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9. The IgG1 MAbs used were DA4.4 anti-IgM (American Type Culture Collection), MOPC-21 control antibody (Organon Teknika), HD37 anti-CD19 (Dako, Carpentaria, CA, and G. Moldenhauer), BU32 anti-CR2/CD21 (Binding Site), 5A6 anti–TAPA-1 (*11*), MHM24 anti–LFA-1/CD11a (Dako), L27 anti-CD20 (Becton-Dickinson), 4KB128 anti-CD22 (Dako), BU38 anti-CD23 (Binding Site), Yz-1 anti-CR1/CD35 [P. S. Changelian, R. M. Jack, L. A. Collins, D. T. Fearon, *J. Immunol.* **134**, 1851 (1985)], and BU25 anti-MHC class II (Binding Site).
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Participation of Non–Zinc Finger Residues in DNA Binding by Two Nuclear Orphan Receptors

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Steroid-thyroid hormone receptors typically bind as dimers to DNA sequences that contain repeated elements termed half-sites. NGFI-B, an early response protein and orphan member of this receptor superfamily, binds to a DNA sequence that contains only one half-site (5′-AAAGGTCA-3′). A domain separate from the NGFI-B zinc fingers, termed the A box, was identified and is required for recognition of the two adenine-thymidine (A-T) base pairs at the 5′ end of the NGFI-B DNA binding element. In addition, a domain downstream of the zinc fingers of the orphan receptor H-2 region II binding protein, termed the T box, determined binding to tandem repeats of the estrogen receptor half-site (5′-AGGTCA-3′).

The steroid-thyroid hormone receptor superfamily is a class of ligand-activated transcription factors that mediates cellular response to hydrophobic ligands (*1*). Much of our knowledge concerning the DNA binding function of these nuclear receptors comes from studies of the estrogen and glucocorticoid receptors. The DNA recognition sites of these proteins contain two copies of protein-specific six-base sequences termed half-sites that are oriented as inverted repeats (*2*, *3*). Mutagenesis (*4*, *5*), nuclear magnetic resonance (*6*), and x-ray crystallography (*7*) studies show that characteristic residues within the Cys_2-Cys_2 Zn finger domains of these receptors make base-specific contacts with the half-sites. High binding affinity and a further degree of sequence specificity based on half-site spacing are achieved by dimerization of two protein monomers, which is a function of the Zn finger (*4*, *7*) and COOH-terminal (*2*, *8*) domains. This model is generally applicable, except that several receptors bind preferentially to promoter elements that contain direct rather than inverted repeats of half-site sequences (*9*, *10*), with the specificity of binding determined by the nucleotide spacing between half-sites (*11*, *12*).

The mammalian protein NGFI-B [(*13*), also called nur77 (*14*)] is an orphan receptor because it shares the structural features of nuclear receptors but has no known ligand specificity. An NGFI-B response element (NBRE) has been identified by genetic selection in yeast (*15*) that confers NGFI-B–dependent transcriptional activity to a heterologous promoter and consists of the 9-nucleotide sequence 5′-AAAAGGTCA-3′. The NBRE contains an estrogen receptor element–thyroid hormone receptor element (ERE-TRE) half-site (5′-AGGTCA-3′). It is likely that the NGFI-B Zn fingers recognize the ERE-TRE half-site like an estrogen or thyroid hormone receptor monomer through the conserved base-contacting residues in these proteins (*4*, *13*). The precise mechanism of binding must be distinct, however, because there is no half-site repeat in the NBRE and nucleotides outside of the half-site are required for binding (*15*).

To characterize nucleotide requirements in the sequence adjacent to the half-site in the NBRE, we synthesized three oligonucleotide pools in which each of the three positions nearest the 5′ end of the sequence 5′-AAAAGGTCA-3′ were replaced with equimolar mixtures of C, G, and T (*16*). These oligonucleotide pools were then used as probes in an electrophoretic mobility shift assay with NGFI-B protein synthesized in Chinese hamster ovary (CHO) cells (*15*). NGFI-B recognized the oligonucleotide pool with C, G, and T at position 1 and the NBRE itself with approximately equal affinity (Fig. 1A). In contrast, replacement of the A at either position 2 or 3 resulted in a reduction of the shifted complex, suggesting that NGFI-B makes base-specific contacts at these nucleotides. To assess the magnitude of this effect, we used the electrophoretic mobility shift assay to perform a Scatchard binding analysis (*17*). NGFI-B bound to a probe that contained the NBRE sequence with a dissociation constant (K_d) of 1.0 nM (Fig. 1B), a physiologically relevant affinity similar to that of estrogen and thyroid hormone receptors for their DNA elements (*8*, *18*). In contrast, a probe that had a substitution of G for A at position 2 of the NBRE was bound by NGFI-B with a K_d of 3.3 nM (Fig. 1B).

Most nuclear receptors do not bind with high affinity to DNA sequences that contain a single half-site because the interaction of one monomer with one half-site does not provide sufficient free energy to stabilize the protein-DNA complex. In contrast, three results support the model that NGFI-B binds to the NBRE as a monomer. (i) The high-affinity NBRE is limited to eight contiguous nucleotides with a single half-site [(*15*); Fig. 1]. (ii) When full-length and truncated NGFI-B

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were cotranslated in vitro and subjected to an electrophoretic mobility shift assay with the NBRE, we did not detect a complex with an intermediate mobility (*19, 20*). (iii) Deletion of the COOH-terminal domain of NGFI-B, which is typically required for high-affinity dimerization of nuclear receptors (*2, 8*), did not reduce the affinity of the NGFI-B–NBRE interaction (*19*). It is possible that NGFI-B might bind to the NBRE as a dimer, with one monomer binding to the extended half-site and a second monomer binding to the remaining nucleotides in a non–sequence-specific fashion. In either instance, it is likely that the two A-T base pairs at the 5′ end of the NBRE confer high-affinity NGFI-B binding because of the free energy contributed by additional base-specific protein-DNA contacts.

To localize within NGFI-B the residues that confer recognition of the 5′ A-T base pairs of the NBRE, we made fusion proteins with NGFI-B and the H-2 region II binding protein from mouse [H-2RIIBP (*21*), also called RXR-β (*22*)], an orphan receptor whose Zn fingers have a 72% similarity with those of NGFI-B. Despite this similarity, a TrpE fusion protein that contained the H-2RIIBP DNA binding domain [TrpE–H-2RIIBP (*23*)] did not bind to the NBRE in an electrophoretic mobility shift assay, whereas a fusion protein that contained the NGFI-B DNA binding domain did (TrpE–NGFI-B) (Fig. 2A). However, H-2RIIBP might bind to the NBRE if the NGFI-B residues that recognize the 5′ A-T base pairs are present. If so, this assay could provide a way of identifying these residues.

Chimeras of the H-2RIIBP and NGFI-B DNA binding domains (Fig. 2B) were produced in bacteria as TrpE fusion proteins (*23*) and used in an electrophoretic mobility shift assay to assess NBRE binding activity (Fig. 2C). Replacement of the entire H-2RIIBP Zn finger structural domain (*6, 7*) with the corresponding region of NGFI-B did not confer NBRE-specific binding to the chimera (chimera BH3). However, replacement of only 16 non–Zn finger residues at the COOH-terminus of the expressed H-2RIIBP fragment with the corresponding NGFI-B residues resulted in strong NBRE binding (chimera HB4). Conversely, the NGFI-B Zn fingers could be replaced with those of H-2RIIBP with no loss of NBRE binding (chimera HB3), while alteration of only five residues at the COOH-terminus of NGFI-B completely eliminated binding (chimera BH6). The lack of NBRE binding by some chimeric proteins was not a result of lack of expression, as determined by Coomassie staining of SDS-polyacrylamide gels of the bacterial extracts (*23*). Thus, the NGFI-B domain critical for NBRE recognition, the A box, was localized to seven residues between chimera positions 5 and 7.

Because both the BH and HB chimeras whose junctions lay within the A box did not bind to the NBRE, we examined which

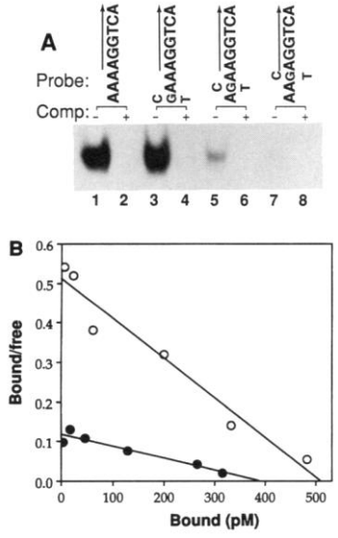

Fig. 1. High-affinity binding of NGFI-B to the sequence AAAGGTCA. (**A**) ^{32}P-labeled oligonucleotides (~0.5 ng) that contained the indicated sequences were used in an electrophoretic mobility shift assay with 5 μg of total protein from an NGFI-B–containing CHO whole-cell extract (*15, 16*). The ERE-TRE half-site in each oligonucleotide is indicated with an arrow. In lanes 2, 4, 6, and 8 a 100-fold molar excess of unlabeled oligonucleotide (that contained the parent sequence AAAAGGTCA) was added. All protein-DNA complexes are shown. Comp, competitor. (**B**) Protein (0.5 μg) from an NGFI-B–containing CHO whole-cell extract (*15*) was used in a series of electrophoretic mobility-shift assays with varying concentrations (between 16 pM and 17 nM) of probes that contained either the sequence AAAAGGTCA (B1a) or AGAAGGTCA (B1b). The protein-DNA complexes and free probe were detected by autoradiography, excised from the gel, and counted directly in a scintillation counter (*17*). Scatchard plots of the results are shown. White dots, B1a (K_d = 1.0 nM); black dots, B1b (K_d = 3.3 nM).

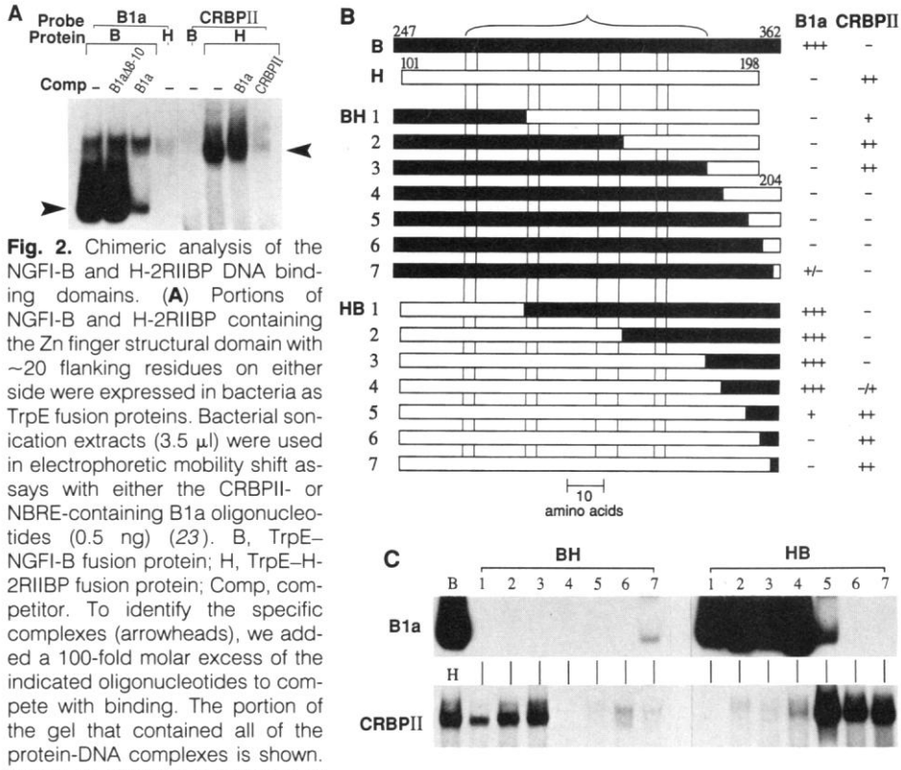

Fig. 2. Chimeric analysis of the NGFI-B and H-2RIIBP DNA binding domains. (**A**) Portions of NGFI-B and H-2RIIBP containing the Zn finger structural domain with ~20 flanking residues on either side were expressed in bacteria as TrpE fusion proteins. Bacterial sonication extracts (3.5 μl) were used in electrophoretic mobility shift assays with either the CRBPII- or NBRE-containing B1a oligonucleotides (0.5 ng) (*23*). B, TrpE–NGFI-B fusion protein; H, TrpE–H-2RIIBP fusion protein; Comp, competitor. To identify the specific complexes (arrowheads), we added a 100-fold molar excess of the indicated oligonucleotides to compete with binding. The portion of the gel that contained all of the protein-DNA complexes is shown. B1aΔ8-10 contains a mutation in the three A-T base pairs 5′ to the ERE-TRE half-site in the NBRE and is not bound by NGFI-B (*15*). (**B**) Schematic showing the composition of the DNA binding domain chimeras. NGFI-B sequences, in black; H-2RIIBP sequences, in white. The numbers of the terminal residues of the fragments are indicated above the bars. Chimeras are labeled BH or HB to indicate the origins of the NH_2- and COOH-terminal portions (where B = NGFI-B and H = H-2RIIBP) followed by a number corresponding to a specific junction point (Fig. 4). The location of residues that compose the Zn finger structural domain are enclosed by the bracket and Zn-chelating cysteines are indicated by vertical lines. To the right of the schematic are the results of the electrophoretic mobility-shift analyses shown in (C), using a scale where +++ indicates binding equivalent to the TrpE–NGFI-B–B1a combination, and – indicates no detectable binding. (**C**) The polypeptides in (B) were expressed in bacteria as TrpE fusion proteins, the bacteria were lysed by sonication, and the extracts (3.5 μl) used in electrophoretic mobility-shift assays with the CRBPII or NBRE-containing B1a oligonucleotides (0.5 ng) (*23*).

of these residues participated in adjacent sequence recognition. A trimeric protein that contains the DNA binding domains of NGFI-B and the bacterial repressor LexA and the transcriptional activating domain of the yeast transcription factor GAL4 (LexA–NGFI-B–GAL4, or LBG) activates expression of a *HIS3* reporter gene in yeast that has two NBREs in its promoter (*15*). To use this protein in a detailed mutational analysis of the A box, we individually randomized the codons encoding these seven amino acids using polymerase chain reaction (PCR) and cloned the resulting fragments to create libraries of mutant LBG derivatives. These seven libraries were then transformed into yeast bearing the NBRE-*HIS3* reporter, and individual clones that contained functional amino acids at a given randomized position were identified by virtue of their His^+ phenotype and sequenced (*24*).

At the last A box residue, Ser^{350}, a variety of functional amino acids was recovered, including positively and negatively charged as well as hydrophobic amino acids, which indicates that there was no specific function for this serine in DNA binding (Fig. 3A). In contrast, at positions 1 and 4 of the A box, Arg^{344} and Arg^{347}, respectively, only the positively charged amino acids lysine and arginine were recovered as functional, suggesting that these residues make ionic contacts, perhaps with the phosphate backbone of the DNA. At position 3, only the wild-type glycine was recovered as functional. Although the lack of a side chain in this amino acid precludes it from participating directly in DNA recognition, it is possible that the addition of any side chain sterically disrupts the protein-DNA complex. Similarly, the preference for proline at position 6 of the A box might reflect a conformational requirement. Only the wild-type arginine and leucine were recovered as functional at positions 2 and 5, respectively.

To examine further the amino acid requirements at positions 2 and 5 of the A box, we made TrpE–NGFI-B DNA binding domain fusion proteins with conservative substitutions of Arg^{345} and Leu^{348} (*23*) and tested them for NBRE binding in an electrophoretic mobility shift assay. Replacement of Arg^{345} with lysine decreased NBRE binding, despite the retention of the positive charge (Fig. 3B). Similarly, replacement of Leu^{348} with the hydrophobic residue valine resulted in an almost complete loss of NBRE binding. When isoleucine was substituted for Leu^{348}, there was less of a decrease in NBRE binding as compared to the valine substitution, which may be a result of the similar chain length of leucine and isoleucine. These results demonstrate that positive charge and hydrophobicity at positions 345 and 348, respectively, are not sufficient to support wild-type DNA binding activity. Rather, the side chains of the wild-type amino acids are essential for full activity. This is consistent with the hypothesis that these residues contact the 5′ adjacent A-T base pairs of the NBRE (*25*), although we cannot rule out the possibility that they make intra- (or inter-) peptide contacts that position other residues of the same (or a second) monomer near the 5′ adjacent base pairs for contact.

The TrpE–H-2RIIBP fusion protein did bind with high affinity to an oligonucleotide that contained five estrogen receptor half-sites present as tandem repeats with a single nucleotide spacer (Fig. 2A). This probe, from the cellular retinol binding protein II (CRBPII) gene promoter, is a binding site for the closely related protein RXR-α (*10*). The fact that TrpE–H-2RIIBP required more than one half-site for binding suggests that multiple subunits of this protein bound to the CRBPII probe in a fashion similar to RXR-α (*10*). Because the NGFI-B DNA binding domain did not bind to the CRBPII probe (Fig. 2A), the panel of DNA binding domain chimeras could be used in an electrophoretic mobility shift assay to identify the H-2RIIBP residues that confer CRBPII binding (Fig. 2C). When the H-2RIIBP Zn fingers were replaced with the NGFI-B Zn fingers, CRBPII site specificity was maintained (chimera BH3). Conversely, replacement of only 16 COOH-terminal non–Zn finger residues in the expressed H-2RIIBP fragment with NGFI-B residues eliminated CRBPII binding (chimera HB4). Although these results were similar to those obtained with the NBRE, the H-2RIIBP residues required for CRBPII recognition did not colocalize with the NGFI-B A box residues. The critical domain in H-2RIIBP, the T box, was localized to 12 residues between chimera positions 3 and 5.

Residues downstream of the Zn finger structural domain, which are not necessary for sequence-specific DNA binding by many nuclear receptors (*4–7, 26*), are not

A

	344						350
Wild-type residues	R_1-	R_2-	G_3-	R_4-	L_5-	P_6-	S_7
Recovered (functional) residues	R(5) R(8) K(3)		G(6)	R(6) K(4)	L(8)	P(8) K(1)	R(4) H(1) E(1) A(1) V(1) G(1)

B

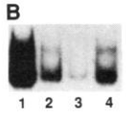

Fig. 3. Codon randomization analysis of the NGFI-B A box. (**A**) The seven wild-type NGFI-B A box residues are shown (*28*) with the numbers of the terminal residues indicated above (*13*). Subscripts indicate the relative position of each residue within the A box. Listed below are all amino acids that were recovered from His^+ yeast colonies after randomization of the individual codons in the LBG transcriptional activator (*24*). Number of times each individual amino acid was recovered is in parentheses. (**B**) Bacterial sonication extracts (3.5 μl) that contained TrpE–NGFI-B derivatives with conservative substitutions at A box positions 2 and 5 were used in an electrophoretic mobility shift assay with the NBRE-containing B1a oligonucleotide (0.5 ng) (*23*). Lane 1, nonmutated TrpE–NGFI-B protein; lane 2, TrpE–NGFI-B with an Arg to Lys mutation at position 345 (345, R → K); lane 3, 348, L → V; lane 4, 348, L → I (*28*).

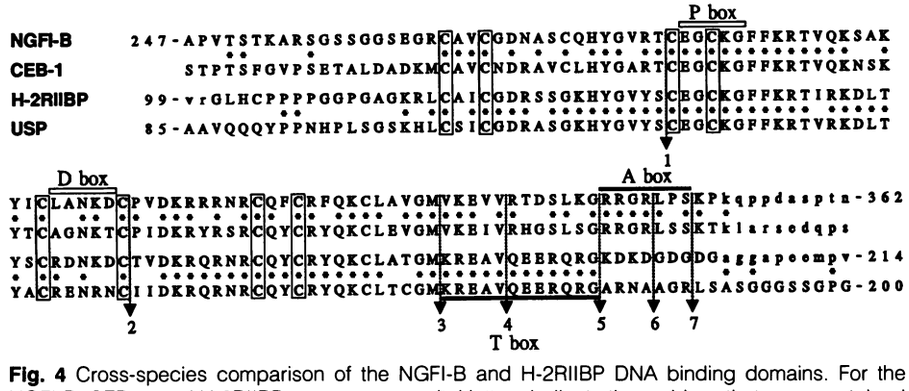

Fig. 4 Cross-species comparison of the NGFI-B and H-2RIIBP DNA binding domains. For the NGFI-B, CEB-1, and H-2RIIBP sequences, capital letters indicate the residues that were contained in the TrpE fusion proteins used in this study (*28*). For comparison, lowercase letters are used at the ends of the fragments to show residues that were not included in the fusion proteins. Ultraspiracle [USP (*27*)] was not tested specifically for binding to the CRBPII sequence and is therefore shown all in capital letters. The terminal residue numbers of the published sequences are shown (*13, 21, 27*). For the NGFI-B–CEB-1 and H-2RIIBP–USP pairs, asterisks mark the identical residues. Zinc-chelating cysteines are boxed and the A and T boxes described here are indicated with solid bars. Open bars indicate the residues that correspond to base-contacting (P box) and dimerization contact–forming (D box) regions of the estrogen and glucocorticoid receptors (*4, 7*). Broken vertical lines with arrowheads and numbers indicate the precise location of the different chimera positions, which correspond to the chimera numbers in Fig. 2B.

only critical for sequence recognition by NGFI-B and H-2RIIBP but also appear to function by different mechanisms. The NGFI-B A box residues are necessary for 5′ adjacent sequence recognition, whereas the H-2RIIBP T box residues may be part of a dimerization interface between protein monomers. In the estrogen and glucocorticoid receptor Zn fingers, dimerization is a function of contacts between amino acids on the same face of adjacent monomers because of inverted repeats in their DNA elements (*4*, *6*, *7*). H-2RIIBP monomers probably bind to the CRBPII oligonucleotide in the same orientation as a result of its direct half-site repeats. We predict that T box residues make contacts with as yet undefined residues on the opposite face of an adjacent monomer.

Although residues downstream of the Zn fingers between H-2RIIBP and the closely related *Drosophila* protein Ultraspiracle (*27*) are conserved, this conservation is lost immediately COOH-terminal to the T box (Fig. 4). In addition, binding of a *Caenorhabditis elegans* nuclear receptor–like gene (termed CEB-1) to the NBRE is dependent on the 5′ adjacent A-T base pairs (*19*). This protein has a 74% amino acid sequence similarity with NGFI-B between the first Zn finger cysteine and the end of the A box, and six of their seven A box residues are identical. This conservation ends immediately COOH-terminal to the A box (Fig. 4). Thus, the critical downstream domains of both NGFI-B and H-2RIIBP show evolutionary conservation, which suggests they are of biological importance.

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16. We synthesized four single-stranded oligonucleotides that contained either the wild-type B1a sequence, 5′-GAGTTTTAAAAGGTCATGCTCAATTT-GGAT-3′ (*15*), or three variants in which the appropriate A nucleotides were replaced with equimolar mixtures of the nucleotides C, G, and T. A primer of the sequence 5′-ATCCAAATTGAGCATGACCT-3′, labeled with [γ-^{32}P]adenosine 5′-triphosphate (ATP) (Amersham) and polynucleotide kinase (New England Biolabs), was then annealed to each of the four oligonucleotides and the second strand of the annealed pairs was completed with Klenow polymerase (U.S. Biochemical) in the presence of all four deoxynucleotides.
17. For Scatchard binding analysis, PCR primers were designed so that one pair yielded a portion of the B1 genomic fragment (*15*) that contained the B1a sequence, but excluded the B1b sequence (B1 nucleotides 1 to 127), and another pair yielded a product that contained the B1b sequence (B1 nucleotides 124 to 229). One primer of each pair was quantified with an extinction coefficient of 20 μg per absorbance unit at 260 nM and labeled with [γ-^{32}P]ATP, and its specific activity determined. The PCR products were purified from an agarose gel with MerMaid (BIO 101) and the concentration of the probes was calculated with the specific activity of the relevant primers. The gels from the electrophoretic mobility shift assay were dried onto Whatman paper, and we exposed them to film to localize the free probe and protein-probe complexes, which were excised and counted directly in a scintillation counter. We determined the quenching factor, consistently ~1.5, by comparing the total counts from gel slices of an individual reaction to the total counts added to that reaction, and used it to correct the counts-per-minute measurements of the experimental points. The concentrations of the free probe and protein-probe complexes were then calculated with the previously determined specific activities. For each protein-probe combination, at least three experiments were performed, all of which gave similar results.
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23. Fragments encoding the NGFI-B and H-2RIIBP DNA binding domains were obtained as a PCR products (with NGFI-B cDNA and reverse-transcribed mouse thymus RNA as templates, respectively) and ligated into the pATH3 plasmid [C. L. Dieckmann and A. Tzagoloff, *J. Biol. Chem.* **260**, 1513 (1985); D. C. Williams, R. M. Van Frank, W. L. Muth, J. P. Burnett, *Science* **215**, 687 (1982)]. We constructed DNA binding domain chimeras at positions 1 to 4 by generating two PCR products corresponding to each portion of the chimera, which were ligated into pATH3 in a three-part ligation. DNA binding domain chimeras at positions 5 to 7 and the A box point mutations were obtained in single PCR reactions with 3′ primers that spanned the affected regions. In all cases, the resulting pATH expression plasmids were verified by restriction digest and sequencing. TrpE fusion proteins were expressed in bacteria as described [M. L. Day, T. J. Fahrner, S. Ayken, J. Milbrandt, *J. Biol. Chem.* **265**, 15253 (1990)], and the bacteria lysed by sonication followed by centrifugation in a microfuge. The concentration of fusion protein in these sonication extracts was estimated by SDS–polyacrylamide gel electrophoresis followed by Coomassie blue staining.
24. The A box residues were individually randomized by PCR with 3′ primers that contained equimolar mixtures of the nucleotides A, C, G, and T at each of the three bases of the target codon. The resulting fragments were ligated into a derivative of the pLG1519 LexA-GAL4 yeast expression vector (*15*). A library of at least 125 clones was obtained for each randomized codon position. We then sequenced DNA corresponding to each library to verify that each position of the target codon contained an approximately equimolar mixture of the four nucleotides, and transformed the DNA into *Saccharomyces cerevisiae* strain YM2632 (*a, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, met$^-$, gal4-542, can^R, leu2:GAL1-lacZ*) that bore the B1-*HIS3* reporter plasmid (*15*). Transformants that grew at or near wild-type rates on histidine-selective medium were picked and purified to single colonies. The NGFI-B DNA binding domain in these clones was then analyzed by PCR sequencing as described (T. E. Wilson, T. Pexton, K. Padgett, M. Johnston, J. Milbrandt, *J. Biol. Chem.*, in press).
25. Although DNA base recognition is typically achieved through hydrogen bond interactions with polar residues such as arginine, there is precedent for the involvement of nonpolar residues, such as leucine, in base recognition, most notably Val^{462} in the glucocorticoid receptor (*7*) and Ala^{49} in the λ repressor [N. D. Clarke, L. J. Beamer, H. R. Goldberg, C. Berkower, C. O. Pabo, *Science* **254**, 267 (1991)]. In both, a methyl group from the base-contacting residue makes a van der Waals interaction with the methyl group at position 5 of a T nucleotide.
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28. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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