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Interaction of *GAL4* and *GAL80* Gene Regulatory Proteins In Vitro

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The *GAL80* protein of *Saccharomyces cerevisiae*, synthesized in vitro, bound tightly to *GAL4* protein and to a *GAL4* protein-upstream activation sequence DNA complex, as shown by (i) coimmunoprecipitation of *GAL4* and *GAL80* proteins with anti-*GAL4* antiserum, (ii) an electrophoretic mobility shift of a *GAL4* protein-upstream activation sequence DNA complex upon the addition of *GAL80* protein, and (iii) *GAL4*-dependent binding of *GAL80* protein to upstream activation sequence DNA immobilized on Sepharose beads. Anti-*GAL4* antisera were raised against a *GAL4-URA3* fusion protein, which could be purified to homogeneity in a single step with the use of an affinity chromatographic procedure for the *URA3* gene product.

GAL4 and *GAL80* proteins regulate the expression of a set of genes in *Saccharomyces cerevisiae* in response to galactose in the growth medium (30). *GAL4* protein causes transcription through binding to upstream activation sequences (UASs) (12–15, 21, 36, 37), whereas *GAL80* protein prevents transcription during growth on nonfermentable carbon sources in the absence of galactose (29, 34). The UASs associated with various genes contain one or more *GAL4*-binding sites of about 20 base pairs (bp), and the sequences of 11 sites so far identified are highly homologous (5, 6, 11, 18). Synthetic oligonucleotides of 17 or 21 bp with such sequences bind *GAL4* protein in vitro and support galactose-dependent transcription in vivo (11, 25). The latter observation not only indicates *GAL4* binding in vivo, but also suggests that *GAL80* protein acts directly on *GAL4* protein or on the *GAL4*-binding region of UAS DNA. *GAL80* protein may associate with (31) or modify *GAL4* protein, for example, forming a protein-protein complex unable to bind UAS DNA or forming a *GAL80-GAL4*-DNA complex unable to activate transcription. Alternatively, *GAL80* protein may compete with *GAL4* protein for binding to UAS DNA.

There are a number of additional observations supporting the notion of *GAL80-GAL4* and *GAL80-GAL4*-UAS DNA interactions in vivo. (i) A truncated *GAL4* molecule lacking the DNA binding domain, when overexpressed, appears to block repression by *GAL80*, indicative of a protein-protein interaction in the absence of DNA (16). (ii) *GAL4*-dependent DNA-binding in vivo persists under conditions of repression by *GAL80* protein, suggesting that a *GAL4*-DNA complex is the target of *GAL80* action (11, 24). (iii) Multiple *GAL4*-binding sites in UASs facilitate repression by *GAL80* protein, again pointing to the involvement of a *GAL4* protein-DNA complex in *GAL80* action (6). Here we present direct evidence for *GAL80* protein binding to *GAL4* protein and to a *GAL4*-UAS DNA complex in vitro.

MATERIALS AND METHODS

Yeast strains and plasmids. *S. cerevisiae* BY2 was described previously (5). Strain 15c/pUG4Is (α *leu2-31,12 ura3-52 Δtrp1 pep4-3*::pUG4Is) is protease deficient and harbors pUG4Is, a derivative of the centromeric vector YCp50 containing the *GAL4* gene under control of the *GAL1* promoter.

For the construction of pUG4Is, the *Sph*I-*Hin*dIII fragment of the *GAL4* gene from pSJ4 (15), lacking the sequence encoding the first 10 amino acids of the protein, was modified with a synthetic *Bgl*II-*Sph*I oligonucleotide that restored the amino-terminal region. The resulting *Bgl*II-*Hin*dIII fragment containing the entire *GAL4* gene was inserted between the *Bam*HI and *Sal*I sites of pBM125 (14).

pGU2, a vector for the expression of *URA3* fusion proteins, carried the *URA3* gene under control of the *GAL1* promoter, along with a region of the 2μm plasmid needed for maintenance at a high copy number in yeast, and with the entire *GAL4* gene to assure a level of *GAL4* protein sufficient for maximal transcription from the *GAL1* promoter (Fig. 1). A 0.2-kilobase (kb) *Xho*I-*Cla*I fragment of the *GAL4* gene was inserted at a *Taq*I site in the second codon of the *URA3* gene, fusing the two coding regions in frame and conserving the Ser codon of the *URA3* gene. The resulting plasmid (pGU2-0.2G4) encoded a *GAL4-URA3* fusion protein extending from Met-79 to Val-146 of *GAL4* and from Ser-2 to the end of the *URA3* protein.

pSP65-G80, used to transcribe the *GAL80* gene in vitro, was constructed by inserting an *Acc*I-*Hin*dIII fragment containing the entire coding sequence of the *GAL80* gene (29) between the *Eco*RI and *Hin*dIII sites of pSP65 (28). pSP65-G4, used to transcribe the *GAL4* gene in vitro, was constructed by inserting the *GAL4*-containing *Bgl*II-*Hin*dIII fragment described above between the *Bam*HI and *Hin*dIII sites of pSP65.

***URA3* and *GAL4-URA3* proteins.** Plasmids pGU2 and pGU2-0.2G4 were introduced into *S. cerevisiae* Sf657-2D (**a** *pep4-3 his4-580 ura3-52 leu2-31,12*) (from C. Fields, Berkeley, Calif.) by a standard transformation procedure (2) with selection for growth on minimal medium supplemented with histidine and uracil. For production of proteins, cells were grown at 30°C in minimal medium–2% sucrose supplemented with histidine to an absorbance at 600 nm of 2, diluted 20-fold with YP medium (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories]), and further grown to an absorbance at 600 nm of 3. Galactose was added to a final concentration of 2%, and the cells were grown for an additional 2 h. Cells were harvested by centrifugation at 6,000 × *g* for 10 min, washed, and suspended in an amount of buffer containing 50 mM potassium phosphate (pH 7.0), 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 0.6 μM leupeptin equal in volume to the cell pellet. Cells were broken at 0°C with an

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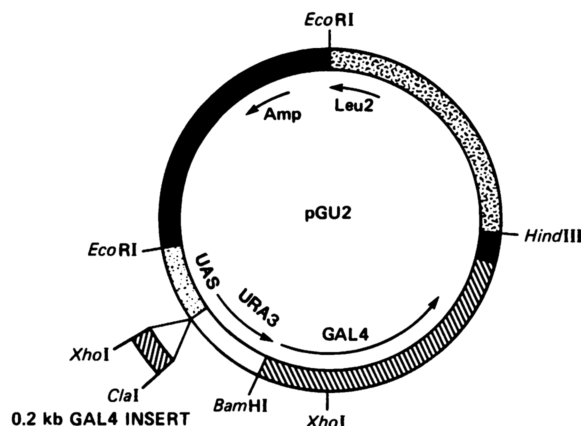

FIG. 1. Expression vector for *URA3* fusion proteins (pGU2), comprising five DNA segments: a 0.8-kb *Eco*RI-*Bam*HI fragment from pBM125 (14), containing the *GAL1* and *GAL10* promoters and intergenic region; a 0.9-kb *Pst*I-*Sma*I fragment containing the entire coding region of the *URA3* gene (33); a 3.6-kb *Bam*HI-*Hin*dIII fragment from pSJ4, containing the entire *GAL4* gene and a short flanking region of pBR322 (15); a 3.3-kb *Hin*dIII-*Eco*RI fragment from pJDB207 (3), containing the *LEU2* gene and 2μm plasmid replication region; and a 2.3-kb *Eco*RI-*Pvu*II fragment of pBR322, containing the β-lactamase (Amp^r) gene and replication origin. The various regions are symbolized as follows: *URA3* gene (▭); *GAL4* gene (▧); 2μm plasmid (▨); pBR322 (■); *GAL1-GAL10* promoters and intergenic region (UAS) (▨). A 0.2-kb *Xho*I-*Cla*I *GAL4* fragment (see text) was inserted to form pGU2-0.2G4 as indicated.

equal volume of glass beads (0.45-mm diameter) with eight 30-s pulses of a bead beater (Biospec Products). Cell lysates were clarified by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 20 min at 4°C. The specific activities of orotidine monophosphate (OMP) decarboxylase (7) in the clarified lysates were 8 U/mg for pGU2-containing cells and 0.4 U/mg for pGU2-0.2G4-containing cells. Similar extracts from wild-type yeast were reported to have specific activities of 0.003 to 0.006 U/mg of protein (7, 32, 35). The clarified lysates were further centrifuged in a Beckman Ti60 rotor at 50,000 rpm for 3 h at 0°C, and the supernatants were adjusted to a concentration of 10 mg of protein per ml. Chromatography on Affi-Gel Blue (Bio-Rad Laboratories) was performed as described previously (23, 32). Samples were loaded onto the column and washed with 10 column volumes of buffer containing 50 mM Tris hydrochloride (pH 8.0), 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 0.6 μM leupeptin. OMP decarboxylase and *GAL4-URA3* fusion protein were specifically eluted from the column in the same buffer with 50 μM 6-aza-UMP (Sigma Chemical Co.). Column fractions were monitored for protein by the method of Bradford (4) and assayed for OMP decarboxylase activity. Peak fractions were combined and concentrated with Centricon-30 filters (Amicon). Specific activities of the purified OMP decarboxylase and *GAL4-URA3* fusion protein were about 40 U/mg, which compares favorably with values for the homogeneous enzyme of 35 to 40 U/mg reported previously (7, 35). Analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (19) revealed a single polypeptide (>98% pure) of 29 kilodaltons from pGU2-containing cells and a polypeptide of 37 kilodaltons from pGU2-0.2G4-containing cells, in good agreement with the sizes expected for OMP decarboxylase and *GAL4-URA3* fusion protein. Yields were 30 and 1.5 mg/liter of culture for OMP decarboxylase and fusion protein, respectively.

Antisera. New Zealand White rabbits were given subcutaneous and intramuscular injections of 250 μg of protein in Freund complete adjuvant. The rabbits were injected 30 days later with 250 μg of protein in Freund incomplete adjuvant and were bled 10 to 14 days afterward. The presence of the desired antibodies in the serum was detected with small amounts of OMP decarboxylase or *GAL4-URA3* fusion protein dotted on nitrocellulose filters and with the use of an indirect immunoperoxidase staining procedure (Vectastain ABC horseradish peroxidase system, Vector Laboratories).

***GAL4* and *GAL80* proteins.** Transcription reactions (20 μl) contained 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine, 500 μM ATP, 500 μM CTP, 500 μM UTP, 100 μM GTP, 500 μM GpppG, 0.5 μCi of [α-^{32}P]GTP, 100 μg of linearized template per ml, 5 U of RNAsin, and 5 U of SP6 polymerase (New England Biolabs). Reactions were carried out at 39°C for 0.5 to 1.0 h and stopped by phenol-chloroform extraction (28). Typically, 0.5 μg of RNA was obtained, based on trichloroacetic acid-precipitable radioactivity. Translation was carried out with rabbit reticulocyte lysates (Promega Biotec) in the presence of [^{35}S]methionine (Amersham Corp.) according to the manufacturer's directions.

Immunoprecipitation of *GAL4*-UAS DNA and *GAL80-GAL4* complexes. Extracts (60 μg of protein) from *GAL4*-overproducing (BY2) cells or from the parental strain (Sf657-2D) were treated with 1 μl of either preimmune serum or antiserum as indicated for 1 h at 0°C. One-sixth of each mixture was added to 20 μl of buffer A (5) containing 1 μg of sonicated salmon sperm DNA and 20,000 cpm each of the following: a 250-bp *Eco*RI-*Bam*HI fragment of UAS DNA from pGF1 (5), labeled by T4 DNA polymerase replacement synthesis (27); and a 529-bp fragment of DNA from the silent mating type locus (a 490-bp *Xho*I-*Xba*I fragment containing the *HMRE* region [1], with additional sequences at the ends from a pUC18 polylinker) labeled in the same way. After 10 min at room temperature, 10 μl of *Staphylococcus aureus* cells (Pansorbin; Calbiochem) was added, and the incubation was continued for 15 min. The mixtures were diluted with cold buffer A (100 μl) and centrifuged in a microfuge for 1.0 min at 4°C. The pellets were washed twice with 100 μl of cold buffer A, suspended in 2% SDS–10 mM EDTA, heated for 10 min at 60°C, and centrifuged. The supernatants were analyzed by electrophoresis in a 2% agarose gel in 0.04 M Tris-acetate–1 mM EDTA. The gel was dried and autoradiographed.

Extracts from strain BY2 and in vitro translation mixtures containing ^{35}S-labeled *GAL80* were added to 20 μl of buffer B (10 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) and incubated for 3 h at 0°C. Antisera (1:400) were added, and the incubation was continued overnight at 0°C. (It was necessary to use less antiserum than in the precipitation of *GAL4*-UAS DNA complexes described above, or else there was nonspecific precipitation, presumably due to a low affinity of *GAL80* protein for immunoglobulins.) Pansorbin (2 μl, prewashed three times in 5 times the volume of buffer B) was added, the incubation was continued for an additional hour at 0°C, and the mixtures were diluted with 150 μl of cold buffer B and centrifuged. The pellets were washed three times in 150 μl of cold buffer B, boiled in SDS-polyacrylamide gel electrophoresis loading buffer, and analyzed by electrophoresis in SDS–10% polyacrylamide gels (19) and fluorography.

Electrophoretic mobility shift experiments. In vitro translation mixtures containing *GAL4* and *GAL80* proteins were combined in the amounts indicated with 1 fmol of a 400-bp *Eco*RI fragment from p10GH (25) containing a single *GAL4*-binding site (labeled with [α-^{32}P]dATP by replacement synthesis with T4 DNA polymerase) and 0.5 μg of sonicated salmon sperm DNA in 10 μl of buffer A. The resulting mixtures were kept for 10 min at room temperature and analyzed by electrophoresis in 4% polyacrylamide gels (acrylamide/bisacrylamide, 80:1) in 0.089 M Tris-borate–0.089 M boric acid–0.002 M EDTA (pH 7.9). Gels were prerun for 2 h at 20 mA, followed by electrophoresis of samples for 4 h at 30 mA (9, 10). Gels were dried and autoradiographed.

Protein binding to DNA-Sepharose beads. The synthetic oligonucleotide G7P1 (Applied Biosystems, 5′-CCA AAA AGC GCT CGG ACA ACT GTT GAC CGT GAT CCG A-3′) and its complement (150 μg each), corresponding in sequence to the *GAL4* binding site nearest the *GAL7* gene (6), were annealed, phosphorylated with T4 polynucleotide kinase, polymerized with T4 DNA ligase, and allowed to react with 4 ml of CNBr-activated Sepharose CL-2B beads as described previously (17). The coupling efficiency, measured spectrophotometrically at 260 nm, was about 40%. DNA-Sepharose beads (10 μl) were combined with *GAL80* translation mixture (0.5 μl) and *GAL4* overproducer extract in the amounts indicated in 50 μl of buffer (20% glycerol, 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.5]) containing 20 μg of poly(dI-dC) per ml and 100 μg of bovine serum albumin per ml. Binding was allowed to proceed overnight at 4°C with constant shaking. The beads were then washed four times in 150 μl of the same buffer and heated for 1 min at 100°C in 40 μl of SDS-polyacrylamide gel electrophoresis loading buffer followed by analysis as described above for immunoprecipitates of *GAL80-GAL4* complexes.

RESULTS

We investigated *GAL80-GAL4* and *GAL80-GAL4*-DNA interactions with the use of anti-*GAL4* antibodies. To avoid inhibition of the interactions of interest by the antibodies, we immunized with a small region of *GAL4* protein, extending from amino acids 79 to 146 (22). This region is thought not to be involved in either DNA binding (16, 18) or transcriptional activation (26), but is likely to be exposed on the surface of the protein (8). The *GAL4* fragment was expressed in yeast as a fusion with OMP decarboxylase, the product of the *URA3* gene (33), which is readily purified by an affinity chromatographic procedure (32). Native OMP decarboxylase and the *GAL4-URA3* fusion protein were expressed in large amounts under galactose regulation (Fig. 1), and antisera against the purified proteins were raised in rabbits.

OMP decarboxylase completely blocked the reaction of anti-OMP decarboxylase serum with OMP decarboxylase (immunodot reactions, revealed by horseradish peroxidase staining) but only partially inhibited the reaction of antifusion protein serum with fusion protein (a 30% reduction in the apparent titer of the antiserum). This indicated the presence of anti-*GAL4* antibodies, whose affinity for wild-type *GAL4* protein was revealed by immunoprecipitation of a complex with UAS DNA as follows. An extract of a *GAL4* overproducer (*S. cerevisiae* BY2, which carries the *GAL4* gene on a high-copy-number plasmid) was treated with anti-fusion protein serum for 1 h at 0°C, followed by the addition of ^{32}P-labeled UAS DNA, under conditions described previously for the detection of specific protein-DNA interactions in a filter-binding assay (5). A second labeled DNA fragment, from the silent mating type locus (*HMRE*), was included as a control for nonspecific binding. Precipitates were formed with *S. aureus* protein A, washed in the cold, solubilized in SDS at 60°C, and analyzed by agarose gel electrophoresis and autoradiography (Fig. 2). The precipitates contained UAS DNA, dependent upon anti-*GAL4* antibodies, as shown by precipitation with anti-fusion protein (lane 3) but not with anti-OMP decarboxylase (lane 4) or preimmune (lane 2) serum, and by blocking with fusion protein (lane 8) but not with OMP decarboxylase (lane 7). The presence of UAS DNA in the immunoprecipitates further required protein from a *GAL4* overproducer, as shown by precipitation with an extract from strain BY2 (*GAL4* on high-copy-number plasmid) but not from the parental strain (lane 5). Specificity for UAS DNA was shown by a lack of precipitation of *HMRE* DNA and by the inhibitory effect of a synthetic *GAL4*-binding oligonucleotide (in the presence of a large excess of carrier DNA; lane 6). The requirement for both anti-*GAL4* antibodies and overproducer protein demonstrated the formation of an antibody-protein-UAS DNA complex. The involvement of anti-*GAL4* antibodies confirmed that the overproduced DNA binding activity in extracts of strain BY2 is the product of the *GAL4* gene and not a secondary activity elicited in the overproducer and capable of binding UAS DNA (5).

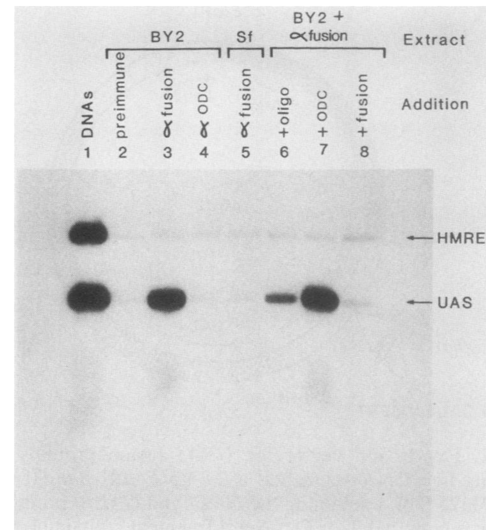

FIG. 2. Immunoprecipitation of a *GAL4* protein-UAS DNA complex. Extracts of a *GAL4* overproducer (strain BY2) or the parental strain (Sf) were prepared as described previously (5) and treated with preimmune serum or the antisera indicated (α fusion, anti-*GAL4-URA3* fusion protein; α ODC, anti-OMP decarboxylase) in the absence (lanes 2 through 6) or in the presence of OMP decarboxylase (5 μg, lane 7) or fusion protein (5 μg, lane 8). A mixture of ^{32}P-labeled *HMRE* and *GAL1-GAL10* UAS DNA fragments and 1 μg of carrier salmon sperm DNA was added, with (lane 6) or without (lanes 2 through 5 and 7 through 8) 10 ng of 25-bp *GAL4*-binding oligonucleotide (5). Immunoprecipitates were formed, washed, solubilized, and analyzed by gel electrophoresis and autoradiography as described in Materials and Methods. A mixture of the two radioactive DNA fragments was run for reference in lane 1.

Immunoprecipitation with anti-fusion protein serum was carried out in a similar fashion to investigate the interaction

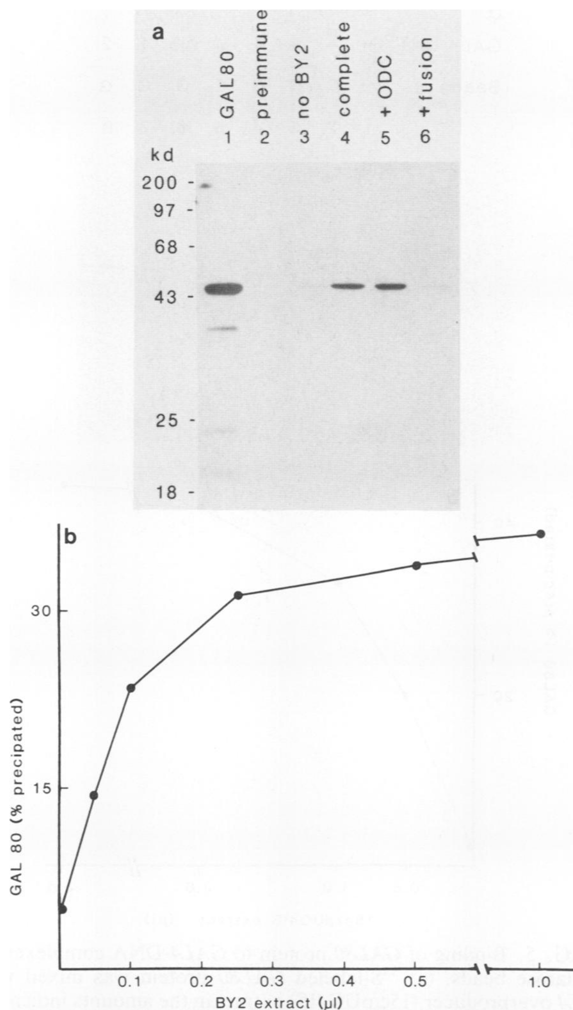

FIG. 3. Immunoprecipitation of a *GAL80-GAL4* protein-protein complex. (a) ^{35}S-labeled *GAL80* protein and *GAL4* overproducer (BY2) extract were incubated (except for the omission of extract in lane 3), followed by treatment with preimmune serum (lane 2) or anti-*GAL4-URA3* fusion serum (lanes 3 through 6) in the absence (lanes 2 through 4) or presence of OMP decarboxylase (5 µg, lane 5) or fusion protein (5 µg, lane 6). Immunoprecipitates were formed, washed, solubilized, and analyzed by gel electrophoresis and fluorography as described in Materials and Methods. The untreated ^{35}S-labeled *GAL80* protein (0.5 µl of translation mixture) was run for reference in lane 1. (b) Immunoprecipitates were formed and analyzed as described for lane 4 above, with the amounts of *GAL4* overproducer (BY2) extract indicated, and bands due to *GAL80* protein were quantitated by densitometry.

of *GAL4* and *GAL80* proteins, with the use of *GAL80* protein labeled by translation in vitro (28). Transcripts of the *GAL80* gene, produced by SP6 RNA polymerase, directed translation by a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The product formed one major band in an SDS-polyacrylamide gel, with an apparent mass of 48 kilodaltons, the same as that expected from the *GAL80* sequence (29) (Fig. 3a, lane 1). The crude translation product was treated with *GAL4* overproducer (BY2) extract and anti-fusion protein antiserum, followed by precipitation with protein A, gel electrophoresis, and fluorography. The precipitate contained *GAL80* protein, dependent upon anti-*GAL4* antibodies, as shown by precipitation with anti-fusion protein (lane 4) but not preimmune (lane 2) serum and by blocking with fusion protein (lane 6) but not with OMP decarboxylase (lane 5). Immunoprecipitation of *GAL80* protein further required *GAL4* overproducer protein, shown by a lack of precipitation in the absence of BY2 extract (lane 3). The extent of precipitation was proportional to the amount of BY2 extract added at low levels of extract (Fig. 3b). These data revealed the formation of an anti-*GAL4* antibody–*GAL4* protein–*GAL80* protein complex. It was possible, then, to investigate the long-standing hypothesis that galactose or one of its metabolites induces *GAL* gene transcription by causing dissociation of the *GAL4-GAL80* protein-protein complex. Immunoprecipitation was carried out as above, except in the presence of 1 mM galactose, galactose-1-phosphate, UDP-galactose, or UDP-glucose, with no change in the results. Either some other molecule serves as inducer, or the effect is simply not revealed in these experiments.

The evidence presented here for both *GAL4* protein-UAS DNA and *GAL4* protein-*GAL80* protein interaction raised the question of whether a ternary complex of the two proteins and DNA could be formed. The first indication of such a complex came from an electrophoretic mobility shift experiment (9, 10). *GAL4* protein was synthesized for these experiments by transcription and translation in vitro, to obtain material free of *GAL80* protein (see Discussion). SP6 transcripts of the *GAL4* gene directed the translation of two products in roughly equal quantities (data not shown), one of about 100 kDa, the molecular mass expected for *GAL4* protein, and the other of about 90 kDa, the size expected for a translation product initiated at the second AUG codon in the *GAL4* sequence (20). The smaller protein should lack the DNA binding domain at the amino terminus (16, 18). When these translation products were mixed with ^{32}P-labeled UAS DNA and analyzed by gel electrophoresis, a single band of mobility less than that of free DNA was observed (Fig. 4, lanes 4 and 9, band a; free UAS DNA ran off the bottom of the gel). This band could be attributed to a *GAL4* protein-UAS DNA complex, since its formation was inhibited by a *GAL4*-binding oligonucleotide (in the presence of a large excess of carrier DNA; Fig. 4, lane 3). The addition of *GAL80* translation product resulted in a second band of slightly lower mobility than the first (Fig. 4, lane 5, band b). Formation of the second band was due to *GAL80* protein, since control translation lysate alone had no effect (lane 9). As more *GAL80* protein was added, the intensity of the second band increased, whereas that of the first band diminished, suggesting that the second band was formed from the first (Fig. 4, lanes 6 and 7). The second band, like the first, was abolished by excess *GAL4*-binding oligonucleotide, showing that it, too, contained *GAL4* protein (Fig. 4, lane 8). Neither band was obtained in the absence of *GAL4* protein (Fig. 4, lane 2). (The faint *GAL4*-dependent smear in lanes 4 through 7 and 9 [lesser mobility] may be due to aggregates of *GAL4* protein and is also observed with *GAL4* protein isolated from yeast [D. Chasman, unpublished].) We conclude that *GAL80* protein acts on a *GAL4* protein-UAS DNA complex, reducing its electrophoretic mobility, very likely through binding to the complex.

Direct evidence for *GAL80* binding to a *GAL4* protein-UAS DNA complex was obtained by a DNA-affinity chromatographic procedure. A *GAL4*-binding oligonucleotide was polymerized with DNA ligase and coupled to cyanogen bromide-activated Sepharose CL-2B beads. High-affinity binding of *GAL4* protein to these beads was demonstrated by their ability to deplete extracts of *GAL4* protein filter binding activity (5). The activity was retained on the beads during

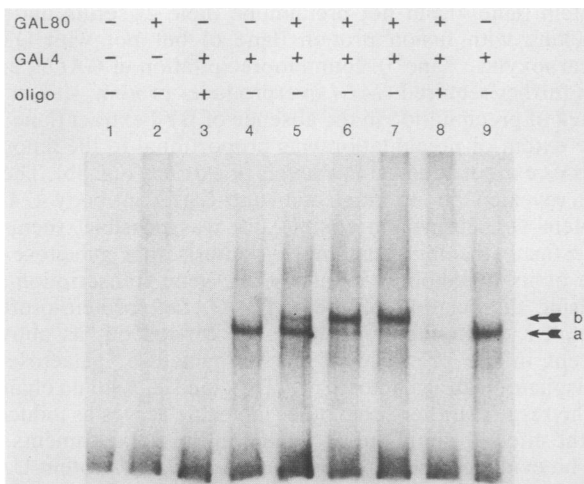

FIG. 4. *GAL4* protein-UAS DNA and *GAL80* protein-*GAL4* protein-UAS DNA complexes revealed by gel electrophoresis. Mixtures of nonradioactive *GAL4* (1 μl) and *GAL80* (0.05, 0.16, 0.5, 0.5, 0.5 μl for lanes 5, 6, 7, 8, and 2, respectively) proteins translated in vitro, ^{32}P-labeled *GAL4*-binding DNA fragment, carrier salmon sperm DNA, and the 25-bp *GAL4*-binding oligonucleotide (oligo, 10 ng) were prepared as described and analyzed by gel electrophoresis and autoradiography. The mixture analyzed in lane 9 contained 0.5 μl of reticulocyte lysate not treated with any RNA in addition to *GAL4*-containing lysate. Bands due to *GAL4* protein-DNA (a) and *GAL80* protein-*GAL4* protein-DNA (b) complexes are indicated by arrows.

washing with 0.1 M KCl (which removed more than 95% of the bound protein) and was eluted with 0.8 M KCl. ^{35}S-labeled *GAL80* protein bound to the beads in a *GAL4*-dependent manner (Fig. 5a, lanes 3 and 6 through 8; Fig. 5b). Specificity of the *GAL80* interaction was further shown by a lack of binding to beads coupled to an unrelated oligonucleotide (Fig. 5a, lane 5) or to beads with no DNA attached at all (Fig. 5a, lane 2).

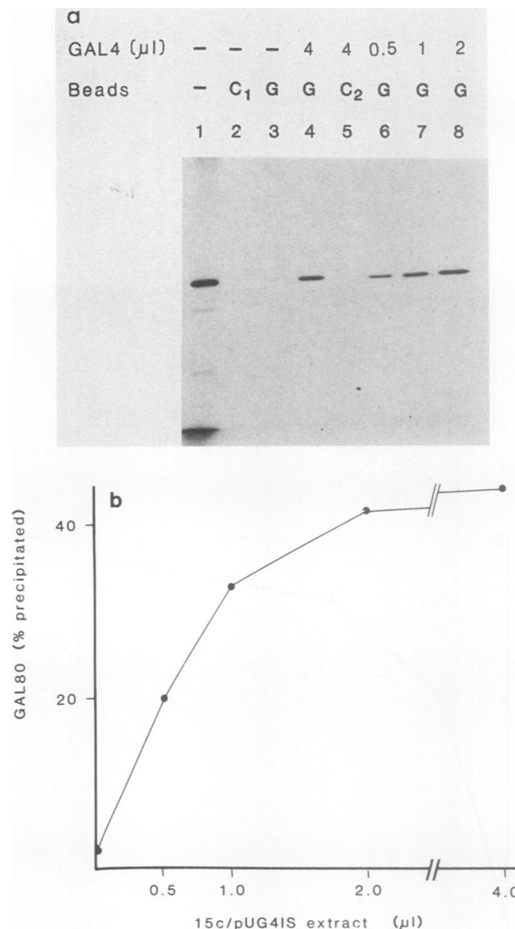

FIG. 5. Binding of *GAL80* protein to *GAL4*-DNA complexes on Sepharose beads. (a) ^{35}S-labeled *GAL80* protein was mixed with *GAL4* overproducer (15c/pUG4IS) extract in the amounts indicated. Sepharose beads coupled to *GAL4*-binding oligonucleotide (G), an unrelated oligonucleotide (C_2), or no oligonucleotide (C_1) were added, along with carrier poly(dI-dC) and bovine serum albumin. Proteins bound to the beads were analyzed by gel electrophoresis and fluorography. (b) Bands due to *GAL80* protein in lanes 3, 4, and 6 through 8 were quantitated by densitometry.

DISCUSSION

The data presented here demonstrate *GAL80* protein binding to free *GAL4* protein and to *GAL4* protein in a complex with UAS DNA. Further interactions between *GAL80* protein and DNA seem unlikely, since no *GAL80*-DNA complex was revealed by gel electrophoresis and since no binding of ^{35}S-labeled *GAL80* protein could be detected with DNA-Sepharose. The possibility that *GAL80* protein caused an electrophoretic mobility shift of a *GAL4*-DNA complex by covalent modification rather than binding also seems unlikely, in view of the stoichiometric nature of the *GAL80* effect and the direct evidence for *GAL80* binding obtained with DNA-Sepharose. The data are most compatible with the formation of a ternary complex of *GAL80* protein, *GAL4* protein, and UAS DNA in the *GAL80*-mediated, repressed state. The stability of such a complex may be augmented by multiple interactions. UASs containing a pair of *GAL4*-binding sites about 52 to 62 bp apart show complete repression by *GAL80* protein, whereas those with a single site allow some escape from repression (6). Perhaps two appropriately positioned *GAL4* molecules can bind a *GAL80* oligomer, increasing the affinity of the interaction.

The strength of *GAL80-GAL4* interaction can be estimated from the electrophoretic mobility shift experiments reported here. The concentration of *GAL80* protein required for half saturation of a *GAL4*-DNA complex corresponds to a dissociation constant of about 5×10^{-9} M for *GAL80-GAL4* interaction (assuming a 1:1 stoichiometry). This agrees well with an estimate made from an immunoprecipitation experiment performed in the absence of DNA with both *GAL80* and *GAL4* proteins synthesized in vitro (data not shown). The high affinity of the interaction raises the possibility that the two proteins occur as an oligomer in vivo. Indeed, mobility shift experiments with extracts of yeast overexpressing *GAL4* reveal a species comigrating with the *GAL80*-*GAL4*-DNA complex described here.

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LITERATURE CITED

1. **Abraham, J., K. A. Nasmyth, J. N. Strathern, A. J. S. Klar, and J. B. Hicks.** 1984. Regulation of mating-type information in yeast: negative control requiring sequences both 5′ and 3′ to the regulation region. J. Mol. Biol. **176:**307–331.

2. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275**:104–109.
3. **Beggs, J. D.** 1981. Multiple-copy yeast plasmid vectors, p. 383–389. *In* D. Von Wettstein, J. Frus, M. Kielland-Brandt, and A. Stenderup (ed.), Molecular genetics in yeast: proceedings of the Alfred Benzon symposium 16. State Mutual Book & Periodical Service, Ltd., New York.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Bram, R., and R. Kornberg.** 1985. Specific protein binding to far upstream activating sequences in polymerase II promoters. Proc. Natl. Acad. Sci. USA **82**:43–47.
6. **Bram, R., N. Lue, and R. Kornberg.** 1986. A *GAL* family of upstream activating sequences in yeast: roles in both induction and repression of transcription. EMBO J. **5**:603–608.
7. **Brody, R. S., and F. H. Westheimer.** 1979. The purification of orotidine-5′-phosphate decarboxylase from yeast by affinity chromatography. J. Biol. Chem. **254**:4238–4244.
8. **Emini, E. A., J. V. Hughes, D. S. Perlow, and J. Boger.** 1985. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J. Virol. **55**:836–839.
9. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
10. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res. **9**: 3047–3060.
11. **Giniger, E., S. Varnum, and M. Ptashne.** 1985. Specific DNA binding of *GAL4*, a positive regulatory protein of yeast. Cell **40**:767–774.
12. **Guarente, L., R. Yocum, and P. Gifford.** 1982. A *GAL10-CYC1* hybrid yeast promoter identifies the *GAL4* regulator as an upstream site. Proc. Natl. Acad. Sci. USA **79**:7410–7414.
13. **Hashimoto, H., Y. Kikuchi, Y. Nogi, and T. Fukasawa.** 1983. Regulation of expression of the galactose gene cluster in *Saccharomyces cerevisiae*: isolation and characterization of the regulatory gene *GAL4*. Mol. Gen. Genet. **191**:31–38.
14. **Johnston, M., and R. W. Davis.** 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:1440–1448.
15. **Johnston, S., and J. Hopper.** 1982. Isolation of yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA **79**:6971–6975.
16. **Johnston, S. A., M. J. Zavortink, C. Debouck, and J. E. Hopper.** 1986. Functional domains of the yeast regulatory protein *GAL4*. Proc. Natl. Acad. Sci. USA **83**:6553–6557.
17. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding protein. Proc. Natl. Acad. Sci. USA **83**:5889–5893.
18. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231**:699–704.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Laughon, A., R. Driscoll, N. Wills, and R. F. Gesteland.** 1984. Identification of two proteins encoded by the *Saccharomyces cerevisiae GAL4* gene. Mol. Cell. Biol. **4**:268–275.
21. **Laughon, A., and R. Gesteland.** 1982. Isolation and preliminary characterization of the *GAL4* gene, a positive regulator of transcription in yeast. Proc. Natl. Acad. Sci. USA **79**:6827–6831.
22. **Laughon, A., and R. Gesteland.** 1984. Primary structure of the *Saccharomyces cerevisiae GAL4* gene. Mol. Cell. Biol. **4**:260–267.
23. **Levine, H. L., R. S. Brody, and F. H. Westheimer.** 1980. Inhibition of orotidine-5′-phosphate decarboxylase by 1-(5′-phospho-β-D-ribofuranosyl) barbituric acid, 6-azauridine 5′-phosphate, and uridine-5′-phosphate. Biochemistry **19**:4993–4999.
24. **Lohr, D., and J. E. Hopper.** 1985. The relationship of regulatory proteins and DNase I hypersensitive sites in the yeast *GAL1-GAL10* genes. Nucleic Acids Res. **13**:8409–8423.
25. **Lorch, Y., and R. D. Kornberg.** 1985. A region flanking the *GAL7* gene and a binding site for *GAL4* protein as upstream activating sequences in yeast. J. Mol. Biol. **186**:821–824.
26. **Ma, J., and M. Ptashne.** 1987. Deletion analysis of *GAL4* defines two transcriptional activating segment. Cell **48**:847–853.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probe from plasmids containing a bacteriophage SP6 promoter. Nucleic Acid Res. **12**:7035–7056.
29. **Nogi, Y., H. Shimada, Y. Matsuzaki, H. Hashimoto, and T. Fukasawa.** 1984. Regulation of expression of the galactose gene cluster in *Saccharomyces cerevisiae*. II. The isolation and characterization of the regulatory gene *GAL80* in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **195**:29–34.
30. **Oshima, Y.** 1982. Regulatory circuits for gene expression: the metabolism of galactose and phosphate, p. 159–180. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), Molecular biology of the yeast *Saccharomyces cerevisiae*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Perlman, D., and J. E. Hopper.** 1979. Constitutive synthesis of the *GAL4* protein, a galactose pathway regulator in *Saccharomyces cerevisiae*. Cell **16**:89–95.
32. **Reves, P., and R. B. Sandquist.** 1978. Purification of orotate phosphoribosyl transferase and orotidylate decarboxylase by affinity chromatography on sepharose dye derivatives. Anal. Biochem. **88**:522–531.
33. **Rose, M., P. Grisafi, and D. Botstein.** 1984. Structure and function of the yeast *URA3* gene: expression in *Escherichia coli*. Gene **29**:113–124.
34. **Torchia, T. E., R. W. Hamilton, C. L. Cano, and J. E. Hopper.** 1984. Disruption of regulatory gene *GAL80* in *Saccharomyces cerevisiae*: effects on carbon-controlled regulation of the galactose/melibiose pathway genes. Mol. Cell. Biol. **4**:1521–1527.
35. **Umezu, K., A. Tadahiro, A. Yoshimoto, and K. Tomita.** 1971. Purification and properties of orotidine-5′-phosphate pyrophosphorylase and orotidine-5′-phosphate decarboxylase from Baker's yeast. J. Biochem. **70**:249–262.
36. **West, R. W., Jr., R. R. Yocum, and M. Ptashne.** 1984. *Saccharomyces cerevisiae GAL1-GAL10* divergent promoter region: location and function of the upstream activating sequences UAS_G. Mol. Cell. Biol. **4**:2467–2478.
37. **Yocum, R., S. Hanley, R. West, Jr., and M. Ptashne.** 1984. Use of lacZ fusions to delimit regulatory elements of inducible divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:1985–1998.