Chemistry & Biology, Vol. 12, 313–321, March, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.01.014

Functional Specificity of Artificial Transcriptional Activators

Chinmay Y. Majmudar,[1] Jenifer K. Lum,[2] Lev Prasov,[1] and Anna K. Mapp[1,2,*]
[1]Department of Chemistry
[2]Department of Medicinal Chemistry
University of Michigan
Ann Arbor, Michigan 48109

Summary

Misregulated transcription is linked to many human diseases, and thus artificial transcriptional activators are highly desirable as mechanistic tools and as replacements for their malfunctioning natural counterparts. We previously reported two artificial transcriptional activation domains obtained from synthetic peptide libraries screened for binding to the yeast transcription protein Med15(Gal11). Here we demonstrate that the transcriptional potency of the Med15 ligands is increased through straightforward structural alterations. These artificial activation domains upregulate transcription via specific Med15 binding interactions and do not function in mammalian cells, which lack Med15. This functional specificity stands in contrast to most natural or artificial activation domains that function across all eukaryotic cell types. The results indicate that the screening strategy holds excellent promise for identifying peptide and small molecule transcriptional activators that function by unique mechanisms with advantageous specificity properties.

Introduction

Transcriptional activators play an essential role in gene regulation by recruiting the RNA polymerase II holoenzyme to the genes with which they are associated (Figure 1) [1]. Many human diseases are characterized by aberrant gene transcription patterns linked to malfunctioning transcriptional regulators [2, 3]. For example, in the case of medulloblastoma, one of the most malignant pediatric cancers, the concentration of the transcriptional repressor REST/NRSF is abnormally high, resulting in the suppression of genes critical for proper differentiation of neuronal cells [4, 5]; recent evidence suggests that upregulating the transcription of REST-regulated genes can mitigate the tumorigenic potential of medulloblastoma cells [6, 7]. Thus, artificial transcriptional activators composed of small molecules or proteins have emerged as important tools to better characterize the relationship between aberrant transcription patterns and disease and in the longer term, to define key characteristics of transcription-based therapeutics [8–10].

Natural activators are exquisitely specific in their function, upregulating cognate genes in particular cell types to predetermined levels upon demand [1]. Part of that specificity is derived from one of the two essential components of an activator, a DNA binding domain (DBD) that recognizes cognate DNA sequences (Figure 1B). The other key component is an activation domain (AD) that interacts with a variety of proteins that constitute the transcriptional machinery and dictates the level of gene upregulation [1]. The AD-transcriptional machinery interactions are tightly regulated by signaling pathways because natural ADs exhibit promiscuous binding behavior [11] that leads to uncontrolled transcriptional stimulation. An additional layer of functional specificity is imposed by transcriptional regulatory networks that dictate when and where a given activator is expressed [12, 13], leading to cell-type specific function.

The functional specificity profile of artificial transcriptional activators is much less sophisticated, and the most success has been attained in gene targeting specificity [9, 10, 14]. For example, artificial activators that upregulate predetermined genes have been constructed by the replacement of endogenous DBDs with novel protein DBDs [15] or with synthetic variants such as peptide nucleic acids [16], triplex-forming oligonucleotides [17, 18], and hairpin polyamides [19]. In addition, artificial activators that function only in the presence of a small molecule have been developed and offer some control over the timing of gene activation, thus serving as a substitute for the signaling pathways that regulate natural AD function [20, 21]. As in natural activators, the AD of artificial activators contacts the transcriptional machinery to upregulate transcription. However, it is typically difficult to predict the level of transcriptional stimulation that will be elicited by a given artificial activator due to many additional factors that impact AD function. These factors include the DBD to which the AD is attached, the position of the DNA binding site relative to the gene, the concentration of the AD present at the gene, and the affinity of the AD for the transcriptional machinery [11, 18, 19, 22–27]. Finally, artificial activators that target particular cell types or organisms remain elusive. This lack of specificity can largely be attributed to the activation domains employed in artificial activator construction. These are typically ADs derived from or closely related to natural activators that in the context of an artificial activator operate outside of the endogenous regulatory pathways [9, 15, 25, 28–30]. Thus, the ADs interact with a wide range of protein targets and, as a result, function in all eukaryotic systems [16, 24].

We previously described a strategy for identifying artificial ADs that employs a screen for ligands of an individual transcriptional machinery protein (Figure 2) [31]. The focal protein of that study was Med15(Gal11) [32], a common target of natural ADs that resides in the mediator complex of the yeast transcriptional machinery [33, 34]. Among the ligands identified from two synthetic peptide combinatorial libraries were two 8 residue peptides that function as activation domains when attached to a DBD and have sequence compositions distinct from any known ADs (Figure 2). In this article, we show that the activity of ADs discovered via the

*Correspondence: amapp@umich.edu

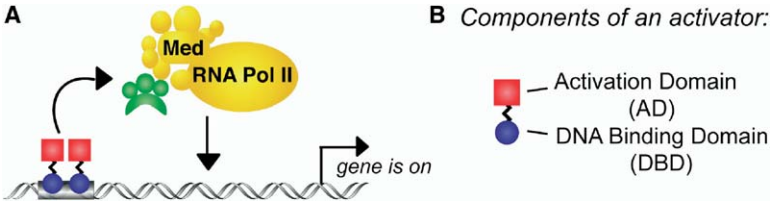

Figure 1. Transcriptional Activation in Eukaryotes

(A) Schematic of transcriptional activation.
(B) The architecture of a transcriptional activator.

screen can be increased by simple modifications such as altering the DNA binding domain to which the AD is attached, consistent with the function of most ADs. However, we also find that the ADs are functionally dependent on the presence of a single protein, the original target Med15. This functional specificity stands in contrast to typical natural or artificial ADs, and, as demonstrated with one of the Med15-dependent ADs, leads to cell-type-specific transcriptional activation. Further, although the experiments described here were carried out using peptide-based activators, the screening strategy is readily extendable to small molecule combinatorial libraries, leading in the future to new classes of small-molecule-based transcriptional activators [35, 36] that function in a cell-type-specific or organism-specific manner.

Results and Discussion

The Med15 ligands were initially evaluated in several functional contexts in order to increase transcriptional potency and facilitate subsequent experiments testing the requirement for Med15. Among the factors that affect the potency of ADs are the DNA binding domain to which the AD is attached and the position of the DNA binding site relative to the gene being upregulated. Both of these factors were first investigated using the more potent of the Med15 ligands, 28 (Figure 2). In addition, artificial transcriptional activators of greater potency were constructed using dimers and trimers of the Med15 ligands as the activation domains.

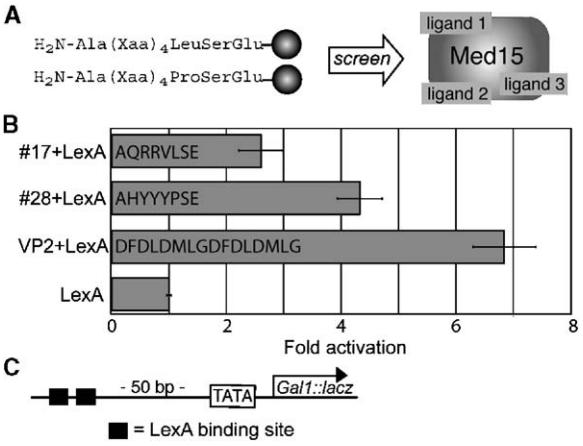

Figure 2. Discovery of Artificial Transcriptional Activators through a Screening Strategy [31]

(A) Two libraries of synthetic peptides were screened for their ability to bind to the transcription factor Med15.
(B) Two of the resulting ligands showed good activity as transcriptional activation domains in *Saccharomyces cerevisiae* compared to a natural AD sequence, VP2, when they were attached to a protein DNA binding domain (LexA).
(C) A schematic of the integrated reporter gene used for the in vivo activation studies. As indicated, the two DNA binding sites for LexA were positioned 50 bp from the TATA box. The transcriptional activity of each LexA fusion protein was measured by assaying β-galactosidase activity.

Exchanging the DNA Binding Domain

Natural transcriptional activators are modular proteins, and activation domains typically function independently of the identity of the DNA binding domain. ADs derived from the potent viral protein VP16, for example, activate transcription efficiently when fused to a wide range of DBDs, including entirely nonnatural versions such as polyamides [22, 25] or triplex forming oligonucleotides [18], although the level of transcription varies. The ability of natural ADs to interact with multiple protein targets may account for this functional flexibility. Although changing the DBD and hence presentation of the AD might affect interactions with a subset of transcription protein targets, it is unlikely to affect all. However, given that our ligands were identified based upon their ability to bind to a single transcription protein, we initially chose a DBD that most closely mimicked the presentation of the ligands in the binding screen. Thus, because the peptides were attached to solid support with the amino terminus free, the selected ligands were fused to the LexA DBD at the amino terminus of the protein (Figure 2).

To test if the Med15 ligands require a particular presentation to function as ADs, we first investigated the function of 28 fused to a commonly used DBD derived from the *S. cerevisiae* protein Gal4, Gal4(1-147). The first 50 residues of Gal4 form a Cys_6-Zn cluster that binds to DNA and the subsequent 50 mediate dimerization through helix-helix interactions that enhance sequence-specific DNA binding [37, 38]. The remaining 47 amino acid residues serve as a linker region of undefined structure between the AD and the DBD. A plasmid was constructed encoding ligand 28 fused to the carboxyl terminus of Gal4(1-147). Thus, ligand 28 is displayed in the opposite orientation (carboxy terminus free) relative to the original LexA fusion. This plasmid and the original 28+LexA construct were each transformed into yeast strains containing two cognate binding sites for either LexA or Gal4 positioned 191 bp upstream of a *lacZ* reporter gene. Activation levels mediated by each of the DBD+28 fusions were then quantitated by measuring β-galactosidase activity [39].

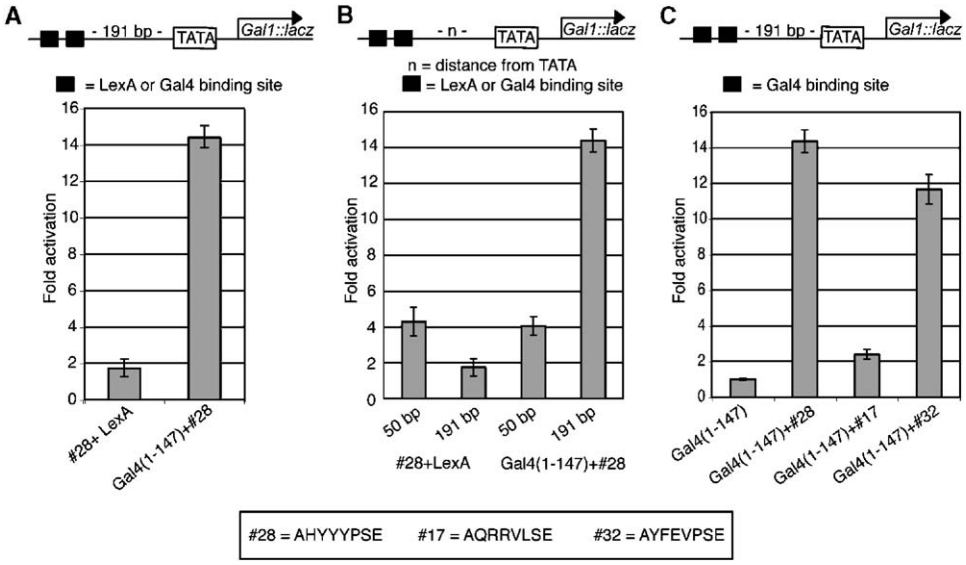

Figure 3. Increasing the Activity of the Med15 Ligands

The transcriptional potency of each artificial transcriptional activator was measured by quantitating β-galactosidase activity using standard methods [39] and fold activation levels were obtained by dividing that activity by that obtained with DNA binding domain alone. Each activity bar represents the average of at least 3 independent experiments with the indicated errors (SDOM). See Table 1 for yeast strain details.

(A) The activity of ligand 28 fused to different DNA binding domains. Note that the position of the DNA binding sites in this yeast strain is different than in the strain in which the results shown in Figure 2 were obtained.

(B) Promoter position-dependence of ligand 28 fused either to LexA (left two bars) or Gal4(1-147) (right two bars).

(C) The activity of 28, 17, and 32 fused to Gal4(1-147).

As shown in Figure 3A, the Gal4(1-147)-based activator was more potent than the 28+LexA fusion protein, with 14.5-fold activation compared to the DBD alone. Thus, the Med15 ligand does not require a free amino terminus to function as an activator despite the original screening conditions. The change in orientation from amino terminus to carboxy terminus is not the only factor contributing to the increase in function. In preliminary experiments, attachment of 28 to the carboxy terminus of the DBD Gal4(1-100) produced an activator approximately 4-fold less active than Gal4(1-147)+28. Thus, the additional linker residues present in the Gal4(1-147)+28 fusion protein likely play a role in the activity increase, projecting the ligand from the DNA more effectively and providing more favorable conditions for the Med15 interaction. A similar reliance upon linkers has been noted with natural transcriptional activation domains such as ATF14 and VP2, both acid-rich ADs derived from the potent viral coactivator VP16 [22, 25], as well as nonnatural activation domains [24]. Future studies of a wider range of DNA binding domains and linkers of varying composition will be employed to quantify this effect.

DNA Binding Site Location

The position of the DNA binding site relative to the transcription start site can also affect how much an activator upregulates a gene [23, 40–42]. Gal4(1-147)+28 was tested in yeast strains bearing 2 binding sites either 50 bp or 191 bp upstream of the TATA box, again using quantitative β-galactosidase assays. As illustrated in Figure 3B, the relationship between potency and binding site position varied with the DNA binding domain employed. For 28+LexA, function was better at a binding site distance of 50 bp from the TATA box, with fold activity dropping by half when moved to 191 bp. The opposite trend was observed with Gal4(1-147) as the DBD. In that case, lower levels of activation were obtained when the binding sites were closer to the TATA box. These latter results parallel the activity of the

Table 1. Yeast Strains Used in This Study

Strain	Genotype	Source
JPY9::pZZ41	Matα *his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4Δ11*::pZZ41[a]	[23]
JPY52::JP185	Matα *his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4Δ11 med15*::LYS2, URA::pJP185[b]	[23]
JPY52::JP188	Matα *his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4Δ11 med15*:LYS2, URA::pJP188[c]	[23]
ZL2	Matα *his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4Δ11 med15*:TRP1, URA::pJP169[d]	[34]

[a] pZZ41 contains two LexA binding sites 50bp upstream of the TATA box.
[b] pJP185 contains two Gal4 binding sites 50bp upstream of the TATA box.
[c] pJP188 contains two Gal4 binding sites 191bp upstream of the TATA box.
[d] pJP169 contains two LexA binding sites 191bp upstream of the TATA box.

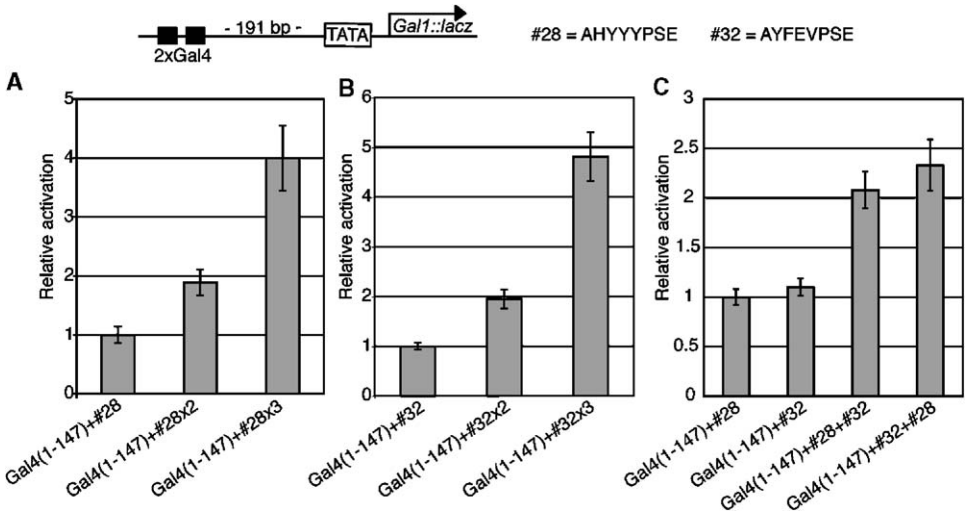

Figure 4. Tuning the Potency of the Artificial Activation Domains

The transcriptional activity of each construct was measured by quantitating β-galactosidase activity in the yeast strain JPY52::JP188 and is reported as a ratio to the activity of Gal4(1-147)+28 or Gal4(1-147)+32 (defined as 1). The activity shown is the average of 3 separate experiments with the indicated error (SDOM). See the Experimental Procedures section for additional details. Multimers containing 4 copies of each peptide were also constructed; however, yeast bearing these plasmids grew poorly and Western blots of the extracts indicated that the tetramer constructs were expressed at very low levels.

(A) The relative fold activation of multimers of 28 on Gal4(1-147).

(B) The relative fold activation of multimers of 32 on Gal4(1-147) DBD.

(C) The relative fold activation of mixed dimeric constructs.

Med15 protein itself when it is employed as an artificial activator. The fusion protein Gal4(1-147)+Med15 is a potent activator at the 191 bp distance; at 50 bp, however, activity drops by half [23]. The results obtained with Gal4(1-147)+28 are suggestive of a mechanism by which 28 specifically recruits Med15 to DNA in an orientation analogous to the positioning in the Gal4 (1-147)+Med15 fusion protein.

Ligands 17 and 32

For ligand 28, the best activity was obtained when Gal4(1-147) was used as a DBD and the DNA binding sites were positioned 191 bp upstream relative to the TATA box. Two additional activating ligands were then tested in this functional context (Figure 3C). One of these is ligand 17, shown in Figure 2. The second is ligand 32 (sequence AYFEVPSE), the next most active ligand identified in the original screen that interacts with Med15 with an affinity similar to 28 and 17 (K_D 1.3 μM versus 4.8 and 2.2 μM, respectively). In addition, the binding site of ligand 28 is distinct from those of 32 and 17 ([31] and Figure S3). Although the sequence of 32 is different from the other two artificial ADs, it bears the most resemblance to the largest class of natural activation domains, the so-called acid-rich ADs that typically contain polar residues interspersed with hydrophobic amino acids [1]. An example of this class of ADs is VP2, a positive control used in the original binding screen and functional assays (Figure 2B). Plasmids encoding either 17 or 32 attached to the carboxyl terminus of the Gal4(1-147) DBD were prepared by standard methods and then transformed into the yeast strains used in earlier experiments. As shown in Figure 3C, the two ligands showed quite different effects. In the case of 17, only 2-fold activity relative to the DBD alone was observed, comparable to the results obtained with the LexA DBD (Figure 2B). Similar results were obtained when 17 was fused to Gal4(1-100); in addition, moving the binding sites closer to the transcriptional start site did not provide an increase in activity (see Figure S1 for details). In contrast, ligand 32 exhibited quite modest activity when fused to the amino terminus of LexA (1.4-fold) but, as shown in Figure 3C, the activity increased to 11.5-fold when it was attached to Gal4(1-147), comparable to the activity of 28. Overall, these experiments provided two artificial activators with improved functional profiles, Gal4(1-147)+28 and Gal4(1-147)+32, and these two activators were used for all further investigations. The results further indicate that the transcriptional activity of ligands obtained from future screening experiments (small molecules or peptides) can be readily improved by straightforward optimization experiments, analogous to typical artificial activation domains.

Activation Domain Multimers

One of the principle mechanisms by which levels of gene transcription can be increased is through augmenting the local concentration of ADs present at a given gene, although the origin of this effect is poorly understood [43–46]. This can be accomplished by increasing the number of activators bound to DNA, leading to transcriptional levels greater than the sum of those observed with the individual activators [44, 46, 47]. Alternatively, the AD itself can be oligomerized within a single transcriptional activator to produce a similar effect [27, 47, 48]. In one example, an artificial

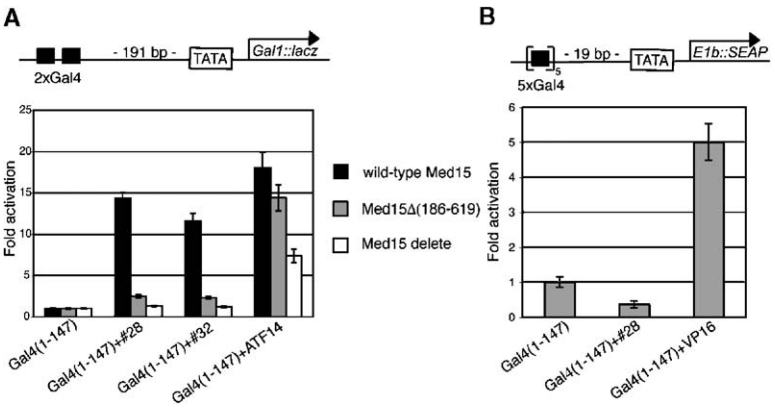

Figure 5. Specificity of Med15 Ligands

(A) The fold activity of 28 and 32 in yeast strains with Med15, Med15Δ(186-619), or with no Med15 present (Med15 delete). Each activity value is the average of three individual experiments with the indicated error (SDOM) See the Experimental Procedures section for additional information.

(B) Fold activation in human embryonic kidney 293 cells. HEK293 cells were transiently transfected with plasmids coding for each construct and SEAP activity was measured using standard methods [59]. See Experimental Procedures for details.

activator containing two copies of a well-studied AD derived from VP16 upregulated transcription 5-fold more effectively than an activator containing a single copy of the AD [48]. From the standpoint of artificial activator design, the latter approach is particularly attractive because it requires a change only in the structure of the artificial transcriptional activator rather than alteration of the regulatory DNA sequences associated with the targeted gene in order to produce greater levels of transcription.

To test if the potency of artificial activators constructed with our Med15 ligands could be further increased by oligomerization, plasmids encoding the Gal(1-147) DBD fused to one, two, or three copies of either peptide 28 (Figure 4A) or peptide 32 (Figure 4B) were constructed such that each peptide sequence was separated from the next by a flexible linker sequence (see Experimental Procedures). In addition, constructs containing a single copy of 28 and 32 separated by the linker sequence were also prepared (Figure 4C). The resulting plasmids were then transformed into the yeast strain used for the experiments shown in Figure 3, and, as before, the activity of each construct was measured as a function of β-galactosidase activity. The results of the experiments are displayed as activation relative to the level obtained with an artificial activator containing a single copy of the ligand (Figure 4). For all of the dimeric constructs, an approximately linear increase in function was observed upon expanding the number of peptide modules from 1 to 2, indicating that this is an effective strategy for tuning potency. Similarly, the trimer of 28 was 3-fold more active than Gal4(1-147)+28. Among all of the constructs, only the trimer of 32 (Figure 4B) showed activity beyond a simple additive increase with greater than 95% confidence (more than three times the level of 32 alone); however, this is still well below the cooperative levels observed with multimers of acid-rich ADs [48]. This was somewhat unexpected because of the three Med15 ligands, 32 bears the greatest sequence resemblance to natural ADs with a balance of polar/acidic and hydrophobic amino acids. Comparable results were obtained when the number of DNA binding sites was increased (data not shown). As illustrated more fully in the following section, the overall lack of cooperative function observed in these experiments may be indicative of a distinct mechanistic difference between the artificial ADs isolated from our binding screen and most natural ADs.

Specificity of Function

Natural activation domains typically interact with a number of transcriptional machinery proteins. The well characterized yeast transcriptional activator Gal4, for example, has more than 10 identified target proteins, although the physiological relevance of all the interactions has yet to be established [11, 26, 49–54]. One consequence is that deletion or mutation of a single transcription protein target rarely leads to complete loss of activator function [34, 53, 55]. Our activator peptides were identified based upon their ability to interact with a single transcriptional machinery protein, however, and perhaps the most compelling question surrounding their function is if the protein target Med15 is required for them to activate transcription. To evaluate this possibility, β-galactosidase assays were carried out in a yeast strain in which Med15 had been deleted from the genome. This experiment is possible because Med15 is not an essential protein, although yeast bearing this alteration exhibit a slow growth phenotype [56]. As shown in Figure 5, we compared the function of Gal4(1-147)+28 and Gal4(1-147)+32 in yeast strains either bearing Med15 (dark bars) or bearing no Med15 (white bars) and noted a nearly complete loss of function. This was in contrast to the positive control, Gal4 (1-147)+ATF14, a sequence taken from the potent viral coactivator VP16, that showed only a 2-fold decrease in activation levels. Although ATF14 is known to interact with Med15, it has several additional putative targets in the transcriptional machinery, and thus its function is attenuated rather than abrogated in the absence of Med15 [57].

This point was further investigated by carrying out the same set of experiments using a yeast strain in which the central region of Med15 (residues 186–619) had been deleted [58]. This mutation minimized the deleterious phenotype of the Med15 delete strain and enabled us to test if the binding sites for ligands 28 and 32 were in this region because the original binding screen was carried out with this fragment. Gratifyingly, nearly identical results were obtained, with 28 and 32 showing little or no activity in this strain while the fold activation of ATF14 was similar to the strain with Med15 present

(light bars). Taken together, these data suggest that both 28 and 32 are dependent upon a binding interaction with Med15 for transcriptional activation to occur.

One interesting feature of acid-rich ADs such as Gal4 or VP16 is that they function in all eukaryotes tested, from yeast through humans [60–62]. Despite differences in RNA polymerase II holoenzyme composition, there is evidently significant conservation across species with regard to activator targets. It has been challenging, however, to identify metazoan homologs of Med 15 [33]. Recently, compelling evidence for homology between the amino terminus of Med15 and the mammalian protein ARC105 was reported; both proteins contain a so-called GACKIX domain often found in targets of activators [63]. The significance of this similarity has yet to be determined, however, because the amino terminus of Med15 can be removed and overall function is maintained [34]. In addition, the two proteins have sequence similarities in the carboxy terminal region, including a glutamine-rich stretch of amino acids. However, the region of Med15 used in our original binding screen (residues 186–619) shares little sequence similarity with ARC105 or any other identified metazoan protein, and we thus anticipated that activators that function through interaction with this region would not be able to function in mammalian cells. To test this idea, a plasmid encoding the most active of the peptides (28) was transiently transfected into human embryonic kidney cells (HEK293 cells) along with a reporter plasmid bearing five Gal4 binding sites within an E1b promoter upstream of a SEAP reporter gene following standard protocols. As a positive control, we also examined the activity of a VP16-derived activation domain fused to Gal4(1-147), known to function well in this system. As indicated in Figure 5B, no activation by ligand 28 was observed while the VP16-derived AD functioned well in this context. This data reinforces the earlier results indicating that 28 is dependent upon Med15 for function, and further, indicates that the ligand screening strategy can be used to identify artificial ADs that are specific for a particular cell type, depending on the target protein. Given the emerging role of cell-type-specific transcription factors and factors expressed only at certain points in development (for example, see [64, 65]), ligands for those proteins will be particularly valuable for functionally specific artificial activator construction.

Significance

The results presented here indicate that artificial activation domains discovered through a binding screen differ from typical natural or artificial ADs in several key respects. Similar to natural ADs, the potency of artificial activators constructed from the Med15 ligands can be readily increased by simply reiterating the AD sequences within the construct. In contrast, a strong synergistic increase in transcriptional levels is not observed. This is most likely related to the functional specificity of the ADs, as subsequent experiments revealed that for at least two of the artificial ADs, Med15 is required for transcription function. In the future, artificial transcriptional activators constructed with the Med15-specific ligands used in combination with ligands targeting other individual transcriptional machinery proteins will thus be outstanding tools for probing the mechanistic origins of transcriptional synergy. Further, since the screening strategy provides activation domains that function through binding interactions with individual transcriptional machinery proteins, targeting other cell-type-specific or organism-specific proteins provides a mechanism for the creation of artificial activators whose functional specificity extends beyond that imposed by the DNA binding domain. Finally, as the screening strategy is equally applicable to combinatorial libraries of small molecules, these results provide a framework for building tunable, uniquely specific small molecule transcriptional regulators.

Experimental Procedures

General Methods

Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs and used as recommended. Oligonucleotides were obtained from Invitrogen. The plasmid YCplac111 and all of the yeast strains used to test the activity of our activator constructs were generously provided by Dr. A. Ansari (University of Wisconsin) [66]. The plasmids pGBKT7, pG5SEAP, pM, and pM3-VP16 were obtained from BD Biosciences. The human embryonic kidney 293 cells used for testing the activity of 28 were purchased from the American Type Culture Collection (ATCC) and maintained as recommended. The QuikChange Site-Directed Mutagenesis Kit used to generate the YCplac111 Med15Δ(186-619) plasmid was purchased from Stratagene. All other chemicals and supplies were purchased from Fisher unless otherwise noted. All techniques used for yeast manipulations were carried out in accordance with standard protocols [39]. All other general molecular biology techniques were carried out as described [67].

Plasmid Construction

17+LexA, 28+LexA, and 32+LexA

Plasmids encoding 17+LexA, 28+LexA, and 32+LexA were generated from pNLexA (Origene) as previously described [31].

Gal4(1-147)+17, Gal4(1-147)+28, and Gal4(1-147)+32

Plasmids encoding Gal4(1-147)+17, Gal4(1-147)+28, and Gal4 (1-147)+32 were generated from pGBKT7 by first annealing oligonucleotides encoding each peptide ligand, (5′-AA TTC GGT TCT GGT GGT TCT GGT NNN NNN NNN NNN NNN NNN NNN TAA-3′ and 5′-TCGA NNN NNN NNN NNN NNN NNN NNN NNN ACC AGA ACC ACC AGA ACCG-3′ or 5′-AA TTC TGT GGT TCT GAT GCT TTG GAT GAT TTT GAT TTG GAT ATG TTG TAA-3′ and 5′-TC GA TTA CAA CAT ATC CAA ATC AAA ATC ATC CAA AGC ATC AGA ACC ACA G-3′) resulting in sticky ends corresponding to the restriction sites EcoRI/SalI. The duplex oligonucleotides were phosphorylated with T4 polynucleotide kinase and ligated with T4 DNA ligase into pGBKT7 predigested with EcoRI/SalI. The resulting plasmids were amplified in DH5α *E. coli* (Invitrogen), selected on LB-agar plates containing 50 μg/ml kanamycin, and isolated from cultures using a QIAprep Spin Miniprep Kit (Qiagen). The sequences of the isolated plasmids were verified by sequencing at the University of Michigan Core Facility.

pM+28

For use in the human cell experiments, a plasmid encoding Gal4 (1-147)+28 was generated from pM by ligation of an oligonucleotide pair encoding peptide 28 (5′-AA TTC GGT TCT GGT GGT TCT GGT GCT CAT TAT TAT TAT CCA TCT GAA TAA-3′ and 5′-TCGA TTA TTC AGA TGG ATA ATA ATA ATG AGC AGA ACC ACC AGA ACCG-3′) into pM that had been predigested with EcoRI/SalI. The resulting plasmid was amplified in DH5α *E. coli* (Invitrogen), selected on LB-agar plates containing 0.1 mg/ml ampicillin and isolated from cultures using a QIAprep Spin Miniprep Kit (Qiagen). The sequence

of the isolated plasmids was verified by sequencing at the University of Michigan Core Facility.

Gal4(1-147)+28x2, Gal4(1-147)+32x2, Gal4(1-147)+28+32, and Gal4(1-147)+32+28

To construct plasmids encoding the Gal4(1-147)+28x2, Gal4 (1-147)+32x2, Gal4(1-147)+28+32, and Gal4(1-147)+32+28 fusions, two pairs of oligonucleotides coding for each of the peptides and linkers were designed and separately annealed. The annealed oligonucleotides for either peptide 28 or peptide 32 (5′-AATTT GGT TCT GGT GGT TCT GGT NNN NNN NNN NNN NNN NNN NNN GGT ACC G-3′ and 5′-GATCC GGTACC NNN NNN NNN NNN NNN NNN NNN NNN ACC AGA ACC ACC AGA ACC A-3′) contained sticky ends complementary to EcoRI/BamHI sites and an additional KpnI site upstream of the BamHI sticky end. The second set of annealed oligonucleotides for peptide 28 or peptide 32 (5′-GATCC GGT TCT GGT GGT TCT GGT NNN NNN NNN NNN NNN NNN NNN TAA G-3′ and 5′-TCGAC TTA NNN NNN NNN NNN NNN NNN NNN NNN ACC AGA ACC ACC AGA ACCG-3′) contained sticky ends complementary to BamHI/SalI sites. The two sets of annealed oligonucleotides were 5′ phosphorylated using T4 polynucleotide kinase. Both pairs of annealed oligonucleotides were then ligated simultaneously with T4 DNA ligase into EcoRI/SalI digested pGBKT7. The resulting plasmids were amplified in SMART *E. coli* cells (Gene Therapy Systems), selected on LB-agar plates containing 50 μg/ml kanamycin, and isolated from cultures using a QIAprep Spin Miniprep Kit (Qiagen). The sequences of the isolated plasmids were verified by sequencing at the University of Michigan Core Facility.

Gal4(1-147)+28x3 and Gal4(1-147)+32x3

To construct plasmids encoding Gal4(1-147)+28x3 and Gal4 (1-147)+32x3, the respective Gal4(1-147) dimer construct, either Gal4(1-147)+28x2 or Gal4(1-147)+32x2 was sequentially digested with KpnI and BamHI. Oligonucleotides corresponding to 28 or 32 (5′-T GGT TCT GGT GGT TCT GGT NNN NNN NNN NNN NNN NNN NNN TTC G-3′ and 5′-GATCC GAA NNN NNN NNN NNN NNN NNN NNN ACC AGA ACC ACC AGA ACC AGTAC-3′) were annealed, resulting in sticky ends complementary to KpnI/BamHI sites and forming an EcoRI site upstream of the BamHI site. These annealed oligonucleotides were 5′ phosphorylated using T4 polynucleotide kinase and then ligated with T4 DNA ligase into KpnI/BamHI digested Gal4(1-147)+28x2 or Gal4(1-147)+32x2, amplified in SMART *E. coli* cells (Gene Therapy Systems), isolated from cultures using a QIAprep Spin, Miniprep Kit (Qiagen), and sequenced at the University of Michigan Core Facility.

Med15Δ(186-619)

The YCplac111+Med15Δ(186-619) plasmid was generated from the parent YCplac111 full-length Med15 plasmid using site-directed mutagenesis. Briefly, two sets of oligonucleotides were designed to insert XhoI restriction sites either after nucleotide 558 (last nucleotide in codon for amino acid 186) or before nucleotide 1858 (first nucleotide in amino acid 620 of Med15). The first set, (5′-CAA TTA CTG CAA AGA ATT CTC GAG CCT AAC ATT CCA CCC-3′ and 5′-GGG TGG AAT GTT AGG CTC GAG AAT TCT TTG CAG TAA TTG-3′) with homology to the region surrounding nucleotide 558, was used to amplify the parent YCplac111 plasmid that encodes full-length Med15. The methylated parent plasmid was then digested with DpnI and the nicked mutagenized plasmid was amplified in SMART *E. coli* cells (Gene Therapy Systems), selected on LB-agar plates containing 0.1 mg/ml ampicillin and isolated from cultures using a QIAprep Spin Miniprep Kit (Qiagen). This modified plasmid was then subjected to the same mutagenesis procedure using the second set of oligonucleotides, (5′-GGG AAA GTA TGA GAA TTC TCG AGC AAA TTT TAA GAA GAC-3′ and 5′-GTC TTC TTA AAA TTT GCT CGA GAA TTC TCA TAC TTT CCC-3′) designed to insert an XhoI restriction site upstream of nucleotide 1858 of Med15. After insertion of both of the XhoI restriction sites, the amplified plasmid was digested with XhoI then gel purified and the resulting YCplac111+Med15Δ(186-619) plasmid was religated with T4 DNA ligase, amplified in SMART *E. coli* cells and selected on LB-agar plates containing 0.1 mg/ml ampicillin. The new YCplac111+Med15Δ(186-619) plasmid was subsequently isolated using a QIAprep Spin Miniprep Kit (Qiagen) and the sequence was verified at the University of Michigan Core Facility.

β-Galactosidase Assays

The function of the ligand-DBD fusions were examined in yeast by a quantitative liquid β-galactosidase assay in accordance with established methods [39]. Briefly, the plasmids encoding the ligand+DBD fusions and the DBD plasmid (negative control) were transformed into yeast using the LiOAc method or by electroporation, and transformed colonies were selected by growth on synthetic complete (SC) media containing 2% raffinose and lacking the appropriate amino acid(s) for selection. Freshly transformed colonies were used to inoculate 5 ml cultures of SC media containing 2% raffinose and lacking the appropriate amino acids. The cultures were incubated overnight at 30°C with agitation. Following incubation, these cultures were used to inoculate 5 ml cultures of SC media containing 2% raffinose, 2% galactose and lacking the appropriate amino acids that were subsequently incubated overnight at 30°C with agitation to an OD_{660} of 0.6–0.9. The yeast cells were harvested and resuspended in breaking buffer (100 mM Tris-HCl (pH 8.0), 20% glycerol) containing the Complete Protease Inhibitors cocktail (Roche). The cells were lysed by vortexing with glass beads. A portion of the cell extract was used to measure β-galactosidase activity via incubation with *o*-nitrophenyl-β-D-galactopyranoside (1 mg/ml) in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4 \bullet 7H_2O$, and 50 mM 2-mercaptoethanol [pH 7]). The reaction was stopped by adding 1 M Na_2CO_3 and the OD_{420} was measured on a Varian Cary 300 UV-vis spectrometer. The activity reported was normalized to the total protein concentration of the extract, measured using a Bradford assay kit (Bio-Rad) with BSA as the standard. Western blot analysis was conducted on each reaction to confirm appropriate expression of each of the constructs.

Secreted Alkaline Phosphatase Assay

The function of Gal4(1-147)+28 was examined in human cells using a quantitative secreted alkaline phosphatase (SEAP) assay in accordance with standard protocols [59]. For this purpose, 4 μg of plasmid encoding Gal4(1-147)+28, the Gal4(1-147) plasmid (pM, negative control), or the Gal4(1-147)+ VP16(411-455) fusion (pM3-VP16, positive control) were transiently transfected into an equal number of human embryonic kidney 293 cells (ATCC) using the PolyFect reagent (Qiagen) according to the manufacturer's protocol. Each transfection reaction also contained 2 μg of the SEAP reporter plasmid pG5SEAP. The transfected cells were cultured at 37°C in a 5% CO_2 incubator in DMEM (Mediatech) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (290 μg/ml) and heat-inactivated FBS (Hyclone). After 72 hr, supernatant from the culture was removed and assayed for SEAP activity. Briefly, 250 μl of the supernatant was heated to 65°C to inactivate any endogenous phosphates, after which it was added to an equal volume of 2× SEAP buffer (1 mM $MgCl_2$, 20 mM L-homoarginine, and 2 M diethanolamine [pH 9.8]) and incubated at 37°C for 10 min. Finally, 20 μl of 20 mM *p*-nitrophenol phosphate in 1× SEAP buffer was added and the OD_{405} was measured at 5 min intervals for 1.5 hr using a plate reader (Molecular Devices). The activity was calculated as the change in light absorbance per minute per sample. The fold activity of Gal4(1-147)+28 and of the positive control were derived by comparison to activity obtained for Gal4(1-147). Each value represents the average of three individual experiments and the error is reported as the standard deviation of the mean.

Immunofluorescence Staining

To verify that the Gal4(1-147) peptide ligand fusions were being expressed and transfected in approximately equal amounts in HEK293 cells, immunofluorescence staining was performed. Briefly, the transiently transfected cells were fixed on glass slides using 2% paraformaldehyde. After multiple washes using blocking buffer (0.05% saponin, 5% BSA, and PBS [pH 7.2]) anti-Gal4 antibody (Covance) was added (1:2000 dilution) and incubated for 2 hr at room temperature. After six 5 min washes, an FITC-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech) was added (1:150 dilution) and incubated for 45 min at room temperature. The slides were then washed with blocking buffer 6 times for 5 min each and Hoechst (Chemicon), a nuclear stain that enables visualization of all cells, was added to the slides. The cells were visual-

ized under a microscope (Leica DM LB connected to Spot RT slider camera, Diagnostic Instruments). The Supplemental Data (Figure S2) shows images of cells transfected with Gal4(1-147), Gal4 (1-147)+28, or Gal4(1-147)+VP16(411-455). The green signal in the images is due to FITC, indicating the cells that express Gal4, while the blue signal is due to the Hoechst stain, showing all the cells. An overlay of both these images shows that >95% of the cells expressed the Gal4 fusion proteins.

Supplemental Data

Supplemental Data for this article is available online at http://www.chembiol.com/cgi/content/full/12/3/313/DC1/.

Acknowledgments

A.K.M is grateful for financial support from the National Institutes of Health (GM65330), the Burroughs Wellcome Fund, the March of Dimes, and the Alfred P. Sloan Foundation. J.K.L. was supported by the Michigan C.B.I. Training Program (NIH GM09597-07). We thank Prof. A. Ansari for yeast strains and helpful discussions. We are grateful to Prof. G. Glick, J. Rush, and A. Boitano for technical assistance with the human cell culture experiments and for a critical reading of this manuscript.

Received: December 9, 2004
Revised: January 19, 2005
Accepted: January 20, 2005
Published: March 25, 2005

References

1. Ptashne, M., and Gann, A. (2001). Genes & Signals (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
2. Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Aksien, L.A., et al. (2000). Molecular portraits of human breast tumours. Nature *406*, 747–752.
3. Duncan, S.A., Navas, M.A., Dufort, D., Rossant, J., and Stoffel, M. (1998). Regulation of a transcription factor network required for differentiation and metabolism. Science *281*, 692–695.
4. Lawinger, P., Venugopal, R., Guo, Z.S., Immaneni, A., Sengupta, D., Lu, W.Y., Rastelli, L., Carneiro, A.M.D., Levin, V., Fuller, G.N., et al. (2000). The neuronal repressor REST/NRSF is an essential regulator in medulloblastoma cells. Nat. Med. *6*, 826–831.
5. Schoenherr, C.J., Paquette, A.J., and Anderson, D.J. (1996). Identification of potential target genes for the neuron-restrictive silencer factor. Proc. Natl. Acad. Sci. USA *93*, 9881–9886.
6. Huang, Y.F., Myers, S.J., and Dingledine, R. (1999). Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. Nat. Neurosci. *2*, 867–872.
7. Immaneni, A., Lawinger, P., Zhao, Z.Y., Lu, W.Y., Rastelli, L., Morris, J.H., and Majumder, S. (2000). REST-VP16 activates multiple neuronal differentiation genes in human NT2 cells. Nucleic Acids Res. *28*, 3403–3410.
8. Pandolfi, P.P. (2001). Transcription therapy for cancer. Oncogene *20*, 3116–3127.
9. Ansari, A.Z., and Mapp, A.K. (2002). Modular design of artificial transcription factors. Curr. Opin. Chem. Biol. *6*, 765–772.
10. Gottesfeld, J.M., Turner, J.M., and Dervan, P.B. (2000). Chemical approaches to control gene expression. Gene Expr. *9*, 77–91.
11. Melcher, K. (2000). The strength of acidic activation domains correlates with their affinity for both transcriptional and non-transcriptional proteins. J. Mol. Biol. *301*, 1097–1112.
12. Shi, Y.G., and Massague, J. (2003). Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell *113*, 685–700.
13. Levy, D.E., and Darnell, J.E. (2002). STATs: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. *3*, 651–662.
14. Faria, M., and Giovannangeli, C. (2001). Triplex-forming molecules: from concepts to applications. J. Gene Med. *3*, 299–310.
15. Beerli, R.R., and Barbas, C.F. (2002). Engineering polydactyl zinc-finger transcription factors. Nat. Biotechnol. *20*, 135–141.
16. Liu, B., Han, Y., Ferdous, A., Corey, D.R., and Kodadek, T. (2003). Transcription activation by a PNA-peptide chimera in a mammalian cell extract. Chem. Biol. *10*, 909–916.
17. Kuznetsova, S., Ait-Si-Ali, S., Nagibneva, I., Troalen, F., Le Villain, J.P., Harel-Bellan, A., and Svinarchuk, F. (1999). Gene activation by triplex-forming oligonucleotide coupled to the activating domain of protein VP16. Nucleic Acids Res. *27*, 3995–4000.
18. Stanojevic, D., and Young, R.A. (2002). A highly potent artificial transcription factor. Biochemistry *41*, 7209–7216.
19. Mapp, A.K., Ansari, A.Z., Ptashne, M., and Dervan, P.B. (2000). Activation of gene expression by small molecule transcription factors. Proc. Natl. Acad. Sci. USA *97*, 3930–3935.
20. Lin, Q., Barbas, C.F., and Schultz, P.G. (2003). Small-molecule switches for zinc finger transcription factors. J. Am. Chem. Soc. *125*, 612–613.
21. Belshaw, P.J., Ho, S.N., Crabtree, G.R., and Schreiber, S.L. (1996). Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. Proc. Natl. Acad. Sci. USA *93*, 4604–4607.
22. Arora, P.S., Ansari, A.Z., Best, T.P., Ptashne, M., and Dervan, P.B. (2002). Design of artificial transcriptional activators with rigid poly-L-proline linkers. J. Am. Chem. Soc. *124*, 13067–13071.
23. Gaudreau, L., Keaveney, M., Nevado, J., Zaman, Z., Bryant, G.O., Struhl, K., and Ptashne, M. (1999). Transcriptional activation by artificial recruitment in yeast is influenced by promoter architecture and downstream sequences. Proc. Natl. Acad. Sci. USA *96*, 2668–2673.
24. Han, Y., and Kodadek, T. (2000). Peptides selected to bind the Ga180 repressor are potent transcriptional activation domains in yeast. J. Biol. Chem. *275*, 14979–14984.
25. Ansari, A.Z., Mapp, A.K., Nguyen, D.H., Dervan, P.B., and Ptashne, M. (2001). Towards a minimal motif for artificial transcriptional activators. Chem. Biol. *8*, 583–592.
26. Wu, Y.B., Reece, R.J., and Ptashne, M. (1996). Quantitation of putative activator-target affinities predicts transcriptional activating potentials. EMBO J. *15*, 3951–3963.
27. Natesan, S., Molinari, E., Rivera, V.M., Rickles, R.J., and Gilman, M. (1999). A general strategy to enhance the potency of chimeric transcriptional activators. Proc. Natl. Acad. Sci. USA *96*, 13898–13903.
28. Nyanguile, O., Uesugi, M., Austin, D.J., and Verdine, G.L. (1997). A nonnatural transcriptional coactivator. Proc. Natl. Acad. Sci. USA *94*, 13402–13406.
29. Ma, J., and Ptashne, M. (1987). A new class of yeast transcriptional activators. Cell *51*, 113–119.
30. Giniger, E., and Ptashne, M. (1987). Transcription in yeast activated by a putative amphipathic α-helix linked to a DNA-binding unit. Nature *330*, 670–672.
31. Wu, Z.Q., Belanger, G., Brennan, B.B., Lum, J.K., Minter, A.R., Rowe, S.P., Plachetka, A., Majmudar, C.Y., and Mapp, A.K. (2003). Targeting the transcriptional machinery with unique artificial transcriptional activators. J. Am. Chem. Soc. *125*, 12390–12391.
32. Bourbon, H.M., Aguilera, A., Ansari, A.Z., Asturias, F.J., Berk, A.J., Bjorklund, S., Blackwell, T.K., Borggrefe, T., Carey, M., Carlson, M., et al. (2004). A unified nomenclature for protein subunits of Mediator complexes linking transcriptional regulators to RNA polymerase II. Mol. Cell *14*, 553–557.
33. Myers, L.C., and Kornberg, R.D. (2000). Mediator of transcriptional regulation. Annu. Rev. Biochem. *69*, 729–749.
34. Barberis, A., Pearlberg, J., Simkovich, N., Farrell, S., Reinagel, P., Bamdad, C., Sigal, G., and Ptashne, M. (1995). Contact with a component of the polymerase-II holoenzyme suffices for gene activation. Cell *81*, 359–368.
35. Minter, A.R., Brennan, B.B., and Mapp, A.K. (2004). A small molecule transcriptional activation domain. J. Am. Chem. Soc. *126*, 10504–10505.
36. Kwon, Y., Arndt, H.-D., Mao, Q., Choi, Y., Kawazoe, Y., Dervan, P.B., and Uesugi, M. (2004). Small molecule transcription factor mimic. J. Am. Chem. Soc. *126*, 15940–15941.
37. Marmorstein, R., Carey, M., Ptashne, M., and Harrison, S.C.

(1992). DNA recognition by Gal4—structure of a protein DNA complex. Nature *356*, 408–414.
38. Hidalgo, P., Ansari, A.Z., Schmidt, P., Hare, B., Simkovich, N., Farrell, S., Shin, E.J., Ptashne, M., and Wagner, G. (2001). Recruitment of the transcriptional machinery through GAL11P: structure and interactions of the GAL4 dimerization domain. Genes Dev. *15*, 1007–1020.
39. Burk, D., Dawson, D., and Stearns, T. (2000). Methods in Yeast Genetics,, First Edition. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
40. Kunzler, M., Braus, G.H., Georgiev, O., Seipel, K., and Schaffner, W. (1994). Functional differences between mammalian transcription activation domains at the yeast GAL1 promoter. EMBO J. *13*, 641–645.
41. Seipel, K., Georgiev, O., and Schaffner, W. (1992). Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. EMBO J. *11*, 4961–4968.
42. Ruden, D.M., Ma, J., and Ptashne, M. (1988). No strict alignment is required between a transcriptional activator binding site and the 'TATA box' of a yeast gene. Proc. Natl. Acad. Sci. USA *85*, 4262–4266.
43. Herschlag, D., and Johnson, F.B. (1993). Synergism in transcriptional activation—a kinetic view. Genes Dev. *7*, 173–179.
44. Vashee, S., Willie, J., and Kodadek, T. (1998). Synergistic activation of transcription by physiologically unrelated transcription factors through cooperative DNA-binding. Biochem. Biophys. Res. Commun. *247*, 530–535.
45. Chi, T.H., Lieberman, P., Ellwood, K., and Carey, M. (1995). A general mechanism for transcriptional synergy by eukaryotic activators. Nature *377*, 254–257.
46. Carey, M., Lin, Y.S., Green, M.R., and Ptashne, M. (1990). A mechanism for synergistic activation of a mammalian gene by Gal4 derivatives. Nature *345*, 361–364.
47. Tanaka, M. (1996). Modulation of promoter occupancy by cooperative DNA binding and activation-domain function is a major determinant of transcriptional regulation by activators in vivo. Proc. Natl. Acad. Sci. USA *93*, 4311–4315.
48. Ohashi, Y., Brickman, J.M., Furman, E., Middleton, B., and Carey, M. (1994). Modulating the potency of an activator in a yeast in-vitro transcription system. Mol. Cell. Biol. *14*, 2731–2739.
49. Klein, J., Nolden, M., Sanders, S.L., Kirchner, J., Weil, P.A., and Melcher, K. (2003). Use of a genetically introduced cross-linker to identify interaction sites of acidic activators within native transcription factor IID and SAGA. J. Biol. Chem. *278*, 6779–6786.
50. Ansari, A.Z., Koh, S.S., Zaman, Z., Bongards, C., Lehming, N., Young, R.A., and Ptashne, M. (2002). Transcriptional activating regions target a cyclin-dependent kinase. Proc. Natl. Acad. Sci. USA *99*, 14706–14709.
51. Chang, C., Gonzalez, F., Rothermel, B., Sun, L.P., Johnston, S.A., and Kodadek, T. (2001). The Gal4 activation domain binds Sug2 protein, a proteasome component, in vivo and in vitro. J. Biol. Chem. *276*, 30956–30963.
52. Jeong, C.J., Yang, S.H., Xie, Y.Q., Zhang, L., Johnston, S.A., and Kodadek, T. (2001). Evidence that Gal11 protein is a target of the Gal4 activation domain in the mediator. Biochemistry *40*, 9421–9427.
53. Park, J.M., Kim, H.S., Han, S.J., Hwang, M.S., Lee, Y.C., and Kim, Y.J. (2000). In vivo requirement of activator-specific binding targets of mediator. Mol. Cell. Biol. *20*, 8709–8719.
54. Koh, S.S., Ansari, A.Z., Ptashne, M., and Young, R.A. (1998). An activator target in the RNA polymerase II holoenzyme. Mol. Cell *1*, 895–904.
55. Lu, X.Y., Ansari, A.Z., and Ptashne, M. (2000). An artificial transcriptional activating region with unusual properties. Proc. Natl. Acad. Sci. USA *97*, 1988–1992.
56. Suzuki, Y., Nogi, Y., Abe, A., and Fukasawa, T. (1988). Gal11 protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in Saccharomyces-cerevisiae. Mol. Cell. Biol. *8*, 4991–4999.
57. Balciunas, D., Hallberg, M., Bjorklund, S., and Ronne, H. (2003). Functional interactions within yeast mediator and evidence of differential subunit modifications. J. Biol. Chem. *278*, 3831–3839.
58. Sakurai, H., Kim, Y.J., Ohishi, T., Kornberg, R.D., and Fukasawa, T. (1996). The yeast GAL11 protein binds to the transcription factor IIE through GAL11 regions essential for its in vivo function. Proc. Natl. Acad. Sci. USA *93*, 9488–9492.
59. Cullen, B.R., and Malim, M.H. (1992). Secreted placental phosphatase as a eukaryotic reporter gene. Methods Enzymol. *216*, 362–368.
60. Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). GAL4 activates transcription in *Drosophila*. Nature *332*, 853–856.
61. Kakidani, H., and Ptashne, M. (1988). GAL4 activates gene expression in mammalian cells. Cell *52*, 161–167.
62. Ma, J., Przibilla, E., Hu, J., Bogorad, L., and Ptashne, M. (1988). Yeast activators stimulate plant gene expression. Nature *334*, 631–633.
63. Novatchkova, M., and Eisenhaber, F. (2004). Linking transcriptional mediators via the GACKIX domain super family. Curr. Biol. *14*, R54–R55.
64. Kim, U., Qin, X.F., Gong, S.C., Stevens, S., Luo, Y., Nussenzweig, M., and Roeder, R.G. (1996). The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. Nature *383*, 542–547.
65. Hiller, M.A., Lin, T.Y., Wood, C., and Fuller, M.T. (2001). Developmental regulation of transcription by a tissue-specific TAF homolog. Genes Dev. *15*, 1021–1030.
66. Lu, Z., Ansari, A.Z., Lu, X.Y., Ogirala, A., and Ptashne, M. (2002). A target essential for the activity of a nonacidic yeast transcriptional activator. Proc. Natl. Acad. Sci. USA *99*, 8591–8596.
67. Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning, a Laboratory Manual, 2nd Edition. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).