Nuclear Accumulation of the Small GTPase Gsp1p Depends on Nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p, and the Acetyl-CoA Carboxylase Acc1p*

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The small GTPase Ran/Gsp1p plays an essential role in nuclear trafficking of macromolecules, as Ran/Gsp1p regulates many transport processes across the nuclear pore complex (NPC). To determine the role of nucleoporins in the generation of the nucleocytoplasmic Gsp1p concentration gradient, mutations in various nucleoporin genes were analyzed in the yeast *Saccharomyces cerevisiae*. We show that the nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p, and the enzyme acetyl-CoA carboxylase (*MTR7*) control the distribution and cellular concentration of Gsp1p. At the restrictive temperature the reporter protein GFP-Gsp1p, which is too large to diffuse across the nuclear envelope, fails to concentrate in nuclei of *nup133Δ, rat2-1, nup85Δ, nic96ΔC*, and *mtr7-1* cells, demonstrating that GFP-Gsp1p nuclear import is deficient. In addition, the concentration of Gsp1p is severely reduced in mutants *nup133Δ* and *mtr7-1* under these conditions. We have now identified the molecular mechanisms that contribute to the dissipation of the Gsp1p concentration gradient in these mutants. Loss of the Gsp1p gradient in *nup133Δ* and *rat2-1* can be explained by reduced binding of the Gsp1p nuclear carrier Ntf2p to NPCs. Likewise, *nup85Δ* cells that mislocalize GFP-Gsp1p at the permissive as well as non-permissive temperature have a diminished association of Ntf2p-GFP with nuclear envelopes under both conditions. Moreover, under restrictive conditions Prp20p, the guanine nucleotide exchange factor for Gsp1p, mislocalizes to the cytoplasm in *nup85Δ, nic96ΔC*, and *mtr7-1* cells, thereby contributing to a collapse of the Gsp1p gradient. Taken together, components of the NPC subcomplex containing Rat2p/Nup120p, Nup133p, and Nup85p, in addition to proteins Nic96p and Mtr7p, are shown to be crucial for the formation of a nucleocytoplasmic Gsp1p gradient.

Transport of macromolecules across the nuclear envelope is mediated by nuclear pore complexes (NPCs) and requires soluble factors as well as nucleoporins, the components of the NPC (reviewed in Refs. 1 and 2). Although yeast NPCs are less complex than their mammalian counterparts (3), the mechanisms of nucleocytoplasmic trafficking are conserved among eukaryotes. The key component believed to control the directionality of transport between nucleus and cytoplasm is the small GTPase Ran in higher eukaryotes or its yeast homolog Gsp1p (reviewed in Refs. 1, 2, and 4). Under normal growth conditions cells display a Ran/Gsp1p concentration gradient, with high levels in the nucleus and low concentration in the cytoplasm. Ran/Gsp1p occurs in two different forms in the cell, predominantly bound to GTP in the nucleus or associated with GDP in the cytoplasm. The different nucleotide-bound states are generated by the unequal distribution of GTPase modulating factors; the GTPase-activating protein Rna1p (or the higher eukaryotic homolog RanGAP1) is preferentially located in the cytoplasm, where it generates Gsp1p-GDP. In contrast, the GTP/GDP exchange factor Prp20p (RCC1 in higher eukaryotes) is concentrated in the nucleus, where it binds to chromatin (5). Prp20p catalyzes the production of nuclear Gsp1p-GTP, which is believed to associate with nuclear anchors. The association with anchors would then prevent Gsp1p diffusion into the cytoplasm and help maintain high Gsp1p concentrations in the nucleus. Members of the β-importin family of transporters and other factors may retain Ran/Gsp1p in the nucleus (6, 7). Furthermore, Mog1p, a nuclear binding protein for Gsp1p-GTP, is required for the generation or maintenance of a nucleocytoplasmic Gsp1p gradient (7). It is believed that the asymmetric distribution of Ran/Gsp1p-GTP and Ran/Gsp1p-GDP fuels protein import as well as nuclear export of proteins and some RNAs.

In higher eukaryotes, translocation of Ran across the nuclear envelope requires Ntf2, a small protein that specifically binds to Ran-GDP (6, 8, 9). Ntf2 transports Ran-GDP into the nucleus where RCC1 converts it to Ran-GTP, thereby dissociating the Ran-Ntf2 interaction. The nucleoporin p62 is likely to play an important role for the translocation of Ran across the NPC, as both Ran and Ntf2 bind to p62. In particular, the FXF repeats present in the N-terminal domain of p62 are involved in these binding reactions (10). Similarly, the yeast nucleoporin Nsp1p, a functional homolog of p62, binds to Ran and Ntf2 (10). As well, the repeat domain of Nsp1p interacts with Gsp1p in vitro (11), indicating that the interactions Ran-p62 and Nsp1p-Gsp1p have been conserved and Gsp1p accumulates in yeast nuclei by the same mechanism as in metazoan cells (6, 8, 9). This idea is further supported by our recent results, which

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§ The abbreviations used are: NPC, nuclear pore complex; DAPI, 4′,6-diamidino-2-phenylindole; Nup, nucleoporin; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; GFP, green fluorescent protein.
show that mutations in the yeast NTF2 gene interfere with Gsp1p nuclear accumulation (7).

In addition to soluble factors, nuclear trafficking requires nucleoporins. With about 30 different nucleoporins, the NPC overall organization in yeast is less complex but similar to higher eukaryotes (3, 12). Various yeast nucleoporins have been shown to be involved in nuclear transport of proteins, RNA, or both types of macromolecules. For instance, Nup133p and Nsp1p play a role in protein import as well as mRNA export (13–18). For example, mutants nsp1-5 and nsp1-W644C (coil 2) fail to accumulate GFP-Nsp1p in nuclei at the non-permissive temperature. Likewise, nsp1-5 and nsp1-ala6 (coil 2) are impaired in export of the 60 S ribosomal subunit (24, 31). Furthermore, mutant Nsp1p fails to incorporate into biochemically distinct protein complexes (12, 18, 19, 22, 23, 27, 28).

### Table I

| Strain      | Genotype                  | Phenotype                        | Ref. |
|-------------|---------------------------|----------------------------------|------|
| RS453       | MAT a ade2 his3 leu2 trp1 ura3 | Wildtype                         | 16   |
| nsp1-133Δ   | MAT a ade2 his3 leu2 trp1 ura3 nsp1-133::HIS3 | NPC clusters, mRNA export | 13   |
| CCY292      | MAT a ade2 his3 leu2 trp1 ura3 nsp84::HIS3 | NPC clusters, mRNA export | 21   |
| nsp84–1    | ade2 his3 leu2 trp1 ura3 nsp84::HIS3 | NE organization | 34   |
| nsp85Δ      | ade2 his3 leu2 trp1 ura3 nsp85::HIS3 | mRNA export, NPC biogenesis | 34   |
| T019        | MAT a ade2 leu2 ura3 lys2 met7–1 | mRNA export | 45   |
| nsp1-C      | MAT a ade2 his3 leu2 trp1 ura3 nsp1::HIS3 | Protein import | 18   |
| nsp1–5      | MAT a ade2 his3 leu2 trp1 ura3 nsp1::HIS3 | Protein import 60 S SU export | 14, 31 |
| nsp1-ala6   | ade2 his3 leu2 trp1 ura3 nsp1::HIS3 (pSB32-LEU2-nsp1-ala6) | Protein import 60 S SU export | 26   |
| nsp1-L640S  | MAT a ade2 his3 leu2 trp1 ura3 nsp1::HIS3 (pSB32-LEU2-nsp1-L640S) | mRNA export | 31   |
| nsp1-ts18   | MAT a ade2 his3 leu2 trp1 ura3 nsp1::HIS3 (pUN100-LEU2-ProT-A-nsp1-ts18) | Protein import | 18   |
| nsp1-W644C  | MAT a ade2 his3 leu2 trp1 ura3 nsp1::HIS3 (pSB32-LEU2-nsp1-W644C) | Protein import | 18   |
| nsp1-L697P  | MAT a ade2 his3 leu2 trp1 ura3 nsp1::HIS3 (pSB32-LEU2-nsp1-L697P) | Protein import | 16   |
| nic96       | MAT a ade2 his3 leu2 trp1 ura3 nic96-6::HIS3 (pUN100-LEU2-nic96-6) | Protein import | 16   |
| nic96ΔN     | MAT a ade2 his3 leu2 trp1 ura3 nic96-6::HIS3 (pUN100-LEU2-nic96-6ΔN) | Protein import | 16   |
| nic96–1     | MAT a ade2 his3 leu2 trp1 ura3 nic96-6::HIS3 (pUN100-LEU2-nic96-6–1) | Protein import, 60 S SU export | 16   |
| nic96ΔC     | MAT a ade2 his3 leu2 trp1 ura3 nic96-6::HIS3 (pUN100-LEU2-nic96-6ΔC) | Protein import, NPC formation | 20   |
| rat7–1      | MAT a his3 leu2 ura3 | mRNA export | 20   |

* U. Nehrbass and E. C. Hurt, unpublished data.

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The markers of yeast strains and their previously described phenotypes with respect to nuclear trafficking are listed. NE, nuclear envelope.

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envelope organization (34, 35). In addition, mutant alleles of RAT2/NUP120 or deletions of nucleoporin genes like NUP133 induce clustering of NPCs (13, 21, 36, 38). However, cluster formation does not correlate with transport defects, and both mutants show clustering under permissive conditions (13, 21). Mutations in Nup133p are synthetic lethal with nup85, rat2/nup120, and nsp1 (see Ref. 39 and references therein), and hybrid genomes have identified Nup84p as a component that interacts with Nup133p (40). Furthermore, Nup133p associates with the Nup84p complex in vitro and in vivo (41, 42).

Previous studies have revealed that several nucleoporins, in particular Nsp1p, participate in nuclear trafficking. This prompted us to determine their role in the nuclear accumulation of Gsp1p, a GTPase implicated in various nuclear transport reactions. Although the interaction of Gsp1p/Ran and Nt2 with FXF repeats of nucleoporins is well established, other components of the NPC involved in Gsp1p nuclear transport have yet to be defined. For instance, zinc finger-containing nucleoporins that bind Ran-GDP in mammalian cells have not been found in *Saccharomyces cerevisiae* (reviewed in Ref. 4).

We have now identified several non-repeat yeast nucleoporins that play a role in the nuclear accumulation of Gsp1p, thereby regulating trafficking across the NPC.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Yeast strains used in this study are listed in Table I. Cells growing logarithmically in YEPD at room temperature were exposed to 37 °C for the times indicated in the figure legends. Standard procedures were used for yeast transformation and selection of transformants.

**Plasmids**—To monitor classical nuclear transport, the gene encoding SV40-GFP (43) was transferred into a centromeric plasmid carrying the *URA3* marker. Expression of SV40-GFP is controlled by the ADH1 promoter. Nuclear accumulation of SV40-GFP requires classical nuclear import to be constitutively active as described previously (7). A centromeric plasmid carrying the GFP-GSP1 gene and a *LEU2* marker was kindly provided by D. Lau and E. Hurt (Heidelberg, Germany). To allow expression in mutant strains that are *Leu* −, we have transferred the ADH1 promoter and the GFP-GSP1 coding sequence into a centromeric plasmid containing the GAL1 promoter and the *URA3* or *LEU2* marker. Expression of NTF2-GFP was induced by overnight growth at room temperature in selective medium supplemented with 2% galactose.

**Generation and Affinity Purification of Antibodies**—His11-tagged Gsp1p was affinity-purified to raise antibodies in mice (7). Polyclonal antibodies were generated against purified His6-tagged Rna1p essentially as described (7). For immunofluorescence studies antibodies were preadsorbed to the His6-tag and affinity-purified with immobilized His6-Rna1p.

**Fluorescence Microscopy**—Fixing of yeast cells, generation of spheroplasts, and incubation with various antibodies have been described previously (7). Affinity-purified secondary antibodies (Jackson ImmunoResearch, West Grove, PA; Molecular Probes, Eugene, OR) were preadsorbed to the His6-tag and affinity-purified with immobilized His6-Rna1p.

**RESULTS**

**Heat Stress Transiently Collapses the Nucleocytoplasmic Gradient of Gsp1p**—We have demonstrated recently that heat stress affects the Gsp1p concentration gradient across the nuclear envelope. Heat shock at 37 °C for 10 min transiently increases the cytoplasmic concentration of Gsp1p (Fig. 1D and Ref. 7). However, as cells adapt to heat, the Gsp1p gradient is reestablished in wild type cells, and Gsp1p again becomes concentrated in nuclei. In various wild type strains the Gsp1p gradient was rebuilt after about 1 h of exposure to 37 °C and remained stable when cells were kept at 37 °C (Fig. 1F, I, H, J, and L). When wild type cells were incubated in the presence of cycloheximide for 6 h, the Gsp1p gradient collapsed, even at room temperature (Fig. 1L). With the assay described in Fig. 1, we have shown previously (7) that mutations in NTF2, PRP20, and MOG1 alter the nucleocytoplasmic distribution of Gsp1p. We have now used this protocol to determine whether mutations in nucleoporins or the *MTR7* gene, which is involved in
nuclear envelope organization, affect the Gsp1p gradient formation when cells are treated with heat. As long periods of exposure to non-permissive conditions may have secondary effects on the Gsp1p gradient, we have exposed cells for a maximum of 6 h to elevated temperatures. Therefore, our studies have identified only mutants that display a rapid collapse of the Gsp1p concentration gradient under non-permissive conditions.

The Nucleoporin Mutants Nup133Δ, rat2-1, and nup85Δ Mislocalize Gsp1p upon Heat Stress—When the clustering strain nup133Δ was incubated for 3 h at 37 °C, the non-permissive temperature, cytoplasmic levels of Gsp1p increased as compared with cells kept at room temperature (Table II). Moreover, after 6 h at 37 °C the Gsp1p concentration gradient had collapsed in most cells (Fig. 2D and Table II). Likewise, in the clustering mutant rat2-1 the Gsp1p nucleocytoplasmic gradient dissipated at the restrictive temperature (Fig. 2H). Double immunofluorescence labeling with antibodies against nucleoporins and Gsp1p demonstrated that the GTPase associated with NPC clusters in both mutants under permissive conditions (data not shown). To characterize further the defect of nup133Δ and rat2-1 in maintaining a nucleocytoplasmic gradient of Gsp1p at 37 °C, we compared levels of the GTPase in control and heat-treated cells by Western blot analysis. When equal amounts of protein from unstressed and heat-shocked cells were analyzed in parallel, Gsp1p levels were reduced in nup133Δ, but no drastic changes were observed for rat2-1 (Table II and Fig. 3).

Additional members of the Nup84p-Nup133p NPC module were studied for Gsp1p distribution. As such, 6 h of heat exposure abolished the gradient formation in nup85Δ cells but did not alter the Gsp1p concentration gradient in nup84Δ cells, which carry a complete disruption of the NUP84 gene (Fig. 2 and Table II). Although the deletion of NUP85 prevented Gsp1p gradient formation at elevated temperatures, concentrations of the GTPase were similar to unstressed cells (Fig. 3).

Taken together, our results demonstrate that three members of the Nup84p-Nup133p NPC subcomplex which in vitro are essential for its assembly are involved in concentrating Gsp1p in nuclei of stressed cells.

The Nucleocytoplasmic Gradient of Gsp1p Collapses in the mRNA Transport Mutant mtr7-1 under Restrictive Conditions—Yeast cells carrying the mtr7-1 mutation, also called acc1-7-1, fail to synthesize very long chain fatty acids under non-permissive conditions. At the restrictive temperature NPCs appear as “spots,” and increased cytoplasmic concentrations of nucleoporins are detected (data not shown and see Ref. 45). Furthermore, the integrity of the nuclear envelope and nuclear export of mRNAs are defective at 37 °C (45). Thus, the mtr7-1 allele has a more general effect on nuclear envelope organization that could also alter nucleocytoplasmic trafficking and retention of Gsp1p. In line with this idea, we found elevated cytoplasmic concentrations of the GTPase upon shift to 37 °C for 6 h (Table II). After 6 h at 37 °C, Gsp1p was no longer accumulated in nuclei. Furthermore, the total concentration of the GTPase was reduced (Fig. 3).

Mutations in the Essential C-terminal Domain of NSP1 Have Only Minor Effects on the Distribution of Gsp1p—We have analyzed the role of several NSP1 mutations (see Introduction) in establishing a Gsp1p concentration gradient. Despite pronounced consequences for nuclear transport, mutations in the essential C-terminal domain of NSp1 had less severe effects on Gsp1p distribution and concentration (Table III). For instance, at room temperature nsp1-5 displayed a poor nucleocytoplasmic Gsp1p gradient, with elevated cytoplasmic levels of Gsp1p (Fig. 4B). Upon exposure to heat, however, Gsp1p gradients improved, and the GTPase became restricted to nuclei (Fig. 4D and Table II). One possible interpretation of these results is the preferential degradation of Gsp1p in the cytoplasm in response to heat stress. Indeed, when equal amounts of protein from unstressed and stressed cells were analyzed by Western blotting, the concentration of Gsp1p was found to be slightly decreased at 37 °C (Fig. 3 and Table II).

Improved nucleocytoplasmic Gsp1p gradients at the non-permissive temperature were also detected in other strains mutated in different coils of the C-terminal Nsp1p domain, i.e. mutants nsp1-ala6, L640S, ts18, or W644C. By contrast, Gsp1p protein levels did not change noticeably at 37 °C in these strains (Table II). Taken together, mutations in different coils of the C-terminal segment, which have strong defects in nuclear trafficking, did not drastically alter the concentration gradient of Gsp1p or levels of the GTPase.

Deletion of the C-terminal NIC96 Domain Changes the Distribution of Gsp1p—We tested the distribution of Gsp1p

### Table II

| Nup Allele | Localization upon heat shock | Protein levels upon heat shock |
|------------|-----------------------------|-----------------------------|
|            | 3 h 37 °C | 6 h 37 °C | 3 h 37 °C | 6 h 37 °C |
| Wild type | N>C | N>C | No change | No change |
| NUP133 nup133Δ | N>C, (N>C) | N>C | Increased | Reduced |
| RAT2 rat2-1 | N>C, (N>C) | N>C | No change | No change |
| NUP84 nup84Δ | N>C, (N>C) | N>C | No change | No change |
| NUP85 nup85Δ | N>C, (N>C) | N>C | Reduced | Reduced |
| MTR7 mtr7-1 | N>C | N>C | No change | Slightly reduced |
| NSP1 nsp1-C | N>C | N>C | No change | No change |
| NSP1 nsp1-5 | N>C, (N>C) | N>C | No change | Slightly reduced |
| NSP1 nsp1-ala6 | N>C, (N>C) | N>C | No change | No change |
| NSP1 nsp1-L640S | N>C, (N>C) | N>C | No change | No change |
| NSP1 nsp1-ts18 | N>C | N>C, (N>C) | No change | No change |
| NSP1 nsp1-W644C | N>C | N>C, (N>C) | No change | No change |
| NSP1 nsp1-L697P | N>C | N>C, (N>C) | No change | Increased |
| NIC96 Wild type | N>C | N>C | No change | No change |
| NIC96 nic96 ΔN | N>C | N>C, (N>C) | No change | No change |
| NIC96 nic96-1 | N>C | N>C, (N>C) | No change | No change |
| NIC96 nic96 ΔC | N>C, (N>C) | N>C, (N>C) | Slightly reduced | Slightly reduced |
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Fig. 3. Western blots with anti-Gsp1p for unstressed controls and heat-stressed cells. Strains shown in the figure were kept for 6 h at room temperature (RT) or at 37°C. Equal amounts of protein from unstressed and stressed cells were analyzed side-by-side by Western blotting with antibodies against Gsp1p.

Fig. 2. Distribution of Gsp1p in the clustering mutants nup133Δ and rat2-1, nup85Δ, mtr7-1, and nup84Δ. Yeast cells carrying a deletion of NUP133 (A–D), the mutation rat2-1 (E–H), a partial disruption of NUP85 (I–L), the mtr7-1 (M–P) allele, or nup84Δ (Q–T) cells were incubated at room temperature (RT) or for 6 h at 37°C. Gsp1p was localized by indirect immunofluorescence. Nuclei were visualized with DAPI.

In different nic96p mutants (see Introduction), with nic96ΔN missing residues 28–63, nic96-1 carrying the mutations L260P and P332L, and nic96ΔC lacking residues 532–839 of the C-terminal domain. All of the mutants mislocalized Gsp1p after prolonged heat treatment, but nic96ΔC showed the most severe effect (Fig. 4L). By contrast, levels of Gsp1p were not drastically altered in any of the nic96 mutants under restrictive conditions (Fig. 3 and Table II). In summary, our results show that the C-terminal domain of Nic96p plays a critical role in generating or maintaining a high concentration of Gsp1p in nuclei.

Effect of Mutant Nucleoporins on Classical Nuclear Protein Import—To determine whether classical nuclear protein import is altered in nucleoporin mutants under the conditions used for our experiments, we have introduced the fluorescent reporter protein SV40-GFP. To concentrate SV40-GFP in nuclei, classical nuclear protein import has to be constitutively active, and the inhibition of import can be monitored by the appearance of SV40-GFP in the cytoplasm (7). At room temperature, all strains accumulated SV40-GFP in nuclei, although some cytoplasmic localization was detected for several of the mutant strains (summarized in Table III). Upon exposure to 37°C, wild type cells adapted, and the reporter protein was concentrated in nuclei when cells were inspected after 3 and 6 h of heat treatment (Table III). By contrast, cells carrying the mutations nup133Δ, rat2-1, mtr7-1, nsp1-1, nsp1-ala6, nsp1-ts18, nic96ΔC, nup84Δ, and nup85Δ failed to accumulate SV40-GFP in nuclei after heat exposure. However, this is not a general defect in nucleoporin mutants. For instance, rat7-1, a mutant impaired in mRNA export (20), did not show a defect in classical nuclear import when incubated for 6 h at 37°C (Table III and Supplemental Material Fig. 1). Based on the Gsp1p distribution upon incubation at 37°C (see above), we have assigned the classical transport mutants to two different groups: group A, cells for which the Gsp1p gradient collapsed in response to heat stress (this includes nup133Δ, rat2-1, mtr7-1, nic96ΔC, and nup85Δ); group B, several nsp1 mutants and nup84Δ display an intact Gsp1p gradient after 6 h at 37°C. Defects in classical nuclear protein transport for members of group A can be explained by the failure of cells to build a nucleocytoplasmic gradient of Gsp1p. By contrast, different mechanisms of import inhibition operate for mutants in group B. In the following, we have further characterized members of group A.

Import of Gsp1p in Mutant Nucleoporin Strains—With a molecular mass of ~25 kDa, Gsp1p is small enough to diffuse in and out of the nucleus. Once diffused through the NPC, retention could concentrate Gsp1p in nuclei. However, transport of Gsp1p across the nuclear envelope could also result in nuclear accumulation of the GTPase. In the second scenario, elevated levels of Gsp1p in the cytoplasm will indicate a defect in Gsp1p nuclear import. To determine whether Gsp1p nuclear import plays a role in mutants of group A, we have monitored the nuclear accumulation of GFP-Gsp1p, a protein of ~52 kDa,
which is too large to diffuse efficiently across NPCs. To this end, cells synthesizing the reporter protein were incubated at room temperature or 37 °C (Fig. 5 and Table III), and the fusion protein was subsequently localized by fluorescence microscopy. In wild type cells, GFP-Gsp1p accumulated in nuclei of unstressed and stressed cells, and the same was observed for several of the mutant strains such as nsp1-5 (Fig. 5). By contrast, strains nup133Δ, rat2-1, nsp96ΔC, and mtr7-1 showed increased amounts of GFP-Gsp1p in the cytoplasm upon exposure to heat. Interestingly, nup133Δ and mtr7-1 also had elevated levels of GFP-Gsp1p in the cytoplasm at the permissive temperature (Fig. 5 and Table III), suggesting defects in Gsp1p nuclear transport even at room temperature. Likewise, in mutant nup85Δ cells GFP-Gsp1p was only slightly accumulated in nuclei when cells were grown at room temperature. Exposure of nup85Δ to heat stress did not drastically change the GFP-Gsp1p distribution (Fig. 5 and Table III).

An alternative model for Gsp1p mislocalization in mutant cells would predict that the failure to retain the GTPase in nuclei increased Gsp1p cytoplasmic concentrations. Augmented cytoplasmic GTPase levels would then simply reflect Gsp1p release from nuclei into the cytoplasm. This redistribution should be independent of de novo Gsp1p synthesis. We attempted to test this possibility by incubating cells at the non-permissive temperature in the presence of the protein synthesis inhibitor cycloheximide. Although incubation with cycloheximide for up to 3 h does not collapse the Gsp1p gradient (7), 6 h of exposure to cycloheximide abolished Gsp1p gradient formation, even in wild type cells (Fig. 1f). We were therefore unable to determine whether nuclear retention defects contribute to changes in the Gsp1p localization when cells were kept for 6 h at non-permissive conditions. Nevertheless, we have clearly shown that several of the mutants studied are deficient in Gsp1p nuclear import (see above).

Nuclear Envelope Association of Ntf2p-GFP Is Altered in Several Nucleoporin Mutants—A possible explanation for the elevated cytoplasmic levels of GFP-Gsp1p under non-permissive conditions could be a failure of Ntf2p, the nuclear carrier for Gsp1p, to properly interact with NPCs. We have addressed this question with Ntf2p-GFP, a reporter protein that concentrates at the nuclear rim in wild type cells at room temperature. A similar distribution is also observed when wild type cells have been incubated at 37 °C for 6 h (Fig. 6A). Although

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Localization of Gsp1p in cells carrying a mutant allele of NSP1 or NIC96. Yeast strains nsp1-5 (A–D), NIC96 (E–H), and nic96ΔC (I–L) were grown at room temperature (RT) or for 6 h at 37 °C as indicated. Gsp1p was located by indirect immunofluorescence and nuclei were stained with DAPI.
Cells kept for 6 h at room temperature (37°C) were fixed and stained with DAPI as described under "nsp1-5" (Wild type yeast, mutants A–D nup133 and strains that mislocalize Gsp1p under restrictive conditions.

nup133 mutant strains to clusters (Fig. 6, exposure to heat significantly reduced Ntf2p-GFP localization of Ntf2p-GFP with NPC clusters under permissive conditions, and Ran1p. In summary, our studies have identified several nucleoporin genes or in nup84. Even though nup84 cells showed reduced binding of Ntf2p-GFP to the nuclear envelope under normal and stress conditions, the difference to wild type cells or between control and heat-treated nup84 cells was not statistically significant.

The Guanine Nucleotide Exchange Factor Prp20p Mislocalizes to the Cytoplasm in nup85Δ, mtr7-1, and nic96ΔC—Collapse of the Gsp1p gradient may also be caused by the mislocalization of Gsp1p interacting factors, i.e. the guanine nucleotide exchange factor Prp20p or the GTPase-activating protein Ran1p. Under normal growth conditions Prp20p is concentrated in nuclei, where it is essential to generate Gsp1p-GTP. By contrast, most of Ran1p resides in the cytoplasm, although nuclear pools of Ran1p have been detected (46). When analyzed by immunofluorescence, Prp20p was concentrated in nuclei of wild type and mutant cells at room temperature (Fig. 7). However, upon heat treatment a significant redistribution of Prp20p is seen in nup85Δ, mtr7-1, and nic96ΔC. Prp20p mislocalization was most prominent in mtr7-1, and after 3 h of heat stress less than 35% of the cells had Prp20p concentrated in nuclei. Moreover, elevated cytoplasmic levels of Prp20p were detected even after 1 h of incubation at 37°C. A difference in Prp20p distribution was also detected for rat2-1, but the effect was less significant for rat2-1 (p = 0.02) as compared with nup85Δ, mtr7-1, and nic96ΔC (p < 0.01).

We have also determined the distribution of Ran1p in wild type and mutant cells under different growth conditions. As reported by others (46) most of Ran1p was cytoplasmic, and the association with nuclei became increased upon heat shock (data not shown). However, we did not detect drastic differences between wild type and mutant cells in the localization of Ran1p. In summary, our studies have identified several nucleoporin mutants in which a collapse of the Gsp1p concentration gradient under stress conditions can be attributed, at least in part, to a mislocalization of Prp20p.

**DISCUSSION**

Gsp1p/Ran is an essential protein that plays a pivotal role in nucleocytoplasmic trafficking of macromolecules (1, 2, 4, 47). As such, conditions that prevent the formation of a Gsp1p/Ran gradient across the nuclear envelope interfere with classical nuclear transport in yeast and in higher eukaryotes (7, 48). Nuclear accumulation of Gsp1p/Ran depends on the Gsp1p/Ran-GDP-binding protein Ntf2p which imports the GTPase into the nucleus, possibly followed by nuclear retention (6, 8). During passage through the NPC, Gsp1p and Ntf2p are believed to interact with nucleoporins. In support of this idea, FXF repeats of nucleoporins such as yeast Nsp1p or mammalian p62 bind Ntf2p and Gsp1p in vitro (10). By contrast, we show that mutations in the C-terminal essential domain of Nsp1p do not cause mislocalization of Gsp1p under restrictive conditions.

To identify novel components of the nuclear envelope that are involved in Gsp1p nuclear accumulation, we have analyzed yeast strains that carry a deletion or mutation in various nucleoporin genes or in MTR7. So far, components of the nuclear envelope other than FG repeat-containing nucleoporins have not been shown to participate in Gsp1p trafficking. Our results demonstrate for the first time that the non-repeat nucleoporins Nup133p, Nup120p/Rat2p, Nup85p, and Nic96p, as well as acetyl-CoA carboxylase regulate the distribution of the small GTPase.

To define the mechanisms that lead to the collapse of the Gsp1p gradient in mutant strains, we have determined whether nuclear import of the fusion protein GFP-Gsp1p is compromised under non-permissive conditions. GFP-Gsp1p is too large to diffuse efficiently across the NPC, and elevated levels of this reporter protein in the cytoplasm suggest that
nuclear import is impaired in nup133Δ, rat2-1, nup85Δ, nic96ΔC, and mtr7-1. Interestingly, nup133Δ, rat2-1, nup85Δ, and mtr7-1 showed elevated cytoplasmic levels of GFP-Gsp1p even at room temperature, suggesting that import is already affected under these conditions.

Nup84p, Nup85p, and Nup120p/Rat2p are present in the same NPC subcomplex, called the Nup84p complex, which also contains Nup145p-C, Seh1p, and Sec13p (35). Furthermore, Nup133p associates with the Nup84p complex, indicating that the Nup84p-Nup133p unit represents a building block of the NPC (41, 42). To assemble the Nup84p module Nup85p, Nup120p/Rat2p and Nup145p-C are required, whereas Nup84p and Seh1p are dispensable (35). Deletion of NUP84, NUP85, or NUP120/RAT2 changes nuclear membrane and NPC organization (34, 35). In particular, a complete disruption of NUP84 in nup84Δ cells leads to an altered distribution of NPCs (34). Despite the effects of the nup84Δ allele on nuclear envelope and NPC assembly, Gsp1p gradients did not collapse in this mutant at the non-permissive temperature. These results emphasize that the changes in Gsp1p localization observed by us for several nucleoporin mutants cannot simply be ascribed to altered nuclear organization. In contrast to nup84Δ cells, Gsp1p gradients collapsed in strains nup85Δ, rat2-1, and nup133Δ under restrictive conditions, which points to a specific role of Nup85p, Nup120p/Rat2p, and Nup133p in nuclear accumulation of the GTPase. We have now demonstrated that the defect in rat2-1 and nup133Δ cells can be attributed to the inefficient association of Ntf2p with NPCs upon exposure to heat stress. However, Nup133p is not essential for binding Ntf2p to the NPC under non-stress conditions, demonstrating the presence of redundant binding sites. Nevertheless, failure of Ntf2p to interact with mutant NPCs in stressed cells will ultimately dissipate the Gsp1p gradient, as Ntf2p is required for Gsp1p nuclear import. In contrast to rat2-1 and nup133Δ mutants, nup85Δ, missing another component of the Nup84p complex, displayed reduced Ntf2p-GFP binding even at room temperature. This is in line with the observation that GFP-Gsp1p in nup85Δ cells already mislocalized at room temperature, similar to what was detected at 37 °C. Furthermore, Prp20p, the guanine nucleotide exchange factor for Gsp1p, redistributed in nup85Δ at elevated temperature (see below). Thus we have identified multiple defects for this mutant that will contribute to the collapse of the Gsp1p gradient (summarized in Table IV).

We have also carried out experiments to further analyze the interaction between Ntf2p and Nup133p. In line with the idea that Ntf2p forms complexes with this nucleoporin, we were able to co-purify both components (not shown). However, results for this co-purification were variable, most likely reflecting the transient nature of this interaction.

Even though nuclear export of the ribosomal 60 S subunit depends on the Gsp1p-GTPase cycle, members of the Nup84p complex did not show a major defect in this export reaction (24). These apparent differences can be explained by the distinct conditions used in our analyses. To study export of the 60 S ribosomal subunit, cells were exposed to 33 °C, followed by a 4-h shift to 20 °C. It is presently not known whether the Gsp1p concentration gradient dissipates at 33 °C. Furthermore, it is possible that a collapsed Gsp1p concentration gradient can be
The nuclear accumulation of GFP-Gsp1p, association of Ntf2p-GFP with NPCs, and nuclear concentration of Prp20p is compared in mutants that collapse the Gsp1p gradient when incubated for 6 h at 37 °C. NE, nuclear envelope association. See “Results” and “Discussion” for details. RT, room temperature.

| Strain          | GFP-Gsp1p | Ntf2p-GFP | Prp20p |
|-----------------|-----------|-----------|--------|
| Wild type       | N>C       | NE        | N>C    |
| nup133Δ         | N≤C, N=C  | N=C       | N>C    |
| rat2-1          | N=C       | N=C       | N>C    |
| mtr7-1          | N=C       | N=C       | N>C    |
| nic96ΔC         | N=C       | N=C       | N>C    |

rebuilt when cells are returned to 20 °C.

Our data clearly demonstrate a deficiency of rat2-1 cells in the formation of a Gsp1p gradient and of Ntf2p binding to the NPC under non-permissive conditions. Moreover, a weak defect was also detected for the nuclear concentration of Prp20p upon exposure to heat. However, it should be noted that previous studies of rat2-1 did not reveal a nuclear protein import defect (21). In these analyses nuclear import was monitored with a reporter protein containing the N-terminal 33 amino acid residues of yeast histone H2B fused to β-galactosidase (21). Yeast histones H2A and H2B were recently shown to be imported into nuclei by several members of the β-importin family, including Kap114p, Kap121p, and Kap95p (49). These β-importins directly bind to N-terminal histone nuclear localization signals (49). By contrast, SV40-GFP, the reporter protein used by us, is expected to accumulate in nuclei via the classical Srp1p/Kap95p import pathway. The specific requirements for nuclear import may differ for the two substrates, thereby explaining the distinct effects of the rat2-1 mutation on their nuclear accumulation. Our results reveal the discrete functions of Rat2p in transport of different cargoes. Although non-classical import mediated by the histone H2B nuclear localization signal is not impaired at the non-permissive temperature, a drastic effect is seen for classical nuclear protein import and Gsp1p nuclear concentration. Our data are in accordance with the idea that individual members of the Nup84p-Nup133p NPC module can selectively affect specific aspects of nucleocytoplasmic trafficking.

Nup133p, Nup85p, Nup120p/Rat2p, and Nic96p are located on both the nuclear and the cytoplasmic side of the NPC (12). None of these nucleoporins contains FXFG or GLFG repeats, and only Nic96p carries heptad repeats implicated in coiled-coil interactions (reviewed in Ref. 39). In line with the latest models for nuclear trafficking (reviewed in Refs. 47 and 50) several mechanisms, not mutually exclusive, can be proposed for the Gsp1p gradient collapse and failure to import GFP-Gsp1p in mutant nucleoporin strains. (a) The organization of NPCs may be changed in a fashion that alters the binding or translocation of Ntf2p-Gsp1p to the NPC. (b) Mislocalization of Gsp1p-interacting factors could affect the Gsp1p gradient. (c) Mutations might modulate the nuclear pore channel size, thereby preventing passage of macromolecules across the NPC. (d) Nuclear retention of the GTPase could be altered by an unknown mechanism. Because SV40-GFP, a protein of ~45 kDa molecular weight, is not impaired at the non-permissive temperature, a drastic effect is seen for classical nuclear protein import and Gsp1p nuclear concentration. Our data are in accordance with the idea that individual members of the Nup84p-Nup133p NPC module can selectively affect specific aspects of nucleocytoplasmic trafficking.
mass, was still able to exit the nucleus in mutants that collapsed the Gsp1p gradient, a more general obstruction of the channel seems unlikely. Moreover, we have demonstrated that nuclear translocation of GFP-Gsp1p was changed in several of the nucleoporin mutants, even at permissive conditions. Therefore, we favor the first two scenarios, i.e. changes in the interaction of Nt2p-Gsp1p with nucleoporins and relocation of Gsp1p-interacting factors, and we have identified these defects in several nucleoporin mutants. As a result, the initial binding of Nt2p-Gsp1p to NPCs or its subsequent translocation into the nucleus may be prevented. In addition, the redistribution of Prp20p will affect the Gsp1p-GTPase cycle, generating elevated levels of Gsp1p-GTP in the cytoplasm. In either case, the consequence will be a collapse of the Gsp1p gradient.

Like several nucleoporins, MTR7 is also required for Gsp1p gradient formation. MTR7 encodes an acetyl-CoA carboxylase, an enzyme required for de novo synthesis of long chain fatty acids that were proposed to stabilize the NPC at the pore membrane interface (45). Under restrictive conditions, destabilization of NPCs is likely to affect all trafficking reactions, including the nuclear accumulation of Gsp1p, as observed by us. However, even at room temperature nuclear import of GFP-Gsp1p was impaired in mtr7-1 cells, demonstrating that transport already has been altered by changing the lipid composition of the nuclear envelope.

Factors other than Nt2p, Prp20p, and Rna1p may also be affected and contribute to the dissipation of the Gsp1p gradient in nucleoporin mutants studied by us. As such, members of the β-importin family have been proposed to play a role in nuclear retention of Ran/Gsp1p (6, 7). At this point it is not clear how affected and contribute to the dissipation of the Gsp1p gradient of the nuclear envelope.

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