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Single-chain 434 repressors with altered DNA-binding specificities

Isolation of mutant single-chain repressors by phenotypic screening of combinatorial mutant libraries

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Abstract

Combinatorial mutant libraries of the single-chain 434 repressor were used to discover novel DNA-binding specificities. Members of the library contain one wild type domain and one mutant domain which are connected by a recombinant peptide linker. The mutant domain contains randomized amino acids in place of the DNA-contacting residues. The single-chain derivatives are expected to recognize artificial operators containing the DNA sequence of ACAA – 6 base-pairs – NNNN, where ACAA is bound by the wild-type and NNNN by the mutant domain. An *in vivo* library screening method was used to isolate mutant DNA-binding domains which recognize the TTAA site of an asymmetric operator. Several mutants showed high affinity binding to the selection target and also strong (up to 80 fold) preference for TTAA over the wild type TTGT sequence. Some of the isolated mutants bound with very high affinities (10–50 pM) to operators containing the TTAC sequence, a close homologue of the TTAA selection target.

Introduction

DNA-binding proteins with desired recognition specificities can be used to construct novel reagents for research and for practical applications. Recently, zinc finger proteins containing the Cys2His2 type motif have been studied most extensively and the remarkable success [1–5] achieved in this field is mainly due to the applicability of the filamentous phage display and affinity selection techniques to this type of DNA-binding motif (see also [6–10] for reviews and references).

Another well studied motif, the helix-turn-helix (HTH) motif of prokaryotic and phage transcription factors can also accommodate changes resulting in altered DNA-binding specificities. These proteins, however, function mostly as homodimers and bind to operators with twofold rotational symmetry [11]. This property poses limitations since a specificity change results in the recognition of an altered but symmetric target sequence.

We have proposed previously that such limitations can be circumvented by covalently linking DNA-binding domains of different recognition properties [12]. It was shown previously that a well studied member of the HTH family, the 434 repressor, can be engineered to form a single-chain protein by covalently linking two DNA-binding domains through a recombinant peptide linker, resulting in a homodimeric single-chain molecule, termed RR69 [12, 13]. Specificity changes can also be performed in the individual DNA-binding domains of RR69: by substituting the DNA-contacting amino acids at the -1, 1, 2 and 5 positions of the $\alpha 3$ recognition helix, a heterodimeric (RR*69, changes in one domain) and a new homodimeric (R*R*69, changes in both domains) single-chain mutant with the expected DNA binding properties could be obtained [12]. DNA recognition studies showed that the single-chain repressors generally recognize two tetranucleotide sequences, the so-called contacted regions which are separated by a 6 base-pairs (bp) spacer or non-contacted region. The sequence of the spacer region was shown to indirectly influence the binding affinity of the single-chain repressors, and only a limited set of spacer sequences

can support high affinity binding [14]. These findings suggested that the mutant single-chain 434 repressors, like the zinc finger proteins, can be built from previously isolated, mutant DNA-binding domains to target relatively long DNA sequences and such constructs could also be used in gene fusions to develop artificial transcription factors with desired specificities.

The known repertoire of the mutant 434 DNA-binding domains which could potentially be used in such constructions is limited to a few examples [15, 16]. A permutational approach based on a combinatorial mutant library of the natural, full length 434 repressor and an *in vivo* selection technique did not result in new binding specificities [17]. In principle, combinatorial libraries of the single-chain 434 repressor could be used to explore new DNA binding specificities . In such libraries, one domain could be kept unchanged (wild type domain) whilst the other one is partially randomized at certain positions. The library members are expected to bind the general ACAA – 6 bp – NNNN sequence in a bidentate manner: the wild-type 434 domain binds to its cognate ACAA sequence facilitating the interaction of the mutant domain with the NNNN target. In this work, combinatorial libraries were tested by using an *in vivo* screening technique to identify library members that interact with an artificial 434-P22 hybrid operator. This operator, termed $O_{R^*}1$ [12, 14] contains the ACAATAAAACTTAA sequence, where the narrow selection target TTAA is underlined. A number of the isolated mutants are expressed and characterized by *in vitro* DNA binding techniques. This study shows that combinatorial single-chain libraries and phenotypic, *in vivo* library screening techniques can be successfully used to isolate mutants with altered DNA-binding properties. This approach may also prove useful for other DNA-binding proteins, especially in cases when the *in vitro* affinity selection and phage display techniques are not easily applicable.

Results and discussion

Combinatorial libraries of single-chain repressors and in vivo *selection for protein–DNA interaction by phenotypic screening*

The principle of the one-plasmid system used to detect protein–DNA interaction *in vivo* has been described [12]. Figure 1 shows the selection scheme and the components of the detection/screening system. Combinatorial libraries were constructed in two operator vectors, pRIZ′$O_{R^*}1$ and pRIZ′$O_{R^*}2$.

Randomizations were performed in the second domain and included those amino acids which were used in the helix redesign experiment [15] to change the DNA binding specificity of the 434 repressor to that of the P22 repressor and which were also shown to result in similar specificity change in the single-chain protein framework [12, 14]. The amino acid residues 27, 28, 29 and 32, corresponding to -1, 1, 2 and 5 positions of the $\alpha 3$ helix, were randomized as shown (Figure 1). Residue 33 is Gln in both repressors and was kept unchanged in the libraries.

Screening of the libraries (10 to 15 thousand colonies of the $O_{R^*}1$ and a few thousand of the $O_{R^*}2$ library) was performed by using X-Gal indicator plates and it was observed that a relatively high proportion of the colonies, at least 1–2%, appeared paler blue than the average. A number of these clones were sequenced and the amino acid sequences are shown in Table 1.

Certain mutants were isolated more than once and these were often encoded by different codons. Residue 5 of the $\alpha 3$ helix was most often Ser, Trp or Gly, and the sequences are listed as homology groups mainly according to the identity of this residue. Arg was often found in position 1 when residue 5 was Ser or Trp. With Gly in position 5, generally small and/or hydrophobic residues were found in positions 1 and 2. A general preference for either Ser or Thr could not be observed in the last position of the turn (or -1 position of the $\alpha 3$ helix) and certain combinations of 1, 2 and 5 residues were obtained with both Ser and Thr in this position. The selected vectors are abbreviated as pRIZ′$O_{R^*}1RR_{XXXX}$, where Xs stand for the selected amino acid residues (one-letter code) in the -1, 1, 2 and 5 positions of the recognition helix.

The previously designed RR*69 heterodimer was not found among the selected clones, although it is a high-affinity binder to the selection target $O_{R^*}1$ *in vitro* [12, 14]. Interestingly, many of the selected and tested mutants seemed to cause stronger repression than RR*69. In order to see if these values could be correlated with the *in vitro* target binding, several mutants were expressed, purified and characterized by *in vitro* DNA binding assays.

DNA binding properties of the isolated mutant single-chain repressors

The *in vitro* interaction between the mutant single-chain repressors and DNA probes was analyzed by gel

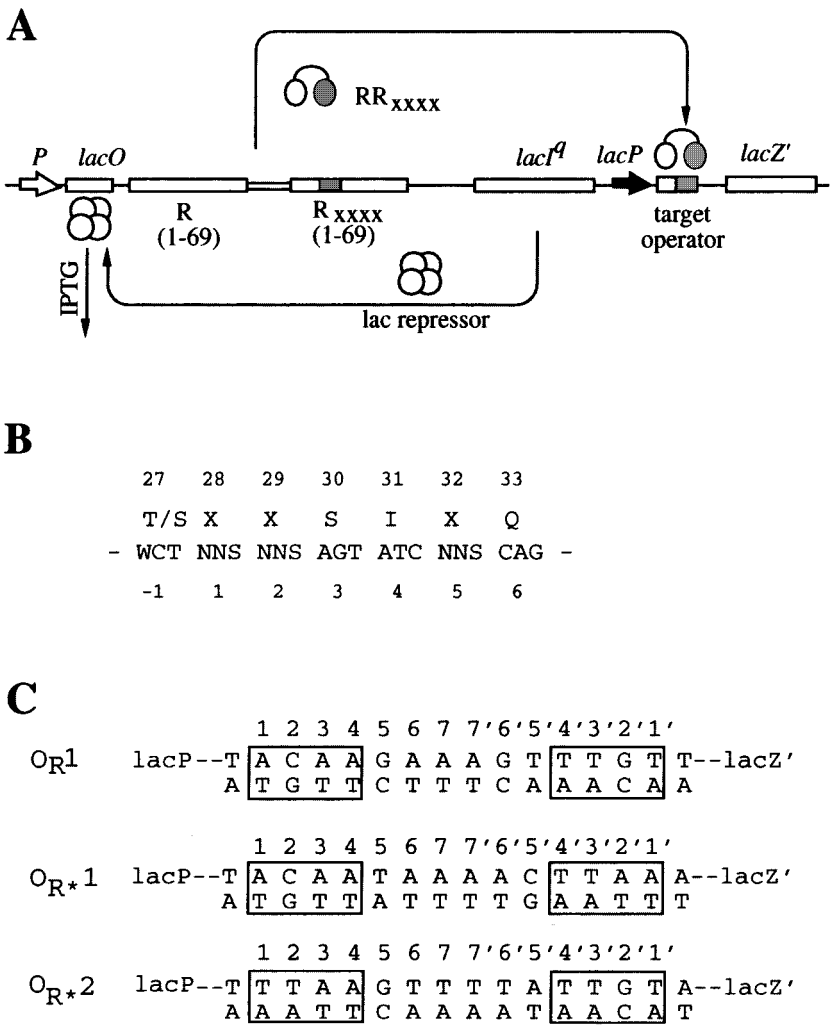

Figure 1. Selection of single-chain repressor mutants that bind to a given target operator. **A**, scheme of the *in vivo* selection based on phenotypic screening of the library members expressed in the pRIZ′ vector [12]. The expression of the *lacZ′* gene and the detectable β-galactosidase activity is dependent on the interaction between the upstream target operator and the single-chain repressor mutant. The mutant DNA regions and protein domains are shaded. **B**, nucleotide and amino acid sequences showing the randomized regions in the $\alpha 3$ recognition helix. **C**, sequences and the numbering scheme of the O_R1 operator of 434 repressor and the target hybrid operators $O_{R*}1$ and $O_{R*}2$. The latter two differ only in the orientation of the operator half-sites with respect to *lacP*.

electrophoretic mobility shift assay (EMSA). Purified mutant proteins were used in protein titration experiments and the equilibrium dissociation constant (K_d) was obtained as the protein concentration present at half-maximal binding, that is when 50% of the DNA probe was shifted. Examples of such titration experiments are shown in Figure 2. The DNA probe was 125 bp long and contained the $O_{R*}1$ sequence, the target of the selection. In order to test the binding specificity change due to the combinatorial mutations, the symmetric O_R1 operator, the target of the wild type RR69 was also used. The O_R1 and $O_{R*}1$ operators are highly homologous: six out of eight bases are identical within the two directly contacted, 4 bp long regions of operator half-sites (see Figure 1). The results (Table 1) show that all tested mutants had lower affinity than the designed, reference RR*69 to the $O_{R*}1$ containing DNA probe. At the same time, many of the mutants showed stronger binding to O_R1 than to $O_{R*}1$. For example, the mutant with the TAT..G sequence (abbreviated as RR_{TATG}) showed over 40 fold preference for O_R1. Only a few mutants showed higher affinity to $O_{R*}1$ than to O_R1 and this preference was in the best case (for the mutant RR_{TRPS}) about five fold. We note

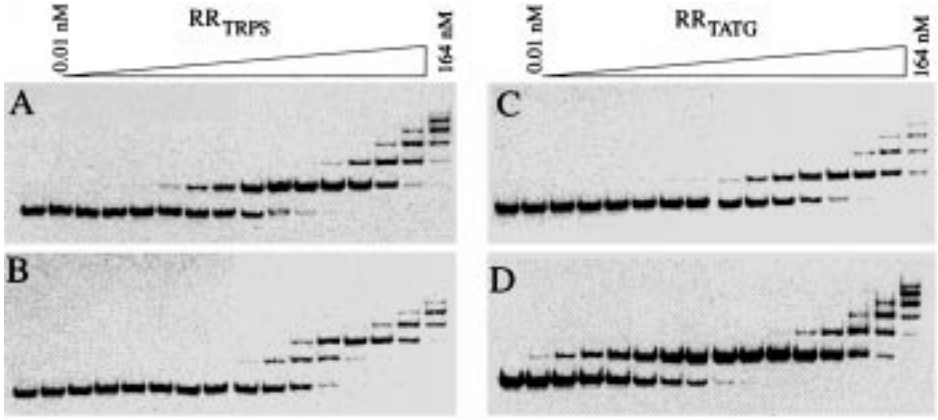

Figure 2. Examples of protein titration experiments performed with single-chain repressor mutants which show preference either for $O_{R*}1$ (RR_{TRPS}) or for O_R1 (RR_{TATG}).The protein concentrations were increased in 2 fold serial increments in the range shown. The interactions shown are **A**, RR_{TRPS}-$O_{R*}1$; **B**, RR_{TRPS}-O_R1; **C**, RR_{TATG}-$O_{R*}1$ and **D**, RR_{TATG}-O_R1.

here that since these binding data were obtained by using long DNA probes, we performed DNase I footprinting with most of the characterized mutants (not shown). These and other experiments with even longer DNA probes showed that the probes were protected against DNase I cleavage only at the operator sites. Both the EMSA and the footprinting assays were performed in the presence of large excess of non-specific DNA (see Materials and methods).

We assumed that the generally higher affinities obtained for O_R1 as compared to $O_{R*}1$ can be explained partly by the preference of the wild type R domain for G (present in O_R1) over T (present in $O_{R*}1$) at position 5 of the operator. Such a preference was previously observed for the wild type RR69 and mutant RR*69 single-chain repressors [14] and for the natural 434 repressor [18]. We have tested this possibility by placing the narrow recognition target TTAA in the place of the TTGT of the O_R1, or in other words into the sequence context of O_R1. This TACAAGAAAGTTTAAT operator, termed O_R1-2′A1′A, differs only in two bases (underlined) from O_R1. Preliminary, qualitative binding experiments show that most of the mutants bind substantially (10–25 fold) stronger to this operator than to $O_{R*}1$. This is substantiated with a few quantitative data: the affinities of RR_{SPSS}, RR_{TRPS} and the RR_{TRES} mutants for the O_R1-2′A1′A operator are 36 pM, 52 pM and 110 pM, respectively. These data mean that a part of the mutants selected for $O_{R*}1$ show in fact strong (up to 80 fold) preference for the TTAA narrow target over the TTGT target of the wild type molecule, therefore they can be categorized as new-specificity mutants. Many of the selected mutant domains show similar affinities to the TTGT and TTAA sites of O_R1 and $O_{R*}1$, respectively, when these sites are tested in identical sequence context. They are probably loss of contact mutants (with respect to the TTGT site) with relaxed specificities.

The fact that both new, altered specificity and broadened specificity mutants can be isolated by the *in vivo* screening method is not surprising, since the screening is based on the *in vivo* detection of an interaction which can be either specific or non-specific. Therefore, complementary specificity studies, as shown for example for the affinity-selected zinc finger motifs [19, 20], are often needed to characterize and to discover mutant DNA binding domains with new specificities.

Testing the DNA-binding specificities of the selected mutant repressors by using rationally designed probes

As observed above and described by others [4, 9, 10, 19, 20], selected DNA-binding proteins often recognize more than one sequence and the optimal recognition sequence could be different from the selection target. The optimal sequence can be found by binding site selection from random DNA pools, but high affinity sites may also be identified by using rationally designed test sequences. Here, we designed test operators simply by considering the recognition pattern observed in the 434 repressor-O_R1 complex [18] and the general chemical recognition principles [21–24]. The test operators were derivatives of O_R1 and contained the TTNC sequence instead of the TTGT

Table 1. Sequences and DNA-binding properties of the single-chain repressors selected by *in vivo* library screening. The wild type (R) and designed mutant (R*) domains are also shown for comparison

Sequence in α3 helix −1 1 2 5 $^{T}_{S}$XXSIXQ	No. of isolates[a]	β-Galactosidase activity[b]	Affinity[c] (nM) for $O_{R*}1$	O_R1
TQQ..E (R)	–	120	>40	0.017
SNV..S (R*)	–	50	0.038	4.80
SPS..S	1	42	0.41	1.65
TRS..S	2 (1)	16	1.90	1.80
SRS..S	– (1)			
TRP..S	2	15	0.77	4.14
SRP..S	– (1)			
TRE..S	1	18	2.71	1.20
TRV..S	1	21	2.35	3.20
TRA..W	2	30	6.60	0.95
SRA..W	– (1)			
TRE..W	2	40	2.10	2.20
SRN..W	2	16	7.60	0.25
SRV..W	2 (1)	18	1.14	1.80
TRM..W	1	22	4.60	4.10
SRM..W	– (1)			
TRI..W	1	20	5.02	5.65
TRT..W	1	18	1.43	0.36
SRQ..W	1	16	6.20	1.57
SRV..A	1 (1)	28	10.6	2.26
TRV..G	1	95	18.0	1.50
SGV..G	1	47		
SLG..G	1	92		
TLA..G	1	60	32.0	2.40
TVA..G	1	30	10.6	0.72
TAT..G	1	39	6.00	0.14
SAA..G	1	45		
SNS..G	1	2	2.30	0.43
TVN..S	1	56	7.5	1.50
SRW..G	5	12	28.0	6.70
SRW..M	2	25		
SRW..V	1	5	34.0	2.40
STW..V	1	22	26.0	5.00
TGE..G	1	85		
SYG..G	1	98		
TQA..W	1	103		
SLL..S	1	78		

[a]Figures in parenthesis are derived from the $O_{R*}2$ selection experiments.
[b]Relative β-galactosidase activities are calculated, value 100 corresponds to the non-repressed state as described in [12].
[c]Apparent dissociation constants (K_d) were given for 124 or 125 bp long DNA probes.

Table 2. A comparison of the binding affinities of various mutants for operator derivatives of O_R1 containing TTNC sequence (-ACAAGAAAGTTTNC-). Affinities are given as percentage of bound operator band observed in EMSA at 0.2 nM protein concentration

Protein	Percentage bound operator when *N* is			
	A	C	G	T
RR_{TRTW}	74	21	74	59
RR_{SRNW}	22	10	23	10
RR_{TRAW}	53	14	64	52
RR_{SRVW}	82	6	26	46
RR_{SRQW}	17	2	44	19
RR_{TRSS}	72	12	23	32
RR_{TRVS}	88	17	15	51
RR_{TRES}	93	77	7	3
RR_{TREW}	91	51	9	7
RR_{TRMW}	46	9	36	42
RR_{TRIW}	50	8	36	13
RR_{TRVG}	78	28	44	66
RR_{SRVA}	77	19	28	44
RR_{SRWG}	10	5	11	5
RR_{SRWV}	8	5	25	4
RR_{TRPS}	92	15	12	31

in the 4′ to 1′ positions. These four operators were cloned and individually tested by using 0.2 nM proteins in EMSA. The results obtained with 16 mutants, all containing Arg in the first helix position, are summarized in Table 2 as percentage of the shifted DNA probe at one protein concentration. Although, this preliminary screening does not provide correct binding affinities for quantitative comparison, it shows that practically all 16 mutants bind to one or more of the test operators with high affinity. The highest affinity interactions were generally observed with the operator containing the 4′-TTAC-1′ sequence, which is the closest homologue to the selection target TTAA. The strong binding in most cases is probably due to a contact between Arg28 and the G residue of the 1′ C-G base pair. The Arg-G contact [21] is observed in many complexes [23, 24] and its occurrence is theoretically possible since a topologically similar contact was observed between Gln28 and the A of the 1′ T-A pair in the 434 repressor–operator complexes [18, 25, 26].

Preliminary affinity determinations show that several of these interactions are at least as strong as those obtained with the wild type RR69 and with the rationally designed RR*69 single-chain repressors. For example, the TRE..S, TRE..W, TRP..S, and TRV..S mutants bound the 4′-TTAC-1′ operator derivative with 10–50 pM affinities. Detailed specificity studies including selection of binding sites for these mutants are in progress.

Such studies should provide novel mutant DNA-binding domains with high binding affinities and well characterized subsite recognition specificities, which can be used to build extended recognition surfaces in the single-chain protein framework.

Materials and methods

Plasmid vectors and bacterial strains

The pRIZ′ vectors were described previously [12]. The pRIZ′O_{R*}1RR(KOX)69 and pRIZ′O_{R*}2RR(KOX)69 vectors were used for library construction. These were obtained from the corresponding RR*69 vectors by cloning a 1.1 kbp long stuffer fragment (KOX) between the *Kpn*I and *Xho*I sites. This fragment replaced the recognition helix of the second domain. The T7 promoter-based expression vectors were derived from the pSETRR90 vector [12]. The *Escherichia coli* strain XL1-Blue (Stratagene) was used for library construction, screening and for β-galactosidase assay [27]. The expression host BL21(DE3)pLysS was obtained from Novagen.

Library construction and phenotypic screening

The single-stranded, partially randomized oligonucleotide TATTCTCTGGTACCWCTNNSNNSAGTATCNNS**CAGCTCGAGCTG** (AT443, W = A or T; N = A, C, G or T; S = G or C) containing cleavage sites for *Kpn*I and *Xho*I (underlined) and a 12 nucleotide long self-complementary 3′-terminal region (bold) was converted into a mixture of homoduplexes by self-annealing followed by a Klenow polymerase fill-in reaction in the presence of dNTP. The resulting duplex was cleaved with *Kpn*I and *Xho*I and the product containing the randomized region was cloned to replace the KOX fragment. Libraries containing 5.8×10^4 ($O_{R*}1$ vector) and 4.4×10^4 ($O_{R*}2$ vector) independent transformants were obtained by electrophoration.

For *in vivo* screening, the libraries were plated onto LB agar plates containing 75 mg/l ampicillin and 10 mg/l tetracycline to obtain about one to two thousand colonies per plate. After plating, the agar was covered with a nitrocellulose filter (BA 85 type, Schleicher & Schuell) and the plates were incubated at 37°C for 10–12 h. The nitrocellulose filter was then

placed (colonies facing up) onto LB agar plates containing antibiotics as above, 1 mM IPTG and 25 mg/l X-gal. The plates were further incubated for 8–12 h at 37°C. The colonies turned slightly blue, and the differences in colour intensity were already visible. For better colour discrimination, the plates were usually kept for 2–4 days at 4°C. Colonies which were paler blue than the average were picked for β-galactosidase assay and for sequencing. Nucleotide sequencing of the region coding for the second, selected mutant domain was performed by using a T7 sequencing kit (Pharmacia Biotech) and a 'forward' sequencing primer AGCATGGTTAGAGCTGGATC (AT446) which is located in the linker coding region, and/or by the backward, vector-specific AT419 primer [12].

Protein expression and purification

The *Bam*HI–*Hin*dIII fragments coding for mutant domains R_{XXXX} were isolated from the pRIZ′ selection vectors and cloned into pSETRR90 [12] to obtain pSETRR$_{XXXX}$ vectors. These were freshly transformed into BL21(DE3)pLysS strain and small scale (50–200 ml) expressions were performed as described [12] with minor modifications as follows. The sonication buffer TE (10 mM Tris–HCl, 2mM EDTA, pH 8.0) was supplemented with 100 mM KCl. After batch absorption onto SP-Sepharose, the enriched fractions were purified by HPLC on a Shodex SP 825 column with a linear gradient of KCl (100–400 mM) in TE. The fractions containing at least 95% pure protein, as judged by SDS-polyacrylamide gel electrophoresis, were stored in the elution buffer at −20°C. Protein concentrations were estimated by using the calculated molar extinction coefficients as described [28].

DNA-binding assays

The long DNA probes (124 bp O_R1 and 125 bp $O_{R*}1$) were obtained by PCR amplification of operators cloned in the pRIZ′ selection vectors [12] by using 5′-^{32}P labeled AGGCTTTACACTTTATGCTTCCG (AT477) and unlabeled GTTTTCCCAGTCACGACGTT (AT474) primers. DNA probes for DNase I protection assay were 157 bp (O_R1) and 158 bp ($O_{R*}1$) long as described [12]. The EMSA and DNase I protection assays were performed as previously described [12] by using a slightly different binding buffer which contained 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 25 mM Tris-HCl (pH 7.0), 6% (v/v) glycerol, 2.5 μg/ml sonicated salmon sperm DNA, 0.1 mg/ml bovine serum albumin (BSA) and 0.02% (w/v) Triton X-100. The protein titration experiments were performed by using serially two-fold diluted proteins. All protein dilutions were performed by using this buffer (without glycerol, BSA and carrier DNA) just before the binding assays were started. The concentration of the ^{32}P-labeled DNA probes was generally between 1 to 10 pM and always significantly lower than the K_d of the analyzed interaction. The binding reactions were performed at room temperature, generally for 2–3 h. Fixed and dried gels were evaluated by using an Instant Imager or a Cyclone Storage Phosphor System (Packard). The equilibrium dissociation constant (K_d) was obtained by determining the protein concentration present at half-maximal binding. The fraction of bound DNA was plotted against the total protein concentration and the data were evaluated by nonlinear least-squares analysis using the KaleidaGraph software (version 3.08, Synergy Software).

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