunoprecipitation with sera raised against either p24^{gag} or gp120^{env} (Fig. 2). Both the p55^{gag} polyprotein precursor and one of its processed products, p24^{gag}, were readily detected in the acutely infected cells (lane 2) but not the parental T cells (lane 1). As expected, Gag was also expressed at high levels in the Rev-expressing C/IIIB/N cells (lane 3) but was undetectable in either the C/IIIB/ Δ 2Rev/N or the C/IIIB/ Δ 12Rev/N cell line (lanes 4 and 5). Not surprisingly, the gp160^{env} glycoprotein precursor and its mature derivative gp120^{env} displayed the same pattern of expression as Gag. Hence, Env glycoproteins were observed in IIIB-infected cells (lane 7) and the Rev-producing transduced cell line (lane 8) but not in either of the Δ Rev cell lines (lanes 9 and 10). The absence of detectable Gag synthesis in either of the Δ Rev cultures was further confirmed by performing ELISAs for soluble p24^{gag}. Repeated analyses of culture supernatants over a period of 4 months showed the level of p24 always to be less than 3 pg/ml, the detection limit for the assay. In contrast, the C/IIIB/N

FIG. 2. Immunoprecipitation analyses of HIV-1 Gag and Env proteins. Metabolically labeled CEM-SS (lanes 1 and 6), IIIB-infected CEM-SS (lanes 2 and 7), C/IIIB/N (lanes 3 and 8), C/IIIB/ Δ 2Rev/N (lanes 4 and 9), and C/IIIB/ Δ 12Rev/N (lanes 5 and 10) cells were lysed in radioimmunoprecipitation assay buffer and subjected to immunoprecipitation with either a rabbit anti-p24^{gag} serum (lanes 1 to 5) or a sheep anti-gp120^{env} serum (lanes 6 to 10). Precipitated proteins were resolved on sodium dodecyl sulfate–10% (anti-Gag) or –7.5% (anti-Env) polyacrylamide gels and visualized by autoradiography. Also indicated is the presence (+) or absence (–) of Rev and whether the culture is acutely infected (AC) or stably transduced (ST).

culture maintained a level of soluble p24 between 50 and 200 ng/ml (data not shown). The induction of p24^{gag} expression following introduction of a *rev* expression vector into each Δ Rev line confirmed that the defect in these integrated proviruses was restricted solely to their *rev* genes (data not shown).

Rev is required for the cytoplasmic expression of intron-containing HIV-1 mRNAs. Previous analyses with a variety

of transiently transfected primate and nonprimate cells have demonstrated that Rev induces the redistribution of viral transcripts within a cell such that incompletely spliced HIV-1 mRNAs, which are otherwise confined to the nucleus, are translocated to the cytoplasm (3, 5, 11, 15, 20, 23, 25, 39, 40). Having established that the stably transduced human T cells faithfully reproduced, at the level of Gag and Env protein synthesis, the phenotype expected for proviruses either expressing or not expressing the Rev *trans*-activator, we wanted to determine how the various classes of viral transcript were distributed within these cells.

Total, nuclear, and cytoplasmic RNAs isolated from each transduced line were therefore analyzed by quantitative S1 nuclease protection. We chose to employ a double-probe strategy that combined one of two different HIV-1-specific DNA probes end labeled upstream of 5' splice sites with a second probe that detected endogenous *c-myc* mRNA and served as an internal control for both RNA loading and specificity. This approach makes it possible to draw both qualitative and quantitative conclusions regarding the expression of viral transcripts in these T-cell lines. The first probe spanned the 5' splice site of the nontranslated leader sequence that is 5' to *gag* (Fig. 3A), whereas the second spanned the 5' splice site at the 3' terminus of the first coding exon of *tat* (Fig. 3B). Because the splice donor in the leader specific probe is utilized during the processing of all singly and fully spliced transcripts (51), this probe is rescued as a 211-nucleotide fragment by the 4- and 2-kb mRNAs and as a 260-nucleotide fragment by the 9-kb mRNA. By using this probe, two predominant fragments with the expected mobilities were rescued by total RNA isolated from each transduced line (Fig. 3A, lanes 4 to 6) and from a culture acutely infected with the IIIB virus (Fig. 3A, lane 3). This demonstrated, firstly, that the 9-kb as well as the 4- and 2-kb viral transcripts were expressed at readily detectable levels in each culture and, secondly, that the transcripts present in the transduced lines were an accurate representation of

FIG. 3. S1 nuclease protection analyses of HIV-1 transcripts in fractionated human T cells. (A) Total (T), nuclear (N), and cytoplasmic (C) RNAs were isolated from IIIB acutely infected (AC) CEM-SS (lane 3), C/IIIB/N (lanes 4, 7, 10, and 13), C/IIIB/ Δ 2Rev/N (lanes 5, 8, 11, and 14), and C/IIIB/ Δ 12Rev/N (lanes 6, 9, 12, and 15) cells as indicated. The presence (+) or absence (–) of Rev in the cultures is also shown. The HIV-1-specific input DNA probe, I(HIV), was used for lanes 1 and 3 to 15; it spans the splice donor 5' to the *gag* gene and is therefore rescued as a 260-nucleotide fragment by the unspliced (9-kb) mRNAs and a 211-nucleotide fragment by the singly (4-kb) and fully (2-kb) spliced mRNAs, as indicated. The *c-myc*-specific input DNA, I(*myc*), was used for lanes 2 to 12 and is rescued as a 319-nucleotide fragment by the endogenous *c-myc* mRNAs. The RNA used for lanes 1 and 2 was yeast transfer RNA. Cytoplasmic samples from a second experiment were analyzed with the HIV probe alone, and the gel was subjected to prolonged autoradiographic exposure (lanes 13 to 15). (B) The same RNA samples used in panel A (lanes 1 to 12) were analyzed here with the *c-myc* probe in combination with an end-labeled probe spanning the 5' splice site which is at the 3' termini of the first coding exons of *tat* and *rev* and, therefore, 5' to the *env* gene. This probe is rescued as a 506-nucleotide fragment by the unspliced (9-kb) and singly spliced (4-kb) mRNAs and a 202-nucleotide fragment by the fully spliced (2-kb) mRNAs.

TABLE 1. Quantitation of HIV-1 transcripts in fractionated cells

Cell type	Relative expression (%) of 9-kb viral mRNA ^a		
	Total	Nucleus	Cytoplasm
Stably transduced T cells			
C/IIIB/N	42	45	42
C/IIIB/Δ2Rev/N	9	17	<1
C/IIIB/Δ12Rev/N	5	19	<1
Transiently transfected COS cells			
pIIIB/N	40	51	32
pIIIB/Δ2Rev/N	19	32	<1

^a The relative level of the 9-kb transcript in each sample is given as a percentage of the combined level of all viral mRNAs (9-, 4-, and 2-kb species) in that sample. Quantitation was determined by densitometric scanning of autoradiographs either similar to the one shown in Fig. 3A (except that the *c-myc* probe was omitted) or of Fig. 4 itself.

those expressed during a normal HIV-1 infection. Closer inspection revealed, however, that the relative proportion of viral transcripts composing the 9-kb species was sixfold higher in the transduced culture synthesizing Rev (Fig. 3A, lane 4) than in either of the ΔRev cultures (Fig. 3A, lanes 5 and 6) (Table 1). Both classes (9 kb and 4 kb plus 2 kb) of transcript were also detected in the nuclear fraction of each cell line (lanes 7 to 9), though the relative levels of the 9-kb mRNA in the ΔRev lines were again lower (approximately threefold) than in the Rev-expressing culture. In contrast to these quantitative differences in nuclear mRNA expression, a distinct qualitative difference in the pattern of viral mRNA expression was observed in the RNA samples obtained from the cytoplasmic fractions. Whereas both transcript classes were present in the cytoplasm of cells synthesizing Rev (Fig. 3A, lanes 10 and 13), no 9-kb mRNA could be detected in the cytoplasmic RNA from either of the ΔRev lines (Fig. 3A, lanes 11 and 12) even after prolonged autoradiographic exposure (Fig. 3A, lanes 14 and 15). The minimal variations in the level of the *c-myc* mRNA that were detected among the total, nuclear, or cytoplasmic samples indicates that equivalent quantities of RNA had been used in each set of three analyses and that the effects of Rev were specific for the viral transcripts (Fig. 3A, lanes 4 to 6, 7 to 9, and 10 to 12, respectively).

We next analyzed identical quantities of the same RNA samples used in Fig. 3A (lanes 3 to 12) with the second end-labeled HIV-1 specific probe described earlier in conjunction with the *c-myc* probe (Fig. 3B). Because the *tat*-specific probe spans a 5' splice site utilized during the processing of singly spliced transcripts to fully spliced transcripts, it is rescued as a 506-nucleotide fragment by 9- and 4-kb mRNAs and as a 202-nucleotide fragment by 2-kb mRNAs. Probe fragments with the predicted mobilities were rescued by total RNA samples derived from acutely infected cells (Fig. 3B, lane 3) and each of the transduced lines (Fig. 3B, lanes 4 to 6). As with the leader-specific probe, expression of the transcripts that rescue the more slowly migrating band was much lower in the ΔRev cultures (Fig. 3B, lanes 5 and 6) than in the Rev-expressing line (Fig. 3B, lane 4). Both classes of transcript were also present in the nuclear fractions of each transduced line (Fig. 3B, lanes 7 to 9), and predictably, expression of the 9- plus 4-kb class was much higher in the culture synthesizing Rev. Consistent with our earlier findings, the intron-containing mRNAs were expressed in the cytoplasm of Rev-expressing cells at a level

FIG. 4. S1 nuclease protection analysis of HIV-1 transcripts in fractionated COS cells. RNAs were isolated from 100-mm cultures of COS cells 48 h after transfection with either 5 μg of the replication-competent virus expression vector pIIIB/N (lanes 2, 4, and 6) or 5 μg of the Rev-defective vector pIIIB/Δ2Rev/N (lanes 3, 5, and 7). At this time, the levels of soluble p24^{gag} in the culture supernatants were shown by ELISA to be ~900 pg/ml (pIIIB/N) and <3 pg/ml (pIIIB/Δ2Rev/N). The same DNA probe was used for analysis as in Fig. 3A; abbreviations are as in the legend to Fig. 3. The RNA used for lane 1 was isolated from mock-transfected cells.

that was indistinguishable from that observed in the nuclei (Fig. 3B, compare lanes 7 and 10), whereas their expression was virtually undetectable in the cytoplasmic fractions obtained from the ΔRev cultures (Fig. 3B, lanes 11 and 12). In striking contrast to the dramatic consequences of Rev expression upon the 9- and 4-kb viral mRNAs, Rev had no discernible quantitative effect upon expression of the completely spliced 2-kb HIV transcripts in either the nuclei or the cytoplasm of these infected T cells.

The data in Fig. 3A and B showing the nuclear confinement of intron-containing HIV-1 transcripts in the absence of Rev are in complete agreement with several previously published studies with transiently transfected proviral and subgenomic expression vectors (3, 5, 11, 15, 20, 25, 39, 40). Nevertheless, we wished to compare the patterns of viral mRNA expression obtained following transient transfection of the fibroblastic cell line COS with the full-length HIV-1 proviral expression vectors used here for construction of the transduced T-cell lines with those described above for the T-cell lines themselves. S1 nuclease protection assays were therefore performed with the leader-specific end-labeled probe and RNA samples isolated from unfractionated and fractionated COS cells 48 h after transfection with either pIIIB/N or pIIIB/Δ2Rev/N (Fig. 4 and Table 1). As with the T-cell lines, both probe fragments were rescued by total and nuclear RNA isolated from Rev-expressing and ΔRev cultures (lanes 2 to 5). In further agreement with the findings in T cells, expression of the 9-kb unspliced mRNA in the cytoplasm was largely restricted to cells producing Rev (lanes 6 and 7). Two quantitative differences between these data and Fig. 3A were, nevertheless, apparent. First, in Rev-expressing COS cells, the appearance of the 9-kb transcript in the cytoplasm is coincident with decreased expression, relative to cultures lacking Rev, of the 4- plus 2-kb class of mRNAs (compare lanes 6 and 7). Second, the relative proportion of viral transcripts consisting of the 9-kb species was approximately twofold higher in the nuclear (and total) RNA samples derived from COS cells lacking Rev than in the corresponding samples from T cells (Table 1; compare Fig. 4, lane 5, with Fig. 3A, lanes 8 and 9). In summary, however, it is clear that the Rev protein is required for the cytoplasmic expression of unspliced HIV-1

FIG. 5. Stability of HIV-1 transcripts in the nucleus. (A) Nuclear RNAs were isolated from C/IIIB/N (lanes 1 to 4, 9, and 10) or C/IIIB/Δ2Rev/N (lanes 5 to 8, 11, and 12) cultures either before (lanes 1, 5, 9, and 11) or after exposure to 5 μ g of actinomycin D per ml or 200 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (lanes 2 to 4, 6 to 8, 10 and 12) for 1, 3, or 6 h as indicated. The probe strategy used was the same as for Fig. 3A (lanes 1 to 12). (B) Nuclear RNAs were isolated from 100-mm cultures of COS cells transfected as for Fig. 4, treated (or not) with actinomycin D as in panel A and analyzed as for Fig. 4. The RNA used for lane 0 was isolated from mock-transfected cells.

transcripts in both infected human T cells and transfected COS cells.

Rev increases the nuclear stability of intron-containing HIV-1 mRNAs. As noted above, cultures lacking Rev displayed clear cell-line-dependent differences in the steady-state level of expression of the 9-kb viral transcript in the nucleus. To address the possibility that cell-type-specific variations in the stability of this mRNA species could contribute to these differences, we used an inhibitor of RNA polymerase II-dependent transcription, actinomycin D (45), to block the synthesis of new viral transcripts. RNA samples were derived from isolated T-cell nuclei at various time-points after the addition of actinomycin D to the lines C/IIIB/N and C/IIIB/Δ2Rev/N (Fig. 5A, lanes 1 to 8) and analyzed by S1 nuclease protection with the HIV-1 leader-specific and *c-myc* end-labeled probes described above. Nuclear RNA samples isolated from COS cells were subjected to an identical actinomycin D time course, following transfection with either pIIIB/N or pIIIB/Δ2Rev/N, and similarly analyzed except that only the leader-specific probe was used (Fig. 5B). In the presence of Rev, the patterns of viral mRNA expression in both the stable T-cell lines and the transient transfectants were indistinguishable from each other prior to the addition of actinomycin D and for the duration of the experiment (Fig. 5, lanes 1 to 4). We therefore deduce that the 9-kb and the 4- plus 2-kb classes of viral transcript are both relatively stable in the nuclei of Rev-expressing T cells and COS cells. In contrast, the addition of actinomycin D had quite different consequences in cultures not expressing Rev (Fig. 5, lanes 5 to 8). Specifically, the 9-kb viral mRNA was barely detectable 1 h after, and undetectable 6 h after, the cessation of viral transcription in stably transduced T-cell cultures (Fig. 5A, lanes 6 and 8), whereas it remained readily detectable, albeit at a modestly reduced level, even after 6 h of drug treatment in transfected COS cells (Fig. 5B, compare lanes 4 and 8). The rapid turnover of the *c-myc* transcripts both in the presence and in the absence of Rev is consistent with the established half-life of ~21 min for this mRNA species (61) and demonstrates that the observed stabilization of the 9-kb viral mRNA is a specific rather than a general consequence

of Rev expression. Further analysis of the T-cell nuclear RNA samples with the *tat*-specific end-labeled probe revealed that the incrementally decreasing rescue of the 211-nucleotide fragment from the C/IIIB/Δ2Rev/N cultures was primarily, though not entirely, caused by the reduced expression of the partially spliced 4-kb, rather than the fully spliced 2-kb, mRNAs (data not shown). We also noted that the stabilities of the different viral transcripts in the cytoplasm of the T-cell cultures treated with actinomycin D were indistinguishable from those of their nuclear counterparts (data not shown). To confirm that the above findings could be attributed to the inhibition of RNA polymerase II-dependent transcription rather than the inclusion of actinomycin D in the cultures, the time course experiment using the C/IIIB/N and C/IIIB/Δ2Rev/N T-cell lines was repeated with a second inhibitor of transcription, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (54), that is both structurally and mechanistically distinct (Fig. 5A, lanes 9 to 12). As with actinomycin D addition, both the 9-kb viral and *c-myc* transcripts were virtually absent from the ΔRev culture 3 h after the inhibition of transcription (lane 12), whereas only the *c-myc* transcript had been turned over in the Rev-expressing culture (lane 10). Taken together, these data demonstrate that Rev can markedly increase the stability, or half-life, of intron-containing HIV-1 transcripts in the nucleus. This effect is manifested far more dramatically in the stably transfected T cells than in the transiently transfected COS cells and is likely to be responsible for the low steady-state levels of the 9- and 4-kb viral mRNAs observed in the nuclei of T cells harboring *rev*-defective integrated proviruses.

DISCUSSION

The precursor of mRNA (pre-mRNA) is transcribed by RNA polymerase II and extensively modified by capping, splicing, methylation, and polyadenylation in the nucleus before being exported to the cytoplasm. Following synthesis, the introns of pre-mRNAs are recognized by nuclear factors that confine them to the nucleus and assemble them into spliceosomes (2, 16, 18). By using yeast cells as the

experimental system, both *cis*-acting sequences and *trans*-acting factors that impart nuclear retention upon pre-mRNA have been genetically identified (32). Although all of these elements are also important for splicing, it remains unclear whether intron recognition and nuclear sequestration necessarily guarantee that a pre-mRNA be incorporated into a complex committed to productive splicing (the commitment complex) (26, 41, 55) or whether, instead, an intron can be excluded from the cytoplasm by alternative mechanisms. For the majority of cellular pre-mRNAs, however, the removal of introns by splicing is an efficient process leading to nucleocytoplasmic transport and translation. For the purpose of this discussion, we will use the term commitment complex to describe a pre-mRNA-containing complex that develops into a functional spliceosome.

Retroviruses use alternative splicing of their initial full-length (or genomic) transcript to place their open reading frames in positions where they can be efficiently translated. The pattern of splicing displayed by HIV-1 is particularly complex, as there are nine distinct gene products to be expressed (7, 44). As a result, many of the mature transcripts have retained intronic sequences that would ordinarily preclude cytoplasmic expression and translation. The function of one of the viral regulatory proteins, Rev, is to induce the expression of proteins, including Gag and Env, from these incompletely spliced mRNAs (13, 56). As noted earlier, the purpose of this study was to determine, for the first time, precisely how Rev influences unspliced HIV-1 mRNA expression in human CD4⁺ T lymphocytes containing stably integrated proviruses.

In this paper we demonstrate that expression of unspliced 9-kb, or genomic, HIV-1 transcripts is restricted entirely to the nuclei of human T cells in the absence of the *rev* gene product (Fig. 3A). Although Rev is required for the detectable expression of proteins from the singly spliced 4-kb mRNAs (Fig. 2), a very low level of these transcripts was observed in the cytoplasm of cells not expressing Rev (Fig. 3B). It is unclear whether this low level represents authentic cytoplasmic 4-kb mRNA expression or instead reflects a low level of RNA leakage from the nucleus during the fractionation of T cells. In the presence of Rev, high levels of both the 9- and 4-kb mRNAs are expressed in the cytoplasm, viral structural protein synthesis is activated, and consequently, virus production is induced. These findings in transduced T lymphocytes are in excellent agreement with what we observe here in transfected COS cells (Fig. 4) and that we and others have previously observed in transfected human and nonhuman cells (3, 5, 11, 15, 20, 23, 25, 39, 40). We conclude, therefore, that the HIV-1 Rev protein is essential for the export of unspliced HIV-1 transcripts from the nucleus to the cytoplasm and that, once transported, these transcripts become available for translation into viral proteins. This hypothesis is in conflict with observations made in other laboratories that are more consistent with Rev's induction of nucleocytoplasmic export being secondary to its translational activation of intron-containing mRNAs that are constitutively expressed in the cytoplasm (1, 9). These data have been derived primarily by quantitative PCR and we note that, in one case (1), the investigators used a subcellular fractionation procedure that also resulted in the detection of cellular intron-containing mRNAs in the cytoplasmic samples. This observation is inconsistent with the generally accepted view that such pre-mRNAs are prevented from entering the cytoplasm (3, 32) and suggests, instead, that the cytoplasmic fractions used for these assays might have contained material originating from the nucleus.

T Lymphocyte

COS Cell

FIG. 6. Summary of the effects of Rev in human T lymphocytes and COS cells.

Having established that Rev is essential for the expression of intron-containing transcripts in the cytoplasm of both T lymphocytes and COS cells, we then performed experiments with inhibitors of RNA polymerase II to determine whether differences in the half-lives of these transcripts in the absence of Rev could account for their increased levels of expression in COS cell nuclei relative to that in T-cell nuclei (Table 1). Our data demonstrate that the unspliced 9-kb viral mRNA is extremely stable in the nuclei of infected T cells expressing Rev but markedly unstable in T cells lacking Rev (Fig. 5A). These findings are consistent with earlier reports in which Rev was shown to enhance the stability of transcripts containing sequences derived from intronic regions of HIV-1 in transient transfection assays (15, 53). In contrast, we found Rev to have only a minor effect on viral mRNA stability in our transfected COS cells (Fig. 5B). We attribute this far less dramatic, and presumably less significant, effect to the relatively high stability of the 9-kb transcripts in the absence of Rev in this system. To summarize, we propose that the low level of 9- and 4-kb HIV-1 mRNAs observed in the nuclei of T lymphocytes not expressing the *rev* gene product is a direct consequence of viral mRNA instability. This hypothesis predicts, therefore, that mRNA degradation contributes substantially to the fate of viral transcripts in HIV-1-infected cells in which Rev function is either suboptimal or absent (47).

To summarize our findings, we propose the schemes depicted in Fig. 6 to account for the effects of the Rev *trans*-activator on the subcellular distribution and stability of HIV-1 mRNAs in CD4⁺ T lymphocytes and COS cells. In T cells not expressing Rev, the 9- and 4-kb mRNAs are identified as being intron containing, excluded from the cytoplasm, and rapidly degraded. In the presence of Rev, these intron-containing mRNAs are both stabilized and

exported to the cytoplasm. In contrast, the fully spliced 2-kb mRNAs are constitutively exported to the cytoplasm, presumably by the same mechanism responsible for the export of fully processed cellular transcripts. Of particular significance, however, is the observation that the extent of 2-kb transcript expression in the nuclear and cytoplasmic compartments of infected T cells is unaffected by the Rev protein (Fig. 3B). If Rev was directly inhibiting splicing, as has been suggested (30), it would be predicted that expression of the fully spliced transcripts would decrease in the presence of Rev. As there is no evidence of any Rev-induced diminution of 2-kb mRNA expression in either compartment of T cells, we suggest that Rev must influence nuclear processes that occur either prior to, or are distinct from, the commitment of newly transcribed mRNAs to productive splicing. On the basis of the data presented here, we propose that these processes are the stabilization and nucleocytoplasmic transport of 9- and 4-kb HIV-1 mRNAs that are otherwise sequestered in the nucleus by inhibitory sequences located in their unprocessed introns.

In COS cells, as in T cells, the 9- and 4-kb transcripts are relatively stable and exported to the cytoplasm only in the presence of Rev. The key difference between the COS cells and the T cells is that, in the absence of Rev, the intron-containing transcripts are relatively stable in the nuclei of COS cells. Consequently, in cells lacking Rev, these transcripts are eventually spliced, rather than degraded, and as such augment expression of the fully spliced 2-kb viral mRNAs (Fig. 4). However, when Rev is present, a population of viral transcripts is diverted away from splicing and translocated to the cytoplasm as the 9- and 4-kb mRNAs. This model provides a clear explanation for the Rev-mediated down-regulation of 2-kb viral mRNA expression and viral regulatory protein synthesis that have been observed in cells transfected with intron-containing HIV-1 expression vectors (1, 14, 17, 38) and suggests, moreover, that the nuclear degradation of HIV-1 pre-mRNAs not committed to productive splicing is far more rapid in infected cells than in transfected cells.

Assuming that our observations made in human CD4⁺ T lymphocytes harboring transduced HIV-1 proviruses accurately portray the situation in an infected individual, we propose that the viral Rev protein functions as a potent activator of the nucleocytoplasmic transport of unspliced viral mRNAs and, coincident with this, acts to stabilize these mRNAs in the nuclei of expressing cells. We suggest, however, that these two processes could well be linked and that an early step in mRNA transport might be the incorporation of nascent transcripts into nuclear ribonucleoprotein complexes refractory to RNA degradation. In addition, because HIV-1 pre-mRNA is initially confined to the nucleus and, to a significant degree in T cells, rapidly degraded in the absence of Rev, we further propose that intron recognition need not commit a pre-mRNA to productive splicing. If intron recognition were invariably to result in splicing, then the Rev-mediated translocation of pre-mRNA from the nucleus would, by reducing the availability of splicing substrates in the nucleus, always result in an inhibition of viral mRNA splicing. Because Rev does not induce such an inhibition in infected T cells, we conclude that the unspliced viral mRNA observed in the cytoplasm of Rev-expressing T cells must originate from a nuclear pool of pre-mRNA that is unavailable for splicing and, in the absence of Rev, is instead targeted for rapid degradation. This hypothesis is consistent with models in which Rev function requires the nuclear retention of HIV-1 introns by host cell factors and the

inefficient utilization of HIV-1 splice sites (3) but inconsistent with models in which Rev is proposed to inhibit directly the formation of functional spliceosomes on viral mRNA (30). An excellent candidate for a host factor involved in the retention of HIV-1 pre-mRNA in the nucleus is the U1 small nuclear ribonucleoprotein particle splicing factor which has been shown by Lu et al. (33) to be essential for Rev function in primate cells and by Legrain and Rosbash (32) to be important for the nuclear confinement of unspliced mRNA in yeast cells. In addition to the splice sites themselves, other *cis*-acting HIV-1 sequences that contribute to the nuclear sequestration of mRNA have been identified (35), although the cellular factors that interact with these elements have yet to be identified. The complex regulation of mRNA expression characteristic of HIV-1 and the other lentiviruses continues, therefore, to make these viruses attractive experimental systems for the analysis of pre-mRNA fate and processing in eukaryotes.

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