

Gal80–Gal80 interaction on adjacent Gal4p binding sites is required for complete *GAL* gene repression

Karsten Melcher^{1,2,3} and H.Eric Xu^{1,4}

¹Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8573, USA

²Present address: Institute of Mikrobiologie, J.W.Goethe-Universität Frankfurt, Marie-Curie Straße 9, N250, D-60439 Frankfurt, Germany

⁴Present address: GlaxoWellcome Inc., V213, 5 Moore Drive, Research Triangle Park, NC 27709, USA

³Corresponding author
e-mail: K.Melcher@em.uni-frankfurt.de

Regulation of the *GAL* genes of *Saccharomyces cerevisiae* is determined by the interplay of the transcriptional activator Gal4p and the repressor Gal80p, which binds and masks the activation domain of Gal4p under non-inducing conditions. Here we demonstrate that Gal80p dimerizes with high affinity and that this dimerization appears to stabilize the Gal4p–Gal80p interaction and also, indirectly, the Gal4p–DNA interaction in a (Gal4p)₂(Gal80p)₂DNA complex. In addition, Gal80 dimers transiently interact with each other to form higher order multimers. We provide evidence that adjacent Gal4p binding sites, when correctly spaced, greatly stabilize Gal80p dimer–dimer interactions and that this stabilization results in the complete repression of *GAL* genes with multiple Gal4p binding sites. In contrast, *GAL* genes under the control of a single Gal4p binding site do not stabilize Gal80p multimers, resulting in significant and biologically important transcriptional leakage. Cooperative binding experiments indicate that Gal80p dimer–dimer interaction probably does not lead to a stronger Gal4p–Gal80p interaction, but most likely to a more complete shielding of the Gal4p activation domain.

Keywords: *GAL4/GAL80/Saccharomyces cerevisiae* transcriptional repression

Introduction

Expression of the galactose catabolic genes of the yeast *Saccharomyces cerevisiae* is determined by the carbon source due to the action of three regulatory proteins: the transcriptional activator Gal4p, the repressor Gal80p and the signal transducer Gal3p (for reviews see Johnston and Carlson, 1992; Melcher, 1997). In the absence of glucose, the transcriptional activator Gal4p binds constitutively to 17 bp recognition sequences upstream of the *GAL* genes. While binding of Gal4p to DNA is necessary for transcriptional activation, it is not sufficient. Rather, Gal4p forms a tight poised complex with the repressor Gal80p. The repressor binds to a 28 amino acid region at

the very C-terminus of Gal4p, which coincides with Gal4p's core activation domain and thereby physically blocks interactions of the activation domain with the transcriptional machinery (e.g. Wu *et al.*, 1996; Koh *et al.*, 1998).

Upon induction by galactose, the inhibitory effect of Gal80p is relieved by the action of the signal transducer Gal3p. Gal3p is a homologue of the galactokinase Gal1p, and in the presence of the galactokinase substrates, galactose and ATP, Gal3p physically binds Gal80p. This interaction is believed to cause a conformational change in Gal80p, leading to a release of Gal80p masking of the Gal4p activation domain (Zenke *et al.*, 1996; Yano and Fukasawa, 1997; Platt and Reece, 1998).

There are two types of *GAL* gene: the catabolic genes *GAL1*, *-2*, *-7* and *-10*, which are extremely tightly regulated with essentially no expression in the absence of galactose, and the *GAL3*, *GAL80* and *MEL1* genes, which have significant and biologically important basal expression. The physiological role for the two types of uninduced states, which arose from a gene duplication, is particularly well illustrated for the *GAL1* and *GAL3* genes. The enzymatic and regulatory functions were separated into the catalytically active protein Gal1, whose production needs to be tightly regulated to avoid accumulation of toxic intermediates (Meyer *et al.*, 1991), and into the catalytically inactive regulator Gal3p. Gal3p, as well as the repressor Gal80p, needs to be already expressed at significant levels in the absence of galactose to keep the system both repressed and inducible. Induction of *GAL3* and *GAL80* is necessary to reach and maintain full induction of the *GAL* genes and to allow rapid stalling of transcription in the fully induced state. A third example of a *GAL* gene with critical basal expression is the melibiase (α -galactosidase)-encoding *MEL1* gene. Mel1p is a secreted enzyme necessary for extracellular breaking down of melibiose into galactose and glucose. Galactose enters the cell and triggers induction of the *GAL* genes, including *MEL1*, via the Gal3p/Gal80p/Gal4p pathway.

Bram *et al.* (1986) observed that *GAL* genes with basal expression are driven by a single Gal4p binding site, while the tightly regulated *GAL* genes are driven by multiple Gal4p sites. Moreover, duplication of an artificial consensus Gal4p binding site in the context of the *GAL2* promoter led to decreased uninduced mRNA production, suggesting that binding site architecture modulates the level of basal *GAL* gene expression. Using a combination of *in vivo* and quantitative *in vitro* analyses, we confirmed these observations and investigated the molecular mechanism of this unusual regulation. Our results indicate that correctly spaced Gal4p binding sites stabilize otherwise transient higher order Gal80p multimers and that these multimers are probably necessary for complete shielding of the Gal4p activation domain.

Table I. Basal expression of *GAL* genes and synthetic reporters depends on the number of Gal4p binding sites

	gly/lac (U)	gly/lac + gal (U)	% basal (corrected) ^a
A. Natural promoters			
<i>GALI-lacZ</i> (four sites)	≤1	3000	<0.1
<i>MELI</i> (one site)	16	880	1.8
B. Inserted into ΔUAS- <i>CYC1-lacZ</i> reporter pJlb (Finley <i>et al.</i> , 1990)			
1× 17mer <i>MELI</i>	84	570	12
2× 17mer <i>MELI</i>	14	2300	0.2
Four sites of <i>GALI</i>	43	5000	0.7
Vector alone	10	10	–

gly/lac: 3% glycerol, 2% lactic acid; gly/lac + gal: 3% glycerol, 2% lactic acid, 2% galactose.

Integrated *Gal1-lacZ* and the *CYC1-lacZ* reporter: units β-galactosidase activity.

MELI: units α-galactosidase activity.

^a[U(gly/lac) – U(gly/lac)_{vector}]:[U(gly/lac + gal) – U(gly/lac + gal)_{vector}]

Standard deviations were ≤20%.

Table II. Basal expression of *MELI* is completely dependent on Gal4p and is due to incomplete repression by Gal80p

Genotype	Gly/lac (U)	gly/lac + gal (U)
<i>GAL4 Gal80</i>	16	880
<i>Δgal4 GAL80</i>	≤1	≤1
<i>GAL4 mcGAL80</i>	1	180

mcGAL80: multicopy *GAL80* plasmid; U: units of α-galactosidase activity.

Standard deviations were ≤20%.

Results

Basal expression of *GAL* genes and synthetic reporters depends on the number of Gal4p binding sites

Table I recapitulates the basal elements of *GAL* regulation. The *GALI* promoter is driven by four Gal4p binding sites, and the *MELI* promoter by a single Gal4p binding site. Expression from a *GALI* promoter-*lacZ* reporter integrated at the chromosomal *GALI* locus is extremely tightly regulated with no detectable basal expression (<0.1%), while basal expression from the endogenous *MELI* (α-galactosidase) gene is readily detectable under non-inducing conditions (1.8% of induced expression).

To confirm that basal expression of *MELI* is determined solely by the number of Gal4p binding sites and not by their particular context, one or two Gal4p binding sites from *MELI* were cloned into an upstream activating sequence (UAS)-less *CYC1-lacZ* reporter. As expected, the reporter gene driven by a single Gal4p binding site has higher basal activity than the one driven by multiple binding sites. Note that the reporter plasmids give high basal activity relative to the endogenous genes and therefore only follow the same trend approximately. The data argue that basal expression is determined by the number of Gal4p binding sites and not by the particular type of Gal4p binding site or by promoter structural context.

Basal expression of *Mel1p* is Gal4p dependent and due to incomplete repression by Gal80p

If basal expression of *MELI* is indeed independent of promoter context, we would expect it to be completely

dependent on Gal4p and not on some other unidentified element. In agreement with a previous report (Post-Beittenmiller *et al.*, 1984), this has been confirmed in Table II. We reasoned that Gal4p-dependent basal expression may be due to incomplete repression by Gal80p. Overexpression of *GAL80* may then partially compensate for weakened repression. Indeed, overexpression of *GAL80* from a multicopy 2μ plasmid strongly reduced basal *MELI* expression (Table II). Overexpression of *GAL80* also led to reduced *MELI* expression under inducing conditions, as expected, since induction functions by shifting the equilibrium of the interaction between Gal80p and the Gal4p activation domain (Sil *et al.*, 1999). The decrease in basal *Mel1* activity upon moderate overexpression of *GAL80* argues that basal expression is at least partially due to incomplete repression by Gal80p.

Gal80p and Gal4p form a complex with 2:2 stoichiometry

To explore the molecular basis of differential repression, we first determined the kinetic parameters of Gal4p–DNA, Gal4p–Gal80p and Gal80p–Gal80p interactions. We purified from *Escherichia coli* full-length Gal80p as well as Gal4p(1–147+34), and carefully determined their concentrations. Gal4p(1–147+34) is a truncated version of Gal4p in which the N-terminal 147 amino acids of Gal4p, which include the DNA binding/dimerization domain, were fused to a 34 amino acid peptide (amino acids 841–875) corresponding to the Gal80p interaction/transcriptional activation site. We first determined the DNA binding activity of Gal4p(1–147+34) by titrating a known amount of protein (15 nM) above the dissociation constant (K_D) (see below) with increasing amounts of a radiolabelled consensus Gal4p binding site oligonucleotide (Figure 1A). The total DNA binding capacity was 14–15 nM, indicating that Gal4p(1–147+34) was >90% active in specific DNA binding. We determined a K_D of 1.3 nM (Figure 1B), in good agreement with published results for both the truncated Gal4p DNA binding domain (Reece and Ptashne, 1993; Vashee *et al.*, 1993) and for full-length Gal4p (Parthun and Jaehning, 1990). However, the affinity of Gal4p(1–147+34) for the same consensus binding site in a longer fragment (108 bp as opposed to a 23 bp

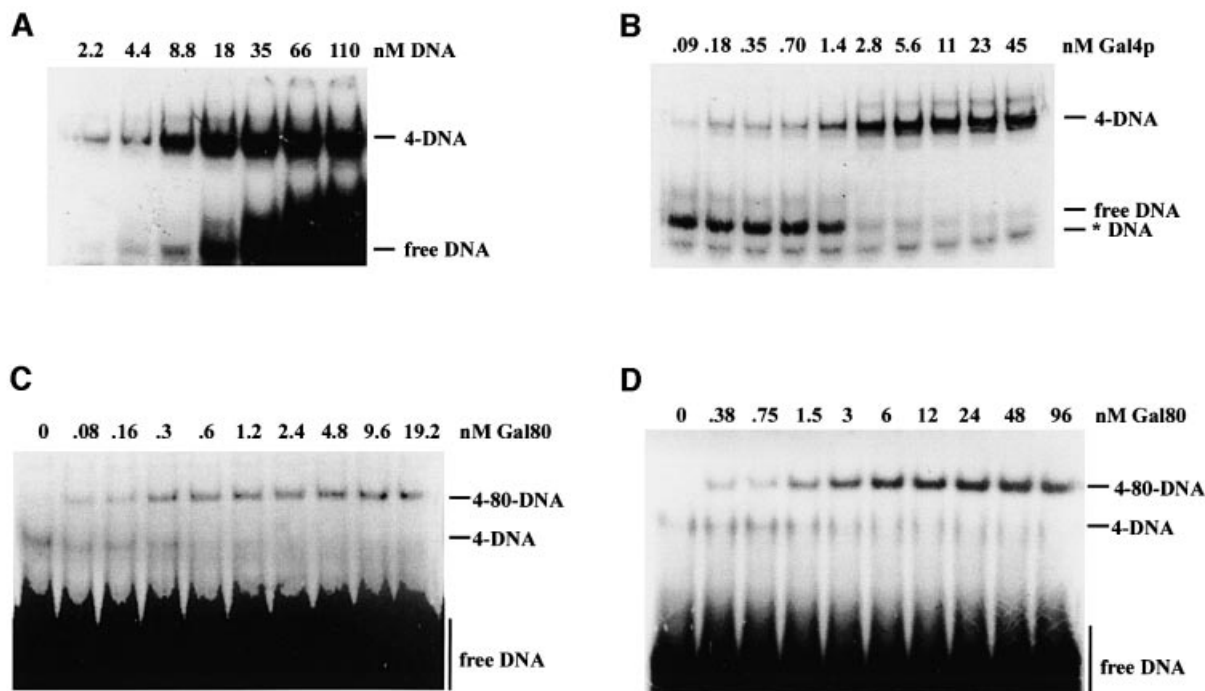


Fig. 1. Kinetic determinations of interactions between DNA, Gal4p(1-147+34) and Gal80p. Binding reactions were separated by native PAGE and subjected to autoradiography. Free and bound complexes were quantitated by PhosphorImager densitometry (Molecular Dynamics). DNA: radiolabelled consensus Gal4p binding site oligonucleotide; 4 and Gal4p: Gal4p(1-147+34); 80: Gal80p; *DNA: radiolabelled unannealed oligonucleotide. (A) Quantitative measurement of the DNA binding capacity of Gal4p(1-147+34). Fifteen nanomoles of Gal4p(1-147+34) dimers were incubated with increasing amounts (nM DNA) of the binding site oligonucleotide. The maximum binding capacity B_{\max} and the apparent dissociation constant K_{app} were calculated according to $[B] = B_{\max} \times [F]/(K_{\text{app}} + [F])$, with $[B]$ and $[F]$ representing the experimentally determined concentrations of bound and free DNA, respectively. (B) Determination of the K_{app} of Gal4p(1-147+34) to the consensus site oligonucleotide. Labelled duplex oligonucleotide (0.4 nM) was incubated with increasing amounts of Gal4p(1-147+34). (C) Determination of the K_{app} of Gal80p to a Gal4p(1-147+34)-DNA complex. Gal4p(1-147+34) (90 pM) was incubated with 20 nM radiolabelled oligonucleotide in the presence of increasing amounts of Gal80p. (D) Gal80p stabilizes a Gal4p(1-147+34)-DNA complex in EMSA. Gal4p(1-147+34) at a concentration above the K_D (5.7 nM) was incubated with excess DNA and increasing amounts of Gal80p. To accentuate the stabilization, electrophoresis was started after loading of samples, rather than loading reactions onto running gels as for the K_D determinations.

oligonucleotide) was ~10 times higher (see Figure 5), suggesting that Gal4p, similar to many restriction endonucleases (Moreira and Noren, 1995), prefers a more rigid DNA structure for optimal binding, and indicating that Gal4p DNA binding affinity may have previously been underestimated.

To determine the affinity of the Gal4p-Gal80p interaction, we first saturated a limiting amount of Gal4p(1-147+34) (0.09 nM) with a large excess of the radiolabelled consensus Gal4p binding site oligonucleotide (20 nM) to drive this very small amount of Gal4p(1-147+34) into a complex with DNA completely. By titrating this complex with increasing amounts of Gal80p, we determined a K_D of 0.3 nM (Figure 1C). We determined a binding capacity of >90% for the Gal80p preparation by titrating a saturating amount of Gal4p(1-147+34)-DNA complex (15 nM) with increasing amounts of Gal80p. Binding was stoichiometric, indicating that two Gal80 molecules bind one Gal4p dimer complexed with DNA.

Gal80p dimerizes with high affinity

Surprisingly, we found that the repressor Gal80p actually stabilized the binding of Gal4p to DNA (Figure 1D). One mechanism by which Gal80p could affect Gal4p-DNA complex formation would be by stabilization of Gal4p

dimers. Gal4p binds DNA exclusively as a dimer (Carey *et al.*, 1989), and it is likely that the combination of Gal4p-Gal4p and Gal4p-DNA interactions is important for a low off-rate. If Gal80p would form stable dimers, then Gal80p dimers could stabilize Gal4p dimers, and thereby Gal4p-DNA complexes. In gel filtration experiments, however, Gal80p appeared to migrate as a monomer (Yun *et al.*, 1991). In order to probe for Gal80p-Gal80p interactions using a more sensitive method, we *in vitro* translated a radiolabelled fusion protein consisting of Gal80p and the 78 amino acid activation domain of herpes simplex VP16 (Leuther and Johnston, 1992). Due to the high negative net charge of the VP16 activation domain, Gal80pVP16 migrated significantly faster than Gal80p in a native polyacrylamide gel. Co-translated Gal80p and Gal80pVP16 migrate on a native polyacrylamide gel as three distinct complexes (Figure 2A) (Leuther, 1993). One complex corresponded to Gal80p, one to Gal80pVP16, and the more abundant complex to a Gal80p-Gal80pVP16 heteromer. The formation of only one additional complex as well as the 1:2:1 stoichiometry of the complexes indicates that the heteromeric complex is a dimer. This stoichiometry represents the statistical distribution of all equally probable dimerizations (Gal80-Gal80, Gal80-Gal80VP16, Gal80VP16-Gal80 and Gal80VP16-Gal80VP16). We conclude that

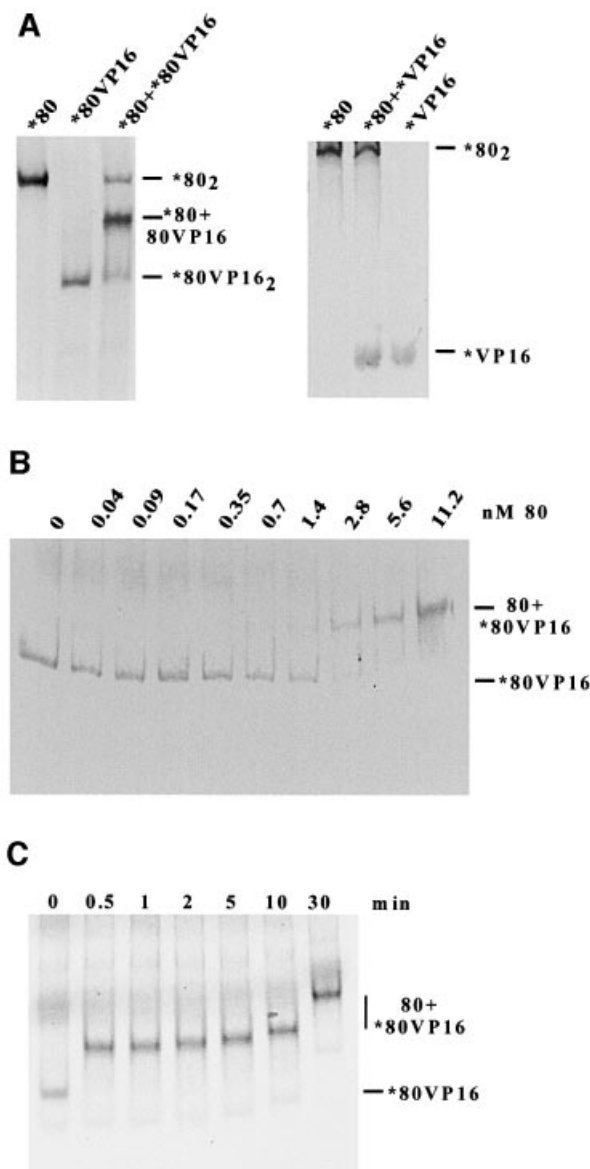


Fig. 2. Gal80p dimerizes *in vitro*. (A) Gal80p (80) and a fusion between Gal80p and the acidic activation domain of herpes simplex virus VP16 (80VP16) were either translated separately or co-translated *in vitro* in the presence of ³⁵S-labelled methionine. Translations (1 μ l, ~1 ng) in the absence of DNA were separated by native PAGE and subjected to autoradiography. Homodimeric proteins and the heterodimeric complex are indicated on the right. As a control for the specificity of complex formation, co-translated Gal80p and VP16 activation domain alone (VP16) were also tested under the same conditions as above for interaction (right panel). (B) Determination of the K_{app} of a Gal80p–Gal80pVP16 complex. Labelled, *in vitro* translated Gal80pVP16 (*80VP16; ~50 pM) was incubated with increasing amounts of unlabelled recombinant Gal80p (80). (C) Time course of the dissociation of Gal80pVP16 dimers. Approximately 1 nM labelled, *in vitro* translated Gal80pVP16 (*80VP16) was incubated with a large molar excess (120 nM) of unlabelled recombinant Gal80p (80). Reactions were incubated for the indicated amount of time before they were loaded onto a running native polyacrylamide gel (note, therefore, the relatively shorter migration of the 80+*80VP16 complex loaded 30 min after the start of electrophoresis). The t_0 time point represents the reaction before the addition of unlabelled Gal80p.

Gal80p is dimeric at the concentration of the *in vitro* translated proteins in the reaction and that it can form a heterodimer with Gal80pVP16. No additional complex is formed when Gal80 is co-translated with the VP16

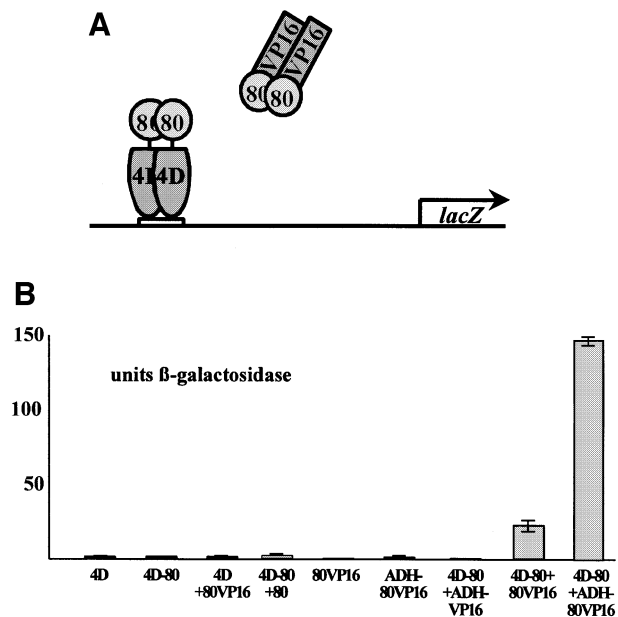


Fig. 3. Gal80p-modified two-hybrid assay. (A) Outline of the assay. A fusion protein in which Gal80p replaces the activation domain of Gal4p activates transcription in the presence of a Gal80pVP16 hybrid (see text for details). (B) Specific reporter gene activities in different transformants. Constructs were either integrated or transformed on single-copy plasmids. 4D: Gal4p(1–841); 4D-80: fusion between Gal4p(1–841) and Gal80p; 80VP16: fusion between Gal80p and the activation domain of VP16; 80: Gal80p; ADH-80VP16: Gal80pVP16 expressed from the *ADHI* promoter; ADH-VP16: His₆-tagged VP16 activation domain expressed from the *ADHI* promoter. When not indicated otherwise, proteins were expressed from the natural *GAL4* and *GAL80* promoters.

activation domain alone (Figure 2A), indicating that Gal80p and VP16 do not interact directly with each other.

We titrated radiolabelled Gal80pVP16 with increasing amounts of unlabelled recombinant Gal80p to determine an apparent dissociation constant (K_{app}) of 2×10^{-9} M by native polyacrylamide gel electrophoresis (PAGE; Figure 2B). We also titrated a minimal amount of radiolabelled Gal80p with increasing amounts of unlabelled recombinant Gal80p to follow the transition from a faster migrating monomeric form into a slower migrating dimeric form by native PAGE. Signals were weak and somewhat diffused due to the very low protein concentration and total radioactivity, but we estimate a K_D for Gal80p homodimerization of $1\text{--}3 \times 10^{-10}$ M (data not shown). Therefore, Gal80p may homodimerize with ~10-fold higher affinity than required to form heterodimers with Gal80pVP16 under equilibrium conditions.

To reconcile the apparent instability of Gal80p dimers during gel filtration with the very low K_D , we determined the half-life of the dimer (Figure 2C). *In vitro* translated, ³⁵S-labelled Gal80pVP16 was incubated with a large molar excess of unlabelled recombinant Gal80p and reactions were loaded onto a running native polyacrylamide gel. As seen on the autoradiogram in Figure 2C, even only 0.5 min after addition of unlabelled Gal80p, essentially all Gal80pVP16 migrated to a position expected for the Gal80p–Gal80pVP16 heterodimer. We note that the stability of the Gal80pVP16 dimer does not necessarily have to reflect the stability of Gal80p dimers. Nevertheless, our results together with the migration of

Gal80p on a gel filtration chromatogram (Yun *et al.*, 1991) strongly indicate that Gal80p dimerizes with high affinity, but that dimers have an unusually high off-rate when not bound to Gal4p. In contrast, Gal80 dimers are significantly stabilized by Gal4p dimers and have a long half-life when bound to Gal4p–DNA complexes (see below). In summary, a series of strong protein–protein and protein–DNA interactions appears to stabilize all components of a DNA–(Gal4p)₂–(Gal80p)₂ complex.

Gal80p–Gal80p interaction *in vivo*

We employed a modified two-hybrid system to probe *in vivo* for Gal80p self-association. We expressed Gal4p(1–841)–Gal80p in a strain deleted for the wild-type *GAL4* and *GAL80* genes. In this hybrid, the 34 amino acid activation/Gal80p interaction domain of Gal4p is deleted and replaced by Gal80p directly fused to the truncated protein. Neither Gal4p(1–841)–Gal80p nor Gal80pVP16 alone produced significant β -galactosidase activity from an integrated *GAL1* promoter–*lacZ* reporter (Figure 3A and B). Co-expression of Gal4p(1–841)–Gal80p and Gal80pVP16, however, produced 33 U of β -galactosidase activity, clearly demonstrating that Gal80p also self-associates *in vivo*. Unlike typical two-hybrid systems, fusion proteins were expressed in single copy from the natural *GAL4* and *GAL80* promoters, respectively, indicating that the interaction occurs under normal physiological conditions.

Gal4p binds DNA exclusively as dimers (Carey *et al.*, 1989). If DNA-bound Gal4p(1–841)–Gal80p represents a stable dimer as well, then the two-hybrid interaction would point to a Gal80p dimer–dimer interaction. In support of this interpretation, overexpression of integrated Gal80pVP16 from an *ADHI* promoter directed significantly higher β -galactosidase activity (Figure 3B). This indicates that the intracellular concentration of the fusion proteins expressed from their own promoters is below the K_D of the two-hybrid interaction, consistent with a relatively weak affinity rather than the high affinity Gal80p monomer–monomer interaction. Co-expression of the VP16 activation domain alone with Gal4p(1–841)–Gal80p did not yield detectable β -galactosidase activity (Figure 3B).

Gal80p dimers transiently associate with each other

Complete repression by Gal80p on multiple Gal4p sites versus leaky repression on a single site suggest a cooperative repression by Gal80p. Cooperative effects are often mediated by direct protein–protein interactions, such as between a Gal80p dimer assembled on one binding site and a component, most likely Gal80p again, associated with the other binding site. While the two-hybrid interaction pointed to a weak Gal80p dimer–dimer interaction, we could detect no higher order Gal80p complexes stable to native electrophoresis (Figures 1C and D and 2A). Therefore, if Gal80 dimers do associate with each other, the interaction must be relatively transient.

To detect potentially transient Gal80p dimer–dimer interactions physically, we used a novel chemical crosslinking technique (Brown *et al.*, 1995). The reagent employed is a high valency peptide–nickel complex activated by a peracid, because this agent allows efficient

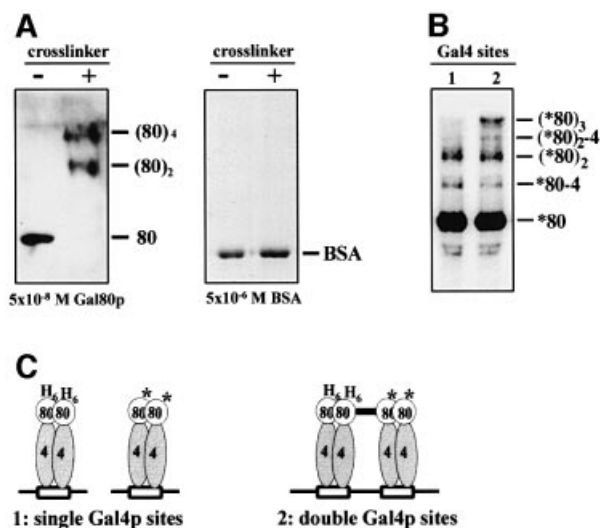


Fig. 4. Gal80p multimerization is stabilized by adjacent Gal4p binding sites. 80/(80)₂/(80)₃/(80)₄: Gal80p monomer/dimer/trimer/tetramer; 80–4/(80)₂–4: Gal80p–Gal4p(1–147+34) heterodimer/heterotrimer; asterisk: radioactively labelled. Compositions of complexes are indicated and are based on molecular weight standards, immunoblots and on control titrations using different concentrations of Gal80p and different crosslinking efficiencies. (A) Gal80p tetramerizes in solution. Gal80p (5×10^{-8} M) or, as a control, BSA (5×10^{-6} M) was incubated for 2 min in the presence or absence of crosslinking reagent (crosslinker). Reactions were separated by SDS–PAGE. Gal80p complexes were visualized by immunoblotting, and BSA by Coomassie Blue staining. (B) Reactions with labelled Gal80p as outlined in (C) were separated by SDS–PAGE and subjected to autoradiography. Asterisks indicate labelled Gal80p and Gal80p–His₆Gal80p complexes. Note that with the low crosslinking efficiency in (C), more assembled tetramers are crosslinked to trimers than to tetramers. (C) Schematic outline of the experiment in (B). Gal4p and Gal4p–His₆Gal80p complexes were allowed to assemble on single or double Gal4p binding sites. Reactions were briefly incubated with labelled Gal80p (*80) and crosslinking was initiated. The thick bar indicates a crosslink between His₆–80 and *80.

and rapid formation of essentially 0 Å crosslinks. A 2 min incubation of 5×10^{-8} M Gal80p with these crosslinking reagents resulted in essentially 100% dimer formation, as detected by SDS–PAGE (Figure 4A). The same result was obtained with Gal80p purified from yeast (data not shown). As a control for the specificity of crosslinking, the reaction was also performed with acetylated bovine serum albumin (BSA), which is strictly monomeric in solution. As shown in the right panel of Figure 4A, incubation of acetylated BSA, even at a 100-fold higher concentration (5×10^{-6} M) than Gal80p (5×10^{-8} M), with the crosslinking reagents did not yield any detectable unspecific complexes. Incubation with a completely different crosslinker, 0.01% glutardialdehyde, led to the formation of dimeric, trimeric and tetrameric Gal80p complexes, and still exclusively monomeric BSA (data not shown).

Given the essentially 100% crosslinking efficiency of the peptide–nickel reagent under these conditions, the lack of monomeric Gal80p at a concentration of 5×10^{-8} M is consistent with our K_D determination for Gal80p dimerization, which predicts that at this concentration, <1% of Gal80p is monomeric. Almost half of the dimers were covalently connected to tetramers, suggesting that the K_D

for the Gal80p dimer–dimer interaction is in the range of 5×10^{-8} M. No trimers were detectable, consistent with all higher order complexes arising from covalently cross-linked dimers (Figure 4A). With lower crosslinking efficiency (data not shown and Figure 4B), monomers and trimers were also visible. We conclude that Gal80p dimerizes with high affinity, and in solution it transiently multimerizes with moderate affinity.

Gal80 dimer–dimer interaction is stabilized by adjacent Gal4p binding sites

If locking into a completely repressed form is due to Gal80p dimer–dimer interactions, then this interaction should be weak or transient for a complex assembled on a single Gal4p binding site, but stabilized by the vicinity of two complexes assembled on adjacent Gal4p binding sites. In order to address this important prediction, we performed the experiment outlined in Figure 4C. We constructed and purified two variants of Gal80p, one of which has a His₆ tag at its N-terminus (His₆-Gal80p) and the other with a five-amino-acid heart muscle kinase (HMK) site for radiolabelling (Blanan and Rutter, 1992) introduced at its C-terminus (Gal80p–HMK). We then incubated Gal4p(1–147+34) dimers with an equimolar amount of consensus Gal4p binding sites and an approximately half-equimolar amount of His₆-Gal80p. In one reaction, oligonucleotides with single Gal4p binding sites were used, and in the other reaction, DNA fragments with the same molar amounts of binding sites, but with two adjacent binding sites on each fragment, were used. Complexes were allowed to assemble and assembly reactions were monitored in parallel reactions by shift-westerns. Assembled complexes were then briefly incubated with ³²P-labelled Gal80p–HMK and crosslinking was initiated. Reactions were separated by SDS–PAGE and subjected to autoradiography. Figure 4B shows that formation of, and crosslinking to, Gal80p dimers was, as expected, approximately equally efficient in the 1× and 2× binding site reactions. In contrast, efficient crosslinking to trimers and to traces of tetramers was dependent on adjacent Gal4p binding sites, consistent with the prediction that the Gal80p dimer–dimer interaction needs to be stabilized by the close proximity of Gal80p dimers assembled on adjacent Gal4p binding sites. Note that with the low crosslinking efficiency under these conditions, more assembled tetramers were crosslinked to trimers than to tetramers, and only a fraction of complexes were crosslinked to Gal4p(1–147+34) as well.

In addition, His₆-Gal80p with covalently linked proteins was purified on Ni–agarose beads under denaturing conditions. Direct measurement of Cerenkov radiation confirmed that co-purification of radioactivity depended on crosslinking and that significantly more radioactivity co-purified with His₆-Gal80p in the 2× than in the 1× binding site reaction (data not shown). Incubation times for radiolabelled Gal80p–HMK with pre-assembled complexes 2–14 min before crosslinking yielded essentially indistinguishable products (data not shown), indicating that Gal80p dimers complexed with DNA-bound Gal4p(1–147+34) dimers are highly stable with little if any exchange during the incubation period.

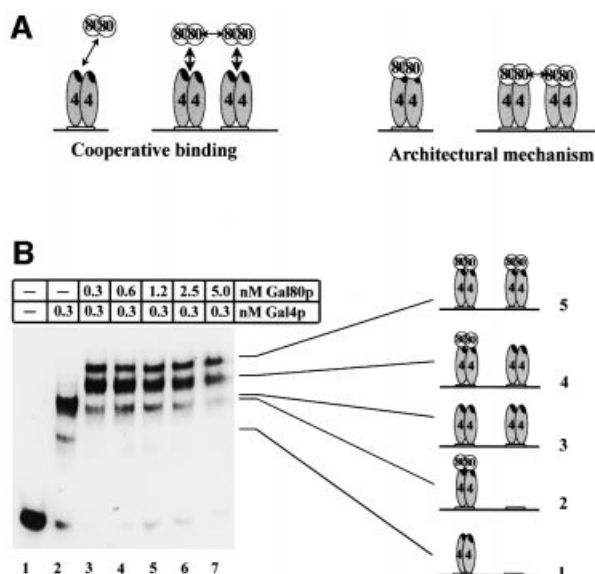


Fig. 5. Gal80p dimers bind non-cooperatively adjacent Gal4p dimers. (A) Cartoon depicting quantitative and qualitative mechanisms on how the Gal80p dimer–dimer interaction could modulate repression. 4: Gal4p; 80: Gal80p; the black oval within each Gal4p monomer represents the activation/Gal80p interaction domain. (B) Gal4p(1–147+34) (0.3 nM) was incubated with a substoichiometric amount of a ³²P-labelled, gel-isolated, 108 bp restriction fragment containing two Gal4p binding sites, spaced 10 bp apart, and with increasing amounts of Gal80p. Reactions were separated by native electrophoresis and subjected to autoradiography. Note that with higher concentrations of Gal80p, total visible labelling decreases, presumably because higher order Gal80p complexes are unstable under native electrophoresis. The five complexes formed are presented as cartoons next to the bands of the autoradiogram.



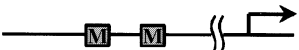







Complete repression depends on the spacing of Gal4p binding sites

Multiple Gal4p binding sites in all constructs used so far were separated 10 bp from each other. We chose this distance to position adjacent Gal4p–Gal80p complexes on the same side of the helix (one helical turn equals ~10.4 bp in B-form DNA). We noticed that all natural adjacent Gal4p binding sites are either 1, 2 or >47 bp apart, i.e. they are either close enough to contact each other directly on the same side of the DNA or are sufficiently far away from each other to allow looping or bending of the intervening DNA.

Spacing of Gal4p binding sites by one half-helical turn (6 bp) would position Gal80p dimers on opposite sides of a rigid helix, the least favourable position for protein–protein interactions. If complete repression depends on direct binding of adjacent Gal80p dimers, then a 6 bp spacing between Gal4p binding sites would be expected to eliminate or at least reduce dimer–dimer interactions and thereby complete repression. If, on the other hand, complete repression were a consequence of interactions of the transcriptional machinery with either the repressor or the activator, then the positioning of Gal4p binding sites towards each other should have little, if any, effect.

We inserted either strong consensus Gal4p binding sites or the weak Gal4p binding site from the *MEL1* promoter into the single copy reporter plasmid pMEL-β (Melcher *et al.*, 2000). pMEL-β contains a UAS-less *MEL1* promoter fused to *lacZ*. Introduced binding sites replace the weak natural Gal4p binding site of the *MEL1* promoter, i.e. are

Table III. Basal expression depends on the spacing of Gal4p binding sites

Arrangement and spacing of Gal4p binding sites	Units α -galactosidase activity		% basal activity (corrected for vector)
	Gly/lac	Gal + gly/lac	
vector only	99 \pm 7	30 \pm 4	—
 -	108 \pm 7	202 \pm 11	(5.2%)*
 1bp	99 \pm 11	663 \pm 36	<0.1%
 10bp	93 \pm 6	819 \pm 28	<0.1%
 10bp - 6bp	312 \pm 30	1507 \pm 167	14.4%
 10bp - 10bp	107 \pm 13	1917 \pm 86	0.4%
 -	157 \pm 13	1085 \pm 21	5.5%
 6bp	494 \pm 101	2141 \pm 96	18.7%
 10bp	91 \pm 13	2192 \pm 286	<0.1%
 6bp - 6bp	536 \pm 16	2120 \pm 135	25.3%
 10bp - 10bp	70 \pm 21	1908 \pm 241	<0.1%

*Because of low total activity, the value cannot be calculated exactly.

M: Gal4p binding site of *MEL1* promoter; C: consensus Gal4p binding site.

in the same context and distance from the transcriptional initiation site as the natural site. As expected and shown in Table III, transcriptional activation as measured by β -galactosidase activity in galactose grown cells does not depend on the spacing of high affinity consensus Gal4p binding sites towards each other. For instance, two consensus Gal4p binding sites spaced 6 bp apart direct 2141 U of β -galactosidase activity, while the same two sites spaced 10 bp apart direct 2192 U. In contrast, complete repression strictly depended on the spacing of adjacent Gal4p binding sites with respect to each other. Binding sites spaced 1 or 10 bp apart had no detectable basal activity (<0.1%), while the same sites separated by 6 bp directed high basal activity (>5%), independent of the type and number of Gal4p binding sites. We conclude that full repression is abolished when adjacent Gal4p binding sites are on opposite sides of a rigid helix, strongly implying that full repression depends on the direct interaction between adjacent repressor complexes.

Mechanism of differential repression

Gal80p binds non-cooperatively to adjacent Gal4p–DNA complexes. The repressor–repressor interaction could affect the Gal4p–Gal80p interaction either quantitatively

(i.e. increases the strength of the Gal4p–Gal80p binding; ‘cooperative binding’ in Figure 5A) or qualitatively (i.e. changes the nature of the Gal4–Gal80 interaction; ‘architectural mechanism’ in Figure 5A). In the first case, a weakened binding could lead to leaky repression by partial dissociation of the complex between Gal80p and the Gal4p activation domain. Although attractive, this model would imply that the Gal80p dimer–dimer interaction has to be kinetically sufficiently stable to allow stabilization of the Gal4p–Gal80p interaction, for which we determined a K_D of 3×10^{-10} M. To address this issue experimentally, cooperative binding experiments were performed.

We incubated a radiolabelled restriction fragment with two consensus Gal4p binding sites, separated by 10 bp, with 0.3 nM recombinant Gal4p(1–147+34) and increasing concentrations of Gal80p (Figure 5B). The composition of retarded complexes on a native gel was unambiguously determined by titration with different amounts of Gal4p and Gal80p, and by anti-Gal4p and anti-Gal80p antibody supershifts (data not shown). If the Gal80p dimer–dimer interaction would stabilize the Gal4p–Gal80p interaction, then there should be a fast transition from neither of the two Gal4p dimers being occupied by Gal80p (complex 3 in Figure 5B) to both

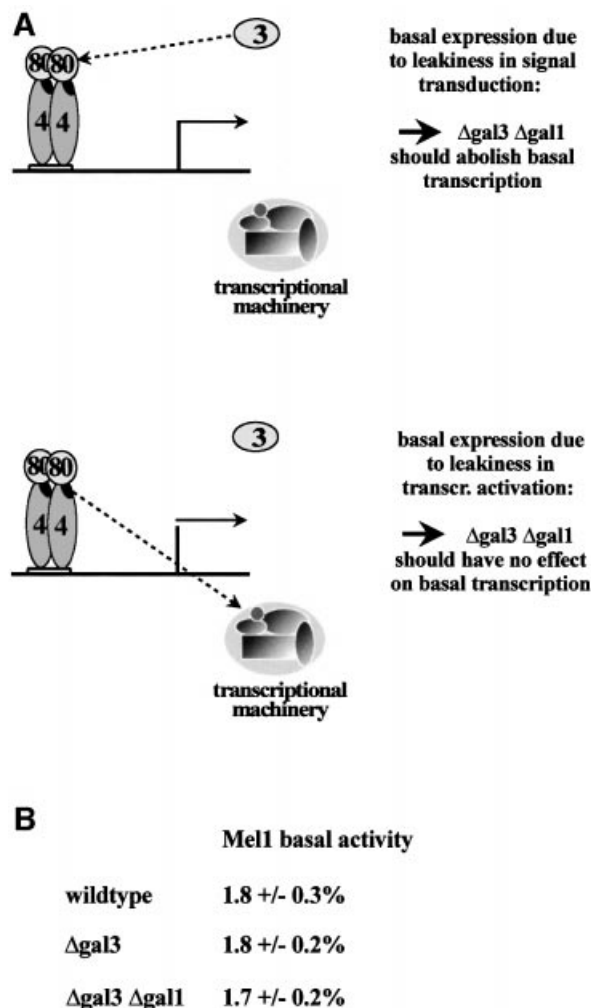


Fig. 6. Basal expression is not affected by disruption of the *GAL3 GAL1* signal transduction pathway. (A) Experimental outline to distinguish between two models for *MEL1* basal expression. 4: Gal4p; 80: Gal80p; 3: Gal3p. (B) Percentage basal Mel1p activities (relative to galactose-grown, wild-type cells) in strains that are isogenic except for *GAL1* and *GAL3*.

complexes being fully occupied by Gal80p (complex 5 in Figure 5B). The fraction of complexes with only one Gal4p dimer being occupied by Gal80p (complex 4 in Figure 5B) should then be under-represented. However, we detect a slow transition from the fraction of unoccupied Gal4p dimers to the fraction of completely occupied dimers and a large fraction of complexes in which only one of the two Gal4p dimers is bound by Gal80p (Figure 5B), indicating that under the *in vitro* conditions used, Gal80p does not bind cooperatively to adjacent Gal4p dimers. This result is consistent with the Gal80 dimer-dimer interaction being transient and much weaker than the Gal4p-Gal80p and the Gal4p-DNA interactions. Note that with higher concentrations of Gal80p, total visible labelling decreases, presumably because higher order Gal80p complexes are unstable during electrophoresis. In summary, under the conditions used we find no evidence for a stabilization of the Gal4p(1-147+34)-Gal80p complex by Gal80p dimer-dimer interaction.

Incomplete repression is not due to leakiness of the signal transduction pathway. We can envisage two qualitative

(architectural) models by which Gal80p multimerization on adjacent Gal4p binding sites could abolish basal transcription (Figure 6A). In one model, the signal transducer Gal3p would still have a low residual affinity for Gal80p in the absence of galactose, sufficient to allow some low level expression from genes regulated by a single Gal4p binding site. Locking of Gal80p dimers into multimers might then occlude accessibility for Gal3p. In the second model, signal transduction to Gal80p would be unaffected, but rather Gal4p's activation domain would not be completely masked by dimeric Gal80p. Formation of Gal80p multimeric complexes would then completely block accessibility of the activation domain and hence fully prevent the activation domain from making protein-protein contacts with the transcriptional machinery (Figure 6A). To distinguish between these models we completely disrupted the signal transduction pathway by deleting both the *GAL3* and *GAL1* genes. Mel1p expression under non-inducing conditions was unaffected by the lack of Gal1p and Gal3p (Figure 6B). We conclude that basal expression is not a consequence of a leaky signal transduction pathway, but of a process subsequent to Gal3p's activation of Gal80p.

Discussion

The *GAL* genes of *S.cerevisiae* fall into two classes. One group is completely repressed under non-inducing conditions and characterized by multiple Gal4p binding sites. Genes of the other class have biologically important basal expression and single Gal4p binding sites. We demonstrated that basal expression can be strongly reduced by moderate overexpression of the repressor Gal80p. An increase in the concentration of Gal80p can therefore partially overcome the need for adjacent Gal4p-Gal80p complexes for complete repression. This observation suggested a simple model. Adjacent Gal4p binding sites could stabilize weak or transient interactions between Gal80 molecules. Gal80p dimer-dimer interaction could in turn stabilize the Gal4p-Gal80p interaction or could prevent residual interactions of the complex with the transcription apparatus or with components of the signal transduction pathway. This model makes several specific predictions and we used a combination of *in vivo* and quantitative *in vitro* assays to test them.

First, Gal80 molecules on adjacent binding sites must be capable of interacting with each other. We established that the unit of Gal80p binding to a Gal4p-DNA complex is a homodimer. We could then trap interactions between Gal80p dimers by chemical crosslinking. Secondly, this interaction must be weak or transient without the proximity arising from adjacent Gal4p binding sites. Moreover, an increase in the concentration of Gal80p should drive dimer-dimer interaction in solution or on a single Gal4p binding site. In a series of *in vitro* experiments, we demonstrated that the Gal80p dimer-dimer interaction is weak, with a K_D that appears to be at least two orders of magnitude higher than the monomer-monomer interaction, that it is not stable to native electrophoresis, and that it is kinetically too transient to promote cooperative binding of Gal80p dimers on adjacent Gal4p-DNA complexes. In addition, overexpression of the Gal80pVP16 hybrid resulted in strongly increased reporter

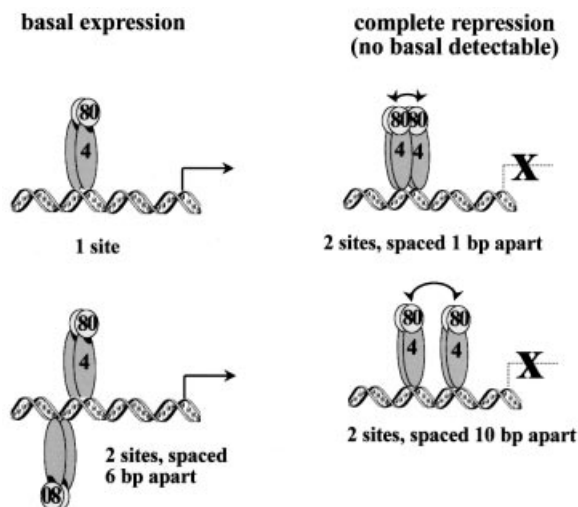


Fig. 7. Model for differential repression in the yeast *GAL* system. On a single Gal4p binding site or on two binding sites on opposite sides of the DNA helix, Gal80p dimers do not interact with each other and only partially mask the Gal4p activation domain. On binding sites on the same side of the DNA helix (1 and 10 bp spacing), the Gal80p dimer–dimer interaction is stabilized, causing complete shielding of the activation domain.

gene activity (Figure 3), as predicted if the expression level from integrated *GAL80VP16* under the control of the wild-type *GAL80* promoter is so low that only a fraction of Gal80p hybrids engage in dimer–dimer interactions on a single Gal4p binding site. Thirdly, assembly of Gal4p–Gal80p complexes on adjacent DNA sites must stabilize the inherently transient interaction between Gal80p dimers. We confirmed this prediction by using a 0 Å crosslinker in two assembly reactions that differed only in having the same concentration of Gal4p binding sites above the K_D either as isolated sites or as neighbouring sites. Finally, the ability or inability of adjacent Gal80p dimers to interact with each other should correlate with complete or incomplete repression. We used a DNA spacing assay to probe for the importance of protein–protein interactions. Complexes assembled right next to each other or one helical turn apart are on the same side of the DNA. In this orientation they can interact with each other, which correlates with complete repression of transcription. Complexes assembled a half-helical turn from each other are on opposite sides of a rigid helix. This orientation was incompatible with complete repression, providing strong evidence for an architectural mechanism that links full repression with a direct interaction between the assembled complexes (Table III).

How could a stabilized Gal80p dimer–dimer interaction increase repression? The simplest explanation would be that it does so by stabilizing the Gal4p–Gal80p interaction and thereby the masking of the Gal4p activation domain. However, cooperative binding assays indicated that Gal80p dimer–dimer interactions may be kinetically too labile to increase the strength of the Gal4p–Gal80p interaction significantly. We therefore favour a mechanism in which Gal80p multimerization reduces residual accessibility of the Gal4p–Gal80p complexes either to the signal transducer or to the transcriptional machinery. Since basal expression was independent of a functional Gal3p signal transduction pathway, we believe that it most

probably results from partial access of the activation domain to the transcriptional machinery (Figure 7).

Our analysis of the mechanism of differential repression clearly points to the potential of weak interactions that are difficult to detect by standard biochemical methods, yet can have profound regulatory consequences. In order to understand the interactions between the regulatory factors, it was important to put them into a quantitative framework. We have shown that Gal80p dimerizes in solution with high affinity and that Gal4p forms a heterotetramer with Gal80p on DNA. We have found that in this complex, the half-life of the Gal80p dimer is greatly extended relative to that of the free dimer and that, in turn, Gal80p stabilizes the interaction between Gal4p and DNA. While our K_D determination for Gal4p bound to a consensus site oligonucleotide agreed well with that of several other laboratories, our K_D for the Gal4p–Gal80p interaction is more than one order of magnitude lower than that determined by Lue *et al.* (1987; 5×10^{-9} M) using an electrophoretic mobility shift assay (EMSA), and two orders of magnitude lower than that determined by Ptashne and colleagues using a BIAcore microchip (Wu *et al.*, 1996). The apparent difficulty in determining this binding constant probably reflects the complicated linkage of equilibria between Gal4p and DNA, Gal4p and Gal80p, and Gal80p and Gal80p. In particular, we needed to DNA-drive the lower affinity interaction of Gal4p to its consensus site oligonucleotide at a Gal4p concentration that is below the K_D of its higher affinity interaction with Gal80p. We therefore believe that the lowest value, determined at a Gal4p–DNA concentration below the 0.3 nM K_D of the Gal4p–Gal80p interaction, is probably the correct one. With complexes assembled on a consensus site restriction fragment, rather than a minimal oligonucleotide, all pairwise interactions within the DNA-bound complexes have K_D s in the subnanomolar range and are stabilized further by neighbouring interactions. These strong interactions stabilize transient dimer–dimer interactions by positioning dimers in suitable close proximity.

Activator–repressor systems that involve activation domain masking are not a peculiarity of the yeast *GAL* system, but rather have been found in a number of biologically important systems, including the binding of the tumour suppressor RB to the activation domains of E2F (Nevins, 1992) and E1A (Trouche and Kouzarides, 1996), and the masking of the p53 activation domain by MDM2 (Momand *et al.*, 1992; Kussie *et al.*, 1996). Therefore, the type of differential repression characterized here may apply to other systems as well.

Finally, we would like to point out that while *MEL1* basal expression is abolished in the absence of Gal4p, other *GAL* genes may be regulated by additional elements (see, for instance, Sakurai *et al.*, 1994). While basal expression can be achieved either by only a single Gal4p binding site or by introducing additional regulators, complete repression of *GAL* genes can only be achieved by correctly spaced multiple Gal4p binding sites.

Materials and methods

Strains, enzyme assays and genetic techniques

Yeast strains used were 21R (*GAL4 GAL80 MEL1 ura3-52 leu2-3,112 ade1*) (Johnston and Hopper, 1982) for testing expression driven from

Gal4p binding sites (Tables I–III), and YJ0Z (*Gal4 Gal80 MEL1 ura3-52 leu2-3,112 his3 ade2-101 trp1* with an integrated *GAL1-lacZ* reporter) (Leuther and Johnston, 1992) for two-hybrid experiments and disruption of the signal transduction pathway. Derivatives of YJ0Z were described previously (Leuther and Johnston, 1992; Ding and Johnston, 1997). *GAL4* integrants were constructed by transforming linearized YIp351-*GAL4* into YJ0Z80 and YJ0ZΔ*gal3*. *GAL1* was disrupted by using pYES-TRP1-*GAL1*-3' untranslated region (Ding and Johnston, 1997). Single-site integration and disruptions were confirmed by PCR. α - and β -galactosidase assays were performed as described previously (Melcher *et al.*, 2000).

Protein purification and labelling

Gal4p(1–147+34), Gal80p and Gal80p–HMK were purified as glutathione *S*-transferase (GST) fusion proteins using standard protocols. GST tags were removed with TEV protease as described (Melcher, 2000). His₆-Gal80p was expressed from plasmid pPROEX-Gal80 (U.Kuchibhotla and S.A.Johnston, unpublished) and purified by Ni-chelate chromatography. Gal80p–HMK was radioactively labelled with [γ -³²P]ATP and 10 U of HMK (Sigma) for 70 min at room temperature in buffer as described previously (Blanar and Rutter, 1992).

Determination of protein concentrations

Concentrations of the purified proteins were determined by Bradford assay, by SDS–PAGE/Coomassie Blue titration with BSA standards and by absorbance at 280 nm with $\epsilon_{\text{Gal4p}(1-147+34)} = 17\,700\text{ cm}^2/\text{mol}$ (2W, 2F, 4Y) and $\epsilon_{\text{Gal80p}} = 41\,570\text{ cm}^2/\text{mol}$ (3W, 20F, 22Y). Results of all three assays agreed well with each other.

Gel retardation assays (EMSA) and DNA-free gel shifts

For EMSA with a single Gal4p binding site, oligonucleotides 1 and 2 (see Table IV, available supplementary data at *The EMBO Journal* Online) were annealed to each other and end-labelled with [γ -³²P]ATP and T4 kinase. Oligonucleotides 3 and 4, containing two completely palindromic consensus Gal4p binding sites, annealed almost exclusively intramolecularly. We therefore first cloned the mixture with a fraction of intermolecularly annealed oligonucleotides into the polylinker of YEp351. Only intermolecularly annealed oligonucleotides had correct ends for cloning. We then isolated a 160 bp 2× binding site fragment by PCR with standard M13 primers. The PCR fragment was partially digested with *Xba*I and *Pst*I, and end-labelled with Klenow polymerase and [α -³²P]dATP. Restriction fragments together with end-labelled pUC19/*Sau*3A standards were separated on a 12% polyacrylamide gel and visualized by autoradiography. The band corresponding to the 108 bp fragment was excised, and the DNA isolated by the crush and soak method (Sambrook *et al.*, 1989).

Binding reactions (15 μ l) were performed with the indicated concentration of proteins in buffer A(50) [25 mM Tris–HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.125 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT)]. Reactions also contained 1 mg/ml unprogrammed rabbit reticulocyte lysate proteins (except for Figure 1D) and 1–2 μ g of sheared salmon sperm DNA. For antibody supershifts, reactions were incubated with 1 μ l of purified IgGs. Protein–DNA complexes were resolved by running 6% polyacrylamide gels in 0.5× TBE (12.5 mM Tris, 95 mM glycine, 0.5 mM EDTA) at 10 V/cm and 4°C.

For DNA-free gel shifts, Gal80p and Gal80pVP16 were *in vitro* translated with [³⁵S]methionine. Indicated amounts of translated and recombinant proteins were incubated in 15 μ l reactions in buffer A(50) for 15 min at room temperature (Figure 2A and B) or for the indicated amount of time (Figure 2C) before complexes were resolved as above. Gels were fixed for 30 min in 40% methanol/10% acetic acid prior to drying and autoradiography.

Immunoblots and shift-westerns

SDS or native polyacrylamide gels were blotted to polyvinylidene difluoride (PVDF) membranes. Shift-westerns were briefly immersed in 100% methanol, air-dried, and subjected to autoradiography prior to re-wetting and blocking. Blots were incubated with polyclonal rabbit anti-Gal80p serum (a kind gift from T.Fukasawa) or anti-Gal4p(729–875) serum (a kind gift from K.K.Leuther) at 1:10 000 dilutions. Secondary antibodies were horseradish peroxidase conjugated (Bio-Rad) for use with the chemiluminescence kit from Dupont.

Crosslinking

GlyGlyHis-Ni-mediated crosslinking was described by Brown *et al.* (1995). All crosslinking reactions were performed in buffer A(50). Reactions were allowed to equilibrate before incubation with 100 μ M

freshly Ni-complexed (or as control, uncomplexed) GlyGlyHis tripeptide and 100 μ M magnesium monoperoxyphthalic acid (MMP) for 2 min (crosslinking in solution) or 90 s (crosslinking of assembled complexes) at room temperature. Acetylated BSA was purchased from Promega.

For the assembly reaction, 3.1 pmol of Gal4p(1–147+34) dimers and 1.5 pmol of His₆-Gal80p dimers were incubated in 80 μ l of buffer A(50), with either 3 pmol of the annealed consensus site oligonucleotide or 1.5 pmol of the 163 bp PCR fragment with two adjacent consensus Gal4p binding sites (3 pmol sites in total) for 20 min at room temperature. Expected molar ratios and quantitative binding were confirmed by titrating all components of the reaction in parallel shift-westerns using anti-Gal80p and anti-Gal4p antibodies for detection. ³²P-labelled Gal80p–HMK (0.3 pmol) was incubated with pre-assembled complexes for 3 min prior to crosslinking. Aliquots (10 μ l) per reaction were quenched by the addition of SDS sample buffer, separated by SDS–PAGE, blotted to PVDF membranes, and subjected to autoradiography. Aliquots (40 μ l) of each reaction, together with 40 μ l of 'no crosslinking' controls were quenched by the addition of 600 μ l of denaturing wash buffer (6 M guanidium hydrochloride, 20 mM Tris pH 7.9, 500 mM NaCl, 20 mM imidazole) and 10 μ l of Ni-charged Sepharose (Qiagen) for denaturing Ni-affinity chromatography as described (Fancy *et al.*, 1996). Ni-bound complexes were washed as described previously (Fancy *et al.*, 1996) and Cerenkov radiation of retained radioactivity determined by scintillation counting.

Plasmid constructions and oligonucleotides

For detailed information see the Supplementary data, available at *The EMBO Journal* Online.

Supplementary data

Supplementary data to this paper are available at *The EMBO Journal* Online.

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