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Targeting the Transcriptional Machinery with Unique Artificial Transcriptional Activators

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Transcriptional activators play a central role in the regulation of gene expression by controlling access of chromatin-remodeling enzymes and the transcriptional machinery to the genes with which they are associated (Figure 1).[1] The correlation between a growing number of human diseases and misregulation of gene expression has spurred intense efforts toward the development of artificial transcriptional activators that could be used to restore controlled expression of affected genes.[2,3] Activators are modular proteins, minimally containing a DNA binding domain (DBD) that recognizes a specific site in DNA and an activation domain (AD) that interacts with the transcriptional machinery.[3] Artificial activators that target specific genes have been constructed by replacement of endogenous DBDs with novel protein DBDs or with synthetic variants such as peptide nucleic acids, triplex-forming oligonucleotides, and hairpin polyamides.[3,4] In contrast to the diversity of DBDs available, the activation domains incorporated into artificial activators are typically peptides derived from endogenous activators or peptides similar to natural ADs.[3,5,6]

To expand the repertoire and functional diversity of available artificial ADs, we employed a screen to identify ligands for Gal11, an important component of the transcriptional machinery. We describe here two new AD motifs identified from synthetic peptide libraries using this approach that bear little resemblance to endogenous activating domains. The transcriptional levels stimulated by the new ADs and two well-characterized ADs also examined in this study do not correlate with the binding affinity of the ADs for the target protein, Gal11; these findings illustrate that factors outside of binding affinity contribute to the functional potency of transcriptional activators.

It has proven difficult to conclusively identify the transcriptional machinery proteins that activators target to initiate transcription in vivo. This is partially due to the characteristic "stickiness" of many eukaryotic ADs, which at least in vitro interact with a number of proteins that constitute the transcriptional machinery.[1] A common target emerging from studies of transcriptional activators is Mediator, a multiprotein complex proposed to act as a bridge between DNA-bound activators and RNA polymerase II.[7] The overall structure and function of Mediator appears highly conserved from yeast through human, although the identity of the constituent proteins varies.[8] Gal11 is a Mediator component unique to yeast that has been implicated as a target of endogenous activators.[7,9] Particularly relevant for our purposes, when Gal11 is fused to a protein DNA binding domain it activates transcription robustly.[10] It thus seemed likely that ligands for this protein could be used to localize Gal11 to DNA and thereby activate transcription.

Conditions for identifying Gal11 ligands were optimized using two well-characterized ADs known to interact with the central region of the protein, VP2[9,11] and P201[6] (Figure 2). The ADs were attached to solid support and incubated with varying concentrations of a GST-tagged version of Gal11 to identify the lowest protein

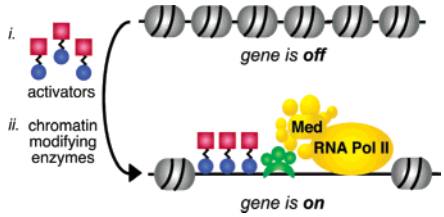

Figure 1. A model of transcription initiation. Activators localize at specific sites on DNA (black line) and participate in the recruitment of chromatin remodeling enzymes that alter the chromatin structure (gray) along with the RNA polymerase II holoenzyme (gold) and general transcription factors (green) to initiate transcription.

a) **VP2 :** DFDLDMLGDFDLDMLG
P201: LTGLFVQDYLLPTCIP

b)

Library 1: AXXXXLSE			Library 2: AXXXXPSE		
1. NITY	9. RARV	17. QRRV	22. SFHR	30. VLGW	
2. ETVS	10. SHRT	18. SLNR	23. FWLF	31. IWLF	
3. RRGV	11. GRRA	19. EDRR	24. NQQW	32. YFEV	
4. TWRR	12. RVYR	20. RWTI	25. DLTM	33. VHPV	
5. HTRH	13. AHRR	21. NLRT	26. FYRN	34. FNWE	
6. RDRT	14. SRHR		27. MHFP	35. FTLW	
7. FARR	15. RQRN		28. HYYY	36. LWFF	
8. QRRT	16. YHRT		29. PVLG	37. IFFF	

Figure 2. (a) Sequences of ADs used as positive controls. (b) Composition of peptide libraries and isolated ligands.

concentration necessary for readily detectable binding: 2.5 μM GST+Gal11(186-619). These conditions were then used to select Gal11 ligands from two eight-residue synthetic peptide libraries, each with four randomized positions for a total of 320 000 total unique sequences (Figure 2) (see Supporting Information for details). The C-terminal residues of Library 1 are similar to sequences often found in eukaryotic ADs, thought to form amphipathic helices upon interaction with the transcriptional machinery.[1,3] Library 2 has a proline at position 3 to bias the screen against the isolation of peptides similar to endogenous activators. From the two libraries, 37 ligands for Gal11 were identified (Figure 2). The majority fall into three categories: amphipathic peptides similar in composition to endogenous activators, hydrophobic peptides, and a number bearing an excess of positive charge. These data suggested that the ligands were targeting at least three different binding surfaces of Gal11.

To test if the Gal11 binding interaction would lead to activator function, a series of plasmids were constructed, each of which encoded a fusion protein consisting of a Gal11 ligand attached to a DNA binding domain (LexA). The plasmids were then transformed into the yeast strain LS41,[12] which bears two LexA binding sites 50 bp upstream of a *lacZ* reporter as part of an inducible Gal1 promoter. In an X-gal plate assay,[13] most ligands functioned as weak activators, but #17 and #28 appeared nearly as active as the positive control, a VP2+LexA fusion. This was surprising as both ligands have sequences unlike any known eukaryotic activators. Quantitative β-galactosidase assays[13] confirmed their activity, with

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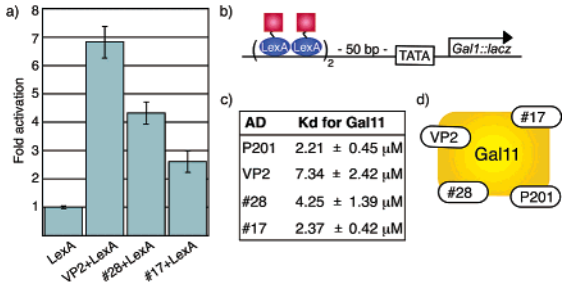

Figure 3. (a) Quantitative β-galactosidase assays with the activity of VP2, #28, and #17 displayed as fold activity relative to the negative control, LexA. Each value represents the average of three individual experiments. (b) Integrated template in the *S. cerevisiae* strain LS41.[12] (c) Dissociation constants for the four ADs. (d) Depiction of the 4 ADs bound to Gal11.

#28 activating transcription 63% as well as VP2 and #17 38% as well as VP2, impressive activity for such small functional units.

The functional potency of an activator is commonly linked to its affinity for the transcriptional machinery.[14] So, for example, because the control activating domain P201[6] is unusually potent, it would be expected to bind to Gal11 more tightly than VP2 or any of the selected Gal11 ligands. On the basis of this, the differences in activation levels obtained with VP2, #17, and #28 could thus be explained by differences in binding affinity for Gal11. To probe this question, the four ADs were synthesized and labeled with fluorescein. Dissociation constants for each with Gal11 were then measured using fluorescence polarization (Figure 3c).[15] Surprisingly, all of the ADs bind to Gal11 with similar K_d values, with even the potent P201 exhibiting a K_d in the micromolar range.

Because each of the four ADs have a unique composition, we hypothesized that the differences in potency could be related to differences in target binding site rather than binding affinity.[16] To test if the activators target unique sites within Gal11, competition experiments were carried out. Each labeled AD was combined with Gal11 such that approximately saturated binding was observed, and unlabeled competitor was then titrated in while changes in binding were monitored by fluorescence polarization. Decreased binding of the labeled activators was observed when unlabeled activator of the same identity was titrated in, as would be expected. However, when any of the other combinations were examined, no decrease in binding was observed (see Supporting Information for details). These data suggest that the four activators each target a unique site (Figure 3d) and, furthermore, that these different sites are likely linked to the alterations in function observed in vivo.

The binding affinities of the Gal11 ligands that did not activate transcription or activated only weakly were also investigated. Six (27, 30, 31, 34, 35, 37) were observed to bind weakly (K_d's > 10 μM), and the remainder bound with affinities comparable to those of the activating peptides (1−10 μM). This suggests that the nonactivating peptides target surfaces of Gal11 that are not accessible in the context of the transcriptional machinery. In addition, these surfaces may be involved in other protein−protein interactions, and thus some of the nonactivating ligands may function as transcription inhibitors in a different context.

In summary, we have identified two artificial transcriptional activating domains of unusual sequence composition. This significantly expands the repertoire of activation domains available for the design of artificial transcriptional regulators. These ADs bind to unique sites within the Mediator complex, and the binding sites appear to be responsible for differences in potency, demonstrating for the first time that binding affinity is not the sole determinant of regulator potency. Finally, because the strategy was developed using synthetic peptide libraries, it can readily be extended to synthetic libraries of nonpeptidic structures, paving the way for the discovery of nonbiopolymer-based activating regions.[17]

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Supporting Information Available: Details of library construction, library screens, fluorescence polarization experiments, and plasmid construction and yeast strain information (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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