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Isolation and Characterization of Dominant Mutations Resistant to Carbon Catabolite Repression of Galactokinase Synthesis in *Saccharomyces cerevisiae*

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Seven dominant mutations showing greatly enhanced resistance to the glucose repression of galactokinase synthesis have been isolated from *GAL81* mutants, which have the constitutive phenotype but are still strongly repressible by glucose for the synthesis of the Leloir enzymes. These glucose-resistant mutants were due to semidominant mutations at either of two loci, *GAL82* and *GAL83*. Both loci are unlinked to the *GAL81- gal4*, *gal80*, or *gal7 · gal10 · gal1* locus or to each other. The *GAL83* locus was mapped on chromosome V at a site between *arg9* and *cho1*. The *GAL82* and *GAL83* mutations produced partial resistance of galactokinase to glucose repression only when one or both of these mutations were combined with a *GAL81* or a *gal80* mutation. The *GAL82* and *GAL83* mutations are probably specific for expression of the Leloir pathway and related enzymes, because they do not affect the synthesis of α-D-glucosidase, invertase, or isocitrate lyase.

In the on-off control of the *lac* operon in *Escherichia coli*, there is dual control of enzyme synthesis (9): negative control by a repressor coded by the *lacI* gene, and positive control by a complex molecule consisting of a specific catabolite gene activator protein (CAP protein) and cyclic adenosine 3',5'- monophosphate. The repressor detects the presence or absence of β-galactoside in the cytoplasm, and the CAP protein detects glucose through the alteration of the intracellular level of cyclic adenosine 3',5'-monophosphate. These signals are conveyed to the appropriate sites in the promoter region of the *lac* operon.

Glucose repression or carbon catabolite repression is also commonly observed in yeasts. Recent biochemical studies clearly demonstrate that glucose repression of cytochrome *c* synthesis in *Saccharomyces cerevisiae* is at the level of gene transcription (32, 33), whereas the contribution of cyclic adenosine 3',5'-monophosphate is, in general, ambiguous in yeasts. To elucidate the genetic mechanism of carbon catabolite repression in yeast, mutants with altered regulatory properties have been isolated and characterized by several workers. These include mutations producing resistance to carbon catabolite repression in invertase synthesis (15, 24), pleiotropic mutations conferring resistance to carbon catabolite repression (5, 6), the *hex* mutation (13) with reduced activity of glucose phosphorylation, which contributes to carbon catabolite repression, and the *ADR* mutations (4, 7) in a regulatory system for glucose repression of alcohol dehydrogenase synthesis. The genetic data, however, are still insufficient to construct a genetic model for carbon catabolite repression.

In previous communications (22, 23), we proposed a genetic model for the role of the inducer in the synthesis of the galactose pathway enzymes (the Leloir enzymes) in *S. cerevisiae*. The structural genes for the Leloir enzymes, the *gal1* locus (encodes galactokinase [EC 2.7.1.6]), *gal7* (encodes α-D-galactose-1-phosphate uridylyltransferase [EC 2.7.7.12]), and *gal10* (encodes uridine diphosphoglucose 4-epimerase [EC 5.1.3.2]) (11), are tightly linked to each other in the order centromere-*gal7-gal10-gal1* on chromosome II (3). These clustered genes are coordinately controlled by a positive and a negative cytoplasmic factor which are coded and constitutively produced by the *GAL81-gal4* and *gal80* genes, respectively. These factors interact in the cytoplasm or nucleoplasm and convey signals of the presence or absence of inducer (galactose) to the structural genes. However, activity of the Leloir enzymes in *S. cerevisiae* also shows carbon catabolite regulation, (1, 21, 31). Presumably there is a control circuit conveying the signals due to the presence or absence of glucose or other carbon catabolite effectors to the structural genes.

Synthesis of galactose transport protein, which is coded for by *gal2* (8, 10), is probably controlled coordinately with the Leloir enzymes under the same *GAL81-gal4* and *gal80* genes (V.

P. Cirillo, unpublished data [see 18]). Although there is no clear evidence on glucose repression of the *gal2* expression, Matern and Holzer (21) reported that, in the galactose-utilizing system, galactose uptake is the most sensitive to carbon catabolite inhibition, whereas galactokinase is insensitive to glucose inhibition but sensitive to glucose repression (1). Glucose inhibition of galactose uptake would prevent intracellular accumulation of galactose and thus prevent induction of the galactose transport protein and the Leloir enzymes. To overcome this difficulty in the isolation of mutants affecting carbon catabolite repression in the *gal* system, we tested *GAL81* mutants, which have the constitutive phenotype for production of the Leloir enzymes (12, 22) and possibly for the galactose transport protein.

This paper describes seven independent dominant mutations which define two galactose-specific genes, *GAL82* and *GAL83*. Mutations in either of these genes give rise to decreased sensitivity to carbon catabolite repression in the synthesis of galactokinase, but only when the mutations are combined with the constitutive *GAL81* or *gal80* mutation, or with both. The expression of the *gal* structural genes requires a certain amount of intracellular galactose, a *GAL81* mutation, or a *gal80* mutation, even in the *GAL82* and *GAL83* mutants.

MATERIALS AND METHODS

Yeast strains. Genotypes and sources of the yeast strains used are listed in Table 1.

Media. General usage and composition of nutrient, minimal, EBGal (20 g of polypeptone, 10 g of yeast extract, 20 g of galactose, and 20 mg ethidium bromide dissolved in and made up to 1 liter with distilled water), YPGly (20 g of polypeptone, 10 g of yeast extract, and 20 g of glycerol dissolved in and made up to 1 liter with distilled water), YPGlyGal (20 g of galactose added per liter of YPGly), YPEth (5 g of ethanol per liter replaced the glycerol in YPGly), and YPEthGal (2 g of galactose added per liter of YPEth) media were described previously (23, 27). YPGlu medium contained 50 g of glucose, 20 g of polypeptone, and 10 g of yeast extract per liter. YPGluGal medium was YPGlu medium plus 10 g of galactose per liter. YPGluGly medium contained 20 g of glycerol per liter of YPGlu medium. YPGluGlyGal medium was YPGluGly medium plus 20 g of galactose per liter. YPGluGlyMal medium was YPGluGly medium plus 20 g of maltose per liter.

Selection of mutants. Respiratory competent cells of a *gal7* mutant cannot grow on YPEthGal medium, but can grow on YPEth medium because they are sensitive to galactose (11). However, the same mutant can grow on YPGluGal medium. This can be explained by the impaired expression of galactokinase in the presence of glucose or inhibition of galactose uptake by glucose. Catabolite repression-resistant mutants in galactokinase synthesis can be isolated by picking up colonies which fail to grow on YPGluGal medium. As discussed above, a *GAL81* mutation was included with the *gal7* mutation in the parent strains in the mutant isolation. The *GAL81 gal7* double mutants could gorw on YPGluGal medium. This is because glucose inhibits the galactose uptake system or catabolite repression is still effective in the *GAL81* cells or both. Mutagenesis was performed with ethyl methane sulfonate as described by Lindegren et al. (19). The ethyl methane sulfonate-treated cells were inoculated into many test tubes, each with 5 ml of YPGlu medium, to give approximately 10^4 surviving cells per ml, and the tubes were shaken at 30°C for 2 days. Then each culture was diluted 10-fold with fresh YPGluGal medium. After incubation of the culture of 7 h at 30°C, 10 μg of nystatin per ml (30) was added, and the culture was further incubated for 90 min. The cells were washed and plated on YPGlu medium after an appropriate dilution. Since the galactose effect is not fatal for the *gal7* mutants, whereas it is for the epimerase less *gal10* mutant cells tested so far, the nystatin screening method was effective for enrichment of catabolite-resistant mutants. The colonies which failed to grow on the YPGluGal plates were isolated and purified by repeated spreading on nutrient plates. Only one mutant was saved from each of the original subcultures to ensure the independence of the mutants.

Assay of enzyme activities. For assay of galactokinase and α-D-glucosidase (EC 3.2.1.20) activities, cells permeabilized for the substrates were prepared with dimethyl sulfoxide (2). During the logarithmic phase of cell growth, 1 ml of culture was taken and centrifuged. The cell pellet was chilled on ice, and 0.5 ml of 40% (vol/vol) dimethyl sulfoxide solution in distilled water was added. The suspension was incubated at 30°C for 20 min. Then cells were washed twice with 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8) as described previously (27). This suspension was used as an enzyme source for the galactokinase assay. Methods for galactokinase assay using cells permeabilized to the substrate were described previously (27). One unit of enzyme activity was defined as the amount which catalyzed the conversion of 1 μmol of galactose to galactose-1-phosphate per h under the specified conditions.

For the α-D-glucosidase assay, the cells were treated with dimethyl sulfoxide solution (40%) at 30°C for 30 min, washed with 0.075 M phosphate buffer (pH 6.8), and suspended in the appropriate amount of the same buffer. α-D-Glucosidase activity was assayed by using *p*-nitrophenyl-α-D-glucoside as substrate, according to the method described by Halvorson and Ellias (16). One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of *p*-nitrophenol per min under these conditions. *p*-Nitrophenyl-α-D-glucoside was synthesized in our laboratory.

Invertase (β-D-fructofuranoside fructohydrolase; EC 3.2.1.26) was measured as described (14), using a cell suspension as the enzyme source. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of glucose assayed with Glucostat (Worthington Diagnostics) per min under the specified conditions.

Isocitrate lyase (EC 4.1.3.1) activity was determined

TABLE 1. *List of yeast strains*[a]

Strain	Genotype	Source
P-28-24C	**a** Wild type	Our stock culture
F10D	α Wild type	**a** to α mutant of P-28-24C
G174-8A	**a** *GAL81-16*	Constructed by tetrad dissection of hybrid prepared by a cross between F10D and a haploid segregant of a constitutive galactose-positive mutant from G99 (**a**/α *GAL80^c-2/GAL80^c-2* [22])
G183-1C	α *gal80-2*	Segregant from Y268-2BR-36 (a constitutive galactose-positive revertant [*gal80-2*] from Y268-2B [27] × N61-2A (23) cross
G211-6A	α *gal7-2*	Matsumoto et al. (22)
G250-4C	α *gal4-2 gal7-2*	Matsumoto et al. (22)
G298-2A	α *GAL81-16 gal7-2*	Segregant from G174-8A × G250-4C cross
G373-1B	**a** *GAL81-12 gal7-2*	Constructed by repeated crosses and tetrad dissections among haploid segregants of a constitutive galactose-positive mutant from diploid strain, G99 (**a**/α *GAL80^c-2/GAL80^c-2* [22]), P-28-24C, and G250-3C (**a** *gal4-2 gal7-2*; a haploid clone from the same family of G250-4C)
G373-3A	**a** *GAL81-12*	Constructed by repeated crosses and tetrad dissections among haploid segregants of a constitutive galactose-positive mutant from diploid strain, G99 (**a**/α *GAL80^c-2/GAL80^c-2* [22]), P-28-24C, and G250-3C (**a** *gal4-2 gal7-2*; a haploid clone from the same family of G250-4C)
G373-4B	α *GAL81-12*	Constructed by repeated crosses and tetrad dissections among haploid segregants of a constitutive galactose-positive mutant from diploid strain, G99 (**a**/α *GAL80^c-2/GAL80^c-2* [22]), P-28-24C, and G250-3C (**a** *gal4-2 gal7-2*; a haploid clone from the same family of G250-4C)
G373-6B	α *GAL81-12 gal7-2*	Constructed by repeated crosses and tetrad dissections among haploid segregants of a constitutive galactose-positive mutant from diploid strain, G99 (**a**/α *GAL80^c-2/GAL80^c-2* [22]), P-28-24C, and G250-3C (**a** *gal4-2 gal7-2*; a haploid clone from the same family of G250-4C)
G373-1BR-1	**a** *GAL81-12 gal7-2 GAL82-R1*	Mutant from G373-1B
G373-1BR-4	**a** *GAL81-12 gal7-2 GAL83-R4*	Mutant from G373-1B
G374-1D	**a** *GAL81-16 gal7-2*	Segregant from G298-2A × G174-8A cross
G397-1C	α *gal80-2 gal7-2*	Segregant from G183-1C × G251-5B (**a** *GAL80^c-1 gal7-2* [22]) cross
CR3-1B	**a** *GAL81-12 GAL82-R1*	Segregant from G373-1BR-1 × G373-4B cross
CR3-3A	α *GAL81-12 GAL82-R1*	Segregant from G373-1BR-1 × G373-4B cross
CR4-5C	**a** *GAL81-12 GAL83-R4*	Segregant from G373-1BR-4 × G373-4B cross
CR4-6C	α *GAL81-12 GAL83-R4*	Segregant from G373-1BR-4 × G373-4B cross
CR5-3C	**a** *gal7-2 GAL82-R1*	Segregant from G373-1BR-1 × G211-6A cross
CR8-1D	α *GAL82-R1*	Segregant from CR5-3C × F10D cross
CR9-3A	α *GAL81-12 GAL82-R1 GAL83-R4*	Contructed by repeated crosses and tetrad dissections among G373-1BR-1, G373-1BR-4, and G373-3A
CR10-9D	α *gal7-2 GAL83-R4*	Segregant from G373-1BR-4 × G211-6A cross
CR11-1B	**a** *GAL83-R4*	Segregant from CR10-9D × P-28-24C cross
CR14-17D	**a** *gal80-2 GAL82-R1*	Constructed by repeated crosses and tetrad dissections among CR5-3C, G397-1C, and G183-1C
CR15-11C	α *gal80-2 GAL83-R4*	Constructed by repeated crosses and tetrad dissections among CR10-9D, G397-2A (**a** *gal80-2 gal7-2*; a haploid clone from the same family of G397-1C), and G183-1C
CR19-3D	α *GAL81-12*	Segregant from CR4-5C × G373-6B cross

[a] The genetic symbols are those proposed by Plischke et al. (29), whereas the symbols for mating types (**a** and α) follow conventional usage. In the constructed strains, genotypes for markers other than the mating type and *gal* were omitted from the table, except for strains XG41-8D and XG42-19C.

TABLE 1—*Continued*

Strain	Genotype	Source
XG41-8D	**a** *GAL81-12 gal7-2 arg9*	Constructed by repeated crosses and tetrad dissections among X1265-2A (**a** *his4 leu2 thr4 ade6 his1 ura1 ade2 arg9*; obtained from Lindegren's stock), F10D, and G373-6B
XG42-19C	α *GAL81-12 GAL83-R4 gal7-2 ura3-1 lys1-1*	Constructed by repeated crosses and tetrad dissections among X2383-5A (α *his5-2 lys1-1 can1-100 trp5-48 ade2-1 ura3-1 SUP7*; obtained from Berkeley Yeast Genetics Stock Center), P-28-24C, F10D, G373-1B, G373-1BR-4, and G373-6B

according to Dixon and Kornberg (Biochem. J. **72**:3p, 1959) in a cell extract. Cells were cultivated, harvested in 20 mM potassium phosphate buffer (pH 7.4) with 0.1 mM ethylenediaminetetraacetic acid, and passed once through a French pressure cell (Ohtake) with a pressure of 500 kg/cm^2. The supernatant obtained after centrifugation of the homogenate at 35,000 × *g* for 15 min at 4°C was used as the enzyme source. One unit of enzyme activity was the amount of enzyme which consumed 1 μmol of isocitrate per min under the conditions described. Protein was determined according to Lowry et al. (20), using bovine serum albumin as a standard.

Genetic techniques. The methods for genetic analysis were described previously (27).

RESULTS

Isolation of *GAL82* and *GAL83* mutations. According to the protocol described in Materials and Methods, strains G373-1B (**a** *GAL81-12 gal7-2*) and G298-2A (α *GAL81-16 gal7-2*) were subjected to ethylmethane sulfonate mutagenesis followed by nystatin selection, and the treated cells were spread on YPGlu plates. Colonies developed on the plates were screened for inability to grow on YPGluGal by replica plating, whereas the original strains could grow on YPGluGal medium. We isolated 39 independent colonies from strain G373-1B and 13 colonies from G298-2A. Since our initial aim was to isolate mutations resistant to the glucose repression occurring at the promoter of *gal1*, we tested the isolated colonies as to whether their mutations were dominant or recessive over the wild-type counterpart. Each mutant clone was crossed to the *GAL81 gal7* strains, G373-6B (α) or G374-1D (**a**), having the complementary mating type of the mutants, and the resultant diploids were tested for sensitivity to glucose repression by testing their growth on YPGluGal medium. Seven dominant mutants were selected from the 52 isolates.

Four dominant mutants isolated from strain G373-1B (G373-1BR-1 to -4; mutant alleles were designated *R1* to *R4*) were crossed to an α *GAL81-12 gal7-2* strain (G373-6B), and the three mutants from strain G298-2A (G298-2AR-1 to -3; mutant alleles were designated *R5* to *R7*) were crossed to the **a** *GAL81-16 gal7-2* strain (G374-1D). The resultant diploids were subjected to tetrad analysis. All of the tetrads showed 2+:2− segregation for growth on YPGluGal medium for each of the 7 to 11 asci examined from each diploid. This indicates that each mutant bears a single mutation.

The seven mutants were localized to two loci by pairwise crosses between different mutants (or their segregants). We observed that the combinations of *R1* × *R2*, *R1* × *R6*, *R3* × *R4*, *R4* × *R5*, and *R4* × *R7* always gave a 0+:4− segregation for growth on YPGluGal medium, whereas other combinations *R1* × *R3*, *R1* × *R4*, *R1* × *R5*, *R1* × *R7*, and *R4* × *R6*, showed three different segregations, 0+:4−, 1+:3−, and 2+:2−, for growth on the same medium. The segregation patterns indicated that mutants G373-1BR-1, G373-1BR-2, and G298-2AR-2 (i.e., *R1*, *R2*, and *R6*) bear mutations on one of the two loci (called *GAL82*), whereas mutants G373-1BR-3, G373-1BR-4, G298-2AR-1, and G298-2AR-3 (i.e., *R3*, *R4*, *R5*, and *R7*) have mutations at the other locus (called *GAL83* because they were specific for galactose metabolism, as described below). Since the compiled data from the heterozygous combinations of the above crosses showed 3:25:9 to be the ratio of 0+:4−, 1+:3−, and 2+:2− asci for growth on YPGluGal medium at 30°C, the *GAL82* and *GAL83* loci are unlinked to each other.

***GAL82* and *GAL83* loci are not linked to *GAL81-gal4*, *gal80*, and the *gal7*•*gal10*•*gal1* cluster.** Crossing *GAL81-12 gal7-2 GAL82-R1* (G373-1BR-1) and *GAL81-12 gal7-2 GAL83-R4* (G373-1BR-4) strains with a *GAL81-12 GAL7*$^+$ strain produced 2+:2−, 3+:1−, and 4+:0− segregations on YPGluGal medium in ratios of 7:22:7 for the first cross and 13:18:16 for the second cross. These results indicate that neither *GAL82* nor *GAL83* is linked to the *gal7* gene. Since the *gal1* and *gal10* genes are composed of a cluster with the *gal7* gene, *GAL82* and *GAL83* are also unlinked to the *gal1* and *gal10* genes.

The 2+:2− asci in the above crosses are the

parental ditype asci (PD), and the 4+:0− and 3+:1− asci are nonparental ditype (NPD) and tetratype (T) asci, respectively. The tetrad distribution for the G373-1BR-4 × G373-4B cross with a PD/NPD/T ratio of 13:16:18, indicates that both the *gal7* and *GAL83* loci are centromere-linked genes on two different chromosomes. This was further confirmed by tetrad analysis of the diploid XG41-8D (a *GAL81-12 gal83*$^+$ *gal7-2 arg9 URA3*$^+$) × XG42-19C (α *GAL81-12 GAL83-R4 gal7-2 ARG9*$^+$ *ura3-1*). The *GAL83* locus was located on chromosome V approximately 5 centimorgans distal from *arg9*, which is approximately 3 centimorgans to the right of the centromere (25). The tetrad data showed a PD/NPD/T ratio of 80:0:9 for the *GAL83* and *arg9* combination and 62:0:27 for *GAL83* and *ura3*, whereas the *ura3* and *arg9* combination showed a 69:0:20 ratio. Although we did not test for *cho1* (26), these data clearly indicated that the order of these genes on chromosome V is *ura3*-centromere-*arg9-GAL83-cho1*.

Since the *GAL81* mutations occur inside of the *gal4* locus (22) and the *GAL81* mutation segregated independently from the *GAL82* mutation, as described below, the *GAL82* locus should be different from *gal4*. To confirm this, strain G373-1BR-1 was crossed with strain G250-4C (α *gal4-2 gal7-2*), and the diploid was subjected to tetrad analysis. Strain G250-4C can grow on YPGluGal, whereas G373-1BR-1 cannot. The diploid showed slow growth on YPGluGal, and the ratio of PD (2+:2−) to NPD (4+:0−) to T (3+:1−) tetrads was 3:3:7 in the 13 asci examined. This indicates that the *GAL82* locus is not linked to *gal4*. To test linkage between the *GAL82* and *gal80* loci, strain CR5-3C (a *gal7-2 GAL82-R1*) was crossed with the *gal80-2 gal7-2* strain G397-1C (α). The resultant diploid could grow on YPGluGal medium. It was subjected to tetrad analysis. Testing growth on YPGluGal, three ascus types, showing 4+:0−, 3+:1−, and 2+:2− segregation in 2, 7, and 1 asci, respectively, were found, indicating that *GAL82* is not linked to the *gal80* locus. The segregation pattern further indicates that a cell having the *gal80 GAL82-R1 gal7* genotype is sensitive to galactose by the *gal7* mutation even in the presence of glucose, so that the *gal80 GAL82-R1* genotype presumably gives rise to the glucose resistance phenotype for galactokinase synthesis. This is confirmed in later experiments. In conclusion, the *GAL82* and *GAL83* loci are different from the *gal4* and *gal80* genes and the *gal7·gal10·gal1* gene cluster. The *GAL83* locus is on chromosome V between *arg9* and *cho1*.

GAL81 GAL82 and GAL81 GAL83 double mutants are resistant to glucose repression. It is possible that the *gal7* mutation gives rise to some unusual effects on the expression of the other structural genes for the Leloir enzymes. To eliminate this possibility, we examined CR3-1B (a *GAL81-12 GAL82-R1 GAL7*$^+$) and CR4-5C (a *GAL81-12 GAL83-R4 GAL7*$^+$), strains selected from asci showing both 2+:2− segregation on EBGal medium and 4+:0− segregation on YPGluGal medium, from the crosses G373-4B (α *GAL81-12*) × G373-1BR-1 or G373-1BR-4. The *GAL81-12 GAL82-R1 GAL7*$^+$ (CR3-1B), *GAL81-12 GAL83-R4 GAL7*$^+$ (CR4-5C), and *GAL81-12* (G373-4B) strains were grown at 30°C in YPGly and YPGluGly media. The time course of the galactokinase appearance was followed by using suspensions of permeabilized cells as the enzyme source (Fig. 1). The *GAL81-12* strain (G373-4B) produced about 4% as much galactokinase activity in YPGluGly medium as in YPGly medium. On the other hand, the *GAL81-12 GAL82-R1* strain (CR3-1B) showed 46% as much enzyme activity and the *GAL81-12 GAL83-R4* strain (CR4-5C) produced 53% as much in YPGluGly as in YPGly medium. No essential difference in cellular growth rates was observed among these strains, whereas higher growth rates were observed in YPGluGly medium than in YPGly medium for each strain.

To investigate possible interactions between the *GAL82* and *GAL83* mutations, strain CR9-3A (α *GAL81-12 GAL82-R1 GAL83-R4*) was constructed as described in Table 1. When this strain was cultivated in YPGly and YPGluGly media, it showed essentially the same levels of galactokinase activity on the respective media as those of the *GAL81-12 GAL82-R1* and *GAL81-12 GAL83-R4* mutants (Fig. 1). This indicates that the *GAL82-R1* and *GAL83-R4* mutations have no additive effect on resistance to glucose repression.

Dominance of the *GAL82-R1* and *GAL83-R4* mutations over the wild-type counterpart at the level of galactokinase activity was also confirmed by comparing galactokinase activity of a diploid having the *GAL81-12 GAL82-R1* (or *GAL83-R4*)/*GAL81-12 gal82*$^+$ *gal83*$^+$ genotype with the corresponding homozygous diploids (*GAL81-12*/*GAL81-12* and *GAL81-12 GAL82-R1* [or *GAL83-R4*]/*GAL81-12 GAL82-R1* [or *GAL83-R4*]). Apparently reduced rates of galactokinase synthesis were observed in the heterozygous diploids in comparison with those of the homozygous diploids in YPGluGly medium. However, the level of the enzyme activities was significantly higher than that observed in the cells homozygous for the *GAL81-12 gal82*$^+$ *gal83*$^+$ genotype (Fig. 2).

The *GAL82* or *GAL83* single mutation is sensitive to glucose repression. All *GAL82*

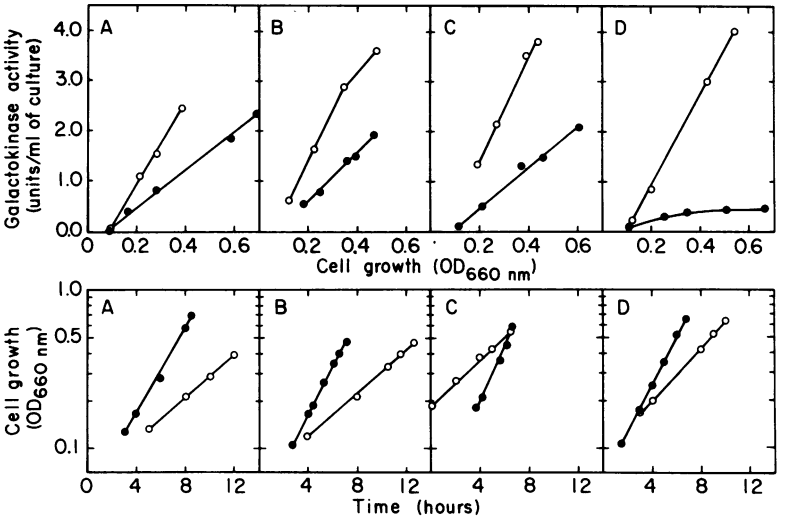

FIG. 1. *Time courses of galactokinase synthesis in strains having the GAL81-12 GAL82-R1, GAL81-12 GAL83-R4, GAL81-12 GAL82-R1 GAL83-R4, and GAL81-12 gal82$^+$ gal83$^+$ genotypes. Cells of strain (A) CR3-1B (GAL81-12 GAL82-R1), (B) CR4-5C (GAL81-12 GAL83-R4), (C) CR9-3A (GAL81-12 GAL82-R1 GAL83-R4), and (D) G373-4B (GAL81-12) were shaken at 30°C for 48 h in YPGly medium. A 0.5-ml portion of each culture was inoculated into 100 ml of YPGly (○) or YPGluGly (●) medium and shaken at 30°C. Galactokinase activity (upper panels) as a function of cell growth (optical density [OD] of the culture at 660 nm; lower panels) was determined by using permeabilized cell suspension as the enzyme source.*

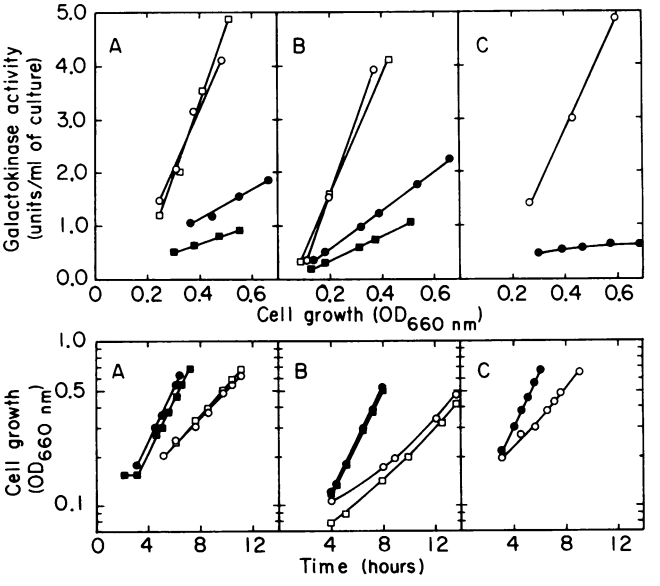

FIG. 2. *Time courses of galactokinase synthesis in diploids having the genotypes homozygous or heterozygous for the GAL82-R1 or GAL83-R4 alleles and homozygous for the GAL81-12 allele. Cells of diploid CR3-1B × CR3-3A (GAL81-12 GAL82-R1/GAL81-12 GAL82-R1) (A; ○, ●), CR3-1B × G373-4B (GAL81-12 GAL82-R1/GAL81-12 gal82$^+$) (A; □, ■), CR4-5C × CR4-6C (GAL81-12 GAL83-R4/GAL81-12 GAL83-R4) (B; ○, ●), CR4-5C × G373-4B (GAL81-12 GAL83-R4/GAL81-12 gal83$^+$) (B; □, ■), and G373-4B × G373-3A (GAL81-12/GAL81-12) (C; ○, ●) crosses were shaken at 30°C for 48 h in YPGly medium. A 0.5-ml portion of each culture was inoculated into 100 ml of YPGly (open symbols) or YPGluGly (closed symbols) medium and shaken at 30°C. Galactokinase activity (upper panels) as a function of cell growth (optical density [OD] of the culture at 660 nm; lower panels) was determined by using permeabilized cell suspension as the enzyme source.*

and *GAL83* mutations were isolated from and investigated in strains carrying the *GAL81* mutation. Next, we studied the *GAL82* and *GAL83* mutations in *gal81*$^+$ cells. Two mutants, G373-1BR-1 (a *GAL81-12 GAL82-R1 gal7-2*) and G373-1BR-4 (a *GAL81-12 GAL83-R4 gal7-2*), were crossed with strain G211-6A (α *gal81*$^+$ *gal7-2*), the diploids were sporulated, and four-spored asci were dissected. Three ascus types, showing 4+:0−, 3+:1−, and 2+:2− segregations on YPGluGal medium, were observed for 1, 5, and 0 asci, respectively, from the G373-1BR-1 × G211-6A cross and for 2, 7, and 6 asci for the G373-1BR-4 × G211-6A cross. These observations indicated that the catabolite repression-resistant phenotype might occur by a combination of the *GAL82* or *GAL83* mutation with the *GAL81* mutation. To confirm this possibility, strains CR8-1D (α *GAL82-R1*) and CR11-1B (a *GAL83-R4*) were constructed as described in Table 1. Cells of strains CR8-1D, CR11-1B, and P-28-24C (wild type) were shaken in YPGlyGal and YPGluGlyGal media, and their time courses of galactokinase appearance were followed by using suspensions of permeabilized cells as the enzyme source. Induction of galactokinase occurred in YPGlyGal medium to almost the same level in all strains, whereas they were fully repressed in YPGluGlyGal medium (Fig. 3). No galactokinase activity appeared in those strains when they were cultivated in YPGluGly and YPGly media (data not shown). Thus, the *GAL82* and *GAL83* mutations could neither overcome catabolite repression in the combination with the *gal81*$^+$ allele nor give rise to constitutive synthesis of the enzyme.

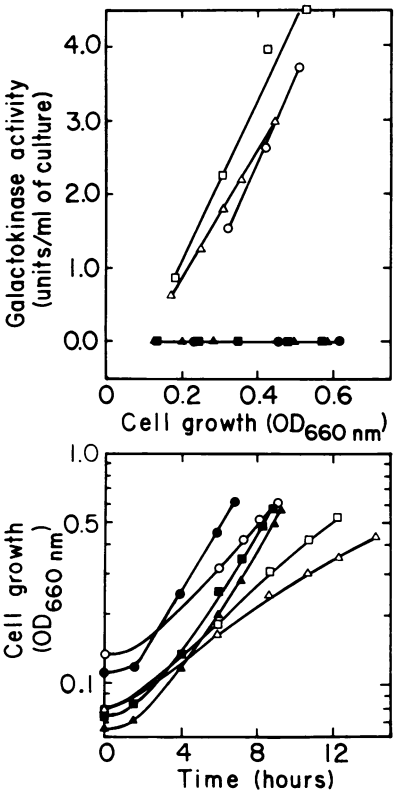

FIG. 3. *Time courses of galactokinase synthesis in strains having the GAL82-R1 or the GAL83-R4 genotype. Cells of strains CR8-1D (GAL82-R1; △, ▲), CR11-1B (GAL83-R4; □, ■), and P-28-24C (wild type; ○, ●) were shaken at 30°C for 48 h in YPGly medium. A 0.5-ml portion of each culture was inoculated into 100 ml of YPGlyGal (open symbols) or YPGluGlyGal (closed symbols) medium and shaken at 30°C. Galactokinase activity (upper panel) as a function of cell growth (optical density [OD] of the culture at 660 nm; lower panel) was determined by using permeabilized cell suspension as the enzyme source.*

***gal80 GAL82* and *gal80 GAL83* double mutants are resistant to glucose repression.** We observed high levels of galactokinase production in the *GAL81-12 GAL82-R1* (or *GAL83-R4*) strains in the presence of glucose but not in the *gal81*$^+$ *GAL82-R1* (or *GAL83-R4*) strain. However, the results of the linkage study with *gal80* suggested that the *gal80* mutation, the other constitutive mutation for synthesis of the Leloir enzymes, is also effective for overcoming the glucose repression in combination with the *GAL82* or *GAL83* mutation. To confirm this we constructed strains CR14-17D (a *gal80-2 GAL82-R1*) and CR15-11C (α *gal80-2 GAL83-R4*), as described in Table 1. These strains were cultivated in YPGly and YPGluGly media, and their galactokinase activities were determined. It was apparent that synthesis of the enzyme in these strains is resistant to glucose repression (Fig. 4).

***GAL82* and *GAL83* mutations are specific for the *gal* system.** The catabolite-sensitive enzymes α-D-glucosidase, invertase, and isocitrate lyase were examined in *GAL82* and *GAL83* mutants. It is known that maltose and sucrose utilization in *S. cerevisiae* are controlled by polymeric genes, and each polymeric gene system for maltose utilization might consist of a number of complementary genes (28; Y. Oshima, unpublished data). Although we do not know the exact genotype for maltose and sucrose utilization in strains CR3-1B (a *GAL81-12 GAL82-R1*), CR4-5C (a *GAL81-12 GAL83-R4*), and G373-4B (α *GAL81-12*), these strains are able to utilize maltose and sucrose. They were cultivated at 30°C in YPGlyMal and YPGluGlyMal media to test α-D-glucosidase activity or in YPGly and YPGluGly media to test isocitrate lyase and invertase activities. The time course of appearance of α-D-glucosidase activity was followed,

and we observed an almost constant specific activity of the enzyme during growth to an optical density at 660 nm of 0.6. α-D-Glucosidase formation in all three strains was fully repressed by the presence of glucose in the medium (Table 2). Similarly, invertase and isocitrate lyase were repressed by glucose in those strains, whereas strain CR4-5C (a *GAL81-12 GAL83-R4*) showed a considerable level of invertase activity in the presence of glucose. However, the constitutive level of invertase should not be related to the *GAL83-R4* mutation, because a meiotic segregant (CR19-3D) having the *GAL81-12 gal83*$^+$ genotype, isolated from the CR4-5C × G373-6B cross, showed almost the same level of invertase activity found in strain CR4-5C (Table 2). These results indicate that the *GAL82* and *GAL83* mutations do not affect the α-D-glucosidase, invertase, and isocitrate lyase activities. These observations strongly suggest that the *GAL82* and *GAL83* genes are specific for the Leloir enzymes.

DISCUSSION

The data described in this communication are summarized in Table 3 along with additional

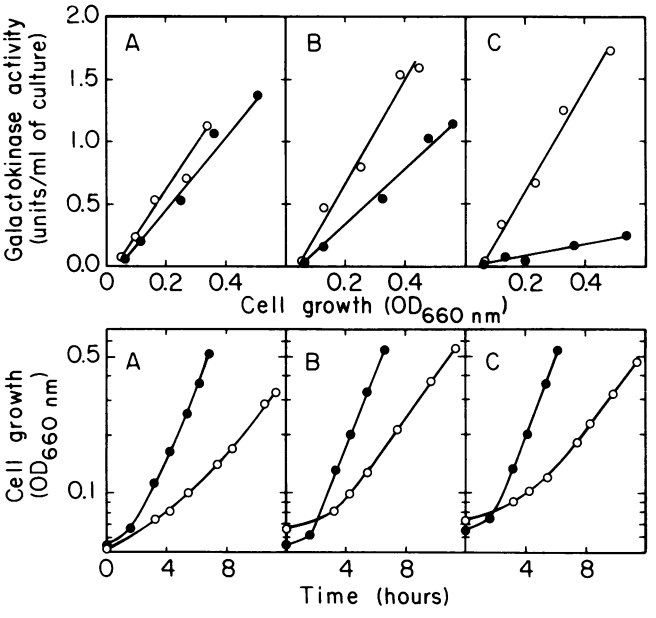

FIG. 4. *Time courses of galactokinase synthesis in strains having the gal80 GAL82 or the gal80 GAL83 genotype. Cells of strains (A) CR14-17D (gal80-2 GAL82-R1), (B) CR15-11C (gal80-2 GAL83-R4), and (C) G183-1C (gal80-2) were shaken at 30°C for 48 h in YPGly medium. A 0.5-ml portion of each culture was inoculated into 100 ml of YPGly* (○) *or YPGluGly* (●) *medium and shaken at 30°C. Galactokinase activity (upper panels) as a function of cell growth (optical density [OD] of the culture at 660 nm; lower panels) was determined by using permeabilized cell suspension as the enzyme source.*

TABLE 2. *Specific activities of α-D-glucosidase, isocitrate lyase, and invertase in cells having the GAL81-12 GAL82-R1 or the GAL81-12 GAL83-R4 genotype*[a]

Strain	Genotype	Sp act					
		α-D-Glucosidase (mU/ml per OD_{660})		Isocitrate lyase (mU/mg of protein)		Invertase (U/ml per OD_{660})	
		YPGlyMal	YPGluGlyMal	YPGly	YPGluGly	YPGly	YPGluGly
G373-4B	*GAL81-12*	165	1	32.8	0	0.74	0.07
CR3-1B	*GAL81-12 GAL82-R1*	161	1	105.1	0	0.80	0.12
CR4-5C	*GAL81-12 GAL83-R4*	200	10	80.4	0	1.29	0.56
CR19-3D	*GAL81-12*	—[b]	—	—	—	1.23	0.45

[a] Cells of each strain were shaken at 30°C for 48 h in YPGly medium. A 0.5-ml portion of each culture was inoculated into 100 ml of the indicated medium and shaken at 30°C. At appropriate intervals, samples were taken and activities of the enzymes as a function of cell growth were determined. The highest specific activity attained was listed. OD_{660}, Optical density at 660 nm.

[b] —Not tested.

TABLE 3. *Effect of the GAL82 and GAL83 mutations on galactokinase synthesis in various media*

Genotype		Growth on:			
GAL82 (or *GAL83*)	*GAL81* (or *gal80*)	Medium	Effector		Galactokinase act (test strain)[a]
			Glucose	Galactose	
Wild type	Wild type	YPGluGly	+	−	− (<0.01 [P-28-24C])
		YPGluGlyGal	+	+	− (<0.01)
		YPGlyGal	−	+	+++ (10.1)
		YPGly	−	−	− (<0.01)
Wild type	Mutant	YPGluGly	+	−	+ (0.4 [G373-4B])
		YPGluGlyGal	+	+	+ (0.6)
		YPGlyGal	−	+	+++ (9.6)
		YPGly	−	−	+++ (9.6)
Mutant	Wild type	YPGluGly	+	−	− (0.01 [CR8-1D])
		YPGluGlyGal	+	+	− (0.01)
		YPGlyGal	−	+	+++ (8.9)
		YPGly	−	−	− (<0.01)
Mutant	Mutant	YPGluGly	+	−	++ (3.8 [CR3-1B])
		YPGluGlyGal	+	+	++ (2.8)
		YPGlyGal	−	+	+++ (8.1)
		YPGly	−	−	+++ (8.0)

[a] Specific activity of galactokinase in a certain genotype is symbolized as follows: +++, induced level of the wild-type cells; ++, medium level; +, low level; −, no activity. Specific galactokinase activity (units per milliliter per optical density unit at 660 nm of the culture) observed with the strain given in brackets is listed as an example.

data. The *GAL82* and *GAL83* mutations are effective for expression of galactokinase activity only when these mutations are combined with the *GAL81* or *gal80* mutation, the mutations giving rise to constitutive synthesis of the Leloir enzymes. In wild-type cells, synthesis of galactokinase occurred when the cells were cultivated in a medium with galactose and without glucose (or any other material which exerts carbon catabolite repression). In the *GAL81* or *gal80* mutant, however, the presence of galactose in the medium is not a prerequisite for enzyme synthesis, although the mutants still show significant sensitivity to glucose repression. Since galactokinase is insensitive to glucose inhibition (1), lower levels of the enzyme activity in YPGluGlyGal or YPGluGly medium than in YPGlyGal or YPGly medium are due to glucose repression. When the *GAL82* or *GAL83* mutation was combined with the *GAL81* or *gal80* mutation, sensitivity of the cells to glucose repression was greatly reduced, and cells showed significant galactokinase activity even with glucose in the medium.

The simplest interpretation of the above results is that glucose repression of galactokinase activity is mediated by two regulatory circuits. One of the circuits consists of the *GAL80* and *gal81*$^+$-*GAL4* proteins, the same proteins which transmit the galactose signals. The other circuit involves the *gal82*$^+$ and *gal83*$^+$ gene products. This double circuit system for glucose repression provides a safeguard to prevent unnecessary gene expression in the presence of glucose. Since the *GAL81* (or *gal80*) single mutants (e.g., G373-4B) showed almost the same low but significant level of galactokinase activity in YPGluGlyGal medium as in YPGluGly medium, whereas the *GAL82* (or *GAL83*) single mutants (e.g., CR8-1D) showed uninduced levels of activity (Table 3), the *GAL80* and *gal81*$^+$-*GAL4* circuit exerts the major role in glucose repression in the *gal* system. This accounts for the observation that the *GAL82* (or *GAL83*) mutants showed the same phenotype as the wild-type cells. Whether the *gal82*$^+$-*gal83*$^+$ circuit conveys the glucose signal to a presumptive promoter region of the structural genes for the enzymes or to the *gal81*$^+$-*GAL4* protein, the positive factor for *gal* gene expression, is not known.

Alternatively, it is possible to envisage another mechanism to explain the glucose sensitivity of the *gal81*$^+$ *GAL82* strain (Table 3), namely, that the glucose signal is conveyed to the regulatory factors (the *gal81*$^+$-*GAL4* and *GAL80* products) or directly to the promoter of the structural genes by a single circuit consisting of the *gal82*$^+$ and *gal83*$^+$ products and that the galactose uptake system is inhibited by glucose much more severely than suggested by Matern and Holzer (21). For example, although the *GAL82* mutant (CR8-1D) may be insensitive to

glucose repression, the intracellular amount of galactose may be lower than the threshold level necessary for the expression of the *gal* structural genes with the wild-type alleles of the *GAL81* and *gal80* loci even in YPGluGlyGal medium, as the galactose uptake system is strongly inhibited by glucose. Since the *GAL81 GAL82* (or *GAL83*) *gal7* mutants could not grow on YPGluGal medium, the *GAL81 GAL82* (or *GAL83*) genotype must give rise to a significant level of galactose uptake activity by the cells, probably by enhanced synthesis of the galactose transport protein due to the *GAL81* mutation, and the cells must retain substantial transport activity even under glucose inhibition.

All the above arguments require the assumption that the *GAL82* and *GAL83* genes are involved in regulating the transcription of the structural genes for the Leloir enzymes, as has been shown for *gal4* regulation (17). Confirmation of these ideas will require further study.

At the beginning of this study, we supposed that mutants insensitive to carbon catabolite repression would occur at a promoter region of the structural genes. Hence we selected dominant mutations. However, no dominant mutations linked to the *gal7·gal10·gal1* cluster were detected. In the process of selecting the *GAL82* and *GAL83* mutations, we obtained seven dominant mutations among the 52 primary isolates. Although we did not investigate the remaining 45 primary isolates, all of them were recessive mutations. This fact strongly suggests that there might be other genes, in which mutations are recessive to the wild-type allele, in the system for glucose repression. Recently, Ciriacy (7) suggested the same kind of gene, *adr4*, in a regulatory system for synthesis of glucose-repressible alcohol dehydrogenase in *S. cerevisiae*. This type of mutation is under investigation.

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