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Rev-mediated nuclear export of RNA is dominant over nuclear retention and is coupled to the Ran–GTPase cycle

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ABSTRACT

The human immunodeficiency virus type-1 Rev protein induces the nuclear export of intron-containing viral mRNAs that harbor its binding site, the Rev response element (RRE). A leucine-rich region of Rev, the activation domain, is essential for function and has been shown to be a nuclear export signal (NES). Although Rev exports viral RNAs that resemble cellular mRNAs, competition studies performed using microinjected *Xenopus laevis* oocytes have previously indicated that Rev utilizes a non-mRNA export pathway. Here, we show that Rev is able to induce the export of both spliceable and non-spliceable RRE-containing pre-mRNAs and that this activity is not dependent on the location of the RRE within the RNA. Importantly, even RNA molecules of different classes, such as U3 snoRNA and U6 snRNA, which are retained in the nucleus by non-pre-mRNA mechanisms, are exported to the cytoplasm in response to Rev. Consistent with the notion that Rev-mediated export of RRE-containing RNA is mechanistically distinct from the export of processed cellular mRNA, a chimeric Rev protein in which its NES is replaced by the NES of hnRNP A1 does not induce the export of a Rev-responsive mRNA. Finally, we demonstrate that Rev/RRE-activated RNA export is, like other nuclear export pathways, linked to the Ran–GTPase cycle.

INTRODUCTION

Many nuclear encoded RNAs are post-transcriptionally exported to the cytoplasm in the form of ribonucleoprotein (RNP) complexes. Microinjection experiments performed using *Xenopus laevis* oocytes have shown that different classes of RNA including messenger, transfer, ribosomal and small nuclear RNAs (mRNA, tRNA, rRNA and snRNA, respectively) are exported to the cytoplasm via distinct pathways (1–4). These findings suggest that distinct class-specific factors (proteins) are important for the export of given RNA species. For certain RNAs, in particular tRNA, it has been demonstrated that the RNA itself binds directly to a member of the importin-β-like superfamily of nucleocytoplasmic transport (import and export) receptors (5,6). For other RNAs, the RNA may either be bridged to an export receptor by an RNA-binding adaptor protein, or be exported by mechanisms that are less well defined and might not involve proteins related to these receptors (7–12; for reviews see 13–15).

Cellular mRNA export appears, at least in part, to be mediated by heterogeneous nuclear RNP (hnRNP) proteins. In particular, hnRNP A1, a protein which is present at ~10^8 copies per somatic cell nucleus, shuttles rapidly between the nucleus and the cytoplasm and is bound to mRNA during mRNA nuclear export, and is presumed to play a major role in the export process (10,12). Importantly, a 38-amino acid region of A1 termed M9—residues 268 to 305—has been shown to be sufficient for protein nuclear export, thus defining this element as a nuclear export signal (NES) (16). Recently, nuclear microinjection of excess A1, but not of an NES-deleted derivative, was found to inhibit the export of a dihydrofolate reductase (dhfr) mRNA in *Xenopus* oocytes (17). Thus, the binding of A1 [and possibly also other hnRNP proteins (18)] to mRNA, as well as ensuing NES function, appears to facilitate the transport of cellular mRNA to the cytoplasm.

Most RNAs are retained in the nucleus and extensively modified/processed prior to being targeted to the cytoplasm. The relationship between nuclear export and RNA processing has been most extensively analyzed for pre-mRNA. For instance, studies in yeast have shown that the removal of introns by splicing markedly enhances export and that it is the interaction of spliceosomal factors with pre-mRNA that is responsible for retention in the nucleus (19,20). In other words, splicing must be completed before mature mRNA is released from nuclear retention and transported to the cytoplasm. In contrast to mRNA, other RNA species, such as the nucleolar U3, U8 and U14 snoRNAs and the spliceosomal U6 snRNA, are entirely confined to the nucleus and are never transported to the cytoplasm (21–23). The mechanisms by which these RNAs are retained in the nucleus remain largely unknown but

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are likely to differ from those that sequester incompletely processed pre-mRNAs.

While most cellular mRNAs only exit the nucleus after being fully spliced, the regulated transport of the intron-containing mRNAs of complex retroviruses such as human immunodeficiency virus type-1 (HIV-1) constitutes an important violation of this 'rule' (for reviews see 24,25). Following the onset of viral transcription, only fully spliced viral mRNAs are transported to the cytoplasm. At later times, however, both unspliced and partially spliced (intron-containing) viral mRNAs must also be exported; not only do these mRNAs encode essential virion proteins, but unspliced full-length RNA also serves as the viral genome. Importantly, this temporal regulation of viral mRNA expression is strictly dependent on the virally encoded activator of RNA export, Rev (24,25).

Numerous mutational analyses have helped to define the essential functional elements of the 116-amino acid HIV-1 Rev protein (25). The N-terminal 66 amino acids harbor sequences that mediate (i) the direct binding of Rev to all intron-containing viral RNAs via a complex stem–loop structure, the Rev response element (RRE), that is located in the *env* gene, (ii) the multimerization of Rev on the RRE and (iii) the nuclear import of Rev. Towards the C-terminus is a leucine-rich domain (previously termed the activation or effector domain) that functions as an NES and is dispensable for RRE binding and multimerization (25). More recently, it has been demonstrated that the NES of Rev binds to the export receptor exportin 1/Crm1p (7,11,26) such that intron-containing Rev–RNP complexes are targeted to and through the nuclear pore complex (NPC). To achieve this, however, Rev must also overcome the interactions that impart nuclear retention; for intron-containing HIV-1 RNAs, these are thought to be mediated by spliceosomal components (24).

Microinjection studies using *Xenopus* oocytes have shown that synthetic Rev NES peptides that are coupled to BSA (BSA-R) can inhibit the Rev-mediated nuclear export of RNA, presumably by the titration of exportin 1 (and perhaps other factors involved in Rev-mediated export) (1). The finding that BSA-R did not inhibit the nuclear export of a cellular mRNA indicated that these two export processes may require different limiting factors (1). In this study, we have examined Rev-mediated RNA export in further detail by evaluating the susceptibilities of a variety of different nuclear restricted RNAs to Rev/RRE-dependent export. Using microinjections of *Xenopus* oocytes, it was found that all RRE-containing pre-mRNAs tested, whether spliceable or non-spliceable, were exported to the cytoplasm in response to Rev. Moreover, two non-mRNA molecules, U3 small nucleolar (sno) RNA and U6 snRNA, were also exported from the nucleus in response to the Rev/RRE interaction. Consistent with the notion that the export of mRNA and Rev occur via different pathways, it was also noted that the NES of Rev could not be functionally substituted with the transferable NES of the hnRNP A1 protein. Finally, and as observed for other nuclear transport pathways, Rev activated RNA export function was shown to be linked to the Ran–GTPase cycle. The implications of these results for the interplay between nuclear retention and nucleocytoplasmic transport are discussed.

MATERIALS AND METHODS

Plasmid construction

The transcription template plasmids pAd46, pAd48 and pU6 have been described (1). pAd50 and pAd52 were similarly generated except that the RRE was located at positions 258 and 345 of the pre-mRNA. p9001 was derived from pAd50 by introducing a G to A point mutation in the 5′-splice site at intron position +5 by site directed mutagenesis. p9003 was also derived from pAd50 and lacks the 5′-exon and most of the intron up to position 234. The RRE (nt 7362–7595) was added to pU3 and pU6 at the 3′-termini of the transcript coding regions to form pU3-RRE and pU6-RRE.

The eukaryotic expression vectors pgTat, pcRev and pcRevM10 have been described (27). The Rev(wild type):A1 vectors were constructed by PCR-mediated amplification of *Bgl*II–*Hin*dIII fragments that encoded residues 268–305 (the M9 fragment or minimal NES), 235–320 (the M3 fragment) or 181–320 (M3 plus the glycine-rich region) of hnRNP A1 (16) followed by their insertion into pcRevM9 (27). The resulting Rev:A1 chimeric proteins therefore retain the N-terminal 66 residues of Rev and comprise 106, 154 and 208 amino acids, respectively. These were subsequently modified by introduction of the RevM6 mutation (27) to create the $Rev_{\Delta NLS}$:A1 chimeras.

In vitro transcription

DNAs were linearized with *Bam*HI (pAd46, pAd48, pAd50, pAd52, U6, p9001 and p9003) or *Hin*dIII (U6-RRE and U3-RRE) and used as templates for *in vitro* transcription reactions using T7 RNA polymerase and the m^7GpppG cap as described (1). ^{32}P-labeled RNAs were extracted with phenol, precipitated with ethanol and dissolved in water.

Microinjection of *X.laevis* oocytes and analysis of RNA transport

As described previously (1), *X.laevis* ovaries were incubated for 2–3 h in calcium-minus modified Barth's medium containing 0.2% collagenase type II (Sigma). Defulliculated stage V and VI oocytes were collected and stored in small fractions in Barth's medium at 18°C. For injection of Rev–RNA complexes, ^{32}P-labeled RNAs (typical specific activity 0.5×10^6 c.p.m./μl; total concentration of 0.7 μM) were incubated for 30 min on ice in Rev binding buffer [1 M NaCl, 20 mM HEPES–KOH (pH 7.9), 2.5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol and 1 mM DTT] in the presence of a 10-fold molar excess of recombinant Rev or RevM10 to allow formation of RNA–Rev complexes (1). ^{32}P-labeled U6 RNA and a 10 mg/ml solution of dextran blue were added to all mixtures to monitor the site of injection. In some experiments, purified recombinant histidine tagged wild-type Ran or RanQ69L (28) were also included in the injection cocktails at a final concentration of 2 μg/μl. Typically, 10–15 nl of solution was injected per nucleus, and oocytes were then incubated at 18°C for various times. Oocytes were manually dissected into nuclear and cytoplasmic fractions, RNAs isolated, fractionated on urea–polyacrylamide gels and visualized by autoradiography (29).

Analysis of Rev function in transfected COS cells

As described elsewhere (27), 35-mm subconfluent COS cell monolayers were transiently transfected using DEAE-dextran. Tat and Rev proteins were detected by metabolic labeling

using [^{35}S]methionine, -cysteine followed by immunoprecipitation using specific polyclonal antisera raised in rabbits against synthetic peptides, SDS–polyacrylamide gel electrophoresis, fluorography and autoradiography.

Nucleocytoplasmic shuttling in interspecies heterokaryons

The evaluation of shuttling was performed as described (30) except that transiently transfected COS cells were used to express Rev or the Rev:A1 fusions. COS cell monolayers were transfected in plastic culture dishes, replated onto poly-D-lysine treated glass coverslips at 48 h and cultured for a further 24 h. Mouse L cells were then added to the medium and allowed to settle for 3.5 h, the cultures treated with 100 µg/ml cycloheximide for 30 min to inhibit protein synthesis and the cells fused by floating the coverslips on 50% polyethylene glycol (average relative molecular mass, 1450 *g*) for ~150 s. Following extensive washing, the cultures were maintained in the presence of cycloheximide for a further 3 h, fixed and stained with the Rev-specific rabbit antiserum and 5 µg/ml Hoechst 33258. Bound antibody was detected with a fluorescein isothiocyanate conjugated anti-rabbit antibody raised in goats and the samples viewed by epifluorescence.

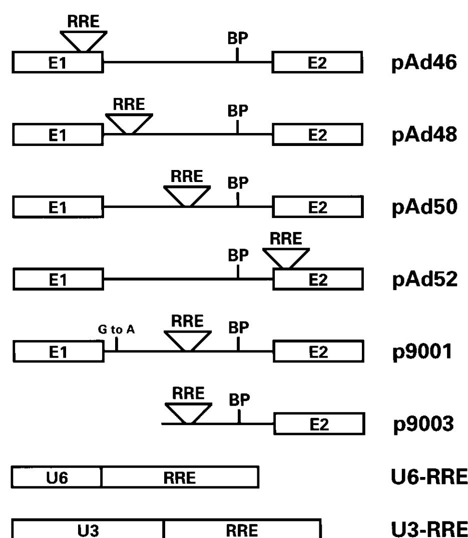

Figure 1. Schematic representation of the RNA transcripts used for *Xenopus* oocyte injections.

RESULTS

The RRE functions in both intronic and exonic positions in pre-mRNAs

In *X.laevis* oocytes, the HIV-1 Rev protein induces the nuclear export not only of an adenovirus derived pre-mRNA that harbors the RRE within its intron but also of the intron lariat itself (1,29). Although the RRE can function at different intronic locations and in the 3′-exon in transfected mammalian cells (25), we wished to determine whether this is also the case in *Xenopus* oocytes. Accordingly, the RRE was inserted in to the first exon (pAd46), the second exon (pAd52) or at two different positions of the intron (pAd48 and pAd50, respectively) of the pre-mRNA (Fig. 1). ^{32}P-labeled transcripts were generated *in vitro* and injected directly into the nuclei of oocytes either with or without a 10-fold molar excess of recombinant Rev protein. Ensuing splicing and nuclear export were determined at 90 min by cell fractionation, RNA isolation and visualization following gel electrophoresis (Fig. 2). As controls for nuclear injection and integrity, U6 snRNA was also included in all injected samples.

As expected, pAd48 pre-mRNA was efficiently spliced in the absence of Rev; the excised intron lariat was entirely restricted to the nucleus and 30–50% of the spliced product was exported to the cytoplasm (Fig. 2, lanes 3 and 4). The unspliced pre-mRNA was also restricted to the nucleus and could only be detected at early timepoints and prior to the completion of splicing (1,29) (data not shown). In contrast, the addition of Rev resulted in efficient translocation of the RRE-containing unspliced pre-mRNA and excised intron lariat to the cytoplasm (Fig. 2, lanes 11 and 12). Essentially identical results were obtained when the RRE was moved to a position more distal to the 5′-splice site in pre-mRNA pAd50 (Fig. 2, lanes 5, 6, 13 and 14).

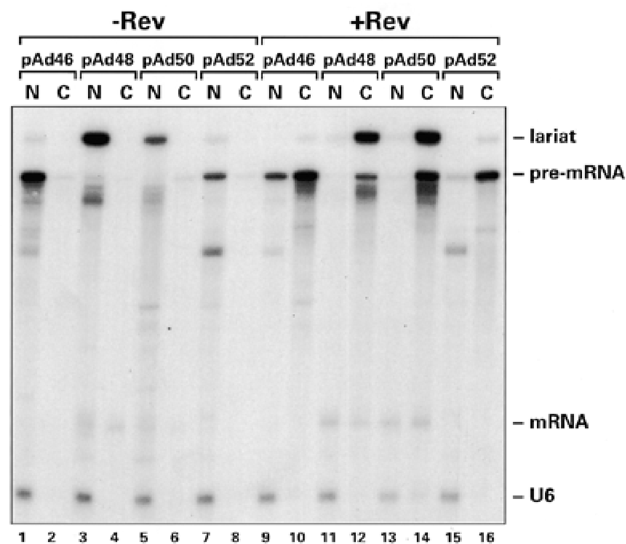

Figure 2. Rev-mediated export of adenovirus pre-mRNA is independent of the position of the RRE. ^{32}P-labeled adenovirus pre-mRNAs containing the RRE in the intron (pAd48 and pAd50) or in the first or second exon (pAd46 and pAd52, respectively) were injected into the nuclei of *Xenopus* oocytes either in the absence (lanes 1–8) or presence (lanes 9–16) of recombinant Rev protein. At 90 min, the oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions and the RNA was analyzed using denaturing polyacrylamide gels and autoradiography. U6 snRNA was co-injected in all experiments to ensure that the injection occurred exclusively into the nucleus. The bands that correspond to pre-mRNA, lariat, spliced mRNA and U6 are indicated to the right of the gel. The same results were also obtained when Rev was injected 1 h later than pAd46 or pAd52 RNAs, confirming that Rev does not function by inhibiting the interaction of nuclear retention factors.

To address RRE function in exonic locations, oocyte nuclei were injected with the pAd46 or pAd52 pre-mRNAs with or without Rev. In contrast to the RNAs described above, both of these pre-mRNAs were spliced very inefficiently (Fig. 2, lanes 1, 2, 7 and 8); this finding was further confirmed using *in vitro* splicing extracts (data not shown). We speculate that

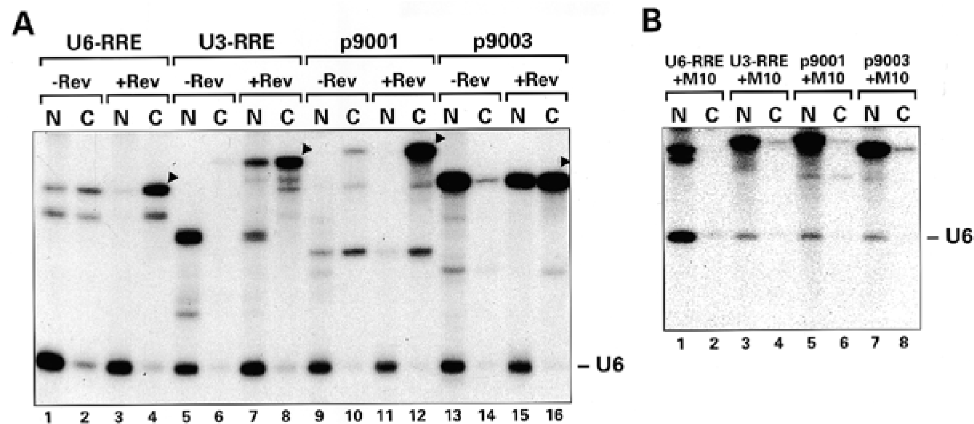

Figure 3. Rev is dominant over nuclear retention of different classes of RNA. (**A**) Radiolabeled U6-RRE, U3-RRE, p9001 and p9003 RNAs were mixed with U6 snRNA and injected either alone (lanes 1, 2, 5, 6, 9, 10, 13 and 14, respectively) or along with recombinant Rev protein (lanes 3, 4, 7, 8, 11, 12, 15 and 16, respectively) into the nucleus of oocytes. Nuclear export was analyzed after 90 min as in Figure 2. The exported full-length RNAs in lanes 4, 8, 12 and 16 are indicated with arrowheads. (**B**) Using the same set of oocytes, the same RNAs as in (A) were also nuclear injected with RevM10 and export analyzed.

these splicing defects are most likely to be due to the close proximity of the RRE to the 5′- or 3′-splice site and the subsequent inhibition of RNA recognition (and binding) by spliceosomal factors. Nevertheless, both pAd46 and pAd52 pre-mRNAs were confined to the nucleus in the absence of Rev (Fig. 2, lanes 1, 2, 7 and 8) even after prolonged incubations of up to 3 h (data not shown). Importantly, when these RNAs were co-injected with Rev they were efficiently exported to the cytoplasm (Fig. 2, lanes 9, 10, 15 and 16). We therefore conclude that the presence of the RRE in a pre-mRNA provides Rev responsiveness independent of its specific localization.

Rev activates the nuclear export of pre-mRNAs with deleted consensus splice site signals

The results obtained with the pAd46 and pAd52 pre-mRNAs suggest that the capacity to be spliced is not required for the Rev response. This would appear to be consistent with the earlier observation that a non-spliceable RNA that carried a non-conventional ApppG 5′-cap was rendered Rev-responsive by the addition of the RRE (29). To evaluate further the importance of splicing in the oocyte system, we next examined the ability of Rev to export pre-mRNA mutants that are defective in splicing and that, as a result, are retained in the nucleus as non-productive splicing complexes. It was anticipated that such experiments would indicate whether Rev can actively dissociate pre-mRNAs from nuclear retention sites in oocyte nuclei. The p9001 and p9003 pre-mRNA mutants were therefore generated and their export phenotypes determined by nuclear injection of *Xenopus* oocytes (Figs 1 and 3). The p9001 mRNA contains a G to A point mutation at the +5 position of the intron but is otherwise identical to pAd50 pre-mRNA; p9003 mRNA is a deletion mutant that lacks the 5′-exon and most of the intron but retains the RRE, branch point and 3′-splice site (Fig. 1). Importantly, both RNAs form a splicing commitment complex *in vivo* and *in vitro* (also called complex A) but fail to either dissociate or form additional spliceosomal complexes (20) (data not shown).

In the absence of Rev, nuclear injected pAd9001 RNA was highly unstable (Fig. 3A, lanes 9 and 10). p9003 RNA was stable, however, and, as would be expected for an RNA that assembles into a stable commitment complex, was entirely nuclear (Fig. 3A, lanes 13 and 14). These two RNAs were then co-injected with a 10-fold molar excess of Rev. In both cases, the RNAs were not only stable but were also efficiently transported to the cytoplasm (Fig. 3A, lanes 11, 12, 15 and 16). To confirm that the induction of nuclear export was due to the action of a functional Rev protein and not the result of masking of the RNA from retention factors, a non-functional mutant Rev protein, RevM10, was also co-injected with these RNAs. This particular protein has a disrupted NES but is still able to bind to the RRE as efficiently as wild-type Rev; its defect in activity is therefore due to an inability to interact with exportin 1 and, possibly, other cellular cofactors (25). As shown in Figure 3B, the p9001 and p9003 RNAs remained entirely nuclear in the presence of RevM10 (Fig. 3B, lanes 5–8). In contrast to oocytes that received p9001 RNA alone, the addition of RevM10 appeared to confer RNA stabilization (compare Fig. 3A, lane 9 with Fig. 3B, lane 5), implying that RevM10 does indeed bind to the RRE under these *in vivo* conditions. Taken together, these results show that Rev can activate the export of RNAs that are retained in the nucleus as stable 'dead-end' splicing complexes.

Rev-dependent nuclear export of U6 snRNA and U3 snoRNA

As discussed earlier, U3 snoRNA, U6 snRNA and pre-mRNA are all retained in the nucleus. Importantly, however, the mechanism by which this occurs for U3 and U6 appears to be very different than for the spliceosome-mediated retention of pre-mRNA. Thus, the RNA polymerase III transcribed U6 snRNA is retained (at least in part) by its association with the La protein while U3 snoRNA localizes to nucleoli where it is retained by one or several (as yet unidentified) saturable Box D interacting factor(s) (21–23,31). It was therefore of interest to test whether human U3 or U6 could be programed for nuclear export by Rev. The RRE was fused to the 3′-termini of both

RNAs (Fig. 1) and the resulting U3-RRE or U6-RRE chimeric RNAs were injected into the nuclei of oocytes with an equal amount of U6 RNA in the presence or absence of Rev (Fig. 3A).

Both RNAs were unstable in the absence of Rev with the RRE portion of each being degraded; as expected, the resulting 'mature' U3 and U6 RNAs still localized predominantly to the nucleus (Fig. 3A, lanes 1, 2, 5 and 6; in the case of U6-RRE, the degraded U6 product co-migrates with the co-injected unit length U6). In oocytes that also received Rev, both chimeric RNAs were stabilized as full-length species and efficiently exported to the cytoplasm (Fig. 3A, lanes 3, 4, 7 and 8). A potential explanation for the observed export of the U3-RRE and U6-RRE RNAs was that the interaction of Rev with the RRE might have interfered with the appropriate association of these RNAs with their respective nuclear retention factors. To exclude this possibility, we also injected U3-RRE or U6-RRE together with RevM10 into nuclei and analyzed the patterns of localization. As illustrated in Figure 3B, RevM10 mediated the stabilization of both chimeric RNAs in the nucleus but failed to induce the nuclear export of either (lanes 1–4). In conclusion, Rev is not only able to override the nuclear retention of pre-mRNA but is also capable of activating the nuclear export of non-pre-mRNAs that would otherwise be restricted to the nucleus.

The NES of hnRNP A1 cannot functionally replace Rev's NES

The HIV-1 Rev NES can be functionally substituted by the NESs of other lentivirus Rev proteins or the Rex protein of human T-cell leukemia virus type-1 (HTLV-1) as well as by the leucine-rich NESs of non-viral proteins such as the inhibitor of protein kinase A (PKI), the fragile X mental retardation (FMR) 1 protein and the inhibitor of NF-κB (IκBα) (24,25). To determine whether the NES of A1, which displays no discernible primary sequence similarity to leucine-rich NESs (32), could also function in this context, Rev:A1 chimeric proteins were constructed in which NES-containing regions of A1 that comprised residues 268–305 (the M9 fragment), 235–320 or 181–320 were appended to the N-terminal 66 amino acids of HIV-1 Rev. As noted above, this region of Rev is fully sufficient for nuclear import and RRE binding (24,25). The *trans*-activation potential of each of these chimeric proteins was evaluated in transfected COS cells using a reporter plasmid, gTat, which expresses a genomic version of the HIV-1 *tat* gene. This vector expresses two *tat* mRNAs, a fully spliced transcript that encodes an 86-amino acid form of Tat and an unspliced transcript that contains the RRE and encodes a foreshortened Tat protein of 72 amino acids. Because the unspliced mRNA only enters the cytoplasm in the presence of a functional Rev, or Rev chimerum, synthesis of the 72-amino acid Tat protein serves as a sensitive indicator of Rev function (27).

COS cell monolayers were transiently cotransfected with gTat and vectors that expressed wild-type Rev (positive control), RevM10, the Rev:A1 fusion proteins or an irrelevant protein (negative control). At 48 h, the cells were metabolically labeled with [^{35}S]cysteine, -methionine for 2 h and lysed in RIPA buffer. The Tat and Rev proteins were then immunoprecipitated with relevant antisera, electrophoretically resolved and visualized by autoradiography (Fig. 4, upper and lower panels). As described previously, gTat transfected cells only expressed the 86-amino acid form of Tat (Fig. 4, lane 2) whereas cells containing Rev also synthesized the 72-amino acid Tat protein (Fig. 4, lane 3). In contrast, neither RevM10

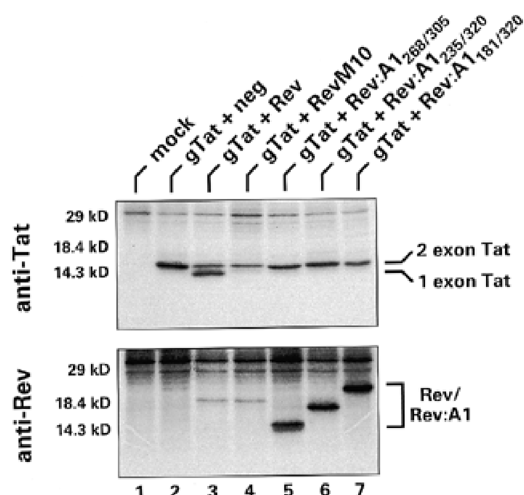

Figure 4. The NES domains of HIV-1 Rev and hnRNP A1 are not functionally interchangeable. COS cell monolayers were mock transfected (lane 1) or transfected with gTat + negative control vector (lane 2), gTat + Rev (lane 3), gTat + RevM10 (lane 4), gTat + Rev:A1$_{268/305}$ (lane 5), gTat + Rev:A1$_{235/320}$ (lane 6) or gTat + Rev:A1$_{181/320}$ (lane 7). Following metabolic labeling, cell lysates were immunoprecipitated with anti-Tat (**top**) or anti-Rev (**bottom**) antisera and visualized following electrophoresis through SDS-14% polyacrylamide gels.

nor any of the Rev:A1 chimeras were capable of inducing expression of 72-amino acid Tat (Fig. 4, lanes 4–7) and, presumably, of activating the export of unspliced *tat* transcripts. Importantly, insufficient expression levels cannot account for the lack of Rev function in these samples as the various Rev proteins were readily detected by the Rev-specific antiserum (Fig. 4, lower panel).

To address possible explanations for the inability of these Rev:A1 chimeras to activate the nuclear export of a Rev-responsive RNA, we evaluated the export capabilities of these fusion proteins using interspecies heterokaryons (Fig. 5). COS cell monolayers were initially transfected with vectors expressing wild-type Rev or each of the three Rev:A1 proteins. At 72 h, each culture was fused to untransfected mouse L cells, maintained in cycloheximide-containing medium for 3 h and subjected to indirect immunofluorescence and Hoechst staining (the L cell nuclei are distinguished from the COS cell nuclei by their smaller size and regions of intense Hoechst staining). As shown previously, Rev shuttles rapidly between the nucleus and the cytoplasm and was therefore efficiently relocalized to the L cell-derived nuclei of expressing heterokaryons (Fig. 5a–c) (30). In marked contrast, all three Rev:A1 fusion proteins, which each localized to the nuclei/nucleoli of the transfected COS cells, remained in the COS cell-derived nuclei for the duration of the experiment (representative results for Rev:A1$_{181/320}$ are shown in Fig. 5d–f). Two potential explanations for these results are that the nature of the fusions between Rev and A1 inactivates the A1 NES, or that interactions between Rev and nuclear components cannot be overcome by A1's NES. To examine these possibilities, NES-containing fragments of A1 were joined to the N-terminal region of a mutant Rev protein, RevM6, that does not accumulate in the

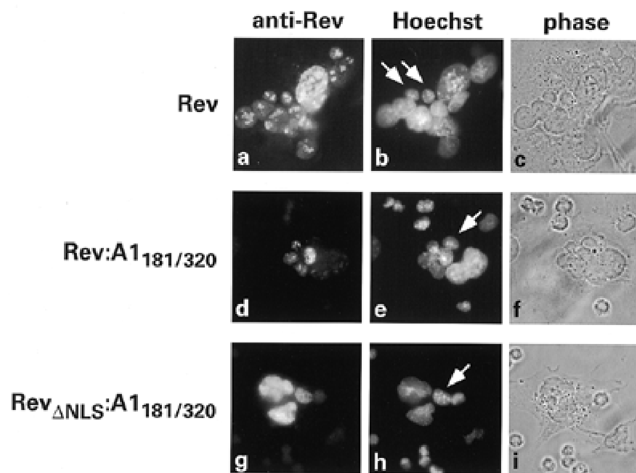

Figure 5. The NES of hnRNP A1 does not confer nuclear export on the HIV-1 Rev protein. COS cell monolayers were transfected with vectors encoding either Rev (**a**, **b** and **c**), Rev:A1$_{181/320}$ (**d**, **e** and **f**) or Rev$_{\Delta NLS}$:A1$_{181/320}$ (**g**, **h** and **i**) and fused to mouse L cells to form heterokaryons. Following incubation for 3 h in the absence of protein synthesis, fixed cells were stained with an anti-Rev antiserum (a, d and g) and Hoechst 33258 (b, e and h, L cell-derived nuclei are indicated with arrows). The corresponding phase contrast analyses are also shown (c, f and i).

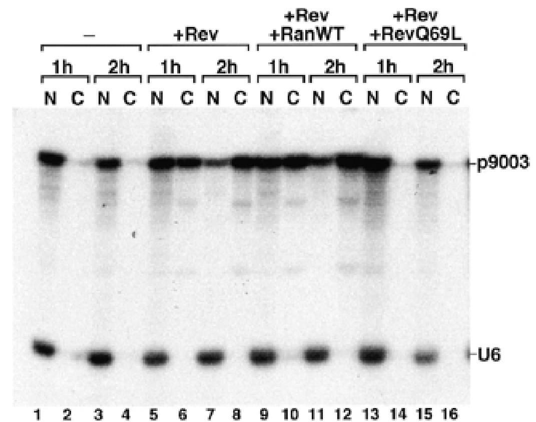

Figure 6. Sensitivity of Rev-mediated RNA export to RanQ69L. A mixture of p9003 RNA and U6 snRNA was injected into *Xenopus* oocyte nuclei either alone (lanes 1–4), with wild-type Rev (lanes 5–8), with Rev and wild-type Ran (lanes 9–12) or with Rev and RanQ69L (lanes 13–16). Nuclear export was measured 1 and 2 h later as in Figure 2.

nucleus (25,27), and their respective shuttling capabilities tested in heterokaryon assays. Importantly, and in contrast to the fusions with wild-type Rev sequences, the Rev$_{\Delta NLS}$:A1 fusions not only localized to the nucleus, but also shuttled efficiently (results for Rev$_{\Delta NLS}$:A1$_{181/320}$ are shown in Fig. 5h and i). Of note, the nuclear localization of these chimeras is explained by the fact that the NES of A1 also functions as a nuclear localization signal (NLS) (16,33). Thus, the NES of A1, while clearly transferable, is not capable of conferring export activity on either wild-type Rev or intron-containing, Rev-responsive RNA.

Rev-mediated RNA export is linked to the Ran–GTPase cycle

It has been demonstrated that the export of RNA out of the nucleus is functionally linked to the Ran–GTPase cycle (4,5,34–37). To test whether this relationship extends to Rev-mediated RNA export, we assessed the effect of perturbation of the Ran–GTPase system in *Xenopus* oocytes (Fig. 6). For this purpose, we purified both wild-type Ran and a mutated derivative in which the glutamine at position 69 has been substituted for leucine (RanQ69L). Because this mutant is deficient for GTPase activity (35,37), export processes which are either linked to, or dependent on, Ran-mediated GTP hydrolysis should be inhibited by the addition of RanQ69L. The export potential of p9003 derived RNA was therefore determined 1 or 2 h following nuclear injection alone, with Rev, with Rev and RanQ69L or with Rev and wild-type Ran. As expected, the RNA was restricted to the nucleus in the absence of Rev (Fig. 6, lanes 1–4), or transported to the cytoplasm in its presence (Fig. 6, lanes 5–8). In contrast to excess wild-type Ran which had no discernible effect on Rev function (Fig. 6, lanes 9–12), RanQ69L was able to inhibit the Rev-mediated export of RRE-containing RNA to the cytoplasm to a significant degree (Fig. 6, lanes 13–16). We conclude, therefore, that Rev-mediated RNA export is coupled to the Ran–GTPase cycle.

DISCUSSION

It has been established that the HIV-1 Rev *trans*-activator contains an NES that directly promotes the nuclear export of RRE-containing viral RNAs (25). Despite this generally accepted view of Rev-regulated transport, many of the specific aspects of this export process remain to be elucidated. In particular, it is unclear how Rev releases RRE-containing mRNA from nuclear retention. Using microinjections of *Xenopus* oocytes, we demonstrate that Rev function is not restricted solely to mRNA, but that the transport of sn/snoRNAs to the cytoplasm can also be rendered sensitive to Rev (Fig. 3). Thus, RNAs that are retained in the nucleus by functional pre-mRNA splice sites and interacting factors (Fig. 2) (19,20), 'dead-end' non-functional splicing complexes (Fig. 3), an inappropriate 5′-cap structure (20,29) or non-mRNA related mechanisms (Fig. 3), can each be induced to undergo nuclear export in response to the Rev/RRE interaction. The mechanism of Rev-mediated RNA export therefore appears to be independent of the mode of nuclear retention and can, accordingly, be considered 'dominant' over all such nucleus restricted fates. In other words, it appears unlikely that specific interactions between Rev and individual RNA retention factors are required for Rev function.

Of particular interest concerning the nuclear export of RNA are the potential commonalties and differences that may exist between the Rev-regulated export of HIV-1 mRNAs and the apparently constitutive transport of fully processed cellular mRNAs. For instance, the Rev NES can saturate the nuclear export of both U snRNAs and 5S rRNA but not of mRNA in *Xenopus* oocytes (1). To address this issue further, we have examined whether the leucine-rich NES of Rev is functionally interchangeable with the NES of the mRNA export factor hnRNP A1. The finding that various Rev:A1 chimeric proteins

were devoid of Rev function (Fig. 4) suggests that these two pathways of RNA export may be fundamentally different, a result that is consistent with the aforementioned competition experiments. Moreover, the fact that Rev can induce the nuclear export of any RNA that is confined to the nucleus whereas hnRNP A1—which binds to both intron-containing pre-mRNA and mature mRNA—appears only to be capable of mediating the export of mature mRNA, is also indicative of important dissimilarities between these pathways.

In addition to being inactive in RNA export, the wild-type Rev:A1 fusions examined here were, themselves, also unable to exit the nucleus (Fig. 5). In contrast, however, derivative $Rev_{\Delta NLS}$:A1 fusions were clearly capable of efficient nuclear export. This result, together with the earlier demonstration that the A1 NES is sufficient to confer nuclear export on the nucleoplasmin core domain (16), suggests that the RNA binding/NLS domain of Rev may be retained in the nucleus by interactions that are dominant over the export capabilities of A1's NES. Since it has been suggested that A1 has a role in the export of fully processed mRNA, it is possible that nuclear retention that is mediated by the interaction of splicing factors with pre-mRNA may also be dominant over export that is mediated by A1. An alternative, though in our view less likely, explanation for the retention of Rev:A1 fusion proteins might be that the precise nuclear location(s) at which these chimeras accumulate may not be accessible to the A1 NES export machinery.

Despite the differences that appear to exist between the Rev/RRE and mRNA export pathways, both are inhibited in *Xenopus* oocytes by the nuclear injection of the GTPase-deficient Ran mutant Q69L (Fig. 6). These results, therefore, further support the idea that the nuclear export of diverse RNPs is linked to the activity of the Ran–GTPase cycle. Interestingly, it has recently been demonstrated that the nuclear export of a leucine-rich NES that is not associated with RNA requires Ran to be in the GTP-bound state but can be uncoupled from Ran-mediated GTP hydrolysis (35). Understanding this striking difference between protein-mediated RNA (RNP) export and protein export will no doubt be aided by a more detailed appreciation of the roles of Ran, its associated factors and GTP hydrolysis in nuclear transport.

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