SEC62 Encodes a Putative Membrane Protein Required for Protein Translocation into the Yeast Endoplasmic Reticulum

Raymond J. Deshaies and Randy Schekman
Division of Biochemistry and Molecular Biology, Barker Hall, University of California, Berkeley, California 94720

Abstract. Yeast sec62 mutant cells are defective in the translocation of several secretory precursor proteins into the lumen of the endoplasmic reticulum (Rothblatt et al., 1989). The deficiency, which is most restrictive for α-factor precursor (ppαF) and preprocarboxypeptidase Y, has been reproduced in vitro. Membranes isolated from mutant cells display low and labile translocation activity with ppαF translated in a wild-type cytosol fraction. The defect is unique to the membrane fraction because cytosol from mutant cells supports translocation into membranes from wild-type yeast. Invertase assembly is only partly affected by the sec62 mutation in vivo and is nearly normal with mutant membranes in vitro.

A potential membrane location for the SEC62 gene product is supported by evaluation of the molecular product. DNA sequence analysis reveals a 32-kD protein with no obvious NH2-terminal signal sequence but with two domains of sufficient length and hydrophobicity to span a lipid bilayer. Sec62p is predicted to display significant NH2- and COOH-terminal hydrophilic domains on the cytoplasmic surface of the ER membrane. The last 30 amino acids of the COOH terminus may form an α-helix with 14 lysine and arginine residues arranged uniformly about the helix. This domain may allow Sec62p to interact with other proteins of the putative translocation complex.

We previously described a selection for yeast mutants defective in translocation of secretory protein precursors into the lumen of the ER. Mutant cells are selected by a procedure that requires a signal peptide-containing cytoplasmic enzyme chimera to remain in contact with the cytosol. To date, we have isolated temperature-sensitive (Ts') mutations in three genes (sec61, sec62, and sec63) that disrupt translocation of secretory protein precursors into the ER lumen (Deshaies and Schekman, 1987; Deshaies et al., 1988b; Rothblatt et al., 1989). Meyer and his colleagues have also isolated a translocation-defective mutant, ptl1, using a similar selection procedure (Toyn et al., 1988). Allelism tests indicate that PTL1 and SEC63 are the same gene (Rothblatt et al., 1989). Yeast cells bearing a mutant allele of either sec61, sec62, or sec63 accumulate untranslocated precursors of a subset of soluble proteins destined for the secretory pathway, suggesting that the SEC61, SEC62, and SEC63 genes product are required for the proper translocation of soluble proteins through the ER bilayer (Deshaies and Schekman, 1987; Rothblatt et al., 1989; Toyn et al., 1988). sec62 Strains exhibit a pronounced defect in the translocation of a subset of precursor proteins even at 24°C, a temperature that is permissive for cell growth (Rothblatt et al., 1989). The strong translocation defect observed with sec62 mutants at temperatures compatible with those used to assay protein translocation in vitro suggested that sec62 would be a good candidate for reconstitution and biochemical dissection of a specific protein translocation defect. In this paper, we describe the reconstitution of protein translocation in vitro with components prepared from a sec62 strain, and the molecular cloning and sequence analysis of the SEC62 gene. A preliminary account of this work was reported elsewhere (Deshaies et al., 1988b).

Materials and Methods

Strains, Materials, Plasmids, and General Methods

Bacterial and yeast strains used in this study are listed in Table I. Yeast cells were grown in rich or synthetic minimal media as described (Deshaies and Schekman, 1987).

PMSF, creatine phosphokinase (type I), ATP, proteinase K (protease, type XI), diethyl pyrocarbonate, kanamycin, ampicillin, ethidium bromide, agarose and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO); restriction enzymes, S1 nuclease, T2 DNA ligase, calf intestinal alkaline phosphatase, Klenow fragment of DNA polymerase I, random primer DNA labeling kit, phosphocreatine, and staphylococcal nuclease S7 were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); SP6 RNA polymerase, RQI DNase, and ribonuclease inhibitor (RNasin) were purchased from Promega Biotech (Madison, WI); α-[32P]dCTP (>3,000 Ci/mmol), β-[32P]dATP (>40 Ci/mmol), [35S]α-thio-dATP (1,200 Ci/mmol), [35S]methionine (>1,200 Ci/mmol), and Amplify were purchased from Amersham Corp. (Arlington Heights, IL); SDS-PAGE reagents (electrophoresis grade) were purchased from Bio-Rad Laboratories (Richmond, CA); GTP was purchased from P-L Biochemicals (Milwaukee, WI);
Table 1. Yeast and Bacterial Strains

| Strain                  | Genotype                   | Source or reference                  |
|-------------------------|----------------------------|-------------------------------------|
| Saccharomyces cerevisiae|                            |                                     |
| RDM 15-9B               | ade2, pep4-3, MATa          | Deshaies and Schedeman, 1987         |
| RDM 42-3C               | leu2-3, -112, ura3-52, trp1, ade2, sec62-1, MATa | This study                          |
| RDM 43-9C               | his4, ura3-52, pep4: URA3, sec62-1, MATa | This study                          |
| RDM 50-94C              | leu2-3, -112, his4, ura3-52, sec62-1, MATa | This study                          |
| X2180-1B                | gal2, mal, mel, SEC2, CUP1, MATa | YGSC                                 |
| DBY 2060                | leu2-3, -112, MATa          | D. Botstein, Genentech, South San Francisco, CA |
| W303-Leu†               | leu2-3-112, his3-11, -15, trp1-1, ura3-1, ade2-1, can1-100, MATa | Borkovich et al., 1989               |
| RPD 99-101§             | Same as W303-Leu, except sec62:: His3 | This study                          |
| RPD 95-971†             | Same as W303-Leu, except sec62:: URA3 | This study                          |
| Escherichia coli        |                            |                                     |
| TG1                     | Δ(lac, pro) supE thi hsds5 F° [traD36 pro+ lacI4 lacZΔM15] | Mark Biggin, unpublished observations, Department of Molecular Biology and Biophysics, Yale University |
| MC1061                  | F° hisdR′ hisdM′ araD139 Δ (araAB01C-λeuf7679 Δ(lac)X74 galU galK rpsL | Emr et al., 1986                      |

*Yeast Genetic Stock Center, University of California, Berkeley, CA 94720.
† W303-Leu was derived by transformation of W303 with a linear LEU2 fragment. Segregation of leucine prototrophy among W303-Leu spore progeny suggests that LEU2 integrated at more than one site in the W303 genome.
§ RPD 99, RPD 100, and RPD 101 are three independent clones of W303-Leu transformed with pRD14 (sec62:: H163).
¶ RPD 95, RPD 96, and RPD 97 are three independent clones of W303-Leu transformed with pRD13 (sec62::URA3).

Sephadex G25 (medium), G50 (medium), and 5-fluoroorotic acid were purchased from Pharmacia Fine Chemicals (Piscataway, NJ); exonuclease III and low melting point agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD); 7T RNA polymerase was purchased from New England Biolabs, (Beverly, MA); α-thio-dNTP mixture was purchased from Stratagene (La Jolla, CA), and Sequenase version 1.0 DNA sequencing kit was purchased from United States Biochemical Corp. (Cleveland, OH). Lyticase (fraction II, 60,000 U/ml) was prepared as described (Scott and Schekman, 1980). M13K07 helper phage (Vieira and Messing, 1987) was kindly provided by Linda Silveira (Division of Biochemistry and Molecular Biology, University of California, Berkeley).

Escherichia coli plasmids pUC19 (Vieira and Messing, 1987), and the E. coli–yeast shuttle plasmids YEp551, YEp351 (Hill et al., 1986), and YEp24 (Botstein et al., 1979) have been described previously. E. coli plasmids pPM2, used for producing RNA transcripts in vitro, are described in the Promega Biotech catalogue. The E. coli–yeast shuttle plasmid pSE658 is identical to pSEV58 (Emr et al., 1986), except for the multiple-cloning site that was derived from pUC18 (Yanisch-Perron et al., 1985). Plasmids used for in vitro transcription/translation of prepro carboxypeptidase Y (pOZCP516), preinverase (pG2SUC23), and preinverase fragment (pG2SUC91) have been described previously (Rothblatt et al., 1987), and were kindly provided by J. Rothblatt (Division of Biochemistry and Molecular Biology, University of California, Berkeley). Plasmid pDH100, which was used to synthesize prepro–α-factor (ppαF) transcripts in vitro, was constructed by David Julius and has been described (Hansen et al., 1986). The yeast genomic library constructed by Rose et al. (1988) contains 10–20 kb fragments resulting from partial digestion of Saccharomyces cerevisiae genomic DNA with Sau IIA inserted into the Bam HI site of the E. coli–yeast shuttle vector YCP80 (CEN4, ARSI, URA3). The YCP50-based genomic library was a generous gift of J. Rine, (Division of Biochemistry and Molecular Biology, University of California, Berkeley).

Common recombinant DNA techniques, including Southern and Northern transfer hybridization, enzymatic modification of DNA, fragment purification, bacteriophage and plasmid isolation were performed essentially as described by either Maniatis et al. (1982) or Ausubel et al. (1987). Yeast strains were constructed by standard genetic techniques (Sherman et al., 1983). RDM 42-3C, RDM 43-9C, and RDM 50-94C were derived from the fourth, fifth, and sixth outcrosses, respectively, of the original sec62 isolate. All yeast transformations (except for introduction of the YC850 gene bank into RDM42-3C) were done by the lithium acetate method (Ausubel et al., 1987). Total protein was measured by the Markwell modification of the Lowry method (Markwell et al., 1978).

Preparation of a Translation Competent Yeast Lysate

Protease deficient SEC (RDM 15-9B) and sec62 (RDM 43-9C) strains were grown at 24°C or 30°C in 12 liters of 2% bacto-yeast, 1% yeast extract, 3% dextrose (rich medium) to an OD50 of 1.5–5.0. Cells were harvested in a continuous flow rotor (Sharples Corp., Worthingham, MA), washed twice by resuspending in 250 ml distilled water (all solutions used subsequently were treated with 0.1% diethyl pyrocarbonate and autoclaved to quench RNase activity), and centrifuged for 5 min at 5,000 rpm in a GSA rotor (DuPont Co., Wilmington, DE). Washed cell pellets (26–45 g wet weight) were resuspended in a minimal volume (~25 ml) of buffer A (100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 20 mM Hepes, pH 7.4) supplemented with 0.3 mM PMSF, and disrupted at 4°C by agitation in a 100-ml bead beater (Biospec Products, Bartlesville, OK) as described by the manufacturer. The crude lysate was centrifuged at 6,500 rpm for 6 min in an HB-4 rotor (Sorvall) at 4°C, and the resulting supernatant fraction was centrifuged for 30 min at 100,000 g in a Ti45 rotor (Beckman Instruments, Palo Alto, CA) at 4°C. The clear, yellow-colored supernatant fraction (S100) was collected and chromatographed at 4°C on a 100 ml Sephadex G25 column (12 × 3.8 cm) equilibrated with buffer A plus 14% glycerol. The column was eluted with equilibration buffer and 2 ml fractions were collected. Fractions with an A280 > 30 were pooled, diluted to a final A280 of 60 with buffer A +14% glycerol, frozen as 1 ml aliquots in liquid nitrogen, and stored at −85°C. Typically, 35–45 ml of translation competent extract was obtained from a single preparation. Endogenous mRNA was degraded in thawed lysates by adding CaCl2 (0.8 mM) and staphylococcal nuclease S7 (500 U/ml). Nuclease digestion proceeded for 15 min at 20°C, and was terminated by adding EGTA to a final concentration of 2 mM.

Preparation of Translation Competent Yeast Microsomes

Protease deficient SEC (RDM 15-9B) and sec62 (RDM 43-9C) strains were...
grown at 17°C, 24°C or 30°C in 2 liters of rich medium to an OD₆₀₀ of 1.5-5.0. Membranes were isolated as described by Rothblatt and Meyer (1986b) with the following modifications: cells were treated with DTT for 10 min at 24°C. Spheroplasts were prepared by digesting cell walls with 10-20 U of lytase/OD₆₀₀ U of cells for 30-60 min at the growth temperature; spheroplasts were resuspended at 0.25 g/ml in lysis buffer containing 0.25 M sorbitol in place of sucrose; membranes were collected by centrifugation at 30000 g in a Ti45 rotor (Beckman Instruments); membranes were washed with 10 ml membrane storage buffer containing 0.3 M sorbitol in phosphate buffered by centrifugation and resuspended in membrane storage buffer to an A₂₈₀ of 45. Endogenous mRNAs in the membrane suspension were degraded by treatment with CaCl₂ (0.8 mM) and staphylococcal nuclease (250 U/ml) for 5 rain at 20°C. The nuclease reaction was terminated by adding EGTA to a final concentration of 2 mM and membranes were frozen as 30-µl aliquots in liquid nitrogen. Typically, 0.75-1.25 ml of membranes with an A₂₈₀ of 45 were obtained in a single preparation.

The extra wash with membrane storage buffer employed in this modified procedure was critical. pppF synthesized in a sec62 S100 was glycosylated more efficiently by washed wild-type membranes. Unwashed membranes sustained poor glycosylation that was exacerbated in co-translational import assays. This effect was reconstituted by combining washed SEC membranes and the membrane wash, suggesting that wild-type membrane fractions contained a soluble compound that differentially influenced glycosylation of precursors produced in SEC and sec62 S100s. The molecular nature of the glycosylation defect seen with sec62 S100 and unwashed SEC membranes has not been determined.

In vitro Transcription and Translation

Plasmids pDI100 (Xba I), pG2CPY16 (Hind III), pG2SUC23 (Pvu II), and pG2SUC91 (Pvu II) were linearized with the indicated restriction enzymes and transcribed (20 µg of plasmid DNA/reaction) by SP6 polymerase as described (Hansen et al., 1986). Following transcription, the mRNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in water to an A₀₂₈₀ of 6. Messenger RNAs (~250 ng/15-µl reaction) were translated for 30 min at 20°C as described by Kepes and Schekman (1988). Translation reactions contained 33% (vol/vol) yeast S100.

Co- and Posttranslational Protein Translocation

Co-translational translocation was assayed in 15-µl translation reactions supplemented with 0.5-1.5 µl of yeast membranes (A₀₂₈₀ = 45). Samples were incubated for 30 min at 20°C and reactions were terminated by adding SDS-PAGE sample buffer and heating to 95°C. For posttranslational translocation assays, mRNAs were translated for 30 min at 20°C, and translation was quenched by adding 1 µl 16 mM cycloheximide. Cycloheximide was not added to preprocarboxypeptidase Y or preinvertase-fragment translation reactions. Reactions were adjusted to 25 µl with 6 µl buffer A (14% glycerol, 1 µl energy mix (8.3 mM ATP, 265 mM creatine phosphate, 1.7 M GTP, 580 mM potassium acetate, 10 mM magnesium acetate), and 0-2 µl of yeast membranes or membrane storage buffer. Posttranslational import assays were conducted for 30 min at 20°C-25°C. Enclosure of precursors within complexes was assessed by treatment of import reactions with protease K as described (Kepes and Schekman, 1988).

Aliquots of quenched reactions (typically, one quarter to one third of a sample was used) were subjected to SDS-PAGE and the gels were processed as described (Kepes and Schekman, 1988). Samples containing radiolabeled preprocarboxypeptidase Y were immunoprecipitated with anti-carboxypeptidase Y (CPY) serum (Deshales and Schekman, 1987) before SDS-PAGE. Autoradiograms were quantified by densitometry as described (Deshales and Schekman, 1987).

Cloning and DNA Sequencing

sec62 cells were transformed by the spheroplast method (Ausubel et al., 1987) with 4 µg of a library of yeast genomic DNA in the single copy vector YCp50 (Rose et al., 1988). The transformants were plated onto eight plates containing selective minimal medium (-uracil) and incubated overnight at 24°C. Seven of the plates were then shifted to the restrictive temperature of 37°C for 3-4 d, and one plate of transformants was left at 24°C to allow an estimation of the transformation frequency. Among ~66000 Ura⁺ RDM 42-3C colonies, seven grew at 37°C. Plasmid DNA was isolated from these Ts⁻ transformants (Ausubel et al., 1987), amplified in E. coli, and retransfected into RDM 42-3C. Plasmids from four of the seven original Ts⁻ transformants conferred Ts⁺ growth to RDM 42-3C in the rescree, indicating that their yeast DNA insertions complemented sec62. Restriction mapping of these plasmids indicated that they contained overlapping inserts (A. Ein, unpublished results).

Plasmid pSEC6240 contained a 12-kb insert of yeast DNA that complemented the Ts growth defect of sec62 strains. pSEC6240 was partially digested with Hind III and religated to yield pSEC6207, which contained a 4.4-kb insert that complemented sec62 strains. A restriction map of this insert is shown in Fig. 4 (top line). The 1.7-kb Eco Rv–Sph I and 3.2-kb Pst I–Sal I fragments from the pSEC6207 insert were subcloned (in opposite orientations) into pUC119 to generate pRD8 and pRD9, respectively. Unidirectional deletions were constructed by digesting pRD8 and pRD9 with Sst I plus Bam HI, and treating the linearized vectors sequentially with exonuclease III, S₁ nuclease, Klenow fragment and T₄ DNA ligase as described (Henikoff, 1987). Deletion derivatives of pRD8 and pRD9 were propagated in bacterial strain TG-1, and single stranded sequencing templates were prepared from transformants superinfected with M13K07 as described (Vieira and Messing, 1987). The nucleotide sequence of a 1.9 kb region of the pSEC6207 insert was determined by the dideoxy chain termination method (Sanger et al., 1977). The sequence depicted in Fig. 5 was deduced on both strands except for a small gap extending between nucleotides 37-74 in the noncoding region upstream of the SEC62 gene. The SEC62 sequence was evaluated with the assistance of the programs Testcode and CodonPreference (Grishkov et al., 1984), developed by the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

DNA and RNA Hyridization Analyses

To determine whether SEC62 is a single copy gene S. cerevisiae genomic DNA was prepared from strain W303-Leu as described (Ausubel et al., 1987). It was digested with either Eco RI, Cla I, Eco RV, or Hind III, separated electrophoretically on a 0.8% agarose gel and transferred to a nitrocellulose membrane. Plasmid pSEC6207 was digested with Eco RV and Cla I and the 1.4-kb Eco RV–Cla I fragment containing the SEC62 gene (see Fig. 4) was radioactively labeled with α-[³²P]-dCTP as described in the instructions supplied with the random-primed DNA labeling kit. This probe was hybridized at high stringency (50% formamide, 5 x SSC, 37°C) to the immobilized genomic DNA fragments, and the filter was subsequently washed and exposed as described (Maniatis et al., 1982). Inspection of the autoradiogram revealed a single, intensely labeled band in each lane. SEC62 sequences were present on a 3.2-kb Pst I fragment, a 1.9-kb Cla I fragment, a 1.5-kb Eco RV fragment, and a 2.8-kb Hind III fragment. The size of the Cla I fragment detected agreed with that predicted from the restriction map of the pSEC6207 insert (Fig. 4).

Genomic DNA samples prepared from diploids heterozygous for a sec62 disruption allele (strain RPD99 and RPD95) were also hybridized to the 32P-labeled Eco Rv–Cla I fragment of pSEC6207 at high stringency as described above. In these cases, hybridization to the fragment bearing the wild-type SEC62 allele always decreased in intensity by ~2 fold (relative to W303-Leu), and one or two additional bands were seen. In each instance, the additional bands migrated as predicted from the restriction map of the plasmid used to generate the disrupted allele.

The direction of transcription of the SEC62 gene was determined with single-stranded RNA probes, prepared in vitro, by hybridization to poly A-selected yeast mRNA (Melton et al., 1984). pGEM2 was digested with Sma I and Sal I allowing the 1.8-kb Eco RV–Sal I fragment of pSEC6207 to be introduced. The resulting plasmid (pRDI0) was linearized by digestion with the indicated restriction enzyme and transcribed with T7 polymerase (Eco RI) or SP6 polymerase (Hind III). Reaction transcriptions contained α-[³²P]-dCTP, and were performed as described in the Promega Biotech catalogue. Total yeast RNA was isolated from yeast strain X2180-1B and fractionated on oligo-dT cellulose as described (Maniatis et al., 1982). Two micrograms of poly A-enriched RNA were loaded per lane and separated electrophoretically on a 1.2% agarose, 2.2 M formaldehyde gel. RNA was transferred by blotting onto a nitrocellulose filter, which was then probed, washed, and exposed as described (Maniatis et al., 1982). A single mRNA species of ~1-kb was detected by the T7 probe, whereas the SP6 probe failed to hybridize to any poly-A-selected RNA species. These results indicate that the SEC62 locus expressed a ~1.5-kb mRNA that was transcribed in the direction Eco Rv to Cla I.

Construction of Deletion-Replacement Vectors

A plasmid (pRD12) containing an internal deletion of SEC62 sequences was constructed by combining Ts⁺ and Sec⁺ derivatives of the SEC62 locus that were generated by exonuclease III digestion (see Cloning and DNA sequencing). Plasmid pRD8 derivative Δ12 (the endpoint of this deletion mapped within...
Results

Microsomes Extracted from sec62 Cells Are Deficient in Protein Translocation

Translocation of pppF into microsomes has been reconstituted with membrane and soluble fractions (S100; supernatant derived from centrifuging a crude yeast lysate at 100,000 g for 30 min) isolated from wild-type yeast (Hansen et al., 1986; Rothblatt and Meyer, 1986a; Waters and Blobel, 1986). Radiolabeled pppF synthesized in a yeast S100 is co- or posttranslationally translated into yeast microsomes in an ATP-dependent manner, rendering it resistant to digestion by exogenously added proteases (Hansen et al., 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986). Upon import into the ER, pppF experiences signal peptide cleavage, core glycosylation, and trimming of glucose residues from its core oligosaccharides (Waters et al., 1988).

Before examining membrane and soluble fractions isolated from mutant cells, we assayed translocation of pppF into ER microsomes using components extracted from wild-type cells. Translocation of pppF was monitored by subjecting reactions to SDS-PAGE followed by autoradiography. Since pppF was the only labeled molecule produced during in vitro translation, all labeled species detected were derived from it. pppF mRNA was translated in an S100 isolated from wild-type yeast. If microsomes were included either co- (Fig. 1, lane 1) or post- (Fig. 1, lane 4) translationally, ~70% of the pppF molecules were modified, yielding glycosylated pre-α-factor (pppF). Whereas unmodified pppF was sensitive to proteolysis, pppF was resistant to added protease (Fig. 1, lanes 2 and 5), suggesting that it resided within the lumen of the ER. Protease resistance was abolished in the presence of detergent, presumably because of solubilization of the ER membrane barrier (Fig. 1, lanes 3 and 6). The data described above were obtained with membrane and soluble fractions prepared from wild-type yeast propagated at 30°C. Virtually identical results were obtained with fractions isolated from wild-type cells cultured at 24°C or 17°C (data not shown).

Cell-free translocation reactions were also performed with cytosolic and membrane fractions derived from sec62 strains grown at 24°C. S100 prepared from sec62 cells efficiently translated pppF mRNA. If sec62 microsomes were added co- or posttranslationally, only a small amount of pppF was detected (Fig. 1, lanes 7 and 10). This deficit of glycosylated molecules did not result from a failure to core glycosylate translocated precursors, since unmodified pppF was completely sensitive to added protease (Fig. 1, lanes 8 and 11). As in the wild-type reaction, glycosylated precursors were sensitive to proteolysis only in the presence of detergent (Fig. 1, lanes 9 and 12). Quantitation of this autoradiogram revealed that translocation of pppF was reduced ~fourfold in a sec62 reaction (Table II). The severe translocation defect observed with components extracted from sec62 cells grown at 24°C was consistent with the effect of the sec62 mutation in vivo: >50% of newly synthesized pppF accumulated in an unglycosylated, untranslocated form in sec62 cells pulse

Table II. Quantitation of Prepro-α-factor, Preinvertase, and PreproCPY Import into SEC+ and sec62 Microsomes

| Precursor                  | SEC+ microsomes | sec62 microsomes |
|---------------------------|-----------------|------------------|
| prepro-α-factor†          | experiment 1    | 66               |
|                           | experiment 2    | 67               |
| preinvertase-fragment†    | experiment 1    | 19               |
|                           | experiment 2    | 36               |
| intact preinvertase†      |                 | 26               |
| prepro-CPY†               |                 | 49               |

*Translocation reactions were conducted as detailed in the legend to Fig. 3.
†The data shown were obtained from posttranslational import reactions. Qualitatively similar results were obtained with co-translational translocation assays.
‡Import of intact preinvertase was conducted co-translationally.
labeled at 24°C (Rothblatt et al., 1989). Thus, the SEC62 gene product was apparently required for the maximal activity of a membrane bound or soluble constituent of the protein translocation apparatus.

Since protein import into the yeast ER in vitro requires the participation of both soluble components (Chirico et al., 1988; Deshaies et al., 1988) and a membrane fraction, mixing experiments were performed to test whether the activity of one or both of these fractions was compromised by the sec62 mutation. ppαF mRNA was translated in a wild-type or sec62 lysate, and wild-type microsomes were added co- or posttranslationally to allow import of ppαF. Precursor synthesized in either a wild-type or sec62 S100 was efficiently translocated and core glycosylated by wild-type membranes (Fig. 2 A). This result suggested that SEC62 did not encode one of the soluble factors required for efficient protein import into the yeast ER.

To test the hypothesis that the sec62 mutation impaired the translocation capacity of microsomal membranes, ppαF translated in a wild-type S100 was incubated with wild-type or sec62 membranes in a posttranslational import reaction. ppαF produced in a wild type S100 was efficiently translocated into and glycosylated by wild-type membranes (Fig. 2 B, lanes 1–6). In contrast, ppαF was poorly translocated into membranes isolated from sec62 cells (Fig. 2, lanes 7–12). This import deficiency was not constrained to posttranslational reactions, as sec62 membranes were also defective in cotranslational assays (data not shown).

The small fraction of ppαF that was translocated into sec62 microsomes was core-glycosylated (Fig. 2 B, compare plus and minus protease digestion, lanes 7 and 8, lanes 10 and 11), but migrated aberrantly on SDS polyacrylamide gels. A novel species that migrated slower than the triply-glycosylated pro-α-factor (ppαF) was detected in sec62 microsomes. This form may represent an intermediate in core oligosaccharide trimming, since inhibition of glucosidase I and II activity by deoxynojirimycin in an in vitro translocation assay results in the accumulation of a similar high molecular mass species (Waters et al., 1988). ppαF contained within sec62 microsomes migrated more heterogeneously than that formed by wild-type microsomes. Perhaps the sec62

Figure 2. The translocation defect of sec62 extracts resides in the membrane fraction. (A) Washed microsomes isolated from sec (RDM 15-9B) cells were added co- (0.05 A260 U, lanes 3–8) or post- (0.068 A260 U, lanes 9–14) translationally to sec62 (lanes 6–8, 12–14) or sec (lanes 3–5, 9–11) S100 fractions programmed with ppαF mRNA. Translations depicted in lanes 1 and 2 were not supplemented with membranes. SEC microsome and S100 fractions were prepared from cells grown at 30°C. The sec62 S100 fraction was prepared from cells grown at 24°C. Reactions were followed by protease treatment, SDS-PAGE, and fluorography. Asterisk refers to unglycosylated ppαF. (B) ppαF mRNA was translated in batch in a SEC* (RDM 15-9B) S100 fraction for 30 min at 20°C. Cycloheximide was added to arrest polypeptide elongation, and the sample was subdivided into equal portions which received 0.017 A260 U (lanes 1–3, 7–9) or 0.05 A260 U (lanes 4–6, 10–12) of SEC* (RDM 15-9B, lanes 1–6) or sec62 (RDM 43-9C, lanes 7–12) washed microsomes. SEC* microsomes and S100 fractions were prepared from cells grown at 30°C, whereas sec62 microsomes were prepared from cells grown at 24°C. Following a posttranslational import incubation (30 min at 20°C), samples were treated with protease and subjected to SDS-PAGE and fluorography.
mutation perturbs the assembly of glucosidases and other enzymes required for normal synthesis and trimming of core oligosaccharides (see Discussion). This effect was exaggerated in vitro; intermediates of this form were not seen in sec62 cells (Rothblatt et al., 1989).

Translocation of ppoF into wild-type membranes was diminished up to twofold when an equal concentration of sec62 membranes was present (data not shown). Therefore, defective import of ppoF into sec62 membranes was not because of the presence of a potent translocation inhibitor in the mutant microsomes. Rather, this observation suggested that ppoF that was unable to translocate was retained on the cytoplasmic face of sec62 microsomes. Whereas ppoF was poorly translocated into the ER lumen in sec62 cells maintained at 24°C, cells grown at 17°C exhibited little or no mutant phenotype (Rothblatt et al., 1989). Membranes prepared from sec62 and wild-type strains propagated at 17°C were equally active in posttranslational translocation assays (data not shown). The effect of growth temperature on the import activity of sec62 microsomal membranes suggested that the translocation defect exhibited by membranes from cells grown at 24°C was a consequence of the conditional nature of the sec62 mutation.

A more direct relationship between the inferred thermolability of the sec62 gene product and the in vitro translocation defect of sec62 membranes was probed by measuring the thermal inactivation kinetics of membranes isolated from wild-type and sec62 cells grown at 24°C and 17°C. Whereas microsomes derived from sec62 cells grown at 24°C were inactivated by incubation at 31°C more rapidly than microsomes from wild-type strains (t1/2 = 10 min for sec62, 40 min for SEC membranes) no dramatic difference was observed with membranes isolated from wild-type and sec62 cells cultured at 17°C (data not shown). Perhaps the partial imposition of the sec62 block at 24°C in vivo labilized the microsomes, rendering them more sensitive to a thermal stress. The relative thermal resistance of membranes prepared from 17°C-grown sec62 cells suggests that the sec62 gene product is Ts+ for synthesis or assembly into the membrane, rather than Ts− for function.

**sec62 Membranes Discriminate between Different Precursors**

Mutant sec62 cells exhibit differential defects in the translocation of secretory precursor proteins. PreproCPY and ppoF are poorly translocated into the ER of sec62 cells, whereas preinvertase import is minimally perturbed (Rothblatt et al., 1989). The molecular basis of this discrimination is not understood. PreproCPY and an amino terminal fragment of preinvertase can be posttranslationally imported into yeast microsomes in vitro (Hansen and Walter, 1988). Intact preinvertase is translocated into yeast microsomes in vitro only during translation (Rothblatt et al., 1987; Hansen and Walter, 1988). If the inability of sec62 microsomes to import ppoF was a faithful representation of the translocation defect observed with sec62 cells, then sec62 microsomes should retain the ability to translocate preinvertase, but should be unable to translocate preproCPY. To test this prediction, we compared import of preproCPY, preinvertase, an amino terminal fragment of preinvertase, and ppoF into wild-type and sec62 microsomes. Messenger RNA encoding each preprotein was translated in a wild-type S100, and import was allowed to proceed posttranslationally in the presence of wild-type or sec62 microsomes. As was shown in Fig. 2 B, ppoF synthesized in a wild-type S100 was efficiently translocated into and glycosylated (gpoF) by wild-type microsomes (Fig. 3, lanes 1 and 2), but only poorly translocated into sec62 microsomes (Fig. 3, lanes 3 and 4). In contrast, a 31-kD amino-terminal fragment of preinvertase (plnv) was assembled into wild-type (Fig. 3, lanes 5 and 6) and sec62 (Fig. 3, lanes 7 and 8) microsomes with equivalent efficiency. PreproCPY resembled ppoF in import competence: ~50% of preproCPY was imported into wild-type microsomes (Fig. 3, lanes 9 and 10), but only a small fraction was imported into sec62 microsomes (Fig. 3, lanes II and 12). Quantitative results from two import experiments are presented in Table II. On average, sec62 microsomes exhibited a three- to fourfold defect (relative to wild type) in translocation of ppoF, but were equally proficient in import of preinvertase-fragment. sec62 microsomes were severely deficient in assembly of preproCPY, as seen in pulse-labeling studies with sec62 cells (Rothblatt et al., 1989). A similar discrimination between these three precursors was also observed in cotranslational import assays that, in addition, allowed analysis of the import of intact preinvertase into wild-type and sec62 microsomes. As with the preinvertase-fragment (Fig. 3 and Table II), intact preinvertase was translocated into wild-type and sec62 microsomes with equal efficiency.

**Figure 3. sec62 microsomes are selectively defective in import of ppoF and preproCPY, but not preinvertase-fragment.** mRNA encoding either ppoF (A, lanes 1–4) preinvertase fragment (B, lanes 5–8), or preproCPY (C, lanes 9–12) was translated in a SEC+ (RDM 15-9B) S100 fraction for 30 min at 20°C, at which point each translation was split into two samples and supplemented with 0.05 A260 U of either SEC+ (RDM 15-9B), lanes 1, 2, 5, 6, 9, and 10) or sec62 (RDM 43-9C, lanes 3, 4, 7, 8, II, and 12) washed microsomes (cycloheximide was not added). After a 30 min incubation at 20°C to allow import, each reaction divided in half and either mock-treated (odd-numbered lanes) or digested with proteinase K (even-numbered lanes). Both SEC+ and sec62 microsomes were isolated from cells grown at 24°C. After protease treatment, samples were processed for SDS-PAGE and fluorography. gplnv, core-glycosylated invertase-fragment; plnv, unglycosylated preinvertase-fragment; gpCPY, core-glycosylated proCPY; ppCPY, unglycosylated preproCPY.
efficiency (Table II). Glycosylated preinvertase-fragment and intact preinvertase molecules that were formed within sec62 microsomes migrated heterogeneously on SDS polyacrylamide gels, presumably as a result of some defect in core-oligosaccharide synthesis or processing (see Discussion).

**Isolation of the SEC62 Gene**

The results described above indicated that microsomes isolated from sec62 cells were defective in the import of a subset of secretory precursor proteins. This implied that the SEC62 gene product was required for the proper function of the ER membrane translocation machinery. To distinguish between these models, we isolated the SEC62 gene, determined its nucleotide sequence, and examined the structure of the predicted SEC62 polypeptide (Sec62p).

Yeast genomic DNA sequences that complemented the Ts- growth defect of sec62 strains were isolated by transformation with a gene bank contained in the single-copy plasmid YCp50 (Rose et al., 1988). A detailed restriction map of a sec62-complementing subclone (pSEC6207) is shown in Fig. 4. Five fragments of the 4.4-kb pSEC6207 insert were independently subcloned into a centromere-containing plasmid (pSEYc68) and tested for their ability to complement sec62 strains (Fig. 4). A 1.7-kb fragment (Eco RV–Sph I) of the pSEC6207 insert complemented both the Ts growth and ppcF accumulation phenotypes of sec62 cells (data not shown).

A chromosomal integration experiment was performed to determine whether pSEC6207 contained the authentic SEC62 gene or another gene capable of suppressing the sec62 mutation. Plasmids that lack an autonomously replicating sequence (ARS) are not stably maintained in yeast cells unless they integrate into the genome. Integration is achieved by cleaving such a plasmid within yeast DNA sequences exposing free ends that direct homologous recombination with the corresponding chromosomal sequences (Orr-Weaver et al., 1981). The 4.6-kb Hind III–Sph I fragment of pSEC6207 was subcloned into the yeast integration vector YIp351, which contains the LEU2 gene but lacks an ARS. The resulting plasmid (pRD7) was digested with Hpa I and used to transform a SEC62 leu2 strain (DBY2060) to leucine prototrophy. Four independent Leu+ DBY2060 clones were mated to a leu2 sec62 strain (RDM 50-94C), and the diploids were sporulated and ascii dissected into tetrads. The segregation of the LEU2 and SEC62 genes among the spore progeny from each cross is documented in Table III. In each case, temperature resistant growth and leucine prototrophy segregated 2:2 and cosegregated in 44 of 45 tetrads. These data indicate that pRD7 integrated at the SEC62 locus. Therefore, pSEC6207 contains the authentic SEC62 gene.

**DNA Sequence of SEC62**

The nucleotide sequence of each strand of the Eco RVb–Sph I fragment of pSEC6207 was determined by sequencing two sets of ordered deletions using the dideoxynucleotide chain-termination method (Sanger et al., 1977). The nucleotide sequence of most of the region between the Eco RV site and the Sau IIIA/Bam HI boundary of genomic and vector sequences; Sph, Sph I; Sal, Sal I. The dotted line indicates sequences derived from the ‘Tet’ region of YCp50.

**Table III. pSEC6207 Contains the Authentic SEC62 Gene**

| Sporulated diploid* | Asci dissected | Segregation of temperature sensitivity and leucine prototrophy among spore progeny |
|---------------------|----------------|----------------------------------------------------------------------------------|
|                     |                | Ts+                                 | Ts-                                 | Ts+                                 | Ts-                                 |
| RPD 91              | 12             | 24                                  | 0                                  | 0                                   | 24                                  |
| RPD 92              | 10             | 20                                  | 0                                  | 0                                   | 20                                  |
| RPD 93              | 12             | 24                                  | 0                                  | 0                                   | 24                                  |
| RPD 94              | 12             | 24                                  | 1                                  | 0                                   | 23                                  |

* Haploid strain DBY 2060 (SEC62, leu2) was transformed with Hpa I-linearized pRD7, and four independent Leu+ transformants were mated with RDM 50-94C (sec62, leu2). Each diploid (RPD 91–94) was sporulated and subjected to tetrad analysis.
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The nucleotide sequence of the SEC62 gene and the predicted amino acid sequence of Sec62p. Both strands of the SEC62 coding sequence and flanking regions were sequenced entirely, except for the stretch between nucleotides 37-74, which was only sequenced on the strand shown. Numbers in the right-hand column refer to the nucleotide number, with the first nucleotide shown designated as 1. Sequences potentially involved in the initiation (TATA) boxes or termination of transcription are denoted by straight or squiggly underlines, respectively. Carets mark the boundaries of the exonuclease III deletions used to construct the sec62::HIS3 and sec62::URA3 deletion-replacement alleles. The exact boundary of the deletion endpoint at the 3' end of SEC62 was unknown, but probably mapped within the 9-nucleotide region shown. The longest open reading frame started with the ATG codon at nucleotide 315, though another potential initiation codon was noted at nucleotide 342. Potential acceptor sites for asparagine-linked oligosaccharides are marked with an asterisk, and the two potential membrane-spanning domains of Sec62p are shaded; note that the second domain contains a charged amino acid (arginine 207). The Eco RV site (RVc) used for various plasmid constructions is boxed. The initiation codon (nucleotide 27) of a divergent open reading frame located upstream is indicated.

Figure 5. Nucleotide sequence of the SEC62 gene and the predicted amino acid sequence of Sec62p.
son, 1985) revealed no apparently meaningful homologies. Similarly, a search of the Genbank plant and eukaryotic organelle nucleotide data bases (version 58.0, December 1988) with the tFastn algorithm failed to detect any convincing homologies.

Hydropathy analysis (with window sizes of 9 and 19 residues) revealed that the predicted Sec62p contained two stretches of amino acids that were sufficiently long and hydrophobic to span a lipid bilayer (Kyte and Doolittle, 1982). These putative membrane-spanning domains (shown shaded in Fig. 5) were separated by eight amino acids, three of which were arginine. The second hydrophobic domain of Sec62p (amino acids 187-218) was longer than common transmembrane domains, and contained a positively charged residue (arginine 207). Perhaps this segment exhibits a more complex interaction with the lipid bilayer. Notably, Sec62p lacked an amino-terminal hydrophobic stretch characteristic of signal peptides. Another feature of Sec62p was the extremely basic character of its carboxy terminus. 14 of the last 29 residues were either arginine or lysine; only three acidic residues were present in this same region. Analysis of Sec62p with the secondary structure-predicting algorithm of Chou and Fasman (1978) revealed that the highly basic carboxy terminus may form an a-helix with basic residues displayed nearly uniformly about the helix. Based on the hydropathy analysis, we predict that Sec62p is a membrane protein. A model for the membrane topology of Sec62p and speculations on the function of its extremely basic carboxy terminus are developed in the Discussion section.

**SEC62 Is an Essential Single-Copy Gene**

The existence of Ts- lethal sec62 alleles implied that the SEC62 gene product performed an essential function. However, null mutations in several genes lead to heat or cold sensitive growth, indicating that some genes identified by temperature-conditioned growth mutations are only essential at the nonpermissive temperature (Craig and Jacobsen, 1984; Novick et al., 1989). To test whether SEC62 was essential for vegetative growth at optimal growth temperatures (24-30°C) we deleted the chromosomal copy of SEC62 and examined the effect of this deletion on cell growth. Two plasmids containing an internal deletion within the 3.1-kb Pst I-Sph I fragment of pSEC6207 were constructed (see Materials and Methods). These plasmids contained DNA flanking both the 5' and 3' ends of SEC62, but lacked the SEC62 coding region located between nucleotides 726 and 1245 (Fig. 5, deletion endpoints are marked with carets) that was replaced by either the URA3 or HIS3 gene of *S. cerevisiae*. Both plasmids were treated with Cla I, which in each case liberated a fragment containing the sec62 deletion/replacement allele plus 1,000 bp of 5' and 220 bp of 3' flanking sequences.

These fragments were used to transform yeast diploid strain W303-Leu to either histidine (RPD 99-101) or uracil (RPD 95-97) prototrophy. Southern hybridization analyses of genomic DNA prepared from transformants RPD 99 and RPD 95 indicated that they each contained one wild-type and one disrupted chromosomal copy of SEC62 (see Materials and Methods). The RPD 99-101 and RPD 95-97 transformants were sporulated, and asci were dissected into tetrads and germinated at 17, 24, or 30°C. Every individual transformant examined segregated 2 live spores and two dead spores at either 24°C (Table IV), 17, or 30°C (data not shown). No colonies bearing the replacement marker (HIS3 or URA3) were recovered, suggesting that the observed spore inviability resulted from the deletion of SEC62. To confirm this hypothesis, diploid RPD 99 (which is heterozygous for the HIS3 deletion/replacement allele of sec62) was transformed with a centromeric URA3 plasmid either containing (pR5D, see Fig. 4) or lacking (pSEYc68) the SEC62 gene. Several RPD 99 transformants bearing either plasmid were sporulated, dissected into tetrads, and germinated at 30°C. As expected, tetrads derived from RPD 99 cells transformed with pSEYc68 consistently yielded two live and two dead spore clones (Table IV). In contrast, tetrads derived from RPD 99 cells transformed with pR5D most commonly yielded 3-4 viable spores (Table IV). Although spore clones bearing both the sec62::HIS3 disruption and pR5D were recovered, no spores containing the disruption but lacking the SEC62 plasmid were found (Table IV). His+ colonies (wild-type allele of SEC62 in the chromosome) containing pR5D segregated Ura- clones on 5-fluoroorotic acid-containing medium (Boeke et al., 1987) at high frequency, whereas His+ (sec62::HIS3 chromosomal allele) colonies containing pR5D failed to yield Ura- papillae. Taken together, these data indicate that the SEC62 gene is essential for spore germination and mitotic growth.

Genomic DNA from yeast strain W303-Leu was digested with several restriction enzymes and subjected to Southern hybridization analysis using the Eco RV-Cla I fragment of pSEC6207 as a probe. Hybridizations performed under conditions of high stringency revealed a single chromosomal species, indicating that SEC62 was a single-copy gene in *S. cerevisiae* (data not shown). Hybridizations performed at lower stringency failed to detect any additional fragments, suggesting that the *S. cerevisiae* genome did not harbor other genes homologous to SEC62. Homologous sequences were detected by low stringency Southern hybridization analyses of genomic DNA isolated from *Schizosaccharomyces pombe* and *Kluyveromyces lactis* (data not shown). Perhaps SEC62 encodes a protein that is conserved among different organisms.

**Discussion**

In an accompanying report, we describe a recessive mutation in the sec62 gene that results in Ts- yeast cell growth and defective import of a subset of precursor polypeptides into the ER (Rothblatt et al., 1989). sec62 cells accumulate significant levels of untranslocated preproCPY and pppF at 24°C and 37°C, but not at 17°C. In contrast, preinvertase import in sec62 cells is only partially blocked at 24 or 37°C (Rothblatt et al., 1989). In this paper, we show that pppF synthesized in a sec62 soluble extract is poorly assembled into sec62 microsomes. Mixing experiments with wild-type cytosol and membranes indicated that this translocation defect is attributable to the sec62 microsomal fraction. The membrane-localized import defect is related to the sec62 mutation, since membranes prepared from cells grown at 17°C are competent for translocation, whereas membranes isolated from cells grown at 24°C exhibit reduced activity. Just as sec62 cells show a selective defect in the membrane assembly of pppF and preproCPY, but not preinvertase, sec62 microsomes fail to import preproCPY, but efficiently import...
intact preinvertase and an amino-terminal fragment of preinvertase.

Defective import of ppαF and preproCPY into sec62 microsomal membranes suggests either that Sec62p is a constituent of the ER membrane translocation apparatus, or that wild-type Sec62p is a cytosolic or membrane protein required for the synthesis, membrane insertion or posttranslational modification of a membranous component(s) of the translocation machinery. The predicted protein sequence of Sec62p suggests that it is embedded within a membrane.

Impaired translocation into sec62 microsomes may be a direct or indirect consequence of mutant Sec62p. Untranslocated precursors accumulated in sec62 cells may jam translocator pores or signal sequence receptors (Wiedmann et al., 1987), rendering sec62 membranes incompetent for ppαF import in vitro. Alternatively, a reduction of Sec62p activity may subtly alter the structure or composition of the ER bilayer, resulting in pleiotropic defects in ER membrane physiology. We consider these possibilities unlikely, since sec62 microsomes retain the capacity to import invertase precursors efficiently. Further investigation will be required however to demonstrate a direct role for wild-type Sec62p in protein translocation.

The selective translocation defect imposed by the sec62 mutation may be explained by alternative assembly pathways or by distinct thresholds of requirement for Sec62p. Completely distinct assembly pathways seem unlikely in light of the observation that sec double mutants that include sec62 are more severely deficient in invertase translocation (Rothblatt et al., 1989). As suggested in the accompanying paper, the hydrophobicity, position, or structure of import signals may influence the interaction of precursor molecules with components of the translocation apparatus. Experiments performed in other systems indicate that the signal sequences of invertase and CPY do not contain equivalent information. Whereas preinvertase translocates into mammalian ER in vivo (Bergh et al., 1987) and in vitro (Perlman and Halvorson, 1981), preproCPY is not imported into mammalian ER in vivo or in vitro (Bird et al., 1987). The inability of preproCPY to penetrate mammalian ER membranes may be solely because of the relative hydrophilicity of preproCPY's signal peptide; hydrophobic amino acid substitutions within the preproCPY secretion signal improve translocation into mammalian ER in vivo and in vitro (Bird et al., 1987). The sec62 mutation may reduce the affinity of interaction between some component(s) of the translocation machinery and the signal sequences of secretory precursors, thereby increasing the selectivity of translocation and discriminating against precursor molecules with less hydrophobic signal sequences (such as preproCPY).

ppαF molecules translocated into sec62 microsomes are apparently modified on all three acceptor sites for N-linked glycosylation, but the glycosylated precursors migrate aberrantly on SDS polyacrylamide gels. Also, ppαF synthesized in a sees2 S100 fraction is inefficiently glycosylated by un-washed wild-type microsomes, whereas precursor synthesized in a wild-type S100 fraction is properly glycosylated by the same membrane preparation (see Materials and Methods). One interpretation of these results is that the sec62 mutation primarily blocks core oligosaccharide synthesis, transfer, or trimming. If any components of the translocation apparatus require core-glycosylation for their assembly and function, such a lesion might indirectly result in inactive translocation complexes. This is unlikely, since mutations that block the biosynthesis and processing of core oligosaccharides do not affect protein translocation (Feldman et al., 1987; Bernstein et al., 1989; for review, see Deshaies et al., 1989). Rather, the constitutive translocation defect in sees2 cells cultured at 24°C may result in reduced assembly of components of the multienzyme pathway that catalyzes core oligosaccharide assembly and trimming.

The sequence of the SEC62 gene predicts a protein (Sec62p) that lacks an amino-terminal signal sequence, but contains two stretches of amino acids sufficiently long and hydrophobic to span a lipid bilayer. Based on the in vitro reconstitution data and the primary structure of Sec62p, we speculate that Sec62p is a 32-kD integral membrane protein that spans the ER bilayer two times, with highly charged amino- and carboxy-terminal domains emerging from either the cytoplasmic or luminal face of the ER membrane. Evaluation of the Sec62p sequence with an algorithm designed to assess membrane protein topology predicts that the amino terminal domain protrudes into the ER lumen (Hartmann et al., 1989). It is difficult to predict the disposition of the basic carboxy-terminal domain of Sec62p, as the second putative transmembrane anchor is unusually long (30 amino acids) and may adopt a novel conformation in the bilayer (e.g., it may be sufficiently long to span the membrane twice in a β-sheet configuration). The topology of Sec62p can be rigorously determined once specific antibodies are available. A search of protein and nucleotide sequence databases failed to identify any proteins homologous to Sec62p. However, the mo-
The hydrophilic amino- and carboxy-terminal domains of Sec62p may facilitate translocation by interacting with cytosolic factors, precursor molecules, or membrane-associated components of the translocation apparatus. In light of the third possibility, it is interesting to consider the predicted sequence of the SEC63 gene product (Sec63p) which also has been identified as a gene involved in nuclear protein import (Sadler et al., 1989). SEC63 has also been cloned (Sadler et al., 1989), and its DNA sequence predicts a polypeptide with 1–3 potential transmembrane domains and an extremely acidic carboxy terminus (26 of the last 52 residues are aspartate or glutamate). This contrasts with the extremely basic carboxy terminus of Sec62p (14 of the last 29 residues are lysine or arginine). The highly charged COOH-terminal domains of both Sec62p and Sec63p are predicted to adopt α-helical secondary structures (Chou and Fasman, 1978). Perhaps the opposite charges of these putative α-helical domains allows the formation of extensive interprotein contacts between Sec62p and Sec63p. A potential physical association of Sec62p and Sec63p is consistent with the observed genetic interaction of the sec62 and sec63 mutant alleles, whereas sec62 or sec63 single mutants grow normally at 24°C and are inviable at 37°C, sec62 sec63 double mutants are inviable at 24°C, and grow only at lower temperatures (Rothblatt et al., 1989). Alternatively, Sec62p and Sec63p may interact only indirectly such as in the display of the charged domains on opposing membrane surfaces. Such localized charge asymmetry could create a surface potential capable of influencing the opening or closing of a membrane channel.

Our intention in adopting a genetic approach was to discover novel membrane-associated components of the protein translocation machinery, since these have proved to be especially intractable to conventional biochemical analysis. The data shown here and in the accompanying papers suggest that our approach has indeed led to the identification of membrane-localized translocation factors. Besides Sec62p and Sec63p, the polypeptide predicted by the nucleotide sequence of the SEC63 gene also contains several potential transmembrane domains (C. Stirling and R. Schekman, unpublished data). The availability of cloned DNA encoding Sec61p, Sec62p, and Sec63p will allow the production of specific antisera, determination of transmembrane topologies, and mapping of functional domains of these polypeptides. This information will provide a more refined picture of the structure and function of the secretory protein translocator in the ER membrane.

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