Yeast Nucleoporins Involved in Passive Nuclear Envelope Permeability

Nataliya Shulga,* Nima Mosammaparast,* Richard Wozniak,‡ and David S. Goldfarb*

*Department of Biology, University of Rochester, Rochester, New York 14627; and ‡Department of Cell Biology, University of Alberta, Alberta, Canada T6G 2H7

Abstract. The vertebrate nuclear pore complex (NPC) harbors an ∼10-nm diameter diffusion channel that is large enough to admit 50-kD polypeptides. We have analyzed the permeability properties of the Saccharomyces cerevisiae nuclear envelope (NE) using import (NLS) and export (NES) signal-containing green fluorescent protein (GFP) reporters. Compared with wild-type, passive export rates of a classical karyopherin/importin (Kap) Kap60p/Kap95p-targeted NLS-GFP reporter (cNLS-GFP) were significantly faster in nup188-D and nup170-D cells. Similar results were obtained using two other NLS-GFP reporters, containing either the Kap104p-targeted Nab2p NLS (rgNLS) or the Kap121p-targeted Pho4p NLS (pNLS). Elevated levels of Hsp70 stimulated cNLS-GFP import, but had no effect on the import of rgNLS-GFP. Thus, the role of Hsp70 in NLS-directed import may be NLS- or targeting pathway-specific. Equilibrium sieving limits for the diffusion channel were assessed in vivo using NES-GFP reporters of 36–126 kD and were found to be greater than wild-type in nup188-Δ and nup170-Δ cells. We propose that Nup170p and Nup188p are involved in establishing the functional resting diameter of the NPC’s central transport channel.

Key words: nuclear pore complex • import/export signals • green fluorescent protein • diffusion channel • Saccharomyces cerevisiae

Introduction

The nuclear pore complex (NPC)1 spans both membranes of the nuclear envelope (NE) and mediates the receptor-mediated transport of macromolecules and the passive exchange of ions, metabolites, and intermediate sized macromolecules (Mattaj and Englmeier, 1998; Ohno et al., 1998). The bulk of the NPC is comprised of a donut-shaped annulus inserted at sites of fusion between the inner and outer nuclear membranes. Signal-directed nuclear transport occurs through the middle of the octagonally symmetric annulus (Feldherr et al., 1984; Akey and Goldfarb, 1989; Feldherr and Akin, 1997). The signal-directed channel has been proposed to be housed within an ∼12-MD apparatus called the central transporter (Akey and Goldfarb, 1989; Akey and Radermacher, 1993; Yang et al., 1998; however, see Rout et al., 2000).

The yeast NPC is a massive structure (>50 MD) composed of, at minimum, 30 different nucleoporins (nups), most of which are probably represented in multiples of eight (Yang et al., 1998; Stoffler et al., 1999; Rout et al., 2000). 12 of the 30 known nups contain degenerate phenylalanine-glycine repeats (FG-nups), which serve as docking sites for transport receptors (Radu et al., 1995; Seedorf et al., 1999; Rout et al., 2000). Eight of the 12 FG-nups are symmetrically distributed on both faces of the NPC. The other four FG-nups are located either on the nuclear (Nup1p and Nup60p) or cytoplasmic (Nup159p and Nup42p) side of the NPC (Rout et al., 2000).

The compact structure of the NPC (Eichelt et al., 1990; Hinshaw et al., 1992; A key and Radermacher, 1993; Yang et al., 1998) requires that nups form multiple connections with other nups. Biochemical and genetic evidence indicates that the NPC is organized into discrete interconnecting subcomplexes. Several putative subcomplexes have been identified using biochemical approaches (for review see Stoffler et al., 1999). Nup120p is part of one subcomplex that includes Sec13p, Seh1p, Nup84p, and Nup85p (Siniossoglou et al., 1996). A nther subcomplex is composed of at least three nups, Nup53p, Nup59p, and Nup170p (Marelli et al., 1998). Nup53p and Nup59p (but not Nup170p) contain FG repeats that act as binding sites for the import karyopherin, Kap121p. The components of
this subcomplex genetically interact with another group of physically associated NPC proteins that includes two nups, Nup170p and Nic96p, and an integral membrane protein, Pom152p (Aitchison et al., 1995; Nehrbbas et al., 1996; Zabel et al., 1996; Marelli et al., 1998; Tcheperegine et al., 1999). The multiple genetic interactions between the individual components of these latter two complexes suggest that they are functionally intertwined. Consistent with this hypothesis, immunoelectron microscopy studies have shown that components of these complexes, including Pom152p, Nup170p, Nup188p, Nup53, and Nup59, are constituents of symmetrical structures located on both the cytoplasmic and nuclear faces of the NPC core (Wozniak et al., 1994; Nehrbbas et al., 1996; Marelli et al., 1998).

Although the annular subunits and most of the FG-nups are symmetrically distributed across the membrane, distinctly nonsymmetrical structures extend out into the nucleus and cytoplasm. The initial docking of import cargo (Richardson et al., 1988) and the dissociation of export cargo (Kehlenbach et al., 1999) occur on filaments that protrude from the cytoplasmic face of the NPC. A basket-like structure formed of eight fibrils joined by a ring at their distal ends extends into the nucleoplasm (Ris, 1997; Stoffler et al., 1999). The nuclear basket is a flexible structure that may serve to anchor substrates to the NPC and guide them along the translocation route (Kiseleva et al., 1996). Tpr (Bangs et al., 1998) and its nonessential yeast homologues, Mlp1p and Mlp2p (Strambio-de-Castillia et al., 1999), are filamentous proteins that extend from the basket structure into the nuclear interior (Kosova et al., 2000).

Signal-bearing proteins are bound by soluble targeting receptors, most of which belong to the karyopherin/imporin (Kap) β family of A rm/H E A T repeat-containing factors (O’hno et al., 1998; Wozniak et al., 1998; A dam, 1999). These importins and exportins, of which there are at least 14 in yeast (Wozniak et al., 1998; Nakaiyln and Dreyfuss, 1999), mediate transport by binding selectively to different classes of signal sequences. For example, classical nuclear localization signals (cNLS) are recognized by K αp60p (Srp1p), the Nab2p NLS (rgNLS) by K αp104p, and the Pho4p NLS (pNLS) by K αp121p (Nakaiyln and Dreyfuss, 1999). Many proteins that are exported out of the nucleus for various reasons contain leucine-rich nuclear export signals (NES; M attaj and Englemier, 1998). Crm1/X po1p is the major exportin responsible for the export of most NES cargo (M attaj and Englemier, 1998).

The small GTPase R an plays a central role in determining the directionality of nuclear transport. R an functions in conjunction with several regulatory proteins, including GTPase activating (R anGAP) and exchange (R anG EF) factors. Current models suggest that directionality is achieved at one level by two preexisting conditions. First, R an-GTP is concentrated in the nucleus and R an-GDP in the cytoplasm. Second, import cargo-receptor complexes are destabilized by R an-GTP and export cargo-receptor complexes are stabilized by R an-GTP. A s a result, export cargo-receptor complexes form in the nucleus and dissociate in the cytoplasm. Conversely, import cargo-receptor complexes form in the cytoplasm and dissociate in the nucleus (O’hno et al., 1998; A dam, 1999). This model does not explain at a molecular level how vectorial translocation through the transporter occurs. However, translocation probably involves serial interactions of cargo-receptor complexes with various FG-nups that are displayed along the interior of the transporter. R recently, R out et al. (2000) proposed a B rownian affinity gating mechanism for signal-directed transport. A although translocation per se may not require hydrolysis of GTP or A TP (Nakaiyln and Dreyfuss, 1999), phosphate bond energy is required in the cytoplasm to dissociate export complex from the NPC (Kehlenbach et al., 1999) and to maintain the nucleocytoplasmic gradient of R an-GTP/R an-GDP.

The vertebrate NPC can accommodate the transport of karyophilic coloidal gold particles up to 26-nm diameter (Feldherr and A kin, 1997), as well as large natural substrates, such as ribosomal subunits (H urt et al., 1999; Moy and Silver, 1999), Balbiani ring pre-mRNPs (D aneholt, 1999), and the HIV-1 preintegration complex (Fouchier and M alim, 1999). The functional diameter of the vertebrate channel is under physiological control (Feldherr and A kin, 1994a,b).

In addition to the mammoth signal-directed channel, the NPC harbors the largest known membrane diffusion channel in nature (Paine et al., 1975; Zimmerberg, 1999). In vitro studies indicate that each NPC harbors a single diffusion channel with a diameter of 10.7 nm and a length of 89 nm (K eminer and P eters, 1999). The diffusion channel appears to be unaffected by receptor-mediated trafficking (see D anker et al., 1999). U sing microinjected coloidal gold particles, Feldherr and A kin (1997) determined that the diffusion channel is located at the center of the NPC. These results are consistent with the notion that the diffusion channel may be the resting state of the transporter. The molecular mechanism and control of the transporter gating mechanism remains a major unresolved problem (see R out et al., 2000).

In this study, we demonstrate that Nup170p and Nup188p are involved in establishing the diameter of the diffusion channel. We have quantified NE permeability in wild-type (wt), nup170-Δ, and nup188-Δ cells, and conclude that Nup170p and Nup188p normally restrict the diameter of the diffusion channel. In nup170-Δ and nup188-Δ cells, both the rates of passive transport, and the size limit of GFP reporter proteins that can cross the NE are increased. Because the diffusion channel is probably a structural property of the transporter, these findings are directly relevant to the gating mechanism of signal-directed transport channel.

**Materials and Methods**

**Strains, Plasmids, and Cell Culture**

All yeast strains used in this study were derived from a W303 genetic background. (MATa ade2-1 leu2-3,112 his3-11, 15 trpl-1 ura3-1 can1-100), nup188-Δ (Nehrbbas et al., 1996) and nup170-Δ (Aitchison et al., 1995) null strains were previously described. The plasmids pGA D-NLS GFP (Shulga et al., 1996) and YCpGAL1-SSA1 (Werner-Washburne et al., 1987), which contain SSA1 under the control of the GAL1 inducible promoter, were used as described. pPHO4-4-GFP was constructed by fusing DNA sequence encoding residues 120-140 of PHO 4 (K affman et al., 1998) to the NH2 terminus of GFP (S65T). pGFPC-Bc was constructed from pGFPC-N-FUS (Niedenthal et al., 1996) by inserting a PCR-amplified B-KD COOH-terminal portion of SSB1 (Shulga et al., 1999). GFP-NE S reporter constructs were produced by fusing tandem copies of the protein A Z-domain.
Nuclear Envelope Permeability

Results

Passive Diffusion of cNLS-GFP Across the Yeast Nuclear Envelope

The permeability properties of the yeast NE can be probed in living cells using small NLS-GFP reporter proteins (Shulga et al., 1996; Roberts and Goldfarb, 1998). The following short discussion lays out a few of the kinetic and thermodynamic considerations that are prerequisite to an analysis of the passive and receptor-mediated transport properties of small signal-bearing cargo. cNLS-GFP (43 kD) contains the SV40 large T antigen NLS and is imported by the Kap60p/Kap95p receptor-mediated pathway (Shulga et al., 1996). Small NLS-cargo like cNLS-GFP accumulate in nuclei because they diffuse out of nuclei more slowly than they are imported. To a first approximation, the steady-state nucleocytoplasmic distribution \( \left[ \text{N} \right]/\left[ \text{C} \right] \) of cNLS-GFP is determined by the ratio of the rate constants \( k_{\text{Pi}} \) for its passive export and \( k_{\text{Fi}} \) for its receptor-mediated import, over the rate constant for its passive export \( k_{\text{Pe}} \), such that \( \left[ \text{N} \right]/\left[ \text{C} \right] = \frac{k_{\text{Pi}} + k_{\text{Fi}}}{k_{\text{Pe}}}. \)

To study the permeability properties of the yeast NE, we sought conditions that would allow us to measure passive transport without interference from receptor-mediated transport. Inhibitors of glycolysis and mitochondrial respiration have been widely used to inhibit receptor-mediated nuclear transport. In the presence of sodium azide and 2-deoxyglucose (azide/deoxyglucose), cNLS-GFP, which is initially concentrated in the nucleus, rapidly equilibrates across the NE \( \left[ \text{N} \right]/\left[ \text{C} \right] = 1 \); Shulga et al., 1996). This observation provided a method to directly measure apparent rates of NE permeability in vivo. The kinetics of cNLS-GFP export in azide/deoxyglucose were studied over a range of temperatures and occurred with apparent first order kinetics between 0–37°C (Fig. 1). A good first indication that cNLS-GFP export is passive is that it occurred 4.5 kcal/mole, which is in the range for diffusion-limited processes. Previously, we estimated the apparent \( E_a \) for facilitated cNLS-GFP import at 11–12 kcal/mole (Shulga et al., 1996). These estimated temperature coefficients are important because they support the conclusion that the mode of cNLS-GFP export in azide/deoxyglucose is passive (Shulga et al., 1996; Roberts and Goldfarb, 1998). In addition, these results show that the bulk of cNLS-GFP is free to diffuse in the nucleus.
are strong inhibitors of facilitated nuclear transport. The premise of their use is that they inhibit ATP production. For example, the addition of antimycin (another inhibitor of mitochondrial respiration) and 2-deoxyglucose to Saccharomyces carlsbergensis reduced endogenous respiration rates and ATP levels by 90% within 1–2 min (Eddy et al., 1970). Such a precipitous drop in the cell’s nucleotide triphosphate pool may explain the immediate onset of net NLS-GFP export after addition of azide/deoxyglucose (Fig. 1). However, the physiological effects of these drugs extends beyond their better known activities. 2-deoxyglucose, for example, has effects that compound its activity as a competitive inhibitor of hexokinase. The addition of 2-deoxyglucose to S. carlsbergensis cells actually stimulates respiration rates by ~50% (Eddy et al., 1970). Even more perplexing was the observation that sodium azide continued to cause the net export of cNLS-GFP in rho8 cells, which are deficient in mitochondrial respiration and are unable to grow on glycerol as the sole carbon source (Pan, X. and D.S. Goldfarb, unpublished results). Therefore, mitochondrial respiration is unlikely to be the relevant target of azide in this particular assay. Regardless of azide’s mechanism of action, it is nevertheless clear that the export of cNLS-GFP occurs by simple diffusion under these conditions.

Concerns such as these led us to use chilling (0°C) as a less problematic means to preferentially inhibit receptor-mediated transport. As discussed, the large difference between the temperature coefficients of receptor-mediated import and passive export allows passive transport to be studied at 0°C with little interference from receptor-mediated transport (see Fig. 3).

Effects of Chilling on the Nucleocytoplasmic Distribution of cNLS-GFP in Wild-type and Mutant Cells

The effects of chilling on the steady-state nucleocytoplasmic localization of cNLS-GFP were determined in wt and nup-deficient cells. Fig. 2 shows the localization of cNLS-GFP in wt, nup170-Δ, and nup188-Δ cells grown at 23°C, and incubated for 1 h at 23 or 0°C after the induction of GAL1-SSA1 expression. At 23°C, before induction of GAL1-SSA1 expression, nup170-Δ and nup188-Δ cells exhibited abnormally high cytoplasmic levels of cNLS-GFP (low [N]/[C]) compared with wt cells (Fig. 2, compare a, c, and e). nup188-Δ cells exhibited a particularly low [N]/[C] (Fig. 2, c). Immunoblot analysis using anti-GFP antibodies revealed that cNLS-GFP was full length in these strains (not shown).

Although nup188-Δ and nup170-Δ cells mislocalize a significant portion of cNLS-GFP to the cytoplasm, they grow, mate, sporulate, and germinate at parental levels (not shown, see Aitchison et al., 1995; Nehrbass et al., 1996; Zabel et al., 1996). It is unlikely that these cells could survive if native nuclear proteins were mislocalized to the same extent as cNLS-GFP. The mislocalization of cNLS-GFP in nup188-Δ and nup170-Δ cells is more consistent with an innocuous increase in NE permeability.

Also shown in Fig. 2 are the effects of inducing GAL1-SSA1 expression on the localization of cNLS-GFP in wt, nup188-Δ, and nup170-Δ cells. Ssa1p is a cytoplasmic Hsp70 that we previously implicated in nuclear transport (Shulga et al., 1996). Specifically, induction of GAL1-SSA1 expression increased both the rates of cNLS-GFP import and its steady-state nuclear accumulation (higher

---

Figure 2. Steady-state localization of cNLS-GFP in wt, nup170-Δ, and nup188-Δ cells was determined before induction of GAL1-SSA1 (23°C), after induction (23°C + SSA1), and incubated on ice after induction (0°C + SSA1). GFP fluorescence and Hoechst stain images were obtained by confocal microscopy (see Materials and Methods).
and m). These results are consistent with the notion that GFP in caused only a mild increase in cytoplasmic levels of cNLS-

fact, equilibration in complete within 15 min at 0°C (see Figs. 3 and 4). Chilling on the nuclear localization of cNLS-GFP in nup188-Δ cells was remarkable. Incubation on ice caused the virtually complete equilibration of cNLS-GFP in nup188-Δ cells (Fig. 3 A). The dynamic effects of temperature shifts on the [N]/[C] of cNLS-GFP in nup188-Δ cells are also shown in Fig. 3 A. In a reversible fashion, cNLS-GFP diffused out of nuclei down its concentration gradient at 0°C, and, after shifting back to room temperature (~22°C), was imported back up its concentration gradient into nuclei. Fig. 3 B shows the effects of various downward temperature shifts on the rate of export and eventual steady-state distribution of cNLS-GFP in nup188-Δ cells. Basically, export rates increased and steady state [N]/[C] levels decreased as the temperature dropped (Fig. 3 B). These results support the hypothesis that the [N]/[C] of cNLS-GFP is determined by the relative rates of receptor-mediated import and passive exchange, and they reinforce the fact that receptor-mediated transport has a significantly higher temperature coefficient.

It is helpful to understand the effects that GAL 1-SSA1 expression have on the facilitated and passive transport of cNLS-GFP. It should be noted that the GAL 1-driven overexpression of SSA1 results in a moderate three- to fourfold increase in total Ssa1-4p levels. These levels are similar to those found in normal heat-shocked cells (Shulga et al., 1996). In that study, we concluded that Ssa1p stimulated the rate of Kap60p-mediated import of cNLS-GFP. At that time, we could not absolutely rule out the alternative possibility that high levels of Ssa1p increased the apparent rate of cNLS-GFP import not by a direct stimulation of import, but rather by reducing the rate of cNLS-GFP passive export. We have now shown that the rate of cNLS-GFP passive export is also stimulated by GAL 1-SSA1 induction. Therefore, Ssa1p stimulates both the facilitated and passive transport of cNLS-GFP. Importantly, GAL 1-SSA1 expression stimulates receptor-mediated import rates relatively more than passive export rates.

Rates of passive export of cNLS-GFP at 0°C were also determined in nine additional null strains, including NUP1, NUP2, NUP100, NUP120, NUP157, NUP170, MIP1, MP2, and POM152 (Fig. 3 C). The localization of cNLS-GFP was normal at 23°C in every strain (not shown) except nup170-Δ, which showed somewhat higher than normal cytoplasmic levels (Fig. 2 e). Initial [N]/[C] levels of cNLS-GFP in each of these strains were sufficiently

![Figure 3. Temperature dependence of cNLS-GFP nuclear accumulation in wild-type and mutant cells.](image)
high to allow the measurement of export kinetics without having to overexpress SSA1. At 0°C, cNLS-GFP rapidly equilibrated across the NE in nup170-Δ cells, but remained mostly nuclear in wt and all eight other null strains (Fig. 3). These results indicate that Nup188p and Nup170p play specific roles in the size of the diffusion channel.

Even after prolonged incubation on ice, cNLS-GFP remained slightly more concentrated in the nuclei of nup170-Δ and nup188-Δ cells (Fig. 2, o and p). Two factors may explain why complete equilibration was never reached. First, past experience suggests that a small amount of non-specific nuclear retention of cNLS-GFP should not be unexpected. For example, small diffusible proteins such as lysozyme (14.4 kD) and soybean trypsin inhibitor (21 kD) accumulate to low levels in tissue culture cell nuclei (Breeuwer and Goldfarb, 1990), as does native GFP in yeast nuclei (Zanchin and Goldfarb, 1999). Second, receptor-mediated transport is not completely inhibited at 0°C. In fact, we could quantify the rate of cNLS-GFP import in wt and mutant cells at 0°C. In the experiment shown in Fig. 4, wt, nup170-Δ, and nup188-Δ cells grown at 23°C in complete medium were treated for 40 min with 2-deoxyglucose to induce equilibration of cNLS-GFP (Fig. 4, bottom). The cells were then washed and resuspended in ice-cold complete medium containing glucose and placed in an ice bath. While on ice, cNLS-GFP was imported into wt nuclei with a half-time of ~3.5 h (Fig. 4). For comparison, the half-time for cNLS-GFP import in wt cells at 30°C is ~5 min (Shulga et al., 1996). A’s expected, no nuclear accumulation of cNLS-GFP was observed at 0°C in nup170-Δ and nup188-Δ cells (Fig. 4).

**Effect of NLS Targeting Pathway on Nuclear Envelope Permeability**

To this point, it has been implicitly assumed that the diffusion of cNLS-GFP across the NE is proportional to the size of the reporter and is unaffected by the targeting characteristics of particular NLSs. This is not necessarily so. The proteins that comprise the diffusion channel may not be indifferent to NLS sequences. To test for NLS-specific effects, we performed steady-state permeability studies with the Pho4p (pNLS) and Nab2p (rgNLS; Lee and Ashitch, 1999) NLSs. Distinct members of the karyopherin β family mediate the nuclear transport of pNLS-GFP and rgNLS-GFP. As shown in Fig. 5 A, the behavior of rgNLS-GFP in wt, nup170-Δ, and nup188-Δ cells was almost indistinguishable from cNLS-GFP. At 23°C, rgNLS-GFP accumulated to high levels in wt nuclei and to lesser extents in nup170-Δ and nup188-Δ cells (compare Fig. 5 A, a, b, and c). Incubation on ice had little effect on the [N]/[C] of rgNLS-GFP in wt cells (Fig. 5 A, compare a and g), but caused virtual equilibration in nup170-Δ and nup188-Δ cells (Fig. 5 A, compare b and h, and c and i). Also similar to cNLS-GFP, azide/deoxyglucose induced the near complete equilibration of rgNLS-GFP across the NEs of both wt and mutant cells (Fig. 5 A, j, k, and l).

Curiously, the induction of GAL1-SSA1 expression did not rescue the poor steady-state nuclear localization of rgNLS-GFP in nup170-Δ and nup188-Δ cells (Fig. 5 A, compare b and e, and c and f). Because SSA1 induction had a striking effect on the [N]/[C] of cNLS-GFP in nup170-Δ and nup188-Δ cells (Shulga et al., 1996; Fig. 2), we looked for possible quantitative effects on rgNLS-GFP using a more sensitive kinetic assay. As shown in Fig. 5 B, elevated levels of Ssa1p did not even partially rescue the import defect of rgNLS-GFP in nup170-Δ and nup188-Δ cells. Thus, Hsp70 may act selectively on different NLS targeting pathways (see Discussion).

The steady-state localizations of pNLS-GFP at 23 and 0°C in wt, nup170-Δ, and nup188-Δ cells are shown in Fig. 6. In these experiments, pNLS-GFP behaved as if it were a poorer karyophile than either cNLS-GFP or rgNLS-GFP, but with regard to relative NE permeability it was similar. Specifically, at 23°C pNLS-GFP localized only moderately well in the nuclei of wt and mutant cells (Fig. 6, a–f). Upon shifting to 0°C, pNLS-GFP equilibrated across the NE of nup170-Δ and nup188-Δ cells (Fig. 6, i–l). pNLS-GFP remained mostly nuclear in wt cells at 0°C (Fig. 6, g and h). We were unable to determine if GAL1-SSA1 expression could rescue the poor localization of pNLS-GFP in these strains because, for unknown reasons, the fluorescence of
pNLS-GFP dimmed after galactose induction (not shown). We conclude that the NE permeability properties of wt, nup170-Δ, and nup188-Δ cells are not significantly influenced by the category of NLS presented by the GFP reporter.

**Increased Nuclear Envelope Sieving Limits in nup170-Δ and nup188-Δ Cells**

The steady-state and kinetic experiments described above suggest that cNLS-GFP (43 kD) is barely able to fit through wt diffusion channels, but is small enough to readily pass through the mutant diffusion channels of nup170-Δ and nup188-Δ cells. The hypothesis that the functional size of the diffusion channel(s) in nup170-Δ and nup188-Δ cells are larger than wt was directly tested by studying the passive import at 0°C of 36, 51, 66, 81, and 126 kD GFP-NES reporter proteins (see Materials and Methods). GFP-NES reporters were used instead of NLS-GFP reporters for technical reasons, including the fact that at 23°C they were all strongly excluded from both wt and mutant nuclei. After shifting cells to 0°C, GFP-NES66 rapidly diffused down its concentration gradient from the cytoplasm into the nucleus of nup170-Δ and nup188-Δ cells (Fig. 7A, compare c and i, and e and k). In contrast, all GFP-NES reporters including GFP-NES66 remained excluded from the nuclei of wt cells at 0°C (Fig. 7A, compare a and g). In Fig. 7A and B, GFP and Hoechst fluorescence is shown as dark instead of light tones. These data indicate that the diffusion channels of wt cells are too...
small to admit GFP-NE S66. This is not surprising since the diffusive export of cNLS-GFP (43 kD) in wt cells was very slow (Fig. 4). GFP-NE S36 appeared in wt nuclei after 3 h at 0°C, but never equilibrated (not shown). Thus, GFP-NE S36, like NLS-GFP (43 kD), is apparently only barely able to diffuse through the wt diffusion channel. As shown in Fig. 7 B, the passive equilibration of GFP-NE S66 at 0°C in nup188-Δ cells was reversible. These images show a four-minute time course in four cells (two of them mitotic) for the facilitated export of nuclear NES-GFP66 at 23°C after equilibration on ice.

The NE sieving properties of wt, nup170-Δ, and nup188-Δ cells were addressed by quantifying the steady-state $[C]/[N]$ ratios of 66, 81, and 126 kD GFP-NE S reporters at 23 and 0°C. In these experiments, the steady state $[C]/[N]$ ratios of the different GFP-NE S reporters were quantified at both 23 and 0°C. If a particular GFP-NE S reporter was too large to diffuse across the NE, then its $[C]/[N]$ ratio will be the same at 23 and 0°C. In this case, the ratio of the two ratios $([C]/[N])_{23°C}/([C]/[N])_{0°C}$ will be $\sim 1.0$. For GFP-NE S reporters that are small enough to equilibrate across the NE, the $[C]/[N]$ ratio will be greater than one. As shown in Fig. 7 C, the $[C]/[N]$ ratio of GFP-NE S66 in wt cells was $\sim 4.3$ at both 23 and 0°C. The ratio of these values (1.01) indicates that NES-GFP66 is too large to diffuse across the NE of wt cells. In contrast, the $[C]/[N]$ ratios of GFP-NE S66 in nup188-Δ and nup170-Δ cells at 23 and 0°C are significantly greater than one (2.73 and 3.85, respectively), confirming that the mutant NEs are permeable to NE S-GFP66. Furthermore, GFP-NE S81 and GFP-NE S126 are free to diffuse across the NE of nup170-Δ cells. The 81- and 126-kD reporters were, however, excluded from the nuclei of nup188-Δ cells. These data demonstrate that the diffusion channels in nup188-Δ and nup170-Δ cells are both permeable to larger proteins than wt diffusion channels. Interestingly, nup170-Δ diffusion channels are permeable to larger reporters than the diffusion channels of nup188-Δ cells.

**Discussion**

The key finding of this study is that the permeability of the yeast NE is greatly affected by the deletion of NUP170 and NUP188. These results establish the role of Nup170p and Nup188p in determining the functional diameter of the diffusion channel which, at $\sim 10$-nm diameter, is the largest known channel in nature (Zimmerberg, 1999). Although the hypothesis that the diffusion channel is part of the NPC is considered fact, there is actually no genetic or biochemical evidence to support it. The present results are the first to directly link particular NPC components to the diffusion channel.

The diffusion channel is an enigma. Many karyophilic proteins that are small enough to diffuse across the NE are imported by receptor-mediated pathways (Breeuwer and Goldfarb, 1990; Pruschy et al., 1994). What, if not for the transport of small proteins, is the function of the $\sim 10$-nm diameter diffusion channel? A much smaller channel would suffice for the diffusion of ions, metabolites, and nucleotides. There is evidence that passive diffusion is the preferred pathway for the nuclear transport of some proteins (see A dachi et al., 1999). Alternatively, the diffusion channel may be an innocuous consequence of pore complex architecture. For example, Hinshaw et al. (1992) proposed that the eight peripheral channel features that appear in three dimensional reconstructions of the annulus of the Xenopus oocyte NPC might provide a route for membrane proteins to traffic between the inner and outer nuclear membranes. It was noted that these channels were $\sim 10$-nm diameter and, as a result, could serve double duty as the elusive diffusion channels. More recent studies, which conclude that a single diffusion channel resides in the middle of the NPC, have virtually ruled out this possibility (Feldherr and Akin, 1997; Keminer and Peters, 1999).

The receptor-mediated translocation channel is also located at the center of the NPC (Feldherr et al., 1984; A key
A key and Goldfarb (1989) proposed that this channel is a property of an apparatus called the transporter that fills the space created by the pore annulus (see also Kiseleva et al., 1996). Using cryo-electron microscopy and single particle imaging techniques, Akey and his colleagues subsequently obtained low resolution structures of an $\sim 12$ MD transporter (Akey and Radermacher, 1993; Yang et al., 1998). Even so, the transporter remains a controversial structure whose physiological association with the NPC annulus is at best labile, as it is often missing from the pores in NE preparations (see Ris, 1997). This fact, as well as variations in the appearance of the plug when it does appear, has led some to suggest that the transporter is either the remnant of a collapsed basket or cargo caught in transit (Ohno et al., 1998; Stoffler et al., 1999). If true, then something else is needed to explain how it is that the gaping 40-nm diameter annular hole behaves in vivo like a much smaller 10-nm diameter channel. One possibility is that the nuclear basket, which is also centered over the annulus, is the true diffusion barrier. However, this hypothesis is specifically excluded by the observation that 4–7-nm diameter colloidal gold particles, following their injection into the nucleus, accumulated as they encountered their rate-limiting diffusion barrier at the NE (Feldherr and Akin, 1997). If the basket were the diffusion barrier, then these gold particles would have accumulated outside rather than inside the fishtrap. Thus, in addition to localizing the diffusion channel somewhere along the axis that extends through the middle of the pore annulus, the results of Feldherr and Akin (1997) show that the diffusion channel lies in the plane of the NE.

A remarkable feature of the NPC is its capacity to regulate the transport of both small and very large cargo. The notion of a gated channel was proposed by Bonner (1978) who considered the possibility that large proteins might enter the nucleus by increasing the nuclear pore radius through specific interactions. A gated channel is supported...
by physiological (Feldherr and Akin, 1994a,b) and image reconstruction studies (Akey and Goldfarb, 1989). The basket structure has also been ascribed with dynamic properties akin to gating (Jarnik and Aebi 1991; Kiseleva et al., 1996).

The notion that the diffusion channel and transporter are both located at the hub of the NPC leads to the interesting proposition that the diffusion channel may be a structural consequence of the architecture of the transporter. If the diffusion channel and transporter are features of the same channel-forming apparatus, then it follows that they are composed of the same nups. Nup170p is a good candidate for this class of nups. Nup170p was previously implicated in receptor-mediated transport through its interaction with the Nup53p. Nup53p, which contains FG repeats, is a docking site for the NLS-cargo receptor Kap121p, and is required for efficient Kap121-mediated import (Marelli et al., 1998).

In vivo steady-state and kinetic experiments were used in this study to support the conclusion that nup170- and nup188Δ cells contain enlarged diffusion channels. Our strategy was to initially target GFP by signal-directed transport to either the nucleus (NLS-GFP) or the cytoplasm (NES-GFP). Subsequent chilling allowed the passive equilibration of reporters to be studied without significant interference from receptor-mediated transport. The differential effect of temperature on receptor-mediated and passive transport is due to the large apparent difference in their temperature coefficients: \( E_a \sim 12 \text{ kcal/mole} \) for receptor-mediated import and \( E_a \sim 5 \text{ kcal/mole} \) for passive export. At 0°C in wt cells, the import of cNLS-GFP occurred with a half time of 3.5 h, which is \(~40\) times slower than at 30°C. The rapid rate of passive equilibration of cNLS-GFP in chilled nup188Δ and nup170Δ cells (t_{1/2} \sim 2 \text{ m}) demonstrates the passive diffusion across the NE proceeds at appreciable rates at 0°C. All of these results support the hypothesis that the distribution across the NE of small NLS- and NES-GFP reporters, at any time and temperature, is determined largely by competing rates of receptor-mediated transport and passive diffusion.

True rate constants for passive and receptor-mediated transport and actual \( E_a \) cannot be determined in vivo. However, for the purposes of this study only relative (apparent) values are necessary. These estimates are proportional to the true values and are, therefore, sufficient to support the central conclusion that the NE permeability is increased in nup188Δ and nup170Δ cells. A iso, quantitative comparisons between the diffusive transport of NLS and NES reporters in wt and mutant strains is unimportant and potentially misleading. Because we do not know the shapes of the different reporters, the functional diameter of the different reporters may or may not vary in direct proportion to their mass. The purpose of this analysis is neither to determine actual channel diameters or transport rates. What is important to the central conclusions of this study are the relative differences between the diffusive transport of individual reporters within various strains.

There were significant differences between the effects of azide/deoxyglucose and chilling on cNLS-GFP export in wt cells. Even on ice, azide and/or deoxyglucose induced the virtually complete equilibration of cNLS-GFP in wt cells. In contrast, chilling alone caused only a slight increase in cytoplasmic cNLS-GFP levels, even after induction of GAL1-SSA1 expression. If azide, deoxyglucose and chilling were each acting only as inhibitors of receptor-mediated transport, then we would not expect to observe differences between their effects on cNLS-GFP localization at 0°C. It will be important to monitor the effects of these treatments on cellular levels of ATP and GTP levels. The situation is further complicated by the inhibitory effect of azide on cNLS-GFP export in rho+ cells, which lack a functional electron transport chain (mitochondrial cytochrome oxidase is the classical target for azide). The differential effects of azide, deoxyglucose, and chilling on cNLS-GFP localization are not likely to be resolved until after their specific effects on cellular levels of ATP and GTP are determined. The fact that the incubation of wt cells at 0°C did not evoke cNLS-GFP equilibration, but did so in nup170Δ and nup188Δ cells, is among the strongest evidence that these nups are involved in determining the functional diameter of the diffusion channel.

It is interesting that the apparent NE sieving limit for GFP-NES reporters in nup170Δ cells was higher than in nup188Δ cells (Fig. 7 C). This was unexpected because nup188Δ cells exhibited a more striking steady-state cNLS-GFP nuclear localization defect at 23°C than nup170Δ cells (Fig. 2). It is possible that NLS-GFP and GFP-NES reporters differ with respect to their passive exchange properties. For example, NLS and NES signals could interact differently with soluble and/or NPC-associated transport factors in the different genetic backgrounds. Alternatively, the accessibility, dimensions, or shape of the diffusion channels could be different when approached from either side. For example, the entrance to the diffusion channel could be funnel shaped at one end and not the other (see Keminer and Peters, 1999). Interference by associated structures could also conceivably influence the accessibility of the diffusion channel. For example, nuclear baskets and cytoplasmic filaments could impede bulk diffusion in the vicinity of the diffusion channel. It should be noted a nup188Δ disruption strain (Zabel et al., 1996) and a temperature-conditioned allele of NUP188 (Nehrbass et al., 1996) exhibited nuclear envelope abnormalities. A iso, the deletion of NUP184, a putative NUP188 homologue in S. pombe, caused the nuclear accumulation of poly(A)+ RNA in nutrient rich medium (Whalen et al., 1999). In contrast, no defects of any type have been reported previously in nup170Δ cells.

NPC sieving limits were evaluated by determining the potential of different size GFP-NES reporters to equilibrate across the NE at 0°C. Equilibration of a particular GFP-NES reporter can only occur when the rate of its diffusive import equals or exceeds the rate of its facilitated export. Because receptor-mediated transport is incompletely inhibited at 0°C (see Fig. 2 B), slowly diffusing reporters may never equilibrate. This effect could result in underestimates of sieving limits. Even so, this caveat would not affect the central conclusion that the NEs in nup188Δ and nup170Δ cells are more permeable than wt NEs.

The induction of GAL1-SSA1 expression was used in this study to improve the steady-state nuclear localization of cNLS-GFP in nup188Δ cells. However, in addition to increasing rates of receptor-mediated import, GAL1-SSA1 induction also increased, albeit to a lesser extent, the
permeability of wt nuclei to cNLS-GFP (Figs. 2, 3, and 4). The net effect of GAL 1-SSA1 induction on growing cells was to increase fK/fKp, which results in faster import rates and higher steady-state [N/C] levels of cNLS-GFP. How could Ssa1p stimulate to different degrees receptor-mediated import and passive export? It is likely that Ssa1p stimulates cNLS-GFP import by promoting complex formation between the cNLS and its targeting receptor, Kαp60p. The best evidence for this particular hypothesis is that the induction of GAL 1-SSA1 expression completely suppressed the cNLS-GFP import defect of srp1-31ls cells (Shulga et al., 1996). Also, studies using permeabilized cells indicates that H sp70 is coimported with cNLS cargo—Kαp60 (PTA C60) complexes (O’kuno et al., 1993).

Because the induction of GAL 1-SSA1 is such a strong stimulator of cNLS-GFP import, we were surprised to see that it had no effect on the otherwise poor localization of rgNLS-GFP. This result suggests that Ssa1p acts selectively on the Kap60-mediated import of cNLS cargo. Kap60 is unique among the NLS binding karyopherins, most of which are members of the karyopherin β family (Wozniak et al., 1998). Kap60 contains tandem armadillo repeats, whereas the karyopherin βs contain tandem HEAT motifs. While armadillo and HEAT repeats are related (Malki et al., 1997), they fold into somewhat different structures. Individual armadillo repeats contain three α-helical segments that pack in tandem in right-handed superhelices. Individual HEAT motifs contain two α-helical segments that pack in tandem into left-handed superhelical domains. Otherwise, both types of proteins provide similar cargo binding surfaces. Ssa1p might also act differentially on individual cNLSs, which are quite variable and include sequences of different lengths with either one or two basic motifs (see Conti et al., 1998). The relatively small stimulation of passive export by GAL 1-SSA1 induction may be mediated by the activity of H sp70 chaperones like Ssa1p to reduce nonspecific protein–protein interactions. In this case, Ssa1p may reduce the transient retention of cNLS-GFP in the nucleus and increase its freedom to diffuse to the diffusion channel. Ssa1p may also have this solubilizing effect on cytoplasmic cNLS-GFP, but it is doubtful that this mechanism could entirely account for the large stimulation of import.

In conclusion, these experiments establish an in vivo methodology for the study of NE permeability in yeast. The finding that Nup170p and Nup188p are involved in NE permeability provides the first evidence for the role of specific nups in the structure and function of the diffusion channel. Because the diffusion channel and the transporter may be properties of the same apparatus, these findings may be directly relevant to the structure, function, and gating mechanism of the signal-directed translocation channel.

We are grateful to Laura Davis, Mike Rout, Susan Wente, and John Aitchison for null strains. This work was supported by a merican Cancer Society grant BE-104C to D.S. Goldfarb, and the Medical Research Council of Canada and Alberta Heritage Foundation for Medical Research to R. Wozniak.

Submitted: 12 January 2000
Revised: 24 March 2000
Accepted: 10 April 2000

References

A dachi, M., M. Fukuda, and E. Nishida. 1999. Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer. EMBO J. 18:3547–3558.
A dam, S.A. 1999. Transport pathways of morphocycles between the nucleus and the cytoplasm. Curr. Opin. Cell Biol. 11:402–406.
Aitchison, J.D., M.P. Rout, M. Marelli, G. Blobel, and R.W. Wozniak. 1995. Two novel related yeast nucleoporins Nup170p and Nup157p: complementation with the vertebrate homologues Nup155p and functional interaction with the yeast nuclear pore membrane protein Pom152p. J. Cell Biol. 131:1133–1148.
A key, C.W., and D.S. Goldfarb. 1989. Protein import through the nuclear pore complex is a multistep process. J. Cell Biol. 109:971–982.
A key, C.W., and M. Radermacher. 1993. Aarchitecture of the Xenopus nuclear pore complex revealed by three-dimensional cryo-electron microscopy. J. Cell Biol. 122:1–19.
Bangs, P., B. Burke, C. Powers, R. Craig, A. Purohit, and S. Doxsey. 1998. Functional analysis of Tpr: identification of nuclear pore complex association and nuclear localization domains and a role in mRNA export. J. Cell Biol. 143:1801–1912.
Bonner, W.M. 1978. Protein migration and accumulation in nuclei. In The Cell Nucleus. Vol. 6. H. Bush, editor. Academic Press, New York. 97–148.
Breeuwer, M., and D.S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. Cell. 60:999–1008.
Conti, E., M. Uy, L. Leighton, G. Blobel, and J. Kuriyan. 1998. Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. Cell. 94:193–204.
Dahm, B. 1999. Pre-mRNA particles from gene to nuclear pore. Curr. Biol. 9:R412–R415.
Danker, T., H. Schillers, J. Storck, V. Shahin, B. Kramer, W. Wilhelmi, and H. Oberleithner. 1999. Nuclear hourglass technique: an approach that detects electronically open nuclear pores in Xenopus laevis oocytes. Proc. Natl. Acad. Sci. USA. 96:13530–13535.
Eddy, A.A., K. Backen, and G. Watson. 1970. The concentration of amino acids by yeast cells depleted of adenine triphosphate. Biochem. J. 120:853–858.
Feldherr, C.M., and D. Akin. 1994a. Variations in signal-mediated nuclear transport during the cell cycle in B A L b (S3T3) cells. Exp. Cell. Res. 215:206–210.
Feldherr, C.M., and D. Akin. 1994b. Role of nuclear trafficking in regulating cellular activity. Int. Rev. Cytol. 151:183–228.
Feldherr, C.M., and D. Akin. 1997. The location of the transport gate in the nuclear pore complex. J. Cell Sci. 110:3065–3070.
Feldherr, C.M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. J. Cell Biol. 99:2216–2222.
Fouchier, R.A., and M.H. Malim. 1999. Nuclear import of human immunodeficiency virus type-1 preintegration complexes. Adv. Virus Res. 52:275–299.
Hindshaw, J.E., B.O. Carragher, and R.A. Milligan. 1992. Architecture and design of the nuclear pore complex. Cell. 69:1133–1141.
Hurt, E., S. Hannus, B. Schmelz, D. Lalu, D. Tollervey, and G. Simos. 1999. A novel in vivo assay reveals inhibition of ribosomal nuclear export in ran-cycle and nucleoporin mutants. J. Cell Biol. 144:389–401.
Jarnik, M., and U. Aebi. 1991. Evidence for a more complete 3-D structure of the nuclear pore complex. J. Struct. Biol. 107:291–308.
Kaffman, A., N.M. Rank, and E.K. O’Shea. 1998. Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev. 12:2673–2683.
Kellenbach, R.H., A. Dickmanns, A. Kellenbach, T. Guan, and L. Gerace. 1999. A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. J. Cell Biol. 145:645–657.
Kemminer, O., and R. Peters. 1999. Permeability of single nuclear pores. Biophys. J. 77:217–228.
Kiseleva, E., M.W. Goldberg, B. Dahm, and T.D. Ailen. 1996. RNP export is mediated by structural reorganization of the nuclear pore basket. J. Mol. Biol. 260:304–311.
Kosova, B., N. Pante, C. Rollenhagen, A. Podtelejnikov, M. Mann, U. Aebi, and E. Hurt. 2000. Mi2p2, a component of nuclear pore attached intranuclear filaments, associates with Nic96p. J. Biol. Chem. 275:343–350.
Lee, D.C., and J.D. Aitchison. 1998. Kap104p-mediated nuclear import. Nuclear localization signals in mRNAs–binding proteins and the role of R an and RNA J. Biol. Chem. 274:29031–29037.
Malik, H.S., T.E. Eckhardt, and D.S. Goldfarb. 1997. Evolutionary specialization of the nuclear targeting apparatus. Proc. Natl. Acad. Sci. USA. 94:13738–13742.
Marelli, M.J.D. Aitchison, and R.W. Wozniak. 1998. Specific binding of the karyopherin Kap121p to a subunit of the nuclear pore complex containing Nup153p, Nup50p, and Nup170p. J. Cell Biol. 143:1813–1830.
Matja, I.W., and L. Engelmeier. 1998. Nucleocytoplasmic transport of the soluble phase. Annu. Rev. Biochem. 67:265–306.
May, T.J., and P.A. Silver. 1999. Nuclear export of the small ribosomal subunit requires the ran-GTPase cycle and certain nucleoporins. Genes Dev. 13:2118–2133.
Nakielny, S., and G. Dreyfuss. 1999. Transport of proteins and RNA in and out of the nucleus. Cell. 99:677–690.
Nehrbass, U., M.P. Rout, S. Maguire, G. Blobel, and R.W. Wozniak. 1996. The...
yeast nucleoporin Nup188p interacts genetically and physically with the core structures of the nuclear pore complex. J. Cell Biol. 133:1153–1162.

Niedenthal, R. K., L. Riles, M. J. Johnston, and J. H. Hegemann. 1996. Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast. 12:773–786.

Nilsson, B., T. Moks, B. J. Janson, A. Brahmsen, A. Emlad, E. Holm gren, C. Henrichson, T. A. Jones, and M. Uhlén. 1987. A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng. 1:107–113.

O hno, M., M. Fornerod, and I. W. Mattaj. 1998. Nucleocytoplasmic transport: the last 200 nanometers. Cell. 92:327–336.

O'kun, Y., N. I. Mamonto, and Y. Yoneda. 1993. 70-kD heat shock cognate protein colocalizes with karyophilic proteins into the nucleus during their transport in vitro. Exp. Cell Res. 206:134–142.

Paine, P. L., L. C. More, and S. B. Horowitz. 1975. Nuclear envelope permeability. Nature. 254:109–114.

Pruschy, M., Y. Ju, L. Spitz, E. Carafoli, and D. S. Goldfarb. 1994. Facilitated nuclear transport of calmodulin in tissue culture cells. J. Cell Biol. 127:1527–1536.

Radu, A., M. S. Moore, and G. Blobel. 1995. The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. Cell. 81:215–222.

Richter, R., A. Holzenburg, E. L. Buhle, Jr., M. Jarnik, A. Engel, and U. Aebi. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell. 52:655–664.

Ris, H. 1997. High-resolution field-emission scanning electron microscopy of nuclear pore complex. Scanning. 19:368–375.

Roberts, P. M., and D. S. Goldfarb. 1998. In vivo nuclear transport kinetics in Saccharomyces cerevisiae: a role for heat shock protein 70 during targeting and translocation. J. Cell Biol. 135:329–339.

Shulga, N. P., P. Roberts, Z. G., L. Spitz, M. M. Tabb, M. Nomura, and D. S. Goldfarb. 1996. In vivo nuclear transport kinetics in Saccharomyces cerevisiae: a role for heat shock protein 70 during targeting and translocation. J. Cell Biol. 135:329–339.