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SEC59 Encodes a Membrane Protein Required for Core Glycosylation in *Saccharomyces cerevisiae*

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When incubated at a restrictive temperature, *Saccharomyces cerevisiae sec59* mutant cells accumulate inactive and incompletely glycosylated forms of secretory proteins. Three different secretory polypeptides (invertase, pro-α-factor, and pro-carboxypeptidase Y) accumulated within a membrane-bounded organelle, presumably the endoplasmic reticulum, and resisted proteolytic degradation unless the membrane was permeabilized with detergent. Molecular cloning and DNA sequence analysis of the *SEC59* gene predicted an extremely hydrophobic protein product of 59 kilodaltons. This prediction was confirmed by reconstitution of the *sec59* defect in vitro. The α-factor precursor, which was translated in a soluble fraction from wild-type cells, was translocated into, but inefficiently glycosylated within, membranes from *sec59* mutant cells. Residual glycosylation activity of membranes of *sec59* cells was thermolabile compared with the activity of wild-type membranes. Partial restoration of glycosylation was obtained in reactions that were supplemented with mannose or GDP-mannose, but not those supplemented with other sugar nucleotides. These results were consistent with a role for the Sec59 protein in the transfer of mannose to dolichol-linked oligosaccharide.

Studies of protein localization in *Saccharomyces cerevisiae* have benefited from the isolation of conditional mutations that block protein transport at defined stages of the secretory pathway (34). The initial events, protein translocation into and glycosylation, processing, and folding within the endoplasmic reticulum, have been defined by several different genetic selection procedures. Secretory (*sec*) mutations selected on the basis of accumulation of inactive precursors of secreted proteins resulted in the identification of two genes, *SEC53* and *SEC59* (12, 13). Other selections yielded mutations that block polypeptide translocation from the cytoplasm (8, 9), mutations that interfere with core oligosaccharide synthesis and trimming (10, 17, 18), and a defect in signal peptide processing (3).

The *sec53* and *sec59* mutations cause plasma membrane, vacuolar, and secretory proteins to remain associated with the endoplasmic reticulum (ER). Initial characterization of these mutants suggested that secretory precursor polypeptides accumulate in a transmembrane orientation, consistent with a defect in the completion of translocation into the ER lumen (12). A more detailed analysis of the *sec53* phenotype and the *SEC53* gene product (Sec53p) has led to a different conclusion. Refined conditions of yeast spheroplast rupture and proteolysis of homogenates have shown that secretory polypeptides accumulate within the lumen of the ER in *sec53* cells (11). Sec53p is a 29-kilodalton protein in the cytosolic fraction (2) and has been identified as phosphomannomutase (21), the enzyme that produces mannose-1-phosphate, a direct precursor of GDP-mannose. Hence, the *sec53* phenotype results from a deficiency in the assembly of the precursor of N-linked oligosaccharide.

Core oligosaccharides are transferred to protein on the lumenal surface of the ER membrane (37). When core oligosaccharide synthesis is blocked with tunicamycin, invertase and acid phosphatase fold incorrectly and are poorly secreted (12, 27). These effects are exaggerated at elevated temperatures. This almost certainly explains the pleiotropic and temperature-sensitive nature of the *sec53* mutation.

Unlike *sec53* cells, which produce a spectrum of truncated core oligosaccharides (K. Runge, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1985), *sec59* cells produce discrete but incomplete core units (13, 20). Both mutants transfer a reduced number of oligosaccharides to secretory polypeptides (13, 20). In order to explore further the relationship between these two genes, we evaluated the nature of the *sec59* defect in vitro and the structure of the *SEC59* gene product (Sec59p). In this report we describe the results of biochemical and molecular cloning analysis that suggest that Sec59p acts within the ER membrane to facilitate glycosylation of translocated polypeptides.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and materials. *S. cerevisiae* X2180-1A (*MAT***a** *gal2*$^-$) (Yeast Genetic Stock Center, Berkeley, Calif.), X2180-1B (*MATα gal2*$^-$) (Yeast Genetic Stock Center), SF629-3C (*MATα sec59-1 suc2-Δ9 ura3-52*), MBY9-8D (*MATα sec59-1 leu2-3 leu2-112 trp1-289 his*$^-$), MBY18-4D (*MATα sec59-1 ura3-52 trp1-289 his*$^-$), and GPYD1004 (*MAT***a**/*MATα leu2-3*/*leu2-3 leu2-112*/*leu2-112 ura3-52*/*ura3-52 CAN1*/*can1 TRP1*/*trp1-289 PEP4*/*pep4-3 MNN4*/*mnn4-1*) were used in this study. Bacterial transformations were done with *Escherichia coli* SE10 (*pyrF*::Tn*5 araΔ*(*lac-pro*) *rpsL thi* [ϕ80d*lacZ*ΔM15]) (from S. Emr, California Institute of Technology, Pasadena, Calif.).

The yeast *E. coli* shuttle plasmids YEp13, YIp5, and YCp50 have been described previously (4, 6). pSEYC68 (obtained from S. Emr) is a yeast centromere plasmid which contains the yeast *URA3* gene and the multiple cloning site and α-complementation fragment from pUC18 (43). pDB31 is a yeast *E. coli* shuttle plasmid which contains a fusion of the invertase structural gene (*SUC2*) to the triose phosphate isomerase gene (*TPI*) promoter. This construct produces the secreted form of invertase constitutively (5, 7). pGEM1 (Promega Biotech, Madison, Wis.) contains a multiple clon-

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ing site flanked by the bacteriophage T7 and SP6 promoters. The yeast genomic library contains DNA fragments obtained by a partial *Sau*3a restriction digest inserted into YEp13 (28).

YPD medium contained 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% Bacto-Peptone (Difco), and 2% glucose. Minimal medium (42) was used with 2% glucose, and sulfate salts were replaced with chloride salts, with ammonium sulfate added to the desired concentration. The absorbances of dilute cell suspensions were measured in 1-cm cuvettes at 600 nm in a spectrophotometer (PMQII; Zeiss).

Other reagents were obtained as indicated. Deoxynucleoside triphosphates, NAD^+, NADPH, cytochrome *c*, trypsin, GDP-mannose, and saponin were from Sigma Chemical Co. (St. Louis, Mo.). Restriction endonucleases, exonuclease III, and DNA modification enzymes were from Bethesda Research Laboratories (Gaithersburg, Md.). Dideoxynucleoside triphosphates were from P-L Biochemicals (Milwaukee, Wis.). Proteinase K and S1 nuclease were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). T7 and SP6 RNA polymerases were from Promega Biotech. Protein A-Sepharose was from Pharmacia Fine Chemicals (Piscataway, N.J.). [α-^{35}S]dCTP (1,200 Ci/mmol), [α-^{32}P]dATP (800 Ci/mmol), [^{35}S]methionine, and Amplify were from Amersham Corp. (Arlington Heights, Ill.). Nitrocellulose was from Schleicher & Schuell Inc. (Keene, N.H.). Oligo(dT)-cellulose was from Collaborative Research, Inc. (Waltham, Mass.). Carrier-free [^{35}S]Na_2SO_4 was from ICN Radiochemicals (Irvine, Calif.). Preparations of lyticase (35), anti-invertase antiserum (33), and anti-carboxypeptidase Y (anti-CPY) antiserum (38) have been described previously. Anti-α-factor antibody reacted with both the prepropeptide and mature regions of the α-factor precursor molecule (31).

Cloning and DNA methods. *SEC59* was cloned by transformation of MBY9-8D with the yeast genomic library that was constructed in YEp13. Plasmids which enabled MBY9-8D to grow at 37°C (restrictive temperature) in the absence of leucine were selected and isolated as described previously (2). Total yeast RNA and poly(A)$^+$ RNA isolation procedures have been described previously (2, 26). Standard techniques for plasmid isolation, agarose gel electrophoresis, transformation of *E. coli* and *S. cerevisiae*, transfer of RNA to nitrocellulose filters, and preparation and hybridization of nick-translated probes to nitrocellulose filters have been described elsewhere (19, 26, 36). DNA sequencing was performed by the dideoxy chain-termination method (32). Nested deletions were generated by the action of *E. coli* exonuclease III and S1 nuclease (15). Both strands of *SEC59* were sequenced entirely.

Radiolabeling, proteolysis of *sec59* extracts, and immune precipitation. Strain SF629-3C containing plasmid pDB31 was grown to an optical density at 600 nm (OD_{600}) of 0.5 at 24°C in minimal medium containing 2% glucose and 0.1 mM $(NH_4)_2SO_4$. Cells (20 OD_{600} units) were sedimented, washed in medium without sulfate, suspended at 10 OD_{600} units per ml in medium containing 0.01 mM $(NH_4)_2SO_4$, and placed at 37°C to impose the *sec59* block. After 30 min, $Na_2{}^{35}SO_4$ (2 mCi) was added and incubation was continued for 15 min. NaN_3 was added to 10 mM and incubation was continued for 5 min. Cells were centrifuged at 22°C, suspended in 5 ml of 0.1 M Tris sulfate (pH 9.4)–30 mM 2-mercaptoethanol–10 mM NaN_3, and incubated for 15 min at 22°C. Treated cells were sedimented and suspended in 0.6 ml of spheroplast buffer (1.4 M sorbitol, 25 mM Tris hydrochloride [pH 7.5], 2 mM $MgCl_2$, 5 mM NaN_3) containing 30 mM 2-mercaptoethanol. Lyticase (10 U of lyticase/OD_{600} U of cells) was used to convert cells to spheroplasts during a 20-min incubation period at 30°C. The sample was then chilled on ice and spheroplasts were collected by centrifugation through a cushion of spheroplast buffer (containing 1.9 M sorbitol), at 8,000 × *g* (HB-4 rotor; Dupont/Sorvall) for 5 min at 4°C.

To prepare cell extracts, spheroplasts were suspended in 1 ml of 0.7 M mannitol–50 mM Tris hydrochloride (pH 7.5)–0.1 M KCl–1 mM EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and homogenized in three 1-min bursts at 0°C in a 2-ml tissue grinder (Potter-Elvehjem) fitted with a Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) pestle. For proteolysis, the extract was divided, one portion was adjusted to 0.2% saponin, a protease mixture was added to both samples (final concentrations of 0.2 mg of proteinase K per ml and 0.1 mg of trypsin per ml), and incubations were continued on ice. At various time points portions were removed, and reactions were terminated by the addition of trichloroacetic acid to 15% (wt/vol). Samples were left on ice for 15 min, followed by centrifugation for 10 min in a microcentrifuge (Fisher Scientific Co., Pittsburgh, Pa.) at room temperature. Supernatant fractions were removed, and precipitated material was washed with 0.5 ml of 5% trichloroacetic acid. Pellets were solubilized by heating them for 5 min at 95°C in 4% sodium dodecyl sulfate (SDS)–0.125 M Tris (unbuffered)–10% glycerol. This was followed by agitation with a vortex mixer.

Resolubilized extracts were diluted to 1 ml with phosphate-buffered saline (12.5 mM sodium phosphate [pH 7.4], 0.2 M NaCl) containing 1.0% Triton X-100 and 0.1% SDS, and portions were removed for immune precipitation. Immune precipitations and washing of immune complexes coupled to protein A-Sepharose were performed as described previously (9). Pro-α-factor and CPY immune precipitations contained 0.4 OD_{600} U of cell equivalent of radiolabeled material per proteolyzed extract, while immune precipitations of invertase contained 0.1 OD_{600} U of cell equivalent of radiolabeled material. Immune precipitations were performed in the presence of a nonradioactive yeast cell extract made from cells (SEY2108) that contained no invertase, CPY, or prepro-α-factor cross-reacting material (final concentration, 1 mg of protein per ml) (1). Samples were subjected to two sequential rounds of immune precipitation prior to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% (for invertase and CPY) or 13.8% (for pro-α-factor) polyacrylamide gels. Following electrophoresis, gels were fixed and treated with Amplify, and proteins were visualized by fluorography at −85°C.

Other assays and procedures. Methods for SDS-PAGE (24) and assay of NADPH cytochrome *c* reductase activity (22) have been described previously. Hydropathy analysis was done by the program of Kyte and Doolittle (23), with a window of 10 amino acids. Comparison between the deduced *SEC59* amino acid sequence and the protein data base of the National Biomedical Research Foundation (National Institutes of Health) was done with the FASTP program (25). Generation of ^{32}P-labeled RNA probes with SP6 and T7 RNA polymerase was done by the method provided by the supplier (Promega Biotech). Methods for the preparation of mutant and wild-type S-100 protein synthesis lysates and translocation-competent mutant and wild-type membranes and in vitro translation and translocation of prepro-α-factor have been described elsewhere (21).

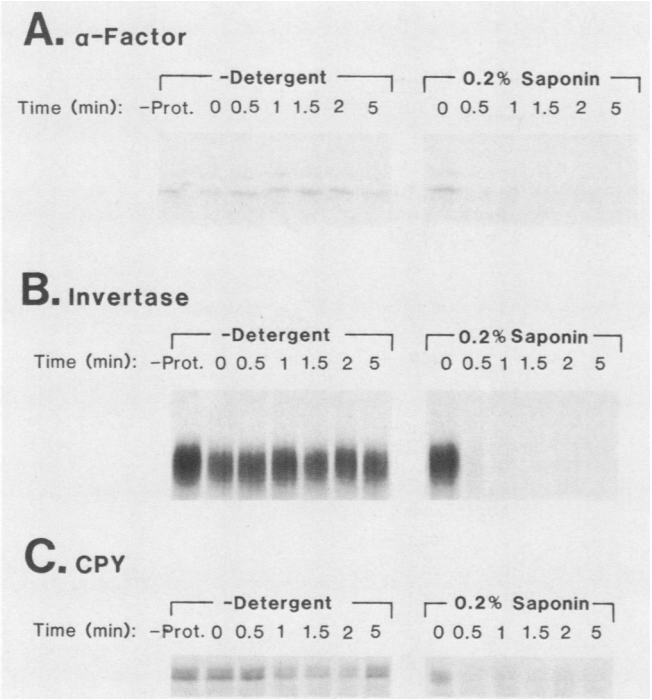

FIG. 1. Proteolysis of a *sec59* lysate. Strain SF629-3C(pDB31) (*sec59*) was radiolabeled in vivo at 37°C with $[^{35}S]SO_4^{2-}$. Cells were converted to spheroplasts and lysed. The lysate was subjected to proteolysis in the absence or presence of 0.2% saponin. At the indicated times, portions were removed and proteolysis was terminated by the addition of trichloroacetic acid. Samples were split and subjected to immune precipitation with antibody directed against α-factor precursor (A), invertase (B), or CPY (C), followed by SDS-PAGE and fluorography. The α-factor and CPY immune precipitations contained 0.4 OD_{600} unit of cell equivalent radiolabeled extract; invertase immune precipitations contained 0.1 OD_{600} unit of cell equivalent of radiolabeled extract.

RESULTS

Secretory proteins accumulate in a membrane-enclosed form in *sec59*. The initial evaluation of the orientation of secretory precursor proteins that accumulated in *sec53* and *sec59* membranes suggested a significant exposure of incompletely glycosylated invertase on the cytoplasmic surface of the ER (12). However, the conditions of proteolysis that were used to draw this conclusion have since been shown to be incompatible with ER organelle integrity (11). Using refined conditions of spheroplast lysis and protease treatment, we judged secretory polypeptides to be completely sequestered within the ER lumen in *sec53* lysates (11).

We reexamined the location of three glycoprotein precursors that accumulated in *sec59* using the new conditions of membrane preparation. Cells of strain SF629-3C(pDB31) were transferred to 37°C for 30 min to impose the *sec59* block and then radiolabeled for 15 min with $[^{35}S]SO_4^{2-}$. Under these conditions pro-α-factor, invertase, and proCPY accumulate with variable numbers of N-linked oligosaccharide side chains (13, 20). Radiolabeled cells were converted to spheroplasts and lysed by homogenization, and membranes were exposed to a mixture of proteinase K-trypsin in the presence or absence of the nonionic detergent saponin. Figure 1 shows SDS-PAGE of pro-α-factor (Fig. 1A), invertase (Fig. 1B), and proCPY (Fig. 1C) immunoprecipitated from the protease-treated lysates. In the absence of detergent, all three proteins remained intact, while the addition of saponin allowed all three proteins to be degraded. These results indicate that secretory polypeptides are sequestered within a membrane-bounded compartment, most likely the ER. Thus, the defect in *sec59* must follow completion of polypeptide translocation into the ER lumen.

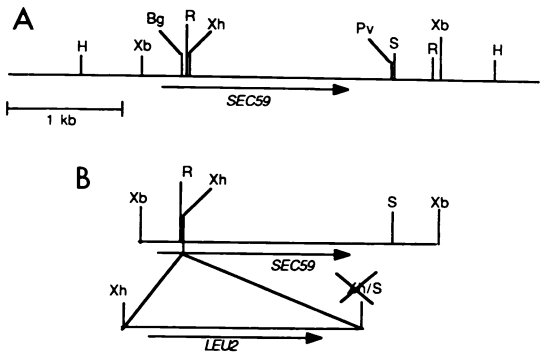

FIG. 2. Restriction map of *SEC59*. (A) Restriction map of the *SEC59* genomic region. (B) Restriction map of a *sec59* insertion mutation. A 2.0-kb *Xho*I-*Sal*I restriction fragment containing the yeast *LEU2* gene was inserted into the *Xho*I site within the *SEC59*-coding region. The *Xho*I site 5′ of *LEU2* was preserved, but the *Sal*I site immediately 3′ of *LEU2* was destroyed. Restriction site abbreviations: H, *Hin*dIII; Xb, *Xba*I; Bg, *Bgl*II; R, *Eco*RI; Xh, *Xho*I; Pv, *Pvu*II; S, *Sal*I. Arrows indicate the orientations and approximate limits of the putative *SEC59*-coding region and *LEU2*.

An alternate interpretation of this result is that the conditions of lysis produced a multilamellar structure that occluded the cytoplasmic surface of the ER. This possibility was tested by assaying the accessibility of NADPH cytochrome *c* reductase, an integral, cytoplasmically oriented ER marker protein (22), to its substrate cytochrome *c*. No latency of this enzyme was detected in assays that were performed in the presence or absence of detergent. Hence, the ER membrane cytoplasmic surface is accessible to exogenous proteins such as trypsin.

Cloning of *SEC59*. We cloned *SEC59* in an effort to reveal, through analysis of the sequence of the gene product, the most likely intracellular location of Sec59p. MBY9-8D (*sec59 leu2*) was transformed with a yeast genomic DNA library, and transformants were selected for leucine prototrophy and growth at 37°C. Approximately 15,000 Leu^+ colonies were obtained, of which 40 were also Ts^+. Plasmid DNAs isolated from four random Ts^+ colonies were propagated in *E. coli* and reintroduced into MBY9-8D to confirm that single plasmids conferred both the Leu^+ and Ts^+ phenotypes. Restriction enzyme mapping showed that the plasmids contained overlapping yeast DNA inserts in both orientations with respect to the YEp13 vector. The restriction map of one clone that contained a 4.5-kilobase (kb) insert is shown in Fig. 2A.

Genetic analysis was used to prove that the complementing genomic fragment contained the authentic *SEC59* gene. A 2.5-kb *Hin*dIII-*Sal*I fragment that complemented *sec59* was inserted into the yeast integrating plasmid YIp5, yielding pSEC5929. An *Xba*I site within the genomic insert was cleaved, yielding linear DNA which was introduced into MBY18-4D (*sec59 ura3*). All stable Ura^+ transformants were Ts^+. One such transformant was mated to another *sec59 ura3* strain, and progeny haploid spores were evaluated by tetrad analysis. Among 20 tetrads, the Ura^+:Ura^- and Ts^+:Ts^- phenotypes segregated 2:2, with all Ts^+ progeny also being Ura^+. This result demonstrates that pSEC5929 integrates intact at a single locus. When the

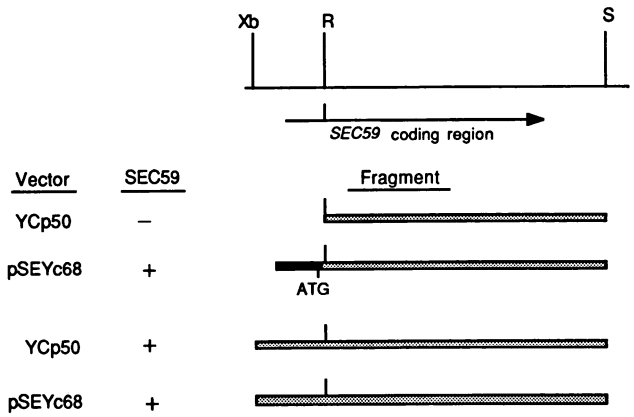

FIG. 3. Complementation of *sec59-1*. Fragments of the *SEC59* genomic region were subcloned into the vector YCp50 or the β-galactosidase α-complementation-based vector pSEYc68, and the ability of each plasmid to complement the *sec59-1* mutation was tested. Stippled bars are *SEC59* restriction fragments subcloned into the indicated vector; the solid bar indicates the β-galactosidase α-complementation fragment of pSEYc68; ATG represents the initiation codon of the α-complementation fragment; vertical lines above the fragments represent the *Eco*RI site that is present in the *SEC59*-coding region. Restriction site abbreviations: Xb, *Xba*I; R, *Eco*RI; S, *Sal*I.

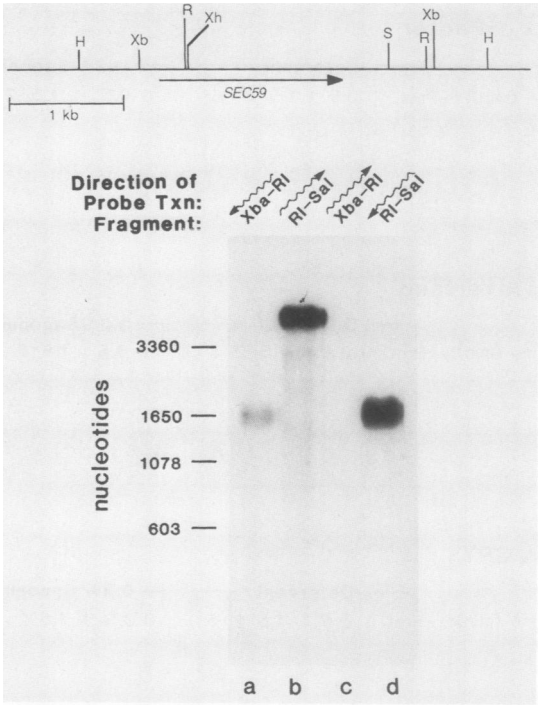

FIG. 4. *SEC59* RNA analysis. Poly(A)$^+$ RNA (15 μg per lane) was subjected to electrophoresis under denaturing conditions on 1.2% agarose gels and transferred to nitrocellulose. Lanes were probed with strand-specific probes obtained by cloning the indicated restriction fragments into the plasmid pGEM1, followed by transcription with either T7 or SP6 RNA polymerase. Restriction site abbreviations: H, *Hin*dIII; Xb, *Xba*I; R or RI, *Eco*RI; Xh, *Xho*I; S, *Sal*I.

original Ura$^+$ Ts$^+$ transformant was crossed to a *SEC59 URA3* strain, all the progeny of 40 tetrads were Ts$^+$ and one-quarter of the progeny were Ura$^-$. These data demonstrate that pSEC5929 directs integration at the *SEC59* locus and not at the *URA3* locus, and establish that the insert contains the authentic *SEC59* gene.

Further subcloning of the insert established the limits and orientation of the *SEC59* gene. The 2.1-kb *Xba*I-*Sal*I fragment cloned into the single-copy vectors pSEYC68 or YCp50 complemented *sec59-1* (Fig. 3). However, the 1.75-kb *Eco*RI-*Sal*I fragment complemented *sec59-1* only when the fragment was cloned in pSEYC68. Further examination of the *SEC59* sequence indicated that this fragment was missing the promoter and N-terminal coding region (see below), resulting in vector-dependent complementation.

Analysis of *SEC59* mRNA was used to establish the direction of transcription. Subclones of the *Xba*I-*Eco*RI and *Eco*RI-*Sal*I fragments inserted into transcription vectors were used to generate radiolabeled, single-stranded RNA probes. Hybridization of electrophoretically resolved poly(A)$^+$ RNA showed that *SEC59* was represented in a single mRNA of ~1,650 nucleotides that was initiated in the 0.35-kb *Xba*I-*Eco*RI restriction fragment and extended into the 1.75-kb *Eco*RI-*Sal*I restriction fragment (Fig. 4). A second mRNA, which was transcribed from the opposite strand, extended into the *Eco*RI-*Sal*I fragment. At most only about half of this transcript (>3,400 nucleotides) could be accommodated in the *sec59*-complementing fragment.

SEC59* is required for spore germination.** The conditional lethality of *sec59-1* could be caused by a thermosensitive *sec59* gene product or a thermosensitive process uncovered by a null allele of *sec59*. These possibilities were distinguished by tetrad analysis of a diploid strain carrying a disruption of one *SEC59* locus (29). A 2.0-kb *Xho*I-*Sal*I fragment containing the *LEU2* gene was cloned into the *Xho*I site within the *SEC59* gene (Fig. 2B). The enlarged (4.1-kb) *Xba*I-*Sal*I fragment was excised and introduced by transformation into strain GPYD1004. Disruption of a *SEC59* locus was selected by formation of Leu$^+$ transformants. Integration at the correct locus was confirmed by Southern hybridization analysis of DNA isolated from diploid transformants (data not shown). A Leu$^+$ transformant was sporulated, and each of 43 tetrads produced two viable spores, each of which was Leu$^-$ and contained an intact *SEC59* locus, as judged by genomic Southern hybridization. Other markers in the cross (*MATa**/*MATα can1*/*CAN1 trp1*/*TRP1*) were unaffected and segregated independent of the lethal phenotype. We conclude that a null allele of *sec59* is lethal; the phenotype of the *sec59-1* mutant most likely derives from a thermosensitive Sec59p.

Sequence analysis of *SEC59*. Both strands of the *sec59-1*-complementing 2.1-kb *Xba*I-*Sal*I restriction fragment were sequenced. A single open reading frame of 1,557 nucleotides (519 codons) that was uninterrupted by introns was predicted to encode a protein of 58,916 daltons (Fig. 5). The size and orientation of the open reading frame was consistent with the characteristics of *SEC59* mRNA. Furthermore, a gene fusion in which *lacZ* was introduced 5′ to the *Xho*I site in *SEC59* was expressed in *E. coli* and generated a hybrid protein of the expected size (165 kilodaltons) and reading frame (data not shown).

The authentic 5′ end of *SEC59* mRNA, which was determined by primer extension analysis, showed major transcripts initiated at nucleotide positions −24 to −57 with respect to the first start codon (data not shown). Other potential start codons at positions 70 and 100 were noted.

Translation initiation at the first start codon was not essential for expression of functional Sec59p. The *Eco*RI-*Sal*I fragment that complemented *sec59-1* when it was

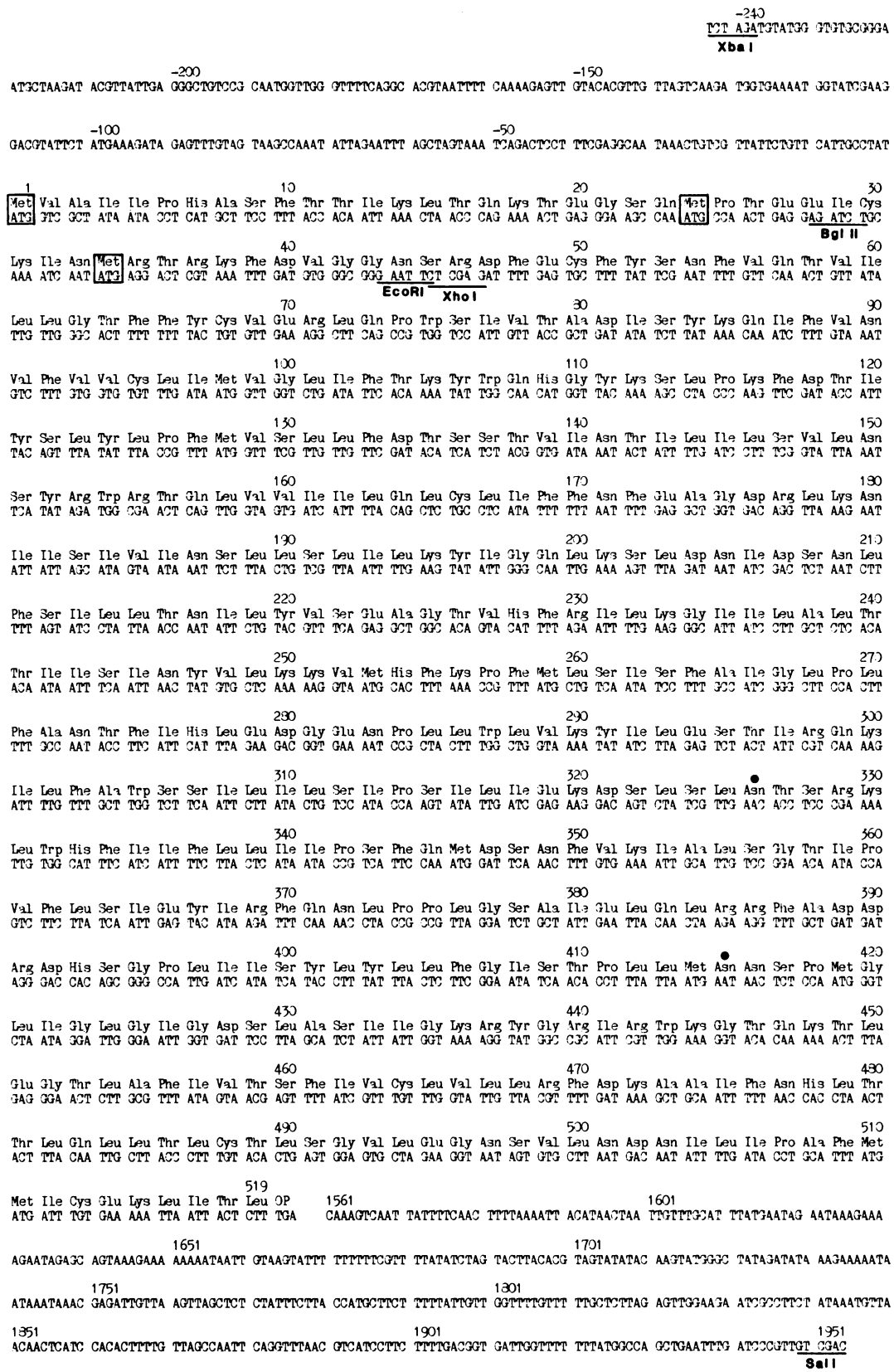

FIG. 5. *SEC59* gene sequence. Numbers above the nucleotides refer to their positions relative to the A of the first in-frame initiation codon of *SEC59*. Numbers above amino acids refer to positions relative to the first AUG codon of the *SEC59* open reading frame. Possible initiation codons are boxed; underlined sequences correspond to the indicated restriction sites; possible N-glycosylation sites are indicated by dots over the asparagine attachment site. The translated molecular weight was 58,915.81.

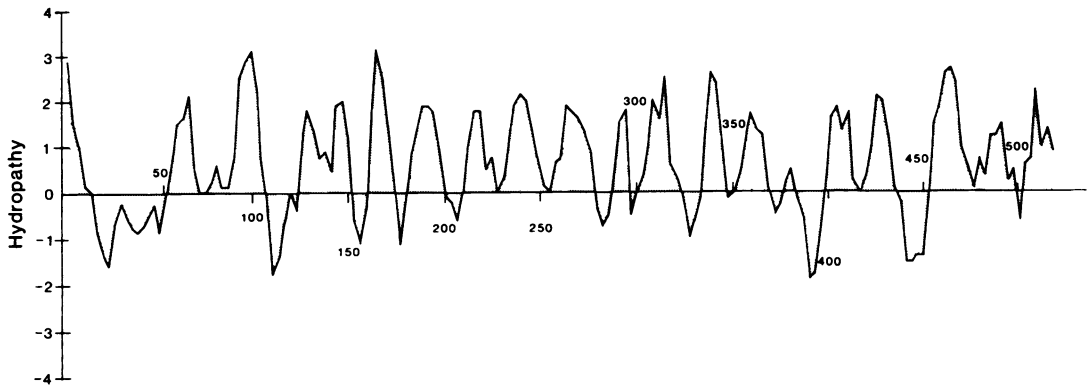

FIG. 6. Hydropathy analysis of *SEC59*. Hydropathy analysis was performed by using the program of Kyte and Doolittle (23), with a window value of 10 amino acids. Numbers represent amino acids.

cloned into pSEYC68 (Fig. 3) contained only a portion of *SEC59*: 44 amino acids (8.5% of the *SEC59*-coding region) were represented between the *Eco*RI site and the putative start codon (Fig. 5). Apparently, the cloning vector pSEYC68 provided a promoter and start codon which functioned to express a partly functional Sec59p fragment that was missing the N-terminal 8.5% of the coding sequence.

Hydropathic analysis of the putative Sec59p was performed (Fig. 6). According to the predictions of Kyte and Doolittle (23), this protein would start with a hydrophilic N terminus of about 55 amino acids, but would follow with extremely hydrophobic regions making up 90% of the molecule. The hydrophobic domains ranged from 12 to 20 amino acids in length and were punctuated by clusters of charged residues. Two potential N-glycosylation sites were detected at amino acids 326 and 415. Neither the hydrophilic N-terminal region nor the sequences immediately following the second and third AUG codons resembled a secretory signal peptide. Finally, no significant protein sequence homology was found between Sec59p and any other sequence in the data base of the National Biomedical Research Foundation, National Institutes of Health.

Defective glycosylation of pro-α-factor by *sec59* membranes in vitro. The predicted hydrophobic character of Sec59p suggested that the protein might reside and function in the ER membrane. To test this prediction we used a cell-free assay that measures translocation and glycosylation of α-factor precursor (14, 31, 40). Membrane and soluble S-100 fractions were prepared from wild-type and *sec59-1* cells grown at a permissive temperature (24°C). [^{35}S]methionine-labeled prepro-α-factor was synthesized in mutant and wild-type S-100 fractions during a 20-min incubation at 20°C. After the addition of cycloheximide to terminate protein synthesis, translation products were mixed with wild-type or mutant membranes and further incubated for 60 min at 20°C. Portions of translocation reaction mixtures were then treated with or without proteinase K at 0°C in the presence or absence of 0.2% saponin. In a typical reaction with wild-type components, four species that resisted proteinase K digestion, and that therefore were sequestered within the ER, were detected (Fig. 7, lanes 1 to 3). These corresponded to precursor forms bearing 0, 1, 2, or 3 core oligosaccharides (31). The translocated but unglycosylated form (indicated as 0) migrated more slowly than the primary translation product. This form represents signal peptide-processed pro-α-factor (41). A similar spectrum of translocated species was seen when the α-factor precursor, which was made in a *sec59* S-100 fraction, was translocated into wild-type membranes (Fig. 7, lanes 7 to 9). A different pattern of translocated species appeared when mutant membranes were used either with wild-type (lanes 4 to 6) or mutant S-100 (lanes 10 to 12) fractions. Material migrating faster than the singly or doubly glycosylated precursor predominated; no triplyglycosylated species was produced. On the basis of the altered electrophoretic mobility, the glycosylated forms may contain incomplete core oligosaccharide units. In contrast, as judged by protection against proteinase K, the extent of precursor sequestered within vesicles was not decreased in *sec59* membranes. Similar results were obtained in experiments in which the α-factor precursor was synthesized in the presence of membranes (data not shown). This phenotype reproduces that seen in *sec59* cells radiolabeled at 37°C (20). We interpreted these results to mean that the *sec59* mutation primarily affects the process of glycosylation by the membrane fraction.

A direct effect of the *sec59* mutation on the membrane fraction was established by examining the thermolability of mutant and wild-type translocation and glycosylation activities. Separate membrane fractions were incubated at 37°C, and portions were withdrawn at intervals for assay of the translocation and glycosylation activity at 20°C by using the α-factor precursor made in a wild-type S-100 fraction. Production of total translocated species, as judged by the sum of

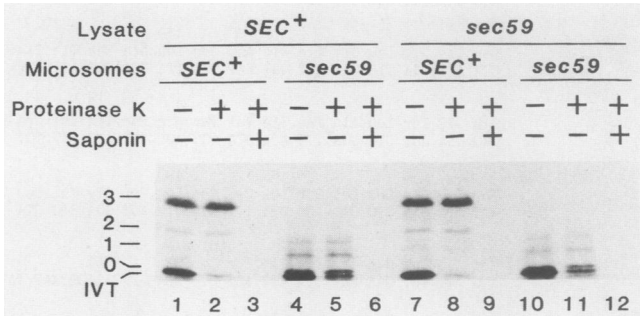

FIG. 7. Posttranslational translocation of prepro-α-factor in a combination of wild-type or *sec59-1* extracts. Translations were performed either in a wild-type- (lanes 1 to 6) or in a mutant (lanes 7 to 12)-derived lysate and were terminated with cycloheximide. Translocation reactions were done with either wild-type- (lanes 1 to 3 and 7 to 9) or mutant (lanes 4 to 6 and 10 to 12)-derived microsomes. After incubation, all reaction products were incubated in the presence or absence of proteinase K, with or without saponin. Species of prepro-α-factor: IVT, In vitro translation product; 0 to 3, translocated forms bearing 0 to 3 core sugars, respectively.

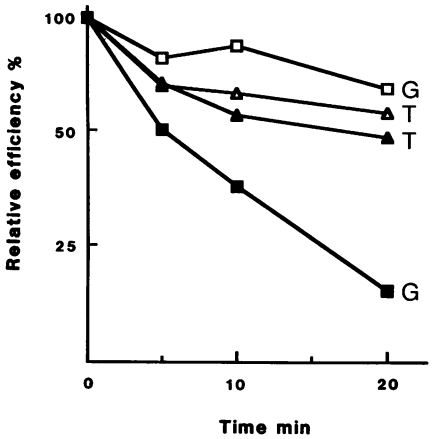

FIG. 8. Thermosensitivity of yeast microsomes for translocation and glycosylation of prepro-α-factor. Either wild-type- (open symbols) or *sec59-1* (closed symbols)-derived microsomes were incubated at 37°C for various times and were then assayed at 20°C in the presence of translation products made in a wild-type lysate. After the autoradiogram was scanned, the sum of all protease-protected species (0 to 3) was taken as an index of translocation (T) (triangles), and the sum of the 3 core glycosylated species was taken as an index of glycosylation (G) (squares). The relative indices are plotted here versus the incubation time on a semilogarithmic scale.

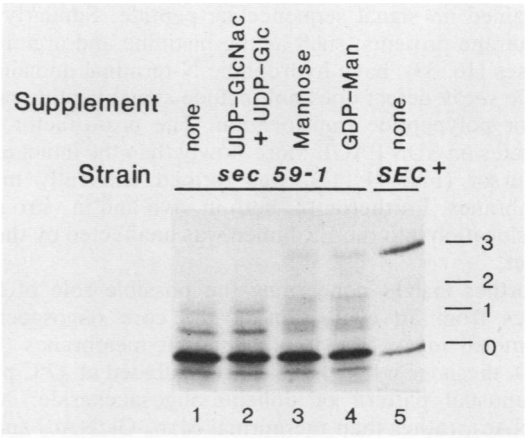

FIG. 9. In vitro complementation of the *sec59* glycosylation defect by sugar derivatives. Translation of prepro-α-factor was performed in a wild-type lysate and was terminated with cycloheximide. Translocation reactions were performed with mutant or wild-type membranes in the presence of 0.1 mM GTP with the indicated supplements (UDP-*N*-acetylglucosamine [UDP-GlcNac], UDP-glucose [UDP-Glc], or GDP-mannose [GDP-Man], 10 μM, or mannose, 100 μM). Similar results were obtained when prepro-α-factor was translated in a *sec59* lysate (data not shown). Incubations containing *sec59* membranes were treated with proteinase K after completion of the translocation reaction. The numbers 0 to 3 indicate translocated forms of α-factor precursor bearing 0 to 3 core oligosaccharides, respectively.

the protease-protected forms, decayed similarly in mutant and wild-type membranes (Fig. 8). On the other hand, the production of core-glycosylated material (forms 1 to 3) decayed significantly faster in *sec59* versus that in wild-type membranes. Parallel incubations of mutant and wild-type soluble S-100 fractions at 37°C revealed no significant difference in thermosensitivity when assayed with wild-type membranes at 20°C to measure translocation and glycosylation (data not shown).

We have shown previously that the addition of 1 μM GDP-mannose fully restores the formation of normal core-glycosylated α-factor precursor in lysates of *sec53* cells (21). The glycosylation activity of *sec59* extracts was stimulated by the addition of GDP-mannose (10 μM) or mannose (100 μM) but not by UDP–N-acetylglucosamine or UDP-glucose (Fig. 9). Although the effect was partial, it resulted in the formation of a small amount of triply core-glycosylated pro-α-factor and in the conversion of singly and doubly glycosylated precursors to forms that migrated along with the corresponding forms produced by wild-type membranes. No enhanced formation of triply core-glycosylated pro-α-factor was seen with wild-type membranes supplemented with sugar nucleotides (21). These results suggest that Sec59p may participate in mannose transfer to core oligosaccharide rather than in sugar nucleotide synthesis.

DISCUSSION

The block in secretion in *sec59* mutant cells followed polypeptide translocation into the ER lumen. Underglycosylated forms of invertase, pro-α-factor, and proCPY accumulated, apparently in an improperly folded form. The blocks produced by the *sec53* mutation and by the treatment of wild-type cells with tunicamycin superficially resembled the *sec59* lesion. Based on further characterization of the *SEC59* gene product (Sec59p) and the behavior of membranes isolated from *sec59* mutant cells, we suggest that Sec59p acts either directly or indirectly in the biosynthesis of dolichol-linked core oligosaccharides.

Two observations direct attention to the ER membrane as the locus of Sec59p function. First, the forms and location in which secretory polypeptides accumulated in *sec59* cells, coupled with the putative hydrophobic character of Sec59p, suggest that Sec59p is an ER membrane protein that facilitates glycosylation and folding of translocated polypeptides. Second, the mutant phenotype was reproduced in an in vitro translocation and glycosylation reaction when mutant membranes were incubated with the α-factor precursor made in a wild-type soluble fraction.

Localization of the putative *SEC59* gene product to the membrane was inferred from the sequence of the gene. The complete *SEC59* gene and a fragment that was missing the hydrophobic N-terminal 8.5% of the coding sequence were capable of restoring growth at 37°C to *sec59-1* cells. Translation of *SEC59* from the first AUG codon would produce a 59-kilodalton polypeptide. The C-terminal 90% of Sec59p was predicted to contain many short stretches of hydrophobic amino acids separated by clusters of charged and hydrophilic peptides. Most of the hydrophobic stretches were of insufficient length (<17 amino acids) to span a membrane in the form of an α helix. Alternative structures or a limited tolerance for charged residues in the membrane may allow Sec59p to be almost entirely embedded within the bilayer.

Complementation of *sec59-1* by a gene fragment that was missing 5'-regulatory and -coding sequences deserves special comment. Vector (pSEYC68) sequences must allow expression of the *SEC59* fragment. The *E. coli lac* promoter is known to function in *S. cerevisiae* (S. Emr, personal communication), and the start codon of the β-galactosidase α-complementation fragment of pSEYC68 is fused in-frame to the *SEC59*-coding region at the *Eco*RI site in the subclone. Complementation by this gene fragment may result from production of a functional protein, the combined activity of the fragment and a crippled but possibly not inert *sec59-1* mutant protein, or both. The dispensable N-terminal region

contained no signal sequencelike peptide. Similarly, other membrane proteins, such as the histidine and arginine permeases (16, 39), have hydrophilic N-terminal domains.

The *sec59* defect does not include signal peptide processing or polypeptide translocation. The pro-α-factor, which migrates on SDS-PAGE more slowly than the intact α-factor precursor (Fig. 7) (41), was formed efficiently in *sec59* membranes. Furthermore, both in vivo and in vitro protein translocation into the ER lumen was unaffected by the *sec59* defect.

Further insight concerning the possible role of Sec59p comes from an evaluation of the core oligosaccharides assembled in *sec59* cells and mutant membranes (Runge, Ph.D. thesis) (Fig. 7). *sec59* cells incubated at 37°C produce an unusual pattern of dolichol-oligosaccharide: Man_{5-6}-$GlcNAc_2$, rather than the normal Man_9-$GlcNAc_2$ and Glc_3-Man_9-$GlcNAc_2$, where Man is mannose, GlcNAc is *N*-acetylglucosamine, and Glc is glucose (18; Runge, Ph.D. thesis). These truncated oligosaccharides may account for the anomalous increased electrophoretic mobility of singly and doubly glycosylated pro-α-factor translocated into mutant membranes in vitro (Fig. 7; compare lanes 1, 2, 7, and 8 with lanes 4, 5, 10, and 11, respectively). Core-glycosylated forms of invertase and pro-α-factor that accumulate in *sec59* are sensitive to endoglycosidase H (13, 20). Thus, the transferred oligosaccharide must be different from the Man_5-$GlcNAc_2$ heptasaccharide biosynthetic intermediate which, when transferred to protein (e.g., in the *alg3* mutant [18]), cannot be removed by endoglycosidase H. In this regard the *sec59* phenotype resembles that produced when MDCK tissue culture cells are treated with mannosamine, which inhibits an α1→2 mannosyltransferase that operates in core oligosaccharide synthesis, leading to aberrant Man_{5-6}-$GlcNAc_2$ species that are sensitive to endoglycosidase H (30). On the other hand, neither mannosamine treatment nor the *alg3* mutation compromise cell growth.

The *sec59* block also results in the transfer of a reduced number of oligosaccharides per secretory polypeptide (13, 20). This defect may arise from a reduction in the amount of available oligosaccharide or in the rate of its transfer to protein. Indirect evidence suggests that the oligosaccharide biosynthetic precursors GDP-mannose and dolichol-P-mannose are not limiting in *sec59* extracts. Formation of fully glycosylated pro-α-factor is only partly restored by the addition of GDP-mannose to an in vitro translocation and glycosylation reaction consisting of *sec59* membranes and wild-type cytosol. Dolichol-phosphate-mannose synthesis, which is required to complete core oligosaccharide assembly from the heptasaccharide intermediate, is normal in *sec59* membranes (L. Lehle, personal communication).

A defect in one of the mannosyl transferases involved in assembling the dolichol-pyrophosphate-$GlcNAc_2$-Man_5 intermediate on the cytoplasmic surface of the ER may explain the effect of *sec59*. These enzymes, like Sec59p, are integral membrane proteins. An increase in the concentration of the substrate GDP-mannose could enhance the activity of a mutant transferase. In the absence of the proper addition of an α-linked mannose, improper growth of the core may result in an aberrant oligosaccharide that is transferred inefficiently to protein. Retarded transfer of shortened core oligosaccharide to protein has been seen in certain *alg* mutants (18). Alternatively, the glycosylation defect may be secondary to some other problem with the ER membrane structure and function that accounts for the lethality of the *sec59-1* mutation. Further characterization of the structure of the core oligosaccharide produced in *sec59* may define the exact function of Sec59p.

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