The Gal4 Activation Domain Binds Sug2 Protein, a Proteasome Component, in Vivo and in Vitro*

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An in vivo protein interaction assay was used to search a yeast cDNA library for proteins that bind to the acidic activation domain (AD) of the yeast Gal4 protein. Sug2 protein, a component of the 19 S regulatory particle of the 26 S proteasome, was one of seven proteins identified in this screen. In vitro binding assays confirm a direct interaction between these proteins. Sug2 and Sug1, another 19 S component, were originally discovered as a mutation able to suppress the phenotype of a Gal4 truncation mutant (Gal4Δp) lacking much of its AD. Sug1p has previously been shown to bind the Gal4 AD in vitro. Taken together, these genetic and biochemical data suggest a biologically significant interaction between the Gal4 protein and the 19 S regulatory particle of the proteasome. Indeed, it is demonstrated here that the Gal4 AD interacts specifically with immunopurified 19 S complex. The proteasome regulatory particle has been shown recently to play a direct role in RNA polymerase II transcription and the activator-19 S interaction could be important in recruiting this large complex to transcriptionally active GAL genes.

The Gal4 protein (Gal4p) of the yeast Saccharomyces cerevisiae stimulates the expression of several genes involved in galactose metabolism in response to an increase in the concentration of the sugar (1). Gal4p is a prototypical “acidic” activator that has been shown to function in a large variety of cell types, indicating that its mechanism of action closely mimics that of activators found in almost all eukaryotic cells (2, 3). Gal4p has a sequence-specific DNA-binding domain at its N terminus (4) and an acidic activation domain (AD) at its C terminus (5, 6). The AD is thought to bind one or more proteins in the RNA polymerase II holoenzyme, chromatin remodeling machines, and perhaps other complexes involved in transcription. Considerable effort has been directed toward attempting to elucidate the direct target(s) of the Gal4 AD, mostly using various in vitro binding assays (7–9). Whereas these studies have provided several candidate proteins, the physiological relevance of these Gal4 AD-transcription factor complexes remains uncertain. There is only one case of which we are aware in which a protein has been implicated as a Gal4 AD-binding partner both biochemically and genetically. This is the Sug1 protein, a constituent of the 19 S regulatory particle (10–13) of the 26 S proteasome (14). Swaffield et al. (15) isolated an allele of Sug1 (sug1-1) that suppressed the “no growth on galactose” phenotype of gal4Δp, a truncation that removes most, but not all, of the C-terminal Gal4 AD and results in a protein with ~3% activity of the wild-type activator. Subsequent biochemical analysis demonstrated that a GST fusion protein containing the intact Gal4 AD binds Sug1p in vitro (7). Another suppressor of gal4Δp, called sug2-1, was isolated along with sug1-1. The wild-type Sug2 gene was cloned subsequently (11) and was also found to be a component of the 19 S complex.

Whereas these data are consistent with a biologically relevant interaction between the Gal4 AD and the proteasome, a concern is that the genetic data could reflect an indirect effect. For example, it is possible that suppression of gal4Δp could be due to decreased proteasome activity in the sug1-1 and sug2-1 strains, possibly leading to very high levels of Gal4p protein. Since many weak activators function more efficiently when expressed at high levels, this model could explain suppression without invoking a Gal4 AD-19 S interaction, and gross overexpression of Gal4p does compensate for the growth on galactose defect (16). Therefore, one must view the idea that Sug1p and Sug2p are true coactivators with caution.

A useful and unbiased approach to understanding the protein-binding chemistry of the Gal4 AD would be to use a two-hybrid assay or some similar approach to search for proteins that bind the Gal4 AD directly in vivo. Of course, one cannot use a potent activation domain as bait in the standard two-hybrid assay, which relies on reconstitution of the activity of a transcriptional activator (17). Recently, Karin and co-workers (18) have reported an alternative in vivo protein interaction assay in which the interaction of fusion proteins containing the factors of interest occurs on the cytoplasmic side of the plasma membrane and results in activation of Ras. Therefore, one can employ transcriptional activators as bait in this system. We report here that the Sug2 gene product was one of seven putative Gal4 AD-binding proteins isolated from this unbiased in vivo screen. Gal4 AD-Sug2p binding was confirmed in vitro. Furthermore, it is demonstrated that the Gal4 AD can bind the native 19 S regulatory particle containing Sug1p and Sug2p in vitro. These data, when considered along with the previous studies mentioned above and other recent results from our laboratories (21) (see “Discussion”), suggest an important physical and functional interaction between the Gal4 protein and the 19 S regulatory particle.
MATERIALS AND METHODS

CytoTrap Assays—The yeast strain used is cdc25H (MATa, ura3, lys2, leu2, trp1, his200, ade1, cdc25–2, GAL+). Media—YPD was made by mixing 1% yeast extract, 2% Bacto-peptone, and 2% dextrose. For plates, 2% Bacto-agar was added. Synthetic defined glucose media with a complete supplement mixture (CSM) except uracil and leucine (SD/glucose (−UL)) were made as follows. 1.7 g of yeast nitrogen base, 5 g of ammonium sulfate, 20 g of galactose, 0.67 g of CSM-LEU-URA, 17 g of agar were mixed in 1 liter total volume and autoclaved. Synthetic defined galactose media (1 liter) with complete supplement mixture except uracil and leucine (SD/galactose (−UL)) contained 1.7 g of yeast nitrogen base, 5 g of ammonium sulfate, 20 g of galactose, 0.67 g of CSM-LEU-URA, 17 g of agar.

Plasmids—To generate pSOS-Gal4 AD, Gal4 AD (residues 841–874) was PCR-amplified from pHKT-34 with primers containing engineered BamHI sites and was subcloned into the BamHI site of pSOS. This plasmid was called pSOS-Gal4.

To generate pMyr-Gal80, Gal80 (amino acids 1–435) was PCR-amplified from pTL37N with primers containing engineered EcoRI sites and was subcloned into the EcoRI site of pMyr. This plasmid is called pMyr-Gal80.

To generate pSOS-cl-wt, pSOS-cl-E233K, pSOS-cl-A152T, pMyr-cl-wt, pMyr-cl-E233K, and pMyr-cl-A152T, the papain fragment C of cI (amino acid residues 123–236) was PCR-amplified from Escherichia coli lysogen N99 clt (number 127) using primers containing engineered BamHI and SalI sites for cloning into pSOS and EcoRI and XhoI sites for cloning into pMyr. The PCR product of cl was cloned into pCR-Scriber mutagenesis vector (Stratagene), mutagenesis was used to introduce E233K and A152T, and the resulting DNA was used to transform pMyr-cl-wt, pSOS-cl-E233K, and pSOS-cl-A152T and subcloned into the EcoRI and XhoI sites of pSOS to make pSOS-cl-wt, pSOS-cl-E233K, and pSOS-cl-A152T and subcloned into the EcoRI and XhoI sites of pMyr to make pMyr-cl-wt, pMyr-cl-E233K, and pMyr-cl-A152T.

Yeast Transformation and Detection of Protein-Protein Interaction—Yeast cdc25H cells were made competent as described by the manufacturer of the Cytotrap kit (Stratagene). 5×10^6 of each plasmid was added to 100 μl of yeast competent cells in separate microcentrifuge tubes. The contents of each microcentrifuge tube were mixed thoroughly but gently. The contents were then centrifuged at 13,000 g for 3 min. The supernatant was then incubated with immobilized anti-FLAG antibody again for 90 min. The beads were then pelleted and washed 4 times in 1.5 ml of bead buffer. After the final wash, the supernatant was carefully removed and 20 μl of SDS loading buffer was added to the beads. The beads were then boiled for 5 min and loaded onto a 10% SDS-PAGE gel. The gels were transferred to nitrocellulose for 45 min at 20 V using a Genie electroblotter (Idea Scientific). The membranes were then used for measurement of labeled protein by phosphorimaging (Molecular Dynamics). Inputs represent 10% of total input.

Purification of the 19 S Regulatory Particle—The purification of 19 S was carried out according to the method of Verma et al. (19) with minor modifications. The protocol involves initial purification of the intact 26 S proteasome carrying a FLAG tag on the C terminus of the Pre1 proteasome (a 19 S subunit) by immunosorbent chromatography and FLAG peptide elution. Specifically, eight liters of a culture derived from the yeast strain RJD1144 (MATa his3200 leu2–3, 112 lys2–801 trp3–63 FFE1FLAG::Yiplac211(URA3); kindly provided by Prof. R. Deshaies and R. Verma, Caltech) was grown in synthetic uracil dropout medium to an A_600 of 2–3. The cells were pelleted, washed with distilled water, and frozen in liquid nitrogen. The frozen pellet was ground in mortar filled with liquid nitrogen until a very fine powder was obtained. Three volumes of buffer A (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl_2 plus an ATP-regenerating system (20) were added. The solution was centrifuged in an SS34 rotor at 13,000 × g for 20 min. After centrifugation, the cleared lysate was transferred to another tube and incubated with immobilized anti-FLAG monoclonal antibody (Sigma) for 90 min. The beads were then pelleted and washed in buffer A + 2 mM ATP + 0.1% Triton X four times and with buffer A + 2 mM ATP twice. Beads were then incubated with 150 μg/ml FLAG peptide for at least 4 h at 4 °C. The supernatant was collected. Typically, this protocol yields approximately 1 mg of highly purified 26 S proteasome.

To obtain the 19 S regulatory complex, this 26 S preparation was then loaded in buffer A + 0.1 M NaCl, an ATP regeneration mixture and FLAG peptide overnight to favor dissociation of the 19 S regulatory particle and the 20 S core complex, as well as to remove free FLAG peptide. This was then followed by dialysis in buffer A for another hour. The preparation was then incubated with immobilized anti-FLAG antibody again for 90 min to bind the free FLAG-tagged 20 S core proteasome. The supernatant was collected, concentrated in a Centricon filter device, and found by gel electrophoresis and Western blotting (21) to be largely free of fuge tube, 2 μg of pSOS plasmid was added to 2 μg of pMyr-cDNA plasmid library and 5.4 μl of β-mercaptoethanol to 500 μl of yeast competent cells. The transformations were incubated at room temperature for 30 min with occasional mixing, heat-shocked at 42 °C for 10 min, and then plated on ice for 5 min. For each transformation, the mixture was then plated in 150-mm LB/galactose plates. The plates were kept at 37 °C for 6 days. Colonies may appear much later (10 days). Colonies arising from the pSOS and pMyr-cDNA transformation provide an estimate of the number of false-positive clones from the cDNA library and the temperature-sensitive revertants.

The colonies that grew at 37 °C were patched onto two SD/glucose (−UL) and one SD/galactose (−UL) agar plates. One SD/glucose (−UL) and one SD/galactose (−UL) agar plate were transferred to 37 °C. The other SD/glucose (−UL) agar plate was kept at room temperature for further patching. Colonies that grew at 37 °C on SD/galactose (−UL) agar plates, but not on SD/glucose (−UL) agar plates, were repatched. Only those colonies that grew at 37 °C on SD/galactose (−UL) agar plates, but not on SD/glucose (−UL) agar plates, both times were considered putative-temperature-sensitive revertants. Isolation of pMyr-cDNA plasmids from yeast was done as suggested by the manufacturer of the Cytotrap kit (Stratagene, Inc.).
20 S complex. Approximately 300 µg of the 19 S complex was obtained from the 8-liter culture.

Association of the Gal4 AD with the Yeast 19 S Complex—7.5 µg of GST-Gal4 AD was incubated with 12 µg of the 19 S preparation in 100 µl of MTB buffer for 1 h. Glutathione-agarose beads were then added, and 30 min later the bead-bound material was isolated by a brief centrifugation. The beads were washed three times with MTB buffer, and the bound proteins were analyzed by SDS-PAGE/Western blotting. The same protocol was employed in a control reaction using green fluorescent protein (GFP) fused to GST rather than the Gal4 AD.

Immunoprecipitation of the Mammalian 19 S Complex—Mammalian 19 S was immunoprecipitated from an in vitro translated (radiolabeled) ySup2-containing lysate according to the method described by Hendil and co-workers (22). 10 µl of rabbit reticulocyte lysate containing in vitro translated yeast Sug2p lysate was pre-cleared by the addition of 2 µl of 10% protein A + Staphylococcus aureus cells (Roche Molecular Biochemicals) and 400 µl of IP buffer (25 mM Tris, pH 7.6, 2 mM ATP, 1 mM dithiothreitol, 2 mM MgCl2, 5 mM KCl, 17% glycerol, 40 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase). The suspension was incubated at 4 °C for 1 h. The pre-cleared lysate was centrifuged briefly, and the supernatant was used for the subsequent immunoprecipitation experiments. Equal 130-µl aliquots of the cleared lysate were kept as an input fraction or incubated with 270 µl of IP buffer and 20 µl of protein A + S. aureus cells that had been loaded with either mouse monoclonal anti p45/110 (mSug1p, courtesy of Dr. K. B. Hendil, University of Copenhagen) or anti-trinitrophenol (Phar-Mingen). Following a 1-h incubation at 4 °C, the suspensions were centrifuged at 8000 × g for 2 min. The supernatant was retained, and then total protein was precipitated with trichloroacetic acid. The pellet was washed 3 times in 1 ml of IP buffer. Each sample was then resuspended in SDS-containing loading buffer supplemented with 8 M urea, boiled for 2 min, and electrophoresed through a 10% SDS-PAGE. Gels were then transferred to nitrocellulose membranes (Osmontics) using a Genie blotter (Idea Scientific). The presence of radiolabeled yeast Sug2p was assessed by exposing the nitrocellulose membrane to a PhosphorImager screen and reading the screen on the Storm system (Molecular Dynamics). Following visualization of radioactive proteins, Gels were then transferred to nitrocellulose membranes (Osmonics) using a Genie blotter (Idea Scientific). The presence of radiolabeled yeast Sug2p was assessed by exposing the nitrocellulose membrane to a PhosphorImager screen and reading the screen on the Storm system (Molecular Dynamics). Following visualization of radioactive proteins, the membranes were washed throughly in TBST to remove residual radioactivity. Western blot analysis, nitrocellulose membranes were then subjected to Western blot analysis to assess the quality of the immunoprecipitation experiments. Membranes were probed first with rabbit anti-Mss1p antisera (Affinity Research), washed extensively, and then probed with horseradish peroxidase-conjugated donkey anti-rabbit antisera (Pierce). The blots were developed using Supersignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

An in Vivo Protein Interaction Assay for Finding Gal4 AD-binding Partners—The “Cytotrap”®, or SOS, protein-interaction assay developed by Aronheim et al. (18) was used to screen a yeast cDNA library for polypeptides that interact with the core Gal4 AD (residues 841–875). As shown schematically in Fig. 1A, the “bait” protein (here the Gal4 AD) was fused to the human son of sevenless protein (SOS), the analogue of yeast Cdc25p, an activator of Ras, and the other was tagged with a myristoylation sequence. Unlike yeast Cdc25p, hSOSp lacks specific interactions with native yeast proteins that can recruit it to the plasma membrane. Therefore, in cells carrying a temperature-sensitive cdc25 allele, Ras can be activated at the restrictive temperature only if hSOSp is recruited to the membrane via an interaction between the protein fused to it and the membrane-localized myristoylated protein.

To ensure that this assay would be useful for the identification of AD-binding proteins, the well characterized interaction between the Gal4 AD and Gal80 repressor protein (5, 23–25) was employed. The Gal4 AD was fused to hSOSp, and Gal80p was tagged with a myristoylation signal. Cells expressing either pSOS-Gal4 AD or pMyr-Gal80p did not grow at 37 °C, but cells expressing both proteins did (Fig. 1B).

Identification of Gal4 AD-Binding Proteins in Vivo—With these preliminary results in hand, SOS-Gal4 AD was used as bait in an experiment using a yeast cDNA library fused to a myristoylation tag under the control of the GAL1 promoter. Approximately 5 × 10^5 transformants containing library plasmids and the pSOS-Gal4 AD plasmid were grown on SD/galactose (∼UL) agar plates at 25 °C, replica-plated onto SD/galactose (∼UL) agar plates, and transferred to 37 °C. 344 colonies that grew at 37 °C were isolated and tested for growth on SD/glucose (∼UL) and SD/galactose (∼UL) agar plates at

![Fig. 1. Verification that the Gal4 AD is a viable "bait" protein in the Cytotrap system.](http://www.jbc.org/)

- **A.** Schematic diagram of the Cytotrap assay (18). B, known protein-protein interactions support activation of Ras at the restrictive temperature. cdc25H cells were transformed with the indicated plasmids and patched onto galactose minimal plates as described under "Material and Methods." Only cells expressing both Sos-Gal4 AD and Myr-Gal80 fusion proteins grew robustly at 37 °C. Left panel, cells were grown at 25 °C; right panel, cells were grown at 37 °C.

- **B.** Expression of the Sos-Gal4 AD fusion proteins were confirmed by Western blot analysis using an anti-SOS monoclonal antibody (data not shown). JZ, c-Jun leucine zipper domain. The different columns represent replicate stamps.
cells were grown at 25 °C or 37 °C. Library plasmids were isolated from 53 clones that exhibited galactose-dependent growth at 37 °C. These were retransformed into cdc25H cells with either the original bait (pSOS-Gal4 AD) or pSOS. Twenty five isolates did not exhibit Gal4 AD-dependent growth and thus represent the background of the cDNA library. These plasmids include members of the Ras family and components of the Ras signaling pathway such as protein kinase C, etc. Fourteen plasmids suppressed the cdc25 phenotype only in the presence of pSOS-Gal4. These were sequenced.

Seven different putative Gal4 AD-binding polypeptides were identified. Four were known proteins as follows: Hap5p, a fragment of TATA-binding protein-associated factor 60 (TAFII60), Apl1p, and Sug2p. Three corresponded to putative protein complex (36). Of these proteins, Hap5p and TAFII60 are members of the heterotrimeric CCAAT-binding complex and is involved in the activation of a number of genes in response to a non-fermentable carbon source (30). TAFII60 is a member of the transcription factor IID complex that binds to core promoters in mRNA-encoding genes (31) and is also one of many proteins in the SAGA complex (32). It is interesting to note that while many TAFs are not required for the expression (33, 34) of most genes, recent evidence indicates that TAFII60 is one of a few “histone-like” TAFs that is essential for the expression of most genes (35). Sug2p, as mentioned in the introduction, is a component of the 19 S proteasome regulatory particle and is also an essential gene in yeast (11, 13). Apl1p is thought to be a member of a clathrin-associated protein complex (36). Of these proteins, Hap5p and TAFII60 are transcription factors, and Sug2p has been implicated genetically as being involved in Gal4p-mediated transcription (15). There is no obvious connection between Apl1p and transcription.

These interactions appear to be specific. As mentioned previously, growth was not supported by SOS-Gal4 AD and the Myr-repressor dimerization domain (Fig. 1). Furthermore, Hap5p did not bind to SOS itself or a fusion of SOS to the leucine zipper from c-Jun (Fig. 3). Sug2p also did not bind the SOS parent protein (Fig. 3).

A Dimeric Gal4 AD Fusion Protein Also Binds Sug2p in Vivo—The Gal4 AD is displayed as a dimer in the context of native Gal4p (4), and hSOSp is not known to dimerize. Therefore, it was of interest to ask if these interactions are retained when the AD is displayed in a more biologically relevant manner. Myr-Sug2p was tested against a fusion of SOS to GST-Gal4AD. GST is a native dimer (37,39) and so should provide a Gal4 AD dimer. Furthermore, this construct has been used extensively for in vitro binding experiments (7). As is shown below, binding of Sug2p to the Gal4 AD was observed in vivo in this context as well. Fig. 4 demonstrates that Sug2p binds GST-Gal4 AD in yeast. In this figure, the cells were serially diluted across each row. The GST-Gal4 AD/Sug2p interaction supported growth about as well as the well characterized GST-Gal4 AD/Gal80p interaction at 37 °C when the cells were plated on dextrose. GST-AD/Sug2p also supported robust growth on galactose at this temperature, indeed better than did the GST-Gal4 AD/Gal80p combination. We conclude that Sug2p can bind a dimeric Gal4 AD-containing protein.

Some of the details of the results shown in Fig. 4 deserve

The yeast strain cdc25H was cotransformed with the indicated pairs of pMyr and pSOS constructs. Single transformants were picked, diluted in a 5-fold series, and stamped to selective plates containing either dextrose or galactose as the carbon source. Plates were then grown for 5 days at either 22 or 37 °C. Growth at 37 °C indicates a positive interaction between the membrane-bound pMyr product and the SOS fusion. The Gal4 AD-Sug2p interaction is observed in the context of the dimeric GST fusion protein. Note that the apparent strength of the Gal4 AD-Sug2p interaction, as manifested by cell survival in the serially diluted stamps, is approximately the same when the cells were plated on dextrose or galactose. However, the Gal4 AD-Gal80p interaction appears much more robust on dextrose than galactose. See text for details.

![Fig. 2. Estimation of the affinity between proteins required to generate a positive signal in the Cytotrap assay. The wild-type λ repressor dimerization domain (d) or two different point mutants, which form homodimers with known equilibrium dissociation constants, were tested in the Cytotrap system. The results indicate that a Kd value of somewhere between 10⁻⁴ and 10⁻³ M is necessary for a protein-protein interaction to be detected by this assay. The different columns represent replicate stamps.](http://www.jbc.org/)

| Kd (M) | pSOS | pMyr |
|-------|-------|-------|
| 20 aM | λ Repressor (wt) | λ Repressor (wt) |
| 100 nM | λ Repressor (E235K) | λ Repressor (E235K) |
| 1 μM | λ Repressor (A152T) | λ Repressor (A152T) |

![Fig. 3. The Gal4 AD binds Sug2p and Hap5p in vivo, verification of the results of the screen through pairwise experiments. pMyr plasmids were isolated from cells identified in the screen as harboring Gal4 AD-binding proteins. DNA sequencing was employed to identify the open reading frame fused to the myristoylation tag. Two of the seven proteins identified in this assay were Sug2p and Hap5p. To verify these results, cells were transformed with the indicated plasmids and patched onto galactose minimal plates as described under “Material and Methods.” Only cells expressing both Sos-Gal4 AD and Myr-Sug2p or Sos-Gal4 AD and Myr-Hap5 fusion proteins grew at 37 °C. Left panel, cells were grown at 25 °C; right panel, cells were grown at 37 °C. JZ, c-Jun leucine zipper domain. The different columns represent replicate stamps.](http://www.jbc.org/)

| Fusions | pSOS | pMyr |
|---------|-------|-------|
| None | None |
| None | GST |
| Gal4pH | GST |
| Gal4pH | GST-Gal4AD |
| Sug2p | GST |
| Sug2p | GST-Gal4AD |

![Fig. 4. A dimeric GST-Gal4 AD fusion protein binds Sug2p in yeast. The yeast strain cdc25H was cotransformed with the indicated pairs of pMyr and pSOS constructs. Single transformants were picked, diluted in a 5-fold series, and stamped to selective plates containing either dextrose or galactose as the carbon source. Plates were then grown for 5 days at either 22 or 37 °C. Growth at 37 °C indicates a positive interaction between the membrane-bound pMyr product and the SOS fusion. The Gal4 AD-Sug2p interaction is observed in the context of the dimeric GST fusion protein. Note that the apparent strength of the Gal4 AD-Sug2p interaction, as manifested by cell survival in the serially diluted stamps, is approximately the same when the cells were plated on dextrose or galactose. However, the Gal4 AD-Gal80p interaction appears much more robust on dextrose than galactose. See text for details.](http://www.jbc.org/)
comment, since they raise important detailed points regarding the use of the Cytotrap system for probing Gal4 AD interactions. First, it might seem surprising that any growth would be observed at 37 °C on dextrose-containing media since expression of the myristoylated protein is under the control of the GAL1 promoter, which is normally silent in the absence of galactose. Of course, GAL1 is normally silent because of tight Gal80p-mediated repression (40). In the present study, the hSOS-Gal4 AD is expressed constitutively at high levels, so one would expect that much of the native Gal80p would be titrated from GAL1 promoter-bound Gal4p, allowing significant expression of the GAL1 promoter-regulated Myr fusion gene even in the absence of the inducer (41). The other seemingly unusual result in Fig. 4 is the fact that the apparent strength of the SOS-GST-Gal4 AD/Myr-Gal80p interaction is weaker on galactose than on dextrose. Whereas the current model is that Gal4p and Gal80p do not dissociate upon induction (25, 42, 43), a recent study (44) indicates that this association might be weakened upon induction. In addition, there is evidence that Gal4p-Gal80p binding under induced conditions might also involve another region of Gal4p outside of the core AD (44), which is not present on the fragment employed in the Cytotrap experiment. Thus, our observation of apparently weaker binding in vitro in the presence of galactose may reflect the true biochemical properties of this complex. However, since Myr-Gal80p will act as repressors of its own transcription in this case (the gene is under GAL1 promoter control), it could be that this result is an artifact of the particular ratios of the SOS-GST-Gal4 AD, Myr-Gal80p, and native Gal4p. In any case, this could explain why Gal80p was not isolated in the initial screen for Gal4 AD-binding proteins.

A Sug1p-Gal4 AD Interaction Cannot Be Detected in a Cytotrap Assay—Sug1p was also not identified as a Gal4 AD-binding protein in the initial screen even though there is genetic and biochemical evidence for an interaction (7, 15, 45). Of course, this negative result could be due to many things, including the possibility that SUG1 was not represented in the library. To probe this point more carefully, Sug1p was cloned into the pMyr vector and cotransfected into yeast with the SOS-GST-Gal4 AD-expressing plasmid as bait. No interaction between Sug1p and the Gal4 AD was observed under conditions identical to those in which a clear Gal4 AD-Sug2p interaction was registered (Fig. 5). Western blotting revealed the presence of the myristoylated Sug1p, which was distinguishable electrophoretically from wild-type Sug1p (data not shown), so this negative result was not due to poor expression of the fusion protein. However, very little native Sug1p was detected in this strain. For some reason, the high level expression of the myristoylated derivative resulted in loss of most of the wild-type protein. One possible explanation is that the myristoylated Sug1p competes with wild-type Sug1p for incorporation into the 19 S complex and that free, wild-type Sug1p is then degraded. Since most, or all, of the 19 S complex in yeast is nuclear (12), it could be that proteasome-incorporated Myr-Sug1p is unable to localize to the plasma membrane. In any case, the failure to observe a positive Gal4 AD-Sug1p interaction in this experiment argues that the AD-Sug2p interaction is probably direct and not due to Gal4 AD binding to some other protein in a 19 S complex containing Myr-Sug2p. This conclusion is further buttressed by the lack of an interaction between the Gal4 AD and Rpt1, another ATPase in the 19 S complex (Fig. 5).

The Gal4 AD Binds Sug2p in Vitro—To test whether a Gal4 AD-Sug2p interaction could be detected biochemically, 35S-labeled Sug2p was prepared by in vitro transcription and translation in a rabbit reticulocyte extract. This solution was then probed with a GST-Gal4 AD fusion protein or, as a control, GST lacking the Gal4 fusion. As shown in Fig. 6, labeled Sug2p was retained by the Gal4 AD but not by GST alone. Furthermore, neither GST nor GST-Gal4 AD bound labeled human Rpt1 (Mss1/S7), a component of the human 19 S complex (also known as PA700 (46)). This experiment confirms a Gal4 AD-Sug2p interaction and is also consistent with the idea that it is direct. However, given the very high level of identity between Sug2 proteins in different species, a potential complication in this experiment was the possibility that the yeast Sug2p might load into the rabbit proteasome and that retention of labeled yeast Sug2p by the Gal4 AD could reflect an indirect interaction. To address this possibility, the experiment shown in Fig. 7 was conducted. The extract containing the in vitro translated yeast Sug2p was exposed to an anti-mSug1 antibody capable of immunoprecipitating the rabbit 19 S complex (22). This is demonstrated in the lower panel of Fig. 7, which displays a Western blot using, as a probe, an antibody against mRpt1, another 19 S constituent. Essentially all of the mRpt1 was immunoprecipitated by the immobilized anti-mSug1 antibody, whereas this was not the case when a control antibody (anti-trinitrophenol) was employed in the immunoprecipitation. Analysis of the pelleted and supernatant fractions from the anti-mSug1 immunoprecipitation by autoradiography revealed that all of the yeast
Sug2 protein was present in the supernatant. In other words, no detectable labeled yeast Sug2p was incorporated into the native rabbit 19 S complex. This result, when taken together with the Fig. 6 data, supports the idea that the interaction between the Gal4 AD and in vitro translated Sug2p is direct and rules out the possibility that the Fig. 6 result is due to an indirect AD-19 S interaction bridged by other components of the rabbit 19 S complex.

The Gal4 AD Binds the Yeast 19 S Complex in Vitro—Since Sug1p and Sug2p reside in the 19 S regulatory particle of the proteasome, it was important to ask if the Gal4 AD-Sug1 (7) and Gal4 AD-Sug2p (this study) interactions observed using the free polypeptides could be recapitulated in the context of the 19 S complex. To probe this point, the yeast 19 S complex was immunopurified using a variation of the protocol reported by Verma et al. (19) (see “Materials and Methods”). In this procedure, the intact 26 S proteasome is first immunopurified by virtue of a FLAG tag placed at the C terminus of the Pre1 protein, a core proteasome constituent. After elution of the proteasome from the antibody column with FLAG peptide, the complex was incubated in a buffer that promotes dissociation of the 19 S and 20 S sub-complexes. This solution was then passed over another anti-FLAG antibody column to remove most of the 20 S complex, leaving predominantly the 19 S regulatory particle in the flow through. A well defined and limited set of Coomassie Blue-stained bands in the 26 S and 19 S preparations (Fig. 8) was observed that corresponded to that reported by Verma et al. (19), indicating a high level of purity.

Fig. 9 shows the results of two different in vitro binding assays using the GST-Gal4 AD fusion protein. In Fig. 9A, immobilized GST-Gal4 AD was incubated with a crude yeast whole cell extract, and the beads were pelleted and washed thoroughly. The presence or absence of Sug2p in the AD-associated fraction was assessed by SDS-PAGE and Western blotting. As shown, ~10% of the total Sug2p in the extract was associated with the Gal4 AD under the conditions employed. As mentioned above, it has been shown previously that in a similar assay, Sug1p is retained by the Gal4 AD (7). These data therefore argue that the retention of Sug1p and Sug2p reflect the association of the AD with the intact proteasome regulatory particle. This conclusion is buttressed by the experiment shown in Fig. 9B in which binding of the immunopurified 19 S preparation with the Gal4 AD was assessed by the same method. Western blots show that GST-Gal4 AD bound Sug1p, Sug2p, and Cim5p/Rpt1, again indicating binding of the intact complex to the AD. A control fusion protein, GST-GFP, did not retain detectable quantities of the 19 S. These experiments verify that the Gal4 AD is capable of binding to the 19 S regulatory particle of the proteasome directly. Of course, these data do not prove that the Gal4 AD-19 S association is mediated by direct AD-Sug2p and/or AD-Sug1p interactions, although this is obviously a reasonable hypothesis.

DISCUSSION

In this study, an alternative type of two-hybrid assay that allows the use of potent activators as bait (18) was employed to
screen a yeast cDNA library for Gal4 AD-binding proteins. Seven “hits” were obtained, the Sug2, Hap5, TAF,60, and Apl1 proteins as well as three open reading frames of unknown function. Given these results, the Cytotrap assay can be considered as a promising tool for beginning to characterize the binding partners of activators that cannot be used as bait in standard two-hybrid formats. Its strengths include its unbiased nature and the fact that the binding must occur in vivo in the presence of a large excess of competitor proteins that would presumably block nonspecific interactions. Furthermore, all of the necessary materials are now available in the form of a kit (Stratagene, Inc.), and recent technical developments have simplified the screening protocol (47). Nonetheless, it is important to keep in mind that this assay, as well as other alternative two-hybrid systems capable of using potent activators as bait (48–50), employ artificial fusion proteins and take the AD out of its normal context in the nucleus. Thus, the hits obtained should be considered as interesting leads worthy of further investigation but nothing more. Indeed, we focused on further analysis of Sug2p as a potential Gal4 AD binding partner because of previous genetic evidence for an interaction between these proteins (15). More work will be required to assess the potential biological relevance of the other AD-protein interactions identified in this study.

Another limitation of the Cytotrap-based strategy, common to any genetic assay, is that proteins or protein fragments that are toxic when expressed at high levels are unlikely to be identified. For example, recent experiments have indicated that the Gal11 protein (51–53), a holoenzyme component, is a target of the Gal4 AD as well as other activators (54). Over-expression of the Gal4 AD-binding domain of Gal11p is highly toxic to yeast, possibly because it interferes with many activator-holoenzyme interactions. Thus, it is not surprising that Gal11p failed to turn up in the screen reported here. There will undoubtedly prove to be other quirks in this system, for example the inability of some Myt tag-containing fusion proteins to localize to the membrane as might have been the case for Sug1p. The major point is that there are several mechanisms that might result in activator-binding partners being missed in this type of assay. Nonetheless, although the Cytotrap-based approach has clear limitations, it should find use as a relatively convenient and unbiased preliminary tool in the search for the binding partners of potent acidic activators.

In this study, we focused subsequent biochemical efforts on better characterizing the Gal4 AD-Sug2p interaction since there was previous genetic evidence for a Gal4p-Sug2p interaction (15). Experiments using in vitro translated Sug2p demonstrated that association with the AD is apparently direct (Figs. 6 and 7). This result, in combination with a previous report of Gal4 AD-Sug1p binding (7), led us to ask if the Gal4 AD could bind the intact 19 S proteasome regulatory particle, since most or all cellular Sug1p and Sug2p is thought to be incorporated into this complex. As is shown in Fig. 9, this is indeed the case. Sug2p was retained by the Gal4 AD in the context of a crude yeast extract as well as in an experiment using a highly purified 19 S preparation. Based on the data presented here and elsewhere (7) for interaction between the Gal4 AD and the isolated Sug1 and Sug2 proteins, we propose a model that the 19 S-Gal4 AD association is mediated by direct contacts with Sug1p and Sug2p. It is worthwhile to note that native Gal4p functions as an obligate homodimer (4); thus it is possible that Sug1p and Sug2p could be bound simultaneously in the context of the 19 S complex, assuming the complex contains a single copy of each protein.

An obvious concern in evaluating protein interaction data of any kind is whether the binding events are biologically relevant. As mentioned previously, the relevance of the putative Gal4 AD-Sug2p interaction was supported by the observation that an allele of SUG2, sug2-1, could suppress the growth on galactose defect of the gal4D mutation. Recent results of Russell and Johnston (16) have further strengthened this conclusion. In a study that employed two sug2 alleles, it was demonstrated that the rescue of gal4D by sug2-1 is allele-specific (16). Indeed, the same study also demonstrated that rescue of gal4D by sug1-1 is allele-specific as well. Thus, the genetic evidence for a functionally relevant interaction between Gal4p, Sug1p, and Sug2p is quite strong and, when combined with the data reported in this paper, presents a compelling case for biological relevance.

What might be the role of Gal4 AD-Sug2 and Gal4 AD-Sug1p interactions? Sug1p and Sug2p are two of the six AAA proteins (55) that are thought to mediate the unfolding of proteins, a necessary step in the introduction of the protein substrate into the internal cavity of the core 20 S proteolytic complex (13, 56–58). Therefore, an obvious model is that Gal4 AD-Sug2p/Sug1p binding somehow is critical in regulating the proteolytic turnover of Gal4p. It is interesting with regard to this type of model that several reports have appeared recently that correlate the potency of an activation domain with the efficiency of its proteasome-mediated proteolysis (59–61).

However, other models can be considered as well. First, Russell and Johnston (16) demonstrated clearly that the suppression of gal4D by sug2-1 or sug1-1 cannot be explained simply by reduced proteolysis, indicating that the 19 S complex can play some other role in some aspect of Gal4p-mediated transcription. Most importantly, we have shown recently that the 19 S complex, and Sug1p and Sug2p in particular, are required for efficient RNA polymerase II elongation in vitro and in vivo and that this effect is not the result of proteasome-mediated proteolysis (21). For example, mutations in SUG1 and SUG2 confer extreme sensitivity to 6-azauracil, a phenotype correlated closely with elongation defects (62), whereas mutations in proteolytic subunits of the proteasome do not. In vitro, inactivation of Sug1p by antibodies or through the use of temperature-sensitive mutants greatly reduces the efficiency of polymerase II elongation. This effect can be reversed by the addition of immunopurified 19 S to the extract. Mutations in 20 S core subunits that strongly reduce peptidase activity or the addition of highly specific protease inhibitors have no effect on transcription. These data have allowed us to conclude that the 19 S plays a previously unanticipated, direct functional role in RNA polymerase II elongation (21) (and perhaps other phases of the transcription cycle as well). Since Gal4p has been implicated in activating the elongation phase of transcription (63, 64) as well as the formation of preinitiation complexes (65, 66), it is possible that a direct Gal4 AD-19 S interaction, perhaps through direct contacts with Sug2p and Sug1p, is important in mediating these processes.

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The Gal4 Activation Domain Binds Sug2 Protein, a Proteasome Component, in Vivo and in Vitro

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