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Functional Domains of a Negative Regulatory Protein, GAL80, of *Saccharomyces cerevisiae*

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To study the functional domains of a transcriptional repressor encoded by the *GAL80* gene of *Saccharomyces cerevisiae*, we constructed various deletion and insertion mutations in the *GAL80* coding region and determined the ability of these mutations to repress synthesis of galactose-metabolizing enzymes as well as the capacity of the mutant proteins to respond to the inducer. Two regions, from amino acids 1 to 321 and from amino acids 341 to 423, in the total sequence of 435 amino acids were required for repression. The internal region from amino acids 321 to 340 played a role in the response to the inducer. The 12 amino acids at the carboxy terminus were dispensable for normal functioning of the GAL80 protein. Using indirect immunofluorescence and subcellular fractionation techniques, we also found that two distinct regions (amino acids 1 to 109 and 342 to 405) within the putative repression domain were capable of directing cytoplasmically synthesized *Escherichia coli* β-galactosidase to the yeast nucleus. In addition, three *gal80* mutations were mapped at amino acid residues 183, 298, and 310 in the domain required for repression. On the basis of these results, we suggest that the GAL80 protein consists of a repression domain located in two separate regions (amino acid residues 1 to 321 and 341 to 423) that are interrupted by an inducer interaction domain (residues 322 to 340) and two nuclear localization domains (1 to 109 and 342 to 405) that overlap the repression domains.

Expression of many yeast genes is primarily under positive control mediated by a specific activator protein that binds to upstream activating sequences (UASs). Regulation can be accomplished by changing either the amount (13) or the activity of the activator protein. The latter type of regulation acts on the *GAL1*, *-7*, and *-10* genes, which encode three galactose-metabolizing enzymes in *Saccharomyces cerevisiae*. These genes are clustered on chromosome II (7). Their transcripts are undetectable in yeast cells grown in the absence of galactose and induced within 15 min after addition of this sugar to a level of 1 to 2% of the total poly(A)$^+$ RNA (51). This tight repression and the high level of expression result from the interaction of two regulatory proteins, GAL4 (14, 20, 26, 27) and GAL80 (29, 38, 40, 53, 58). The GAL80 protein is proposed to inhibit the function of the GAL4 protein by a direct interaction (36, 41). When the GAL80 protein is inactivated by binding to the inducer, the GAL4 protein is liberated and functions to cause efficient expression of *GAL1*, *-7*, and *-10*. This model has been supported by the following crucial findings. First, sequences designated UAS_G were found in regions upstream of *GAL1*, *-7*, and *-10* and were shown to be necessary for enhancement of transcription by the GAL4 protein (9, 18, 44, 52, 55, 56). Second, footprinting experiments in vitro (2, 3, 28) and in vivo (10) demonstrated that the GAL4 protein does in fact bind to UAS_G. Third, the GAL80 protein was shown to bind the GAL4 protein in vitro (31).

Recently, the functional aspects of the GAL4 protein have been studied extensively and shown to consist of four domains that are individually responsible for nuclear targeting (49, 50), DNA binding (4, 19, 24), stimulation of transcription (4, 8, 21, 22, 24, 32, 33), and interaction with the GAL80 protein (21, 22, 33). In contrast, little is known about the functional domains of the GAL80 protein. This study has revealed that the GAL80 protein is composed of at least three functional domains: a domain involved in repression, a domain for interaction with inducer, and two domains for accumulation of the protein in the nucleus. It also appears that the localization of the GAL80 protein in the nucleus is a process separate from repression.

MATERIALS AND METHODS

Yeast and bacterial strains. *S. cerevisiae* MT81-1 (*MATa gal80::LEU2 ade ura3 trp1 his3 leu2*) was used for assays of repressor activity of variants of *GAL80*. In this strain, the *LEU2* fragment was inserted into *GAL80*, replacing a 0.6-kilobase (kb) region between two *Bgl*II sites (Fig. 1C). Strain MT8-1 (*MATa ade ura3 trp1 his3 leu2*) was used as the wild type with respect to *GAL80*. Diploid strains N625 (*MATa/MATα gal80::LEU2/gal80::LEU2 ura3/ura3 trp1/trp1 ade/ade* and N689 (*MATa/MATα GAL4^c/GAL4^c ura3/ura3 met/MET thr/THR trp/TRP his/his ade/ade*) were used for immunofluorescence determinations of the intracellular distribution of the GAL80 protein. *Escherichia coli* M15 (d[*lac-pro*] *thi* φ80d*lacZM15 ara recA rpsL*) was used for cloning of genes and propagation of plasmids.

Media and growth conditions. Yeast media SGlyLac and SGlyLacGal, for noninducing and inducing conditions, respectively, have been described previously (52). SGlyLac supplemented with 0.1% 2-deoxygalactose and 2% agar was used for determination of repressor activity of mutant genes of *GAL80* on plates (40). Yeasts constitutively synthesizing the galactose-metabolizing enzymes do not grow on plates containing 2-deoxygalactose, whereas wild-type yeasts do. To study β-galactosidase synthesis of yeast cells on the plates, SGlu or SGal agar (52) was supplemented with 5′-bromo-4′-chloro-3′-indoyl-β-D-galactoside (X-Gal; 40 μg/ml) and K_2HPO_4 (50 mM). *E. coli* cells were grown in LB medium, and ampicillin was added to a final concentration of 30 μg/ml when necessary.

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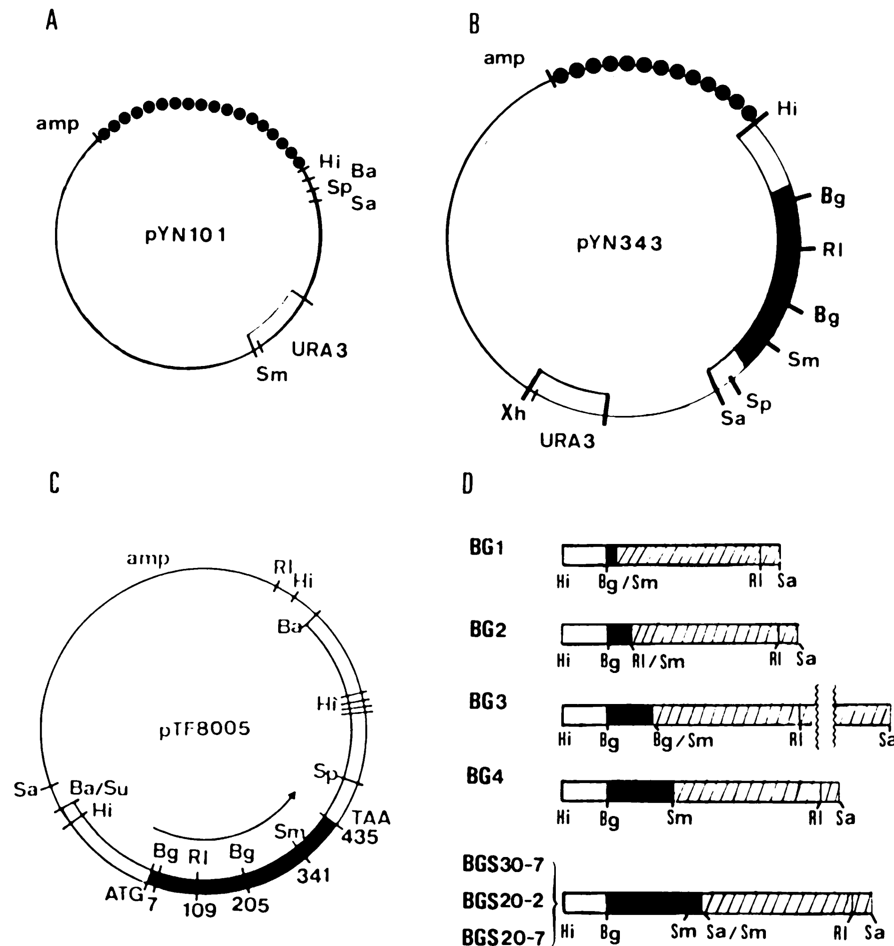

FIG. 1. Structure of pYN101 (A), pYN343 (B), pTF8005 (C), and gene fusions of *GAL80* and *E. coli lacZ* (D). Symbols: —, pBR322 DNA; ●●●●, 2μm DNA; ▭, yeast or bacterial DNA; ■, *GAL80* reading frame (1,345 base pairs); ▨, *lacZ*. Abbreviations: Bg, *Bgl*II; RI, *Eco*RI; Sm, *Sma*I; Sp, *Sph*I; Hi, *Hin*dIII; Sa, *Sal*I; Su, *Sau*3A; Xh, *Xho*I. Each number in panel C indicates the number of codons, taking the first ATG triplet as +1. The extra 3.2-kb region in pBG3 represents *lacY* and *lacA* DNA downstream of *lacZ*, which was derived from pMC874 (6).

Plasmids. To construct pYN101 (Fig. 1A), the *Eco*RI-*Hin*dIII fragment of YIp5 (1) was replaced with the *Eco*RI-*Hin*dIII fragment containing 2μm DNA excised from YEp24 (1), and the resulting plasmid was cleaved with *Eco*RI. The ends were filled in with Klenow fragment and self-ligated.

To construct pYN343 (Fig. 1B), the *Hin*dIII-*Sph*I fragment of pTF8005 (Fig. 1C), containing the entire *GAL80* gene (38), was inserted into pYN105, a derivative of pYN101 (Fig. 1A), by replacing the *Hin*dIII-*Sph*I region. To construct pYN105, the *Sma*I site in *URA3* of pYN101 was eliminated by insertion of an *Xho*I linker.

Carboxy-terminal deletions. pBG1, pBG2, pBG3, and pBG4 (Fig. 1D) were constructed by replacing carboxy-terminal portions of *GAL80* derived from pTF8005, at the indicated positions, with an amino-terminally truncated *lacZ* gene (*'lacZ*) from *E. coli* as follows. To construct pBG1, the following three fragments were ligated: the 1.3-kb *Hin*dIII-*Bgl*II (blunt ended by filling in with Klenow enzyme) fragment encoding the amino-terminal 7 amino acids of GAL80 protein from pTF8005; the 3.0-kb *Sma*I-*Sal*I fragment of the *'lacZ* gene from pMC1871 (5), in which the *Bam*HI site was filled in; and the 7.0-kb *Hin*dIII-*Sal*I fragment of pYN101. pBG2 and pBG4 were constructed in the same way except that the 1.6-kb *Hin*dIII-*Eco*RI (blunt ended) fragment encoding the 109 amino-terminal amino acids and the 2.3-kb *Hin*dIII-*Sma*I fragment encoding 342 amino acids of GAL80 from pTF8005, respectively, were used. To construct pBG3, the 1.9-kb *Hin*dIII-*Bgl*II fragment encoding the 205 amino-terminal amino acids of GAL80, the 6.2-kb *Bam*HI-*Sal*I fragment of *'lacZ* from pMC874 (6), and the 7.0-kb *Hin*dIII-*Sal*I fragment of pYN101 were ligated.

To construct pBGS30-7, pBGS20-2, and pBGS20-7, pYN 343 was digested with *Sal*I and treated with BAL 31 nuclease. The ends were filled in with Klenow fragment and self-ligated with *Sal*I linker. Three of six candidates were chosen by DNA sequencing. The *Hin*dIII-*Sal*I (blunt ended) fragments containing carboxy-terminally truncated *GAL80* were prepared from each plasmid. Each fragment was ligated together with the *Hin*dIII-*Sal*I fragment of pYN105, to the 3.0-kb *Sma*I-*Sal*I fragment from pMC1871.

To construct pBGSD20-7, pBGS20-7 was cleaved at the *Bgl*II and *Sma*I sites. The *Bgl*II site was blunt ended by filling in and ligated to the *Sma*I site, with elimination of the 1.0-kb internal fragment. pBGSD20-2 was constructed similarly but starting with pBGS20-2.

In-frame insertion mutations. For pBG32, the 1.2-kb *Eco*RI-*Sal*I fragment was excised from pYN343 and substituted for the *Eco*RI-*Sal*I fragment of pBG2. For pBG36, the

0.5-kb *Sma*I-*Sal*I fragment was excised from pYN343 and substituted for the *Eco*RI (blunt ended)-*Sal*I fragment of pBG4 at the carboxy terminus of the '*lacZ* portion. For pIE20, the *Sma*I-*Sal*I fragment of pYN343 was subcloned into the *Sma*I-*Sal*I sites of pUC118 (54). The resulting plasmid was cleaved at the *Eco*RI site in the polylinker of pUC118, and the ends were filled in with Klenow fragment and self-ligated. From the resulting plasmid, the *Eco*RI (blunt ended)-*Sal*I fragment bearing an 18-base-pair in-frame insertion in the *GAL80* coding region was excised and substituted for the *Sam*I-*Sal*I region of pYN343.

Internal deletion mutations. For pDE11, pDE12, and pDE13, the 7.9-kb *Hin*dIII-*Sal*I fragment excised from pBG3 was inserted between the *Hin*dIII and *Sal*I sites of pBR322 to construct pYN200. Plasmid pYN200 was successively digested with *Bgl*II and BAL 31. The resulting DNA was cleaved with *Hin*dIII, and a 10.5-kb fragment lacking the 5' portion of the *GAL80* coding region was obtained. This fragment was ligated to the 1.3-kb *Bgl*II (blunt ended)-*Hin*dIII fragment that encoded the 7 amino-terminal amino acids of the GAL80 protein derived from pYN200. The resulting plasmid was introduced into *E. coli* M15, and transformants showing blue color on X-Gal-containing agar were selected as candidates for *gal80'-'lacZ* fusions. Three internal deletions of eight candidates were chosen by DNA sequencing. The *Hin*dIII-*Eco*RI fragment containing a deletion mutation was substituted for the corresponding fragment of pYN343.

For pDE21, pDE22, and pDE23, the 5.3-kb *Hin*dIII-*Sal*I fragment from pBG4 was inserted between the *Hin*dIII and *Sal*I sites of pBR322. The resulting plasmid, pYN243, was cleaved with *Sma*I and treated with BAL 31. The plasmid was further digested with *Hin*dIII, and the 2.3-kb fragment of the *Hin*dIII-*Sma*I fragment was inserted into pUC118 in the presence of the *Sma*I linker. Of six candidates, three internal deletions were selected by DNA sequencing. The *Hin*dIII-*Sma*I fragment containing a deletion was substituted for the wild-type *GAL80* gene in pYN343.

For pDE31, pDE11 was cleaved with *Hin*dIII and *Eco*RI. The resulting 1.6-kb fragment was substituted for the *Hin*dIII-*Eco*RI fragment of pDE21.

Fusions of *gal7'-'lacZ-'gal80* (pBG73). The 6.9-kb *Bam*HI-*Sal*I fragment encoding *gal7'-'lacZ* from pMT24-271 (52) was inserted between the *Bam*HI and *Sal*I sites of pYN105. The *Eco*RI (blunt ended)-*Sal*I fragment carrying the carboxy terminus of '*lacZ* was substituted for the 0.5-kb *Sma*I-*Sal*I fragment of pYN343 to generate *gal7'-'lacZ-'gal80* in a continuous reading frame.

Immunoblot analysis. Yeast cells carrying the indicated plasmids were grown to the logarithmic phase of growth in SGlyLac or SGlyLacGal medium. About 5×10^7 cells were withdrawn, suspended in 0.1 ml of extraction buffer (1 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris hydrochloride, pH 8.0) and disrupted in the presence of 0.1 g of glass beads by vigorous agitation on a Vortex mixer (The Vortex Manufacturing Co.) for 10 min at 4°C. After centrifugation at 10,000 rpm for 10 min in a microfuge, the clear supernatant was removed. The concentration of protein was determined by the method of Lowry et al. (30), and each sample, containing a defined amount of protein, was subjected to 8% polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Fractionated proteins were electrophoretically transferred to a Durapore membrane (Millipore Corp.). Detection of chimeric proteins was carried out as described previously (12) except that β-galactosidase-specific immunoglobulin G (IgG) of mouse origin (Promega Biotec) was used as the first antibody and skim milk (Snow-Brand Co.) was used at a concentration of 5% in place of gelatin in blocking or reaction buffers. When the mouse IgG was used, alkaline phosphatase-conjugated anti-mouse IgG of goat origin (Promega Biotec) was used as the second antibody. To detect the GAL80 protein, GAL80-specific antiserum from rabbits was used as the first antibody. The antiserum was raised against a synthetic peptide composed of amino acids 247 to 264 of the GAL80 protein.

Indirect immunofluorescence microscopy. Indirect immunofluorescence staining was performed essentially by the method of Kilmartin and Adams (25), using β-galactosidase-specific IgG from mice (Promega Biotec) and fluorescein isothiocyanate-conjugated anti-mouse IgG raised in goats (Organon Teknika). Nuclei were identified by staining with propidium iodide under a fluorescence microscopy (I. Uno, personal communication).

Subcellular fractionation of yeast lysate. Nuclei were isolated as described by Sherman et al. (46), except that the spheroplast lysate was fractionated directly in a 50% Percoll (Sigma Chemical Co.) gradient after disruption in a Dounce homogenizer. Fractions were collected from the bottom of the gradient, and those containing nuclei were determined by staining a portion of each fraction with propidium iodide. Each fraction was assayed for β-galactosidase activity as described previously (52).

Isolation of *gal80* mutants. To isolate recessive constitutive *gal80* mutants the *gal7'-'lacZ* gene fusion was integrated at the chromosomal *HIS3* locus of MT8-1. The resulting yeast strain (N583) yielded blue colonies on SGal plates that contained X-Gal but not on X-Gal-containing SGlu plates, indicating that the integrated *gal7'-'lacZ* was under normal control by galactose. The N583 strain was mutagenized with sufficient ethyl methane-sulfonate to permit 10% survival and plated on SD plates containing X-Gal. Colonies showing blue color were tested for sensitivity to 2-deoxygalactose. In this way, nine constitutive mutants were selected from approximately 20,000 colonies. All of the mutants were found to be recessive to the wild type, which suggested that they were *gal80* but no *GAL4*^{c} mutants. Three mutant alleles were retrieved by the gap repair method (15), using pYN343 that had been linearized by deletion of the *GAL80* region between the *Bgl*II and *Sma*I sites (Fig. 1B). Plasmids carrying retrieved alleles were designated pGAL80-x, where x indicates the allele number. The plasmids were reintroduced into MT81-1, and the resulting transformants were tested for 2-deoxygalactose sensitivity and high galactokinase activity in the uninduced state (data not shown). The nucleotide sequence of the 1.0-kb DNA fragment between the *Bgl*II and *Sma*I sites was determined in each of the retrieved genes.

Other procedures. Galactokinase activity was assayed in cells permeabilized with dimethyl sulfoxide as described previously (39). The lithium acetate method was used for transformation of yeast cells (17). DNA sequencing was performed by the dideoxynucleotide chain termination method, using bacteriophages M13mp18 and -mp19 (45, 57).

RESULTS

Principles of the experiments. We assumed initially that the 435-amino-acid GAL80 protein (38) consisted of at least two functional domains: one for repression and one for interaction with the inducer. We further predicted that the GAL80 protein would have a domain for localization to the nucleus, because interaction with the GAL4 protein may take place in the nucleus. To examine these hypotheses, we constructed

TABLE 1. Effect of carboxy-terminal deletions on GAL80 function

Plasmid	Amino acids retained	Growth on 2-deoxy-galactose[a]	Galactokinase activity[b]		β-Galacto-sidase activity[c]
			Uninduced	Induced	
pYN343	1–435	+	0.2	1.9	ND
pYN105	0	−	2.2	1.8	ND
pBG1	1–7	−	2.2	2.1	3851
pBG2	1–109	−	2.5	1.7	447
pBG3	1–205	−	2.2	1.9	843
pBG4	1–342	−	2.6	2.1	857
pBGS30-7	1–383	−	2.4	2.7	694
pBGS20-2	1–405	−	2.1	2.4	891
pBGS20-7	1–423	+	0.5	1.7	<1[d]

[a] Five independent transformants harboring the respective plasmids were fully grown in SGlyLac medium and streaked on SGlyLac agar containing 0.1% 2-deoxygalactose. Growth was examined after 48 h of incubation at 30°C.

[b] Each of the five transformants tested for growth on 2-deoxygalactose was grown to logarithmic phase in SGlyLac (uninduced) or SGlyLacGal (induced). Enzyme activity is expressed as nanomoles of galactose phosphorylated per hour per milliliter of culture with an A_{660} of 1.0. Empirically, the error range of this assay method is within 10%.

[c] Yeast cells harboring the indicated plasmids were grown in SGlyLac. A crude extract of protein was prepared from the cells, β-galactosidase activity was determined as described by Tajima et al. (52). Values are averages of two independent assays, with a variation of less than 15%. ND, Not determined.

[d] When the transformant was grown in the presence of galactose, activity increased to 207.

various *gal80* mutations and introduced them on a multicopy vector into yeast cells that carried a null mutation of *gal80* (*gal80::LEU2*). The resulting transformants were studied for the ability to grow in the presence of 2-deoxygalactose as well as for the ability to repress *GAL1* expression. If a mutated *gal80* gene is defective in repression, transformants should not grow on 2-deoxygalactose-containing medium and should exhibit a high level of galactokinase activity under noninducing conditions. By contrast, a *gal80* mutant defective solely in interaction with the inducer should grow on 2-deoxygalactose plates and exhibit a low level of galactokinase activity which would not increase in response to induction.

We also investigated the relative amounts of mutant protein by immunoblot analysis to rule out the possibility that deficiency in repression was due merely to instability of the mutant protein. Previously, we found that a UAS_G is located upstream of *GAL80*, causing the gene to be regulated by GAL4 and GAL80 proteins at the transcriptional level (16, 47). Therefore, a GAL80 protein that retains repressor activity should repress transcription of the *GAL80* gene as well as of *GAL1*, *-7*, and *-10*. In fact, we were able to detect wild-type GAL80 protein prepared from induced cells but not from uninduced cells in our immunoblot analysis (see Fig. 3A, lane 1; unpublished data).

Carboxy-terminal deletion. We constructed a series of *gal80* deletions that replace various lengths of the carboxy-terminal portion with the *E. coli lacZ* gene such that the fusion gene encoded a chimeric protein with β-galactosidase activity (Table 1). All deletions except that which lacked the carboxy-terminal 12 amino acids (pBGS20-7) were defective in repression of *GAL1*, as judged by the inability to grow on a 2-deoxygalactose plate and by higher galactokinase activity under noninducing conditions compared with the uninduced level in pYN343. The unexpectedly high uninduced level in pYN343 was accounted for by loss of the plasmid,

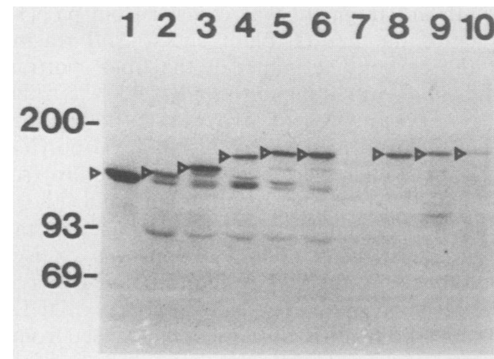

FIG. 2. Immunoblots of proteins from yeast cells transformed with the following plasmids: lane 1, pBG1; lane 2, pBG2; lane 3, pBG3; lane 4, pBG4; lane 5, pBGS30-7; lane 6, pBGS20-2; lane 7, pBGS20-7; lane 8, pBGS20-7; lane 9, pBG32; and lane 10, pBG36. Transformants were grown on either SGlyLac (lanes 1 to 7, 9, and 10) or SGlyLacGal (lane 8). The predicted (observed) molecular sizes of the respective proteins were 118 (120), 127 (130), 137 (140), 152 (150), 157 (155), 160 (155), 162 (not detected), 162 (160), 163 (165), and 163 (165) kilodaltons. Arrowheads indicate positions of those proteins. A 100-μg sample of total protein was loaded on each lane. Positions of molecular size markers (in kilodaltons) (Amersham International plk) are indicated on the left.

which was observed in 5 to 10% of culture population under out experimental conditions.

Immunoblot analysis (Fig. 2, lanes 1 to 6) showed that the repression-defective deletions produced proteins that reacted with β-galactosidase-specific IgG under noninducing conditions. (From now on, we shall refer to a mutant GAL80 protein by using the designation of the plasmid that carries the corresponding mutant gene; for example, BG4 stands for the *gal80'-'lacZ* fusion protein produced by plasmid pBG4.) BGS20-7 was detected in extracts from cells grown under inducing but not noninducing conditions (Fig. 2, lanes 7 and 8), indicating that the fusion protein repressed its own production, a result that is in accordance with previous findings (16, 47). These proteins exhibited sizes predicted from the respective constructions, although most of the extracts also contained degradation products.

Even though BGS20-7 was immunologically undetectable, it was sufficient for repression. This fact eliminates the possibility that the observed loss of repressor activity in BG1, BG2, BG3, BG4, BGS30-7, or BGS20-2 was due to instability in yeast cells.

Analysis of in-frame insertion mutations. We constructed in-frame insertion mutations in which approximately 1,000 amino acids derived from β-galactosidase from *E. coli* were

TABLE 2. Effect of in-frame insertions on GAL80 function[a]

Plasmid	Position inserted	Sequence inserted	Growth on 2-deoxy-galactose	Galactokinase activity	
				Uninduced	Induced
pBG32	109	β-Galactosidase	−	2.1	2.5
pBG36	341	β-Galactosidase	+[b]	1.1	1.5
pIE20	341	IRARYP[c]	+	0.2	1.2

[a] Experimental conditions were as given in Table 1, footnotes *a* and *b*.

[b] Growth was poorer than that of yeast cells carrying pIE20, and the colony size of yeast cells with pBG36 was approximately 50% of that of cells with pIE20.

[c] Derived from the polylinker region of pUC118 as described in Materials and Methods.

TABLE 3. Effect of internal deletions on GAL80 function[a]

Plasmid	Amino acids retained	Growth on 2-deoxy-galactose	Galactokinase activity	
			Uninduced	Induced
pDE11	1–7, 24–435	–	2.6	1.9
pDE12	1–7, 26–435	–	2.6	2.3
pDE13	1–7, 32–435	–	2.1	1.9
pDE21	1–321, 341–435	+	0.4	0.6
pDE22	1–312, 341–435	–	2.3	1.7
pDE23	1–299, 341–435	–	2.4	2.0
pDE31	1–7, 24–321, 341–435	–	2.2	2.0

[a] Experimental conditions were as given in Table 1, footnotes *a* and *b*.

inserted in frame at amino acid 109 or 341 of GAL80. Insertion of the large portion of β-galactosidase at residue 341 (BG36) still allowed incomplete retention of repressor activity, whereas insertion at residue 109 (BG32) led to a complete loss of such activity (Table 2). Immunoblot analysis (Fig. 2, lanes 9 and 10) showed that both insertions produced fusion proteins of the predicted size (approximately 165 kilodaltons). The relative amounts of the proteins were similar (Fig. 2, lanes 9 and 10). Insertion of a heterologous peptide of 6 amino acids at residue 341 (IE20) did not appreciably affect the normal repressor ability (Table 2). These results suggested that the sequence near amino acid 109 is involved in repression and that residue 341 is not part of the repression domain.

Analysis of internal deletions. We next studied internal deletions that lacked various amino acids (Table 3). All proteins with deletions except the protein that lacked 19 amino acids between residues 322 and 340 (DE21) were defective in repression. By contrast, repressor activity but not interaction with the inducer remained intact in DE21. When two internal deletions, between residues 8 to 23 (DE11) and 322 to 340 (DE21), were combined, the resulting mutant (DE31) totally lost repressor activity.

We examined, by immunoblot analysis, whether the loss of repressor activity after introduction of internal deletions was attributable to the instability of the mutant proteins. Mutant proteins DE11, DE12, and DE13 (Fig. 3A, lanes 3 to 5) and DE22 (Fig. 3B, lane 3) were visualized as bands migrating slightly faster than the wild-type protein (Fig. 3A, lane 2; Fig. 3B, lane 1), as expected from the constructions. We were unable to detect DE21 protein under noninducing conditions (Fig. 3B, lane 2), presumably because of self-repression. This result again indicated that just a trace amount of the functional peptide of GAL80 was sufficient for repression of GAL4 function. IE20, having repressor activity, was also not detected under noninducing conditions (data not shown). DE23 was barely detectable and was presumed to be less stable than the wild-type protein (Fig. 3B, lanes 4 and 1). These results suggested that deletions of residues 8 to 23, 8 to 25, 8 to 31, or 313 to 340 did not affect the stability of the mutant proteins. We concluded, therefore, that these regions, with the exception of region 322 to 340, were prerequisites for repressor function, whereas region 322 to 340 was required for recognition of the inducer.

Analysis of missense mutations. To define more accurately the functional domain involved in the repressor activity of the GAL80 protein, we determined the nucleotide sequences of genes with three independently isolated *gal80* mutations. We were able to identify single-amino-acid alterations at residues 298, 310, and 183 in the *gal80-11*, *gal80-31*, and *gal80-46* genes, respectively (Table 4). The amount of mutant protein relative to that of the wild-type protein was investigated by immunoblot analysis (Fig. 3C). The amount of protein encoded by *gal80-31* or *gal80-46* was similar to or

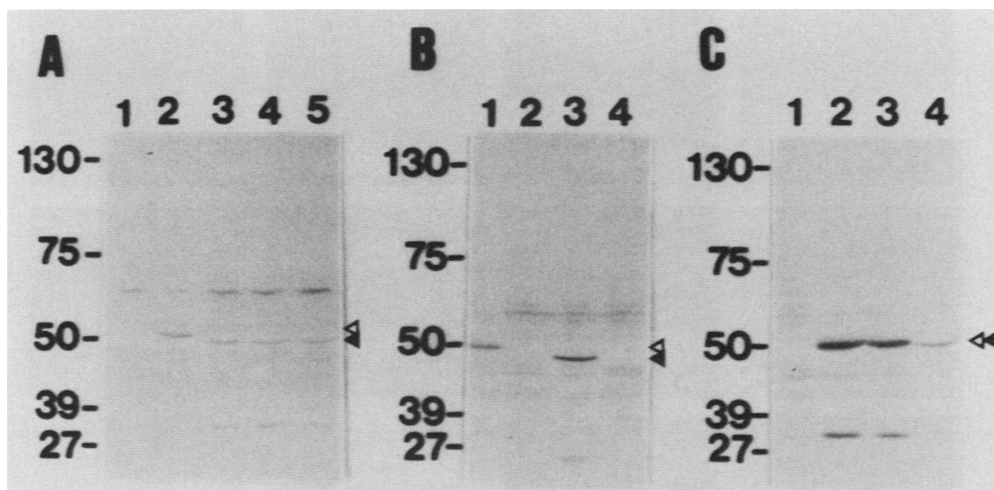

FIG. 3. Immunoblot analysis of wild-type (◁) and mutant (◀) GAL80 proteins from yeast cells transformed with the following plasmids: (A) lanes 1 and 2, pYN343; lane 3, pDE11; lane 4, pDE12; lane 5, pDE13. The predicted (observed) molecular sizes of the respective proteins were 48 (not detected), 48 (50), 47 (49), 47 (49) and 46 (49) kilodaltons. Yeast cells were grown on SGlyLac (lanes 1, 3, 4, and 5) or SGlyLacGal (lane 2). Crude extracts were prepared as described in Materials and Methods, and 120 (lanes 1 and 2) or 240 (lanes 3 to 5) μg of protein was loaded per lane. (B) Lanes: 1, pYN343; 2, pDE21; 3, pDE22; 4, pDE23. The predicted (observed) molecular weights of the respective proteins were 48 (50), 47 (not detected), 46 (48), and 44 (not detected) kilodaltons. Yeast cells were grown in SGlyLacGal (lane 1) or SGlyLac (lanes 2 to 4). Crude extracts were prepared as described above, and 120 (lane 1) or 240 (lanes 2 to 4) μg of total protein was loaded in each lane. (C) Lanes: 1, pGAL80-11; 2, pGAL80-31; 3, pGAL80-46; 4, pYN343. The predicted (observed) molecular sizes of the respective proteins were 48 (50) kilodaltons. Yeast cells were grown on SGlyLac (lanes 1 to 3) or SGlyLacGal (lane 4), and 120 (lanes 1 to 3) or 170 (lane 4) μg of total protein was loaded per lane. Positions of molecular size markers (in kilodaltons) are indicated to the left of each panel. Since we used prestained molecular size markers (Bio-Rad Laboratories), the observed values were slightly larger than those obtained with the usual markers.

TABLE 4. Nucleotide and amino acid changes in the coding region of *gal80* mutant genes

Mutant allele	Amino acid position	Nucleotide change[a]	Amino acid change
gal80-11	298	GGT→ GAT	Gly→Asp
gal80-31	310	GGA→GAA	Gly→ Glu
gal80-46	183	GGT→ GAT	Gly→Asp

[a] Altered nucleotides are underlined.

greater than that of the wild-type GAL80 protein. The Gal80-11 protein was not detectable, presumably because of instability of the protein. Therefore, the loss of repressor activity in Gal80-31 and Gal80-46 proteins indicated that the amino acid changes Gly-183 to Asp and Gly-310 to Glu diminish repressor activity, suggesting that the amino acids located at residues 183 and 310 are involved in repression.

Intracellular distribution of the GAL80 protein. We examined whether a domain required for accumulation of protein in the nucleus is present in the GAL80 protein, as it is in other yeast regulatory proteins, such as GAL4 (50), MATα2 (11), and HAP2 (43). We used gene fusions of various portions of *GAL80* to *lacZ*. The β-galactosidase protein, encoded by the *E. coli lacZ* gene, should be too large to diffuse freely into the yeast nucleus. If a protein containing a region of GAL80 accumulated in the nucleus, that region would presumably be responsible for accumulation of GAL80 in the nucleus. To study accumulation in the nucleus, we used two techniques: indirect immunofluorescence analysis (Fig. 4) and subcellular fractionation in a Percoll gradient (Fig. 5).

Results of the immunofluorescence analysis indicated that a chimeric protein with amino acid residues 1 to 109 of the GAL80 protein (BG2) accumulated in yeast nuclei (Fig. 4C), whereas one having only residues 1 to 7 (BG1) was unable to do so (Fig. 4A), suggesting that a region involved in accumulation in the nucleus was present in the amino acid segment 1 to 109. Next, we examined whether a carboxy-terminal portion also had the ability to direct β-galactosidase into the nucleus. Both chimeric proteins, containing residues 1 to 7 and 342 to 405 (BGSD20-2) and residues 1 to 7 and 342 to 423 (BGSD20-7), accumulated in the nucleus (Fig. 4E and G), indicating that the region between residues 342 and 405 is also responsible for accumulation in the nucleus. BGSD20-7 was defective in repression because the internal segment 8 to 341 was missing. Furthermore, when the carboxy-terminal residues were attached to codon 1007 of the β-galactosidase in a continuous reading frame, the product of the resulting *gal7'-'lacZ-'gal80* fusion (BG73) accumulated in the nucleus (Fig. 4I). Since the fusion protein encoded by

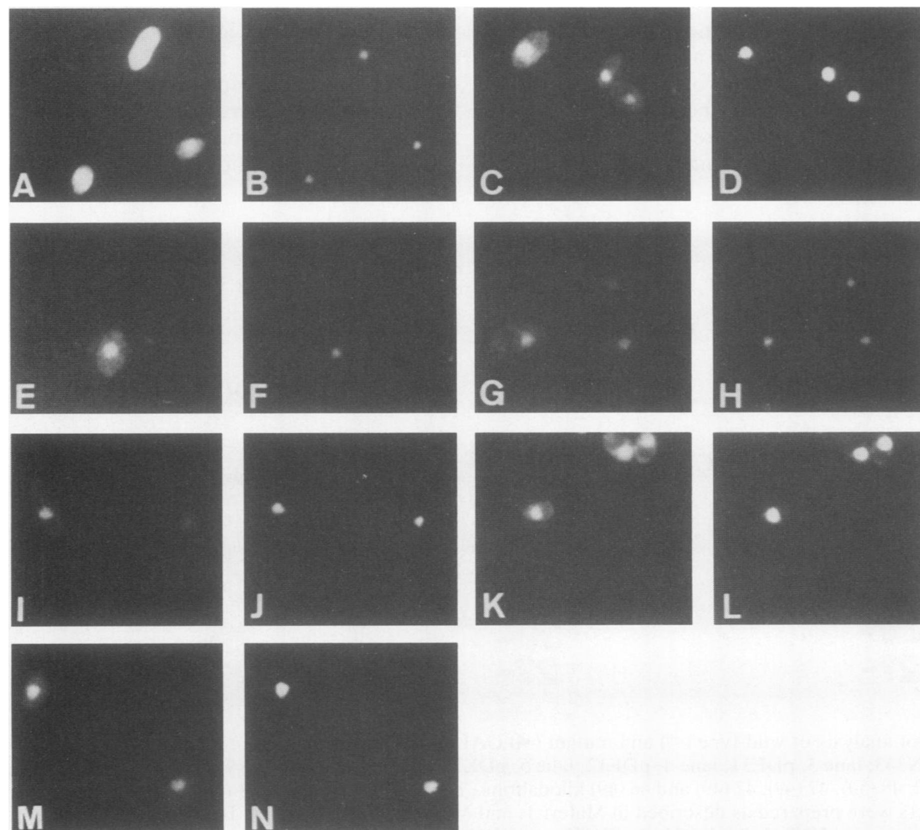

FIG. 4. Indirect immunofluorescence of yeast cells producing chimeric proteins. Cells carrying the indicated plasmids were grown in SGlyLac. (A and B) pBG1; (C and D) pBG2; (E and F) pBGSD20-2; (G and H) pBGSD20-7; (I and J) pBG73 (these plasmids were carried in diploid yeast cells of strain N625, which carried *GAL4/GAL4*); (K and L) pBG2; (M and N) pBGSD20-2 (these plasmids were carried in diploid yeast cells of strain N689, which carried *GAL4ᶜ/GAL4ᶜ*). Samples were incubated with mouse β-galactosidase-specific monoclonal antibody as the primary antibody and then with fluorescein isothiocyanate-conjugated anti-mouse IgG of goat origin. Nuclei were stained with propidium iodide. Panels A, C, E, G, I, K, and M show fluorescein-stained fields to demonstrate the distribution of Gal80–β-galactosidase fusion proteins; panels B, D, F, H, J, L, and N show the corresponding fields stained with propidium iodide to allow visualization of nuclei.

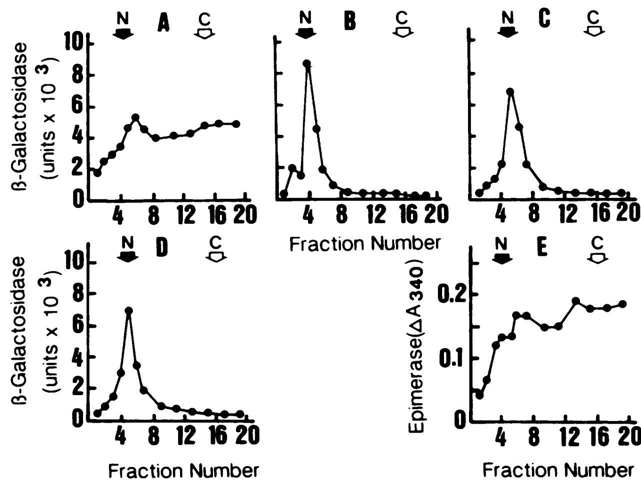

FIG. 5. Subcellular fractionation analysis of Gal80–β-galactosidase fusion proteins. Yeast cells transformed with the indicated plasmids were grown in SGlyLac. (A) pBG1; (B) pBG2; (C) pBGSD20-2; (D) pBGSD20-7. Gradients were fractionated and assayed for β-galactosidase activity. As a control, the activity of UDPglucose-4-epimerase, a cytoplasmic enzyme, was assayed in the same fractions of the gradient shown in panel B (E). N and C, Approximate locations of nuclei and cytosolic materials, respectively.

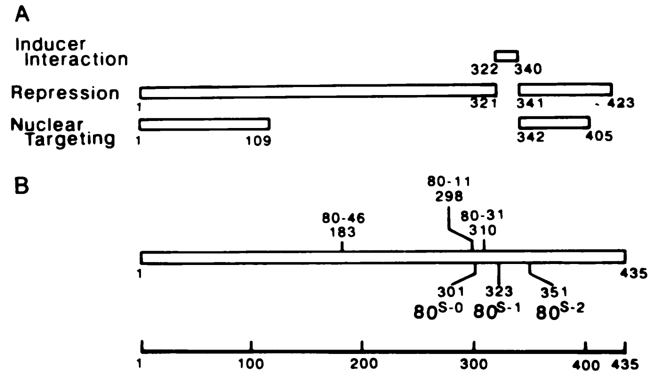

FIG. 6. (A) Proposed functional domains of the GAL80 protein. The boxes delimit regions (the boundary amino acids are listed below the boxes) suggested by results of this study to be involved in each function of the protein. (B) Physical map of *gal80* and *GAL80*^{s} mutations. The length of the GAL80 protein is marked off in amino acid residues at the bottom. The open box represents the reading frame of the GAL80 protein. Positions of recessive constitutive mutations, *gal80* (this work), are denoted above the box, and those of dominant uninducible mutations, *GAL80*^{s} (38), are shown below the box.

gal7'-'lacZ could not be localized in the nuclei (unpublished data), these results indicate that the carboxy-terminal segment of residues 342 to 405 was sufficient for localization to the nucleus.

One might argue that the GAL80 protein entered the nucleus as a complex with the GAL4 protein, since GAL4 is localized to the nucleus (50). To determine whether the nuclear localization of the *gal80'-'lacZ*-encoded protein was dependent on wild-type GAL4 protein, we introduced either pBG2 or pBGSD20-2 into *GAL4*^{c} cells. The *GAL4*^{c} mutation encodes a mutant protein that can no longer interact with the GAL80 protein (21, 36, 41). Immunofluorescence studies using β-galactosidase-specific IgG showed that both fusion proteins were able to accumulate in nuclei of *GAL4*^{c} as well as *GAL4* cells (Fig. 4K and M).

The subcellular localization of each fusion protein was also determined. The fusion proteins BG2, BGSD20-2, and BGSD20-7 were localized to the nucleus (Fig. 5B to D), whereas BG1 was not (Fig. 5A). These results are consistent with those of the immunofluorescence analysis described above.

DISCUSSION

We have presented molecular genetic evidence that the 435-amino-acid GAL80 protein consists of at least three types of functional domains (Fig. 6A). Removal of either 12 amino acids of the carboxy terminus or 19 amino acids between residues 322 and 340 did not affect repression. Surprisingly, we were able to separate the GAL80 protein into two components at that position by inserting a large fragment (approximately 1,000 amino acids) of β-galactosidase without completely destroying repressor activity. It will be of interest to study the molecular mechanism by which the two regions function to repress transcriptional activation of the GAL4 protein. In accordance with our findings, Ma and Ptashne (35) inserted an acidic peptide of 74 amino acids, derived from the *E. coli* genome, into GAL80 at position 341. The resulting chimeric protein works as a strong transcriptional activator when it is synthesized in yeast cells, producing a derivative of the GAL4 protein. This result suggests that the GAL80 protein with an insertion of these 74 amino acids retains the ability to interact with the GAL4 derivative.

We suggest that two regions, one between the amino terminus and residue 321 and the other between residues 341 and 423, are required for repression for the following reasons. (i) The deletion between residues 8 and 31 as well as that between residues 313 and 340 led to a defect in repression. (ii) The in-frame insertion of β-galactosidase at residue 109 resulted in loss of repressor activity. (iii) Yeast cells bearing missense mutations at amino acid 183 or 310 exhibited the constitutive phenotype (Fig. 6B). (The mutation at amino acid 298 led to production of a GAL80 protein that was undetectable in our immunoblot analysis, possibly because of metabolic instability in yeast cells, and therefore could not be presented as data to identify the functional domain.) (iv) All of the carboxy-terminal deletions thus far studied that extend beyond residue 423 have caused loss of repressor activity.

Induction of galactose-metabolizing enzymes by galactose was severely impaired in yeast cells producing a GAL80 protein lacking residues 322 to 340 (DE21). Because the repressor ability of that protein was indistinguishable from that of wild-type GAL80, we conclude that the region 322 to 340 is solely responsible for interaction with the inducer. The uninducible phenotype of DE21 was semidominant over wild-type *GAL80* (data not shown), as is the case for missense *GAL80*^{s} mutations (39). Three missense mutations, *GAL80*$^{s\text{-}0}$, *GAL80*$^{s\text{-}1}$, and *GAL80*$^{s\text{-}2}$, were previously mapped to residues 301, 323, and 351, respectively (38; Fig. 6B). We assume that the inducer-interactive domain partially overlaps the repressor domain, since the *GAL80*$^{s\text{-}0}$ and *GAL80*$^{s\text{-}2}$ mutations were located outside the region 322 to 340.

We also suggest that GAL80 is a nuclear protein. Two distinct regions are involved in localization of the protein to the nucleus: one encompasses residues 1 to 109, and the other encompasses residues 342 to 405. Either region was

sufficient to direct *E. coli* β-galactosidase to the nucleus. Both regions were located within the repressor domains, although neither alone exhibited repressor function, indicating that nuclear localization alone was not sufficient for repression. The domains showed no obvious homology to one another or to the signal peptide identified in T antigen from simian virus 40 (23) or the MATα2 protein from yeast cells (11). It is unknown why some nuclear proteins carry multiple signals for nuclear localization. For example, multiple signals in the MATα2 protein may be required for localization of the entire protein (49). In the case of the glucocorticoid receptor, one of two determinants may act predominantly for entry into the nucleus (42). One of two domains of yeast histone H2B was shown (37) to be an apparent nuclear-targeting domain via interaction with histone H2A. We were unable to distinguish between the two domains of the GAL80 protein with respect to function.

We considered the possibility that nuclear localization of the GAL80 protein is, at least in part, dependent on the GAL4 protein. We have not yet succeeded in determining the location of GAL80 protein in yeast cells with the *gal4* null mutation because of poor expression of *GAL80* in that mutant (data not shown). Nevertheless, we believe it unlikely that the bulk of the GAL80 protein enters the nucleus as a complex with the GAL4 protein for the following reasons. (i) The number of GAL80 molecules seems to be at least 10 times that of GAL4 molecules in uninduced yeast cells (14). Silver et al. (48) argued that the amount of the GAL4 protein expressed from the chromosomal gene is so small that it cannot be detected by the standard immunofluorescence technique. Furthermore, GAL80 molecules would be more abundant in induced than in noninduced yeast cells (47), whereas the concentration of GAL4 would remain constant (26). Therefore, more than 90% of the GAL80 molecules should remain unbound to GAL4 in induced yeast cells if we assume that one molecule of GAL80 binds one molecule of GAL4. (ii) We showed that either of the two nuclear localization domains of GAL80 could lead β-galactosidase into the nuclei of $GAL4^c$ yeast cells. Ma and Ptashne showed (33) that overproduction of the peptide of 30 carboxy-terminal amino acids, one of the two transcriptional activating sequences of the GAL4 protein, causes constitutive expression of *GAL1-lacZ*, presumably by titrating out all of the GAL80 molecules. Johnston et al. (21) showed that the $GAL4^c$ mutation results from deletion of 28 carboxy-terminal amino acids of GAL4. These results strongly suggest that $GAL4^c$ cells would indeed produce an altered GAL4 protein that lacks the domain required for interaction with GAL80. Therefore, it appears that the GAL80 protein is not conveyed to the nucleus by the GAL4 protein.

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