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

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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 clone. DNA sequence analysis reveals a 32-kD protein with no obvious NH_2-terminal signal sequence but with two domains of sufficient length and hydrophobicity to span a lipid bilayer. Sec62p is predicted to display significant NH_2- 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^-)[1] 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., 1988*b*; Rothblatt et al., 1989). Meyer and his colleagues have also isolated a translocation-defective mutant, *ptl1-1*, 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* gene products 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., 1988*b*).

1. *Abbreviations used in this paper*: ARS, autonomously replicating sequence; CPY, carboxy peptidase Y; gpαF, core-glycosylated pro-α-factor; pαF, pro-α-factor; ppαF, prepro-α-factor; ORF, open reading frame; Ts^-, temperature sensitive.

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 1), 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, S_1 nuclease, T_4 DNA ligase, calf intestinal alkaline phosphatase, Klenow fragment of DNA polymerase 1, random primed DNA labeling kit, phosphocreatine, and staphylococcal nuclease S7 were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); SP6 RNA polymerase, RQ1 DNase, and ribonuclease inhibitor (RNasin) were purchased from Promega Biotech (Madison, WI); α-[^{32}P]dCTP (~3,000 Ci/mmol), α-[^{32}P]CTP (>400 Ci/mmol), [^{35}S]-α-thio-dATP (1,200 Ci/mmol), [^{35}S]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 I. Yeast and Bacterial Strains

Strain	Genotype	Source or reference
Saccharomyces cerevisiae		
RDM 15-9B	*ade2, pep4-3, MATα*	Deshaies and Schekman, 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, MATα*	This study
RDM 50-94C	*leu2-3, -112, his4, ura3-52, sec62-1, MATα*	This study
X2180-1B	*gal2, mal, mel, SUC2, CUP1, MATα*	YGSC*
DBY 2060	*leu2-3, -112, MATa*	D. Botstein, Genentech, South San Francisco, CA
W303-Leu‡	$\frac{leu2\text{-}3\text{-}112}{LEU2}$, $\frac{his3\text{-}11,\text{-}15}{his3\text{-}11,\text{-}15}$, $\frac{trp1\text{-}1}{trp1\text{-}1}$, $\frac{ura3\text{-}1}{ura3\text{-}1}$, $\frac{ade2\text{-}1}{ade2\text{-}1}$, $\frac{can1\text{-}100}{can1\text{-}100}$, $\frac{MATa}{MAT\alpha}$	Borkovich et al., 1989
RPD 99-101§	Same as W303-Leu, except $\frac{sec62::HIS3}{SEC62}$	This study
RPD 95-97‖	Same as W303-Leu, except $\frac{sec62::URA3}{SEC62}$	This study
Escherichia coli		
TG1	Δ(*lac, pro*) *supE thi hsdD5 F' [traD36* pro$^+$ *lacI*^{q1} *lacZΔM15]*	Mark Biggin, unpublished observations, Department of Molecular Biology and Biophysics, Yale University
MC1061	F$^-$ *hsdR*$^-$ *hsdM*$^+$ *araD139* Δ (*araAB01C-leu*)*7679* Δ(lac)X74 *gal1U 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::HIS3*).
‖ 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); T7 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 plasmid pUC119 (Vieira and Messing, 1987), and the *E. coli*-yeast shuttle plasmids YEp351, YIp351 (Hill et al., 1986), and YEp24 (Botstein et al., 1979) have been described previously. *E. coli* plasmid pGEM2, used for producing RNA transcripts in vitro, is described in the Promega Biotech catalogue. The *E. coli*-yeast shuttle plasmid pSEYc68 is identical to pSEYc58 (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 (pG2CPY16), preinvertase (pG2SUC23), and preinvertase fragment (pG2-SUC91) 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 pDJ100, 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 IIIA inserted into the Bam HI site of the *E. coli*-yeast shuttle vector YCp50 (*CEN4, ARS1, 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, bacterial transformation 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 YCp50 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-peptone, 1% yeast extract, 3% dextrose (rich medium) to an OD_{600} of 1.5–5.0. Cells were harvested in a continuous flow rotor (Sharples Corp., Warminster, PA), 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 0.5 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 A_{260} >30 were pooled, diluted to a final A_{260} 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 $CaCl_2$ (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 Translocation 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 OD_{600} of 1.5–5.0. Membranes were isolated as described by Rothblatt and Meyer (1986*b*) 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 lyticase/OD_{600} 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 30,000 *g* in a Ti45 rotor (Beckman Instruments); membranes were washed with 10 ml membrane storage buffer containing 0.3 M sorbitol in place of sucrose, collected by centrifugation, and resuspended in membrane storage buffer to an A_{280} (in 1% SDS) of 45. Endogenous mRNAs in the membrane suspension were degraded by treatment with $CaCl_2$ (0.8 mM) and staphylococcal nuclease S7 (250 U/ml) for 5 min 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 A_{280} of 45 were obtained in a single preparation.

The extra wash with membrane storage buffer employed in this modified procedure was critical. ppαF 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 pDJ100 (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 A_{260} 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 (A_{280} = 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 translations. 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 mM 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 microsomal vesicles was assessed by treatment of import reactions with proteinase 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 (Deshaies and Schekman, 1987) before SDS-PAGE. Autoradiograms were quantified by densitometry as described (Deshaies 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 ~66,000 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 retransformed into RDM 42-3C. Plasmids from four of the seven original Ts^+ transformants conferred Ts^+ growth to RDM 42-3C in the rescreen, indicating that their yeast DNA inserts complemented *sec62*. Restriction mapping of these plasmids indicated that they contained overlapping inserts (A. Eun, 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 RVc–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, S_1 nuclease, Klenow fragment and T_4 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 M13KO7 as described (Vieira and Messing, 1987). The nucleotide sequence of a 1,911-bp 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 (Gribskov et al., 1984), developed by the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

DNA and RNA Hybridization 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), digested with either Pst I, 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 RVc–Cla I fragment containing the *SEC62* gene (see Fig. 4) was radioactively labeled with α-[^{32}P]-dCTP as described in the instructions supplied with the random-primed DNA labeling kit. This probe was hybridized at high stringency (50% formamide, 5 × 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 >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 ^{32}P-labeled Eco RVc–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 RVc–Sal I fragment of pSEC6207 to be introduced. The resulting plasmid (pRD10) was linearized by digestion with the indicated restriction enzyme and transcribed with T7 polymerase (Eco RI) or SP6 polymerase (Hind III). Transcription reactions contained α-[^{32}P]CTP, 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 polyA-selected RNA species. These results indicate that the *SEC62* locus expressed a ~1.0-kb mRNA that was transcribed in the direction Eco RVc to Cla I.

Construction of Deletion-Replacement Vectors

A plasmid (pRD12) containing an internal deletion of *SEC62* sequences was constructed by combining 5′ and 3′ deletions 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

10-bp 5′ of nucleotide 1,243 in Fig. 5) was digested with Hind III, blunted with Klenow enzyme plus dNTPs, then digested with Eco RI. The ~550-bp fragment released by this double digestion contained the Δ12 deletion endpoint and 300 bp of DNA flanking the 3′ end of *SEC62* (up to the Sau IIIA/Bam HI boundary of pSEC6207). Plasmid pRD9 deletion derivative Δ26 (the endpoint of this deletion lay exactly between nucleotides 275 and 276 in Fig. 5) was digested with Nar I, blunted with Klenow enzyme plus dNTPs, and then digested with Eco RI. This double digestion yielded a vector containing the Δ26 deletion endpoint and ~1,650 bp of DNA 5′ of *SEC62*. The ~550-bp fragment derived from Δ12 was inserted into the double-digested Δ26 vector. The product of this manipulation, plasmid pRD12, contained the region between the Pst I and Sph I sites of pSEC6207, except for the *SEC62* coding sequences located between nucleotides 276 and ~1,243 (Fig. 5), which were replaced by the Eco RI–Sma I portion of the pUC119 polylinker. Plasmid pRD13 was generated by inserting a blunt-ended 1.2-kb Hind III fragment containing the *URA3* gene (from plasmid YEp24) into the unique Sma I site of pRD12. Plasmid pRD14 was constructed by inserting a blunt-ended 1.3-kb Bam HI–Xho I fragment containing the *HIS3* gene (Struhl, 1985) into the unique Sma I site of pRD12.

pRD13 and pRD14 were digested with Cla I, and the released fragments bearing the *sec62* deletion-replacement alleles were purified by electrophoresis through low melting point agarose gels and used to transform yeast strain W303-Leu to histidine (pRD14 fragment) or uracil (pRD13 fragment) prototrophy.

Results

Microsomes Extracted from sec62 Cells are Deficient in Protein Translocation

Translocation of ppαF 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, 1986*a*; Waters and Blobel, 1986). Radiolabeled ppαF synthesized in a yeast S100 is co- or posttranslationally assembled into yeast microsomes in an ATP-dependent manner, rendering it resistant to digestion by exogenously added proteases (Hansen et al., 1986; Rothblatt and Meyer, 1986*b*; Waters and Blobel, 1986). Upon import into the ER, ppαF experiences signal peptide cleavage, core glycosylation, and trimming of glucose residues from its core oligosaccharides (Waters et al., 1988).

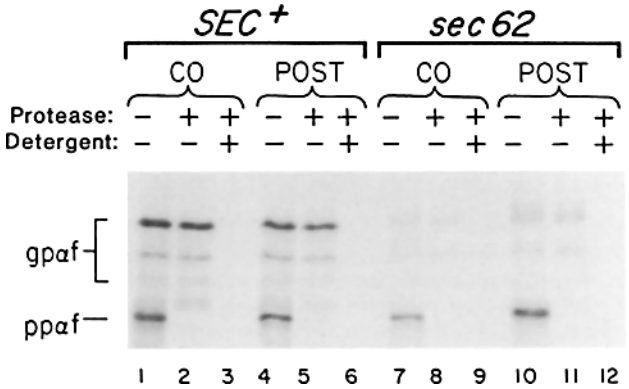

Figure 1. sec62 extracts are deficient in protein translocation. Co- and posttranslational translocation of ppαF was reconstituted with S100 and washed membrane fractions prepared from *SEC*+ (RDM 15-9B, lanes *1–6*) and *sec62* (RDM 43-9C, lanes *7–12*) strains grown at 30°C and 24°C, respectively. Co-translational import reactions were conducted at 20°C for 30 min in the presence of 0.05 A_{200} U of membranes (*CO*, lanes *1–3*, *7–9*). For posttranslational translocation, MFα1 mRNA was translated 30 min at 20°C, translation was quenched with cycloheximide, 0.068 A280 U of membranes were added, and reactions were incubated 30 min at 20°C (*POST*, lanes *4–6*, *10–12*). Reactions were followed by protease treatment, SDS-PAGE, and fluorography.

Before examining membrane and soluble fractions isolated from mutant cells, we assayed translocation of ppαF into ER microsomes using components extracted from wild-type cells. Translocation of ppαF was monitored by subjecting reactions to SDS-PAGE followed by autoradiography. Since ppαF was the only labeled molecule produced during in vitro translation, all labeled species detected were derived from it. ppαF 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 ppαF molecules were modified, yielding glycosylated pro-α-factor (gpαF). Whereas unmodified ppαF was sensitive to proteolysis, gpαF 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 ppαF mRNA. If *sec62* microsomes were added co- or posttranslationally, only a small amount of gpαF 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 ppαF 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 ppαF 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 ppαF 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	Percent translocation*	
	SEC+ microsomes	*sec62* microsomes
prepro-α-factor‡		
experiment 1	66	20
experiment 2	67	17
preinvertase-fragment‡		
experiment 1	19	14
experiment 2	36	38
intact preinvertase§	26	28
prepro CPY‡	49	<10

* 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.

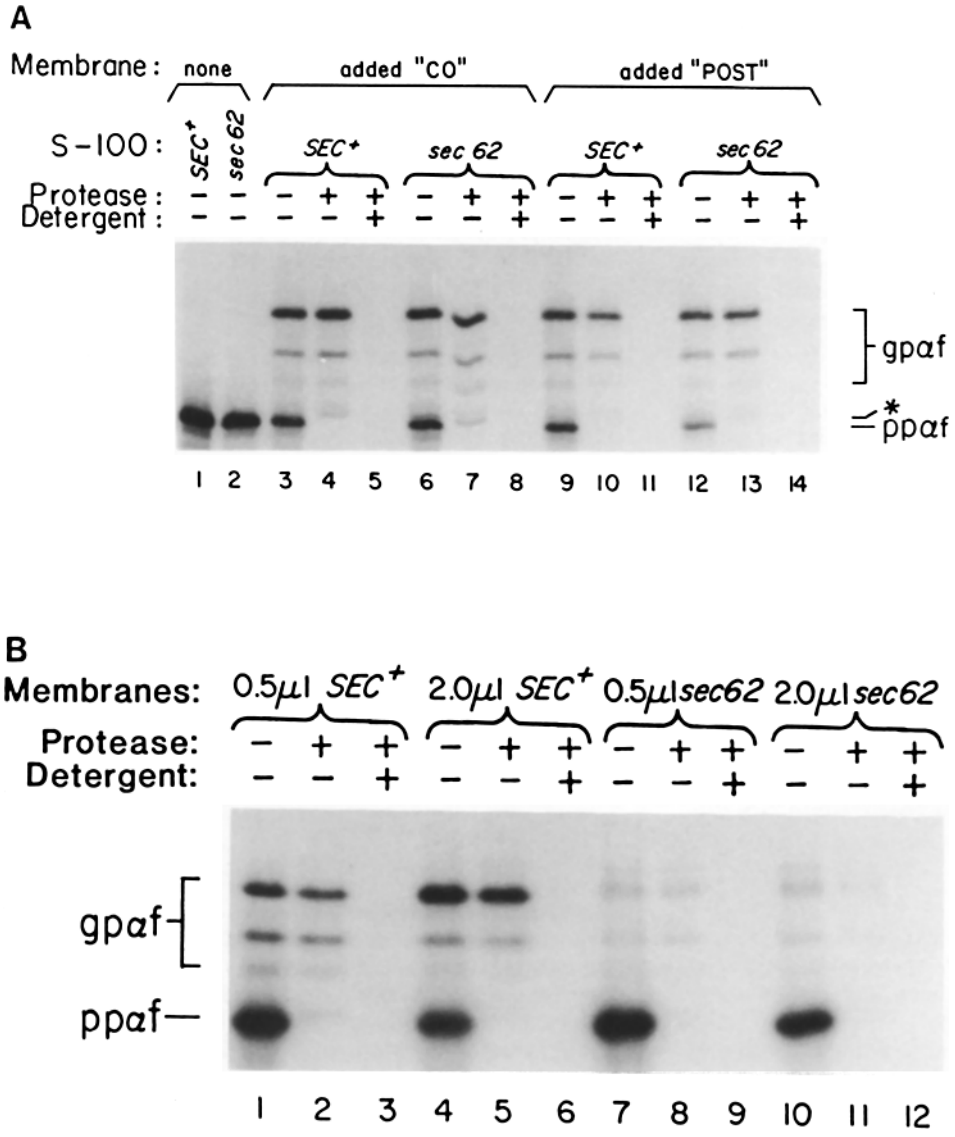

Figure 2. The translocation defect of *sec62* extracts resides in the membrane fraction. (*A*) Washed microsomes isolated from *SEC* (RDM 15-9B) cells were added co- (0.05 A_{280} U, lanes *3–8*) or post- (0.068 A_{280} U, lanes *9–14*) translationally to *sec62* (lanes *6–8*, *12–14*) or *SEC* (lanes *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 pαF. (*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 A_{280} U (lanes *1–3*, *7–9*) or 0.05 A_{280} 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.

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 microsomes in co- and posttranslational import reactions (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 (gpαF) 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). gpαF contained within *sec62* microsomes migrated more heterogeneously than that formed by wild-type microsomes. Perhaps the *sec62*

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 ppαF 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 ppαF into *sec62* membranes was not because of the presence of a potent translocation inhibitor in the mutant microsomes. Rather, this observation suggested that ppαF that was unable to translocate was retained on the cytoplasmic face of *sec62* microsomes. Whereas ppαF 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 ($t_{½}$ = 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 ppαF 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 ppαF 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 ppαF 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*, ppαF synthesized in a wild-type S100 was efficiently translocated into and glycosylated (gpαf) 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 (pInv^f) 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 ppαF 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 *11* 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 ppαF, 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

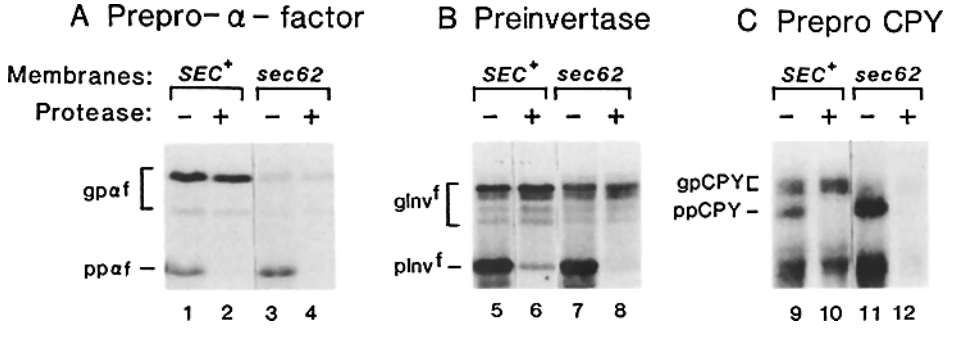

Figure 3. *sec62* microsomes are selectively defective in import of ppαF and preproCPY, but not preinvertase-fragment. mRNA encoding either ppαf (*A*, lanes *1–4*) preinvertase fragment (*B*, lanes *5–8*), or preproCPY (*C*, lanes *9–12*) was translated in a *SEC*⁺ (RDM 15-9B) S100 fraction for 30 min at 20°C, at which point each translation was split into two samples and supplemented with 0.05 A_{280} 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*, *11*, 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. *gInv^f*, core-glycosylated invertase-fragment; *pInv^f*, unglycosylated preinvertase-fragment; *gpCPY*, core-glycosylated proCPY; *ppCPY*, unglycosylated preproCPY.

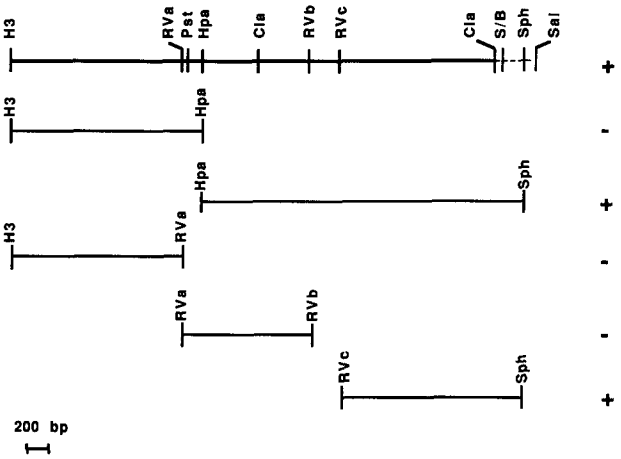

Figure 4. Complementation of the *sec62* Ts⁻ growth defect by different subclones of pSEC6207. A restriction map of the genomic yeast DNA insert of the *sec62*-complementing plasmid pSEC6207 is shown on the top line. Different regions of this insert were subcloned into the centromere-containing plasmid pSEYc68 to generate plasmids pRD1-pRD5 (shown in descending order), which were then tested for their ability to complement the Ts⁻ growth defect of the *sec62* strain RDM 50-94C. Results of the complementation experiments are indicated in the right-hand column. (+), complements the Ts⁻ growth defect of *sec62* strains; (−), fails to complement the Ts⁻ growth defect of *sec62* strains; *H3*, Hind III; *RVabc*, distinct Eco RV sites; *Pst*, Pst I; *Hpa*, Hpa I; *Cla*, Cla I; *S/B*, 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^r region of YCp50.

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 execution of a membrane-associated function involved in translocation of ppαF and preproCPY into the ER lumen. To fulfill this role, *SEC62* may encode either a soluble protein required for the sustained translocation-promoting activity of a membrane component (e.g., Sec62 protein may activate some membrane component by covalent modification), or a membrane protein that is 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 RVc–Sph I) of the pSEC6207 insert complemented both the Ts growth and ppαF 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 asci 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 RVc site and the Sau IIIA/Bam HI junction at the clone boundary is shown in Fig. 5. This 1,385 nucleotide stretch contained a single long open reading frame (ORF) of 849 bp that started at nucleotide 315 and terminated at nucleotide 1,164. Potential transcription initiation signals (TATA boxes) were noted at positions 118, 125, and 293, which are 22–197 nucleotides upstream of the putative initiator AUG, respectively. Though not all yeast genes possess TATA elements, they are commonly found 40–120 bp upstream of the site(s) of transcript initiation (Struhl, 1987). A putative transcription termina-

Table III. pSEC6207 Contains the Authentic SEC62 Gene

Sporulated diploid*	Asci dissected	Segregation of temperature sensitivity and leucine prototrophy among spore progeny			
		Ts⁺ Leu⁺	Ts⁺ Leu⁻	Ts⁻ Leu⁺	Ts⁻ Leu⁻
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.

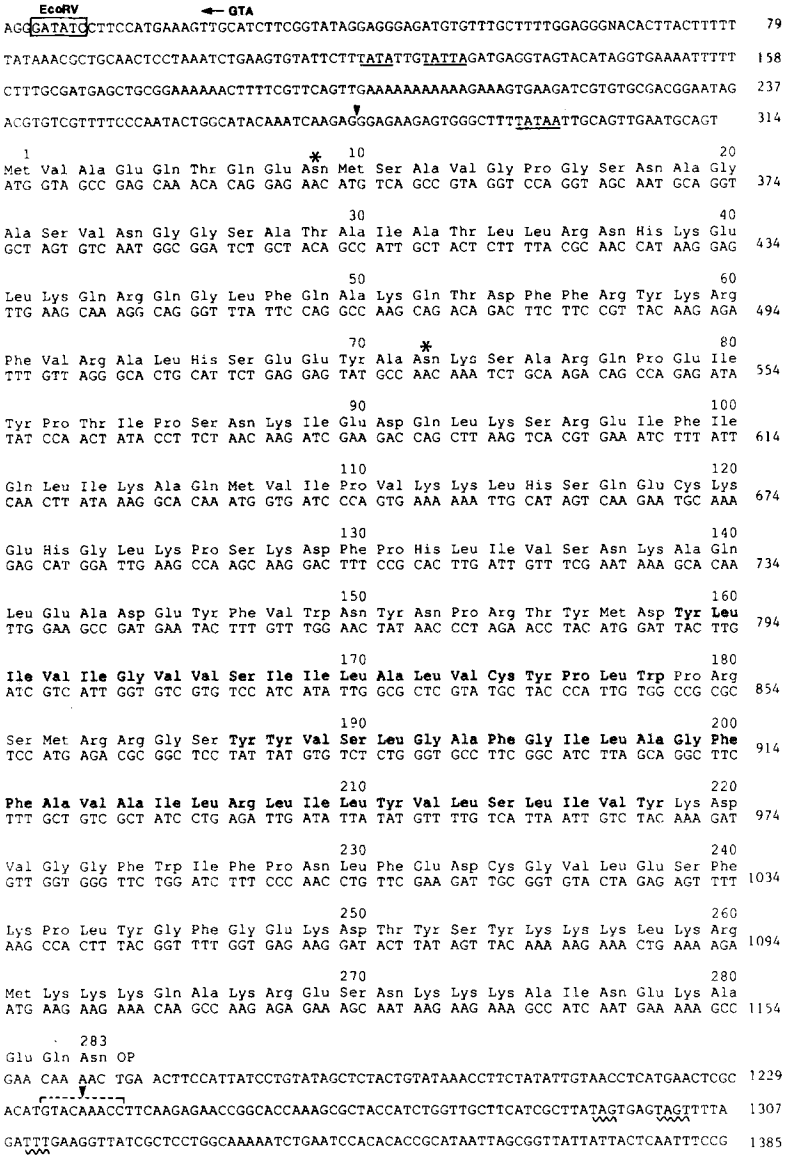

Figure 5. Nucleotide sequence of the *SEC62* gene and the predicted amino acid sequence of Sec62p. Both strands of the *SEC62* coding sequence and flanking regions were sequenced entirely, except for the stretch between nucleotides 37–74, which was only sequenced on the strand shown. Numbers in the right-hand 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.

tion signal reminiscent of that found at the 3′ end of the *CYC1* gene (Zaret and Sherman, 1982) was detected at positions 1,293–1,312. The *SEC62* transcript apparently is not spliced, since no conserved splicing elements were noted in the sequenced region (Langford and Gallwitz, 1983). Two lines of evidence suggested that the long ORF encoded a polypeptide. First, the only region of the sequenced fragment shown in Fig. 5 that exhibited a codon bias similar to that of previously sequenced yeast genes corresponded exactly to the long ORF (Gribskov et al., 1984). Second, hybridization of single-stranded radiolabeled RNA probes (transcribed in vitro from a plasmid containing the Eco RVc–Sal I fragment of pSEC6207) to poly (A)$^+$ mRNA purified from wild-type yeast cells indicated that the *SEC62* locus encoded a single, ~1.0-kb mRNA species that was transcribed in the same direction as the long ORF (Eco RVc to Sal I).

An unusual feature of the long ORF was the presence of an out-of-frame ATG immediately preceding the proposed site of translation initiation. Comparison of the DNA sequence of 96 yeast genes reveals that ATG triplets rarely occur upstream of the initiation codon (Hamilton et al., 1987). Sherman and Stewart (1982) have shown that the generation of out-of-frame AUG codons in the 5′ leader of the *CYC1* message potently inhibits translation of iso-1-cytochrome c. The *KAR1* gene of *S. cerevisiae* contains a long ORF preceded by an out-of-frame ATG. KAR1 protein is normally present at low levels in yeast, and its overexpression causes cell death (Rose and Fink, 1987). Perhaps ATG codons located 5′ of the site of translational initiation serve to regulate the expression of genes that encode polypeptides whose synthesis must be maintained at low levels. Unlike *KAR1*, *SEC62* carried on a high copy number 2-μm–based plasmid (YEp351) did not compromise cell growth or protein translocation (data not shown).

A polypeptide with a molecular mass of 32,381-kD and an unusually basic isoelectric point of 10.7 was predicted from the ORF (Fig. 5). Comparison of the sequence of the predicted Sec62p with proteins entered in the National Biomedical Research Foundation database (version 16.0 March 1988, 7,396 entries) using the Fastp algorithm (Lipman and Pear-

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 α-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-conditional 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 276 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 (pRD5, 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 pSEY c68 consistently yielded two live and two dead spore clones (Table IV). In contrast, tetrads derived from RPD 99 cells transformed with pRD5 most commonly yielded 3–4 viable spores (Table IV). Although spore clones bearing both the *sec62*::*HIS3* disruption and pRD5 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 pRD5 segregated Ura$^-$ clones on 5-fluoroorotic acid-containing medium (Boeke et al., 1987) at high frequency, whereas His$^+$ (*sec62*::*HIS3* chromosomal allele) colonies containing pRD5 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 RVc–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 ppαF 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 ppαF 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 ppαF and preproCPY, but not preinvertase, *sec62* microsomes fail to import preproCPY, but efficiently import

Table IV. SEC62 Is an Essential Gene

Sporulated diploid	Spore viability in tetrads (live/dead)				Segregation of *sec62::HIS3* allele (His$^+$) and Centromeric (CEN) plasmids (Ura$^+$)			
	4:0	3:1	2:2	1:3	Ura$^+$ His$^+$	Ura$^+$ His$^-$	Ura$^-$ His$^-$	Ura$^-$ His$^+$
RPD 99-101*	0	1‡	15	0	0	0	33	0
pSEYc68 in RPD 99-101§	0	0	30	0	0	38	22	0
pRD5 in RPD 99-101‖	7	13	4	0	28	29	18	0

* The results shown represent pooled data from three independent clones of W303-Leu transformed with the *sec62::HIS3* disruption allele (RPD 99-101).
‡ All three viable spores in this tetrad were His$^-$.
§ The results shown here represent pooled data from three independent clones (RPD 99-101) transformed with the *CEN4-ARS1-URA3* plasmid pSEYc68.
‖ The results shown here represent pooled data from three independent clones (RPD 99-101) transformed with a pSEYc68 vector containing the *SEC62* gene (pRD5).

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. Untraslocated 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 *sec62* S100 fraction is inefficiently glycosylated by unwashed 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 *sec62* 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 lumenal 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-

lecular mass of Sec62p is similar to two recently identified dog pancreas ER membrane proteins (SRP receptor β-subunit and mp30) that are thought to function in protein translocation (Tajima et al., 1986).

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 *SEC61* 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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