Rna1p, a Ran/TC4 GTPase Activating Protein, Is Required for Nuclear Import

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Abstract. The *Saccharomyces cerevisiae* gene, *RNA1*, encodes a protein with extensive homology to the mammalian Ran/TC4 GTPase activating protein. Using indirect immunofluorescence microscopy, we have demonstrated that *rna1-1* mutant cells are defective in nuclear import of several proteins. The same result is obtained when nuclear import is examined in living cells using a nuclear protein fused to the naturally green fluorescent protein. These findings suggest a role for the Rna1p in trafficking of proteins across the nuclear membrane. To investigate this role more directly, an in vitro import assay that monitors the import of a fluorescently labeled substrate into the nuclei of semi-intact yeast cells was used. Import to the nucleus requires the addition of exogenous cytosol. Results indicate that, in contrast to wild-type cytosols, extracts made from *rna1-1* mutant cells are unable to support import of the fluorescently labeled substrate into competent nuclei. Immunoblotting demonstrates that these mutant-derived extracts are depleted of Rna1p. However, when purified Rna1p is added back to these extracts the import activity is restored in a dose-dependent manner. These results demonstrate that Rna1p plays a direct role in the import of proteins into the nucleus.

MACROMOLECULAR transport across the nuclear envelope occurs at nuclear pores (Forbes, 1992; Rout and Wente, 1994) and consists both of the import of protein and snRNAs and the export of mature RNA and ribosomes (Garcia-Bustos et al., 1991; Nigg et al., 1991; Osborne and Silver, 1993; Fabre and Hurt, 1994). Since these processes are linked by their site of action it is likely that a number of components of the transport system are directly involved in both processes. For example, the nucleoporins, which are the structural components of the pore complex (Panté and Aebi, 1993; Rout and Wente, 1994), are likely to participate directly in both import and export. Although the nuclear pores embedded in the nuclear membrane serve as the site of translocation, proteins found both in the cytoplasm and the nucleus are required for the bi-directional transport of macromolecules (Powers and Forbes, 1994). It is not clear whether these accessory proteins function in a single concerted cycle of import and export or comprise two independent cycles, one that mediates the import of proteins to the nucleus and one that mediates the export of RNA from the nucleus.

Recent work has focused on the role of the small GTP-binding protein Ran/TC4 in nuclear import. This protein, which was originally identified on the basis of its homology to Ras (Drivas, 1990), has been shown to be a cytosolic factor that is absolutely required for import into isolated nuclei (Melchior et al., 1993a; Moore and Blobel, 1993, 1994a). Highly conserved homologues of Ran/TC4 have been identified in a number of organisms including *Xenopus* (Moore and Blobel, 1993), fission yeast (Matsumoto and Beach, 1991), and budding yeast (Belhumeur et al., 1993). In *Saccharomyces cerevisiae*, there are two copies of the Ran/TC4 homologue, *GSP1* and *GSP2*, which differ only by two amino acids. *GSP1* is an essential gene which is expressed at a higher level than the nonessential *GSP2* (Belhumeur et al., 1993).

A recent study demonstrated that in budding yeast, overexpression of mutant Gsp1p locked in the GTP-bound form causes defects both in protein import and poly(A)$^+$ RNA export (Schlenstedt et al., 1995). These results indicate that the ratio of GTP-/GDP-bound Gsp1p within the cell must be meticulously regulated. Biochemical studies have shown that isolated Ran/TC4 hydrolyzes GTP very slowly (Bischoff et al., 1994; Schlenstedt et al.,

1995). The rate of GTP hydrolysis is enhanced in the presence of cytosolic extracts. Fractionation of cytosolic extracts has resulted in purification of a GTPase activating protein or (GAP)[1] activity for Ran/TC4 and identified it as a mammalian homologue of *S. cerevisiae* Rna1p (Bischoff et al., 1995a). Other studies have demonstrated that Rna1p from *Schizosaccharomyces pombe* , which complements the *rna1-1* mutation in *S. cerevisiae* (Melchior et al., 1993b), also functions as a GTPase activating protein (Bischoff et al., 1995a).

The *RNA1* gene was initially identified in *S. cerevisiae* in a genetic screen for mutants defective in RNA processing (Hartwell, 1967) and mutations in this gene have subsequently been shown to result in defective pre-tRNA splicing, processing of pre-rRNA, and production and export of poly (A)$^+$ RNA from the nucleus (Hopper et al., 1978; Amberg et al., 1992). As localization studies of Rna1p determined that it was a cytoplasmic protein that was excluded from the nucleus (Hopper et al., 1990), it was not clear how defects in nuclear events such as RNA processing and transport were manifested. Its identification as a GAP suggests that Rna1p may be a component of a multifaceted system that uses a cycle of GTP hydrolysis to regulate the transport of macromolecules both into and out of the nucleus. Thus, the phenotypes observed in the mutants might result from alterations in the regulation of this hydrolysis cycle rather than from a direct involvement of the Rna1p in RNA processing.

The loss of a functional GAP protein would be reflected in a higher ratio of GTP/GDP-bound protein within the cell. Experiments that artificially altered this ratio by overexpression of the GTP-bound form of Gsp1p (Schlenstedt et al., 1995) demonstrated that the localization of proteins to the nucleus, as well as the export of poly (A)$^+$ RNA, are affected by changes in this ratio. Since RNA transport defects in the *rna1-1* mutant have been characterized, we were interested in determining whether this mutant would also display defects in protein import as would be expected if it is involved in regulation of GTP hydrolysis by Gsp1p.

Results presented here indicate that the temperature-sensitive mutant *rna1-1* is unable to efficiently target proteins to the nucleus at the nonpermissive temperature. To determine whether Rna1p is directly involved in protein import, we use an in vitro import assay (Schlenstedt et al., 1993) and demonstrate that cytosols prepared from *rna1-1* mutant cells are unable to support import of a fluorescently labeled nuclear localization sequence (NLS)-containing substrate. Addition of purified Rna1p is able to rescue the defective cytosols. These findings strongly suggest that Rna1p plays a direct role in the import of macromolecules to the nucleus.

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1. *Abbreviations used in this paper*: ECL, enhanced chemiluminescence; GAP, GTPase activating protein; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; GT, glutathione; HSA, human serum albumin; IPTG, isopropylthio-β-D-galactoside; NLS, nuclear localization sequence; RT, room temperature.

Materials and Methods

Strains and Plasmids

The wild-type strain employed for indirect immunofluorescence microscopy was the haploid strain FY86 ([*MATα, ura3-52, leu2Δ1, his3Δ200*]; a gift from F. Winston, Harvard University Medical School). Strain PSY714 (*MATa, rna1-1 ura3-52, leu2Δ1*) was created by crossing the original strain EE1b (Atkinson et al., 1985) twice to FY86. For in vitro import assays a wild-type sister spore of PSY714 (PSY715 [*MATα, ura3-52, leu2Δ1, his3Δ200*]) was used as the control. The diploid wild-type strain PSY223 (*MATa/MATα, ura3-52/ura3-52, leu2-3/leu2-3, his3/HIS3, his4/HIS4*) was used to prepare semi-intact cells for the in vitro assay.

The glutathione-*S*-transferase (GST)-Rna1p fusion plasmid was constructed by ligating PCR-amplified *RNA1* DNA with engineered BglII-EcoRI ends into the BamHI-EcoRI sites of pGEX-2T (Pharmacia, Piscataway, NJ). For experiments that examined the localization of a reporter protein, two constructs were employed. The first was a *LEU2, GAL1* promoter plasmid encoding the SV-40–NLS fused to the *SUC2* gene (Nelson and Silver, 1989), and the second was a *URA3, GAL1* promoter plasmid that consists of a portion of the nuclear protein Npl3p (Bossie et al., 1992) fused to the NH$_2$ terminus of the naturally fluorescent green fluorescent protein (GFP) (Chalfie et al., 1994) (Lee, M. S., and P. A. Silver, unpublished data).

Protein Localization

Indirect immunofluorescence microscopy was used to examine the localization of either an endogenous nuclear protein, Npl3p (Bossie et al., 1992; Flach et al., 1994), or an artificial reporter protein. For experiments that examined the cellular localization of Npl3p, cells were grown in YEPD at room temperature (RT) to a density of 10^7 cells/ml. Cultures were then split and half was maintained at RT and half was shifted to 36°C for 2 h. For inducible reporter proteins the cells were grown to a density of 5 × 10^6 cells/ml in media lacking uracil or leucine (depending on the plasmid employed) with 2% raffinose. Cultures were then induced by the addition of 2% galactose and growth was continued at RT for 2–4 h. Following this induction, cultures were shifted to 36°C. Samples were then either prepared for immunofluorescence microscopy as previously described (Flach et al., 1994) or viewed directly by microscopy using the FITC channel to examine the cellular localization of the fusion to GFP. For immunofluorescence microscopy, incubation with antibodies against Npl3p (1:500 dilution) (Bossie et al., 1992) or invertase (1:20,000 dilution) (Nelson and Silver, 1989) was followed by incubation with FITC-labeled antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:1,000 dilution and with DAPI.

In Vitro Import Assay

This assay was performed as described previously (Schlenstedt et al., 1993). Semi-intact cells were prepared from the wild-type strain PSY223. Cytosols were made from *rna1-1* mutant cells (strain PSY714) or a wild-type spore from the same tetrad (PSY715) grown at RT or 36°C. Cultures were grown to a density of 10^7 cells/ml at RT and then shifted to the nonpermissive temperature (36°C) (or maintained at the permissive temperature) for 2 h prior to the preparation of cytosols. Cells were pelleted, washed in buffer A (0.25 M sorbitol, 20 mM Pipes-KOH, pH 6.8, 150 mM K-acetate, 5 mM Mg-acetate), and resuspended in 1/75 vol buffer A containing 1 mM DTT, 0.5 mM PMSF, 3 μg/ml leupeptin, aprotinin, chymostatin, and pepstatin. Acid washed 0.5 mm glass beads were added at 1/3 vol of the cell suspension. Cells were lysed by a series of six to eight 120-s pulses in an ice-cold mini-bead beater. The resulting lysate was centrifuged at 30,000 g for 10 min. The resulting supernatant was then centrifuged at 100,000 g for 45 min. Samples were frozen in liquid nitrogen and stored at −80°C. The final protein concentration was ∼5–10 mg/ml as determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as a standard.

Import assays (30 μl total reaction vol) were performed in buffer A and contained 5 × 10^7 semi-intact cells/ml, 1 mM ATP, 0.1 mg/ml creatine kinase, 10 mM creatine phosphate, 1.5–2 mg/ml cytosolic proteins, and 5 μg/ml of a fluorescent substrate consisting of NLS peptide (CTPPKKKRKV) conjugated to human serum albumin (HSA). Some experiments also contained purified GST-Rna1p at a concentration of 10–50 μg/ml. Reactions were incubated at 30°C for 10 min, stained with DAPI (final concentration 0.25 μg/ml), and viewed by fluorescence microscopy. For all import data,

experiments were performed at least three times and 200 cells were scored in each experiment. The means and standard deviations shown were calculated from three independent experiments.

Purification of GST-Rna1p

The GST-Rna1 fusion protein was expressed in *Escherichia coli* DH5α cells. Cultures were grown overnight in Luria broth containing 100 μg/ml ampicillin at 37°C and then diluted into fresh media and grown until the OD 600 reached 0.6–0.7. Cultures were then induced with 0.5 mM isopropylthio-β-D-galactoside (IPTG) for 2 h at 30°C. Cells were pelleted at 6000 g at 4°C for 15 min and pellets were stored at −20°C overnight. The cells were then thawed and resuspended in 1 ml (per 50 ml culture) lysis buffer (200 mM NaCl, 50 mM Tris, pH 8.0, 2.5 mM EDTA, 0.1% Tween 20) containing protease inhibitors (0.5 mM PMSF, 3 μg/ml leupeptin, aprotinin, chymostatin, and pepstatin). Cells were lysed by sonication with six to eight 30-s pulses at a medium setting on a Heat Systems Ultrasonicator (Heat Systems Inc. Farmingdale, NY). The lysate was centrifuged at 30,000 g at 4°C for 15 min to pellet cell debris. The soluble fraction was diluted to a concentration of 5 mg/ml and bound to GT-sepharose (Pharmacia) (1 ml bed vol/500 ml culture). The binding was carried out for 1–3 h at 4°C. 10-min washes of 10 vol were performed at 4°C in the following order: 1× lysis buffer; 1× wash buffer (50 mM Tris, pH 8.0, 200 mM NaCl); 1× high salt wash buffer (50 mM Tris, pH 8.0, 500 mM NaCl); 2× wash buffer. The fusion protein was eluted in three 10-min washes (1 vol) of 10 mM glutathione in 50 mM Tris, pH 8.0. The pooled eluates were then dialyzed for 1.5 h at 4°C against T buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% β-mercaptoethanol) and overnight in T buffer containing 10% glycerol. The protein was concentrated with a Centriprep-30 (Amicon, Beverly, MA). Purification and concentration of the fusion protein were monitored by SDS-PAGE (Laemmli, 1970). Glycerol was added to the purified protein to a final concentration of 10% and aliquots were stored at −80°C. Final protein concentration was 0.2–0.3 mg/ml as determined by Bio-Rad protein assay employing BSA as a standard.

GAP Assay

Purification of GST-Gsp1p from yeast lysates was performed as previously described (Schlenstedt et al., 1995). Gsp1p was further purified after cleavage with thrombin (Sigma, St. Louis, MO) as previously described (Smith and Johnson, 1988). Gsp1p (160 nM) was labeled with 1.4 μl [γ-³²P]GTP (6,000 Ci/mmol; NEN DuPont, Boston, MA), 1 mM DTT, 1 mg/ml BSA in a volume of 10 μl for 20 min at room temperature. GTPase assays were performed in 50-μl reactions in the presence of 40 nM Gsp1p-[γ-³²P]GTP with or without GST-Rna1p or GST-Rna1-1p diluted in GAP buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA). The amount of non-hydrolyzed GTP was determined by a filter binding assay. After incubation for 10 min at 30°C, 1 ml ice-cold GAP buffer was added. Triplicate samples were vacuum filtered through nitrocellulose, filters were washed 10 times with 0.5 ml GAP buffer. The remaining Gsp1p-bound GTP was determined by scintillation counting. No radioactivity was removed under identical conditions when [α-³²P]GTP-labeled Gsp1p was used.

Immunoblotting

Protein samples were resolved by SDS-PAGE (Laemmli, 1970) on 10% gels. Proteins were then transferred by standard methods to nitrocellulose (Towbin et al., 1979). The anti-Rna1p antibody (Hopper et al., 1990) was used at a concentration of 1:10,000. Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL).

Results

Rna1-1 Mutant Cells Are Defective in Protein Import In Vivo

To investigate the role of Rna1p in protein import to the nucleus, we used the well-characterized mutant *rna1-1*. This temperature-sensitive, conditionally lethal mutation prevents growth at temperatures of 30°C and above. Mutant cells display pleiotropic defects in RNA processing

and transport. The defect in RNA transport occurs rapidly following a shift to the nonpermissive temperature and results in the accumulation of approximately 60% of poly(A)⁺ RNA within the nucleus (Hutchinson et al., 1969; Amberg et al., 1992).

Initial experiments examined the localization of the nuclear protein Npl3p (Bossie et al., 1992). As evidenced by co-localization with the DAPI staining of chromatin, this protein is efficiently targeted to the nucleus in wild-type cells maintained at RT or shifted to 36°C (Fig. 1, *a–f*). While *rna1-1* cells target Npl3p to the nucleus when maintained at RT (Fig. 1, *g–i*), they are unable to do so following a two hour shift to the nonpermissive temperature (Fig. 1, *j–l*). The same results were obtained when cells were shifted to the nonpermissive temperature for one hour (data not shown).

Since Npl3p is an RNA binding protein that shuttles between the nucleus and the cytoplasm (Flach et al., 1994; Wilson et al., 1994), we considered the possibility that the aberrant targeting of this protein in the *rna1-1* mutant
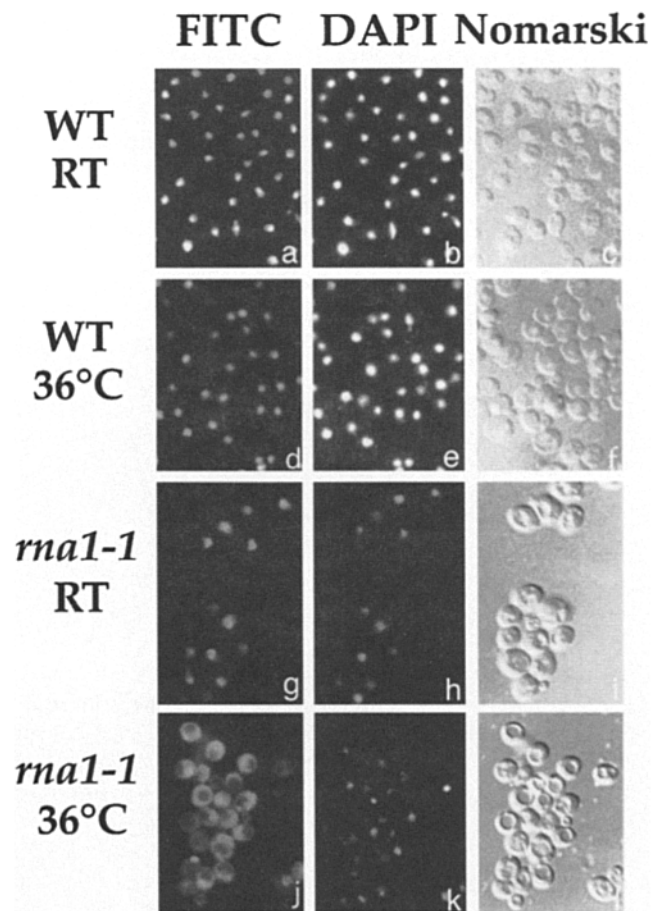

Figure 1. The endogenous nuclear protein, Npl3p, is mislocalized in *rna1-1* mutant cells at the nonpermissive temperature. Wild-type (*a–f*) or *rna1-1* mutant cells (*g–l*) were maintained at RT (*a–c* and *g–i*) or shifted to 36°C for 2 h (*d–f* and *j–l*) and prepared for immunofluorescence microscopy as described in experimental procedures. Cells were stained with anti-Npl3p followed by FITC-labeled anti–rabbit antibody (*a, d, g,* and *j*), stained with DAPI (*b, e, h,* and *k*), or viewed by Nomarski optics (*c, f, i,* and *l*).

might be a reflection of the function of Npl3p in RNA metabolism rather than an indication of a general defect in protein localization. To address this point, we examined the localization of a galactose-induced reporter protein containing an NLS. Cells were grown at the permissive temperature in selective media containing 2% raffinose. Cultures were then induced for 3–4 h by the addition of 2% galactose followed by a one to 2-h shift to 36°C. Since *rna1-1* mutants are defective in the export of poly(A)$^+$ RNA from the nucleus, it was critical that reporter constructs be induced at the permissive temperature and shifted to the nonpermissive temperature only after sufficient induction. Experiments in which cultures were induced and shifted simultaneously showed no signal for the reporter protein (data not shown) as would be expected if the reporter construct is transcribed but message is unable to be efficiently transported to the cytoplasm for translation.

Results are shown for an inducible reporter construct that encodes the SV-40–NLS fused to invertase. As observed with the endogenous Npl3p, the protein is efficiently localized to the nucleus in wild-type cells at both RT and 36°C (Fig. 2, *a–f*). This is not the case for the *rna1-1* mutants which localize the protein predominantly to the nucleus at the permissive temperature (Fig. 2, *g–i*) but show marked mislocalization of the reporter protein to the cytoplasm following a shift to the nonpermissive temperature (Fig. 2, *j–l*). Similar results were observed when another reporter construct, H2B-NLS β-galactosidase (Moreland et al., 1987), was employed for this experiment (data not shown). Since the RNA export defect in *rna1-1* cells can be observed following a 20-min shift to the nonpermis-

FITC   DAPI   Nomarski

WT RT

WT 36°C

rna1-1 RT

rna1-1 36°C

Figure 2. Rna1-1 mutant cells mislocalize the reporter protein, SV-40–NLS invertase, at the nonpermissive temperature. Wild-type (*a–f*) and *rna1-1* mutant (*g–l*) cells expressing the fusion protein SV-40–NLS invertase were either maintained at RT (*a–c* and *g–i*) or shifted to 36°C (*d–f* and *j–l*) for two hours prior to preparation of the cells for immunofluorescence microscopy. Cells are shown stained with anti-invertase followed by a FITC-labeled anti–rabbit antibody (*a, d, g,* and *j*), stained with DAPI (*b, e, h,* and *k*), or viewed by Nomarski optics (*c, f, i,* and *l*).
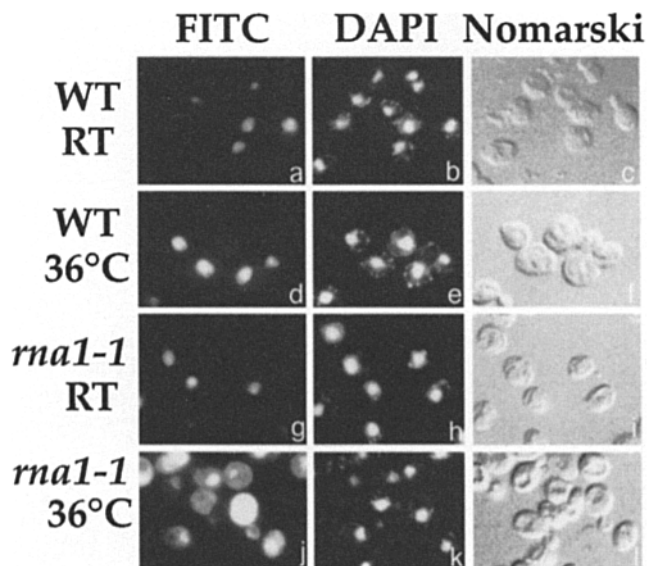
sive temperature (Amberg et al., 1992), we examined the time of onset of the protein import defect. Using the SV-40–invertase reporter protein expressed as described above, mislocalization to the cytoplasm was observed as early as 15 min following a shift to 36°C (data not shown). These data suggest that cells containing a conditional allele of *RNA1* are unable to efficiently target proteins to the nucleus at the nonpermissive temperature and that this defect occurs on the same time scale as the previously reported RNA transport defect.

Nuclear protein import was also examined directly in living cells. To accomplish this, GFP (Chalfie et al., 1994) was targeted to the nucleus by constructing a fusion to a small portion of Npl3p. These experiments have the advantage over indirect immunofluorescence microscopy that cells are not fixed, thus results obtained cannot be attributed to fixation procedures or to processing of the cells. For these experiments, cells were grown and induced at RT as described above. Cultures were shifted to the nonpermissive temperature when the GFP signal of the fusion protein became visible by fluorescence microscopy (approximately three to four hours after induction). Following the shift to the nonpermissive temperature, samples were examined directly by fluorescence microscopy. The GFP-Npl3 fusion protein was properly targeted to the nucleus in wild-type cells at either RT or 36°C (Fig. 3, *a–d*) and in mutant cells maintained at the permissive temperature (Fig. 3, *e* and *f*). However, when mutant cells were shifted to the nonpermissive temperature, protein mislocalization to the cytoplasm was observed in a manner similar to that observed by indirect immunofluorescence microscopy (Fig. 3, *g* and *h*). Cells containing the GFP-Npl3 fusion protein were also stained with DAPI to demonstrate that the fusion protein co-localized with DAPI staining of the nucleus (data not shown). Taken together, the results presented above show that cells containing mutant Rna1p are unable to efficiently target proteins to the nucleus.
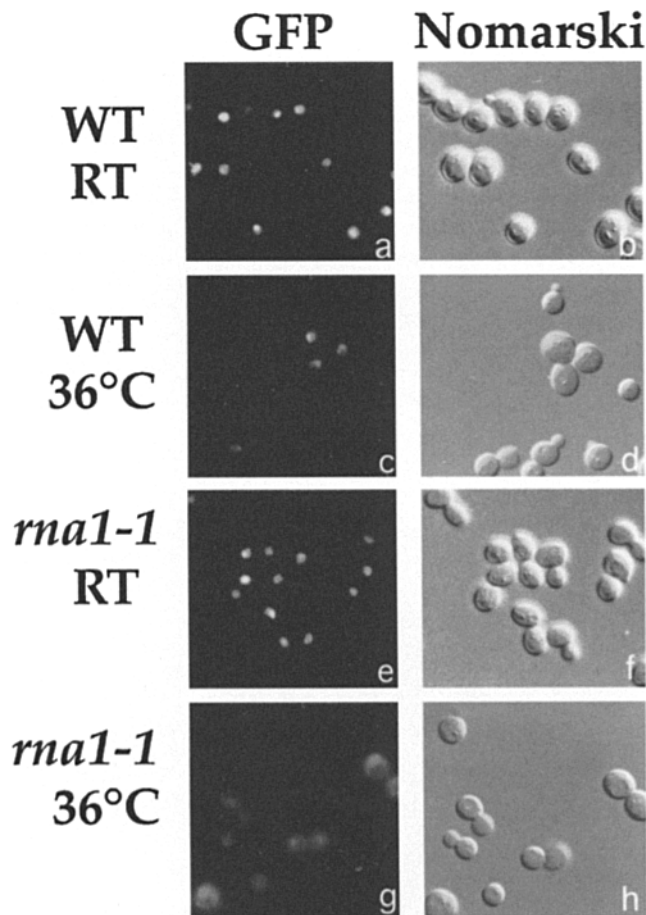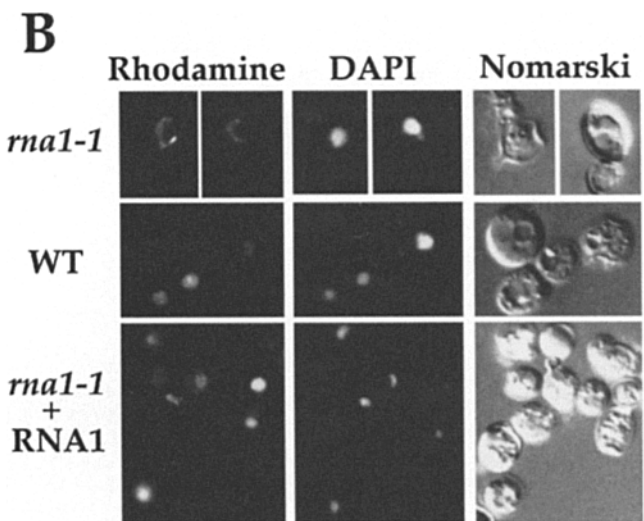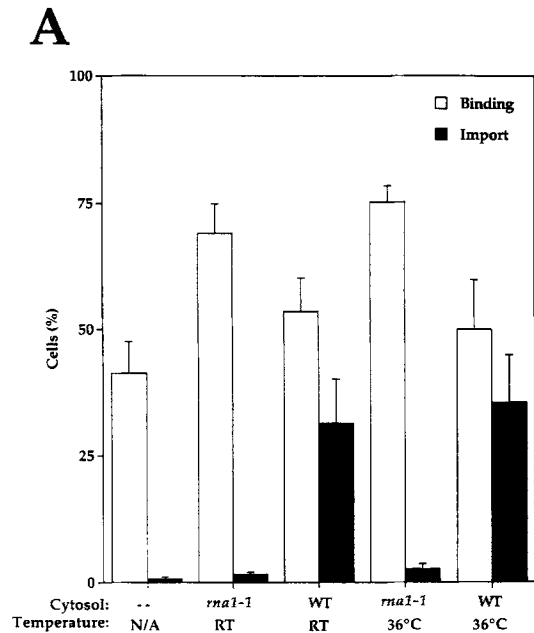
Rna1-1 Mutant Cytosols Are Unable to Support Nuclear Import of a Fluorescently Labeled Substrate In Vitro

In order to examine the role of the Rna1p in protein import more directly, we employed an in vitro import assay (Schlenstedt et al., 1993). The assay faithfully reproduces all aspects of nuclear import. Both exogenous cytosol and an energy regenerating system are required for import of a fluorescently labeled NLS-containing substrate into the nuclei of semi-intact yeast cells. Hence, this assay can be combined with yeast genetics to investigate the role of nuclear components in import by examining the function of mutant semi-intact cells or to investigate the function of cytosolic factors by examining cytosols derived from mutant cells. Using this approach mutations in several nucleoporins have been shown to block import to the nucleus (Schlenstedt et al., 1993); however, no mutants with import defective cytosols had previously been described.

Since Rna1p is located in the cytoplasm (Hopper et al., 1990), we prepared cytosols from both *rna1-1* mutant (PSY714) and wild-type (PSY715) cells either maintained at the permissive temperature or shifted to the nonpermis-

Figure 3. An Npl3p-GFP fusion protein is mislocalized in living *rna1-1* cells shifted to the nonpermissive temperature. Both wild-type (*a–d*) and *rna1-1* (*e–h*) cells expressing a fusion protein consisting of a portion of Npl3 fused to GFP are shown. Wild-type cells efficiently target this protein to the nucleus at either RT (*a*) or following a 2-h shift to 36°C (*c*). Corresponding Nomarski images are shown in panels *b* and *d*, respectively. In contrast, *rna1-1* cells which localize the protein to the nucleus efficiently at RT (*e*) are unable to do so after a 2-h shift to 36°C (*f*). Nomarski images are shown in *g* and *h*, respectively.

sive temperature for 2 h. The cytosols were tested for the ability to stimulate import of the fluorescently labeled substrate into the nuclei of wild-type semi-intact cells. At a final concentration of 2 mg/ml, cytosol prepared from shifted or unshifted wild-type cells stimulated import into the nuclei of semi-intact cells (Fig. 4 *A*). In contrast, little or no import was observed upon addition of an equal amount of the *rna1-1* mutant cytosols prepared either from cells maintained at the permissive temperature or shifted to the nonpermissive temperature. No import was observed in the absence of cytosol (data not shown). Fig. 4 *B* shows examples of semi-intact cells with fluorescently labeled substrate bound to the nuclear envelope in the presence of *rna1-1* mutant cytosol (*rna1-1*) and import

tors the uptake of a fluorescently labeled NLS-containing substrate. In the absence of exogenous cytosol import does not occur, although binding to the nuclear envelope is observed. When cytosol prepared from wild-type cells maintained at RT or shifted to 36°C is added to import reactions to a final concentration of 2 mg/ml, ~40% of the cells accumulate substrate within the nucleus. In contrast, at the same concentration cytosols made from *rna1-1* mutant cells at RT or at 36°C were unable to support import. The height of each bar represents the mean of three separate experiments in which 200 cells were counted. Standard deviations are indicated by the error bars. (*B*) Examples of the binding and import reactions observed in the in vitro assay are shown. In the presence of mutant *rna1-1* cytosols (final protein concentration 1.5 mg/ml) binding of the NLS-containing fluorescently-labeled substrate is observed (*rna1-1*). As previously demonstrated (Schlenstedt et al., 1993), in the presence of wild-type cytosol import to the nucleus is observed (*WT*). Import is also observed when mutant *rna1-1* cytosol is supplemented with 50 μg/ml purified GST-Rna1p (*rna1-1 + RNA1*). The *rhodamine* signal indicates the NLS-containing fluorescent substrate and the nucleus is indicated by *DAPI* staining of chromatin. *Nomarski* images of the corresponding cells are shown.

Figure 4. (*A*) Cytosols made from *rna1-1* mutant cells are unable to support import in vitro. Cytosolic extracts made from wild-type and *rna1-1* cells were included in an in vitro assay that moni-

into the nucleus in the presence of wild-type cytosol (*WT*).

It is not surprising that results obtained in vitro differ from those obtained in vivo. Often temperature-sensitive proteins in yeast function adequately at the permissive temperature in vivo but are unable to function in in vitro assays even at the permissive temperature (e.g., Minvielle-Sebastia et al., 1994).

In contrast to cytosolic extracts prepared from *rna1-1* cells, the semi-intact cells prepared from this mutant were competent to support import in the presence of wild-type cytosol (data not shown). However, the combination of *rna1-1* mutant semi-intact cells and mutant cytosols was not able to do so. These results lend further credence to the hypothesis that Rna1p is a cytosolic factor required for protein import to the nucleus.

Rna1p Is Absent from Mutant Cytosols

To address the inability of *rna1-1* mutant cytosols to support nuclear import, an analysis of Rna1p in wild-type and mutant extracts was carried out. Immunoblot analysis with anti-Rna1p antibody (Hopper et al., 1990) demonstrated that, as previously reported (Hopper et al., 1990; Tung et al., 1992), the amount of Rna1p present in *rna1-1* mutant cells (Fig. 5, lanes *3* and *4*) is similar to the amount found in wild-type cells (Fig. 5, lanes *1* and *2*). While cytosolic extracts made from wild-type cells contain as much Rna1p as found in whole cells (Fig. 5, lanes *5* and *6*), cytosolic extracts made from *rna1-1* mutant cells and utilized in the in vitro assay almost completely lack Rna1p (Fig. 5, lanes *7* and *8*) which is found exclusively in the pelleted material
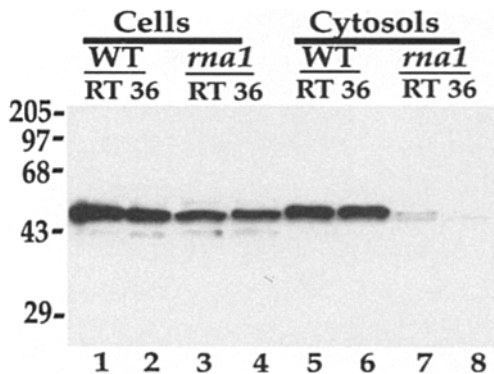

Figure 5. Immunoblotting demonstrates that the Rna1p is depleted from cytosolic extracts made from *rna1-1* mutant cells. Whole cell extracts were made as described in experimental procedures. Cytosolic extract samples were those prepared for the in vitro assay. Equal amounts of protein were loaded in each lane as determined both by BioRad protein assay and by staining blots with Ponceau S following transfer of proteins. Samples were resolved on a 10% SDS-PAGE gel and immunoblotted with anti-Rna1p antibodies. Wild-type cells grown at RT (lane *1*) and shifted to 36°C (lane *2*) contain approximately equal amounts of Rna1p. Mutant cells grown at either RT (lane *3*) or shifted to 36°C (lane *4*) contain approximately as much protein as wild-type cells. Rna1p is present in wild-type cytosols (*RT*, lane *5*; 36°C, lane *6*) at levels approximately equal to those observed in whole cells. In contrast, mutant cytosols prepared from *rna1-1* cells grown at either temperature (*RT*, lane *7*; 36°C, lane *8*) contained barely detectable levels of Rna1p. The position of molecular weight markers (*kD*) is indicated to the left.

(data not shown). Quantitation of the amount of protein by scanning densitometry of immunoblots and comparison to known amounts of purified protein indicates that Rna1p is very abundant in cytosol. The protein is present at a level that is approximately 1% of total soluble protein in wild-type cytosolic extracts used for these experiments. Mutant cytosols, however, have at least 50-fold less Rna1p than found in wild-type cytosols when prepared from cells grown at the permissive temperature. The protein is virtually undetectable in cytosolic extracts made from cells grown at the nonpermissive temperature.

Addition of Purified Rna1p Restores the Import Activity of rna1-1 Mutant Cytosols

Since *rna1-1* mutant cytosols that were not competent to stimulate import into semi-intact cells were depleted of Rna1p, we wanted to determine whether adding back purified Rna1p would rescue the defective cytosols. To facilitate purification of Rna1p, a GST-fusion protein was expressed in *E. coli*. In yeast, expression of a galactose-inducible GST-Rna1p fusion protein rescues the temperature-sensitive phenotype of the *rna1-1* mutant (data not shown), indicating that Rna1p can function as a GST fusion. In addition, the GST-Rna1p fusion protein is localized to the cytoplasm and excluded from the nucleus in a manner similar to that observed for endogenous Rna1p (data not shown). A purification profile for the *E. coli* fusion protein is shown in Fig. 6. Briefly, the protein was induced in DH5α cells (lane *2*), a soluble lysate was made (lane *3*), and the protein was purified by incubation with glutathione (GT)-Sepharose (lane *4*). The GST fusion protein was eluted with excess glutathione, dialyzed against buffer, and concentrated to 0.2 mg/ml. The purified fusion protein was specifically recognized by antibodies directed toward either Rna1p or GST (data not shown).

Purified GST-Rna1p was then added to import assays at various concentrations (Fig. 7). Addition of 50 μg/ml (final concentration) of purified GST-Rna1p had no effect on the stimulation of import observed with 1.5 mg/ml wild-type cytosol (data not shown). A final concentration of 50 μg/ml of the fusion protein did not stimulate import in the absence of exogenous cytosol (data not shown). However, when GST-Rna1p fusion protein was added in conjunction
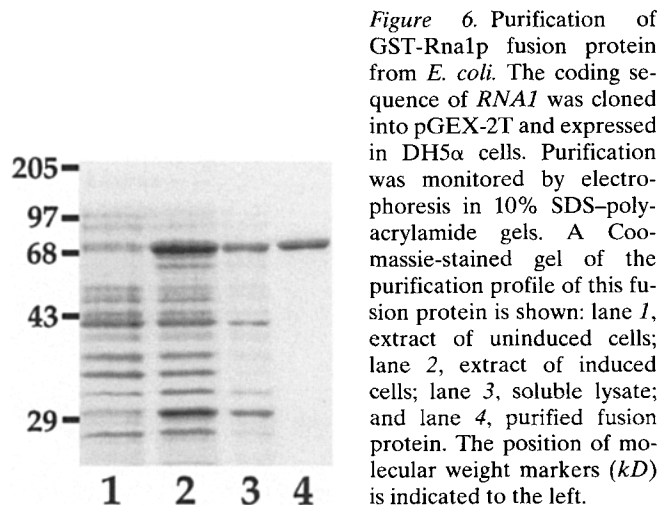
Figure 6. Purification of GST-Rna1p fusion protein from *E. coli*. The coding sequence of *RNA1* was cloned into pGEX-2T and expressed in DH5α cells. Purification was monitored by electrophoresis in 10% SDS–polyacrylamide gels. A Coomassie-stained gel of the purification profile of this fusion protein is shown: lane *1*, extract of uninduced cells; lane *2*, extract of induced cells; lane *3*, soluble lysate; and lane *4*, purified fusion protein. The position of molecular weight markers (*kD*) is indicated to the left.

with 1.5 mg/ml *rna1-1* mutant cytosol, a dose-dependent increase in protein import was observed. As the amount of import to the nucleus increases, the amount of binding at the nuclear envelope decreases (Fig. 7, *inset*; also see Fig. 4 *B*). The assumption is that although in the absence of functional Rna1p import to the nucleus is blocked, binding to the nuclear envelope can still occur. Thus, upon addition of functional Rna1p a portion of the nuclear envelope-bound substrate is imported to the nucleus and a subsequent decrease in the overall level of binding results. Maximal stimulation (approximately equal to that observed with wild-type cytosol) was observed at a final concentration of 50 μg/ml of the fusion protein. Import of the fluorescently-labeled substrate observed in the presence of 50 μg/ml GST-Rna1p in conjunction with mutant cytosol is shown in Fig. 4 *B* (*rna1-1* + *RNA1*). The maximal amount of GST-Rna1p added back to experiments (50 μg/ml) corresponds to ~2% of total cytosolic protein in these reactions, an amount that correlates well with the observed level of Rna1p found in wild-type cytosols, approximately 1% of total protein. Slightly less import was observed in the experiments described in Fig. 7 than in those presented in Fig. 4. This is due to the fact that cytosol was added at a final concentration of 1.5 mg/ml (rather than 2 mg/ml) in
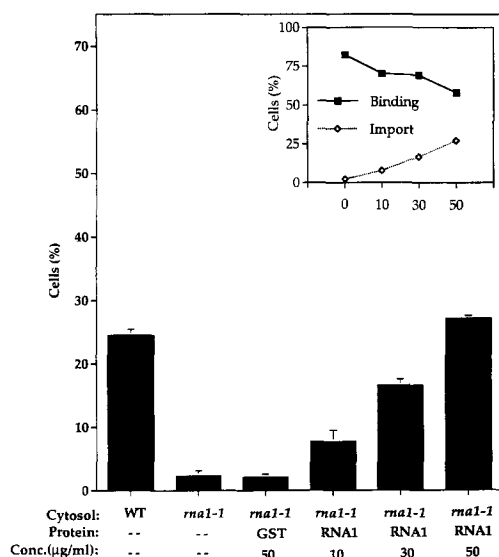
Figure 7. Addition of purified GST-Rna1p fusion protein from *E. coli* to *rna1-1* cytosols results in the stimulation of import in the in vitro assay. The in vitro assay was performed and quantitated as described in Fig. 4 with the exception that cytosols were added to a final concentration of 1.5 mg/ml. No import was observed in the absence of cytosol (not shown) or in the presence of *rna1-1* mutant cytosol prepared from cells shifted to 36°C. Addition of purified GST (final concentration of 50 μg/ml) to *rna1-1* cytosol did not stimulate import. However, when purified GST-Rna1p was added to *rna1-1* cytosol at 10 μg/ml, 30 μg/ml, or 50 μg/ml, a dose-dependent increase in import of the fluorescently labeled substrate was observed. The height of each bar represents the mean of three separate experiments in which 200 cells were counted. Standard deviations are indicated by the error bars. (*Inset*) As import of the fluorescent substrate increases a consequent decrease in the level of binding to the nuclear envelope is observed. The percentage of cells showing either binding (■) or import (◇) is plotted versus the amount of purified GST-Rna1p added to the reaction.

these experiments to allow for the addition of appropriate concentrations of purified GST-Rna1p. This amount of cytosol is limiting for import and thus results in the observed decrease in the percentage of cells importing substrate to the nucleus. Purified GST alone had no effect on protein import at either concentration. Finally, addition of the purified mutant protein, GST-Rna1-1p, to a concentration of 50 μg/ml did not stimulate import in the presence of mutant cytosol (data not shown).

To examine the biochemical activity of both GST-Rna1p and GST-Rna1-1p, a GAP assay was performed. This assay monitors the stimulation of GTP hydrolysis by a GTPase in the presence of a GAP (Schlenstedt et al., 1995). It has recently been demonstrated that homologues of Rna1p serve as GAPs for Ran/TC4 (Coutavas et al., 1993; Bischoff et al., 1994, 1995a). Thus, Gsp1p purified from yeast was labeled with [γ-^{32}P]GTP as described in Materials and Methods. A GST fusion to either wild-type or mutant Rna1p was added to reactions and the rate of GTP hydrolysis was monitored by following the loss of the labeled γ-phosphate. This is plotted as the percent radioactive GTP that remained bound to Gsp1p (Fig. 8) vs. concentration of Rna1p (or Rna1-1p) added to reactions. GST-Rna1p induces GTP hydrolysis by Gsp1p in a manner comparable to that previously reported for the mammalian proteins (Bischoff et al., 1994, 1995a). As determined by three independent titrations, 0.5 ± 0.2 nM wild-type Rna1p was required to hydrolyze 50% of the bound GTP in a 10-min incubation. In contrast, the mutant protein purified from *E. coli* was unable to stimulate GTP hydrolysis by Gsp1p under conditions identical to those used for the wild-type protein. Even at a concentration of 90 nM, mutant protein was unable to significantly stimulate GTP hydrolysis (data not shown). Thus, data obtained from the GAP assay confirms the biochemical functionality of the wild-type Rna1p fusion protein employed for these stud-
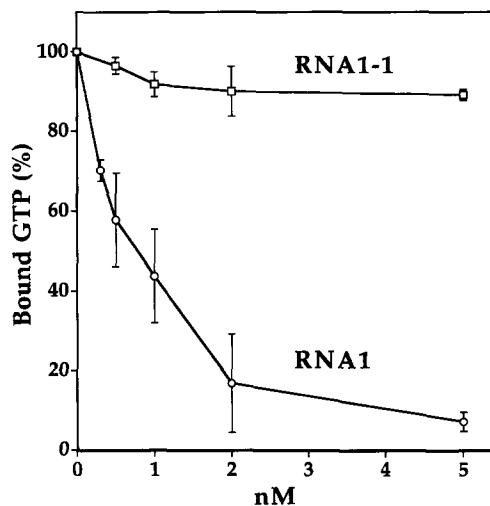
Figure 8. Rna1p is a GTPase activating protein for Gsp1p. [γ-^{32}P] GTP-labeled Gsp1p was incubated for 10 min with various concentrations of GST fused to either wild-type (○) or mutant (□) Rna1p as indicated. Experiments were carried out as described in Materials and Methods. Results are plotted as the percent [γ-^{32}P]GTP that remained bound to Gsp1p. Each point shown on the graph represents the mean of three experiments. Standard deviations are indicated by the error bars.

ies. These results also provide a probable explanation for the inability of the mutant fusion protein to induce import of the NLS-containing substrate to the nucleus and suggest that GAP activity is required for nuclear import.

Discussion

The results presented above demonstrate that Rna1p is involved in the import of proteins into the nucleus. The temperature-sensitive mutant *rna1-1* exhibits defects in protein localization at the nonpermissive temperature. Furthermore, an in vitro assay demonstrates that cytosols lacking Rna1p are unable to support import into the nuclei of semi-intact yeast cells. Only when purified Rna1p is added back to *rna1-1* cytosols do they become competent to support import.

The *rna1-1* strain was originally isolated as a yeast mutant with defects in RNA processing and transport (Hartwell, 1967; Hopper et al., 1978; Amberg et al., 1992). The subsequent observation that Rna1p was located in the cytoplasm and excluded from the nucleus (Hopper et al., 1990) made it difficult to assign a cellular function to Rna1p. However, recent biochemical studies have identified a role for this protein in the regulation of the small GTP-binding protein Ran/TC4. The Rna1p homologues from both HeLa cells (Coutavas et al., 1993; Bischoff et al., 1994) and *S. pombe* (Bischoff et al., 1995a) were purified as stimulators of Ran GTPase activity. Data presented here demonstrate that the *S. cerevisiae* protein also functions as a GAP for Gsp1p. Furthermore, it has been shown that the *S. pombe* homologue of Rna1p is able to function in *S. cerevisiae* (Melchior et al., 1993b). Thus, Rna1p is a highly conserved protein that regulates the ratio of GTP-/GDP-bound Ran/TC4 within the cell by enhancing the rate of Ran/TC4/Gsp1p-mediated GTP hydrolysis. This explains the similar phenotypes observed for *rna1-1* mutant cells shifted to the nonpermissive temperature and wild-type cells overexpressing a GTP-bound form of the budding yeast Ran/TC4 homologue, Gsp1p (Schlenstedt et al., 1995). In both cases, cells are defective in the localization of nuclear proteins and in the export of poly(A)$^+$ RNA from the nucleus. Taken together, these findings suggest that the cytoplasmic Rna1p does not function solely in RNA processing but rather affects other aspects of cellular metabolism. Thus, Rna1p may play two distinct roles within the cell, one that affects export of RNA and one that affects import of proteins. Conversely, it may function through a more general role in regulation of macromolecular transport, a suggestion that is supported by its identification as the GAP for Ran/TC4.

To address the possibility that the Rna1p functions directly in nuclear import we employed an in vitro import assay. The in vitro assay has the advantage over in situ methods that protein import can be separated from RNA export; the assay monitors uptake of substrate into nuclei of semi-intact cells and requires the addition of exogenous cytosol. Since cells and cytosol can be derived from different sources (i.e., wild-type or mutant), it is possible to determine whether mutant extracts directly affect protein import in the absence of improperly processed or transported RNA molecules. It is difficult to uncouple protein import and RNA export in vivo as they are both mediated by the nuclear pores, a fact that complicates the determination of which phenotype is direct and which is indirect. For this reason, the in vitro assays are critical. In experiments that examine the function of mutant cytosols, the nuclei employed are derived from wild-type cells; thus, the results obtained in this assay are not clouded by the possibility that poly(A)$^+$ RNA is aberrantly accumulated in the nucleus or within the nuclear pore resulting in an indirect blockage of protein import. In addition, the GAP assay directly examines the biochemical function of Rna1p.

Results indicate that cytosolic extracts that lack Rna1p are unable to support import into wild-type nuclei; however, in the presence of wild-type cytosol, *rna1-1* mutant cells are able to support import. Furthermore, addition of purified Rna1p to mutant cytosols depleted of Rna1p restores the import activity. This is not the case for mutant protein. A GAP assay confirms the biochemical activity of the wild-type protein purified from *E. coli* but also demonstrates that the mutant protein does not serve as a functional GAP when purified from *E. coli*. The *rna1-1* mutant consists of two point mutations, both of which are required to obtain the conditional phenotype (Traglia et al., 1989). Further structure-function analyses will be carried out to determine whether either or both of these mutations specifically alter the GAP activity of Rna1p. Thus, results obtained for the GAP activity of Rna1p parallel those obtained in the in vitro import assay. Wild-type Rna1p which functions as a GAP in vitro is able to efficiently restore protein import activity to depleted cytosols whereas the nonfunctional mutant protein is not. These findings strongly support the hypothesis that Rna1p is an essential cytosolic factor required for nuclear import.

A recent study that demonstrated that the *rna1-1* mutant was synthetically lethal with a conditional allele of the nucleoporin *NUP1* provides further evidence that Rna1p is involved in transport of macromolecules across the nuclear envelope (Bogerd et al., 1994). In contrast to data presented in this report, Bogerd et al. (1994) cited unpublished data that found no protein import defect in *rna1-1* mutant cells under conditions in which RNA export is defective. Since the experimental methods were not detailed, it is impossible to make a direct comparison to the results presented in this manuscript. In order to explain the RNA export defect and the synthetic lethality with *nup1*, the authors invoke a model where the cytoplasmic Rna1p might transiently associate with the nuclear pore to facilitate RNA export. However, our finding that Rna1p plays a direct role in import of proteins into the nucleus raises the possibility that this synthetic lethality results from a loss of an essential interaction between cytosolic components of the protein import system and the nuclear pore.

Although the *rna1-1* mutant was originally isolated on the basis of defective RNA processing, it has not yet been demonstrated that Rna1p has a primary function in the maturation and export of RNA. It is plausible, in light of the role of Rna1p in protein import, that RNA export defects observed in the *rna1-1* mutant may be indicative of the loss of some short-lived nuclear factor that is required for RNA processing/transport rather than a direct function in RNA export. Until an in vitro assay for the export of poly(A)$^+$ RNA from the nucleus is developed, it will not be feasible to examine RNA export in the absence of

protein import and distinguish between these two possibilities.

Rna1p restores import activity to mutant cytosols but alone is not able to substitute for wild-type cytosol in import reactions. This suggests that Rna1p is part of a multicomponent system that functions in protein import through regulation of the nucleotide-bound state of Ran/TC4. Other members of this system have been identified (Coutavas et al., 1993; Lee et al., 1993; Moore and Blobel, 1993, 1994b; Bischoff et al., 1994; Lounsbury et al., 1994; Powers and Forbes, 1994; Bischoff et al., 1995b; Ouspenski et al., 1995; Schlenstedt, G., D. H. Wong, D. M. Koepp, and P. A. Silver, manuscript submitted). For example, the nuclear protein RCC1 (Ohtsubo et al., 1989; Dasso, 1993) serves as the nucleotide exchange factor for Ran/TC4 mediating the interchange of the GDP-bound state to the GTP-bound state (Bischoff and Ponstingl, 1991a). In fact, in the nucleus Ran/TC4 exists as a complex with RCC1 (Bischoff and Ponstingl, 1991b). Interestingly, the yeast homologue of RCC1, *PRP20*, was identified as a temperature-sensitive mutant with pleiotropic defects in RNA processing/transport (Aebi et al., 1990; Fleischmann et al., 1991; Kadowaki et al., 1992). Like *rna1-1*, the *prp20-1* mutant is unable to efficiently localize NLS-containing proteins to the nucleus (Corbett, A. H., D. M. Koepp, and P. A. Silver, unpublished results). In support of a direct role for Prp20p/RCC1 in nuclear import it has also been demonstrated that in mammalian cells loss of RCC1 causes a defect in protein import to the nucleus in vivo (Tachibana et al., 1994).

Previous fractionation studies have not identified Rna1p as an essential cytosolic factor required for nuclear import (Melchior et al., 1993a; Moore and Blobel, 1993, 1994b). It is possible that at sufficiently high Ran/TC4 concentration, the level of GTP hydrolysis obtained is able to compensate for the lack of GAP activity. This might also be the case if mechanistic differences between Ran/TC4 and Gsp1p result in different intrinsic rates of GTP hydrolysis. Alternatively, wild-type nuclei employed for other in vitro assays could contain an amount of Rna1p that is adequate to support import under the conditions employed.

Several models can be constructed based on the present data regarding Rna1p, Ran/TC4, and RCC1. Since Ran/TC4 is found both in the cytoplasm and in the nucleus and interacts with proteins in both the nucleus and the cytoplasm, it is possible that: (a) a single pool of protein shuttles between the nucleus and cytoplasm; or that (b) distinct protein pools function separately in the different compartments. In either case, a role for Rna1p can be envisioned. If Ran/TC4 functions by shuttling between the nucleus and the cytoplasm, GTP hydrolysis could be required for transport into the nucleus. Ran/TC4 would be targeted to the nuclear membrane in its GTP-bound state, and then upon hydrolysis it would be transported into the nucleus. Since a nucleotide exchange factor, RCC1, is located in the nucleus, it is likely that import would be followed by an interchange between the GDP-bound state of Ran/TC4 and the GTP-bound state. It is possible that an as of yet unidentified nuclear GAP is required for export of Ran/TC4 from the nucleus which may or may not be accompanied by the export of poly(A)$^+$ RNA or proteins. In this model, a cytoplasmic nucleotide exchange factor would be required to return GDP-bound Ran/TC4 to its GTP-bound state and complete the cycle. Alternatively, it is possible that Ran/TC4 is simply exported in its GTP-bound state. A similar scenario would occur if Ran/TC4 exists as separate, compartmentalized pools. In this case, GTP hydrolysis would support transport of the substrate alone rather than a substrate·Ran/TC4 complex, and the Ran/TC4 would simply cycle to and from the nuclear membrane rather than across it.

At this point it is not possible to distinguish between these models. Based on the data presented in this manuscript it is, however, possible to ascribe a direct role in protein import to Rna1p. Thus far the combination of biochemical and genetic approaches has been extremely useful in the identification of factors involved in nuclear trafficking and will continue to be effective in the elucidation of the mechanism of transport across the nuclear envelope.

Note Added in Proof: After submission of this manuscript, J. Becker et al. demonstrated that *S. cerevisiae* Rna1p functions as a GTPase activating protein for Gsp1p (Becker, J., F. Melchior, V. Gerke, F. R. Bischoff, H. Ponstigl, and A. Wittinghoffer. 1995. *RNA1* encodes a GTPase-activating protein specific for Gsp1p, the Ran/TC4 homologue of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 270:11860–11865).

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