Intragenic Revertants of Yeast Invertase Variants with Secretion-Defective Leader Sequences

DAPHNE PREUSS[1] AND DAVID BOTSTEIN[1,2]*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139,[1] and Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080[2]

Several secretion-defective variants of invertase from *Saccharomyces cerevisiae* were generated by replacement of the wild-type signal sequence codons with DNA fragments with random sequences. Strains encoding these proteins failed to grow on medium containing sucrose as the sole source of carbon. The invertase that was made in these strains was found to fractionate with soluble, cytoplasmic proteins, and indirect immunofluorescence confirmed that the mutant invertase was located throughout the cytoplasm. To define the defects in the secretion-defective leader sequences, we selected revertants by requiring growth on sucrose. Surprisingly, most of the reversion events consisted of point changes and duplications in the upstream noncoding portion of the gene. Each of these changes introduced several hydrophobic residues into the nonfunctional leader sequences, suggesting that the defective random leader peptides might simply lack adequate hydrophobicity to be effective signal peptides.

Yeast invertase, the product of the *SUC2* gene of *Saccharomyces cerevisiae*, is necessary for the extracellular hydrolysis of sucrose, thus permitting wild-type yeast to utilize sucrose as the sole source of carbon and energy (8). Two distinct mRNAs are transcribed from the *SUC2* gene: A constitutive 1.8-kilobase message encodes cytoplasmic invertase, and a regulated, 1.9-kilobase message encodes a secreted form of invertase (5). Under conditions of glucose deprivation, the regulated form of invertase is rapidly induced and is secreted from the cell, while the constitutive form fails to enter the secretory pathway (7, 29).

The two messages differ at their 5′ ends. The regulated mRNA encodes 20 additional amino-terminal residues that are mostly hydrophobic (7). When this message is synthesized in vitro and translocated by dog pancrease membranes (28) or yeast membranes (30), the first 19 amino acids are cleaved from the protein, generating mature invertase. Likewise, these residues are absent from mature invertase that is secreted in vivo and are, therefore, removed during export (29). Finally, these 19 residues, when fused to the amino terminus of β-galactosidase, are sufficient to target the fusion protein to the endoplasmic reticulum (ER), the first step in the secretory pathway (C. A. Kaiser, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1987). Thus, these amino-terminal residues constitute a signal sequence for secretion. They are necessary for invertase secretion, since the constitutive form of invertase remains in the cytoplasm, and they are sufficient to target a cytoplasmic protein such as β-galactosidase to the ER.

In vitro mutagenesis of the invertase signal sequence has yielded several secretion-defective mutants. In general, only large deletions or substitutions lead to severe defects in invertase secretion (17). To further define the requirements for a functional signal sequence, Kaiser et al. (18) replaced the wild-type invertase signal sequence with random peptides. Surprisingly, about one-fifth of all random leader sequences allowed invertase to be secreted. Furthermore, these random signal sequences not only allowed invertase secretion but they also efficiently targeted β-galactosidase to

the ER (Kaiser, Ph.D. thesis). These random sequences have properties similar to those of naturally occurring signal sequences; they are quite hydrophobic and have few charged residues (16).

Although one-fifth of the random peptides function as signal sequences, the majority of the random sequences fail to properly target invertase to the secretory pathway. These sequences tend to be charged and have relatively few hydrophobic residues (18). Yeast strains containing these variant proteins fail to secrete invertase and, consequently, do not grow on sucrose. To better understand the secretion defect of these proteins and to gain insight into the evolution of functional signal sequences, we isolated several revertants by selecting for growth on sucrose. In every case, the revertant leader sequences were more hydrophobic than those of the nonfunctional leaders, thus supporting the idea that only general properties, such as hydrophobicity, and not specific amino acid sequences are required for signal sequence function.

MATERIALS AND METHODS

Strains, plasmids, and growth media. *S. cerevisiae* DBY1034 was *MATa his4-539 lys2-801 ura3-52 SUC2*; strain DBY2356 was *MATα leu2-3 leu2-112 ura3-52 suc2-Δ9 sec18-1* (10); DBY2449 was *MATα ade2-101 ura3-52 suc2-Δ9*; DBY2617 was *MATa his4-539 lys2-801 ura3-52 suc2-438*; and DBY5266, DBY5267, and DBY5268 were *MATα ade2-101 ura3-52 suc2-Δ9*, with yeast episomal (YEp) plasmids encoding, respectively, *suc2-317*, *suc2-527*, and *suc2-756*.

Escherichia coli DB6507 was HB101 (*thr leu pro hsdR hsdM recA*) with a Tn*5* insertion in the *pyrF* gene (*pyrF74 ::*Tn*5*).

Plasmid pRB420 is a YCp50 derivative with a wild-type *SUC2* clone (17). Plasmid pRB576 is a YCp50 derivative that encodes *suc2-450* (18).

YEP and SD media were prepared as described previously (17). YEPD was YEP medium supplemented with 2% glucose; YEP-sucrose medium contained 2% sucrose and 1 µg of antimycin A (Sigma Chemical Co., St. Louis, Mo.) per ml; antimycin A makes *S. cerevisiae* grow fermentatively. YEP-lactate contained 2% lactic acid and was adjusted to pH 5.5

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* Corresponding author.

with potassium hydroxide. SD-CAS liquid medium was 6.7g of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) per liter, 0.2% vitamin-free Casamino Acids (Difco) adsorbed with charcoal (fine; Norite), and 2% glucose. Cell density was determined by spectrophotometry (model Lambda 3B; The Perkin-Elmer Corp., Norwalk, Conn.). One A_{600} unit was equal to approximately 10^7 haploid yeast cells.

DNA methods. Purification of plasmid DNA and the isolation of DNA fragments was performed as described by Maniatis et al. (25). Restriction enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.), and were used as recommended by the supplier. YEp plasmids were constructed from pRB576 by in vivo recombination in *S. cerevisiae* by using DNA fragments derived from YEp420 as a source of 2μm sequences (24). Plasmids were recovered from *S. cerevisiae* as described by Hoffman et al. (15), and yeast transformations were performed by the techniques of Ito et al. (16). DNA sequencing was carried out as described by Maxam and Gilbert (26) on plasmid DNA that was end labeled at the *Hind*III site 5' of the *SUC2*-coding sequence or at the *Ava*II site within the coding sequence.

Invertase activity gels. Cells were grown overnight in liquid YEP-lactate medium to induce invertase synthesis, and cell extracts were prepared and fractionated on 5% polyacrylamide gels as described previously (6). Invertase activity was detected by first incubating the gel in 100 mM sucrose–100 mM sodium acetate (pH 4.5) at 37°C for approximately 90 min and then by staining any liberated glucose by immersing the gel in 0.1% 2,3,5-triphenyl tetrazolium chloride–0.5 M sodium hydroxide at 90°C (13).

Cell fractionation on sucrose gradients. Strains DBY2356 and DBY2449 were transformed, respectively, with pRB420 or derivatives of pRB576 and were grown in SD-CAS medium at 26°C. A total of 100 A_{600} units of *S. cerevisiae* was pelleted and suspended in 50 ml of YEP medium with 0.1% glucose and incubated at 37°C (for the *sec18* strain) or at 30°C for the DBY2449 strains. Following a 2-h induction period, cell extracts were prepared as described previously (12). Spheroplasted cells were lysed by agitation with glass beads, and the homogenate was layered onto a linear 10 to 50% (wt/wt) sucrose gradient, and centrifugation and invertase assays were performed as described before (17).

Immunofluorescence. Cells were grown to the exponential phase in SD-CAS medium and then transferred to SD medium with 0.1% glucose–0.2% clear Casamino Acids at a density of 1 A_{600} unit per ml for 2 h at 30°C. The cells were fixed, spheroplasted, and attached to slides as described by Adams and Pringle (1). Affinity-purified rabbit anti-invertase antibody was added at a 1:50 dilution in 20 μl of TSA buffer (100 mM sodium chloride, 20 mM Tris hydrochloride [pH 7.5], 0.1 mg of bovine serum albumin per ml) and was incubated with the spheroplasts for 45 min at room temperature. The wells were then washed three times with TSA buffer, fluorescein-conjugated goat anti-rabbit immunoglobulin G was added at a 1:500 dilution in 20 μl of TSA buffer, and the slide was incubated for 45 min at room temperature. 4′,6-Diamidine 2-phenylindole (DAPI) was added at 1 μg/ml in TSA buffer, and after a 5-min incubation, the wells were washed three times with TSA buffer. Ten microliters of 1 mg of p-phenylenediamine per ml–90% glycerol–0.85% (wt/vol) sodium chloride–66 mM sodium phosphate (pH 9.0) was added and a cover slip was placed on the slide. The cells were visualized with a standard fluorescence photomicroscope (Zeiss).

Mutagenesis. Approximately 10^7 cells were spread onto a YEP-sucrose plate and then mutagenized by treatment with ethyl methanesulfonate (EMS) or UV irradiation. For EMS treatment, a 1-cm sterile filter disk was placed on the center of each plate and 30 μl of EMS was absorbed onto the filter. This volume of EMS was sufficient to kill all of the cells within 1 cm of the filter. For UV mutagenesis, the plate was irradiated at 1 J/s for 100 s, which killed about 75% of the cells.

Invertase assays. Cultures were grown in liquid YEP-lactate medium to the exponential phase. Two A_{600} units of cells was centrifuged, and the culture supernatant was held on ice until the invertase assay was performed. The cell pellet was washed and suspended in 1 ml of cold 10 mM sodium azide–25 mM Tris hydrochloride (pH 7.5). Half of this cell suspension was held on ice, and the remaining half was spheroplasted by adding 0.5 ml of 2.8 M sorbitol–50 mM potassium phosphate (pH 7.5)–50 mM 2-mercaptoethanol–50 μg of Zymolyase 100000 (Kirin Brewery, Tokyo, Japan) and by incubating the suspension at 30°C for 1 h. The spheroplasts were pelleted by low-speed centrifugation and were washed and suspended in 1 ml of 1.4 M sorbitol–25 mM potassium phosphate (pH 7.5). The spheroplasts were then lysed by the addition of 0.5 ml of 1% Triton X-100. Assays were performed on the spheroplast lysates, whole cells, and culture supernatants as described previously (17, 18).

Pulse-labeling and immunoprecipitation. Cells were grown to the exponential phase in SD medium with 5% glucose. To induce invertase synthesis, cells were washed and suspended in SD medium with 0.1% glucose at a density of 1 A_{600} unit per ml and were incubated at 30°C for 1 h. The cultures were pulse-labeled by the addition of 100 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.), and after 6 min the chase was initiated by the addition of cold methionine to a final concentration of 1 mg/ml. Periodically, samples containing 1 A_{600} unit of cells were removed, and the cells were precipitated by brief centrifugation at 12,000 × g. The cell pellet was heated to 95°C for 3 min, and 20 μl of 1% (wt/vol) sodium dodecyl sulfate (SDS)–50 mM dithiothreitol–50 mM Tris hydrochloride (pH 7.5) and 0.1 g of glass beads were added. The cells were lysed by vigorous agitation, and the samples were diluted into 1 ml of 1% (vol/vol) Triton X-100–50 mM Tris hydrochloride (pH 7.5)–0.1 M sodium chloride. Cell debris was removed by centrifugation at 12,000 × g for 10 min. The extracts were then incubated with 20 μl of protein A conjugated to Sepharose CL-4B (50%; vol/vol; Sigma) for 30 min at 4°C, and the remaining debris and beads were removed by centrifugation at 12,000 × g. Immunoprecipitation of labeled proteins and washing of immune complexes was performed as described previously (9), and the final pellet was suspended in 40 μl of sample buffer (80 mM Tris hydrochloride [pH 6.8], 2% SDS, 0.01% bromphenol blue, 0.1 M dithiothreitol, 10% glycerol) and heated to 95°C for 3 min. Labeled proteins were resolved on SDS-polyacrylamide gels (7.5%), and the gels were fixed and treated with Amplify (Amersham). Radioactive proteins were visualized by exposure of XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C.

Denaturing gels and immunoblots. Cells were grown to the exponential phase in YEP medium with 5% glucose to repress invertase synthesis. Two A_{600} units of cells (2 × 10^7 cells) were collected and suspended either in YEP with 5% glucose (repressing conditions) or in YEP with 0.1% glucose (derepressing conditions) for 3.5 h. Tunicamycin (Sigma) was added at 10 μg/ml during induction to inhibit glycosylation. Following invertase induction, the cells were collected, washed in 1 ml of 10 mM sodium azide–25 mM Tris

hydrochloride (pH 7.5), and then heated to 95°C for 3 min. To the cell pellet, 20 µl of sample buffer and 0.1 g of glass beads were added, and the cells were lysed by vigorous agitation for approximately 2 min. Samples treated with endo-β-N-acetylglucosaminidase H (endo H) were diluted with 3 volumes of 20 mM sodium citrate (pH 5.5) and were incubated for 12 h at 37°C in the presence of 3 µg of endo H per ml. An additional 80 µl of sample buffer was added to the remaining samples, and each lysate was heated to 95°C for 3 min prior to loading on an SDS-polyacrylamide gel (7.5%). Electrophoresis, transfer to a nitrocellulose filter, and immunoblotting were performed as described previously (17).

Mapping of the Suc⁺ revertants. Each of the Suc⁺ revertants was mapped by the recombinational method described by Kunes et al. (19). Plasmid pRB576 (18) (see Fig. 7A) was digested extensively with SmaI, which linearized the plasmid at a unique site within the leader sequence of the suc2-450 allele. The linearized plasmid was transformed into DBY2449 along with homologous restriction fragments from the Suc⁺ revertants. Approximately 0.5 µg of linearized pRB576 was used for each transformation, and in general, the fragments were mixed with the linearized vector at molar ratios of between 10:1 and 0.3:1. Transformants were selected by requiring a Ura⁺ phenotype, and then the transformants were screened for growth on YEP-sucrose plates. Since the plasmid pRB576 encodes the Suc⁻ allele suc2-450, any Suc⁺ transformants that arose incorporated the Suc⁺ mutation that was present on the fragment. By using several fragments, one can narrowly define the region in which the Suc⁺ mutation occurred. This method was limited by convenient restriction sites and by the requirement for homology on both ends of the linearized vector. The smallest overlapping homology used in these experiments was 50 base pairs.

RESULTS

Isolation and characterization of mutants. In order to identify the essential components of signal sequences, we replaced the wild-type signal sequence with random peptides, thus generating a large collection of proteins with nonfunctional leader sequences as well as a surprisingly large number of functional ones (18). Strains containing the defective proteins failed to grow on sucrose because they did not secrete invertase, yet they produced active, internal enzyme containing the altered leader sequence. Furthermore, the mutants failed to produce detectable glycosylated invertase (18). Since invertase is normally glycosylated when it enters the ER (11), the lack of glycosylation of the mutant proteins suggests that they have not yet entered the secretory pathway.

Mutant proteins interact with cytoplasmic invertase. Cytoplasmic invertase assembles into an active, stable dimer, and when more than one form of invertase is present in the cytoplasm, both homodimers and heterodimers can assemble (14, 17, 35). To determine whether the mutant proteins were localized in the cytoplasm, we observed the interaction of cytoplasmic invertase with invertase made in the mutant strains. Invertase dimers remain intact during electrophoresis on a nondenaturing gel (17). Thus, if the mutant proteins interact with cytoplasmic invertase, novel heterodimers should be detected on native gels.

To test this hypothesis, centromere plasmids encoding each of the suc2 alleles with secretion-defective random leaders were transformed into the suc2-438 strain DBY2617, which makes only cytoplasmic invertase (17). Extracts from
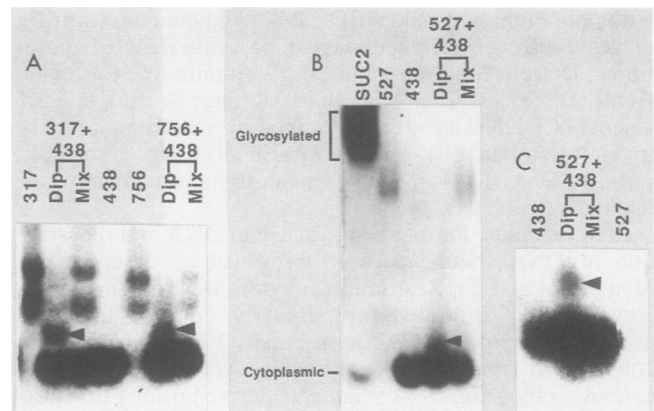

FIG. 1. Nondenaturing gels stained for invertase activity. Cells were grown in YEP-lactate medium and lysed by agitation in the presence of glass beads. Extracts from suc2-317 and suc2-756 had three distinct bands of invertase activity, presumably representing inducible, mutant invertase; constitutive, cytoplasmic invertase; and a heterodimer of these two forms. The suc2-438 strain had a single band with a high mobility, and the suc2-527 strain had a single band with a much lower mobility. Plasmids encoding each of the mutant alleles shown on this gel were introduced into the suc2-438 strain, and extracts of the heteroallelic diploids (Dip) were compared with mixtures of the haploid extracts (Mix). In every case, the diploid strain had a novel form of invertase (see arrowheads). The gels in panels A and B were run in parallel, and the gel in panel C was run two times longer to obtain improved resolution.

these mutant heteroallelic diploids as well as from the mutant haploids were electrophoresed on nondenaturing gels, which were then stained for active invertase (Fig. 1). Extracts from cells carrying the suc2-438 allele produced invertase with a mobility similar to that of cytoplasmic invertase made in a wild-type strain (Fig. 1B). Each of the other haploid strains (suc2-317, suc2-527, and suc2-756) produced invertase with a lower mobility than that found in the suc2-438 strain, yet this invertase ran faster and was less diffuse than the glycosylated invertase found in a SUC2 control. In all cases, the heteroallelic diploid strains had a novel form of invertase that was absent from both the haploids and a mixture of haploid extracts. For suc2-527, this was best visualized in the gel shown in Fig. 1C, which was run two times longer for improved resolution. Since invertase within the secretory pathway did not form dimers with cytoplasmic invertase, these results indicate that all of the mutant alleles encode invertase that has access to the cytoplasmic compartment.

Fractionation of mutant proteins. To determine whether the mutant proteins were soluble or were membrane associated, extracts from strains containing each of the Suc⁻ alleles were fractionated on sucrose gradients. Invertase encoded by a suc2-527 strain was entirely in the soluble fraction of the gradient (Fig. 2). Identical results were observed in parallel gradients of strains carrying either suc2-317 or suc2-756 (data not shown). Parallel fractionation of extracts from a sec18 SUC2 strain (DBY2356 transformed with pRB420), which accumulated invertase within the ER, were used to identify the ER membrane (Fig. 2). In this background, a significant portion of the invertase fractionated at a much higher density and was separated from soluble invertase. Thus, the localization of the mutant forms of invertase suggests that these proteins are soluble in the cytoplasm. If the proteins are at all associated with a membrane fraction, this association is weak enough to be
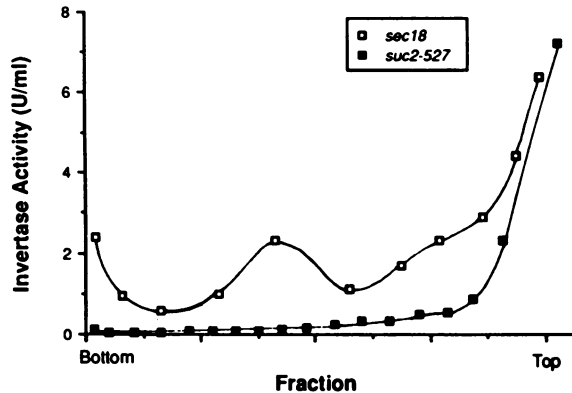
FIG. 2. Invertase activity in fractions of a linear sucrose gradient loaded with crude cell extracts. Invertase encoded by a *sec18 SUC2* strain (induced at the nonpermissive temperature) was used as a marker for the ER. All of the invertase encoded by *suc2-527* (as well as *suc2-317* and *suc2-756*) fractionated at the top of the gradient. A similar fractionation pattern has been observed with *suc2-438* (17). The soluble invertase found in the *sec18* strain was probably caused by enzyme that was released during preparation of the extracts.

disrupted during preparation of the extracts or cell fractionation.

Immunofluorescence of mutant invertase. Finally, to confirm that the invertase produced by the mutants was indeed in the cytoplasm rather than associated with a membranous compartment, we used indirect immunofluorescence to visualize the invertase produced by the mutant strains. Strains with the alleles *suc2-317*, *suc2-527*, or *suc2-756* on high-copy-number plasmids were grown under conditions that simultaneously selected for the plasmid and induced invertase production. The cells were fixed, spheroplasted, and mounted onto slides, as described above. The mutant strains exhibited diffuse staining throughout the cells, except in the region of the nucleus (Fig. 3, arrowheads). No fluorescence was observed in a strain with a complete deletion of the *SUC2* gene, and identical but much fainter staining was observed in mutant strains containing centromere plasmids (data not shown). These results, along with the formation of heterodimers and cell fractionation, strongly indicate that these mutant invertase proteins are soluble in the cytoplasm.

Selection of Suc+ revertants. In order to better understand the defects in the mutants, as well as the requirements for a functional signal sequence, we selected Suc+ revertants of Suc− strains containing the alleles *suc2-317*, *suc2-527*, or *suc2-756*. The *suc2* deletion strain DBY2449 was transformed with derivatives of the centromere plasmid pRB576, which encode each of the mutant alleles. A small portion of each mutant strain was used to inoculate several independent cultures of liquid YEPD medium. A portion of approximately 10^7 cells from each independent culture was then spread onto a YEP-sucrose plate with 1 μg of antimycin A per ml to select for revertants that fermented sucrose. Only one revertant per plate was selected for further analysis.

In the first screen, 10^8 cells encoding each mutant allele were spread and were incubated at 30°C for approximately 10 days. From this screen, the spontaneous revertants *527-r1* and *527-r2*, as well as *317-r1* and *756-r2*, were identified. Subsequent screens attempted to identify several more revertants of *suc2-527*; UV mutagenesis yielded *527-r4*, and mutagenesis with EMS yielded 17 more revertants of *suc2-527*. The spontaneous reversion frequency was quite low; it

was estimated to be about 10^{-8}, and UV or EMS mutagenesis increased that frequency about 10-fold.

As each revertant was isolated, single colonies were purified on a YEPD plate and retested for growth on sucrose. Plasmids were isolated from each revertant that was retested, and these plasmids were introduced into *E. coli* DB6507. The mutations were verified as being plasmid-linked by transforming DBY2449 with plasmids that were isolated from *E. coli*. All of the revertants identified in this fashion allowed strain DBY2449 to grow as well on sucrose as if it contained a wild-type invertase allele.

Revertants secrete active invertase. To determine the efficiency of secretion of the revertant invertase proteins, we quantitated the extracellular and intracellular invertase levels in each revertant strain. The cultures were grown in liquid YEP-lactate medium, both to induce invertase synthesis and to permit an assay of any invertase that was released into the culture medium. The external activity (Table 1) represents the sum of the invertase activity found in the medium and on the cell surface. The internal activity was determined from spheroplasts that were washed and then lysed with detergent. Each Suc− mutant secreted little active invertase, accumulating most of the activity internally. In contrast, all of the revertants released substantial levels of invertase, secreting between 35 and 92% of the total activity.

Overexpression of mutant invertase. Since each of the revertants produced substantially more active invertase than the Suc− strains did (Table 1), we tested whether simply increasing the level of mutant invertase alters the Suc− phenotype. We converted centromere plasmids containing each of the mutant alleles to high-copy-number, 2μm plasmids using in vivo recombination as described by Ma et al. (24). Briefly, the centromere plasmids were linearized within the *CEN4* sequence, and then were mixed with DNA fragments containing both 2μm replication sequences and homologous sequences flanking the centromere insertion site. The mixture was used to transform the yeast strain DBY2449, and plasmids were reisolated from the transformants to verify their structure. Even when expression was increased in this manner, the mutant invertase encoded by *suc2-317*, *suc2-527*, or *suc2-756* failed to allow yeast strains to grow on sucrose.

TABLE 1. Invertase activity in Suc− mutants and their revertants[a]

SUC2 allele	Intracellular activity[b]	Extracellular activity[c]	% Secreted
SUC2	40	730	95
317	89	0.1	0.1
317-r1	220	250	53
527	6	0.1	1.6
527-r1	55	680	92
527-r2	140	560	80
527-r4	100	440	81
527-r39	34	320	90
527-r50	31	310	91
527-r89	32	140	81
527-r98	120	200	62
756	13	0.1	0.8
756-r2	89	47	35

[a] Cells were grown in YEP-lactate medium to the exponential phase. Invertase activity is expressed in nanomoles of glucose released per minute per absorbancy unit (A_{600}) at 37°C. Values represent the average of measurements from two separate experiments that did not differ by more than 20%.
[b] Intracellular activity was obtained from spheroplast lysates.
[c] Extracellular activity was the sum of the activity found in the culture supernatant and that found on the surface of intact cells.
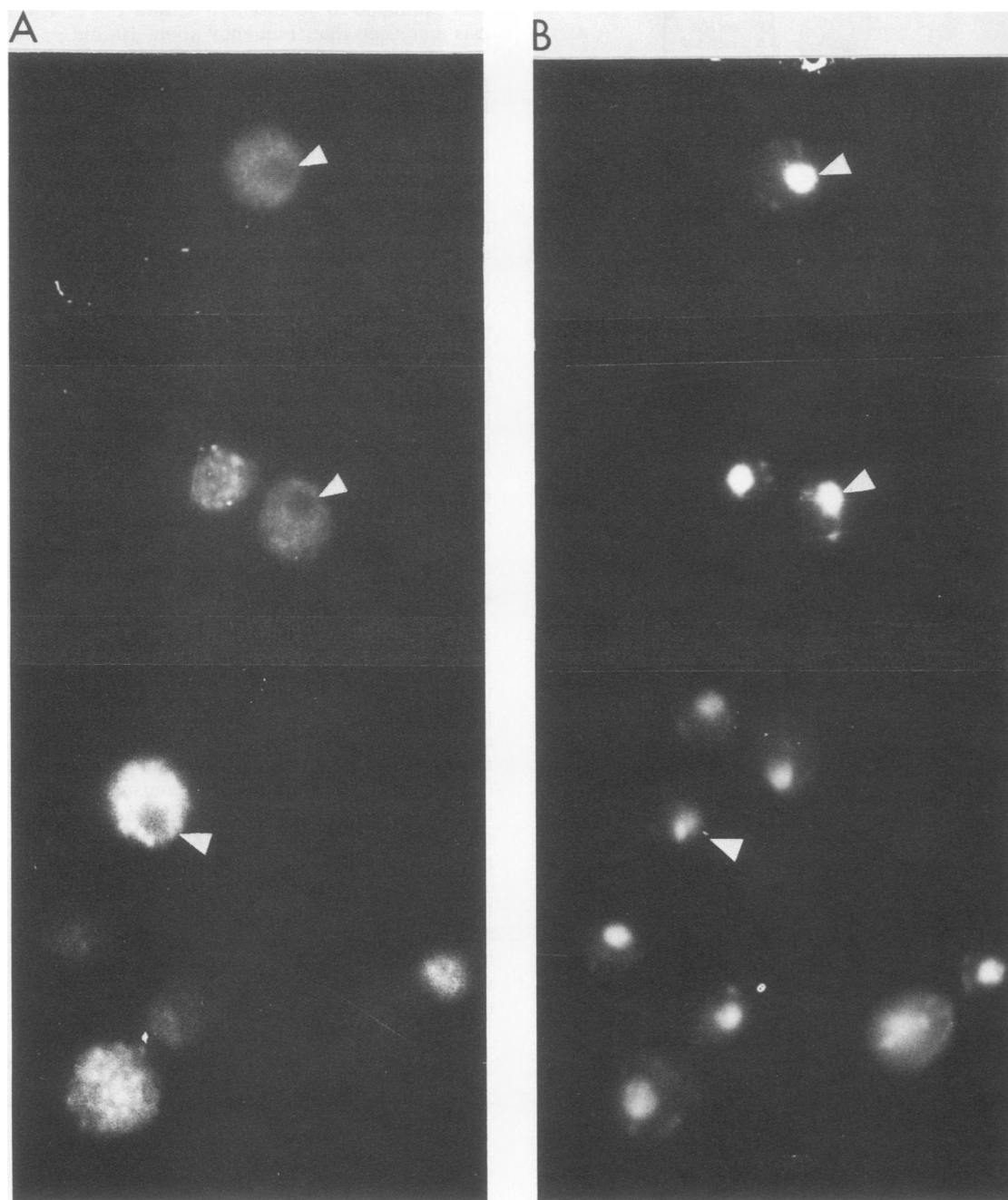
FIG. 3. Immunofluorescent localization of invertase in cells expressing *suc2-317* (A). Similar patterns were observed in *suc2-527* and *suc2-756* strains. Invertase was detected throughout the cytoplasm, but was not in the nucleus (arrowheads). DAPI staining of the DNA in combination with Nomarski optics is also shown (B).

To verify that these strains had elevated levels of invertase relative to those in strains that carried the corresponding centromere plasmids, we performed quantitative invertase assays (Table 2). For this experiment, the yeast strains were grown in minimal medium containing 2% lactate, thus continuously selecting for the plasmids and inducing invertase synthesis. As expected, the strains that carried 2μm-based plasmids produced more invertase than the strains with centromere plasmids did. This increase in invertase activity ranged between 7- and 18-fold. Furthermore, this invertase was efficiently retained within the cells (Table 2). Thus, a substantial increase in the total level of mutant invertase does not significantly increase the level of external invertase and, consequently, fails to suppress the sucrose growth defect. Therefore, the suppression observed in the revertant strains cannot be attributed to a change in the level of invertase alone.

Mutant proteins are not rapidly degraded. The low, steady-state levels of invertase in the mutant strains relative to the levels in the revertant strains raises the possibility that these various forms of invertase are less stable in the cytoplasm than they are within the secretory pathway. To test this, we

FIG. 4. Pulse-labeling and immunoprecipitation of invertase made in the Suc⁻ mutants (A) or in their revertants (B), followed by fractionation through SDS-polyacrylamide gels (7.5%). Cells were induced for invertase synthesis and were labeled with [^{35}S]methionine for 6 min before the addition of excess, nonradioactive methionine. Samples were removed immediately after the labeling period and at 5, 10, and 30 min after the initiation of the chase. (B) P represents the sample that was removed just after the 6-min pulse-labeling period; C is the sample that was removed after a 5-min chase. All gels shown here were run in parallel, and wild-type controls appeared as expected. Core glyc, Core glycosylation.

pulse-labeled cells that expressed the mutant proteins and collected samples throughout a 30-min chase period. The labeled invertase was immunoprecipitated and then electrophoresed through a denaturing polyacrylamide gel (Fig. 4A). The invertase made in each of the mutant strains was found to be quite stable; mutant protein levels did not vary by more than twofold throughout the chase period. Furthermore, similar results were obtained when the invertase produced in the revertant strains was examined (data not shown). Thus, the steady-state invertase levels shown in Table 1 accurately reflect the levels of invertase synthesis in both the mutant and revertant strains.

Revertants produce glycosylated invertase. To ascertain whether the revertants produced active, glycosylated invertase, extracts from each revertant strain were electrophoresed through a nondenaturing gel, which was then stained for invertase activity (Fig. 5). On continuous induction of invertase synthesis, most of the invertase produced by the revertant strains migrated with the glycosylated invertase made in a wild-type strain. Furthermore, most of the strains no longer produced invertase with the mobility of that found in the parent strains. The revertant 527-r98 was an exception; it produced glycoslyated invertase as well as some nonglycosylated invertase with the mobility of that made in the suc2-527 strain. These results are directly

comparable to the quantitative measurements of invertase levels, since the yeast cultures that were used in each experiment were grown under identical conditions. Thus, the invertase activity that was measured in the revertant strains (Table 1) represents, for the most part, glycosylated invertase (Fig. 5).

To carefully determine the extent of glycosylation of the invertase made in the revertant strains, we electrophoresed extracts from each revertant on denaturing SDS-polyacryl-

TABLE 2. Comparison of invertase activity in YEp and YCp derivatives of Suc⁻ mutants[a]

SUC2 allele	Intracellular activity[b]	Extracellular activity[c]	% Secreted
SUC2	26	300	92
317-YCp	85	2.0	2.2
317-YEp	640	8.8	1.4
527-YCp	7	0.3	3.5
527-YEp	130	4.0	3.1
756-YCp	13	0.9	6.2
756-YEp	150	2.7	1.8

[a] Activity was determined as described in footnote a of Table 1. The cells were grown in SD-CAS medium with 2% lactate.
[b] See footnote b in Table 1.
[c] See footnote c in Table 1.

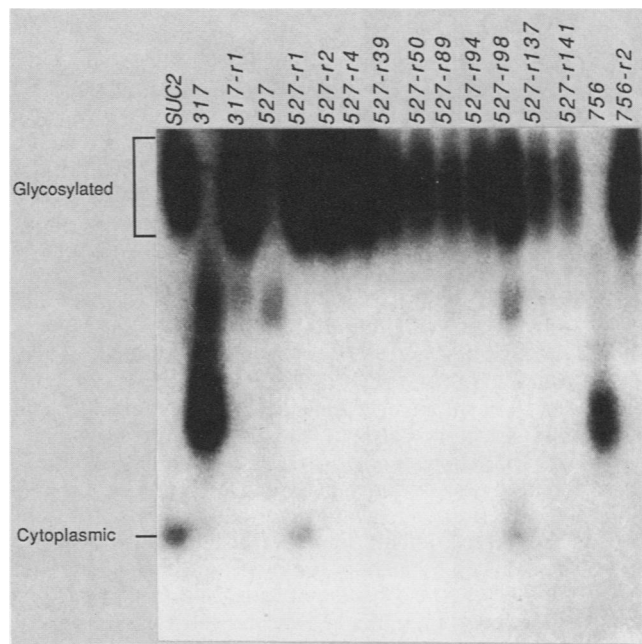FIG. 5. Nondenaturing gel stained for invertase activity. Cells were grown in YEP-lactate medium, and extracts were made from cells that were lysed by agitation with glass beads. The lanes containing the Suc⁻ mutants (suc2-317, suc2-527, and suc2-756) were loaded with twice as much crude extract as all of the other lanes. Each of the Suc⁺ revertants had invertase that migrated with glycosylated invertase from a SUC2 strain.
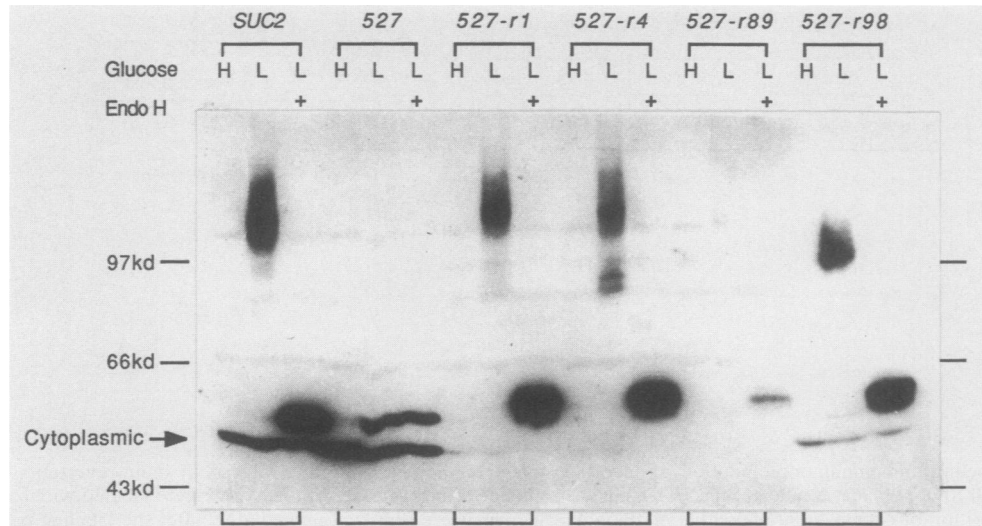
FIG. 6. Denaturing gel of a Suc⁻ mutant and several Suc⁺ revertants. Cells were grown in 5% YEP-glucose medium and then transferred to YEP with 5% glucose (H) or 0.1% glucose (L). Endo H was added to extracts from induced strains in order to remove most of the oligosaccharide. Following transfer to a nitrocellulose filter, invertase antigen was detected by incubation with invertase antiserum and ¹²⁵I-labeled protein A. The *suc2-527* allele was encoded by a high-copy-number YEp vector; all other alleles were on YCp vectors. kd indicates the molecular size in kilodaltons.

amide gels and identified invertase antigen by immunoblotting (Fig. 6). As expected, almost all of the invertase made in the revertant strains had a mobility characteristic of glycosylated protein. This glycosylation could be removed by the glycolytic enzyme endo H (Fig. 6). Furthermore, invertase glycosylation was effectively inhibited in both wild-type and revertant strains by treatment with tunicamycin (data not shown). Since tunicamycin blocks the synthesis of N-linked oligosaccharides (20, 22), the glycosylated invertase produced by the revertant strains, like that made by wild-type strains, contains oligosaccharides that are strictly N-linked.

Wild-type invertase rapidly transits the secretory pathway, receiving core glycosylation in the ER, followed by outer-chain glycosylation in later stages of the pathway. As demonstrated by Schauer et al. (32) after they pulse-labeled wild-type cells for only 5 min, all of the labeled invertase is in a glycosylated state; that which is only core glycosylated receives outer-chain glycosylation with a half-time of about 1.5 min. Since the extent of glycosylation is variable, a heterogeneous population of invertase proteins with an average molecular weight of 120 kilodaltons is generated (11). To determine whether the revertant proteins rapidly entered the secretory pathway, we briefly pulse-labeled cells that carried each of the revertant alleles. After only 6 min, essentially all of the invertase made in each revertant strain entered the secretory pathway and received core glycosylation (Fig. 4B). However, after an additional chase period, most of this invertase had not moved on to receive outerchain glycosylation. When the revertant proteins were observed after much longer time periods, the glycosylated invertase produced by most of the revertant strains migrated like wild-type invertase on denaturing gels (Fig. 6, *527-r1* lanes). Some revertants, like *527-r4*, had substantial levels of glycosylated invertase that migrated between 85 and 90 kilodaltons, indicating that these proteins received only core glycosylation. In fact, three revertant strains, *317-r1*, *527-r98*, and *756-r2*, accumulated only core glycosylated invertase. The same three strains had a relatively high fraction of internal invertase activity, as determined by a quantitative assay (Table 1). Thus, although all of the revertants pro-

duced invertase that efficiently entered the secretory pathway, some strains accumulated substantial levels of invertase at intermediate stages of that pathway and are, therefore, phenotypically distinct from wild-type strains.

Mapping of the reversion events. To determine whether the reversion mutations were indeed at the *SUC2* locus, we mapped each Suc⁺ reversion event using the techniques described by Kunes et al. (19). First, we determined that plasmids isolated from each Suc⁺ revertant carried the reversion mutation, since transformation of these plasmids into the *suc2* deletion strain DBY2449 generated strains with Suc⁺ phenotypes. Next, we isolated several restriction fragments from the revertant plasmids, mixed each fragment with a linearized plasmid bearing a Suc⁻ allele, and transformed DBY2449 with the mixture. Since the restriction fragments were homologous to both ends of the linearized plasmid, efficient in vivo recombination generated a recombinant, circular plasmid. Furthermore, since DBY2449 lacked chromosomal *SUC2* homology, the presence of Suc⁺ transformants indicated that the restriction fragment in question contained the Suc⁺ reversion mutation.

As a source of linearized plasmid for the experiment described above, we used the centromere plasmid pRB576. This plasmid carries *suc2-450*, an allele with an extensive deletion of the wild-type signal sequence, and a *Sma*I restriction site 11 base pairs downstream of the regulated initiation codon (18). When transformed with this plasmid, DBY2449 produces only cytoplasmic invertase and is, therefore, a Suc⁻ strain. Except for the region of the signal sequence, pRB576 was homologous to the plasmids that encoded each of the Suc⁻ mutants and the Suc⁺ revertants. Thus, pRB576, which is linearized by *Sma*I digestion, can be repaired in vivo by restriction fragments from the revertant plasmids, as long as these fragments are homologous to regions on either side of the *Sma*I site (Fig. 7A).

Several appropriate restriction fragments containing different portions of the *SUC2* locus were isolated from each revertant plasmid and, as a negative control, from a plasmid containing the Suc⁻ allele *suc2-527* (Fig. 7B). DBY2449 was then transformed with each of these fragments in combina-
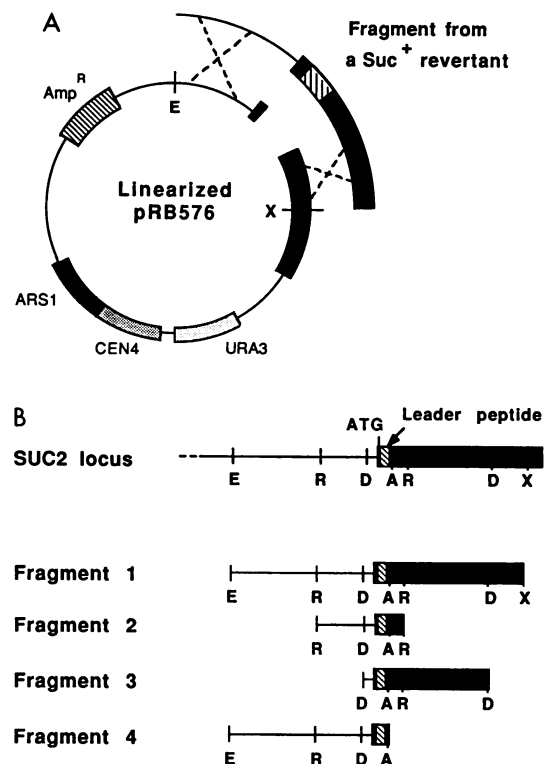
FIG. 7. (A) Strategy for mapping Suc⁺ revertants. Plasmid pRB576 was linearized at a unique *Sma*I site, and fragments from Suc⁺ revertants were added. Transformation of the mixture into *S. cerevisiae* yielded Suc⁺ recombinants if the Suc⁺ mutation was on the fragment. (B) Restriction fragments from the Suc⁺ revertant plasmids used to localize the reversion mutations. Abbreviations: A, *Ava*II; D, *Dde*I; E, *Eco*RI; R, *Rsa*I; X, *Xba*I. All of the restriction sites are present in the wild-type sequence (34), as well as in each of the mutants.

tion with pRB576 that was linearized at the *Sma*I site, and the transformants were tested for growth on sucrose. None of the restriction fragments obtained from the plasmid encoding *suc2-527* converted pRB576 to a Suc⁺ plasmid. However, restriction fragments from each revertant plasmid did generate Suc⁺ recombinants, indicating that the reversion mutations map to the *SUC2* locus. Furthermore, the use of several restriction fragments localized the mutations to a very small region of the gene (Fig. 7B). In this manner, all of the reversion mutations were mapped to a small region of the gene consisting of 50 base pairs of the *SUC2* 5'-noncoding region and just 10% of the region that encodes invertase.

Characterization of the reversion events. As each revertant plasmid was isolated, it was compared, following restriction digestions, with plasmids from the Suc⁻ parent strain by electrophoresis on agarose gels. Three types of mutations were apparent immediately. First, the revertants *527-r39*, *527-r50*, *527-r94*, *527-r137*, and *527-r141* had fairly long insertions or duplications that were either upstream of or included the *Hind*III site located 26 base pairs before the initiation codon; while *527-r1*, *527-r2*, *527-r68*, and *756-r2* had insertions or duplications that were downstream of this *Hind*III site. Second, some revertants appeared to be simple point mutations that either retained the *Hind*III site, as in *527-r89*, or lost it, as in *317-r1* and *527-r4*. Nine additional *suc2-527* revertants of this latter type were identified, but none was characterized further. Finally, one revertant,

527-r98, had a short deletion downstream of the *Hind*III site. These preliminary results indicate that the predominant type of reversion event involves alterations, at least in part, of the 5'-noncoding portion of the *SUC2* gene.

Sequence of the revertant leaders. Several of the revertant leaders were sequenced by the method of Maxam and Gilbert (26), and the predicted amino acid sequences of the revertant leader peptides as well as the sequences of the Suc⁻ mutants are presented in Fig. 8. Those revertants that had alterations downstream of the *Hind*III site were sequenced by end-labeling them at that site. All other revertants were sequenced by end-labeling them at the *Ava*II site at position +89 or at the *Ava*II site that was present in all leader sequences derived from *suc2-527* (Fig. 8A). In every case, the bulk of the region that was mapped was also sequenced, and for each revertant, only a single alteration was detected.

The 5'-noncoding region of the *SUC2* gene contains an open reading frame that, if translated, is quite hydrophobic (Fig. 8). Most of the reversion mutations allowed this region to be translated, thus increasing the hydrophobicity of the amino termini of the mutant proteins. Two general mechanisms, point mutations and duplications, resulted in translation of a portion of this upstream region. First, point mutations generated new, in-frame initiation codons, as demonstrated by the revertants *527-r4*, *527-r89*, and *317-r1* (Fig. 8A). A frequent location for this point change was an A to T transversion at the *Hind*III site at position −26. Although only two revertants of this type were sequenced, nine others probably had a similar change, as indicated by the loss of the *Hind*III restriction site. Alternatively, a new initiation codon was generated, again by an A to T transversion, even further upstream at position −38, as demonstrated in *527-r89*. We found no revertants with alterations upstream of the transcriptional initiation site at position −40 (7). Downstream of this site there were only three positions, −38, −26, and −8, at which a single point mutation would generate a new initiation codon in the proper reading frame. Although we found 12 independent revertants that had point mutations at position −38 or −26, none were found with point changes at position −8. This suggests that the amino-terminal addition of more than two or three hydrophobic residues is required for efficient suppression of the Suc⁻ phenotype.

In addition to point mutants, we identified several revertants that arose by duplication of a portion of the 5'-noncoding region of the *SUC2* gene (Fig. 8B). Two of these revertants, *756-r2* and *527-r2*, had identical, short duplications that resulted in the addition of the same hydrophobic residues to the amino termini of the mutant proteins. Much longer duplications were observed in *527-r1* and *527-r50*, which added 18 and 13 residues, respectively, to the *suc2-527* leader. Although a variety of point mutations and duplications were observed, each resulted in the addition of nearly the same group of hydrophobic residues near or at the amino terminus of invertase.

Finally, the revertant *527-r98* arose by a deletion of 10 residues from the *suc2-527* leader sequence (Fig. 8C). This deletion removed three charged residues, as well as several polar ones, and juxtaposed two fairly short, hydrophobic stretches. Although this leader peptide allowed invertase to be secreted, it may, in fact, be an inefficient signal sequence, since a strain carrying this revertant allele produced some nonglycosylated invertase with the same mobility as that of the cytoplasmic invertase made by a *suc2-527* strain (Fig. 5).

Denaturing gels confirm sequence predictions. The se-

A

```
                          HindIII                                    AvaII
                            ↓                                          ↓
              + +  ++              +        +-        +    -
527           nrnkqnkklffsltymMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...


              + +  +                +        +-        +    -
527-r4        nrnkqnkMLFFSLTYMMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...
                     ↑


              +    ++               +        +-        +    -
527-r89       nrnMQNKKLFFSLTYMMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...
                 ↑


              + +  ++               +         +-  +
317           nrnkqnkklffsltymMLFPHTATYRAPPCLVGGGRDGRVWLSTGGM...


              + +  +                 +        +-  +
317-r1        nrnkqnkMLFFSLTYMMLFPHTATYRAPPCLVGGGRDGRVWLSTGGM...
                     ↑
```

B

```
                        + +  ++                       ++   +
756         nrnkqnkklffsltymMLFPPPPRRWGCRPAGGM...
                        ───────────►


                        + +  ++                       ++   +
756-r2      nrnkqnkklffsltymMLFFSLTYMMLFPPPPRRWGCRPAGGM...
                        ─────────►         ───────►


                        + +  ++               +    +-     +    -
527         nrnkqnkklffsltymMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...


                      + +  ++                 +    +-     +    -
527-r2      nrnkqnkklffsltymMLFFSLTYMMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...
                        ────────►     ────────►


            + +  ++                           +    +-     +    -
527-r1      nrnkqnkklffsltymMLFPHHQWRVLFFSLTYMMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...
                        ────────►     ────────►


              + +  ++                 +  ++    +    +-     +    -
527-r50     nrnkqnkklffsltymMKNKKLFFSLTYMMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...
                        ────────►      ───────►
```

C

```
              + +  ++                 +    +-     +    -
527         nrnkqnkklffsltymMLFPHHQWRVQSSKDCCTIPSSGRLVPEGGM...


              + +  ++                              +    -
527-r98     nrnkqnkklffsltymMLFPH----------CCTIPSSGRLVPEGGM...
```
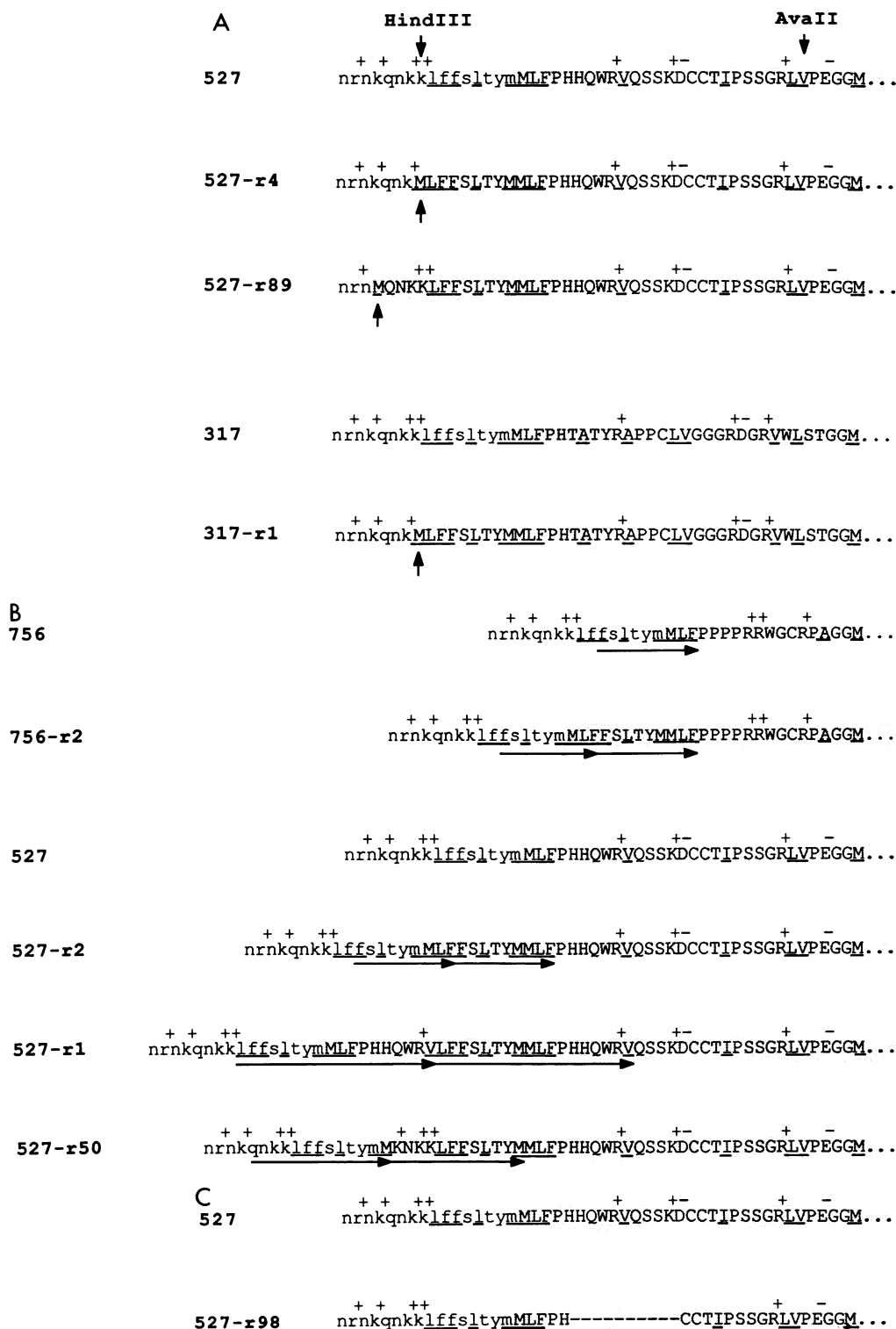
FIG. 8. Predicted amino acid sequence of nonfunctional and revertant leaders. The upstream open reading frame is represented by lowercase letters; the coding portion of the gene is in capital letters and is shaded. Charges on amino acids (Arg, Asp, Glu, and Lys) are indicated above each sequence, and hydrophobic residues (Ile, Leu, Met, Phe, and Val) are underlined. The last residue in each sequence is the initiator methionine for constitutive, cytoplasmic invertase. (A) Revertants that arose by point mutations (arrows), which generated a new initiation codon. (B) Revertants that arose by duplication of the nonfunctional leader sequence. The arrow under the *suc2-756* sequence indicates the region that was duplicated to form *756-r2*. (C) A deletion of a portion of the *suc2-527* sequence generated *527-r98*. Abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
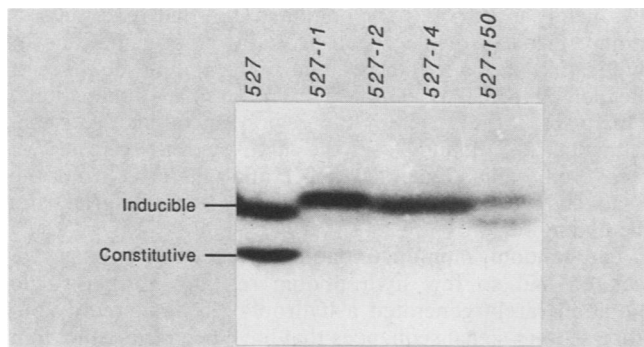
FIG. 9. Denaturing gel of crude extracts. Cells were induced for invertase synthesis in the presence of tunicamycin. The *suc2-527* allele was on a YEp vector; all others were on YCp vectors. The invertase observed in each strain migrated as predicted by the DNA sequence.

quence changes observed in the revertants predicted that the invertase that was made in the revertant strains would have a different molecular weight than the invertase that was made in the Suc⁻ mutant strains. To test this prediction, invertase synthesis was induced in several revertant strains in the presence of tunicamycin, which inhibits glycosylation, and, consequently, permitted us to observe the size of the protein moiety alone. Extracts from each strain were electrophoresed on a denaturing gel, and the mobility of invertase was compared with that of invertase produced by the Suc⁻ parent (Fig. 9). Each of the revertants that were sequenced were analyzed in this manner, and in every case the mobility of invertase was altered as expected. Most revertants produced invertase that migrated on these gels as a single band. However, some extracts contained invertase that migrated as two discrete bands (Fig. 9, lane *527-r50*). This may have been caused by some degradation within the extract or, since the lower band migrated with regulated invertase from a *suc2-527* strain, could be attributed to the loss of the tandem duplication during growth of the culture. Finally, the regulated invertase from each of the revertants migrated more slowly than the constitutive invertase from wild-type strains. This indicates that each of the revertant proteins retains its leader sequence. Thus, even though the revertant proteins are secreted, none undergoes the signal cleavage that is characteristic of wild-type secreted invertase.

DISCUSSION

Characteristics of functional signal sequences. Replacing the wild-type invertase signal sequence with a variety of random peptides allowed us to make a detailed comparison of leader sequences that targeted invertase to the secretory pathway with those that did not. Leaders that function as signal sequences are enriched in hydrophobic residues, while those that fail to target invertase to the secretory pathway have numerous charged and polar residues (18). This generalization is further supported by the revertants that were obtained from strains with nonfunctional leader sequences. Each reversion event either increased the number of hydrophobic amino acids or decreased the number of charged amino acids in the nonfunctional leader sequence (Fig. 8). This implies that the secretion defect of the nonfunctional leader sequences is simply due to a charge density that is too high and a degree of hydrophobicity that is too low to be consistent with function as a secretion signal.

Previously, Kaiser et al. (18) demonstrated that signal sequences derived from random DNA fragments are quite similar to naturally occurring yeast signal sequences. When the hydrophobicity and charge density of the leaders were quantitated, naturally occurring signal sequences, as well as randomly derived signal sequences, were separated from the amino termini of yeast cytoplasmic proteins (Fig. 10A and B). A similar separation was observed when the hydrophobicity and charge density of the revertant leader sequences were compared with those of the nonfunctional leaders (Fig. 10C). In no case did we find a sequence that did not behave in a predictable manner. Furthermore, since most of the revertant leaders arose by the addition of a few hydrophobic residues to a nonfunctional leader sequence, we conclude that the defective leader sequences, if preceded by hydrophobic residues, can cross the ER membrane. This reinforces the idea that the nonfunctional leader sequences are simply not hydrophobic enough to allow ER targeting, and the requirements for a functional signal sequence are most likely limited to a combination of adequate hydrophobicity and a low charge density or to closely related properties.

Most of the Suc⁺ revertants had leaders that were more hydrophobic and had a lower charge density than those of the Suc⁻ mutants. However, *527-r50* and *527-r89* acquired several charged residues in the process of incorporating a hydrophobic domain, simultaneously increasing both hydrophobicity and charge density. This additional charge apparently has no detrimental effects, since these leaders efficiently targeted invertase to the ER. Furthermore, these revertant leaders had properties that were similar to those of naturally occurring signal sequences (Fig. 10C). Thus, a functional signal sequence may contain several charged amino acids; as long as a domain of adequate hydrophobicity is present, efficient targeting to the secretory pathway occurs.

Position of the hydrophobic domain. The invertase produced by a *suc2-527* strain differed from that made by the revertants *527-r2* and *527-r4* by only nine residues at the amino terminus of the protein. This short stretch of hydrophobic residues also corrected the defect in the leader sequences encoded by *suc2-317* and *suc2-756*, as demonstrated by the revertants *317-r1* and *756-r2*, respectively. Thus, these amino acids are sufficient to allow the secretion of several mutant proteins and are, therefore, not allele specific in their suppression. Furthermore, since these nine residues are responsible for the secretion of the invertase made by *527-r2* and *527-r4*, it is likely that they constitute the functional part of the signal sequences encoded by *527-r1*, *527-r50*, and *527-r89*. Each of these latter revertants had charged and polar residues at the amino terminus, which placed the hydrophobic domain between five and nine residues downstream of the initiation codon. Thus, the hydrophobic residues that allow a leader to function as a signal sequence need not be at the immediate amino terminus of the protein. Many naturally occurring signals also have amino-terminal charges preceding a hydrophobic domain (36). The revertants described here demonstrate that these charged residues are not required for, nor do they inhibit, secretion.

New signals are generated by duplications and upstream rearrangements. Each of the 22 independent revertants that we isolated obtained a functional signal sequence by the addition or removal of several amino acids from a nonfunctional leader sequence. Although 12 revertants arose by point mutations in the 5′-noncoding portion of the gene, in no case did we observe single or multiple point mutations within the nonfunctional leaders themselves. Previous stud-
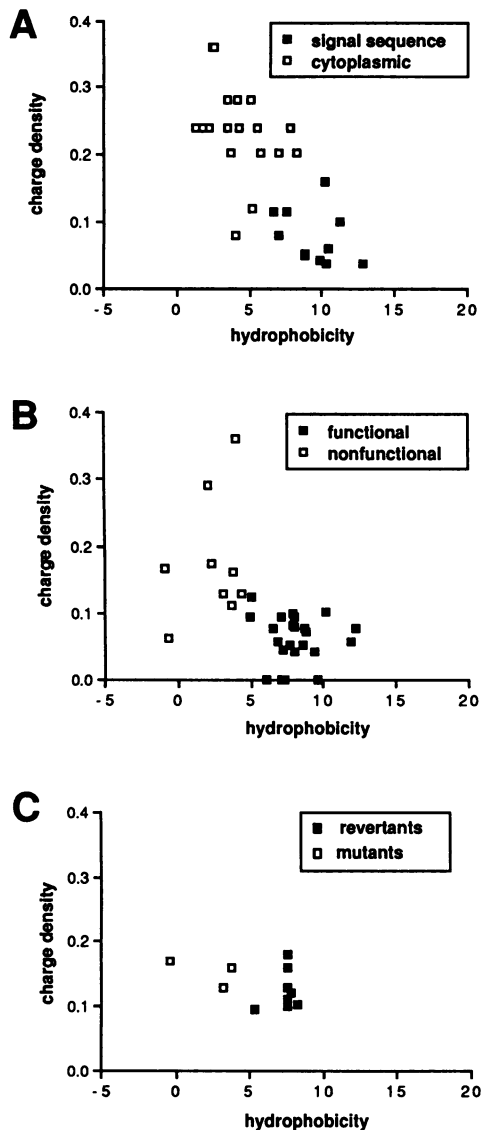
FIG. 10. Plot of the hydrophobicity and charge density of the amino-terminal sequences of various proteins. For panels A and B, data from Kaiser et al. (18) were used. For each sequence, the most hydrophobic 10-amino-acid segment was identified, and the hydrophobicity was quantitated by using the parameters of Kyte and Doolittle (21), which were normalized to a mean of 0.0 and a standard deviation of 1.0. The charge density is the ratio of the number of charged residues (Arg, Asp, Glu, and Lys) to the total number of residues in the sequence. (A) Comparison of yeast signal sequences to the amino termini of yeast cytoplasmic proteins. The signal sequences were encoded by *MFα1*, *MFα2*, *PHO5*, *SUC2*, *KEX2*, *PRC1*, *STE3*, *BAR1*, *KIL-K*, *STA1*, and *MEL1*; and the cytoplasmic proteins were alcohol dehydrogenase II, glyceraldehyde 3-phosphate dehydrogenase, superoxide dismutase (CuZn), phosphoglycerate kinase, anthranilate synthase component I, anthranilate synthase component II, ribosomal protein S33, ribosomal protein 51, ATP phosphoribosyltransferase, pyruvate kinase, thymidylate kinase, phosphoribosyl-AMP cyclohydrolase, enolase, tryptophan synthase, triosephosphate isomerase, phosphoglyceromutase, methionyl-tRNA synthetase, and elongation factor 1-αA (18, 23; Protein Sequence Database of the Protein Identification Resource, Division of Research Resources, National Institutes of Health). (B) Comparison of random sequences that targeted yeast invertase to the secretory pathway (functional) with those that did not (nonfunctional). (C) Comparison of several nonfunctional leader sequences (mutants) with Suc⁺ revertants.

ies, mainly in *E. coli*, have demonstrated that revertants of mutant signal sequences can arise by point mutations or duplication of two to four residues in the coding sequence of the defective leader (3, 4, 31). These small duplications, which are entirely within the region encoding the signal sequence, are distinct from the large duplications that we recovered, which included substantial material from the 5'-noncoding region of the gene and little or no material from the coding region.

The random, nonfunctional leader sequences that we isolated had so few hydrophobic residues that a single mutation rarely generated a hydrophobic domain. In contrast, mutant signal sequences that have been used to obtain revertants in previous studies (3, 4, 31) retain substantial portions of the wild-type signal sequence. In our case, we required alterations that simultaneously change several residues, rather than single amino acids, in order to obtain enough hydrophobicity to generate a functional signal sequence. For a gene, such as *SUC2*, with a nearby DNA sequence that is capable of encoding hydrophobic residues, the predominant evolutionary mechanism for obtaining a functional signal sequence is likewise likely to be simple incorporation of these codons into the coding sequence of the gene. Furthermore, since one-fifth of all random leader sequences generate a functional yeast signal sequence (18), rearrangements, such as those observed here, have a high a priori probability of forming a signal sequence. Gene duplications play a major role in the evolution of new proteins; similarly, alterations like those we observed here may represent a common mechanism for the evolution of naturally occurring signal sequences.

Revertants produce more invertase than do Suc⁻ mutants. Each of the revertants not only secreted active invertase but they also produced substantially more invertase than the Suc⁻ mutants did. As determined by invertase activity assays (Table 1), this increase in invertase production ranged between 6- and 100-fold. Furthermore, these levels correlated well with the amounts of invertase observed on immunoblots, indicating that the differences cannot be attributed to changes in specific activity (data not shown). We demonstrated that increasing the level of the mutant proteins fails to suppress the sucrose growth defect. However, it is striking that every revertant isolated in this study produced more invertase than the Suc⁻ mutants did.

We can eliminate the following explanations for this observation. First, several Suc⁻ mutants that express high levels of active, cytoplasmic invertase had been isolated (17, 18). Although these strains were not chosen for reversion analysis, they nevertheless demonstrate that yeast cells are capable of synthesizing large amounts of cytoplasmic invertase. Second, degradation of the mutant proteins cannot account for the observed differences in levels since pulse-labeling experiments demonstrated that the mutant invertase proteins are quite stable. Finally, the differences in invertase levels cannot be attributed to transcriptional regulation. Essentially all of the invertase made by both the Suc⁻ mutants and the revertants is induced under conditions of glucose deprivation. This suggests that the inducible *SUC2* promoter is unaffected by these downstream mutations. Furthermore, preliminary measurements of the *SUC2* mRNA levels in these strains support this conclusion. Therefore, the invertase levels observed in the Suc⁻ mutants and in the revertants are most likely affected by translational regulation.

Alterations near the initiation codon, such as those observed in the revertants, could potentially affect translation.

Extensive analysis of mutations in yeast iso-1-cytochrome *c* has led to the conclusion that alterations in nucleotides 5', but not 3', of the initiation codon affect the efficiency of translation (2, 33). In contrast, all of the Suc⁻ mutants and several of the revertants differed from the wild-type gene only in sequences that were downstream of the initiation codon. Nonetheless, we cannot eliminate the possibility that these changes directly influence translation. In addition, any secondary structure that is present in the mRNA transcripts may also influence the efficiency of translation, thus accounting for the observed differences in protein levels. Alternatively, the signal recognition particle found in higher cells binds, in the absence of membranes, to ribosomes that synthesize secretory proteins and arrests translation (27, 37). Although an analog of the signal recognition particle has not yet been found in *S. cerevisiae*, a similar function which allows translational regulation of secreted proteins may exist.

Revertant proteins do not behave like wild-type invertase. Finally, although the revertant leader sequences efficiently targeted invertase to the secretory pathway, they were not cleaved like wild-type signal sequences. In addition, some of the revertant proteins accumulated at intermediate stages within the secretory pathway. Thus, all of the revertant proteins allowed strains to grow on sucrose, but none behaved like wild-type invertase when examined in detail. This result emphasizes the multiple steps that are required for efficient secretion. Hydrophobicity satisfies the requirement for membrane targeting, the first event in secretion. However, naturally occurring signals have undergone further evolutionary changes that permit proteins to efficiently transit the secretory pathway. The cleavage of the signal sequence plays an important role in the secretion of invertase (32), yet the revertants are secreted with various efficiencies, even though none of their leader sequences was cleaved. We are testing the effect of additional substitutions of amino acids downstream of the hydrophobic domain in order to identify residues that influence the secretory process after membrane targeting has occurred.

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