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Galactokinase Encoded by *GAL1* Is a Bifunctional Protein Required for Induction of the *GAL* Genes in *Kluyveromyces lactis* and Is Able To Suppress the *gal3* Phenotype in *Saccharomyces cerevisiae*

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We have analyzed a *GAL1* mutant (*gal1-r* strain) of the yeast *Kluyveromyces lactis* which lacks the induction of β-galactosidase and the enzymes of the Leloir pathway in the presence of galactose. The data show that the *K. lactis GAL1* gene product has, in addition to galactokinase activity, a function required for induction of the lactose system. This regulatory function is not dependent on galactokinase activity, as it is still present in a galactokinase-negative mutant (*gal1-209*). Complementation studies in *Saccharomyces cerevisiae* show that *K. lactis GAL1* and *gal1-209*, but not *gal1-r*, complement the *gal3* mutation. We conclude that the regulatory function of *GAL1* in *K. lactis* soon after induction is similar to the function of *GAL3* in *S. cerevisiae*.

The genes for lactose and galactose metabolism in *Kluyveromyces lactis* are organized and regulated analogously to the galactose system in *Saccharomyces cerevisiae* (10, 23). The similarity between both systems is extensive: the upstream activating sequences mediating galactose induction show a high degree of homology (29), and the positive regulatory proteins LAC9 and GAL4 can function in both organisms (4, 17, 24, 28, 29).

The genes in *S. cerevisiae* encoding enzymes of galactose and melibiose metabolism are induced shortly after a cell grown on a nonfermentable carbon source receives galactose (1, 6, 33). The induction mechanism is dependent on the function of *GAL4*, *GAL80*, and *GAL3* (reviewed in reference 13). *GAL4* and *GAL80* encode proteins which are directly involved in transcription activation and repression of galactose-governed promoters (13). The *GAL3* gene has been recently analyzed in detail, but its function remains unknown (2, 33). Most noticeably, *GAL3* mutants exhibit an induction time increased by several days (31, 33, 38), leading to the hypothesis that *GAL3* is responsible for the synthesis of the inducer molecule (6). No *K. lactis* mutations with a phenotype similar to that of *S. cerevisiae gal3* have been described.

In a study on the regulation of the lactose metabolism in *K. lactis*, we have recently isolated a number of new regulatory mutants. One of these mutants carries a recessive mutation which leads to loss of induction of β-galactosidase, galactokinase, and lactose permease. In this report, we show that this mutation is located in the *GAL1* gene. By gene disruption, we show that *GAL1* from *K. lactis* (*kl-GAL1*) is required for induction of the lactose genes and therefore plays a double role in the lactose system. Furthermore, we show that *kl-GAL1* and a galactokinase-negative allele can complement the *gal3* mutation in *S. cerevisiae*. This finding suggests that the additional function of galactokinase encoded by *kl-GAL1* is able to substitute a defective *GAL3* function in *S. cerevisiae*.

After the experiments described here were completed, Bhat et al. (3) showed that *gal3* long-term induction can be shortened by introduction of additional copies of the *S. cerevisiae GAL1* gene. They also showed that *Escherichia coli* galactokinase does not have an effect on the induction time in *gal3* cells and speculate that the *GAL1* gene or its product has, in addition to galactokinase activity, a function involved in *gal3* complementation. We demonstrate here that the *gal3* phenotype can be completely suppressed by the presence of *GAL1* from *S. cerevisiae* under control of the *PDC1* promoter.

(Part of this work was presented elsewhere [19a].)

MATERIALS AND METHODS

Yeast strains. *K. lactis* SD11R3 (*GAL*$^+$ *trp1*) and RWJ5d (*gal1-r trp1*) were isolated by Walker-Jonah et al. (35a). Strain KB6-2C (*GAL*$^+$ *ade ura3 his*) was provided by K. Breunig. Strain KB6-2C-1 (*ura3 ade his gal1::URA3*) was isolated as a *GAL1* disruptant (*gal1-d*) of strain KB6-2C; strain RWJ5d-2 (*ura3 his gal1-r*) resulted from a cross between KB6-2C-1 and RWJ5d. Strain 22A295 (*ade1-1 trp1-1 gal1-209*) was a gift of R. C. Dickson; strain 22A295-1 (*ura3 ade1-1 gal1-209*) resulted from a cross between 22A295 and a *GAL*$^+$ *ura3* strain. *S. cerevisiae* α-MZ4 (α *trp1 leu2 ura3 gal1*) and MC45-5A (α *leu2-3,112 ura3-52 trp1-289*) were kindly provided by R. Zitomer and M. Ciriacy, respectively. Strain TTD1-13A (*gal3 MEL1 trp1 ura3-52 leu2-3,112*) was kindly provided by J. Hopper. MC45-5A-1 (α *leu2-3,112 ura3-52 trp1-289 gal3::LEU2*) was isolated as a *GAL3* disruptant (*gal3-d*) of strain MC45-5A.

Bacterial strains. *E. coli* DH5α F′ *endA1 hsdR17* r_K^- m_K^- *supE44 thi-1 recA1 gyrADΔ* (*lacZYA-argF*)*U169* (ϕ80d*lacZ* ΔM15) was used.

Plasmids and plasmid constructions. pAWJ9 (Fig. 1) is a derivative of plasmid pEK2 (26) carrying a partially restricted *Sau*3A genomic fragment of *K. lactis* DNA inside a single *Bam*HI site. The recombinant *K. lactis* genomic library of partially *Sau*3A-digested DNA fragments of wild-type strain CBS2360 in pEK2 was provided by S. Das. Plasmids pL1-U, pLRG-S, pBM-LRG, Y7PDC-G1 (Fig. 1), and pISB (Fig. 1) are described in this report. Plasmid pGK090 (Fig. 1), which carries the *E. coli* galactokinase gene under control of the *S. cerevisiae PDC1* promoter, was provided by E. Kellermann. Plasmid pBM48 was kindly

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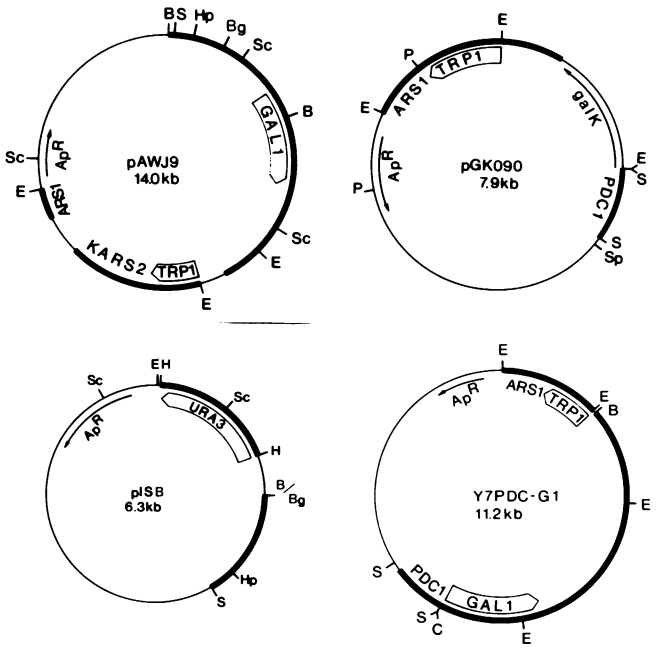

FIG. 1. Maps of plasmids used. In the map of pAWJ9, bold lines represent *K. lactis* sequences (autonomous replicating sequence *KARS2* and the complementing gene with flanking regions) and *S. cerevisiae* sequences (*ARS1* and *TRP1*). Thin lines represent pBR322 sequences. In plasmid pGK090, the *PDC1* promoter is fused with the *E. coli* galactokinase gene *galK*. Yeast *CYC1* terminator sequences are located between the *galK* gene and the *Eco*RI site (27). Plasmid Y7PDC-G1 contains the *PDC1* promoter of *S. cerevisiae*, of which 15 nucleotides at the 3′ end are deleted. This promoter is fused to the *S. cerevisiae GAL1* gene after introduction of a *Cla*I site at positions −25 to −30 in front of the ATG by oligonucleotide-directed mutagenesis. Flanking regions reach to the *Bam*HI site. The rest of the plasmid consists of YRp7 sequences. The small fragment between the *S. cerevisiae TRP1* gene and the *Bam*HI site consists of the pUC19 polylinker region. Plasmid pISB consists of pBR322 sequences (thin lines), the *S. cerevisiae URA3* gene, and the *Sal*I-*Bgl*II fragment of *K. lactis* DNA (see text). Yeast sequences are shown as bold lines. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; S, *Sal*I; Sc, *Sca*I; Sp, *Sph*I. In the case of pAWJ9, only the *Bgl*II and *Sal*I sites present in the upstream region of *GAL1* are indicated.

provided by M. Johnston. Plasmid pT1 (33) was kindly provided by J. Hopper and was used for *GAL3* disruption in MC45-5A.

Transformations were performed as described elsewhere for yeasts (11) and for *E. coli* (19).

Media and growth conditions. Yeast strains were maintained in synthetic media based on yeast nitrogen base without amino acids and ammonium sulfate. Supplements to meet nutritional requirements were added as specified by Zimmermann (39); in addition, ammonium sulfate (5 g/liter) was added as a nitrogen source. Glucose, galactose, or glycerol served as a carbon source. Strains carrying plasmids were maintained in corresponding media lacking either uracil or tryptophan, depending on the selectable marker present on the plasmid. For measurements of galactokinase or α-galactosidase activity, the cells were pregrown in medium containing glucose as a carbon source. After washing, they were transferred into galactose-containing medium. For β-galactosidase measurements under inducing conditions, the cells were maintained in medium containing 0.5% glucose and 2% galactose or 3% glycerol and 2% galactose, depending on whether the strain was glucose repressible (KB6-2C and derived strains) or not (5).

For the propagation of *E. coli* cells, LB medium containing bacterial peptone (10 g/liter), yeast extract (5 g/liter), and NaCl (5 g/liter) was used. If required, penicillin G potassium salt (150 μg/ml) or tetracycline (12 μg/ml) was added.

Enzyme measurements. α-Galactosidase levels were measured as described by Kew and Douglas (16), with the following modifications. The cells were washed twice with buffer containing 31 mM citric acid and 39 mM KH_2PO_4 (pH 4.0). After suspension of the cells in the same buffer and addition of glass beads, crude extracts were prepared in a Braun homogenizer. The reactions were carried out at 30°C in 2.7 ml of buffer containing 5.5 mM *p*-nitrophenyl-α-D-galactopyranoside. The reaction was terminated by adding 0.3 ml of 1 M Na_2CO_3. The amount of p-nitrophenol was measured at 440 nm. In cells growing on solid medium, α-galactosidase activity was visualized as described by Buckholz and Adams (7).

Galactokinase measurements were performed as described by Rymond et al. (27). Crude extracts were prepared in a solution of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5)–1 mM dithiothreitol–300 μg of bovine serum albumin per ml. Cell debris was removed by 10 min of centrifugation at 10,000 rpm. The supernatant was used for activity measurements. The reaction mixture contained 125 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1.25 mM dithiothreitol, 4 mM NaF, 2 mM ATP, and 1 mM [^{14}C]galactose (specific activity, 2.17 μCi/μmol). A 100-μl volume of the reaction mixture was preincubated for 30 s at 30°C; then the crude extract was added, and at different time points aliquots were pipetted into 2 μl of a solution containing 10% galactose and 250 mM EDTA. After the samples were spotted on DE81 filters and washed with four changes of a 0.1% galactose solution for 2 h and once with ethanol, the amount of [^{14}C]galactose was determined in a liquid scintillation counter.

β-Galactosidase measurements were performed as described by Miller (20), with the following modifications. Cells were washed twice in a buffer containing 5 mM Tris-HCl (pH 7.5), 5% glycerol, and 10 mM KCl. Crude extracts were prepared as described above, using the same buffer containing 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation. The supernatant was used for activity measurements. The reaction was performed at 30°C in 1.0 ml of buffer containing 4 mg of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per ml. After the addition of crude extract, the reaction was measured for 10 min photometrically. Protein amounts were measured according to the method of Lowry et al. (18).

Genetic techniques. Mating of *K. lactis* strains was performed on solid medium containing 5% malt extract as described by Wickerham (37). Diploids growing on selective medium were incubated overnight in liquid rich medium containing 1% yeast extract and 2% peptone supplemented with 2% glucose (YEPD). Sporulation was then performed on malt extract plates. Dissection of spores was achieved by micromanipulation. YEPD master plates containing spore progeny were replica plated on selective media.

Mutagenesis. Mutagenesis was performed by incubating cells overnight in 0.1 M sodium phosphate buffer (pH 7.4) containing freshly prepared 1 mM 1-methyl-3-nitro-1-nitrosoguanidine (NNG) at 30°C for 10 min. Mutagenized cells were washed several times, grown in YEPD medium for 24 h, and then plated on complete medium. Mutants defective

TABLE 1. Induction of β-galactosidase in *K. lactis* mutants and transformants

Strain	*Gal* genotype	Plasmid	Carbon source	β-Galactosidase activity (nmol/min/mg)	Galactokinase activity (nmol/min/mg)	Lactose permease (U/mg)
SD11R3	*GAL1*		Glucose	34	3.0	5.0
SD11R3	*GAL1*		Glucose + galactose	2,700	22.0	180.0
RWJ5d	*gal1-r*		Glucose + galactose	70	0	2.3
KB6-2C	*GAL1*		Glycerol + galactose	3,500	ND[a]	ND
RWJ5d-2	*gal1-r*		Glycerol + galactose	80	ND	ND
KB6-2C-1	*gal1-d*		Glycerol + galactose	50	ND	ND
22A295	*gal1-209*		Glycerol + galactose	2,500	0	ND
RWJ5d	*gal1-r*	pgal1-209	Glucose + galactose	2,000	0	ND
RWJ5d	*gal1-r*	pgal1-r	Glucose + galactose	50	0	ND

[a] ND, not determined.

in lactose metabolism were isolated on yeast nitrogen base medium plus 0.5% glucose, 2% lactose, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg/ml). Cells from pale blue colonies were incubated in minimal medium containing glucose plus galactose or lactose. Mutants able to grow on glucose plus galactose but not on lactose were further analyzed.

Gene disruption was performed as described by Rothstein (25). The disrupted *kl-GAL1* gene (*gal1-d*) was constructed by replacement of the internal *Cla*I-*Stu*I fragment by a *Cla*I-*Nru*I DNA fragment from plasmid pBR-URA carrying the *S. cerevisiae URA3* gene in the *Hin*dIII site of pBR322. Strain KB6-2C *ade ura3 his* was transformed with *Eco*RI-*Xba*I-digested plasmid pL1-U to uracil prototrophy. pL1-U consists of pUC19 and the *Sal*I-*Eco*RI fragment carrying the disrupted *kl-GAL1*. Transformants not able to grow on galactose were subjected to Southern analysis. Strain KB6-2C-1 showed the restriction map expected for the desired *gal1* disruption.

Isolation of the mutant alleles *gal1-209* and *gal1-r*. Plasmid pISB (Fig. 1) was cut with *Hpa*I and integrated into the genome of strain RWJ5d-2 (*gal1-r*) or 22A295-1 (*gal1-209*) by selection for Ura$^+$ transformants. The mutant alleles were excised by *Eco*RI digestion of the genomic DNA isolated from the respective transformants and propagated in *E. coli*. Restriction analysis with *Sca*I and *Eco*RV showed the expected fragments. Plasmids were cut with *Eco*RI and ligated into a yeast vector consisting of the *Eco*RI fragment of pAWJ9 (Fig. 1) bearing *ARS1*, *KARS2*, and *TRP1*, resulting in plasmids pgal1-209 and pgal1-r.

Preparation and analysis of DNA. Isolation and purification of plasmids from *E. coli* were carried out as described by Maniatis et al. (19). For preparation of total yeast DNA, the method of Struhl et al. (32) was used. Southern analysis was performed as described previously (19). Nonradioactive labeling of DNA was achieved by using the DNA labeling and detection kit of Boehringer Mannheim.

DNA sequencing of both DNA strands was performed as described elsewhere (30).

Nucleotide sequence accession number. The EMBL accession number for *K. lactis kl-GAL1* is M74111.

RESULTS

A lactose regulatory mutant can be complemented by *GAL1*. After mutagenesis of *K. lactis* SD11R3 by NNG, mutants that grew poorly on lactose or galactose but well on glucose plus galactose were isolated. By testing these mutants for growth on glucose plus galactose, mutants defective in *GAL7* or *GAL10* were excluded. The Lac$^-$ mutants which were not complemented by transformation with *LAC4* (encoding β-galactosidase), *LAC12* (encoding Lac permease), or *LAC9* (encoding Lac activator) were further tested. One mutant, RWJ5d, showed only basal levels of lactose permease, β-galactosidase, and galactokinase (Table 1) and was therefore possibly affected in a novel gene of *LAC* gene expression.

To clone this putative gene, mutant RWJ5d was transformed by a *K. lactis* partial *Sau*3A genomic library in plasmid pEK2 (26) carrying the *S. cerevisiae TRP1* gene as a selective marker. Approximately 19,700 Trp$^+$ transformants were screened, of which one had the Lac$^+$ phenotype restored. The Lac$^+$ transformant contained a plasmid, pAWJ9 (Fig. 1), which was isolated after amplification in *E. coli* and able to transform RWJ5d to Lac$^+$ and Trp$^+$ with a high frequency. These transformants contained wild-type β-galactosidase activities. pAWJ9 contained a 6-kb insert (Fig. 1) of which a *Sca*I subclone but not *Sca*I-*Bam*HI subclones were able to complement the Lac$^-$ mutation in RWJ5d. The 3.3-kb *Sca*I fragment was sequenced (Fig. 2). It contains an open reading frame of 504 amino acids with an N-terminal sequence identical to the published N-terminal 184-amino-acid sequence of the *GAL1* gene from *K. lactis* (36). The cloned gene, therefore, appeared to be the *kl-GAL1* gene. This conclusion is supported by the high degree of homology between the sequenced reading frame and the sequence of the *Saccharomyces carlsbergensis GAL1* gene (9).

The fact that the uninducible Lac$^-$ mutation could be complemented by the *kl-GAL1* gene raised the question of whether the mutant harbors a *GAL1* mutation or the complementing *kl-GAL1* gene suppresses the uninducible phenotype. The former possibility would mean that this mutant represents a new class of *gal1* mutants. Until now, *K. lactis gal1* mutants have been described as lacking only galactokinase activity and being normal in the other enzyme activities of the lactose metabolic pathway (23).

To resolve this question, the *kl-GAL1* gene was disrupted (see Materials and Methods) by the technique of Rothstein (25). The *gal1* disruption mutant KB6-2C-1 was crossed with strain RWJ5d carrying the recessive mutation that prevents induction of *LAC* gene expression. The diploids were Gal$^-$, indicating that the uninducible mutant carries a *gal1* mutation. Spores of six tetrads were all Gal$^-$. Southern analysis of the spores of two tetrads showed a 2:2 segregation of *gal1-d* and the wild-type gene (data not shown). Furthermore, Table 1 shows that *gal1-d* prevented induction of

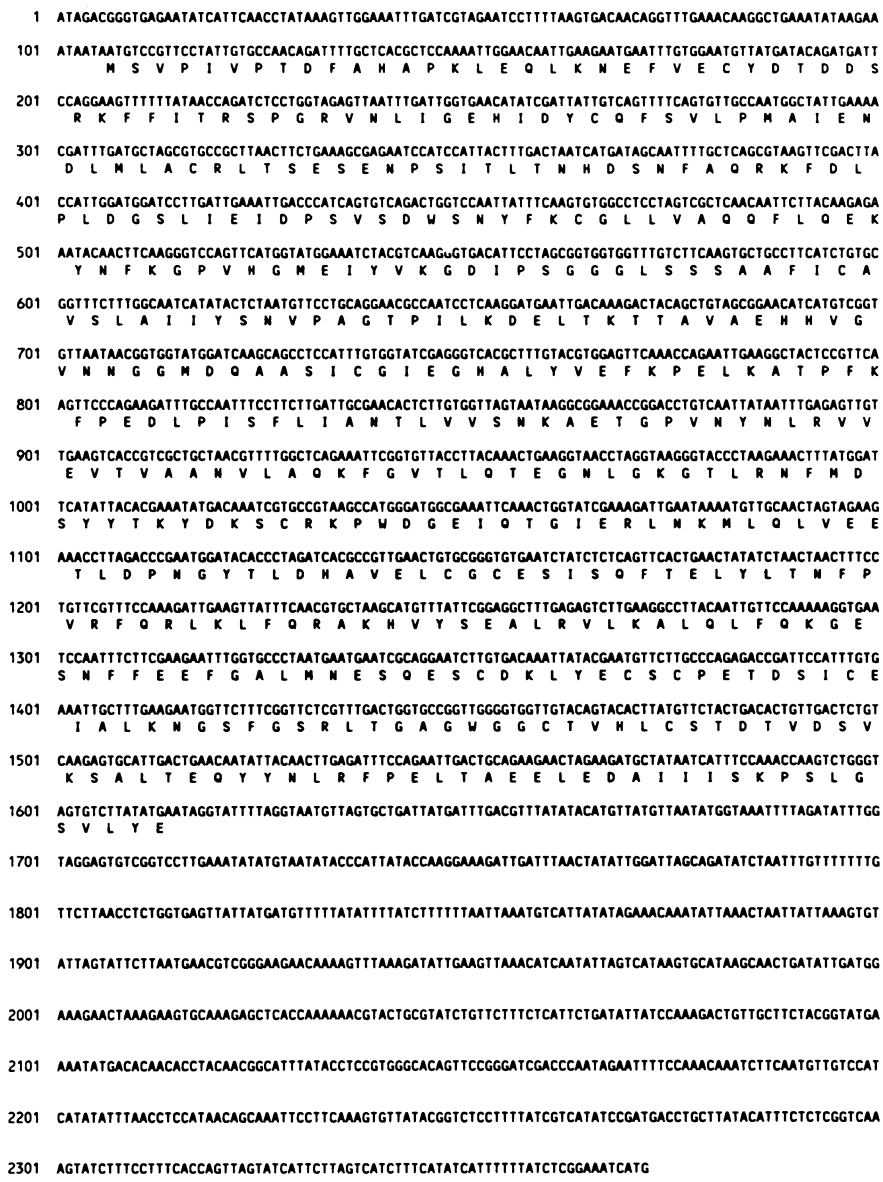

FIG. 2. DNA sequence of *kl-GAL1*. The deduced amino acid sequence is given under the open reading frame.

β-galactosidase, as observed for the mutation in the uninducible mutant RJW5d. The previously described *K. lactis gal1-209* strain 22A295 (23) was tested as a control and showed normal β-galactosidase activity.

Crosses of *gal1-209* (22A295) with the uninducible mutant (RWJ5d-2) or the *gal1* disruption mutant (KB6-2C-1) produced Gal$^-$ diploid cells. Spores from six tetrads of 22A295/RWJ5d-2 diploid cells were all Gal$^-$, confirming that all three mutations affected the same gene. Only in the cross between strains 22A295 (*gal1-209*) and RWJ5d-2 (uninducible mutant) did a few Gal$^+$ diploids arise, probably as a result of intragenic recombination. In summary, the genetic analysis shows that we are dealing with two different mutant alleles of the *kl-GAL1* gene: *gal1-209* which leads only to a galactokinase-negative phenotype, and the mutant allele, which prevents the induction of β-galactosidase and the Leloir enzymes, which we therefore designate *gal1-r*.

The mutant alleles *gal1-209* and *gal1-r* were isolated by integrative cloning using vector pISB (Fig. 1) carrying the *Sal*I-*Bgl*II fragment located upstream from the *kl-GAL1* gene (see Materials and Methods). The mutant alleles were then ligated into a yeast vector consisting of the *Eco*RI fragment from pAWJ9 (Fig. 1) bearing *ARS1*, *KARS2*, and *TRP1*, resulting in plasmids pgal1-209 and pgal1-r. Each plasmid was introduced into strain RWJ5d, and the induction of β-galactosidase was tested (Table 1). The presence of pgal1-209 led to wild-type β-galactosidase activity, whereas the presence of pgal1-r did not lead to induction of β-galactosidase. The observed phenotypes are in agreement with those of the genomic mutant alleles in strains 22A295 and RWJ5d, indicating that the respective mutations had been cloned.

Complementation of *S. cerevisiae gal3* by the *kl-GAL1*. The *GAL3* gene has a striking homology with the galactokinase-encoding genes from *S. carlsbergensis*, *E. coli* (2), and *K. lactis*. We therefore tested whether *kl-GAL1* can suppress the *gal3* phenotype in *S. cerevisiae*. A 3.3-kb *Sca*I fragment

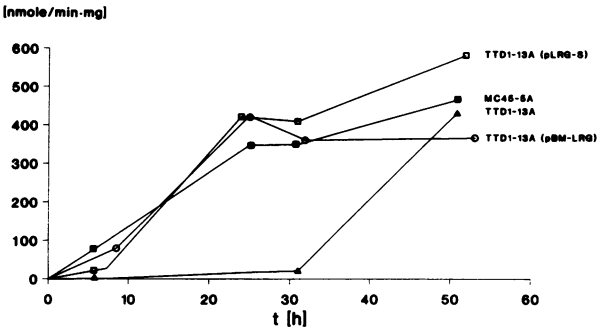

FIG. 3. Induction profiles of α-galactosidase activity in transformants of TTD1-13A (*gal3*) carrying plasmid pLRG-S or plasmid pBM-LRG in comparison with those of the untransformed strain TTD1-13A and Gal$^+$ strain MC45-5A. The cells were pregrown in synthetic medium containing 2% glucose as a carbon source, washed, and transferred to galactose medium at time zero.

from pAWJ9 (Fig. 1) encompassing *kl-GAL1* was subcloned in YRp7, resulting in plasmid pLRG-S. An *S. cerevisiae gal3* mutant (TTD1-13A) was transformed with pLRG-S to tryptophan prototrophy. The transformants showed wild-type induction of α-galactosidase (Fig. 3), indicating that the 3.3-kb fragment, and therefore most probably the *kl-GAL1* gene, is responsible for the phenotypic complementation of *gal3*.

Furthermore, we tested whether the complementation was also exerted when *kl-GAL1* was present on a *CEN* plasmid. The 5.3-kb *Eco*RI-*Sal*I fragment from pAWJ9 (Fig. 1), bearing *kl-GAL1*, was inserted into the *CEN*-containing plasmid pBM150 (14) under removal of the *GAL1-GAL10* promoter fragment, resulting in plasmid pBM-LRG. The α-galactosidase activity curve in Fig. 3 shows that even low-copy-number *kl-GAL1* can restore the normal induction time.

We next examined whether the constitutive presence of galactokinase activity was responsible for the complementation of the *gal3* mutation. If this were true, the *E. coli galK* gene, known to be able to complement *gal1* in *S. cerevisiae*, should also be able to cause normal induction in a *gal3* mutant. The *gal3* mutant TTD1-13A was transformed with plasmid pGK090 (Fig. 1) carrying the *E. coli galK* gene under control of the *PDC1* promoter of *S. cerevisiae*. However, these *galK* transformants still displayed the *gal3* phenotype (data not shown). Measurements of galactokinase activity in these *E. coli galK*-containing transformants showed enzyme activities similar to those of transformants containing *kl-GAL1* (22.4 mU/mg of protein). Thus, the absence of short-term induction cannot be due to the lack of galactokinase activity but apparently results from the absence of an additional function present on the kl-GAL1 protein and required for fast induction of *LAC* gene expression.

***gal3* can be complemented by mutant allele *gal1-209* but not by the *gal1-r* allele.** As shown above, the *gal3* mutation cannot be complemented by only galactokinase activity, at least not by the *E. coli* enzyme activity. Since the *K. lactis* galactokinase enzyme is able to complement *gal3*, we speculated that the *K. lactis* protein carries an additional function or activity not present in the *E. coli* enzyme. The availability of two mutant alleles of *K. lactis GAL1* with different phenotypes allowed us to test whether the regulating activity can be separated from the galactokinase activity.

TABLE 2. Complementation of the *gal3* disruption strain by plasmid pgal1-209

Strain	Gal genotype	Plasmid	α-Galactosidase activity (nmol/min/mg)[a]
MC45-5A	Wild type		560.5
MC45-5A-1	*gal3-d*		0.0
MC45-5A-1	*gal3-d*	pgal1-209	42.0
MC45-5A-1	*gal3-d*	pgal1-r	0.0

[a] Measurements were performed after incubation of cells for 16 h in galactose-containing medium.

In plate tests, only TTDI-13A (*gal3*) transformants carrying pgal1-209 showed induction of α-galactosidase by galactose after 24 h. Untransformed TTDI-13A (*gal3*) as well as transformants containing pgal1-r did not show any detectable α-galactosidase activity under those conditions. α-Galactosidase activities were measured 16 h after induction in cultures of the *gal3* disruption strain MC45-5A-1 transformed with pgal1-209 or pgal1-r. The results presented in Table 2 show that the presence of the *gal1-209* allele led to a partial complementation of about 10% of the wild-type activity, whereas the presence of the *gal1-r* allele had no effect. We conclude that the regulatory function defective in *gal1-r*, but unaffected in *gal1-209*, is responsible for the complementation of *gal3*. This finding further supports the conclusion that the enzymatic activity of the *K. lactis* galactokinase is not required for the *gal3* complementation. However, we cannot completely exclude the possibility that allele *gal1-209*, although defective in encoding an active galactokinase, still encodes an unknown additional enzyme activity also possessed by the wild-type enzyme and involved in the induction process. The *E. coli* galactokinase would not have this enzyme activity. Allele *gal1-209* was able to restore β-galactosidase induction in the *K. lactis gal1-r* mutant RWJ5d, implicating the second function as well.

***gal3* can be also complemented by *S. cerevisiae GAL1*.** *gal3* complementation was initially performed by using the *kl-GAL1* gene. Having excluded that galactokinase activity by itself can suppress the *gal3* phenotype, we investigated whether *gal3* can be complemented by the *S. cerevisiae GAL1* gene. The *gal3* mutant TTD1-13A was transformed with plasmid pBM48, which carries the *S. cerevisiae GAL1* gene under its own promoter. Tryptophan prototrophic transformants were isolated and tested for induction by galactose. As shown in Fig. 4, the presence of pBM48 in *gal3* cells reduced the induction time from 48 h to 24 h. Assuming that the *GAL1* gene on the plasmid is regulated normally, this means that a *GAL1* gene dose that is a few times higher is able to partly repress the *gal3* phenotype. *GAL1* is strictly regulated with a very low noninduced expression level; apparently, raising this level significantly affects the induction process. These data are in agreement with a similar observation of Bhat et al. (3).

To test whether the level of *GAL1* expression at the time of induction determines the induction time in a *gal3* cell, we replaced the *GAL1* promoter with the *PDC1* promoter (15), yielding Y7PDC-G1 (Fig. 1). The *PDC1* promoter leads to transcription under the conditions used before and after induction, and therefore galactokinase should be produced constitutively. As expected, the presence of Y7PDC-G1 in the *gal3* mutant TTD1-13A completely suppressed the mutant phenotype (Fig. 4). The α-galactosidase activity had increased about 8 h after transfer of the cells into galactose

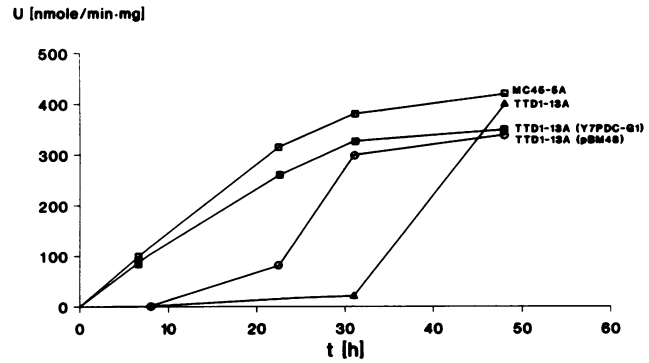

FIG. 4. Induction profile of α-galactosidase activity of TTD1-13A (*gal3*) transformants carrying plasmid Y7PDC-G1 in comparison with those of the untransformed strain, a transformant carrying the *S. cerevisiae GAL1* gene under control of its own promoter on the *CEN* plasmid pBM48, and Gal$^+$ strain MC45-5A.

medium, in a fashion indistinguishable from that in wild-type cells. Clearly, a high level of *GAL1* expression removes the need for the GAL3 protein.

DISCUSSION

The induction mechanisms of the lactose-galactose regulon and of the comparable melibiose-galactose regulon in *K. lactis* and *S. cerevisiae*, respectively, are largely unknown. The *S. cerevisiae gal3* mutation leads to the so-called long-term adaptation phenomenon: a 2- to 5-day delay in the appearance of the enzymes involved in the catabolism of melibiose and galactose (12, 31, 33, 34). In *K. lactis*, a mutation of the type of *gal3* has never been observed. Although preliminary in nature, this finding could mean that the induction mechanism is different in this yeast.

We had set out to obtain more data on the regulation of the lactose system in *K. lactis* and isolated a number of mutants belonging to the constitutive and to the down-regulated type. Here, we describe one regulatory mutant unable to induce all measured enzyme activities of the lactose system.

It was surprising to discover that this mutation could be complemented by the cloned *kl-GAL1* gene, since the phenotype of a *gal1* mutant such as *gal1-209* had been described only as galactokinase minus (23). The genetic analysis of *gal1-r* has shown that indeed it is an allele of *kl-GAL1*. This means that we have identified a novel mutant *kl-GAL1* allele, *gal1-r*, having a phenotype different from that of *gal1-209*. In a strain carrying *gal1-r*, the lactose genes are not inducible, whereas a *gal1-209* mutant lacks only galactokinase activity. This suggests that the *kl-GAL1* protein is bifunctional: it catalyzes phosphorylation of galactose and has a function required for induction of the lactose system. As expected, both functions are absent in the *gal1-d* strain. We further showed that allele *gal1-209* cloned from the mutant defective in galactokinase activity was able to restore induction of *LAC* gene expression in a *gal1-r* strain. Thus, the function of the kl-GAL1 protein required for induction is apparently unaffected in allele *gal1-209* and is not dependent on galactokinase activity. We speculate that it is a regulatory rather than an enzymatic function, although we cannot exclude the possibility that the gene product of *gal1-209* has an unknown enzymatic activity.

The regulatory function of *kl-GAL1* could also be observed in *S. cerevisiae*, which means that in addition to

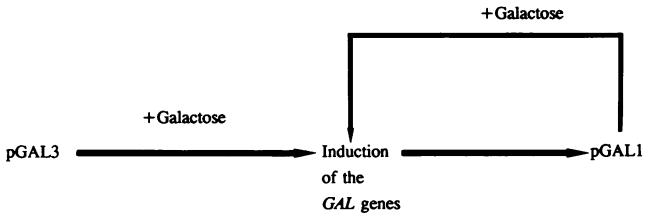

FIG. 5. Scheme for the induction of *GAL* genes.

LAC9 (4, 24, 28, 29), a second regulatory protein is functional in both organisms. The regulatory function can be performed by *S. cerevisiae GAL1* as well. We showed, in agreement with data of Bhat et al. (3), that approximately one additional copy of *GAL1* in an *S. cerevisiae gal3* cell shortens the induction time significantly. Moreover, constitutive expression of the *S. cerevisiae GAL1* gene completely suppressed the *gal3* phenotype.

In *S. cerevisiae*, the GAL3 protein is required only during induction. Therefore, another protein has to be able to maintain the induced state in response to the presence of galactose. This function could be performed by the *GAL1*-encoded galactokinase. The scheme in Fig. 5 summarizes the conclusions from our data. At normal induction, galactose in the presence of the GAL3 protein leads to the induction of the galactose genes, including *GAL1*. Then the galactokinase protein in the presence of galactose reinforces the induction signal, leading to a higher expression level. Upon depletion of galactose, the inducing activity of the galactokinase protein decreases, after which transcription of the *GAL* genes, including *GAL1*, decreases.

Why does *S. cerevisiae* have *GAL3*, the function of which can also be fulfilled by *GAL1*? We assume that the GAL3 protein is present in noninduced cells in levels high enough to enable fast induction. At high concentrations, galactose uptake is constitutive. The inducible *GAL2* gene, encoding a galactose permease (8), is required for growth only on low levels of galactose (less than 0.5%) (3). If galactokinase activity were present in uninduced cells, the presumably toxic galactose 1-phosphate would be formed immediately after the cells are exposed to galactose. Therefore, a similar protein without galactokinase activity, i.e., the GAL3 protein, has acquired this function.

In *K. lactis*, the situation might be different. The natural substrate of this yeast species is lactose, which after being taken up into the cell is hydrolyzed into galactose and glucose. The generation of galactose is dependent on at least one inducible gene product, β-galactosidase. Assuming that galactose is not a natural substrate, the presence of galactokinase in noninduced cells would not be harmful. The mode of *GAL1* expression in *K. lactis* is such that its gene product could play this role in induction. Noninduced wild-type cells already contain a basal level of galactokinase activity which is much higher than that in noninduced *S. cerevisiae* (33) cells. If in evolution lactose metabolism was derived from galactose metabolism, we believe that in *K. lactis*, *GAL3* has been lost and its function has been taken over by *GAL1*.

It has been proposed that *GAL3* is involved in the synthesis of the inducer or coinducer (34, 35) and that in *gal3* cells the Leloir enzymes are required for inducer synthesis (6). However, we have shown that the function of the *GAL3* product can be fulfilled by a galactokinase-minus *gal1* allele. This makes it unlikely that *GAL3* enzymatically produces an

inducer. Moreover, Bhat et al. (3) were not able to measure any galactokinase activity in the presence of overexpressed *GAL3*. We propose that no metabolites of galactose are involved in induction and that the requirement of the Leloir enzymes for induction in *gal3* mutants (3, 6, 33, 35) could be explained by the impairment of the *GAL1* regulatory function by accumulating galactose 1-phosphate.

Recently, Mylin et al. (21) presented evidence showing that the GAL4 protein occurs in at least three forms which differ in degree of phosphorylation. It is tempting to speculate that phosphorylation or dephosphorylation of the GAL4 protein is the end point of the induction pathway. The protein kinase responsible for the phosphorylation could be activated directly or indirectly by the GAL3 or GAL1 protein in response to the interaction with galactose within the cell or at the cell membrane. Evidence from Ramos et al. (22) showing that *GAL1* is required for functional high-affinity galactose uptake could indicate a close association between the galactokinase and the carrier in the membrane. Alternatively, in the presence of galactose, the GAL1 protein could directly or indirectly lead to the activation of the GAL4 positive regulator, probably by overcoming *GAL80* repression.

A comparison of the GAL1 proteins from *S. carlsbergensis* and *K. lactis* with the *E. coli galK* gene product indicates a long central region lacking in the *E. coli* galactokinase and containing regions of homology with the GAL3 protein. We are presently studying the function of *GAL1* by in vitro mutagenesis and mutant analysis.

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