A Yeast Mutant Defective at an Early Stage in Import of Secretory Protein Precursors into the Endoplasmic Reticulum

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Abstract. We have devised a genetic selection for mutant yeast cells that fail to translocate secretory protein precursors into the lumen of the endoplasmic reticulum (ER). Mutant cells are selected by a procedure that requires a signal peptide–containing cytoplasmic enzyme chimera to remain in contact with the cytosol. This approach has uncovered a new secretory mutant, *sec*61, that is thermosensitive for growth and that accumulates multiple secretory and vacuolar precursor proteins that have not acquired any detectable post-translational modifications associated with translocation into the ER. Preproteins that accumulate at the *sec*61 block sediment with the particulate fraction, but are exposed to the cytosol as judged by sensitivity to proteinase K. Thus, the *sec*61 mutation defines a gene that is required for an early cytoplasmic or ER membrane–associated step in protein translocation.

THE first step in the biogenesis of proteins destined for the secretory pathway is their insertion into the membrane of the endoplasmic reticulum (ER).[1] This process has been studied intensively in mammalian cells through the use of an in vitro assay that faithfully reproduces cotranslational translocation of secretory proteins into the lumen of the ER (2). Dissection of the components required for this activity has revealed the existence of both soluble and membrane-bound factors that participate in protein translocation. The signal recognition particle is a soluble ribonucleoprotein particle consisting of six polypeptides (54) and one molecule of 7SL RNA (55). The signal recognition particle binds to the signal sequence of a nascent preprotein (28, 56), thereby forming a complex that interacts with an integral membrane protein of the ER known as docking protein or signal recognition particle receptor (13, 33). This targeting event is followed by cotranslational translocation of the preprotein into the ER lumen. Either during or shortly after the translocation event, the signal sequence is cleaved by the enzyme signal peptidase (10) and core oligosaccharides are transferred to specific asparagine residues (44) of the translocated polypeptide. The mechanism of protein permeation across the hydrophobic core of the ER membrane is not understood. Experiments with intermediates artificially blocked at various stages of membrane penetration suggest that this process is mediated by proteins, though they have yet to be identified by the existing assays (14).

Recently, several groups have reconstituted protein translocation into the yeast ER in vitro (15, 42, 57). A yeast translation extract programmed with prepro-α-factor mRNA directs the synthesis of an intact precursor which can insert co- or posttranslationally into yeast microsomes and become core-glycosylated. The existence of a posttranslational reaction has allowed these investigators to demonstrate that protein translocation into the yeast ER is energy dependent (15, 43, 57). In addition, fractionation experiments suggest that the import reaction requires cytosolic components (58). The reconstitution of yeast protein translocation in vitro presents an opportunity to combine a biochemical analysis of protein translocation with a genetic approach aimed at identifying genes whose products participate in the reconstituted reaction.

Among a large collection of temperature-sensitive, secretion-defective mutants of *Saccharomyces cerevisiae* isolated in this laboratory (12, 36, 37), members of only two complementation groups (*sec*53 and *sec*59) affect early events in protein secretion, though neither mutation blocks protein translocation into the ER (10a). In an attempt to identify genes required for the translocation event, we have developed a direct selection for temperature-sensitive (Ts), import mutants of *Saccharomyces cerevisiae*.

The protocol described in this report is similar to that used by Oliver and Beckwith (39) to isolate mutants of *Escherichia coli* defective in the export of periplasmic and cell wall proteins from the cytoplasm. Strains expressing abortively translocated fusions of the periplasmic maltose-binding protein (MBP) to β-galactosidase produced much less β-galactosidase activity than strains harboring similar fusions with mutations in the maltose-binding protein signal sequence. By selecting for mutants that expressed high levels of β-galactosidase activity from the wild-type fusion protein (presumably owing to retention of the fusion protein in the cytoplasm), Oliver and Beckwith (39) were able to isolate mutations in

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; ER, endoplasmic reticulum; proCPY, procarboxypeptidase Y; Ts, temperature sensitive; YPD, 1% yeast extract, 2% peptone, 2% dextrose.

two genes (*sec*A and *sec*B) that blocked export and maturation of multiple secretory precursors.

We have modified this strategy to select positively for mutants of *Saccharomyces cerevisiae* that are defective in protein translocation. In this report, we describe the isolation and phenotypic characterization of mutants that define two complementation groups (*sec*61 and *sec*62). Mutations in both genes cause temperature-sensitive growth and accumulation of α-factor precursor. Detailed analysis of *sec*61 strains has revealed that this mutation results in the accumulation of cytoplasmically exposed and unmodified precursors of multiple secretory and vacuolar proteins.

Materials and Methods

Strains, Growth Conditions, and Materials

The bacterial and yeast strains used in this study are listed in Table I. All plasmids were propagated in HB101, except those sensitive to restriction by BclI, which were propagated in NK 5772. Yeast strains were constructed by standard genetic techniques (48). Original mutant isolates were backcrossed at least three consecutive times to RDB 103 to test for cosegregation of the Ts growth and α-factor accumulation phenotypes. All experiments were performed with these backcrossed derivatives. Haploid *sec*61 strains were recovered from the original *sec*61 diploid isolates by mating the diploid mutant to diploid S395D-1, sporulating this tetraploid strain, and then sporulating a Ts *MAT*a/*Mat*α diploid spore clone derived from the tetraploid. Haploid *MAT*α mutant strains were then backcrossed with RDB 103.

YPD liquid broth contained 1% Bacto-Yeast extract, 2% Bacto-Peptone (Difco Laboratories, Detroit, MI), and 2–5% glucose. Wickerham's minimal medium (59) was used with 2–5% glucose. Solid media were supplemented with 2% Bacto-Agar. For pulse labeling of cells with $[^{35}S]SO_4^{2-}$, sulfate salts were replaced by chloride salts, and ammonium sulfate was supplemented at 200 μM for overnight growth and at 0–10 μM during radiolabeling. Liquid cultures were grown in flasks with vigorous agitation, and experiments were initiated with cells in logarithmic phase. The optical density at 600 nm (OD_{600}) of dilute cell suspensions was measured in 1-cm quartz cuvettes using a Zeiss PMQII spectrophotometer (Carl Zeiss, Inc., Thornwood, NY); 1 OD_{600} of cells corresponds to 0.15 mg of dry weight.

The following reagents were obtained as indicated: histidinol, tunicamycin, ethyl methanesulfonate, concanavalin A(Con A)-Sepharose 4B, α-methylmannoside, NADPH, cytochrome *c*, proteinase K, protein A, bovine serum albumin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co., St. Louis, MO; restriction endonucleases, DNA modification enzymes, and nuclease-treated wheat germ in vitro translation components were from Bethesda Research Laboratories, Gaithersburg, MD; T4 DNA ligase and glyceraldehyde-3-phosphate (diethylacetyl form) were from Boehringer Mannheim Biochemicals, Indianapolis, IN; Amplify, [^{35}S]methionine (1,200 Ci/mmol), and ^{125}I-NaI (highest specific activity) were from Amersham Corp., Arlington Heights, IL; SDS-polyacrylamide gel electrophoresis reagents (electrophoresis grade) were from Bio-Rad Laboratories, Richmond, CA; nitrocellulose was from Schleicher & Schuell, Inc., Keene, NH; protein A-Sepharose was from Pharmacia Fine Chemicals, Piscataway, NJ; carrier-free $[^{35}S]Na_2SO_4$ was from ICN Radiochemicals, Irvine, CA; IgG Sorb was from The Enzyme Center, Boston, MA; and nonfat dry milk was from Safeway, North Berkeley, CA. Lyticase (fraction II, 30,000–90,000 U/ml) was prepared as described by Scott and Schekman (46). Invertase (45) and carboxypeptidase Y (50) antisera were prepared as described previously. Anti-α-factor sera was generously provided by J. Rothblatt, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany (42). Rabbit reticulocyte lysate was prepared as described (1).

Construction of *HIS4* Gene Fusions

A *SUC2-HIS4* gene fusion was constructed in the yeast shuttle vector YCp50, which is a low copy number plasmid that contains *URA3* as a selectable marker and *CEN4 ARS1* for mitotic stabilization and replication competence, respectively (51). In the first step, the 1.6-kb Eco RI-Bam HI fragment from the 5′ portion of the *SUC2* gene (from pRB58 [5]) was inserted between the unique Eco RI and Bam HI sites in YCp50 to generate $YCp50^{EB}$. Next, the 1.6-kb Xho II fragment of pYAH-I2 (40), which contains the 5′ portion of *HIS4*, was inserted into the Bam HI site of $YCp50^{EB}$ to generate YCp501. The remainder of the *HIS4* gene was introduced by replacing the 1,350-bp Cla I-Sph I fragment of YCp501 with the corresponding 2.7-kb fragment from pYAH-I2 resulting in plasmid YCp502, which contains almost the entire *HIS4* gene (except for the first 33 codons of *HIS4*A) fused out of frame to the 5′ half of the *SUC2* gene. An in-frame *SUC2-HIS4* fusion was created by inserting the 250-bp Bam HI-Bcl I fragment of *SUC2* into the unique Bam HI site of YCp502, yielding YCp503. Unfortunately, this fusion plasmid failed to complement *his4C* mutations, even though a hybrid protein of the proper size was synthesized constitutively.

We reasoned that an increase in the copy number of the *SUC2-HIS4* fusion might allow complementation of *his4C* strains. Thus, the fusion gene was introduced into a multicopy 2 μm-based vector. The junction sequences and *HIS4* coding portion of the *SUC2-HIS4* fusion were transferred to

Table I. Bacterial and Yeast Strains

Strain	Genotype	Source or reference
Saccharomyces cerevisiae		
RDB 103	*leu2*-3,-112 *ade2 MAT*a	This study
RDM 15-5B	*leu2*-3,-112 *ade2 ura3*-52 *pep4*-3 *sec61*-2 *MAT*α	This study
RDM 15-9B	*ade2 pep4*-3 *Mat*α	This study
RDM 15-10D	*leu2*-3,-112 *ade2 sec18*-1 *MAT*α	This study
RDM 15-3A	*leu2*-3,-112 *his4 pep4*-3 *sec18*-1 *sec61*-2 *MAT*α	This study
FC2-12B	*leu2*-3,-112 *ura3*-52 *trp1*-1 *his4*-401 *HOL*1-1 *MAT*α	R. Parker (40)
DYFC2-12B	*leu2*-3,-112/*leu2*-3,-112 *ura3*-52/*ura3*-52 *trp1*-1/*trp1*-1 *his4*-401/*his4*-401 *HOL*1-1/*HOL*1-1 *MAT*α/*MAT*α	This study*
S395D-1	*his1*/*his1 leu1*/*leu1 trp2*/*trp2 MAT*a/*MAT*a	YGSC‡
PBY404C	*suc2*-Δ9 *MAT*a	P. Böhni
165/7	*his4*-25 (*his4*A$^-$) *MAT*a	R. Parker (40)
E331	*his4*-331 (*his4*B$^-$) *MAT*a	R. Parker (40)
S942-1Ca	*his4*-864 (*his4*C$^-$) *MAT*a	R. Parker (40)
Escherichia coli		
NK5772	*dcm*-6 *dam*-3 *gal*K2 *gal*T22 *met*B1 *leu*Y1 *tsx*-78 *thi*-1 *ton*A31 *mtl*-1	J. Kadonaga
HB101	F$^-$ *hsdS20* (r_B^-, m_B^-) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 (S_m^r) *xyl*-5 *mtl*-1 *sup*E44	(30)

* Spontaneously derived from FC2-12B.
‡ Yeast Genetic Stock Center, University of California, Berkeley, CA.

pRB58 by digesting YCp503 to completion with Bam HI, and partially with Sal I, to generate a 3.4-kb Bam HI-Sal I fragment. This fragment was inserted in place of the 4.2-kb Bam HI-Sal I fragment (containing the 3' half of *SUC2*) of pRB58. The resulting plasmid, pSHE1, produced more fusion protein and complemented mutations in *his*4A, 4B, and 4C.

Expression of the cytoplasmic invertase-*HIS4* fusion protein was eliminated by replacing the extreme 5' portion of the *SUC2* coding region with the promoter elements and coding sequences for the prepro region of the yeast *MFα1* gene. This replacement was accomplished by inserting the 1.9-kb Bgl II-Bam HI fragment of pSEY210 (8) into the site vacated by complete digestion of pSHE1 with Bam HI, and partial digestion with Bcl I (the relevant Bcl I site is at the extreme 5' end of the *SUC2* insert; the excised fragment is 2.7 kb). The product of this manipulation was designated pαSHF8. In contrast to the *SUC2-HIS4* fusion, which contained amino acid residues 1–289 of secretory invertase, pαSHF8 encoded a fusion protein in which the first 88 amino acids of the prepro region of α-factor were fused in frame to invertase starting at amino acid 5 of the signal peptide sequence. This resulted in the deletion of four amino acids from the NH_2 terminus of the invertase portion of the *MFα1-SUC2-HIS4* fusion (see Emr et al., [8]).

Plasmids were introduced into yeast strains either by the spheroplast (17) or lithium acetate (19) procedure. Agarose gel electrophoresis, plasmid purification, fragment isolation, transformation of bacteria, and other recombinant DNA manipulations were performed by standard methods (30). All recombinant DNA modifying enzymes were used according to the suppliers' instructions.

Mutant Isolation and Screening

FC2-12B and DYFC2-12B cells containing pαSHF8 were grown to stationary phase in minimal medium supplemented with histidine, tryptophan, and leucine. 12 OD_{600} U of cells were harvested by centrifugation, washed with sterile 50 mM potassium phosphate buffer, pH 7.0, and resuspended at 2 OD_{600}/ml in potassium phosphate buffer. Ethyl methanesulfonate was added to a final concentration of 3%, and the cells were incubated with the mutagen for 60 min (round II) or 75 min (round I) at 30°C (% killing = 50% in round I, 73% in round II). The mutagen was quenched by adding an equal volume of sterile 12% sodium thiosulfate, and cells were collected by centrifugation, washed two times consecutively with potassium phosphate buffer and resuspended in 30 ml of minimal medium supplemented with leucine, tryptophan, and histidine. After a 20-h recovery period at 24°C, the mutagenized cells were centrifuged, resuspended to 5 OD_{600}/ml in potassium phosphate buffer and plated onto minimal medium supplemented with leucine, tryptophan, and 3 mM histidinol (1–3 × 10^6 cells per plate). After incubation at 30°C for 5–10 d, mutant clones were picked and steaked onto YPD plates. After 2 d at 30°C these patches were replica-plated onto YPD plates and individual replicas were incubated at 30 and 37°C. Clones that grew at 30°C but not at 37°C were picked and retested for Ts growth by streaking onto YPD plates at 37°C. Confirmed histidinol$^+$, Ts mutants were cured of the fusion plasmid by streaking them three times consecutively on nonselective medium (YPD). Uracil auxotrophs were isolated, rescreened for Ts growth, and retransformed with fresh pαSHF8 to assess the linkage of the Ts and histidinol$^+$ phenotypes to the original plasmid.

Ura$^-$ derivatives of the original isolates were screened by immunoblotting for the accumulation of intracellular forms of prepro-α-factor. Briefly, mutant colonies were inoculated into 3 ml of YPD and grown at 30°C to an OD_{600} of 1.5–15. 4 OD_{600} U of cells were collected and diluted to a volume of 3 ml with fresh YPD. These cultures were incubated for 2–3 h at 37°C and then diluted with an equal volume of ice-cold 20 mM NaN_3. The cells were collected by centrifugation, washed with ice cold 10 mM NaN_3, and resuspended in 0.3 ml Laemmli sample buffer (29) supplemented with 1 mM PMSF. Glass beads (~0.3 g of 0.3–0.5-mm diam) were added and the mixture was vortexed vigorously for 2 min, then immediately heated in boiling water for 5 min. Aliquots (24 μl) of these samples were applied to 12.5% SDS polyacrylamide gels and electrophoresis was performed as described by Laemmli (29).

Electrophoretically separated proteins were transferred to nitrocellulose filters (4), and the filters were processed essentially as described by Kaiser and Botstein (23), except that the blocking incubation was for 30 min at 24°C, and anti-α-factor antiserum (1/500 dilution) was used to probe the filters. An unfractionated lysate, prepared from *MATa* cells (PBY404C), was included at a final concentration of 3–5 mg of protein/ml during the antibody incubation to block antibody association with filter-bound proteins not related to α-factor. After decoration of bound antibodies with ^{125}I-labeled protein A, filters were exposed to X-ray film with an intensifying screen at −70°C.

Radiolabeling and Immunoprecipitation

Cells were grown overnight in minimal medium supplemented with 200 μM $(NH_4)_2SO_4$ to an OD_{600} of 0.5–1.0. An aliquot of cells was sedimented for 2–3 min at room temperature in a clinical centrifuge, washed with distilled water, and resuspended to 1–2 OD_{600}/ml in minimal medium supplemented with 0–10 μM $(NH_4)_2SO_4$. For radiolabeling of invertase, cells were first derepressed for invertase production by incubation in minimal medium (200 μM ammonium sulfate) plus 0.1% glucose for 30 min at 30 or 37°C, then collected and resuspended in minimal medium containing 0–10 mM $(NH_4)_2SO_4$ and 0.1% glucose. Radioactive sulfate ($H_2{}^{35}SO_4$) was added at a concentration of 200–300 μCi/OD_{600}, and incorporation was carried out for 20–40 min at 30 or 37°C. Labeling was terminated by the addition of an equal volume of ice-cold 20 mM NaN_3, and samples were chilled on ice for 5 min. Radioactive cells were sedimented in a clinical centrifuge, washed with ice-cold 10 mM NaN_3, and resuspended at 5–20 OD_{600}/ml in spheroplasting buffer, which contains 1.4 M sorbitol, 60 mM β-mercaptoethanol, 25 mM MOPS, pH 7.4, 10 mM NaN_3, 5 mM $MgCl_2$. Lyticase was added (25 U/OD_{600}) and cells were converted to spheroplasts during a 45-min incubation at 30°C. Spheroplasts were sedimented for 20 s in a microcentrifuge, resuspended to 5–10 OD_{600}/ml in 1% SDS, heated to 100°C for 4 min, and centrifuged for 10 min in a microcentrifuge (Oscar Fisher Co., Inc., Newburgh, NY) to remove insoluble material. Aliquots of the supernatant fractions were diluted to 1.0 ml in 200 mM NaCl, 12.5 mM sodium phosphate, pH 7.4 (PBS), 1% Triton X-100, 0.25% SDS. α-Factor (1 μl/OD_{600} cell equivalent), invertase (1.5 μl/OD_{600} cell equivalent) or carboxypeptidase Y (CPY) (2 μl/OD_{600} cell equivalent) antiserum was added in saturating amounts, and immunoprecipitations were carried out for 3–16 h at 4°C. For α-factor and invertase immunoprecipitations, samples were supplemented with an unfractionated lysate of nonradioactive cells (2 mg protein/ml) from strain PBY404C (*suc2*Δ, *MATa*). After the antibody incubation, protein A-Sepharose was added in saturating amounts (1 μl beads/μl antiserum) and the incubation was continued for 1–2 h at room temperature. Immune complexes were sedimented in a microcentrifuge, and washed sequentially with PBS, 1% Triton X-100, 0.1% SDS (two times), and 50 mM NaCl, 10 mM Tris, pH 7.4 (two times). Bound antigens were then dissociated by heating in Laemmli sample buffer, and samples were applied to 7.5% (for invertase and CPY) or 12.5% (for prepro-α-factor) SDS polyacrylamide gels. After SDS-PAGE, the gels were fixed and treated with Amplify. Radioactive proteins were visualized by exposure to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) 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).

Glycoprotein Precipitation with Con A-Sepharose 4B

Cells were labeled with [^{35}S]SO_4^{2-} and extracts were diluted 10 fold with concanavalin A reaction buffer (0.5 M NaCl, 20 mM Tris, pH 7.4, 2% Triton X-100) and split into two equal portions. One half was immunoprecipitated directly with antibodies against α-factor or CPY, and the other was treated with Con A-Sepharose 4B beads (30 μl packed volume of beads for the amount of whole-cell extract derived from 1 OD_{600} of cells) for 3 h at room temperature. Con A-coated beads were then sedimented in a microcentrifuge. The supernatant fraction (unbound) was removed, adjusted to 0.2% SDS, and heated in boiling water for 3 min; the beads (bound fraction) were washed two times with con A reaction buffer and Con A-bound glycoproteins were eluted by heating the beads in 200 μl 1% SDS at 100°C. The eluate was clarified by centrifugation and diluted to 1.0 ml in Con A reaction buffer. Unbound and bound fractions were then immunoprecipitated with α-factor or CPY antiserum as described in the preceding section.

Subcellular Fractionation and Proteolysis of sec18 sec61 Extracts

Fractionation of RDM 15-3A extracts was performed as described by Bernstein et al. (1) except that prior to spheroplast formation the culture was incubated for 60 min at 30°C in YPD containing 5% glucose. Immunoblotting was performed as described above in *Mutant Isolation and Screening*. NADPH cytochrome *c* reductase (25), glyceraldehyde-3-phosphate dehydrogenase (1), and total protein (31) were assayed as previously described. The latency of the NADPH cytochrome *c* reductase activity was determined by performing assays in the absence and presence of 0.1% Triton X-100.

Determinations of the proteolytic sensitivity of accumulated prepro-α-

factor and proCPY in lysates of *sec*18 *sec*61 cells were performed starting with spheroplasts prepared as described in the previous section. All subsequent operations were performed at 4°C. Spheroplasts (25 OD_{600} U) were layered over a 10-ml cushion of SPB and 1.9 M sorbitol, and sedimented at 5,000 rpm for 5 min in a Sorvall HB-4 rotor (DuPont/Sorvall, Newtown, CT). The spheroplast pellet was resuspended in 0.73 ml lysis buffer (0.3 M mannitol, 0.1 M KCl, 50 mM Tris, pH 7.5, 1 mM EGTA), transferred to a 2.0 ml Potter-Elvejem homogenizer tube, and lysed by three consecutive cycles of homogenization (1 min of homogenization followed by 1 min on ice) using a motor-driven Potter-Elvejem teflon pestle. After centrifugation at 2,000 rpm for 4 min in a Sorvall HB-4 rotor, the resulting cell-free extract was split into two 0.3-ml aliquots. One sample was adjusted to 0.4% Triton X-100, and 40-μl aliquots (without protease control) from each sample were transferred to 0.56 ml of 20% trichloroacetic acid (TCA). Proteinase K was added to the remainder of both samples at a final concentration of 0.3 mg/ml, and at 0, 1.5, 3, 10, and 20 min, 42-μl aliquots from each sample were quenched in 0.56 ml of 20% TCA (processing of the zero time points required 15 s). TCA precipitates were collected by centrifugation and washed with −20°C acetone. Precipitated proteins were solubilized in 50 μl of Laemmli sample buffer, and samples were diluted and immunoprecipitated with anti-α-factor and anti-CPY sera.

In Vitro Transcription and Translation

mRNA coding for secreted invertase was prepared by in vitro transcription of pGEM2-*SUC*2-23 (provided by J. Rothblatt), which contains the entire *SUC*2 gene inserted into pGEM2 downstream of the bacteriophage SP6 promoter (32). The template was cut with Pvu II, followed by phenol extraction and ethanol precipitation. Linear pGEM2-*SUC*2-23 (1.2 μg) was transcribed in a 20-μl reaction using SP6 polymerase as described by Rothblatt and Meyer (42). A 1-μl aliquot of the transcription mix was translated (10 μl reaction) in the BRL nuclease-treated wheat germ in vitro translation system, and the reaction was terminated by adding SDS to 2% and heating at 95°C for 4 min. Translation products were immunoprecipitated with anti-invertase serum as described above in *Radiolabeling and Immunoprecipitation*. The in vitro translation products shown in Fig. 6, lanes *7* and *9* each represent one-sixth of a 10-μl reaction.

Hybrid-selected α-factor mRNA was translated in a rabbit reticulocyte lysate as described by Bernstein et al. (1).

Results

A Selection Scheme for Yeast Translocation Mutants

Numerous experiments have demonstrated that a signal sequence can direct cytoplasmic proteins to the secretory pathway in vivo (7, 47, 49). Based on these data, we reasoned that if a signal sequence were attached to a cytoplasmic enzyme required for the formation of an essential nutrient, the hybrid protein would be directed to the ER. If the substrate of the enzyme were limited to the cytoplasm, the cells would not grow unless they were supplied with the nutrient. Mutations that block hybrid protein import into the ER would allow cells to grow on the substrate.

We chose the *HIS*4 gene because it encodes a trifunctional cytoplasmic polypeptide that can sustain amino-terminal protein fusion events and retain histidinol dehydrogenase activity associated with the carboxy-terminal domain (24, 40). Histidinol dehydrogenase catalyzes the last step in histidine biosynthesis, the conversion of histidinol to histidine. Cells that express wild-type *HIS*4 protein and contain the mutant allele *HOL*1-1 (this mutation increases the efficiency of histidinol uptake) are able to convert exogenously supplied histidinol to histidine.

We predicted that if a signal sequence were fused to the amino terminus of the *HIS*4 protein, it would be translocated into the lumen of the ER and glycosylated, as shown schematically in Fig. 1 *A*. Wild-type cells (bearing the *his*4-401 deletion which overlaps the A, B, and C regions of *HIS*4) expressing this plasmid-encoded fusion protein would not convert histidinol to histidine in that the histidinol dehydrogenase would be sequestered from the cytoplasm, and charged, polar molecules such as histidinol or histidine are unlikely to penetrate across the ER membrane. Therefore, these cells would not grow on medium containing histidinol in place of histidine. If cells containing this gene fusion were selected for growth on histidinol, mutants that mislocalized the fusion protein to the cytoplasm would convert the histidinol to histidine (Fig. 1 *B*) and grow.

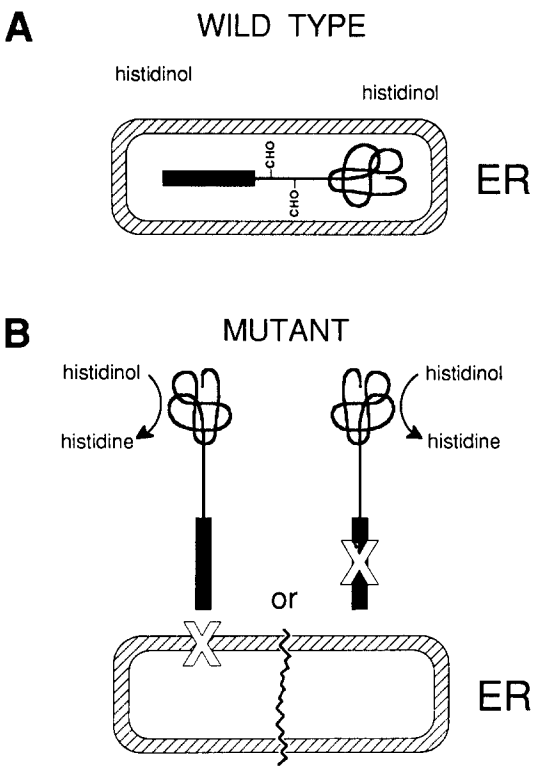

Figure 1. Predicted topology of a signal peptide–containing *HIS*4 hybrid protein. The predicted subcellular location and enzymatic properties of a histidinol dehydrogenase fusion protein are shown for both (*A*) wild-type and (*B*) mutant cells. (*CHO*) Asparagine-linked carbohydrate; (▬) a signal sequence; and (*folded black trace*) the catalytic domain of the fusion protein. (*X*) A mutation that inactivates either the signal sequence or some cellular component (membrane-bound or cytosolic) required for protein translocation.

Potentially this procedure would select for mutations that disrupted signal sequence function or mutations that disabled the cellular machinery responsible for targeting secretory proteins to the ER. These alternative possibilities are indicated by *X* marks in Fig. 1 *B*. Genetic tests (i.e., plasmid linkage) may be used to distinguish between these two classes of mutations. The strains described in this report contained recessive mutations that caused pleiotropic defects in the ER targeting apparatus.

In order to recover viable, translocation-defective cells, it was necessary to strike a balance between the lethal effect of secretory mutations and the requirement for sufficient cytosolic histidinol dehydrogenase activity. For this purpose, the growth temperature (30°C) represented a semipermissive condition where the mutant phenotype was only partially ex-

pressed. To identify mutants with more severe mislocalization defects, clones selected for growth on histidinol at 30°C were screened on rich medium for Ts growth at 37°C, presumably owing to an exaggeration of the partial defect expressed at 30°C.

Expression of HIS4 Hybrid Proteins

Two conditions were required for the approach to succeed: first, protein products of *HIS*4 gene fusions must retain histidinol dehydrogenase activity; and second, such a fusion protein must be directed to the yeast secretory apparatus in vivo, rendering the enzyme unable to supply histidine for growth. To test the first requirement, a gene fusion that encodes both a cytoplasmic and a signal peptide-containing hybrid was produced by ligating the 5′ half of the *SUC*2 coding region to a fragment containing the bulk of the *HIS*4 coding region (Fig. 2 *A*). The *SUC*2 gene codes for two different species of the yeast enzyme invertase (5): a cytoplasmic, unglycosylated form of the enzyme is expressed constitutively, and a secreted, highly glycosylated form is derepressed by growth in medium containing low concentrations of glucose. The primary structure of these two polypeptides differs only in the transitory presence of a signal peptide at the amino terminus of the secreted preenzyme. Yeast cells (*his*4Δ, *HOL*1-1) transformed with a multicopy plasmid containing the *SUC*2-*HIS*4 gene fusion (pSHE1) directed the synthesis of two different fusion proteins (data not shown). As expected, a nonglycosylated 130-kD species was produced constitutively. When transformants were shifted to derepression medium, a glycosylated fusion protein was also synthesized. To determine whether the cytoplasmic hybrid protein possessed histidinol dehydrogenase activity, pSHE1 transformants (*his*4Δ, *HOL*1-1) were assayed for their ability to grow on minimal medium containing histidinol and high concentrations of glucose. As a control, untransformed cells and cells transformed with vector sequences lacking the *HIS*4 insert were also analyzed. The results shown in Fig. 2 *B* demonstrated that the *SUC*2-*HIS*4 fusion allowed *his*4Δ, *HOL*1-1 cells to grow on histidinol. This capacity was dependent on the *HIS*4 insert, because clones transformed with vector sequences did not grow.

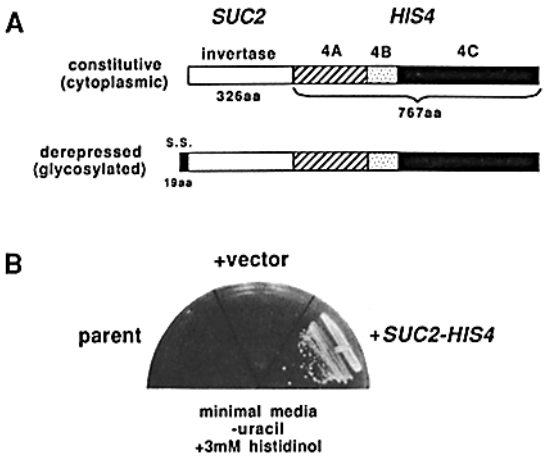

Figure 2. A *SUC*2-*HIS*4 gene fusion confers growth on histidinol. (*A*) The predicted structure of the protein products of the *SUC*2-*HIS*4 fusion are shown. *4A*, *4B*, and *4C* refer to separate domains of the trifunctional *HIS*4 protein; histidinol dehydrogenase activity is encoded by *4C*. The signal sequence of secretory invertase is shown as a small black rectangle at the NH_2 terminus of the derepressed fusion protein. (*B*) DYFC2-12B cells without plasmid (parent), transformed with vector sequences alone (+vector), or transformed with a multicopy plasmid containing the *SUC*2-*HIS*4 gene fusion (+*SUC*2-*HIS*4) were streaked onto minimal medium supplemented with 5% glucose, leucine, tryptophan, and 3 mM histidinol. These growth conditions should allow production of only the cytoplasmic fusion protein. The plate shown was incubated at 30°C for 3.5 d.

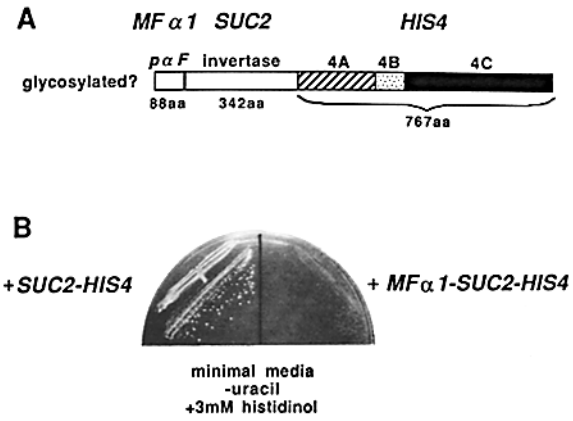

Figure 3. A *MFα1-SUC*2-*HIS*4 gene fusion does not confer growth on histidinol. (*A*) The predicted structure of the protein product of a *MFα1-SUC*2-*HIS*4 gene fusion is shown. The signal sequence-containing prepro region of α-factor is abbreviated as *pαF*. *4A*, *4B*, and *4C* are as described in Fig. 2 *A*. (*B*) DYFC2-12B cells transformed with multicopy plasmids containing either the *SUC*2-*HIS*4 (pSHE1) or *MFα1-SUC*2-*HIS*4 (pαSHF8) gene fusion were analyzed as described in Fig. 2 *B*.

A test of the second condition required elimination of the cytoplasmic invertase-histidinol dehydrogenase hybrid protein. This requirement was met by replacing the promoter of the *SUC*2 gene with upstream and coding sequences from the yeast *MFα1* gene (Fig. 3 *A*). *MFα1* encodes the precursor of the secreted mating pheromone α-factor (27). Multicopy plasmids (pαSHF8) bearing this tripartite fusion were introduced into yeast cells identical to those used in the previous experiment. In contrast to the pSHE1 transformants, cells containing pαSHF8 were not able to grow on minimal medium plus histidinol (Fig. 3 *B*).

Production of the glycosylated hybrid protein encoded by pαSHF8 was evaluated with transformed cells that were pulse-labeled with $[^{35}S]SO_4^{2-}$ in the presence or absence of tunicamycin, which inhibits asparagine-linked glycosylation. Extracts prepared from these cells were treated with invertase antiserum, which recognizes determinants encoded by the *SUC*2 portion of the fusion. Cells labeled in the absence of tunicamycin synthesized two species of fusion protein (Fig. 4, lane *1*). The predominant species migrated with a molecular mass of ~160 kD. In addition, a minor product of 140 kD was detected. When the transformant was labeled in the presence of tunicamycin (lane *2*), a single polypeptide that comigrated with the lower M_r form seen in lane *1* was made. The molecular mass of the unglycosylated material in lane *2* agreed with that predicted for an unmodified prepro-α-factor-invertase-*HIS*4 hybrid protein. Both polypeptides were plasmid encoded, in that cells containing vector se-

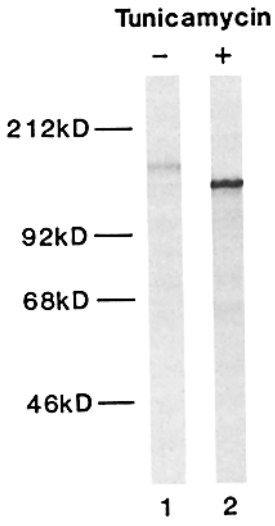

Figure 4. The *MFα1-SUC2-HIS4* fusion plasmid directs the synthesis of a glycoprotein. DYFC2-12B cells (2 OD_{600} per sample) transformed with pαSHF8 were labeled for 20 min at 30°C with $[^{35}S]SO_4^{2-}$ in the presence or absence of 10 μg/ml tunicamycin after a 15-min pretreatment with or without tunicamycin, respectively. Whole-spheroplast extracts were treated with anti-invertase serum and the immunoprecipitates were fractionated on a 7.5% SDS-polyacrylamide gel. Lanes *1* and *2* are individual tracks from a single polyacrylamide gel.

quences did not express any high M_r cross-reactive material (data not shown). Also, both products were under mating-type regulation, because neither *MATa* haploids nor *MATa/MATα* diploids transformed with pαSHF8 expressed any hybrid protein as expected for a gene under the control of *MATα* (data not shown). The nature of the 140-kD species seen in lane *1* was not examined further. In any event, this plasmid did not complement the chromosomal *his4* deletion. These data suggested that the pαSHF8 transformants were unable to grow on histidinol because the majority of hybrid protein was localized to the secretory pathway.

Isolation of Temperature-sensitive Histidinol Prototrophs

Inasmuch as the primary assumptions were confirmed by these experiments, the histidinol selection scheme was applied to yeast cells (DYFC2-12B; see Table I) containing the *MFα1-SUC2-HIS4* fusion plasmid. Transformants were mutagenized with ethyl methanesulfonate, allowed to recover overnight in minimal medium (-uracil) at 30°C, and plated on minimal medium (-uracil) with histidinol substituted for histidine. After incubation for 5–7 d at 30°C, plates were examined for the presence of histidinol prototrophs (histidinol$^+$). The frequency of mutations that allowed growth on histidinol was ~2.5×10^{-5} (Table II, round I). 440 histidinol prototrophs were then screened for Ts growth on rich medium at 37°C. Five clones exhibited both Ts and histidinol$^+$ phenotypes. Derivatives of all five isolates that had been cured of the plasmid were not able to grow on histidinol, confirming that the histidinol$^+$ phenotype was dependent on the plasmid. These cells also exhibited Ts growth on rich medium, demonstrating that thermosensitivity was caused by a chromosomal mutation. Retransformation of these clones with unmutagenized pαSHF8 restored growth on histidinol medium, demonstrating that this phenotype was also due to a chromosomal mutation, as opposed to plasmid-linked defects such as signal sequence mutations.

Secretion defects were tested directly by assaying for the accumulation of secretory precursors in plasmid-cured derivatives from four of the original isolates. Whole-cell extracts were fractionated by SDS-PAGE and immunoblotted with antiserum that reacts with prepro-α-factor. Three of the mutants accumulated a precursor form of α-factor that was not seen in wild-type cells.

A genetic relationship between the thermosensitive growth and α-factor accumulation phenotypes was evaluated by backcrossing two mutants to wild-type strains. Tetrads derived from these crosses exhibited low spore viability, and we discovered that this was because the original isolates were diploid. The unmutagenized parent strain was also diploid, possibly as a result of the transformation procedure. Haploid mutants were obtained from two of the isolates by mating with *MATa/MATα* diploids to form tetraploids, followed by two consecutive rounds of meiosis and tetrad analysis. Backcrosses of these haploid mutants to wild-type strains revealed that both Ts defects segregated as single mutations (two Ts and two wild-type spores in 19/19 tetrads analyzed in each cross). Thermosensitivity was inseparable from the prepro-α-factor accumulation property in that 10 out of 19 *MATα* spore clones were Ts and each accumulated prepro-α-factor. All nine wild-type progeny, however, failed to accumulate prepro-α-factor.

Heterozygous diploids, derived by mating both Ts mutants to wild-type strains, grew at 37°C, demonstrating that the Ts mutations were recessive. Precursor forms of carboxypeptidase Y and invertase detected in haploid mutant cells were absent in the heterozygotes, indicating that the accumulation phenotypes were recessive also. Because the parental strain was diploid, these recessive mutations may have been recovered by gene conversion or mitotic recombination events that occurred during or shortly after mutagenesis. Complementation analysis revealed that all five Ts mutations were allelic,

Table II. Histidinol Selection Scheme Enriches for Temperature-sensitive Translocation Mutants

	Round I*		Round II‡	
Stage of mutant isolation	Colonies analyzed/ total colonies	Fraction§ of total	Colonies analyzed/ total colonies	Fraction§ of total
Cells plated on histidinol medium	2×10^7	–	2.4×10^7	–
Histidinol prototrophs (histidinol$^+$)	492	2.5×10^{-5}	1,600	6.7×10^{-5}
Temperature-sensitive for growth	5‖/440	2.8×10^{-7}	58/600	6.4×10^{-6}
Accumulation of α-factor precursor¶	3/4	2.1×10^{-7}	7/40	1.1×10^{-6}

* DYFC2-12B diploid cells were used for mutant isolation.
‡ FC2-12B haploid cells were used for mutant isolation.
§ This value represents the number of colonies exhibiting a given phenotype (or the number expected to exhibit that phenotype, if all colonies were analyzed) divided by the number of cells plated on histidinol.
‖ All of these isolates are allelic.
¶ Immunoblots of whole cell lysates were probed with α-factor antiserum as described in Materials and Methods.

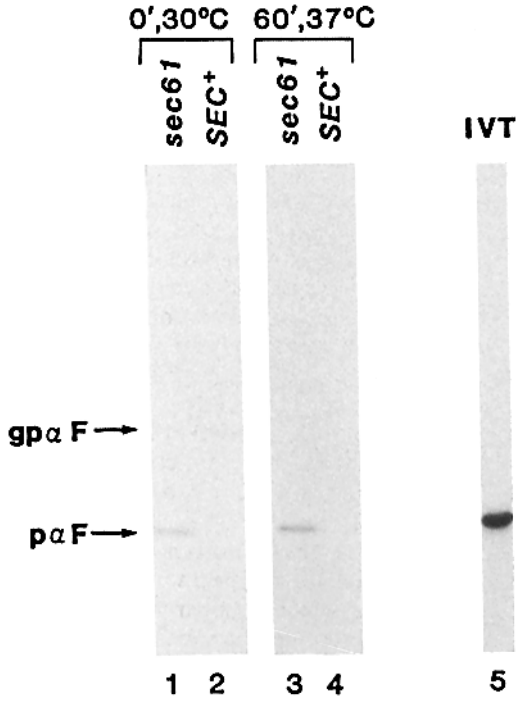

Figure 5. Unmodified prepro-α-factor accumulates at both the nonpermissive and semipermissive temperatures in *sec*61 strains. RDM 15-5B (*sec*61) and RDM 15-9B (*SEC*$^+$) cells were labeled with [^{35}S]SO_4^{2-} at 30°C for 30 min (lanes *1* and *2*) or at 37°C for 30 min after a 60-min incubation at 37°C (lanes *3* and *4*). Spheroplast pellet lysates were prepared and incubated with anti-α-factor serum. Immunoprecipitates were evaluated by SDS-PAGE on a 12.5% polyacrylamide gel. Each lane contains the material derived from 0.5 OD_{600} U of cells. Glycosylated prepro-α-factor in transit through the secretory pathway is designated *gpαF*; *pαF* refers to prepro-α-factor. *IVT* (lane *5*) is the in vitro translation product of prepro-α-factor mRNA. Lanes *1–5* were all derived from a single polyacrylamide gel.

although it was not certain that the mutations were of independent origin. The gene defined by these mutations was designated *sec*61.

To assess whether the proportion of Ts, histidinol$^+$ mutants among a population of mutagenized haploid cells might be substantially greater, the procedure was repeated using haploid FC2-12B cells transformed with pαSHF8. As expected, the frequency of "*sec*" mutants obtained from the histidinol selection was about fivefold greater when haploid cells were used (Table II, round II). All seven candidates obtained from round II complemented *sec*61. At least one additional complementation group was been identified among these isolates. The phenotype of strains bearing a mutant allele of this gene, *sec*62, is being investigated.

sec61 Cells Accumulate Unprocessed α-Factor Precursor

More information on the effect of the *sec*61 mutation was obtained by assessing the fate of prepro-α-factor expressed at the semipermissive and nonpermissive temperatures. Wild-type cells labeled for 30 min at either 30 or 37°C contained very little α-factor cross-reactive material (Fig. 5, lanes *2* and *4*) because of the rapid rate of α-factor secretion (22). At 30°C, a trace amount of core-glycosylated prepro-α-factor (*gpαF*) in transit through the early stages of the secretory pathway was detected (lane *2*).

When *sec*61 cells were labeled for 30 min at 30°C or 30 min at 37°C after a 60-min preshift to the nonpermissive temperature, a distinct species of α-factor precursor (*pαF*) was accumulated (Fig. 5, lanes *1* and *3*). This form of α-factor was not detected in wild-type cells at either temperature. The expression of a secretory defect at both 30 and 37°C was predicted by the selection scheme, which required that cells be partially defective at the growth temperature. Mutant cells also accumulated substantial amounts of α-factor precursor at 24°C. Additionally, *sec*61 strains grew slower than wild-type yeast at several temperatures, suggesting that there was no completely permissive temperature for *sec*61 function. Though a preshift to 37°C exaggerated the mutant phenotype, it did not completely inhibit α-factor secretion, in that even after a 60-min preshift to the nonpermissive temperature, newly synthesized biologically active pheromone was detected in the growth medium (not shown). The precursor that accumulated at the *sec*61 block comigrated with the in vitro translation product of hybrid selected *MFα1* mRNA electrophoresed on the same SDS-polyacrylamide gel (lane *5*). This result demonstrated that *sec*61 cells accumulated a form of α-factor that was not detectably modified either by glycosylation or proteolytic processing (22). One possible interpretation was that the *sec*61 mutation blocked an early event in protein translocation, prior to addition of core oligosaccharides in the lumen of the ER.

Multiple Unprocessed Secretory Proteins Accumulate in sec61 Cells

α-Factor shares early stages of the secretory pathway with other cell surface and vacuolar proteins (22), hence *sec*61 should interrupt the processing and localization of these proteins as well. This point was tested for the cell wall enzyme invertase and the vacuolar enzyme CPY by radiolabeling wild-type and mutant cells at 30 and 37°C, and treating spheroplast lysates with antisera reactive with each protein.

Evaluation of CPY precursor forms was complicated by the coincident electrophoretic mobilities of the unglycosylated precursor and the glycosylated mature species (50). This problem was circumvented by introduction of the *pep*4-3 mutation which blocks proenzyme cleavage (16). In *SEC pep*4-3 strains radiolabeled at 30 or 37°C (Fig. 6 *A*, lanes *2* and *4*), two forms of proCPY were seen: plCPY, a core glycosylated 67-kD precursor in transit through the ER, and a mature (*M*) 69-kD species that is found in the Golgi body and vacuole (50).

A novel form of proCPY was seen when *sec*61 *pep*4-3 strains were radiolabeled at 30 or 37°C after a 2-h incubation at the nonpermissive temperature. The new 58-kD species migrated as predicted for the primary translation product of the gene encoding CPY (53). At 30°C the *sec*61 defect was incomplete, in that both pl and mature forms of proCPY were also detected. After a 120-min preshift to 37°C, however, all of the newly synthesized proCPY accumulated at the *sec*61 block. With shorter 37°C preincubations (30 or 60 min), a fraction of the proCPY synthesized at 37°C escaped the *sec*61 block and appeared as the pl and mature forms (data not shown). These data suggested that the *sec*61 mutation was leaky, and required long temperature shifts to be fully expressed.

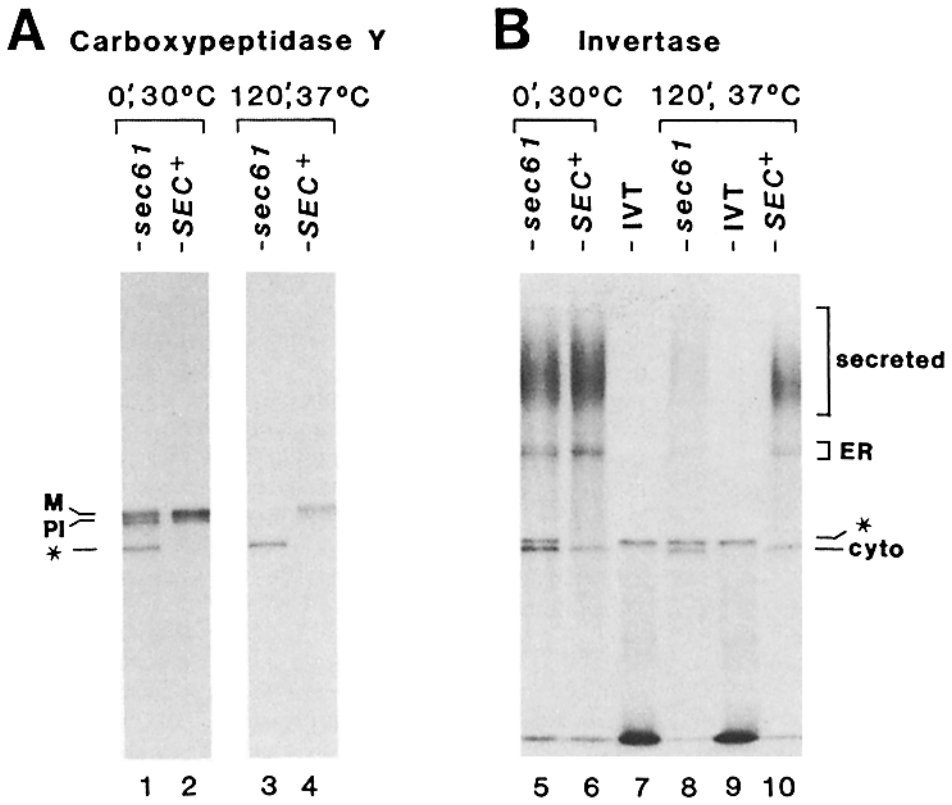

Figure 6. Unmodified precursors of invertase and CPY accumulate in *sec*61 cells. (*A*) The same extracts that were used for the experiment described in Fig. 5 were treated with anti-CPY serum. Immunoprecipitates were fractionated on a single 7.5% polyacrylamide gel. Lanes *1* and *2*, cells were labeled at 30°C for 30 min; lanes *3* and *4*, cells were labeled at 37°C for 30 min after a 120-min preincubation at 37°C. Lanes *1–4* contain the material derived from 0.2 OD_{600} U of cells. *PI*, *M*, and * refer to glycosylated proCPY in transit through the ER, mature proCPY in the Golgi body or vacuole, and proCPY accumulated at the *sec*61 block, respectively. (*B*) RDM 15-5B (*sec*61) and RDM 15-9B (*SEC*+) cells were labeled with $[^{35}S]SO_4^{2-}$ for 30 min at 30°C (lanes *5* and *6*) or for 30 min at 37°C after a 120-min incubation at 37°C (lanes *8* and *10*). Before each labeling, cultures were derepressed for invertase production in 0.1% glucose minimal medium at 30°C for 30 min (lanes *5* and *6*) or at 37°C for 30 min (lanes *8* and *10*). Whole-spheroplast lysates were prepared and treated with anti-invertase serum. Immune complexes were harvested and analyzed by SDS-PAGE on a 7.5% polyacrylamide gel. Lanes *5*, *6*, *8*, and *10* represent the total invertase derived from 0.5 OD_{600} U of cells. *IVT* (lanes *7* and *9*) is the in vitro translation product of secreted invertase mRNA. *ER*, *cyto*, and * refer to core glycosylated invertase in transit through the ER, cytoplasmic (unglycosylated) invertase, and secretory preinvertase accumulated by the *sec*61 mutation, respectively.

Additional information concerning the position of the *sec*61 block was revealed by analysis of radiolabeled invertase. Wild-type cells produced three discrete types of invertase at 30 or 37°C (Fig. 6 *B*, lanes *6* and *10*). Core-glycosylated intermediates in transit through the ER and highly glycosylated cell wall molecules were synthesized in response to glucose deprivation. A nonglycosylated cytoplasmic enzyme was made constitutively (9). Besides these species, *sec*61 mutant cells labeled at 30°C or after a 2-h incubation at 37°C accumulated an additional form (lanes *5* and *8*) that comigrated with the in vitro translation product of secretory invertase mRNA (Fig. 6 *B*, lanes *7* and *9*). The difference in M_r between the secreted and cytoplasmic primary translation products is due to the presence of a signal peptide at the amino terminus of the secretory preprotein (41). Comigration of *sec*61-specific invertase with the signal sequence-containing in vitro translation product indicated that the *sec*61 defect was imposed prior to signal peptide cleavage.

Based on the SDS gel mobility of precursors accumulated in *sec*61 cells, the block appeared to precede the addition of asparagine-linked core oligosaccharides, which are transferred to protein in the lumen of the ER. This prediction was tested directly by treating extracts from $[^{35}S]SO_4^{2-}$-labeled *sec*61 cells with the mannose-binding lectin Con A immobilized on Sepharose beads. Beads were recovered by centrifugation, pellet and supernatant fractions were treated with SDS, and the distribution of accumulated precursors was assessed by immune precipitation with α-factor and CPY antisera. The data in Fig. 7 *A*, lane *1* show the total complement of α-factor present in the *sec*61 extract derived from the same sample depicted in Fig. 5, lane *1*. When mixed with Con A-Sepharose and separated into pellet (lane *3*) and supernatant (lane *2*) fractions, glycosylated prepro-α-factor in transit through the ER was quantitatively recovered in the bound fraction, whereas prepro-α-factor accumulated by the *sec*61 mutation remains in the supernatant. The results obtained for proCPY were similar to those for α-factor. The data in Fig. 7 *B*, lane *4* shows the proCPY present in the total extract (same sample as Fig. 6, lane *1*). Mature and plCPY were bound to the Con A-Sepharose (lane *5*), although some material was lost in sample preparation. In contrast, the *sec*61-specific proCPY (*) was quantitatively recovered in the unbound fraction (lane *6*). Preinvertase accumulated in *sec*61 mutants behaved identically to proCPY and prepro-α-factor (data not shown). In all cases, the association was specific for mannose, as binding was prevented by the competitor, α-methylmannoside (data not shown).

sec61-Accumulated Preproteins Are Exposed to the Cytoplasm

The histidinol selection scheme demanded that a prepro-α-factor-invertase-*HIS*4 chimeric protein accumulate in a loca-

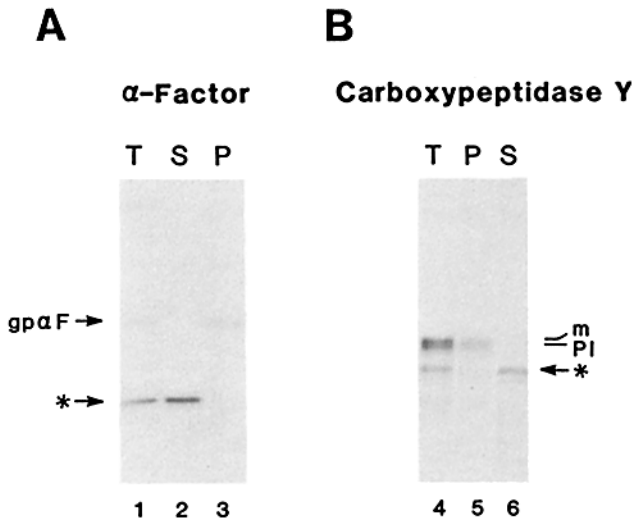

Figure 7. CPY and α-factor precursors accumulated at the *sec*61 block do not bind to Con A. (*A*) The $[^{35}S]SO_4^{2-}$ labeled extract (0.5 OD_{600} per lane) used to generate the sample shown in Fig. 5, lane *1* was directly immunoprecipitated with anti-α-factor serum (lane *1*), or was treated with Con A-Sepharose 4B and separated by sedimentation into bound (lane *3*) and free (lane 2) fractions prior to immunoprecipitation. *T*, *S*, and *P* are abbreviations for the total, supernatant, and pellet fractions. The position of the glycosylated α-factor precursor is indicated by *gpαF*; sec61-specific prepro-α-factor is indicated by *. (*B*) Same as in *A*, except the sample used (0.3 OD_{600} per lane) is identical to Fig. 6, lane *1*. Lane *4*, total CPY antigen; lane *5*, bound (pellet) proCPY species; lane *6*, free (supernatant) proCPY material. *PI*, *m*, and * are as described in Fig. 6 *A*.

tion accessible to histidinol. The most likely location of this fusion protein, and of preproteins whose localization was perturbed by the *sec*61 mutation, was in the cytoplasm or on the cytoplasmic face of the ER. The lack of detectable modifications on preproteins accumulated by the *sec*61 cells was consistent with this prediction. To test directly whether these precursors were exposed to the cytoplasmic compartment, extracts of $[^{35}S]SO_4^{2-}$-labeled *sec*61 cells were exposed to protease in the absence and presence of detergent. Proteolysis was terminated by the addition of TCA, and the quenched reactions were evaluated by immune precipitation with anti-α-factor and anti-CPY sera, followed by SDS-PAGE.

Extracts were prepared from a strain in which the disposition of precursor polypeptides and the integrity of ER vesicles could be evaluated simultaneously. For this purpose, a strain bearing mutant copies of the *sec*18 and *sec*61 genes was constructed. The *sec*18 mutation, which is nonpermissive at 30°C, causes core-glycosylated secretory proteins to accumulate within the lumen of the endoplasmic reticulum (9, 36). When *sec*18 *sec*61 mutants grown at 24°C were shifted to 30°C, a fraction of the newly synthesized prepro-α-factor was accumulated at the *sec*61 stage. The prepro-α-factor that escaped the *sec*61 block became core-glycosylated and arrested by the *sec*18 mutation. This glycosylated prepro-α-factor served as a reference for the lumen of the endoplasmic reticulum. The results in Fig. 8 *A* document the sensitivity of glycosylated prepro-α-factor and prepro-α-factor to proteinase K. When no protease was added (lanes *1* and *7*), glycosylated and unglycosylated forms of prepro-α-factor were seen. Upon exposure to protease for increasing lengths of time in the absence of detergent (lanes *2–6*), the amount of *sec*61 accumulated prepro-α-factor diminished gradually while the lowest mobility *sec*18 form (glycosylated prepro-α-factor) remained resistant to digestion. After a 20-min incubation with proteinase K, 90% of the prepro-α-factor was degraded, while 85% of the glycosylated form was resistant to proteolysis. The residual amount (10%) of prepro-α-factor that resisted protease action was similar to the fraction of NADPH cytochrome *c* reductase activity that was latent in this extract (see below). In the presence of detergent (lanes *8–12*), all species of prepro-α-factor were degraded rapidly; the digestion was essentially complete after 3-min (lane *10*).

As a control to estimate the proportion of lysed membranes in the extract, a mock-digested aliquot was centrifuged to obtain pellet and supernatant fractions. α-Factor immunoprecipitates of these fractions are displayed in lanes *13* and *14*. The extent of protection of *sec*18 prepro-α-factor was proportional to the amount of material that sedimented (compare lane *6* with lane *13*). Prepro-α-factor accumulated at the *sec*61 block, however, was almost completely protease sensitive in the absence of detergent (lane *6*), even though it sedimented quantitatively (lane *13*). Though clearly accessible to exogenous protease, the rate of prepro-α-factor digestion was accelerated two- to threefold by detergent. This may reflect increased exposure of this material to protease in the presence of detergent, implying that prepro-α-factor retained at the *sec*61 block was associated with some membrane-bound component, or directly with the phospholipid bilayer.

Protease sensitivity of accumulated prepro-α-factor was also examined in a homogenate prepared from a *sec*61 *SEC*18 strain. Although these samples did not contain the glycosylated control, the unglycosylated precursor was degraded at a rate similar to that seen in Fig. 8 *A*. Hence, proliferated ER membrane produced by the *sec*18 block (36) did not alter the behavior of the *sec*61 species.

Fig. 8 *B* documents the protease sensitivity of the *sec*18 and *sec*61 forms of proCPY present in the same extract. In the absence of detergent, proCPY that accumulated at the *sec*18 block was refractory to proteolysis over a 20-min incubation, while proCPY held at the *sec*61 block was completely digested within 1.5 min (lanes *2–6*). In the presence of detergent, all species of proCPY were susceptible to proteolysis, though the glycosylated proCPY was only partially digested (lanes *8–12*). The results in lane *13* demonstrate that the majority of proCPY present in this extract was sedimentable.

To ensure that the surface of the ER membrane was accessible to proteinase K, assays were performed to determine the latency of the cytoplasmically exposed ER membrane enzyme, NADPH cytochrome *c* reductase (25), to its substrate cytochrome *c*. Assays performed with the same extract used in these experiments indicated that only 10% of the reductase activity was latent.

Prepro-α-Factor Accumulated in sec61 Cells Is Particulate

The particulate nature of prepro-α-factor in extracts prepared from *sec*18 *sec*61 mutant cells (Fig. 8 *A*, lane *13*) was examined in more detail. Spheroplast lysates were subjected to differential centrifugation and subcellular fractions were assayed for NADPH cytochrome *c* reductase and glyceralde-

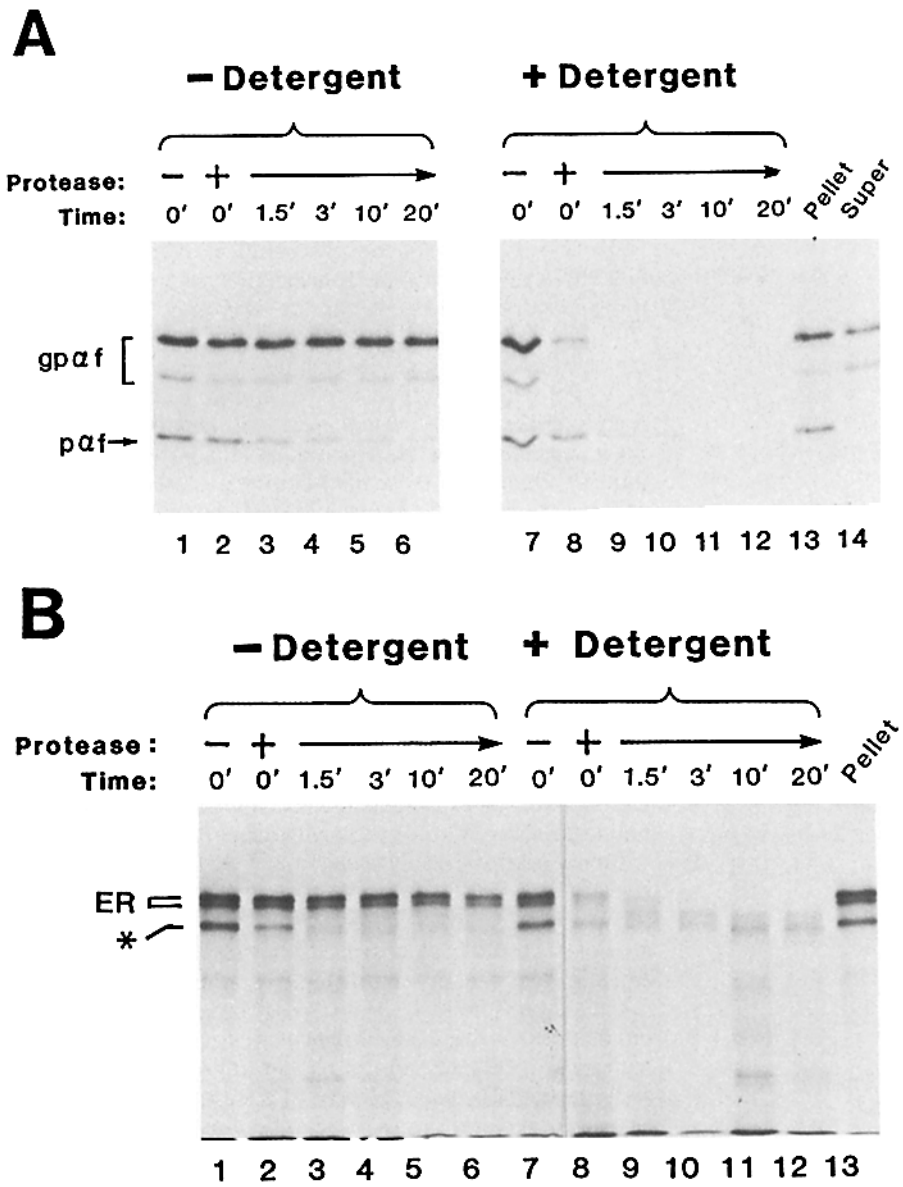

Figure 8. Proteolysis of prepro-α-factor and proCPY in *sec*18 *sec*61 cell extracts. RDM 15-3A cells were labeled and lysates were prepared as described in Materials and Methods. Aliquots of the lysate were mock digested (lanes *1* and *7*) or were treated with 300 μg/ml proteinase K in the absence (lanes *2–6*) or presence (lanes *8–12*) of 0.4% Triton X-100 for 0–20 min on ice. A separate aliquot was fractionated by centrifugation into pellet (lane *13*) and supernatant (lane *14*) fractions. (*A*) Samples were quenched with TCA, immunoprecipitated with anti-α-factor serum, and evaluated by SDS-PAGE on a 12.5% polyacrylamide gel. Each lane contains the amount of prepro-α-factor precipitated from 1.2 OD_{600} U of cells. α-Factor precursors accumulated by the *sec*18 and *sec*61 mutations are designated *gpαF* and *pαF*, respectively. (*B*) Aliquots from the proteolysis reactions were treated with anti-CPY serum and immunoprecipitates were analyzed by SDS-PAGE on a 7.5% polyacrylamide gel. Each lane contains total CPY antigen derived from 0.4 OD_{600} U of cells. ProCPY accumulated at the *sec*18 and *sec*61 stages is designated *ER* and *, respectively.

hyde-3-phosphate dehydrogenase, a cytoplasmic enzyme. Aliquots of each fraction were also evaluated by SDS-PAGE and immunoblotting with α-factor antiserum to monitor the fractionation properties of prepro-α-factor accumulated at the *sec*18 and *sec*61 blocks. As in the previous experiment, precursors accumulated by the *sec*18 mutation served as a marker for the lumen of the ER. The enzyme assay results are presented in Table III. As expected, the cytosolic marker was quantitatively recovered in the high-speed supernatant (HSS). NADPH cytochrome *c* reductase sedimented predominantly in the high speed pellet fraction; ~30% of the reductase activity sedimented in the low speed pellet fraction. An anti-α-factor immunoblot is shown in Fig. 9. The three lower mobility forms of α-factor precursor seen in the extract (lane *1*) corresponded to different glycosylated species (22) that were accumulated in *sec*18 mutants (compare the *sec*18 extract in lane *7* with the wild-type extract in lane *6*). The highest mobility band in lanes 1–5 corresponded to prepro-α-factor accumulated at the *sec*61 block, and comigrated with the in vitro translation product. The unglycosylated prepro-α-factor sedimented exclusively with the particulate fractions, whereas lumenal glycosylated prepro-α-factor was distributed between the high speed supernatant and pellet fractions, presumably due to some rupture of the ER membrane during cell lysis. The sedimentation of *sec*61-accumulated prepro-α-factor was not influenced by the *sec*18 mutation, in that extracts of *sec*61 mutants yielded similar results. In addition, proCPY accumulated in this mutant was recovered in the sedimentable fraction of a lysate (data not shown).

Discussion

A genetic selection has been devised to identify genes required for secretory protein translocation into the ER. This selection procedure demands that a signal peptide-containing cytoplasmic enzyme, whose location has been diverted to the secretory pathway, be retained in contact with the

Table III. Distribution of Marker Enzymes in sec18 sec61 Subcellular Fractions

Enzyme	Cellular location	Subcellular fraction	% Activity	Specific activity
Glyceraldehyde-3-P dehydrogenase	Cytoplasm	Extract	100	2.55
		HSS	112	5.32
		HSP	0.2	0.013
NADPH cytochrome *c* reductase	ER membrane	Extract	100	0.178
		HSS	5.6	0.026
		HSP	80.5	0.307

Subcellular fractions from the experiment depicted in Fig. 9 were assayed for their content of glyceraldehyde-3-P dehydrogenase and NADPH cytochrome *c* reductase activities as described in Materials and Methods. HSS and HSP, high-speed supernatant and pellet fractions, respectively. Each value presented is the average of the initial rates of activity measured in three independent trials. Specific activity is expressed as $\Delta A_{550\ nm}$ per minute per milligram of protein for NADPH cytochrome *c* reductase and $\Delta A_{340\ nm}$ per minute per milligram of protein for glyceraldehyde-3-P dehydrogenase.

cytosol because of a defect in the cellular translocation machinery. Mutations in two genes have been isolated that cause temperature sensitive growth and accumulation of prepro-α-factor. Because the selection demands that mutant cells express a partial defect at a temperature that permits growth (30°C), accumulation of α-factor precursor is seen at 30°C and is enhanced at the restrictive temperature (37°C). These phenotypes are the result of single lesions, inasmuch as temperature sensitivity and secretory protein accumulation cosegregate when mutants are backcrossed to wild type strains.

*sec*61, the mutant described in this report, also accumulates preinvertase and procarboxypeptidase (proCPY). Presumably other secretory proteins that are essential for yeast cell growth are also blocked in this mutant. We have not yet examined membrane protein precursors, many of which do not contain classical amino-terminal signal sequences (18, 34, 52). These properties could alter the assembly pathway of a protein. This possibility may be tested by evaluating the integration of membrane proteins into the ER membrane of *sec*61 mutant cells.

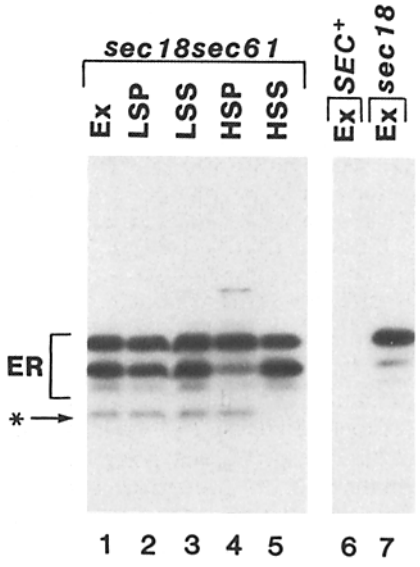

Figure 9. Fractionation of prepro-α-factor species accumulated in *sec*18 *sec*61 cells. Extracts of RDM 15-3A cells (*sec*18 *sec*61) were prepared and fractionated as described in Materials and Methods. Protein (30 μg) from each fraction was electrophoresed on a 12.5% polyacrylamide gel and immunoblotted with anti-α-factor serum. Lane *1*, whole extract; lane *2*, 660 - *g* pellet; lane *3*, 660 - *g* supernatant; lane *4*, 100,000 - *g* pellet; lane *5*, 100,000 - *g* supernatant. *Ex*, *LSP*, *LSS*, *HSP*, and *HSS* refer to extract, low-speed and high-speed pellet, and supernatant fractions, respectively. *ER* and * indicate forms of prepro-α-factor arrested by the *sec*18 and *sec*61 mutations. Unfractionated extracts of RDM 15-9B (*SEC*$^+$) and RDM 15-10D (*sec*18) strains were prepared, subjected to SDS-PAGE, and immunoblotted as described above. Lane *6*, whole extract from RDM 15-9B; lane *7*, whole extract from RDM 15-10D.

Preproteins accumulated at the *sec*61 block are not glycosylated, and their signal sequences have not been removed. These properties are expected for a molecule that has not yet engaged, or has only partly penetrated the ER membrane bilayer. Precursor forms of two secretory proteins are sedimentable but susceptible to exogenous proteolytic attack though the ER membrane remains largely intact.

α-Factor precursor made in an in vitro yeast protein synthesis reaction is soluble and becomes sedimentable only after translocation into ER vesicles (15). In contrast, both α-factor and CPY precursors sediment along with membranes in extracts of *sec*61 cells. This property could represent an intermediate stage in the translocation process in which precursors become firmly associated with the ER membrane (6), or could simply result from aggregation or nonspecific binding of precursors to membranes within mutant cells. Synthetic signal peptides and intact secretory precursor proteins have the capacity to insert into and at least partly through lipid monolayers and vesicle bilayers in vitro (3, 38). The physical basis of the sedimentability of precursor proteins may be established by fractionation of membranes from *sec*61 mutant cells.

Comparable blocks in secretion have been explored with *Escherichia coli*. Secretory precursors accumulate in thermosensitive mutants such as SecA, SecB, and SecY (21, 26, 39), or in wild-type cells that express high levels of maltose binding protein-β-galactosidase hybrid proteins (20). Cell fractionation experiments have shown that the precursors distribute between the periplasmic, membrane, and cytosolic fractions in proportions that are influenced by the monovalent ion concentration (20). In these conditions the preproteins may associate with the cytoplasmic membrane nonspecifically via the hydrophobic signal peptide.

A specific effect on the initial step in the secretory pathway, as opposed to a more general disruption of protein transport, is indicated by the pattern of precursors that appear in *sec*61 cells at the semirestrictive temperature, 30°C. Accumulation of core- or highly-glycosylated forms of α-factor, invertase, or CPY is expected in mutants with impaired intercompartmental protein transport from the ER or the Golgi body (9, 22, 50). In contrast, *sec*61 mutants show no such accumulation.

*sec*61 Mutant cells require 37°C preincubation periods of at least 2 h before the secretion block becomes complete. This long lag time suggests that the *sec*61 mutant protein is thermolabile for synthesis or association with an oligomeric complex, rather than thermosensitive for function. Alternatively, the mutant protein may be thermolabile in performing a modification that is required for the activity of another component of the translocation machinery. In either case, the defect would become more pronounced only as active species are replaced by inactive forms.

Two other yeast mutants, *sec*53 and *sec*59, are defective in an early stage of secretory protein biogenesis (12). Phenotypic characterization demonstrated that these Ts mutants accumulate underglycosylated, inactive precursors at the nonpermissive temperature (12). Initial protease protection experiments suggested that preinvertase is abortively translocated, remaining tightly associated with the ER membrane but accessible to trypsin in a homogenate (11). More refined methods for performing proteolysis experiments, however, have now shown that the partially glycosylated prepro-α-factor, invertase, and proCPY that accumulate in these mutants are completely protected against trypsin and protease K attack in the absence of, but not in the presence of detergent (10a). Because invertase accumulated in *sec*53 and *sec*59 mutants has had its signal peptide removed, is partially glycosylated and apparently resides within the lumen of the ER (10a), the *sec*61 defect must precede the block imposed by *sec*53 or *sec*59. In addition, the *sec*61 mutation complements both *sec*53 and *sec*59 mutations, indicating that *sec*61 defines a new function in the secretory pathway.

By characterizing the phenotypes of additional import mutants we may be able to reconstruct the events that occur during protein translocation in vivo (35). An examination of the properties of these mutants in the yeast in vitro protein translocation assay should further our understanding of the molecular mechanism of protein translocation into the yeast endoplasmic reticulum.

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