SELF-ASSOCIATION OF THE GAL4 INHIBITOR PROTEIN, GAL80, IS IMPAIRED BY GAL3: EVIDENCE FOR A NEW MECHANISM IN THE GAL GENE SWITCH

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ABSTRACT

The DNA-binding transcriptional activator Gal4 and its regulators Gal80 and Gal3 constitute a galactose-responsive switch for the GAL genes of yeast. Gal4 binds to GAL gene UAS\textsubscript{GAL} sites as a dimer via its N-terminal domain and activates transcription via a C-terminal transcription activation domain (AD). In the absence of galactose a Gal80 dimer binds to a dimer of Gal4, masking the Gal4AD. Galactose triggers Gal3-Gal80 interaction to rapidly initiate Gal4-mediated transcription activation. Just how Gal3 alters Gal80 to relieve Gal80 inhibition of Gal4 has been unknown, but previous analyses of Gal80 mutants suggested a possible competition between Gal3-Gal80 and Gal80 self-association interactions. Here we assayed Gal80-Gal80 interactions and tested for effects of Gal3. Immunoprecipitation, cross-linking, denaturing and native PAGE analyses of Gal80 in vitro and fluorescence imaging of Gal80 in live cells show that Gal3-Gal80 interaction occurs concomitantly with a decrease in Gal80 multimers. Consistent with this, we find that newly discovered nuclear clusters of Gal80 dissipate in response to galactose-triggered Gal3-Gal80 interaction. We discuss the effect of Gal3 on the quaternary structure of Gal80 in light of the evidence pointing to multimeric Gal80 as the form required to inhibit Gal4.
Regulation of gene expression by promoter-specific DNA-binding transcription activator proteins is a common strategy cells use to modulate production of proteins adaptively. In the activated state a transcriptional activator mediates a host of binding reactions to recruit and assemble RNA polymerase and associated transcriptional factors at the promoter. Typically, such transcriptional activators are inhibited or activated by signaling processes that involve ligand binding, protein-interaction cascades and chemical modifications (4, 9, 13, 22, 42, 44). Regulation of several well-studied transcriptional activators involves masking and unmasking of their activation domains through protein-protein interactions (11, 14, 17, 25, 26, 27).

A prominent example is Gal4, the DNA-binding transcriptional activator of the GAL gene switch that controls expression of the galactose pathway genes in Saccharomyces cerevisiae (10, 18, 19, 24, 35). Alternative interactions among the three GAL gene switch proteins, Gal4, Gal80 and Gal3, determine whether the Gal4 transcriptional activation domain (Gal4AD) is masked or not masked by Gal80 (34, 39). In the absence of galactose the Gal80 protein binds to a small peptide (aa 855-870) within the Gal4AD and thereby prevents Gal4-mediated promoter activation (25, 26, 43).

Galactose converts Gal3 to a form that readily binds to Gal80. It is well established that Gal3-Gal80 complex formation is required for the relief of Gal80 inhibition of Gal4AD and Gal4-mediated transcription activation of the GAL genes (5, 6, 40, 45). These events occur rapidly, resulting in readily detectable GAL mRNA within 3 to 4 minutes of exposure to galactose (8, 38, 46).
Understanding exactly how galactose-activated Gal3 binding to Gal80 alters Gal80 to overcome inhibition of Gal4 has been a goal of researchers in the field for the past several years. Two questions concerning discrete mechanistic events in the GAL gene switch have driven such research. First: Does Gal80 dissociate from Gal4 or remain associated with Gal4 in response to the Gal3-Gal80 interaction? On this issue there is evidence in support of dissociation from our lab (17, 31, 37) as well as evidence for non-dissociation from other labs (1, 23, 33). Second: How does the binding of Gal3 to Gal80 alter Gal80 such as to relieve inhibition of Gal4? It is this second question that is the focus of the work presented here.

Just how the binding of Gal3 to Gal80 alters Gal80 might come down to simple competition between Gal4 and Gal3 for binding to Gal80 as has been proposed for the somewhat similar GAL gene switch of the distantly related yeast, Kluyveromyces lactis (K. lactis or Kl) (28, 36, 41). In that system, the binding of KlGal1, a Gal3 homologue, to KlGal80 overcomes its inhibition of KlGal4 activity (47). The experimental evidence indicates that KlGal1 and KlGal4 binding to KlGal80 are mutually exclusive and a heterotetrameric KlGal802-KlGal12 complex forms in response to galactose. Based on mathematical modeling of that system it was suggested that two KlGal1 monomers somehow compete with KlGal80-KlGal4 dimer-dimer interactions (3). Possibly, in the S. cerevisiae GAL gene switch as well, it could be simple competition between Gal80-Gal4 and Gal80-Gal3 binding events that mechanistically couples Gal3-Gal80 binding to activation of Gal4. The findings that over-expression of either Gal4AD or Gal3 relieves Gal80 inhibition of Gal4 in the absence of galactose (5, 16, 20, 21, 26) and increasing Gal80 concentration reverses the effect (30) are consistent with such a possibility.
However, there is no direct physical evidence to date showing that such simple competition occurs in the *S. cerevisiae* GAL gene switch. Here we report the results of *in-vitro* molecular studies and spinning disk confocal fluorescence imaging of live yeast cells showing that Gal3 interaction with Gal80 reduces levels of Gal80 self-assemblies. Additionally, we report novel intra-nuclear clusters of Gal80 that dissipate under conditions where Gal3 binds to Gal80. We propose that the Gal3-mediated decrease in Gal80 multimers revealed by these studies represents a mechanistic event leading to relief of inhibition of Gal4. Our proposal is consistent with the data of others pointing to a correlation between the Gal80 self-association interactions and inhibition of Gal4 (28).
MATERIALS AND METHODS

Yeast strains and plasmids:

Yeast strain Sc952 (MATa ade1 ile leu2-3,112 ura3-52 trpl-HIII his3-Δl

LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' gal4Δ::NAT) was used for the *in-vivo* microscopy assays of Gal80-Gal80 and Gal80-Gal3 interactions. Sc952 was derived from yeast strain Sc902 (19) by deleting *GAL4* with a deletion cassette PCR amplified from pFJ68N (17) with primers OE174 (ATC ATT TTA AGA GAG GAC AGA GAA GCA AGC CTC

CTG AAA GGT AAT ATA GAT CTG TTT AGC) and OE175 (GAA GTG AAC TTG

CGG GGT TTT TCA GTA TCT ACG ATT CAT TGG CGG CGT TAG TAT CGA

ATC). Protein extracts for the *in-vitro* protein-protein binding assays were obtained from yeast strain Sc745 cells (MATa ade1 ile leu2-3,112 ura3-52 trpl-HIII his3-Δl MEL1

LYS2::P_{GAL1}-HIS3 gal4Δ::LEU2). The plasmids used in this study were generated using standard molecular cloning and PCR techniques. A more detailed description for construction of each plasmid will be provided upon request. A list of all the plasmids can be found in the table below.
<table>
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<tr>
<th>Plasmids</th>
<th>Genotype</th>
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<tr>
<td>pOE145</td>
<td><em>CEN ARS1 TRP1</em> $p_{ADH2}\cdot GAL80\cdot 2mYFP$</td>
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<td>pGP23A</td>
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<td><em>CEN ARS1 TRP1</em></td>
<td>NE biolabs</td>
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<td>pXT102</td>
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<td>15</td>
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Table 1. Plasmids used in this study.
Microscopy:

All microscopy experiments were carried out with a Nikon TE-2000U spinning disk confocal microscope that was equipped with a 100x /1.4 NA objective lens (Nikon, Melville, NY), 488-, 514-, and 568-nm argon ion lasers, and a charge-coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). The following protocol was used to observe the effect of galactose on protein interactions in single cells. Cells were grown to mid-log phase in non-inducing media and immobilized in the Y04C microfluidics plates (Cellasic). The temperature of the media in the plates was kept at 28°C with a Biotech lens heater. Images were acquired before and after adding galactose to the media circulated around the immobilized cells.

For the determination of the number of molecules in small foci clusters we followed a previously described procedure of photon counting (13). Briefly; eleven Z-section images were taken of each cluster with a step size of 0.4 μm. Three Z-sections around the brightest of each foci were quantified and added using Image J software. The number of molecules was estimated based on fluorescence intensity comparison of the test samples to 120 Cse4-mYFP (monomeric yellow fluorescent protein) molecules in anaphase centromeres of JW2687 cells (13).

Pulldown assays:

Sc745 cells expressing HA-Gal80 (pGP23A) and Gal80-2mYFP (pOE145) were grown to mid-log phase in the appropriate synthetic drop out media. The cells were pelleted and re-suspended in lysis buffer (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 200 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 5 mM MgCl2) with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 mM PMSF, 0.5 μg/ml
benzamidine, and 1 μg/ml aprotinin). Acid washed beads (0.5 μm diameter) were added to cells in 2ml microfuge tubes, and the cells were mechanically lysed by vortexing as previously described (6, 29, 33). An aliquot of whole cell extract containing 1 μg of total protein was mixed with 20 μl of E. coli extract containing Gal3 (expressed from pXT52) in a binding buffer (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.125 mM EDTA, 10% glycerol, 1 mM DTT) that was previously shown suitable for Gal80 self-association interactions (28). Agarose beads conjugated to anti-HA antibody were added, and the samples were rotated in 4°C for 2 hours. The targeted proteins were precipitated at 3000 x g with the beads, and the beads were washed once with lysis buffer with or without galactose. The final pellets were subjected to SDS-PAGE, and were assessed on a western blot probed with a 1:5000 dilution of anti-Gal80 (VVA883 from Simpson lab, Janelia Farm Research Campus, HHMI) and 1:1000 dilution of anti-Gal3 (#138).

**Protein expression and purification:**

Affinity purification was used to purify 6xHis tagged Gal3 and Gal80. Gal80 from pXT49, Gal3 from pXT52, 6xhis-Gal80 from pClaks82 and 6xhis-Gal3 from pAKS123 were expressed in the Rosetta (DE3) strain using standard protocols. The cells were resuspended in 45 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl and 10 mM imidazole, pH 8.0) with one tablet of protease inhibitor cocktail (Roche) and were lysed in a French press with pressure of 1200 psi.

Purified 6xhis-tagged proteins were pulled down with Talon beads according to manufacturer’s protocol (Clontech). They were then buffer-exchanged into a buffer of composition 15 mM KH2PO4, 150 mM KCl, 10% glycerol, 2 mM DTT and 1 mM PMSF. They were then concentrated using the Amicon Ultra-4 system (EMD Millipore -
Billerica, MA; catalog no. UFC800308). The untagged Gal80 and Gal3 molecules were purified with the following procedure. The 6xhis-Gal3 extract was mixed with extract containing untagged Gal80, and the 6xhis-Gal80 extract was mixed with the extracted containing untagged Gal3. Galactose and ATP were added to the mixtures to the final concentrations of 25 mM and 2 mM, respectively. A 2 ml volume of Ni-NTA resin (Qiagen) was added into the extracts and the samples were rocked/rotated for 2 hours at 4°C in 15 ml falcon tubes. Samples were centrifuged, and the precipitate resin was washed once with wash buffer (20 mM imidazole, 2 mM ATP, 25 mM Galactose, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0). The resin was then transferred to a gravity chromatography column (BioRad) and washed twice with the wash buffer. The untagged proteins were then eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0) lacking ATP and galactose.

Cross-linking of Gal80 and Gal3:

The cross-linking reaction was carried out as described elsewhere (2). Briefly, 2 μM of Gal80 molecules were incubated with Gal3 at indicated concentrations in the presence of 500 μM ATP and 25 mM galactose in 30 μl total reaction volume at 4°C for 1 hr. Formaldehyde was then added to the samples to a final concentration of 30 mM, and the samples were incubated at 4°C for an additional 2 hr to crosslink the proteins. A volume of 10 μL 4xSDS electrophoresis loading buffer was added to each sample, and the samples were incubated at room temperature for additional 10 min. Proteins were separated by 7.5% SDS-PAGE and visualized by coomassie blue staining.
Discontinuous blue native protein gel Electrophoresis:

The native gel electrophoresis was carried out using the Niepmann and Zheng procedure (30) that allows the separation of proteins according to their size, oligomeric state, and shape. A fixed amount of Gal80 (2.5 μM final concentration) was incubated with increasing amounts of Gal3 in the presence or absence of 2 mM ATP and 25 mM galactose at 4°C for 2 hr. The samples were then mixed with gel loading buffer (100 mM Tris-Cl pH 8.0, 40% glycerol, 0.5% Serva Blue G) and incubated for an additional 10 min at room temperature. The protein species in the samples were analyzed on a non-denaturing 4-16% polyacrylamide gradient gel. Histidine (100 mM final concentration, pH 8.0) and 0.002% Serva Blue G were added to the cathode buffer prior to electrophoresis. Catalase (230 kDa), GAPDH (143 kDa) and BSA (69 kDa) were co-run as molecular markers. The gels were destained with several changes of 7.5% acetic acid and 5% ethanol. To check if 6xhis-Gal80S-2 has a higher propensity to oligomerize compared to 6xhis-Gal80 we repeated the above analyses using precast 4-20% BN-PAGE gels from Jule Inc. (Milford, CT, USA). Compositions of loading buffer, cathode buffer, anode buffer, method of destaining as well as the molecular markers were kept the same as above.
RESULTS

Conditions favoring Gal3-Gal80 interaction reduce the level of HA-Gal80-Gal80-2mYFP complexes in yeast extracts: To determine whether Gal3-Gal80 association reduces Gal80 self-association we performed co-immunoprecipitation assays (co-IP). Yeast extracts containing HA-Gal80 and Gal80-2mYFP and plus or minus galactose were incubated with *E. coli* extracts containing wild type (WT) Gal3, or no Gal3, or a mutant Gal3 (H199R) that is defective in Gal80 binding (15). Approximately two-fold less Gal80-2mYFP was co-precipitated with anti-HA Ab-conjugated agarose beads under conditions favoring Gal3-Gal80 interaction compared to conditions that did not (Figure 1). Importantly, we detected Gal3 in the co-precipitates only in the presence of galactose. These results suggest a mechanism whereby Gal3 binding to Gal80 reduces Gal80 self-association and/or the stability of pre-existing Gal80 oligomers.

Increasing [Gal3] vs. a fixed [Gal80] leads to Gal3:Gal80 complexes at the expense of Gal80 oligomers: Previously it was shown that Gal80 dimerizes very strongly (Kd = 0.2 nM) and tetramerizes moderately strongly (Kd = 50 nM) *in-vitro* (30). To determine whether the reduction in Gal80 self-association interactions observed in our co-IP experiments reflects a direct impact of Gal3-binding on Gal80 oligomeric structures, we employed cross-linking and native poly-acrylamide gel experiments. We could readily detect multiple bands in SDS-PAGE corresponding to cross-linked Gal80 molecules in the absence of Gal3 (Figure 2, lane 3), confirming oligomerization of Gal80 *in-vitro*. While the mobility of the most prominent band was consistent with the molecular weight of Gal80 dimer, the dimer is not necessarily the most populated quaternary Gal80 species under native conditions, as it requires only one cross-link to yield dimers but more than...
one to capture higher order oligomers. Nevertheless, we also observed species with mobilities consistent with higher order multimeric species. We nominally label these as trimeric (80₃) and tetrameric (80₄) complexes. However, their mobilities could as well be reflective of tetrameric and hexameric species. Strikingly, the abundance of cross-linked Gal80 species decreased in response to increasing amounts of Gal3 (Figure 2, lanes 4-9), as a new band corresponding to the expected size of a Gal3-Gal80 heterodimer appeared. Next, we wanted to verify that the gradual disappearance of Gal80 oligomers and corresponding appearance of Gal3-Gal80 complex was a consequence of Gal3-binding to Gal80. We addressed this by performing similar cross-linking experiments using the Gal3-E116G mutant that does not bind to Gal80 (15). For comparing the mutant to WT Gal3 we had to use tagged Gal3 proteins, 6xHis-Gal3-E116G and 6xHis tagged WT Gal3 (6xHis-Gal3), because the mutant molecules could not be purified by binding to 6xHis-Gal80. As expected, cross-linked 6xHis-Gal80 oligomers were diminished in presence of 6xHis-Gal3 (Figure S1A), but not in presence of 6xHis-Gal3-E116G (Figure S1B). These gels did not exhibit a distinct band corresponding to the cross-linked Gal3-Gal80 complexes like we observed for untagged Gal80 and Gal3 molecules. We do not know the basis for this. Perhaps 6His-Gal3-6His-Gal80 complexes migrate similarly to Gal80-Gal80 complexes and are obscured on the gel. Alternatively, the 6xHis tags on Gal3 and/or Gal80 might inhibit cross-linking of the two species.

Native gels also showed a reduction in Gal80 self-association in response to formation of Gal3-Gal80 complexes. Two separate binding reaction series were carried out in which [Gal3] was increased against a constant 2.5 μM of Gal80 (Figure 3A). We detected Gal80 oligomers on 4-16% gradient non-denaturing polyacrylamide gels.
containing ATP and galactose (Figure 3A). These oligomeric species of Gal80 diminished in response to increased levels of Gal3, while a new mobility species appeared. The mobility of this newly appearing species corresponds to that expected of a Gal3-Gal80 heterodimer, the galactose/ATP-dependent Gal3-Gal80 complex first detected by analytical filtration chromatography (42). We cut out the newly appearing band (marked 80-3 in Figure 3A) and applied it to SDS PAGE. We found that the cut out band contained two polypeptides; one had the expected size of Gal80 and the other had the expected size of Gal3 (Figure 3B).

When we repeated the native gel analysis with the Gal80S-2 variant that does not bind to Gal3, we did not observe a decrease in the levels of the Gal80 oligomers and a new band corresponding to Gal3-Gal80 complexes (Figure 3C). Surprisingly, in comparing the two gels, the Gal80S-2 showed more prominent higher order oligomers than did wild type Gal80 (Figure 3A and 3C). To verify this observation, we ran both wild type Gal80 and Gal80S-2 on the same precast 4-20% BN-PAGE gel. Again, the Gal80S-2 mutant showed more prominent higher order oligomers than did WT Gal80 (Figure S2).

**Gal80-2mYFP dissociates from DBD-Gal80 in a Gal3 and galactose-dependent manner in live cells:** Taken all together the results presented above constitute strong evidence that under *in-vitro* conditions Gal3 association with Gal80 reduces Gal80 self-association. To test whether Gal3 has such an effect in live yeast cells we set up a spinning disk confocal microscopy-based experiment that takes advantage of a previously developed UAS$_{GAL}$ array system (19). The UAS$_{GAL}$ array in yeast S. cerevisiae consists of 8 tandem P$_{GAL1}$-GST-T$_{ADH1}$ fusion genes that each have four Gal4 binding sites (32...
UAS$_{GAL}$ sites total). This array is located close to an array of 64 LacO elements at the genomic LEU2 locus. Gal80 fused to the Gal4 DNA binding domain (DBD-Gal80), Gal80-2mYFP, and Gal3-mCherry (Figure 4 and Figure S3A-C) from yeast ADH2 promoters were expressed in the array cells bearing a gal4-deletion (Sc952). We monitored the effect of galactose and Gal3 on the DBD-Gal80-Gal80-2mYFP interaction in a single cell using a microfluidics system (Onix, Cellasic inc.). In response to galactose addition, the fluorescence intensity due to Gal80-2mYFP binding to DBD-Gal80 at the array diminished while fluorescence intensity due to Gal3-mCherry increased (Figure 4 and Figure S3A-C). We quantified the mYFP/mCherry exchange at the arrays of the 4 cells in figures 4 and S4A-C (Figure S3D-E). Comparison of these cells revealed a substantial cell-to-cell variation, which might be attributed to the variation in the plasmid-based expression levels of Gal80-2mYFP and Gal3-mCherry in each cell, indicating the sensitivity of these molecular interactions to cellular Gal80-Gal3 ratio. Galactose did not affect the intensity of the DBD-Gal80-Gal80-2mYFP spots in cells expressing no Gal3 protein (Figure S4A), and Gal3-GFP co-localized with LacI-mCherry at the array only in galactose treated cells (Figure S4B). These results along with the results of our in-vitro assays above provide strong evidence that Gal3-Gal80 complexes arise at the expense of Gal80 self-association in live cells.

Nuclei of live cells display clusters of Gal80-2mYFP that dissipate in response to galactose-triggered Gal3-Gal80 interaction: During the course of spinning disc confocal imaging of WT cells (except for the fluorescence tags) expressing Gal80-2mYFP, we discovered that the YFP fluorescence formed densely packed foci (clusters) in the nuclei of glycerol-lactic acid grown cells (Figure 5A). In a set of 70 cells, we found...
approximately 29% of the cells with one cluster, 47% with two clusters and 24% showing 3 or more clusters. Using photon-counting calculations based on Cse4-mYFP as a standard (13), we estimated that each cluster contained approximately 40-60 molecules. Addition of galactose to these cells resulted in complete dissipation of the clusters within 30 minutes (Figure 5B). Similar results were observed with cells maintained in the presence of cycloheximide at levels that prevent protein synthesis (data not shown). When the galactose was removed after dissipation of clusters, the clusters reappeared within 30 minutes (Figure 5B). Dissipation of clusters did not occur in isogenic cells expressing Gal80S-2-mYFP (Sc857) (Figure 5C) or in the gal3 deletion strain (Sc858) (Figure 5D). Furthermore, when a mutant of Gal3, Gal3C-D368V, which is capable of binding to Gal80 in the absence of galactose (6), was expressed in the gal3 deleted cells (Sc858), we did not detect clusters in glycerol-lactic acid medium (Figure S5C). On the other hand, the same host strain with a variant of Gal3 (Gal3D111C) that does not bind to Gal80 exhibited Gal80 clusters even 2 hours after addition of galactose (Figure S5D). Thus, these results revealed that there is a coalescence of Gal80-2mYFP within sub-nuclear regions in the absence of galactose and that these clusters are dispersed in response to galactose-triggered Gal3-Gal80 interaction.
DISCUSSION

The in-vitro and in-vivo studies presented here reveal for the first time that the binding of Gal3 to Gal80 reduces the levels of dimer and higher-order forms (oligomers or multimers) of Gal80. Our in-vitro data was obtained using three approaches. One approach utilized co-IP of two differently tagged versions of Gal80 as a measure of the various Gal80 self-association assemblies. The other two approaches involved detection of Gal80 monomer, dimer and oligomer species by gel electrophoresis with or without chemical cross-linking. All approaches allowed detection of Gal3-Gal80 complexes and clearly demonstrated substantial reductions in the levels of Gal80 dimer and multimer species under conditions favoring the Gal3-Gal80 interaction.

Both native and formaldehyde treated samples of Gal80 showed multiple forms of Gal80 having electrophoretic mobilities consistent with dimers and oligomers. These forms of Gal80 were diminished in response to increases in the concentration of Gal3 in the presence of galactose. In these experiments we noted discrepancies between the relative yields of various cross-linked species observed on SDS-PAGE and the yields of the apparently corresponding species evident on native gels. Such discrepancies are expected due to the inherently low cross-linking efficiency and the fact that a higher number of cross-links are required to capture trimers and tetramers relative to dimers. Nevertheless, these results highlight the occurrence of dimer and multimer forms of Gal80 in the absence of galactose, and document the emergence of Gal3-Gal80 complexes at the expense of Gal80 oligomers.

Our in-vivo data was obtained using fluorescence microscopy of live yeast cells that contain a UAS\textsubscript{GAL} array and express two interacting forms of Gal80; Gal80-2mYFP
and DBD-Gal80. The Gal80-2mYFP is known to retain all normal Gal80 regulatory functions (19), and the DBD-Gal80 binds to the UAS\textsubscript{GAL} site, Gal4 and Gal3 (33). Using such cells we observed galactose- and Gal3-dependent dissociation of Gal80-2mYFP from UAS\textsubscript{GAL}-associated DBD-Gal80. Gal3-mCherry association with DBD-Gal80 at the array followed the dissociation of Gal80-2mYFP closely. We do not know the detailed composition of the UAS\textsubscript{GAL} array-associated DBD-Gal80-Gal80-2mYFP complexes. Considering the high affinity of Gal4-DBD for the UAS\textsubscript{GAL} site (2 nM; 36, 44) and strong dimerization of DBD and Gal80, independently (12, 28, 33), it is likely that a DBD-Gal80 dimer associates with each UAS\textsubscript{GAL} and a dimer of Gal80-2mYFP binds to each DBD-Gal80 dimer. As well, it is also possible that a single dimer of DBDGal80 associated with a single UAS\textsubscript{GAL} acts to nucleate binding of more than one dimer of Gal80-2mYFP through higher order Gal80-2mYFP self-associations. In either case, our \textit{in-vivo} results establish that the interaction of Gal3 with Gal80 either competes with or destabilizes dimer-dimer and possibly higher order, multimeric assemblies of Gal80. Given that at least a single DBD-Gal80 dimer is expected to remain at each UAS\textsubscript{GAL} site within the array under galactose conditions, the Gal3-mCherry fluorescent dot we observe is likely due to either one or two Gal3-mCherry molecules bound to each DBD-Gal80 dimer.

Taken together, our \textit{in-vitro} and \textit{in-vivo} experiments provide compelling evidence that Gal3 binding to Gal80 reduces the levels of Gal80 dimers and multimers. Mechanistically how the binding of Gal3 to Gal80 brings about such a reduction is not clear. This could occur through destabilization of pre-existing Gal80 multimers, competition with Gal80 self-association, or both.
What might be the biological relevance of this action of Gal3 on Gal80-Gal80 self-association? Melcher and Xu obtained both in-vitro and in-vivo data documenting Gal80 self-association and implicating roles for Gal80 dimers and tetramers in inhibition of Gal4 (28). Their in-vitro experiments revealed that Gal80 dimerizes with high affinity ($K_D$~0.1 to 0.3 nM), Gal80 dimers self-associate with a $K_D$ of ~50 nM, and Gal80 assembles into a 2:2 complex with a Gal4 dimer residing at its target UAS$_{GAL}$ DNA site. Their in-vivo experiments entailed measuring promoter activation as a function of the spacing between neighboring UAS$_{GAL}$ sites. Their results indicated that Gal80 dimer-dimer interaction is stabilized under the natural spacings of multiple UAS$_{GAL}$ sites located upstream of the very tightly repressed $GAL1$, $GAL10$, $GAL7$ and $GAL2$ genes (7). Such evidence provided a plausible explanation for why the $MEL1$ and $GAL80$ genes that have only a single UAS$_{GAL}$ site (7) show weaker repression (in absence of galactose) and consequently, appreciable Gal4-dependent basal level of expression (i.e., weaker Gal80 inhibition) (28). Additional support for the notion that Gal80 self-association is important for its ability to inhibit Gal4 comes from recent x-ray crystallographic studies from the Joshua-Tor lab. Their crystal structures for the dimeric and tetrameric forms of Gal80 and a Gal80-Gal4AD complex support the notion that Gal80 and Gal4 can interact to form a 2:2 heterotetramer (22). That work also identified an amino acid substitution (N230R) within the Gal80 dimerization surface that impairs Gal80 self-association also impairs Gal4AD-Gal80 interaction and Gal80-inhibition of Gal4 transcriptional activity (22). Taken together, the results from the Melcher and Joshua-Tor labs lend support to the idea that Gal80 self-association assemblies play a physiological role in the GAL gene.
switch. By extension, we propose that Gal3’s effect on the levels of Gal80 dimers and oligomers is an important mechanistic event in the galactose-triggered activation of Gal4. Our discovery of Gal80 intra-nuclear clusters and their dissipation by galactose-activated Gal3 may well represent an additional illustration of Gal3’s effect on Gal80 self-assemblies. Tracking of these clusters over time indicated that they are very dynamic assemblies. Our ability to image these Gal80 formations is no doubt due to the capability of the spinning-disk confocal microscope to resolve highly dynamic objects within a small space. Because the clusters dissipate in response to galactose only under conditions when Gal3-Gal80 interaction occurs we hypothesize that Gal80 clusters represent higher order self-assemblies of Gal80. We further postulate that it is because of the higher order assemblies that we were able to detect Gal80-2mYFP. Indeed, our photon-counting data estimated 40 to 60 Gal80-2mYFP molecules per cluster, consistent with higher order Gal80 self-assembly hypothesis. Further work will be required to determine whether Gal80 lacking a YFP tag also forms similar intra-nuclear clusters. We did not observe clustering of 2mYFP-tagged Gal3, a protein that is at higher abundance than Gal80 (17). Moreover, we did not observe clustering of the very low abundance Gal4-2mYFP in wild type yeast cells (data not shown). This outcome could not be simply attributed to insufficient expression of the fluoro-tagged Gal4 molecules, since the isogenic strain that carries an array of UAS\textsubscript{GAL} sites exhibited coalescence of Gal4-2mYFP (19). So, if the Gal80-2mYFP in the clusters is bound to Gal4 in cells lacking this UAS\textsubscript{GAL} array, they are observable presumably due to signal amplification by Gal80 multimerization at Gal4-nucleated sites. We have attempted to see whether Gal80-2mYFP molecules clustered independently of Gal4; however, gal4 deletion cells were severely impaired for \textit{GAL80}
gene expression (data not shown), consistent with previous findings (41, 43). When we expressed Gal80-2mYFP in gal4 deletion cells from two Gal4-independent promoters, ADH2 and MET25, we did not observe Gal80 clusters (data not shown). These preliminary results suggested that Gal80 clustering might require Gal80-Gal4 interactions.

In summary, the results presented here reveal that the binding of Gal3 to Gal80 reduces the levels of Gal80 oligomers in-vitro and in-vivo. This alteration of the quaternary structure of Gal80 could occur through Gal3’s capacity to interfere or compete with Gal80 self-association, reduce the stability of Gal80 oligomers, or both. In light of the evidence from others that Gal80 self-association is important for inhibition of Gal4 we propose that Gal3’s effect on Gal80’s quaternary structure represents a physiologically relevant mechanism in the GAL gene regulatory system.

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REFERENCES:


FIGURE LEGENDS:

Figure 1. Co-immunoprecipitation assays with two differently tagged Gal80 species show that Gal3 binding to Gal80 interferes with formation and/or stability of Gal80 multimers. (A) HA-Gal80 (pGP23a) and Gal80-2mYFP (pOE145) were expressed in Sc745 cells. Gal80 self-association was assayed by co-immunoprecipitation (co-IP) with anti-HA antibody-conjugated agarose beads in the presence or absence of galactose and/or wild-type (pXT52) or mutant Gal3 (H199R) that was expressed in *E. coli* (n=3). (B) The intensity of the bands corresponding to co-precipitated Gal80-2mYFP in (A) was quantified and normalized to lane 1 using the Image J software. (C) The HA-Gal80-Gal80-2mYFP interaction (as in A) was tested in the presence of increasing Gal3 (pXT52) concentration (n=2).

Figure 2. Cross-linking followed by SDS-PAGE shows that the levels of Gal80 multimers are reduced in response to Gal3-Gal80 interaction. Untagged Gal3 (3 μM) alone when incubated with 500 μM ATP and 25 mM galactose in 30 mM formaldehyde did not show any Gal3 oligomers (lane 1). A fixed amount of Gal80 (2 μM) was incubated with increasing amount of Gal3 (lanes 4-9; 0.3 μM, 0.6 μM, 1.2 μM, 3 μM, 6 μM, 12 μM) in the presence of 500 μM ATP, 25 mM galactose and 30 mM formaldehyde at 4°C (lanes 4-9). The samples were resolved on a 7.5% SDS-polyacrylamide gel, and were subsequently stained with coomassie blue (n=2) (Note: The Gal3 band in lane 6 is more than the double of the one in lane 5, which is not consistent with an increase from 0.3 μM to 0.6 μM. This discrepancy is likely due to a pipetting error.)

Figure 3. Native PAGE shows that the levels of Gal80 multimers decrease in response to Gal3-Gal80 complex formation. (A) 2.5 μM untagged Gal80 was incubated in series with increasing amounts of untagged Gal3 (0.4 μM, 1 μM, 2 μM, 4 μM, 10 μM) in the presence of 2 mM ATP and 25 mM galactose at 4°C for 2 hr. The samples were then incubated with loading buffer (100 mM Tris-Cl pH 8.0, 40% glycerol, 0.5% Serva Blue G) for 10 minutes, and were subsequently resolved at 4°C on a 4–16% polyacrylamide gradient gel containing 2 mM ATP and 25 mM galactose (n=2). (B) The band marked as 80-3 in (A) was cut out from the native gel. The peptides from this cut-out were analyzed in a 10% SDS-polyacrylamide gel. (C) The native gel in (A) was repeated with a Gal80S-2 mutant that is defective in Gal3-binding (n=2).

Figure 4. Spinning disk confocal imaging of live cells shows that Gal3-Gal80 association interferes with formation and/or stability of Gal80 multimers. DBD-Gal80 (pOE165), Gal80-2mYFP (pOE145), and Gal3-mCherry (pOE208) were expressed in array cells lacking Gal4 (Sc952). The cells were grown to the mid-log phase in glycerol-lactic acid media, and loaded into Cellasic microfluidics plate chambers. Cells were initially imaged in absence of galactose. After galactose addition images were acquired during a period of 2 hours as indicated (n=4).

Figure 5. Gal80-2mYFP forms nuclear clusters that dissipate in response to galactose-activated Gal80-Gal3 interaction. (A) Gal80-2GFP and Sec63-mCherry were expressed from genomic loci in Sc1029 cells. Sec63-mCherry marked the nuclear and...
cellular peripheries (n>10). (B) Sc856 cells expressing Gal80 fused to 2 monomeric YFP molecules (Gal80-2mYFP) were grown to mid-log phase in glycerol-lactic acid media and were loaded into Cellasic microfluidics plate chambers. Localization of Gal80-2mYFP in the uninduced cells was imaged. Galactose was then added to the media in the chambers together with cycloheximide (to prevent galactose-induced synthesis of new Gal80 molecules), and images at indicated time points were acquired. Galactose was then removed from the media, and the same cells were imaged again at indicated time points (n>5). (C) The Gal80S-2-2mYFP cells (Sc857) were grown to mid-log phase in glycerol-lactic acid media. Images were acquired either in absence of galactose, or after the cells were incubated in 2% galactose for 1 hour (n>10). (D) Genomic Gal80 was tagged with 2mYFP in gal3Δ cells (Sc858). The cells were prepared, and images were acquired as in (C) (n>5).
FIGURES:

Figure 1:

A

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anti-Gal80

anti-Gal80

B

Normalized intensity of Gal80-2mYFP band

0.2 0.4 0.6 0.8 1 1.2

1 2 3 4 5

Lanes

C

Gal80

Gal80-2mYFP

Gal3

HA-Gal80
Figure 2:
Figure 3

A

B

C

2.5 μM of Gal80 in each lane
No Gal80

250 kDa
150 kDa
80 kDa
69 kDa

0 μM 1 μM 2 μM 4 μM 10 μM 2 μM 4 μM 10 μM

Gal3

Gal3

Gal80

- 75 kDa
- 50 kDa

2.5 μM of Gal80 in each lane
No Gal80

0 μM 1 μM 2 μM 4 μM 10 μM 2 μM 4 μM 10 μM

Gal3
Figure 4: 

![Image of figure 4 showing pre-galactose and time points with arrows indicating changes]

**Gal80-2mYFP**

**Gal3-mCherry**

**Composite**
Figure 5:

A

Gal80-2mYFP  |  Sec63-mCherry  |  Composite  |  Bright field

B

Galactose  |  Gly-Lac

Gly-Lac  |  10 min  |  30 min  |  10 min  |  30 min

C

Galactose

Gal80<sup>S-2</sup>-2mYFP

Bright-Field
Galactose

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