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# *GAL2* Codes for a Membrane-Bound Subunit of the Galactose Permease in *Saccharomyces cerevisiae*

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**The gene encoding the galactose permease of *Saccharomyces cerevisiae* (*GAL2*) was cloned. The clone restores galactose permease activity to *gal2* yeasts and is regulated by galactose in a manner similar to other *GAL* gene products (*GAL1*, *-7*, and *-10*). Experiments with temperature-conditional secretory mutants indicated that transport of the *GAL2* gene product to the cell surface requires a functional secretory pathway. In addition, gene fusions were constructed between the *GAL2* gene and the *Escherichia coli lacZ* gene. The *GAL2-lacZ* gene fusions code for galactose-regulated β-galactosidase activity in yeasts. The β-galactosidase activity was found to be membrane bound.**

In the yeast *Saccharomyces cerevisiae*, the uptake of galactose is mediated by a specific permease (15, 24) whose expression is regulated by galactose (27). We showed previously that the transport of galactose permease to the cell surface requires a functional secretory pathway (28). It is not clear, however, whether plasma membrane proteins are transferred through the same series of membrane-bounded structures as secretory proteins (8, 9, 19, 20) or whether there are branch points along the pathway, as is found for vacuolar carboxypeptidase Y (26). The galactose permease protein may contain specific information within its polypeptide chain that directs its delivery to the plasma membrane.

As a first step in characterizing export and assembly into the plasma membrane of galactose permease, we describe here the cloning of the *GAL2* gene, analysis of galactose permease export in temperature-conditional secretion mutants, and the construction and characterization of *GAL2-lacZ* gene fusions.

## MATERIALS AND METHODS

**Strains and media.** *Escherichia coli* MC1061 [*F' araD139* Δ(*lacIPOZYA*)*X74* Δ(*araABOIC-leu*)*7697 galK hsdR hsdM rpsL*] was provided by M. Casadaban. SE10 [*pyrF*::Tn*5 ara* Δ(*lac pro*) *rpsL thi* (φ80 d *lacZM15*)] was used as the host for subcloning. Initial cloning of the galactose permease was done in the host sf731-8B (*MATα leu2-3 leu2-112 pep4 prc1 cps1 gal2*). Biochemical studies were done with yeast strains SEY2102 (*MATα leu2-3 leu2-112 ura3-52 his4-519 suc2-Δ9 gal2*) (7), pSEY5016 (*sec-1*), pSEY5076 (*sec-7*), and pSEY5186 (*sec-18*), which were all *MATα leu2-3 leu2-112 ura3-52 gal2* (6), and CFY-1 (*MATa gal4 leu2-3 leu2-112 ura3-52*) and CFY-2 (*MATa gal80 leu2-3 leu2-112 ura3-52*). All strains were constructed by standard genetic methods (23).

*E. coli* was grown in LB medium (17), and ampicillin was added when required to a final concentration of 100 μg/ml. Bacterial indicator plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were prepared as described (17). Yeast cells were grown in YEP or synthetic minimal medium supplemented with the indicated carbon source (23). Yeast X-Gal indicator plates containing 2% galactose and 0.1% glucose were prepared as described by Rose et al. (22).

**Transformation and plasmid analysis.** Transformation of *E. coli* (16) and *S. cerevisiae* (1, 14) with plasmid DNA was done as described. A DNA library in the *E. coli*-yeast hybrid plasmid YEp13, prepared by partial *Sau*3A digestion of total genomic DNA from *S. cerevisiae* AB320 cloned into the *Bam*HI site of the vector, was a gift from K. Nasmyth (18). Small-scale preparation of plasmid DNA from *E. coli* and yeast spheroplasts was done by using an alkaline sodium dodecyl sulfate method (6). Vectors pSEY101 and pSEY8 used for subcloning are described elsewhere (5, 6; S. D. Emr, A. Vassarotti, J. Garrett, B. L. Geller, M. Takeda, and M. G. Douglas, manuscript submitted for publication). Restriction endonuclease and exonuclease digestions and ligations with T4 DNA ligase were done as recommended by the suppliers. DNA restriction digests were analyzed by electrophoresis in 50 mM Tris-borate (pH 8.3) and 2 mM EDTA on 1% agarose gels.

**Other procedures.** Galactose permease assays (24, 28) and β-galactosidase assays (17) were performed as described previously. Labeling of cells, immunoprecipitations with antisera directed against β-galactosidase, and electrophoresis on sodium dodecyl sulfate–7% polyacrylamide gels were done as described previously (6, 26). For rapid lysis, cells were suspended at $2 \times 10^9$ cells per ml in lysis buffer (50 mM Tris hydrochloride [pH 8.7], 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride), 0.5 g of glass beads per ml were added, and samples were vortexed four times for 1 min, with 1-min intervals of cooling on ice. The cell extract (crude extract) was centrifuged in an Eppendorf microfuge for 15 min (10,000 × *g*). The supernatant was removed, and the pellet (total membrane fraction) was suspended in lysis buffer.

## RESULTS

**A 2.55-kb DNA fragment containing the *GAL2* promoter and structural gene.** A YEp13-based gene bank made from partial *Sau*3A digests of total genomic DNA from a galactose-fermenting yeast strain, AB320 ($Gal^+$), was used to transform the $Leu^-$ *S. cerevisiae gal2* mutant strain sf731-8B. Approximately 18,000 $Leu^+$ transformants were screened on galactose as the sole carbon source in a second step for accelerated growth (compared to the *gal2* parent).

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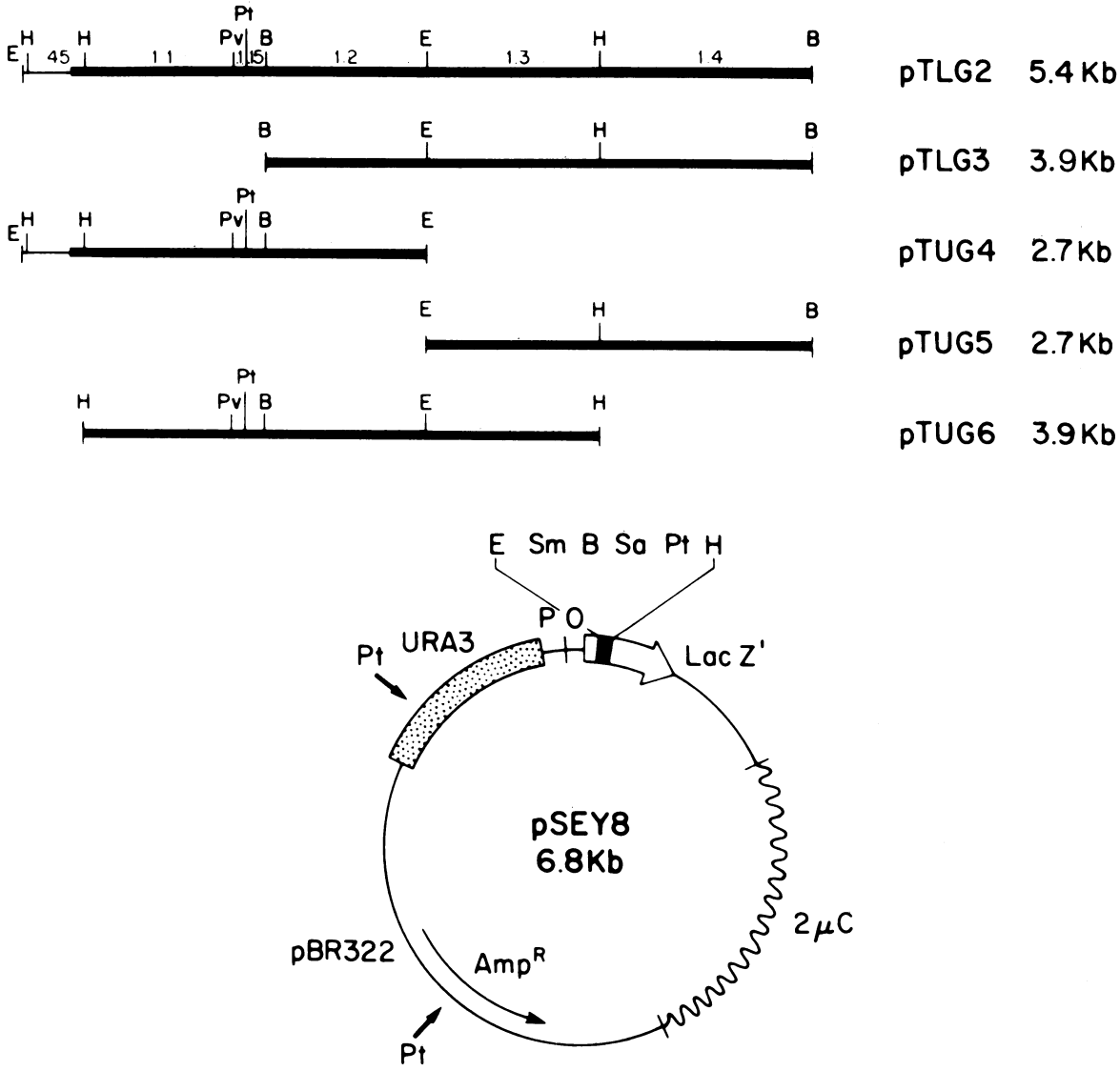


FIG. 1. Subcloning of *GAL2* into YEp13 (pTLG clones) and pSEY8 (pTUG clones). The 2.7-kb-pair DNA fragment of pTUG4 is the smallest subclone that complements the *gal2* mutation. pTLG2 is the original clone from the YEp13-based gene bank. pTLG3 is a YEp13-based *Bam*HI subclone. The *GAL2* gene was further subcloned into the polylinker of pSEY8, yielding pTUG4 (*Eco*RI subclone), pTUG5 (*Eco*RI-*Bam*HI subclone), and pTUG6 (*Hin*dIII subclone). Abbreviations: E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II; Pt, *Pst*I; B, *Bam*HI; Sa, *Sal*I; Sm, *Sma*I.

Of 12 faster-growing transformants, 2 were further characterized. Plasmid DNA was isolated from these transformants and subjected to restriction analysis. Both plasmids contained inserts with restriction maps that overlapped. The shorter insert was designated pTLG2 (Fig. 1) and was further used for subcloning into YEp13 and pSEY8. The 3.9-kilobase (kb) *Bam*HI fragment was subcloned into the unique *Bam*HI site of YEp13, yielding pTLG3. The 2.7-kb *Eco*RI fragment, the 2.7-kb *Hin*dIII fragment, and the 2.7-kb *Eco*RI-*Bam*HI fragment were all subcloned into the polylinker of pSEY8, yielding pTUG4, pTUG6, and pTUG5, respectively (Fig. 1). All subclones were used to transform the *gal2* yeast strain SEY2102 and tested for accelerated growth on galactose as the sole carbon source. Subclones pTUG4 and pTUG6, sharing a common 2.1-kb DNA fragment, confer a $Gal^+$ phenotype to this *gal2* mutant, while pTLG3 and pTUG5 do not.

Plasmid pTLG3 does not complement the *gal2* mutation in strain SEY2102. However, we found that this plasmid does rescue this strain to $Gal^+$ at high frequency. Strain SEY2102 reverts to $Gal^+$ at a frequency of $10^{-7}$. When this strain contains plasmid pTLG3, $Gal^+$ colonies are detected at a frequency of $3 \times 10^{-5}$. Apparently, the 1.2 kb pairs of DNA that pTLG3 shares in common with the complementary plasmid pTUG4 covers part of the *GAL2* gene and can recombine with it to give $Gal^+$ recombinants of strain SEY2102. Confirmation that the clone derives from the *GAL2* locus comes from the demonstration by Carle and Olson (4) that the 3.9-kb *Bam*HI fragment of pTLG2 hybridizes to chromosome XII, resolved by orthogonal-field-alternation gel electrophoresis. Taken together, these results strongly suggest that the above clones carry the chromosomal *GAL2* locus.

**Galactose permease induction in transformants comparable**

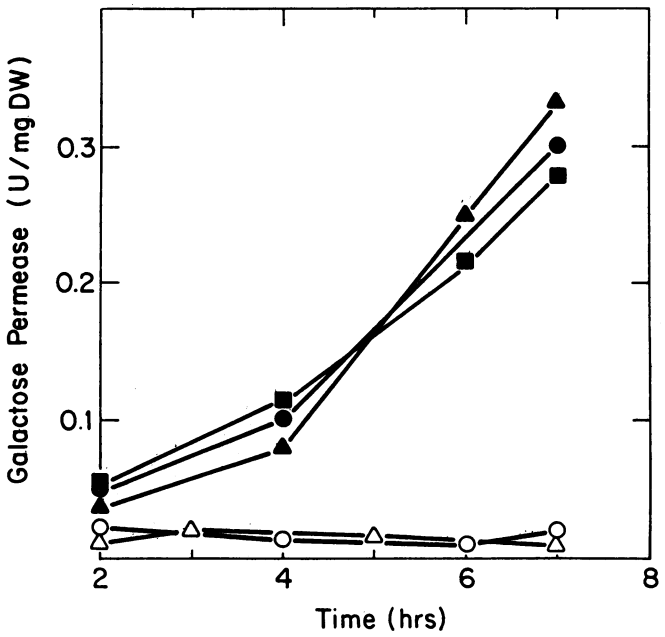


FIG. 2. Incorporation of galactose permease activity in strain SEY2102 carrying different *GAL2* subclones is indicated. Symbols: ●, pTLG2; ▲, pTUG4; ■, pTUG6; ○, pTLG3; △, pTUG5. Cells were grown at 24°C in minimal medium containing 2% glucose and 20 μg of leucine and histidine per ml. At time zero the cells were transferred into minimal medium containing 2% galactose and 20 μg of leucine and histidine per ml, and incubation was continued at 24°C. At the time indicated in the figure, cell samples were withdrawn and assayed for galactose permease as described previously (24, 28).

**to wild type.** All subclones were further tested in a transport assay for the ability to express a functional galactose permease. Subclones pTUG4 and pTUG6 containing the 2.1-kb region were able to express galactose permease activity in the *gal2* mutant strain SEY2102 when shifted into a galactose-containing medium (Fig. 2). Overexpression of galactose permease activity is not detected in strains harboring these clones on the multicopy plasmids YEp13 or pSEY8. The specific galactose permease activity was comparable to wild-type strain AB320. Sodium dodecyl sulfate-gel analysis of crude extracts and plasma membrane fractions (unlabeled or $^{35}SO_4^{2-}$ labeled) of cells grown on glucose or galactose did not permit detection of a candidate protein band corresponding to galactose permease (data not shown).

TABLE 1. Galactose permease activity is blocked in *sec* mutants[a]

| Strain | *sec* group | Galactose permease activity (U/mg [dry wt]) at: | | |
|---|---|---|---|---|
| | | 2 h at 24°C | 2 h at 37°C | 4 h at 37°C → 24°C |
| SEY5016 | 1 | 0.43 | 0.073 | 0.45 |
| SEY5076 | 7 | 0.61 | 0.17 | 0.52 |
| SEY5186 | 18 | 0.57 | 0.064 | 0.40 |

[a] Cells were grown at 24°C in YP medium with 2% ethanol for three to four generations, transferred to YP medium with 0.5% galactose and 0.05% glucose, and shifted to 37°C or kept at 24°C. At 2 h, the 37°C culture was shifted to 24°C for another 2 h with cycloheximide (0.2 mg/ml). At the end of each experiment, cells were chilled on ice and washed with 1 ml of ice-cold 20 mM sodium azide. The cells (10 optical density units at 600 nm) were then used to perform the galactose permease assay (24, 28).

**Plasmid-expressed galactose permease requiring a functional secretory pathway for transport to the cell surface.** Conditional mutants defective at different stages in the secretory pathway (20) were used to construct *gal2-sec* double mutants (6). These mutants were used to test whether galactose permease expressed from a plasmid-borne *GAL2* gene requires a functional yeast secretory pathway for its localization to the cell surface.

In galactose-fermenting yeast cells grown for two to three generations in a nonfermentable carbon source, such as ethanol, galactose permease and other *GAL* genes are induced in 15 min after a shift into a galactose-containing medium (28). A similar induction was observed at a permissive growth temperature (24°C) in *gal2-sec* double mutants (6) harboring pSEY8 with the 2.7-kb-pair *Eco*RI subclone (pTUG4) as an insert. However, all *gal2-sec* mutants failed to show this activity during a 2-h induction at a nonpermissive growth temperature (37°C). Upon return to the permissive temperature in the presence (or absence) of cycloheximide, galactose permease activity was detected in the *gal2-sec* mutants (Table 1).

**Expression of *GAL2-lacZ* gene fusions regulated by galactose.** The gene fusion approach in *S. cerevisiae* has been employed successfully to study various features of molecular events in the process of secretion (2, 3, 6, 7) import into the mitochondria (5, 12, 13) and into the nucleus (11, 25). We

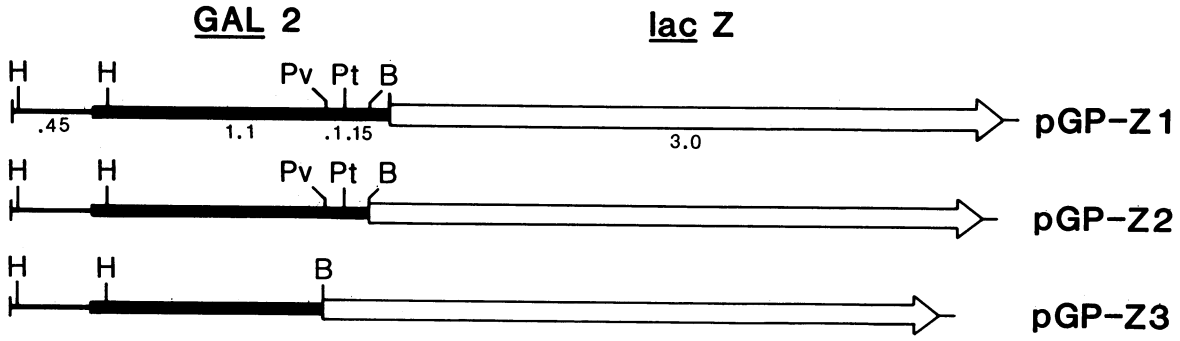


FIG. 3. Gene fusions between *GAL2* and *lacZ*. pGP-Z1 was obtained from a library of truncated DNA fragments ligated into the *Eco*RI-*Sma*I linker of pSEY101. pGP-Z2 and pGP-Z3 were obtained by subcloning of the *Eco*RI-*Bam*HI or *Eco*RI-*Pvu*II fragment into the *Eco*RI-*Bam*HI or *Eco*RI-*Sma*I sites of pSEY101, respectively. This generated uninterrupted open reading frames with the *lacZ* gene. Abbreviations are as in the legend to Fig. 1.

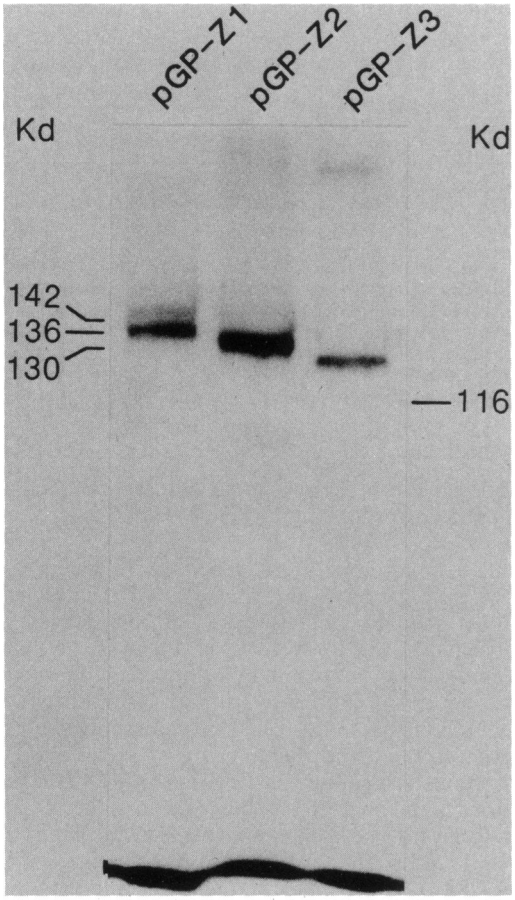


FIG. 4. Identification of the *GAL2-lacZ* hybrid proteins in yeast. Yeast strain SEY2102 harboring the indicated plasmids was grown to mid-logarithmic phase under uracil selection in 2% glucose. Cells were shifted into 2% galactose, incubated for 2 h, labeled for 1 h with $^{35}SO_4^{2-}$, and processed for immunoprecipitation. See the text for further explanations.

chose this approach for the following reasons: (i) to determine the direction of transcription of the *gal2* gene and (ii) to study the delivery of galactose permease to the plasma membrane. pSEY101, an *E. coli*-yeast shuttle vector carrying a truncated *lacZ* gene, was used for construction of the *GAL2-lacZ* gene fusions (5). Vector pTUG4, a derivative of vector pSEY8 containing the *GAL2* gene within the 3-kb-pair DNA *Eco*RI fragment, was digested with *Sal*I, treated with BAL31, and digested with *Eco*RI. The library of truncated DNA fragments was ligated into the *Eco*RI-*Sma*I site of the *lacZ* fusion vector pSEY101, which resulted in several potential fusions of the *GAL2* gene to *lacZ*, one of which is shown in Fig. 3 (pGP-Z1). The other two fusions, pGP-Z2 and pGP-Z3, were obtained by subcloning of the *Eco*RI-*Bam*HI or *Eco*RI-*Pvu*II fragment into the polylinker of vector pSEY101. When yeast is transformed with pGP-Z1, -2, and -3, β-galactosidase expression can be detected on plates with the chromophoric substrate X-Gal or by liquid assays. The level of β-galactosidase expression directed by the *GAL2-lacZ* hybrid genes was determined after growth in either glucose or galactose in *gal2*, *gal80*, and *gal4* mutant strains (Table 2). The steady-state levels of β-galactosidase increased up to three orders of magnitude when cells were shifted from glucose into galactose, suggesting a tight control of regulation. The fact that in *gal4* mutants only low and in

TABLE 2. Expression of β-galactosidase by *GAL2-lacZ* fusions is regulated by galactose

| Plasmid[a] | Enzyme activity[b] and growth conditions[c] | | | |
|---|---|---|---|---|
| | *gal2* | | *gal80*, 0.1% glucose | *gal4*, 2% galactose |
| | 0.1% Glucose | 2% Galactose | | |
| pGP-Z1 | 0.1 | 283.0 | 158.0 | 0.1 |
| pGP-Z2 | 0.07 | 154.0 | 120.0 | 0.1 |
| pGP-Z3 | 0.08 | 189.0 | 171.0 | 0.15 |
| pSEY101 | 0.03 | 0.03 | 0.02 | |

[a] Plasmids are in host SEY2102 (*gal2*), CFY-1 (*gal4*), and CFY-2 (*gal80*).
[b] 1 Optical density unit at 600 nm of cells was withdrawn from the culture, washed with 1 ml of water, and assayed in Z buffer in the presence of chloroform and sodium dodecyl sulfate. Activity is expressed as nanomoles of *O*-nitrophenyl-β-D-galactoside hydrolyzed per minute per optical density unit at 600 nm of cells at 30°C.
[c] All cultures were grown at 30°C to an absorbance of 1 to 2 at 600 nm in minimal medium (yeast nitrogen base) supplemented with leucine, histidine, and adenine, but lacking uracil to maintain selection for the plasmid. In 2% galactose media, sufficient galactose enters the cells to induce *GAL* gene expression.

*gal80* mutants constitutive expression of β-galactosidase were found suggests that regulation of the *GAL2* promoter is similar to other *GAL* promoters (21, 27).

**GAL2-lacZ fusions direct the synthesis of hybrid proteins.** The β-galactosidase expression studies described above for yeast cells harboring the plasmids pGP-Z1, -2, and -3 all indicate that such cells are synthesizing a hybrid galactose permease–β-galactosidase protein. We tested for the presence of such a hybrid protein by first radioactively labeling SEY2101 cells (carrying pGP-Z1, -2, -3) with $^{35}SO_4^{2-}$, followed by immunoprecipitation with antiserum directed against β-galactosidase. The antisera specifically recognize proteins with apparent molecular weights of 130,000 to 142,000 on sodium dodecyl sulfate-polyacrylamide gels (Fig. 4). The molecular weight of β-galactosidase is 116,000, suggesting that in the fusions pGP-Z1, pGP-Z2, and pGP-Z3 14,000, 20,000, and 26,000 daltons of galactose permease are present, respectively. A $^{35}SO_4^{2-}$ labeling experiment performed with pGP-Z2 in the presence of tunicamycin, an inhibitor of asparagine N-linked glycosylation (6), revealed a fusion protein identical in molecular weight to that in the absence of the drug, suggesting that the N-terminal portion of galactose permease does not carry N-linked glycosyl residues (data not shown).

**Cellular location of the *GAL2-lacZ* hybrid gene product.** Attempts have been made to determine the location of β-galactosidase activity in yeast cells expressing *GAL2-lacZ* gene fusions. As a control in this analysis, soluble β-galactosidase activity expressed from the plasmid carrying the *CYC1-lacZ* gene fusion pLG669Z (10) and endoplasmic reticulum-associated β-galactosidase activity expressed from a *SUC2-lacZ* gene fusion pSEY124 (6) were also monitored. To compare the subcellular distribution of β-galactosidase activity in each of the above cases, total membrane fractions and plasma membrane fractions from induced cells were isolated. Total membrane fractions contained a high yield of β-galactosidase activity (86 to 89%) for the *GAL2-lacZ* gene fusions, and a lower but significant level of β-galactosidase activity (40%) was associated with this fraction for the *SUC2-lacZ* fusion (Table 3). The *CYC1-lacZ* peptide is soluble. Qualitatively, the same results were found in plasma membranes isolated as described earlier (29). Plasma membrane-enriched fractions contained a high yield

TABLE 3. β-Galactosidase activity expressed from *GAL2-lacZ* gene fusions copurifies with a membrane fraction enriched for plasma membranes[a]

| Plasmid | Fusion | Crude extract [U/10$^7$ cells (%)] | Total membrane fraction [U/10$^7$ cells (%)] | Plasma membrane fraction [U/10$^7$ cells (%)] |
|---|---|---|---|---|
| pGP-Z1 | *GAL2-lacZ* | 78.6 (100) | 67.6 (86) | 20.3 (26) |
| pGP-Z3 | *GAL2-lacZ* | 46.9 (100) | 41.7 (89) | 9.9 (21) |
| pSEY124 | *SUC2-lacZ* | 8.4 (100) | 3.4 (40) | 1.1 (13) |
| pLG669Z | *CYC1-lacZ* | 210 (100) | 10.5 (5) | 0.8 (0.4) |

[a] Cells were grown in yeast nitrogen broth containing 20 μg of histidine and leucine per ml and 2% glucose to mid-log phase at 30°C. Cells were then shifted to yeast nitrogen broth containing 20 μg of histidine and leucine per ml and 2% galactose for 5 h at 30°C. Plasma membranes were prepared as described in reference 29 and refer to the membrane fractions from the Renografin density gradient. Total membrane fractions were prepared as described in the text.

of β-galactosidase activity (20 to 26%) for the *Gal2-lacZ* gene fusions and a lower but significant level of β-galactosidase activity associated with this membrane fraction (13%) for the *SUC2-lacZ* fusion (Table 3). The *CYC1-lacZ* fusion peptide is totally soluble. This experiment shows that the *GAL2-lacZ* hybrid protein is strongly associated with a membrane fraction enriched for plasma membranes.

Although the *GAL2-lacZ* fusion proteins are largely associated with a membrane fraction enriched for plasma membranes, induced cells carrying *GAL2-lacZ* fusion plasmids do not show a Lac$^+$ phenotype in that they do not have external β-galactosidase activity, nor can they form individual colonies on plates with lactose as the sole carbon source. As found for *SUC2-lacZ* gene fusions (6), *GAL2-lacZ* fusion proteins might not be localized correctly and instead be retained in the endoplasmic reticulum, some of which may copurify in the plasma membrane fraction (29).

## DISCUSSION

The yeast *GAL2* gene has been cloned by complementation of a yeast *gal2* mutant. Evidence to support this includes: (i) the clone restores galactose permease activity to *gal2* mutant yeast; (ii) a noncomplementing DNA fragment of the *GAL2* clone can rescue a *gal2* mutant to Gal$^+$ phenotype; and (iii) regulation of the cloned gene mimics that of other previously characterized *GAL* genes. Furthermore, our results indicate that the *GAL2* gene is the structural gene for the galactose permease. Fusions of *GAL2* to *lacZ* direct the synthesis of a galactose-regulated β-galactosidase activity which is membrane associated. Data presented here demonstrate that the *GAL2* gene, like the *GAL1*, *GAL10*, and *GAL7* genes, has no detectable basal level of expression under noninducing conditions. The wild-type *GAL4* function has been shown to be necessary for the expression of induced levels of *GAL2*, and the wild-type *GAL80* function has been shown to be required for repression of *GAL2* in the absence of galactose, showing an interplay of the *GAL4* and *GAL80* proteins to be a central feature of *GAL2* regulation.

Also, the β-galactosidase activity expressed from hybrid *GAL2-lacZ* genes is membrane associated, which is consistent with *GAL2* coding for a permease. Among a large number of *gal* mutants, no candidate for the galactose permease other than *gal2* has been reported. This suggests that the *GAL2* gene probably codes for a functional galactose permease. *GAL2* function is not required for galactose control of the *GAL2-lacZ* gene fusions. In view of these data, it is unlikely that the cloned *GAL2* sequences encode a regulatory protein whose expression is required for production of galactose permease activity.

Delivery of the galactose permease to the cell surface requires a functional secretory pathway. A permease intermediate accumulated at the nonpermissive temperature in secretory mutants is reversibly incorporated into the plasma membrane upon return to the permissive temperature. The endoplasmic reticulum, the Golgi apparatus, and secretory vesicles are essential intracellular organelles for transport of galactose permease.

Gene fusions pGP-Z1, pGP-Z2, and pGP-Z3 direct the synthesis of hybrid proteins. Properties related to the expression and synthesis of these hybrid gene products have been analyzed. Expression of β-galactosidase from the hybrid genes are regulated by galactose in a manner similar to that observed for the wild-type *GAL2* gene, other *GAL* genes (27), and *MEL1* (5). The gene fusions direct the synthesis of hybrid proteins of 130, 138, and 142 kilodaltons, which are recognized specifically by antiserum directed against β-galactosidase. Using the published molecular weight of β-galactosidase (116,000) as a reference, these hybrids contain about 14, 22, and 26 kilodaltons of *GAL2* N-terminal protein sequence, respectively. Radiolabeling of fusion proteins in the presence of tunicamycin did not alter their apparent molecular weights, suggesting the absence of N-linked oligosaccharides at the N-terminal portion of galactose permease. An RNA species of ca. 2,000 bases was observed on Northern blots of total RNA from galactose-induced cells using the *GAL2* gene as a probe (M. E. Digan, personal communication). This transcript could accommodate a 60-kilodalton polypeptide chain.

Galactose permease, as judged by activity assays, expressed from a 2μ circle-based plasmid (present at multiple copies per yeast cell) is not more abundant than in wild-type cells where the gene is expressed from one copy. Hence, the *GAL2* clone may not be of assistance in the purification of functional permease from yeast cells. However, isolation of the hybrid protein with the goal of raising antibodies directed against the galactose permease, construction of other gene fusions, and sequence information of the *GAL2* gene should permit a more detailed study of galactose permease biogenesis.

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