The yeast galactose genetic switch is mediated by the formation of a Gal4p–Gal80p–Gal3p complex

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***Saccharomyces cerevisiae* responds to galactose as the sole source of carbon by activating the *GAL* genes encoding the enzymes of the Leloir pathway. Here, we show *in vitro* that the switch from repressed to activated gene expression involves the interplay of three proteins [an activator (Gal4p), a repressor (Gal80p) and an inducer (Gal3p)] and two small molecules (galactose and ATP). We also show that the galactose- and ATP-dependent interaction between Gal3p and Gal80p occurs without disruption of the Gal80p–Gal4p interaction. Thus, Gal3p-mediated activation of transcription occurs via the formation of a tripartite protein complex.**

Keywords: Gal4p/galactose/gene regulation/genetic switch/*in vitro* transcription

Introduction

The response of a yeast cell to being challenged with galactose as the sole carbon source is to rapidly activate expression of the *GAL* genes, by several thousand-fold (Johnston, 1987; Lohr *et al.*, 1995; Reece and Platt, 1997). This activation is dependent on Gal4p, a prototypical transcriptional activator (Hashimoto *et al.*, 1983). Gal4p is bound upstream of the *GAL* genes in a variety of carbon sources, and its transcriptional activity is controlled by a negative regulator, Gal80p (Nogi *et al.*, 1984; Giniger *et al.*, 1985), which interacts directly with the activation domain of Gal4p to inhibit its activity (Lue *et al.*, 1987; Wu *et al.*, 1996). How is this repressed state overcome when the yeast are grown on galactose? Some clues to this process have been gained from the analysis of the *GAL3* gene product. Mutations in this gene lead to a slow induction of *GAL* gene expression with *gal3* yeast cells reaching full expression 3–5 days after the addition of galactose to the culture (Rotman and Spiegelman, 1953). Gal3p shares high sequence homology to galactokinase, Gal1p, but does not possess a galactokinase activity itself (Bhat *et al.*, 1990). Over-production of either Gal3p or Gal1p in yeast cells gives rise to galactose-independent *GAL* gene expression (Bhat and Hopper, 1991, 1992). Therefore, Gal3p is thought to play a direct role in the process of induction, and Gal1p is able to at least partially complement this function. Recent data have shown that Gal80p and Gal3p interact with each other in a galactose-dependent fashion (Suzuki-Fujimoto *et al.*, 1996; Zenke *et al.*, 1996; Blank *et al.*, 1997; Yano and Fukasawa,

1997). The association between the two proteins is also dependent upon a nucleotide, although nucleotide hydrolysis is not essential (Zenke *et al.*, 1996). The effect of the Gal80p–Gal3p interaction on the ability of Gal4p to activate transcription has not been investigated.

Does the interaction between Gal80p and Gal3p allow Gal4p-mediated transcription to occur? To address this question, and to discern the fate of Gal80p upon induction, we have established the galactose genetic switch *in vitro*. By addition of Gal3p to a reconstituted transcription reaction containing Gal4p and Gal80p, we demonstrate the ability of Gal3p to mediate galactose induction of transcription *in vitro* in a galactose-dependent manner. Furthermore, the galactokinase, Gal1p, is shown to functionally substitute, albeit weakly, for Gal3p in the induction process *in vitro*. Mutant versions of Gal3p and Gal80p are used to demonstrate the specificity and galactose dependency of Gal3p in galactose induction. Finally, we show Gal3p regulation of galactose induction is mediated by the formation of a tripartite complex consisting of Gal4p, Gal80p and Gal3p.

Results

In vitro transcription with Gal4p, Gal80p and Gal3p

We have previously described the purification of functional truncated versions of Gal4p (comprising the DNA-binding domain fused to the activation, and Gal80p interaction, domain) (Reece *et al.*, 1993). Figure 1A shows Gal80p, Gal3p and Gal1p purified from yeast cells. These proteins were used to modulate the transcriptional activity of Gal4p using a yeast nuclear extract-based *in vitro* transcription system (Wu *et al.*, 1996) (Figure 1B). Gal4p activates transcription ~10-fold using this system, and this activity is specifically inhibited by approximately equimolar amounts of Gal80p (Figure 1B, lanes 2 and 3). Gal3p has no effect on the basal level of transcription (Figure 1B, lane 5) or on the transcriptional activity of Gal4p in the absence of Gal80p (Figure 1B, lane 4), but increasing amounts of Gal3p relieve the inhibitory effect of Gal80p on Gal4p (Figure 1B, lanes 6–10). We note that a 20- to 30-fold molar excess of Gal3p over Gal80p is required to alleviate Gal80p repression. The *in vitro* transcription experiments of Figure 1B were performed in the presence of 1.5 mM galactose. The presence or absence of galactose has no effect on either the basal or activated levels of transcription generated by Gal4p (data not shown).

Gal1p is able to relieve Gal80p repression

The galactokinase, Gal1p, is able to activate the Gal4p–Gal80p complex *in vitro* in a galactose-dependent manner. It has previously been shown that over-production of Gal1p *in vivo* promotes galactose-independent expression

Fig. 1. Gal3p and Gal1p alleviate Gal80p repression *in vitro*. (A) Purified fractions (100 ng) of Gal80p, Gal80^{G152D}p, Gal3p, Gal3^cp and Gal1p were separated by SDS-PAGE and stained with Coomassie brilliant blue. The size of the molecular weight markers (lanes 1 and 7), expressed in kDa, are indicated. (B) Gal3p alleviates the repression of Gal80p on Gal4p *in vitro*. *In vitro* transcription and primer extension reactions were performed as described previously (Wu *et al.*, 1996) using pG₅E4 as a template, shown above the figure. Reactions contained 12 nM Gal4p [amino acids 1–93 fused to 768–881 (Reece *et al.*, 1993)] and 15 nM Gal80p as indicated. Gal3p was present at the concentrations shown. All reactions contained 1.5 mM galactose. (C) Gal1p alleviates the repression of Gal80p on Gal4p *in vitro*. Reactions contained 12 nM Gal4p, 15 nM Gal80p and Gal1p as indicated. Galactose was added to a final concentration of 1.5 mM where indicated.

of the *GAL* genes (Bhat and Hopper, 1992). We tested the ability of Gal1p to activate Gal4p–Gal80p complex *in vitro* (Figure 1C). Like Gal3p, Gal1p has no effect on the ability of Gal4p to activate transcription in the absence of Gal80p (Figure 1C, lane 4). Increasing amounts of Gal1p will, however, promote activation of a Gal4p–Gal80p complex in the presence of galactose (Figure 1C, lanes 5–9). At the highest concentration of Gal1p tested (15 μ M; Figure 1C, lane 9) we observe ~50% of the transcriptional

Fig. 2. The influence of galactose on (A) Gal3p and (B) Gal3^cp function. *In vitro* transcription reactions contained, where indicated, 12 nM Gal4p, 15 nM Gal80p, 1.0 μ M Gal3p and 1.0 μ M Gal3^cp. The final concentration of galactose in each reaction is indicated. (C) A comparison of the effect of galactose on Gal3p and Gal3^cp. Phosphoimage analysis was used to quantitate the major E4 extension product. Relative activation is expressed as a percentage of the activation observed in the presence of 1.5 mM galactose.

activity of Gal4p alone as determined by phosphoimage analysis. This level of Gal1p corresponds to a 40-fold molar excess compared with the amount of Gal3p required to achieve the same level of transcriptional activity (Figure 1B). In the absence of galactose we are unable to observe Gal1p-mediated activation of Gal4p–Gal80p (Figure 1C, lane 10).

Galactose-dependent relief of transcriptional repression

The ability of Gal3p to relieve the inhibitory effect of Gal80p on Gal4p is dependent upon galactose. Decreasing the amount of galactose in the transcription reactions reduces the ability of Gal3p to influence the inhibitory activity of Gal80p (Figure 2A, lanes 4–10). To test the specificity of the effect of galactose on the ability of

Fig. 3. The effect of Gal80p mutants on transcription *in vitro*. All reactions contained 12 nM Gal4p. (A) Gal80 proteins were added at concentrations indicated. Galactose and additional ATP were not included in the transcription reactions. (B) Reactions contained 15 nM Gal80p, 12 nM Gal80^{S-2}p, 1.0 μ M Gal3p and 1.0 μ M Gal3^{cp}p as indicated. Galactose was added to a final concentration of 1.5 mM.

Gal3p to influence transcription, we used a constitutive mutant of Gal3p, Gal3^{cp}. Gal3^{cp} is derived from the *GAL3^{c-322}* mutation (Asp368Val) which activates *GAL* gene expression in the absence of galactose *in vivo*, and constitutively interacts with Gal80p *in vitro* (Blank *et al.*, 1997). We find that Gal3^{cp} relieves Gal80p-mediated repression of Gal4p activity in the absence of galactose (Figure 2B, lane 10). Low levels of galactose, however, stimulate the ability of Gal3^{cp} to relieve Gal80p repression ~2-fold (Figure 2B, lanes 5–9). The effect of galactose on the activity of Gal3p and Gal3^{cp} are represented graphically in Figure 2C. It is clear that very low concentrations of galactose (50 μ M) promote full activity of Gal3^{cp}, whereas a 30-fold higher concentration of galactose is required to promote full activity of the wild-type protein.

We are unable to test the effect of ATP on the ability of Gal3p to relieve Gal80p-mediated repression of Gal4p activity using this *in vitro* transcription system since ATP is required for transcription itself. In the presence of 1.5 mM galactose, ATP at a concentration of 1 mM allows full relief of Gal80p repression by Gal3p (data not shown).

Gal80p mutants

Mutant versions of Gal80p respond appropriately in the *in vitro* transcription system. We investigated two mutants of Gal80p. The first, Gal80^{G152D}p, was isolated as a null allele of the *GAL80* gene (see Materials and methods). This protein will only repress Gal4p-mediated transcription when present at a concentration 20-fold greater than the wild-type protein (Figure 3A, compare lanes 3–5 and 6–8). The second Gal80p mutant has an uninducible phenotype. Gal80^{S-2}p (Glu351 converted to Lys) (Nogi and Fukasawa, 1984) is able to interact with, and repress

Fig. 4. Gal3p forms a complex with Gal4p-Gal80p in the presence of galactose and ATP. Electrophoretic mobility shift assay showing the formation of a Gal4p-Gal80p-Gal3p complex. Assays were performed as described previously (Reece and Ptashne, 1993) using a double-stranded oligonucleotide containing a single Gal4p recognition site. Reactions, containing as indicated Gal4p (150 nM), Gal80p (124 nM) and Gal3^{cp}p in the presence or absence of ATP (1 mM) and galactose (1%).

the transcriptional activity of, Gal4p indistinguishably from wild-type Gal80p (Figure 3A, compare lanes 3–5 and 9–11). Gal80^{S-2}p is, however, refractory to the effect of Gal3p (Figure 3B, lane 7) or Gal3^{cp}p (Figure 3B, lane 8).

Gal4p-Gal80p-Gal3p complex formation

How does Gal3p exert its effect on the Gal4p-Gal80p complex? We investigated this using an electrophoretic mobility shift assay (Figure 4). Gal4p forms a complex with DNA in this assay (Figure 4, lane 5) that is further retarded upon addition of Gal80p (Figure 4, lane 7). Gal3^{cp}p does not bind DNA itself (Figure 4, lane 2) and has no effect on a Gal4p-DNA complex (Figure 4, lane 6) but will interact with a Gal4p-Gal80p complex and retard its mobility further (Figure 4, lanes 8 and 9). Approximately the same amount of Gal3^{cp}p, relative to the concentration of Gal4p and Gal80p, is required to observe the formation of this complex as is needed to observe transcriptional activation of Gal4p-Gal80p (Figure 2). In the absence of either galactose or ATP, the efficiency of formation of the Gal4p-Gal80p-Gal3^{cp}p complex is diminished (Figure 4, lanes 10–12). The absence of either galactose or ATP reduces Gal4p-Gal80p-Gal3^{cp}p complex formation by 30–35% (Figure 4, lanes 10 and 11). In the absence of both galactose and ATP, formation of the tripartite complex is reduced by 50% (Figure 4, compare lane 9 with 12).

We have found that wild-type Gal3p precipitates when electrophoretic mobility shift assays are loaded onto polyacrylamide gels. We have therefore been unable to observe an interaction between wild-type Gal3p and the Gal4p-Gal80p complex. Attempts to identify suitable buffer conditions where this precipitation does not occur have been unsuccessful (data not shown). Gal3^{cp}p is able to promote activation of a Gal4p-Gal80p complex as efficiently as wild-type Gal3p (Figure 2) and therefore we believe that the Gal4p-Gal80p-Gal3p complex represents the induced state.

Fig. 5. Diagrammatic representation of galactose induction. In the absence of galactose, the transcriptional activity of Gal4p is inhibited by Gal80p. The association of galactose and ATP with Gal3p promote its interaction with the Gal4p–Gal80p complex. This interaction allows Gal4p to be freed from Gal80p inhibition and activate transcription.

Discussion

These data, together with previous results, suggest a model for the yeast galactose genetic switch (Figure 5). Gal4p is bound to DNA upstream of its target genes and, in the absence of galactose, its transcriptional activity is repressed by Gal80p. The binding of ATP and galactose to Gal3p promotes its association with Gal80p (Suzuki-Fujimoto *et al.*, 1996; Zenke *et al.*, 1996; Blank *et al.*, 1997; Yano and Fukasawa, 1997). We show that this interaction results in the activation of transcription. The association of Gal3p with Gal80p does not dissociate Gal80p from Gal4p, but rather results in the formation of a tripartite Gal4p–Gal80p–Gal3p complex. The fate of Gal80p upon induction of the Gal4p–Gal80p complex has been the focus of much debate. Genetic evidence suggests that the two proteins remain associated after induction (Leuther and Johnston, 1992), but the precise destination of Gal80p remains unresolved (Yano and Fukasawa, 1997). Our data suggest that, upon interaction with Gal3p, Gal80p undergoes a conformational change to release the activation domain of Gal4p such that it can interact with its targets within the transcriptional machinery. It is conceivable that Gal80p dissociates from Gal4p but is held in the complex by Gal3p. Such a model would necessitate direct interaction between Gal3p and Gal4p. We do not observe this interaction (Figure 4, lane 6), but it is possible that it only occurs in the presence of Gal80p, and our assays would not distinguish between these different complexes.

How does the transcriptional activation domain of Gal4p become available to the transcriptional machinery? Perhaps the simplest mechanism to explain the available data would be if Gal80p contacted Gal4p in, at least, two regions; one overlapping the activation domain (Wu *et al.*, 1996), and one elsewhere in Gal4p. Activation by Gal3p may release the point of contact overlapping the activation domain while not affecting the other interaction site. Presumably, the second interaction site is close to the activation domain itself since the version of Gal4p used

in these experiments (amino acids 1–93 fused to 768–881) is missing much of the full-length protein sequence.

Why is such a large molar excess of Gal3p required to observe activation of the Gal4p–Gal80p complex? We have found that a 30-fold molar excess of Gal3p is required to relieve the inhibitory effects of Gal80p. It is formally possible that much of the Gal3p we have purified is inactive. However, we do not believe this to be the case, since a weak interaction between Gal3p and Gal80p, judged by the efficiency of pull-down experiments from yeast extracts, has been observed in other experiments (Suzuki-Fujimoto *et al.*, 1996; Zenke *et al.*, 1996; Blank *et al.*, 1997; Yano and Fukasawa, 1997). Gal4p is known to be present in yeast cells at very low levels (Johnston, 1987), but the levels of Gal3p in relation to Gal4p have not been studied. Our *in vitro* data suggest that Gal3p must be present in a considerable excess over Gal4p levels for the induction process to occur. It is possible, therefore, that the yeast cell uses this low-affinity interaction to modulate its response to galactose. The intracellular levels of galactose may have to exceed a threshold, to saturate the available Gal3p, before activation of *GAL* gene expression occurs.

Gal1p is also capable of activating the Gal4p–Gal80p complex, although less efficiently than Gal3p (Figure 2). It is possible that the relatively low levels of induction we observe using Gal1p could be due to the metabolism of galactose. Induction by Gal1p can directly explain how induction of the *GAL* genes occurs in the absence of Gal3p (Bhat and Hopper, 1991). When yeast cells lacking Gal3p are placed into media which contains galactose as the sole carbon source, the *GAL* genes will be activated 4–5 days later. It is possible that leaky expression of *GAL1* will eventually build up sufficiently high levels of Gal1p to induce all of the *GAL* genes. With the exception of Gal3p, we have been unable to observe galactose-independent activation of Gal4p–Gal80p. *In vivo*, overproduction of either Gal3p or Gal1p gives rise to a constitutive *GAL* gene expression (Bhat and Hopper, 1992). We would anticipate that *in vitro* experiments performed with a higher ratio of Gal3p to Gal4p–Gal80p would yield galactose-independent expression.

Mapping the precise interactions between Gal3p and Gal80p, and the concomitant effects on Gal4p will be vital to understanding the molecular basis of the *GAL* genetic switch. We also need to understand the role of galactose and ATP in converting Gal3p into a protein able to interact with Gal80p. How does the binding of ATP and galactose to Gal3p allow the interaction with Gal80p? Now that we have been able to reconstitute the entire *GAL* genetic switch *in vitro*, experiments are in progress to elucidate the precise interactions and conformational changes that occur in this complex of proteins and small molecules during transcriptional induction.

Materials and methods

Media and strains

Escherichia coli strain DH5 α was used for all DNA manipulations. The following *Saccharomyces cerevisiae* strains were used: yeast nuclear extract was prepared from BJ2168 (MAT α *ura3 leu2 trp1 gal2 prb1 pep4 prc1*) grown in YPD media (Ohashi *et al.*, 1994); proteins were over-produced in strain MC2 (MAT α *trp1 ura3-52 leu2-3 prc1-407 prb1-*

112 *pep4-3*); genetic analysis was performed in strain JPY5 (MAT α *ura3-52 his3 Δ 200 leu2 Δ 1 trp1 Δ 63 lys2 Δ 385*).

Selection of a *GAL80* null mutation

JPY5 yeast strain deleted for endogenous *GAL80*, and harbouring a *GALI-lacZ* reporter gene was transformed with plasmids bearing mutated versions of the *GAL80* gene. Null alleles were isolated by searching for blue yeast colonies on plates containing 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside, supplemented with raffinose as the sole carbon source. *GAL80*^{G152D} is a double point mutation (converting Gly152 to Asp, and producing a silent mutation at amino acid Lys287). The encoded protein only weakly interacts with Gal4p using in electrophoretic mobility shift assays (data not shown).

Plasmids

Gal80p, Gal3p and Gal1p yeast expression vectors were constructed using the polymerase chain reaction (PCR; oligonucleotide sequences are available on request). Amplified DNA fragments were cloned into pYEX-BX (Clontech), such that the genes were under the control of the *CUP1* promoter. All plasmids were subjected to DNA sequencing to ensure that no mutations arose during the amplification process. Mutant versions of each protein were constructed by PCR-mediated site directed mutagenesis of the appropriate gene and insertion of the mutated product into pYEX-BX. Gal4p(1–93)+(768–881) was produced from the plasmid pRJR113 as described (Reece *et al.*, 1993).

Protein purification

Plasmids over-producing Gal80p, Gal3p or Gal1p were transformed into MC2 yeast cells and grown in minimal media containing 2% glucose, but lacking leucine. Cells (3 L) were grown at 30°C until an absorbance of 1.0 at 600 nm was reached. Protein production was induced by the addition of CuSO₄ to a final concentration of 0.5 mM, and cell growth continued for a further 23 h. Cells were harvested by centrifugation, washed in 40 ml of Buffer A (20 mM HEPES pH 8.0, 0.5 M NaCl, 1.4 mM β -mercaptoethanol), centrifuged again and resuspended in 60 ml of Buffer A containing 10% glycerol and Complete protease inhibitors (Boehringer Mannheim). The cells were broken open using a bead-beater (Biospec Products) and centrifuged at 27 000 *g* for 20 min. The supernatant was further diluted with 140 ml Buffer A containing 10% glycerol before application to a 2 ml Ni²⁺-NTA-agarose column (Qiagen) and washed with 20 column volumes of Buffer B (20 mM HEPES pH 8.0, 150 mM NaCl, 20% glycerol, 30 mM imidazole). Protein was eluted from the column using Buffer B containing 250 mM imidazole. Fractions were analysed by SDS-PAGE, pooled, and dialysed against Buffer C (20 mM HEPES pH 7.5, 100 mM potassium glutamate, 10 mM MgSO₄, 20% glycerol, 5 mM DTT). Each of the proteins were isolated with >95% purity. The purification of Gal4p(1–93)+(768–881) has been described previously (Reece *et al.*, 1993).

In vitro transcription

In vitro transcription reactions were performed as previously described (Wu *et al.*, 1996). Briefly, reactions (25 μ l) contained 10 mM HEPES (pH 7.5), 10 mM MgSO₄, 5 mM EGTA, 10% glycerol, 2.5 mM DTT, 100 mM potassium glutamate, 10 mM magnesium acetate, 2% polyvinylalcohol, 8 mM phosphoenolpyruvate, 0.31 nM of pG5E4 (Wu *et al.*, 1996), 4 nM pGEM3Z (Promega), 3 μ l yeast nuclear extract (60 mg/ml). Recombinant proteins were added together with, where appropriate, galactose (at the concentrations shown) and ATP (1.5 mM). The reactions were incubated for 10 min at 22°C. Nucleotide triphosphates were added (1 mM) and the reactions allowed to proceed for an additional 45 min at 22°C. Primer extension analysis of the RNA produced during these reactions was performed using an oligonucleotide complementary to the E4 coding sequence (5'-GCGGCAGCCTAACAGTCAGCCTTACCAGTA-3') (Lillie *et al.*, 1986; Lin *et al.*, 1988). The extension products were separated on a 10% polyacrylamide gel containing 1 $\times$ TBE and 5 M urea, and analysed by autoradiography. Phosphorimage analysis was performed on a Fuji BAS2000 phosphorimager to quantitate the major E4 extension product.

Mobility shift assay

The DNA probe used was a double-stranded (5'-TCCGGAGGACTGT-CCTCCGGT-3'), representing the consensus Gal4p binding (Wu *et al.*, 1996). Reactions mixes (10 μ l) contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 5 mM MgCl₂, 10 μ M ZnSO₄, 50 pM ³²P-labelled probe DNA, 100 μ g/ml BSA and protein at the indicated concentrations. Reactions were incubated at 4°C for 20 min prior to loading onto a pre-electrophoresed 6% (w/v) polyacrylamide gel con-

taining 45 mM tris base, 45 mM boric acid, 1 mM EDTA, 1% (w/v) glycerol. Electrophoresis was carried out at 20 V/cm for 2 h, and the gels were subjected to autoradiography.

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