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A General Method for Selection and Screening of Coiled Coils on the Basis of Relative Helix Orientation

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Abstract: We have developed a method for selecting coiled coils that associate with a given relative helix orientation from a randomized pool of proteins. To select for antiparallel dimers, we have designed a model basic region-leucine zipper (bZip) heterodimer capable of binding DNA only when the coiled coil associates with an antiparallel relative helix alignment. The dimerization domain for this bZip heterodimer is the model antiparallel coiled coil Acid-a1-Base-a1 (Oakley, M. G.; Kim, P. S. *Biochemistry* **1998**, *37*, 12603), and both monomers contain the GCN4 basic region. Although the basic regions in naturally occurring bZip proteins are located N-terminal to the leucine zipper, we have attached the GCN4 basic region to the C-terminus of Acid-a1 to allow both basic regions to contact DNA in an antiparallel heterodimer. The resulting heterodimer, BR-Base-a1-Acid-a1-BR, can bind to a direct repeat of the GCN4 half-site in vivo, leading to spectinomycin resistance in the transcription interference assay of Elledge et al. (Elledge, S. J.; Sugiono, P.; Guarente, L.; Davis, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3689). A buried interhelical polar interaction between two Asn residues in the Acid-a1-Base-a1 heterodimer is known to specify an antiparallel helix orientation. The position of one of these buried Asn residues was randomized, and bZip heterodimers containing antiparallel coiled coils were selected using the transcription interference assay. All of the selected colonies contained Asn at the randomized position, suggesting that the selection is specific for antiparallel coiled coils.

Introduction

The coiled coil[1] is a widespread structural motif found both as the dominant structure in fibrous proteins and as an oligomerization domain in a wide variety of proteins.[2] Coiled coils consist of two or more α-helices, supercoiled around one another, that associate in a parallel or an antiparallel orientation. The sequences of both parallel and antiparallel coiled coils are characterized by a heptad repeat of amino acid residues, denoted **a**−**g**.[3] The residues at positions **a** and **d** are predominantly apolar, forming a 4-3 hydrophobic repeat, with charged residues occurring frequently at the **e** and **g** positions.[3−7] This sequence regularity has led to the successful development of computer algorithms to identify potential coiled-coil domains from primary amino acid sequences.[6−8] Indeed, the application of coiled-coil prediction algorithms to genome and protein databases has highlighted the biological importance of this class of proteins. Analyses of sequenced genomes have led to the estimate that over 5% of all putative open reading frames contain coiled-coil-forming domains.[9,10] Similarly, an analysis of protein sequence databases suggests that 1−3% of all amino acid residues in proteins occur in coiled coils.[7,8] Thus, the coiled coil appears to be the most common motif in nature for mediating protein oligomerization.

Due to the prevalence of coiled-coil domains and to the recent explosion in genome sequence information, the ability to predict potential interaction partners from coiled-coil sequences would be extremely valuable. However, a number of possible quaternary structures have been observed for coiled coils, differing in the number of strands and in the relative orientation of the constituent helices.[2,11] Because the primary amino acid sequence of a coiled-coil-forming region determines not only its partner specificity, but also the oligomerization state and helix orientation of the resulting coiled coil, the variety of coiled-coil quaternary structures complicates efforts to predict interaction partners.

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The α-helices of naturally occurring coiled coils have previously been assumed to be parallel. However, a growing number of structurally characterized proteins contain intramolecular or intermolecular antiparallel coiled-coil domains.[11] A striking example is the class of structural maintenance of chromosomes (SMC) proteins that contain fibrous antiparallel coiled-coil domains of 600−800 amino acid residues.[12−14] Indeed, the discovery that these proteins contain antiparallel coiled-coil domains has led to a new understanding of their mechanism of action.[12−17] Given the growing importance of antiparallel coiled coils, it is clear that an understanding of the interactions that affect helix orientation is necessary to accurately predict the biochemical function of a coiled coil from its primary amino acid sequence.

A number of powerful methods are available for identifying protein−protein interaction partners from protein libraries (for a review, see ref 18). These techniques often involve the construction of hybrid proteins in which successful protein−protein interactions result in a change in transcriptional activity[19] or a reconstitution of enzyme activity through the reassembly of enzyme fragments.[20] Indeed, both types of genetic selection assays have been used to study parallel coiled coils. In a pioneering experiment, Hu et al. fused the leucine zipper of the homodimeric yeast transcriptional activator GCN4 to the DNA-binding domain of the bacteriophage λ repressor, which requires a separate dimerization domain for function.[21] Functional dimerization domains were identified from randomized GCN4 leucine zipper sequences due to effective transcriptional repression by the hybrid proteins. This and similar bacterial transcription assays have been used to study the importance for parallel coiled-coil stability and dimerization specificity of charged residues at the **e** and **g** positions, of hydrophobic residues at interior **a** and **d** positions, and of buried polar residues.[21−26] More recently, Michnick and co-workers have developed a genetic selection using oligomerization-assisted reassembly of engineered fragments of dihydrofolate reductase.[27] This selection has also been applied to the study of coiled-coil dimerization specificity, identifying potential pairing partners from separate randomized coiled-coil libraries that would result in 2×10^6 possible heterodimers.[28,29] Importantly, although these experiments have enriched our understanding of the behavior of parallel coiled coils, these techniques do not yet allow for the selection of coiled coils that interact in an antiparallel orientation.

We describe here a strategy for selecting antiparallel coiled coils from a randomized pool of coiled-coil proteins, using a readily selectable function, DNA binding. The basic region-leucine zipper (bZip) transcription factors contain a bipartite DNA-binding motif consisting of a coiled-coil leucine zipper dimerization domain and a highly charged basic region that directly contacts DNA (for reviews, see refs 30 and 31). Because the dimerization regions of all naturally occurring bZip proteins form parallel coiled coils, we have designed a functional, DNA-binding bZip heterodimer with an *antiparallel* coiled coil. Due to the arrangement of subdomains in this heterodimer, formation of a parallel coiled coil does not lead to formation of a stable protein−DNA complex, allowing us to use sequence-specific DNA binding to probe for antiparallel coiled-coil formation. To demonstrate the utility of this approach, we have randomized a residue in a model antiparallel coiled coil that participates in a buried polar interaction known to be important for helix orientation.[32−34] Using the transcription interference assay of Elledge et al.,[35] we have selected for heterodimers that bind the appropriate DNA site. After four rounds of selection, we obtain only those heterodimers in which the buried polar interaction is reconstituted, suggesting that this assay preferentially identifies dimerization sequences that can form antiparallel coiled coils.

Results

Design of a Functional bZip Heterodimer Containing an Antiparallel Coiled Coil. We used a model coiled coil, Acid-a1-Base-a1,[32] to construct a functional bZip protein containing an antiparallel coiled coil (Figure 1). Specificity for heterodimer formation in this coiled coil is controlled by interhelical Coulombic interactions: Acid-a1 contains only Glu residues and Base-a1 only Lys residues at both the **e** and **g** positions (Figure 1A).[32,33] The helix orientation is controlled by a buried polar interaction.[32−34] Both peptides contain all hydrophobic Leu residues at the interior **a** and **d** positions, with the exception of a single polar Asn residue in each peptide. The interior Asn residues in Base-a1 and Acid-a1 are positioned to interact only when the helices align in an antiparallel orientation (Figure 1B), and these peptides associate predominantly with an antiparallel alignment of helices.[32] In contrast, the Asn residues of the designed peptides, Acid-p1 and Base-p1 (not shown), are positioned to interact only when the peptides associate in a parallel relative orientation, and these peptides form a parallel coiled coil.[33]

Formation of stable protein−DNA complexes by bZip proteins is dependent on dimerization, and mutations that prevent dimerization also prevent DNA binding.[30,31] However, the leucine zipper domain does not contact the DNA directly,[36,37]

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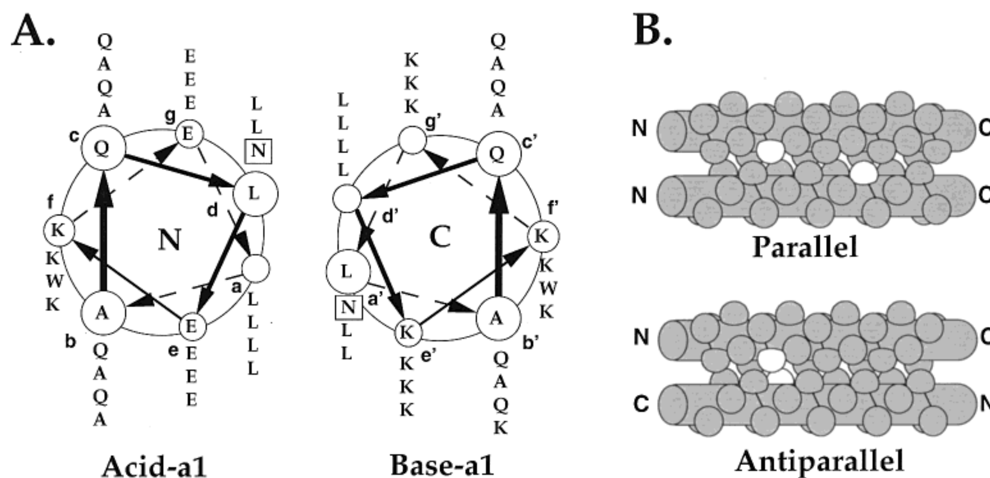

Figure 1. (A) Helical wheel representation of the antiparallel Acid-a1-Base-a1 heterodimer. The view is shown looking down the superhelical axis from the N-terminus of Acid-a1 and the C-terminus of Base-a1. The peptide sequence of Acid-a1 is Ac-KQLEKELQANEKELAQLEWELQALEKELAQ-NH_2. The sequence for Base-a1 is Ac-KQLKKKLQALKKKLAQLKWKNQALKKKLAQ-NH_2. These sequences differ from those previously reported[32] by substitution of a Lys residue for Ala at the N-terminus of both peptides. These substitutions occur at **b** positions and are not expected to affect interhelical interactions in this coiled coil. In addition, the positions of the Asn residues have been changed from position 21 to position 10 of Acid-a1 and from position 10 to position 21 of Base-a1, maintaining the potential for an **a**−**d′** buried polar interaction only in an antiparallel coiled coil. (B) Schematic view of parallel and antiparallel complexes of Acid-a1 and Base-a1, with the Asn residues represented by white semicircles. In the antiparallel complex, the two Asn residues can interact, but in the parallel complex, the Asn residues are separated by more than three turns of α-helix.

	basic region	linker	coiled coil
a) GCN4	MKDPAALKRARNTEAARRSRARK	LQRV	KQLEDKVEELLSKNYHLENEVARLKKLVGER
b) BR-Acid-p1	MKDPAALKRARNTEAARRSRARK	LQRV	KQLEKELQALEKENAQLEWELQALEKELAQ
c) BR-Base-p1	MKDPAALKRARNTEAARRSRARK	LQRV	KQLKKKLAQLKKKNAQLKWKLQALKKKLAQ
d) BR-Base-a1	MKDPAALKRARNTEAARRSRARK	LQRV	KQLKKKLQALKKKLAQLKWKNQALKKKLAQ

	coiled coil	linker	basic region
e) Acid-a1-BR	MKQLEKELQANEKELAQLEWELQALEKELAQ	LQRV	KRARNTEAARRSRARKAALKG

Figure 2. Sequences, from N- to C-termini, of the bZip peptides discussed in this paper. Standard single-letter abbreviations are used for each amino acid residue. The basic, linker, and coiled-coil-forming regions are indicated. Asn residues important for determining the relative helix orientation of the coiled-coil domains are underlined. (a) The bZip domain of GCN4, residues 226−281. (b) BR-Acid-p1, in which the leucine zipper of GCN4 has been replaced by the model coiled-coil-forming peptide, Acid-p1.[33] (c) BR-Base-p1, in which the leucine zipper of GCN4 has been replaced by the model coiled-coil-forming peptide, Base-p1.[33] (d) BR-Base-a1, in which the leucine zipper region of GCN4 has been replaced by the model peptide, Base-a1.[32] (e) Acid-a1-BR, in which the coiled-coil sequence of Acid-a1[32] is placed N-terminal to the basic region of GCN4.

and its primary role is to position the two basic regions appropriately for sequence-specific DNA contacts. Indeed, the coiled-coil dimerization domain can be replaced by a flexible, disulfide-containing linker in vitro.[38] Similarly, in domain-swapping experiments using naturally occurring bZip proteins, the dimerization properties of the resulting chimeras are determined by the leucine zipper regions, while the DNA-binding specificity is determined by the basic regions.[30,31] Thus, a heterodimer formed by BR-Acid-p1 (Figure 2b), which contains the GCN4 basic region and the Acid-p1 coiled coil, with BR-Base-p1 (Figure 2c), which contains the GCN4 basic region and the Base-p1 coiled coil, should recognize the optimum DNA sites for GCN4 (Figure 3A).

In contrast, if the Acid-p1 and Base-p1 sequences in the BR-Acid-p1-BR-Base-p1 heterodimer are replaced by those of Acid-a1 or Base-a1 (Figure 1A), which associate in an *antiparallel* orientation, the resulting heterodimer will have basic regions on opposite sides of the coiled coil (Figure 3B). Because two basic regions are required for DNA binding, the basic region for one partner in the complex must be placed C-terminal to the leucine zipper to result in sequence-specific DNA binding (Figure 3C). As all naturally occurring bZip proteins have the basic region N-terminal to the leucine zipper, it was necessary to design a bZip peptide with a C-terminal basic region.

We considered the bZip domain of GCN4 to contain four subdomains (Figure 2a). We defined the leucine zipper as residues 251−281 of GCN4 and the basic region as residues 231−246. The four residues between these regions were considered the "linker" region. Finally, The N-terminal residues, DPAAL, though not part of the conserved basic region, are important for DNA binding.[39] Asp and Pro are often found at the N-termini of α-helices, and they are followed by the excellent helix-forming residues, Ala and Leu.[40] In the Acid-a1-BR sequence (Figure 2e), we have placed the Acid-a1 sequence at the N-terminus, followed by the "linker" and basic region defined for GCN4−56. At this stage of our design, we used the same "linker" found in GCN4, even though it was not clear that this linker would provide the optimal subdomain spacing for a bZip protein containing a C-terminal basic region and an N-terminal leucine zipper. Finally, the *C*-terminal end of the basic region, which no longer abuts the leucine zipper, is stabilized by helix-forming residues, followed by a lysine

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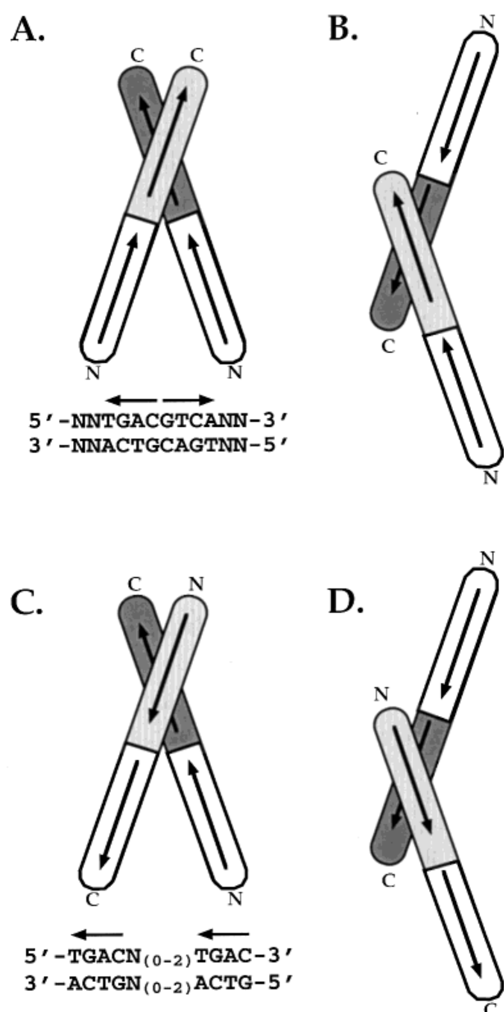

Figure 3. Schematic representations of model bZip heterodimers. (A) A parallel bZip heterodimer containing the GCN4 basic regions binding to the CRE recognition site, an inverted repeat. (B) The heterodimer from (A) with its coiled-coil domain in an antiparallel relative orientation, leaving the basic regions too far apart to bind DNA. (C) An antiparallel bZip heterodimer in which the basic region for one monomer has been placed C-terminal to the coiled-coil domain. (Both basic regions are now in position to bind DNA, recognizing a direct repeat of the CRE half-site. (D) The heterodimer from (C) with its coiled-coil domain in a parallel relative orientation, leaving the basic regions too far apart to bind DNA. The coiled-coil domains are shown in light and dark shades of gray, and the GCN4 basic regions are shown in white. The arrows above the DNA sequences indicate the relative orientations of each half-site within the full binding site.

and a glycine, both of which occur preferentially at the C-termini of α-helices.[40] Importantly, although the *order* of the basic region and coiled-coil subdomains is reversed, the sequence of amino acid residues *within* each subdomain is maintained.

The peptides Acid-a1-BR and BR-Base-a1 (Figure 2) should form a functional bZip heterodimer with an antiparallel coiled coil (Figure 3C). Because we expect the basic region for the Acid-a1-BR monomer to lie in the major groove in the opposite orientation to that found for GCN4, the DNA-binding specificity of this heterodimer should differ from that of GCN4 (Figure 3C). GCN4 binds to the pseudosymmetrical AP-1 site, 5′-TGACTCA-3′,[41−43] or to the fully symmetrical CRE site, 5′-TGACGTCA-3′,[44] with each basic region contacting a 5′-TGAC-3′ half-site.[36,37] In the antiparallel complex, the BR-Base-a1 basic region will recognize this half-site, while we expect Acid-a1-BR to recognize the opposite sequence, 5′-GTCA-3′. We have recently shown that a variant of GCN4 with a C-terminal basic region and an N-terminal leucine zipper binds to an inverted repeat of this half-site.[45] The resulting recognition site for the antiparallel heterodimer is a direct repeat of the GCN4 half-site (Figure 3C), rather than the wild-type inverted repeat. Because GCN4 contacts bases outside the conserved half-sites, the appropriate spacing for a direct repeat must be determined empirically. Similar binding to a direct repeat of half-sites has been observed for bZip basic regions constrained in a head-to-tail fashion by a flexible disulfide-containing linker.[46]

Expression of bZip Heterodimer. The Acid-a1, Acid-p1, Base-p1, and Base-a1 peptides all fail to form stable homodimers in solution and are therefore predominantly unfolded at micromolar concentrations at physiological temperature.[32,33] Because expression of these bZip peptides in vivo is likely to result in much lower protein concentrations, we expected that the individual Acid and Base bZip proteins would be highly susceptible to protease digestion and therefore difficult to express.[47] In our initial studies, we therefore explored several strategies for expressing both monomers in the same cell, using the T7 expression system in the BL21(DE3) strains of *Escherichia coli*.[48] We failed to achieve reproducible expression levels when separate vectors with different antibiotic resistance markers were transformed into the same cell. In addition, we could not reliably express BR-Acid-p1 and BR-Base-p1 from separate T7 promoters on the same plasmid. Indeed, to achieve even modest levels of expression, especially of the BR-Acid peptides, it was necessary to place both Acid and Base bZip coding sequences under the control of a single promoter, with ribosome-binding sites adjacent to both open reading frames (Kim, B.-M.; Woods, H. L.; Huff, L. M.; Jones, C.; Oakley, M. G., unpublished results). We assume that efficient translation of both peptides from a polycistronic RNA increases the local concentration of monomers, allowing heterodimer formation to compete with proteolysis. Because *E. coli* JM109 cells proved to be far superior to the BL21 strains for in vivo selection experiments, both peptides in the heterodimer were expressed from a polycistronic RNA under the control of the *lac* promoter (see Experimental Section).

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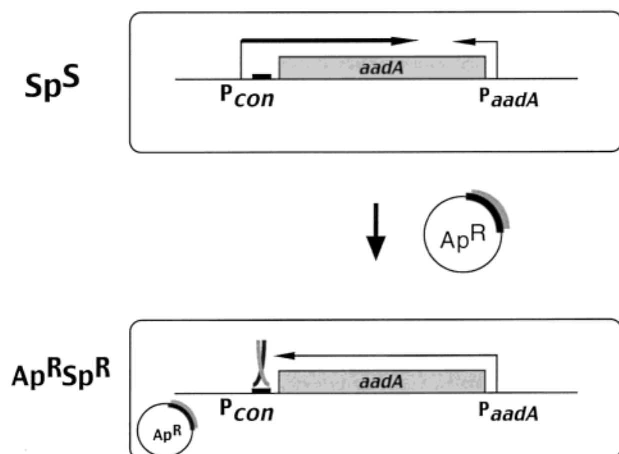

Figure 4. Schematic view of the transcription interference assay used to detect DNA binding. (Top) Transcription interference reporter plasmid of Davis and co-workers.[35] A strong constitutive promoter is positioned to interfere with the transcription of the *aadA* gene, which confers spectinomycin resistance on the host bacterial cell. The DNA site of interest is positioned to behave as an operator site. In the absence of a DNA-binding protein that targets this site, transcription from the constitutive promoter prevents transcription of *aadA*, and the cells are sensitive to spectinomycin. (Bottom) If our antiparallel bZip protein is expressed in the same cells as the reporter plasmid, it forms a complex with the target DNA site, repressing transcription from the constitutive promoter and allowing transcription of the *aadA* gene. Cells are now resistant to spectinomycin.

Transcription Interference Assay. We have employed an in vivo selection procedure for sequence-specific protein−DNA complex formation previously described by Davis and co-workers.[35] This transcription interference assay uses a reporter plasmid in which a strong, constitutive bacterial promoter, the *conII* promoter,[49] interferes with transcription of the spectinomycin resistance gene, *aadA*, encoded on the opposite strand (Figure 4). A DNA-binding site of interest is placed near the *conII* transcription start site, creating a repressible promoter. In the absence of a protein that can bind to this artificial operator sequence, transcription from the *aadA* gene is disrupted, and cells are sensitive to the antibiotic spectinomycin. However, if a protein that binds selectively to the operator site is simultaneously expressed in a given cell, a spectinomycin-resistant (Sp^R) phenotype is conferred. In this way, proteins binding a given DNA sequence can be selected from libraries.[35] Sera and Schultz have applied this in vivo selection strategy to bZip proteins, successfully identifying proteins with altered DNA-binding specificity from a randomized pool of C/EBP mutants.[50]

As a first step to applying this strategy to our parallel and antiparallel model bZip heterodimers, we placed an 11 base pair sequence, 5′-GATGACTCATC-3′, containing the AP-1 site with the 5′-G of this sequence in the +1 position relative to the *conII* transcription start site. This reporter plasmid, pAP1, was transformed into *E. coli* JM109 cells, and no colonies were observed in the presence of spectinomycin (Table 1). When the plasmid pVS12, which encodes the expression of the GCN4 bZip domain, was transformed into pAP1-containing cells, an average of 1200 colonies were observed in the presence of spectinomycin for every 10^6 colonies observed in its absence, indicating a Sp^R phenotype (Table 1). Although the ratio of colonies found in the presence and in the absence of spectinomycin is smaller than that observed by other workers,[35,50] the absence of background makes this assay very sensitive. Similarly, when the plasmid pARA04, which contains coding sequences for BR-Acid-p1 and BR-Base-p1, is transformed into pAP1-containing cells, approximately 440 colonies are obtained in the presence of spectinomycin for every 10^6 colonies observed in its absence, suggesting that the Acid-p1-Base-p1 coiled coil mediates formation of a functional parallel bZip heterodimer in vivo. The somewhat lower efficiency with which this parallel heterodimer interferes with transcription relative to GCN4 is probably due to less efficient expression of the model heterodimer than of the naturally occurring bZip homodimer. Finally, when an expression vector encoding an antiparallel bZip heterodimer, for which AP-1 is not the optimal binding site, is expressed in pAP-1 containing cells, ≤5 colonies are obtained in the presence of spectinomycin for every 10^6 colonies observed in its absence (data not shown), suggesting that the antiparallel heterodimer does not bind selectively to the AP-1 site in vivo.

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Table 1. Growth of Cells in the Presence of Spectinomycin

DNA site	protein	no. of colonies[a]
AP-1	none[b]	0
AP-1	GCN4	1200[c]
AP-1	BR-Acid-p1/BR-Base-p1	440[c]
DIR0	none[d]	31
DIR0	Acid-a1-BR/BR-Base-a1	400
DIR1	none[d]	2.5
DIR1	Acid-a1-BR/BR-Base-a1	0.3
DIR2	none[d]	1.4
DIR2	Acid-a1-BR/BR-Base-a1	0.1

[a] Colonies observed in the presence of spectinomycin (60 μg/mL), ampicillin (40 μg/mL), and IPTG (500 μM) per 10^6 colonies observed in the presence of chloramphenicol (25 μg/mL) and ampicillin (40 μg/mL). [b] No protein-encoding plasmid present; no ampicillin used in this assay. [c] No IPTG used in this assay. Inclusion of IPTG results in a 3−5-fold increase in the number of colonies observed. [d] The parent expression plasmid, pUC19, was used in these assays.

In Vivo Screen for a Functional DNA-Binding Site for the Antiparallel bZip Heterodimer. Three DNA sites containing a direct repeat of the GCN4 half-site 5′-TGAC-3′ were screened for binding to the antiparallel heterodimer Acid-a1-BR-BR-Base-a1 (Figure 3C). These three sites, termed DIR0, DIR1, and DIR2, contain zero, one, or two base pairs, respectively, between the half-sites. The plasmid pVS16N, which contains coding sequences for Acid-a1BR and BR-Base-a1 (Figure 2d,e) was transformed into *E. coli* JM109 cells containing the reporter plasmid pDIR0, pDIR1, or pDIR2. The plasmid pUC19, which does not encode a DNA-binding protein, was used as a negative control. The number of colonies obtained for each protein with each of the three reporter strains is listed in Table 1. A significant number of colonies were obtained in the presence of spectinomycin only for pDIR0, indicating that the best DNA-binding site for the antiparallel heterodimer Acid-a1-BR-BR-Base-a1 contains two adjacent 5′-TGAC-3′ half-sites. Because all three DNA sites contain potential binding sites for both monomers, the strong preference for the DIR0 site suggests that functional dimer formation is required for spectinomycin resistance.

The antiparallel heterodimer appears to have a strong preference for the DIR0 site, as very few colonies are observed when pDIR1 or pDIR2 is used as a reporter plasmid. In fact, expression of the antiparallel bZip protein appears to reduce the number of colonies observed relative to the negative control

by a factor of approximately 10. A similar reduction in the number of colonies for all three binding sites in the absence of spectinomycin was also observed (data not shown), suggesting that the expression of Acid-a1-BR-BR-Base-a1 is somewhat toxic to cells. Thus, the use of pUC19 as a negative control is likely to lead to a 10-fold overestimate of the background spectomycin resistance.

The highest background for colony growth in the presence of spectinomycin was observed for the pDIR0 reporter plasmid. It is not clear why this plasmid allows higher levels of spectinomycin-resistant cell growth than do the other reporter plasmids listed in Table 1. However, it is clear that the number of colonies obtained in the presence of the pDIR0 reporter plasmid and the antiparallel heterodimer is at least an order of magnitude higher than that observed in the absence of Acid-a1-BR-BR-Base-a1. Indeed, because the background levels measured in the presence of the pUC19 expression vector do not take into account the toxicity of the antiparallel bZip heterodimer, it is likely that a 100-fold enrichment of proteins that bind to the DIR0 site can be obtained in each round of selection.

Selection Strategy. The identification of a functional in vivo binding site for the antiparallel bZip heterodimer, Acid-a1-BR-BR-Base-a1, allows us to use this coiled coil as a starting point to probe for interhelical interactions important for specifying an antiparallel alignment of helices within a coiled coil. When a randomized pool of proteins is transformed into cells containing the pDIR0 reporter plasmid, those heterodimers that can form antiparallel coiled coils bind to the DIR0 site, leading to a spectinomycin-resistant phenotype (Figure 3C). Because the basic region in the Acid-a1-BR monomer is C-terminal to the coiled-coil domain while the basic region in the BR-Base-a1 monomer is N-terminal to the coiled-coil domain, formation of a parallel heterodimer places the basic regions on opposite ends of the coiled coil (Figure 3D). Thus, those heterodimers that can form only parallel coiled coils are not expected to bind to DNA, leading to a spectinomycin-sensitive (Sp^S) phenotype. Similarly, those heterodimers with poor oligomerization properties should also fail to bind DNA.

Test Selection. This selection strategy, which uses DNA binding as an indicator of coiled-coil helix orientation, is indirect. We therefore tested its efficacy using an interaction known to specify the helix orientation of the Acid-a1-Base-a1 coiled coil. The Asn residue at position 11 in the Acid-a1-BR coiled coil was randomized to all 64 possible codons by site-directed mutagenesis. The resulting pool of heterodimers was transformed into pDIR0-containing *E. coli* JM109 cells and transferred to spectinomycin-containing agar plates after 2 h of nonselective growth. First-round-selected cells were pooled and isolated by washing the agar plate. After purification of the plasmid DNA (see Experimental Section), the selection process was repeated. After the first round of selection, plasmid DNA from the 10 largest colonies was harvested and sequenced. Five of these colonies contained Asn codons at the randomized site, while three contained codons for Lys, one for Leu, and one for Pro. After the fourth round of selection, the plasmid DNA from 11 colonies was sequenced. All 11 selected colonies contained Asn at the randomized site.

Discussion

A buried polar interaction between two interior Asn residues is sufficient to specify a preference for a parallel or an antiparallel orientation in the Acid−Base coiled coil. When the Asn residues are positioned so that they can interact only in a parallel orientation, as in Acid-p1-Base-p1, a parallel coiled coil is formed.[33] Similarly, when the Asn residues are positioned so that they interact only in the antiparallel orientation, a predominantly antiparallel coiled coil is formed.[32] This strong preference for pairing of buried Asn residues is also a factor in the dimerization specificity in parallel coiled-coil libraries,[26,29] and it has been proposed that the potential for pairing Asn residues at interior **a** residues is important for the dimerization specificity of naturally occurring bZip proteins.[26,51] Because the variable position in Acid-a1-BR packs against the Asn residue in BR-Base-a1 only when an antiparallel coiled coil is formed, the strong preference for Asn at this position suggests that our selection strategy successfully identifies coiled coils with a preference for an antiparallel relative helix orientation.

We are now in a position to extend this selection to the study of other interactions that influence helix orientation preference in coiled coils. Because the structural database is currently smaller for antiparallel coiled coils than for their parallel counterparts, selections can play a key role in identifying important interactions that have not yet been observed for structurally characterized, antiparallel coiled coils. Selections can also be targeted to the examination of a particular type of interaction. For example, although residues at the **a** and **d** positions make up the hydrophobic interior in both parallel and antiparallel coiled coils, there are differences in the packing interactions at these positions. In parallel coiled coils, **a** residues pack against **a′** residues, and **d** residues pack against **d′** residues, giving rise to two layers with distinct geometric requirements.[52,53] In antiparallel coiled coils, **a** residues always pack against **d′** residues, resulting in a single type of hydrophobic layer.[54] Subtle changes in the structure of hydrophobic residues at the **a** and **d** positions strongly influence the oligomerization state in parallel coiled coils.[53,55,56] Due to the still different geometrical constraints of the **a**−**d′** interactions found in antiparallel coiled coils, the identity of hydrophobic residues at these positions is also likely to influence helix orientation preference in ways that are difficult to predict. A selection strategy is well suited to addressing these and similar issues.

Elledge and Davis estimate that libraries of up to 10^8 clones can be screened by their transcription interference assay.[49] Indeed, Sera and Schultz have successfully screened a library of 3.2×10^7 gene sequences, corresponding to 3.2×10^6 protein sequences or five completely randomized residues, using this method.[50] Michnick and co-workers have used similarly sized libraries in which nine residues in each monomer were partially randomized, allowing them to probe interactions involving 18 positions in a heterodimer.[28] While previous experience with

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coiled-coil selections suggests that the use of smaller, targeted libraries also leads to important insights,[23,26] the ability to perform multiple rounds of selection is an important asset for identifying trends in larger libraries.

Previous genetic selections involving coiled-coil proteins have used hybrid proteins with a flexible linker between the coiled-coil domain and the DNA-binding domain or enzyme fragment used to detect dimerization. For example, Hu et al. have incorporated a 30-residue flexible linker sequence between the coiled-coil and DNA-binding domains.[21] This linker is sufficiently long that both parallel and antiparallel coiled-coil formation should lead to transcriptional repression. This type of system has been used effectively to study variants of the bZip peptides GCN4, Fos, and Jun, all of which associate preferentially in a parallel orientation.[21−23,25,26] However, in large libraries containing many randomized residues, interactions that determine a parallel orientation may be removed. Thus, larger, more diverse libraries may identify both parallel and antiparallel coiled coils, complicating the interpretation of the resulting data.

Michnick and co-workers have also used flexible sequences to connect coiled-coil libraries to enzyme fragments. In their initial work, these investigators made use of engineered dihydrofolate reductase fragments in which the N-termini of both fragments are close in space. The coiled-coil sequences in their heterodimer library were attached to the N-terminal ends of these fragments through 11-residue, glycine-rich linkers.[27] Because both coiled-coil sequences are at the N-terminus of the resulting hybrid protein, parallel coiled-coil formation was expected. Indeed, the selected heterodimers all have interactions consistent with parallel, in-register coiled-coil formation.[28,29] However, in other experiments, hybrid proteins containing the GCN4 coiled-coil sequence and fragments of aminoglycoside phosphotranferases were generated in which the GCN4 leucine zipper is at the N-terminus of one fragment and at the C-terminus of the other.[20] In both cases, a 15-residue flexible linker was used to separate the coiled-coil domain and the enzyme fragment. If a fixed orientation were required for functional reassembly of enzyme activity, an antiparallel alignment of helices would be needed due to the order of subdomains in the hybrid proteins. However, GCN4 mediates reassembly of the fragments, suggesting that coiled coils of both orientations are functional in this assay. Using a similar strategy, Ghosh et al. have recently described the functional reassembly of N- and C-terminal domains of the green fluorescent protein (GFP) using a model antiparallel coiled coil.[57] Because their design uses much shorter, six-residue linkers between the coiled coil and GFP fragment, antiparallel coiled-coil formation may indeed be required for functional protein reassembly, and this system may also prove useful for probing the features that lead to a given helix orientation in coiled coils.

Our selection strategy is convenient because it can be readily applied to both parallel and antiparallel coiled coils. Indeed, Pu and Struhl have previously used a GCN4 library in which residues at the **e** and **g** positions of the coiled coil were randomized to probe the determinants for dimerization specificity.[24] These workers found that a large number of sequences were functional in their screen for transcriptional activation,[24] similar to the results observed in early rounds of selection by Michnick and co-workers.[28] Our method can also use the bZip domain of naturally occurring coiled coils, and it has the added advantage that multiple rounds of selection are possible, greatly enhancing its stringency. In addition, in vivo selection methods have been developed that select *against* formation of a specific protein−DNA complex.[58] This negative selection can be adapted to provide a means not only to select coiled coils that can associate in a given relative helix orientation, but also to exclude those that are capable of associating in the opposite orientation. Finally, because this system allows selection for either parallel or antiparallel coiled coils, a combination of these methods can be used to determine the orientation of naturally occurring coiled coils of unknown structure. Proteins that contain coiled-coil domains often have additional binding partners through other interaction domains. Defining the relative orientation of the coiled-coil domain can therefore provide important information about the overall structure and regulation of large multiprotein complexes.

Experimental Section

General. *E. coli* strain JM 109 was used for all in vivo control and selection experiments. Plasmids pNN396 and pNN388[35] were a generous gift from R. W. Davis. DNA manipulations were carried out by standard methods.[59] Oligonucleotides were supplied by Genosys Biotechnologies, Inc. All mutations were made by Kunkel mutagenesis[60] or through the use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The primary sequence of each construct was confirmed by DNA sequencing.[61]

Construction of Reporter Plasmids. The oligonucleotides 5′-cgATGACTCATctgca-3′ and 5-gATGAGTCATcggtac-3′, containing the AP-1 site (capitalized), were annealed and ligated into *Kpn*I/*Pst*I-digested pNN396 to yield the ampicillin-resistant plasmid pAP1a. The *Hin*dIII/*Not*I fragment of pAP1a was ligated into *Hin*dIII/*Not*I-digested pNN388 to yield the chloramphenicol-resistant plasmid pAP1. The reporter plasmids pDIR0, pDIR1, and pDIR2 were constructed by analogous steps. The oligonucleotides used to construct the DIR0 site were 5′-cTGACTGACgctgca-3′ and 5-gcGTCAGTCAggtac-3′. Those used to construct the DIR1 site were 5′-cTGACgTGACgctgca-3′ and 5-gcGTCAcGTCAggtac-3′, and those used to construct the DIR2 site were 5′-cTGACgaTGACgctgca-3′ and 5-gcGTCAtcGTCAggtac-3′.

Construction of Protein Expression Vectors. The plasmid pBR-Acid-p1, encoding the basic region of GCN4 (residues 226−250) and the coiled-coil sequence Acid-p1,[33] was constructed from the vector pGCN4−56, which contains the coding sequence for residues 226−281 of GCN4, preceded by Met and Lys residues.[62] The coding sequences for the Acid-p1 coiled-coil region were amplified using the polymerase chain reaction (PCR) from overlapping oligonucleotides[63] with the oligonucleotides 5′-AGCTGCAGCGTGTTAAGCAGCTG-GAGAAAGAACTCCAAGCGTTGGAAAAGGAAAACGCTCAGCTG-3′, 5′-ACGGATCCTCACTGCGCTAACTCCTTCTCCAGTGCTTGA-AGTTCCCACTCCAGCTGAGC,GTTTTCC-3′,5′-AGCTGCAGCGTGTTA-3′, and 5′-ACGGATCCTCACTGC-3′. The resulting fragment was subcloned into the *Pst*I-*Bam*HI site of pGCN4−56, replacing the GCN4 leucine zipper coding sequences. The resulting plasmid, pBR-Acid-a1, contains the coding sequence for the 57-residue peptide BR-Acid-

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p1 (Figure 2b). The plasmid pBR-Base-p1 (Figure 2c) was constructed through analogous steps, using the following peptides for the amplification of the Base-p1 coiled-coil coding sequence: 5′-AGCTGCAGCGTGTTAAGCAGCTGAAAAAGAAACTCCAAGCGTTGAAGAAGAAGAACGCTCAGCTG-3′, 5′-ACGGATCCTCACTGCGCTAATTTTTTCTTCAGTGCTTGAAGTTTCCATTTCAGCTGAGCGTTCTTC-3′, 5′-AGCTGCAGCGTGTTA-3′, and 5′-ACGGATCCTCACTGC-3′.

The plasmid pAcid-p1-BR was constructed by subcloning the Acid-p1 coding sequences into the *Nde*I-*Pst*I site of pBRBR.[45] The resulting plasmid encodes a 56-residue peptide with the Acid-p1 coiled-coil sequence N-terminal to the GCN4 basic region. The positions of the Asn codons in the vectors pBR-Base-p1 and pAcid-p1-BR were moved by successive rounds of site-directed mutagenesis to produce plasmids encoding the amino acid sequences of BR-Base-a1 and Acid-a1-BR, respectively (Figure 2d,e).

Acid and Base bZip peptides were expressed from a polycistronic RNA containing ribosome-binding sites adjacent to both open reading frames. The parent expression vector was constructed in several steps. The BR-Base coding sequences were subcloned into the *Nde*I-*Eco*RI site of pET22b, followed by subcloning of the Acid-BR sequences into the *Eco*RI site of the resulting plasmid. The *Eco*RI site between the two coding sequences was replaced by a ribosome-binding site through site-directed mutagenesis. Subsequent vectors were constructed by amplifying the BR-Base coding sequences followed by a ribosome-binding site and subcloning the resulting fragment into the *Nde*I site of the appropriate Acid expression vector. For in vivo experiments, protein coding sequences were cloned into the *Xba*I/*Eco*RI sites of pUC19 so that proteins were expressed under the control of the *E. coli lac* promoter. The plasmid pVS16N contains coding sequences for BR-Base-a1 and Acid-a1-BR (Figure 2d,e), and the plasmid pARA04 contains coding sequences for BR-Base-p1 and BR-Acid-p1 (Figure 2b,c).

In Vivo Screening and Selection Experiments. Protein expression vectors were transformed into freshly competent *E. coli* JM109 cells containing the appropriate reporter plasmid. The resulting cell cultures were incubated overnight in LB medium supplemented with chloramphenicol (25 μg/mL) and ampicillin (40 μg/mL). Serial dilutions were then prepared from each culture and spread onto LB-agar plates supplemented with ampicillin (40 μg/mL) and chloramphenicol (25 μg/mL) to monitor nonselective growth or supplemented with ampicillin (40 μg/mL), spectinomycin (60 μg/mL), and IPTG (0.5 mM) to monitor selective growth. The number of colonies observed under selective conditions was found to vary significantly with minor changes in experimental conditions. Each data point in Table 1 represents the average of at least three separate experiments. In addition, all experiments using the pAP1 reporter plasmid were performed at the same time, allowing an accurate comparison between these values (lines 1−3, Table 1). Similarly, all experiments involving the DIR-binding sites (lines 4−9, Table 1) were run in parallel.

In the selection experiment, the codon for Asn 11 of Acid-a1BR was randomized by QuikChange mutagenesis (Stratagene), using 100 pmol each of the oligonucleotides 5′-caagcgNNNgaaaaggaactggct-cagCTCGAGtgggaattgcaagcac-3′ and its complement, where N is an equimolar ratio of the four bases A, T, G, and C. A silent mutation incorporated a unique *Xho*I site (capitalized) to differentiate between starting material and library DNA. Following digestion by *Dpn*I, one half of the resulting mixture was transformed into freshly competent *E coli.* JM109 cells. After 1 h of nonselective growth, cells were plated on LB-ampicillin (40 μg/mL) plates. More than 1000 colonies were obtained, suggesting 10−100-fold coverage of the 64-member library. Plasmid DNA was isolated from 10 of these colonies, and each plasmid was found to contain an *Xho*I site.

The remaining half of the *Dpn*I-digested mutagenesis reaction was transformed into freshly competent *E. coli.* JM109 cells containing the reporter plasmid pDIR0. Cells were incubated in LB containing ampicillin (40 μg/mL) and chloramphenicol (25 μg/mL) for 2 h before the resulting culture was spread onto LB-agar containing ampicillin (40 μg/mL), spectinomycin (60 μg/mL), and IPTG (0.5 mM). The plates were incubated at 37 °C for 36 h and then washed to harvest spectinomycin-resistant cells. Plasmid DNA was purified from these pooled cells, and the selected library DNA was separated from the reporter by agarose (0.5%) gel electrophoresis following selective cleavage of the reporter plasmid by *Kpn*I. Three further rounds of selection were carried out. Individual colonies were sequenced after the first and fourth rounds of selection. Each of the sequenced colonies contained the unique *Xho*I site found only in the library DNAs.

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