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**MEMBRANE TRANSPORT STRUCTURE  
FUNCTION AND BIOGENESIS:**  
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## The Mechanism of Ran Import into the Nucleus by Nuclear Transport Factor 2\*

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The small GTPase Ran is essential for virtually all nucleocytoplasmic transport events. It is hypothesized that Ran drives vectorial transport of macromolecules into and out of the nucleus via the establishment of a Ran gradient between the cytoplasm and nucleoplasm. Although Ran shuttles between the nucleus and cytoplasm, it is concentrated in the nucleus at steady state. We show that nuclear transport factor 2 (NTF2) is required to concentrate Ran in the nucleus in the budding yeast, *Saccharomyces cerevisiae*. To analyze the mechanism of Ran import into the nucleus by NTF2, we use mutants in a variety of nuclear transport factors along with biochemical analyses of NTF2 complexes. We find that Ran remains concentrated in the nucleus when importin-mediated protein import is disrupted and demonstrate that NTF2 does not form a stable complex with the transport receptor, importin- $\beta$ . Consistent with a critical role for NTF2 in establishing and maintaining the Ran gradient, we show that NTF2 is required for early embryogenesis in *Caenorhabditis elegans*. Our data distinguish between two possible mechanisms for Ran import by NTF2 and demonstrate that Ran import is independent from importin- $\beta$ -mediated protein import.

Protein import into the nucleus is a multi-step process involving recognition of the protein substrate, targeting of the substrate to the nuclear pore complex (NPC),<sup>1</sup> translocation of the substrate through the pore, release into the nucleus, and finally recycling of the import factors back to the cytoplasm (1–3). Recognition of the transport substrate in the cytoplasm requires both intrinsic sequences in the protein to be imported

(4) and soluble receptor proteins (3, 5, 6). Proteins containing a classical nuclear localization signal (NLS) are first recognized by the heterodimeric importin- $\alpha$ /importin- $\beta$  NLS receptor (3, 7–10). Once bound, the importin- $\alpha$ /importin- $\beta$  receptor directs the entire complex to the NPC via interactions between importin- $\beta$  and the pore (3, 11). Efficient translocation through the pore requires both the small GTP-binding protein, Ran (Gsp1p/Gsp2p or scRan in *Saccharomyces cerevisiae*), and the homodimeric Ran-binding protein, nuclear transport factor 2 (NTF2) (3).

Ran is a 25-kDa Ras-like GTP-binding protein that cycles between the GTP- and GDP-bound states (2, 12). The nucleotide bound state and the cellular localization of Ran are both essential to coordinate nucleocytoplasmic transport (1, 2). These properties are regulated by a number of Ran-interacting factors including the GTPase-activating protein (GAP) RanGAP1 (Rna1p in *S. cerevisiae*) (13, 14), the guanine nucleotide exchange factor (GEF) RCC1 (Prp20p in *S. cerevisiae*) (15, 16), and NTF2 (17–19). The strict compartmentalization of the RanGAP to the cytoplasm (20) and the RanGEF to the nucleus (21) has led to the hypothesis that nucleocytoplasmic transport is driven by a Ran gradient. In this model, RanGDP levels would be high in the cytoplasm because of the activity of the RanGAP, conversely, RanGTP levels would be high in the nucleus because of the activity of the RanGEF (16, 21). Consistent with the Ran compartmentalization model is the fact that RanGDP is required for protein translocation through the pore into the nucleus (22, 23), and RanGTP appears to be involved in the final release of the cargo complex into the nucleus (5, 24). In addition, RanGTP is required for re-export of importin- $\beta$  to the cytoplasm and thus recycling of the soluble import factors (23, 25).

NTF2 was originally identified as an activity that stimulates import of proteins into nuclei of permeabilized mammalian cells (26). *In vitro* binding assays demonstrate that NTF2 binds to RanGDP, importin- $\beta$ , and nuclear pore proteins containing phenylalanine-glycine (XFXFG) repeats (17, 27, 28). Although NTF2 is highly conserved and deletion of the *S. cerevisiae* homologue is lethal (29, 30), in some mammalian *in vitro* permeabilized import assays NTF2 is not required. This observation has been used to suggest that NTF2 may not be essential in higher eukaryotes. However, recent studies demonstrate that NTF2 acts as a mediator of RanGDP import into the nucleus to replenish the nuclear stores of RanGTP (18, 19, 31). These data suggest a critical role for NTF2 in establishing and maintaining the Ran gradient *in vivo*.

Two possible pathways for Ran import into the nucleus by NTF2 can be derived from previous studies (Fig. 1). In model A, Ran is imported into the nucleus as an import complex containing import cargo/importin- $\alpha$ /importin- $\beta$ /RanGDP/NTF2. Once inside the nucleus this complex is disassociated by the exchange of RanGDP to RanGTP by the nuclear RanGEF. In this model

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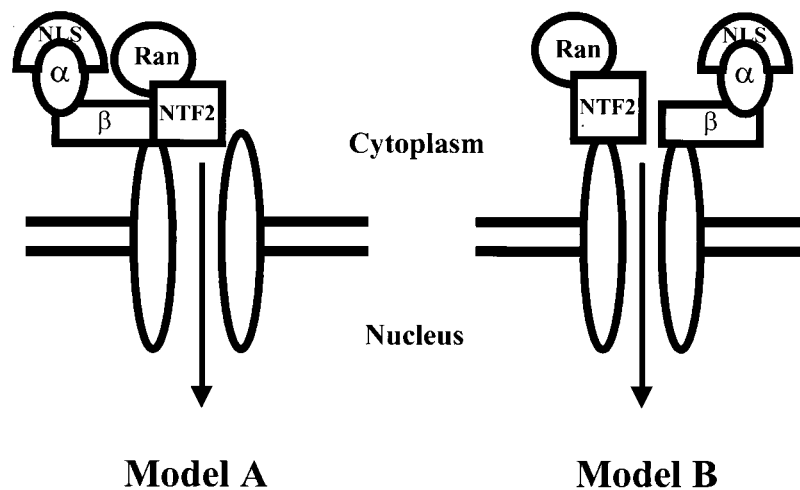
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<sup>1</sup> The abbreviations used are: NPC, nuclear pore complex; NTF2, nuclear transport factor 2; NLS, nuclear localization signal; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; GTP $\gamma$ S, guanosine 5'-3-O-(thio)triphosphate; GDP $\beta$ S, guanyl-5'-yl thio-phosphate; dsRNA, double-stranded RNA.

**FIG. 1. Two models for the import of RanGDP into the nucleus by NTF2.** In *model A*, NTF2 imports Ran into the nucleus in a large complex with NLS cargo/importin- $\alpha/\beta$ . In *model B*, NTF2 imports Ran into the nucleus in a complex that is distinct from the NLS cargo/importin- $\alpha/\beta$  complex.



RanGDP and NTF2 would form a complex that includes importin- $\beta$ . This model is consistent with *in vitro* binding assays demonstrating that NTF2 interacts directly with importin- $\beta$  (27). However, *in vitro* binding assays have shown that importin- $\beta$  has a low affinity for RanGDP unless the Ran-binding protein RanBP1 (Yrb1p in *S. cerevisiae*) is present (32). In model B, import of Ran by NTF2 is independent of the importin- $\alpha/\beta$ /cargo complex. Once RanGDP reaches the nuclear face of the NPC, the exchange factor binds and exchanges GDP for GTP, and RanGTP, for which NTF2 has no detectable affinity, is released into the nucleus. This model predicts that two separate and distinct complexes are formed, the RanGDP/NTF2 complex and the importin- $\alpha/\beta$ /cargo complex, and that these complexes need never interact.

In this study, we investigate the mechanism of Ran import into the nucleus to distinguish between the two models presented in Fig. 1. First, we show that NTF2 is required for Ran import into the nucleus in the budding yeast *S. cerevisiae*. We then demonstrate that Ran import is maintained in the absence of importin- $\beta$ -mediated import of NLS cargo. Next, we show that NTF2 forms a complex containing Ran and nucleoporins but does not form a stable complex with importin- $\beta$ . These results provide experimental evidence that the Ran/NTF2 import complex is separate from the importin- $\beta$  import complex and show that Ran compartmentalization in the nucleus is primarily controlled through NTF2-mediated import. Consistent with the critical role of NTF2 in establishing and maintaining a Ran gradient, we also show that NTF2 is essential in the multicellular organism *Caenorhabditis elegans*, demonstrating for the first time that NTF2 is required for viability in higher eukaryotes.

#### MATERIALS AND METHODS

All chemicals were obtained from Sigma or U.S. Biological unless otherwise noted. All DNA manipulations were performed according to standard methods (33), and all media were prepared by standard procedures (34). All yeast strains and plasmids used in this study are described in Table I.

**Depletion of Ntf2p *In Vivo***—The plasmids, pAC611 (pGAL1-10-myc-NTF2) and pAC410 (scRan-GFP) were introduced into the NTF2 deletion strain ACY114. Transformants were maintained in galactose media to induce continuous expression of the myc-NTF2 protein then shifted to glucose to shut off expression. The localization of scRan-GFP was monitored by directly viewing the GFP signal in living cells through a GFP optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera.

**Localization of GFP-LacZ-NLS, Ntf2p-GFP, and scRan-GFP in *rsl1-1* Cells**—The plasmid pAC46 (*CEN*, *LEU*, *rsl1-1*) was transformed into the importin- $\beta$  deletion strain ACY208. Transformants were streaked onto 5-fluoroorotic acid (5-FOA) plates and incubated at 25 °C

to select for cells that had lost the *URA3* plasmid containing the wild-type *RSL1* (importin- $\beta$ ) gene. These cells were then transformed with either pAC697 (*GFP-LacZ-NLS*) (35), pAC410 (*scRan-GFP*), or pAC709 (*NTF2-GFP*). Transformants were grown to log phase at 25 °C and split, half was shifted to 37 °C for 2.5 h, and GFP signals were detected as described above.

**Ntf2p Purification and Immobilization**—All NTF2 proteins were purified from *Escherichia coli* as described previously for rat NTF2 (28). Expression plasmids were transformed into *E. coli* BL21 (DE3). Transformants were inoculated into 2 $\times$  tryptone-yeast extract medium containing 100  $\mu$ g of ampicillin/ml and grown overnight at 30 °C. It was not necessary to induce expression as the basal level of expression of the T7 polymerase yielded a large amount of Ntf2p. Bacteria were harvested by centrifugation and stored at -80 °C until required.

Ntf2p was isolated by thawing the cell pellet and resuspending in 25% sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed in a French pressure cell and treated with DNase I for 30 min at room temperature. The soluble fraction was isolated by centrifugation at 40,000  $\times$  g for 20 min and dialyzed overnight against 20 mM Tris-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 0.1 mM PMSF (NTF2 buffer A). The lysate was clarified at 40,000  $\times$  g for 30 min at 4 °C and applied to DE52 ion exchange column (10  $\times$  3 cm) and washed with NTF2 buffer A. Ntf2p was eluted from the column with a gradient of 0–400 mM NaCl. Fractions containing Ntf2p, as determined by SDS-polyacrylamide gel electrophoresis (PAGE), were pooled, concentrated using a Centrprep-10 (Amicon) concentrator and applied to a column of Sephacryl SR100 pre-equilibrated in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 0.1 mM PMSF (NTF2 buffer B). Fractions containing Ntf2p were collected and pooled.

Purified Ntf2p was cross-linked to CNBr-Sepharose beads as described previously (28). Briefly, CNBr-Sepharose beads (Amersham Pharmacia Biotech) were swollen and washed in 1 mM HCl. Beads were transferred to coupling buffer (100 mM NaHCO<sub>3</sub>, pH 8.3, and 500 mM NaCl) and added to 2–5 mg of Ntf2p in coupling buffer. Coupling was carried out at 4 °C overnight. Residual active groups were blocked with 1 M Tris-HCl, pH 8.0, for 2 h at room temperature. Beads were then washed successively and extensively four times in coupling buffer and acid wash buffer (0.1 M sodium acetate, pH 4.0, and 500 mM NaCl).

**Binding Assays**—Yeast cell extracts were prepared from cultures grown overnight at 30 °C or at room temperature (for temperature-sensitive strains) in yeast extract-peptone dextrose (YEPD) medium. Cells were harvested by centrifugation and washed once with water. Cells were then resuspended in one volume of PBSMT (phosphate-buffered saline, 2.5 mM MgCl<sub>2</sub>, and 0.5% Triton X-100) supplemented with protease inhibitors (0.5 mM PMSF and 3  $\mu$ g each of aprotinin, leupeptin, chymostatin, and pepstatin/ml). One volume of glass beads was added, and cells were lysed with 10–15 60-s pulses in a bead beater (lysis was monitored by light microscopy to >70% lysis). The resulting lysate was clarified by centrifugation and assayed for protein concentration with a Bio-Rad protein assay kit.

Two mg of yeast lysate was incubated with 50  $\mu$ l of either Ntf2p-Sepharose beads or bovine serum albumin (BSA)-Sepharose beads. Binding was carried out in PBSM (phosphate-buffered saline, 2.5 mM MgCl<sub>2</sub>) (total volume, 500  $\mu$ l) at 4 °C for 1 h. Beads were then washed two times for 10 min in PBSM and one time for 10 min in PBSMT.

TABLE I  
Yeast strains and plasmids used

Strain or plasmid	Description
<b>Strains</b>	
ACY192	MAT $\alpha$ <i>ura3-52 trp1<math>\Delta</math>63 leu2<math>\Delta</math>1 his3<math>\Delta</math>200</i>
ACY114	MAT $\alpha$ <i>ura3-52 trp1<math>\Delta</math>63 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 NTF2<math>\Delta</math>::HIS3 pAC626</i>
ACY212	MAT $\alpha$ <i>ura3-52 trp1<math>\Delta</math>63 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 GSP1<math>\Delta</math>::HIS3 GSP2<math>\Delta</math>::HIS3 pAC629 (49)</i>
ACY60	MAT $\alpha$ <i>ura3-52 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 ade2-1 rna1-1 (13)</i>
ACY109	MAT $\alpha$ <i>ura3-52 trp1<math>\Delta</math>63 leu2<math>\Delta</math>1 prp20-1 (42)</i>
ACY208	MAT $\alpha$ <i>ura3-52 trp1<math>\Delta</math>63 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 ade2-IRSL1<math>\Delta</math>::HIS3 pAC710 (42)</i>
BQY104	MAT $\alpha$ <i>ura3-52 trp1<math>\Delta</math>63 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 GSP1<math>\Delta</math>::HIS3 GSP2<math>\Delta</math>::HIS3 RSL1::RSL1-GFP (importin-<math>\beta</math>95)-URA3 pAC627</i>
<b>Plasmids</b>	
pAC19	2 $\mu$ , <i>LEU2, GAL1-10, amp</i>
pAC626	<i>NTF2, CEN, URA3, amp (29)</i>
pAC160	<i>myc-NTF2, CEN, LEU2, amp (29)</i>
pAC78	<i>GSP1, CEN, TRP1, amp (45)</i>
pAC627	<i>myc-GSP1, CEN, TRP1, amp (45)</i>
pAC628	yeast Ntf2 bacterial expression plasmid (pMW172) (45)
pAC629	<i>GSP1, CEN, URA3, amp (45)</i>
pAC45	C-terminal GFP fusion plasmid (56)
pAC410	1.4-kilobase coding region of <i>GSP1</i> amplified with <i>XhoI-Pst1</i> oligonucleotides inserted into <i>XhoI-Pst1</i> sites of pAC45
pAC611	1.0-kilobase coding region of <i>myc-NTF2</i> (pAC160) amplified with <i>BamHI-XhoI</i> oligonucleotides inserted into <i>BamHI-XhoI</i> sites of pAC19
pAC619	267 base pairs including exons 2, 3, and 4 of <i>C. elegans NTF2</i> amplified from a <i>C. elegans</i> cDNA library with <i>BamHI-HindIII</i> oligonucleotides inserted into the TOPOI vector (Invitrogen)
pAC211	rat Ntf2 bacterial expression vector (44)
pAC630	rat Ntf2W7A bacterial expression vector (44)
pAC46	<i>rsl1-1, CEN, LEU2, amp (42)</i>
pAC710	<i>RSL1, CEN, URA3, amp (42)</i>
pAC709	780 base pairs coding region of <i>NTF2</i> amplified with <i>EcoRI-XhoI</i> oligonucleotides inserted into <i>EcoRI-XhoI</i> sites of pAC45
pAC697	<i>GFP-LacZ-NLS, URA3 (35)</i>

Bound proteins were eluted with 100  $\mu$ l of sample buffer, and 5  $\mu$ l was resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblotting.

**Immunoblot Analysis**—Immunoblot analysis was performed essentially as described (36). Importin- $\beta$  was detected by incubation with a 1:1000 dilution of a rabbit polyclonal antibody against yeast importin- $\beta$ 95 (the generous gift of Dr. D. M. Koepp and Dr. P. Silver). Nsp1p was detected by incubation with a 1:5000 dilution of a rabbit polyclonal antibody against the XFXFG repeats of Nsp1p (the generous gift of Dr. M. Stewart). scRan was detected by incubation with a 1:1000 dilution of a rabbit polyclonal antibody against scRan (the generous gift of Dr. D. H. Wong and Dr. P. Silver). Yrb1p was detected by incubation with a 1:10,000 dilution of a rabbit polyclonal antibody against Yrb1p (the generous gift of Dr. G. Schlenstedt and Dr. P. Silver). Importin- $\alpha$  was detected by incubation with a 1:5000 dilution of a rabbit polyclonal antibody raised against recombinant yeast importin- $\alpha$ . For quantitation, films were analyzed using a Molecular Dynamics Personal Densitometer SI scanning laser densitometer.

**Sizing Column and Sucrose Gradient**—The importin- $\beta$ 95GFP/*myc-GSP1/GAL1-10-myc-NTF2* yeast strain BQY104 was constructed by integrating importin- $\beta$ 95GFP as described (37) into the genome of the *GSP1 $\Delta$ /GSP2 $\Delta$*  yeast (PSY962) maintained with a plasmid containing *myc-scRan* (pPS966) and transformed with *pGAL1-10-myc-NTF2* (pAC611). BQY104 (importin- $\beta$ 95GFP/*myc-GSP1/GAL1-10-myc-NTF2*) cells were grown in galactose to induce expression of *myc-NTF2*. Lysates were made as described above, and 10 mg of BQY104 yeast extract was loaded onto a Sephacryl S-200 column, and 1-ml fractions were collected. Equal amounts of each fraction were loaded onto SDS-PAGE gels and immunoblotted with anti-*myc* to detect Ntf2p and scRan, anti-GFP to detect importin- $\beta$ 95GFP, and anti-importin- $\alpha$  to detect importin- $\alpha$ .

**C. elegans Methods and Strains**—*C. elegans* strains were cultured at 20  $^{\circ}$ C as described previously (38). The single strain used in this study was *ceh-22::GFP*, which contains the *ceh-22* promoter fused to the GFP coding sequence and integrated onto linkage group V (39).

**RNA-mediated Interference of NTF2 in C. elegans**—The *C. elegans* NTF2 homologue was identified on cosmid R05D11.3. RNA corresponding to exons 2, 3, and 4 of the *C. elegans NTF2* gene (see Fig. 8) was transcribed from pAC619 (*ceNTF2*) using the RiboMAX large scale RNA production system (Promega). Twenty *ceh-22::GFP* adult hermaphrodites were injected with dsRNA (40). As a negative control, 10 *ceh-22::GFP* adult hermaphrodites were injected with dsRNA corresponding to the cloned *spe-9* gene (41), which exhibits no detectable RNA interference phenotype. Injected adults were allowed to recover and lay eggs for 12 h post-injection on individual agar growth plates.

Each animal was then transferred to a fresh growth plate and allowed to lay eggs for 8 h. Adults were removed, and these eggs were allowed to incubate for 24 h. At the end of this period, unhatched eggs were transferred to an agarose-coated slide and examined under a Zeiss Axiophot microscope equipped with differential interference contrast optics. Eggs were assayed for pharyngeal organogenesis by scoring for GFP expression.

## RESULTS

**NTF2 Is Required to Concentrate Ran in the Nucleus in S. cerevisiae**—To test whether Ntf2p is required to concentrate Ran in the nucleus in the budding yeast *S. cerevisiae*, the localization of Ran was analyzed as Ntf2p was depleted from yeast cells. Myc-tagged Ntf2p (*myc-Ntf2p*) under the control of the inducible *GAL1-10* promoter and scRan fused to the green fluorescent protein (*scRan-GFP*) were introduced into *NTF2 $\Delta$*  yeast cells (ACY114). These cells were maintained in galactose to induce continuous expression of the *myc-NTF2* gene and maintain viability. At time 0, cells were shifted to glucose to repress *myc-Ntf2p* expression and the localization of scRan-GFP was monitored by microscopy (Fig. 2A). At 0 h in glucose scRan was localized throughout the cell with a clear concentration in the nucleus. After 4 h in glucose-containing media, scRan localization was shifted toward the cytoplasm. By 6 h scRan appeared evenly distributed throughout the cell with no clear concentration in the nucleus (Fig. 2A). As shown in Fig. 2B, the expression of *myc-Ntf2p* was concomitantly reduced at 4 and 6 h as scRan was redistributed. Thus, Ntf2p is required to concentrate Ran in the nucleus in *S. cerevisiae*.

**scRan Is Concentrated in the Nucleus in the Importin- $\beta$  Mutant, *rsl1-1***—If NTF2-mediated Ran import occurs in concert with importin- $\alpha$ / $\beta$ /cargo, then Ran should not concentrate in the nucleus in either importin- $\alpha$  or importin- $\beta$  mutants that are unable to support NLS cargo import. Because both Ran and NTF2 have been shown to interact with importin- $\beta$ , we analyzed Ran localization in the importin- $\beta$  temperature-sensitive mutant, *rsl1-1* (42). The *rsl1-1* allele of importin- $\beta$  contains a nonsense mutation at amino acid 851 resulting in the loss of 10 amino acids from the C terminus. The Rsl1-1 protein is unable to support NLS-mediated protein import. In addition, the

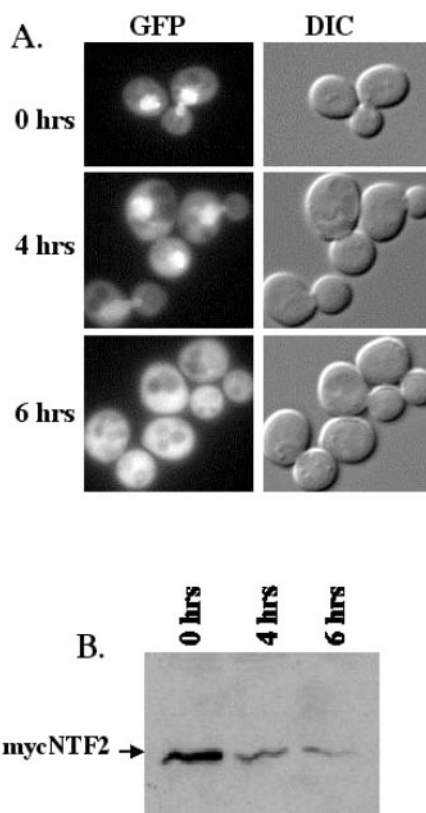


FIG. 2. Ntf2p is required for scRan import into the nucleus in *S. cerevisiae*. *pGAL1-10-myc-NTF2* (pAC611) was transformed into ACY114 (*NTF2Δ*) cells. Transformants were maintained in galactose to induce continuous expression of Ntf2p. To deplete Ntf2p, cells were washed and transferred to glucose media to repress Ntf2p expression. Samples were taken at 0, 4, and 6 h in glucose for observation of scRan-GFP localization (A) and myc-Ntf2p immunoblot analysis (B).

Rsl1-1 protein does not efficiently interact with nucleoporins and mislocalizes to the cytoplasm.<sup>2</sup> We analyzed the localization of both scRan-GFP and Ntf2p-GFP in the *rsl1-1* mutant. In addition, to monitor NLS-mediated protein import in *rsl1-1* cells, the localization of GFP-LacZ-NLS was analyzed. Consistent with previous reports, NLS-mediated import is reduced at 37 °C in *rsl1-1* mutant cells (42) (Fig. 3). However, as previously shown with myc-Ntf2p (29), Ntf2p-GFP is localized throughout the cell with a clear concentration at the nuclear rim at both 25 and 37 °C. Furthermore, scRan-GFP remains concentrated in the nucleus in *rsl1-1* mutant cells at 37 °C. These results show that import of Ran into the nucleus is maintained in the absence of importin- $\beta$ -mediated NLS cargo import and suggest that import of the NTF2/Ran complex is independent of the importin- $\alpha/\beta$ /NLS cargo complex as diagrammed in Fig. 1 (model B).

**Yeast Ntf2p Interacts with scRan, Nucleoporins, and Importin- $\beta$  in Vitro**—To determine whether the NTF2/Ran complex interacts with importin- $\beta$  biochemically, we performed bead binding assays using bacterially expressed yeast Ntf2p covalently coupled to CNBr-Sepharose beads. Purified recombinant importin- $\beta$  bound to Ntf2p-CNBr beads as well as to myoglobin-CNBr beads and BSA-CNBr beads in the presence or absence of Ran (data not shown). This nonspecific binding was observed even in the presence of 1 M NaCl.

Because recombinant importin- $\beta$  bound nonspecifically to Ntf2p beads, we analyzed Ntf2p beads incubated with yeast cell lysates. Components of the lysate that bound to the beads

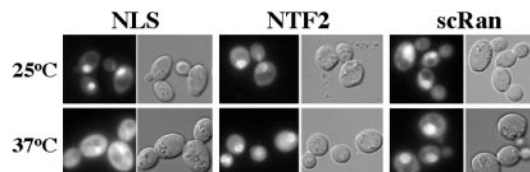
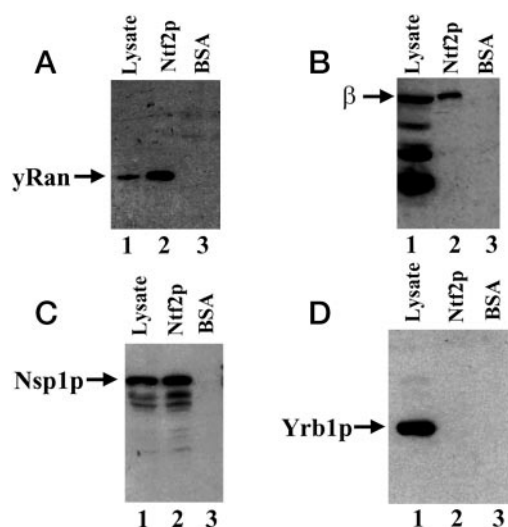


FIG. 3. Localization of Ran is independent of NLS-mediated protein import by importin- $\beta$ . Cells expressing the importin- $\beta$  temperature-sensitive allele, *rsl1-1* (42), were transformed with plasmids encoding GFP-LacZ-NLS (pAC697), NTF2-GFP (pAC709), or scRan-GFP (pAC410). Transformants were grown to log phase at 25 °C and split, and half were shifted to 37 °C for 2.5 h. Cells were observed by directly viewing the GFP signal in living cells through a GFP optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera. Corresponding differential interference contrast images are shown.

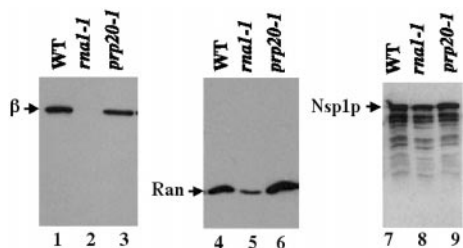
were analyzed by SDS-PAGE. Immunoblots of these gels show that yeast Ntf2p forms complexes containing scRan, the XFXFG-containing nucleoporin Nsp1p, and importin- $\beta$  (Fig. 4, A, lane 2; B, lane 2; and C, lane 2). However, Yrb1p, a protein previously shown to form a macromolecular complex with importin- $\beta$  and RanGDP (32), was not present in any of our Ntf2p affinity purified complexes (Fig. 4D, lane 2). The lower bands observed in yeast cell lysate with the importin- $\beta$  polyclonal antibody (Fig. 4A, lane 1) are due to nonspecific antibody interactions. BSA coupled to CNBr-Sepharose beads did not bind scRan, Nsp1p, or importin- $\beta$  (Fig. 4, A, lane 3; B, lane 3; and C, lane 3), demonstrating that these interactions are specific in the presence of yeast lysate. Silver staining of SDS-PAGE gels of Ntf2p affinity purified complexes reveals approximately 20 distinct proteins that interact specifically with Ntf2p as compared with BSA (data not shown). Given the number of proteins that interact with Ntf2p in these bead binding experiments, it is important to note that these experiments do not distinguish between direct or indirect interactions between Ntf2p and any of the proteins identified in the complexes isolated.

**The Interaction between Ntf2p and Importin- $\beta$  Is via Nucleoporins**—*In vitro* studies have shown that importin- $\beta$  interacts with several different nucleoporins (3, 5, 30, 43) and that this interaction can be disrupted by RanGTP (5). Perhaps Ntf2p beads bind to importin- $\beta$  because this interaction is mediated by nucleoporins. If so, the nucleotide bound state of scRan should affect the level of importin- $\beta$  found in the Ntf2p complexes. To test this hypothesis, wild-type Ntf2p beads were incubated with *rna1-1* and *prp20-1* yeast cell lysates. Rna1p is the scRan GAP (13, 14), and Prp20p is the GEF for scRan (16). Therefore, *rna1-1* cell lysates that are defective in GAP activity (13) contain more scRan in the GTP bound state than wild-type cell lysates; conversely, *prp20-1* cell lysates contain less scRanGTP than wild-type cell lysate and, hence, more scRanGDP. Immunoblots of beads incubated with *rna1-1* yeast cell lysates indicate that importin- $\beta$  does not form a complex with Ntf2p in these cell extracts where RanGTP levels are elevated (Fig. 5, lane 1 versus lane 2). Furthermore, because Ntf2p specifically binds RanGDP, the binding of scRan to Ntf2p in these extracts is reduced (Fig. 5, lane 4 versus lane 5); however, Nsp1p binding to Ntf2p is not significantly altered (Fig. 5, lane 7 versus lane 8). Immunoblots show a slight increase in scRan binding to Ntf2p beads incubated with cell extracts from *prp20-1* mutant yeast consistent with an increase in scRanGDP (Fig. 5, lane 4 versus lane 6). However, the level of bound importin- $\beta$  was only slightly decreased (Fig. 5, lane 1 versus lane 3), and Nsp1p binding was unchanged. Similar results were obtained in extracts that were preincubated with either the nonhydrolyzable form of GTP, GTP $\gamma$ S, to lock the available scRan into the GTP bound state, or GDP $\beta$ S, to lock

<sup>2</sup> D. M. Koepp and P. A. Silver, unpublished data.



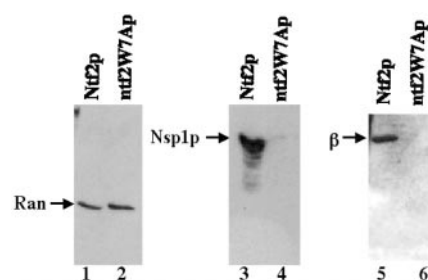
**FIG. 4. Ntf2p forms a complex containing scRan, nucleoporins, and importin- $\beta$ .** Recombinant Ntf2p was immobilized on CNBr-Sepharose beads and incubated with wild-type yeast (ACY192) cell lysate (2 mg of total protein; see “Materials and Methods”). Proteins bound to Ntf2p were identified by immunoblotting (lanes 2 in A–D) with scRan antisera (A), importin- $\beta$  antisera (B), Nsp1p antisera (C), or Yrb1p antisera (D, negative control). Total lysate was also analyzed by immunoblotting (lanes 1; A, 20  $\mu$ g; B, 60  $\mu$ g; C, 10  $\mu$ g; and D, 10  $\mu$ g). As a control for specificity of binding, BSA was immobilized on CNBr-Sepharose beads and incubated with wild-type yeast cell lysate, and bound proteins were detected by immunoblotting (lane 3 in A–D).



**FIG. 5. RanGTP disassociates importin- $\beta$  from the Ntf2p complex.** The interaction of importin- $\beta$  with Ntf2p is decreased in an *rna1-1* mutant (increased RanGTP) but is unchanged in a *prp20-1* mutant (increased RanGDP). Yeast cell lysates were prepared from wild-type (WT), *rna1-1*, and *prp20-1* cells. Proteins from these lysates that bound recombinant Ntf2p were detected by immunoblotting with anti-importin- $\beta$  (lanes 1–3), anti-scRan (lanes 4–6), and anti-Nsp1p (lanes 7–9) antibodies.

scRan in the GDP state (data not shown). These data suggest that RanGTP disassociates the importin- $\beta$ /Ntf2p complex and suggest that importin- $\beta$  interacts with Ntf2p via nucleoporins rather than binding directly to Ntf2p.

The interaction between Ntf2p and XFXFG-containing nucleoporins was recently analyzed (44). The crystal structure of NTF2 allowed the design of a mutant in rat NTF2, Ntf2W7A, with reduced affinity for XFXFG repeat nucleoporins. To further test the possibility that importin- $\beta$  forms a complex with Ntf2p via nucleoporins, we purified both the rat wild-type and rat Ntf2W7A proteins and analyzed interactions with nucleoporins and importin- $\beta$ . Rat NTF2 forms complexes with scRan, Nsp1p, and importin- $\beta$  comparable to that observed with yeast Ntf2p (45) (Fig. 6, lanes 1, 3, and 5). As previously reported (44), Ntf2W7A interacts with scRan in a manner comparable to that observed for wild-type rat NTF2 but has reduced affinity for Nsp1p (Fig. 6, lanes 2 and 4). Furthermore, Ntf2W7A exhibits a reduced ability to interact with importin- $\beta$  (Fig. 6, compare lanes 5 and 6), providing further evidence that the interaction observed between Ntf2p and importin- $\beta$  occurs via nucleoporins.



**FIG. 6. Importin- $\beta$  interacts with Ntf2p via nucleoporins.** Recombinant rat Ntf2 and rat Ntf2W7A were immobilized on CNBr-Sepharose beads and incubated with wild-type yeast (ACY192) cell lysate. Proteins bound to rat Ntf2 (lanes 1, 3, and 5) and rat Ntf2W7A (lanes 2, 4, and 6) were identified by immunoblotting with anti-scRan (lanes 1 and 2), anti-Nsp1p (lanes 3 and 4), and anti-importin- $\beta$  (lanes 5 and 6) antibodies.

*In Vivo Analysis of Ntf2p Complexes*—Because the previous experiments relied on interactions between bound recombinant Ntf2p and proteins in cell lysates, we examined Ntf2p complexes formed *in vivo* by separating yeast cellular protein complexes using an S-200 sizing column. Fractions were analyzed for the presence of Ntf2p, scRan, and importin- $\beta$ . Fractions containing Ntf2p were shown to contain scRan but not importin- $\beta$  (Fig. 7). Ran was present in the majority of fractions analyzed, indicative of the high abundance of this protein and its ability to form a large number of complexes (3). Therefore, the NTF2/Ran complex most likely represents a minor Ran complex. As a control for another known nuclear transport interaction, the importin- $\alpha$ /importin- $\beta$  complex was examined by analyzing fractions for the presence of importin- $\alpha$ . The majority of importin- $\alpha$  co-purifies with importin- $\beta$ , demonstrating that the importin- $\alpha$ /importin- $\beta$  complex is intact. Similar results were obtained by analyzing cell fractions in sucrose gradients (data not shown). These data demonstrate that Ntf2p does not form a complex containing importin- $\beta$  *in vivo* that is sufficiently stable to detect and support model B (Fig. 1), where Ntf2p facilitates import of Ran into the nucleus separate from the cargo/importin- $\alpha$ /importin- $\beta$  complex.

*NTF2 Is Essential in Multicellular Eukaryotes*—If Ntf2p is the primary factor required to establish and maintain a concentration of nuclear Ran, this function should be essential in higher eukaryotes. To test this hypothesis, we performed RNA-mediated interference of the *C. elegans* NTF2 homologue. This technique has been used successfully to obtain gene-specific loss-of-function phenotypes for a variety of genes (40, 46–49). A search of the *C. elegans* genomic data base identified the *ceNTF2* gene. A TBLASTN search using human NTF2 as the query sequence revealed one gene on cosmid R05D11.3 that encodes a predicted protein with 42% identity to human NTF2 (Fig. 8A). A search of the *C. elegans* genomic data base using the *ceNTF2* sequence as the query sequence revealed no other sequences with significant homology, indicating that *C. elegans* contains a single NTF2 homologue. The *ceNTF2* is 528 nucleotides in length and contains three introns and four exons (Fig. 8B). Double-stranded RNA corresponding to exons 2, 3, and 4 (as indicated in Fig. 8B) was injected into gonads of transgenic adult hermaphrodites expressing GFP under the regulation of the *C. elegans* homeobox CEH22 promoter. The CEH22 promoter is active in a subset of pharyngeal cells, allowing us to examine the differentiation of this organ in the broods of the injected worms (39). As a negative control, the CEH22-GFP transgenic adult hermaphrodites were injected with dsRNA corresponding to the cloned *spe-9* gene, which exhibits no detectable RNA interference phenotype (41). Injection of dsRNA corresponding to *ceNTF2* produced embryonic lethality among injected worms. After 24 h none of the NTF2

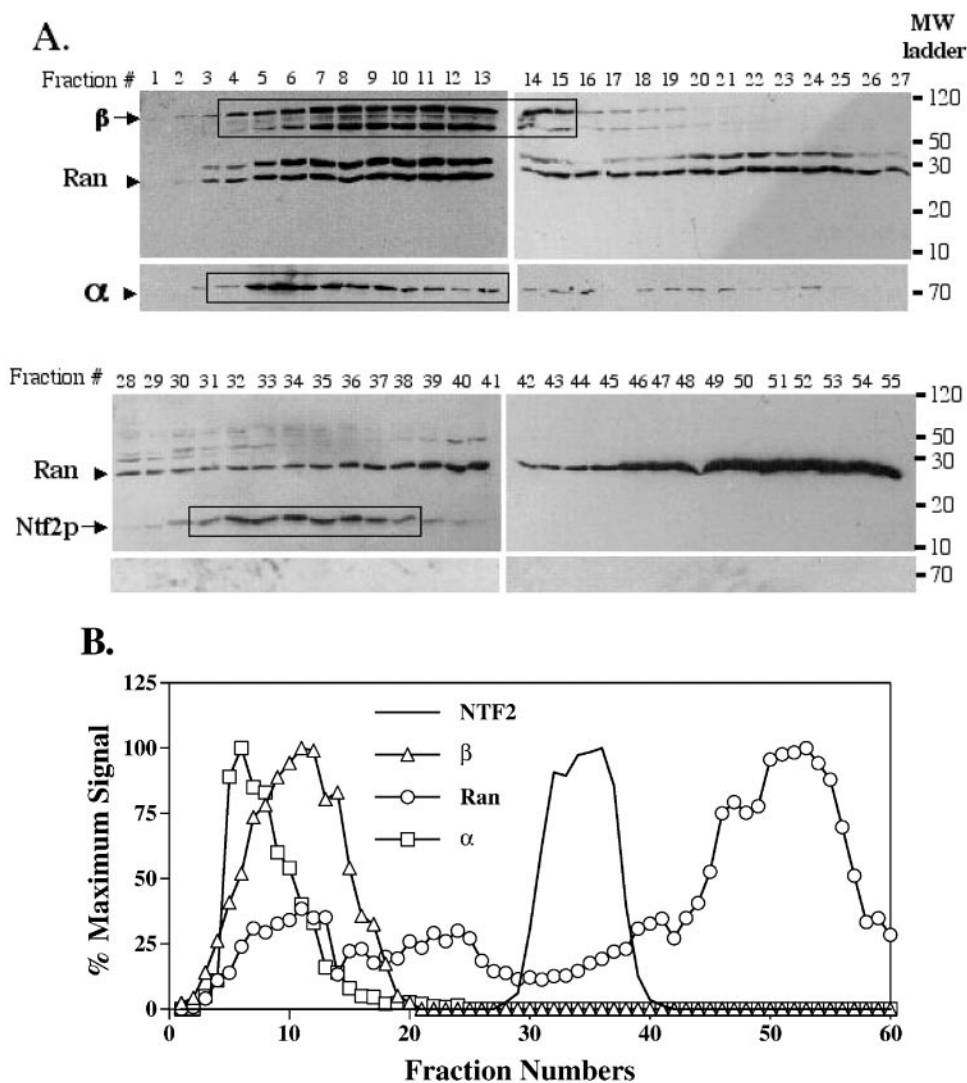


FIG. 7. Ntf2p copurifies with scRan but not importin- $\beta$  *in vivo*. A, 10 mg of total yeast protein was loaded onto an S-200 sizing column, and fractions were analyzed by SDS-PAGE. Proteins were detected by immunoblotting as described under "Materials and Methods." The importin- $\beta$ , importin- $\alpha$ , and Ntf2p peaks are boxed. B, the distribution of the proteins in A was plotted as fraction number *versus* maximal signal as determined by densitometric analysis of the films shown in A.

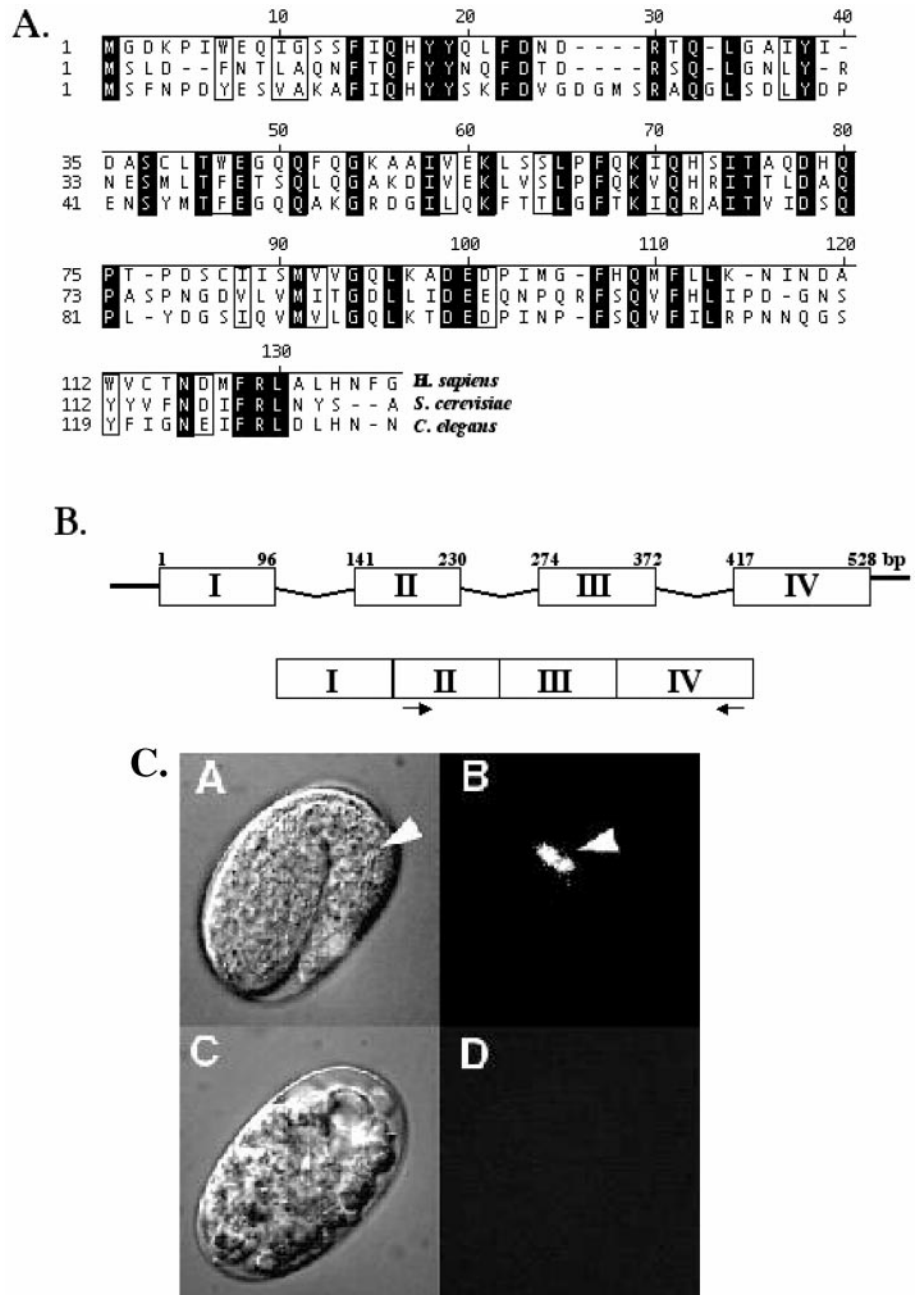
injected embryos had differentiated into the comma, 2-fold, or 3-fold structures associated with wild-type embryogenesis. Instead, the embryos arrested as a multicellular mass with no obvious structure. Control injected *spe-9* (41) eggs had all hatched after 12 h of incubation. As shown in Fig. 8C, all NTF2 injected embryos (>50 individual arrested embryos examined) lacked GFP fluorescence, unlike control *spe-9* injected embryos. These data suggest that proper pharyngeal differentiation did not occur, indicating that *ceNTF2* is an essential gene for early *C. elegans* embryogenesis and represents the first demonstration that NTF2 is required in higher eukaryotes.

#### DISCUSSION

*In vitro* studies show that NTF2 can act as a mediator of RanGDP import into the nucleus to replenish the nuclear stores of RanGTP (18, 19). More recently, it was shown that monoclonal antibodies to NTF2 injected into mammalian cells block the import of Ran into the nucleus (31). Two possible mechanisms for Ran import into the nucleus by NTF2 can be derived from these studies. NTF2 may import Ran into the nucleus in a large complex including importin- $\alpha/\beta$ /NLS cargo/Ran/NTF2 (Fig. 1, *model A*), or, alternatively, Ran import by NTF2 could be independent of importin- $\beta$ -mediated import of cargo (Fig. 1, *model B*). Here we use the budding yeast *S.*

*cerevisiae* to investigate the mechanism of Ran import into the nucleus by NTF2. We found that NTF2 is required to import Ran into the nucleus in *S. cerevisiae* and that this import is independent of importin- $\beta$ -mediated nuclear transport. This is consistent with the hypothesis that nucleocytoplasmic transport is driven by the Ran gradient. In this hypothesis, it would be necessary for the cell to set up and maintain the gradient prior to and independent of the onset of any cycles of import or export. Thus, NTF2-mediated import of Ran must be independent from other nucleocytoplasmic transport processes.

The two models presented in Fig. 1 focus on NTF2-mediated import of Ran into the nucleus. However, to complete the model, a mechanism for recycling NTF2 back to the cytoplasm for another round of RanGDP import should be incorporated. RanGTP exits the nucleus complexed with importin- $\beta$  proteins (3). Once in the cytoplasm RanGTP is converted to RanGDP when the cytoplasmically localized RanGAP stimulates the Ran GTPase. NTF2 is then required to re-import RanGDP into the nucleus. Therefore, there must be some mechanism for re-export of NTF2 to replenish the cytoplasmic pool. NTF2 is a small homodimeric protein (28 kDa) well below the predicted 60-kDa diffusion size of nuclear pores (3). Thus, NTF2 could move back through the pore by diffusion independent of any



**FIG. 8. NTF2 is essential in *C. elegans*.** *A*, amino acid sequence alignment of *Homo sapiens*, *S. cerevisiae*, and *C. elegans* NTF2 proteins. Identical residues are shaded in black, and conserved residues are boxed. *B*, schematic representation of the *C. elegans* NTF2 coding region. Boxes represent exons, and the arrows represent the primers used for polymerase chain reaction amplification of exons 2, 3, and 4 (see "Materials and Methods"). *C*, adult hermaphrodites with an integrated *ceh-22::GFP* gene were injected with dsRNA corresponding to the *spe-9* (panels *A* and *B*) or *ceNTF2* (panels *C* and *D*). Panel *A*, differential interference contrast image of *spe-9* control embryo at 4 h. Panel *B*, GFP fluorescence of *spe-9* control embryo as in panel *A*. Arrows indicate areas of pharyngeal morphogenesis. Panel *C*, differential interference contrast image of an arrested *ceNTF2* embryo at 24 h. Panel *D*, GFP fluorescence of *ceNTF2* embryo as in panel *C* showing a lack of detectable pharyngeal morphogenesis. Pictures shown are representative of >50 embryos observed.

other transport factors. Alternatively, it could be exported in a complex with a member of the importin- $\beta$  family of transport receptors. NTF2 has been shown to be concentrated at the NPC (our data and Refs. 18, 29, 50, and 51). However, in all cases this localization has been based on epitope-tagged NTF2 proteins that, in most cases, are not functional proteins. Recent *in vivo* localization of endogenous NTF2 in mammalian cells suggests that NTF2 is concentrated in the nucleus with no observed localization at the NPC (31) and that earlier reports of NTF2 concentrated at nuclear pores may represent a transient stage of NTF2 localization. In addition, isolation and analysis of the yeast NPC identified several members of the importin- $\beta$  family of proteins but not NTF2 or Ran (52), which is consistent with the rather weak  $1 \mu\text{M}$  binding constant calculated for the interaction between NTF2 and nucleoporins (53). Prior to these reports one could envision a model with NTF2 concentrated at the NPC where it bound to RanGDP on the cytoplasmic face of the pore, transported it through the pore, released Ran once exchange to RanGTP occurred, and traveled back through the

pore to repeat the cycle. This model is based on NPC localization of NTF2 and assumes that NTF2 remains tightly associated with the pore at all times. However, placing a tag on NTF2 may slow down the import/export process so that the tag itself skews the localization of NTF2 and slows its transit through the pore. If NTF2 were only transiently associated with the pore one might predict that recycling of NTF2 would require additional factors. Nuclear export signals are not well conserved (54) making it difficult to predict whether NTF2 contains a functional nuclear export signal. Further studies will be required to determine the mechanism of NTF2 export and complete the model for RanGDP import by NTF2.

If NTF2 is wholly responsible for importing Ran into the nucleus and maintaining the critical Ran gradient, then NTF2 would be predicted to be required in all organisms. The essential nature of NTF2 has only been demonstrated in the single cell eukaryote, *S. cerevisiae*. However, in many *in vitro* permeabilized import assays NTF2 is not absolutely required. This has led to the hypothesis that in higher eukaryotes NTF2 may



be a nonessential accessory factor. An alternative explanation is that the addition of excess Ran in these assays compensates for the lack of NTF2. This is supported by the observation that a deletion of the yeast *NTF2* gene can be suppressed by a mere 2-fold increase in scRan expression (55). To resolve this question, we show that NTF2 is essential in the multicellular organism, *C. elegans*, indicating, for the first time, that NTF2 is required in higher eukaryotes.

Ran constantly shuttles between the nucleus and the cytoplasm. However, most studies only observe the steady state localization of any given protein. Localization of shuttling proteins at steady state is a result of an equilibrium state where, for primarily nuclear proteins, there is a faster rate of import than export, and for proteins localized to the cytoplasm, there is a faster rate of export than import. In either case, some mechanism is required to increase the rate in one direction or the other. In the case of Ran, NTF2 determines the extent to which Ran is imported into the nucleus, thus controlling the dynamic compartmentalization of Ran and establishing the gradient, which is required for efficient nucleocytoplasmic transport.

Although we focus our experiments on classic importin- $\alpha/\beta$ -mediated NLS-protein import, our model of Ran import can be extended to other nucleocytoplasmic transport pathways. If the Ran gradient drives nuclear transport, then it would be predicted that this gradient must be established and maintained prior to any rounds of nuclear transport. Therefore, NTF2 must import Ran independent of all nucleocytoplasmic transport pathways. Here, we present experimental evidence for the independent import of Ran into the nucleus and provide a mechanistic understanding of the essential nature of the Ran import factor NTF2.

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