Pit-1 is a member of the POU family of transcription factors, which contain a bipartite DNA binding domain. The DNA binding domain consists of a POU-specific domain and a POU homeodomain. Each of the subdomains can interact with DNA independently, but both subdomains are required for high affinity, sequence-specific DNA binding. To examine the contributions of individual amino acids to the function of the DNA binding domain of Pit-1, we developed an approach involving random, in vitro mutagenesis followed by functional screening in Saccharomyces cerevisiae. Using this strategy, we identified a number of point mutations that altered the function of the Pit-1 DNA binding domain. Mutations that altered Pit-1 function were found in both the POU-specific and the POU homeodomain. Most of the mutations involve amino acid residues that are conserved in POU factors. One of the more frequent kinds of mutation affected residues located in the hydrophobic core of the protein. Another common mutation involved amino acids that are thought to make specific contacts with DNA. These mutations define a number of amino acid residues that are important for the function of the DNA binding domain of Pit-1.

Pit-1 is a tissue-specific transcription factor, which is important for the expression of the prolactin and growth hormone genes (1–6). Pit-1 appears to play a crucial role in both the pituitary (4, 7–10). Comparison of the amino acid sequence of Pit-1 with the sequence of the mammalian factors, Oct-1 and Oct-2 as well as the Caenorhabditis elegans gene product, unc-86, led to the identification of a gene family containing an approximately 150-amino acid conserved motif, which is referred to as the POU domain (11). Subsequently, more than 20 POU factors have been identified (12). The POU domain consists of two conserved subdomains, which are joined by a segment of variable length and sequence. The amino-terminal subdomain has been found only in POU proteins and is designated the POU-specific domain. The carboxy-terminal subdomain contains a region that is homologous to the homeodomain DNA binding motif and is designated the POU homeodomain. Preparation of truncated proteins and mutagenesis studies have demonstrated that the POU domain is a bipartite DNA binding motif and that both the POU-specific and the POU homeodomain contribute to high affinity, specific DNA binding (3, 13–17). The variable length and sequence of the region connecting the POU-specific and the POU homeodomain has led to the suggestion that this region functions as a flexible linker. This view has been reinforced by the finding that insertion of six alanine residues into the connecting link of Oct-1 has no effect on DNA binding (13). Pit-1 can bind to DNA as either a monomer or a dimer, with the dimer forming cooperatively (3). Cross-linking studies have suggested that Pit-1 is probably a monomer in solution (3). On the other hand, protein interaction assays have indicated that Pit-1 may be able to form a dimer in solution (18).

The structure of the Oct-1 POU-specific domain has been determined by NMR spectroscopy (19, 20). The POU-specific domain of Oct-1 consists of four α-helices surrounding a hydrophobic core and is organized in a structure that is similar to the DNA binding domains of bacteriophage λ, 434 repressor, and 434 cro. Recently, the crystal structure of the POU domain of Oct-1 bound to DNA has been resolved (21). This crystal structure reveals that the POU-specific domain and the POU homeodomain contact separate sites on different sides of the helix. The structure of the POU homeodomain demonstrates similar features and DNA contacts as have been determined for other homeodomains. Interestingly, there are no apparent protein-protein contacts between the POU-specific and POU homeodomains. The structure of the linker region could not be resolved in the crystal structure consistent with the view that this region may be a disordered, flexible part of the protein.

Although a substantial amount of information is available concerning the structure of Pit-1 and other POU factors, relatively little is known concerning the contribution of individual amino acids to maintaining the structure and function of the POU homeodomain. To study the contributions of individual amino acid residues to the structure and function of Pit-1, it would be useful to develop a method to screen for alterations in Pit-1 function. It has been demonstrated that Pit-1, like many mammalian transcription factors, can activate gene expression in Saccharomyces cerevisiae (22). The ability of Pit-1 to activate transcription in yeast suggests that genetic selection techniques could be used to identify mutations that altered the function of Pit-1. To identify mutations in the Pit-1 DNA binding domain, we coupled in vitro mutagenesis and phenotypic screening in yeast. This approach has permitted the identification of a number of point mutations, which alter the ability of the Pit-1 POU domain to bind to DNA.

MATERIALS AND METHODS

Preparation of a GCN4-Pit-1 Yeast Expression Vector—A fusion gene was prepared in which the DNA binding domain of Pit-1 was linked to the transcriptional activation domain of GCN4. To construct the expression vector, the Pit-1 DNA binding domain coding sequence (amino acid residues 117–291) was amplified by the polymerase chain reaction using an upstream primer that created a KpnI site and permitted

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To whom correspondence should be addressed: Dept. of Cell and Developmental Biology, L215, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97201.
in-frame fusion to a KpnI site at Pro-152 of GCN4. The upstream primer also created a SpeI site, which overlapped the coding sequence for Leu-117 of Pit-1 without changing the amino acid sequence. This SpeI site facilitated reconstruction of full-length Pit-1 for subsequent studies in mammalian cells. The amplified fragment was subcloned, and the correct coding sequence was confirmed by nucleotide sequence analysis. The DNA binding domain of Pit-1 was then used to replace the DNA binding domain of GCN4 in the yeast expression vector YCP88-GCN4, which was a gift of Dr. Kevin Struhl (23). The carboxyl-terminal region of GCN4, which contains the DNA binding domain of GCN4, was replaced with a KpnI-SstI fragment containing the DNA binding domain of Pit-1 to create YCP88-GCN4-Pit-1 (Fig. 1). An indicator plasmid, containing 4 tandem binding sites for Pit-1 upstream of a minimal CYC1 promoter linked to the lacZ reporter gene, has been described previously (22).

Chemical Mutagenesis of the Pit-1 DNA Binding Domain—The YCP88-GCN4-Pit-1 plasmid was treated with either nitrous acid or formic acid essentially as described (24). For these studies, 20 μg of plasmid DNA was dissolved in 100 μl of 250 mM sodium acetate (pH 4.3) and 1 mM sodium nitrite. For formic acid treatment, 20 μg of DNA was dissolved in 100 μl of 45% formic acid. The samples were incubated at room temperature for varying times (5-40 min), and then 10 μl aliquots were removed and reactions were stopped by adding 30 μl of 10 MTris (pH 7.4), 1 mM EDTA, 40 μl of 2.5 M sodium acetate (pH 7.0), and 2 μg of transfer RNA followed by 0.2 ml of ethanol. The precipitated DNA was washed by centrifugation, washed in 70% ethanol, and finally resuspended in 400 μl of water. The DNA binding domain of Pit-1 was isolated from the mutagenized DNA by polymerase chain amplification using the primers described above. The amplified DNA was then inserted into the YCP88-GCN4 vector as described above. The ligation mixture was transformed into Escherichia coli, and plasmid DNA was prepared from a pool of approximately 20,000 bacterial transformants.

Yeast Screen for Pit-1 Mutants—S. cerevisiae strain 1783 (ura3-52, trp1-1, his4, can1) was obtained from Dr. David Levin (The Johns Hopkins University, Baltimore). Cultures were propagated in standard yeast media. Yeast cells were transformed as described (25). Yeast strains were first transformed with the indicator plasmid pCS10–4X1P, containing 4 tandem binding sites for Pit-1 upstream of a minimal promoter, which is linked to lacZ gene (26). This strain was then transformed with the pool of mutagenized YCP88-GCN4-Pit-1 DNA, which carries a Ura3 gene. Transformants were selected on minimal medium deficient for uracil and leucine. Colonies from both wild type and mutagenized DNA transformants were picked randomly and streaked on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside. White and dark blue colonies were picked for further analysis.

Recovery of Plasmids from Yeast and DNA Sequence Analysis—A crude preparation of yeast plasmid DNA was isolated as described (26). This DNA was then used to transform E. coli strain MC1066 (pyrF, leuB, trpC, thi-1) by electrotransformation (27). The yeast plasmid DNA mixture contains both the high copy number reporter plasmid, pCS10–4X1P, and the low copy number expression plasmid, YCp88-GCN4-Pit-1. To select for the Pit-1 expression vector, the Ura3 gene of the expression vector was used to complement the pyrF mutation of strain MC1066. The nucleotide sequence of the Pit-1 coding sequence was determined by the dideoxy-chain termination method (28).

Analysis of DNA Binding by Mutant Pit-1 Proteins—Purified Ycp88-GCN4-Pit-1 DNA was used as a template for in vitro transcription and translation to produce [3H]leucine-labeled GCN4-Pit-1 fusion using an SP6 transcription/translation reaction mixture (Promega). To determine the relative synthesis of individual proteins, incorporation of [3H]leucine was quantitated. For this assay, 5-μl aliquots of the transcription/translation reaction mixture were combined with 1 μl of freshly prepared 0.25 M hydroxyl peroxide, 0.1 M NaOH, and 1 μl of 10% trichloroacetic acid. After incubation for 10 min on ice, precipitates were collected on glass fiber filters by vacuum filtration. The filters were dried, and radioactivity was determined by liquid scintillation counting. A gel mobility shift assay was used to test the DNA binding activity of the GCN4-Pit-1 fusion proteins. DNA binding reactions contained 100,000 cpm of 32P-labeled DNA probe, 1 μg of salmon sperm DNA, 10 μM Tris, pH 7.5, 5% glycerol, 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol in a total volume of 25 μl. The DNA probe consists of the sequence CTGGGGATATATTTATATCATGAGACTGG, representing the most proximal Pit-1 binding site of the rat prolactin gene designated the 1P site (29) with the addition of Aval sites at the termini. Each binding reaction contained a constant amount of GCN4-Pit-1 fusion protein (250 ng) and 1 μl of 32P-labeled DNA probe. To adjust the content of radiolabeled GCN4-Pit-1 protein, unprogrammed cell lysate was added to reactions. Binding reactions were incubated for 20 min at room temperature, and DNA:protein complexes were resolved by non-denaturing gel electrophoresis as described previously (30). The DNA binding activities of the mutant and the wild type Pit-1 chimera were determined using a Molecular Dynamics PhosphorImager to quantitate protein:DNA complexes and free DNA.

Analysis of Pit-1 Mutants in Mammalian Cells—For studies in mammalian cells, the Pit-1 DNA binding domains were reassembled with the Pit-1 transcriptional activation domain. For this purpose, an SpeI site was placed at Leu-117 of the Pit-1 coding sequence in a manner that did not change the predicted amino acid sequence. The mutant Pit-1 DNA binding domain as an SpeI-SstI fragment was used to replace the wild-type Pit-1 DNA binding domain sequence in an Rous sarcoma virus-luciferase reporter vector (26). For transfection studies, HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum. For transfection by electroporation, an 0.5-ml suspension of cells in Dulbecco’s phosphate-buffered saline was combined with 0.5 μg of Rous sarcoma virus-luciferase expression vector and 5 μg of a reporter gene containing 7 copies of a Pit-1 binding site upstream of a minimal promoter, which is linked to luciferase coding sequence in pUC-luc-link (31). The cells received a single electrical pulse at 220 V delivered from a total capacitance of 960 microfarads and were immediately placed in serum-containing medium. The cells were harvested 24 h after transfection, and luciferase activity was determined as described elsewhere (32).

RESULTS

To develop a screen for mutations that alter the function of the DNA binding domain of Pit-1, we took advantage of the fact that Pit-1 has been shown to activate transcription in yeast (22). To identify mutations in the POU domain that either decrease or increase function, we employed random mutagenesis coupled with a genetic screen in yeast. Although Pit-1 can activate transcription in yeast, it is a relatively weak activator (22). In an effort to enhance transcriptional activation and to improve the sensitivity of the screens, we replaced the amino-terminal transactivation domain of Pit-1 with the transactivation domain of the yeast transcription factor GCN4. This was accomplished by insertion of the POA domain of Pit-1 into a yeast expression vector for GCN4 so that the POA domain of Pit-1 replaced the DNA binding domain of GCN4 (Fig. 1). Because we have utilized a GCN4-Pit-1 fusion for this analysis, it is unlikely that we would detect any mutations that affect interactions between the DNA binding domain and the transcriptional activation domain. Thus, the screen is most likely to identify mutations that simply modify the DNA binding activity of the GCN4-Pit-1 fusion protein. The Ycp88-GCN4-Pit-1 expression vector was introduced into yeast cells with the pCS10–4X1P reporter gene, which contains a lacZ gene linked to four copies of Pit-1 binding site (22). The GCN4-Pit-1 fusion protein was found to activate the reporter gene about 3-fold better than the wild-type Pit-1. The GCN4-Pit-1 fusion was used for all of the yeast mutagenesis studies. The Pit-1 POA domain was mutagenized in vitro with nitrous acid or formic acid and then inserted into an unmutagenized GCN4 expression vector. This should generate a pool of mutagenized plasmids bearing mutations only in the coding region of the Pit-1 POA domain. The mutagenized plasmids were transformed into a yeast strain containing the indicator plasmid, and mutants were identified by screening transformants for expression of the lacZ indicator gene. Using this screen, 170 yeast clones that exhibited altered β-galactosidase activities were isolated. The nature and location of the mutation was then determined by DNA sequence analysis. A number of the clones contained frameshifts or termination codons, and these clones were not analyzed further. Several of the clones contained multiple point mutations, and these were also not analyzed further. Sequence analysis led to the identification of 42 different point mutations within the Pit-1 DNA binding domain, which resulted in altered Pit-1 function in yeast (Table 1 and Fig. 2). Many of the point mutations were found more than once, and several mu-
tions were found three or four times, suggesting that we are approaching saturation with this screen. To designate the location and nature of mutations, the wild-type residue is listed using the single letter code followed by the position of the residue and then the predicted amino acid present in the mutant. Thus, the Y148D mutation indicates the substitution of Tyr-148 with Asp. Two of the mutations increased Pit-1 function in yeast. The other mutations decreased Pit-1 function. These mutations are distributed throughout the Pit-1 DNA binding domain. Each of the mutations was tested for the ability to bind DNA in vitro (Table I and Fig. 3). The mutant DNA binding domains from several of the clones were also used to construct mammalian expression vectors for Pit-1 and tested by transient transfection of HeLa cells (Fig. 4).

As the mutations were targeted to the DNA binding domain of Pit-1, it was expected that the altered transcriptional activity of the mutants would likely be due to changes in DNA binding activity. To test the DNA binding activity of the mutants, the GCN4-Pit-1 expression vectors were recovered from yeast and used as templates for in vitro synthesis of the GCN4-Pit-1 fusion protein. The DNA binding activity of the chimeric protein was assessed using a gel mobility shift assay with equal amounts of the cell-free synthesized protein in each reaction. A representative mobility shift assay is shown in Fig. 3, and the DNA binding activity of all of the mutants is summarized in Table I. Most of the mutations that reduced transcriptional activation in yeast rather severely inhibited DNA binding activity. This screen is probably biased toward isolation of mutations that substantially reduce DNA binding activity. For a few of the mutations, partial inhibition of DNA binding activity was observed (T149A, E230K, R246P, R268G). A number of the mutations that had decreased DNA binding activity in vitro were used to prepare mammalian expression vectors and tested for transcriptional activation in HeLa cells (Fig. 4). As expected, all of these mutations substantially decreased the ability of Pit-1 to activate transcription in mammalian cells.

Two mutations were found to modestly increase reporter gene expression in yeast. The two activating mutations are located rather close to each other, but within different subdomains. The E212K mutation is located at the extreme carboxyl terminus of the linker, and the T220A mutation is located in the amino-terminal arm of the homeodomain. Thr-220 has previously been shown to be phosphorylated by the cAMP-dependent protein kinase, and phosphorylation of this site alters DNA binding activity (33–35). This site may be phosphorylated in yeast, and the T220A mutation would block phosphorylation of the site. The T220A and E212K mutations were not found to increase DNA binding activity in vitro or transcriptional activation in mammalian cells (data not shown). Thus, it is not clear how these mutations enhance reporter gene activation in yeast.

**DISCUSSION**

By using a strategy involving random, in vitro chemical mutagenesis and phenotypic screening in yeast, we have identified a series of point mutations that alter the function of the Pit-1 DNA binding domain (POU domain). All of the point mutations that result in decreased transcriptional activation in yeast also have decreased DNA binding activity, and these mutations result in decreased transcriptional activation in mammalian cells. Point mutations that decrease transcriptional activation were found in both the POU-specific domain and POU homeodomain. Most of the mutations occur in residues that are highly conserved among POU proteins, and this conservation likely reflects the contribution of these residues to the formation of major structural motifs of the DNA binding domain. One of the residues identified in this screen, Trp-261, has previously been shown to be crucial for Pit-1 function (7). Analysis of mutations that occur at the dwarf locus of mice has shown that a point mutation in the codon for Trp-261 occurs in the Snell dwarf mouse, and the inability of the mutant Pit-1 to bind DNA and stimulate transcription apparently accounts for the loss of expression of the prolactin, growth hormone, and thyroid-stimulating hormone genes in these mice. Two other point mutations that disrupt Pit-1 function have been detected by analysis of DNA from patients with pituitary deficiencies. These mutations, A158P (10) and R271W (9) clearly disrupt Pit-1 function but have little or no effect on DNA binding activity. Presumably, these mutations interfere with protein–protein interactions that are important for transcriptional activation in mammalian cells. The fact that these mutations do not affect DNA binding probably accounts for our failure to identify the mutations in our screen. The use of a GCN4-Pit-1 fusion likely biases the screen to detect mutations that simply alter DNA binding activity.

A number of previous studies have demonstrated that both the POU-specific domain and POU homeodomain are involved in high affinity, sequence-specific DNA binding (3, 13, 15, 36). It has been shown that both the POU-specific and the POU homeodomain are each able to interact with DNA (16, 37, 38). Thus, high affinity binding by the complete DNA binding domain of Pit-1 factors appears to involve the interaction of each subdomain with specific DNA sites. The finding that mutations in both the POU-specific and the POU homeodomain are able to
As structural information has recently been obtained for both the POU-specific domain and POU homeodomain, it is possible to evaluate the mutations that we have identified relative to the structural features of the protein. The availability of both structural and mutation data provides increased insight into the significance of particular structural features. The structure of the POU-specific domain of Oct-1 has been determined by both multidimensional nuclear magnetic resonance spectroscopy (19, 20) and by crystallography (21). The overall structure and docking of the POU homeodomain are similar to those observed in other homeodomain-DNA complexes. The first nine residues of the homeodomain form an extended amino-terminal arm that fits into the minor groove of the DNA.

The crystal structure of Oct-1 revealed the presence of a network of hydrogen-bonded amino acids within the POU-specific domain, which appeared to play an important role in contacting DNA and stabilizing the structure of the Oct-1 (21). Gln-44, which is the first residue of helix 3 of the Oct-1 POU-specific domain, was found to make contacts with adenine in the major groove, and this interaction is stabilized by hydrogen bonds to Gln-27, the first residue of helix 2. Gln-27 also hydrogen bonds to Glu-51 (near the carboxyl-terminal end of helix 3), which in turn is hydrogen bonded to Arg-20 (helix 1). Arg-20 also makes phosphate contacts with DNA. All of the corresponding amino acids of Pit-1 were isolated as mutations (R143G, Q150R, Q167R, E174G), which decrease function of Pit-1 in our screen. Therefore, our random mutagenesis and screen has confirmed the functional importance of this hydrogen-bonded network within the POU-specific domain.

As with the POU-specific domain, mutations in the POU homeodomain that decrease Pit-1 function include several different classes of mutations. For instance, some of the mutations replace a hydrophobic residue, it is not clear if the effects of the mutations are due to disruption of the hydrophobic core as opposed to disruption of a helical structure. Several of the mutations in the Pit-1 POU-specific domain involve residues that make specific base contacts or phosphate contacts with DNA (R143G, T149A, Q150R, Q167R, T169G, C171R, K185E). Two of these, T149A and T169G, might be considered to be very conservative replacements. However, the fact that these threonine residues make phosphate contacts that would be disrupted by the mutations provides a possible explanation for the effects on DNA binding. The conservative nature of these replacements likely results in minimal perturbations of the POU domain structure and argues that changes in a single phosphate contact may substantially reduce DNA binding activity.

The present, non-directed selection approach has permitted the identification of sites in which proline substitutions interfere with function. However, as these mutations replace a hydrophobic residue, it is not clear if the effects of the mutations are due to disruption of the hydrophobic core as opposed to disruption of a helical structure. Several of the mutations in the Pit-1 POU-specific domain involve residues that make specific base contacts or phosphate contacts with DNA (R143G, T149A, Q150R, Q167R, T169G, C171R, K185E). Two of these, T149A and T169G, might be considered to be very conservative replacements. However, the fact that these threonine residues make phosphate contacts that would be disrupted by the mutations provides a possible explanation for the effects on DNA binding. The conservative nature of these replacements likely results in minimal perturbations of the POU domain structure and argues that changes in a single phosphate contact may substantially reduce DNA binding activity.

As with the POU-specific domain, mutations in the POU homeodomain that decrease Pit-1 function include several different classes of mutations. For instance, some of the mutations likely disrupt the hydrophobic interior of the homeodomain (L229S, F233S, W261R, F262S) or involve residues that make base or phosphate contacts with DNA (K226E, K238E, V260E, W260A, N264D, N264S, R266G). Glu-230 is conserved in most homeodomain proteins (39). We found that substitution of Glu-230 with lysine (E230K) reduced the DNA binding activity of Pit-1 to 35% of wild type. Interestingly, lysine is substantially decrease DNA binding activity of Pit-1 is consistent with the important contribution of each of these subdomains to the functional properties of the protein.
Mutation of the DNA Binding Domain of Pit-1

Fig. 2. Summary of point mutations that alter the function of the Pit-1 DNA binding domain. The amino acid sequence of the DNA binding domain of Pit-1 is shown using the single letter amino acid code. Arrows indicate amino acid replacements predicted by specific mutations. Two mutations that increase reporter gene activity in yeast are indicated by asterisks; the other mutations decrease reporter gene activity in yeast. The predicted positions of major helices are indicated below the sequence based on the solution structure of the POU-specific domain of Oct-1 (19, 20) and the crystal structure of the DNA binding domain of Oct-1 (21).

![Diagram of amino acid replacements](image)

Fig. 3. Mobility shift analysis of the interaction of Pit-1 mutants with a DNA binding site from the rat prolactin gene. GCN4-Pit-1 fusion protein was prepared by in vitro transcription and translation, and the products were incubated with a 32P-labeled DNA representing the 1P Pit-1 binding site, the most proximal Pit-1 binding site from the rat prolactin gene (29). Equal amounts of GCN4-Pit-1 fusion protein were added to each binding reaction based on incorporation of [3H]leucine into the products. The amount of translation mixture added to the binding reaction was maintained at a constant amount by addition of the unprogrammed reaction mixture. Protein interactions with DNA were resolved by electrophoresis on a nondenaturing polyacrylamide gel.

In summary, we have used a genetic screen to identify mutations that alter the function of the Pit-1 DNA binding domain. These studies have identified a number of amino acid replacements predicted by specific mutations. Two mutations that increase reporter gene activity in yeast are indicated by asterisks; the other mutations decrease reporter gene activity in yeast. The predicted positions of major helices are indicated below the sequence based on the solution structure of the POU-specific domain of Oct-1 (19, 20) and the crystal structure of the DNA binding domain of Oct-1 (21).

Fig. 4. Transcription activation by Pit-1 mutants in mammalian cells. HeLa cells were transfected with 10 µg of an indicator plasmid, which contains seven copies of 1P Pit-1 binding site from the rat prolactin gene upstream of the luciferase gene and 0.5 µg of either a control plasmid (Bluescript) or expression vectors for wild-type or mutant Pit-1. Cells were collected 24 h after transfection, and luciferase activity was determined. Values are means ± S.E. for three separate transfections.

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