# Evidence That Gal11 Protein Is a Target of the Gal4 Activation Domain in the Mediator†

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ABSTRACT: The mediator is an approximately 20 protein complex that is essential for the transcription of most genes in yeast. It is contacted by a number of gene-specific activators, but the details of these interactions are not well understood in most cases. Here, evidence is presented that the mediator component Gal11 represents at least one target of the Gal4 activation domain (AD). Deletion of Gal11 is shown to decrease the affinity of the Gal4 AD for the mediator, and direct binding of an N-terminal domain of Gal11 with the Gal4 AD is demonstrated. Quantitative studies, however, indicate that the $K_D$ of the 1:1 Gal4 AD−Gal11 complex is modest. Combined with in vivo data showing that Δ*gal11* cells exhibit reduced, but still significant, Gal4-mediated gene expression, these results suggest that the dimeric activator might also contact another protein in the mediator in addition to Gal11.

Gene-specific transcriptional activators function by recognizing specific sites in the promoter of their target gene and by interacting directly with one or more multiprotein transcription complexes. Over the past several years, it has become clear that the mediator (*1*), an approximately 20 protein complex (*2*, *3*), is a critical target of most (*4*−*10*), but not all (*11*), activators in yeast and probably in all eukaryotic cells (*12*, *13*). It is therefore of considerable interest to probe the chemistry of activator−mediator interactions further.

Biochemical studies using mediator complexes purified from various mutant yeast strains have shown that it consists of stable Srb4 and Rgr1 subcomplexes (*6*). Some mediators also contain a "Gal11 module" which consists of the Gal11, Sin4, Med2, and Med3 (Hrs1) proteins and which appears to be substoichiometric in yeast with respect to the Srb proteins. The Gal11 module is connected to the core mediator via contacts with the Rgr1 subcomplex and is required for activated, but not basal, transcription in vitro (*6*). Gal11 itself has emerged as a candidate for the direct target of several activators. For example, the VP16 and GCN4 activation domains (ADs)[1] bind Gal11 in a Far Western blot experiment (*6*). However, the generality of Gal11 as an activator target is unclear. *GAL11* is not essential, though deletion of the gene in yeast results in altered expression of a large number of genes (*14*−*16*). Furthermore, there are hints that different activators exhibit different mediator binding chemistries. For example, mutations have been isolated in mediator components which differentially affect transcription mediated by different activators (*10*).

In this report, a study of the interaction of the potent yeast activator Gal4 protein and the mediator is presented. When Gal11 is absent, Gal4 AD−mediator binding is compromised. Conversely, when Gal11 is overexpressed, Gal4 AD−mediator binding is accentuated, consistent with this coactivator being a direct target of Gal4. Biochemical studies are presented that confirm a direct interaction between the Gal4 AD and a well-defined domain in Gal11 comprised of approximately residues 1−351. Quantitative measurements demonstrate that the Gal4 AD−Gal11(1−351) complex has a stoichiometry of 1:1 and a modest $K_D$ of $>10^{-7}$ M. Models for Gal4−mediator interactions that accommodate these new data are discussed. A point of particular interest is that Gal4 functions as a dimer (*17*). Thus, the observed 1:1 stoichiometry of the Gal4 AD−Gal11(1−351) complex suggests that the second Gal4 AD in the dimer could contact a different surface of the mediator and that cooperativity between two modest affinity contacts could result in high-affinity activator−mediator binding.

## MATERIALS AND METHODS

*Gal4 Activation Domain Binding to Mediator in Extracts.* "Pull-down" experiments were conducted as described previously (*24*), except that the GST−Gal4 AD concentration was $10^{-8}$ M. Srb4 was detected using an anti-Srb4 antibody kindly provided by Prof. R. Young (MIT). HA−Gal11 was detected using a monoclonal antibody raised against the HA epitope. The strains deleted for *GAL11* and which express either native or elevated levels of HA−Gal11 were produced by standard insertion/substitution methods (*46*) and were congenic.

*Gal4 AD Binding to Gal11 and Gal11 Fragments in Vitro.* A $His_6$-tagged derivative of full-length Gal11 was purified from JM109 cells harboring the expression plasmid pQE32-

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[1] Abbreviations: AD, activation domain; DBD, DNA-binding domain; $K_D$, equilibrium dissociation constant; GST, glutathione *S*-transferase.

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GAL11 (kindly provided by Prof. T. Fukasawa) according to the protocol of Sakurai et al. (*47*), which involved chromatography over Ni-saturated NTA−agarose and DE-52 columns. To monitor binding of the protein to the Gal4 core AD, GST−Gal4 AD (residues 847−881) (1 $\mu$M) (*24*) was mixed with 250 $\mu$L of the DE-52 fraction in PBS buffer. After a 30 min incubation, glutathione−agarose beads were added, and the bead-bound material was isolated by brief centrifugation followed by thorough washing of the beads and analysis by SDS−PAGE. His6-Gal11 was detected by Western blotting using an antibody that recognizes the six histidine tag and three flanking residues.

To identify the Gal4 AD−binding domain of Gal11, various fragments of the *GAL11* gene (see Figure 4) were amplified using the PCR and cloned in-frame into the pFLAG vector (Sigma). The resulting constructs were then transformed into BL21(DE3), and the transformants were grown in LB media with ampicillin at the final concentration of 50 $\mu$g/mL. Protein expression was induced with IPTG (1 mM) when the culture reached an $OD_{600}$ of 0.6. The cells were then incubated for a further 2 h at 37 °C. A cleared extract (20 mM sodium phosphate, pH 7.5, 50 mM NaCl, and 15% glycerol) was made from these cells using standard procedures. The lysate (400 $\mu$L) was mixed with glutathione bead-bound GST or GST−Gal4 AD (1 $\mu$M) and incubated at 4 °C for 1 h with rotation. The beads were washed three times with PBS (300 mM NaCl) plus 0.1% Triton X-100 and once without the detergent. SDS-containing gel loading dye was added to the beads, which were then boiled for 3 min. After a brief centrifugation, the supernatant was loaded onto a 10% SDS−polyacrylamide gel. Western blotting using a monoclonal antibody raised against the FLAG tag was employed to visualize the Gal11 fragments.

*Purification of GST−Gal11(1−351) and Gal11(1−351).* The DNA encoding Gal11 residues 1−351 was amplified by the PCR and cloned in-frame into pGEX3X to provide pGEX3X−Gal11(1−351). GST−Gal11(1−351) fusion protein was purified by glutathione affinity chromatography from an extract prepared from *Escherichia coli* harboring pGEX3X−Gal11(1−351). This fusion protein was cleaved using the factor Xa protease to provide Gal11(1−351). The GST fragment was removed by passing the products over glutathione−Sepharose. This polypeptide was >90% pure as evidenced by SDS−PAGE and Coomassie Blue staining.

*Fluorescence Polarization Assay.* Purified Gal4(1−93 + 768−881) (40 nM) was mixed with 10 nM 5′-fluoresceinated, double-stranded oligonucleotide (5′-GAC GGA GGA CTG TCC TCC GAG-3′) in the following buffer: 20 mM HEPES, pH 7.5, 75 mM potassium acetate, 0.02 mM zinc sulfate, 4 mM magnesium acetate, 1 mM $\beta$-mercaptoethanol, 0.05 mM EDTA, 10% glycerol, and 0.1 mg/mL BSA. The solution was incubated at 4 °C for 15 min and then split into several aliquots. Then, purified GST−Gal11(1−351) or Gal11(1−351) was added to each reaction to give the final concentrations indicated in Figure 6. After a 30 min incubation at room temperature, the sample was placed into the cavity of a Beacon 2000 instrument (PanVera Corp.), and the degree of fluorescence polarization was measured. The data are presented as $\Delta mA$ ($\Delta mA = mA_{Gal11} - mA_0$), i.e., normalized to a value in which the polarization of the Gal4−DNA complex is set to zero. Controls were performed in which either the Gal4 derivative was omitted or GST was added to the Gal4 derivative−DNA complex. In neither case was a significant change in fluorescence polarization observed.

*Determination of the GST−Gal4 AD−Gal11 Complex Affinity Using a Pull-Down Assay.* A DNA fragment encoding the full-length yeast *GAL11* gene was cloned into *Bam*HI/*Sal*I-cut pTL37N. The resulting plasmid was linearized by *Pst*I digestion and then blunt-ended using T4 DNA polymerase to provide the template for in vitro transcription. The mRNA was synthesized using T7 RNA polymerase and purified with the Qiagen RNeasy mini kit. Six hundred nanograms of the purified mRNA was used in a 150 $\mu$L in vitro translation reaction using rabbit reticulocyte lysate (Promega) and [$^{35}$S]Met. Five microliters of this reaction was used in each GST−Gal4 AD pull-down assay. The pull-down assay was performed according to the published protocol (*23*) with modifications. Various amounts of GST−Gal4 AD (see Figure 5) were mixed with in vitro translated full-length Gal11 for 45 min at 4 °C. Then 20 $\mu$L (50% slurry) of glutathione−agarose beads was incubated with each reaction for 45 min to pull down the GST−Gal4 AD and the complexed Gal11. The radiolabeled Gal11 pulled down by GST−Gal4 AD was resolved on SDS−PAGE and quantified using a phosphorimager. The data in Figure 5 represent the results obtained in two independent experiments.

## RESULTS

*Binding of the Gal4 Activation Domain to the Mediator Is Gal11-Dependent.* An experiment was designed to determine if the mediator is a high-affinity Gal4 target. Since the exact composition of the mediator, as defined by biochemical purification, is a matter of some controversy, it was desirable to carry out the experiment in a crude whole cell extract in order to employ complexes as close to the native state as possible. Thus, an extract prepared from wild-type yeast was incubated with bead-bound GST−Gal4 AD [a fusion of glutathione *S*-transferase and the core GAL4 AD, residues 841−875 (*18*)]. The retained proteins were analyzed by gel electrophoresis and Western blotting. In this experiment, low levels of bead-bound GST−GAL4 AD were employed ($\approx 10^{-8}$ M) in order to reduce low-affinity, potentially nonspecific interactions. As shown in Figure 1, mediator was retained by the Gal4 AD, as demonstrated by the presence of the known mediator components Srb4 and Gal11 in the bead-bound fraction.

To test the Gal11 dependence of AD−mediator binding, the experiment was repeated using extract prepared from a Δ*gal11* strain or one which overexpresses Gal11 at a level about 5-fold higher than wild-type cells. The essential protein Srb4 was used as a marker for mediator retention by GST−Gal4 AD. As seen in Figure 1, deletion of *GAL11* resulted in about a 10-fold decrease in AD-bound Srb4 relative to the experiment carried out with the wild-type extract. Conversely, 5-fold overexpression of Gal11 increased the amount of Srb4 retained by GST−Gal4 AD by about the same amount (recall that the Gal11 module is apparently substoichiometric with respect to the Srb complex). These results were not due to effects of Gal11 on Srb4 levels, as demonstrated by the almost identical amounts of Srb4 protein in the input lanes.

To ask if this effect of Gal11 was specific for the AD−mediator interaction, the same experiment was repeated

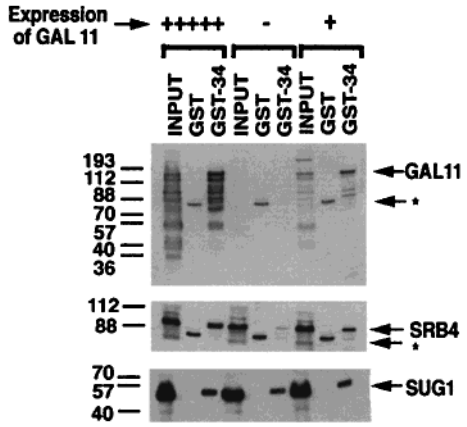


FIGURE 1: Gal4 activation domain to the mediator complexes is dependent on the Gal11 protein. A low level of bead-bound GST fusion protein containing the core Gal4 AD, or GST alone as a control, was incubated with a crude yeast extract in the presence of excess *E. coli* proteins. The levels of Gal11, Srb4, and Sug1 retained on the beads were analyzed by SDS−PAGE and Western blotting. The +, −, and +++++ designations indicate experiments done with extracts made from wild-type, Δgal11, and Gal11 overexpressing cells, respectively. The bands indicated by the asterisk represent an unknown factor that cross-reacts with the antibodies employed and is enriched by binding to GST. The many bands in the lanes probed with anti-Gal11 antibodies represent proteolytic degradation products.

except that retention of the Sug1 protein was analyzed. Sug1 is a component of the 19S regulatory particle of the 26S proteasome (*19*−*21*) and has previously been shown to be retained by the Gal4 AD (*22*−*24*). The level of Gal11 had little or no effect on the amount of Sug1 retained by the Gal4 AD (Figure 1). These data argue that Gal11 is critical for high-affinity Gal4 AD−mediator interactions but not for all AD−protein interactions.

*The Gal80 Repressor Blocks Gal4 AD−Mediator Interactions.* In the absence of the inducer galactose, the *GAL* genes are silenced by the Gal80 repressor, which binds tightly and specifically to the Gal4 AD, blocking its interaction with transcription factors (*18*). To ask if the Gal80 repressor blocked Gal4 AD−mediator interactions under the conditions employed here, the degree of Gal11 retention by the Gal4 AD or a preformed Gal4 AD−Gal80 complex was determined. As shown in Figure 2, preincubation of the AD with the repressor blocked binding of Gal11, as expected for a biologically relevant interaction.

*The Gal4 AD Binds Gal11 Directly.* The results shown in Figure 1 are consistent with direct Gal4−Gal11 binding in the context of the mediator but could also be due to association of the AD with other proteins in the Gal11 module if Gal11 is necessary for its structural integrity. To probe for direct Gal4−Gal11 interactions, GST−Gal11 was expressed, purified, and mixed with in vitro transcribed and translated Gal4 or fragments of Gal4. Parallel experiments were conducted with GST and GST−Gal80 as negative and positive controls, respectively. As shown in Figure 3, GST−Gal80 and GST−Gal11 both retained $^{35}$S-labeled full-length Gal4 and a fragment containing the DNA-binding and dimerization domains fused to the C-terminal AD. Neither GST−Gal80 nor GST−Gal11 retained a fragment lacking the AD. None of the three Gal4 proteins bound to GST alone. These experiments show that Gal11 binds the Gal4 AD directly. Comparison of the levels of Gal4 retained by GST−

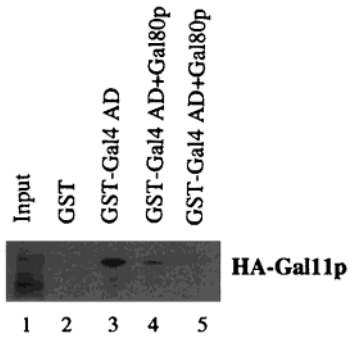


FIGURE 2: The Gal80 repressor blocks Gal4 activation domain−Gal11 binding. A pull-down experiment was performed with either bead-bound GST or GST−Gal4 AD and an extract prepared from yeast cells that express HA-tagged Gal11. A Western blot of the bead-bound material was probed with anti-HA antibody. For lanes 4 and 5, purified $His_6$-Gal80 was preincubated with the Gal4 AD prior to introduction of the extract. In lane 4, the Gal80 concentration was approximately half the $K_D$ of the complex, and in lane 5, the AD was saturated completely.

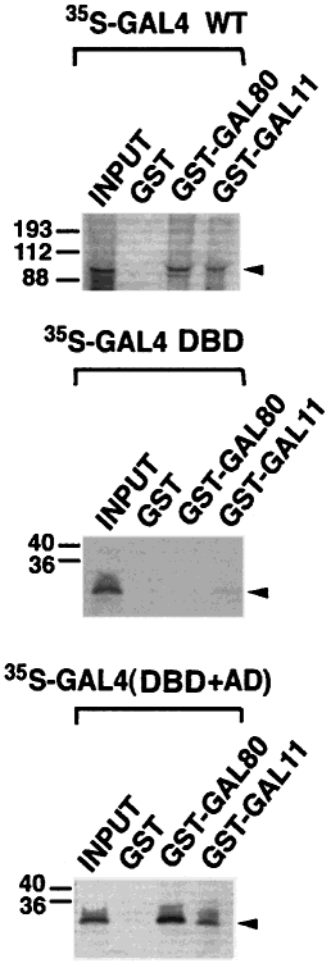


FIGURE 3: The Gal4 activation domain binds Gal11 directly. Full-length $^{35}$S-labeled Gal4 protein, or the fragments shown in the figure, was produced by in vitro transcription and translation. Pull-down experiments demonstrated that full-length Gal4 and a derivative containing the core AD bind to Gal11, but a fragment lacking the AD does not. The pattern of binding of the Gal4 derivatives to GST−Gal11 is identical to that observed for binding to Gal80, which is known to target the core activation domain. DBD = DNA-binding domain.

Gal80 and GST−Gal11 suggests that the GST−Gal11 fusion binds Gal4 roughly 5-fold more weakly than Gal80. The $K_D$

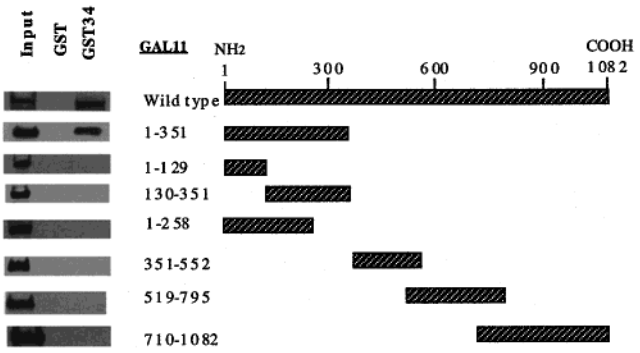


FIGURE 4: An N-terminal domain of Gal11 protein binds the Gal4 activation domain. Epitope-tagged fragments of Gal11 protein were expressed in *E. coli* and tested for binding to a GST−Gal4 AD (GST34) fusion protein. Only full-length Gal11 and an N-terminal fragment containing the first 351 residues were observed to bind tightly.

of the Gal4−Gal80 complex has been estimated to be approximately $2 \times 10^{-10}$ M (K. Melcher, T. Kodadek, and S. A. Johnston, in preparation). This suggests that the Gal4 AD−Gal11 interaction is of high affinity, though this assay does not represent true equilibrium binding conditions and so this conclusion should be taken with a grain of salt.

*The Gal4 AD Binds an N-Terminal Domain in Gal11.* Gal11 is a large polypeptide of 1082 residues. To localize the Gal4-binding domain, various fragments of Gal11 were expressed in *E. coli*, and their binding to the GST−Gal4 AD fusion protein was probed in pull-down experiments. As shown in Figure 4, a fragment comprised of residues 1−351 bound the Gal4 AD well, while more C-terminal fragments did not. Furthermore, fragments containing only part of the 1−351 region also failed to bind the Gal4 AD, for example, 1−129, 1−258, and 130−351. We conclude that approximately the first 351 residues of Gal11 form a distinct structural domain that is the target of the Gal4 AD.

*Quantitative Studies of the Gal4 AD−Gal11 Interaction.* To measure the equilibrium dissociation constant of the Gal4 AD for Gal11, a titration experiment was carried out using $^{35}$S-labeled, in vitro translated full-length Gal11 and the GST−Gal4 AD fusion protein. As shown in Figure 5, analysis of the fraction of labeled Gal11 retained at various AD concentrations provided a smooth binding curve. The $K_D$ calculated from these data is approximately $4 \times 10^{-7}$ M. A similar number was obtained when Gal11(1−351) was employed rather than the full-length protein (data not shown). This polypeptide was obtained by factor Xa-mediated cleavage of a GST−Gal11(1−351) fusion protein produced in *E. coli*.

A concern with these pull-down assays is that they are not completely homogeneous and therefore do not represent a true equilibrium situation. To examine the Gal4−Gal11 interaction in a completely solution-based assay, fluorescence polarization was employed. A Gal4 derivative containing the DNA-binding and dimerization domains (residues 1−93) fused to a C-terminal fragment (residues 768−881) of the protein including the core AD was expressed in *E. coli* and purified. The Gal4 derivative was bound to a fluorescein-labeled, double-stranded oligonucleotide containing a consensus Gal4 binding site (*17*, *25*). This complex was then titrated with Gal11(1−351), and the binding of the Gal11 N-terminal domain to the Gal4 derivative was monitored by

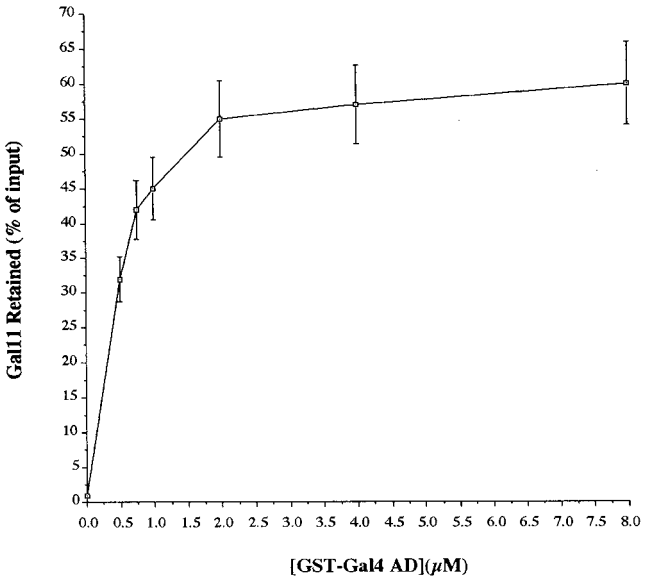


FIGURE 5: The Gal4 AD binds in vitro translated Gal11 protein with moderate affinity. $^{35}$S-Labeled, full-length Gal11 produced by in vitro transcription/translation was titrated with increasing amounts of the GST−Gal4 AD fusion protein. The fraction of the Gal11 input retained at each concentration of AD was measured by pull-down with glutathione−agarose beads, SDS−PAGE, and phosphoimagery. The titration curve suggests a $K_D$ for the complex of approximately $4 \times 10^{-7}$ M.

fluorescence anisotropy (*26*). As shown in Figure 6 (top graph), the fluorescence polarization data also suggest a modest $K_D$ for the Gal4 AD−Gal11(1−351) complex, although a precise value cannot be assigned from the data available. As can be seen from the graph, the Gal4−DNA complex was not saturated even at 140 nM Gal11(1−351). Unfortunately, solubility considerations precluded continuing this titration experiment to higher concentrations of Gal11-(1−351). No change in polarization was observed when GST was added to the protein−DNA complex rather than Gal11-(1−351), nor did Gal11(1−351) bind to the labeled DNA in the absence of Gal4(1−93 + 768−881) (data not shown). Thus, a GST pull-down assay using full-length Gal11 and a fluorescence polarization experiment using the Gal4 AD-binding fragment of Gal11 both indicate a modest affinity Gal4 AD−Gal11 complex.

A strikingly different result was obtained when a GST fusion of the Gal11 N-terminal domain was employed in the titration experiment. Gal4(1−93 + 768−881) (40 nM) bound to fluorescein-labeled DNA (10 nM) was titrated with increasing amounts of GST−Gal11(1−351) (see Figure 6, bottom graph). Half-saturation under these conditions was observed at only ≈9 nM GST−Gal11(1−351). The true $K_D$ of the complex is clearly significantly lower than this since the Gal4 derivative was present at a concentration of 40 nM. In other words, this titration was done under near-stoichiometric conditions. It was not possible to reduce the Gal4 derivative−DNA complex concentration further to attempt to measure the true binding constant due to sensitivity limitations. It is informative that under the conditions utilized (40 nM Gal4 derivative) saturation required between 35 and 65 nM GST−Gal11(1−351), arguing that the stoichiometry of the Gal4 AD−Gal11(1−351) complex is 1:1.

At first glance, the different behavior of Gal11(1−351) and the analogous GST fusion protein in this assay seemed odd. However, given the 1:1 stoichiometry of binding

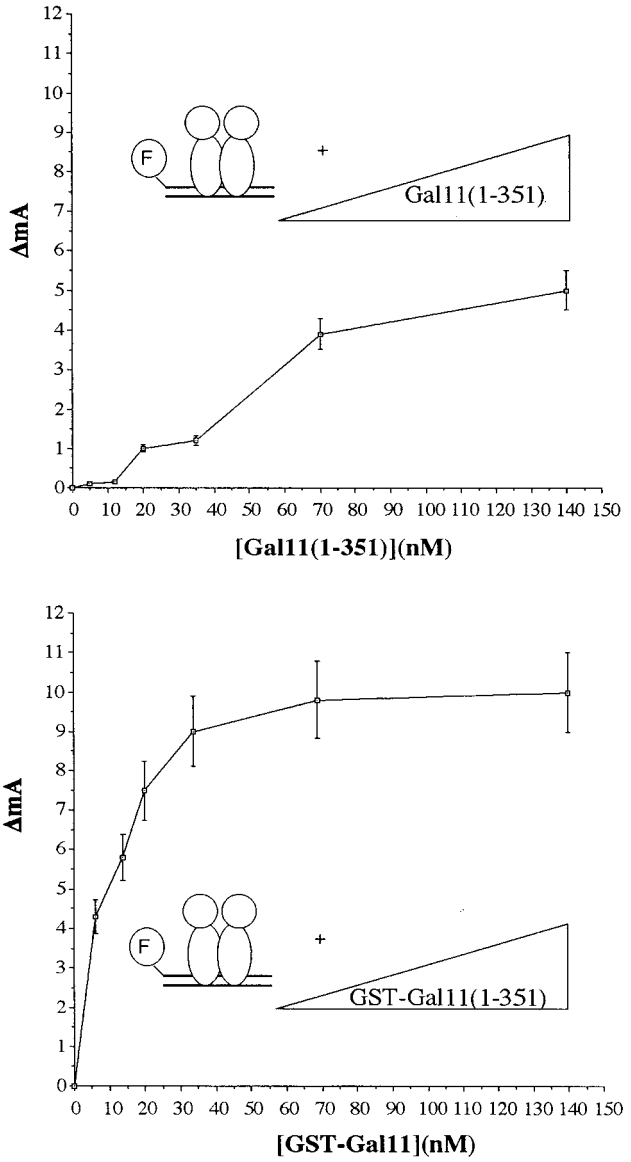


FIGURE 6: The Gal4 AD binds to monomeric and dimeric forms of Gal11(1−351) with widely different affinities. A complex comprised of a fluorescently labeled oligonucleotide containing a consensus Gal4 binding site and purified Gal4(1−93 + 768−881) (40 nM) was titrated with increasing amounts of purified Gal11-(1−351) (top graph) or GST−Gal11 (1−351) (bottom graph). Binding was measured by monitoring the fluorescence anisotropy, which increases as the labeled DNA tumbles more slowly due to association of the Gal11 domain with the bound Gal4 derivative. The equilibrium dissociation constants derived from these data are >150 nM and approximately 9 nM for the Gal4 derivative with Gal11(1−351) and GST−Gal11(1−351), respectively. The latter value represents an upper limit for the real $K_D$ (see text).

revealed in the latter experiment, this result can be easily rationalized on the basis of an avidity effect. GST is a native dimer, as is the Gal4 construct. Therefore, it is highly likely that two modest affinity Gal4 AD−Gal11(1−351) contacts cooperate to provide a tight dimer−dimer contact.

## DISCUSSION

The major conclusion of this study is that the Gal4 AD contacts Gal11 protein, a mediator component. A well-defined AD-binding domain was identified in the N-terminal region of Gal11, comprising approximately residues 1−351. Biophysical analysis of this interaction using recombinant purified proteins indicates that the binding affinity is modest [typical of AD−coactivator interactions (*9*, *24*, *27*)] and that the proteins form a 1:1 complex. The results shown in Figure 1 strongly support the idea that the interactions observed between the recombinant proteins are relevant to AD−mediator binding. Deletion of Gal11 reduces AD−mediator binding significantly, whereas overexpression of this protein stimulates mediator retention by the AD. It is interesting to note that fusions between the Gal4 DNA-binding domain and Gal11 or C-terminal Gal11 fragments are unusually potent and general artificial activators (*28*−*31*). We suggest that this may reflect the fact that the Gal11 is accessible on the surface of the mediator and that these unnatural fusions probably mimic native activator−mediator interactions better than other DNA-binding domain−transcription factor fusions. It is also important to note that while this study was in progress, Kim and co-workers reported evidence, from different types of experiments, that also suggest that Gal11 is a target of the Gal4 AD, as well as other activators (*32*, *33*).

However, several lines of evidence, some emerging from this study, indicate that while Gal4 AD−Gal11 binding is critical for efficient activator−mediator binding, it is unlikely to be the whole story. It has been known for some time that deletion of *GAL11* reduces, but does not abolish, Gal4-mediated transcription (*34*). The level of *GAL* gene expression is approximately 10%−15% in a Δ*gal11* strain of that observed in a wild-type strain. If one assumes that Gal4−mediator contacts are required for any transcription to be observed, which seems reasonable (*4*), then this observation would indicate that lower affinity Gal4−mediator contacts exist in a Δ*gal11* strain. This is consistent with our observation that Gal4 AD−mediator binding is weakened considerably, but not abolished, in a Δ*gal11* extract (Figure 1). One possibility is that Gal4, which functions as a homodimer (*17*), makes a second contact with the mediator complex. Since the stoichiometry of the Gal4 AD−Gal11-(1−351) complex is 1:1, a second equivalent of the AD will be available in the context of a promoter-bound complex. It is possible that this second AD could contact another region of Gal11 or a second molecule of Gal11 in the mediator, but proposing Gal11 as the sole target of Gal4 in the mediator makes it difficult to explain why Gal4-mediated transcription is reduced, but not abolished, in Δ*gal11* strains. It seems more likely that there is a second target of Gal4 in the mediator and that this putative contact and the Gal4 AD−Gal11 interaction cooperate with one another to provide an overall tight binding event (Figure 7). An attractive feature of the two-point contact model is that it would explain why single point mutations in the Gal4 AD have never been found that disrupt transcription grossly (*35*). If AD contacts with Gal11 and the other binding partner are nonidentical, which almost certainly must be true, then no single point mutation would completely disrupt the overall interaction. A single contact model would predict that seriously debilitating point mutations should have been discovered. It is interesting that even the well-known $Phe_{442}$ mutations in the VP16 AD (*36*) have a marked effect on activation only in the context of chimeric factors that contain only half of the full 78-residue AD (*37*). It is possible that each half of the VP16 AD binds different targets in the mediator as is apparently the case for the Gal4 dimer. Finally, we note that our observation of very

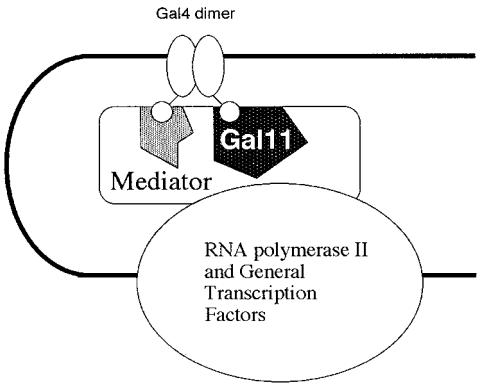


FIGURE 7: Schematic model of the proposed mode of interaction of Gal4 with the mediator. It is postulated that one of the two activation domains in the Gal4 dimer contacts Gal11 and that the other activation domain binds a different protein, whose identity is currently not known. See text for details.

high affinity contacts between the Gal4 AD and the GST−Gal11(1−351) fusion protein (Figure 6) certainly validates the idea that a dimeric activator can bind a target complex tightly through a two-point contact. While the $K_D$ of the Gal4−mediator interaction (or for that matter any activator−mediator interaction) is unknown, it generally assumed that potent activators probably bind the mediator tightly, and this is consistent with our observation of almost complete retention of Gal11 from an extract by low levels of GST−Gal4 AD (Figure 1). However, this experiment employed bead-bound protein and does not represent a homogeneous solution at true equilibrium, so it is unwise to attempt to infer a true $K_D$ from these data. Nonetheless, given this important caveat, if dimeric Gal4 does bind the mediator with high affinity, then this cannot be explained simply on the basis of a single modest affinity Gal4 AD−Gal11(1−351) contact.

If there is a second Gal4-binding protein in the mediator, a possible candidate is Srb4. Indeed, Koh et al. have argued that Srb4 is the primary target of the Gal4 AD in the mediator (*9*). This would appear to be at odds with the fact that retention of Srb4 by the Gal4 AD from a crude extract is strongly dependent on Gal11, as demonstrated in Figure 1. We note that almost all of the biochemical experiments described in that paper employed purified, recombinant proteins (Srb2p, Srb4, Srb5p, and Srb6p) or a complex of these four factors. In that context, Srb4 was found to bind to the Gal4 AD. Therefore, these experiments may not accurately reflect the in vivo situation. In the single experiment where a purified holoenzyme complex was employed, very weak "label transfer" of a radioactive tag from the Gal4 AD to Srb4 was observed, indicating proximity of the two polypeptides. It was not clear whether this holoenzyme preparation contained Gal11. It is possible that Srb4 could be a second contact point of Gal4 in the mediator, though more work will have to be done in the context of Gal11-containing complexes to address this possibility more rigorously.

Alternatively, it is conceivable that there is no single specific binding partner of Gal4 in the mediator other than Gal11. Acidic activation domains appear to be relatively "sticky", with the unusual characteristic of having hydrophobic side chains well displayed in solution due to intervening charged residues. Several genetic studies have implicated these hydrophobic groups as being the residues most important for activation (*35*, *36*, and references therein). This may be why so many different recombinant proteins have been reported to bind with modest affinity to the Gal4 AD and related domains such as the acidic AD of VP16 (*8*, *38*−*45*). Thus, one can imagine that the Gal4−mediator interaction is anchored by a modest affinity, but specific, Gal11 interaction, while the second AD equivalent could contact any of several molecular surfaces in the complex to cement tight binding. Indeed, basic physical chemistry dictates that the second Gal4 AD−mediator protein contact need not be a high-affinity interaction in order to support tight activator−mediator binding. For a two-point contact, $K_D$(overall) approaches $K_D1 \times K_D2$, assuming that the stereochemistry of the complex allows each individual interaction to assume an optimal geometry. Given a Gal4 AD−Gal11(1−351) $K_D$ of $10^{-6}$−$10^{-7}$ M, the second contact would only have to have a $K_D$ in the millimolar range to provide an overall nanomolar interaction. Even assuming nonideal linking geometry, it is unlikely that the second contact would have to be tighter than approximately micromolar. Especially in light of this possibility, it will be important to devise methods by which Gal4 AD−transcription factor contacts can be probed in the context of complete, physiologically relevant complexes containing Gal11.

Finally, the data reported here do not speak to the potential importance of several other Gal4 AD−protein interactions that have been reported in the literature. While the Gal4 AD−Gal11 interaction appears to be important for achieving full activation by Gal4, it is clear that the activator can interact with other proteins in the absence of Gal11 (*9*, *24*, *27*), as described above. Even in wild-type cells, it may be that the Gal4 AD associates sequentially with several proteins over the course of the transcription cycle, some of which may be outside the context of the mediator. It will be important to examine each of these activator−transcription complexes biochemically and to set up definitive mechanistic tests for their physiological relevance in vivo.

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