The Galactose Switch in *Kluyveromyces lactis* Depends on Nuclear Competition between Gal4 and Gal1 for Gal80 Binding*[^1,2]^†

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The Gal4 protein represents a universally functional transcription activator, which in yeast is regulated by protein-protein interaction of its transcription activation domain with the inhibitor Gal80. Gal80 inhibition is relieved via galactose-mediated Gal80–Gal1–Gal3 interaction. The Gal4–Gal80–Gal1/3 regulatory module is conserved between *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Here we demonstrate that *K. lactis* Gal80 (KlGal80) is a nuclear protein independent of the Gal4 activity status, whereas KlGal1 is detected throughout the entire cell, which implies that KlGal80 and KlGal1 interact in the nucleus. Consistently KlGal1 accumulates in the nucleus upon *KIGAL80* overexpression. Furthermore, we show that the KlGal80–KlGal1 interaction blocks the galactokinase activity of KlGal1 and is incompatible with KlGal80–KlGal4–AD interaction. Thus, we propose that dissociation of KlGal80 from the AD forms the basis of KlGal4 activation in *K. lactis*. Quantitation of the dissociation constants for the KlGal80 complexes gives a much lower affinity for KlGal1 as compared with Gal4. Mathematical modeling shows that with these affinities a switch based on competition between Gal1 and Gal4 for Gal80 binding is nevertheless efficient provided two monomeric Gal1 molecules interact with dimeric Gal80. Consistent with such a mechanism, analysis of the sedimentation behavior by analytical ultracentrifugation demonstrates the formation of a heterotetrameric KlGal80–KlGal1 complex of 2:2 stoichiometry.

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Regulation of transcription requires coupling of gene-specific transcription activators to regulatory signals. The galactose switch, which induces the synthesis of galactose metabolic enzymes in response to this sugar in the environment, serves as a textbook model of how such coupling can be achieved in a eukaryotic cell (for reviews see Refs. 1–3). In *Saccharomyces cerevisiae* the regulatory module consists of three proteins, the DNA binding activator Gal4, its negative regulator Gal80, and the signal-transducing Gal3 protein. Gal4 is bound to its target genes in induced as well as noninduced cells (4–6). Its C-terminal activation domain overlaps with the binding site for Gal80 (7–13), which in the absence of galactose binds to Gal4 and prevents recruitment of coactivators and the formation of the preinitiation complex at the promoters (14). Gal80 inhibition is relieved by a mechanism that requires direct interaction with Gal3 (15–18), which is a sensor of intracellular galactose. Gal3 is a galactokinase-related protein that has lost enzymatic activity but retained the ability to bind the galactokinase substrates galactose and ATP, both of which are required to increase the affinity of Gal3 for Gal80 (15, 19). In the absence of a functional Gal3 protein, the *GAL1* gene product, galactokinase (Gal1), can partially substitute for Gal3 in relieving Gal80 inhibition (20, 21). Apparently, Gal3 and Gal1, which are 78% identical, are the products of divergent evolution after gene duplication (22) and in recent *S. cerevisiae* are dedicated to regulatory and enzymatic functions, respectively.

In the lactose-fermenting yeast *Kluyveromyces lactis*, which has not undergone genome duplication like *S. cerevisiae*, a Gal3 ortholog is lacking (22). Instead, KlGal1 is a bifunctional protein, which fulfills enzymatic as well as regulatory function in the *K. lactis* lactose/galactose (*LAC*/*GAL*) regulon (23). In this yeast, intracellular galactose not only arises from uptake of this sugar from the medium but also from cytosolic hydrolysis of the disaccharide lactose, a major carbon source in the natural habitat of *K. lactis*. Together with the galactose metabolic genes, the genes *LAC12* and *LAC4*, encoding lactose permease and β-galactosidase, respectively, are subject to transcriptional regulation by KlGal4 (Lac9), a member of the fungal specific Gal4 family of zinc cluster transactivators (24–27). KlGal4 can substitute for ScGal4 in inducing the *GAL* regulon and vice versa despite the fact that, apart from the DNA binding domain and the C-terminal Gal80 binding domain, overall similarity between both proteins is low (26, 28, 29). Although *KIGAL1* can substitute for *GAL3* in *S. cerevisiae* (23), neither Gal3 nor ScGal1 can complement the noninducible phenotype of a *K. lactis* gal1 deletion mutant unless the *KIGAL80* gene is exchanged by ScGal80 (15). Only one out of the three characterized dominant alleles of *ScGal80* that confer a super-re-

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pressed phenotype (GAL80) on ScGal4 (30) has a similar effect in K. lactis upon substitution of equivalent amino acids in KlGAL80 (31). These data indicate differences between both yeasts either in the Gal1/Gal3-Gal80 interface and/or in the mechanism that relieves Gal80 inhibition.

It has been suggested that in S. cerevisiae compartmentation of regulators contributes to Gal4 activation (6). ScGal80p is detectable throughout the cell indicating that it shuttles between the nucleus and cytoplasm (32). When tethered in the cytoplasm either via a membrane-anchored Gal3 variant or by surrogate protein interaction of fusion proteins, its inhibitory function is relieved (6). However, ScGal80 can be detected at Gal4 target promoters in both uninduced and induced cells, although the level is apparently decreased in induced cells (6, 33).

A crucial question is whether Gal4 and Gal3/Gal1 binding to Gal80 is mutually exclusive. A heterotrameric ScGal4-ScGal80-Gal3 complex can form in vitro under conditions of high excess of Gal3 (34), but it remains to be shown that this complex is able to activate transcription. Apparently, Gal3 destabilizes the Gal4-Gal80 complex in vitro (35). Attempts to map subdomains of Gal80 sufficient for binding to either one of its interaction partners have been unsuccessful so far. Both Gal80 orthologs are remarkably sensitive to mutations, and many single amino acid exchanges in ScGal80 simultaneously eliminate binding to Gal4, Gal3, and homodimerization (36, 37). These mutations do not cluster in particular regions and apparently affect the overall structural integrity of Gal80 rather than specific functions. However, close proximity of Gal3/Gal1 and Gal4 binding interfaces on Gal80 is suggested by the few amino acid substitutions that selectively influence Gal4 or Gal3/Gal1 binding activity, like G302R (KlGal1 binding deficiency) and G311E (Gal4 binding deficiency) (31).

Here we present evidence that KlGal1 and Gal4-AD binding to KlGal80 is incompatible, and we propose that the galactose switch is based on competition between its two interaction partners in the nucleus. Mathematical modeling supports a mechanism, in which two KlGal1 monomers compete against the cooperative binding of the KlGal80 dimer to the Gal4 dimer. The formation of a heterotrameric KlGal802-KlGal12 complex and the nuclear interaction of the partners is confirmed experimentally. With the qualitative and quantitative data presented, a full mechanistic understanding of an important model transcriptional switch is within reach.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—The K. lactis strains JA6/D801 (gal80-D1::ScURA3), JA6/D1R (gal1::Scura3), JA6/D1D802R (gal80-D2::Scura3 gal1::Scura3), and JA6/80HisR (KlGAL80-IHIS) are isogenic to JA6 (α trp1-1 1 ura3-12 ade1-600 adeT-600) (27) except for the indicated alleles. In JA6/80HisR, the wild type KlGAL80 gene was replaced by the KlGAL80 allele coding for the internally His₆-tagged KlGal80 protein IHGal80 (the gene sequence is identical to that on plasmid pETIHG80). Yeasts were grown in synthetic minimal medium (SM: 0.67% yeast nitrogen base without amino acids) mixed with amino acid/base supplement according to Sherman et al. (38). The carbon sources glucose, galactose, and glycerol were added to a final concentration of 2, 2, and 3%, respectively. For selection of transformants, the corresponding amino acid or base was omitted.

Plasmids—Plasmid pETIHG80 was constructed by cloning the KlGAL80-IHIS sequence from pEAG80His15, which codes for the internally His₆-tagged KlGal80 variant IHGal80, into vector pET21c (Novagen). In IHGal80 the amino acid Val¹⁴⁹ of KlGal80 protein is replaced by sequence LRSHHHHH-HHG. Plasmid pETNHG80 is based on vector pET15b (Novagen) and codes for an N-terminal His₆-tagged KlGal80 variant (NHGal80), in which the first methionine is replaced by sequence MGSSHHHHHSGGLVPRGSHMLEDRS (recognition site for thrombin is underlined). Plasmids pETNHG80-S2 and pETNHG80-31 are derivatives of pETNHG80 and code for the N-terminal His₆-tagged KlGal80 mutant variants NHGal80-S2 (amino acid exchange E367K; position is given for KlGal80 wild-type protein without His₆ tag) and NHGal80-31 (G311E), respectively. Plasmid pETNHG1 codes for an N-terminal His₆-tagged KlGal1 protein variant (NHGal1) and was constructed by cloning the KlGAL1 gene sequence into pET15b. In NHGal1 the sequence of the KlGal1 wild-type protein is preceded by amino acid sequence MGSSHHHHHSGGLVPRGSH. Plasmid pGstGal1 codes for a glutathione S-transferase-KlGal1 fusion protein (31). The centromeric plasmid pCGFPAG1 contains a GFP-KlGAL1 fusion gene under control of the ScADH1 promoter and the KICEN2-ARS1-KARS12-ScTRP1-ScURA3 cassette from pKATUC4 (39). Plasmid pCGFPAG1-ura3Δ is a Scura3 derivative of pCGFPAG1 and was obtained by introducing a frameshift mutation into the ScURA3 gene. Centromeric plasmids pCQRS80 and pCQRS80 are derivatives of pKATUC4 containing a GFP-KlGAL80 and a GFP-ScGAL80 fusion gene under control of the ScADH1 promoter, respectively. Construction of multicopy plasmid pEAG80 containing the KlGAL80 gene under control of the ScADH1 promoter was described previously (31). Further details of plasmid constructions and sequences are available on request.

Synthetic Peptide—The AD-22 peptide consisting of the 22 C-terminal amino acids of KlG4 (amino acid sequence: TQQLFNTTTTMDVYNYIFDNDE) was produced by solid phase synthesis using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) strategy (F. Bordusa, Max Planck Research Unit for Enzymology of Protein Folding, Halle, Germany).

Protein Expression and Purification—KlGal80 variants with either an N-terminal (NHGal80, NHGal80-S2, and NHGal80-31) or an internal (IHGal80) His₆ tag were expressed in Escherichia coli strain Rosetta(DE3)-pLys (Novagen) from plasmids pETNHG80, -S2, -31, and pETIHG80, respectively. The transformed cells were grown in LB-camp-amp medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 35 mg/liter chloramphenicol, 100 mg/liter ampicillin) to an A₆₀₀ of 1–2. Protein expression was induced by diluting the cells 1:10 into induction medium (LB-camp-amp with 200 mM KH₂PO₄ and 1.5% lactose). Cells were grown for 12–15 h at 26 °C to an A₆₀₀ of 4–8. After harvesting the cells, the pellet was frozen in liquid nitrogen and resuspended in lysis buffer (50 mM Tris, 20 mM sodium phosphate, 1 mM EDTA, 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 1% Triton X-100, 100 mg/liter EDTA, 0.5% sodium deoxycholate, and protease inhibitors). Cells were sonicated (three cycles, 20 s each) and centrifuged (3,500 × g, 30 min). The supernatants were applied to glutathione S-transferase columns. Bound proteins were eluted with glutathione. Pure AD-22 peptide was prepared by cleaving the His₆ tag by thrombin (1 unit /μg of AD-22).
citrate, 10 mM NaCl, 10 mM imidazole, 0.1% Tween 20, pH 8.0). Cell lysis was performed by sonication. The crude cell extract was mixed with 0.05% protease sulfate for precipitation of nucleic acids and centrifuged at 35,000 × g for 20 min. The resulting supernatant was loaded on to a nickel-nitrilotriacetic acid-Sepharose (Qiagen)-containing column. After washing the column with lysis buffer containing 50 mM imidazole, the bound proteins were eluted with lysis buffer containing 250 mM imidazole. The protein solution was dialyzed overnight against buffer TCG (20 mM Tris/HCl, pH 8.0, 10% w/v glycerol, 20 mM sodium citrate) or the running buffer for ultracentrifugation experiments (see below).

KGal1p was expressed either as a glutathione S-transferase fusion protein (GstGal1) in K. lactis (for enzyme activity measurements) or as an N-terminal His₆-tagged protein (NHGal1) in E. coli (for enzyme activity and ultracentrifugation experiments). For expression of GstGal1 K. lactis gal1 gal80 deletion strain JA6/D1D802R (40) transformed with plasmid pGstGal1 was grown overnight in selective SM-glucose medium. A crude extract of the cells was prepared by glass bead lysis in buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% w/v glycerol, 0.1% Tween 20, 1.4 mM β-mercaptoethanol). The extract was loaded on a column packed with glutathione-Sepharose Fast Flow (GE Healthcare) and washed with buffer A. GstGal1 was eluted with buffer A containing 20 mM reduced glutathione and dialyzed overnight against buffer B (20 mM Tris/HCl, pH 7.5, 20% w/v glycerol). Protein NHGal1 was expressed in E. coli Rosetta(DE3)-pLys (Novagen) transformed with plasmid pETNHG1. Expression and purification procedures were essentially the same as described for the KGal80 proteins with the exception that the washing buffer contained 20 mM imidazole. Subsequently a second purification step by gel filtration chromatography (running buffer: 20 mM Tris/HCl, pH 7.5, 10% w/v glycerol, 500 mM NaCl) was carried out. Fractions with highly purified NHGal1 were combined and dialyzed overnight against the buffer used in the ultracentrifugation experiments. MiniSacGal4p (amino acids 1–100 + 840–881) was expressed from pRJR229 (11) in E. coli strain BL21(DE3)-pLys (Novagen) and purified as described (41).

Galactokinase and Inhibition Assay—Galactokinase activity (galactose + ATP → galactose-1-phosphate + ADP) was measured by means of a coupled enzymatic assay where the production of ADP was coupled to the reactions of pyruvate kinase and lactate dehydrogenase, and the consumption of NADH was monitored (42). The reaction mixture contained 100 mM Tris/HCl, pH 7.9, 5 mM MgCl₂, 10 mM KCl, 0.3 mg/ml bovine serum albumin, 1 mM fructose 1,6-bisphosphatase, 0.25 mM NADH, 2 mM phosphoenolpyruvate, 1 mM ATP, 6.6 units/ml pyruvate kinase, and 13.5 units/ml lactate dehydrogenase. In the case of high salt conditions 100 mM potassium acetate was added. The reaction mixture was prewarmed to 30 °C and mixed with purified KGal1 protein (GstGal1 or NHGal1) to a final concentration of 5–15 nM. After transferring the reaction mixture to the cuvettes, KGal80 and Gal4 variants were added to the individual samples as indicated. Galactokinase reaction was started by the addition of 50 mM galactose. The Kₘ,app values of KGal1 for galactose and ATP are about 5 mM (data not shown) and 90 mM (43), respectively, which means that in our experiments KGal1 was saturated with both substrates. The reaction proceeded at 30 °C, and NADH consumption was monitored photometrically (Photometer Beckman DU 640) at a wavelength of 340 nm over a period of 3–5 min. The activities did not differ significantly between both purified KGal1 variants (GstGal1 and NHGal1), and kₘ values were always in the range of 60–70 s⁻¹. For galactokinase inhibition assays, five samples containing the same KGal1 and varying KGal80 and Gal4 concentrations were measured against a sample with KGal1 giving relative galactokinase activities.

Analytical Gel Filtration—Gel filtration experiments were carried out on the SMART System with column Superdex 200 PC 3.2/30 (GE Healthcare). KGal80 in buffer TCG was preincubated at room temperature for at least 30 min. 10-μl samples were applied to the column equilibrated in the running buffer as indicated. The runs were performed at 100 μl/min and 25 °C. The column was calibrated using a set of molecular mass standards (Sigma).

Analytical Ultracentrifugation—Proteins NHGal1 and IHGal80 were dialyzed overnight against buffer C (20 mM Tris/HCl, 30 mM EDTA, 35 mM MgCl₂, pH 8.2). The protein solutions (with the individual proteins or a mixture of both) were supplemented with 50 mM galactose and 1 mM ADP. Measurements were performed in an analytical ultracentrifuge Optima XL-A (Beckman Coulter). For sedimentation equilibrium experiments, double sector cells were used at 6000 rpm and 20 °C in an An50Ti rotor. The apparent molecular mass of the proteins was calculated using the software provided by Beckman Coulter. In the case of the isolated NHGal1 and IHGal80, both proteins could be shown to behave as a single homogeneous species, respectively. Thus, the apparent molecular mass reflects the mass and stable association state of the two proteins in solution. In another experiment 5 μM Gal80 was titrated with increasing concentrations of Gal1. Here the calculated apparent molecular mass is an average value describing a mixture of the Gal80-Gal1 complex and the nonassociated species. In the first part of this titration the apparent molecular mass increases with increasing Gal1 concentration up to an equimolar ratio of Gal80 and Gal1, which reflects the stoichiometry of the complex (44). Further addition of Gal1 leads to a decrease of the apparent molecular mass because of the excess of the nonassociated Gal1.

Preparation of K. lactis Cells for Fluorescence Microscopy—The cell suspension taken from a freshly grown culture was mixed with an equal volume of 4% formaldehyde in PBS⁶ (10 mM sodium phosphate, pH 7.2, 150 mM NaCl) and incubated for 3 min. 10 μl of this suspension were spotted on a slide and adjusted by slightly pressing on with a coverslip. The slide was immersed in liquid nitrogen for 30 s and washed in PBS. The cells were coated with DAPI solution (3 μM DAPI in PBS), covered with a coverslip, and observed microscopically.

Calculations of Intracellular Levels of KGal1 and KGal80—KGal1 and KGal80 concentrations were determined from galactose-induced cells of the strains JA6 and JA6/80HisR, respectively. Cells were pre-grown overnight in SM-galactose

⁶ The abbreviations used are: PBS, phosphate-buffered saline; AD, activation domain; DAPI, 4',6'-diamidino-2-phenylindole.
Competitive Mechanism of the Galactose Switch in K. lactis

Model 0

\[ [G80] = [G80]^2 / K_{d1} \]

\[ [G1-G80] = [G1] [G80] / K_d \]

Mass balance equations:

\[ [G80]_2 = [G80] + [G1-G80] + 2[G80] \]

\[ [G1] = [G1] + [G1-G80] \]

Model 1 (active Gal4 is calculated as: [G4]):

\[ [G4-G80] = [G4] [G80] / K_{d1} \]

\[ [G4-G80] = [G4] [G80] / K_{d1} \]

\[ [G4-G80] = [G4] [G80] / K_d \]

\[ [G1-G80] = [G1] [G80] / K_d \]

\[ [G1-G80]_2 = [G1] [G80] / K_d \]

\[ [G4-G80] = [G4] [G80] / K_{d1} \]

\[ [G4-G80]_2 = [G4] [G80] / K_{d1} \]

\[ [G1-G80] = [G1] [G80] / K_d \]

\[ [G1-G80]_2 = [G1] [G80] / K_d \]

\[ [G4] = [G4] + 2[G4] \]

\[ [G80]_2 = [G80] + [G1-G80] + 2[G80] \]

\[ [G1] = [G1] + [G1-G80] + 2[G1] \]

\[ [G80] = [G80] + [G1-G80] + 2[G80] \]

Simulations with the mathematical steady state models were performed using the program Octave version 2.1.36 by solving the equation system with the integrated fsolve function. In the galactokinase inhibition experiments, the relative activity of KlGal1 was taken as the fraction of Gal1 not bound to Gal80. Quantitative analyses of the experiments with KlGal80 and Gal4 (miniSGal4 and synthetic KlGal4 activation domain peptide) were carried out on the basis of model 1. To estimate the binding constants, we minimized the summed square of residuals (deviations between measured and simulated data) using the functions mnsmax and mdsmax of the Matrix Computation Toolbox (N. J. Higham, data available online).

Simulations of the galactose switch of K. lactis were carried out with mathematical models 1 and 2. The proportion of active Gal4 was taken as the Gal4 fraction not bound to Gal80. Two states according to the noninduced and induced state were simulated. The “switch efficiency” is given by the ratio of active Gal4 in induced state relative to noninduced state. The \( K_D \) values for the Gal1-Gal80 (\( K_1 = 83 \text{ nm} \)) and the Gal4-Gal80 (\( K_2 = 1 \text{ nm} \)) complexes were determined by the galactokinase inhibition assay. \( K_1 \) was set to 0.5 nm for model 2 because dimer-dimer interactions are considered in this model. The \( K_D \) value for Gal80 dimerization (\( K_d \)) was set to 0.1 nm as determined for dimerization of ScGal80 (47); \( K_D \) of Gal4 dimerization (\( K_d \)) was 0.01 nm. The total Gal4 concentration was estimated to be 80 nm in the noninduced state, as described for S. cerevisiae Gal4 (48) based on a nuclear volume of 2 fl (46), and 200 nm in the induced state because KLIG44 is autoregulated and the protein level increases by a factor of 2–3 in galactose-grown cells (49). Gal80 and Gal1 concentrations in the induced state were determined in this work. Gal80 concentration in the noninduced state was assumed to be 20-fold lower than in the induced state (50). A summary of the model parameters and their values is given in Table S1. Influences of parameter variations on the simulation results are illustrated in Fig. S1.

RESULTS

KLGal80 Blocks Galactokinase Activity—The K. lactis Gal1 protein combines enzymatic activity with the ability to bind KlGal80. To address the question whether KLGal80 binding would influence catalysis, galactokinase activity was assayed in the presence of increasing concentrations of KLGal80. For this purpose KLGal1 was purified from K. lactis as a Gst-KLGal1 fusion protein (GstGal1) and KLGal80 from E. coli as His\(_{6}\)-tagged proteins. In the KLGal80 variant NHGal80, the His\(_{6}\) tag was fused to the N terminus of KLGal80, and in IHHGal80 the tag was inserted at an internal position, in the so-called linker region. The linker is a nonconserved segment that had been shown to tolerate insertion without affecting KLGal80 function.
Compartmentalized and modified 

# Competitive Mechanism of the Galactose Switch in K. lactis

**FIGURE 2.** **KlGal80 inhibits the enzymatic activity of KlGal1.** The relative galactokinase activities of purified GstGal1 are plotted against the KlGal80 concentration. Measurements were performed with KlGal80 variants NHGal80 (circles), IHGal80 (squares), and NHGal80-S2 (triangles) in the absence (empty symbols) or presence (filled symbols) of 100 mM potassium acetate (Kac). For each set, the results of at least three independent measurements are shown, except for measurements with NHGal80-S2 (one experiment). Solid lines are best fits for each experimental data set used to determine the apparent dissociation constant KD,app. The dotted lines represent alternative best fits to one of these data sets (NHGal80 with 100 mM potassium acetate) on the basis of a mathematical competitive model, which assumes a competition of KlGal80 dimerization against the KlGal1-KlGal80 interaction (see model 0 under “Experimental Procedures” and see Fig. 1) with KD,app values for Gal80 dimerization of 100 nM (broken line) and 1 nM (dotted line), respectively.

To rule out nonspecific effects on galactokinase activity by Gal80 addition, NHGal80-S2, a mutant variant lacking the ability to bind KlGal1 (31), was used as a control. As shown in Fig. 2, addition of KlGal80 to the galactokinase assay reduced enzymatic activity in a concentration-dependent manner, whereas NHGal80-S2 had no influence. Because binding of KlGal80 to KlGal1 led to the inhibition of its enzymatic activity, the inhibition curve offered the possibility to determine the apparent affinity between both proteins. For this purpose the data were fitted to the following equation: relative activity = KD,app/(KD,app + [KlGal80]), where [KlGal80] is the total concentration of KlGal80. The equation was derived assuming a simple monomer-monomer interaction between KlGal1 and KlGal80 (see “Experimental Procedures”). The resulting fits are shown in Fig. 2 (solid lines), and the apparent dissociation constants for the complex are listed in Table 1 for IHGal80 and NHGal80, respectively. The inhibitory effect and thus the binding to KlGal1 was stronger with IHGal80 than NHGal80. Cleavage of the His6 tag from NHGal80 by thrombin did not affect the affinity of this variant (data not shown). We thus believe that the modification of the N terminus (nine additional amino acids remain at the wild type protein body after cleavage) rather than the His6 tag itself reduces the affinity for KlGal1.

The Gal4 Transcription Activation Domain Relieves Galactokinase Inhibition—If the Gal4 activation domain (Gal4-AD) competes with KlGal1 for binding to KlGal80, it should be possible to monitor this competition in the galactokinase inhibition assay. We used a 22-amino acid synthetic peptide (AD-22) identical to the KlGal4 C terminus containing the AD. A similar peptide of ScGal4 had been shown to be sufficient for binding ScGal80 in vitro (9). As shown in Fig. 3A, the KlGal4-AD peptide relieved KlGal80-mediated inhibition of galactokinase activity in a concentration-dependent way. No influence of the peptide was observed when wild type KlGal80 was replaced by NHGal80-31, a mutant variant unable to interact with KlGal4 in vitro and defective in KlGal4 inhibition in vivo (31). We conclude that AD-22 interacts with KlGal80 in a similar way as full-length KlGal4 and that this interaction is incompatible with KlGal80-KlGal1 interaction, which means that KlGal1 and KlGal4-AD compete for binding to KlGal80. The experimental data could be simulated with a mathematical competitive model, in which Gal80 exclusively interacts with either Gal1 or Gal4 (see model 1 under “Experimental Procedures” and Fig. 1 and see Fig. 3A, solid lines). By fitting this model to the data points, a KD,app of about 1.5 μM was determined for both complexes AD-22-NHGal80 and AD-22-IHGal80.

A high excess of the peptide over KlGal80 was necessary to achieve significant effects in the kinase assay. Because ScGal4 and ScGal80 interact as dimers (47), cooperativity may increase the affinity between both proteins. We therefore tested whether more effective binding could be observed with miniScGal4 (1–100 + 840–881). This protein, which consists of ScGal4-AD fused to the ScGal4 DNA binding and dimerization domain, had been tested extensively for its ability to substitute for full-length Gal4 (11) and was shown to be fully repressed by Gal80 and fully competent of activating transcription in response to galactose. With this protein the inhibitory influence of KlGal80 on KlGal1 could be relieved very efficiently with a sigmoidal dose-response curve. In Fig. 3B, the results obtained with NHGal80 are shown. Conducting the experiment with IHGal80 and/or under higher salt conditions (100 mM potassium acetate) led to very similar results (data not shown). Again the mutant variant NHGal80-31, which was used as negative control, was not affected (Fig. 3B). As in the case of AD-22, the experimental data could be fitted quite well to the mathematical competitive model (solid lines in Fig. 3B), allowing for estimation of the KD,app values for the KlGal80-miniScGal4 interaction. The KD,app values obtained did not differ significantly between the experiments (NHGal80 and IHGal80 each under low and high salt conditions) and were always in the range between 0.5 and 2.5 nM with an average of 1.0 ± 0.8 nM, which is close to the value obtained previously for miniScGal4-ScGal80 interaction (0.3 nM; Ref. 47).

Because the DNA binding domain of Gal4 is unlikely to contribute to Gal80 binding, we hypothesize that the higher affinity

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**TABLE 1**

| KlGal80 variant | KD,app (without Kac) | KD,app (100 mM Kac) |
|-----------------|----------------------|---------------------|
| IHGal80        | 30                   | 83                  |
| NHGal80        | 75                   | 292                 |

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Because the DNA binding domain of Gal4 is unlikely to contribute to Gal80 binding, we hypothesize that the higher affinity...
of miniScGal4 for KlGal80 as compared with AD-22 is because of cooperativity resulting from dimerization of the Gal80 binding domains via the DNA binding domain-linked dimerization motif (12). Consistently, a Gst-KlGal4-AD fusion protein had a much higher affinity for NHGal80 than AD-22 ($K_D \approx 15$ nM; data not shown), suggesting that in this case the dimerization via the glutathione $S$-transferase moiety can substitute to a certain degree for the dimerization motif of Gal4 and facilitates cooperative binding to KlGal80 dimers.

Stoichiometry and Composition of the KlGal1-KlGal80 Complex—Based on gel filtration experiments, it was proposed that Gal3 is monomeric (51). To examine the oligomerization of the homologous K. lactis protein KlGal1, we used an N-terminal His$_6$-tagged protein variant (NHGal1) because glutathione $S$-transferase itself can dimerize (52). Analysis of the molecular weight of NHGal1 by gel filtration (data not shown) and analytical ultracentrifugation (Fig. 4B) confirmed its monomeric state. In contrast, neither NHGal80 nor IHGal80 eluted from the column as a distinct peak suggesting heterogeneity of its oligomeric state and/or conformation (Fig. 4A, first profile). Similar results were obtained with ScGal80 (53). However, upon addition of 30–50 mM citrate or EDTA, KlGal80 (both variants) could be converted into a distinct molecular species that eluted from the column as a sharp symmetrical peak compatible with a dimeric molecule (see Fig. 4A for effect of EDTA). Under these buffer conditions an apparent molecular mass of 118 kDa was determined by analytical ultracentrifugation of IHGal80 (52.2 kDa) indicating that KlGal80 can be stabilized as a dimer in solution (Fig. 4B).

To analyze the stoichiometry and composition of the KlGal1-KlGal80 complex, analytical ultracentrifugation was performed under equilibrium conditions with 5 $\mu$M of KlGal80 and increasing concentrations of KlGal1 in the presence of galactose. To avoid enzymatic activity of KlGal1 during these experiments, the binding buffer contained ADP, which was shown previously to function as a substitute for ATP in triggering the KlGal1-KlGal80 interaction (15). Furthermore the buffer contained 30 mM EDTA to stabilize dimeric KlGal80 (see above).

As shown in Fig. 3C, with increasing concentrations of KlGal1, the apparent molecular weight increased reaching a maximum when equimolar amounts (5 $\mu$M each) of both proteins were present. With excess of KlGal1 the apparent molecular mass decreased again. These data clearly indicate complex formation of 1:1 stoichiometry. The apparent molecular mass of the complex at equimolar amounts of both proteins was about 200 kDa (Fig. 3B) indicating that dimeric KlGal80 associates with two KlGal1 molecules (theoretical mass of 220 kDa).

Competition of KlGal80 Oligomerization against KlGal1 Binding—Based on mutational analysis it has been suggested that the Gal3-binding site on ScGal80 may overlap with the dimerization region such that dimerization of ScGal80 would compete against Gal3 binding (36). Our data presented above, which rather point to a binding of KlGal1 to dimeric KlGal80, argue against a competition between KlGal80 dimerization and binding to KlGal1. Furthermore, ScGal80 dimerizes with high affinity (0.1 nM; Ref. 47). We have not determined the $K_D$ value for KlGal80 dimerization systematically, but at a concentration of 400 nM and in the presence of citrate it was essentially com-

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**FIGURE 3.** Gal4 competes against KlGal80-dependent inhibition of KlGal1. A, influence of a synthetic activation domain peptide (AD-22) on KlGal80-mediated KlGal1 inhibition. Relative galactokinase activities of GstGal1 mixed with KlGal80 (circles, 175 and 330 nM NHGal80; squares, 95 and 240 nM IHGal80) were determined as a function of the concentration of a 22-amino acid synthetic peptide (AD-22) identical to the KlGal4 C terminus. Measurements were done in the absence of potassium acetate. Solid lines show simulation results on the basis of a mathematical dissociation model (see model 1 under “Experimental Procedures” and see Fig. 1); $K_D$ values for the best fits are as follows: $K_D = 1.65$ and 1.35 $\mu$M (Gal4-Gal80) and $K_D = 151$ and 49 nM (Gal1-Gal80) with NHGal80 and IHGal80, respectively. The inset shows experimental results obtained with NHGal80 (circles) and the Gal4 binding-deficient variant NHGal80-31 (triangles). The concentration of the synthetic peptide in the latter case is given in relative arbitrary units (AU). Broken lines are visual guides. B, influence of miniScGal4 on KlGal80-dependent KlGal1 inhibition. Experiments were performed as in A except that peptide AD-22 was replaced by purified miniScGal4. Results are shown for NHGal80-31 (triangles) and different concentrations of NHGal80 (black and gray circles). Solid lines are simulation results on the basis of a dissociation model, the $K_D$ values of the best fit are as follows: $K_D = 1.5$ nM (Gal4-Gal80) and $K_D = 65$ nM (Gal1-Gal80).
Evidence for weak competition of oligomeric KGal80 against KGal1 binding was obtained by mixing wild type NGal80 protein with the KGal1 binding-deficient NGal80-S2 variant. As shown above (see Fig. 2), this protein on its own had no influence on galactokinase activity. However, when the two KGal80 variants (NGal80-S2 at 5-fold molar excess) were preincubated before starting the galactokinase assay, enzymatic activity was higher than with NGal80 alone (Fig. 5). The simplest interpretation of this finding is formation of hetero-oligomeric complexes consisting of wild type NGal80 protein and the nonbinding mutant variant competing against KGal1 binding. With the 5-fold molar excess of NGal80-S2 over NGal80, the reduction of the inhibitory efficiency was relatively small indicating that competition of KGal80 oligomerization against KGal1 binding is weak and unlikely to be caused by KGal80 dimerization. We therefore propose that only the formation of higher oligomeric KGal80 complexes (e.g. tetramers) competes against KGal1 binding. However, the weakness of this competition suggests negligible relevance for the galactose switch.

Nuclear Localization of KGal80 and Nucleocytoplasmic Localization of KGal1—Of crucial importance for the operation of the galactose switch is the intracellular compartmentation of its regulators. To analyze the localization of KGal80 in the cell, a GFP-

KGal80 fusion protein was expressed from a constitutive promoter. As shown in Fig. 6A, the protein was detectable only in the nucleus, both in induced and noninduced cells. The localization was confirmed by immunofluorescence of wild type KGal80 using KGal80-specific antibodies (data not shown) ruling out artificial localization caused by the GFP construct. The predominant nuclear localization of KGal80 was surprising because ScGal80 had been detected throughout the cell (32). To confirm the difference in equilibrium distribution of ScGal80 and KGal80, GFP-ScGal80 was expressed in K. lactis (see Fig. 6B) and GFP-KGal80 was expressed in S. cerevisiae. The results show that KGal80 is nuclear in S. cerevisiae and K. lactis, whereas ScGal80 is detectable in the nucleus and cytoplasm of both hosts. Thus, our results clearly identify differences in the intracellular distributions of the Gal80 orthologs from S. cerevisiae and K. lactis. Moreover, they demonstrate that these differences are determined by the proteins themselves and not by the host cell.

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Nuclear Localization of KGal80 and Nucleocytoplasmic Localization of KGal1—Of crucial importance for the operation of the galactose switch is the intracellular compartmentation of its regulators. To analyze the localization of KGal80 in the cell, a GFP-
Competitive Mechanism of the Galactose Switch in K. lactis

The nuclear localization of KlGal80 was not affected by the presence or absence of KlGal1 (data not shown). However, conversely, KlGal80 affected the cellular distribution of KlGal1. A GFP-KlGal1 fusion protein, expressed in a Klgal1 Klgal80 mutant from a constitutive promotor, gave fluorescence throughout the entire cell with no evidence for nuclear exclusion (Fig. 6C, left column). In a Klgal1 mutant with the wild type KlGAL80 gene, a similar distribution of GFP-KlGal1 was seen in noninduced cells, whereas in galactose-grown cells nuclear enrichment occurred (Fig. 6C, middle column). These data suggest that interaction between KlGal1 and KlGal80 takes place in the nucleus. Nuclear accumulation of KlGal1 would result from the higher affinity between both proteins in the presence of galactose and/or the increase in KlGal80 concentration. Consistently, upon overexpression of KlGAL80 GFP-KlGal1 was found to be nuclear in galactose and strongly enriched in the nuclei of glycerol-grown cells (Fig. 6C, right column). These data rule out an induction mechanism depending on tethering of KlGal80 in the cytoplasm and indicate that competition between KlGal4 and KlGal1 for KlGal80 binding occurs in the nucleus.

Mathematical Modeling of the Galactose Switch—Our findings of competition between KlGal4 and KlGal1 for KlGal80 binding in vitro supports a competition model for the galactose switch. However, the high affinity of the Gal4-Gal80 interaction and the relatively low affinity of the KlGal80-KlGal1 interaction even in the presence of galactose raises the question whether the cellular concentrations of the three regulators would allow the efficient operation of such a switch and, in particular, whether KlGal1 could efficiently dissociate the Gal4-Gal80 complex under inducing conditions.

Cultures induced by 2% galactose were used to determine in parallel the intracellular concentrations of IHGAL80 and KlGal1. For the latter, the enzymatic activity in the cell extract and the experimentally determined $k_{cat}$ of the purified enzyme gave a concentration of $\sim 11 \mu M$ corresponding to $1.5 \times 10^5$ molecules per cell. The IHGAL80 concentration was determined by quantitative Western blotting using the purified protein as a standard and was calculated to range between 2 and 4 $\mu M$ in the nucleus (2.4 to $4.9 \times 10^3$ molecules). Thus, in induced cells there was a 3–6-fold excess of KlGal1 over KlGal80 in the nucleus assuming a distribution throughout the cell of the former and an exclusive nuclear localization of the latter protein as indicated by the localization studies. Because the concentrations of both proteins in noninduced cells were too low to be accurately determined, these values could only be estimated from the fold induction determined previously (50).

On the basis of these concentrations and the affinities between the proteins as determined by the galactokinase inhibition experiments (see Table S1), the galactose switch was described in mathematical terms in a simple model (see model 1 under “Experimental Procedures” and see Fig. 1) treating the regulators as three competing units. Two equilibrium conditions representing the induced and noninduced state were considered. Simulations of both conditions differed in two points as follows: (i) the concentrations of the regulators because GAL4, GAL80, and GAL1 themselves are galactose-regulated genes in K. lactis, and (ii) the affinity between Gal1 and Gal80. The latter is regulated by galactose and couples the switching process to an intracellular signal. The affinity determined by the galactokinase inhibition experiments at saturating galactose concentrations was used to simulate the induced state. For the noninduced state, the Gal1-Gal80 interaction was neglected. These two assumptions certainly overestimate the influence of galactose because overexpression of KlGal1 indicates weak interaction with KlGal80 even in the absence of galactose (40). However, the experimental data allow us to analyze whether in principle a competition mechanism could result in an efficient switching given the quantitative constraints determined in this work. Here the term switch efficiency is defined as the ratio of simulated active Gal4 concentrations in the induced state relative to the noninduced state.

The data rule out a competition mechanism. The switch efficiency increases when the $K_D$ value for Gal1-Gal80 approaches that of the Gal4-Gal80 complex (simulations not shown), but our experimental data argue against such a low $K_D$ value for the KlGal1-KlGal80 complex. Varying the concentrations of Gal4, Gal80, and Gal1 in a biologically meaningful parameter space did not significantly improve the switch efficiency.
We then described the interactions between the regulators in a slightly more sophisticated competitive model (see model 2 under “Experimental Procedures” and see Fig. 1), incorporating the mechanistic details presented in the previous sections. In this model, Gal1 was monomeric and could interact with monomeric and dimeric Gal80, whereas Gal4 and Gal80 could solely interact as dimers. Therefore, this model implies that the high affinity between Gal4 and Gal80 results from cooperative dimer-dimer interaction. As the simpler mathematical description, model 2 can simulate the experimental data from the galactokinase inhibition experiments (data not shown). In addition, the more complicated model can achieve much higher switch efficiencies with the same parameter values (Fig. 7, solid lines).

For the simulations shown in Fig. 7, the $K_D$ for Gal4 dimerization was set...
to an artificially low value (0.01 nM) to account for Gal4 binding to DNA almost exclusively as a dimer (54). Thus, in vivo essentially all Gal4 protein involved in activation is dimeric. The dissociation constant for the Gal80 dimer was based on measurements for ScGal80 dimerization (0.1 nM; Ref. 47). Varying this parameter by a factor of 10 had hardly any influence on the simulation results (Fig. S1).

Comparing the switch efficiency with the proportion of active Gal4 in the induced state shows that with increasing Gal80 concentration in the induced state (which in our simulations is concomitantly coupled to an increase in the noninduced state) the latter, as expected, decreases but at the same time the switch efficiency increases (see Figs. 6 and S1). Increasing the ratio of Gal80 concentrations between the induced and noninduced state strongly decreases the switch efficiency (Fig. S1), illustrating the impact of negative feedback provided by GAL80 autoregulation on the efficiency of the switch. However, the model has the property to partially compensate for this negative influence on Gal4 activation. Simulation results illustrating this effect are presented in Fig. 8, in which the fraction of active Gal4 at increasing Gal80 concentrations and constant Gal1 to Gal80 ratios are shown. As expected, an increase in Gal80 initially decreases the fraction of active Gal4. However, at a Gal1 to Gal80 ratio above 1 there is a threshold, above which active Gal4 increases with a further increase in Gal80. This non-intuitive result stresses the value of mathematical models. Because both genes, GAL80 and GAL1, are under control of Gal4, the parallel increase of both regulators during induction is exactly the situation observed in vivo. Apparently, high concentrations of Gal1 and Gal80 favor formation of the Gal1-Gal80 complex thus stabilizing the induced state, whereas at low concentrations of both proteins Gal80 is “trapped” in the high affinity Gal4 interaction and thereby stabilizes the noninduced state.

DISCUSSION

The simplest model to mechanistically explain the galactose switch is competition between Gal4 and Gal1/Gal3 for binding to overlapping sites on Gal80. Here we present evidence that this is indeed the basis for the galactose switch, at least in K. lactis.

We found that KiGal80-dependent inhibition of the galactokinase activity of KiGal1 is reversed by a miniGal4 variant or a peptide derived from the KiGal4 activation domain. Assuming that inactive galactokinase molecules reflect those that are bound by KiGal80 and active ones are free of KiGal80, we conclude that interaction between Gal4-AD and KiGal80 is incompatible with the KiGal80-KiGal1 interaction. Our model does not exclude the transfer of KiGal80 to a second binding site on KiGal4 upon galactose induction as suggested for the S. cerevisiae proteins (35). However, it implies dissociation of the activation domain of KiGal4 from KiGal80 as a result of KiGal1-KiGal80 complex formation and suggests that KiGal1- and Gal4-AD-binding sites on KiGal80 are overlapping. On the basis of the galactokinase inhibition assay, we were able to quantify the parameters underlying competition between both partners and to describe the switch by computational simulation. Our mathematical models did not attempt to describe the switch in detail but rather focused on the question whether the specific modes of interactions between the three main regulators could support an efficient switching despite the large difference in the affinities of the competing interactions. With the simple model (model 1), in which the three regulators were treated as three competing units, no efficient switch could be simulated with the determined $K_D$ value of the Gal1-Gal80 complex. However, with the extended model (model 2) that implied monomeric Gal1 competing against cooperative Gal4-Gal80 dimer-dimer interaction the switch efficiency was greatly increased. Furthermore, this model has the intriguing property that an increase of Gal4 activation can be achieved by a parallel increase of the Gal1 and Gal80 concentrations (Fig. 8). This property could partially compensate for the negative influence of GAL80 negative autoregulation on the switch efficiency (see Fig. S1).

The two key assumptions in our extended mathematical model, i.e. (i) highly cooperative dimer-dimer interaction between Gal4 and Gal80 and (ii) binding of monomeric Gal1 to dimeric Gal80, are both in line with the experimental results presented in this work. It had been proposed that dimerization of ScGal80 competes against ScGal80-Gal3/ScGal1 binding (36). However, stabilization of the KiGal80 dimer by EDTA allowed us to demonstrate the formation of a KiGal80-KiGal1 heterotetramer in solution (Fig. 4) ruling out this possibility for the K. lactis proteins. Because KiGal1, like its orthologs, is a monomeric protein, we propose a structural organization in the heterotetramer of KiGal1-KiGal80-KiGal1 implying that the KiGal80 dimer interacts with two separate monomers. Presumably, the S. cerevisiae proteins form a similar complex, detection of which has been hampered by the instability of dimeric ScGal80 at the conditions applied (51). The sequence of Gal80 suggests a structure related to that of the glucose-fructose oxidoreductase from Zymomonas mobilis that has been shown to form tetramers (55, 56). Likewise, ScGal80 tetramer formation on adjacent DNA-bound Gal4 dimers has been
A marked and surprising difference between KlGal80 and ScGal80 lies in the cellular localization. We have shown here that KlGal80 is only detected in the nucleus, no matter which growth conditions or KlGal1 status, whereas ScGal80 can be detected in cytoplasm and nucleus (6, 32). Sequestration of ScGal80 in the cytoplasm during induction may therefore contribute to Gal4 activation in S. cerevisiae, whereas such a mechanism can be excluded for K. lactis. There is no evidence that the equilibrium of KlGal80 would be shifted in favor of cytoplasmic localization upon induction. On the contrary, the presence of galactose and/or high concentrations of KlGal80 leads to a nuclear accumulation of KlGal1. We therefore propose that the galactose switch in K. lactis is exclusively operated in the nuclear compartment. KlGal1 might enter the nucleus piggybacking on KlGal80. However, we did not observe a nuclear exclusion of GFP-KlGal1 in the absence of KlGal80. Thus, a KlGal80-independent nucleocytoplasmic transport should be considered.

In Fig. 9 a model is presented that summarizes our findings. It implies compatible interactions of the KlGal80 dimer with monomeric KlGal1 and the incompatibility of a KlGal80 tetramer to interact with KlGal1. A KlGal1-KlGal80-KlGal1 complex is proposed on the basis of our experimental data and the results coming from mathematical simulations. The latter have shown that formation of such a complex results in high switching efficiency even when KlGal1-KlGal80 affinities are much lower than those of the KlGal4-KlGal80 complex. Any previous descriptions of the galactose switch in mathematical terms assumed comparable affinities of Gal80 to Gal3 and Gal4 (61, 62), because so far experimental data allowing the calculation of $K_D$ values for the Gal1/Gal3-Gal80 complex have been lacking. The combination of experiments and mathematical modeling has enabled us to identify modes of interactions between the regulatory proteins as crucially important factors for the operation of the molecular switch.

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