Nucleic Acids Research, 1997, Vol. 25, No. 18 **3657–3664**

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 *KlSNF1*-independent pathway(s) also regulates induction. *MIG1* is required for full glucose repression of the regulon, but there must be a *KlMIG1*-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 *KlGAL1* expression. Regulating *KlGAL1* 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 *KlLAC4* (β-galactosidase; 8) and *KlLAC12* (lactose permease), which are transcribed in opposite directions from a common promoter (9), and *KlGAL1*, *KlGAL7* and *KlGAL10* [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 *KlGAL1* and *KlGAL10* transcribed in opposite directions from a common promoter (reviewed in 1).

The transcription induction pathway centers around the DNA-binding protein KlGal4p (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 KlGal80p which is bound to and modulates the transcription activator activity of KlGal4p (12). Induction of the regulon also requires an uncharacterized activity of the KlGal1 protein that is independent of its galactokinase activity (13). This uncharacterized activity may be responsible for the galactose and ATP-dependent binding of KlGal1p to KlGal80p, an interaction that permits KlGal4p 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 *KlGAL4* promoter (4). How this region of the promoter modulates glucose repression is unknown. The KlGal80 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 ScSnf4p 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 *ScGAL1* and ScGAL4 *promoters* (22,23). Cells lacking Mig1p show partial derepression of the galactose regulon and this phenotype is epistatic to loss of ScSnf1p, suggesting that ScMig1p acts downstream of ScSnf1p (reviewed in 18). DNA-bound Mig1p represses transcription by forming a complex with Tup1p and Ssn6p (24,25). It is not yet known how Snf1p communicates with the Mig1p–Tup1p–Ssn6p 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 eucaryotes. 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 JSD1 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 pBDsnf1 by cleaving the *Eco*RI 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 JSD1/R is a Ura$^-$ derivative of strain JSD1 isolated for resistance to 5′-fluoroorotic acid (27).

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

Name	Genotype	Source
JA6	*MATα ade trp1 ura3*	(3)
SD12	*MATα trp1 ura3 lac4*	(3)
JSD1	Derivative of JA6 carrying *snf1-Δ1*	this work
JSD1/R	Ura$^-$ derivative of JSD1	this work
JSD2	Derivative of JA6 carrying *snf1-Δ1 mig1-Δ1*	this work
JSD2/R	Ura$^-$ derivative of JSD2	this work
JSD3	*MATa ade ura3 mig1-Δ1*	this work
JSD4	Derivative of JA6 carrying *snf1-Δ1 gal1-10*	this work
JSD5	Derivative of JA6 carrying *gal1-10*	this work
JSD6	JA6 carrying *GAL1-11*	this work
JSD7	JA6 carrying *snf1-Δ1 GAL1-11*	this work
MCY1845	*MATa snf1-Δ10 ade2-101 ura3-52 SUC2*	(44)

The *snf1 mig1* double deletion strain JSD2 was derived from strain JSD1. The *KlMIG1* gene was obtained by amplification of JA6 chromosomal DNA using the PCR and two primers, 5′-CGGAATTCCGTGCGATTAGGTCAGTTCA and 5′-CGGAATTCCGGTGTTCATCGATAGTCGT, which have an *Eco*RI site added to their 5′-end to facilitate cloning. The sequence of the primers was based on the published *KlMIG1* DNA sequence (28). The amplified *KlMIG1* gene was cloned into the *Eco*RI site of pBLUESCRIPT (In Vitrogen, San Diego, CA) to give pBDMIG1. The region between the two *Nde*I sites within *KlMIG1*, nucleotides +87 to +1109, was replaced with a 1.4 kb DNA fragment carrying the *ScTRP1* gene to yield the *klmig1-Δ1* allele which is carried in pBDmig1. pBDmig1 DNA was digested with *Eco*RI and transformed into strain JSD1 with selection for Trp$^+$ transformants. Southern blot analysis confirmed that the *KlMIG1* locus had been replaced by the *klmig1-Δ1* allele. A Ura$^-$ derivative of strain JSD2, JSD2/R, was isolated by resistance to 5′-fluoroorotic acid.

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 *KlGAL1-11* allele in which the putative Mig1 binding site, the GC–AT–BOX, of the *GAL1* promoter are 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 *KlGAL1* 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 *Xba*I–*Bam*HI DNA fragment of *KlGAL1* into the cognate sites of pBLUESCRIPT, yielding pBSKlgal, which was cut at the unique *Bgl*II site (+119 relative to the *GAL1* start codon) and the *Bsp*MI 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 pBSKlgalURA3. The *klgal1-10* allele, released from pBSKlgalURA3 as a *Sma*I and *Sac*II DNA fragment, was transformed into strain JSD1/R with selection for Ura$^+$ cells, followed by screening for Gal$^-$ Lac$^-$ cells. Only two out of nearly 2000 Ura$^+$ transformants were Gal$^-$ Lac$^-$, and 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*

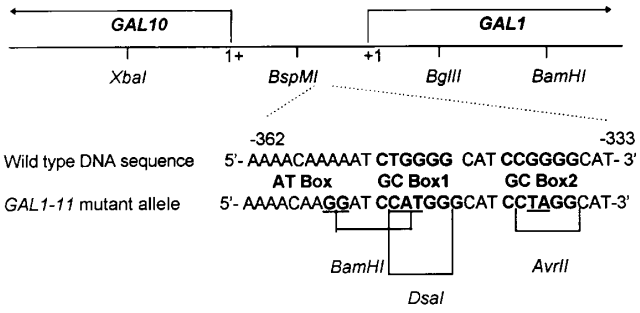

Figure 1. The *KlGAL1-10* promoter. A diagrammatic representation of the *KlGAL1-10* promoter is shown at the top of the figure with the direction of transcription indicated by arrows. The wild-type DNA sequence containing a putative Mig1p binding site composed of an AT-box and GC-boxes is shown between 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 *Bam*HI, *Dsa*I and *Avr*II restriction sites were used to determine the presence of the mutant allele in strains JSD6 and JSD7.

allele in strains JSD4 and JSD5 was verified by Southern blotting (data not shown).

In the second step of constructing strains JSD6 and JSD7, the *KlGAL1-11* 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′-CGGAATGAGCGGAAGACTATGCCTAGGATGCCATGG-ATCCTTGTTTTCTCAGCAGGCAAA-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 *Bsp*MI restriction site just upstream of the GC box in the *KlGAL1* promoter to the *Bgl*II site in the *KlGAL1* coding region (Fig. 1). The 0.6 kb fragment was made by using the PCR and primers containing an *Eco*RI or a *Bam*HI site so that the PCR product could be cloned into the cognate sites of pBLUESCRIPT.

The product of the first PCR was cleaved with *Kpn*I 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 *Bam*HI and *Eco*RI and cloned into the cognate sites of pBLUESCRIPT to give pBSM0.6Kgal1. Mutation of the putative GC and AT boxes (Fig. 1) was verified by DNA sequence and restriction site analysis of pBSM0.6Kgal1. The 0.6 kb *Bsp*MI–*Bgl*II fragment in pBSKlgal was replaced with the corresponding fragment from pBSM0.6Kgal1, containing the mutated bases shown in Figure 1, to yield pBSMKlgal1.

Strain JSD7 was made by cleaving pBSMKlgal1 at its *Xba*I and *Hin*dIII sites, transforming the DNA into strain JSD4, and selecting for transplacement of the *klgal1-10* allele (marked with *ScURA3*) with the mutant *KlGAL1-11* 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. The correct transplacement event in Ura$^-$ Lac$^+$ cells was first identified by using the PCR and then verified by Southern blot analysis. At least three independent isolates of each strain were assayed for β-galactosidase, galactokinase and CAT activity as described in the text.

PYED and defined media (4) were supplemented with the carbon sources indicated in table and figure legends. ME medium 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 *KlSNF1* gene was selected from a *K.lactis* genomic library carried on the multi-copy vector pAB24 (31). Portions of the original plasmid carrying *KlSNF1* were subcloned into YEp352 (32) and tested for complementation of the Suc$^-$ phenotype of strain MCY1845. pBSNF1 carries the *KlSNF1* gene on a 3.1 kb *Eco*RI DNA fragment inserted into the *Eco*RI site of pBLUE-SCRIPT. 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).

Enzyme assays and miscellaneous procedures

For assaying CAT activity, transformed yeast cells were pre-grown 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 (OD_{600}) of 0.25–0.30 and grown to an OD_{600} of 0.7–0.8. Cells were centrifuged for 5 min at 5000 *g* at 4°C, and suspended in 300 µl of ice-chilled breaking buffer (50 mM sodium phosphate, pH 7.5, 5% glycerol, 1 mM EDTA and 1 mM PMSF; this buffer was found to give more reproducible assays than a previously described buffer (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).

RESULTS

Isolation of a *SNF1* homolog from *K.lactis*

To isolate the *KlSNF*1 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 localized within a 3.1 kb *Eco*RI 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 *Ssp*I showed one band of hybridization with the *KlSNF1*-containing 3.1 kb *Eco*RI 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 *SNF*1 (sucrose non-fermenting) is required for utilization of sucrose (37). The *Klsnf1* mutant strain JSD1, like a *Scsnf1* 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

Strains	Genotype	Doubling time (min)[a]			
		Glucose	Sucrose	Lactose	Galactose
JA6	*SNF1*	102 ± 6	88 ± 15	98 ± 6	103 ± 4
JSD1	*snf1*	143 ± 11	154 ± 10	318 ± 13	391 ± 25

[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.

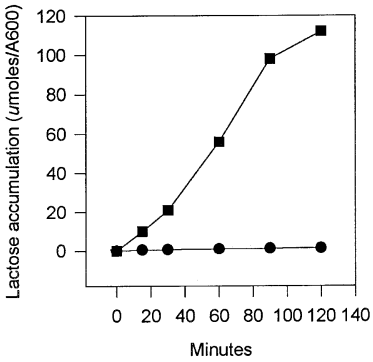

Figure 2. Kinetics of lactose accumulation. The amount of lactose transported into cells by the Lac12 permease was measured in lactose-induced, log phase wild-type JA6 (squares) and *Klsnf1*-deleted JSD1 (circles) cells using the procedures described in the legend to Table 4.

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

To begin to understand why the *KlSNF1* gene is necessary for rapid growth on lactose and galactose, we determined which structural gene(s) in the lactose–galactose regulon requires the KlSnf1 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 *KlSNF1* 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 *KlSNF1* 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

Strain	Relevant genes	Specific activities (nmol product/mg protein/min)[a]								
		Galactokinase (*GAL1*)			Epimerase (*GAL10*)			Transferase (*GAL7*)		
		U	I	R	U	I	R	U	I	R
JA6	*SNF1MIG1*	10 (100)	190 (100)	22	67 (100)	591 (100)	63	129 (100)	1092 (100)	157
JSD1	*snf1MIG1*	10 (100)	43 (23)	16	56 (84)	183 (31)	59	106 (82)	334 (31)	128
JSD2	*snfmig1*	10 (100)	116 (61)	18	74 (110)	482 (82)	190	101 (78)	577 (53)	194
JSD3	*SNF1mig1*	31 (310)	232 (122)	103	91 (136)	799 (135)	729	367 (284)	1662 (152)	741
JSD6	*SNF1GAL1-11*	14 (140)	213 (112)	70	ND	ND	ND	ND	ND	ND

[a]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.

Table 4. *LAC4* and *LAC12* expression is controlled by KlSnf1p and KlMig1p[a]

Strains	Relevant genotypes	β-Galactosidase activity			Permease activity		
		U	I	R	U	I	R
JA6	*SNF1MIG1*	75 (100)	4469 (100)	265	1.0 (100)	18.7 (100)	1.9
JSD1	*snf1MIG1*	17 (23)	415 (9)	23	0.4 (40)	0.5 (3)	0.4
JSD2	*snf1mig1*	30 (40)	1737 (39)	56	0.5 (50)	3.2 (17)	1.0
JSD3	*SNF1mig1*	245 (327)	7546 (169)	2271	1.2 (120)	29.6 (158)	7.1
JSD6	*SNF1GAL1-11*	85 (113)	5319 (119)	1566	ND	ND	ND

[a]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 OD_{600} unit per 20 min. Cells were grown overnight in defined medium at 30°C, diluted into fresh medium to give an OD_{600} of 0.2–0.3 and grown to an OD_{600} of 0.7–0.8. After washing once with cold medium, 1 mM [^{14}C]lactose was added to each culture and the intracellular lactose accumulation (μm/A_{600}) 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.

Table 5. *KlGAL4* expression in mutant strains

Strain	Relevant genes	Units of CAT activity[a]		
		Uninduced	Induced	Repressed
JA6	*SNF1MIG1*	11.4 ± 0.9 (100)	71.5 ± 5.9 (100)	28.4 ± 1.5 (100)
JSD1	*snf1MIG1*	9.6 ± 1.2 (84)	15.6 ± 1.6 (22)	14.1 ± 1.1 (50)
JSD2/R	*snf1mig1*	10.7 ± 1.0 (94)	40.3 ± 2.2 (56)	40.8 ± 4.1 (144)
JSD3	*SNF1mig1*	10.3 ± 0.9 (90)	75.8 ± 5.2 (106)	73.7 ± 7.0 (259)
JSD6	*SNF1GAL1-11*	13.4 ± 1.0 (118)	61.7 ± 4.8 (86)	48.2 ± 5.3 (170)

[a]Strains JA6, 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.

Table 6. *KlGAL80* expression in mutant strains

Strain	Genotypes	GUS activity(nmol/mg protein/min)[a]		
		Uninduced	Induced	Repressed
JA6	*SNF1MIG1*	12 ± 2 (100)	575 ± 60 (100)	55 ± 4
JSD1	*snf1SNF1*	11 ± 1 (92)	148 ± 20 (26)	17 ± 2
JSD2/R	*snf1mig1*	12 ± 1 (100)	162 ± 18 (28)	44 ± 8
JSD3	*SNF1mig1*	15 ± 2 (125)	224 ± 16 (39)	39 ± 3

[a]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 JA6. Mean values ± standard deviation represent at least three independent determinations.

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 coding region of the *CAT* gene. This reporter gene, when carried on a single-copy *CEN* vector, has been shown to be a very sensitive way to measure small changes in *KlGAL4* expression (6). Induction of *KlGAL4* expression in mutant strain JSD1 was reduced to 22% of the level seen in wild-type strain JA6 (Table 5). Viewed another way, the level of *KlGAL4* expression in the mutant strain under inducing conditions (15.6 CAT units) was

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 *KlGAL80* expression. A reporter plasmid, pC80GUS, containing the *KlGAL80* promoter fused to the β-glucuronidase (GUS) coding region was used to measure the effect of *KlSNF1* on *KlGAL80* expression. The *klsnf1*-deleted strain JSD1 had about the same GUS activity as the wild-type strain under the uninduced condition, indicating that mutation of *klsnf1* had no effect on basal expression of *KlGAL80* (Table 6). In contrast, GUS activity was induced only 13-fold in mutant strain JSD1 compared with the 48-fold induction seen in 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 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.

We next 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 *klsnf1* 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 *klsnf1* 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 *klsnf1 klmig1* double mutant strain JSD2 gave an 11.6-fold induction, showing that the *klmig1* mutation can partially reverse the effect of the *klsnf1* 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 KlGal4 (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 Mig1 binding site consisting of a GC box (G/C C/T G G G/A G) 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 KlMig1p 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-galactosidease 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

JSD6, 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. Goffrini *et al*. (15) also noted that a *Klsnf1* (*fog2*) mutant strain fails to utilize numerous carbon sources including galactose. Our data agree with Goffrini *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 Goffrini *et al*.

Snf1p is necessary for full induction of the regulon

Our data show that KlSnf1p 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 KlMig1p 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 KlMig1p acts downstream of KlSnf1p 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 situtation 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, KlSnf1 is epistatic to KlMig1. One interpretation of the glucose repression data is that there is a Mig1p-independent, KlSnf1p-dependent glucose repression pathway operating on the *GAL* genes in *K.lactis*.

If KlSnf1p worked solely through KlMig1p 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 KlSnf1p has a second, KlMig1p-independent pathway, for activating expression of the regulon or that there is a KlSnf1p-independent pathway. Of these two hypotheses, the KlMig1p-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.

KlMig1p acts at the *KlGAL1* 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 *KlGAL1* and *KlGAL10* promoter (Fig. 1). Mutation of this site resulted in a strain, JSD6, that behaved qualitatively like the *klmig1* deletion strain JSD3 as measured by expression of *KlGAL1* (galactokinase activity, Table 3) and *KlGAL4* (β-galactosidase activity, Table 4). The value for these two enzymes was derepressed almost as much in strain JSD6 as in strain JSD3. The difference between the two strains could reflect low affinity binding of KlMig1 to the mutated promoter site in strain JSD6. Thus, these data support the hypothesis that KlMig1p regulates expression of the lactose–galactose regulon primarily by binding to the *KlGAL1* promoter. Although it seems unlikely that KlMig1p 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 KlSnf1p acts in a signaling pathway that terminates with the KlMig1 repressor protein bound to the divergently transcribed *KlGAL1-10* promoter (Fig. 1). When glucose is present in the culture medium KlMig1p is bound to the *KlGAL1* promoter and transcription is repressed, even if inducer is present also in the culture medium (glucose repressing

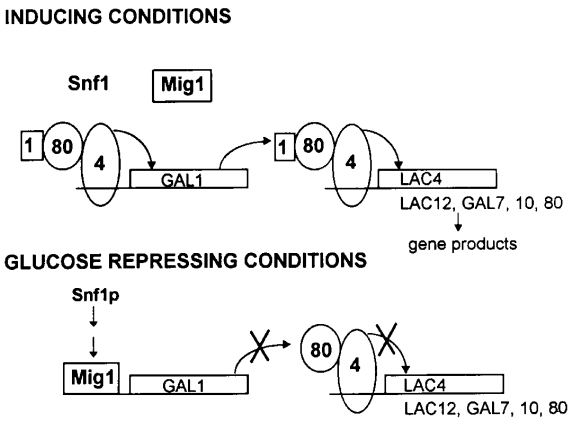

Figure 3. Role of KlSnf1p and KlMig1p in glucose repression. This model attempts to explain the function of the KlMig1 binding site (Fig. 1) in the *KlGAL1* promoter and its role in mediating glucose repression of the lactose–galactose regulon in *K.lactis* by the KlSnf1 and KlMig1 proteins. The KlSnf1p–KlMig1p signaling pathway acts to switch off transcription of *KlGAL1* when glucose is present in the culture medium thereby preventing *KlGAL4*-mediated transcription activation which is necessary for induction of the lactose–galactose regulon.

conditions), by a pathway requiring KlSnf1p. In the absence of glucose and the presence of inducer, KlMig1 repression is switched off by a KlSnf1p-dependent pathway and transcription of *KlGAL1* and perhaps *KlGAL10* is increased to produce klGal1p. Regulating the concentration of KlGal1p in this manner provides a mechanism for switching expression of the rest of the genes in the regulon on and off.

KlGal1p has an activity, besides galactokinase activity, that is essential for induction of the regulon (13). This second activity may enable KlGal1p to bind KlGal80p in a galactose and ATP-dependent fashion as has been shown (12). Interaction between KlGal1p 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 *KlGAL4* because the concentration of the KlGal4p 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, KlGal1p might target the KlGal80–KlGal4p complex bound to the *KlLAC12* promoter so that synthesis of the lactose–galactose transporter would begin. This hypothesis is based upon our observation that expression of *KlLAC12*, more than any other gene in the regulon, is dependent upon the KlSnf1p–KlMig1p pathway. Dependence upon the KlSnf1p–KlMig1p pathway is supported by the slow growth of the *Klsnf1* 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 KlSnf1p 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 ScSnf1p. Finally, the ScSnf1p–KlMig1p repression pathway interfaces to the lactose–galactose regulon by binding of KlMig1p to the *KlGAL1* promoter. These data provide a framework for uncovering other signaling pathways that govern expression of the lactose–galactose regulon.

ACKNOWLEDGEMENTS

This work was support 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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