MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2566–2575 Vol. 17, No. 5
0270-7306/97/$04.00+0

Novel Gal3 Proteins Showing Altered Gal80p Binding Cause Constitutive Transcription of Gal4p-Activated Genes in *Saccharomyces cerevisiae*

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Received 23 September 1996/Returned for modification 18 November 1996/Accepted 24 February 1997

Gal4p-mediated activation of galactose gene expression in *Saccharomyces cerevisiae* normally requires both galactose and the activity of Gal3p. Recent evidence suggests that in cells exposed to galactose, Gal3p binds to and inhibits Gal80p, an inhibitor of the transcriptional activator Gal4p. Here, we report on the isolation and characterization of novel mutant forms of Gal3p that can induce Gal4p activity independently of galactose. Five mutant *GAL3*^{c} alleles were isolated by using a selection demanding constitutive expression of a *GAL1* promoter-driven *HIS3* gene. This constitutive effect is not due to overproduction of Gal3p. The level of constitutive *GAL* gene expression in cells bearing different *GAL3*^{c} alleles varies over more than a fourfold range and increases in response to galactose. Utilizing glutathione *S*-transferase–Gal3p fusions, we determined that the mutant Gal3p proteins show altered Gal80p-binding characteristics. The Gal3p mutant proteins differ in their requirements for galactose and ATP for their Gal80p-binding ability. The behavior of the novel Gal3p proteins provides strong support for a model wherein galactose causes an alteration in Gal3p that increases either its ability to bind to Gal80p or its access to Gal80p. With the Gal3p-Gal80p interaction being a critical step in the induction process, the Gal3p proteins constitute an important new reagent for studying the induction mechanism through both in vivo and in vitro methods.

Galactose-induced expression of the galactose pathway genes (*GAL* genes) in the yeast *Saccharomyces cerevisiae* is determined by the functional interplay of three pathway-specific regulatory proteins: Gal3p, Gal4p, and Gal80p. The way these proteins function in repressing *GAL* gene transcription in the absence of galactose and activating *GAL* gene transcription in the presence of galactose has been worked out in broad outlines (reviewed in references 31, 32, and 43). Gal4p binds to a specific regulatory DNA site (UAS_{Gal}) upstream of the *GAL* genes (12, 25, 47) and causes transcriptional activation through functions specified by amino acids distinct from those in the DNA binding domain (39, 45). Although Gal4p remains bound at the UAS_{Gal} site in the absence of galactose (25, 29, 42, 66), it cannot activate transcription because of the particular nature of its association with Gal80p (15, 35, 37, 46). The presence of galactose in the absence of glucose triggers an alteration of the Gal80p-Gal4p relationship (38, 48, 58, 59) that results in rapid activation of Gal4p-dependent transcription. Gal3p is the key to understanding this alteration. Without Gal3p, or its redundant function within Gal1p (a galactokinase protein) (7, 8, 10, 49), the *GAL* system is not inducible. Gal3p somehow responds to the presence of galactose by transmitting an induction signal. The ultimate target of this signal must be the Gal80p-Gal4p complex, since in the absence of Gal80p function neither galactose nor Gal3p (nor Gal1p) is necessary for Gal4p-dependent gene expression (19, 20, 75).

Until recently it was thought that Gal3p acted catalytically in response to galactose to produce an inducer or coinducer molecule (13, 76) that would presumably interact with Gal80p (31, 55, 56, 59). However, the discovery that high-level production of Gal3p (or Gal1p) leads to constitutive transcription of the *GAL* genes ruled out such a model (8, 73, 80). The new findings suggested that galactose binds to Gal3p and facilitates its transition to an active form that under the simplest model would bind to the Gal4p-Gal80p complex (7, 8). Support for this notion has recently been obtained through the discovery that Gal3p and its Gal1p homolog in the yeast *Kluyveromyces lactis* can interact with Gal80p (73, 80).

The overall role of Gal3p in the induction of *GAL* gene transcription now seems clear. However, we still lack a mechanistic understanding of how galactose affects the activity of Gal3p and how the Gal3p-Gal80p interaction leads to activation of Gal4p. To learn more about Gal3p activity, we set out to isolate *GAL3* mutants conferring galactose-independent activation of Gal4p. In this report, we describe the isolation and characterization of such *GAL3* constitutive (*GAL3*^{c}) mutants. The *GAL3*^{c} mutations cause constitutive expression of the *GAL* genes in a manner that is not dependent upon overproduction of Gal3p. Each of the mutations changes the Gal80p-binding properties of Gal3p, altering the manner in which galactose and ATP affect Gal80p-binding activity. We discuss our findings in terms of the current models of the Gal3p-Gal80p-Gal4p induction switch. We also discuss the potential utility of the novel Gal3 proteins for dissecting the induction mechanism through both in vivo and in vitro methods.

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotide primers used in this study include BLNK01, 5′-GCC-TCA-AGT-CGT-CAA-GTA-AAG-3′; BLNK02, 5′-TAA-CAA-ATG-CGG-ACC-CTA-AAT-3′; BLNK03, 5′-ACG-AAA-ACC-AAG-CCC-AAC-CAT-3′; BLNK04, 5′-GTC-TGC-CAG-CGC-AAG-TAA-ATG-3′; BLNK05,

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5′-GGG-AAA-AGT-TGT-CAG-GTA-ATC-3′; BLNK07, 5′-GGG-AAG-CCG-AAA-GAA-CCA-TTG-3′; BLNK09, 5′-TTC-ACA-CGG-ACG-AAG-ATT-3′; BLNK11, 5′-TGG-AGG-GCG-GTG-CTT-CTT-3′; BLNK16, 5′-TCA-GCG-GGA-TTT-GTG-TAG-3′; BLNK17, 5′-TGG-CAT-TAT-TCC-CTC-CTA-3′; BLNK18, 5′-GTC-GTG-ACT-GGG-AAA-ACC-CTG-GCG-TAA-TTA-CCC-CAG-AAA-TAA-3′; BLNK19, 5′-TCC-TGT-GTG-AAA-TTG-TTA-TCC-GCT-AGC-ATT-GGG-CAG-CTG-TCT-3′; G1, 5′-CGG-ATC-CGC-ATG-AAT-ACA-AAC-GTT-CCA-ATA-3′; G2, 5′-CGG-ATC-CGC-TCA-TTC-ATT-AGT-CGG-CCA-AAA-3′; MYLI22, 5′-AGC-GGA-TAA-CAA-TTT-CAC-ACA-GGA-3′; WOOD11, 5′-GGA-ACG-TTG-AGC-TCC-ATA-CTA-TG-3′; WOOD12, 5′-GAT-CGT-AAA-AGC-TTT-TAC-3′; and WOOD20, 5′-CAA-CAG-AGC-TCA-TGA-ATA-CAA-ACG-3′.

Plasmid constructions. A *GAL3* deletion/disruption allele (*gal3Δ-3*::*LEU2*) was constructed as follows. First, a 3.9-kb *Nhe*I fragment was deleted from pT2 (75), a *GAL3* clone in YEp24 (11), to create pTEB5. Next, the 580-bp *Bgl*II-*Xho*I fragment of pTEB5 containing codons 151 to 342 of the *GAL3* gene was replaced with a 2.6-kb *Bgl*II-*Xho*I *LEU2* fragment from YEp13 (14), generating pTEB10.

A low-copy plasmid carrying the *GAL3* gene was constructed as follows. pTEB8 was generated by inserting the 2.7-kb *Nhe*I fragment from pT1-3B (75), a *GAL3* clone in YEp24, into the *Spe*I site of pRS414 (*TRP1 CEN6 ARSH4*) (69). pTEB8 was then linearized with *Cla*I. The ends were treated with BAL 31 nuclease, rendered flush with Klenow fragment and T4 polynucleotide kinase, and religated. A selected plasmid resulting from this procedure, pTEB16, has a deletion of 53 bp that removed all restriction sites from *Xho*I through *Sma*I in the multiple-cloning site on the 3′ side of *GAL3*.

The coding region of glutathione *S*-transferase (GST) was fused to *GAL3* as follows. Initially, codons 1 through 424 of *GAL3* were amplified by PCR from a derivative of pT1-3B with primers G1 and G2. This PCR product was digested with *Bam*HI, and the ends were filled in with Klenow fragment. The resulting 1.2-kb blunt-ended fragment was introduced into the *Sma*I site of the GST-containing vector pEG(KT) (50), forming pEG(KT)3-3 (78). The 3′ end of the *GAL3* gene was subsequently introduced into pEG(KT)3-3 by replacing its 853-bp *Bgl*II-*Sal*I fragment with the 1.7-kb *Bgl*II-*Sal*I fragment of pTEB8, forming pEG(KT)3-3+CT. The resulting full-length GST-*GAL3* fusion encodes a protein of 771 amino acids. pMPW50, which expresses this GST-*GAL3* fusion from the *GAL3* promoter, was constructed by three-way ligation. A 1.8-kb *Sac*I-*Xho*I fragment from pEG(KT)3-3 containing GST plus *GAL3* 5′ sequences was ligated to a 5.8-kb *Xho*I-*Not*I fragment from pTEB16 containing *GAL3* 3′ and vector sequences and to a 2.4-kb *Not*I-*Sac*I *GAL3* promoter fragment from a PCR product generated with pTEB16 as template and primers MYLI22 and WOOD11. To construct a control plasmid containing only GST regulated by the *GAL3* promoter, the WOOD12 oligonucleotide was self annealed and phosphorylated to yield a double-stranded DNA fragment having *Bam*HI-compatible ends, an internal *Hin*dIII site, and in-frame stop codons in either orientation. This fragment was substituted for the 2.0-kb *Bam*HI fragment of pMPW50 to yield pMPW51, which expresses a GST protein of 248 amino acids.

To place *GAL3* under *ADH2* promoter control, a 6.3-kb *Bgl*II-*Sac*I fragment from pTEB16 containing vector sequences plus the 3′ end of the *GAL3* gene was ligated to a 704-bp *Sac*I fragment containing the *ADH2* promoter from YEpC-PADH2a (62) and to a 451-bp *Sac*I-*Bgl*II fragment containing the 5′ end of the *GAL3* gene. The latter fragment was derived from a PCR product generated with primers BLNK04 and WOOD20 and with pTEB16 as template. This three-way ligation yielded plasmid pMPW66. To place the GST-*GAL3* and GST genes under *ADH2* promoter control, the 704-bp *Sac*I fragment from YEpC-PADH2a was used to replace the 695-bp *Sac*I fragments of pMPW50 and pMPW51, creating pMPW60 and pMPW61, respectively.

To delimit mutation sites involved in the constitutive phenotype, fragments were subcloned from constitutive mutant *GAL3^c* genes in pTEB16 plasmid derivatives and were used to replace the equivalent fragments of the unmutagenized *GAL3* gene in pTEB16, generating the pCL-3C series of plasmids. The fragments used were the 687-bp *Eco*RI-*Bgl*II fragment of pTEB16-303, the 580-bp *Bgl*II-*Xho*I fragment of pTEB16-311, the 253-bp *Xho*I-*Stu*I fragment of pTEB16-322, and the 458-bp *Stu*I-*Bst*EII fragments of pTEB16-362 and pTEB16-371. To place the *GAL3^c* and GST-*GAL3^c* alleles under control of the *ADH2* promoter, the 1.65-kb *Nsi*I-*Bst*EII fragments of pMPW66 and pMPW60 were replaced with equivalent fragments from the pCL-3C series of plasmids, generating the pCLAC and pCLAGC series of plasmids.

A low-copy plasmid containing *GAL80* expressed under the control of the *ADH2* promoter was constructed in three steps. First, the vector pRS426 (*URA3*; 2μm) (17) was gapped with *Sac*I and *Sma*I to yield a 5.7-kb vector fragment which was ligated to a 695-bp *Sac*I-*Sma*I *ADH2* promoter fragment from YEpC-PADH2a, yielding pMPW79. Next, pMPW80 was constructed by using a 1.7-kb *Sna*I-*Hin*dIII fragment containing the *GAL80* gene originally derived from pTT808 (74) to replace the 24-bp *Sma*I-*Hin*dIII fragment of pMPW79. Finally, high-copy plasmid pMPW80 was converted to low-copy plasmid pMPW82 by replacing the 2.6-kb *Sca*I fragment containing 2μm sequences with a 1.8-kb fragment from the vector pRS416 (69) containing *CEN6* and *ARSH4* sequences.

A plasmid expressing a TrpE-Gal80p fusion protein used for production of anti-Gal80p antisera (9) was prepared by replacing the *Bam*HI-*Hin*dIII fragment from the multiple-cloning site of pATH11 (57) with a 1.7-kb *Bgl*II-*Hin*dIII fragment containing codons 7 to 435 of *GAL80*.

Strain constructions. Transformation of DNA into yeast cells was carried out by using the procedures of Gietz et al. (24) or Chen et al. (16). Chromosomal loci were altered via the one-step (64) or two-step (65) gene replacement method.

The yeast strains used in this work were derived through a series of genetic modifications of strain SJ21R (Sc252), which has the genotype *MAT***a** *ade1 ile leu2-3,112 MEL1 ura3-52* (34). First, the chromosomal *TRP1* gene of SJ21R was replaced with the nonfunctional *trp1-HIII* allele (79) by the two-step method with *Xba*I-digested plasmid L328 (provided by R. Keil). The resulting strain was ScPJB644 (9). The chromosomal *HIS3* gene of ScPJB644 was replaced with the nonfunctional *his3Δ-1* allele (65) by the two-step method with an *Eco*RI-digested derivative of pJJ42 (54) from which the 189-bp *Hin*dIII fragment had been removed (9), resulting in strain ScTEB652. A construct carrying portions of the *GAL1* promoter fused to the *HIS3* open reading frame was integrated downstream of the chromosomal *LYS2* locus of ScTEB652 as described by Flick and Johnston (23) by using plasmid pGH1 (5) digested with *Pvu*II. The resulting strain was ScTEB723.

ScTEB723 was modified further by disruption of the *GAL1*, *GAL3*, *GAL4*, or *GAL80* gene. Disruptions were confirmed by PCR of yeast genomic DNA by the procedure of Ling et al. (41). The chromosomal *GAL3* locus of ScTEB723 was replaced by the one-step method with the *gal3Δ-3*::*LEU2* deletion/disruption allele contained on the 6.7-kb *Sph*I-*Hin*dIII fragment of pTEB10, resulting in *gal3Δ* strain ScTEB724. The *gal3Δ gal80Δ* strain ScPX726 was constructed from ScTEB724 by two-step replacement of the chromosomal *GAL80* locus as previously described (53) with an *Xho*I-digested derivative of pTT801 (74) whose 596-bp *Bgl*II fragment was deleted. The *gal4Δ* strain ScPX745 was constructed from ScTEB723 by one-step replacement of the chromosomal *GAL4* locus by using a *GAL4* deletion allele disrupted with *LEU2* as previously described for strain Sc413 (54). To construct *gal1Δ GAL3* and *gal1Δ gal3Δ* strains ScTEB753 and ScTEB756, respectively, a *GAL1* deletion/disruption allele (*gal1Δ-1740*::*URA3*) was used to replace the chromosomal *GAL1* loci of strains ScTEB723 and ScTEB724 via the one-step method. The *gal1Δ-1740*::*URA3* allele had been prepared by the double-fusion PCR method of Amberg et al. (2). pJK1 (7, 8, 10) served as a template for amplifying regions upstream and downstream of the *GAL1* open reading frame with primer pairs BLNK16-BLNK18 and BLNK17-BLNK19. These fragments were joined to a *URA3* marker amplified from pJJ215 (36).

Mutagenesis of *GAL3* and selection of *GAL3^c* mutants. Four overlapping segments covering the entire *GAL3* gene were subject to PCR mutagenesis with pTEB16 as template and primer pairs which hybridize to locations flanking unique restriction sites in *GAL3*. Primers BLNK01 and BLNK04 flank the *Eco*RI and *Bgl*II sites and gave a PCR product of 928 bp. BLNK02 and BLNK05 flank the *Bgl*II and *Xho*I sites and gave a product of 863 bp. BLNK03 and BLNK07 flank the *Xho*I and *Stu*I sites and gave a product of 429 bp. BLNK09 and BLNK11 flank the *Stu*I and *Bst*EII sites and gave a product of 573 bp. The PCR reaction conditions were as described by Zhou et al. (81). pTEB16 that had been gapped with one pair of restriction enzymes was used along with the appropriate PCR product pool to cotransform yeast strain ScTEB724, producing intact plasmids according to the gap repair procedure of Muhlrad et al. (51). Trp$^+$ prototrophs were pooled and replated onto synthetic complete medium lacking histidine and tryptophan and containing glycerol, lactic acid, and 0.05% glucose as carbon sources. The selection medium was supplemented with 10 mM 3-amino-1,2,4-triazole (an inhibitor of the His3p enzyme) to prevent background growth due to low-level expression from the P_{GAL1}-*HIS3* reporter (22). Plasmids were isolated from individual colonies that grew on the selection medium according to the method of Hoffman and Winston (27).

DNA sequencing. Both strands of the entire *GAL3* gene between the *Eco*RI and *Bst*EII sites of pTEB16 were subjected to DNA sequencing with Sequenase version 2.0 (Amersham Life Science). A major revision in the *GAL3* sequence has recently been reported by Suzuki-Fujimoto et al. (73). In agreement with their findings, our *GAL3* sequence contains a number of corrections from the originally published sequence (3) and is identical to that submitted to sequence databases (GenBank accession numbers Z48008 and X95966) by groups sequencing the relevant region of chromosome IV (21, 52). The revised *GAL3* sequence predicts that Gal3p is a protein of 520 amino acids (73), rather than 425 amino acids as previously reported (3).

Each of the pCL-3C plasmids, constructed by replacing fragments of the wild-type *GAL3* gene with the corresponding fragments from *GAL3^c* mutant plasmids, was sequenced on both strands across the relevant region, and the resulting sequence was compared with that of the wild-type *GAL3* gene. The *GAL3^c-303* allele of pCL-3C-303 was sequenced between the *Eco*RI and *Bgl*II sites and carried five mutations: changes of T to C at nucleotides (nt) 56, 90, and 135 of the *GAL3* open reading frame, a change of T to A at nt 206, and a change of A to T at nt 209. Of these, only the mutations at nt 56, 206, and 209 are predicted to alter the amino acid specificity. The change at nt 56 is not indicated in Fig. 2 as it was not required for constitutive expression (this mutation was excluded from plasmid pCLAC-303). It was not determined whether the mutation at either nt 206 or 209 could individually produce the constitutive phenotype. The *GAL3^c-311* allele of pCL-3C-311 was sequenced between the *Bgl*II and *Xho*I sites and found to contain a single change of T to A at nt 710. The *GAL3^c-322* allele of pCL-3C-322 was sequenced between the *Xho*I and *Stu*I sites and found to contain a single change of A to T at nt 1103. The *GAL3^c-362* allele of pCL-3C-362 and the *GAL3^c-371* allele of pCL-3C-371 were sequenced be-

tween the *Stu*I and *Bst*EII sites. These alleles were found to contain single changes of C to T at nt 1526 and T to C at nt 1525, respectively.

Protein sequence comparison. The Gal3p sequence was aligned with multiple galactokinase sequences with the Gene Inspector software package (Textco, Inc.), which utilizes the Clustal V algorithm (26). The protein sequences used were as follows (Swiss-Prot database accession numbers are in parentheses): *S. cerevisiae* Gal3p (P13045), *S. cerevisiae* Gal1p (P04385), *K. lactis* Gal1p (P09608), *Bacillus subtilus* GalK (P39574), *Escherichia coli* GalK (P06976), *Lactobacillus helveticus* GalK (Q00052), *Streptomyces lividans* GalK (P13227), *Salmonella typhimurium* GalK (P22713), *Haemophilus influenzae* GalK (P31767), human GALK1 (P51570), and mouse Glk1 (1).

Media and culture conditions. Solid and liquid growth media for yeast cells were prepared essentially as described by Rose et al. (63). Plasmids were maintained in yeast by culture in synthetic complete (SC) medium lacking tryptophan and/or uracil. Carbon sources were included at the following concentrations: glucose (2% or 0.05% [wt/vol]), galactose (2% [wt/vol]), glycerol (3% [wt/vol]), and lactic acid (2% [vol/vol]; pH was adjusted to 5.7 with KOH).

To obtain uninduced and induced samples, yeast cells were cultured overnight at 30°C to an optical density at 600 nm of 0.20 to 0.35 and then divided into two equal portions. To one portion, galactose was added to a final concentration of 2%. An equivalent volume of water was added to the other portion. Either 3 or 6 h after the addition of galactose, the cells were harvested by centrifugation and frozen at −80°C.

Preparation of whole-yeast-cell extracts. Whole-cell extracts for the immunoblots shown in Fig. 3 were prepared essentially as described by Mylin et al. (53), following the procedure used to prepare metabolically labeled yeast cell extracts. Whole-cell homogenates for alpha-galactosidase enzyme assays were prepared by resuspending cell pellets in 300 μl of citrate-phosphate buffer (31 mM citric acid, 39 mM Na_2HPO_4), adding an equal volume of 0.45-mm-diameter glass beads, and vortexing with alternating 30-s pulses for a total of 12 min. The homogenate was removed, and the beads were washed with 1.2 ml of buffer, with the wash being added to the homogenate. Whole-cell extracts for the immunoblots shown in Fig. 4 were prepared as follows. Yeast cell pellets were suspended in 425 μl of lysis buffer (1% Triton X-100, 10 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, 2 mM dithiothreitol, 20 μg of aprotinin per ml, 200 mM NaCl). One gram of glass beads was added per sample, and the sample was then vortexed in alternating pulses of 30 s for a total of 8 min. The extract was eluted from the beads and clarified for 20 min at 9,980 × *g* in a microcentrifuge at 4°C. The supernatant was withdrawn and saved for immunoblots and interaction assays. All extracts were stored at −80°C until use. Protein concentrations in the extracts were estimated by the procedure of Peterson (60) or by using the Bio-Rad protein assay.

Gal3p-Gal80p interaction assay. Whole-yeast-cell extract (310 μg) was brought up to a volume of 225 μl with lysis buffer. An additional volume of 225 μl of lysis buffer alone or lysis buffer supplemented with 4 mM ATP and/or 4% galactose was added. The extract was rotated in the presence of 40 μl of 50% glutathione Sepharose beads (Pharmacia) for 2 h at 4°C. The beads were pelleted and washed four times with 500 μl of either lysis buffer alone or lysis buffer supplemented with 4 mM ATP and/or 4% galactose. The beads were then boiled for 8 min in 44 μl of 1× electrophoresis sample buffer (54).

Immunoblotting. For the immunoblots shown in Fig. 3, equivalent amounts of whole-cell extract (60 μg for detection of Gal3p and Gal80p, 40 μg for detection of Mel1p, 30 μg for detection of Gal7p) were loaded in each lane and proteins were fractionated on sodium dodecyl sulfate–10% (Gal3p and Mel1p) or –12.5% (Gal7p and Gal80p) polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with rabbit polyclonal antiserum, followed by an antirabbit horseradish peroxidase-linked secondary antibody (Amersham Life Science). Antisera against Gal80p (9) were produced by methods similar to those used for preparation of anti-Gal3p antisera (10). Both anti-Gal3p and anti-Gal80p antisera were used at a dilution of 1:1,000. Affinity-purified antiserum against Mel1p (provided by R. Bozzato of Allelix Inc.) was used at a dilution of 1:1,000. Antiserum raised against Gal7p (28) was used at a dilution of 1:30,000. Proteins were visualized with a chemiluminescence reagent (RENAISSANCE; DuPont NEN Research Products).

For the immunoblots shown in Fig. 4, sodium dodecyl sulfate–7.5% polyacrylamide gels were used to fractionate 14 μl (for GST-Gal3p detection) or 18 μl (for Gal80p detection) of glutathione Sepharose eluate (Fig. 4A) or 37.5 μg of whole-cell extract (Fig. 4B). Immunodetection was carried out by the alkaline phosphatase method as described previously (54). Anti-Gal80p antiserum was used at a dilution of 1:200, and anti-GST antiserum (provided by Andrew Waskiewicz) was used at a dilution of 1:3,500.

Enzyme activity determination. Alpha-galactosidase enzyme activities were determined by spectrophotometric assay as described previously (61).

RESULTS

Isolation of novel *GAL3* constitutive mutants. Based on a recent working model of Gal3p's induction activity (7, 8), we sought *GAL3* mutants which require neither galactose nor overproduction of Gal3p to induce Gal4p activation of *GAL* gene transcription. To generate *GAL3* mutations, four consecutive segments accounting for the entire coding region of *GAL3* were subjected to PCR mutagenesis (see Materials and Methods). The PCR products were used in conjunction with the gapped *GAL3* gene on a *TRP1 CEN* plasmid (pTEB16) to cotransform yeast strain ScTEB724 (*gal3*Δ *his3*Δ *trp1*Δ). Four pools of gap-repaired plasmid were established, one corresponding to each mutagenized segment.

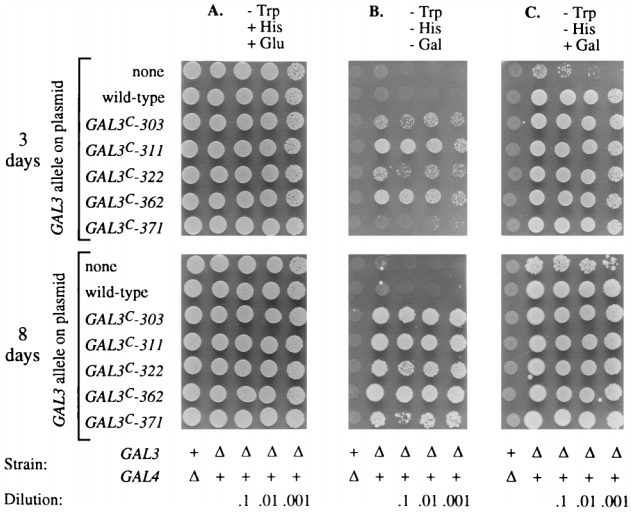

FIG. 1. *GAL3*^{c} alleles enable expression of a P_{GAL1}-*HIS3* reporter construct in the absence of galactose in a *GAL4*-dependent manner. Plasmids containing no *GAL3* (pRS414) or containing the allele *GAL3* (pTEB16), *GAL3*^{c}*-303* (pCL-3C-303), *GAL3*^{c}*-311* (pCL-3C-311), *GAL3*^{c}*-322* (pCL-3C-322), *GAL3*^{c}*-362* (pCL-3C-362), or *GAL3*^{c}*-371* (pCL-3C-371) were introduced into yeast strains which carry the P_{GAL1}-*HIS3* reporter. The resulting plasmid-containing strains were grown to saturation in SC liquid medium lacking tryptophan and containing 2% glucose. These cultures were spotted (8 μl) onto solid growth medium and allowed to grow at 30°C for 3 days (upper row of results) or 8 days (lower row of results). In each panel, the first column of spots is composed of plasmid transformants of strain ScPX745 (*gal4*Δ *his3*Δ *trp1*Δ *LYS2*::P_{GAL1}-*HIS3*). The second through fifth columns are composed of transformants of strain ScTEB724 (*gal3*Δ *his3*Δ *trp1*Δ P_{GAL1}-*HIS3*) which were serially diluted 10-fold three times. (A) Cultures were spotted onto SC medium lacking tryptophan and containing 2% glucose. This was a positive control to ensure that all transformant cultures were viable. (B) Cultures were spotted onto SC medium lacking both tryptophan and histidine and containing 3% glycerol, 2% lactic acid, and 0.05% glucose to assay for constitutive expression of the P_{GAL1}-*HIS3* reporter. (C) Cultures were spotted onto a medium identical to that used for panel B but containing 2% galactose to assay for galactose-induced expression of the P_{GAL1}-*HIS3* reporter.

Selection of constitutive mutants relied upon a reporter construct in which the open reading frame for the *HIS3* selectable marker is expressed under the control of the galactose-inducible *GAL1* promoter (P_{GAL1}) (5, 23, 33). ScTEB724 carries a chromosomal copy of the P_{GAL1}-*HIS3* reporter, allowing *HIS3* transcription to be regulated by Gal3p. ScTEB724 carrying the wild-type *GAL3* gene on pTEB16 could grow on medium lacking histidine when galactose was present but could not grow when galactose was absent (Fig. 1). The P_{GAL1}-*HIS3* reporter provides a genetic selection for *GAL* regulatory mutants that permit *HIS3* expression in the absence of galactose (33). To select for such constitutive mutants, the four yeast plasmid pools were plated onto synthetic medium lacking both histidine and galactose. Mutant colonies arose at a frequency of approximately 1 per 50,000 plasmid-containing colonies. Plasmids were isolated from individual mutant colonies and then transferred back into ScTEB724 to determine if constitutive *HIS3* expression could be attributed to the *GAL3* plasmid. Twenty-three mutant plasmids conferring the constitutive phenotype were identified. One plasmid was derived from the pool

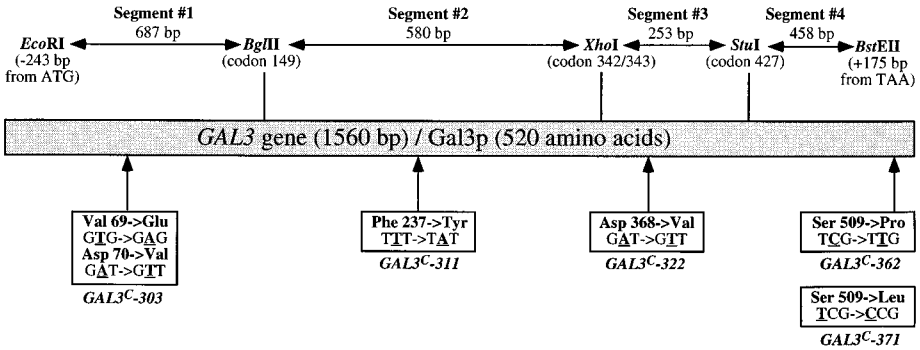

FIG. 2. *GAL3*^{c} mutations are widely distributed. The shaded rectangle represents both the *GAL3* gene and the Gal3p protein it encodes. The *GAL3*^{c} mutations were derived by PCR mutagenesis of four segments of the *GAL3* gene. The relative locations and sizes of these segments are noted above the gene, along with the locations of restriction sites which separate the segments and which were used for subcloning them. The names of the five *GAL3*^{c} alleles described in this report are noted below the gene, along with a description of the codon change(s) present in the alleles and the amino acid change(s) it specifies. Three additional mutations present in the *GAL3*^{c}*-303* allele are not shown, either because they do not produce amino acid changes or because an amino acid change was not required for the constitutive phenotype.

in which the first *GAL3* segment was mutagenized. Seven plasmids were from the second pool, 5 were from the third pool, and 10 were from the fourth pool.

Partial DNA sequencing of the mutant plasmids showed that those originating from the same pool often contained identical mutations in their *GAL3* genes. Five plasmids which had different mutation sites were chosen for further analysis. Complete double-stranded sequencing of the appropriate subcloned fragments of these plasmids confirmed that each contains a different single or double point mutation. These *GAL3* constitutive (*GAL3*^{c}) mutations are widely distributed across the *GAL3* gene, rather than being clustered together in a particular region (Fig. 2). The *GAL3*^{c}*-303* allele specifies alterations in two adjacent amino acids, changing valine 69 to glutamic acid and aspartic acid 70 to valine. The mutation in the *GAL3*^{c}*-311* allele specifies a change of phenylalanine 237 to tyrosine. The mutation in the *GAL3*^{c}*-322* allele specifies a change of aspartic acid 368 to valine. Two of the *GAL3*^{c} mutations (*GAL3*^{c}*-362* and *GAL3*^{c}*-371*) specify different amino acid changes at the same position in Gal3p, residue 509 (Ser). In *GAL3*^{c}*-362*, this serine codon is changed to a proline codon, while in *GAL3*^{c}*-371* it is changed to a leucine codon.

When expressed from a *CEN* plasmid in ScTEB724, each of the *GAL3*^{c} alleles was sufficient to cause growth of ScTEB724 on medium lacking both histidine and galactose (Fig. 1). When expressed in a *gal4*Δ strain (ScPX745) isogenic to ScTEB724, however, the *GAL3*^{c} alleles were unable to cause growth of ScPX745 on medium lacking histidine regardless of the presence of galactose (Fig. 1). This indicates that, as expected, the *GAL3*^{c} mutants act through a Gal4p-dependent pathway.

The *GAL3*^{c} alleles cause constitutive expression of the *GAL* and *MEL* genes independently of *GAL1*. To determine whether the *GAL3*^{c} alleles cause constitutive expression of endogenous *GAL* and *MEL* genes, we performed immunoblot analyses of Gal7p (galactose-1-phosphate uridylyltransferase), Gal80p, and Mel1p (alpha-galactosidase) from yeast strain ScTEB756 (*gal3*Δ *gal1*Δ) carrying wild-type or mutant *GAL3* alleles on plasmids. The *GAL7*, *GAL80*, and *MEL1* genes are known to be regulated at the level of transcription by galactose and by Gal3p, Gal4p, and Gal80p (4, 28, 30, 61, 68, 71, 72).

The Gal7p, Gal80p, and Mel1p proteins were not detected in extracts from ScTEB756 carrying the wild-type *GAL3* allele and grown in the absence of galactose (Fig. 3A through C, lane 3). In contrast, these proteins were present in extracts from ScTEB756 carrying the *GAL3*^{c}*-303*, *GAL3*^{c}*-311*, or *GAL3*^{c}*-322*

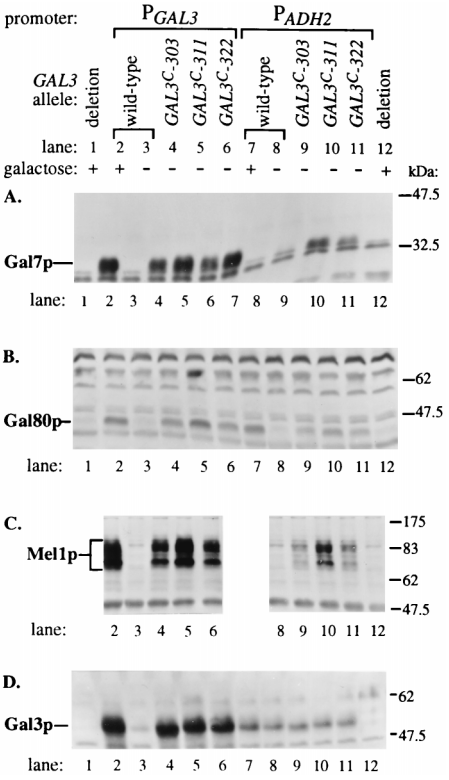

FIG. 3. *GAL3*^{c} alleles cause the constitutive expression of the *GAL7*, *GAL80*, and *MEL1* genes. This effect is not dependent upon the accumulation of mutant Gal3p proteins to a level greater than that of wild-type Gal3p. Immunoblots of whole-yeast-cell extracts from cultures grown in the absence of galactose (SC medium lacking tryptophan and containing 3% glycerol, 2% lactic acid, and 0.05% glucose) or induced with 2% galactose for 6 h were probed with polyclonal antisera raised against Gal7p (A), Gal80p (B), Mel1p (C), or Gal3p (D). The locations of these proteins are indicated, along with the positions of molecular mass markers. Extracts are from strain ScTEB756 (*gal1*Δ *gal3*Δ) carrying plasmid pRS414 (lanes 1 and 12), pTEB16 (lanes 2 and 3), pCL-3C-303 (lane 4), pCL-3C-311 (lane 5), pCL-3C-322 (lane 6), pMPW66 (lanes 7 and 8), pCLAC-303 (lane 9), pCLAC-311 (lane 10), or pCLAC-322 (lane 11). The plasmid-based *GAL3* alleles present in the yeast cells which provided the extracts, the promoter controlling expression of those alleles, and the presence or absence of galactose in the cell cultures are indicated above the immunoblots.

alleles and grown in the absence of galactose (lanes 4 through 6). These results demonstrate that the mutant Gal3p proteins specified by these *GAL3^c* alleles cause constitutive expression of the *GAL* and *MEL* genes. Moreover, since ScTEB756 is a *gal1*Δ *gal3*Δ strain, these results establish that Gal1p, a Gal3p-like protein, is not required for the constitutive phenotype. When *GAL3^c* alleles carried on a *CEN* plasmid were introduced into strain ScTEB753 (*gal1*Δ *GAL3*), the cells exhibited the constitutive phenotype (data not shown), indicating that the *GAL3^c* alleles are dominant over the wild-type *GAL3* allele.

The *GAL3^c* phenotype is not caused by overproduction of Gal3p. Overproduction of Gal3p can cause constitutive *GAL* gene expression (8, 73). Therefore, it was conceivable that the mutations in the *GAL3^c* alleles might produce the constitutive phenotype by increasing the amount of Gal3p in the cell, through either increased synthesis or decreased turnover. To determine if this was the case, it was necessary to replace the *GAL3* promoter, as Gal3p positively regulates its own expression through a Gal4p-binding site in its promoter (3). Uninduced yeast cells carrying a *GAL3^c* allele are expected to have an increased amount of Gal3p compared to cells carrying the wild-type allele due to this promoter effect.

We used immunoblot analysis to assay the level of Gal3p in strain ScTEB756 (*gal1*Δ *gal3*Δ) carrying *GAL3* wild-type, *GAL3^c-303*, *GAL3^c-311*, or *GAL3^c-322* alleles on plasmids. (The *GAL3^c-362* and *GAL3^c-371* alleles were not tested.) As expected, the amounts of Gal3p detected in extracts from yeast cells grown in the absence of galactose and carrying one of the mutant alleles were found to be greatly increased above the amounts detected in extracts from yeast cells carrying wild-type alleles (Fig. 3D, compare lanes 4 through 6 with lane 3). The amounts of Gal3p detected in noninduced cells carrying mutant alleles were similar to the amounts detected in galactose-induced cells carrying wild-type alleles (compare lanes 4 through 6 with lane 2). When the *ADH2* promoter (P_{ADH2}) replaced the *GAL3* promoter, both wild-type and mutant Gal3p proteins were detected at similar levels in the presence or absence of galactose (lanes 7 through 11; also data not shown). The P_{ADH2}-*GAL3* wild-type allele did not cause the production of detectable amounts of Gal7p, Gal80p, or Mel1p in the absence of galactose (Fig. 3A through C, lanes 8), although more Gal3p was produced than when expressed under *GAL3* promoter control (Fig. 3D, compare lanes 8 and 3). In contrast, each of the mutant P_{ADH2}-*GAL3^c* alleles elicited constitutive *GAL* and *MEL* gene expression (Fig. 3A through C, lanes 9 through 11), demonstrating that each mutant Gal3p protein retained its constitutive property despite being expressed at the same level as wild-type Gal3p. From these results, we conclude that the *GAL3^c* mutations give rise to the constitutive phenotype through a means other than an increase in the level of Gal3p in the cell.

Cells bearing different *GAL3^c* alleles differed in their levels of constitutive response, but all retained galactose responsiveness. To quantify the level of Gal3p activity in yeast carrying the wild-type and mutant *GAL3* plasmids, the level of *MEL1* gene expression was determined by performing alpha-galactosidase enzyme assays (Table 1). Noninduced ScTEB756 (*gal1*Δ *gal3*Δ) yeast carrying different P_{ADH2}-*GAL3^c* alleles produced different levels of constitutive alpha-galactosidase activity that ranged from about 12% (P_{ADH2}-*GAL3^c-371*) to 54% (P_{ADH2}-*GAL3^c-311*) of the activity level found in galactose-induced yeast carrying wild-type P_{ADH2}-*GAL3*. This quantitative data taken together with the *HIS3* assay and immunoblot data (Fig. 1 and 3) revealed that different *GAL3^c* alleles confer different levels of constitutive response for *GAL* and *MEL* gene expression. The addition of galactose to yeast cells carrying any of the five P_{ADH2}-*GAL3^c* alleles increased the alpha-galactosidase activity to a level even greater than that obtained from galactose-induced cells carrying the P_{ADH2}-*GAL3* wild-type allele (Table 1). This result confirmed that the mutant Gal3p proteins are still capable of responding to galactose.

TABLE 1. Effect of *GAL3^c* mutations on alpha-galactosidase expression[a]

Plasmid	*GAL3* allele	Alpha-galactosidase activity[b]	
		Without galactose	With galactose (6 h)
pRS414	None	3 ± 3	4 ± 2
pMPW66	P_{ADH2}-*GAL3*	3 ± 4	1,026 ± 7
pCLAC-303	P_{ADH2}-*GAL3^c-303*	175 ± 3	1,106 ± 37
pCLAC-311	P_{ADH2}-*GAL3^c-311*	552 ± 4	1,237 ± 43
pCLAC-322	P_{ADH2}-*GAL3^c-322*	269 ± 4	1,229 ± 7
pCLAC-362	P_{ADH2}-*GAL3^c-362*	522 ± 2	1,111 ± 19
pCLAC-371	P_{ADH2}-*GAL3^c-371*	125 ± 10	1,108 ± 58

[a] Values determined from whole-cell extracts of strain Sc756 (*gal1*Δ *gal3*Δ) carrying the indicated plasmids.
[b] Activities are expressed as picomoles of PNP (*p*-nitrophenyl-α-D-galactopyranoside) liberated per milligram of protein per minute.

Each of the five *GAL3^c* mutations results in altered Gal80p-binding characteristics. We developed a Gal3p-Gal80p interaction assay to determine whether the constitutive phenotype of the mutant Gal3p proteins might relate to changes in their Gal80p-binding properties. Wild-type and mutant *GAL3* genes were fused at their 5′ ends to plasmid-based GST genes expressed under control of the *ADH2* promoter. These GST-*GAL3* constructs could complement a *gal3*Δ strain for induction activity (data not shown). To prevent galactose regulation of the level of Gal80p in the cell (30, 68), the *ADH2* promoter was used to replace the *GAL80* promoter of a plasmid-based *GAL80* gene. After growth in the presence or absence of galactose, whole-yeast-cell extracts were prepared from strain ScPX726 (*gal3*Δ *gal80*Δ) carrying the P_{ADH2}-GST-*GAL3* and/or P_{ADH2}-*GAL80* plasmids. GST-Gal3p fusions were isolated from the extracts with glutathione Sepharose (Fig. 4A). The amount of Gal80p that copurified with each of the GST-Gal3p fusions was analyzed by immunoblotting (Fig. 4A). Since the interaction of Gal1p and Gal80p from galactose-induced *K. lactis* cells is improved by adding galactose and ATP to the buffers used in an in vitro binding assay (80), we tested the effects of these compounds on the Gal3p-Gal80p interaction in our assay.

All five of the novel Gal3p mutants isolated in this study differed from wild-type Gal3p in their Gal80p-binding characteristics (Fig. 4A). Furthermore, a comparison among the five revealed a range of Gal80p-binding properties with respect to galactose and/or ATP dependence (Fig. 4A). For each Gal3p protein, the observed differences in Gal80p binding in response to different conditions of exposure to galactose and/or ATP were not due to differences in the levels of GST-Gal3p (compare lanes 3 through 10 of each GST-Gal3p immunoblot in Fig. 4A). Also, for each GST-Gal3p mutant strain the levels of Gal80p in extracts did not vary noticeably between extracts from cells cultured in the presence or absence of galactose (Fig. 4B). In cells lacking the Gal80p protein, there was a noticeable tendency for reduced recovery of GST-Gal3p (Fig. 4A; compare lane 2 with lanes 3 through 10).

Considering first the absence of galactose in the cell growth media, no Gal80p was found complexed with wild-type GST-Gal3p, unless galactose was added during the in vitro binding and wash steps (Fig. 4A; compare lanes 3 and 4 with lanes 5

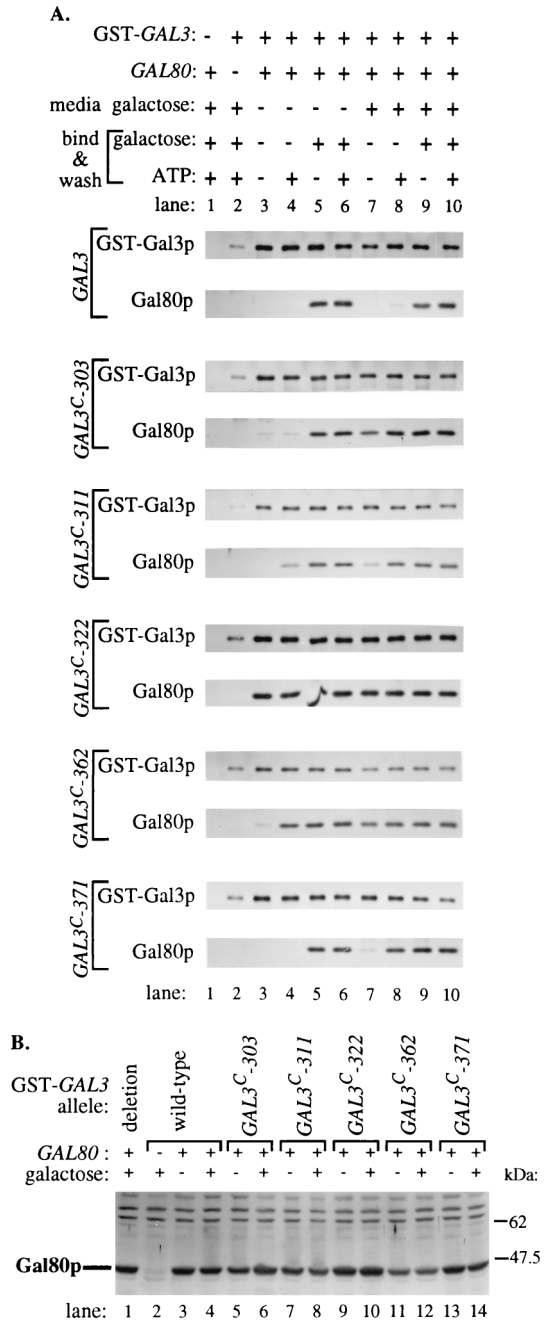

FIG. 4. Mutant GST-Gal3p fusion proteins exhibit altered Gal80p-binding properties compared to wild-type GST-Gal3p. (A) Extracts were prepared from cultures of yeast strain ScPX726 (*gal3*Δ *gal80*Δ) carrying P_{ADH2}-GST plasmid pMPW61 (lane 1), P_{ADH2}-GST-*GAL3* plasmid pMPW60 (top pair of immunoblots, lanes 2 through 10), or P_{ADH2}-GST-$GAL3^c$ plasmid pCLAGC-303, pCLAGC-311, pCLAGC-322, pCLAGC-362, or pCLAGC-371 (lower five pairs of immunoblots, lanes 2 through 10; the *GAL3* allele is indicated to the left of the figure) plus either P_{ADH2}-*GAL80* plasmid pMPW82 (lanes 1 and 3 through 10) or vector pRS416 (lane 2). The plasmid-containing yeast cells were either grown in the absence of galactose (lanes 3 to 6; SC medium lacking tryptophan and uracil and containing 3% glycerol, 2% lactic acid, and 0.05% glucose) or induced with 2% galactose for 3 h (lanes 1, 2, and 7 through 10). Glutathione Sepharose was used to isolate GST-Gal3p, GST, and any associated proteins from the extract. As indicated, galactose (lanes 1, 2, 5, 6, 9, and 10) and ATP (lanes 1, 2, 4, 6, 8, and 10) were included either alone or in combination in the buffer used during the binding and washing steps of this procedure. The glutathione Sepharose eluate was examined by immunoblotting with anti-GST polyclonal antisera (upper immunoblot of each pair) or anti-Gal80p polyclonal antisera (lower immunoblot of each pair). (B) Immunoblot of the whole-cell extracts probed directly with anti-Gal80p antisera.

and 6). In contrast, GST-Gal3p-322, GST-Gal3p-303, and GST-Gal3p-362 all showed binding to Gal80p without the addition of galactose and/or ATP to the in vitro binding and washing buffers (lane 3). Under those conditions we detected no Gal80p binding by the GST-Gal3-311 and GST-Gal3-371 proteins. When ATP alone was added to the binding buffer, both GST-Gal3p-311 and GST-Gal3p-371 now showed Gal80p binding, but in the latter case the binding was very weak and barely detectable on the blots (Fig. 4A, lane 4). The addition of ATP alone also markedly increased the Gal80p binding of the GST-Gal3-362 protein but had little or no effect on the Gal80p binding of GST-Gal3p-303 and GST-Gal3p-322 (compare lanes 3 and 4). For both the wild-type GST-Gal3 protein and mutant GST-Gal3 proteins 303, 311, and 371, the addition of galactose to the binding and wash buffers resulted in either a modest or strong increase in Gal80p binding that did not change noticeably in response to the addition of ATP to the binding and wash buffers (compare lanes 3 and 4 with lanes 5 and 6).

When the mutant Gal3 proteins from galactose-grown cell extracts were analyzed for Gal80p binding, all mutants again behaved differently from wild-type Gal3p. Wild-type GST-Gal3p showed no detectable binding when both galactose and ATP were omitted from the binding and wash buffers (lane 7). The addition of ATP alone did result in weak binding of wild-type GST-Gal3p to Gal80p (lane 8). When galactose was added to the binding and wash buffers, the binding of wild-type GST-Gal3p to Gal80p was increased considerably (lanes 9 and 10). All five mutant GST-Gal3 proteins exhibited some binding to Gal80p even in the absence of galactose and ATP addition to the binding and wash buffers (lane 7). For three of the mutant GST-Gal3 proteins (Gal3p-303, Gal3p-322, and Gal3p-362), the addition of ATP in the absence of added galactose did not markedly increase the Gal80p binding (lanes 7 and 8). In contrast, GST-Gal3 mutant proteins 311 and 371 showed a marked increase in Gal80p binding in response to ATP addition in the absence of added galactose (lanes 7 and 8).

DISCUSSION

The discovery that overproduction of either Gal3p or Gal1p in *S. cerevisiae* can overcome Gal80p inhibition of Gal4p-mediated transcription activation led to a model wherein the function of galactose is to convert Gal3p to a form able to bind to Gal80p and alter the Gal80p-Gal4p complex (7, 8). One prediction of the model, direct interaction between Gal3p (or Gal1p) and Gal80p, has been realized (73, 80). The Gal3p-Gal80p interaction is detected in extracts from non-galactose-grown *S. cerevisiae* cells in which Gal3p is overproduced (73). Further consideration of the model led to a second prediction: that it should be possible to identify a Gal3 mutant protein that is better able than the wild type to bind Gal80p in the absence of galactose. Evidence presented here supports such a prediction. We identified five dominant $GAL3^c$ alleles that confer Gal4p-mediated activation of the galactose/melibiose regulon genes independently of galactose. The constitutive phenotype is not due to increased levels of Gal3p. Each of the five mutant Gal3p proteins differs from wild-type Gal3p in Gal80p-binding properties with respect to galactose and/or ATP dependency.

The $GAL3^c$ alleles we isolated map at dispersed sites throughout approximately 86% of the *GAL3* coding region (Fig. 2). In an attempt to infer the possible significance of the sites affected, we aligned the sequences of the three yeast proteins known to possess *GAL* induction activity (Gal3p and Gal1p of *S. cerevisiae* and Gal1p of *K. lactis*) with the sequences of six bacterial and two mammalian galactokinase proteins

(data not shown). Of the two adjacent residues altered in Gal3p-303, Asp 70 is conserved in the other yeast proteins, corresponding to aspartic acids at residue 76 of *S. cerevisiae* Gal1p and residue 66 of *K. lactis* Gal1p. However, an aspartic acid is not found at a corresponding position in the other galactokinases. Val 69 of Gal3p is not conserved in the other yeast proteins, being replaced by Phe 75 in *S. cerevisiae* Gal1p and Asn 66 in *K. lactis* Gal1p. This suggests that the Asp-70-to-Val change is more likely than the Val-69-to-Glu change to cause the constitutive phenotype demonstrated for Gal3p-303. In addition to Asp 70, two other residues that are altered in the mutants (Phe 237 in Gal3p-311 and Ser 509 in Gal3p-362 and Gal3p-371) were found to be conserved among each of the yeast proteins but not among the remaining galactokinases (data not shown). Thus, these three residues may be specifically important to the induction but not to the galactokinase activity of these proteins. Asp 368, which is altered in Gal3p-322, is conserved only between Gal3p and Gal1p of *S. cerevisiae*. Leucine is the amino acid at the corresponding position in *K. lactis* Gal1p.

The locations of the amino acids affected by the *GAL3^c* mutations provide no clue to their significance with respect to the altered Gal80p-binding properties of the mutant proteins. Even for the known galactokinase proteins, binding sites for galactose and ATP have not been determined experimentally or clearly defined by sequence alignment analyses. Nor has the region or regions of Gal3p (or Gal1p) involved directly in Gal80p interaction been mapped.

The *GAL3^c* mutations could alter amino acids directly involved in the Gal80p-binding site. Alternatively, the amino acid substitutions could cause Gal3p to mimic a ligand-induced conformation that is better able to bind to Gal80p at a separate site. Candidate ligands for such an allosteric model of Gal3p function are galactose and ATP (see below). The binding of sugars to other proteins, such as the *lac* repressor (40) and the D-glucose/D-galactose and maltose/maltodextrin chemosensory receptors (44, 67) of *E. coli* and yeast hexokinase (6), has been demonstrated to cause conformational changes that modify the activities of these proteins. Clearly, future genetic analyses as well as physical analyses will be necessary to assign functions to the *GAL3^c* sites and establish whether the amino acids at these dispersed sites communicate or act independently as determinants in Gal3p-Gal80p binding.

The molecular basis for the positive effect of galactose on Gal3p's activity has not been directly established. However, direct binding of galactose to Gal3p is expected to be involved for several reasons. The sequences of the *GAL1*-encoded galactokinase proteins of *S. cerevisiae* and *K. lactis* are, respectively, 74 and 62% identical to that of Gal3p (3, 9, 18, 21, 49, 52, 70, 73, 77). Both of these galactokinase proteins have Gal3p-like induction activity (7, 8, 10, 49) and must bind galactose and ATP as substrates for their galactokinase activities. Recent work with *K. lactis* Gal1p and Gal80p proteins purified substantially from whole-cell extracts showed that an in vitro interaction between these proteins was dependent upon the addition of both galactose and ATP (80). Thus, the simplest working model is that Gal3p in *S. cerevisiae*, like Gal1p in *K. lactis*, binds galactose and ATP. However, direct proof that galactose and/or ATP directly bind to Gal3p has not yet been established.

The results presented here for *S. cerevisiae* wild-type GST-Gal3p, like those of Zenke et al. (80) for *K. lactis* Gal1p, indicate that the ability to interact with Gal80p is enhanced in the presence of galactose. Zenke et al. observed only weak binding of Gal1p and Gal80p in extracts prepared from galactose-grown *K. lactis* cells when neither galactose nor ATP was added to the in vitro binding assay mixture. When they included ATP and galactose in their in vitro binding assay, strong binding between Gal1p and Gal80p was observed. In our experiments, growth of *S. cerevisiae* cells in galactose-containing media was not by itself sufficient to permit us to detect the interaction of wild-type Gal3p with Gal80p in vitro. But when galactose with or without ATP was added to the binding assay, we detected relatively strong Gal3p-Gal80p binding (Fig. 4A). Thus, in this work and in the previous work of Zenke et al. (80), a strong galactose dependence for *S. cerevisiae* Gal3p binding to *S. cerevisiae* Gal80p and for *K. lactis* Gal1p binding to *K. lactis* Gal80p, respectively, has been established. Additionally, cross-species complex formation between *S. cerevisiae* Gal3p and *K. lactis* Gal80p was shown to occur in the presence but not in the absence of galactose and ATP (80), but ATP and galactose were not tested separately. Suzuki-Fujimoto et al. (73) previously reported *S. cerevisiae* Gal3p binding to *S. cerevisiae* Gal80p, but in their work they were not able to distinguish between a possible Gal80p copy level effect and a possible galactose binding effect on the amount of Gal3p-Gal80p complex in extracts from galactose-cultured cells versus that in extracts from non-galactose-cultured cells.

ATP addition to the binding and wash buffers in our experiments in which galactose was added did not produce an appreciable increase in the amount of Gal80p bound by wild-type Gal3p compared to that observed when ATP was not added (Fig. 4A; compare lanes 5 and 6 and lanes 9 and 10). This result contrasts with that of Zenke and colleagues (80) working with Gal1p and Gal80p from noninduced cultures of *K. lactis*. They did not detect a Gal1p-Gal80p complex in vitro unless ATP as well as galactose was added to the binding assay mixture. *S. cerevisiae* Gal3p and *K. lactis* Gal1p are not identical proteins, so perhaps these proteins differ in either their responses to ATP or their abilities to retain cellular ATP during extract preparation. Alternatively, sufficient amounts of endogenous ATP may have remained in the extracts in our experiments but not in the experiments of Zenke and colleagues. The fact that we do observe increased Gal3p-Gal80p interaction with the addition of ATP for wild-type Gal3p as well as for three of the five mutant Gal3p proteins (Fig. 4A) suggests that for both the *S. cerevisiae* Gal3p-Gal80p and the *K. lactis* Gal1p-Gal80p interactions, ATP is a likely effector molecule.

Among the five Gal3p mutant proteins we observe a range of Gal3p-Gal80p-binding properties with respect to galactose and ATP dependency. Our comparisons are based primarily on differences between the profiles of the mutants, as compared to each other and to that of the wild type, where the profile is defined as the pattern observed reading from lanes 3 through 10 in Fig. 4A. Since our assays are not quantitative for comparisons between mutants, we can only make qualitative statements regarding any perceived differences in the magnitudes of the Gal3p-Gal80p complexes recovered under similar conditions. Based on such an analysis, the behaviors of mutants Gal3p-322, Gal3p-303, and Gal3p-362 appear most dramatically different from that of wild-type Gal3p. The mutants exhibit binding to Gal80p even when neither galactose nor ATP is added (culture media and binding assay). In the case of Gal3p-322 and Gal3p-303 the level of binding is not obviously affected by added ATP (Fig. 4A, lanes 3 and 4). In contrast, for Gal3p-362, the level of Gal80p binding appears to be markedly increased when ATP is added to the binding and wash buffers (lanes 3 and 4). And, under the same conditions of no galactose added (culture media and binding assay), the other mutant Gal3p proteins exhibit either no detectable binding to Gal80p (Gal3p-371) or binding only under conditions of ATP addition (Gal3p-311) (lanes 3 and 4). Our interpretation is that

among the various Gal3p mutants, the Gal3p-322, Gal3p-303, and Gal3p-362 proteins are the ones that are the most effectively stabilized in a Gal80p-binding-competent form that is normally favored when galactose (and/or ATP) binds. Because these proteins potentially could retain ATP which bound at the time of cell extract preparation, our experiments have not determined whether or not the Gal80p binding we observe in the absence of added ATP (lane 3) is absolutely ATP independent. However, Gal3p-362, in contrast to Gal3p-322 and Gal3p-303, exhibits a marked increase in Gal80p binding in response to added ATP (compare lanes 3 and 4). Gal3p-311 and Gal3p-371 proteins show no detectable Gal80p binding in the absence of added galactose and ATP (lane 3) but do show weak (Gal3p-311) to very weak (Gal3p-371; not readily reproduced in the photo) Gal80p binding in response to added ATP (compare lanes 3 and 4).

When galactose is present in the media but neither galactose nor ATP is added to binding and wash buffers, there is a marked increase in Gal3p-Gal80p complex recovered for all mutants but not for wild-type Gal3p (Fig. 4A, compare lanes 3 and 7). Under the same conditions, except that ATP is added to the binding and wash buffers, a readily detectable increase in Gal3p-Gal80p complex recovery is observed for wild-type Gal3p and for Gal3p mutants 311 and 371 (compare lanes 7 and 8). The blots are also suggestive of a slight effect of ATP on Gal3p-303 binding to Gal80p (lanes 7 and 8). In these experiments in which galactose is present in the cell culture media, the results may reflect differences in the extent to which the different Gal3p proteins retain galactose through the binding assay and wash steps. Taken all together, this data suggests a range of Gal80p-binding properties among the Gal3p mutants that might simply reflect quantitative and/or qualitative differences with respect to dependency on galactose and ATP. To address these issues, it will be necessary to first establish whether galactose and ATP bind to Gal3p. If they do, then a quantitative assay can be developed for determination of binding constants for the galactose-Gal3p- and ATP-Gal3p-binding reactions; this assay can be applied to the analysis of the various mutant Gal3p proteins.

Based on the work of Zenke et al. (80) and our work here, the emergent model is that galactose and ATP act to effect a form of Gal3p (Gal1p) that is enhanced in its ability to bind to Gal80p and to relieve the Gal80p inhibition of Gal4p-dependent transcription activation. This model is consistent with the fact that overexpression of Gal3p or Gal1p causes a constitutive response (8, 73). In wild-type *GAL3* cells it is likely that only a very small fraction of Gal3p statistically explores the active conformation at any time. This might give rise to Gal4p-dependent basal expression (61). When Gal3p is considerably overproduced, the number of Gal3p molecules in the active fraction might exceed the critical threshold needed for full-fledged induction. Gal3p overproduction in the absence of galactose does result in Gal3-Gal80p complex formation (73).

For the Gal3p mutant proteins we expected to observe a correlation between galactose-independent Gal80p binding and galactose-independent Gal4p activity. Indeed, although we have not carried out strictly quantitative determinations of the binding of the various Gal3p proteins to Gal80p, some comparisons from Fig. 4A and 4B are suggestive of differences in Gal80p-binding strengths among the Gal3p mutant proteins. The suggestive differences roughly parallel the determined differences in Gal4p activities as inferred from *MEL1* gene expression (Table 1). For example, Gal3p mutants 303 and 371 appear to bind Gal80p more weakly than mutants 362, 322, and 311 in the absence of galactose and show lower-level constitutive responses (Fig. 4A, lanes 3 and 4, and Table 1). These results are thus consistent with the notion that increased complex formation between Gal3p (or Gal1p) and Gal80p in response to galactose and ATP activation of Gal3p is a critical step leading to the alteration of the Gal80p-Gal4p complex, allowing Gal4p to activate target gene transcription. Development of a more quantitative assay for Gal3p-Gal80p binding will be crucial to establishing a rigorous test of this notion. This is not to exclude the possibility that one Gal3p mutant protein showing greater Gal4p activation than another could conceivably show weaker Gal80p binding. Such a deviation could reflect complexity in the induction pathway wherein the Gal3p-Gal80p-binding step would be just the initial event in a multistep mechanism leading to activation of the Gal4p protein.

The Gal3p mutant proteins may prove useful in future studies aimed at dissecting the pathway of induction. One goal is to determine the effect of Gal3p on Gal80p and how that in turn alters the Gal80p-Gal4p interaction. Use of some of the mutant proteins in physical studies might allow us to begin to differentiate between a Gal3p-Gal80p-binding step and subsequent steps in the induction pathway. Additionally, a challenging goal in the pursuit of the induction mechanism in the galactose/melibiose system is to obtain a regulatable Gal4p-dependent yeast in vitro transcription system (58). One requirement for such a system will be faithful Gal3p-mediated alteration of the Gal80p-Gal4p interplay. For this purpose one of the Gal3 mutant proteins, such as Gal3p-322, might be useful as the initiating induction signal in an in vitro transcription reaction. In contrast to the use of the wild-type Gal3p, use of the Gal3p-322 protein would not require galactose and ATP, which may be metabolized in the yeast extract.

ACKNOWLEDGMENTS

Michael P. Woods and T. Eric Blank contributed equally to this work.

We thank Anita Hopper and Alok Sil for critical reading of the manuscript and Jonathan Raser, Lori Knackstedt, and Ling Zou for technical assistance. We thank Paul Bartel and Stanley Fields, Jayadeva Bhat, Richard Bozzato, Tom Christianson, Bob Deschenes, Phil Hieter, Judith Jaehning, Ralph Keil, Duane Mooney and Ted Young, Larry Mylin, Louise Prakash, Tim Torchia, Andrew Waskiewicz, and Ling-wen Yuan for providing plasmids, strains, oligonucleotides, and/or antisera used in this work.

This work was supported by Public Health Service grant GM27925 to James E. Hopper.

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