Nucleic Acids Research, 1996, Vol. 24, No. 10 **1829–1836**

In vitro and *in vivo* evidence that protein and U1 snRNP nuclear import in somatic cells differ in their requirement for GTP-hydrolysis, Ran/TC4 and RCC1

Christopher Marshallsay, Achim Dickmanns[1], F. Ralf Bischoff[2], Herwig Ponstingl[2], Ellen Fanning[1] and Reinhard Lührmann*

Institute for Molecular Biology and Tumour Research, Philipps University, Emil-Mannkopffstrasse 2, 35037 Marburg, Germany;[1]Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235, USA and [2]Division of Molecular Biology of Mitosis, German Cancer Research Center, Im Neuenheimer Feld 280, 69009 Heidelberg, Germany

Received February 26, 1996; Revised and Accepted April 4, 1996

ABSTRACT

GTP-hydrolysis, the small ras-related GTP-binding protein Ran and its cognate guanosine nucleotide exchange factor, the *RCC1* gene product, have recently been identified as essential components of the protein nuclear import pathway. In this report we use three independent approaches to investigate the role of these components in U1 snRNP nuclear import in somatic cells. (i) Using a somatic cell based *in vitro* nuclear import system we show that U1 snRNP nuclear import, in marked contrast to protein transport, is not significantly inhibited by non-hydrolyzable GTP-analogs and is therefore unlikely to require GTP-hydrolysis. (ii) Using the dominant negative Ran mutant RanQ69L, which is defective in GTP-hydrolysis, we show that Ran-mediated GTP-hydrolysis is not essential for the nuclear import of U1 snRNP in microinjected cultured cells. (iii) Using a cell line expressing a thermolabile *RCC1* gene product, we show that the nuclear accumulation of microinjected U1 snRNP is not significantly affected by RCC1 depletion at the non-permissive temperature, indicating that RCC1 function is not essential for U-snRNP nuclear import. Based on these observations we conclude that protein and U-snRNP nuclear import in somatic cells differ in their requirements for GTP-hydrolysis, and Ran or RCC1 function. Based on these results, the substrates for nucleocytoplasmic exchange across the NPC can be divided into two classes, those absolutely requiring Ran, including protein import and mRNA export, and those for which Ran is not essential, including U-snRNP nuclear import, together with tRNA and U1 snRNA nuclear export.

INTRODUCTION

The bi-directional movement of macromolecules between the cytoplasmic and nuclear compartments occurs solely through the nuclear pore complexes (NPC) embedded in the nuclear envelope (NE). Although many smaller molecules can passively diffuse across the NPC, others, particularly those larger than 40 kDa are translocated by signal mediated mechanisms. Both the recognition of the nuclear localisation signals (NLS) by their cognate receptors and docking at the NE precede the energy dependent movement through the nuclear pores (reviewed in 1). NLS recognition and NE docking requires two proteins, denoted importin-α and importin-β (also referred to as karyopherin-α and -β, respectively) (2–6). The importin-α/β-karyophile complex is presumed to dock with proteins on the cytoplasmic face of the NPC (5,7). Subsequent nuclear entry requires an additional protein complex containing at least the two proteins p25 [Ran/TC4, ras-related nuclear protein (8,9), a small GTP-binding protein] and p10/NTF2, as well as GTP-hydrolysis (10–15).

The U1, U2, U4 and U5 small nuclear ribonucleoprotein particles (U-snRNPs), essential components of the splicing machinery, are assembled in a complex sequence of events in both the nuclear and cytoplasmic compartments. The RNA components of these U-snRNPs are co-transcriptionally capped with a 7-methyl-guanosine-cap (m^7G-cap) structure which constitutes part of their nuclear export signal (16). Once in the cytoplasm these U-snRNAs, which also share a single-stranded uridine rich sequence referred to as the Sm-binding site, are assembled into an RNA–protein complex, referred to as the Sm-core domain, which contains members of the Sm-protein family (17). The m^7G-cap is then hypermethylated to a 2,2,7-trimethylguanosine-cap (m_3G-cap), an event which effectively inactivates the nuclear export signal. Additional RNP specific proteins are then added to such Sm-core particles prior to, during or after re-entry into the nucleus (reviewed in 18).

From studies using microinjected *Xenopus* oocytes, we know that these U-snRNPs possess a complex bipartite nuclear localisation signal (NLS) composed of the Sm-core domain and the m_3G-cap (19–22). However, based on recent results obtained using both microinjected cultured cells and an *in vitro* snRNP nuclear import system, it is now clear that the Sm-core domain alone is both necessary and sufficient to mediate the nuclear targeting of U-snRNPs in somatic cells (23,24). The reported

* To whom correspondence should be addressed

m_3G-cap dependence is a characteristic unique to oocytes (24). U-snRNP nuclear import has the following features (23): (i) it is ATP and temperature dependent, (ii) it is a saturable process, and (iii) it requires soluble cytosolic factors. At least some components of the U-snRNP and protein nuclear import pathway differ since these two karyophile classes do not cross compete for limiting cytosolic factors. Nevertheless, both U-snRNPs and proteins enter the nucleus through the same or structurally similar nuclear pores, since antibodies directed against NPC proteins inhibit the transport of both karyophile classes. However, the two karyophile classes appear to interact differently with the NPC during translocation from the cytoplasm to the nucleus, since wheat germ agglutinin (WGA) has only a limited inhibitory effect on U-snRNP nuclear accumulation under conditions which completely abolish protein import (23,25).

The recent observation that protein nuclear import requires Ran-mediated GTP-hydrolysis has lead to the suggestion that such small ras-related GTP-binding proteins are general components of the nucleocytoplasmic transport machinery (11–13,15). GTP-binding proteins are a super family of proteins known to function as molecular switches in diverse cellular events including vesicle targeting (26), protein synthesis and targeting to the ER by the signal recognition particle (27), as well as mRNA export (28–30). Ran (M_r ~25 kDa) is a predominately nuclear protein representing ~0.36% of total HeLa cell protein (31). At ~10^7 copies/cell Ran is ~20-fold more abundant than its cognate guanosine nucleotide exchange factor, RCC1, and its GTPase activating protein, RanGAP1 (32). The phenotypes associated with mutations in Ran or Ran-interacting proteins are pleiotropic and relate to diverse cellular functions which include cell cycle progression, nuclear structure, RNA processing and export as well as protein import (reviewed in 33,34–36). Whereas in most cases it remains to be established whether these effects are the direct or indirect consequence of Ran or Ran-interacting protein dysfunctions, in protein nuclear import, the direct participation of Ran-mediated GTP-hydrolysis has been demonstrated (11,13,15,35). Recently, Ran-mediated GTP-hydrolysis has been mapped to an early step in protein nuclear import observed exclusively on the cytoplasmic face of the NPC (36).

One mutation affecting a Ran-interacting protein involves the evolutionarily conserved *RCC1* (regulator of chromosome condensation 1) gene which was initially reported as the mutant gene responsible for the pleiotropic temperature sensitive phenotypes of the tsBN2 cell line (reviewed in 33). Recently *RCC1* was implicated in RNA export (28–30) and protein import (37,38). Incubation of tsBN2 cells at the restrictive temperature leads to the rapid degradation of the *RCC1* gene product (RCC1). At this time RCC1 is no longer detectable using immunological assays (31,37,39). Loss of RCC1 function can be relieved by the re-introduction of wild type RCC1 (37) or either GDP- or GTP-bound Ran (38).

In this report we present *in vitro* and *in vivo* evidence that protein and U-snRNP nuclear import in somatic cells differ in their requirement for GTP-hydrolysis, RCC1 and Ran. These results suggest that the early steps of the protein and U-snRNP nuclear import pathways differ, but do not exclude the possibility that the two pathways converge at a later step prior to or at the site of the actual NPC translocation.

MATERIALS AND METHODS

Preparation of microinjected proteins

SV40 T-antigen was purified as described (41). Recombinant human Ran and RanQ69L were expressed in *Escherichia coli* and purified as described (38). After loading with GTP, the identity of the nucleotide bound to the recombinant protein was confirmed by HPLC (42).

Cell culture

HeLa and tsBN2 cells (43) were grown in Dulbecco's modified Eagle medium (Gibco, Eggenstein, Germany) supplemented with antibiotics and 10% fetal calf serum (FCS) (BioChrom, Berlin, Germany) in a humidified incubator at 33.5°C (tsBN2, permissive temperature) or 37°C (HeLa) under 10% atmospheric CO_2.

Microinjection

For microinjection experiments, cells were plated at least 36 h before microinjection on glass coverslips (Eppendorf, Hamburg, Germany). An Eppendorf microinjector (model 5242/5170) mounted on an IM35 inverted microscope (Carl Zeiss, Oberkochen, Germany) was used to deliver samples. The karyophiles, SV40 T-antigen (0.5 mg/ml), and U1 snRNP (1 mg/ml), were centrifuged for 15 min at 14 000 *g*, prior to microinjection. For co-injections, karyophile was mixed 1:1 with recombinant Ran (4 mg/ml). The volume injected was estimated to be 5–10 × 10^{-15} litres. Microinjection needles were pulled from glass capillaries on an automatic pipette puller (David Kopf Instruments, Tujunga, USA).

Immunofluorescent staining

Microinjected cells were washed three times with PBS (pH 7.4), fixed in 4% ice cold paraformaldehyde in PBS for 15 min, permeabilized for 20 min in 0.2% Triton X-100 in PBS, and blocked for at least for 1 h in 10% FCS in PBS to reduce non-specific staining. T-antigen was visualised by staining for 1 h with an antibody mix containing mouse monoclonal antibodies Pab 101, Pab 221, Pab 416 and Pab 419 (10 µg/ml each) (38) followed by FITC-conjugated second antibody (Jackson Immuno-Research Laboratories, West Grove, USA) (1:50) for 1 h. Antibodies were diluted with 10% FCS in PBS, the incubations were carried out at room temperature. After each antibody incubation, cells were washed three times quickly and then another three times for 10 min each. After the last wash step, the coverslips were air-dried, mounted in 90% glycerol containing 0.1 mg/ml *p*-phenylenediamine, and viewed on an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) using a 63× objective.

Preparation of fluorescently labelled karyophiles

Fluorescently labelled BSA–NLS conjugates were prepared as described (23). As judged by SDS–PAGE, fluorescein-labelled conjugates do not contain free label and have 10–20 NLS peptides (peptide sequence, PKKKRKV^{132}EDPYC) per BSA molecule. Fluorescently labelled dextran, M_r 70 kDa, was obtained from Sigma (Deisenhofen, Germany). Isolated HeLa U1 snRNPs were purified and labelled as described (23) except the NHS

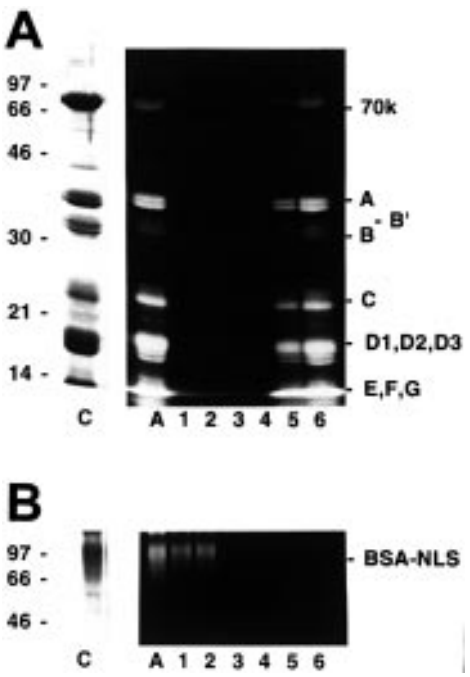

Figure 1. Fluorescently labelled HeLa U1 snRNPs sediment as 10-12S particles and are labelled on all constituent proteins. Fluorescently labelled U1 snRNP (**A**) and BSA–NLS peptide conjugates (**B**), 50 µg samples were sedimented on 5–20% glycerol gradients and fractions analysed using SDS–PAGE and visualised using UV light induced fluorescence (lanes A and 1–6) and Coomassie blue stain (lanes C). Analysis of glycerol gradient fractions (top to bottom, lanes 1–6), and ~25% of the material loaded onto the gradients (lanes A).

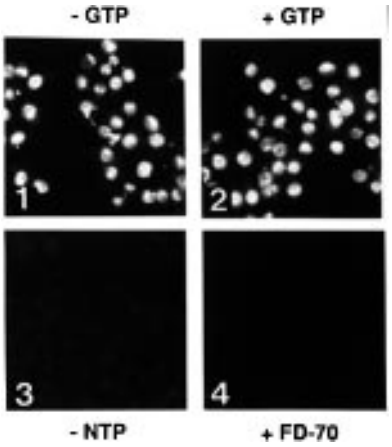

Figure 2. *In vitro* nuclear import of U1 snRNP requires ATP. *In vitro* import of fluorescently labelled U1 snRNPs into the nuclei of permeabilized HeLa cell nuclei was assayed with the standard import mix without (**1**) or with (**2**) exogenously added GTP (1 mM), (**3**) under hexokinase/glucose mediated ATP depletion conditions at 4°C, and (**4**) with fluorescently labelled dextran, M_r 70 kDa, instead of U1 snRNP.

(*N*-hydroxy-succinimidyl)-Cy3 dye was used for labelling (Biological Detection Systems Inc., Pittsburgh, USA). Labelled U1 snRNPs were purified using Centricon C-100 units (Amicon) and dialysed for 6 h against T-buffer (molecular weight cut off, 8 kDa). The purity and integrity of labelled particles was confirmed by SDS–PAGE and sedimentation analysis on 5–20% glycerol gradients in T-buffer at 260 000 *g* at 4°C for 6 h, in a Beckmann TLS-55 rotor as described (23).

In vitro nuclear import assay

Unless indicated otherwise, nuclear import assays were performed essentially as described (23). Untreated reticulocyte lysate (Serva, Heidelberg, Germany), pre-dialysed in T-buffer, was typically 50% of the transport mix volume. Karyophiles were added to give a working concentration of 0.1 mM. ATP depleted conditions were obtained by pre-incubation of the transport mix, without ATP, phosphocreatine and creatinephosphokinase, for 30 min at 37°C in the presence of either 10 U/ml apyrase or 10 U hexokinase/10 mM glucose and then incubation of subsequent transport assays at 4°C. Standard transport assays were incubated for 60 min at 37°C, before termination by washing the coverslips 3 × 5 min in ice-cold PBS (pH 7.4) followed by 15 min fixation in 4% paraformaldehyde/PBS on ice. Coverslips were then washed for 5 min each in PBS and PBS containing 50 ng/ml bisbenzimide DNA dye (Hoechst 33258), and finally three times in PBS, before air drying and mounting. Nuclear fluorescence was quantified using a Kappa video camera (Kappa Messtechnik, Germany) linked to a Quantimet 570 running with customised Leica Q570 Software (Leica, Bensheim, Germany) as described (23). For each mean fluorescence value ~100 nuclei were measured in at least two independent experiments.

RESULTS

In vitro nuclear import of U1 snRNP requires ATP and is independent of exogenously added GTP

We have recently established an *in vitro* system which accurately reproduces U-snRNP nuclear import *in vivo* (23). The recent demonstration that GTP is required for protein nuclear import suggested that GTP may also be needed for U-snRNP nuclear import. Since our previous studies were routinely performed in the absence of exogenously added GTP we have tested whether the inclusion of GTP has a stimulatory effect on U-snRNP nuclear import.

For these studies we have used fluorescently labelled HeLa U1 snRNPs as model karyophile. Isolated 10-12S U1 snRNP preparations contained the common Sm proteins (E, F, G, D1, D2, D3, B and B′), the specific A, C and 70k proteins and also ~1% contaminating U5 snRNP proteins. These U1 snRNPs were fluorescently labelled via primary amine groups of proteins exposed on the intact particles and purified from excess dye and any proteins which dissociate during labelling by microfiltration on Centricon C-100 units and dialysis. After this procedure, all of the U1 snRNP proteins (E, F, G, D1, D2, D3, C, A, B, B′ and 70k) are labelled although the labelling of the 70k and B, B′ proteins is reproducibly weaker (Fig. 1A, lane A). To confirm that labelled U1 snRNPs are intact 10-12S RNPs, we sedimented such particles on 5–20% glycerol gradients and analysed gradient fractions using SDS–PAGE. Consistent with previous studies (23), labelled U1 snRNP were found in fractions 5 and 6 as 10-12S particles (Fig. 1A). A very small (<1%) amount of free unlabelled protein, in fractions 1 and 2, migrating at 70 kDa, was also detectable with Coomassie blue staining but not with UV induced fluorescence. For comparison Figure 1B shows that free fluorescently labelled proteins (BSA–NLS conjugates) sedimented in fractions 1 and 2 on

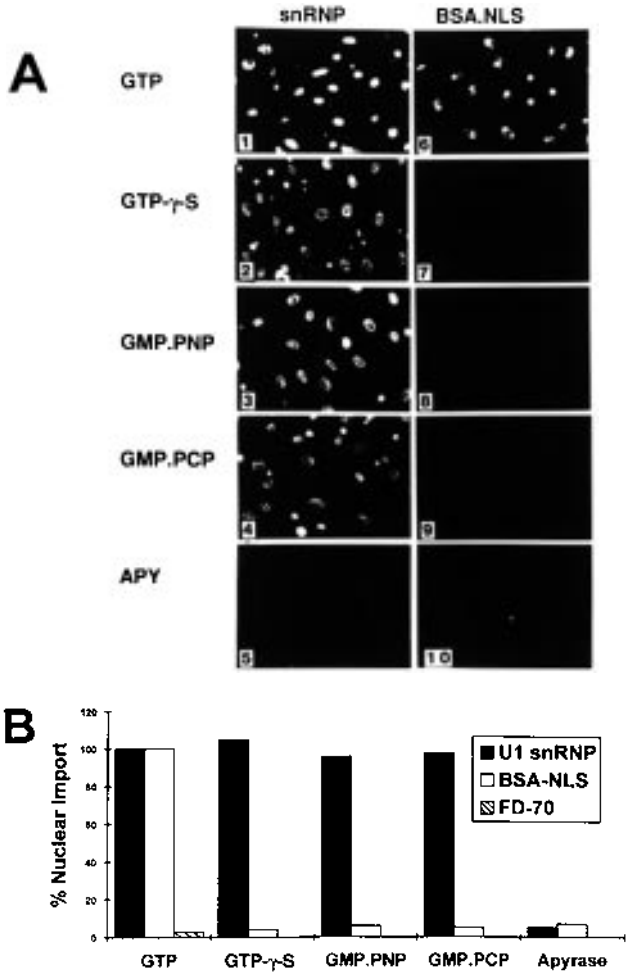

Figure 3. GTP-hydrolysis is essential for protein, but not U1 snRNP, nuclear import. (**A**) *In vitro* import of fluorescently labelled U1 snRNPs or BSA–NLS conjugates into the nuclei of permeabilized HeLa cell nuclei supplemented with 1 mM nucleotide as indicated: (1, 6) GTP; (2, 7) GTP-γ-S; (3, 8) GMP.PNP; (4, 9) GMP.PCP; and (5, 10) with apyrase and incubation at 4°C. Panels 1–5, U1-snRNP transport. Panels 6–10, BSA-NLS transport. Import was for 60 min at 37°C. (**B**) Quantitation of the nuclear fluorescence in (A). Typically ~100 randomly selected cells, located at various locations on the coverslip, were analysed. FD-70, fluorescence measured when fluorescently labelled dextran, M_r 70 kDa, was used instead of U1 snRNP.

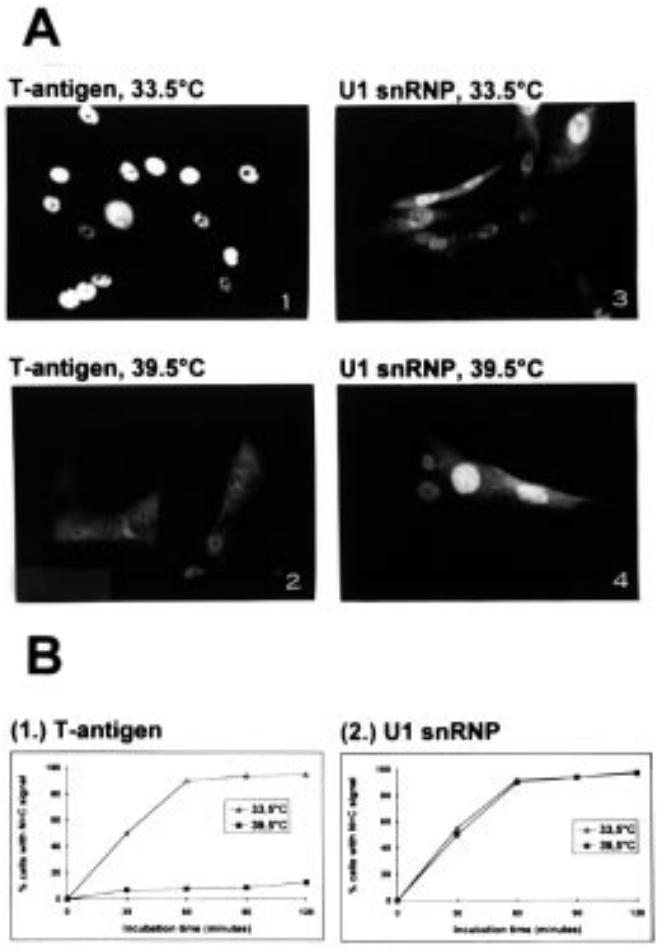

Figure 4. RCC1 depletion inhibits protein, but not U-snRNP, nuclear accumulation *in vivo*. Nuclear import of microinjected SV40 T-antigen (T-antigen; 0.5 mg/ml final concentration) or fluorescently labelled U1 snRNP (U1 snRNP; 1 mg/ml final concentration) was assayed in cells expressing a thermolabile *RCC1* gene product. Cells were pre-incubated at either the permissive (33.5°C) or the restrictive (39.5°C) temperature for 6 h prior to cytoplasmic injection and continued incubation for the indicated times. The cells were then processed and evaluated using fluorescence microscopy. (**A**) Representative nuclear accumulation of T-antigen (panels 1 and 2) and U1 snRNP (panels 3 and 4) after a 60 min incubation at the permissive and restrictive temperatures respectively. (**B**) Kinetics of T-antigen (graph 1) and U1 snRNP (graph 2) nuclear import under the conditions used in (A). Nuclear import was evaluated by counting the number of cells stained predominantly in either the nucleus (N>C) or in the cytoplasm (N<C), at each time point and expressing the values as the percent of total stained cells counted.

equivalent gradients. Therefore our labelled U1 snRNP preparations contain intact ribonucleoprotein particles and only very few free labelled proteins. Cytoplasmically injected labelled U1 snRNPs accumulate in the nuclei of microinjected cultured cells incubated at 37°C but not at 4°C, indicating that this import is an active process (data not shown). In contrast, free dye does not accumulate in nuclei either in the *in vitro* system or when microinjected into the cytoplasm of cultured cells (23). Several experiments performed *in vitro* and *in vivo*, demonstrate that labelled U-snRNPs are transported as such and do not undergo disassembly/reassembly events (22,23). Whereas the U1 snRNP specific proteins are known to enter the nucleus by the conventional protein import pathway (18), the free Sm core proteins are known to enter the nucleus only in the form of RNPs (18).

As shown in Figure 2, fluorescently labelled U1 snRNPs are equally well imported in permeabilized HeLa cell nuclei supplemented with somatic cell cytosol, in the standard assay with (panel 2) or without (panel 1) the inclusion of 1 mM GTP. Consistent with our previous report (23) this transport requires ATP since ATP depletion using hexokinase and glucose inhibits nuclear translocation (panel 3). This nuclear fluorescence is not the result of simple diffusion since other large molecules such as fluorescein-labelled dextrans (FD-70, M_r 70 kDa) (panel 4) do not accumulate in these nuclei. We conclude that fluorescently labelled U1 snRNPs are

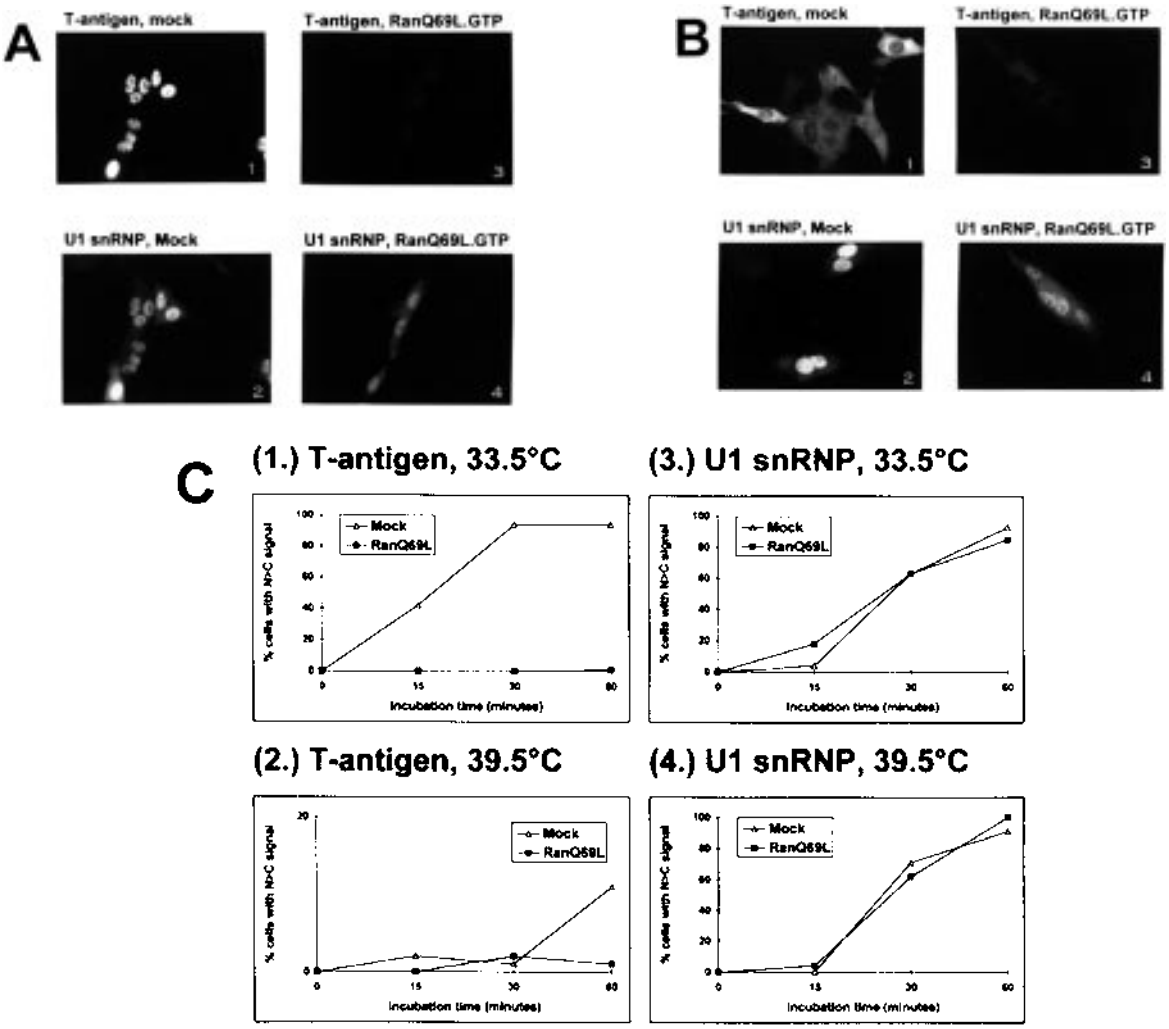

Figure 5. Ran mediated GTP-hydrolysis is essential for protein but not for U1 snRNP nuclear translocation *in vivo*. Nuclear import of microinjected SV40 T-antigen (T-antigen; 0.25 mg/ml final concentration) or fluorescently labelled U1 snRNP (U1 snRNP; 0.5 mg/ml final concentration), co-injected with recombinant RanQ69L.GTP (2 mg/ml final concentration) in tsBN2 cells. Microinjection and quantification were performed as described in Figure 4. (**A**) Nuclear import of T-antigen and U1 snRNP (panels 3 and 4, respectively) after 60 min incubation at the permissive temperature (33.5°C) when co-injected with RanQ69L.GTP and GTP alone in buffer (mock) (panels 1 and 2, respectively). (**B**) As in (A) but with incubation at the restrictive temperature (39.5°C). (**C**) Nuclear import kinetics of T-antigen (graphs 1 and 2), and U1 snRNP (graphs 3 and 4) at the permissive (33.5°C; graphs 1 and 3) and restrictive (39.5°C; graphs 2 and 4) temperatures, respectively, when co-injected with RanQ69L.GTP.

imported into the nuclei of permeabilized cultured cells in an ATP dependent manner with or without exogenously added GTP.

GTP-hydrolysis is essential for protein but not U1 snRNP nuclear import *in vitro*

Although the inclusion of exogenously added GTP had no stimulatory effect on U1 snRNP nuclear import, endogenous GTP derived from the permeabilized cells or pre-bound to cytosolic GTP-binding proteins could suffice to mediate U1 snRNP nuclear import. To address this possibility we have examined the effects of non-hydrolyzable GTP-analogs on U1 snRNP nuclear accumulation *in vitro*. As seen in Figure 3A, U1 snRNP and protein karyophile, a fluorescently labelled BSA–NLS peptide conjugate, are actively imported in the presence of 1 mM GTP (panels 1 and 6). When GTP is substituted with the non-hydrolyzable analogs GTP-γ-S, GMP.PNP or GMP.PCP, the nuclear import of U1 snRNP is not significantly altered (panels 2, 3 and 4, respectively). In contrast the nuclear accumulation of BSA–NLS was markedly inhibited by the inclusion of the same non-hydrolyzable GTP-analogs (panels 7, 8 and 9, respectively). Consistent with Figure 2, and as seen in panels 5 and 10, both U1 snRNP and protein nuclear accumulation are inhibited when NTP is depleted by apyrase treatment, confirming that the observed nuclear import is the result of active transport. Similar results were obtained when using HeLa cell S-100 extracts (data not shown) instead of rabbit reticulocyte lysate, indicating that the source of somatic cell cytosol is not critical.

Consistent with the visual evaluations (Fig. 3A), quantitative analysis using video-linked-fluorescence-microscopy revealed a reduction of 80% or more in the protein nuclear accumulation in the presence of three different non-hydrolyzable GTP-analogs when compared with transport in the presence of GTP (Fig. 3B). In contrast, inclusion of GTP or non-hydrolyzable GTP-analogs

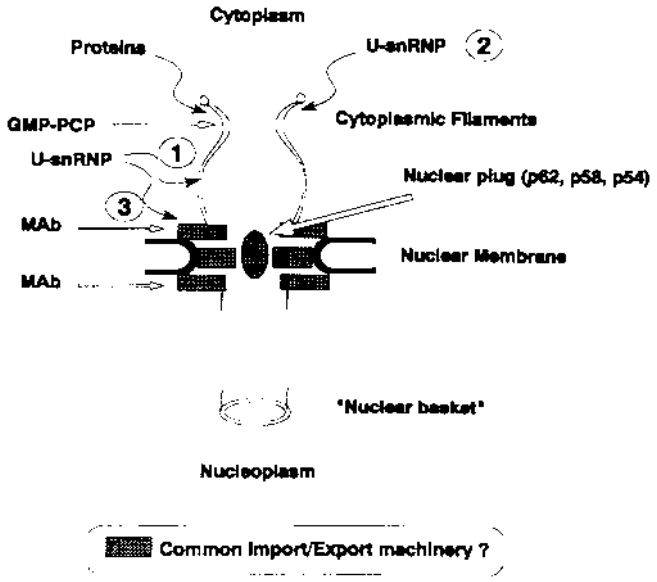

Figure 6. Early steps of protein and U-snRNP nuclear import: a working model for protein and U-snRNP nuclear import. (**1**), (**2**) and (**3**) indicate possible U-snRNP entry points to the nuclear import machinery. GMP-PCP indicates the observed site of protein karyophile accumulation in the presence of non-hydrolyzable GTP analogs, mutant RanQ69L, or in the absence of ATP (1,36,46). (MAb) and (Nuclear plug) indicate the vicinity of antigens recognised by anti-pore protein p62 antibodies (reviewed in 1).

did not significantly impair the observed U1 snRNP nuclear accumulation in this system (Fig. 3B).

Although we cannot exclude the possibility that GTP persisting in the permeabilized cells or GTP contamination of the ATP in the energy regenerating system, could suffice for the U1 snRNP nuclear import observed in our assays, these results do suggest that GTP-hydrolysis is essential for the nuclear accumulation of protein, but not U1 snRNP. They are therefore consistent with the established role of GTP-binding proteins, such as Ran, in protein nuclear import (11,13,15) and suggest that such proteins are not essential for U1 snRNP transport.

RCC1 depletion inhibits the nuclear accumulation of microinjected protein, but not U1 snRNP

Independent evidence supporting our earlier conclusion that GTP-binding proteins, in particular Ran, are not essential for U-snRNP nuclear import, was obtained using the mutant baby hamster kidney cell line tsBN2 which expresses a temperature sensitive *RCC1* gene product (43). Incubation of these cells at the restrictive temperature leads to the rapid degradation of the RCC1, thereby disrupting Ran GTP/GDP cycling and leading to a defect in protein nuclear import (see Introduction) (37,38). If Ran GTP/GDP cycling is essential for U1 snRNP nuclear import, then RCC1 depletion might be predicted to lead to a U1 snRNP transport defect.

We therefore pre-incubated tsBN2 cells for 6 h at either the permissive (33.5°C) or restrictive (39.5°C) temperatures prior to cytoplasmic microinjection of karyophile. After injection the incubation was continued at the same temperature for 60 min prior to fixation and preparation for fluorescence-microscopy. After 6 h incubation at the non-permissive temperature, little or no RCC1 is detectable immunologically (31,37–39). As seen in Figure 4A, the observed nuclear accumulation of U1 snRNP was equally efficient at both the permissive and restrictive temperatures (compare panels 3 and 4). In contrast, the nuclear import of the SV40 T-antigen was drastically inhibited at the restrictive, but not the permissive, temperature (compare panels 1 and 2).

To quantitatively evaluate the ability of tsBN2 cells to import karyophile, a kinetic analysis of karyophile nuclear accumulation was performed and the microinjected cells displaying predominantly nuclear or cytoplasmic signals were counted and expressed as a percent of the total number of stained cells. As shown in Figure 4B, this quantitational analysis confirmed our interpretation of Figure 4A. Consistent with our *in vitro* data, these results suggest that RCC1, and therefore indirectly also Ran, is not essential for U1 snRNP nuclear import.

Ran-mediated GTP-hydrolysis is essential for protein but not for U1 snRNP nuclear translocation *in vivo*

To directly test the possible involvement of Ran-mediated GTP-hydrolysis in U1 snRNP nuclear import, we have studied the effects of co-injecting a dominant negative Ran mutant, designated RanQ69L, into cultured cells. As a consequence of changing glutamate residue 69 into a leucine, RanQ69L is GTPase deficient and therefore accumulates in the GTP-bound form (31,32,44). Thus, RanQ69L would be expected to induce the same phenotype as the addition of non-hydrolyzable GTP-analogs. Indeed, RanQ69L dramatically inhibits protein nuclear import both *in vitro* (36) and *in vivo* (38), presumably by acting as a competitive inhibitor that non-productively binds to Ran interacting proteins, such as nuclear pore components and RanGAP1.

Recombinant GTP-bound human Ran (Ran.GTP) and RanQ69L (RanQ69L.GTP), was prepared and charged with GTP as described previously (32,38; data not shown). Based on HPLC analysis, >95% of nucleotide bound to these recombinant proteins was GTP, the rest being GDP (data not shown). Both recombinant proteins display the expected functional phenotypes: the GTPase activity of the human Ran, but not the mutant RanQ69L, was stimulated several orders of magnitude by RanGAP *in vitro* (32; data not shown).

As seen in Figure 5A, co-injection of RanQ69L.GTP together with karyophile into tsBN2 cells incubated at the permissive temperature resulted in a drastic inhibition of protein transport (compare panels 1 and 3), but induced only a minimal effect on the observed U1 snRNP nuclear import after a 60 min incubation (compare panels 2 and 4). Control co-injections of recombinant wild-type Ran.GTP (data not shown) or GTP alone in buffer (panels 1 and 2) had no significant effect on either protein or U1 snRNP transport. We estimate that the amount of injected recombinant Ran is equivalent to ~5% of total cellular Ran and is equimolar with cytoplasmic Ran (32).

Conceivably only weak effects of the mutant Ran, or even the RCC1 depletion, on U1 snRNP transport could be missed by our assay. In contrast the combined effects of RanQ69L.GTP co-injection and RCC1 depletion could be expected to have a more obvious effect on U1 snRNP transport. We therefore also tested the effects of RanQ69L.GTP co-injection into tsBN2 cells incubated at the restrictive temperature. As shown in Figure 5B the inhibitory effect on protein import, after 60 min incubation, was even more drastic (compare panels 1 and 3) than with either treatment alone (Figs 4A and 5A), whereas U1 snRNP transport

remained largely unperturbed (compare panels 2 and 4). A kinetic analysis of the co-injection experiments (Fig. 5C) confirmed our interpretation that RanQ69L.GTP inhibits protein (graphs 1 and 2), but not U1 snRNP, nuclear import (graphs 3 and 4). Together with our earlier results (Fig. 4), these results indicate that neither RCC1 nor Ran-GDP/GTP cycling are essential for U1 snRNP transport.

DISCUSSION

Small GTP-binding proteins are essential components of many fundamental transport pathways. The identification of the small GTP-binding protein Ran, as an essential component of the protein nuclear import (11,13,15) and RNA export (28–30) machinery, provided further evidence that this class of proteins play crucial roles in cellular targeting events. The recent report that tRNA and U1 snRNA nuclear export is not dependent on RCC1 function (40) indirectly challenged the proposed universal role of Ran in nucleocytoplasmic exchange.

Using three independent approaches, we provide *in vivo* and *in vitro* evidence that Ran-GDP/GTP cycling is not essential for U1 snRNP nuclear import in somatic cells: (i) using a homologous *in vitro* transport system supplemented with non-hydrolyzable GTP-analogs we show that GTP-hydrolysis is not essential for U1 snRNP nuclear import; (ii) using the dominant negative Ran mutant, RanQ69L, which displays a defective GTP-hydrolysis, we provide *in vivo* evidence that Ran mediated GTP-hydrolysis is not essential for U1 snRNP nuclear import; and finally, (iii) using the temperature sensitive cell line tsBN2 which expresses a thermolabile *RCC1* gene product, the only known guanosine nucleotide exchange factor for Ran, we show that RCC1 mediated exchange of Ran bound GDP with GTP *in vivo* is also not essential for U1 snRNP nuclear import. Our *in vitro* studies with the non-hydrolyzable GTP-analogs also argue against the possible involvement of a further, as yet unidentified, GTP-binding protein in U1 snRNP nuclear import. In sum, our data argue against a role for GTP-binding proteins, such as Ran, as universal regulators of nucleocytoplasmic exchange (11–13,33). Instead our results, together with the observation that tRNA and U1 snRNA nuclear export are RCC1-independent, and therefore probably also Ran-independent (40), suggest that at least two pathways exist for nucleocytoplasmic exchange, one of which is absolutely Ran-dependent and the other which is not.

Available data suggest that at least some early components of the protein and U-snRNP nuclear import pathways differ. These include (i) their different sensitivity to wheat germ agglutinin which binds to *N*-acetyl-glucosamine modified nuclear pore proteins, (ii) the inability of these two karyophile classes to cross compete for limiting transport factors (23,25,45), and now also (iii) their differential Ran requirements. Despite these differences, based on the ability of an antibody directed against the NPC protein p62 to inhibit the nuclear import of both karyophile types, both proteins and U-snRNPs are believed to be translocated through the same or structurally similar NPCs (23,25). As shown schematically in Figure 6, consistent with the concept of a common translocating machinery, p62 has been localised to both the cytoplasmic and nuclear faces of the NPC (reviewed in 1). Current data suggest that the initial energy independent docking of karyophilic protein at the NPC occurs at sites some 60 nm from the central plane, a region corresponding with the NPC-fibrils projecting into the cytoplasm (36,46, reviewed in 1; Fig. 6). This region also coincides with the sites of protein karyophile accumulation induced by non-hydrolyzable GTP analogs or by the GTPase deficient Ran mutant, RanQ69L, suggesting that at least one site of GTP-hydrolysis is at or very close to the initial docking site (36,46, reviewed in 1; Fig. 6). Conceivably additional sites of GTP-hydrolysis events, not detected in this study (36), could exist along the protein import pathway to, and through, the central channel of the NPC.

The data presented in this study suggest that GTP-hydrolysis or Ran-GDP/GTP cycling is not essential for U1 snRNP nuclear import in somatic cells. Therefore, if the protein and U-snRNP import pathways utilise common components at some point, these must lie beyond the sites of transport arrest induced by Ran dysfunction, as depicted in route 1 (Fig. 6). Alternatively U-snRNPs may enter the import machinery via a completely different route, involving an initial docking with U-snRNP specific NPC structures (Fig. 6, route 2), or alternatively have an abbreviated import pathway and access the common import machinery at a point downstream of the Ran-mediated checkpoint (Fig. 6, route 3). In this context, based on our observation that fluorescently labelled U-snRNP can accumulate at the NE under ATP-depletion conditions in a modified *in vitro* import assay (unpublished data), much as described for karyophilic proteins (36,46, reviewed in 1), it will be interesting to compare the sites of karyophile accumulation under these conditions at the ultrastructure level. Likewise, using available NPC protein mutants and a yeast based U-snRNP *in vitro* nuclear import system, it should soon be possible to directly address the role of specific NPC proteins in protein and U-snRNP nuclear import.

ACKNOWLEDGEMENTS

We thank A. Brunahl and M. Hauser for preparation of antibodies, A. Badouin and M. Wick for technical assistance, and C. Cole for providing tsBN2 cells. Support from the Deutsche Forschungsgemeinschaft (Fa 138/4-3 to E.F., SFB 286 to R.L., Po 152/8-2 to H.P.), the Vanderbilt University and the Fonds der Chemischen Industrie is gratefully acknowledged.

REFERENCES

1 Melchior,F. and Gerace,L. (1995) *Curr. Opin. Cell Biol.*, **7**, 310–318.
2 Adam,E.J.H. and Adam,S.A. (1994) *J. Cell Biol.*, **125**, 547–555.
3 Görlich,D., Prehn,S., Laskey,R.A. and Hartmann,E. (1994) *Cell*, **79**, 767–778.
4 Görlich,D., Kostka,S., Kraft,R., Dingwall,C., Laskey,R.A., Hartmann,E. and Prehn,S. (1995) *Curr. Biol.*, **5**, 383–392.
5 Görlich,D., Vogel,F., Mills,A.D., Hartmann,E. and Laskey,R.A. (1995) *Nature*, **377**, 246–248.
6 Weis,K., Mattaj,I.W. and Lamond,A.I. (1995) *Science*, **268**, 1049–1053.
7 Radu,A., Moore,M.S. and Blobel,G. (1995) *Cell*, **81**, 215–222.
8 Drivas,G.T., Shih,A., Coutavas,E., Rush,M.G. and Eustachio,P.D. (1990) *Mol. Cell Biol.*, **10**, 1793–1798.
9 Bischoff,F.R. and Ponstingl,H. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10830–10834.
10 Moore,M.S. and Blobel,G. (1992) *Cell*, **69**, 939–950.
11 Moore,M.S. and Blobel,G. (1993) *Nature*, **365**, 661–663.
12 Moore,M.S. and Blobel,G. (1994) *Trends Biochem. Sci.*, **19**, 211–216.
13 Melchior,F., Paschal,B., Evans,J. and Gerace,L. (1993) *J. Cell Biol.*, **123**, 1649–1659.
14 Paschal,B.M. and Gerace,L. (1995) *J. Cell Biol.*, **129**, 925–937.
15 Schlenstedt,G., Saavedra,C., Loeb,J.D.J., Cole,C.N. and Silver,P.A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 225–229.
16 Hamm,J. and Mattaj,I.W. (1990) *Cell*, **63**, 109–118.
17 Mattaj,I.W. and De Robertis,E.M. (1985) *Cell*, **40**, 111–118.
18 Mattaj,I.W., Boelens,W., Izaurralde,E., Jarmolowski,A. and Kambach,C. (1993) *Mol. Biol. Rep.*, **18**, 79–83.

19 Hamm,J., Darzynkiewicz,E., Tahara,S.M. and Mattaj,I.W. (1990) *Cell*, **62**, 569–577.
20 Fischer,U. and Lührmann,R. (1990) *Science*, **249**, 786–790.
21 Fischer,U., Darzynkiewicz,E., Tahara,S.M., Dathan,N.A., Lührmann,R. and Mattaj,I.W. (1991) *J. Cell Biol.*, **113**, 705–714.
22 Fischer,U., Sumpter,V., Sekine,M., Satoh,T. and Lührmann,R. (1993) *EMBO J.*, **12**, 573–583.
23 Marshallsay,C. and Lührmann,R. (1994) *EMBO J.*, **13**, 222–231.
24 Fischer,U., Heinrich,J., Van Zee,K., Fanning,E. and Lührmann,R. (1994) *J. Cell Biol.*, **125**, 971–980.
25 Michaud,N. and Goldfarb,D. (1992) *J. Cell Biol.*, **116**, 851–861.
26 Preifer,S.R. (1992) *Trends Cell. Biol.*, **2**, 41–46.
27 Miller,J.D., Wilhelm,H., Gierasch,L., Gilmore,R. and Walter,P. (1993) *Nature*, **366**, 351–354.
28 Kadowaki,T., Zhao,Y. and Tartakoff,A.M. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2312–2316.
29 Kadowaki,T., Goldfarb,D., Spitz,L.M., Tartakoff,A.M. and Ohno,M. (1993) *EMBO J.*, **12**, 2929–2937.
30 Amberg,D.C., Fleischmann,M., Stagljar,I., Cole,C.N. and Aebi,M. (1993) *EMBO J.*, **12**, 233–241.
31 Ren,M., Drivas,G., Eustachio,P.D. and Rush,M.G. (1993) *J. Cell Biol.*, **120**, 313–323.
32 Bischoff,F.R., Klebe,C., Kretschmer,J., Wittinghofer,A. and Ponstingl,H. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 2587–2591.
33 Dasso,M. (1993) *Trends Cell. Biol.*, 18, 96–101.
34 Corbett,A.H., Koepp,D.M., Schlenstedt,G., Lee,M.S., Hopper,A.K. and Silver,P.A. (1995) *J. Cell Biol.*, **130**, 1017–1026.
35 Schlenstedt,G., Wong,D.H., Koepp,D.M. and Silver,P.A. (1995) *EMBO J.*, **14**, 5367–5378.
36 Melchior,F., Guan,T., Yokoyama,N., Nishimoto,T. and Gerace,L. (1995) *J. Cell Biol.*, **131**, 571–581.
37 Tachibana,T., Imamoto,N., Seino,H., Nishimoto,T. and Yoneda,Y. (1994) *J. Biol. Chem.*, **269**, 24542–24545.
38 Dickmanns,A.,Bischoff,R.F., Bischoff,F.R., Marshallsay,C., Lührmann,R., Ponstingl,H. and Fanning,E. (1996) *J. Cell. Sci.*, in press.
39 Nishitani,H., Ohtsubo,M., Yamashita,K., Iida,H., Pines,J., Yasudo,H., Shibata,Y., Hunter,T. and Nishimoto,T. (1991) *EMBO J.*, **10**, 1555–1564.
40 Cheng,Y., Dahlberg,J.E. and Lund,E. (1995) *Science*, **267**, 1807–1810.
41 Schneider,C., Weisshart,K., Guarino,L.A., Dornreiter,I. and Fanning,E. (1994) *Mol. Cell. Biol.*, **14**, 3176–3185.
42 Tucker,J., Sczakiel,G., Feuerstein,J., John,J., Goody,R.S. and Wittinghofer,A. (1986) *EMBO J.*, **5**, 1351–1358.
43 Nishimoto,T. and Basilico,C. (1978) *Som. Cell. Genet.*, **4**, 323–340.
44 Klebe,C., Prinz,H., Wittinghofer,A. and Goody,R.S. (1995) *Biochemistry*, **34**, 12543–12552.
45 Van Zee,K., Dickmanns,A., Fischer,U., Lührmann,R. and Fanning,E. (1993) *J. Cell Biol.*, **121**, 229–240.
46 Richardson,W.D., Mills,A.D., Dilworth,S.M., Laskey,R.A. and Dingwall,C. (1988) *Cell*, **52**, 655–664.