Characterization of a Gene Product (Sec53p) Required for Protein Assembly in the Yeast Endoplasmic Reticulum

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ABSTRACT *SEC53*, a gene that is required for completion of assembly of proteins in the endoplasmic reticulum in yeast, has been cloned, sequenced, and the product localized by cell fractionation. Complementation of a *sec53* mutation is achieved with unique plasmids from genomic or cDNA expression banks. These inserts contain the authentic gene, a cloned copy of which integrates at the *sec53* locus. An open reading frame in the insert predicts a 29-kD protein with no significant hydrophobic character. This prediction is confirmed by detection of a 28-kD protein overproduced in cells that carry *SEC53* on a multicopy plasmid. To follow Sec53p more directly, a *LacZ-SEC53* gene fusion has been constructed which allows the isolation of a hybrid protein for use in production of antibody. With such an antibody, quantitative immune decoration has shown that the *sec53-6* mutation decreases the level of Sec53p at 37°C, while levels comparable to wild-type are seen at 24°C. An eightfold overproduction of Sec53p accompanies transformation of cells with a multicopy plasmid containing *SEC53*. Cell fractionation, performed with conditions that preserve the lumenal content of the endoplasmic reticulum (ER), shows Sec53p highly enriched in the cytosol fraction. We suggest that Sec53p acts indirectly to facilitate assembly in the ER, possibly by interacting with a stable ER component, or by providing a small molecule, other than an oligosaccharide precursor, necessary for the assembly event.

The secretory pathway in yeast has been defined genetically by a series of temperature-sensitive mutant strains blocked at various stages in protein transport (1, 2). Class B *sec* mutants (*sec53* and *sec59*) represent the earliest block we have detected (3). At the nonpermissive temperature (37°C), *sec53* mutant cells accumulate precursors of secreted proteins (such as invertase) and vacuolar proteins (such as carboxypeptidase Y) firmly associated with the endoplasmic reticulum (ER)[1] membrane (4). Although mutant cells accumulate full-length invertase polypeptide at 37°C, normal core glycosylation and folding to an enzymatically active configuration are blocked. On return to a permissive temperature (24°C), precursor invertase is glycosylated, restored to an active form, and secreted. Hence, the *sec53* block may represent an authentic intermediate step in the process of assembly in the ER. These characteristics are unlike the phenotype expected for mutations in subunits of the signal recognition particle or docking protein, which would result in accumulation of truncated or complete secretory polypeptides in the cytoplasm.

On the basis of these and other results, we proposed that the *SEC53* gene product acts directly to facilitate the complete penetration of polypeptides into the ER lumen. According to this view, the *SEC53* gene product (Sec53p) would either be an integral component of the ER membrane, or peripherally associated on the lumenal surface of the membrane. To test this hypothesis, we have identified Sec53p by the application of molecular cloning techniques. In contrast to our expectation, we report here that Sec53p is a hydrophilic, cytoplasmic protein.

MATERIALS AND METHODS

Strains, Plasmids, Growth Conditions, and Materials: The bacterial and yeast strains used in this study are listed in Table I.

Escherichia coli plasmids pUC9 and pUR290, used to make carboxyl terminal fusions to β-galactosidase, have been described by others (7, 8). The

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; PCI, phenol/chloroform/isoamyl alcohol (25:24:1); TBS, Tris-buffered saline (50 mM Tris-HCl, pH 8.0, 150 mM NaCl).

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TABLE I. *Strains*

Strain	Genotype	Source or reference
S. cerevisiae		
X2180-1B	*MATα gal2⁻*	YGSC*
MBY7-5C	*MATα sec53-6 trp1-289 leu2-3,112 ura3-52 his⁻*	This study
MBY21	*MATα/MATα SEC53/sec53-6 CAN1/can1 trp1-289/trp1-289 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his⁻/his⁻*	This study
HMSF 176	*MATα sec18-1*	reference 1
JRY9	*MATα trp1-289 leu2-3,112 ura3-52 his3⁻ his4⁻*	J. Rine
E. coli		
MC 1061	*F⁻ araD139 Δ(araABOIC-leu)7679 ΔlacX74 galU galK rpsL hsdR*	reference 5
MC 1061[chr::Tn5]	MC 1061 containing a chromosomal Tn5 insertion	R. Foster and J. Rine
BMH 71-18	*Δ(lac pro) F' lacI^qZΔM15 pro⁺*	reference 6

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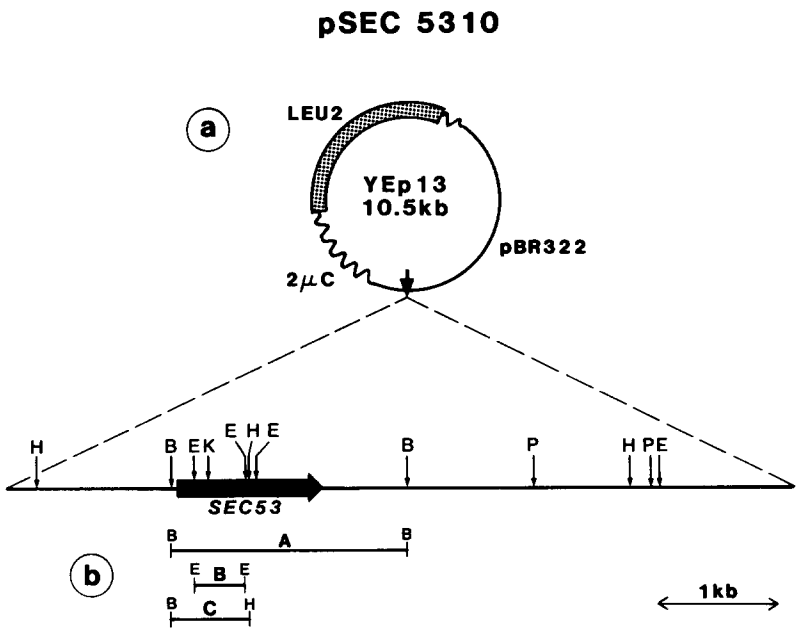

FIGURE 1 Restriction map of plasmid pSEC5310. (*a*) pSEC5310 contains a 7-kb insert in YEp13 that complements *sec53* cells. The heavy arrow indicates the direction and approximate limits of the *SEC53* mRNA, as determined from a cDNA clone. (*b*) Fragments from pSEC5310 that are subcloned into different vectors referred to in the text. *E*, *Eco*RI; *K*, *Kpn*I; *B*, *Bgl*II; *H*, *Hin*dIII; *P*, *Pst*I.

E. coli–yeast shuttle plasmid YEp13 (9) contains the yeast *LEU2* gene and sequences needed for autonomous replication in both hosts. YIp5 (10), an integrating plasmid, contains the yeast *URA3* gene but lacks a sequence for replication in yeast. The yeast genomic DNA library, constructed by K. A. Nasmyth, contains DNA fragments obtained by a partial *Sau3A* restriction enzyme digest inserted into YEp13 (11). The yeast cDNA expression library of McKnight and McConaughy contains inserts adjacent to the *ADH1* promoter on a plasmid that also carries the yeast *TRP1* gene (12).

YPD medium contained 1% Bacto-Yeast extract, 2% Bactopeptone, and varying amounts of glucose. Wickerham's minimal medium (13) was used with 5% glucose as a carbon source. For $^{35}SO_4^{2-}$ labeling of cells, sulfate salts were replaced by chloride salts and ammonium sulfate was added to the desired concentration. The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a Zeiss PMQII spectrophotometer; 1 OD_{600} unit of cells corresponds to 0.15 mg dry weight.

Other reagents were obtained as indicated: ATP, GTP, deoxynucleoside triphosphates, NAD^+, NADH, cytochrome *c*, O-nitrophenyl-β-D-galactopyranoside, acetylphenylhydrazine, hemin (type I, bovine), phosphocreatine, ε-aminocaproic acid, creatine phosphokinase (type I), micrococcal nuclease, spermidine (free base), diethyl pyrocarbonate, bovine serum albumin (BSA) (radioimmunoassay grade), protein A, isopropyl-thio-β-D-galactoside, and Freund's complete and incomplete adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO); restriction endonucleases and DNA modification enzymes were from Bethesda Research Laboratories (Gaithersburg, MD); dideoxynucleoside triphosphates were from P-L Biochemicals (Milwaukee, WI); glyceraldehyde-3-phosphate (diethylacetal form) and calf liver tRNA were from Boehringer Mannheim Biochemicals (Indianapolis, IN); $H_2{}^{35}SO_4$ (1,255 Ci/mmol), [^{35}S]methionine (1,200 Ci/mmol), and ^{125}I-Na (highest specific activity) were from Amersham Corp. (Arlington Heights, IL); nitrocellulose was from Schleicher & Schuell, Inc., Keene, NH); RNAsin was from Promega Biotec (Madison, WI); Oligo dT cellulose was from Collaborative Research (Waltham, MA); DEAE cellulose (DE-52) was from Whatman Ltd. (Maidstone, Kent, UK); IgG Sorb was from The Enzyme Center (Boston, MA); EN^{3}HANCE was from New England Nuclear (Boston, MA). Lyticase and anti-invertase antiserum were prepared as described before (14, 15). Protein A was labeled with ^{125}I by the chloroamine T/NaI method (16).

Cloning, Sequence Analysis, and Transposon-mediated Mutagenesis of SEC53: Yeast strain MBY7-5C was grown in YPD (5% glucose) medium to an OD_{600} of 1–2 and converted to spheroplasts which were transformed with the genomic DNA library of Nasmyth (see reference 11) or the cDNA library of McKnight and McConaughy (12). Transformants were selected on minimal medium lacking an amino acid (leucine for the genomic library; tryptophan for the cDNA library) at 24°C. After 24 h, at which time transformants began to appear, plates were transferred to the *sec* restrictive temperature (37°C). Colonies that continued to grow were selected, replated, and plasmid DNA was isolated from each. Yeast plasmid DNA was used to transform *E. coli* strain MC1061. Plasmid DNA was isolated from individual transformants and complementation of both *sec53* and amino acid auxotrophy was confirmed by transformation of the original yeast host.

Plasmid pSEC5310 was isolated from the genomic DNA bank and contains a 7-kb insert in YEp13 that includes the *SEC53* gene (Fig. 1 *a*). pSEC5315 contains a 1.1-kb *Bgl*II fragment (Fig. 1 *b*, fragment *A*) that includes *SEC53* in YIp5. pSEC5319 contains the 0.4-kb *Eco*RI fragment of *SEC53* (Fig. 1 *b*, fragment *B*) subcloned into YIp5. pSEC5316 contains the 5′ half of *SEC53* (Fig. 1 *b*, fragment *C*) subcloned into pUC9.

Restriction endonuclease digestions and T_4 DNA ligase reactions were carried out according to the supplier's instructions. Reactions with T_4 DNA polymerase were performed as described by Maniatis et al. (17). Standard techniques of plasmid isolation, agarose gel electrophoresis, and DNA transformations of *E. coli* and *S. cerevisiae* have been described elsewhere (17–19). DNA sequencing was performed using the dideoxy chain termination method

of Sanger et al. (20). M13 primer was a gift of W. Rottman and E. Penhoet (Department of Biochemistry, University of California at Berkeley).

Transposon-mediated mutagenesis of *SEC53* was performed in *E. coli* strain MC1061[chr::Tn5] transformed with pSEC5310. Cells were selected on ampicillin-containing plates and 24 separate resistant transformants were grown to stationary phase in 6-ml L broth cultures. Plasmid preparations from each were suspended in 0.03 ml of 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, and 0.01 ml of each was used to transform strain MC1061. Cells containing plasmids that sustained a transposition event were selected on LB plates containing ampicillin (100 μg/ml) and Kanamycin (25 μg/ml). Out of 10^5 ampicillin-resistant transformants, from 0 to 14 were doubly resistant. One amp^r kan^r colony from each transformation was used to prepare plasmid, which was used to retransform MC1061 to insure that the drug resistance phenotype was plasmid linked. Eleven independent Tn5 insertions into pSEC5310 were obtained.

RNA Preparation and In Vitro Translation: RNA was isolated from strain X2180-1B. Cells (~2,000 OD_{600} units) were sedimented, resuspended in 40 ml of water, distributed into 10-ml aliquots, and centrifuged again. Cell pellets were resuspended in 3 ml each of Buffer A (0.2 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.01 M EDTA, 1% SDS). Glass beads (0.5 mm) were added to ~¾ of the final volume, followed by 3 ml of PCI (phenol/chloroform/isoamyl alcohol; 25:24:1). Samples were agitated on a vortex mixer for 2 min at room temperature, pooled into two samples, and each diluted with 6 ml of water. After mixing, samples were centrifuged at 8,000 *g* for 10 min in a Sorvall HB-4 rotor at 24°C. The aqueous layer was removed and re-extracted with PCI, and the organic layer was re-extracted with 5 ml of water. After centrifugation, the aqueous layers were pooled, and the PCI extraction was repeated four to five times until the interface was free of particulate material. Nucleic acid was precipitated with ethanol, sedimented, resuspended in water, and precipitated again prior to use. Approximately 30 mg of nucleic acid were obtained in this preparation. Aqueous solutions were treated with 0.01% diethylpyrocarbonate and autoclaved prior to use.

Hybrid selection from total yeast RNA was performed as described elsewhere (21). Nitrocellulose filters containing plasmid DNA were exposed to RNA individually with one filter per container.

Rabbit reticulocyte lysates were prepared by the method of Pelham and Jackson (22), as modified by Appling and Rabinowitz (23). Translation reactions contained 100 mM potassium acetate, 0.8 mM magnesium acetate, and other components as described (23). After incubation for 60 min at 30°C, one-tenth (3 μl) of each sample was removed, mixed with 17 μl of SDS gel sample buffer, and heated in boiling water for 3 min. The remainder of the translation mix was prepared for immune precipitation by addition of SDS to 2%, followed by heating as above. Samples were diluted 30-fold in phosphate-buffered saline (PBS) (12.5 mM sodium phosphate, pH 7.5, 0.2 M NaCl) + 1% Triton X-100 and incubated with 0.1 ml of IgG Sorb at 0°C for 15 min. A clarified supernatant fraction, obtained by centrifugation for 2 min in a microfuge, was split in half and mixed with antibody. For immune serum, precipitations contained 0.02 ml of an IgG fraction (6.5 mg protein/ml); preimmune controls contained 0.01 ml of crude serum. Samples were incubated overnight at 0°C followed by addition of 75 μl of IgG Sorb. Immune complexes were washed as before (15). Final pellets were resuspended in 0.04 ml of SDS gel sample buffer and heated as before. Clarified supernatant fractions were analyzed by SDS gel electrophoresis on 12.5% polyacrylamide gels as described by Laemmli (24). Gels were fixed, treated with EN^{3}HANCE, and radioactive proteins localized by exposure to Kodak X-OMAT AR Film at −70°C.

Sec53p Antiserum: The 0.4-kb *Eco*RI fragment from pSEC5310 (Fig. 1 *b*, fragment *B*) was isolated and converted to a fully duplex form by treatment with T_4 DNA polymerase and the four deoxynucleoside triphosphates. This fragment was inserted by blunt end ligation into vector pUR290 that had been converted to a linear completely duplex form by treatment with *Bam*H1 endonuclease and T_4 DNA polymerase plus deoxynucleoside triphosphates. *E. coli* BMH71-18 was transformed with the ligated sample and ampicillin-resistant colonies were selected. Plasmids from individual transformants were isolated and evaluated by restriction analysis for insertion of the *SEC53* fragment. Promising transformants were further screened for production of a protein of the size expected for the β-galactosidase-Sec53p hybrid (~135 kD). A plasmid (pSCZ5350) that satisfied these criteria was isolated and the *LacZ/SEC53* DNA junction was sequenced to confirm that fragment B had been inserted in the proper orientation and reading frame. The fusion protein retained β-galactosidase activity.

To purify the hybrid protein, BMH71-18/pSCZ5350 was grown at 37°C in LB/amp medium (2L) to an OD_{540} of ~0.5 isopropyl-thio-β-D-galactoside was added to a final concentration of 1 mM and incubation continued for 65–75 min. Cells were centrifuged at 4°C and broken as described elsewhere (25). After a brief sonication, cell extracts were centrifuged for 1 h at 100,000 *g* in a Beckman Type 40 rotor at 4°C. Over 75% of the β-galactosidase activity was in the 100,000-*g* pellet fraction. The sediment was suspended in 5 ml of 0.02% SDS and centrifuged for 20 min at 39,000 *g* in a Sorvall SS-34 rotor at 4°C. About 50% of the total β-galactosidase activity was recovered in the final pellet fraction which represents between 10 and 15 mg of hybrid protein. This material was resuspended in 7 ml of SDS gel sample buffer and heated for 5 min in a boiling water bath. Solubilized samples were applied to preparative SDS gels made of 5% polyacrylamide. After electrophoresis, gels were stained with Coomassie Blue and the hybrid protein band was cut from the gel. Gel pieces were suspended in SDS stacking gel buffer, and protein was electroeluted into dialysis tubing. Eluted protein was dialyzed extensively against water and lyophilized.

Rabbits were immunized by multiple intradermal injections (26). Primary injections contained ~200 μg of hybrid protein in Freund's complete adjuvant. The same amount of protein in Freund's incomplete adjuvant was used in boosts. After two boosts, blood samples were collected and fresh boosts given at intervals of 7 to 10 days. Crude serum was used directly or after isolation of the IgG fraction by the DEAE cellulose method (26). Two rabbits gave a significant immune response after five boosts.

Cell Fractionation and Detection of Sec53p: For detection of metabolically radiolabeled Sec53p, yeast strain MBY7-5C, containing different plasmids, was grown at 24°C to an OD_{600} of 0.5–1.5 in minimal medium containing 0.1 mM ammonium sulfate. Aliquots (0.5 OD_{600} unit of cells) were centrifuged and cells resuspended in 2 ml of minimal medium containing 0.05 mM ammonium sulfate. $H_2{}^{35}SO_4$ (0.25 mCi) was added and incubation continued for 6 h at 24°C. Labeled cells were centrifuged at room temperature, washed once with 5 ml cold 10 mM NaN_3, and resuspended in 0.1 ml of SDS gel sample buffer. Proteins were extracted by heating for 5 min in a boiling water bath. Debris was removed by sedimentation in a microfuge for 5 min and samples of the supernatant fraction (3×10^6 cpm/lane) applied to SDS gels containing 12.5% polyacrylamide. After electrophoresis, gels were exposed to X-ray film as above.

For immune detection of Sec53p in unfractionated extracts, strain MBY7-5C containing different plasmids was grown to an OD_{600} of 0.5–2.5 in minimal medium containing 0.2 mM ammonium sulfate. Cells (10 OD_{600} units) were sedimented, washed once with cold 10 mM NaN_3, and resuspended in 0.1 ml of 2% SDS, 1 mM phenylmethylsulfonyl fluoride. Glass beads (0.3 g) were added and samples agitated at full speed for 2 min on a vortex mixer, followed by heating for 3 min in boiling water. The extract solution was removed and the glass beads washed three times with 0.05 ml of 0.1% SDS. Pooled extracts contained between 1.5 and 3.4 mg protein/ml.

Subcellular localization was performed on a *sec18* mutant strain to allow detection of Sec53p and ER-localized invertase in the same fractions. HMSF176*a* was grown at 24°C to an OD_{600} ~2 in YPD with 5% glucose. Two aliquots (300 OD_{600} units cells each) were centrifuged and cells were resuspended in 150 ml YPD with 0.1% glucose (conditions which derepress synthesis of invertase); one sample was incubated at 24°C, the other at 37°C. After 40 min, NaN_3 was added to 10 mM, and cells were sedimented and resuspended in 10 ml of 10 mM Tris-$SO_4{}^{2-}$-, pH 9.4, 30 mM 2-mercaptoethanol, 10 mM NaN_3. Samples were incubated for 10 min at room temperature, centrifuged, and washed once with 10 mM NaN_3. Cell pellets were resuspended in 3 ml spheroplast buffer (1.5 M sorbitol, 50 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 10 mM NaN_3), lyticase was added to 20 units/OD_{600} unit of cells, and cells were converted to spheroplasts during a 30-min incubation at 30°C with gentle agitation. This solution was layered on a cushion of spheroplast buffer that contained 1.8 M sorbitol, and spheroplasts were sedimented at 8,000 *g* for 5 min at 4°C in a Sorvall HB-4 rotor. The pellet was resuspended in 1.5 ml of Buffer B (0.3 M mannitol, 0.1 M KCl, 20 mM 2, (*N*-morpholino)ethane sulfonic acid, pH 6.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride) and glass beads were added to ¾ of the final volume. Lysis was achieved by three 15-s agitations on a VWR vortex mixer (setting 3). Liquid was removed and the glass beads were washed five times with 0.75 ml Buffer B. Solutions from the same lysate were pooled and adjusted to 5 ml with Buffer B. An aliquot (1 ml) of the extract was removed, and the remainder centrifuged at 660 *g* for 3 min at 4°C in a Sorvall HB-4 rotor. 3 ml of the supernatant fraction was removed and centrifuged at 100,000 *g* for 1 h at 4°C in a Beckman type 40 rotor (Beckman Instruments, Inc., Palo Alto, CA). The high speed supernatant fraction was removed, and the pellet was gently washed with Buffer B and resuspended in Buffer B + 0.1% Triton X-100.

Immunoblotting was used to quantify the subcellular distribution of Sec53p and of the cytoplasmic and ER-accumulated forms of invertase. Fractions subjected to SDS gel electrophoresis were transferred to nitrocellulose filters (27), which were then blocked in Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 1% BSA. Antibody reactions were also performed in the buffer. After exposure to antibody, filters were washed for 10 min each in TBS,TBS + 0.4% Triton X-100 + 0.05% SDS, and TBS. Washed filters were treated with ^{125}I-labeled protein A (2.5×10^4 cpm/ml, 5×10^6 cpm/μg protein)

(27). Filters were again washed sequentially and reactive proteins visualized by autoradiography using an intensifying screen at −70°C. Autoradiograms were quantified by scanning with a Kratos model SD3000 spectrodensitometer coupled to a Kratos SDS300 density computer (Kratos Analytical Instruments, Ramsey, NJ) and Hewlett-Packard 3380A integrator (Hewlett-Packard Co., Palo Alto, CA).

Assays and Other Procedures: NADPH cytochrome *c* reductase was assayed as described by Kubota et al. (28). Glyceraldehyde-3-phosphate dehydrogenase was assayed as described elsewhere (29). The diethylacetal form was converted to glyceraldehyde-3-phosphate by heating a 10-mM solution containing 0.1 g/ml Amberlite IR120 (H^+ form) for 2 min in a boiling water bath. After filtration, aliquots were stored frozen at −20°C. β-Galactosidase activity was measured at described by Miller (30). Protein was measured by a modification of the Lowry procedure (31).

RESULTS

Cloning and Sequence Analysis of SEC53

Yeast strain MBY7-5C was transformed separately with a yeast genomic DNA library and a cDNA library. Transformants were selected simultaneously for growth on a medium minus the relevant amino acid and for growth at 37°C, the restrictive temperature for *sec53* cells. From the genomic library, ~15,000 Leu^+ colonies were obtained, of which 75 were also Ts^+. The cDNA library yielded ~15,000 Trp^+ transformants of which ~30 were also Ts^+.

Plasmids that conferred a Ts^+ phenotype were isolated, used to transform *E. coli*, and re-isolated. Individual plasmids were then used to transform yeast to confirm that the initial Ts^+ and prototrophic phenotypes were plasmid linked. Eight complementing plasmids (four each from the genomic and cDNA libraries) were analyzed by restriction mapping; all contained DNA from the same genomic region. A restriction map of one genomic clone that complements *sec53* mutations, pSEC5310, is shown in Fig. 1 *a*. The direction of transcription and approximate limits of the *SEC53* gene were determined by comparing the restriction map of pSEC5310 with a cDNA clone that also conferred a Ts^+ phenotype to a *sec53* strain.

Genetic analysis was used to prove that plasmid pSEC5310 contains the structural gene for *sec53*. Fragment A (Fig. 1 *b*), a 1,700 *Bgl*II restriction fragment that complements *sec53*,[2] was subcloned into the yeast integrating plasmid YIp5, forming pSEC5315. This construct was used to transform strain MBY7-5C, and stable Ura^+/Ts^+ transformants were selected. A transformant was crossed to another *ura3*-52, *sec53*-6 strain to form a diploid cell, which was then sporulated and subjected to tetrad analysis. The Ura^+:Ura^- and Ts^+:Ts^- phenotypes showed 2:2 segregation patterns as expected if pSEC5315 had integrated into the genome at a single site. In addition, analysis of 29 asci, each containing four viable spores, showed that the Ura^+/Ts^+ phenotypes were tightly linked with a genetic distance of ≤1 cM. When the Ura^+/Ts^+ transformant was crossed with a *URA3, SEC53* strain, all progeny spores of 20 tetrads were Ts^+, proving that integration had occurred at the *SEC53* locus. Though both parents in this cross were Ura^+, approximately one-fourth of the progeny were Ura^-, consistent with integration of pSEC5315 at a locus distinct from *URA3*.

The conditional lethality of *sec53* mutations is consistent with either a thermosensitive *sec53* product, or with a null mutation that exposes an independent thermosensitive process. These possibilities were distinguished by disruption of the *SEC53* chromosomal locus in a diploid cell, followed by tetrad analysis. The 0.4-kb *Eco*RI fragment of *SEC53* (Fig. 1 *b*, fragment *B*) was subcloned into YIp5 (pSEC5319) and cut at the unique *Kpn*I site within *SEC53* to direct integration at the *SEC53* locus (32). Linear DNA was used to transform diploid strain MBY21 and stable Ura^+ transformants were selected. Plasmid integrations at the wild-type locus (12 Ura^+/Ts^- transformants) and *sec53*-6 locus (20 Ura^+/Ts^+ transformants) were obtained. Transformants that had integrated pSEC5319 at the *sec53* locus were sporulated and only asci with two viable spores (24 total) or one viable spore (8 total) were recovered. Ths spores were all Ura^-/Ts^+, while other loci (*MATa/MATα* and *CAN1/can1*) were unaffected and assorted independently of the lethal phenotype. From these results we conclude that disruption of the *SEC53* locus is a lethal event and that this gene is required for spore germination.

The DNA sequence of the *SEC53* gene, shown in Fig. 2 *a*, contains a single long open reading frame, uninterrupted by introns, starting at the nucleotide designated +1 and terminating with an amber codon after nucleotide 762. This open reading frame predicts a hydrophilic polypeptide of 254 amino acids (30% charged) with a molecular weight of 29,050. Hydropathic analysis, based on the program of Kyte and Doolittle (33), shows no hydrophobic region long enough to span a lipid bilayer. No significant sequence homology to proteins in the Dayhoff protein sequence bank at the University of California, San Francisco, was found for the *SEC53* coding region.

Examination of the DNA sequence upstream from *SEC53* reveals TATA sequences, which are thought to be involved in positioning the start of transcription (34), centered around positions -13, -85, and -162. The first *SEC53* nucleotide identifiable in our cDNA clone is at position -47, thus the TATA sequences around nucleotides -85 or -162 may function in vivo. Also highlighted in Fig. 2 *a* are sequences that may be involved in transcription termination and polyadenylation. The sequence TAG ..... 16 nucleotides ..... TACGT ..... 10 nucleotides .... AAA, located between nucleotides 763–800 (80 nucleotides upstream of the poly A site found in our cDNA insert), is homologous to a conserved sequence that may function in termination and polyadenylation of yeast mRNAs (35). Another sequence, AAUAAA, thought to function in polyadenylation of eukaryotic mRNAs (36), is found 38 nucleotides upstream from the cDNA poly A site.

Detection of Sec53p

To identify directly the *SEC53* gene product (Sec53p), strain MBY7-5C containing pSEC5310, YEp13, or no plasmid, was labeled for 6 h with $^{35}SO_4^{2-}$. Labeled proteins were extracted and evaluated by SDS gel electrophoresis and autoradiography. A species of ~28-kD was overproduced in cells transformed with the multicopy *SEC53* plasmid (Fig. 3, lane *B*), as compared with control cells. The size of this polypeptide is consistent with the molecular weight predicted for the *SEC53* gene product. Detection of Sec53p by this technique indicates that this protein is abundant. This correlates with the level of *SEC53* mRNA, which was shown by Northern

[2] Fragment A contains only 35 bp upstream from the first nucleotide identified in the cDNA insert. This fragment complements *sec53* when present on a multicopy plasmid; consequently, some promoter function may be provided by vector sequences.

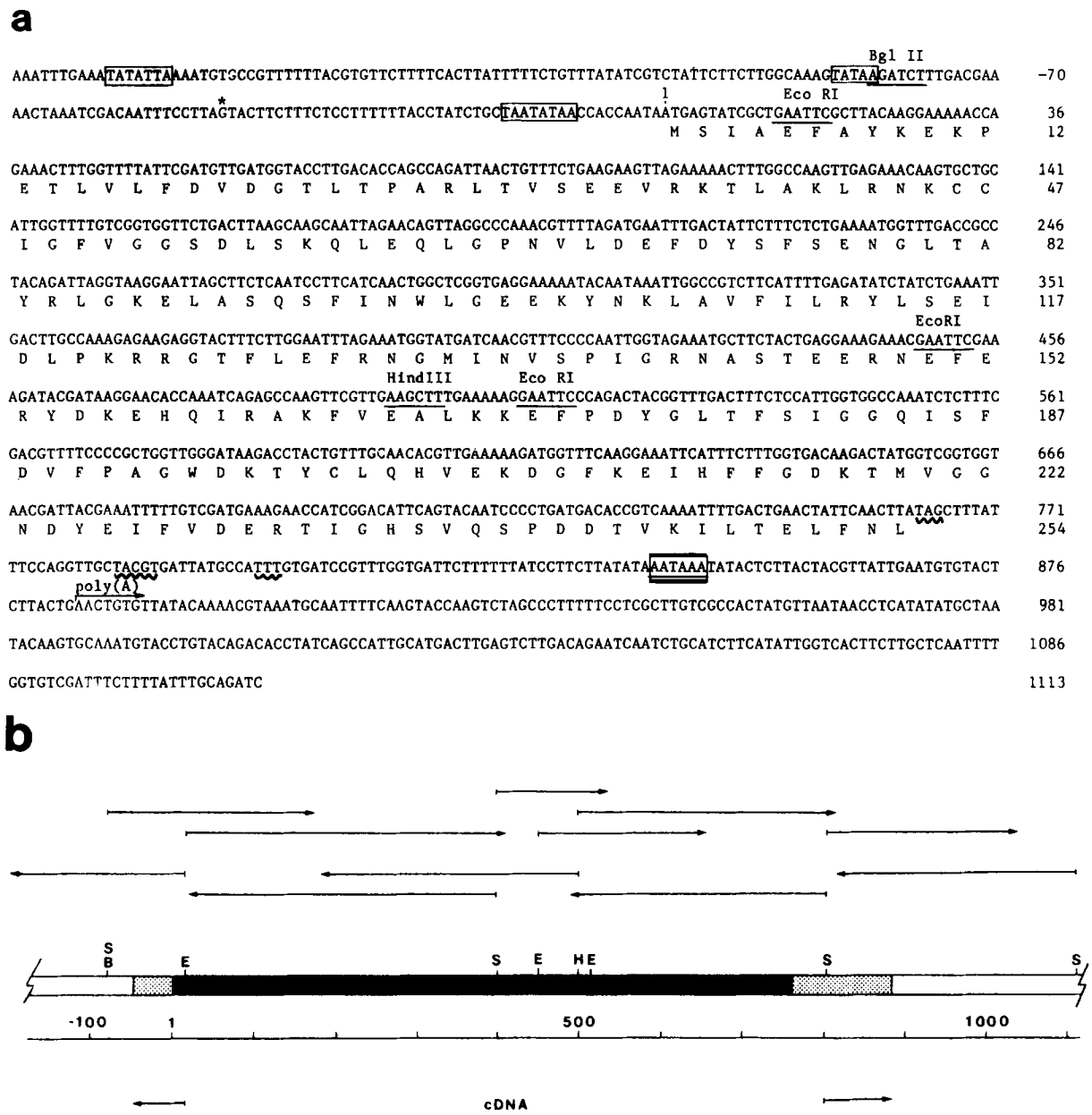

FIGURE 2 Sequence of the *SEC53* gene. (a) Primary sequence of the *SEC53* gene. (*b*) Sequencing strategy for *SEC53*. The solid bar represents the coding region, dotted areas the 5′ and 3′ non-coding regions from the *SEC53* mRNA. Arrows above show sequencing strategy for genomic DNA subcloned into M13; arrows below indicate sequences obtained from the cDNA clone. Numbers refer to the nucleotides relative to the initiating A of the coding sequence. Light boxes indicate TATA sequences; heavy box indicates a poly A addition signal; asterisk is first nucleotide found in the cDNA; poly A indicates site of poly A addition found in the cDNA; underlined sequences correspond to the restriction sites indicated.

hybridization to be present at 15–30 copies per cell (data not shown).

Overproduction of the 28-kD protein was further correlated with *SEC53* by analysis of Tn5 transposon insertion mutations introduced into pSEC5310. Four insertions were evaluated, two of which inactivated *SEC53* complementing activity by disruption or deletion. The 28-kD protein was overproduced only in the two transformants that contained Tn5 insertions outside of *SEC53* (Fig. 4, lanes *B* and *D*). These data suggest that the 28-kD protein is the product of *SEC53*, or of another protein whose expression is regulated by *SEC53* gene dosage.

A more direct connection between the 28-kD protein and *SEC53* was made by isolation and in vitro translation of *SEC53* mRNA. The 0.5-kb *Bgl*II/*Hind*III fragment, which encompasses the 5′ half of *SEC53* (Fig. 1 *B*, fragment *C*), was subcloned into the *E. coli* vector pUC9 (pSEC5316). pSEC5316 was used to hybrid select *SEC53* mRNA from total yeast RNA, followed by translation of the purified message in a rabbit reticulocyte protein synthesis system. Radioactive protein products from hybrid-selected and control translation reactions were compared by SDS gel electrophoresis and autoradiography. Fig. 5 (lane *C*) shows the product of the reaction was the 28-kD protein previously associated

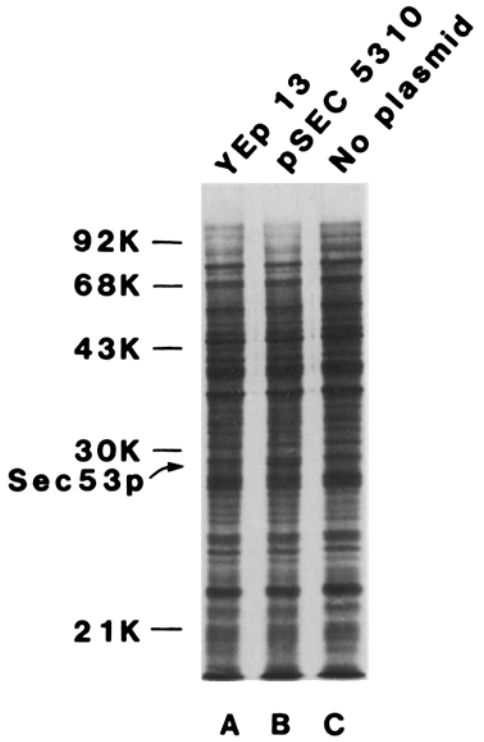

FIGURE 3 Overproduction of Sec53p. Yeast cells (strain MBY7-5C) containing different plasmids were labeled with $^{35}SO_4$ for 6 h. Whole cell extracts were subjected to SDS PAGE on 12½% gels (3 × 10^6 cpm/lane) followed by autoradiography.

with *SEC53*. (The 43-kD protein observed in this experiment is an artifact of translation with a rabbit reticulocyte system in the presence of [^{35}S]methionine.)

Immune Detection and Localization of Sec53p

Having detected Sec53p, we developed an antibody probe suitable for identification of the protein in subcellular fractions. A portion of the *SEC53* gene representing amino acids 6 through 151 was fused to the 3′ end of the *E. coli LacZ* gene. A hybrid protein product with the predicted size was produced in *E. coli*, and the exact nature of the fusion junction was confirmed by DNA sequence analysis. Because of the high level of expression of the hybrid in *E. coli*, a simple fractionation scheme, concluding with preparative SDS gel electrophoresis, was used for purification of the protein. A polyclonal serum directed against β-galactosidase and Sec53p epitopes was developed in response to immunization with the hybrid protein.

Recognition of Sec53p by the antiserum was tested in two ways: decoration of the antigen in SDS gels of untransformed and pSEC5310-transformed yeast strains, and immune precipitation of hybrid-selected mRNA translation products. Fig. 6 shows that at 24°C, the 28-kD protein was detected in both mutant and wild-type cells, and that similar levels of overproduction (eightfold) were achieved by introduction of pSEC5310. At 37°C, the level of Sec53p in *sec53-6* cells is diminished relative to wild-type, indicating a lability expected of a denatured polypeptide (data not shown). Immune precipitation of Sec53p was performed on the translation product of *SEC53* mRNA enriched by hybrid selection shown in Fig. 5 (lane *C*). This protein was precipitated by Sec53p antiserum (lane *D*), while preimmune serum did not react (lane *E*).

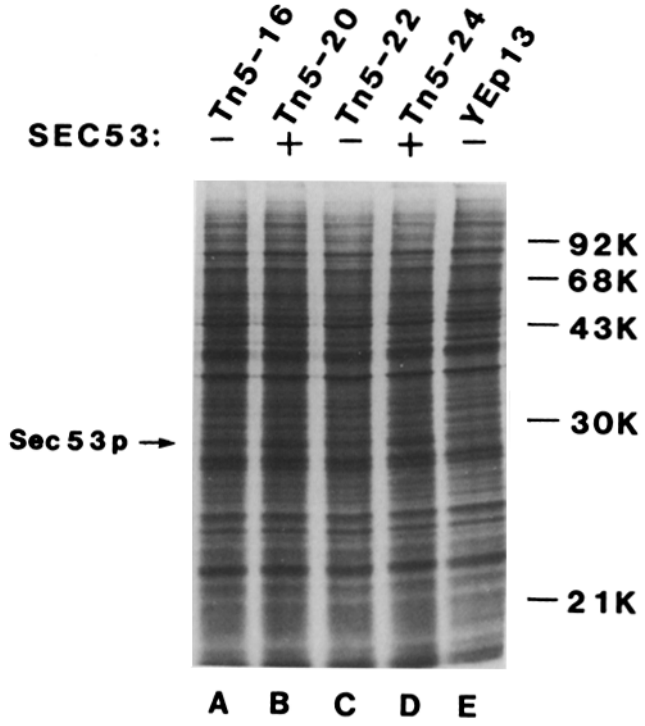

FIGURE 4 Effect of Tn5 insertions in pSEC5310. Yeast cells (strain MBY7-5C) containing plasmid pSEC5310 with different Tn5 insertions were labeled with $^{35}SO_4$ for 6 h. Whole cell extracts were subjected to SDS PAGE on 12½% gels (3 × 10^6 cpm/lane) followed by autoradiography. *SEC53* heading indicates the ability of a plasmid to complement a *sec53* mutation. Tn5 insertions in pSEC5310 were found for Tn5-16, in the 400-bp *Eco*RI fragment of the *SEC53* coding region; Tn5-20, in the 50-bp *Pst*I-*Eco*RI fragment of yeast DNA 3′ to *SEC53*; Tn5-22, in *SEC53* (also deletes the 3′ half of *SEC53*); Tn5-24, in the *LEU2* region of pSEC5310; YEp13, control plasmid.

Parallel evaluation of the in vitro translation product and immune decorated cell lysates showed coincident migration of Sec53p on SDS PAGE (not shown).

Sec53p in the Cytosol Fraction

Yeast cells were fractionated to determine if Sec53p was localized in the cytosolic or microsomal fractions. Fractionation was performed with *sec18* mutant cells incubated at permissive (24°C) and restrictive (37°C) temperatures under conditions which derepressed synthesis of the secreted enzyme, invertase. In this mutant, protein transport from the ER is blocked and soluble forms of invertase accumulate within the ER lumen (15). Although the lumenal contents are easily released from *sec18* ER membranes, conditions of spheroplast lysis have been developed that retain this structure in an intact, sedimentable form (15). Such conditions were used to establish the location of Sec53p.

Cells were converted to spheroplasts, lysed, and aliquots centrifuged at 100,000 *g* for 1 h. Samples of the extract, high speed supernatant, and high speed pellet fractions were evaluated by SDS gel electrophoresis and immunodecoration with Sec53p and invertase antibodies. Fig. 7 *a* shows the distribution of Coomassie Blue staining protein species in the three fractions prepared from *sec18* cells incubated at the restrictive and permissive temperatures. Fig. 7 *b* shows that most of the Sec53p was in the high speed supernatant fraction prepared from cells incubated at either temperature. The same distri-

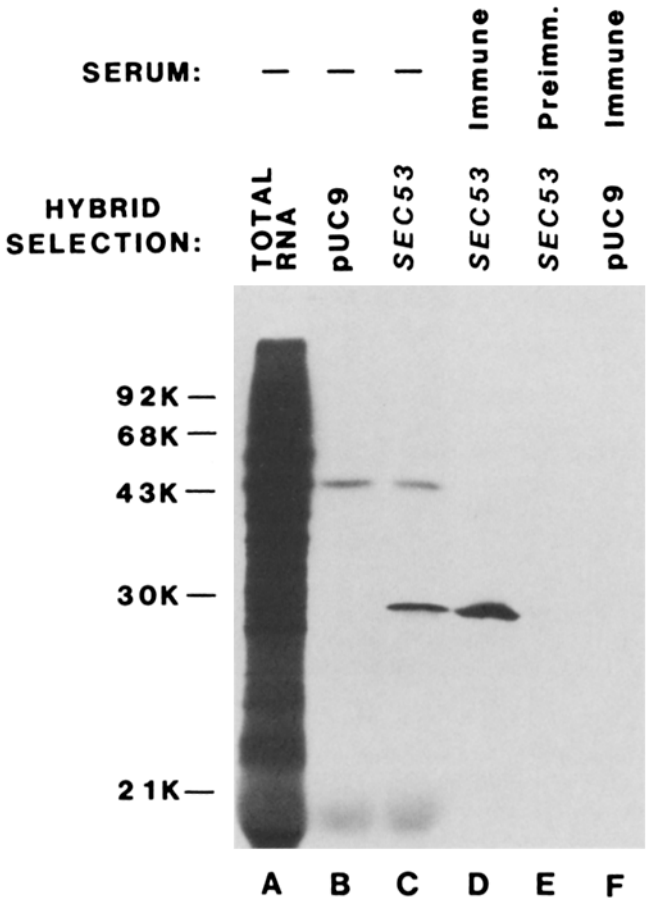

FIGURE 5 Hybrid selection, in vitro translation, and immune precipitation of Sec53p. Plasmid pSEC5316 (lanes *C–E*) or pUC9 (lanes *B* and *F*) were used to hybrid select total yeast RNA for translation in vitro. Translation products were untreated (lanes *A–C*), treated with immune IgG (lanes *D* and *F*), or treated with preimmune serum (lane *E*), and subjected to SDS PAGE on 12½% gels followed by fluorography. Lane *A* contains the translation products obtained from 10 μg of total yeast RNA.

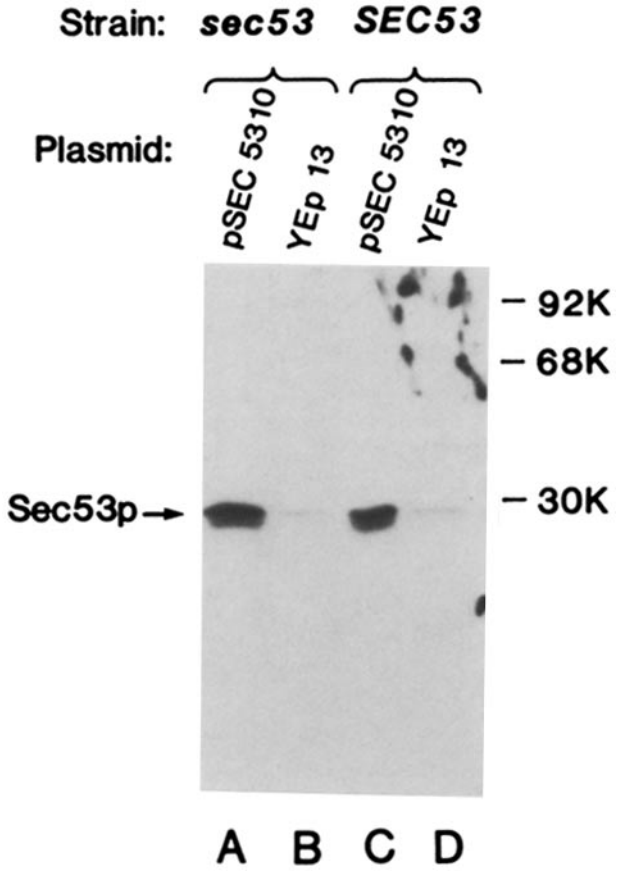

FIGURE 6 Steady state levels of Sec53p. Cell extracts from strains MBY7-5C (*sec53*) and JRY9 (*SEC53*) containing different plasmids were subjected to SDS PAGE on 12½% gels (40 μg protein/lane). Proteins were transferred to nitrocellulose and probed with anti-Sec53p IgG diluted 1:50 and ^{125}I-protein A followed by autoradiography.

bution of Sec53p was found in fractions from wild-type cells (not shown). A small amount of two higher mobility species was found in the high speed supernatant and high speed pellet fractions. These may represent proteolytic fragments of Sec53p; they were less apparent in samples of the crude extract. Fig. 7*c* shows the distribution of invertase immunoreactive forms. Extracts of cells incubated at 37°C show the ER-accumulated transit form of invertase exclusively in the high speed pellet fraction. This form was too transient to detect in cells incubated at 24°C. An alternate transcript of the invertase gene (*SUC2*) gives rise to a cytoplasmic form of the enzyme (37) which appeared in the high speed supernatant fraction in both cell samples.

Densitometric scanning of immunoblots performed with varying levels of antigen was used to quantify the fractionation. These values are compiled in Table II along with the recovery and enrichment of known markers of the ER membrane (NADPH cytochrome *c* reductase) and cytosol (glyceraldehyde-3-phosphate dehydrogenase). On the basis of these data we conclude that Sec53p is a cytosolic protein, though it could be detached from the cytoplasmic surface of a membrane by our method of cell lysis.

DISCUSSION

The *SEC53* gene has been cloned from yeast genomic and cDNA expression libraries. A single insert that integrates at the *sec53* locus contains an open reading frame that predicts a 29-kD protein. Part of the cloned gene was used in a gene disruption protocol to show that *SEC53* is essential for germination of yeast spores. Although the essential nature of *SEC53* is implied by the conditionally lethal alleles described previously (3), this phenotype could be explained by a null allele of *sec53* replaced in a temperature-sensitive fashion by another protein. Such cases have been reported for other yeast genes (38). The gene disruption results show that *SEC53* is required directly.

A protein of ~28-kD, as predicted from the DNA sequence, is overproduced by cells that contain *SEC53* on a multicopy plasmid. Additional evidence identifying this protein as Sec53p has come from transposon mutagenesis of plasmid DNA, from hybrid selection of mRNA and translation in a reticulocyte reaction, and from production of antibody directed against a portion of the *SEC53* coding sequence.

These techniques have been used to monitor the level and cellular location of Sec53p. In rich medium, *SEC53* mRNA is present in 15 to 30 copies per cell which accounts for expression of the protein at a high level. At 24°C, the steady state level of Sec53p is not affected by the *sec53-6* mutation

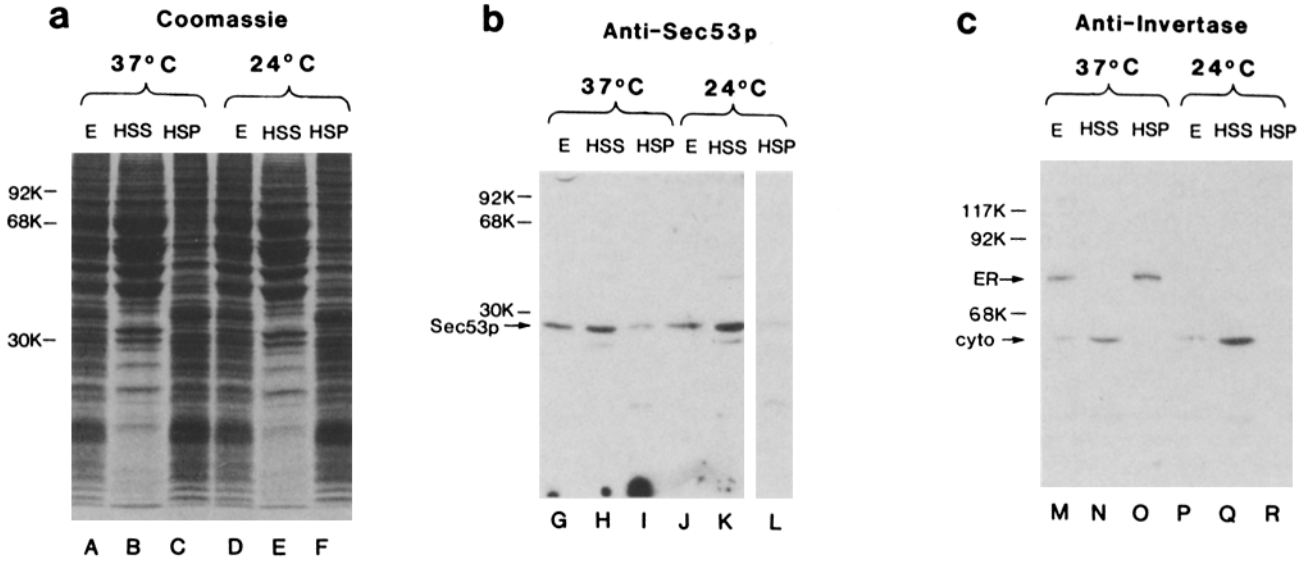

FIGURE 7 Fractionation of *sec18* cells. *sec18* cells incubated at 37°C or 24°C were fractionated and Extract (*E*), 100,000-*g* supernatant (*HSS*), and 100,000-*g* pellet (*HSP*) fractions were subjected to SDS PAGE (40 μg protein/lane). (*a*) Coomassie Blue–stained gel (12½% gel). (*b*) Autoradiogram of immunoblot using anti-Sec53p IgG diluted 1:50 (12½% gel). (*c*) Autoradiogram of immunoblot using anti-invertase antibody diluted 1:400 (7½% gel).

TABLE II. *Recovery* (and Enrichment‡) in Fractions of sec18 Cells*

	Sec53p	Cytoplasmic invertase	ER invertase	GAP	NADPH Cytochrome *c* reductase
37°C					
Extract	100 (1.0)	100 (1.0)	100 (1.0)	100 (1.0)	100 (1.0)
HSS	98 (2.2)	144 (3.2)	5 (0.11)	70 (1.6)	4 (0.09)
HSP	11 (0.24)	5 (0.12)	57 (1.2)	3 (0.06)	64 (1.4)
24°C					
Extract	100 (1.0)	100 (1.0)	NA	100 (1.0)	100 (1.0)
HSS	77 (2.6)	78 (2.6)	NA	59 (2.0)	4 (0.11)
HSP	4 (0.11)	6 (0.18)	NA	3 (0.06)	62 (1.7)

Sec18 cells were fractionated as described in the legend to Fig. 7. Levels of sec53p, cytoplasmic invertase, and ER-accumulated invertase in each fraction were determined by quantitative immunoblotting and densitometry of autoradiograms. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAP) and NADPH cytochrome *c* reductase were determined by enzymatic assays.

* Recovery is percent of activity found in Extract.

‡ Enrichment, shown in parentheses, is specific activity in each fraction.

NA, not applicable.

nor by changes in growth medium that cause derepressed synthesis of invertase. At 37°C, however, the mutant protein is labile. An eightfold overproduction of Sec53p is obtained in cells transformed with a multicopy plasmid that carries *SEC53*. Overproduction at this level has no harmful effect on protein secretion or cell growth. Under the conditions explored here, expression of *SEC53* appears to be constitutive and is not limited by the availability of regulatory factors.

DNA sequence analysis has shown Sec53p to be a hydrophilic protein with 30% charged amino acids and no significant length of hydrophobic peptide such as would be found in a membrane anchor segment. In addition to no signal peptide-like sequence, other forms of proteolytic maturation of Sec53p are unlikely. The primary translation product made in vitro and the mature polypeptide detected in cells are the same size and are consistent with the size calculated from the coding sequence. These characteristics are compatible with a cytosolic location of Sec53p.

Direct evidence for a cytosolic location of Sec53p has come from evaluation of cell fractions with an antibody directed against part of the *SEC53* coding sequence. Hence, models that rely on an integral membrane association or ER lumenal location of Sec53p are eliminated.

The function of Sec53p in translocation remains elusive. Our previous suggestion that Sec53p plays a direct role in this process came from the observation that invertase accumulated in *sec53* at 37°C was exposed on the cytoplasmic surface of the ER membrane and was largely susceptible to trypsin digestion (4). In this orientation, cytosolic Sec53p could act directly on the secretory polypeptide during the translocation event. We have recently found, however, that alternative conditions of *sec53* membrane isolation and trypsin treatment preserve an apparently complete form of invertase (Feldman, R., and R. Schekman, unpublished results). These new data suggest that secretory molecules may accumulate on the lumenal surface of the ER membrane when the *sec53* block is imposed. In this circumstance, Sec53p would act indirectly to allow folding and glycosylation of secretory precursors within the ER lumen. Moreover, as the *sec53* block is thermoreversible, mutant Sec53p function must be restored in the cytoplasm in a fashion that allows interrupted assembly to commence on the other side of the ER membrane. Sec53p may execute its function by producing a small molecule required for protein assembly in the ER lumen, or by influencing the

activity of an ER membrane component that serves this role.

It seems unlikely that defective oligosaccharide synthesis or transfer can account directly for the effect of *sec53* on protein assembly in the ER. First, unlike Sec53p, all the enzymes of oligosaccharide biosynthesis and transfer to protein are integral membrane proteins (39, 40). Second, alleles of *SEC53*, identified as deficient in asparagine-linked glycosylation (*alg4*), show a spectrum of dolichol-oligosaccharides (from man, to man_8) accumulated at the restrictive temperature in vivo and in vitro (41; also K. Runge and P. Robbins, personal communication). At least some of the smaller oligosaccharides may be transferred to protein at 37°C because invertase accumulated in *sec53* has a slightly reduced electrophoretic mobility which is not increased by treatment with endoglycosidase H. Oligosaccharides smaller than $Man_5GlcNAc$ are resistant to cleavage by endoglycosidase H. That any of the oligosaccharide precursors are produced in the mutant argues that the sugar-nucleotides UDP-GlcNAc and GDP-man are available for glycosylation. Finally, the vanadate-sensitive plasma membrane ATPase, another molecule whose assembly is blocked in *sec53*, appears not to be glycosylated at any state in its biosynthesis (Hansen, W., and R. Schekman, unpublished results). Hence, though Sec53p appears to influence oligosaccharide synthesis, this in itself cannot account for the role it plays in protein assembly.

Glutathione is another small molecule that could play a role in proper assembly in the ER. Scheele and Jacoby (42) showed that oxidized glutathione stimulates proper disulfide bond formation and protein folding in an in vitro protein synthesis reaction coupled to protein translocation into dog pancreas microsomal vesicles. If the generation of oxidized glutathione is deficient in *sec53*, the defect is unlikely to be directly related to translocation because the α-factor precursor molecule, whose assembly is blocked in *sec53* mutant cells (43), contains no cysteine residues.

Many other indirect influences on ER membrane structure can be invoked to explain the *sec53* phenotype. For example, although the major phospholipids are made in normal amounts in *sec53* (S. Henry, personal communication), their intracellular distribution could be altered to the detriment of ER assembly processes. Models such as this are constrained by the observation that assembly of mitochondrial membrane and matrix proteins are not delayed in *sec53* at 37°C (R. Hay and G. Schatz, personal communication).

Now that Sec53p has been located in the cytosol, it should be possible to detect restoration of some aspect of secretory protein assembly in mixtures containing *sec53* membrane and wild-type cytosol fractions.

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