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Mutations in the Signal Sequence of Prepro-α-Factor Inhibit Both Translocation into the Endoplasmic Reticulum and Processing by Signal Peptidase in Yeast Cells

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The effects of five single-amino-acid substitution mutations within the signal sequence of yeast prepro-α-factor were tested in yeast cells. After short pulse-labelings, virtually all of the α-factor precursor proteins from a wild-type gene were glycosylated and processed by signal peptidase. In contrast, the signal sequence mutations resulted in the accumulation of mostly unglycosylated prepro-α-factor after a short labeling interval, indicating a defect in translocation of the protein into the endoplasmic reticulum. Confirming this interpretation, unglycosylated mutant prepro-α-factor in cell extracts was sensitive to proteinase K and therefore in a cytosolic location. The signal sequence mutations reduced the rate of translocation into the endoplasmic reticulum by as much as 25-fold or more. In at least one case, mutant prepro-α-factor molecules were translocated almost entirely posttranslationally. Four of the five mutations also reduced the rate of proteolytic processing by signal peptidase in vivo, even though the signal peptide alterations are not located near the cleavage site. This study demonstrates that a single-amino-acid substitution mutation within a eucaryotic signal peptide can affect both translocation and proteolytic processing in vivo and may indicate that the recognition sequences for translocation and processing overlap within the signal peptide.

Most proteins destined to be secreted from eucaryotic cells must first be imported into the endoplasmic reticulum (ER). Secreted proteins are generally synthesized with a transient amino-terminal sequence 15 to 30 amino acids in length, the signal peptide (6, 34), which is necessary for translocation and is removed by signal peptidase in the ER. Although the sequences of signal peptides are not conserved, they usually have a basic N-terminal region, a central hydrophobic core region, and one of a particular set of residues at positions −1 and −3 relative to the signal peptidase cleavage site (43, 44). Translocation of proteins into the ER also involves several *trans*-acting components. Some of these have been defined biochemically in mammalian in vitro translocation systems (15, 32, 41, 47). Recently, it was shown that translocation of some proteins into the ER of the simple eucaryote *Saccharomyces cerevisiae* (yeast) requires the presence of 70-kilodalton heat shock-related proteins both in vivo (12) and in vitro (11, 12). In addition, several proteins in yeast cells that are essential both for import of precursors into the ER and for cellular growth recently were identified genetically (13, 40). A detailed understanding of the import process requires knowledge of which components of the translocation machinery interact with the signal peptide. One approach to this problem is to first identify single-amino-acid substitution mutations within a signal sequence that disrupt the normal function of the signal peptide. Although a large number of deleterious substitution mutations within procaryotic signal sequences have been identified (4, 33), previous attempts to identify similar mutations within two different yeast proteins were unsuccessful (5, 22). Furthermore, a large fraction of random sequences were able to function to some extent as secretion signal sequences (22). The results of these studies might indicate that the specificity with which signal peptides are recognized is low and therefore that point mutations within a signal sequence may not be expected to impair translocation to a significant extent.

Yeast cells of the α mating type secrete a peptide pheromone, α-factor, which is derived from a precursor polypeptide (prepro-α-factor) that is translocated into the ER, glycosylated, and proteolytically processed in the Golgi and secretory vesicles (21). We recently described a selection scheme for the isolation of signal sequence mutants of a secreted protein and used this procedure to identify single-amino-acid substitutions within the signal sequence of prepro-α-factor that severely inhibited translocation into microsomes in vitro (1). Preliminary experiments in the previous study indicated that the effect of the signal sequence mutations on secretion of α-factor was significantly less than the effect observed in vitro. However, by using a direct assay of ER translocation—radiolabeling of proteins for short intervals and determination of the extent of glycosylation after immunoprecipitation—we show here that the α-factor signal sequence mutations in fact do have a significant deleterious effect on translocation of prepro-α-factor in vivo. In addition to having effects on translocation, four of the five mutations also reduced the rate of processing by signal peptidase.

MATERIALS AND METHODS

Strains. *S. cerevisiae* DA102-1 (*MATα sec18-1 ura3 leu2-3,112 trp1 his4 ade2-1 can1-100 mfα1::LEU2 mfα2::URA3*) was constructed by first obtaining a diploid (DA102) from strains mfα1::LEU2C mfα2::URA3 (25) and MBY2-2A (*MATα sec18-1 ura3-52 leu2-3,112 his4 pep4-3 mnn4-1*). The diploid DA102 was sporulated, and canavanine-resistant cells were selected among random germinated spores and screened for the desired genetic markers.

Plasmid constructions. The pTCAF2 plasmids, which carry wild-type (WT) or mutant α-factor genes, are identical to the

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pTCAF1 series (1) except that a DNA fragment containing a complete transcription termination sequence was inserted 35 base pairs 3′ to the stop codon of the α-factor gene. The pTCAF2 plasmids contain yeast *CEN3*, *TRP1*, and *ARS1* sequences for selection and maintainance of the plasmid. To construct pTCAF2, a 270-base-pair *SalI-XhoI* fragment containing mostly 3′-flanking sequences of the *STE2* gene (9) was ligated into the *SalI* site of pTCAF1 (1) just 3′ to the α-factor gene (27). The *SalI* site in the 270-base-pair fragment is located 70 base pairs 5′ to the termination codon of *STE2*. The *XhoI* site in the 270-base-pair fragment was created, using Klenow fragment and *XhoI* linkers (30), from the *HindIII* site 200 base pairs 3′ to the termination codon (9).

Production of antisera. Antisera were raised by first constructing a *trpE*–α-factor fusion gene after ligating a 470-base-pair *EcoRI-SalI* fragment from pAF2 (1) into the *EcoRI-SalI* site of pATH3 (pATH vectors were constructed by T. J. Koerner by modification of the plasmid described in reference 38). The resulting fusion protein contains pro-α-factor sequences fused on the carboxy-terminal side of TrpE. The fusion protein was overproduced as described previously (23); the insoluble fraction was solubilized in sample buffer containing 6% sodium dodecyl sulfate (SDS) and electrophoresed through an SDS–10% polyacrylamide slab gel. The protein band was visualized by staining the gel briefly in 75 mM Tris chloride (pH 7.5)–20% methanol–0.25% brilliant blue G. Protein was electroeluted from the excised gel slice into dialysis tubing in the presence of SDS running buffer. The fusion protein (375 μg) was mixed with an equal volume of Freund complete adjuvant, which was then used for intramuscular injection of rabbits. Three boosts of 250 μg of fusion protein in Freund incomplete adjuvant were given at 3-week intervals thereafter.

Cell labeling and immunoprecipitation. DA102-1 cells transformed (19) with pTCAF2 plasmids were grown at 23°C to an optical density at 600 nm of between 0.5 and 0.8 in SD medium lacking tryptophan (SDM−Trp) (0.67% yeast nitrogen base without amino acids plus 2% glucose, adenine, uracil, and amino acids [except tryptophan] [26]). Cells were centrifuged, washed once in SD medium lacking methionine (SDM−Met), and resuspended in SDM−Met to 4×10^7 cells per ml. The cells were incubated at 37°C for 10 min, after which 100 to 500 μCi of Tran^{35}S-label (ICN Radiochemicals, Irvine, Calif.) was added, and the cells incubated at 37°C for various time intervals. Proteins were extracted essentially as described previously (48). A 1-ml sample of cell culture was added to microcentrifuge tubes containing 150 μl of 1.85 N NaOH–7.4% 2-mercaptoethanol, and the tubes were incubated on ice for 10 min. The proteins were precipitated by addition of 150 μl of 50% trichloroacetic acid, incubated on ice for 10 min, and centrifuged at 12,000 × *g* for 5 min. The pellets were rinsed with 1 ml of ice-cold acetone, dried in a Speed-Vac (Savant Instruments, Inc.), and dispersed by addition of 60 μl of 3.6% SDS–140 mM Tris chloride (pH 8.8)–0.7 mM EDTA and extensive boiling with occasional agitation. Insoluble material was removed by centrifugation in a microcentrifuge for 5 min. The supernatants (50 μl) containing extracted labeled protein were added to microcentrifuge tubes together with 1 ml of TNET (50 mM Tris chloride [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.02% sodium azide). α-Factor antisera (25 μl) were added, and the mixtures were incubated at 4°C overnight. Then 80 μl of settled protein A-Sepharose beads (CL-4B; Pharmacia, Inc., Piscataway, N.J.) was added, and the tubes were incubated with mixing at 4°C for 30 min. The beads were pelleted, using minimal centrifugation in a microcentrifuge, and washed four to five times with 0.9 ml of TNET. Proteins were eluted from the beads by addition of sample buffer, boiling for 3 min, and brief centrifugation. The supernatants were electrophoresed through an SDS–14% polyacrylamide gel (28) that also contained 2 mM EDTA. In contrast to previous studies in which 12% polyacrylamide gels were used (21, 46), we found that it was unnecessary to include urea in our 14% gels in order to resolve pro-α-factor from prepro-α-factor. To prevent glycosylation, log-phase cells in SDM−Trp were first incubated for 30 min at 23°C in the presence of 10 μg of tunicamycin (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were then washed and resuspended in SDM−Met as described above in preparation for labeling with ^{35}S.

Proteolysis of extracts. DA102-1 cells carrying pTCAF2 plasmids (WT or mutant m3) were grown at 23°C in SDM−Trp to an optical density at 600 nm of 0.6. The cells were pelleted and suspended in 5 ml of 1 M sorbitol–0.6× SDM–Trp. NaOH was added to neutralize the pH, dithiothreitol added to 10 mM, and 0.5 mg of Zymolyase 100000 (Seikagaku Kogyo, Tokyo, Japan) was added. After incubation at 23°C with gentle shaking for 30 min, the spheroplasts were pelleted, washed twice in 1 M sorbitol–0.6× SDM−Met, and resuspended in 10 ml of the same solution. The spheroplasts were then incubated at 23°C with gentle shaking for 1 h. A 4-ml sample of spheroplasts was transferred to 37°C, incubated for 10 min, and labeled for 5 min with gentle shaking after addition of 0.6 mCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.). Protein from 1 ml of spheroplasts was immediately extracted by the NaOH–2-mercaptoethanol procedure described above. The remaining 3 ml was added to a tube containing an equal volume of ice-cold 20 mM sodium azide and incubated on ice for 5 min. All following steps were at 4°C or less. The spheroplasts were then pelleted (4,000 × *g*, 5 min), suspended in 3 ml of lysis buffer [0.3 M sorbitol, 0.1 M KCl, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES-KOH; pH 7.4), 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)], and lysed in a Dounce homogenizer, using a tight-fitting pestle. Cellular debris was removed by centrifugation at 700 × *g* for 4 min, and the supernatant (2.7 ml) was divided into three equal portions. The first portion received no additions. To the second was added 250 μg of proteinase K (Sigma). The third portion received proteinase K as described above and Triton X-100 to 0.36%. All samples were incubated on ice for 30 min, after which the proteins were precipitated by addition of trichloroacetic acid to 12%. All remaining steps, including immunoprecipitation, were performed as described above.

Endo H reactions and protein sequencing. Samples were immunoprecipitated as described above. After the last wash with TNET, the tubes were spun briefly and as much as possible of the remaining liquid above the beads was removed. Then 20 μl of 1% SDS was added to the beads, and the samples were boiled for 3 min; 180 μl of distilled water was added, the samples were reboiled and centrifuged, and the supernatant was recovered. To 50 μl of each sample was added 2.5 μl of 3 M sodium acetate (pH 5.5) and 1 μl (25 ng) of endoglycosidase H (endo H; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). In control samples, the endo H was omitted. All samples were incubated at 37°C for 20 h. The samples were then frozen, lyophilized, and solubilized in sample buffer for electrophoresis in SDS–14% polyacrylamide gels.

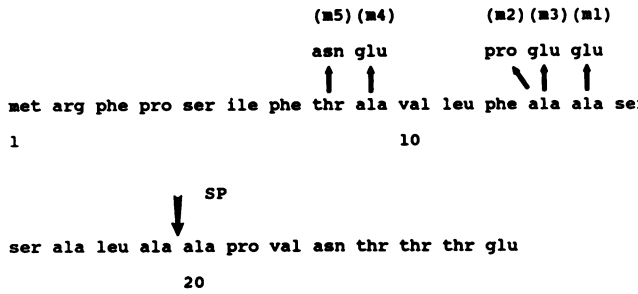

FIG. 1. Amino-terminal acid sequence of prepro-α-factor and mutations. Protein sequence is deduced from DNA sequence. Large arrow indicates cleavage site of signal peptidase (SP).

Radiolabeled mutant m4 α-factor precursor proteins were prepared for sequencing by labeling cells as described above except that the cells were labeled with 1 mCi of Tran^{35}S-label for 3 min. The immunoprecipitated proteins were electrophoresed through an SDS–14% polyacrylamide gel that had been polymerized the day before. The proteins were electroblotted onto a polyvinylidine difluoride membrane (Immobilon; Millipore Corp., Bedford, Mass.) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid–10% methanol (pH 10.5) for direct radiosequencing of membrane-bound protein (31). Slices of membrane-containing glycosylated forms of the mutant m4 α-factor precursor were excised by using an autoradiogram as a template. Three Edman degradation cycles were carried out with an amino acid sequencer (model 890C; Beckman Instruments, Inc., Fullerton, Calif.). The derivative samples were lyophilized, solubilized in 1.5 ml of Aquasol scintillation cocktail (Dupont, NEN Research Products, Boston, Mass.), and counted in a Beckman model LS-230 scintillation counter.

Miscellaneous procedures. In vitro translation reactions and translocation into yeast microsomes were done as described previously (1). Halo assays were performed as described previously (1) except that cells to be tested for secretion of α-factor were in exponential-phase growth. A semiquantitative value was obtained by the following procedure. A *MAT*α strain (W303-1B [25]) was diluted in various proportions with an isogenic strain (*mfα1 mfα2* [25]) that has disrupted α-factor genes, and the cell mixture was spotted onto plates seeded with a yeast strain (6360-17-2a [1]) that is supersensitive to and growth inhibited by low levels of α-factor. The resulting radius of zone of inhibition was found to be proportional to the logarithm of the fraction of W303-1B cells in the cell mixture, a result similar to that observed previously with purified α-factor (20). The graph generated could then be used to estimate the amount of α-factor secreted from each mutant on the basis of the radius of zone of inhibition. The amount of α-factor secreted from undiluted W303-1B cells was arbitrarily set at 10,000 U.

RESULTS

Secretion of α-factor. Shown in Fig. 1 are the α-factor signal sequence mutations we identified previously and tested in an in vitro translocation system (1). After construction of yeast *CEN* plasmids (pTCAF2 series) carrying the α-factor genes and transformation of a yeast strain (DA102-1) that contains disrupted copies of both α-factor genes, the effects of the mutations on secretion of α-factor were tested by the halo assay (27). In all cases, a detectable difference was observed between the mutant and WT α-factor genes (Table 1). As measured by this method, yeast cells carrying the mutant genes produced 2- to 12-fold less α-factor than did the WT gene. In preliminary experiments in the previous study, three of the mutations tested had only a one- to fourfold negative effect on steady-state secretion of α-factor (1). There are probably two reasons for the difference between the results of the two assays. First, the α-factor genes in the original plasmids (pTCAF1 series [1]) apparently lacked a complete transcription termination sequence. After insertion of a complete terminator downstream of the α-factor genes (pTCAF2 plasmids), secretion of α-factor from the WT gene was increased about 40-fold. Second, in the experiments described here, cells to be tested for secretion of α-factor were grown only to mid-exponential-phase density rather than to stationary phase as was done previously.

TABLE 1. Effects of mutations on secretion of α-factor

Plasmid	Amt (U) of α-factor secreted[a]
pTCAF2 (WT)	5,000
pTACF2-ml	1,000
pTCAF2-m2	1,000
pTCAF2-m3	400
pTCAF2-m4	800
pTCAF2-m5	2,500

[a] Expressed as arbitrary units based on a halo assay; see Materials and Methods.

Pulse-labeling in *sec18* cells. Secretion of α-factor is the final step in a series of reactions required for normal biosynthesis, which include translation, translocation into the endoplasmic reticulum (ER), removal of the signal sequence, glycosylation, and further proteolytic processing (21, 46). We wanted to determine, using radiolabeling assays, which of these steps is affected by the signal sequence mutations. Because transport of glycosylated pro-α-factor through the secretory pathway is rapid (21), we decided to utilize the *sec18* mutation in order to trap α-factor precursor proteins within the ER. Temperature-sensitive mutations in the *sec18* gene prevent transport of proteins from the ER to the Golgi at the restrictive temperature (21, 35) and thus permit a direct comparison of the relative amounts of translocated protein (core glycosylated pro-α-factor) and untranslocated prepro-α-factor. Therefore, a yeast strain (DA102-1) was constructed that carries the temperature-sensitive *sec18-1* mutation and disrupted copies of both chromosomal α-factor genes (25). DA102-1 transformants carrying pTCAF2 plasmids were then pulse-labeled with [^{35}S]methionine at the restrictive temperature, α-factor precursor proteins were immunoprecipitated with antisera against pro-α-factor, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

Cells synthesizing WT prepro-α-factor accumulated predominantly glycosylated pro-α-factor when labeled for 30 s (Fig. 2A, lane 2) or for only 15 s (data not shown). The absence of significant amounts of unglycosylated WT prepro-α-factor could result from a cotranslational mode of translocation, even though translocation of prepro-α-factor into ER membranes can occur posttranslationally in vitro (16, 45). Glycosylated pro-α-factor from a 30-s pulse migrated more slowly than did glycosylated pro-α-factor derived from an in vitro translocation reaction (compare lanes 1 and 2 in Fig. 2A), probably because of incomplete trimming of the core oligosaccharides from $Glc_3Man_9GlcNAc_2$ to

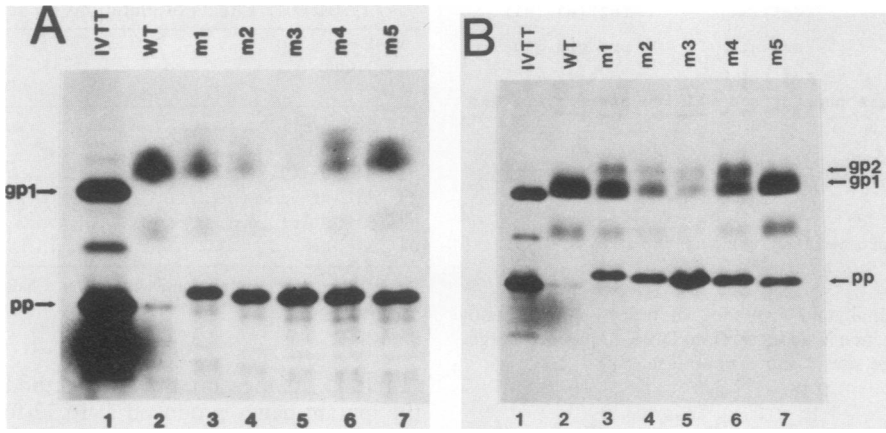

FIG. 2. Radiolabeling of wild-type and mutant α-factor precursor proteins. DA102-1 cells (4×10^7 in 1 ml) carrying WT or mutant (m1 to m5) α-factor genes were labeled at 37°C with 0.5 mCi of Tran^{35}S-label for 30 (A) or 90 (B) s. The α-factor proteins were immunoprecipitated and electrophoresed through an SDS–14% polyacrylamide gel. Samples in lanes 1 were used as markers for prepro-α-factor (pp) and glycosylated pro-α-factor (gp1) and were derived from in vitro translation of prepro-α-factor and translocation (IVTT) into yeast microsomal membranes. The identity of gp2 is discussed in Results.

$Man_8GlcNAc_2$, which occurs within the lumen of the ER (10, 46). After a longer pulse (Fig. 2B, lanes 1 and 2) or during a pulse-chase experiment (see Fig. 4A), the mobility of the in vivo-derived glycosylated pro-α-factor was seen to approach that of the fully processed in vitro translocated protein. The faint band running between prepro-α-factor and glycosylated pro-α-factor is probably protein that was not yet core glycosylated at all three Asn-X-Thr sites in pro-α-factor.

The signal sequence mutations did not alter significantly the rate of synthesis of prepro-α-factor (Fig. 2A). However, all five mutations led to an accumulation of mostly unglycosylated, labeled prepro-α-factor after a 30-s pulse with [^{35}S]methionine (Fig. 2A, lanes 3 to 7). Some of the mutant proteins (m1 to m3) had a lower gel mobility than did WT prepro-α-factor, and the mutations m1 to m3 also reduced the gel mobility of in vitro-synthesized prepro-α-factor (1; unpublished data). This phenomenon may be related to the aberrant mobility of prepro-α-factor in some polyacrylamide gel systems (46). After a 90-s pulse, all of the mutant α-factor genes produced at least some glycosylated pro-α-factor. Therefore, the mutations had a moderate to severe effect on translocation, although none of these single-amino-acid substitutions completely abolished the function of the signal peptide. The signal sequence of prepro-α-factor is necessary for translocation, however, because several different deletion variants of the precursor protein that lack the signal sequence failed to be transported into the ER (1; data not shown). In addition to the glycosylated pro-α-factor band (gp1) seen in cells carrying the WT α-factor gene, cells carrying mutant genes m1 to m4 also accumulated a more slowly migrating protein (gp2) after a 90-s pulse-labeling (Fig. 2B). This mutant phenotype will be discussed further below.

It was formally possible that the nonglycosylated proteins that predominated in pulse experiments with mutant α-factor genes were in fact translocated but failed to be glycosylated. This alternative was ruled out in a protease digestion experiment. Spheroplasts containing either the WT or mutant m3 α-factor gene were pulse labeled with [^{35}S]methionine, lysates were made, and proteinase K was added to digest any proteins not contained within membrane vesicles such as microsomes. As expected, both WT glycosylated pro-α-factor and glycosylated m3 protein (both gp1 and gp2) were mostly protected from protease digestion (Fig. 3, compare lanes 3 to 4 and 7 to 8) unless the vesicles were disrupted with detergent (lanes 5 and 9). In contrast, unglycosylated m3 protein was susceptible to exogenous protease (lanes 7 and 8). This result demonstrates that the unglycosylated mutant m3 protein was in a cytosolic location.

Pulse-chase experiments. Pulse-chase studies were carried out to (i) obtain an estimate of the in vivo translocation rates of mutant prepro-α-factor proteins and (ii) determine

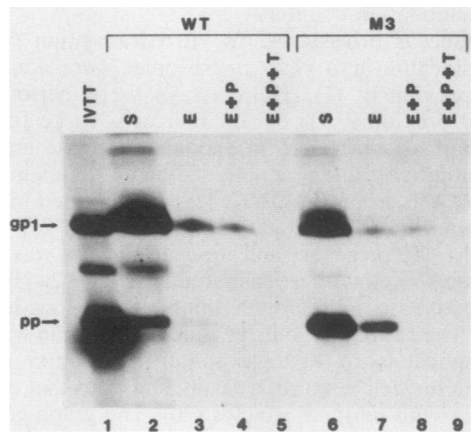

FIG. 3. Protease treatment of extracts from cells expressing normal or mutant prepro-α-factor. DA102-1 cells expressing WT or mutant m3 prepro-α-factor were converted to spheroplasts and labeled for 5 min with 150 μCi of [^{35}S]methionine (Amersham) per ml at 37°C (see Materials and Methods). Total proteins (S) were extracted from one sample of labeled cells, and α-factor precursor proteins were immunoprecipitated (lanes 2 and 6). The remaining spheroplasts were homogenized, and a low-speed supernatant (extract) was obtained. The extracts (E) were divided into three equal portions, and the following components were added: lysis buffer only (lanes 3 and 7); proteinase K (P) to 280 μg/ml (lanes 4 and 8); and proteinase K (280 μg/ml) and Triton X-100 (T) to 0.36% (lanes 5 and 9). After digestion, the proteins were immunoprecipitated and gel electrophoresed as described in the legend to Fig. 2. Lane 1 corresponds to lane 1 in Fig. 2. Prepro-α-factor (pp) and glycosylated pro-α-factor (gp1) were derived from in vitro translation-translocation reactions (IVTT) as in Fig. 2.

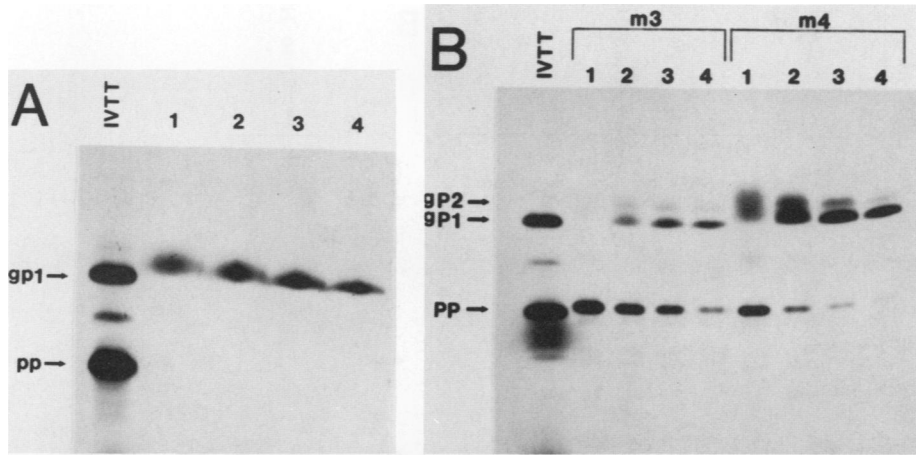

FIG. 4. Pulse-chase analysis of normal and mutant α-factor precursor proteins. DA102-1 cells (4×10^7/ml) with pTCAF2 plasmids were labeled for 1 min with 120 μCi of Tran^{35}S-label. Cycloheximide was added to 0.33 mM, and samples were removed at the times indicated below for protein extraction, immunoprecipitation, and gel electrophoresis. Prepro-α-factor (pp) and glycosylated pro-α-factor (gp1) were derived from in vitro translation-translocation reactions (IVTT) as in Fig. 2. The identity of gp2 is discussed in Results. (A) Pulse-chase experiment with cells containing the wild-type α-factor gene. Length of chase was 5 s (lane 1), 20 s (lane 2), 1 min (lane 3), or 2 min (lane 4). (B) Pulse-chase experiment with cells carrying mutant α-factor gene m3 or m4. Length of chase was 5 s (lanes 1), 1 min (lanes 2), 2 min (lanes 3), or 5 min (lanes 4).

whether there was a precursor-product relationship between the two major glycosylated forms (gp1 and gp2) deriving from α-factor mutants m1 to m4. Cells expressing the WT or mutant α-factor genes were labeled for 1 min, at which time cycloheximide was added; samples were taken at various time intervals for immunoprecipitation of α-factor precursor proteins. In cells expressing the WT α-factor gene, an increase in mobility due to trimming of the oligosaccharide residues was observed to occur during the chase interval (Fig. 4A). As shown above, very little of mutant m3 was translocated and glycosylated after 1 min of labeling, but after a chase period of 2 min approximately half of the mutant precursor had been glycosylated and thus posttranslationally transported into the ER (Fig. 4B). The translocation rate of the m3 precursor can be estimated to be at least 25-fold slower than that of the WT prepro-α-factor, assuming a conservative estimate of a 5-s average half-life for translocation of the WT precursor. An effect on translocation of this magnitude would be similar to that seen in vitro (50-fold) for the m3 mutant protein (1). It appeared that m4 precursor was translocated in vivo with at least a fivefold-lower rate than was WT prepro-α-factor, which is close to the eightfold-lower translocation rate previously observed in vitro (1).

As mentioned above, two species of glycosylated proteins derived from mutants m1 to m4 (bands gp1 and gp2 in Fig. 2B and 4B). Most clearly seen in the case of mutant m4, both gp1 and gp2 apparently underwent the oligosaccharide-trimming reaction during the chase period in the experiment shown in Fig. 4B. In addition, it appeared that gp2 was converted to gp1 during the chase period. This result and the relative mobilities of gp1 and gp2 suggested that gp2 may be glycosylated prepro-α-factor, i.e., protein that had been translocated and glycosylated but not yet cleaved by signal peptidase.

Endo H digestions and radiosequencing. To determine whether the gp2 protein appearing in cells expressing mutant α-factor genes m1 to m4 was glycosylated prepro-α-factor, we first treated pulse-labeled glycosylated α-factor precursor proteins with endo H. Endo H removes all Asn-linked core carbohydrate except one GlcNAc residue per site initially glycosylated (39). Therefore, if gp2 is in fact glycosylated prepro-α-factor, then the gel mobility of the endo H-digested gp2 protein should be slightly slower than that of the primary translation product, prepro-α-factor. On the other hand, gp2 conceivably could differ from gp1 only in the nature of its Asn-linked oligosaccharides (e.g., extent of trimming). In this case, endo H treatment of gp2 should produce a protein identical to endo H-digested gp1, which should have a slightly slower mobility than does pro-α-factor.

When DA102-1 cells expressing the WT α-factor gene at the restrictive temperature were labeled in the presence of tunicamycin, which prevents Asn-linked glycosylation, pro-α-factor accumulated within the ER (Fig. 5A, lane 2). In the gel system we used, pro-α-factor had a greater mobility than did prepro-α-factor (Fig. 5A, lanes 1 and 2). As expected, endo H-digested WT glycosylated pro-α-factor migrated somewhat more slowly than pro-α-factor (lanes 2 and 3). Endo H digestion of immunoprecipitated proteins derived from labeled cells expressing the m4 mutant α-factor gene gave rise to the appearance of three bands (Fig. 5A, lane 7). The middle band of lane 7 corresponds to untranslocated mutant prepro-α-factor (compare lanes 7 and 9), whereas the mobility of the lower band matches that of endo H-digested WT glycosylated pro-α-factor (Fig. 5B, lanes 3 and 7). Finally, the upper band of lane 7 had a mobility slightly lower than that of prepro-α-factor, which would be the expected migration rate of endo H-digested glycosylated prepro-α-factor. Mutant proteins m1 to m3 also appeared as two species of glycosylated protein (Fig. 2B) and also formed the same pattern of three bands as did mutant m4 protein after digestion with endo H (Fig. 5A, lanes 4 to 7). In contrast, expression of the m5 mutant α-factor gene did not lead to the appearance of the gp2 band (Fig. 2B, lane 7). Furthermore, digestion of m5 proteins with endo H (Fig. 5A, lane 8) did not produce the upper band (pp*) seen after endo H treatment of the other four mutant proteins (Fig. 5A, lanes 4 to 7). These experiments therefore strongly support the idea that the gp2 protein is glycosylated prepro-α-factor.

To confirm the identity of the gp2 protein, cells expressing the m4 mutant prepro-α-factor were first labeled with

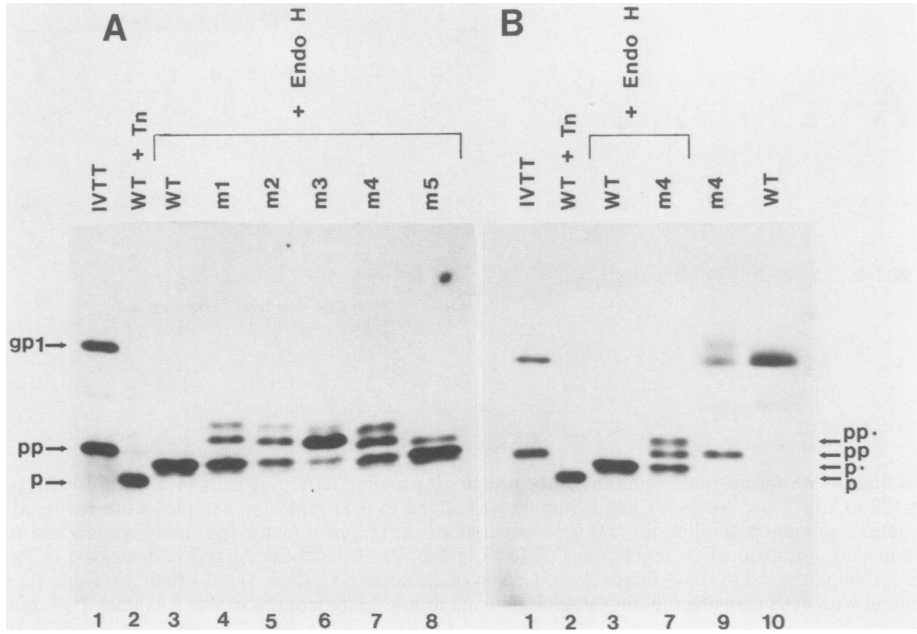

FIG. 5. Endoglycosidase H treatment of wild-type and mutant glycosylated α-factor precursor proteins. (A) Proteins were labeled for 90 s and immunoprecipitated as described in the legend to Fig. 2. Endoglycosidase H was added to (lanes 3 to 8) or omitted from (lanes 9 and 10) the reaction mixtures. The proteins in lanes 3 to 8 of panel B were derived from the same samples of labeled-immunoprecipitated proteins displayed in lanes 2 to 7 of Fig. 2 except that those of Fig. 2 were subsequently only mock treated with endo H. Proteins in lane 2 were from cells containing the wild-type α-factor gene that had been incubated in the presence of tunicamycin (10 μg/ml) for 30 min at 23°C before labeling. Identities of the bands are as follows: pro-α-factor (p), endo H-treated glycosylated pro-α-factor (p*), prepro-α-factor (pp), endo H-treated glycosylated prepro-α-factor as deduced from this work (pp*), and glycosylated pro-α-factor (gp1). IVTT, Products of in vitro translation-translocation of prepro-α-factor as in Fig. 2.

[^{35}S]methionine, and the m4 mutant proteins were immunoprecipitated; after gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes for direct protein sequencing (31). Slices of membrane containing either gp1 or gp2 were then excised and subjected to automated Edman protein sequencing reactions. A significant fraction of the total radioactivity was released from the gp2 band in the first sequencing turn (Table 2), showing that a methionine is the N-terminal residue of gp2. The first of four methionine residues following the N-terminal methionine of prepro-α-factor is at position 101, and therefore gp2 must retain the signal sequence. As expected, only a small percentage of the total radioactivity in the gp1 band was released in the first turn, and the small amount released may have been due to contamination with gp2 protein. These studies show, therefore, that four of the five signal sequence mutations inhibited signal peptidase processing of translocated prepro-α-factor.

DISCUSSION

In a previous study, we showed that signal sequence mutations within prepro-α-factor decreased translocation efficiency by 5- to 50-fold in vitro (1). Here we have described experiments showing that these mutations also had a deleterious effect on translocation into the ER in vivo. To a first approximation, the relative magnitude of effect of each mutation on translocation seems to be about the same in vivo as that observed in vitro. The Ala-13→Glu mutation (m3), for example, reduced the rate of translocation about 50-fold in vitro and at least 25-fold in vivo. A major difference between the two systems is, of course, that translocation of both WT and mutant prepro-α-factor is much more efficient within intact cells than in vitro. In fact, the high efficiency of translocation that exists in vivo apparently can obscure significant differences between normal and mutated signal sequences if the assay for translocation is not sufficiently sensitive. The particular conditions of the halo assays we used in the previous study to test the in vivo effects of the α-factor signal sequence mutations showed only slight differences between WT and two of three mutants in steady-state secretion of α-factor (1). In contrast, by using a direct assay of translocation—short pulse-labeling of cells followed by immunoprecipitation of α-factor precursors—we found in this study that most of the mutations had at least a 5- to 25-fold negative effect on translocation in vivo. Increasing the expression level of each α-factor gene and altering the conditions of the halo assays somewhat revealed that all of the mutations also decrease secretion of α-factor.

Unlike the findings of this study, replacement with a charged amino acid within the hydrophobic core region of the signal peptide of the yeast carboxypeptidase Y precursor apparently did not affect translocation in vivo (5). In our case, we selected for mutations affecting translocation, and it is unlikely that all signal sequence mutations within

TABLE 2. Results of radiosequencing m4 mutant protein

Protein	cpm released in sequencing reactions at given turn			Total cpm on membrane[a]
	1	2	3	
gp1	140	22	20	19,040
gp2	1,120	43	18	12,520

[a] Counts per minute remaining on membrane after sequencing reactions plus counts per minute released during sequencing.

prepro-α-factor would lead to a detectable effect on translocation. Nevertheless, that small changes within the signal sequence of prepro-α-factor can have large effects on translocation implies that there is a fairly high degree of specificity in the interaction between the signal peptide and components of the translocation machinery. How can this view be reconciled with the observation that 25% of all random sequences attached to the amino terminus of yeast invertase are able to function as secretion sequences (22)? The study with random sequences shows that the translocation apparatus is relatively flexible and helps to define minimal structural features (hydrophobic sequences) required of a signal sequence. However, the membrane translocation rates of the random sequence-invertase hybrids were not determined, and thus it cannot yet be concluded that these sequences are functionally equivalent to normal signal peptides with respect to translocation efficiency. Our studies have shown that a decreased rate of translocation may not lead to a corresponding effect on secretion, presumably because other steps in the secretion pathway can be rate limiting.

When proteins were radioactively labeled for even very short intervals (15 s), we observed only slight amounts of unglycosylated WT prepro-α-factor. This result is consistent with a cotranslational mode of translocation for WT prepro-α-factor, although we cannot exclude that translocation is posttranslational and occurs very rapidly after completion of the polypeptide chain. In addition, nearly all of the WT α-factor precursor had been processed by signal peptidase to pro-α-factor (data not shown). This result indicates that the signal sequence is cleaved either during or immediately after translocation into the ER. On the other hand, translocation of the Ala-13→Glu (m3) mutant protein into the ER appeared to be almost completely posttranslational. Thus, prepro-α-factor can be transported into the ER posttranslationally in vivo as well as into microsomes in vitro (16, 36, 45). Indeed, it seems likely that the effects of these signal sequence mutations would have been more severe if prepro-α-factor were incapable of posttranslational translocation. It would be interesting in this regard to compare the in vivo translocation activity of the WT and mutant α-factor signal sequences when attached to a protein that is unable to translocate posttranslationally (in vitro), such as yeast preinvertase (17, 36). Preinvertase may be incapable of posttranslational transport because it folds into a highly stable structure that is not easily unfolded into a translocation-competent form (17, 42). If this is true, then the efficiency of translocation of invertase by a mutated signal sequence would depend on how quickly the polypeptide folds into a stable, translocation-incompetent form.

As well as adversely affecting translocation, four of the five signal sequence mutations also were found to reduce the rate of processing by signal peptidase. This is a surprising result because none of these mutations is located near the signal peptidase cleavage site (46; Fig. 1). In fact, the mutation with the largest effect on processing, Ala-9→Glu (m4), is at position −11 relative to the processing site. These results suggest that the yeast signal peptidase recognizes a larger portion of the prepro-α-factor signal sequence than just the region adjacent to the cleavage site. Among the lesions affecting processing is the Ala-13→Pro mutation, which would be predicted to disrupt the secondary structure of the signal peptide (8) without significantly altering its hydrophobicity. Optimal cleavage rates by signal peptidase may depend on the secondary structure of the signal peptide as well as on the sequences at the cleavage site. These data suggest that the translocation apparatus and signal peptidase may recognize overlapping regions and a similar secondary structure within the signal peptide. This hypothesis is contrary to a previous model which proposed that the translocation and cleavage specificity sequences within a signal peptide function independently of each other (43). A prediction that can be made from our model is that additional mutations within the hydrophobic core of one of the mutant prepro-α-factor signal sequences generally would have the effect of further inhibiting both translocation and processing by signal peptidase.

Precursors with mutations that block translocation completely would, of course, never be exposed to the action of signal peptidase. However, by using an in vitro signal peptidase assay (2, 14), it is possible to bypass the translocation step and examine directly the effects of signal sequence mutations on the cleavage reaction. Although there is not yet an in vitro signal peptidase system from yeasts (as far as we know), a gene that is required for removal of signal peptides in vivo has been identified (7). An in vitro processing analysis is also required to rule out the possibility that the effect of the α-factor signal sequence mutations on signal processing is indirect. It is possible, for example, that the mutations not only decrease the rate of translocation but also alter in a qualitative way transfer of the protein into the ER such that processing by signal peptidase is less efficient. It is not obvious how translocation of mutant proteins could be altered in such a way as to affect processing. As discussed above, it may be that one effect of the α-factor signal sequence mutations is to convert (at least partly) translocation of the precursors from a cotranslational to a posttranslational mode. However, there does not appear to be a strong correlation between the effect of a mutation on translocation and the effect on processing. Furthermore, the m5 mutation apparently has no effect on processing.

Previously, signal sequence mutations were identified in proteins secreted from yeast cells (37) or bacteria (24, 29) that blocked or severely inhibited processing by signal (leader) peptidase. These mutations were usually located proximal to the processing site and apparently had no effect on translocation. Numerous bacterial signal sequence mutations that affect translocation have been identified (4, 33), and there are at least two possible reasons why single mutations affecting both translocation and processing have not been identified among them. (i) A mutation that severely inhibits translocation prevents most of the precursors from becoming exposed to leader peptidase. (ii) Procaryotic systems lack a generally useful landmark for translocation (other than processing by leader peptidase), such as glycosylation, thus necessitating more difficult and less easily quantifiable procedures such as protection against digestion by added protease (24). This would be especially difficult when the mutation has only a transient effect on processing, as was the case with the α-factor signal sequence mutants. Among mitochondrial targeting sequences, there are several examples known in which mutations in the mid-region or amino-terminal region of the peptide adversely affect both import function and processing by the specific protease localized in the mitochondrial matrix (3, 18; D. T. Mooney, D. B. Pilgrim, and E. T. Young, submitted for publication). Thus, it may be a general feature of proteases that remove targeting signals that they recognize an extensive region along the N-terminal signal.

In addition to their value in defining features of the signal peptide important for translocation and processing, these mutants also should be useful in further studies on secretion.

For example, using genetic techniques such as suppression of a mutant phenotype (i.e., suppression of the impaired α-factor secretion phenotype), it might be possible to identify proteins of the translocation apparatus that interact with the signal peptide. Alternatively, a translocation factor that interacts directly with the signal peptide would be expected to cross-link in vitro (47) with WT but not mutant prepro-α-factor.

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LITERATURE CITED

1. **Allison, D. S., and E. T. Young.** 1988. Single-amino-acid substitutions within the signal sequence of yeast prepro-α-factor affect membrane translocation. Mol. Cell. Biol. **8**:1915–1922.
2. **Baker, R. K., G. P. Bentivoglio, and M. O. Lively.** 1986. Partial purification of microsomal signal peptidase from hen oviduct. J. Cell. Biochem. **32**:193–200.
3. **Bedwell, D. M., S. A. Strobel, K. Yun, G. D. Jongeward, and S. D. Emr.** 1989. Sequence and structural requirements of a mitochondrial protein import signal defined by saturation cassette mutagenesis. Mol. Cell. Biol. **9**:1014–1025.
4. **Benson, S. A., M. N. Hall, and T. J. Silhavy.** 1985. Genetic analysis of protein export in *Escherichia coli* K12. Annu. Rev. Biochem. **54**:101–134.
5. **Blachly-Dyson, E., and T. H. Stevens.** 1987. Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal sequence. J. Cell Biol. **104**:1183–1191.
6. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835–851.
7. **Böhni, P. C., and R. J. Deshaies.** 1988. Sec11 is required for signal peptide processing and yeast cell growth. J. Cell Biol. **106**:1035–1042.
8. **Briggs, M. S., D. G. Cornell, R. A. Dluhy, and L. M. Gierasch.** 1986. Conformations of signal peptides induced by lipids suggest initial steps in protein export. Science **233**:206–208.
9. **Burkholder, A. C., and L. H. Hartwell.** 1985. The yeast α-factor receptor: structural properties deduced from the sequence of the Ste2 gene. Nucleic Acids Res. **13**:8463–8475.
10. **Byrd, J. C., A. L. Tarentino, F. Maley, P. H. Atkinson, and R. B. Trimble.** 1982. Glycoprotein synthesis in yeast. J. Biol. Chem. **257**:14657–14666.
11. **Chirico, W. J., M. G. Waters, and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **332**:805–810.
12. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor proteins. Nature (London) **332**:800–805.
13. **Deshaies, R. J., and R. Schekman.** 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. J. Cell Biol. **105**: 633–645.
14. **Evans, E. A., R. Gilmore, and G. Blobel.** 1986. Purification of microsomal signal peptidase as a complex. Proc. Natl. Acad. Sci. USA **83**:581–585.
15. **Gilmore, R., and G. Blobel.** 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. J. Cell Biol. **95**:470–477.
16. **Hansen, W., P. D. Garcia, and P. Walter.** 1986. *In vitro* protein translocation across the yeast endoplasmic reticulum: ATP-dependent post-translational translocation of the prepro-α-factor. Cell **45**:397–406.
17. **Hansen, W., and P. Walter.** 1988. Prepro-carboxypeptidase Y and a truncated form of pre-invertase, but not full-length invertase, can be posttranslationally translocated across microsomal vesicle membranes from *Saccharomyces cerevisiae*. J. Cell Biol. **106**:1075–1081.
18. **Hurt, E. C., D. S. Allison, U. Müller, and G. Schatz.** 1987. Amino-terminal deletions in the presequence of an imported mitochondrial protein block the targeting function and proteolytic cleavage at the carboxy terminus. J. Biol. Chem. **262**: 1420–1424.
19. **Ito, H., Y. Kukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
20. **Julius, D., L. Blair, A. Brake, G. Sprague, and J. Thorner.** 1983. Yeast α factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. Cell **32**:839–852.
21. **Julius, D., R. Schekman, and J. Thorner.** 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell **36**:309–318.
22. **Kaiser, C. A., D. Preuss, P. Grisafi, and D. Botstein.** 1987. Many random sequences functionally replace the secretion signal sequence of yeast invertase. Science **235**:312–317.
23. **Konopka, J. B., R. L. Davis, S. M. Watanabe, A. S. Ponticelli, L. Schiff-Maker, N. Rosenberg, and O. N. Witte.** 1984. Only site-directed antibodies reactive with the highly conserved *src*-homologous region of the v-*abl* protein neutralizes kinase activity. J. Virol. **51**:223–232.
24. **Koshland, D., R. T. Sauer, and D. Botstein.** 1982. Diverse effects of mutations in the signal sequence on the secretion of β-lactamase in *Salmonella typhimurium*. Cell **30**:903–914.
25. **Kurjan, J.** 1985. α-Factor structural gene mutations in *Saccharomyces cerevisiae*: effects on α-factor production and mating. Mol. Cell. Biol. **5**:787–796.
26. **Kurjan, J., and B. D. Hall.** 1982. Mutation at the *Saccharomyces cerevisiae SUP4* $tRNA^{Tyr}$ locus: isolation, genetic fine-structure mapping, and correlation with physical structure. Mol. Cell. Biol. **2**:1501–1513.
27. **Kurjan, J., and I. Herskowitz.** 1982. Structure of a yeast pheromone gene (MFα): a putative α-factor precursor contains four tandem copies of mature α-factor. Cell **30**:933–943.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
29. **Lin, J. J. C., H. Kanazawa, and H. C. Wu.** 1980. Purification and characterization of the outer membrane lipoprotein from an *Escherichia coli* mutant altered in the signal sequence of prolipoprotein. J. Biol. Chem. **255**:1160–1163.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidine difluoride membranes. J. Biol. Chem. **262**:10035–10038.
32. **Meyer, D. I., E. Krause, and B. Dobberstein.** 1982. Secretory protein translocation across membranes—the role of the "docking protein." Nature (London) **297**:647–650.
33. **Michaelis, S., and J. Beckwith.** 1982. Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Annu. Rev. Microbiol. **36**:435–465.
34. **Milstein, C., G. G. Brownlee, T. M. Harrison, and M. D. Mathews.** 1972. A possible precursor of immunoglobulin light chains. Nature (London) New Biol. **239**:117–120.
35. **Novick, P., C. Field, and R. Schekman.** 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21**:205–215.
36. **Rothblatt, J. A., and D. I. Meyer.** 1986. Secretion in yeast: translocation and glycosylation of prepro-α-factor in vitro can occur via an ATP-dependent post-translational mechanism. EMBO J. **5**:1031–1036.
37. **Schauer, I., S. Emr, C. Gross, and R. Schekman.** 1985. Invertase signal and mature sequence substitutions that delay inter-com-

partmental transport of active enzyme. J. Cell Biol. **100**:1664–1675.

38. **Spindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**:132–141.
39. **Tarentino, A. L., T. H. Plummer, and F. Maley.** 1974. The release of intact oligosaccharides from specific glycoproteins by endo-β-N-acetyl-glucosaminidase H. J. Biol. Chem. **249**:818–824.
40. **Toyn, J., A. R. Hibbs, P. Sanz, J. Crowe, and D. I. Meyer.** 1988. In vivo and in vitro analysis of pt11, a yeast ts mutant with a membrane associated defect in protein translocation. EMBO J. **7**:4347–4353.
41. **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.
42. **Verner, K., and G. Schatz.** 1988. Protein translocation across membranes. Science **241**:1307–1313.
43. **von Heijne, G.** 1983. Patterns of amino acids near signal sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
44. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184**:99–105.
45. **Waters, M. G., and G. Blobel.** 1986. Secretory protein translocation in a yeast cell-free system can occur post-translationally and requires ATP hydrolysis. J. Cell Biol. **102**:1543–1550.
46. **Waters, M. G., E. A. Evans, and G. Blobel.** 1988. Prepro-α-factor has a cleavable signal sequence. J. Biol. Chem. **263**: 6209–6214.
47. **Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport.** 1987. A signal sequence receptor in the endoplasmic reticulum membrane. Nature (London) **328**:830–833.
48. **Yaffe, M. P., and G. Schatz.** 1984. Two nuclear mutations that block mitochondrial protein import in yeast. Proc. Natl. Acad. Sci. USA **81**:4819–4823.