

Research Paper

Towards a minimal motif for artificial transcriptional activators

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Received 24 January 2001; revisions requested 9 March 2001; revisions received 30 March 2001; accepted 20 April 2001

First published online 8 May 2001

Abstract

Background: Most transcriptional activators minimally comprise two functional modules, one for DNA binding and the other for activation. Several activators also bear an oligomerization region and bind DNA as dimers or higher order oligomers. In a previous study we substituted these domains of a protein activator with synthetic counterparts [Mapp et al., *Proc. Natl. Acad. Sci. USA* 97 (2000) 3930–3935]. An artificial transcriptional activator, 4.2 kDa in size, comprised of a DNA binding hairpin polyamide tethered to a 20 residue activating peptide (AH) was shown to stimulate promoter specific transcription [Mapp et al., *Proc. Natl. Acad. Sci. USA* 97 (2000) 3930–3935]. The question arises as to the general nature and the versatility of this minimal activator motif and whether smaller ligands can be designed which maintain potent activation function.

Results: Here we have replaced the 20 amino acid AH peptide with eight or 16 residues derived from the activation domain of the potent viral activator VP16. The 16 residue activation module coupled to the polyamide activated transcription over two-fold better than the analogous AH conjugate. Altering the site of attachment of the activation module on the polyamide allowed

reduction of the intervening linker from 36 atoms to eight without significant diminution of the activation potential. In this study we also exchanged the polyamide to target a different sequence without compromising the activation function further demonstrating the generality of this design.

Conclusions: The polyamide activator conjugates described here represent a class of DNA binding ligands which are tethered to a second functional moiety, viz. an activation domain, that recruits elements of the endogenous transcriptional machinery. Our results define the minimal structural elements required to construct artificial, small molecule activators. If such activators are cell-permeable and can be targeted to designated sites in the genome, this series of conjugates may then serve as a tool to study mechanistic aspects of transcriptional regulation and eventually to modulate gene expression relevant to human diseases. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Small molecule activator; Hairpin polyamide; Chemical regulator; Targeted gene control

1. Introduction

Positive regulation (activation) of gene expression requires factors called transcriptional activators. An economical ‘recruitment’ model posits that activator proteins bind to DNA and recruit the transcriptional machinery to a proximal promoter, thereby stimulating gene expression [2]. These steps define the initial regulatory decisions in a transcriptional circuit, and misregulation at any stage can

result in a variety of human diseases. Given the critical role of activators in transcriptional regulation, the development of artificial counterparts that could be used to rectify errors in gene expression has been a long-standing goal at the interface of chemistry and biology. Activators achieve specificity in targeting genes by a DNA recognition module which binds to cognate DNA sequences near a promoter and in most cases binding specificity is further enhanced by dimerization [1,2]. The key functional module, the activating region, is thought to bind several components of the transcriptional machinery ([2,3] and references therein). Many of these components of the machinery exist in large multi-subunit complexes which associate with the RNA polymerase II, and are known as the RNA polymerase II holoenzyme [4,5]. The holoen-

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zyme along with a few additional factors, like TBP, constitutes the transcriptional machinery that is recruited by activators to most promoters *in vivo* [6]. It is believed that weak interactions between an activating region and several components of the holoenzyme result in high avidity ‘multi-dentate’ binding by the activator to the holoenzyme. In addition, acidic activating regions (e.g. those used here) are believed to contact and recruit nucleosome modifying activities to promoters [7,8].

1.1. Artificial transcriptional activators

Attempts to generate artificial activators have relied on this principle of recruitment. In one example, a dimer of the natural product FK506 was used to couple a chimeric DNA binding protein to an activating region, thus up-regulating a gene bearing upstream cognate binding sites [9–11]. More recently attachment of an activating region to a triplex forming oligonucleotide or designed Zn(II) finger motifs was shown to up-regulate gene expression [12,13]. Our approach towards creating a general synthetic motif for artificial activators that are capable of targeting a wide variety of DNA sequences relies upon a versatile non-protein DNA binding motif, namely the polyamides which are composed of heterocyclic residues [14]. Obeying a set of pairing rules, the polyamides bind to pre-determined DNA sequences in the minor groove with affinities and specificities comparable to typical DNA binding proteins [14–16]. Finally, polyamides are cell-permeable and can mediate their regulatory effects on gene expression in primary cells and even *Drosophila* larvae [17–19].

In our previous studies, we substituted key functional domains of naturally occurring activator proteins with synthetic counterparts to create an entirely synthetic transcriptional activator [1]. One focus of that study was to test the importance of dimerization of activators in their ability to activate transcription. In that study a flexible polyether linker (the linker domain) replaced the typical protein dimerization domain and it served to tether the designed activating region AH to a hairpin polyamide that binds the cognate sequence 5'-TGTTAT-3' (Fig. 1). This created our smallest functional activator, with a molecular weight of 4.2 kDa [1]. Given that polyamides composed of pyrrole, imidazole, and hydroxypyrrole heterocycles can be designed to target a wide variety of DNA

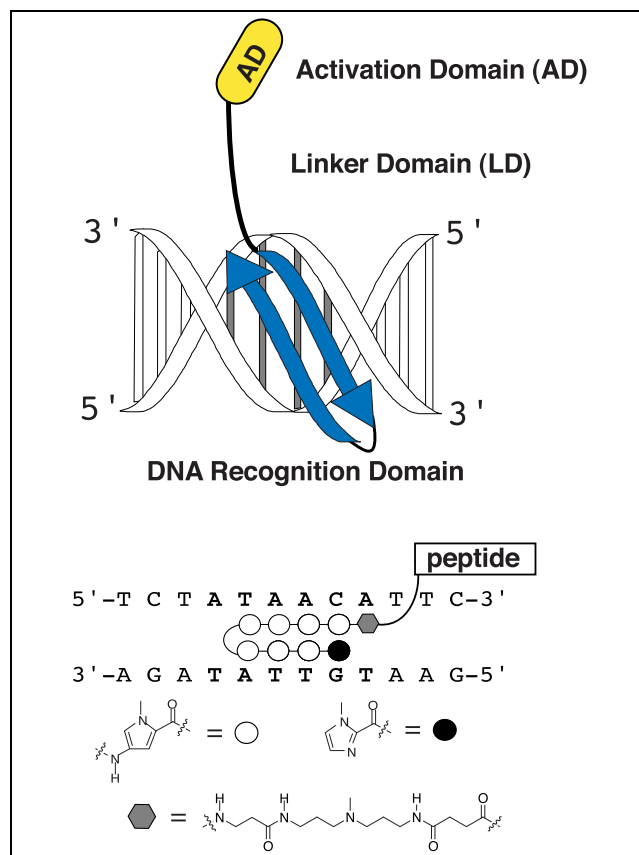


Fig. 1. Design of a synthetic activator. The synthetic activator is composed of three functional domains. The activation domain (AD), a peptide, is tethered to the DNA binding region by a linker domain (LD). The DNA recognition domain is a hairpin polyamide composed of imidazole and pyrrole amino acids that selectively binds the sequence 5'-TGTTAT-3'.

sequences, this motif can potentially be used to generate compounds that regulate expression of designated genes [14].

1.2. A smaller, more potent artificial activator

Our original conjugate (PA-1L-AH, **2**) functioned demonstrably (15–20-fold over polyamide levels) in a cell-free system [1] and the next long-term goal was to use this motif for upregulation in cell culture (Fig. 2). Some immediate concerns remained to be addressed prior to undertaking these experiments, however. Although our activator is much smaller than a typical protein activator such as Gal4 (~101 kDa versus 4.2 kDa), it was desirable to further decrease the molecular weight of the motif in order to increase the probability of membrane permeability. Ideally, this would be accomplished with no loss in function. This could be carried out by varying the identity of the activating region or by shortening the linker domain. In addition, we wished to characterize the binding of the conjugates to the cognate sites within the promoter region and correlate this with function. Finally it remained

Table 1
Summary of conjugate size containing identical eight ring polyamide but different activation domains and tether lengths

Conjugate	Molecular weight
PA-1L-AH (2)	4164
PA-1L-VP1 (3)	2764
PA-1L-VP2 (4)	3670
PA-VP1 (7)	2375
PA-VP2 (8)	3282
PA-(py)-VP2 (9)	3282

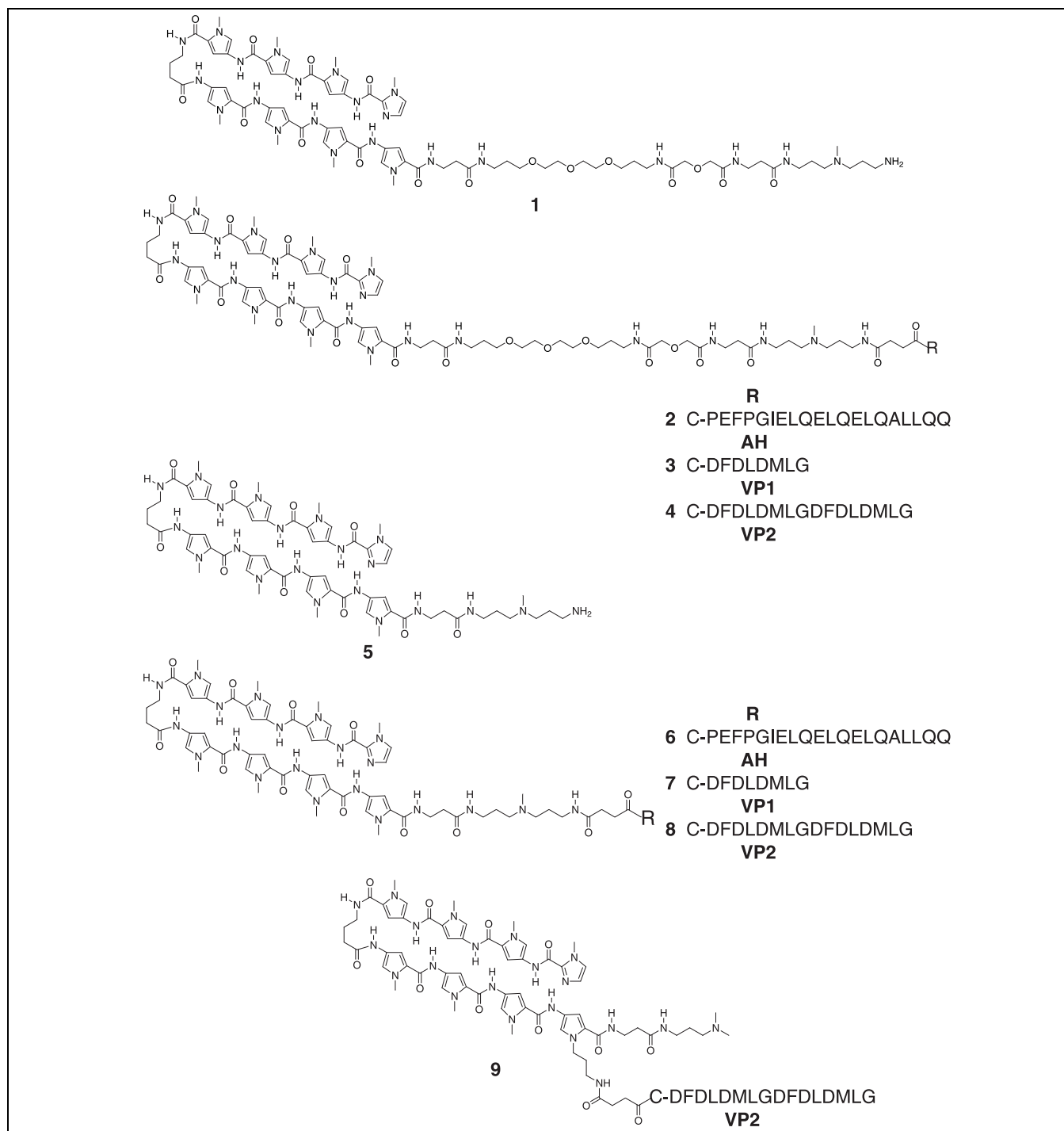


Fig. 2. Structures of hairpin polyamides **1** and **5** and conjugates **2–4** and **6–9** prepared to explore the effect of activating region identity, linker domain motif, and site of attachment, on activation potential. All are designed to bind to the DNA sequence 5'-TGTTAT-3' and differ in the identity of the activating region and the linking domain. In conjugates **2–4**, the activating peptide is covalently linked to the hairpin polyamide by a polyether tether 36 atoms in length. Conjugates **6–8** have a shorter, 12 atom tether. The three different activating regions are AH [29], a designed peptide used in our earlier study, and one (VP1) or two (VP2) tandem repeats of an eight amino acid sequence derived from the viral activator protein VP16 [23,24]. For conjugate **9**, the activator peptide VP2 was attached to an internal pyrrole residue. The C at the junction between the peptide and the tether is cysteine, a key residue for the ligation of peptides with polyamide substrates [34].

to be demonstrated that the identity of the DNA recognition module, the polyamide, could be changed without compromising function.

Here we describe artificial activators with tunable po-

tency affected by the size and identity of the activating region as well as the site of attachment to the polyamide. Most notably, we demonstrate a potency of 50–150% greater than PA-1L-AH (**2**) with concomitant decreases

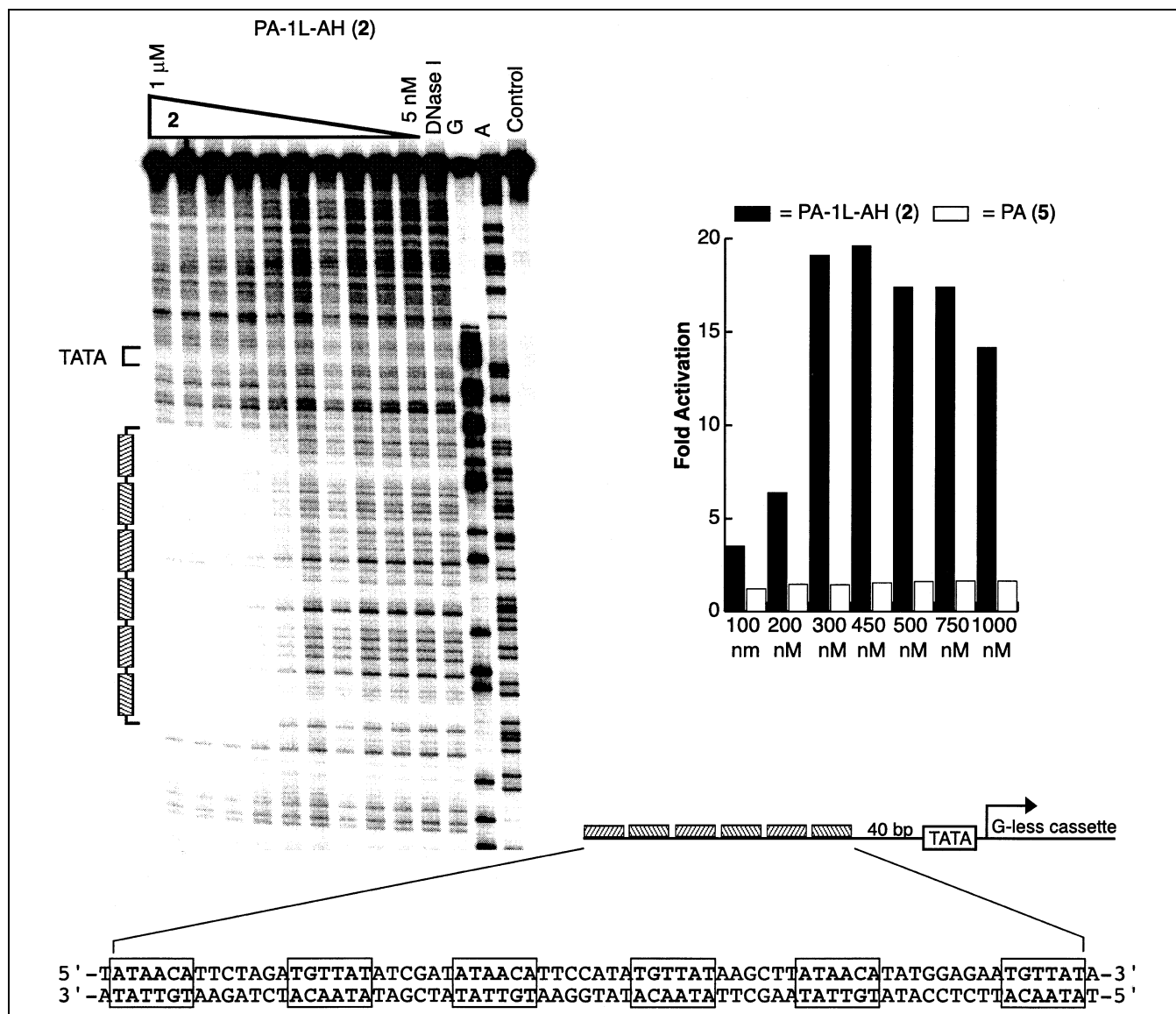


Fig. 3. Promoter occupancy corresponds to activator function. (Upper left) Storage phosphor autoradiogram of a DNase I footprinting titration of **2** (PA-1L-AH) carried out under transcription conditions on a 5'-³²P labeled 363 bp DNA fragment containing both the promoter region and 140 bp of the G-less cassette reporter. To each footprinting reaction, unlabeled plasmid DNA was added to bring the total concentration of polyamide binding sites to a level equivalent to those utilized in transcription assays. From left to right the lanes are DNase I digestion products in the presence of **2** at concentrations of 1 μ M, 750 nM, 500 nM, 300 nM, 200 nM, 100 nM, 50 nM, 20 nM, 10 nM, and 5 nM respectively; DNase I digestion products with no **2** present; the G sequencing lane; the A sequencing lane; undigested DNA. (Upper right) Titration of conjugate **2** in transcription assays shows that maximal activation corresponds to maximal occupancy of the binding sites. Relative fold activation was determined by comparing the levels of transcript produced by increasing concentrations of **2** or **5** with that obtained from transcription reactions performed in the absence of any compound (basal levels). Increasing concentrations of **5** did not have any effect on transcription whereas conjugate **2** shows maximal activation at 300–450 nM. (Lower) Design of the plasmid DNA template used for in vitro transcription experiments and the DNase I footprinting titration experiments. The promoter region contains six cognate binding sites for the hairpin polyamide upstream of a G-less cassette reporter.

in size of 21% and 12%, respectively (Table 1). We also report that all polyamide–peptide conjugates of this minimal motif bind to their cognate sites upstream of the AdML promoter. Consistent with our predictions, we find that the concentration of conjugate required for full promoter occupancy corresponds to the concentration required to elicit maximal activation in vitro. Finally, we show that changing the DNA recognition domain can be accomplished while retaining function.

2. Results and discussion

2.1. Activation modules and their site of attachment

The activation domain (residues 411–490) of VP16, a viral protein, fused to a DNA binding protein yields a chimeric activator with the potency comparable to strong natural activators [20]. Dissection of this and other activation domains has identified minimal units that activate

transcription; these modules are often surreptitiously reiterated in natural activating regions [2,21,22]. In VP16 one such minimal module consists of eight amino acids [23,24]. When reiterated the activation potential of the consequent peptide increased in a synergistic rather than an additive manner [23,25]. This was also reported for other activating modules [26–28]. We adopted this property of activating regions in order to design artificial activators of varying strengths. It appeared likely that decreasing the activating region size to a minimal eight amino acid module (VP1) would provide a small activator that would retain some level of function. In order to probe these questions, we designed a series of polyamide–peptide conjugates, each of which consists of a hairpin polyamide designed to target the cognate sequence 5'-TGTTAT-3', a flexible tether of varying length (12 or 36 atoms), and one of three different activating regions (Fig. 2). The three activating regions are AH [29], a designed peptide used in our earlier studies, and one (VP1) or two (VP2) tandem repeats of an eight amino acid sequence derived from VP16 [24,25].

Next, from our earlier studies we determined that a critical role of the linking or dimerization domain is to facilitate projection of the activating module fully away from the DNA for productive interaction with the transcriptional machinery [1]. To date this had been accommodated by conjugation through a polyether linker at the C-terminus of the hairpin polyamides, but conjugation via linkage at an internal pyrrole residue appeared an attractive alternative. Solution studies as well as X-ray crystallographic data have demonstrated that the *N*-methyl group of the pyrrole residues is directed outward from the minor groove [30–32]. We hypothesized that extension to an eight atom linker at this position as in conjugate **9** (Fig. 2) should provide effective projection of any peptide conjugated at that position, perhaps eliminating the need for a longer tethering linker.

Polyamides **1** and **5** were prepared by solid phase synthesis and subsequently elaborated to provide conjugates **2–4** and **6–9** in accordance with established procedures [1,34]. All polyamides and conjugates thus prepared were purified by reversed phase HPLC and their identities were confirmed by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. Conjugates **2**, **3**, and **4** incorporate a 36 atom polyether tether as the linker domain. Conjugates **6**, **7**, and **8** utilize a shorter 12 atom linker.

2.2. Promoter occupancy under transcriptional conditions

The dissociation constant (K_D) for conjugate **2** binding to its cognate site (5'-TGTTAT-3') has been measured as 32 nM using quantitative DNase I footprinting titrations [1]. However, almost 10-fold higher concentrations of this conjugate were required to achieve maximal transcriptional activation in vitro (Fig. 3). The conditions for these experiments are substantially different than those of the

quantitative footprinting assays. Perhaps the most pertinent difference is the concentration of DNA. In quantitative DNase I footprinting experiments, a greater than 10-fold excess of polyamide relative to DNA is necessary for accurate determination of K_D for the ligand–DNA complex [35]. In vitro transcription assays, however, employ a higher concentration of plasmid DNA (0.8 ng/μl), and this has been demonstrated in other cases to decrease the occupancy of a binding site by 10–100-fold [36]. To investigate the binding behavior of **2** under such conditions, a 5'-³²P labeled 363 bp DNA fragment containing both the promoter region and 140 bp of the G-less cassette reporter was generated. To each footprinting reaction, unlabeled plasmid DNA was added to bring the total concentration of polyamide binding sites to a level equivalent to those utilized in transcription assays. As anticipated, DNase I footprinting titrations performed under the conditions of the in vitro transcription assays revealed that 50% occupancy of the three dimeric binding sites occurs at a concentration of 215 nM, approximately a seven-fold increase over the measured K_D (Fig. 3).

2.3. Promoter occupancy and the level of activation

An in vitro transcription titration experiment with conjugate **2** demonstrates that full occupancy of the promoter is necessary for maximal activator function (Fig. 3). At a concentration of 100 nM, detectable levels (more than four-fold) of transcriptional activation are observed, and as the concentration increases to provide full saturation of the dimeric sites, activation levels climb to almost 20-fold over basal transcription. Transcription levels were not influenced by a similar increase in the concentrations of **5** which does not bear an activation module. Taken together, the data suggest that optimal conjugate concentration for in vitro transcription assays can be predicted using data from DNase I footprinting titrations under such conditions. Therefore, additional DNase I footprinting titration experiments were carried out, revealing that the poly-

Table 2
Summary of data from DNase I footprinting titration experiments for conjugates **2–4** and **7–9** and polyamide **5**

Conjugate	50% occupancy (nM)
PA-1L-AH (2)	215
PA-1L-VP1 (3)	75
PA-1L-VP2 (4)	210
PA (5)	15
PA-VP1 (7)	110
PA-VP2 (8)	75
PA-(py)-VP2 (9)	175

The reported 50% occupancies are the average values obtained from a minimum of two DNase I footprint titration experiments. 50% occupancies were determined by best fit Langmuir binding titration isotherms obtained from nonlinear least squares algorithm ($n=1$) as previously described [35]. All experiments were carried out under transcription conditions as described in Section 4 and depicted in Fig. 3.

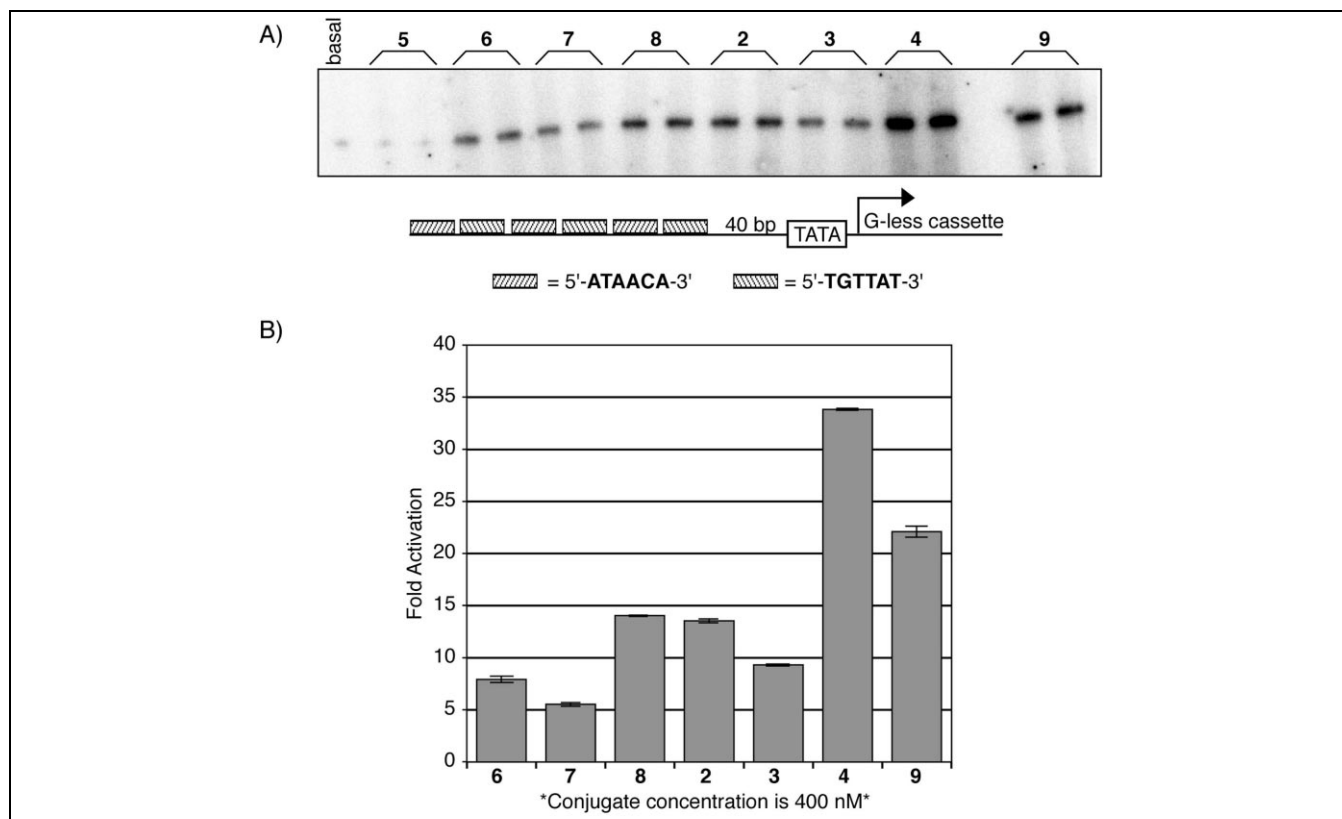


Fig. 4. In vitro transcription reactions with the compounds listed in Fig. 2. Transcription reaction conditions are described in Section 4. (A) Storage phosphor autoradiogram of the reactions which were performed in duplicate with 400 nM concentrations of each compound including 9, the 'internal pyrrole' conjugated polyamide. The template configuration showing the sequence and number of the polyamide binding sites is depicted below the gel. (B) Fold activation was determined by comparing the amount of transcription elicited by each compound with that of the parent polyamide, 5. The standard deviations were determined from the measurement of the duplicates of each compound.

amide-peptide conjugates require concentrations of 75–215 nM to attain 50% occupancy (Table 2). Consistent with previous studies we observe that tethering a peptide to the C-terminus of the hairpin polyamide leads to a 5–14-fold decrease in overall binding affinity. We had anticipated that attachment of a peptide through an internal pyrrole residue, as in conjugate 9, would have a lesser impact upon DNA binding. However, the concentration of conjugate 9 required for 50% occupancy, 175 nM, is similar to that of its C-terminus linked counterparts. Based on these data, all subsequent in vitro transcription experiments were carried out at conjugate concentrations of 300–400 nM.

2.4. Activating potential corresponds to composition and the site of attachment of the activation module

The in vitro transcription experiments of Fig. 4 reveal that the strength of the activator-polyamide conjugates is proportional to the size and composition of the activating region, and that projection of the activating region away from the DNA enhances its functional potency. Results from five independent sets of reactions summarized in Fig. 5 show that when compared with conjugate 2 (PA-1L-AH) conjugate 7 (PA-VP1) is a weaker activator and

that substituting VP1 with VP2 (conjugate 8) increases the activation strength of the resulting conjugate. While the composition of the activating region plays a role in the determining transcriptional potency it was surprising that reiteration of the VP1 module in this context only improved transcriptional potency by two-fold. The absence of appreciable synergy in transcriptional activation in vitro suggests that the synergy observed when a similar motif was reiterated in vivo may arise from the relative accessibility of the two VP1 modules when presented in the context of a chimeric fusion protein [23] or that there is an activation potency threshold in vivo below which activators do not function efficiently. Another possible explanation is the differences in the secondary structure of the eight versus the 16 residue peptide. However, based on studies demonstrating resilience of the activating regions to structural perturbations [10,37], it is more likely that difference in activation potential of these peptides stems from the fact that there is simply insufficient surface area available for high avidity interactions with the targets in the transcriptional machinery.

Consistent with the requirement for an activating module to access targets in the transcriptional machinery, projecting the VP1 or VP2 module via a longer linker further improves their activation potential (Fig. 4B). The normal-

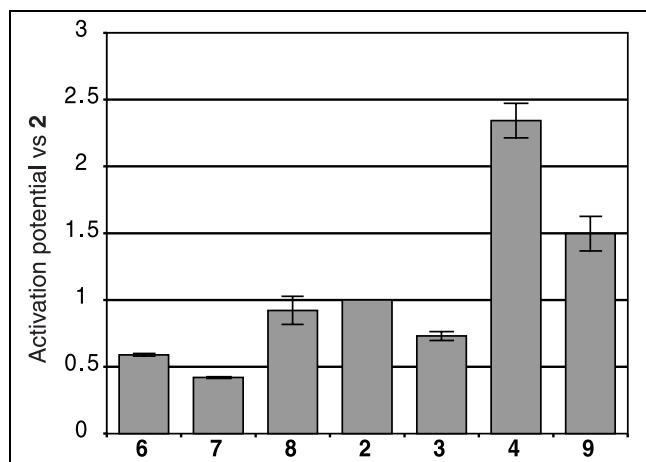


Fig. 5. Summary of five independent in vitro transcription reactions showing the relative potency of each compound in comparison to PA-1L-AH. The absolute fold activation mediated by all conjugates varies slightly between different experiments. To effectively compare the activation potential of the various conjugates we normalized data from five separate experiments for each conjugate to that of **2**.

ized data from five independent experiments summarized in Fig. 5 demonstrate that all of the activator peptides benefit from the inclusion of a flexible hydrophilic linker. In this configuration, VP2 is now more than three-fold

more potent than VP1. It is possible that the additive increase in transcriptional potency, upon reiteration of the VP1 module, in the absence of a linker occurs because of less than optimal access of the activation modules to their targets in the transcriptional machinery. Simply improving the reach of the activator modules now leads to a more than additive (about three-fold) increase in activation potency as the VP1 module is reiterated. Moreover, from Fig. 5 it is apparent that VP2 peptide attached to the C-terminus of the polyamide via a 36 atom linker (PA-1L-VP2, **4**) is the most potent of the polyamide activating region conjugates synthesized.

Figs. 4 and 5 also show that conjugate **9**, where VP2 is attached to an internal pyrrole residue via an eight atom linker, activates transcription robustly despite the absence of a long linker. Structural studies of polyamide–DNA complexes predict that projection of the activating region from this internal position should be particularly effective. The ability of this conjugate (**9**) to activate ~60% better than PA-VP2 (**8**), which does not bear a linker, and ~50% less well than one that has a long polyether linker (**4**) further supports the suggestion that projection of the activating module a certain distance away from the DNA plays an important role in determining the efficacy of activation.

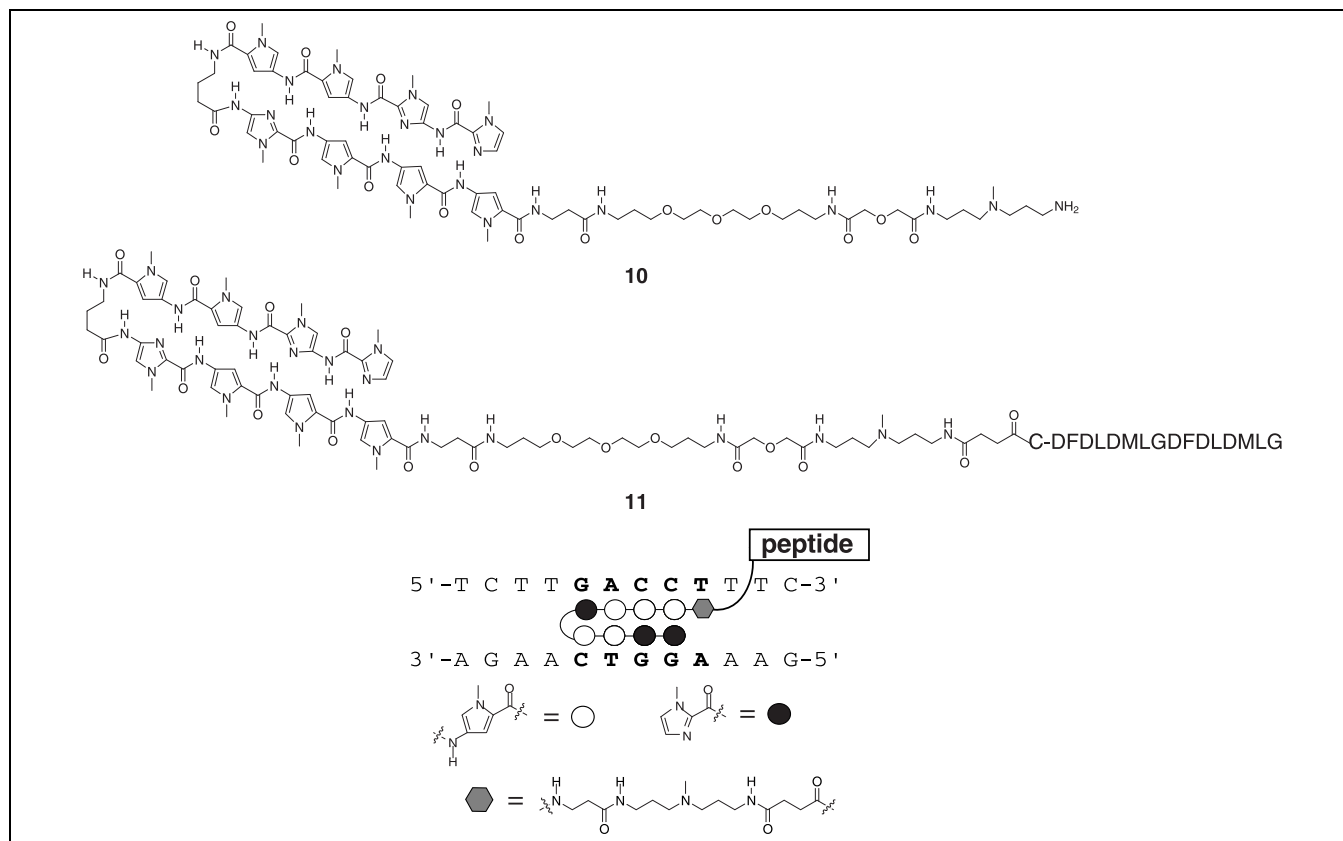


Fig. 6. Structures of hairpin polyamide **10** and conjugate **11**. Both polyamide **10** and conjugate **11** were designed to target the DNA sequence 5'-TGACCT-3'.

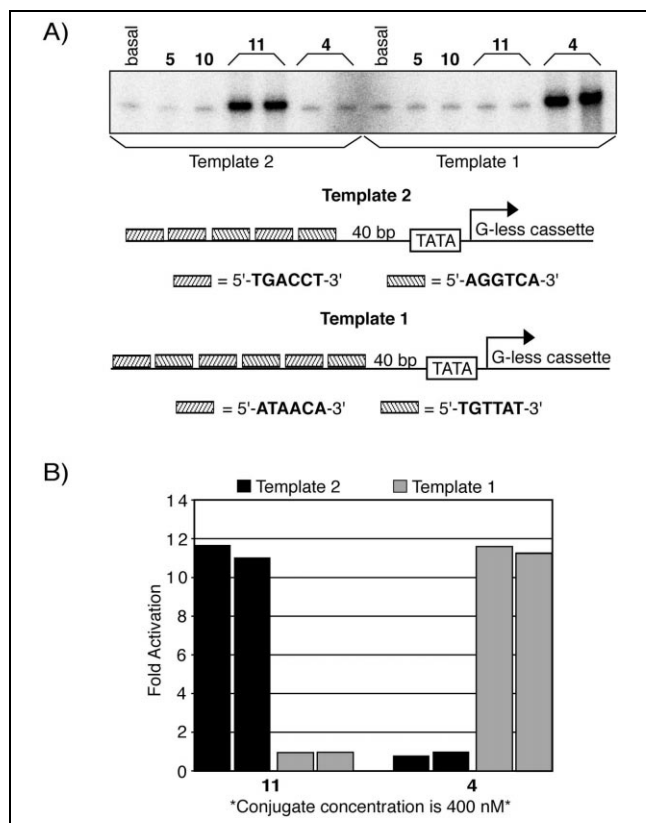


Fig. 7. Each conjugate activates transcription from a template bearing its own cognate DNA binding site. Conjugates bearing identical linker and activator modules but differing hairpin polyamides target and stimulate transcription from their respective templates. Both templates bear the AdML TATA box and identical transcriptional units. The sequences and the orientation of each polyamide binding site on either template is depicted below the gel. Compounds **10** and **11** are designed to target template 2 whereas **3** and **5** bind template 1. Transcription reactions were performed as described in Section 4 using 400 nM concentrations of the respective compounds.

2.5. Replacing the DNA binding module alters specificity

The final requirement for generality of the polyamide-peptide motif of artificial activators is the ability to alter the identity of the DNA recognition domain without compromising function. To test this we designed conjugate **11** to target the sequence 5'-AGGTCA-3', incorporated the polyether linker as the linking domain, and chose VP2 as the activating region since this motif had proven to be the most active of all tested (Fig. 6). Control polyamide **10** and conjugate **11** were prepared by established protocols [33]. As shown in Fig. 7 a template bearing five cognate binding sites 40 bp upstream of the AdML G-less cassette reporter was constructed. As in earlier studies, conjugate **11** activated transcription 15–20-fold on template 2 bearing the cognate binding sites whereas polyamide **10**, which lacks an activation module, had little impact on basal transcription. Furthermore, transcription was dependent upon the presence of cognate DNA binding sites as **11** failed to activate transcription on template 1 at 400 nM (Fig. 7). Conversely, conjugate **4** activated transcription

from template 1 but not template 2. In additional experiments, it was found that when the concentration of conjugate **4** or **11** was increased to 600 nM or greater, low levels (two- to three-fold) of transcription stimulation were observed on templates lacking the cognate polyamide binding sites (data not shown). Taken together, these data suggest that it is possible to change the identity of the polyamide DNA binding domain without functional penalty.

3. Significance

Here we describe a series of artificial regulators which were generated by tethering one or two tandem repeats of the minimal activating module of the viral activator VP16. The activating region is either conjugated directly to the polyamide via a short linker or has a longer polyethylene glycol linker between the two modules. These features generate a family of regulators with tunable activation potentials. We also show that tethering the VP2 activating module to an internal pyrrole projects the peptide directly out of the minor groove of the DNA thereby increasing its activation potential despite the absence of an extended linker. While further increasing the number of reiterated minimal VP modules would increase the activation potency of these conjugates we sought to maintain the compactness of activator and in this respect conjugate **9** represented a perfect compromise between strength and size. The results described here thus provide evidence that artificial activators of differing strengths can be generated by varying the activation module and its accessibility and that these activators can be targeted to different sites by altering the composition of the polyamides. Taken together our results define the basic design principles for general construction of a minimal artificial regulatory factor. Whether these minimal design elements will be sufficient to allow specific gene activation in cell culture remains to be determined and will be reported in due course.

4. Materials and methods

4.1. Synthesis of polyamide-peptide conjugates

Polyamides **1**, **5**, and **10** were prepared according to established solid phase synthesis protocols [33] and subsequently transformed into conjugates **2–4**, **6–8**, and **11** by previously reported methods [1,34]. Conjugate **9** was prepared in an analogous fashion. The identity of all conjugates was verified by MALDI-TOF mass spectrometry. Characterization: **2** (PA-1L-AH): MALDI-TOF [M+H] (average mass) calc. 4164.7, obs. 4164.6; **3** (PA-1L-VP1): MALDI-TOF [M+H] (monoisotopic mass) calc. 2763.25, obs. 2763.53; **4** (PA-1L-VP2): MALDI-TOF: [M–H] (average mass) calc. 3670.1, obs. 3670.1; **6** (PA-AH): MALDI-TOF: [M+H] (average mass) calc. 3774.2, obs. 3774.5; **7** (PA-VP1):

MALDI-TOF [M+H] (monoisotopic mass) calc. 2374.0, obs. 2374.0; **8** (PA-VP2): MALDI-TOF [M+H] (monoisotopic mass) calc. 3280.4, obs. 3280.5; **9** (PA-(py)-VP2): MALDI-TOF [M+H] (monoisotopic mass) calc. 3280.4, obs. 3280.7; **11**: MALDI-TOF [M+H] (monoisotopic mass) calc. 3670.6, obs. 3670.7.

4.2. DNase I footprinting titration experiments

A 363 bp 5'-³²P labeled PCR fragment was generated from template plasmid pAZA812 in accordance with standard protocols and isolated by nondenaturing gel electrophoresis. All DNase I footprinting reactions were carried out in a volume of 40 µl. We note explicitly that 0.8 ng/µl of plasmid pPT7 was used in these reactions as unlabeled carrier DNA. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were 50 mM HEPES, 100 mM KOAc, 15 mM Mg(OAc)₂, 5 mM CaCl₂, 6.5% glycerol, 1 mM dithiothreitol, pH 7.0 and 15 kcpm 5'-radiolabeled DNA. The solutions were equilibrated for 75 min at 22°C. Cleavage was initiated by the addition of 4 µl of a DNase I stock solution and was allowed to proceed for 7 min at 22°C. The reactions were stopped by adding 10 µl of a solution containing 2.25 M NaCl, 150 mM EDTA, 0.6 mg/ml glycogen, and 30 µM base pair calf thymus DNA and then ethanol precipitated. The cleavage products were resuspended in 100 mM Tris-borate-EDTA/80% formamide loading buffer, denatured at 85°C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% crosslink, 7 M urea) at 2000 V for 2 h 15 min. The gels were dried under vacuum at 80°C and quantitated using storage phosphor technology.

4.3. In vitro transcription assays

Template plasmid pAZA812 was constructed by cloning a 78 bp oligomer bearing three cognate palindromic sequences for conjugates **2–4** and **6–9** into a *Bgl*/II site 30 bp upstream of the TATA box of pMLΔ53. This plasmid has the AdML TATA box 30 bp upstream of a 277 bp G-less cassette. Template pAZA813 was constructed by cloning a 78 bp oligomer containing five cognate sites for conjugate **11** into a *Bgl*/II site 30 bp upstream of the TATA box of pMLΔ53 [1]. For each reaction, 20 ng of plasmid (30 fmol of palindromic sites) was pre-incubated with 400 nM concentrations of the compound for 75 min prior to the addition of 90 ng of yeast nuclear extract in a 25 µl reaction volume. The reactions were performed as previously described and resolved on 8% 30:1 polyacrylamide gels containing 8 M urea [1]. Gels were dried and exposed to photostimulatable phosphorimaging plates (Fuji Photo Film Co.). Data were visualized using a Fuji phosphorimager followed by quantitation using MacBAS software (Fuji Photo Film Co.).

Acknowledgements

We are grateful to the National Institutes of Health for research support.

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