Chemistry & Biology, Vol. 10, 909–916, October, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2003.09.008

Transcription Activation by a PNA-Peptide Chimera in a Mammalian Cell Extract

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Summary

Synthetic activators that mimic the ability of native transcription factors to recruit the RNA polymerase holoenzyme to specific promoters could, in principle, be constructed by joining a sequence-specific DNA binding moiety with a molecule able to bind the holoenzyme. We report here that a peptide nucleic acid (PNA)-peptide chimera is capable of activating transcription in vitro in a HeLa nuclear extract. Specifically, a promoter-targeted PNA alone acts as a strong inhibitor of basal transcription in a HeLa nuclear extract, presumably due to structural modification of the promoter. However, the fusion of a Gal80-binding peptide to the PNA, but not control peptides, reactivates transcription. The Gal80-binding peptide was selected solely on the basis of its ability to bind the yeast repressor.

Introduction

In eukaryotic cells, the expression of most genes is regulated by gene-specific transactivators. Most of these proteins are comprised of sequence-specific DNA binding and activation domains (ADs). ADs bind to one or more proteins in various transcription complexes, such as the RNA polymerase II holoenzyme [1–3] and chromatin remodeling/modification machines [4]. These two binding properties allow activators to function, at least in part, by attracting these enzymatic complexes to nearby promoters, thus stimulating gene expression [5–8]. Synthetic molecules that promote gene-specific activation would be powerful tools for biological research and could potentially be of medical interest as well. It has been suggested that artificial activators which reconstitute the recruitment activity of native transcription factors could be made by linking together appropriate synthetic DNA- and protein binding moieties [9]. To execute such a plan of course, one must have in hand the appropriate specific DNA- and protein binding molecules. There exist certain classes of molecules capable of binding DNA sequences specifically, such as the hairpin polyamides developed by Dervan and colleagues [10–12] and the peptide nucleic acids (PNAs) pioneered by Nielsen and coworkers [13, 14]. Synthetic activation domains could perhaps be obtained by screening combinatorial libraries for molecules that bind coactivators or other relevant protein targets.

Recently, there have been several advances in this area. Dervan, Ptashne, and colleagues reported that a synthetic molecule comprised of a hairpin polyamide linked to a peptide was able to activate transcription in vitro in a yeast nuclear extract [15–17]. Stanojevic and Young found that activating peptides tethered to a triple-helix-forming oligonucleotide could induce transcription activation [18]. This work is significant in that it represents the first example of activation of transcription by a synthetic molecule in cultured cells. These results demonstrate the feasibility of making synthetic activators, though the peptide activation domains in these molecules were not derived from simple binding studies, but instead were taken from known activators or derived from functional genetic selections.

Two studies have demonstrated that peptides selected solely on the basis of their ability to bind a target protein can function as activation domains in cells when fused to an appropriate DNA binding domain. In the first such report, we demonstrated that 20 residue peptides selected from a phage-displayed library for binding to the transcriptional repressor Gal80 had activity in yeast [19]. Subsequently, Frangioni and colleagues showed that eight residue peptides, again isolated by phage display, that bind the KIX domain of the coactivator Creb binding protein (CBP) have activity in mammalian cells [20].

Recently, we communicated the synthesis and preliminary characterization of a chimeric molecule comprised of a “clamp-type” PNA fused via a flexible linker to one of the Gal80 binding peptides derived from phage display [21]. It was shown that this molecule could bind DNA and recruit either the Gal80 protein or the Gal11 protein [22, 23], a yeast coactivator, to the bound DNA, thus reconstituting a fundamental property of activators. In this study, we report the further characterization of this novel molecule. In particular, we show that a Gal4 DBD-Gal80 binding peptide is capable of transcriptional activation in mammalian cells, even though the protein against which the peptide was selected is from yeast. We also analyze the activity of the PNA-peptide chimera in transcription experiments in vitro using human nuclear extracts. Interestingly, the PNA alone has a potent repressive effect on the basal level of transcription. It is shown that fusion of the Gal80 binding peptide reactivates robust transcription whereas control peptides do not. A number of other controls and DNA binding experiments are presented which, together, argue that this synthetic molecule indeed has transactivation activity. These results represent the first observation of activity of this type using a molecule whose components were obtained completely by design and binding-based screening experiments. Thus, these experiments, combined with the previous work mentioned above, complete an important proof of principle for a pathway to the development of cell-permeable, nonpeptidic synthetic transcription factors.

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Table 1. Transcription Activities of Polypeptides Measured by Luciferase Assay

Polypeptide Expressed	Fold Activation
Gal4(1-147)	1
Gal4(1-147)-G80BP-A	6.4
Gal4(1-147)-20un	2.3
Gal4(1-147)-20un-G80BP-A	46 ± 4
Gal4(1-147)-(G80BP-A)$_2$	653 ± 60
Gal4(1-147)-(G80BP-A)$_3$	4680 ± 500
Gal4	3007 ± 500
Gal4(1-147)-VP16	9220 ± 550

The fold activation is defined as the ratio of transcription driven by the indicated construction and the DNA-binding domain [Gal4(1-147)], which is normalized to one.

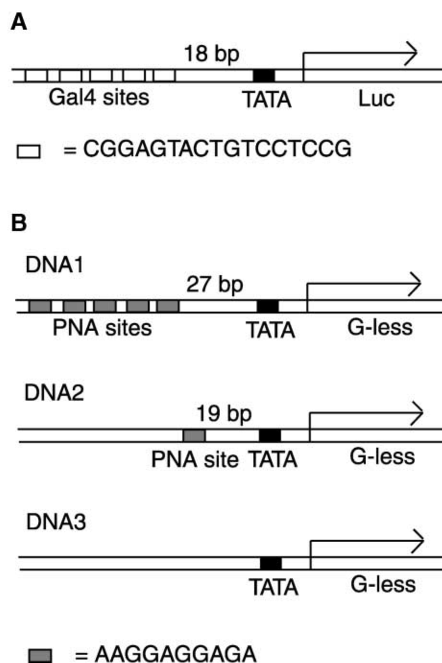

Figure 1. Schematic View of the DNA Templates Used in This Study

(A) Firefly luciferase-encoding reporter gene for mammalian cell culture experiments with five consensus Gal4 binding sites in the promoter-proximal region.

(B) G-less cassette reporters for in vitro transcription experiments containing zero, one, or five BisPNA binding sites in the promoter-proximal region. The distance between the PNA binding sites and the TATA box is indicated for DNAs 2 and 3.

Results

The Gal80 Binding Peptide Functions as an Activation Domain in Mammalian Cells

In previous work [19], we used phage display to identify two peptides that bind tightly and specifically to the Gal80 protein. Gal80 is a transcriptional repressor that blocks activation of the Gal4 transactivator by binding tightly to the activation domain of that protein [24]. The hope was that a Gal80 binding peptide would serve as a mimic of the potent Gal4 AD. Both peptides, when expressed as a fusion with the Gal4 DNA binding domain (DBD), indeed supported transcriptional activation in yeast [19]. As a first step toward the development of a synthetic activator that would function in mammalian cells, we asked if one of these peptides, Gal80 binding peptide A (G80BP-A) (Table 1) [19], would activate transcription in cultured mammalian cells, even though it was isolated against a yeast transcription factor.

A number of constructs that encode Gal4(1-147)-peptide(s) fusion proteins were constructed by subcloning the protein-encoding fragments employed for our previous yeast studies into the mammalian expression vector pEGFP-N$_3$ (Promega). These expression plasmids were cotransfected into 293 cells along with a reporter plasmid, pG5B, which has five Gal4 binding sites upstream of the promoter driving the firefly luciferase (*Luc*) gene (Figure 1A). A *Renilla* luciferase expressing vector with the SV40 enhancer, pRL-SV40, was also added to the transfection mixture to provide an internal control for transfection efficiency.

The normalized Luc activities driven by each Gal4-peptide(s) construct are shown in Table 1, with the level observed in the presence of Gal4(1-147) set to one. The Gal4(1-147)-G80BP-A fusion gave a 6-fold increase of the transcription level, whereas fusion of a control peptide, 20un (a 20-mer picked randomly from the library) provided a 2-fold increase. For comparison, fusion of the native Gal4 or VP16 activation domains to Gal4(1-147) provided much higher levels of activation (about 3000- and 9200-fold, respectively). However, when the 20un peptide was inserted between the Gal4 DBD and G80BP-A, a more robust 46-fold increase in Luc expression was observed, suggesting that a spacer between the two functional motifs is required for full activity. Finally, as we had observed in yeast cells [19], multimerization of G80BP-A resulted in a synergistic increase in activation. Activators with two or three copies of G80BP-A drove 650- and 4700-fold higher levels, respectively, of luc expression than did the DBD alone. The peptide with three consecutive G80BP-A units has transcription activity close to that of Gal4 and VP16 activation domain. We conclude that G80BP-A functions as a weak activation motif in mammalian cells, but that potent activation can be achieved through multimerization.

Characterization of PNA-Peptide Chimeras

As reported previously [21], we have synthesized and purified a PNA-G80BP-A chimera and demonstrated that it is capable of recruiting transcription proteins to the DNA to which it is bound. Here we examine the transcription activation activity of this molecule in a mammalian nuclear extract.

To facilitate these experiments, a number of PNA-peptide conjugates (Figure 2) were created and their DNA binding properties characterized. BisPNA was designed to bind the double-stranded DNA sequence 5′-AAGGAGGAGA-3′, a favorable site for PNA complexation. MisPNA has four mismatched bases relative to BisPNA and is therefore not expected to associate tightly with this DNA sequence. PNAs were synthesized using automatic solid-phase synthesis. G80BP-A and 20un peptides were added to the PNAs by manual solid-phase synthesis.

To assess the DNA binding properties of the PNA-

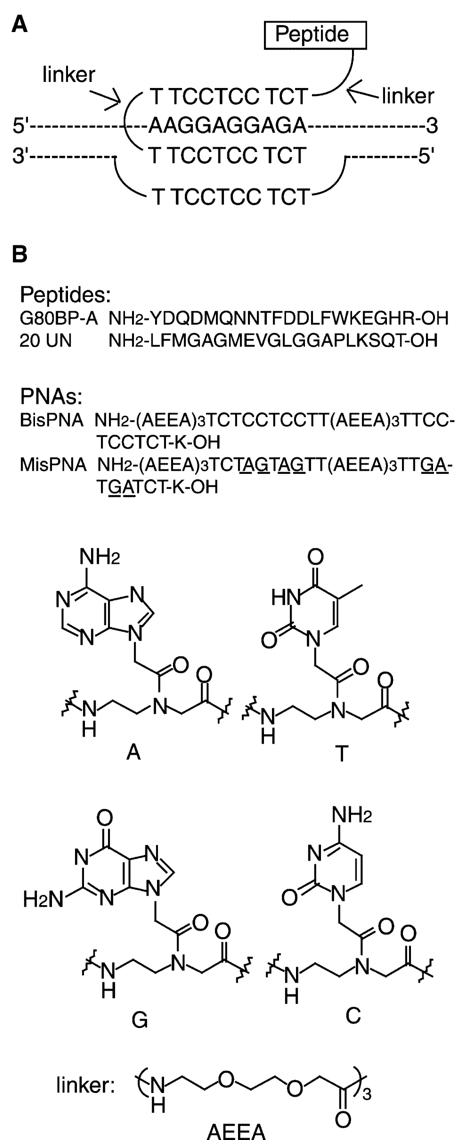

Figure 2. Structures of the Synthetic Activators

(A) Schematic view of the BisPNA-peptide·DNA complex illustrating the strand invasion mode of binding.

(B) Chemical structures of the components employed to make the PNA-peptide chimeras.

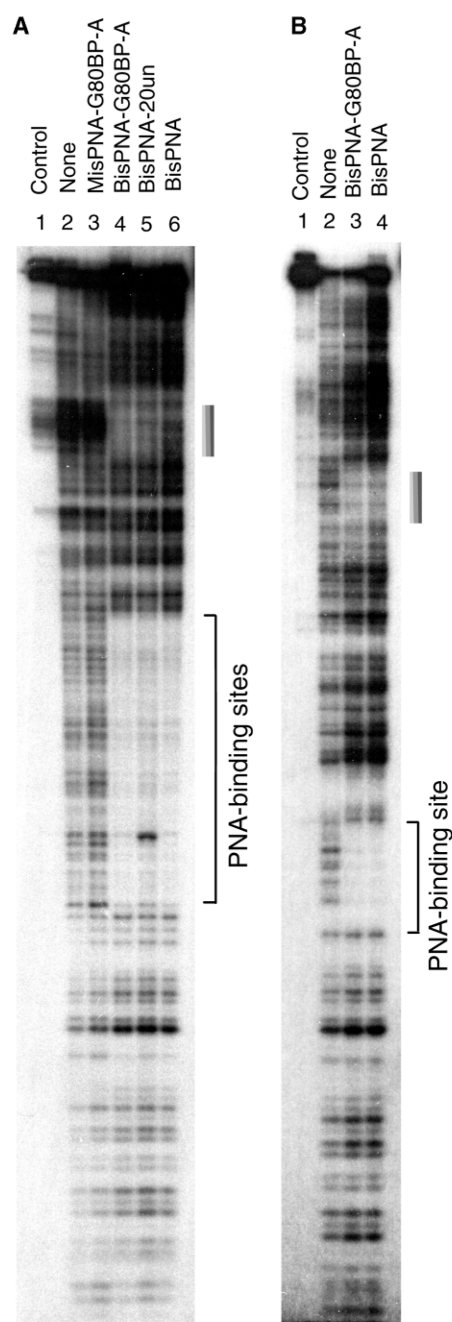

Figure 3. Footprinting Analysis of PNA·5′-32P-Labeled DNA Complexes

Complexes were preformed as described in the text and Experimental Procedures section, and these complexes were probed with DNaseI.
(A) DNA template containing five PNA binding sites (DNA1, Figure 1B). Lane 1, undigested DNA. Samples in lanes 2–6 were all digested with DNaseI. Lane 2, DNA alone; lane 3, DNA + MisPNA-G80BP-A; lane 4, DNA + BisPNA-G80BP-A; lane 5, DNA + BisPNA-20un; lane 6, DNA + BisPNA (no peptide fusion).
(B) DNA template with one PNA binding site (DNA2, Figure 1B). Lane 1, undigested DNA. Samples in lanes 2–4 were digested with DNaseI. Lane 2, DNA alone; lane 3, DNA + BisPNA-G80BP-A; lane 4, DNA + BisPNA (no peptide fusion). In both gels, the major site of PNA binding is indicated and corresponds to the expected protection of the target site(s). A lighter footprint (striped bar) was also observed for the BisPNA constructs, indicating a weaker association with a region of the G-less cassette.

peptide conjugates, ^{32}P-labeled double-stranded DNAs of about 500 base pairs were produced by PCR. These DNAs contained either one or five binding sites for the BisPNA-peptide molecules, as well as a core promoter and a 377 bp G-less cassette (Figure 1B). A 200-fold molar excess of PNA-peptide was incubated with the DNA for 16 hr at room temperature. The large excess of PNA-peptide conjugate was necessary because strand invasion (Figure 2A) of PNA-peptides into linear DNAs is a slow process at neutral pH [25, 26]. After binding, the excess PNA-peptide molecules were removed by a PCR-like purification protocol [21].

The purified PNA-peptide·DNA complexes were subjected to DNase I footprinting to examine their binding chemistry. In Figure 3A, the DNA template containing

five BisPNA binding sites (DNA1; Figure 1B) was employed. As anticipated, the BisPNA molecule lacking a fused peptide provided a strong footprint (compare lanes 2 and 6) at the expected site (as determined by sequencing; data not shown), whereas the control molecule, MisPNA, did not provide such a footprint (compare lanes 2 and 3). Lanes 4 and 5 show that the BisPNA molecules with either the G80BP-A or 20un peptides fused to them exhibit a footprint nearly identical to that of the parent BisPNA (compare with lane 6). The only significant difference is a single site of nuclease hypersensitivity in the BisPNA-20un·DNA complex, which we cannot explain. These data prove that the PNA molecules exhibit the anticipated sequence specificity and that fusion of the activating or control peptides does not compromise DNA binding.

In Figure 3B the DNA template contained one PNA binding site (DNA2; Figure 1B). The BisPNA·DNA and BisPNA-G80BP-A·DNA had similar footprints (compare lanes 3 and 4 with lane 2). The indicated binding region was determined by DNA sequencing (data not shown). These results prove that BisPNA and BisPNA-peptide can bind to a single site on DNA.

The footprinting experiments also provided some evidence for lower affinity binding of the BisPNA-containing molecules to the G-less cassette region (striped bar in Figure 3). This was not completely unexpected since there were sites in the cassette very similar to the designed target site (5′-AAGGAGGAGA).

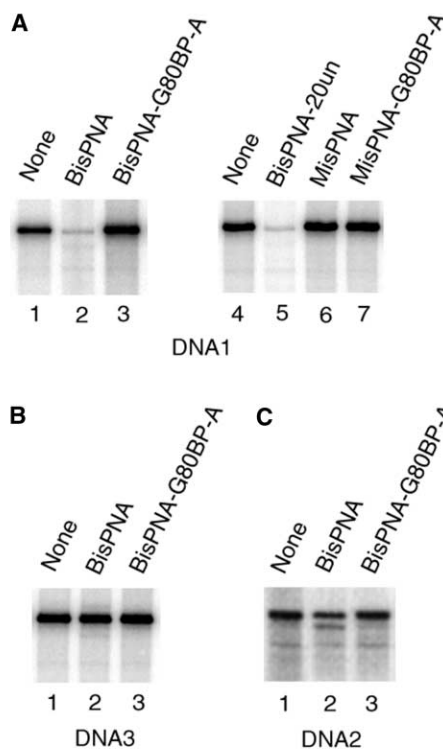

Figure 4. Results of In Vitro Transcription Reactions Carried Out in HeLa Nuclear Cell Extracts

The identities of the DNA and synthetic construct (if present) are indicated.

In Vitro Transcription

For in vitro transcription experiments, reporter plasmids containing either zero, one, or five PNA binding sites, a core promoter, and a G-less cassette were linearized with HindIII. These linear DNAs of about 3000 bps were incubated with a 170-fold molar fold excess of PNA or PNA-peptide chimera for 16 hr at room temperature. The PNA-peptide·DNA complexes were purified by PCR purification protocol as discussed above for the footprinting experiments. The preformed complexes were then added to HeLa nuclear extract and transcription was measured [27].

Figure 4A shows the results of experiments conducted with DNA1 (Figure 1B), the template that contains five binding sites for the BisPNA molecule upstream of the core promoter.

The distance between PNA binding sites and the TATA box is 27 base pairs. Lanes 1 and 4 in Figure 4A reflect the level of basal (unactivated) transcription observed in this system, as no PNA construct was bound to the DNA in these experiments. As shown in lane 2, binding of BisPNA lacking an appended peptide strongly repressed transcription, possibly due to PNA-mediated interference of the assembly of the transcription complex and/or perturbations in the DNA structure due to PNA invasion. When the BisPNA-G80BP-A hybrid was bound to the DNA template, much more robust transcription was observed. The level was slightly higher than that observed in reactions lacking a PNA (lane 1). This result shows that the presence of the Gal80 binding peptide can rescue a repressed template and reactivate transcription.

To confirm that the transcription activation was caused by G80BP-A tethered to the DNA via the PNA moiety, similar experiments were conducted with various other PNA-containing constructs. As shown in lane 5 of Figure 4A, the BisPNA-20un chimera repressed transcription. This important control demonstrates that the fusion of a control peptide to the PNA does not derepress transcription and that the special activating characteristics of G80BP-A are required to obtain strong RNA synthesis. Second, the level of transcription observed when the DNA template had been incubated with MisPNA or MisPNA-G80BP-A was identical within error to that observed in the basal reaction (compare lanes 6 and 7, respectively, to lane 4 in Figure 4A), as expected from the fact that MisPNA does not bind the sites in this template (see Figure 3).

To further probe the correlation between PNA-DNA binding and repression of transcription, transcription experiments were conducted with the DNA3 template (Figure 1B) that lacks PNA binding sites. In this case, the addition of BisPNA or the BisPNA-G80BP-A chimera had no effect on transcription, the levels observed being identical to those seen in the basal reaction (Figure 4B, compare lanes 2 and 3 with lane 1). Thus, we conclude that sequence-specific recognition of the DNA template by the PNA moiety is essential for both PNA-mediated repression of transcription as well as G80BP-A-dependent reactivation of transcription. Figure 4C shows the results of experiments using a template containing a single BisPNA binding site, DNA2. In this case, BisPNA

also repressed basal transcription (lane 2 compared with lane 1), but much less severely than was the case for the multiple PNA binding site template. The transcription level observed when the BisPNA-G80BP-A molecule was bound to the template was similar to that seen in the basal experiment.

Discussion

In this paper, we have demonstrated that G80BP-A, a 20-mer peptide selected against Gal80 in yeast, is able to act as a transcription activation domain in mammalian cells. As was the case in yeast cells [19], a single copy of G80BP-A fused to the Gal4 DBD supports only modest levels of activation, but multimerization of the peptide resulted in a synergistic increase in activation. Three copies of the peptide supported a level of activation similar to that of the potent Gal4 and VP16 native activation domains. We note that there is good evidence that native ADs themselves are probably comprised of synergistically interacting peptide units [28, 29], so we suspect that the Gal80 binding peptide is a mimic of just such a functional native unit. These results spurred us to examine the activity of the Gal80 binding peptide in vitro in mammalian nuclear extracts in the context of a completely synthetic molecule.

The results of these experiments, shown in Figure 4, were striking. First, binding of the PNA molecule(s) to the promoter region of the transcription template severely repressed the relatively high level of basal transcription observed in these experiments. The strand invasion of BisPNA forms a P loop structure that disrupts the local DNA double helix (Figure 2) [30, 31]. Therefore, a reasonable model is that the PNA·DNA complex is a less optimal substrate for binding the transcription machinery and basal activity is thus repressed. One would imagine that the structural disruption in the promoter region would be more severe when the template contains five, rather than one, binding sites, and this correlates with the much greater repression observed for the DNA1 versus the DNA2 template. Again, no effect of any of the PNA constructs was observed on transcription from template DNA3, which lacks PNA binding sites.

Whatever the detailed mechanism of PNA-mediated repression, it was advantageous in evaluating the activity of the activation potential of G80BP-A in this assay. Transcription reactions on naked (i.e., nonchromatin) templates typically exhibit relatively high basal activity since the general transcription machinery need not compete with histones for the promoter. It is generally acknowledged that most of the activation effect on chromatin templates is due to derepression, with so-called "true activation" (stimulation beyond the basal level) [32] providing a much more modest contribution. We were concerned that this would make it difficult to evaluate the activity of what we anticipated would be an artificial AD of only modest potency. Indeed, in our hands, even the potent VP16 AD, when fused to the Gal4 DBD, supports only a 3- to 5-fold increase in transcription from naked templates in transcription under these conditions (A.F. and T.K., unpublished data). However, the very low level of transcription from the PNA-bound template provided a much "cleaner" background against which to evaluate the activity of G80BP-A.

As shown in Figure 4, the level of transcription in vitro supported by BisPNA-G80BP-A was at least 10-fold greater than that which was obtained from the BisPNA-bound template (compare lanes 2 and 3 in Figure 4A). However, fusion of a control peptide, 20un, to the PNA did not stimulate transcription (lane 5), showing the specific effect of the Gal80 binding peptide. The level of transcription supported by the BisPNA-G80BP-A conjugate was consistently higher than that of the basal reaction, but only modestly so. Therefore we were initially concerned that the apparent reactivation of repressed transcription mediated by BisPNA-G80BP-A might be due to poor DNA binding of this construct and that the activity observed was essentially the basal reaction. However, this model was refuted completely by the DNaseI footprinting experiments shown in Figure 3. The binding characteristics of the BisPNA lacking a peptide, BisPNA-G80BP-A, and BisPNA-20un were almost identical. Fusion of the peptide to the PNA did not compromise DNA binding activity in any way. We note that the conditions under which the PNA·DNA complexes were formed in the footprinting and transcription assays were identical. Thus, we conclude that the BisPNA-G80BP-A indeed functions as an activator in the mammalian nuclear extract.

As mentioned in the Introduction, several papers have now made clear the feasibility of making synthetic transcription factors [15–18]. These molecules consisted of hairpin polyamide or triplex-forming oligonucleotide DNA binding elements fused to activating peptides derived from native activators or obtained in functional genetic screens [33, 34]. The results reported here advance the field further by demonstrating that a synthetic activator can be constructed from components that were either designed (the PNA moiety) or obtained through simple screening assays based on binding alone (the Gal80 binding peptide). This is important, because the ultimate goal in this area is to develop synthetic activators that could be used routinely to manipulate gene expression in cultured cells and perhaps in animals. Thus, it will be desirable in the future to move away from peptides and oligonucleotides and to employ more "drug-like" molecules in these constructs. Although we have employed a peptide AD mimic in this study, it was isolated solely on the basis of a physical binding assay [19], suggesting that nonpeptidic AD surrogates could be obtained by screening appropriate combinatorial libraries against Gal80 [19] or the KIX domain of CBP[20]. Of course, genetic and other functional screens cannot easily provide nonpeptidic moieties. Finally, these results also represent the first report of a synthetic activator that functions in a mammalian nuclear extract.

The importance of moving toward synthetic activators that lack native peptide elements is perhaps highlighted by unpublished work from our laboratory. We have invested considerable effort in looking for PNA-G80BP-A-mediated activation in cultured mammalian cells by preforming reporter plasmid (both circular and linear)·PNA-G80BP-A complexes in vitro and transforming these species into cells. In no case was significant activation of the reporter gene observed (B.L. and T.K.,

unpublished data). Of course, it is difficult to interpret negative results of this type, but one possible reason for the lack of activity is the destruction of the unstructured peptide in cells. The PNA-DNA complex is so stable that if the peptide were degraded, there would be no chance of exchanging the now inactive species for another molecule of an artificial activator. We note that this model is not necessarily in conflict with the reported activity of the oligonucleitde-peptide conjugate of Stanojevic and Young [18] in cultured cells. The peptides employed in that study lack a lysine residue, which is required for degradation via the ubiquitin-proteasome pathway [35] that operates on native activators [36], the kinetic half-life of the synthetic activator·DNA complex is unknown, and there was excess activator in the cells, whereas we transfected stoichiometric activator·DNA complexes. Efforts to isolate nonpeptidic ligands for Gal80 and KIX domain are underway in our laboratory.

Significance

We demonstrate here that it is possible to construct a synthetic activator that functions in a mammalian nuclear extract from components that were either designed or isolated in simple binding assays. These results are important because, together with earlier efforts from other laboratories, they complete the proof of principle for a pathway for the generation of synthetic activators lacking any native peptide segments.

Experimental Procedures

Synthesis

Peptide nucleic acids (PNA) monomers Fmoc-T-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH, Fmoc-A(Bhoc)-OH, and Fmoc-AEEA-OH; carboxyl activators O-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HoAt) were from Applied Biosystems. Diisopropylethylamine (DIPEA) and 2,6-lutidine were from Sigma-Aldrich. Fmoc-XAL-PEG-PS resin was from NOVAbiochem. All Fmoc amino acid monomers were from BACHEM and Advanced Chemtech.

BisPNA and MisPNA (see Figure 2B for sequences) were synthesized on an Expedite 8900 Nucleic Acid Synthesis System (Applied Biosystems), using standard fluorenylmethoxy-carbonyl (Fmoc) chemistry. Fmoc-XAL-PEG-PS resin was used as solid phase at 2 μmol scale. For each coupling reaction, 10 μmol of each monomer, 10 μmol of HATU, 10 μmol of HoAt, 2.5 μl of DIPEA, and 1 μl of 2,6-lutidine were used. The reaction was carried in 1 ml of 1-methyl-2-pyrrolidinone at ambient temperature for 20 min. When PNA synthesis was finished, the resins with PNA attached were taken off the synthesizer and placed in a 25 ml frit funnel. G80BP-A and 20un peptides (see Figure 2B for sequences) were added to the PNA sequences manually by linear solid-phase Fmoc peptide synthesis. The reaction condition was the same as in the PNA automatic synthesis except that each coupling time was 40 min instead of 20 min. The PNA-peptide conjugates were cleaved by trifluoroacetic acid, purified by reverse-phase HPLC on a C18 column, and lyophilized to dryness. MALDI-TOF: BisPNA, [M+1] calculated 6193.2, found 6188.4; BisPNA-G80BP-A, [M+H] calculated 8731.8, found 8729.4; BisPNA-20un [M+1] calculated 8138.4, found 8140.3; MisPNA [M+1] calculated 6449.1, found 6447.5; MisPNA-G80BP-A [M+1] calculated 8986.8, found 8988.9.

Plasmids

The Gal(1-147)-peptide(s) fusion oligonucleotides for mammalian cell expression were made by PCR using pGBT9-peptide(s) vectors [19] as templates. The PCR products were purified using the QIAGEN gel extraction kit, digested with PstI/NotI, and inserted into PstI/NotI-cleaved pEGFP-N_3 (Promega) vector. The resultant plasmids were named pEDBD-Peptide(s).

The Gal4(1-147)-VP16 fusion oligonucleotide for mammalian cell expression was made by PCR using pGEX-GSTCS-GAL4(1-147)-VP16(78aa) vector as the template. The PCR product was purified by QIAGEN gel extraction kit, digested with PstI/NotI, and inserted into PstI/NotI-cleaved pEGFP-N_3 vector. The resultant plasmid was named pEDBD-VP16.

The firefly luciferase reporter vector pG5B that contains five Gal4 binding sites in the proximal region of a TATA box was a kind gift from Dr. Marc R. Montminy (Salk Institute).

The original G-less cassette reporter plasmids pTF-C_2AT for in vitro transcription were kindly provided by Dr. Stephen A. Johnston (UT Southwestern). The reporter plasmids with a single PNA binding site (pTF-C_2AT-P1) and five PNA binding sites (pTF-C_2AT-P5) close to TATA box were constructed by annealing synthetic oligonucleotides and inserting the fragment into the EcoR1/BamH1-cleaved pTF-C_2AT. The plasmid pTF-C_2AT-NS containing no PNA binding site was made by cleaving pTF-C_2AT with EcoR1 and BamH1, filling with Klenow fragment, and religating.

Strain

Human embryonic kidney 293 cells (American Type Culture Collection, CRL-1573).

Cell Culture, Transfection, and Luciferase Assay

293 cells were grown in 6-well plates at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 400 μg/ml ampicillin, and 100 μg/ml streptomycin. Cells were passed every 24 hr and reached 70% confluence before transfection. The cells were transfected by the Lipofectamine Plus method (Promega protocol) with 1.52 μg of total DNA, including 750 ng of pG5B reporter vector, 750 ng of the respective pEDBD-peptide(s) vector, and 20 ng of *Renilla* luciferase plasmid (pRL-SV40, Promega) for normalization. All transfected cultures were maintained at 37°C under 5% CO_2. Luciferase assays were conducted 40 hr after transfection with a dual-luciferase reporter assay system (Promega). 20% of the total cellular extract was used in each luciferase reaction.

Footprinting

PNA-Peptide·DNA Complexes Preparation

500 bp DNA sequences containing bisPNA binding sites, a TATA box, and a 377 bp G-less cassette were made by PCR using pTF-C_2AT-P5 or pTF-C_2AT-P1 as the templates. The PCR products were purified by PCR purification kit (Qiagen). After purification, the DNAs were labeled with [γ-^{32}P]ATP. 10 μg of DNA templates was mixed with 5 μl of [γ-^{32}P]ATP (6000 Ci/mmol, 10 μCi/μl, Amersham), and 1 μl (10 unit) T4 polynucleotide kinase (NE Biolabs) in 100 μl of reaction buffer (NE labs). The reaction mixture was incubated at 37°C for 60 min. The solution was then extracted with phenol/chloroform. The labeled DNA was separated from the unincorporated [γ-^{32}P]ATP by centrifugation through a Sephadex G-50 column. After labeling, DNAs were digested with HindIII to remove the ^{32}P labels at the G-less cassette end. The singly ^{32}P end-labeled DNAs were isolated by 0.8% agarose gel electrophoresis and extracted from the agarose gel by a gel extraction kit (Qiagen).

30 nM of the singly ^{32}P end-labeled DNA was mixed with 6 μM of PNA-peptide in 10 mM (pH 7.5) phosphate buffer and 1 mM EDTA at ambient temperature for 16 hr. The PNA-peptide·DNA complexes were purified by Qiagen nucleotide purification kit and stored at −20°C. The complexes were stable, as no dissociation was found within one week.

Footprinting Assay

0.5 μg of DNA or 1 μg of PNA-peptide·DNA complex was digested by 1 μl of DNase1 (Invitrogen, 1×10^{-3} U/μl) in 200 μl of assay buffer (10 mM Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, and 100 mM KCl [pH 8.0]) at 37°C for 2 min. The reaction solution was immediately mixed with 700 μl of precipitation buffer (648 μl of ethanol, 2 μl of 3 mg/ml yeast tRNA, and 50 μl of 7.5 M ammonium acetate) at −70°C. The pellets were washed with 70% ethanol twice. Samples were electrophoresed on an 8% (5% cross-link) urea-acrylamide gel at 1300V for 1.5 hr. Urea was removed by 15% methanol

and 5% acetic acid before the gel was dried at 80°C. The gel was autographed by exposing to a Kodak X-OMAT film.

PNA binding sites were confirmed by DNA sequencing using a standard DNA polymerase protocol (Sequenase 2.0, USB, Amersham Pharmacia).

In Vitro Transcription

PNA-Peptide·DNA Complexes Preparation

Plamids pTF-C_2AT-P1, pTF-C_2AT-P5, and pTF-C_2AT-NS were digested with HindIII. The products were purified with Qiagen nucleotide purification kit. Linear DNAs were stored in nuclease-free water. 20 nM linear DNA and 3.4 μM of BisPNA-peptide (or MisPNA-peptide) were mixed in 10 mM (pH 7.5) phosphate buffer and 1 mM EDTA at ambient temperature for 16 hr. The PNA-peptide·DNA complexes were purified by Qiagen PCR purification kit.

In Vitro Transcription

In 30 μl reaction volume, 2 nM specific template (DNAs or PNA-peptide·DNA complexes), 6 μl of (50 μg proteins) HeLa nuclear extract (Promega), the A/C/UTP mixture (final concentrations: 100 μM ATP and CTP, 5 μM UTP), and 0.5 μl [α-^{32}P]UTP (3000 Ci/mmol, 10 μCi/μl, Amersham) were mixed in transcription buffer (10 mM HEPES [pH 7.9], 10% glycerol, 50 mM KCl, 10 mM EDTA, 250 μM PMSF, 250 μM DTT, 5 mM $MgCl_2$). The reaction mixture was incubated at 30°C for 60 min. After that, 5 μl of Rnase T1 (30U, Roche) in buffer 0.1 M EDTA was added, and the mixture was incubated for an additional 10 min at 30°C. All reactions were terminated by adding 100 μl of stop buffer (7 M urea, 350 mM NaCl, 10 mM Tris [pH 7.4], 10 mM EDTA, and 1% SDS). The reaction products were purified by phenol/chloroform extraction and precipitated in a precipitation solution containing 500 μl of ethanol, 100 μl of 1 M ammonium acetate, and 1 μl of 10 mg/ml yeast tRNA. The pellets were suspended in formamide loading dye and electrophoresed on 8% urea-polyacrylamide gels. The gels were dried and the bands visualized on a phosphor screen (Molecular Dynamics).

Acknowledgments

This work was funded by a grant from the NIDDK/NIH (P01-DK58398).

Received: June 22, 2003
Revised: July 22, 2003
Accepted: July 30, 2003
Published: October 17, 2003

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