MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7566–7576
0270-7306/93/127566-11$02.00/0

Vol. 13, No. 12

Gal80 Proteins of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* Are Highly Conserved but Contribute Differently to Glucose Repression of the Galactose Regulon

FRANK T. ZENKE, WOLFGANG ZACHARIAE, ASTRID LUNKES,† AND KARIN D. BREUNIG*

Institut für Mikrobiologie, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany

Received 13 May 1993/Returned for modification 8 July 1993/Accepted 23 August 1993

We cloned the *GAL80* gene encoding the negative regulator of the transcriptional activator Gal4 (Lac9) from the yeast *Kluyveromyces lactis*. The deduced amino acid sequence of *K. lactis GAL80* revealed a strong structural conservation between *K. lactis* Gal80 and the homologous *Saccharomyces cerevisiae* protein, with an overall identity of 60% and two conserved blocks with over 80% identical residues. *K. lactis gal80* disruption mutants show constitutive expression of the lactose/galactose metabolic genes, confirming that *K. lactis* Gal80 functions in essentially in the same way as does *S. cerevisiae* Gal80, blocking activation by the transcriptional activator Lac9 (*K. lactis* Gal4) in the absence of an inducing sugar. However, in contrast to *S. cerevisiae*, in which Gal4-dependent activation is strongly inhibited by glucose even in a *gal80* mutant, glucose repressibility is almost completely lost in *gal80* mutants of *K. lactis*. Indirect evidence suggests that this difference in phenotype is due to a higher activator concentration in *K. lactis* which is able to overcome glucose repression. Expression of the *K. lactis GAL80* gene is controlled by Lac9. Two high-affinity binding sites in the *GAL80* promoter mediate a 70-fold induction by galactose and hence negative autoregulation by Gal80. Gal80 in turn not only controls Lac9 activity but also has a moderate influence on its rate of synthesis. Thus, a feedback control mechanism exists between the positive and negative regulators. By mutating the Lac9 binding sites of the *GAL80* promoter, we could show that induction of *GAL80* is required to prevent activation of the lactose/galactose regulon in glycerol or glucose plus galactose, whereas the noninduced level of Gal80 is sufficient to completely block Lac9 function in glucose.

Regulation of transcription in eukaryotic cells by gene-specific activators in many cases is influenced by interaction of these regulators with non-DNA-binding proteins. One of the first examples of a protein controlling the activity of an activator by direct interaction was the Gal80 protein of *Saccharomyces cerevisiae*, which regulates the activation function of the prototype transcriptional activator Gal4, but the details of the regulation are far from being understood (for a review, see reference 20).

Gal4 activates genes involved in galactose metabolism in response to galactose in the medium. In the absence of inducing sugars, induction is prevented by the regulatory protein Gal80 (12, 16). Loss of Gal80 activity confers constitutive expression of Gal4-regulated genes under noninducing conditions, demonstrating that transcriptional activation by Gal4 is independent of Gal80 function (45, 57).

Gal80 has been shown to directly interact with a subdomain of Gal4 adjacent to and partly overlapping an activation domain (23, 31–33, 51). According to the current model, binding of Gal80 protein blocks activation by covering this domain (22, 31, 33, 45). The signal generated by the presence of galactose in the medium may cause dissociation of the complex and release Gal4 from inhibition or, alternatively, induce a conformational change in the complex from an inactive to an active state. Recently, the finding that Gal80 is associated with Gal4 under inducing conditions was taken as evidence for the latter possibility (6, 28, 43). Phosphorylation of Gal4 was found to accompany the active state (36, 38, 44) and may be involved in the hypothetical transition.

* Corresponding author.
† Present address: Institut für Neuropathologie, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany.

An understanding of the induction process requires a detailed analysis of the Gal4-Gal80 interaction. Dissection of Gal4 into functional domains has shown that the C-terminal 30 amino acids are sufficient to bind Gal80 protein (33). In contrast, deletion analysis of the *GAL80* gene indicated that most of the coding sequence is required for expression of a functional repressor (41), and relatively little information on the structural organization of this repressor could be obtained in this way.

We therefore undertook an approach to clone the *GAL80* homolog of *Kluyveromyces lactis* and examine its sequence for evolutionary conserved domains. Comparison between homologous genes of this budding yeast and *S. cerevisiae* had provided useful information in a number of cases (14, 29, 37, 48, 49, 55). The *GAL4* homolog of *K. lactis LAC9* had previously been cloned both by complementation of an *S. cerevisiae gal4* mutant (49) as well as by complementation in *K. lactis* (61). The transcriptional activator Lac9 regulates galactose as well as lactose metabolism in *K. lactis* (53) and can be functionally replaced by Gal4 (46). Like Lac9, Gal4 function in *K. lactis* is regulated by lactose and galactose. A region with 88% identical residues to the Gal80 binding domain of Gal4 is located at the C terminus of Lac9 (49, 61), and mutations in that region result in a phenotype resembling that of equivalent mutations in Gal4 (9). All of these findings strongly suggested that a *GAL80* homolog exists in *K. lactis*, although attempts to clone that gene by complementation of the available constitutive *lac10* (10) and *lrg4* mutants (58) have failed so far.

Here we report on the isolation of the *GAL80* gene of *K. lactis* in an alternative constitutive mutant, *rlm1* (*K. lactis gal80-1*), using a screening procedure for reduced expression of the β-galactosidase gene. Sequencing of the cloned *K.*

lactis GAL80 gene revealed a much higher conservation of the negative regulators of Gal4 between *K. lactis* and *S. cerevisiae* than exists for the activators. The phenotype of disruption mutants confirmed that the *K. lactis GAL80* gene is required to block activation of the genes involved in galactose and lactose metabolism in the absence of inducing sugars and thus is functionally equivalent to *S. cerevisiae GAL80*. However, in contrast to that in *S. cerevisiae gal80* mutants, glucose repression is ineffective in *gal80* mutants of *K. lactis*. We propose that this difference in phenotype is caused by differences in the activator concentrations between the two yeasts rather than by any qualitative differences in the mode of regulation. In line with this proposal, we demonstrate by analysis of *K. lactis GAL80* gene expression that like *S. cerevisiae GAL80*, *K. lactis GAL80* is a galactose-inducible gene, but the level of induction is 70-fold, as opposed to 5-fold for *S. cerevisiae GAL80* (17). Thus, the principles of the regulatory circuits that control galactose metabolism seem to be conserved between the two yeasts, but quantitative differences lead to modifications of the gene expression patterns.

MATERIALS AND METHODS

Yeast strains and transformation. All *K. lactis* strains except KB101 were isogenic to JA6 (α *ade ura3 trp1-11 LAC9-2*) (5). The two *gal80* deletion mutants JA6/D801 (*gal80Δ1::URA3*) and JA6/D802 (*gal80Δ2::URA3*) and the promoter mutant JA6/DUAS80 (*GAL80-Δ349/178*) were obtained by one-step gene displacement (47) with *Xho*I-cleaved plasmids pD801, pD802, and pDUAS80, respectively. JA6/D801R (α *ade trp1 gal80Δ1::ura3*) and JA6/D802R (α *ade trp1 gal80Δ2::ura3*) are *ura3* mutants of JA6/D801 and JA6/D802, respectively, isolated by selection for resistance to 5-fluoro-orotic acid (2). The deletion mutant JA6/D80DL9 (α *ade trp1 gal80Δ1::ura3 lac9::URA3*) was constructed by one-step gene displacement in JA6/D801R with *Eco*RI- and *Hpa*I-cut plasmid pDL9 (5). JA6/2-2 (α *ade trp1 LAC9-2::URA3-LAC9-2*) (25) and DL9R (α *ade trp1 lac9::ura3*) (63) have been described previously. JA6/1c (*gal80-1*) resulted from UV mutagenesis of JA6. KB101 (**a** *ade trp1 ura3 gal80-1*) is a meiotic segregant resulting from a cross between JA6/1c and KB6-2C (**a** *ade ura3 hisB-35*). KB6-2C is a meiotic segregant from a cross between JA6 and HA35 (*hisB-35*), which in turn was derived from CBS2360 by ethyl methanesulfonate mutagenesis (58). The 1.6 μm plasmid pKD1 (7) was introduced into KB101 by cotransformation with KEp6 to increase the stability of the KEp6-based plasmids (1). The presence of pKD1 in the transformants was confirmed by Southern analysis. Strain KB101/I80 (*gal80-1::URA3-GAL80*) was constructed by integrating pI80 at the homologous locus after cleavage with *Sau*I. Yeast transformation and selection for recombinants were performed as described previously (11, 24, 63).

Culture conditions. For Northern (RNA) analysis and enzyme measurements, yeast strains were grown in synthetic medium (SM; 0.67% yeast nitrogen base without amino acids supplemented with an amino acid-purine-pyrimidine mixture [54]). For selective growth, the corresponding component was omitted from the mixture. For immunoblot analysis, strains were grown in rich medium (YEP) (1% yeast extract, 2% peptone). As a carbon source, 2% glucose, 2% galactose, 2% glucose plus 2% galactose, or 3% glycerol was added after autoclaving. Yeast cultures were pregrown in glucose-containing medium to an optical density at 600 nm (OD_{600}) of 0.5 to 1. Cells were washed with prewarmed medium lacking the carbon source and resuspended in the appropriate medium to an OD_{600} of 0.1. The main cultures were then grown to an OD_{600} of 0.5 to 0.8, and cells were harvested by centrifugation. Analysis of β-galactosidase expression was performed on SM agar plates containing 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml.

Plasmids. The *K. lactis GAL80*-containing plasmids pKB1-1 and pKB2-1 were isolated from a genomic library of *K. lactis* CBS 2359/152 (60) based on the episomal plasmid KEp6 (1).

K. lactis GAL80 was subcloned from pKB1-1 on a 4.1-kb *Xho*I fragment into pBluescript KS(+) (Stratagene, Heidelberg, Germany), giving pKlGAL80. The integration plasmid pI80 was constructed by inserting the *S. cerevisiae URA3* gene on a 1.2-kb *Hin*dIII fragment into the *Sma*I site located in the polylinker region of pKlGAL80. The *gal80::URA3* disruption plasmid pD801 was obtained after cutting pKlGAL80 with *Nde*I (+448) and *Nco*I (+1094; the numbering system of *K. lactis GAL80* refers to the ATG as +1) and inserting the *S. cerevisiae URA3* gene on a 1.2-kb *Hin*dIII fragment. In the case of pD802, sequences from the *Sau*I site at −349 to the *Nco*I site at +1094 were replaced with the *URA3* gene. The two Lac9 binding sites in the *K. lactis GAL80* promoter were deleted by cleaving pKlGAL80 with *Sau*I (−349) and *Bst*NI (−178), followed by filling in and religation to give plasmid pDUAS80.

The *GAL80* gene from *S. cerevisiae* was subcloned as a 3.2-kb *Hin*dIII fragment from YCp50GAL80 (50) into the *Hin*dIII site of KEp6.

K. lactis Gal80–β-glucuronidase (GUS) fusions were constructed as follows. A 0.9-kb *Cla*I fragment (−660 to +262) from pKlGAL80 containing the wild-type *K. lactis GAL80* promoter or the equivalent 0.7-kb fragment from pDUAS80 containing the deleted promoter was subcloned into pT7T3 19U (Pharmacia, Freiburg, Germany) cut with *Cla*I. These plasmids were cut with *Hpa*I and *Eco*RI, and a 1.8-kb *Sma*I-*Eco*RI fragment from pBI101.1 (19) was integrated to fuse the first 24 amino acids of *K. lactis* Gal80 to the N terminus of bacterial GUS. The resulting plasmids, p80GUS and pD80GUS, were cleaved with *Sac*I and *Asp* 718, and a 6.1-kb *Sac*I-*Asp* 718 fragment from pKATUC41 containing ARS1, KARS12, *S. cerevisiae TRP1* and *URA3*, and *K. lactis* CEN2 sequences (KATUC cassette) was inserted, giving pC80GUS and pCD80GUS, respectively. pKATUC41 was constructed by cloning the 6.1-kb KATUC cassette as an *Eco*RI-*Sal*I fragment from pKATUC3 (62) via *Xho*I linkers into pBluescript KS(+). The resulting plasmid, pKATUC4, was cleaved with *Sph*I and *Bam*HI and then religated, thereby recreating the *Bam*HI site to give pKATUC41.

Plasmids pKR7-Gal (59) and pJ2B3 (55) used to detect mRNAs of the *GAL1-GAL10-GAL7* gene cluster and the histone H3 gene *HHT1* have been described elsewhere.

Sequencing of *K. lactis GAL80*. Different fragments of pKB1-1 were subcloned into the vector pT7T3 19U. Sequencing of the *GAL80* gene was performed on both strands with a T7 sequencing kit (Pharmacia). Homology comparison between the deduced Gal80 protein sequences of *S. cerevisiae* and *K. lactis* was carried out with the program GAP from the University of Wisconsin Genetics Computer Group package (8) on a μVAX Station II.

Enzyme measurements. Enzyme activities were measured in crude extracts prepared by glass bead disruption as described previously (63). β-Galactosidase was assayed in a buffer (5 mM Tris-HCl [pH 7.5], 10 mM KCl, 5% glycerol)

TABLE 1. Influence of *gal80* mutations on *LAC4* gene expression

Strain	Relevant genotype	β-Galactosidase activity (mU/mg)[a]			
		Glucose	Galactose	Glucose-galactose	Glycerol
JA6	*GAL80*	1.3 ± 0.5	2,610 ± 740	15 ± 3	82 ± 20
KB101	*gal80-1*	2,570 ± 870	3,030 ± 1,170	2,110 ± 940	8,260 ± 670
JA6/D801	*gal80Δ1::URA3*	1,310 ± 210	2,920 ± 1,250	1,130 ± 150	8,250 ± 3,310
JA6/D802	*gal80Δ2::URA3*	1,540 ± 140	4,350 ± 460	1,370 ± 30	8,570 ± 2,480
JA6/D80DL9	*gal80Δ1::ura3/lac9::URA3*	0.6 ± 0.1	11 ± 1	0.6 ± 0.3	44 ± 10
DL9	*lac9::URA3*	0.5 ± 0.4	15 ± 4.2	0.5 ± 0.1	50 ± 6

[a] Cells were grown in SM medium containing the indicated carbon sources. The mean value ± standard deviation of three or two (JA6/D802, JA6/D80DL9, and DL9) independent measurements is given.

containing 4 mg of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 0.25 mg of bovine serum albumin per ml.

GUS measurements were performed in GUS extraction buffer (10 mM sodium phosphate [pH 7.0], 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100 [19]) containing 1 mM *p*-nitrophenyl-β-D-glucuronide, and the reaction was monitored photometrically. The extinction coefficient of *p*-nitrophenol in GUS extraction buffer was 8,800 M^{-1} cm^{-1}, as determined by three independent measurements.

Protein concentration in the crude extract was determined by the method of Lowry et al. (30).

Northern blot analysis. Total RNA was prepared by extraction with phenol-sodium dodecyl sulfate (SDS) as described by Schmitt et al. (52). Poly(A)$^+$ RNA was isolated by oligo(dT) column chromatography (18). RNA was separated in 1% agarose gels containing 6% formaldehyde and blotted onto nylon membranes (Hybond N; Amersham, Braunschweig, Germany). Electrophoresis, transfer, hybridization, and washing were performed as indicated by the supplier. Hybridization probes were labeled with [α-^{32}P]dATP by random priming. For detection of *GAL1*, *GAL7*, and *GAL10* mRNAs in total RNA, plasmid pKR7-GAL (59) was used. Plasmid pJ2B3 (55) containing the *HHT1*, *HHT2*, *TRP1*, and *IPP* genes served as a control in this experiment. To detect the *K. lactis GAL80* transcript, three intragenic *Eco*RV fragments were hybridized to poly(A)$^+$ RNA. In this case, a 0.7-kb *Asp* 718 fragment from plasmid pJ2B3 containing only sequences of the histone H3 and H4 genes, *HHT1* and *HHT2*, respectively, served as an internal standard. Northern blots were quantitated by densitometric scanning of different exposures to ensure a linear response of the film, using a Molecular Dynamics model A300 laser densitometer.

Gel retardation assays. Preparation of *K. lactis* S100 extracts and binding experiments were performed essentially as described previously (63). The relative affinities of Lac9 for the binding sites in the *K. lactis GAL80* promoter were determined by competition experiments using a labeled UASI fragment from the *LAC4* promoter and unlabeled fragments containing different Lac9 binding sites as competitor DNA (62). A 397-bp *Sau*I-*Acc*I fragment (−349 to +45) from the *K. lactis GAL80* promoter containing UASI and UASII was used (fGAL80). The fragment was cleaved by *Mbo*II or *Ava*II to assay for binding to UASI and UASII, respectively. Complete digestion was confirmed by analysis on a silver-stained polyacrylamide gel. Retarded bands were cut out from the dried gel and quantitated by Cerenkov counting.

Immunoblot analysis. Western blot (immunoblot) analysis was performed as described previously (63). Polyclonal antisera from rabbit raised against Lac9 and Gal1 fused to glutathione *S*-transferase were used for immunological detection. Densitometric analysis was performed as described above.

Nucleotide sequence accession number. The nucleotide sequence accession number of *K. lactis GAL80* in the EMBL data bank is Z21512.

RESULTS

The *rlm1* mutation results in constitutive expression of lactose/galactose metabolic genes. In *K. lactis* JA6, the endogenous β-galactosidase gene *LAC4* is expressed at a very low level during growth in the absence of galactose or lactose; thus, cells form white colonies on glucose plates containing X-Gal. In an attempt to isolate negative regulators of the regulon, we mutagenized these cells and screened for expression of higher levels of β-galactosidase on this medium which would result in cleavage of the chromogenic substrate and the formation of blue colonies. Among the mutants that were obtained, the *rlm1* (regulator of lactose metabolism) mutation showed the most pronounced phenotype and was characterized further. The blue phenotype was recessive in a cross to the wild-type strain KB6-2C and segregated 2:2 in all of eight tetrads analyzed. Measurements of β-galactosidase activity in KB101 *rlm1*, a meiotic segregant, confirmed that the blue color of the colonies on X-Gal reflected elevated enzyme levels. In the mutant, β-galactosidase activity on glucose-containing medium was almost identical to the fully induced level on galactose of the wild type; on glycerol, enzyme activities were even higher (Table 1, KB101).

To determine whether other genes of the *GAL* regulon were also affected by the mutation, the RNA levels of the *GAL1*, *GAL10*, and *GAL7* genes were compared between the wild-type JA6 and the mutant. Northern blotting (Fig. 1) revealed that in KB101, RNA concentrations were similar between repressed (glucose- or glucose-galactose-grown) and induced (galactose-grown) cells, indicating that all three genes were expressed constitutively in the mutant (Fig. 1). Thus, a negative regulator of the entire *LAC/GAL* regulon seemed to be affected by the mutation.

Complementation of the *rlm1* mutant by the *GAL80* gene of *S. cerevisiae*. Coordinate regulation of the genes of the *LAC/GAL* regulon is achieved by the transcriptional activator Lac9. The phenotype of the *rlm1* mutant suggested that a negative regulator of Lac9 (*K. lactis* Gal4) function could be inactive. The *GAL80* gene encodes such a negative regulator in *S. cerevisiae*.

To examine whether a Gal80-like function was affected in

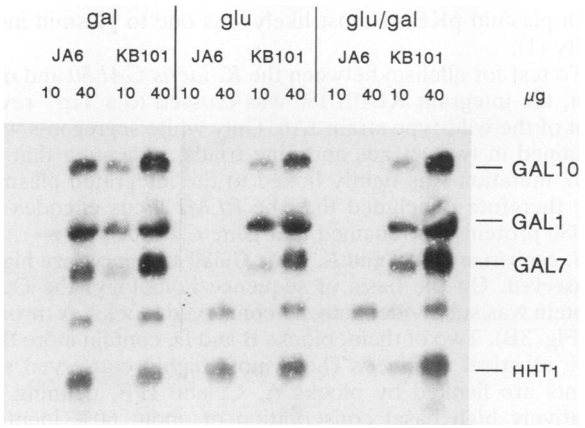

FIG. 1. Northern analysis of *GAL1*, *GAL10*, and *GAL7* mRNAs in the constitutive mutant. Total RNA from yeast strains JA6 and KB101 (*rlm1*) grown in SM medium containing the indicated carbon sources was isolated and separated on an agarose gel containing formaldehyde. After blotting onto a nylon membrane, the RNA was hybridized with [α-^{32}P]dATP-labeled plasmid pKR7-Gal (59), which contains the entire *GAL1-GAL10-GAL7* gene cluster and plasmid pJ2B3 (55), containing the *HHT1* (histone H3), *HHT2*, and *IPP* genes.

KB101, we tested the ability of the *S. cerevisiae GAL80* gene to complement the *rlm1* mutation. The gene was cloned in the *K. lactis* vector KEp6, which is based on the 2μm-like multicopy plasmid pKD1 (7). After transformation of KB101 with KEp6GAL80 and plasmid pKD1, which is required for stability of the KEp6 vector (see Materials and Methods), Ura$^+$ transformants formed light blue-green colonies on X-Gal plates containing glucose and were clearly distinguishable from control transformants with the vector plasmid KEp6. The presence of the *S. cerevisiae GAL80* gene on the plasmid reduced β-galactosidase activities in glucose-grown cells from 2,000 mU/mg in the control to about 400 mU/mg. To exclude the possibility that this reduction was due to titration of the Lac9 activator by the Lac9 binding site in front of the *GAL80* gene (3, 5, 56), the *GAL80* reading frame was disrupted by filling in the *Eco*RI site in the coding region (40). KB101 transformed with the mutated plasmid KEp6GAL80m showed the same color as did the KEp6 transformants. Thus, the observed partial complementation of the *rlm1* mutant phenotype was not due to a titration effect but depended on an intact reading frame of the *S. cerevisiae GAL80* gene. Therefore, the *rlm1* mutation was a good candidate for a mutation in the *K. lactis* equivalent of the *S. cerevisiae GAL80* gene.

Isolation of the *K. lactis GAL80* gene. To isolate DNA fragments that would complement the *rlm1* mutation, we transformed KB101 with a *K. lactis* genomic library (60) and screened for reduced β-galactosidase activity on X-Gal plates. Since the presence of X-Gal in the plates drastically reduced the number of transformants, probably as a result of toxic effects of the blue dye, Ura$^+$ transformants were first selected on YNB-glucose plates and subsequently replica plated on X-Gal plates.

Among 5,000 dark blue transformants, two light blue colonies were obtained. Plasmids isolated from these transformants were characterized by restriction analysis. Two plasmids, pKB1-1 and pKB2-1, that consisted of the vector KEp6 and inserts, both approximately 6 kb in length, were obtained. The two inserts had an overlap of more than 5 kb. Retransformation of these plasmids into KB101 confirmed that the light blue phenotype of colonies on X-Gal plates was coupled to the presence of the plasmid.

The complementing activity was mapped on the insert by deletion analysis of pKB1-1. As shown in Fig. 2, all sequences left to the *Sau*I site could be deleted without loss of activity, whereas a deletion in the right half or filling in of the

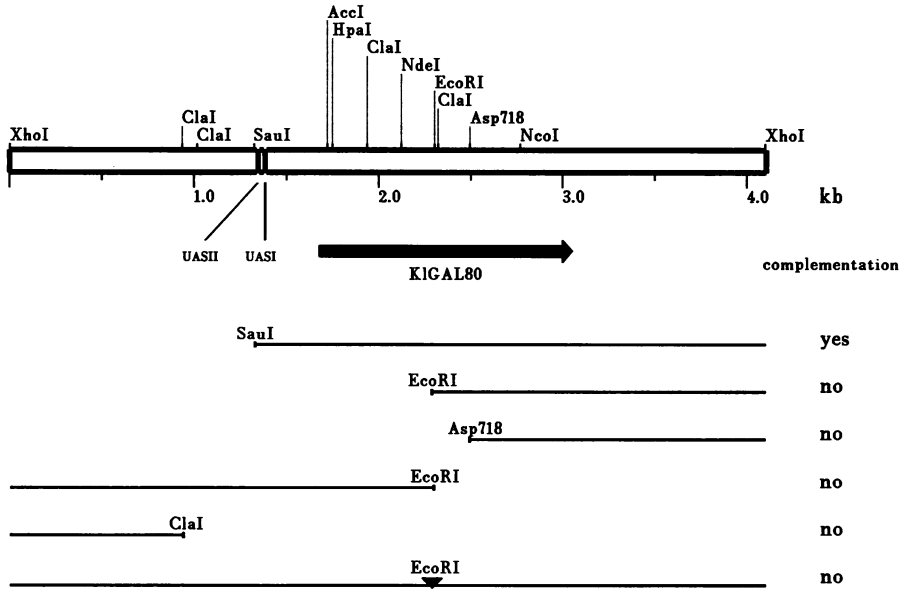

FIG. 2. Restriction map of the 4.1-kb *Xho*I genomic fragment containing the *K. lactis GAL80* gene. The relevant restriction sites used for complementation analysis, deletion, and reporter gene fusion are shown. The Lac9 binding sites UASI (5′-CGGCTCATAGTCTTCCG-3′) and UASII (5′-CGGACCTCTCCATTCCG-3′) at positions −295 and −333, respectively, found in the *K. lactis GAL80* promoter are marked by black boxes. Deletions were constructed in plasmid pKB1-1, and the indicated segments were tested by transformation into the constitutive strain KB101 (*rlm1*) for the ability to complement the constitutive phenotype on SM-glucose medium containing X-Gal.

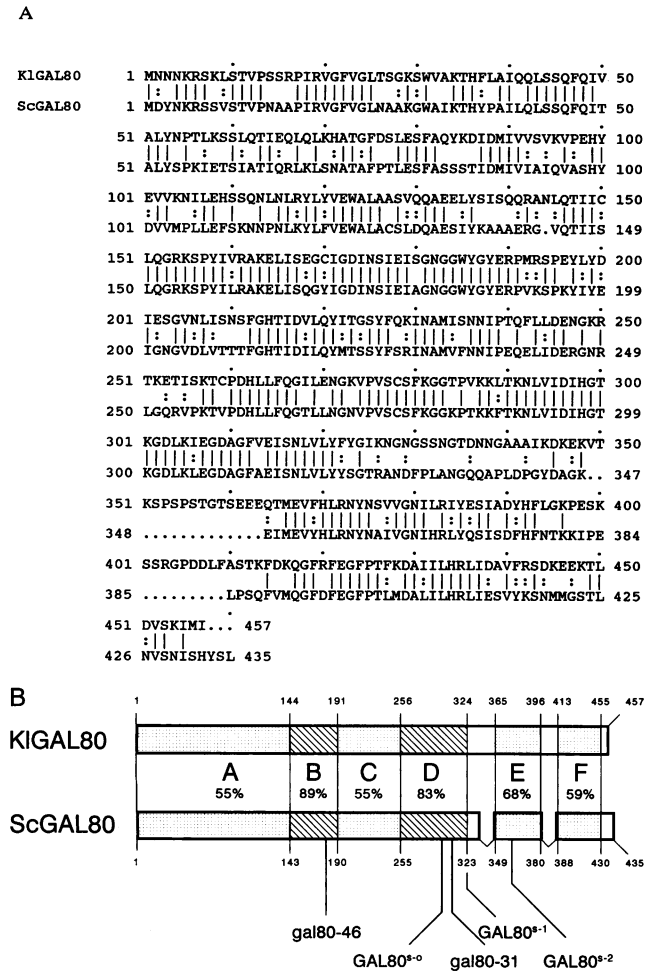

FIG. 3. Comparison between the deduced amino acid sequences of *K. lactis* Gal80 (KlGAL80) and *S. cerevisiae* Gal80 (ScGAL80). (A) The alignment was performed with the program GAP (8) of the University of Wisconsin Genetics Computer Group package. Lines indicate identical amino acids, and conservative exchanges are marked by dots. (B) Schematic representation of the alignment. The proportion of identical amino acids is given in percentage of the length of each block of *K. lactis* Gal80. The hatched regions indicate the most highly conserved blocks, stippled regions consist of 55 to 68% identical residues, and unmarked areas show no significant similarity.

*Eco*RI site abolished complementation. Sequencing of a 2.2-kb region downstream from the second *Cla*I site gave an open reading frame of 1,371 nucleotides encoding a protein of 457 residues and molecular weight of 51,000.

Comparison of the sequence with entries in a data bank (see Materials and Methods) revealed a high similarity with the sequence of the *S. cerevisiae GAL80* gene. Over the entire coding region, 60% of both nucleotides and deduced amino acid residues are identical, and there is an overall similarity at the protein level of 75% (Fig. 3A). From these data and the complementation tests, we conclude that the isolated gene is the *K. lactis* homolog of *GAL80*.

Integration of the *K. lactis GAL80* gene at the homologous chromosomal locus in the *rlm1* mutant strain KB101 gave white colonies, indicating that full complementation could be obtained in single copy. The partial complementation seen with plasmid pKB1-1 most likely was due to plasmid instability (1).

To test for allelism between the *K. lactis GAL80* and *rlm1* loci, the integrant KB101/I80 was crossed to a Trp^+ revertant of the wild-type strain JA6. Only white segregants were obtained in two tetrads and nine triads, indicating that the *rlm1* mutation was tightly linked to the integrated plasmid. We therefore concluded that the *RLM1* locus encodes the Gal80 protein and renamed that gene *GAL80*.

***S. cerevisiae* Gal80 and *K. lactis* Gal80 sequences are highly conserved.** On the basis of sequence homology, the Gal80 protein was subdivided into six conserved blocks, A through F (Fig. 3B). Two of them, blocks B and D, contain more than 80% identical residues. These most highly conserved segments are flanked by blocks A, C, and E/F, defining the relatively high basal conservation of about 60% identity. Blocks E and F, although rather similar to each other, have been assigned as two blocks since they are separated by an insertion resulting from the longer *K. lactis* Gal80 protein. Another length difference exists in the linker region that separates block D from block E. This segment from residues 325 to 365 of *K. lactis* Gal80 and 324 to 349 of *S. cerevisiae* Gal80 is the only one in which the two proteins show no significant similarity. A sharp boundary separates the linker from the highly conserved block D. The N-terminal two-thirds of the two proteins (blocks A to D) can be almost perfectly aligned, with only one extra residue in *K. lactis* Gal80.

All positions to which *S. cerevisiae GAL80* mutations have been mapped (41, 42) are conserved in the *K. lactis* protein (Fig. 3B). In block D, constitutive *gal80*$^-$ as well as noninducible *GAL80*^{s} mutations are located. This region may thus play an important role in interaction with Gal4 or Lac9, since both mutant phenotypes have been ascribed to changes in the affinity to the activator (50).

Outside the protein-coding region of the gene, the only sequence homology which may be significant is a potential Lac9/Gal4 binding site. Whereas in the *GAL80* promoter region of *S. cerevisiae* there is one such site at position −165 from the ATG (17), two sequences, 5′-CGGCTCATAGTCT TCCG-3′ and 5′-CGGACCTCTCCATTCCG-3′, conforming to the consensus sequence $CGGN_5A/TN_5CCG$-3′ (13), could be found in *K. lactis GAL80* at positions −333 and −295 (central base pair). This finding suggested that *K. lactis GAL80* expression, like *S. cerevisiae GAL80* expression (17), is controlled by Lac9/Gal4 (see below).

Disruption of the *K. lactis GAL80* gene results in constitutive expression of Lac9-controlled genes and a loss of repressibility by glucose. Analysis of the original mutant KB101 *gal80-1* had already shown that in *K. lactis*, as in *S. cerevisiae*, the Gal80 function was required to block expression of all Lac9-controlled genes in the absence of galactose (Fig. 1; Table 1). These results were confirmed in two independent *gal80* deletion mutants, JA6/D801 and JA6/D802 (see Materials and Methods). All three mutant strains expressed high levels of β-galactosidase under noninducing (glycerol) and even under repressing (glucose and glucose-galactose) conditions (Table 1, K101, JA6/D801, and JA6/D802). Under conditions of glucose repression, the situation in *K. lactis* contrasts sharply with that in *S. cerevisiae*. Whereas in *S. cerevisiae gal80* mutants, enzyme activities in glucose were around 5% of the induced wild-type levels (39), *gal80* mutants of *K. lactis* were induced up to at least 35%. This finding implies that in *K. lactis*, the Gal80 protein is required for the maintenance of the glucose-repressed state. There was no difference regardless of whether galactose was added

to the glucose medium, indicating that regulators other than Gal80 which might be capable of mediating a galactose-specific effect are negligible under these growth conditions.

Additional disruption of the *LAC9* gene in a *gal80* deletion mutant abolished constitutivity, and Lac9-dependent gene expression in the double mutant was indistinguishable from that in the *lac9* single mutant (Table 1, JA6/D80DL9 and DL9), confirming that Gal80 functions only through Lac9.

The β-galactosidase activities in the *lac9* mutants were low under all growth conditions, but in the presence of glucose (in glucose and glucose-plus-galactose media), they were still more than 10-fold lower than in its absence. This finding indicates that a *lac9*-independent form of glucose repression exists. If this repression is suppressed by a highly active Lac9 activator, the involvement of Gal80 in glucose repression could be an indirect consequence of its influence on Lac9 activity (see also Discussion). Some negative influence of glucose can still be observed in the *gal80* single mutants (Table 1, JA6/D801 and JA6/D802), that is, when Lac9 is highly active. It is hard to distinguish whether this reflects residual glucose repression of the *LAC4* gene, glucose repression of *LAC9* gene expression (see below), or a combination of both. In glycerol-grown cells, the β-galactosidase activities were two- to threefold higher than on galactose, suggesting that even galactose may exert some repression which in the mutants becomes evident as a result of the absence of galactose induction.

Expression of the autoregulated *LAC9* activator gene is moderately elevated in *K. lactis gal80* mutants. Since the *LAC9* gene is controlled by autoregulation (62), we have analyzed the influence of *K. lactis GAL80* on Lac9 synthesis. In contrast to the *LAC* and *GAL* genes, which encode metabolic enzymes, induction of the regulatory gene *LAC9* in the wild type is only a factor of 2 to 3 (26, 62, 63). Lac9-specific antibodies were used to compare the Lac9 protein concentration between JA6 and JA6/D802R (*gal80Δ2::ura3*) by immunoblotting. We have shown before that this method is sufficiently accurate to quantify the Lac9 concentration within a confidence range of less than 20% (62, 63). A typical experiment is shown in Fig. 4, and the data of three independent experiments are summarized in Table 2. Under noninducing conditions (in the absence of galactose), the *gal80* mutant contained about 1.7-fold-higher Lac9 levels than did the isogenic wild type in both repressed and derepressed cells, confirming the dependence of *LAC9* gene regulation on the Gal80 function.

Two other conclusions can be drawn from these data. First, despite the positive feedback loop, no further amplification of Lac9 occurs in the absence of *K. lactis* Gal80, indicating that additional mechanisms that limit activation of the *LAC9* promoter by Lac9 must exist. Second, the Lac9 concentration is still carbon source regulated in a *gal80* mutant, although to a lower extent than in the presence of Gal80. The moderate variations in Lac9 concentration in the mutant correlate with the activity of Lac9-controlled genes. Concentrations of galactokinase and β-galactosidase, which were determined in parallel to the Lac9 protein concentration by Western blotting and enzyme assays, respectively (Table 2), showed the behavior typical of all genes of the regulon, with lower levels on glucose and glucose-galactose than on galactose-glycerol and glycerol.

The *K. lactis GAL80* gene is regulated by Lac9 and Gal80, as are the other lactose/galactose metabolic genes. The presence of two potential Lac9 binding sites in the *GAL80* promoter (Fig. 2) suggested that the *K. lactis GAL80* gene may also be controlled by Lac9 and in that case should be induced by galactose. To address this question, RNA was isolated from cells grown in glucose, galactose, glucose plus galactose, and glycerol and analyzed by Northern blotting (Fig. 5). The band detected with a *K. lactis GAL80* probe had a size of 1.7 kb, in good agreement with the size of the *GAL80* open reading frame (1.37 kb), and was not present in the *gal80* disruption strain JA6/D802.

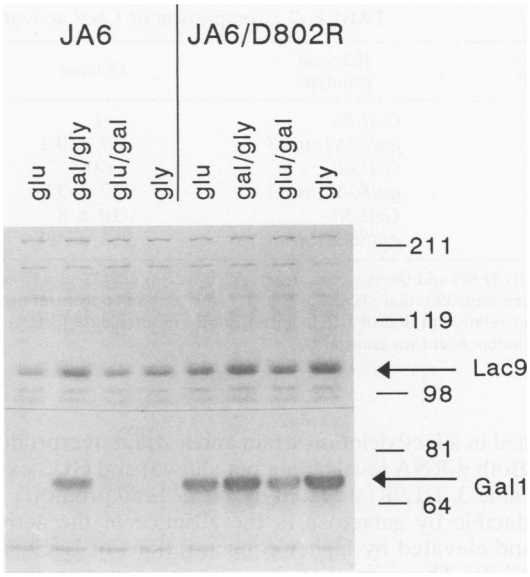

FIG. 4. Comparison of Lac9 and Gal1 concentrations in JA6 (wild type) and JA6/D802R (*gal80Δ2::ura3*) by immunoblotting. Strains were pregrown in glucose-containing medium, transferred to medium containing 2% glucose (glu), 2% galactose–3% glycerol (gal/gly), 2% glucose–2% galactose (glu/gal), or 3% glycerol (gly), and harvested after 4.5 h of growth (OD_{600}, 0.5 to 0.7). After preparation of crude extracts, samples containing 50 μg of protein were resolved on an SDS–7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was cut into upper and lower halves, which were incubated with Lac9 and Gal1 antisera, respectively. Bands were visualized by a secondary antibody linked to alkaline phosphatase. The positions of prestained proteins (Sigma) used as molecular weight standards are indicated in kilodaltons.

K. lactis GAL80 gene expression was strongly regulated. Surprisingly, in the absence of galactose (in glucose or glycerol), a condition in which the function of the negative regulator of the lactose/galactose metabolic genes is required, mRNA levels were about 50-fold lower than in galactose medium. In glucose-galactose, the *K. lactis GAL80* mRNA level was 12-fold higher than on glucose but clearly lower than on galactose, implying that *GAL80* gene expression was subject to glucose repression.

To quantify the expression levels by an independent method, the *K. lactis GAL80* promoter and 5′ end of the coding region were fused to a reporter gene encoding the bacterial GUS (see Materials and Methods). Enzyme activities were determined in a wild-type strain transformed with a centromere plasmid expressing the gene fusion (Table 3, JA6). In line with the analysis of *K. lactis GAL80* mRNA, the *GAL80* promoter directed a 70-fold-higher level of fusion gene expression in galactose than in glucose. The presence of glucose in addition to galactose reduced induction to about 10%. The noninduced level in glycerol was very similar to that in glucose-galactose.

The dependence of *GAL80* gene induction on Lac9 was

TABLE 2. Comparison of Lac9 activator concentrations in wild-type and *gal80* mutant strains

Parameter assayed[a]	Relevant genotype	Glucose	Glycerol-galactose	Glucose-galactose	Glycerol
Lac9	*GAL80*	1	2.2 ± 0.1	1.2 ± 0.2	1.5 ± 0.2
	gal80Δ2::ura3	1.7 ± 0.1	2.7 ± 0.3	1.9 ± 0.1	2.7 ± 0.2
Gal1	*GAL80*	<0.5	100	5 ± 3	<0.5
	gal80Δ2::ura3	137 ± 33	179 ± 33	130 ± 21	205 ± 68
β-Gal	*GAL80*	10 ± 8	2,030 ± 1,340	84 ± 82	157 ± 80
	gal80Δ2::ura3	1,751 ± 488	3,320 ± 940	1,660 ± 530	5,270 ± 1,200

[a] JA6 (*GAL80*) and the isogenic strain JA6/D802R (*gal80Δ2::ura3*) were used. Relative amounts of Lac9 and Gal1 were determined by densitometric scanning of Western blots like that shown in Fig. 4. Lac9 protein concentrations were normalized to that of JA6 in glucose; Gal1 protein concentrations were expressed in percent relative to that of JA6 in galactose. β-Galactosidase (β-Gal) activity is given in milliunits per milligram. Each value is the mean ± standard deviation of three independent measurements.

examined in a *lac9* deletion strain and a strain overproducing Lac9. Both mRNA levels (data not shown) and GUS expression (Table 3, DL9R) directed by the *GAL80* promoter were not inducible by galactose in the absence of the activator Lac9 and elevated by higher concentrations of Lac9 (Table 3, JA6/2-2). The regulation by carbon source in the *lac9* deletion strain resembles that of the *LAC4* promoter (Table 1) and again clearly shows a repressing effect of glucose in the absence of Lac9. Glucose repression was largely suppressed by the higher concentration of strain JA6/2-2 (Table 3), as has been shown for other Lac9-controlled genes in this strain (25, 62). Thus, activation of the *GAL80* gene depends on the activator Lac9, and its pattern of regulation resembles that of the other genes of the lactose/galactose regulon despite its function as a negative regulator of these genes.

When the *GAL80-GUS* fusion gene was introduced into a *gal80*$^-$ mutant (Table 3, JA6/D801R), the *GAL80* promoter was induced under all growth conditions, demonstrating that the dependence of *GAL80* regulation on Lac9 results in a negative regulation by Gal80, thereby generating an autoregulatory feedback loop.

The enzymatic activities were again highest in glycerol-containing and lowest in glucose-containing media, as described above for β-galactosidase (Table 1).

The *K. lactis GAL80* promoter contains two high-affinity Lac9 binding sites. The most likely *cis*-acting control elements conferring Lac9/Gal80-dependent regulation are the two Lac9 consensus binding sites located at positions −295 and −333 of the *K. lactis GAL80* promoter. To determine whether either or both of these sites could bind the activator in vitro, we tested them in a competition experiment. A labeled fragment containing a well-characterized Lac9 binding site (UASI of the *LAC4* promoter [13, 62]) was incubated with Lac9-containing protein extracts in the presence of increasing amounts of a *K. lactis GAL80* promoter fragment. This fragment containing both potential Lac9 binding sites competed for Lac9 binding to UASI about five to six times better than to unlabeled $UASI_{LAC4}$ (Fig. 6). The ability of fragments containing only a single site to compete was reduced by a factor of about 2, indicating that both sites had roughly the same affinity for Lac9 in vitro. Each of these sites therefore had a two- to threefold-higher affinity to the activator than to $UASIV_{LAC4}$, the strongest Lac9 binding site characterized so far (13).

Inducibility of *K. lactis GAL80* gene expression renders the *LAC/GAL* regulon susceptible to glucose repression. To demonstrate the in vivo function of these Lac9 binding sites in the *K. lactis GAL80* promoter, we deleted these sites (from positions −349 to −178) in the Gal80-GUS fusion construct. Determination of GUS activity in different media showed that induction by galactose was abolished in the wild-type strain carrying the mutant plasmid (Table 4, JA6 carrying pCD80GUS), confirming the function of the deleted region as galactose-dependent upstream activation sequences (UAS_{GAL80}). The Lac9 dependence of the deleted region is shown by the identical behavior of the *LAC9* strain containing the plasmid with the deleted promoter and the *lac9* mutant strain with the wild-type promoter (compare DL9R in Table 3 with JA6 carrying pCD80GUS in Table 4). The basal level of expression was not significantly affected by the deletion and was independent of Gal80 and Lac9 function, as demonstrated by transformation of the mutated plasmid into *gal80* and *lac9* deletion strains (Table 4, JA6/D801R and DL9R).

This finding allowed us to address the question whether the basal level of *K. lactis GAL80* expression was sufficient to block Lac9 function and, if so, what the function of induction by galactose might be. By gene replacement, the noninducible *K. lactis GAL80*-ΔUAS promoter was introduced into the chromosome, replacing the wild-type *GAL80* promoter, giving strain JA6/DUAS80, and the influence of the promoter mutation on β-galactosidase gene expression

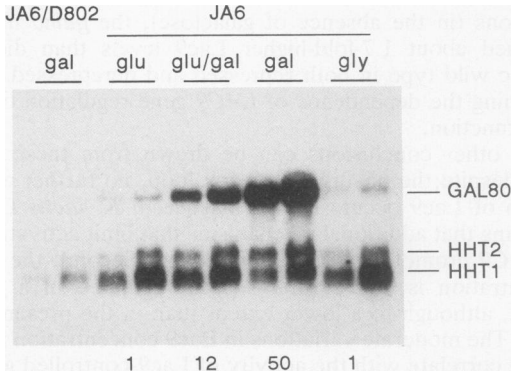

FIG. 5. Transcriptional regulation of the *K. lactis GAL80* gene. Cells were grown in SM medium containing the indicated carbon sources, and then poly(A)$^+$-enriched RNA was prepared. About 5 and 10 μg of RNA for each sample were separated in an agarose gel containing formaldehyde and blotted onto a nylon membrane. For detection of *K. lactis GAL80* mRNA, the three intragenic *Eco*RV fragments were labeled radioactively. *HHT1* and *HHT2* mRNAs encoding histones H3 and H4 (55) were detected with a 0.7-kb *Asp* 718 fragment isolated from plasmid pJ2B3 (55). The *GAL80* signal was normalized to the *HHT1* and *HHT2* signals, and the relative concentration of the *GAL80* mRNA is given in relation to that of JA6 grown on glucose.

TABLE 3. Regulation of *K. lactis GAL80* gene expression

Strain	Relevant genotype	GUS activity (mU/mg)[a]			
		Glucose	Galactose	Glucose-galactose	Glycerol
JA6	*LAC9 GAL80*	1.5 ± 0.3	108 ± 28	10 ± 2.5	6 ± 1.4
DL9R	*lac9::ura3 GAL80*	0.9 ± 0.2	5.7 ± 1	0.9 ± 0.1	7.7 ± 3
JA6/2-2	*LAC9*(2×) *GAL80*	5.9 ± 2.4	134 ± 23	95 ± 27	15 ± 7.5
JA6/D801R	*LAC9 gal80Δ1::ura3*	126 ± 28	170 ± 17	119 ± 34	302 ± 43

[a] Strains were transformed with pC80GUS containing the *K. lactis GAL80*-GUS fusion and were grown in selective minimal medium with the indicated carbon sources. Mean values ± standard deviations of at least three independent measurements are presented. DL9R was grown on galactose plus 3% glycerol instead of galactose. No GUS activity was detectable in untransformed strains.

was analyzed (Table 4, JA6/DUAS80). In cells grown in glucose, sufficient Gal80 protein was produced to keep the *LAC4* gene repressed. In galactose, β-galactosidase activities were slightly higher than the wild-type level, indicating that even under inducing conditions, high levels of Gal80 protein exert a certain negative influence on *LAC4* gene expression. In glucose-galactose, the most obvious effect of the *GAL80* promoter mutation on the *LAC4* gene was seen. Whereas the wild-type strain was repressed, β-galactosidase activities in the mutant were 20- to 50-fold higher. The values showed a much higher variation than in the wild type; between 220 and 1,180 mU/mg was measured in four independent experiments. Clearly, inducibility of the *GAL80* gene is required for the maintenance of glucose repression in the presence of galactose.

High variations of β-galactosidase activities were also observed in glycerol, with values of between 100 and 1,500 mU/mg. When these measurements were repeated with precultures grown in glycerol instead of glucose medium, the β-galactosidase activities were more reproducible, reaching 2,700 ± 600 mU/mg. Since we could not detect a significant influence of the mutation on *GAL80* gene expression under noninducing conditions, the reason for the higher activity in the absence of galactose is not obvious (see Discussion).

DISCUSSION

We have isolated the *K. lactis GAL80* gene by complementation of a mutation which leads to constitutive expression of the lactose/galactose metabolic genes. The deduced amino acid sequence is similar to that of Gal80 of *S. cerevisiae* throughout the entire protein. With 60% identical residues, the sequence conservation is much higher than that of their target activators, Lac9 and Gal4 (49, 61). Despite the high overall homology, the structural comparison was very informative by revealing two blocks that are more strongly conserved than the rest of the protein (Fig. 3B). No such information had been obtained from attempts to dissect functional domains of *S. cerevisiae* Gal80 (41). From these data, it seemed that most of the protein is required for its repressor activity. The high sequence conservation supports that conclusion and indicates that the function of Gal80 imposes strong constraints on the sequence. Only the last 12 amino acids were shown to be dispensable in a series of C-terminal deletions of *S. cerevisiae* Gal80 (41). This deletion removed 5 of the 25 conserved residues of block F (Fig. 3A). Only one additional region could be mutated without loss of the repression function: at position 341 (Fig. 3B), large insertions were tolerated, and a 19-amino-acid deletion between residues 322 and 340 still retained repressor activity but was uninducible. Both of these mutations fall into the only nonconserved region of the protein, which separates the N-terminal two-thirds from the C-terminal blocks E and F. In contrast to the rest of the protein, neither length nor sequence seems to be important in this linker region.

The noninducible phenotype of the 19-amino-acid deletion mentioned above may be due to the removal of residue 323, which forms the C-terminal end of one of the two highly conserved blocks. A Gly→Arg mutation of this residue is responsible for noninducibility of the $GAL80^{s-1}$ mutant (42). This $GAL80^{s-1}$ mutation is special among the three known noninducible (or superrepressing) $GAL80^{s}$ mutants (42): its noninducible phenotype is lost when wild-type *GAL4* is replaced by the $GAL4^{c}$ missense allele *GAL4-Ile859* (51) and

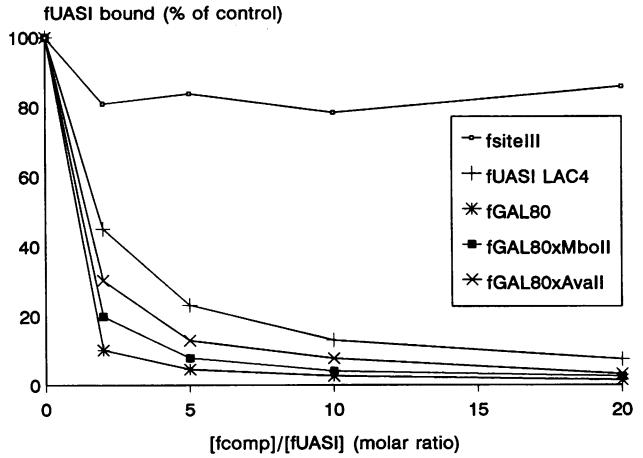

FIG. 6. Equilibrium competition experiment with the UASI fragment from the *LAC4* promoter ($fUASI_{LAC4}$) for Lac9 binding. Binding reaction mixtures contained 0.6 ng of labeled $fUASI_{LAC4}$ (4.7 fmol; 4.45 × 10^4 cpm), 0- to 20-fold molar excess of unlabeled competitor fragment (fcomp), and 1 μg of S100 protein. Sonicated calf thymus DNA was added to give a total of 100 ng of DNA. The S100 extract was prepared from the induced wild-type strain *K. lactis* JA6 transformed with plasmid pEXL9G10-3, which allows inducible overexpression of Lac9 (62). Competitor fragments were derived from the *K. lactis GAL80* promoter as described in Materials and Methods. fGAL80 was cleaved with the restriction endonucleases *Mbo*II or *Ava*II prior to competition in order to investigate $UASI_{GAL80}$ or $UASII_{GAL80}$ alone. Fragment fsiteIII contains a UAS-related sequence from the *LAC4* promoter which is not bound by Lac9 (13). Samples were subjected to gel electrophoresis, and the Lac9-UASI complex was quantitated by Cerenkov counting. The amount of fUASI retarded in the reactions with a competitor fragment in relation to a reaction without competitor fragment (9,200 cpm) was plotted against the ratio of competitor fragment to labeled fUASI. Reactions without Lac9 protein were used to calculate background radioactivity (<65 cpm). The data represent the averages of two independent experiments.

TABLE 4. Effect of UAS_{GAL80} deletion on *K. lactis GAL80* and *LAC4* gene expression

Enzyme and strain	Relevant genotype	Transformed plasmid	Enzyme activity (mU/mg)[a]			
			Glucose	Galactose	Glucose-galactose	Glycerol
GUS						
JA6	*LAC9 GAL80*	pC80GUS	1.5 ± 0.3	108 ± 28	10.2 ± 2.5	6.1 ± 1.4
JA6	*LAC9 GAL80*	pCD80GUS	1 ± 0	2.5 ± 0.7	0.8 ± 0.4	8.2 ± 0.3
JA6/D801R	*LAC9 gal80Δ1R*	pCD80GUS	0.9 ± 0.3	1.4 ± 0.1	0.7 ± 0.2	8.6 ± 0.3
DL9R	*lac9::ura3 GAL80*	pCD80GUS	1.3 ± 0.5	8.8 ± 0.6	1.1 ± 0.4	9.2 ± 1.7
β-Galactosidase						
JA6	*LAC9 GAL80*		1.3 ± 0.2	2,430 ± 790	18 ± 5	87 ± 48
JA6/DUAS80	*LAC9 GAL80*ΔUAS[b]		20 ± 18	3,480 ± 1,260	820 ± 431	610 ± 670

[a] Mean value ± standard deviation of two (pCD80GUS transformants) or five (pC80GUS transformants) independent measurements. For strain DL9R, glycerol was added in addition to galactose. pC80GUS contains the wild-type *K. lactis GAL80* promoter, whereas in pCD80GUS, the Lac9 binding sites were deleted.
[b] In the *GAL80*ΔUAS allele (*GAL80-349/178*), sequences from −349 to −178 including the two Lac9 binding sites have been deleted.

also when *GAL4* is replaced by *LAC9* in *S. cerevisiae* (50). Interestingly, the s-1 mutation is located right at the position where *S. cerevisiae* Gal80 and *K. lactis* Gal80 start to diverge into the linker region. All three of the noninducible *GAL80*^{s} mutations are in a narrow region flanking the linker (Fig. 3B). Blocks D and E therefore may play an important role not only in interaction with Gal4/Lac9 but also in the induction process.

Not only the structure but also the function of Gal80 is conserved between *K. lactis* and *S. cerevisiae*. Null mutants of *gal80* express the Gal4/Lac9-controlled genes under noninducing conditions both in *S. cerevisiae* and in *K. lactis*. *S. cerevisiae GAL80* is able to complement the *K. lactis gal80* mutation, and as shown before (46), *K. lactis GAL80* is able to regulate Gal4 function in *K. lactis*. *LAC9* in *S. cerevisiae* is active but not regulated (49, 50). In these experiments, higher concentrations of Lac9 than of Gal4 were found, suggesting that the concentration of Gal80 may be insufficient to repress Lac9 activity in *S. cerevisiae*. In line with this finding, it was shown that Lac9 can be blocked in *S. cerevisiae* strains overexpressing Gal80 or containing a *GAL80*^{s} mutant allele (50). Thus, the repressing activity of Gal80 depends on both its concentration and its affinity for the activator. The Gal80 repressor of both yeasts is able to interact with the heterologous activator, and the lack of Lac9 regulation in *S. cerevisiae* seems to be due to quantitative rather than qualitative differences.

However, there is a major difference between the phenotypes of *gal80* mutants of *K. lactis* and *S. cerevisiae* with respect to glucose repression of the regulon. Whereas in *S. cerevisiae*, the Gal4 activity is low in the absence of Gal80 when the medium contains glucose (21, 34, 35, 39). Lac9 is able to almost fully induce the β-galactosidase and galactokinase genes even when glucose is the sole carbon source. We propose that this difference in sensitivity to glucose is a result of a higher concentration of the activator in *K. lactis* than in *S. cerevisiae*. In *K. lactis*, it was shown that glucose repression can be suppressed even in the presence of Gal80 by a slight increase in the Lac9 concentration as it occurs in some natural strains (4, 26, 63) or by a duplication of the *LAC9* gene (25) (Table 3). In *S. cerevisiae*, expression of *GAL4* on a multicopy plasmid produces a similar phenotype (21). In an *S. cerevisiae gal80* mutant, glucose repression can also be overcome when *GAL4* gene expression is moderately elevated as a result of a *mig1* mutation (39) or when the gene is fused to a stronger promoter (15, 27). Activation of Gal4 by mutating Gal80 is therefore not sufficient for induction unless the concentration of Gal4 is elevated at the same time (15, 27, 39). In contrast, a *gal80* mutant of *K. lactis* contains sufficient Lac9 activator to allow for induction. The influence of the *gal80* mutation on activator gene expression in *K. lactis* but not in *S. cerevisiae* (62) may contribute to the phenotypic differences between the two species, but we assume that even the noninduced Lac9 level is higher than that of Gal4.

The fact that glucose repression also occurs in the absence of the Lac9 activator as shown by the regulation of *LAC4* and *K. lactis GAL80* gene expression by the carbon source in a *lac9* deletion strain (Tables 1 and 3) suggests that glucose does not act through the Lac9 molecule (although such an effect cannot be excluded as an additional mechanism). We propose that a glucose-dependent, Lac9-independent repression mechanism counteracts the induction process. The efficiency of induction is determined by the total activity of the activator in the cell, which in turn is a function of its concentration and its specific activity. The latter is controlled by the negative regulator Gal80, which responds only to the presence or absence of galactose.

The *GAL80* genes of *K. lactis* and *S. cerevisiae* are both controlled by autoregulation. However, a much higher level of induction is observed in *K. lactis*. Accordingly, two binding sites for the transcriptional activator are located in the *K. lactis GAL80* promoter, whereas only one Gal4 binding site is present in *S. cerevisiae GAL80*. Lac9 binds with high affinity to these sites in vitro and probably also in vivo, as indicated by the fact that overexpression of Lac9 by a factor of 4 to 5 (62) gives only 30% higher *K. lactis GAL80* gene expression levels under inducing conditions (Table 3; compare JA6 and JA6/2-2) but 60% higher *LAC4* levels (62). At first glance, it is amazing that the *GAL80* gene is induced in galactose, that is, under conditions in which its function does not seem to be required. Eliminating inducibility by deleting the Lac9 binding sites had shown that the noninduced level of the negative regulator is clearly sufficient to inhibit Lac9 function in glucose-grown cells. This is in line with data demonstrating that in *S. cerevisiae*, trace amounts of a functional Gal80 peptide are sufficient to confer repression (41).

What is the function of the strong induction of *K. lactis GAL80* in galactose then? Since *LAC9* gene expression is induced only 2- to 3-fold, the 70-fold induction of *K. lactis GAL80* leads to a large excess of the negative regulator over Lac9 in galactose. Nevertheless, a further increase in *K. lactis GAL80* gene expression reduces Lac9 activity (64),

and similar results have been reported for *S. cerevisiae* (41, 42). A lowering of the Gal80 concentration as in strain JA6/DUAS80 resulted in a small increase of Lac9-dependent gene activation even in galactose (Table 4). Thus, despite the large excess of Gal80 over the activator, variations in Gal80 concentration still have an influence on Lac9 activity. However, this effect is small during galactose induction, since the negative regulator becomes inactivated by a galactose-dependent signal. Accordingly, because of the low inhibitory activity of Gal80, a reduction or even loss of function only has a moderate effect, but a high concentration still provides some negative control on the highly active Lac9 molecule. We have shown that a noninducible *K. lactis GAL80* gene is not sufficient to allow for glucose repression in the presence of galactose. Since under conditions of glucose repression, gene activation by Lac9 very crucially depends on the total activity of the activator (26, 63), any change not only in the concentration but also in the specific activity of the activator has a profound influence on the efficiency of glucose repression. Obviously, the specific activity of Lac9 being regulated by Gal80 protein is influenced by the *K. lactis GAL80* gene expression rate.

The high variability of β-galactosidase activities obtained in the *K. lactis GAL80*-ΔUAS mutant also suggests that induction of the *GAL80* gene may serve to stabilize the regulon even in the absence of galactose, perhaps by counterbalancing fluctuations in Lac9 levels. In addition, *GAL80* induction may supply the cell with sufficient repressor to be able to switch off the *GAL* regulon when galactose is exhausted. Experiments are under way to test this prediction.

The fact that the concentration of Gal80 protein has an influence on the activator activity even under conditions in which Gal80 is in excess over Lac9 is hard to reconcile with a simple allosteric transition model for induction in which a stable Gal4-Gal80 complex is converted from an inactive to an active state upon induction. Any such model has to take into account that active and inactive states of the activator are in an equilibrium that is influenced by the Gal80 concentration.

ACKNOWLEDGMENTS

We thank M. J. R. Stark, S. A. Johnston, M. Ciriacy, and R. C. Dickson for plasmids pJ2B3, YCp50GAL80, pBI101.1, and pKR7-Gal, respectively, M. Wésolowski-Louvel for the *K. lactis* genomic library, and J. Meyer and C. P. Hollenberg for galactokinase-specific antibodies. We are indebted to H. Bojar for use of the densitometer, and we thank R. Kölling and M. Ramezani-Rad for critical comments on the manuscript and P. Kuger for excellent technical assistance.

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft to K.D.B. and by EG grant BIOT-CT91-0267 (DSCN).

REFERENCES

1. **Bianchi, M. M., C. Falcone, C. X. Jie, M. Wésolowski-Louvel, L. Frontali, and H. Fukuhara.** 1987. Transformation of the yeast *Kluyveromyces lactis* by new vectors derived from the 1.6 μm circular plasmid pKD1. Curr. Genet. **12:**185–192.
2. **Boeke, J. D., F. LaCroute, and G. F. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:**345–346.
3. **Bram, R. J., N. F. Lue, and R. D. Kornberg.** 1986. GAL family of upstream activating sequences in yeast: roles in both induction and repression of transcription. EMBO J. **5:**603–608.
4. **Breunig, K. D.** 1989. Glucose repression of LAC gene expression in yeast is mediated by the transcriptional activator LAC9. Mol. Gen. Genet. **216:**422–427.
5. **Breunig, K. D., and P. Kuger.** 1987. Functional homology between the yeast regulatory proteins GAL4 and LAC9: LAC9-mediated transcriptional activation in *Kluyveromyces lactis* involves protein binding to a regulatory sequence homologous to the GAL4 protein-binding site. Mol. Cell. Biol. **7:**4400–4406.
6. **Chasman, D. I., and R. D. Kornberg.** 1990. GAL4 protein: purification, association with GAL80 protein, and conserved domain structure. Mol. Cell. Biol. **10:**2916–2923.
7. **Chen, X. J., M. Saliola, C. Falcone, M. M. Bianchi, and H. Fukuhara.** 1986. Sequence organization of the circular plasmid pKD1 from the yeast *Kluyveromyces drosophilarum*. Nucleic Acids Res. **14:**4471–4481.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Dickson, R. C., C. J. Gerardot, and A. K. Martin.** 1990. Genetic evidence for similar negative regulatory domains in the yeast transcription activators GAL4 and LAC9. Nucleic Acids Res. **18:**5213–5217.
10. **Dickson, R. C., M. Sheetz, and L. R. Lacy.** 1981. Genetic regulation: yeast mutants constitutive for β-galactosidase activity have an increased level of β-galactosidase messenger ribonucleic acid. Mol. Cell. Biol. **1:**1048–1056.
11. **Dohmen, R. J., A. W. M. Strasser, C. B. Höner, and C. P. Hollenberg.** 1991. An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast **7:**691–692.
12. **Douglas, H. C., and D. C. Hawthorne.** 1966. Regulation of genes controlling synthesis of the galactose pathway enzymes in yeast. Genetics **54:**911–916.
13. **Gödecke, A., W. Zachariae, A. Arvanitidis, and K. D. Breunig.** 1991. Coregulation of the *Kluyveromyces lactis* lactose permease and β-galactosidase genes is achieved by interaction of multiple LAC9 binding sites in a 2.6 kbp divergent promoter. Nucleic Acids Res. **19:**5351–5358.
14. **Gonçalves, P. M., K. Maurer, W. H. Mager, and R. J. Planta.** 1992. *Kluyveromyces* contains a functional ABF1-homologue. Nucleic Acids Res. **20:**2211–2215.
15. **Griggs, D. W., and M. Johnston.** 1991. Regulated expression of the *GAL4* activator gene in yeast provides a sensitive genetic switch for glucose repression. Proc. Natl. Acad. Sci. USA **88:**8597–8601.
16. **Hopper, J. E., J. R. Broach, and L. B. Rowe.** 1978. Regulation of the galactose pathway in *Saccharomyces cerevisiae*: induction of uridyl transferase mRNA and dependency on GAL4 gene function. Proc. Natl. Acad. Sci. USA **75:**2878–2882.
17. **Igarashi, M., T. Segawa, Y. Nogi, Y. Suzuki, and T. Fukasawa.** 1987. Autogenous regulation of the *Saccharomyces cerevisiae* regulatory gene GAL80. Mol. Gen. Genet. **207:**273–279.
18. **Jacobsen, A.** 1987. Purification and fractionation of poly(A) RNA. Methods Enzymol. **152:**254–261.
19. **Jefferson, R. A.** 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5:**387–405.
20. **Johnston, M.** 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. **51:**458–476.
21. **Johnston, M., and J. E. Hopper.** 1982. Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effect on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA **79:**6971–6975.
22. **Johnston, S. A., J. M. Salmeron, Jr., and S. S. Dincher.** 1987. Interaction of positive and negative regulatory proteins in the galactose regulon of yeast. Cell **50:**143–146.
23. **Johnston, S. A., M. J. Zavortink, C. Debouck, and J. E. Hopper.** 1986. Functional domains of the yeast regulatory protein GAL4. Proc. Natl. Acad. Sci. USA **83:**6553–6557.
24. **Klebe, R. J., J. V. Harris, Z. D. Smart, and M. G. Douglas.** 1983. A general method for polyethylene-glycole induced genetic transformation of bacteria and yeast. Gene **25:**333–341.
25. **Kuger, P., A. Gödecke, and K. D. Breunig.** 1990. A mutation in the Zn-finger of the GAL4 homolog LAC9 results in glucose repression of its target genes. Nucleic Acids Res. **18:**745–751.

26. **Kuzhandaivelu, N., W. K. Jones, A. K. Martin, and R. C. Dickson.** 1992. The signal for glucose repression of the lactose-galactose regulon is amplified through subtle modulation of transcription of the *Kluyveromyces lactis KI-GAL4* activator gene. Mol. Cell. Biol. **12**:1924–1931.
27. **Lamphier, M. S., and M. Ptashne.** 1992. Multiple mechanisms mediate glucose repression of the yeast *GAL1* gene. Proc. Natl. Acad. Sci. USA **89**:5922–5926.
28. **Leuther, K. K., and S. A. Johnston.** 1992. Nondissociation of GAL4 and GAL80 in vivo after galactose induction. Science **256**:1333–1335.
29. **Lewis, M. J., and H. R. B. Pelham.** 1990. The sequence of the *Kluyveromyces lactis* BiP gene. Nucleic Acids Res. **18**:6438.
30. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
31. **Lue, N. F., D. I. Chasman, A. R. Buchman, and R. D. Kornberg.** 1987. Interaction of *GAL4* and *GAL80* gene regulatory proteins in vitro. Mol. Cell. Biol. **7**:3446–3451.
32. **Ma, J., and M. Ptashne.** 1987. The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. Cell **50**:137–142.
33. **Ma, J., and M. Ptashne.** 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell **48**:847–853.
34. **Matsumoto, K., A. Toh-e, and Y. Oshima.** 1981. Isolation and characterization of dominant mutations resistant to carbon catabolite repression of galactokinase synthesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **1**:83–89.
35. **Matsumoto, K., T. Yoshimatsu, and Y. Oshima.** 1983. Recessive mutations conferring resistance to carbon catabolite repression of galactokinase synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **153**:1405–1414.
36. **Mylin, L. M., J. P. Bhat, and J. E. Hopper.** 1989. Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator. Genes Dev. **3**:1157–1165.
37. **Mylin, L. M., C. J. Gerardot, J. E. Hopper, and R. C. Dickson.** 1991. Sequence conservation in the *Saccharomyces* and *Kluveromyces* GAL11 transcription activators suggests functional domains. Nucleic Acids Res. **19**:5345–5350.
38. **Mylin, L. M., M. Johnston, and J. E. Hopper.** 1990. Phosphorylated forms of GAL4 are correlated with ability to activate transcription. Mol. Cell. Biol. **10**:4623–4629.
39. **Nehlin, J. O., M. Carlberg, and H. Ronne.** 1991. Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. EMBO J. **10**:3373–3377.
40. **Nogi, Y., and T. Fukasawa.** 1984. Nucleotide sequence of the yeast regulatory gene *GAL80*. Nucleic Acids Res. **12**:9287–9298.
41. **Nogi, Y., and T. Fukasawa.** 1989. Functional domains of a negative regulatory protein, GAL80, of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:3009–3017.
42. **Nogi, Y., H. Shimada, Y. Matsuzaki, H. Hashimoto, and T. Fukasawa.** 1984. Regulation of expression of the galactose gene cluster in *Saccharomyces cerevisiae*. II. The isolation and dosage effect of the regulatory gene *GAL80*. Mol. Gen. Genet. **195**:29–34.
43. **Parthun, M. R., and J. A. Jaehning.** 1990. Purification and characterization of the yeast transcriptional activator GAL4. J. Biol. Chem. **265**:209–213.
44. **Parthun, M. R., and J. A. Jaehning.** 1992. A transcriptionally active form of GAL4 is phosphorylated and associated with GAL80. Mol. Cell. Biol. **12**:4981–4987.
45. **Perlman, D., and J. E. Hopper.** 1979. Constitutive synthesis of the GAL4 protein, a galactose pathway regulator in *Saccharomyces cerevisiae*. Cell **16**:89–95.
46. **Riley, M. I., J. E. Hopper, S. A. Johnston, and R. C. Dickson.** 1987. GAL4 of *Saccharomyces cerevisiae* activates the lactose-galactose regulon of *Kluyveromyces lactis* and creates a new phenotype: glucose repression of the regulon. Mol. Cell. Biol. **7**:780–786.
47. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.
48. **Saliola, M., J. R. Shuster, and C. Falcone.** 1990. The alcohol dehydrogenase system in the yeast, *Kluyveromyces lactis*. Yeast **6**:193–204.
49. **Salmeron, J. M., and S. A. Johnston.** 1986. Analysis of the *Kluyveromyces lactis* positive regulatory gene *LAC9* reveals functional homology to, but sequence divergence from, the *Saccharomyces cerevisiae GAL4* gene. Nucleic Acids Res. **14**:7767–7781.
50. **Salmeron, J. M., S. D. Langdon, and S. A. Johnston.** 1989. Interaction between transcriptional activator protein LAC9 and negative regulatory protein GAL80. Mol. Cell. Biol. **9**:2950–2956.
51. **Salmeron, J. M., Jr., K. K. Leuther, and S. A. Johnston.** 1990. *GAL4* mutations that separate the transcriptional activation and GAL80-interactive functions of the yeast GAL4 protein. Genetics **125**:21–27.
52. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18**:3091–3092.
53. **Sheetz, R. M., and R. C. Dickson.** 1980. Mutations affecting synthesis of β-galactosidase activity in the yeast *Kluyveromyces lactis*. Genetics **95**:877–890.
54. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Stark, M. J. R., and J. S. Milner.** 1989. Cloning and analysis of the *Kluyveromyces lactis TRP1* gene: a chromosomal locus flanked by genes encoding inorganic pyrophosphatase and histone H3. Yeast **5**:35–50.
56. **Tajima, M., Y. Nogi, and T. Fukasawa.** 1986. Duplicate upstream activating sequences in the promoter region of the *Saccharomyces cerevisiae GAL7* gene. Mol. Cell. Biol. **6**:246–256.
57. **Torchia, T. E., R. W. Hamilton, C. L. Cano, and J. E. Hopper.** 1984. Disruption of regulatory gene *GAL80* in *Saccharomyces cerevisiae*: effects on carbon-controlled regulation of the galactose/melibiose pathway genes. Mol. Cell. Biol. **4**:1521–1527.
58. **Walker-Jonah, A.** 1988. Studies on the regulation of lactose utilisation in the yeast *Kluyveromyces lactis*. PhD. thesis. University of Duesseldorf, Duesseldorf, Germany.
59. **Webster, T. D., and R. C. Dickson.** 1988. The organization and transcription of the galactose gene cluster of *Kluyveromyces lactis*. Nucleic Acids Res. **16**:8011–8028.
60. **Wésolowski-Louvel, M., C. Tanguy-Rougeau, and H. Fukuhara.** 1988. A nuclear gene required for the expression of the linear DNA-associated killer system in the yeast *Kluyveromyces lactis*. Yeast **4**:71–81.
61. **Wray, L. V., Jr., M. W. Witte, R. C. Dickson, and M. I. Riley.** 1987. Characterization of a positive regulatory gene, *LAC9*, that controls induction of the lactose-galactose regulon of *Kluyveromyces lactis*: structural and functional relationships to *GAL4* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:1111–1121.
62. **Zachariae, W., and K. D. Breunig.** 1993. Expression of the transcriptional activator *LAC9* (*KlGAL4*) in *Kluyveromyces lactis* is controlled by autoregulation. Mol. Cell. Biol. **13**:3058–3066.
63. **Zachariae, W., P. Kuger, and K. D. Breunig.** 1993. Glucose repression of lactose/galactose metabolism in *Kluyveromyces lactis* is determined by the concentration of the transcriptional activator LAC9 (KlGAL4). Nucleic Acids Res. **21**:69–77.
64. **Zenke, F.** Unpublished data.