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Invertase β-Galactosidase Hybrid Proteins Fail To Be Transported from the Endoplasmic Reticulum in *Saccharomyces cerevisiae*

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The yeast *SUC2* gene codes for the secreted enzyme invertase. A series of 16 different-sized gene fusions have been constructed between this yeast gene and the *Escherichia coli lacZ* gene, which codes for the cytoplasmic enzyme β-galactosidase. Various amounts of *SUC2* NH_2-terminal coding sequence have been fused in frame to a constant COOH-terminal coding segment of the *lacZ* gene, resulting in the synthesis of hybrid invertase-β-galactosidase proteins in *Saccharomyces cerevisiae*. The hybrid proteins exhibit β-galactosidase activity, and they are recognized specifically by antisera directed against either invertase or β-galactosidase. Expression of β-galactosidase activity is regulated in a manner similar to that observed for invertase activity expressed from a wild-type *SUC2* gene: repressed in high-glucose medium and derepressed in low-glucose medium. Unlike wild-type invertase, however, the invertase-β-galactosidase hybrid proteins are not secreted. Rather, they appear to remain trapped at a very early stage of secretory protein transit: insertion into the endoplasmic reticulum (ER). The hybrid proteins appear only to have undergone core glycosylation, an ER process, and do not receive the additional glycosyl modifications that take place in the Golgi complex. Even those hybrid proteins containing only a short segment of invertase sequences at the NH_2 terminus are glycosylated, suggesting that no extensive folding of the invertase polypeptide is required before initiation of transmembrane transfer. β-Galactosidase activity expressed by the *SUC2-lacZ* gene fusions cofractionates on Percoll density gradients with ER marker enzymes and not with other organelles. In addition, the hybrid proteins are not accessible to cell-surface labeling by ^{125}I. Accumulation of the invertase-β-galactosidase hybrid proteins within the ER does not appear to confer a growth-defective phenotype to yeast cells. In this location, however, the hybrid proteins and the β-galactosidase activity they exhibit could provide a useful biochemical tag for yeast ER membranes.

Protein secretion in *Saccharomyces cerevisiae* is mediated by the same set of organelle intermediates employed by higher eucaryotes. Proteins destined for secretion are initially cotranslationally translocated into the endoplasmic reticulum (ER) lumen, where they receive N-glycosidically-linked core oligosaccharides (4, 33, 36). Next, the proteins are transferred to the Golgi complex, where additional glycosyl modifications take place before packaging into secretory vesicles and final delivery to the cell surface. Yeast temperature-sensitive mutants defective for distinct steps in the secretory pathway have demonstrated clearly the essential role and the order of events of the pathway in *S. cerevisiae* (29, 30). Proteins destined for delivery to other cellular compartments, such as the vacuole, also traverse the secretory pathway (40). Secretory proteins must possess information within their sequence or structure that is responsible for distinguishing them from these other proteins, thereby allowing for their efficient delivery to the cell surface. To better understand the nature of this information, we employed the technique of gene fusion to analyze the secretion of the yeast *SUC2* gene product, invertase.

Fusions between genes encoding *Escherichia coli* exported proteins and *lacZ* have already proved useful in the study of bacterial protein secretion (1–3, 13, 14, 27; reviewed in reference 38). Also, it has been demonstrated clearly that the *lacZ* gene, when fused to a variety of yeast genes, can lead to the synthesis of hybrid proteins that exhibit β-galactosidase activity in *S. cerevisiae* (*S. cerevisiae* normally lacks any endogenous β-galactosidase activity) (17, 20, 26, 32, 35, 39). Such gene fusions have been used extensively to study the regulation of gene expression in *S. cerevisiae*. We attempted to extend the use of the *E. coli lacZ* gene in *S. cerevisiae* to study the yeast secretory pathway.

The yeast *SUC2* gene codes for the sucrose-cleaving enzyme invertase. This gene encodes two distinct mRNAs: a larger mRNA that carries an additional sequence and its 5′ end (containing the coding sequence for the 19-amino-acid signal peptide of invertase) which directs the synthesis of secreted invertase and a smaller mRNA lacking this sequence which directs the synthesis of a cytoplasmic unglycosylated form of invertase. The expression of the larger mRNA and secreted invertase are subject to glucose repression. The smaller mRNA is constitutively expressed, as is the internal form of invertase (7, 9, 34). The transit of secreted invertase through the ER, the Golgi Complex, and secretory vesicles has been documented clearly (36). Glycosyl modifications of this polypeptide include the addition of approximately nine N-linked core oligosaccharides during delivery into the ER lumen (43). After limited glycosyl processing, high-mannose chains (outer chains) are added to the core oligosaccharides in the Golgi complex (15). The glycosylated invertase is then packaged into secretory vesicles that deliver invertase, as well as other secretory proteins, to the cell surface (30). Secreted invertase remains trapped in the compartment bounded by the plasma membrane and the cell wall, the yeast periplasm, presumably because of its high molecular mass (~250 kilodaltons [kd] as the active glycosylated dimer; 24).

The yeast *SUC2* gene has been cloned (7), and its entire nucleotide sequence has been determined (42). We have

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taken advantage of this clone to construct and characterize 16 different size classes of *SUC2-lacZ* gene fusions. We report here on the regulation, the delivery, and the glycosylation of the different-sized invertase-β-galactosidase hybrid proteins coded for by these gene fusions.

MATERIALS AND METHODS

Strains and plasmids. Yeast and *E. coli* strains used are listed in Table 1. The plasmids employed are also listed. Standard genetic crosses were used to construct the yeast strains (28, 37). Strains containing the *suc2-Δ9* marker are deleted for their chromosomal *SUC2* gene (C. Falco and M. Carlson, personal communication). In addition, these strains also lack all other invertase structural genes (*SUC1*, *SUC3-SUC7*) (8).

Media. Yeast and *E. coli* media have been described previously (15, 25, 37).

DNA methodology. Restriction endonuclease digestions and ligations with T4 DNA ligase were done as recommended by the suppliers. Bal 31 nuclease was from Bethesda Research Laboratories, Inc. Digestions with Bal 31 were performed at 23°C as recommended by the supplier, except the digestion buffer contained 200 mM NaCl rather than the recommended 600 mM NaCl. Other standard techniques of plasmid DNA isolation, agarose gel electrophoresis, and DNA transformation of *E. coli* and *S. cerevisiae* were done as described (6, 21, 25, 37).

Enzyme assays. β-Galactosidase activity was measured as described (13) (1 unit equals the amount of enzyme that hydrolyzes 1 nmol of *o*-nitrophenyl-β-D-galactosidase per min at 28°C). Invertase was assayed according to Goldstein and Lampen (19) (units are given as nanomoles of glucose released per minute at 30°C). NADPH cytochrome *c* reductase was assayed as described by Kubota et al. (22) (units are given as nanomoles of cytochrome *c* reduced per minute).

Labeling and immunoprecipitation. For labeling with $^{35}SO_4^{2-}$ (Amersham Corp.), strains containing the various *SUC2-lacZ* fusions were grown to mid-logarithmic-phase in minimal medium containing 5% glucose and 0.1 mM $(NH_4)_2SO_4$. Cells (2 absorbance units at 600 nm) were collected by centrifugation and resuspended in 1 ml of the same medium with or without 10 μg of tunicamycin (Sigma Chemical Co.) per ml. After incubation at 25°C for 20 min, cells were harvested by centrifugation, washed once with water, and resuspended in 1 ml of minimal medium containing 0.1% glucose, without sulfate and with or without 10 μg of tunicamycin per ml. After 15 min of incubation at either 25 or 37°C, $^{35}SO_4^{2-}$ was added to 400 μCi/2 absorbance units of cells at 600 nm. Cultures were further incubated at the same temperature for 30 min, harvested by centrifugation, and washed with 10 mM NaN_3. For labeling in 5% glucose, cells were simply resuspended in fresh growth medium, without sulfate, and label was added as above. Labeled cell pellets were handled in one of two ways.

For rapid lysis, cells were resuspended in 50 μl of 1% sodium dodecyl sulfate (SDS), 0.15 g of glass beads was added, and samples were vortexed for 90 s, heated in a boiling water bath for 3 min, and diluted to 1 ml with 2% Triton X-100 in phosphate-buffered saline (12.5 mM, potassium orthophosphate [pH 7.0], 200 mM NaCl). For separation of cells into secreted and internal fractions, labeled cell pellets were resuspended in 200 μl of 1.4 M sorbitol–50 mM potassium orthophosphate (pH 7.5)–10 mM NaN_3–40 mM 2-mercaptoethanol–75 U of lyticase per absorbance unit at 600 nm (15). After 60 min at 30°C, spheroplasts were harvested by centrifugation for 10 min at 3,000 × *g*. Spheroplast supernatant fractions were adjusted to 0.5% SDS and heated in a boiling water bath for 3 min. Spheroplast pellets were resuspended in 50 μl of 1% SDS and heated in a boiling water bath for 3 min. Samples were diluted to 1 ml with 2% Triton X-100 in phosphate-buffered saline and pretreated with 50 μl and IgG Sorb (Enzyme Center), prepared as described by the manufacturer. After 30 min on ice, samples were sedimented for 20 min in a microfuge. Supernatant fractions were transferred to new tubes and immunoprecipitated with antibody directed against either invertase or β-galactosidase. Affinity-purified anti-invertase antibody (20 μl) (16) or 5 μl of anti-β-galactosidase antibody (an amount sufficient to precipitate >95% of the hybrid protein present in each sample) was added, and samples were incubated overnight at 4°C. IgG Sorb (30 μl) was added, and samples were incubated at 4°C for 30 min. Immune complexes were washed and solubilized as described elsewhere (40). Samples were then subjected to gel electrophoresis on 7.5% polyacrylamide-SDS slab gels according to the procedure of Laemmli (23). Gels were fixed, dried, and allowed to expose Kodak X-Omat AR film at −70°C.

Surface labeling of yeast cells with $Na^{125}I$ was done as described (31), except that Iodo-Beads (Pierce Chemical) were used instead of Iodogen in the iodination reactions.

Cell fractionation. Wild-type (M1-2Bα), *sec18* (SEY5186), *sec7* (SEY5076), and *sec1* (SEY5016) yeast cells carrying

TABLE 1. Strains and plasmids

Strains and plasmids	Genotype or relevant markers	Source or reference
S. cerevisiae		
M1-2Bα	*MATα ura3-52 trp1-289 SUC2*	41
SEY2101	*MATa ura3-52 leu2-3 leu2-112 suc2-Δ9 ade2-1*	12
SEY2102	*MATα ura3-52 leu2-3 leu2-112 suc2-Δ9 his4-519*	12
SEY5188	*MATα sec18-1 ura3-52 leu2-3 leu2-112 suc2-Δ9*	12
SEY5186	*MATα sec18-1 ura3-52 leu2-3 leu2-112 SUC2*	This study
SEY5076	*MATα sec7-1 ura3-52 leu2-3 leu2-112 SUC2*	This study
SEY5016	*MATα sec1-1 ura3-52 leu2-3 leu-112 SUC2*	This study
E. coli MC1061	F⁻ *araD139 Δ(araABOIC-leu)7679 ΔlacX74 galU galK rpsL hsdR*	10
Plasmids		
pSEY101	*lacZ*, *URA3*, 2μm circle DNA, *bla*	11
pRB58 (YEp13-*SUC2*)	*SUC2*, *URA3*, 2μm circle DNA, *bla*	7

SUC2-lacZ gene fusions on the plasmids pSEY110, pSEY119, or pSEY124 were grown in 500 ml of minimal medium containing 5% glucose. Cultures were grown at 25°C to an absorbance value at 600 nm of 1.0. Cells were collected by centrifugation (3,000 × *g*, 5 min) and resuspended in 500 ml of minimal medium at 37°C containing 0.1% glucose. The cultures were incubated for 2 h at 37°C, NaN_3 was added to 10 mM, and incubation was continued for 10 more min at 37°C. Cells were chilled on ice, washed once with 50 mM Tris (pH 7.5)–10 mM NaN_3, and converted to spheroplasts as described (see above). The remaining steps were carried out at 4°C. Spheroplasts were centrifuged (3,000 × *g*, 5 min) through 10 ml of 1.8 M sorbitol–50 mM Tris (pH 7.5)–1 mM NaN_3 onto 2 g of glass beads. The supernatant was discarded. Spheroplasts and glass beads were vortexed together for ca. 1 min. Glass beads were washed five times (2 ml each) with 0.5 M mannitol–50 mM Tris (pH 7.5)–1 mM NaN_3. The washes were pooled and centrifuged (800 × *g*, 10 min) to remove unlysed spheroplasts and whole cells. The cell-free lysate was centrifuged (12,000 × *g* 10 min) through 1 ml of 0.8 M mannitol onto 1 ml of 30% Percoll–0.5 M mannitol to isolate cellular organelles and membranes. The supernatant above the mannitol-Percoll interface was removed. The material at the mannitol-Percoll interface (membrane pellet) was resuspended in the Percoll remaining at the bottom of the centrifuge tube. This material was then fractionated on a self-forming Percoll gradient which permits resolution of yeast ER, Golgi, and secretory vesicle organelle fractions. Percoll and mannitol were added to the membrane pellet to 18% and 0.5 M, respectively (final volume, 8 ml). This solution was mixed, underlaid with 1 ml of 30% Percoll–0.5 M mannitol, and overlaid with 1 ml of 0.5 M mannitol. The Percoll density gradient was formed during centrifugation (39,000 × *g*, 30 min). Fractions (1 ml each) were collected with an auto Densi-flow 2c fractionator (Searle Buchler) from the top of the gradient (P. Esmon, unpublished data).

Yeast vacuoles were isolated from osmotically lysed spheroplasts on a discontinuous Ficoll gradient (40).

RESULTS

***SUC2-lacZ* gene fusion construction.** A set of gene fusions containing different-sized portions of the NH_2-terminal coding region for the yeast *SUC2* gene were fused in frame to a unique COOH-terminal segment of the *E. coli lacZ* gene. Initially, these fusions were constructed by using a number of different *lacZ* plasmids that were designed specifically for gene fusion construction both in *E. coli* and *S. cerevisiae*. We have limited discussion here to the use of a single plasmid, pSEY101 (11). The *E. coli*-yeast shuttle vector carries selectable markers (*bla* [Amp^r] and *URA3*) and DNA segments (ColE1 *ori* of pBR322 and the *FLP* region of the 2 μm circle DNA) that permit maintenance of the plasmid in both *E. coli* and *S. cerevisiae*. In addition, pSEY101 contains a *lacZ* gene lacking all of its 5′ regulatory sequences, as well as the coding sequence for the first eight amino acids of the *lacZ* gene product, β-galactosidase. In their place, restriction enzyme sites for *Eco*RI, *Sma*I, and *Bam*HI are present and unique in this vector.

The yeast *SUC2* gene has been cloned, and its entire nucleotide sequence has been determined (7, 42). The *SUC2* gene was isolated from a YEp13 yeast library. The plasmid carrying the *SUC2* gene, pRB58, contains a unique *Sal*I DNA restriction site that maps to a position ca. 3.5 kilobases to the 3′ side of the *SUC2* gene. Deletions that extend to various degrees into the COOH-terminal coding region of the *SUC2* gene were made by treating *Sal*I-restricted pRB58 plasmid DNA with the double-stranded exonuclease Bal 31. The appropriate digestion times for Bal 31 were determined empirically by sizing the digested DNAs on agarose gels (in general the rate of digestion was ~200 base pairs [bp]/min). The deleted plasmids next were restricted with *Eco*RI. This enzyme restricts at a site mapping ca. 900 bp to the 5′ side of the *SUC2* gene and thereby generates a series of fragments, all with an *Eco*RI site on one end and blunt or nearly blunt ends generated by the Bal 31 exonuclease at the other. These fragments were ligated directly into the *lacZ* fusion vector pSEY101, which had been digested with both *Eco*RI and *Sma*I (which generates blunt ends). Because of the orientation of these restriction sites and the method used to generate the deletions extending into *SUC2*, all of the *SUC2* gene fragments are inserted into pSEY101 such that both *SUC2* and *lacZ* are in the same orientation. The ligated plasmids were transformed into *E. coli* MC1061, and some 5,000 Amp^r transformants were isolated and pooled, and plasmid DNA was purified from the mixture of transformants. This DNA was used to transform the yeast strain SEY2102 to Ura$^+$. After 48 h of incubation, the overlaid regeneration agar containing the Ura$^+$ transformants was transferred onto pH-adjusted yeast 5-bromo-4-chloro-3-indolyl-β-D-galactoside indicator plates (35). Colonies containing active β-galactosidase turned blue on these plates in 24 to 48 h. These colonies were picked, and plasmid DNA was isolated from each and amplified by passage through *E. coli* before restriction mapping. All of the β-galactosidase-positive colonies analyzed (20) in this way were found to carry various-sized portions of the *SUC2* gene inserted in front of the *lacZ* gene. In addition, each of these 20 plasmids also was found to confer a β-galactosidase-positive phenotype to *E. coli*. We took advantage of this observation and directly screened for β-galactosidase-positive *E. coli* colonies derived by transforming the bacteria with the above-described pSEY101-*SUC2* DNA ligation mix. Restriction analysis of the β-galactosidase-positive *E. coli* transformants revealed that many (an additional 20) contained *SUC2* DNA inserted in front of the *lacZ* gene. When these constructs were transformed into *S. cerevisiae*, all 20 were found to confer a β-galactosidase-positive phenotype to the yeast cells. When tested for invertase expression however, none of the *SUC2-lacZ* gene fusions gave rise to detectable levels of invertase activity when present in a Δ*suc* yeast strain (SEY2102), indicating that in each of the hybrid constructs, invertase is inactive. The 40 total putative *SUC2-lacZ* gene fusions were divided by DNA restriction analyses into 16 distinct size classes, each containing slightly more invertase coding sequence inserted in front of the *lacZ* gene (pSEY125 through pSEY110; smallest to largest, respectively). For all further characterizations described below, six representative size classes of the *SUC2-lacZ* gene fusions were chosen (Fig. 1).

Expression and regulation of *SUC2-lacZ* gene fusions. The expression of secreted invertase from the yeast *SUC2* gene is subject to glucose regulation (7). We tested whether β-galactosidase activity expressed from the *SUC2-lacZ* gene fusions exhibits a similar pattern of control. The yeast strain M1-2Bα, harboring each of the *SUC2-lacZ* fusion plasmids (pSEY125, pSEY124, pSEY122, pSEY119, pSEY114, and pSEY110), was grown in minimal medium containing 4% glucose. The cells were shifted for 3 h into medium containing either 0.1% glucose or 4% glucose before the enzyme assays. In every case, the β-galactosidase activity expressed by these cells was repressed in medium containing high glucose and derepressed in medium containing low glucose

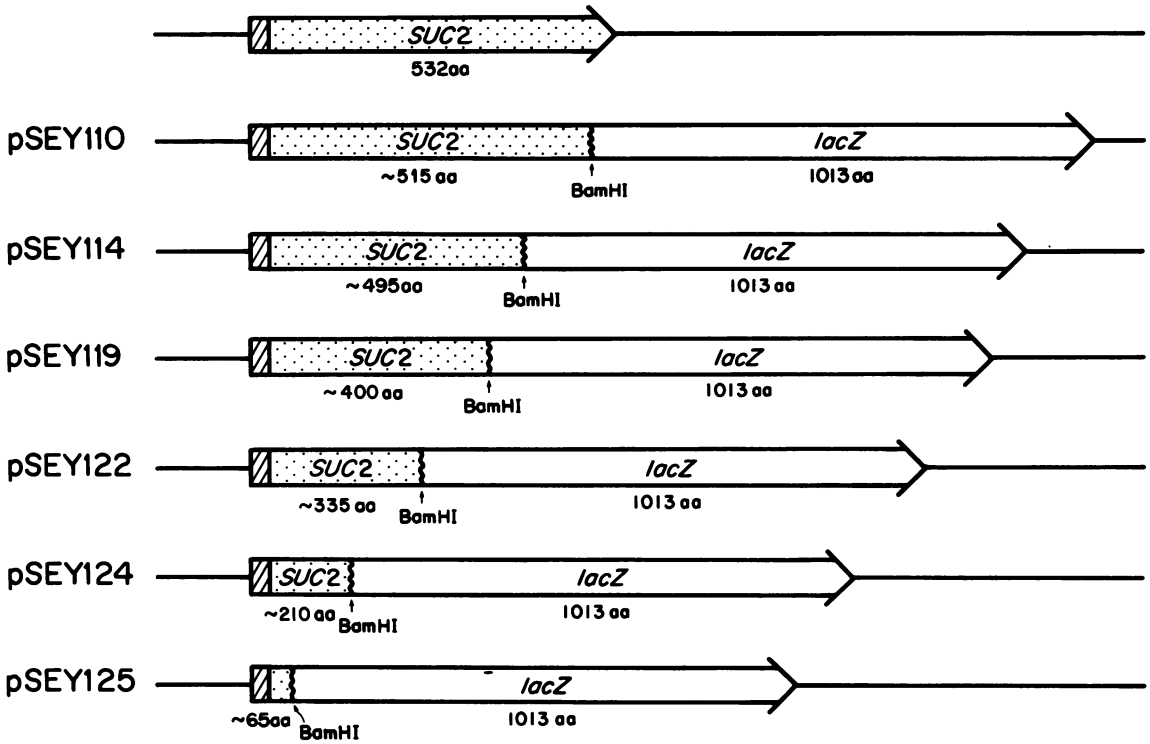

FIG. 1. *SUC2-lacZ* gene fusions. Six representative fusion size classes are shown. Each fusion is contained in the multiple copy *E. coli*-yeast shuttle vector pSEY101. The approximate number of amino acids coded for by the *SUC2* sequences present in each gene fusion is indicated. These numbers were determined by DNA restriction mapping each *SUC2-lacZ* gene fusion and comparing these maps to the known sequence of the *SUC2* gene. Hatched boxes indicate the portion of the *SUC2* gene that encodes the 19-amino-acid signal sequence. Stippled boxes correspond to *SUC2* sequences encoding mature invertase. *lacZ* sequences encoding an active COOH-terminal fragment of β-galactosidase are indicated by open boxes. Because of the method used to construct the *SUC2-lacZ* gene fusions, each fusion contains a *Bam*HI restriction site at the joint between *SUC2* and *lacZ* sequences (see the text).

(Table 2). The level of derepression was similar to that seen with invertase when it was expressed from a *SUC2* gene contained on the multiple-copy yeast plasmid pRB58 (Table 2). Indeed, even those *SUC2-lacZ* fusions that carried only the very NH_2-terminal coding sequence of *SUC2* fused to *lacZ* exhibited this regulation, suggesting that little, if any, of the *SUC2* structural gene was required for the catabolite control of *SUC2* gene expression. The variation seen in the total levels of β-galactosidase activity expressed by different size classes of *SUC2-lacZ* gene fusions probably was due to differences in the specific activity of β-galactosidase for the different hybrid proteins. The level of expression of each size class of hybrid proteins and their relative stabilities were similar (see below). Fusions containing a large amount of invertase coding sequence fused to *lacZ* encode hybrid proteins with the lowest levels of β-galactosidase activity.

TABLE 2. β-Galactosidase expression from *SUC2-lacZ* gene fusions

Plasmid[a]	β-Galactosidase activity (U per OD_{600})[b]		Derepression ratio
	4% Glucose	0.1% Glucose	
pSEY125	2	14	7
pSEY124	8	32	4
pSEY122	4	36	9
pSEY119	4.5	32	7
pSEY114	0.4	7	18
pSEY110	0.45	6	13
pSEY101	<0.02	<0.02	
pRB58[c]	150	1,200	8

[a] All plasmids were in the yeast strain M1-2Bα, except plasmid pRB58 which was in a Δ*suc* strain, SEY2102.

[b] U per OD_{600}, Enzyme activity units per one absorbance unit of cells at 600 nm.

[c] Values represent total invertase activity (units per OD_{600}). Cells were permeabilized by treatment with 0.2% Triton X-100 before assay.

SUC2-encoded invertase is made in two forms, an unglycosylated cytoplasmic form and a glycosylated secreted form (7, 9, 34). Only the secreted form of the enzyme is subject to glucose repression. The *SUC2-lacZ*-encoded hybrid proteins behaved in a similar way. Figure 2 demonstrates this for the invertase-β-galactosidase hybrid protein coded for by plasmid pSEY124. Yeast strain SEY2102 (pSEY124) was labeled with $^{35}SO_4^{2-}$ for 30 min in medium containing either 4 or 0.1% glucose. The labeled cells were lysed, extracts were treated with anti-invertase antibodies, and the immunoprecipitates were analyzed by electrophoresis in polyacrylamide slab gels containing SDS. Two forms of the invertase-β-galactosidase hybrid protein were detected in these cells. The protein band with the higher apparent molecular weight corresponded to the glycosylated hybrid protein, and the band with the lower apparent molecular weight corresponded to the unglycosylated hybrid (see below). Only expression of the glycosylated form of the hybrid protein was repressed by high glucose; the unglycosylated hybrid was expressed constitutively. Because this result was observed with all *SUC2-lacZ* gene fusions tested (pSEY124, pSEY119, and pSEY110), it supports the contention that catabolite control of the *SUC2* gene is directed by sequences mapping to the 5′ side of the structural gene.

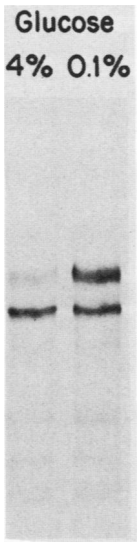

FIG. 2. Invertase-β-galactosidase hybrid proteins expressed in high- and low-glucose medium. Yeast cells (SEY2102) containing gene fusion plasmid pSEY124 were labeled with $^{35}SO_4^{2-}$ for 30 min in medium containing either high (4%) or low (0.1%) glucose. Cells were lysed with glass beads and subjected to immunoprecipitation with anti-invertase antibodies. Samples were then electrophoresed on a 6% polyacrylamide-SDS gel.

Finally, we determined whether regulation of a normal chromosomal copy of the *SUC2* gene was affected by the presence in the same cell of any of the *SUC2-lacZ* gene fusions on multiple-copy plasmids. For every fusion tested (pSEY125, pSEY124, pSEY122, pSEY119, pSEY114, and pSEY110), including the control plasmid pSEY101 which lacks all *SUC2* related sequences, we found that expression of the chromosomal *SUC2* gene was regulated normally (invertase expression was derepressd 100-fold by growth in low glucose [0.1%] medium for 3 h). Therefore, multiple copies of *SUC2* regulatory sequences do not appear to affect the glucose repressibility of a wild-type chromosomal *SUC2* gene. This was somewhat surprising since the wild-type *SUC2* gene, when present on the multiple-copy plasmid pRB58, did not exhibit this stringent glucose control (Table 2). Apparently, chromosomal and plasmid *SUC2* gene copies remain differentially regulated by glucose independent of the presence of one another in the same cell.

Hybrid proteins expressed from *SUC2-lacZ* gene fusions. The DNA restriction and expression data presented above all indicated that cells harboring the various *SUC2-lacZ* gene fusions were synthesizing hybrid proteins with invertase sequences at their NH_2 terminus and β-galactosidase sequences at their COOH terminus. To confirm this, cells containing the *SUC2-lacZ* gene fusions were labeled for 30 min with $^{35}SO_4^{2-}$ in medium containing 0.1% glucose. The cells were lysed by breakage with glass beads and subjected to immune precipitation with either anti-invertase or anti-β-galactosidase antisera. The same set of cells also were labeled in the presence of tunicamycin, a drug that blocks the synthesis of N-linked oligosaccharide chains. These cells were lysed and analyzed as described above. Figure 3 shows that each of the *SUC2-lacZ* gene fusions directed the synthesis of hybrid proteins. In the absence of tunicamycin, two protein bands were detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), whereas in the presence of the drug only the band with the lower apparent molecular weight was seen. This, together with data described above (Fig. 2), indicated that the higher-molecular-weight protein species represented in each case the glucose-regulated, glycosylated form of the protein. The lower-molecular-weight band corresponded to the constitutive, unglycosylated hybrid protein. There was good agreement between the predicted size of each of the hybrid proteins based on DNA restriction analyses and the observed molecular weight of the unglycosylated hybrids (Table 3). In addition, since each of the hybrid proteins could be immunoprecipitated specifically with either invertase or β-galactosidase-specific antisera, they contained both invertase and β-galactosidase protein sequences. With neither antisera was there evidence of rapid proteolytic breakdown of the hybrid proteins; they appeared to be quite stable in both the glycosylated and unglycosylated forms. Also, the level of synthesis of each of the hybrids was similar. We found that equivalent numbers of labeled cells gave rise to similar quantities of immunoprecipitable

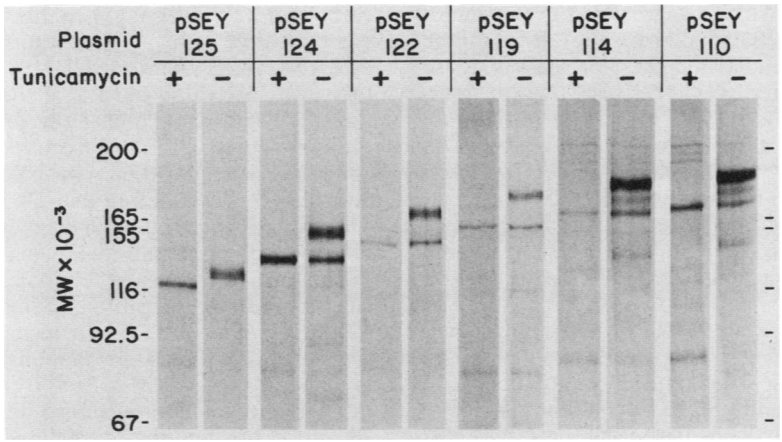

FIG. 3. *SUC2-lacZ* gene fusions direct the synthesis of hybrid proteins. Yeast strain SEY2102 harboring the indicated plasmids was labeled in low-glucose (0.1%) medium for 30 min with $^{35}SO_4^{2-}$ either in the presence (+) or absence (−) of tunicamycin (10 μg/ml). The labeled cells were lysed with glass beads before immunoprecipitation with anti-invertase sera and electrophoresis on a 6% polyacrylamide-SDS gel. Molecular mass markers include: myosin heavy chain (200 kd), *E. coli* RNA polymerase β (155 kd) and β′ (165 kd) subunits, phosphorylase *A* (92.5 kd), and bovine serum albumin (67 kd). MW, Molecular weight.

TABLE 3. Hybrid proteins encoded by *SUC2-lacZ* gene fusions

Plasmid	bp of invertase-coding sequence present in *SUC2-lacZ* gene fusions[a]	Predicted mol wt of *SUC2-lacZ*-encoded hybrid proteins[b]	Observed mol wt of invertase-β-galactosidase hybrid proteins[c]	
			Unglycosylated	Glycosylated[d]
pSEY125	~200	121,000	120,000	125,000 (2)
pSEY124	~640	137,000	135,000	150,000 (6)
pSEY122	~1,000	151,000	150,000	165,000 (8)
pSEY119	~1,200	158,000	155,000	175,000 (12)
pSEY114	~1,480	168,000	165,000	185,000 (12)
pSEY110	~1,550	171,000	170,000	190,000 (13)
WT *SUC2*	1,596			

[a] Based on restriction mapping of each plasmid with *Eco*RI, *Hin*dIII, and *Bam*HI.

[b] Based on an average molecular weight for each *SUC2* amino acid of 110. A molecular weight of 116,000 for β-galactosidase was used (18). All predicted molecular weights do not include the 19-amino-acid signal sequence of invertase. It was presumed that this is removed from the hybrid proteins during ER insertion (12).

[c] Molecular weights were obtained from the autoradiogram shown in Fig. 3. Measurements were made to the center of each hybrid protein band and compared with the indicated molecular weight markers. All molecular sizes are rounded off to the nearest 5 kd.

[d] The number of available potential *SUC2* glycosylation sites (asparagine X threonine/serine) present in the *SUC2-lacZ* fusions is indicated in parentheses for each fusion. Each core oligosaccharide unit attached to a protein leads to an apparent molecular size shift of ~2 to 3 kd.

hybrid protein, as detected by autoradiography (data not shown).

Cellular location of the invertase β-galactosidase hybrid proteins. Like wild-type invertase, the invertase-β-galactosidase hybrid proteins appeared to be synthesized in two forms: a cytoplasmic unglycosylated hybrid and a glycosylated, possibly secreted hybrid protein. Several experiments were performed to determine the location of the hybrid proteins. First, we found that yeast cells harboring the different gene fusions did not exhibit extracellular β-galactosidase activity even when grown in low-glucose (0.1%) medium (assays done with whole cells showed less than 5% of the β-galactosidase activity seen with lysed cells; data not shown). This was further demonstrated by the fact that these cells were unable to grow on lactose (the substrate of β-galactosidase) as the sole carbon source. If the β-galactosidase activity were extracellular (periplasmic), it should be available to cleave extracellular lactose to release glucose and galactose that then should permit cell growth. Apparently, the β-galactosidase activity was intracellular, or there was insufficient enzyme to allow even very slow growth on lactose. Several attempts were made to select for Lac^+ mutants; however, no such mutants were found. Apparently, these cells could not mutate to secrete the invertase-β-galactosidase hybrid proteins or mutate to transport lactose into the cell, where the β-galactosidase activity accumulated.

Secreted glycoproteins are accessible to cell surface labeling by ^{125}I (31); internal invertase is not. We tested to see whether the hybrid invertase-β-galactosidase proteins coded for by plasmids pSEY125 and pSEY110 could be labeled in this way. Although the yeast cells harboring these plasmids were efficiently labeled with ^{125}I, β-galactosidase-specific antisera did not detect any labeled hybrid protein in the cells. As a control, before ^{125}I labeling, yeast cells were permeabilized with 0.2% SDS and then, after labeling and lysis, extracts were treated with anti-β-galactosidase antibodies.

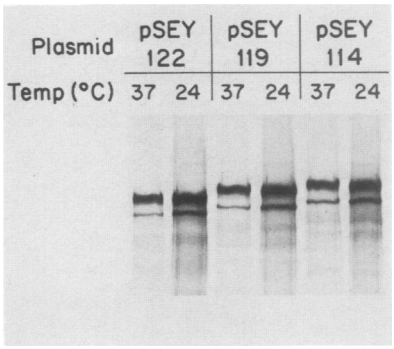

FIG. 4. Hybrid invertase-β-galactosidase proteins detected in a *sec18* temperature-sensitive ER-accumulating mutant. A *sec18* mutant (SEY5188) containing the indicated plasmids was labeled with $^{35}SO_4^{2-}$ for 30 min at the permissive (24°C) and nonpermissive (37°C) temperatures before hybrid protein immunoprecipitation with anti-invertase antibody and electrophoresis on an 8% polyacrylamide-SDS gel. The incorporation of radiolabel is approximately twofold higher at 24°C than at 37°C.

In this case, labeled hybrid protein was detected by SDS-PAGE (data not shown). This demonstrated that neither the invertase nor the β-galactosidase domains of the hybrid proteins encoded by either plasmid were exposed at the cell surface.

Further evidence that the hybrid invertase-β-galactosidase proteins were intracellular came from analysis of the glycosylation pattern. Wild-type invertase, during transit to the cell surface, is modified in the ER by the addition of N-linked core oligosaccharides, and then in the Golgi body large mannose-rich outer chains are added to the core oligosaccharides (15). This Golgi modification causes a dramatic shift in the apparent molecular weight of invertase. When evaluated by SDS-PAGE, core glycosylated invertase migrates as several discrete species which are converted to very diffuse, low-mobility forms by the addition of outer-chain carbohydrate. The hybrid proteins, unlike invertase, did not show this pattern (Fig. 3). Rather, the glycosylated form of the hybrid proteins migrated as fairly well-defined bands on SDS-PAGE. This indicated that the hybrids were only core glycosylated and did not receive outer-chain carbohydrate. Indeed, when the *SUC2-lacZ* gene fusions were placed into a temperature-conditional, secretion-defective yeast mutant (SEY5188) that accumulates secretory proteins within the ER at the nonpermissive temperature (34°C), we found that the immunoprecipitated hybrid proteins showed the same pattern of migration on polyacrylamide gels after labeling with $^{35}SO_4^{2-}$ at either the permissive (23°C) or nonpermissive (34°C) temperatures (Fig. 4). This implies that little or no modification beyond ER core oligosaccharide addition takes place.

The results are most consistent with the invertase-β-galactosidase hybrid proteins being initially inserted into the ER, core glycosylated, and terminally sequestered in this organelle. We analyzed the intracellular location of the invertase-β-galactosidase hybrid proteins by employing cellular fractionation procedures designed to isolate specific secretory organelles accumulated in different conditional secretion-defective yeast mutants (see above). Three plasmids carrying different *SUC2-lacZ* gene fusions (pSEY124, pSEY119, and pSEY110) were transformed into wild-type *S. cerevisiae* (M1-2Bα), an ER-accumulating mutant (SEY5186), a Golgi-accumulating mutant (SEY5076), and a

secretory vesicle-accumulating mutant (SEY5016). The mutant cells were derepressed for synthesis of the glycosylated hybrid proteins at 37°C for 2 h in 0.1% glucose medium before cell breakage and fractionation on Percoll density gradients. In addition to β-galactosidase activity being monitored, ER membranes were detected by assay of NADPH-cytochrome *c* reductase, and accumulated secretory organelles were assessed by assay of invertase expressed from the normal chromosomal *SUC2* gene. In each case tested, membrane-associated β-galactosidase activity was found to cofractionate with the ER and not with invertase contained in Golgi membranes or secretory vesicles (Fig. 5). Because ER membranes fractionated near the top of the gradient (lower density) at a position where yeast vacuoles also banded, we tested for the presence of β-galactosidase activity within yeast vacuoles by employing a separate fractionation procedure (see above). Only a very small fraction of the β-galactosidase activity (1%) could be detected in vacuole fractions that contained 17% of the vacuole marker enzyme α-mannosidase. This amount was well within the normally observed contamination of ER markers with vacuoles (40). Finally, by labeling cells with $^{35}SO_4^{2-}$ before breakage and fractionation on Percoll density gradients, we confirmed that not only the β-galactosidase activity but also, as expected, the hybrid invertase-β-galactosidase proteins themselves were detected by immunoprecipitation followed by SDS-PAGE only in the gradient fractions containing the ER and not in Golgi or secretory vesicle fractions (data not shown).

DISCUSSION

We characterized a series of gene fusions constructed between the yeast *SUC2* gene and the *E. coli lacZ* gene. Variable amounts of the *SUC2* NH_2-terminal coding sequence were fused in frame to a constant fragment of the *lacZ* gene (Fig. 1). The gene fusions encode active β-galactosidase in both *S. cerevisiae* and *E. coli*. In *S. cerevisiae* the β-galactosidase activity expressed from the various gene fusions is repressed by glucose in a way similar to that observed for invertase expression from a wild-type *SUC2* gene (Table 2). In addition, we demonstrated that the *SUC2-lacZ* gene fusions direct the synthesis of hybrid proteins in *S. cerevisiae* (Fig. 3). The hybrid invertase-β-galactosidase proteins can be immunoprecipitated specifically by antisera directed against either the invertase or the β-galactosidase portions of the hybrid proteins. The hybrid proteins are fairly stable in *S. cerevisiae* and migrate on polyacrylamide gels with apparent molecular weights similar to those expected based on DNA restriction mapping of the different sized gene fusions (Table 3).

A wild-type *SUC2* gene directs the synthesis of both a cytoplasmic unglycosylated form of invertase (lacking the NH_2-terminal signal peptide) and a secreted (periplasmic) glycosylated form of the enzyme (synthesized with the NH_2-terminal signal peptide) (7, 9, 34). The *SUC2-lacZ* gene fusions behave similar in that they all direct the synthesis of an unglycosylated, presumably cytoplasmic form of the hybrid invertase-β-galactosidase proteins and in that they also direct the synthesis of a glycosylated form of the hybrid protein. In addition, as with wild-type invertase, expression of the glycosylated form of the hybrid proteins is repressed in high-glucose-containing medium (Fig. 2). However, unlike invertase, the glycosylated hybrid proteins are not secreted. Rather, we have demonstrated by cell fractionation, by cell surface labeling with ^{125}I, and by growth studies with lactose medium that the invertase-β-galactosidase hybrid proteins

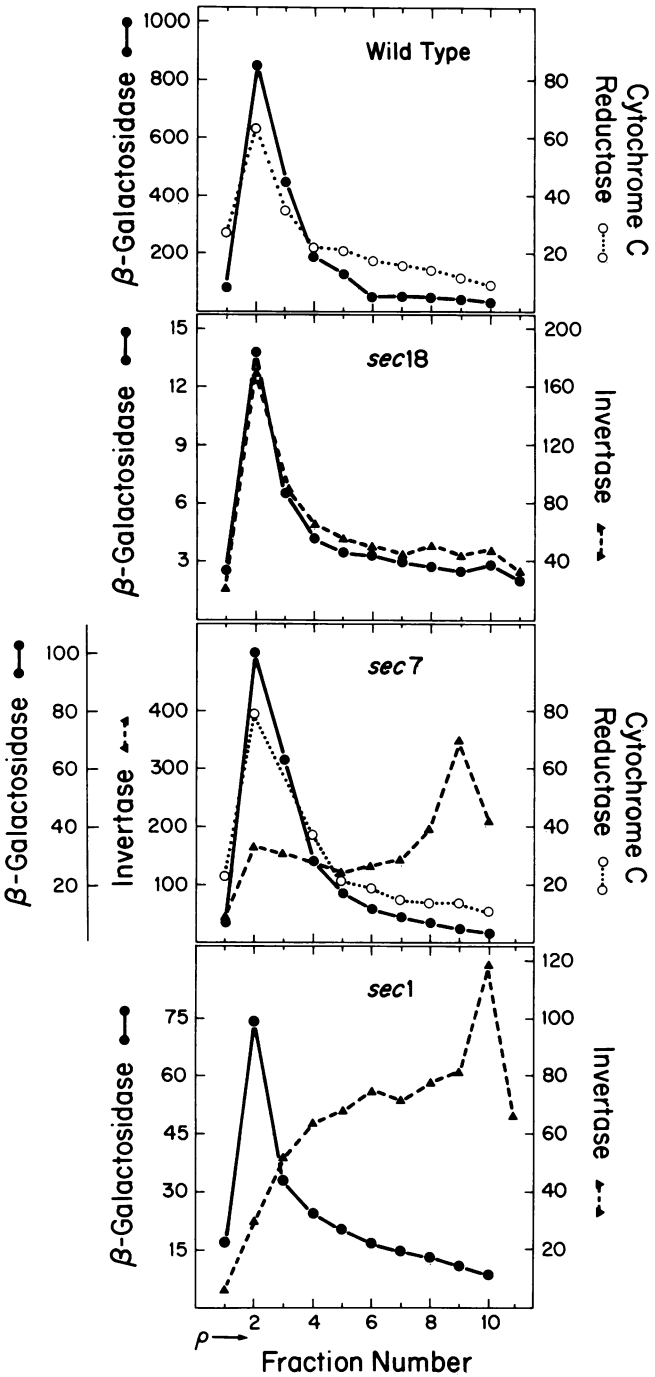

FIG. 5. Subcellular location of invertase-β-galactosidase hybrid proteins. Wild-type and temperature-conditional secretion-defective mutants that accumulate either ER (*sec18*), Golgi (*sec7*), or secretory vesicles (*sec1*) were transformed with plasmids pSEY124, pSEY119, and pSEY110. The cells were shifted to 37°C in 0.1% glucose medium for 90 min before enzymatic spheroplast formation and cell breakage with glass beads. Isolated cell membranes and organelles were loaded on top of 15% Percoll gradients. After centrifugation, 1-ml fractions were collected from the top of the gradient and used for subsequent enzyme assays. Invertase expressed from a wild-type chromosomal *SUC2* gene was used as a marker enzyme for the accumulated organelle in each *sec* mutant. NADPH cytochrome *c* reductase is an ER marker enzyme. Data are shown only for gradients run with *S. cerevisiae* cells containing plasmid pSEY124. Similar results were obtained with *S. cerevisiae* cells containing plasmid pSEY119 and pSEY110.

are located in the ER (Fig. 5). Subsequent steps of secretory protein delivery to the Golgi complex and then to secretory vesicles before release at the cell surface are apparently absent for the hybrid proteins. Even those hybrid genes that contain more than 90% of the *SUC2* structural gene fused to *lacZ* do not encode secreted hybrid proteins. It seems unlikely that these fusions lack the *SUC2* information responsible for allowing efficient secretion of invertase. Certain *lacZ* sequences may interfere with the targeting process. This has been observed already for the secretion of protein hybrids constructed between secretory proteins and LacZ in *E. coli* (38). In these cases, the hybrid proteins become jammed in the inner membrane of *E. coli*. It is assumed that it is the LacZ portion of the hybrid proteins that becomes jammed during translocation of the hybrids across the membrane. This also may be the case for the invertase-β-galactosidase hybrid proteins. Because the hybrid proteins are glycosylated and because N-linked oligosaccharide addition takes place on the lumenal side of the ER membrane, we can assume that, at least, the amino-terminal portion of the invertase-β-galactosidase hybrid proteins is being transferred into the lumen of the ER. Further experiments will be necessary to determine whether the hybrid proteins are being transferred completely into the ER lumen or if they become jammed during transmembrane transfer, as appears to occur in *E. coli*.

It has been proposed in the membrane trigger hypothesis that the signal sequence present at the NH_2 terminus of secretory proteins allows the protein to assume a conformation that permits it to penetrate a membrane without the assistance of a translocation machinery (44). One might expect that sequences mapping outside the NH_2-terminal signal peptide would have constraints on them so as to stabilize the membrane insertion conformation of a polypeptide. It appears unlikely that this model applies to the mechanism of invertase insertion into ER membranes. We substituted all but approximately the first 60 amino acids (including the 19-amino-acid signal sequence) of invertase with the completely heterologous cytoplasmic protein, β-galactosidase. This short invertase sequence is still competent both for insertion into the ER membrane and for glycosyl modification, suggesting that little NH_2-terminal sequence information is necessary for initiation of invertase translocation across the ER membrane. This is most consistent with the signal hypothesis model for protein secretion, which delegates the ER insertion information to the signal peptide of a secretory protein (5).

β-Galactosidase expressed from each of the *SUC2-lacZ* gene fusions is repressed in glucose-containing medium similarly to wild-type invertase when it is expressed from the cloned *SUC2* gene (Table 2). The sequences responsible for this catabolite control must be present in each of the gene fusions, even those fusions containing only a small amount of the *SUC2* structural gene. This suggests that the *SUC2* promoter and other regulatory sequences map to the 5′ side of the *SUC2* structural gene and are apparently completely contained in the 900 bp of 5′ *SUC2*-associated DNA present in each of the *SUC2-lacZ* gene fusions cnstructed. The regulatory molecule(s) responsible for glucose control of *SUC2* does not appear to be limiting in cells that contain multiple copies of *SUC2* regulatory sequences. We found that none of the *SUC2-lacZ* multicopy gene fusion-containing plasmids have any effect on the levels of invertase expressed or secreted in either high- or low-glucose medium from a chromosomal wild-type *SUC2* gene.

It is not clear why the *SUC2-lacZ*-encoded hybrid proteins remain associated with the ER. The hybrid proteins may be blocked in subsequent delivery steps because invertase misfolds in the hybrids, causing the proteins to aggregate and mask export signals present within the invertase molecule, thereby making them unavailable for binding to a specific receptor(s) or to a secretory protein packaging machinery (all of the hybrids characterized lack invertase activity). Whatever the reason, having the hybrid proteins accumulate within the ER does not seem to be detrimental to the cell. Other proteins continue to be secreted, and the cells grow normally. In this location, the hybrid invertase-β-galactosidase proteins should provide a convenient marker for the ER. The β-galactosidase activity associated with the hybrid proteins can be followed during ER isolation quite easily.

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