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Secretion-Defective Mutations in the Signal Sequence for *Saccharomyces cerevisiae* Invertase

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Nine mutations in the signal sequence region of the gene specifying the secreted *Saccharomyces cerevisiae* enzyme invertase were constructed in vitro. The consequences of these mutations were studied after returning the mutated genes to yeast cells. Short deletions and two extensive substitution mutations allowed normal expression and secretion of invertase. Other substitution mutations and longer deletions blocked the formation of extracellular invertase. Yeast cells carrying this second class of mutant gene expressed novel active internal forms of invertase that exhibited the following properties. The new internal proteins had the mobilities in denaturing gels expected of invertase polypeptides that had retained a defective signal sequence and were otherwise unmodified. The large increase in molecular weight characteristic of glycosylation was not seen. On nondenaturing gels the mutant enzymes were found as heterodimers with a normal form of invertase that is known to be cytoplasmic, showing that the mutant forms of the enzyme are assembled in the same compartment as the cytoplasmic enzyme. All of the mutant enzymes were soluble and not associated with the membrane components after fractionation of crude cell extracts on sucrose gradients. Therefore, these signal sequence mutations result in the production of active internal invertase that has lost the ability to enter the secretory pathway. This demonstrates that the signal sequence is required for the earliest steps in membrane translocation.

The secreted *Saccharomyces cerevisiae* enzyme invertase is a convenient model for the study of protein localization. The wild-type *SUC2* gene of *S. cerevisiae* encodes both a cytoplasmic and an extracellular glycosylated form of invertase (5, 14, 30). The extracellular enzyme is responsible for the extracellular hydrolysis of sucrose (7) and permits yeasts to use sucrose as a carbon and energy source. The function, if any, of the normal cytoplasmic enzyme is not yet known.

The *SUC2* gene expresses two distinct mRNAs (27) that differ at their 5′ ends (4). Expression of both the longer mRNA and the external form of invertase are regulated by glucose repression. The shorter mRNA and the cytoplasmic form of invertase are expressed constitutively at lower levels. Mapping of the 5′ ends of both mRNAs relative to the nucleotide sequence of the *SUC2* gene has shown that the regulated mRNA contains a coding sequence for 20 mostly hydrophobic amino acids that is not present in the shorter, constitutively expressed transcript (6) (see Fig. 1).

When mRNA from a *SUC2* strain is translated in vitro, a precursor form of invertase is synthesized that appears to be processed and translocated by dog pancreas membranes (27). A partial amino acid sequence of this precursor contains 19 amino-terminal residues that correspond to the additional coding sequence of the longer mRNA (29). These sequences are not found in the mature extracellular form of invertase and therefore must be removed during export. Thus, the amino-terminal leader peptide exhibits the hallmarks of a signal sequence: it is required for invertase export (the form of invertase synthesized without it remains in the cytoplasm) and it is removed during the maturation of extracellular invertase.

We conducted a mutational analysis of the invertase signal peptide with the aim of rigorously demonstrating its role in invertase export and of improving our understanding of the mechanism of its action. Our approach was to generate deletion and substitution mutations within the signal sequence by manipulating a cloned segment of the *SUC2* gene in vitro and then to return the interesting alleles to the chromosomal locus and to analyze their phenotypic consequences.

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MATERIALS AND METHODS

Yeast strains, plasmids, and culture media. Yeast strain DBY1034 is a *his4-539 lys2-801 ura3-52 SUC2*. Strain DBY1701 is a *his4-539 lys2-801 ura3-52 SUC2-Δ9*. YT455 is α *ura3-52 ade2-101 suc2-Δ9* (obtained from M. Carlson). The *sec18-1* allele used in these studies was in strain HMSF176 (25) obtained from P. Novick.

Plasmids pRB410 and pRB420 were constructed by inserting an *Eco*RI-to-*Hin*dIII fragment including a functional *SUC2* gene into the *Eco*RI-to-*Hin*dIII sites of YIp5 and YCp50, respectively. These plasmids were constructed such that the *Hin*dIII site at the junction between the *SUC2* sequences and the plasmid sequences is lost.

YEP liquid medium was 10 g of yeast extract (Difco Laboratories, Detroit, Mich.) per liter and 20 g of Bacto-Peptone (Difco) per liter. Solid medium contained 20 g of Bacto-Agar (Difco) per liter. Growth on sucrose as the sole carbon source was assessed on YEP-sucrose solid medium that contained YEP medium with 2% sucrose and 1 μg of antimycin A (Sigma Chemical Co., St. Louis, Mo.) per ml (used as a substitute for anaerobic growth conditions to inhibit the growth of Suc$^-$ strains). SD liquid medium was 6.7 g of yeast nitrogen base without amino acids (Difco) per liter and 2% glucose. Solid medium to select against *URA3* (2) contained 0.5 mg of 5-fluoroorotic acid per ml, 6.7 g of yeast nitrogen base per ml, 5.5 μg of uracil per ml, 30 μg of lysine per ml, 18 μg of histidine per ml, 2% glucose, and 2% agar. Cell density was measured in a 1-cm quartz cuvette in a Beckman model 25 spectrophometer. One A_{600} unit corresponds to approximately 10^7 haploid yeast cells.

DNA methods. Standard plasmid DNA construction and analysis were carried out by the methods of Maniatis et al. (20). Nuclease S1 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) digestions were done in 30 mM sodium acetate (pH 4.8)–100 mM sodium chloride–5 mM zinc sulfate at a linear plasmid DNA concentration of 100 μg/ml. For digestion of single-stranded DNA only, 100 U of S1 per ml were used. To generate deletions by the limited digestion of duplex DNA, 1,500 U of S1 per ml were used. All digestions were carried out for 20 min at 25°C. Digestion of DNA by T4 DNA polymerase was done in 33 mM Tris acetate (pH 7.9)–66 mM potassium acetate–10 mM magnesium acetate–0.5 mM dithiothreitol–200 μM dTTP–60 U of T4 DNA polymerase (Bethesda Research Laboratories) per ml. Linear plasmid DNA was added at a concentration of 100 μg/ml, and the reaction was carried out for 30 min at 25°C. DNA sequencing was carried out by the method of Maxam and Gilbert (21) on plasmid DNA that was 3′ end labeled at the *Hin*dIII site that is 5′ to the *SUC2* coding sequence; this *Hin*dIII site was the only *Hin*dIII site that remained in the mutant plasmids (see below).

Introduction of mutations into the chromosome. The plasmidborne signal sequence mutations that blocked the ability of cells to utilize sucrose were crossed onto the chromosome by a modification of the method of Scherer and Davis (31). Integration of the plasmid pRB410 carrying a mutation in the *SUC2* signal sequence was directed to the chromosomal *SUC2* locus of strain DBY1034 by cutting the plasmid DNA at a unique *Xba*I restriction site within the *SUC2* gene before transformation. Ura^+ transformants were isolated, and then segregants that had lost the plasmid sequences and presumably one copy of the *SUC2* gene were selected by growth on medium containing 5-fluoroorotic acid to select against *URA3* (2). Individual transformants were found to give rise to both Suc^+ and Suc^- segregants, indicative of the segregation of a heteroallelic duplication. One Suc^- segregant was recovered for each transformant. The invertase band pattern on a nondenaturing gel was the same for these strains as for strains carrying the same mutant *SUC2* allele on a centromere plasmid (data not shown).

Fine structure mapping of chromosomal *SUC2* mutations. The chromosomal *SUC2* mutations were shown to lie within the signal sequence by the plasmid-chromosome marker rescue mapping method of Falco et al. (10). A strain carrying a chromosomal *SUC2* mutation was transformed with DNA from each of a set of plasmids carrying different amounts of the early part of the wild-type *SUC2* gene. These plasmids are identical to pRB400 except that the fusion junction endpoints are in the vicinity of the *SUC2* signal sequence (data not shown). Transformants were grown to saturation in SD medium supplemented with histidine and lysine. Approximately 5×10^7 cells were spread onto solid YEP-sucrose medium and were exposed to 5 min of sunlamp radiation under four FS20TI2 fluorescent sunlamps (Westinghouse) to stimulate mitotic recombination. Plates were then incubated in the dark at 30°C for several days. For all mutants tested, when the transformed deletion plasmid carried approximately 100 base pairs of the wild-type *SUC2* coding sequence, 10 to 100 Suc^+ colonies were produced, whereas none were produced with the same mutant strain carrying a plasmid with the *SUC2* coding sequence deleted.

Invertase assays. Cells were grown to the exponential phase in YEP with 2% glucose. Two A_{600} units of cells were centrifuged and suspended in YEP medium with either 2% glucose (repressing conditions) or 0.1% glucose (derepressing conditions) and aerated at 30°C for 2 h. Cells were pelleted and suspended in 10 mM sodium azide. External invertase activity was determined by assaying intact cells as described previously (15). Internal invertase activity was determined by assaying spheroplast lysates. Spheroplasts were prepared by mixing 0.5 ml of a cell suspension (1 A_{600} unit) with 0.5 ml of 2.8 M sorbitol–50 mM potassium phosphate (pH 7.5)–50 mM 2-mercaptoethanol–50 μg of Zymolyase 60000 (Kirin Brewery, Tokyo, Japan). After 1 h of incubation at 37°C, the spheroplasts were pelleted by low-speed centrifugation and then lysed by suspension in 0.5 ml of Triton X-100. 2-Mercaptoethanol was prevented from interfering with the second stage of the invertase assay by the addition of 0.2 mM *N*-ethylmaleimide. Invertase activity units are defined as nanomoles of glucose released per minute at 37°C.

Denaturing gels and immunoblots. Cells repressed and derepressed for invertase synthesis were prepared as described above, and two A_{600} units were rinsed once in 25 mM Tris hydrochloride (pH 7.5)–10 mM sodium azide. Cell pellets were suspended in 20 μl of sample buffer (80 mM Tris hydrochloride [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate, 100 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 0.01% bromphenol blue), and glass beads (0.5 mm) were added up to the miniscus. This slurry was agitated on a vortex mixer at top speed for 1 min. An additional 130 μl of sample buffer was added, and samples were heated to 95°C for 3 min. After centrifugation for 5 min in a microcentrifuge, 10 μl of the supernatant of each sample (about 30 μg of protein by the Bradford assay using bovine serum albumin as a standard) was loaded onto a 7.5% polyacrylamide slab gel and subjected to electrophoresis by the method of Laemmli (19). Proteins were electrophoretically transferred to nitrocellulose filters (32) which were then incubated in blocking buffer (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 10 mM sodium azide, 5% nonfat dry milk, 0.05% Tween 20) for 2 h at 37°C. Antibody binding was carried out in this buffer with 50 μl of invertase antiserum (a generous gift of R. Schekman) and 4 mg of a crude cell extract of YT455 to competitively block antibody association with filter-bound proteins not related to invertase. Filters were washed four times with blocking buffer. Filter-bound antibodies were labeled by incubation in blocking buffer containing 5 μCi of ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, Ill.). Filters were washed as above except in the absence of dried milk and then were exposed to X-ray film with intensifying screens at −70°C. An EC Apparatus Corp. densitometer was used for quantitation of band intensities on the exposed film.

Invertase activity gels. Two A_{600} units of cells derepressed for invertase were prepared as for invertase assays. Cell extracts were prepared and fractionated on 5% polyacrylamide gels as described previously (5). To resolve closely related forms of invertase, 40-cm gels were run at 5 V/cm for 40 h at 4°C. Invertase activity was detected within the gel by incubation in 100 mM sucrose–100 mM sodium acetate (pH 4.5) at room temperature for 20 min followed by staining with 0.1% 2,3,5-triphenyltetrazolium chloride in 0.5 M sodium hydroxide at 100°C (12).

Cell fractionation on sucrose gradients. Cells were grown to the exponential phase in YEP medium with 2% glucose at 26°C. Cells (100 A_{600} units) were centrifuged and suspended in 100 ml of prewarmed YEP medium with 0.1% glucose and incubated at 37°C (for the *sec18* strain) or 30°C (for the *SEC* strains) for 90 min. Cell extracts were prepared as described previously (11), and the homogenate was layered onto a linear 10 to 50% (wt/wt) sucrose gradient containing 100 mM

sodium chloride and 10 mM Tris hydrochloride (pH 7.5). Gradients were centrifuged at 25,000 rpm for 2 h in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C. About 16 fractions (10 drops each) were collected from the bottom of each tube. Each fraction was diluted 10-fold into 1% Triton X-100 and assayed for invertase activity as described above. In gradient fractions that contained significant levels of invertase activity, a measurable amount of sucrose within the gradient was hydrolyzed before the enzyme assay. Corrected values for the invertase activity were obtained by subtracting the level of background hydrolysis that was determined for each fraction.

Protease sensitivity of invertase. Cells were derepressed for invertase synthesis as for invertase assays and were stored as frozen cell pellets at −20°C. To 50 A_{600} units of frozen cells was added 50 μl of 300 mM mannitol–100 mM sodium chloride–10 mM Tris hydrochloride (pH 7.5)–5 mM magnesium chloride. Glass beads (0.5 mm) were added up to the miniscus, and the slurry was vortexed at top speed for 1 min, 200 μl of the same buffer was added, the samples were vortexed briefly, and the liquid above the beads was drawn off. This extract was cleared of cell debris by centrifugation for 2 min in a microcentrifuge. To 20 μl of this extract was added either *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin (Millipore Corp., Bedford, Mass.) or alpha-chymotrypsin (Worthington Diagnostics, Freehold, N.J.). The digestions were stopped by the addition of 0.5 mg of soybean trypsin inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml for trypsin digestion or 1 mM phenylmethylsulfonyl fluoride for chymotrypsin digestion. Samples were loaded onto nondenaturing gels after the addition of 5 μl of 50% glycerol and 0.01% bromphenol blue. Gels were run and stained for invertase activity as described above.

RESULTS

Isolation of signal sequence mutations. To obtain specifically mutations of the signal sequence, *SUC2* sequences were altered in vitro on the plasmid pRB400. In this plasmid, the beginning of the *SUC2* gene is fused to the *lacZ* gene of *Escherichia coli*. This plasmid was constructed in a manner closely analogous to that described by Emr et al. (8). About one half of the *SUC2* gene was included in the hybrid gene, and the structure of the plasmid is shown in Fig. 1. The hybrid gene specified the synthesis of beta-galactosidase activity in yeasts which is regulated by glucose repression. When a frameshift mutation was introduced into the signal sequence at the position of the *Hin*dIII site (*suc2-113*), little or no beta-galactosidase activity was expressed (data not shown). On the basis of these observations, we believe that this hybrid gene encodes a protein composed of the amino-terminal sequences of the secreted form of invertase and an active beta-galactosidase moiety. The use of this hybrid gene facilitates the construction of useful insertion and deletion mutations in the *SUC2* gene sequences since alterations that conserve the correct reading frame can be identified easily by their ability to express beta-galactosidase activity in yeasts.

The first step in the mutagenesis of the signal sequence was to replace the *Hin*dIII site in the signal sequence on plasmid pRB400 with a unique *Sal*I site. The plasmid was partially digested with *Hin*dIII, treated with S1 nuclease to remove the single-stranded ends, and then religated in the presence of a molar excess of *Sal*I linkers. Eight *Sal*I linker insertions in the *Hin*dIII site in the signal sequence were identified by restriction digestion analysis. When these mutant plasmids were introduced into yeast cells, three of them (*suc2-321*, *suc2-331*, and *suc2-301*) expressed beta-galactosidase activity. DNA sequence analysis of each of these plasmids showed that all had short deletions within the *SUC2* signal sequences, presumably owing to S1 digestion, in addition to the insertion of one or more *Sal*I linkers. The mutation *suc2-321* had three tandem *Sal*I linkers, and the mutations *suc2-301* and *suc2-331* both had single linker insertions.

Secondary mutations were then generated at the newly created *Sal*I site in several different ways. The *suc2-321* mutation was altered by digestion with *Sal*I (this removes two of the three tandem linkers), filling in the cohesive ends with DNA polymerase I, and religation. The result of these operations is the shortening of the inserted sequences by 12 base pairs. This mutation is designated *suc2-506*. Other short deletions were generated by digesting the plasmid DNA carrying the *suc2-331* allele with *Sal*I and then treating the DNA with S1 nuclease and ligase. Under the conditions used, one to eight base pairs of duplex DNA were removed from each end in addition to the *Sal*I cohesive ends. The alleles *suc2-210*, *suc2-211*, and *suc2-236* were isolated in this manner. Longer deletions were generated by linearization of plasmid DNA carrying the *suc2-331* allele with *Sal*I followed by digestion of nucleotides from the 3′ ends of the linear molecule by treatment with T4 DNA polymerase. Excess dTTP was added to this reaction to suppress exonucleolytic degradation past adenine residues on the template DNA chains. The plasmid DNA was then treated with S1 nuclease to remove single-stranded DNA and ligated to reform circular molecules. These manipulations generated the deletion mutations *suc2-437* and *suc2-438*. The DNA sequence and the derived amino acid sequence of the signal sequence region specified by each of the mutations are shown in Fig. 2.

Introduction of mutations into the *SUC2* gene and fine structure mapping of the mutant sites. Each signal sequence mutation was introduced into the same wild-type *SUC2* plasmid (no fusion) to assess the effect of the mutation on the invertase phenotype. A restriction fragment carrying the mutant signal sequence was subcloned into a copy of the *SUC2* gene carried on the yeast centromere plasmid pRB420 (Fig. 1). Each mutant allele could be distinguished from the wild type by either the presence of a new restriction site or a change in the size of a restriction fragment. The expected restriction digestion pattern of these plasmids confirmed in every case that these manipulations reconstructed a mutant *SUC2* gene. The initial characterization of the mutant phenotypes was carried out with these plasmidborne alleles.

To control for the possibility that expression of invertase from a plasmid would in some way alter the phenotype, some of the signal sequence mutations were introduced into the chromosomal *SUC2* locus. Each mutant signal sequence that failed to produce extracellular invertase and therefore failed to grow on sucrose as the sole carbon source when expressed from pRB420 (a single-copy plasmid) was also subcloned into the integrating yeast vector pRB410. Again, the presence of the mutant allele was checked by restriction digestion analysis. The plasmidborne mutation was then recombined into the chromosomal *SUC2* locus by a modification of the method of Scherer and Davis (31) as described in the Materials and Methods.

To verify that the failure of the resulting mutant strains to grow on sucrose was due to changes within the signal sequence, the marker rescue mapping method of Falco et al.

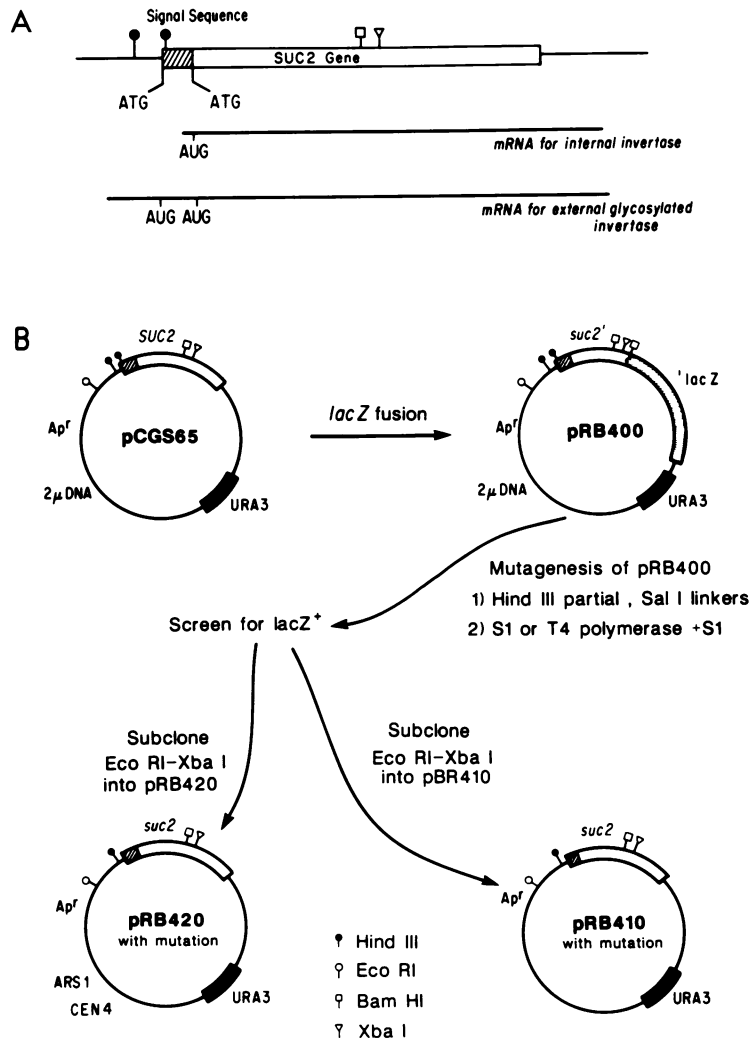

FIG. 1. (A) Working model for the synthesis of both secreted and cytoplasmic forms of invertase from the *SUC2* gene (4). (B) Diagram of plasmid manipulations carried out in this study. The plasmid pCGS65 carries a yeast *Eco*RI fragment that contains a functional *SUC2* gene. An amino-terminal segment of *SUC2* DNA generated by *Bal* 31 digestion was ligated into the plasmid pCGS63 at the 5' end of the carboxy-terminal domain of *lacZ*. Plasmid pRB400 is one of the hybrid plasmids generated by this method that specifies beta-galactosidase activity in yeasts. Details of the in vitro mutagenesis of the *SUC2* signal sequence are described in the text.

(10) was carried out. Strains carrying the chromosomal Suc$^-$ alleles were transformed with DNA from each member of a set of deletion plasmids carrying segments of the *SUC2* gene. In fact, Suc$^+$ (growth on sucrose) recombinants were produced by each of the mutants only when the plasmid with which they were transformed included the wild-type *SUC2* signal sequence region (see Materials and Methods). Therefore, the observed failure to grow on sucrose must have been due to mutations in that region.

Invertase activity produced by mutants. To investigate the nature of the block to formation of extracellular invertase, invertase protein in the mutants that grew on sucrose as well as those that failed to grow on sucrose was examined. This examination included analyses of enzyme activity, structure, and location. Centromere plasmids carrying each of the mutant alleles of the *SUC2* gene were introduced into the yeast strain YT455 which carries a deletion of the entire chromosomal *SUC2* gene. Table 1 shows the ability of these strains to use sucrose as a carbon source and the levels of internal and external invertase activity. The mutations *suc2-437*, *suc2-438*, *suc2-321*, and *suc2-506* blocked the ability of the cells to utilize sucrose. All other mutations allowed cells to grow on sucrose. The levels of invertase activity expressed in cells containing different plasmidborne alleles of the *SUC2* gene were assayed such that the intracellular and extracellular invertase activities were determined independently (Table 1). As expected, strains able to grow on sucrose produced external invertase, whereas the sucrose-negative strains did not produce detectable enzyme on their surface. Intracellular invertase activity was detected in all of these mutants.

There is a second criterion for distinguishing two forms of the enzyme that is independent of their cellular location, namely, the difference in their expression in response to glucose levels in the medium (5, 13, 26). When wild-type cells are grown in the presence of high glucose levels, only the (constitutive) cytoplasmic enzyme is expressed; when cells are grown in the presence of low glucose levels, both forms of the enzyme are produced. Thus, the forms of invertase that are expressed by the glucose-regulated pro-

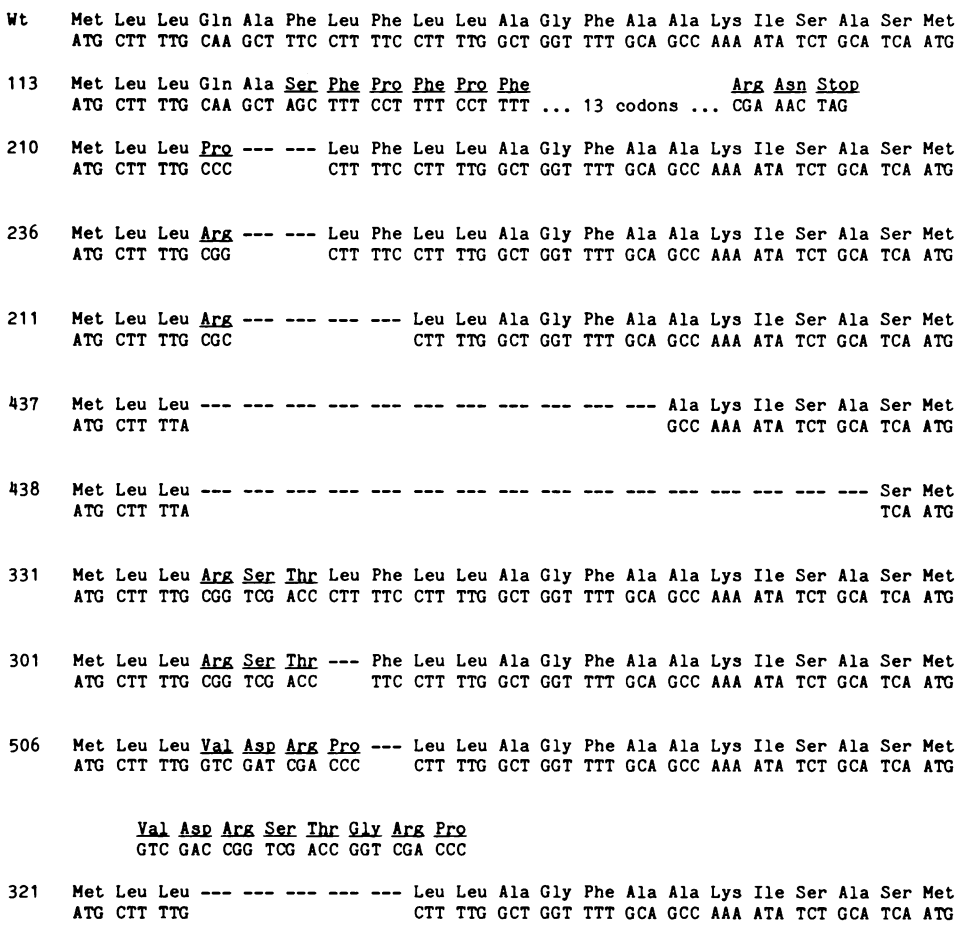

FIG. 2. Nucleotide sequence of the signal sequence mutations in the *SUC2* gene and the derived amino acid sequences. The amino acid substitutions are underlined, and the deletions are indicated by dashed lines. The first 21 amino acids of the proposed precursor form of the secreted form of invertase are shown. No other sequence changes were found for any of these mutants.

moter can be identified as forms of the enzyme that are differentially produced under these two growth conditions. Since the regulation of a wild-type copy of the *SUC2* gene on the plasmid is similar to that of the chromosomal gene (Table 1, footnote *a*), *SUC2* alleles on either centromere plasmids or chromosome can be examined. Table 1 and footnote *a* show that all of the signal sequence mutants that fail to grow on sucrose (Suc^-), except for *suc2-321*, specify the synthesis of inducible internal invertase activity which is above the level of the constitutive internal enzyme found in wild-type cells. The presence of inducible intracellular invertase shows that these mutants are producing an intracellular form of the normally secreted enzyme. Experiments described below directly demonstrate biochemically the existence of novel intracellular forms of invertase that are produced by these mutants.

The mutant *suc2-211* also showed induction of high levels of intracellular invertase activity, although it produced sufficient external enzyme to allow growth on sucrose. The nature of the intracellular form of the enzyme produced by this mutant is currently under investigation.

Invertase antigen specified by mutant genes. Ordinarily, after invertase enters the endoplasmic reticulum (ER) it is glycosylated and the signal peptide is cleaved (9). The mobility of invertase in a denaturing gel can be used to indicate whether either of these modifications has occurred. The proteins specified by the Suc^- signal sequence mutants were analyzed by separating cell extracts by denaturing gel electrophoresis and then identifying invertase antigen with invertase antiserum (32). Figure 3 shows the forms of invertase present in the mutant strains grown under both glucose repressing and derepressing conditions. We expect that mutant cells grown under repressing conditions (high glucose) will produce only a nonglycosylated form of invertase, which has the same mobility as nonglycosylated wild-type enzyme. Cells grown under derepressing conditions (low glucose) should produce both a nonglycosylated form of invertase and a form corresponding to the mutant enzyme. The mutants *suc2-506*, *suc2-437*, and *suc2-438* each produced a regulated form of invertase not found in wild-type cells. All of these invertase-related proteins had the mobilities expected of proteins which have retained a defective signal sequence and are otherwise unmodified. The large increase in molecular weight characteristic of glycosylation was not seen for any of these forms, suggesting that none of these proteins entered the ER.

The mutant *suc2-321* did not express a regulated form of invertase antigen, explaining the absence of regulated invertase enzyme activity in this strain. All of the mutants expressed a form of invertase that comigrated with the cytoplasmic enzyme under conditions of glucose repression. Other experiments (data not shown) revealed that under

TABLE 1. Ability of strain YT455 carrying different *SUC2* alleles on the centromere plasmid pRB420 to grow on YEP-sucrose medium[a]

Plasmid allele	Growth on sucrose	Invertase activity			
		Internal		External	
		2% glucose	0.1% glucose	2% glucose	0.1% glucose
SUC2	+	16	23[b]	6.5	95
suc2-Δ9	−	<1	<1	<1	<1
suc2-113	−	17	18	<1	<1
suc2-210	+	13	36[b]	2.3	152
suc2-236	+	14	20[b]	1.8	79
suc2-211	+	15	46	3.4	24
suc2-437	−	15	41	<1	<1
suc2-438	−	5	123	<1	<1
suc2-301	+	19	18[b]	1.8	57
suc2-331	+	16	22[b]	3.0	72
suc2-506	−	21	32	<1	<1
suc2-321	−	14	14	<1	<1

[a] In addition, the intracellular and extracellular invertase activity produced under glucose-repressing and -derepressing conditions is shown for these mutants. Activity is expressed in units/A_{600}; the assay procedure is given in the Materials and Methods. The values given are the average of two independent assays of each strain that did not differ by more than 20%. For comparison, strain DBY1034 that carries a wild-type chromosomal *SUC2* allele produces 48 internal and 260 external units of activity under glucose derepressing conditions.

[b] These values may be elevated owing to the presence of extracellular enzyme not removed during spheroplasting.

more stringently repressing conditions (growth in 5% glucose), all of the mutants continued to produce this comigrating species except for *suc2-438* which did not appear to make any form of the enzyme under these conditions. Therefore, the band of invertase antigen produced by *suc2-438* under the repressing conditions seen in Fig. 3 is probably the result of incomplete repression and not invertase expression from the constitutive promoter. These results suggest that, except for the *suc2-438* allele, none of the signal sequence mutations significantly alter transcription from the constitutive promoter under conditions of glucose repression.

Quantitation of mutant protein levels. The immunoblots were also used to quantitate the level of invertase antigen produced by the mutant alleles. The amount of invertase antigen produced by the *suc2-438* mutant was comparable to that produced by the wild type, as judged by the band intensities on an immunoblot (Fig. 3). This mutant also expressed levels of internal invertase enzyme activity equal to the external activity produced by the wild type (Table 1). Therefore, the enzyme specified by *suc2-438* has a specific activity similar to that of wild-type invertase.

A dilution series of the antigen produced by the *suc2-438* mutant was used as the standard for determining the levels of invertase antigen synthesized by other mutant strains (Fig. 4). The amounts of inducible invertase antigen produced by the *suc2-506* and *suc2-437* alleles relative to that produced by *suc2-438* were 25 and 50%, respectively. The comparable ratios of inducible invertase activity expressed by these alleles were about 10 and 20%, respectively (Table 1). Therefore, the specific enzyme activities of the inducible forms of invertase produced by *suc2-437* and *suc2-506* are roughly half of the specific activity of the wild-type enzyme. This is close enough to allow substantial conclusions by following either invertase activity or antigen.

Mutant proteins dimerize with the cytoplasmic enzyme. It is known that the active internal form of invertase is composed

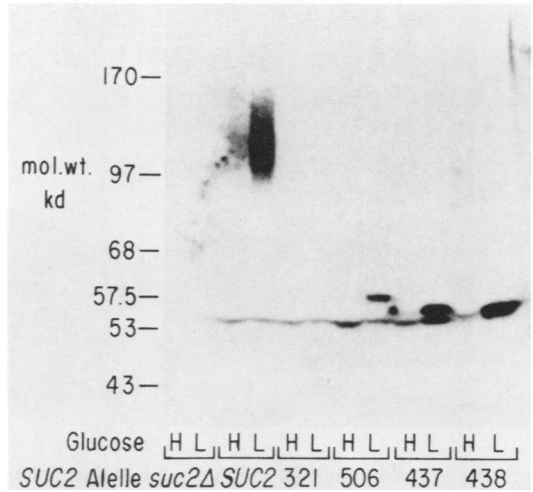

FIG. 3. Denaturing gel electrophoresis of mutant invertase proteins. Cells were grown in YEP medium with 2% glucose and then transferred to YEP medium with either 0.1% glucose (L) or 2% glucose (H) at 30°C for 2 h. Cell extracts were subjected to denaturing gel electrophoresis and transferred to a nitrocellulose filter. Invertase antigen was identified by incubation of the filter in antiinvertase serum and ^{125}I-labeled protein A followed by autoradiography. mol.wt., Molecular weight, kd, kilodalton.

of stable dimers (33). A direct test of whether the mutant forms of invertase are cytoplasmic is to see whether these proteins coassemble with the internal form of the enzyme. Indeed, when the mutant enzymes are isolated in their native

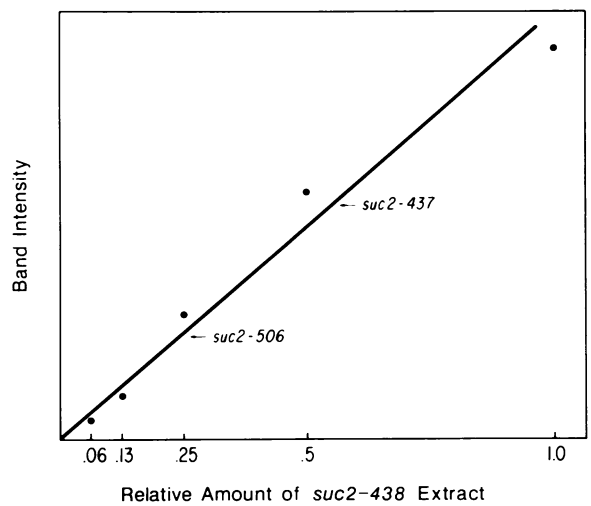

FIG. 4. Comparison of the quantities of invertase antigen produced by different *SUC2* alleles. An extract prepared from cells expressing the *suc2-438* allele under derepressing conditions was serially diluted with extract from a *SUC2* strain (YT455) such that each sample contained a constant amount of cellular protein with different amounts of invertase antigen. Samples were resolved on a denaturing gel, invertase antigen was detected as described in the legend to Fig. 3, and the intensities of the invertase bands were determined by densitometry. The intensities of the bands are proportional to the amount of invertase in the sample over the range examined. Extracts from cells expressing *suc2-437* and *suc2-506* under derepressing conditions were run on the same gel. The intensity of the band of induced invertase antigen for each of these extracts is shown relative to the *suc2-438* dilution series.

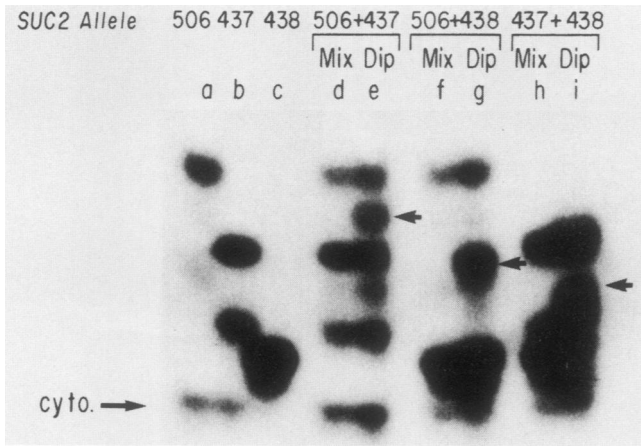

FIG. 5. Portion of a nondenaturing acryalamide gel stained for invertase activity that shows the properties of the mutant forms of invertase in their native conformation. Cells were grown in YEP with 2% glucose and then were transferred to YEP with 0.1% glucose for 2 h. Extracts were electrophoresed on a nondenaturing gel and stained for invertase activity. Extracts from *suc2-506* and *suc2-437* cells show three distinct bands of invertase activity (the middle band of invertase activity in the *suc2-506* extract is very faint in this figure and can be better seen in Fig. 7). The highest mobility band in these extracts comigrates with the wild-type cytoplasmic enzyme (cyto.). Lanes d to i show the result of pairwise combinations of the different mutant alleles. Lanes d, f, and h contain extracts of two haploid strains expressing different mutant genes that were mixed after cell lysis (Mix). Lanes e, g, and i contain extracts of partial heteroallelic diploids (Dip). The bands of activity that are unique to the extracts from the heteroallelic diploid strains are indicated by arrows. No glycosylated invertase (which has a much lower mobility than any of the forms shown here) was found on this gel.

conformation, they can be found as dimers with the cytoplasmic form of invertase, as shown by the following experiment. Extracts from cells carrying either the *suc2-506* or *suc2-437* allele were resolved on a nondenaturing polyacrylamide gel, and three distinct bands of invertase activity were found (Fig. 5, lanes a and b). We have already shown with denaturing gels (Fig. 3) that each of these mutants specifies only two invertase polypeptides, one of which is the constitutive, cytoplasmic wild-type polypeptide. The presence of three rather than two bands on a nondenaturing gel implies an additional form of invertase that is only present when the enzyme is in a native conformation and when there are two distinct invertase proteins expressed in the cell. The most obvious explanation of this finding is that the new band of intermediate mobility on the native gel is composed of heterodimers between the inducible mutant proteins and the constitutive subunits of the internal enzyme. This is related to the observation of Grossman and Zimmerman (16) who found that when two invertase isozymes are expressed in the same cell, mixed dimers between the different cytoplasmic enzymes are formed.

Additional support for this conclusion comes from an experiment that shows that two different mutant subunits form mixed dimers with one another when both mutant alleles are expressed in the same cell. Heteroallelic diploids for the *SUC2* gene were constructed by transforming strains carrying a chromosomal *SUC2* mutant allele with a centromere plasmid bearing a different *SUC2* mutant allele. Figure 5 shows that in every case tested the diploid strains expressed a form of the enzyme with both of the properties expected of a heterodimer composed of two different mutant subunits. This new form of the enzyme was not found in either of the haploid parents or in a mixture of their extracts. Second, the mobility of the novel form of the enzyme was the average of the mobilities of the two different homodimers composed of mutant subunits. The formation of these mixed dimers demonstrates that, unlike the wild-type secreted form of invertase, these mutant proteins are assembled in the same compartment as the constitutive cytoplasmic enzyme.

Cell fractionation. We confirmed by cell fractionation experiments that the mutant forms of invertase are found in the cytoplasmic compartment. Extracts from cells expressing the mutant enzyme were resolved on a sucrose gradient under conditions designed to separate soluble and membrane-associated cellular components. The invertase activity expressed by the *suc2-506* mutant was found entirely in the soluble fraction of the gradient (Fig. 6). Invertase from the *suc2-437* and *suc2-438* mutants gave the same profile as that of *suc2-506* and was also in the soluble fraction (Fig. 6, legend). Material from the peak fractions of each gradient was resolved on a native gel and was found to consist of both the internal (no signal peptide) and mutant forms of the enzyme, having a signal peptide (data not shown). The gradient fractions containing material derived from the endoplasmic reticulum were identified in a parallel gradient by the position of invertase activity expressed by a wild-type gene in the conditional secretion-defective mutant *sec18* induced at the nonpermissive temperature. The soluble fractions were clearly distinguishable from this membrane form. Therefore, the mutant forms of the enzyme are either soluble in the cytoplasm or they have such a weak association with a membrane compartment as to be efficiently disassociated during cell fractionation.

Structure of mutant subunits. The mobilities of mutant invertase proteins on denaturing gels described above strongly suggest that these proteins retain their (defective) signal sequence. The structure of the mutant enzymes was studied further by analyzing the protease sensitivity of the mutant proteins in their native state. The enzymatic activities of all forms of invertase were found to be highly resistant to treatment with both trypsin and chymotrypsin (data not shown). We discovered that, under the appropriate conditions, the mobilities of the mutant proteins specified by *suc2-506* and *suc2-437* in native gels are altered by protease treatment. These cleaved proteins retain their full enzyme activity. This differential effect of protease on mobility and enzyme activity can be understood as proteolytic cleavage within the defective signal sequences retained by these mutant proteins.

The effects of protease treatment on the different mutant invertases is shown in Fig. 6 and can be interpreted in the following manner. If trypsin cleavage occurs only at the lysine residue of the signal peptide in the proteins specified by *suc2-506* and *suc2-437*, enzymes with the same size and charge as that specified by the *suc2-438* allele should be generated (Fig. 7A). When extracts expressing these alleles were treated with trypsin, the forms of invertase produced by the *suc2-506* and *suc2-437* alleles were both converted into a form with the same mobility as the form produced by the *suc2-438* allele which was itself unaltered by trypsin treatment (Fig. 7B). An analogous interpretation can be made of the results of chymotrypsin digestion, except that in this case cleavage presumably occurs at the phenylalanine that is present in the signal peptide of the protein produced by the *suc2-506* allele but not in those specified by either the *suc2-437* or *suc2-438* allele. The results of the chymotrypsin

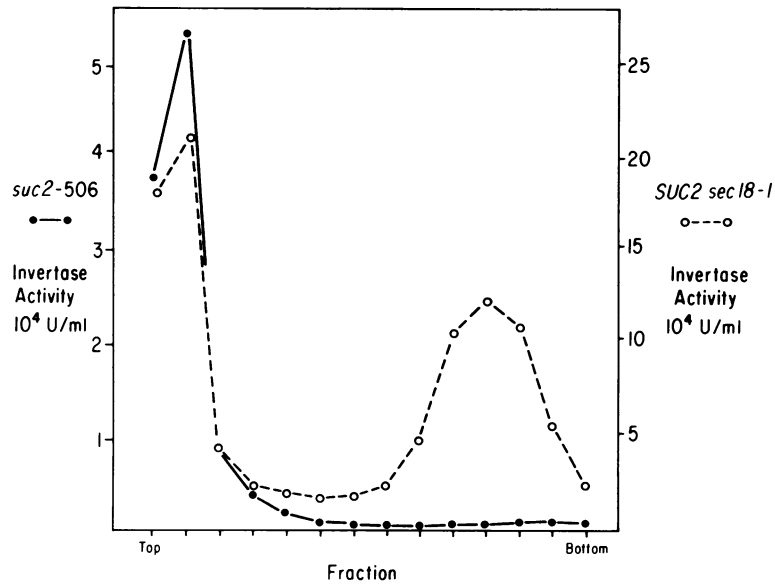

FIG. 6. Sucrose gradient profile of invertase activity in crude cell extracts. The distribution of activity for the *suc2-437* and *suc2-438* alleles is the same as that for *suc2-506*. Invertase expressed by a *sec18* strain at 37°C was used as an ER marker enzyme. The soluble activity found in the *sec18* extract is probably due to enzyme released during cell disruption.

treatment were also found to be fully consistent with the hypothesis that protease cleavage occurs solely within the signal peptide moiety of these proteins (Fig. 7C). These observations further support the conclusion that each of these mutant proteins carries a defective signal peptide. In addition, they show that when the mutant invertase precursors are in a native conformation the signal peptide is uniquely sensitive to proteases.

DISCUSSION

Mutations define stages at which signal sequences act. These experiments constitute a critical test of the hypothesis that secreted invertase is synthesized in vivo as a precursor with an amino-terminal signal peptide. Our results are fully consistent with this idea. We showed that it is possible to isolate mutations in the signal sequence of the *SUC2* gene whose only apparent phenotypic effect is to alter the cellular localization of invertase and to leave the (altered) signal peptide joined to invertase. The existence of this type of mutation demonstrates that the signal sequence plays a direct role in the export of invertase to the cell surface. Furthermore, these sequences were found not to be essential for the synthesis of the regulated enzyme since the *suc2-438* deletion which removes almost all of the signal sequence expresses normal levels of active enzyme.

The site of action of the mutated domain of the signal sequence in the secretory process can be inferred from the cellular location of the mutant proteins. The alleles *suc2-506*, *suc2-437*, and *suc2-438* which block invertase export show a common location with the normal, constitutive intracellular enzyme by a number of criteria. These enzymes are able to form heterodimers with the cytoplasmic form of invertase and are found in the soluble fraction of crude cell extracts. Thus, the mutant enzymes must be assembled and sequestered within the cytoplasm. In addition, the mutant proteins have not undergone either glycosyl modification or proteolytic processing of the signal peptide. Both of these modifications are associated with entry of secretory proteins into the lumen of the ER (1, 9, 22). On the basis of these results we can conclude that there is little, if any, significant interaction of the mutant proteins with cell membranes and that these signal sequence lesions block the earliest steps in translocation across the ER.

Native structure of mutant invertase precursors. The mutant *suc2-506* specifies an active enzyme which retains a full-length signal peptide. The presence of the signal peptide does not significantly alter either the enzymatic activity of these proteins or their capacity to form dimers. In addition, the signal peptide is uniquely sensitive to digestion by both trypsin and chymotrypsin, strongly suggesting that when these proteins are in the native conformation, the signal peptide is exposed to solution. Therefore, the presence of a defective signal peptide does not appear to influence either the structure or assembly of invertase within the cytoplasm.

Mutations define functional elements of the signal sequence. Some of the functional requirements for a signal sequence in yeasts are defined by this collection of mutations. One of the common characteristics of both procaryotic and eucaryotic signal sequences is a domain of hydrophobic amino acids (28, 35, 36). The wild-type *SUC2* signal sequence contains a series of 11 apolar amino acids; however, the phenotype of some of the mutants demonstrates that not all of these residues are necessary for the correct localization of invertase. The mutant *suc2-236* has a segment of nine hydrophobic amino acids and shows no secretory defect, and the mutant *suc2-211* retains only seven contiguous hydrophobic residues and retains partial function for membrane translocation.

Most of the mutations that are limited to the amino-terminal end of the signal sequence that we examined do not prevent function. Both substitutions of charged and polar residues, in the case of *suc2-301* and *suc2-331*, and the addition of a positive charge with the deletion of two hydrophobic residues, in the case of *suc2-236*, had no detectable effect on the function of the signal sequence. These results suggest that the amino-terminal region of the signal sequence is not specifically recognized by secretory

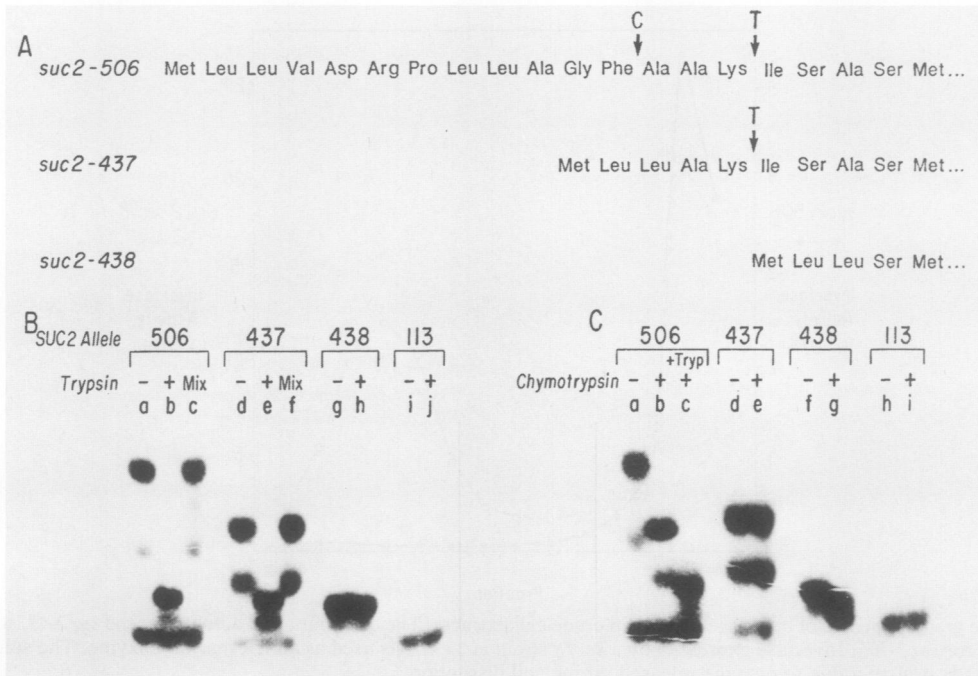

FIG. 7. Nondenaturing gel showing the effects of protease digestion on invertase. (A) Primary trypsin and chymotrypsin cleavage sites expected to occur within the mutant signal peptides. (B) Crude extracts of cells expressing internal forms of invertase were incubated in the presence (+) or absence (−) of 0.1 mg of trypsin per ml at 25°C for 30 min. As a control to show that the digestion of invertase is not due to trypsin activation of a secondary enzymatic activity, mixing experiments were performed (lanes c and f, labeled Mix). An extract from the *SUC2* deletion strain DBY1701 was incubated with trypsin for 20 min, and then trypsin was inactivated with 0.5 mg of soybean trypsin inhibitor per ml. This extract was then mixed with the extracts containing the mutant invertases, and incubation was continued for 20 min. All samples were subjected to nondenaturing gel electrophoresis and stained for invertase activity. (C) Same as above except that 0.2 mg of chymotrypsin per ml was used. Lane c is the result of treatment of the *suc2-506* extract with both trypsin and chymotrypsin. The band pattern characteristic of cleavage by trypsin alone is produced, supporting the hypothesis that chymotrypsin cleavage occurs within the peptide sequences that are removed by trypsin cleavage.

factors. Brown et al. (3) have also shown that a number of insertion mutations at the *Hin*dIII site of the signal sequence have no effect on invertase activity or localization. The *suc2-506* allele also is altered in this early region of the signal sequence but in contrast leads to an absolute defect in translocation. The altered domain of this particular mutant may disrupt a nonspecific interaction with the signal peptide or may influence another part of the polypeptide structure which is required for function.

Signal sequence mutations and *SUC2* gene expression. A number of inferences concerning the expression of invertase can be drawn from this work. As a consequence of the unique structure of the *SUC2* gene, all mutations in the signal sequence are also mutations in the region of the constitutive promoter for the cytoplasmic form of invertase (6). All of the mutants isolated with the exception of *suc2-438* constitutively express normal levels of the cytoplasmic enzyme. This means that the promoter region of this gene is rather insensitive to mutations in the vicinity of the transcriptional start sites. Further, the mRNA start sites for the constitutive promoter that have been defined by S1 mapping (6) are removed only by the *suc2-438* deletion. We can conclude that deletions upstream of the mRNA start sites which are as long as 33 base pairs do not impair promoter function if they do not remove the start sites themselves. These findings are consistent with experiments on the *HIS4* (23) and *CYC1* (17) genes that show that correct mRNA initiation can take place after the distance between the initiation sites and the TATA sequences is changed substantially.

A number of the signal sequence mutations reduce the level of expression of the regulated enzyme. Some of these mutations may reduce the efficiency of translation of the *SUC2* mRNA. In particular, the triple linker insertion in mutant *suc2-321* may inhibit translation because of the considerable secondary structure that the tandem linkers could impart to the mRNA. A more speculative possibility is that a factor exists in yeasts which is equivalent to the mammalian signal recognition particle (37) in its putative capacity to arrest translation of nacent secretory proteins. If the mutant signal sequence allows binding to a signal recognition particle but prevents interaction with a release factor, a reduction in invertase synthesis could result. This model has been suggested to explain the properties of certain signal sequence mutations in two bacterial systems (18, 34).

We defined a number of different phenotypes that result from signal sequence mutations. This knowledge will facilitate the isolation of additional mutations which, in principle, will further define the functional regions of the signal peptide and the different stages of the secretory pathway in which these elements act. In addition, the mutations which are phenotypically Suc^- may allow the isolation of suppressor mutations which may identify new elements of the secretory apparatus.

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