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review

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Regulation of Transcription Activation by Gal4p

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Summary

Gal4p, a member of a fungal trans-activator family, regulates transcription of genes controlling metabolism of galactose and galactose disaccharides like lactose in yeast. This article reviews recent work on the function and regulation of the Gal4p activation domain. The molecular switch that activates Gal4p in response to galactose consists of two proteins, Gal80p and Gal1p or Gal3p. Gal80p can directly interact with Gal4p and Gal1p/Gal3p. The latter interaction depends on galactose and ATP and relieves the inhibitory effect of Gal80p on Gal4p. How Gal80p is inactivated by this interaction is unknown. Since the synthesis of Gal80p and Gal1p/Gal3p is under control of Gal4p and the concentration of both proteins is crucial for Gal4p activity, the regulon is entirely under feedback control. On the basis of comparison between homologous regulators from *Saccharomyces cerevisiae* and *Kluyveromyces lactis* it is proposed that the dynamics of the intermolecular interactions is important for the transcriptional switch. Differences in the mode of regulation that are observed between the two yeasts are likely to be caused by different kinetic parameters rather than by mechanistic differences. However, the phosphorylation of Gal4p in *S. cerevisae* and Gal80p in *K. lactis* suggests that different ways exist to integrate additional signals into the regulon.

Key words: Gal4p, transcriptional activity, *Saccharomyces cerevisiae*

Introduction

Due to the pioneering genetic analysis of Oshima and coworkers, Gal4p together with Pho4p belonged to the first eukaryotic transcription activators studied in detail (reviewed in *1,2*). Mutations in the regulatory genes *GAL4* and *PHO4* defined Gal4p and Pho4p as positive regulatory factors in the yeast *Saccharomyces cerevisiae* required for induction of galactose and phosphate metabolism, respectively. The constitutive induction of these pathways in recessive *gal80* and *pho80* mutants identified Gal80p and Pho80p as negative regulatory elements. The similar nomenclature reflects the formal genetic analogy whereas the molecular mechanisms by which Gal80p and Pho80p prevent Gal4p and Pho4p from activating transcription under non-inducing growth conditions turned out to be rather different (see below). The focus of this review will be the function and regulation of the activation domain of Gal4p by Gal80p. We will not address the issue of glucose repression here. Glucose repression affects Gal4p controlled genes through factors other than Gal4p, reduces *GAL4* gene expression and also controls inducer uptake. However, except for an early report there is little evidence that glucose affects the Gal4 protein directly.

The Gal4p Protein Family

Gal4p belongs to a protein family with >80 members defined by a conserved DNA-binding domain (DBD) so far only found in fungi (*3,4*). All of them are apparently regulators of transcription with a DBD located at or close to the N-terminus. There is little sequence similarity throughout the rest of the proteins except for

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a subfamily that shares a moderately conserved central region of homology, also called middle homology domain (MHD) (*5*, compare ref. *6* for a recent compilation of these proteins). The proteins sharing the MHD form the Gal4p subfamily (*4*). The function of this region is unknown and it has not been shown that it represents a domain in the sense of an autonomously folding unit. In a few family members including Gal4p, activating mutations have been mapped to this region. In Gal4p the MHD overlaps with an „inhibitory domain« identified by deletion analysis of the *S. cerevisiae GAL4* gene. In the Leu3 protein the central region was shown to form intramolecular interactions with the activation domain (*7*). Whether this property is conserved in other family members remains to be seen.

In members of the Gal4p subfamily activation domains are mostly located at the C-terminus where no sequence similarity exists. An additional internal activation region defined by deletion analysis of Gal4p may not be of functional significance in the context of the entire protein.

Only for the DBD detailed structural information is available. For four family members, Gal4p from *S. cerevisiae*, Gal4p from the related budding yeast *Kluyveromyces lactis* (also called KlGal4p or Lac9p) and the *S. cerevisiae* Ppr1 and Put3 proteins the structures of the DBDs have been solved at atomic resolution (*8–11*). The DNA-contact site is formed by a protein fold in which two zinc ions are complexed by six conserved cysteine residues in a $Cys_6Zn(II)_2$ zinc-cluster. The proteins form homodimers through a coiled-coil motif that is connected to the zinc cluster by a linker region. The linker region determines the spacing of the DNA contact sides and contributes to binding specificity (*12,13*). At the DNA level two conserved 5'-CGG-3' trinucleotides in direct or inverted orientation make contact to the zinc clusters. Whereas the Gal4p and Ppr1p homodimers bind symmetrically to their recognition sites, Put3p binds asymmetrically to a similar sequence (*10*). Nevertheless Gal4p can activate the *PUT* structural genes in a strain lacking Put3p (*14*).

Transcription Activation by Gal4p

Gal4p has been shown to be able to function as an activator in a wide variety of eukaryotic cells. Together with the VP16 protein from Herpes simplex it is the prototype of the class of so-called »acidic activators«. The mechanism of transcription activation for this class of activators, in contrast to *e.g.* glutamine-rich activators, must involve a highly conserved aspect of the transcription process and has been studied extensively. At least one rate limiting step in Gal4p-induced transcription in yeast seems to be recruitment of the RNA polymerase II holoenzyme to promoters of target genes (*15*). Deletion analysis of the Gal4p activation domain resulted in a gradual decrease in activation potential and gave no clue about specific residues essential for activation. This finding correlates with the lack of sequence similarity on different ADs. Although the activation domain of acidic activators is characterised by a high content of negatively charged amino acid residues, hydrophobic rather than acidic side chains seem to be crucial for activating activity. *In vitro* interaction studies revealed numerous components of the transcription apparatus that bind to the activation domain but which of these contacts can occur *in vivo* and which ones are crucial for the activation function is still a controversial issue. A series of mutations in Gal4p showed a strong correlation between the level of activation measured *in vivo* and the affinities of Gal4p for TBP and TFIIB *in vitro* (*16,17*). Gal4-AD and DNA binding to TBP is competitive indicating that the activator binds to the same face of TBP as the TATA-box (*18*). According to the »hand-off« model for TBP-activator interaction Gal4p may compete an inhibitor of TBP and then hand it off to DNA (*18,19*). Biochemical and genetic analysis also provided strong evidence that SRB4, a component of the mediator complex of the RNA polymerase II holoenzyme, is a target of the Gal4p activation domain (*20*). The mediator apparently functions as a coactivator for various activators (*21*). Thus, multiple activator-target interactions may play synergistic roles in eliciting high levels of gene expression *in vivo*.

Chromatin remodelling also contributes to Gal4p-mediated gene activation. The well-studied *GAL1-GAL10* promoter that contains four binding sites for Gal4p undergoes a striking chromatin transition upon galactose induction (*22*). A window of nuclease accessible chromatin becomes wider and encompasses the entire intergenic region including the transcription initiation sites. The activation domain of Gal4p is required for chromatin remodelling whereas the SWI/SNF chromatin remodelling complex is probably not, at least not at this particular promoter (*23*). The issue of recruitment of remodelling or chromatin modifying factors has not been solved for Gal4p yet.

Inhibition of the Gal4p Activation Function

The activation domain of Gal4p overlaps with a region that is essential for down-regulation of the activation function under non-inducing conditions. It serves as the binding site for the negative regulator Gal80p. It was thus proposed that Gal80p binding interferes with the interaction of Gal4p and an activator target. Biochemical and genetic evidence that support such a model has been reviewed earlier (*1,24–26*).

The Gal80p-binding domain is highly conserved between the Gal4p homologues from *S. cerevisiae* and *K. lactis* (*27*). Among the 30 C-terminal amino acids that are sufficient for Gal80p binding (*28*) there is a block with 14 out of 15 residues identical in Gal80 from *K. lactis* (KlGal80p) and Gal80 from *S. cerevisiae* (ScGal80p). Thus, there is a higher sequence constraint for the Gal80p binding face than for any activator target protein. Mutations that separate Gal4p transcription activation and Gal80p binding function have been identified (*29,30*). The sequence conservation in the Gal80p binding domain is reflected in the conserved sequence of the Gal80p homologues from both yeasts (see below, *31*).

Mini-Gal4p variants consisting of the DBD fused to the AD were shown to behave very similar to the full-length protein with respect to regulation (*32*). They were used in most *in vitro* studies on Gal4p regulation. Gal80p is sufficient to block activated transcription of a mini-

Gal4p variant *in vitro* and inhibits interaction of mini-Gal4p with TBP (*17,32*).

However, *in vivo*, Gal4p inhibition may be more complex as indicated by several findings. Gal4p is subject to regulated phosphorylation at multiple sites (*33,34*). Of these, position S699, which is not contained in the mini-Gal4p variants, has an influence on Gal4p activity. A serine to alanine substitution at this site reduced induced gene expression levels, in particular at low inducer concentrations. Since this reduction is only observed in a *GAL80*$^{+}$ background, it was suggested that phosphorylated S699 interferes with Gal80p inhibition (*35*). The protein kinase responsible for phosphorylation at this site is a component of the RNA polymerase II holoenzyme, the CDK8-like cyclin dependent kinase encoded by the *SRB10* (kinase) and *SRB11* (cyclin) genes (*36*). The SRB10 protein level decreased in response to nutrient limitation and other stressors (*37*). Possibly, phosphorylation of Gal4p serves to integrate physiological signals other than galactose availability.

There is no evidence for phosphorylation of Gal4p in *K. lactis* but in this yeast Gal80p may serve as a signal integration point. In contrast to ScGal80p, KlGal80p is subject to regulated phosphorylation. The phosphorylated form is detected under non-inducing conditions whereas it disappears upon galactose induction and the non-phosphorylated form accumulates (*38*). Reduction of the phosphorylated form requires KlGal1 protein function (see below). We have preliminary evidence that phosphorylation is not directly involved in the regulation of KlGal80p inhibitory activity by galactose and thus, it may serve a more subtle fine-tuning similar to phosphorylation of ScGal4p (Kapp and Breunig, unpublished data). Interestingly, a specific mutation in the *KlGAL80* gene, *KlGAL80* $^{s\text{-}0,}$ resulted in a constitutive phenotype. Even when overexpressed, the mutant gene product has very low inhibitory activity although it can still bind efficiently to mini-Gal4p *in vitro*. In this mutant, the phosphorylated form of KlGal80p is under-represented (*38*).

It is interesting to compare the sequences of the two Gal80p homologues from *S. cerevisiae* and *K. lactis*. They display a much higher overall conservation than Gal4p (*39*). There seems to be a strong structural constraint on Gal80p since spontaneously arising recessive loss-of-function alleles can easily be recovered. However, insertions are tolerated in a particular region that is not conserved between KlGal80p and ScGal80p (*31,40*). This region has therefore been named »linker region« (*39*). It is flanked on both sides by the most highly conserved sequence blocks and mutations that do not allow Gal80p inhibitory activity to be relieved (dominant *GAL80*^{s} alleles) are located in these two blocks (Fig. 1). The linker may serve as a flexible loop that connects two parts of the protein that come together in the 3D-structure providing the interface for protein binding. All three characterised *GAL80*^{s} mutations, eliminate the interaction with Gal1p/Gal3p (see below) both in KlGal80p and in ScGal80p, but retain the Gal4p binding activity. The reverse phenotype is conferred by the *gal80-31* mutation that maps between two *GAL80*^{s} sites. Gal80-31p is unable to bind Gal4p but still interacts with Gal1p/Gal3p. Thus, Gal80p binding to its two partners is separable by mutation but residues crucial for each of these interactions are located close to each other.

Relieving Inhibition

Early genetic studies had indicated that activation of Gal4p requires Gal3p function, however, the nature of Gal3p function has remained elusive for a long time. *S.*

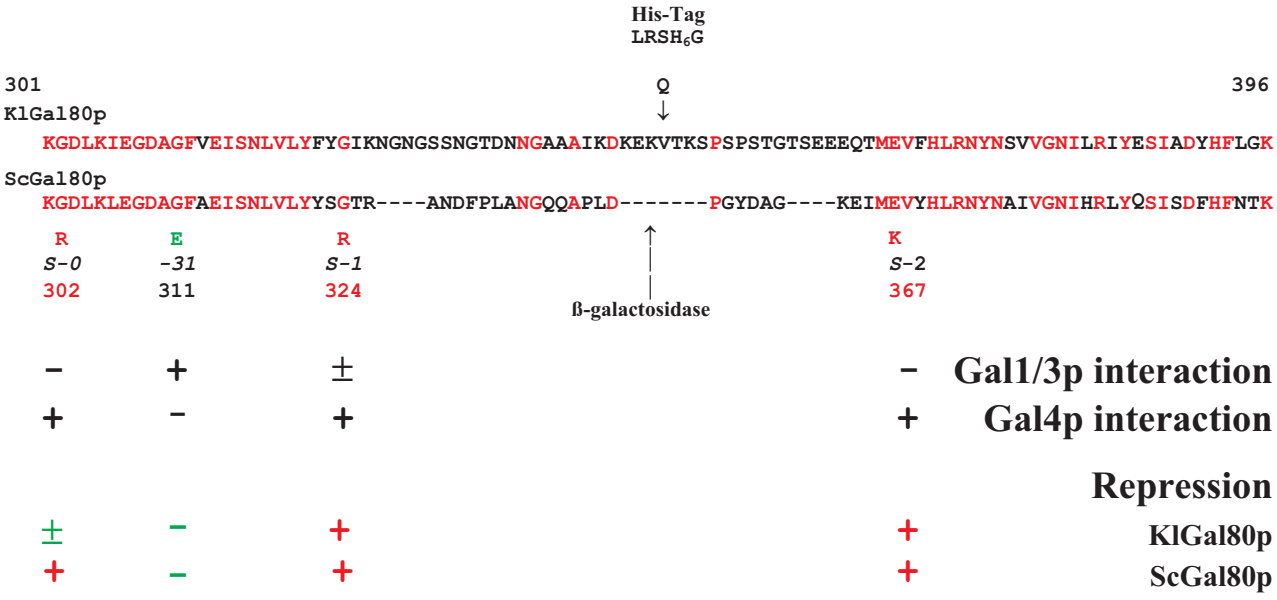

Fig. 1. Comparison of ScGal80p and KlGal80p sequences. A central segment of the protein sequence with the least conserved »linker region« embedded in highly conserved sequence blocks is shown. Identical amino acids are shown in red. Arrows above and below the sequence indicate positions where insertions do not affect the Gal80p inhibitory activity. Amino acid exchanges that alleviate either Gal1p/Gal3p or Gal4p binding activity are marked in red and green, respectively. The influence of these mutations on Gal4p repression is different for the *GAL80*$^{s\text{-}0}$ allele of KlGal80p and ScGal80p

cerevisiae gal3 mutants show a so-called long-term adaptation (LTA) phenotype (*41*). Induction in these mutants is delayed some 3–4 days but after adaptation growth in galactose is almost like wild-type. Since *gal1 gal3* mutants were unable to adapt, it was initially proposed that Gal3p converts galactose into an inducer molecule by a reaction that can be circumvented or substituted by the action of the *GAL1* gene product, a galactose-ATP-α-D--galactose-1-phophotransferase (galactokinase). However, no evidence for any enzymatic activity of Gal3p could be obtained. Since *S. cerevisiae gal1* mutants are unable to utilize galactose, but are not affected in galactose induction, it came as a surprise that some *K. lactis gal1* mutants were not inducible. Detailed analysis of a specific mutant, *Klgal1-209*, that lacked galactokinase activity, but was not affected in Gal4p activation, revealed that KlGal1p is a bifunctional protein with a regulatory and a catalytic activity. Both activities are separable by mutation (*42*). The regulatory function was retained in the KlGal1-209p variant and was able to complement the LTA phenotype of the *S. cerevisiae gal3* mutant. This indicated that KlGal1-209p and Gal3p have equivalent regulatory activities. In *S. cerevisae* Gal1p can substitute for Gal3p but in this case induction is delayed partly because the non-induced level of *ScGAL1* gene expression is extremely low. Indeed, overexpression of ScGal1p reduces the time required for induction in a *gal3* mutant background (*42*,*43*).

ScGAL1, *ScGAL3* and *KlGAL1* share over 80 % sequence identity and ScGal3p is apparently an enzymatically inactive variant of ScGal1p. Insertion of two amino acids that are missing in ScGal3p in a region highly conserved between all known galactokinases was sufficient to convert Gal3p into a galactokinase (*44*). Obviously, the two genes *GAL3* and *GAL1* arose by a duplication event in *S. cerevisae* and subsequently diverged functionally. *K. lactis* is lacking a *GAL3* homologue.

As already mentioned above, the nature of the Gal1p/Gal3p regulatory activity lies in the ability to interact with Gal80p. Genetic evidence for a direct protein-protein-interaction between Gal1p or Gal3p and Gal80p was obtained from gene shuttle experiments between *S. cerevisiae* and *K. lactis* (*31*). Whereas the *KlGAL1* gene could complement the *Scgal3* mutant phenotype neither *ScGAL3* nor *ScGAL1* could restore induction in a *Klgal1* mutant unless in the same *K. lactis* mutant *KlGAL80* was replaced by *ScGAL80*. Formation of a specific KlGal1p-KlGal80p or Gal3p-ScGal80p complex could be demonstrated by pull-down experiments and co-immunoprecipitation (*31*,*45*). The affinity of KlGal80 for Gal3p is weak, which probably contributes to the lack of cross-complementation in *K. lactis*, but is high enough to allow for complex formation *in vitro* (*31*). Importantly, complex formation required both, galactose and ATP. Galactose and ATP are substrates of galactokinase and thus must bind to Gal1p. Probably, binding results in a conformational change in Gal1p that increases the affinity for Gal80p (Fig. 2). This conformational change is still hypothetical and formally it cannot be excluded that galactose and ATP affect Gal80p rather than Gal1p. However, earlier experiments addressing the possibility of inducer binding to Gal80p were negative. It should be stressed that galactokinase sequences from bacteria to

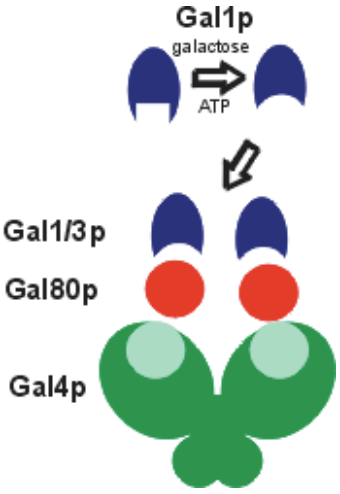

Fig. 2. The hypothetical conformational change resulting from binding of galactose and ATP to Gal3p/Gal1p increasing the affinity for Gal80p

mammals are evolutionarily related. However, they belong to a protein family unrelated to hexokinases for which a conformational change resulting from substrate binding has been well characterized (*46*). Since binding of Gal3p and KlGal1-209p to Gal80p is still regulated by galactose the hypothetical conformational change in yeast galactokinases apparently occurs upon substrate binding in the absence of enzymatic activity. To analyse the specificity of the inducer molecules several other metabolites had been tested in the *in vitro* Gal80p binding assay. Whereas ATP could be replaced by GTP, ADP or ATP-γ-S indicating that nucleotide hydrolysis is not required, no other sugar could substitute for galactose (*31*).

Several laboratories have addressed the question which region of Gal3p/Gal1p interacts with Gal80p. Point mutations in *KlGAL1* and *GAL3* that prevented Gal80p binding or resulted in galactose-independent binding were isolated by screening for non-inducible mutants. These mutations mapped to different locations over the entire length of the coding region indicating that a Gal80p-binding interface cannot be assigned to a separate domain (*47*,*48*). Likewise deletion analysis and two-hybrid experiments with subfragment of the gene did not reveal such a region. It appears that Gal1p and Gal3p are essentially one-domain proteins and 3D-structural information is required to assign the part of the protein binding to Gal80p.

The Galactose Switch: Allosteric *vs.* Dissociation Model

Gal80p interacts with two partners, Gal1p/Gal3p on the one hand and Gal4p on the other hand. Whether or not these interactions are mutually exclusive is an important question when trying to understand the mechanism by which Gal1p/Gal3p alleviates the inhibitory activity of Gal80p on Gal4p (Fig. 3). Platt and Reece could reconstitute the switch from active to inactive Gal4p and back *in vitro* (*26*). Adding Gal80p to a Gal4p-dependent *in vitro* transcription assay was sufficient to block trans-

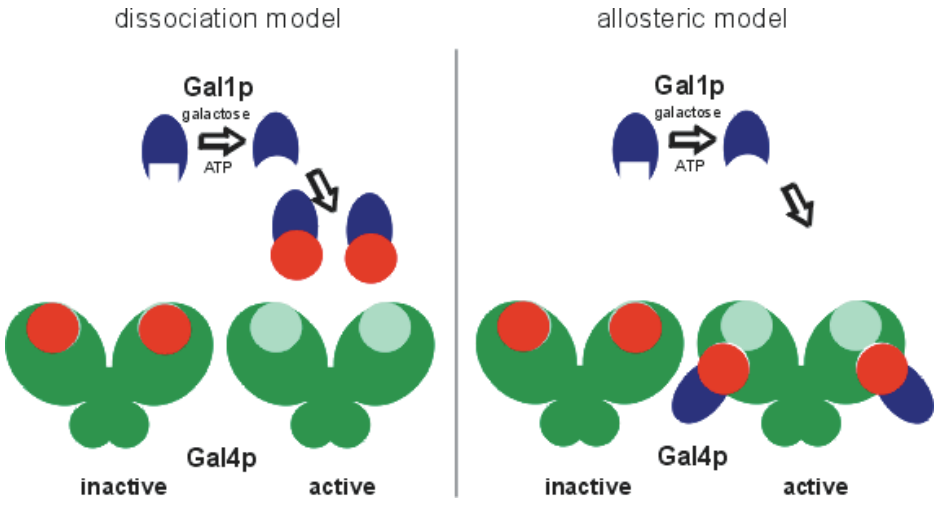

Fig. 3. Two alternative models how Gal4p may be activated in response to Gal1p/Gal3p-Gal80p interaction

criptional activation and addition of Gal3p together with Gal80p restored activation in the presence of galactose and ATP. Moreover, they could show by gel retardation assay that Gal4p, Gal80p and Gal3p are able to form a ternary complex on DNA. These data strongly supported a model proposed much earlier by Leuther and Johnston (*49*). Based on the finding that a Gal80-VP16 fusion protein could form two-hybrid interaction with a Gal4p derivative in induced cells these authors suggested that an allosteric transition occurs in the Gal4p-Gal80p complex upon activation of Gal4p. In agreement with the allosteric model Gal4p purified from induced cells was found associated with Gal80p (*5,50*)

However, the allosteric model was challenged by several recent findings. First, the ternary complex formed between Gal4p, Gal80p and Gal3p *in vitro* required a 30-fold molar excess of Gal3p over Gal80p and a tight-binding mutant Gal3p variant (*26*). Secondly, experimental approaches to monitoring Gal80p-Gal4p interaction indicated that Gal3p destabilized the complex in the presence of galactose and ATP (*51*). Third, evidence was provided that the subcellular distribution dynamics of Gal3p and Gal80p in *S. cerevisae* may play a role in regulating Gal4p (*52*). These authors demonstrated cytoplasmic localisation of Gal3p and dual cytoplasmic-nuclear location for Gal80p. Fourth, Gal4p activity responds very sensitively to the elevated concentrations of Gal80p in induced cells (*39,53,54*) suggesting a dynamic interaction between molecules. The older dissociation model (*55,56*) is therefore not yet ruled out and a multi-step process may emerge.

The Dynamic Aspect of Induction

The dynamic aspect of the induction process has been neglected in most studies. Both Gal3p and Gal80p are encoded by Gal4p controlled genes. The increase of Gal80p concentration upon galactose induction is surprising given that very low concentrations are sufficient for repression (*39*). Thus, very efficient mechanisms must be operative to maintain the induced state. Induced Gal4p activity is still sensitive to Gal80p levels and *gal80* null mutants have higher Gal4p activity than *GAL80* wild-type cells. Therefore, the regulation of Gal80p synthesis must play an important role in controlling the induction process. In *K. lactis* this was indeed observed (*39,54*). In

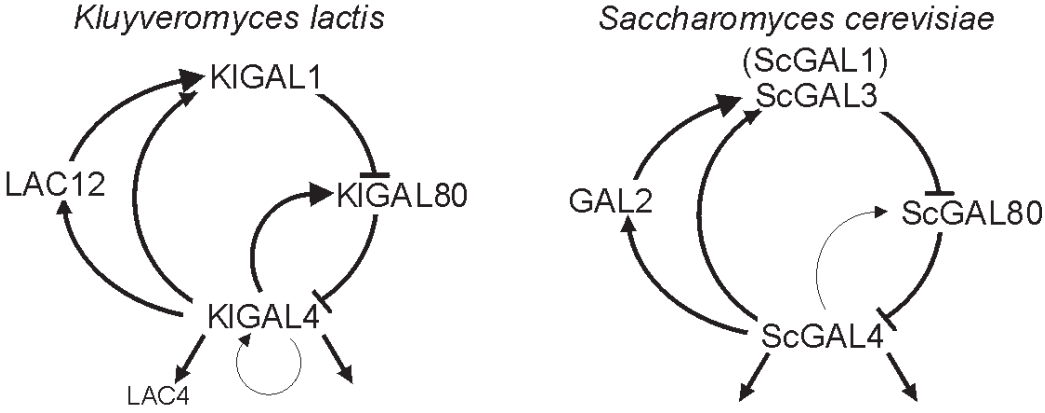

Fig. 4. The regulatory circuits that control Gal4p activity in *S. cerevisae* and *K. lactis* respectively

this lactose utilising yeast, the *GAL* genes are co-regulated with the lactose metabolic genes and the Gal4p levels are higher than in *S. cerevisiae* (*57*), possibly as a consequence of adaptation to a lactose-rich environment. As a consequence KlGal80p plays a more important role in keeping KlGal4p repressed (*39*). When glucose and galactose are simultaneously present in the medium, induction of Gal80p is essential to prevent Gal4p activation in *K. lactis* but not in *S. cerevisae*. There are two KlGal4p binding sites located in the *KlGAL80* promoter and these binding sites have the highest affinity of all known KlGal4p binding sites indicating that this gene can compete efficiently for limiting amounts of activator (*54,58*). Deleting these sites resulted in unbalanced regulatory activity with highly variable Gal4p controlled gene expression rates.

It is thus important to understand the relationship between the rate of Gal80p accumulation, the rate of Gal1p accumulation, the rate of galactose uptake and metabolism and the rate of Gal80p inactivation during establishment of the induced state. The regulatory circuits controlling these processes are similar but not identical between *S. cerevisae* and *K. lactis* (Fig. 4): *(i)* inducer is taken up through an active transporter, Lac12p, and can accumulate intracellularly against a concentration gradient in *K. lactis* whereas *S. cerevisae* Gal2p is a diffusion facilitator (*59,60*), *(ii)* the lack of the regulatory protein Gal3p in *K. lactis* results in a convergence of metabolic and signalling function at KlGal1p, and *(iii)* KlGal4p but not ScGal4p is subject to autoregulation (*61, 62*). These differences certainly affect the kinetic parameters of the regulatory process. Whether they also required different regulatory mechanisms remains to be seen.

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Regulacija aktivacije transkripcije s Gal4p

Sažetak

Gal4p, jedan je od transaktivatora koji dolazi u gljiva, a regulira transkripciju gena što kontroliraju metabolizam galaktoze i galaktoznih disaharida kao što je laktoza u kvascu. U radu je dan pregled novijih istraživanja o djelovanju i regulaciji aktivacijske domene Gal4p. Molekularni poticaj kojim se aktivira Gal4p, kao odgovor na prisutnost galaktoze, sastoji se od dva proteina, Gal80p i Gal1p odnosno Gal3p. Gal80p može izravno reagirati s Gal4p i Gal1p/Gal3p. Interakcije Gal80p i Gal1p/Gal3p, koje ovise o galaktozi i ATP-u dovode do gubitka inhibitornog djelovanja Gal80p prema Gal4p. Međutim, nije poznat mehanizam inaktivacije Gal80p ovim interakcijama. Budući da je sinteza Gal80p i Gal1p/Gal3p pod kontrolom Gal4p, a koncentracija ovih dvaju proteina je odlučujuća za aktivnost Gal4p, ovaj je regulon kontroliran povratnom spregom. Uspoređujući homologne regulatore iz *Saccharomyces cerevisiae* i *Kluyveromyces lactis* uočena je važnost dinamike intermolekularnih interakcija za uključivanje transkripcije. Razlike u načinu regulacije, opažene između ta dva kvasca, vjerojatno su uzrokovane različitim kinetičkim parametrima, a ne razlikom u mehanizmu djelovanja. Međutim, fosforilacija Gal4p u *S. cerevisiae* i Gal80p u *K. lactis* upućuje na to da postoje različiti putevi kojima se dodatni signali uključuju u ovaj regulon.