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

I. Sadler, A. Chiang, T. Kurihara, J. Rothblatt,‡ J. Way, and P. Silver*

*Department of Biology, Princeton University, Princeton, New Jersey 08544, and ‡Department of Biochemistry, University of California, Berkeley, California 94720

Abstract. When nuclear localization sequences (termed NLS) are placed at the N terminus of cytochrome c_1, 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_1, but expressing the hybrid NLS–cytochrome c_1 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_1 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. *npl1-1* 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 preprocarboxypeptidaseY 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 (Pfaller and Neupert, 1987; Pfanner 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)[1] are stretches of amino acids that are capable of redirecting 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., 1984*a*) to β-galactosidase also yields a hybrid protein that is predominantly associated with the nucleus 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., 1984*b*).

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_1 in the yeast *Saccharomyces cerevisiae*. Cytochrome c_1 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_1 are respiratory deficient and cannot

1. *Abbreviations used in this paper*: DAPI, diamidinophenylindole; EMS, ethyl methanesulfonate; NLS, nuclear localization sequences; preproCPY, preprocarboxypeptidase Y.

© The Rockefeller University Press, 0021-9525/89/12/2665/11 $2.00

grow on nonfermentable carbon sources such as glycerol. Cytochrome c_1 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 c_1 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 c_1 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-CYT1 Gene Fusions

pIS144 and pIS145. The Ban II-Bam HI fragment of the plasmid YEp13-41 (Sadler et al., 1984), which contains the complete cytochrome c_1 gene (*CYT1*) except the first 26 base pairs, was cloned into pucl9 (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 S1 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 S1 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 pAAH5 (Ammerer, 1983). The resulting fusions are shown in Fig. 1.

pIS157. For constructing the SV40-CYT1 gene fusion, a Bam HI–Xho I fragment of the plasmid pPS144 (containing the *ADH1* promoter and *GAL4*) was replaced by a Bam HI–Xho I fragment with the *ADH1* promoter and the *SV40* NLS (see Fig. 1).

pIS341. For constructing the C terminal truncated GAL4-CYT1, a 700-bp Xho I–Asp 718 fragment from pIS34 was placed into pPS63 in place of the intact *CYT1*.

Immunoblots

Cell extracts were prepared as previously described (Yaffe and Schatz, 1984) with the following modifications. Cells were grown in leucine dropout medium with glucose (Sherman et al., 1983); for growth of the strains without plasmids, leucine was added. 1 ml of cells (1×10^8 cells/ml) was mixed with 160 μl of fresh 1.85 M NaOH/7.4% β-mercaptoethanol and incubated in ice for 10 min. 160 μl 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, 0.15 M NaCl; Johnson et al., 1984), incubated for 12 h with anti-cytochrome c_1 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). After washing for 30 min in PBS, the protein-antibody complexes were visualized by treatment with horse-radish peroxidase color developing reagent (Bio-Rad Laboratories).

Immunofluorescence

Cells were grown in 5 ml of minimal selective media with 2% glucose to a cell density of 1–2 × 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 μl of glusulase (Dupont Co., Wilmington, DE) and 5 μl of zymolyase (10 mg/ml zymolyase 20,000 (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 μl solution P. 20 μl 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 mg/ml BSA (Sigma Chemical Co.) in PBS were applied. To visualize cytochrome c_1 and its derivatives, rabbit anti-cytochrome c_1 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-nucleolar antibody (Armstrong and Broach, 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 1,000× (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 × 10^7 cells/ml of W303Δ*cyt1* containing pIS 157) 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 pH7 to a final concentration of 1 × 10^8 cells/ml. The cells were treated with 10 or 40 μl of ethyl methanesulfonate (EMS) for 1 h at 30°C and the mutagenesis stopped by the addition of 0.2 ml of the cells to 8 ml of 5% sodium thiosulfate. The cells were diluted 1:10 and 1:100 in H_2O and 50–100 μl plated onto leucine dropout plates with 2% glucose. After 4 d at 30°C, the colonies were replica plated to yeast peptone (YEP) plates (Sherman et al., 1983) with 3% glycerol. Potential mutants were restreaked onto YEP/3% glycerol, leucine dropout/2% glucose, and YEP/2% glucose (YEPD) plates at 23°C, 30°C, and 37°C. Mutants that failed to grow at 37°C under all three conditions were cured of plasmids by cultivating cells from a single colony in 5 ml YEPD for more than 30 generations, plating cells onto YEPD plates, and replica plating the resultant colonies to leucine dropout plates to check for plasmid loss. Cells that failed to grow in the absence of leucine were rechecked for the inability to grow on glycerol and for temperature sensitive growth at 37°C.

Cloning and Sequence Analysis of NPL1/SEC63

DNA encoding NPL1 was identified as follows. Yeast strain *npl1-1* was grown in YEPD to ~1 × 10^8 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 (PIS525; Fig. 6) with 250 *NPL1* base pairs at the 5' end and 630 *NPL1* bp at the 3' end. The *NPL1-URA3* fragment was excised by Ssp I–Sph I 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 c_1 (Fig. 1). DNA encoding the first 74 amino acids of either wild-type or mutant GAL4* (serine 6 to phenylalanine) was fused to cytochrome c_1 at the 12th codon. For the SV40-cytochrome c_1 hybrid, an oligonucleotide encoding the SV40 NLS was fused to cytochrome c_1 at the same codon. The strong constitutive promoter from *ADH1* was used to express all gene fusions.

GAL4-Cytochrome c_1 and SV40-Cytochrome c_1 Fusion Proteins Failed to Complement a Cytochrome c_1 Deficient Yeast Strain

We anticipated that cells would be unable to grow on glycerol if cytochrome c_1 were directed to the nucleus instead of the mitochondria. To confirm this, plasmids bearing *GAL4-CYT1* and *SV40-CYT1* gene fusions were introduced into two different cytochrome c_1 deficient strains (W303α Δ*cyt1* and 1165/11, Table I), which by themselves do not grow on glycerol. Cells producing the GAL4-cytochrome c_1 or the SV40-cytochrome c_1 fusion also failed to grow on glycerol. In contrast, cells containing GAL4*-cytochrome c_1 grew on glycerol as did cells transformed with a plasmid bearing wild-type cytochrome c_1 (data not shown).

The inability of cells bearing NLS–cytochrome c_1 to grow on glycerol is not because of failure to produce intact fusion proteins. GAL4–cytochrome c_1 and SV40–cytochrome c_1 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 c_1 was detected only for the GAL4*-cytochrome c_1 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 c_1 antibody. The untransformed *cyt1* strain (Table I) produced no detectable cytochrome c_1 (Fig. 2, lane *1*). Lysates from cells producing wild-type cytochrome c_1 (Fig. 2, lane *5*) contained mature cytochrome c_1 (apparent molecular mass of 31 kD; cytochrome c_1 does not run true to size

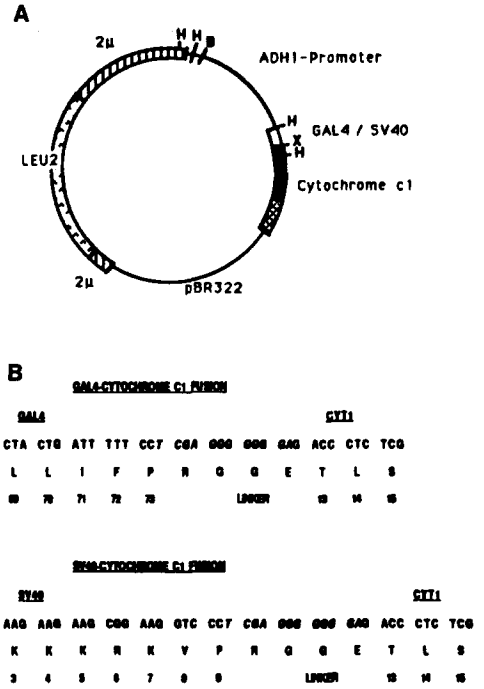

Figure 1. Plasmids encoding GAL4- and SV40–cytochrome c_1 fusion proteins. *A*, Diagram of the gene fusions under the control of the *ADH1* promoter. In pIS 144 and 145, cytochrome c_1 (*black area*) on a Xho I–Pvu II fragment (*black and shaded areas*) was fused to DNA encoding the first 74 GAL4 amino acids (*white area*). pIS145 contains a mutation at GAL4 amino acid serine 6. pIS 157 contains the SV40 NLS in place of GAL4 (*white area*). *H*, *B*, and *X* indicate sites cut by the restriction enzymes Hind III, Bam HI, and Xho I, respectively. *B*, The sites of the fusion betwen the nuclear localization sequences and *CYT1* are illustrated.

Table I. Strains Used in This Study

Species and strain *S. cerevisiae*	Genotype	Source
1165/11	*MATa leu2-3 leu2-112 his3 ilv cyt1-1*	Lang and Kaudewitz (1982)
W303αΔ*cyt1*	*MATa leu2-3 leu2-112 ura3-52 trp1 ade2 his3 cyt1::HIS3*	A. Tzagaloff
W303aΔ*cyt1*	*MATa leu2-3 leu2-112 ura3-52 trp1 ade2 his3 cyt1::HIS3*	A. Tzagaloff
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	MATα *leu2-3 leu2-112 trp1 ura3-52 his3*	J. Broach
MS810	*MATa/α ura3-52/ura3-52 ade1-101/+ leu2-3, leu2-112/leu2-3, leu2-112*	M. Rose
E. coli		
JM101	D(*lac pro*)*thi supE F' proABlacIq*ZDM15	Messing (1979)
MM294	*end hsdR thi pro*	Lauer et al. (1981)

in this gel system (Wakabayashi et al., 1980) and the major processing intermediate (cytochrome c_1 is processed in two steps). Lysates from cells producing GAL4–cytochrome c_1 and SV40–cytochrome c_1 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 c_1 contained the full-length fusion protein and the intermediate and mature forms (Fig. 2, lane *3*).

Localization of Cytochrome c_1 Fusion Proteins

The inability of cells bearing NLS-cytochrome c_1 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 c_1 are still localized to mitochondria, but, for some reason, only the GAL4*–cytochrome c_1 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 c_1 fusion proteins were associated with the nucleus at the rim, as determined by indirect immunofluorescence. Cells producing cytochrome c_1 fusion proteins or wild-type cytochrome c_1 were examined by indirect immunofluorescence with an antibody directed against cytochrome c_1. Wild-type cytochrome c_1 was localized exclusively to mitochondria (Fig. 3, *1–3*) as judged by the coincidence of the diamidinophenylindole (DAPI) staining with mitochondrial DNA and the ribbon-like appearance of the immunofluorescence that is characteristic of mitochondria. GAL4–cytochrome c_1 fusion proteins were at the nucleus and opposed to the nuclear envelope (Fig. 3, *4* and *7*) as judged by comparison to the same cells stained with DAPI (Fig. 3, *5* and *8*). SV40–cytochrome c_1 was similarly localized (data not shown). Conversely, the mutant GAL4*–cytochrome c_1 was not localized to the nucleus. Instead, the protein was cytoplasmic and often concentrated near the cell periphery (Fig. 3, *13*). A few cells showed what appeared to be mitochondrial-associated protein, although this was difficult to confirm because of the high cytoplasmic staining. However, based on the amount of processing to mature cytochrome c_1 seen on Western blots, we estimate that localization to the mitochondria is at least 10% (Fig. 3, lane 3). Attempts to quantify the amount of fusion protein associated with specific organelles by cell fractionation were unsuccessful because of the instability of fusion proteins when cells were lysed under nondenaturing conditions.

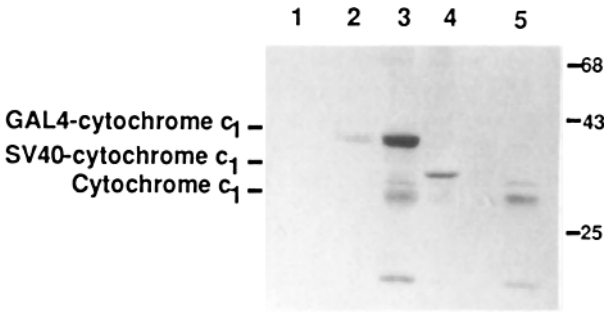

Figure 2. Production of GAL4- and SV40–cytochrome c_1 proteins. Cell extracts from 1165/11 containing plasmids encoding intact cytochrome c_1 and cytochrome c_1 fusion proteins were subjected to SDS-PAGE (12% gel). Proteins were transferred to nitrocellulose and probed with rabbit anticytochrome c_1 antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase. Lane *1*, no plasmid; lane *2*, pIS144 (GAL4–cytochrome c_1); lane *3*, pIS145 (GAL4*–cytochrome c_1); lane *4*, pIS157 (SV40–cytochrome c_1); lane *5*, YEp13-41 (wild-type cytochrome c_1). 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 c_1 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 c_1 defective strain (W303-$\alpha\Delta cyt1$) bearing the SV40–cytochrome c_1 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 c_1 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 c_1 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 (W303a$\Delta cyt1$) was no longer temperature sensitive and failed to grow on glycerol when transformed with the plasmid bearing the SV40- or GAL4–cytochrome c_1 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) reconfirmed 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 W303a$\Delta cyt1$. The resulting haploids were then retransformed with plasmids bearing either GAL4–cytochrome c_1, GAL4*–cytochrome c_1, or SV40–cytochrome c_1 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–

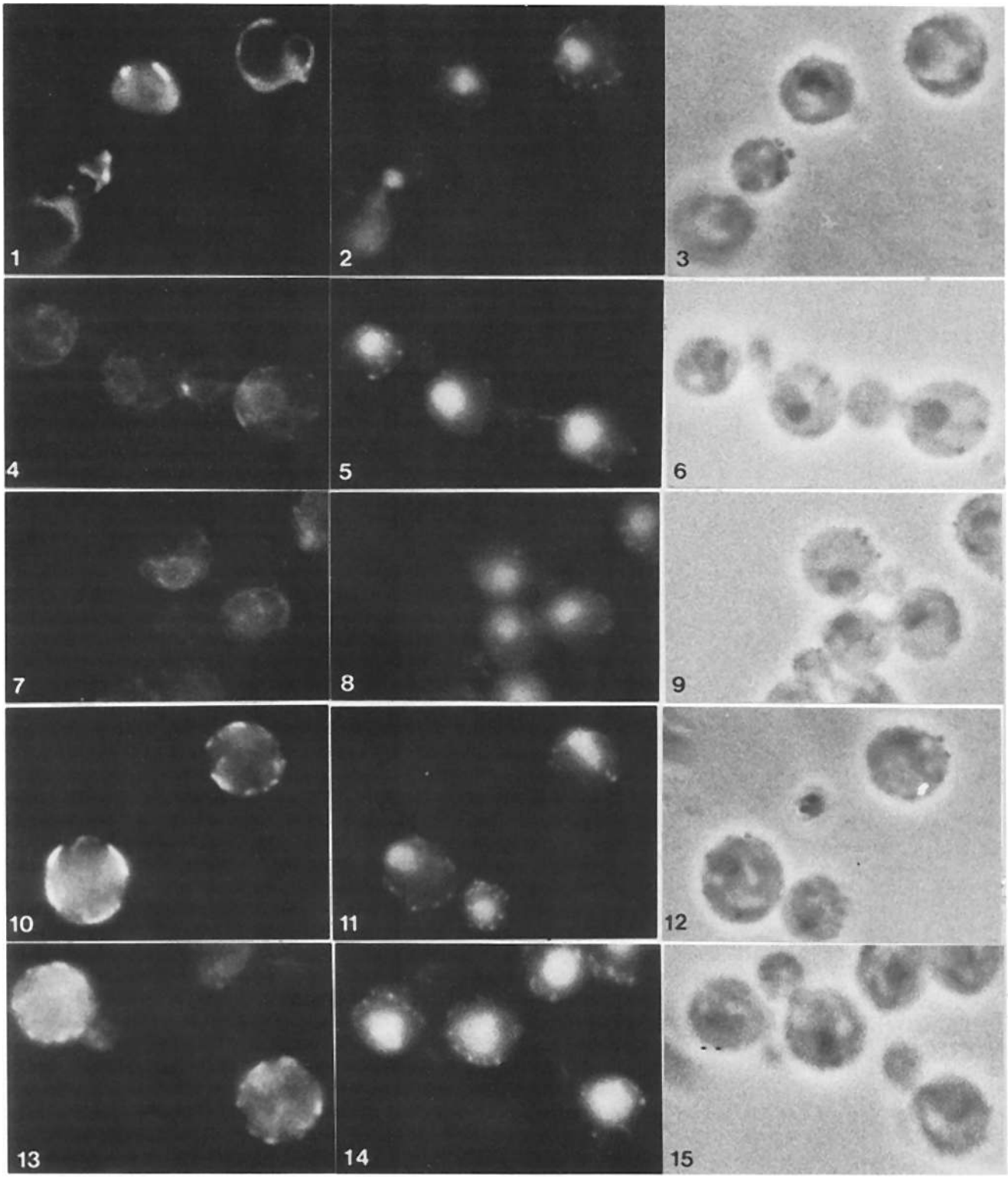

Figure 3. Immunofluorescence of cells producing GAL4- and SV40-cytochrome c_1. Cells were grown at 30°C, prepared for immunofluorescence and treated with a rabbit anticytochrome c_1 antibody, followed by FITC-conjugated anti-rabbit IgG, to localize the cytochrome c_1 and the derivatives, and DAPI to stain cell DNA. W303Δ*cyt1* cells producing intact cytochrome c_1 (*1–3*); producing GAL4–cytochrome c_1 (4–9); and producing GAL4*–cytochrome c_1 (10–15). *1*, *4*, *7*, *10*, and *13* are stained with anticytochrome c_1, *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_1 since Ts^+/Ts^- segregated 2:2, all Ts^- were Gly^+, and all Ts^- were Gly^-. The ten wild-type spores (*cyt1*) grew normally at 37°C but failed to grow on glycerol when transformed with GAL4–cytochrome c_1 or SV40–cytochrome c_1. All 20 spores grew on glycerol when bearing the plasmid encoding GAL4*–cytochrome c_1. The properties of *npl1* are summarized in Table II.

Both *npl1-1* and *npl1-2* showed altered localization of the GAL4- and SV40–cytochrome c_1 proteins by immunofluorescence. Cells grown at 30°C were examined by immunofluorescence with the anticytochrome c_1 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 c_1 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, *sec 63-1* 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 *npl1-1* and *sec63-1* was sporulated. Spores from 14 complete asci were all viable at 23°, 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 *npl1-1, npl1-2* and *sec63-1*. Cells were grown at 24°C, shifted to 37°C for 2 h, pulse labeled with $^{35}SO_4^{2-}$ and assayed for processing of preprocarboxypeptidaseY (preproCPY). *sec63-1* cells accumulated unprocessed preproCPY at both 24°C and 37°C (Fig. 4, lanes *1* and *2*). In contrast, *npl1-1* and *npl1-2* 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 NPL^+ 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

Table II. Growth Characteristics of npl1

	30°C Glycerol				30°C	36°C
	SV40-CYT1	GAL4-CYT1	CYT1	YEP213	YEPD	YEPD
npl1	+	+	+	−	+	−
W303 (*NPL1*)	−	−	+	−	+	+

Summary of growth properties of *npl1-1*. *npl1-1* or wild-type (W303) cells bearing different plasmids were streaked on plates containing glycerol as the sole carbon source and incubated at 30°C and growth was recorded after 5 d. Growth on YEPD was scored after 3 d at the indicated temperatures.

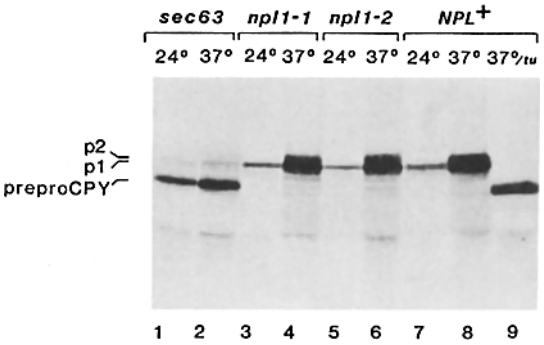

Figure 4. Pulse-radiolabeling analysis of preproCPY accumulation in *sec63-1* and *npl1* mutants. JRM 151-1B (*sec63-1*), PSY5 (*npl1-1*), PSY8 (*npl1-2*), and W303α (*NPL1*) cells were radiolabeled with $^{35}SO_4^{2-}$ for 6 min at 24°C, or after a 2-h preincubation at 37°C, or for 6 min at 37°C. Inhibition of asparagine-linked core glycosylation in W303α cells was achieved by incubating the cells with 10 μg/ml tunicamycin for 15 min before and for the duration of the pulse-labeling (lane *9*). SDS-solubilized, heat-denatured extracts were prepared and incubated with CPY antiserum and the resulting immunoprecipitates analyzed by gel electrophoresis as described (Rothblatt et al., 1989). PreproCPY indicates unglycosylated preproCPY accumulated in cells during the pulse-labeling. ER and Golgi forms of glycosylated proCPY are designed p1 and p2, respectively.

library by complementation of the ts-lethal phenotype of *npl1-1*. 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 *npl1-1* 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 *npl1-1* 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 (*sec63-1 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

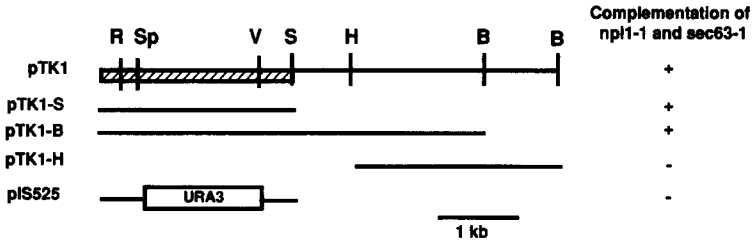

Figure 5. Plasmids containing the NPL1/SEC63 region of the yeast genome. pTK1 contains the 6-kb fragment of DNA shown, inserted at the Bam-HI site of YEP13 so that transcription of the *TetR* gene would proceed from left to right on the diagram shown. pTK1-S, pTK1-B, and pTK1-H were generated by elimination of the Sph I, Bam HI, and Hind III fragments respectively from pTK1, pTK1, pTK1-S, and pTK1-B rescue the TS-lethality for *npll-1*, while pTK1-H does not. pIS525 contains *URA3* inserted between the Spe I and Eco RV sites in the *NPL1* coding sequence. Restriction site code: *R*, Eco RI; *Sp*, Spe I; *V*, Eco RV; S, Sp I; H, Hind III; and B, Bam HI.

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 *URA3* 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 asci 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 *npll* complementing region of pTK1 was sequenced by the dideoxy 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 *npll* 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 nondescript. It contains one internal repeat, Lys-Gln-Pro-Leu-Ile/Val-Pro-X-Ser-X-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

npll/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 *sec61* and *sec62* cells (Fig. 8, *3* and *4; sec61* 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 *npll-1* cells in contrast to *sec63-1* cells. In separate experiments, mislocalization of the SV40-invertase in *npll-1* at 30°C varied from 2–10%. This is consistent with other observations that *sec63-1* is a stronger allele than *npll-1* and *npll-2*.

Endogenous nuclear protein localization is affected in *npll-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 *npll-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 *npll-1* cells bearing *NPL1* on a multi-copy plasmid no longer showed mislocalization of the nucleolar antigen. 5–10% of *npll-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 c_1 contains both a NLS and a portion of a mitochondrial localization sequence, it is not processed to mature cytochrome c_1 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 (*npl* mutants). These mutants allowed GAL4- and SV40–cytochrome c_1 to enter mitochondria and, thus, for cells to grow on glycerol. One mutant *npll* is allelic to *sec63*, a gene identified as important for translocation of proteins across the ER (Rothblatt et al., 1989).

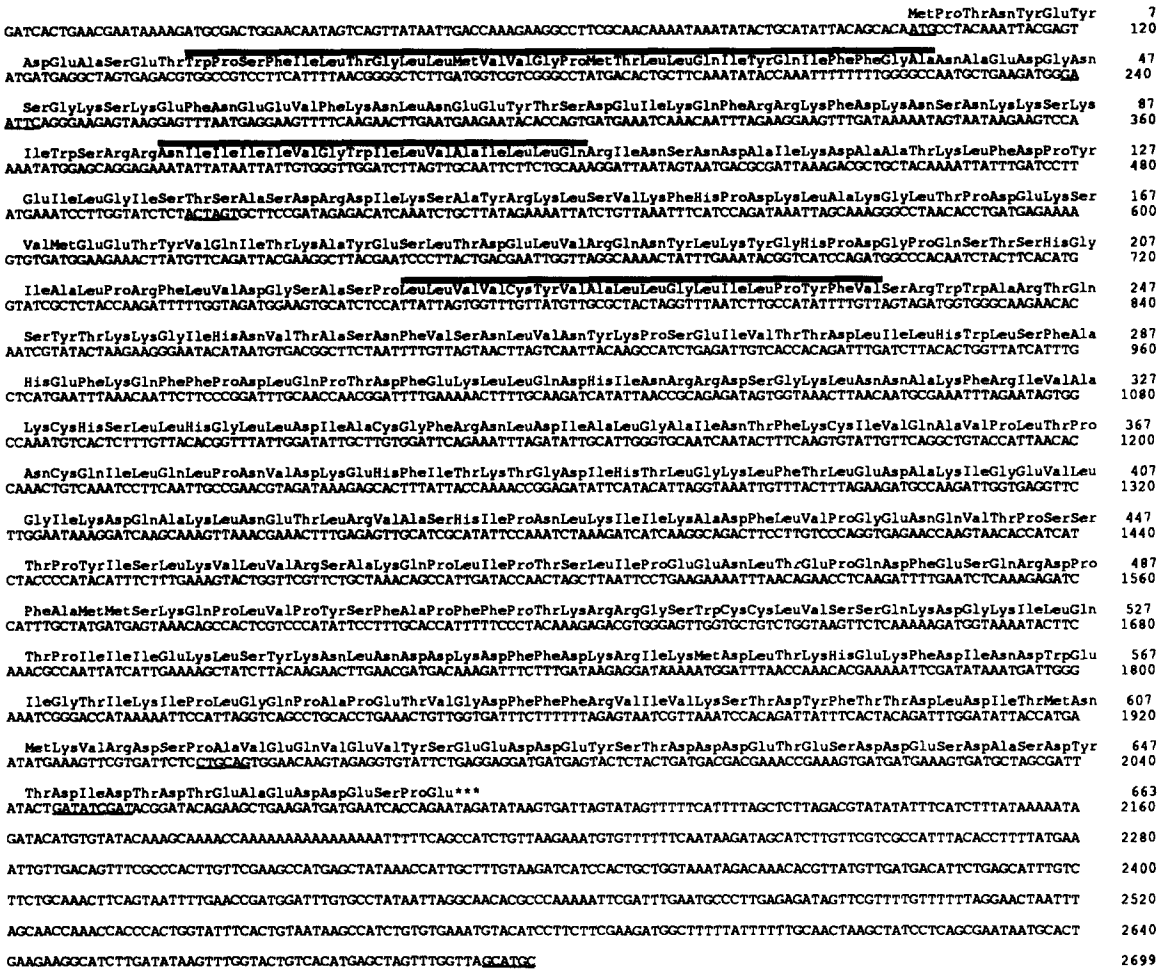

Figure 6. The sequence of the *NPL1/SEC63* gene. The sequence shown represents the insert in plasmid pTK1-S, which contains the entire *NPL1/SEC63* gene, as defined by complementation of *npll* and *sec63* mutants, and by linkage analysis (see Results). Also shown is the putative protein sequence encoded by this gene. Unique restriction sites are underlined (bp 239: Eco RI; bp 502: Spe I; bp 1943: Pst I; bp 2047: Eco RV; bp 2050: Cla I; bp 2695: Sph I). Sequences shown by bold underline indicate hydrophobic stretches of amino acids that may form potential transmembrane domains.

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 strengthens the idea that it constitutes a distinct domain. Within the DnaJ protein, the region of homology occurs at the very N terminus, and is followed by the glycine-rich sequence Gly-Gly-Met-Gly-Gly-Gly-Gly-Phe-Gly-Gly-Gly (Ohki et al., 1986; Bardwell et al., 1986), which may act as a spacer to loosely connect this domain to the rest of the protein.

DnaJ is an *E. coli* heat-shock protein that functions with GrpE and the *E. coli* HSP70 cognate, DnaK. These proteins have been implicated in many processes, including proteolysis of abnormal proteins (Straus et al., 1988), synthesis of certain membrane proteins (Ohki 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 destabilizing 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., 1988; Zimmerman et al., 1988; Chirico et al., 1988).

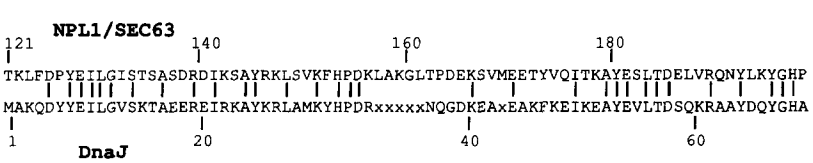

Figure 7. The homology between NPL1/SEC63 and DnaJ. The region of homology between these genes, with amino acid identities indicated with dots. Xs represent gaps introduced by the fastA program to optimize the alignment.

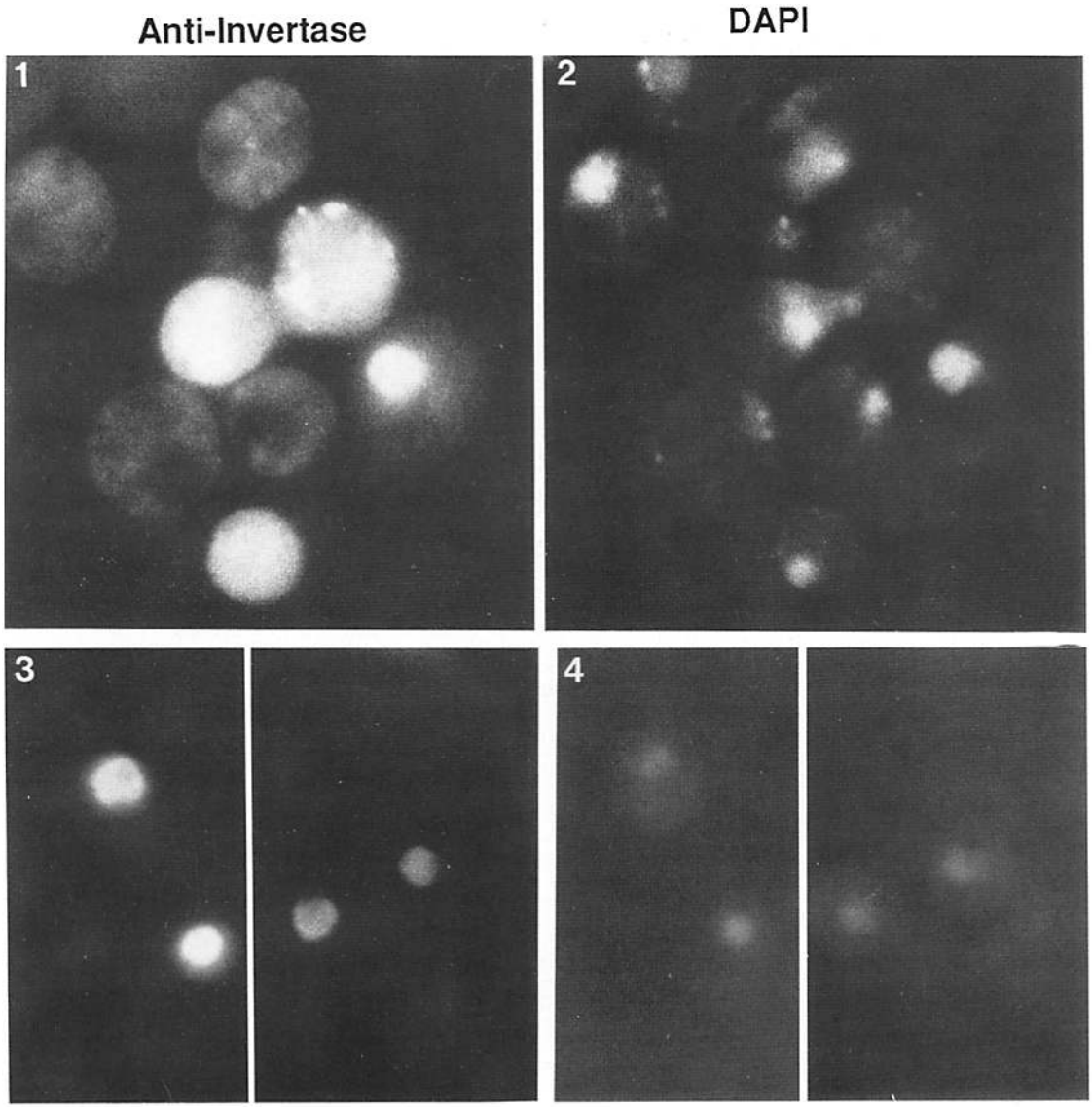

Figure 8. Localization of SV40-invertase in *sec62* and *sec63*. JRM151-1B (*sec63-1*) cells (*1–2*) and RDM 50-94C (*sec62-1*) cells (*3–4*) bearing pMN8 (a plasmid encoding the SV40 NLS fused to cytoplasmic invertase [Nelson and Silver, 1989]) were grown to a cell density of 5×10^6 cells/ml in minimal 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 *SUC2*, 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_1 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

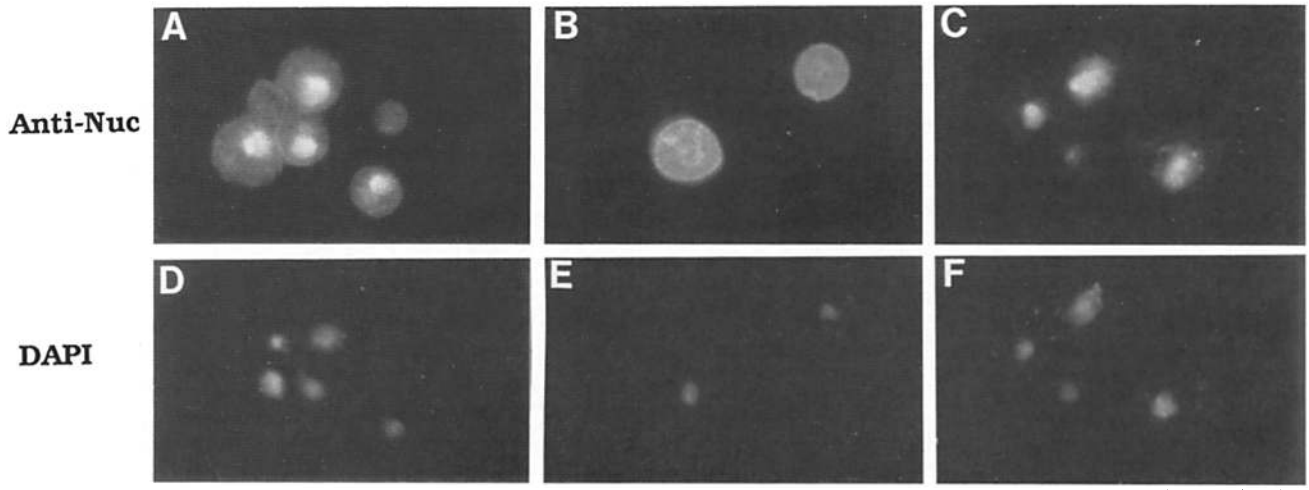

Figure 9. Localization of a nucleolar antigen in *npl1/sec63* and *sec62* cells. JRM151-1B (*sec63-1*) cells (*A*, *B*, *D*, and *E*) and RDM50-94C (*sec62-1*) cells (*C* and *F*) were grown at 23°C, shifted to 30°C or 37°C for 2 h, prepared for immunofluorescence, and treated with mouse antinucleolar antibody followed by FITC-conjugated anti-mouse IgG to localize a normally nucleolar-associated antigen (*A–C*) and with DAPI to visualize nuclear DNA (*D–F*).

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 *sec61* and *sec62* cells, also defective in ER assembly (Deshaies 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 c_1 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*.

We especially thank A. Haid, A. Tzagaloff, G. Schatz, R. Schekman, and J. Broach for gifts of strains and antibodies, and Mark Osborne, Jim Rothman, Mitch Bernstein, Walter Neupert, and R. Schekman for comments on the manuscript, and Ray Deshaies for extensive helpful discussions.

This work was supported by National Institutes of Health grant GM36373-01 and, in part, by a Basil O'Connor grant from the March of Dimes and a National Science Foundation Presidential Young Investigator Award (to P. Silver). I. Sadler was supported by a Deutscher Akademischer Austauschdienst and Deutsche Forschungsgemeinschaft Postdoctoral Fellowship and A. Chiang by National Institutes of Health Cell and Molecular Biology Training grant GM07312. J. Way is a Merck Research Fellow. J. Rothblatt is a Postdoctoral Fellow of the California Division of the American Cancer Society.

Received for publication 7 June 1989 and in revised form 24 July 1989.

References

Ammerer, G. 1983. Expression of genes in yeast using the *ADC1* promoter. *Methods Enzymol.* 101:192–211.

Bardwell, J. C. A., K. Tilly, E. Craig, J. King, M. Zylicz, and C. Georgopoulos. 1986. The nucleotide sequence of the *Escherichia coli* K12 DnaJ gene. *J. Biol. Chem.* 261:1782–1785.

Burglin, T. R. 1988. The yeast regulatory gene *PHO2* encodes a homeo box. *Cell.* 53:339–340.

Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.).* 332:1–5.

Deshaies, R. J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105:633–646.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.).* 322: 800–805.

Dodson, M., H. Echols, S. Wickner, C. Alfano, K. Mensa-Wilmot, B. Gomes, J. LeBowitz, J. D. Roberts, and R. McMacken. 1986. Specialized nucleoprotein structures at the origin of replication of bacteriophage lambda: localized unwinding of duplex DNA by a six-protein reaction. *Proc. Natl. Acad. Sci.* 83:7638–7642.

Hemmingsen, S. M., C. Woolford, M. van der Vies Saskia, K. Tilly, D. T. Dennis, C. P. Georgopoulus, R. W. Hendrix, and R. J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature (Lond.).* 333:330–334.

Ito, H., Y. Fukado, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells with alkali cations. *J. Bacteriol.* 153:163–168.

Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3–8.

Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith. 1984*a*. Sequence requirements for nuclear location of simian virus 40 large T-antigen. *Nature (Lond.).* 311:33–38.

Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984*b*. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499–509.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lang, B. F., and F. Kaudewitz. 1982. Cytochrome c_1 deficient mutants in *Saccharomyces cerevisiae*. *Curr. Genet.* 6:229–235.

Lauer, G., R. Pastrana, J. Sherley, and M. Ptashne. 1981. Construction of

overproducers of the bacteriophage 434 repressor and cro proteins. *J. Mol. and Appl. Genet.* 1:139–147.

Mandel, M., and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol.* 53:159–162.

Maniatas, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Messing, J. 1979. Recombinant DNA technical bulletin. National Institutes of Health Publication No. 79-99. 2:43–48.

Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes; the role of the docking protein. *Nature (Lond.).* 297:647–650.

Nasmyth, K. A., and S. I. Reed. 1980. Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci.* 77:2119–2123.

Nelson, M. N., and P. Silver. 1989. Context affects nuclear protein localization in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 9:384–389.

Ohashi, A., J. Gibson, I. Gregor, and G. Schatz. 1982. Import of proteins into mitochondria. The precursor of cytochrome c_1 is processed in two steps, one of them heme-dependent. *J. Biol. Chem.* 257:1302–47.

Ohki, M., F. Tamura, S. Nishimura, and H. Uchida. 1986. Nucleotide sequence of the *Escherichia coli* dnaJ gene and purification of the gene product. *J. Biol. Chem.* 261:1778–1781.

Ohki, M., H. Uchida, F. Tamura, R. Ohki, and S. Nishimura. 1987. The *Escherichia coli* dnaJ mutation affects biosynthesis of specific proteins, including those of the lac operon. *J. Bacteriol.* 169:1917–1922.

Pain, D., Y. S. Kanwar, and G. Blobel. 1988. Identification of a receptor for protein import into chloroplasts and its localization to envelope contact zones. *Nature (Lond.).* 331:232–237.

Pelham, H. R. B. 1986. Speculations of the functions of the major heat shock and glucose-regulated proteins. *Cell.* 46:959–961.

Pfaller, R., and W. Neupert. 1987. High affinity binding sites involved in the import of porin into mitochondria. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2635–42.

Pfanner, N., H. K. Muller, M. A. Harmey, and W. Neupert. 1987. Mitochondrial protein import: involvement of the mature part of a cleavable precursor protein in the binding to receptor sites. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3449–3454.

Rose, M. D., L. M. Misra, and J. P. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homologue of mammalian BiP/GRP78. *Cell.* 57:1211–1221.

Rothblatt, J. A., R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* 109:2641–2652.

Sadler, I., K. Suda, G. Schatz, F. Kaudewitz, and A. Haid. 1984. Sequencing of the nuclear gene for the yeast cytochrome c1 precursor reveals an unusually complex amino-terminal presequence. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2137–2143.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Sanger, F., A. R. Coulson, B. F. Barrell, A. Z. H. Smith, and B. Roe. 1980. Cloning in a single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161–178.

Sherman, F., G. Fink, and C. Lawrence. 1983. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 163 pp.

Silver, P., and M. Hall. 1988. Transport of proteins into the nucleus. *In* Protein Transport and Organelle Biogenesis. R. C. Das and P. W. Robbins, editor. Academic Press, San Diego. 749–769.

Silver, P. A., L. P. Keegan, and M. Ptashne. 1984. Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization. *Proc. Natl. Acad. Sci. USA.* 81:5951–5955.

Silver, P. A., A. Chiang, and I. Sadler. 1988. Mutations that alter both localization and production of a yeast nuclear protein. *Genes & Dev.* 2:707–717.

Silver, P., I. Sadler, and M. Osborne. 1989. Yeast proteins that recognize nuclear localization sequences. *J. Cell Biol.* 109:983–989.

Straus, D. B., W. A. Walter, and C. A. Gross. 1988. *Escherichia coli* heat shock gene mutants are defective in proteolysis. *Genes & Dev.* 2:1851–1858.

Tajima, S., L. Lauffer, L. Rath, and P. Walter. 1986. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. *J. Cell Biol.* 103:1167–1178.

Verner, K., and G. Schatz. 1988. Protein translocation across membranes. *Science (Wash. DC.).* 241:1307–1313.

Wakabayashi, S., H. Matsubara, C. H. Kim, K. Kawai, and T. E. King. 1980. The complete amino acid sequence of bovine cytochrome c_1. *Biochem. Biophys. Res. Commun.* 97:1548–1554.

Walter, P., and G. Blobel. 1980. Purification of a membrane-associated protein complex required for protein translocation across the ER. *Proc. Natl. Acad. Sci USA.* 27:7112–7116.

Wiedmann, M., T. V. Kurzchalia, H. Bielka, and T. A. Rapoport. 1987. Direct probing of the interaction between the signal sequence of nascent preprolactin and the SRP by specific cross linking. *J. Cell Biol.* 104:201–207.

Yaffe, M. P., and G. Schatz. 1984. Two nuclear mutations that block mitochondrial protein import in yeast. *Proc. Natl. Acad. Sci. USA.* 81:4819–4828.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and puc19 vectors. *Gene (Amst.).* 33:103–119.

Zimmerman, R., M. Sagstetter, M. J. Lewis, and H. R. B. Pelham. 1988. Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat into microsomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2875–2880.