JOURNAL OF VIROLOGY, Dec. 1996, p. 8332–8339 Vol. 70, No. 12
0022-538X/96/$04.00+0

Human Immunodeficiency Virus Type 1 Rev Function Requires Continued Synthesis of Its Target mRNA

SANDRA IACAMPO AND ALAN COCHRANE*

Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Received 2 May 1996/Accepted 13 August 1996

Synthesis of human immunodeficiency virus structural proteins is dependent on expression of the virus-encoded Rev protein due to the constitutive nuclear sequestration of mRNAs coding for the structural proteins. The pathway by which Rev, through interaction with the Rev-responsive element (RRE) within the mRNA, achieves export of the mRNA remains unclear. To probe the mechanism by which Rev induces nuclear export of its target mRNAs, the effect of inhibiting mRNA synthesis on the function of Rev was examined. Two approaches to address this issue were pursued: (i) the use of general transcription inhibitors such as 5,6-dichlorobenzimidazole riboside (DRB) and actinomycin D, and (ii) the more selective modulation of target gene transcription permitted by the use of a tetracycline-regulated promoter. Addition of either DRB or actinomycin D inhibited Rev action despite the presence of significant quantities of the target mRNA throughout the course of drug treatment. Furthermore, prolonged DRB treatment was found to improve rather than diminish the induction observed. Subsequent analysis using the tetracycline-modulated promoter demonstrated that Rev function was dependent on the transcription rate of the target mRNA and independent of target mRNA concentration. These data strongly indicate that Rev functions through interaction with newly synthesized target mRNA, facilitating its export by preventing its interaction with the host factors that effect nuclear sequestration.

The control of human immunodeficiency virus type 1 replication has been demonstrated to occur at multiple levels involving the accessory proteins (Vif, Vpr, Vpu, Tat, Rev, and Nef) encoded by the virus (8, 57). The Rev protein has been shown to be essential for the expression of the structural proteins of the virus because of its requirement for the transport of the unspliced (9-kb) and singly spliced (4-kb) viral mRNAs which encode these proteins from the nucleus to the cytoplasm so that translation can occur (13, 14, 22, 34). In the absence of Rev, the unspliced and singly spliced mRNAs are sequestered in the nucleus of the cell (13, 14, 22, 34). Mediation of export of the RNA is achieved by direct interaction of Rev with a 240-nucleotide sequence designated the Rev-responsive element (RRE) within the target RNA (4, 10, 59). Although binding to the target RNA is essential for function, it is not sufficient given that mutations in amino acids 75 to 85 of Rev impede function but do not affect the capacity of the protein to bind to the RRE (33, 36, 40).

In parallel with the identification and analysis of Rev-RRE RNA interaction, studies have demonstrated that Rev is localized to the nucleus, with significant localization to the nucleolus (6, 28, 33, 41). However, more recent studies have shown that Rev localization is more dynamic given that Rev is capable of shuttling between the nucleus and the cytoplasm (9, 26, 37, 38, 43, 53, 54, 58), a property that is dependent on the 75- to 85-amino-acid domain of Rev and essential for function (9, 37, 53). This domain is capable of conferring a shuttling phenotype to heterologous proteins upon conjugation (15, 38), indicating that it functions independent of the RNA binding domain of Rev. Further studies have identified a cellular factor (designated hRIP [16] or Rab [1]) that specifically interacts with the transactivation domain of Rev and appears to mediate the Rev-dependent movement of viral structural protein mRNAs from the nucleus to the cytoplasm.

While these studies have provided a detailed picture of how Rev functions, it remains unclear as to how the interaction of Rev with its target mRNAs overcomes the constitutive suppression of their transport to the cytoplasm. Sequestration in the nucleus of the unspliced and singly spliced viral mRNAs has been proposed to be due to one of two mechanisms: (i) the inefficiency of the splicing of these mRNAs (2, 30), the mRNAs becoming trapped in spliceosome complexes, or (ii) the action of *cis*-acting repressive sequence elements present within the Rev-regulated mRNAs (5, 31, 46, 50). In either case, Rev must dramatically alter the nuclear metabolism of its target mRNAs in order to circumvent or modify their sequestration within the nucleus. In vitro experiments have demonstrated that Rev is capable of interacting with RNA containing the RRE (4, 10, 59), which is essential for conferring Rev responsiveness to the viral structural protein mRNAs. Mutations that affect the in vitro interaction dramatically affect in vivo function (23, 24, 35, 39, 40, 60), indicating that the Rev-RRE interaction observed is of physiological significance. However, it remains unclear how the interaction of Rev with the RRE portion of the target mRNA alters the effect of the splice sites or *cis*-acting sequence elements, some of which have been mapped several kilobases away from the RRE (5, 31, 46, 48–50). Two simple models are that (i) by interaction with the RRE, Rev releases the mRNA from the sequestering complexes in the nucleus or (ii) by interaction with the RRE, Rev prevents the entrapping complexes from forming, thus modifying the metabolism of the target mRNAs in the nucleus. One prediction of the latter hypothesis is that mRNAs already within sequestering complexes would not be efficiently rescued by expression of Rev. As the blockage of the expression of viral structural mRNAs is virtually complete in the absence of Rev (14, 21, 46), all unspliced and singly spliced viral mRNAs must eventually enter the sequestering complexes. Consequently, if the latter model is correct, only newly synthesized RNA or

* Corresponding author. Phone: (416) 978-2500. Fax: (416) 978-6885.

RNA not yet within the sequestering complexes would be capable of being efficiently rescued by Rev, and Rev function would necessitate continued target mRNA synthesis. If the former model is correct, Rev function should be independent of RNA synthesis provided that the target mRNAs are relatively stable within the nucleus.

To test the predictions of these models, two approaches were pursued: (i) We assessed the effects of 5,6-dichlorobenzimidazole riboside (DRB) (51) and actinomycin D (42), potent inhibitors of mRNA synthesis, on the function of Rev. To eliminate the possible effects of DRB on plasmid maintenance or amplification inherent in transient expression systems, we used stable cell lines that accurately reproduce the regulation of viral structural protein mRNA expression by Rev. Using these cell lines, we observed that Rev function was extremely sensitive to the addition of DRB and actinomycin D and that the effect of DRB cannot be readily explained by either instability of the preexisting mRNA or a labile host cofactor. (ii) We used a tetracycline-regulated promoter (18) permitting specific modulation of the transcriptional rate of the gene of interest. Using these systems, we found that Rev function was independent of the concentration of its target mRNA but was determined solely by its rate of synthesis.

MATERIALS AND METHODS

Plasmids and transfections. Plasmids pDM128 and pRSVRevGR were the generous gift of T. Parslow and have been described previously (25). To generate plasmid pCMV*DM128 used in the latter set of experiments described in this report, the simian virus 40 promoter of pDM128 was excised using *Sal*I-*Xba*I and replaced with an *Xho*I-*Xba*I fragment containing a tetracycline-regulated cytomegalovirus (CMV) promoter, CMV*, generously supplied by H. Bujard (18).

In experiments using transient transfections, cells were transfected by the DEAE-dextran protocol as previously described (7) and harvested 2 days after transfection. Dexamethasone and tetracycline were added at concentrations of 10 μM and 0 to 1 μg/ml, respectively, where indicated. In experiments examining the effect of DM128 RNA concentration and transcription rates on Rev function, cells were transfected with pCMV*DM128, pRSVRevGR, and pCMVtTApolyA and incubated for 2 days in the absence of dexamethasone and in the tetracycline concentrations indicated. Cells were washed three times to remove any tetracycline present, and medium was then switched to one containing either dexamethasone alone or dexamethasone and indicated doses of tetracycline. Cells were harvested at indicated times, and chloramphenicol acetyltransferase (CAT) enzyme levels were determined as previously described (17).

Effects of DRB and actinomycin D on Rev function. Cell lines were generated in two stages by first cotransfecting pRSVRevGR and pSV_2neo, and cells displaying regulated expression of Rev were used for a subsequent round of transfection with pDM128 and pSVhygro. Cells were screened for induction of CAT activity by administration of 10 μM dexamethasone to the medium. All stable transfection were carried out by the calcium phosphate protocol (27). To determine the effects of DRB and actinomycin D on the function of Rev, cells were grown in 24-well dishes to greater than 80% confluency prior to treatment. To initiate induction of Rev activity, medium containing 10 μM dexamethasone was added to the cells; the control was treated with medium containing 0.1% ethanol. Cells were treated with 100 μM DRB, a concentration sufficient to block greater than 90% of mRNA synthesis in the cell, or with actinomycin D at a concentration of 4 or 0.04 μg/ml. To remove DRB, cells were washed twice with complete medium and then placed in fresh medium containing 10 μM dexamethasone. At indicated times, cells were washed once with phosphate-buffered saline (PBS) and detached from the plate by incubation in PBS plus 2 mM EDTA. Cells were collected, pelleted, and resuspended in 100 μl of 0.25 M Tris-HCl (pH 8.0), and extracts were used for CAT assays as previously described (17). Percent acetylation was determined by excising unacetylated and acetylated regions of the thin-layer chromatogram, and radioactivity was determined by scintillation counting. All values were subsequently normalized for levels of protein present in each assay.

Effects of DRB on protein synthesis and RNA transport. To evaluate any possible effects of DRB on protein synthesis, cells were incubated with 25 μCi of ^{35}S-Translabel (ICN) in the presence of no additions, 100 μM DRB, or 20 μg of cycloheximide per ml. At indicated times, cells were harvested and pelleted. Cells were resuspended in 0.25 M Tris-HCl (pH 8.0) to which 10 volumes of 10% trichloroacetic acid was subsequently added. Protein precipitates were collected by filtration through Whatman GFA filters, and the filters were washed three times with 10% trichloroacetic acid. Filters were then dried and counted in a scintillation counter.

To assess whether DRB affected RNA transport, cells were treated as follows. At $t = 0$, medium containing 200 μCi of [^{3}H]UTP was added to cells. Forty-five minutes later, DRB was added to a final concentration of 100 μM. At indicated times, cells were harvested and fractionated into nuclear and cytoplasmic fractions by Nonidet P-40 lysis (20), and RNA was extracted by the guanidine-phenol-chloroform protocol (3). Purified RNA obtained was then quantitated, aliquots were spotted onto DE81 filters, and radioactivity in each fraction was determined by liquid scintillation.

RNA analysis. For the analysis of pDM128-generated RNA, stable cell lines were grown on 100-mm-diameter tissue culture plates to greater than 80% confluency. Cells were harvested following treatment by incubation in PBS plus 2 mM EDTA and collected by centrifugation at 1,000 × *g* for 5 min at 4°C. In the case of transient transfection into Cos cells, cells were transfected with 5 μg of pDM128 according to the DEAE-dextran protocol. Two days posttransfection, cells were treated with 4 μg of actinomycin D per ml or 150 μM DRB and harvested at the times indicated. Total RNA was prepared by either the guanidine-phenol-chloroform protocol (3) or use of an RNAeasy kit (Qiagen).

To detect pDM128-generated mRNA, S1 nuclease analysis was carried out with either an antisense RNA probe spanning the entire CAT gene or a labeled DNA probe spanning the CAT sequence between *Hin*dIII and *Eco*RI sites (nucleotides 1 to 251). RNA probes were generated from *Hin*dIII-linearized BI-CAT, a plasmid which contains the entire CAT gene in the antisense orientation relative to the T7 promoter of Bluescript (Stratagene). Probe synthesis was carried out as outlined by the manufacturer (Promega), with the following modifications: incubations were at 15°C for 30 min in the presence of 12.5 μM UTP and 50 μCi of [^{32}P]UTP (800 Ci/mmol), using 1 μg of template; and reactions were terminated by addition of 2 U of RQ_1 DNase (Promega) and incubation for 30 min at 37°C. DNA probe was prepared by subcloning of the *Hin*dIII-*Eco*RI fragment of CAT into Bluescript SK+ (Stratagene) and excising the CAT sequence with a *Hin*dIII-*Sac*I digest. Isolated DNA fragment was end labeled by using Klenow enzyme, [α-^{32}P]dCTP, and unlabeled dGTP, dATP, and TTP as previously detailed (47). Unincorporated nucleotides were removed by passage of the sample through a Sephadex G-50 spin column, and the eluant was phenol-chloroform extracted. Probe was then added to 10 μg of total RNA, and the samples were lyophilized, resuspended in 30 μl of 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES pH 6.4)–400 mM NaCl–1 mM EDTA, and incubated at 75°C for 10 min then 42°C for 16 h. Subsequently, 300 μl of S1 buffer (50 mM sodium acetate [pH 4.6], 280 mM NaCl, 4.5 mM $ZnCl_2$, 20 μg of denatured salmon sperm DNA per ml, 1 U of S1 nuclease per μl) was added, and the mixture was digested at 25°C. Reactions were stopped by addition of 20 μg of tRNA and EDTA to a final concentration of 5 mM. Samples were phenol-chloroform extracted, ethanol precipitated, resuspended in 80% formamide–10 mM EDTA, and analyzed on 4% polyacrylamide–8 M urea–1× Tris-borate-EDTA gels. Detection of protected bands was by autoradiography or exposure to PhosphorImager screens.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was analyzed by Northern (RNA) blotting. Total RNA was run on 1.2% formaldehyde agarose gels and blotted onto nitrocellulose filters. Blots were blocked and hybridized as detailed by Promega, using as the probe antisense RNA generated from a plasmid containing rat GAPDH cDNA (generously provided by N. Chaulifour). Blots were washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and analyzed with a PhosphorImager.

RESULTS

Effects of DRB and actinomycin D on Rev function. It has been observed that significant quantities of unspliced mRNA, the putative target of Rev, are found within the nucleus in the presence or absence of Rev (13, 14, 22, 34). This observation raises questions as to the efficiency with which sequestered mRNA can be rescued by Rev and whether all of the unspliced and singly spliced viral RNA in the nucleus can be equally rescued by Rev. To examine this issue in more detail, the effects of general inhibitors of RNA polymerase II activity on the ability of Rev to induce transport of the nucleus-entrapped, unspliced mRNA into the cytoplasm were examined. The drugs actinomycin D and DRB (51) were used to inhibit RNA synthesis by RNA polymerase II. If all RRE-containing RNAs in the nucleus are equally targeted by Rev, then inhibition of the synthesis of nascent transcripts should have no effect on the ability of Rev to induce mRNA transport provided that the preexisting mRNAs are relatively stable over the time course of the experiment. To monitor Rev function, the vectors pRSVRevGR and pDM128 (25) were used to generate stable cell lines. The vector pRSVRevGR provides for posttranslational regulation of Rev function, the Rev-glucocorticoid receptor

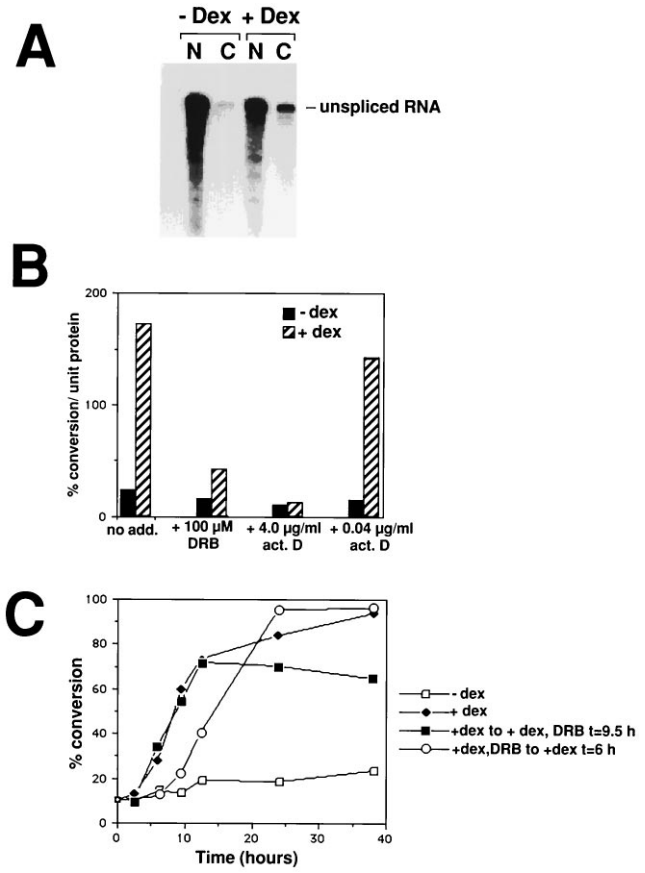

FIG. 1. Effects of DRB and actinomycin D on Rev function. (A) To assay the effect of Rev expression on the subcellular distribution of unspliced DM128 RNA, cells were grown in the absence or presence of dexamethasone (Dex) and then harvested. Nuclear (N) and cytoplasmic (C) fractions were prepared as previously outlined (20), and total RNA was extracted. Aliquots of the RNA were then probed with an antisense CAT riboprobe specific to the unspliced form of DM128 RNA as detailed in Materials and Methods. (B) Cells were grown to confluency and then treated as indicated overnight. Cells were harvested, and CAT assays were performed. Data shown are the averages of triplicate determinations for each datum point. act. D, actinomycin. (C) Effect of DRB on the induction and maintenance of CAT expression. Cell lines were grown to confluency and then exposed to the following treatment regimens: − dex, no addition of either DRB or dexamethasone; + dex, treatment with dexamethasone and no DRB; +dex to + dex, DRB t=9.5 h, treatment with dexamethasone and no DRB, DRB added at $t = 9.5$ h, and incubation continued; +dex,DRB to +dex t=6 h, treatment with both dexamethasone and DRB at $t = 0$, DRB removed at $t = 6$ h, and incubation continued in the presence of dexamethasone. At indicated times, cells were harvested for subsequent CAT assay. Values indicated are the averages of three independent determinations.

(RevGR) fusion protein being held in an inactivate state within the cytoplasm until the addition of dexamethasone to the medium. This manner of regulation permits activation of Rev function independent of either translation or transcription in the short term. Plasmid pDM128 was generated from a modification of the *env* gene of human immunodeficiency virus type 1 in which the CAT reading frame has been placed 3′ of the 5′ splice site of *env*. This organization results in CAT expression being dependent on the Rev-mediated export of the unspliced form of the mRNA from the nucleus to the cytoplasm.

In the absence of Rev, unspliced DM128 RNA is not efficiently exported to the cytoplasm, leading to low levels of CAT expression (Fig. 1A and B). Dexamethasone addition induced CAT expression over the next 24 h by facilitating export of unspliced DM128 RNA to the cytoplasm (Fig. 1A) (25). Addition of 100 μM DRB or 4 μg of actinomycin D per ml at the same time as dexamethasone addition strongly suppressed CAT induction (Fig. 1B). However, addition of 0.04 μg of actinomycin D per ml, sufficient to inhibit rRNA synthesis but not mRNA synthesis (data not shown) (42), did not significantly affect the capacity of Rev to induce CAT expression. Therefore, it can be concluded that while inhibition of RNA polymerase I activity has little effect on Rev function, inhibition of RNA polymerase II activity blocks Rev function, consistent with previous observations (9, 43).

To further examine the effect of transcription inhibitors on Rev function, subsequent work used DRB, whose effect on transcription is rapid and readily reversible. The effect of delayed addition or removal of DRB on the kinetics of CAT induction was studied in a number of independent Rev-inducible cell lines, and a representative data set is shown (Fig. 1C). Little or no CAT activity could be detected 3 h after addition of dexamethasone; CAT activity increased linearly from 6 to 12 h after induction and then more slowly up to 40 h. Addition of DRB at the same time as dexamethasone led to nearly complete suppression of CAT induction (Fig. 1B and C). Addition of DRB 9.5 h after dexamethasone administration interrupted the increase in CAT activity, showing that continued RNA synthesis is required for continued induction of CAT activity. When DRB was added at the same time as dexamethasone and subsequently removed, induction of measurable CAT activity was observed within 3 h. CAT activity continued to increase after removal of DRB to attain, by 20 h, levels at least equivalent to those obtained with dexamethasone treatment alone.

To confirm that the effect of DRB treatment is due to inhibition of mRNA synthesis and not to a secondary effect of the drug, the effects of DRB treatment on protein synthesis and RNA transport were examined. As shown in Fig. 2A, no difference in [^{35}S] methionine incorporation into total protein was discernible between untreated and DRB-treated cells up to 4 h after administration of the drug. After 4 h, incorporation was less than that observed in untreated cells, but the extent of incorporation continued to increase over the time period tested. This difference has been attributed to the loss of the pool of unstable mRNAs within the cell (19, 51). The effect of DRB is also in stark contrast to the effect of cycloheximide, which completely blocked incorporation of label into protein. To test for possible effects of DRB on total RNA (including rRNA, mRNA, and tRNA) transport from the nucleus to the cytoplasm, cells were labeled with [^{3}H]uridine and DRB was added 45 min after addition of label. Cells were collected at various times after addition of label, and total RNA was isolated from the nuclear and cytoplasmic fractions. As shown in Fig. 2B, treatment with DRB inhibited further incorporation of label into nuclear RNA, but the accumulation of radiolabeled RNA in the cytoplasm continued in a linear fashion throughout the experiment. Therefore, although DRB treatment inhibits RNA synthesis, there is no observable effect on total RNA transport or protein synthesis.

To eliminate alternative explanations for the inhibition of Rev function by DRB, the effect of DRB on the steady-state levels of unspliced pDM128 mRNA was next examined. As shown in Fig. 3A, addition of DRB alone or in the presence of dexamethasone resulted in no significant decrease in unspliced pDM128 RNA levels over the course of 9 h, indicating that this mRNA is relatively stable over the time course of the experiments described above. Therefore, the inability of Rev to act in the presence of DRB cannot be ascribed to the loss of Rev-responsive RNA in the nucleus. To address the possibility that the observed effect of DRB on Rev function could be attrib-

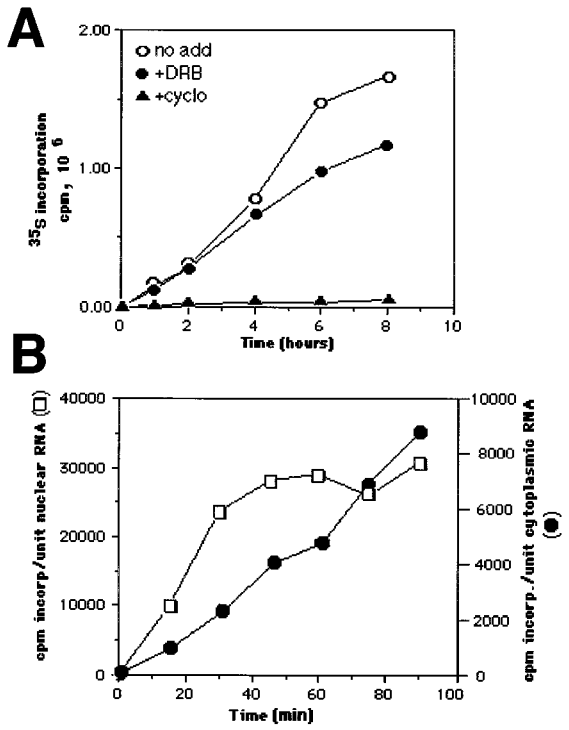

FIG. 2. Effect of DRB on protein synthesis and RNA nuclear/cytoplasmic transport. (A) The cell line CRCAT4B2 was grown to confluency and then incubated in medium containing 25 μCi of ^{35}S-Translabel (ICN) with or without either DRB (100 μM) or cycloheximide (cylo; 20 μg/ml). At indicated time points, cells were harvested and total incorporation of label was determined by trichloroacetic acid precipitation. (B) The cell line CRCAT4B2 was grown to confluency and then incubated in medium containing [^{3}H]uridine (200 μCi/ml). Forty-five minutes after addition of label, DRB was added to a final concentration of 100 μM and incubation continued. At the indicated times after addition of radiolabel, cells were harvested, nuclear and cytoplasmic fractions prepared, and total RNA was isolated from each. Total radioactive RNA in each fraction was determined by absorption onto DE81 filters and scintillation counting.

uted to inactivation of the target RNA or inactivation or loss of a cellular cofactor, the effect of the length of DRB treatment on the kinetics of induction was examined (Fig. 3B). The data demonstrate that prolonged DRB treatment (as long as 12 h), rather than impairing the response, actually results in the elimination of the lag phase and increases the extent of the response (DRB treatment for >8 h increasing induction over cells treated with dexamethasone alone by greater than twofold).

Effect of specifically modulating target gene promoter activity on Rev function. The results of the experiments using DRB suggest that ongoing RNA synthesis is required for Rev to act. However, these results cannot distinguish between a requirement for new target RNA, a requirement for another cellular RNA whose synthesis or abundance is sensitive to treatment with DRB, or other effects of the drug. To test directly for the requirement for newly synthesized DM128 RNA, we carried out experiments using the tetracycline-modulated promoter developed by Gossen and Bujard (18). In this system, the activity of the promoter (here designated CMV*) is dependent on the binding of a transcriptional activator, tTA (comprised of Tet^r fused to the transactivation domain of the herpes simplex virus transcriptional activator, VP16), to Tet^r binding sites located in the CMV* promoter. tTA is unable to transactivate its target gene in the presence of tetracycline, which blocks the capacity of tTa to bind its target DNA sequence. Consequently, the transcriptional activity of the CMV* promoter can be modulated by the concentration of tetracycline in the medium, with high doses (1 μg/ml) effecting a complete inactivation of the promoter.

To verify the functional characteristics of this tetracycline-regulated system, the constitutive promoter of pDM128 was replaced with the CMV* promoter and the effect of tetracycline on the Rev response was assayed by transfection into Cos 7 cells. As shown in Fig. 4A, addition of tetracycline to cells cotransfected with pDM128, pRSVRevGR, and pREPtTA (a plasmid which expresses the Tet^r-VP16 fusion protein) had no effect on induction of CAT activity by Rev, as anticipated given that tetracycline would not affect the activity of the constitutive promoter present in pDM128. Failure to observe an effect of tetracycline in this instance indicates that tetracycline addition does not directly impair Rev function. However, addition of tetracycline to cells transfected with pCMV*DM128, pRSVRevGR, and pREPtTA completely blocked induction of CAT expression. In parallel with these functional analyses, immunofluoresence was also performed to assess the effect of tetracycline on Rev distribution. No effect of tetracycline (1 μg/ml) on the distribution of Rev between the nucleus and cytoplasm was observed, nor did the drug impair the ability of RevGR to translocate into the nucleus upon the addition of dexamethasone (data not shown).

Having established that the system functions as desired, the

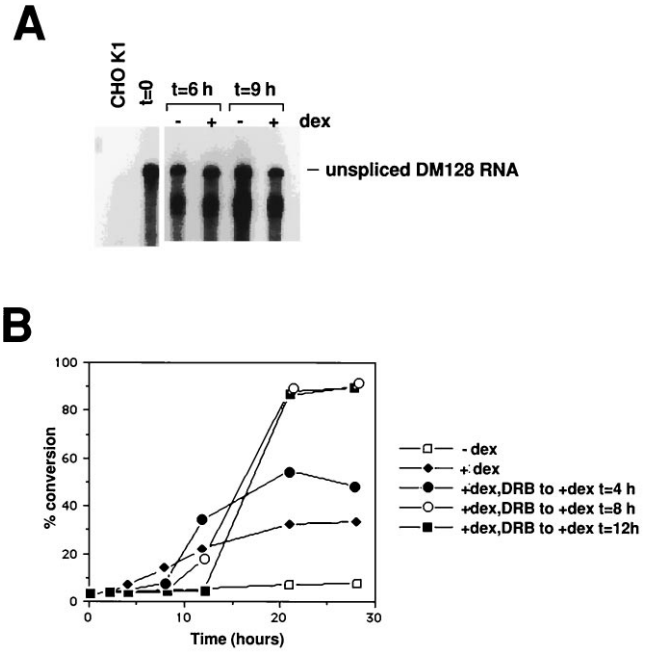

FIG. 3. Effect of DRB on unspliced mRNA stability and kinetics of Rev response. (A) To evaluate the effect of DRB treatment on CAT mRNA abundance, cells were treated with DRB in the presence (+) and absence (−) of dexamethasone (dex). At the indicated times after DRB addition, cells were harvested, total RNA was prepared, and CAT mRNA was detected by S1 analysis. (B) To determine the effect of the duration of DRB treatment on the subsequent induction kinetics following DRB removal, the following treatment regimens were used; −dex, no DRB and no dexamethasone added; +dex, dexamethasone added at $t = 0$; +dex,DRB to +dex t=4 h, DRB and dexamethasone added to the cultures at $t = 0$ and DRB removed at $t = 4$ h; +dex,DRB to +dex t=8 h, DRB and dexamethasone added to the cultures at $t = 0$ and DRB removed at $t = 8$ h; +dex,DRB to + dex t=12h, DRB and dexamethasone added to the cultures at $t = 0$ and DRB removed at $t = 12$ h. At the indicated times, cells were harvested and CAT was assayed as previously outlined. Each point is the average of three independent determinations.

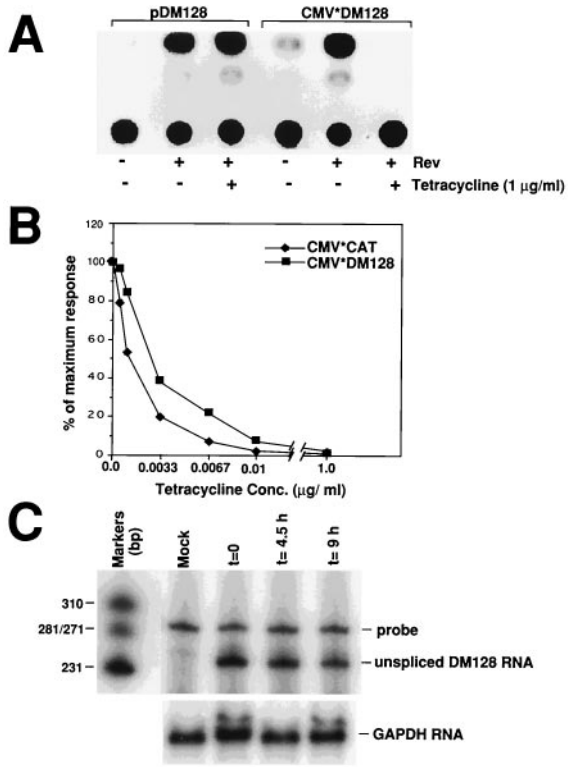

FIG. 4. Use of tetracycline-responsive promoter for analysis of Rev mechanism. (A) Analysis of the expression system. Either pDM128 or pCMV$_T$DM128 was transfected into cells along with pRSVRevGR and pREPtTA (expression vector for tTA). Immediately following transfection, cells were incubated in the presence (+Rev) or absence (−Rev) of dexamethasone and in the presence (+Tet) or absence (−Tet) of tetracycline (1.0 μg/ml). Cells were harvested 2 days posttransfection, and CAT assays were performed as previously described. (B) To assay the effect of tetracycline concentration on CMV* promoter activity in general and on the Rev response in particular, Cos cells were transfected with pCMV*CAT or pCMV*DM128 and pRSVRevGR and pCMVtTApolyA and, immediately after transfection, incubated with dexamethasone and various concentrations of tetracycline. After incubation for 2 days, cells were harvested and levels of CAT expression were determined. Data shown are the averages of a minimum of three independent determinations and are expressed as percentages of the response observed in the absence of tetracycline. (C) Determination of DM128 RNA stability. Cos cells were transfected with pDM128 and, 2 days posttransfection, treated with 4 μg of actinomycin D per ml. Cells were harvested at indicated times after addition of actinomycin D, and total RNA was extracted. Levels of DM128 RNA and GAPDH RNA were determined as outlined in Materials and Methods.

dose-response characteristics of the tetracycline-regulated system were defined. To differentiate effects of tetracycline on the CMV* promoter alone and those due to possible limitations of the Rev response, the responses of two vectors were analyzed; the first, pCMV*CATpolyA, is a Rev-independent vector and thus, its response to changes in tetracycline concentration will directly reflect changes in promoter activity alone; the second, pCMV*DM128, is a Rev-dependent vector and thus will reflect alterations in promoter function, within the limitations of Rev function, in response to changes in tetracycline concentration. Analysis of the response of pCMV*CATpolyA revealed that little or no CAT activity was detectable at concentrations of tetracycline between 0.01 and 1.0 μg/ml. Further reduction of tetracycline concentration resulted in increased CAT activity, with 50% of maximum activity reached at a concentration of 0.001 μg/ml and 90% of maximum activity achieved at 0.0005 μg/ml. In contrast to the dose-response curve of pCMV*CATpolyA, the dose-response curve of pCMV*DM128 was more rapid, reaching 50% of maximum response at a tetracycline concentration of 0.0033 μg/ml (compared with the 20% of maximum activity for pCMV*CAT polyA at this tetracycline concentration), and began to plateau at 0.001 μg/ml. The discrepancy between the dose-response curves for the two reporters despite the fact that they utilize the same promoter could be attributed to a saturation of Rev function at lower levels of RNA synthesis.

With the dose-response characteristics of the system defined, the requirement for continued transcription of pCMV*DM128 for a Rev response can be examined. However, key to the evaluation of the hypothesis is that the target mRNA be sufficiently stable to allow discrimination between a requirement for transcription and a dependence on the target RNA itself. Consequently, the half-life of DM128 RNA was determined following transfection and treatment with actinomycin D (4 μg/ml) (Fig. 4C). Analysis of the rate of DM128 loss following initiation of actinomycin D treatment indicated that this RNA is relatively stable in Cos cells, in agreement with previous findings of Malim and Cullen (32). Similar results were obtained upon inhibition of transcription with 150 μM DRB (data not shown). Therefore, following inhibition of transcription, a significant amount of DM128 RNA is present to serve as target for Rev, assuming that Rev can effect transport of this pool of RNA.

To directly test the requirement for continued synthesis of target RNA on Rev function, cells were cotransfected with pRSVRevGR, pCMV*DM128, and pCMVtTApolyA and incubated in the absence of both dexamethasone and tetracycline for 2 days to permit accumulation of DM128 mRNA. The medium was then changed to one containing dexamethasone and various concentrations of tetracycline. Analysis of the effect of tetracycline concentration on the extent of the Rev response (Fig. 5A) revealed that it was similar in this circumstance to that obtained when tetracycline was added immediately after transfection (Fig. 4A and B). A high dose of tetracycline (1 μg/ml) resulted in little or no Rev response, doses of 1 ng/ml or less resulted in a maximal response, and an intermediate dose (3.3 ng/ml) resulted in an intermediate level of response.

As a further test, the rate of response to Rev activation was examined when significantly different levels of target RNA were present prior to addition of dexamethasone. Following transfection of cells with pRSVRevGR, pCMV*DM128, and pCMVtTApolyA, cultures were incubated in either the absence or presence of tetracycline (0.01 μg/ml) for 2 days in the absence of dexamethasone. Medium was then changed to one containing dexamethasone with or without tetracycline, and the rate of CAT induction was determined. As shown in Fig. 5B, the presence of tetracycline (at either 1.0 or 0.01 μg/ml) inhibited the Rev response, consistent with the previous observation. However, of greatest significance was the finding that the rate of response after Rev activation in cultures previously maintained in 0.01 μg of tetracycline per ml was equivalent to that of cultures that were maintained in the absence of tetracycline.

DISCUSSION

Experimental systems used to date have proved successful in outlining the elements involved in Rev function. Research has resulted in the demonstration that Rev is capable of shuttling between the nucleus and cytoplasm (26, 37, 43) and the characterization of the host factor (hRIP/Rab) that is capable of interacting with the transactivation domain of Rev (1, 16). However, questions as to how Rev overrides the sequestration

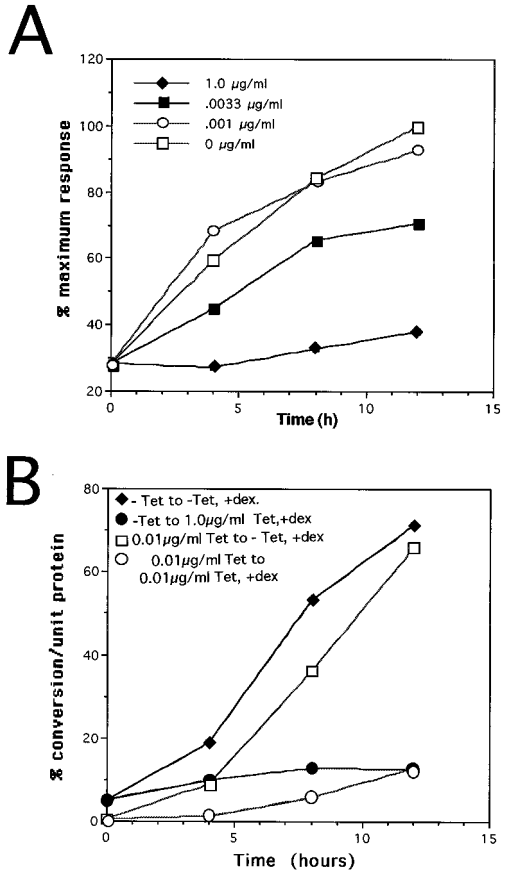

FIG. 5. Correlation of Rev Function with target RNA abundance and transcription rate. (A) Effect of transcription rate on Rev function. Cells were transfected with pCMV*DM128, pRSVRevGR, and pCMVtTApolyA. After transfection, cells were incubated in the absence of both tetracycline and dexamethasone for 2 to 3 days. Following this period of incubation, cells were treated with either dexamethasone alone or dexamethasone and the indicated concentration of tetracycline (0.0033, 0.001, or 1 μg/ml). Following treatment with dexamethasone, cells were harvested at the indicated times and CAT enzyme levels were determined. Data shown are plotted as percentages of the level of CAT expression observed in cultures incubated for 12 h with dexamethasone alone. (B) Independence of Rev function of the initial DM128 RNA concentration. Cells were transfected with pCMV*DM128, pRSVRevGR, and pCMVtTApolyA. Immediately after transfection, cells were incubated in either the absence or the presence of tetracycline (0.01 μg/ml) in the absence of dexamethasone. After 2 days, cells incubated in the absence of tetracycline were treated with dexamethasone alone (− Tet to −Tet, +dex.) or dexamethasone and 1 μg of tetracycline per ml (−Tet to 1.0 μg/ml Tet,+dex). Cells incubated with 0.01 μg of tetracycline per ml were washed and subsequently treated with either dexamethasone alone (0.01 μg/ml Tet to − Tet, +dex) or dexamethasone and 0.01 μg of tetracycline per ml (0.01 μg/ml Tet to 0.01 μg/ml Tet, +dex). Cells were harvested at indicated times, and CAT levels were determined.

of its target mRNA remain to be addressed. At least two populations of viral structural protein mRNAs exist within the nucleus: those already within sequestering/splicing complexes (probably representing the bulk of the viral structural protein RNA present within the nucleus) and newly synthesized RNA that has not yet interacted with the host factors comprising the sequestration/splicing complex. If viral structural protein RNAs present within sequestering complexes are bona fide targets for Rev, their rescue necessitates their removal from the complexes which retain them in the nucleus, a problem not associated with the rescue of newly synthesized transcripts. The demonstration that the levels of unspliced virus structural protein mRNAs in the nucleus are not significantly altered upon activation of Rev (13, 14, 22, 34) indicates that only a fraction of the RNA present is ever targeted by Rev for export, consistent with the hypothesis that only a subpopulation of the viral structural protein mRNA in the nucleus is affected by Rev expression.

To address the possibility that newly synthesized RNA may actually be the sole target for Rev, the effect of inhibition of their formation on Rev function was examined. As demonstrated in Fig. 1B, administration of DRB or actinomycin D, inhibitors of RNA polymerase II function, resulted in suppression of Rev function. Furthermore, DRB was effective at inhibiting the Rev response whether added before or after activation of the RevGR protein (Fig. 1C). Failure of actinomycin D at low concentrations to inhibit Rev function suggests that the effect observed is specific to RNA polymerase II transcripts and does not require continued synthesis of rRNA, consistent with previous experiments (9, 43). The observation that DRB has no detectable effect on total RNA transport and a limited effect on protein synthesis indicate that these steps cannot be the source of the inhibition of Rev function. Consequently, mRNA synthesis alone appears to be essential for all phases of the Rev response, consistent with newly synthesized RNA being the target for Rev.

Alternative explanations of the DRB effect, such as target RNA instability and instability of a cofactor, appear unlikely in light of the data shown in Fig. 3. Addition of DRB does not result in a dramatic loss of unspliced pDM128 RNA since significant quantities of this RNA are present up to 9 h after DRB addition. In addition, if loss or inactivation of the preexisting RNA or a host factor was the basis for the DRB effect, the duration of DRB treatment should affect the properties of induction following DRB removal. With increasing duration of treatment with DRB, one would anticipate a slower and more limited response upon removal of DRB given that a more extensive inactivation or loss would have occurred. However, in contrast to these predictions, increased length of exposure to DRB actually increases both the rate and extent of induction (Fig. 3B). That the effect is opposite what is predicted discounts the hypothesis that the ability of DRB to block Rev function can be attributed to loss or inactivation of either the RNA or a cofactor. The increase in the extent of the Rev response following DRB treatment is similar to the previously characterized superinduction phenomenon effected by treatment with antimetabolites in other systems and has been attributed to increases in mRNA stability or transcription rates or the loss of a labile repressor (11, 12, 29, 44, 45, 55).

One explanation for the data presented is that Rev functions to induce transport of newly synthesized target RNAs prior to their interaction with host components that sequester them in the nucleus (Fig. 6). That Rev is capable of interacting with nascent transcripts has been previously demonstrated by the successful replacement of the Tat-TAR interaction by substitution of RRE for TAR and use of a Tat-Rev fusion protein to confer Tat transactivation onto a promoter (52, 56). Given that interaction with RNA must occur shortly after its synthesis in order for Tat to function, the successful use of the Tat-Rev fusion to target Tat to a promoter indicates that Rev is also capable of interaction with nascent transcripts in vivo. The proposed model for Rev function explains the failure of Rev to dramatically reduce the levels of preexisting target mRNAs in the nucleus since interaction with these mRNAs would not be of any consequence. This model also predicts that the capacity of Rev to induce gene expression would be proportional to the rate of formation of the nascent target transcripts and independent of the total amount of RNA present. As a direct test of this model, the effect of specifically modulating the target

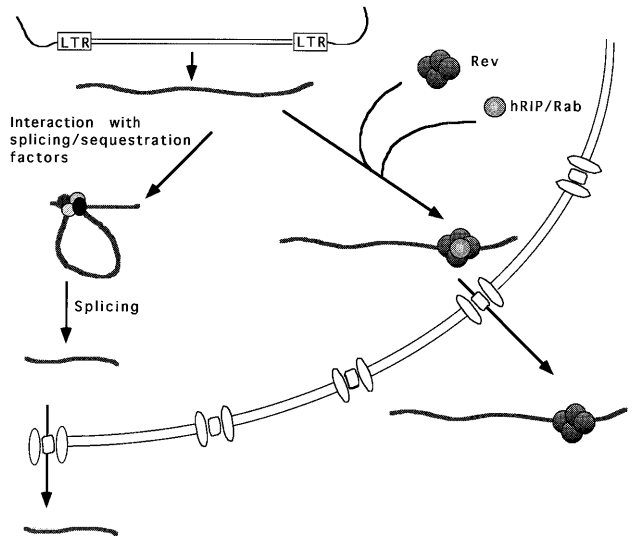

FIG. 6. Model for the mechanism of Rev-mediated export of target mRNAs. Following synthesis and in the presence of Rev, two mutually exclusive pathways are available to the nascent viral structural protein mRNA. Should interaction with Rev occur, the RNA is shuttled to the cytoplasm following interaction of the Rev-mRNA complex with hRIP/Rab. Alternatively, the nascent transcript can interact with the splicing/sequestering complexes of the nucleus. Once this latter pathway is initiated, the RNA either undergoes complete splicing prior to transport or is degraded but is unable to be rescued by interaction with Rev. LTR, long terminal repeat.

gene promoter on Rev function was investigated. Using the tetracycline-modulated promoter developed by Gossen and Bujard (18), we found (Fig. 5) that inhibition of transcription of pCMV*DM128 by addition of tetracycline resulted in loss of the Rev response even though previous experiments had established no direct effect of tetracycline on Rev function (Fig. 4A). Furthermore, the extent of response to Rev activation was found to be dependent on the rate of transcription of the target gene and independent of target mRNA concentration within the nucleus of the cell. In experiments shown in Fig. 5A, the rate of response following Rev activation was found to be proportional to the rate of transcription of the target gene despite the presence of significant levels of DM128 RNA within the nucleus. This latter point is inferred from the relative stability of DM128 RNA within the nucleus (Fig. 4C) and the finding that prior to Rev activation and tetracycline administration, DM128 RNA was being produced at twice the rate required for maximum Rev response (Fig. 4B). In further support of this hypothesis are the data in Fig. 5B. From the data on the dose-response curve of pCMV*CATpolyA in Fig. 4B, there is a 50-fold difference in transcription rates of the CMV* promoter in the absence and presence of 0.01 μg of tetracycline per ml. Since only the rate of transcription is being modulated in this system, the difference in transcription rates should directly translate into equivalent differences in RNA levels given that $d[\mathrm{R}]/dt = k1 - k2[R]$, where $[R]$ corresponds to DM128 RNA concentration, $k1$ is the rate of synthesis, and $k2$ is the rate of degradation. Therefore, after 2 days of incubation, cultures exposed to 0.01 μg of tetracycline per ml should have significantly reduced levels of DM128 RNA compared with untreated samples. The observation that the rate of induction following Rev activation was equivalent in cultures previously incubated in the presence or absence of tetracycline (Fig. 5B) indicates that the Rev response is independent of the initial level of DM128 RNA but dependent on the existing rate of transcription of the gene. The rapidity with which changes in the rate of transcription affect the capacity of Rev to rescue its target RNA suggests that it is interacting with a subpopulation of RNA with an extremely short half-life whose abundance is tightly linked to the transcription status of the gene. Such a population would be nascent transcripts prior to their interaction with the host splicing/sequestration apparatus. The failure to obtain a response to Rev activation in the absence of ongoing transcription, despite the presence of significant levels of unspliced DM128, also indicates the commitment to splicing or sequestration is not reversible. Within a short period of time after synthesis, the Rev-responsive RNA enters into a compartment or state at which point it can no longer be rescued by the binding of Rev to the RRE.

Consequently, balanced expression of proteins from spliced and unspliced forms of the primary viral transcript is achieved by the competition between entry into the splicing/sequestration pathway and Rev-mediated export to the cytoplasm (Fig. 6). The failure of Rev to alter the fate of the unspliced mRNA once committed to the splicing pathway prevents futile cycles within the cell. It also relieves Rev of the requirement to disrupt existing protein-RNA complexes that are the basis for the observed sequestration, some of which may exist several kilobases from the RRE (5, 31, 46, 50).

ACKNOWLEDGMENTS

A. Cochrane has been supported by an AmFAR/F.L. and E.L. Cummings Scholar award, as a chercheur boursier by the Federation de Recherche en Santé de Quebec (FRSQ), and as an MRC Scholar. This work was supported by grants from the Medical Research Council of Canada, the FRSQ, and National Health and Welfare Canada.

We acknowledge N. Acheson, who aided in the writing of the manuscript.

REFERENCES

1. **Bogerd, H., R. Fridell, S. Madore, and B. Cullen.** 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. Cell **82:**485–494.
2. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59:**789–795.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Cochrane, A. W., C.-H. Chen, and C. Rosen.** 1990. Specific interaction of the HIV Rev transactivator protein with a structured region in the env mRNA. Proc. Natl. Acad. Sci. USA **87:**1198–1201.
5. **Cochrane, A. W., K. S. Jones, S. Beidas, P. J. Dillon, A. M. Skalka, and C. A. Rosen.** 1991. Identification and characterization of intragenic sequences which repress human immunodeficiency virus structural gene expression. J. Virol. **65:**5305–5313.
6. **Cochrane, A. W., A. Perkins, and C. A. Rosen.** 1990. Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. J. Virol. **64:**881–885.
7. **Cullen, B. R.** 1988. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. **152:**684–704.
8. **Cullen, B. R., and W. C. Greene.** 1990. Functions of the auxiliary gene products of the human immunodeficiency virus type 1. Virology **178:**1–5.
9. **D'Agostino, D., V. Ciminale, G. Pavlakis, and L. Chiecho-Bianchi.** 1995. Intracellular trafficking of the human immunodeficiency virus type 1 Rev protein: involvement of continued rRNA synthesis in nuclear retention. AIDS Res. Hum. Retroviruses **11:**1063–1070.
10. **Daly, T., K. Cook, G. Gray, T. Maione, and J. Rusche.** 1989. Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element in vitro. Nature (London) **342:**816–819.
11. **Dinter, H., and H. Hauser.** 1987. Superinduction of the human interferon-beta promoter. EMBO J. **6:**599–604.
12. **Edwards, D., and L. Mahadevan.** 1992. Protein synthesis inhibitors differentially superinduce c-fos and c-jun by three different mechanisms: lack of evidence for labile repressors. EMBO J. **11:**2415–2424.
13. **Emerman, M., R. Vazeux, and K. Peden.** 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. Cell **57:**1155–1165.
14. **Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. The rev protein of HIV-1 affects the stability and

transport of the viral mRNA. Proc. Natl. Acad. Sci. USA **86:**1495–1499.
15. **Fischer, U., J. Huber, W. Boelens, I. Mattaj, and R. Luhrmann.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific RNAs. Cell **82:**475–483.
16. **Fritz, C., M. Zapp, and M. Green.** 1995. A human nucleoporin-like protein that specifically interacts with HIV Rev. Nature (London) **376:**530–533.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
18. **Gossen, M., and H. Bujard.** 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA **89:**5547–5551.
19. **Granick, D.** 1975. Nucleolar necklaces in chick embryo fibroblasts. 1. Formation of necklaces by dichlororibobenzimidazole and other adenosine analogs that decrease RNA synthesis and degrade preribosomes. J. Cell Biol. **65:**398–417.
20. **Greenberg, M., and E. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311:**433–438.
21. **Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis.** 1989. The Rev (Trs/Art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a *cis*-acting sequence in the *env* region. J. Virol. **63:**1265–1274.
22. **Hammarskjöld, M. L., J. Heimer, B. Hammarskjöld, I. Sangwan, L. Albert, and D. Rekosh.** 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. J. Virol. **63:**1959–1966.
23. **Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. M. Green, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element. Cell **60:**685–693.
24. **Holland, S. M., N. Ahmad, R. K. Maitra, P. Wingfield, and S. Venkatesan.** 1990. Human immunodeficiency virus Rev protein recognizes a target sequence in Rev-responsive element RNA within the context of RNA secondary structure. J. Virol. **64:**5966–5975.
25. **Hope, T. J., X. Huang, D. McDonald, and T. G. Parslow.** 1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping of cryptic functions of the arginine-rich motif. Proc. Natl. Acad. Sci. USA **87:**7787–7791.
26. **Kalland, K., A. M. Szilvay, K. Brokstad, W. Saetrevik, and G. Haukenes.** 1994. The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. Mol. Cell. Biol. **14:**7436–7444.
27. **Kriegler, M.** 1990. Gene transfer and expression: a laboratory manual. Stockton Press, New York.
28. **Kubota, S., H. Siomi, T. Satoh, S. Endo, M. Maki, and M. Hatanaka.** 1989. Functional similarity of HIV-1 Rev and HTLV-1 Rex proteins: identification of a new nucleolar-targeting signal in Rev protein. Biochem. Biophys. Res. Commun. **162:**963–970.
29. **Lebendiker, M. A., C. Tal, S. Dror, S. Pilo, A. Eilon, Y. Banai, and R. Kaempfer.** 1987. Superinduction of the human gene encoding immune interferon. EMBO J. **6:**585–589.
30. **Lu, X., J. Heimer, D. Rekosh, and M.-L. Hammarskjold.** 1990. U1 small nuclear RNA plays a direct role in the formation of a rev-regulated human immunodeficiency virus env mRNA that remains unspliced. Proc. Natl. Acad. Sci. USA **87:**7598–7602.
31. **Maldarelli, F., M. A. Martin, and K. Strebel.** 1991. Identification of post-transcriptionally active inhibitory sequences in human immunodeficiency virus type I RNA: novel level of gene regulation. J. Virol. **65:**5732–5743.
32. **Malim, M., and B. Cullen.** 1993. Rev and the fate of Pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. Mol. Cell. Biol. **13:**6180–6189.
33. **Malim, M. H., S. Bohnlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 rev trans-activator—derivation of a trans-dominant repressor of rev function. Cell **58:**205–214.
34. **Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338:**254–257.
35. **Malim, M. H., L. S. Tiley, D. F. McCarn, J. R. Rusche, J. Hauber, and B. R. Cullen.** 1990. HIV-1 structural gene expression requires binding of the Rev trans-activator to its RNA target sequence. Cell **60:**675–683.
36. **Mermer, B., B. Felber, M. Campbell, and G. N. Pavlakis.** 1990. Identification of transdominant HIV-1 Rev protein mutants by direct transfer of bacterially produced proteins into human cells. Nucleic Acids Res. **18:**2037–2044.
37. **Meyer, B., and M. Malim.** 1994. The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. Genes Dev. **8:**1538–1547.
38. **Meyer, B., J. Meinkoth, and M. Malim.** 1996. Nuclear transport of human immunodeficiency virus type 1, visna virus, and equine infectious anemia virus Rev proteins: identification of a family of transferable nuclear export signals. J. Virol. **70:**2350–2359.
39. **Olsen, H., P. Nelbock, A. Cochrane, and C. Rosen.** 1990. Secondary structure is the major determinant for interaction of HIV Rev protein with RNA. Science **247:**845–848.
40. **Olsen, H. S., A. W. Cochrane, P. J. Dillon, C. M. Nalin, and C. A. Rosen.** 1990. Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in env mRNA is dependent upon multimer formation mediated through a basic stretch of amino acids. Genes Dev. **4:**1357–1364.
41. **Perkins, A., A. W. Cochrane, S. M. Ruben, and C. A. Rosen.** 1989. Structural and functional characterization of the human immunodeficiency virus rev protein. J. Acquired Immune Defic. Syndr. **2:**256–263.
42. **Perry, R., and D. Kelley.** 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. J. Cell Physiol. **76:**127–140.
43. **Richard, N., S. Iacampo, and A. Cochrane.** 1994. HIV-1 Rev is capable of shuttling between the nucleus and cytoplasm. Virology **204:**123–131.
44. **Ringold, G., B. Dieckmann, J. Vannice, M. Trahey, and F. McCormick.** 1984. Inhibition of protein synthesis stimulates the transcription of human beta-interferon genes in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA **81:**3964–3968.
45. **Ringold, G. M., B. Dieckmann, J. L. Vannice, M. Trahey, and F. McCormick.** 1984. Inhibition of protein synthesis stimulates the transcription of human beta-interferon genes in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA **81:**3964–3968.
46. **Rosen, C. A., E. Terwilliger, A. Dayton, J. G. Sodroski, and W. A. Haseltine.** 1988. Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **85:**2071–2075.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Schwartz, S., B. K. Felber, D. M. Benko, E.-M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64:**2519–2529.
49. **Schwartz, S., B. K. Felber, E. V. Fenyo, and G. N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. **64:**5448–5456.
50. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1992. Distinct RNA sequences in the *gag* region of human immunodeficiency virus type 1 decreases RNA stability and inhibit expression in the absence of Rev protein. J. Virol. **66:**150–159.
51. **Sehgal, P. B., and I. Tamm.** 1978. Halogenated benzimidazole ribosides: novel inhibitors of RNA synthesis. Biochem. Pharmocol. **27:**2475–2485.
52. **Southgate, C., M. L. Zapp, and M. R. Green.** 1990. Activation of transcription by HIV-1 Tat protein tethered to nascent RNA through another protein. Nature (London) **345:**640–642.
53. **Stauber, R., G. Gaitanaris, and G. Pavlakis.** 1995. Analysis of trafficking of Rev and transdominant Rev proteins in living cells using green fluorescent protein fusions: transdominant Rev blocks the export of Rev from the nucleus to the cytoplasm. Virology **213:**439–449.
54. **Szilvay, A. M., K. Brokstad, R. Kopperud, G. Haukenes, and K.-H. Kalland.** 1995. Nuclear export of the human immunodeficiency virus type 1 nucleocytoplasmic shuttle protein Rev is mediated by its activation domain and is blocked by transdominant negative mutants. J. Virol. **69:**3315–3323.
55. **Tan, Y., J. Armstrong, Y. Ke, and M. Ho.** 1970. Regulation of cellular interferon production: enhancement by antimetabolites. Proc. Natl. Acad. Sci. USA **67:**464–471.
56. **Tiley, L., S. Madore, M. Malim, and B. Cullen.** 1992. The VP16 transcription activation domain is functional when targetted to a promoter-proximal RNA sequence. Genes Dev. **6:**2077–2087.
57. **Trono, D.** 1995. HIV accessory proteins: Leading roles for the supportive cast. Cell **82:**189–192.
58. **Wolff, B., G. Cohen, J. Hauber, D. Meshcheryakova, and C. Rabeck.** 1995. Nucleocytoplasmic transport of the Rev protein of human immunodeficiency virus type 1 is dependent on the activation domain of the protein. Exp. Cell Res. **217:**31–41.
59. **Zapp, M., and M. Green.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature (London) **342:**714–716.
60. **Zapp, M. L., T. J. Hope, T. G. Parslow, and M. R. Green.** 1991. Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. Proc. Natl. Acad. Sci. USA **88:**7734–7738.