Glucose represses the lactose–galactose regulon in *Kluyveromyces lactis* through a *SNF1* and *MIG1*-dependent pathway that modulates galactokinase (*GAL1*) gene expression

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Received May 21, 1997; Revised and Accepted July 17, 1997

**ABSTRACT**

Expression of the lactose–galactose regulon in *Kluyveromyces lactis* is induced by lactose or galactose and repressed by glucose. Some components of the induction and glucose repression pathways have been identified but many remain unknown. We examined the role of the *SNF1* (*KlSNF1*) and *MIG1* (*KlMIG1*) genes in the induction and repression pathways. Our data show that full induction of the regulon requires *SNF1*; partial induction occurs in a *Klsnf1*-deleted strain, indicating that a *KISNF1*-independent pathway(s) also regulates induction. *MIG1* is required for full glucose repression of the regulon, but there must be a *KIMIG1*-independent repression pathway also. The *Klmig1* protein appears to act downstream of the *KlSnf1* protein in the glucose repression pathway. Most importantly, the *Klsnf1*-*Klmig* repression pathway operates by modulating *KIGAL1* expression. Regulating *KIGAL1* expression in this manner enables the cell to switch the regulon off in the presence of glucose. Overall, our data show that, while the *Snf1* and *Mig1* proteins play similar roles in regulating the galactose regulon in *Saccharomyces cerevisiae* and *K. lactis*, the way in which these proteins are integrated into the regulatory circuits are unique to each regulon, as is the degree to which each regulon is controlled by the two proteins.

**INTRODUCTION**

*Kluyveromyces lactis* is one of the few yeasts that can use the milk sugar lactose as a carbon and energy source, which suggests that this yeast may have evolved under different and unique selection pressures, particularly for carbon sources, than have many other yeasts including *Saccharomyces cerevisiae* (reviewed in 1). *Kluyveromyces lactis* grows slightly more rapidly with lactose as a carbon source than with glucose (2), but at least in some strains, glucose is the preferred carbon source since it represses expression of the genes necessary for utilization of lactose or galactose (3). Few components of the glucose repression pathway have been identified and we have only a rudimentary outline of the way in which the pathway represses expression of the genes necessary for lactose and galactose utilization—the lactose–galactose regulon (4–7). To further our understanding of the repression pathway, we examined the role of the *SNF1* (*KlSNF1*) and the *MIG1* (*KlMIG1*) genes in glucose repression and induction. We show here that at least one glucose repression pathway contains both the *KlSNF1* and the *KlMIG1* gene products and we identify a way in which this pathway modulates expression of genes in the lactose–galactose regulon.

Utilization of lactose or galactose requires induction of transcription of *KILAC4* (β-galactosidase; 8) and *KILAC12* (lactose permease), which are transcribed in opposite directions from a common promoter (9), and *KIGAL1, KIGAL7* and *KIGAL10* [coding for galactokinase (EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (EC 2.7.7.12, transferase) and uridine diphosphoglucose 4-epimerase (EC 5.1.3.2, epimerase), respectively], which are tightly linked, with *KIGAL1* and *KIGAL10* transcribed in opposite directions from a common promoter (reviewed in 1).

The transcription induction pathway centers around the DNA-binding protein KIGal4p (10,11) whose concentration is tightly regulated by an autoregulatory loop that produces a 2–3-fold increase in its concentration, an essential ingredient in the induction pathway (4,7). Activation of transcription is also controlled by the negative regulator KIGal80p which is bound to and modulates the transcription activator activity of KIGal4p (12). Induction of the regulon also requires an uncharacterized activity of the KIGal1 protein that is independent of its galactokinase activity (13). This uncharacterized activity may be responsible for the galactose and ATP-dependent binding of KIGal1p to KIGal80p, an interaction that permits KIGal4p to activate transcription (12).

Glucose represses expression of the lactose–galactose regulon in some but not all strains of *K.lactis* (3). Repressing and non-repressing strains differ by two bases in the *KIG4* promoter (4). How this region of the promoter modulates glucose repression is unknown. The KIGal80 protein is another known component of the glucose repression pathway. Expression of the lactose–galactose regulon is only slightly (10–20%) repressed in a *Klgal80* deletion strain (14). Finally, the *FOG1/GAL83* gene

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may be necessary for the glucose repression pathway (15), but this inference relies heavily upon what we know about GAL83 in S. cerevisiae.

Many genes necessary for glucose repression of the galactose regulon in S. cerevisiae have been identified and their role in the pathway is becoming clearer. A central component is ScSnf1, encoding a serine/threonine protein kinase (16). The ScSnf1 protein regulates many cellular functions (17) and is particularly critical for governing carbon metabolism (reviewed in 18). The protein has been conserved in organisms ranging from yeasts to plants to man where the ScSnf1 homolog, termed the AMP-dependent protein kinase, plays roles in cellular stress responses (19) and regulation of cholesterol and fatty acid biosynthesis (20).

Much of what is known about ScSnf1p function has come from studying its role in glucose starvation. These studies have shown that the protein kinase activity of ScSnf1p is regulated in response to glucose by ScSnf1p and by other proteins (17).

Another key component necessary for glucose repression of the galactose regulon in S. cerevisiae is the Mig1 protein (reviewed in 18). Mig1p acts to repress transcription of the galactose regulon by binding to GC-boxes (21) present in the S. GAL1 and S. GAL4 promoters (22,23). Cells lacking Mig1p show partial derepression of the galactose regulon and this phenotype is epistatic to loss of ScSnf1p, suggesting that Sc Mig1p acts downstream of ScSnf1p (reviewed in 18). DNA-bound Mig1p represses transcription by forming a complex with Tup1p and Snf6p (24,25). It is not yet known how Snf1p communicates with the Mig1p–Tup1p–Snf6p complex.

While a great deal is known in S. cerevisiae about the mechanisms Snf1p and Mig1p use to regulate galactose and other gene expression, it remains to be determined if these proteins function in similar signal transduction pathways and similar mechanistic modes in other fungi and in more complex eukaryotes. Data from mammals demonstrate that the Snf1p homolog, AMP-activated protein kinase, performs unique functions and regulates isoprenoid and fatty acid biosynthesis (19). Although K. lactis is closely related to S. cerevisiae on an evolutionary time scale (26), the two organisms have experienced different selective pressures and are not likely to use Snf1p and Mig1p in identical ways, particularly to regulate galactose metabolic genes, since K. lactis but not S. cerevisiae evolved to utilize lactose as a carbon source.

**MATERIALS AND METHODS**

**Yeast strains and growth media**

The S. cerevisiae (MCY1845) and K. lactis strains used in these studies are listed in Table 1. Strain JS1 was derived from strain JA6 by one step gene replacement (4) of the wild-type KLSNF1 chromosomal allele with the klsnf1-Δ1 deletion allele, which has nucleotides –143 to +1629 replaced with a 1.1 kb DNA fragment carrying the S. cerevisiae URA3 (ScURA3) gene. The klsnf1-Δ1 allele, released from pBSdnf1 by cleaving the EcoRI sites, was transformed into strain JA6 followed by selection for Ura+ transformants. Because homologous recombination is less frequent in K. lactis than in S. cerevisiae, Ura+ transformants were screened for Lac+ and Gal+ cells by replica plating and cells with these phenotypes were then analyzed by Southern blotting to confirm that the SNF1 locus had been replaced by the klsnf1-Δ1 deletion allele. Strain JS1/D1/R is a Ura– derivative of strain JS1D1 isolated for resistance to 5′-fluoroorotic acid (27).

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**Table 1. Genotype and origin of yeast strains used in these studies**

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JA6</td>
<td>MATα ade trp1 ura3</td>
<td>(3)</td>
</tr>
<tr>
<td>SD12</td>
<td>MATα trp1 ura3 lac4</td>
<td>(3)</td>
</tr>
<tr>
<td>JSD1</td>
<td>Derivative of JA6 carrying snf1-Δ1</td>
<td>this work</td>
</tr>
<tr>
<td>JSD1/R</td>
<td>Ura– derivative of JSD1</td>
<td>this work</td>
</tr>
<tr>
<td>JSD2</td>
<td>Derivative of JA6 carrying snf1-Δ1 mig1-Δ1</td>
<td>this work</td>
</tr>
<tr>
<td>JSD2/R</td>
<td>Ura– derivative of JSD2</td>
<td>this work</td>
</tr>
<tr>
<td>JSD3</td>
<td>MATα ade ura3 mig1-Δ1</td>
<td>this work</td>
</tr>
<tr>
<td>JSD4</td>
<td>Derivative of JA6 carrying snf1-Δ1 gal1-10</td>
<td>this work</td>
</tr>
<tr>
<td>JSD5</td>
<td>Derivative of JA6 carrying gal1-10</td>
<td>this work</td>
</tr>
<tr>
<td>JSD6</td>
<td>JA6 carrying GAL1-11</td>
<td>this work</td>
</tr>
<tr>
<td>JSD7</td>
<td>JA6 carrying snf1-Δ1 GAL1-11</td>
<td>this work</td>
</tr>
<tr>
<td>MCY1845</td>
<td>MATa snf1-Δ10 ade2-101 ura3-52 Suc2</td>
<td>(44)</td>
</tr>
</tbody>
</table>

The snf1 mig1 double deletion strain JSD2 was derived from strain JS1. The KIM1G1 gene was obtained by amplification of JA6 chromosomal DNA using the PCR and two primers, 5′-CGGAATTCTTCGCGAATTCCGTTCA and 5′-CGGAGATTCTCGGTGTTACATCATTCA, which have an EcoRI site added to their 5′-end to facilitate cloning. The sequence of the primers was based on the published KIM1G1 DNA sequence (28). The amplified KIM1G1 gene was cloned into the EcoRI site of pBLUESCRIPT (In Vitrogen, San Diego, CA) to give pBDMIG1.

The mig1 mutant strain JSD3 was constructed by crossing strains SD12 and JSD2, selecting diploids on medium lacking uracil and adenine (29), sporulating diploids and dissecting tetrads. Haploid Trp+ offspring were identified and the presence of the ScTRP1-marked klmig1-Δ1 allele was verified by Southern blot analysis.

Strains JSD6 and JSD7 carry the KGLAL1-11 allele in which the putative Mig1 binding site, the GC–AT–BOX, of the GAL1 promoter is inactivated by multiple mutations (Fig. 1). These strains were constructed in several steps. First, intermediate strains JSD4 and JSD5 were made by replacing the wild-type KGLALI promoter with the klgal1-10 allele, having nucleotides –488 to +119 replaced with the ScURA3 gene. This allele was made by cloning a 1.4 kb XbaI–BamHI DNA fragment of KGLALI into the cognate sites of pBLUESCRIPT, yielding pBSKgalI, which was cut at the unique XbaI site (+119 relative to the GAL1 start codon) and the BspMI site (–488 relative to the GAL1 start codon), treated with Klenow DNA polymerase I to make the ends blunt, and ligated to a 1.1 kb DNA fragment carrying ScURA3 to give pBSKgalIURA3. The klgal1-10 allele, released from pBSKgalIURA3 as a Smal and SacII DNA fragment, was transformed into strain JSD1/D1/R with selection for Ura+ cells, followed by screening for Gal+ Lac– cells. Only two out of nearly 2000 Ura+ transformants were Gal– Lac–, one of these was designated strain JSD4. Strain JSD5 was made by crossing strains SD12 and JSD4, sporulating diploids, and identifying Ura+ Gal– Lac– offspring. The presence of the klgal1-10
Figure 1. The KIGAL1-10 promoter. A diagrammatic representation of the KIGAL1-10 promoter is shown at the top of the figure with the direction of transcription indicated by arrows. The wild-type DNA sequence containing a putative Mga1p binding site composed of an AFbox and OC-boxes is shown between nucleotides –362 and –333 relative to the ATG (+1) start codon of GAL1. The GAL1-11 promoter allele is shown at the bottom of the figure with the mutated bases underlined. The indicated BamHI, Dsal and AvrII restriction sites were used to determine the presence of the mutant allele in strains JSD6 and JSD7.

In the second step of constructing strains JSD6 and JSD7, the KIGAL1-10 allele (Fig. 1) was made by using site-directed mutagenesis and a two step PCR protocol (30). The first PCR used pBS0.6kgal as a DNA template, a mutagenic primer corresponding to the bases –278 to –218 (5’ to 3’) of the GAL1 promoter (5’-CGAAGATCCGCAGAAGACTGCAGCTAGGCGCATGG-ATCCCTTGTTCCTCACGACGGCAA-3’), and universal primer 1211 (New England BioLabs, Beverly, MA) corresponding to bases in the pBluescript vector.

The plasmid pBS0.6kgal contains a 0.6 kb fragment extending from the BspMII restriction site just upstream of the GC box in the KIGAL1 promoter to the BglII site in the KIGAL1 coding region (Fig. 1). The 0.6 kb fragment was made by using the PCR and primers containing an EcoRI or a BamHI site so that the PCR product could be cloned into the cognate sites of pBluescript.

The product of the first PCR was cleaved with KpnI to remove the 1211 sequence, and then used in conjunction with universal primer 1201 (New England BioLabs) in a second PCR. The second PCR product was cleaved with BamHI and EcoRI and cloned into the cognate sites of pBluescript to give pBSM0.6kgal. Mutation of the putative GC and AT boxes (Fig. 1) was verified by DNA sequence and restriction site analysis of pBSM0.6kgal. The 0.6 kb BspMII–BglII fragment in pBSKgal was replaced with the corresponding fragment from pBSM0.6kgal, containing the mutated bases shown in Figure 1, to yield pBSMKgal1.

Strain JSD7 was made by cleaving pBSMKgal1 at its XbaI and HindIII sites, transforming the DNA into strain JSD4, and selecting for transplacement of the kigal1-10 allele (marked with Sc:URA3) with the mutant KIGAL1-11 allele. Selection was done by plating cells on plates containing 5-fluoroorotic acid. Strain JSD6 was made in the same manner by transforming strain JSD5.

RESULTS

Enzyme assays and miscellaneous procedures

For assaying CAT activity, transformed yeast cells were pregrown overnight to saturation in defined medium supplemented with the carbon sources indicated in the text. Saturated overnight cultures were diluted into 10 ml of fresh medium to an optical density at 600 nm (OD600) of 0.25–0.30 and grown to an OD600 of 0.7–0.8. Cells were centrifuged for 5 min at 5000 C for 10–15 min. Samples were centrifuged for 4°C for 5 min, and the supernatant fluid was used immediately for enzyme assay (6). An equal volume of 0.5 mm diameter acid-washed glass beads was added and the cells were disrupted by vortexing at 4°C for 10–15 min. Samples were centrifuged at 4°C for 5 min, and the supernatant fluid was used immediately for enzyme assay (6). A unit of CAT activity is defined in Table 5.

GUS activity was measured as described by Jefferson (33) using cells and extracts prepared as for the CAT assay. Previously described assays were used to measure β-galactosidase activity (4), transferase, epimerase and galactokinase activity (34) and lactose transport (35).

Yeast cells were transformed using the procedure of Gietz et al. (36).

Isolation of a SNF1 homolog from K.lactis

To isolate the KISNF1 gene, S.cerevisiae strain MCY1845 (relevant features: snf1-Δ10, Suc–) was transformed with a K.lactis genomic DNA library, Ura+ transformants were selected, pooled, and re-selected for Suc+ cells. To determine if a plasmid-borne gene was responsible for Suc+ colonies, plasmid DNA from 10 Suc+ transformants was recovered by transformation into and purification from Escherichia coli, followed by retransformation into strain MCY1845. All MCY1845 Ura+ transformants were Suc+, indicating that a plasmid-borne gene was responsible for the Suc+ phenotype. The plasmids carried the same 10 kb insert as determined from restriction endonuclease digestion. The complementing gene was used for sporulating diploids according to previously published procedures (29). Plates containing 5-fluoroorotic acid were made by mixing 500 ml autoclaved 4% agar with 500 ml of a filter sterilized solution containing 7 g of yeast nitrogen base (Difco), 1 g of 5-fluoroorotic acid, 50 mg of uracil and 20 g of glucose.

Gene isolation and reporter plasmids

The KISNF1 gene was selected from a K.lactis genomic library carried on the multi-copy vector pAB24 (31). Portions of the original plasmid carrying KISNF1 were subcloned into YEp352 (32) and tested for complementation of the Suc– phenotype of strain MCY1845. pBSNF1 carries the KISNF1 gene on a 3.1 kb EcoRI DNA fragment inserted into the EcoRI site of pBluescript. The nucleotide sequence of both strands of the 3.1 kb fragment was determined using a commercial DNA sequencing kit (United States Biochemical Corp., Cleveland, OH).

The reporter plasmid pKlgal4CAT contains the Klgal4 promoter fused to the coding region of the chloramphenicol acetyl transferase (CAT) gene (6). The reporter plasmid pC80GUS contains the Klgal80 promoter fused to the β-glucuronidase (GUS) gene (14).
was localized within a 3.1 kb EcoRI restriction fragment by subcloning and complementation testing (data not shown).

The DNA sequence of the 3.1 kb fragment was determined and, when analyzed, showed one open-reading frame, predicted to encode a protein of 602 amino acids with a mass of 68 463 Da. This predicted protein is identical to one recently identified as the *K.lactis* Snf1 protein (Klsnf1p) (15). Klsnf1p shows 75% amino acid identity with the *S.cerevisiae* Snf1 protein (ScSnf1p), indicating that the two proteins are structural homologs.

There appears to be only one *SNF1* coding sequence in *K.lactis*, since a Southern blot made using genomic DNA cut with SspI showed one band of hybridization with the *KISNF1*-containing 3.1 kb EcoRI DNA fragment radiolabeled with $^{32}$P (data not shown).

**Impaired carbon utilization in a Klsnf1 mutant strain**

To determine if the Klsnf1 protein is necessary for expression of the lactose–galactose regulon, the growth rate of a *Klsnf1* deletion strain (JSD1) was measured in a medium having lactose or galactose as the carbon source. The deleted strain grew much slower than the non-deleted strain on both sugars (Table 2), indicating that full expression of the lactose–galactose regulon requires Klsnf1p. Strain JSD1 grew, albeit slower than wild-type strain JA6, with sucrose as the carbon source. This result is in contrast to the situation in *S.cerevisiae* where SNF1 (sucrose non-fermenting) is required for utilization of sucrose (37). The *Klsnf1* mutant strain JSD1, like a Scsnf1p mutant strain, grew slightly slower on glucose than did the wild-type strain JA6. Strain JSD1 failed to grow at all when sorbitol, raffinose, maltose, glycerol or ethanol were used as the carbon source (data not shown). These results demonstrate that Snf1p plays a central role in carbon metabolism in *K.lactis*, as it does in *S.cerevisiae*.

**Table 2. Effect of the Klsnf1 deletion on cell growth**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Doubling time (min)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>JA6</td>
<td>SNF1</td>
<td>102 ± 6</td>
</tr>
<tr>
<td>JSD1</td>
<td>snf1</td>
<td>143 ± 11</td>
</tr>
</tbody>
</table>

$^a$Cells were grown in defined medium supplemented with the indicated carbon source added to a final concentration of 2% (w/v), sonicated to dissociate clumped cells, and diluted into fresh medium to give a starting OD$_{600}$ of 0.2–0.3. The doubling time is defined as the time in min for the OD$_{600}$ to double. Mean values ± the standard deviation represent data from three independent determinations.

**Figure 2.** Kinetics of lactose accumulation. The amount of lactose transported into cells by the Lac12 permease was measured in lactose-induced, log phase wild-type JA6 (squares) and Klsnf1-deleted JSD1 (circles) cells using the procedures described in the legend to Table 4.

**Deletion of Klsnf1 reduces expression of the linked GAL1, GAL7 and GAL10 genes**

To begin to understand why the *KISNF1* gene is necessary for rapid growth on lactose and galactose, we determined which structural gene(s) in the lactose–galactose regulon requires the Klsnf1p protein for normal expression under uninduced (basal), induced and glucose-repressed conditions. The Klsnf1-deleted strain JSD1 had about the same uninduced level for the three enzymes as the wild-type strain JA6, but the induced level was reduced to 23–31% of that wild-type strain (Table 3). These results show that *KISNF1* is essential for full induction of expression of these three linked genes. In addition, the activities of the three enzymes are still repressed by glucose in the deletion strain, implying that *KISNF1* is not essential for maintaining glucose repression of these three genes.

**Deletion of Klsnf1 reduces expression of LAC4 and LAC12**

We next measured expression of the *LAC4* (β-galactosidase) and the *LAC12* (lactose permease) genes which are transcribed in opposite direction from the same promoter (9). Deletion of *Klsnf1* greatly reduced β-galactosidase activity under both uninduced and induced conditions (Table 4), but the induction mechanism was still operating on the *LAC4* gene, although only about half as effectively as in wild-type cells (60-fold induction in wild-type JA6 cells compared with 24-fold in JSD1 cells).

**Table 3. GAL1, GAL10 and GAL7 expression is controlled by Klsnf1p and Klmig1p**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genes</th>
<th>Specific activities (nmol product/mg protein/min)$^b$</th>
<th>Epimerase (GAL10)</th>
<th>Transferase (GAL7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Galactokinase (GAL1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>U I R</td>
<td>U I R</td>
<td>U I R</td>
</tr>
<tr>
<td>JA6</td>
<td>SNF1MIG1</td>
<td>10 (100) 90 (100) 22</td>
<td>67 (100) 591 (100) 63</td>
<td>129 (100) 1092 (100) 157</td>
</tr>
<tr>
<td>JSD1</td>
<td>snf1MIG1</td>
<td>10 (100) 43 (23) 16</td>
<td>56 (84) 183 (31) 59</td>
<td>106 (82) 334 (31) 128</td>
</tr>
<tr>
<td>JSD2</td>
<td>snf1mig1</td>
<td>10 (100) 116 (61) 18</td>
<td>74 (110) 482 (82) 190</td>
<td>101 (78) 577 (53) 194</td>
</tr>
<tr>
<td>JSD3</td>
<td>SNF1mig1</td>
<td>31 (310) 232 (122) 103</td>
<td>91 (136) 799 (135) 729</td>
<td>367 (284) 1662 (152) 741</td>
</tr>
<tr>
<td>JSD6</td>
<td>SNF1GAL1−11</td>
<td>14 (140) 213 (112) 70</td>
<td>ND ND ND</td>
<td>ND ND ND</td>
</tr>
</tbody>
</table>

$^b$Specific activities were determined in cell-free extracts made from log-phase cells grown in defined medium supplemented with the indicated carbon sources: uninduced (U), 2% sucrose; induced (I), 2% sucrose plus 2% galactose; repressed (R), 2% sucrose plus 2% galactose plus 2% glucose at 30°C. Values represent the average of at least three independent determinations and the standard deviation was < ± 20%. Numbers in parenthesis represent the percentage of enzyme activity relative to the value for JA6, ND, not determined.
The KlSnf1 mutation had its greatest effect on lactose transport activity; basal activity was reduced and no induction occurred (Table 4). To verify that lactose transport was not induced in JSD1 mutant cells, the kinetics of lactose uptake were followed over a 2 h period. During this time JSD1 cells failed to accumulate lactose (Fig. 2), indicating greatly reduced expression of LAC12.

We conclude from the data presented in Table 4 and Figure 2 that the KlSNF1 gene is required for a normal basal level of LAC4 and LAC12 expression. Full induction of LAC4 expression requires KlSNF1 but ~10% of the inducible expression is independent of KlSNF1. Induction of LAC12 expression is entirely dependent upon KlSNF1. KlSNF1 is not necessary for glucose repression of LAC4 expression since β-galactosidase activity was nearly the same under uninduced and glucose repressed conditions in the Klsnf1 mutant JSD1 (Table 4). Because LAC12 expression was not induced in strain JSD1, glucose repression could not be evaluated (Table 4).

**Effect of the KlSnf1 deletion on expression of KlGAL4**

Full induction of the lactose–galactose regulon requires autoactivation of KlGAL4 expression (6). To determine if KlSNF1 is necessary for autoactivation we measured KlGAL4 expression using a reporter gene in which the KlGAL4 promoter is fused to the CAT coding region. Transformants were grown in selective medium lacking Trp or Ura and supplemented with the carbon sources as described in the legend to Table 2. Numbers in parenthesis represent the percentage of enzyme activity relative to the value for strain JA6. Mean values ± standard deviation represent at least three independent determinations.

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### Table 4. LAC4 and LAC12 expression is controlled by KlSnf1p and KlMig1p

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotypes</th>
<th>α-Galactosidase activity</th>
<th>Permease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U</td>
<td>I</td>
</tr>
<tr>
<td>JA6</td>
<td>SNF1MIG1</td>
<td>75 (100)</td>
<td>4469 (100)</td>
</tr>
<tr>
<td>JSD1</td>
<td>snf1MIG1</td>
<td>17 (23)</td>
<td>415 (9)</td>
</tr>
<tr>
<td>JSD2</td>
<td>snf1Mig1</td>
<td>30 (40)</td>
<td>1737 (39)</td>
</tr>
<tr>
<td>JSD3</td>
<td>SNF1mig1</td>
<td>245 (327)</td>
<td>7546 (169)</td>
</tr>
<tr>
<td>JSD6</td>
<td>SNF1GAL1-11</td>
<td>85 (113)</td>
<td>5319 (119)</td>
</tr>
</tbody>
</table>

Cell culture conditions were the same as described in the legend to Table 2. Lactose permease activity is defined as μmol of lactose accumulated per OD600 unit per 20 min. Cells were grown overnight in defined medium at 30°C, diluted into fresh medium to give an OD600 of 0.2–0.3 and grown to an OD600 of 0.5–0.8. After washing once with cold medium, 1 m[M1-14C]lactose was added to each culture and the intracellular lactose accumulation (μmol/μg protein) was measured. The values shown represent the average of at least three independent determinations. The standard deviations were < ±15%. Numbers in parenthesis represent the percentage of enzyme activity relative to the value for strain JA6. ND, not determined.

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### Table 5. KlGAL4 expression in mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genes</th>
<th>Units of CAT activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uninduced</td>
</tr>
<tr>
<td>JA6</td>
<td>SNF1MIG1</td>
<td>11.4 ± 0.9 (100)</td>
</tr>
<tr>
<td>JSD1</td>
<td>snf1MIG1</td>
<td>9.6 ± 1.2 (84)</td>
</tr>
<tr>
<td>JSD2/R</td>
<td>snf1Mig1</td>
<td>9.7 ± 1.0 (94)</td>
</tr>
<tr>
<td>JSD3</td>
<td>SNF1mig1</td>
<td>10.3 ± 0.9 (90)</td>
</tr>
<tr>
<td>JSD6</td>
<td>SNF1GAL1-11</td>
<td>13.4 ± 1.0 (118)</td>
</tr>
</tbody>
</table>

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### Table 6. KlGAL80 expression in mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypes</th>
<th>GUS activity (μmol/mg protein/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uninduced</td>
</tr>
<tr>
<td>JA6</td>
<td>SNF1MIG1</td>
<td>12 ± 2 (100)</td>
</tr>
<tr>
<td>JSD1</td>
<td>snf1SNF1</td>
<td>11 ± 1 (92)</td>
</tr>
<tr>
<td>JSD2/R</td>
<td>snf1Mig1</td>
<td>12 ± 1 (100)</td>
</tr>
<tr>
<td>JSD3</td>
<td>SNF1mig1</td>
<td>15 ± 2 (125)</td>
</tr>
</tbody>
</table>

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*a Strains JSD1, JSD2 and JSD3 were transformed with the reporter plasmid pKlGAL4CAT containing the full length KlGAL4 promoter fused to the CAT coding region. The transformants were grown in the selective medium with the indicated carbon source: Uninduced: 2% sucrose; Induced: 2% sucrose + 2% galactose; Repressed: 2% sucrose + 2% galactose + 2% glucose. Units of CAT activity are % conversion of substrate (c.p.m. measured in the organic phase expressed as a percentage of total c.p.m.) per mg protein per 45 min. Numbers in parenthesis represent the percentage of enzyme activity relative to the value for strain JA6. Mean values ± standard deviation represent at least three independent determinations.

*b Strains were transformed with pC80GUS which carries the KlGAL80 promoter fused to the GUS coding region. Transformants were grown in selective medium lacking Trp or Ura and supplemented with the carbon sources as described in the legend to Table 2. Numbers in parenthesis represent the percentage of enzyme activity relative to the value for strain JA6. Mean values ± standard deviation represent at least three independent determinations.
only slightly above the uninduced level of the wild-type strain (11.4 CAT units). We conclude from the data shown in Table 5 that KlSNF1 is required for activation of KlGAL4 expression during induction of the lactose–galactose regulon.

Effect of the KlSnf1 deletion on expression of KlGAL80

KlGal4p binds to two UAS sequences in the KlGAL80 promoter and regulates its expression (14,38). Thus, we expected a KlSnf1 deletion strain to show impaired expression (11.4 CAT units). We conclude from the data shown in Table 5 that deletion of KlSnf1 had no effect on basal expression of KlGAL80 expression. The KlSnf1-deleted strain JSD1 had about the same GUS activity as the wild-type strain JA6. We conclude from these data that full induction of KlGAL80 expression requires the KlSNF1 gene. Glucose repressed GUS activity, indicating that KlSNF1 plays no role in maintaining repression of KlGAL80 expression (Table 6).

Role of the KlMig1 protein in expression of the lactose–galactose regulon

The data presented thus far show that KlSnf1p is needed for full induction of the lactose–galactose regulon but they do not indicate how the protein is working in the induction pathway. The ScSnf1 protein is known to exert some of its effects on induction of the lactose–galactose regulon but they do not indicate how the protein is working in the induction pathway. The KlSnf1 protein is known to exert some of its effects on transcription through the ScMig1 protein, thought to act by repressing transcription (reviewed in 18). The ScMig1 protein is known to bind the ScGAL4 and the ScGAL1 promoters, thereby repressing expression of the galactose regulon (23).

We first determined if expression of the lactose–galactose regulon is regulated by KlMig1p. This was done by measuring expression of the lactose–galactose genes in a klmig1-deleted strain, JSD3. The klmig1 mutation had the same general effect on expression of the GAL1, GAL7, GAL10, LAC4 and LAC12 genes; expression increased under uninduced and induced conditions and glucose did not repress expression as well as in the wild-type strain JA6 (Tables 3 and 4). These data indicate that KlMig1p normally acts to repress expression of these genes under uninduced, induced and glucose-repressed growth conditions.

Next we determined if KlMig1 acts downstream of KlSnf1, as does the ScMig1 protein when it regulates the galactose regulon of S.cerevisiae, or whether it acts upstream. Action downstream of KlSnf1 would be indicated if a klmig1 mutation restored induction of LAC–GAL gene expression in a klnsf1 strain (23). The same trends were observed for expression of the GAL1, GAL7, GAL10, LAC4 and LAC12 genes and we will focus on GAL1, since as we show below, its expression appears to be of central importance to the regulon. The klnsf1 mutant strain JSD1 showed a 4.3-fold induction of GAL1 expression (Table 3), much less than the 19-fold induction seen in wild-type JA6 cells. The klnsf1 klmig1 double mutant strain JSD2 gave an 11.6-fold induction, showing that the klnsf1 mutation can partially reverse the effect of the klnsf1 mutation. Thus, KlMig1p acts downstream of KlSnf1p in the signaling pathway for induction of the LAC–GAL genes.

The klmig1 mutation (strain JSD3) had no effect on expression of KlGAL4 in the uninduced and induced states but it caused a complete loss of glucose repression (Table 5). Similar trends were seen for KlGAL80 expression (Table 6). The implications of these results will be considered in the Discussion. Lastly, uninduced or basal expression of both KlGAL4 and KlGAL80 was not changed significantly by deletion of either snf1 or mig1 or both genes (Tables 5 and 6), indicating that basal expression of KlGAL4 and KlGAL80 is regulated in a manner independent of SNF1 and MIG1.

KlMig1p acts through the GAL1 promoter to govern expression of the lactose–galactose regulon

KlGAL1 encodes the Leloir pathway enzyme galactokinase, necessary for phosphorylation of galactose (34). In addition, the protein has a second, independent activity that is necessary for induction of the regulon (13). This second activity probably enables KlGAL1p to bind KlGAL80p, a reaction requiring both galactose and ATP (12). One model that explains these data envisages KlGAL1p acting as a molecular sensor of galactose that switches KlGAL4p between transcriptionally inactive and active forms. In the uninduced state, KlGAL80p would complex with KlGAL1 (39), thereby preventing transcription activation. During induction of the lactose–galactose regulon the inducer galactose would bind to KlGAL1p and this complex would then bind to KlGAL80p thereby switching KlGAL4p from an inactive to an active form capable of turning on transcription of genes in the lactose–galactose regulon (12).

If this model is correct, it provides an explanation for our observation (strain JSD3, Tables 3 and 4) that deletion of klmig1 increases the basal and induced level of LAC–GAL gene expression and partially abrogates glucose repression. We imagine that in the uninduced state KlMig1p binding to the KlGAL1 promoter prevents expression. Early during induction, the repressive effect of KlMig1p is switched off so that transcription of KlGAL1 begins, followed by production of KlGAL1p. KlGAL1p in conjunction with galactose and ATP then complexes with KlGAL80p, thereby enabling KlGAL4p to activate expression of the other genes in the regulon.

As first pointed out by Cassart et al. (28), the KlGAL1 promoter contains a potential M1g1 binding site consisting of a GC box (G/C/C/T/G/G/G/A/G/A) preceded on the 5' side by an A-rich region (21); we found no other promoters in the regulon with a Mig1p binding site. If this model is correct, it predicts that mutation of the Mig1p binding site in the KlGAL1 promoter (Fig. 1) should partially abrogate glucose repression and cause a small increase in basal and induced expression of the regulon. As predicted by this hypothesis, we found that glucose repression of β-galactosidase and galactokinase activity was partially abolished and both basal and induced expression were slightly increased in mutant strain JSD6 compared with wild-type strain JA6 (Tables 3 and 4).

We also determined if mutation of the GC–AT box region of the KlGAL1 promoter abrogated glucose repression of KlGAL4 expression as was seen in the klmig1 deletion strain JSD3 (Table 5). Glucose repression of KlGAL4 expression was abrogated in strain JSD6 compared with wild-type strain JA6 but not to the same extent as in strain JSD3 (Table 5). The difference between strains JSD3 and JSD6 could result from low affinity binding of KlMig1p to the mutant KlGAL1 promoter sequence in strain
JS6, whereas deletion of klmig1 would completely abolish promoter binding (strain JSD3).

**DISCUSSION**

The *S.cerevisiae* SNF1 gene plays a global role in regulating carbon utilization (18,40). One aim of our research was to determine if SNF1 plays a similar role in *K.lactis* and, in addition, if it plays specific roles in induction and glucose repression of the lactose–galactose regulon. Based upon the inability of the Klsnf1-deleted strain JSD1 to utilize a variety of fermentable and non-fermentable carbon sources (Table 2 and data not shown) we conclude that SNF1 is a global regulator of carbon utilization in *K.lactis*. One difference between *S.cerevisiae* and *K.lactis* is that utilization of sucrose requires SNF1 in *S.cerevisiae* whereas this is not the case in *K.lactis* (Table 2). The physiological reason for this difference is not apparent. Goffini et al. (15) also noted that a Klsnf1 (fog2) mutant strain fails to utilize numerous carbon sources including galactose. Our data agree with Goffini et al. except that our klsnf1 mutant grew slowly on galactose. This difference may be due to the higher concentration of galactose (2%) we used compared with the lower concentration (0.5%) used by Goffini et al.

**Snf1p is necessary for full induction of the regulon**

Our data show that KISnF1p is essential for full induction of the lactose–galactose regulon. This conclusion is based both upon the slow growth rate of a klsnf1-deleted strain when lactose or galactose are the carbon source (Table 2) and upon analysis of the expression of the structural genes in the regulon including the GAL1, GAL7, and GAL10 gene cluster (strain JSD1 compared with JA6, Table 3) and the divergently transcribed LAC4 and LAC12 genes (Table 4), plus the positive regulator GAL4 (Table 5) and the negative regulator GAL80 (Table 6).

Since cells deleted for klsnf1 grow when lactose or galactose are the only carbon source and partially induce most genes in the regulon (Tables 3–6), there must be a SNF1-independent mechanism that can partially activate expression of the regulon. In contrast, snf1-deleted *S.cerevisiae* cells do not grow on galactose (40) indicating that expression of the regulon is completely dependent upon SNF1.

**Mig1p is necessary for full repression by glucose**

The conclusion that KIMIG1p is necessary for full glucose repression of the lactose–galactose regulon is based upon the inability of the klmig1 deletion strain JSD3 to repress expression of the structural and regulatory genes as well as the wild-type strain JA6 under glucose repressing conditions (Table 3). Because the expression level of GAL1, GAL7, LAC4, LAC12 and GAL80 (Tables 3–5) under glucose repressing conditions (glucose plus galactose) is still below the level seen under inducing conditions, there must be a MIG1-independent mechanism for glucose repression. This mechanism does not affect expression of GAL10 (Table 3) or GAL4 (Table 5). A MIG1-independent mechanism for glucose repression of SUC2 expression has also been seen in *S.cerevisiae* (41).

Data for the behavior of the snf1 mig1 double mutant strain in comparison with the single mutant strains (Tables 3 and 4) argue that KIMIG1p acts downstream of KISnF1p in the induction (derepression) pathway. A similar epistatic relationship has been found for the two proteins in the pathway for derepressing expression of the galactose regulon in *S.cerevisiae* (22,23) and many other experiments argue that ScMig1p acts downstream of ScSnf1p (reviewed in 18). However, the situation in *K.lactis* is probably not this simple, because the Klmig1 mutant strain (JSD3) does not fully repress galactose gene expression under glucose repressing conditions, while the Klsnf1 Klmig1 double mutant does fully repress (Tables 3 and 4). These data indicate that, with respect to glucose repression, KLSnF1p is epistatic to KLMIG1p. One interpretation of the glucose repression data is that there is a Mig1p-independent, KISnF1p-dependent glucose repression pathway operating on the GAL genes in *K.lactis*.

If KISnF1p worked solely through KIMIG1p we would expect that under inducing conditions the snf1 mig1 double mutant strain would have gene expression levels that are similar to the wild-type values, but this is not the case for any of the genes in the regulon (compare strains JSD2 and JA6 in Table 3). These data add further support to the hypothesis that KISnF1p has a second, KIMIG1p-independent pathway, for activating expression of the regulon or that there is a KISnF1p-independent pathway. Of these two hypotheses, the KIMIG1p-independent pathway is supported by the data for the klmig1 mutant strain JSD3.

Expression of the structural genes in strain JSD3 under inducing conditions is above the wild-type level (Tables 3 and 4), indicating that when KLMIG1p is removed, expression of the structural genes can be fully induced. Recent data identify an ScSnf1p pathway that does not require ScMig1p. In this pathway ScSnf1p modulates the activity of the ScSip4 transcription activator (42). Genetic evidence suggests that ScSnf1p interacts with two other transcription activators, Msn2p and Msn4p (43), so there may be homologs of one or more of these proteins in *K.lactis* which might be necessary for full induction of the lactose–galactose regulon.

**KIMIG1p acts at the KIGAL1 promoter**

In *S.cerevisiae*, Mig1p confers glucose repression on the galactose regulon by binding to the ScGAL4 and ScGAL1 promoters (22,23). A search of the known promoters in the lactose–galactose regulon of *K.lactis* identified only one putative Mig1p binding site located in the divergently transcribed KIGAL1 and KIGAL10 promoter (Fig. 1). Mutation of this site resulted in a strain, JS6D, that behaved qualitatively like the klmig1 deletion strain JSD3 as measured by expression of KIGAL1 (galactokinase activity, Table 3) and KIGAL4 (β-galactosidase activity, Table 4). The value for these two enzymes was derepressed almost as much in strain JS6D as in strain JSD3. The difference between the two strains could reflect low affinity binding of KIMIG1p to the mutated promoter site in strain JS6D. Thus, these data support the hypothesis that KIMIG1p regulates expression of the lactose–galactose regulon primarily by binding to the KIGAL1 promoter. Although it seems unlikely that KIMIG1p regulates the LAC–GAL genes in some additional way, our data do not eliminate this possibility.

**A model for regulation of the lactose–galactose regulon**

Based upon the data presented here and upon data derived from *S.cerevisiae*, we propose (Fig. 3) that KISnF1p acts in a signaling pathway that terminates with the KIMIG1p repressor protein bound to the divergently transcribed KIGAL1–10 promoter (Fig. 1). When glucose is present in the culture medium KIMIG1p is bound to the KIGAL1 promoter and transcription is repressed, even if inducer is present also in the culture medium (glucose repressing
To activate transcription of genes in the regulon (12), which from the inhibitory effect of KlGal80p thereby allowing KlGal4p between KlGal1p and KlGal80p is thought to release KlGal4p in an ATP-dependent fashion as has been shown (12). Interaction may enable KlGal1p to bind KlGal80p in a galactose-and-glucose-repressing conditions, by a pathway requiring KISnflp. In the absence of glucose and the presence of inducer, KlGal1p repression is switched off by a KISnflp-dependent pathway and transcription of KIGAL1 is increased to produce KlGal1p. Regulating the concentration of KIGAL1p in this manner provides a mechanism for switching expression of the rest of the genes in the regulon on and off.

KIGAL1p has an activity, besides galactokinase activity, that is essential for induction of the regulon (13). This second activity may enable KIGAL1p to bind KlGal80p in a galactose-and-glucose-repressing pathway as has been shown (12). Interaction between KIGAL1p and KlGal80p is thought to release KlGal4p from the inhibitory effect of KlGal80p thereby allowing KlGal4p to activate transcription of genes in the regulon (12). Which gene(s) might be activated first by KlGal4p? We favor KIGAL4 because the concentration of the KIGAL4p limits expression of the regulon and a 2–3-fold induction of expression of this gene is known to be essential for maximal induction of the regulon (6,7) and escape from glucose repression (4,5).

Alternatively or simultaneously, KIGAL1p might target the KlGal80–KlGal4p complex bound to the KILAC12 promoter so that synthesis of the lactose–galactose transporter would begin. This hypothesis is based upon our observation that expression of KILAC12, more than any other gene in the regulon, is dependent upon the KISnflp–KlMig1p pathway. Dependence upon the KISnflp–KlMig1p pathway is supported by the slow growth of the KISnfl deletion strain on either galactose or lactose (Table 2) and failure of the strain to transport lactose during a 2-h incubation period (Fig. 2).

In summary, our data show that KISnflp is necessary for maximal induction of the lactose–galactose regulon, but there must be other induction pathways that remain to be identified. Second, the KlMig1 protein is an essential element if the pathway for glucose repression of the regulon, but there must be another repression pathway. KlMig1 works downstream of ScSnflp. Finally, the ScSnflp–KlMig1p repression pathway interfaces to the lactose–galactose regulon by binding of KlMig1p to the KIGAL1 promoter. These data provide a framework for uncovering other signaling pathways that govern expression of the lactose–galactose regulon.

ACKNOWLEDGMENTS

This work was supported by grant MCB–9219839 from the National Science Foundation. We thank Drs Karin Breunig and Wolfgang Zachariae for strains and plasmids.

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