Recent developments in the engineering of zinc finger proteins

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INTRODUCTION
The expression of genes in all organisms is primarily controlled by the interactions of protein complexes with DNA. Transcription factors are important mediators of these interactions and generally contain one or more DNA-binding motifs. The structures of well-characterised DNA-binding domains such as the leucine zipper, helix-turn-helix, homeodomain and zinc finger show that these domains have similar modes of specific base recognition via amino acid side-chains protruding from an α-helix, known as a ‘recognition helix’, into the major groove of DNA. It was anticipated that these domains could be engineered into ‘designer’ transcription factors to target any DNA sequence for custom gene regulation.1–8 One domain, the zinc finger, emerged as the obvious choice to engineer because of its small size, simplicity of structure and its relatively simple interactions with DNA. Now, as biology enters the post-genomic era, with the sequences of many eukaryotic genomes unveiled, the technology to regulate genes using these ‘designer’ zinc finger transcription factors exists. This paper will first provide an overview of zinc finger structure and will then focus on the current status of efforts to regulate gene expression at will. Progress made towards using ‘designer’ transcription factors in the treatment of human disease will also be considered.

STRUCTURE OF CYS₂HIS₂ ZINC FINGERS
The zinc finger motif, originally recognised by Klug and co-workers,9 contains approximately 30 amino acids, which fold into a compact βαβ domain stabilised by a zinc ion and a cluster of hydrophobic residues. The zinc ion is chelated between two cysteine residues within the β-strands and two histidine residues in the α-helix.10 Hence, zinc fingers of this type are known as Cys2His2 zinc fingers. This domain is found in over 700 human proteins, making it the second most abundant motif found in the human
Zinc finger proteins have a simple ββα structure

Zinc finger interactions with DNA are well understood for model proteins

3 zinc fingers bind 9–10 bp of DNA

Cys2His2 zinc fingers have a simple ββα structure

Figure 1: A schematic representation of the interaction between a single zinc finger and its double-stranded DNA target site, as inferred from X-ray crystal structures and biochemical studies. The simple ββα structure of the zinc finger can clearly be seen. Straight arrows indicate contacts from zinc finger amino acid side-chains at helical positions –1, 3 and 6 to consecutive nucleotides on the top DNA strand. The curved arrow demonstrates a ‘cross-strand’ interaction from the amino acid side-chain at position 2 to the complementary DNA strand. Note that a single zinc finger interacts with double-stranded DNA over a 4 bp span.

These cross-strand interactions can create synergy between neighbouring fingers for the recognition of the 5’ base of each triplet-binding site, and have been explored in detail. In general, adjacent zinc fingers within a transcription factor are connected by the consensus linker peptide –TGEKP–. Such a high degree of conservation implies a significant role for this linker sequence in zinc finger function. Indeed, mutagenesis studies on the first three fingers of transcription factor IIIA (TFIIIA) demonstrated that mutations in this linker could reduce DNA-binding affinity up to 20-fold. Also, it has been shown that the disordered TGEKP-linker becomes structured on binding to DNA and forms a C-terminal helix-capping motif which helps stabilise the zinc finger–DNA complex. The importance of the linker sequence is further demonstrated by the natural Wilms’ tumour suppressor protein, WT1. This protein has two isoforms, characterised by the presence (+WTS) or absence (–WTS) of a three-amino acid insertion in the TGEKP-linker between the third and fourth fingers. The WT1–WTS isoform binds DNA through four fingers and is associated mainly with active transcription, whereas the WT1+WTS isoform binds DNA far more weakly, and is associated instead with regions of RNA processing.
ENGINEERING OF ZINC FINGER PROTEINS TO BIND DOUBLE-STRANDED DNA

Background
Two approaches were originally used for zinc finger protein (ZFP) engineering in order to expand the DNA recognition code and to create zinc fingers that bind desired base triplets — a combinatorial approach using libraries of zinc fingers displayed on the surface of filamentous phage that were selected against target DNA sequences, and a rational design approach that used databases to predict rules for amino acid–base interactions. Methods to design and select zinc finger units and assemble them to create three-finger DNA-binding domains have been reviewed thoroughly by the authors’ group and others and only recent advances will be discussed in detail here.

Phage selections
Initial combinatorial experiments were based on the assumption that the zinc finger was an isolated unit that bound a DNA triplet. Randomisations of residues within an individual zinc finger of the three-finger protein Zif268 and selections against DNA triplets of the sequence 5′-GNN-3′ were successful and led to a three-finger assembly that bound a 9 bp sequence within an oncogenic gene. Selections of this type have since been extended so that small libraries of zinc finger modules that bind every possible sequence of the form 5′-GNN-3′ have been made and validated. Triplet sequences other than 5′-GNN-3′ proved problematic to select until the cross-strand interaction between zinc fingers was studied in detail. Again, similar experiments have resulted in a library of zinc fingers that bind 5′-ANN-3′. The obvious limitation of this strategy was that it ignored the potential for synergy between adjacent fingers. At best, this was likely to result in suboptimal binding, and, at worst, unfavourable cross-strand interactions could considerably reduce affinity. In contrast, strategies which exploited the cross-strand interactions have revealed zinc finger pairs that display high specificity for a particular base in the 5′ position.

Bipartite method to phage selection
In a recent publication, a method for the rapid, high throughput engineering of sequence-specific zinc finger DNA-binding domains which addresses the need for synergy between adjacent zinc fingers has been developed. In this method, clones were selected in parallel from two phage display libraries of three-finger domains which recognised sequences of the form 3′-IJK LMG GCG-5′ (Lib12) and 3′-GCC GMN OPQ-5′ (Lib23), where IJKLMNPOQ were any specified bases (Figure 2). Small pools of fingers from each of the selections were then cleaved within the middle finger and the desired 1.5 finger units were recombined to create diversity among the final population of three-finger peptides (Figure 2). These were then re-selected against the 3′-IJK LMN OPQ-5′ target site to find the optimal three-finger peptide in each case. Hence, selection of zinc finger domains was achieved in the context of the final target site, which is favourable since the fine structure of DNA is dependent on its sequence context. This ‘bipartite’ procedure has been illustrated by the selection of several three-finger peptides which specifically target the human immunodeficiency virus (HIV)-1 promoter region. Furthermore, since there are no sequence restrictions on the DNA–binding sites for proteins produced by this method, potentially any genomic sequence can be rapidly and specifically targeted.

Bacterial two-hybrid selections
As an alternative to phage display, a bacterial two-hybrid system has been developed which is analogous to the yeast two-hybrid system (Figure 3). This had the advantage of being an in vivo system
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Figure 2: A representation of the ‘bipartite’ approach to the construction of novel three-finger peptides for binding desired 9–10 bp target sites. 1.5 finger units are selected in parallel from two phage libraries, Lib12 (which contains randomisations in finger one and the first half of finger two) and Lib23 (which contains randomisations in the second half of finger two and finger three). These 1.5 finger units are then recombined to generate a novel three-finger protein.

Figure 3: A simple representation of the bacterial two-hybrid system for selecting zinc finger variants from large randomised libraries. In the example described, finger three (F3) of the three-finger protein is randomised at positions 1, 2, 3, 5 and 6. In (A), F3 is unable to bind the DNA subsite, the binding of RNA polymerase to its promoter is not stabilised and insufficient selection marker is produced to allow bacterial growth on selective media. In (B), bacteria grow on the selective media when F3 binds tightly to the DNA subsite causing RNA polymerase to be recruited to the DNA complex and the selection marker is expressed.
where zinc finger functionality and specificity were selected in the context of the bacterial genome. A three-finger Zif268 peptide library of $2 \times 10^9$ members was created which contained randomisations at positions $-1, 1, 2, 3, 5$ and $6$ of finger $3$. This gave more diversity in these helical positions than the phage libraries described previously. Library members were screened against desired target sites of the form $3'\text{--GCG GGG XXX--5'}$ (in which the fixed positions are bound by fingers $1$ and $2$ of Zif268) (Figure 3). Selection was based on the ability of tightly bound clones to activate transcription of a downstream selection marker, which allowed bacterial growth on selective media. The sequences of the selected ZFPs agreed well with those selected by phage display methods; additionally, in one selection it appeared that a zinc finger of better affinity and specificity had been recovered.

**Limitations of rational design**

Analyses of zinc finger databases and selections have suggested a DNA recognition ‘code’, particularly with respect to certain seemingly well-conserved amino acid–base contacts, however, the ‘code’ is clearly ambiguous and further confused by the context dependence of DNA and protein structure. As a result, attempts to design site-specific zinc finger DNA-binding domains, solely by rational design or by selection followed by site-directed mutagenesis, can be both time consuming and unsatisfactory. The potential limitations of rational design were highlighted in a study by Wolfe et al. of the high-resolution structures of two phage-selected Zif268 variants bound to their target DNA sites, which incorporated a eukaryotic TATA box sequence. Briefly, standard base contacts were found at positions $-1$ and $3$ of the zinc fingers helices but residues at position $6$ often made no obvious interactions with their predicted base partner. Secondary contacts were made from the residues at positions $1$ and $2$ in the helix of all fingers. The relatively flexible and slightly deformed structure of the TATA element, in comparison with ideal B-DNA, may help to explain some of these unusual interactions. Zinc fingers could be rationally designed to bind the same TATA sequence but the code would not allow for the same richness of interactions between protein and DNA and may lead to reduced affinity and specificity.

**ENGINEERING OF ZINC FINGER PROTEINS TO OTHER NUCLEIC ACID STRUCTURES**

**Binding of telomeric DNA**

The utility of the zinc finger module has been further demonstrated by the phage selection of a three-finger Zif268 variant, which bound single-stranded human telomeric DNA in the G-quadruplex conformation. The exact nature of the binding interaction was unknown; however, binding was both sequence dependent and structure specific, and provided the first example of an engineered protein that bound to G-quadruplex DNA. Such proteins may find in vivo uses for investigating the structure and maintenance of telomeric DNA.

**Binding of RNA**

As stated earlier, many ZFPs are known to bind RNA. In the case of the nine-finger protein, TFIIIA, the main contribution to DNA binding is from fingers $1–3$, while fingers $4–7$ contribute most of the RNA-binding energy. In contrast, WT1 is believed to bind both DNA and RNA through the same fingers. Therefore, binding to RNA may require recognition of a combination of sequence and three-dimensional structure as opposed to DNA recognition which is essentially base sequence specific.

The potential to use zinc fingers to bind RNA has been developed to target the double-stranded HIV Rev Response
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Element (RRE). This element is found within the env gene and was chosen as a target because zinc fingers that competitively inhibit the binding of the Rev protein to this sequence could potentially inhibit HIV propagation. Friesen and Darby\textsuperscript{56} isolated, by a combination of phage display and gene shuffling, two-finger proteins that bound with high affinity to HIV RRE stem–loop IIB and 5S RNA. It was later demonstrated that most of the binding affinity for the stem–loop IIB was provided by the C-terminal finger of the selected peptide which recognised the isolated stem–loop far better than the full-length RRE.\textsuperscript{55} Phage selections and recombination were then used to generate novel zinc fingers that bound with high affinity to the full-length HIV RRE. In a different approach against the same target, the recognition helices of single fingers of Zif268 were engineered to contain residues from the ß-helix in Rev responsible for RRE binding. The new proteins bound RRE and activated a HIV-1 LTR-CAT reporter when fused to the HIV-1 Tat activation domain.\textsuperscript{57}

Polydactyl ZFPs

Early attempts to build six-finger peptides joined all fingers with the canonical linker sequence, −TGEKP−, between units.\textsuperscript{58,59} These proteins displayed only modest increases in affinity over their constituent three-finger peptides. Whereas, six-finger proteins constructed by joining two three-finger domains with linkers, that were either 4 or 7 amino acids longer than the canonical linker, bound their extended target sites up to 6,000-fold tighter than either three-finger site alone.\textsuperscript{60} These longer linkers were designed to relieve some of the strain within the DNA-bound complex and to allow the three-finger DNA-binding domains to bind sequences separated by 1 or 2 bp.

Engineering of linkers to improve specificity

Not all DNA sequences provide favourable targets for recognition by zinc finger modules, and ZFPs that can skip short (>2 bp) stretches of DNA can avoid unsuitable genomic sequences. A long, flexible linker sequence may serve this purpose, but may also result in loss of affinity for DNA.\textsuperscript{61} This problem has been approached by the use of the following novel methods.

The crystal structure of the first six fingers of TFIIIA bound to its DNA target had shown that the fourth zinc finger did not bind DNA but crossed the minor groove, allowing the third and fifth zinc fingers to bind in the major groove some 6 bp apart.\textsuperscript{55} Hence, the fourth zinc finger of TFIIIA acted as a ‘structured’ linker. This property was applied to an engineered transcription factor comprising of the first four fingers of TFIIIA joined N-terminally to the three fingers of Zif268. This protein bound to its optimal sites approximately 1,000-fold tighter than to the Zif268 site alone and showed a 5–to 10-fold preference for its optimal sites in comparison with sites with shorter or longer gaps than 7 or 8 bp.\textsuperscript{60} In the same study, finger 2 of Zif268 was also engineered so it could
also act as a structured linker. Surprisingly, the protein could bind continuous target sequences as tightly as it could sites with 1–10 bp (a turn of the DNA double helix) gaps of unbound DNA. This fact suggested that a zinc finger could ‘flip’ out of the major groove when a suitable binding triplet was absent to allow adjacent fingers to bind their optimal sequences.

Although zinc fingers that bind DNA with higher affinity are desirable, such as those discussed above, it is important that specificity should not be compromised. Clearly, if a six-finger peptide binds very tightly to its target sequence but also binds tightly to a host of non-target sequences, it may in fact be less specific than a weaker binding protein containing fewer fingers. To address the problem of specificity, six-finger peptides have been constructed from strings of two-finger units (termed 3 × 2 F peptides) using linkers which were 1 or 3 amino acids longer than the canonical linker. These proteins displayed far greater specificity for their full-length target site over partial or mutated sites when compared with similar ZFPs, which were formed from two three-finger units (termed 2 × 3 F peptides). This was exemplified by a 3 × 2 F peptide’s 10⁶-fold preference for its 18 bp site over a 9 bp half-site, in contrast to the 2 × 3F peptide’s 10³-fold selectivity. In addition, it was demonstrated that a 3 × 2F peptide could bind with high affinity to target sites which include one or two suitably placed gaps between recognition sequences (Figure 4).

Dimerisation domains

Another system for extending the recognition site of ZFPs is to use existing dimerisation domains. The first scheme fused the Gal4 dimerisation domain to the first two fingers of Zif268. An alternative dimerisation scheme fused a zinc finger domain (fingers 2 and 3 of Zif268) to the leucine zipper region of the GCN4 protein. In this approach, the recognition helix of the C-terminal zinc finger was extended to make it continuous with the leucine zipper helix of GCN4 (Figure 4).

A novel dimerisation scheme has also been developed by using phage display to select from libraries of random 15-residue polypeptides those that promoted dimer formation. One such N-terminal peptide enhanced the binding of a two-zinc finger domain for a palindromic site by almost 1,000-fold in comparison with the two-finger units alone. The structure of this dimer revealed that the 15 amino acid peptide mediates dimerisation by interacting with a hydrophobic patch on the opposite ZFP (Figure 4).

Figure 4: Strategies for engineering zinc finger protein recognition of extended DNA target sequences. (A) Connecting three-finger domains to create six- (or nine-) finger peptides which recognise 18 bp of DNA. The type of linker used determines the binding mode of the peptide, with short canonical linkers such as –TGEKP– directing binding to contiguous sequences, longer flexible linkers allowing binding across a range of non-bound DNA stretches and structured linkers, which show a preference for a particular span of non-bound DNA. (B) Connecting two-finger domains to create six-finger peptides which bind 18 bp of DNA. The potential for two extended linker peptides allows the protein to bind target sequences incorporating two stretches of non-bound DNA. (C) Homodimerisation of two-finger peptides by linking the Gal4 dimerisation domain, or the leucine zipper region of GCN4 to the C-terminus of the zinc finger domains. The resulting dimers bind inverted repeats of the target site. Heterodimers of zinc finger domains can be created in the same way to bind target sequences that do not contain inverted repeats. (D) Homodimers of two-finger peptides have been created using an N-terminal, phage-selected, 15-residue peptide. The dimer binds an inverted repeat of the zinc finger-binding site.
to be seen whether this peptide can promote dimer formation of different combinations of zinc fingers.

**IN VIVO APPLICATIONS AND REGULATION OF ENDOGENOUS GENES**

**Effector domains**

In order for designer transcription factors to have a desired function *in vivo*, it is generally considered to be necessary to attach an effector domain to the DNA-binding component. These effector domains may be a TATA box-binding protein, a methylase, an integrase or a nuclease domain, or they may be activators or repressors of transcription.

**Recombination**

Restriction endonuclease domains are popular appendages to engineered zinc finger domains and provide a means of directing nuclease activity to particular regions of DNA. The efficient homologous recombination of plasmids *in vivo* has been demonstrated by a zinc finger–FokI fusion injected into *Xenopus laevis* oocyte nuclei. The zinc finger–FokI fusion cut the plasmids at a specific site and activated the DNA molecules for recombination, which was 100 per cent efficient in optimal conditions. Due to its high efficiency, it is hoped that such a procedure will allow the targeted recombination of desired DNA molecules for many *in vivo* applications.

**Activation and repression of endogenous genes and functional genomics**

Activation and repression domains fused to ZFPs have been shown to regulate effectively the expression of reporter constructs, containing binding sites for six-finger peptides. For functional genomics, the regulation of endogenous genes in a chromosomal environment has proved to be a more complex task. The potential difficulties of targeting artificial transcription factors to chromatin have been clearly demonstrated by Zhang et al. Here, a series of nine three-finger proteins (including HSV–1 VP16 activation domains) were targeted to the 5′ flanking sequence of the human erythropoietin gene. In reporter assays, which use an extra-chromosomal template, gene activation was found to be proportional to zinc finger-binding affinity. At the chromosomal locus, accessibility of the binding site was an important determinant of ZFP activity. In a subsequent study, Liu et al. mapped DNase I hypersensitive regions in the locus of the vascular endothelial growth factor A (VEGF–A) gene. Such regions are known to be more accessible to DNA-binding proteins than surrounding sequences and so may provide favourable target sequences. Three ZFPs targeted to these DNase I hypersensitive regions and fused to the VP16 or p65 activation domains activated expression of VEGF–A between 2- and 15-fold. Furthermore, all major splice variants of VEGF–A were activated in physiological proportions. This result underlines one of the advantages of ‘designer’ transcription factor technology versus overexpression of the gene from complementary DNA (cDNA). In another example, two different six-finger ‘designer’ transcription factors separately regulated the proto-oncogenes *erb-2* and *erb-3* despite the fact that their binding sites shared 15 out of 18 bases. The repressor of *erb-2* specifically induced growth arrest and the accumulation of G1 Erb-2-overexpressing tumour cells. Ren et al. have further demonstrated the use of zinc fingers in functional genomics. ZFP repressors were made against two splice variants of mouse PPARγ (γ1 and γ2). One ZFP repressed the γ2 isoform by 50 per cent, and not the γ1 isoform, leading to a 50 per cent reduction in adipogenesis. The other ZFP completely repressed both variants and completely blocked adipogenesis, but only when γ2 was overexpressed in the cells was adipogenesis completely restored. In the experiments, the zinc fingers caused a phenotypic change in the
cells and the function of the γ2 isoform was clearly identified.

**Progress towards gene therapy**

An ultimate goal in this field of research is for ZFP transcription factors to be used in gene therapy, where tight control of target gene expression and delivery to the correct target tissue without eliciting a host immune reaction are important considerations.

As mentioned previously, in theory, a binding site of at least 16 bp should be unique within the human genome assuming that the sequence is random; however, this is not the case, especially in sequences upstream of promoters where conserved motifs are located. While many potential ZFP-binding sites will be inaccessible because of the effects of chromatin, binding of designer transcription factors to sites other than the target gene may lead to inappropriate gene activity. Methods of testing ZFP specificity exist, such as selection of an appropriate target site from a library of oligonucleotides. This method, known as ‘SELEX’, can provide a consensus binding site sequence which may then be used in an *in silico* specificity search. Microarrays also provide means of testing specificity and have been developed such that immobilised DNA sequences can be bound by engineered ZFPs. Alternatively, expression profiling by conventional DNA microarrays can provide a measure of global gene activity upon induction of ZFP expression or ZFP delivery. Results of such tests for any engineered ZFP are yet to be published.

A means of inducing expression is extremely valuable for regulating the level of designer transcription factor or for controlling the time of expression. A system that used a doxycycline-inducible promoter to control ZFP expression has been successfully implemented; however, small molecules may be used to interact directly with the ZFP and regulate activity. Two alternatives to this latter approach exist. In the first, a ZFP that activates VEGF-A, see above, was fused to a FK506-binding protein (FKBP). On a separate peptide, an activation domain was fused to a domain of FKBP rapamycin-associated protein (FRAP). Activation of VEGF-A only occurred in the presence of the rapamycin analogue which caused the FKBP and FRAP domains to dimerise and form an active transcription complex (Figure 5). In the second example of this approach, the ligand-binding domain (LBD) of the oestrogen or progesterone (steroid hormone) receptors was inserted between a three-finger DNA-binding domain and a VP64 activation domain. In theory, addition of the ligand to the fusion protein would cause the protein to be released from an inactivating complex, to dimerise and to bind DNA. In the experiment, the three-finger LBD fusions formed a dimer in the presence of ligand and demonstrated a 1,000-fold ligand-dependent activation of the reporter gene. The authors were also able to show activation from a six-finger peptide attached to two steroid hormone ligand-binding domains joined by a linker. A 3–10-fold activation of a reporter gene under the control of the endogenous *erb-2* gene promoter was achieved in this single-chain dimer system. Xu et al. have used a similar steroid hormone system to regulate successfully an adenovirus-delivered transgene in mice. Recently, the first example of regulation of an endogenous gene in an animal model by an engineered ZFP transcription factor has been presented. The transcription factor, which activated VEGF-A in culture (see above), was delivered using an adenoviral vector into an angiogenesis mouse model. The protein induced healthier and more mature vasculature than that stimulated by a cDNA encoding the VEGF 164 isoform of the gene.

**CONCLUSION**

In the post-genomic era, the wilful regulation of genes is highly desirable. By using native zinc finger sequences as a structural backbone and manipulating the
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key amino acids that form sequence specific contacts with DNA, the regulation of endogenous genes has been achieved. With the growing number of genes that have been regulated using these ZFP transcription factors, it is obvious that this is a powerful technology, although it remains to be seen whether the advances that have occurred in cell culture systems can be translated into animal and plant models and, ultimately, humans. Progress towards this goal is already under way and the regulation of an endogenous gene in an animal model\(^7\) is a major step. While it is dangerous to speculate on future progress, it is anticipated that future developments in the understanding of gene regulation, in delivery systems and in the methods to regulate expression will lead to the exciting prospect of ‘designer’ transcription factors being used in human therapy and many other applications.

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\(\text{Figure 5: Schematic representation of strategies used to create ligand-responsive artificial zinc finger transcription factors. (A) A ligand-dependent dimerisation domain (DD1) is fused to the C-terminus of a three-zinc finger peptide. An effector domain (ED) is fused to another ligand-dependent dimerisation domain (DD2) for the control of gene expression. On addition of the ligand, the dimerisation domains DD1 and DD2 are able to heterodimerise. The effector domain is in a complex with the zinc finger domain to allow target specific gene regulation.}\(^8\) (B) Three-zinc finger peptides are fused to the N-terminus of steroid hormone receptor ligand-binding domains (LBDs) using long (18–36 residue) flexible linkers.\(^8\) An effector domain is then fused to the C-terminus of the protein. On addition of ligand, an intermolecular dimerisation event takes place, which allows the zinc finger complex to enter the nucleus, bind its DNA target site and regulate gene activity. The zinc finger domains of the dimer are able to bind direct repeats, inverted repeats or everted repeats of their target subsites. (C) A ‘single-chain’ fusion protein is created, comprising a six-zinc finger DNA-binding domain attached to two steroid hormone receptor LBDs and an effector domain.\(^8\) The six-finger DNA-binding domain targets an 18 bp site for gene-specific regulation.\)
NOTE

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