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FUNCTION AND BIOGENESIS:
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Nucleus by Nuclear Transport Factor 2

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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-β. 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-β-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),1 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-α/importin-β NLS receptor (3, 7–10). Once bound, the importin-α/importin-β receptor directs the entire complex to the NPC via interactions between importin-β 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 Ran gradient to the cytoplasm (20) and the RanGAP 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-β 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-β, 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-α/β/RanGDP/NTF2. Once inside the nucleus this complex is disassociated by the exchange of RanGDP to RanGTP by the nuclear RanGEF. In this model...
RanGDP and NTF2 would form a complex that includes importin-β. This model is consistent with in vitro binding assays demonstrating that NTF2 interacts directly with importin-β (27). However, in vitro binding assays have shown that importin-β 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-α/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-α/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-β-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-β. These results provide experimental evidence that the Ran/NTF2 import complex is separate from the importin-α/cargo complex, and that Ran compartmentalization in the nucleus is primarily controlled through NTF2-mediated import. Consistently, from 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-myctf2) 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 and show that NTF2 forms a complex containing Ran and nucleoporins but does not form a stable complex with importin-β.

**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-β 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-β) 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.

**Ntf2 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× tryptone-yeast extract medium containing 100 μg of ampicillin/ml and grown overnight at 30 °C. Ntf2p was isolated by thawing the cell pellet and resuspending in 25% sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 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 × g for 20 min and dialyzed overnight against 20 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM PMSF (NTF2 buffer A). The lysate was clarified at 40,000 × g for 30 min at 4 °C and used for gel filtration (see below) or centrifugation for 1 h. Residual active groups were blocked with 1 M Tris-HCl, pH 8.0, 2 mM MgCl₂, 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 NaHCO₃, 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 mM 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 mM 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 (YPED) 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 MgCl₂, and 0.5% Triton X-100) supplemented with protease inhibitors (0.5 mM PMSF and 3 μg 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 μl of either NTF2-Sepharose beads or bovine serum albumin (BSA)-Sepharose beads. Binding was carried out in PBSM (phosphate-buffered saline, 2.5 mM MgCl₂) at 4 °C for 1 h. Beads were then washed twice for 10 min in PBSM and one time for 10 min in PBSMT.
Bound proteins were eluted with 100 μl of sample buffer, and 5 μl was resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblotting.

**Immunoblot Analysis**—Immunoblot analysis was performed essentially as described (36). Importin-β was detected by incubation with a 1:1000 dilution of a rabbit polyclonal antibody against yeast importin-β95 (the generous gift of Dr. D. M. Koepp and Dr. P. Silver). Nap1p was detected by incubation with a 1:5000 dilution of a rabbit polyclonal antibody to Nap1p (the generous gift of Dr. M. Stewart). scRan was detected by incubation with a 1:10,000 dilution of a rabbit polyclonal antibody against yeast importin-β (the generous gift of Dr. G. Schlenstedt and Dr. P. Silver). Yrb1p was detected by incubation with a 1:1000 dilution of a rabbit polyclonal antibody against yeast Yrb1p (the generous gift of Dr. D. H. Wong and Dr. P. Silver). Ntf2p was detected by incubation with a 1:5000 dilution of a rabbit polyclonal antibody against Ntf2p (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. M. Stewart).

**Sizing Column and Sucrose Gradient**—The importin-β95GFP/myc-GSP1/XhoI oligonucleotides inserted into the TOPOI vector (Invitrogen) were amplified from a pAC46 yeast bacterial expression plasmid (pMW172) (45) and used as a sizing column and sucrose gradient.

**TABLE I**

| Strain or plasmid | Description |
|------------------|-------------|
| **Strains**      |             |
| ACY192           | MATa ura3-52 trp1Δ363 leu2Δ1 his3Δ200                           |
| ACY114           | MATa ura3-52 trp1Δ363 leu2Δ1 his3Δ200 NTF2Δ::His3 pAC626     |
| ACY212           | MATa ura3-52 trp1Δ363 leu2Δ1 his3Δ200 GSP1Δ::His3 GSP2Δ::His3 pAC629 (45) |
| ACY90            | MATa ura3-52 trp1Δ363 leu2Δ1 ade2-1 rna-l-1 (13)             |
| ACY109           | MATa ura3-52 trp1Δ363 leu2Δ1 prp20-1 (42)                     |
| ACY208           | MATa ura3-52 trp1Δ363 leu2Δ1 his3Δ200 ade2-1-RLS1Δ::His3 pAC710 (42) |
| BQY104           | MATa ura3-52 trp1Δ363 leu2Δ1 his3Δ200 GSP1Δ::His3 GSP2Δ::His3 RSL1::RSL1-GFP (importin-β95)-URA3 pAC627 |
| **Plasmids**     |             |
| pAC19            | 2μ, LEU2, GAL1-10, amp                                       |
| pAC26            | NTF2, CEN, URA3, amp                                         |
| pAC160           | myc-NTF2, CEN, LEU2, amp                                      |
| pAC78            | GSP1, CEN, TRP1, amp                                         |
| pAC267           | myc-GSP1, CEN, TRP1, amp                                      |
| pAC268           | yeast Ntf2 bacterial expression plasmid (pMW172) (45)        |
| pAC269           | GSP1, CEN, URA3, amp                                         |
| pAC45            | C-terminal GFP fusion plasmid                                 |
| pAC410           | 1.4-kiolbase coding region of GSP1 amplified with Xhol-Pst1 oligonucleotides inserted into Xhol-Pst1 sites of pAC45 |
| pAC611           | 1.0-kiolbase coding region of myc-NTF2 amplified with BamHI-HindIII oligonucleotides inserted into the TOPOI vector (Invitrogen) |
| pAC619           | 267 base pairs including exons 2, 3, and 4 of C. elegans NTF2 amplified from a C. elegans cDNA library with BamHI-HindIII oligonucleotides inserted into the TOPOI vector (Invitrogen) |
| pAC211           | rat Ntf2 bacterial expression vector (44)                    |
| pAC360           | rat Ntf2WTA bacterial expression vector (44)                  |
| pAC46            | ral1Δ, CEN, LEU2, amp                                         |
| pAC710           | RSL1, CEN, URA3, amp                                         |
| pAC709           | 780 base pairs coding region of NTF2 amplified with EcoRI-XhoI oligonucleotides inserted into EcoRI-XhoI sites of pAC45 |
| pAC697           | GFP-LacZ-NLS, URA3 (35)                                      |

Each animal was then transferred to a fresh growth plate and allowed to lay eggs for 8 h. Animals 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 Axioshot 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 NTF2Δ 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 in glucose 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-β Mutant, ral1-1**—If NTF2-mediated Ran import occurs in concert with importin-αβ/cargo, then Ran should not concentrate in the nucleus in either importin-α or importin-β mutants that are unable to support NLS cargo import. Because both Ran and NTF2 have been shown to interact with importin-β, we analyzed Ran localization in the importin-β temperature-sensitive mutant, ral1-1 (42). The ral1-1 allele of importin-β contains a nonsense mutation at amino acid 851 resulting in the loss of 10 amino acids from the C terminus. The Ral1-1 protein is unable to support NLS-mediated protein import. In addition, the
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Rsl1-1 protein does not efficiently interact with nucleoporins and mislocalizes to the cytoplasm. 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-β-mediated NLS cargo import and suggest that import of the NTF2/Ran complex is independent of the importin-α/NLS cargo complex as diagrammed in Fig. 1 (model B).

Yeast Ntf2p Interacts with scRan, Nucleoporins, and Importin-β in Vitro—To determine whether the NTF2/Ran complex interacts with importin-β biochemically, we performed bead binding assays using bacterially expressed yeast Ntf2p covalently coupled to CNBr-Sepharose beads. Purified recombinant importin-β 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-β bound nonspecifically to Ntf2p beads, we analyzed Ntf2p beads incubated with yeast cell lysates. Components of the lysate that bound to the beads were analyzed by SDS-PAGE. Immunoblots of these gels show that yeast Ntf2p forms complexes containing scRan, the XFG-containing nucleoporin Nps1p, and importin-β (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-β 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-β 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-β (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-β Is via Nucleoporins—In vitro studies have shown that importin-β 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-β because this interaction is mediated by nucleoporins. If so, the nucleotide bound state of scRan should affect the level of importin-β found in the NTF2 complex. 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-β 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). Immunobinding showed 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-β 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, GTPγS, to lock the available scRan into the GTP bound state, or GDPβS, to lock

FIG. 2. Ntf2p is required for scRan import into the nucleus in S. cerevisiae. pGAL1-10-myc-NTF2 (pAC611) was transformed into ACY114 (NTF2) 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 (lane 1) and myc-Ntf2p immunoblot analysis (B).

FIG. 3. Localization of Ran is independent of NLS-mediated protein import by importin-β. Cells expressing the importin-β temperature-sensitive allele, rsl1-1 (42), were transformed with plasmids encoding GFP-LacZ-NLS (pAC879), 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.
that bound recombinant Ntf2p were detected by immunoblotting with scRan antisera (A), importin-β antisera (B), Nsp1p antisera (C), or Yrb1p antisera (D). Total lysate was also analyzed by immunoblotting (lanes 1–3) for the presence of Ntf2p, scRan, and importin-β. Fractions containing Ntf2p were shown to contain scRan but not importin-β (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-α/importin-β complex was examined by analyzing fractions for the presence of importin-α. The majority of importin-α co-purifies with importin-β, demonstrating that the importin-αβ 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-β 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-αβ complex.

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Recombinant rat Ntf2 and rat Ntf2W7A were immobilized on CNBr-Sepharose beads and incubated with wild-type yeast (ACY192) cell lysate. Proteins bound to Ntf2p were identified by immunoblotting (lanes 1, 3, and 5) and rat Ntf2W7A (lanes 2, 4, and 6) were identified by immunoblotting with anti-scRan antibodies. 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-β (Fig. 6, compare lanes 5 and 6), providing further evidence that the interaction observed between Ntf2p and importin-β occurs via nucleoporins.

**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-β. Fractions containing Ntf2p were shown to contain scRan but not importin-β (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.
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-α/β/NLS cargo/Ran/NTF2 (Fig. 1, model A), or, alternatively, Ran import by NTF2 could be independent of importin-β-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-β-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 should be incorporated. RanGTP exits the nucleus complexed with importin-β 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...
other transport factors. Alternatively, it could be exported in a complex with a member of the importin-β 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-β family of proteins but not NTF2 or Ran (52), which is consistent with the rather weak 1 μM 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 the 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-\(\beta\)-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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