**The EMBO Journal** Vol.16 No.21 pp.6535–6547, 1997

# The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus

**Elisa Izaurralde[1,2] Ulrike Kutay[3], Cayetano von Kobbe[1], Iain W.Mattaj[2] and Dirk Görlich[3,4]**

[1]University of Geneva, Department of Molecular Biology, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland, [2]European Molecular Biology Laboratory, Meyerhofstraße 1, 69117 Heidelberg and [3]Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany

[4]Corresponding author
e-mail: dg@mail.zmbh.uni-heidelberg.de

**The GTPase Ran is essential for nuclear import of proteins with a classical nuclear localization signal (NLS). Ran's nucleotide-bound state is determined by the chromatin-bound exchange factor RCC1 generating RanGTP in the nucleus and the cytoplasmic GTPase activating protein RanGAP1 depleting RanGTP from the cytoplasm. This predicts a steep RanGTP concentration gradient across the nuclear envelope. RanGTP binding to importin-β has previously been shown to release importin-α from -β during NLS import. We show that RanGTP also induces release of the M9 signal from the second identified import receptor, transportin. The role of RanGTP distribution is further studied using three methods to collapse the RanGTP gradient. Nuclear injection of either RanGAP1, the RanGTP binding protein RanBP1 or a Ran mutant that cannot stably bind GTP. These treatments block major export and import pathways across the nuclear envelope. Different export pathways exhibit distinct sensitivities to RanGTP depletion, but all are more readily inhibited than is import of either NLS or M9 proteins, indicating that the block of export is direct rather than a secondary consequence of import inhibition. Surprisingly, nuclear export of several substrates including importin-α and -β, transportin, HIV Rev and tRNA appears to require nuclear RanGTP but may not require GTP hydrolysis by Ran, suggesting that the energy for their nuclear export is supplied by another source.**

*Keywords*: importin/nuclear transport/RanBP1/RCC1/Rna1/transportin

## Introduction

Transport between nucleus and cytoplasm proceeds through the nuclear pore complexes (NPCs) that allow diffusion of small molecules of up to 40 kDa and can accommodate active, energy-dependent transport of particles as large as several million daltons. Import and export of different classes of transport substrates through the NPC appears to be mediated by distinct, saturable transport receptors that are thought to shuttle between nucleus and cytoplasm (for review see Gerace, 1995; Görlich and Mattaj, 1996; Koepp and Silver, 1996; Nigg, 1997). According to this model, an import receptor would bind the substrate in the cytoplasm then carry it through the NPC into the nucleus. After the release of the import substrate into the nucleoplasm, the receptor would return to the cytoplasm without the cargo and accomplish the next round of import. Conversely, an export factor has to bind the export substrate in the nucleus and release it in the cytoplasm. This predicts asymmetric import/export cycles and implies that the binding of the substrate to its receptors is somehow regulated by the different environments of nucleus and cytoplasm.

The import of proteins with a classical, basic-type nuclear localization signal (NLS) serves as a paradigm for transport with a shuttling receptor (for reviews see Görlich and Mattaj, 1996; Panté and Aebi, 1996; Schlenstedt, 1996). The NLS of the import substrate is bound in the cytoplasm by the heterodimeric importin-α/β complex. Importin-α provides the NLS-binding site, whereas importin-β accounts for the subsequent interactions with the NPC that drive translocation. The trimeric NLS/importin-α/β complex is transferred through the NPC probably as a single entity, and becomes disassembled upon termination of translocation. The two subunits are returned to the cytoplasm, most likely separately. The small GTPase Ran appears to fulfil at least two distinct functions in the import process. First, Ran's GTP cycle appears to supply at least a substantial proportion of the energy required for translocation into the nucleus (Melchior *et al.*, 1993a; Moore and Blobel, 1993; Weis *et al.*, 1996). This is presumed to involve nucleotide exchange and GTP hydrolysis on NPC-bound Ran. One nucleoporin, RanBP2, has been shown to bind RanGTP and RanGAP1 specifically (Wu *et al.*, 1995; Yokoyama *et al.*, 1995; Mahajan *et al.*, 1997). However, the identity of the NPC component(s) that would translate Ran's GTP cycle into a directed movement through the NPC is not yet known. The second function of Ran, which has been more clearly established, is to regulate the interaction between importin-α and -β. The direct binding of RanGTP to importin-β dissociates the importin heterodimer (Rexach and Blobel, 1995; Chi *et al.*, 1996; Görlich *et al.*, 1996a). That this dissociation is a specific nuclear event that follows translocation into the nucleus can be explained by free RanGTP being available only inside the nucleus. Ran itself is predominantly, but not exclusively nuclear (Bischoff and Ponstingl, 1991a). Ran's major nucleotide exchange factor is the chromatin-bound RCC1, that generates RanGTP inside the nucleus (Ohtsubo *et al.*, 1989; Bischoff and Ponstingl, 1991b). In contrast, the principal GTPase activating protein RanGAP1 is excluded from the nucleoplasm (Hopper *et al.*, 1990; Melchior *et al.*, 1993b;

Bischoff *et al.*, 1994, 1995a; Matunis *et al.*, 1996; Mahajan *et al.*, 1997). RanGAP1 stimulates conversion of RanGTP to the GDP-bound form and thereby depletes RanGTP from the cytoplasm. The Ran-binding protein RanBP1 (Coutavas *et al.*, 1993) is also cytoplasmic (Richards *et al.*, 1996). It binds specifically to the GTP-bound form of Ran and stimulates the GTPase activation by RanGAP1 10-fold (Beddow *et al.*, 1995; Bischoff *et al.*, 1995b). Thus the asymmetric distribution of Ran, RCC1, RanGAP1 and RanBP1 should result in a steep RanGTP gradient across the nuclear envelope with a high nuclear concentration and a very low level in the cytoplasm. Ran and its effector proteins have also been studied in yeast and all major aspects required for the RanGTPase cycle were found to be conserved (for a review see Koepp and Silver, 1996).

The second protein import pathway thus far characterized is mediated by transportin, which is related to importin-β (Nakielny *et al.*, 1996; Pollard *et al.*, 1996; Fridell *et al.*, 1997). hnRNPA1 and several closely related hnRNP proteins are targeted into the nucleus by virtue of the M9-domain, the import signal of the transportin-dependent pathway (Michael *et al.*, 1995; Siomi and Dreyfuss, 1995; Weighardt *et al.*, 1995). The M9 signal binds to transportin directly, with no equivalent of importin-α being involved (Nakielny *et al.*, 1996; Pollard *et al.*, 1996; Fridell *et al.*, 1997). A yeast homologue of transportin that also appears to be involved in the import of hnRNP-like proteins has been characterized (Aitchison *et al.*, 1996). It had not been established whether Ran played a direct role in transportin-mediated import.

The export of RNAs out of the nucleus is not yet well understood, but it is thought to require the association with specific RNA-binding proteins that carry the actual export signals (reviewed by Izaurralde and Mattaj, 1995). The paradigm for this model is the export of HIV-1 viral RNAs containing a Rev response element. The HIV Rev protein binds to this element and the complex is then exported by virtue of Rev's leucine-rich nuclear export signal (NES) (Bogerd *et al.*, 1995; Fischer *et al.*, 1994, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995; Wen *et al.*, 1995).

Pre-mRNAs (hnRNAs), the primary RNA polymerase II transcripts, associate in the nucleus with hnRNP proteins that assist RNA processing and probably mediate export of the matured mRNA out of the nucleus (reviewed in Dreyfuss *et al.*, 1993). Indeed, some hnRNP proteins carry nuclear export signals and shuttle rapidly between nucleus and cytoplasm. The shuttling of hnRNP A1 is the best understood. Its M9 domain confers not only transportin-dependent import, it is also essential for nuclear export of hnRNP A1 (Michael *et al.*, 1995). Saturation experiments in *Xenopus* oocytes provide evidence for a factor (or factors) that recognizes hnRNPA1 through an interaction that includes, but is not confined to, the M9 domain and is required for the export of at least some mRNAs (Izaurralde *et al.*, 1997; Saavedra *et al.*, 1997). So far it had been unclear if transportin would only be involved in the import of M9 proteins or if it would also play a role in M9-dependent export of proteins like hnRNP A1 and of RNA. In addition to the M9 signal other signals like that recently found in hnRNP K protein (Michael *et al.*, 1997) confer shuttling and may well contribute to mRNA export, which in turn would imply that mRNA export may not occur by a single homogenous mechanism.

The nuclear export of U snRNAs is distinct from mRNA export. The 7-methyl cap structure and its interaction with the heterodimeric nuclear cap binding protein complex (CBC) is critical for U snRNA export (Hamm and Mattaj, 1990; Izaurralde *et al.*, 1995). In contrast to mRNA export, export of U snRNAs can be inhibited by saturation of the HIV-1 Rev NES export pathway, suggesting that a leucine-rich export signal plays a role in U snRNA export (Fischer *et al.*, 1995). The asymmetric distribution of RanGTP has been proposed to be required for the directionality of CBC-dependent export of U snRNAs. CBC exists in complex with importin-α. This heterotrimer binds capped U snRNAs. However, when importin-β interacts with the CBC-bound importin-α, capped RNA is released (Görlich *et al.*, 1996b). As discussed above, the asymmetric distribution of RanGTP only allows the importin-α/β interaction to occur in the cytoplasm. Thus, CBC releases RNA in the cytoplasm, and nuclear RanGTP prevents this release in the nucleus.

A considerable body of genetic evidence suggests that Ran is involved in nuclear export of RNA. For example, expression of a GTPase-deficient Ran has been shown to cause an mRNA export defect in yeast (Schlenstedt *et al.*, 1995). Furthermore, *in vivo* studies in yeast and in mammalian cells have suggested that Ran's nucleotide exchange factor RCC1 (Prp20p in yeast) is required for nuclear export of U snRNAs and of the majority of mRNAs (Forrester *et al.*, 1992; Amberg *et al.*, 1993; Kadowaki *et al.*, 1993, 1994; Cheng *et al.*, 1995), and that the yeast RanGAP1, Rna1p, is also required for mRNA export (Traglia *et al.*, 1989). However, these genetic experiments do not distinguish whether Ran is directly involved in export or is required solely for the re-import of shuttling export mediators from the cytoplasm.

Here we show that Ran is involved directly not only in the import of NLS proteins, but also in M9-dependent nuclear import, in the recycling of transportin, importin-α and -β back to the cytoplasm, and in the export of HIV Rev, tRNA, U snRNA, and several mRNAs. Our data suggest that the requirement of Ran for nuclear export is direct rather than for re-import of potential export mediators. We show that the asymmetric distribution of the Ran system across the nuclear envelope is crucial for nucleocytoplasmic transport and that major transport pathways are blocked when RanGAP1 or RanBP1 are mislocalized into the nuclear compartment. The sensitivity towards these treatments and the requirement for GTP hydrolysis by Ran is different for different import or export pathways. Furthermore, we show that RanGTP releases the M9 signal from transportin and provide evidence that this happens upon entry into the nucleus. This indicates that transportin can import an M9-containing substrate but has to return to the cytoplasm without the cargo. This suggests that the export function of the M9 signal is probably mediated by a receptor distinct from transportin.

## Results

### *RanGTP dissociates the M9/transportin complex*

RanGTP binding to importin-β precludes the binding of importin-α. This interaction, together with the asymmetric distribution of the components of the Ran system, is critical in making importin-β a uni-directional import

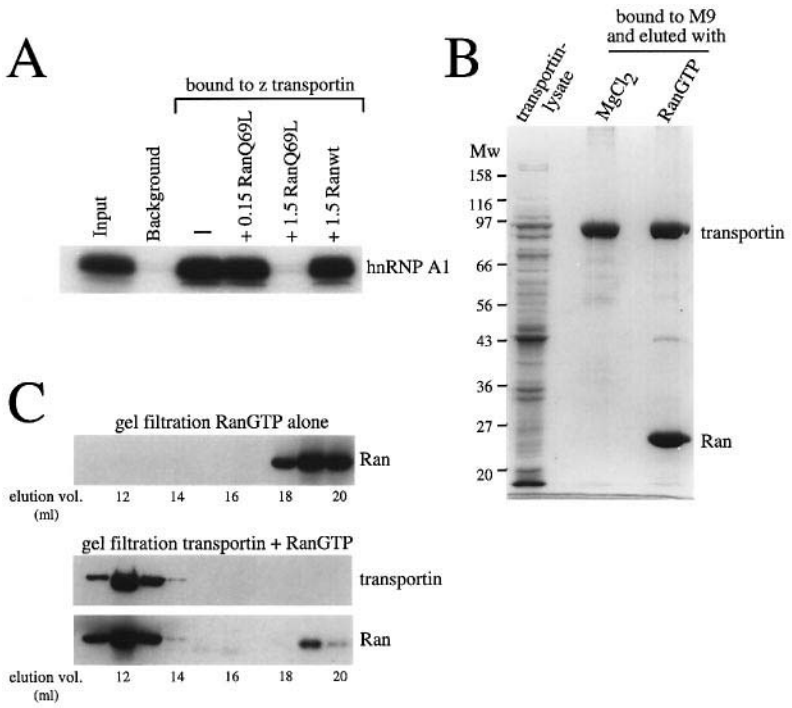


**Fig. 1.** (**A**) Regulation of the M9/transportin interaction by Ran. hnRNP A1 was translated *in vitro* (Input) and subjected to binding to IgG Sepharose alone (Background) or to IgG Sepharose to which z-tagged transportin had been pre-bound. Where indicated, 0.15 μM or 1.5 μM RanQ69L (GTP-form), or 1.5 μM Ran wt (GDP-form) were added before binding. The input and the bound fractions were analysed by SDS–PAGE followed by fluorography. (**B**) RanGTP dissociates transportin from M9. Transportin was expressed at 15°C in *E.coli.* A lysate was prepared in binding buffer: 50 mM Tris–HCl pH 7.5, 400 mM NaCl, 10 mM magnesium acetate. 1 ml post-ribosomal supernatant (corresponding to 20 ml culture) was subjected to binding to 30 μl Streptavidin agarose to which the biotinylated nucleoplasmin core-M9 fusion had been pre-bound. After washing with 5 times 1 ml equilibration buffer and 1 h wash in Ran buffer (20 mM potassium phosphate pH 7.0, 100 mM NaCl, 1 mM magnesium acetate), the bound protein was either eluted with 100 μl of 1 M magnesium chloride or 100 μl of 10 μM RanQ69L (GTP form). Analysis was by SDS–PAGE followed by Coomassie staining. (**C**) RanGTP and transportin form a stable complex. Two nmoles RanQ69L GTP either alone or together with 2 nmoles z-tagged transportin were incubated in 200 μl for 30 min on ice and applied at 0.4 ml/min to a 25 ml Superdex 200 column (Pharmacia) which was equilibrated in Ran buffer. One ml fractions were collected of which 10 μl were analysed by Western blotting with an rabbit anti Ran antibody. Transportin was detected by virtue of its z-tag (the IgG binding domain from Protein A). Note that free RanGTP runs at an elution volume of ~19 ml whereas in the presence of transportin, most RanGTP elutes as a complex with transportin ~12 ml.

factor for NLS-proteins. To date, it has not been clear whether transport mediators other than importin-β might be regulated in a similar way. Transportin is responsible for nuclear import of proteins with an M9 domain (Pollard *et al.*, 1996; Fridell *et al.*, 1997). The M9 domain, however, is not only an import signal, it is also essential for nuclear export of hnRNP A1 (Michael *et al.*, 1995) which might mean that transportin is responsible for both import and export. However, it is also possible that transportin mediates import, but that the export receptor for hnRNP A1 is distinct from transportin. If transportin was both import and export receptor for hnRNP A1 then it should also bind this substrate in the nucleus, i.e. in the presence of RanGTP. To test this, we performed the experiments shown in Figure 1A. *In vitro* translated hnRNP A1 efficiently binds to immobilized transportin. However, binding was completely prevented if assayed in the presence of saturating amounts of RanQ69L (GTP form), whereas wild-type RanGDP had no effect on the interaction (Figure 1A). The RanQ69L mutant is GTPase deficient and remains in the GTP-bound form even in the presence of cytoplasmic RanGAP (Bischoff *et al.*, 1994; Klebe *et al.*, 1995). Figure 1B shows that RanGTP can also dissociate a pre-formed M9/transportin complex. Transportin was expressed in *Escherichia coli* and the total lysate was passed over an immobilized M9 domain to allow transportin to bind. Transportin could be eluted from this column not only with 1 M magnesium chloride, but also specifically with RanGTP demonstrating that RanGTP releases the M9 domain from transportin. As transportin and RanGTP form a stable complex under the conditions of elution as judged by gel filtration (Figure 1C), the release is probably the consequence of the direct binding of RanGTP to transportin. This would also be consistent with the observation that transportin closely resembles importin-β in its effects on the RanGTPase (F.R.Bischoff, S.Nakielny and G.Dreyfuss, personal communication).

### *M9 import is Ran-dependent*

If the interaction between RanGTP and transportin was significant for M9 import, one would predict that M9 import is Ran dependent. Figure 2A confirms this assumption and shows that the efficient import of a fluorescently labelled M9 fusion protein into the nuclei of permeabilized HeLa cells requires the addition of both transportin and Ran. Wild type Ran cannot be substituted by two mutant forms, RanT24N or RanQ69L that are unable to stably bind GTP or unable to hydrolyse the bound GTP respectively (see below). This is consistent with a previous report that RanQ69L can inhibit M9-dependent protein import (Nakielny *et al.*, 1996).

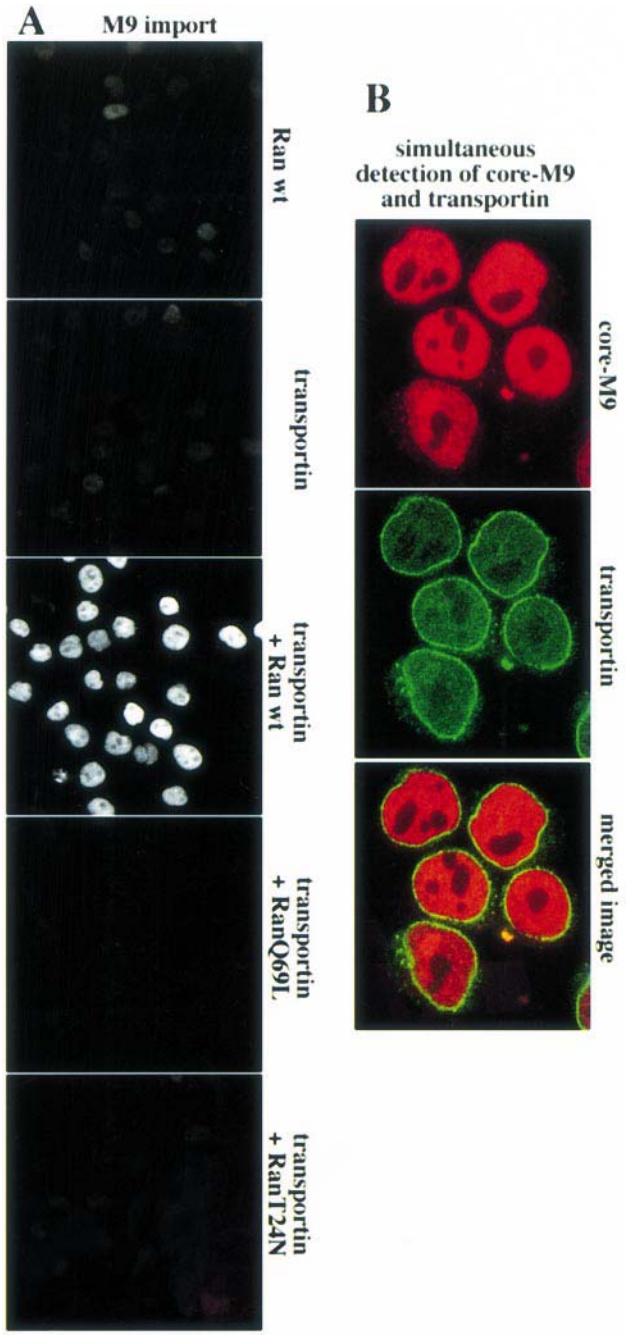


**Fig. 2.** (**A**) Ran-dependence of M9 import. The substrate for import into nuclei of permeabilized cells was a fluorescently labelled fusion between the M9 domain from hnRNP A1 and the core domain from nucleoplasmin. Where indicated, 0.8 μM transportin and 1.5 μM Ran wild type, RanQ69L, or RanT24N were added. Import in the presence of an energy-regenerating system was allowed for 10 min at 20°C. Confocal sections through the equators of the permeabilized cell nuclei are shown. For more details see Materials and methods. (**B**) The M9 substrate and transportin separate after entry into the nucleus. Import in the presence of wild type Ran was essentially as in (A), with the modifications that the core M9 fusion (1 μM) was labelled with Texas Red maleimide and transportin (0.1 μM) was used in a fluorescein-labelled form. After fixation, the distribution of the M9 substrate and transportin was determined by confocal laser scanning microscopy. The panels shows the two channels separately and the merged image. Note that the M9 fusion strongly accumulated inside the nucleus, whereas transportin gave a weak intranuclear signal and was bright at the nuclear envelope.

One would predict from these data that the M9 protein would dissociate from transportin upon nuclear entry, in a manner analogous to the dissociation of importin-β from importin-α. To show this directly, we used a limiting concentration of fluorescein-labelled transportin (0.1 μM) together with the Texas Red labelled M9 reporter to perform an *in vitro* import reaction in the presence of Ran and an energy-regenerating system. After fixation, the distribution of transportin and the import substrate were determined by confocal fluorescence microscopy (Figure 2B). Whereas the M9 fusion (shown in red) had strongly accumulated inside the nuclei, transportin (green) was bright at the nuclear envelope but gave only a weak signal inside the nuclei. These results suggest that, although capable of entry into the nucleoplasm, transportin does not remain tightly associated with the M9 import substrate inside the nucleus. Rather, it is likely to be efficiently returned to the cytoplasm, leaving the M9 protein behind.

It should be noted that the distribution of transportin in these experiments depended on the amounts added. At a limiting concentration, i.e. when import was dependent on the recycling of transportin back to the cytoplasm, transportin showed the typical nuclear pore staining pattern and only a weak intranuclear signal. At a saturating concentration (2 μM), transportin strongly accumulated inside the nuclei and the nuclear signal then obscured the NPC staining (not shown), and resembled the transportin staining pattern found in intact cells (Fridell *et al.*, 1997). We assume that at high transportin concentration re-export is more readily saturated than entry into the nucleus.

### *mRNA and U snRNA export, but apparently not tRNA export, require GTP hydrolysis by Ran*

We next wanted to know if Ran also plays a direct role in any of the various processes of export from the nucleus. To address this question it is essential to separate the requirement for export from that for re-import of export mediators. In addition, it is necessary to investigate two possible functions for Ran in a given export pathway. First, the process could rely on GTP hydrolysis by Ran as an energy source. Second, Ran could be required to regulate compartment-specific interactions of the transport factors, as it does for importin-β and transportin. The export of various substrates can be followed by injecting them into nuclei of *Xenopus* oocytes and analysing their distribution between nucleus and cytoplasm after dissecting the oocytes at various time points. Likewise, nuclear import can be studied following cytoplasmic injection of the substrate of interest. With these assays one can potentially measure the effects of dominant-negative Ran mutants or that of a disturbed asymmetry of the Ran system on a given transport process.

The RanQ69L mutant is GTPase deficient (Bischoff *et al.*, 1994; Klebe *et al.*, 1995) and should block processes that require GTP hydrolysis by Ran in a dominant-negative way, as has been shown for NLS-dependent nuclear import (Palacios *et al.*, 1996). We therefore used the sensitivity towards RanQ69L as a criterion to test if DHFR or histone H4 mRNA, U1 or U5 snRNA, or tRNA would require GTP hydrolysis by Ran for their export out of the nucleus. A mixture of these RNAs was injected into nuclei of *Xenopus* oocytes together with U6 snRNA, which stays in the nucleus and was the internal control for nuclear

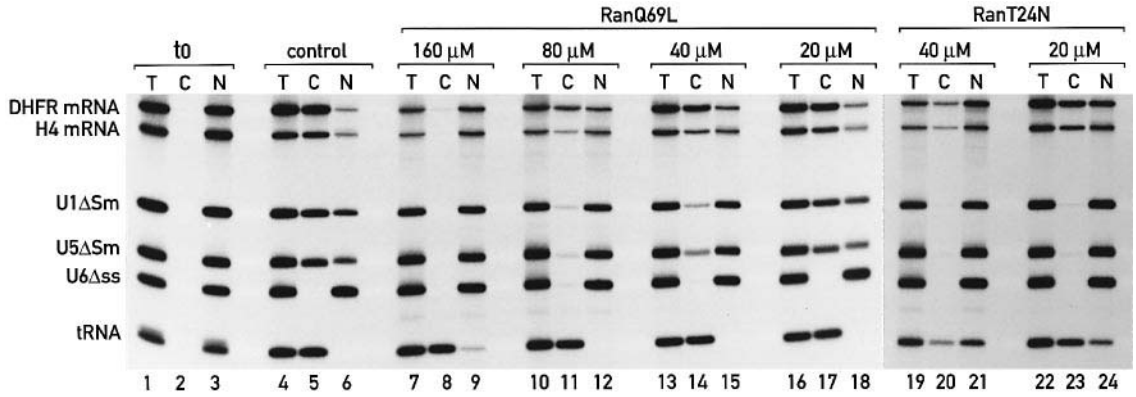


**Fig. 3.** Effects of nuclear injection of Ran mutants on RNA export. *X.laevis* oocyte nuclei were co-injected with a mixture of $^{32}$P-labelled RNAs and recombinant RanQ69L or RanT24N proteins as indicated above the lanes. The mixture of RNAs consisted of: DHFR mRNA, histone H4 mRNA, U1ΔSm, U5ΔSm, U6Δss and human initiator methionyl tRNA. The ΔSm U snRNAs lack the Sm binding site required for re-import into the nucleus. U6Δss does not leave the nucleus and is an internal control for nuclear integrity. Synthesis of DHFR, histone H4, U1ΔSm and U5ΔSm 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) or cytoplasmic (C) and nuclear (N) fractions were collected 210 min after injection in lanes 4–24 or immediately after injection in lanes 1–3. RNAs were resolved on 8% acrylamide–7 M urea denaturing gels. The concentration of the recombinant proteins in the injected samples were as indicated above the lanes.

integrity. Without inhibitor, the mRNAs, U1 and U5 snRNA and tRNA were all efficiently exported to the cytoplasm within the 210 min of incubation (Figure 3, compare lanes 1–3 with 4–6). The co-injection of 160 μM RanQ69L completely blocked export of U snRNA and mRNA, but left tRNA export essentially unaffected (Figure 3, lanes 7–9). The 160 μM in the injected sample should result in a final nuclear concentration of ~20 μM RanQ69L which is the same order of magnitude one would expect for endogenous wild type Ran in the nucleus. Export of U snRNAs was severely affected by injection of RanQ69L at 40 μM (lanes 13–15) and is thus the most sensitive export process. Nuclear injection of 160 μM RanQ69L does not block NLS-dependent or M9-dependent protein import (not shown). The export inhibition is therefore unlikely to be a secondary consequence of an import defect. These experiments therefore suggest that U snRNA export and mRNA export require GTP hydrolysis by Ran whereas tRNA export may not have such a requirement. The two classes of RNA that are affected are however differentially sensitive to this treatment.

It should be noted that RanQ69L only has a dominant-negative effect on NLS- and M9-dependent protein import when injected into the cytoplasm (not shown). There it probably causes premature disassembly of import complexes formed by transportin or importin. That nuclear RanQ69L is not inhibitory for protein import is consistent with termination of import requiring the presence of nuclear RanGTP but not GTP hydrolysis by Ran.

### *The RanT24N mutant inhibits export of mRNA, U snRNA and tRNA from the nucleus*

Nuclear injection of 20 μM RanT24N blocked U snRNA export completely, and severely reduced the rate of export of the two mRNAs and that of tRNA (Figure 3, lanes 22–24). At 40 μM the block was also essentially complete for mRNA and tRNA export (lanes 19–21). The dominant-negative effect might be explained by the failure of this mutant to bind GTP (Klebe *et al.*, 1995). However, the T24N mutation in Ran also reduces the affinity for GDP ~500-fold. As a consequence, the complex of (nucleotide-free) RanT24N with the exchange factor RCC1 is neither dissociated by GTP nor by GDP. This makes RanT24N an effective competitive inhibitor of RCC1-mediated nucleotide exchange ($K_i$ = 39 nM) (Klebe *et al.*, 1995). The dominant-negative effect of T24N on mRNA, U snRNA and tRNA export can thus also be explained by a blockage of the RCC1-dependent production of nuclear RanGTP. These results suggest an involvement of Ran in the export of all three categories of RNA.

### *Mislocalization of RanGAP1 to the nucleus blocks RNA export*

RanGAP1 (Rna1p in yeast) is normally excluded from the nucleoplasm (see Introduction). When injected into nuclei it should result in depletion of RanGTP from this compartment and thus would be expected to affect RNA export similarly to the RanT24N mutant. To test this we used Rna1p, the RanGAP1 from *Saccharomyces pombe* (Melchior *et al.*, 1993b; Bischoff *et al.*, 1995a) which is a highly efficient activator of Ran from higher eukaryotes. For these experiments *S.pombe* Rna1p has two further advantages: it is very stable and it can easily be produced in a recombinant form. Figure 4A shows the dose-dependence of export inhibition by nuclear Rna1p. U snRNA export is essentially blocked by nuclear co-injection of as little as 0.5 μM Rna1p in the injection mixture and is thus the most sensitive of the pathways tested. To inhibit tRNA export to a similar extent, ~10 times more Rna1p had to be injected. Note however that tRNA export occurs at a ~5-fold higher rate than U snRNA export, and that its inhibition is thus underestimated in Figure 4A. Even the smallest amount of Rna1p reduced the export of DHFR and histone H4 mRNA significantly. However the residual export occurring on injection of 1 μM or more Rna1p was rather resistant to further inhibition. This may indicate that two or more mechanisms with different requirement for RanGTP contribute to mRNA export. Alternatively, some mRNA export mediators might have pre-bound RanGTP which is resistant to Rna1p. Interestingly, not all mRNAs whose export was

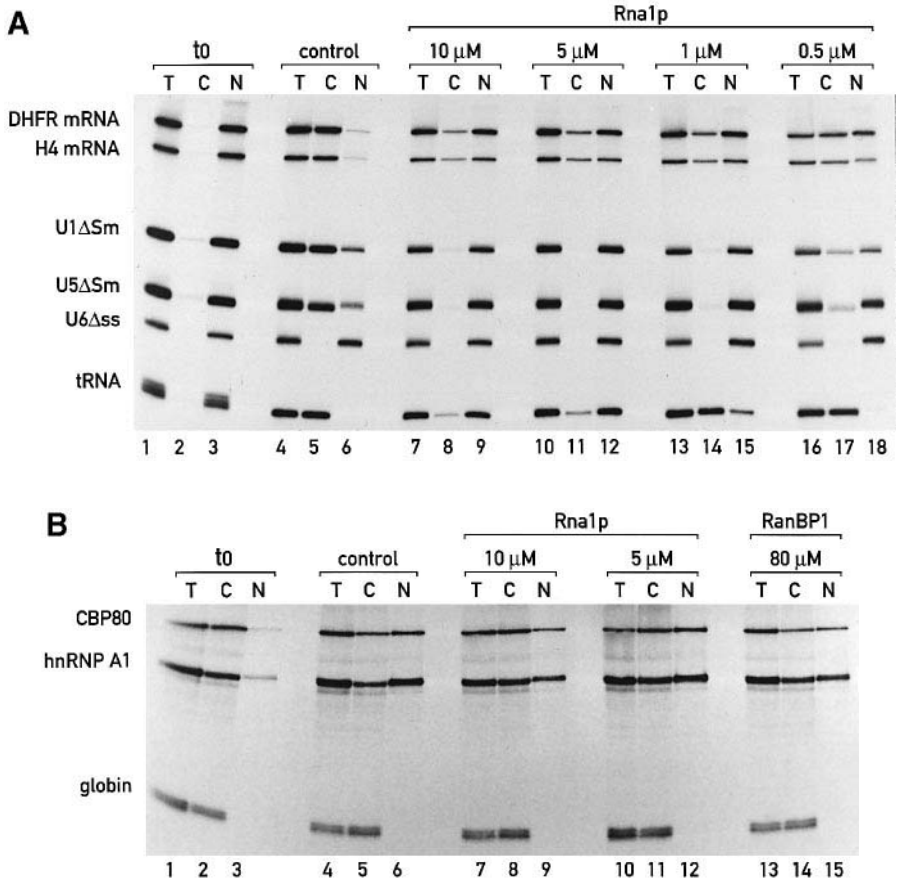


**Fig. 4.** (**A**) Nuclear injection of Rna1p (RanGAP1) inhibits RNA export. *Xenopus* oocyte nuclei were co-injected with the mixture of radiolabelled RNAs described in Figure 3A together with various concentrations of recombinant Rna1p as indicated above the lanes. Rna1p stays in the nucleus and its resulting nuclear concentration is ~1/8 of the concentration in the injection mix. Transport was analysed after 3 h incubation as described in Figure 3A. (**B**) Effect of nuclear Rna1p on protein import. *Xenopus* oocyte nuclei were injected with recombinant Rna1p or RanBP1 as indicated above the lanes. One h later, a mixture of *in vitro* translated $^{35}$S-labelled human CBP80 and hnRNP A1 were injected into the oocyte cytoplasm. Proteins were extracted after 4 h incubation in lanes 4–15 or immediately after injection in lanes 1–3. Note the concentration of nuclear Rna1p that blocks RNA export only reduces but does not prevent nuclear protein import.

tested were similarly affected by nuclear injection of Rna1p (see Discussion below).

NLS- and M9-dependent protein import are only slightly affected by nuclear injection of 5 μM Rna1p (Figure 4B) and still occur to an easily detectable extent on injection of 10 μM Rna1p, suggesting that the export defects described above are direct and not the secondary consequence of an import block. Block of nuclear import of an NLS-protein or of hnRNP A1 requires more stringent conditions, namely the injection of 40 μM Rna1p into the nucleus (data not shown). At this high Rna1p concentration, nuclear RanGTP probably drops below the level required for efficient termination of protein import.

In order to distinguish between a requirement for the presence of nuclear RanGTP per se and a requirement for GTP hydrolysis by Ran in the export of the different RNAs, we assayed the effects of the GTPase-deficient RanQ69L on the Rna1p-induced export block. Because a high RanQ69L concentration blocks mRNA and U snRNA export (see Figure 3) a low concentration (25 μM) of the mutant was used. Some of the effects of nuclear RanGAP1 can be rescued by the GTPase deficient Ran mutant. Figure 5A shows that the block of tRNA and mRNA export caused by nuclear injection of 10 μM Rna1p was essentially relieved by the co-injection of the RanQ69L mutant. This confirms that tRNA and mRNA export require the presence of nuclear RanGTP. It also suggests that at least some functions of RanGTP in tRNA and mRNA export are independent of the hydrolysis of the bound nucleotide. In contrast, GTP hydrolysis by Ran appears obligatory for U snRNA export as the transport block caused by nuclear Rna1p was not detectably restored by RanQ69L.

As further evidence that the export defects caused by nuclear Rna1p were a direct effect on Ran, we next tested if they could be compensated by co-injecting RCC1. As detailed in the introduction, the RanGEF RCC1 catalyses the production of RanGTP and is thus a direct antagonist of RanGAP1. Nuclear RanGTP levels should be restored if the nuclear concentration of RCC1 is sufficiently elevated. Indeed, nuclear co-injection of 20 μM RCC1 restored export of tRNA, mRNA and U snRNA (Figure 5B). This experiment confirms that the inhibition of RNA export by nuclear Rna1p is indeed due to the depletion of nuclear RanGTP and can be overcome by an increased RanGTP production.

### *Effects of mislocalization of RanBP1 to the nucleus*

In order to reduce the nuclear concentration of RanGTP without necessarily inducing the production of high levels

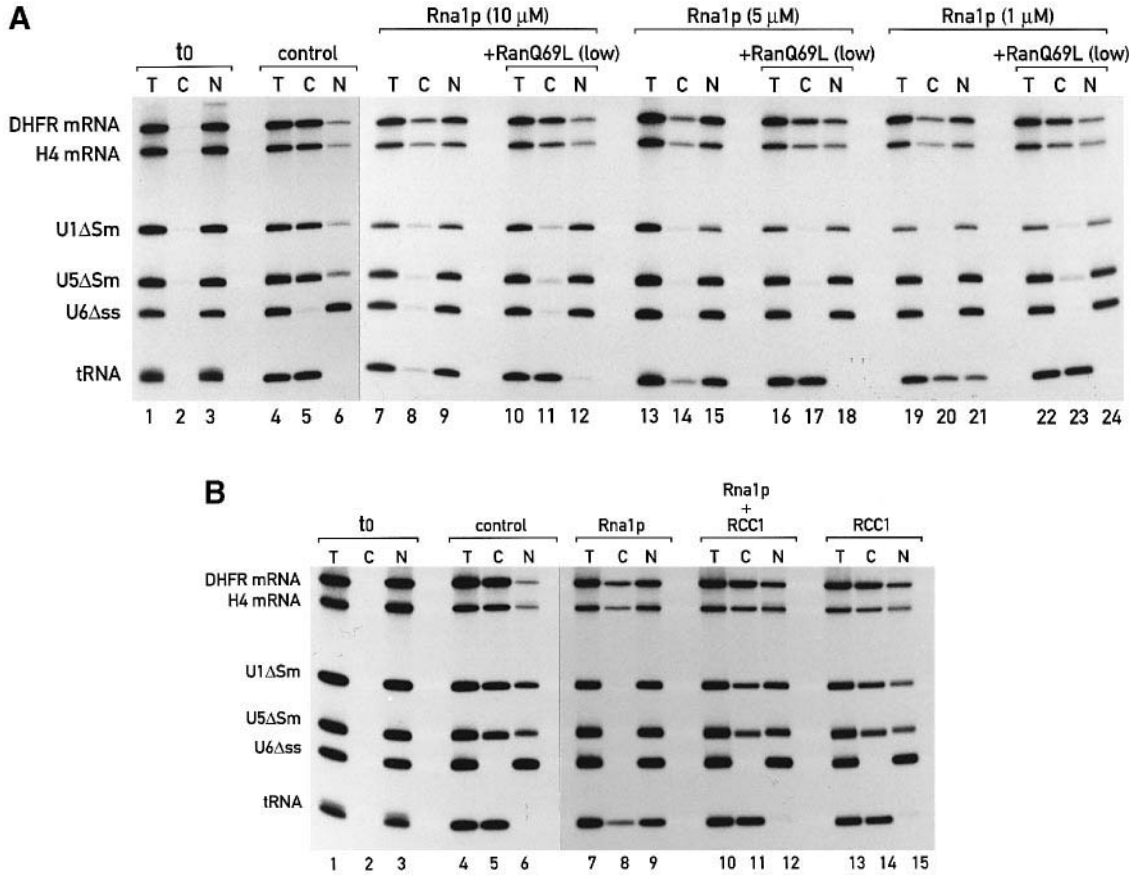


**Fig. 5.** (**A**) The block of tRNA and mRNA export induced by nuclear injection of Rna1p (RanGAP1) can be relieved by coinjection of RanQ69L. *Xenopus* oocyte nuclei were co-injected with the mixture of labelled RNAs described on Figure 1 and increasing concentrations of RanGAP1 (Rna1p) as indicated above the lanes. In lanes 10–12, 16–18 and 22–24, RanQ69L was included in the injection mixtures at 25 μM. This concentration does not inhibit RNA export (see Figure 3). (**B**) The export block caused by nuclear injection of RanGAP1 (Rna1p) can be relieved by coinjection of the RanGEF RCC1. *Xenopus* oocyte nuclei were co-injected with the mixture of radiolabelled RNAs described above and the following recombinant proteins: Rna1p (lanes 7–9), Rna1p and RCC1 (lanes 10–12) or RCC1 alone (lanes 13–15). The concentration of Rna1p in the injection mixture was 10 μM and that of RCC1 20 μM. In lanes 4–15 RNAs were extracted after 210 min incubation; in lanes 1–3 immediately after injection.

of RanGDP, the cytoplasmic RanGTP binding protein RanBP1 was next introduced into the nucleus. RanBP1 is normally kept in the cytoplasm by virtue of a C-terminal domain which contains a NES for rapid nuclear export and also appears to confer cytoplasmic retention (Richards *et al.*, 1996; Zolotukhin and Felber, 1997). Figure 6A shows that nuclear injection of 40 μM RanBP1 severely inhibits the export of U snRNA and causes a significant reduction in tRNA and mRNA export. As injection of twice this concentration of RanBP1 has only a minor effect on import of either the NLS reporter or hnRNP A1 (Figure 4B) we conclude that export is directly inhibited by nuclear RanBP1.

U snRNA export can be competed by injection of saturating amounts of a BSA-Rev NES peptide conjugate (Fischer *et al.*, 1995). As RanBP1 also contains a functional Rev-like NES (Richards *et al.*, 1996; Zolotukhin and Felber, 1997) and is exported when injected into nuclei of *Xenopus* oocytes (not shown) we had to rule out that the inhibition of U snRNA export was simply due to saturation of the NES export pathway. Figure 6B shows that the Ran-binding domain alone (i.e. without the NES) injected at 20 μM inhibits U snRNA export. The export inhibition is therefore not due to the NES, and is most simply explained by RanGTP binding of the injected RanBP1.

### *Nuclear export of HIV Rev and importin-α are Ran-dependent*

Major nuclear transport activities include the export of proteins that carry a leucine-rich nuclear export signal (NES) like that found in HIV Rev and the recycling of shuttling import receptors like importin-α back to the cytoplasm. To study the Ran-dependence of nuclear export of HIV Rev and that of the 'recycling' of importin-α, the proteins were translated *in vitro* and injected into oocyte nuclei. Without inhibitor and in the presence of even the highest concentration of RanQ69L (80 μM) both Rev and importin-α were exported to the cytoplasm. RanQ69L even appears to accelerate Rev and importin-α export. However, the export of both was inhibited by any treatment that lowered the nuclear level of free RanGTP, i.e. by nuclear injection of either 40 μM RanT24N, 80 μM Ran binding domain from RanBP1 and 20 or 10 μM Rna1p (Figure 7). These inhibitory effects of nuclear Rna1p on Rev and importin-α export could be partially relieved by co-injecting 25 μM RanQ69L or 20 μM RCC1 (Figure 7). This suggests that importin-α and Rev export require

the presence of nuclear RanGTP but apparently not the hydrolysis of the bound nucleotide. The requirement of nuclear RanGTP for importin-α export is consistent with data from *S.cerevisiae* (Koepp *et al.*, 1996) showing that SRP1p (yeast importin-α) accumulates in the nucleus if Prp20p (the yeast nucleotide exchange factor for Ran) is defective, which probably also causes a decrease in the nuclear RanGTP concentration.

It should be noted that even though importin-α and NES-dependent export have similar requirements for Ran, importin-α export is not inhibited by saturating amounts of NES conjugates (E.Izaurralde, unpublished), suggesting that the export of Rev and of importin-α are mediated by distinct factors. In fact, these distinct export mediators for Rev-like NESs and importin-α have recently been identified as Crm1p (Fornerod *et al.*, 1997b; Stade *et al.*, 1997) and CAS (Kutay *et al.*, 1997b) respectively.

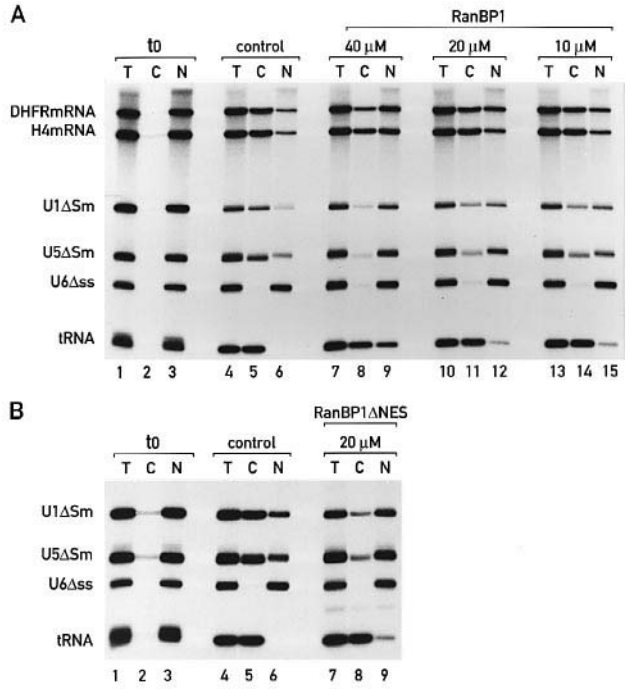


**Fig. 6.** (**A**) Effects of mislocalization of RanBP1 to the nucleus on RNA export. Increasing concentrations of recombinant RanBP1 were co-injected into oocyte nuclei together with the mixture of radiolabelled RNAs described in Figure 3. The concentration of the recombinant protein in the injection mixtures was as indicated above the lanes. RNA export was analysed following 4 h incubation. (**B**) Effects of RanBP1 lacking the NES on U snRNA export. A deletion mutant of RanBP1 (RanBP1ΔNES) lacking the last 43 amino acids including the NES was injected into oocyte nuclei together with the following labelled RNAs: U1 and U5 ΔSm RNAs, U6Δss and tRNA. Export was analysed after 3 h incubation.

### *Importin-β and transportin are probably exported from the nucleus as complexes with RanGTP*

When injected into oocyte nuclei, importin-β and transportin are readily exported (Figure 8); note that the time scale of export of the two proteins is different. The export of importin-β is so rapid that already after 5 min (t0, Figure 8, lanes 1–3) ~50% importin-β has reached the cytoplasm. The 90 min time point represents the steady state distribution with ~95% importin-β in the cytoplasm (lanes 4–6). Nuclear export of importin-β and transportin is resistant to inhibition by 80 μM RanQ69L (lanes 10–12), indicating that GTP hydrolysis by Ran is not rate-limiting for their export. However, nuclear injection of either Rna1p or RanBP1 significantly inhibited export (lanes 7–9 and 16–21). The export inhibition caused by nuclear Rna1p was completely relieved if importin-β and transportin were co-injected with RanQ69L. This suggests that these export processes require the presence of nuclear RanGTP but may not need the hydrolysis of the bound nucleotide. As importin-β and transportin both bind RanGTP with high affinity (Rexach and Blobel, 1995 and Figure 1; Görlich *et al.*, 1996a), the results not only suggest that importin-β and transportin export requires nuclear RanGTP, but also that the two import factors are most likely exported as complexes with RanGTP.

## Discussion

The striking feature of the Ran system is the asymmetric distribution of its constituents between nucleus and cytoplasm. The expected result of the nuclear location of Ran's major nucleotide exchange factor RCC1 and of the cytoplasmic location of both RanBP1 and the RanGTPase activating protein is a high concentration of RanGTP inside the nucleus and low levels in the cytoplasm. Here we show that free, nuclear RanGTP is directly required for major export processes out of the nucleus. As an experimental approach we microinjected three different effectors, which are expected to reduce the concentration of free nuclear RanGTP, into the nucleus. The first was the RanT24N mutant (Klebe *et al.*, 1995) which should prevent RanGTP production by blocking RCC1 in a stable complex with (nucleotide-free) RanT24N. In addition we also mislocalized both Rna1p, the *S.pombe* RanGAP1

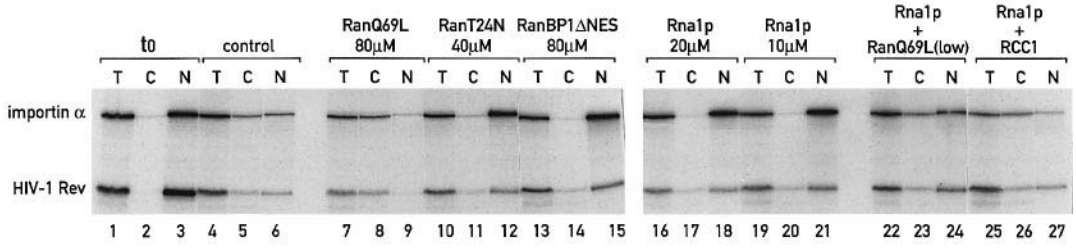


**Fig. 7.** Nuclear export of importin-α and HIV1 Rev protein are dependent on the RanGTPase system. *In vitro* translated $^{35}$S-labelled *Xenopus* importin-α and HIV-1 Rev protein were injected into *X.laevis* oocyte nuclei. In lanes 1–6, labelled proteins were diluted in PBS, in lanes 7–21 the labelled proteins were co-injected with indicated concentrations of RanQ69L (lanes 7–9), RanT24N (lanes 10–12), RanBP1ΔNES (lanes 13–15) and Rna1p (lanes 16–21). In lanes 22–27 the export inhibition caused by nuclear injection of Rna1p (at 10 μM) was relieved by co-injection of 25 μM RanQ69L (lanes 22–24) or 20 μM RCC1 (lanes 25–27). Transport was analysed 4 h after injection.

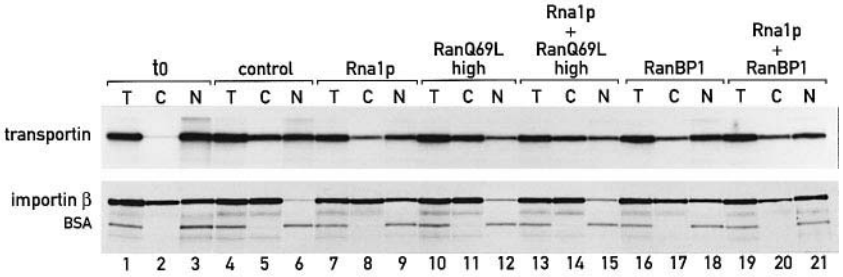


**Fig. 8.** Nuclear export of transportin and importin-β. *In vitro* translated $^{35}$S-labelled human importin-β or transportin were injected into oocyte nuclei along with the recombinant proteins indicate above the lanes. The concentration of recombinant proteins in the injected samples was 40 μM for Rna1p and RanBP1, and 80 μM (high) for RanQ69L. Oocytes injected with transportin were dissected 4 h after injection. Export of importin-β was analysed 90 min after injection. Since export of importin-β is extremely fast, $^{14}$C-labelled BSA was included in the injection mixtures as an internal control for nuclear injection.

(Melchior *et al.*, 1993b; Bischoff *et al.*, 1995a) and RanBP1 (Coutavas *et al.*, 1993) to the nucleus by microinjection. Rna1p depletes RanGTP by converting it into the GDP-bound form. RanBP1 has probably two effects: it titrates RanGTP and it forms a stable trimeric complex together with RCC1 and (nucleotide-free) Ran (Bischoff *et al.*, 1995b; Hayashi *et al.*, 1995) and thus should inhibit nucleotide exchange similar to the RanT24N mutant (Klebe *et al.*, 1995). Nuclear injections of either Rna1p, RanBP1 or RanT24N have similar effects on nuclear transport. They block or at least inhibit nuclear export of tRNA, DHFR or histone H4 mRNA, U snRNA, export of an NES-containing reporter protein, as well as the recycling of importin-α, importin-β and transportin from the nucleus back to the cytoplasm. We have confirmed that the export inhibition is due to a direct effect on Ran. We show that the inhibitory effect of nuclear Rna1p on U snRNA, mRNA, tRNA, Rev and importin-α export can be compensated for by co-injection of RCC1, the direct antagonist of Rna1p, i.e. by an increased production of RanGTP. For export of importin-α and -β, transportin, HIV Rev and tRNA, the block can be relieved also by co-injecting the GTPase-deficient and Rna1p-resistant RanQ69L mutant. This Ran mutant has no inhibitory effect on export of these substrates even when injected at the highest concentration tested. This makes GTP hydrolysis by Ran unlikely to be the energy source for these export processes. That HIV Rev export requires nuclear RanGTP but not GTP hydrolysis by Ran has also been shown by Richards *et al.* (1997) by microinjection experiments in cultured cells. In contrast, the export of U snRNA is highly sensitive to inhibition by RanQ69L, and in this case export inhibition caused by nuclear Rna1p injection is not restored by RanQ69L, indicating that GTP hydrolysis by Ran is probably required for U snRNA export. The export of mRNA shows intermediate behaviour (see below).

### *Ran is directly, but differentially, required for nuclear export processes*

The inhibition of RNA export and HIV Rev export by RanGTP depletion from the nucleus appears to be direct and not the secondary consequence of an import defect. These export processes are considerably more sensitive than NLS- and M9-dependent protein import to nuclear RanGTP depletion and conditions for export inhibition could be found which hardly affected NLS- and M9-dependent protein import. In addition, the onset of the export block is immediate. When, for example, Rna1p is co-injected into the nucleus with the rapidly exported tRNA, no tRNA export is seen. This suggests that Ran is directly required for export of NES-containing proteins, tRNA, U snRNA and at least some mRNAs. Considering that NLS- and M9-dependent import and snRNP import are also Ran-dependent (Melchior *et al.*, 1993a; Moore and Blobel, 1993; Palacios *et al.*, 1996; this study) one can say that Ran plays a key role in the majority of nuclear transport pathways. There is evidence so far for only one major exception. The export of heat shock mRNA out of the yeast nucleus appears to be independent of the Ran system at 42°C (Saavedra *et al.*, 1996). It will be of interest to see whether the nuclear accumulation of heat shock proteins upon heat shock (Munro and Pelham, 1984) is also Ran independent.

The different pathways show a different sensitivity towards RanGTP depletion from the nucleus which probably reflects the different affinities of the respective transport mediators for Ran. U snRNA export is the most sensitive process and is essentially blocked by nuclear injection of as little as 0.5 μM Rna1p, resulting in a nuclear concentration of ~60 nM. This is more than an order of magnitude less than the normal cytoplasmic concentration of RanGAP1 (Bischoff *et al.*, 1994). Previous studies have shown that U snRNA export can be competed with Rev-NES-conjugates, suggesting that a leucine-rich NES plays a role U snRNA export (Fischer *et al.*, 1995). Our observation that RanQ69L can substitute for wild type Ran in NES-dependent export but not in U snRNA export suggests, however, a higher complexity for U snRNA export. Ran might have three targets in U snRNA export: first the Ran-sensitive factor required for NES-dependent export; second the component that requires GTP hydrolysis by Ran; and finally the previously reported requirement for RanGTP to prevent the dissociation of CBC from capped RNA in the nucleus (Görlich *et al.*, 1996b).

The export of tRNA is also very sensitive towards RanGTP depletion from the nucleus. Nuclear injection of 1 μM Rna1p increased the half-time for export from ~10 min (Zasloff, 1983; Jarmolowski *et al.*, 1994) to ~2 h, at 5 μM the export block was nearly complete even after 4 h of incubation. The striking difference to U snRNA export is that tRNA export is insensitive to injection of high concentration of the Ran Q69L mutant, and therefore

may be independent of GTP hydrolysis by Ran. This would in turn suggest that the energy required for the nuclear pore passage of tRNA is provided by another source. In experiments with the tsBN2 cell line, that is temperature sensitive for RCC1, the export of both mRNA (Kadowaki *et al.*, 1993) and U snRNA (Cheng *et al.*, 1995) was blocked at non-permissive temperature. In contrast, tRNA export was still observed and therefore suggested to occur independently of the Ran system (Cheng *et al.*, 1995; see also Her *et al.*, 1997). One possible explanation for this observation might be that the nuclear RanGTP concentration in the tsBN2 cells was not sufficiently reduced to completely prevent tRNA export, which is a very rapid process, over a period of 6 h. In fact, Richards *et al.* (1997) have shown that even at non-permissive temperature the tsBN2 cells still produce sufficient nuclear RanGTP to allow efficient Rev-NES-dependent export to occur, whereas the more stringent depletion of nuclear RanGTP by nuclear injection of RanGAP caused a complete block of NES-export.

The export of mRNAs shows signs of being the most complex process studied here. The export of DHFR and histone H4 mRNA was inhibited by nuclear injection of a high concentration of RanQ69L, but the Rna1p-induced inhibition could be reversed by the injection of 25 μM RanQ69L. This could be explained by the existence of two Ran-dependent steps. Nuclear RanGTP may be required in one process e.g. to stabilize export complexes. In addition, the results suggest that a process contributes to mRNA export which requires GTP hydrolysis by Ran but can tolerate a low concentration of RanQ69L.

Even the smallest amount of nuclear Rna1p reduced the export of DHFR and histone H4 mRNA significantly. Surprisingly however, the residual export was then rather resistant to further inhibition by a higher Rna1p concentration. In additional experiments, we determined that the export of an mRNA derived from the adenovirus major late transcript (Hamm *et al.*, 1989) was hardly affected by nuclear Rna1p (not shown). This could indicate that several mechanisms with different requirements for Ran are involved in mRNA export. The contribution of a particular mechanism to the export of an individual mRNA might depend on the pattern of associated hnRNP proteins and the kind of export signals they carry. *In vitro* binding experiments have indeed shown that individual primary transcripts each associate with a unique set of hnRNP proteins (Bennett *et al.*, 1992). The relative amounts of specific hnRNP proteins bound to individual nascent transcripts was also found to vary considerably *in vivo* (Matunis *et al.*, 1993). Another indication for the existence of several distinct mRNA export mechanisms is that excess of hnRNPA1 competitively inhibits export of DHFR mRNA, U1A mRNA and hnRNP C mRNA, but does not block the export of histone H4 mRNA or that of the adenovirus major late transcript derived mRNA (Izaurralde *et al.*, 1997; E.Izaurralde, unpublished data).

### *The roles of Ran in the shuttling of import mediators*

We show here that transportin-mediated nuclear protein import, like the importin pathway, depends on Ran and requires GTP hydrolysis by Ran. We assume that GTP hydrolysis by Ran at the NPC provides energy for the actual translocation of both types of substrate into the nucleus. Consistent with a requirement for free nuclear RanGTP to terminate NLS and M9 import, these pathways can be inhibited by nuclear injection of Rna1p. That a rather high concentration of Rna1p is required for inhibition agrees with the very high affinities for RanGTP of importin-β and transportin whose dissociation constants are ~1 nM (Görlich *et al.*, 1996a; Figure 1; this manuscript; F.R.Bischoff, personal communication). We did not see a severe inhibition of protein import by nuclear RanBP1, consistent with the observation that not only RanGTP but also the RanGTP/RanBP1 complex can dissociate the importin heterodimer and thus probably mediate termination (Chi *et al.*, 1996; Görlich *et al.*, 1996a). However, nuclear RanBP1 does severely inhibit the recycling of importin-α back to the cytoplasm. A shortage of cytoplasmic importin-α could explain the decline in protein import when cells were transfected with a RanBP1 mutant that accumulates in the nucleus (Richards *et al.*, 1996). Note that in *Xenopus laevis* oocytes the enormous cytoplasmic store of importin-α and -β, which is found at roughly 3 μM, ensures that these components will not become limiting in our experiments.

NLS import is terminated at the nuclear side of NPC by direct binding of RanGTP to importin-β which dissociates the importin heterodimer (Rexach and Blobel, 1995; Chi *et al.*, 1996; Görlich *et al.*, 1996a). M9 import appears to occur similarly. We show here that free RanGTP dissociates the transportin/M9 complex and we observe that M9 and transportin indeed separate from each other after NPC passage. Transportin appears to be rapidly returned to the cytoplasm, leaving the M9 reporter behind. Export of transportin and importin-β requires the presence of nuclear RanGTP, but not GTP hydrolysis by Ran. Transportin and importin-β are probably exported as complexes with RanGTP, which would preclude the binding of the M9 domain and importin-α respectively, and thereby prevent the re-export of the corresponding import substrates. To allow binding of another substrate molecule in the cytoplasm and thus the next round of import, Ran needs to be dissociated from importin-β and transportin. This might be attained by the concerted action of RanGAP1 and RanBP1 (Lounsbury and Macara, 1997; F.R.Bischoff and D.Görlich, unpublished results). Our data suggest that transportin and importin-β act as uni-directional import factors that can accomplish multiple rounds of import and thereby achieve nuclear accumulation of a molar excess of import substrates.

An implication from our data is that transportin cannot mediate the export function of the M9 signal and that it is therefore most likely not an mRNA export factor. This means that the mediators of export of the various RNA classes still remain to be identified. An entire class of potential transport factors has recently been identified which show distant similarity to importin-β (Fornerod *et al.*, 1997a; Görlich *et al.*, 1997) and in particular to importin-β's Ran-binding motif (Görlich *et al.*, 1997). Some of them indeed bind RanGTP and/or interact with the NPC (Fornerod *et al.*, 1997a; Görlich *et al.*, 1997). Two of them, CAS and Crm1p, have recently been shown to mediate nuclear export of importin-α (Kutay *et al.*, 1997b) or of NES-containing proteins (Fornerod *et al.*, 1997b; Stade *et al.*, 1997) respectively. CAS and Crm1p

appear to bind their substrates only in a nuclear environment, i.e. in the presence of RanGTP and form then trimeric substrate/exportin/RanGTP complexes. It might well be that some of the other factors of the importin-β/CAS/Crm1p superfamily will turn out to be export mediators whose substrate binding is regulated by Ran.

## Materials and methods

All enzymes used for DNA manipulations were purchased from New England Biolabs. T7 RNA polymerase and RNAsin were from Promega. The cap analogue $m^7$GpppG (Darzynkiewicz *et al.*, 1985, 1988) used to prime the synthesis of DHFR, Histone H4, U1ΔSm and U5ΔSm RNAs and the modified nucleotide γ-mGTP used to synthesize U6Δss RNA were a kind gift of E.Darzynkiewicz. Labelled nucleoside triphosphates and [$^{35}$S]methionine were from Amersham Corp.

### *Recombinant proteins*

Expression of wild type Ran, core-M9 fusion, transportin and z-tagged transportin has been described (Görlich *et al.*, 1997; Kutay *et al.*, 1997a). Transportin was purified on nickel agarose followed by MonoQ. RanQ69L and RanT24N were expressed from the pQE32 (Qiagen) and purified on nickel agarose followed by S-Sepharose FF. The coding region of Rna1p was amplified from *S.pombe* genomic DNA, cloned into the zz60 vector (Görlich *et al.*, 1997), and was expressed with an N-terminal Z-tag and a C-terminal his-tag. Purification was on nickel agarose. RCC1 was expressed from the pQE60 vector (Qiagen) and purified with nickel agarose followed by Mono Q. The enzymatic activity of Rna1p and RCC1 was verified by enzymatic assays (kindly performed by F.R.Bischoff). Full length RanBP1 and a fragment comprising of residues 1–160 were expressed from the zz60 vector and purified on nickel agarose followed by Mono Q.

### *In vitro translation*

The CBP80 and hnRNP A1 constructs used for *in vitro* translation have been described previously (Izaurralde *et al.*, 1994, 1997). Importin-α, importin-β and transportin were cloned into the T7–70 vector (Görlich *et al.*, 1997) which contains a T7 promoter and the 5′UTR from *Xenopus* beta globin in front of the multicloning site. For generation of $^{35}$S-labelled *in vitro* translated proteins the combined *in vitro* transcription/translation (TNT) kit from Promega was used. In brief, 1 μg of plasmid DNA containing the gene to be transcribed and translated was incubated in a total volume of 50 μl containing 25 μl reticulocyte lysate, 2 μl TNT-polymerase, 2 μl reaction buffer, 2 μl amino-acid mix lacking methionine, 40 μCi [$^{35}$S]methionine (Amersham) and 1 μl (10U) RNAsin (Promega). The reaction was carried out at 30°C for 2 h. Translation was checked by SDS–PAGE and subsequent fluorography using intensify solutions from Amersham (Amplify). *In vitro* translated proteins were diluted 10 times in PBS, concentrated to 50 μl using microconcentrator devices (Nanosep, Pall Filtron) and washed again by dilution with 500 μl of PBS and concentration to a final volume of 25 μl. This procedure removes the non-incorporated [$^{35}$S]methionine.

### *Oocyte injections*

Oocyte injections and analysis of microinjected RNA by denaturing gel electrophoresis, autoradiography or phosphoimager analysis were performed as described (Jarmolowski *et al.*, 1994). The concentration of the recombinant proteins in the injected samples are indicated in the figure legends. Microinjection of *in vitro* translated protein into oocytes, isolation of protein from oocytes and SDS–PAGE analysis were all carried out as described (Kambach and Mattaj, 1992). The mutant RNAs used (U1ΔSm, U5ΔSm) lack protein binding sites required for the nuclear import of these RNAs (Hamm and Mattaj, 1990; Jarmolowski *et al.*, 1994) and thus remain in the cytoplasm after export from the nucleus.

### *Import assays with permeabilized HeLa cells*

The basic method has been described previously (Görlich *et al.*, 1994, 1996a). The assay contained: 20 mM HEPES/KOH pH 7.5, 140 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 250 mM sucrose and 2 mg/ml nucleoplasmin-core to block non-specific binding, 150 nM RanBP1, 100 nM Rna1p, 200 nM NTF2 and an energy-regenerating system containing each 0.5 mM GTP and ATP, 10 mM creatine phosphate and 50 μg/ml creatine kinase. Where indicated 0.8 μM transportin, 1.5 μM Ran wild type or RanQ69L, or RanT24N were added. Incubation was for 10 min at 20°C. Fluorescence labelling of Transportin and core-M9 has been described (Görlich *et al.*, 1997; Kutay *et al.*, 1997a).

## Acknowledgements

We would like to thank Petra Schwarzmaier for technical help, Nicolas Roggli and Yves-Michel Cully for help with the figures. We are grateful to Dr F.R.Bischoff for the RanBP1 cDNA clone and many helpful discussions, Dr E.Darzynkiewicz for the gift of the 7-methyl GpppG and γ-methyl GTP cap analogues, and Dr D.Schümperli for the mouse Histone H4 clone. This work was supported by the Deutsche Forschungsgemeinschaft (DFG), the Swiss National Science Foundation (grant 3100-046841), the State of Geneva and the Human Frontier Science Programme Organisation.

## References

Aitchison,J.D., Blobel,G. and Rout,M.P. (1996) Kap104: a karyopherin involed in the nuclear transport of messenger RNA binding proteins. *Science*, **274**, 624–627.

Amberg,D.C., Fleischmann,M., Stagljar,I., Cole,C.N. and Aebi,M. (1993) Nuclear PRP20 protein is required for mRNA export. *EMBO J.*, **12**, 233–241.

Beddow,A.L., Richards,S.A., Orem,N.R. and Macara,I.G. (1995) The Ran/TC4 GTPase-binding domain: identification by expression cloning and characterization of a conserved sequence motif. *Proc. Natl Acad. Sci. USA*, **92**, 3328–3332.

Bennett,M., Pinol Roma,S., Staknis,D., Dreyfuss,G. and Reed,R. (1992) Differential binding of heterogeneous nuclear ribonucleoproteins to mRNA precursors prior to spliceosome assembly *in vitro*. *Mol. Cell. Biol.*, **12**, 3165–3175.

Bischoff,F.R. and Ponstingl,H. (1991a) Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. *Proc. Natl Acad. Sci. USA*, **88**, 10830–10834.

Bischoff,F.R. and Ponstingl,H. (1991b) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature*, **354**, 80–82.

Bischoff,F.R., Klebe,C., Kretschmer,J., Wittinghofer,A. and Ponstingl,H. (1994) RanGAP1 induces GTPase activity of nuclear ras-related Ran. *Proc. Natl Acad. Sci. USA*, **91**, 2587–2591.

Bischoff,F.R., Krebber,H., Kempf,T., Hermes,I. and Ponstingl,H. (1995a) Human RanGTPase activating protein RanGAP1 is a homolog of yeast RNA1p involved in messenger RNA processing and transport. *Proc. Natl Acad. Sci. USA*, **92**, 1749–1753.

Bischoff,F.R., Krebber,H., Smirnova,E., Dong,W.H. and Ponstingl,H. (1995b) Coactivation of RanGTPase and inhibition of GTP dissociation by Ran GTP binding protein RanBP1. *EMBO J.*, **14**, 705–715.

Bogerd,H.P., Fridell,R.A., Madore,S. and Cullen,B.R. (1995) Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell*, **82**, 485–494.

Cheng,Y., Dahlberg,J.E. and Lund,E. (1995) Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. *Science*, **267**, 1807–1810.

Chi,N.C., Adam,E.J.H., Visser,G.D. and Adam,S.A. (1996) RanBP1 stabilises the interaction of Ran with p97 in nuclear protein import. *J. Cell Biol.*, **135**, 559–569.

Coutavas,E., Ren,M., Oppenheim,J.D., D'Eustachio,P. and Rush,M.G. (1993) Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. *Nature*, **366**, 585–587.

Darzynkiewicz,E., Ekiel,I., Tahara,S.M., Seliger,L.S. and Shatkin,A.J. (1985) Chemical synthesis and characterisation of 7-methylguanosine Cap analogues. *Biochemistry*, **24**, 1701–1707.

Darzynkiewicz,E., Stepinski,J., Ekiel,I., Jin,Y., Haber,D., Sijuwade,T. and Tahara,S.M. (1988) Beta-globin mRNAs capped with m7G, m2.7(2)G or m2.2.7(3)G differ in intrinsic translation efficiency. *Nucleic Acids Res.*, **16**, 8953–8962.

Dreyfuss,G., Matunis,M.J., Pinol Roma,S. and Burd,C.G. (1993) hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.*, **62**, 289–321.

Fischer,U., Meyer,S., Teufel,M., Heckel,C., Lührmann,R. and Rautmann,G. (1994) Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J.*, **13**, 4105–4112.

Fischer,U., Huber,J., Boelens,W.C., Mattaj,I.W. and Lührmann,R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–483.

Fornerod,M., van Deursen,J., van Baal,S., Reynolds,A., Davis,D., Gopal,M., Fransen,J. and Grosveld,G. (1997a) The human homologue of yeast Crm1 is in a dynamic subcomplex with Can/Nup214 and a novel nuclear pore component. *EMBO J.*, **16**, 807–816.

Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997b) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, in press.

Forrester,W., Stutz,F., Rosbash,M. and Wickens,M. (1992) 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.*, **6**, 1914–1926.

Fridell,R.A., Truant,R., Thorne,L., Benson,R.E. and Cullen,B.R. (1997) Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β. *J. Cell Sci.*, **110**, 1325–1331

Fritz,C.C., Zapp,M.L. and Green,M.R. (1995) A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature*, **376**, 530–533.

Gerace,L. (1995) Nuclear export signals and the fast track to the cytoplasm. *Cell*, **82**, 341–344.

Görlich,D. and Mattaj,I.W. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.

Görlich,D., Prehn,S., Laskey,R.A. and Hartmann,E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*, **79**, 767–778.

Görlich,D., Panté,N., Kutay,U., Aebi,U. and Bischoff,F.R. (1996a) Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.*, **15**, 5584–5594.

Görlich,D., Kraft,R., Kostka,S., Vogel,F., Hartmann,E., Laskey,R.A., Mattaj,I.W. and Izaurralde,E. (1996b) Importin provides a link between nuclear protein import and U snRNA export. *Cell*, **87**, 21–32.

Görlich,D., Dabrowski,M., Bischoff,F.R., Kutay,U., Bork,P., Hartmann,E., Prehn,S. and Izaurralde,E. (1997) A novel class of RanGTP binding proteins. *J.Cell Biol.*, **138**, 65–80.

Hamm,J. and Mattaj,I.W. (1990) Monomethylated cap structures facilitate RNA export from the nucleus. *Cell*, **63**, 109–118.

Hamm,J., Dathan,N.A. and Mattaj,I.W. (1989) Functional analysis of mutant *Xenopus* U2 snRNAs. *Cell*, **59**, 159–169.

Hayashi,N., Yokoyama,N., Seki,T., Azuma,Y., Ohba,T. and Nishimoto,T. (1995) RanBP1, a ras-like nuclear G protein binding to Ran/TC4, inhibits RCC1 via Ran/TC4. *Mol. Gen. Genet.*, **247**, 661–669.

Her,L.-S., Lund,E. and Dahlberg,J.E. (1997) Inhibition of Ran guanosine triphosphatase-dependent nuclear transport by the matrix protein of vesicular stomatitis virus. *Science*, **276**, 1845–1848.

Hopper,A.K., Traglia,H.M. and Dunst,R.W. (1990) The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. *J. Cell Biol.*, **111**, 309–321.

Izaurralde,E. and Mattaj,I.W. (1995) RNA export. *Cell*, **81**, 153–159.

Izaurralde,E., Lewis,J., McGuigan,C., Jankowska,M., Darzynkiewicz,E. and Mattaj,I.W. (1994) A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell*, **78**, 657–668.

Izaurralde,E., Lewis,J., Gamberi,C., Jarmolowski,A., McGuigan,C. and Mattaj,I.W. (1995) A cap-binding protein complex mediating U snRNA export. *Nature*, **376**, 709–712.

Izaurralde,E., Jarmolowski,A., Beisel,C., Mattaj,I.W., Dreyfuss,G. and Fischer,U. (1997) A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. *J. Cell Biol.*, **137**, 27–35.

Jarmolowski,A., Boelens,W.C., Izaurralde,E. and Mattaj,I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.*, **124**, 627–635.

Kadowaki,T., Goldfarb,D., Spitz,L.M., Tartakoff,A.M. and Ohno,M. (1993) Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. *EMBO J.*, **12**, 2929–2937.

Kadowaki,T. *et al*. (1994) Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (mtr) mutants. *J. Cell Biol.*, **126**, 649–659.

Kambach,C. and Mattaj,I.W. (1992) Intracellular distribution of the U1A protein depends on active transport and nuclear binding to U1 snRNA. *J. Cell Biol.*, **118**, 11–21.

Klebe,C., Bischoff,F.R., Ponstingl,H. and Wittinghofer,A. (1995) Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1. *Biochemistry*, **34**, 639–647.

Koepp,D.M. and Silver,P.A. (1996) A GTPase controlling nuclear trafficking: running the right way or walking randomly? *Cell*, **87**, 1–4.

Koepp,D., Wong,D., Corbett,A. and Silver,P. (1996) Dynamic localisation of the nuclear import receptor and its interactions with transport factors. *J. Cell Biol.*, **133**, 1163–1176.

Kutay,U., Izaurralde,E., Bischoff,F.R., Mattaj,I.W. and Görlich,D. (1997a) Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. *EMBO J.*, **16**, 1153–1163.

Kutay,U., Bischoff,F.R., Kostka,S., Kraft,R. and Görlich,D. (1997b) Export of importin-α is mediated by a specific nuclear transport factor. *Cell*, in press.

Lounsbury,K.M. and Macara,I.G. (1997) Ran-binding protein 1 (RanBP1) forms a ternary complex with Ran and Karyopherin b and reduces GTPase-activating protein (RanGAP) inhibition by karyopherin b. *J. Biol Chem.*, **272**, 551–555.

Mahajan,R., Delphin,C., Guan,T., Gerace,L. and Melchior,F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell*, **88**, 97–107.

Matunis,E.L., Matunis,M.J. and Dreyfuss,G. (1993) Association of individual hnRNP proteins and snRNPs with nascent transcripts. *J. Cell Biol.*, **121**, 219–228.

Matunis,M.J., Coutavas,E. and Blobel,G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein between cytosol and nuclear pore complex. *J. Cell Biol.*, **135**, 1457–1470.

Melchior,F., Paschal,B., Evans,E. and Gerace,L. (1993a) Inhibition of nuclear protein import by nonhydrolyzable analogs of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.*, **123**, 1649–1659.

Melchior,F., Weber,K. and Gerke,V. (1993b) A functional homologue of the RNA1 gene product in *Schizosaccharomyces pombe*: purification, biochemical characterization, and identification of a leucine-rich repeat motif. *Mol. Biol. Cell*, **4**, 569–581.

Michael,W.M., Choi,M. and Dreyfuss,G. (1995) A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell*, **83**, 415–422.

Michael,W.M., Eder,P.S. and Dreyfuss,G. (1997) The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. *EMBO J.*, **12**, 3587–3598.

Moore,M.S. and Blobel,G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature*, **365**, 661–663.

Munro,S. and Pelham,H.R. (1984) Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of *Drosophila* hsp 70. *EMBO J.*, **3**, 3087–3093.

Nakielny,S., Siomi,M.C., Siomi,H., Michael,W.M., Pollard,V. and Dreyfuss,G. (1996) Transportin: nuclear transport receptor of a novel nuclear protein import pathway. *Exp. Cell Res.*, **229**, 261–266.

Nigg,E.A. (1997) Nucleocytoplasmic transport: signals, mechanism and regulation. *Nature*, **386**, 779–787.

Ohtsubo,M., Okazaki,H. and Nishimoto,T. (1989) The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J. Cell Biol.*, **109**, 1389–1397.

Palacios,I., Weis,K., Klebe,C. and Dingwall,C. (1996) Ran/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus. *J. Cell Biol.*, **133**, 485–494.

Panté,N. and Aebi,U. (1996) Towards the molecular dissection of protein import into nuclei. *Curr. Opin. Cell Biol.*, **8**, 397–406.

Pollard,V.W., Michael,W.M., Nakielny,S., Siomi,M.C., Wang,P. and Dreyfuss,G. (1996) A novel receptor mediated nuclear protein import pathway. *Cell*, **88**, 985–994.

Rexach,M. and Blobel,G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell*, **83**, 683–692.

Richards,S.A., Lounsbury,K.M., Carey,K.L. and Macara,I.G. (1996) A nuclear export signal is essential for the cytosolic localisation of the Ran binding protein, RanBP1. *J. Cell Biol.*, **134**, 1157–1168.

Richards,S.A., Carey,K.L. and Macara,I.G. (1997) Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. *Science*, **276**, 1842–1844.

Saavedra,C., Tung,K.S., Amberg,D.C., Hopper,A.K. and Cole,C.N. (1996) Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae*. *Genes Dev.*, **10**, 1608–1620.

Saavedra,C., Felber,B. and Izaurralde,E. (1997) The simian retrovirus-1 constitutive transport element, unlike the HIV-1 RRE, uses factors required for cellular mRNA export. *Curr. Biol.*, **7**, 619–628.

Schlenstedt,G. (1996) Protein import into the nucleus. *FEBS Lett.*, **389**, 75–79.

Schlenstedt,G., Saavedra,C., Loeb,J.D.J., Cole,C.N. and Silver,P.A. (1995) 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*, **92**, 225–229.

Siomi,H. and Dreyfuss,G. (1995) A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.*, **129**, 551–560.
Stade,K., Ford,C.S., Guthrie,C. and Weis,K. (1997) CRM1/Exportin 1 (XPO1) is an essential nuclear export factor. *Cell*, in press.
Stutz,F., Neville,M. and Rosbash,M. (1995) Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell*, **82**, 495–506.
Traglia,H.M., Atkinson,N.S. and Hopper,A.K. (1989) Structural and functional analyses of *Saccharomyces cerevisiae* wild-type and mutant RNA1 genes. *Mol. Cell. Biol.*, **9**, 2989–2999.
Weighardt,F., Biamonti,G. and Riva,S. (1995) Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains of hnRNP A1. *J. Cell Sci.*, **108**, 545–555.
Weis,K., Dingwall,C. and Lamond,A.I. (1996) Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities. *EMBO J.*, **15**, 7120–7128.
Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.
Wu,J., Matunis,M.J., Kraemer,D., Blobel,G. and Coutavas,E. (1995) Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, ran-GTP binding sites, zinc fingers, a cyclophilin a homologous domain, and a leucine-rich region. *J. Biol. Chem.*, **270**, 14209–14213.
Yokoyama,N. *et al*. (1995) A giant nucleopore protein that binds Ran/TC4. *Nature*, **376**, 184–188.
Zasloff,M. (1983) tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Natl Acad. Sci. USA*, **80**, 6436–6440.
Zolotukhin,A.S. and Felber,B.K. (1997) 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.*, **272**, 11356–11360.

*Received on June 18, 1997; revised on August 14, 1997*