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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The DNA-binding transcriptional activator Gal4 and its regulators Gal80 and Gal3 constitute a galactose-responsive switch for the *GAL* genes of *Saccharomyces cerevisiae*. Gal4 binds to *GAL* gene UAS_{GAL} (upstream activation sequence in *GAL* gene promoter) 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, and 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 (1–7). Regulation of several well-studied transcriptional activators involves masking and unmasking of their activation domains through protein-protein interactions (8–12).

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* (13–18). 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 (19, 20). In the absence of galactose, the Gal80 protein binds to a small peptide (amino acids [aa] 855 to 870) within the Gal4AD and thereby prevents Gal4-mediated promoter activation (10, 11). Galactose converts Gal3 to a form that readily binds to Gal80 (21). 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 (22–25). These events occur rapidly, resulting in readily detectable *GAL* mRNA within 3 to 4 min of exposure to galactose (26–28).

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. The first question is 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 (19, 29, 30) as well as evi-

dence for nondissociation from other labs (20, 31, 32). The second question is 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* (18, 33–35). In that system, the binding of *K. lactis* Gal1 (KlGal1), a Gal3 homologue, to KlGal80 overcomes its inhibition of KlGal4 activity (33, 36). The experimental evidence indicates that KlGal1 and KlGal4 binding to KlGal80 are mutually exclusive and that 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 (33). Possibly, in the *S. cerevisiae* *GAL* gene switch as well, it is simple competition between Gal80-Gal4 and Gal80-Gal3 binding events that mechanistically couples Gal3-Gal80 binding to activation of Gal4. The findings that overexpression of either Gal4AD or Gal3 relieves Gal80 inhibition of Gal4 in the absence of galactose (11, 15, 22, 37) and that increasing Gal80 concentration reverses the effect (38) are consistent with such a possibility. However, to date, there is no

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TABLE 1 Plasmids used in this study

Plasmid	Relevant genotype or phenotype	Reference or source
pOE145	CEN ARS1 TRP1 P _{ADH2} -GAL80-2mYFP	This study
pGP23A	CEN ARS1 URA3 P _{ADH2} -HA-GAL80	19
pMPW66	CEN ARS1 TRP1 P _{ADH2} -GAL3	44
pRS414	CEN ARS1 TRP1	New England BioLabs
pOE165	CEN ARS1 URA3 P _{ADH2} -DBD-GAL80	This study
pOE167	CEN ARS1 URA3 P _{ADH2} -DBD-GAL3	This study
pOE169	CEN ARS1 URA3 P _{ADH2} -DBD-GAL80-GFP	This study
pOE170	CEN ARS1 URA3 P _{ADH2} -DBD-GAL3-GFP	This study
pME9	CEN ARS1 HIS3 P _{ADH2} -LACI-mCHERRY	41
pOE208	CEN ARS1 HIS3 P _{ADH2} -GAL3-mCHERRY	This study
pCLYGFP1-3	CEN ARS1 TRP1 P _{ADH2} -GAL3-GFP	This study
pXT52	GAL3 Amp ^r	This study
pXT102	GAL80-6×His Kan ^r	This study
pCLAKS82	6×His-GAL80 Kan ^r	This study
pXT49	GAL80 Kan ^r	This study
pAKS118	6×His-GAL80 ^{S-2} Kan ^r	This study
pPXGSGT4AD	GST-GAL4AD Amp ^r	This study
pAKS123E116G	6×His-GAL3-E116G	This study
pAKS123H199R	6×His-GAL3-H199R	This study
pTEB16	CEN ARS1 TRP1 P _{GAL3} -GAL3	23
pTEB16D368V	CEN ARS1 TRP1 P _{GAL3} -GAL3-D368V	47
pTEB16D111C	CEN ARS1 TRP1 P _{GAL3} -GAL3-D111C	47

direct physical evidence showing that such simple competition occurs in the *S. cerevisiae* GAL gene switch. In fact, a consideration of the Gal3-Gal80p-galactose-ATP structure (21) together with the Gal80p-Gal4AD structures from *S. cerevisiae* (4) and *K. lactis* (39) led Lavy and coworkers to suggest that Gal3 and Gal4AD do not compete for the same target surface on Gal80 (21).

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 intranuclear 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 (40).

MATERIALS AND METHODS

Yeast strains and plasmids. *S. cerevisiae* strain Sc952 [*MATa ade1 leu2-3,112 ura3-52 trp1-HIII his3-Δ1 LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan^r gal4Δ::NAT*] was used for the *in vivo* microscopy assays of Gal80-Gal80 and Gal80-Gal3 interactions. Sc952 was derived from yeast strain Sc902 (29) by deleting *GAL4* with a deletion cassette PCR amplified from pJF68N (41) 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 leu2-3,112 ura3-52 trp1-HIII his3-Δ1 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 plasmids used in this study can be found in Table 1.

Microscopy. All microscopy experiments were carried out with a Nikon TE-2000U spinning-disk confocal microscope that was equipped with a 100×/1.4-numerical-aperture (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 noninducing medium and immobilized in the Y04C microfluidics plates (Cellasic). The temper-

ature of the medium in the plates was kept at 28°C with a Biotech lens heater. Images were acquired before and after adding galactose to the medium circulated around the immobilized cells.

For the determination of the number of molecules in small focus clusters, we followed a previously described procedure of photon counting (42). Briefly, 11 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 (42).

Pulldown assays. *S. cerevisiae* Sc745 cells expressing hemagglutinin (HA)-tagged Gal80 (pGP23A) and Gal80-2mYFP (Gal80 fused to two monomeric YFP molecules (Gal80-2mYFP) (pOE145) were grown to mid-log phase in the appropriate synthetic dropout medium. The cells were pelleted and resuspended in lysis buffer (20 mM HEPES [pH 7.4], 0.5% Triton X-100, 200 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol [DTT], 5 mM MgCl₂) with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 μg/ml benzamide, and 1 μg/ml aprotinin). Acid-washed beads (0.5-μm diameter) were added to cells in 2-ml microcentrifuge tubes, and the cells were mechanically lysed by vortexing as previously described (23, 43, 44). An aliquot of whole-cell extract containing 1 μg of total protein was mixed with 20 μl of *Escherichia 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 (40). Agarose beads conjugated to anti-HA antibody were added, and the samples were rotated at 4°C for 2 h. The targeted proteins were precipitated at 3,000 × 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 assessed on a Western blot probed with a 1:5,000 dilution of anti-Gal80 (VVA883 from J. Simpson's lab, Janelia Farm Research Campus, Howard Hughes Medical Institute [HHMI]) and 1:1,000 dilution of anti-Gal3 (number 138).

Protein expression and purification. Affinity purification was used to purify 6×His-tagged Gal3 and Gal80. Gal80 from pXT49, Gal3 from pXT52, 6×His-Gal80 from pClaks82 and 6×His-Gal3 from pAKS123 were expressed in the *E. coli* 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 a pressure of 1,200 lb/in².

Purified 6×His-tagged proteins were pulled down with Talon beads according to the manufacturer's protocol (Clontech). They were then buffer exchanged into a buffer composed of 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 (catalog no. UFC800308; EMD Millipore, Billerica, MA). The untagged Gal80 and Gal3 molecules were purified by using the following procedure. The 6×His-Gal3 extract was mixed with extract containing untagged Gal80, and the 6×His-Gal80 extract was mixed with the extract 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 nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) was added to the extracts, and the samples were rocked/rotated for 2 h at 4°C in 15-ml Falcon tubes. The samples were centrifuged, and the precipitated 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 (Bio-Rad) 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 conducted as described elsewhere (45). Briefly, 2 μM Gal80 molecules were incubated with Gal3 at the concentrations indicated in the figures in the

presence of 500 μM ATP and 25 mM galactose in a total mixture reaction volume of 30 μl at 4°C for 1 h. 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 h to cross-link the proteins. A volume of 10 μl of 4 $\times$ SDS 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. Native gel electrophoresis was conducted by the Niepmann and Zheng procedure (46) that allows the separation of proteins according to their size, oligomeric state, and shape. A fixed amount of Gal80 (final concentration of 2.5 μM) 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 h. 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 nondenaturing 4 to 16% polyacrylamide gradient gel. Histidine (final concentration of 100 mM; pH 8.0) and 0.002% Serva Blue G were added to the cathode buffer prior to electrophoresis. Catalase (230 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (143 kDa), and bovine serum albumin (BSA) (69 kDa) were used as molecular markers. The gels were destained with several changes of 7.5% acetic acid and 5% ethanol. To check whether 6 $\times$ His-Gal80^{S-2} has a higher propensity to oligomerize compared to 6 $\times$ His-Gal80, we repeated the above analyses using precast 4 to 20% blue native (BN)-polyacrylamide gels from Jule Inc. (Milford, CT, USA). The compositions of loading buffer, cathode buffer, and anode buffer, the method of destaining, and the molecular markers were kept the same as described 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 coimmunoprecipitation (co-IP) assays. Yeast extracts containing HA-Gal80 and Gal80-2mYFP and with or without 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 (47). Approximately 2-fold-less Gal80-2mYFP was coprecipitated with anti-HA antibody (Ab)-conjugated agarose beads under conditions favoring Gal3-Gal80 interaction compared to conditions that did not (Fig. 1). Importantly, we detected Gal3 in the coprecipitates 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 preexisting Gal80 oligomers.

Increasing the concentration of Gal3 versus a fixed concentration of Gal80 leads to Gal3-Gal80 complexes at the expense of Gal80 oligomers. Previously it was shown that Gal80 dimerizes very strongly (K_d [dissociation constant] of 0.2 nM) and tetramerizes moderately strongly (K_d = 50 nM) *in vitro* (40). 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 polyacrylamide gel experiments. We could readily detect multiple bands on SDS-polyacrylamide gels corresponding to cross-linked Gal80 molecules in the absence of Gal3 (Fig. 2, lane 3), confirming oligomerization of Gal80 *in vitro*. While the mobility of the most prominent band was consistent with the molecular mass of the 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

FIG 1 Coimmunoprecipitation 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 coimmunoprecipitation (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 intensities of the bands corresponding to coprecipitated Gal80-2mYFP in panel A were quantified and normalized to lane 1 using the Image J software. (C) The HA-Gal80–Gal80-2mYFP interaction (as in panel A) was tested in the presence of increasing Gal3 (pXT52) concentrations (indicated by the height of the triangle above the lanes) ($n = 2$).

capture higher-order oligomers. Nevertheless, we also observed species with mobilities consistent with higher-order multimeric species. We nominally label these as trimeric (80_3) and tetrameric (80_4) complexes. However, their mobilities could also be reflective of tetrameric and hexameric species. Strikingly, the abundance of cross-linked Gal80 species decreased in response to increasing amounts of Gal3 (Fig. 2, lanes 4 to 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 the 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 (47). To compare

FIG 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 μ M) alone when incubated with 500 μ M ATP and 25 mM galactose in 30 mM formaldehyde did not show any Gal3 oligomers (lane 1). A fixed amount of Gal80 (2 μ M) was incubated with increasing amounts of Gal3 (lanes 4 to 9 have 0.3 μ M, 0.6 μ M, 1.2 μ M, 3 μ M, 6 μ M, 12 μ M Gal3, respectively) in the presence of 500 μ M ATP, 25 mM galactose, and 30 mM formaldehyde at 4°C (lanes 4 to 9). The samples were resolved on a 7.5% SDS-polyacrylamide gel and were subsequently stained with Coomassie blue ($n = 2$) (Note that the Gal3 band in lane 6 is more than double that of the one in lane 5, which is not consistent with an increase from 0.3 μ M to 0.6 μ M. This discrepancy is likely due to a pipetting error.).

the mutant to WT Gal3, we had to use tagged Gal3 proteins, 6 $\times$ His-Gal3-E116G and 6 $\times$ His-tagged WT Gal3 (6 $\times$ His-Gal3), because the mutant molecules could not be purified by binding to 6 $\times$ His-Gal80. As expected, cross-linked 6 $\times$ His-Gal80 oligomers were diminished in the presence of 6 $\times$ His-Gal3 (see Fig. S1A in the supplemental material) but not in the presence of 6 $\times$ His-Gal3-E116G (see Fig. 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 6 $\times$ His-Gal3–6 $\times$ His-Gal80 complexes migrate similarly to Gal80-Gal80 complexes and are obscured on the gel. Alternatively, the 6 $\times$ His 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 performed in which the concentration of Gal3 was increased against a constant concentration (2.5 μ M) of Gal80 (Fig. 3A). We detected Gal80 oligomers on 4 to 16% gradient nondenaturing polyacrylamide gels containing ATP and galactose (Fig. 3A). These oligomeric species of Gal80 diminished in response to increased levels of Gal3, while a new 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 (48). We cut out the newly appearing band

FIG 3 Native PAGE shows that the levels of Gal80 multimers decrease in response to Gal3-Gal80 complex formation. (A) 2.5 μ M untagged Gal80 was incubated in series with increasing amounts of untagged Gal3 (0.4 μ M, 1 μ M, 2 μ M, 4 μ M, and 10 μ M) in the presence of 2 mM ATP and 25 mM galactose at 4°C for 2 h. The samples were then incubated with loading buffer (100 mM Tris-Cl [pH 8.0], 40% glycerol, 0.5% Serva Blue G) for 10 min and subsequently resolved at 4°C on a 4 to 16% polyacrylamide gradient gel containing 2 mM ATP and 25 mM galactose ($n = 2$). (B) The band labeled 80-3 in panel A was cut out from the native gel. The peptides from this cutout band were analyzed in a 10% SDS-polyacrylamide gel. The positions of molecular mass markers (MM Markers) are indicated to the right of the gel. (C) The native PAGE in panel A was repeated with a Gal80^{S-2} mutant that is defective in Gal3 binding ($n = 2$).

FIG 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 medium containing glycerol-lactic acid and loaded into Cellasic microfluidics plate chambers. The cells were initially imaged in the absence of galactose. After the addition of galactose, images were acquired during a 2-h period as indicated ($n = 4$).

(labeled 80-3 in Fig. 3A) and applied it to an SDS-polyacrylamide gel. We found that the cutout band contained two polypeptides; one had the expected size of Gal80, and the other had the expected size of Gal3 (Fig. 3B).

When we repeated the native gel analysis with the Gal80^{S-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 (Fig. 3C). Surprisingly, in comparing the two gels, Gal80^{S-2} showed more-prominent higher-order oligomers than did wild-type Gal80 (Fig. 3A and C). To verify this observation, we ran both wild-type Gal80 and Gal80^{S-2} on the same precast 4 to 20% BN-polyacrylamide gel. Again, the Gal80^{S-2} mutant showed more-prominent higher-order oligomers than did WT Gal80 (see Fig. S2 in the supplemental material).

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} (upstream activation sequence in GAL gene promoter) array system (29). The UAS_{GAL} array in yeast *S. cerevisiae* consists of eight 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 (Fig. 4; see Fig. S3A to C in the supplemental material) from yeast *ADH2* promoters were expressed in the array cells bearing a *gal4* deletion (Sc952). We monitored the effects of galactose and Gal3 on the DBD-Gal80-Gal80-2mYFP interaction in a single cell using an Onix microfluidics system (Cellasic Inc.). In response to the addition of galactose, the fluorescence intensity due to Gal80-2mYFP binding to DBD-Gal80 at the array diminished, while fluorescence intensity due to Gal3-mCherry increased (Fig. 4; see Fig. S3A to C in the supplemental material). We quantified the mYFP/mCherry exchange at the arrays of the four cells in Fig. 4 and Fig. S4A to C in the supplemental material (see also Fig. S3D and E). Comparison of these cells revealed 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 the cellular Gal80-Gal3 ratio. Galactose did not affect the intensity of the DBD-Gal80-Gal80-2mYFP spots in cells expressing no Gal3 protein (see Fig. S4A), and Gal3-GFP colocalized with LacI-mCherry at the array only in galactose-treated cells (see Fig. 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.

The nuclei of live cells display clusters of Gal80-2mYFP that dissipate in response to galactose-triggered Gal3-Gal80 interaction. During the course of spinning-disk 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 cells grown in glycerol-lactic acid (Fig. 5A). In a set of 70 cells, we found approximately 29% of the cells with one cluster, 47% with two clusters, and 24% showing three or more clusters. Using photon-counting calculations based on Cse4-mYFP as a standard (42), we estimated that each cluster contained approximately 40 to 60 molecules. The addition of galactose to these cells resulted in complete dissipation of the clusters within 30 min (Fig. 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 min (Fig. 5B). Dissipation of clusters did not occur in isogenic cells expressing Gal80^{S-2}-2mYFP (Sc857) (Fig. 5C) or in the *gal3* deletion strain (Sc858) (Fig. 5D). Furthermore, when a mutant of Gal3, Gal3^C-D368V, which is capable of binding to Gal80 in the absence of galactose (23), was expressed in the cells in which *gal3* was deleted (Sc858), we did not detect clusters in glycerol-lactic acid medium (see Fig. S5C in the supplemental material). 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 h after the addition of galactose (see Fig. S5D). Thus, these results revealed that there is a coalescence of Gal80-2mYFP within subnuclear 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 dimers and higher-order forms (oligomers or multimers) of Gal80. Our *in vitro* data were obtained by 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 with electrophoretic mobilities consistent with those of 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-polyacrylamide gels and the yields of the apparently corresponding species evident on native gels.

FIG 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 two monomeric YFP molecules (Gal80-2mYFP) were grown to mid-log phase in medium containing glycerol-lactic acid and were loaded into CellASIC microfluidics plate chambers. Localization of Gal80-2mYFP in the uninduced cells was imaged. Galactose was then added to the medium in the chambers together with cycloheximide (to prevent galactose-induced synthesis of new Gal80 molecules), and images at the indicated time points were acquired. Galactose was then removed from the medium, and the same cells were imaged again at the indicated time points ($n > 5$). (C) The Gal80^{S-2}-2mYFP cells (Sc857) were grown to mid-log phase in glycerol-lactic acid-containing medium. Images were acquired either in the absence of galactose or after the cells were incubated in 2% galactose for 1 h ($n > 10$). (D) Genomic Gal80 was tagged with 2mYFP in *gal3Δ* cells (Sc858). The cells were prepared, and images were acquired as described above for panel C ($n > 5$).

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 were obtained using fluorescence microscopy of live yeast cells that contain a UAS_{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 (29), and the DBD-Gal80 binds to the UAS_{GAL} site, Gal4, and Gal3 (44). Using such cells, we observed galactose- and Gal3-dependent dissociation of Gal80-2mYFP from UAS_{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_{GAL} array-associated DBD-Gal80-Gal80-2mYFP complexes. Considering the high affinity of Gal4-DBD for the UAS_{GAL} site (3 nM [49]) (50, 51) and strong dimerization of DBD and Gal80 (40, 44, 52) independently, it is likely that a DBD-Gal80 dimer associates with each UAS_{GAL} and a dimer of Gal80-2mYFP binds to each DBD-Gal80 dimer. It is also possible that a single dimer of DBD-Gal80 associated with a single UAS_{GAL} acts to nucleate binding of more than one dimer of Gal80-2mYFP through higher-order Gal80-2mYFP self-associations. In either case, our *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_{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.

In contrast to a previously proposed structural model (21), the results of our *in vitro* and *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 preexisting 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 (40). The results of their *in vitro* experiments revealed that Gal80 dimerizes with high affinity (K_d of ~ 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 (53). Such evidence provided a plausible explanation for why the *MEL1* and *GAL80* genes that have only one UAS_{GAL} site (53) show weaker repression (in the absence of galactose) and consequently, appreciable Gal4-dependent basal level of expression (i.e., weaker Gal80 inhibition) (40). 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 (4). That work also identified an amino acid substitution (N230R) within the Gal80 dimerization surface that impairs Gal80 self-association also impairs Gal4AD-Gal80 inter-

action and Gal80 inhibition of Gal4 transcriptional activity (4). 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 intranuclear 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 intranuclear clusters. We did not observe clustering of 2mYFP-tagged Gal3, a protein that is at higher abundance than Gal80 (41). 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_{GAL} sites exhibited coalescence of Gal4-2mYFP (29). Therefore, if the Gal80-2mYFP in the clusters is bound to Gal4 in cells lacking this UAS_{GAL} array, they are observable presumably due to signal amplification by Gal80 multimerization at Gal4-nucleated sites. We have attempted to determine whether Gal80-2mYFP molecules clustered independently of Gal4; however, *gal4* deletion cells were severely impaired for *GAL80* gene expression (data not shown), consistent with previous findings (54, 55). 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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