A two-base change in a POU factor-binding site switches pituitary-specific to lymphoid-specific gene expression

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The structurally related POU homeo domain proteins Pit-1 and Oct-2 activate pituitary- and lymphoid-specific transcription, respectively, by binding to similar AT-rich motifs in their target genes. In this study we identify bases critical for recognition and activation by Pit-1 and examine how small differences in Pit-1- and Oct-2-binding sites can impart differential transcriptional responses in pituitary and B-lymphoid cells. Scanning mutagenesis of Pit-1 response elements in both the rat prolactin and growth hormone genes reveals a critical binding motif recognized in an identical manner by the native Pit-1 protein and cloned Pit-1 gene product. This motif, ${}^A_TTAT^T_CCAT$, differs by only two bases from the octamer element, $ATTGTCAT$, required for Oct-2-dependent activation of immunoglobulin genes. Cross recognition of Pit-1 and Oct-2 sites by both factors can be demonstrated in competitive binding assays, in which an oligomeric Pit-1 site from the prolactin gene is converted to an Oct-2 site by a double point mutation. In contrast to the binding data, no cross activation of transcription is detectable in cultured cell lines. When inserted immediately 5' to a prolactin TATA box, the wild-type prolactin element enhances transcription strongly in pituitary cells but is inactive in B cells, whereas the octamer variant of the prolactin site activates expression in B cells but is silent in pituitary lines. Both elements are nonfunctional in heterologous cell lines that lack Pit-1 and Oct-2. The selective *trans*-activation by two POU factors that recognize related response elements may thus be comparable to the differential activation by steroid and thyroid hormone receptors of closely related response elements.

[Key Words: Pit-1; Oct-2; POU homeo domain; pituitary]

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Regulation of the network of genes that controls the development and physiology of eukaryotes involves the selective interactions of *trans*-acting proteins with specific genomic response elements. In mammals, the polypeptide hormone genes of the anterior pituitary have provided a developmental system in which to examine mechanisms of transcriptional control. Sequence- and cell-specific protein-DNA interactions have been observed in both proximal (Elsholtz et al. 1986; Cao et al. 1987; Gutierrez-Hartmann et al. 1987; Nelson et al. 1988; Schuster et al. 1988) and distal (Nelson et al. 1986, 1988) regulatory elements of the rat prolactin gene, which is expressed exclusively in this tissue. Correct targeting to the pituitary in transgenic mice (Crenshaw et al. 1989) and restricted expression of the prolactin gene in cultured lactotrophs (Nelson et al. 1986) appears to be dependent on these regulatory elements. The growth hormone gene is expressed in pituitary somatotrophs, which embryonically share a cell lineage with

prolactin-producing lactotrophs (Behringer et al. 1988; Borelli et al. 1989). Appropriate expression of this gene is determined by specific promoter elements (Nelson et al. 1986; Lira et al. 1988) that exhibit structural similarity to those of the prolactin gene (Nelson et al. 1988). We have previously used competitive DNA binding analysis and in vitro transcription to demonstrate that a common transcription factor, Pit-1, activates expression of both pituitary genes (Nelson et al. 1988).

Cloning of the Pit-1 cDNA (Bodner et al. 1988; Ingraham et al. 1988) has facilitated the investigation of the role of Pit-1 in regulation of pituitary-specific genes. A member of the recently described POU homeo domain family of transcriptional activators (for review, see Herr et al. 1988; Levine and Hoey 1988; Robertson 1988), Pit-1, is structurally related to the factor Oct-2 (Clerc et al. 1988; Muller et al. 1988; Scheidereit et al. 1988; Staudt et al. 1988), which regulates lymphoid-specific expression of immunoglobulin genes. This regulation is affected by the interaction of Oct-2 with AT-rich genomic elements containing the octamer motif $ATTGTCAT$ (Landolfi et al. 1986; Staudt et al. 1986). A third member of the POU family, Oct-1 (Sturm et al.

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1988), recognizes the identical ATTTGCAT motif but differs from Oct-2 in its ubiquitous tissue distribution and its ability to activate certain eukaryotic promoters that lack a TATA box, for example, small nuclear RNA genes. The similarity of some Pit-1-binding sites (Nelson et al. 1988) to the octamer motif raises the possibility of cross recognition of sites by these factors. Although the extent of binding site degeneracy has not been determined for Pit-1, both octamer-binding proteins have been shown to bind with high affinity to several AT-rich elements, in addition to those that include the ATTTGCAT consensus (for review, see Garcia-Blanco et al. 1989). The POU proteins may thus exhibit functional similarity to the *Drosophila* family of homeo domain-containing transcription factors, which recognize and probably compete for related binding elements (Desplan et al. 1988; Hoey and Levine 1988; Jaynes and O'Farrell 1988), providing a system for the coordinate activation and repression of sets of developmental genes. Although expressed in discrete cell types in mature animals, a number of POU proteins, including Pit-1 and Oct-2, appear to be coexpressed embryologically in the rat neural tube (He et al. 1989), suggesting potential regulation of common genes during a crucial phase of mammalian development.

As a first step toward understanding the regulatory interplay of the POU transcription factors, we have investigated the relatedness of Pit-1- and Oct-2-binding elements. In this study mutational analysis of Pit-1 response elements in the rat prolactin genes reveals a critical binding motif, $A_TTAT^T_CCAT$, in these sites. The motif differs by only 2 nucleotides from the Oct-1/Oct-2 consensus sequence. We find that Pit-1 and Oct-2 bind to both Pit-1 and Oct-2 response elements but that the 2-bp difference between elements is sufficient to impart differential transcriptional responsiveness in pituitary and B cells, respectively.

Results

Requirement of the octamer-like motif in Pit-1 response elements for binding and transcriptional activation

To define the potential interrelationships of response elements for POU domain factors, it was first necessary to delineate the critical nucleotides in the Pit-1 response element. Based on the alignment of several Pit-1-binding sites in the rat prolactin and growth hormone genes, Nelson et al. (1988) proposed that a sequence, $A^A_TTAT^T_CCAT$, was required for high-affinity binding of Pit-1. Of these sites, the most proximal (1P) Pit-1-binding site in the rat prolactin gene (−62 to −38), which confers *trans*-activation by the cloned factor both in a stably transfected cell line (Ingraham et al. 1988; Mangalam et al. 1989) and in vitro transcription assays (Mangalam et al. 1989; V. Albert, unpubl.), was selected for mutational analysis. Figure 1A illustrates a series of scanning clustered mutations introduced in the proximal wild-type element, WT-1P. In gel shift experiments, this binding site yielded a major 'bound' band that was competed by <10-fold molar excess of WT-1P

site, but not by >300-fold excess of a nonspecific site (Fig. 1B). Clustered point mutations located at the extreme 5' end of the site or 3' of the TATTCAT sequence did not appreciably affect binding of Pit-1 (see CM-1 and CM-7 in Fig. 1C). However, 3-bp mutations within or overlapping the TATTCAT motif markedly reduced or abolished binding of the factor (see CM-4, CM-5, and CM-6 in Fig. 1C). The importance of the TATTCAT motif in Pit-1 binding was further demonstrated by a mutant site, PAL-1P (Fig. 1B), in which TATTCAT was reiterated in the antisense strand, creating a short segment of dyad symmetry. PAL-1P bound Pit-1 two to three times more efficiently than did the wild-type site (Fig. 1C) and, because of its high affinity, was used in our initial screening of λ expression libraries to correctly identify a Pit-1-positive clone (Ingraham et al. 1988). In transfected GC cells, PAL-1P stimulated activity of a minimal prolactin promoter (−36 to +34) four to five times more efficiently than the wild-type element (data not shown). Our analysis indicated that in addition to the critical TATTCAT core of the 1P element, A or T base pairs immediately 5' of the core were also required for high-affinity binding of Pit-1. Mutations in this region reduced the affinity of Pit-1 5- to 10-fold (cf. CM-2 and CM-3 to WT-1P in Fig. 1C).

The highly sensitive in vitro transcription system was used to assess function of single copies of mutant sites CM-1 to CM-6, inserted 5' of the prolactin TATA box, that was not possible by transfection. Consistent with the binding analysis, core mutant sites CM-4 and CM-5 were completely inactive in this assay, whereas intermediate levels of transcriptional activity were seen in the variants (CM-2, CM-3, CM-6) that bind Pit-1 at lower affinity than WT-1P (Fig. 1D). Competition experiments confirmed that only mutant sites that bound Pit-1 caused a decrease in Pit-1-dependent stimulation of transcription.

Scanning mutational analysis (Fig. 2A) of a Pit-1 site in the rat growth hormone gene (GH2, −132 to −105) yielded a strikingly similar profile to that of the prolactin WT-1P site. In the GH2 element, the sequence TATCCAT formed a critical recognition motif with a preceding AT-rich region contributing to high-affinity binding of Pit-1 (Fig. 2B). These data therefore provide evidence that a core TATNCCAT flanked 5' by AT residues constitutes a strong Pit-1-binding site in the prolactin and growth hormone promoters. Identical data for the growth hormone GH2 sites has been obtained using bacterially expressed Pit-1 holoprotein or affinity-purified Pit-1 from GC cells.

A finer resolution analysis of the WT-1P core sequence TATTCAT (Fig. 3A) was performed using native Pit-1 or the cloned gene product expressed either in HeLa cells or in a reticulocyte lysate translation system. In all three cases, a transversion at position 1, 2, 3, 6, or 7 of the heptanucleotide core substantially reduced or abolished Pit-1 binding (Fig. 3B). Conversion of TATTCAT to TATGCAT (PM-4) caused only a minor decrease in binding, as expected from the core consensus TATNCCAT. Unexpectedly, replacement of the highly

Figure 1. Binding and transcriptional analysis of clustered point mutations in the prolactin 1P region. (A) Map of the proximal rat prolactin promoter and illustration of mutations scanning the 1P element. The core sequence for binding of Pit-1, TATTCAT (1P), is shown in boldface type. (B) Gel shift assay of Pit-1 binding to 1P mutants using GC nuclear extracts. The molar excess of mutant oligonucleotide used to compete binding of Pit-1 to the WT-1P probe is indicated (fold). (C) Quantitative binding analysis of 1P mutant sites using GC extracts. In the absence of competition (B, lane 2), the fraction of bound/free represents 100% total binding. The amount of probe in this bound fraction was 2000 dpm. (D) *Cis*-activity of 1P mutants in an in vitro transcription system derived from GC nuclear extracts. The upper band represents transcripts correctly initiated from the prolactin promoter. An RSV promoter construct is included as an internal reference (lower transcript).

conserved C (see Nelson et al. 1988) in TATTCAT (PM-5) also caused only a small reduction in binding efficiency. In the context of the wild-type site, however, our data suggest that Pit-1 may closely contact DNA within the major groove at the C position of the TATTCAT core; methylation of the core G (ATGAATA, antisense strand) prevented binding of the factor completely, whereas similar modification of Gs elsewhere in the binding site (including the G immediately 3' of TATTCAT) had no effect (Fig. 3C). Identical patterns of interference were observed using pituitary cell (GC) nuclear extracts or purified Pit-1 (data not shown).

Shared DNA recognition motifs among POU proteins *Pit-1, Oct-1, and Oct-2*

The similarity of the core sequences in Pit-1 (ATATTCAT)- and Oct-1/Oct-2 (ATTTGCAT)-binding sites predicts potential cross recognition of sites by these POU domain proteins. A related motif, AT-

TATCAT, is reportedly bound as efficiently as the canonical octamer sequence by the lymphoid factor Oct-2 (Scheidereit et al. 1988). To examine reciprocal binding, we first converted the Pit-1 site WT-1P to an octamer element (OCT-1P), in which all flanking bases from the rat prolactin gene were conserved (see Fig. 1A). As shown in Figure 4A (gels 1 and 4), both Oct-1 and Oct-2 bound specifically to this modified Pit-1 site; the relative mobility of these Oct/OCT-1P complexes is consistent with earlier work in which a sequence from the immunoglobulin heavy-chain gene served as the binding site for Oct-1 and Oct-2 (Landolfi et al. 1987). In competition assays, Oct-1 bound the prolactin Pit-1 element WT-1P with apparent affinity three to five times lower (at 50% competition) than the OCT-1P element [Fig. 4A (gel 4), B]. Identical results were obtained for Oct-2 (data not shown). Unrelated sequences such as a cAMP response element competed for the octamer-binding factors only at very high concentrations. Native Pit-1 [Figs. 1C and 4A (gels 2 and 3)] and cloned Pit-1 (Fig. 4A,

Figure 2. Binding analysis of clustered point mutations in the growth hormone GH2 region. (A) Map of growth hormone promoter and illustration of clustered point mutations in the GH2-binding site. (B) Binding of Pit-1 from GC nuclear extracts to the GH2-binding site. Graph shows percent maximal binding to the GH2-WT probe in the presence of 50-fold molar excess mutant oligonucleotides.

gels 1–3), which exhibited similar mobilities in nondenaturing gels, bound to the OCT-1P variant 8–10 times less efficiently than to the wild-type site, based on competition analysis. Even at this reduced affinity, however, the OCT-1P site bound Pit-1 at least 50–100 times more strongly than mutant sites CM-4, CM-5, or unrelated DNA response elements (Fig. 1C). The mobility of Pit-1/OCT-1P complexes is faster than the mobility of Pit-1/WT-1P complexes. The faster migrating species has also occasionally been observed for Pit-1/WT-1P complexes. These different species probably represent monomeric and dimeric forms of Pit-1. Although monomeric in solution, Pit-1 appears to bind cooperatively to form dimers on DNA (H. Ingraham, in prep.). Pit-1 dimers could bind to the WT-1P site, with one molecule contacting the TATNCAT sequence and the second molecule contacting the AT-rich region. Alternatively, the entire TATNCAT + AT-rich region could be contacted by a single molecule, whereas the second molecule may contact DNA directly. Experiments are currently in progress to address this question.

Analysis of the interaction of Pit-1 and OCT-1P by methylation interference (Fig. 4C) revealed that contact points in the octamer core were identical to those of Oct-1 (Staudt et al. 1986; Fig. 4C) and Oct-2 (Staudt et al. 1986; Scheidereit et al. 1987), with methylation of either the sense (ATTTGCAT) or antisense (ATGCAAT) G preventing binding. Conversely, it appears that the sequence TATTCAT constitutes the primary core for interaction of octamer-binding factors with the wild-type prolactin element 1P, as judged by the ability of only mutations present in CM-4 and CM-5 to completely disrupt binding (data not shown). These data demonstrate that although differences exist in binding site preference, the points of DNA–protein interaction are similar for Pit-1, Oct-1, and Oct-2, consistent with the closely related DNA recognition domains of these proteins (Herr et al. 1988).

Functional restriction of POU factor-binding elements

Although mutant sites devoid of Pit-1-binding activity

Figure 4. Cross recognition of binding sites by POU proteins Pit-1, Oct-1, and Oct-2. (A) (Gel 1) Binding of Oct-1 and cloned Pit-1 expressed in HeLa cells to WT-1P and OCT-1P probes; (gels 2 and 3) binding of native and bacterially expressed Pit-1 to WT-1P and OCT-1P probes; (gel 4) competition of WT-1P and OCT-1P for binding of lymphoid-specific Oct-2 from BJA-B nuclear extracts. (B) Quantitative comparison of Oct-1 (HeLa cell extract) binding to the 32 P-labeled OCT-1P site in the presence of competing oligomeric sites OCT-1P, WT-1P, and CRE. (C) Methylation interference of Pit-1 (GC extract) and Oct-1 (B82 cell extract) binding to the 28-bp OCT-1P sequence. (D) In vitro transcription analysis of WT-1P and OCT-1P elements fused to the prolactin TATA box using GC nuclear extracts.

(CM-4, CM-5) did not stimulate transcription in pituitary nuclear extracts, several variants (CM-2, CM-3, CM-6) that bound Pit-1 at much reduced affinity relative to WT-1P (Fig. 1C) activated the prolactin promoter in vitro (Fig. 1D). On this basis, it was expected that OCT-1P would exhibit at least partial activity in enhancing promoter function. To test *cis*-activation by OCT-1P, it was important to use conditions in which Oct-1, expressed ubiquitously and with a high affinity for OCT-1P, did not exclude Pit-1 from binding to the octamer site. Binding studies suggested that HeLa-Pit-1⁺ nuclear extracts, which contain about one-tenth the level of Pit-1 present in GC extracts (Mangalam et al. 1989), would be unsuitable for this transcriptional analysis because many of the OCT-1P sites would be occupied by Oct-1 and not Pit-1 (see Fig. 4A, gel 1). In GC extracts, however, the higher levels of Pit-1, lower levels of Oct-1, or both, permitted effective competition of binding of Oct-1 to OCT-1P such that by gel shift analysis, >90% of the bound fraction represented Pit-1/OCT-1P complexes (see Fig. 4A, gel 3). Therefore, GC extracts were used to study Pit-1 responsiveness of the OCT-1P element in vitro. Unexpectedly, transcription units containing the OCT-1P site upstream of the prolactin TATA box were completely inactive in GC nuclear extracts (Fig. 4D). Insertion of up to three tandem

copies of the site adjacent to the prolactin promoter had no stimulatory effect. However, when used in vitro as a competitor of the WT-1P/prolactin promoter transcription unit, the OCT-1P element specifically decreased the response to Pit-1, as expected from its ability to bind the factor.

Transcriptional specificity of the OCT-1P and WT-1P elements was examined in intact cells by DNA-mediated gene transfer. Pituitary (GC) and lymphoid (S-194, BJA-B) cells were transiently transfected with plasmids that contained one or more copies of the WT-1P-binding site (Fig. 5A). As in the in vitro assay, transcription units containing the octamer variant were inactive in pituitary cells, regardless of the number of inserts (Fig. 5B, left), whereas the wild-type prolactin element enhanced transcription up to 100-fold, depending on copy number (Fig. 5B, right). In contrast, the OCT-1P *cis*-acting element enhanced activity of the prolactin promoter in a dose-dependent manner when transfected into the S-194 myeloma cell line, which expresses the factor Oct-2; stimulation by OCT-1P was even more pronounced in a second myeloma cell line, BJA-B (Fig. 5B, left). In view of its ability to compete binding of the octamer-binding factors at 20–30% the efficiency of OCT-1P, the WT-1P element was predicted to be *cis*-active in Oct-2-positive myeloma cells, in addition to pitu-

Figure 5. Cell-specific function of the WT-1P and OCT-1P elements in vivo. (A) Illustration of WT-1P- and OCT-1P-containing constructions used for transfection of pituitary (GC) and myeloma cell lines (S-194 and BJA-B). (B) Cell-specific, binding site-dependent activation of the luciferase reporter gene in transfected cells. Results are the mean ($\pm$ SEM) of three independent experiments.

itary cells. Surprisingly, even three tandem copies of WT-1P have no detectable stimulation of promoter activity in S-194 cells, as opposed to >50-fold stimulation by the same number of OCT-1P elements (Fig. 5B). A weak WT-1P-dependent signal was observed in BJA-B cells, but only at 1–5% that of the OCT-1P-binding site. A small stimulatory effect on transcription by the WT-1P site was also observed in vitro, using BJA-B nuclear extracts (data not shown), although the difference between OCT-1P and WT-1P activity in this cell-free system was less pronounced than in transfected lymphoid cells.

To examine the specificity of *cis*-activation by WT-1P in pituitary and lymphoid cells, respectively, heterologous cell lines with high transfection efficiencies were tested. These cells were unable to express constructs in which WT-1P or OCT-1P elements were fused to the prolactin TATA box (Fig. 5B) but responded when a cAMP or thyroid hormone response element was substituted, suggesting that functional restriction was at a level of the binding site rather than the prolactin promoter. Furthermore, the inability of OCT-1P to activate transcription in these Oct-1-positive cell lines is consistent with recent studies on the promoter selectivity of Oct-1 (Tanaka et al. 1988) and agrees with the conclusions of others (Wirth et al. 1987) that the presence of Oct-2, specifically, accounts for octamer-dependent activation in transfected lymphoid cells.

Discussion

In *Drosophila*, the program for embryogenesis is controlled by a family of genes that contain homeo boxes (for review, see Akam 1987; Gehring 1987; Scott and Carroll 1987; Ingham 1988). Cotransfection analyses have demonstrated that interaction of the homeo domain-containing proteins with specific genomic elements can synergistically enhance or inhibit gene ex-

pression (Jaynes and O'Farrell 1988; Han et al. 1989; Krasnow et al. 1989). However, the mechanism by which these DNA-binding proteins selectively activate or repress genes is complicated by the promiscuity of binding site recognition; for example, at least six *Drosophila* homeo domain proteins are known to bind the 10-bp consensus sequence TCAATTAAAT (Desplan et al. 1988; Hoey and Levine 1988), which appears to suggest diminished stringency in transcriptional control. Because of the overlapping specificity at the level of DNA binding, developmental regulation may be determined by competition among homeo domain proteins for related sites, with the net rate of transcription reflecting the interaction of promoter- or enhancer-bound factors (Desplan et al. 1988; Hoey and Levine 1988; Jaynes and O'Farrell 1988). Competition would be affected by the abundance of the homeo domain proteins and their binding affinities for multiple related *cis*-acting elements. Among the mammalian POU homeo domain proteins Pit-1, Oct-1, and Oct-2, DNA binding activity appears to require both the 60-amino-acid homeo domain, found in *Drosophila*, and the upstream POU-specific region (Ko et al. 1988; Sturm and Herr 1988; H. Ingraham and M.G. Rosenfeld, unpubl.). The highly conserved primary structure of these domains may explain, in part, the similarities in DNA-binding sites recognized by these factors and predicts that novel members of the family could possess similar binding specificities. Recent cloning of four new mammalian POU cDNAs confirms remarkable conservation of the POU region among these factors (He et al. 1989). Examination of the expression of seven POU domain genes in the developing fetal rat brain demonstrated marked spatial and temporal restrictions in the appearance or disappearance of each POU mRNA. Although the role of the gene products in developmental regulation is undefined, it is tempting to propose that specific patterns of gene transcription, regulated by a combinatorial interaction

of POU proteins, contribute to the phenotypic maturation of cell groups in the developing organism.

As in the *Drosophila* system, regulation of tissue differentiation by multiple POU factors having nearly identical DNA-binding domains will require mechanisms to ensure the appropriate interactions of transcriptional regulators with the correct target genes. Because of structural divergence outside the POU homeo region, it is likely that members of the POU family will exhibit protein–protein interactions, thereby evoking different transcriptional responses. In the case of Oct-1 and Oct-2, which bind to identical DNA sequences, such interactions permit activation of different promoter types. Promoters containing the octamer motif and a TATA element are generally responsive to Oct-2, but not Oct-1, whereas those having an octamer and a PE (proximal element) are activated by Oct-1; TATA box promoters can become Oct-1-responsive in the presence of VP16, a viral *trans*-activator that interacts with the homeo domain protein (Gerster and Roeder 1988; Stern et al. 1989). Other unique protein–protein interactions are suggested by the primary structures of POU factors revealing a heptameric leucine repeat (Landschulz et al. 1988) only in Oct-2 and by binding analyses demonstrating that Oct-2 contacts DNA as a monomer (Clerc et al. 1988), whereas Pit-1 forms dimers on DNA (V.R. Albert, unpubl.; H. Ingraham, in prep.).

In this study we investigated the contribution of the DNA-binding site toward selective *trans*-activation by pituitary- and lymphoid-specific POU homeo proteins. Two possible modes of differential gene activation by related POU factors are suggested by our data. The first is consistent with a general dependence of *trans*-activation on the affinity of POU factor-binding sites. High affinity Pit-1 sites, WT-1P and CM-1, display marked Pit-1-responsiveness, whereas 1P mutants devoid of binding are completely inactive in transcription assays. Those variants that merely decrease the affinity of Pit-1 binding partially reduce the transcriptional efficacy of the site. In a similar manner, the OCT-1P element is a strong binding site for Oct-2 and serves as a response element for the lymphoid factor but fails to confer activation by Pit-1, which it binds less strongly.

A second site-dependent mode of transcriptional control is suggested by several cases in which the *in vitro* affinity of the site (as measured by competitive binding assays) is a poor predictor of its transcriptional function. The mutant sites CM-3 and CM-6, e.g., bind Pit-1 with apparent affinities at least 10-fold lower than WT-1P but demonstrate significant *cis*-activation in response to the factor (see Fig. 1C,D). This may be similar to the case of Oct-2, where degenerate binding sites from *Drosophila* and yeast homeo box genes are bound by the factor at ~10-fold lower affinity than elements containing the octamer sequence ATTTGCAT (Ko et al. 1988; Scheidereit et al. 1988); however, in transfected cells, cloned Oct-2 activates two *Drosophila* gene promoters that contain degenerate octamer motifs (Ultrabithorax and Abdominal-B) with about the same efficiency as ATTTGCAT-containing constructs (Thali et al. 1988). The ability of weaker binding sites to confer regulation may reflect

stabilization of POU protein–DNA interaction by other proteins in transcription complex. Given the transcriptional activity of these lower affinity Pit-1 sites, it is surprising that the OCT-1P site is completely inactive, either in Pit-1-containing nuclear extracts or in transfected GC cells. The explanation may be that *in vitro* binding assays with short elements do not accurately reflect the affinity of POU protein–DNA interactions during assembly of transcription complexes; however, in this case (unlike that of CM-2, CM-3, and CM-6), binding of Pit-1 to an OCT-1P/promoter unit would require *destabilization* to account for the absence of transcription. A second explanation, that Pit-1 is excluded from binding to OCT-1P by high-affinity Oct-1/OCT-1P interactions, appears unlikely (discussed in Results), given the predominance of Pit-1/OCT-1P, rather than Oct-1/OCT-1P complexes in GC nuclear extracts. A third explanation is that Pit-1 binds stably to OCT-1P-containing promoters but in a transcriptionally inactive conformation. Although recent studies with transcriptionally active GAL4- or LexA-homeo proteins fusions have suggested that *trans*-activation by homeo domain-containing proteins is a simple consequence of their binding to DNA and might not require the homeo domain (Fitzpatrick and Ingles 1989; Samson et al. 1989), the potential regulatory role of homeo domain–DNA interactions is still unclear. There is evidence that the transcriptional activity of some factors is dictated, in part, by the primary structure of the DNA-binding site. In the case of the steroid/thyroid hormone receptor family, receptors for thyroid hormone bind to thyroid hormone response elements or to estrogen response elements with equally high affinity, but estrogen response elements, which resemble gapped mutants of the thyroid hormone response element, cannot confer thyroid hormone-dependent gene activation (Glass et al. 1988). More recently, the lymphoid factor NF- κ B was shown to bind with equally high affinity to sites in the interleukin-2 receptor α -chain gene and immunoglobulin κ -chain gene but to activate transcription only in the latter case (Cross et al. 1989). A 2-base mutation yielding the high affinity site of the interleukin receptor gene within the context of the κ -chain gene was able to block the transcriptional response to NF- κ B. These studies demonstrate that neither binding site affinity nor flanking DNA sequences in the promoter can completely account for *trans*-activation by some factors and suggest that the primary structure of the site may impact on the ability of the factor to be configured in a transcriptionally active mode.

In conclusion, we have determined, by mutagenesis, sequences in the rat prolactin and growth hormone genes necessary for high-affinity binding of native and cloned Pit-1 and have defined bases in the prolactin site critical for *trans*-activation. A double point mutation in this binding site created a strong response element for the B-cell factor, Oct-2. Cross recognition of Pit-1 and Oct-2 sites was observed, whereas cross activation of sites by these factors was not. Our data demonstrate that high-affinity binding sites for the POU factors Pit-1 and Oct-2 function as response elements but suggest that

not all binding sites are transcriptionally active. The domains of the Pit-1 protein required for binding to DNA in a transcriptionally competent form are currently under investigation to further define the contribution of the recognition element at a molecular level.

Materials and methods

Mutant Pit-1 sites

Mutagenesis of the prolactin 1P region (−64 to −38) was performed using 28-bp binding sites. Gel-purified templates (sense strand) containing clustered point mutations in the 5′ half of the sequence (−62 to −52; CM-1, CM-2, CM-3, PAL-1P) were annealed to a primer, 5′-CACCTTCATGAATA (antisense strand), and filled with Klenow to generate the binding site. Antisense templates containing mutations in the 3′ half of the 1P element (−51 to −40; CM-4, CM-5, CM-6, CM-7) were annealed to a second primer, 5′-GCTGATTATATATA, and filled. The sequence of the cAMP response element (CRE) from the α -subunit of the human chorionic gonadotropin gene is 5′-TGACGTCAATCGTACCATGACGTCAC.

Binding analysis

Gel shift analysis (Fried and Crothers 1981), using 32 P-labeled WT-1P (−64 to −38) or OCT-1P sites, was performed as described (Elsholtz et al. 1986), except that (1) the reaction buffer contained 4% Ficoll, 25 mM HEPES (pH 7.8), 60 mM NaCl, 1 mM DTT, and 67 μ g/ml poly[d(I-C)], and (2) electrophoresis of samples was carried out for 20 min. Nuclear extracts and phosphocellulose-fractionated bacterial lysates were prepared as outlined previously (Mangalam et al. 1989). Reticulocyte lysates (Promega) were primed with 0.02 μ g Pit-1 mRNA (synthesized using a T3 polymerase/Bluescript system), and 3 μ l of the 50- μ l reaction mixture used in the DNA-binding reaction. Probe used was 5–10 fmoles per reaction. Concentration of competitor oligomers was determined by fluorimetry (SLM, Model II). For quantitation of competition data, labeled protein–DNA complexes and unbound probe were excised from the gel following autoradiography, and the fraction of total radioactivity in each species was measured by scintillation counting.

Methylation interference

WT-1P or OCT-1P sites, 32 P-labeled on one 5′ terminus, were partially methylated with dimethylsulfate, incubated with nuclear extracts, and separated into bound and free forms on native 5% polyacrylamide gels. After brief autoradiography, both forms were purified from excised gel sections using DE-81 paper, cleaved with 0.5 M piperidine, and analyzed on denaturing gels (Siebenlist and Gilbert 1980; Glass et al. 1988).

In vitro transcription and transfection analyses

For in vitro transcription assays, blunt-ended 1P variants were inserted in direct orientation 5′ to a truncated prolactin promoter (−36 to +34) fused to the luciferase gene (De Wet et al. 1987). Promoter activity was assayed in the presence of 15 μ l GC nuclear extract (~5 mg/ml) and 0.5 μ g plasmid, using primer extension (Nelson et al. 1988). GC cells were transiently transfected as described (Nelson et al. 1986). HeLa and B82 cells were transfected using the calcium–phosphate procedure (Chen and Okayama 1987). Lymphoid cell lines S-194 and BJA-B were transfected at a density of 4×10^6 to 5×10^6 cells per plate using 10 μ g plasmid and a final DEAE–dextran concentration of 200 μ g/ml. After a 30-min incubation, cells were chloroquine-shocked (100 μ M) and incubated for an additional 2

hr before washing and resuspension in fresh serum-containing medium. All cells were harvested 48 hr post-transfection, and luciferase activity was measured by luminometry (Nelson et al. 1988).

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References

- Akam, M. 1987. The molecular basis for metamereric pattern in the *Drosophila* embryo. *Development* **101**: 1–22.
- Behringer, R.R., L.S. Mathews, R.D. Palmiter, and R.L. Brinster. 1988. Dwarf mice produced by genetic ablation of growth hormone-expressing cells. *Genes Dev.* **2**: 452–461.
- Bodner, M., J.-L. Castrillo, L.E. Theill, T. Deerinck, M. Ellisman, and M. Karin. 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55**: 505–518.
- Borrelli, E., R.A. Heyman, C. Arias, P.E. Sawchenko, and R.M. Evans. 1989. Transgenic mice with inducible dwarfism. *Nature* **339**: 538–541.
- Cao, Z., E.A. Barron, A.J. Carrillo, and Z.D. Sharp. 1987. Reconstitution of cell-type-specific transcription of the rat prolactin gene in vitro. *Mol. Cell. Biol.* **7**: 3402–3408.
- Chen, C. and H. Okayama. 1987. High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**: 2745–2752.
- Clerc, R.G., L.M. Corcoran, J.H. LeBowitz, D. Baltimore, and P. Sharp. 1988. The B-cell specific Oct-2 protein contains POU box and homeo box-type domains. *Genes Dev.* **2**: 1570–1581.
- Crenshaw, III, E.B., K. Kalla, D.M. Simmons, L.W. Swanson, and M.G. Rosenfeld. 1989. Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. *Genes Dev.* **3**: 959–972.
- Cross, S.L., N.F. Halden, M.J. Lenardo, and W.J. Leonard. 1989. Functionally distinct NF- κ B binding sites in the immunoglobulin κ and IL-2 receptor α chain genes. *Science* **244**: 466–469.
- Desplan, C., J. Theis, and P.H. O'Farrell. 1988. The sequence specificity of homeodomain–DNA interaction. *Cell* **54**: 1081–1090.
- DeWet, J.R., K.V. Wood, M. Deluca, D.R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: Structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**: 725–737.
- Elsholtz, H.P., H.J. Mangalam, E. Potter, V.R. Albert, S. Supowit, R.M. Evans, and M.G. Rosenfeld. 1986. Two different cis-active elements transfer the transcriptional effects of both EGF and phorbol esters. *Science* **234**: 1552–1557.
- Fitzpatrick, V.D. and C.J. Ingles. 1989. The *Drosophila* fushi tarazu polypeptide is a DNA-binding transcriptional activator in yeast cells. *Nature* **337**: 666–668.
- Fried, M. and D.M. Crothers. 1981. Equilibrium and kinetics of Lac repressor operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* **9**: 6505–6525.

- Garcia-Blanco, M.A., R.G. Clerc, and P.A. Sharp. 1989. The DNA binding homeo domain of the Oct-2 protein. *Genes Dev.* 3: 739–745.
- Gehring, W.J. 1987. Homeo boxes and the study of development. *Science* 236: 1245–1252.
- Gerster, T. and R.G. Roeder. 1988. A herpes virus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc. Natl. Acad. Sci.* 85: 6347–6351.
- Glass, C.K., J.M. Holloway, O.V. Devary, and M.G. Rosenfeld. 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* 54: 313–323.
- Gutierrez-Hartmann, A., S. Siddiqui, and S. Loukin. 1987. Selective transcription and DNase I protection of the rat prolactin gene by GH3 pituitary cell-free extracts. *Proc. Natl. Acad. Sci.* 84: 5211–5215.
- Han, K., M.S. Levine, and J.L. Manley. 1989. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* 56: 573–583.
- He, X., M.N. Treacy, D.M. Simmons, H.A. Ingraham, L.W. Swanson, and M.G. Rosenfeld. 1989. Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* 340: 35–42.
- Herr, W., R.A. Sturm, R.G. Clerc, L.M. Corcoran, D. Baltimore, P.A. Sharp, H.A. Ingraham, M.G. Rosenfeld, M. Finney, G. Ruvkun, and H.R. Horvitz. 1988. The POU domain: A large conserved region in the mammalian Pit-1, Oct-1, Oct-2, and *Caenorhabditis elegans unc-86* gene products. *Genes Dev.* 2: 1513–1516.
- Hoey, T. and M. Levine. 1988. Divergent homeobox proteins recognize similar DNA sequences in *Drosophila*. *Nature* 332: 858–861.
- Ingham, P.W. 1988. The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature* 335: 25–34.
- Ingraham, H.A., R. Chen, H.J. Mangalam, H.P. Elsholtz, S.E. Flynn, C.R. Lin, D.M. Simmons, L. Swanson, and M.G. Rosenfeld. 1988. A tissue-specific transcription factor containing a homeo domain specifies a pituitary phenotype. *Cell* 55: 519–529.
- Jaynes, J.B. and P.H. O'Farrell. 1988. Activation and repression of transcription by homeo domain-containing proteins that bind a common site. *Nature* 336: 744–749.
- Ko, H., P. Fast, W. McBride, and L.M. Staudt. 1988. A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. *Cell* 55: 135–144.
- Krasnow, M.A., E.E. Saffman, K. Kornfeld, and D.S. Hogness. 1989. Transcriptional activation and repression by ultrathorax proteins in cultured *Drosophila* cells. *Cell* 57: 1031–1043.
- Landolfi, N.F., J.D. Capra, and P.W. Tucker. 1986. Interaction of cell type-specific nuclear proteins with immunoglobulin V promoter region sequences. *Nature* 323: 548–551.
- . 1987. Protein-nucleotide contacts in the immunoglobulin heavy-chain promoter region. *Proc. Natl. Acad. Sci.* 84: 3851–3855.
- Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA-binding proteins. *Science* 240: 1759–1764.
- Levine, M. and T. Hoey. 1988. Homeobox proteins as sequence-specific transcription factors. *Cell* 55: 537–540.
- Lira, S.A., E.B. Crenshaw III, C.K. Glass, L.W. Swanson, and M.G. Rosenfeld. 1988. Identification of rat growth hormone genomic sequences targeting pituitary expression in transgenic mice. *Proc. Natl. Acad. Sci.* 85: 4755–4759.
- Mangalam, H.J., V.R. Albert, H.A. Ingraham, M. Kapiloff, L. Wilson, C. Nelson, H. Elsholtz, and M.G. Rosenfeld. 1989. A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. *Genes Dev.* 3: 946–958.
- Muller, M.M., S. Ruppert, W. Schaffner, and P. Matthias. 1988. A cloned octamer transcription factor stimulates transcription from lymphoid-specific promoters in non-B cells. *Nature* 336: 544–551.
- Nelson, C., V.R. Albert, H.P. Elsholtz, L.I.-W. Lu, and M.G. Rosenfeld. 1988. Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. *Science* 239: 1400–1405.
- Nelson, C., E.B. Crenshaw III, R. Franco, S.A. Lira, V.R. Albert, R.M. Evans, and M.G. Rosenfeld. 1986. Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression in rat prolactin and growth hormone genes. *Nature* 322: 577–562.
- Robertson, M. 1988. Homeo boxes, POU proteins and the limits to promiscuity. *Nature* 336: 522–524.
- Samson, M.-L., L. Jackson-Grusby, and R. Brent. 1989. Gene activation and DNA binding by *Drosophila* Ubx and abd-A proteins. *Cell* 57: 1045–1052.
- Scheidereit, C., A. Heguy, and R.G. Roeder. 1987. Identification and purification of a human lymphoid-specific octamer-binding protein (OTF-2) that activates transcription of an immunoglobulin promoter in vitro. *Cell* 51: 783–793.
- Scheidereit, C., J.A. Cromlish, T. Gerster, K. Kawakami, C. Balmaceda, R.A. Currie, and R.G. Roeder. 1988. A human lymphoid-specific transcription factor that activates immunoglobulin genes is a homeobox protein. *Nature* 336: 551–557.
- Schuster, W.A., M.N. Treacy, and F. Martin. 1988. Tissue-specific trans-acting factor interaction with proximal rat prolactin promoter sequences. *EMBO J.* 7: 1721–1733.
- Scott, M.P. and S.B. Carroll. 1987. The segmentation and homeotic gene network in early *Drosophila* development. *Cell* 51: 689–698.
- Siebenlist, U. and W. Gilbert. 1980. Contacts between *Escherichia coli* RNA polymerase and an early promoter of phage T7. *Proc. Natl. Acad. Sci.* 77: 122–126.
- Staudt, L.M., H. Singh, R. Sen, T. Wirth, P.A. Sharp, and D. Baltimore. 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. *Nature* 323: 640–643.
- Staudt, L.M., H. Singh, R. Sen, J.H., LeBowitz, P.A. Sharp, and D. Baltimore. 1988. Cloning of a lymphoid-specific cDNA encoding a protein binding the regulatory octamer DNA motif. *Science* 241: 577–580.
- Stern, S., M. Tanaka, and W. Herr. 1989. The Oct-1 homeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* 341: 624–630.
- Sturm, R.A. and W. Herr. 1988. The POU domain is a bipartite DNA binding structure. *Nature* 336: 601–604.
- Sturm, R.A., D. Gokul, and W. Herr. 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. *Genes Dev.* 2: 1582–1599.
- Tanaka, M., U. Grossniklaus, W. Herr, and N. Hernandez. 1988. Activation of the U2 snRNA promoter by the octamer motif defines a new class of RNA polymerase II enhancer elements. *Genes Dev.* 2: 1764–1778.
- Thali, M., M.M. Müller, M. Delorenzi, P. Matthias, and M. Benz. 1988. *Drosophila* homeotic genes encode transcriptional activators similar to mammalian OTF-2. *Nature* 332: 858–861.
- Wirth, T., L.M. Staudt, and D. Baltimore. 1987. An octamer oligonucleotide upstream of the TATA motif is sufficient for lymphoid-specific promoter activity. *Nature* 329: 174–178.

A two-base change in a POU factor-binding site switches pituitary-specific to lymphoid-specific gene expression.

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References

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