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Gal80 Dimerization and the Yeast *GAL* Gene Switch

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ABSTRACT

The *Saccharomyces cerevisiae* Gal80 protein has two binding partners: Gal4 and Gal3. In the absence of galactose, Gal80 binds to and inhibits the transcriptional activation domain (AD) of the *GAL* gene activator, Gal4, preventing *GAL* gene expression. Galactose triggers an association between Gal3 and Gal80, relieving Gal80 inhibition of Gal4. We selected for *GAL80* mutants with impaired capacity of Gal80 to bind to Gal3 or Gal4AD. Most Gal80 variants selected for impaired binding to Gal4AD retained their capacity to bind to Gal3 and to self-associate, whereas most of those selected for impaired binding to Gal3 lost their ability to bind to Gal4AD and self-associate. Thus, some Gal80 amino acids are determinants for both the Gal80-Gal3 association and the Gal80 self-association, and Gal80 self-association may be required for binding to Gal4AD. We propose that the binding of Gal3 to the Gal80 monomer competes with Gal80 self-association, reducing the amount of the Gal80 dimer available for inhibition of Gal4.

TRANSCRIPTION of the galactose pathway genes (*GAL* genes) in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* is induced by galactose through the activities of the regulatory proteins, Gal4, Gal80, and Gal3 (*S. cerevisiae*) or Gal1 (*K. lactis*) (Carlson 1987; Johnston 1987; Lohr *et al.* 1995; Reece and Platt 1997; Schaffrath and Breunig 2000). The Gal4 transcriptional activator binds as a dimer to sites in the promoter regions of the *GAL* genes (Silver *et al.* 1984; Bram and Kornberg 1985; Giniger *et al.* 1985; Bram *et al.* 1986; Ma and Ptashne 1987b; Silver *et al.* 1988; Carey *et al.* 1989; Marmorstein *et al.* 1992). Although Gal4 binds to its binding sites in both the absence and the presence of galactose (Selleck and Majors 1987), its capacity to activate transcription is inhibited in the absence of galactose by Gal80, a protein that associates with the Gal4 transcriptional activation domain (Gal4AD) (Torchia *et al.* 1984; Yocum and Johnston 1984; Giniger *et al.* 1985; Lohr and Hopper 1985; Johnston *et al.* 1987; Lue *et al.* 1987; Ma and Ptashne 1987a; Selleck and Majors 1987; Chasman and Kornberg 1990; Leuther and Johnston 1992; Mizutani and Tanaka 2003). The evidence suggests that a dimer of Gal80 masks the Gal4AD (Melcher and Xu 2001).

Inhibition of the Gal4AD by Gal80 is reduced in response to galactose by binding of Gal3 (Gal1 in *K. lactis*) to Gal80 (Bajwa *et al.* 1988; Bhat and Hopper 1992; Suzuki-Fujimoto *et al.* 1996; Zenke *et al.* 1996; Blank *et al.* 1997; Yano and Fukasawa 1997; Platt and Reece 1998; Vollenbroich *et al.* 1999; Menezes *et al.* 2003). It is not known how the galactose-triggered binding of Gal3 to Gal80 relieves inhibition of Gal4AD. Data from some experiments appear to support the idea that Gal80 does not dissociate from Gal4 in response to galactose (Leuther and Johnston 1992; Platt and Reece 1998; Bhaumik *et al.* 2004). This would necessitate the entry of Gal3 into the nucleus, as the binding of Gal3 to Gal80 is required for activation of Gal4 (Blank *et al.* 1997). However, Gal3 is not detectable in the nucleus (Peng and Hopper 2000) and, when Gal3 was tethered to membranes outside the nucleus, galactose-mediated activation of Gal4 was normal (Peng and Hopper 2002). Moreover, the amount of Gal80 bound to Gal4 was found to decrease shortly after cells are exposed to galactose (Peng and Hopper 2002). Thus, the entry of Gal3 into the nucleus to form a Gal3-Gal80-Gal4 complex is questionable.

The evidence that galactose causes Gal3 to modulate the Gal4-Gal80 interaction is compelling, and the occurrence of Gal3-Gal80 and Gal4-Gal80 complexes is undisputed (Johnston *et al.* 1987; Lue *et al.* 1987; Ma and Ptashne 1987a; Chasman and Kornberg 1990; Yun *et al.* 1991; Leuther and Johnston 1992; Parthun and Jaehning 1992; Suzuki-Fujimoto *et al.* 1996; Wu *et al.* 1996; Zenke *et al.* 1996; Blank *et al.* 1997; Yano and Fukasawa 1997; Platt and Reece 1998; Vollenbroich *et al.* 1999; Melcher and Xu 2001; Timson *et al.* 2002; Menezes *et al.* 2003). Clearly, understanding the two binding reactions of Gal80 will be important to an overall understanding of the operation of the *GAL* gene switch.

There has been no systematic and focused effort to identify *GAL80* mutations that impair Gal80's capacity to bind Gal3 or Gal4AD. The only mutations of *GAL80* known to affect Gal80's binding to either Gal3 or Gal4

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TABLE 1

Oligonucleotide sequences used in this study

VP7	5′-AGCCATTATGAAGTTGTTATGCCTCTCTTGGAATTCTCCAAAAATAATCCGAACCTCAA GTATCTTTTCGTAGAATGGGCC-3′
VP8	5′-TTGAAAACCATTGCATTTATCCTGGAAAAGTACGAACTTGTCATGTATTGTAAAATATCG-3′
VP9	5′-GCCTGTTAAATCACCAAAATACATCTATGAAATCGGGAACGGTGTAGATCTGGTAACCAC AACATTTGGTCACACAATCG-3′
VP10	5′-GATATACTTCCATGATTTCTTTACCTGCATCATACCCCGGGTCTAAAGGAGCTTGTTGT CCATTGGCTAG-3′
VP11	5′-TTGAAACTTGAAGGCGATGCCGGCTTCGCAGAAATTTCAAATCTGGTCCTTTACTACAG TGGAACTAGAGCAAACGACTTCCCGCTAG-3′
VP12	5′-CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCCCGGAATTAGCTTGGCTGCAG GTCGA-3′
VP20	5′-AACAAAGGTCAAAGACAGTTGACTGTATCGCCGGAATTCCCGGGGATCCGCATGGACTAC AACAAGAGATC-3′
VP17-1	5′-AATAGTTCTTTCTTGAACATAACCTTCTGGCATGGCAGACTTGAAAAAGTCATGTTGTTT CATA-3′
VP33	5′-ATTAGCGGAATCAGGAGTGCAAAAAGAGAAAATAAAAGTAAAAAGGTAGGGCAACACAT AGTAACTTTTCTTAAAAGAATCAAAGACA-3′
VP34-1	5′-TTGTTTCTTTATAGAGTGTAAGAGGTATGAGTAAACTTTTAATATTTAAAGGTTGTTCC AAGAAGGTGTTTAGTGTTAAAGGGAACATTATAGGGTGTTAAGA-3′

were identified on the basis of one of two phenotypes: constitutive or uninducible expression of the *GAL* genes (Douglas and Pelroy 1963; Douglas and Hawthorne 1964, 1966; Nogi *et al.* 1977). We undertook a mutational analysis of the binding activities of the *S. cerevisiae* Gal80 protein. Our results suggest that dimerization of Gal80 and binding of a Gal80 monomer to Gal3 utilizes some of the same features of Gal80, whereas the binding of a Gal80 dimer to Gal4AD utilizes features of Gal80 that are unique to its dimer form. This distinction in the Gal80 binding modes could constitute a central element of the *GAL* gene switch mechanism.

MATERIALS AND METHODS

Yeast strains, media, and transformation: Yeast strain VP2-103 (*MAT*a *leu2-3,112 rtp1-901 his3*Δ*200 ade2-101 gal3*Δ*::ADE2 gal4*Δ *gal80*Δ *SPAL::URA3 GAL1::LacZ GAL1::HIS3@LYS2 can1*^{R} *cyh2*^{R}) was used as the host strain for all two-hybrid assays and selections. VP2-103 was derived from strain Mav103 (Vidal *et al.* 1996a,b) by disruption/replacement (Guldener *et al.* 1996) of the *GAL3* gene with a gal3Δ::*ADE2* cassette that was generated with PCR with primers VP33 and VP34-1 and *ADE2* template DNA. Yeast strain Sc725 (*MAT*a *ade1 ile leu2-3,112 ura3-52 trp1-HindIII his3-*Δ*1 MEL1 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 gal80-*Δ*BglII*) was used to evaluate the *GAL* gene switch phenotype conferred by all isolated *GAL80* mutations. The construction of Sc725 was described previously (Blank *et al.* 1997) following the method of Flick and Johnston (1990). The oligonucleotide sequences used in this study are listed in Table 1.

Yeast cells were propagated at 30°. YEP, synthetic complete (SC), or synthetic nutrient drop-out media were prepared as described by Rose *et al.* (1990) and supplemented with 2% (w/v) glucose or, in the case of the two-hybrid assays, 2% (w/v) glucose and 1% (w/v) galactose as indicated. Yeasts were transformed according to standard procedures (Chen *et al.* 1992). The 5-fluoroorotic acid (5-FOA)-containing media used for the reverse two-hybrid assay were prepared as described elsewhere (Vidal *et al.* 1996a).

Plasmids and bacteria: pAKS42 (2μ *TRP1, P*$_{ADH1}$*::DBGAL80*) is a 2μ, *TRP1* plasmid expressing amino acids 1–147 of Gal4 (DB) fused in frame with Gal80. pAKS37 (2μ, *LEU2 P*$_{ADH1}$*:: G4ADVP16*) is a 2μ, *LEU2* plasmid expressing amino acids 768–881 of Gal4 (Gal4AD) fused in frame with the VP16 transcriptional activation domain (VP16AD) consisting of the last 78 aa of the VP16 protein, an HSV type 1 transcriptional activation protein (Durfee *et al.* 1993; Vojtek and Hollenberg 1995). pG3VP16 (2μ, *LEU2, P*$_{ADH1}$*::GAL3VP16*) is a 2μ, *LEU2* plasmid expressing Gal3 fused in frame to VP16AD. pVP16Gal80 (2μ, *LEU2 P*$_{ADH1}$*::VP16Gal80*) is a 2μ, *LEU2* plasmid expressing VP16AD fused in frame to Gal80. All of these fusion proteins were expressed from the yeast ADH1 promoter. Plasmid constructions were carried out using standard recombinant DNA methods and involved multiple steps. All constructs were confirmed by DNA sequencing. The details are available upon request. *Escherichia coli* DH5α was used as the bacterial host.

PCR mutagenesis of *GAL80* and gap-repair generation of libraries in yeast: Mutagenesis of the *GAL80* gene was performed using Taq DNA Polymerase (Sigma, St. Louis) and the manganese (Mn)-dITP error-prone PCR method (Xu *et al.* 1999; Fenton *et al.* 2002). pAKS42 (2μ *TRP1*, *P*$_{ADH1}$*::DBGAL80*) encoding the two-hybrid bait fusion protein, DBGal80, was used as template for the Mn-dITP error-prone PCR reactions.

Each of four separate regions of the *GAL80* ORF was amplified to create four PCR product pools, which together represented the entire *GAL80* ORF. The four regions, together with the PCR primer sets were as follows: ~600 bp flanked by *Bgl*II sites (aa 5–204) with primers VP20 and VP17-1, ~270 bp flanked by *Apa*I and *Cla*I sites (aa 122–214) with primers VP7 and VP8, ~330 bp flanked by *Cla*I and *Nhe*I sites (aa 214–333) with primers VP9 and VP10, and ~300 bp flanked by *Nhe*I and *Sal*I sites (aa 333–435) with primers VP11 and VP12. Each of the four separated PCR product pools was used to reconstitute a cognate gapped *GAL80* gene in pAKS42 using the homologous recombination-based gap-repair method of Muhlrad *et al.* (1992). The four cognate gapped plasmids were created by the following digestions, respectively: *Bgl*II, *Apa*I and *Cla*I, *Cla*I and *Nhe*I, and *Nhe*I and *Sal*I. Each PCR

product pool and cognated gapped plasmid DNA were combined and used to transform cells of yeast VP2-103 bearing either the prey plasmid pAKS37 (encoding Gal4ADVP16) or the prey plasmid pG3VP16 (encoding Gal3VP16). Approximately 1×10^5 primary Leu^+ Trp^+ yeast prototrophs from the gap-repair transformation were obtained for each of the four PCR product pools. The Trp prototrophy arises as a consequence of homologous recombination between PCR product and its cognate gapped plasmid. These transformants were then subjected to the 5-FOA/*URA3*-based reverse two-hybrid selection (see below) to identify transformants expressing a DBGal80 fusion protein impaired for interaction with Gal4AD or Gal3.

Yeast two-hybrid assays: To detect Gal80-Gal4AD, Gal80-Gal3, and Gal80-Gal80 interactions with the classical (forward) yeast two-hybrid assay (Fields and Song 1989; Chien *et al.* 1991) we substituted the VP16AD for the Gal4AD, as the Gal4AD is one of Gal80's natural binding partners. DBGal80 chimeras consisting of the Gal4 DNA-binding domain fused to the N terminus of the wild-type or variant Gal80 and expressed from plasmid pAKS42 (*DBGAL80*) served as the UAS_{GAL}-binding bait for all two-hybrid interaction assays. The corresponding two-hybrid binding partners (preys) were as follows: the G4ADVP16 (Gal4VP16) chimera consisting of Gal4 amino acids 768–881 fused to the VP16AD and expressed from the ADH1 promoter on pAKS37, the Gal3VP16 chimera consisting of Gal3 fused to VP16AD and expressed from ADH1 promoter on pG3VP16, and VP16AD fused to the wild-type or variant Gal80 protein and expressed from the ADH1 promoter on pVP16Gal80. Yeast VP2-103, which contains the UAS_{GAL}-based reporter genes, *URA3* and *LacZ*, was used as the two-hybrid host strain.

The 5-FOA/*URA3*-based two-hybrid selection (reverse two-hybrid) (Vidal *et al.* 1996a) was used for the isolation of Gal80 variants defective in interaction with Gal4AD or Gal3, respectively. The two-hybrid reporter gene (*SPAL::URA3*) in strain VP-103 cells couples expression of *URA3* to an interaction between bait and prey. The *URA3*-encoded enzyme converts 5-FOA to a toxic product that inhibits cell growth and thus provides the selection for cells in which there is no or weak interaction of bait and prey proteins. The reverse two-hybrid selections, the subsequent forward two-hybrid screens, and the mutation confirmations were performed as follows. Primary Leu^+ Trp^+ yeast prototrophs from each of the gap-repair transformations (see section above) were separately plated at 1×10^4 cells per plate on two 15-mm agar plates lacking leucine and tryptophan and containing 0.2% (w/v) 5-FOA. Approximately 200–500 viable yeast colonies per each of two 5-FOA selection plates were recovered. Approximately 3000 survivors representing all four PCR product pools were obtained. These were subsequently scored for interaction in the forward direction two-hybrid system using *URA3* reporter-based colony growth on uracil-deficient media and a standard *LacZ* reporter-based β-galactosidase colony color assay (Montano 2001). The colonies showing no or weak interaction were processed for Western blot analyses. Approximately 300 isolates were shown by Western blot analyses to express normal cellular levels of full-length Gal80 protein. Plasmids were isolated from those colonies and were sequenced across the DNA region involved in the gap-repair event. Many were found to have no mutation or multiple mutations within the region representing the PCR product pool. Purified plasmids found to bear a single mutation within the gap-repaired region were retransformed into yeast, and the transformants were retested by the reverse and forward two-hybrid assays. Gal80 variant proteins were further evaluated by pull-down assays and phenotype analysis (see below).

Evaluation of the capacity of Gal80 variants to self-associate and to associate with wild-type Gal80: Each Gal80 variant was reconstituted as a two-hybrid prey construct, VP16Gal80, and tested in yeast ScVP2-103 (*SPAL10::URA, GAL1::lacZ gal4Δ, gal80Δ, gal3Δ*) for interaction with itself as bait (DBGal80) or with the wild-type Gal80 as bait. As well, we tested each Gal80 variant as a prey, VP16Gal80, for interaction with the wild-type Gal80 as bait (DBGal80). The expression levels of the two reporter genes in ScVP2-103 (*SPAL10::URA* and *GAL1::lacZ*) were independently scored to evaluate the relative levels of interaction exhibited by the various bait-prey pairs. Transformants containing bait and prey plasmids were grown to late log phase and the cell density was adjusted to 5×10^7 cells/ml. Seven-microliter samples of 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were spotted onto solid growth media containing 2% glucose and 1% galactose or onto Schleicher & Schuell (Keene, NH) Optitran filters on growth media containing 2% glucose and 1% galactose. The relative expression level of the *SPAL10::URA*3 reporter gene in colonies grown at 30° on media lacking uracil, tryptophan, and leucine was determined by the size of the colony, relative to that for the wild-type Gal80 self-association, attained at 3 and 4 days following spotting. The relative expression of the *GAL1::lacZ* reporter gene in colonies grown at 30° on solid media lacking tryptophan and leucine was determined by the intensity of blue color development, relative to that for wild-type Gal80 self-association, at 15-min intervals throughout a 3-hr period after initiating the colorometric colony assay for β-galactosidase. The assays were performed in triplicate using independent transformants.

Evaluation of the GAL gene switch phenotype conferred by Gal80 variants: Gal80 variants were reconstituted in the context of otherwise native Gal80 in plasmid $pGP15^{\Delta}$ and evaluated in yeast strain Sc725 for ability to inhibit Gal4 and respond to galactose-activated Gal3. The $GAL1_{UAS}$-controlled *HIS3* reporter gene in this strain couples growth on histidine-deficient media to *GAL* gene switch operation (Flick and Johnston 1990). The *GAL* gene switch phenotype was scored on dropout (d.o.) media lacking uracil and histidine (ura d.o., his d.o.) media containing the 10 mm 3-AT (3-amino-1,2,4-triazole from Sigma) and carbon sources glycerol (3% v/v)/lactic acid (2% v/v) or galactose (2% w/v)/glycerol (3% v/v)/lactic acid (2% v/v) as indicated.

Yeast whole-cell extracts: Yeast whole-cell extracts from mid-log phase yeast cells were prepared by vortexing with glass beads as previously described (Mylin *et al.* 1989; Blank *et al.* 1997). For Western blots, cell extracts from 1.5 ml of culture were prepared in 200 μl of buffer A (40 mm Tris-HCl, pH 7.4, 2 mm EDTA, pH 8.0, 2 mm DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/liter chymostatin, 1 mm PMSF, 0.157 μg/ml benzamidine, and 1 μg/ml bestatin). Extracts for pull-down assays were prepared in binding buffer: 20 mm HEPES, pH 7.6, 200 mm NaCl, 0.5% Triton X-100, 0.5 mm EDTA, 2 mm DTT, 5 mm $MgCl_2$, leupeptin; 1 μg/ml pepstatin; 1 μg/liter chymostatin; 1 mm PMSF; 0.157 μg/ml benzamidine; and 1 μg/ml bestatin. For the Gal80-Gal3 interaction assays ATP (at 4 mm) and galactose (at 100 mm) were present. Extracts were stored at −80°. Protein concentrations were estimated by the method of Peterson (1977).

SDS-PAGE and Western blots of yeast whole-cell extracts: SDS-PAGE gels (9%, acrylamide/bisacrylamide ratio, 37.5:1) were used to fractionate 80 μg of whole yeast cell protein. Proteins were electro-transferred to a nitrocellulose membrane. For detection of Gal80 or DBGal80 the blot was probed with rabbit anti-Gal80 polyclonal serum (Blank *et al.* 1997) at 1:200 dilution or monoclonal anti-Gal4 DNA-binding domain antibody (Santa Cruz Biotechnology) at a dilution of 1:4000, respectively. The secondary antibody used was horseradish peroxidase-linked anti-mouse antibody (Amersham Life Science). Probed blots were developed with the chemilumines-

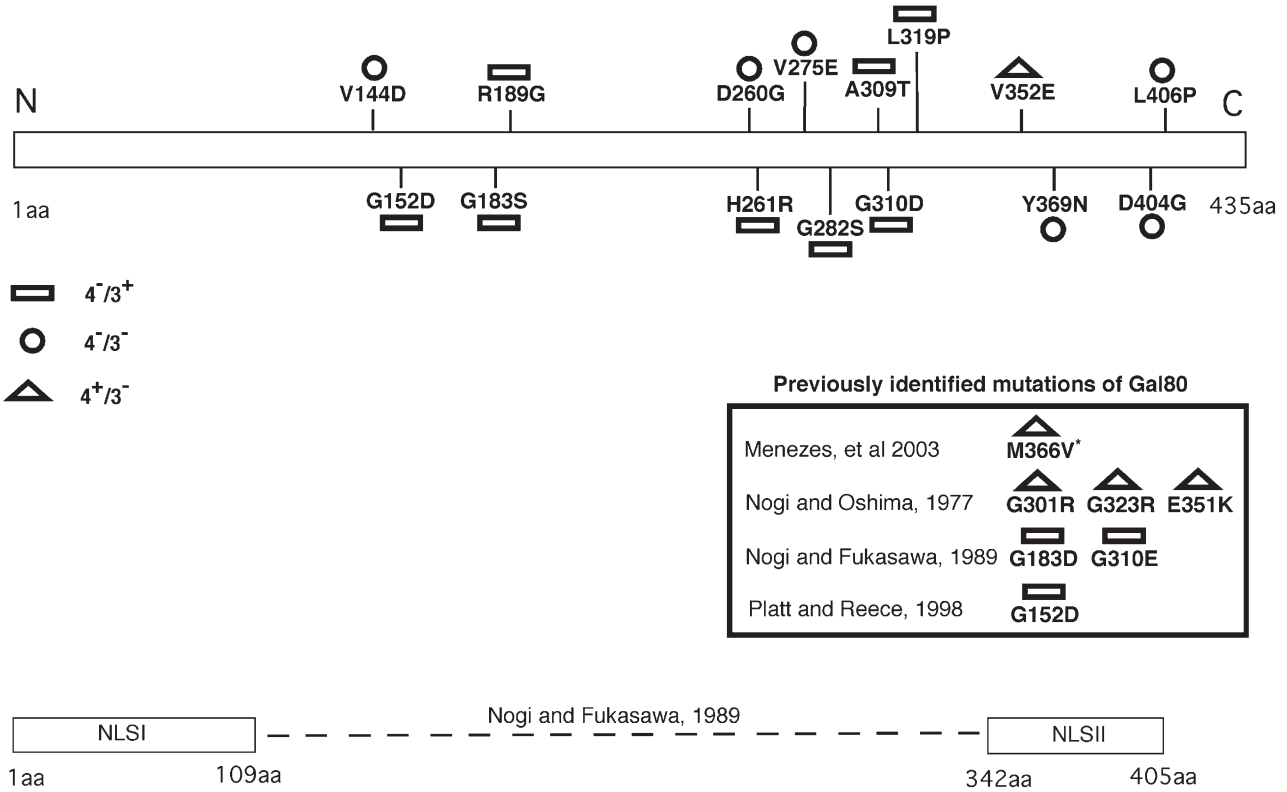

FIGURE 1.—Linear map of Gal80 showing distribution of new and previously isolated mutations and known motifs. (Rectangles) $4^-/3^+$ (Gal80 mutant variants that failed to bind to Gal4AD but retained binding to Gal3); (circles) $4^-/3^-$ (Gal80 mutant variants that failed to bind to Gal4AD and Gal3); and (triangles) $4^+/3^-$ (Gal80 mutant variant that retained binding to Gal4AD and failed to bind Gal3).

cence Western Lightning kit reagent from Perkin Elmer (Norwalk, CT).

***In vitro* pull-down assays:** Physical confirmation of the yeast two-hybrid genetic selection and screen was performed using the pull-down assay (KRAICHELY and MACDONALD 2001). The source of Gal80 for the pull-down assays was yeast strain Sc725 cultured in media containing the carbon sources glycerol, lactic acid, and galactose. The pull-down assays for physical interaction between Gal80 (wt and variants) and Gal4AD expressed in *E. coli* as GSTGal4AD(pEGSTG4AD) were performed essentially as described previously (SIL *et al.* 1999). The pull-down assays for physical interaction between Gal80 (wt and variants) and Gal3 were performed with extracts from yeast strain Sc725 (*gal80*Δ) expressing Gal80 on plasmid pGP15$^{\Delta}$ (*CEN ARS1 URA3 GAL80*) (PENG and HOPPER 2000) and extracts from yeast strain Sc787 (*gal80*Δ, *gal1*Δ, *gal3*Δ) expressing GSTGal3 on plasmid pMPW60 (P_{ADH2}-*GST-GAL3*) essentially as described previously (BLANK *et al.* 1997). The final pellet was subjected to SDS-PAGE fractionation (8% gel) and Western immunoblot analysis. To detect GSTGal3 and Gal80 the blots were probed with a mixture of rabbit anti-GST polyclonal antiserum at 1:1000 dilution and rabbit anti-Gal80 polyclonal antiserum at 1:200 dilution.

Homology modeling: Multiple sequence alignments were performed using ClustalX (HIGGINS and SHARP 1988). A homology model for *S. cerevisiae* Gal80 based on *Zymomonas mobilis* glucose fructose oxidoreductase (1h6d chain A) (NURIZZO *et al.* 2001) was derived using the MODWEB Modeling Server (FISER and SALI 2003). The models were rendered for illustration using the GRASP program (NICHOLLS *et al.* 1991).

RESULTS

Most Gal80 variants selected for impaired interaction with Gal4AD retained the capacity to interact with Gal3: To identify Gal80 amino acids that are required for binding to Gal4AD we used mutagenized libraries of DBGal80 as bait and Gal4ADVP16 as prey in the reverse two-hybrid selections. We identified nine single-amino-acid substitutions that cause severely reduced interaction with Gal4AD when retested in both the reverse and the forward two-hybrid assays. The positions of these amino acid substitutions within the Gal80 protein are illustrated in Figure 1. The forward and reverse two-hybrid assay results for all nine Gal80 variants tested with Gal4ADVP16 are shown in Figure 2A. The nine DBGal80 variants are full length and are present in the cell at levels similar to those in the wild type (Figure 3A). All nine Gal80 variants showed impaired physical interaction with GSTGal4AD in a pull-down assay (Figure 3C).

We next determined whether these amino acids are important for the interaction of Gal80 with Gal4AD or Gal3. All variants except D260G retained an appreciable capacity to interact with Gal3 in the two-hybrid assay (Figure 2B) and in a pull-down assay (Figure 3D). The eight variants that fail to bind to Gal4AD but retain binding to Gal3 are referred to as $4^-/3^+$ variants (Figure

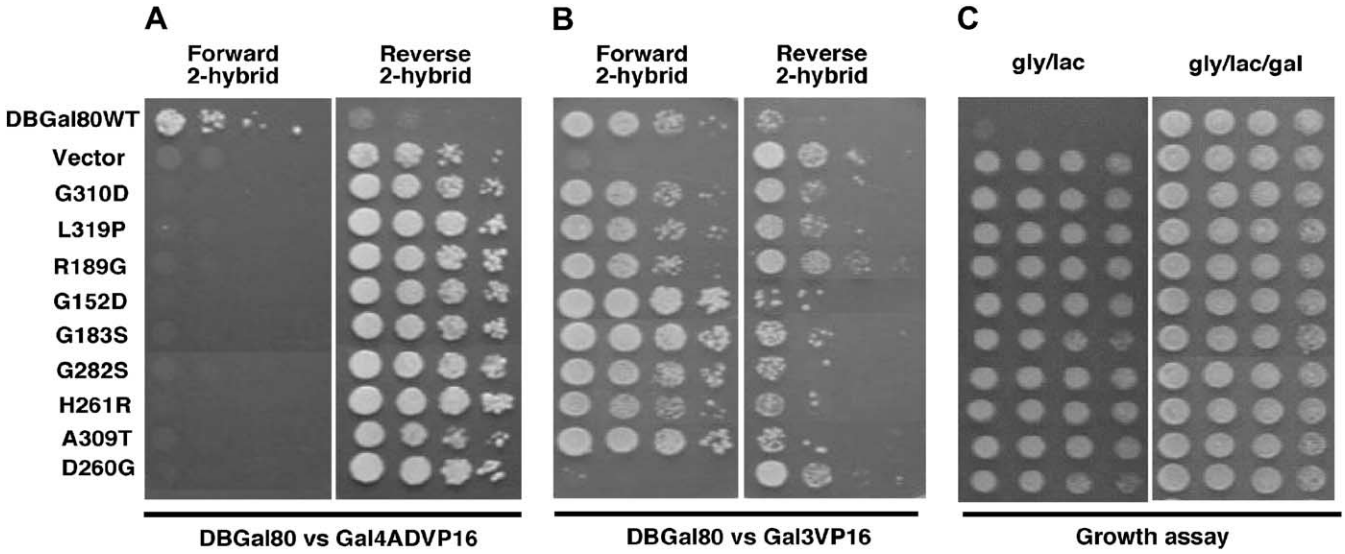

FIGURE 2.—Eight of the nine Gal80 single-amino-acid substitution variants selected as severely impaired in two-hybrid interaction with Gal4AD retain two-hybrid interaction with Gal3. Shown are forward and reverse two-hybrid interactions of Gal80 wild type and Gal80 single-amino-acid substitution variants as baits (DBGal80) with (A) Gal4AD (Gal4ADVP16) and (B) Gal3 (Gal3VP16) as prey. A 10-fold dilution series of yeast ScVP2 (*SPAL10::URA, gal4*Δ, *gal80*Δ, *gal3*Δ) transformants containing bait and prey plasmids were spotted onto leu trp ura d.o. synthetic media agar plates containing 2% glucose and 1% galactose for the forward two-hybrid assay or 2% glucose and 1% galactose and 0.2% 5-FOA for the reverse two-hybrid assay. (C) The phenotype conveyed by each Gal80 variant as determined by *GAL1*::*HIS3* reporter-dependent colony growth in the absence of histidine. Each Gal80 variant was expressed from the wild-type *GAL80* promoter on plasmid pGP15Δ in yeast strain Sc725.

1). The exceptional variant, D260G, that is impaired for interaction with both Gal4AD and Gal3, is referred to as a $4^-/3^-$ variant (Figure 1).

To determine the effects of these Gal80 single-amino-acid substitutions on the *GAL* gene switch we tested the ability of each variant Gal80 to complement *gal80*Δ. As

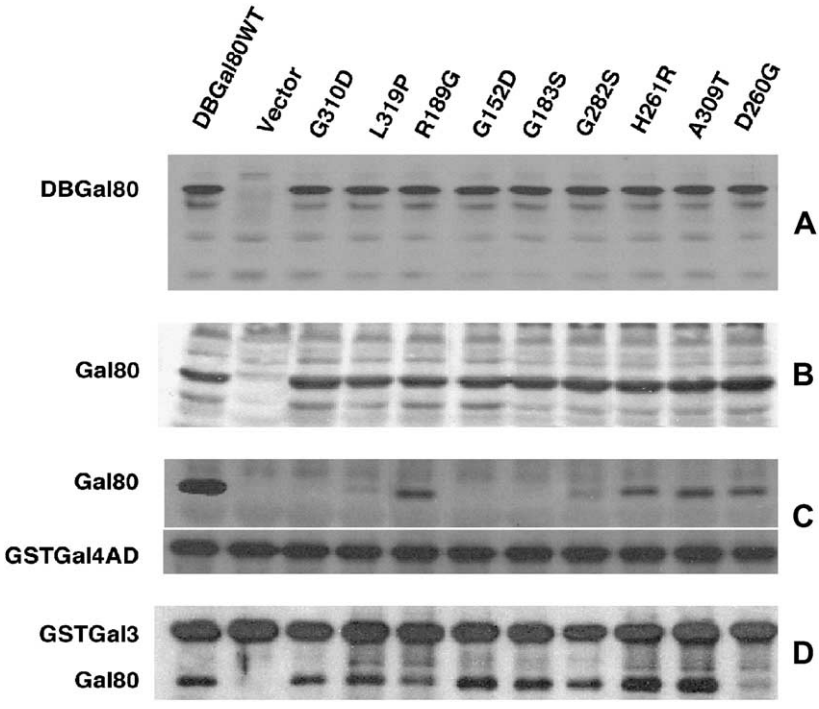

FIGURE 3.—Gal80 single-amino-acid substitution variants selected as impaired in interaction with Gal4AD (those in Figure 2) are full length, expressed at equivalent levels to wild-type Gal80, and behave physically in a pull-down assay in a manner consistent with their behavior in the two-hybrid assay. (A) SDS-PAGE immunoblot of Gal80 variants expressed as DBGal80 fusion proteins (two-hybrid bait form) from the ADH1 promoter on plasmid AKS42. DBGal80 proteins were probed with anti-DBGal4 antisera. (B) SDS-PAGE immunoblot of Gal80 variants (no fusion) expressed from the GAL80 promoter on plasmid pGP15Δ. Gal80 proteins were probed with anti-Gal80 antisera. (C) Pull-down assays for physical interactions of Gal80 variants (shown in Figure 2) with Gal4AD. (D) Pull-down assays for physical interactions of Gal80 variants (shown in Figure 2) with Gal3. For all pull-down assays the source of Gal80 was yeast strain Sc725 expressing Gal80 wild type or the designated Gal80 variant. For determination of Gal80 interaction with Gal4AD (C) the Gal80 extract was incubated with a preformed complex consisting of glutathione (GT)-Sepharose and GSTGal4AD (expressed in *E. coli*). For determination of Gal80 interaction with Gal3 (D) the Gal80 extract was incubated in the presence of 2% galactose with an aliquot of yeast extract (strain Sc787) containing GSTGal3. Gal80 and GSTGal3 were detected on the same blot by using a mixture of rabbit polyclonal anti-Gal80 (1:200 dilution) and rabbit polyclonal anti-GST (1:3000 dilution).

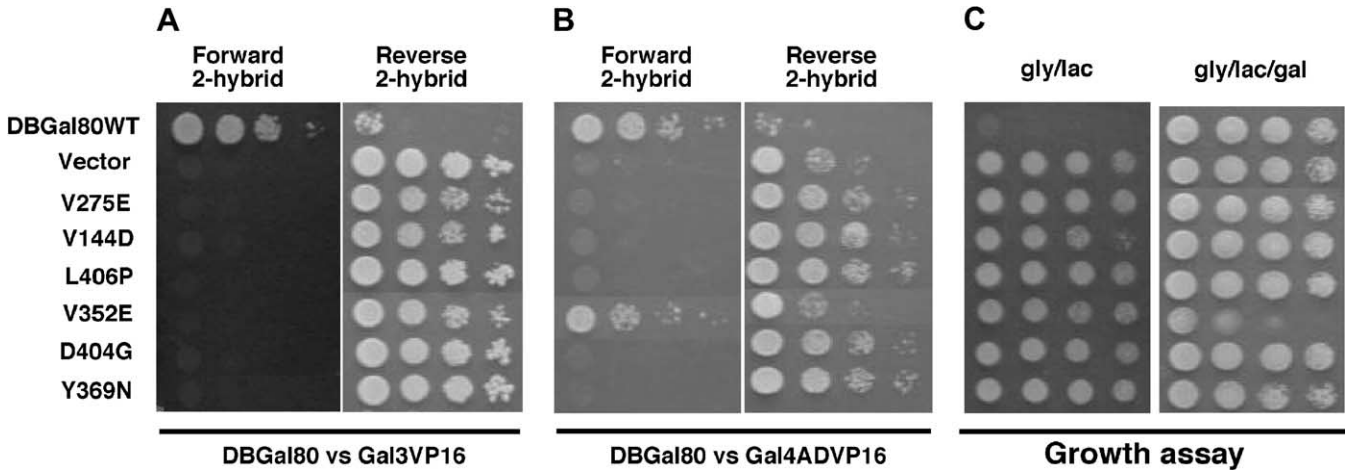

FIGURE 4.—Five of the six Gal80 single-amino-acid substitution variants that were selected as severely impaired for two-hybrid interaction with Gal3 were also severely impaired for interaction with Gal4AD. Shown are forward and reverse two-hybrid interactions of Gal80 wild type and Gal80 single-amino-acid substitution variants as baits (DBGal80) with (A) Gal3 (Gal3VP16) and (B) Gal4AD (Gal4ADVP16) as prey. A 10-fold dilution series of yeast ScVP2 (*SPAL10::URA, gal4*Δ*, gal80*Δ*, gal3*Δ) transformants containing bait and prey plasmids were spotted onto leu trp ura d.o. synthetic media agar plates containing 2% glucose and 1% galactose for the forward two-hybrid assay or containing 2% glucose and 1% galactose and 0.2% 5-FOA for the reverse two-hybrid assay. (C) The phenotype conveyed by each Gal80 variant, as determined by *GAL1::HIS3* reporter gene-dependent colony growth in the absence of histidine (his d.o. media). Each Gal80 variant was expressed from the wild-type *GAL80* promoter on plasmid pGP15Δ in yeast Sc725 cultured on uracil- and histidine-deficient synthetic media containing 10 mM 3-AT and either glycerol (3%)/lactic acid (2%) (gly/lac) or glycerol (3%)/lactic acid (2%)/galactose (2%) (gly/lac/gal).

expected, each of the nine Gal80 variants that fail to bind to Gal4AD produced the constitutive phenotype in which Gal4 is active in the absence of galactose (Figure 2C). Each of the variant Gal80 proteins was found to be present in the cell at a steady-state level similar to that in wild-type Gal80 (Figure 3B). Thus, each Gal80 variant produces a cellular *GAL* gene switch phenotype that is consistent with its binding activities.

Most Gal80 variants selected directly for impaired interaction with Gal3 were also severely impaired for interaction with Gal4AD: By a selection procedure similar to the one described above except using Gal3Vp16 as the prey plasmid we identified six Gal80 single-amino-acid substitutions that weaken the Gal80-Gal3 interaction (Figure 4A; amino acid changes shown in Figure 1). These six DBGal80 variants are full length and are present in the cell at levels similar to those in wild type (Figure 5A). All six variants showed impaired interaction with GSTGal3 by a pull-down assay (Figure 5C). We conclude that these six Gal80 amino acids are determinants of the Gal80 interaction with Gal3.

Each of these six DBGal80 variants was tested as bait for ability to interact with Gal4AD. All variants except the V352E were severely impaired for interaction with Gal4AD (Figures 4B and 5D). The five variants that failed to bind to both Gal4AD and Gal3 are referred to as $4^-/3^-$ variants (Figure 1). The exceptional variant, V352E, that was severely impaired for interaction with Gal3 but not appreciably impaired for interaction with Gal4AD is referred to as a $4^+/3^-$ variant (Figure 1).

When introduced into native Gal80, each of the six Gal80 variants produced the constitutive phenotype in which Gal4 is active in the absence of galactose (Figure 4C, gly/lac). Variant V352E interacts with Gal4AD as well as the wild-type Gal80 protein in a pull-down assay, but it is slightly impaired for binding to Gal4AD in the two-hybrid test. Because even a slight impairment in binding to Gal4AD would be expected to give rise to the constitutive phenotype, the results for variant V352E are not unexpected. All six of these Gal80 variants are expressed in the cell at a steady-state level similar to that in wild-type Gal80 (Figure 5B). Thus, overall, each Gal80 variant produces a cellular *GAL* gene switch phenotype that is consistent with its binding activities.

The major finding from these results is that five of the six Gal80 variants selected as Gal3 nonbinders are also severely defective in binding to Gal4AD. These results are in striking contrast to our observation that eight of the nine Gal80 variants selected as Gal4AD nonbinders retained the capacity to bind to Gal3.

Most Gal80 variants selected for impaired interaction with Gal3 fail to self-associate, whereas most variants selected for impaired interaction with Gal4AD retain the capacity to self-associate: Because Gal80 binds to Gal3 as a monomer (TIMSON *et al.* 2002) and to Gal4 as a dimer (MELCHER and XU 2001) we considered the possibility that the pronounced difference in the behavior of the two classes of Gal80 variants stems from differences in the Gal80 monomer and dimer binding preferences. We observed a striking difference in self-association capacities of those Gal80 mutants selected for impaired binding to Gal4AD (listed in Figure 2) and

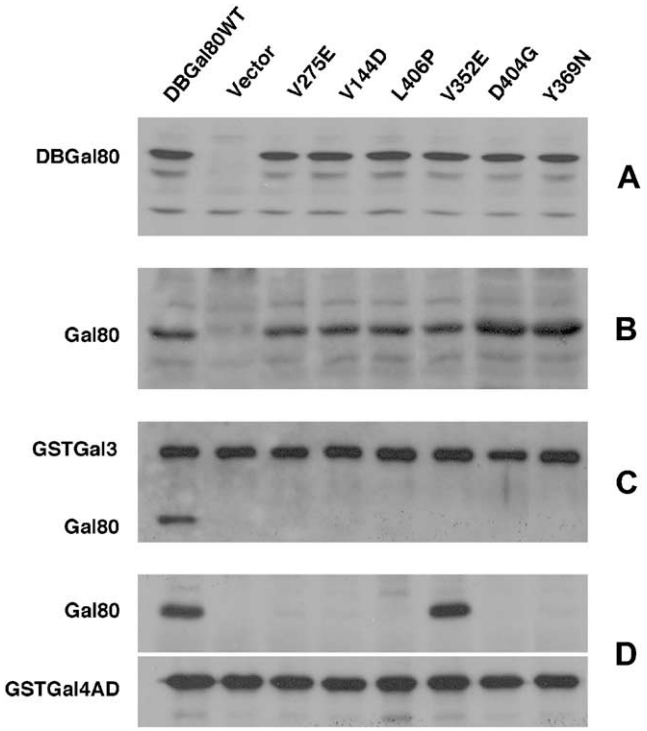

FIGURE 5.—Gal80 single-amino-acid substitution variants selected as impaired for interaction with Gal3 (those in Figure 4) are full length, expressed at equivalent levels to wild-type Gal80, and behave physically in a pull-down assay in a manner consistent with their behavior in the two-hybrid assay. (A) SDS-PAGE immunoblot of Gal80 variants in the form of DBGal80 fusion proteins (two-hybrid bait form) expressed from the *ADH1* promoter on plasmid AKS42. DBGal80 proteins were probed with anti-DBGal4 (aa 1–147) antisera. (B) SDS-PAGE immunoblot of Gal80 variants in the native Gal80 form (no fusion) expressed from the *GAL80* promoter on plasmid pGP15Δ. Gal80 proteins were probed with anti-Gal80 polyclonal rabbit antisera. (C) Pull-down assays for physical interactions of Gal80 variants (shown in Figure 4) with Gal3. (D) Pull-down assays for physical interactions of Gal80 variants (shown in Figure 4) with Gal4AD. Details for pull-down assays are the same as given in the Figure 3 legend.

TABLE 2

Self-association tests for Gal80 variants

Original Gal4 nonbinders	Mutant *vs.* mutant	Mutant *vs.* WT
G310D	++	+++
L319P	+	+++
R189G	++	+++
G152D	++	+++
G183S	+	+++
G282S	++	+++
H261R	++	+++
A309T	+	+++
D260G	−	−
Original Gal3 nonbinders	**Mutant *vs.* mutant**	**Mutant *vs.* WT**
V275E	−	++
V144D	−	++
L406P	−	++
V352E	++++	++++
D404G	−	++
Y369N	−	++
Controls		
DBGal80 WT	++++	++++
Vector	−	−

Gal80 single-amino-acid substitution variants were tested for self-association and for association with the wild-type Gal80 in the standard yeast two-hybrid assay. Each variant was reconstituted as a prey construct, VP16Gal80, and tested in yeast ScVP2-103 (*SPAL10::URA, GAL1::lacZ gal4*Δ, *gal80*Δ, *gal3*Δ) for interaction with itself and with the wild-type Gal80, respectively, as baits (DBGal80). As well, we tested each Gal80 variant as a prey, VP16Gal80, for interaction with the wild-type Gal80 as bait, DBGal80. Yeast transformants containing bait and prey plasmids were spotted, with 10-fold dilutions, onto leu trp d.o. for LacZ reporter or leu trp ura d.o. for URA3 reporter. Synthetic media agar plates containing 2% glucose and 1% galactose were used.

those selected for impaired binding to Gal3 (listed in Figure 4) (Table 2). Eight of the nine Gal80 variants selected as Gal4AD nonbinders showed measurable self-association (homodimerization) in the two-hybrid assay, although at levels appreciably lower than those observed for wild-type Gal80 (Table 2). The one exceptional variant that showed no self-interaction, D260G, was the one that had been determined to also be impaired for interaction with Gal3 (listed in Figure 2). In contrast, five of the six Gal80 variants selected as Gal3 nonbinders showed no detectable two-hybrid self-association (Table 2). Those five were the ones that were determined to also be impaired for interaction with Gal4AD (listed in Figure 4). The one exceptional variant that showed a wild-type level of self-interaction, V352E, was the one that retained an appreciable capacity to interact with Gal4AD (Figure 4). Thus, without exception, *GAL80* mutations that severely impair the capacity of Gal80 to bind to Gal3 and Gal4AD also impair the capacity of Gal80 to self-associate.

Gal80 variants that are defective in binding to Gal3 and Gal4AD and in self-association might represent grossly misfolded proteins (although this seems unlikely as all are present in yeast at levels equivalent to the wild-type protein; Figures 3 and 5). Each variant was tested as bait (DBGal80 form) and prey (Gal80VP16 form) against a Gal80 wild-type prey (DBGal80) or a Gal80 wild-type bait (Gal80VP16), respectively (Table 2). All of the Gal80 variants except D260G were capable of interacting with native Gal80 protein. The five Gal80 variants that were selected as Gal3 nonbinders and shown not to interact with Gal4AD or to self-associate (homodimerize) showed a level of interaction with Gal80 that appears to be about half that observed for native Gal80 self-association (Table 2). The eight Gal80

variants that were selected as Gal4AD nonbinders and shown to retain the capacity to bind to Gal3 and to self-associate (homodimerize) showed a level of interaction with Gal80 that we estimate to be about three-fourths that observed for the Gal80 self-association (Table 2). These results suggest that the Gal80 variant proteins, except possibly D260G, are not grossly misfolded.

Gal80 functional domains affected by the mutations: All eight of the $4^-/3^+$ class of Gal80 substitutions lie between amino acids 152 and 319 (Figure 1). Substitutions at residues 152, 183, and 310 had been isolated previously as mutations that confer constitutive expression of the *GAL* genes (Nogi and Fukasawa 1989; Platt and Reece 1998). Also within this region of *GAL80* are two mutations of the $4^-/3^-$ class. One of those, variant D260G, was originally selected as a Gal4AD nonbinder; the other, V275E, was originally selected as a Gal3 nonbinder. Three $4^-/3^-$ mutations, Y369N, D404G, and L406P, fall in the region of Gal80 between amino acids 351 and 406.

The region spanning aa 350–406 appears to be a hotspot for Gal3 nonbinder mutations. Three $4^-/3^-$ Gal80 variants identified in this work (Y369N, D404G, and L406P), together with the previously identified *S. cerevisiae GAL80S*$^{-2}$ variant (E351K), lie in this region. The *GAL80S*$^{-2}$ allele was identified as a dominant, non-inducible mutant and was shown to result in a Gal80 protein that binds to Gal4 but not to Gal3 (Nogi *et al.* 1977; Nogi and Fukasawa 1984; Yano and Fukasawa 1997). The V352E Gal80 variant ($4^+/3^-$) we identified is yet another example of a Gal3 nonbinder mutation affecting amino acids in this region. Moreover, the *K. lactis* Gal80 M366V substitution (corresponding to the *S. cerevisiae* Gal80 residue M350) impairs the capacity of the *K. lactis* Gal80 protein to bind the *K. lactis* Gal1 protein, a Gal3 ortholog (Menezes *et al.* 2003).

It is striking that this region rich in Gal3 nonbinder mutations coincides with a nuclear localization sequence, NLSII (Nogi and Fukasawa 1989) (Figure 1). However, none of the variants that alter this region of Gal80 change the subcellular distribution of Gal80 (determined by fluorescence microscopy of Gal80-GFP; V. Pilauri and J. Hopper, data not shown).

DISCUSSION

Gal3-binding determinants and nuclear localization sequences overlap: It is striking that five of eight Gal80 single-amino-acid substitutions shown to impair Gal80's capacity to bind to Gal3 map within or in close proximity to NLSII, one of the two previously identified nuclear localization sequences (Nogi and Fukasawa 1989). This finding suggests the possibility that a surface involved in binding to Gal3 overlaps with the NLSII. If this were the case, the binding of Gal3 to Gal80 could potentially mask NLSII. Because the galactose-triggered Gal3-Gal80 association takes place predominantly, if not exclusively, in the cytoplasm (Peng and Hopper 2000, 2002), masking of the Gal80 NLSII by Gal3 would be expected to interfere with the nuclear entry of a Gal80-Gal3 complex established in the cytoplasm. This notion is consistent with the observation that in cells expressing a Gal3GFP fusion no GFP fluorescence is detectable in the nucleus up to 90 min following the addition of galactose (G. Peng and J. Hopper, unpublished observations), long after the initiating step in *GAL* gene induction has been executed (Yarger *et al.* 1984; Torchia and Hopper 1986; Schultz *et al.* 1987; Dunn *et al.* 1999; Bryant and Ptashne 2003). Unfortunately, our tests for Gal3 inhibition of Gal80 nuclear entry in the presence of galactose have been hampered by the low level of Gal80 protein in cells prior to and shortly after galactose induction (P. Darminio and J. Hopper, unpublished observations).

No single-amino-acid substitutions causing impairment of Gal80's capacity to bind to Gal3 were identified within the region spanning aa 321–341. This region has been suggested to be the inducer response domain on the basis of the observation that Gal80 lacking this domain is unable to bind to Gal3 but retains its capacity to bind to Gal4 (Nogi and Fukasawa 1989; Yano and Fukasawa 1997). However, a possible direct involvement of amino acids within this domain in the binding of Gal80 to Gal3 has recently been called into question by results showing that a *K. lactis* Gal80 peptide containing aa 267–404, the region corresponding to the *S. cerevisiae* Gal80 aa 321–341, does not interact with the *K. lactis* Gal1 protein (Menezes *et al.* 2003). Consistent with this result, we observed that Gal80 aa 1–390, which lacks only the C-terminal 45 amino acids, fails to interact with Gal3 (data not shown). Thus, it seems unlikely that the region spanning aa 321–341 binds to Gal3. An alternative possibility is that the region spanning aa 321–341 might provide structural elements required for proper presentation of Gal80 residues in a separate region that compose part of the Gal3-binding surface. Whether the region spanning aa 351–406 that is a hotspot for Gal3 nonbinder variants is part of the Gal3-binding surface remains to be determined.

The behavior of Gal80 variants suggests a role for the Gal80 monomer-dimer equilibrium in the *GAL* gene switch: Most of the *GAL80* mutations selected for impairment of Gal80's capacity to bind Gal4AD do not severely impair Gal80's capacity to bind Gal3 or to self-associate, whereas most of the *GAL80* mutations selected for impairment of Gal80's capacity to bind Gal3 also severely impair Gal80's capacity to bind Gal4AD and to self-associate. A parsimonious interpretation of these results is that the latter class of mutations identifies Gal80 amino acids that are determinants for both Gal3 binding and self-association and that Gal80 self-association is in turn required for wild-type levels of binding to Gal4AD. We further propose that a surface of Gal80 involved in binding Gal3 is also involved in self-association and that

a surface required for Gal80 binding to Gal4AD is created upon Gal80 self-association. These notions predict that at least some mutations selected on the basis of impaired binding of Gal80 to Gal4AD would not severely impair the binding of Gal80 to Gal3 or self-association, as the latter binding activities would not depend on features unique to the Gal80 dimer. Indeed, of the nine variants obtained in our selection for *GAL80* mutations that impair Gal80's binding to Gal4AD, only one, D260G, was found to also severely impair Gal80's binding to Gal3.

We note that our selection for Gal4AD nonbinder variants of Gal80 did not yield the variants V144D, V275E, Y369N, D404G, and L406P, even though these Gal3 nonbinders were shown by secondary screen to be considerably impaired for binding to Gal4AD. A likely possibility is that these Gal3 nonbinders are impaired for Gal4AD binding only as a consequence of their defects in self-association and that their intrinsic defects in self-association are suppressed by the very strong dimerization domain contained within the Gal4 amino acids 1–147 (Carey *et al.* 1989; Marmorstein *et al.* 1992; Hidalgo *et al.* 2001) that constitute the N terminus of the two-hybrid bait, DBGal80. Suppression of the self-association defects of these DBGal80 variant proteins could be sufficient to result in 5-FOA toxicity.

A working hypothesis: *How Gal80 dimer and monomer binding modes might affect the GAL gene switch:* On the basis of our results and the previously observed composition of complexes of Gal80-Gal3 and Gal80-Gal4AD, we propose that Gal3 competes with Gal80 self-association and that the galactose-triggered binding of Gal3 to Gal80 shifts Gal80 from the dimer to the monomer. A decrease in the level of the dimer, the form of Gal80 we hypothesize to be capable of binding to and inhibiting Gal4AD, would lead to active Gal4 and *GAL* gene transcription. A role of a Gal80 monomer-dimer equilibrium in the *GAL* gene switch can be readily integrated with the previously proposed galactose-triggered Gal3-Gal80 association in the cytoplasm and reduced binding of Gal80 to Gal4 (Peng and Hopper 2000, 2002) (Figure 6).

A speculative Gal80 homology model depicting the putative distribution of identified amino acid substitutions: The structure of Gal80 is currently unknown. Gal80 has been predicted to belong to the GFO/IDH/MOCA superfamily of proteins, which contain a Rossman fold (Aravind and Koonin 1998). Two Gal80 sequences, Gal80 of *S. cerevisiae* and Gal80 of *K. lactis*, were submitted independently to the MODWEB server. In each case, the MODWEB server selected glucose-fructose oxidoreductase (GFOR), which is identical to Gal80, as the single template. Although Gal80 is a homodimer, whereas GFOR is a homotetramer, these proteins have been predicted to share the same fold, or tertiary structure (Aravind and Koonin 1998). This prediction is warranted, as the fold of the GFOR monomer is largely unperturbed when the first 22 residues are removed,

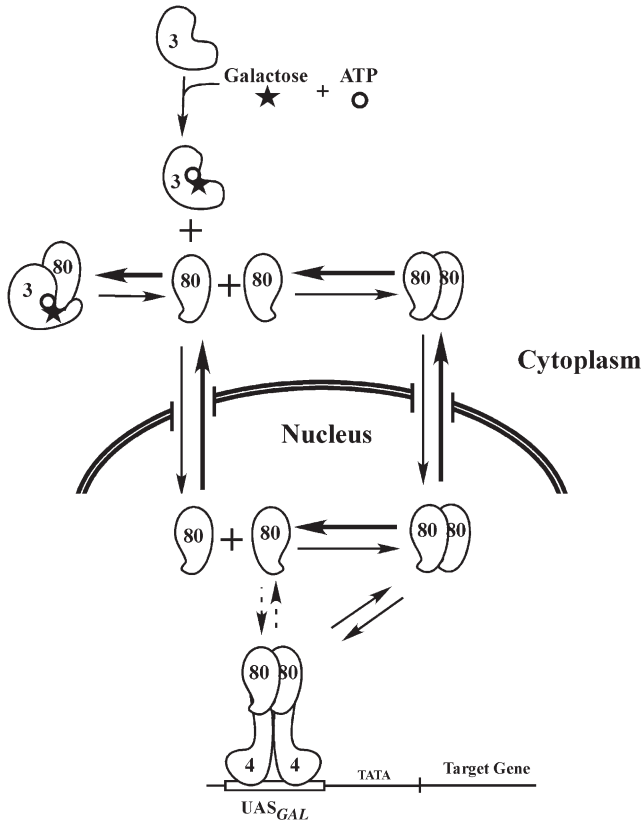

Figure 6.—Schematic of proposed *GAL* gene switch model depicting Gal80's monomer-dimer equilibria and Gal80 monomer-Gal3 and Gal80 dimer-Gal4 binding preferences in the context of the previously proposed linked-equilibrium scheme of Peng and Hopper (2002). We have indicated shuttling for both the monomer and the dimer forms of Gal80, but it is unknown which form or forms of Gal80 shuttle between nucleus and cytoplasm.

even though the quaternary fold has been affected (Lott *et al.* 2000). Such behavior suggests that the oligomeric state of the GFOR enzyme does not have a large effect on the tertiary structure (Lott *et al.* 2000).

Residues 6–433 of *S. cerevisiae* Gal80 and 6–312 of *K. lactis* Gal80 could be modeled onto the structure of GFOR. The residues identified by the binding data from this study were mapped onto the Gal80 homology model (Figure 7) along with the positions of three previously identified amino acid substitution variants of *S. cerevisiae* Gal80 (G301R, G323R, and E351K) that are defective for binding to Gal3 (Nogi *et al.* 1977). We also show the putative position of M350, which corresponds to a *K. lactis* Gal80 single-amino-acid substitution, M366V (Menezes *et al.* 2003), that impairs the capacity of the *K. lactis* Gal80 protein to bind to the *K. lactis* Gal1 protein. The positions of G152 and D260, representing mutations isolated in this study, are not visible on the surface of the homology model, as these residues are buried.

Most notably, the single-amino-acid substitutions that map to the surfaces of the model segregate onto two

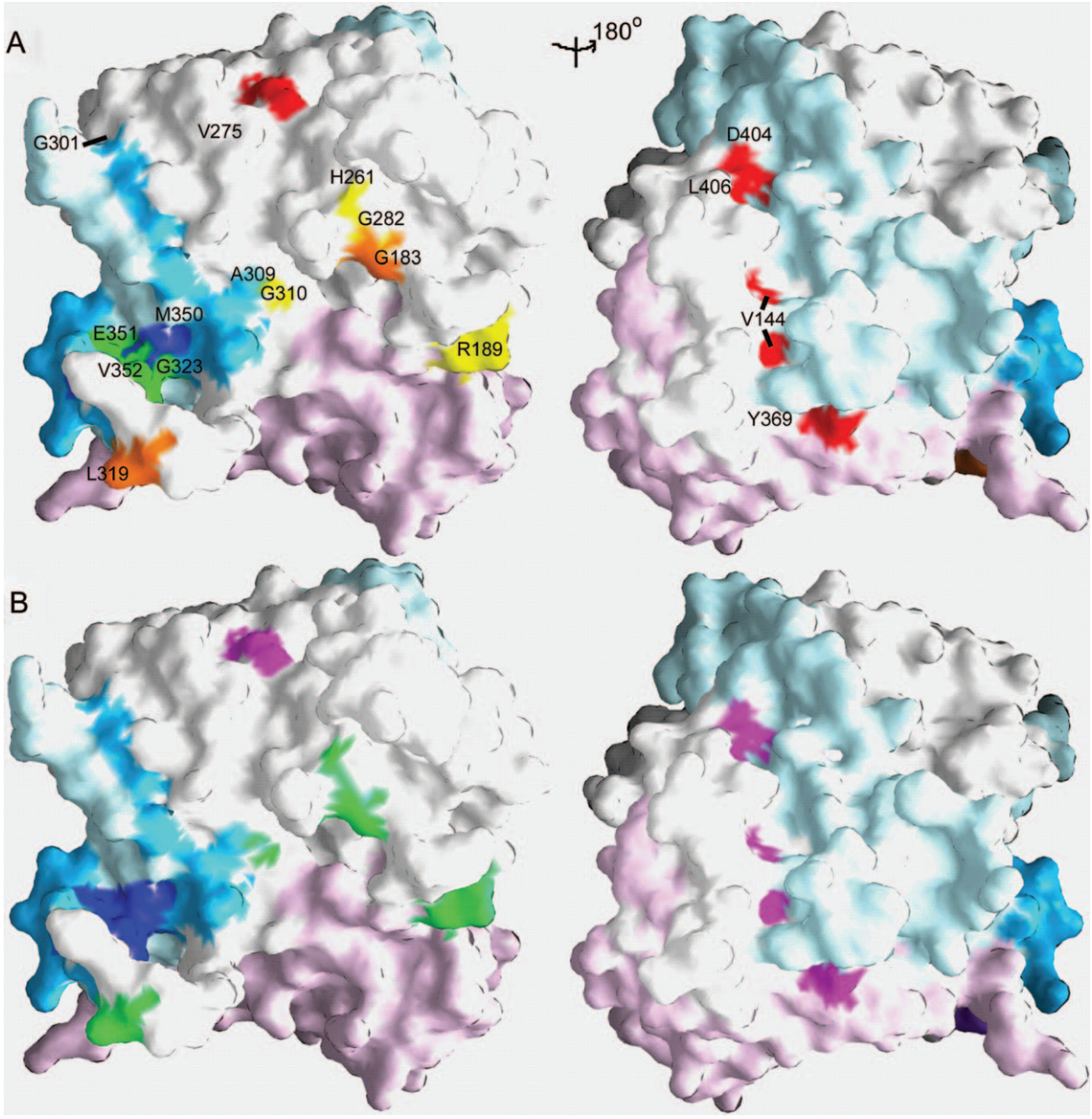

FIGURE 7.—Many of the mutations map to the putative molecular surface of Gal80 and co-localize. The molecular surface of the Gal80 monomer is illustrated; NLSI and NLSII are tinted light pink and light blue, respectively. The previously identified inducer region is colored mid-blue (residues 322–340). They are adjacent in space. (A) Mutations that affect Gal80:Gal80 self-association are colored according to the following scheme: red, complete impairment; orange, severe impairment; yellow, moderate impairment; and green, no impairment. Mutations identified in previous studies for which no self-association data are available are colored according to their Gal4- and Gal3-binding character. (B) Mutations that affect the interaction of Gal3 and/or Gal4 are colored according to the following scheme: Gal4−/Gal3+, green; Gal4−/Gal3−, magenta; and Gal4+/Gal3−, deep blue. The right-hand panel has been rotated 180° along the *y*-axis relative to the left-hand panel, to show the back of the model. Amino acid residues are labeled according to the aa sequence of *S. cerevisiae* Gal80.

opposite faces. Four of the five single-amino-acid substitutions selected for impairment of Gal80's binding to Gal3 and found to also impair its binding to Gal4AD and self-association lie on one face of the model (see V144D, Y369N, D404G, and L406P) (red residues, Figure 7, right). These mutations lie between NLSI and NLSII. Thus, NLSI and NLSII are adjacent to residues implicated in the binding of Gal80 to Gal3. Seven of the nine single-amino-acid substitutions selected for impairment of Gal80 binding to Gal4AD fall on the opposite face of the model (Figure 7, left), and they retain Gal80's capacity to bind to Gal3 and to self-associate. The distribution of the two distinct classes of Gal80 variants on the model of the monomer is consistent with the implications from our genetic data concerning the Gal80 monomer and dimer binding activities.

In summary, our evaluation of the binding profiles of the Gal80 variants identified by this work leads us to

propose that the *GAL* gene switch is due to the galactose-induced binding of the Gal3 protein to a Gal80 monomer, which competes with Gal80 self-association. Since the binding of Gal4 to Gal80 requires a multimeric form of Gal80, most likely a dimer, this would prevent Gal80 from binding to and inhibiting Gal4.

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