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Artificial Sequence-Specific DNA Binding Peptides: Branched-Chain Basic Regions

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Abstract: Branched-chain-basic-region peptides ("bcBR") consisting of two bZIP basic regions connected through a C-terminal N^{α},N^{ϵ}-lysine linkage bind to DNA with high affinity and high specificity. bcBR with two identical basic regions recognize 2-fold-symmetric DNA sites, and bcBR with two nonidentical basic regions recognize asymmetric DNA sites. Resin-linked bcBR ("bcBR beads"), which are compatible with one-bead/one-peptide combinatorial chemistry approaches, retain high-specificity DNA binding activity and permit high-throughput screening of DNA recognition properties.

Introduction

bZIP sequence-specific DNA binding proteins, which bind to DNA as dimers, contain a ~20 amino acid "basic region" involved in DNA binding and a ~30 amino acid "leucine zipper" involved in dimerization.[1,2] bZIP sequence-specific DNA binding proteins recognize 2-fold-symmetric DNA sites, with the basic region of one subunit interacting with one half-site and the basic region of the other subunit interacting with the other half-site.[1,2] Different bZIP basic regions have different half-site recognition specificities; the best-characterized bZIP basic regions are the GCN4 basic region, which recognizes the half-site 5′-ATGAC-3′ (here referred to as "*G*")[3−9] and the C/EBP basic region, which recognizes the half-site 5′-ATTGC-3′ (here referred to as "*C*").[7,10]

The bZIP basic region is among the smallest peptide entities able to bind DNA in a sequence-specific fashion and, as such, is an attractive starting point for design of artificial sequence-specific DNA binding peptides.[2,11−17] A bZIP basic region

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peptide as short as 30 amino acids is able to bind to DNA in a sequence-specific fashion when maintained as a dimer by a C-terminal disulfide bond,[11] a C-terminal Fe:$(terpy)_2$ complex,[13,14] a C-terminal cyclodextrin−adamantyl complex,[15] or a C-terminal organic template.[14,16]

Here, we show that a branched peptide consisting of two bZIP basic regions connected through a C-terminal N^{α},N^{ϵ}-lysine linkage is able to bind to DNA in a sequence-specific fashion. We refer to such a branched peptide as a "branched-chain-basic-region peptide" ("bcBR"). In principle, bcBR have three interesting features: (*i*) bcBR can be prepared by conventional automated peptide synthesis (cf. ref 18). (*ii*) bcBR can be prepared with two identical bZIP-derived basic regions (using identical protecting groups on the branch-point lysine N^{α} and N^{ϵ} atoms) or with two nonidentical bZIP-derived basic regions (using different protecting groups on the branch-point lysine N^{α} and N^{ϵ} atoms). The ability to prepare bcBR with two nonidentical bZIP-derived basic regions permits straightforward construction of molecules able to recognize nonsymmetric DNA sites. For example, a bcBR with one GCN4 basic region and one C/EBP basic region is predicted to have specificity for a "hybrid" DNA site with one *G* half-site and one *C* half-site. (*iii*) bcBR can be prepared in soluble form or in resin-linked form. Availability in resin-linked form permits construction and affinity-sorting of "one-bead/one-peptide" combinatorial peptide libraries[19,20] and, therefore, permits use of efficient combinatorial chemistry approaches[19,20] to isolate bcBR having novel DNA recognition specificities.

Results

bcBR with Two Identical Basic Regions. In initial work, we synthesized and analyzed two bcBR: one having two GCN4 basic regions ("bcBR-GG") and one having two C/EBP basic regions ("bcBR-CC") (Figure 1A). Electrophoretic-mobility-shift DNA binding experiments establish that the peptides have the predicted DNA recognition specificities (Figures 2A,B and 3A,B, Table 1). Thus, bcBR-GG binds well to a DNA site with two *G* half-sites ($K_d = 4-5$ nM; cf. $K_d = 3$ nM for intact, native GCN4 bZIP) but binds substantially less well to DNA sites with one *G* half-site and one *C* half-site, two *C* half-sites, or two nonspecific half-sites. Analogously, bcBR-CC binds well to a DNA site with two *C* half-sites ($K_d = 100$ nM; cf. $K_d = 30$ nM for intact, native C/EBP bZIP) but binds substantially less well to DNA sites with one *G* half-site and one *C* half-site, two *G* half-sites, or two nonspecific half-sites. Similar results are obtained using a broad range of buffer compositions and reaction temperatures, including—in apparent contrast to results with other basic-region peptides[11−17]—reaction temperatures >20 °C (Figure 3 and data not shown). DNase I footprinting experiments confirm that bcBR-GG and bcBR-CC bind at, and only at, the predicted sites (Figure 4).

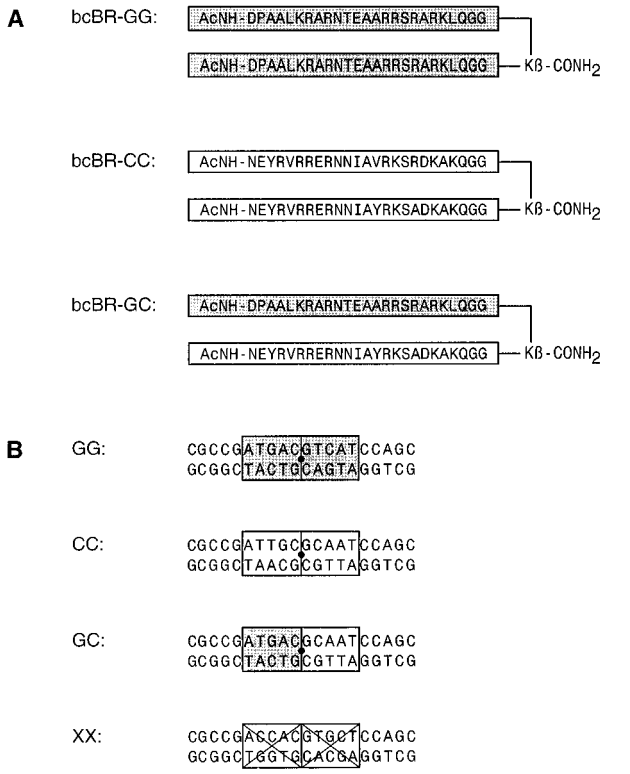

Figure 1. (A) Peptides synthesized. bcBR-GG, bcBR-CC, and bcBR-GC contain, respectively, two GCN4 basic regions, two C/EBP basic regions, and one GCN4 basic region and one C/EBP basic region. Each peptide contains an N^{α},N^{ϵ}-lysine branch (N^{ϵ} at top in figure) and a C-terminal β-alanine residue. (B) DNA fragments synthesized. GG, CC, GC, and XX contain DNA sites with, respectively, two *G* half-sites, two *C* half-sites, one *G* half-site and one *C* half-site, and two arbitrary, nonspecific half-sites.

GCN4 binds not only to a DNA site with two *G* half-sites but also, with comparable affinity, to a derivative of the site lacking one central bp (the "AP1" or "GCRE" site).[6,9] bcBR-GG—unlike Fe:$(terpy)_2$-linked peptides[13,14]—likewise binds with comparable affinity to the derivative of the site lacking one central bp (Table 2).

bcBR with Two Nonidentical Basic Regions. To prepare a bcBR able to recognize an asymmetric DNA site, we synthesized a branched-chain-basic-region peptide with one GCN4 basic region and one C/EBP basic region ("bcBR-GC") (Figure 1A). To synthesize this peptide, we used Fmoc-Lys-(Dde)-OH[21] as the branch amino acid, deprotected N^{α} with 20% piperidine, synthesized the C/EBP basic region, and then deprotected N^{ϵ} with 2% hydrazine in DMF and synthesized the GCN4 basic region. Electrophoretic-mobility-shift DNA binding experiments establish that bcBR-GC has the predicted, novel DNA recognition specificity; i.e., bcBR-GC binds well to a DNA site with one *G* half-site and one *C* half-site ($K_d = 30$ nM) but binds substantially less well to DNA sites with two *G* half-sites, two *C* half-sites, or two nonspecific half-sites (Figures 2C and 3C, Table 1). DNase I footprinting experiments confirm that bcBR-GC binds at, and only at, the predicted site (Figure 4).

Resin-Linked bcBR ("bcBR Beads"). To demonstrate that bcBR synthesized and deprotected without cleavage from the resin retain DNA binding activity and are compatible with one-bead/one-peptide[19,20] high-throughput screening, we prepared bcBR-GG beads and bcBR-CC beads, and we analyzed DNA

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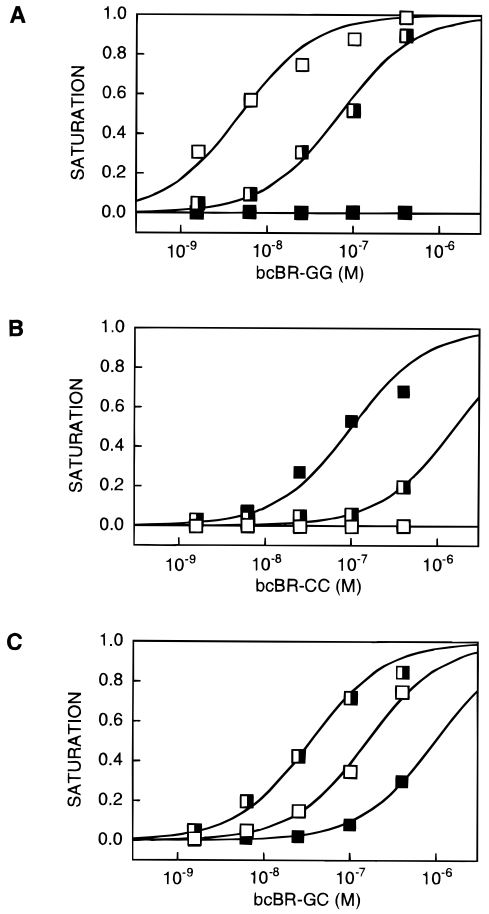

Figure 2. Electrophoretic-mobility-shift DNA binding experiments (4 °C): (A) bcBR-GG, (B) bcBR-CC, (C) bcBR-GC. Data for titrations of DNA fragments GG, CC, and GC are presented as open squares, filled squares, and half-filled squares, respectively. Data for titrations with DNA fragment XX are omitted for clarity (no data points above 0).

Table 1. Equilibrium Dissociation Constants for bcBR−DNA Interaction

	K_d (nM)			
	GG	CC	GC	XX
bcBR-GG	*5* (±3)[a]	>10 000	70 (±2)	>10 000
bcBR-CC	>10 000	*100* (±50)	2000 (±500)	>10 000
bcBR-GC	200 (±40)	1000 (±500)	*30* (±9)	>10 000

[a] Mean (±1 SEM).

Table 2. Equilibrium Dissociation Constants for bcBR−DNA and GCN4p−DNA Interaction

	K_d (nM)	
	GG	GG_{AP1}[a]
bcBR-GG	4 (±2)[b]	20 (±9)
$GCN4_p$[c]	3 (±3)	2 (±2)

[a] GG_{AP1}, which contains the AP1 DNA site,[3,6−8] has the sequence 5′-CGCCGATGACTCATCCAGC-3′/5′-GCTGGATGAGTCATCGGCG-3′. [b] Mean (±1 SEM). [c] $GCN4_p$, a 58 amino acid peptide corresponding to the bZIP motif of GCN4, contains all determinants of GCN4 for dimerization and DNA binding.[3]

binding activity in agarose-embedment one-bead/one-peptide screens[20] (Figure 5). We mixed bcBR-GG beads and bcBR-CC beads in various ratios, incubated the beads with ^{32}P-labeled DNA fragment GG or CC in the presence of excess unlabeled competitor DNA, washed the beads, embedded the beads in agarose, and phosphorimaged the agarose-embedded beads (Figure 5). In the resulting phosphorimages, black points

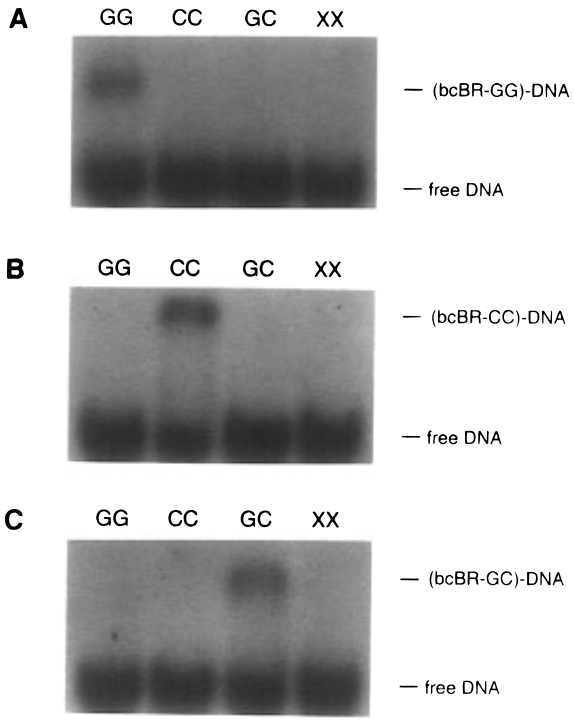

Figure 3. Electrophoretic-mobility-shift DNA binding experiments (25 °C): (A) bcBR-GG (10 nM), (B) bcBR-CC (25 nM), (C) bcBR-GC (25 nM).

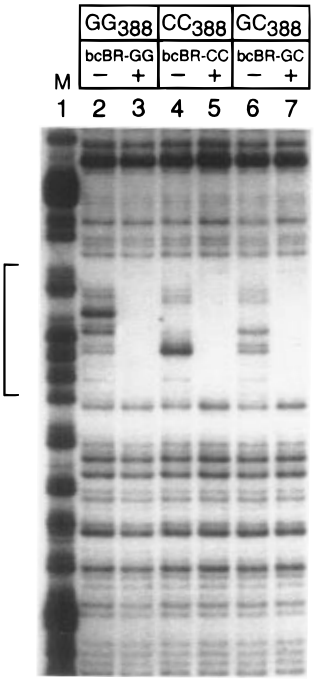

Figure 4. DNase I footprinting: lane 1, markers (Maxam-Gilbert G>A DNA-sequencing reaction[25] of DNA fragment GG_{388}); lanes 2 and 3, DNase I reactions of DNA fragment GG_{388} in the absence and presence of bcBR-GG; lanes 4 and 5, DNase I reactions of DNA fragment CC_{388} in the absence and presence of bcBR-CC; lanes 6 and 7, DNase I reactions of DNA fragment GC_{388} in the absence and presence of bcBR-GC.

indicated bcBR beads with high specific DNA binding activity for the ^{32}P-labeled DNA probe ("positives"), and gray points indicated bcBR beads with low specific DNA binding activity for the ^{32}P-labeled DNA probe ("negatives"). Distinction between positives and negatives was unambiguous, with a >20-fold difference in binding of the ^{32}P-labeled DNA probe between positives and negatives (>10^7 vs 5×10^5 phosphorimager units).

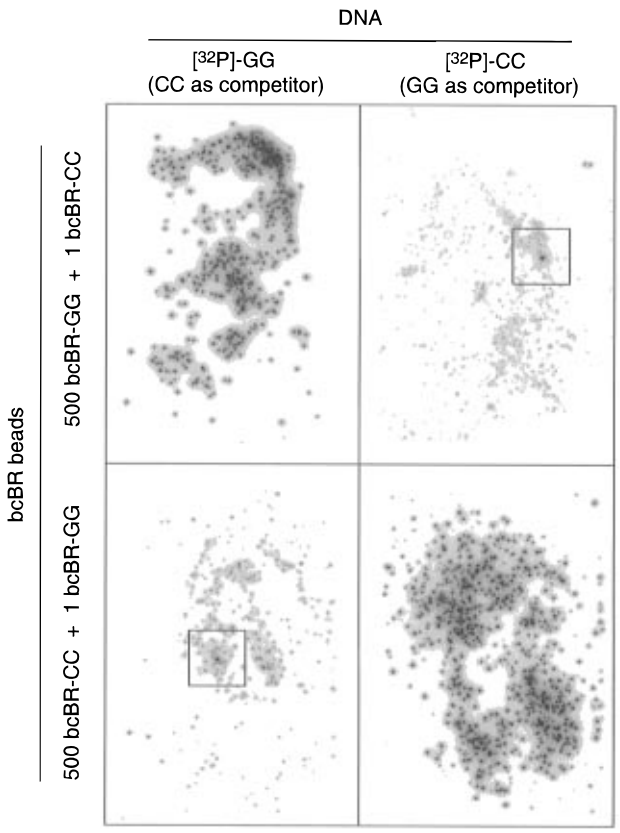

Figure 5. Resin-linked bcBR (bcBR beads). Single bcBR beads with high specific DNA binding activity are readily detectable (squares in top right and bottom left panels), even in the presence of large numbers of bcBR beads with low specific DNA binding activity. For the top panels, 500 bcBR-GG beads were mixed with 1 bcBR-CC bead, incubated with ^{32}P-labeled DNA fragment GG (top left panel) or CC (top right panel) in the presence of excess unlabeled competitor DNA, washed, embedded in agarose, and phosphorimaged. For the bottom panels, 1 bcBR-GG bead was mixed with 500 bcBR-CC beads, incubated with ^{32}P-labeled DNA fragment GG (bottom left panel) or CC (bottom right panel) in the presence of excess unlabeled competitor DNA, washed, embedded in agarose, and phosphorimaged.

Single positives were readily detectable, even in the presence of 500-fold excesses of negatives.

Discussion

Our results establish that bcBR are robust, accurate models of bZIP sequence-specific DNA binding proteins (Figures 2−4 and Tables 1 and 2). Our results further establish that bcBR synthesized and deprotected without cleavage from the resin retain high-specificity DNA binding activity and permit high-throughput screening of DNA recognition properties (Figure 5). bcBR open the way to one-bead/one-peptide combinatorial chemistry approaches[19,20] for analysis of bZIP−DNA interaction and for identification of bZIP-derived peptides with novel DNA recognition properties.

In the crystallographic structure of the GCN4-DNA complex, four amino acids within the GCN4 basic region contact DNA base edges within the DNA site (Asn235, Ala238, Ala239, and Arg243; ref 5; see also refs 3 and 4), and genetic experiments establish that these four amino acids determine DNA binding specificity.[2,10,22] It should be possible to construct a bcBR-GG-derived one-bead/one-peptide combinatorial library having all 160 000 possible amino acid sequences at the four DNA-base-contacting positions, to screen the library with each of the 1024 possible 2-fold-symmetric 10 bp DNA sites (performing 1024 parallel screens with the ^{32}P-labeled DNA site as probe and the unlabeled DNA site GG as competitor; see Figure 5), and, thereby, to identify *all* functional combinations of amino acid sequences and DNA sites. Such an analysis would provide the first comprehensive description of the DNA recognition repertoire—the "range"—of a DNA binding motif and would yield multiple novel-specificity DNA binding peptides.

Efforts to determine the solution structure of the bcBR-GG−DNA complex and to construct and screen a bcBR-GG-derived one-bead/one-peptide combinatorial library are in progress.

Experimental Section

bcBR-GG and bcBR-CC. bcBR-GG and bcBR-CC (sequences in Figure 1A) were prepared on an AB430A automated synthesizer (Applied Biosystems, Inc.) using Boc chemistry and were purified by reversed-phase HPLC. The composition and homogeneity were verified by analytical reversed-phase HPLC, amino acid analysis, and electrospray MS [bcBR-GG, expected $m = 5764$, observed $m = 5765$ (± 3); bcBR-CC, expected $m = 6267$, observed $m = 6268$ (± 1)].

bcBR-GC. bcBR-GC (sequence in Figure 1A) was prepared by manual synthesis using Fmoc chemistry and using Fmoc-Lys(Dde)-OH[21] (Novabiochem, Inc.) as the branch point lysine precursor and was purified by reversed-phase HPLC. The composition and homogeneity were verified by analytical reversed-phase HPLC, amino acid analysis, and electrospray MS [expected $m = 6016$, observed $m = 6016$ (± 0.5)].

Resin-Linked bcBR (bcBR Beads). bcBR-GG and bcBR-CC beads were prepared by manual synthesis using Fmoc chemistry using (aminomethyl)-PEG4000-derivatized polystyrene resin beads (NovaSyn TG amino resin; Novabiochem, Inc.). bcBR-GG and bcBR-CC beads had diameters of ~0.1 mm and substitution levels of 0.1 mmol/g (0.2 nmol/bead).

Before use, bcBR beads (15 mg) were hydrated by incubation for 15 h at 4 °C in 60 mL of buffer A [10 mM Mops−NaOH (pH 7.2), 100 μg/mL bovine serum albumin, and 100 μg/mL gelatin] with mechanical agitation (Clay Adams Nutator, Becton-Dickinson, Inc.), collected by centrifugation (500*g*, 2 min at 4 °C), washed three times with 15 mL of buffer A containing 300 mM NaCl and 10 mM $MgCl_2$, and resuspended in 15 mL of buffer A containing 300 mM NaCl and 10 mM $MgCl_2$.

Electrophoretic-Mobility-Shift DNA Binding Experiments. Reaction mixtures for experiments in Figure 2 and Tables 1 and 2 contained (20 μL) the following: 0−400 nM bcBR-GG, bcBR-CC, bcBR-GC, or GCN4p,[3a] 0.5 nM ^{32}P-labeled DNA fragment GG, CC, GC, XX, or GG_{AP1} (sequences in Figure 1B and notes to Table 2; prepared as in ref 23; 4 Bq/fmol), and 7 μg/mL poly(dI-dC)·poly(dI-dC) ($MW_{av} = 3\ 000\ 000$) (Sigma, Inc.), in buffer A containing 300 mM NaCl, 10 mM $MgCl_2$, and 5% glycerol. Reaction mixtures were incubated for 1 h at 4 °C. Reaction products were analyzed by electrophoresis in 45 mM Tris borate (pH 8.0) and 0.1 mM EDTA through 5% polyacrylamide slab gels (25 cm × 7 cm × 0.15 cm; 20 V/cm; prerun for 10 min at 4 °C; run for 30 min at 4 °C), followed by phosphorimaging (Model 425E PhosphorImager; Molecular Dynamics, Inc.). Equilibrium dissociation constants were extracted by nonlinear regression using the program SigmaPlot 4.0 (Jandel Scientific, Inc.) and the following equation (cf. ref 23):

$$\theta = \frac{[\mathrm{bcBR}]_F}{K_d + [\mathrm{bcBR}]_F} \quad (1)$$

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where θ denotes fractional saturation of the DNA (i.e., radioactivity in the band corresponding to the bcBR−DNA complex divided by the sum of radioactivity in the band corresponding to the bcBR−DNA complex and radioactivity in the band corresponding to free DNA), $[bcBR]_F$ denotes the free bcBR concentration (approximately equal to the total bcBR concentration under our conditions), and K_d denotes the equilibrium dissociation constant. θ and $[bcBR]_F$ were inputs to the nonlinear regression; K_d was an unconstrained output.

Reaction mixtures for experiments in Figure 3 contained (20 μL) 10 nM bcBR-GG, 25 nM bcBR-CC, or 25 nM bcBR-GC, 0.5 nM ^{32}P-labeled DNA fragment GG, CC, GC, or XX, 10 mM Mops−NaOH (pH 7.2), 100 mM NaCl, and 5% glycerol. Reactions were carried out as above except that reaction and electrophoresis temperatures were 25 °C.

DNase I Footprinting. Reaction mixtures contained (100 μL) the following: 0 or 90 nM bcBR, 0.5 nM immobilized ^{32}P-labeled DNA fragment GG_{388}, CC_{388}, GC_{388}, or XX_{388} [388 bp DNA fragments; prepared as in ref 24 using derivatives of plasmid pBluescript-SKII$^-$ (Stratagene, Inc.) having DNA fragments GG, CC, and GC inserted at the *Eco*RI site; 50 Bq/fmol], 80 ng/mL DNase I (Worthington, Inc.), 10 mM Mops−NaOH (pH 7.2), 100 mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. Reaction components except DNase I, $CaCl_2$, and $MgCl_2$ were incubated for 1 h at 4 °C. Reactions were initiated by addition of DNase I, $CaCl_2$, and $MgCl_2$ and were terminated after 1 min at 4 °C by addition of 100 μL of 200 mM NaCl, 20 mM EDTA, and 1% SDS. Products were isolated as in ref 24 and analyzed by electrophoresis through 6% polyacrylamide 7 M urea[25] followed by autoradiography and phosphorimaging.

High-Throughput Screening. Reaction mixtures contained 500 hydrated, washed bcBR beads (1 mg, 0.1 μmol), 0.25 nM ^{32}P-labeled DNA fragment (4 Bq/fmol), and 5 μM unlabeled competitor DNA fragment, in 1 mL of buffer A containing 300 mM NaCl and 10 mM $MgCl_2$. After incubation 2 h at 4 °C with mechanical agitation, bcBR beads were collected by centrifugation (500*g*, 2 min at 4 °C), washed four times with 1 mL of buffer A containing 300 mM NaCl and 10 mM $MgCl_2$ (1 min at 4 °C with mechanical agitation in each wash), resuspended in 1 mL of 0.5% ultralow-gelling-temperature agarose (SeaPrep agarose, FMC, Inc.; preheated to 50 °C), and immediately plated onto a liquid layer of 4 mL of 0.5% ultralow-gelling-temperature agarose (preheated to 50 °C) on a 0.02 cm polyester film (GelBond film, FMC, Inc.) in a 7 cm × 10 cm acrylic casting tray (Hoefer, Inc.). After cooling to 25 °C and air-drying for 15 h at 25 °C, the resulting films containing agarose-embedded reaction products were analyzed by phosphorimaging. bcBR beads exhibiting high specific DNA binding activity readily can be identified by high incorporation of radioactivity and readily can be recovered from the films by excision, heating, and washing.

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