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Mapping Critical Residues in Eukaryotic DNA-Binding Proteins: A Plasmid-Based Genetic Selection Strategy with Application to the Oct-2 POU Motif†

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ABSTRACT: Discrimination between allowed and disallowed amino acid substitutions provides a powerful method for analysis of protein structure and function. Site-directed mutagenesis allows specific hypotheses to be tested, but its systematic application to entire structural motifs is inefficient. This limitation may be overcome by genetic selection, which allows rapid scoring of thousands of randomly (or pseudorandomly) generated mutants. To facilitate structural dissection of DNA-binding proteins, we have designed two generally applicable bacterial selection systems: pPLUS selects *for* the ability of a protein to bind to a user-defined DNA sequence, whereas pMINUS selects *against* such binding. Complementary positive and negative selections allow rapid mapping of critical residues. To test and calibrate the systems, we have investigated the bipartite POU domain of the human B-cell-specific transcription factor Oct-2. (i) An invariant residue (Asn347) in the DNA-recognition helix of the POU-specific homeodomain (POU_{HD}) was substituted by each of the 19 other possible amino acids. The mutant proteins each exhibited decreased specific DNA binding as defined *in vivo* by genetic selection and *in vitro* by gel retardation; relative affinities correlate with phenotypes in the positive and negative selection systems. An essential role for Asn347 in wild-type POU_{HD}–DNA recognition is consistent with homologous Asn–adenine interactions in cocrystal structures of canonical homeodomains. (ii) Extension of pPLUS/pMINUS selection to the POU-specific subdomain (POU_S) is demonstrated by analysis of mutations in its putative helix-turn-helix (HTH) element. The altered DNA-binding properties of the mutant proteins *in vivo* and *in vitro* are in accord with a structural analogy between POU_S and the operator-binding domain of phage λ repressor. Together, our results provide a foundation for future biochemical studies of the structure and evolution of POU_{HD} and POU_S HTH sequences.

Protein–DNA recognition is mediated by families of related structural motifs (Pabo & Sauer, 1992). Insight into the structure and function of such motifs can be obtained by comparative biochemical studies of mutant proteins. DNA-binding surfaces may be mapped, for example, by analysis of mutations that alter DNA binding but permit native folding (Hecht et al., 1983). Specific interactions between amino acid side chains and DNA bases may be inferred from altered or relaxed sequence specificity (Ebright, 1991). Application of such methods to bacterial and yeast DNA-binding proteins has been facilitated by the availability of genetic selection strategies (Youderian et al., 1983; Ebright et al., 1984; Lehming et al., 1990; Mossing et al., 1991; Strubin & Struhl, 1992; Wilson et al., 1991). To extend this approach to higher organisms, we have developed two plasmid-based genetic selection systems for use in *Escherichia coli*. These systems select for or against the ability of a heterologous DNA-binding protein to recognize a user-defined sequence. The selection system is used to characterize the human Oct-2 POU domain, a B-cell-specific transcription factor important in the regulation of immunoglobulin gene expression [for review, see Verrijzer and Van der Vliet (1993)].

Each selection strategy is based on cotransformation of *E. coli* with two plasmids—one expressing the DNA-binding protein of interest and the second containing a repressible reporter gene. Reporter expression is regulated by a user-defined DNA sequence that is recognized by the DNA-binding protein (Figure 1). Repression of the reporter gene is detected by growth on media containing selective antibiotics. The selection plasmids are derived from pACYC184 (Chang & Cohen, 1978), which permits stable cotransformation with any pBR322-derived expression vector. Positive selection is achieved through repressible expression of a gene conferring streptomycin sensitivity (*str*): cells expressing the *str* product are sensitive to streptomycin. Repression of *str* expression by a functional "repressor" permits cell growth (Mossing et al., 1991). Negative selection is achieved through repressible expression of chloramphenicol acetyltransferase (CAT):[1] cells expressing CAT are resistant to chloramphenicol. Repression of CAT expression by a functional repressor inhibits cell growth. Together, the systems permit selection of mutations that confer wild-type, decreased, increased, or altered specificity. Analogous two-plasmid selection systems have been used in bacterial genetics (Lehming et al., 1990).

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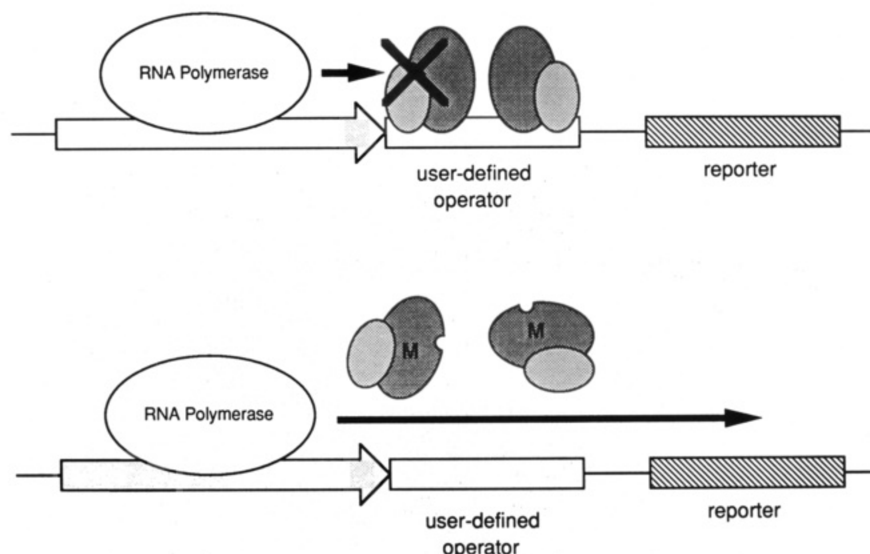

FIGURE 1: Schematic representation of selection strategy based on *lac* promoter and operator. In the presence of an appropriate DNA-binding activity, transcription is repressed (upper panel), yielding a selectable phenotype *via* the linked reported gene. Mutations in the DNA-binding protein (M) that impair function (lower panel) permit transcription, yielding a complementary selectable phenotype. The user-defined operator is shown to contain two binding sites as defined in Figure 2; the bipartite POU motif is depicted as two globular domains.

The utility of the selection systems in analysis of eukaryotic DNA-binding motifs is illustrated by analysis of mutations in human Oct-2 (Singh et al., 1986; Staudt et al., 1988; Clerc et al., 1988; Ko et al., 1988; Muller et al., 1988; Scheidereit et al., 1988). This application was motivated by the biological importance of its novel bipartite structure, designated the POU motif (Herr et al., 1988; Finney et al., 1988; Ingraham et al., 1988). This motif, conserved among a family of higher eukaryotic transcription factors, contains a divergent homeodomain (POU_{HD}) and a POU-specific subdomain (POU_S). Both subdomains are required for high-affinity DNA recognition (Sturm & Herr, 1988; Ingraham et al., 1990; Kristie & Sharp, 1990; Verrijzer et al., 1990a,b, 1992). Biochemical studies of the Oct-2 POU domain have shown that the two subdomains fold independently and are flexibly tethered by a proteolytically sensitive linker (Botfield et al., 1992). Both subdomains contain helix-turn-helix (HTH) recognition elements. Although the structure of the bipartite POU domain has not been determined, NMR studies demonstrate that POU_{HD} retains the secondary structure (Morita et al., 1993) and the tertiary structure (Sivaraja et al., 1994) of a canonical homeodomain (Qian et al., 1989); the related Oct-1 POU_S is analogous in structure to the DNA-binding domain of bacteriophage λ repressor (Assa-Munt et al., 1993; Dekker et al., 1993). The intact POU domain is therefore likely to combine these two classes of HTH recognition.

Here, we focus on quantitative calibration of the selection system by biochemical study of substitutions in the DNA-recognition helix of Oct-2 POU_{HD}. The site of substitution, Asn347, was chosen for its biological interest. Among homeodomains, this residue is invariant (Scott, 1989; Laughon, 1991). In crystal structures of Engrailed and MATα2 homeodomain–DNA complexes—but not in the solution structure of the Antennapedia complex (Otting et al., 1990)—the homologous Asn forms bidentate hydrogen bonds with an adenine (Kissinger et al., 1990; Wolberger et al., 1991). Phenotypes observed in the selection system are shown to reflect *in vitro* DNA-binding properties: each of the 19 substitutions significantly reduces the affinity of the Oct-2 POU domain for an octamer DNA site, demonstrating the importance of the native residue to DNA recognition. The selection system is also used to test aspects of the proposed functional analogy between the POU_S HTH and that of bacteriophage λ repressor (Assa-Munt et al., 1993; Dekker et al., 1993). The present studies provide a foundation for analysis of the interrelation of sequence, structure, and function in a novel eukaryotic DNA-binding motif.

[1] Abbreviations: P, POU-specific subdomain of Oct-2 (amino acids 194–270); BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast-protein liquid chromatography; GRA, gel-retardation assay; GST, glutathione-*S*-transferase of *Schistosoma japonica* (amino acids 1-226); GST-H, fusion protein of glutathione-*S*-transferase and the Oct-2 homeodomain; GST-P, fusion protein of glutathione-*S*-transferase and the Oct-2 POU-specific subdomain; GST-PI, fusion protein of glutathione-*S*-transferase and the Oct-2 POU-specific subdomain and intervening linker region; GST-PIH, fusion protein of glutathione-*S*-transferase and the intact Oct-2 POU motif; H, homeodomain of Oct-2 (amino acids 295–359); HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HTH, helix-turn-helix; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PI, POU-specific subdomain with intervening linker region of Oct-2 (amino acids 194–294); PIH, POU-specific subdomain, intervening linker region, and homeodomain of Oct-2 (amino acids 194–359); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; POU, acronym designating eukaryotic DNA-binding motif; POU_{HD}, POU-specific homeodomain; POU_S, POU-specific subdomain; rp-HPLC, reverse-phase high-performance liquid chromtography; SDS, sodium dodecyl sulfate. Amino acids are designated by standard three-letter or single-letter codes.

MATERIALS AND METHODS

Strains. E. coli strains HB101 (supE44 *hsd*S20(r_B^- m_B^-)*rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5-*mtl*-1) and DH5A (F^- φ80d Δ*lac*Z ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_κ^-,m_κ^+) supE44 λ^- *thi*-1 *gyr*A *rel*A1) were used as indicated. Competent cells were prepared, stored, and transformed by the method of Nishimura et al. (1990). Transformations with single plasmids (the pGEX2T expression vector or either selection plasmid; see below) yielded only the expected antibiotic resistances (Table 1). For selections with dual transformants (Table 2), the cells were exposed to 0.1 mM IPTG immediately after heat shock and prior to plating on selective media (see below).

Media and Buffers. LB medium consisted of 1% Bacto Tryptone, 0.5% yeast extract, and 1% NaCl. STG medium consisted of 2% Bacto Tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, and 50 mM KPO_4 (pH 7.4). When present,

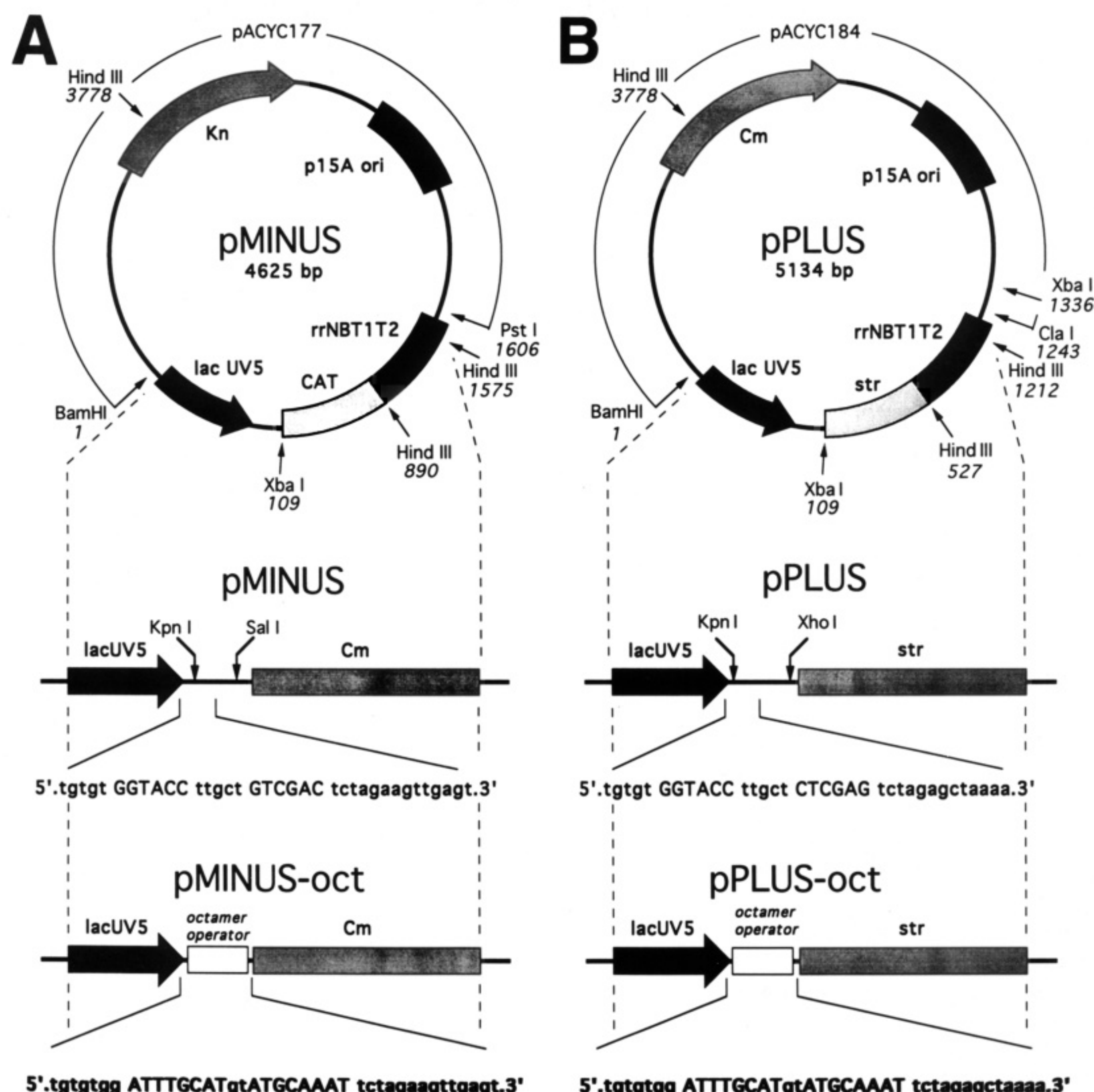

FIGURE 2: Structures of (A) pMINUS and (B) pPLUS. Cloning sites for insertion of user-defined operator sequences and sequences of head-to-head octamer sites (5-ATGCAAAT and complement) are shown in capital letters in bottom panels.

antibiotic concentrations were the following: ampicillin, 50 μg/mL; carbenicillin, 50 μg/mL; chloramphenicol, 20 μg/mL; kanamycin, 30 μg/mL. A range of streptomycin concentrations were used as described below and in the figure legends.

Construction of Expression Plasmids. The Oct-2 POU motif [pGEX-PIH; nucleotides 646-1143 as defined by Clerc et al. (1988), 166 aa], isolated POU-specific subdomain with intervening linker (pGEX-PI; nucleotides 646-948, 101 aa), and isolated homeodomain (pGEX-H; nucleotides 949–1143, 65 aa) were expressed as thrombin-cleavable fusion proteins as described (Botfield et al., 1992). Expression is directed by a *tac* promoter under the control of the *lac* repressor. Expression plasmids encode resistance to ampicillin (*amp*) and contain the pBR322-derived origin of replication *ori1*.

Construction of pMINUS-Octamer and pMINUS. pMINUS-octamer was constructed by ligation of three DNA cassettes with the 3020-bp *Bam*HI/*Pst*I fragment of pACYC177 (Chang & Cohen, 1978). This plasmid is a low-copy number *E. coli* vector containing the p15A origin of replication, which allows it to coexist with plasmids containing the *ColE1* origin (such as pBR322 and derivatives). The three DNA cassettes were (i) a 114-bp *Bam*HI/*Xba*I fragment encoding the *lacUV5* promoter and head-to-head octamer site (see Figure 2A), (ii) a 787-bp *Xba*I/*Hin*dIII fragment encoding the *chloramphenicol acetyltransferase* gene from

Table 1: Antibiotic Resistance and Sensitivity of Single Transformants[a]

plasmid	streptomycin	carbenicillin	kanamycin	chloramphenicol
host strain	R	S	S	S
pGEX	R	R	S	S
pMINUS	R	S	R	R
pPLUS	S	S	S	R

[a] All selections are performed in host strain HB101; S indicates sensitive, and R indicates resistant. HB101 is insensitive to streptomycin up to a concentration of 450 μg/mL; pGEX transformants are also resistant to carbenicillin at 50 μg/mL; the pMINUS transformants are resistant to kanamycin at 30 μg/mL and to chloramphenicol at 100 μg/mL; and the pPLUS transformants are resistant to chloramphenicol at 25 μg/mL and sensitive to streptomycin above 1.5 μg/mL.

pKK232-8 (Pharmacia) (Brosius, 1984), and (iii) a 722-bp *Hin*dIII/*Pst*I fragment containing the transcription terminator *rrnBT1T2* from pKK223-3 (Pharmacia) (Brosius & Holy, 1984). The promoter/octamer cassette was synthesized from complementary, overlapping synthetic oligonucleotides, which generated the following sequence: 5′-**ggatcc**gcgcaacgcaattaatgtgagttagctca-ctcattaggcaccccaggctttacactttatgcttccggctcgtatgatgtgtgg ATTTGCAT gt ATGCAAAT **tctaga**-3′. Restriction sites are indicated by boldface; the head-to-head octamer site is capitalized. Both the CAT and *rrnBT1T2* cassettes were harvested from the indicated sources using PCR to introduce the desired restriction sites. pMINUS was

constructed from pMINUS-octamer by replacing the *Bam*HI/*Xba*I promoter/octamer cassette with a 111-bp *Bam*HI/*Xba*I cassette containing a *lacUV5* promoter and unique *Kpn*I and *Sal*I restriction sites. The cassette was synthesized from complementary, overlapping synthetic oligonucleotides that generated the following sequence: 5′-ggatccgcgcaacgcaat-taatgtgagttagctcactcattaggcaccccaggct-ttacactttatgcttccg-gctcgtatgatgtgtgg **GGTACC** ttgct **GTCGAC** tctaga-3′. The *Kpn*I and *Sal*I restriction sites are shown in boldface. Inclusion of these unique sites in pMINUS permits insertion of a user-defined operator sequence. Because digestion with *Kpn*I results in a 3′-overhang and digestion with *Sal*I a 5′-overhang, a single-stranded synthetic oligonucleotide can be directly ligated in place following 5′-phosphorylation. Both pMINUS and pMINUS-octamer confer constitutive resistant to kanamycin and conditional resistance to chloramphenicol (see Results).

Construction of pPLUS-Octamer and pPLUS. pPLUS-octamer was constructed by ligation of three DNA cassettes with the 3892-bp *Bam*HI/*Cla*I fragment of pACYC184 (Chang & Cohen, 1978). As with pACYC177, this plasmid contains the p15A origin of replication, which alllows it to coexist with plasmids containing the *ColE1* origin. The three DNA cassettes were (i) a 114-bp *Bam*HI/*Xba*I fragment encoding the *lacUV5* promoter and head-to-head octamer site (Figure 2B), (ii) a 424-bp *Xba*I/*Hin*dIII fragment encoding the streptomycin-sensitivity gene from pNO1523 (Pharmacia) (Post et al., 1978), and (iii) a 722-bp *Hin*dIII/*Pst*I fragment containing transcription terminator *rrnBT1 T2* from pKK223-3 (Pharmacia) (Brosius & Holy, 1984). The promoter/octamer cassette was identical to that used in the construction of pMINUS-octamer (see above). Both the streptomycin-resistance cassette and the *rrnBT1 T2* cassette were harvested from the indicated sources using PCR to introduce the desired restriction sites. pPLUS was constructed from pPLUS-octamer by replacing the *Bam*HI/*Xba*I promoter/operator cassette with a 111-bp *Bam*HI/*Xba*I cassette containing a *lacUV5* promoter and unique *Kpn*I and *Xho*I restriction sequences. The cassette was synthesized from complementary, overlapping synthetic oligoncleotides that generated the following sequence: 5′-ggatccgcgcaacgcaattaatgtgagttagctcac-tcattaggcaccccaggcttt-acactttatgcttccggctcgtatgatgtgtgg **GG-TACC** ttgct **CTCGAG** tctaga-3′. The *Kpn*I and *Xho*I restriction sites, shown above in boldface, permit insertion of a user-defined operator sequence; following dual restriction, a single-stranded synthetic oligonucleotide can be directly ligated in place following 5′-phosphorylation. The same oligonucleotide can be used to introduce the operator sequence into both pPLUS and pMINUS. Both pPLUS and pPLUS-octamer confer constitutive resistance to chloramphenicol and conditional resistance to streptomycin (see Results).

Mutagenesis. The sequence of pGEX-PIH was randomized at Asn347 [numbering scheme of Clerc et al. (1988)] by site-directed mutagenesis to yield 19 mutant proteins representing the complete set of possible amino acid substitutions. Mutations were introduced by the phosphorothioate method, using the "oligonucleotide-directed mutagenesis kit" of Amersham, according to the instructions of the vendor. Fidelity of each construct was verified by direct DNA sequencing of the entire PIH sequence. In each case, only the desired mutation was present.

pMINUS Selection. To select for mutants with decreased specific DNA-binding activity, cotransformants were grown at 30 °C on LB plates containing kanamycin, carbenicillin, and chloramphenicol (50–100 μg/mL; see Results). Plates were incubated for 36-48 h. The cotransformants were also plated on kanamycin and carbenicillin to assess transformation efficiency.

pPLUS Selection. To select for mutants that retain specific DNA-binding activity, cotransformants were grown at 30 °C on LB plates containing chloramphenicol, carbenicillin, and streptomycin. The cotransformants were also plated on chloramphenicol and carbenicillin to assess transformation efficiency. The positive selection system was tested at streptomycin concentrations of 1–80 μg/mL; ordinarily, a streptomycin concentration of 2–5 μg/mL yielded selective growth of cells expressing an Oct-2 POU domain with retained specific DNA-binding activity. Selection plates were incubated for 72–96 h.

Protein Purification. Conditions for protein expression and purification were as previously described (Botfield et al., 1992). In brief, *E. coli* strain DH5α containing the appropriate expression plasmid was grown at 30 °C in STG medium with 50 μg/mL ampicillin to midlog phase, induced with 1 mM IPTG, grown for an additional 3 h, harvested by centrifugation, and lysed by sonication. The fusion protein was purified by glutathione-affinity chromatography and cleaved by thrombin. The Oct-2 fragment was isolated by reverse-phase HPLC (25-mm × 10-cm Waters DeltaPak C18 column), using a 10–70% acetonitrile gradient in 0.1% TFA as described (Botfield et al., 1992). The elution positions of the native and mutant proteins were similar. The purity of the protein was >98% as evaluated by SDS–PAGE. Protein concentrations were determined from calculated extinction coefficients at 280 nm (ϵ_{280} PIH: 16 807); native and mutant proteins were assumed to have identical extinction coefficients.

Gel-Retardation Assay. Specific DNA-binding activity was evaluated using a gel-retardation assay (Fried & Crothers, 1981). One strand of the duplex oligonucleotide (5′-GT<u>ATGCAAAT</u>GG-3′; the consensus octamer element is underlined) was end labeled with [γ-^{32}P]ATP (New England Nuclear) using T4 polynucleotide kinase (Boehringer-Mannheim, Ltd.) and annealed to the second strand of the duplex (5′-CC<u>ATTTGCAT</u>AC-3′), and unincorporated label was removed by gel filtration. Specific activity of the resulting probe was approximately 10^6 dpm nM^{-1}. Reactions (typically in 10 uL) contained 50 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, 0.1 mg/mL BSA, 0.1 mg/mL double-stranded poly(dI-dC) (Pharmacia cat. no. 27-7880), 1 nM labeled specific DNA, and the indicated protein. Reactions were incubated for 2–4 h at 4 °C, and bound versus unbound DNA resolved by gel electrophoresis at 4 °C as described (Botfield et al., 1992).

RESULTS

Our results are presented in three parts. In part I, a general bacterial selection system is described for analysis of sequence-specific DNA-binding proteins. Selections are designed for either loss or retention of specific DNA binding. In part II, phenotypes are calibrated by analysis of the Oct-2 POU domain, isolated POU-specific homeodomain (POU_{HD}), and isolated POU-specific subdomain (POU_S). In part III, this system is applied to a library of variant POU domains in which an invariant residue in the POU_{HD} recognition α-helix is randomized. The 19 mutant domains are each purified, and their octamer-binding properties *in vitro* are correlated with genetic phenotypes. The selection system is then used to characterize additional mutations on the surface of POU_S.

(I) Design of a Bacterial Selection System

Overview. Positive and negative selection schemes are illustrated in Figure 1; selection plasmids pPLUS and

pMINUS are shown in Figure 2. The plasmids contain the p15A origin of replication from pACYC-184 (Chang & Cohen, 1978) and thus permit stable cotransformation of host cells with pPLUS or pMINUS, and any expression plasmid containing an *oriI* (pBR322-derived) origin. A user-defined DNA-operator sequence (corresponding to the DNA-recognition sequence of the test protein) can be inserted into unique *Kpn*I and *Xho*I restriction sites (see Materials and Methods). pPLUS confers constitutive resistance to chloramphenicol and repressible sensitivity to streptomycin; pMINUS confers constitutive resistance to kanamycin and repressible resistance to chloramphenicol. Expression of a functional DNA-binding protein represses transcription of the reporter gene and thus permits or inhibits growth on media containing the appropriate antibiotic (streptomycin for pPLUS, chloramphenicol for pMINUS).

(i) Loss of Function. Selection for decreased specific DNA-binding activity is obtained by cotransformation with pMINUS. pMINUS encodes the enzyme chloramphenicol acetyltransferase (CAT) under the control of a *lac* promoter in which the *lac* operator has been replaced by a DNA-target site of the protein of interest. CAT expression is thus expected to be downregulated by formation of a heterologous protein–DNA complex near the start site of transcription. Selection is expected to be more stringent at higher concentrations of chloramphenicol or IPTG.

(ii) Retention of Function. Complementary selection for retention of specific DNA-binding activity is obtained by cotransformation with pPLUS. This plasmid encodes the variant ribosomal protein SRP, which confers sensitivity to streptomycin (Post et al., 1978; Mossing et al., 1991). SRP expression is under the transcriptional control of an analogously modified *lacUV5* promoter. Downregulation of SRP expression (and hence insensitivity to streptomycin) would be expected following formation of the heterologous protein–DNA complex. pPLUS constitutively expresses CAT as a selectable marker. The streptomycin selection used with pPLUS requires that the *E. coli* host strain be otherwise insensitive to streptomycin (as is strain HB101 used in the present study). Stringency of selection is expected to increase with increasing concentration of streptomycin and to decrease with increasing concentrations of IPTG.

Application to a Human DNA-Binding Motif. Positive and negative selection systems were constructed for the Oct-2 POU domain. pGEX expression plasmids encoding the intact POU domain, POU_S, and POU_{HD} have previously been described (Materials and Methods; Botfield et al., 1992). Octamer-specific pPLUS and pMINUS plasmids each contain an 18-bp tandem octamer site as shown in Figure 2 (bottom panels). This site contains head-to-head octamer elements separated by two base pairs; its length (8 + 2 + 8) is identical to that of the *lac* operator (18 base pairs) in the native *lac* promoter. Design of this synthetic octamer is based on the studies of LeBowitz et al. (1989), which demonstrated by DNase protection that such tandem sites exhibit cooperative and high-affinity Oct-2 binding. Gel-retardation assays of the isolated 18-mer (Figure 3) verify enhanced binding of Oct-2 POU domain to the head-to-head (lanes c and d) relative to head-to-tail (lanes a and b) octamer sites. In addition, the mobilities of the two 2:1 complexes differ; the increased mobility of 2:1 complex a suggests formation of a more compact protein–DNA complex. Such compaction is in accord with the known DNA-bending properties of POU domains (Verrijzer et al., 1991; Verrijzer & Van der Vliet, 1993) and may contribute to transcriptional repression in our constructs.

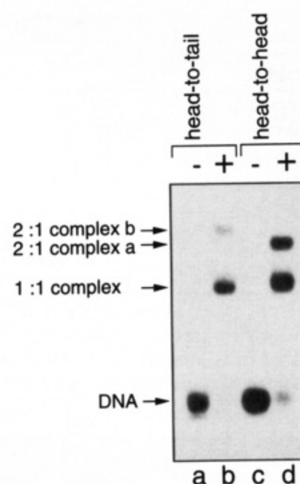

FIGURE 3: DNA-binding assay comparing head-to-tail (lanes a and b) and head-to-head (lanes c and d) octamer sites. The two probes each form 1:1 complexes of identical mobility but distinct 2:1 complexes (arrows at left). The sequence of the head-to-tail site is 5′-aattctgATGCAAATgtATGCAAATcatgCATG; the sequence of the head-to-head site is 5′-aattctgATTTGCATgtATGCAAATcatg (consensus octamer sites are shown in capital letters). At the top, + (or –) indicates the presence (or absence) of 500 nM wild-type Oct-2 POU domain in the assay.

(II) Calibration

A key parameter of a genetic selection system is its stringency: what range of biochemical activities will score as positive (pPLUS), and conversely, what loss of function is required to score as negative (pMINUS). The selection systems were initially tested by comparison of bacterial phenotypes in the presence of pGEX-PIH (expressing the intact POU domain), pGEX-PI (expressing POU_S), pGEX-H (expressing POU_{HD}), or a control pGEX vector expressing native glutathione-*S*-transferase. In Table 2 are summarized the resulting phenotypes of antibiotic resistance or sensitivity; representative plates are shown in Figure 4 (pPLUS) and 5 (pMINUS). Results obtained with the control pGEX plasmid are as predicted on the basis of the two individual plasmids (rows 1 and 2 of Table 2). This finding demonstrates that neither plasmid interferes with antibiotic resistance or sensitivity conferred by the other. A similar result is obtained with pGEX-PI (rows 3 and 4 of Table 2; panels A and D of Figures 4 and 5), consistent with the absent or weak affinity of POU_S for an octamer DNA site (Sturm & Herr, 1988; Kristie & Sharp, 1990; Botfield et al., 1992; Verrijzer et al, 1992). In contrast, cotransformation with pGEX-PIH yields distinct phenotypes (rows 5 and 6 of Table 2). Cotransformants with pMINUS are sensitive to chloramphenicol, presumably due to specific downregulation of CAT expression in *trans*. Similarly, pPLUS/pGEX-PIH cotransformants are insensitive to streptomycin, presumably due to specific downregulation of SRP. Cotransformation with pGEX-H yields phenotypes intermediate between those of pGEX-PI (inactive) and pGEX-PIH (active), in overall accord with their relative octamer affinities *in vitro* (Botfield et al., 1992).

Detailed responses of pGEX-PIH cotransformants depend on the order of transformation. The negative selection system is most stringent when bacteria are first transformed with the expression plasmid and then with the selection plasmid. Under these conditions, a chloramphenicol concentration of 20–25 μg/mL is sufficient to inhibit the cell growth, whereas a chloramphenicol concentration of at least 50 μg/mL is required to suppress the growth of cells cotransformed in the opposite order. An opposite effect should be seen in the positive selection system. We attribute these differences to effects of "pre-

Table 2: Phenotypes of Cotransformants with Wild-Type Oct-2 Fragments[a]

plasmid	streptomycin	carbenicillin	kanamycin	chloramphenicol
pGEX-2T/pMINUS-oct	R	R	R	R
pGEX-PIH/pMINUS-oct	R	R	R	**S**
pGEX-H/pMINUS-oct	R	R	R	R
pGEX-PI/pMINUS-oct	R	R	R	R
pGEX-2T/pPLUS-oct	S	R	S	R
pGEX-PIH/pPLUS-oct	**R**	R	S	R
pGEX-H/pPLUS-oct	**I**	R	S	R
pGEX-PI/pPLUS-oct	S	R	S	R

[a] All selections are performed in host strain HB101; S indicates sensitive, R indicates resistant, and I indicates intermediate. HB101 is insensitive to streptomycin up to a concentration of 450 μg/mL; pGEX transformants are also resistant to carbenicillin at 50 μg/mL; the pMINUS transformants are resistant to kanamycin at 30 μg/mL and to chloramphenicol at 100 μg/mL; and the pPLUS transformants are resistant to chloramphenicol at 25 μg/mL and sensitive to streptomycin above 1.5 μg/mL. Changes in antibiotic resistance or sensitivity due to *trans* regulation are shown in boldface.

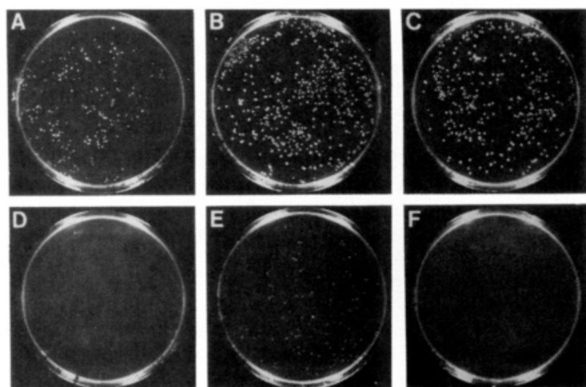

FIGURE 4: pPLUS selection plates. Upper panels (A–C) do not contain streptomycin and so do not select for specific DNA binding by the Oct-2 POU domain; lower panels (D–F) contain 10 μg/mL streptomycin as the selective agent. Plates A and D (left) were cotransformed with pPLUS and pGEX2T-PI; plates B and E (middle) were cotransformed with pPLUS and pGEX2T-PIH; plates C and F (right) were cotransformed with pPLUS and pGEX2T-(N347X)-PIH (equimolar mixture of 19 possible non-native substitutions). Under selective pressure, only cells expressing the wild-type Oct POU domain grow.

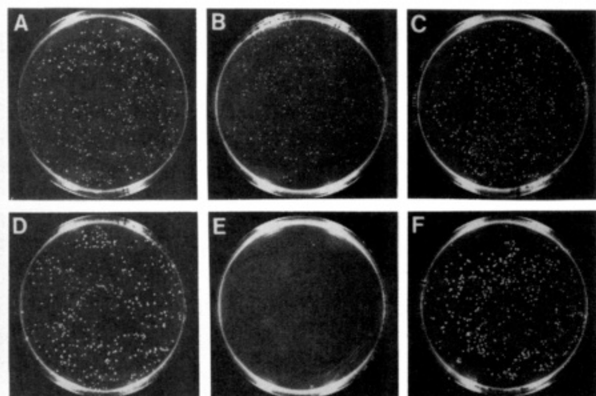

FIGURE 5: pMinus selection plates. Upper panels (A–C) do not contain chloramphenicol and so do not select against specific DNA binding by the Oct-2 POU domain; lower panels (D–F) contain 50 μg/mL chloramphenicol as the selective agent. Plates A and D (left) were cotransformed with pMINUS and pGEX2T-PI; plates B and E (middle) were cotransformed with pMINUS and pGEX2T-PIH; plates C and F (right) were cotransformed with pMINUS and a representative mutant (pGEX2T-(N347E)PIH). Under selective pressure, only cells expressing the wild-type Oct-2 POU domain fail to grow.

loading" the host with the DNA-binding protein when pGEX-PIH is first introduced. We also note that pGEX-PIH cotransformants do not grow on plates containing 0.1 mM IPTG even in the absence of the selective antibiotic, presumably due to a toxic effect of elevated GST-PIH concentrations in this context. Growth of cells containing pGEX (control vector), pGEX-PI, or pGEX-H is less affected. We further note that differences in growth rates in the pMINUS system in the presence of 0.1 mM IPTG may also be used to distinguish between missense and nonsense mutations. Expression of the mutant GST-PIH fusion proteins retards bacterial growth relative to expression of GST-PI, considered as a model nonsense mutation. This distinction is likely to be useful in screening colonies isolated by pMINUS selection following random mutagenesis.

(III) Analysis of Mutations in Oct-2 POU_{HD} and POU_S

The selection systems were used to test the importance of invariant residues in the POU_{HD} and POU_S HTH elements. Characterization of mutations in these subdomains is described in turn.

A. POU_{HD} Recognition Helix. A library of POU_{HD} point mutants was constructed by randomizing an invariant DNA-contact position in its putative recognition α-helix (Asn347). All 19 substitutions were tested *in vivo* and *in vitro*. In X-ray structures of canonical homeodomain–DNA complexes (Kissinger et al., 1990; Wolberger et al., 1991)—*but not in an NMR structure* (Otting et al., 1990)—the homologous Asn forms bidentate hydrogen bonds with an adenine in the major groove (Figure 6 A–C). In the NMR structure (Antennapedia), the side chain is not in direct contact with DNA and in fact appears not to be well ordered (Billeter et al., 1993). The inequivalent role of this side chain among structures of classical homeodomain complexes renders uncertain prediction of its importance in POU_{HD}.

(i) Phenotypes with pPLUS. Each mutant scores as inactive as illustrated in Figure 4. Wild-type GST-PIH confers insensitivity to streptomycin at a concentration of 80 μg/mL (panel E), whereas none of the 19 mutants grow at this or 50-fold lower streptomycin concentrations (panel F). Quantitative titration of the streptomycin concentration yielded no conditions under which phenotypes of the mutants could be distinguished from that of GST-PI. In contrast, wild-type GST-H yields an distinguishable phenotype relative to GST-PI and GST-PIH (not shown): at a low concentration of streptomycin (1.5 μg/mL), the pGEX-H cotransformants are insensitive, indicating at least partial retention of octamer binding. Unlike wild-type pGEX-PIH, pGEX-H cotransformants are sensitive to streptomycin at higher concentrations (>2 μg/mL).

(ii) Phenotypes with pMINUS. Quantitative titration of chloramphenicol concentration established that at least 20–25 μg/mL chloramphenicol is required to supress growth of wild-type pGEX-PIH while permitting growth of pGEX-PI. (At lower concentrations, differences in growth rates are observed, leading to distinguishable colony sizes on agar plates.) In the presence of 25 μg/mL chloramphenicol, all mutants grow as illustrated in Figure 5. The phenotype of a

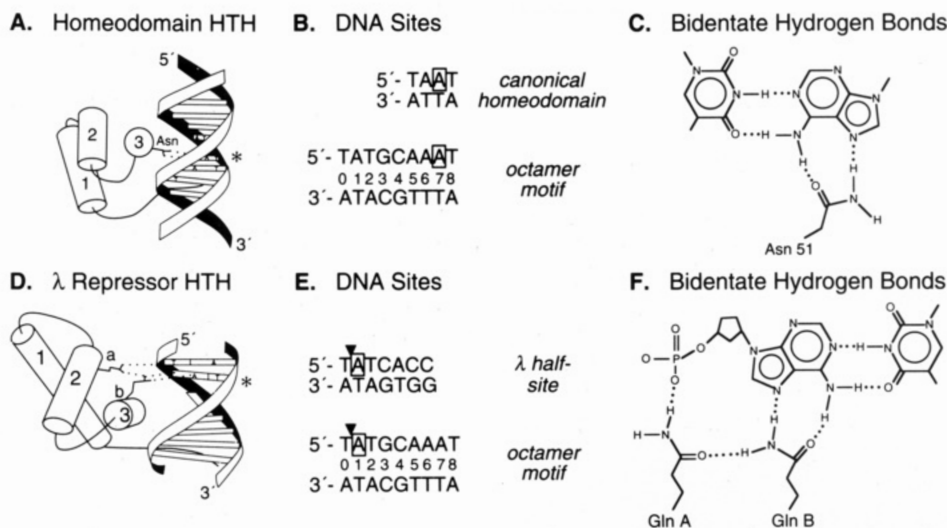

FIGURE 6: Overview of POU structural motifs. (A) Cylinder model of homeodomain–DNA complex showing putative Asn347–DNA contact (Asn51 in consensus homeodomain numbering); the panel is adapted from Kissinger et al. (1990). (B) Proposed alignment between canonical homeodomain core site (TAAT; upper sequence) and octamer motif (5'-ATGCAAAT; lower sequence); homologous adenine contacts are boxed. (C) Bidentate hydrogen bonds between adenine and Asn51 in cocrystal structures (Kissinger et al., 1990; Wolberger et al., 1991). (D) α-Helices 1-3 of the N-terminal domain of λ repressor (Jordan & Pabo, 1988) provide a model of POU_S (Assa-Munt et al., 1993; Dekker et al., 1993); invariant Gln side chains at the N-terminus of each HTH α-helix (a and b) are proposed to contact the DNA backbone and adenine base, respectively. For clarity, α-helix 4 is not shown. (E) Proposed alignment between λ half-operator site (upper sequence) and octamer motif (lower sequence); analogous phosphate and adenine contacts are indicated by arrowhead and box, respectively. (F) Network of side-chain-, phosphate-, and base-specific hydrogen bonds involving analogous Gln side chains in cocrystal structures of phage repressor and Cro proteins (in the λ repressor, Gln A and B are residues 33 and 44, respectively).

representative analogue (N347E) is shown in panel F. The individual substitutions differ, however, in extent of growth, providing an approximate indicator of residual specific DNA-binding activity *in vivo*. Those colonies which exhibit the least growth presumably retain the greatest activity; conversely, those that grow best presumably retain the least activity. By this criterion, the 19 substitutions may be grouped into three classes: (a) the poorest growth (most active protein) is exhibited by strains containing N347R and N347A mutations; (b) the best growth (least active protein) is exhibited by strains containing N347P, N347D, and N347E mutations; and (c) the remaining 14 mutations (including N347E; panel F) confer intermediate growth characteristics. Wild-type pGEX-H scores for loss of function with poor growth comparable to that of the N347R and N347A analogous. These qualitative differences are also seen on quantitative titration of chloramphenicol; i.e., the least active variant exhibits the greatest tolerance to increasing chloroamphenicol challenge.

Biochemical Studies of Mutant Proteins. Phenotypes of mutant proteins represent the integrated result of several possible effects *in vivo*, including changes in specific or nonspecific DNA-binding, altered specificity for other DNA-target sites, decreased protein stability, or level of expression. In the present case, relative stabilities and levels of expression under conditions of genetic selection were not determined. Upon IPTG induction, levels of overexpression were similar, suggesting that no gross differences occur. To exclude such confounding influences, however, it is important to correlate phenotypes with the biochemical properties of purified mutant proteins. To accomplish such calibration, each of the 19 mutant fusion proteins was purified and cleaved with thrombin to yield the isolated POU fragment as described (Botfield et al., 1992). Relative octamer affinities of the native and 19 mutant POU domains were evaluated using a gel mobility-shift assay as shown in Figure 7. In overall accord with the observed phenotypes, all mutant domains exhibit reduced affinity for the octamer site. As inferred from differences in growth rates in the pMINUS system, the residual specific DNA-binding activity was greatest for the N347R and N347A analogues (upper panel).

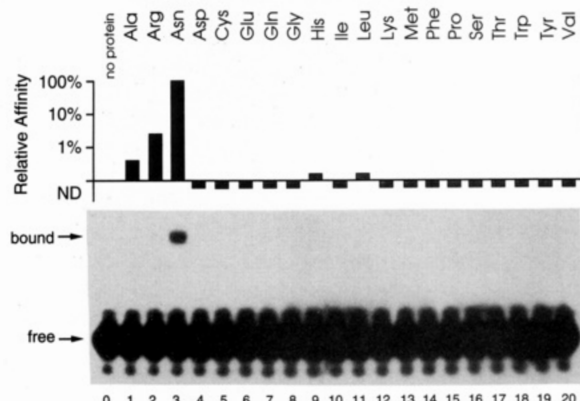

FIGURE 7: DNA-binding assay comparing the activities of wild-type and the 19 Asn347 mutants. (Lower panel) Gel-retardation autoradiogram demonstrates that only the wild-type side chain permits significant POU binding (lane 3). Relative affinities are shown by log in upper panel; quantitation was obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). ND indicates no detectable complex.

B. POU_S HTH Element. The POU-specific domain is analogous in structure to phage repressor and Cro proteins (Assa-Munt et al., 1993; Dekker et al., 1993), but its mode of DNA recognition has not been characterized. As a first step toward testing the predicted functional analogy between POU_S and the prokaryotic helix-turn-helix motif, surface mutations were introduced into POU_S and their effects on specific DNA binding were tested *in vivo* and *in vitro*. These mutations were also designed to test whether the selection systems may be used to characterize mutations in POU_S (as well as POU_{HD}) so as to permit genetic dissection of bipartite DNA recognition by the intact POU motif.

Gln → Ala substitutions were individually introduced into POU_S at positions 205, 212, 221, and 238. Gln205 and Gln212 are on the surface of α-helix 1 and are not conserved among POU_S sequences; Gln221 and Gln238 occur at the N-terminus of each HTH α-helix and are invariant among POU_S sequences (Assa-Munt et al., 1993; Dekker et al., 1993). In models developed by analogy to the crystal structure of a specific

Table 3: Phenotypes and *in Vitro* Activities of POU$_S$ Mutants[a]

mutation	pMINUS system	pPLUS system	gel-shift assay
wild-type	–	+	+
Q205A	–	+	+
Q212A	–	+	+
Q221A	+	–	–
Q238A	+	–	–

[a] pMINUS selections were performed on LB plates containing 50–75 μg/mL chloramphenicol (selectable marker), carbenicillin, and kanamycin; pPLUS selections were performed on LB plates containing 2.5 μg/mL streptomycin (selectable marker), carbenicillin, and chloramphenicol. In columns 1 and 2, symbols + and – designate growth or no growth; in column 3, the symbols refer to the presence or absence of specific DNA-binding activity. Mutations that decrease specific DNA-binding activity are indicated in boldface.

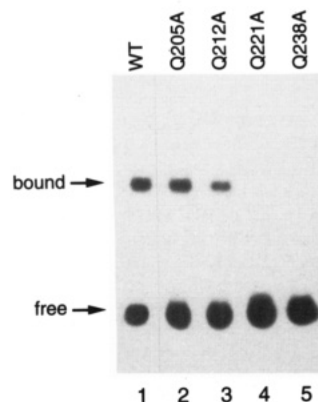

FIGURE 8: DNA-binding assay comparing the activities of wild-type (lane 1) and four Gln → Ala analogues in POU$_S$ (lanes 2–5). The octamer-binding activities of the Q221A and Q238A analogues are reduced by at least 20-fold.

complex between the N-terminal domain of λ repressor and an operator side (Jordan & Pabo, 1988), Gln221 and Gln238 are predicted to participate in a network of phosphate- and base-specific hydrogen bonds (Assa-Munt et al., 1993; Dekker et al., 1993) as illustrated in Figure 6 (D–F). These models also predict that the glutamine side chains in helix 1 are, in contrast, directed away from the DNA surface.

The four Gln → Ala mutant POU domains were individually tested in the pPLUS and pMINUS selection systems; the resulting phenotypes are given in Table 3. As predicted by analogy to λ repressor, the substitutions in the POU$_S$ HTH permit growth in the pMINUS system but not in the pPLUS system, implying loss of function in each case. The substitutions in α-helix 1 yield the opposite pattern of phenotypes, implying retention of function. The functional implications were verified by purifying the four mutant proteins and testing their specific DNA-binding activities by gel-retardation assay (Figure 8). Whereas the substitutions in α-helix 1 do not significantly affect octamer binding (lanes 2 and 3), the HTH substitutions reduce binding by at least 20-fold (lanes 4 and 5), in accord with analogous mutations in the HTH of phage repressors (Hecht et al., 1983; Hochschild & Ptashne, 1986; Wharton & Ptashne, 1987).

DISCUSSION

Genetic selection permits identification of residues critical for structure or function. Random cassette mutagenesis, for example, may be used to efficiently explore regions of sequence space (Reidhaar-Olson & Sauer, 1988) and has been applied to prokaryotic (Bowie & Sauer, 1989; Lim & Sauer, 1991; Mossing et al., 1991) and yeast (Strubin & Struhl, 1992; Cormack & Struhl, 1993) systems. Here, we have described a simple and general method to select for loss or gain of function in a general DNA-binding protein of interest. Complementary positive and negative selections in principle permit rapid isolation of critical residues from libraries of random mutations. Complementary selections also enable negative phenotypes to be distinguished from the general toxity of overexpressed proteins in *E. coli*, since growth is always expected in one system or the other. Because transcription factors commonly belong to structural motifs (Klug & Rhodes, 1987), such analysis may provide broad insight into conserved families of eukaryotic regulatory proteins. Its implementation in a bacterial system excludes studies of eukaryotic posttranslational modification, such as phosphorylation.

This study focuses on the human Oct-2 POU motif, which consists of an N-terminal domain (POU$_S$) and a C-terminal homeodomain (POU$_{HD}$). The POU motif is representative of a large family of eukaryotic transcription factors that regulate both housekeeping and developmental pathways. POU$_S$ and POU$_{HD}$ are of particular structural interest, as they contain distinct HTH motifs. Their dual use in DNA recognition provides a model for the cooperative assembly of protein–DNA complexes. The utility of the selection system is illustrated by mutagenesis of the POU$_S$ and POU$_{HD}$ HTH α-helices. In the POU$_{HD}$ recognition, α-helix Asn347 (Asn51 in canonical homeodomain numbering; Otting et al., 1988, 1990) is invariant. The cocrystal structures of Engrailed and MATα2 homeodomains (Kissinger et al., 1990; Wolberger et al., 1991) would predict that Asn347 contacts an adenine. This contact, which involves bidentate hydrogen bonds (Figure 6C), is adenine specific. Extension of the cocrystal models to POU$_{HD}$ is not clear, however, since the homologous Asn51 contact is not observed in the 3D-NMR structure of the Antennapedia complex (Otting et al., 1990; Billeter et al., 1993). To our knowledge, the importance of Asn at this position of a homeodomain-recognition α-helix has been tested by mutagenesis only in one case (Bicoid) (Hanes & Brent, 1991). Ala or Gln substitution reduces specific transcription of a reporter gene by 20- or 60-fold, respectively; changes in DNA affinity *in vitro* were not measured. Our results demonstrate that Asn347 in Oct-2 POU$_{HD}$ is required for octamer binding *in vivo* and *in vitro*: none of the other 19 native amino acids can substitute. Elsewhere, we show that the corresponding adenine base in the octamer DNA site (boxed in Figure 6B) is also required for function and is the site of relaxed specificity for certain Asn347 substitutions (Botfield et al., 1994). Accordingly, we propose that in the Oct-2 complex, Asn347 contacts an adenine, as in Engrailed and MATα2, at a corresponding position in the DNA site.

Bidentate hydrogen bonds between a side-chain carboxamide and an adenine are also predicted between an invariant Gln in the POU$_S$ HTH (Gln238). This prediction is motivated by a structural analogy between the Oct-1 POU$_S$ and the DNA-binding domains of bacteriophage repressor and Cro proteins (Dekker et al., 1993; Assa-Munt et al., 1993) (Figure 6D). In the latter, a specific network of hydrogen bonds is observed between Gln residues at the N-terminus of each HTH α-helix, the contacted adenine, and the 5′-phosphate as shown (Figure 6E). Our results do not test the details of this model. Nevertheless, it is striking that the homologous Gln residues in Oct-2 POU$_S$ are required for function, whereas Gln residues in α-helix 1 may be substituted by Ala without loss of octamer affinity. Mutations in the analogous Gln residues of the λ repressor yield corresponding phenotypes. In fact, the Q238A mutation results in relaxed sequence specificity at position 2 of the octamer (boxed in Figure 6E) (Jancso et al., 1994) in accord with studies of "loss of function" mutations in prokaryotic HTH proteins (Hochschild &

Ptashne, 1986; Wharton & Ptashne, 1987; Ebright, 1986; Zhang & Ebright, 1990). Further studies of mutations that confer altered or relaxed sequence specificity will enable POU_S- and POU_{HD}-recognition codes to be defined.

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