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# Nuclear Preservation and Cytoplasmic Degradation of Human Immunodeficiency Virus Type 1 Rev Protein

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**Rev, a major regulatory protein of human immunodeficiency virus type 1, has been demonstrated to shuttle between the nucleus and cytoplasm of infected cells. The fate of the Rev protein in living cells was evaluated by pulse-chase experiments using a transient Rev expression system. Sixteen hours after chasing with unlabelled amino acids, 45% of the labelled Rev was still present, which clearly indicates a long half-life of Rev in living cells. A Rev mutant which is deficient in the ability to migrate from the nucleus to the cytoplasm was degraded more slowly than the wild-type Rev protein. As well, another Rev mutant protein, which lacks a functional nucleolar targeting signal (NOS) and is unable to enter the cell nucleus, was rapidly degraded and undetectable 16 h after chasing. Nuclear-nucleolar targeting properties provided by a divergent NOS from a related retrovirus, which was used to substitute for the NOS of Rev, increased the intracellular half-life of this Rev mutant. Moreover, coexpression of an intracellular anti-Rev single-chain antibody (SFv), which has been shown to interfere with the nuclear translocation of Rev, accelerated the degradation of the wild-type Rev protein. Differential degradation of Rev in the nucleus and cytoplasm may play a critical role in determining and maintaining different stages of human immunodeficiency virus type 1 infection, in conjunction with the shuttling properties of the Rev protein.**

The Rev protein of human immunodeficiency virus type 1 (HIV-1) is an indispensable posttranscriptional *trans*-acting regulator of viral gene expression (26). Rev enables unspliced or incompletely spliced HIV-1-specific mRNA containing the Rev responsive element (RRE), which is a secondary structured RNA segment located in the *env* region, to migrate out of the cell nucleus. Thus, HIV-1 structural proteins translated from these mRNAs can eventually be synthesized (17). It has been demonstrated that Rev directly binds to the RRE (8, 14, 28) and is capable of shuttling between the cell nucleus and cytoplasm (9, 19, 24, 27). Recent studies have shown that Rev conveys both RRE-containing unspliced HIV-1-specific RNA and an RRE-containing lariat of an intron after splicing out of the cell nucleus in *Xenopus* oocytes (6). These cumulative findings clearly indicate that Rev plays a positive role in translocation of RRE-containing mRNA to the cytoplasm during protein shuttling, although possible roles for Rev in splicing events and nuclear degradation of viral mRNAs may not be excluded (15).

Rev is a key protein for virus replication, as expression of all the structural proteins is dependent on the expression of the Rev protein (26). Therefore, in different stages of HIV-1 infection, various levels of Rev expression should be critically maintained. Since HIV-1 has only one promoter in the 5′ long terminal repeat for expression of all the viral proteins, regulation of the quantity of Rev protein may occur after the transcriptional level, exploiting various cellular biological mechanisms to alter Rev function. However, little is known about the cellular factors that may affect the fate of Rev after protein synthesis. Particularly, the molecular mechanisms involved with the turning off of Rev function by cellular factors and the degradation of the Rev protein in living cells remain almost unknown, although these may be critical aspects of the regulation of Rev. In this study, we evaluated the fate of the Rev protein, after expression in Cos-1 cells, by metabolic radiolabelling followed by immunoprecipitation analyses. These studies allowed further dissection of the dynamic process of Rev function in living host cells.

**Half-life of intracellular HIV-1 Rev.** A simian virus 40-transformed monkey kidney cell line, Cos-1, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For the expression of HIV-1 Rev protein, a cytomegalovirus promoter-driven Rev expression plasmid, pcREV (17), and a simian virus 40 promoter-driven Rev expression plasmid, pH2rev (10, 12), were utilized. For single labelling, 1 μg of either pcREV or pH2rev was transfected into $2 \times 10^5$ Cos-1 cells, seeded into 35-mm-diameter tissue culture wells. Transfections were carried out by an established DEAE-dextran method, as previously described (3). Fifty hours after transfection, cells were starved in DMEM without cysteine and then the medium was supplemented with 5% dialyzed fetal bovine serum for 1 h, after which cells were metabolically labelled by adding 200 μCi of L-[$^{35}$S]cysteine for 2 h at 37°C. After being labelled, cells were washed twice with DMEM for the chase phases of these experiments and maintained in a growth medium. At 0, 4, and 16 h after chasing, the cells were washed once with phosphate-buffered saline and harvested in 1 ml of a radioimmunoprecipitation assay (RIPA) buffer (1). The Rev protein was immunoprecipitated by using either rabbit anti-HIV-1 Rev serum 1/20 (raised against a synthetic peptide corresponding to amino acid residues 1 to 20 of HIV-1 Rev) or anti-Rev serum 27/51 (raised against a synthetic

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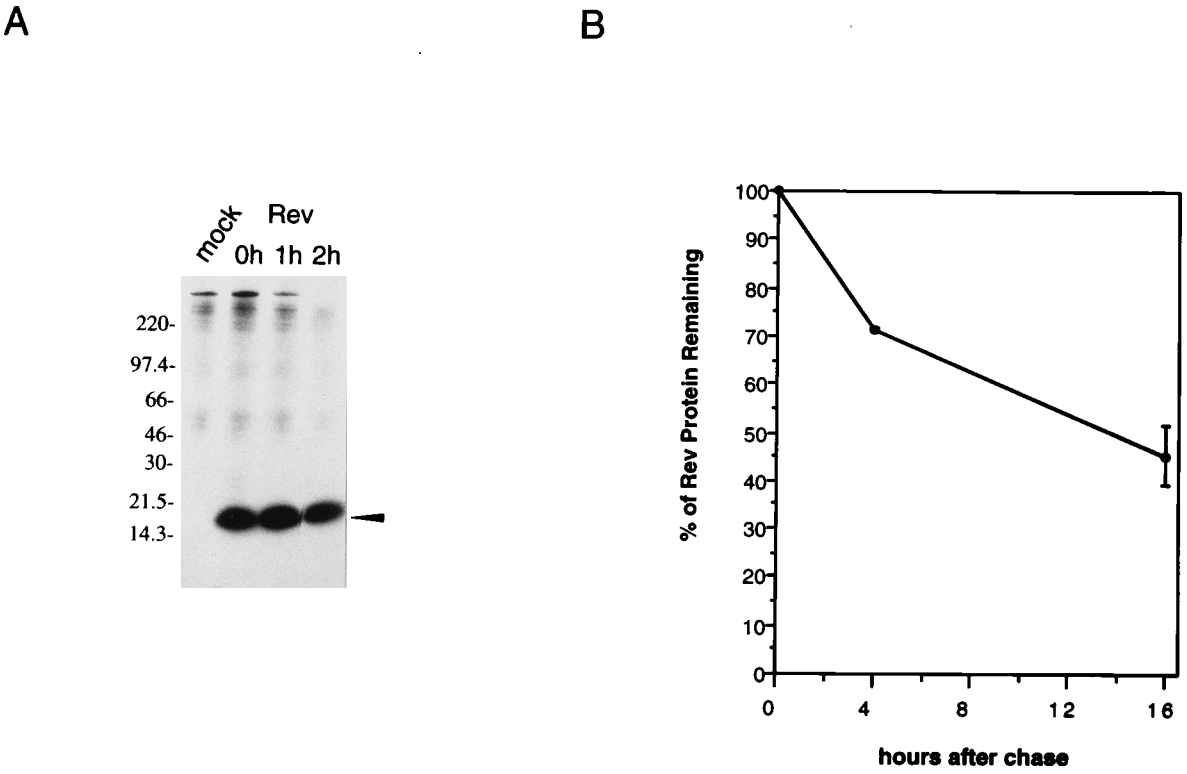


FIG. 1. Intracellular degradation of the HIV-1 Rev protein. (A) HIV-1 Rev remains intact up to 2 h after chasing in pulse-chase experiments with transfected cells. L-[$^{35}$S]cysteine-labelled Rev was immunoprecipitated with an antiserum (Rev 27/51) and analyzed by SDS-PAGE in 8 to 16% Tris-glycine gels (Novex) after pulse-chase analyses. Specific signals for Rev are indicated with an arrowhead. Of note, most of the background signals disappeared 2 h after chasing. Positions for molecular mass markers (in kilodaltons) are shown on the left. (B) Quantitative analysis of the degradation of HIV-1 Rev. Relative signal intensity was quantified and calculated by using a phosphorimager (Molecular Dynamics). The value at 4 h after chasing represents a mean for three independent experiments. The error bar at this time point is not visible, as the variations in the data were extremely small. The value at 16 h represents a mean for five independent experiments, and the error bar indicates the standard deviation.

peptide consisting of residues 27 to 51 of HIV-1 Rev) (kindly provided by Bryan R. Cullen) (13) and protein A-Sepharose 6 MB (Pharmacia LKB). After being washed twice with RIPA buffers, twice with HSWB (20 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1% sodium deoxycholate [pH 7.6]) and twice with a low-salt wash buffer (1), immunocomplexes were dissociated by a sodium dodecyl sulfate (SDS) sample buffer (Novex) and the labelled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography with a commercially available reagent (Amplify; Amersham). Quantitation of signals was performed by using a phosphorimager (Molecular Dynamics). While most of the labelled background signals decreased rapidly within 2 h after chasing, no significant decrease of the Rev signal was observed at the same time point (Fig. 1A). Repeated experiments, using pcREV and pH2rev, revealed that the labelled Rev protein decreased to 71% of the baseline level at 4 h after chasing and to 45% of the baseline level 16 h after chasing, regardless of the expression plasmid used (Fig. 1B). Thus, the intracellular half-life of the Rev protein was demonstrated to be nearly 16 h.

**Intracellular degradation of Rev mutants.** Rev is known to shuttle between the nucleus and cytoplasm of HIV-1-infected cells (9, 19, 24, 27). To investigate which subcellular compartment, eukaryotic nucleus or cytoplasm, is primarily responsible for the degradation of Rev, we utilized vectors expressing Rev protein mutants. The RevM10 mutant possesses a point mutation in the conserved leucine-rich–activation domain (18). The mutation deprives Rev of the ability to migrate out of the cell nucleus (19). First, we compared the intracellular fates of the wild-type Rev and RevM10 by pulse-chase analyses. As shown in Fig. 2, the quantity of labelled Rev protein was slightly greater for RevM10 immediately after chasing and the difference became more prominent 16 h after chasing. These

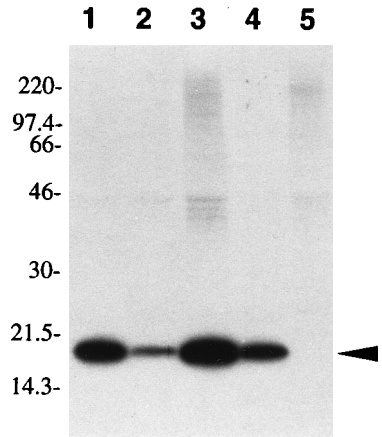


FIG. 2. Comparative analysis of the metabolic labelling and degradation profiles of Rev and RevM10. Cos-1 cells were transfected with either pH2rev or pH2RevM10 and labelled with L-[$^{35}$S]cysteine. Rev proteins were immunoprecipitated with an antiserum (Rev 27/51) and analyzed by SDS-PAGE on 14% Tris-glycine gels (Novex). Lanes: 1, Rev, 0 h after chasing; 2, Rev, 16 h after chasing; 3, RevM10, 0 h after chasing; 4, RevM10, 16 h after chasing; 5, mock-transfected cells, 0 h after chasing. Positions of molecular mass markers (in kilodaltons) and Rev proteins are shown on the left and right, respectively.

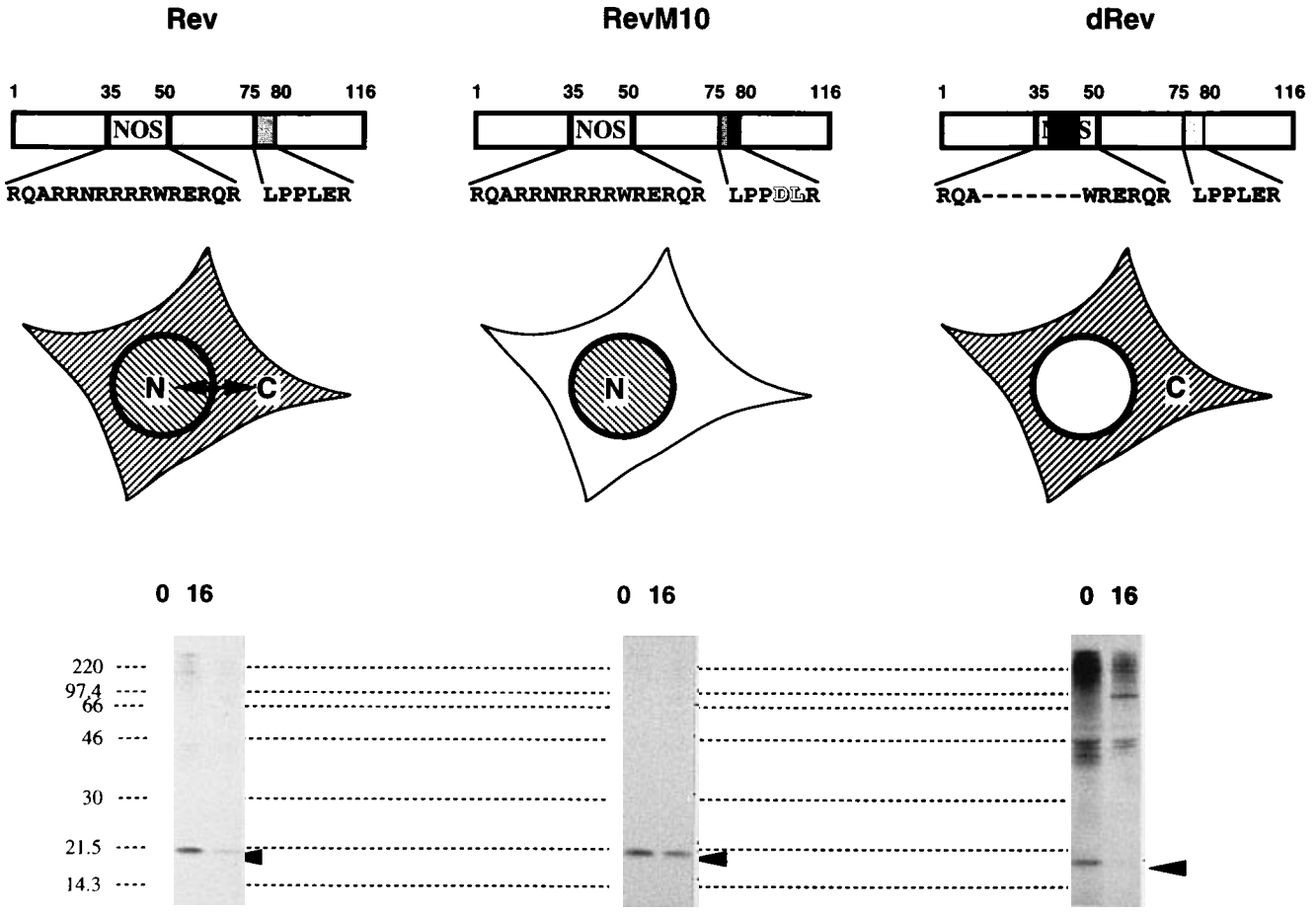


FIG. 3. Alteration of the intracellular rate of degradation of Rev with or without mutations in structure. (Top) Primary structures of the wild-type and mutant Rev molecules. NOS, the nucleolar targeting signal, also contains the RRE-binding domain. Amino acid sequences of the mutated areas are shown in single-letter code below the illustrations. Numbers on the top represent the first and last residues of corresponding areas and of the entire Rev protein. Mutated and deleted residues are indicated by outlined letters and dashes, respectively. Such alterations are represented as small, shadowed boxes in the corresponding regions. (Middle) Subcellular localization of the wild-type and mutated Rev proteins. "N" and "C" represent the cell nuclei and cytoplasm, respectively. Areas in which each Rev moiety is located are hatched. A bidirectional arrow in the left most illustration denotes Rev's shuttling property. (Bottom) Pulse-chase analyses of each Rev moiety. Radiolabelled Rev was immunoprecipitated with an antiserum (Rev 1/20) and analyzed by SDS-PAGE on 14% Tris-glycine gels. Numbers indicate time, in hours, after chasing. Specific signals are denoted by arrowheads. Positions of molecular mass standards, in kilodaltons, are shown by dotted lines. These data are representative of two independent sets of experiments.

findings suggest that RevM10 is more stable than wild-type Rev in living cells. For further analyses, we have employed another Rev mutant, termed dRev. In contrast to RevM10, dRev, which has a 7-amino-acid deletion in the core of the nuclear-nucleolar localization signal (NOS), is unable to enter the cell nucleus (7, 10). The backbones of both plasmids, pH2revM10 and pH2drev, are precisely the same as that of pH2rev. Previous studies have described the construction of pH2drev (10). The other plasmid, pH2revM10, was constructed by substituting the *Hin*dIII fragment containing *rev* cDNA with a *Sac*I-*Hin*dIII 540-bp fragment of pcRevM10 (13). Further, pulse-chase analyses of these Rev mutants were performed, as detailed above. The nucleus-confined RevM10 moiety was much less efficiently degraded during the time course studies than the wild-type Rev moiety was. At the time point 4 h after chasing, degradation of RevM10 was not significantly detected (data not shown). At the 16-h time point, 71% of baseline RevM10 levels were still detectable (mean value for two independent experiments) (Fig. 3). This value for RevM10 at 16 h corresponds to that for the wild-type Rev protein at 4 h after chasing, suggesting a prolonged half-life of RevM10 in the cell. On the contrary, the mutant dRev demonstrated strikingly rapid degradation in the cells. Four hours after chasing, the signal of dRev decreased to below 25% of its baseline level (data not shown). Sixteen hours after chasing, the signal of dRev was barely detectable in all experiments (Fig. 3). Comparing the data obtained with these mutant and wild-type Rev proteins, we concluded that rapid degradation may occur mainly in the cytoplasm and Rev could be stably stored in the cell nucleus.

**Degradation profile of a Rev-Rex chimeric mutant.** If the rapid degradation of dRev was due to cytoplasmic retention, nuclear-nucleolar targeting provided by another NOS may rescue this Rev mutant from degradation, to some extent. To examine this hypothesis, we utilized one more mutant, termed NOXRev. As described in a previous report, NOXRev possesses the NOS of the Rex protein of human T-cell leukemia virus type 1 substituted for that of the HIV-1 Rev protein (11). As a result, NOXRev was targeted to the cells' nuclei or nucleoli, but with reduced affinity (11). We analyzed the fate of expressed NOXRev by similar experiments using an expression plasmid termed pH2NOXRev, whose backbone is also exactly

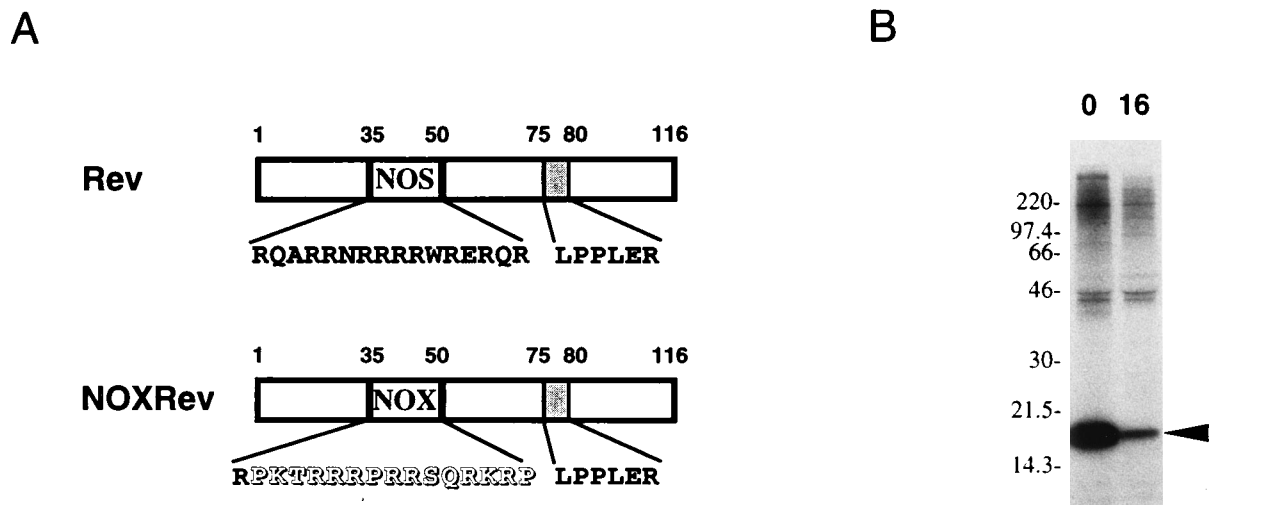


FIG. 4. Expression and degradation profile of a Rev-Rex chimeric protein, NOXRev. (A) Primary structure of NOXRev in comparison with wild-type Rev. The NOS of Rex is abbreviated as NOX, and its amino acid sequence is shown in outlined single-letter code. (B) Radioimmunoprecipitation analyses. Cos-1 cells were transfected with pH2NOXrev and metabolically labelled. The NOXRev protein expression was analyzed with an antiserum (Rev 1/20) by SDS-PAGE on 14% Tris-glycine gels. Positions of molecular mass standards (in kilodaltons), time after chasing (in hours), and the position of NOXRev are shown on the left, top, and right, respectively.

the same as those of pH2rev and other Rev mutants used in this study. As expected, NOXRev remained detectable 16 h after chasing (Fig. 4). The remaining quantity of NOXRev was 14.9% of the initial NOXRev level at the 16-h time point. This value is consistent with partial recovery of the nuclear-nucleolar targeting property, provided by the NOS of Rex, as described previously (11). As such, the NOS of Rex can increase the intracellular half-life of dRev.

**Alterations of HIV-1 Rev half-life by an intracellular anti-Rev SFv.** To confirm and extend the above-described findings, alternate but complementary experiments were carried out. It has been reported that a single-chain monoclonal antibody variable fragment (SFv) is able to inhibit Rev function by interfering with the nuclear localization of the Rev protein, when coexpressed intracellularly (4). The cDNA of the anti-Rev SFv (designated D8) was subcloned into a eukaryotic expression vector, pCI (Promega), and termed pCILHD8. Expression of the anti-Rev SFv by this plasmid, driven by the cytomegalovirus promoter, had been confirmed by a Rev functional inhibition assay (5, 23) (data not shown). By utilizing this plasmid in cotransfection studies, the wild-type Rev molecules could be sequestered in the cytoplasm. For these experiments, 500 ng of pH2rev and 1 μg of pCILHD8 were cotransfected into Cos-1 cells by the same protocol as that described above. Pulse-chase analyses of the labelled Rev protein in the presence of the anti-Rev SFv molecules demonstrated an accelerated degradation of Rev, in comparison with the rate for cells solely transfected with pH2rev (Fig. 5). These results clearly support our conclusion that Rev is rapidly degraded in the cytoplasm and can be preserved for a considerable time in the cell nucleus. In addition, we have constructed an RRE expression plasmid and performed experiments similar to those as described above. Coexpression of the RRE led to no significant difference in the degradation profile of the Rev protein (data not shown).

In these studies, we have found that the intracellular half-life of the HIV-1 Rev protein was unexpectedly long. There have been only a few reports describing the cellular half-lives of viral transactivators. For example, the half-life of the human T-cell leukemia virus type 1 transactivator Tax protein had been shown to be approximately 4.5 h (25). In comparison, the half-life of Rev seems to be approximately three times as long. However, once the Rev protein was maintained outside the cell nucleus, its half-life was dramatically shortened to less than 4 h. In contrast, a shuttling-deficient mutant of Rev, sequestered in the nucleus, demonstrated a prolonged half-life which was significantly longer than 16 h. Mutation of the NOS of Rev caused rapid degradation of the Rev molecule, and addition of the NOS of Rex led to a partial rescue of intracellular stability. These findings suggest that the fate of the HIV-1 Rev protein is under the control of its subcellular localization. Thus, the fact that coexpression of RRE did not alter the intracellular

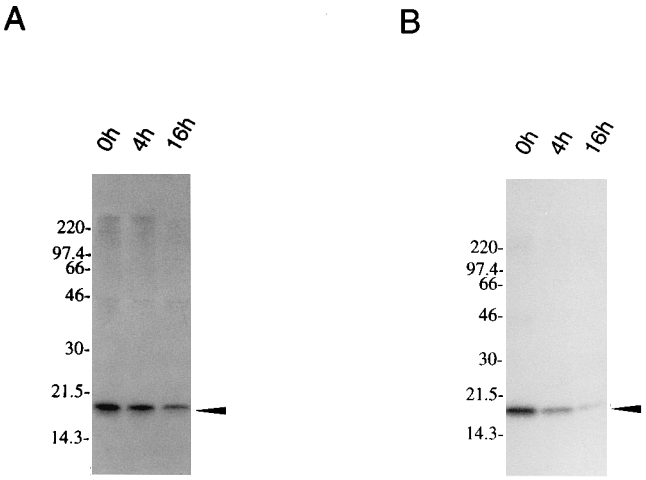


FIG. 5. Effects of an anti-Rev SFv on the degradation of Rev. Degradation profiles in the absence (A) and presence (B) of SFv are displayed. Rev, immunoprecipitated by an antiserum (Rev 1/20), was analyzed by 14% Tris-glycine SDS-PAGE and fluorography. Time periods after chasing are indicated above the lanes. Positions of molecular mass markers (in kilodaltons) and the Rev protein are shown on the left and the right, respectively. Relative intensities of the signals at 4 and 16 h after chasing, in comparison with intensities at the zero time point in each experiment, are as follows: without SFv, 72.0% (4 h) and 46.6% (16 h); with SFv, 40.4% (4 h) and 9.2% (16 h).

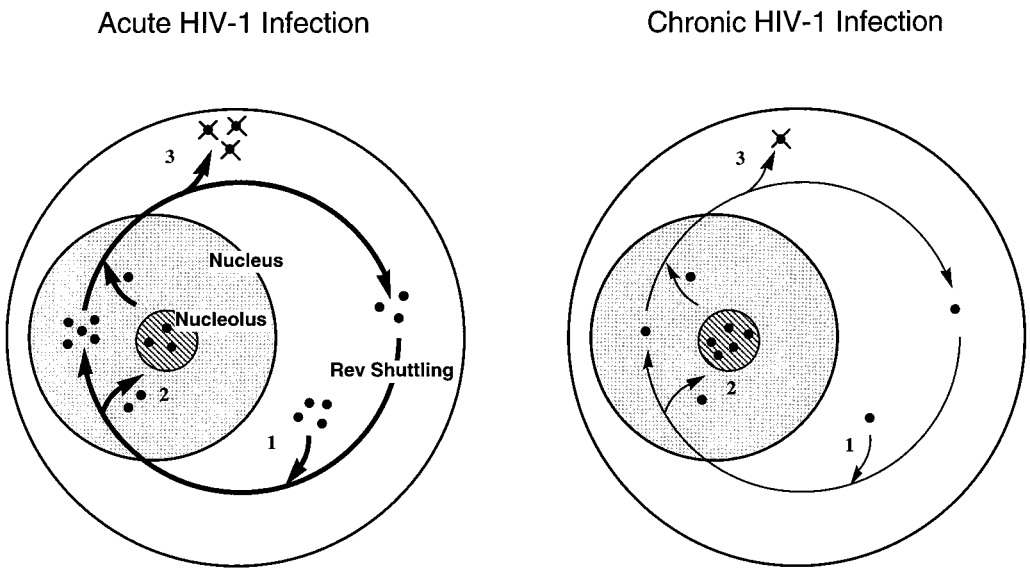


FIG. 6. Fate of the Rev protein functioning in two different stages of HIV-1 infection. The large; grey, medium; and hatched, small circles represent the infected cell membrane, cell nucleus, and cell nucleoli, respectively. Solid grains denote intact Rev molecules. Rev is synthesized in the cytoplasm (step 1), stored in the cell nucleoli (step 2), and then transported and degraded in the cytoplasm (step 3), while constantly shuttling between two cellular compartments, the nucleus and cytoplasm. In the acute stage (left panel), relatively large quantities of Rev are synthesized, shuttle, and are degraded, whereas some of these Rev molecules are directed to the nucleoli for storage. After progression to the chronic stage of cellular HIV-1 infection (right panel), a functional level of Rev still shuttles continuously to support viral production, with Rev being stored in the cell nucleoli. Additional HIV-1 Rev may be produced, at various times, by de novo synthesis.

stability of Rev is consistent with a previous report that has shown the RRE independency of Rev export (27). Rather, the shuttling property of HIV-1 Rev is likely to be dependent on the cellular background. Of interest, in an astrocytoma cell line in which Rev function was shown to be blocked, Rev was found predominantly in the cytoplasm (21). The blockade of Rev function in these cells might not only be due to a lowered opportunity for Rev to bind the RRE in the cell nucleus but also might be secondary to the rapid degradation of Rev in the cytoplasm.

As discussed above, if Rev is sequestered in the cytoplasm it is rapidly degraded. In contrast, if Rev is maintained in the cell nucleus it can be conserved for a relatively long time period and can be utilized as a stable supply to maintain efficient HIV-1 structural gene expression. It has been reported that in most cell lines, Rev is located predominantly in the cell nucleoli (2, 13). Even in the above-described astrocytoma cell line, Rev was visible in the cell nucleoli as well as in the cytoplasm by immunofluorescent staining, which indicates Rev's strong affinity for the cell nucleolus (21). The signal required for nucleolar localization of Rev has been identified (2, 12). Another report has also demonstrated that the nucleolar accumulation of Rev protein (or RevM10) was so strong that some Rev-induced nucleolar deformity and dysfunction might occur (22). A recent report has also shown that transiently expressed Rev prevents the progression of mitosis and inhibits cell division, through accumulation of Rev in the cell nucleoli (20). In our laboratories, Western blotting (immunoblotting) analysis of the accumulated wild-type Rev and RevM10 48 h posttransfection demonstrated very little difference in the quantities of these moieties. On the basis of theoretical calculations, if Rev and RevM10 are expressed constantly and degraded exponentially with their own half-lives, the amount of accumulated RevM10 would only be up to 1.85-fold greater than the amount of wild-type Rev 48 h after transfection. This small discrepancy may be explained by the effect of overexpressed Rev or RevM10 on cell proliferation. Namely, the more Rev or RevM10 accumulates, the worse cell viability would be. This would decrease the rate of synthesis and increase the loss of Rev or RevM10. Consequently, the relatively small difference between the total levels of Rev and RevM10 would be further decreased as time passes after transfection (data not illustrated).

However, the positive role of the nucleolar localization of the Rev protein is still obscure. It is possible that the cell nucleoli may be used as a storage site or reservoir for intracellular Rev preservation. Thus, nucleolar localization may play a passive, but positive, role in the homeostatic control of the intracellular circulation of Rev, throughout various stages of HIV-1 infection. As illustrated in Fig. 6, at the stage of acute HIV-1 infection a vast quantity of Rev molecules would be synthesized from fully spliced viral mRNAs (2) and a portion of these Rev moieties would be directed to storage in the cell nucleoli. As the levels of Rev would soon overflow the storage capacity of the nucleoli, a relatively large portion of Rev molecules would shuttle out to the cytoplasm and would be rapidly degraded. If degradation became faster than the synthesis of new Rev molecules from multiply spliced mRNAs, which were further decreased by the function of Rev, shuttling of Rev would also decrease. In parallel with the decrease in Rev, the acute HIV-1 infection stage would be terminated and chronic infection would be established.

In the chronic stage of HIV-1 infection at the cellular level, a reduced quantity of Rev should be continuously shuttling between the nucleus and cytoplasm, while conveying unspliced and incompletely spliced viral mRNA to the cytoplasm. The overall cellular supply of the Rev protein would be stabilized by the Rev protein in the cell nucleoli, stored during the acute stage of HIV-1 infection. The degradation of Rev would then be relatively slow, as the cytoplasmic levels of Rev would be relatively low. The supply of new Rev molecules to maintain the level of working Rev moieties may not be continuous or stable, being based on the augmenting effect of the Rev reservoir in the nucleoli. Previous studies have revealed the poten-

tial utility of critical levels of Rev for efficient HIV-1 replication in certain cell types. These findings may be based on the requirement for multimerization to generate efficient Rev function (14, 23). Our hypothesis, presented here, may explain how the HIV-1 provirus maintains the stable expression of the Rev protein at efficient levels during chronic infection. From the point of view of anti-HIV-1 genetic therapies, it also implies that we may be able to induce restricted HIV-1 replication in infected cells, simply by perturbing the well-balanced supply circuit of Rev. These data strengthen the rationale of using anti-Rev strategies in genetic therapies against HIV-1 infections (4, 5, 16).

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