In Vivo Nuclear Transport Kinetics in *Saccharomyces cerevisiae*: A Role for Heat Shock Protein 70 during Targeting and Translocation

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Abstract. The transport of proteins into the nucleus is a receptor-mediated process that is likely to involve between 50–100 gene products, including many that comprise the nuclear pore complex. We have developed an assay in *Saccharomyces cerevisiae* for the nuclear transport of green fluorescent protein fused to the SV-40 large T antigen nuclear localization signal (NLS-GFP). This assay allows the measurement of relative NLS-GFP nuclear import rates in wild-type and mutant cells under various physiological conditions. Probably the best understood component of the nuclear transport apparatus is Srp1p, the NLS receptor, which binds NLS-cargo in the cytoplasm and accompanies it into the nucleus. When compared to *SRP1⁺* cells, NLS-GFP import rates in temperature-sensitive *srp1-31* cells were slower and showed a lower temperature optimum. The in vivo transport defect of the *srp1-31* cells was correlated with the purified protein's thermal sensitivity, as assayed by in vitro NLS peptide binding. We show that the kinetics of NLS-directed nuclear transport in wild-type cells is stimulated by the elevated expression of *SSA1*, which encodes a cytoplasmic heat shock protein 70 (Hsp70). Elevated Hsp70 levels are sufficient to suppress the NLS-GFP import defects in *srp1-31* and *nup82-3* cells. *NUP82* encodes a protein that functions within the nuclear pore complex subsequent to docking. These results provide genetic evidence that Hsp70 acts during both targeting and translocation phases of nuclear transport, possibly as a molecular chaperone to promote the formation and stability of the Srp1p-NLS–cargo complex.

THE nuclear pore complex (NPC)[1] catalyzes the bidirectional transport of macromolecules across the nuclear envelope (Fabre and Hurt, 1994; Melchior and Gerace, 1995; Davis, 1995). The trafficking of proteins and RNAs across the NPC involves several distinct pathways that are directed by various targeting signals. Protein import is the best understood pathway and begins with the binding of nuclear localization signal (NLS)-containing proteins (Dingwall and Laskey, 1991) to a targeting apparatus that includes an ∼60-kD NLS-binding protein (Adam and Gerace, 1991; Görlich et al., 1994) and an ∼90-kD protein (Adam and Adam, 1994; Görlich et al., 1995) that is required to direct the targeting complex to docking sites at the nuclear envelope (Melchior and Gerace, 1995). EM evidence indicates that initial docking occurs at the periphery of the NPC (Akey and Goldfarb, 1989), probably on or near filaments that extend from the NPC into the cytoplasm (Richardson et al., 1988; Melchior and Gerace, 1995). In contrast to targeting, which is an ATP-independent process, translocation requires metabolic energy (Adam and Adam, 1994; Richardson et al., 1988). While a specific requirement during translocation for ATP hydrolysis has not been demonstrated, GTP hydrolysis by the Ran GTPase is required (Moore and Blobel, 1993; Melchior et al., 1993; Schlendstedt et al., 1995). Ran may function as a molecular switch to mediate the transfer of the targeting complex from the filamentous docking sites to downstream intermediates (Melchior and Gerace, 1995). Although kinetic analysis indicates that most karyophilic proteins compete for the same NLS receptors (Michaud and Goldfarb, 1993), which are presumably members of the importin/karyopherin family (Görlich et al., 1994; Powers and Forbes, 1994), U1-like snRNPs use a kinetically distinct targeting apparatus (Michaud and Goldfarb, 1991; Fischer et al., 1993). Recently, it was shown that both the basic domain NLSs and U1 snRNPs require Ran for step(s) subsequent to docking (Palacios et al., 1996).

NPCs harbor both diffusion channels and NLS-directed gated channels (Davis, 1995). The NLS-directed channels dilate to accommodate substrates of various sizes (Akey

1. *Abbreviations used in this paper: E*$_a$, coefficient of apparent energy activation; GFP, green fluorescent protein; GST, glutathione *S*-transferase; Hsp 70, heat shock protein 70; NLS, nuclear localization signal; NPC, nuclear pore complex; ts, temperature sensitive.

and Goldfarb, 1989). Several reports from the Feldherr laboratory indicate that the NLS-directed channel is under physiological control (Feldherr and Akin, 1991, 1993, 1994; Feldherr et al., 1994). Phosphorylation may regulate the function of Srp1p, the yeast NLS receptor (Azuma et al., 1995). The nuclear transport of many proteins is regulated by substrate-specific control mechanisms such as anchoring, NLS-masking, and NLS covalent modification. The import of many nuclear kinases and transcription factors is, for example, controlled by phosphorylation (Karin and Hunter, 1995). Control can be exercised during development, as with Drosophila dorsal protein (Karin and Hunter, 1995; Belvin et al., 1995), or during the cell cycle in mammalian cells (Karin and Hunter, 1995; Tagawa et al., 1995) and in yeast cells (Moll et al., 1991; Yan et al., 1993). Many proteins shuttle back and forth across the nuclear envelope; a phenomenon that is better understood now with the recent discovery of nuclear export signals (see Gerace, 1995).

A role for heat shock protein 70 (Hsp70) in nuclear transport has been suggested by several studies (reviewed in Melchior and Gerace, 1995). Hsp70 is a molecular chaperone that plays multiple housekeeping functions in several cellular compartments, mainly to assist in aspects of protein folding, targeting, and assembly. With regard to its role in nuclear transport, the import of proteins containing either the SV-40 large T antigen NLS or the nucleoplasmin NLS was inhibited by the microinjection of anti-HSP70 antibodies into tissue culture cells (Imamoto et al., 1992). In vitro studies showed that the depletion of Hsp70 from cytosolic extracts prevented karyophile import (Shi and Thomas, 1992; Okuno et al., 1993; Yang and De-Franco, 1994), although in the latter study, the hormone-dependent import of glucocorticoid receptor did not require Hsp70. Finally the ectopic expression of human Hsp70 in mouse cells complemented the defective function of a mutant SV-40 large T antigen NLS (Jeoung et al., 1991). This result indicates that Hsp70 may act to facilitate the binding of the NLS by the NLS receptor during the earliest stages of targeting.

A curious feature of the nuclear envelope is the existence of aqueous channels that are large enough to facilitate the passive transport of small (<60 kD) reporter proteins (Peters, 1986; Dingwall, 1991). Small NLS-containing proteins do not diffuse through these channels, but instead are complexed in the cytoplasm by the NLS-directed targeting apparatus and imported via an energy-dependent process (Breeuwer and Goldfarb, 1990). It is not known how generally the aqueous channels are used by soluble proteins, although a few cases have been reported (see Harootunian, et al., 1993). In image reconstructions of the pore annulus, eight putative diffusion channels appeared at the periphery of the structure, adjacent to the membrane (Hinshaw et al., 1992). It was proposed that these channels might facilitate the transfer of integral membrane proteins between the inner and outer nuclear membranes, although another NPC reconstruction suggests that these channels are normally occluded (Akey, 1995). In fact, both studies could be correct because Greber and Gerace (1995) showed that the diffusion channels in tissue culture cells may be gated by a mechanism involving luminal calcium.

Genetic and biochemical approaches in yeast have led to the identification and functional analysis of genes that have direct or regulatory roles in one or more of the nuclear transport subprocesses outlined above (Osborne and Silver, 1993; Fabre and Hurt, 1994; Davis, 1995). The mislocalization of nuclear proteins in mutant yeast cells has been visualized by immunofluorescence using either endogenous or reporter proteins, and by direct fluorescence using green fluorescent protein (GFP; Schlendstedt et al., 1995). Normal and defective import can also be partially reconstituted using semiintact cells (Schlendstedt et al., 1993). Kinetic approaches to the study of nuclear transport have not been possible in yeast, mostly because yeast cells are small and difficult to microinject. The bright visible emission of GFP fluorescence has been exploited for in situ studies in cell, developmental, and molecular biology (Marshall et al., 1995; Heim et al., 1994; Rizzuto et al., 1995). Here, we describe a quantitative nuclear import assay that uses the SV-40 large T antigen NLS (see Dingwall, 1991) fused to the 238–amino acid GFP from the jellyfish Aequorea victoria (Cody et al., 1993). The assay allows the measurement of NLS-directed import rates and passive export rates. For example, we use the assay to show that a temperature-sensitive mutation in the yeast SRP1 gene (Yano et al., 1992, 1994), which is the homologue (Azuma et al., 1995; Enenkel et al., 1995; Loeb et al., 1995) of the vertebrate 60-kD NLS-receptor family (Powers and Forbes, 1994), slows the rate and decreases the temperature optimum of NLS-GFP import. This new assay is applicable to a range of studies where the measurement of mediated import rates and passive export rates are informative.

Materials and Methods

Strains, Growth Conditions, and Materials

Previously described methods and media were used for testing and genetic analysis of general yeast mutations (Sherman et al., 1987, Sherman, 1991). Standard YPD medium, denoted glucose medium in this paper, and synthetic media (SC medium) used for growing and testing yeast strains have been described by Sherman (1991). NLS-GFP–expressing strains containing substituted srp1-31 (NOY612) and srp1-49 (NOY613) genes (Yano et al., 1994; Loeb et al., 1995) are derivatives of W303-1a (NOY388) (MATa ura3-1 leu2-3,112 his3-11 trp1-1, can1-100). nle4^{ts} strain LDY463 (Hoffman et al., manuscript submitted for publication) is a derivative of W303 (WHY12) (MATa ura3-1 leu2-3,112 his3-11 trp1-1, can1-100).

Preparation of GST-Srp1 Fusion Proteins

GST-Srp1, GST-srp1-31, and GST-srp1-49 fusion proteins were expressed in Escherichia coli from the plasmids pNOY3198 (GST-Srp1), pNOY3209 (GST-srp1-31), and pNOY3210 (GST-srp1-49) essentially described as in Azuma et al. (1995). Briefly, pNOY3209 and pNOY3210 were prepared by replacing the SacI–SpeI fragment in pNOY3198 (Azuma et al., 1995) with the SacI–SpeI fragment from pNOY163 (srp1-31) or pNOY166 (srp1-49) (Yano et al., 1994).

In Vitro NLS Peptide-binding Assay

Synthetic SV-40 T antigen NLS peptides containing the protein kinase A site (LRRASL) GYGPKKKRKVEDLRRASLG was prepared by Bio-Synthesis (Lewisville, TX). Peptide labeling was carried out in 40 μl of 20 mM Hepes, pH 7.4/1 mM EDTA/1 mM DTT/5 mM MgCl$_2$/10 μCi [γ-^{32}P]ATP containing 50 μM NLS peptide and 6 U cAMP-dependent protein kinase catalytic subunit from bovine heart (Sigma Immunochemicals, St. Louis, MO). After incubation at 23°C for 1 h, 200 μl of 20 mM Hepes, pH 7.4/1 mM EDTA/1 mM DTT/5 mM MgCl$_2$ was added, and the reaction was terminated by treating for 10 min at 80°C.

For heat treatment, GST-Srp1, GST-srp1-31, or GST-srp1-49 proteins (0.75 μM) were incubated in buffer containing 20 mM Hepes, pH 7.4/1 mM EDTA/1 mM DTT/5 mM MgCl₂ at 42°C for the prescribed times, after which 28-μl portions were removed to tubes on ice. For the NLS-binding assay, 12 μl of 8.3 μM ^{32}P-NLS peptide was added to the heat-treated proteins, and the mixture was incubated at 23°C for 20 min. 5 μl of 5 mM disuccinimidyl suberate in dimethyl sulfoxide was then added, followed by incubation at 23°C for 20 min (Partis et al., 1983). The ^{32}P-NLS peptide cross-linked to the GST fusion proteins was separated from free peptide by SDS-PAGE and analyzed by autoradiography followed by scintillation counting of gel slices.

Plasmids and DNA Manipulations

pGADGFP was constructed by Greg Hannon (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) from pGAD (Chien et al., 1991) by the fusion of the 238–amino acid (26,888 D) GFP (Prasher et al., 1992) downstream of Gal4p, the SV-40 large T antigen NLS (PKKKRKV) and amino acids 768–881 of Gal4 (Ma and Patashne, 1987). The predicted mass of the fusion protein including linker sequences is 42,818 D. The NLS-GFP fusion gene on pGADGFP is transcribed from an alcohol dehydrogenase promoter. pGAL1-SSA1, which contains the yeast SSA1 Hsp70 gene transcribed by the inducible GAL1 promoter was a gift from Elizabeth Craig (Werner-Washburne et al., 1987).

Fluorescence Microscopy

Fluorescence photomicroscopy was performed using an C-35AD-2 camera mounted on a BH-2 microscope (both from Olympus Corp., Lake Success, NY). Cells were viewed using a fluorescene excitation filter with either an Olympus SPlan or APlan 100 oil immersion objective. Kodax TMAX 400 film was processed with TMAX developer, indicator stop bath, and rapid fixer (Eastman Kodak, Rochester, NY).

NLS-GFP Import Assay

Cells were grown to early–mid-log phase (A_{600} = 0.1–0.4) in a shaking water bath or rotator in SC medium containing 2% glucose, unless otherwise noted. In some strains, the growth exceeding these densities resulted in higher cytoplasmic NLS-GFP fluorescence. 8–20 ml of cells were harvested at room temperature by pelleting in a Sorvall GLC. Cell pellets were resuspended in 1 ml ddH₂O, and the cells were repelleted at room temperature in a microcentrifuge. Adherence of the cells to the walls of the microfuge tubes was prevented by rinsing the tubes with a 0.1% BSA solution. The washed cell pellets were resuspended in 1 ml of 10 mM sodium azide ("azide"), 10 mM 2-deoxy-D-glucose ("deoxyglucose") in glucose-free SC medium and incubated for 45 min at 30°C to allow nucleocytoplasmic equilibration of NLS-GFP fluorescence. Similar results were obtained when the cells were incubated in azide/deoxyglucose on ice. The cells were then pelleted by centrifugation for 1 min in a Hermle Z229 centrifuge at 4°C, washed once with 1 ml ice-cold ddH₂O, and repelleted at 4°C. Unless indicated, import was initiated at time 0 by suspending the cells in 20–100 μl glucose-containing SC medium prewarmed to the assay temperature. For scoring, 2-μl samples of cells in solution were placed on a glass slide and mounted with a coverslip for counting at each time interval. Cells were scored during a 1-min window that terminated at the reported assay times. At least 30 cells were counted at each time point. A particular cell was scored as "nuclear" if the nucleus was both brighter than the surrounding cytoplasm and a clear nuclear–cytoplasmic boundary was visible. Consistent scoring required that the focus be adjusted onto the plane of the nuclei of each cell. Individual cells of unusually bright or weak fluorescence, or of aberrant morphology were excluded. Linear regression lines, drawn through the linear portions of the import time courses, were used to calculate relative rates.

Heat Shock and SSA1 Induction

For heat shock induction, cells grown at 30°C were pelleted and resuspended directly into medium prewarmed to 39°C, where they were maintained for 1 h. Heat-shocked cells were then processed and assayed for NLS-GFP import at appropriate temperatures, as described above. The induction of SSA1 in cells containing pGAL1-SSA1 was performed by resuspending glucose-grown cells in SC medium containing 2% galactose. Galactose induction was performed at 30°C for 1 h before performing the import assays.

Results

Relative Import Kinetics of NLS-GFP

Constitutively expressed NLS-GFP accumulated in yeast nuclei of logarithmically growing cells (Fig. 1 A). Incubating NLS-GFP–expressing cells in glucose-free medium in the presence of 10 mM azide and 10 mM deoxyglucose inhibited active NLS-directed import and resulted in the equilibration of GFP fluorescence across the nuclear envelope, presumably by the diffusive export of NLS-GFP through the nuclear pores (Fig. 1 B). The equilibration of NLS-GFP occurred at all temperatures tested, including 0°C, but only in the presence of azide/deoxyglucose. Azide or deoxyglucose alone were sufficient to promote export, although the export kinetics were slower than in the presence of both drugs. The export of NLS-GFP at 0°C indicates that it occurred by passive diffusion, which is reasonable for a 43-kD protein (Dingwall, 1991). In cells placed on ice at 0°C in the absence of azide/deoxyglucose, however, NLS-GFP remained concentrated in the nucleus for at least 3 h (Mosammaparast, N., unpublished results). This result suggests that NLS-GFP export does not occur at nearly the same rate in actively metabolizing cells as in cells that are suspended in metabolic poisons. Alternatively, NLS-GFP import was not completely inhibited by chilling on ice.

NLS-GFP import kinetics were obtained by fluorescence microscopic monitoring of the reimport of NLS-GFP after passive export. Import was initiated by washing away the azide/deoxyglucose and resuspending the cell pellet in SC medium containing a carbon source, usually glucose, at the appropriate temperature. Fig. 1 C shows cells 15 min after reimport was initiated by suspending the cells in glucose-containing medium prewarmed to 30°C. Relative import rates were quantified by counting the percent of normal cells that showed NLS-GFP nuclear accumulation as a function of time (see Materials and Methods). Relative rates, while not true rates, can be used to compare import kinetics between trials where a single variable, such as temperature or genetic background, is changed (see below). The entire assay procedure could be performed in the presence of 50 μg/ml cyclohexamide, indicating that new protein synthesis is not required for efficient posttranslational NLS-GFP import (data not shown). We also found that cells that were treated with azide/deoxyglucose could be stored on ice for up to 3 h after washing without significantly affecting the relative NLS-GFP import rate when subsequently assayed at 25°C (data not shown). NLS-GFP was excluded from the large vacuolar vesicles that often coalesced during azide/deoxyglucose treatment (Fig. 1, B and C), although large vacuoles were often present in untreated cells.

We measured NLS-GFP import in cells grown in glucose, galactose, and raffinose because these fermentable carbon sources are commonly used in the analysis of gene function using GAL-inducible promoters (see below). Time courses for NLS-GFP import in W303-1a cells, grown and processed at 30°C in SC medium containing as the sole carbon source either 2% glucose, 2% raffinose, or 2% galactose are shown in Fig. 2, A–C, respectively. Because the initial slopes of these curves are superimposable,
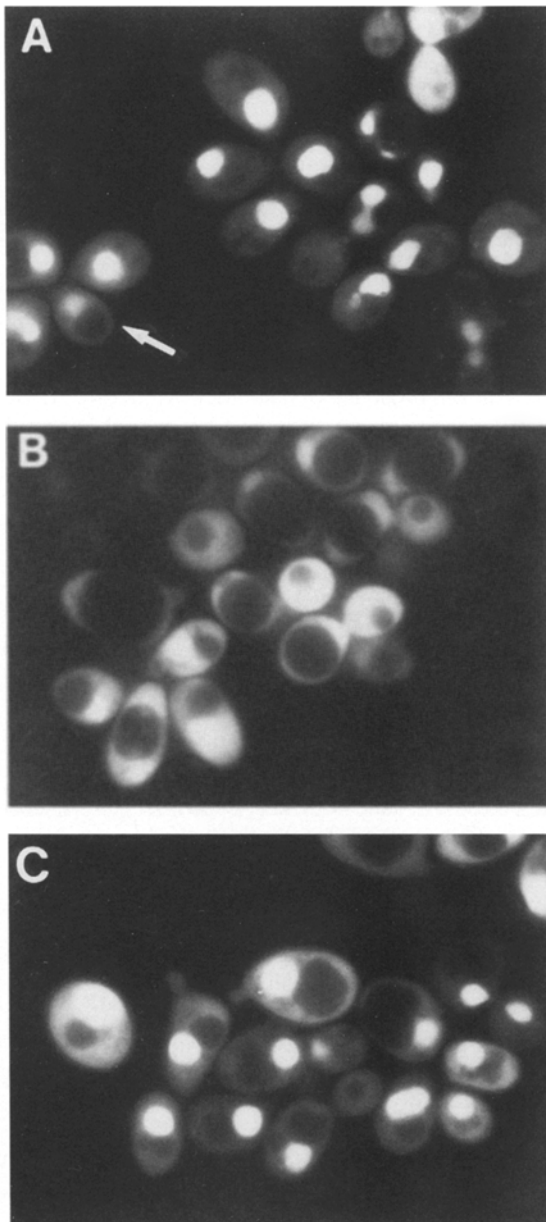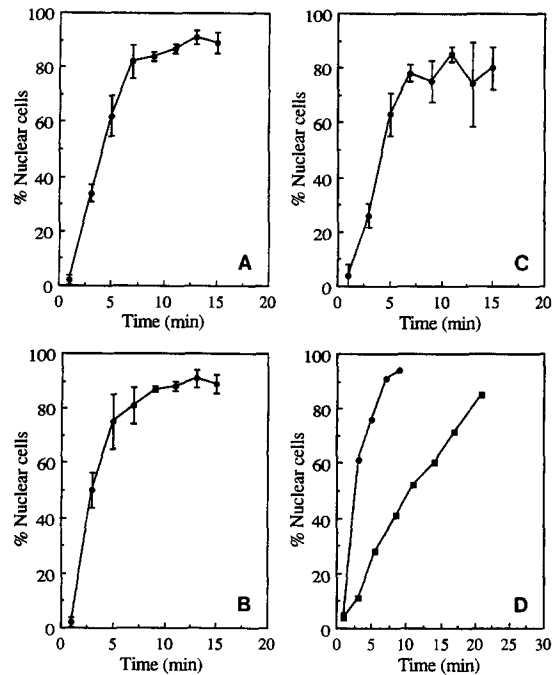
Figure 2. NLS-GFP import kinetics in cells grown in different carbon sources and in *srp1-31* cells at 35°C. W303-1a cells were cultured to OD_{600} = 0.1–0.3 at 30°C in SC medium containing either 2% glucose (A), 2% raffinose (B), or 2% galactose (C). Each point is the average of four trials. The cultures were treated identically as described in the Materials and Methods and assayed for import at 30°C in medium containing the same carbon source as used in the growth medium. Time courses for import into *srp1-31* (*filled squares*) and *SRP1* (*filled circles*) cells grown in SC-glucose medium at 30°C and assayed at 35°C are also shown (D).

Figure 1. Intracellular localization of NLS-GFP fluorescence before and after drug treatment. (A) Logarithmically growing W303-1a [pGADGFP] cells before (A), after azide and deoxyglucose treatment (B), and after recovery from drug treatment in glucose-containing medium (C). The arrow in A points to a cell that would not be scored as "nuclear."

we conclude that NLS-GFP import kinetics do not depend on whether the cells are cultured in glucose, galactose, or raffinose. Growth and metabolic rates are different in cells grown in these three carbon sources, so NLS-GFP import kinetics are not exquisitely sensitive to this parameter. A carbon source was necessary, however, since NLS-GFP import did not occur in cells resuspended in SC medium lacking a carbon source (data not shown).

For these import kinetics to be useful for the study of the transport apparatus, the rate-limiting step in NLS-GFP import should be a step directly along the transport pathway, and not be limited by the rate of ATP production af-

ter depleted cells are resuspended in complete medium. We investigated whether switching the carbon source after ATP depletion might cause a delay in ATP production and limit the rate of NLS-GFP import. As shown in Fig. 3, cells grown in glucose and shifted to either raffinose or glucose at the beginning of the time course (see legend for details) showed approximately the same relative import rates, 14 ± 1.3%/min (glucose to glucose) vs 12 ± 2.1%/min (glucose to raffinose). However, import was slower in cells shifted from glucose to galactose (6 ± 1.7%/min). Presumably, NLS-GFP import rates in cells shifted from glucose to galactose were slower because these cells, which are GAL+, required some time to adapt to galactose catabolism. We predict, therefore, that conditions or mutations that significantly reduce the capacity of the cell to rapidly regenerate ATP after energy depletion may result in slower apparent NLS-GFP import rates.

Temperature Dependence of NLS-GFP Import in Normal and Mutant Cells

Fig. 2 D shows NLS-GFP import kinetics in a temperature-sensitive (ts) NLS receptor mutant, *srp1-31* (NOY612), and its isogenic *SRP1+* parental strain, W303-1a (NOY388). Both strains were grown at 30°C and assayed at 35°C, where the relative rate of NLS-GFP import in *srp1-31* cells
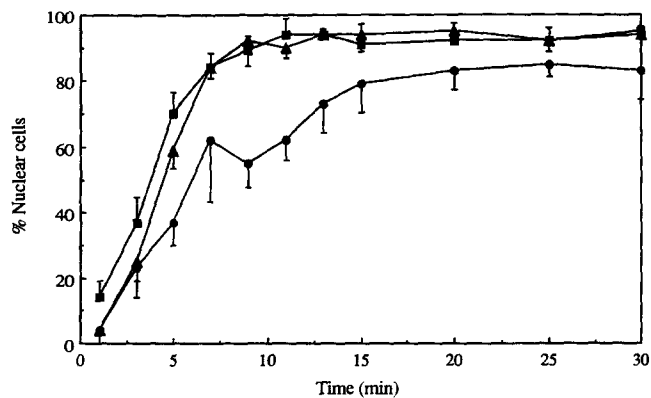
Figure 3. Effects on import rates of different carbon sources after growth in glucose. W303-1a cells cultured at 30°C in glucose were treated with drugs, as described in Materials and Methods, and then assayed at 30°C in fresh medium containing either 2% glucose (*filled triangles*), 2% raffinose (*filled squares*), or 2% galactose (*filled circles*).

was significantly slower than in the W303-1a normal strain. This result demonstrates that the temperature dependence of relative NLS-GFP import rates is an especially informative parameter for comparing nuclear import in normal and ts mutant strains.

To extend this analysis, we measured import rates in cells that were grown at the permissive temperature of 30°C and then shifted to different temperatures at T_o (see Materials and Methods). For two normal strains, W303-1a (shown in Fig. 4) and AD5 (not shown), maximum import rates occurred around 35°C. In a different W303 laboratory strain (WHY12, used below), however, import rates increased between 35 and 38°C (Hoffman et al., manuscript submitted for publication). Both relative import rates and temperature optima are strain dependent, and comparisons between mutant and normal strains should, therefore, be performed with isogenic or extensively backcrossed strains. For AD5 cells, the Arrhenius plot for import between 15 and 35°C is shown in Fig. 4 *B*. The apparent energy of activation (E_a) for the rate-limiting–mediated step in nuclear import was calculated from these data to be 11.1 kcal/mol. The E_a for import in W303-1a cells between 20–35°C was 11.9 kcal/mol (not shown), even though the relative rates of import at each temperature were higher than in AD5 cells. These high E_a values indicate that the rate limiting step in the import of the 43-kD NLS-GFP protein is mediated and not diffusion rate limited. In contrast, NLS-GFP export kinetics proceed with an E_a of ∼5 kcal/mol, which is consistent with export being either a diffusion rate–limited process or limited by dissociation from nuclear retention sites (Mosammaparast, N., personal communication).

Defective Import in Cells Containing a Temperature-conditional Mutation in the Yeast NLS-receptor Gene SRP1

The temperature dependence of import in ts mutations of the *SRP1* gene, *srp1-31* (strain NOY612), and *srp1-49* (strain NOY613) are shown in Fig. 4 *A*. NOY388 (W303-1a), NOY612, and NOY613 are isogenic strains. Maximum im-
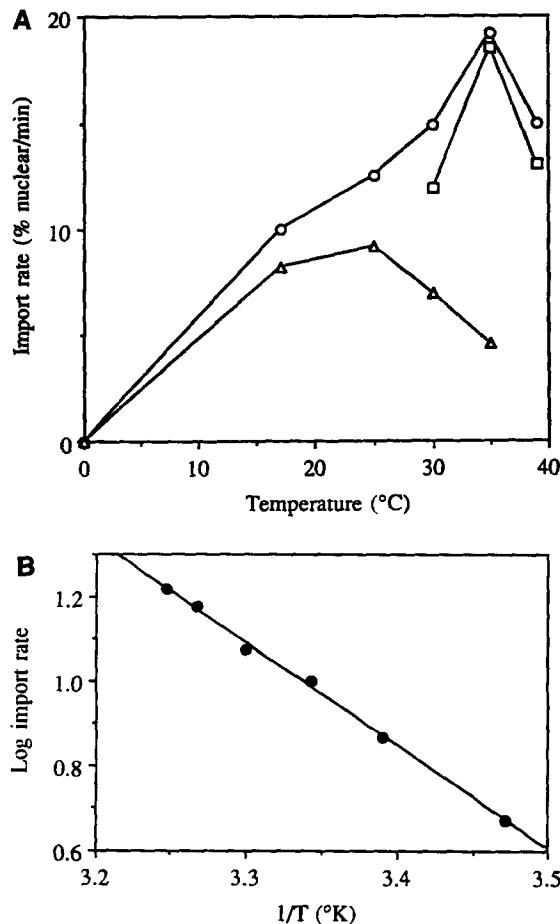

Figure 4. NLS-GFP import as a function of assay temperature. (*A*) Import rates as a function of assay temperature in isogenic *SRP1*+(W303-1a), *srp1-31*, and *srp1-49* cells. *SRP1*+ (*circles*), *srp1-31* (*triangles*), and *srp1-49* (*squares*) cells were cultured at 30°C and assayed for import at different temperatures. The initial slopes of the import kinetics were used to calculate relative rates. (*B*) Arrhenius plot of import rates in AD5 cells at different temperatures.

port rates in *srp1-31* cells occurred at ∼25°C, ∼10°C lower than in W303-1a cells (Fig. 4). In contrast, the temperature optimum for *srp1-49* cells was similar to the optimum in W303-1a cells (Fig. 4). The ts *srp1-49* growth defect, which does not become apparent until after 4 h at 38°C, is obviously less severe than the *srp1-31* growth defect, which is apparent even at 25°C (Yano et al., 1994). Because the protocol we used to assess NLS-GFP import measures import kinetics immediately upon shifting the temperature, it is possible that the *srp1-49* defect developed too slowly to be detected by this assay protocol. Alternatively, the assay may not be sensitive enough to detect the *srp1-49* defect, or the *srp1-49* mutation may affect an essential function of Srp1p that is separate from its role in nuclear import.

NLS Peptide Binding by Wild-type and Mutant Srp1 Proteins Correlates with In Vivo Transport Efficiencies

Wild-type Srp1, as well as mutant srp1-31 and srp1-49 proteins, were purified from *E. coli* as glutathione *S*-transferase (GST) fusions (see Materials and Methods) to as-

sess their NLS peptide-binding activity by in vitro assay. Previously, this assay was used to show that purified Srp1p bound specifically to NLS peptides, and that the NLS binding was competed by wild-type, but not mutant NLS peptides (Azuma et al., 1995). As shown in Fig. 5, A and B, the NLS peptide-binding efficiency of GST-Srp1p, GST-srp1-31p, and GST-srp1-49p decreased following preheat treatment at 42°C. Fig. 5 A shows normalized binding data for each protein, indicating that the NLS peptide-binding capacity of GST-srp1-31p is more sensitive to heat treatment than either the wild-type protein or GST-srp1-49p. Thus, after 5 min at 42°C, GST-srp1p lost 70% of its binding activity, whereas GST-srp1-49 lost 29% activity, and the wild-type protein, GST-Srp1p, lost only 9%. Fig. 5 B shows the results before normalization to indicate the severity of the NLS peptide-binding defect of GST-srp1-31p relative to GST-Srp1p and GST-srp1-49p. These results indicate that the in vitro NLS peptide-binding activity and thermal sensitivity of purified wild-type Srp1 and mutant srp1 proteins correlate with the temperature sensitivity of nuclear import in their respective host cells.

Heat Shock and Induction of Hsp70 Suppresses the srp1-31 NLS-GFP Import Defect

In the NLS-GFP import assay protocol described above, cells were not exposed to nonpermissive temperatures until the start of the import time course. This strategy minimizes pleotropic effects that occur when cells are kept for long times at nonpermissive growth temperatures. However, many ts mutants that express their defects only after new protein synthesis, assembly of disfunctional complexes, or during a specific cell cycle stage need to be incubated at nonpermissive temperatures for a period before a functional defect becomes apparent. With this in mind, we investigated whether or not the preincubation of srp1-31 cells at nonpermissive temperatures before performing the NLS-GFP import assay would enhance the NLS-GFP import defect. Unexpectedly, srp1-31 cells preincubated for 1 h at 39°C displayed bright nuclei and clear cytoplasms, and reimported NLS-GFP significantly faster than those maintained at 30°C before the assay. Fig. 6 shows an NLS-GFP import time course for srp1-31 cells grown at 30°C and either maintained at 30°C or shifted to 39°C for 1 h before the assay, which was performed at 37°C. Preincubation of srp1-31 cells at 39°C resulted in import kinetics that were at least as rapid as in non–heat-shocked normal cells. At 39°C, general protein synthesis is inhibited, and several heat shock proteins are induced. One of these heat shock proteins, Hsp70, has been previously implicated in nuclear transport in mammalian cells (see Melchior and Gerace, 1995). Therefore, we tested whether the induction of Hsp70 alone could emulate the heat shock suppression of the srp1-31 import defect. As shown in Fig. 6, the galactose induction of a plasmid-encoded copy of SSA1 was sufficient to suppress the NLS-GFP import defect in srp1-31 cells at 37°C. SSA1 encodes one of the four major cytosolic Hsp70s in yeast (Craig et al., 1994).

We then asked whether Hsp70 induction could stimulate nuclear transport rates in SRP1+ cells. Fig. 7 shows NLS-GFP import kinetics in SRP1+ cells containing pGAL1-SSA1 grown at 30°C and assayed at 15°C. Clearly, the ap-
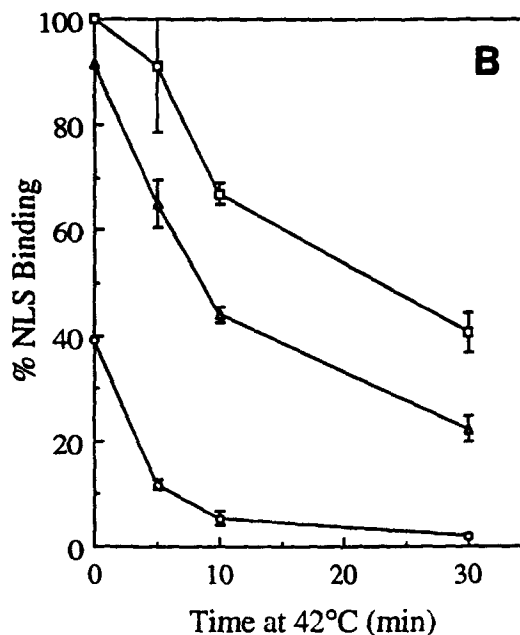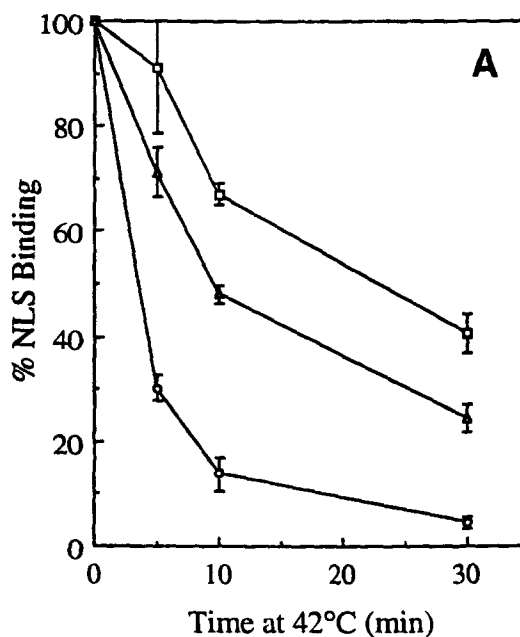

Figure 5. Thermal sensitivity of NLS peptide-binding to GST fusion proteins. (A) Binding of ^{32}P-NLS peptides by GST-Srp1 (squares), GST-srp1-31 (circles), and GST-srp1-49 (triangles) fusion proteins was assessed after treatment at 42°C (see Materials and Methods). Initial binding efficiencies were normalized to 100% for each protein. (B) Binding data plotted without normalization. Before heat treatment, the NLS peptide binding by GST-srp1-31 relative to GST-Srp1 was 39 ± 3.6%, and that of GST-srp1-49 compared to GST-Srp1 was 92 ± 7.6%.

parent NLS-GFP import rate was significantly faster in cells overexpressing Hsp70. Because we show below that the induction of SSA1 can also suppress mutations that function downstream of targeting, we do not know which stage(s) along the import pathway was stimulated by the
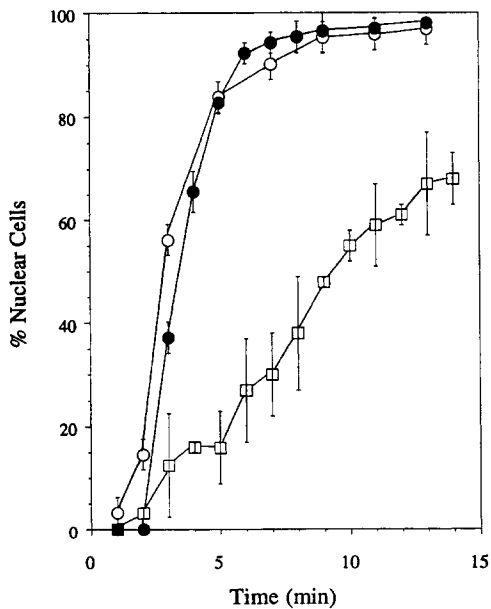
Figure 6. Heat shock or the induction of *SSA1* suppress the NLS-GFP import defect in *srp1-31* cells. NLS-GFP import in *srp1-31* cells was analyzed at 39°C before (*squares*) and after either heat shock (*circles*) or *SSA1* induction (*filled circles*; see Materials and Methods).

Figure 7. Elevated Hsp70 level stimulates the rate of NLS-GFP import in wild-type cells. W303 cells were grown at 30°C and assayed at 15°C. p*GAL-SSA1*-transformed cells were grown in glucose (*circles*) or galactose-containing medium (*filled circles*). Cells lacking the p*GAL-SSA1* plasmid were grown in the presence of galactose (*filled squares*).

induction of Hsp70 in wild-type cells. The stimulation of NLS-GFP import kinetics in *SRP1*⁺ cells does, however, demonstrate that Hsp70 can stimulate nuclear transport by a mechanism that is independent from its postulated capacity to refold mutant or damaged proteins, for example, srp1-31p.

When NLS-GFP import was assayed in *SRP1*⁺ cells at 25, 30, or 35°C after a 1-h heat shock at 39°C, less striking but statistically significant increases in relative NLS-GFP import rates were observed (Roberts, P., unpublished observations). We can explain why Hsp70 induction stimulates import better at low temperatures if we begin with the reasonable assumption that nuclear import is a multistep process composed of several relatively slow steps. Then, if we postulate that some of these steps have different temperature coefficients (E_as), the rate-limiting step in the overall process will have the potential to switch between steps as a function of temperature. Hsp70 may stimulate steps that are rate limiting at 15°C, but less so at higher temperatures at which other steps become rate limiting.

Heat Shock and Induction of Hsp70 Suppresses the nup82-3 NLS-GFP Import Defect

Our working hypothesis for the function of Hsp70 in nuclear transport includes its participation during both the targeting and translocation phases of NLS-directed nuclear import. This notion was suggested by the competition import studies (Okuno et al., 1993), which showed that the import of Hsp70 into permeabilized cell nuclei required the concomitant import of NLS cargo. Hsp70 did not accumulate in these nuclei unless NLS cargo was included in the soluble extract. Furthermore, Hsp70 import was not competed by saturating concentrations of NLS
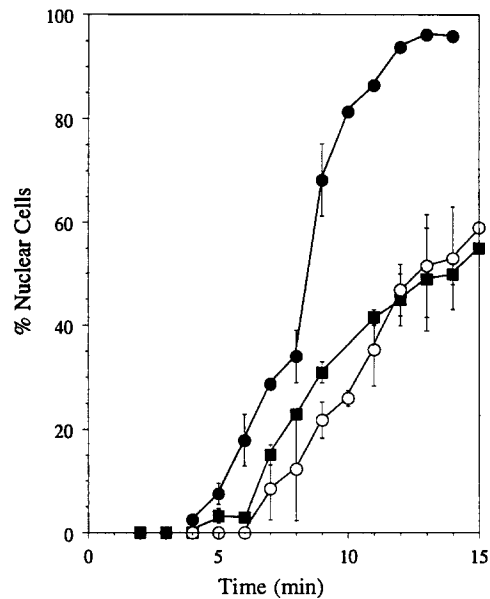
cargo (Okuna et al., 1993). These observations are consistent with Hsp70 functioning as a shuttling transport factor whose import is stimulated, but not competed, by the NLS cargo import. By analogy, we predict that the import of Srp1p, a shuttling transport factor, will be noncompetitively stimulated by NLS-cargo. To test if Hsp70 participated during steps subsequent to targeting and docking, we asked if *SSA1* induction would suppress NLS-GFP import defects caused by a ts mutation in a nucleoporin gene, *NUP82*, that functions downstream of Srp1p-mediated targeting.

NUP82 encodes an FG nucleoporin that was identified by genetic and biochemical methods (Grandi et al., 1995; Hurwitz and Blobel, 1995; Belanger et al., 1994). Genetic evidence (Hoffman et al., manuscript submitted for publication) indicated that Nup82p functions downstream of Nup2p, which is a NPC-associated XFXFG nucleoporin that has been proposed to serve as a docking site for Srp1p-NLS cargo-targeting complexes (Belanger et al., 1994). The hypothesis that Nup82p functions during translocation is supported by the observations that after temperature shifts, the ts allele *nup82-3* caused rapidly developing defects in both NLS-directed protein import (Hoffman et al., manuscript submitted for publication) and mRNA export (Grandi et al., 1995; Hurwitz and Blobel, 1995; Hoffman et al., manuscript submitted for publication).

We showed previously that *nup82-3* cells displayed a severe temperature conditional NLS-GFP import defect (Hoffman et al., manuscript submitted for publication). Fig. 8 shows that either heat shock or *SSA1* induction suppressed the *nup82-3* import defect at 39°C. These data indicate that Hsp70 has access to and functions within the NPC translocation apparatus at sites downstream of docking. We have also observed that Hsp70 induction sup-
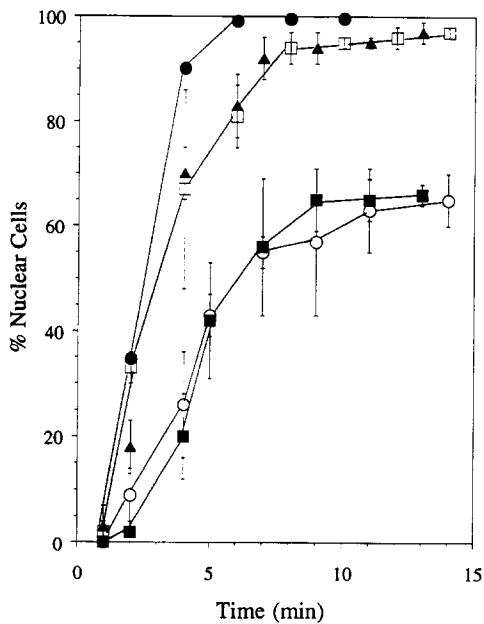
Figure 8. Heat shock or the induction of *SSA1* suppress the NLS-GFP import defect in *nup82-3* cells. Cells were treated as described in Materials and Methods. Except where indicated all cells were grown at 30°C, heat shocked at 39°C, and assayed at 39°C. Non–heat-shocked *NUP82*^{+} cells (W303 strain WHY12) grown in glucose and assayed at 37°C (*closed circles*), non–heat-shocked *nup82-3* cells (LDY463; *open circles*), heat-shocked *nup82-3* cells (*open squares*), and non–heat-shocked *nup82-3* cells containing the p*GAL-SSA1* plasmid grown in glucose (*closed squares*) or grown in galactose (*closed triangles*).

presses the NLS-GFP import defects caused by deletion or ts mutation of the *NUP188* gene (Shulga, N., R. Wozniak, D.S. Goldfarb, manuscript in preparation). Biochemical, structural, and genetic evidence indicates that Nup188p, a nonessential nucleoporin, is associated with Pom152p, an integral nuclear membrane protein, and with other non–repeat-containing nucleoporins that may form the octagonal core structure of the NPC (Aitchison et al., 1995; Nehrbass et al., 1996). Although it is possible that Nup188p plays a direct role in NLS cargo translocation, mutations in *NUP188* may affect NLS-GFP import by indirectly impairing the function of the more centrally located translocation apparatus. Based on the suppression of the NLS-GFP import defects in *nup82-3* (Fig. 8) and *nup188Δ* and ts mutant cells, we conclude that Hsp70 participates in nuclear transport during both targeting and subsequent translocation steps.

Discussion

NLS-GFP Import Kinetics

The NLS-GFP import assay described here allows the measurement of relative import rates by a simple protocol that involves scoring the localization of NLS-GFP fluorescence in individual cells as a function of time. The practical advantages of this assay over previous methods to assess nuclear transport in yeast include the capacity to quantify relative import rates in normal cells under a variety of

physiological conditions, for example, before and after heat shock, and the demonstration of temperature conditional import defects in mutant cells, for example, in *srp1-31* and *nup82-3* cells.

For purposes such as those summarized above, the relative rates obtained by the statistical counting method described in the Materials and Methods provide an adequate measure of import efficiency in normal and mutant strains under various physiological conditions. The calculation of the apparent energy of activation for import is a good example of an analysis where relative rates are applicable (Fig. 4 *B*). The Arrhenius energy of activation, E_a, is determined by the slope of rates obtained at different temperatures, and is independent of the magnitude of the actual rates. The values obtained, 11–12 kcal/mol, indicate that the rate-limiting step for NLS-GFP import is mediated and not diffusion limited. It should be noted, however, that the apparent straight line in the Arrhenius plots do not prove the existence of a single rate-limiting step, and as discussed above in connection with the temperature dependency of the Hsp70 stimulation of NLS-GFP import rates, the possibility exists that rate-limiting steps are different at different temperatures.

The effect of different carbon sources on the rate of NLS-GFP import (Fig. 3) suggests that the rate at which the cells regenerate ATP after drug treatment can influence the apparent rate of NLS-GFP import. Although complicated and poorly understood, aerobically grown *Saccharomyces cerevisiae* oxidizes glucose predominantly via anaerobic fermentation instead of aerobic respiration, which is more efficient but slower (Lagunas, 1986). While raffinose and galactose are metabolized less rapidly than glucose, the rates of NLS-GFP import were virtually identical in cells grown in these three carbon sources. Therefore, the NLS-GFP assay is not sensitive to the different rates of raffinose and galactose catabolism. The slower import rates observed in cells grown in glucose and shifted to galactose at the beginning of the import time course is presumably caused by a delay in ATP production while the cell adapts to galactose catabolism. Although this specific hypothesis has not been tested, it is fair to predict that physiological conditions or mutations that significantly reduce rates of ATP generation will cause apparent reductions in the relative rate of NLS-GFP import.

We do not fully understand the dynamic kinetic relationship between active NLS-GFP import and passive NLS-GFP export. We have only observed NLS-GFP export in cells treated with metabolic inhibitors and not in metabolically active cells, even at 0°C, a temperature at which import but not passive export should be inhibited. Thus, the passive export of NLS-GFP may not occur to a significant level in metabolically active cells. It is also possible, however, that passive export does take place at 0°C, and that it is counterbalanced by a small residual import in the absence of metabolic inhibitors, leading to the apparent absence of export. This question is currently under investigation.

Relative Import Rates in ts Cells

As shown above, we found that the absolute rates and temperature optima for import in *srp1-31* cells were lower

than in normal strains. We conclude that these lower apparent rates reflect a defect in the nuclear transport apparatus. An attractive feature of the NLS-GFP import assay protocol is that the cells are grown at permissive temperatures, then shifted to the assay temperature only during the 10–15-min period of the assay. Thus, defects in import reflect an almost instantaneous response to the assay temperature. By visual inspection, the nucleocytoplasmic distribution of NLS-GFP in cells grown at 30°C is virtually identical in wild-type and *srp1-31* mutant cells. The apparent initial rate of NLS-GFP import in *srp1-31* cells at 30°C, however, is reproducibly only ~50% of the rate in normal cells (Fig. 4 *A*). Since *srp1-31* cells (doubling time = 296 min) grow at about one third of the rate of wild-type cells (doubling time = 107 min) at 30°C in glucose-SC medium, one would not necessarily expect a decreased import rate in these cells to result in the steady-state cytoplasmic accumulation of NLS-GFP. It is clear that merely inspecting the nucleocytoplasmic distribution of nuclear proteins in mutant cells, as has been done often in earlier studies, is an insensitive method to detect defects in nuclear transport rates. The present method, which measures the rate of nuclear protein import directly and independently of growth rates, can circumvent this problem.

The demonstration of a significant import rate defect in *srp1-31* cells validates the application of the NLS-GFP rate assay to the study of nuclear transport in temperature-conditional mutants. Initially identified as a suppressor of a mutation in a subunit of RNA polymerase I (Yano et al., 1992), the *SRP1* gene is one of only a few yeast genes whose function in nuclear transport has been elucidated. Genetic analysis indicates that the Srp1 protein interacts with the NPC proteins Nup1p and Nup2p (Belanger et al., 1994), and the *srp1-31* mutation was recently shown to cause the mislocalization of nuclear proteins both in vivo and in vitro (Loeb et al., 1995). Furthermore, purified Srp1p binds NLS peptides in vitro (Görlich et al., 1995; Azuma et al., 1995), and using a permeabilized tissue culture cell assay, Srp1p was able to functionally replace the vertebrate cytosolic NLS receptor (Enenkel et al., 1995).

The defect in import kinetics in *srp1-31* cells is apparent immediately upon shifting the cells from permissive to nonpermissive temperatures (Fig. 4). This suggests that the ts defect in nuclear transport in *srp1-31* cells is a direct result of the temperature-induced disfunction of srp1-31p in its role as the NLS receptor. This conclusion is supported by the NLS peptide-binding data, which demonstrate that purified GST-srp1-31p is less active and more sensitive to heat treatment than either GST-srp1-49p or wild-type GST-Srp1p.

It is interesting that a mutation in *SRP1* causes a reduction in the rate of NLS-directed import. This may occur because Srp1p mediates the rate-limiting step in import, or the *srp1-31* mutation slows an otherwise non–rate-limiting, Srp1p-mediated step enough for it to become the rate-limiting step. In vertebrate tissue culture cells, the slow step in import is thought to occur after docking because microinjected NLS proteins transiently accumulate at the nuclear periphery before concentrating in the nucleoplasm (Richardson et al., 1988; Breeuwer and Goldfarb, 1991; Pruschy et al., 1995). This does not prove, however, that the step

after docking is the rate-limiting step in yeast, or for that matter in tissue culture cells. Because the minimal substrate for both targeting and translocation is likely to be the Srp1p–NLS cargo complex, the maintenance of this complex will be critical for progression along the multistep import pathway. In cells containing mutant Srp1p receptor, the dissociation or steric alteration of the mutant Srp1p–NLS cargo complex might be sufficient to confer rate-limiting status upon any targeting, docking, or translocation step that recognizes the complex as substrate.

Role of Hsp70 in Nuclear Transport

It has been apparent for some time that Hsp70 is present in and functions in the nucleus during heat shock (see Pelham, 1984). A general nuclear housekeeping function for Hsp70 was also suggested by the observation that Hsp70 shuttles between the nucleus and cytoplasm of *Xenopus oocytes* (Mandell and Feldherr, 1990). There is evidence that one of these functions is to chaperone NLS cargo across the nuclear envelope (see Melchior and Gerace, 1995). In the present study, we show that the NLS-GFP import defects caused by the *srp1-31* and *nup82-3* mutations are suppressed either by heat shock or by the elevated expression of an Hsp70. Furthermore, induction of Hsp70 stimulates NLS-GFP import kinetics in wild-type cells. These gain-of-function phenotypes provide an assay for the in vivo molecular analysis of Hsp70 function in nuclear transport.

The suppression of the ts NLS-GFP import defect of *srp1-31* cells by elevated Hsp70 levels can be explained by several mechanisms. First, Hsp70 may function as a molecular chaperone to facilitate the binding of NLS cargo by Srp1p by binding directly to NLS peptides (Dingwall and Lasky, 1992). Several independent lines of research support the hypothesis that Hsp70 functions to facilitate the presentation of NLSs to the NLS receptor during the targeting phase of nuclear transport (see below). The hypothesis we favor is that Hsp70 binds and dissociates from the NLS, thereby maintaining its solubility and accessibility to free cytoplasmic Srp1p. In this fashion, Hsp70 increases the available concentration of the NLS. A peptide-binding and release cycle for Hsp70 has been proposed to explain its chaperone function (Palleros et al., 1993). In this model, the ADP form of Hsp70 binds stably to peptide targets. Subsequent exchange of ATP for ADP induces a conformational change in the enzyme that promotes dissociation. ATP hydrolysis then converts Hsp70 back to the high affinity ADP form, which may rebind and repeat the cycle. Second, elevated levels of Hsp70 might function to maintain mutant srp1-31p in a functional conformation at nonpermissive temperatures. This hypothesis is consistent with the proposed mechanism for Hsp70 function during stress response, where it is thought to assist in the refolding of denatured polypeptides. It is a fact, though, that few ts mutations are suppressed by heat shock. The one case we know of where overexpression of Hsp70 suppressed a ts mutation involved the specific interaction of *E. coli* dnaK, a procaryotic Hsp70 homologue, with a mutant dnaA protein (Hwang and Kaguni, 1991). Also, the stimulation of NLS-GFP import by Hsp70 in normal *SRP1+* yeast cells argues that Hsp70 cannot be acting only through

folding or repair pathways. Third, using its high affinity for NLS peptides and its shuttling capacity, Hsp70 might replace the defective srp1p as the NLS-targeting receptor. In the same vein, Hsp70 might facilitate the binding of the NLS-GFP to a second, as-yet undiscovered NLS receptor. An Srp1p-independent parallel targeting pathway has been proposed for U1-like snRNPs (See Michaud and Goldfarb, 1991).

In vitro binding studies support the notion that Hsp70 functions during the import process by binding directly to the NLS. Hsp70 target sequences located within a few proteins have been delineated and, in addition to containing the expected hydrophobic amino acids, most contain basic amino acids, including the COOH-terminal 23 amino acids of p53 (SS<u>HLK</u>S<u>KK</u>GQSTS<u>RHKK</u>LMF<u>K</u>TE GPDSD), which resembles the bipartite nucleoplasmin NLS (see Hightower et al., 1994). Screening of a random peptide library revealed that bovine Hsp70 binds tightly to both hydrophobic (e.g., FYQLALT) and basic heptapeptides (e.g., NIV<u>RKKK</u>), although only the hydrophobic heptapeptides stimulated the Hsp70 ATPase (Hightower et al., 1994; Takenaka et al., 1995).

In a separate analysis, a 69-kD rat liver nuclear envelope–associated protein that turned out to be Hsp70 (Imamoto et al., 1992) bound specifically to synthetic NLS peptides (Imamoto-Sonobe et al., 1990). In a permeabilized cell nuclear transport assay, rabbit RBC Hsp70 accumulated in nuclei coincident with the import of NLS substrate (Okuno et al., 1993). While this is consistent with the idea that Hsp70 crosses the nuclear envelope in a complex with the NLS substrate, the import of Hsp70 under these conditions was not competed by the addition of saturating concentrations of NLS substrate, suggesting that Hsp70 import occurs by a distinct mechanism. One explanation for this observation is that Hsp70 is itself a transport factor that enters the nucleus in association with the Srp1p-NLS cargo (see below). The import of transport factors that accompany NLS cargo into the nucleus should be stimulated, but not competed, by the availability of NLS cargo, even at saturating concentrations, where the import of rates approach maximum velocity. Finally, the observation that human Hsp70 complemented a T antigen NLS mutation in mouse cells (Jeoung et al., 1991) suggests that Hsp70 can stimulate the presentation of a mutant NLS to its receptor. Together with our findings in srp1-31 cells, these results suggest that Hsp70 acts to facilitate the initial interaction between the NLS and the NLS-receptor. The suppression of the nup82-3 and nup188Δ NLS-GFP import defects by elevated Hsp70 levels indicates that Hsp70 also participates in steps subsequent to Srp1p-mediated targeting.

How could elevated levels of Hsp70 suppress mutations at multiple steps along the import pathway? As described above, it is likely that NLS cargo is escorted across the nuclear envelope in a multicomponent complex that minimally includes Srp1p. We have already proposed a mechanism that explains how Hsp70 could promote the initial formation of the NLS-Srp1p–targeting complex. Hsp70 induction may suppress downstream defects, such as those caused by nup82-3 and nup188Δ mutations, by helping to maintain the integrity of the Srp1p–NLS complex during translocation. Mutations such as nup82-3 that reduce the efficiency of translocation may directly or indirectly cause

the destabilization of the translocation-competent NLS cargo–Srp1p complex. The premature dissociation of the translocation complex could derail the sequential translocation process and reduce import rates. Hsp70 could counter these effects by promoting the stable association between the NLS and Srp1p. Elevated Hsp70 levels could therefore suppress both targeting and translocation defects by the same mechanism. Alternatively, Hsp70 may interact with different transport components along the import pathway, and suppression could result from these unique interactions. Either way, the suppression of both targeting and translocation defects indicates that Hsp70 has access to transport intermediates deep within the NPC translocation channel. This notion is consistent with Hsp70 either being a stoichiometric component of the NLS cargo–containing translocation complex or, alternatively, being a soluble factor that is available to transiently interact with the complex. The stimulatory effects of elevated Hsp70 levels on NLS-GFP import are most consistent with the latter hypothesis, which would predict a concentration dependence.

In conclusion, the present work demonstrates the utility of the newly developed NLS-directed import assay system in yeast, and emphasizes the physiological importance of the NLS peptide-binding reaction carried out by the NLS receptor protein Srp1p, presumably with the help of Hsp70, in determining the overall rate of nuclear protein import. This in vivo assay may be adaptable to other cell types.

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