A Mutant Nuclear Protein with Similarity to RNA Binding Proteins Interferes with Nuclear Import in Yeast

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We have isolated mutants of the yeast *Saccharomyces cerevisiae* that are defective in localization of nuclear proteins. Chimeric proteins containing the nuclear localization sequence from SV40 large T-antigen fused to the N-terminus of the mitochondrial F_1F_0 -ATPase are localized to the nucleus. *Npl* (nuclear protein localization) mutants were isolated by their ability to grow on glycerol as a consequence of no longer exclusively targeting SV40- F_1F_0 -ATPase to the nucleus. All mutants with defects in localization of nucleolar proteins and histones are temperature sensitive for growth at 36°C. Seven alleles of *NPL3* and single alleles of several additional genes were isolated. *NPL3* mutants were studied in detail. *NPL3* encodes a nuclear protein with an RNA recognition motif and similarities to a family of proteins involved in RNA metabolism. Our genetic analysis indicates that *NPL3* is essential for normal cell growth; cells lacking *NPL3* are temperature sensitive for growth but do not exhibit a defect in localization of nuclear proteins. Taken together, these results indicate that the mutant forms of *Npl3* protein isolated by this procedure are interfering with nuclear protein uptake in a general manner.

INTRODUCTION

Certain proteins enter the nucleus by recognition of a nuclear localization sequence (NLS) in the transported protein (Silver, 1991). After NLS-dependent binding either in the cytoplasm or at the nuclear pore complex, proteins are transported through the pore in an ATP-dependent manner. This two-step model is based on observations made on the behavior of proteins microinjected into cells (Richardson *et al.*, 1988) or introduced into *in vitro* nuclear import reactions derived from extracts of *Xenopus* oocytes (Newmeyer and Forbes, 1988) or semipermeabilized mammalian cells (Adam *et al.*, 1990; Adam and Gerace, 1991).

Many nuclear proteins contain their own NLS, whereas some enter the nucleus coupled to another NLS-bearing protein (Garcia-Bustos *et al.*, 1991; Silver, 1991). Proteins have been identified that specifically interact with NLSs and have the properties expected for receptors for nuclear imported proteins (Adam *et al.*, 1989; Benditt *et al.*, 1989; Lee and Melese, 1989; Li and Thomas, 1989; Silver *et al.*, 1989; Yamasaki *et al.*, 1989; Imamoto-Sonobe *et al.*, 1990; Meier and Blobel, 1990; Stochaj *et al.*, 1991). Recent experiments indicate that a cytoplasmic NLS-binding protein is necessary for

faithful nuclear protein uptake in semi-permeabilized cells (Adam and Gerace, 1991; Stochaj and Silver, 1992), lending support to the notion of a receptor that shuttles proteins from the cytoplasm to the nucleus. Further support for this comes from the observation that certain mutant forms of SV40 T-antigen block import of other nuclear proteins (Schneider *et al.*, 1988). Disruption of import by these mutated proteins depends on the presence of a wild-type NLS in T-antigen. One interpretation of these findings is that the mutant T-antigens are sequestering some component (such as a receptor) necessary for nuclear protein import.

Recent evidence indicates that there is at least one other pathway distinct from recognition of certain NLSs by their corresponding receptors (reviewed in Goldfarb and Michaud, 1991). Microinjection experiments in *Xenopus* oocytes showed that titration of the components that are required for SV40 T-antigen NLS-dependent import did not alter the rate of import of U2 snRNPs (Michaud and Goldfarb, 1991). Further analysis has led to the proposal that the M_3 GpppN cap present on some RNAs is part of a unique NLS, which in turn has its own receptor for nuclear import (Fischer *et al.*, 1991).

A third class of proteins move both in and out of the nucleus. For example, Borer *et al.* (1989) showed that nucleolin, which in steady state is found mainly in the nucleolus, actually shuttles between the nucleus and the cytoplasm. They suggest that such proteins may play a role in the import of ribosomal proteins from the cytoplasm. Mandell and Feldherr (1991) also have shown that two HSP70 proteins recycle across the nuclear envelope in *Xenopus* oocytes.

Thus far, all macromolecules have in common that they pass through the pore to enter the nucleus (Feldherr *et al.*, 1984). Nuclear pores are formed by large protein complexes of $\sim 10^8$ Da (Reichelt *et al.*, 1990) that are embedded in the nuclear membranes. Although pore complexes can be isolated in semipure form, only a few component proteins have been characterized and little is known about their function (Berrios *et al.*, 1983; Gerace *et al.*, 1984; Davis and Blobel, 1986; Finlay *et al.*, 1987; Hanover *et al.*, 1987; Snow *et al.*, 1987; Davis and Fink, 1990; Nehrbass *et al.*, 1990).

To study nuclear protein import in more detail, a scheme for isolating yeast mutants defective in nuclear protein localization (termed *npl*) was implemented by this laboratory several years ago (Sadler *et al.*, 1989). Our approach relied on several assumptions: 1) import of nuclear proteins is an essential process and, as such, we should seek conditional mutants; 2) we could isolate mutants at a semipermissive temperature that demonstrated only a partial defect in protein localization; and 3) we could select for the mislocalization of a normally nuclear protein to another cellular compartment. Three genes, *NPL1* (Sadler *et al.*, 1989), *NPL2*, and *NPL6* (Chiang and Silver, unpublished data), have been identified by the original screening procedure.

We now report the isolation of seven alleles of a new gene, *NPL3*, one allele of *NPL4*, as well as one new allele of *NPL1* using a modification of the original screen (Sadler *et al.*, 1989). The new scheme uses the missorting of a fusion protein containing an NLS fused to $F_1\beta$ -ATPase, a soluble enzyme of the mitochondrial matrix. All mutants are temperature sensitive for growth and show mislocalization of nucleolar proteins and histone H2B. The *NPL3* gene has been isolated and encodes a nuclear protein with sequences associated with RNA binding and a region of identity to fibrillarins (Schimmang *et al.*, 1989; Henriquez *et al.*, 1990; Lapeyre *et al.*, 1990), nucleolins (Lapeyre *et al.*, 1987), the yeast Ssb1 (Jong *et al.*, 1987), Nsr1 (Lee *et al.*, 1991), and Gar1 (Girard *et al.*, 1992). The genetic analysis presented here demonstrates that *NPL3* is essential for normal cell growth. The mutations in *NPL3* isolated in this study result in a general block of protein import into the nucleus, perhaps by interacting with a component required for nuclear protein uptake.

MATERIALS AND METHODS

Construction of SV40-ATP2 Gene Fusions

The HindIII site of pWT (1-14) (Bedwell *et al.*, 1989), which contains the *ATP2* gene encoding the mature $F_1\beta$ -ATPase with the first 14 amino acids of the mitochondrial signal sequence, was converted to an *Xho* I site. A fragment from pMN8 (Nelson and Silver, 1989) containing the yeast *ADH1* promoter and DNA encoding the SV40 T-antigen NLS was inserted into the *Xho* I site so that the SV40 NLS was in frame with pre- $F_1\beta$ -ATPase. The resulting plasmid (pNLS- $F_1\beta$; Figure 2) contained the SV40-*ATP2* gene fusion, the yeast *URA3* gene, and 2 μ yeast origin of replication. An identical construct in which a mutant form of the SV40 NLS (Kalderon *et al.*, 1984) replaces the wild-type sequence was constructed in similar fashion (pNLS*- $F_1\beta$; Figure 2).

To alter the ATG codon at the start of the *ATP2* coding sequence, a synthetic oligonucleotide (5'-TGGTAGAACCCCTCT-3') was used for site-directed mutagenesis to change an AT to GG in the DNA encoding the mitochondrial targeting sequence, resulting in a GGG codon for glycine instead of the ATG methionine codon. The HindIII-Kpn I fragment from pNLS- $F_1\beta$ was placed into M13mp19, and the oligonucleotide was used for site-directed mutagenesis (Sambrook *et al.*, 1989) using the dual primer method with the -40 M13 sequencing primer. The labeled oligonucleotide was used to screen for plaques containing the appropriate base changes and confirmed by DNA sequencing. RF-DNA was prepared from the phage and used to transfer this region back into pNLS- $F_1\beta$ and is now referred to as pNLS- $F_1\beta^{Gly}$. An equivalent change was made to pNLS*- $F_1\beta$ resulting in plasmid pNLS*- $F_1\beta^{Gly}$ (Figure 2).

Isolation of Mutants

Cells (20 ml at $\sim 2 \times 10^7$ cells/ml of SEY6215 or AVY4-1 [see Table 3] containing pNLS- $F_1\beta$ [see Figure 2]) cultivated in uracil dropout media with 2% glucose were collected by centrifugation ($2600 \times g$, 3 min) and resuspended in 3 ml 0.1 M sodium phosphate, pH 7. Cells were mutagenized with 10 or 50 μ l ethyl methanesulfonate (EMS) for 1 h at 30°C, and the mutagenesis was stopped by the addition of 9 ml 5% sodium thiosulfate. The levels of mutagen used resulted in 10 and 90% cell death, respectively. After addition of sodium thiosulfate, cells were collected by centrifugation ($2600 \times g$, 5 min), resuspended in 1 ml H₂O, and 50–100 μ l plated onto yeast extract/peptone (YEP) plates with 3% glycerol. After 4–5 d at 30°C, potential mutants (Gly+ colonies) were restreaked onto YEP/2% glycerol at 30 and 36°C. Mutants that failed to grow at 36°C were checked for growth on YEP/2% (YEPD) at 36°C. Clones that failed to grow at 36°C under both growth conditions were examined by immunofluorescence with antibodies to histone H2B and nucleolar proteins after growth at 30°C. Mutants that also showed defects in nuclear protein localization were cured of plasmids and rechecked for the ability to grow on glycerol after transformation with nonmutagenized pNLS- $F_1\beta$ and pNLS- $F_1\beta^{Gly}$.

Immunofluorescence Analysis of Mutants

Cells were prepared for immunofluorescence as previously described (Sadler *et al.*, 1989). To visualize histone H2B, rabbit anti-histone H2B (from M. Grunstein, University of California at Los Angeles) was used at a dilution of 1:1000 followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Miles Scientific, Naperville, IL) at 1:1000 dilution. To visualize nucleolar-associated proteins, a mix of three mouse monoclonal antibodies that react with two nucleolar proteins of unknown identity (from J. Broach, Princeton University, Princeton, NJ) was used at a 1:1000 dilution followed by FITC-conjugated anti-mouse IgG at 1:1000. To visualize SV40-invertase chimeras, rabbit antiserum raised against the cytoplasmic form of yeast invertase was used at 1:1000 dilution followed by FITC anti-rabbit IgG as described above. To visualize Kar2, rabbit anti-Kar2 serum (Rose *et al.*, 1989), at a dilution of 1:10 000, or a mouse monoclonal anti-Kar2 antibody (from M. Rose and J. Vogel, Princeton Uni-

versity), at a dilution of 1:10 followed by the appropriate FITC or Texas Red (Jackson ImmunoResearch, West Grove, PA) conjugated secondary antibodies, were used. Nuclei were visualized by treatment with DAPI (Sadler *et al.*, 1989). Cells were viewed at $\times 1000$ with a Zeiss (Thornwood, NY) Axioskop equipped for fluorescence. Exposure times for immunofluorescence pictures were 15–30 s.

For nuclear protein retention experiments, cells were grown to a density of $\sim 5 \times 10^6$ cells/ml in uracil dropout media with 2% ethanol at 22°C to induce expression of nuclear associated SV40-invertase under the control of the *ADH2* promoter. Cells were then collected by centrifugation at $2600 \times g$ for 5 min and resuspended in YEPD. After incubation at 30°C for 4 h, cells were prepared for immunofluorescence and probed with anti-invertase and anti-Kar2 antibodies. To examine nuclear import of SV40-invertase, cells were grown in uracil dropout media with 2% ethanol at 30°C for 4 h.

Genetic Analysis of *NPL3*

Genetic crosses indicate that the *npl3* alleles are recessive and the thermosensitivity, growth on glycerol, and nuclear protein mislocalization phenotypes cosegregate. A diploid strain obtained by backcrossing *npl3* cells to an isogenic strain of opposite mating type was no longer temperature sensitive for growth, failed to grow on glycerol when transformed with the plasmid bearing *SV40-ATP2*, and showed normal nuclear localization of nucleolar and histone proteins. To sporulate the resulting diploid, it had to be transformed with a normal copy of *ATP2* because Gly[−] cells will not sporulate. For all *npl3* mutants, the Ts defect segregated as a single mutation (2 Ts[−] and Ts⁺ spores per 8–12 tetrads tested for each allele).

To show linkage between the Ts[−] growth and the ability to grow on glycerol, haploids from the cross of *npl3-250* to SEY6215 were cured of the *ATP2*-containing plasmid, transformed with plasmids bearing the *SV40-ATP2* fusion, and tested for growth on glycerol. The inability to grow at 36°C was tightly linked to the ability to grow on glycerol at 30°C because Ts⁺ and Ts[−] segregated 2:2, all Ts[−] spores were Gly⁺, and all Ts⁺ spores were Gly[−]. Twelve wild-type spores (*Δatp2*) grew normally at 36°C but failed to grow on glycerol when transformed with pNLS-F₁β, and the 12 Ts[−] spores did grow on glycerol when producing SV40-F₁βATPase.

Molecular Analysis of *NPL3*

Npl3 cells were transformed (Ito *et al.*, 1983; Rose *et al.*, 1990) with a YCp50-based yeast genomic DNA library (Rose *et al.*, 1987). The cells were plated on uracil dropout medium at room temperature (RT), and the resulting Ura⁺ colonies were replica-plated to uracil dropout medium at RT and 36°C. From 3776 Ura⁺ colonies screened, we obtained 7 colonies that grew at 36°C. Plasmid segregants were generated by successive culturing in nonselective medium (YEPD), and all colonies reverted to Ts[−] upon loss of the plasmid. Plasmid DNA was rescued from three individual transformants and, when introduced back into *npl3* cells, conferred the Ts⁺ phenotype. Defects in the nuclear localization of histone H2B and nucleolar proteins was also reverted to wild-type by the presence of the plasmid DNA. The inserts in all three plasmids shared common restriction sites, and one with the smallest insert (YCpNPL3-3 [Figure 8A]) was chosen for further studies.

A ~ 5 -kilobase (kb) *Hpa* I fragment was cloned into the *EcoRV* site of Bluescript KS⁺ (Stratagene, La Jolla, CA) and designated pB3, and an ~ 2.8 -kb *Bgl* II fragment was cloned into the *Bam*HI site of Bluescript KS⁺ and designated pA2 (see Figure 8A). A set of nested deletions was generated from the *Cla* I to the distal *Hpa* I site with exonuclease III and mung bean nuclease (Henikoff, 1987; Sambrook *et al.*, 1989). The complementary strand was sequenced by preparing synthetic oligonucleotides as primers, and DNA sequence of double-stranded DNA was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase reagents from United States Biochemical (Cleveland, OH). DNA sequence analysis was performed using the University of Wisconsin GCG software package (Devereux *et al.*, 1984).

To delete *NPL3*, pB3 was digested with *Sma* I and *Sac* II that occur only in the polylinker. The *Sac* II site was made blunt with Klenow and the plasmid religated, effectively removing the *Xba* I site in the polylinker. The resulting plasmid (pB3Δ*Xba* I) was digested with *Xba* I and *EcoRV* to remove a 2.6-kb fragment containing the entire *NPL3* gene and replaced with a 1.5-kb *Xba* I-*Hpa* I *URA3* containing fragment from YEp352 (Hill *et al.*, 1986) (see Figure 8B). The resulting plasmid pB3Δ*NPL3*::*URA3* was digested with *Xho* I and *EcoRI*, and the linear DNA was used to transform diploid strains MS810 and PSY198 to replace a single copy of the chromosomal *NPL3* gene (Rothstein, 1983). The gene replacement was confirmed by Southern blot analysis.

Preparation of Antibodies to *Npl3*

The *Bsa*AI-*Hind*III fragment of *NPL3* encoding amino acids 9–252 (Npl3ΔGR) was inserted into the *Sma* I-*Hind*III site of pATH10 (from A. Tzagaloff, Columbia University, New York, NY). The TrpE fusion was induced with indolacrylic acid, and total *Escherichia coli* lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A protein band of the predicted molecular mass was present in induced cultures but not in noninduced cultures or induced cells bearing only the native pATH vector. The band was excised and eluted into one times gel running buffer. An emulsion was prepared with Freund's adjuvant and injected into a New Zealand white rabbit to generate polyclonal antibodies. The rabbit was boosted once 3 wk after the initial immunization, and serum was collected 10 d after the boost. The resulting antiserum was absorbed against an extract from cells deleted for *NPL3* and used for immunofluorescence experiments.

Western Blots

Yeast were grown in YEPD or uracil dropout media to late log phase for preparation of total cell proteins. Cells were pelleted at 2500 rpm and washed once with cold distilled water. The cells were resuspended in 0.5 ml of lysis buffer (50 mM tris(hydroxymethyl)aminomethane-HCl, pH 8, 0.1% TritonX-100, 0.5% SDS) per 10 ml of original culture. Protease inhibitors were added to a final concentration of 0.1 μg/ml each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin. The cells were transferred to microfuge tubes, glass beads (0.5 mm) were added, and the cells were lysed by repeated vortexing. Aliquots of the total cell extract were removed for protein determination using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA).

Samples were mixed 1:1 with two times sample buffer, and proteins were separated by SDS-PAGE (Laemmli, 1970) on 10% polyacrylamide gels. Portions of gels were stained with Coomassie blue to visualize the protein loaded for each sample. After electrophoresis, proteins were blotted to nitrocellulose filters and incubated in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST), 0.005% SDS, and 5% nonfat dry milk for 1 h at RT. Subsequently, the filters were incubated for 12 h at 4°C with antibodies diluted in 5% nonfat dry milk/PBST and washed with PBST/0.005% SDS. Bound antibodies were detected with secondary antibodies coupled to horseradish peroxidase (HRP) and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Bands were not detected on blots incubated with preimmune serum.

Genetic Mapping of *NPL3*

The *Hpa* I fragment (Figure 8) was used to probe a blot of yeast chromosomes (Rose *et al.*, 1990) as well as the prime clone lambda filter library (Olson *et al.*, 1986). Genetic crosses were set up between *npl3* and *trp4*, *ade8*, and *snf1* strains. The resulting tetrads were scored for the appropriate auxotrophy, temperature sensitivity, and mating type to ensure that complete tetrads were being examined. To determine the map distance between *NPL3* and *ADE8*, Y256 was crossed to *npl3-101*, *npl3-110*, and *npl3-322* and FW468 was crossed to *npl3-250*. Eighty-one total tetrads were analyzed, and the individual computations for each cross were essentially identical (19, 20, 18, and 20

cM). A cross between FY468 and *npl3-250* was also scored for temperature-sensitive growth versus *TRP4* as well as *TRP4* versus *ADE8*. We also mapped *ADE8* with the prime clone filters, and there was good agreement between the physical and genetic mapping data. *NPL3* was mapped relative to *SNF1* (*snf1::LEU2*) by crosses between *npl3* and MCY2369. Forty-four complete tetrads were analyzed from this cross.

RESULTS

Mutant Isolation

To identify cellular components that affect nuclear protein uptake, we have developed a genetic screen that relies on the missorting of a nuclear-localized protein to another cellular compartment, in this case the mitochondria. The basic scheme is outlined in Figure 1. This screen is similar to the one we used to identify *NPL1*, as well as several other *npl* mutants (Sadler *et al.*, 1989; Chiang and Silver, unpublished data). Previously, the mutant isolation scheme used the mistargeting of a fusion protein containing an NLS fused to cytochrome *c*₁. Cytochrome *c*₁ is normally located in the mitochondrial inner membrane. However, the NLS-cytochrome *c*₁ is localized to the nucleus with a preference for the nuclear envelope. Because cytochrome *c*₁ is a membrane protein, it could have presented a certain bias in the original screen.

The current screen makes use of a plasmid that encodes a chimeric protein containing the SV40 T-antigen NLS fused to pre-*F*₁ β -ATPase (Figure 2). *F*₁ β -ATPase is a soluble enzyme of the mitochondrial matrix (Takeda *et al.*, 1985). It is normally made as a higher molecular weight precursor that is translocated into the mitochondrial matrix where it is required for respiration.

Several lines of evidence support the validity of this approach. The NLS from SV40 T-antigen is functional in this context because the fusion protein is efficiently localized to the nucleus, as determined by indirect immunofluorescence (Figure 4, A–C). Cells deleted for *ATP2* (which encodes *F*₁ β -ATPase), but containing SV40-*F*₁ β -ATPase, are unable to grow on glycerol (Figure 3). Furthermore, failure to grow on glycerol is a direct result of localization of the protein to the nucleus and not simply that the presence of the NLS inhibits uptake of the protein into mitochondria. A mutation that reduces the nuclear localization activity of the SV40 NLS in both animal and yeast cells (Kalderon *et al.*, 1984; Lanford and Butel, 1984; Nelson and Silver, 1989) results in cells that can grow on glycerol (Figure 3). In addition, when viewed by immunofluorescence, the mutant SV40-*F*₁ β -ATPase is no longer exclusively found in the nucleus (Figure 4, D–F), indicating that nuclear localization and failure to grow on glycerol depend on an active NLS. When the NLS is mutant, enough *F*₁ β -ATPase must reach the mitochondria to allow cells to grow on glycerol. Mutation of the SV40 NLS in these fusions does not affect the amount of fusion protein produced. The mitochondrial uptake of the fusion protein is also dependent on the presence of the mitochondrial targeting sequence in the fusion protein. Proteins consisting of either the normal or mutant NLS fused to mature *F*₁ β -ATPase failed to grow on glycerol. Taken together, these results show that the intracellular location of the SV40-*F*₁ β -ATPase in *atp2* cells can be monitored by growth on glycerol; when the protein is nuclear, cells are Gly[–], when the NLS is inactive, cells grow on glycerol (Gly⁺). It is this growth difference

Figure 1. Scheme for the isolation of *npl* mutants. Chimeric proteins containing the NLS from SV40 large T-antigen (SV40-NLS) fused to the mitochondrial *F*₁ β -ATPase are preferentially localized to the nucleus, which results in failure to grow on glycerol in cells missing their endogenous *F*₁ β -ATPase ($\Delta atp2$). Mutation of the SV40 NLS (indicated by the asterisk) or inactivation of a gene important for nuclear protein import results in missorting of some of the normally nuclear-targeted SV40-*F*₁ β -ATPase to the cytoplasm and mitochondria and growth on glycerol.

Figure 2. Plasmids encoding the SV40 NLS fused to pre-F₁β-ATPase. Diagram of gene fusions under the control of the ADH1 promoter (P_{ADH1} indicated by broad hatched lines). The construction of the plasmids is described in MATERIALS AND METHODS. The region of ATP2 encoding mature F₁β-ATPase is indicated by a solid black line, the portion of the mitochondrial presequence by small hatched lines, and the SV40 NLS by the dark shaded area. The amino acid sequence corresponding to the SV40 NLS and the region created by insertion of a linker to generate an in-frame fusion with pre-F₁β-ATPase is shown at the bottom of the figure. The asterisk indicates the mutated form of the SV40 NLS where the lysine at position 4 has been converted to a threonine.

that forms the basis for the search for additional *npl* mutants.

We hypothesized that an incomplete block in nuclear protein localization would result in uptake of enough F₁β-ATPase by the mitochondria to allow cells to grow on glycerol, whereas a complete block in nuclear protein

localization would result in cell death. Thus, we isolated mutants that were Gly⁺ at 30°C, but failed to grow (Ts⁻) at 36°C. *Atp2* defective strains (Gly⁻) of both *a* and *α* mating types bearing the SV40-ATP2 gene fusion were mutagenized with EMS, and mutants were selected by their ability to grow on glycerol at 30°C (see MA-

Figure 3. Growth of yeast cells on glycerol. Yeast cells (AVY4-1 [*Δatp2*]) transformed with pNLS-F₁β (SV40 NLS-ATP2/AVY4-1 in the figure), pNLS*-F₁β (SV40 NLS*-ATP2/AVY4-1 in the figure), a plasmid containing the intact ATP2 gene encoding wild-type F₁β-ATPase (ATP2/AVY4-1 in the figure), and YEp24 (YEp24/AVY4-1) streaked on YEP medium containing 3% glycerol as the only carbon source and incubated at 30°C for 7 d.

Figure 4. Immunofluorescence of cells producing NLS- $F_1\beta$ -ATPase. A single colony was used to inoculate 5 ml of ura dropout media containing 2% glucose, and cells were grown at 30°C to a density of $\sim 1 \times 10^7$ cells/ml, prepared for immunofluorescence as described in MATERIALS AND METHODS, and treated with a rabbit anti- $F_1\beta$ -ATPase antibody followed by FITC-conjugated anti-rabbit IgG, to localize $F_1\beta$ -ATPase and the NLS-bearing derivatives, and DAPI to stain cell DNA. SEY6215 ($\Delta atp2$) cells producing SV40- $F_1\beta$ -ATPase (A–C); producing SV40*- $F_1\beta$ -ATPase (D–F); and intact $F_1\beta$ -ATPase (G–I). A, D, and G are stained with anti- $F_1\beta$ -ATPase; B, E, and H are the corresponding cells stained with DAPI; and C, F, and I are the corresponding cells viewed by phase contrast.

TERIALS AND METHODS). The Gly⁺ colonies were further screened for temperature sensitivity at 36°C on both glycerol and glucose-containing medium. Only mutants that were Ts[–] on both were subjected to further analysis.

The resulting mutants were placed into complementation groups with regard to temperature sensitivity and ability to grow on glycerol (Table 1). Six independently isolated mutants failed to complement each other for both of these phenotypes. Because the ability to grow on glycerol was recessive and not plasmid-linked, these six define a new complementation group designated *NPL3*.

All mutants were crossed to *npl1* and *npl2*, as well as a number of other yeast *sec* and *cdc* mutants. Although one new allele of *NPL1* was identified, none of the remaining mutants appeared to be members of multiallelic complementation groups when the diploids were tested

for complementation of the temperature-sensitive growth defect.

All Ts[–] Gly⁺ mutants were examined by immunofluorescence for defects in nuclear protein localization using antibodies to histone H2B and nucleolar proteins. All *npl3* mutants showed alterations in nuclear protein localization. In wild-type cells grown at 30°C, staining with the antibodies is restricted to the nucleus, consistent with an exclusive nuclear localization of these proteins (Figures 5, A–C and 6, A–C). For the *npl3* mutants, growth at the semipermissive temperature (30°C) results in nuclear proteins being distributed throughout the cells (Figures 5, D–F and 6, D–F). In some (but not all) cells, the 4′6-diamidino-2-phenylindole · 2HCl (DAPI) staining also showed alterations in the morphology of the nuclear DNA after continuous growth at 30°C. The aberrant protein localization is not due to degraded fragments accumulating in the cytoplasm because im-

Table 1. *NPL* complementation group analysis

Complementation group	Growth on glycerol		Growth on YEPD		Alleles isolated	Chromosome location
	30°C	36°C	30°C	36°C		
<i>npl1</i>	+	—	+	—	1	XV
<i>npl3</i>	+	—	+	—	7	IVR
<i>npl4</i>	+	—	+	—	1	II

Mutants were isolated as described in MATERIALS AND METHODS. Members of a given complementation group were determined by crossing mutants of opposite mating types, selecting for the resulting His⁺ Lys⁺ diploids, and testing them for growth at 36°C on YEPD plates and for growth on YEP plates with 2% glycerol at 30 and 36°C. The primary classification of complementation groups is based on the ability of diploids to grow on glycerol at 30°C. The chromosome assignments for *NPL1*, *NPL3*, and *NPL4* were made by hybridization of the corresponding cloned gene to blots of intact yeast chromosomes. The isolation of the *NPL1* gene has been reported by Sadler et al. (1989), and the isolation of *NPL4* will be reported elsewhere (DeHoratius and Silver, unpublished results).

munoblot analysis of all mutants indicated that the histone and nucleolar proteins were intact. Sometimes the immunofluorescence signal for the mutants was brighter when compared with wild-type cells. We do not understand the reason for this because by immunoblot analysis there does not appear to be a significant increase in the amount of histone or nucleolar protein present in the cell. Perhaps protein mislocalized in the cytoplasm is more reactive with the antibodies than nuclear localized protein. The degree of mislocalization of nuclear proteins was approximately the same for all cells analyzed from a particular mutant strain (i.e., the pictures in Figures 5 and 6 are representative of the entire cell population). *Npl3* mutant cells often tend to be larger after continuous growth at 30°C.

One other Ts[−] Gly⁺ mutant, *npl4-1*, also showed mislocalization of nucleolar and histone H2B proteins (Figure 5, G–I). The temperature-sensitive growth and protein mislocalization are genetically linked for this mutation but unlinked to *npl3*.

Taken together, this search for *npl* mutants has yielded six alleles of *npl3*, one new allele of the previously identified *npl1*, and a single allele of *npl4*. Although this screen is not saturated, there is a clear bias toward uncovering alleles of *npl3*.

Characterization of *npl3* Mutants

We have conducted a detailed genetic characterization of *npl3*. Mutations that allowed growth on glycerol are chromosomal and not plasmid linked. When mutants were cured of the plasmid and retransformed with unmutagenized plasmid bearing the *SV40-ATP2* fusion, the ability to grow on glycerol was restored. However, *npl3* mutants without the plasmids were still unable to grow on glycerol, showing that the Gly⁺ phenotype depends on the presence of the *SV40-F₁β-ATPase* fusion protein.

All *npl3* mutations are recessive and responsible for temperature-sensitive growth, the ability to grow on glycerol using *SV40-ATP2* and mislocalization of nuclear proteins. These phenotypes cosegregate in all genetic crosses (see MATERIALS AND METHODS). All Ts[−] spores tested showed abnormal localization of nucleolar and histone proteins. In all Ts⁺ spores, the nucleolar and histone proteins were located only at the nucleus.

One possible explanation for the ability of *npl3* (and *npl4*) cells to grow on glycerol was that they were starting translation at a second internal ATG after the sequence encoding the *SV40 NLS* (see Figure 1). If this were the case, a form of pre-F₁β-ATPase would be produced that contained only the mitochondrial signal sequence. The resulting protein would be targeted only to the mitochondria and allow the cells to grow on glycerol. To rule out this possibility, we eliminated the second ATG in the NLS-fusion proteins by site-directed mutagenesis (Figure 2; see MATERIALS AND METHODS). *Npl3* cells were still Gly⁺ when harboring pNLS-F₁β^{Gly}, thus ruling out the possibility that translational restarts were the cause of the Gly⁺ phenotype. A second mutagenesis was carried out with cells bearing this new gene fusion and one allele of *NPL3* was obtained, further confirming the notion that the ability to grow on glycerol is indeed due to improper nuclear targeting of *SV40-F₁β-ATPase*.

We sought to test whether or not the *npl3* alleles we had isolated were of an unusual class or whether the defects we observed were typical for a Ts[−] defect in *NPL3*. To accomplish this, we identified a new allele of *npl3* from a collection of randomly generated Ts[−] mutants. *Npl3* mutant cells were mated to 480 independently isolated Ts[−] mutants (Klyce and McLaughlin, 1973). The resulting diploids were tested for the ability to grow at 36°C. Two mutants were identified that could no longer complement the *npl3* Ts[−] phenotype. One of

Figure 5. Localization of histone H2B in *npl* cells. SEY6215 (wild-type) cells (A–C), *npl3-250* cells (D–F), *npl4-1* cells (G–I), and *npl3-1* cells (J–L) were grown from inoculation of a single colony into YEPD media at 30°C to $\sim 1 \times 10^7$ cells/ml, prepared for immunofluorescence as described in MATERIALS AND METHODS, and treated with rabbit anti-histone antibody followed by FITC-conjugated anti-rabbit IgG to localize normally nuclear-associated histones (B, E, H, and K), DAPI to visualize DNA (A, D, G, and J), and viewed by Nomarski optics (C, F, I, and L).

these mutants would not sporulate when crossed either to wild-type or *npl3* cells and was not studied further. However, the diploids from crosses of the other mutant and *npl3* cells did sporulate, and all resulting spores were temperature sensitive (no tetratypes were obtained), suggesting a linkage between the new Ts mutant and *npl3*. The linkage was confirmed by rescue of the Ts[−] defect with a plasmid bearing the *NPL3* gene.

This new allele of *npl3* (*npl3-1*) also showed a defect in nuclear protein localization at 30°C. The original mutant and Ts[−] spores from a backcross to wild-type cells were examined by indirect immunofluorescence with anti-nucleolar and anti-histone antibodies. In cells grown at 22°C, only nuclear staining with both antibodies was observed. However, in cells grown at 30°C, nucleolar and histone proteins were distributed throughout the cytoplasm (Figures 5, J–L and 6, G–I). In fact, the improper localization of these proteins was strongest in this independently isolated allele when compared with the other 6 *npl3* alleles.

A new allele of *npl1* was also identified by the same method. Linkage to *NPL1* was confirmed genetically,

and these cells also showed a nuclear localization defect similar to other *npl1* alleles when grown at 30°C.

Nuclear Protein Localization Defect of *npl3* is Temperature Sensitive and due to a Failure in Import

The nuclear localization defect in *npl3* cells could result from a failure to correctly import proteins into the nucleus or to retain them once they are there. To distinguish between these possibilities, we conducted the following experiments and show that the defect in nuclear protein localization in *npl3* is due, at least in part, to incorrect import of proteins into the nucleus.

Npl3 cells were transformed with a plasmid encoding a fusion protein containing the SV40 T-antigen NLS fused to the normally cytoplasmic form of invertase (Nelson and Silver, 1989) and under transcriptional control of the inducible *ADH2* promoter (Eisen *et al.*, 1988). When wild-type cells containing this plasmid are grown at 30°C in ethanol, the SV40-invertase fusion protein is found entirely at the nucleus (Figure 7A, A–

Figure 6. Localization of nucleolar antigens in *npl* cells. SEY6215 (wild-type) cells (A–C), *npl3-250* cells (D–F), and *npl3-1* cells (G–I) were grown from inoculation of a single colony into YEPD at 30°C to a density of $\sim 1 \times 10^7$ cells/ml, prepared for immunofluorescence as described in MATERIALS AND METHODS, and treated with mouse anti-nucleolar antibodies followed by FITC-conjugated anti-mouse IgG to localize normally nucleolar-associated antigens (B, E, and H), DAPI to visualize DNA (A, D, and G), and viewed by Nomarski optics (C, F, and I).

C). However, when *npl3* cells are grown under the same conditions, SV40-invertase is found distributed throughout the cell (Figure 7A, G–I). When the same cells are grown at 22°C, SV40-invertase is mainly at the nucleus (Figure 7A, D–F). The amount of SV40-invertase protein induced is the same at 22°C as at 30°C, further confirming that the nuclear localization defect is not due to protein overproduction. Thus, failure of correct nuclear protein localization is temperature sensitive. Unfortunately, we could not sufficiently induce expression of SV40-invertase from the *ADH2* promoter (or other genes from the *GAL1* promoter) at the completely nonpermissive temperature of 36°C, so we were unable to accurately measure the onset of the nuclear localization defect.

To test for a defect in nuclear protein retention, *npl3* cells containing the *ADH2-SV40-SUC2* plasmid were grown in ethanol at 22°C to fully induce the expression and nuclear localization of SV40-invertase (Figure 7A, D–F). The cells were then simultaneously shifted to glucose-containing medium (to repress expression from the *ADH2* promoter) and 30°C and examined by immunofluorescence with anti-invertase antibody. The

SV40-invertase was still localized at the nucleus by comparison with the DAPI staining (Figure 7B, A–C as compared with Figure 7A, D–F before the temperature shift. In some cells, the distribution of the SV40-invertase was slightly greater than that indicated by the DAPI stain. To demonstrate that the region that corresponds to the nuclear interior is greater than that defined by the DNA staining, we analyzed *npl3* cells with antibodies to Kar2. In yeast, the nuclear envelope and the endoplasmic reticulum (ER) are continuous as evidenced by the distribution of the Kar2 protein (Rose *et al.*, 1989). In *npl3* cells, staining with anti-KAR2 antibodies defines the periphery of the nucleus (Figure 7B, D–F). Note that the area occupied by the whole nucleus is greater than that defined by DAPI staining alone. This can be explained, in part, by the presence of the nucleolus, which is not stained by DAPI. Moreover, in dividing cells, the nuclear envelope extends into the daughter bud, but the interior is not stained by DAPI. This could suggest some defect in nuclear division in *npl3* cells.

We further confirmed the retention of SV40-invertase within the nuclear interior by the following double-labeling experiment. To show that the SV40-invertase is

A

B

Figure 7

Figure 7. (A) *Npl3* cells have a temperature-sensitive defect in nuclear protein localization. SEY6215 (wild-type) cells (A–C) grown at 30°C in 3% ethanol, *npl3-1* cells grown at 22°C in 3% ethanol (D–F), *npl3-1* cells grown at 22°C in 2% glucose and shifted to 30°C for 4 h in 3% ethanol (G–I). All cells are transformed with pMN8 a plasmid encoding the SV40 NLS fused to cytoplasmic invertase (Nelson and Silver, 1989). Samples were prepared for immunofluorescence and treated with rabbit anti-invertase, followed by FITC-conjugated anti-rabbit IgG, to localize the SV40-invertase fusion proteins (B, E, and H), DAPI to visualize DNA (A, D, and G), and viewed by Nomarski optics (C, F, and I). (B) Nuclear protein retention and nuclear envelope morphology in *npl3* cells. *Npl3* cells were grown at 22°C in uracil dropout media with 3% ethanol. At 6×10^6 cells/ml, cells were collected by centrifugation, and resuspended in prewarmed YEPD and incubated at 30°C for 4 h. Cells were then prepared for immunofluorescence as described in MATERIALS AND METHODS and probed with rabbit anti-invertase antibodies followed by FITC-conjugated anti-rabbit antibodies (B) and rabbit anti-Kar2 antibodies followed by FITC-conjugated anti-rabbit antibodies (E). A and D are the corresponding cells treated with DAPI to visualize DNA and C and F are cells viewed by Nomarski optics. (C) Nuclear proteins are retained in *npl3* cells. The same cells as in Part B were also probed with a mixture of mouse monoclonal anti-Kar2 and rabbit anti-invertase antibodies followed by Texas Red-conjugated anti-mouse and FITC-conjugated anti-rabbit antibodies. (A) Cells stained with the anti-Kar2 antibodies; (B) the same cells stained with anti-invertase antibodies; (C) the same cells stained with DAPI to localize the DNA. The corresponding negative controls show that in the absence of the corresponding primary antibodies, the signal from the secondary antibodies alone is equal to the slight degree of nonnuclear staining for B. The exposure times for Panel B were longer (30 s) than for the same cells in Part B (15 s).

indeed within the confines of the nucleus, we stained the same cells with anti-Kar2 mouse monoclonal antibodies and rabbit polyclonal anti-invertase antibodies after a 4-h shift to 30°C in glucose. SV40-invertase was always located only within the region defined as the nuclear envelope by the localization of Kar2 (Figure 7C). (The slight nonnuclear staining is equal to that seen in cells treated only with secondary antibodies or in cells deleted for the *SUC2* gene.) These results lead us to conclude that *npl3* mutants are not defective in nuclear protein retention but rather fail to correctly import proteins into the nucleus.

Characterization of the *NPL3* Gene

To further clarify the role of *NPL3* in nuclear protein localization, we cloned the corresponding gene from a yeast genomic DNA library (Rose *et al.*, 1987) by complementation of the Ts-lethal phenotype of the *npl3* mutant. Deletion analysis of one clone localized the *npl3* complementing region within a ~5-kb DNA fragment (Figure 8A).

The cloned DNA encodes the *NPL3* structural gene as demonstrated by linkage analysis. DNA from YCpNPL3-3 was subcloned into a yeast integrating

vector. To direct integration of *URA3* at *NPL3*, the resulting plasmid (YIpNPL3), was digested at a unique *Kpn* I site within *NPL3* and used to transform W303 (*NPL3 ura3*). The correct integration event was verified by Southern analysis. The proper transformants were crossed to *npl3* strains to form diploids and sporulated. In 23 asci analyzed, each containing four viable spores, two spores were Ura⁺/Ts⁺ and two spores were Ura[−]/Ts[−], thus confirming the linkage between the cloned DNA and *NPL3*. *Npl3* cells bearing the cloned DNA also showed normal nuclear protein localization (like wild-type cells in Figures 5 and 6) when cells were examined by immunofluorescence with antibodies to histone H2B and nucleolar proteins.

The location of *NPL3* in the yeast genome was determined by physical and genetic means. An initial chromosome blot indicated that *NPL3* resides on chromosome IV. Probing of a prime cloned lambda library (Olson *et al.*, 1986) with the cloned DNA confirmed the initial chromosome assignment and allowed us to further narrow the location of *NPL3* to IVR in the vicinity of *SPT3*. Genetic crosses between *npl3* and *trp4*, *ade8*, and *snf1* strains (see MATERIALS AND METHODS) were used to confirm and more precisely determine the

Figure 8. Plasmids containing the *NPL3* region of the yeast genome. (A) The entire insert isolated from the YCp50 clone (YCpNPL3-3) was ~8.5 kb. The enzymes listed are as follows: Bc, *Bcl* I; Bg, *Bgl* II; Bs, *Bsa* AI; C, *Cla* I; D, *Dra* I; H, *Hind*III; Hp, *Hpa* I; K, *Kpn* I; Pm, *Pml* I; RI, *Eco*RI; RV, *Eco*RV; Sa, *Sac* I; Sn, *Sna* BI; and Xb, *Xba* I. The lines below the map represent the portions of the cloned DNA that were tested for complementing the Ts⁻ defect of *npl3-250*. The results of these tests are shown in the column at the right. (B) Deletion of *NPL3*. The entire coding region of *NPL3* is contained within the central *Dra* I fragment of the cloned DNA and indicated by the bold arrow. The *Xba* I-*Eco*RV fragment was removed and replaced with the *Xba* I-*Hpa* I fragment from YEp352 containing the *URA3* gene.

location of *NPL3* (Table 2). This analysis indicates that *NPL3* is located between *ADE8* and *SNF1* at a map distance of 19 and 28 cM, respectively.

NPL3 Encodes a Protein with Homology to a Class of Proteins Involved in RNA Metabolism

The *NPL3* gene sequence predicts a protein of 414 amino acids (45.4 kDa) with an acidic pI of 5.35 (Figure 9). The deduced amino acid sequence of Npl3 has several interesting features. This sequence can be divided into three regions: an N-terminal region that has four repeats of the peptide APQE and is enriched in proline residues, a central region that may recognize RNA, and a C-terminal region containing several glycine-arginine rich repeats. These regions are separated by proline-rich stretches, suggesting that they constitute independent domains.

The central region of *NPL3* (amino acids 126–195) contains a RNA recognition motif, which is distinguished by the presence of the RNP-2 and RNP-1 consensus sequences and a number of other highly con-

served residues typical of this motif (reviewed in Kenan *et al.*, 1991). Secondary structure analysis (Chou and Fasman, 1978) predicts a structural organization of the Npl3 protein similar to other RNA binding proteins, such as hnRNPB1 and A2 (reviewed in Cobiainchi *et al.*, 1990).

The C-terminal third of the protein (starting at amino acid 284) is comprised of a series of simple repeats, RGGF/Y. After 7 repeats of RGGF, a similar set of 10 near-perfect repeats of RGGY occurs. In total, the carboxy-terminal 130 amino acids is composed of 17 sets of R-G-G-F/Y. This repeated sequence is found in other eukaryotic RNA and single-stranded DNA binding proteins. In yeast, proteins with the R-G-G-F/Y repeats include Ssb1 (Jong *et al.*, 1987), Nop1 (Schimmang *et al.*, 1989; Henriquez *et al.*, 1990), Nsr1 (Lee *et al.*, 1991), and the recently identified Gar1 (Girard *et al.*, 1992).

We generated an antibody against a portion of Npl3 lacking the glycine-arginine rich C-terminus (see MATERIALS AND METHODS). This antibody recognized a protein doublet that migrates slightly slower than the

Table 2. Genetic mapping of *NPL3*

Cross	Parental ditype	Tetratypes	Nonparental ditypes	Linkage (cM)
<i>npl3</i> × <i>trp4</i>	6	35	1	48
<i>npl3</i> × <i>snf1</i>	19	25	0	28
<i>npl3</i> × <i>ade8</i>	35	21	0	19
<i>ade8</i> × <i>trp4</i>	6	35	1	29
	<i>TRP4</i>	<i>ADE8</i>	<i>NPL3</i>	<i>SNF1</i>
10 cM				

The position of *NPL3* on chromosome IV was determined as described in MATERIALS AND METHODS. After the initial chromosome assignment, DNA corresponding to the *NPL3* gene was used to probe the prime clone lambda filter library (Olson et al., 1986), which indicated linkage to the region near *ADE8*. The indicated genetic crosses were then set up, the diploids sporulated, and the indicated numbers of tetrads analyzed for their genotype. The results of this analysis are summarized below the table where *NPL3* is shown to reside between *ADE8* and *SNF1*.

predicted molecular mass of Npl3 when used to probe yeast extracts by Western blotting (Figure 10). Neither band was observed in extracts prepared from cells deleted for *NPL3* ($\Delta npl3::URA3$), indicating that both bands are products of the *NPL3* gene and suggestive of some form of posttranslational modification. Both bands are present in *npl3* mutants, indicating the mutations are likely to be single amino acid substitutions and not truncations of the protein. After absorption against an extract from cells deleted for *NPL3*, the anti-Npl3 antibody was used to localize the protein by immunofluorescence. The antibody reacted only with the nucleus, indicating the nuclear location of Npl3 protein (Figure 11, A–C).

Npl3^{ts} Mutants Block Nuclear Protein Import

Several possibilities exist for how mutations in *npl3* affect import of nuclear proteins. 1) Npl3 might be directly involved in the process, for instance as a carrier of proteins into the nucleus. This has been proposed as a possible role for proteins such as nucleolin, which are known to shuttle in and out of the nucleus (Borer et al., 1989). 2) Mutations in *NPL3* somehow disrupt the integrity of the nuclear pore complex and hence its function. 3) Certain mutations in *NPL3* generate mutant proteins that interfere with the transport of other nuclear proteins. This could occur by binding to and subsequent sequestering of some component of the import machinery.

The first test for which all of the above might explain how *npl3* mutants alter nuclear protein import is to analyze the phenotype of cells lacking *NPL3*. The *NPL3* chromosomal locus was deleted and replaced with *URA3*

in a diploid cell (Figure 8B; see MATERIALS AND METHODS) and the resulting transformants subjected to tetrad analysis. Stable Ura⁺ transformants containing one deleted and one normal copy of *NPL3* (verified by Southern analysis) were sporulated at 23°C, and two Ura[–] viable spores and two very small Ura⁺ spores were recovered from all complete asci. The Ura⁺ cells were confirmed to be deleted at the *NPL3* locus by Southern analysis and by the fact that no Npl3 protein

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-97 TTTAAAAAGGACATGAGAAAAATAATTTCTCTCTCTCTAAATATATATCTTTGAAG -40
-39 GAATCAAAATTAAGCAATTACGCTAAAACCATAGGATAATGTCTGAAGCTCAAGAAACT 21
    M S E A Q E T
22 CACGTAGAGCAACTACCAGAATCTGTTGTCGATGCCCGACGTCGAAGAACAGCACAAGAA 81
    H V E Q L P E S V V D A P V E E Q H Q E
82 CCACCACAGGCTCCAGATGCTCCACAAGAACCAAGTTCACAGGAATCTGCTCCACAG 144
    P P Q A P D A P Q E P Q V P Q E S A P Q
145 GAATCTGCTCCACAAGAACCAAGCTCCACAAGAACCAAGTTCAGTTCTCTCCACCATCT 204
    E S A P Q E P P A P Q E Q N D V P P P S
205 AATGCTCCAATTTATGAAGGCGAAGATCCCAAGTGTCCAAGACTACCAAGAGGCCAC 264
    N A P I Y E G E E S H S V Q D Y Q E A H
265 CAGCACCACCAACCACTGAACCCCAACCATATTATCTCTCTCTCCAGGTGAACAC 324
    Q H H Q P P E P P Q P Y Y P P P P P G E H
325 ATGCACGACGCCCAACCAATGCACCCGCTCAAGAGGAGAATCTCGAACACCAAGTTG 384
    M H G R P P M H H R Q E G Q L S N T R
385 TTTGTTAGACCTTTCCCATTTGACGTTCAAGAATCCGAGTTGGAATGAATCTTTGGTCCA 444
    F V R P F P L D V O E S E D N E F G
445 TTTGACCAATGAAGGAAGTCAAGATCTTGAACGCTTCGGTTTGTGAATTTGAAGAA 504
    F G P M K E V K I L N G F A V F E F
505 GCAGAATCCGCTGCCAAAGCCATTGAAGAAGTTCACGGTAAGAGTTTGTCTAACCAACCT 564
    A E S A A K A I E E V H G K S F A N O P
565 TTGGAAGTTGTTTACTCTAAATTCGCTGCCAAGAGATACCGTATCACCATGAAGAACTTA 624
    L O V V Y S K L P A K R Y R I T M K N L
625 CCAGAAGTTGTTTCTAGCGCAAGATCTTAAAGATTATGCCAGGGAATAGTTTAGAACT 684
    P E G C S W Q D L K D L A R E N S L E T
685 ACTTTTCTAGCGTCAATACCAAGAGATTTGATGGTACCGGTGCTCTAGAAATCCCTAGT 744
    T F S S V N T R D F T G D T G A L E F F S
745 GAAGAAATCTTGGTCAAGCTTTGGAGAGATTAAACAATATTGAATTCAGAGGTTCTGTG 804
    E E I L V E A L E R L N N I E F R G S V
805 ATTACTGTTGAAAGAGATGACAATCTCCACCAATCAGAAGATCAATAGAGGTGGCTTC 864
    I T V E R D D N P P P I R R S N R G G F
865 AGAGGTGCGGGCGGCTTCAGAGGCGGCTTCAGAGGTGGCTTCAGAGGCGGTTCTCCAGA 924
    R G R G G F R G G F R G G F R G G F S
925 GCGGGCTTCGGTGGCCCGCAGAGTGGATTGTTGGTCCAAGAGTGGTTACGGTGGCTAT 984
    G G F G G P R G G F G G P R G G Y G G Y
985 TCCAGAGGTGGCTACGGTGGCTACTCCAGAGGCGGATATGGTGGCTCCAGAGGTGGTTAC 1044
    S R G G Y G G Y S R G G Y G G S R G G Y
1045 GATAGTCTTAGAGGTGGTTACGATAGTCCAAGAGGTGGTTATTCAGAGGTGGCTATGCT 1104
    D S P R G G Y D S P R G G Y S R G G Y G
1105 GGTCCAAGAAATGATTACGGTCTCCAAGAGGTAGCTACGGTGGTTCAAGAGGTGGTTAT 1164
    G P R N D Y G P P R G S Y G G S R G G Y
1165 GATGGTCCAAGAGGCGATTATGGTCTCCAAGAGATGCATACAGAACCAAGAGATGCTCCA 1224
    D G P R G D Y G P P R D A Y R T R D A P
1225 CGTGAAGATCACCAACCAAGGTAAGCCATTATATAGTTGAGAAAAAAGGAGAAATT 1284
    R E R S P T R Stop

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Figure 9. The sequence of *NPL3*. The sequence shown represents the region between the central *Dra* I fragment encoding the open reading frame in DNA capable of complementing the Ts[–] defect of *npl3* mutants. The putative TATA box is shown by the underlying box beginning at position –56. The glycine-arginine rich C-terminus is composed of two types of repeats shown by the single underlines. The RNA recognition motif (RRM) in the central portion of the protein is shown by cross-hatched underlining with the RNP-1 and RNP-2 consensus sequences marked by an additional solid underline. The four repeats of APQE in the amino terminus are indicated by single underlines. The entire *NPL3* DNA sequence is available in the GenBank Database under accession number M86731.

was observed on immunoblots probed with anti-Npl3 antibodies (Figure 10). When restreaked at 23°C, the $\Delta npl3::URA3$ cells formed very small colonies and at 37°C the null mutants did not grow (Figure 12A). Taken together, the phenotype of cells lacking *NPL3* is poor growth at 23°C and failure to grow at 37°C.

Because cells missing *NPL3* were viable, we were able to examine them for defects in nuclear protein import. $\Delta npl3$ cells were grown at 30°C, and the distribution of nuclear proteins was analyzed by immunofluorescence. Histone H2B proteins (Figure 12B, A–C) were localized to the nucleus when cells were analyzed with the appropriate antibodies. In addition, when cells grown at 22°C were shifted to 36°C for 4 h, histone H2B also remained nuclear. Thus, the nuclear protein localization pattern was normal in cells lacking *NPL3*. This result indicates that Npl3 protein is not essential for nuclear protein uptake by the assays used here. We cannot rule out the possibility that there is a slight defect that cannot be detected by immunofluorescence analysis.

On the other hand, the mutant *npl3* alleles identified by our screen showed mislocalization of several nuclear proteins at 30°C (Figures 5–7). To explain these findings, we reasoned that mutations in *npl3* might generate a mutant protein that blocks import of other nuclear proteins. To test this hypothesis, we created diploids containing one copy of a temperature-sensitive *npl3* allele and one null allele. These diploids (*npl3^{ts}/Δnpl3*) behaved like the *npl3^{ts}* alleles: they were temperature sensitive for growth at 37°C and showed mislocalization of nucleolar proteins and histone H2B (Figure 12B, D–F). This is in contrast to heterozygous diploids containing one wild-type and one Ts– allele (*npl3^{ts}/NPL3*) where protein localization remains normal.

Nuclear localization of Npl3 itself is disrupted in *npl3^{ts}* mutants. When *npl3* cells are grown continuously at 30°C, Npl3 protein is distributed in both the nucleus and the cytoplasm (Figure 11, D–F). This is similar to

Figure 10. Identification of Npl3 protein. Yeast cell lysates were prepared from wild-type, *NPL3* deleted, and *npl3-101* Ts– mutant cells. The proteins were separated by SDS-PAGE and blotted to nitrocellulose as described in MATERIALS AND METHODS. The blot was probed with a 1:1000 dilution of anti-NPL3ΔGR antiserum. Bound antibody was detected with HRP-conjugated secondary antibody and visualized by enhanced chemiluminescence. No bands were seen in blots probed with pre-immune serum. In the experiment shown, approximately five- to sixfold more protein was loaded in the $\Delta npl3$ lane.

the mislocalization of histone H2B and nucleolar proteins observed in *npl3* mutants under similar conditions.

In summary, *NPL3* is essential for normal cell growth. Temperature-sensitive mutations in *NPL3* generate a mutant protein that can disrupt the import of other nuclear proteins, including those containing an NLS from SV40 large T-antigen. The implications of these results are discussed below.

DISCUSSION

We have isolated yeast mutants defective in localization of proteins to the nucleus, using a modification of an existing approach (Sadler *et al.*, 1989). When the β subunit of the mitochondrial F_1 ATPase contains the SV40 T-antigen NLS preceding the signal for mitochondrial import, the resulting fusion protein is preferentially located at the nucleus. In contrast to the NLS-cytochrome c_1 protein fusion, which concentrated at the nuclear envelope, the SV40- $F_1\beta$ -ATPase appears completely intranuclear. Cells mutated for the wild-type *ATP2*, but bearing the SV40- $F_1\beta$ -ATPase, lack mitochondrial

Table 3. Yeast strains used in this study

Name	Genotype	Source
SEY6215	Mat a <i>ura3-52 leu2-3,112 trp1-Δ901 lys2-801 suc2-Δ9 Δatp2::LEU2</i>	S. Emr ^a
AVY4-1	Mat α <i>ura3-52 leu2-3,112 suc2-Δ9 his4-519 gal2 Δatp2::LEU2</i>	S. Emr ^a
W303	Mat a <i>ade2-1 trp1-1 ura3-1 leu2-3, 112 his3-11,15 can1-100</i>	B. Thomas ^b
YPH149	Mat α <i>ura3-52 lys2 ade- his7 trp1-Δ1</i> [CF VII/ <i>URA3</i> / <i>RAD2</i> distal] [CF VII/ <i>TRP1</i> / <i>RAD2</i> proximal]	P. Hieter ^c
FW468	Mat a <i>his4-912Δ ade8 trp4</i>	F. Winston ^d
MCY2369	Mat a <i>snf1-15::LEU2 his3-Δ200 ura3-52 leu2-3,112</i>	M. Carlson ^e
Y256	Mat α <i>ura3 leu2 his3 ade8 ras1::URA3</i>	J. Broach
MS810	a/α <i>ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-101/ADE2 trp1Δ1/TRP1</i>	M. Rose
PSY198	a/α <i>ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3Δ200/his3Δ200 ade2-101/ADE2 trp1Δ1/TRP1</i>	J. Broach

Strains are described by their respective names, mating types, genotypes, and source (designated by principal investigator).

^aUniversity of California, San Diego; ^bColumbia University, NY; ^cJohns Hopkins, Baltimore, MD; ^dHarvard Medical School, MA; ^eColumbia University, NY.

Figure 11. Location of Npl3 protein in wild-type and *npl3* mutant cells. Wild-type cells (A–C) and *npl3* mutant cells (D–F) were grown from inoculation of a single colony into YEPD at 30°C to a cell density of $\sim 1 \times 10^7$ cells/ml, prepared for immunofluorescence, and probed with rabbit anti-Npl3 antibodies followed by FITC-conjugated anti-rabbit antibodies (B and E). A and D are the corresponding cells stained with DAPI, and C and F are the same cells viewed by Nomarski optics.

function (are Gly[−]) as a consequence of the nuclear location of the fusion protein. We isolated *npl* mutants that alter the nuclear localization of this hybrid protein. The mutants were selected based on their ability to restore growth on glycerol and secondarily screened for temperature sensitivity and defects in localization of endogenous nuclear proteins.

Several genes were identified by the scheme presented here. One, *npl1*, was previously identified by the screen that used missorting of cytochrome *c*₁ to the nucleus (Sadler *et al.*, 1989). The second new type of mutant is represented by *npl3* and *npl4*. Mutants in this class are temperature sensitive for growth and show defects in nuclear localization of histone H2B and several nucleolar proteins. It is unlikely that this collection of mutants represents all the genes that could be identified by our screen.

The identification of *NPL1* in two different (albeit similar) screens (Sadler *et al.*, 1989; this report) indicates that this gene is also essential for proper nuclear location of proteins. However, the fact that some alleles behave differently from *NPL3* in the import versus retention experiment (Chiang and Silver, unpublished data) suggests that *NPL1* may act at a different stage. The fact that *NPL1* is allelic to *SEC63*, an ER membrane protein involved in protein translocation across the ER (Rothblatt *et al.*, 1989), led us to suggest that Npl1 is either distinctly involved in both processes or is important for assembly of membrane proteins of the pore complex (Sadler *et al.*, 1989). Recently, Greber and Gerace (1992) found that antibodies to the luminal portion of the membrane-associated nuclear pore GP210 protein altered the structure and function of the pore complex. This, together with our observations of *NPL1/SEC63*, further supports the idea that components of the ER lumen can affect nuclear transport.

We isolated seven alleles of the previously unidentified *NPL3* gene. All *npl3* mutants identified by our

screen are temperature sensitive for growth, mislocalize nucleolar and histone proteins, and have a defect in nuclear import of proteins with SV40 NLSs. *Npl3^{ts}* mutants do not show defects in retention of proteins once they are inside the nucleus. One of the *npl3* alleles was isolated from a collection of independently generated temperature-sensitive yeast mutants. This allele also shows defects in nuclear protein import, suggesting that we have not identified a particularly unusual type of *npl3* allele.

The sequence of *NPL3* predicts a protein with similarity to proteins that bind RNA and are involved in RNA metabolism. Npl3 contains the RNP consensus sequences as well as a series of RGGF/Y repeats—both common features of some RNA binding proteins. Proteins that share these motifs have been implicated in a wide variety of processes, including RNA splicing, pre-rRNA processing, and assembly of ribosomes. The yeast nucleolar protein, Nop1, and its mammalian counterpart, fibrillarin, have been studied extensively in this regard (Jansen *et al.*, 1991; Tollervey *et al.*, 1991). Yeast cells require Nop1 for viability, but human fibrillarin can partially substitute at low temperatures. Interestingly, the nuclear morphology is also disrupted in *nop1* mutants, indicating that it may also play a role in nuclear organization. In yeast, the nucleolus is adjacent to the nuclear envelope and may be involved in maintaining its structure.

Npl3 protein is located in the nucleus. However, unlike other yeast proteins with sequence similarities to Npl3, such as Nop1 (Tollervey *et al.*, 1991) and Ssb1 (Jong *et al.*, 1987), Npl3 is not restricted to the nucleolus. The Npl3 protein does not contain a prototypical SV40 T-antigen-like or a readily apparent bipartite NLS (Dingwall and Laskey, 1991). Thus, Npl3 may contain a unique NLS or enter the nucleus by interaction with other NLS-containing proteins.

A

B

Figure 12. Characterization of cells lacking *NPL3*. (A) One copy of *NPL3* was deleted and replaced with the *URA3* gene in a diploid strain that was sporulated, and the resulting germinated spores were tested for growth on YEPD at 23 and 37°C. B and D are cells that contain the *NPL3* deletion ($\Delta npl3::URA3$), and A and C are cells that have an intact copy of *NPL3*. (B) Cells lacking *NPL3* ($\Delta npl3::URA3$ [A–C]) and a diploid with one copy of a temperature-sensitive *npl3* allele, and the other copy of *NPL3* deleted (*npl3^{ts}/Δnpl3::URA3* [D–F]) were grown at 30°C and prepared for immunofluorescence. B and E are cells treated with anti-histone H2B antibody followed by FITC anti-rabbit antibody, A and D are the corresponding cells stained with DAPI, and C and F are the cells viewed by Nomarski optics.

Npl3 is only absolutely required for growth at high temperatures. Yeast deleted for *NPL3* can still grow at room temperature, although their growth rate is impaired. Relevant to the studies presented here, there is no obvious defect in nuclear localization of several normally nuclear proteins in cells lacking *NPL3*. The defect in nuclear protein localization is confined to the tem-

perature-sensitive alleles isolated in our screen for *npl* mutants. This would suggest that *Npl3* is not essential for transporting proteins into the nucleus. However, other similar proteins may exist that can substitute for *Npl3* action.

We propose that the mutant forms of *Npl3* isolated in this study block the passage of proteins into the nu-

cleus in a general manner. This is based on four results: 1) we identified temperature-sensitive mutations in *NPL3* by a screen that relies on the missorting of nuclear proteins, 2) *Npl3^{ts}* mutants show mislocalization of several different nuclear proteins, 3) *Npl3^{ts}* mutants are blocked in import and not retention of proteins bearing an SV40 NLS, and 4) only *npl3^{ts}* alleles show defects in nuclear protein import; cells lacking *NPL3* ($\Delta npl3$) do not. The Npl3 protein produced in the mutants is not truncated or dramatically overproduced, suggesting that the mutant Npl3 proteins contain single amino acid changes. Taken together, these results indicate that Npl3 is not required for protein import, but the mutant forms of Npl3 can interfere with the process. This could happen by, for instance, mutant protein binding at the nuclear pore complex on its way in or out of the nucleus. Such a blockage could result in a recessive growth defect if wild-type Npl3 competes for the proposed pore complex binding sites. These alleles of *NPL3* are classified as neomorphic because they lead to products that interfere with nuclear protein localization only when the wild-type *NPL3* activity is reduced. Examples of this type of mutant behavior can be found, for instance, in the *cactus* gene of *Drosophila* (Roth *et al.*, 1991).

In some respects, results with Npl3 are similar to those of Schneider *et al.* (1988), who found mutant forms of large T-antigen that interfered with import of another nuclear protein. However, we do not know how Npl3 is targeted to the nucleus, i.e., whether or not it uses the same apparatus as proteins bearing the SV40 NLS. No sequence similar to the SV40 or other NLSs is present in Npl3. However, Npl3 could block import of SV40 NLS-containing proteins at a common step, such as translocation through the pore complex. Interestingly, import of Npl3 itself is blocked in *npl3* mutants. Extragenic suppressors of *npl3* temperature-sensitive mutants might then reside in components of the nuclear import machinery.

Results over the past 10 years indicate that translocation of proteins across the nuclear envelope is highly conserved. Similar NLSs function in mammals, yeast, and plants (Kalderon *et al.*, 1984; Nelson and Silver, 1989; Howard *et al.*, 1992). Antibodies raised against mammalian nucleoporins (Davis and Blobel, 1986) recognize similar proteins in yeast (Davis and Fink, 1990). And a highly conserved phosphoprotein that binds NLSs is found in yeast, animal, and plant cells (Stochaj and Silver, 1992). We propose that proteins such as mutant forms of Npl3 may block import in all cell types and, as such, may serve as valuable reagents for not only further dissecting the process but also for selectively blocking nuclear-cytoplasmic exchange of macromolecules.

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