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$$F_{inc} = \frac{(R^2_{wi} - R^2_{wo})/M}{(1 - R^2_{wi})/df_{res}}$$

where F_{inc} is the incremental F ratio, R^2_{wi} is the multiple R^2 achieved with the new parameter in the equation, R^2_{wo} is the multiple R^2 without the new parameter in the equation, M is the number of new parameters, and df_{res} is the residual degrees of freedom in the final analysis of variance table [B. G. Tabachnick and L. S. Fidell, *Using Multivariate Statistics* (HarperCollins, New York, ed. 2, 1989), p. 157]. The percentage of variance accounted for by each parameter was calculated using Type III (or partial) sums of squares, which are invariant to the order of parameters in the model [*GLM-VARCOMP*, vol. 2 of *SAS/STAT User's Guide* (Statistical Analysis System Institute, Cary, NC, 1990), p. 936].
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Zinc Finger Phage: Affinity Selection of Fingers with New DNA-Binding Specificities

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A phage display system was developed and used to select zinc finger proteins with altered DNA-binding specificities. The three zinc fingers of the Zif268 protein were expressed on the surface of filamentous phage, and a library of variants was prepared by randomizing critical amino acids in the first zinc finger. Affinity selections, using DNA sites with base changes in the region recognized by the first finger, yielded Zif268 variants that bound tightly and specifically to the new sites. This phage system provides a tool for the study of protein-DNA interactions and may offer a general method for selecting zinc finger proteins that recognize desired target sites on double-stranded DNA.

Designing and selecting proteins with new DNA-binding specificities can test and extend our understanding of protein-DNA interactions. The zinc finger motif, of the type first discovered in transcription factor IIIA (*1*), offers an attractive framework for these studies. This zinc finger is one of the most common eukaryotic DNA-binding motifs (*2*), and this family of proteins can recognize a diverse set of DNA sequences (*3*). Zinc finger proteins also exhibit a modular organization which suggests that it may be possible to "mix and match" fingers to obtain proteins with novel DNA-binding specificities (*4*, *5*). Crystallographic studies of the Zif268-DNA complex (*4*) and other zinc finger–DNA complexes (*3*, *6*) show that residues at four positions make most of the base contacts, and there has been some discussion about rules or codes that may explain zinc finger–DNA recognition (*7*). However, the recently reported structures of the GLI-DNA complex (*3*) and of the Tramtrack-DNA complex (*6*) show that zinc fingers can dock against the DNA in a variety of slightly different ways. This complexity makes model building and rational design more difficult, but it also reemphasizes the versatility of the zinc finger motif.

Phage display systems have provided powerful selection methods for many studies of peptides and proteins (*8*, *9*). To explore the usefulness of phage display for studying zinc finger–DNA interactions, we first expressed the three Zif268 zinc fingers (*10*) on the surface of filamentous phage (Fig. 1, A and B). The resulting construct—fd-tet.Zif—produced useful titers of "Zif phage" (*11*), and these phage bound specifically to the nine–base pair site recognized by Zif268 (*12*). We then created a library of Zif variants by randomizing the four positions of the first finger that appear most important for making base contacts (*3*, *4*, *6*). These randomized positions include the residue immediately preceding the α helix and include the second, third, and sixth residues of this helix (*13*).

Affinity selection methods were then used to search the library for phage that would recognize altered binding sites. In each round of affinity selection, phage were equilibrated with biotinylated target DNA and then applied to streptavidin-coated microtiter wells. After washing, the retained phage were eluted in high salt buffer, amplified in *Escherichia coli*, and purified to prepare for the next cycle. The target DNA duplexes for these selections contained modified Zif268 binding sites with changes in the region recognized by finger one (*4*), and we refer to each duplex by the sequence of this region (Fig. 1C). Initially, we performed five rounds of selection with each of the target sites (Fig. 1D) (*14*). During these initial selection series, retention efficiencies in the GACC- and GCAC-selected phage pools increased about 100 times, whereas retention efficiencies for the CCTG pool remained low (*15*). We then used these enriched GACC and GCAC pools as a starting point for additional, more stringent selection cycles (Fig. 1D) (*14*). The CCTG pool was not studied further.

Phage pools from critical stages of the GACC and GCAC selections were characterized by sequencing (Fig. 1D), and amino acid preferences were apparent in each pool. For the GACC pool, sequencing after the initial selection series showed that all of the phage (12/12) could be characterized by the consensus sequence (S/D/T)_NR (Table 1). Three additional rounds of selection using high salt washes did not substantially change this consensus (Table 2). For the GCAC selections, sequencing revealed notable changes in the later pools. After the initial selection series, many of the phage belonged to a group characterized by the consensus sequence R_DR (18/22), but there also was a group characterized by the sequence _G(S/T)R (4/22) (Table 1). After additional rounds of selection with high salt washes, a single sequence—RADR—from the first group predominat-

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ed (Table 2). However, when the additional rounds of selection used both high salt washes and competitor Zif268 site in the binding reactions, we found that a single sequence—QGSR—from the second group predominated.

We studied three Zif268 variants in more detail by recloning and overexpressing them in *E. coli*, then purifying the resultant peptides (*16*) and measuring DNA-binding affinities. We studied the predominant Zif268 variants obtained in each of the three later selection series—DSNR, RADR, and QGSR (Table 2)—and also included the wild-type peptide RDER as a control. Peptide affinities for each of three binding sites [GACC, GCAC, and GCGC (wild type)] were determined by quantitative gel-shift analysis (Table 3) (*17*). Each of the variant peptides binds with high affinity to the site used for its selection (Table 3, boxed entries). Moreover, the DSNR and QGSR peptides exhibit new specificities in that they bind to these new sites substantially better than they bind to GCGC. The RADR peptide (unlike the QGSR peptide) does not discriminate well between GCAC and GCGC. The only difference in the selection conditions for these two variants was the use of competitor Zif268 site in the selections that yielded QGSR.

Although our main goal was to test the feasibility of this selection system, these experiments have several implications:

1) Our results show how this phage system can be used to help identify critical protein-DNA contacts. Thus, the arginine selected at position 6 of the α helix was present in each of the selected phage (72/72), and the crystal structure of the Zif268 complex readily explains this preference (*4*). We presume that this arginine makes a pair of hydrogen bonds with the guanine at the 5′ end of the GACC and GCAC subsites. Likewise, every Zif variant selected with GACC has an asparagine at position 3, and, as seen in the Tramtrack-DNA complex (6), this asparagine could make critical contacts with the adenine.

2) The apparent failure of the CCTG selections raises some important questions. Is this sequence inherently more difficult to recognize with a zinc finger? Would further

Table 1. Amino acid sequences of phage from the GACC and GCAC pools after the initial selection series (Fig. 1D). The four randomized positions in the α-helical region of finger one are denoted as −1, 2, 3, and 6. Consensus sequences are indicated in bold. An underscore (_) indicates that there is no clear preference at the corresponding position. The numbers in parentheses indicate the total number of times this amino acid sequence was recovered and the number of distinct DNA sequences that encoded this amino acid sequence (*20*).

GACC	GCAC
-1 2 3 6	-1 2 3 6
SQNR (4,2)	RSDR (4,2)
DANR (2,1)	RPDR (3,2)
DRNR	RGDR (3,1)
DSNR	HSDR (2,2)
SSNR	RVDR (2,2)
STNR	AADR
TANR	KSDR
TPNR	RADR
	RAER
	R_DR
	NGSR (2,2)
	SGSR
	TGTR
s/d/t_NR	**_G s/r R**

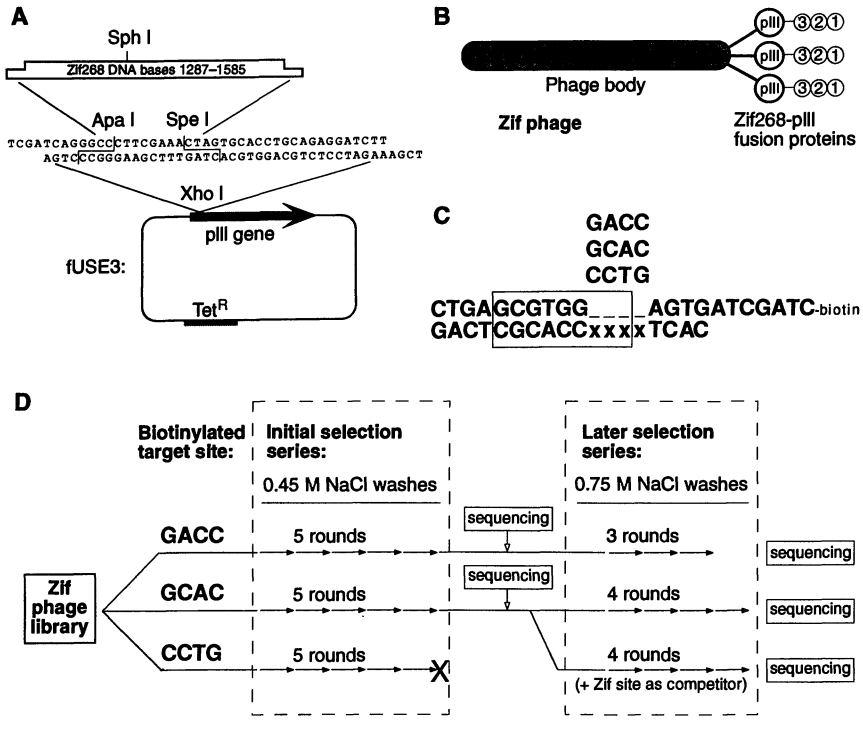

Fig. 1. (**A**) Construction of the Zif phage vector fd-tet.Zif. The phage vector fUSE3 (*19*) was converted into fd-tet.Zif in two steps: (i) A polylinker was inserted into the Xho I site of fUSE3 and (ii) a PCR-amplified fragment of Zif268 complementary DNA encompassing bases 1287 through 1585 (*10*) was cut with Apa I and Xba I and then ligated into the Apa I and Spe I sites of the polylinker. (**B**) Sketch of the Zif phage. The Zif268 zinc finger peptide, which contains three fingers (denoted by the numbered circles), is fused to the NH_2-terminal end of the phage coat protein pIII. Three to five copies of this fusion protein should be present at one end of the virion (*8*). (**C**) The three biotinylated DNA sites used for affinity selections. The sequences of the underscored region were GACC, GCAC, or CCTG (where xxxx indicates the appropriate complementary sequence), and these duplexes are referred to as GACC, GCAC, and CCTG. Zif normally recognizes the consensus sequence GCGTGGGCG (with the first finger contacting the underlined "GCG" subsite) (*4*), and the box marks the corresponding region of the duplexes. (**D**) Overview of selections. Samples from the phage library were subjected to five rounds of selection with the biotinylated DNA duplexes GACC, GCAC, or CCTG. The GACC and GCAC pools were then used in additional rounds of selection under more stringent conditions (the washes contained more salt and, for one of the GCAC selections, the binding reactions contained nonbiotinylated Zif268 binding site as a specific competitor) (*14*). Pools were characterized at the indicated stages by sequencing of randomly chosen phage. The X indicates that there were no further selections with the CCTG pool.

GACC	GCAC	GCAC (+ competitor)
-1 2 3 6	-1 2 3 6	-1 2 3 6
DSNR (8,4)	RADR (7,4)	QGSR (16,3)
SSNR (4,3)		
DRNR (2,1)		
NSNR		
D/S SNR	**RADR**	**QGSR**

Table 2. Amino acid sequences in the final phage pools (after the later selection series shown in Fig. 1D). The designation "+ competitor" indicates that specific competitor DNA (nonbiotinylated wild-type Zif268 site) was added to the binding mixes during the later selection series (*14*). Symbols are as described in Table 1.

Finger one sequence -1 2 3 6	Apparent K_d (nM) GACC	GCAC	GCGC (wt)
DSNR	[0.019]	2.5	1.8
RADR	9.3	[0.068]	0.035
QGSR	1.8	[0.055]	0.54
RDER (wt)	33.0	5.6	2.7

Table 3. Apparent K_d's for the binding of zinc finger peptides to DNA fragments containing the GACC, GCAC, and GCGC (wild type) forms of the Zif268 binding site. Each peptide is specified by the amino acid residues at the four positions of finger one that were randomized in the library (−1, 2, 3, and 6). RDER is wild type. The three DNA duplexes share the sequence AGCAGCTGA[GCGTGG_ _ _]_AGTGAGCT and are specified by the bases at the four underscored positions. [The bracketed region marks the position of the Zif268 binding site GCGTGGGCG (*4*).] Boxes mark the interaction of each variant with the site used for its selection.

variation (for example, randomizing additional residues to change base contacts and phosphate contacts) have allowed recognition of this subsite?

3) Results from the GCAC selections suggest that adding specific competitor DNA can aid in the recovery of variants with altered specificities, and we presume that the specific competitor screened out variants that still recognized the wild-type site.

The experiments reported in this paper demonstrate that the phage display system can be used to select zinc fingers with novel DNA-binding specificities, and extensions of these strategies will allow us to explore the limits of zinc finger design: Is it possible to select a zinc finger peptide that recognizes any desired sequence on double-stranded DNA? What are the limits of affinity and specificity in DNA binding? This phage display system offers a tool for addressing fundamental questions about protein-DNA recognition and may also provide a means for generating DNA-binding proteins that can be used for research, diagnosis, and therapy.

Note added in proof: Jamieson *et al.* (*18*) also have developed a zinc finger phage display system and have selected Zif268 variants with altered DNA-binding specificity.

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11. For production of Zif phage, MC1061 cells (*9*) that contained fd-tet.Zif were grown to saturation (room temperature, ~40 hours, no agitation) under anaerobic conditions in 0.05 liter of Zif phage broth [tryptone (32 g liter^{-1}), yeast extract (20 g liter^{-1}), glucose (8 g liter^{-1}), casamino acids (1 g liter^{-1}), tetracycline (0.02 g liter^{-1}), thiamine (0.5 mg liter^{-1}), 100 μM $ZnCl_2$, 50 μM dithiothreitol, 86 mM NaCl, and 40 mM Hepes (pH 7.8)]. To increase titer, we grew cultures in dialysis tubes (50-kD cutoff) suspended in 1 liter of broth. Phage were titered as tetracycline-transducing units (TTU) essentially as described (*9*) except that starved K91 cells had been stored at −80°C in a buffer containing 67 mM NaCl, 42 mM $NH_4H_2PO_4$, and 14% glycerol. Titers of Zif phage cultures were 0.5 × 10^9 to 14 × 10^9 TTU/ml.
12. E. J. Rebar and C. O. Pabo, unpublished data.
13. To construct the library, we synthesized two oligonucleotides: 5′-GGAATCGATTCCATGGGGCCCCATGAACGGCCGTACGCTTGCCCTGTCGAGTCCTGCGATCGTCGATTTTCG and 5′-CCATCTCGATCGCATGCATATTCGACACTGGAAGGGCTTCTGGCCTGTGTGGATCCGGATATGSNNGGTGAGSNNSNNAGASNNCGAAAATCGACG (**N** = A, T, G, and C; **S** = G and C), with complementary 12-base 3′ ends. These were annealed and then extended with sequenase 2.0 (United States Biochemical). The resulting duplex was digested with Apa I and Sph I (sites are underlined) and ligated with the large Apa I–Sph I fragment of fd-tet.Zif. Ligation products were electroporated into MC1061 cells (*9*), and this yielded ~2.8 × 10^7 independent transformants. This library was grown essentially as described (*11*). Phage were purified by ultracentrifugation (171,000*g*, 4°C, 6 hours), and phage pellets were resuspended in ~1/100 volume of binding buffer [50 mM NaCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5% glycerol, bovine serum albumin (BSA; 0.1 mg/ml), and 15 mM Hepes (pH 7.8)]. This final phage library preparation (~4.7 × 10^{11} TTU) was stored anaerobically (<1 ppm O_2) on ice. Because of concerns about oxidation, all phage manipulations were done so as to minimize exposure to O_2. To estimate library complexity, we sequenced 20 unselected clones. (Single-stranded templates were sequenced with sequenase 2.0 and protocols from United States Biochemical.) Three corresponded to the parent construct (fd-tet.Zif) and appear to have resulted from the reinsertion of the fragment excised during library construction. Seventeen phage contained the correct library insert, but there was a significant cytosine bias at the randomized codons. Base ratios were C:A:T:G = 48:19:19:15 at the first two codon positions and C:G = 74:26 at position 3.
14. Our selection protocol is based on the "biopanning" procedure (*9*). The first round in each initial selection series (the leftmost arrow in each of the three pathways in Fig. 1D) was done as follows: Binding reactions (121 μl) were made that contained ~3.5 × 10^{10} TTU of library phage, 39 nM of biotinylated target DNA [GACC, GCAC, or CCTG (Fig. 1C)], and sheared calf thymus DNA (0.059 mg/ml) in 0.9× binding buffer (*13*). Each sample was preincubated for 50 min, diluted into 3.6 volumes of 0.05 M NaCl wash buffer [0.05 M NaCl with 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5% glycerol, 0.5% w/v Triton X-100, and 15 mM Hepes (pH 7.8)], and applied to streptavidin-coated wells (six wells, 30 μl per well) of a Pro-Bind plate (Becton Dickinson). After 50 min the samples were removed from the wells and then (i) over a period of 35 min, the wells were rinsed 10 times with 0.25 ml of 0.45 M NaCl wash buffer (identical to 0.05 M NaCl wash buffer except for the higher NaCl concentration) and (ii) 40 μl of elution buffer [binding buffer (*13*) with 4 M NaCl] was added to each well. After eluting for 2 hours, each set of six eluates was pooled, titered, and used to infect K91 cells (*11*). Transduced cells were incubated for 1 hour at 37°C in 5 ml of LB broth containing tetracycline (0.2 μg/ml), centrifuged (15 min, 1600*g*), and resuspended in 50 ml of degassed Zif phage broth. Each culture was then grown anaerobically in a 50-ml centrifuge tube and purified essentially as described (*13*). Other rounds of selection in the initial series were similar except that, starting at round 3, sonicated salmon sperm DNA was substituted for sheared calf thymus DNA in the binding reactions. Selections in the later series were similar except that 0.75 M NaCl washes were used and the binding reactions in one of the GCAC selection series included a nonbiotinylated Zif268 binding site (0.36 μM) as a specific competitor. All phage manipulations, except for elution and infection of K91 cells, were carried out in an anaerobic chamber with <1 ppm of O_2.
15. In the first round of selection, <0.009% of library phage applied to the streptavidin-coated wells was recovered in the eluates. By the fifth round this retention efficiency had risen to 0.6 to 0.8% for the GACC and GCAC phage pools, but was <0.001% for the CCTG pool. For comparison, control experiments using Zif phage and a biotinylated wild-type Zif site typically yielded retention efficiencies of 0.5 to 1.0%.
16. The zinc finger regions from the phage variants [corresponding to residues 333 to 421 of Zif268 (*10*)] were subcloned into the T7 expression vectors pET-3d or pET-21d (Novagen). These expression constructs were transformed into BL21 cells containing the pLysS plasmid and then induced as recommended (Novagen). Additionally, the corresponding wild-type peptide (RDER) was expressed as described (*4*). Zinc finger peptides were purified by reversed-phase batch extraction and reversed-phase high-performance liquid chromatography (*4*). The final peptide preparations were reconstituted in water in an anaerobic chamber and adjusted to 2.75 mM $ZnSO_4$ and 50 mM bis-tris propane (pH 6.8). Peptide samples were stored at −80°C. To estimate purity, we subjected peptides to SDS–polyacrylamide gel electrophoresis and silver staining. No impurities staining as intensely as 2% of the purified peptide were observed in any preparation (*12*).
17. To derive apparent dissociation constants (K_d's) we (i) used quantitative gel-shift analysis to determine the fraction of DNA fragment bound at a series of peptide concentrations, (ii) estimated the K_d at each point in the transition region of the resulting "binding curve," and (iii) averaged these K_d's. We used those points for which 0.1 ≤ fraction DNA bound ≤ 0.9 (six or seven points). Standard deviations were always < K_d (average)/4. Binding reactions contained radioactive DNA fragment (~2.5 pM or ~25 pM), peptide (from a twofold dilution series), and poly (dI-dC)–poly (dI-dC) (14.7 μg/ml; Pharmacia) in degassed gel-shift buffer [50 mM NaCl, 5 mM $MgCl_2$, 10 μM $ZnSO_4$, 5% glycerol, BSA (0.1 mg/ml), 0.1% NP-40, and 15 mM Hepes (pH 7.8)]. Binding reactions were equilibrated at room temperature for either 30 min (for RDER) or 4 hours (for the variant peptides) and electrophoresed on 10% polyacrylamide gels in 0.03 M tris-Hepes (pH 7.8). [Control experiments showed that the variant peptides required longer equilibration times (*12*).] Dried gels were quantitated with the use of a PhosphorImager system (Molecular Dynamics). A freshly thawed sample of peptide was used for each set of gel-shift experiments, and we determined the binding activity by titrating a portion of each sample against a defined concentration of binding site (150 μM or 300 μM). Each sample was titrated twice, with two different DNA fragments (of the three in Table 3), and the calculated activities always agreed within 20%.
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19. The fUSE3 vector is identical to fUSE2 [S. F. Parmley and G. P. Smith, *Gene* **73**, 305 (1988)], except that the two vectors differ in a small region near the 5′ end of the pIII gene. The DNA sequences of this region in fUSE2 and fUSE3 are, respectively, 5′-GCTGAAGATCTTGAAAGTTGT and 5′-GCTGAAA**CTCGAG**TTGT (differences are underlined; bold letters show the Xho I site of fUSE3). The polymerase chain reaction (PCR) primers used to clone the Zif fingers were 5′-GGATCGATTCCATGGGGCCCCATGAACGCCCATATGC and 5′-GGGTCGACTGCAGATCTAGAGGCCACCACACTTTTGTC (the Apa I and Xba I sites are underlined).
20. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; H, His; K, Lys; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.

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