Dynamic Localization of the Nuclear Import Receptor and Its Interactions with Transport Factors

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Abstract. Characterization of the interactions between soluble factors required for nuclear transport is key to understanding the process of nuclear trafficking. Using a synthetic lethal screen with the mal-1 strain, we have identified a genetic interaction between Rnalp, a GTPase activating protein required for nuclear transport, and yeast importin-β, a component of the nuclear localization signal receptor. By the use of fusion proteins, we demonstrate that Rnalp physically interacts with importin-β. Mutants in importin-β exhibit in vivo nuclear protein import defects, and importin-β localizes to the nuclear envelope along with other proteins associated with the nuclear pore complex. In addition, we present evidence that importin-α, but not importin-β, mislocalizes to the nucleus in cells where the GTPase Ran is likely to be in the GDP-bound state. We suggest a model of nuclear transport in which Ran-mediated hydrolysis of GTP is necessary for the import of importin-α and the nuclear localization signal–bearing substrate into the nucleus, while exchange of GDP for GTP on Ran is required for the export of both mRNA and importin-α from the nucleus.

Transport of proteins from the cytoplasm to the nucleus is a regulated process that depends not only on an intrinsic signal in the protein to be imported, but also on a number of cellular proteins. Many nuclear proteins have a nuclear localization signal (NLS) that is required for their proper targeting to the nucleus (Hall et al., 1984; Kalderon et al., 1984; Silver et al., 1984). Alternatively, nuclear proteins lacking an NLS may be carried into the nucleus in a complex with an NLS-bearing protein (Dingwall et al., 1982). Generally, nuclear protein import can be divided into two steps: (a) binding of the NLS-bearing protein to a cytosolic NLS receptor and translocation to the nuclear pore complex (NPC) and (b) transport of the NLS-bearing protein into the nucleus.

The initial binding step of nuclear protein import requires at least two proteins in addition to the NLS-bearing protein substrate. The first is a 60-kD protein that will be referred to here as importin-α (also known as karyopherin-α in vertebrates and as Kap60p and Srp1p in yeast) (Adam and Gerace, 1991; Görlich et al., 1994; Yano et al., 1994; Radu et al., 1995). The yeast importin-α was originally identified as a suppressor of RNA polymerase I (Yano et al., 1992). Mutants in importin-α show defects in nuclear protein import and nuclear structure and also exhibit a G2/M cell cycle arrest (Yano et al., 1994; Küssel and Frasch, 1995; Loeb et al., 1995). Purified importin-α binds NLSs and targets an NLS-bearing substrate to the nuclear pore in in vitro import assays in the presence of a second NLS receptor subunit, importin-β (also known as karyopherin-β in vertebrates and Kap95p in yeast) (Görlich et al., 1995b; Imamoto et al., 1995; Moroianu et al., 1995). The importin-β protein has a molecular weight of ~95,000 and has a weaker affinity for NLSs than importin-α (Chi et al., 1995; Görlich et al., 1995a; Imamoto et al., 1995; Moroianu et al., 1995). Importin-β is most likely responsible for targeting of the complex to the NPC, as it has been shown that importin-α cannot deliver NLS-containing substrates to the NPC in the absence of importin-β in vitro (Enenkel et al., 1995; Görlich et al., 1995b; Moroianu et al., 1995). In addition, importin-β binds specific nucleoporins (Iovine et al., 1995; Kraemer et al., 1995; Radu et al., 1995; Rexach and Blobel, 1995).

The second step of import requires the small GTPase, Ran (the yeast homolog is called Gsp1p), and a number of proteins that associate with it (Drivas, 1990; Belhumeur et al., 1993; Moore and Blobel, 1993, 1994). In in vitro assays, the addition of Ran stimulates transport of NLS substrate already bound at the pore (Melchior et al., 1993; Moore and Blobel, 1993, 1994). Overexpression of mutant Ran locked in the GTP-bound state in yeast leads to defects in both nuclear protein import and mRNA export (Schlenstedt et al., 1995a). Rnalp, a cytoplasmic protein, has recently been shown to be directly involved in nuclear import and to act as a GTPase activating protein (GAP) for...
yeast Ran (Becker et al., 1995; Bischoff et al., 1995a; Corbett et al., 1995). In vivo assays, mutants in Rna1p exhibit defects in both protein import and mRNA export (Hopper et al., 1978; Amberg et al., 1992; Corbett et al., 1995), similar to the defects seen when the GTP-stabilized form of Ran (Ran-GTP) is overexpressed. These results are consistent, since both situations lead to an excess of free Ran-GTP in the cell. A second protein associated with Ran is yeast Ran (Becker et al., 1995; Bischoff et al., 1995a; Corbett et al., 1995b). Presumably, importin-α is later exported to the cytoplasm, and exchange of GDP for GTP occurs only in the cytoplasm as well as the nucleus. Exchange occurs only in the nucleus and hydrolysis occurs only in the cytoplasm if Ran is free to move between the nucleus and cytoplasm.

It is not clear how the proteins required for the binding of an NLS-bearing protein interact with proteins required for translocation, or indeed, if they directly interact at all. Only very recently has it been shown that Ran associates with the NLS receptor (Rexach and Blobel, 1995). In this study we report both a genetic and a physical interaction between the NLS receptor and importin-β. We show that the importin-β protein localizes to the nuclear envelope and colocalizes with importin-α and Nup159p. Importin-β mutants exhibit defects in nuclear protein import in vivo. In addition, by investigating the localization of importin-α and importin-β in a number of mutants defective for nuclear protein import and/or mRNA export, we have found that when exchange of GDP for GTP on Ran is blocked, importin-α is mislocalized to the nucleus. This supports a model in which hydrolysis of GTP by Ran occurs only in the cytoplasm, and exchange of GDP for GTP occurs only in the nucleus.

### Materials and Methods

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. All DNA manipulations were performed according to standard methods (Sambrook et al., 1989), and all yeast media were prepared by standard procedures (Rose et al., 1990). All strains used are described in Table I, and all plasmids novel to this work are described in Table II.

### Synthetic Lethal Screen

#### Strain Construction

The parental strains for the screen were created by

| Name | Genotype | Reference |
|------|----------|-----------|
| PSY137 | ade2 ade3 can1 leu2 lys2 ura3 MAT a | Kranz and Holm, 1990 |
| EE1b (PSY544) | rna1-1 his4 his7 tyr1 ura3 MAT a | Traglia et al., 1989 |
| FY23 (PSY580) | leu2Δ trp1 Δ65 ura3-52 MAT a | Winston et al., 1995 |
| FY86 (PSY581) | his3Δ200 leu2Δ trp1 Δ52 MAT a | F. Winston, unpublished data |
| PSY615 | ade2Δ ade3 ade8ΔADH3 his3Δ3 leu2Δ lys1Δ lys1Δ | Henry et al., 1996 |
| FY23 (PSY580) | ade2Δ ade3 ade8ΔADH3 his3Δ3 leu2Δ lys1Δ lys1Δ | Henry et al., 1996 |

All strains used for this study are shown. For detailed information on the construction of strains refer to the Materials and Methods section.
Table II. Plasmids Generated for This Study

| Plasmid | Description |
|---------|-------------|
| pPS709  | 5.5-kb BamHI-Sall ADE3 fragment from pDRK225 (Johnson and Kolodner, 1995) inserted into the BamHI-Sall sites of YEp352 |
| pPS714  | 2.3-kb RNAI SacI fragment of YCPRNA1 (Traglia et al., 1989) inserted into the SacI site of pPS709 |
| pPS779  | 5.5-kb BamHI-Sall ADE3 fragment from pDRK225 (Johnson and Kolodner, 1995) inserted into the BamHI-Sall sites of YEp351 |
| pPS783  | 0.7-kb coding region of yeast Ran amplified with BamHI-HindIII oligonucleotides inserted into pAlter-1; threonine 26 was changed to arginine using a mutated oligonucleotide |
| pPS888  | 2.3-kb RNAI SacI fragment of YCPRNA1 (Traglia et al., 1989) inserted into the SacI site of pPS779 |
| pPS889  | Original clone identified in screen. 10-kb Sau3AI fragment inserted into the BamHI site of YCP50 (Rose et al., 1987) |
| pPS890  | 3.5-kb RSL1 EcoRV-KpnI fragment of pPS889 inserted into Smal-KpnI sites of pRS316 (Sikorski and Hieter, 1989) |
| pPS891  | 33-bp c-myc oligonucleotide inserted into AfII site of pPS890 |
| pPS892  | XbaI oligonucleotide inserted into pGEX.2TB (Lee et al., 1994) to convert XbaI site to BglII; 0.7-kb Bgl II-BamHI fragment of pGEX.2TB inserted into the BamHI site of pPS293 (Lee et al., 1996) |
| pPS893  | 2.5-kb coding region of RSL1 amplified with BamHI-XhoI oligonucleotides inserted into the BamHI-Sall sites of YEp352 |
| pPS894  | 2.5-kb coding region of RSL1 amplified with BamHI-XhoI oligonucleotides inserted into the BamHI-XhoI sites of pGEX.4T-1 |
| pPS898  | 3.4-kb RSL1 BamHI-PstI fragment of pPS890 inserted into pRS305 (Sikorski and Hieter, 1989) |
| pPS900  | 2.3-kb RNAI SacI fragment of YCPRNA1 (Traglia et al., 1989) inserted into the SacI site of YEp351 |
| pPS909  | 1.0-kb coding region of HIS3 using Ncol-PstI oligonucleotides inserted into the Ncol-PstI sites of pRU49 (Traglia et al., 1989) |

All plasmids generated for this study are described.

crossing an rna1 strain EE1 (PSY54, gift of A. Hopper, Pennsylvania State University, Hershey, PA) to the wild-type strain FY86, (PSY581, gift of F. Winston, Harvard Medical School, Boston, MA) to produce PSY688. PSY688 was crossed to PSY137 (gift of C. Holm, University of California, San Diego) to create PSY689 and PSY670, carrying ade2 and ade3 alleles. The diploid 2bx3b (PSY543, gift of A. Hopper), containing a disruption of the RNA1 locus and the YCP50RNA1 plasmid (pPS350, gift of A. Hopper), was sporulated to generate PSY873.

Plasmid Construction. pPS709 was generated by inserting a 5.5-kb BamHI-Sall ADE3 fragment from pDRK225 (Johnson and Kolodner, 1995) into the BamHI and SalI sites of YEpl52 (New England Biolabs, Beverly, MA). The same strategy was used to create pPS711, except that YEp351 was the vector used. pPS714 was then created by inserting a 2.3-kb RNAI SacI fragment into the SacI site of pPS709. Similarly, pPS884 was generated by insertion of the RNAI SacI fragment into the SacI site of pPS714. pPS888 was created by insertion of a 0.7-kb RNAI SacI fragment into the SacI site of pPS889.

Screening. PSY569 and PSY870 transformed with pPS714 were grown to 1 × 106 cells per ml and mutagenized with ethylmethanesulfonate as described (Lawrence, 1991) to produce 50% cell death. Approximately 100,000 colonies were screened for a nonsectoring phenotype (Sect -). Sect colonies were restreaked on synthetic complete media containing 1 mg/ml 5' fluoro-orotic acid (FOA). 30 consistently Sect - and FOA-sensitive (FOA s) strains were isolated. These mutants were backcrossed to determine dominance/recessivity. Backcrosses were sporulated to determine if the mutants carried synthetic lethality. Both PSY680 and PSY871 were transformed with pPS889.

Construction of rna1A359-397 Strains. A strain containing the rna1 A359-397 allele was constructed using pPS899. pPS899 was linearized by digestion with NsiI and transformed into PSY874. Correct integration of the construct in the chromosome was verified by hybridization using enhanced chemiluminescence (ECL) as a detection method (Amersham Life Science Inc., Arlington Heights, IL). The strain bearing the integration construct was backcrossed once to PSY869 to generate PSY885, which was then crossed to PSY875 and PSY876.

Construction of rna1A359-397 Strains. A strain containing the rna1Δ359-397 allele was constructed using pPS909. pPS909 (3.5-kb EcoRV-KpnI genomic fragment inserted into the Smal-KpnI sites of pPS320) rescued the Sect phenotype.

Linkage. An integration construct, pPS898, was generated by insertion of the 3.5-kb BamHI-PstI fragment of pPS890 into the BamHI and PstI sites of pPS749 (pRS305). pPS898 was linearized by digestion with NsiI and transformed into PSY874. Correct integration of the construct in the chromosome was verified by hybridization using enhanced chemiluminescence (ECL) as a detection method (Amersham Life Science Inc., Arlington Heights, IL). The strain bearing the integration construct was backcrossed once to PSY869 to generate PSY885, which was then crossed to PSY875 and PSY876.
Fink Lab, Massachusetts Institute of Technology, Cambridge, MA), using a PCR method (Baudin et al., 1993). Oligonucleotides were generated that contained 19-bp DNA complementary to the HIS3 coding region and 45-bp DNA complementary to RSL1. Bacteriological medium was transformed into the pPS293 (Lee et al., 1996) downstream of the Gal1 promoter to generate pPS892. The RSL1 coding region was amplified by PCR with VENT polymerase (New England Biolabs) using oligonucleotides DK13 5' GCCTAACCTCACTCGTTCAGAATG 3' and DK14 5' CGGCTAGGCTCTCTCTGCTGGAAGG 3'. DNA was amplified from pPS729 using DK11 and DK12 and transformed into PSY878. Correct integration of the gene was verified by PCR analysis using DK17 5' CTGGAAAT- TATTCTGCCTTTTTC 3' and the internal HIS3 oligonucleotide 5' GC- CTTACTCAAAGGC 3', and by Southern blotting using ECL for detection as described above.

**Generation of Myc-tagged Importin-β**

Oligonucleotides encoding the c-myc epitope with Affi-oxime-compatible ends were generated. They are DK15 5' TTAATTTGCAGAAGCTGATTAC- ATAAGTCCCGAGCAGAAGCT 3' and DK16 5' TTAAGG- CGGCTAGGCTCTCTCTGCTGGAAGG 3'. The oligonucleotides were mixed 1:1 and denatured for 2 min at 65°C followed by a shift to 37°C for 5 min to anneal. The double-stranded oligonucleotide was then inserted into the AflII site of pPS890. Correct orientation was verified by DNA sequencing.

**Construction of Ran mutants**

The GDP-bound form of yeast Ran, T26N, was cloned downstream of the GALl promoter in plasmid pGEX.2TB (Lee et al., 1994), was digested with XbaI, and the oligo 5' CGGCTCGAGCTCTCCTATGACGGAGAAGGT 3'. The 2.5-kb PCR fragment was digested with BamHI and Aval and inserted into the pGEX.2TB plasmid. The GDP-bound form of yeast Ran, G21V, has been described (Schlenstedt et al., 1994). After mutagenesis, the coding region was sequenced to confirm the point mutation. The T26N mutant was cloned downstream of the GALl promoter in plasmid pPS310 (Schlenstedt et al., 1995a) as a BamHI–HindIII fragment. The GTP-bound form of yeast Ran, G21V, has been described (Schlenstedt et al., 1995a).

**Purification of Protein.** Cells were grown overnight in Ura- synthetic complete medium with 2% glucose at room temperature. Cells were diluted into Ura- synthetic complete medium with 2% glucose. Cultures were diluted at ~2 × 10³ cells/ml with 2% galactose for 3 h at room temperature. Lysates were made in PBSMT by glass bead lysis, and then centrifuged in a microfuge at 13,000 × g for 10 min at 4°C. Lysates were incubated with GT-Sepharose (Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 h. The beads were washed once in PBSMT and twice in PBSM. Sample buffer was added directly to the beads, and samples were resolved by SDS-PAGE analysis on 10% gels (Laemmli, 1970). For immunoblotting, protein was transferred to nitrocellulose by standard methods (Towbin et al., 1979). Anti-importin-α was used at 1:5,000. Immunoreactive bands were detected by ECL (Amersham Life Science, Inc.).

**Indirect Immunofluorescence Microscopy**

Cells requiring galactose induction were first grown in glucose-supplemented medium overnight, and then diluted into 2% galactose medium overnight. Cells were induced with 2% galactose for 3 h, and cells without a galactose-inducible construct were grown overnight in the appropriate medium. In experiments with cells shifted to the nonpermissive temperature, the cells were shifted to 36°C for 2 h. All cells were washed in 0.1 M potassium phosphate buffer, pH 6.5, and resuspended in P solution (0.1 M potassium phosphate buffer, pH 6.5, 1.2 M sorbitol). Cell walls were digested with 15–30 μg of zymolyase (10 mg/ml in P solution plus 0.5% B-mercaptoethanol) at 30°C until the cells appeared dark under phase microscopy. Cells were adhered to slides treated with 0.3% poly-L-lysine. Fixation conditions differed depending on the experiment.

**Formaldehyde Fixation.** 0.1- vol 37% formaldehyde was added to cultures 30 min before digestion with zymolyase. Cells adhered to slides were also fixed in methanol for 6 min at −20°C and dried in cold acetone for 30 s.

**Methanol Fixation.** No formaldehyde was added to cells before washing. After cells were adhered to slides, the cells were fixed in methanol for 30 min at −20°C. Slides were dried in cold acetone for 30 s before incubation with antibody. In all experiments, primary antibody was incubated overnight in PBS plus 0.5% BSA, while secondary antibody was incubated for 2 h in PBS plus 0.5% BSA. Antibody dilutions for all experiments are as follows: anti-myc (9E10) tissue-culture supernatant was used undiluted; anti-importin-α, 1:1,000 (gift of D. Görlich, Wellcome/CRC Institute, Cambridge, UK); anti-Nup159, 1:2,000 (Gorlich et al., 1995); anti-β-galactosidase, 1:800 (Bosse et al., 1992); anti-importin-β, 1:500. All secondary antibodies (Jackson Immuno Research Laboratories, Inc., West Grove, PA) were used at 1:1,000. 4', 6-diamidino-2-phenylindole was used at a final concentration of 1 μg/ml to label DNA.

**Immunoprecipitation.** Lysates were made from cells grown to 1 × 10⁶ cells/ml in PBSMT (PBS, 5 mM MgCl₂, 0.5% Triton X-100) plus protease inhibitors (0.5 mM PMSF, 3 μg/ml each leupeptin, aprotinin, chymostatin, and pepstatin) by glass bead lysis. Lysates were centrifuged at high speed in a microfuge to pellet cell debris. Lysates were incubated with an anti-myc agarose conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at 4°C. The beads were washed as follows: once in PBSMT and twice in PBSM. Sample buffer was added directly to the agarose conjugate, and samples were resolved by SDS-PAGE analysis using 10% gels (Laemmli, 1970).

**Purification of Glutathione-S-transferase (GST)–importin-β from Yeast**

**Plasmid Construction.** The Escherichia coli GST expression vector, pGEX.2TB (Lee et al., 1994), was digested with XbaI, and the oligo 5' TAAGGAATCTA 3' was inserted to convert the XbaI site to BgIII. A 673-bp BgIII–BamHI fragment containing the GST open reading frame was then inserted into pPS293 (Lee et al., 1996) downstream of the GAL1 promoter to generate pPS892. The RSL1 coding region was amplified by PCR with VENT polymerase (New England Biolabs) using oligonucleotides DK13 5' GCCGGATCCATGGCAGGCTAGTTTGGCT 3' and DK14 5' CGGCCTAGGCTCTCTCTGCTGGAAGG 3'. The 2.5-kb PCR fragment was digested with BamHI and Aval and inserted into the BamHI–Aval site of pPS890.
10 min, and once in Ab2 for 30 min. 4',6-diamidino-2-phenylindole (1 μg/ml in Ab2) was used to visualize DNA.

**Generation of Anti-importin-β and Anti-Rnalp Antibodies**

The open reading frame of RSL1 was amplified using the oligonucleotides DK13 and DK14. The 2.5-kb PCR product was digested with BamHI and Aval and inserted into the BamHI and Xhol sites of pGEX4T-1 (Pharmacia Biotech) to create pF894. The GST–RNA1 fusion construct has already been described (Corbett et al., 1995).

DH5α carrying the fusion constructs were grown overnight in Luria Bertani broth containing ampicillin (100 μg/ml) at 37°C, and then diluted 1:200 in fresh Luria Bertani broth with ampicillin (100 μg/ml) and grown at 30°C until OD600 = 0.6–0.7. Cultures were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 2 h, cells were pelleted at 6,000 g at 4°C for 15 min, and pellets were stored at ~20°C overnight. The cells were resuspended in (1 ml per 50 ml culture) lysis buffer (200 mM NaCl, 50 mM Tris, pH 8.0, 2.5 mM EDTA, pH 8.0, 0.1% Tween-20, 0.5 mM PMSF, 3 μg/ml each leupeptin, aprothin, chymostatin, and pepstatin). Cells were lysed by sonication with six to eight 30-s bursts at a medium setting on a Heat Systems Ultrasonicator (Heat Systems, Inc., Farmington, NY). The lysate was centrifuged at 30,000 g for 15 min at 4°C to pellet cell debris. The soluble fraction (at concentration of 5 mg/ml) was bound to GT-Sepharose (Pharmacia Biotech) (at 1-ml bed vol per 500 ml culture) previously washed with lysis buffer. The binding step was performed at 4°C for 1–3 h. 10-min washes (10 vol) were performed at 4°C in the following order: once in lysis buffer, once in wash buffer (200 mM NaCl, 50 mM Tris, pH 8.0), once in high salt wash buffer (300 mM NaCl, 50 mM Tris, pH 8.0), and twice in wash buffer. The fusion protein was eluted in three 10-min washes (1 vol) 10 mM glutathione in 50 mM Tris, pH 8.0, at room temperature.

The proteins were concentrated using Centricon-30 (Amicon Inc., Beverly, MA). Purification and concentration was monitored by SDS-PAGE. Final protein concentration was determined by the Bio-Rad protein assay (Richmond, CA) using BSA as a standard. Purified protein was sent to HRP, Inc. (Denver, PA) for injection into New Zealand White rabbits. Titration of antiserum was performed using previously described immunoblotting techniques (Towbin et al., 1979).

**Results**

**The Rnal-1 Protein Accumulates at the Nuclear Envelope**

The rnal-1 mutation exhibits temperature-sensitive defects in RNA processing, mRNA export, and nuclear protein import (Hopper et al., 1978; Amberg et al., 1992; Corbett et al., 1995). Rnalp is a normally cytoplasmic protein; however, Rnal-1p is not observed in immunoblots of cytosolic extracts, even though the protein is present at wild-type levels in whole cell lysates (Hopper et al., 1990; Tung et al., 1992; Corbett et al., 1995). We generated antiserum specific for the Rnal1 protein using an E. coli–purified GST fusion. When this antiserum is used for indirect immunofluorescence on wild-type cells, the Rnal protein is found throughout the entire cytoplasm but is absent from the nucleus, as has been previously reported (Fig. 1, top row) (Hopper et al., 1990). However, when the anti-Rnalp antibody is used on rnal-1 cells, the mutant protein is found to be associated with the nuclear envelope with only slight cytoplasmic staining (Fig. 1, bottom row; only cells at permissive temperature are shown). This localization of the rnal-1 mutant protein suggests that it is binding with high affinity to a protein associated with the nuclear envelope, thus explaining the absence of Rnal-1p from cytosolic extracts. Given the interesting phenotypes of the rnal-1 mutant, we chose to identify other factors with which it might interact by performing a synthetic lethal screen (Kranz and Holm, 1990; Bender and Pringle, 1991).

**Figure 1.** The Rnal-1 protein accumulates at the nuclear envelope. Wild-type (top row) and rnal-1 (bottom row) cells were grown to log phase, and cultures were split in half and either shifted to 36°C for 3 h or kept at room temperature. Cells were prepared for immunofluorescence without formaldehyde fixation. The α-Rnalp antibody was used at 1:1,000 dilution.

**Genetic Interactions with Mutant Alleles of RNA1**

Synthetic lethality refers to the phenomenon observed when two conditional mutations are lethal in combination under otherwise normally permissive conditions for each mutant allele. An example is when the double mutant of two temperature-sensitive mutations is dead at permissive temperature, conditions under which each allele is viable. One interpretation of this phenomenon is that the cell can survive a slight malfunction in the first gene, but upon a defect in an interacting gene, the cell dies. This strategy can be used to identify potential genetic interactions.

Toward this end, a synthetic lethal screen was performed with the rnal-1 allele using the yeast sectoring assay (Kranz and Holm, 1990; Bender and Pringle, 1991). Two rnal-1 ade2 ade3 strains of opposite mating type transformed with a plasmid carrying wild-type alleles of RNA1 and ADE3 were sufficiently mutagenized with ethyl methanesulfonate to kill 50% of the cells (Lawrence, 1991). After screening ~100,000 colonies, 30 nonsectoring (Sect⁻), FOA-sensitive mutants were identified. Of those 30, 15 became Sect⁺ and FOA resistant when an additional plasmid carrying the RNA1 allele was introduced into the mutants, indicating that they were dependent on RNA1 for viability. These 15 mutants were backcrossed to the parental strain to determine if the mutations were dominant or recessive. The backcrosses were also sporulated to determine if the mutation segregated as a single gene. In addition, the 15 mutants were crossed to an RNA1 disruption strain to identify any null alleles of RNA1.

Two mutants, PSY871 and PSY872, were identified that were not RNA1 null alleles. Both were recessive, and their Sect⁻, FOA⁺ phenotypes segregated as a single locus. When the mutants were crossed to each other, the diploid was Sect⁻ and FOA⁺, indicating noncomplementation. When tetrads were dissected from this diploid, only Sect⁻, FOA⁺ colonies were obtained, indicating that PSY871 and
PSY872 were mutated in the same gene. PSY871 was unable to grow at 36°C, while PSY872 did not demonstrate any temperature sensitivity. We refer to these mutants as \( rsl1-1 \) (PSY871) and \( rsl1-2 \) (PSY872) for \( rna1-1 \) synthetic lethals.

**Rsl Mutants Are Not Allele Specific**

To determine allele specificity, both mutants were crossed to a strain containing another temperature-sensitive allele of \( rna1 \), \( rna1Δ359-397 \). The \( rna1Δ359-397 \) allele is missing amino acids 359–397 and exhibits defects in RNA processing (Traglia et al., 1989). When the diploids were sporulated, two Sect− and two Sect+ spores were obtained in every complete tetrad, indicating that the \( rsl \) mutants are also synthetically lethal with the \( rna1Δ359-397 \) allele (data not shown).

**Cloning of the RSL1 Gene**

The gene responsible for the mutations was cloned by complementing the nonsectoring phenotype. Both \( rsl1 \)
mutants were transformed with a yeast genomic library (Rose et al., 1987). Approximately 10,000 transformants were screened for a Sect<sup>+</sup> phenotype, and plasmid DNA was recovered from three that were identified as Sect<sup>+</sup> and FOA<sup>-</sup>. When the plasmid DNA was retransformed into rsl1-1 and rsl1-2 strains, only two of the clones rescued the nonsectoring phenotype. Restriction analysis indicated that the clones contained overlapping sequences and that neither clone contained the RNAI gene. Several HindIII fragments of the genomic clone were subcloned and subjected to DNA sequence analysis. Sequences obtained indicated that the clone mapped to chromosome XII, whose sequence had already been completed by the Yeast Genome Sequencing Project. Several open reading frames were identified on the clone, and subclones designed to isolate specific open reading frames were created (Fig. 2A). Only the open reading frame between the EcoRV and KpnI sites rescued the nonsectoring phenotype of rsl1-1 and rsl1-2, as well as the temperature sensitivity of rsl1-1 (data not shown).

The open reading frame identified was 2.5 kb long and predicted a protein of ~95 kD. When a database search was performed with the protein sequence, the most homologous protein was the human importin-β protein, with 34% identity (Fig. 2B). Given the reasonably high percent identity between human importin-β and the RSL1 gene product, this protein has been identified as the yeast importin-β homolog (Görlich et al., 1995a). The yeast importin-β protein also shows a limited region of homology to both vertebrate and yeast importin-α in the first arm repeat of these proteins. In addition, there are two uncharacterized yeast proteins and the previously identified protein Pselp (Chow et al., 1992) that exhibit a moderate level of identity to importin-β, all of which might have a similar function to importin-β (Görlich et al., 1995a).

To show that the RSL1 gene is the same gene that is mutated in the synthetic lethal mutants, the LEU2 gene was integrated next to the RSL1 gene. Correct integration of the LEU2 gene was verified by Southern blotting. After crossing to the rsl1-1 and rsl1-2 mutants, tetrads were dissected. Of eight complete tetrads, none of the Leu<sup>+</sup> colonies exhibited a nonsectoring phenotype, indicating that RSL1 was the gene mutated in the mutants identified in the synthetic lethal screen (data not shown).

**The RSL1 Gene Is Essential for Cell Viability**

The RSL1 coding region was replaced with the HIS3 gene using a PCR method (Baudin et al., 1993). The HIS3 gene was amplified using oligonucleotides with flanking RSL1 sequence. The PCR product was transformed into a wild-type diploid and His<sup>+</sup> transformants were recovered. Correct integration at the RSL1 locus was determined by Southern blotting and PCR analysis (data not shown). The diploid strain was sporulated, and tetrads were dissected. Only two spores were recovered from each of the twelve tetrads, and these spores were His<sup>+</sup>, indicating that the RSL1 gene is essential for viability. When the diploid transformed with a plasmid carrying the RSL1 gene was sporulated, four spores were recovered in each of the twelve tetrads. Only His<sup>-</sup> spores were FOA resistant, indicating that the RSL1 gene was rescuing the lethality of the His<sup>+</sup> spores. Similar results have recently been shown (Iovine et al., 1995).

**rsl1 Mutants Exhibit Import Defects In Vivo**

Given the similarity of the RSL1 gene product with mammalian importin-β, we predicted that the RSL1 gene product would be involved in nuclear protein import. Because importin-β has only been shown to function in import using in vitro methods, we conducted in vivo import assays on the rsl1 mutants. The rsl1 mutants and the parental strain from the mutagenesis were transformed with a construct that expresses a nuclear reporter protein under a galactose-inducible promoter. This reporter protein is a fusion of the SV40 large T antigen NLS to yeast invertase, a normally secreted protein (Nelson and Silver, 1989). Cells were grown in media that selected for a plasmid with the RNAI gene as well as the reporter construct, as all three strains carry an rna1-1 allele, which shows import defects in vivo (Corbett et al., 1995). Cells expressing the reporter

![Figure 3. The rsl1 mutants exhibit nuclear protein import defects in vivo. Expression of the reporter protein SV40-invertase was induced by the addition of 2% galactose to log-phase cultures of wild-type (top row), rsl1-1 (middle row), and rsl1-2 (bottom row) cells for 2 h at room temperature. Cultures were split in half and shifted to 36°C for 2 h. Cells were fixed with formaldehyde and prepared for immunofluorescence. The α-invertase antibody was used at 1:20,000, and FITC-conjugated secondary antibodies were used to visualize the reporter protein (left column). 4',6-diamidino-2-phenylindole staining indicates the location of DNA in the cells (middle column). Only cells shifted to 36°C are shown.](image-url)
immediate-β was split in half, with half remaining at room temperature and the other half shifted to 36°C for 2 h. In the parental strain, the reporter protein is nuclear at both room temperature and at 36°C (Fig. 3, top row; only cells at 36°C are shown). However, both rsll alleles show gross mislocalization of the SV40NLS-invertase protein to the cytoplasm at both room temperature and 36°C (Fig. 3, middle and bottom rows; only cells at 36°C are shown).

Since other nuclear import mutants also exhibit mRNA export defects (Amberg et al., 1992; Schlenstedt et al., 1995a), we tested the rsll mutants for nuclear accumulation of polyA+ RNA. The mRNA export phenotype of both rsll mutants was determined using in situ hybridization with a digoxigenin-labeled poly dT probe (Amberg et al., 1992). No mRNA export defect was observed in either mutant after a 2-h shift to 36°C, a time at which they did exhibit protein import defects (data not shown). This is consistent with what is seen with importin-α mutants, which exhibit protein import defects both in vivo and in vitro but do not show mRNA export defects (Loeb et al., 1995).

Importin-β Localizes to the Nuclear Envelope

To determine the intracellular localization of importin-β, the importin-β protein was epitope tagged by inserting an oligonucleotide coding for the c-myc epitope between codons 14 and 15 in the RSL1 gene. When transformed into rsll-1 and rsll-2 strains, the myc-tagged construct was able to rescue the nonsectoring phenotype of both mutants and the temperature sensitivity of rsll-1, indicating that the myc-tagged protein is functional. When immunoprecipitations using anti-myc-conjugated agarose are performed on cells expressing the myc-tagged protein, a single band of ~95 kD is observed on SDS gels (Fig. 4 A, lane 4). This band is not seen in immunoprecipitations from cells expressing the untagged importin-β protein (Fig. 4 A, lane 2). The other two bands present in the immunoprecipitations correspond to the heavy and light chains of the α-myc antibody. When monoclonal anti-myc antibody (9E10) is used for indirect immunofluorescence microscopy, the importin-β protein localizes to the nuclear envelope in a punctate pattern similar to nucleoporin stain-
Nup159p suggests a possible in vivo interaction as well.

Interacts with the repeats of Nup159p in vitro (Enenkel et al.,

importin-β antisera is used (data not shown). These results

is not complete. Similar results are seen when the anti-

in Fig. 4 C, both Nup159p and importin-β are found at the

the nucleoporin Nup159p and importin-β in cells express-

Importin-fl Colocalizes with Nup159p

To establish that the localization of importin-β was indeed

consistent with nuclear pore staining, localization of both

the nucleoporin Nup159p and importin-β in cells expressing

the myc-tagged importin-β was observed. Cells pre-

pared for immunofluorescence were incubated with both

anti-myc and anti-Nup159p (Gorsch et al., 1995). As shown

in Fig. 4 C, both Nup159p and importin-β are found at the

nuclear envelope, although the overlap of the two proteins

is not complete. Similar results are seen when the anti-

importin-β antisera is used (data not shown). These results

indicate that importin-β localizes to the nuclear pore com-

plex, as would be expected of a component of the NLS re-

ceptor. In addition, it has been shown that importin-β in-

teracts with the repeats of Nup159p in vitro (Enenkel et al.,

1995); our observation of colocalization of importin-β and

Nup159p suggests a possible in vivo interaction as well.

Importin-β Interacts with Import Factors In Vitro

As the RSL1 gene was obtained in a rna1-1 synthetic le-
thal screen, it is possible that importin-β and Rna1p physi-

cally interact. To investigate this possibility, a GST–importin-β

fusion was expressed under a galactose-inducible promoter in

wild-type yeast cells. The fusion protein was purified from

yeast cell lysates using glutathione-Sepha-

rose, and samples were resolved by SDS-PAGE, trans-

ferred to nitrocellulose, and blotted with an anti-Rna1p

antibody. However, no copurifying Rna1p was seen (data

not shown).

Conversely, when a wild-type yeast lysate is mixed with

E. coli–purified GST–importin-β protein bound to gluta-

thione-Sepharose, immunoblotting indicates that Rna1p

and Ran are bound, as well as importin-α (Fig. 5, compare

lanes 3 and 5). No Rna1p or Ran is seen when GST is incu-

bated with wild-type lysates, indicating that the interaction

is dependent on importin-β (Fig. 5, lane 4). We did not ob-

serve binding of importin-β to Npl3p, another factor impli-

cated in nuclear transport, indicating that the interaction

between importin-β and Rna1p and Ran is specific (Fig. 5).

Importin-β Colocalizes with Importin-α

To better understand the interaction between importin-β

and importin-α, we more closely investigated the localiza-

tion of the two proteins. In cells expressing the myc-tagged

importin-β, both proteins localize to the nuclear envelope,

but the overlap of the two proteins did not appear to be

complete (Fig. 6 A). To investigate this localization more

thoroughly, confocal microscopy was used on cells labeled

for both importin-α and importin-β (Fig. 6 B). In the con-

focal micrographs of Fig. 6 B, importin-α is represented by

red, importin-β is represented by green, and yellow is the

overlap of the two proteins. The two proteins do indeed

overlap to a significant extent at the nuclear envelope,

although they are not completely coincident. Some cells

show more importin-α staining at what appears to be in-

side the nucleus, but it is possible that this staining is actu-

ally at the surface of the nuclear envelope.

Importin-α Is Mislocalized to the Nucleus

in prp20-1 Cells

The importin-α protein is predicted to enter the nucleus

with the NLS substrate in the process of nuclear import

and then presumably exit the nucleus at a later time. Re-

cently, it has been demonstrated that importin-α accumu-

lates in the nucleus of nup120Δ cells, a mutant that exhib-

its an mRNA export defect (Aitchinson et al., 1995). This

result suggests that when mRNA export is blocked, impor-

tin-α cannot exit the nucleus. Therefore, we chose to in-

vestigate the localization of importin-α in a number of mu-

tants that exhibit transport defects.

In wild-type cells, importin-α is primarily cytoplasmic at

room temperature and at 36°C (Fig. 7 B, top row) when

cells are prepared for indirect immunofluorescence mi-

scopy using formaldehyde fixation. However, when cells

are not fixed with formaldehyde before immunofluores-

cence microscopy, importin-α localizes primarily to the

nuclear envelope, as was shown in the previous section

(Fig. 6 A). The difference in importin-α localization de-

pending on fixation conditions may reflect the possibility

that there are distinct pools of importin-α inside the cell.

For example, a population of importin-α may be in the cy-

toplasm awaiting binding of an NLS protein, while another

population is complexed with importin-β and is bound to

the NPC. Similar differences in localization depending on

fixation conditions are also seen with importin-β, but not

with other transport factors such as Rna1p (data not

shown), indicating that this phenomenon may be specific

to components of the NLS receptor.

When the localization of importin-α was investigated in

a number of transport mutants that exhibit either a nu-

clear protein import defect (srp1-31, srp1-49, rsl2-1, rsl2-2) defect

or both a nuclear protein import defect and an mRNA ex-

port defect (rnl1-1, nup49-313), importin-α staining ap-
Figure 6. (A) Importin-β colocalizes with importin-α. Cells expressing myc-tagged importin-β were prepared for immunofluorescence without formaldehyde fixation. The α-myc antibody was used undiluted, while the anti-importin-α antibodies were used at a concentration of 1:1,000. (B) Importin-α and importin-β overlap at the nuclear envelope. Confocal microscopy was used on cells prepared as in Fig. 6 A. Importin-β (green), importin-α (red), and overlap of the two proteins (yellow).

Figure 7. (A) prp20-1 cells exhibit a protein import defect in vivo. Wild-type (top row) and prp20-1 (bottom row) cells carrying the SV40-invertase reporter construct were induced to express the reporter protein with 2% galactose for 2 h at room temperature. Cultures were then shifted to 36°C for 2 h and fixed with formaldehyde. Cells were then prepared for immunofluorescence. Anti-invertase was used at 1:20,000 dilution. Only cells shifted to 36°C are shown. (B) prp20-1 cells mislocalize importin-α to the nucleus. Wild-type (top row) and prp20-1 (bottom row) cells were grown to log phase, and cultures were split in half and then either shifted to 36°C or kept at room temperature. After formaldehyde fixation, cells were prepared for immunofluorescence. Antibodies to importin-α were used at 1:1,000 dilution, while α-Nup159p was used at 1:2,000.
peared identical to that observed in wild-type cells (data not shown). The only transport mutant which exhibited mislocalization of importin-α to the nucleus was prp20-1 (see Fig. 7 B, bottom row). Costaining of importin-α and the nucleoporin Nup159p in these cells indicates that importin-α is indeed accumulating inside the nucleus and not simply at the nuclear envelope. Importin-β was not mislocalized in any of the mutant strains tested (data not shown).

The prp20-1 allele shows both a nuclear protein import and an mRNA export defect in vivo (Aebi et al., 1990; Fleischmann et al., 1991). Fig. 7 A shows the results of an in vivo nuclear protein import assay using the SV40NLS-invertase reporter protein. The reporter protein is properly localized to the nucleus in wild-type cells (top row) but is grossly mislocalized in prp20-1 cells after 2 h at 36°C (bottom row). Additional nuclear reporter proteins (i.e., Npl3p and H2B-α-galactosidase) are mislocalized in prp20-1 cells at the nonpermissive temperature (data not shown). It is difficult to determine which defect, import or export, occurs first in prp20-1 cells; thus, it is difficult to determine which defect causes the accumulation of importin-α in the nucleus.

### Importin-α Is Nuclear in Cells Expressing Ran-GDP

Because Prp20p is the homolog of RCC1 (Fleischmann et al., 1991), the exchange factor for Ran, we reasoned that if importin-α accumulates in the nucleus when the prp20-1 protein is nonfunctional, then perhaps importin-α accumulates in the nucleus of prp20-1 cells because of the lack of exchange of GTP for GDP on Ran. To further investigate this idea, we localized importin-α in cells expressing mutant forms of Ran. It has been shown that expression of Ran-GTP (RanG21V) causes defects in both nuclear import and mRNA export (Schlenstedt et al., 1995a) (Fig. 8 A, middle row). In addition, when Ran is expressed in GDP form (RanT26N), an mRNA export defect results (Fig. 8 A, bottom row). In cells expressing wild-type Ran, no mRNA export defect results (Fig. 8 A, top row). Importin-α was also localized in these cells. In cells expressing Ran-GTP (RanG21V) or wild-type Ran, importin-α is found primarily in the cytoplasm (Fig. 8 B, top and middle rows), while in cells expressing Ran-GDP (RanT26N), importin-α accumulates in the nucleus (Fig. 8 B, bottom row). This result suggests that the lack of exchange results in the accumulation of importin-α in the nucleus. The importin-β protein is not mislocalized in cells expressing the GDP form of Ran (data not shown).

### Discussion

In this study we have shown that mutants of importin-β are synthetically lethal with mutant RNA1 alleles, and importin-β interacts physically with importin-α, Rnalp, and Ran. Mutants in importin-β show defects in nuclear protein import in vivo, as would be expected for mutant al-

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**Figure 8.** (A) Ran mutants exhibit an mRNA export defect. Wild-type cells expressing wild-type Ran (top row), Ran-GTP (middle row), and Ran-GDP (bottom row) were fixed with formaldehyde and prepared for the in situ mRNA export assay. Cells were probed with a digoxigenin-labeled oligonucleotide followed by incubation with FITC-conjugated α-digoxigenin antibodies. (B) Cells expressing Ran-GDP mislocalize importin-α to the nucleus. Cells prepared as in Fig. 7 A were incubated with α-importin-α (1:1,000) followed by FITC-conjugated secondary antisera.
leles of components of the NLS receptor. Importin-β localizes to the nuclear envelope and colocalizes with other proteins that are known to associate with the nuclear pore complex, namely importin-α and Nup159p. We also find that importin-α mislocalizes to the nucleus in prp20-1 cells and in cells expressing Ran in the GDP-bound form.

Importin-β corresponded to the single complementation group identified in the rna1-1 synthetic lethal screen. The rsl1 mutants identified in the screen were also synthetically lethal with another temperature-sensitive allele of RNA1, rna1Δ59-397. Why would mutations in importin-β lead to lethality when present in combination with mutant RNA1 alleles? Synthetic lethality can result from a number of situations. For example, mutations in two genes with redundant functions might lead to cell death, or mutations in two genes that function in the same pathway, but do not physically interact, could lead to cell death. We have tested synthetic lethality between the rna1-1 allele and alleles of other transport factors (Prp20, Yrb1) and have found that in each case the double mutants were viable (Koepp, D.M., and P.A. Silver, unpublished observations). Thus, we believe that a more specific interaction between rna1-1 and the rsl1 mutants is the cause of their synthetic lethality. Mutations in two genes that cause their respective gene products to no longer physically interact might also lead to lethality. We have shown that importin-β is able to physically interact with Rna1p in vitro using a GST-importin-β fusion protein to probe wild-type lysates. Given the result of the rna1-1 synthetic lethal screen, it seems likely that importin-β and Rna1p also interact in vivo. However, it is also possible that Rna1p and importin-β interact using Ran as an intermediary. Investigation of the nature of the in vitro interaction between Rna1p and importin-β is currently underway.

There are several possible reasons why Rna1p does not copurify with GST-importin-β in yeast. One alternative is that the yeast importin-β protein is modified in some way that prevents interaction with Rna1p; the E. coli protein would not have this modification. Another possibility is that the interaction between Rna1p and importin-β is either weak or transitory; thus, it can only be seen with large amounts of GST-importin-β.

Given the interaction between Rna1p and importin-β, the synthetic lethality between mutant alleles of RNA1 and importin-β mutants can be explained in one of two ways. First, mutations in both Rna1p and importin-β cause the two proteins to no longer interact (or to no longer interact with the appropriate intermediary), leading to a lack of nuclear protein import, which results in lethality. Second, the mutations in both Rna1p and importin-β cause the two proteins to associate in a nonfunctional complex that also results in cell death.

Previously, it has only been shown that importin-β is necessary for nuclear protein import through the use of in vitro import assays (Enenkel et al., 1995; Görlich et al., 1995b; Imamoto et al., 1995; Moroianu et al., 1995). We now show that importin-β is required for proper nuclear protein import in vivo as well. The rsl1 mutants grossly mislocalize the nuclear reporter protein, SV40NLS–invertase, at both room temperature and at 36°C. Interestingly, the strength of the import defect depends on the reporter protein used in the in vivo import assay. For example, neither rsl1-1 nor rsl1-2 mislocalizes the RNA-binding protein Npl3p (Koepp, D.M., and P.A. Silver, unpublished observations), which is known as the shuttle between the nucleus and cytoplasm (Flach et al., 1994). When the reporter is β-galactosidase fused to the histone H2B NLS, rsl1-1 mislocalizes the protein only at the nonpermissive temperature, while rsl1-2 slightly mislocalizes it at both room temperature and 36°C (Koepp, D.M., and P.A. Silver, unpublished observations).

Such differences in import defects with respect to reporter proteins are not seen with other transport mutants such as prp20-1 and rna1-1 (Corbett et al., 1995). It is tempting to speculate that the reason for this difference in import activity lies in the differences in the types of NLS present in the reporter proteins. This could also explain why there appears to be more than one importin-β-like protein in yeast.

We have shown that importin-β localizes to the nuclear envelope by two methods, using a myc-tagged protein and by generating importin-β–specific polyclonal antibodies. Importin-β also colocalizes with two nuclear envelope–associated proteins, Nup159p and importin-α, although in each case the overlap of two proteins is not complete. Using confocal microscopy, importin-α appears to localize to the nuclear envelope and to the cytoplasm. This is consistent with importin-α moving from the cytoplasm with the NLS-bearing substrate into the nucleus during nuclear protein import.

Further evidence for this movement of importin-α into the nucleus comes from the localization of importin-α in cells expressing Ran-GDP (RanT26N). In these cells, importin-α is found to accumulate in the nucleus. It is possible that Ran-GDP (RanT26N) is titrating an essential factor that is required for the export of importin-α; one candidate might be Prp20p. When PRP20 on a high copy plasmid is introduced into cells expressing Ran-GDP (RanT26N), however, the aberrant localization of importin-α is not corrected (Koepp, D.M., and P.A. Silver, unpublished observations). This suggests that there might be another factor, aside from Prp20p, that Ran-GDP (Ran T26N) is titrating that causes the mislocalization of importin-α. We find a similar accumulation of importin-α in the nucleus of prp20-1 cells. Because both the prp20-1 mutation and the Ran-GDP (RanT26N) mutant presumably result in the lack of exchange activity, these data suggest that exchange of GDP for GTP on Ran is necessary for the proper exit of importin-α from the nucleus. In addition, it has been shown that a mutant that has defects in mRNA export also accumulates importin-α in the nucleus (Aitchinson et al., 1995). However, the prp20-1 mutant and cells expressing Ran-GDP exhibit both mRNA export and nuclear protein import defects.

To put this data in a broader perspective, recall that the GAP for Ran, Rna1p, is located exclusively in the cytoplasm, while the exchange factor, Prp20p, is located in the nucleus. Evidence concerning the lack of GAP activity and the effects of nonhydrolyzable GTP analogs suggests that hydrolysis of GTP by Ran is directly required for import into the nucleus (Melchior et al., 1993; Corbett et al., 1995; Schlenstedt et al., 1995a). When importin-α is localized in either rna1-1 strains or cells expressing Ran-GTP (Ran G21V), it is found to be in the cytoplasm. However, both the rna1-1 mutant and cells expressing Ran in the GTP...
form also exhibit both protein import and mRNA export defects. How then might we explain the aberrant localization of importin-$\alpha$ in only certain transport mutants? Perhaps importin-$\alpha$ is found in the cytoplasm of cells that lack GAP activity because the import defect is the primary defect, and the mRNA export defect is a result of an import blockage. Similarly, in cells where exchange activity is compromised, the primary defect might be in mRNA export. If this is the case, then importin-$\alpha$ would accumulate in the nucleus of such cells because the import defect takes place later as a result of the blockage of mRNA export. Taken together, this suggests a model in which hydrolysis of GTP by Ran is necessary for the import of NLS-bearing proteins complexed with importin-$\alpha$, and exchange of GDP for GTP on Ran is necessary for the exit of importin-$\alpha$ and mRNA from the nucleus (Fig. 9). Further investigation will be necessary to test the many predictions of this model.

How does the interaction between Rna1p and importin-$\beta$ fit into such a model for nuclear transport? There are two simple ways that this interaction might be explained: (a) The Ran-mediated hydrolysis of GTP, which is activated by Rna1p, causes a conformational change that leads to dissociation of importin-$\alpha$ from importin-$\beta$. This dissociation then allows importin-$\alpha$ to move into the nucleus with the NLS-bearing substrate. (b) The Ran-mediated hydrolysis of GTP is not necessary for the dissociation of importin-$\alpha$ from importin-$\beta$, but is instead necessary for the recycling of importin-$\beta$ from the NPC back to the cytoplasm (Rexach and Blobel, 1995). The combined use of genetics and biochemistry should allow us to distinguish between these two alternatives.

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