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Autoregulation of *GAL4* transcription is essential for rapid growth of *Kluyveromyces lactis* on lactose and galactose

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ABSTRACT

Transcriptional induction of genes in the lactose-galactose regulon of the yeast *Kluyveromyces lactis* requires the GAL4 transcription activator protein. Previous data indicated that the concentration of GAL4 was tightly regulated under basal, inducing, and glucose repressing conditions but the mechanisms were unknown. In this paper we demonstrate that transcription of the *GAL4* gene (*Kl-GAL4*) increases 3- to 4-fold during induction of the regulon. This increase requires a Kl-GAL4 binding site, UAS_G, in front of the *Kl-GAL4* gene, indicating that the Kl-GAL4 protein autoregulates transcription of its own gene. Our data demonstrate that the autoregulatory circuit is essential for full induction of the lactose-galactose regulon and, hence, for rapid growth on lactose or galactose. Other data indicate that basal transcription of the *Kl-GAL4* gene is governed by unidentified promoter elements. The existence of the autoregulatory circuit reveals an important difference between the lactose-galactose regulon and its homologue in *Saccharomyces cerevisiae*, the melibiose-galactose regulon. This difference may have evolved in response to different selective pressures encountered by the two organisms.

INTRODUCTION

Survival of most organisms requires appropriate responses to extracellular signals. One common response to such signals is to increase or decrease the initiation rate of gene transcription. Changes in the rate of transcription initiation are often produced by modulating the concentration or activity of *trans*-acting regulatory proteins. Knowledge of these vital modulatory mechanisms is limited in eucaryotes and much remains to be uncovered (reviewed in 1, 2). In the work presented here we have examined how the concentration of the GAL4 transcription activator protein of *Kluyveromyces lactis* (Kl-GAL4) is modulated. We find that the Kl-GAL4 protein activates transcription of its own gene 3 to 4-fold in response to the extracellular signals lactose or galactose. This small increase is essential for maximal growth rate on these two sugars and has probably evolved to give the organism a selective advantage in its natural habitat.

K.lactis is one of the few yeasts that can use lactose as a carbon and energy source. Utilization requires induced transcription of a group of genes, the lactose-galactose (lac-gal) regulon, that includes *LAC12* (lactose/galactose permease), *LAC4* (β-galactosidase), *GAL1* (galactokinase), *GAL7* (galactoepimerase), and *GAL10* (galactotransferase)(reviewed in 3). Cells growing in the absence of lactose or galactose have a low but measurable level of these proteins. Addition of either sugar to the culture medium produces an increase in the proteins within 15 minutes and the final level of some proteins increases up to a hundred fold (e. g. β-galactosidase). The increase is primarily due to increased transcription.

Transcription of genes in the lac-gal regulon is controlled by four known genes. *Kl-GAL4* (also called *LAC9*) encodes an activator protein that binds a DNA sequence element, 5′ CGG(N)11GCC 3′ (UAS_G), located upstream of all genes in the regulon (3). The UAS_Gs are always found in multiple copies (2 or 4) with the exception, as shown here, of *Kl-GAL4*, which has only one. The *LAC10* gene (4) is hypothesized to encode a negative regulatory protein that binds the carboxyl terminus of the Kl-GAL4 protein (5). In the absence of an inducer such as lactose or galactose this protein–protein interaction is hypothesized to inhibit or conceal the transcription activation activity of the Kl-GAL4 protein. The presence of lactose or galactose in the culture medium reverses the inhibition, in an unknown manner, so that transcription is activated. Maximum expression of the lac-gal regulon also requires the *GAL11* gene which encodes a transcription co-activator necessary for high level expression of genes not only in the lac-gal regulon, but also of many other genes in *K.lactis* (6). Finally, the *GAL1* gene appears to encode a bifunctional protein necessary not only for catabolism of galactose (galactokinase) but also for induction of the regulon. This conclusion is based upon a strain carrying the *gal1-r*, mutation (7) which does not affect basal level galactokinase activity but does prevent induction of the regulon. The role of the GAL1 protein in induction remains to be determined.

In seeking to more fully understand how transcription is induced, we have examined how the concentration of the Kl-GAL4 protein is controlled. Two lines of evidence indicated that the concentration of this protein was tightly controlled. First, the level of the Kl-GAL4 mRNA decreased about 3-fold, compared to fully induced cells, when glucose was added to the culture

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medium (8). Second, insertion of a second copy of the *Kl-GAL4* gene into a wild type strain disrupted normal expression of the lac-gal regulon, as measured by β-galactosidase activity and mRNA level, because both the uninduced and induced level of regulon expression were doubled (8, 9). In addition, glucose no longer repressed expression of the lac-gal regulon in such a strain, implying that glucose normally causes repression by modulating the concentration of the Kl-GAL4 protein.

In this paper we demonstrate that the 3- to 4-fold induction of transcription of the *Kl-GAL4* gene is governed by an autoregulatory circuit composed of the single UAS_G in front of the gene and the Kl-GAL4 protein which binds the UAS_G. This circuit is essential for maximal induction of the regulon and for rapid growth on lactose and galactose. The *Kl-GAL4* promoter is thus a genetic switch for controlling induction of the lac-gal regulon. Other data suggest for the first time that the Kl-GAL4 protein is governing expression of a gene(s) unrelated to the lac-gal regulon.

MATERIALS AND METHODS

Strains and media

Strain JA6 (*MATα ade trp1 ura3* UAS_G) was obtained from K. Breunig (10). Strain SDU2 (*MATα ade trp1 ura3 gal4Δ3-18* UAS_G) was constructed from strain JA6 by deleting the 5′ terminal one-third of the *Kl-GAL4* gene and replacing the deleted region with *URA3* as described (8). Strain SDU2G is a Ura^- derivative of strain SDU2 selected by using 5-fluoroorotic acid (11). The construction of strain SDU1140 (*MATα ade trp1 ura3* UAS_G) has been described (8)). Strains SDU3 and SDU4 (*MATα ade trp1 ura3* $mUAS_G$), Lac^+ Ura^-, were constructed in the same manner as strain SDU1140 using a *Kl-GAL4* allele from strain Y1140 in which the UAS_G had been replaced with the $mUAS_G$ allele (Fig 1.). Replacement of the UAS_G allele with the $mUAS_G$ allele was verified by Southern blotting, The parental strain SDU2 gives a 1.6kb BglII-CspI fragment, indicative of the UAS_G allele which contains a CspI site, while strains carrying the $mUAS_G$ fail to cleave with CspI and give a 2kb BglII fragment (data not shown). Several independent isolates of this construction were shown to have the same level of uninduced β-galactosidase activity and two, SDU3 and SDU4, were arbitrarily chosen for the more detailed studies described in Results. All *K.lactis* strains used in these studies are isogenic derivatives of JA6 that differ only at the *Kl-GAL4* locus. The UAS_G element discussed in this work refers to the one in front of the *Kl-GAL4* locus unless noted otherwise.

Defined medium (Min) (8) was supplemented with the carbon sources indicated in Tables and Figures. To measure doubling times, defined as the time in minutes for the optical density at 600 nm (OD_{600}) unit to double, cells were pregrown to saturation in Min containing 2% sorbitol, sonicated to dissociate clumped cells, and diluted into fresh medium so that after overnight growth the OD_{600} was 0.1−0.3. At various times thereafter the OD_{600} was measured on sonicated samples. All yeast cultures were grown at 30°C.

Plasmid constructions

Plasmid pSK+L9pCAT, containing the *Kl-GAL4* promoter fused to the coding sequence of chloramphenicol acetyl transferase gene (bp 243 to 904, (12)), was constructed by inserting a 5.4 kb EcoRI fragment carrying the *Kl-GAL4* gene from *K.lactis* strain Y1140 (13) into the EcoRI site of pBluescript SK^+ (Stratagene, La Jolla, CA).. To facilitate construction, an NdeI site was created by site-directed mutagenesis of the ATG initiation codon of *Kl-GAL4*. The NdeI−BstEII fragment, containing all of the *Kl-GAL4* coding region, was replaced with a CAT gene which had been engineered by PCR to contain an NdeI restriction site at the ATG start codon and a BstEII restriction site following the STOP codons. The PCR primers used for this construction were 5′-GG-AGCTAAGGAAGGTCATATGGAGAAAAAAATCAC-TGG-3′ and 5′-CCCGCTGGTTACCTTATTACGCCCCGC-CCTG-3′, respectively. To construct a series of centromere-based plasmids, the SmaI−BstEII fragment of pSK+L9pCAT (1620 bp carrying the *Kl-GAL4* promoter-CAT fusion) was cloned into the SmaI site of pKlCEN2-II.8 (14) to give pKlGAL4CAT. Prior to this step, the BglII and HindIII restriction sites were removed from pKlCEN2-II.8 by treatment with the Klenow fragment of DNA polymerase I. A 3-base pair insertion mutation was made in the *UAS*G (Fig. 1) to give the $mUAS_G$ by cleaving pSK+L9pCAT with CspI (cleavage sequence = CGGTCCG, Stratagene), filling in the overhanging ends using the Klenow fragment of DNA polymerase I, and religating the DNA. The Bsp106−HindIII restriction fragment of pKlGAL4CAT was replaced with the corresponding fragment containing the $mUAS_G$ to yield pMC2. Deletion mutations in the *Kl-GAL4* promoter were verified by restriction analysis and insertion mutations were verified by DNA sequence analysis.

Enzyme assays

β-galactosidase activity was assayed using permeabilized whole cells (15). One unit of activity equals a nanomole of product produced per minute per OD_{600} unit.

For assaying chloramphenicol acetyltransferase (CAT) activity, KlGAL4CAT reporter plasmids were transformed into yeast cells and plated on Min medium containing 2% glucose but lacking uracil. One to four days after plating, transformants were used to start cultures (8 ml) which were grown to 0.8−1.2 OD_{600} units in PYE medium (8) containing the carbon sources indicated in Figure and Table legends. Cell-free extracts were prepared as described previously (16). CAT activity was measured using a phase extraction procedure (17). [^{14}C]-chloramphenicol, (Du Pont cat# NEC-408A, specific activity 54.4mCi/mmol) and n-butyrylCoenzymeA, (Sigma, St Louis, MO) were incubated at 37°C with 30μg of protein extract in a volume of 100μl. The reaction mixture was extracted twice with aqueous buffer prior to counting in a liquid scintillation spectrometer. Units of CAT activity were calculated as% conversion of substrate (cpm measured in the organic phase expressed as a percentage of total cpm) per μg protein per minute.

RESULTS

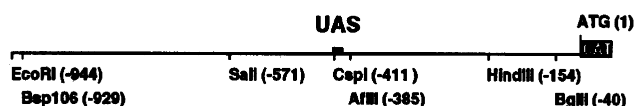
The Kl-GAL4 protein controls transcription of its own gene

The ability of the Kl-GAL4 activator protein to control transcription of its own gene was examined first using a reporter plasmid in which the *Kl-GAL4* promoter was fused to the coding region of the CAT gene. The reporter plasmid also carried the *CEN2* sequence (14) to maintain a copy-number of about one per cell and the *S.cerevisiae URA3* gene for selection of Ura^+ transformants. CAT activity was measured in isogenic strains that had the wild type *Kl-GAL4* allele or a deletion allele. CAT activity (Table 1) was slightly higher in the deletion strain for cells grown under conditions (glycerol) that do not induce expression of the lac-gal regulon. In contrast, when cells were

Table 1. The *Kl-GAL4* protein activates transcription of its own gene.

Strain	*Kl-GAL4*	UNITS CAT ACTIVITY Glycerol	Galactose
JA6	wild type	2.58 ± 0.26	12.33 ± 3.84
SDU2G	deleted	4.16 ± 0.45	5.79 ± 0.67

Strains JA6 and SDU2G were transformed with reporter plasmid pKIGAL4CAT containing the full length *Kl-GAL4* promoter fused to the *CAT* coding region. Cells were grown in PYE medium containing either 5% glycerol and 0.1% glucose or 2% galactose. Mean values ± the standard deviation represent data from at least three experiments.



UAS: CGGACCGGAACAGACCG (-413,-396)

mUAS: CGGACGACCGGAACAGACCG

Figure 1. Diagram of the *Kl-GAL4* promoter. The position of the UAS_G promoter element is indicated by a small filled box and its sequence is shown. The promoter was fused to the *CAT* gene. Restriction endonuclease sites used in plasmid constructions are indicated.

Table 2. A UAS_G modulates increased transcription of *Kl-GAL4* during induction.

Plasmid	*Kl-GAL4* Promoter	UNITS CAT ACTIVITY Glycerol	Galactose
pKIGAL4CAT	UAS_G	2.58 ± 0.26	12.33 ± 3.84
pMC2	$mUAS_G$	4.01 ± 1.67	4.54 ± 0.67
pMC3	ΔUAS_G	1.12 ± 0.16	1.67 ± 0.28
pMC4	Δpromoter	0.08	0.10
pKICEN2-II.8	none (vector)	0.10	0.10

JA6 cells were transformed with the indicated reporter plasmid containing the CAT gene fused to the *Kl-GAL4* promoter. Plasmid pKIGAL4CAT contains the full length promoter (Fig. 1). pMC2 is the same as pKIGAL4CAT except that the UAS_G was mutated by a 3-base pair insertion to give the $mUAS_G$ (Fig. 1). In pMC3 the 544bp region from Bsp106 to AflII was deleted and in pMC4 the 889 bp region from Bsp106 to BglII was deleted (Fig. 1.). Cells were grown in PYE medium supplemented with 2% glycerol or 2% galactose.

grown under inducing conditions (galactose) CAT activity was two-fold higher in the strain with the *Kl-GAL4* gene than in the strain with the deletion allele. These data argue that the Kl-GAL4 protein activates transcription of its own gene when the lac-gal regulon is induced.

Transcription of the *Kl-GAL4* gene is modulated by a UAS_G

To activate transcription of structural genes in the lac-gal regulon the Kl-GAL4 protein must bind a UAS_G (18, 19, 20). We had noted previously that there is one potential UAS_G in the *Kl-GAL4* promoter (13). To determine if this UAS_G was part of an autoregulatory circuit we mutated it ($mUAS_G$, Fig. 1) by inserting three base pairs in the 5′ end of the sequence thereby disrupting the spacing between the 3-base terminal sequences that are essential for binding by the Kl-GAL4 protein (21). The effect of this mutation on transcription directed by the *Kl-GAL4* promoter was examined using a CAT reporter plasmid. When grown under inducing conditions (galactose), cells carrying a reporter plasmid with the $mUAS_G$ gave about three-fold lower CAT activity that an identical plasmid carrying the normal UAS_G (Table 2). An insignificant difference in CAT activity was observed for these cells grown under uninduced (glycerol) conditions. These data demonstrate that the UAS_G is necessary for a 3- to 4-fold induction of *Kl-GAL4* transcription.

The effect on CAT gene expression of deleting the UAS_G was also examined using the pMC3 reporter plasmid which contains the ΔUAS_G allele (Table 2). This allele lacks a 544 bp region of the *Kl-GAL4* promoter (from Bsp106 to AflII, Fig. 1). The ΔUAS_G mutation reduced CAT activity even more than the three base pair $mUAS_G$ insertion mutation for cells grown in the presence of an inducer (galactose). These results support those obtained with the $mUAS_G$. The ΔUAS_G mutation also reduced CAT activity under uninduced conditions (glycerol) suggesting that the deleted region contains one or more promoter elements necessary for basal promoter activity.

As a control to show that CAT gene expression was due to the *Kl-GAL4* promoter, a reporter plasmid (pMC4) with a nearly complete deletion of the promoter was shown to have barely detectable CAT activity under all growth conditions (Table 2); the level of CAT activity was similar to that shown by cells transformed with the parent vector pKICEN2-II.8.

Table 3. Effect of a chromosomal $mUAS_G$ allele on regulon expression

Strain	Units β-galactosidase activity[a] Lactose	Galactose	Sorbitol
SDU1140A (UAS_G)	165 ± 16	167 ± 13	6.9 ± 1.3
SDU1140B (UAS_G)	188 ± 16	175 ± 10	6.4 ± 0.8
SDU3($mUAS_G$)	84 ± 2.2	106 ± 7.4	25 ± 3.5
SDU4 ($mUAS_G$)	87 ± 6.3	110 ± 10	26 ± 3.5

[a]Cells were grown as described for the measurement of doubling times in a defined medium (Min) supplemented with the indicated carbon source (2%, w/v). Samples were sonicated to dissociate clumped cells before the OD_{600}nm (0.1 to 0.8) was measured. Values are the mean plus or minus the standard deviation for 3 or 4 determinations. Strains SDU1140A and SDU1140B are different isolates generated from the same strain construction experiment as are strains SDU3 and SDU4.

The UAS_G is essential for full induction of β-galactosidase activity

To determine if the UAS_G in the *Kl-GAL4* promoter influences expression of structural genes in the lac-gal regulon, we examined expression of *LAC4* by measuring the activity of its product, β-galactosidase, in isogenic strains containing either the UAS_G or the $mUAS_G$ allele in front of the chromosomal *Kl-GAL4* gene. Maximal, fully induced β-galactosidase activity was 49% less in strains containing the $mUAS_G$ allele compared to the UAS_G allele for cells induced by growth on lactose (compare strains SDU3 and SDU4 with strains SDU1140A and SDU1140B, Table 3) and 37% less for cells induced by growth on galactose (Table 3). We conclude from these data that the UAS_G in the *Kl-GAL4* promoter is essential for full induction of *LAC4* expression.

Unexpectedly, the $mUAS_G$ allele in the *Kl-GAL4* promoter caused basal expression of *LAC4*, as measured by β-galactosidase activity, to increase 3.8 times above the level produced by the UAS_G allele (sorbitol values, Table 3). This observation suggests

Table 4. Effect of the $mUAS_G$ on cell growth

Strain (*Kl-GAL4*)	Doubling Time in Minutes[a] Lactose	Galactose	Glucose	PYED
SDU1140A (UAS_G)	96±10 (7)	95±6.6 (8)	95±11 (4)	79±1.7(3)
SDU1140B (UAS_G)	94±12 (7)	95±6 (8)	99±7.9(4)	80±0 (3)
SDU3 ($mUAS_G$)	126±7.9(6)	160±19 (7)	110±10 (4)	80±.58(3)
SDU4 ($mUAS_G$)	124±8.6(6)	153±11 (8)	110±8.4(4)	79±1.7(3)
SDU2A (*gal4*Δ3-18)	NG	NG	109±10 (4)	80 (1)
SDU2B (*gal4*Δ3-18)	NG	NG	107±8.3(4)	80 (1)

[a]Cells were grown in a defined medium (Min) supplemented with the indicated carbon source added to a final concentration of 2% (w/v) except for PYED which is a complex medium containing 2% glucose. Standard deviations are shown with the number of determinations in parentheses. NG = no growth.

that the Kl-GAL4 protein is necessary for maintaining basal level *Kl-GAL4* gene expression.

The UAS_G is essential for rapid growth on lactose and galactose

Based upon the results presented in Tables 2 and 3 we hypothesized that the UAS_G in the *Kl-GAL4* promoter was essential for maximal expression of the lac-gal regulon under inducing conditions. To further examine this hypothesis we compared growth rates in isogenic strains of *K.lactis* containing either the UAS_G or the $mUAS_G$ allele in front of the chromosomal *Kl-GAL4* gene.

Growth rates were expressed as doubling times and were determined for two separate isolates of each strain as described in Materials and Methods. As predicted by our hypothesis, strains with the $mUAS_G$ mutation (strains SDU3 and SDU4, Table 4) grew about 30% slower than their isogenic UAS_G counterparts (strains SDU1140A and SDU1140B) with lactose as the carbon source and about 65% slower with galactose as the carbon source. To demonstrate that the difference in growth rates was due to reduced expression of the lac-gal regulon imposed by the $mUAS_G$ mutation, strains were also grown in defined medium using a preferred, non-inducing carbon source, glucose (Table 4), or a complex medium, PYED (Table 4). In these media the strains grew at the same rate, indicating that the slower growth rate of strains carrying the $mUAS_G$ mutation on lactose or galactose was due to impaired catabolism of the two sugars and not to some general effect on energy metabolism.

DISCUSSION

We noted previously the presence of a single UAS_G in the promoter of the *Kl-GAL4* gene (13) and hypothesized that it might be necessary for normal expression of the lac-gal regulon, possibly by forming part of an autoregulatory loop in which the Kl-GAL4 protein would bind the UAS_G and activate transcription of its own gene. Data presented here verify this hypothesis by showing that the Kl-GAL4 protein is essential for activating expression of the *Kl-GAL4* promoter (Table 1), and that the UAS_G mediates a 3- to 4-fold increase in transcription of the *Kl-GAL4* gene that occurs when the lac-gal regulon is induced (Table 2). This increase in turn is essential for maximal induction of the lac-gal regulon as measured by β-galactosidase activity (Table 3). Most importantly, the UAS_G is necessary for rapid growth since a mutation in it decreases the doubling time of cells by 30% with lactose as the carbon source and by 65% with galactose as the carbon source (Table 4).

The reduced doubling time for growth on lactose may be due to a 49% reduction in the induced level of β-galactosidase activity observed in strains carrying the mutant $mUAS_G$ allele (Table 3), but other unmeasured factors including reduced lactose permease activity (22) may limit the growth rate. Growth limitation on galactose could be due to reduced galactose permease (22) or one of the galactose catabolic enzymes. Further studies will be needed to identify the rate limiting protein. Whichever protein is limiting, we envision that its concentration is reduced because expression of its gene is lowered due to at least one low affinity UAS_G in the gene's promoter which is only occupied if the concentration of the Kl-GAL4 protein is increased 3- to 4-fold, as occurs when the lac-gal regulon is fully induced. If the 3- to 4-fold increase in Kl-GAL4 protein is blocked by mutation of the UAS_G in front of the *Kl-GAL4* gene, the low affinity UAS_G is not occupied and transcription of the gene(s) it governs fails to reach a normal induced level. Any gene in the lac-gal regulon will be affected in a similar manner since their expression, like that of *LAC4*, is dependent upon binding of the Kl-GAL4 protein to multiple UAS_Gs. The large, divergent promoter governing LAC4 and LAC12 expression is known to have 4 UAS_Gs with differing binding affinities (23).

The lac-gal regulon of *K.lactis* is related to the melibiose-galactose regulon of *Saccharomyces cerevisiae* based upon amino acid similarity in the GAL1, GAL4, GAL7, GAL10 (3), GAL11 (6) and GAL80 (24) proteins, and in the sequence, number and relative location of UAS_Gs in the *GAL1-10* promoter region (3). Interestingly, the promoter of the *S.cerevisiae GAL4* gene does not contain a UAS_G and the gene is not autoregulated (16), so autoregulation of the *Kl-GAL4* gene is a unique feature of the lac-gal regulon. A possible reason for this difference is based upon a difference in selective pressure during evolution. *K.lactis* is one of the few yeasts that can grow on lactose and it most likely evolved in close contact with mammals under conditions where it often used lactose and thereby gained a selective advantage over competitors. Savageau (25) has argued that genes for often used metabolic pathways tend to use autoregulatory circuits while seldom used genes do not. Since galactose and melibiose are not abundant sugars it may be that *S.cerevisiae* seldom needed to use them and, therefore, did not evolve an autorgeulatory circuit to govern *GAL4* expression. In addition, enzymes in often used metabolic pathways tend to have a higher basal level than seldom used enzymes (25). The basal level of most of the enzymes encoded by the lac-gal regulon is high and easily detectable while those encoded by the melibiose-galactose regulon is low and barely detectable (3).

We observed that the $mUAS_G$ caused the basal level of β-galactosidase activity, a measure of *LAC4* expression, to increase

3.8-fold (Table 3) over the level seen in strains with the UAS_G allele. The data suggest that the Kl-GAL4 protein is bound to the UAS_G in uninduced cells and is helping to maintain basal transcription of the *Kl-GAL4* gene, possibly acting as a repressor.

Recently published data (26) agree with the results presented in this paper. Together these data establish the importance of controlling the concentration of the Kl-GAL4 protein. The critical role played by the concentration of the GAL4 protein in regulating the galactose regulon of *S.cerevisiae* has been established also (16 and references therein). Reports to date have focused on transcriptional controls and much remains to be done in this area. But additional levels of control including posttranslational modifications of the Kl-GAL4 protein, which appear to regulate the activity of the *S.cerevisiae* GAL4 protein (27, 28, 29), need to be examined.

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