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A genetic method for defining DNA-binding domains: Application to the nuclear receptor NGFI-B

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ABSTRACT **A method is described that allows for rapid and efficient generation of functional mutations in DNA-binding domains of proteins. The target DNA-binding domain is attached to the Gal4p transcriptional-activating domain and expressed in yeast. The binding site recognized by the target domain is placed upstream of a gene that produces a protein toxic to yeast cells, so that the chimeric protein activates its expression, providing a selection against DNA-binding domain function. The chimeric protein also activates expression of a gene necessary for histidine prototrophy, using a second DNA-binding domain included in the chimera (lexA), providing a selection against general activator mutations. Therefore, requiring growth in the absence of histidine focuses mutations to the target DNA-binding domain. This method was applied to the DNA-binding domain of the nuclear receptor NGFI-B. Nearly all mutations obtained concurred with previous studies of NGFI-B and other nuclear receptors, verifying the functional validity of the mutational profile obtained. In addition, by coupling this selection scheme with the two-hybrid system [Chien, C.-t., Bartel, P. L., Sternglanz, R. & Fields, S. (1991) *Proc. Natl. Acad. Sci. USA* 88, 9578–9582], mutations that alter protein interaction domains could also be obtained.**

The regulation of transcriptional initiation is critical for the appropriate expression of genes. Although the transcription factors responsible for this control are multifunctional proteins with several domains, it is the DNA-binding domains that give the greatest degree of specificity, because they determine which promoters will respond to these proteins (1–3). Many DNA-binding domains can be classified into recognizable structural classes [e.g., zinc finger, helix–turn–helix, and B-zip (4–8)]. Such classification can provide great insight, because the structures of many prototypic examples have been solved (9–11). Nonetheless, significant questions have arisen regarding (*i*) the origins of functional differences between members of a given class and (*ii*) the limitations of structural analyses in examining the dynamic features of DNA recognition. Further, many transcription factors have DNA-binding domains that do not fall into one of the recognizable classes, and for these little or no structural information is available (2, 6). It is thus clear that extensive mutational analysis is important for understanding the functional aspects of DNA recognition by transcription factors.

We previously reported an extensive mutational analysis of the zinc-finger DNA-binding domain of the mammalian transcription factor NGFI-A, achieved by using genetic selection in yeast (12). The method was not general, though, because it relied on the unusual growth-repressive property of a fusion protein containing the NGFI-A zinc fingers. To extend the usefulness of this technique, we sought to make such genetic selection applicable to virtually any DNA-binding domain by (*i*) expressing the domain in yeast as a chimera with a known transcriptional activator and (*ii*) causing this chimera to activate expression of a gene that produces a protein toxic to yeast cells. We chose as a test case the DNA-binding domain of the NGFI-B protein, a member of the class of eukaryotic transcription factors known as nuclear receptors (13–15). A significant amount of molecular detail can be inferred regarding this domain by comparison with the crystal structure of the similar glucocorticoid receptor "zinc modules" (9). The NGFI-B DNA-binding domain is clearly more complex, though, because it binds to an atypical nuclear receptor response element as a monomer using amino acids outside of the zinc modules (refs. 16–19; see *Discussion*). We show that application of our technique to NGFI-B yields a mutational profile consistent with known structural information that accurately predicts many of the functional aspects of NGFI-B binding to its unusual site.

MATERIALS AND METHODS

Plasmids and Yeast Strains. Growth and transformation of yeast were accomplished by standard techniques (20). The plasmid used to express the chimeric activator lexA–NGFI-B–GAL4 (LBG) in yeast (pLBG) and the *lexO-HIS3* reporter plasmid [p(lexO)^2] have been described (16). The yeast strain containing *GAL1* with NGFI-B recognition sites in its promoter integrated into the yeast genome was created in two steps. (*i*) Six copies of oligonucleotide B1a (16), which contains the NGFI-B response element (NBRE), were ligated into the *Eco*RI site in the *GAL1* promoter in pBM85 (21). This plasmid contains sequences upstream of the *GAL1* promoter fused to the *GAL1* promoter and coding sequences such that the UAS_{GAL} (upstream activating sequence of the *GAL1* gene, which consists of four binding sites for GAL4p, the activator of *GAL1* expression), but not the TATA box, is removed. Hence, expression of the *GAL1* gene present on this plasmid depends on the addition of activator-binding sites. (*ii*) The normal chromosomal *GAL1* gene was replaced with this engineered version by transplacement (22). This transplacement was accomplished by transforming yeast to Ura$^+$ with the plasmid generated in the first step (pBM85 carries *URA3*), cleaved at the single *Pvu* II site within the *GAL1* coding sequence to direct integration of the plasmid at *GAL1*. The plasmid sequences were subsequently removed by selection for Ura$^-$ colonies on medium containing 5-fluoroorotic acid (23). This selection can occur by either of two recombinational events: one leaves a wild-type *GAL1* gene in the chromosome; the other leaves the altered *GAL1* gene with NBREs in its promoter. These two types of colonies can be discerned because the *GAL1*-promoter deletion that removes UAS_{GAL} also removes a portion of the adjacent *GAL10* gene, rendering the strain Gal$^-$. The resultant strain (*gal10*$^-$ with six NGFI-B-binding sites upstream of *GAL1*) is YM4096.

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Abbreviations: LBG, lexA–NGF1-B–GAL4; GST, glutathione *S*-transferase; NBRE, NGFI-B response element.

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Mutagenesis of LBG Plasmid. LBG plasmid DNA was prepared using a MaxiPrep DNA-purification column from Qiagen (Chatsworth, CA), according to the manufacturer's directions. One milligram of plasmid DNA was treated for 2 hr at 75°C with 104.5 mg of hydroxylamine monochloride (Sigma) in 1.5 ml (final volume) of HA buffer (50 mM sodium pyrophosphate/100 mM sodium chloride/2 mM EDTA, adjusted to pH 7.0 with 50% NaOH after addition of the hydroxylamine monochloride). The DNA was then precipitated with 0.7 vol of isopropanol, washed with 70% (vol/vol) ethanol, and resuspended in Tris/EDTA buffer.

Selection of Galactose-Resistant Yeast Mutants. Yeast strain YM4006 [genotype: *MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 tyr1-501 gal10Δ147-(BRE)6-GAL1 (gal10Δ147* is described in ref. 21)] containing pHR1528 (16) [p(lexO)^2 with *TRP1* selectable marker] was transformed to Ura$^+$ (24) with hydroxylamine-mutagenized pLBG (*URA3* selectable marker). Individual transformants were picked and patched on plates containing glucose and lacking uracil, allowed to grow, and then replica-plated to minimal medium (20) lacking uracil, tryptophan, and histidine with 2% raffinose as the carbon source, and 2% galactose was added to select against LBG function. Yeast patches that grew within 12 days were purified on the same selective plates. Approximately 5% of the plasmid-containing transformants were His$^+$ (i.e., retained activator function) but galactose resistant (i.e., lost NGFI-B DNA-binding activity). The NGFI-B DNA-binding domain was recovered by PCR and sequenced by using primers and described techniques (12). All mutants arose independently.

Gel Shift Analysis. For gel-shift analysis, the wild-type and mutant NGFI-B DNA-binding domains were amplified by PCR using oligonucleotides that prime DNA synthesis at the ends of this domain [nt 849–1166 (13)], such that *Bam*HI and *Eco*RI restriction sites were included at the 5′ and 3′ ends of the coding strand, respectively. The PCR products were digested with these restriction enzymes and ligated into the *Bam*HI and *Eco*RI sites of vector pGEX-1 (Pharmacia). The resulting plasmids directed expression in bacteria of fusion proteins, containing glutathione *S*-transferase (GST) and the NGFI-B DNA-binding domain after induction with isopropyl β-D-thiogalactoside (25). Crude lysates (containing only soluble proteins) of such bacteria were prepared for gel shift as described for the trpE–NGFI-B fusion protein (17). To estimate the amount of soluble GST fusion protein, 50 μl of each lysate was incubated for 5 min with glutathione-agarose beads (corresponding to 25 μl of packed resin) in 1 ml of phosphate-buffered saline. The beads were washed with 1 ml of phosphate-buffered saline three times and then boiled in protein sample buffer to release the fusion proteins. The beads were spun out, and the supernatant was loaded on an SDS/10% PAGE gel followed by staining with Coomassie brilliant blue. Gel-shift analyses were done by using the NBRE-containing B1a oligonucleotide as described (16, 19).

RESULTS

The scheme that we devised for selecting functional mutations in DNA-binding domains is illustrated in Fig. 1. A trifunctional chimeric protein is created that contains the DNA-binding domain of interest sandwiched between the DNA-binding domain of the bacterial transcriptional repressor lexA and the transcriptional-activating domain of the yeast protein GAL4 (26, 27). Chimeras of this type activate transcription in yeast from promoters that bear sites for either of the DNA-binding domains (16). Recognition sites of the test DNA-binding domain are inserted upstream of an inactive variant of the *GAL1* gene promoter that lacks binding sites for its transcriptional activator (Gal4p). In this way, *GAL1* expression is made dependent on the expression and

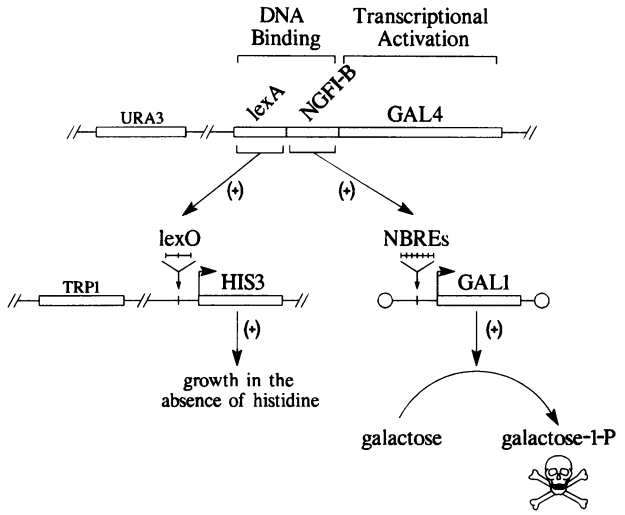

FIG. 1. Scheme for selecting mutations in DNA-binding domains. The relevant regions of the episomal plasmids and chromosomal replacement are shown. Boxes indicate transcribed regions, double slash marks indicate discontinuities in plasmid DNA fragments, and circles indicate insertion into the yeast chromosome. Arrows and (+)'s indicate the actions of the encoded proteins, specifically activation of transcription of *lexO–HIS3* or NBRE–*GAL1* and conversion of galactose to the toxic product galactose 1-phosphate. *URA3* and *TRP1* serve as selectable markers for the LBG and *lexO–HIS3* plasmids, respectively.

function of the intact chimeric activator. The *GAL1*-encoded protein converts galactose to galactose 1-phosphate, which accumulates to toxic levels in yeasts that lack the *GAL10* gene product, the next enzyme in the galactose-utilization pathway. The growth of a *gal10*$^-$ yeast strain expressing the chimeric activator will thus be sensitive to the addition of galactose to the medium (21, 28).

In this situation, three types of mutations could lead to a loss of *GAL1* expression and allow growth in the presence of galactose (assuming a different carbon source is provided). Undesired mutations could occur in the *GAL1* gene itself. However, we found that the frequency of *GAL1* mutations was vanishingly small in comparison with other types of mutations when the plasmid that expressed the chimeric activator was mutagenized *in vitro* before its transformation into yeast. Frequent undesired mutations could also occur in the Gal4p activation domain such that its function is abolished, and mutations could occur in the plasmid carrying the chimeric activator such that its expression is prevented. To select against this class of mutants, the yeast strain also carries a plasmid that contains the recognition sequence of the LexA protein inserted into an inactive variant of the *HIS3* promoter (*lexO–HIS3*). This addition makes expression of the *HIS3* gene also dependent on the presence of the functional chimeric activator but via a DNA-binding domain different from that used by the *GAL1* gene. Because the His3 protein is required for growth in the absence of histidine, selection on medium lacking this nutrient will prevent the recovery of general activator mutations. Hence, recovered mutations are targeted as desired to the test DNA-binding domain. Such mutations eliminate *GAL1* expression in a manner that leaves the remainder of the chimeric protein intact, allowing for the continued expression of the *HIS3* gene and, therefore, growth on plates lacking histidine but containing galactose.

We have previously demonstrated that the LBG chimeric activator behaves as a bifunctional transcriptional activator in yeast, making it suitable for use in our scheme. We therefore inserted six copies of the NBRE sequence into the *GAL1* promoter as shown in Fig. 1, and integrated this gene

into the yeast chromosome, as described. The resultant yeast strain (YM4096, *gal10*$^-$ with NBRE–*GAL1* in the chromosome) was itself resistant to galactose but became sensitive to galactose when the LBG plasmid was transformed into the cells. The galactose sensitivity caused by LBG clearly depended on the NGFI-B DNA-binding domain because a plasmid that expressed only the *lexA* and *Gal4* portions had no effect (data not shown). We next mutagenized the entire LBG plasmid by treatment with hydroxylamine and transformed it into a derivative of strain YM4096 that bore the *lexO–HIS3* plasmid. After establishing growth of these transformants on galactose-free medium, they were replica-plated to medium containing galactose but lacking histidine. Yeast that grew into visible colonies within 12 days were cloned on this same medium. The DNA encoding the NGFI-B DNA-binding domain was then recovered from these yeast cells by PCR and sequenced (see *Materials and Methods*).

The NGFI-B DNA-binding domains from 47 independent galactose-resistant yeast colonies were analyzed to determine the profile of mutations in this domain (Fig. 2). Thirty-nine of these colonies contained base substitutions that altered the encoded NGFI-B polypeptide. The other eight did not contain mutations that altered the polypeptide, presumably because these plasmids contained additional mutations that limited expression of the LBG protein enough to prevent *GAL1*-mediated toxicity, while allowing sufficient *HIS3* expression for growth in the absence of histidine. Twenty unique mutants were recovered. Of these, 15 were single missense mutations, and 5 were double missense mutations. No nonsense or frameshift mutations were recovered. All mutations substituted an adenine for a guanine on one of the DNA strands, as expected for hydroxylamine-mutagenized DNA. Mutations were seen along virtually the entire span of the NGFI-B polypeptide in amino acids known to define the function of this DNA-binding domain (Figs. 2 and 4, see *Discussion* for details).

To verify that the NGFI-B polypeptides obtained from galactose-resistant yeast were impaired in their ability to bind the NBRE, we expressed a representative sample of these mutants in bacteria as GST fusion proteins. Crude lysates of these bacteria were then prepared for use in a gel-shift assay. Interestingly, a subset of the mutant GST fusions gave very small amounts of soluble protein, in contrast to the wild-type fusion and most mutants. Specifically, Gly-264 → Asp and Gly-285 → Ser yielded very poorly soluble fusion proteins (data not shown), possibly due to disruption of the tertiary structure of the DNA-binding domain. Lysates containing the remaining soluble proteins were tested for binding to the NBRE (Fig. 3). Nine of these 10 mutations completely abolished DNA binding, demonstrating the validity of the selection technique. A GST fusion protein with a Ser-259 → Leu (S259L) substitution bound essentially as well as the wild-type NGFI-B polypeptide.

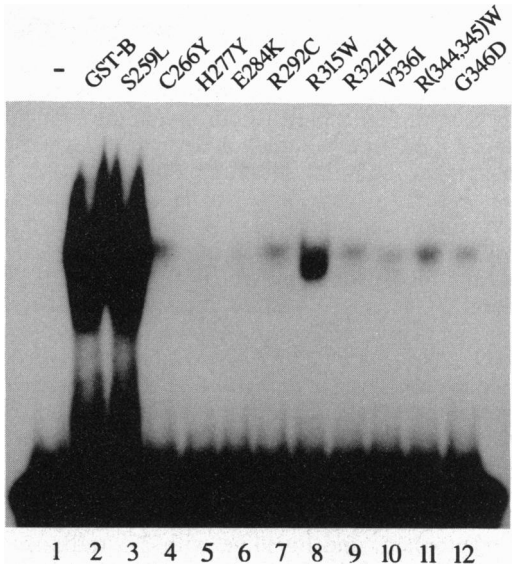

FIG. 3. Gel-shift analysis of mutants recovered from galactose-resistant, His$^+$ yeast. A gel-shift analysis was done with the NBRE and crude bacterial lysates containing GST fusions of the indicated wild-type and mutant NGFI-B DNA-binding domains. Mutant proteins are labeled as follows: wild-type amino acid (single-letter code) and its position number followed by the mutant amino acid. Lanes 2 and 3 are somewhat overexposed in this autoradiograph to demonstrate the lack of protein–DNA complexes in the other lanes. Lane 1 is probe alone with no added protein.

DISCUSSION

Nuclear receptors typically bind as dimers to repeats of a 6-nt sequence known as a half-site, using the highly conserved zinc-module domain (29–31). NGFI-B is unusual because it binds as a monomer to a response element that contains only one half-site (16). This is accomplished by virtue of a zinc module–half-site interaction similar to other nuclear receptors, in addition to the interaction of amino acids downstream of the zinc modules (the A box, see Fig. 4 *Upper*) with 2 A·T bp 5′ to the half-site (17, 19). The mutational profile reported here is consistent with these previous observations with NGFI-B and with a similar study of the related glucocorticoid receptor (42). We obtained mutations in both the NGFI-B zinc modules (aa 267–331) and A box (aa 343–350). The zinc-module mutations occurred primarily in highly conserved amino acids that define the structure of this domain (aa 266, 282, and 322) and in amino acids that interact with the phosphate backbone and bases of the half-site (aa 277, 284, 285, 288, 292, 314, 315, and 322); see comparisons in Fig. 4 and refs. 9, 41, and 43. Interestingly, we obtained only one mutation in the portion of the zinc modules that makes dimerization contacts [the D box (43)]. This change is unlikely to be responsible for the mutant phenotype of this

```
                <----------------------Zinc Modules----------------------------><--T Box--> <A Box>
          260        270        280        290        300        310        320        330        340        350
           |          |          |          |          |          |          |          |          |          |
          GSSGGSEGRCAVCGDNASCQHYGVRTCEGCKGFFKRTVQKSAKYICLANKDCPVDKRRRNRCQFCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKP
single    --L----D-Y--|----(6)Y---(5)KD|-S---C(3)------|-----|------W--|--|C(2)----------I---------D(7)---
          ---------|--|-------------|-S|---------------|-----|---------|--|H(2)--------------------S------
double    ---R-----|--|------------I|--|---------------|-----|---------|--|------------------------------
          ---------|--|-------------|-S|-----H---------|-----|---------|--|------------------------------
          ---------|--|-------------|--|---------------|---N-|---------|--|-----------------------S------
          ---------|--|-------------|--|---------------|-----|-----W---|--|----------------------W-------
          ---------|--|-------------|--|---------------|-----|---------|--|----------------------WW------
```

FIG. 2. NGFI-B DNA-binding domain mutations recovered from galactose-resistant, His$^+$ yeast. Amino acid sequence of the NGFI-B DNA-binding domain polypeptide is shown at top; numbers indicate amino acid position in the native NGFI-B protein (13). Lines beneath show amino acid substitutions recovered from galactose-resistant His$^+$ yeast, where " | " indicates a zinc-chelating cysteine and "-" indicates all other wild-type amino acids. All single-amino acid substitutions are shown on two lines toward the top, where a number in parentheses next to a substituted amino acid indicates the number of times it was recovered. All double substitutions are shown on lines at the bottom, where each line represents a unique double substitution.

```
  domains-                           <P Box>            <D Box>                       <--T Box--> <A Box>
   NGFI-B- GSSGGSEGRCAVCGDNASCQHYGVRTCEGCKGFFKRTVQKSAKYICLANKDCPVDKRRRNRCQFCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKP
  mutated-   **   * *          *    * **  *   *             *       **      *             *       ***
 residues
                                L                     L      H              L
 contacts-          Z  Z H  H  PP    ZHHZBBLL B       PDZD D DZD   D PPDZL ZPL  L    L
       GR- ATTGPPPKLCLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGMNLEARKTKKKIKGIQQATTGV
consensus-          C VC D  SG HYG  SC  CK FFRR       Y C     C VDK  R  C  CR  KC   GM
 sequence             I     T       T        K                  I
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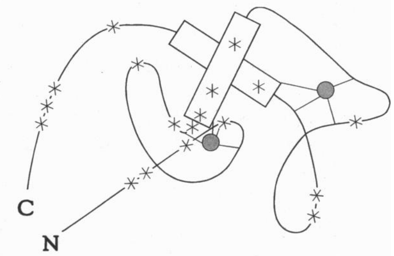

FIG. 4. Comparison of the NGFI-B DNA-binding domain mutational profile with known structural information. (*Upper*) Sequences of the NGFI-B and glucocorticoid receptor (GR) DNA-binding domains are shown. Stars under the NGFI-B sequence indicate the positions where mutations were recovered from galactose-resistant yeast. Above the GR sequence are letters indicating the contacts observed in a crystal of the GR DNA-binding domain (9), according to the following code: B, base contact; D, interpeptide dimerization contact; H, intrapeptide hydrogen bond; L, lipophilic pocket; P, phosphate backbone contact; Z, zinc-chelation. Below the GR sequence are amino acids that illustrate the consensus of the nuclear receptor superfamily, derived from the sequences of NGFI-B, SF-1, COUP, and the receptors for glucocorticoids, progesterone, androgens, estrogen, all-*trans* and 9-*cis*-retinoic acid, and thyroid hormone (13, 32–40). A double underline indicates the glucocorticoid receptor amino acids involved in α-helix formation. (*Lower*) A two-dimensional representation of the peptide backbone of a nuclear receptor DNA-binding domain monomer, patterned after results of NMR analysis of the estrogen receptor (41). Zinc atoms are indicated as circles, and α-helices are represented as rectangles. N and C indicate the amino and carboxyl termini, respectively. The region at the carboxyl terminus is shown in the extended conformation because no structural information is known for this area. Asterisks indicate positions of recovered mutations in the NGFI-B DNA-binding domain.

clone, because it was coupled with a second mutation in the A box (mutation Lys-306 → Asn, Gly-346 → Ser). This is consistent with the fact that dimerization is not used in NBRE binding (19). It must be noted, though, that our mutational profile is clearly incomplete (e.g., a mutation was recovered in only one of the eight zinc-chelating cysteines). The observed A box mutations are also consistent with previous observations because they cluster in the amino acids shown to define the sequence specificity 5′ to the half-site [aa 344–346, (19)]. Specifically, mutation of Arg-344 and Arg-345 eliminates functional groups that interact with the minor groove at these A·T bp.

We also recovered mutant NGFI-B polypeptides with substitutions of unanticipated amino acids. One of these was a conservative substitution just upstream of the A box in a region termed the T box (aa 336). This mutant did not bind DNA *in vitro* (Fig. 3), consistent with our hypothesis that the T box forms a secondary structure that stabilizes the A box–DNA interaction (19). Unanticipated mutations were also recovered in a region upstream of the zinc modules (aa 259, 260, and 264). One of these mutations produced a domain that was insoluble when expressed in bacteria (Gly-264 → Asp), but another was soluble and bound to the NBRE *in vitro* (Ser-259 → Leu). The significance of the recovery of these mutations is not clear at present, but it could reflect an interference with the stability of the LBG protein expressed in yeast. The genetic selection system described here provides a simple, efficient, and broadly applicable method for deriving mutational profiles of DNA-binding domains. The broad applicability of the technique stems from the fact that virtually any DNA-binding domain is likely to function as part of a transcriptional activator in yeast when linked to the strong Gal4p activating domain, due to the modular nature of transcription factors (1, 2, 44). Specifically, we have created functional L*X*G chimeras with three different types of DNA-binding domains: TFIII-A-type zinc fingers (12), nuclear receptor zinc modules (ref. 16; this work), and a B-zip domain (unpublished observations). The simplicity of our technique stems from the ease of yeast manipulation and the fact that the necessary plasmids are constructed and readily available. Because the lexA portion of the hybrid protein (aa 1–87) does not include a dimerization domain (45), mutations affecting both the DNA-binding and dimerization functions of the test protein should be obtained. It is only necessary to insert the test DNA-binding domain (or the entire test protein) into the *lexA–GAL4* plasmid and the relevant recognition sequence into the *GAL1* promoter. For proteins with no known recognition sequence, we have also described a yeast genetic selection system for identifying the binding sites of DNA-binding proteins that is readily applied with the plasmids used here (16). The efficiency of genetic selection of DNA-binding domain mutants is demonstrated by the large panels of functional missense mutations that have been rapidly generated (ref. 12; Fig. 2). The greatest drawback of the current technique in this regard is that it was necessary to apply chemical mutagenesis to the LBG plasmid, which limits the range of possible mutational outcomes. Other mutagenesis protocols, such as PCR-based mutagenesis (46) or growth of the L*X*G plasmid in a mutator strain of bacteria (47, 48) would likely yield a more complete mutational profile. In addition to its use in obtaining mutations altering DNA-binding domains, the method described here can also be used to analyze genetically protein surfaces that interact with other proteins. This analysis could be accomplished by coupling the approaches developed by Fields and colleagues (49, 50) for identifying interacting proteins with our technique.

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