A Highly Potent Artificial Transcription Factor†

Dusan Stanojevic*,‡ and Richard A. Young§

Crosslink Genetics Corporation, One Kendall Square, Building 600, PMB 255, Cambridge, Massachusetts 02139, and Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142

Received October 31, 2001; Revised Manuscript Received February 18, 2002

ABSTRACT: The use of synthetic chemical moieties to design fully functional analogues of transcription factors will give rise to novel molecular tools for targeted gene regulation. Here we demonstrate that a synthetic molecule based on a nonpeptidic DNA-binding domain can be engineered to function as a highly potent transcription factor in vitro and in an intracellular context. The structure of this artificial transcription factor (ATF) consists of three parts: (i) triple-helix-forming oligonucleotide as a DNA-binding domain; (ii) composite linker moiety; and (iii) short synthetic peptide. The direct comparison of ATFs with natural transcription factors in in vitro assays reveals the ability of ATFs to initiate RNA transcription at the correct initiation site. In addition, the transcriptional activation potency of ATFs in vitro matches or exceeds the potency of GAL4-VP16, one of the strongest natural transcriptional activators. This remarkable biological activity is explained as a function of ATF's chemical structure. We also demonstrate for the first time that ATFs possess substantial ability to activate transcription in tissue culture cells, thus opening a prospect for practical applications in basic and applied research. The specific molecular design employed in the synthesis of ATFs may lead to the development of novel gene-targeting pharmaceuticals for treatment of fatal and chronic diseases.

The control of RNA synthesis by transcription factors is a crucial step in regulation of gene expression in all eukaryotes. Transcription factor proteins typically contain at least two functionally independent parts: a DNA-binding domain and an effector (activation or repression) domain. The DNA-binding domain anchors the transcription factor to the promoter while the effector domain binds other proteins to activate or repress RNA synthesis (*1*−*4*). Conceptually, such a modular structure implies that each transcription factor molecule could be regarded as a bidentate ligand. This raises the possibility of replacing the DNA-binding and/or effector domain with synthetic chemical moieties to yield a novel class of molecules—synthetic (or "artificial") transcription factors (ATFs).[1] The main goal of the ATF molecular design is to introduce new, drug-like chemical properties such as a lower molecular weight, resistance to enzymatic degradation, and cell membrane permeability while preserving the crucially important biological function—the ability to regulate RNA transcription of specific genes. Previous attempts to design transcriptional activators from synthetic compounds have yielded molecules having a barely detectable activity (*5*) or showing a relatively weak and limited activation only in in vitro (cell-free) assays (*6*, *7*). In contrast, the goal of the present study is to design much more potent synthetic transcriptional activators whose biochemical activity would be comparable to that of natural transcription factors. Here we describe a unique ATF molecular design assembled from simple, commercially available components. These ATFs possess biochemical properties inherent in natural transcription factors such as the ability to initiate RNA transcription at the correct start site and extremely high transcriptional activation potency in vitro. In addition, the evidence of a substantial ATF activity in an intracellular context is presented for the first time. These findings illustrate the significant potential of ATFs as novel tools for gene manipulation at the level of transcription and are likely to point the way toward new strategies for development of gene-targeting pharmaceuticals for treatment of cancer and many other diseases.

EXPERIMENTAL PROCEDURES

ATF Synthesis. The introduction of a polylinker into the oligonucleotide was achieved by synthesizing a molecule of the general formula CYXCTTGTGGTGGGTGGGGTGT-GGGT, where X represents the Spacer Phosporamidite 18 (Glenn Research) and Y represents the modified T residue bearing the primary amine group on a short tether (Amino-Modifier C2-dT, Glenn Research). This primary amine is the only such chemical group in the oligonucleotide, and it is able to react with various kinds of chemicals that do not affect the rest of the molecule.

The two effector peptides (29-mer and 14-mer) were synthesized on an automated peptide synthesizer using standard FMOC chemistry. The chemical conjugation of each peptide to the rest of the ATF molecule was accomplished through the use of the bifunctional cross-linker succinimidyl

† This work was supported by National Institute for General Medical Sciences STTR Grant R41 GM57712.

* To whom correspondence should be addressed. E-mail: ds@crosslinkgenetics.com.

‡ Crosslink Genetics Corp.

§ Whitehead Institute for Biomedical Research.

[1] Abbreviations: ATF, artificial transcription factor; AD, activation domain; TFO, triple-helix-forming oligonucleotide; C, degrees Celsius; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; SEM, standard error of the mean.

10.1021/bi015906b CCC: $22.00
Published on Web 05/15/2002

6-maleimidylhexanoate (EMCS, Molecular Probes). This cross-linker has two functional groups separated by a six carbon chain: an ester of succinic acid reacts specifically with primary amines, and a maleimide functional group reacts specifically with thiols. The coupling reaction is performed in two successive steps. Since the ester group is the more labile of the two, the cross-linker is first coupled to the amine in a buffer containing 100 mM NaHCO (pH 8) at room temperature for 30 min. The excess cross-linker is removed by chloroform extraction and ethanol precipitation. The second step involves the coupling between the thiol group in the peptide and the maleimide functional group of the cross-linker. The coupling is performed in 100 mM $NaPO_4$ (pH 7) at room temperature for 3 h. The purification of the conjugate was accomplished by reverse-phase HPLC.

Gel Mobility Shift Assays. ATF(29) (10 pmol) was labeled with ^{32}P by incubation with [γ-^{32}P]ATP and kinase (Promega) at 37 °C for 30 min. The reaction was terminated by phenol/chloroform extraction, followed by ethanol precipitation (2 times). The final pellet was dissolved in Tris−EDTA buffer (pH 8) and stored at −70 °C. The 150 bp DNA fragment containing five ATF-binding sites was obtained by digestion of 2 μg of plasmid DNA containing ATF transcription template with *Hin*dIII and *Pst*I restriction enzymes (Promega) and purification on an agarose gel, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in Tris−EDTA buffer and stored at −20 °C. The control DNA fragment of similar size containing no ATF-binding sites was obtained from the G5E4T plasmid (*8*) after digestion with *Hin*dIII and *Bam*HI and following the procedure described above.

The triple-helix formation between the ^{32}P-labeled ATF and the 150 bp DNA fragment (which was not labeled) was initiated by mixing an aliquot of each (containing approximately 1 pmol) in the binding buffer containing 10 mM Tris, pH 8, 40 mM $MgCl_2$, and 100 mM KCl and incubation at room temperature for up to 12 h. The triple-helical complex was resolved by nondenaturing polyacrylamide gel electrophoresis (20% gel) in TBE buffer supplemented with 20 mM $MgCl_2$. The dried gel was exposed on a Fuji Phoshpoimager plate and was visualized by Fuji MacBAS software.

In Vitro Transcription. Two 26-base oligonucleotides, 5′GTTCTCCTCCCTCCCCTCTCCCTCTT3′ and 5′CAAGAGGGAGAGGGGAGGGAGGAGAA3′, were annealed and ligated to yield a series of double-stranded DNA fragments containing multiple binding sites for triple-helix formation. A fragment containing 5 copies of the site was purified by agarose gel electrophoresis and inserted into the *Hin*dIII restriction site in the polylinker of the GnE4T series of transcription templates (*8*). The resulting plasmids are linearized by digestion with *Eco*RI and *Hin*dIII restriction enzymes (Promega). The transcription assay is initiated by incubation of 20−100 ng of linearized transcription templates with ATFs under the same experimental conditions used for gel mobility shift assays described above (10 mM Tris, pH 8, 40 mM $MgCl_2$, and 100 mM KCl at room temperature for several hours). At the end of the incubation period, an aliquot of commercially obtained HeLa nuclear extract (Promega) was added to all reactions, and GAL4-VP16 protein (calibrated to yield maximal activation) was added to the control templates (*8*, *16*, *20*, *23*). All reactions were incubated for 10 min at 30 °C. The transcription was subsequently initiated by the addition of 1 μL of a mixture of ribonucleotide triphosphates (ATP, CTP, GTP, 10 mM each) and 0.5 μL of [α-^{32}P]UTP (NEN, Boston, MA). After further incubation at 30 °C for 30 min, all transcription reactions were terminated by the addition of 100 μL of stop buffer (0.5 M sodium acetate, 0.2% SDS, 10 mM EDTA, 1 μg/mL glycogen, 1 μg/mL Proteinase K). The reactions were vortexed, incubated at 37 °C for 10 min, extracted with phenol/chloroform, and precipitated with 250 μL of ice-cold ethanol. The pellets were dissolved in 20 μL of standard denaturing formamide/dye mix and were loaded and run on a denaturing 6% polyacrylamide gel. Dried gels were exposed both on Kodak Biomax MS film and on Fuji Phosphoimager plates. The quantitation of signals was performed with Fuji MacBAS software.

Cell Permeability Studies. Aliquots containing 100 pmol of ATF(14) or ATF(29) were labeled with AlexaFluor 488 fluorescent tag using the ULYSIS Nucleic Acid Labeling Kit (Molecular Probes) according to the manufacturer's protocol. HeLa tissue culture cells were plated on a glass 16-well chamber slide (Lab-Tec Chamber Slide System, Nalge Nunc International) in DMEM medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies) (*9*) until about 50% of each chamber was covered with cells. The cells were subsequently washed twice with DMEM medium containing no serum. An aliquot of 60 μL of DMEM medium (no serum) containing the labeled ATFs at a concentration of 160 nM was added to each chamber. Some of the reactions also included LipofectAMINE (Life Technologies) in the amount of 0.5 μL per reaction. After 3−12 h incubation at 37 °C, the cells were washed twice with PBS and covered with mounting solution (SlowFade Light Antifade Kit with DAPI, Molecular Probes). Mounted cells were observed with a Nikon Fluorescence microscope using appropriate filers, and digital images were analyzed with OpenLab sofware.

Tissue Culture Transcription Assays. As a control template, we used a reporter construct containing the minimal HSV thymidine kinase promoter driving the expression of CAT reporter gene (*10*). The ATF transcription template was constructed by incorporating the oligonucleotide with 5 copies of ATF-binding sites between the *Hin*dIII and *Bam*HI restriction sites of the polylinker upstream of the control template promoter (Figure 5A). The standard transfection mixture contained 1 μg of the plasmid DNA and 10 or 50 nM ATFs. Transfections were performed with the polycationic lipid LipofectAMINE (Life Technologies) as described in (*9*). Cells were harvested 48 h after transfection, and CAT assays were performed using FAST CAT (Molecular Probes, Inc.) as a substrate. The assays were visualized and quantified on a Fluorimager (Molecular Dynamics).

RESULTS

Design and Synthesis of ATFs. The basic ATF structure and design are described in Figure 1. As a DNA-binding domain, we utilized the 22-mer triple-helix-forming oligonucleotide (TFO). The TFOs have been used to target specific DNA sequences and sites in gene promoters for over a decade (*11*−*14*). This particular 22-mer TFO sequence (Figure 1) has been shown to form a stable triple-helical

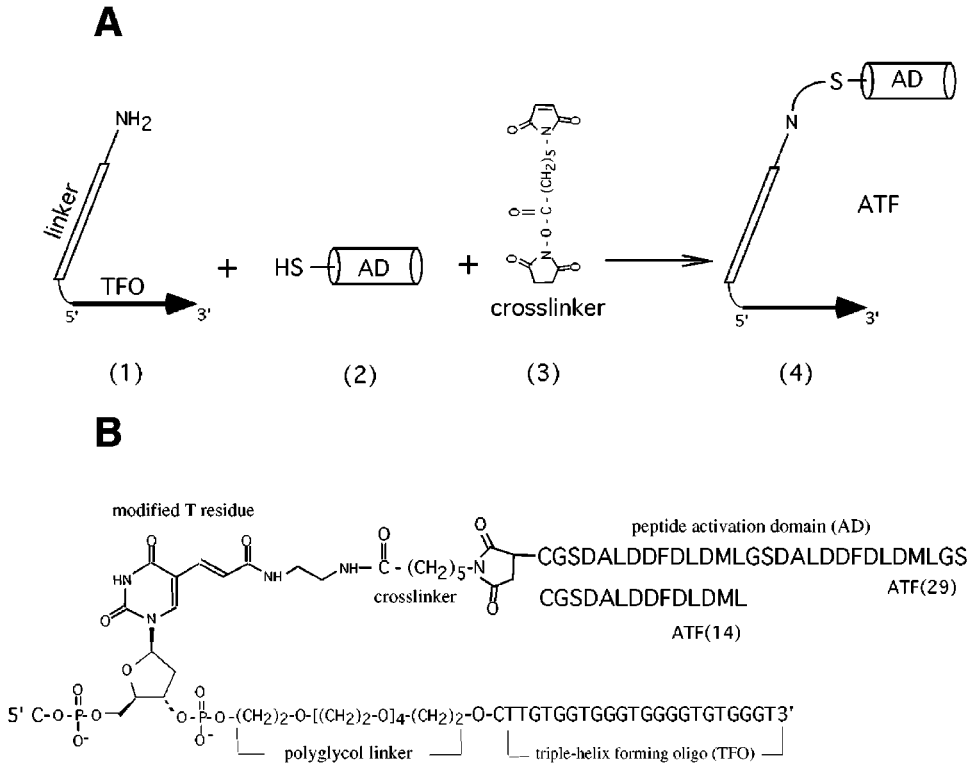

FIGURE 1: ATF structure and design. (A) A schematic diagram for the synthesis of ATFs. Triple-helix-forming oligonucleotide (TFO) is shown as a thick arrow with linker domain attached at the 5′ end. The activation domain (AD) contains a thiol-bearing cysteine residue to serve as the attachment point for chemical conjugation to the amine group of the linker domain through a bifunctional cross-linker. (B) Detailed molecular structure of ATFs. The DNA-binding domain consists of a triple-helix-forming 22-mer oligonucleotide sequence, 5′TTGTGGTGGGTGGGGTGTGGGT3′. The additional C residue and a polyglycol linker were incorporated at the 5′ end of the TFO, followed by the modified T residue carrying an amine group. The first C residue at the 5′ terminus facilitates the labeling of ATFs with phosphate kinase. The effector peptide consists of either one or two repeats of a VP-16-derived sequence, DALDDFDLDML (*17*). The single cysteine residue was incorporated at the amino terminus of each peptide.

complex with target DNA at physiological pH (*15*). A long and flexible polyglycol linker is inserted near the 5′ end of the TFO (Figure 1B). The distal end of the linker bears a modified thymidine residue containing the primary amine group on a short, two-carbon tether (see Experimental Procedures). This primary amine serves as a unique anchoring site for the coupling of transcriptional activation domain (AD). The AD consists of a synthetic 29-mer or 14-mer peptide sequence derived from herpes simplex virus (HSV) protein VP16, one of the strongest transcriptional activators found in nature (*16*). This 29-mer sequence has been shown to retain about 70% of the activity of the full-length VP16 activation domain when attached to the GAL4 DNA-binding domain (*17, 18*). A thiol-bearing cysteine residue is incorporated at the amino terminus to allow for a covalent linkage with the rest of the ATF molecule. The chemical conjugation of the peptide to the rest of ATF molecule was accomplished through a bifunctional cross-linker (Figure 1, Experimental Procedures) (*19*). The coupling reactions resulted in two forms of ATF molecules: ATF(29) carrying the 29-mer VP16 peptide, and ATF(14) containing a shorter version of the VP16 peptide with only 14 amino acid residues (Figure 1B).

ATFs Activate Transcription in Vitro. The transcription templates were synthesized by incorporating five direct repeats of the ATF- or GAL4-binding DNA sequence sites into the polylinker upstream of the basal promoter of adenovirus E4 reporter gene as shown in Figure 3A,B (*8*). The sequence-specific binding of ATFs to the promoter via triplex formation was demonstrated by gel-mobility shift assays (Figure 2). The in vitro transcription runoff assays were performed with crude HeLa nuclear extract following standard protocols (as described under Experimental Procedures).

The results in Figure 3C show that, as expected, GAL4-VP16 strongly activates transcription from the control template containing five GAL4-binding sites. The runoff transcript initiated at the +1 transcription start site is 250 bp long (indicated by arrow). At the same time, ATF(29) is able to activate the transcription from templates containing five ATF-binding sites in the promoter (Figure 3C). The activity of ATF(29) is absolutely dependent on the presence of the corresponding binding sites in the promoter as it does not activate transcription from the control templates or other templates lacking ATF-binding sites (Figure 3D, data not shown). Comparison of RNA transcripts confirms that both GAL4-VP16 and ATF(29) initiate the transcription from the same (+1) site in the promoter adjacent to the TATA box. To our surprise, the maximum level of transcriptional activation by ATF(29) was comparable to the maximal level that could be obtained with GAL4-VP16. For example, the quantitation of RNA transcripts reveals that both GAL4-VP16 and ATF(29) activate transcription 30−40-fold above the basal level (Figure 3, Experimental Procedures). This result corresponds to previously reported maximal levels of in vitro transcriptional activation by GAL4-VP16 from linearized templates containing five GAL4-binding sites (*20*). These levels of activation are obtained with ATF concentra-

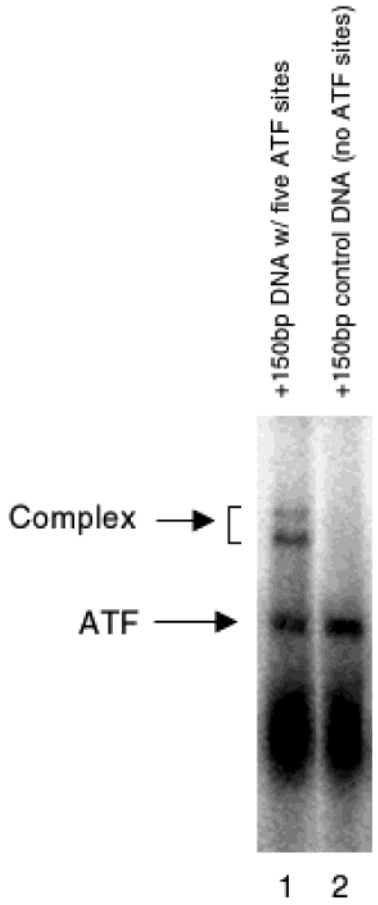

FIGURE 2: Sequence-specific binding of ATF to promoter DNA. Lane 1: The binding reaction of ^{32}P-labeled ATF(29) and unlabeled 150 bp DNA fragment containing five ATF-binding sites. Arrows on the left mark the labeled ATF(29) in the free form and in the complex with the 150 bp promoter DNA fragment. Closely spaced multiple bands corresponding to the triple-helical complex can be attributed to the differences in gel mobility of complexes containing different numbers of bound ATF(29) molecules. Lane 2: The control binding reaction of ATF(29) and the control 150 bp promoter fragment having no ATF-binding sites. It is evident that the formation of triple-helical complex does not occur.

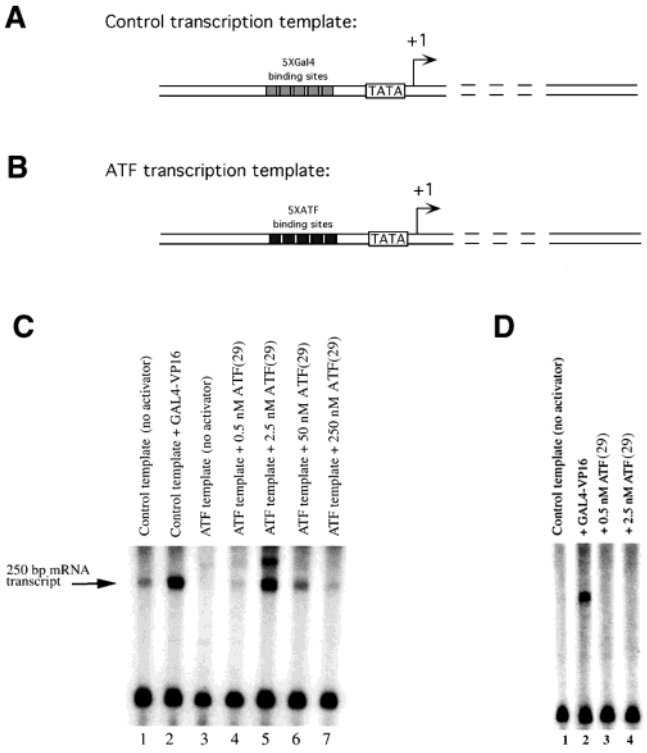

FIGURE 3: Activation of transcription by ATF in vitro. (A) Control transcription template contains five GAL4-binding sites incorporated into the promoter region. (B) The ATF transcription template differs from the control template only in that it contains five ATF-binding sites instead of GAL4-binding sites. (C) The transcriptional activation in vitro by GAl4-VP16 and ATF(29). Lanes 1 and 2 show the typical level of activation by GAL4-VP16. Lane 3−7 represent the transcriptional activation by ATF(29). The 250 bp RNA transcript produced by GAL4-VP16 and ATF(29) is indicated by the arrow. The biological activity of ATF(14) in vitro is very similar to that of ATF(29) (data not shown). (D) The transcriptional activation in vitro from templates containing GAL4-binding sites, but no ATF-binding sites (the control template shown in Figure 3A). As expected, strong transcriptional activation by GAL4-VP16 protein is evident by comparing the signal in lanes 1 and 2. At the same time, ATF(29) is not able to activate the transcription from the same template under the optimal conditions (lanes 3 and 4).

tions between 1 and 40 nM, and further increase in concentration leads to diminishing activation (Figure 3C). This effect indicates another similarity of ATFs with strong natural activators: the ability to "squelch" transcriptional activation, presumably by sequestration of the target components of the transcriptional machinery by excess amounts of strong activators (*21*, *22*). Both the activation and the squelching ability are completely dependent on the presence of the attached AD peptide because the "truncated" ATFs lacking the AD proved completely inactive (data not shown).

Transcriptional Activation by ATFs in Tissue Culture Cells. The cell permeability of ATFs was studied by incubation of labeled ATFs with HeLa tissue culture cells. The results shown in Figure 4 demonstrate that ATFs are able to enter living cells with ease even without the use of carriers such as lipofectamine. After the 3 h incubation period, ATF molecules were located predominantly in nuclei (Figure 4). The addition of lipofectamine increases the cell permeability of all ATFs only about 2-fold (D.S., unpublished data).

The ability of ATFs to activate transcription in vivo was examined by transient cotransfection assays in HeLa or BHK-21 tissue culture cells (*9*). Each reporter construct shown in Figure 5A contains the chloramphenicol acetyltransferase (CAT) reporter gene controlled by the adenovirus thymidine kinase (TK) minimal promoter (*10*). The five direct repeats of ATF-binding sequence were inserted into the polylinker lying immediately upstream of the promoter to yield the in vivo transcription template, while the in vivo control template contains no ATF sites (Figure 5A). Each construct was transfected into cells with and without ATFs, and the activation signal was measured with standard methods (see Experimental Procedures). The results shown in Figure 5 reveal that ATF(29) activates transcription from the in vivo transcription template 5-fold above the basal level. At the same time, ATF(14) caused nearly 30-fold activation of transcription from the same template. This effect is sequence-specific since none of the ATFs were able to activate transcription from the control template lacking the ATF-binding sites (Figure 5C,D). Similarly to in vitro assays, the intact ATF structure is necessary for in vivo function as well. Namely, ATFs having no peptide attached were not able to activate the transcription from either template (data not shown).

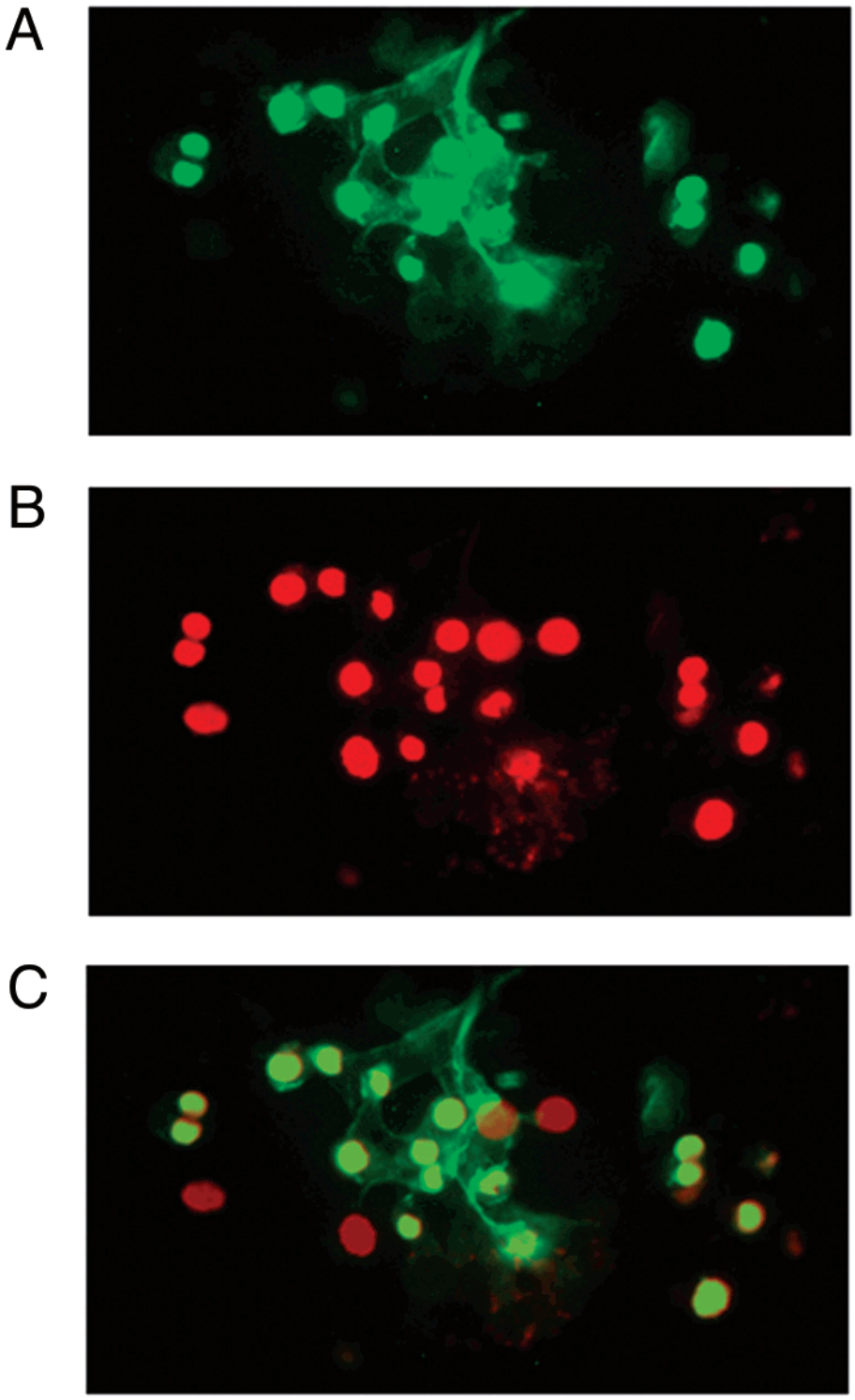

FIGURE 4: Cell permeability of ATFs. (A) HeLa tissue culture cells incubated with labeled ATF(29) (green) for 3 h in the absence of lipofectamine or any other carrier. The intracellular presence of ATF is indicated by the intense green color. (B) The same cells counterstained with DAPI show only nuclei of living cells stained in red. (C) The superimposition of the first two pictures demonstrates the presence of labeled ATFs predominantly in the nuclei of tissue culture cells. About 80% of cells are penetrated by ATF(29). The ATF(14) shows a similar, only 2-fold more intense signal after prolonged, 12 h incubation (data not shown).

DISCUSSION

Significance of Fully Functional ATFs. While there are many successful drugs that target gene products, thus far there is no effective and practical strategy for pharmaceutical targeting of the gene itself. The manipulation of gene expression at the level of transcription has a great potential for drug development because the transcriptional apparatus and basic regulatory mechanisms are common for a great majority of eukaryotic genes (*23*−*26*). In principle, a small-molecule drug designed to modulate transcription of a specific gene can be applied to cause a similar effect on a different gene with only small modifications in its DNA-binding moiety. Therefore, cell-permeable drugs able to turn specific genes on or off will form the basis for a universal

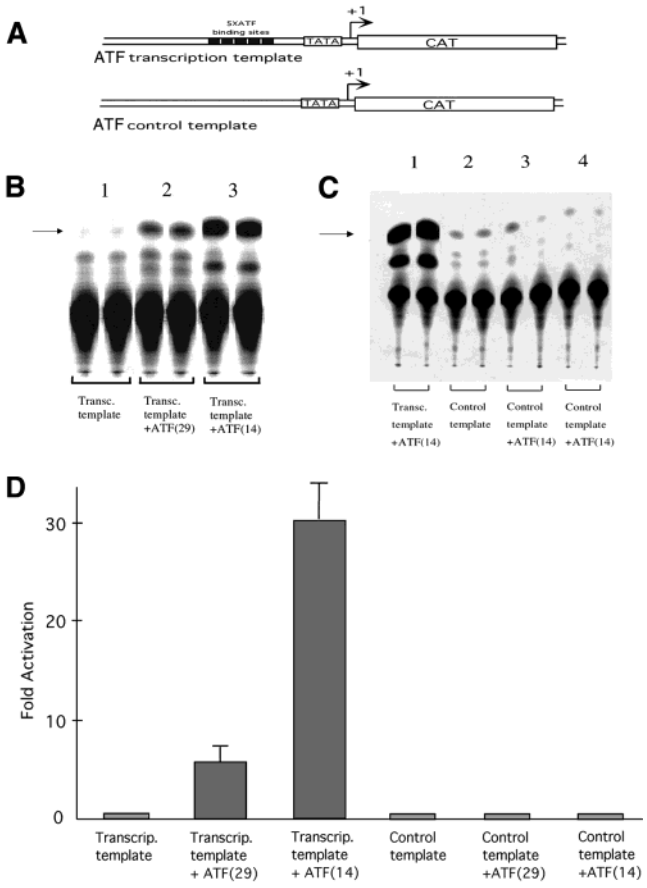

FIGURE 5: Transcriptional activation by ATFs in tissue culture cells. (A) Reporter constructs used in cotransfection assays. The transcription template contains five ATF-binding sites in the promoter, while the control template has no ATF-binding sites. (B) A representative CAT assay whereby tissue culture cells were cotransfected with transcription template alone (lanes 1) or in combination with ATFs (lanes 2 and 3). The ATFs are added to the transfection reaction at a final concentration of 50 nM. (C) A representative CAT assay comparing the activation by ATF(14) from ATF transcriptional templates (lane 1) with the activation from control templates (lanes 2−4). The addition of either 10 nM ATF(14) (lane 3) or 50 nM ATF(14) (lane 4) has no effect on basal transcription from the control template (lane 2). (D) A summary of the biological activity of both ATFs in tissue culture cells. Mean fold transcriptional activation by ATFs for three experiments, each performed in duplicate. Error bars represent SEM ($n = 3$).

therapeutic strategy, applicable to many different genes of medical interest.

The progress in this field has been hampered by the fact that designing an effective ATF proved to be a major challenge. For example, previous attempts to combine synthetic peptides with polyamide DNA binders produced transcriptional activators that bind DNA via a minor groove and exhibit a relatively low activity in in vitro assays (*6*, *7*). Since those studies employ only the reporter gene containing the G-less cassette, it is not clear whether the overall increase in RNA is due to the specific or nonspecific (i.e., random) transcript initiation. However, the development of the first practical applications for ATFs requires the achievement of biological activity and specificity approaching those of natural transcription factors. The present study demonstrates that it is possible to design fully functional ATFs that (i) initiate RNA transcription from the correct promoter site and (ii) possess biological activity comparable to strong transcriptional activator proteins.

Activation Potency Depends on ATF Chemical Structure. The design of highly potent ATF was achieved through several iterations of molecular design and synthesis. For example, we found that various ATF designs containing no linker moiety possess extremely low activity (D.S., unpublished data; ref *5*). However, the addition of linker separating the AD from the TFO drastically improves the ability of ATFs to activate the transcription. The exact chemical configuration of the linker possibly plays a major role in the optimal presentation of the effector moiety to the transcriptional apparatus. Namely, the combination of a long and flexible polyglycol chain with a more rigid and hydrophobic nucleotide cross-linker structure seems be able to mimic the molecular geometry of natural transcription factors particularly well. In fact, the in vitro transcription assays described in the results shown in Figure 3 imply that ATFs could be even more potent transcriptional activators than proteins such as GAL4-VP16. For example, GAL4-VP16 protein binds to each of the five sites in the promoter as a dimer, while ATF molecules bind the corresponding triple-helix target sites as monomers (*27*). However, 5 ATF molecules (carrying 5 activation domains) bound to the promoter cause a similar effect on transcription in vitro compared to 10 GAL4-VP16 molecules (Figure 3C). This high potency of ATF molecules may be due to the simple extended chemical structure involving no bulky protein domains that leaves activation domains much more exposed to interaction with RNA polymerase II holoenzyme and/or other proteins. The exposed activation domain would in effect facilitate the recruitment of the holoenzyme to the promoter and the initiation of RNA transcription. This conclusion is in accord with previous work showing that a 14-mer peptide derived from VP16 (Figure 1B) is inactive when fused to the GAL4 DNA-binding domain through recombinant DNA techniques (*17*). In contrast, the present study demonstrates that the same 14-mer peptide sequence shows a remarkable and specific biochemical activity within the structural context of ATF (Figures 3 and 5).

Toward in Vivo Applications. The results of cotransfection assays in tissue culture cells demonstrate for the first time a substantial biochemical activity of ATFs in an intracellular environment. The ATFs are able to penetrate tissue culture cells with ease and are localized predominantly in nuclei after a relatively short incubation time (Figure 4). Furthermore, ATFs bind to the promoter and elicit a strong (up to 30-fold) activation of reporter gene transcription upon introduction into tissue culture cells (Figure 5). This ATF-generated transcription signal is sequence-specific; namely, the presence of ATF-binding sites in the promoter is absolutely required for transcriptional activation (Figure 5C,D). The introduction of ATFs into tissue culture cells causes no apparent toxic or side effects after 48 h incubation, thus further confirming the specificity and selectivity of ATFs.

The magnitude of the transcriptional activation signal obtained with ATFs should be taken in context of the specific experimental setup. Namely, while in vitro assays allow the direct comparison of activation signals obtained with natural transcription factors and ATFs (Figure 3), it would be very difficult to interpret such comparisons in tissue culture assays. For example, transfection assays require the introduction of GAL4-VP16 expression constructs into tissue culture cells, thus resulting in continuous synthesis of the GAL4-VP16

protein inside the cell (*9*). This "steady supply" of GAL4-VP16 activator protein would be in stark contrast with the introduction of ATFs through the cell membrane at the start of a transient transfection assay. Therefore, it would be very difficult to compare and calibrate intracellular concentrations and activation signals of natural vs artificial activators over the course of these experiments. A similar reasoning could be used to explain the apparent ability of ATF(14) to cause a much stronger effect than ATF(29) in tissue culture assays. Since ATF(14) contains the shorter AD with lower total electrostatic charge, it is expected to have an increased cell permeability, resulting in higher intracellular concentrations compared to the larger ATF(29) (Figure 4).

Gene Targeting Based on Triple-Helix Formation. The formation of triple-helical DNA complexes has been studied extensively over the last 14 years (*28*). All TFOs described thus far bind in the major groove of the DNA through Hoogsteen hydrogen bonding with remarkable specificity and stability (*11*, *12*, *29*, *30*). Although general Hoogsteen base-pairing rules restrict the triplex targeting to DNA sequences containing polypurine or polypyrimidine stretches, the repertoire of potential target DNA sites has been greatly extended through the use of modified bases or linkers (*14*, *31*).

The triple-helix formation has been used to target unique sites in the mammalian genome and repress transcription of a specific gene in tissue culture cells (*13*, *32*, *33*). Conceptually, this kind of targeting strategy employs TFOs (or other synthetic DNA-binding molecules) to interfere with the binding of endogenous transcription factors to specific sites in the target promoter. However, this "passive" gene targeting is severely limited by the requirement that TFO-binding sites in the promoter overlap with binding sites for endogenous transcription factors. This fortuitous arrangement or natural and nonnatural binding sites in native promoters is bound to occur very infrequently, and thus far there are only several described examples, such as *c-myc* or progesterone responsive genes (*13*, *28*, *33*, *40*). In addition, the passive targeting method requires a detailed understanding of the transcriptional regulation of the target gene, including the locations of all binding sites for the key transcriptional factor. This task is generally very difficult given the size and complexity of mammalian promoters as well as a general redundancy of transcription factor binding sites. In contrast, the targeting strategy described in the present study relies on the delivery of "active" effector moieties (activators or repressors) to promoters via TFOs. The binding of ATF delivers the effector moiety to the promoter and triggers the desired effect through interaction with the basal transcriptional machinery. Given that most transcriptional activator or repressor domains act over a distance, the precise location of the ATF-binding site within the promoter is not critical. Consequently, this method allows for a much greater flexibility in the relative position of TFO-binding sites within the target gene promoter. In addition, no detailed knowledge of transcriptional regulation of the target gene is necessary; the only information required in advance is the sequence of the target promoter. For these reasons, we believe that the active gene targeting strategy based on ATFs has a significant potential to expand the number of accessible targets throughout the genome. In addition, ATFs possess some intrinsic advantages over polypeptide-based gene targeting methods such as those involving zinc-finger proteins (*39*). The crucial advantages of ATFs include cell-permeability and the potential for incorporation of effector domains based on nonpeptidic chemical moieties.

CONCLUSIONS

This study demonstrates for the first time that transcription factors can be designed and synthesized from synthetic chemical moieties without the compromise in biological activity. The results presented here make it possible to envision fully functional ATFs based on a variety of different natural or artificial chemical moieties with improved pharmacodynamic and pharmacokinetic properties (*34*−*36*). The successful demonstration of an ATF activity in an intracellular context opens the door toward the application of ATFs as tools for targeting genes in their natural, chromosomal setting (*32*). The progress in this field of research will give rise to new generations of ATFs with possible applications not only as pharmaceuticals, but also as novel tools in molecular biology and functional genomics (*37*−*39*).

ACKNOWLEDGMENT

We are grateful to P. T. Matsudaira, A. Barberis, F. Holstege, F. Robert, D. Pokholok, B. Ren, J. Nau, and V. Stanojevic for help and discussions. D.S. thanks C. Carpenter and N. Hannet for assistance and support.

REFERENCES

1. Brent, R., and Ptashne, M. (1985) *Cell 43*, 729−736.
2. Ma, J., Przibilla, E., Hu, J., Bogorad, L., and Ptashne, M. (1988) *Nature 334*, 631−633.
3. Renkawitz, R. (1990) *Trends Genet. 6*, 192−197.
4. Wolffe, A. P., Wong, J., and Pruss, D. (1997) *Genes Cells 2*, 291−302.
5. Kuznetsova, S., Ait-Si-Ali, S., Nagibneva, I., Troalen, F., Le Villain, J. P., Harel-Bellan, A., and Svinarchuk, F. (1999) *Nucleic Acids Res. 27*, 3995−4000.
6. Mapp, A. K., Ansari, A. Z., Ptashne, M., and Dervan, P. B. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 3930−3935.
7. Ansari, A. Z., Mapp, A. K., Nguyen, D. H., Dervan, P. B., and Ptashne, M. (2001) *Chem. Biol. 8*, 583−592.
8. Lin, Y. S., Carey, M. F., Ptashne, M., and Green, M. R. (1988) *Cell 54*, 659−664.
9. Stoffers, D. A., Stanojevic, V., and Habener, J. F. (1998) *J. Clin. Invest. 102*, 232−241.
10. Ubeda, M., Vallejo, M., and Habener, J. F. (1999) *Mol. Cell. Biol. 19*, 7589−7599.
11. Le Doan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J. L., Thuong, N. T., Lhomme, J., and Helene, C. (1987) *Nucleic Acids Res. 15*, 7749−7760.
12. Moser, H. E., and Dervan, P. B. (1987) *Science 238*, 645−650.
13. Postel, E. H., Flint, S. J., Kessler, D. J., and Hogan, M. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8227−8231.
14. Giovannangeli, C., and Helene, C. (1997) *Antisense Nucleic Acid Drug Dev. 7*, 413−421.
15. Skoog, J. U., and Maher, L. J. F. (1993) *Nucleic Acids Res. 21*, 2131−2138.
16. Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988) *Nature 335*, 563−564.
17. Seipel, K., Georgiev, O., and Schaffner, W. (1992) *EMBO J. 11*, 4961−4968.
18. Nyanguile, O., Uesugi, M., Austin, D. J., and Verdine, G. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13402−13406.
19. Kitagawa, T., Shimozano, T., Aikawa, T., Yoshida, T., and Nishimura, H. (1981) *Chem. Pharm. Bull. 29*, 1130−1135.

20. Haile, D. T., and Parvin, J. D. (1999) *J. Biol. Chem. 274*, 2113−2117.
21. Ptashne, M. (1988) *Nature 335*, 683−689.
22. Tasset, D., Tora, L., Fromental, C., Scheer, E., and Chambon, P. (1990) *Cell 62*, 1177−1187.
23. Lin, Y. S., Carey, M., Ptashne, M., and Green, M. R. (1990) *Nature 345*, 359−361.
24. Struhl, K. (1996) *Biochim. Biophys. Acta 1288*, 1015−1017.
25. Roberts, S. G. (2000) *Cell. Mol. Life Sci. 57*, 1149−1160.
26. Malik, S., and Roeder, R. G. (2000) *Trends Biochem. Sci. 25*, 277−283.
27. Carey, M., Kakidani, H., Leatherwood, J., Mostashari, F., and Ptashne, M. (1989) *J. Mol. Biol. 209*, 423−432.
28. Casey, B. P., and Glazer, P. M. (2001) *Prog. Nucleic Acid Res. Mol. Biol. 67*, 163−192.
29. Rougee, M., Faucon, B., Mergny, J. L., Barcelo, F., Giovannangeli, C., Garestier, T., and Helene, C. (1992) *Biochemistry 31*, 9269−9278.
30. Singleton, S. F., and Dervan, P. B. (1992) *Biochemistry 31*, 10995−11003.
31. Griffin, L. C., Kiessling, L. L., Beal, P. A., Gillespie, P., and Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 7976−7982.
32. Catapano, C. V., McGuffie, E. M., Pacheco, D., and Carbone, G. M. (2000) *Biochemistry 39*, 5126−5138.
33. Postel, E. H. (1992) *Ann. N.Y. Acad. Sci. 660*, 57−63.
34. Izvolsky, K. I., Demidov, V. V., Nielsen, P. E., and Frank-Kamenetskii, M. D. (2000) *Biochemistry 39*, 10908−10913.
35. Larsen, H. J., Bentin, T., and Nielsen, P. E. (1999) *Biochim. Biophys. Acta 1489*, 159−166.
36. Frangioni, J. V., LaRiccia, L. M., Cantley, L. C., and Montminy, M. R. (2000) *Nat. Biotechnol. 18*, 1080−1085.
37. Chubb, J. M., and Hogan, M. E. (1992) *Trends Biotechnol. 10*, 132−136.
38. Helene, C., Giovannangeli, C., Guieysse-Peugeot, A. L., and Praseuth, D. (1997) *Ciba Found. Symp. 209*, 94−102.
39. Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 14628−14633.
40. Ing, N. H., Beekman, J. M., Kessler, D. J., Murphy, M., Jayaraman, K., Zendegui, J. G., Hogan, M. E., O'Malley, B. W., and Tsai, M. J. (1993) *Nucleic Acids Res. 21*, 2789−2796.

BI015906B