MOLECULAR AND CELLULAR BIOLOGY, Aug. 1989, p. 3400–3410
0270-7306/89/083400-11$02.00/0

Vol. 9, No. 8

Cassette Mutagenic Analysis of the Yeast Invertase Signal Peptide: Effects on Protein Translocation

JOHNNY K. NGSEE,[1]† * WILLIAM HANSEN,[2] PETER WALTER,[2] AND MICHAEL SMITH[1]

Department of Biochemistry and the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5,[1] and Department of Biochemistry and Biophysics, University of California Medical School, San Francisco, California 94143-0448[2]

Received 14 December 1988/Accepted 16 May 1989

The coding sequence of the *SUC2* locus was placed under the control of the constitutive *ADH1* promoter and transcription terminator in a centromere-based yeast plasmid vector from which invertase is expressed in a Suc$^-$ strain of *Saccharomyces cerevisiae*. Mutants in the signal peptide sequence were produced by replacing this region of the gene with synthetic oligonucleotide cassettes containing mixtures of nucleotides at several positions. The mutants could be divided into three classes on the basis of the ability to secrete invertase. Class I mutants produced secreted invertase but in reduced amount. The class II mutant, 4-55B, also exhibited reduced a level of invertase, but a significant fraction of the enzyme was intracellular. Class III mutants were partially defective in translocation from the cytoplasm to the endoplasmic reticulum and produced enzymatically active, unglycosylated preinvertase in the cytoplasm. Class III mutant preinvertases were also defective in translocation across canine pancreas microsomes. These results suggested that the reduced level of invertase resulted from proteolytic degradation of inefficiently transported intermediates. Comparison of the sequences of the mutant signal peptides indicated that amino acids at the extreme amino terminus and adjacent to the cleavage site play a crucial role in the secretory process when combined with a mutation within the hydrophobic core.

According to the signal hypothesis, proteins destined for secretion are synthesized with an amino-terminal extension known as the signal peptide (8). The signal recognition particle (SRP), purified from canine pancreas, has been shown to recognize the signal peptide and in some cases to mediate a transient arrest in nascent chain elongation before cotranslational targeting of the precursor to the endoplasmic reticulum (ER) membrane (60, 61). The mechanism by which the signal peptide is recognized by SRP remains elusive. Because of the lack of primary sequence homology among signal peptides (57, 58), recognition by SRP almost certainly must involve certain secondary or tertiary structural features of the signal sequence. These may include features that are common to all signal peptides, such as a hydrophilic amino terminus with one or more basic residues, a central hydrophobic core consisting of 6 to 15 consecutive nonpolar amino acids, and small neutral amino acids adjacent to or at the cleavage site.

In recent years, much effort has been focused on protein secretion in yeast cells. The main attraction is the relative ease with which these organisms can be genetically manipulated. Consequently, a number of temperature-sensitive secretory mutants have been isolated and characterized (15, 21, 22, 44, 45). In addition, the recent development of an in vitro translation-translocation system derived from yeast cells (27, 50, 62) has provided an invaluable tool for studying the biochemical processes and cellular components involved in the early stages of secretion.

The purpose of this study was to define the features encoded within signal peptides that are crucial for proper targeting and translocation in order to complement studies on signal peptide mutations in yeast invertase (29), carboxypeptidase Y (7), and prepro-α-factor (1) and on mutants affecting the machineries of intracellular transport. The yeast invertase gene, encoded by the *SUC2* locus (10), was chosen because yeast cells carrying a wild-type *SUC2* gene have two forms of invertase: a constitutive, unglycosylated cytoplasmic form and a glucose-repressed, glycosylated secreted form. These two forms arise from differential 5′ transcription start sites (11, 46). The longer transcript encodes the signal peptide and gives rise to secreted invertase, whereas the shorter transcript, when translated, yields cytoplasmic invertase. Thus, the information required to direct the enzyme into the secretory pathway is contained within the signal peptide sequence. In addition, the signal sequence of preinvertase functions equally well in a mammalian system (6), suggesting that the behavior of mutants in yeast cells will have more general implications.

Since the target region in the gene is well defined, cassette mutagenesis was used to generate the signal sequence mutations. There are two major advantages of this method. First, a large family of amino acid substitutions can be introduced at any desired position. Second, nonsense mutations can be avoided by carefully selecting the position of the target nucleotides in the codon or by altering the mutant base compositions of the cassette oligonucleotides during synthesis. In this study, mutations were directed mainly at the amino and carboxy termini of the signal peptide, leaving the hydrophobic core largely intact. This approach was designed to minimize perturbation of the hydrophobic core and to focus on the effects of these regions of the signal peptide in the context of an intact hydrophobic core and on its disruption. A Suc$^-$ yeast strain was transformed with a single-copy centromere-containing yeast plasmid into which the altered *SUC2* genes were inserted. The resulting transformants were screened for the inability to synthesize or secrete enzymat-

* Corresponding author.
† Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020.

ically active invertase. The mutants were divided into three classes, which were further characterized by immunological detection, cell fractionation, in vivo pulse-labeling, and in vitro translation and translocation.

MATERIALS AND METHODS

Bacterial and yeast strains. Bacterial vectors were propagated in *Escherichia coli* RRI (9), JM101 (41), and RZ1032 (36). *Saccharomyces cerevisiae* JNY4 (*MATa trp1 ade2-101 suc2-215*) was constructed by crossing MCY36 (12) and C1 (Cold Spring Harbor Laboratory stock) and used as host cells for invertase signal sequence mutants. Unless stated otherwise, yeast cells were grown at 30°C in a synthetic medium containing 0.67% yeast nitrogen base and 2% glucose, supplemented with all amino acids except tryptophan.

Plasmid constructs. The *Pst*I site within the *ARS1* sequence and the two *Eco*RI sites in YRp7 (53) were destroyed by digesting the plasmid with the appropriate restriction enzymes and repairing the ends with T4 DNA polymerase. The remaining *Pst*I site in the *bla* gene was removed by replacing the *Sca*I-to-*Pvu*II fragment with that from pUC13 (56). The *Bam*HI-to-*Cla*I fragment of pYe(CEN3)30 (13) was cloned into the plasmid for stability and copy number control. The *ADH1* promoter-terminator fragment from plasmid pAAR6 (2) was then cloned into the unique *Bam*HI site to give plasmid pJ1.

The 4.3-kilobase *Eco*RI fragment from plasmid pRB58 (54) containing the *SUC2* coding sequence was subcloned into pEMBL8+ (14). By using oligonucleotide-directed mutagenesis (36, 43, 63), an *Eco*RI site was generated at the 5′ untranslated region. The *Hin*dIII site 3′ of the coding sequence was converted into an *Eco*RI site by ligation to an *Eco*RI linker. The 2.2-kilobase *Eco*RI fragment was isolated and cloned into the *Eco*RI site of pJ1 to give plasmid pS1. To facilitate subsequent manipulations, the *Eco*RI site at the junction of the *ADH1* terminator was destroyed.

Cassette mutagenesis. Complementary sets of oligonucleotides homologous to the signal sequence of invertase were synthesized with an Applied Biosystems 380A DNA synthesizer. The wild-type bases at several predetermined positions were synthesized with a mixture of two or the other three bases at a ratio calculated to give an average of two mutant bases per complementary sets of oligonucleotides (39, 48). The phosphorylated oligonucleotides were boiled for 5 min and allowed to anneal at 55°C for 10 min. Oligonucleotides SU1 and SU1-C were cloned into a modified M13mp18 vector with the *Hin*dIII and *Sph*I sites removed but with the β-galactosidase reading frame maintained. Competent JM101 cells were transfected, and replicative-form DNAs from approximately 200 clones were pooled. Annealed oligonucleotides SU2 and SU2-C were then ligated into the *Hin*dIII site of the first set of oligonucleotides and the *Pst*I site of the vector. The religated vectors were restricted with *Kpn*I before transfection to eliminate those not possessing the second set of oligonucleotides. The *Eco*RI-to-*Pst*I fragment from a pool of approximately 200 plaques was transferred to the *SUC2*-containing yeast vector pS5. This pool was used to transform yeast strain JNY4. The transformants were screened for invertase activity. Plasmids from those exhibiting an interesting phenotype were rescued and subjected to DNA sequence analysis to determine the nature of the mutation.

Screening of secretory mutants in yeast cells. Yeast transformants were transferred onto nitrocellulose filters and grown on tryptophan-deficient plates at 30°C for 16 h. To screen for internal invertase, the filters were placed in a chloroform-saturated chamber for 10 min. They were then gently overlaid on 3MM filter paper (Whatman, Inc.) soaked with 100 mM sodium acetate (pH 5)–250 mM sucrose–10 mM NaN_3 for 5 to 10 min. The filters were dried briefly and stained with 0.2% 2,3,5-triphenyl tetrazolium chloride in 0.5 M NaOH. To assay for external invertase activity, the chloroform permeation step was omitted.

Quantitative determination of invertase activity was performed on intact yeast cells as described previously (24). For total invertase activity, the cells were pretreated with 10 μl of chloroform. One unit of invertase activity is defined as one micromole of glucose generated per minute at 37°C per A_{600} unit of cells.

Immunological detection of invertase. Rabbits were immunized with biweekly doses of 200 μg of glycosylated invertase in complete Freund adjuvant. Invertase-specific antibodies were purified by affinity chromatography with internal invertase immobilized on a Sepharose 4B support. The antibodies were eluted from the column with 0.1 M acetic acid (pH 2.9) and immediately neutralized with 1 M Tris base to pH 7. Residual cross-reacting species were removed by adsorption with a crude extract of a Suc^- strain (JNY4).

For Western (immunoblot) analysis, yeast cells were grown to exponential phase in 10 ml of minimal selective medium. The harvested cells were suspended in 500 μl of ice-cold lysis buffer (50 mM Tris hydrochloride [pH 7.5], 20 mM EDTA, 2 mM phenylmethylsulfonyl fluoride) and homogenized in the presence of glass beads (0.45-mm diameter). The extract was centrifuged for 10 min in a microfuge. The level of enzyme activity in the supernatant was determined, and 0.1 U (2 to 35 μl) was fractionated on a 7.5 to 12.5% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nitrocellulose filter (55). The filter was incubated with 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–5% skim milk for 30 to 60 min. Affinity-purified anti-invertase antibodies at a 1:100 dilution were added and incubated at room temperature for 60 to 90 min. The filter was washed three times with 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–0.25% *N*-lauryl sarcosine for 5 to 10 min each time. Bound antibodies were detected with alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G antiserum as specified by the supplier.

Cell fractionation and protease protection. Cells grown to exponential phase in 100 ml of selective medium at 30°C were spheroplasted with Zymolase 60000 (Kirin Brewery), and the extract was fractionated on Percoll (Sigma Chemical Co.) gradient (27). The fractions were assayed for invertase and NADPH-cytochrome *c* reductase (34). The ER-enriched fractions were pooled, and 10 μl was diluted twofold with ice-cold 20 mM *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–250 mM sucrose–10 mM $CaCl_2$–2 mM dithiothreitol. Tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and proteinase K, preincubated at 37°C for 15 min, were added to final concentrations of 750 and 200 μg/ml, respectively. The digestion was carried out at 0°C for 60 min and stopped with the addition of 2 mM phenylmethylsulfonyl fluoride. The samples were immediately added to preheated SDS sample buffer, fractionated on SDS-polyacrylamide gels, and subjected to Western analysis as described above.

The oligosaccharide chains were removed by using the enzyme *N*-glycanase (Genzyme). SDS and 2-mercaptoethanol were added to the ER-enriched sample to final concen-

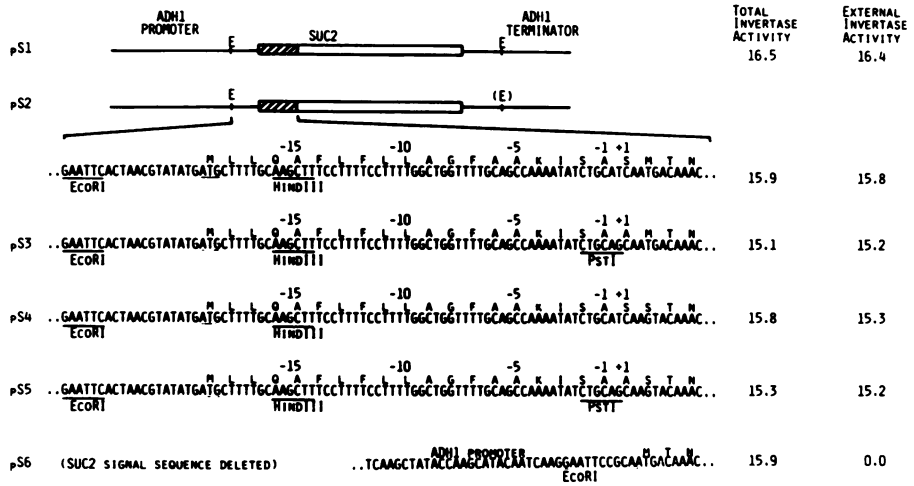

FIG. 1. Sequence and invertase activities of the modified *SUC2* gene. The modifications were introduced with oligonucleotide-directed mutagenesis. The *Eco*RI site 3′ to the *SUC2* coding sequence in plasmid pS1 was deleted to facilitate subsequent subcloning (plasmid pS2). A *Pst*I site was created at the cleavage junction in plasmid pS3. The internal methionine (ATG) codon was mutated to that coding for a serine (AGT) in plasmid pS4. Plasmid pS5 combined these two changes, and the coding sequence for the signal peptide was deleted in plasmid pS6. The modified expression plasmids were transformed into the Suc yeast strain JNY4. Invertase activities are expressed as micromoles of glucose generated per minute per A_{600} unit of cells at 37°C.

trations of 0.5 and 1%, respectively. The sample was denatured at 100°C for 5 min and spun in a microfuge for 5 min to remove the Percoll particle. Deglycosylation was carried out in 0.2 ml of 0.2 M sodium phosphate (pH 8.6)–10 mM 1,10-phenanthroline–1.25% Nonidet P-40–1 U of *N*-glycanase per ml at 37°C for 18 h. The protein was then precipitated with 10% trichloroacetic acid, fractionated on an SDS-polyacrylamide gel, and subjected to Western analysis.

Cell labeling and immunoprecipitation. Exponentially grown cells were spheroplasted with Zymolase 60000 and collected by centrifugation through 3 volumes of sorbitol cushion (50 mM potassium phosphate [pH 7.5], 1.6 M sorbitol) at 3,000 × *g* for 5 min. The cells were suspended at 5 A_{600} units per ml in synthetic medium without tryptophan and methionine but maintained isotonically with 1.4 M sorbitol. The cells were preincubated at 30°C for 30 min. L-[^{35}S]methionine (>1,000 Ci/mmol) was added at 20 μCi per A_{600} unit of cells. After 4 min, the chase period was initiated by the addition of 0.2 mM unlabeled methionine and 0.1 mg of cycloheximide per ml. Equal portions of cells were removed at various times and chilled on ice with 10 mM NaN_3 until all samples had been collected. The cells were centrifuged through 3 volumes of sorbitol cushion as described above. The cell pellet was homogenized with glass beads and subjected to centrifugation at 12,000 × *g* for 10 min. The supernatant was adjusted to a 1-ml volume with 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–5 mg of bovine serum albumin per ml–1% aprotinin. Affinity-purified anti-invertase antibodies were added to the samples, which were incubated at 0°C overnight. Protein A-Sepharose was added; after an incubation period of 60 min at room temperature, the beads were washed twice with 10 mM Tris hydrochloride (pH 7.5)–200 mM NaCl–0.5 M urea–0.5% *N*-lauryl sarcosine, once with 10 mM Tris hydrochloride (pH 7.5)–200 mM NaCl, and once with 0.5% 2-mercaptoethanol. The sample was subjected to SDS-polyacrylamide gel electrophoresis, fluorographed with Enlightning (Dupont, NEN Research Products), and autoradiographed with Kodak X-Omat XAR-5 film (Eastman Kodak Co.) at −70°C. Autoradiograms were quantified with a model 620 densitometer (Bio-Rad Laboratories, Richmond, Calif.).

In vitro transcription and translation. The *Bam*HI-to-*Hin*dIII fragment of pRB58 was cloned into pSP65 (32, 40). The amino-terminal regions of all invertase mutants were subcloned into the *Eco*RI-to-*Bam*HI site of this plasmid. For the truncated preinvertase transcripts, the plasmids were linearized with *Bam*HI, and the recessed ends were repaired with DNA polymerase I (Klenow fragment) and religated with T4 DNA ligase. This generated a termination signal eight codons downstream of the deleted *Bam*HI site. In vitro transcription using SP6 RNA polymerase was performed as described previously (32, 40), and the transcribed mRNAs were translated with the wheat germ (19, 59) or yeast in vitro translation system (27).

RESULTS

Oligonucleotide-directed mutagenesis of *SUC2*. The *SUC2* coding sequence was placed under the control of the constitutive *ADH1* promoter-terminator because the wild-type *SUC2* promoter responsible for expression of the secreted form of invertase is repressed in the presence of glucose (11). It is more convenient for screening of invertase activity to have a constitutively expressed enzyme.

Several other modifications in the *SUC2* gene were introduced to facilitate cassette replacement of the signal sequence and to eliminate the internal methionine codon responsible for translation initiation of the cytoplasmic form of the enzyme. First, a *Pst*I site was constructed adjacent to the normal cleavage site (Fig. 1). This resulted in a missense mutation of the serine residue at position +1 of the mature sequence to an alanine (plasmid pS3). Second, the internal methionine (ATG) codon used in the translation initiation of the cytoplasmic form of invertase was mutated to a serine (AGT) codon (plasmid pS4). This change ensured that all invertase produced initially contained the signal peptide. Third, these two mutations were combined in plasmid pS5, which now contained the amino acid sequence Ser-Ala-Ala-Ser at the cleavage junction. This plasmid was subsequently used in the cassette replacement of the signal peptide. A protein with a fully functional signal peptide would be rapidly transported through the secretory pathway,

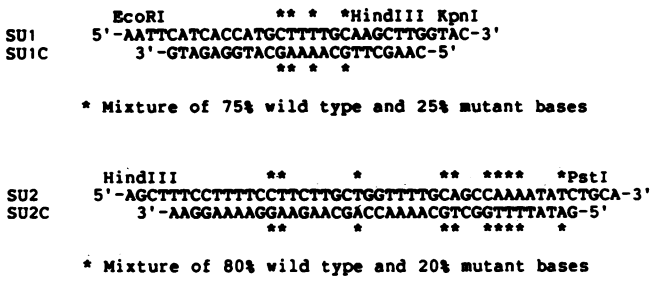

FIG. 2. Cassette oligonucleotides used to generate the invertase signal sequence mutants. During synthesis, mixtures of two or three of the other nucleotides were added to the wild-type nucleotide at the ratio indicated. This ratio was chosen to give an average of one to two mutations per clone for each complementary oligonucleotide. The mutant nucleotide mixtures were designed to avoid the introduction of termination codons. Their positions in the sequence are indicated (*).

hence showing little or no significant steady-state internal accumulation. All invertase activity was secreted (Fig. 1), indicating that (i) the three changes made had no significant effect on invertase secretion and (ii) the sequestration of invertase into the secretory pathway was extremely efficient. As expected, when the signal peptide was deleted (plasmid pS6), invertase secretion was completely abolished and all invertase activity was intracellular. Since synthesis of the cytoplasmic form of invertase was abolished by replacement of the internal methionine codon and its cognate promoter and all invertase produced initially contained the signal peptide, any internal accumulation of the protein resulting from cassette mutagenesis must have resulted from a failure at some stage of the secretory pathway to efficiently process the protein for secretion.

Cassette mutagenesis of the *SUC2* signal sequence. The cassette oligonucleotides shown in Fig. 2 were subcloned into a modified M13mp18 vector. The *Eco*RI-to-*Pst*I fragment from a pool of 200 clones was then transferred to plasmid pS5 and transformed into the Suc$^-$ yeast strain JNY4. The transformants were screened for external invertase activity and subsequent DNA sequence determination. The mutants were divided into three classes (Table 1). Most of the mutants (4-6 to 7-27 in Table 1) belonged to a group (class I) in which almost all detectable invertase activity was external. All members in this group were able to grow on sucrose under anaerobic conditions, a test used to select for Suc$^+$ phenotype (12), further confirming the presence of substantial amounts of external invertase. An unexpected feature of the class I mutants is that mutations in the signal peptide caused up to a threefold decrease in the level of invertase activity. The class II mutants had one representative (4-55B in Table 1). This mutant also had a reduced level of invertase, but a significant fraction (30%) of the enzyme was intracellular. It exhibited a Suc$^+$ phenotype by the ability to grow on sucrose under anaerobic conditions. The class III mutants (28* to 4-56 in Table 1) also produced

TABLE 1. Invertase activities of signal sequence mutants

Plasmid or mutant	Signal sequence mutation																				Invertase activity (U/A_{600} unit of cells)	
	−15								−10					−5				−1			Total[a]	External
pS5	M	L	L	Q	A	F	L	F	L	L	A	G	F	A	A	K	I	S	A		15.3	15.2
Class I																						
4-6		H																			15.9	14.8
31		H			L											N					13.2	12.9
4-56B									D												12.4	12.9
27																H					12.1	12.1
30		H	V																		11.1	10.7
34		N																			10.7	10.1
32		G		E												R					10.5	10.5
25		V		K										E							10.2	8.9
M-3		V														N		P			9.8	9.8
4-3		V												V		T					9.4	9.4
3-15		F	M	K												N		P			9.3	8.3
M-2		H							F												9.2	9.5
4-56A		N	V																		9.0	8.7
35		F		E					H												9.0	8.3
26		R		E					F							G					8.7	8.4
3-75		H														T		P			8.3	8.3
3-6																		P			7.4	7.4
3-12				K												D					7.2	6.8
3-13		P							T												6.6	6.1
7-27		N							F												5.0	5.0
Class II																						
4-55B									R					E		Q					8.6	5.8
Class III																						
28*		N		E					P											AKISA	5.4	0.3
28		N		E					P												4.9	0.3
M-1		G							D												4.5	0.7
4-55		G							R					E		Q					4.0	0.2
M-5		V							R					E		Q					2.7	0.1
4-56		N	V						D												2.6	0.2

[a] Total invertase activity of a wild-type *SUC2* strain under glucose-derepressed conditions is between 0.9 and 1.7 U/A_{600} unit of cells.

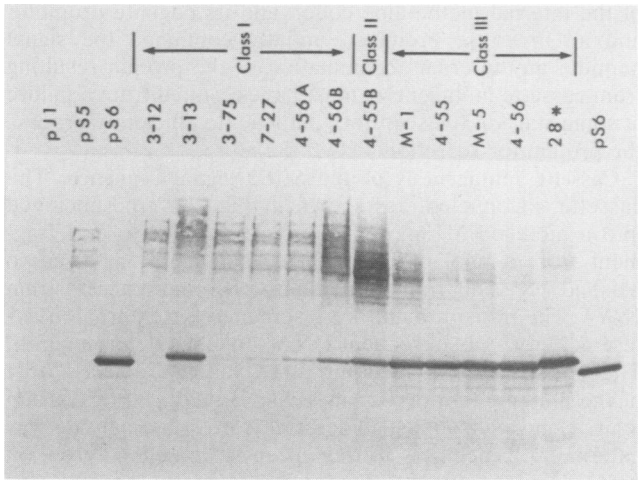

FIG. 3. Western analysis of the signal sequence mutants. Each lane was loaded with cell extract containing 0.1 U of invertase activity. The proteins were transferred to nitrocellulose paper after SDS-polyacrylamide gel electrophoresis, and immunoreactive invertase was detected with affinity-purified antibodies. No immunoreactive invertase could be detected in the strain harboring a plasmid lacking the *SUC2* coding sequence (pJ1). Fully glycosylated invertase appears as a broad band because of the heterogeneous nature of the oligosaccharide chains (e.g., pS5 and the class I mutants 3-12 to 4-56B). Cytoplasmic invertase in the signal peptide-deleted plasmid (pS6) had an apparent molecular size of 56 kilodaltons. A larger unglycosylated preinvertase was present in some of the class I (e.g., 3-13 and 4-56B), the class II (4-55B), and all of the class III (M-1 to 28*) mutants. Core glycosylated invertase, appearing as discrete bands with molecular sizes of 70 to 90 kd, is apparent in the class II mutant 4-55B and the Class III mutants M-1 and 4-55.

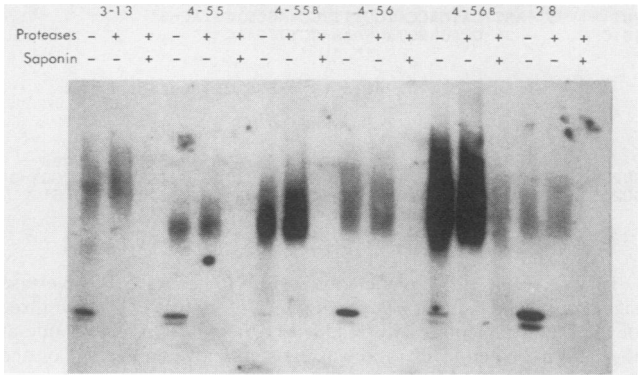

FIG. 4. Western analysis. Cell extracts fractionated on Percoll gradients were treated with TPCK-trypsin (750 μg/ml) and proteinase K (200 μg/ml) at 4°C for 60 min in the presence or absence of 0.3% saponin. After the treatment, the samples were fractionated on an SDS-polyacrylamide gel and subjected to Western analysis. Partial degradation of the unglycosylated forms, as evident by the appearance of a smaller immunoreactive band, was seen in all cases after mock treatment.

a reduced amount of invertase but differed from the class II mutant in that the majority of the enzyme was intracellular. All members of this class showed an extremely slow rate of growth on sucrose under anaerobic conditions and could be readily distinguished, on the basis of colony size, from a Suc^- or a wild-type *SUC2* strain. One of the mutants (28*) had a five-amino-acid duplication near the cleavage site but behaved identically to one lacking this duplication (mutant 28). Strikingly, in some cases a difference of a single amino acid could drastically alter the degree of external localization of the enzyme. This can be seen, for example, by comparing the activities of mutants M-5 and 4-55 with the activity of mutant 4-55B or the activities of mutants M-1 with those of mutants 4-56A, 4-56B, and 4-56.

Mutations in the signal peptide can alter the glycosylation of invertase. To examine the nature of the invertase produced by the signal peptide mutants, cell extracts were subjected to Western analysis in which the proteins were detected with affinity-purified anti-invertase antibodies. Figure 3 shows the Western analysis of cell extracts in which roughly equivalent amounts of invertase, as judged by enzymatic activity, were analyzed. Fully glycosylated invertase, often appearing as a broad band because of its heterogeneous degree of outer-chain glycosylation, was produced in the host carrying the parental plasmid, pS5. As expected, only the cytoplasmic, unglycosylated invertase with an apparent molecular size of 56 kilodaltons was seen with the signal peptide-deleted plasmid, pS6. No immunoreactive invertase was detected in an equivalent amount of protein from the host strain carrying a plasmid lacking the *SUC2* coding sequence (pJ1). In class I mutants such as 3-12, 3-75, 7-27, 4-56A, and 4-56B, almost all immunoreactive invertase appeared fully glycosylated. In contrast to findings for the parental plasmid (pS5), traces amount of unglycosylated preinvertase were also evident in some of the mutants, most notably mutant 3-13. This unglycosylated species exhibited a lower electrophoretic mobility than did the normal cytoplasmic form, suggesting the presence of an uncleaved signal peptide. This finding may reflect a slight deficiency in the ability of the protein to translocate to the luminal side of the ER membrane. Alternatively, the defect may be due to a delay in signal peptide cleavage and core glycosylation of the translocated enzyme. This issue will be addressed in the next section.

In the secretion-defective mutants, classes II and III, both unglycosylated and glycosylated forms of invertase were evident (Fig. 3). As in the class I mutants, the unglycosylated forms had a lower electrophoretic mobility than did the cytoplasmic invertase produced with the signal peptide-deleted plasmid, pS6. Two distinct glycosylated forms were evident in these mutants. A fraction of the glycosylated invertase in the class I (4-55B) and III (M-1 and 4-55) mutants exhibited the characteristic pattern seen when *SUC2* is expressed in a *sec18* mutant, a temperature-sensitive secretion mutant blocked at the transport between the ER membrane and the Golgi complex (20), indicating that glycosylation had not proceeded further than the addition of core oligosaccharides. The second glycosylated form, evident in all class II and III mutants, had a higher molecular weight and was more heterogeneous than the *sec18* type, suggesting processing of the carbohydrate moiety beyond the ER-specific core oligosaccharide addition.

The unglycosylated precursors are localized in the cytoplasm. Cell fractionation studies were undertaken to determine the subcellular localization of the different forms of invertase. Cell extracts were prepared and fractionated on Percoll gradients as described elsewhere (27). Western analysis of the membrane-enriched fractions from selected mutants showed the presence of glycosylated and unglycosylated invertase (Fig. 4). Since the addition of core oligosaccharide chains occurs at the luminal side of the ER membrane, it can be concluded that core glycosylated invertase must have translocated across the ER membrane. Furthermore, proteins containing outer-chain oligosaccharide (e.g., mutants 3-13 and 4-56B) must have translocated

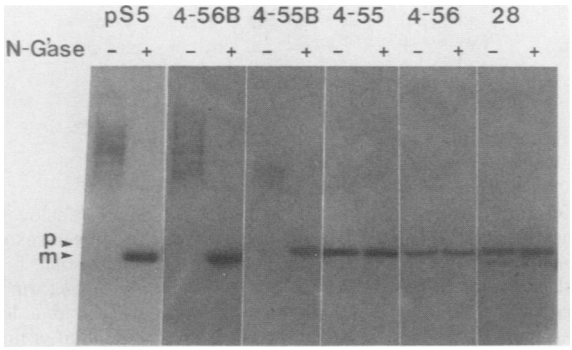

FIG. 5. Enzymatic removal of oligosaccharide chains with *N*-glycanase. Membrane-enriched Percoll gradient fractions were denatured with heat in the presence of SDS and 2-mercaptoethanol. The sample was then mock treated (−) or deglycosylated with *N*-glycanase (+), fractionated on an SDS-polyacrylamide gel, and subjected to Western analysis. The deglycosylated invertase from the wild-type (pS5) and class I mutant (4-56B) exhibited an electrophoretic mobility similar to that of the signal peptide-deleted invertase (m), whereas that from the class II (4-55B) and III (4-55, 4-56, and 28) mutants comigrated with the cytoplasmic unglycosylated preinvertase (p).

across the ER membrane and transported to the Golgi apparatus.

The association of unglycosylated invertase with the membrane-enriched fraction is of interest. One interpretation is that the protein is nonspecifically associated with cytoplasmic components of the ER membrane. Alternatively, the protein is partially or fully translocated across the ER membrane but has undergone neither processing by the signal peptidase nor core glycosylation. To resolve this question, the membrane fractions were treated with proteases (a combination of trypsin and proteinase K) in the presence or absence of detergent (0.3% saponin). Proteins that have completely translocated the membrane bilayer will be resistant to proteolysis unless the integrity of the membrane vesicles is disrupted by the nonionic detergent. The unglycosylated forms in all cases were susceptible to degradation by the proteases (Fig. 4). In contrast, proteolysis of the glycosylated forms was evident only in the presence of detergent. Therefore, it can be concluded that the unglycosylated preinvertases are peripherally associated on the cytoplasmic side of the membrane. Furthermore, the accumulation of the unglycosylated form cannot have resulted from a failure to glycosylate the enzyme after its translocation across the ER membrane. It is not clear whether the mutant preinvertases were specifically targeted to or nonspecifically associated with components on the cytoplasmic side of the ER membrane. However, the presence of a small amount (5 to 10%) of unglycosylated invertase from a host containing the signal peptide-deleted plasmid, pS6, copurifying with the membrane fraction is suggestive of the latter possibility.

To determine whether the signal peptide was cleaved in the glycosylated invertase, the oligosaccharide chains were enzymatically removed with *N*-glycanase, and the protein sample was subjected to Western analysis. Deglycosylation of the invertase intermediate from the class I mutant 4-56B produced a protein with the same electrophoretic mobility as that from the wild type (pS5) (Fig. 5). However, a deglycosylated invertase with an electrophoretic mobility identical to that of the cytoplasmic preinvertase was observed in the class II and III mutants, suggesting retention of the signal peptide.

TABLE 2. Half-lives of the two internal forms of invertase seen in mutants[a]

Plasmid or mutant	Invertase half-life (min)	
	Cytoplasmic 60-kDa form	Core glycosylated 70- to 90-kDa form
pS5	ND	1.8
4-55	>16	ND
4-55B	>16	4.5
4-56	>16	3.8
4-56B	ND	4.5
28	9	ND

[a] Cells were converted into spheroplasts with Zymolase 60000, labeled with L-[^{35}S]methionine for 4 min, and chased for various lengths of time. Invertase was immunoprecipitated, fractionated on an SDS-polyacrylamide gel, and autoradiographed. The amount of immunoreactive invertase was estimated by densitometric scanning of the bands. kDa, Kilodalton; ND, not detectable.

Kinetic analysis of the export of invertase. To determine whether mutations in the signal peptide have any effect on the rate of processing of invertase through the secretory pathway, pulse-chase studies were undertaken. Analysis of the host containing the parental plasmid, pS5, showed the absence of any detectable unglycosylated form at any time. The half-life of the intracellular enzyme (1.8 min; Table 2) was comparable with that observed for the wild-type invertase (1.5 min) reported previously (51).

Pulse-chase studies on the class I mutant 4-56B, which exhibited a near-wild-type phenotype on quantitative enzymatic assays (Table 1) and in Western (Fig. 3) and cell

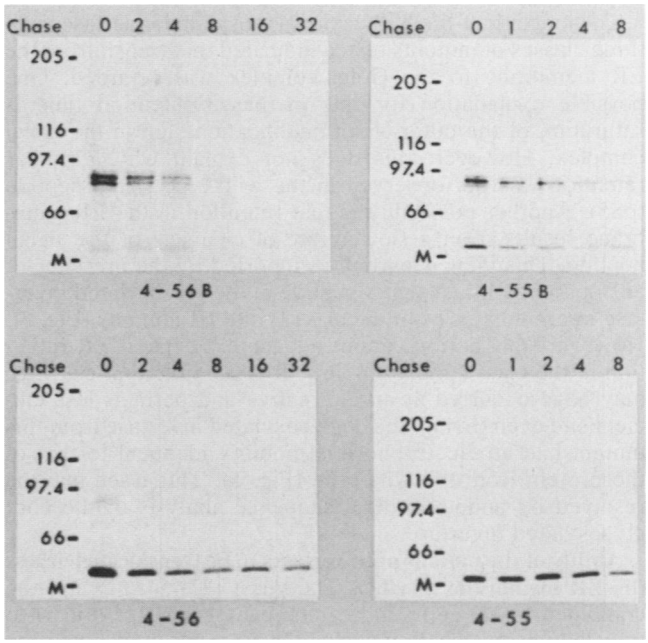

FIG. 6. Autoradiograms of pulse-labeled invertase. The cells were grown to exponential phase in selective medium and converted to spheroplasts with Zymolase 60000. The spheroplasted cells were pulse-labeled with L-[^{35}S]methionine for 4 min and chased for the time periods (in minutes) indicated. Invertase was immunoprecipitated with affinity-purified antibodies and fractionated on an SDS-polyacrylamide gel. Mutant 4-56B was chosen as a representative of the class I mutants, 4-55B is a class II mutant, and 4-55 and 4-56 are class III mutants.

fractionation (Fig. 4 and 5) analyses, revealed a transiently detectable core glycosylated invertase and barely detectable unglycosylated preinvertase (Fig. 6).

In the class II mutant, 4-55B, both core glycosylated and unglycosylated intermediates were evident (Fig. 6), indicating that the preinvertase was inefficiently translocated across the ER membrane and that transport from the ER membrane to the Golgi complex was retarded. It is not clear whether the preinvertase was eventually translocated across the ER membrane or degraded in the cytoplasm. Although the absence of posttranslational translocation of full-length and truncated preinvertase in vitro may argue in favor of the latter view, it does not preclude the possibility that a fraction of the preinvertase may be translocation competent. The fate of the core glycosylated invertase is not apparent in Fig. 6. However, the presence of secreted invertase in this mutant, as determined by a quantitative enzymatic assay (Table 1), indicated that a fraction of the glycosylated enzyme must eventually have reached the cell surface.

In the class III mutants, the unglycosylated preinvertase was the major intermediate detectable at all times (mutants 4-55 and 4-56; Fig. 6), suggesting that the major defect is the inability of the preinvertase to translocate across the ER membrane. In mutant 4-56, a small amount of core glycosylated enzyme was also transiently apparent. The intermediates seen in another class III mutant, 28, were similar to those in mutant 4-55, with the unglycosylated form being the major immunoprecipitated product; no appreciable increase in core glycosylated or externally localized invertase was detected. This cytoplasmic form of the enzyme was found to be less stable than in mutant 4-55 or 4-56, with a half-live of approximately 9 min (Table 2). Thus, the majority of the preinvertase in mutant 28 likely remained in the cytoplasm and perhaps was degraded by cytoplasmic proteases.

The longer half-life of the core glycosylated invertase in all three classes of mutants tested indicated that transit from the ER membrane to the Golgi complex was retarded. One possible explanation for this increased retention time is saturation of the outer-chain modification step in the Golgi complex. However, this does not explain why a similar retention was not observed in the wild-type signal peptide (pS5). Another possibility is that retention in the ER membrane results from a slower rate of cleavage of the signal peptide. This view is partially supported by the presence of an apparently intact signal peptide in the glycosylated invertase intermediates from the class II and III mutants (Fig. 5). However, this notion cannot adequately explain ER retention in the class I mutant 4-56B. It is possible that cleavage may have occurred at an alternative and perhaps less efficient site even though the deglycosylated invertase from this mutant had an electrophoretic mobility identical to that of the protein from the wild-type (Fig. 5). This issue may be resolved by amino-terminal sequence analysis of the core glycosylated invertase.

Ability of the mutant preinvertases to be translocated across the ER membrane in vitro. The yeast (27, 50, 62) and the combined wheat germ and canine pancreas (19, 59) in vitro translation-translocation systems were used to examine the possibility that the defect in the class II and III mutants resides in the ability of the precursor to translocate across the ER membrane. Since full-length invertase is heavily glycosylated, transcripts coding for truncated preinvertases containing the wild-type and mutant signal sequences were used to simplify analysis of the translocated products. The capped transcripts, synthesized by using the in vitro SP6 RNA polymerase system (32, 40), were used to program the yeast translation extract in the presence or absence of yeast microsomal membranes. Core glycosylation of invertase, seen as a shift in the apparent molecular weight on SDS-polyacrylamide gels, was used to monitor translocation.

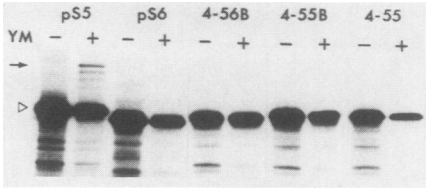

FIG. 7. Homologous yeast in vitro translation and translocation. Transcripts coding for truncated versions of the preinvertases were translated in yeast extract in the presence (+) or absence (−) of yeast microsomal membranes (YM). The unglycosylated form produced by the signal peptide-deleted plasmid, pS6, had a lower electrophoretic mobility than did the wild-type and mutant preinvertases (▷). Translocation and core glycosylation can be seen with the wild-type signal peptide, plasmid pS5 (→), but not with plasmid pS6 or any of the signal peptide mutants.

The wild-type preinvertase (plasmid pS5) was core glycosylated in the presence of yeast microsomal membranes (Fig. 7). The efficiency of translocation was poorer than was found in vivo, as only 5 to 10% of the translated product was translocated. It is likely that factors essential for protein translocation are limiting, missing, or inactive in the yeast in vitro system. In contrast to yeast carboxypeptidase Y, for which translocation is still evident in the absence of an amino-terminal signal peptide (7), deletion of the invertase signal peptide completely abolished translocation. Interestingly, no glycosylated products were detectable in any of the mutants tested. It is probable that the inefficiency of the system precluded the detection of any lower level of translocation, such as might be anticipated with the class I and II mutants. Therefore, the mutants were tested in the more efficient heterologous wheat germ translation and canine pancreas microsomal membrane system (61).

One of the characteristics of this system is its dependence on exogenously added canine SRP, which can impose, in many cases, a transient block in elongation at specific sites in the nascent polypeptide chain of secreted proteins (23, 38, 61). Since SRP has been shown to directly interact with the signal peptide (33, 37), its ability to recognize the invertase signal peptides was examined.

Translational inhibition in the absence of microsomal membranes was observed in preliminary studies for the full-length wild-type protein encoded by pS5 but not for the signal peptide deleted or for any of the class II and III mutants. Since SRP-induced elongation arrest has been shown not to be a prerequisite for translocation across the microsomal membrane (52), and translocation in the absence of translocation inhibition due to a reduction in the binding affinity of SRP has been predicted by a mathematical treatment of the data on the translocation process (49), it is conceivable that the class II and III mutants are competent in translocation despite the absence of a noticeable elongation arrest. To examine this possibility, canine pancreas microsomal membranes were added to the in vitro wheat germ translation system in the presence of two concentrations of canine SRP, 5 and 45 nM.

Preinvertase produced by the parental plasmid, pS5, was efficiently translocated across the canine microsomal membrane (Fig. 8). The efficiency of translocation was dependent on SRP, as increasing the amount of exogenously added SRP increased the amount of translocated product. As would be

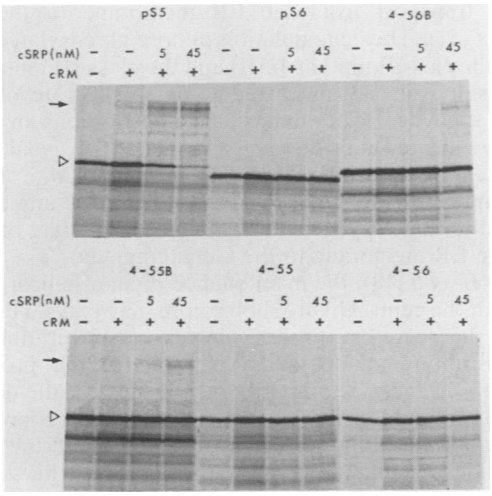

FIG. 8. Translocation of in vitro-translated preinvertase across canine microsomal membranes. The SP6 RNA polymerase-generated transcripts coding for the full-length preinvertases were translated with wheat germ extract in the presence of two concentrations (5 and 45 nM) of purified canine SRP (cSRP) and canine microsomal membranes (cRM). △, Unglycosylated preinvertase. Translocated invertase underwent core glycosylation and appears as a series of higher-molecular-weight bands (→).

expected, no SRP-mediated cotranslational translocation was observed in the signal-deleted invertase, pS6. A small amount of translocated invertase was evident at the higher SRP concentration in the class I (4-56B) and II (4-55B) mutants. Therefore, the results suggest that part of the defect in all three classes of mutants is a reduction in the efficiency of translocation across the ER membrane, with the class III mutants most severely inhibited. This view is consistent with the in vivo pulse-labeling data, which indicate that the class II and III mutant preinvertases are less efficient in translocation.

DISCUSSION

The series of experiments described above demonstrate a range of consequences of replacement of residues in the *SUC2* signal sequence: (i) reduced production of fully glycosylated and secreted invertase, (ii) accumulation of core glycosylated invertase which in some cases retained the signal peptide, and (iii) accumulation of unprocessed preinvertase. A given mutant may have one or more of these phenotypes. Thus, class I mutants, such as 4-6 and 4-56B, produce secreted invertase in close to wild-type amounts, whereas class III mutants, such as M-5 and 4-56, produce mostly internal invertase at about 25% of the secreted wild-type level. In general, other mutants lie between these two extremes both in the amount of enzyme produced and in the points in the secretory pathway at which the enzymes accumulate. The possibility that overexpression of invertase accentuates some of the observed phenotypes cannot be excluded.

Enzymatic assays (Table 1) and Western analysis (Fig. 3) showed that the class I mutants produced fully glycosylated, secreted invertase, but the levels could be as low as one-third of normal. There are several possible reasons for reduced production of secreted invertase without concurrent production of intracellular enzyme. It is unlikely that variation in invertase levels resulted from changes in transcription efficiency, since all mutations were introduced downstream of the *ADH1* promoter and its transcription start sites. Furthermore, Northern (RNA) analysis revealed no correlation between the steady-state level of the invertase transcript and enzymatic activity (data not shown). Although mRNA structural changes affecting translation efficiency cannot be ruled out, there was no correlation between the levels of expression and nucleotide changes adjacent to the translation initiation site nor any obvious increases in the potential for secondary structure resulting from the mutations. Also, no codon bias was shown by those mutants with a high or a low level of expression. Generalized intracellular proteolysis due to the influence of the penultimate amino acid (3) probably is not involved, because no correlation between invertase level and the presence of destabilizing amino acid was evident. However, the possibility that a fraction of the intermediate is degraded by another, unknown mechanism rather than transported to the cell surface cannot be precluded. This possibility suggests that there may exist a mechanism whereby proteolysis is coupled to the rate at which a protein is transported through the secretory pathway and that inefficiently transported intermediates are rendered more susceptible to proteolysis.

Mutant 4-55B (class II) had a distinct phenotype in that it accumulated cytoplasmic preinvertase and core glycosylated invertase (Fig. 3 and 4). Enzymatic removal of the core oligosaccharide chains suggested a protein with an apparently intact signal peptide (Fig. 5). Therefore, a slower rate of cleavage of the signal peptide may account for the kinetic delay in transport out of the ER membrane. Since the mutations in mutant 4-55B are localized amino terminal to the cleavage site, this suggests that residues within the signal peptide not immediately adjacent to the cleavage site may play a role in determining the rate of transport out of the ER membrane. Since this effect is clearly not as severe as a mutation directed at the signal peptide cleavage site (25, 51), it is likely that mutations within the signal peptide have altered its interaction with components in the ER membrane, thus affecting the rate of the cleavage reaction.

The class III mutants showed a partial defect in invertase secretion coupled with a marked internal accumulation of enzymatically active preinvertase and a reduction in the amount of total invertase (Table 1). From the in vivo pulse-chase (Fig. 6) and in vitro translocation (Fig. 7 and 8) experiments, the major defect in this class resides in the inability of the precursor to translocate across the ER membrane, resulting in the accumulation of unglycosylated preinvertase in the cytoplasm.

Several conclusions pertinent to the functional features of signal peptides can be drawn. First, the most essential feature of signal peptides appears to be the presence of a hydrophobic core of 6 to 15 amino acids. In *E. coli*, introduction of a charged residue into the hydrophobic core disrupted signal peptide function (4, 17, 18, 28, 42). In *S. cerevisiae*, the importance of a hydrophobic core in invertase secretion has been demonstrated by partially or completely deleting this segment (29, 47) and by inserting random DNA fragments coding for short stretches of hydrophobic amino acids (30). The in vivo properties of mutant 4-56B suggest that disruption of the continuous stretch of hydrophobic amino acids has a minimal effect on signal peptide function. This notion is consistent with the observation that, provided a minimum number of hydrophobic residues are present in the center of the signal, the sequence can be interrupted by nonhydrophobic residues (30). This view is also consistent with the lack of a readily

noticeable effect on the in vivo processing of the vacuolar enzyme, carboxypeptidase Y, upon introduction of a charged amino acid into the hydrophobic core (7). However, there is clearly a decrease in the ability of this class I preinvertase to translocate across the ER membrane in vitro (Fig. 7 and 8), as was the case with charged amino acid substitutions into the signal peptide of the prepro-α-factor (1). Perhaps the difference between the in vivo and in vitro properties is simply a reflection of the sensitivity of the detection procedures. It is also possible that the position of the charged residue within the amphiphilic, hydrophobic core of the signal peptide is important for translocation and subsequent processing. Therefore, it would be interesting to extend this study through an examination of the positional effect of a charged aspartate residue, since substitution of a charged residue on the hydrophilic side of an amphiphilic helical structure is likely to be less deleterious than one placed on the hydrophobic side.

Second, a large number of signal peptides contain one or more basic amino acids preceding the hydrophobic core. In *E. coli*, this basic residue has been shown to be essential for efficient secretion of exported proteins (26, 28) and for secretion-translation coupling (5). However, the significance of an equivalent basic residue to yeast secretion is unclear, since the wild-type yeast invertase signal does not contain any basic amino acid near the amino terminus. The replacement of other residues with basic residues, such as in the class I mutants 25, 3-15, and 3-12, or with acidic residues, as in mutants 32 and 35, did not produce any dramatic effect on secretion.

Although a basic residue may not be absolutely essential, data from the class III mutants of invertase indicate that the nature of the amino acids at the extreme amino terminus, when combined with amino acid substitutions in the hydrophobic region, plays a crucial role in the secretory process. It appears that the effects of the amino-terminal sequence can be easily masked by a strong hydrophobic core and are evident only when the interaction between the secretory apparatus and the hydrophobic core has been partially disrupted. This can be seen by comparing the properties of the class III mutants M-5 and 4-55 with those of the class II mutant, 4-55B. Replacement of the leucine −18 in mutant 4-55B with valine (mutant 4-55) or with glycine (mutant M-5) resulted in a reduced efficiency of translocation. A similar situation was seen in the class I mutants 4-56A and 4-56B and the class III mutants 4-56 and M-1. Thus, a difference of two amino acids at the amino terminus of mutant 4-56 (valine −17 and asparagine −18) and a single amino acid in mutant M-1 (glycine −18) resulted in a decrease in invertase level, accumulation of cytoplasmic preinvertase, and accumulation of core glycosylated enzyme. Substitution of the amino-terminal residues alone (mutant 4-56A) produced a reduction in secreted invertase but no substantial internal accumulation of preinvertase.

A third feature of signal peptides is the presence of small neutral and α-helix-disrupting amino acids in the vicinity of the cleavage site. In *E. coli*, signal peptide cleavage-defective mutants contain substitutions clustered between the cleavage site and the preceding five to six amino acids (31, 35). In addition, cleavage by the *E. coli* signal peptidase, SPase I, can be demonstrated only in peptide fragments containing the carboxy-terminal five to nine residues of the signal peptide and at least three residues of the mature sequence (16). In yeast invertase, substitution of the alanine residue at position −1 has been shown to alter the signal peptide cleavage site as well as the rate of cleavage and the rate of transport from the ER membrane to the Golgi complex (51). The accumulation of core glycosylated invertase in the class II mutant 4-55B and the class III mutant M-5 may result from a similar reduction in the rate of signal peptide cleavage. The amino acid substitutions in mutant 4-55B are located at positions −4 to −11 of the signal peptide and extend as far as position −18 in mutant M-5. Thus, it would appear that amino acids other than those immediately adjacent to the cleavage site can influence the rate of transit from the ER membrane to the Golgi complex.

As in *E. coli* (18), the maintenance of an α-helical conformation at the central hydrophobic core may play an essential role in the secretory process in yeast. Substitution of α-helix-disrupting amino acids, particularly the proline at position −11, may be primarily responsible for the defect in secretion in mutants 28 and 28*. This view is supported by the isolation of an intragenic revertant in which the proline −11 residue in mutant 28* was mutated back to the wild-type leucine residue (data not shown). This revertant exhibited a class I phenotype with an invertase level approximately one-third that with the wild-type *SUC2* signal peptide (pS5). More important, almost all of the enzyme was in the extracellular compartment. Although the destabilizing influence of proline on the α-helix is intuitively more appealing, the possibility that this effect is mediated by the more hydrophilic nature of proline cannot be excluded.

ACKNOWLEDGMENTS

We thank Caroline Beard for comments on the manuscript.

M.S. is a career investigator of the Medical Research Council (Canada). P.W. was supported by Public Health Service grants GM32384 and GM37485 from the National Institutes of Health. W.H. was supported by Public Health Service grant GM10562 from the National Institutes of Health and by the Giannini Foundation for Medical Research.

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. **Ammerer, G.** 1983. Expression of genes in yeast using the *ADC1* promoter. Methods Enzymol. **101**:192–201.
3. **Bachmair, A., D. Finley, and A. Varshavsky.** 1986. *In vivo* half-life of a protein is a function of its amino-terminal residue. Science **234**:179–186.
4. **Bedouelle, H., P. J. Bassford, Jr., A. V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung.** 1980. Mutations which alter the function of the signal sequence of the maltose-binding protein of *Escherichia coli*. Nature (London) **285**:78–81.
5. **Benson, S. A., M. N. Hall, and B. A. Rasmussen.** 1987. Signal sequence mutations that alter coupling of secretion and translation of an *Escherichia coli* outer membrane protein. J. Bacteriol. **169**:4686–4691.
6. **Bergh, M. L. E., C. L. Cepko, D. Wolf, and P. W. Robbins.** 1987. Expression of the *Saccharomyces cerevisiae* glycoprotein invertase in mouse fibroblasts: glycosylation, secretion and enzymatic activity. Proc. Natl. Acad. Sci. USA **84**:3570–3574.
7. **Blachly-Dyson, E., and T. H. Stevens.** 1987. Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal signal sequence. J. Cell Biol. **104**:1183–1191.
8. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835–851.
9. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68**:245–267.
10. **Carlson, M., B. C. Osmond, and D. Botstein.** 1980. *SUC* genes of

yeast: a dispersed gene family. Cold Spring Harbor Symp. Quant. Biol. **45**:799–804.
11. **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.
12. **Carlson, M., B. C. Osmond, and D. Botstein.** 1981. Mutants of yeast defective in sucrose utilization. Genetics **98**:25–40.
13. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature (London) **287**:504–509.
14. **Dente, L., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single stranded plasmids. Nucleic Acids Res. **11**: 1645–1655.
15. **Deschaies, 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.
16. **Dierstein, R., and W. Wickner.** 1986. Requirements for substrate recognition by bacterial leader peptidase. EMBO J. **5**:427–431.
17. **Emr, S. D., M. N. Hall, and T. J. Silhavy.** 1980. A mechanism of protein localization: the signal hypothesis and bacteria. J. Cell Biol. **86**:701–711.
18. **Emr, S. D., and T. J. Silhavy.** 1983. Importance of secondary structure in the signal sequence for protein secretion. Proc. Natl. Acad. Sci. USA **80**:4599–4603.
19. **Erickson, A. H., and G. Blobel.** 1983. Cell-free translation of messenger RNA in a wheat germ system. Methods Enzymol. **96**:38–50.
20. **Esmon, B., P. Novick, and R. Schekman.** 1981. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell **25**:451–460.
21. **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.
22. **Ferro-Novick, S., P. Novick, C. Field, and R. Schekman.** 1984. Yeast secretory mutants that block the formation of active cell surface enzymes. J. Cell Biol. **98**:35–43.
23. **Gilmore, R., G. Blobel, and P. Walter.** 1982. Protein translocation across the endoplasmic reticulum: isolation and characterization of the signal recognition particle. J. Cell Biol. **95**: 463–469.
24. **Goldstein, A., and J. O. Lampen.** 1975. β-D-Fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42**:504–511.
25. **Haguenauer-Tsapis, M., M. Nagy, and A. Ryter.** 1986. A deletion that includes the segment coding for the signal peptidase cleavage site delays release of *Saccharomyces cerevisiae* acid phosphatase from the endoplasmic reticulum. Mol. Cell. Biol. **6**:723–729.
26. **Hall, M. N., J. Gabay, and M. Schwartz.** 1983. Evidence for a coupling of synthesis and export of an outer membrane protein in *Escherichia coli*. EMBO J. **2**:15–19.
27. **Hansen, W., P. D. Garcia, and P. Walter.** 1986. *In vitro* protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-α-factor. Cell **45**:397–406.
28. **Inouye, S., X. Soberon, T. Franceschini, K. Nakamura, K. Itakura, and M. Inouye.** 1982. Role of positive charge on the amino-terminal region of the signal peptide in protein secretion across the membrane. Proc. Natl. Acad. Sci. USA **79**:3438–3441.
29. **Kaiser, C. A., and D. Botstein.** 1986. Secretion-defective mutations in the signal sequence for yeast invertase. Mol. Cell. Biol. **6**:2382–2391.
30. **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.
31. **Koshland, D., R. T. Sauer, and D. Botstein.** 1982. Diverse effects of mutations in the signal sequence on the secretion of beta-lactamase in *Salmonella typhimurium*. Cell **30**:903–914.
32. **Krieg, P. A., and D. A. Melton.** 1984. Functional messenger RNA produced by SP6 *in vitro* transcription of cloned cDNAs. Nucleic Acids Res. **12**:7057–7070.
33. **Krieg, U. C., P. Walter, and A. E. Johnson.** 1986. Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle. Proc. Natl. Acad. Sci. USA **83**:8604–8608.
34. **Kubota, S., Y. Toshida, 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. Biochem. **81**:197–205.
35. **Kuhn, A., and W. Wickner.** 1985. Isolation of mutants in M13 coat protein that affects its synthesis, processing, and assembly into phage. J. Biol. Chem. **260**:15907–15913.
36. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
37. **Kurzchalia, T. V., M. Wiedmann, A. S. Girshovich, E. S. Bochkareva, H. Bielka, and T. A. Rapoport.** 1986. The signal sequence of nascent preprolactin interacts with 54k-polypeptide of SRP. Nature (London) **320**:634–636.
38. **Lipp, J., B. Dobberstein, and M. T. Haeuptle.** 1987. Signal recognition particle arrests elongation of nascent secretory and membrane proteins at multiple sites in a transient manner. J. Biol. Chem. **262**:1680–1684.
39. **McNeil, J. B., and M. Smith.** 1985. *Saccharomyces cerevisiae CYC1* mRNA 5′-end positioning: analysis by in vitro mutagenesis, using synthetic duplexes with random mismatch base pairs. Mol. Cell. Biol. **5**:3545–3551.
40. **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 bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
41. **Messing, J.** 1979. A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. Recombinant DNA technical bulletin, publication no. 79-99, vol. 2, p. 43–48. National Institutes of Health, Bethesda, Md.
42. **Michaelis, S., H. Inouye, D. B. Oliver, and J. Beckwith.** 1983. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J. Bacteriol. **154**:366–374.
43. **Norris, K., F. Norris, L. Christiansen, and N. Fiil.** 1983. Efficient site-directed mutagenesis by simultaneous use of two primers. Nucleic Acids Res. **11**:5103–5112.
44. **Novick, P., S. Ferro, and R. Schekman.** 1981. Order of events in the yeast secretory pathway. Cell **25**:461–469.
45. **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.
46. **Perlman, D., and H. O. Halvorson.** 1981. Distinct repressible mRNAs for cytoplasmic and secreted yeast invertase are encoded by a single gene. Cell **25**:525–536.
47. **Perlman, D., P. Raney, and D. Halvorson.** 1986. Mutations affecting the signal sequence alter synthesis and secretion of yeast invertase. Proc. Natl. Acad. Sci. USA **83**:5033–5037.
48. **Porter, S. D., and M. Smith.** 1986. Homoeo-domain homology in yeast *MAT 2* is essential for repressor activity. Nature (London) **320**:766–768.
49. **Rapoport, T. A., R. Heinreich, P. Walter, and T. Schulmeister.** 1987. Mathematical modeling of the effects of the signal recognition particle on translation and translocation of proteins across the endoplasmic reticulum membrane. J. Mol. Biol. **195**:621–636, 1987.
50. **Rothblatt, J. A., and D. I. Meyer.** 1986. Secretion in yeast: reconstitution of the translocation and glycosylation of α-factor and invertase in a homologous cell-free system. Cell. **44**:619–628.
51. **Schauer, I., S. Emr, C. Gross, and R. Schekman.** 1985. Invertase signal and mature sequence that delay intercompartmental transport of active enzyme. J. Cell Biol. **100**:1664–1675.
52. **Siegel, V., and P. Walter.** 1985. Elongation arrest is not a prerequisite for protein translocation across the microsomal membrane. J. Cell Biol. **100**:1913–1921.
53. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) **282**:39–43.

54. **Taussig, R., and M. Carlson.** 1983. Nucleotide sequence of the yeast *SUC2* gene for invertase. Nucleic Acids Res. **11**:1943–1954.
55. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
56. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
57. **von Heijne, G.** 1983. Patterns of amino acids near signal sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
58. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184**:99–105.
59. **Walter, P., and G. Blobel.** 1983. Preparation of microsomal membranes for co-translational protein translocation. Methods Enzymol. **96**:84–93.
60. **Walter, P., R. Gilmore, and G. Blobel.** 1984. Protein translocation across the endoplasmic reticulum. Cell **38**:5–8.
61. **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 proteins. J. Cell Biol. **91**:545–550.
62. **Waters, M. G., and G. Blobel.** 1986. Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. J. Cell Biol. **102**:1543–1550.
63. **Zoller, M., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.