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A rapid, generally applicable method to engineer zinc fingers illustrated by targeting the HIV-1 promoter

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Abstract

DNA-binding domains with predetermined sequence specificity are engineered by selection of zinc finger modules using phage display, allowing the construction of customized transcription factors. Despite remarkable progress in this field, the available protein-engineering methods are deficient in many respects, thus hampering the applicability of the technique. Here we present a rapid and convenient method that can be used to design zinc finger proteins against a variety of DNA-binding sites. This is based on a pair of pre-made zinc finger phage-display libraries, which are used in parallel to select two DNA-binding domains each of which recognizes given 5 base pair sequences, and whose products are recombined to produce a single protein that recognizes a composite (9 base pair) site of predefined sequence. Engineering using this system can be completed in less than two weeks and yields proteins that bind sequence-specifically to DNA with *K*d values in the nanomolar range. To illustrate the technique, we have selected seven different proteins to bind various regions of the human immunodeficiency virus 1 (HIV-1) promoter.

Introduction

Customized transcription factors engineered to regulate specific genes will have a significant impact across all medical and agricultural biotechnology1,2. Among other applications, such transcription factors could be used to regulate human disease-related genes, or to produce transgenic animals and plants with advantageous phenotypes. Key to their construction is the engineering of DNA-binding domains with given DNA sequence specificity, to be able to target the appropriate gene(s).

The Cys2-His2 zinc finger is a DNA-binding module that has been used as a scaffold to design DNA-binding proteins with predetermined sequence-specificity3,4. The peptide motif comprises about 30 amino acids that adopt a compact DNA-binding structure on chelating a zinc ion5. Each finger module is capable of recognizing 3-4 base pairs of DNA, such that arrays comprising tandemly repeated modules bind proportionally longer nucleotide sequences. The crystal structure of the Zif268 DNA-binding domain, in complex with its optimal DNA-binding site, shows that the zinc finger array wraps around the DNA, with the α-helix of each finger buried in the major groove6.

Phage-display libraries of zinc fingers have been used to select individual zinc fingers with predetermined DNA-binding specificities1,2,7-16. Two protein-engineering strategies (recently reviewed in ref. 17) have been developed to construct DNA-binding domains using such zinc fingers; however, both fall short of a truly practical, general method. Our original engineering strategy1 and a similar method13,14 simply involve parallel preselection of

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individual zinc fingers and subsequent combination of these modules to produce a polymer. The implementation of this strategy is currently limited to producing proteins that can only bind to DNA sequences with guanine repeated at every third base (i.e. GNNGNN ...). This is a limitation because such binding sites for six-finger proteins would occur extremely rarely in genomic DNA, roughly every $(1/4)^6$ base pairs.

The alternative strategy[2,18] of serial zinc finger selections, though allowing for binding to more diverse DNA targets, may be complicated for high-throughput applications. Nonetheless, the specificity of these fingers has been shown to be generally good[18]. In contrast to these methods, we now present a zinc finger engineering system that (1) yields zinc finger polymers that bind DNA specifically, tightly, and without significant sequence restrictions, (2) can be completed relatively rapidly, and (3) can be easily adapted to a high-throughput automated format. This strategy is based on recent advances in our understanding of zinc finger function, particularly the phenomenon of synergistic DNA recognition by adjacent zinc fingers[11,19], in combination with certain technical advances in zinc finger library design. In this paper we describe the construction of a zinc finger library that functions according to our strategy, and demonstrate the potential of the system by selecting a number of DNA-binding domains that specifically recognize the promoter region (long terminal repeat; LTR) of HIV-1.

Results and discussion

The "bipartite" library strategy

We have devised a "bipartite complementary" system for the construction of DNA-binding domains by phage display (Fig. 1). This system comprises two pre-made master libraries, Lib12 and Lib23, each of which encodes variants of a three-finger DNA-binding domain based on that of the transcription factor Zif268 (refs 6,20). The two libraries are complementary because Lib12 contains randomizations in all the base-contacting positions of F1 and certain base-contacting positions of F2, whereas Lib23 contains randomizations in the remaining base-contacting positions of F2 and all the base-contacting positions of F3 (Fig. 2A). The nonrandomized DNA-contacting residues carry the nucleotide specificity of the parental Zif268 DNA-binding domain.

The design of the bipartite system features two improvements to the conventional zinc finger engineering strategies. First, as already described, each library contains members that are randomized in the α-helical DNA-contacting residues from more than one zinc finger. We have previously shown that the simultaneous randomization of positions from adjacent fingers results in selected zinc finger pairs that can achieve comprehensive DNA recognition, that is, bind DNA without significant sequence limitations[11]. Taking this benefit into account, the proteins produced by these libraries are therefore not limited to binding DNA sequences of the form GNNGNN..., as is the case with other libraries[9,13,21]. Second, the repertoire of randomizations does not encode all 20 amino acids but rather represents only those residues that most frequently function in sequence-specific DNA binding from the respective α-helical positions (Figs. 2B, 6). Excluding the residues that do not frequently function in DNA recognition helps reduce the library size and the "noise" associated with nonspecific binding members of the library.

Phage selections from the two master libraries are performed using the generic DNA sequence 3′-HIJKLMGGCG-5′ for Lib12, and 3′-GCGGMNOPQ-5′ for Lib23, where the underlined bases are bound by the wild-type portion of the DNA-binding domain, and each of the other letters represents any given nucleotide (Fig. 2A). The conserved nucleotides of the Zif268 binding site serve to fix the register of the interaction by binding to the conserved portion of the Zif268 DNA-binding domain in each library. Because the two complementary

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libraries have thus been designed to bind DNA in the same register, the selected DNA-binding portions from each library may be spliced to produce a recombinant three-finger polymer that recognizes the predetermined DNA sequence 3′-HIJKLMNOPQ-5′. This DNA need not contain any of the nucleotide sequences bound by fingers of Zif268, nor does it impose any other DNA sequence limitation.

In order to operate the bipartite strategy, the two zinc finger libraries are subjected to selection in parallel using the DNA sequences described above. The genes of the selected zinc fingers are amplified by PCR, cut using the restriction enzyme *DdeI*, and recombined randomly by re-ligation of the resulting cohesive termini. The enzyme *DdeI* cuts the gene of either library at the same position in the α-helix of F2, allowing for seamless joining of selected zinc finger portions. A further PCR step, performed with selective primers, is used to recover specifically the desired zinc finger product(s) from the pool of recombinants (which contains a number of genes, including wild-type Zif268). The recombined DNA-binding domains are again displayed on phage, to be used in additional rounds of selection to identify the optimal zinc finger product and/or to be used in phage enzyme-linked immunosorbent assay (ELISA) experiments to assess binding to the composite target DNA.

Customized DNA-binding domains that target the HIV-1 promoter

To demonstrate the power of our system, we have targeted a number of sites in the well-characterized promoter of HIV-1. We have tackled this promoter because this has previously been proposed22 and attempted23, albeit without success in producing a three-finger output, indicating that it is a demanding target. Using our selection method, we designed seven DNA-binding domains to bind different loci in the genome of HIV-1 between positions -80 and +60 (Table 1). The different proteins bind tightly and specifically to the DNA sequences against which they were raised (Table 1, Fig. 3). The DNA-binding affinity and specificity of our constructs was tested by phage ELISA (Fig. 3). Additionally, we used bandshift analysis to assay DNA binding and discrimination of three of our engineered proteins in the absence of phage (Figs 4, 5). The proteins were found to bind their target DNA sequences with high affinity and specificity, when tested by either method. Although the various proteins do not share any form of sequence requirement, many of them were designed against the G-rich sequences that occur in the HIV-1 LTR, and we note that their DNA-binding affinity corresponds roughly to the guanine content of their binding sites.

Seven DNA-binding domains were produced, of which six (clones B-G) were engineered according to the full “bipartite” protocol, whereas one protein (clone A) was derived directly by selection from Lib23. This illustrates a further use of the master libraries, namely to select zinc finger domains that bind DNA sequences containing the motif 5′-GCGG-3′ or 5′-GGCG-3′. Four proteins have binding sites that are dispersed upstream of the transcription initiation site (clones A-D), including two that flank the TATA box (clones C, D). Another three proteins bind to a cluster of sites at the beginning of the open reading frame, within the coding region for TAR (clones E-G). One of the motivations for targeting a sequence cluster was to be able to construct six-finger proteins that recognize 18 base pair target sites. It is predicted that such zinc finger polymers would not recognize human DNA sequences, allowing specific targeting of the HIV sequences24-27.

Because the randomizations in the master libraries were restricted to amino acids with validated roles in DNA recognition, many of the recombinant DNA-binding domains make use of contacts that are consistent with the zinc finger-DNA “recognition code”28. For example, the well-known RXD motif found at the N terminus of many zinc finger α-helices was selected in clones A, B, and G. However, it should be noted that it is not unusual for the recombinant proteins to feature idiosyncratic combinations of amino acids that would not have been predicted by any recognition code. This is particularly true of the combinations of

amino acids that are responsible for the inter-finger synergy that allows any base pair to be specified at the interface of zinc finger DNA subsites11. The importance of selecting the optimal amino acid at each base-contacting position was emphasized on analyzing DNA binding by numerous closely related proteins, which often exhibited sizeable differences in DNA-binding affinity.

Conclusion

We have devised a method of engineering zinc finger DNA-binding domains by phage display that has advantages over existing strategies1,2.

The first significant benefit of the present method is that it can produce zinc fingers that bind to diverse DNA-binding sites, whereas certain other methods yield proteins that require the presence of G at every third base position9,13. This improvement arises directly from our recent understanding of the synergistic nature of zinc finger interactions11,19, which have been fully exploited in the bipartite strategy. The ability to bind a variety of DNA sequences is of paramount importance in enabling targeting of any given promoter in the genome, instead of being confined to a very small subset of G-rich DNA sequences.

The second advantage of the method is the unusual speed with which DNA-binding domains can be produced: protein engineering and product validation can be completed in about two weeks. The main reason for the relatively fast turnover is that our new system takes advantage of pre-made phage display libraries, rather than being based on recurring library construction2 in order to assemble a zinc finger polymer. This in turn allows for parallel (cf. serial) selection of zinc fingers from phage display libraries, thus saving time beyond that required simply for cloning. Additionally, the selective PCR protocols allow recombination to be carried out in vitro using a mixed population of zinc finger phage as starting material, thereby circumventing cumbersome clone isolation, DNA preparation and gel purification procedures. The protocol was conceived with high-throughput protein engineering in mind, and the procedure has been automated using liquid-handling robotic systems. The capacity of these systems accommodates engineering of thousands of zinc finger transcription factors per year. With recent developments highlighting the potential of designer zinc fingers29-31, we envisage this system allowing the targeting and manipulation of an ever-increasing number of genes.

Experimental protocol

Construction of phage display libraries

Genes for the two zinc finger phage display libraries were assembled from complementary synthetic DNA oligonucleotides by directional end-to-end ligation. In order to include only the amino acids shown in Figure 2B, a large number of appropriately randomized oligonucleotides (each encoding a subset of a few amino acids) were used in combinations to form gene “minicassettes” that were subsequently ligated together (Fig. 6). Full-length ligation products were amplified by PCR, digested with SfiI and NotI endonucleases, and ligated into the phage vector Fd-Tet-SN (ref. 9). Escherichia coli TG1 cells were transformed with the recombinant vector by electroporation and plated onto TYE medium (1.5% agar, 1% tryptone, 0.5% yeast extract (Sigma-Aldrich Co., St. Louis, MO), 0.8% NaCl, all wt/vol), containing 15 μg/ml tetracycline.

Phage selections

Tetracycline-resistant bacterial colonies were transferred to 2xTY liquid medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 50 μM $ZnCl_2$ and 15 μg/ml tetracycline, and cultured overnight at 30°C in a shaking incubator. Cleared culture

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supernatant containing phage particles was obtained by centrifuging at 300 g for 5 min. Bacterial culture supernatant containing phage was diluted 1:10 in selection buffer (PBS containing 50 μM $ZnCl_2$, 2% (wt/vol) fat-free dried milk (Marvel Premier Brands, UK Ltd., Moreton, UK), 1% (vol/vol) Tween, 20 mg/ml sonicated salmon sperm DNA). One picomole of biotinylated DNA target site was added to 1 ml of phage mixture in sterile 1.5 ml tubes. After incubating for 1 h at 20°C, the zinc finger phage that were bound to the DNA target were captured by binding for 15 min to streptavidin-coated tubes (Roche, Basel, Switzerland). Tubes were emptied and washed 20 times with PBS containing 50 μM $ZnCl_2$, 2% (wt/vol) fat-free dried milk (Marvel), and 1% (vol/vol) Tween. Retained phage were eluted in 0.1 M triethylamine and neutralized with an equal volume of 1 M Tris-HCl (pH 7.4). Logarithmic-phase E. coli TG1 were infected with eluted phage, and cultured overnight at 30°C in 2xTY medium containing 50 μM $ZnCl_2$ and 15 μg/ml tetracycline, to amplify phage for further rounds of selection. After five rounds of selection, E. coli TG1 infected with selected phage were plated and individual colonies were picked and cultured in liquid medium to prepare phage for ELISA DNA-binding assays21. Clones that recognized their target site were retained for subsequent recombination of the two complementary halves recovered from Lib12 and Lib23.

Recombination of complementary clones recovered from Lib12 and Lib23

Following phage panning, the zinc finger genes of the selected clones were recovered by PCR from phage template present in 1 μl eluate. PCR products were diluted in two volumes of *DdeI* buffer (NEBuffer 3; New England Biolabs, Beverly, MA) and digested using 40 units *DdeI* per 100 μl. After heat inactivation of the restriction enzyme, the reaction was made up to T4 ligase buffer (New England Biolabs), and 400 units T4 ligase were added to a 10 μl reaction, incubating for 15 h at 20°C. Recombinants composing the selected portions of Lib12 and Lib23 were amplified selectively by PCR from 1 μl of the ligation mixture, using primers corresponding to unique sequences in the N terminus of Lib12 and the C terminus of Lib23 (30 cycles of amplification with Taq polymerase).

Recombinant DNA-binding domains were cloned into Fd-Tet-SN as described above. Recombinants were tested directly for binding against the composite, final DNA target sequence by phage ELISA (ref. 21). Alternatively, up to two additional rounds of phage selection were carried out using the composite DNA target site as bait before assaying the selected DNA-binding domains.

Expression, purification, and gel shift analysis of zinc finger peptides

Genes for zinc fingers were amplified by PCR and were cloned into the BamHI site of GST-fusion vector pGEX-3X (Amersham Pharmacia, Uppsala, Sweden). Fusion protein expression and purification were carried out from E. coli BL21, with a Redipack GST Purification Module, using Glutathione Sepharose 4B resin according to the manufacturer's instructions (Amersham Pharmacia). Pure three-finger peptides were released from the resin-GST-zinc-finger fusion complex using Factor Xa cleavage (Roche). Zinc finger peptides (<11 kDa) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were quantitated by both absorbance at 280 nm and a dotMetric protein assay kit (Chemicon, Temecula, CA).

For gel shift analysis, 30 bp oligonucleotides, containing the appropriate target sequences, were synthesized, annealed, and end-labeled with [32P]dATP. Appropriate quantities of purified zinc finger peptides were used in 9 μl binding reactions containing: 20 mM Bis-Tris propane (pH 7.0), 100 mM NaCl, 5 mM $MgCl_2$, 50 μM $ZnCl_2$, 0.5% (vol/vol) Nonidet P-40, 5 mM dithiothreitol, 0.1 mg/ml BSA, 5% (vol/vol) glycerol, 1 μg poly-dI-dC, and appropriate quantities of 32P-labeled double-stranded DNA target site (Fig. 4: 0.5 nM DNA

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sites D,F; 0.125 nM sites A, Z. Fig. 5: 0.5 nM sites A,D,F). Gels were developed by autoradiography and analyzed using Molecular Dynamics ImageQuant v1.2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Choo Y, Sanchez-Garcia I, Klug A. *In vivo* repression by a site-specific DNA-binding protein designed against an oncogenic sequence. Nature. 1994; 372:642–645. [PubMed: 7990954]
2. Greisman HA, Pabo CO. A general strategy for selecting high-affinity zinc finger proteins for diverse DNA target sites. Science. 1997; 275:657–661. [PubMed: 9005850]
3. Klug A, Rhodes D. 'Zinc fingers': a novel protein motif for nucleic acid recognition. Trends Biochem. Sci. 1987; 12:464–469.
4. Choo Y, Klug A. Designing DNA-binding proteins on the surface of filamentous phage. Curr. Opin. Biotechnol. 1995; 6:431–436. [PubMed: 7579654]
5. Miller J, McLachlan AD, Klug A. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. 1985; 4:1609–1614. [PubMed: 4040853]
6. Pavletich NP, Pabo CO. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. Science. 1991; 252:809–817. [PubMed: 2028256]
7. Rebar EJ, Pabo CO. Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. Science. 1994; 263:671–673. [PubMed: 8303274]
8. Jamieson AC, Kim S-H, Wells JA. *In vitro* selection of zinc fingers with altered DNA-binding specificity. Biochemistry. 1994; 33:5689–5695. [PubMed: 8180194]
9. Choo Y, Klug A. Toward a code for the interactions of zinc fingers with DNA: selection of randomised zinc fingers displayed on phage. Proc. Natl. Acad. Sci. USA. 1994; 91:11163–11167. [PubMed: 7972027]
10. Wu H, Yang W-P, Barbas CF. Building zinc fingers by selection: toward a therapeutic application. Proc. Natl. Acad. Sci. USA. 1995; 92:344–348. [PubMed: 7831288]
11. Isalan M, Klug A, Choo Y. Comprehensive DNA recognition through concerted interactions from adjacent zinc fingers. Biochemistry. 1998; 37:12026–12033. [PubMed: 9724513]
12. Choo Y. Recognition of DNA methylation by zinc fingers. Nat. Struct. Biol. 1998; 5:264–265. [PubMed: 9546213]
13. Segal DJ, Dreier B, Beerli RR, Barbas CF. Toward controlling gene expression at will: selection and design of zinc finger domains recognising each of the 5′-GNN-3′ DNA target sequences. Proc. Natl. Acad. Sci.USA. 1999; 96:2758–2763. [PubMed: 10077584]
14. Dreier B, Segal DJ, Barbas CF. Insights into the molecular recognition of the 5′-GNN-3′ family of DNA sequences by zinc finger domains. J. Mol. Biol. 2000; 303:489–502. [PubMed: 11054286]
15. Isalan M, Choo Y. Engineered zinc finger proteins that recognise DNA modification by *Hae*III and *Hha*I methyltransferase enzymes. J. Mol. Biol. 2000; 295:471–477. [PubMed: 10623539]
16. Beerli RR, Dreier B, Barbas CF. Positive and negative regulation of endogenous genes by designed transcription factors. Proc. Natl. Acad. Sci. USA. 2000; 97:1495–1500. [PubMed: 10660690]
17. Choo Y, Isalan MD. Advances in zinc finger engineering. Curr. Opin. Struct. Biol. 2000; 10:411–416. [PubMed: 10981627]
18. Wolfe SA, Greisman HA, Ramm EI, Pabo CO. Analysis of zinc fingers optimised via phage display: evaluating the utility of a recognition code. J. Mol. Biol. 1999; 285:1917–1934. [PubMed: 9925775]

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19. Isalan M, Choo Y, Klug A. Synergy between adjacent zinc fingers in sequencespecific DNA recognition. Proc. Natl. Acad. Sci. USA. 1997; 94:5617–5621. [PubMed: 9159121]
20. Christy BA, Lau LF, Nathans D. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA. 1988; 85:7857–7861. [PubMed: 3141919]
21. Choo Y, Klug A. Selection of DNA binding sites for zinc fingers using rationally randomised DNA reveals coded interactions. Proc. Natl. Acad. Sci. USA. 1994; 91:11168–11172. [PubMed: 7972028]
22. Thiesen HJ. From repression domains to designer zinc finger proteins: a novel strategy of cellular immunisation against HIV. Gene Expr. 1996; 5:229–243. [PubMed: 8723389]
23. Wu H, Yang W-P, Barbas CF. Building zinc fingers by selection: toward a therapeutic application. Proc. Natl. Acad. Sci. USA. 1995; 92:344–348. [PubMed: 7831288]
24. Liu Q, Segal DJ, Ghiara JB, Barbas CF. Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proc. Natl. Acad. Sci. USA. 1997; 94:5525–5530. [PubMed: 9159105]
25. Kim JS, Pabo CO. Getting a handhold on DNA: design of poly-zinc finger proteins with femtomolar dissociation constants. Proc. Natl. Acad. Sci. USA. 1998; 95:2812–2817. [PubMed: 9501172]
26. Moore M, Choo Y, Klug A. Design of polyzinc finger peptides with structured linkers. Proc. Natl. Acad. Sci. USA. 2001; 98:1432–1436. [PubMed: 11171968]
27. Moore M, Klug A, Choo Y. Improved DNA-binding specificity from polyzinc finger peptides by using strings of two-finger units. Proc. Natl. Acad. Sci. USA. 2001; 98:1437–1441. [PubMed: 11171969]
28. Choo Y, Klug A. Physical basis of a protein-DNA recognition code. Curr. Opin. Struct. Biol. 1997; 7:117–125. [PubMed: 9032060]
29. Kang JS, Kim JS. Zinc finger proteins as designer transcription factors. J. Biol. Chem. 2000; 275:8742–8748. [PubMed: 10722717]
30. Liu PQ, et al. Regulation of an endogenous locus using a panel of designed zinc finger proteins targeted to accessible chromatin regions. Activation of vascular endothelial growth factor A. J. Biol. Chem. 2001; 276:11323–11334. [PubMed: 11145970]
31. Zhang L, et al. Synthetic zinc finger transcription factor action at an endogenous chromosomal site. Activation of the human erythropoietin gene. J. Biol. Chem. 2000; 275:33850–33860. [PubMed: 10913152]
32. Elrod-Erickson M, Rould MA, Nekludova L, Pabo CO. Zif268 protein-DNA complex refined at 1.6A: a model system for understanding zinc finger interactions. Structure. 1996; 4:1171–1180. [PubMed: 8939742]

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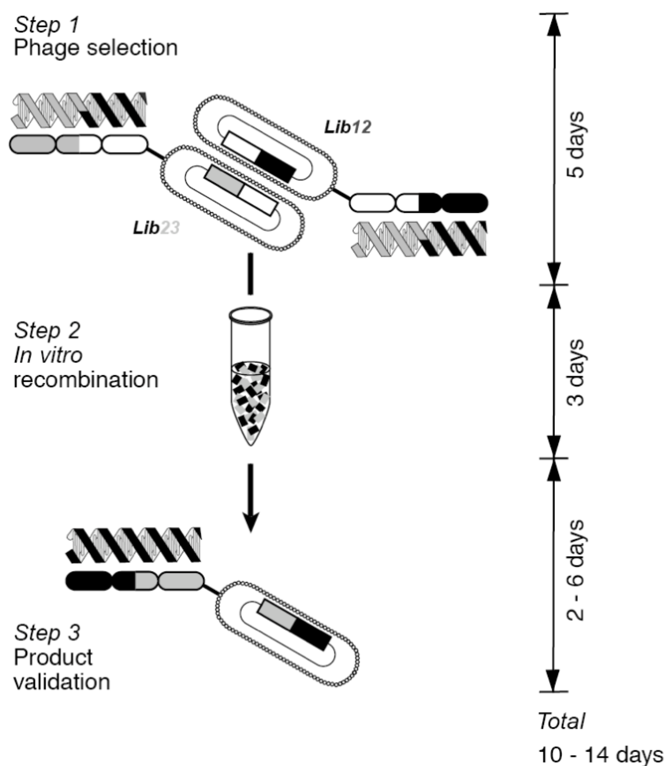

Figure 1.

Overview of the protein engineering strategy. In step 1, two pre-made zinc finger phage-display libraries (Lib12 and Lib23) contain randomized DNA-binding amino acid positions in fingers 1 and 2 (black) or fingers 2 and 3 (gray), respectively. Selections of "one-and-a-half" fingers from each master library are carried out in parallel using DNA sequences in which five nucleotides have been fixed to a sequence of interest. In step 2, zinc finger genes are amplified from the recovered phage using PCR, and sets of "one-and-a-half" fingers are paired to yield recombinant three-finger DNA-binding domains. In step 3, the recombinant DNA-binding domains are cloned back into phage and subjected to additional rounds of selection, or immediately validated for binding to a composite 9 base pair DNA of predefined sequence.

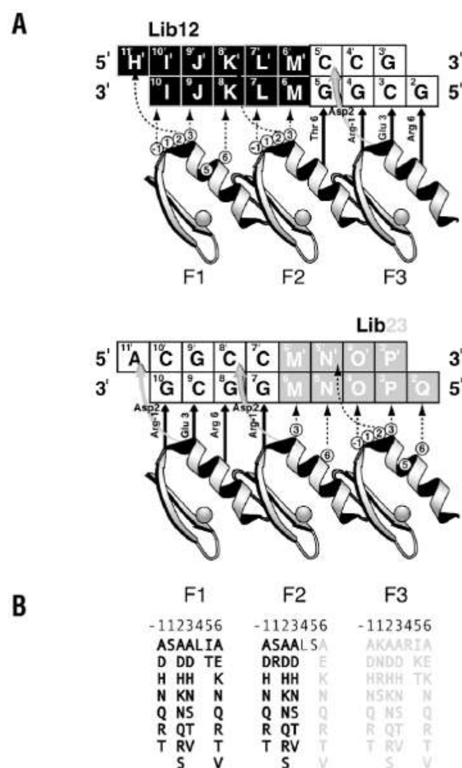

Figure 2.

Composition of the “bipartite” library. (A) DNA recognition by the two zinc finger master libraries, Lib12 and Lib23. The libraries are based on the three-finger DNA-binding domain of Zif268, and the putative binding scheme is based on the crystal structure of the wild-type domain in complex with DNA (refs 6,32). The DNA-binding positions of each zinc finger are numbered, and randomized residues in the two libraries are circled. Broken arrows denote possible DNA contacts from Lib12 to bases H’IJKLM and from Lib23 to bases MNOPQ. Solid arrows show DNA contacts from those regions of the two libraries that carry the wild-type Zif268 amino acid sequence, as observed in the crystal structure. The wild-type portion of each library target site (white boxes) determines the register of the zinc finger-DNA interactions, such that the selected portions of the two libraries can be recombined to recognize the composite site H’IJKLMNOPQ. (B) Amino acid composition of the randomized DNA-binding positions on the α-helix of each zinc finger. A subset of the 20 amino acids was included in each DNA-binding position. Note that positions 4 and 5 of F2 (LS) are specified by the codons CTG AGC, which contain the recognition site of the restriction enzyme *Dde*I (underlined), used as a breakpoint to recombine the products of the two libraries.

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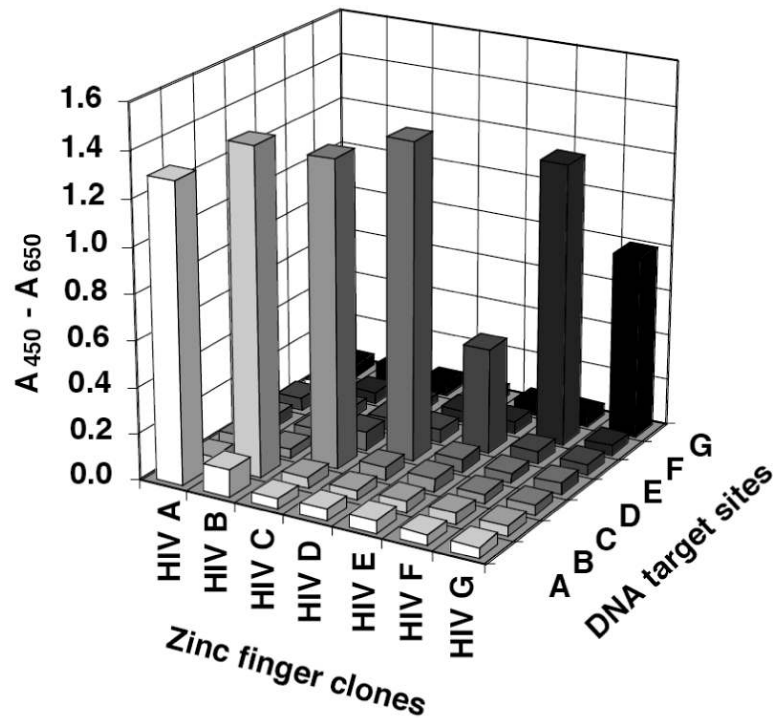

Figure 3.

Matrix specificity assay for seven zinc finger DNA-binding domains designed to bind sequences in the HIV-1 promoter. The seven constructs and their respective binding sites are labeled A-G. Binding of zinc fingers to 0.4 pmol DNA per 50 μl well is plotted vertically from phage ELISA absorbance readings (A450-A650). Each clone was tested using all seven DNA sequences, but strong binding was only observed to those sequences against which they had been designed.

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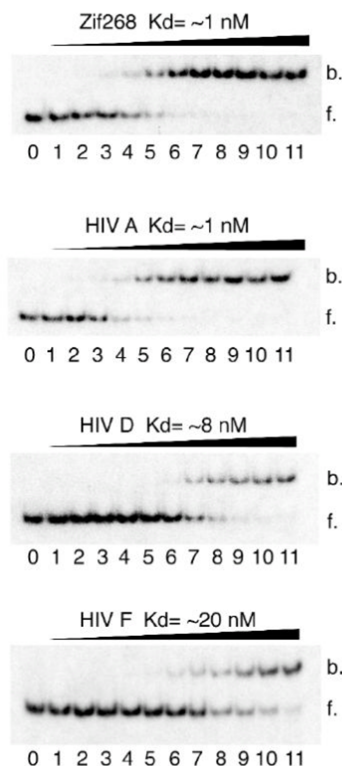

Figure 4.

Gel shift assays to determine the apparent equilibrium dissociation constants (*K*d values) of three engineered zinc finger peptides. Each purified peptide (HIV A, D, and F) was tested for binding to its target DNA site. Two-fold serial dilutions of each peptide resulted in binding reactions ranging from 80 nM peptide (lane 11), down to <80 pM peptide (lane 1). Lane 0 was a control without added protein. The letter “f” denotes free DNA and “b” denotes protein-bound complex. Approximate *K*d values for the three clones are shown above the corresponding figures. The binding behavior of the DNA-binding domain of wild-type Zif268 and its optimal DNA site is shown for comparison.

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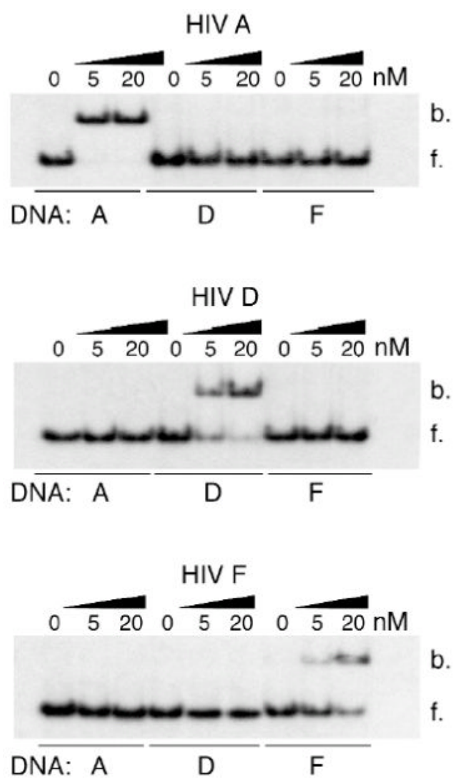

Figure 5.
Matrix sequence-discrimination assay for three-zinc finger peptides generated using three regions of the HIV-1 LTR. 0, 5, and 20 nM of each purified peptide (HIV A, D, and F) were tested against each DNA-binding site (DNA: A, D and F). The letter "f" denotes free DNA and "b" denotes protein-bound complex. DNA binding by each protein was only observed to the sequences against which the zinc fingers had been designed.

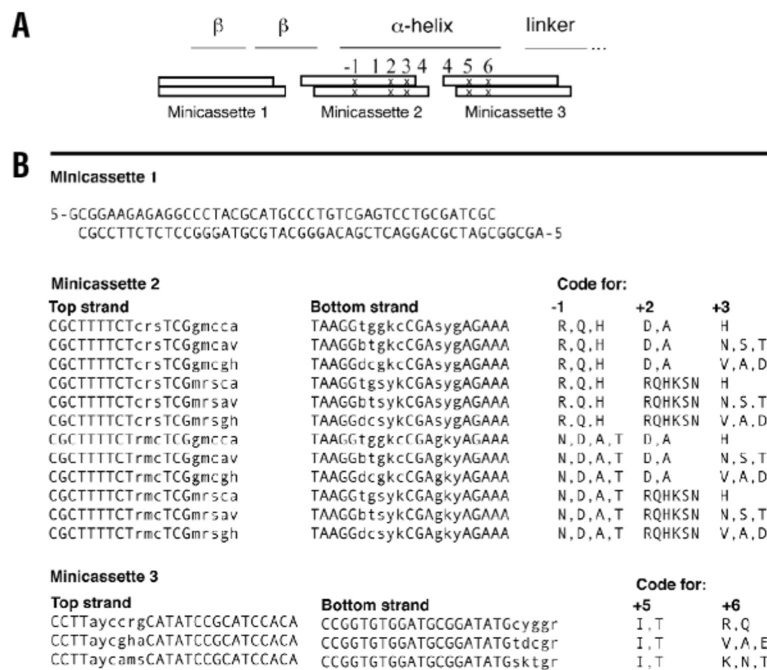

Figure 6.

Construction of a gene cassette coding for the zinc finger phage-display libraries used in this study. (A) The scheme used to generate selective randomization throughout the α-helix of a zinc finger. A set of complementary oligonucleotides is used to construct a series of "minicassettes" that can be annealed and ligated together to construct the randomized portion of the gene. Note that several similar minicassettes, coding for other fingers, need to be constructed to achieve the full randomization scheme outlined in Figure 2B. After ligation of all the minicassettes, the full-length construct is recovered by PCR using primers that contain *Sfi*I/*Not*I restriction enzyme sites for cloning into phage vector. (B) Examples of the oligonucleotides used to achieve the selective randomization of a single zinc finger (Finger1, Lib12). Key to randomized nucleotides: m = A/C; r = A/G; w = A/T; s = G/C; y = T/C; k = T/G; b = C/G/T; n = A/C/G/T; v = A/C/G; h = A/C/T; d = A/G/T.

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Table 1

Selection of DNA-binding domains to recognise the HIV-1 promoter

Clone	DNA target sequence [a]			Position of base Q in LTR	Zinc finger sequence[b]			K_d / nM[c]
	F1	F2	F3		F1	F2	F3	
	3′ -**H IJK**	**LM**N	OPQ -5′		-1123456	-1123456	-1123456	
A	T GCG	GAG	GGA	-79	RSDELTR	RSDNLST	RRDHRTT	1.2±0.2
B	**G AGG**	**GG**T	CAG	-58	**DSAHLTR**	**RSDH**LST	DSANRTK	1.0±0.1
C	**G ACG**	**TC**G	TAG	-36	**ASADLTR**	**NRSD**LSR	TSSNRKK	13.7±3.6
D	**T TCG**	**TC**G	ACG	-22	**HSSDLTR**	**QSSD**LSK	QNATRKR	4.0±0.6
E	**T CCG**	**AG**T	CTA	+22	**DSSSLTK**	**QSAH**LST	DSSSRTK	36.6±15.0
F	**T CTC**	**TC**G	AGG	+33	**ASDDLTQ**	**RSSD**LSR	QSAHRTK	13.3±4.8
G	**G GAT**	**CA**A	TCG	+44	**RSDALTQ**	**DRAN**LST	ASSTRTK	40.3±14.6

[a]Nucleotide sequences from HIV-1 of the form 3′-HIJKLMNOPQ-5′ as recognized by phage clones A-G. Bases that are predicted to be bound by amino acid residues from Lib12 and Lib23, according to the model described in Figure 2, are shown in bold black and gray, respectively. The position of base Q in each site is numbered relative to the transcription start site (+1) in the HIV promoter. Note that the binding site for clone A contains five bases from the binding site of Zif268 (underlined) and that this clone was thus derived directly from Lib23, without the need for recombination.

[b]Amino acid sequences of the helical regions from recombinant zinc finger DNA-binding domains that recognize HIV-1 sequences. The origin of the amino acids is indicated by shading Lib12 and Lib23 residues in bold black and gray, respectively. Clone A, which was derived solely from Lib23, contains wild-type Zif268 residues (underlined).

[c]Apparent K_d for the interaction of the customized DNA-binding domains for their cognate sequences as measured by phage ELISA.