Despite their enormous potential as novel research tools and therapeutic agents, artificial transcription factors (ATFs) that up-regulate transcription robustly in vivo remain elusive. In investigating an ATF that does function exceptionally well in vivo, we uncovered an unexpected relationship between transcription function and a binding interaction between the activation domain and an adjacent region of the DNA binding domain. Disruption of this interaction leads to complete loss of function in vivo, even though the activation domain is still able to bind to its target in the transcriptional machinery. We propose that this interaction parallels those between natural activation domains and their regulatory proteins, concealing the activation domain from solvent and the cellular milieu until it binds to its transcriptional machinery target. Inclusion of this property in the future design of ATFs should enhance their efficacy in vivo.

The expression of specific genes is mediated by transcriptional regulators that bind to DNA and interact with various protein partners to exert transcriptional control. There is increasing evidence that malfunctioning transcriptional regulators play a central role in the etiology of human diseases as diverse as cancer and diabetes (1–5). The development of artificial transcription factors (ATFs), molecules that target specific genes and control their transcriptional levels, has thus become a highly active area of research. ATFs will likely have a clear role in the study and eventual treatment of transcription-based disorders (2, 4–7). A significant challenge has been the creation of fully functional artificial transcriptional activators that seek out and robustly up-regulate specific genes (4, 5, 7).

Endogenous activators are modular proteins minimally composed of two domains as follows: a DNA binding domain that dictates the genes to be targeted and an activation domain that governs the nature and the extent of the transcriptional response through interactions with the transcriptional machinery (Fig. 1A) (6). The two domains typically operate in an independent fashion such that the DNA binding domain of one protein can be attached to the activation domain of another to generate a fully functional activator. The nature of the linker connecting the two domains is variable but most often consists of a dimerization domain that facilitates specific and high affinity DNA binding (6). Inspired by the architecture of natural activators, artificial activators have typically been generated through a modular replacement strategy in which the endogenous DNA binding domain is replaced with an engineered counterpart with desired DNA targeting properties (4, 7, 8). By using this strategy, protein, oligonucleotide, and small molecule DNA binding domains have been used in the construction of ATFs that function well in vitro and/or in cell culture (4–11).

In contrast to the diversity of DNA binding domains used in ATF construction, it has proven much more challenging to identify non-natural activation domains that function robustly in vivo (4, 5, 7). In fact, most ATFs that elicit strong expression of a targeted gene in cells utilize activation domains that are derived from natural transcription factors, such as VP16 or Gal4 (4, 10, 12–16). Attempts to develop non-natural activation domains have predominantly yielded peptides or, more recently, RNA molecules that function only moderately relative to natural systems (11, 17–26). There are, however, two artificial activators that are exceptions to this trend and function strongly in vivo; these thus serve as excellent models for the design or discovery of potent artificial activation domains. One potent activation domain is P201, a hydrophobic peptide (YL-LPTCIP, see Fig. 1B) that when coupled to the DNA binding domain of Gal4 (residues 1–100) activates transcription to levels that are comparable to those obtained by some of the most potent natural activators (Fig. 1) (19, 20). In contrast to natural activators that typically interact with a number of transcriptional machinery proteins, the function of P201 is most likely mediated by targeting the Gal11 component of the transcriptional machinery. In support of this, a single point mutation in Gal11 (T322K) abolishes the ability of P201 to activate transcription in vivo and the ability to bind to that protein in vitro (20).

The second example of a potent artificial activation is observed when Gal4(1–100), a fragment of the transcriptional activator Gal4, is present in a yeast strain bearing a mutated version of Gal11. The first 50 residues of Gal4 compose the minimal DNA binding domain, and the next 50 residues form an extended dimerization domain (see Fig. 1B). In normal yeast strains, Gal4(1–100) is an inert DNA-binding protein. How-
Fig. 1. Scaled illustration of the artificial activator and the yeast RNA polymerase II holoenzyme. A, the construction of a transcriptional activator. The DNA binding domain (DBD) is shown as a green square and the activation domain (AD) is shown in red, the structural model of the Gal4 dimerization domain (residues 53–97, see Ref. 28) with key structural elements denoted (α-helices, α1–3 and loops L1 and L2) is shown along with a scaled representation of FVQD-YLLPTCIP (Gal4(97–100)+P201). C, the composite activator Gal4(1–100)+P201 (in green) was modeled by superimposing the crystal structure of Gal4 residues 1–64 (59) on the NMR-derived structure of Gal4 residues 53–97 (28). P201 and residues 98–100 of Gal4 are shown as fully extended peptides (brown). RNA polymerase II holoenzyme structure (~36 Å) is constructed from electron microscopy (39). The polymerase is colored in turquoise, and the head (H) and middle (M) lobes of the Mediator complex are colored in dark blue. The tail (T) domain of the Mediator is colored in purple; Gal11 the target of the artificial activator as well as several natural activators is a component of this module. The Gal4-binding sites can be separated from the promoter (TATA box) by variable distances, and this is represented by a gap in the DNA template. The interaction between the activator and the holoenzyme is sufficient to elicit the expression from the promoter. However, additional factors that are not shown in this illustration such as the TATA box-binding protein and other general transcription factors (TFIIA, TFIIF, TFIIE, TFIH, and perhaps TFIIB) are required for transcription (27). D, the replacement of asparagine 342 in Gal11 (purple) with valine (shown as a green patch within Gal11) leads to a binding interaction with the dimerization domain of Gal4 (green). This interaction converts Gal11 into a potent artificial activator.

however, when a single point mutation (N342V) is introduced into Gal11, Gal4(1–100) stimulates high levels of transcription (27–29). Several studies indicate that this mutant version of Gal11, called “potentiator” or Gal11P, retains the normal function of Gal11 but is able to interact with the dimerization domain of Gal4 (Gal4dd, residues 50–100; see Fig. 1, B and D) (28). Structure-activity studies indicate that the primary interactions with Gal11P occur in helix 3 and loop 1 of Gal4dd, both of which contain a number of hydrophobic residues (28).

Gal11, a primary target of two of the most potent artificial activators, is also one of the targets of natural yeast activators (20, 27, 29–32). Gal11 resides in the mediator complex; this multiprotein complex interacts with RNA polymerase II and is thought to mediate the positive or negative signals of transcriptional regulators bound to their respective genes (33, 34). In support of this model, a number of mediator subunits have been found to interact with transcriptional activators (35–37), and several of the targeted components, including Gal11, reside in the tail of this tri-lobe complex (Fig. 1C) (38, 39). Because both of the potent artificial activators described above function through an interaction with Gal11, this suggests that Gal11 could be a privileged target for the development of potent activation domains.

Recent evidence has emerged indicating that exclusive targeting of Gal11 does not provide potent transcriptional activation domains. For example, peptides selected specifically for their ability to bind to Gal11 function only modestly as transcriptional activators despite affinities for Gal11 comparable with natural activation domains (23). This suggests that the binding interaction with Gal11 is unlikely to be the sole contributor to the unusually potent function of P201. To gain insight into additional properties of P201 that make it a robust artificial activator, we examined its ability to activate transcription in yeast strains bearing various mutants of Gal11 and to bind these Gal11 mutants in vitro. These experiments indicate the existence of a second binding interaction that is essential for P201-mediated activation. Remarkably, this interaction is not with a transcriptional machinery protein but with the hydrophobic dimerization domain of Gal4 (Gal4dd). We propose that this dynamic interaction between Gal4dd and P201 plays a role analogous to the interactions between natural activation domains and their regulatory proteins, preserving the hydrophobic activation sequences from nonproductive binding events with other cellular proteins. This finding provides an important guiding principle for the future design of robust artificial activators.

MATERIALS AND METHODS

Yeast Strains and Plasmids—The yeast strain used was JPY52-JP188 MATa his3Δ200 leu2Δ1 trp1Δ63 ura3Δ52::P188 lys2Δ385 gal4Δ111 gal11::LYS2. This strain contains a GAL1-lacZ reporter with two GAL4 DNA-binding sites 191 bp upstream of the TATA box integrated at the URA3 locus. The Gal4dd or P201 constructs were on a CEN4 plasmid under the control of a β-actin promoter with a HIS3 selection. The Gal11 constructs were based on YCplac111 (CEN LEU2). Expression and Purification of GST-Gal11(186–619)—The plasmid pGEXVh1 was transformed into chemically competent BL21(D3) E. coli (Invitrogen), and cells were plated onto LB agar plates supplemented with ampicillin (0.1 mg/ml) and chloramphenicol (0.034 mg/ml). Cultures (50 ml) from single colonies were grown overnight at 37° C (275 rpm) in LB supplemented with ampicillin (0.1 mg/ml) and chloramphenicol (0.034 mg/ml) before addition to 1 liter of LB supplemented with ampicillin (0.1 mg/ml). After 3 h, the cultures were cooled to 16 °C, and expression was induced with isopropyl-β-D-galactopyranoside (final concentration 0.5 mM) for 5 h. The cell pellet was lysed using sonication, and the GST-tagged protein was isolated from the cell lysate using glutathione-Sepharose beads (Amersham Biosciences). Elution from the beads was accomplished with 50 mM Tris-HCl buffer, pH 8.0, containing 15 mM glutathione and 0.1% Nonidet P-40. The protein solution thus obtained was concentrated to ~20–25 μM and the buffer exchanged to storage buffer (phosphate-buffered saline, pH 7.4, 1 mM dithiothreitol, 10% glycerol (v/v), and 0.01% Nonidet P-40) using a Millipore Ultrafree centrifugal filter device. The protein solution was stored in 50-μl aliquots at ~80 °C until needed. The protein concentration was measured by using a Bradford
assay (Bio-Rad) with bovine serum albumin as the standard. The identity and purity of the fusion protein was verified by reducing SDS-PAGE with appropriate molecular weight standards.

Beta-galactosidase Assay—For quantitative measurement of activity, freshly transformed colonies were used to inoculate 5-ml cultures of SC media containing 2% raffinose, 2% galactose but lacking uracil, histidine, and leucine. The cultures were incubated overnight at 30 °C for 250 rpm. Following incubation, these cultures were used to inoculate 5-ml cultures of SC media, which were subsequently incubated overnight at 30 °C with agitation to an A\textsubscript{600} of 0.6–0.9. The yeast cells were harvested, and the culture was then lysed with glass beads in Z buffer (for 500 ml: 4.26 g of sodium phosphate, 2.76 g of sodium phosphate monobasic, 0.37 g of potassium chloride, 0.123 g of magnesium sulfate, pH 7.0) for 5 min in the cold. A portion of the cell extract was used to measure beta-galactosidase activity via incubation with o-nitrophenyl-beta-D-galactopyranoside at 4 mg/ml in Z buffer. The reaction was stopped by adding 1 M Na\textsubscript{2}CO\textsubscript{3} and the A\textsubscript{420} was measured. The activity was then normalized to the total protein concentration of the extract, which was measured using a Bradford assay kit (Bio-Rad) with bovine serum albumin as the standard.

Peptide Synthesis and Fluorescent Labeling—XL\textsubscript{Y} (LTGLFVQDRLLPTCIP) and XL\textsubscript{R} (LTGLFVQDRLLPTCIP) were synthesized on Rink albumin as the standard. Peptides were labeled at the N terminus with fluorescein using 5/6-carboxyfluorescein succinimidyl ester (Pierce) in accordance with the manufacturer’s instructions, purified using reversed-phase high pressure liquid chromatography (C18 column with a gradient solvent system: buffer A, 0.1% trifluoroacetic acid; buffer B, CH\textsubscript{3}CN). Each peptide was characterized using electrospray mass spectrometry. The peptides were labeled at the N terminus with fluorescein using 5/6-carboxyfluorescein succinimidyl ester (Pierce) in accordance with the manufacturer’s instructions, purified using reversed-phase high pressure liquid chromatography, and characterized using electrospray mass spectrometry. The labeled peptides were divided into 2-nmol aliquots and stored as dry pellets at –80 °C.

Disassociation Constant Measurements—Disassociation constant measurements were carried out on a Spex Fluoromax-2 fluorometer at room temperature. Each experiment was performed in triplicate. Aliquots of the labeled peptide were incubated with 2-nmol aliquots of fluorescence-labeled XL\textsubscript{Y} or XL\textsubscript{R} (25 nM) in Buffer S at room temperature for 45 min. A 7% acrylamide, 3% glycerol gel was used to make up 10-nM final protein concentration for most measurements). After incubation for 10 min at room temperature, the first measurement was taken (excitation wavelength, 487 nm; emission wavelength, 516 nm). For each successive measurement, the solution was diluted by addition of Buffer S containing 25 nM fluorescein-labeled XL\textsubscript{Y} or XL\textsubscript{R} (to keep the concentration of the labeled component constant throughout the experiment). The solution was then diluted by addition of buffer for 10 min at room temperature. At concentrations above 25 nM, GST + Gal11(186–619) aggregates to a significant degree, and dissociation constants greater than 10 μM thus were not measured. The same procedure was used for binding experiments with GST + Gal4\textsubscript{dd} or GST + Gal4\textsubscript{dd} + P201, except that a 10 nM concentration of XL\textsubscript{Y} or XL\textsubscript{R} was used. The data obtained were plotted in Origin 7.0 and fit to the following equation using the Levenberg-Marquardt least squares method.

$$A = a + b \times e^{-k_{d}t}$$

where $k_{d}$ is the rate constant for the binding of Gal4\textsubscript{dd} to Gal11(186–619) until equilibrium was reached, as determined by anisotropy measurements. Following equilibration, a 1700-fold excess of unlabeled XL\textsubscript{Y} was added by mixing, and anisotropy measurements were taken every 11.5 s to monitor the labeled peptide. An identical procedure was used for the XL\textsubscript{Y} - Gal4\textsubscript{dd} experiment, except that a 10 nM concentration of fluorescein-labeled XL\textsubscript{Y} was used and a 50 μM concentration of GST + Gal4\textsubscript{dd}. A control experiment was carried out to determine the loss in anisotropy due solely to the dilution of the protein and accounted for in the data analysis. All data were plotted using Origin 7.0 and fit to a single phase exponential decay using the Levenberg-Marquardt least squares method.

RESULTS

In Vivo Activation—P201 was originally identified by a screen in Saccharomyces cerevisiae of random 8-residue peptide particles attached to Gal4(1–100). We initially postulated that the potent function of Gal4(1–100) + P201 could be due to some residual affinity of Gal4\textsubscript{dd} (residues 50–100 of Gal4) for wild-type Gal11. Thus the overall level of activation observed with Gal4(1–100) + P201 would be the result of two binding interactions, P201-Gal11 and Gal4\textsubscript{dd}-Gal11. To delineate the relative contributions of P201 and Gal4\textsubscript{dd}, we first looked at the function of Gal4(1–100) + P201 and Gal4(1–100) in yeast strains bearing wild-type Gal11 or its mutants. The first of the key Gal11 mutations is the substitution of threonine 322 to lysine (T322K), a change that disrupts binding with P201. The second mutation results from replacement of asparagine 342 with a valine (N342V, also referred to as the Gal11P mutation), and this alteration promotes an interaction with Gal4\textsubscript{dd342V} (20, 28). We also tested the function of Gal4(1–100) + P201 and Gal4(1–100) in a yeast strain bearing the Gal11 double mutant (Gal11 DM) that has both the T322K and N342V mutations.

Gal4(1–100) + P201 functions well in the wild-type Gal11-bearing strain, and as expected, the T322K substitution in Gal11 abolishes P201 activity (Fig. 2, A and B). In contrast, Gal4\textsubscript{dd} does not activate transcription in yeast strains containing either wild-type Gal11 or Gal11 with the T322K mutation (Fig. 2, C and D). Introduction of a second mutation in Gal11 (N342V, Gal11DM) restores activation function to Gal4\textsubscript{dd342V} to levels nearly identical to those observed with Gal11P (Fig. 2, E and F). The latter result demonstrates that the T322K mutation has little effect on the ability of Gal4\textsubscript{dd} to activate transcription. The Gal11P mutant contains binding sites for both Gal4\textsubscript{dd} and for P201, but as illustrated in Fig. 2G, the levels of transcription elicited by Gal4(1–100) + P201 in this strain are similar to those obtained in the presence of wild-type Gal11 (Fig. 2A).

In the final experiment, we examined the function of Gal4(1–100) + P201 in yeast bearing Gal11DM to decouple the effect of P201 on activation mediated by Gal4\textsubscript{dd} (Fig. 2H). The T322K mutation in Gal11 abrogates P201-mediated activation (Fig. 2B) but has little effect on the function of Gal4\textsubscript{dd} (Fig. 2F). It would be expected, then, that Gal4(1–100) + P201 would activate transcription in Gal11DM yeast primarily because of the interaction of Gal4\textsubscript{dd} with the binding surface generated by the N342V mutation (Fig. 2F). However, activation was significantly diminished in this strain (Fig. 2H). In other words, P201 somehow blocks the function of Gal4\textsubscript{dd}. Identical results were obtained in yeast strains bearing a single binding site for Gal4 (see supplemental Fig. 1 for details). Taken together, these results suggest that although Gal4\textsubscript{dd} and P201 do not appear to function cooperatively, there is a functional interplay between the two peptides. Subsequent experiments were therefore designed to first more clearly define the P201-Gal11 interaction, and second to investigate a possible Gal4\textsubscript{dd} - P201 interaction.

Defining the Minimal Gal11-binding Peptide—The results from the in vivo activation studies above indicate that Gal4\textsubscript{dd} does not interact with wild-type Gal11 in the context of Gal4(1–100) + P201. However, previous mutagenesis studies have shown that residue 97 in helix 3 of Gal4\textsubscript{dd} is important for P201 function, and thus there remained the possibility that some
Interaction of XLY with Gal4dd and Gal11—The data of Fig. 2H suggest that XLY binds directly to Gal4dd and interferes with its ability to interact with Gal11DM. To probe a possible interaction between XLY and Gal4dd, we designed a series of fluorescence polarization assays. For this purpose, XLY was synthesized by standard methods and labeled with carboxyfluorescein (61). Gal4dd and the central region of Gal11, residues 186–619, were expressed and purified as GST fusion proteins. The central region of Gal11 was used in these experiments because it is targeted by XLY as well as Gal4dd; it can be readily generated using a bacterial expression system, and it is the largest fragment of Gal11 that is well behaved at micromolar concentrations in vitro.

As shown in Fig. 3, B and C, XLY binds to both Gal11 and Gal4dd with dissociation constants in the micromolar range and interacts with Gal11 (2-fold more tightly (2.2 versus 5 μM, respectively). We also measured the affinity of XLY for Gal4dd (2.2 μM ± 2-fold) and for Gal11 (3 μM ± 2-fold) sequence containing the fused P201 sequence (compare Fig. 3, C with D). This result indicates that the fused P201 peptide does not inhibit the XLY interaction of Gal4dd. As a control, we also examined the binding properties of a P201 sequence containing an arginine rather than a tyrosine residue (XLY, LTGLFVQDRLLPTCIP); this sequence is inactive as a transcriptional regulator in vitro (19). In our experiments XLY did not bind to Gal11, Gal4dd, or to Gal4dd+P201 under any conditions examined (data not shown). Next, we tested to see if XLY would interact with Gal11 proteins bearing either the 11P (N342V) mutation singly or in combination with the T322K mutation (Gal11DM). Consistent with the in vivo data, we found that introduction of the 11P mutation causes a 3-fold reduction in affinity (Fig. 3E). Also consistent with the lack of activation in vivo (Fig. 2B and H), XLY does not bind to Gal11DM within the limits of our assay. Furthermore, the inactive version of Gal4 (93–100)+P201, XLY, shows no measurable binding interactions with either Gal11P or Gal11DM (data not shown).

NMR studies of Gal4dd suggest that it has several solvent-exposed hydrophobic residues. XLY is also a very hydrophobic peptide. To probe the specificity of the Gal4dd-XLY interaction, we examined the interaction of Gal4dd with another hydrophobic Gal11-binding peptide, AHYYYPSE (23). This peptide has a similar affinity for Gal11 (4.8 μM) relative to XLY (2.2 μM) but is far less robust than XLY as a transcriptional activation domain (23). Moreover, as illustrated in Fig. 3F, this peptide exhibits no detectable binding to Gal4dd. Consistent with the inability to bind Gal4dd in vitro, we find that this peptide, unlike XLY, does not interfere with the ability of Gal4dd to stimulate transcription in strains bearing the Gal11DM mutant (see supplemental Fig. 2 for details). In further experiments we found that XLY does not bind nonspecifically to unrelated proteins such as bovine gamma globulin that display affinity for hydrophobic surfaces (62) (Fig. 3G). Thus, the Gal4dd-XLY binding event cannot simply be ascribed to nonspecific hydrophobic interactions.

Interaction of Gal4dd with Gal11—We next determined the strength of the interaction between Gal4dd and the Gal11 variants by electrophoretic mobility shift assays. Fluorescence polarization was not used for these experiments because the large size of the two components (Gal4dd bound to DNA + Gal11 and its mutants) leads to a minimal change in anisotropy upon complex formation. The Gal11 proteins and the assay conditions (buffer, temperature, and incubation times) employed were identical to those used in the fluorescence polarization assays outlined above to allow for meaningful comparisons of
FIG. 3. Binding behavior of the minimal activator peptide, XLY, A, results from alanine-scanning mutagenesis studies on helix 3 of Gal4dd. The LexA DNA binding domain (1–87) was fused in-frame to Gal4(40–100)+P201, and transcriptional activation was monitored by a reporter bearing two LexA operators 191 bp upstream of the Gal1 promoter driving the expression of the lacZ gene. The use of the LexA DNA binding domain ensures that any loss of activity upon mutagenesis is not due to loss of DNA binding of Gal4dd+P201 (for example see Ref. 28). The β-galactosidase (β-gal) activity averaged from quadruplicate measurements is reported, and the S.D. did not exceed 15%. As shown, residues 93–100 of this region are essential for P201-mediated transcriptional activation. This, in combination with the binding data of 3B, indicates that the minimal functional activator is Gal4(93–100)+P201, named XLY. B, determination of the dissociation constant for the XLY-Gal11 interaction by fluorescence polarization. Fluorescein-labeled XLY (Gal4(93–100)+P201) at a constant concentration was incubated at 25 °C with increasing concentrations of Gal11. The fluorescence polarization at each concentration was measured, and the resulting data fit using the Levenberg-Marquardt least squares method to obtain the dissociation constants. Each experiment was performed in triplicate ($R^2 > 0.98$) with the error indicated. C, determination of the dissociation constant for the XLY-Gal4dd interaction by fluorescence polarization. Conditions were identical to those used for B. D, determination of the dissociation constant for the XLY-Gal4dd+P201 interaction by fluorescence polarization. Conditions were identical to those used for B. E, determination of the dissociation constant for the XLY-Gal11P interaction by fluorescence polarization. Conditions were identical to those used for B. F, the hydrophobic activator peptide AHYYYYPSE does not interact with Gal4dd as monitored by fluorescence polarization. Conditions were identical to those used for B. G, XLY does not interact with bovine gamma globulin as monitored by fluorescence polarization. Conditions were identical to those used for B.
The mobility of a 30-bp oligonucleotide bearing a single Gal4-binding site (17 bp) decreases in the presence of Gal4(1–100) but not Gal11, Gal11P, or Gal11DM (lanes 2, 3, 5, and 11). The mobility of the Gal4-DNA complex is further decreased by increasing concentrations of Gal11P and Gal11DM but not by Gal11 (20 μM in lane 4). The final concentrations of Gal11P and Gal11DM in lanes 6–10 and 12–16 are 1, 3, 9, 18, 20 μM, respectively. Where incubated with DNA, in the absence of Gal4, the concentration of the Gal11 proteins is 20 μM (lanes 3, 5 and 11).

the binding affinities measured by the two techniques. A concentration of 30 nM Gal4(1–100) was sufficient to bind saturably to its cognate DNA site, and this Gal4-DNA complex was incubated with increasing concentrations of wild-type Gal11, Gal11P, or Gal11DM. As demonstrated in Fig. 4, Gal4dd does not interact with wild-type Gal11, consistent with the lack of activation observed in vivo. Introduction of the 11P mutation, however, leads to half of the Gal4-DNA complex being further shifted to a ternary DNA-Gal4-Gal11P complex at a Gal11P concentration of ~18 μM. This binding is virtually unchanged by the introduction of the second (T322K) mutation (compare unnumbered lanes 10 and 16 in Fig. 4). Because Gal11 and its variants begin to aggregate significantly and precipitate at concentrations above 24 μM, the concentration of Gal11 could not be further increased to determine the complete binding isotherm; however, the half-maximal binding at 18 μM provides a reasonable indication of the K_D. Although it is difficult to make a direct quantitative comparison because different techniques were used to assess binding, the results suggest that the affinity of Gal4dd for Gal11P is somewhat weaker than for XLV.

It is thus not surprising that the Gal4dd-XLV interaction inhibits Gal4dd-mediated transcription but does not negatively impact XLV-mediated activation.

Site of the Gal4dd-XLV Interaction—Both the in vivo activation data of Fig. 2 and the in vitro binding data of Fig. 3 are consistent with an interaction between Gal4dd and XLV, but two key questions regarding this interaction remained. 1) Does this interaction contribute to XLV-mediated activation? 2) Where within Gal4dd is the site of this interaction? We addressed these questions by a combination of deletion analysis and mutagenesis experiments (Fig. 5).

In the first set of experiments, the P201 sequence was attached to the LexA DNA binding domain along with fragments of Gal4dd of increasing length. As depicted in Fig. 5A, XLV-mediated transcriptional activation was not observed until the entire Gal4dd region (residues 50–100) was incorporated into the protein construct. The last result of this panel points to the importance of helix 1 and loop 1 for this process, because the construct containing only helices 2 and 3 (Gal4(73–100)) showed no activity.

To refine this picture further, additional alanine-substitution mutagenesis experiments of Gal4dd were carried out. For this purpose, we again employed the chimeric LexA-Gal4 protein described in Fig. 3A to avoid any impact on DNA binding. Using the structural model of Gal4dd to guide these efforts, we mutated residues in loop1 (L1), helix 2 (α2), loop2 (L2), and helix 3 (α3) that are important for Gal4dd/Gal11P interactions (see Fig. 1B and Ref. 28). As shown in Fig. 5B, changing residues 68, 69, 72, and 75 to alanine has a profound effect on XLV-mediated activation, with little or no activity observed, suggesting that loop 1 is the likely site of interaction with XLV.

To probe this further, we examined the binding of XLV to Gal4dd containing one of those mutations, 69A, again using fluorescence anisotropy, and no detectable binding was observed (see supplemental Fig. 3 for details). As summarized in Fig. 5C, several of these residues are also essential for the Gal4dd-Gal11P-binding interaction (28). Most interestingly, not all substitutions affected XLV function; L86A and I89A mutants were functional (>30% active). Both of these residues are essential for the Gal11P-Gal4dd interaction in vitro and for Gal4dd transcriptional activation in vivo (28). In contrast, residues Phe-68 and Leu-77 flanking loop 1 and Asp-84 at the C-terminal end of α2 are essential for XLV function but not for the Gal4dd-Gal11P interaction.

The residues of Gal4dd that are important for XLV function and for Gal11P interaction are also summarized in Fig. 5C. There are several residues in loop 1 that are essential both for XLV function and for the ability of Gal4dd to interact with Gal11P. However, residues that are essential for XLV function are not entirely coincident with those required for the Gal4dd-Gal11P interaction. Taken together, the results suggest that Gal4dd uses distinct but overlapping surfaces along its length to interact with Gal11P and with XLV.

Dynamics of Binding—To further characterize the XLVGal4dd and the XLV-Gal11 interactions, we measured the off-rates again by using fluorescence polarization methods (40). Fluorescein-labeled XLV was incubated with either Gal11 or Gal4(52–100) under conditions of saturated binding (10–25 nM XLV, 18 μM Gal11, or 50 μM Gal4(52–100)). Following equilibration at room temperature, a 1700-fold excess of unlabeled XLV peptide was added as a single aliquot. This leads to a measurable decrease in polarization as the bound fluorescent peptide is exchanged with the unlabeled peptide. As shown in Fig. 6, we find the dissociation of XLV from Gal4(52–100) is faster than the dissociation from Gal11. In fact, virtually all of the exchange occurred prior to the first measurement (11.5 s). Thus, the k_off value of 0.24 s^{-1} for XLV dissociating from Gal4(52–100) calculated from these data represent the lower limit of this rate. In contrast, the dissociation rate for the Gal11XLV interaction is significantly slower, with a k_off of 0.06 s^{-1}. In contrast, the k_off for Gal4dd dissociating from Gal4dd has been measured previously as ~5 s^{-1} by NMR titration (28). Thus, although the measurements between methods are not directly comparable, this off-rate is significantly faster than the XLV-Gal4dd off-rate reported in Fig. 6.

**DISCUSSION**

The hydrophobic octapeptide P201 functions as an unusually robust transcriptional activator when fused to the Gal4 DNA
Our study demonstrates that in addition to the 8 amino acids that make up P201, residues 93–100 of Gal4 are essential for transcriptional activation and also for interaction with Gal11 (Fig. 3A). The minimal functional activator is thus the 16-residue sequence Gal4(93–100)/H11001P201, named XLY. The potency of XLY as an activation domain is particularly remarkable when one considers that other artificial activator peptides of greater size and surface area up-regulate only moderately (17, 22, 23). Moreover, the potency of XLY is not simply a function of targeting Gal11, because artificial activator peptides of similar size and even similar affinity for Gal11 do not activate nearly as well as XLY (23).

The first indication that an interaction with the dimerization domain of Gal4 might contribute to functional potency of XLY came from studies in yeast strains bearing different mutants of Gal11 (Fig. 2). The most revealing result was obtained with Gal4(1–100)/H11001P201 in a yeast strain with Gal11 containing two point mutations (Gal11DM) (Fig. 2H). One of the mutations (N342V) generates a surface that is targeted by Gal4 dd, whereas the second mutation (T322K) abrogates binding to XLY. If in the context of Gal4(1–100)/H11001P201, Gal4dd and XLY each target Gal11 independent of one another, then in the Gal11DM strain one would expect to observe activation levels comparable with those obtained with Gal4dd alone in either the wild type Gal11 or Gal11P background (Fig. 2H).

The rates of dissociation of XLY from Gal11 and Gal4dd were determined by monitoring the change in polarization due to displacement of fluorescently labeled XLY with a 1700-fold excess of unlabeled XLY (Fig. 6). The data thus obtained were fit to a single exponential decay to determine the respective off rates. The data displayed are the average of three individual experiments.
Gal11P or Gal11DM strains. Instead, we observed a significant decrease in activation, suggesting that XLV blocks the interaction between the N342V surface of Gal11DM and Gal4dd. Consistent with this interpretation, the XLV–Gal4dd-binding interaction (5 μM) is qualitatively stronger than that between Gal4dd and Gal11P (∼18 μM by electrophoretic mobility shift assays).

Additional mutagenesis experiments shown in Fig. 5 reveal that the solvent-exposed hydrophobic loop 1 of Gal4dd is the most likely site of the XLV–Gal4dd interaction and, furthermore, that this interaction is essential for the transcriptional activation function of XLV. The mutation of several loop 1 residues abrogates XLV–mediated transcription. Moreover, Gal4dd, in which residue 69 of loop 1 is replaced by an alanine, shows no detectable binding interaction with XLV (see supplemental Fig. 3). Thus, the XLV–Gal4dd interaction is just as essential as the XLV–Gal11-binding interaction for XLV to function as an activator. Consistent with this picture, residues 97–100 of Gal4dd appear conformationally mobile in the NMR structure of Gal4(50–103), and if fully extended, these residues along with the eight residues of P201 would span ∼36 Å, more than sufficient to reach the solvent-exposed hydrophobic loop 1 (Fig. 1B).

These data also provide a clear explanation for how XLV inhibits Gal4dd-mediated activation in yeast strains bearing Gal11DM. Structure-activity studies of Gal4dd employing alanine-scanning mutagenesis as well as binding studies indicate that the hydrophobic residues in loop 1 and the third helix are required for interaction with Gal11P (28). The mutagenesis results of Fig. 5 show that the function of XLV is dependent on several residues of Gal4dd that are also necessary for interaction with Gal11P. The data also demonstrate that although there is significant overlap, not all residues important for XLV function are important for the Gal4dd–Gal11P interaction and vice versa. Thus, Gal4dd interacts with XLV and with Gal11P using two partially overlapping surfaces. More importantly, the results show that interaction with Gal4dd is essential for the ability of XLV to function as a robust activator in vivo. The functional importance of this XLV–Gal4dd interaction is further supported by the observation that a similar peptide (AHYYYPSE) that interacts with Gal11 nearly as well as XLV (23) but does not bind to Gal4dd is not able to activate transcription to the same extent as XLV (Fig. 3F and supplemental Fig. 2). The ~2-fold weaker interaction between XLV and Gal4dd relative to Gal11–XLV (Fig. 3) provides some explanation as to why the XLV–Gal4dd interaction does not inhibit XLV–mediated transcription. In addition, the kinetic data of Fig. 6 are suggestive of a mechanism where rapid interconversion between closed and open states of XLV correlates with inactive and active states of the activator (discussed below). In future experiments, the bound and unbound states of XLV will be characterized in more detail through stopped-flow experiments of the constructs bound to DNA to probe the effects of cooperative DNA binding and interactions between adjacent DNA-bound activators on this process.

By having identified this unexpected XLV–Gal4dd interaction, we are faced with the question as to why this interaction is necessary for XLV to function as a potenter activator. One possible explanation is that the XLV–Gal4dd complex presents XLV in an appropriate conformation for the interaction with Gal11. However, several observations lead us to disfavor this explanation (although further experimentation will be required to rigorously exclude it). Perhaps most important is the transferability of this phenomenon. For example, XLV also functions as a potent transcriptional activator when fused to the DNA binding domain of Pho4 (a helix-loop-helix transcription factor), suggesting that the XLV–Gal4dd complex is not the active component of XLV–mediated transcriptional activation (19). Similarly, we have recently identified several other peptidic activation domains unrelated to XLV that function more strongly (10–100-fold enhancement) when attached to Gal4(1–100) relative to other DNA binding domains; each of these activation peptides interacts with Gal4dd, and some do not require Gal11 to function as transcriptional activators. Furthermore, the hydrophobic residues of XLV that are required for interaction with Gal11 (tyrosine, for example) are also required for XLV to bind to Gal4dd. The simultaneous participation of these residues in interactions with Gal4dd and Gal11, which would be required if the closed complex is the transcriptionally active form, is unlikely.

Taken together, the in vivo activation data and the kinetic and thermodynamic measurements are more consistent with a model in which XLV when bound to Gal4dd is inactive and unable to interact with Gal11 (Fig. 7). We propose that it is only in the open or exposed state that the peptide is functionally active, interacting with Gal11, and facilitating the cooperative assembly of the transcriptional machinery at the promoter (Fig. 7).

The concealment and exposure of XLV are similar to a frequently observed feature of natural activation domains in which the hydrophobic residues that are vital for activator function are masked by either intramolecular or intermolecular interactions with other regulatory proteins (41–53). For example, in the structure of the p53 activation domain complexed with the inhibitor protein MDM-2, the hydrophobic residues that are known to play a critical role in activation are found buried in a hydrophobic cleft on the surface of MDM-2 (41, 42). In response to physiological signals, the residues are released, and the activation domain is capable of stimulating the expression of target genes. There are also several examples of intramolecular concealment of activation domains characterized in yeast such as Leu3 and Puc3. In both of these cases, the full-length activator protein is nonfunctional when tested in vivo or in vitro (49–53). In the presence of a specific metabolite or small molecule, however, the activating region is revealed, and the protein stimulates the expression of genes (49–53).

\[ J. K. Lum, C. Y. Majmundar, A. Z. Ansari, and A. K. Mapp, unpublished results. \]
Thus, in these examples another surface within the activator protein has evolutionarily co-evolved to bind and conceal the activating region in the absence of the relevant signal and to expose it when instructed to do so.

One purpose of the concealment or masking of natural activation domains is regulatory, preventing uncontrolled stimulation of the machinery that degrades them (54–56). This model as-
Examining the Potency of an Artificial Transcription Factor

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