Artificial Transcriptional Activation Domains

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Introduction

Transcriptional regulation entails a complex series of cellular signals and protein–protein interactions that precisely direct sets of genes to be turned on, yielding specific phenotypic effects (Figure 1).[1] It is this process that regulates a cell's fate, utilizing the same genomic content to generate unique gene expression profiles and many different cell types. Given the critical nature of this process, it is not surprising that misregulation at any step can result in aberrant cellular function. Often, this misregulation can be traced back to a malfunctioning transcriptional regulator.[2] For example, in 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.[3,4] However, up-regulation of the REST/NRSF-controlled genes abrogates the tumorigenic potential of treated cells.[5,6] Thus there is growing interest in the identification of molecules that can selectively activate the expression of targeted genes, so-called *artificial transcriptional activators* (ATAs). ATAs are outstanding tools for increasing our understanding of the role of aberrant transcription patterns in disease and might provide a future basis for therapeutic intervention.[7]

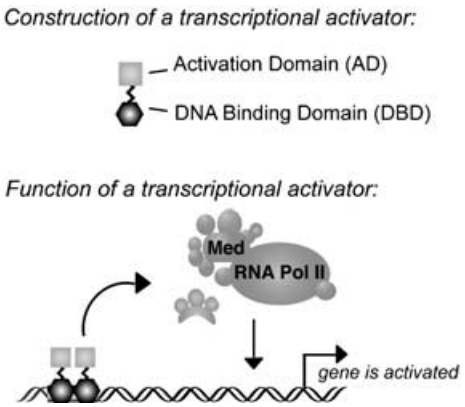

Figure 1. Top: modular domains of a transcriptional activator. Bottom: schematic of the function of a transcriptional activator.

Natural Transcriptional Activators

The endogenous counterparts of ATAs are proteins that are essential contributors to regulated gene expression. Natural transcriptional activators localize to specific sites on DNA in response to extra- or intracellular signals. In addition to binding to DNA, activators interact with a number of proteins associated with RNA polymerase II, recruiting them to the gene of interest, and thus initiating transcription.[1] Activators are modular proteins with two key domains necessary for transcription function: a DNA-binding domain (DBD), which localizes the activator to DNA, and an activation domain (AD), which directly contacts the transcriptional machinery. These two domains can exist within the same protein or assemble into an activator through noncovalent interactions. With these two key domains, transcriptional activators are able to up-regulate selected genes in particular cell types to predetermined levels with exquisite specificity. For example, the yeast activator Gal4 is constitutively present in the cell; however, it binds the transcriptional machinery and induces the *GAL* genes required for galactose metabolism only in the presence of galactose.[1]

There are a number of factors that influence the exquisite gene specificity and tunable functional potency of natural activators. The DBD contributes to the specificity profile by localizing the activator to a particular DNA-binding site. For example, the leucine zipper dimerization domain and the N-terminal basic region of the DBD of the activator Gcn4 permit the recognition of palindromic and pseudopalindromic sites on DNA.[8] In addition, DBDs contribute to overall potency through cooperative DNA binding that can increase the number of activators bound proximal to a gene.[9,10] In contrast, the AD typically contributes little to the specificity of a transcriptional activator but rather dictates the levels of gene up-regulation through binding interactions with transcriptional machinery proteins. The specific AD features that contribute to the functional potency of an activator remain poorly defined. Contributing to this uncertainty, the real protein targets of ADs are largely unknown. For example, Gal4 interacts, at least in vitro, with more than ten proteins in the transcriptional machinery, but it is unclear what percentage of those interactions are functionally relevant.[11–15] In vivo, an elaborate signaling network controls the promiscuous binding behavior of natural ADs such as Gal4 through, for example, dynamic covalent modifications.[16] In addition, many natural ADs have associated repressor proteins that serve to mask their function until the activator is called into action.

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General Design Principles for Artificial Transcriptional Activators

The ideal ATA would exhibit specificity and tunable functional potency to rival that of natural transcriptional regulators. In addition, transportable ATAs that can be employed inside cells and living organisms will be essential for the future development of transcription-based therapeutic agents. On the surface, this is a daunting process, given the functional complexity of natural transcriptional activators and the many mechanistic questions regarding their function that remain. However, the modular organization of natural activators suggests a straightforward approach to generating artificial counterparts: replacement of each of the two key domains with non-natural components. By following this approach, a number of ATAs with unique DNA-targeting properties have been developed by the replacement of the DBD of a natural activator with artificial variants including polyamides, triplex-forming oligonucleotides, peptide nucleic acids, and designer proteins.[7] However, the development of artificial ADs suitable for ATA construction still remains a significant challenge, as most employ ADs taken directly from natural activators. In fact, it was only recently that the first examples of small-molecule ADs were reported.[17,18] In this Minireview, we outline the common approaches taken to identify artificial ADs. This is not a comprehensive treatment of the topic, but rather highlights key challenges in this arena.

Library Selections and Screens

Given the ease with which large libraries of biopolymers can be generated, it is not surprising that most artificial ADs have been discovered through screening of or selection from libraries of molecules. In fact, one of the earliest artificial ADs was isolated from a screen for activator function of random fragments of the *E. coli* genome fused to a DBD.[19] This AD, named B42, shares sequence similarities with the most common class of natural ADs, the so-called acid-rich class. The name of this activator class arises from the typical composition of such activators: hydrophobic amino acids interspersed with polar residues (serine, threonine, glutamic acid, aspartic acid). Examples include the ADs from the viral protein VP16, the yeast activator Gal4, and the human activator c-Myb (Table 1).[1] This seminal experiment revealed that it is at least straightforward to generate peptide-based ADs that resemble natural ADs.

Novel RNA ADs have also been identified in screens. RNA shares several similarities with ADs, including the presence of hydrophobic and acidic functional groups in addition to their ability to form a variety of secondary structures and bind protein and small-molecule targets.[20] The first report of such an AD emerged from a yeast three-hybrid screen developed by the Wickens group to identify novel RNA–protein interactions.[21] Serendipitously, several of their RNA constructs activated transcription in the absence of a protein–AD prey. Subsequently, Ptashne and co-workers screened a RNA hairpin library with a 10-nucleotide variable loop in yeast using a modified version of the yeast three-hybrid system.[22] Their optimized RNA ADs elicited moderate levels of activation, approximately 7–10% of that of the natural activator Gal4. Additionally, a consensus RNA motif was identified (Table 1), unlike similar peptide screens; this suggested that a larger RNA with more conformational freedom might yield more potent activators. Indeed, a study by Buskirk et al. used a similar system to identify longer (40 and 80 nucleotide) RNA ADs with good functional activity.[23] Further characterization revealed a diverse set of potential RNA structures. Additionally, mutagenesis of individual nucleotides in their optimal RNA revealed a critical role of secondary structure in RNA AD function. As mentioned earlier, strict cellular regulation of activator function is critical for normal gene-expression levels and is an important consideration in ATA design. More recently, Liu and co-workers further developed their RNA AD by engineering a ligand-dependent switch to temporally control the structure and, thus, the function of their RNA AD, thereby generating an ATA whose function is regulated at the level of the AD by small-molecule binding.[24]

Table 1. Sequences of activation domains. A) Natural proteins: Short sequences from the viral protein VP16 and the yeast activator Gal4 that mediate activation. B) Biomolecules (peptides or RNA) isolated by screen or selection:[a] KBP 2.20,[25] Gal80BP-A,[26] peptides #28 and #32,[31] and RNA #7.[22] C) Peptide designed to form an amphipathic helix (AH)[46] and function as an artificial activation domain.

	Activation domain	Sequence[b]
A	VP16	^{441}**D**F**D**L**D**M…**D**F**E**F**EQ**^{477}
	Gal4	^{843}**QT**AY**N**AFG…**DD**VY**N**YLF^{869}
B	KBP 2.20	**S**WAVY**E**LLF
	Gal80BP-A	Y**DQD**M**QNNT**F**DD**LFW**KE**G**HR**
	Peptide #28	A**H**YYYP**SE**
	Peptide #32	AYF**E**VP**SE**
	RNA #7	**UGCUGGAUCA**
C	AH	**E**L**QE**L**QE**L**Q**ALL**QQQ**

[a] See text and references for additional information. [b] The side chains of the acids shown in bold are polar, those in normal font are hydrophobic.

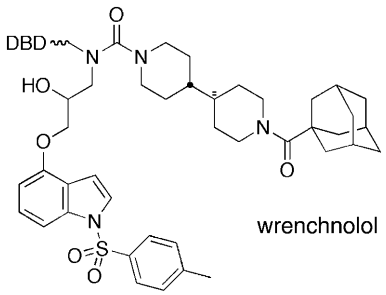
Recently, ligands isolated in screens against particular transcription protein targets have been employed as ADs (Table 1). For instance, Frangioni et al. used phage-display selection to isolate several peptides that bind to the KIX domain of the mammalian co-activators p300 and CREB-binding protein (CBP) with micromolar dissociation constants.[25] When attached to a DBD, these peptides up-regulate transcription efficiently in human cells (ten- to 40-fold relative to background levels). In addition, Kodadek and co-workers have used phage-display selection to identify peptide ligands for the yeast-transcriptional repressor Gal80.[26] These peptides interact with both Gal80 and with a transcriptional machinery protein and, as a result, they function well as ADs when attached to a DBD. In both of these examples, the peptide ADs isolated from the phage-display selections bear a strong resemblance to natural acid-rich ADs and, by all appearances, function in an analogous fashion. For example, Kodadek's Gal80-binding peptides function not just in yeast but also in human cells, thus broadening their applicability.[27] Regardless of their origin, most natural ADs func-

tion in all eukaryotic organisms.[28–30] This probably arises from the earlier-discussed ability of acid-rich ADs, such as Gal4, to interact with a variety of protein partners. In addition, key mechanistic aspects of activator function are conserved across eukaryotic systems. Thus, the functional specificity of ATAs constructed with these peptides exclusively arises from the DBD.

Artificial peptidic ADs that have little sequence similarity to natural ADs have been successfully isolated from binding screens of synthetic peptide libraries.[31] In our laboratory, several such ADs were isolated from a screen of two eight-residue synthetic peptide libraries for binding to an important component of the yeast transcriptional machinery, Med15(Gal11).[31,32] Unlike natural ADs, which interact with many transcriptional machinery proteins, some of these ADs appear to function by targeting a single protein: the original screening target Med15. So, for example, in yeast strains in which Med15 is not expressed or in which the binding site of the AD peptides has been removed from the protein, the ADs show no transcription function.[33] This stands in contrast to natural ADs, which activate transcription in both of those contexts.

The Med15-dependent function of the artificial ADs isolated from the binding screen has an additional interesting implication for ATAs constructed from the Med15 ligands. Unlike many transcriptional machinery proteins, Med15 has no direct homologue in metazoan systems.[32] The closest relative identified thus far is ARC105, with which it shares sequence homology at the amino and carboxy termini of the protein.[34] However, the portion of Med15 that contains the artificial AD binding sites has no homology with ARC105 or any other metazoan protein. Thus, an ATA containing the Med15 ligand #28 (Table 1) as the AD *does not* activate transcription in human cells. So, in this case, an additional level of functional specificity is imposed by the AD, and this leads to the development of a cell-type-specific ATA. Given the emerging role of cell-type-specific transcription factors and factors expressed only at certain points in development (for example see refs. [35, 36]), ligands for those proteins will be particularly valuable for functionally specific artificial activator construction. In addition, screening strategies such as this one can be carried out with synthetic combinatorial libraries of small molecules and thus provide small-molecule ADs with desirable and unique specificity properties.

A screening approach combined with design has been used to develop one of the two examples of a small-molecule transcriptional AD. Small molecules that disrupt the binding interaction between ESX (epithelial-specific transcription factor), a transcriptional activator, and Sur2, one of its protein binding partners, were identified from a screen of a combinatorial library of molecules by Asada et al.[37] This is a particularly interesting interaction to inhibit, since the ESX–Sur2 interaction is known to mediate overexpression of Her2 in malignant breast cancer cells.[38] The initial molecules from the library screen were further refined based upon a solution structure of the ESX activation domain obtained by the authors. This culminated in the identification of the molecule named "wrenchnolol" that effectively inhibits the ESX–Sur2 interaction with an IC_{50} of 6.9 μM in human cells (Scheme 1).[39] This inhibitory behavior is



Scheme 1. The small-molecule activation domain wrenchnolol, which has been shown to activate transcription when attached to a polyamide.[18]

analogous to that exhibited by natural ADs when they are unattached to a DBD and thus not localized to DNA.[40] Not surprisingly, wrenchnolol functions as a transcriptional AD when it is attached to a DBD, a hairpin polyamide.[18] This entirely artificial transcriptional activator upregulates transcription 3.5-fold relative to background in an in vitro system.

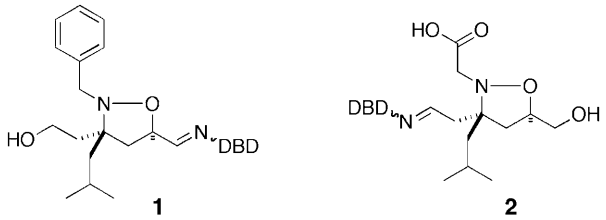
In addition to wrenchnolol, several small-molecule inhibitors of AD–protein target interactions have been described. For example, Vassilev and co-workers recently provided evidence that a series of *cis*-imidazoline analogues, termed the Nutlins, target the p53 activator-binding pocket of MDM2 with submicromolar affinity, thus increasing cellular p53 levels and inducing antiproliferative and apoptotic effects.[41] Attachment of such molecules to DBDs might provide future interesting classes of ATAs.

Design

As outlined in the previous section, the most common class of natural ADs is composed of a combination of polar and hydrophobic amino acid residues. Several lines of evidence suggest that ADs of this class are unstructured in solution but form amphipathic helices upon binding to their target protein in the transcriptional machinery, although the importance of other secondary structural features cannot be ruled out.[42–45] In the late 1980s, Giniger and Ptashne proposed that a peptide designed to form an amphipathic helix should also function as a transcriptional AD. To test this idea, they designed a 20-residue sequence containing a core repeat of glutamine, glutamic acid, and leucine as the amphipathic sequence and fused this sequence to a protein DBD.[46] As predicted, the peptide (named AH for amphipathic helix) functioned as an AD in a cellular system. Furthermore, an ATA containing AH functioned ~20% as well as the potent natural activator Gal4, an impressive level of function for a relatively small peptide. Emphasizing the mechanistic similarities among eukaryotes, AH-containing ATAs function well in yeast as well as in higher eukaryotes.[47] Although the structure of AH when bound to a protein target has not been determined, a peptide containing the AH sequence in a different order does not function as a transcriptional AD; this suggests that secondary structure is an important component of AH function.

Several lines of evidence suggest that reconstituting the exact positioning of the polar and hydrophobic functional groups in an amphipathic helix is not required for AD function. For example, there is little direct sequence homology among the acid-rich activator class; rather, they share the general characteristic of hydrophobic amino acids interspersed with polar residues. Despite the lack of homology, many of the ADs share common protein targets in the transcriptional machinery. For example, Tra1, a member of the chromatin-modifying complexes SAGA and NuA4 in yeast, has been identified as a target of the ADs of at least three natural activators—Gcn4, Gal4, and Hap4—in addition to the viral coactivator VP16.[48] Given that most ADs interact with many transcriptional machinery proteins, it also appears likely that there are a number of similar, permissive binding sites for activators within the transcriptional machinery. Further, relatively small peptides can be used to target those binding sites since sequences as short as 6–8 amino acid residues function as transcriptional ADs.[10,49]

Our laboratory recently took advantage of the lack of specificity in AD-transcriptional machinery interactions to discover the first example of a small-molecule transcriptional AD.[17] In this study, five different combinations of polar and hydrophobic functional groups were grafted onto a five-membered heterocyclic scaffold that provided conformational rigidity. The resulting isoxazolidines ranged from very hydrophobic (three hydrophobic functional groups) to very polar (two polar functional groups, one hydrophobic). (Scheme 2) The goal was to create a radial array of functional groups that very generally mimics that of natural acid-rich ADs. Attachment to a DBD and testing in vitro revealed that three of the five molecules showed excellent activity, with isoxazolidine **1** functioning as well as the well-characterized natural AD ATF14; typically 5–7-fold levels of transcriptional activation were observed with an ATA containing **1** relative to background levels. This is remarkable given the size differential (**1**: 290 Da, ATF14: 1674 Da) and can be at least partially attributed to the stability of the isoxazolidine ring to proteolytic degradation under the assay conditions. Similarly to natural ADs, a balance of polarity and hydrophobicity is the key to the function; for example, isoxazolidine **2**, in which the benzyl group of **1** has been replaced with a carboxylic acid, shows little or no transcriptional activation function. Although a detailed mechanistic study of this AD class has not yet been carried out, the results thus far suggest that other conformationally constrained scaffolds containing similar functional groups should also function as ADs. This provides a straightforward mechanism for the discovery of future classes of small-molecule-based artificial transcriptional activators.



Scheme 2. Isoxazolidine activation domains **1** and **2** were designed by Minter et al. to mimic natural activation domains.[17] Isoxazolidine **1** was found to activate transcription, but isoxazolidine **2** was not.

Future Challenges

One of the greatest remaining challenges in ATA design is the discovery of ADs that confer potency comparable to that of natural ADs in cellular systems. Currently, there is only a single example of a truly potent AD in vivo, a hydrophobic peptide AD named P201 that functions as well as the powerful natural activator Gal4.[50,51] Part of the challenge might lie in the poor cellular stability of typical biopolymer-based ADs. These molecules have many of the same characteristics of natural ADs, such as promiscuous binding, but operate outside of the endogenous regulatory system that controls the binding behavior of natural ADs. Thus, artificial ADs are more prone to premature degradation and unproductive binding interactions that limit their overall effectiveness. One mechanism for increasing the lifetime of ATAs is to use non-biopolymer-based systems as both the AD and the DBD. Future studies of the current suite of small-molecule ADs and the development of additional classes of these molecules will certainly be invaluable for this effort. A second approach is to discover artificial ADs that interface with the endogenous regulatory system in addition to targeting the transcriptional machinery. This could have the added advantage of conferring temporal control over gene up-regulation mediated by an ATA. Given the exciting challenges that lie ahead in this arena, the successful development of fully functional artificial transcriptional activators will require the combined efforts of the chemical, biological, and medical communities in the coming years and will provide a fertile ground for scientific investigation.

Keywords: artificial transcriptional activator · gene expression · gene therapy · peptides · small-molecule activation domain

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