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The Signal for Glucose Repression of the Lactose-Galactose Regulon Is Amplified through Subtle Modulation of Transcription of the *Kluyveromyces lactis Kl-GAL4* Activator Gene

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Induction of the lactose-galactose regulon is strongly repressed by glucose in some but not all strains of *Kluyveromyces lactis*. We show here that in strongly repressed strains, two to three times less *Kl-GAL4* mRNA is synthesized and that expression of structural genes in the regulon such as *LAC4*, the structural gene for β-galactosidase, is down regulated 40-fold or more. Comparative analysis of strains having a strong or weak repression phenotype revealed a two-base difference in the promoter of the *Kl-GAL4* (also called *LAC9*) positive regulatory gene. This two-base difference is responsible for the strong versus the weak repression phenotype. The two base changes are symmetrically located in a DNA sequence having partial twofold rotational symmetry (14 of 21 bases). We hypothesize that this region functions as a sensitive regulatory switch, an upstream repressor sequence (URS). According to our model, the presence of glucose in the culture medium signals, by an unidentified pathway, a repressor protein to bind the URS. Binding reduces transcription of the *Kl-GAL4* gene so that the concentration of the Kl-GAL4 protein falls below the level needed for induction of *LAC4* and other genes in the regulon. For strains showing weak glucose repression, we hypothesize that the two base changes in the URS reduce repressor binding so that the regulon is not repressed. Our results illustrate an important principle of genetic regulation: a small (2- to 3-fold) change in the concentration of a regulatory protein can produce a large (40-fold or greater) change in expression of structural genes. This mechanism of signal amplification could play a role in many biological phenomena that require regulated transcription.

Although small in size, glucose is one of nature's most important effectors of metabolism. Deciphering how glucose regulates diverse metabolic pathways has proven to be a challenging and unfinished task. Multicellular organisms have evolved elaborate and complex mechanisms to control the flow of glucose within and between metabolic pathways that modulate energy homeostasis. Unicellular organisms, particularly bacteria and yeasts, have evolved mechanisms for preferentially using glucose from a mixture of carbon sources, an ability termed glucose or carbon catabolite repression (19). Preferential glucose utilization is accomplished in part by repression and activation of transcription.

The mechanism by which glucose affects transcription is most thoroughly characterized in *Escherichia coli*. Glucose produces a decrease in cyclic AMP (cAMP) which results in the dissociation of cAMP from the catabolite activator (also called the catabolite repressor) protein. Without bound cAMP, the catabolite activator protein is unable to promote transcription of numerous genes (reviewed in reference 30). In this regulatory circuit, glucose acts as a negative effector because transcription is prevented or repressed when glucose is present in the culture medium.

To gain insight into the mechanism of glucose-repressed transcription in eucaryotes, we have examined how glucose represses induction of the lactose-galactose regulon in the common dairy yeast *Kluyveromyces lactis*, a yeast with both research and commercial value. Strains of *K. lactis* fall into three categories with respect to glucose repression of the lactose-galactose regulon. Strains such as Y1140 show weak repression because the fully induced level of the regulon, as measured by β-galactosidase activity, is reduced only twofold by the presence of glucose in the culture medium (7). Strains such as CBS2360 show almost no repression (2), while strains such as JA6 (2) and Y1118 (this report) show strong (50- to 100-fold) repression of the regulon by glucose under inducing conditions. How might glucose affect expression of the regulon? Initial insight into the mechanism of glucose repression was provided by Breunig (2), who showed that a glucose-repressing strain could be converted to a nonrepressing strain by changing the allele carried at the *Kl-GAL4* locus (also called *LAC9* [27]).

Kl-GAL4 codes for a transcription activator protein that regulates and is essential for transcription of the lactose-galactose regulon (33). The Kl-GAL4 protein binds to a family of 17-bp DNA sequences, termed upstream activator sequences (UAS_G; the G refers to the lactose-galactose regulon), located in front of genes comprising the lactose-galactose regulon (3, 9, 17, 25). In the presence of an inducer such as lactose or galactose, the DNA-bound Kl-GAL4 activates transcription of the adjacent structural gene, presumably by contacting the transcription initiation machinery. The transcription activation domain(s) of Kl-GAL4 has not been characterized, but it is most likely a negatively charged domain like those found in the GAL4 protein of *Saccharomyces cerevisiae* (Sc-GAL4 [18]), since Kl-GAL4 and Sc-GAL4 are structural (26, 33) and functional (24, 33) homologs.

What difference in the *Kl-GAL4* alleles accounts for their response to glucose? Kuger et al. (16) examined the *Kl-GAL4* allele in glucose-repressing and nonrepressing strains and concluded that the critical factor for responding to

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glucose was a single amino acid, residue 104, which is leucine in the Kl-GAL4 protein from the glucose-repressing strain JA6 and tryptophan in the Kl-GAL4 protein from the non-glucose-repressing strain Y1140. This amino acid change is notable because it lies in the DNA-binding domain of Kl-GAL4 (31, 32), specifically in the evolutionarily conserved C_6 zinc finger or zinc cluster motif (22, 23). Kuger et al. (16) speculated that in glucose-repressible strains, glucose was indirectly able to reduce the ability of Kl-GAL4 to bind UAS_G. The mechanism for transducing the signal from glucose to the Kl-GAL4 protein is unknown, but from what is known about glucose repression in *S. cerevisiae*, there is likely to be a complex signal transduction pathway (10).

We have examined the difference between a *Kl-GAL4* allele from a glucose-repressible and a nonrepressible strain and have come to a different conclusion than did Kuger et al. (16). Here we show that the difference between the two types of strains is due to a two-base difference in the promoter region of *Kl-GAL4*. We hypothesize that these bases lie in a promoter element, the upstream repression sequence (URS_G; the G refers to the lactose-galactose regulon) that mediates glucose repression of *Kl-GAL4* transcription. The URS provides a sensitive regulatory switch for producing a small (two- to threefold) change in Kl-GAL4 mRNA level. However, this small change is greatly amplified since it causes more than a 40-fold change in expression of one of the genes (*LAC4*) in the lactose-galactose regulon.

MATERIALS AND METHODS

Strains and media. The wild-type *K. lactis* strains Y1140 (*MAT***a**) and Y1118 (*MAT*α) have been described elsewhere (27). Strain JA6 (*MAT*α *ade trp1 ura3*) was obtained from K. Breunig (2). *K. lactis* NRCC2715 was from the National Research Council of Canada. Strain SDU2 (*MAT*α *ade trp1 ura3 gal4*Δ*3-18*; Fig. 1) was constructed by replacing the *Kl-GAL4-2* allele in strain JA6 with a *Sal*I-to-*Bst*EII restriction fragment carrying the *gal4*Δ*3-18* deletion allele. The deletion allele was made by replacing the region of *Kl-GAL4*, from strain Y1118, between the *Bgl*II site (base 906; the numbering system is from reference 33) located upstream of the *Kl-GAL4* initiation codon and replacing the *Kpn*I site (base 1632) with a 1.1-kb DNA fragment carrying the *URA3* gene of *S. cerevisiae*. As shown in this report, the Y1118 *Kl-GAL4* allele is similar to the one in strain JA6, which has been designated *Kl-GAL4-2* because it confers a strong glucose repression phenotype on the lactose-galactose regulon (2), in contrast to the *Kl-GAL4-1* allele from strain Y1140, which confers only a weak glucose repression phenotype (7).

Defined medium (Min) contained double-strength yeast nitrogen base with amino acids (Difco Laboratories) plus the following supplements at 20 mg/liter: adenine sulfate, arginine-HCl, histidine-HCl, leucine, lysine-HCl, methionine, tryptophan, and uracil. One or more supplements were omitted from Min medium for selection of yeast transformants. As a carbon source, glucose (Glu), lactose (Lac), galactose (Gal), or sorbitol (Sorb) was added to a final concentration of 2%. PYED contained 1% peptone, 1% yeast extract, 2% agar (for plates), 50 mM sodium succinate (pH 5), inositol (50 mg/liter), potassium phosphate monobasic (50 mg/ml), and 2% glucose. Cells were grown at 30°C.

Construction of *Kl-GAL4* mutant alleles. The mutant *Kl-GAL4* alleles used in this study are diagrammed in Fig. 1. They were constructed by standard techniques, and the restriction sites used to join fragments are indicated in the figure. The *Kl-GAL4-1L* allele with the Trp-104→Leu amino acid change (strain SDU1140Leu) was constructed by oligonucleotide-directed mutagenesis of the *Kl-GAL4-1* nonrepressing allele in an M13 phage vector. The *Kl-GAL4-755C* and *Kl-GAL4-769G* alleles were constructed by using a two-step polymerase chain reaction (PCR) procedure (15). The first PCR used a primer corresponding to bases 300 to 376 (5′ to 3′) of the *Kl-GAL4* promoter and a second primer corresponding to bases 763 to 745 (5′ to 3′) with base 755 designed to produce a T-to-C mutation. The template for this reaction and the second PCR was a *Sal*I-to-*Bst*EII *Kl-GAL4* DNA fragment (Fig. 1). The second PCR was primed with the first PCR product and a primer corresponding to bases 925 to 909 (5′ to 3′). The resulting PCR product was digested with *Sal*I and *Hin*dIII and cloned into the corresponding region of the *Kl-GAL4-1* allele. By using the same strategy but a different oligonucleotide to prime synthesis of the first PCR product, base 769 was changed from C to G. All mutations were verified by sequencing DNA from individual clones.

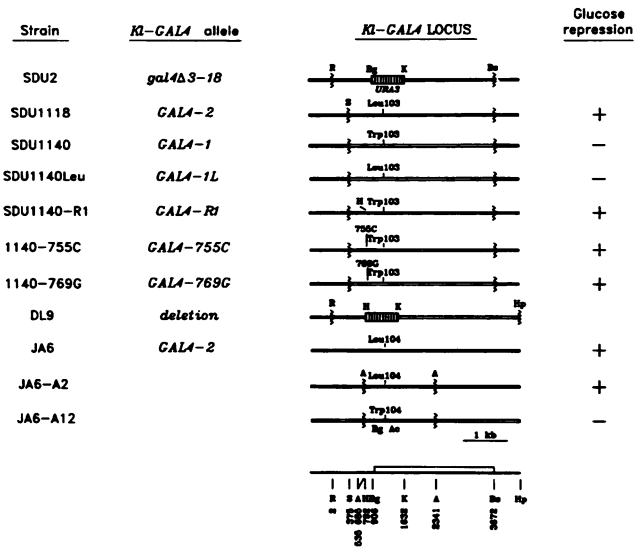

FIG. 1. Diagrammatic representation of the *Kl-GAL4* allele in *K. lactis* strains. The bottom diagram shows restriction sites and the coding region of *Kl-GAL4* (open box). The gene is transcribed from left to right. DNA sequences from a glucose-repressing strain are shown as filled boxes, while those from nonrepressing strains are shown as open boxes. Restriction sites used to promote homologous recombination between a DNA fragment and the chromosome are shown as wavy vertical lines. The nucleotide numbering system is from Wray et al. (33). Strains SDU1118 through 1140-769G were all made by replacement of the *URA3*-deleted *GAL4* allele in strain SDU2 (*gal4*Δ*3-18* allele). The *Bgl*II and *Kpn*I sites were destroyed during construction of the *gal4*Δ*3-18* deletion allele. Strains DL9 through JA6-A12 have been described elsewhere (2, 16). Restriction sites: A, *Ava*II; Ac, *Acc*I; Bg, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I (*Asp* 718); R, *Eco*RI; S, *Sal*I.

Mutant or wild-type *Kl-GAL4* alleles were used to replace the *Δ3-18* allele in strain SDU2 by gene transplacement (33). Ten micrograms of plasmid DNA carrying the mutant allele was digested with *Sal*I and *Bst*EII and transformed into SDU2, using lithium acetate-treated cells (28). Transformed cells were incubated overnight at 23°C in 0.5 ml of PYED before plating on MinLac plates to select for Lac^+ transformants. For reasons that are not clear, Lac^+ transformants often retained the *Δ3-18* allele rather than displacing it with

the mutant allele: the mutant allele was integrated in tandem to the *Δ3-18* allele. To eject the *Δ3-18* allele from the chromosome, cells were plated on MinLac plates containing 5-fluoroorotic acid (1), which selected for loss of the *URA3* gene present in the *Δ3-18* allele. Ura$^-$ Lac$^+$ cells were checked by Southern blotting to verify that the *Δ3-18* allele had been replaced. For example, on a Southern blot (data not shown) containing chromosomal DNA digested with *Bgl*II and probed with the *Sal*I-to-*Bst*EII fragment of *Kl-GAL4*, the wild-type and mutant *Kl-GAL4* alleles showed a 2-kb and a 4.7-kb band of hybridization, while the *Δ3-18* allele gave a single band of about 6 kb because the *Bgl*II site in front of *Kl-GAL4* was destroyed during construction of this deletion allele (diagrammed in Fig. 1).

For all strain constructions, 3 to 12 separate isolates were analyzed by Southern blotting to verify that only one copy of *Kl-GAL4* was present. Then β-galactosidase activity was measured in each strain. Strains were grown in MinGlu medium to saturation, diluted 1/500 to 1/1,000 into MinGluGal medium, grown to a density of 0.5 to 1.5 optical density at 600 nm (OD_{600}) units, and assayed. After verification that all isolates behaved identically, β-galactosidase activity was examined in two strains more carefully by monitoring the kinetics of enzyme induction (e.g., Fig. 2). For this assay, cells were grown to saturation in MinGlu, diluted 1/500 to 1/1,000 into the same medium, grown to a density of 2 to 3 OD_{600} units, centrifuged for 15 s in a microfuge, and resuspended in MinGluGal (prewarmed to 30°C) at a density of 0.5 OD_{600} unit, and the zero time point was taken for assay. After 6 to 8 h of growth, the cells were diluted 1/500 to 1/1,000 in the same medium and grown for a total of 24-h. Additionally, all strains were shown to fully induce β-galactosidase activity when grown under nonrepressing conditions (MinSorbGal; data not shown).

The vector pTZA3-1118, used to introduce a second copy of the repressible *Kl-GAL4-2* allele into strain SDU1118 to create strain SDU1118-2X, was constructed in two steps starting with pTZ19R (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). First, a 3.9-kb *Sal*I-*Eco*RI fragment from p1B3 (33) was inserted into pTZ19R cut with the same two restriction enzymes to give pTZA3. This DNA fragment contains the *URA3* gene of *S. cerevisiae* and the autonomous replication sequence *ARS1* of *K. lactis* derived from pKR1 (28). Second, a 4.7-kb *Eco*RI fragment carrying the *Kl-GAL4-2* allele from strain Y1118 was inserted into the *Eco*RI site of pTZA3 to give pTZA3-1118. Integration of pTZA3-1118 into the chromosomal *ARS1* locus of strain Y1118 was accomplished by digesting the plasmid with *Hpa*I, followed by transformation and selection for Ura$^+$ cells.

RNA isolation; Northern (RNA) blot and primer extension analyses. Yeast cells were grown in Min medium containing an appropriate carbon source to an OD_{600} of 1 to 1.3. Total RNA was isolated from cells as described previously (5). For Northern blot analysis (11), 20 μg of RNA was suspended in 15 μl of 1× MOPS buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA] containing 50% (vol/vol) formamide, 2.1 M formaldehyde, 5% (vol/vol) glycerol, and 0.2% (wt/vol) bromphenol blue. One microgram of ethidium bromide was added, and the sample was heated to 100°C for 2 min, cooled on ice for 5 min, and subjected to electrophoresis for 2 h at 80 Volts on a 1.2% agarose gel containing 1× MOPS buffer and 0.66 M formaldehyde. After electrophoresis, the gel was photographed with 360-nm light. The gel was equilibrated for 20 min in 10× SSPE (1.8 M NaCl, 0.1 M sodium phosphate [pH 7.7], 10 mM EDTA), and RNA was transferred to a Biotrans nylon membrane (ICN Biomedical Inc., Costa Mesa, Calif.) by using a Posiblot apparatus (Stratagene, San Diego, Calif.). The RNA was cross-linked to the membrane by treatment with UV light. The membrane was washed twice for 30 min in 0.1× SSPE containing 0.1% sodium dodecyl sulfate (SDS) at 65°C and prehybridized for 4 h at 57°C in a solution containing 50% formamide, 5× SSPE, 10× Denhardt's solution, 0.1% SDS, and 100 μg of sheared, denatured salmon sperm DNA per ml. The same conditions were used for hybridization except that 2×10^7 cpm of a *Kl-GAL4* riboprobe and 2×10^6 cpm of an *IPP* riboprobe were added, and the sample was incubated for 12 to 16 h. The membrane was washed three times at 65°C for 15 min in 0.1× SSPE containing 0.1% SDS and three times in the same solution at room temperature. The membrane was exposed to preflashed X-ray film (Kodak X-Omat AR) with an intensifying screen for 2 to 12 h at −70°C. Band intensities on the autoradiographs were quantified by two-dimensional scanning (BioImage-Visage 2000; Kodak).

Antisense RNA probes (riboprobes) were synthesized for *Kl-GAL4* (33) and for the *K. lactis IPP* gene (29), using a Stratagene RNA transcription kit. For the *GAL4* riboprobe, [α-^{32}P]CTP at 650 Ci/mmol was used as the radioactive label. For the *IPP* riboprobe, the specific activity of the label was reduced 1/10 by dilution with nonradioactive CTP. The *Kl-GAL4* template DNA was pRSGAL4 cut with *Xmn*I and transcribed by using the T7 promoter. pRSGAL4 carries a 3.4-kb fragment of *Kl-GAL4* in the *Sac*I site of pBSM13$^+$ (Stratagene). The *IPP* template was pKLIPP1 cut with *Xba*I and transcribed by using the T3 promoter. pKLIPP1 carries a 0.75-kb *Eco*RI-*Stu*I fragment of *IPP* cloned into the *Eco*RI-*Hin*cII sites of pBluescript SK+/− (Stratagene).

Isolation of poly(A)$^+$ RNA and primer extension reactions were performed as described previously (33). Strains were grown 12 h in MinGluGal medium to an OD_{600} of 1, and poly(A)$^+$ RNA was prepared. Extension reactions were primed with a LAC9 mRNA primer, 5′-CTCCGATGGCAG TAACGTTTCCGC-3′, and an actin mRNA primer, 5′-TCT TGGAGCGTCGTCACCGGC-3′ (6), used as an internal control for mRNA concentration. About 0.5 pmol of each 5′-^{32}P-end-labeled primer (4×10^5 cpm of the LAC9 primer and 8×10^4 cpm of the actin primer) were hybridized to 7.5 μg of poly(A)$^+$ RNA. Products of the extension reaction were analyzed on a 6% polyacrylamide DNA sequencing gel.

Enzyme assay. β-Galactosidase activity was measured on permeabilized whole cells, using a published procedure (14). Activity is expressed as nanomoles of product produced per minute per OD_{600} unit.

RESULTS

The degree of glucose repression is strain dependent. Breunig (2) first observed that induction of the lactose-galactose regulon in some but not all strains of *K. lactis* is severely repressed by the presence of glucose in the culture medium. We refer to this phenomenon as the glucose repression phenotype. Strains such as JA6 have this phenotype (2), which is assayed as a failure of cells to induce β-galactosidase activity when cultured in medium containing glucose and galactose. In contrast, strains such as Y1140 show a weak glucose repression phenotype because the level of β-galactosidase activity is 50% less than it would be if glucose was omitted from the medium (7). These observations prompted us to examine our strains of *K. lactis* for their glucose repression phenotype. Our results (Table 1) are similar to those presented by Breunig (2). In addition, strain

TABLE 1. Glucose repression phenotype of *K. lactis* strains

Strain	β-Galactosidase activity[a] (U)			Ratio, induced/ repressed
	Uninduced	Induced	Repressed	
Y1140	2.6	525	236	2.2
Y1118	1.6	272	2.8	97
JA6	ND	187	3.9	48
NRCC2715	5.2	276	17	16

[a] Saturated overnight cultures were grown in MinSorb and diluted, to an OD_{600} of 0.1, into fresh MinSorb (uninduced), MinSorb plus Gal (induced), or MinGlu plus Gal (repressed). β-Galactosidase activity was measured when cells reached an OD_{600} of 1.5 to 3.0. Values represent averages of three determinations. Deviations from the average were less than 20%. ND, not determined.

NRCC2715 was unique because glucose repressed induction of β-galactosidase activity to a level intermediate to that for the strongly (JA6) and weakly (Y1140) repressing strains. Furthermore, the wild-type strain Y1118 showed a strong glucose repression phenotype, indicating that the phenotype may be fairly common in wild-type strains.

Glucose repression is not due to amino acid changes in *Kl-GAL4*. Kuger et al. (16) examined the molecular basis for the glucose repression phenotype and concluded that it was due to a single amino acid change in Kl-GAL4: the weakly repressing strain Y1140 had Trp at residue 104, while the strongly repressing strain JA6 had Leu. To verify the results of Kuger et al. (16), we examined the effect of amino acid residue 103 or 104 of Kl-GAL4 on the glucose repression phenotype. The reason why Leu is residue 103 or 104 in Kl-GAL4 is due to variation in the number of asparagine residues in the polyasparagine tract located on the amino-terminal side of the C_6 zinc finger; in some strains of *K. lactis*, Kl-GAL4 has eight Asn, while in other strains, there are nine Asn. Like Kuger et al. (16), we examined the effect of eight versus nine asparagine residues on glucose repression and found no effect (data not shown). To avoid potential problems caused by strains with different genetic backgrounds, we constructed all mutants by using strain JA6. We compared the glucose repression phenotype of a strain with Trp (strain SDU1140) or Leu (strain SDU1140Leu) at residue 103 of Kl-GAL4. The two strains behaved the same and showed the nonrepression phenotype because β-galactosidase activity was induced by galactose when glucose was present in the culture medium (Fig. 2). Four independent transformants (strains) were examined for each *Kl-GAL4* allele, and they all showed the nonrepression phenotype (data not shown). Thus, our data do not support the hypothesis of Kuger et al. (16) that Leu at residue 103 or 104 of Kl-GAL4 creates a glucose repression phenotype (compare our strain SDU1140Leu with strain JA6-A2 of Kuger et al. [Fig. 1]).

The *Kl-GAL4* promoter mediates glucose repression. To begin to understand which region(s) of *Kl-GAL4* mediates glucose repression, we constructed chimeric *Kl-GAL4* genes consisting of sequences from the *Kl-GAL4-1* nonrepressing allele, derived from strain Y1140, and the *Kl-GAL4-2* repressing allele, derived from strain Y1118. Our goal was to locate the base(s) that would convert a nonrepressing *Kl-GAL4* allele into a repressing allele. Because this approach converts a nonfunctional allele to a functional allele, it allows precise arguments to be made about structure-function relationships.

The results from a series of chimeric *Kl-GAL4* genes indicated that DNA sequences in the promoter were responsible for the presence or absence of glucose repression (data not shown). To locate these sequences, we determined the DNA sequence of the glucose-repressing allele, *Kl-GAL4-2* (the gene is from strain Y1118), from base 375 (the *Sal*I site) to the ATG codon (base 977), and compared this sequence with the previously published sequence of the nonrepressing allele *Kl-GAL4-1* (strain Y1140). *Kl-GAL4-2* contained only two base changes: base 755 was a C instead of a T, and base 769 was a G instead of a C (these and other data are summarized in Fig. 8). We used a gain-of-function approach to verify that these two bases were responsible for glucose repression. A DNA fragment from the *Kl-GAL4-2* glucose-repressing allele, defined by the *Sal*I and *Hin*dIII restriction sites (Fig. 1), carrying the two base changes was used to replace the corresponding region of the *Kl-GAL4-1* nonrepressing allele to give the *Kl-GAL4-R1* allele. Two strains, SDU1140-R1 and SDU1140-R2, carrying this allele showed glucose repression, in contrast to the parent strain SDU1140 carrying *Kl-GAL4-1* (Fig. 3). Since the glucose-repressing

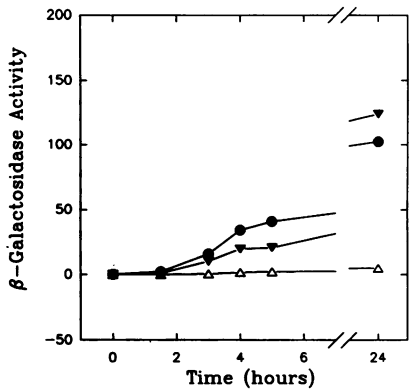

FIG. 2. Evidence that Kl-GAL4 amino acid residue 103 has no effect on glucose repression. Cells were grown overnight to late log phase in MinGlu medium as described in Materials and Methods. Under these conditions, the lactose-galactose regulon is fully glucose repressed. To test for the glucose repression phenotype, cells were diluted into MinGluGal medium at time zero, and the assay was begun. Symbols: circles, SDU1140; closed triangles, SDU1140Leu; open triangles, JA6 or SDU1118.

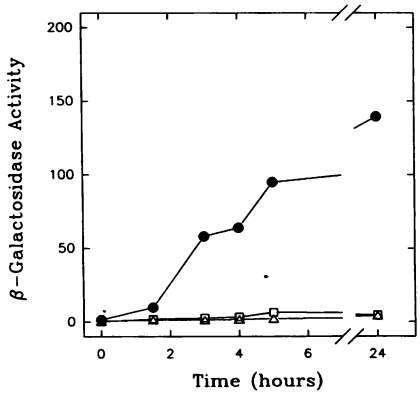

FIG. 3. Evidence that the promoter region of *Kl-GAL4* is responsible for glucose repression of the lactose-galactose regulon. Cells were grown and assayed as described in Materials and Methods and the legend to Fig. 2. Symbols: circles, SDU1140; open triangles, JA6; open squares, SDU1140-R1 and SDU1140-R2.

and nonrepressing strains differ only at positions 755 and 769 of the *Kl-GAL4* promoter, we conclude that one or both of these base pairs is responsible for glucose repression of the lactose-galactose regulon.

The role of bases 755 and 769 in glucose repression was examined by changing each base separately in the *Kl-GAL4-1* (strain SDU1140) nonrepressing allele by using PCR as described in Materials and Methods. As shown in Fig. 4, both bases gave a gain-of-function response since the T-755→C change (strain 1140-755C) produced strong glucose repression, while the C-769→G change (strain 1140-769G) produced some glucose repression of β-galactosidase activity, but repression was not as great as in strain JA6 or in the strain carrying the T-755→C change.

Glucose represses transcription of *Kl-GAL4*. Nucleotides 755 and 769 lie about 110 bases upstream of the major transcription start site in the nontranscribed portion of *Kl-GAL4* (see below and Fig. 6 and 8). Thus, it seemed most likely that they were affecting transcription of *Kl-GAL4*. This hypothesis was examined by using Northern blots. To control for different amounts of RNA per lane, the amount of Kl-GAL4 mRNA was normalized to the amount of the PP_i mRNA encoded by the *IPP* gene. As predicted, there was a two- to threefold-higher amount of Kl-GAL4 mRNA in the two non-glucose-repressing strains SDU1140 and SDU1140Leu than in the two repressing strains JA6 and SDU1140-R1 when cells were grown in MinGalGlu (Fig. 5). To verify that the strains were responding as expected to the presence of glucose, we measured β-galactosidase activity on part of the culture at the same time RNA was extracted (Fig. 5). The nonrepressing strains SDU1140 and SDU1140Leu had 40- and 70-fold more enzyme activity, respectively, than did the repressing strain JA6. In addition, the Northern blots shown in Fig. 5 were stripped and reprobed with a probe for β-galactosidase mRNA to verify that the mRNA level changed to the same extent as the enzyme activity level (data not shown).

Another prediction of our hypothesis is that the level of Kl-GAL4 mRNA should increase when a nonrepressing strain is transferred from a medium containing glucose to a medium containing glucose plus galactose. For a repressing strain, there should be no change. Both predictions were verified. When the nonrepressing strains SDU1140 and SDU1140Leu were grown in medium having only glucose, the Kl-GAL4 mRNA levels were 37 and 42% (data not shown), respectively, below the value shown in Fig. 5 for cells grown in medium containing glucose plus galactose. For the repressing strains JA6 and SDU1140-R1, there was no change (data not shown) in the level of Kl-GAL4 mRNA.

We conclude that *Kl-GAL4* promoters carrying a C at position 755 and a G at position 769 (strains SDU1118 and SDU1140-R1) result in a two- to threefold-lower steady-state level of GAL4 mRNA than do promoters with a T at position 755 and a C at position 769 (strains SDU1140 and SDU1140Leu) when cells are grown under glucose-repressing conditions (glucose and galactose in the medium).

Glucose repression is not due to different transcription start sites in KL-GAL4. The bases at 755 and 769 could affect the level of Kl-GAL4 mRNA by changing the transcription start site(s). Transcription start sites were determined by primer extension analysis, and no difference in start sites or relative abundance of individual Kl-GAL4 mRNA species was observed between a glucose-repressing (strain JA6) and a nonrepressing strain (SDU1140-Leu) (Fig. 6).

Glucose repression is relieved by two copies of *Kl-GAL4*. The preceding results suggest that glucose repression might be overcome by increasing the concentration of Kl-GAL4 mRNA. To examine this hypothesis, strain SDU1118-2X, carrying two copies of a *Kl-GAL4* glucose-repressing allele, *GAL4-2*, was constructed. As predicted, this strain did not glucose repress β-galactosidase activity (Fig. 7). Under glucose-repressing conditions, the strain had 4.6- and 5.7-fold more GAL4 mRNA than did the glucose-repressing strains JA6 and SDU1140-R1, respectively, and 85-fold more β-galactosidase activity (Fig. 5).

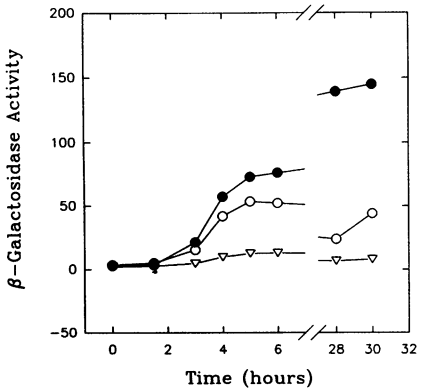

FIG. 4. Evidence that bases 755 and 769 in the *Kl-GAL4* promoter cause glucose repression. Cells were grown and assayed as described in Materials and Methods and the legend to Fig. 2. Symbols: closed circles, SDU1140; open circles, 1140-769G; triangles, 1140-755C.

DISCUSSION

The results presented in this report demonstrate that a region of the *Kl-GAL4* promoter is responsible for most of the glucose repression effect on β-galactosidase activity. The most compelling data for this conclusion come from a comparison of strains that differ by two bases in the promoter. When base 755 is a C and 769 is a G (summarized in Fig. 1 and 8), the strain shows glucose repression of β-galactosidase activity, but when base 755 is a T and 769 is a C, the strain does not show glucose repression (compare strain SDU1140, which does not repress β-galactosidase activity, with strain SDU1140-R1, which does [Fig. 3]).

From these results, we hypothesized that glucose was repressing induction of *Kl-GAL4* transcription. The results of Northern blot analysis summarized in Fig. 5 and the text support our hypothesis. The data can be analyzed in two ways. First, the Kl-GAL4 mRNA level can be compared between a glucose-repressing and a nonrepressing strain. By this comparison, the glucose-repressing strains JA6 and SDU1140-R1 have 35 to 65% (two- to threefold) less *Kl-GAL4* mRNA than do the nonrepressing strains SDU1140 and SDU1140Leu when cells are grown in medium containing glucose and galactose (glucose-repressing conditions). Second, the Kl-GAL4 mRNA level in glucose-grown cells can be compared with the level in glucose/galactose-grown cells. In this comparison, the mRNA level stays the same in glucose-repressing strains but increases two- to threefold in nonrepressing strains.

How might the *Kl-GAL4* promoter mediate glucose repression? We hypothesize that in glucose-repressing strains the presence of glucose in the culture medium is transmitted by an unidentified pathway to a repressor protein that binds to the *Kl-GAL4* promoter in the region of bases 755 and 769.

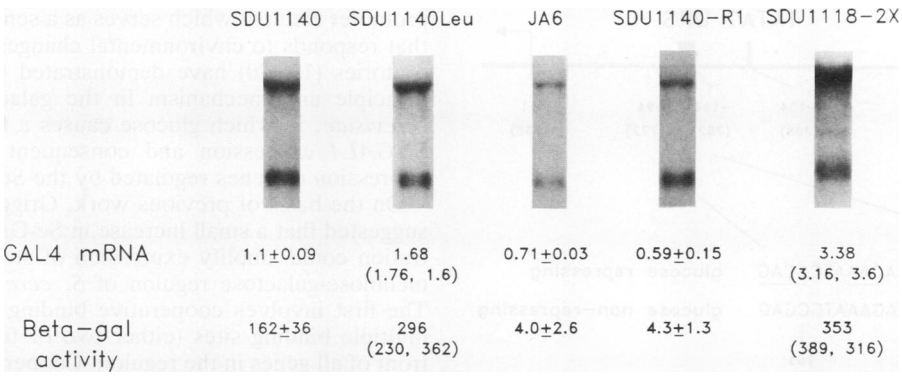

FIG. 5. Transcription of *Kl-GAL4*. Northern blot analysis was used to measure the steady-state level of *Kl-GAL4* transcripts in glucose-repressing and nonrepressing strains. An autoradiogram of a typical Northern blot is shown. The values for Kl-GAL4 mRNA (upper band on the autoradiogram) shown below the blot are presented as a ratio relative to the control *IPP* mRNA (lower band on the autoradiogram). Values represent the average of three or four independent experiments except those for strains SDU1140Leu and SDU1140-R1, which represent two experiments. Beta-gal activity stands for β-galactosidase activity, which was measured at the time of RNA extraction. Cells were grown for about 16 h to an OD_{600} of 1 in MinGalGlu, and RNA was isolated as described in Materials and Methods.

Binding prevents increased transcription. Thus, in glucose-repressing strains, the steady-state level of KL-GAL4 protein remains below the threshold needed for induction of the lactose-galactose regulon. These two bases lie in a hypothetical binding site termed URS_G which is centered about 110 bp upstream of the *Kl-GAL4* transcription start site. The hypothetical URS_G contains 21 bases, 14 of which are related by twofold rotational symmetry about a center, nonsymmetrical base, where the symmetrical bases are indicated as shown in Fig. 8. In nonrepressing strains, the T-755 and C-769 base changes reduce the repressor's affinity for the sequence, which we call $mURS_G$ (mutant URS). Reduced binding uncouples the glucose repression signal so that the steady-state level of Kl-GAL4 increases two- to threefold and the KL-GAL4 protein concentration rises above the threshold needed for induction of the lactose-galactose regulon.

A prediction of our hypothesis is that glucose repression can be overcome by increasing the amount of Kl-GAL4 protein. To test this hypothesis we constructed a strain, SDU1118-2X, having two copies of a *Kl-GAL4-2* glucose-repressing allele: one normal chromosomal allele and a second, unlinked copy integrated at the *ARS1* locus. The strain had a higher level of Kl-GAL4 mRNA than did the parent glucose-repressing strain JA6 (Fig. 5), and as predicted, it no longer glucose repressed expression of *LAC4*, as measured by β-galactosidase activity (Fig. 5 and 7). It is not technically possible to directly measure the amount of active Kl-GAL4 protein, but the increased level of Kl-GAL4 mRNA in the strain suggests that the failure to glucose repress is due to an increased concentration of Kl-GAL4 protein. These results agree with previous results (16) in which glucose repression was relieved when two copies of a non-glucose-repressing *Kl-GAL4* allele were integrated in tandem at the *Kl-GAL4* chromosomal locus.

In contrast to previous results (16), we found that residue 103 or 104, which is Trp in some strains and Leu in others, plays no role in the glucose repression phenotype (compare strains SDU1140 and SDU1140Leu in Fig. 1). We think that our results differ from previous results (16) because of the

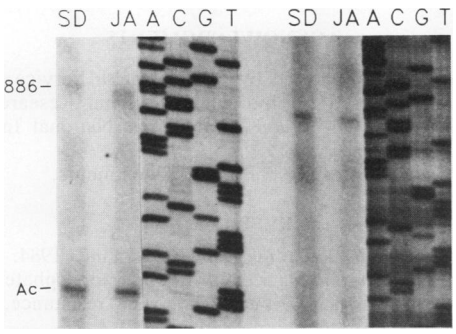

FIG. 6. Transcription start sites in *Kl-GAL4*. *Kl-GAL4* transcription start sites in the nonrepressing strain SDU1140-Leu (SD) and the repressing strain JA6 (JA) were compared by primer extension analysis. The major transcription start site in both strains corresponds to base 886 (numbering system of Wray et al. [33]), which we have redesignated +1 in Fig. 8. Actin mRNA (Ac) served as an internal control for RNA concentration. The length of the primer extension products was determined by comparison with dideoxy DNA sequencing products, labeled A, C, G, and T, obtained by using the LAC9 primer and a *LAC9* DNA template. Reactions on the right half of the figure were the same as those on the left half except that they did not contain the actin primer.

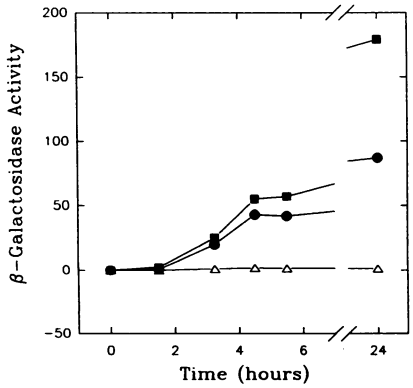

FIG. 7. Evidence that two copies of a *Kl-GAL4* glucose-repressing allele allow cells to escape glucose repression. Cells were grown and assayed as described in Materials and Methods and the legend to Fig. 2. Symbols: circles, SDU1140; squares, SDU1118-2X; triangles, JA6.

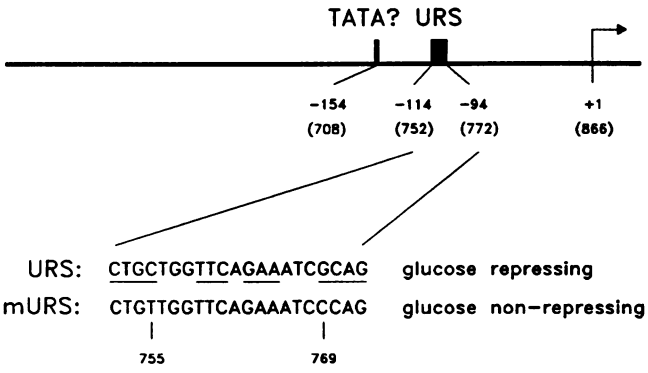

FIG. 8. Location of the URS in the *Kl-GAL4* promoter. The major *LAC9* transcription start site determined by primer extension analysis is shown by an arrow and is labeled +1 to conform with current nomenclature for promoter sequences. The URS, a potential TATA box, and a potential UAS_G are indicated by solid boxes. Numbers in parentheses represent the numbering system of Wray et al. (33). The 14 of 21 bases in the URS related by twofold rotational symmetry are underlined.

way in which strains were constructed. Kuger et al. (16) used an *Ava*II restriction site located 70 bp upstream of the T-755/C base (Fig. 1, strains JA6-A2 and JA6-A12) to promote recombination between the host chromosomal *Kl-GAL4* allele, which came from a non-glucose-repressing allele, and the allele to be examined (this allele was a DNA fragment introduced into the host by transformation). We suspect that recombination sometimes occurred so that the T-755 base in the host (strain DL9; Fig. 1) was not replaced by the C-755 base on the transformed DNA fragment. This would occur when intracellular nucleases removed the ends of the transformed DNA fragment before it recombined with and displaced the chromosomal allele. The consequence of such an unexpected recombination event would be to retain the T-755 base of the host strain, which would give a non-glucose repression phenotype as seen for strain JA6-A12. We avoided this problem by using a *Sal*I site, 380 bp upstream of the T-755/C base, to promote recombination. In addition, we always examined 3 to 12 independently constructed strains for each allele, and for critical experiments we made gain-of-function mutations (nonrepressing → repressing) which required that the final strain have a C at position 755. We have not ruled out the possibility that the glucose repression phenotype of strain JA6 of Kuger et al. is due to promoter mutations different from the ones identified by us in strain Y1118.

Although the URS_G in front of *Kl-GAL4* accounts for most of the glucose repression effect, it is probably not the only element that mediates glucose repression of the lactose-galactose regulon. We noted previously (24) that strains defective in *Kl-gal4* still show a small (two- to threefold reduction) of β-galactosidase activity when glucose is present in the culture medium. This Kl-GAL4-independent glucose repression may involve URS elements located in front of *LAC4* and the other genes in the regulon, as is the case for genes in the melibiose-galactose regulon of *S. cerevisiae* (10, 20).

Our results illustrate an important regulatory principle: small (2- to 3-fold) changes in the concentration of a regulatory protein can produce a large (over 40-fold) change in the expression of structural genes. In the example shown here, the concentration of the regulatory protein is governed by a promoter element which serves as a sensitive genetic switch that responds to environmental changes. Recently two laboratories (13, 20) have demonstrated the same regulatory principle and mechanism in the galactose regulon of *S. cerevisiae*, in which glucose causes a fivefold reduction in *Sc-GAL4* expression and consequent large reduction in expression of genes regulated by the Sc-GAL4 protein.

On the basis of previous work, Griggs and Johnston (13) suggested that a small increase in Sc-GAL4 protein concentration could amplify expression of structural genes in the melibiose-galactose regulon of *S. cerevisiae* in two ways. The first involves cooperative binding of Sc-GAL4 to the multiple binding sites (either two or four sites) present in front of all genes in the regulon. Cooperative binding in vivo has been observed (12). The second mechanism involves cooperative effects on transcription activation produced after GAL4 binds to DNA (4). If both mechanisms were at work, even larger amplification of structural gene expression might occur. For both amplification mechanisms, a small drop in the concentration of GAL4 protein, caused by glucose, would lower the protein concentration enough so that low-affinity DNA-binding sites would not be occupied. The cooperative effects of GAL4 would be lost, and expression of the regulon would be repressed. Similar mechanisms are likely to operate in *K. lactis*, since all genes in the lactose-galactose regulon have multiple Kl-GAL4 binding sites (9).

The proteins that mediate glucose repression by binding to the promoter of the *Kl-GAL4* and the *Sc-GAL4* genes are likely to be different. The URS_G binding site in the *Kl-GAL4* promoter (Fig. 8) is unrelated to the proposed glucose repression sites in the *Sc-GAL4* promoter which bind the MIG1 protein (13, 20, 21). The consensus MIG1 binding site is thought to be WWWWTSYGGGG (W = T or A, S = G or C, and Y = C or T) (20). This or related sequences with fewer than two base differences are not found in the *KL-GAL4* promoter. Comparison of the URS-binding protein, once it is isolated, with MIG1 may reveal a common domain that recognizes the glucose repression signal. Alternatively, *S. cerevisiae* and *K. lactis* may have evolved different glucose signaling pathways so that the signal recognition domains on the URS-binding protein and MIG1 are unique.

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