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, sec61, 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 sec61 block sediment with the particulate fraction, but are exposed to the cytosol as judged by sensitivity to proteinase K. Thus, the sec61 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). 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 reconstructed 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 (sec53 and sec59) 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 (secA and secB) 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 (sec61 and sec62). Mutations in both genes cause temperature-sensitive growth and accumulation of α-factor precursor. Detailed analysis of sec61 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 sec61 strains were recovered from the original sec61 diploid isolates by mating the diploid mutant to diploid S395D-1, sporulating this tetraploid strain, and then sporulating a Ts MATa/Mata diploid spore clone derived from the tetraploid. Haploid MATa 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 at 200 μM for overnight growth and at 0–10 μM during radiolabeling. Liquid cultures were grown in flasks with vigorous agitation, and density at 600 nm (OD600) of dilute cell suspensions was measured in 1-cm quartz cuvettes using a Zeiss PMQII spectrophotometer (Carl Zeiss, Inc., Thornwood, NY); 10D~ of cells corresponds to 0.15 mg of dry weight.

**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 EcoRI-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 YCp50α. 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 YCp50α to generate YCp50HI. The remainder of the HIS4 gene was introduced by replacing the 1.350-bp Cla I-Sph I fragment of YCp50I 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 HIS4A) 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-Bel 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

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**Table I. Bacterial and Yeast Strains**

| Strain       | Genotype                           | Source or reference          |
|--------------|------------------------------------|------------------------------|
| **Saccharomyces cerevisiae** |                                    |                              |
| RDB 103      | leu2-3,-112 ade2 MATa               | This study                   |
| RDM 15-5B    | leu2-3,-112 ade2 ura3-52 pep4-3 sec61-2 MATa | This study                   |
| RDM 15-9B    | ade2 pep3-3 Mata                    | This study                   |
| RDM 15-10D   | leu2-3,-112 ade2 sec18-1 MATa       | This study                   |
| RDM 15-3A    | leu2-3,-112 his4 pep4-3 sec18-1 sec61-2 MATa | This study                   |
| FC2-12B      | leu2-3,-112 ura3-52 trpl-1 his4-401 HOL1-1 MATa | R. Parker (40)               |
| DYFC2-12B    | leu2-3,-112/leu2-3,-112 ura3-52 trpl-1/1/trpl-1 | This study*                  |
| S395D-1      | his4-401/leu2-1/lep2-1/12 MATa      | YGSC‡                        |
| PBY404C      | suc2-Δ9 MATa                        | P. Böhni                     |
| 165/7        | his4-25 (his4A) MATa               | R. Parker (40)               |
| E331         | his4-331 (his4B) MATa              | R. Parker (40)               |
| S942-1Ca     | his4-864 (his4C) MATa              | R. Parker (40)               |
| **Escherichia coli** |                                    |                              |
| NK5772       | dcm-6 dam-3 galK2 galT22 merB1 leu1 y1137-8 thi1-1 | J. Kadonaga                 |
| HBl01        | F- hsdS20 (r*) mcr* recA13 ari-14 proA2 lacY1 galK2 rpsL2O (S*) xyl-5 metl-1 supE44 | (30)                        |

* Spontaneously derived from FC2-12B.
‡ Yeast Genetic Stock Center, University of California, Berkeley, CA.
protein A, filters were exposed to X-ray film with an intensifying screen at related to a-factor. After decoration of bound antibodies with ~25I-labeled incubation to block antibody association with filter-bound proteins not described by Kaiser (23), except that the blocking incubation was for 30 min at 37°C, and the filters were processed essentially as described by Laemmli (29).

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 MFA1 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 pSHF1 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 pSHF28. In contrast to the SUC2-HIS4 fusion, which contained amino acid residues 1-289 of secretory invertase, pSHF28 encoded a fusion protein in which the first 88 amino acids of the prepro region of a-factor were fused in frame to the invertase starting at amino acid 5 of the signal peptide sequence. This resulted in the deletion of four amino acids from the NH2 terminus of the invertase portion of the MFA1-SUC2-HIS4 fusion (see Emer 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 recombiant DNA manipulations were performed by standard methods (30). All recombinant DNA modifying enzymes were used according to the suppliers’ instructions.

### Mutant Isolation and Screening

FC2-2B and DYFC2-2B cells containing pSHF28 were grown to stationary phase in minimal medium supplemented with histidine, tryptophan, and leucine. Cultures of cells were harvested by centrifugation, washed with sterile 50 mM potassium phosphate buffer, pH 7.0, and resuspended to 2 OD600/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 1) or 75 min (round 1) at 30°C (% killing = 50% in round 1, 75% 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 OD600/ml in potassium phosphate buffer and plated onto minimal medium supplemented with leucine, tryptophan, and 3 mM histidinol (1-3 x 106 cells per plate). After incubation at 30°C for 5–10 d, mutant clones were picked and streaked onto YPD plates. After 2 d at 30°C these plates 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 restested 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 media (YPD). Ura” uracil auxotrophs were isolated, rescreened for Ts growth, and retransformed with fresh pSHF28 to assess the linkage of the Ts and histidinol + phenotypes to the insert; the excised frag-
factor and proCPY in lysates of sec18 sec11 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-Elvehjem homogenizer tube, and lysed by three consecutive cycles of homogenization (1 min of homogenization followed by 1 min on a 2.0 ml Potter-Elvehjem 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-Elvehjem 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-SUC2-23 (provided by J. Rothblatt), which contains the entire SUC2 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-SUC2-23 (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 *HIS4* 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 *HIS4* protein and contain the mutant allele *HOLI-1* (this mutation increases the efficiency of histidinol uptake) are able to convert exogenously supplied histidinol to histidine. For this purpose, the 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. 1B) and grow.

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. 1B. 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 HIS4 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 SUC2 coding region to a fragment containing the bulk of the HIS4 coding region (Fig. 2 A). The SUC2 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 (his4Δ, HOLI-1) transformed with a multicopy plasmid containing the SUC2-HIS4 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 (his4Δ, HOLI-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 HIS4 insert were also analyzed. The results shown in Fig. 2 B demonstrated that the SUC2-HIS4 fusion allowed his4Δ, HOLI-1 cells to grow on histidinol. This capacity was dependent on the HIS4 insert, because clones transformed with vector sequences did not grow.

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 SUC2 gene with upstream and coding sequences from the yeast MFA1 gene (Fig. 3 A). MFA1 encodes the precursor of the secreted mating pheromone α-factor (27). Multicopy plasmids (paSHF8) 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 paSHF8 were not able to grow on minimal medium plus histidinol (Fig. 3 B).

Production of the glycosylated hybrid protein encoded by paSHF8 was evaluated with transformed cells that were pulse-labeled with [35S]SO₄⁻ 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 SUC2 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 M₆ 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-HIS4 hybrid protein. Both polypeptides were plasmid encoded, in that cells containing vector se-
sequences did not express any high Mr cross-reactive material (data not shown). Also, both products were under mating-type regulation, because neither MATa haploids nor MATa/MATa diploids transformed with pA3SHF8 expressed any hybrid protein as expected for a gene under the control of MATa (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 pA3SHF8 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 MFa1-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, demonstrating that this phenotype was dependent on the plasmid, 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, 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 pA3SHF8 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 antisera 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. Tetrad 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/MATa diploids to form tetradspores, 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 MATa 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

| Stage of mutant isolation                   | Round I*  | Round II†       |
|--------------------------------------------|-----------|-----------------|
|                                            | Colonies analyzed/total colonies | Fraction of total | Colonies analyzed/total colonies | Fraction of total |
| Cells plated on histidinol medium          | 2 × 10⁷   | –               | 2.4 × 10⁷ | –                     |
| Histidinol prototrophs (histidinol+)       | 492       | 2.5 × 10⁻³      | 1,600    | 6.7 × 10⁻³            |
| Temperature-sensitive for growth           | 51/440    | 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.
§ Immunoblots of whole cell lysates were probed with α-factor antiserum as described in Materials and Methods.
Although it was not certain that the mutations were of independent origin. The gene defined by these mutations was designated sec61.

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 pCaSH8. 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 sec61. At least one additional complementation group was identified among these isolates. The phenotype of strains bearing a mutant allele of this gene, sec62, is being investigated.

sec61 Cells Accumulate Unprocessed α-Factor Precursor

More information on the effect of the sec61 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°C 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 (gpaF) in transit through the early stages of the secretory pathway was detected (lane 2).

When sec61 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 (paF) 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, sec61 strains grew slower than wild-type yeast at several temperatures, suggesting that there was no completely permissive temperature for sec61 function. Though a preshift to 37°C exaggerated the mutant phenotype, it did not completely inhibit α-factor secretion, in that even 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 sec61 block comigrated with the in vitro translation product of hybrid selected MPf1 mRNA electrophoresed on the same SDS-polyacrylamide gel (lane 5). This result demonstrated that sec61 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 sec61 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 sec61 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 pep4-3 mutation which blocks proenzyme cleavage (16). In SEC pep4-3 strains radiolabeled at 30 or 37°C (Fig. 6, lanes 2 and 4), two forms of proCPY were seen: pI, 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 sec61 pep4-3 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 sec61 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 sec61 block. With shorter 37°C preincubations (30 or 60 min), a fraction of the proCPY synthesized at 37°C escaped the sec61 block and appeared as the pl and mature forms (data not shown). These data suggested that the sec61 mutation was leaky, and required long temperature shifts to be fully expressed.
Additional information concerning the position of the sec61 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, sec61 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 Mr 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 sec61-specific invertase with the signal sequence-containing in vitro translation product indicated that the sec61 defect was imposed prior to signal peptide cleavage.

Based on the SDS gel mobility of precursors accumulated in sec61 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 [35S]SO₄²⁻ labeled sec61 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 I show the total complement of α-factor present in the sec61 extract derived from the same sample depicted in Fig. 5, lane I. 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 sec61 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 sec61-specific proCPY (*) was quantitatively recovered in the unbound fraction (lane 6). Preinvertase accumulated in sec61 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-HIS4 chimeric protein accumulate in a loca-
Figure 7. CPY and α-factor precursors accumulated at the sec61 block do not bind to Con A. (A) The $[^{35}S]\text{SO}_4^{2-}$ 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 $\text{gpaF}$; 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. PL, m, and * are as described in Fig. 6 A.

**Prepro-α-Factor Accumulated in sec61 Cells Is Particulate**

The particulate nature of prepro-α-factor in extracts prepared from sec61 sec18 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 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 sec18 prepro-α-factor was proportional to the amount of material that sedimented (compare lane 6 with lane 13). Prepro-α-factor accumulated at the sec61 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 sec61 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 sec61 sec18 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 sec18 block (36) did not alter the behavior of the sec61 species.

Fig. 8 B documents the protease sensitivity of the sec18 and sec61 forms of proCPY present in the same extract. In the absence of detergent, proCPY that accumulated at the sec18 block was refractory to proteolysis over a 20-min incubation, while proCPY held at the sec61 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 sec18 sec61 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 secl8 sec61 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 OD₆₀₀ U of cells. α-Factor precursors accumulated by the secl8 and sec61 mutations are designated gpaF and paF, 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 OD₆₀₀ U of cells. ProCPY accumulated at the secl8 and sec61 stages is designated ER and *, respectively.

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

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 secl8 and sec61 blocks. As in the previous experiment, precursors accumulated by the secl8 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 secl8 mutants (compare the secl8 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 sec61 block, and co-migrated with the in vitro translation product. The unglycosylated prepro-α-factor sedimented exclusively with the particulate fractions, whereas luminal 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 sec61-accumulated prepro-α-factor was not influenced by the secl8 mutation, in that extracts of sec61 mutants yielded similar results. In addition, proCPY accumulated at the secl8 and sec61 stages is designated ER and *, respectively.
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 $\Delta A_{340\,nm}$/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.

sec61, 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 sec61 mutant cells.

Preproteins accumulated at the sec61 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 sec61 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 sec61 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 sec61 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 to the Golgi body (9, 22, 50). In contrast, sec61 mutants show no such accumulation.

![Table III. Distribution of Marker Enzymes in sec18 sec61 Subcellular Fractions](image-url)
sec61 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 sec61 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, sec53 and sec59, 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 shown that the partially glycosylated prepro-a-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 sec53 and sec59 mutants has its signal peptide removed, is partially glycosylated and apparently resides within the lumen of the ER (10a), the sec61 defect must precede the block imposed by sec53 or sec59. In addition, the sec61 mutation complements both sec53 and sec59 mutations, indicating that sec61 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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References
1. Bernstein, M., W. Hoffmann, G. Ammerer, and R. Schekman. 1985. Characterization of a gene product (Sec33p) required for protein assembly in the yeast endoplasmic reticulum. J. Cell. Biol. 101:2374-2382.
2. Biebel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. J. Cell Biol. 67:852-862.
3. Briggs, M. S., L. M. Gierasch, A. Zlotnick, J. D. Lear, and W. F. DeGrado. 1985. In vivo function and membrane binding properties are correlated for Escherichia coli LamB signal peptides. Science (Wash. DC) 230:1096-1099.
4. Burnette, W. N. 1981. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiolabeled protein A. Anal. Biochem. 121:195-203.
5. Carlson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell. 28:145-154.
6. Connolly, T., and R. Gilmore. 1986. Formation of a functional ribosome-membrane junction during translocation requiring the participation of a GTP-binding protein. J. Cell Biol. 103:2253-2262.
7. Emer, S. D., I. Schauer, W. Hansen, P. Eason, and R. Schekman. 1984. Invertase-beta-galactosidase hybrid proteins fail to be transported from the endoplasmic reticulum in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:2347-2355.
8. Emer, S. D., R. Schekman, M. C. Flessel, and J. Thorner. 1983. An MFa1-SUC2 (a-factor-invertase) gene fusion for study of protein localization and gene expression in yeast. Proc. Natl. Acad. Sci. USA. 80:7070-7084.
9. Esmon, B., P. Novick, and R. Schekman. 1981. Componentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell. 25:451-460.
10. Evans, E. A., R. Gilmore, and G. Biebel. 1986. Purification of microsomal signal peptide as a complex. Proc. Natl. Acad. Sci. USA. 83:581-585.
11. Feldman, R. I., M. Bernstein, and R. Schekman. 1987. Product of sec53 is required for folding and glycosylation of secretory proteins in the lumen of the yeast endoplasmic reticulum. J. Biol. Chem. 262:9332-9339.
12. Ferro-Novick, S., W. Hansen, I. Schauer, and R. Schekman. 1984. Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. J. Cell Biol. 98:44-53.
13. Gilmore, R. R. and G. Biebel. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. J. Cell Biol. 95:470-477.
14. Gilmore, R., and G. Biebel. 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbation. Cell. 42:497-505.
15. Hansen, W., P. D. Garcia, and P. Walter. 1986. In vitro translocation across the yeast endoplasmic reticulum: ATP-dependent post-translational translocation of the pre-a-factor. Cell. 45:397-406.
16. Hemmings, B. A., G. S. Zubenko, A. Hasilik, and E. W. Jones. 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 78:435-439.
17. Hinnen, A., J. H. Hicks, and G. R. Fink. 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA. 75:1929-1933.
18. Hoffmann, W. 1985. Molecular characterization of the CAN1 locus in Saccharomyces cerevisiae. J. Biol. Chem. 260:11831-11837.
19. Ito, H., Y. Fukuda, K. Murata, and A. Kumura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168.
20. Ito, K., P. J. Bassford, Jr., and J. R. Beckwith. 1981. Protein localization in E. coli: is there a common step in the secretion of periplasmic and outer-membrane proteins? Cell. 24:707-717.
21. Ito, K., M. Witterkind, M. Nomura, K. Shiba, T. Yura, A. Miura, and H. Nashimoto. 1983. A temperature-sensitive mutant of E. coli exhibiting slow processing of exported proteins. Cell. 28:799-807.
22. Julius, D., R. Schekman, and J. Thorner. 1984. Glycosylation and processing of pre-a-factor through the yeast secretory pathway. Cell. 36:309-318.
23. Kaiser, C. A., and D. Botstein. 1986. Secretion-defective mutations in the signal sequence for Saccharomyces cerevisiae invertase. Mol. Cell. Biol. 6:2382-2391.
24. Keesey, J. K., R. Bigelis, and G. R. Fink. 1979. The product of the his4 gene cluster in Saccharomyces cerevisiae. J. Biol. Chem. 254:7427-7433.
25. Kubota, S., Y. Yoshida, H. Kumaoka, and A. Furumichi. 1977. Studies on the microsomal electron-transport system of anaerobically grown yeast. V. Purification and characterization of NADPH-cytochrome c reductase. J. Biochim. 81:197-206.
26. Kumamoto, C. A., and J. Beckwith. 1983. Mutations in a new gene, secB, cause defective protein localization in Escherichia coli. J. Bacteriol. 154:253-260.
27. Kuriyan, J., and I. Herskowitz. 1982. Structure of a yeast phenome gene (MFa): a putative a-factor precursor contains four tandem repeats of mature a-factor. Cell. 30:931-943.
28. Kurzchalia, T. V., M. Wiedmann, A. S. Girshovich, S. Bochkareva, H. Bieka, and T. A. Rapoport. 1986. The signal sequence of nascent preproa-maltoprotein interacts with the 54K polypeptide of the signal recognition particle. Nature (Lond.). 320:634-636.
29. Lennmii, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. J. Mol. Biol. 47:220-230.
30. Maniatis, T., E. F. Frisch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratoy, Cold Spring Harbor, NY.
31. Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. N. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 7:206-210.
32. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacterio-
33. Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes—the role of the "docking protein." Nature (Lond.) 297:647–650.

34. Nakayama, N., A. Miyajima, and K. Arni. 1985. Nucleotide sequences of STE2 and STE3, cell type-specific sterile genes from Saccharomyces cerevisiae. EMBO (Eur. Mol. Biol. Organ.) J. 4:2643–2648.

35. Novick, P., S. Ferro, and R. Schekman. 1981. Order of events in the yeast secretory pathway. Cell. 25:461–469.

36. Novick, P., C. Field, and R. Schekman. 1985. A point mutation in the conserved hexapeptide motif in yeast. Cell. 41:107–118.

37. Novick, P., and R. Schekman. 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 76:1858–1862.

38. Ohno-Iwasita, Y., P. Wolfe, K. Ito, and W. Wickner. 1984. Processing of preproteins by liposomes: leader peptidase. Biochemistry. 23:6178–6184.

39. Oliver, D. B., and J. Beckwith. 1981. E. coli mutant pleiotropically defective in the export of secreted proteins. Cell. 25:765–772.

40. Parker, R., and C. Guthrie. 1985. A point mutation in the conserved hexanucleotide at a yeast 5′ splice junction uncouples recognition, cleavage, and ligation. Cell. 41:107–118.

41. Perlman, D., H. O. Halvorson, and L. E. Cannon. 1982. Presecretory and cytoplasmic invertase polypeptides encoded by distinct mRNAs derived from the same structural gene differ by a signal sequence. Proc. Natl. Acad. Sci. USA. 79:781–785.

42. Rothblatt, J. A., and D. I. Meyer. 1986a. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertase in a homologous cell-free system. Cell. 44:619–628.

43. Rothblatt, J. A., and D. I. Meyer. 1986b. Secretion in yeast: translocation and glycosylation of prepro-α-factor in vitro can occur via an ATP-dependent post-translational mechanism. EMBO (Eur. Mol. Biol. Organ.) J. 5:1031–1036.

44. Rothman, J. E., and H. F. Lodish. 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. Nature (Lond.) 264:775–780.

45. Schauer, I., S. Emr, C. Gross, and R. Schekman. 1985. Inverse signal and mature sequence substitutions that delay intercompartmental transport of active enzyme. J. Cell Biol. 100:1664–1675.

46. Scott, J., and R. Schekman. 1980. Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. J. Bacteriol. 142:414–423.

47. Sharma, S., L. Rodgers, J. Brandisma, M.-J. Gething, and J. Sambrook. 1985. SV40 T antigen and the exocytotic pathway. EMBO (Eur. Mol. Biol. Organ.) J. 4:1479–1489.

48. Sherman, F., G. R. Fink, and J. B. Hicks. 1983. Methods in Yeast Genetics: A Laboratory Manual. Revised edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

49. Simon, K., E. Perara, and V. R. Lingappa. 1987. Translocation of globin fusion proteins across the endoplasmic reticulum membrane in Xenopus laevis oocytes. J. Cell Biol. 104:1165–1172.

50. Stevens, T., B. Eason, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell. 30:439–448.

51. Stitschohm, D. T., C. Mann, and R. W. Davis. 1982. Centromeric DNA from Saccharomyces cerevisiae. J. Mol. Biol. 158:157–179.

52. Tanaka, J. I., and G. R. Fink. 1985. The histidine permease gene (HIP1) of Saccharomyces cerevisiae. Gene (Amst.). 38:205–214.

53. Vails, L. A., C. P. Hunter, J. H. Rothman, and T. H. Stevens. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the prepeptide. Cell. 48:887–897.

54. Walter, P., and G. Blobel. 1980. Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA. 77:7112–7116.

55. Walter, P., and G. Blobel. 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature (Lond.). 229:691–698.

56. Walter, P., I. Ibrahimi, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum I. Signal recognition particle (SRP) binds to in vitro assembled polysomes synthesizing secretory protein. J. Cell Biol. 91:545–550.

57. Waters, M. G., and G. Blobel. 1986. Secretary protein translocation in a yeast cell-free system can occur post-translationally and requires ATP hydrolysis. J. Cell Biol. 102:1543–1550.

58. Waters, M. G., W. J. Chirico, and G. Blobel. 1986. Protein translocation across the yeast microsomal membrane is stimulated by a soluble factor. J. Cell Biol. 103:2629–2636.

59. Wickerham, L. J. 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. 52:293–301.