A Yeast Gene Important for Protein Assembly into The Endoplasmic Reticulum and the Nucleus Has Homology to DnaJ, an Escherichia coli Heat Shock Protein

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Abstract. When nuclear localization sequences (termed NLS) are placed at the N terminus of cytochrome c, a mitochondrial inner membrane protein, the resulting hybrid proteins do not assemble into mitochondria when synthesized in the yeast Saccharomyces cerevisiae. Cells lacking mitochondrial cytochrome c, but expressing the hybrid NLS-cytochrome c, proteins, are unable to grow on glycerol since the hybrid proteins are associated primarily with the nucleus. A similar hybrid protein with a mutant NLS is transported to and assembled into the mitochondria. To identify proteins that might be involved in recognition of nuclear localization signals, we isolated conditional-lethal mutants (npl, for nuclear protein localization) that missorted NLS-cytochrome c, to the mitochondria, allowing growth on glycerol. The gene corresponding to one complementation group (NPL1) encodes a protein with homology to DnaJ, an Escherichia coli heat shock protein. npl1 is allelic to sec63, a gene that affects transit of nascent secretory proteins across the endoplasmic reticulum. Rothblatt, J. A., R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman. 1989. J. Cell Biol. 109:2641-2652. The npl1 mutants reported here also weakly affect translocation of preprocarboxypeptidase Y across the ER membrane. A normally nuclear hybrid protein containing a NLS fused to invertase and a nucleolar protein are not localized to the nucleus in npl1/sec63 cells at the nonpermissive temperature. Thus, NPL1/SEC63 may act at a very early common step in localization of proteins to the nucleus and the ER. Alternatively, by affecting ER and nuclear envelope assembly, npl1 may indirectly alter assembly of proteins into the nucleus.

Each organelle in a eukaryotic cell has a distinct set of proteins that are necessary for its specific function. Certain peptides can act as signals to localize proteins to particular organelles such as the ER, the mitochondria (Verner and Schatz, 1988) and the nucleus (Silver and Hall, 1988). Several proteins have been identified that mediate the recognition of ER-destined proteins and their subsequent translocation across or assembly into the ER membrane (Walter and Blobel, 1980; Meyer et al., 1982; Tajima et al., 1986; Wiedman et al., 1987). Receptors have been proposed for mitochondrial signal peptides (Pfänder and Neupert, 1987; Pfänner et al., 1987) and recently a receptor for protein import into chloroplasts has been identified (Pain et al., 1988). By analogy, similar components may exist for localization of proteins to the nucleus.

Nuclear localization sequences (NLS) are stretches of amino acids that can redirect nonnuclear proteins such as β-galactosidase to the nucleus. When the first 74 amino acids of the yeast DNA binding protein GAL4 are joined to β-galactosidase, the result is a fusion protein that is found exclusively in the yeast nucleus as determined by immunofluorescence (Silver et al., 1984). A single amino acid change within this sequence (serine 6 to phenylalanine, termed GAL4*) causes the GAL4*β-galactosidase fusion protein to be no longer exclusively nuclear localized (Silver et al., 1988). Fusion of the NLS, PKKKRKV, from SV40 T-antigen (Kalderon et al., 1984a) to β-galactosidase also yields a hybrid protein that co-immunoprecipitates only when the yeast in yeast (Nelson and Silver, 1989). This amino acid sequence has been shown to be both necessary and sufficient for nuclear transport of SV40 T-antigen, as well as a number of other proteins (Kalderon et al., 1984b).

We have examined the localization of hybrid proteins with the SV40 or GAL4 NLS preceding almost all of the mitochondrial signal sequence of cytochrome c, in the yeast Saccharomyces cerevisiae. Cytochrome c, is found in the inner membrane of the yeast mitochondria as part of the complex III of the respiratory chain. Yeast lacking a functional cytochrome c, are respiratory deficient and cannot

1. Abbreviations used in this paper: DAPI, diamidino-phenylindole; EMS, ethyl methane sulfonate; NLS, nuclear localization sequences; preproCPY, preprocarboxypeptidase Y.
grow on nonfermentable carbon sources such as glycerol. Cytochrome c1 is made as a 34-kD molecular mass precursor, which has a 61 amino acid presequence at the amino terminus (Sadler et al., 1984). The presequence is removed in two steps as the protein is assembled into the mitochondria.

The NLS-cytochrome c1 fusion protein is preferentially localized to the nucleus and at the nuclear periphery as determined in immunofluorescence. Conversely, a protein containing a mutated NLS assembles into the mitochondria. The NLS-cytochrome c1 gene fusion fails to support growth on glycerol. We have used this growth defect to isolate mutants that grow on glycerol as a consequence of a defect in protein localization. The phenotypes of one complementation group (NPL1) are described. The gene corresponding to the mutation has been isolated and its DNA sequence determined.

The sequence predicts an open reading frame of 663 amino acids with a region of homology to DnaJ, an Escherichia coli heat shock protein (Ohki et al., 1986). The implications of these findings on protein sorting to the ER and the nucleus are discussed.

Materials and Methods

Construction of GAL4- and SV40-CYTI Gene Fusions

pIS144 and pIS145. The Ban II-Bam HI fragment of the plasmid YEpl3-41 (Sadler et al., 1984), which contains the complete cytochrome c1 gene (CYTI) except the first 26 base pairs, was cloned into puc19 (Yanisch-Perron et al., 1985) cut with Ban II and Bam HI. The resulting plasmid was opened at Ban II, the 3' overhang digested with St I and a Sma I 12mer linker attached to give the appropriate number of bases for an in-frame fusion to the GAL4 NLS. Sequence analysis of the resulting plasmid showed that the St I digest of the 3' overhang had caused an out of frame fusion site. We therefore attached a Xho I 8mer linker to the Sma I site. From the resulting plasmid (pIS34) the Xho I-Hpa I fragment was isolated and cloned into the plasmids pPS63 and pPS73 (Silver et al., 1988) respectively, which were cut with Xho I and Pvu II. These plasmids contain the GAL4 wild-type and mutant nuclear localization sequences under the control of the ADH1 promoter in the parent plasmid pAhA5 (Ammerer, 1983). The resulting fusions are shown in Fig. 1.

pIS157. For constructing the SV40-CYTI gene fusion, a Bam HI-Xho I fragment of the plasmid pSI44 (containing the ADH1 promoter and GAL4) was replaced by a Bam HI-Xho I fragment from the ADH1 promoter and the SV40 NLS (see Fig. 1).

pIS341. For constructing the C terminal truncated GAL4-CYTI1, a 700-bp Xho I-Asp 718 fragment from pIS34 was placed into pPS63 in place of the intact CYTI.

Immunoblotting

Cell extracts were prepared as previously described (Yaffe and Schatz, 1984) with the following modifications. Cells were grown in leucine drop-out medium with glucose (Sherman et al., 1983); for growth of the strains without plasmids, leucine was added. 1 ml of cells (1 x 10^6 cells/ml) was mixed with 160 ml of fresh 1.85 M NaOH/7.4 % mercaptoethanol and incubated in ice for 10 min. 160 ml 50% TCA was then added, incubation on ice continued for another 10 min, followed by centrifugation (14,000 g, 5 min). The resulting pellets were washed with cold acetone, air-dried, resuspended in Laemmli sample buffer, and heated for 5 min at 95°C. The samples were electrophoresed on 10 or 12 % SDS polyacrylamide gels (Laemmli, 1970). The polyacrylamide gels were blotted to nitrocellulose with a Polyblot Electroblotter (American Bionetics, Emeryville, CA). The nitrocellulose was blocked with 3 % BSA (United States Biochemical Corp., Cleveland, OH) or 5 % non-fat milk powder (Carnation Co., Los Angeles, CA) in PBS (50 mM Kphosphate, pH 7.0, 0.15 M NaCl; Johnson et al., 1984), incubated for 12 h with anti-cytochrome c1 antibody (the gift of G. Schatz, Biocenter, Basel, Switzerland) in 3 % BSA in PBS, washed for 2 h in PBS and incubated for 2 h with anti-rabbit IgG conjugated with horse-radish peroxidase (Bio-Rad Laboratories, Cambridge, MA).

Immunofluorescence

Cells were grown in 5 ml of minimal selective media with 2 % glucose to a cell density of 1-2 x 10^7 cells/ml and prepared for indirect immunofluorescence as previously described (Silver et al., 1984) with the following modifications. To each culture 0.6 ml of 37 % formaldehyde was added for fixation and the cultures gently shaken at 30°C for 90 min. The cells were collected by centrifugation (2.500 g, 5 min), washed once in 5 ml of solution P (1.1 M sorbitol, 0.1 M potassium phosphate, pH 6.5), resuspended in 1 ml solution P, transferred to a microcentrifuge tube and washed again. The cell pellet was resuspended in 1 ml of solution P and incubated at 30°C for 1 h with 5 ml of glusulase (Dupont Co., Wilmington, DE) and 5 ml of zymolase (10 mg/ml zymolase 20000 (in solution P; Miles Scientific Div., Naperville, IL). The cells were collected by centrifugation (3,000 g, 5 min), washed with solution P, and resuspended in 100-200 pl solution P. 20 pl of cells were placed in each well of a multiwell slide (Flow Laboratories, Inc., McLean, VA) that had been previously coated with 0.1 % polylysine (Sigma Chemical Co., St. Louis, MO). Following aspiration of the excess cells, the slide was immersed in methanol (~20°C) for 6 min, then transferred to acetone (~20°C) for 30 s. The slide was allowed to air dry and antibodies diluted in 1 ml mg BSA (Sigma Chemical Co.) in PBS were applied. To visualize cytochrome c1 and its derivatives, rabbit anti-cytochrome c1 was used at a dilution of 1:10,000, followed by FITC conjugated goat anti-rabbit IgG (Miles Scientific Div.) at a 1:200 dilution. To visualize SV40-invertase, rabbit antiinvertase (from R. Schekman, University of California, Berkeley, CA) was used at a 1:1,000 dilution followed by FITC conjugated goat anti-rabbit as described above. To visualize a nucleolar-associated antigen, a mouse monoclonal anti-nuclear antibody (A. Frumpton and Broun, Princeton University, unpublished results) was used at 1:1,000 dilution followed by FITC conjugated anti–mouse (Miles Scientific Div.) at 1:500. To visualize nuclei and mitochondria, the same cells were treated with DAPI (1 µg/ml) for 30 s. Cells were viewed at 1,000X (Axioskop equipped for fluorescence; Carl Zeiss Inc., Thornwood, NY). Exposure times for immunofluorescence pictures were 15 or 30 s.

Isolation of Mutants

Cells (10 ml at 4 x 10^7 cells/ml of W303Δcytl containing pIS157) cultivated in leucine dropout media with 2 % glucose were collected by centrifugation (2,600 g, 5 min) and resuspended in 0.1 M sodium phosphate pH 7.0. The cell pellet was resuspended in 0.1 % polylysine (Sigma Chemical Co.) in PBS were applied. To visualize cytochrome c1 and its derivatives, rabbit anti-cytochrome c1 was used at a dilution of 1:10,000, followed by FITC conjugated goat anti–rabbit IgG (Miles Scientific Div.) at a 1:200 dilution. To visualize SV40-invertase, rabbit antiinvertase (from R. Schekman, University of California, Berkeley, CA) was used at a 1:1,000 dilution followed by FITC conjugated goat anti–rabbit as described above. To visualize a nucleolar-associated antigen, a mouse monoclonal anti-nuclear antibody (A. Frumpton and Broun, Princeton University, unpublished results) was used at 1:1,000 dilution followed by FITC conjugated anti–mouse (Miles Scientific Div.) at 1:500. To visualize nuclei and mitochondria, the same cells were treated with DAPI (1 µg/ml) for 30 s. Cells were viewed at 1,000X (Axioskop equipped for fluorescence; Carl Zeiss Inc., Thornwood, NY). Exposure times for immunofluorescence pictures were 15 or 30 s.

Cloning and Sequence Analysis of NPL1/SEC63

DNA encoding NPL1 was identified as follows. Yeast strain npl1-1 was grown in YEPD to ~1 x 10^7 cells/ml and transformed (Ito et al., 1983) with the genomic DNA library of Nasmyth (Nasmyth and Reed, 1980). Transformants were selected on minimal medium lacking leucine at 30°C. Approximately 3,500 colonies were replica-plated at 37°C to minimal medium lacking leucine. Plasmid DNA was prepared from two yeast colonies that could grow at 37°C but failed to grow at 37°C when cured of the plasmid. Both plasmids contained the same size insert and were capable of rescuing the temperature sensitivity when reintroduced into npl1-1 cells. One of these plasmids, pTK1, was used for all further analysis. DNA sequencing was performed using the dideoxy chain termination method of Sanger et al. (1977, 1980). Standard M13 primers or synthetic primers (prepared by a DNA synthesizer; 380A, Applied Biosystems Inc., Foster, CA) corresponding to regions of NPL1 were used.

The homology between NPL1/SEC63 and DnaJ was identified by a computer search using the FASTA algorithm. The mean initial alignment score was 28 (SD, 7.5). The alignment score for the DnaJ versus NPL1/SEC63 homology was 172, while the second highest score was 91. The extent and
length of sequence similarity is similar to that found between homeobox proteins of different families (Burglin, 1988).

The NPL1 gene disruption was constructed as follows. The plasmid pTK1-S was digested with Eco RI and religated to delete the 5' end of NPL1. URA3 was inserted between the Spe I and Eco RV sites to yield a plasmid (PISS525; Fig. 6) with 250 NPL1 base pairs at the 5' end and 630 NPL1 bp at the 3' end. The NPLI-URA3 fragment was excised by SpI-SphI treatment and introduced into MS810. Stable transformants were analyzed by Southern blot analysis to confirm that integration had occurred at the chromosomal NPL1.

**Other Methods**

Published methods were used for yeast transformation (Ito et al., 1983) and bacterial transformation (Mandel and Higa, 1970). DNA manipulations were performed as described by Maniatis et al. (1982). Radiolabeling and immunoprecipitation was performed as described by Rothblatt et al., 1989.

**Results**

To test the effect of a NLS on the intracellular distribution of a mitochondrial protein, we constructed gene fusions between the GAL4 or the SV40 T-antigen nuclear localization sequences and cytochrome c1 (Fig. 1). DNA encoding the first 74 amino acids of either wild-type or mutant GAL4* (serine 6 to phenylalanine) was fused to cytochrome c1 at the 12th codon. For the SV40-cytochrome c1 hybrid, an oligonucleotide encoding the SV40 NLS was fused to cytochrome c1 at the 1st codon. The strong constitutive promoter encoding the SV40 NLS was fused to cytochrome c1 at the 12th codon. For the SV40-cytochrome c1, hybrid, an oligonucleotide encoding the SV40 NLS was fused to cytochrome c1 at the 1st codon. The strong constitutive promoter from ADH1 was used to express all gene fusions.

**GAL4-Cytochrome c1 and SV40-Cytochrome c1, Fusion Proteins Failed to Complement a Cytochrome c1 Deficient Yeast Strain**

We anticipated that cells would be unable to grow on glycerol if cytochrome c1 were directed to the nucleus instead of the mitochondrion. To confirm this, plasmids bearing GAL4-CYT1 and SV40-CYT1 gene fusions were introduced into two different cytochrome c1 deficient strains (W303Δcyt1 and 1165/11, Table I), which by themselves do not grow on glycerol. Cells producing the GAL4-cytochrome c1 or the SV40-cytochrome c1 fusion also failed to grow on glycerol. In contrast, cells containing GAL4*-cytochrome c1 grew on glycerol as did cells transformed with a plasmid bearing wild-type cytochrome c1 (data not shown).

The inability of cells bearing NLS-cytochrome c1 to grow on glycerol is not because of failure to produce intact fusion proteins. GAL4-cytochrome c1 and SV40-cytochrome c1 fusion proteins are synthesized in these strains and are of the predicted sizes. Correct mitochondrial localization, assessed by processing of the fusion protein to mature cytochrome c1, was detected only for the GAL4*-cytochrome c1 hybrid, consistent with the ability of cells synthesizing this protein to grow on glycerol. Cell lysates from transformed cells were analyzed by immunoblots and probed with anti-cytochrome c1 antibody. The untransformed cyt1 strain (Table I) produced no detectable cytochrome c1 (Fig. 2, lane 1). Lysates from cells producing wild-type cytochrome c1 (Fig. 2, lane 3) contained mature cytochrome c1 (apparent molecular mass of 31 kD; cytochrome c1 does not run true to size.

**Table I. Strains Used in this Study**

| Species and strain | Genotype | Source |
|--------------------|----------|--------|
| **S. cerevisiae**   |          |        |
| 1165/11            | MATa leu2-3 leu2-112 his3 ilv cyt1-1 | Lang and Kaudewitz (1982) |
| W303Δcyt1          | MATa leu2-3 leu2-112 ura3-52 trp1 ade2 his3 cyt1::HIS3 | A. Tzagoloff |
| W303Δαcyt1         | MATa leu2-3 leu2-112 ura3-52 trp1 ade2 his3 cyt1::HIS3 | A. Tzagoloff |
| RDM50-94C           | MATa leu2-3 leu2-112 his4 ura3-52 sec62-1 | R. Schekman |
| JRM151-1B           | MATa leu2-3, leu2-112 ura3-52 sec63-1 | R. Schekman |
| JR25A-2A            | MATa leu2-3 leu2-112 trpl ura3-52 his3 | J. Broach |
| MS810               | MATa/α ura3-52/ura3-52 ade1-1011 + leu2-3, leu2-112/leu2-3, leu2-112 | M. Rose |
| E. coli             |          |        |
| JM101               | D(lac pro)thi supE F' proABlacIqZDM15 | Messing (1979) |
| MM294               | end hisR thi pro | Lauer et al. (1981) |
in this gel system (Wakabayashi et al., 1980) and the major processing intermediate (cytochrome c1 is processed in two steps). Lysates from cells producing GAL4–cytochrome c1 and SV40–cytochrome c1 contained primarily the fusion proteins of apparent molecular masses 42 kD and 37 kD, respectively (Fig. 2, lanes 2 and 4). Cells producing GAL4*–cytochrome c1 contained the full-length fusion protein and the intermediate and mature forms (Fig. 2, lane 3).

Localization of Cytochrome c1 Fusion Proteins

The inability of cells bearing NLS-cytochrome c1 proteins to grow on glycerol could be because of localization of the fusion proteins to the nucleus. This is supported by the observation that a fusion protein with a mutated NLS can grow on glycerol. An alternate explanation is that SV40- and GAL4–cytochrome c1 are still localized to mitochondria, but, for some reason, only the GAL4*–cytochrome c1 allows glycerol-dependent growth. To test between these possibilities, immunofluorescence was used to assess the intracellular location of the various fusion proteins. The GAL4– and SV40–cytochrome c1 fusion proteins were associated with the nucleus at the rim, as determined by indirect immunofluorescence. Cells producing cytochrome c1 fusion proteins or wild-type cytochrome c1 were examined by indirect immunofluorescence with an antibody directed against cytochrome c1. Wild-type cytochrome c1 was localized exclusively to mitochondria (Fig. 3, 1–3) as judged by the coincidence of the diaminophenylindole (DAPI) staining with mitochondrial DNA and the ribbon-like appearance of the immunofluorescence that is characteristic of mitochondria. GAL4–cytochrome c1 fusion proteins were associated with the nucleus and opposed to the nuclear envelope (Fig. 3, 5 and 8). SV40–cytochrome c1 was similarly localized (data not shown). Conversely, the mutant GAL4*–cytochrome c1 was not localized to the nucleus. Instead, the protein was cytoplasmic and often concentrated near the cell periphery.

Figure 2. Production of GAL4– and SV40–cytochrome c1 proteins. Cell extracts from 1165/11 containing plasmids encoding intact cytochrome c1 and cytochrome c1 fusion proteins were subjected to SDS-PAGE (12% gel). Proteins were transferred to nitrocellulose and probed with rabbit anticytochrome c1 antibody and goat anti–rabbit IgG conjugated with horseradish peroxidase. Lane 1, no plasmid; lane 2, pLS144 (GAL4–cytochrome c1); lane 3, pLS145 (GAL4*–cytochrome c1); lane 4, pLS157 (SV40–cytochrome c1); lane 5, YEp13-41 (wild-type cytochrome c1). In these experiments, unequal amounts of protein were loaded for each extract. In other experiments, when equal cell equivalents are loaded, the results are the same as those presented here (data not shown).

Isolation of Yeast Mutants Defective in Protein Localization

We took advantage of the inability of cells bearing the SV40- and GAL4–cytochrome c1 to grow on glycerol to isolate mutants that might be defective in nuclear protein localization (npl). We hypothesized that a partial block in nuclear localization would result in assembly of enough protein into mitochondria to allow growth on glycerol. A complete block in nuclear protein localization would, however, result in cell death. Therefore, we sought mutants that were Gly+ at 30°C but Ts- at 36°C. A cytochrome c1 defective strain (W303-Δcyl1) bearing the SV40–cytochrome c1 gene fusion was mutagenized with EMS (see Materials and Methods), and the resulting colonies were screened for the ability to grow on glycerol at 30°C (for details of the mutagenesis see Materials and Methods). The Gly+ colonies were further screened for temperature sensitivity at 37°C on rich medium. Two mutants (Gly+, Ts-) in the same complementation group (npl1) were obtained and analyzed in detail.

Mutations that allowed growth on glycerol are chromosomal and not plasmid-linked. When mutants were cured of the plasmid and retransformed with unmutagenized plasmids bearing either the GAL4- or SV40–cytochrome c1 fusions, the ability to grow on glycerol was restored. The mutagenized strains without the plasmids were still unable to grow on glycerol, showing that the phenotype is not due to reversion of the chromosomal CYT1 but depends on the presence of the cytochrome c1 fusion. A single recessive mutation is responsible for the thermosensitivity and growth on glycerol. A diploid strain obtained by backcrossing to an isogenic strain of the opposite mating type (W303αΔcyl1) was no longer temperature sensitive and failed to grow on glycerol when transformed with the plasmid bearing the SV40- or GAL4–cytochrome c1 fusion. (Since the diploids were phenotypically Gly+, they would not sporulate. To sporulate the diploids, they were transformed with a plasmid bearing a normal copy of CYT1.) For npl1-1 and npl1-2, the Ts defect segregated as a single mutation (2 Ts+ and 2 wild-type spores per tetrad in all 16 tested). Backcrosses of npl1-1 to a wild-type strain (JR25A-2A) confirmed this (two Ts+ and two wild-type spores in 18/18 tetrads analyzed).

To demonstrate linkage between the thermosensitive growth and growth on glycerol, the CYT1 bearing plasmid was segregated from haploids from the cross of npl1-1 with W303αΔcyl1. The resulting haploids were then retransformed with plasmids bearing either GAL4–cytochrome c1, GAL4*–cytochrome c1, or SV40–cytochrome c1, and tested for the ability to grow on glycerol. The inability to grow at 37°C was tightly linked to the ability to grow on glycerol when spores contained plasmids encoding GAL4– or SV40–cytochrome c1 fusion proteins.
Figure 3. Immunofluorescence of cells producing GAL4- and SV40-cytochrome c. Cells were grown at 30°C, prepared for immunofluorescence and treated with a rabbit anticytochrome c antibody, followed by FITC-conjugated anti-rabbit IgG, to localize the cytochrome c and the derivatives, and DAPI to stain cell DNA. W303Acyl cells producing intact cytochrome c (1-3); producing GAL4-cytochrome c (4-9); and producing GAL4*-cytochrome c (10-15). 1, 4, 7, 10, and 13 are stained with anticytochrome c, 2, 5, 8, 11, and 14 are the corresponding cells stained with DAPI, and 3, 6, 9, 12, and 15 are the corresponding cells viewed by phase-contrast.
cytochrome c, since Ts+/Ts- segregated 2:2, all Ts- were Gly+, and all Ts- were Gly-. The ten wild-type spores (cyt/) grew normally at 37°C but failed to grow on glycerol when transformed with GAL4-cytochrome c1 or SV40-cytochrome c1. All 20 spores grew on glycerol when bearing the plasmid encoding GAL4*-cytochrome c1. The properties of npl1 are summarized in Table II.

Both npl1-1 and npl1-2 showed altered localization of the GAL4- and SV40-cytochrome c1 proteins by immunofluorescence. Cells grown at 30°C were examined by immunofluorescence with the anticytochrome c1 antibody. For both mutants, the proteins were no longer localized to the nucleus, but rather distributed throughout the cell (data not shown). In some cells, an increase in mitochondrial associated protein was also observed.

Allelism to SEC63
One possible explanation for the localization of GAL4- and SV40-cytochrome c1 is that they may be localized to the ER, which is continuous with the nuclear envelope. Mutations that affect early steps in translocation of proteins across the ER have been identified (Deshaies and Schekman, 1987; Rothblatt et al., 1989). We tested for complementation between our mutants and mutants defective in early steps of protein translocation into the ER. One such mutation, sec63-1, is allelic to npl1; npl1-1 and sec63-1 failed to complement each other for growth at 37°C. To test for linkage between these two mutations, a diploid between npl1-I and sec63-1 was sporulated. Spores from 14 complete asci were all viable at 23°C but all were Ts- for growth at 38°C. sec63-1 cells are defective for translocation of some secreted proteins across the ER as evidenced by accumulation of unprocessed secretory precursor proteins (Rothblatt et al., 1989). We thus wished to compare translocation of proteins across the ER in npl1-I, npl1-II and sec63-1. Cells were grown at 24°C, shifted to 37°C for 2 h, pulse labeled with [35S]SO42- and assayed for processing of preprocarboxypeptidase Y (preproCPY). sec63-1 cells accumulated unprocessed preproCPY at both 24°C and 37°C (Fig. 4, lanes 1 and 2). In contrast, npl1-I and npl1-II showed much lower levels of preproCPY at 37°C (Fig. 4, lanes 4 and 6). However, the level of preproCPY accumulation was more than that seen in NPL1+ cells (Fig. 4, lane 8). sec63-1 is the strongest of the alleles examined thus far with regards to the ER translocation defect.

Characterization of the NPL1/SEC63 Gene
To understand better the role of NPL1/SEC63 in normal cell function, the gene was cloned from a yeast genomic DNA library by complementation of the ts-lethal phenotype of npl1-I. Transformants were selected for growth on medium minus leucine at 23°C. The resulting transformants were tested for growth at 37°C after replica plating. From ~3,500 Leu+ colonies, we obtained two colonies that grew at 37°C. When introduced back into npl1-I cells, plasmids recovered from both transformants conferred the Ts+ phenotype. One rescuing clone, pTK1, was selected for further study (Fig. 5; the second clone, pTK2, had similar restriction enzyme cleavage sites to pTK1 and has not been studied further.). Deletion analysis of this clone localized the npl1 complementing region to a 2.7-kb DNA fragment.

The cloned DNA in pTK1 encodes the NPL1/SEC63 structural gene. We subcloned the 2.7-kb restriction fragment that complemented npl1 into the yeast integrating plasmid YIp5. To direct integration of URA3 at NPL1, the plasmid was digested with Spe I and transformed into W303a (NPL1 ura3). Integration of intact plasmid at NPL1 was confirmed by Southern analysis (data not shown). The transformant was crossed to the npl1-I strain to form a diploid and sporulated. In 22 asci, each containing four viable spores, two spores were Ura+/Ts+ and two spores were Ura-/Ts-. In addition, 36 additional spores from incomplete asci of the same diploid all showed cosegregation of Ura+ with Ts+ and Ura+ with Ts-.

To confirm further the linkage between SEC63 and NPL1, diploids were constructed between the strain with URA3 integrated at the NPL1 Spe I site and JRM151-1B (sec63-I ura3). In 31 asci containing four viable spores, two Ura- spores were Ts- and two Ura+ spores were Ts+ indicating that sec63 and npl1 are tightly linked. JRM151-1B grew at 37°C when transformed with a plasmid bearing the 2.7-kb fragment encoding NPL1. Finally, the 1.7-kb Eco RI-Pst I NPL1 containing DNA fragment crosshybridized with a 1.7-kb Eco RI-Pst I fragment of a centromere-based plasmid that...
restores growth of sec63 at 37° (A. Eun and R. Schekman, unpublished results).

NPL1 is essential for germination. The NPL1 chromosomal locus was disrupted with URAS in a diploid cell and the resulting transformants subjected to tetrad analysis (see Materials and Methods). Stable Ura+ transformants were sporulated and two viable spores were recovered from each of 20 complete ascii at both 23°C and 17°C. In all cases, the two surviving spores were Ura+.

The DNA Sequence of NPL1/SEC63

The 2.7-kb nplll complementing region of pTK1 was sequenced by the dye-dye chain terminating method of Sanger et al., 1980 (Fig. 6). This sequence contains a 1989 bp open reading frame beginning with a methionine codon at bp 102, preceded by several possible TATA boxes (at bp 15, 45, 75, and 85). A 2.7-kb polyA RNA was detected by Northern blot analysis using the nplll complementing region as a probe (data not shown). It is possible that some regulatory/promoter sequences are missing in the complementing clone, since it contains only ~100 bp upstream from the first potential ATG start codon.

The deduced amino acid sequence of NPL1 has several striking features. There are three potential membrane-spanning regions, from amino acids 14–41, 93–108, and 221–239 (Fig. 6). There is no good signal sequence for ER translocation. Between the second and third hydrophobic regions (amino acids 125–197) is a sequence that shares 43% amino acid identity and many conservative substitutions with respect to the amino terminus of the DnaJ protein of E. coli (Fig. 7; see below; Ohki et al., 1986; Bardwell et al., 1986). The next region (amino acids 240–611) is relatively non-descript. It contains one internal repeat, Lys-Glu-Pro-Leu-Ile/Val-Pro-X-Ser-X-Pro (where the Xs are mostly hydrophobic amino acids), at amino acids 461–471 and 493–503. The C-terminal 52 amino acids are largely acidic; 27 are Asp or Glu, and none are Lys, Arg, or His. Of the remaining 25 amino acids in this region, 15 are Ser, Thr, or Tyr. There are three potential N-linked glycosylation sites following the third hydrophobic stretch.

Nuclear Protein Localization in NPL1/SEC63 Cells

nplll/sec63 cells showed a defect in nuclear protein localization. JRM151-1B (sec63-1) was transformed with a plasmid encoding a hybrid protein containing the SV40 T-antigen-NLS fused to invertase (lacking the signal sequence). In wild-type cells, this protein is efficiently localized to the nucleus (Nelson and Silver, 1989). In JRM151-1B cells grown at 37°C for 4 h, the SV40-invertase was no longer localized to the nucleus in 90% of the cells that were stained with the antibody (Fig. 8, 1 and 2). The mislocalization of SV40-invertase is first detectable after 1.5 h at 37°C and was reversed by transformation of JRM151-1B cells with NPL1 (data not shown). Moreover, the SV40-invertase was localized entirely to the nucleus in sec62 and sec62 cells (Fig. 8, 3 and 4; sec62 and sec62 are also defective in translocation of proteins across the ER [Deshaies and Schekman, 1987; Rothblatt et al., 1989]). Mislocalization of the nuclear SV40-invertase was weak and variable in nplll-1 cells in contrast to sec63-1 cells. In separate experiments, mislocalization of the SV40-invertase in nplll-1 at 30°C varied from 2–10%. This is consistent with other observations that sec63-1 is a stronger allele than nplll-1 and nplll-2.

Endogenous nuclear protein localization is affected in nplll-1 and sec63-1. The localization of a nucleolar protein was examined with an antibody to a 35-kD nucleolar protein. This nucleolar-specific antigen was mislocalized in 95% of sec63-1 cells (Fig. 9 B) and 10% of nplll-1 cells when they were examined by immunofluorescence after 2 h at 37°C. Localization of the same antigen was unaffected in sec62 cells grown under similar conditions (Fig. 9, C). sec63-1 and nplll-1 cells bearing NPL1 on a multi-copy plasmid no longer showed mislocalization of the nucleolar antigen. 5–10% of nplll-1 cells also displayed nonnuclear GAL4 at 30°C (data not shown). Intact GAL4 is normally completely nuclear localized in wild-type cells (Silver et al., 1988).

Discussion

We have identified yeast mutants that are defective in the localization of nuclear proteins and protein assembly into the ER. When the yeast mitochondrial protein cytochrome c1 contains both a NLS and a portion of a mitochondrial localization sequence, it is not processed to mature cytochrome c1 and instead is localized to the nucleus and at the nuclear rim. If a mutant NLS is used, enough of the protein is correctly processed and localized within mitochondria to function and allow growth on glycerol. We isolated mutants that affect the nuclear localization of these hybrid proteins (nplll mutants). These mutants allowed GAL4- and SV40-cytochrome c1 to enter mitochondria and, thus, for cells to grow on glycerol. One mutant nplll is allelic to sec63, a gene identified as important for translocation of proteins across the ER (Rothblatt et al., 1989).
The Sequence of the NPL1/SEC63 Gene

The NPL1/SEC63 protein sequence contains three possible membrane spanning regions, a region of homology to E. coli DnaJ, and a very acidic C terminus. The region of homology between NPL1 and DnaJ consists of only 70 amino acids, suggesting that this is a single common domain within otherwise dissimilar proteins. The fact that this region of homology is between two potential membrane spanning regions, a region of homology to DnaJ, and GrpE have been hypothesized to act by destabilization of abnormal proteins (Straus et al., 1988), synthesis of certain membrane proteins (Okhi et al., 1987), recovery from heat shock (Pelham, 1986), and activation of the unwinding activity of DnaB during lambda DNA replication (Dodson et al., 1986). To explain these observations, DnaK, DnaJ, and GrpE have been hypothesized to act by destabilization of folded proteins (Pelham, 1986; Straus, 1988), perhaps by lowering the transition energy between alternative folded states.

Similarly, yeast heat-shock proteins have been proposed to act as “unfoldases” that alter protein conformation (Deshaies et al., 1986; Pelham, 1986; Straus, 1988). To explain these observations, DnaK, DnaJ, and GrpE have been hypothesized to act by destabilization of folded proteins (Pelham, 1986; Straus, 1988), perhaps by lowering the transition energy between alternative folded states.

The Figure 6. The sequence of the NPL1/SEC63 gene. The sequence shown represents the insert in plasmid pTKI-S, which contains the entire NPL1/SEC63 gene, as defined by complementation of nplI and sec63 mutants, and by linkage analysis (see Results). Also shown is the putative protein sequence encoded by this gene. Unique restriction sites are underlined (bp 239: Eco RI; bp 502: Spe I; bp 1943: Pst I; bp 2672: Hind III).

Figure 7. The homology between NPL1/SEC63 and DnaJ. The region of homology between these genes, with amino acid identities indicated with dots. Xs represent gaps introduced by the fastA program to optimize the alignment.
Figure 8. Localization of SV40-invertase in sec62 and sec63. JRM151-1B (sec63-1) cells (1-2) and RDM 50-94C (sec62-1) cells (3-4) bearing pMN8 (a plasmid encoding the SV40 NLS fused to cytoplasmic invertase [Nelson and Silver, 1989]) were grown to a cell density of 5 × 10^6 cells/ml in minimal selective media with 2% glucose at 23°C, shifted to 37°C for 4 h, prepared for immunofluorescence, and treated with rabbit antiinvertase, followed by FITC-conjugated anti-rabbit IgG, to localize the SV40-invertase fusion proteins (1 and 3), and DAPI to visualize nuclear DNA (2-4). Although all the cells are SUL-~, the level of endogenous invertase is low and undetectable by immunofluorescence with antiinvertase (data not shown).

A family of HSP70s in yeast are necessary for localization of proteins to both the ER and mitochondria (Deshaies et al., 1988; Chirico et al., 1988). In this case, the HSP70 proteins may maintain targeting sequences in an active, translocation-competent conformation. It is not known whether these proteins play a similar role in localization of nuclear proteins.

The region of homology between DnaJ and NPL1/SEC63 may mediate an interaction with HSP70 proteins. This domain in NPL1/SEC63 could allow the formation of a complex that would associate an HSP70 with the membrane. This complex could interact with secreted proteins to expose their signal sequences and associate them with the ER membrane in advance of translocation across this membrane. By this model, the DnaJ region would be exposed to the cytoplasm. On the other hand, the DnaJ region could be exposed to the lumenal surface. In this case, NPL1/SEC63 could interact with yeast BiP (Rose et al., 1989), the ER localized HSP70 homologue thought to be important for passage of proteins through the ER (Pelham, 1986).

**NPL1/SEC63 Affects Assembly of Proteins into the ER and the Nucleus**

One plausible explanation for the dual effect of this mutation is as follows. It may be that the SV40- and GAL4–cytochrome c fusion proteins are actually located at the ER. Somehow, attachment of the NLS caused an endogenous secretory signal sequence to be revealed and some of the protein, by default, binds to or enters the ER. (There are no N-linked
glycosylation sites so this is difficult to analyze.) Hence, mutations that keep proteins out of the ER would also cause the mislocalization of these fusion proteins. The effect on nuclear proteins may be secondary because it requires at least 1–2 h at 37°C before it is observed. sec63 mutants are, on the other hand, already defective at 24°C for ER translocation. But sec62 and sec63 cells, also defective in ER assembly (Deshais and Schekman, 1987), show no abnormal nuclear protein localization when tested under the same conditions, arguing against this view. The inability to observe strong defects in nuclear protein localization at the semipermissive temperature of 30°C may instead be because of the insensitivity of the immunofluorescence assay used to localize nuclear proteins.

An alternate explanation is that the GAL4- and SV40–cytochrome c1 proteins do interact with some component of the nuclear import apparatus, but that their transport is not complete. NPL1/SEC63 could then be a component shared by both ER and nuclear import machinery. NPL1/SEC63 may be a component of the nuclear pore as well as part of a translocator complex involved in protein import into the ER. Alternatively, it may be a receptor for a chaperonin (Hemmingsen et al., 1988) that delivers precursors to the ER and the nuclear envelope. Finally, the mutant reported here may affect assembly of the pore complex, perhaps by disrupting insertion of membrane proteins that are necessary pore components. The intracellular localization of NPL1/SEC63 protein and tests of its role in an in vitro nuclear localization assay (Silver et al., 1989) will address these questions directly. Until the true function of this gene is understood, future publications should refer to it as SEC63.

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