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A Tissue-Specific Transcription Factor Containing a Homeodomain Specifies a Pituitary Phenotype

Holly A. Ingraham,*† Ruoping Chen,† Harry J. Mangalam,† Harry P. Elsholtz,*† Sarah E. Flynn,*† Chijen R. Lin,† Donna M. Simmons,*‡ Larry Swanson,*‡ and Michael G. Rosenfeld*†

* Howard Hughes Medical Institute
†Eukaryotic Molecular Biology Program
School of Medicine
University of California, San Diego
La Jolla, California 92093
‡Neural Systems Laboratory
Salk Institute for Biological Studies
La Jolla, California 92037

Summary

Multiple related *cis*-active elements required for cell-specific activation of the rat prolactin gene appear to bind a pituitary-specific positive transcription factor(s), referred to as Pit-1. DNA complementary to Pit-1 mRNA, cloned on the basis of specific binding to AT-rich cell-specific elements in the rat prolactin and growth hormone genes, encodes a 33 kd protein with significant similarity at its carboxyl terminus to the homeodomains encoded by Drosophila developmental genes. Pit-1 mRNA is expressed exclusively in the anterior pituitary gland in both somatotroph and lactotroph cell types, which produce growth hormone and prolactin, respectively. Pit-1 expression in heterologous cells (HeLa) selectively activates prolactin and growth hormone fusion gene expression, suggesting that Pit-1 is sufficient to confer a characteristic pituitary phenotype. The structure of Pit-1 and its recognition elements suggests that metazoan tissue phenotype is controlled by a family of transcription factors that bind to related *cis*-active elements and contain several highly conserved domains.

Introduction

The precise temporal and spatial patterns of development are controlled by sequential activation of a hierarchy of regulatory genes (Gehring, 1987; Scott and Carroll, 1987). Ultimately, organ identity is dictated by specific patterns of gene expression. In mammals, indirect evidence supports the existence of tissue-specific factors critical for the transcriptional activation of the genes that define cellular phenotype (e.g., Walker et al., 1983; Staudt et al., 1986; Nelson et al., 1986; Bodner and Karin, 1987; Hammer et al., 1987a, 1987b; Courtois et al., 1987; Costa et al., 1988; Nelson et al., 1988; Singh et al., 1988).

The development of the anterior pituitary gland provides an excellent model system in which to study cell-specific gene activation. Pituitary development results in five distinct cell types derived from a common lineage that are distinguished on the basis of the secreted hormone. Somatotrophs and lactotrophs are the last two phenotypically distinct cell types to appear during development, producing the recently diverged hormones, growth hormone and prolactin, respectively (Chetelain et al., 1979; Watanabe and Daikoku, 1979; Cooke et al., 1981; Hoeffler et al., 1985). The transient coexpression of prolactin and growth hormone occurs in a subset of somatotrophs during pituitary development prior to the appearance of lactotrophs (Watanabe and Daikoku, 1979; Chetelain et al., 1979; Hoeffler et al., 1985). Similar or identical mechanisms might therefore be responsible for the activation of both genes.

Cell-specific expression of the rat prolactin gene is dictated by two separate regions, a distal enhancer (−1831 to −1530; Nelson et al., 1988) and a proximal region (−422 to +33; Nelson et al., 1986; Guitierrez-Hartmann et al., 1987; Cao et al., 1987; Lufkin and Bancroft, 1987; Nelson et al., 1988). Lactotroph-specific expression of fusion genes in transgenic mice can be conferred by either the distal enhancer or the proximal promoter region; however, the simultaneous presence of both regions markedly increases both the penetrance and level of transgene expression (E. B. Crenshaw III and M. G. R., unpublished). DNA-mediated gene transfer and mutagenesis experiments reveal that the distal and proximal regulatory regions contain multiple related sequences that appear to bind a tissue-specific nuclear protein(s) and exhibit synergistic interactions (Nelson et al., 1988). Mutation of even a single *cis*-active element can reduce prolactin gene expression by 80%–90% (Nelson et al., 1988).

Expression of growth hormone in somatotrophs of transgenic mice (Behringer et al., 1988; Lira et al., 1988) can be specified by as little as 180 bp of rat growth hormone 5′-flanking genomic information (Lira et al., 1988). This region contains two *cis*-active elements required for cell-specific expression in vitro (Nelson et al., 1986; West et al., 1987; Bodner and Karin, 1987; Ye and Samuels, 1987). The possibility that a single or two related cell-specific positive transcription factors can bind to sites in both the rat prolactin and growth hormone genes is suggested by competition analyses of DNAase I footprints and in vitro transcription (Nelson et al., 1988).

In this paper we report the identification of the factor responsible for cell-specific activation of prolactin gene transcription. It is a 33 kd protein with homology to the homeodomain regions encoded in genes regulating development in Drosophila. These data imply that a distantly related member of the homeobox gene family dictates a cellular phenotype in the anterior pituitary.

Results

Structural Characterization of Pit-1

A series of related elements in the rat prolactin and growth hormone genes that are required for the characteristic pattern of tissue-specific expression are diagrammed in Figure 1A. Based on several analyses, including muta-

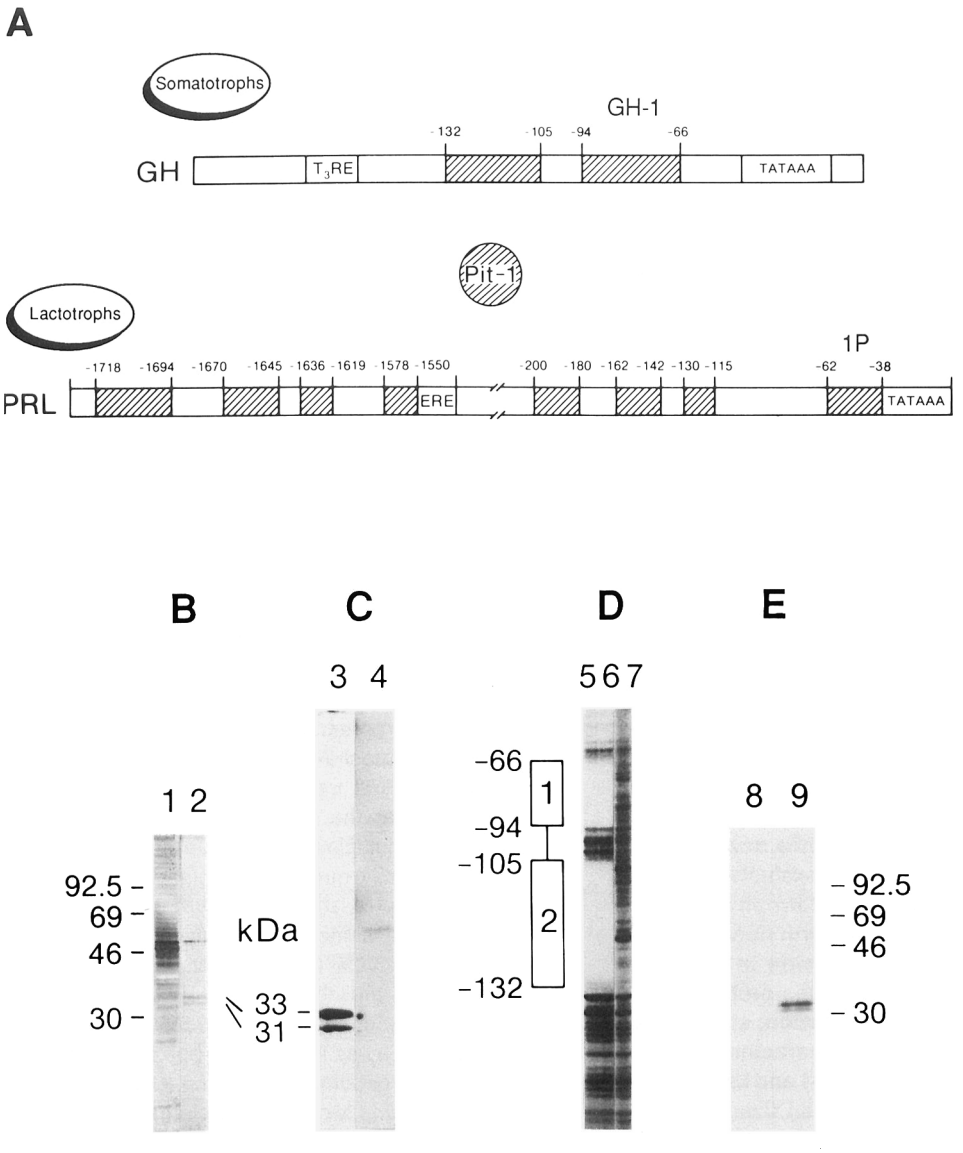

Figure 1. Characterization of a Pituitary Nuclear Protein That Binds to Prolactin Pit-1 *Cis*-Active Elements

(A) Schematic diagram of the cell-specific elements in the rat prolactin and growth hormone genes that exhibit cell-specific DNAase I footprints and are required for cell-specific gene expression. The factor(s) binding to these elements in the prolactin gene is referred to as Pit-1. The GH-1 and Prl-1P elements are indicated. The T_3 and estrogen response elements (T_3RE, ERE) are also indicated.

(B) Purification of Pit-1 from G/C cells indicates that a 33 kd–31 kd protein doublet can bind the tissue-specific *cis*-active element following denaturation and renaturation. Purification of G/C extracts by phosphocellulose and DEAE chromatography yielded a 20-fold purification (lane 1), and two passes over an oligonucleotide affinity column yielded a >5,000-fold purification (lane 2). A silver-stained SDS–10% polyacrylamide gel reveals major bands that migrate at 47, 33, and 31 kd.

(C) Size-fractionated total protein present in G/C nuclear extract (30 μg) was transferred to nitrocellulose filters and probed in the presence of poly(di-dC) (10 μg/ml) with ^{32}P-labeled, ligated oligonucleotides corresponding to the Prl-1P *cis*-active element (10^6 cpm/ml, lane 3) or to a cAMP regulatory element (Montminy et al., 1986; lane 4). Autoradiographs were exposed for 12 hr. Two identical bands bound labeled Prl-1P probe when copurified Pit-1 preparations (10 ng) were used. No binding was observed when HeLa or 208F cell extracts were similarly analyzed.

(D) Purified Pit-1 protein (3 ng, lane 5) and unfractionated nuclear extract (30 μg, lane 6) give identical DNAase I–resistant footprints using a ^{32}P-labeled rat growth hormone fragment (−320 to +8). Lane 7 shows the DNAase I ladder in the absence of extract. Similar footprints were observed when the opposite strand was labeled, and the purified material also gave footprints identical to the crude extract with either distal or proximal prolactin genomic enhancer regions (data not shown).

(E) In vitro [^{35}S]methionine-labeled translation products generated using reticulocyte lysate with or without (lane 8) T3 polymerase–catalyzed, capped Pit-1 transcripts (10 ng), using the Pit-1 cDNA insert described in Figure 3 as template (see Figure 3, lane 9), were subjected to electrophoresis on SDS–10% polyacrylamide gels. The autoradiograph exposure shown is for 6 hr. Migration of protein standards is shown to the right (sizes in kd).

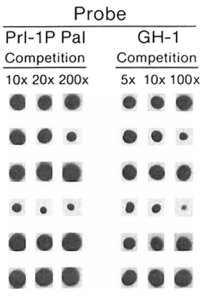

Figure 2. Binding of a Fusion Protein to Pit-1 Recognition Elements: Screening for Pit-1 cDNA

Double-stranded cDNA inserts prepared from G/C cells or estrogen-treated rat pituitary mRNAs were inserted into λgt11 or λZAP (Stratagene), respectively, and 500,000 plaques of each library were screened using ligated, radiolabeled (>10^8 cpm/μg) GH-1 or Prl-1P Pal probes as described (Singh et al., 1988; Vinson et al., 1988). Plaque-purified isolates were tested for specificity of binding radiolabeled, ligated oligonucleotides (~10^6 cpm/ml) corresponding to the Prl-1P Pal or GH-1 sites by competition with the indicated molar excess of double-stranded oligonucleotides corresponding to the GH-1, GH-1 Mut, Prl-1 Pal, Prl-1P Mut, and GH-1 Mut elements, and the cAMP regulatory element (CRE), as shown. Individual plaques were transferred to nitrocellulose and probed as described in Experimental Procedures. The consensus sequence is indicated in boldface; lowercase letters indicate alterations from the wild-type sequence. In other experiments, no binding was observed for T_3 or cAMP regulatory elements.

tions, DNAase I footprints, and in vitro transcription, the eight related *cis*-active sequences in the rat prolactin genes appear to bind a common, or closely related, factor(s), which we refer to as Pit-1 (Nelson et al., 1988). The proposed consensus sequence of these elements ($^{T/T}_{A/A}$TATNCAT) has been confirmed by a mutational analysis of the prolactin 1P (Prl-1P) element (H. P. E. and V. Albert, unpublished observations).

To characterize the tissue-specific factor(s) responsible for the developmental activation of the prolactin gene, purification employing DNA affinity chromatography was performed using nuclear extracts from a rat pituitary cell line (G/C). A highly purified (>5,000-fold) preparation of biologically active protein was obtained (Figure 1B). The major species in this preparation migrated as a doublet (M_r = 33,000 and 31,000) on silver-stained SDS–polyacrylamide gels, although a 47 kd protein was also present in most preparations (Figure 1B). The presence of a species migrating at 43 kd in SDS–polyacrylamide gels following UV cross-linking techniques with crude nuclear extracts (Nelson et al., 1988) appears to reflect aberrant migration of the cross-linked protein due to the covalently linked DNA (Nelson et al., 1988). The identical cell-specific DNAase I footprints observed using total G/C nuclear extract were found using the purified preparation at a 10,000-fold lower protein concentration (Figure 1D and data not shown). Analyses of Western blots following transfer revealed that both proteins in the doublet selectively bound to cell-specific elements from either the growth hormone or prolactin genes (data not shown). Even when crude G/C nuclear extracts were used, only the 33 kd–31 kd protein doublet bound to Pit-1 elements (Figure 1C, lane 3), and binding was selectively competed by a 100-fold molar excess of functional Pit-1 elements. Binding was not observed using an inactive Pit-1 element, such as the Prl-1P element containing a 4 bp mutation of the consensus sequence, or with other, unrelated elements, nor was binding observed using HeLa nuclear extracts.

The ability of Pit-1 to bind to *cis*-active elements of the prolactin and growth hormone genes permitted utilization of this property for screening of rat pituitary and G/C cell cDNA expression libraries, employing a variation of a technique developed by Singh et al. (1988) to isolate a factor binding the H2TF1 element, and modified by Vinson et al. (1988) for isolation of C/EBP. In this method, β-galactosidase fusion proteins are screened based upon their ability to bind with high affinity to specific DNA sequences. A palindromic variant of the Prl-1P binding site (Prl-1P Pal, Figure 2), which provides a high-affinity functional binding site, was used to screen recombinants. Representative cDNA libraries were generated from the G/C rat pituitary cell line and from estrogen-treated pituitary glands. Screening of 10^6 independent recombinants containing size-selected DNA inserts complementary to either G/C or estrogen-treated rat pituitary mRNA yielded one specific positive plaque. The fusion protein bound to three cell-specific elements (Prl-1P Pal and the rat growth hormone 1 element [GH-1] in Figure 2, and Prl-1P) but did not bind mutated, nonfunctional elements (Prl-1P Mut and GH-1 Mut; Figure 2) or unrelated regulatory elements for cAMP or thyroid hormone (data not shown). Competition analyses with excess unlabeled oligomers confirmed the specificity of sequence recognition with only functional Pit-1 elements competing for binding (Figure 2). Based on these analyses, the plaque containing the fusion protein was purified and its insert utilized as a probe for isolation of full-length cDNAs. Using a 370 bp cDNA fragment as probe to rescreen the libraries, reactive inserts were detected at a frequency of approximately 1 in 10,000 for both libraries. Six clones were sequenced in their entirety; all contained a coding sequence identical to that of the clone originally selected.

The entire coding sequence for Pit-1 predicted from sequences of two cDNA clones, derived from pituitary and G/C cells, is shown in Figure 3. The sequence reveals an 873 nucleotide open reading frame corresponding to an encoded protein of 291 amino acids and a predicted M_r = 32,900. The junction of the fusion protein initially derived from the G/C expression library and identified by binding to Pit-1 elements was at amino acid 45. This particular clone exhibited divergent 3′ noncoding information as indicated by boldface italics (Figure 3). Translation of capped in vitro transcripts in reticulocyte lysates produced a doublet of [^{35}S]methionine-labeled proteins of approximately 33 kd and 31 kd when analyzed on SDS–polyacryl-

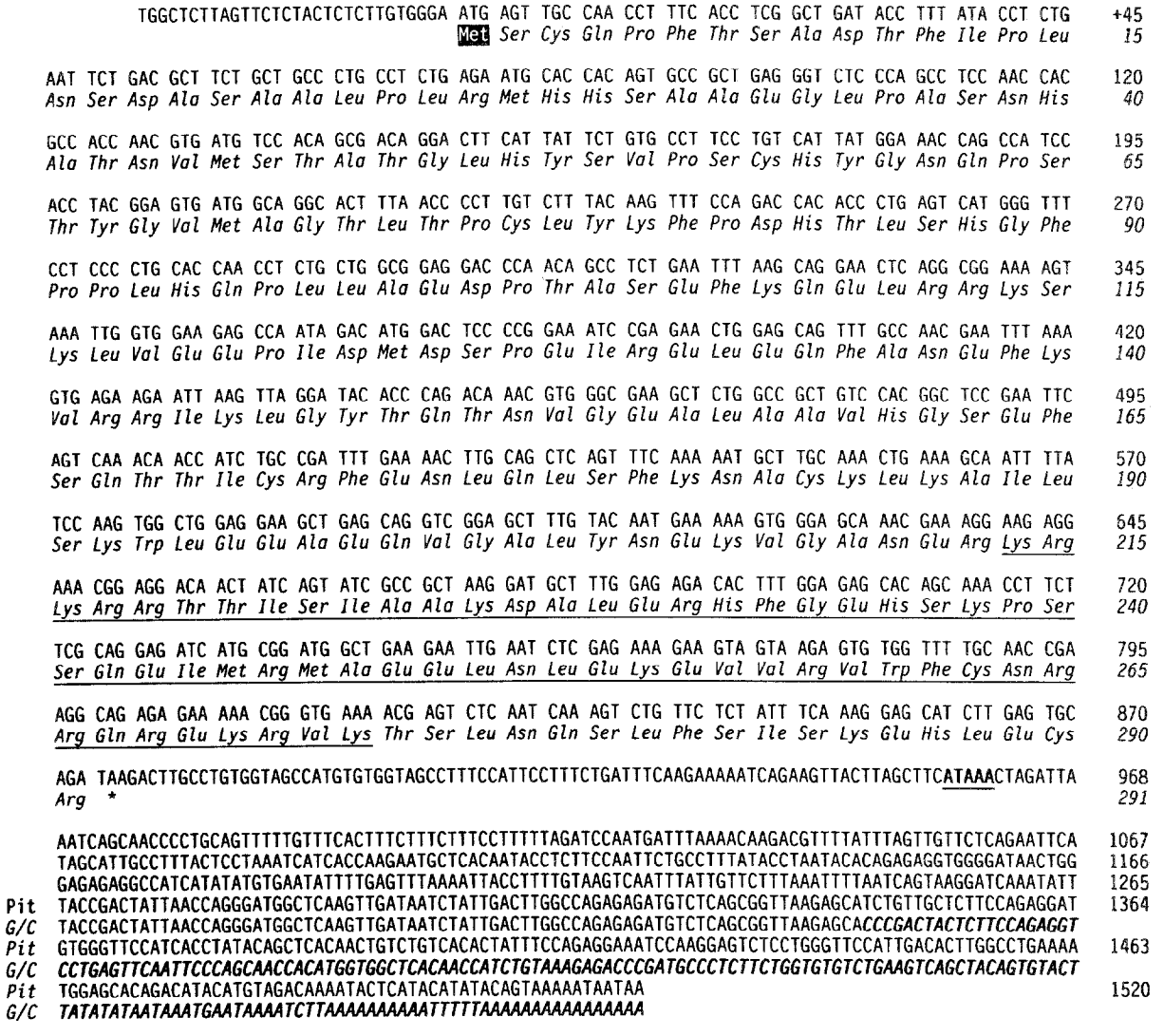

Figure 3. Structure of Pit-1 cDNA and Homology with Other Developmental Factors

Both strands of five independent cDNA inserts were sequenced entirely by enzymatic procedures using dideoxynucleotides and a T7 polymerase (Sequenase) on a double-stranded DNA template (Sanger et al., 1977). A cDNA isolated from a G/C library diverged from the pituitary (Pit) sequence in 3′-noncoding information, as indicated (italics). The region in the predicted open reading frame containing homology to the homeodomains of Drosophila *trans*-acting factors is underlined.

amide gels (Figure 1E). The doublet resembles the doublet of the purified Pit-1 protein (Figure 1B) and is consistent with the possibility that these proteins represent products of a single transcription unit. These two species could result from either alternative use of initiator methionines or posttranslational modifications such as phosphorylation or glycosylation. Using the avidin/biotin complex DNA binding assay (Glass et al., 1987), it was demonstrated that the in vitro translation products specifically bind to a biotinylated Prl-1P *cis*-active elements, confirming this property of the putative Pit-1 protein (data not shown).

While the amino terminus of Pit-1 exhibits no clear homology with other reported proteins, the carboxyl terminus contains a 60 amino acid sequence with similarity to the homeodomain encoded in several Drosophila and vertebrate regulatory genes (Gehring, 1987; Scott and Carroll, 1987). A comparison of the Pit-1 carboxyl terminus with several examples of homeodomain-containing proteins is shown in Figure 4. This domain contains homology in both of the putative helical regions presumed to be the DNA binding domain (McKay and Steitz, 1981; Ohlendorf et al., 1983; Laughon and Scott, 1984). Although Pit-1 clearly represents a divergent homeobox protein, it exhibits homology with the highly conserved region proximal to the third helical turn and an identity in seven of the nine invariant amino acids observed for all Drosophila homeodomains as indicated by the dots in Figure 4. The

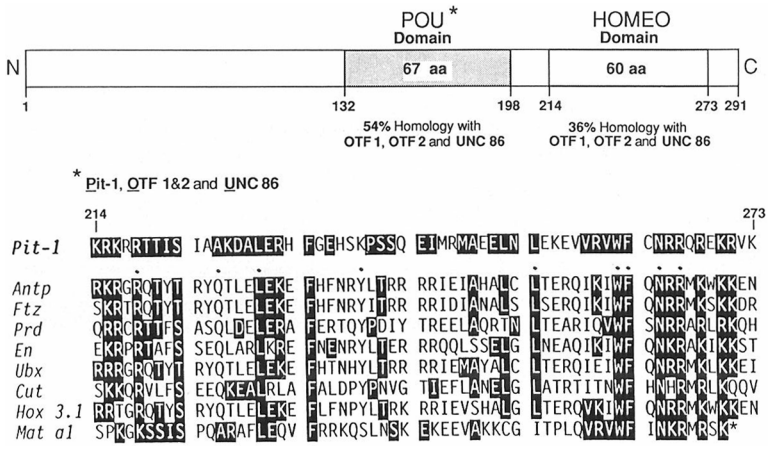

Figure 4. Putative Domains of Pit-1, and Comparison of the Carboxyl Terminus of Pit-1 with the Homeodomains of Several Regulatory Proteins

Pit-1 is compared with the products of *Antennapedia* (*Antp*; McGinnis et al., 1984a), *fushi tarazu* (*Ftz*; Scott and Carroll, 1987), *paired* (*prd*; Frigerio et al., 1986), *engrailed* (*en*; Fjose et al., 1985; Poole et al., 1985), *Ultrabithorax* (*Ubx*; McGinnis et al., 1984a; Scott and Weiner, 1984), *cut* (Blochinger et al., 1988), mouse *Hox-3.1* (Awgulewitsch et al., 1986), and yeast mating type regulatory gene *MATa1* (Astell et al., 1981). Dots indicate nine "invariant" amino acids in Drosophila homeodomain-containing proteins; serine-threonine and lysine-arginine are considered to be equivalent.

Pit-1 homeodomain exhibits a 35% identity with *paired*, although it is likely to exhibit significant homology to other related tissue-specific transcription factors.

Pit-1 Gene Expression Is Restricted to the Anterior Pituitary

The pattern of expression of mature Pit-1 transcripts was examined in rat tissues and permanent cell lines. As shown in Figure 5A, permanent pituitary cell lines (G/C) and pituitaries from untreated or estrogen-stimulated animals express a series of Pit-1–reactive transcripts. The major transcripts are 2.3–2.5 kb long. A minor transcript of 4.5 kb may represent a nuclear precursor, while a 1.25 kb transcript is suggested to represent a transcript that utilizes a proximal poly(A) site (Figure 3, boldface in the 3′ untranslated region). Pit-1 transcripts were not detected in any other cell line or tissue evaluated, even at low stringency or with exposure times 20-fold longer than those shown in Figure 5. These data suggest that the Pit-1 gene product is expressed exclusively in the pituitary gland.

To characterize which pituitary cell types express the Pit-1 transcripts, in situ hybridization was performed in conjunction with immunohistochemical costaining. As shown in Figure 6, Pit-1 expression is restricted to the anterior pituitary, with no detectable hybridization in the intermediate lobe or the posterior pituitary. Both somatotroph and lactotroph cell types express Pit-1 mRNA; examples are shown in Figures 6C and 6D. Hybridization to the Pit-1 probe is observed in a subset of thyrotrophs, while no detectable hybridization is observed in gonadotrophs (data not shown).

Hybridization of a Pit-1 cDNA probe to size-fractionated genomic DNA reveals that, for the four restriction enzymes analyzed, only a single hybridizing band is ob-

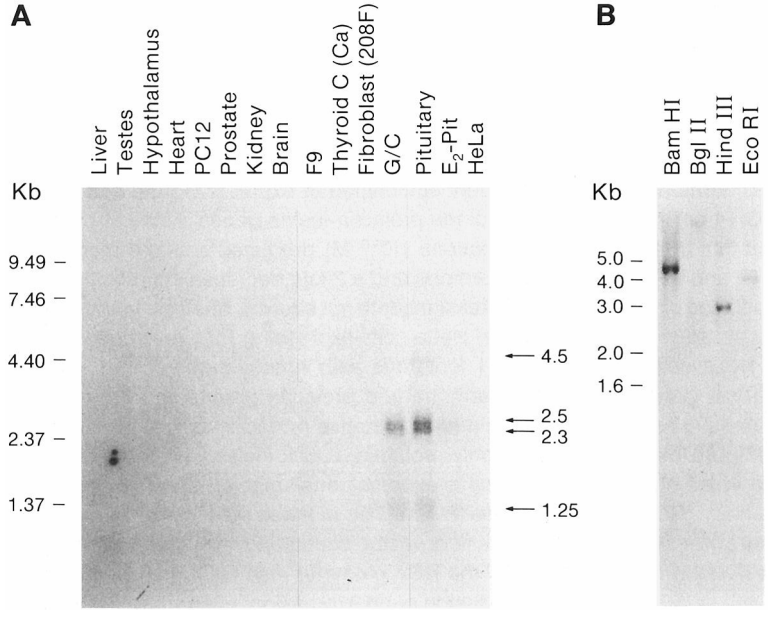

Figure 5. Expression of Pit-1 mRNA Is Limited to Pituitary and Prolactin- and Growth Hormone–Producing Pituitary Cell Lines

(A) Hybridization of a 370 bp radiolabeled cDNA probe to poly(A)$^+$-selected RNA (3 μg) isolated from a series of rat tissues and cell lines. The autoradiograph shown is a 12 hr exposure, but no hybridization was observed in negative lanes even with a 12 day exposure time. Migration of the RNA standards, and sizes of reactive species, are noted.

(B) Hybridization of the identical 370 bp Pit-1 cDNA probe to total rat DNA digested with the four indicated restriction enzymes. Migration of DNA standards is indicated.

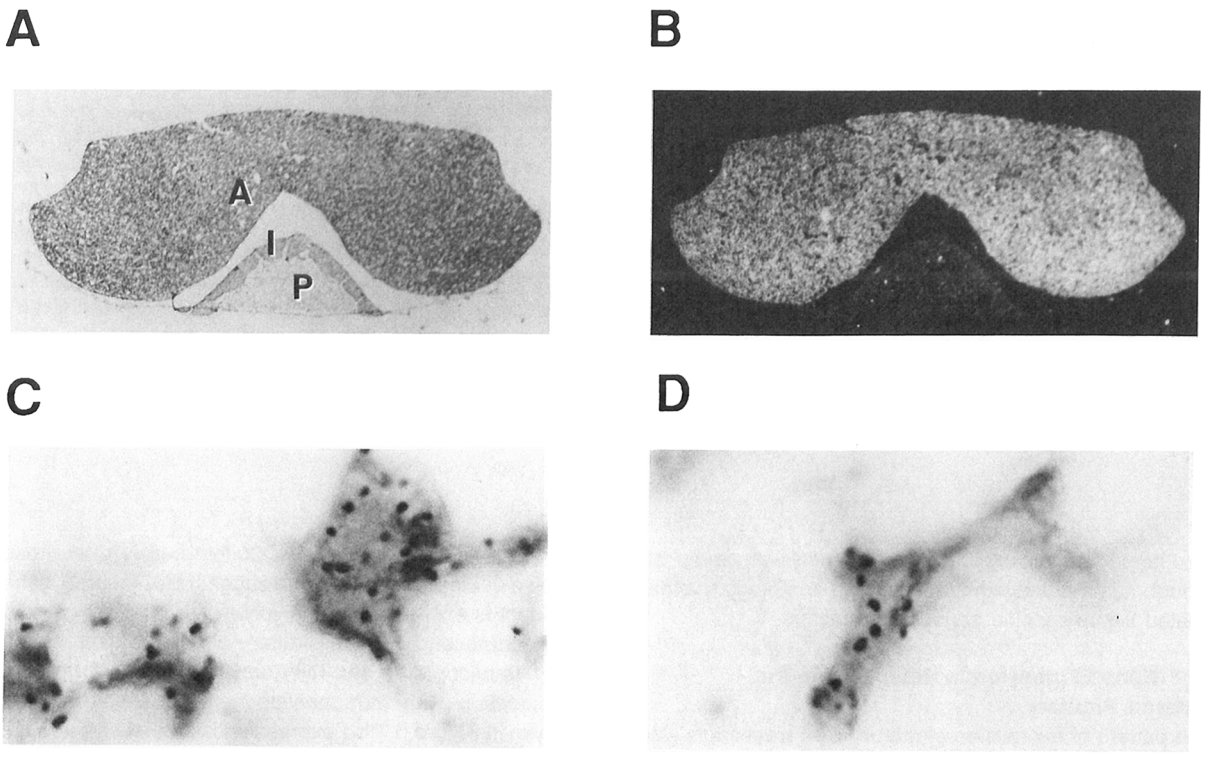

Figure 6. Pit-1 mRNA Is Expressed in Several Cell Types of the Anterior Pituitary

(A) Comparison of anterior (labeled "A"), intermediate ("I"), and posterior ("P") lobes for hybridization of ^{35}S-labeled Pit-1 cDNA probe. A dark-field exposure is shown in (B). Hybridization is confined to the anterior pituitary. Potential costaining of each cell type was evaluated by immunohistochemical analysis using specific anti-LH, -TSH, -prolactin, and -growth hormone antibodies, and hybridization histochemistry using a Pit-1–specific RNA probe. No staining was observed using labeled sense RNA. Costaining was observed in lactotrophs (C) and somatotrophs (D). The cells are visualized on the basis of specific staining with antisera, and the silver grains reflect hybridization of Pit-1 probe. Similar results were obtained in three experiments of similar design, and also using primary cultures of rat pituitary glands.

served (Figure 5B). These data are consistent with the possibility that there is a single Pit-1 gene in the rat capable of generating a series of mature transcripts.

The Pit-1 Gene Product Confers Cellular Phenotype

The functional effects of Pit-1 were assessed by DNA-mediated gene transfer experiments. Pit-1 cDNA was inserted into a transcription unit containing the SV40 early or Rous sarcoma virus (RSV) promoter and CAP site, and cotransfected with a plasmid containing a reporter gene under the control of the rat prolactin or growth hormone promoters or containing several copies of the GH-1 or Prl-1P elements or the T_3 response element fused to a truncated prolactin promoter (−36 to +33; Figures 7A and 7B). These transcription units utilize the correct 5′ initiation site in G/C cells, as previously reported (Nelson et al., 1988), and express only at extremely low levels in HeLa cells (Figure 7A). Cotransfection with a plasmid expressing the Pit-1 transcription unit results in a dramatic stimulation of expression of the rat prolactin fusion genes (Figure 7A). These levels of expression are comparable to those observed for the identical fusion gene in rat pituitary (G/C cells). These transcription units utilize the correct 5′ initiation site in these cells, as previously reported (Nelson et al., 1988).

Fusion genes containing multiple copies of either the GH-1 or Prl-1P cell-specific *cis*-active elements also exhibited marked Pit-1–dependent stimulation of expression (Figure 7A), confirming the ability of Pit-1 to bind in a transcriptionally active conformation to the isolated cell-specific elements of either gene. Pit-1 exerted no effects on expression of other transcription units, such as one containing the T_3 response element (Figure 7A). Fusion genes containing a contiguous 180 or 320 bp of rat growth hormone 5′-flanking information exhibited a lower Pit-1–dependent stimulation of expression than observed in the case of the prolactin fusion genes. Addition of T_3 and dexamethasone (10^{-8} M) produced a 3-fold increase in growth hormone and a 2-fold decrease in prolactin fusion gene expression (data not shown). Analysis using nuclear extracts of HeLa cells expressing Pit-1 revealed identical DNAase I footprints with affinity-purified Pit-1 on both growth hormone and prolactin promoters (Mangalam et al., submitted). A putative rat lactotroph cell line that expresses only prolactin (235-1; Nelson et al., 1988) selectively failed to express transfected rat growth hormone fusion genes (Figure 7B). In these cells, cotransfection with an expression vector containing the Pit-1 cDNA under control of the RSV promoter also failed to induce growth hormone fusion gene expression, even when the 5′-flank-

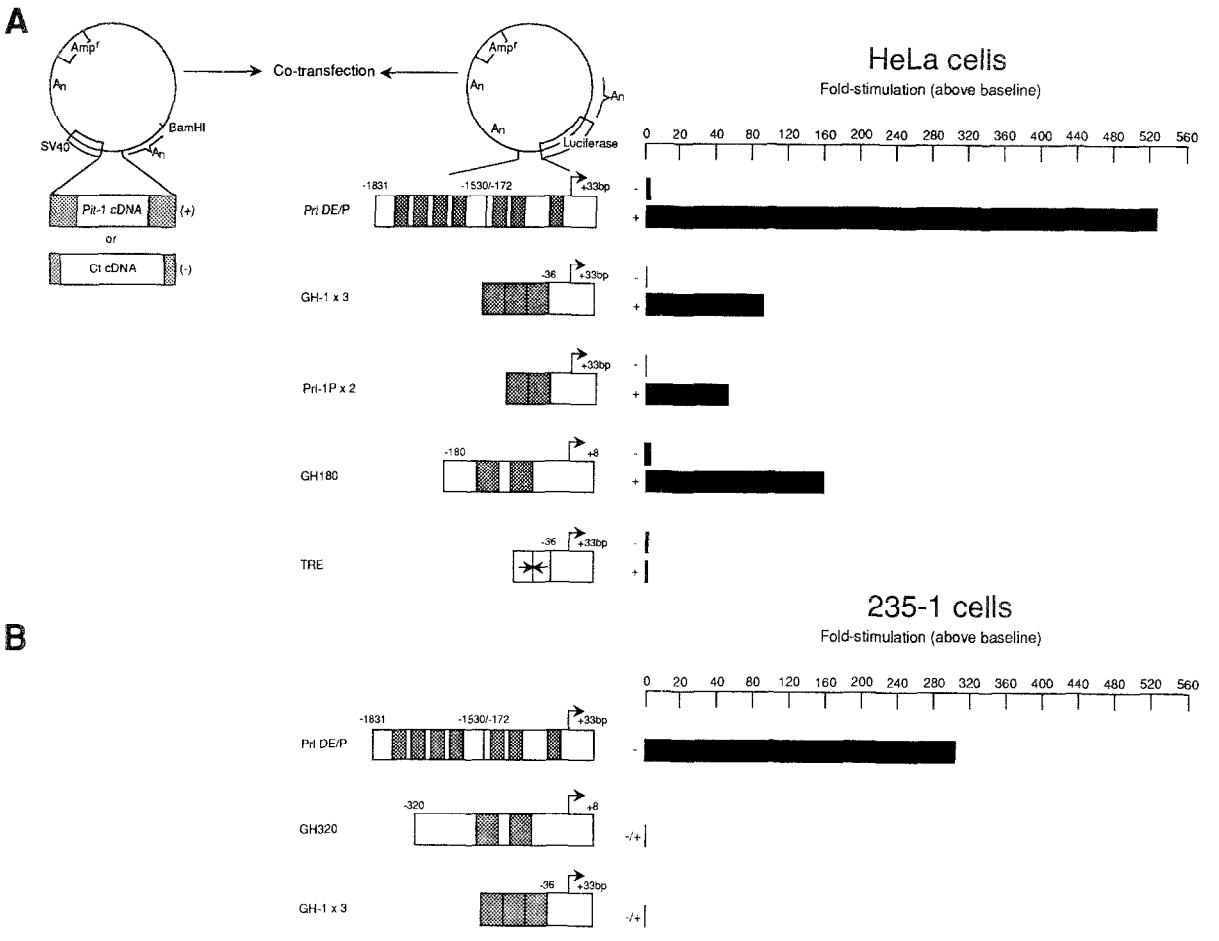

Figure 7. The Pit-1 Gene Product Transfers to Heterologous Cells the Ability to Express Prolactin and Growth Hormone Fusion Genes

(A and B) Cotransfection assay used for expression analyses. Pit-1 cDNA (referred to as +) was placed into an expression vector with an SV40 early or RSV promoter, and SV40 splice and poly(A) information, as previously described (Lin et al., 1987; Nelson et al., 1988). As a control, the expression vector containing a nonexpressing segment of epidermal growth factor receptor cDNA (Ct) was utilized (referred to as −). Reporter plasmids contained either −320 bp of rat GH (−320 to +8; construct GH320), −180 bp of rat GH (−180 to +8; construct GH180), −422 bp the rat prolactin distal enhancer (−1531 to −1830) fused to the proximal promoter (−189 to +35; construct Prl DE/P), or two Prl-1P elements (−36 to +68; construct Prl-1P×2) or three GH-1 elements (−62 to +88) fused to the rat prolactin TATA box region (−36 to +8; construct GH-1×3), all fused to a luciferase reporter gene (Nelson et al., 1986). Results are the average of duplicate determinations differing by <5 %. Similar results were obtained in four independent experiments.

(B). *Growth hormone fusion genes are not expressed in 235-1 cells. Transfection assays using growth hormone fusion genes* (GH320 or GH-1×3) revealed no expression even with cotransfection of the Pit-1 expression vector.

ing region was limited to three contiguous copies of the rat GH-1 element (Figure 7B, GH-1×3).

The identification of the transcriptional activator of the rat prolactin gene as a distant member of the homeobox gene family provides direct evidence that these factors activate gene transcription.

Discussion

The development of the anterior pituitary gland requires a molecular code that promotes and restricts expression of subsets of genes to generate specific cellular phenotypes. In this paper we report the identification, isolation, and molecular cloning of the structural gene encoding a pituitary transcriptional activator (Pit-1) capable of directing tissue-specific expression of the rat prolactin and growth hormone genes. Pit-1 exhibited no obvious structural similarity with transcription factors containing the "leucine zipper" motif or metal-coordinated "fingers" (Landschulz et al., 1988; Evans, 1988).

Unexpectedly, Pit-1 shares significant homology with a 60 amino acid region, referred to as the homeodomain, initially described in three gene products regulating early development in Drosophila (*Antennapedia, Ultrabithorax*, and *fushi tarazu*; Scott and Weiner, 1984; McGinnis et al., 1984b) and present in over 20 related gene products that dictate position-specific determination in Drosophila (Awgulewitsch et al., 1986; Gehring, 1987; Scott and Carroll, 1987). Multigene families containing highly conserved homeobox domains are presumed to direct early developmental events and have been described in C. elegans, amphibians, mice, and humans (Carrasco, 1984; Levine et

al., 1984; Colberg-Poley et al., 1985; Awgulewitsch et al., 1986). The precise molecular mechanisms by which homeodomain-containing gene products serve as developmental regulators is not established. They share homology with the yeast **a**1 and α2 regulatory gene products, known to regulate, individually and in combination with other transcription factors, gene expression that determines yeast cell mating type (Shephard et al., 1984; for review, see Nasmyth and Shore, 1987), suggesting similar functional roles for the homeotic genes. The observation that Pit-1 directly activates prolactin gene transcription as a consequence of binding to *cis*-active elements and generates a pituitary phenotype in heterologous cells links homeodomain-containing proteins to tissue-specific transcriptional activation in mammals.

Pit-1 exhibits an identity of only 7/9 invariant (see dots, Figure 4) and 15/20 of the most highly conserved amino acids present in the Drosophila homeodomains (see Figure 4), thereby representing the most divergent reported homeodomain. Amino acid identities with any of eight other homeodomains are arbitrarily indicated in black shading to facilitate visual comparison of related areas. The sequence variations modify only several of the conserved amino acids predicted to be on one side of the putative α-helical regions (amino acids 12–21, 31–38, and 43–50; McKay and Steitz, 1981; Ohlendorf et al., 1983). A potentially critical divergence is observed in the putative recognition region where cysteine, glutamine, and glutamic acid are uniquely present at residues 50, 54, 56. Recently a number of genes with highly divergent homeobox domains, and often exhibiting tissue-specific and cell-specific patterns of expression, have been identified (e.g., Way and Chalfie, 1988; Barad et al., 1988; Blochinger et al., 1988). The importance of a homeodomain-containing gene product in determining cellular phenotype is established by analysis of *cut* locus mutants in Drosophila. In the absence of *cut* gene activity, external sensory organs are transformed into chorodotonal organs (Blochinger et al., 1988).

The Pit-1 DNA recognition element is a short AT-rich sequence ($^{TT}_{AA}$TATNCAT) differing by only a single nucleotide from the immunoglobulin octamer sequence (ATTTGCAT) that binds different factors in lymphoid and nonlymphoid cells (Table 1). This binding site is sufficient to impart lymphoid-specific promoter activity (Walker et al., 1983; Wirth et al., 1987; Singh et al., 1988). Indeed, Pit-1 can bind at 10-fold lower affinity to the immunoglobulin octamer sequence (H. P. E., unpublished data). Two adjacent regions (POU and HOMEO; Figure 4) are highly conserved between OTF-1, OTF-2, and *unc-86*, consistent with the functional importance of both domains. The putative *cis*-active elements suggested to be critical for binding of Drosophila homeobox gene products have been less well defined. One such binding site of the *eve* protein is AT-rich (Hoey and Levine, 1988), although it differs at 4/8 residues from the Pit-1 recognition element (Table 1). The consensus yeast *MATα2* product binding site (Johnson and Herskowitz, 1985) is remarkably similar to the Pit-1 and OTFII recognition elements (Table 1). Therefore, it is possible that the members of the gene family responsible for developmental activation of gene transcription bind to related, AT-rich elements, possibly inducing critical DNA bending events. This would be analogous to the steroid hormone T_3 receptor gene family, whose members exhibit remarkable similarity of binding sites (Evans, 1988).

Table 1. Comparison of *Cis*-Active Sequences Important in Prolactin, Immunoglobulin, Yeast *MAT***a***1*, and Drosophila *engrailed* Gene Expression

Gene	Recognition Element	*Trans*-Acting Factor
Prolactin	AATATNCAT	Pit-1
Immunoglobulin κ	NATTTGCAT	OTFII
*MAT***a***1*	AATTTACAT	MATα2 protein
engrailed	CAATTAAAT	*eve* protein

Sequences shown bind the discrete factors Pit-1, OTFII (Wirth et al., 1987), yeast MATα2 (Johnson and Herskowitz, 1985), and *eve* protein (Hoey and Levine, 1988), respectively.

The presence of multiple Pit-1–dependent elements in the prolactin gene appears to be an important aspect of its developmental activation, because a single element is insufficient to produce marked increases in gene expression. Such combinatorial effects of multiple Pit-1 elements might be similar to the proposed interactions of small "enhanson" elements in the SV40 promoter (Ondek et al., 1988). We have found that a fusion gene containing three copies of the GH-1 element is expressed in the pituitary gland of transgenic mice, confirming that this element alone is sufficient for developmental targeting of gene expression to the pituitary (E. B. Crenshaw III, S. A. Lira, and M. G. R., unpublished). However, elevated levels of Pit-1 are required for effective stimulation of growth hormone expression. These results are consistent with the observation that Pit-1 levels are higher in somatotrophs than lactotrophs (L. S., unpublished). Pit-1 is also expressed in a subset of thyrotrophs, and these data would suggest that an additional component must account for appropriate cell-type-specific gene expression in the mature pituitary gland. The failure of Pit-1 to stimulate growth hormone fusion gene expression in a putative lactotroph cell line suggests a restrictive mechanism.

These observations define the molecular basis of pituitary-specific activation of rat prolactin and growth hormone gene expression and suggest that a family of factors distantly related to homeodomain-containing proteins are responsible for establishing the patterns of gene transcription that define differentiated phenotypes.

Experimental Procedures

Purification of Pit-1 and DNA Binding Assays

G/C nuclear extract was prepared as previously described (Nelson et al., 1986) and subjected to serial chromatography using phosphocellulose and DEAE (H. J. M., unpublished). The 20-fold enriched material was subjected to three serial passes over an affinity column prepared as previously described (Kadonaga et al., 1987) using ligated GH-1 oligonucleotides (−62 to −89) linked to Sepharose CL-2B by cyanogen bromide (Nelson et al., 1988). After extensive washing with 0.1 M NaCl, elution was performed with a linear NaCl gradient; the most purified Pit-1 protein eluted at 0.3 M NaCl. Identical results were obtained using a Prl-1P oligonucleotide column. DNAase I footprint analysis was per-

formed as previously described using rat growth hormone −320 to +8, rat prolactin −422 to +36, and rat prolactin −1830 to −1531 fragments labeled at either terminus with [γ-^{32}P]ATP (Nelson et al. 1988).

Labeled, ligated probes of 6–12 nucleotides corresponding to the GH-1 or Prl-1P or Prl-1P Pal element (see Figure 2; 10^9 cpm/μg) were used in binding reactions (1 ng/ml) in the presence or absence of the indicated competitor DNA, and 10 μg/ml poly(dI-dC), using the denaturation and renaturation procedure of Vinson et al. (1988). All procedures were performed at 4°C. The procedure used for Western blot analysis involved separation of proteins on an 8%–12% polyacrylamide–SDS gel, transfer to nitrocellulose, binding of ligated oligonucleotide probes (>5 × 10^8 cpm/μg) by incubation at 4°C for 16 hr in 25 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 9), 1 mM dithiothreitol, and washing for 2 min cycles at 4°C, and used the denaturation and renaturation procedure described above. Incubations were performed in the presence of 10 μg/ml poly(dI-dC).

Cloning and Sequencing of Pit-1 cDNA

cDNA was prepared using mRNA from G/C cells or retired breeder female rats treated for 14 days with estrogen implants. Lactotrophs represented >80% of the total cells in these pituitaries. cDNA was rendered double stranded and treated with EcoRI methylase, ligated to EcoRI linkers, size-fractionated to select for inserts longer than 2–3 kb, and then cloned into λgt11 (G/C) or λZAP (pituitary) as we have previously described (Lin et al., 1987). Screening was performed by the method of Singh et al. (1988), using modifications introduced by Vinson et al. (1988). Screening was performed using the Prl-1P Pal probe. Approximately 5 × 10^5 plaques from the G/C line and rat pituitary were screened; more than 100 primary plaques were picked for further screening, and only 1 was positive on tertiary screening. Libraries were rescreened using an insert isolated from this recombinant, and positive clones were obtained at a frequency of 1 in 10,000. A total of 12 clones were analyzed, and 6 were fully sequenced. For any ambiguous regions or compressions, deoxyriboinosine was used to confirm sequence. Both strands of 6 independent cDNA inserts were sequenced entirely by enzymatic procedures using dideoxy nucleotides and a T7 polymerase (Sequenase) on a double-stranded DNA template (Sanger et al., 1977), utilizing oligonucleotide primers.

RNA and DNA Analyses

The 370 bp EcoRI fragment of a Pit-1 cDNA clone corresponding to amino-terminal coding information was used as template for ^{32}P-labeled probes generated using random primers (>8 × 10^8 cpm/μg). Poly(A)-selected RNA was prepared from a series of rat tissues and cell lines, size-fractionated under denaturing conditions using formaldehyde–0.8% agarose gels, transferred to nitrocellulose, and hybridized for 12 hr as previously reported (Lin et al., 1987). Blots were washed in 2× SSC at 65°C for 30 min. Autoradiography was performed for 16 hr. RNAs in each lane were intact in equivalent amounts to within 50% of one another based on staining of ribosomal RNA and hybridization to other cDNA probes. Similar conditions were used for genomic blot analyses, performed by the method of Southern (1975).

In Situ Hybridization; Immunohistochemical Costaining Procedure

Adult male Sprague-Dawley rats were perfused with glutaraldehyde, and 10 μm thick cryostat sections of the pituitary were mounted on gelatin-coated slides and vacuum dried. Slides were then incubated for 1 hr at 41°C in 0.02 M KPBS containing 2% BSA (Sigma fraction V), 0.3% Triton X-100, 1 mM dithiothreitol, 5 mg/ml heparin, and 50 U/ml RNasin (Boyer et al., 1983). After rinsing in KPBS, sections were incubated for 48 hr at 4°C in primary antibodies (rabbit anti-prolactin at 1:400 [NIAMD], rabbit anti-ovine growth hormone at 1:3000 [Immunonuclear], or rabbit anti-rat thyroid-stimulating hormone at 1:1500 [NIADDK]) containing 5 mg/ml heparin. Antibody binding was visualized using the Vector ABC peroxidase procedure and a modification of the cobalt intensification procedure (Sakanaka et al., 1987). Sections were serially placed in 0.1 M Tris, 0.05 M EDTA, and 0.5% Triton X-100; hybridized without probe (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt's solution, 10 mM Tris [pH 8.0], 1 mM EDTA, 500 μg/ml tRNA, 1 mM dithiothreitol); and then hybridized for 48 hr with two changes of hybridization solution containing 5 × 10^6 cpm/ml of Pit-1 single-stranded RNA synthesized with [^{35}S]UTP. Posthybridization washes included treatment with RNAase A (10 μg/ml) and 30 min in 0.1× SSC at 55°C. Dry sections were dipped in Kodak NTB-2 autoradiography emulsion (diluted 1:1) and were developed after 10 days in Kodak D-19.

Cotransfection Analyses

Reporter plasmid vectors containing either rat prolactin 5′-flanking information (−1831 to −1530/−178 to +36), rat growth hormone information (−320 to +8), or multiple boxes of GH-1 or Prl-1P elements (see Figure 2) and the firefly luciferase gene (de Wet et al., 1987) were prepared as previously described (Nelson et al., 1988). Expression plasmids for Pit-1 or a control plasmid containing a noncoding portion of the epidermal growth factor receptor (Ct) were under transcriptional regulation of the SV40 early or RSV promoter region, as previously described (Nelson et al., 1988). An SV40 splice poly(A) site was 3′ of the inserted DNA. HeLa or 235-1 cells were cotransfected with each reporter plasmid (10 μg) using the calcium phosphate coprecipitation procedure (Chen and Okayama, 1987) and 10 μg of the expression vector containing the Pit-1 insert (+) or Ct insert (−). Luciferase assays were performed as previously described (de Wet et al., 1987) 48 hr following transfection.

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Note Added in Proof

The sequence of Pit-1 has been compared by personal communications (initiated by W. Herr) with the sequences of the immunoglobulin octamer binding proteins in B cells, OTF-2 (Roger Clerc, Lynn Corcoran, David Baltimore, and Phillip Sharp, submitted) and OTF-1 (Richard Sturm and Winship Herr, submitted), and the protein encoded by the neuronal developmental regulatory gene *unc-86* (Michael Finney, Gary Ruvkun, and H. Robert Horvitz, Cell, in press). These four gene products exhibit two highly conserved domains (see Figure 4), referred to as the POU domain, exhibiting >50% identity in 67 amino acids, and the HOMEO domain, exhibiting an overall identity of 36% in 60 amino acids.