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The simian retrovirus-1 constitutive transport element, unlike the HIV-1 RRE, uses factors required for cellular mRNA export

Claudio Saavedra*, Barbara Felber† and Elisa Izaurralde*

Background: A hallmark of retroviral gene expression is that unspliced retroviral genomic RNA is exported to the cytoplasm, whereas endogenous intron-containing cellular RNAs are usually retained in the nucleus. In complex retroviruses, such as human immunodeficiency virus-1 (HIV-1), nuclear export is accomplished by the interaction of a virally encoded protein, Rev, with a *cis*-acting RNA element, the Rev-responsive element (RRE). In type D retroviruses, such as the simian retrovirus type 1 (SRV-1), however, genomic RNA is exported by cellular factor(s) that interact with a conserved *cis*-acting RNA element, the constitutive transport element (CTE).

Results: We found that the CTE was exported in a specific and saturable fashion from *Xenopus* oocyte nuclei. When inserted into the intron of an adenovirus-derived pre-mRNA, the CTE did not affect splicing efficiency but promoted the nuclear export of the excised intron lariat that is normally retained within the nucleus. Export of CTE-containing RNAs to the cytoplasm was not affected by the heterogeneous nuclear ribonucleoprotein A1 or an excess of peptides corresponding to the Rev nuclear export signal. Microinjection of saturating amounts of CTE RNA did not affect tRNA export or Rev-mediated export but did inhibit mRNA export. CTE-mediated export was found to be dependent on Ran-mediated GTP hydrolysis.

Conclusion: The Rev–RRE system and the CTE direct intron-containing RNAs to distinct export pathways. Although previous data have suggested that Rev uses the same export pathway as uracil-rich small nuclear RNAs and 5S ribosomal RNA, the CTE seems to interact with evolutionarily conserved factors that are essential for cellular mRNA export.

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Background

RNA export from the nucleus is an energy-dependent process that relies on recognition of the RNA or ribonucleoprotein export substrates by saturable factors [1–3]. Microinjection experiments in *Xenopus* oocytes have revealed that different classes of RNA — transfer RNA (tRNA), uracil-rich small nuclear RNA (UsnRNA), messenger RNA (mRNA) and ribosomal RNA (rRNA) — do not compete for export [3], indicating that their export is mediated at least in part by specific factors [3–6]. These factors are likely to be RNA-binding proteins that recognise a common feature exhibited by RNA molecules of the same class. Several cellular RNA-binding proteins have been suggested to play a role in the export of different classes of RNAs (reviewed in [7]) but, with the exception of the nuclear cap-binding protein complex implicated in the export of RNA polymerase II-transcribed spliceosomal UsnRNAs [8], none of these RNA-binding proteins has been shown to be directly involved in export.

Export of mRNAs has been suggested to be mediated by heterogeneous nuclear ribonucleoproteins (hnRNPs; reviewed in [9]). Of these, the best candidate is hnRNP A1, which shuttles constantly between the nucleus and cytoplasm [10]. Shuttling of hnRNP A1 is determined by a 38 amino-acid sequence, termed M9, located near the carboxyl terminus of the protein [11]; the M9 import receptor has been identified as transportin [12,13]. Recently, it has been shown that an excess of hnRNP A1 in the nucleus saturates export of mRNA, providing evidence of a role for this protein in mRNA export [14]. In order to prevent translation of mRNAs into aberrant cellular proteins, incompletely spliced mRNAs are retained in the nucleus by binding to spliceosomal factors. The mature mRNA, but not the excised intron, can be exported only after splicing is completed [15].

To circumvent the requirement of splicing prior to export, all retroviruses have evolved a mechanism that allows the nuclear export of unspliced forms of viral RNAs, which are essential for viral replication. In the case of complex retroviruses, a highly regulated mechanism has been identified. This mechanism, best characterised in the human immunodeficiency virus type 1 (HIV-1), requires the activity of

the virally encoded Rev protein and the presence of a Rev response element (RRE) in the RNA [16–19]. The appearance of intron-containing viral RNA in the cytoplasm is directly promoted by the association between Rev and the RRE. The complex is then exported by virtue of a leucine-rich nuclear export signal (Rev-NES) present in the Rev carboxy-terminal domain [20,21]. Competition experiments have shown that the Rev-NES promotes RNA nuclear export by interacting with cellular factors that participate in the export of two other classes of cellular RNAs: UsnRNAs and 5S rRNA [4].

Simpler retroviruses, however, do not encode a Rev-like *trans*-acting protein. In the case of the simian type D retroviruses, Mason-Pfizer monkey virus (MPMV) and the simian retroviruses type 1 and 2 (SRV-1 and SRV-2), a *cis*-acting RNA element was recently demonstrated to act as an autonomous export signal for unspliced mRNAs [22–25]. This structured RNA element, termed the constitutive transport element (CTE), shares structural and functional similarities in all three type D retroviruses [23]. Furthermore, the CTEs from MPMV and SRV-1 can functionally replace the Rev–RRE system in the context of RNA export and HIV-1 virus replication [24,25], suggesting that cellular factor(s) could play an analogous role to that of Rev protein.

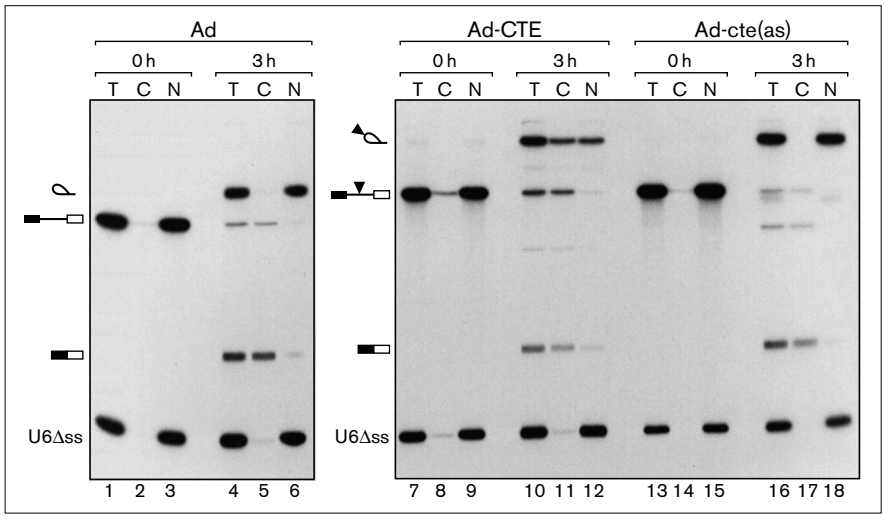
In this study, we report that the SRV-1 CTE, unlike the HIV-1 Rev–RRE system, uses cellular factors that are essential for the export of mRNAs. We propose that, after overcoming nuclear retention, the CTE allows intron-containing RNAs to access a late step in the mRNA export pathway.

Results

The SRV-1 CTE promotes nuclear export of excised intron lariats in *Xenopus* oocytes

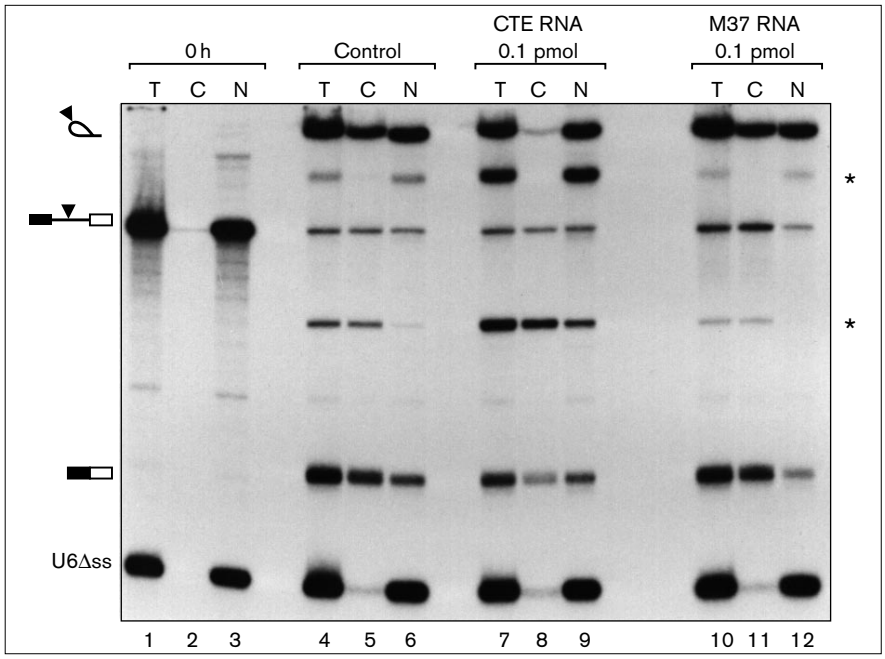
The CTE has been proposed to interact with the cellular transport machinery in order to bypass the retention mechanisms that normally confine pre-mRNAs to the nucleus [22–26]. As a first step towards the identification of the nuclear export pathway used by the CTE, we investigated whether this element would promote the nuclear export of intron-containing RNAs in *Xenopus* oocytes. A 173 bp DNA fragment corresponding to the SRV-1 CTE was cloned into the intron of an adenovirus-derived precursor [27], which has previously been shown to be spliced efficiently in *Xenopus* oocytes [28]. This insertion generated two precursors that had the CTE inserted either in the sense orientation, Ad-CTE, or the antisense orientation, Ad-cte(as). *Xenopus* oocyte nuclei were injected with ^{32}P-radiolabelled RNAs synthesised *in vitro* using the original adenovirus-derived construct (Ad), Ad-CTE or Ad-cte(as) as templates. As an internal control for nuclear injection and nuclear envelope integrity, we included radiolabelled U6Δss RNA in the injection mixtures: this RNA is not exported from the nucleus and lacks the protein-binding sites required for nuclear import, therefore remaining localised at the site of injection [29,30]. After a 3 hour incubation, oocytes were dissected and the nucleocytoplasmic distribution of the precursor RNA and the splicing products was analysed. Figure 1 shows that whereas the intron lariats derived from the splicing of Ad and Ad-cte(as) templates were retained in the nucleus, the excised intron lariat from Ad-CTE was exported to the cytoplasm (Figure 1, compare lanes 4–6 and 16–18 with 10–12). In contrast, the spliced mRNA product (Ad-mRNA), which is

Figure 1



Excised intron lariats harbouring the SRV-1 CTE are exported to the cytoplasm in *Xenopus* oocytes. *Xenopus* oocyte nuclei were injected with a mixture of *in vitro* transcribed ^{32}P-labelled U6Δss RNA and the following precursor RNAs: Ad (lanes 1–6), Ad-CTE (lanes 7–12) and Ad-cte(as) (lanes 13–18). The three precursors are derived from the adenovirus major late transcription unit. Ad-CTE and Ad-cte(as) contain the SRV-1 CTE inserted in the sense and the antisense orientation, respectively. U6Δss does not leave the nucleus and is an internal control for nuclear integrity. Synthesis of precursor RNAs was primed with the m^{7}GpppG cap dinucleotide, whereas synthesis of U6Δss RNA was primed with γ-mGTP. RNA samples from total oocytes (T), cytoplasmic (C) and nuclear (N) fractions were collected immediately after injection (0 h) or 3 h after injection as indicated. Products of the splicing reaction were resolved on 10% acrylamide 7 M urea denaturing gels. The mature products and intermediates of the splicing reaction are indicated diagramatically on the left of the panels. The filled triangle represents the CTE.

Figure 2



Saturation of the CTE nuclear export pathway. A mixture of *in vitro* transcribed radiolabelled Ad-CTE precursor RNA and U6Δss RNA was injected into *Xenopus* oocyte nuclei either without competitor (lanes 1–6) or with unlabelled competitor CTE RNA (lanes 7–9) or unlabelled M37 competitor RNA (lanes 10–12). The M37 RNA is a mutant form of the CTE lacking loops A and B. The amount of competitor RNA injected per oocyte nucleus was 0.1 pmol. Synthesis of competitor RNAs was primed with the ApppG dinucleotide. In lanes 1–3, RNA was extracted immediately after injection, and in lanes 4–12, oocytes were dissected 3 h after injection. RNAs were analysed as described in Figure 1. Symbols are as in Figure 1. The asterisks on the right of the panel indicates the position of RNA molecules that are likely to originate from the degradation of the intron lariat and the precursor RNA. These degraded RNAs are also present in Figures 4,5.

identical for the three precursors, was exported to the cytoplasm with the same efficiency irrespective of the pre-mRNA from which it originated (Figure 1, lanes 4–6, 10–12, 16–18). The efficiency of splicing was similar for the three precursor RNAs (Ad, Ad-CTE and Ad-cte(as); lanes 4, 10 and 16, respectively) indicating that, in agreement with a previous report [22], the CTE does not interfere with spliceosome assembly. However, a small fraction of the precursors remained unspliced after 3 hours incubation: this small fraction was exported to the cytoplasm in all three cases, and therefore was probably not committed to splicing and not retained in the nucleus. Although more of the CTE-containing pre-mRNA was found in the cytoplasm, it is not clear that this difference is significant.

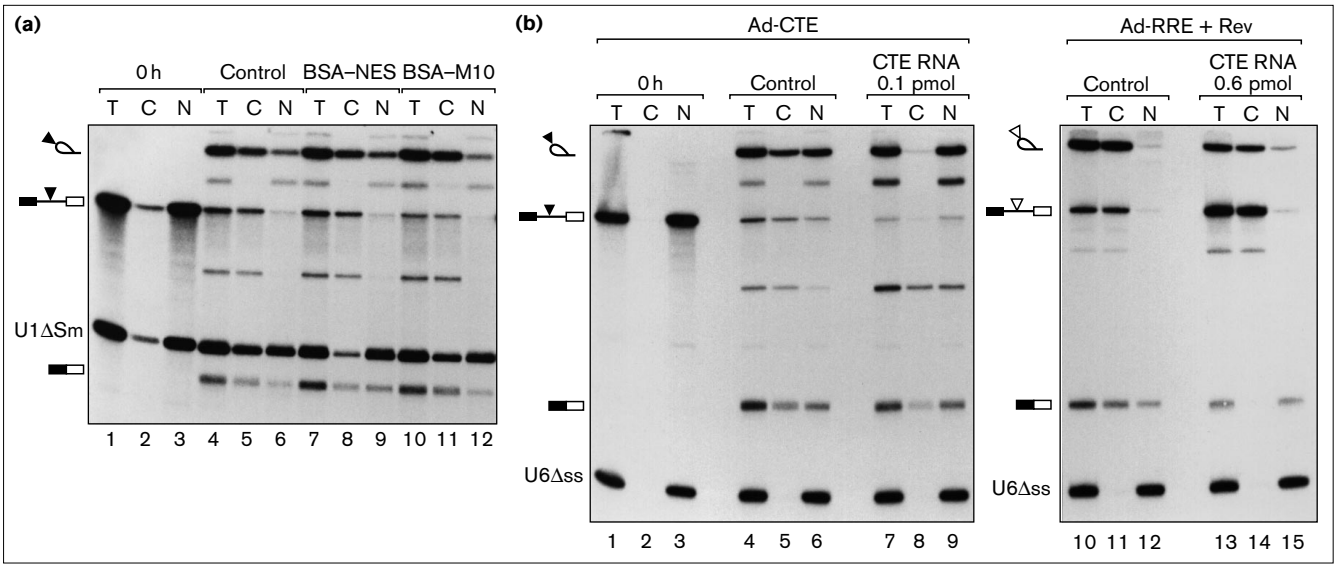
Aside from the mature RNA, only the intron lariat harbouring the CTE is efficiently exported. This *cis*-acting element is sufficient to bypass the mechanism that causes nuclear retention of excised introns and to promote the export of intron-containing RNAs into the cytoplasm. The finding that the CTE functions in *Xenopus* oocytes indicates that the cellular factors that recognise this element are conserved from *Xenopus* to mammals.

CTE-mediated export is saturable

As mentioned above, the CTE element is likely to promote the export of intron-containing RNAs by interacting with cellular factors. In order to test whether these factors are limiting, radiolabelled Ad-CTE pre-mRNA was co-injected into oocyte nuclei together with an excess of unlabelled CTE RNA. The same amount of an RNA corresponding to a CTE mutant (termed M37) that contains a deletion of loop A and loop B was used as a negative control. The M37 mutant is defective in mediating HIV-1 Gag production from an expression plasmid (C. Tabernero and B.F. unpublished observations). In order to avoid any possible competition for the nuclear cap-binding complex that participates in both pre-mRNA processing and export of RNA from the nucleus [8,31], synthesis of the competitor RNAs was primed with the cap analogue ApppG instead of m^7GpppG. As shown in Figure 2, injection of 0.1 picomoles of competitor CTE RNA per oocyte nucleus abolished export of the CTE-containing intron lariat (Figure 2, compare lanes 7–9 with lanes 4–6), whereas co-injection of the same amount of M37 RNA had no effect (Figure 2, lanes 10–12). In the presence of the CTE competitor RNA, the production of two degradation products derived from the intron lariat and from the pre-mRNA was stimulated, suggesting that the CTE-containing RNAs had become unstable (Figure 2, bands indicated by asterisks; see also Figures 4,5). The inhibitory effect of the CTE RNA was independent of the cap structure (data not shown).

Saturation of export of the intron lariat by the presence of excess CTE RNA indicates that the CTE interacts with an essential limiting factor while exporting the lariat. Since the competitor RNA corresponds to the minimal CTE (173 nucleotides) and does not share any other common sequence with the precursor RNA, we conclude that this element is sufficient to interact with the limiting export factor(s), and that no additional RNA sequences are

Figure 3



CTE promotes export of intron-containing RNAs by a distinct pathway from the Rev–RRE system. **(a)** CTE-mediated nuclear export is not inhibited by saturating amounts of Rev-NES peptides conjugated to BSA. A mixture of *in vitro* transcribed ^{32}P-labelled Ad-CTE precursor RNA and U1ΔSm RNA was injected into *Xenopus* oocyte nuclei either with (lanes 7–9) or without (lanes 1–6) BSA–NES competitor. Competition with BSA–M10 is shown in lanes 10–12. The concentration of the BSA–peptide conjugates in the injected samples was 5 mg/ml and the final concentration in the oocyte nucleus was 5–10% of this value. In lanes 1–3, RNA was extracted immediately after injection; in lanes 4–12, splicing and transport were analysed 3 h after injection as described in Figure 1. **(b)** Rev-mediated export of RRE-containing RNAs is not inhibited by saturating amounts of CTE RNA. A mixture of ^{32}P-labelled RNAs consisting of U6Δss RNA and Ad-CTE (lanes 1–9) or Ad-RRE (lanes 10–15) precursor RNAs was injected into *Xenopus* oocyte nuclei. HIV-1 Rev protein was included in the injection mixtures containing the Ad-RRE pre-mRNA. In lanes 1–6 and 10–12, the RNAs were injected in PBS. In lanes 7–9 and 13–15, the injection mixture also contained unlabelled competitor CTE RNA. The concentration of Rev protein in the injected samples was 2.5 pmol/μl. The amount of competitor RNA injected per oocyte nucleus is indicated above the lanes. RNAs were extracted immediately after injection in lanes 1–3 or after 3 h after injection in lanes 4–15. The RRE is represented by an open triangle.

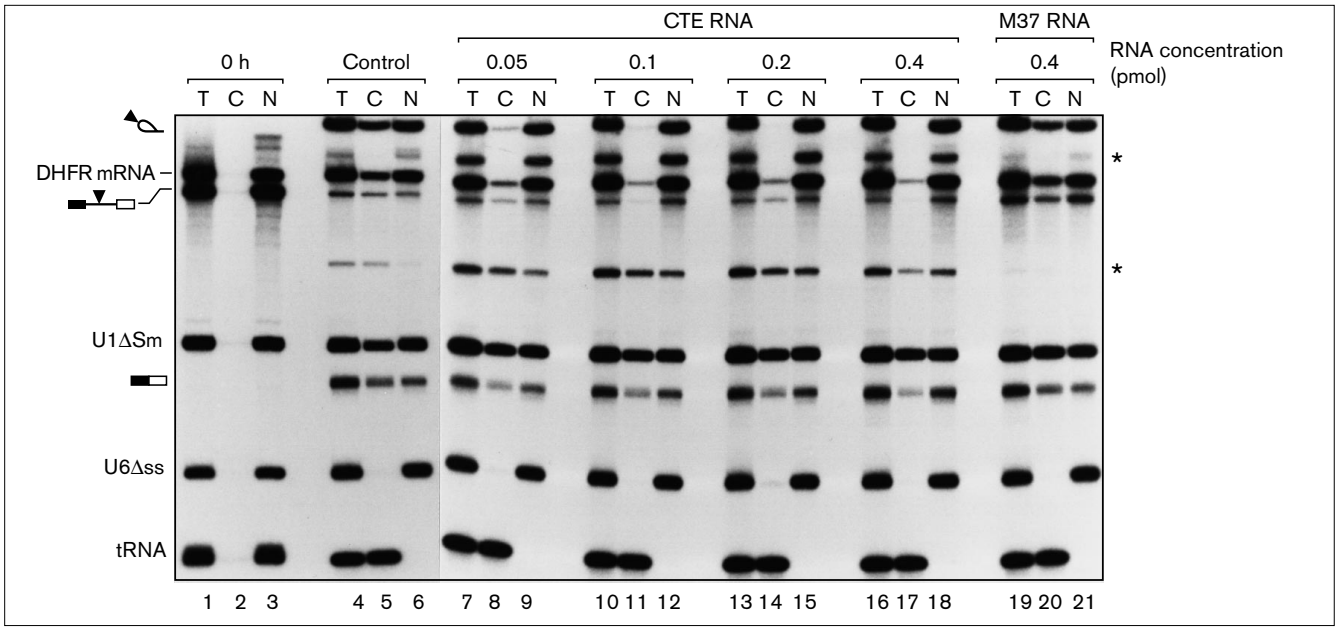
required for this activity. Note that the CTE competitor RNA also interfered with the export of the mature mRNA (Figure 2, compare lanes 7–9 with lanes 4–6). The inhibitory effect of the CTE on the export of cellular RNAs is discussed below.

The CTE directs the excised intron lariat into an export pathway distinct from that used by the HIV-1 Rev NES

Competition studies in *Xenopus* oocytes have shown that the NES of Rev directs RRE-containing RNAs to an export pathway that is used by two other species of cellular RNAs, the 5S rRNA and the UsnRNAs [4]. The observation that substitution of the RRE with either the MPMV or the SRV-1 CTE bypasses the requirement of Rev for HIV-1 and SIVmac expression and replication [24–26,32] suggested that the CTE directs unspliced RNAs to the same export pathway used by the Rev protein. To test this hypothesis, CTE-mediated export was analysed in the presence of Rev-NES peptides cross-linked to bovine serum albumin, BSA (BSA–NES). As a control, we used BSA conjugated to a peptide corresponding to the inactive M10 mutant of the Rev-NES, generating BSA–M10 [33]. *Xenopus* oocyte nuclei were injected with radiolabelled Ad-CTE pre-mRNA along with saturating amounts of BSA–NES or BSA–M10 (Figure 3a). U1ΔSm RNA was included in the injection mixtures as a control because export of this RNA has been shown to be severely inhibited by BSA–NES peptide conjugates [4].

Splicing and export were analysed 3 hours after injection. Surprisingly, saturation of the Rev-NES export pathway did not affect the nuclear export of the excised intron lariat bearing the CTE (Figure 3a, compare lanes 7–9 with lanes 4–6), whereas export of U1ΔSm RNA was inhibited under these conditions, as expected. Injection of BSA–M10 had no effect on the export of any of the RNA species analysed (Figure 3a, lanes 10–12). Thus, CTE-mediated nuclear export is not saturated by BSA–NES conjugates, suggesting that the CTE is likely to direct RNAs to a pathway that is distinct from that used by HIV-1 Rev, 5S rRNA and UsnRNAs. Consistent with this conclusion, we also observed that injection of up to 0.6 picomoles of U1ΔSm RNA per oocyte nucleus had no effect on export of the CTE-containing intron lariat, whereas it completely inhibited export of U5ΔSm RNA (data not shown).

Figure 4



CTE RNA interferes with mRNA nuclear export. *Xenopus* oocyte nuclei were injected with a mixture of the following radioactively labelled RNAs: Ad-CTE, DHFR mRNA, U1ΔSm, U6Δss and human initiator methionyl tRNA. In lanes 7–18, increasing amounts of unlabelled competitor CTE RNA were included in the injection mixtures. The M37 RNA was used as a negative control (lanes 19–21). Synthesis of the competitor RNAs and of labelled precursor Ad-CTE, DHFR mRNA and U1ΔSm RNA was primed with the m^7GpppG dinucleotide, whereas synthesis of U6Δss RNA was primed with γ-mGTP. In lanes 4–21, RNA was extracted 3 h after injection, in lanes 1–3, RNA was extracted immediately after injection. The concentration of the competitor RNA injected per oocyte nucleus is indicated above the lanes. The asterisks indicate the presence of the degraded RNAs.

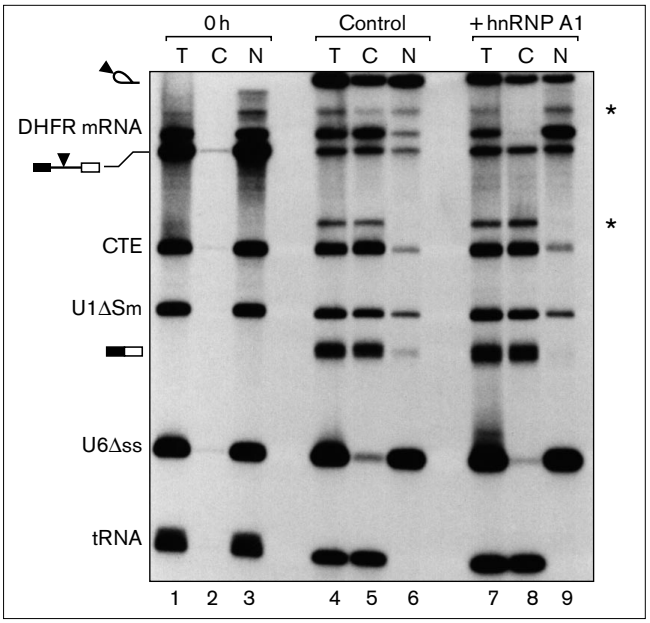
An alternative explanation for the results described above is that the CTE might also interact with the export machinery used by Rev–RRE, but with a much higher affinity. If this were the case, the Rev-NES conjugates would not compete efficiently *in trans* for CTE-mediated export. In order to rule out this hypothesis, we injected an excess of CTE RNA and analysed whether it could compete for Rev-mediated export. Figure 3b shows that Rev-mediated export of the RRE-containing intron lariat was not affected by co-injection of up to 0.6 picomoles of CTE RNA (Figure 3b, lanes 13–15). Note that 0.1 picomoles of CTE RNA per oocyte nucleus were sufficient to completely saturate the export of the intron lariat containing the CTE (Figure 3b, lanes 7–9). Taken together, these results indicate that the CTE signal drives the export of intron-containing RNAs by a pathway different from that used by the Rev–RRE system.

CTE RNA interferes with the export of cellular mRNAs

The experiments described above reveal that the CTE is not using the export pathway used by HIV-1 Rev, 5S rRNA and UsnRNAs. This result, together with the observation that microinjection of saturating amounts of CTE competitor RNA inhibited the export of Ad-mRNA (Figure 2, compare lanes 7–9 with the control lanes 4–6; Figure 3b, compare lanes 7–9 with lanes 13–15), led us to analyse whether the CTE export pathway has any common features with the mRNA export pathway. *Xenopus* oocyte nuclei were injected with a mixture of ^{32}P-labelled RNAs consisting of Ad-CTE pre-mRNA, mouse dihydrofolate reductase (DHFR) mRNA, U1ΔSm, U6Δss and human initiator methionyl tRNA. Export of labelled RNAs was analysed in the presence of increasing amounts of unlabelled competitor CTE RNA. Mutant M37 RNA was used as a negative control. Injection of 0.05 picomoles of CTE RNA per oocyte inhibited the export of the CTE-containing intron lariat (Figure 4, lanes 7–9). As shown in Figure 2, however, injection of 0.1 picomoles of competitor RNA was required to block lariat export completely (Figure 4, lanes 10–12); under these conditions, export of DHFR mRNA was severely inhibited.

Complete inhibition of Ad-mRNA export required higher concentrations of the competitor RNA (Figure 4, lanes 16–18). The inhibitory effect of the CTE RNA on export of DHFR and Ad-mRNAs was specific since the M37 RNA had no effect at the highest concentration used (Figure 4, lanes 19–21). Furthermore, the inhibition was concentration-dependent, indicating that the CTE competitor RNA was titrating a limiting factor required

Figure 5



Injection of hnRNP A1 does not saturates CTE-mediated export. Recombinant hnRNP A1 was injected into oocyte nuclei as indicated above the lanes. After incubation for 1 h, a second microinjection was performed into the oocyte nuclei with the same mixture of the radioactively labelled RNAs described in Figure 4 including CTE RNA. RNA was extracted 200 min after the second injection. The concentration of the recombinant protein in the injected sample was 3 mg/ml. The asterisks indicate the presence of the degraded RNAs.

for export of these mRNA species. Export of U1ΔSm RNA and tRNA was not affected even when the concentration of competitor was 8-fold higher than that required to saturate export of the CTE-containing intron lariat (Figure 4, lanes 16–18). Although the CTE RNA slightly perturbed UsnRNA export in some experiments (data not shown), this effect was not concentration-dependent and thus was probably non-specific. As the kinetics of tRNA export are much faster than those of mRNA and UsnRNA export [3], we also analysed the effect of the CTE competitor RNA 1 hour after injection. Even after this short incubation, the export of tRNA was not affected (data not shown). In conclusion, an excess of CTE RNA specifically titrates a limiting factor(s) required for the efficient export of DHFR mRNA and Ad-mRNA.

CTE-mediated export is not saturated by an excess of hnRNP A1 protein

The competition experiment described above showed that the CTE can block export of Ad and DHFR mRNAs efficiently and specifically, and that the amount of competitor required to obtain this effect was different for each RNA. The different sensitivity of these RNAs to the competition by CTE might imply either that Ad-mRNA and DHFR mRNA export pathways use different limiting factors or that they have different affinities for the same binding factor. Note that different pre-mRNA and mRNA transcripts have binding preferences for different hnRNP proteins *in vivo* and *in vitro* [34,35]. DHFR mRNA export has recently been demonstrated to be saturated by an excess of hnRNP A1 [14]. We therefore examined whether saturating amounts of hnRNP A1 could interfere with CTE RNA export. In agreement with a previous report [14], pre-injection of hnRNP A1 into oocyte nuclei severely compromised export of DHFR mRNA (Figure 5, lanes 7–9). In contrast, the export of Ad-mRNA, CTE RNA and the CTE-containing lariat was unaffected. This result suggests either that hnRNP A1 is not used by the CTE for export or that hnRNP A1 is not the limiting factor for CTE-mediated export. Consequently, the finding that CTE saturates both Ad-mRNA and DHFR mRNA nuclear export indicates that the CTE is accessing a step in common with the export pathway of those RNAs: this step must be downstream of the association between hnRNP A1 and DHFR mRNA.

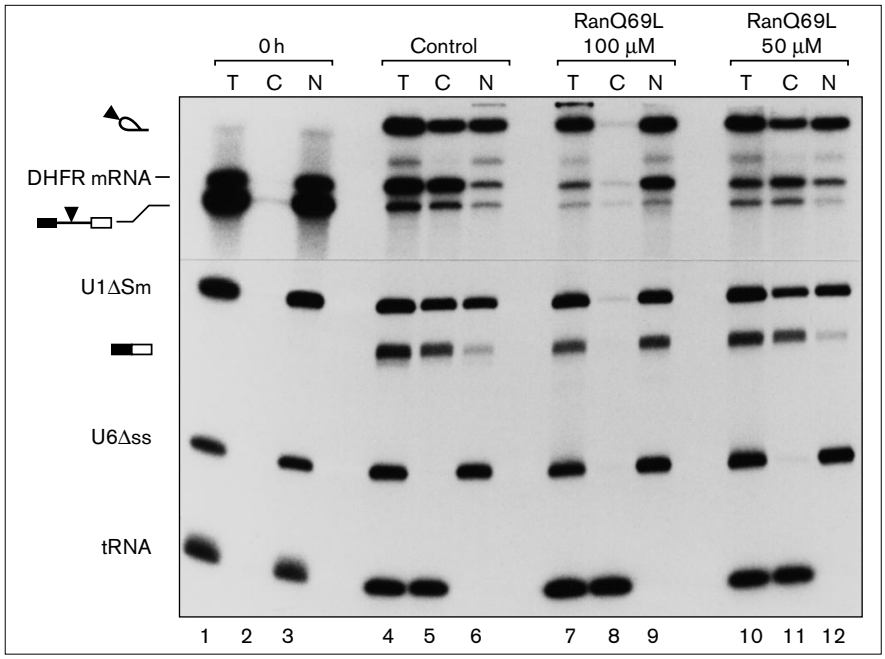
CTE-mediated export is dependent on Ran GTPase activity

The results described above suggest that the CTE allows intron-containing RNAs to access late steps in the mRNA export pathway. As it has been suggested that mRNA export depends on the small GTPase Ran [36–40], we tested whether the export of CTE-containing RNA was also affected by perturbing Ran GTPase activity. We used a mutant of Ran that contains a mutation of the glutamine residue at position 69 to leucine (RanQ69L [41]). This mutant has no GTPase activity and should act as a dominant-negative inhibitor of processes that require Ran-mediated GTP hydrolysis, such as nuclear localisation signal-dependent nuclear import [42,43].

A mixture of radiolabelled RNAs consisting of Ad-CTE pre-mRNA, DHFR mRNA, U1ΔSm, U6Δss and human initiator methionyl tRNA was injected into oocyte nuclei together with RanQ69L protein. Microinjection of this protein into the nuclear compartment completely blocked export of U1ΔSm RNA and severely inhibited the export of most RNA species, including the excised intron lariat containing the CTE (Figure 6, compare lanes 7–9 with lanes 4–6). The export of tRNA was unaffected. The inhibitory effect of RanQ69L was concentration-dependent (Figure 6, compare lanes 7–9 with lanes 10–12). Injection of RanQ69L into the oocyte nucleus did not prevent protein import (data not shown), suggesting that the export defects described above are direct and not a secondary consequence of an import block. The export inhibition by nuclear injection of RanQ69L suggests that CTE-mediated export, like mRNA and UsnRNA export, requires Ran-mediated GTP hydrolysis.

Figure 6

CTE-mediated export is dependent on the small GTPase Ran. A mixture of radioactively-labelled RNAs consisting of Ad-CTE, DHFR mRNA, U1ΔSm, U6Δss and human initiator methionyl tRNA was injected into *Xenopus* oocyte nuclei either with PBS (lanes 1–6) or in the presence of mutant RanQ69L protein (lanes 7–12). Dissection was performed 3 h after injection (lanes 4–12), or immediately after injection (lanes 1–3). RNA analysis was performed as described in Figure 1. The concentration of the recombinant protein in the injection mixtures is indicated above the lanes.



Discussion

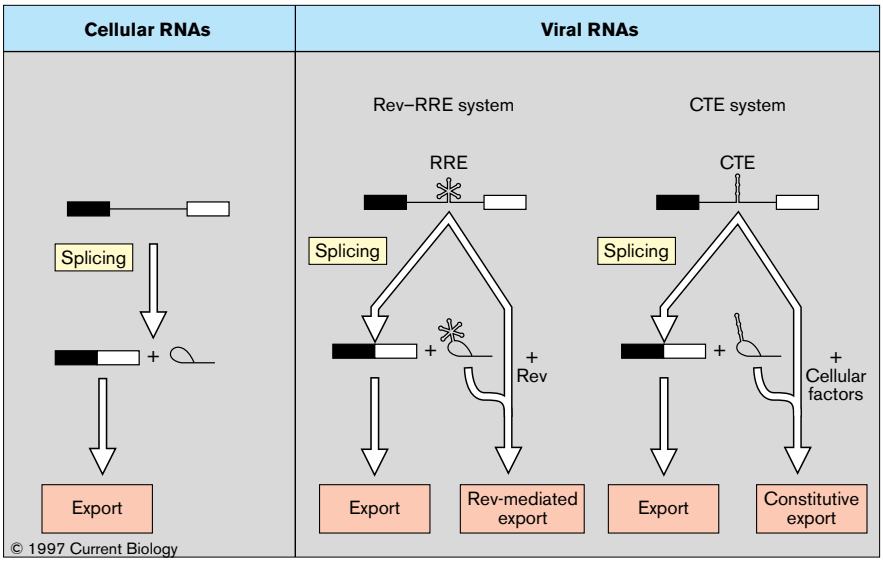
The constitutive transport element (CTE) from type D retroviruses promotes the nuclear export of intron-containing RNAs by interacting with cellular factors (Figure 7). In this report, we have shown that insertion of the SRV-1 CTE into the intron of a precursor mRNA promotes export of the excised intron lariat following splicing in *Xenopus* oocytes. This result suggests that there is conservation of the cellular machinery used by the CTE to bypass the retention mechanisms that usually confine intron-containing RNAs to the nucleus.

Based on the observation that substitution of the HIV-1 RRE by the CTE allows viral replication in the absence of Rev protein [24–26,32], it was plausible to speculate that the CTE could direct intron-containing RNAs to the same cellular pathway used by the HIV-1 Rev protein. Surprisingly, however, our results demonstrated that the CTE directs RNAs to an export pathway different from that used by Rev. Firstly, RNAs bearing the CTE were efficiently transported to the cytoplasm in the presence of saturating amounts of a peptide comprising the Rev-NES. Secondly, an excess of CTE RNA had no effect on Rev-mediated export of an RRE-containing RNA. Furthermore, a recent report has shown that a mutant form of the Ran-binding protein 1 interferes with the Rev–RRE pathway but not with the CTE pathway in mammalian cells [44]. Taken together, these findings demonstrate that the Rev–RRE and the CTE export pathways are distinct.

This observation led us to examine whether the CTE was exported from the nucleus via pathways used by other classes of cellular RNAs. Our competition studies revealed that, although saturating concentrations of the CTE RNA inhibited export of mRNAs, they had only a minor effect on the export of UsnRNAs and no effect on tRNA export. The inhibitory effect of the CTE RNA on export of mRNAs was specific and concentration-dependent. These results suggest that the CTE is titrating a limiting factor that is essential for mRNA export.

Among the hnRNPs described to date, only hnRNP A1 has been directly implicated in mRNA nuclear export [10–14]. An excess of recombinant hnRNP A1 protein has been shown to inhibit export of DHFR mRNA [14]. In agreement with this, when we co-injected hnRNP A1 into oocyte nuclei together with a mixture of different classes of RNAs, only DHFR mRNA was inhibited from exiting the nucleus. Nuclear export of the CTE RNA was not perturbed by high concentrations of hnRNP A1. In the light of these observations, we speculate that the CTE provides an export signal that bypasses one or more steps of the mRNA nuclear transport process. One of these steps would be the association of mRNA with hnRNP A1 and the recognition of this complex by a putative receptor. Consequently, the observation that competitor CTE RNA can also saturate Ad-mRNA export without being affected by hnRNP A1 provides support for the existence of a common late step in the mRNA export process that is used by DHFR, spliced Ad-mRNAs and the CTE RNA.

Figure 7



Export of cellular mRNAs takes place after removal of introns. In contrast with cellular pre-mRNAs, unspliced viral RNAs are exported by virtue of *cis*-acting elements (for example, RRE and CTE). In the case of HIV-1, the RRE interacts with the virally-encoded protein Rev, which harbours a NES. Export of unspliced viral RNA from Type D retroviruses relies on the interaction of the CTE with cellular factors: thus, unspliced CTE-containing RNAs are constitutively exported. As shown in these studies, the Rev–RRE system and the CTE direct intron-containing RNAs to distinct export pathways.

One possible scenario is that the CTE avidly recruits mRNA export factors to generate a export signal that has a dominant effect over retention in the nucleus. Alternatively, the CTE-binding factor(s) that allows nuclear retention to be overcome does not interact with cellular mRNAs; however, after overcoming retention and during export to the cytoplasm, the CTE uses factors that are also involved in cellular mRNA export.

Finally, our results demonstrate that the small GTPase Ran is required for CTE-mediated export. Ran acts in concert with several factors that modulate its nucleotide-bound state (reviewed in [45]). With a few exceptions, almost all nucleocytoplasmic transport processes require functional Ran [46], including CTE-mediated export as shown here. It is interesting that both Rev–RRE-mediated and CTE-mediated export differ in their pathways of delivery of intron-containing RNAs to the cytoplasm. The nature of this difference will be better understood when the factors involved in these cellular pathways begin to be fully identified.

Conclusions

Cellular pre-mRNAs are normally retained in the nucleus. After the removal of introns, the mRNA but not the intron lariat is exported to the cytoplasm. In contrast to cellular pre-mRNAs, unspliced retroviral RNAs are exported from the nucleus by virtue of structured *cis*-acting elements (for example, RRE and CTE). In the case of HIV-1, the RRE interacts with the virally encoded protein Rev, which harbours a NES. Type D retroviruses do not encode a *trans*-acting protein such as Rev, and the export of their unspliced RNAs must therefore rely on the interaction of the CTE with cellular factors. The Rev protein has been previously shown to promote export by interacting with factors that also participate in the export of UsnRNAs and 5S rRNA. In this study, we have demonstrated that the CTE promotes export of intron-containing RNAs by a pathway distinct from that used by the Rev–RRE system. Furthermore, we have shown that the export of CTE RNAs uses factors that are required for the export of cellular mRNAs, corroborating our finding that the CTE-mediated export system is distinct from the Rev–RRE system. Recently, similar results have been obtained using the CTE from MPMV (A. Pasquinelli, E. Lund and J. Dahlberg, personal communication).

Materials and methods

Materials

All enzymes used for DNA manipulations were purchased from New England Biolabs. T7 or T3 RNA polymerases and RNasin were from Promega. AmpliTaq DNA polymerase was from Perkin-Elmer Cetus. The modified nucleotide γ-mGTP used to prime the synthesis of U6Δss RNA, and the cap analogue m^7GpppG used to prime synthesis of all precursor RNAs, DHFR mRNA, U1ΔSm were kind gifts of E. Darzynkiewicz. The ApppG dinucleotide was purchased from Boheringer. Labelled nucleoside triphosphates were from Amersham. Isolation of plasmid DNA, cloning, transformation of *Escherichia coli* and gel analysis of recombinant plasmids were performed as described [47]. PCR analysis was performed as described [48]. Synthesis of NES and M10 peptides and coupling to BSA were performed as described [4]. The sequence (in single-letter amino-acid code) of the Rev-NES peptide was CLPPLERLTL and that of the M10 mutant, CLPPDLRLTL.

Plasmid constructions and synthesis of RNA in vitro

DNA templates for *in vitro* synthesis of DHFR mRNA, tRNA, U1ΔSm and U6Δss RNAs have been previously described [3]. Plasmids p37S12 and Ad-RRE (pAd48) were described elsewhere [21,23]. To obtain plasmids Ad-CTE and Ad-cte(as), a DNA fragment coding for SRV-1 CTE was amplified by PCR using p37S12 as a template and

primers 5′-GGTCCGCGGAGACCACCTCCCCTGCGA-3′ and 5′-CTCCCGCGGCAAATCCCTCGGAAGCTG-3′. The 173 bp PCR product was digested with *Sac*II and inserted in both orientations into the unique *Sac*II site of plasmid pBSAd1 [27]. The plasmid pBt-CTE was constructed by excising a *Bam*HI–*Xho*I DNA fragment from p37S12 (containing the CTE) and inserting it into *Bam*HI–*Xho*I digested pBluescript KS+, thus placing the CTE downstream of the T7 promoter. The plasmid pBt-M37 carries a mutant form of the CTE that has a deletion of loops A and B. For *in vitro* synthesis of radiolabelled RNA, plasmids pBt-CTE and pBt-M37 were linearised with *Xho*I and transcribed using T7 RNA polymerase, whereas plasmids pBS-Ad1, pAd-CTE and pAd-cte(as) were linearised with *Sau*3A and transcribed using T3 RNA polymerase.

Xenopus *oocyte microinjections and RNA analysis*

Oocyte injections and analysis of microinjected RNA by denaturing gel electrophoresis and autoradiography analysis were performed as previously described [3]. The mutant RNAs used (U1ΔSm and U6Δss) lack the protein-binding sites required for the nuclear import of these RNAs [30,49]. Thus U1ΔSm and U5ΔSm remain in the cytoplasm after export from the nucleus. U6Δss is neither exported nor imported and is used as an internal control for nuclear injection and nuclear integrity. The BSA–NES and BSA–M10 conjugates were injected at 5 μg/μl. The concentrations of competitor RNAs in the injected samples are indicated in the figure legends.

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References

1. Zasloff M: **tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process.** *Proc Natl Acad Sci USA* 1983, **80:**6436-6440.
2. Bataillé N, Helser T, Fried HM: **Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: a generalized, facilitated process.** *J Cell Biol* 1990, **111:**1571-1582.
3. Jarmolowski A, Boelens WC, Izaurralde E, Mattaj IW: **Nuclear export of different classes of RNA is mediated by specific factors.** *J Cell Biol* 1994, **124:**627-635.
4. Fischer U, Huber J, Boelens WC, Mattaj IW, Lührmann R: **The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs.** *Cell* 1995, **82:**475-483.
5. Pokrywka NJ, Goldfarb DS: **Nuclear export pathways of tRNA and 40S ribosomes include both common and specific intermediates.** *J Biol Chem* 1995, **270:**3619-3624.
6. Saavedra C, Tung KS, Amberg DC, Hopper AK, Cole CN: **Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae*.** *Genes Dev* 1996, **10:**1608-1620.
7. Izaurralde E, Mattaj IW: **RNA Export.** *Cell* 1995, **81:**153-159.
8. Izaurralde E, Lewis J, Gamberi C, Jarmolowski A, McGuigan C, Mattaj IW: **A cap-binding protein complex mediating UsnRNA export.** *Nature* 1995, **376:**709-712.
9. Dreyfuss G, Matunis MJ, Piñol-Roma S, Burd CG: **hnRNP proteins and the biogenesis of mRNA.** *Annu Rev Biochem* 1993, **62:**289-321.
10. Piñol-Roma S, Dreyfuss G: **Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm.** *Nature* 1992, **355:**730-732.
11. Michael WM, Choi M, Dreyfuss G: **A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway.** *Cell* 1995, **83:**415-422.
12. Pollard VW, Michael WM, Nakielny S, Siomi M, Wang F, Dreyfuss G: **A novel receptor-mediated nuclear protein import pathway.** *Cell* 1996, **86:**985-994.
13. Fridell RA, Truant R, Thorne L, Benson RE, Cullen BR: **Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β.** *J Cell Sci* 1997, **110:**1325-1331.
14. Izaurralde E, Jarmolowski A, Beisel C, Mattaj IW, Dreyfuss G, Fischer U: **A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export.** *J Cell Biol* 1997, **137:**27-36.
15. Legrain P, Rosbash M: **Some *cis*- and *trans*-acting mutants for splicing target pre-mRNA to the cytoplasm.** *Cell* 1989, **57:**573-583.
16. Hammarskjöld ML, Heimer B, Hammarskjöld I, Sangwan I, Albert L, Rekosh D: **Regulation of human immunodeficiency virus *env* expression by the *rev* gene product.** *J Virol* 1989, **68:**951-958.
17. Felber BK, Hadzopoulou-Cladaras M, Cladaras C, Copeland T, Pavlakis GN: **Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA.** *Proc Natl Acad Sci USA* 1989, **86:**1495-1499.
18. Emerman M, Vazeux R, Peden K: **The Rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization.** *Cell* 1989, **57:**1155-1165.
19. Malim MH, Hauber J, Le SY, Maizel J, Cullen BR: **The HIV-Rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA.** *Nature* 1989, **338:**254-257.
20. Wen W, Meinkoth JL, Tsien RY, Taylor SS: **Identification of a signal for rapid export of proteins from the nucleus.** *Cell* 1995, **82:**463-473.
21. Fischer U, Meyer S, Teufel M, Heckel C, Lührmann R, Rautmann G: **Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA.** *EMBO J* 1994, **13:**4105-4112.
22. Ernst RK, Bray M, Rekosh D, Hammarskjöld ML: **A structured retroviral RNA element that mediates nucleocytoplasmic export of intron-containing RNA.** *Mol Cell Biol* 1997, **17:**135-144.
23. Tabernero C, Zolotukhin AS, Valentin A, Pavlakis GN, Felber BK: **The posttranscriptional control element of the simian retrovirus type 1 forms an extensive RNA secondary structure necessary for its function.** *J Virol* 1996, **70:**5998-6011.
24. Bray M, Prasad S, Dubay JW, Hunter E, Jeang KT, Rekosh D, Hammarskjöld ML: **A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent.** *Proc Natl Acad Sci USA* 1994, **91:**1256-1260.
25. Zolotukhin AS, Valentin A, Pavlakis GN, Felber BK: **Continuous propagation of RRE(-) and Rev(-)RRE(-) human immunodeficiency virus type 1 molecular clones containing a *cis*-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes.** *J Virol* 1994, **68:**7944-7952.
26. Tabernero C, Zolotukhin AS, Bear J, Schneider R, Karsenty G, Felber BK: **Identification of an RNA sequence within an intracisternal-A particle element able to replace Rev-mediated posttranscriptional regulation of human immunodeficiency virus type 1.** *J Virol* 1997, **71:**95-101.
27. Konarska MM, Padgett RA, Sharp PA: **Recognition of cap structure in splicing *in vitro* of mRNA precursors.** *Cell* 1984, **38:**731-736.
28. Hamm J, Dathan NA, Mattaj IW: **Functional analysis of mutant *Xenopus* U2 snRNAs.** *Cell* 1989, **59:**159-169.
29. Vankan P, McGuigan C, Mattaj IW: **Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes.** *EMBO J* 1992, **11:**335-342.
30. Hamm J, Mattaj IW: **Monomethylated cap structures facilitate RNA export from the nucleus.** *Cell* 1990, **63:**109-118.
31. Izaurralde E, Lewis J, McGuigan C, Jankowska M, Darzynkiewicz E, Mattaj IW: **A nuclear cap-binding protein complex involved in pre-mRNA splicing.** *Cell* 1994, **78:**657-668.
32. von Gegerfelt A, Felber BK: **Replacement of posttranscriptional regulation in SIVmac239 generated a Rev-independent infectious virus able to propagate in rhesus peripheral blood mononuclear cells.** *Virology* 1997, **232:**291-299.
33. Malim MH, Bohnlein S, Hauber J, Cullen BR: **Functional dissection of the HIV-1 Rev trans-activator: derivation of a *trans*-dominant repressor of Rev function.** *Cell* 1989, **58:**205-214.
34. Matunis MJ, Matunis EL, Dreyfuss G: **Isolation of hnRNP complexes from *Drosophila melanogaster*.** *J Cell Biol* 1992, **116:**245-255.
35. Bennett M, Piñol-Roma S, Staknis D, Dreyfuss G, Reed R: **Differential binding of heterogeneous nuclear ribonucleoproteins to mRNA precursors prior to spliceosome assembly *in vitro*.** *Mol Cell Biol* 1992, **12:**3165-3175.
36. Forrester W, Stutz F, Rosbash M, Wickens M: **Defects in mRNA 3′-end formation, transcription initiation, and mRNA transport associated with the yeast mutation prp20: possible coupling of mRNA processing and chromatin structure.** *Genes Dev* 1992, **6:**1914-1926.

37. Kadowaki T, Goldfarb D, Spitz LM, Tartakoff AM, Ohno M: **Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily.** *EMBO J* 1993, **12:**2929-2937.
38. Amberg DC, Fleischmann M, Stagljar I, Cole CN, Aebi M: **Nuclear PRP20 protein is required for mRNA export.** *EMBO J* 1993, **12:**233-241.
39. Cheng Y, Dahlberg JE, Lund E: **Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport.** *Science* 1995, **267:**1807-1810.
40. Schlenstedt G, Saavedra C, Loeb JDJ, Cole CN, Silver PA: **The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly (A)$^+$ RNA in the cytoplasm.** *Proc Natl Acad Sci USA* 1995, **92:**225-229.
41. Klebe C, Bischoff FR, Ponstingl H, Wittinghofer A: **Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1.** *Biochem* 1995, **34:**639-647.
42. Schlenstedt G, Wong DH, Koepp DM, Silver PA: **Mutants in a yeast Ran-binding protein are defective in nuclear transport.** *EMBO J* 1995, **14:**5367-5378.
43. Palacios I, Weis K, Klebe C, Mattaj IW, Dingwall C: **RAN/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus.** *J Cell Biol* 1996, **133:**485-494.
44. Zolotukhin AS, Felber BK: **Mutations in the nuclear export signal of human Ran-binding protein RanBP1 block the Rev-mediated posttranscriptional regulation of human immunodeficiency virus type 1.** *J Biol Chem* 1997, **272:**11356-11360.
45. Koepp DM, Silver PA: **A GTPase controlling nuclear trafficking: running the right way or walking RANdomly.** *Cell* 1996, **87:**1-4.
46. Izaurralde E, Kutay U, von Kobbe C, Mattaj IW, Görlich D: **The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus**. *EMBO J* 1997, in press.
47. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor. Cold Spring Harbor Laboratory Press, 1989.
48. Innis MA, Gelfand DH, Sninsky JJ, White TJ: *PCR Protocols.* San Diego: Academic Press, 1990.
49. Jarmolowski A, Mattaj IW: **The determinants for Sm protein binding to *Xenopus* U1 and U5 snRNAs are complex and non-identical.** *EMBO J* 1993, **12:2**23-232.

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