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review

Designed transcription factors as structural, functional and therapeutic probes of chromatin *in vivo*

Fourth in review series on chromatin dynamics

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Despite its central importance in gene regulation, chromatin in mammalian cells remains relatively poorly understood—a predicament due to the paucity of robust genetic tools in mammals, the complexity of the chromatin remodeling machinery, and the dynamic properties of chromatin *in vivo*. Here we review recent developments in understanding endogenous mammalian gene regulation via the use of designed transcription factors (TFs). These include mutated forms of naturally occurring TFs that exhibit dominant-negative activity, and designed proteins with novel, predetermined DNA-binding specificities. Systematic targeting of designed TFs to particular promoters is helping to illuminate the complex rules that chromatin imposes on TF access and action *in vivo*. We evaluate the potential applications of these proteins as probes of mammalian chromatin-based regulatory pathways and their potential for the therapy of human disease, highlighting leukemia in particular.

Introduction

Once assembled into chromatin inside the nucleus, the human genome acquires the ability to regulate ontogeny. Of the many 'emergent properties' gained by DNA following chromatin assembly, the regulatory program embedded in the genome is particularly salient. This program is determined by complex interactions between the DNA, the core and linker histones, nonhistone regulators, and a large number of activities that modify and remodel chromatin structure (Wolffe, 1998). In yeast, programming at the chromatin level has been successfully studied by reverse genetics and various genome-wide analysis methods (Gregory, 2001; Wyrick and Young, 2002), and is well understood in specific cases such as the mating type loci, the *PHO* genes or the *HO* endonuclease promoter (Gregory, 2001). With the notable exception of embryonic stem cells, the mammalian genome resists homologous recombination, and thus reverse genetics in human cells is difficult, though feasible (Sedivy, 2002). Understanding the interplay between chromatin and the genome is further hampered by the complications arising from the existence of functionally redundant genes within each family of chromatin regulators, including, for example, the following pairs: CBP and p300, HDAC1 and 2, Brg1 and hBrm, N-CoR and SMRT (Berger, 2001; Fyodorov and Kadonaga, 2001; Khochbin *et al.*, 2001). Consequently, with some exceptions, including the mouse serum albumin gene (Zaret, 1995), the MMTV LTR (Hager, 2001) and the β-interferon enhanceosome (Merika and Thanos, 2001), the relationship between 'packaging' and regulation of mammalian genes is not as well understood as it is for the genes of budding yeast.

This predicament may soon be resolved in part due to improvements in technologies that allow *in vivo* regulation of specific mammalian genes. These include post-transcriptional approaches such as RNA interference (in certain, but not all, cell types; reviewed in Hutvagner and Zamore, 2002), but also techniques that appropriate the cells' own machinery for transcriptional regulation. It is now feasible to change the expression level of a mammalian gene via the use of a transcription factor (TF) that was *designed* to do so. Dominant-negative allelic forms of

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naturally occurring proteins can be used for this purpose and molecules with novel DNA-binding specificities can also be designed. For example, polyamides or peptide-nucleic acids that recognize sequences by engaging the DNA via the major or minor groove can now be synthesized (Braasch and Corey, 2001; Demidov and Frank-Kamenetskii, 2001; Dervan, 2001). Alternatively, a protein domain can be designed to bind to a particular sequence with high specificity (Pavletich and Pabo, 1991); this is convenient from a technical standpoint because the DNA-binding module fused to a functional domain relevant to the specific experimental goal can be encoded by a single chimeric cDNA which the cell transcribes and translates.

This review describes the use of designed TFs to regulate endogenous mammalian genes *in vivo*, the promise and limitations of this approach in basic science and clinical settings, and what its application has taught us about mammalian chromatin.

The positives of being negative

A regulatory pathway can be analyzed by the introduction of proteins that act as repressors of its components (Figure 1). This approach recapitulates regulatory mechanisms used by the cell: for example the basic helix–loop–helix leucine zipper (bHLH-ZIP) protein MAD interacts with MAX and represses the transcription of genes regulated by c-MYC/MAX heterodimers (Grandori *et al.*, 2000), and splicing variants of the estrogen or glucocorticoid receptor act as dominant-negative (DN) repressors (Oakley *et al.*, 1996; Ogawa *et al.*, 1998). In addition, the etiology of various human diseases has been traced to genetic lesions that yield DN proteins. For example, mutated forms of the thyroid hormone receptor β interfere with wild-type receptor function in patients with RTH (resistance to thyroid hormone) syndrome (Figure 1iv; Chatterjee, 1997) and mutations in the homeobox protein PITX2, which heterodimerizes with other homeodomain proteins, cause Rieger syndrome (Figure 1ii or iv, depending on the allele; Cushman and Camper, 2001).

The effects of DN repressors are consequences of the fact that many proteins contain separable functional domains (Figure 1). This property has been exploited to design artificial DN repressors in which a non-functional amphipathic acidic extension is linked to a leucine zipper to yield proteins that dimerize with high affinity and specificity to bHLH-Zip and bZIP TFs and abolish DNA binding (Figure 1ii; Krylov *et al.*, 1997). This type of DN repressor has also successfully been used to ablate various pathways in transgenic animals (e.g. Moitra *et al.*, 1998).

Design of transcription factors with novel DNA-binding specificities

A comprehensive endogenous gene control platform requires the ability to select or design DNA-binding domains (DBDs) with novel, pre-determined DNA-sequence specificities. The Cys_2–His_2 zinc finger—the most common natural DNA-binding motif (Tupler *et al.*, 2001)—has emerged as the domain of choice for this method. Naturally occurring zinc finger proteins (ZFPs) have diverse target sites (Wolfe *et al.*, 2000a), proving that this motif is adaptable. Structural studies of ZFP–DNA complexes (Pavletich and Pabo, 1991; Houbaviy *et al.*, 1996) showed that these proteins use multiple, tandem fingers to

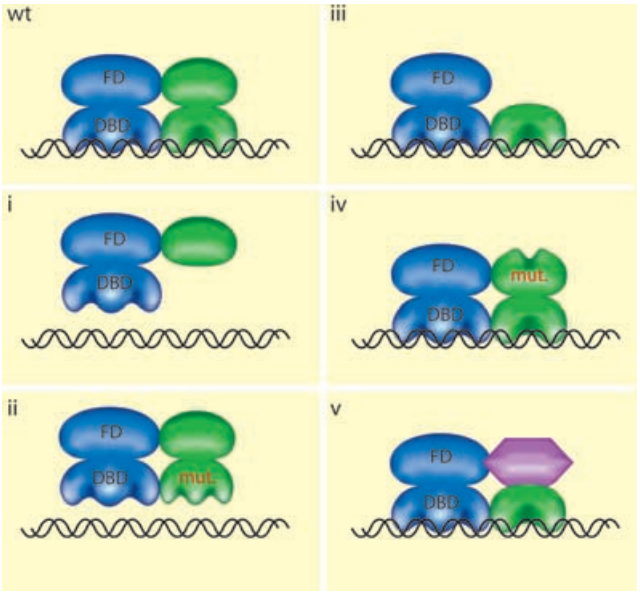

Fig. 1. Schematic representation of potential configurations for a dominant-negative (DN) allelic form of an endogenous wild-type (wt) regulator. In this hypothetical example, based on nuclear hormone receptors, the wt functional TF (upper left) is a heterodimer between two subunits (blue and green), each possessing a distinct DNA-binding domain (DBD) and functional domain (FD). The various DN forms are: (i) a deletion of the DBD; (ii) a mutated DBD; (iii) a deleted FD; (iv) a mutated FD; and (v) a different FD with a functional heterodimerization interface. Impairment in DNA binding is indicated by the 'floating' of the complex above the DNA.

interact with a series of adjacent subsites (typically, 3 or 4 base pairs each) in the major groove of the DNA. Considerable functional autonomy in the recognition of a subsite target by each finger enables 'mix and match' DNA-binding protein design, in which fingers with known subsite preferences are linked to yield multifinger proteins with a high affinity for desired target sequences (reviewed in Choo and Isalan, 2000; Pabo *et al.*, 2001; Segal and Barbas, 2001; Beerli and Barbas, 2002). Numerous fingers with distinct sequence preferences have been described. Some of these have been derived from naturally occurring proteins, but most have been produced by selection or design efforts aimed at modifying zinc finger specificity in order to expand the combined repertoire and, thus, encompass the broadest possible range of target sites (Desjarlais and Berg, 1992; Choo and Klug, 1994; Jamieson *et al.*, 1994; Rebar and Pabo, 1994; Greisman and Pabo, 1997). Multiple fingers (usually 3–6) have been joined to obtain ZFPs which recognize target sequences of 9–18 base pairs (Choo and Isalan, 2000; Wolfe *et al.*, 2000b; Pabo *et al.*, 2001).

Naturally occurring Cys_2–His_2 ZFPs perform a variety of functions (Davidson, 2001): for example, Sp1 is a transcriptional activator, Kox-1 is a transcriptional repressor, and CTCF is an insulator. This indicates that a wide spectrum of functional domains may be used in fusions with a designed ZFP-based DBD. In agreement with this expectation, various modules including activation domains from viral protein 16 (VP16), nuclear hormone receptors, and NF-κB, and repression domains from Krüppel-associated box (KRAB)-containing regulators, have been successfully used in the context of designed ZFP TFs (Table I). Studies in *Drosophila*

Table I. Regulation of endogenous genes resident in their native chromosomal locus using designed ZFP TFs

Target gene	ZFP used	Functional domain	Location of target sequence relative to [+1] and its chromatin organization	Effect on gene	Comments	References
MDR1	5-finger	KRAB (two copies)	[–50]; n.d.	Repression of induction	ZFP target overlaps Sp1 site	Bartsevich and Juliano (2000)
Erythropoietin	3-finger	VP16	[–862]; linker DNA between two nucleosomes	Activation	In cell line used, gene is completely silent and unresponsive to hypoxia	Zhang *et al.* (2000)
VEGF-A	3-finger	VP16 and NF-κB p65	[–500], [+1], [+500]; all 3 in DNase I hypersensitive site	Activation	Multiple splice isoforms upregulated proportionately	Liu *et al.* (2001)
erbB-2	6-finger	VP64 (VP16 × 4)	[+20]; n.d.	Activation	ErbB-2 activators do not activate ErbB-3	Beerli *et al.* (2000a); Dreier *et al.* (2001)
erbB-3	6-finger	VP64 (VP16 × 4)	[+50]; n.d.			
erbB-3	6-finger	KRAB	[+50]; n.d.	Repression	Cells exhibit growth arrest when *erbB-2* is repressed.	Beerli *et al.* (2000a); Dreier *et al.* (2001)
PPARγ1 and 2	6-finger	KRAB	[–200] of γ2; DNase I hypersensitive site	Repression of induction	Repression prevents adipogenesis	Ren *et al.* (2002)

n.d., not determined.

have shown that regulatory domain efficacy can be distance dependent (Mannervik *et al.*, 1999). The active range of functional domains fused to designed ZFPs within a chromatin environment has not been comprehensively investigated, and most published studies (Table I) use designed proteins that target the immediate promoter region, although some exceptions exist. The silent erythropoietin gene has been activated by a single ZFP–VP16 fusion bound to [–862] relative to the transcription start site (Zhang *et al.*, 2000). A ZFP–KRAB fusion that binds to and represses the peroxisome proliferator activated receptor (PPAR) γ2 promoter also affects transcription originating from the PPARγ1 promoter located ~60 kb upstream (Ren *et al.*, 2002), but it is not clear whether this latter repression occurs at the level of transcriptional initiation or elongation. Both for basic science and therapeutic purposes, it will be of interest to investigate how (and if) the behavior of specific functional domains changes depending on the promoter type to which they are targeted, and on the distance from the transcription start site.

The activity of the ZFP–functional domain chimera can be controlled through several strategies. For example, the level of the designed TF in the cell can be modulated by driving its synthesis with a promoter that responds to a small molecule, such as an ecdysteroid or tetracycline, whose titer has been shown to accurately correlate with the activity of the gene targeted by the TF (for example, see Kang and Kim, 2000; Zhang *et al.*, 2000). Alternatively, a synthetic 'nuclear hormone receptor' that fuses a designed ZFP DBD to a functional ligand-responsive module (for example, progesterone- or estrogen-responsive) can be created (Beerli *et al.*, 2000b; Beerli and Barbas, 2002). With respect to the latter, class II nuclear hormone receptors function as repressors in the absence of ligand and as activators in its presence (Urnov and Wolffe, 2001). Thus, in principle, one could even use the same designed TF to either activate or repress the target gene.

Table I summarizes published data on the use of designed ZFP-based TFs to regulate endogenous genes *in vivo* (also reviewed in Reik *et al.*, 2002). As discussed in the next section, it is important to make a distinction between the control of genes residing in their native chromosomal environment, and that of genes in other situations (e.g. *in vitro*, on transiently transfected episomes, etc.).

The challenge of chromatin

Chromatin imposes a complicated set of rules on TF action (Wolffe and Hansen, 2001). For example, locus-wide (Horak *et al.*, 2002) and genome-wide (Ren *et al.*, 2000) analysis of TF binding *in vivo* showed that only a fraction of 'consensus' sites are actually engaged by the TF inside the nucleus. Furthermore, major differences have been observed between the actions of a given TF in transient transfection-based reporter gene assays and its behavior on the chromosomal copy of the same DNA sequence (Smith and Hager, 1997). Interestingly, several ZFP TFs, including Sp1 (Li *et al.*, 1994) and GATA-1 (Boyes *et al.*, 1998), can bind chromatin *in vitro* with only moderate decreases in affinity relative to naked DNA, and in some cases can induce an ATP-independent perturbation in histone–DNA contacts (Boyes *et al.*, 1998; Cirillo *et al.*, 2002). Thus, simple models of the 'chromatin is an obstacle' variety are insufficient (Urnov, 2002).

A growing body of data describes the interplay between designed TFs and chromatin: in several studies (Table I) ZFPs were deliberately designed to bind DNase I hypersensitive sites in gene promoters in order to facilitate access and activation by

Table II. Transcription factor aberrations in cancer and possible 'transcription therapies'

Transcription factor	Aberration	Cancer type	References	Possible transcription therapy?
RARα	Translocation leading to fusion with PML	Acute promyelocytic leukemia	Look *et al*. (1997)	Designed TF (DBD only) mimicking RAR to compete at RAREs
AML1-ETO	″	M2 (acute myelogenous leukemia)	Piazza *et al*. (2001)	Designed TF (DBD only) mimicking AML1 to compete at AML1 sites
c-Myc	Translocation leading to upregulated protein levels	Burkitt's lymphoma	Gaidano and Dalla-Favera (2001)	Designed TF repressor for Ig promoter
BCL6	″	Non-Hodgkin's lymphoma	Gaidano and Dalla-Favera (2001)	Designed TF that activates BCL6-repressed genes
WT1	Nonsense mutations leading to truncation	Renal; soft tissue sarcoma	Lee *et al*. (1999)	Designed TF activator for WT1 targets
TLS/FUS and EWS	Translocation leading to chimeric fusion protein	Sarcoma	Aman (1999)	Unclear
p53 and Rb	Loss-of-function mutations	Many	Hanahan and Weinberg (2000)	Designed TF activator for Rb target genes

the TF. In one notable study, a panel of 3-finger ZFPs was designed against various sites in the *EPO* gene promoter. *In vitro*, these proteins bound their intended target sites with high affinity and robustly activated a transiently transfected reporter plasmid (Zhang *et al.*, 2000), and their efficiency was not significantly affected by the location of the ZFP target site within the promoter. However, in the case of the endogenous promoter, these same proteins exhibited markedly different behavior and only ZFPs designed to target a sequence upstream of an Alu element upregulated EPO to pharmacological levels (Zhang *et al.*, 2000). Because Alu elements are known to direct translational and rotational histone octamer positioning (Englander and Howard, 1995), this suggested that chromatin at the silent *EPO* locus assumes a non-random organization. In fact, nucleosomes were found to assume a specific translational frame over the silent *EPO* gene promoter (Zhang *et al.*, 2000).

Two other studies, which have illustrated the importance of TF target sites, examined designed ZFP regulation of the *HER-2/neu/erbB-2* gene. In the case of this promoter, one ZFP–KRAB fusion directed to a target site located at ~[+100] repressed the endogenous copy of this gene (Beerli *et al.*, 2000a), but a second that was targeted closer to the transcription start site (~[+30]) failed to do so (Dreier *et al.*, 2001). This illuminates a poorly understood role for native nucleoprotein organization of the promoter in controlling regulatory factor access and action, as well as suggesting that designed regulators abide by the rules of chromatin, and may therefore be useful probes of its structure *in vivo*.

Various applications for these proteins in functional studies of the genome can also be envisaged. For example, designed ZFP-based repression of transcription of both isoforms of the PPARγ gene was recently used in a 'mutation-free reverse genetics' study and illuminated a unique contribution made by the PPARγ2 isoform (Ren *et al.*, 2002). Similar conditional 'transcription knockouts' may be used to reversibly up- or downregulate endogenous genes, which code for components of the chromatin-based regulatory machinery, in order to gauge their role in regulating the genome.

Towards 'transcription therapy' with designed regulators

In addition to their applications in basic science, designed TFs also have the potential to effect therapeutic control of specific genes *in vivo*, since aberrant gene transcription underlies a significant proportion of human disease. Remission could potentially be achieved by selectively down- or upregulating a relatively small number of genes. Increased transcription of the fetal γ-globin gene (by inhibiting its normal silencing) in sickle cell anemia patients has already been shown to alleviate the phenotype caused by mutation of the adult β-globin gene (Noguchi *et al.*, 1988), and designed ZFPs may even be capable of activating silenced fetal globin genes (A. Reik, unpublished). The possibility of using ZFPs to regulate vascular endothelial growth factor (VEGF) in pro- and anti-angiogenic therapies (Liu *et al.*, 2001) is also being actively investigated, as is the use of 'transcription therapy' in treating cancer, for which aberrant activity of oncogenic and tumor suppressive TFs has been directly and extensively implicated (see Table II and Pandolfi, 2001).

Oncogenic transcriptional events are potentially amenable to correction through transcription therapy at various levels of intervention (Table II): (i) blockage of the aberrant function of chimeric TFs or the reversal of excessive transcriptional activity through the use of DN proteins; (ii) de-repression of tumor suppressor gene expression when epigenetically silenced through chromatin remodeling, for example by hypermethylation of CpG islands in gene promoters (Baylin *et al.*, 1998); and (iii) repression/de-repression of biologically relevant targets of TFs whose expression is aberrant in cancer pathogenesis. Transcriptional regulatory approaches are already proving to be effective in diseases such as acute promyelocytic leukemia (APL), which is caused by a chromosomal translocation that fuses the retinoic acid receptor α (RARα) gene to heterologous partners (Piazza *et al.*, 2001), forming a DN transcriptional repressor that targets histone deacetylase (HDAC) (Grignani *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998). The overexpression of

inhibitors of HDACs (HDACIs) in combination with RA have induced leukemia remission and prolonged survival (Warrell *et al.*, 1998; He *et al.*, 2001), indicating that the approach in general may be very successful. Nevertheless, the lack of specificity for aberrant transcription complexes and target genes may prove to be a major potential limitation of this approach. Hence there is a need to focus on more targeted methods that use ZFPs, polyamides or other molecules that selectively regulate genes. Not only may this intervention render the therapeutic effects more selective, but possibly also less toxic. Furthermore, ZFPs or other molecules that can bind a DNA sequence with high specificity could be utilized to mimic the transcriptional activity of powerful tumor suppressor proteins such as p53 or Rb, thus exerting a broader antitumor activity in cancers of various histological origins.

The utilization of ZFPs or polyamides for cancer therapy does not preclude, but in fact calls for, the use of 'transcription therapy' modalities, either in combination or sequentially. While combinatorial/sequential regimens for cancer treatment are known to often reach greater efficacy by reducing resistance to therapy, in a 'transcription therapy' setting, such regimens might in fact be essential for successful reactivation of gene expression, since the combinatorial use of transcriptionally active compounds may convey specificity and potentiate efficacy, in turn reducing toxicity and immunogenicity. Finally, transcription therapy for cancer may be combined with known effective chemotherapeutic agents and even tailored to enhance their efficacy. It is conceivable that drug uptake and/or the biological response to a chemotherapeutic agent (i.e. the apoptotic response) in cancer cells may be modulated at the transcription level through repression or induction of relevant target genes (e.g. by modulating the expression of detoxifying pumps or anti-apoptotic molecules). Based on these premises, there is little doubt that chromatin remodeling and transcriptional regulation with transcriptionally active compounds will have a remarkable and immediate impact on the ways in which cancer and other diseases are treated in the post-genomic era.

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