doi.org/10.1002/hlca.202100095

FULL PAPER

HELVETICA chimica acta

Redirection of the Transcription Factor SP1 to AT Rich Binding Sites by a Synthetic Adaptor Molecule

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Dedicated to Professor Dr. *E. Peter Kündig* on the occasion of his 75th birthday

The ubiquitous transcription factor SP1 binds to a GC rich consensus sequence. Here we describe an adaptor molecule that mediates binding of SP1 to a non-cognate DNA site rich in AT. The adaptor is comprised of a *Dervan*-type hairpin polyamide with high affinity to an AT rich hexamer duplex. It also carries a 27mer DNA that contains the SP1 consensus sequence. The synthesis and purification of the polyamide-DNA conjugate is reported. Pulldown experiments and western blot analysis demonstrate adaptor mediated binding of SP1 to the hexamer duplex TTGTTA.

Keywords: adaptor mediated recruitment, DNA conjugate, DNA recognition, hairpin polyamides, protein capture.

Introduction

Transcription factors are key regulators of gene expression. They are normally composed of a binding domain responsible for specific recognition of DNA. The regulatory domain interacts with other proteins that modify chromatin and activate or repress transcription. In chemical biology, the design and evaluation of artificial transcription factors has become a major activity in recent years. One common approach for achieving sequence specific recognition of DNA relies on the chemically induced dimerization of synthetic helical peptides.[1–10] Molecular biology, on the other hand, has provided access to adaptable proteins such as zinc fingers,[11–13] TALEs[14–16] and dCas9.[17–21] These DNA binding proteins are combined with activator parts, typically short peptide sequences or entire domains of natural transcription factors. Alternatively, sequence recognition can be achieved with oligonucleotides binding to genomic DNA by strand displacement[22–24] or triple helix formation.[25–31] A third group of artificial transcription factors contains hairpin polyamides[32–40] as a versatile class of minor groove binding agents. In contrast to these approaches, the aim of our study is to redirect natural transcription factors to non-cognate binding sites using bifunctional adaptor molecules (*Figure 1*). This concept has already been realized with triplex forming oligonucleotides (TFO): A single stranded sector of purines binds to genomic DNA while an appended oligonucleotide hairpin recruits the activator protein.[25,27–29] Interestingly, some naturally occurring long noncoding RNAs are discussed to work exactly in this way.[31] In our hands, however, *Dervan*-type hairpin polyamides revealed distinctly higher affinities to duplex DNA compared to purine TFOs. Thus, in the present study we have synthesized adaptor molecules **1** and **2** (*Figure 2*), conjugates of polyamides and DNA[41] to trap a naturally occurring transcription factor. To avoid the intramolecular association of the polyamide and the DNA part of the adaptor, the attached nucleotide sequence should be as different as possible from TTGTTA, the binding site of the hairpin polyamides used in this study. We have

Supporting information for this article is available on the WWW under https://doi.org/10.1002/hlca.202100095

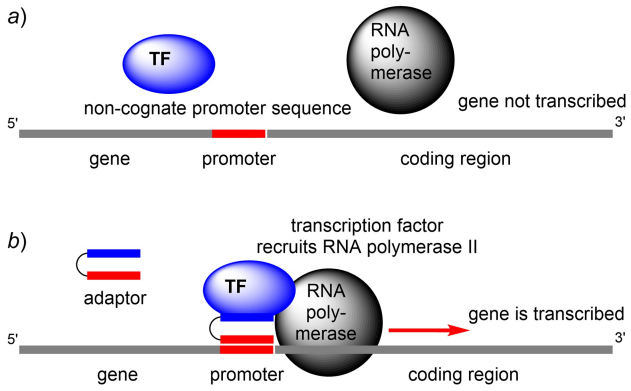


Figure 1. Redirection of a transcription factor. *a*) The protein binds to its cognate sequence specifically, hence transcription of other genes is not affected. *b*) Adaptor mediated binding of the protein to a non-cognate site may cause upregulation of the corresponding gene by recruiting RNA polymerase II. The red and blue colors indicate the reciprocal complementarity of binding sites.

therefore chosen SP1. This ubiquitous transcription factor is known for high affinities to GC rich recognition sites[42,43] expected not to interact with hairpin polyamides.

Results and Discussion

The synthesis of hairpin polyamides[44,45] has been well described by *P. Dervan* and is most often executed on solid support.[46,47] In the initial phase of this study, however, larger quantities of polyamides were needed to optimize the conjugation step with DNA. We therefore preferred a solution phase synthesis. *Scheme 1* depicts the build-up of polyamide **24**, the precursor of adaptor compound **1**. Starting from the known[46,47] heterocycles **4**, **6**, and **8**, repetitive standard procedures were applied leading to intermediates in good yields and purities. Whenever possible, haloform reactions were used for the coupling of building blocks. Only the less nucleophilic amino imidazole obtained by reduction of **7** required more powerful coupling conditions. Hydrogenation products of nitro compounds were directly acylated in most cases with exception of compound **13**. The synthesis of adaptor **2** is shown in the *Supporting Information*.

To convert polyamide **24** into adaptor **1**, we used a method reported for DNA-peptide conjugation.[48] An MMT protected C_6 amino linker was attached to the support-bound DNA and detritylated by mild acid treatment. After neutralization, the support was step by step treated with 1,6-diisocyanatohexane and with polyamide **24**. Roughly 30–45% of the DNA strands reacted as desired, but we also observed crosslinking of two strands with the diisocyanate. After deprotection, purification of adaptor **1** by HPLC was found to be insufficient. Preparative denaturing electrophoresis, however, gave access to pure samples of the conjugate.

Hairpin polyamides derived from netropsin and distamycin associate with double stranded DNA in the minor groove. They fold back forming an intramolecular dimer with antiparallel orientation. The amide NH groups act as H-bond donors with different types of nucleobases as acceptors. In contrast, the amino group of guanosine causes a steric clash with pyrrole derived polyamides. By replacing pyrrole with imidazole, this repulsion can be converted into attractive H-bonds from guanosine NH_2 as donors to imidazole nitrogens as acceptors. Additional H-bonds between polyamide and DNA are formed by the GABA and β-Ala linkers. As a result, adaptor **1** recognizes DNA duplexes of the general sequence WWGWWW where W can be either A or T. From previous studies of the *Dervan* group, TTGTTA was expected to match the structure of polyamide **24**. The relative orientation of DNA *versus* adaptor **1** is shown in *Figure 3*. To invert this orientation, the position of the imidazole moiety within the polyamide had to be transposed leading to adaptor **2**. The affinity of hairpin polyamides to specific variants of the general sequence WWGWWW, however, can vary significantly. We therefore decided not to rely on a single adaptor design exclusively.

The binding of adaptor conjugates **1** and **2** to DNA duplex **25** was tested in gel shift assays (*Figure 4*). Although homogeneous by denaturing gel electrophoresis, both adaptors showed additional minor bands in the native gel that result from self-association (*lanes 2* and *8*). Adaptor **1** in the presence of **25** caused a new band of reduced mobility (*lane 1*, arrow) when compared to the isolated compounds (*lanes 2* and *5*). DNA **3** formed a duplex with both adaptors **1** and **2** (*lanes 4* and *6*). This GC rich duplex (blue in *Figures 2* and *3*) is not directly involved in the adaptor-target complex (red) but intramolecular association with the polyamide part might prevent binding to duplex **25**. This, however, is not the case: When the duplex of **1** and **3** was added to **25** (*lane 3*), the band of **25** became distinctly attenuated (as in *lane 1*) and the band of **1** and **3** was slightly more retarded (compare *lanes 3* and *4*). In contrast, *lane 7* where the duplex of adaptor **2** and **3** was mixed with duplex **25** just looked

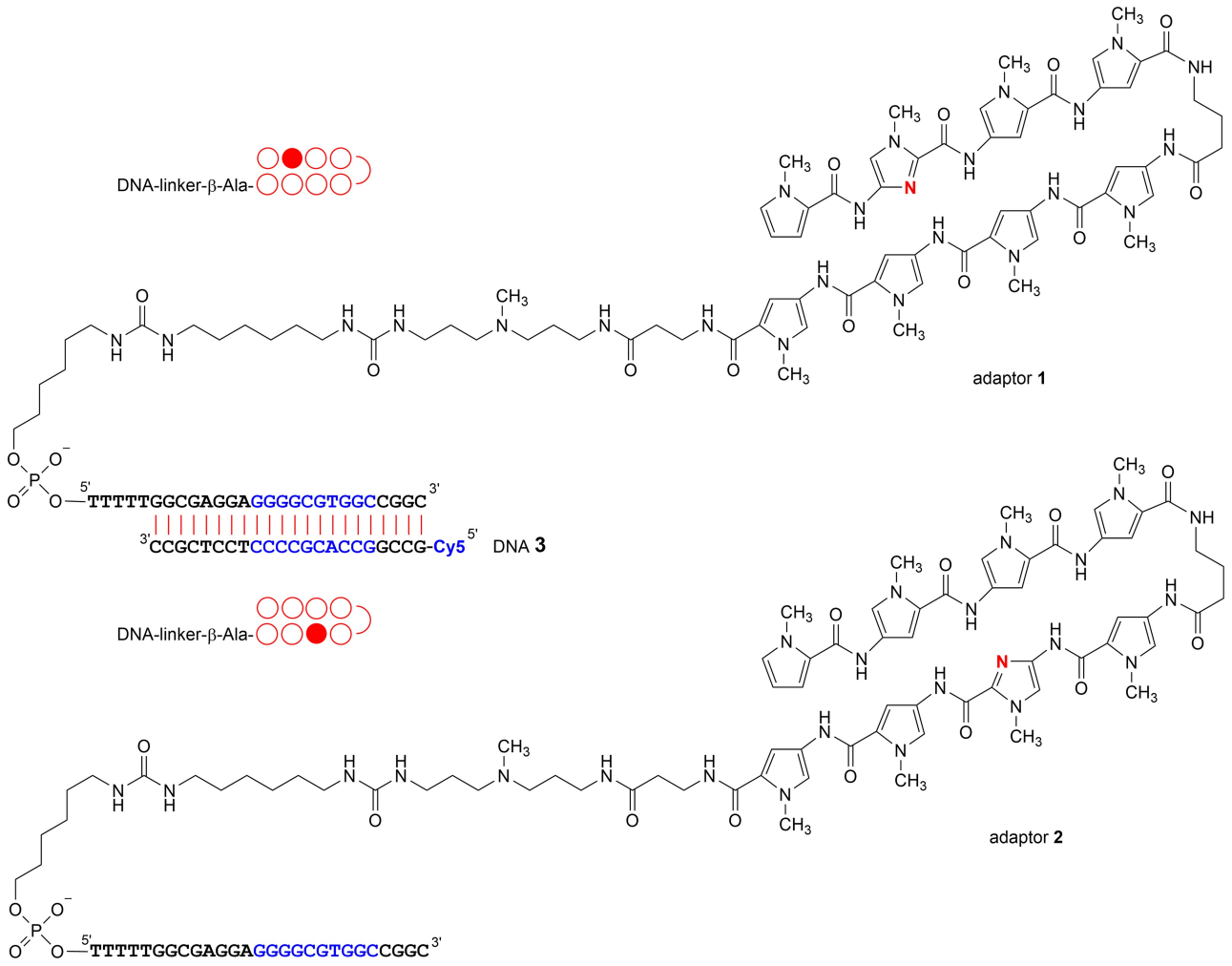
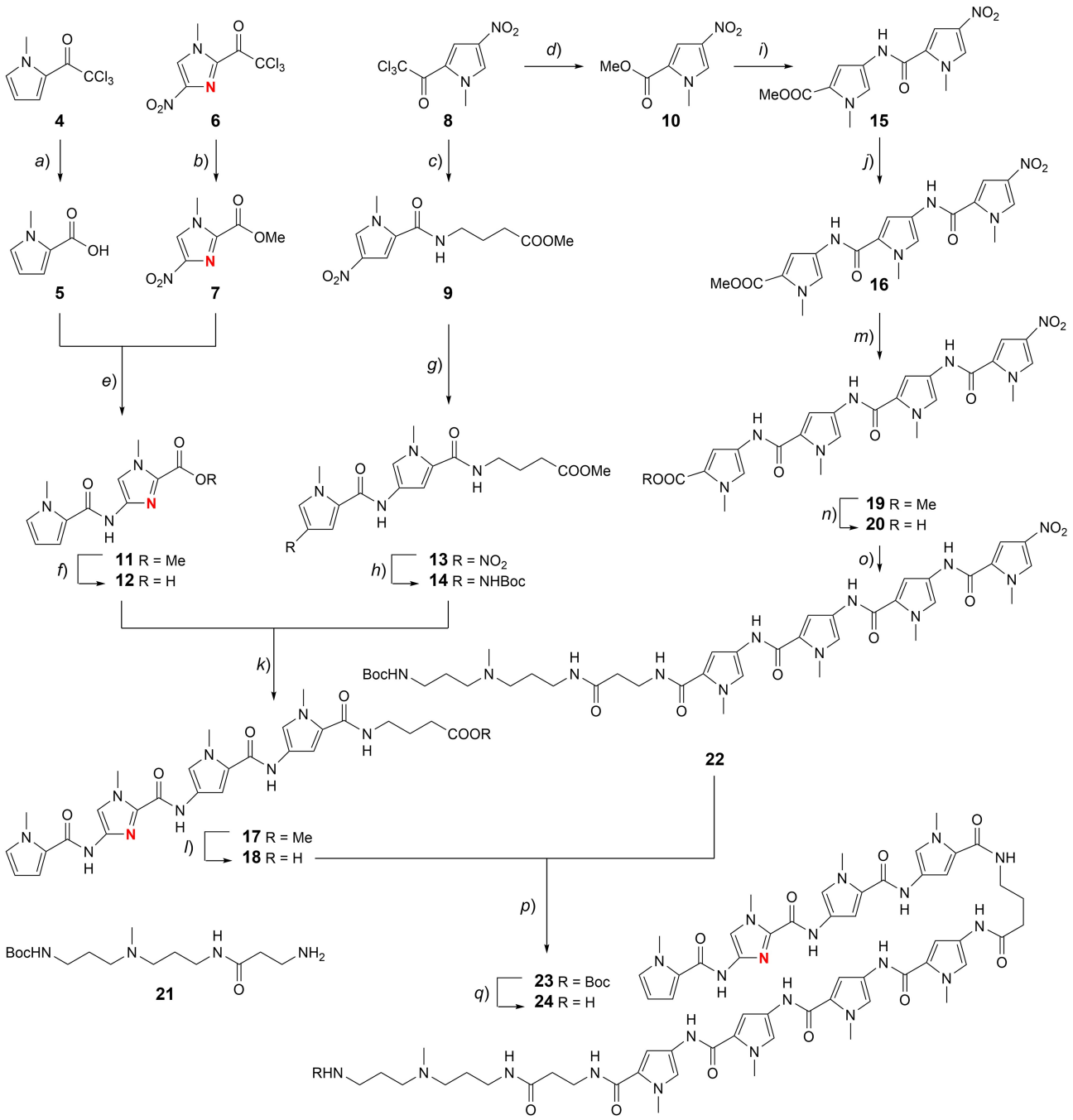


Figure 2. Adaptor **1** shown in complex with the dye-labeled DNA **3** contains a binding site for the transcription factor SP1 (blue). Adaptors **1** and **2** only differ in the position of the imidazole moiety. In the simplified representation, pyrroles are symbolized by open and imidazoles by filled red circles. The color indicates affinity to a DNA sequence different from the SP1 binding site (blue).

like an overlay of *lanes 5* and *6*. To clearly attenuate the band of duplex **25**, a large excess of adaptor **2** was required (*lane 9*). Accordingly, adaptor **1** binds more tightly to DNA duplex **25** than adaptor **2**.

As a second test for adaptor affinities to DNA duplex **25**, we performed a pulldown assay with Cy5-labeled DNA **3**. Duplex **25** (300 nM) was incubated with either DNA **3** alone or a duplex of **3** and the adaptor conjugates **1** or **2** (each 100 nM). The biotinylated duplex **25** was isolated with streptavidin-coated magnetic beads. Then the strands were separated by adjusting the pH to 10. Finally, we quantified the fluorescence of the liberated DNA **3**. Due to the lack of complementarity between **3** and **25**, only low levels of fluorescence were found in the absence of adaptors but strong effects in their presence. The build-up of ternary complexes (adaptor + **3** + **25**) takes some time and they partially dissociate upon washing of the isolated beads. We therefore used a standardized incubation and washing procedure that allowed us to recover 11.6 ± 1.2% of the fluorescence of the initially used complex of **3** and adaptor **1**. In the analogous experiment with adaptor **2**, only 6.5 ± 0.6% of the fluorescence could be reisolated. Accordingly, ternary complexes are formed but, as already seen in the band shift assay, they are distinctly weaker for adaptor **2**.

To demonstrate the redirection of SP1 to the non-cognate binding site TTGTTA, we used pulldown experiments from HeLa nuclear extracts in combination with western blots (*Figure 5*). As a positive control, we incubated the biotinylated duplex **26** containing the SP1 consensus sequence with HeLa extract. After



Scheme 1. Synthesis of polyamide **24**, the precursor of adaptor **1**. *a*) NaOH, MeOH, 79%; *b*) DMAP, MeOH, 85%; *c*) methyl 4-aminobutanoate hydrochloride, DIPEA, CH_2Cl_2, 95%; *d*) DMAP, MeOH, 89%; *e*) **7**, H_2, Pd/C, AcOEt, then **5**, HOBt, DIC, DIPEA, DMF, 79%; *f*) NaOH, MeOH, 86%; *g*) **9**, H_2, Pd/C, AcOEt, then **8**, DIPEA, CH_2Cl_2, 81%; *h*) H_2, Pd/C, Boc_2O, MeOH, 100%; *i*) **10**, H_2, Pd/C, AcOEt, then **8**, DIPEA, CH_2Cl_2, 86%; *j*) **15**, H_2, Pd/C, AcOEt, then **8**, DIPEA, CH_2Cl_2, 79%; *k*) **14**, AcCl, MeOH, then **12**, HBTU, DIPEA, DMF, 85%; *l*) LiOH, MeOH, H_2O, 85%; *m*) **16**, H_2, Pd/C, Boc_2O, MeOH, then TFA, CH_2Cl_2, then **8**, DIPEA, CH_2Cl_2, 71%; *n*) NaOH, EtOH, 89%; *o*) **20**+**21**, DIC, HOBt, DIPEA, DMF, 80%; *p*) **22**, H_2, Pd/C, DMF, then **18**, HBTU, DIPEA, 62%; *q*) TFA, CH_2Cl_2, 100%. For experimental details, CAS registry numbers of known compounds and the synthesis of linker **21** see *Supporting Information*.

pulldown using magnetic beads, a band appeared (*lane 1*) in a region matching the reported size of SP1 (81 kD). It should be noted that the SP1 binding sequence GGGGCGTGGC of both adaptor conjugates is taken from the *c-kit* promoter[49] and slightly differs from the standard consensus sequence GGGGCGGGGC

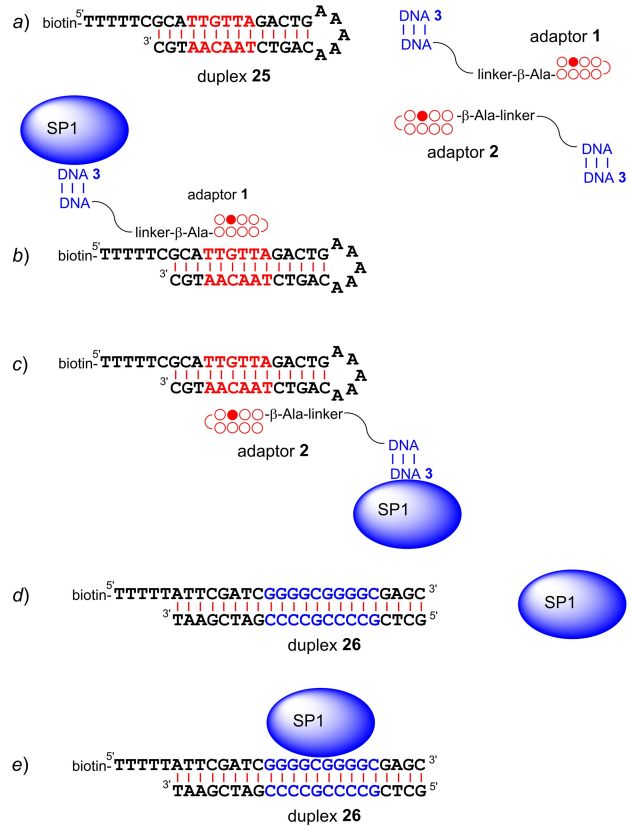


Figure 3. *a*) The biotinylated capture duplex **25** represents the red promoter region shown in *Figure 1*. It does not contain a binding site for SP1. *b*) and *c*) Adaptors **1** and **2** were designed to bind the AT-rich sequence (red) in two different orientations. When hybridized with oligonucleotide **3**, the resulting DNA duplex (sequence see *Figure 2*) interacts with SP1. Indirect binding of the protein to duplex **25** depends on the presence of adaptor molecules. *d*) The consensus sequence for SP1 in the biotinylated DNA duplex **26** is shown in blue. *e*) SP1 binds directly to duplex **26**.

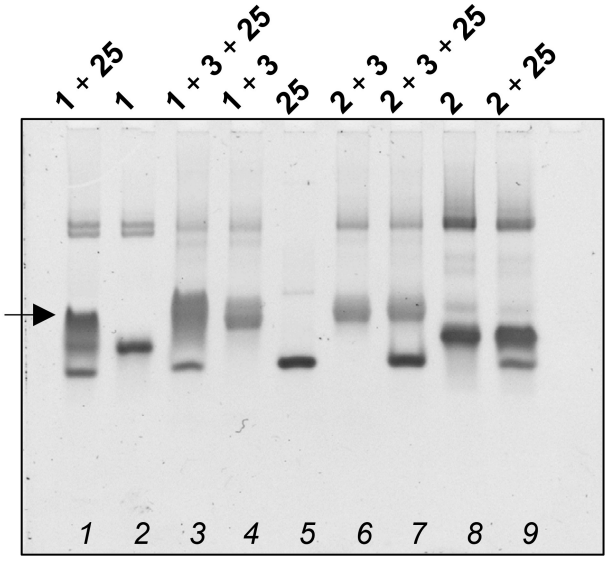


Figure 4. *Lane 1*: adaptor **1** (2 μM) + duplex **25** (0.5 μM); *lane 2*: adaptor **1** (2 μM); *lane 3*: adaptor **1** (2 μM) + DNA **3** (2 μM) + duplex **25** (0.5 μM); *lane 4*: adaptor **1** (2 μM) + DNA **3** (2 μM); *lane 5*: duplex **25** (0.5 μM); *lane 6*: adaptor **2** (2 μM) + DNA **3** (2 μM); *lane 7*: adaptor **2** (2 μM) + DNA **3** (2 μM) + duplex **25** (0.5 μM); *lane 8*: adaptor **2** (4.6 μM); *lane 9*: adaptor **2** (4.6 μM) + duplex **25** (0.5 μM). The samples were incubated for 20 h at r.t. in buffer Z. The 16% native gel was stained with SYBR Gold.

present in DNA **26**. As pointed out by *Balasubramanian*, the c-*kit** sequence as well as many other SP1 binding sequences have a strong tendency to form quadruplex structures. SP1 binds to such quadruplexes with equal affinity as to the corresponding duplexes.[50] Quadruplex formation is also a putative explanation for the additional bands found with adaptors **1** and **2** in the native gel (*Figure 4*). The experiment was therefore repeated with duplex **27**, containing the c-*kit** sequence GGGGCGTGGC (*lane 2*). Both DNAs captured SP1 with comparable results. The biotinylated AT rich duplex **25**, in contrast, gave only a weak band in the pulldown assay (*lane 3*), not more than the unloaded beads (*lane 4*) used as a negative control. The background signal seen in lanes *3* and *4* may indicate some nonspecific binding of SP1 to the beads but is most probably explained by the viscosity of the HeLa extract that limits the efficiency of washing steps. Finally, when duplex **25** was combined with adaptor **1**, again a strong band appeared in the gel (*lane 5*), at least five times more intense as in the controls. Adaptor **1** thus mediated binding of SP1 to the AT rich capture DNA.

Conclusions

The biological function of DNA and RNA is regulated by numerous proteins. Some like restriction enzymes directly change the molecular structure of their targets. The majority, however, act in a more indirect way by recruiting other proteins to initiate the biochemical response. Whenever synthetic molecules are intended to adopt the function of such DNA/RNA regulators, *e.g.*, as artificial transcription factors binding a new recognition site, it is worth considering a redirection strategy: Bifunctional adaptor molecules may capture the protein of interest and guide it to the designed new binding site. This strategy is applicable to many kinds of proteins, *e.g.*, to RISC complexes acting on mRNAs.[51] In the present study, we have

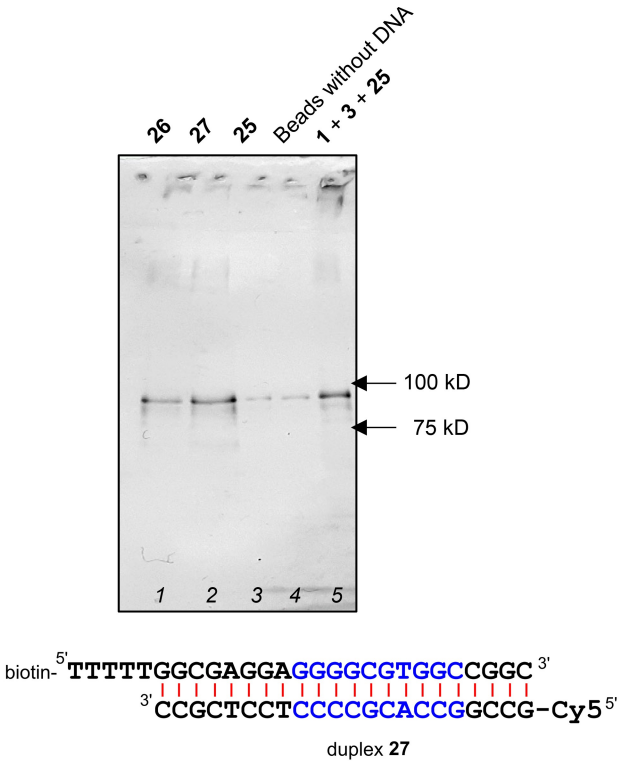


Figure 5. Pulldown of Sp1 from HeLa nuclear extract. Visualization by western blot after electrophoretic separation of proteins using an antiserum against Sp1. *Lane 1*: duplex **26** (300 nM); *lane 2*: duplex **27** (300 nM); *lane 3*: duplex **25** (300 nM); *lane 4*: streptavidin-coated beads, but unloaded with DNA; *lane 5*: adaptor **1** (900 nM) + DNA **3** (900 nM) + duplex **25** (300 nM). The samples were incubated for 3 h at r.t. in buffer Z containing 1% BSA.

investigated the chemical aspects of SP1 redirection. A key question remains still open: Is adaptor mediated association of the transcription factor functionally equivalent to direct binding to a promoter site? A comparison with the yeast-two-hybrid approach, however, shows that the DNA binding and the activating domains may be split into separated molecules without losing the function of transcriptional activation. Before designing cell culture experiments with adaptors such as conjugate **1**, other important aspects have to be considered. Most significant is the stability of the adaptor's GC rich DNA duplex. To avoid strand separation, a hairpin oligonucleotide can be used and stabilized against enzymatic degradation by introducing modified building blocks in selected positions. To act on transcription, the adaptor must not only enter the cell but also the nucleus. This may be accomplished in future studies by adding a nuclear localization sequence to adaptor **1** in a second conjugation step.

Experimental Section

General (*see also* Supporting Information)

Oligonucleotides were purchased from *BioTeZ*, *BioSpring*, and *Iba*, streptavidin-coated magnetic beads from *Promega*. The preparation of HeLa lysate and the enrichment of SP1 by *E. Kalden* and *U. Scheffer* followed published procedures.[52,53] Sterile *MilliQ* water was used for the purification of adaptor compounds and for all band shift and pulldown experiments. Buffer Z: 25 mM HEPES pH 7.5, 100 mM KCl, 12.5 mM $MgCl_2$, 0.01 mM $ZnCl_2$.

Synthesis and Purification of Adaptors **1** *and* **2**

The DNA part including the 5′ amino linker was assembled from fast deprotection amidites on a standard support. 10 mg of the support (*ca.* 250 nmol) were suspended in CH_2Cl_2 (1 mL) for 30 min. The MMT group was then removed by incubation with 3% trichloroacetic acid in dry CH_2Cl_2 (0.5 mL) for 5 min. The support was washed with CH_2Cl_2 (0.5 mL) and the acid treatment was repeated. Afterwards, the support was washed five times with acetonitrile (0.5 mL). Then a solution of DIPEA (1.7 μL, 10 μmol) and 1,6-diisocyanatohexane (8.42 μL, 48 μmol) in 1 mL of dry acetonitrile was added. After incubation for 18 h at room temperature, the support was washed five times with acetonitrile. Subsequently, a solution of the polyamide (*e.g.* **24**; 2.5 μmol, 10 equiv.) in dry MeOH (150 μL) and DIPEA (10–25 μL) in dry acetonitrile (750 μL) were added. The mixture was mildly shaken for 24 h at room temperature and afterwards washed five times with acetonitrile (0.5 mL). To detach the product, the solid support was incubated with aqueous ammonia (32%, 0.5 mL) for 30 min at room temperature. This procedure was repeated three times and the combined ammonia solutions were heated to 55 °C for 5 h. After removal of the solvent in a vacuum centrifuge, the dry residue was dissolved in a small volume of saturated aqueous urea solution, mixed with some 2× loading buffer (8 M urea, 20 mM EDTA, 0.2% orange G), heated to 90 °C for 5–10 min and then purified by electrophoresis in a 16% denaturing polyacrylamide gel (7 M urea, 1x TBE buffer, acrylamide/bisacrylamide 19:1). The electrophoresis was run at 230–250 V in 0.5x TBE buffer until the dye had moved to the mid of the gel. Bands containing the pure adaptors **1** or **2** were visualized by UV shadowing, excised, and extracted from the gel matrix over night with elution buffer (500 mM AcONa pH 7.0, 2 mM EDTA, 0.1% SDS). Afterwards, the solution was

passed through a centrifugal filter (*VWR*, *516–0235*, 13000×*g*), mixed with a threefold volume of EtOH and kept overnight at −20 °C. The precipitated product was isolated by centrifugation, dissolved in water, and desalted by gel filtration (*NAP-10*). Finally, the product was identified by mass spectrometry (*Bruker micrOTOF-Q II* with *Agilent 1200* Series HPLC and *MultoKrom 5-C18* column) and the concentration was determined through UV absorption (*Nanodrop 200c*).

Data of Adaptor **1**: MS (ESI-TOF): 10053.3 (M^-, $C_{340}H_{434}N_{131}O_{179}P_{27}^-$; calc. 10051.2).

Data of Adaptor **2**: MS (ESI-TOF): 10053.3 (M^-, $C_{340}H_{434}N_{131}O_{179}P_{27}^-$; calc. 10051.2).

Electrophoretic Band Shift Experiments (Figure 4)

Oligonucleotide samples were incubated for 20 h at room temperature in buffer Z and then mixed with loading buffer (40% sucrose, 0.25 *Crocein Orange G*). Electrophoretic separation was conducted in a 16% native polyacrylamide gel (acrylamide/bisacrylamide 37.5:1, 1x TBE buffer, 5% glycerol. Sample volumes between 6 and 10 μL and a voltage of 100 V was applied. Electrophoresis was stopped when the dye had reached the center of the gel. After staining (SYBR gold 1:10000 in 10 mM *Tris*-HCl pH 7.5, 1 mM EDTA, 15–20 min) the gel image was recorded with a digital camera equipped with a green filter.

Pulldown Experiments

Loading: Streptavidin-coated magnetic-beads (600 μL aliquot; binding capacity 0.75 pmol/μL) were washed three times with 0.5x SSPE-buffer (5 mM sodium dihydrogen phosphate; 0.5 mM EDTA and 75 mM sodium chloride). Afterwards, a solution of DNA **25** (2 μM) in 0.5x SSPE-buffer (450 μL) with 10% *Roti*®-Block (*Roth*) was added. The suspension was incubated overnight at r.t. After the beads were collected with a magnetic stand, the supernatant was removed, and the beads were washed three times with 0.5x SSPE-buffer.

Fluorescence Based Experiments

An aliquot of beads loaded with capture DNA **25** (corresponding to a DNA concentrations of 300 nM), the adaptor molecule (100 nM) and DNA **3** (100 nM) were suspended in 100 μL of buffer Z. The mixture was incubated for 3 h at r.t., while the beads were kept in suspension. The beads were then collected and washed three times with the same buffer. To separate the strands, 0.5x SSPE-buffer was added followed by the addition of 3 μL of 0.1 M NaOH. After 30 min, the suspension was transferred in a black 96 well microtiter plate (*Corning* costar) and the fluorescence was recorded (λ_{ex} = 649 nm, λ_{em} = 670 nm, *Tecan Safire II*).

Western Blot (Figure 5)

An aliquot of magnetic beads loaded with oligonucleotides (DNA **25**: 900 nM, DNA **26** and **27**: 300 nM) was incubated in buffer Z containing 1% BSA (100 μL). HeLa nuclear extract enriched in SP1 (3 μL) as well as adaptor **1** and DNA **3** (300 nM each) were added and the mixture was incubated for 3 h at r.t. After incubation, the beads were washed four times with buffer Z. Then 6 μL of 2x *Laemmli*-buffer (100 mM *Tris*-HCl pH 6.8, 20% glycerol 4% SDS, 200 mM DTT, 0.1% bromophenol blue) were added and the mixture was heated to 90 °C for 5 to 10 min. The samples were analyzed by SDS-PAGE (6% stacking, 8% resolving gel). Gels were run at 100 to 120 V. After electrophoresis, proteins were transferred to a PVDF membrane at 8 W for 30 min. The membrane was blocked with 10% *Roti*®-Block or 0.5% casein (Hammarsten Grade) for 20 min at r.t. Afterwards, the membrane was incubated with the primary antibody (*Sigma Aldrich*) diluted 1:750 in *Tris*-buffered saline with 0.1% casein (Hammarsten Grade) at 4 °C for overnight. The membrane was washed three times with *Tris*-buffered saline (TBS) containing 0.1% *Tween 20* followed by addition the AP-linked antibody (Cell Signaling) in a dilution of 1:1500 in TBS containing 0.1% casein. The mixture was incubated for 2.5 h at 30 °C and subsequently washed (3×TBS containing 0.1% *Tween 20* and 1× P3 (100 mM *Tris*-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$)). The bands were visualized by a colorimetric stain with NBT and BICP, scanned and quantified with the software TotalLab Quant v12.2.

Acknowledgements

Open access funding enabled and organized by Projekt DEAL.

Author Contribution Statement

M. B. performed the experiments. *U. S.* and *E. K.* cultivated HeLa cells and enriched SP1. *M. B.* and *M. W. G.* participated in writing the manuscript.

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Received June 14, 2021
Accepted August 6, 2021

