The Discovery of Zinc Fingers and Their Applications in Gene Regulation and Genome Manipulation

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gene correction, gene targeting, modular design, protein engineering, transcription activation, transcription inhibition

**Abstract**

An account is given of the discovery of the classical Cys2His2 zinc finger, arising from the interpretation of biochemical studies on the interaction of the *Xenopus* protein transcription factor IIIA with 5S RNA, and of structural studies on its structure and its interaction with DNA. The finger is a self-contained domain stabilized by a zinc ion ligated to a pair of cysteines and a pair of histidines, and by an inner hydrophobic core. This discovery showed not only a new protein fold but also a novel principle of DNA recognition. Whereas other DNA binding proteins generally make use of the two-fold symmetry of the double helix, zinc fingers can be linked linearly in tandem to recognize nucleic acid sequences of varying lengths. This modular design offers a large number of combinatorial possibilities for the specific recognition of DNA (or RNA). It is therefore not surprising that the zinc finger is found widespread in nature, including 3% of the genes of the human genome.

The zinc finger design is ideally suited for engineering proteins to target specific genes. In the first example of their application in 1994, a three-finger protein was constructed to block the expression of an oncogene transformed into a mouse cell line. In addition, a reporter gene was activated by targeting an inserted zinc finger promoter. Thus, by fusing zinc finger peptides to repression or activation domains, genes can be selectively switched off or on. It was also suggested that by combining zinc fingers with other effector domains, e.g., from nucleases or integrases, to form chimeric proteins, genomes could be manipulated or modified. Several applications of such engineered zinc finger proteins are described here, including some of therapeutic importance.
INTRODUCTION

Ten years of research on the structure of chromatin led to the discovery of the nucleosome and an outline of its structure, as well as the next level for the folding of DNA in the 300-Å chromatin fiber (1, 2). This resulted in an interest in what was then called “active chromatin,” the chromatin that is involved in transcription or that was poised to do so, and in finding a tractable system, which offered the possibility of extracting relatively large amounts of material for biochemical and structural studies.

The work of Robert Roeder and Donald Brown on the SS RNA genes of Xenopus laevis, which are transcribed by RNA polymerase III (reviewed in Reference 3) was intriguing. They discovered that the correct initiation of transcription requires the binding of a 40-kDa protein factor, variously called factor A or transcription factor IIIA (TFIIIA), which had been purified from oocyte extracts. By deletion mapping, it was found that this factor interacts with a region about 50 nucleotides long within the gene, called the internal control region. This was the first eukaryotic transcription factor to be described.

Immature oocytes store SS RNA molecules in the form of 7S ribonucleoprotein particles (7S RNPs) (4), each containing a single 40-kDa protein, which was later shown (5) to be identical with TFIIIA. TFIIIA therefore binds both SS RNA and its cognate DNA, and it was consequently suggested that it may mediate autoregulation of SS gene transcription (5). Whether this autoregulation occurred in vivo or not, the dual interaction provided an interesting
structural problem that could be approached because of the presence of large quantities of the protein TFIIIA in immature *Xenopus* oocytes.

In the autumn of 1982, Miller, a new graduate student, began studies on TFIIIA. This led to the discovery of a remarkable repeating motif within the protein, which was later, in laboratory jargon, called a zinc finger because it contained zinc (Zn) and gripped or grasped the DNA (6). This repeating structure was discovered through biochemistry and not, as some reviews have stated, by computer sequence analysis.

**PREPARATION AND CHARACTERIZATION OF TFIIIA FROM THE 7S RNP**

When Miller repeated the published protocols for purifying the 7S RNP, he obtained very low yields, which were attributed to dissociation. Brown and Roeder had used buffers that contained dithiothreitol (DTT) because the protein had a high cysteine content and EDTA to remove any contamination by metals, which hydrolyze nucleic acids. The gel filtration of the complex in 0.1 mM DTT resulted in a separate elution of protein and 5S RNA. However, when the strong reducing agent sodium borohydride did not disrupt the complex, it was realized that the protein was not held together by disulfide bridges and that a metal might be involved. After the particle was incubated with a variety of chelating agents, particle dissociation could be prevented only by prior addition of Zn$^{2+}$ and not by a variety of other metals. Analysis of a partially purified 7S preparation by atomic absorption spectroscopy also revealed a significant concentration of Zn, with at least 5 mol Zn per mol particle.

While these experiments were in progress, Hanas et al. (7) reported the presence of Zn in the 7S RNP at a ratio of two per particle. This seemed to be an underestimate because their buffers contained 0.5 mM or 1 mM DTT, which has a high binding constant for Zn of about $10^{30}$. Miller et al. (6) repeated the analysis with pure and undissociated particle preparations, without DTT, and took great care to ensure no contamination. The conclusion was that the native 7S RNP contains between 7 and 11 Zn ions (6). This result was consistent with the fact that the protein was known to contain large numbers of histidine and cysteine residues, the most common ligands for Zn in enzymes and other proteins. Such a result hinted at some kind of internal substructure.

A natural step was then to see if any such substructure could be revealed by proteolytic digestion. Miller et al. (6), who had already begun such studies, had found two products, an intermediate 33-kDa fragment and a limit 23-kDa fragment. At about that time Brown’s group (8) also showed that, on treatment with proteolytic enzymes, the 40-kDa TFIIIA protein breaks down to a 30-kDa product, which is then converted to a 20-kDa product. They proposed that TFIIIA consists of three structural domains, which they identified as binding to different parts of the 50-base pair (bp) internal control region of the 5S RNA gene.

Carrying proteolytic studies further, Miller et al. (6) found that on prolonged proteolysis the TFIIIA product breaks down further, finally to a limit digest of about 3 kDa. In the course of this breakdown, periodic intermediates differing in size by about 3 kDa were seen. The correspondence in size between these last two values suggested that the 30-kDa domain of TFIIIA might contain a periodic arrangement of small, compact domains each of 3 kDa. If each such domain contained one Zn atom, then the observed high Zn content was explained.

This novel idea of small Zn-stabilized domains was strengthened by the timely publication by Roeder’s group (9) of the amino acid sequence of TFIIIA derived from a cDNA clone. Upon inspection, the large number of cysteines and histidines present in the protein appeared to occur in a more or less regular pattern. A rigorous computer analysis by McLachlan showed that, of the 344 amino acids of the TFIIIA sequence residues, numbers 13–276 form a continuous run of nine tandemly repeated, similar units of about 30 amino acids, each containing...
A REPEATING STRUCTURE FOR TFIIIA

On the basis of the three different lines of evidence described above, namely (a) a 30-amino acid repeat in the sequence, which (b) corresponds in size to the observed periodic intermediates and the limit-digest product of 3 kDa, and (c) the measured Zn content of 7–11 atoms, Miller et al. (6) proposed that the 30-kDa region of the TFIIIA protein has a repeating structure consisting of nine 30-amino acid units (Figure 2a). Twenty-five of the 30 amino acids in the repeat fold around a Zn ion to form a small independent structural domain or module, the “finger,” and the five intervening amino acids provide the linkers between consecutive fingers (Figure 2b). The Zn ion forms the basis of the folding by being tetrahedrally coordinated to the two invariant pairs of cysteines and histidines. In addition to this uniquely conserved pattern of Cys-Cys...His-His, each repeat also contains three other conserved amino acids, namely Tyr6 (or Phe6), Phe17, and Leu23, all of which are large hydrophobic residues (Figure 1). It was suggested that these might interact to form a hydrophobic cluster stabilizing the compact finger module. The 30-amino acid repeat is rich in basic and polar residues, but the largest number are found concentrated in the region between the second cysteine and the first histidine, implicating this region in particular in nucleic acid binding. This was later found to be the case (11).
Formally, when indexed on a 30-amino acid repeat, the repeating structure could be written as

\[1-5 \quad 6 \quad 8 \quad 13 \quad 17 \quad \text{linker} \quad hX_1 \quad CX_{2,4} \quad CX_3 \quad hX_{2,3} \quad 23 \quad 26 \quad 30 \quad HX_{1,4} \quad HX_{3,4} \quad H,\]

where \(h\) represents a conserved (large) hydrophobic residue. The proposal that each 25-amino acid module formed an independently folded, Zn-stabilized domain soon gained support from two lines of research. First, a study using extended X-ray absorption fine structure confirmed that the Zn ligands are two cysteines and two histidines (12). Second, Tso et al. (13) found that, in the DNA sequence of the gene for TFIIIA, the position of the intron-exon boundaries mark most of the proposed finger module domains.

In evolutionary terms, the multifingered TFIIIA may have arisen by gene duplication of an ancestral domain comprising ~30 amino acids. Because one such self-contained small domain would have had the ability to bind to nucleic acids and could be passed on by exon shuffling, Miller et al. (6) suggested that these domains might occur more widely in gene control proteins than in just this case of TFIIIA. The extent to which this prediction has been borne out [3% of the genes of the human genome, at the latest count (14)] is astonishing. Indeed, within months of the paper’s publication, the investigators received word of sequences homologous to the zinc finger motif of TFIIIA. The first two were from Drosophila, the serendipity gene from Rosbash’s group (15) and the Krüppel gene from Jäckle’s group (16).

A NEW PRINCIPLE OF DNA RECOGNITION

The key points that emerged from Miller et al. (6) were that a new protein fold became known for nucleic acid binding and a novel principle of DNA recognition. The overall design for specific DNA recognition was distinctly different from that of the helix-turn-helix motif, found in the first DNA-binding proteins to be described. The latter binds to DNA as a symmetric dimer to a palindromic sequence on the DNA, thus making use of both the twofold symmetry of the DNA helix backbones and also the nucleotide sequence. Heterodimeric variations of this and other twofold symmetric designs were found later, but they still make use of the double-helix symmetry.

In contrast, the zinc finger is a DNA-binding module that can be linked tandemly in a linear, polar fashion to recognize DNA (or RNA) sequences of different lengths. Each finger domain has a similar structural framework but can achieve chemical distinctiveness through variations in a number of key amino acids.
Table 1  Number of Cys2 His2 and Cys4 genes in the genomes of various organisms (14)*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total number of genes</th>
<th>Cys2 His2</th>
<th>Cys4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>23,299</td>
<td>709 (3.0%)</td>
<td>48 (0.21%)</td>
</tr>
<tr>
<td>Mouse</td>
<td>24,948</td>
<td>573 (2.3%)</td>
<td>42 (0.17%)</td>
</tr>
<tr>
<td>Rat</td>
<td>21,276</td>
<td>466 (2.2%)</td>
<td>43 (0.2%)</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>20,062</td>
<td>344 (1.7%)</td>
<td>53 (0.26%)</td>
</tr>
<tr>
<td>Drosophila</td>
<td>13,525</td>
<td>298 (2.2%)</td>
<td>21 (0.16%)</td>
</tr>
<tr>
<td>Anopheles</td>
<td>14,653</td>
<td>296 (2.0%)</td>
<td>20 (0.14%)</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>19,564</td>
<td>173 (0.88%)</td>
<td>270 (1.3%)</td>
</tr>
<tr>
<td>Caenorhabditis briggsae</td>
<td>11,884</td>
<td>115 (0.9%)</td>
<td>167 (1.4%)</td>
</tr>
</tbody>
</table>

*The Cys4 genes refer to the steroid and thyroid hormone family of nuclear receptors, which were also later misleadingly named zinc fingers because of a similarity in the amino acid sequences, suggesting the presence of zinc tetrahedrally ligated to four cysteines. The structures are, however, quite different from the classical Cys2 His2 finger. Note how the proportion of Cys2 His2 genes increases with greater complexity on the evolutionary scale.

The precise pattern of amino acid interactions of zinc fingers with DNA remained unknown. The breakthrough came in 1991 when Pavletich & Pabo (11) solved the crystal structure of a complex of a DNA oligonucleotide specifically bound to the three-finger DNA-binding domain of the mouse transcription factor Zif268, an early response gene. The primary contacts are made by the α-helix, which binds in the DNA major groove through specific hydrogen-bond interactions from amino acids at helical positions –1, 3, and 6 to three successive bases (a triplet) on one strand of the DNA (Figure 4a). Later, the second zinc finger-DNA complex solved, by Fairall & Finch (21) in Rhodes’ group, revealed an important secondary interaction from helical position 2 to the other strand (Figure 4b and Figure 5). This is the canonical docking arrangement, but there are, however, some wide variations from the bacterial helix-turn-helix motif.
ZINC FINGER PEPTIDES FOR THE REGULATION OF GENE EXPRESSION

The mode of DNA recognition by a finger is thus principally a one-to-one interaction between individual amino acids from the recognition helix to individual DNA bases (11). This is quite unlike the case of other DNA-binding proteins, where one amino acid may contact two bases and vice versa. Moreover, because the fingers function as independent modules, fingers with different triplet specificities can be linked to give specific recognition of longer DNA sequences. For this reason, the zinc finger motifs are ideal natural building blocks for the de novo design of proteins for recognizing any given sequence of DNA. Indeed the first

Figure 3
The structure of a zinc finger from a two-dimensional NMR study of a two-finger peptide in solution (20). The same study showed that the linker between the two modules is highly flexible (19).

Figure 4
(a) Schematic diagram (32) of the first model of modular recognition of DNA by a three-zinc finger peptide, illustrating the results of the first crystal structure determination of the complex between the DNA-binding domain of the transcription factor Zif268 and an optimized DNA-binding site (11). Each finger interacts with a 3-bp subsite on one strand of the DNA, using amino acid residues in helical positions $-1$, $3$ and $6$. (b) Refined model of DNA recognition (32, 33).

View of the potential hydrogen bonds to the second strand of the DNA, the so-called cross-strand interactions, emanating from position 2 on the recognition helix. This is based on the crystal structure of the tramtrack-DNA complex (21), the mutagenesis (32) and phage display selection studies of Isalan et al. (33), on the refined structure of the Zif268-DNA complex, and of variants by Pabo and his colleagues (22). The fingers ideally bind 4-bp overlapping subsites, so that adjacent fingers are functionally synergistic though structurally independent.
Figure 5
Another view of the refined model. Contacts with the DNA are made by amino acids at helical positions $-1, 3$, and $6$ to the coding strand and from position $2$ to the noncoding strand. Thus, the binding site for a finger is not simply a triplet of three successive bases, but a 4-bp site overlapping with that of the preceding finger (32). Illustration courtesy of Sangamo BioSciences, Inc.

experiments by Berg (17) and others, using site-directed mutagenesis, showed that it is possible to rationally alter the DNA-binding characteristics of individual zinc fingers when one or more of the amino acids in the $\alpha$-helical positions are varied. As a small collection of these mutants accumulated, it became possible to find some regularities or rules relating amino acids on the recognition $\alpha$-helix to corresponding bases in the bound DNA sequence.

The MRC laboratory adopted a different approach. The reason was that these rules did not take into account the fact that real DNA structures are not fixed in the canonical B form, but as was shown earlier, there are wide departures, depending on the DNA sequence (23, 24). This was further supported by the structure of the zinc finger-tramtrack DNA complex (21). Here the helical position used for the primary contact by the first finger with the $3'$-most base of the cognate triplet (thymine) is not the canonical $-1$, but $2$. The cause is that the DNA helix is deformed from the B form, with a thymine followed by an adenine at a helical rotation angle of $39^\circ$, rather than the canonical $36^\circ$, and preceded by another adenine at an angle of $33^\circ$. The interaction with the finger thus occurs at an ATA sequence, which has unusual flexibility, as noted long ago (23). DNA is not a rigid, passive participant in its interaction with proteins.

**AFFINITY SELECTION FROM A LIBRARY OF ZINC FINGERS BY PHAGE DISPLAY**

The alternative to this rational but biased design of proteins with new specificities was the isolation of desirable variants from a large pool or library. A powerful method, namely phage display, of selecting such proteins is the cloning of peptides (25) or protein domains (26) as fusions to the minor coat protein (pIII) of bacteriophage fd, which leads to their expression on the tip of the capsid. Phages displaying the peptides of interest can be affinity purified by binding to the target and then amplified in bacteria for use in further rounds of selection and for DNA sequencing of the cloned genes. This technology was applied to the study of zinc finger-DNA interactions after Choo demonstrated that functional zinc finger proteins could be displayed on the surface of fd phage and that such engineered phage could be captured on a solid support coated with the specific DNA (27, 28). The phage display method was also adopted by other groups working on zinc fingers, including those of Pabo and Barbas.

Phage display libraries comprising $\sim10^7$ variants of the middle finger from the DNA-binding domain of Zif268 were created. A DNA oligonucleotide of fixed sequence was used to bind and hence purify phage from this library over several rounds of selection, returning a number of different but related zinc fingers which bind the given DNA. By comparing similarities in the amino acid sequence of functionally equivalent fingers, the likely mode of interaction of these fingers with DNA was deduced (27). Remarkably, most base contacts were found to occur from three primary positions on the $\alpha$-helix of the zinc
finger, correlating well with the implications of the crystal structure of Zif268 bound to DNA (11).

This demonstrated ability to select zinc fingers with desired specificity meant that, as the data from the selections accumulated, some wider general rules could be devised for a recognition code (28), and hence, DNA-binding peptides could be made to measure using the combinatorial strategy exemplified by TFIIIA. In other words, these general rules could be used for the rational design of a zinc finger peptide to recognize a short run of DNA sequence by mixing and matching individual specific fingers. Where the general rules for finger specificity led to an ambiguity, as in the case of closely related triplets, e.g., GCG and GTG, Choo & Klug (28) showed that zinc finger modules could nevertheless be selected to discriminate between them.

USE OF ENGINEERED ZINC FINGER PEPTIDES TO REPRESS GENE EXPRESSION IN A MOUSE CELL LINE

One interesting possibility for the use of such zinc finger peptides is to selectively target genetic differences in pathogens or transformed cells. In December 1994, Choo et al. (29) reported the first such application, which built a protein that recognized a specific DNA sequence both in vitro and in vivo. This was a crucial test of the understanding of the mechanism of zinc finger DNA recognition. This proof of the principle led to future zinc finger studies of potential applications in gene regulation for research purposes and for therapeutic correction. It also stimulated the creation of the first biotech companies (Sangamo BioSciences, Inc., in Richmond, California, and later Gendaq Ltd. in Cambridge, United Kingdom) to exploit the new technology.

In summary, a three-finger peptide was created that is able to bind site specifically to a unique 9-bp region of the p190 bcr-abl cDNA: This is a transforming oncogene, which arises by translocation between the tips of chromosomes 9 and 22, of which one product is the Philadelphia chromosome (29). Chromosome 22 contains a novel DNA sequence at the junction of two exons, one each from the two genomic parent bcr and abl genes. The engineered peptide discriminated in vitro against like regions of the parent bcr and c-abl genes, which differ in only a single base out of the 9-bp target, by factors greater than one order of magnitude (29).

This peptide also contained a nuclear localization signal (NLS) fused to the zinc finger domain so that the peptide could accumulate in the nucleus. Consequently, stably transformed mouse cells, made interleukin-3 independent by the action of the oncogene, were found to revert to IL-3 dependency on transient transfection with a vector expressing the peptide. This construct was also engineered to contain an e-myc epitope, which enabled investigators to follow by immunofluorescence the localization of the peptide to the nuclei of the transfected cells. When IL-3 is subsequently withdrawn from cell culture, over 90% of the transfected p190 cells become apoptotic (that is, showing chromosome degradation) within 24 h (Figure 6, left). These experiments were repeated on cells transformed by another related oncogene, p210 bcr-abl, which served as a control. All transfected p210 cells maintained their IL-3 dependency and remained intact on entry of the engineered peptide (Figure 6, right) (29).

Measurements of the levels of p190 bcr-abl mRNA extracted from cells treated with the peptide showed that the repression of oncogenic expression by the zinc finger peptide was due to a transcriptional block imposed by the sequence-specific binding of the peptide, which, with its highly basic NLS, presumably obstructed the path of the RNA polymerase. In later experiments to inhibit gene expression, a repression domain was added, such as the Kox domain from the Xenopus KRAB zinc finger family, and fused to the zinc finger construct (30, 31).
PROMOTER-SPECIFIC ACTIVATION BY A CHIMERIC ZINC FINGER PEPTIDE

These experiments showed that a zinc finger peptide could be engineered to switch off gene expression in vivo. The same paper (29) also described other experiments on a different cell system (cultured mouse fibroblasts) to show that a gene could also be switched on by a zinc finger construct. The same 9-bp sequence was used, but this time as a promoter for a CAT reporter gene contained in a plasmid. The peptide, which recognized the promoter sequence, was fused to a VP16 activation domain from herpes simplex virus and, on transient transfection, stimulated expression of the reporter gene by a factor of 30-fold above controls.

IMPROVING ZINC FINGER SPECIFICITY BY PROTEIN ENGINEERING

First having shown proof of the principle that engineered zinc finger peptides could be used to target DNA, improving the specificity of recognition became the focus of subsequent work. Although the main source of specificity lies in the amino acids at positions –1, 3, and 6 of the recognition α-helix of a zinc finger for successive bases lying on one strand of a DNA triplet, Isalan et al. (32) found that the “cross-strand” interaction, described above, from helical position 2 to the neighboring base pair on the adjacent triplet (Figure 4b) can significantly influence specificity. Therefore, it has been necessary to revise the simple model that zinc fingers are essentially independent modules that bind 3-bp subsites to a model that considers functional synergy at the interface between adjacent independently folded zinc fingers. In this refined model, Zif268-like zinc fingers potentially bind 4-bp overlapping subsites (Figures 4b and 5) (33).

Consequently, Isalan et al. (34) redesigned the method of phage library construction to allow for the optimization of the interaction that a finger makes with the DNA-binding site of the adjacent N-terminal finger. They adopted a bipartite selection strategy in which two halves of a three-finger DNA-binding domain are selected separately and then recombined in vitro to make a complete three-finger domain, which binds 9 bp and automatically allows the interface synergy between its constituent three fingers. These two separate (nonoverlapping) libraries are then used to perform the bipartite selections. Because all the steps are carried out in vitro, the method is rapid and easily adapted to a high-throughput automated format.
was applied commercially by the MRC spin-off company Gendaq (later acquired by Sangamo BioSciences) for up to $2 \times 4^5 = 2048$ binding sites to create a large archive of zinc finger peptides that recognize a vast number of DNA 9-bp sequences. Second, an important step forward was to increase the length of the DNA sequence targeted and hence its degree of rarity; at the same time, an increase was expected in the binding affinity of the longer zinc finger construct. Three zinc fingers recognize a 9-bp sequence, which would occur randomly many thousands of times in a large genome. Therefore, six fingers linked together would recognize a DNA sequence 18 bp in length, which is sufficiently long to constitute a rare address in the human genome. However, one cannot simply go on adding fingers with the conventional linkers because the periodicity of the packed fingers does not quite match the DNA periodicity. They thus tend to get out of register and are strained in doing so, leading to only a small increase in affinity. Investigators have, therefore, learned how to engineer longer runs of zinc fingers that can target longer DNA sequences and have affinities a thousand times greater than three-finger peptides (Figure 7) (35, 36).

An early design by Kim & Pabo (37) was to use a longer more flexible linker between two different preexisting three-finger domains to form a six-finger peptide, but this has not been used much in practical applications. The method of choice in current use is that developed in Cambridge by Moore et al. (36) (and later transferred to Sangamo via Gendaq). This uses two-finger binding domains, which can be obtained from the archive described above. Three of these are assembled into six-finger domains, using longer variants of the conserved six-amino acid linker TGEKP (Figure 7b).

The linkers contain an extra glycine residue or a glycine-serine-glycine tripeptide between the constituent two-finger modules. Such six-finger domains (denoted $3 \times 2F$) bind their 18-bp targets with picomolar affinity, as also do six fingers made by linking two three-finger domains ($2 \times 3F$) made with an extended linker (36). However, the advantage of the $3 \times 2F$ over the $2 \times 3F$ strategy is that it is much more sensitive to a mutation or an insertion in the target sequence, with a loss of affinity of up to 100-fold. Thus, the $3 \times 2F$ peptide discriminates more strongly than the $2 \times 3F$ peptide between closely related DNA target sequences. The logic behind the $3 \times 2F$ design was that the strain in binding longer DNA targets is more evenly distributed than in a $2 \times 3F$ construct, and it indeed turned out to have the advantage expected. These six-finger peptides not only have picomolar affinities for their 18-bp targets but also give virtually single-gene recognition (38) when tested on DNA microarrays displaying 20 thousand different sequences. As mentioned above, a library of two-finger peptides begun in an MRC laboratory, Cambridge, was transferred via Gendaq to Sangamo.

Another strategy Moore et al. (35) developed was to target two noncontiguous 9-bp DNA sequences separated by up to 10 bp of unbound DNA. Using a nonspecific binding finger (in which all key amino acids had been mutated to serine) as a structured linker, investigators found that it could span a gap of 7 to 8 bp and maintain picomolar affinity. In contrast, the use of a flexible linker such as (GSG)$_n$ displayed no preference for a length of span, but the affinity was reduced to $\sim 50$ pM, probably attributable to the increased conformational entropy of the long peptide. These strategies have not yet been deployed.

**SOME APPLICATIONS OF ENGINEERED ZINC FINGER PROTEINS**

Zinc finger proteins (ZFPs) can be engineered with a variety of effector domains fused to polyzinc finger peptides, which can recognize virtually any desired DNA sequence with high affinity and specificity. They thus form the basis of a novel technology, which has increasing uses in research and medicine. An excellent summary of numerous such applications has been given by Pabo and his colleagues (39). A few of these are mentioned here.
Figure 7
Two modes of generating six-zinc finger proteins for specific recognition of 18-bp sequences (35, 36). (a) Two three-finger peptides fused together using an extended canonical linker (2 × 3F scheme). (b) Three two-finger peptides linked using canonical linkers extended by an insertion of either a glycine residue or a glycine-serine-glycine sequence in the canonical linkers between fingers 2 and 3 and fingers 4 and 5, respectively.

VEGF: vascular endothelial growth factor

1. Inhibition of human immunodeficiency virus (HIV) expression (30): It was shown that ZFPs targeted to the HIV promoter long terminal repeat activated by the tat protein effectively repressed expression, and preliminary experiments in a cellular infection assay gave a threefold drop in infectivity.

2. Disruption of the effective cycle of infection of herpes simplex virus (31): A ZFP transcription factor designed to repress the promoter of a viral gene that is normally activated first in the replication cycle produced a tenfold reduction in the virus titer in an infected cell line (Figure 8). This was a good result, considering that there are five other “immediate early” genes that contribute to the infection. Several more of these would have to be repressed to reduce the titer further.

3. Activating the expression of vascular endothelial growth factor (VEGF)-A in a human cell line (40) and in an animal cell line (41)
model (41): These experiments have led to a therapeutic application (see below).

4. Regulation of the level of zinc finger expression by a small molecule (42): This can be used in controlling the dose and/or timing for a therapeutic application. An efficient way of achieving this is by fusing the ZFP to the ligand-binding domain of a steroid hormone nuclear receptor. In the absence of hormone, the ZFP transcription factor is retained in the cytoplasm, but after ligand binding, the ZFP translocates to the cell nucleus in active form.

STIMULATION OF NEW VASCULATURE BY ENGINEERED ZINC FINGER PROTEINS

Following the work at Sangamo on the activation of the transcription factor VEGF by ZFPs in mouse and human cell lines (40), experiments showed that new blood vessels could be formed in a mouse ear (41). These did not leak, unlike the results that had been obtained by earlier workers who had been delivering various cDNA-spliced isoforms of the gene. The reason for the success with ZFP activation is that the latter acts on the promoter of the VEGF gene, and hence, all spliced isoforms are naturally produced when the gene is induced to promote angiogenesis.

Subsequent work by F. Giordano (unpublished results) at Yale and B. Annex (unpublished results) at Duke University showed increased blood flow in hind limbs of ischemic rabbits, with the ZFP delivered by a retrovirus or simply by injecting the DNA. Two years ago Sangamo began clinical trials to evaluate the ability of an appropriate ZFP to stimulate the natural growth of normal blood vessels in treating claudication, a symptom of peripheral arterial obstructive disease that causes poor blood flow in the legs. The phase 2 trials have shown great improvements and, indeed, also in the more serious condition of critical limb ischemia.

Figure 8
Repression of herpes simplex virus (HSV-1) infection (at 0.05 pfu/cell) of FACS-sorted HeLa cells (9 \times 100,000) by zinc finger peptides fused to a KOX1 repression domain (31). The peptides are targeted to the viral gene IE175 K, the first of the six immediate early genes to be expressed by the virus. A six-finger recognition peptide, 6F6KOX, reduces the virus titer tenfold, whereas a three-finger peptide produces only a 20% reduction.

GENE CORRECTION BY HOMOLOGOUS RECOMBINATION USING SEQUENCE-SPECIFIC ZINC FINGER NUCLEASES

Gene correction is the process by which sequence alterations in defective or deleterious genes can be changed or corrected by homologous recombination (HR)-mediated gene conversion between the target locus and a donor construct encoding the corrective sequence (Figure 9, left). Monogenic disorders, such as X-linked severe combined immune deficiency (SCID), sickle-cell anemia, hemophilia, and Gaucher’s disease, are caused by the inher- itance of defective alleles of a single gene. The ability to replace this gene sequence via HR-mediated gene correction has the potential of fully restoring the gene function and providing a permanent cure for patients with these disorders. However, this process is highly inefficient in that the frequency of unaided HR at a specific locus occurs is only about 1 in 10^5 cells (43). This is far below a level that would be considered therapeutic. A double-stranded

ZFP: zinc finger protein
HR: homologous recombination
Gene modification or correction using homologous recombination (43) via "short-path gene conversion" stimulated by a double-stranded break (DSB) (44). The left side schematically depicts the repair of a random X-ray-induced DSB by homologous recombination using the sister chromatid as the repair donor. As shown on the right, a site-specific DSB is created by zinc finger protein nucleases (ZFNs). Abbreviation: HDR, homology-directed repair.

Figure 9
Gene modification or correction using homologous recombination (43) via "short-path gene conversion" stimulated by a double-stranded break (DSB) (44). The left side schematically depicts the repair of a random X-ray-induced DSB by homologous recombination using the sister chromatid as the repair donor. As shown on the right, a site-specific DSB is created by zinc finger protein nucleases (ZFNs). Abbreviation: HDR, homology-directed repair.

Figure 10
Gene correction using a pair of three-zinc finger protein nucleases (ZFNs) (45) to produce a double-stranded DNA break. The zinc finger peptides are linked to the nonspecific catalytic domain of the Fok1 endonuclease by a short amino acid linker. In the three-dimensional structure the two catalytic domains form a dimeric association.

break (DSB) has been demonstrated by Jasin (44) to potentiate HR at a specific genetic locus by ∼5000-fold. Therefore, the introduction of a corrective donor sequence together with a site-specific nuclease that would produce a DSB at or near the location of the mutation could stimulate gene correction to levels that would provide a therapeutic impact.

Jasin’s demonstration was based on artificially introducing into an endogenous gene of a human cell line an 18-bp DNA sequence, which was the specific binding site for the homing endonuclease Sce1 and had only a small probability of occurring naturally elsewhere in the genome. However, to carry out gene correction in native cells requires the specific targeting of the mutated sequence, and a zinc finger peptide fused to a nuclease domain is the natural choice (Figure 9, right). A nuclease of this type has been developed by Chandrasegaran and coworkers (45) using an engineered ZFP fused to the nonspecific cleavage domain of the Fok1 type II restriction enzyme. This type of zinc finger nuclease (ZFN) has been used by Carroll and colleagues (46) to produce mutants in Drosophila by gene correction and by Porteus & Baltimore (47) in a green fluorescent protein model system to study gene modification in a human cell line. Following Chandrasegaran, the three-finger ZFP nucleases were introduced as pairs with tandem binding sites engineered in opposite orientations (Figure 10), with a 6-bp spacing separating the two half sites (45). In both cases, the efficiency of the targeting is not very high, but this was not crucial to those studies. Indeed, some of the Drosophila mutants were lethal, but they are normally selected on the phenotype. Both studies showed that a ZFN-produced DSB can markedly increase the rate of HR between a donor DNA construct and a reporter gene in their two different systems.

The results of Carroll’s and of Porteus’ laboratories stimulated work at Sangamo to apply the gene correction method to tackle monogenic disorders of the human genome. The workers set out to determine whether ZFNs could create a comparable increase in HR frequency at an endogenous human gene. They focused their efforts on the IL2Rγ gene in which loss-of-function point mutations cause X-linked SCID. In the absence of bone marrow transplantation or gene therapy, this malady leads to death in early childhood. Treatment for the disease by gene therapy (48) has been performed by inserting one or more copies of the normal gene in the chromosomes of a number of affected children, that is, by gene addition rather than gene correction. After successful
treatment of the majority of the patients, two of them died some years later of leukemia, probably because the random insertion of the gene led to its coming under inappropriate control.

The middle exon of the IL2Rγ gene contains SCID mutation hot spots. Because maximal homology-driven recombination occurs when a DSB is evoked at or close to the mutated site, the Sangamo workers engineered a pair of ZFNs specific for the exact location of the mutation on the X chromosome. Two DNA-binding domains were assembled from the zinc finger archive, described above, each containing four highly specific zinc finger motifs, and thus simultaneously recognizing two different 12-bp sites, separated by a fixed distance between them. The chance of this particular pattern existing elsewhere in the genome is negligible. Having confirmed in vitro that the proteins bind as intended, they next improved them further by single amino acid substitutions in the zinc finger recognition helices, which gave an additional fivefold increase in potency. The results (49) showed an 18% to 20% rate of gene correction, which was stable after one month in culture, in the target cells (Figure 11).

This accomplishment is dramatic, with an increase by many orders of magnitude over anything achieved in the past by “gene targeting,” particularly as no selection has been used. Moreover, measurements of both the mRNA and protein levels expressed by the corrected IL2Rγ gene showed that the mutation had been efficiently and stably corrected. The next steps in curing SCID disease are to isolate CD34 progenitor cells from the patient’s bone marrow and correct them ex vivo. After this gene correction, the cells can be allowed to expand and then be reintroduced into the bone marrow to repopulate it with corrected cells. Of course, an extensive study of ZFN safety (e.g., verifying that no other DSBs are created outside the target and checking for immunogenicity) must first be undertaken, but there is every prospect of gene correction eventually becoming a reality for SCID and other monogenic diseases.

![Image](https://www.annualreviews.org/doi/10.1146/annurev-biochem-040111-150928)

**Figure 11**

Results of gene correction for severe combined immune deficiency disease by the Sangamo group (49). High-frequency homology-driven repair (HDR) at the endogenous IL2Rγ locus of leukemia K562 cells produced by designed zinc finger nucleases (ZFNs). Cells were transfected with the indicated plasmids (at two different concentrations indicated by low and high to ensure that the exogenous DNA load was not limiting). The donor DNA was marked by a BsrB1 restriction site (not present in the K562 genome), which was created by replacing a single nucleotide of one codon so as to preserve the amino acid code. After one month, genomic DNA was isolated, digested to completion with EcoR1, BsrB1, and Dpn1, and the percentage of BsrB1-carrying chromosomes was measured by Southern blotting with the probe indicated. A Sph1 restriction fragment is also shown as a size marker. Abbreviation: GFP, green fluorescent protein.

**SPECIFICITY OF ZINC FINGER CONSTRUCTS**

Like any conventional small-molecule drug, a ZFN can produce secondary effects, but in this case, it is possible to assess this by measuring the binding to off-target sites on the DNA. This
was done by Sangamo in their experiments on establishing HIV resistance in CD4 T cells by genome editing (50). Here the target was the HIV receptor CCR5, which was disrupted at 36% efficiency, and effects at the top 15 DNA sequence-related sites were read by 454 DNA pyrosequencing technology. The largest off-target effect (5.4%) was at the biologically related CCR2 site, and of the other 13 sites, one was at an intron of a gene AβLIMZ on chromosome 4, which showed a very low frequency of mutations in the two 38,000 sequences examined. Taken together with the “surveyer” Cel-1 nuclease data and the preservation of the biological properties of the cell, all the results support the conclusion that the ZFNs used in the CCR5 work are highly specific.

TARGETED GENE KNOCKOUT

Gene knockout is the most powerful tool for determining the function of a gene and also for permanently modifying the phenotype of a cell. Recent methods use HR (43) where recognition of the target is by the homology of the extrachromosomal donor DNA (see above). The low efficiency of this process can necessitate screening thousands of clones. Rapid gene knockout can be achieved by simply using a ZFN to create a DSB at the target and, in the absence of a DNA donor, allowing it to be repaired by the natural process of nonhomologous end joining (NHEJ). This is an imperfect repair process and usually results in changes to the DNA sequence at the site of the break and hence to mutant (null) alleles of the protein products. In this way, investigators at Sangamo produced a disruption of the dihydrofolate reductase gene in CHO cells at frequencies greater than 1%, thus obviating the need for selection markers. This established a new method for gene knockouts (51), with applications to reverse genetics and recombinant protein productions in mammalian cells in a serum-free media.

As an unnamed reviewer remarked, “it is ironic that the NHEJ pathway, responsible for the annoying background of random integrants that has plagued gene targeting for so long, is the basis of a simple and efficient method for knockouts” (43). A spectacular demonstration of the power of NHEJ-targeted knockout has appeared recently. Meng et al. (52) and Doyon et al. (53) have shown that heritable mutations can be produced in zebrafish by generating mRNAs encoding ZFNs for the locus of interest and injecting them into embryos at the one-cell stage. Both groups also measured off-target effects: Meng et al. found a rate of ~1%, but Doyon et al. detected no off−target cleavage. The most likely (and comforting) explanation of the difference is that the latter used four-finger ZFNs and thus was more specific than the three-finger ZFNs used by Meng et al.

OTHER APPLICATIONS OF ENGINEERED ZINC FINGER PROTEINS

The Sangamo ZFP development programs for obstructive limb disease, diabetic neuropathy, and HIV/AIDS are the most advanced, but other programs underway include amyotrophic lateral sclerosis (ALS, a motor neuron disease) and nerve regeneration. These can be followed on the company’s Web site at http://www.sangamo.com.

It should also be mentioned that customized zinc finger constructs for given DNA sequences can now be obtained commercially from Sigma-Aldrich, as laboratory reagents under the name Compo-Zr® technology.

Another application of zinc finger technology outside “the therapeutic space” is in the strategic partnership Sangamo has with Dow Agricultural Sciences for use of ZFNs in the breeding of enhanced plant crops. One example that has been announced is the layering of traits (quantitative trait loci or QTLs) in which particular alleles of a set of genes can be introduced to give a particular variant of a crop.

Thanks largely due to the development of a robust zinc finger technology by Sangamo BioSciences for therapeutic applications, the power of the new principle of gene recognition, discovered in 1985 (29), has been recognized by other groups. Thus, Sangamo's
recent paper in Nature on genome modification in maize (54) was accompanied by a paper from another group on tobacco (55). A science correspondent for the Daily Telegraph recently called the technology "precision gene surgery." The availability of customized zinc finger reagents from Sigma-Aldrich, mentioned above, means that the technology is now accessible to all researchers without the means to make their own.

Frequent press releases from Sangamo BioSciences chart the progress of the applications, e.g., most recently to diabetic neuropathy, where nerve conduction has been re-established in what were essentially blocked nerves.

DISCLOSURE STATEMENT

I am on the Scientific Advisory Board of Sangamo BioSciences, Inc., a biotech company in Point Richmond, California, which is further developing the zinc finger technology for applications in therapeutics and plant crops engineering.

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