FUNCTIONAL AND DISTRIBUTION STUDIES OF ISOFORMS OF THE PITUITARY-SPECIFIC TRANSCRIPTION FACTOR, PIT-1

by

Brian Kloss

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1995

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

2/3/95
Date

Carter Bancroft, Ph.D.
Chair of Examining Committee

2/3/95
Date

Terry Ann Krulwich, Ph.D.
Executive Officer

Joseph Margiotta, Ph.D.

James J. Bieker, Ph.D.

Leslie Pick, Ph.D.

Lawrence A. Chasin, Ph.D.

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

FUNCTIONAL AND DISTRIBUTION STUDIES OF ISOFORMS OF THE PITUITARY-SPECIFIC TRANSCRIPTION FACTOR, PIT-1

by

Brian Kloss

Advisor: Carter Bancroft, Ph.D.

The pituitary-specific transcription factor Pit-1 (GHF-1, Pit-1α) has been shown to be involved in cell type-specific expression and hormonal regulation of the prolactin and growth hormone genes. Two unique protein products of the pit-1 gene, Pit-1a (GHF-2, Pit-1β) and Δ4Pit-1, have been identified. The transcript encoding the Pit-1a isoform arises by alternate splicing of the Pit-1 mRNA precursor and is detected in all rat anterior pituitary cell lines examined. While Pit-1a binds the same regions of the rat prolactin and growth hormone gene proximal promoters as Pit-1, it is unable to trans-activate the prolactin gene promoter, yet it can trans-activate the growth hormone gene promoter. The Δ4Pit-1 transcript is identical in sequence to Pit-1 except that it lacks exon IV of the pit-1 gene, encoding amino acids 147-201. Therefore, like Pit-1a, Δ4Pit-1 is proposed to arise as a result of alternate splicing of the Pit-1 precursor mRNA. The excised amino acids of Δ4Pit-1 represent a majority of the Pit-1 $POU_{specific}$ domain, necessary for homo- and heterodimerization and sequence-specific, high-affinity DNA binding. The Δ4Pit-1 mRNA and protein are detected only in the GH_3 somatomammotroph cell line and the Δ4Pit-1 protein cannot bind DNA or trans-activate the prolactin and growth hormone gene promoters. Using an extremely sensitive amplification technique, it has been demonstrated that the 235-1 and MMQ cell lines contain readily detectable prolactin mRNA, but no detectable growth hormone mRNA and therefore appear to be true representatives of the mammotroph cell type. Taken together, these data suggest that in

addition to Pit-1, the Pit-1a and Δ4Pit-1 isoforms contribute to restrict expression of the prolactin and growth hormone genes to the appropriate cell types. However, the true functional roles of Pit-1a and Δ4Pit-1 have yet to be determined.

Acknowledgments

I thank my preceptor, Dr. Carter Bancroft for all of his guidance, encouragement and patience during the completion of my dissertation project in his laboratory. I would also like to thank all of the present and former members of the Bancroft laboratory, especially Drs. Daniel Fischberg, Wayne Pan, Arthur Jackson and James Rocco (honorary) for their camaraderie during the past several years. I thank all of the wonderful friends and colleagues I have made over the last several years, especially Drs. Jörg Heierhorst, William B. Thornhill, William Probst, Martin Walsh and Sven Beushausen, whose most beneficial social, technical and intellectual contributions were made almost exclusively outside of the laboratory. Lastly, but certainly not least, I would like to thank my family, without whose support I would never have completed my degree.

Calvin and Hobbes by Bill Watterson

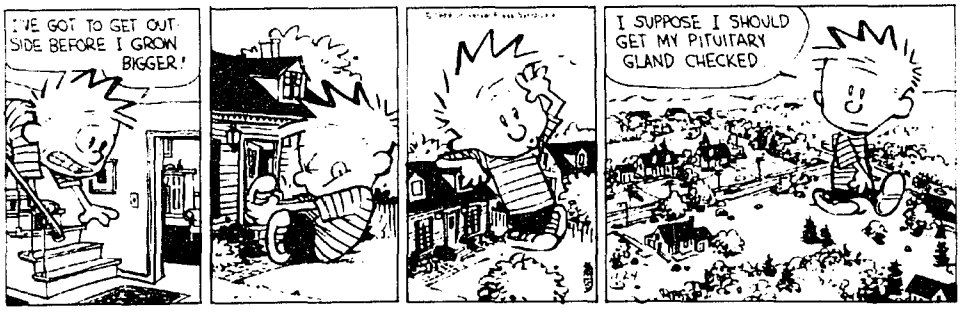


Table of Contents

List of Tables

List of Figures

INTRODUCTION AND SUMMARY

In adult mammals, the anterior pituitary contains at least five different cell types which produce at least six different peptide hormones (reviewed in Karin et. al. 1990). These different cell types arise from a single common progenitor (**Figure 1**, Karin et. al. 1990) and with few exceptions, expression of the genes encoding these hormones is restricted exclusively to the anterior pituitary (Emanuele et. al. 1992, Pellegrini et. al. 1992, Delhase et. al. 1993, Ezzat et. al. 1993, Nowak et. al. 1993). Furthermore, expression of the hormones of the anterior pituitary is hormonally regulated, ultimately involving changes at the transcriptional level (reviewed in Gourdji and Laverriére 1994). For these reasons, the pituitary gland serves as an excellent model for studies of cell type-specific gene expression and signal transduction pathways (reviewed in Ingraham et. al. 1990, Karin et. al. 1990).

While the posterior pituitary originates from neuroectoderm, the anterior pituitary gland arises from an invagination of the oral ectoderm, called Rathke's pouch (Schwind 1928, reviewed in Karin et. al. 1990, Theill and Karin 1993). Initially, the anterior pituitary differentiates from a single primordial stem cell into two cell types, acidophils and basophils (Karin et. al. 1990, Theill and Karin 1993). Development continues, until the cells of the mature pituitary have terminally differentiated into five distinct cell types (Karin et. al. 1990, Theill and Karin 1993): (a) mammotrophs (lacotrophs) which produce prolactin Prl) (b) somatotrophs which produce growth hormone (GH) (c) corticotrophs which produce adrenocorticotropin ACTH) (d) thyrotrophs which produce thyroid stimulating hormone (TSH) and (e) gonadotrophs which produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). While prolactin, growth hormone and ACTH are all single polypeptides, TSH, LH and FSH are glycoprotein hormones which share a common α subunit but have unique β subunits (Theill and Karin 1993). The prolactin and growth hormone producing cells of the anterior pituitary arise

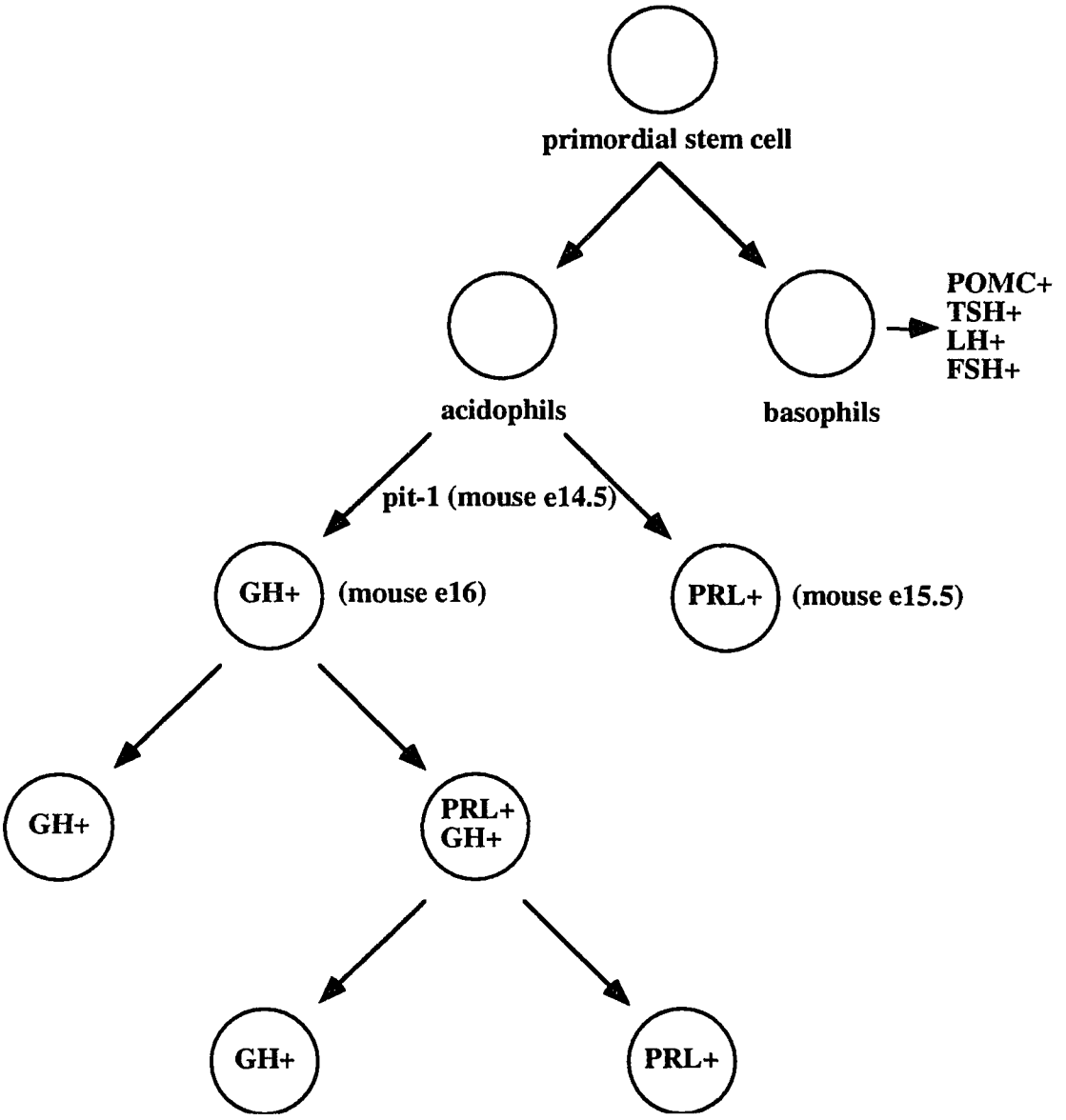


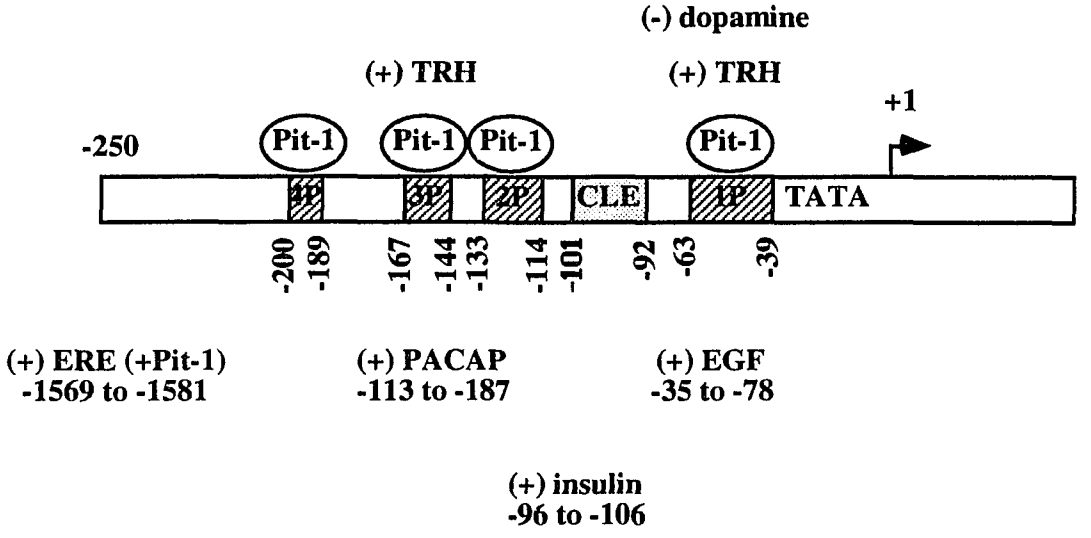
Figure 1. Anterior pituitary development. The development of the pituitary gland is shown schematically. The hormone(s) produced by each cell type is given. The days in which prolactin, growth hormone and pit-1 gene transcripts are first detected during mouse development are indicated. (Redrawn from Karin et. al. 1990).

from acidophils, while all other cell types of the anterior pituitary originate from basophils (Karin et. al. 1990). Therefore, studies of the molecular mechanisms involved in the cell type-specific expression of the prolactin and growth hormone genes are particularly attractive.

Expression of the prolactin and growth hormone genes is regulated at the transcriptional level in response to a wide variety of extracellular cues (reviewed in Karin et. al. 1990, Theill and Karin 1993, Gourdji and Laverriére 1994). Transcription of the rat prolactin gene is increased in response to estrogen (Day et. al. 1990), pituitary adenylate cyclase activating polypeptide (PACAP) (Coleman and Bancroft 1993), epidermal growth factor (EGF) (Jackson et. al. 1990), insulin (Stanley 1992) and thyrotropin releasing hormone (TRH) (Yan et. al. 1991). Transcription of the rat prolactin gene is repressed by dopamine (Elsholtz et. al. 1991) and transforming growth factor β (TGFβ) (Delidow et. al. 1991). Similarly, transcription of the rat growth hormone gene is positively regulated in response to glucocorticoids (Treacy et. al. 1991), retinoic acid (Bedo et. al. 1989), thyroid hormone (Brent et. al. 1989) and growth hormone releasing factor (GRF) (Barinaga et. al. 1983). Transcription of the rat growth hormone is negatively regulated by somatostatin Sugihara et. al. 1993) and activin Struthers et. al. 1992). The hormones which regulate transcription of the prolactin and growth hormone genes and the locations of their respective response elements within the 5'-flanking regions of these two genes are summarized in **Figure 2.**

Regions within 187bp, 5'- of the prolactin gene transcription start site, have been shown to be sufficient for cell type-specific expression of the prolactin gene (Lufkin et. al. 1989). Regions within 235bp, 5'- of the growth hormone gene transcription start site, have been similarly identified (Nelson et. al. 1986, Lira et. al. 1993). Cell fusion studies and transfection analysis of non-pituitary cell lines have convincingly demonstrated that expression of the prolactin and growth hormone genes is dependent on a single, but

A. Rat Prolactin



B. Rat Growth Hormone

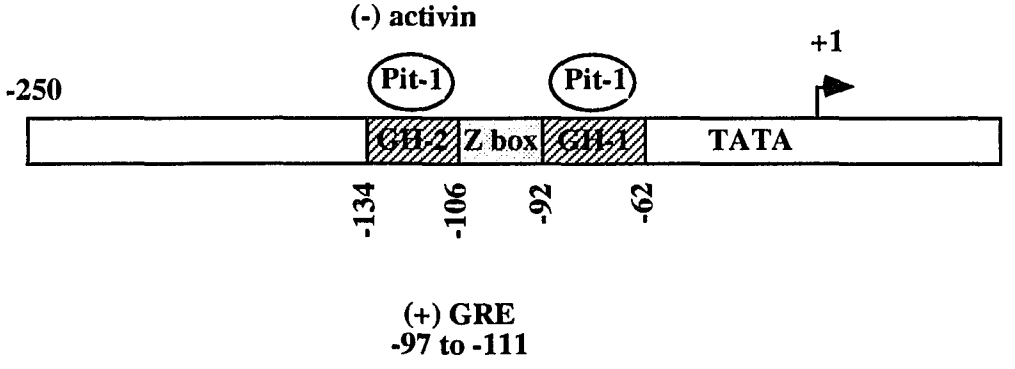


Figure 2. Rat prolactin and growth hormone gene proximal promoters. Schematic representation of the rat prolactin A) and growth hormone (B) gene proximal promoters. Pit-1 binding sites in the rat prolactin sites 1P-4P) and growth hormone gene proximal promoters (sites GH-1 and GH-2) are indicated. Boundaries of the Pit-1 binding sites or other response elements were determined by DNAse I footprinting and 5'-deletion analysis. The rightward facing arrow denotes the transcription start site. The CLE is a CRE-like element. The Z box has been shown to bind the transcription factor Zn-15. Abbreviations are as follows: TRH (thyrotropin releasing hormone), ERE (estrogen response element), PACAP (pituitary adenylate cyclase activating polypeptide), EGF (epidermal growth factor) and GRE (glucocorticoid response element).

functionally diverse, pituitary-specific transcription factor, Pit-1 (Bodner et. al. 1988, Ingraham et. al. 1988, McCormick et. al. 1988, Nelson et. al. 1988, Mangalam et. al. 1989, Fox et. al. 1990). As shown in **Figure 2**, this region of the prolactin gene promoter contains four Pit-1 binding sites, whereas the aforementioned region of the growth hormone gene promoter contains two Pit-1 binding sites (Bodner et. al. 1988, Ingraham et. al. 1988).

During murine development, prolactin and growth hormone gene transcripts are first detected in the pituitary on embryonic day 18 (e18) (Simmons et. al. 1990). However, prolactin gene transcripts reach maximal levels postnatally (Simmons et. al. 1990). The first pit-1 gene transcripts are detected on day e15.5-16, immediately preceding the appearance of prolactin and growth hormone mRNAs (Simmons et. al. 1990). Mice lines expressing a functionally altered Pit-1 protein suffer from pituitary hypoplasia and combined pituitary hormone deficiency, demonstrating the importance of Pit-1 for pituitary cell proliferation during development (Li et. al. 1990, Radovick et. al. 1992, Shibayama et. al. 1993). Interestingly, identification of a missense (Pfäffle et. al. 1992), or a nonsense (Tatsumi et. al. 1992) mutation in the human pit-1 gene results in a combined hormone deficiency without pituitary hypoplasia (see Discussion). It has been suggested that in the Snell and Jackson dwarf mice, the pituitary hypoplasia it the result of a point mutation in Pit-1 that renders it unable to trans-activate the gene encoding the receptor for growth hormone releasing factor (Lin et. al. 1992). Additionally, the Pit-1 protein regulates expression of its own gene (Chen et. al. 1990, McCormick et. al. 1990, Rhodes et. al. 1993), has been demonstrated to be involved in regulation of the prolactin gene by TRH (Yan et. al. 1991) and is required, along with a thyrotroph-specific isoform of the Pit-1 protein, for maximal expression of the TSHβ gene (Haugen et. al. 1993, Haugen et. al. 1994, Lin et. al. 1994).

The pit-1 gene encodes a 31-33kDa protein that is a member of the POU domain family of transcription factors (Bodner et. al. 1988, Ingraham et. al. 1988). The Pit-1

POU domain is an ~160 amino acid region that is highly homologous to regions of the mammalian octamer binding proteins and the C. elegans protein, unc-86 (Clerc et. al. 1988, Finney et. al. 1988, Herr et. al. 1988, Sturm et. al. 1988). The POU domain is subdivided into the $POU_{specific}$ domain, which is unique to POU family members and which is joined by a variable length linker region to the POU_{homeo} domain, which is similar to the homeodomain containing proteins of Drosophila (Herr et. al. 1988). The Pit-1 POU_{homeo} domain has been shown to be necessary for low-affinity, promiscuous DNA binding (Ingraham et. al. 1990), whereas the Pit-1 $POU_{specific}$ domain has been shown to be necessary for high-affinity, sequence-specific DNA binding, as well as homodimer and Pit-1/Oct-1 heterodimer formation (Ingraham et. al. 1990, Voss et. al. 1991a, Verrijzer et. al. 1992). Furthermore, the Pit-1 $POU_{specific}$ domain contains a putative nuclear localization signal (Theill et. al. 1989). Although not extensively characterized, an NH_2-terminal region of Pit-1, rich in serine and threonine amino acids, has been shown to be necessary for trans-activation (Theill et. al. 1989).

Alignment of the Pit-1 binding sites of the rat prolactin and growth hormone gene proximal promoters yields a consensus binding site with the sequence TATNCAT (Nelson et. al. 1988). Interestingly, Pit-1 and several of the octamer binding proteins exhibit overlapping binding specificities in vitro (Elsholtz et. al. 1990, Aurora and Herr 1992). However, in transient transfection assays, Pit-1 is unable to trans-activate a reporter gene containing an octamer binding site and vice-versa (Elsholtz et. al. 1990), suggesting that the function of the POU domain is not limited to DNA binding. The amino acid sequence of the POU domain is extremely similar among POU family members (reviewed in Herr et. al. 1988, Rosenfeld 1991, Wegner et. al. 1993), allowing structural determinations of one POU domain-containing protein to be extended to other POU family members. Nuclear magnetic resonance spectroscopy studies have revealed that the Oct-1 POU domain, like the DNA binding domain of the bacteriophage λ repressor, adopts a helix-loop-helix motif in solution (Assa-Munt et. al. 1993). The

$POU_{specific}$ and POU_{homeo} domains both make specific nucleotide contacts (Verrijzer et. al. 1992, Voss et. al. 1993) and domain swapping experiments between POU family members have demonstrated that both the $POU_{specific}$ and POU_{homeo} domains contribute to DNA binding specificity (Aurora and Herr 1992).

Phosphorylation of transcription factors by cellular kinases has long been known to be important for temporal regulation of DNA binding and trans-activation activities (reviewed in Hunter and Karin 1992, Karin 1992). Pit-1 is phosphorylated in response to cAMP and phorbol esters in vivo and protein kinases A and C in vitro (Kapiloff et. al. 1991). However, phosphorylation of Pit-1 is not required for estrogen-, forskolin- and phorbol ester-dependent increases in prolactin gene transcription (Fischberg et. al. 1994). Treatment of cultured cells with TRH causes Pit-1 to be transiently phosphorylated, but phosphorylation of Pit-1 is not required for increased prolactin gene transcription (Howard and Maurer 1994). Although Pit-1 binding sites have been shown to be capable of conferring responsiveness to cAMP, phosphorylation of Pit-1 is not required for the transcriptional increase (Okimura et. al. 1994). Additionally, phosphorylation of Pit-1 is not required for basal expression of both the prolactin and growth hormone genes (Fischberg et. al. 1994). Phosphorylation of Pit-1 has variable effects on its affinity for DNA depending on which Pit-1 binding site of the rat prolactin or growth hormone gene promoters was examined (Kapiloff et. al. 1991, Okimura et. al. 1994). Furthermore, phosphorylation of Pit-1 has been implicated in hormonal regulation of the TSHβ gene, presumably by altering its affinity for DNA (Steinfelder et. al. 1992). However, phosphorylation of Pit-1 has not yet been directly shown to be important for regulation of prolactin or growth hormone gene expression in vivo.

The liver-enriched, functionally antagonistic transcription factors, LAP and LIP, have been shown to be encoded by the same mRNA (Descombes and Schibler 1991). Protein synthesis can be initiated from one of two in-frame translation initiation sites and preference for either site appears to be developmentally regulated (Descombes and

Schibler 1991). Similarly, by in vitro transcription and translation, or by Western analysis, the Pit-1 protein is routinely seen as a 31-33KDa doublet (see Results). It has been demonstrated that this doublet is the result of alternate translation initiation site usage and both the 31KDa- and 33KDa versions of Pit-1 are equally capable of trans-activating the prolactin gene (Voss et. al. 1991b). Therefore, these two species may be considered isoforms of Pit-1, although no physiological significance of this alternate translation site usage has been determined (see Discussion).

It has been previously shown that transcription factor isoforms can be generated by alternative splicing (reviewed in Koenig et. al. 1989, Foulkes and Sassone-Corsi 1992, Hsu et. al. 1992, Skipper et. al. 1993, Stehle et. al. 1993, Sohn et. al. 1994) and in several cases, these isoforms have been shown to have distinct functional properties (Koenig et. al. 1989, Hsu et. al. 1992, Skipper et. al. 1993, Stehle et. al. 1993). Alternatively spliced isoforms of other POU domain-containing proteins have been identified (Hatzopoulos et. al. 1990, Treacy et. al. 1991, Treacy et. al. 1992). Pit-1T, the aforementioned thyrotrope-specific Pit-1 isoform, is thought to arise as a result of alternative splicing of the pit-1 gene primary transcript (Haugen et. al. 1993). Pit-1T contains an in-frame, 14 amino acid insertion in the trans-activation domain of Pit-1 and has been shown to act synergistically with Pit-1 to trans-activate the TSHβ gene (Haugen et. al. 1993, Haugen et. al. 1994).

More recently, it has been demonstrated that in addition to Pit-1, maximal expression of the growth hormone gene requires the transcription factor Zn-15 (Lipkin et. al. 1993). Zn-15 is an unusual transcription factor that contains fifteen putative zinc fingers (Lipkin et. al. 1993). However, expression of the Zn-15 gene is not restricted to somatotrophs (see Results, Lipkin et. al. 1993) and therefore cannot solely be responsible for cell type-specific expression of the growth hormone gene (see Discussion).

The search for additional factors involved in regulating transcription of the prolactin and growth hormone genes has led to the detection of two additional Pit-1 isoforms. The first isoform of Pit-1 to have been described is Pit-1a (Pit-1β, GHF-2) (Konzak and

Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Pit-1a has been shown to arise as a result of alternative splicing of the pit-1 gene primary transcript and its transcript is detected in the same cell types which contain the Pit-1 mRNA (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). The result is an in-frame, 26 amino acid insertion in the region of Pit-1 shown to be necessary for trans-activation (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Like the rest of the trans-activation domain of Pit-1, the region unique to Pit-1a is serine and threonine rich (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Although the Pit-1a protein has been shown to bind to the same sites of the prolactin and growth hormone gene proximal promoters as Pit-1, Pit-1a is unable to trans-activate the prolactin gene (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). However the Pit-1a protein has been demonstrated to be capable of trans-activating the growth hormone gene (Konzak and Moore 1992, Theill et. al. 1992), in one report, even better than Pit-1 (Konzak and Moore 1992).

The third and perhaps most unusual of the Pit-1 isoforms yet identified, is Δ4Pit-1 (see Results, Voss et. al. 1993, Day and Day 1994b, Day and Day 1994a). Δ4Pit-1 is identical to Pit-1 except that it contains a 54 amino acid, in-frame deletion, representing the loss of a majority of the $POU_{specific}$ domain (Voss et. al. 1993, Day and Day 1994b, Day and Day 1994a). Like the other Pit-1 isoforms, Δ4Pit-1 is thought to arise as a result of alternative splicing of the pit-1 gene precursor RNA, since the deleted region exactly matches exon IV of the mouse pit-1 gene (Lin et. al. 1993, Voss et. al. 1993, Day and Day 1994b, Day and Day 1994a). There are conflicting reports regarding the distribution of Δ4Pit-1. One group detects the Δ4Pit-1 transcript in several rat anterior pituitary cell lines and in the adult rat pituitary (Voss et. al. 1993). However, others suggest that expression of Δ4Pit-1 is restricted to somatomammotropic GH_3 cells passaged as a tumor in rats (Day and Day 1994b, Day and Day 1994a), or to GH_3 cells maintained in culture (see Results). The Δ4Pit-1 protein cannot bind to several of the previously identified

Pit-1 binding sites of the rat prolactin or growth hormone gene proximal promoters (see Results, Voss et. al. 1993), nor can it trans-activate the prolactin or growth hormone genes (Voss et. al. 1993, Fischberg 1994). Using random oligonucleotide selection, one group has identified an artificial Δ4Pit-1 binding site which is also recognized by Pit-1 (Voss et. al. 1993), but a target gene promoter containing this sequence has yet to have been identified. It has been demonstrated that the Δ4Pit-1 protein can specifically repress transcription of the prolactin gene, presumably by forming heterodimers with Pit-1 and preventing it from binding DNA (Day and Day 1994a). The Pit-1 isoforms are shown schematically in **Figure 3**.

In addition to identification of the Pit-1 isoforms, detailed analysis of several rat anterior pituitary cell lines and adult rat pituitaries was performed. The distribution of the Pit-1 isoform transcripts and proteins was compared to the distribution of the prolactin and growth hormone transcripts among the same cell lines in hopes of better understanding how the Pit-1 isoforms are involved in regulating the expression of these two genes. Through these studies two absolute mammotropic cell lines were identified. These two cell lines, 235-1 and MMQ, as well as the adult rat pituitary, all contain the Pit-1 and Pit-1a transcripts, but none express the Δ4Pit-1 transcript. Interestingly, the 235-1 cell line does not contain any detectable Pit-1 protein (see Results and Discussion). The Δ4Pit-1 transcript and protein are detected only in the GH_3 cell line. Furthermore, these cells are also the only ones examined which express both the growth hormone and prolactin genes, providing correlative evidence that the Δ4Pit-1 protein may be involved in the cell type-specific expression of the growth hormone gene (see Results and Discussion).

Although it is unclear by what mechanisms these proteins exert their effect, it appears that complex interplay between the Pit-1 isoforms is responsible for the cell type-specific expression of the prolactin and growth hormone genes. Furthermore, this data supports the hypothesis that additional, positive- and negative-acting factors are necessary for

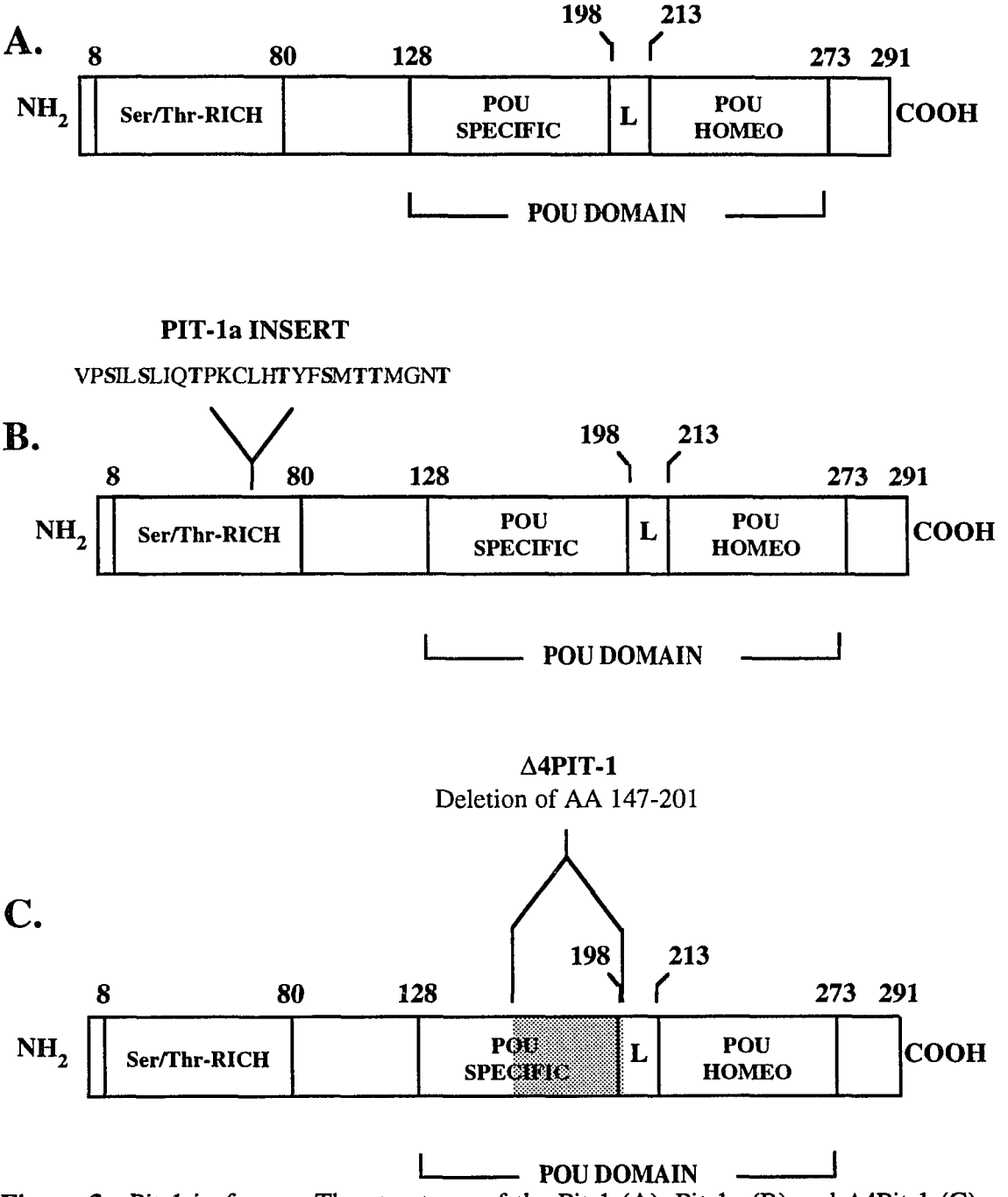


Figure 3. Pit-1 isoforms. The structures of the Pit-1 (A), Pit-1a (B) and Δ4Pit-1 (C) isoforms are shown. The serine and threonine-rich trans-activation domain was identified by inspection of the Pit-1 amino acid sequence. The POU domain and its $POU_{specific}$- and POU_{homeo} subdomains were identified by comparison with the amino acid sequence of other POU domain family members. The amino acid sequence encoded by the exon unique to Pit-1a is indicated. Serine and threonine residues present in the Pit-1a insert are in boldface type. The shaded region in Δ4Pit-1 represents those sequences encoded by exon IV of the pit-1 gene that are lacking in the Δ4Pit-1 isoform.

restricting expression of these genes to the appropriate cell type (see Discussion, Larson et. al. 1986, Pan et. al. 1990, Jackson et. al. 1992).

MATERIALS AND METHODS

All reagents and chemicals were from Fisher or Sigma, unless indicated otherwise, although some specialty items from these same manufacturers are given. All oligonucleotides were synthesized by the Brookdale Center for Molecular Biology DNA Core Facility (Mount Sinai School of Medicine), or by Genset.

Isolation of RNA

Total RNA was isolated from tissue culture cells and adult Wistar rat pituitaries (Rockland) by guanidinium isothiocyanate lysis/cesium chloride gradient centrifugation (Ausubel et. al. 1992). Approximately 10^8 cells, from a 150mm tissue culture dish, were washed twice with PBS (137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H_2O$ and 1.4mM KH_2PO_4, pH~7.3) and lysed directly on the plates by the addition of 3.5ml guanidinium solution (4M guanidinium isothiocyanate (BRL), 20mM NaOAc pH 5.2, 0.1mM DTT and 0.5% Sarkosyl). The cells were removed by scraping and the lysates transferred to 50ml polypropylene centrifuge tubes. Chromosomal DNA was sheared by passing the lysates through a 20 gauge needle several times. To prepare RNA from adult rat pituitaries, the tissue was first homogenized in a motor-driven Teflon/glass homogenizer (3.5ml guanidinium solution per five pituitaries) before shearing of the chromosomal DNA. The lysates were layered on 1.5ml cushions of 5.7M CsCl (BRL) in 0.1M EDTA pH 8.0, in autoclaved 13x51mm ultracentrifuge tubes (Beckman). RNAs were pelleted by centrifugation at 35,000 RPM (150,000xg) in a Beckman L7 ultracentrifuge for at least 12 hours at 18°C. The supernatants were removed and the RNA pellets resuspended in 350ul TES (10mM Tris-HCl pH 7.4, 5mM EDTA and 1% SDS). Following the addition of 40ul 3M NaOAc, the RNAs were extracted twice with phenol/chloroform and precipitated by the addition of 1ml 100% ethanol. The RNA pellets were resuspended in 360ul sterile water, extracted twice with phenol/chloroform and reprecipitated with

ethanol. The final RNA pellets were resuspended in sterile water. RNA concentrations were determined by A_{260}/A_{280} (Beckman) and stored in aliquots at -80°C.

The quality of each RNA preparation was examined by separating 5-10ug of total RNA from each cell line or tissue, alongside RNA size standards (BRL), through 1.4% agarose gels containing 0.66M formaldehyde. 28S and 18S ribosomal RNAs were visualized by ethidium bromide staining (Fourney et. al. 1988).

RT-PCR analysis and Southern hybridization

Prior to their addition to the reverse transcription reactions, one (cultured cells) or five (pituitary) micrograms total RNA were denatured by heating to 75°C for five minutes. The heat denatured RNA, antisense primer (20uM final concentration), dNTPs (Pharmacia, 2mM final concentration) and MMLV reverse transcriptase (BRL) were combined on ice. First strand cDNAs were synthesized by incubation for one hour at 37°C. (See Table I for a list of oligonucleotide primers used for RT-PCR/Southern hybridization analysis.)

First strand cDNA reactions were added directly to the amplification reactions, which had been pre-assembled on ice. Each reaction contained two units Taq DNA polymerase (Promega), dNTPs (Pharmacia, 0.2mM final concentration) and sense primer (2uM final concentration) in a total volume of 100ul. Each reaction was overlaid with two drops of mineral oil (Sigma). cDNAs were amplified by incubation at 95°C, one minute, 55°C, one minute and 72°C, one minute, for fifty cycles, using a thermal cycler (Perkin-Elmer). Amplification reactions were extracted twice with phenol/chloroform and precipitated with ethanol. The DNA was resuspended in TE pH 8.0 (10mM Tris pH 8.0, 1mM EDTA) and a portion of each of the amplified products was separated by electrophoresis through 1.4% agarose gels or 15% native polyacrylamide gels. The electrophoresis buffer for both agarose and polyacrylamide gel electrophoresis was 1X TBE (1X TBE is 89mM Tris, 89mM boric acid and 2mM EDTA).

Amplified products separated through agarose were transferred to Zeta-Probe membranes (Bio-Rad) by capillary transfer in 0.4N NaOH. Amplified products separated through acrylamide were electrophoretically transferred to Zeta-Probe membranes in 0.3X TBE, using a semi-dry transfer apparatus (Hoefer Scientific). Following electrophoretic transfer, the amplified products were denatured and fixed by soaking the membranes in 0.4N NaOH for one hour. All filters were neutralized in 6X SSC (20X SSC is 3M NaCl and 0.3M Na_3citrate·$2H_2O$, pH 7.0 with HCl) for 30 minutes and prehybridized for at least four hours in 6X SSC, 10X Denhardt's (50X Denhardt's is 5% ficoll, 5% polyvinylpyrrolidone and 5% BSA) and 0.2% SDS (Boehringer Mannheim) at 50°C. Oligonucleotide probes were radiolabelled with [γ-^{32}P]ATP (New England Nuclear) and T4 polynucleotide kinase, according to the manufacturer's directions (New England Biolabs). Unincorporated label was removed using NenSorb 20 columns (New England Nuclear) and the labelled oligonucleotide eluted in 1ml 50% ethanol, according to the manufacturer's directions. The probe was evaporated in a Speed-Vac concentrator (Savant) and resuspended in 200ul sterile water. Incorporation of label was determined by liquid scintillation counting (Beckman). The probe was added to a specific activity of $5x10^5$-10^6cpm per milliliter hybridization solution (identical to prehybridization solution) and allowed to hybridize overnight at 50°C. Filters were washed extensively (Ausubel et. al. 1992) and exposed to film for 1-4 hours at -80°C, with an intensifying screen.

Preparation of nuclear and whole cell extracts

All buffers used for preparation of nuclear and whole cell extracts included 1uM each of leupeptin and pepstatin A and 1mM PMSF to inhibit proteolysis, as well as 0.5-1mM DTT (all from Sigma). All procedures were carried out as quickly as possible at 4°C. Nuclear extracts were prepared essentially as described (Dignam et. al. 1983). Cells were removed from tissue culture dishes by treatment with trypsin/EDTA (Gibco), or by scraping into PBS, transferred to a 15ml polypropylene tube and pelleted by

Table I. Oligonucleotides used

The oligonucleotides used throughout this work are provided here for reference. The Bancroft laboratory's code, target gene and sequence of each of the oligonucleotides are indicated. A brief description of each oligonucleotide is provided. Legends to figures throughout this work where oligonucleotides were used will be referred to by their code. Underlined sequences are those that differ from wild type target gene sequences. Target gene sequences contained within each oligonucleotide are given. *Numbering is as follows: 'a', the transcription start site is +1, 'b', the translation start site is +1.

oligo. name	target gene*	oligo. sequence	notes
CB1	rat prolactin -206 to -183[a]	5'-TCGACGCTGTAATTA ATCAAAATCC-3'	CB1 and CB2 are complementary and contain site 4P of the rat prolactin gene proximal promoter for gel-shift analysis.
CB2	rat prolactin	5'-TCGAGGATTTTGATT AATTACAGCG-3'	see above
CB3	rat prolactin -69 to -35[a]	5'-TCGACTGCCTGATTA TATATATATTCATGAA GGTGTCGAAC-3'	CB3 and CB4 are complementary and contain site 1P of the rat prolactin gene proximal promoter for gel-shift analysis.
CB4	rat prolactin	5'-TCGAGTTCGACACCT TCATGAATATATATAT AATCAGGCAG-3'	see above
CB10	rat prolactin -166 to -141[a]	5'-TCGACTTCCTGAATA TGAATAAGAAATAA A-3'	CB10 and CB11 are complementary and contain site 3P of the rat prolactin gene proximal promoter for gel-shift analysis.
CB11	rat prolactin	5'-TCGATTTATTTCTTAT TCATATTCAGGAAG-3'	see above
CB19	rat growth hormone -99 to -67[a]	5'-TCGACTGGCTCCAGC CATGAATAAATGTATA GGGAAAG-3'	CB19 and CB20 are complementary and contain site GH-1 of the rat growth hormone gene proximal promoter for gel-shift analysis.
CB20	rat growth hormone	5'-TCGACTTTCCCTATA CATTTATTCATGGCTGG AGCCAG-3'	see above

CB23	rat pit-1 +277 to +297[b]	5'-GACCACACCCTGAGT CATGGG-3'	Used for detection of rat Pit-1 and Δ4Pit-1 RT-PCR products by Southern hybridization.
CB24	rat prolactin -128 to -112[a]	5-TCGACCATTTGATGT TTAAAATTATTGGGG-3'	CB24 and CB25 are complementary and contain site 2P of the rat prolactin gene proximal promoter for gel-shift analysis.
CB25	rat prolactin	5'-TCGACCCCAATAATT TTAAACATCAAATGG-3'	see above
CB45	rat prolactin -69 to -35[a]	5'-TCGAGTGCCGTCGTA TATATATATTCATGAA GGTGTCGAAC-3'	CB45 and CB46 are complementary and contain a mutated site 1P of the rat prolactin gene proximal promoter (designated *1P) for gel-shift analysis.
CB46	rat prolactin	5'-TCGAGTTCGACACCT TCATGAATATATATAT ACGACGGCAC-3'	see above
CB47	rat prolactin -69 to -35[a]	5'-TCGAGTGCCTGATTA TATATATAGGACTGAA GGTGTCGAAC-3'	CB47 and CB48 are complementary and contain a mutated site 1P of the rat prolactin gene proximal promoter (designated 1P*) for gel-shift analysis.
CB48	rat prolactin	5'-TCGAGTTCGACACCT TCAGTCCTATATATATA ATCAGGCAC-3'	see above
CB52	rat prolactin -69 to -35[a]	5'-TCGAGTGCCGTCGTA TATATATAGGACTGAA GGTGTCGAAC-3'	CB52 and CB53 are complementary and contain a mutated site 1P of the rat prolactin gene proximal promoter (designated *1P*) for gel-shift analysis.
CB53	rat prolactin	5'-TCGAGTTCGACACCT TCAGTCCTATATATATA CGACGGCAC-3'	see above

CB58	rat growth hormone -99 to -67[a]	5'-TCGACTGGCTCCAGC CAGTCCTAAATGTATA GGGAAAG-3'	CB58 and CB59 are complementary and contain a mutated site GH-1 of the rat growth hormone gene proximal promoter (designated *GH-1) for gel-shift analysis.
CB59	rat growth hormone	5'-TCGACTTTCCCTATA CATTTAGGACTGGCTG GAGCCAG-3'	see above
CB62	rat growth hormone -99 to -67[a]	5'-TCGACTGGCTCCAGC CATGAAGCCCGGTATA GGGAAAG-3'	CB62 and CB63 are complementary and contain a mutated site GH-1 of the rat growth hormone gene proximal promoter (designated GH-1*) for gel-shift analysis.
CB63	rat growth hormone	5'-TCGACTTTCCCTATA CCGGGCTTCATGGCTG GAGCCAG-3'	see above
CB74	rat pit-1 +1 to +22[b]	5'-ATGAGTTGCCAACCT TTCACCT-3'	Sense strand primer used for amplification of rat Pit-1 isoform mRNAs by RT-PCR.
CB78	rat pit-1 +643 to +624[b]	5'-TCTTCCTTTCGTTTGC TCCC-3'	Antisense primer used for amplification of rat Pit-1 isoform mRNAs by RT-PCR.
CB80	rat pit-1 +1 to +13[b]	5'-TCGGGATCGAGGGA CATATGAGTTGCCAAC-3'	Sense strand primer for amplification of rat Pit-1 isoforms by PCR. Contains NdeI site for subcloning.
CB81	rat pit-1 +917 to +901[b]	5'-GGAATGGAAAGGGA TCCACACATGGCTACC-3'	Antisense primer for amplification of rat Pit-1 isoforms by PCR. Contains BamHI site for subcloning.
CB92	rat pit-1a +215 to +191[b]	5'-TTTCCCATCGTTGTC ATCGAGAAT-3'	Used for detection of rat Pit-1a RT-PCR products by Southern hybridization.
CB101	rat growth hormone +39 to +58[a]	5'-GTGGACAGATCACTG AGTGG-3'	Sense strand primer for amplification of rat growth hormone mRNA by RT-PCR.

CB102	rat growth hormone +714 to +695[a]	5'-TGCCTAGAAAGCAC AGCTGC-3'	Antisense primer for amplification of rat growth hormone mRNA by RT-PCR.
CB103	rat growth hormone +351 to +370[a]	5'-CATGGAATTGCTTCG CTTCT-3'	Used for detection of rat growth hormone RT-PCR products by Southern hybridization.
CB104	rat prolactin +15 to +34[a]	5'-GACTTCTTG GGGAAGTGTGG-3'	Sense strand primer for amplification of rat prolactin mRNA by RT-PCR.
CB105	rat prolactin +712 to +693[a]	5'-GGACAATTTGGCACC TCAGG-3'	Antisense primer for amplification of rat prolactin mRNA by RT-PCR.
CB106	rat prolactin +339 to +358[a]	5'-ACAAGCCCAGAAAG TCCCTC-3'	Used for detection of rat prolactin RT-PCR products by Southern hybridization.
CB111	rat Zn-15 +2857 to +2876[b]	5'-AGGCTTTAGAAACTG CTGGC-3'	Sense strand primer for amplification of rat Zn-15 mRNA by RT-PCR.
CB112	rat Zn-15 +3842 to +3802[b]	5'-GGAAAGAGAACGAC TGAGAAG-3'	Antisense primer for amplification of rat Zn-15 mRNA by RT-PCR
CB113	rat Zn-15 +3325 to +3389[b]	5'-GGTACCAACTGACTT AACAATGGG-3'	Used for detection of rat Zn-15 RT-PCR products by Southern hybridization.
CB123	rat pit-1a +325 to +344[b]	5'-CCCGTCTATTTTGTC TTTGATCC-3'	Used for detection of rat Pit-1a RT-PCR products by Southern hybridization.
CB135	rat pit-1 +1 to +20[b]	5'-GGACTAGTAATGTAC CCATACGACGTCCCAG ACTACGCTATGAGTTG CCAACCTTTCAC-3'	Sense strand primer for generation of epitope-tagged Pit-1 isoforms by PCR. Contains influenza hemagglutinin epitope 'tag', 'Kozak sequence', translation initiation site and an SpeI site for subcloning.

CB136	rat pit-1 +1186 to +1163[b]	5'-CATATATGGCGGCCG CTCTCCCAG-3'	Antisense primer for generation of epitope-tagged Pit-1 isoforms by PCR. Contains a NotI site for subcloning.
CB143	rat pit-1 +904 to +881[b]	5'-TACCACACGCGGCCG CCACAGGCA-3'	Antisense primer for generation of epitope-tagged Pit-1 isoforms by PCR. Contains a NotI site for subcloning.

centrifugation. The collected cells were then washed twice with ice-cold PBS. The volume of the packed cells was estimated and the cells resuspended in five packed cell volumes of ice-cold hypotonic buffer (10mM Hepes pH 7.9, 1.5mM $MgCl_2$ and 10mM KCl). The cells were pelleted by centrifugation and resuspended in three packed cell volumes of ice-cold hypotonic buffer and allowed to swell on ice for ten minutes. Cells were disrupted by at least ten strokes in a glass/glass Dounce homogenizer, using a tight fitting pestle. Lysis was monitored by examining a portion of the lysate under a microscope and homogenization continued until a majority of the cells had been disrupted. The disrupted cells were transferred to a 15ml Corex™ tube and the nuclei collected by centrifugation at 3300xg for 15 minutes in a Sorvall SS-34 rotor. The supernatant (cytoplasmic fraction) was removed, transferred to a 1.5ml microfuge tube, quick frozen on dry ice and stored at -80°C. The volume of the nuclear pellet was estimated and the nuclei resuspended in a volume of ice-cold low-salt buffer (20mM Hepes pH 7.9, 25% glycerol, 1.5mM $MgCl_2$, 0.02M KCl and 0.2mM EDTA) equal to 1/2 of the packed nuclear volume. While gently vortexing, a volume of ice-cold high-salt buffer (high-salt buffer is identical to low-salt buffer except it contains 1.2M KCl) equal to 1/2 of the packed nuclear volume was added drop-wise. The nuclei were homogenized by one or two strokes in a glass/glass Dounce homogenizer, using a tight fitting pestle and extracted by rocking at 4°C for 30 minutes. The extract was transferred to a 15ml Corex™ tube and nuclear debris removed by centrifugation at 25,000xg for 30 minutes in

a Sorvall SS-34 rotor. The supernatant (nuclear extract) was transferred to dialysis tubing with a 12,000-14,000 molecular weight cutoff (Spectropor) and dialyzed for 2X2 hours against 1000 volumes dialysis buffer (20mM Hepes pH 7.9, 20% glycerol, 100mM KCl and 0.2mM EDTA) at 4°C. The dialysate was transferred to a 1.5ml microfuge tube and insoluble material removed by centrifugation at 14,000rpm in a microcentrifuge (Brinkmann). The protein concentration was determined by the method of Bradford (Bio-Rad, Bradford 1976), using BSA as a standard, divided into aliquots, quick frozen on dry ice and stored at -80°C.

To prepare whole cell extracts, cells from a 150mm tissue culture dish were washed twice with ice-cold PBS, removed by scraping into PBS and collected by centrifugation. The cells were resuspended in 0.75ml hypotonic buffer (10mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 1mM EDTA and 1mM EGTA) and allowed to swell on ice for 30 minutes. Cells were disrupted by sonication for 2X2 minutes at 4°C and homogenized by ten strokes in a glass-glass Dounce homogenizer, using a tight fitting pestle. The homogenate was brought to 1M KCl by the drop-wise addition of 0.25ml 4M KCl and extracted by rocking for 30 minutes at 4°C. Debris was removed by centrifugation and the extracts dialyzed for 2X2 hours against 1000 volumes of 'gel-shift buffer' (10mM Tris pH 8.0, 100mM KCl, 5mM $MgCl_2$ and 10% glycerol). The dialysate was transferred to a 1.5ml microcentrifuge tube and insoluble material pelleted by centrifugation. Protein concentrations of the whole cell extracts were determined by the method of Bradford (Bradford 1976). The extracts were divided into aliquots, quick frozen on dry ice and stored at -80°C.

Western analysis

Western analysis was performed using a polyclonal Pit-1 antibody (generous gift of Dr. Richard Maurer, Oregon Health Sciences University) or an affinity purified antibody. To prepare the affinity purified antibody, the peptide NH_2-ANEFKVRIKLGUTQ-

COOH, corresponding to amino acids 136-150 of Pit-1, was cross-linked to BioGel 10 beads (Bio-Rad) according to the manufacturer's directions. Rabbit antiserum, raised against the same peptide (Pocono Rabbit Farms), was passed three times over the affinity column (1ml bed volume). The affinity column was washed with buffer (100mM Tris pH 7.5, 150mM NaCl and 1mM NaN_3), and bound antibody eluted with 5ml 3.5M $MgCl_2$. The eluted antibody was dialyzed for 2X24 hours against 400 volumes of the column buffer. The affinity purified antibody was then concentrated by ultrafiltration (Amicon), divided into aliquots and stored at -20°C.

Protein samples for Western analysis were separated through denaturing polyacrylamide gels (Laemlli 1970), containing 10-15% acrylamide (Schwarz-Mann Biotech), alongside molecular weight standards (Amersham), using a mini-gel apparatus (Bio-Rad). The separated proteins were then electrophoretically transferred (Hoefer Scientific) to nitrocellulose (Schleicher and Schuell) for two hours in Towbin buffer ((Towbin et. al. 1979), 25mM Tris pH 8.0, 192mM glycine, 0.04% SDS and 20% methanol). The nitrocellulose filters were blocked for at least two hours in 20ml TBST (10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween 20), containing 7.5% Carnation non-fat dry milk. The blocking solution was removed and the primary antibody added at a 1:10,000 dilution in 20ml TBST, containing 1% dry milk and allowed to incubate at room temperature overnight. The filter was washed for 3X10 minutes with 20ml TBST and immunoreactive proteins detected by enhanced chemiluminescence (ECL), according to the manufacturer's directions (Amersham). In order to reprobe filters, bound antibodies were removed by incubating the filter in stripping buffer (62.5mM Tris pH 6.8, 2% SDS and 100mM β-Mercaptoethanol) for 2X30 minutes at 50°C. Antibodies for detection of actin Boehringer Mannheim and Dr. Jörg Heierhorst) were used at a 1:2,000 dilution.

In vitro transcription

Capped mRNAs, encoding each of the Pit-1 isoforms, were transcribed in vitro as previously described (Morris et. al. 1992). The template used for in vitro transcription of the Pit-1 cDNA has been previously described (Yan et. al. 1991). The Pit-1 template was linearized by digestion with restriction enzyme StyI (New England Biolabs) and transcribed in vitro with SP6 RNA polymerase (New England Biolabs) to produce an mRNA of approximately 1500 nucleotides. The cloning of the Pit-1a cDNA has been previously described (Morris et. al. 1992). The Pit-1a template was linearized by digestion with restriction enzyme BamHI (New England Biolabs) and transcribed in vitro with T7 RNA polymerase (New England Biolabs) to produce an mRNA of approximately 1800 nucleotides. The Δ4Pit-1 cDNA was obtained by PCR amplification of GH_3 cell total RNA using oligonucleotide primers specific for sequences within the 5'- and 3'-ends of the published rat Pit-1 cDNA sequence (Ingraham et. al. 1988). The amplified product was digested with PstI and HindIII (New England Biolabs) and the 1015 bp fragment ligated into pGEM4z (Promega) that had been digested with PstI and HindIII (A. E. Jackson and C. Bancroft, unpublished data). The Δ4Pit-1 template was linearized by digestion with SmaI (New England Biolabs) and transcribed in vitro using T7 RNA polymerase (New England Biolabs) to produce an mRNA of approximately 1100 nucleotides. The RSV-CREB construct was kindly provided by Dr. Richard Goodman (Oregon Health Sciences University). The CREB cDNA was excised by digestion with HindIII and XbaI (New England Biolabs) and ligated into pGEM7z (Promega) that had been digested with HindIII and XbaI (J. Tian and C. Bancroft, unpublished data). The CREB template was linearized by digestion with EcoRI (New England Biolabs) and transcribed in vitro using T7 RNA polymerase (New England Biolabs) to produce an mRNA of approximately 1000 nucleotides. Each in vitro transcription reaction contained 40mM Tris-HCl pH 7.9, 6mM $MgCl_2$, 2mM spermidine (Sigma), 15ug linearized template DNA, 1mM DTT, 0.1mg/ml BSA, 0.5mM each ATP, CTP and UTP, 0.02mM

GTP (Pharmacia), 0.5mM $m^7G(5')ppp(5')G$ cap structure analog (New England Biolabs), 1unit/ul RNAsin Promega) and 115 units of the appropriate RNA polymerase. Reactions were incubated at either 37°C (T7 RNA polymerase), or 40°C (SP6 RNA polymerase) for two hours. Template DNA was removed by the addition of 400 units RNAsin and 15 units RQ1 RNAse-free DNAse (Promega) and incubation at 37°C for 15 minutes. The reactions were extracted twice with phenol/chloroform and unincorporated nucleotides removed by spin column chromatography through Sephadex G-25 (Pharmacia). The RNAs were re-extracted twice with phenol/chloroform and precipitated by the addition of 1ml 100% ethanol. The in vitro transcribed mRNAs were resuspended in sterile water. The RNA concentrations were determined by A_{260}/A_{280} and the in vitro transcribed mRNAs were stored at -80°C.

The quality of the RNA preparations was examined by separating 0.5ug of each in vitro transcribed RNA by electrophoresis, alongside RNA size standards (BRL), through 1.4% agarose gels, containing 0.66M formaldehyde. The in vitro transcribed mRNAs were visualized by ethidium bromide staining (Fourney et. al. 1988).

Preparation of wheat germ lysate and in vitro translation

Wheat germ lysate for cell-free translation was prepared essentially as described (Erickson and Blobel 1983). All necessary glassware, tubes, Pasteur pipettes, buffers, etc. were autoclaved to remove any contaminating RNAses and precooled to 4°C. All procedures were carried out as rapidly as possible at 4°C. Seven milliliters of extraction buffer (10mM Tris pH 7.6 with acetic acid, 90mM KOAc, 3mM MgOAc and 1mM DTT) was added to one gram of wheat germ (General Mills, Inc.) in a mortar on ice and ground to a paste with a pestle. The ground wheat germ/extraction buffer slurry was transferred to a 15ml Corex™ tube and insoluble material removed by centrifugation in a Sorvall SS-34 rotor at 14,000rpm for ten minutes at 4°C. The lipid layer was removed by aspiration and the supernatant transferred to a clean 15ml Corex™ tube. The supernatant

volume was measured and the salt concentration adjusted by the addition of 200ul 1M Tris-acetate pH 7.6 and 20ul 1M MgOAc, per 10ml extract. Insoluble material was removed by centrifugation at 14,000rpm for ten minutes at 4°C. The supernatant was applied to a 25x1.5cm column bed of Sephadex G-25 (Pharmacia), pre-equilibrated with column elution buffer (1mM Tris pH 7.6 with acetic acid, 50mM KOAc, 1mM MgOAc and 280ul β-Mercaptoethanol per liter of buffer was added just before use) and 1ml fractions were collected. The first six void volume fractions were pooled (beginning with the first fraction that precipitates in 10% TCA), transferred to a 15ml Corex™ tube and insoluble material removed by centrifugation at 14,000rpm for 10 minutes at 4°C. The supernatant was removed, transferred to a 15ml polypropylene tube, divided into 100ul aliquots and quick frozen in liquid nitrogen. The aliquots were stored at -80°C.

In vitro translation reactions were assembled on ice. Each reaction contained 20mM Hepes pH 7.2, 70mM KOAc, 1.6mM MgOAc, 4mM DTT, 80uM spermine (Sigma), 1.2mM ATP, 0.24mM GTP (Pharmacia), 9.6mM creatine phosphate (Sigma), 64ug/ml creatine phosphokinase (Sigma), 40uM each amino acid (minus methionine), 2.5ul Tran^{35}S-label™ (ICN, ~1000 Ci/mmol), 1ul RNAsin Promega), 10ul wheat germ lysate and 0.1-0.5ug in vitro transcribed, capped mRNA in a final volume of 25ul. Reactions were incubated at 28°C for one hour. In vitro translation reactions using rabbit reticulocyte lysates were carried out according to the manufacturer's directions (Promega). Two microliters of each in vitro translation reaction was spotted on Whatman 3MM cellulose filters. Proteins were precipitated by placing the filters in ice-cold 10% TCA for ten minutes. The filters were then transferred to boiling 5% TCA for five minutes. The filters were washed three times with 10% TCA, once with 95% ethanol and allowed to air dry. TCA precipitable counts were determined by liquid scintillation counting (Beckman). To the remainder of the translation reaction, an equal volume of 20% TCA was added, the proteins precipitated on ice for 20 minutes and pelleted by centrifugation. The pellet was resuspended in 2X sample buffer (100mM Tris pH 6.8,

20% glycerol, 4% SDS (Boehringer Mannheim), 0.2% bromphenol blue and 200mM DTT) and separated by SDS-PAGE. Gels were impregnated with the fluor 2,5-diphenyloxazole (PPO) in DMSO, dried and the in vitro translation products visualized by autoradiography.

For DNA binding studies using in vitro translated Pit-1 and Pit-1a, unlabelled methionine (40uM final concentration) was substituted for [^{35}S]methionine. However, parallel reactions were always carried out using [^{35}S]methionine and incorporation of label was determined by TCA-filter precipitation and liquid scintillation counting as described above. Since Pit-1 is predicted to contain seven methionine residues and Pit-1a is predicted to have nine, this allowed the volumes of unlabelled translation reactions used for subsequent gel-shift analysis to be adjusted so that approximately equal quantities of Pit-1 and Pit-1a could be compared (Morris et. al. 1992).

Preparation of recombinant Pit-1 isoform proteins

Oligonucleotide primers containing an NdeI site (CB80) or a BamHI site (CB81) were synthesized and used to amplify the coding region of each of the Pit-1 isoforms using Vent™ DNA Polymerase (New England Biolabs), according to the manufacturer's specifications. Amplifications were carried out by incubation at 95°C, one minute, 55°C, one minute and 72°C, one minute for 30 cycles. The amplification products were digested with NdeI and BamHI (New England Biolabs) and isolated by electrophoresis on to DEAE paper (Schleicher and Schuell). The DNA was eluted into 400ul high-salt buffer (20mM Tris pH 8.0, 1M NaCl and 0.1mM EDTA), extracted repeatedly with water-saturated butanol and precipitated with ethanol (Ausubel et. al. 1992). Each of the isolated Pit-1 isoform cDNA fragments was ligated into an NdeI/BamHI-digested pET3Xa vector (Novagen). Recombinants were identified by restriction analysis and sequenced by the chain termination method (Sanger et. al. 1977), using Sequenase™ T7

DNA Polymerase (United States Biochemical). Recombinant plasmids were then used to transform the E. Coli expression host BL21(DE3)pLysS (Novagen).

To express each of the recombinant proteins, one liter of TB (25 grams Tryptone (Gibco), 15 grams yeast extract (Gibco) and 4ml glycerol), containing 50ug/ml Carbenicillin Sigma), was inoculated with frozen bacterial stocks expressing each of the isoforms and grown at 37°C to an A_{600} of ~0.6. Isopropyl-1-thio-β-D-galactoside (IPTG, Sigma) was added to a final concentration of 1mM and the cultures incubated for an additional three hours at 30°C. Cells were harvested by centrifugation and resuspended in 20ml of buffer (20mM Tris pH 7.5, 100mM NaCl, 1mM EDTA, 1mM DTT and protease inhibitors) and stored in aliquots at -20°C.

To isolate the recombinant proteins, frozen stocks of induced cells were removed from -20°C and allowed to slowly thaw on ice. The cells were disrupted by sonication for 2X2 minutes at 4°C. Debris was removed by centrifugation and the extract fractionated by precipitation with 20-80% ammonium sulfate (BRL). The precipitated proteins were resuspended in 'gel-shift buffer' and dialyzed for 2X2 hours against 1000 volumes of the same buffer, using 12,000-14,000 molecular weight cutoff dialysis tubing (Spectropor). Protein concentrations were determined by the method of Bradford (Bradford 1976), using BSA as a standard. The partially purified proteins were stored at 4°C.

In order to determine the relative amount of Pit-1 isoform protein present in each extract, varying quantities of each preparation were separated by SDS-PAGE, transferred to nitrocellulose and the recombinant proteins detected by Western analysis as described above. Immunoreactive proteins were quantitated by densitometry of films (LKB 2222-020 Ultroscan XL). The partially purified isoforms were quantitated as relative absorbance units per microgram of protein extract. Equal absorbance units of each isoform were used in DNA binding studies so that estimates of their relative abilities to bind DNA could be made.

Electrophoretic mobility shift analysis (EMSA or gel-shift analysis)

Gel-shift analysis was carried out essentially as described (Pan et. al. 1990), with minor modifications. Double-stranded oligonucleotides were prepared by annealing equimolar quantities of single-stranded oligonucleotides. Oligonucleotides were diluted in 1X Sequenase™ buffer (United States Biochemical, 40mM Tris pH 7.5, 20mM $MgCl_2$ and 50mM NaCl), heated to 95°C for five minutes and allowed slowly cool. Probes were labelled with [α-^{32}P]dNTPs (New England Nuclear) by end-filling, using the Klenow fragment of E. Coli DNA polymerase I (New England Biolabs) under the conditions recommended by the manufacturer. Unincorporated nucleotides were removed by spin column chromatography through Sephadex G-25 (Pharmacia). Whole cell extracts, nuclear extracts, in vitro translated proteins, or recombinant proteins, were combined with 4-8ug Poly(dI·dC)·Poly(dI·dC) (Pharmacia) in gel-shift buffer, with or without unlabelled competitor DNA, in a total volume of ~10ul and incubated for 20 minutes at room temperature. Approximately 0.2-1ng of ^{32}P-labelled probe was added to each reaction and incubated for another ten minutes at room temperature. Reactions were stopped by the addition of 1/4 volume of 6X agarose gel loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol and 30% glycerol). Protein-DNA complexes were separated from unbound probe by electrophoresis through 10% or 15% acrylamide/0.25X or 0.5X TBE, non-denaturing gels at 4°C. Gels were fixed in 30% methanol/10% acetic acid for 20 minutes, dried and exposed to Kodak XAR film for 2-4 hours at -80°C with an intensifying screen.

Construction, expression and immunoprecipitation of epitope-tagged Pit-1

The epitope 'tag', M)YPYDVPDYA from the influenza hemagglutinin (HA) protein (Kolodziej and Young 1991), was added to the NH_2-terminus of Pit-1 by PCR. The sense strand oligonucleotide primer (CB135) used for amplification contained the 'tag' coding sequence, an initiator methionine and 'Kozak' sequence (Kozak 1991) and a NotI

restriction site for subcloning. The antisense primer (CB136) contained an SpeI restriction site. The Pit-1 cDNA was used as template for 30 rounds of amplification at 95°C, one minute, 55°C, one minute and 72°C, one minute using the reaction conditions for Vent™ DNA Polymerase (New England Biolabs) recommended by the manufacturer. The amplified products were digested with NotI and SpeI (New England Biolabs), gel-purified and ligated into NotI/SpeI-digested pRcRSV (Invitrogen). Recombinants were identified by restriction analysis. The epitope-tagged Pit-1 cDNA insert from pRcRSV-HAPit-1 was excised by digestion with HindIII and XbaI (New England Biolabs), gel-purified and ligated into HindIII/XbaI-digested pRcCMV (Invitrogen). Recombinants were identified by restriction analysis.

pRcCMV-HAPit-1 was linearized by digestion with XbaI (New England Biolabs). The linearized DNA was used as template for in vitro transcription to generate a transcript of approximately 1300 nucleotides. In vitro transcription and translation reactions were performed as described above.

In vitro translation reactions were used directly for immunoprecipitation. Each 25ul translation reaction was combined with 1-2ug of the monoclonal antibody 12CA5 in RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 0.5% deoxycholate and 0.1% SDS) in a total volume of 50ul. Immunoprecipitations were carried out at 4°C for two hours. Immune complexes were collected by the addition of 20ul of a 50% slurry of Protein A Sepharose beads (Sigma) in RIPA buffer. The immunoprecipitation reactions were incubated at 4°C for another hour. The beads were pelleted by centrifugation and the supernatant removed. The beads were resuspended in 2X SDS sample buffer, heated to 95°C for five minutes and the samples analyzed by denaturing polyacrylamide gel electrophoresis and fluorography as above.

RESULTS

Chapter I. Detection and functional analysis of Pit-1a

Background

Transcription factor isoforms, originating by alternate splicing of a single precursor RNA, have been previously identified (reviewed in Foulkes and Sassone-Corsi 1992, Hsu et. al. 1992, Skipper et. al. 1993, Stehle et. al. 1993, Sohn et. al. 1994) and in some cases, demonstrated to have unique functions (Koenig et. al. 1989, Skipper et. al. 1993, Stehle et. al. 1993), or DNA binding specificities (Treacy et. al. 1991, Hsu et. al. 1992, Treacy et. al. 1992). As part of a collaboration with the laboratory of Dr. Lawrence Chasin Columbia University), attempts were made to identify any Pit-1 isoforms. A GH_3 cell cDNA library was constructed and screened with a radiolabelled Pit-1 cDNA probe (Morris et. al. 1992). Following sequencing of positive clones obtained from two successive rounds of hybridization, a cDNA clone was identified that is identical in sequence to Pit-1 except that it contains a 78 base pair insertion. This insertion results in the in-frame addition of 26 amino acids in the trans-activation domain of Pit-1 (**Figure 3b**, Morris et. al. 1992). The largest open reading frame of this cDNA is predicted to encode a protein of 35.8KDa (Morris et. al. 1992). This Pit-1 isoform was termed Pit-1a and is the first Pit-1 isoform to have been identified (Morris et. al. 1992). Southern hybridization analysis has demonstrated that both Pit-1 and Pit-1a are encoded by a single gene and the two isoforms originate by alternate splicing of the same primary transcript (Morris et. al. 1992).

Detection of the Pit-1a mRNA in various cell types

A procedure of reverse transcription and PCR amplification (RT-PCR) has been successfully used to detect rare mRNAs from preparations of total RNA (Kawasaki et. al. 1988). Here, this approach was utilized to determine the distribution of the Pit-1 and

Pit-1a transcripts. Total RNA was isolated from several rat cell lines and adult rat pituitaries by guanidinium isothiocyanate lysis and cesium chloride gradient centrifugation. Equal quantities of each total RNA preparation, determined spectrophotometrically, were separated on denaturing agarose gels and ribosomal RNAs visualized by ethidium bromide staining to assess the quality of the RNA preparations (**Figure 4**). Total RNA prepared from the rat cell lines or adult rat pituitaries was used as a template for first strand cDNA synthesis, using an antisense primer complementary to sequences common to both Pit-1 and Pit-1a. First strand cDNAs were then amplified by PCR following the addition of a sense primer which is complementary to sequences of both Pit-1 and Pit-1a. Portions of each amplification reaction were separated on agarose or non-denaturing polyacrylamide gels. The amplified products were transferred to nylon membranes and detected by Southern hybridization using radiolabelled oligonucleotide probes that anneal to sequences located between the two oligonucleotides used for amplification. The additional hybridization step was performed for two reasons: (a) to increase the sensitivity of the assay and (b) to increase the specificity of the technique. Using a radiolabelled oligonucleotide probe complementary to Pit-1a-specific sequences (**Figure 5a**), or a probe capable of recognizing both the Pit-1 and Pit-1a amplified products (**Figure 5b**), the transcripts of both isoforms were detected in total RNA prepared from all of the rat anterior pituitary cell lines examined (**Figure 5a**, lanes 3-5 and **Figure 5b**, lanes 4-6), as well as in total RNA prepared from adult rat pituitaries (**Figure 5a**, lane 6 and **Figure 5b**, lanes 7-9). Neither the Pit-1 (**Figure 5b**, lane 3), nor the Pit-1a (**Figure 5a**, lane 2) transcripts could be detected in total RNA prepared from the Rat 6 fibroblast cell line. In both experiments, in vitro transcribed mRNAs (see below) encoding either the Pit-1 (**Figure 5b**, lane 1), or Pit-1a (**Figure 5a**, lane 1), isoforms served as controls for identifying the amplified products of the Pit-1 isoform transcripts from the total RNA preparations. The detection of the Δ4Pit-1 isoform (**Figure 5b**, lanes 2 and 6), will be discussed in the following chapter.

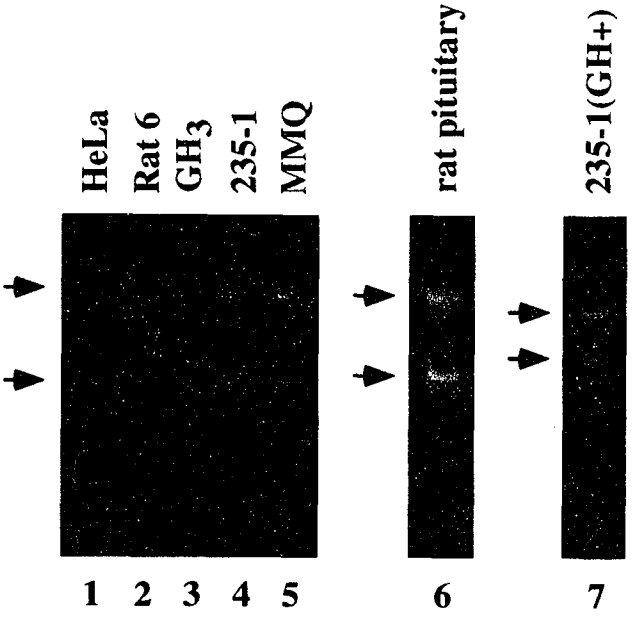


Figure 4. Preparation of total RNA. Total RNA was prepared from each of the indicated cell lines or tissue as described in Materials and Methods. Five to ten micrograms of each total RNA preparation was separated by denaturing agarose gel electrophoresis. The 18S and 28S ribosomal RNAs were visualized by ethidium bromide staining and are indicated by arrows.

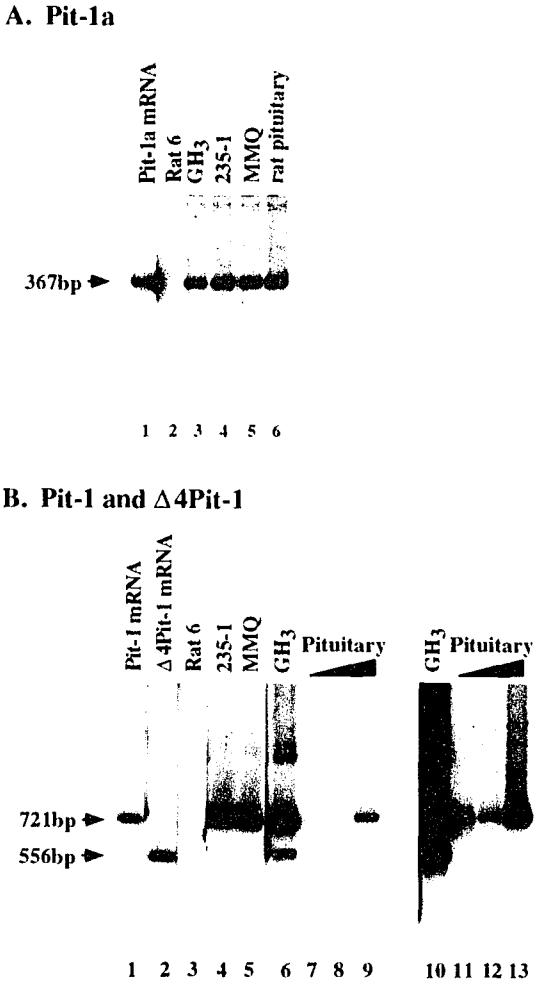


Figure 5. Distribution of the Pit-1 isoform mRNAs. The Pit-1a (A) or Pit-1 and Δ4Pit-1 (B) transcripts were detected by RT-PCR/Southern hybridization analysis as described in Materials and Methods. (A) To detect Pit-1a transcripts, one (cell lines), or five (pituitary) micrograms of total RNA was used as template for amplification using oligonucleotides CB36 and CB 74. Amplified products were detected with CB92. The expected size of the Pit-1a-specific amplified product is 367 base pairs and is indicated with an arrow. (B) To detect Pit-1 and Δ4Pit-1 transcripts, one (cell lines), or one, two or five (pituitary) micrograms total RNA was used as template for amplification using oligonucleotides CB74 and CB78. Amplified products were detected with CB23. The expected size of the Pit-1-specific amplified product is 721 base pairs. The expected size of the Δ4Pit-1-specific amplified product is 556 base pairs. The amplified products of the Pit-1 and Δ4Pit-1 mRNAs are indicated by arrows.

Ability of Pit-1a to bind to Pit-1 binding sites in the prolactin gene promoter

Pit-1 has been shown to bind to four sites within the rat prolactin gene proximal promoter (**Figure 2**, Ingraham et. al. 1988). In order to test the ability of Pit-1a to bind to these same sites, it was necessary to obtain a source of both the Pit-1 and Pit-1a proteins. To this end, an in vitro transcription/translation system was developed. Capped mRNAs were transcribed in vitro, using the cDNAs encoding either the Pit-1 or Pit-1a isoform as template. The in vitro transcribed mRNAs were purified and equal quantities of each mRNA were separated on denaturing agarose gels alongside RNA size standards. Visualization of the RNAs by ethidium bromide staining demonstrated that full length transcripts had been obtained (**Figure 6a**). Either a commercially available rabbit reticulocyte lysate (see Materials and Methods), or a wheat germ lysate (Erickson and Blobel 1983) system was then used to translate the mRNAs in the presence of [^{35}S]methionine. Incorporation of radioactivity was determined by TCA precipitation of a portion of each translation reaction on filters and liquid scintillation counting (data not shown). Equal TCA-precipitable material from either the reticulocyte lysate or wheat germ lysate systems were separated by SDS-PAGE and the in vitro translation products visualized by fluorography (**Figure 6b**). No radiolabelled proteins were detected when the in vitro transcribed mRNAs were omitted (**Figure 6b**, lanes 1 and 4). The in vitro translation products of Pit-1 are of the expected sizes of 31- and 33KDa (**Figure 6b**, lanes 2 and 5, Voss et. al. 1991b). Addition of the in vitro transcribed Pit-1a to either system produces two major in vitro translation products differing by approximately 3KDa (**Figure 6b**, lanes 3 and 6), consistent with alternate translation initiation site usage (Voss et. al. 1991b). However, in both the reticulocyte lysate and the wheat germ lysate systems, the Pit-1a in vitro translation products are smaller than the predicted 35.8KDa (**Figure 6b**, lanes 3 and 6). While the cause of the aberrant migration of the in vitro translated Pit-1a protein was never determined, it is not likely to be a result of premature translation termination since the regions of the Pit-1 protein necessary for DNA binding

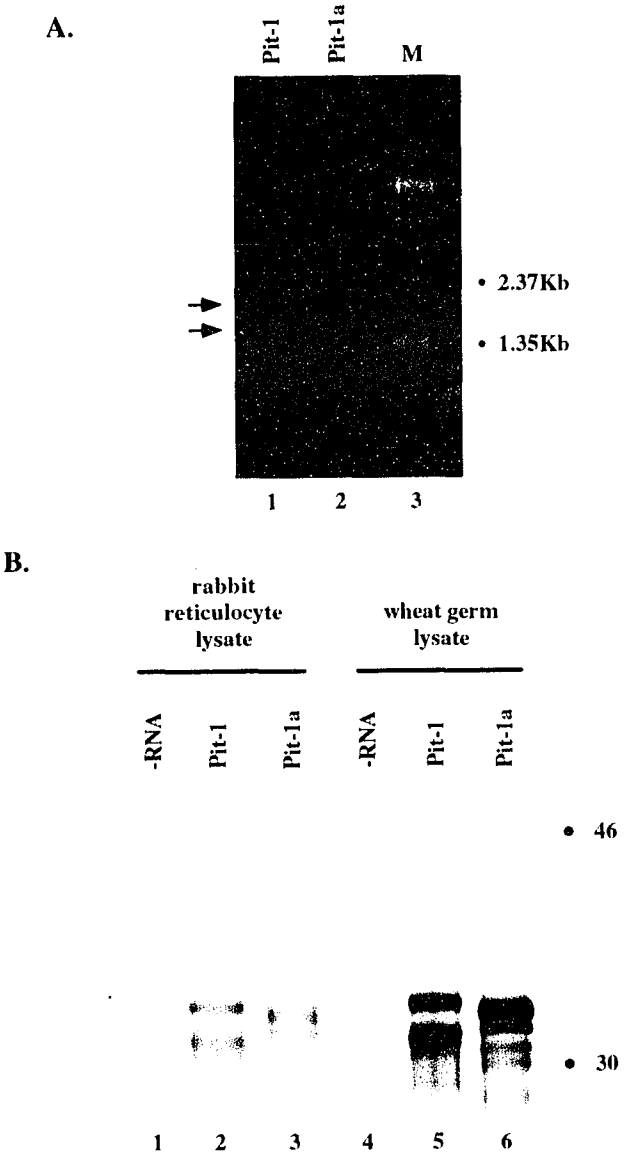


Figure 6. Expression of Pit-1 and Pit-1a in vitro. (A) The Pit-1 and Pit-1a mRNAs were transcribed in vitro as described in Materials and Methods. Approximately 0.5 micrograms of each mRNA was separated alongside RNA size standards by denaturing agarose gel electrophoresis. RNAs were visualized by ethidium bromide staining. The in vitro transcribed products are indicated by arrows. The sizes of the relevant RNA standards are given. (B) Pit-1 and Pit-1a were translated in vitro using a rabbit reticulocyte or wheat germ lysate system as described in Materials and Methods. [^{35}S]methionine-labelled translation products were visualized by fluorography. The 30- and 46KDa standards are indicated.

have been localized to the carboxy-terminal region of Pit-1 (Ingraham et. al. 1990) and the in vitro translated Pit-1a protein is still capable of binding DNA (see below).

An electrophoretic mobility shift assay (EMSA or gel-shift assay) was utilized to compare the DNA binding properties of the in vitro translated Pit-1 and Pit-1a isoforms. Radiolabelled, double-stranded oligonucleotides, containing sequences of the rat prolactin gene proximal promoter shown to be bound by Pit-1 (Ingraham et. al. 1988), were used as probes. Approximately equal quantities of the unlabelled, in vitro translated Pit-1 and Pit-1a proteins (see Materials and Methods) were combined with each of the radiolabelled probes and the protein-DNA complexes separated from the free probe by native acrylamide gel electrophoresis. The gels were fixed and dried and the protein-DNA complexes visualized by autoradiography (**Figure 7a**). While the wheat germ lysate did exhibit some binding activity in the absence of any exogenous mRNA (**Figure 7a**, lanes 7, 12 and 17), these results demonstrate that Pit-1a (**Figure 7a**, lanes 5, 10, 15 and 20) is capable of binding to the same sites of the rat prolactin gene proximal promoter as Pit-1 (**Figure 7a**, lanes 2, 8, 13 and 18), although it appears that Pit-1a binds with a somewhat lower affinity than Pit-1. Binding of both Pit-1 (**Figure 7a**, lanes 3, 9, 14 and 19) and Pit-1a (**Figure 7a**, lanes 6, 11, 16 and 21) to these sites was competed by the addition of a 100-fold molar excess of unlabelled oligonucleotide probe, demonstrating that these protein-DNA complexes are sequence-specific.

Nuclear extracts were prepared from CHO cells that had been stably transfected with a Pit-1 or Pit-1a expression vector (Morris et. al. 1992). These extracts were used for gel-shift analysis using a radiolabelled, double-stranded oligonucleotide, containing site 1P of the rat prolactin gene proximal promoter (**Figure 7b**). Extracts prepared from untransfected CHO cells showed no detectable binding activity (**Figure 7b**, lanes 1-3). However, extracts prepared from CHO cells stably transfected with Pit-1 (**Figure 7b**, lane 4), or Pit-1a (**Figure 7b**, lane 7), yields several readily detectable protein-DNA complexes. These protein-DNA complexes are competed by the addition of a 100-fold

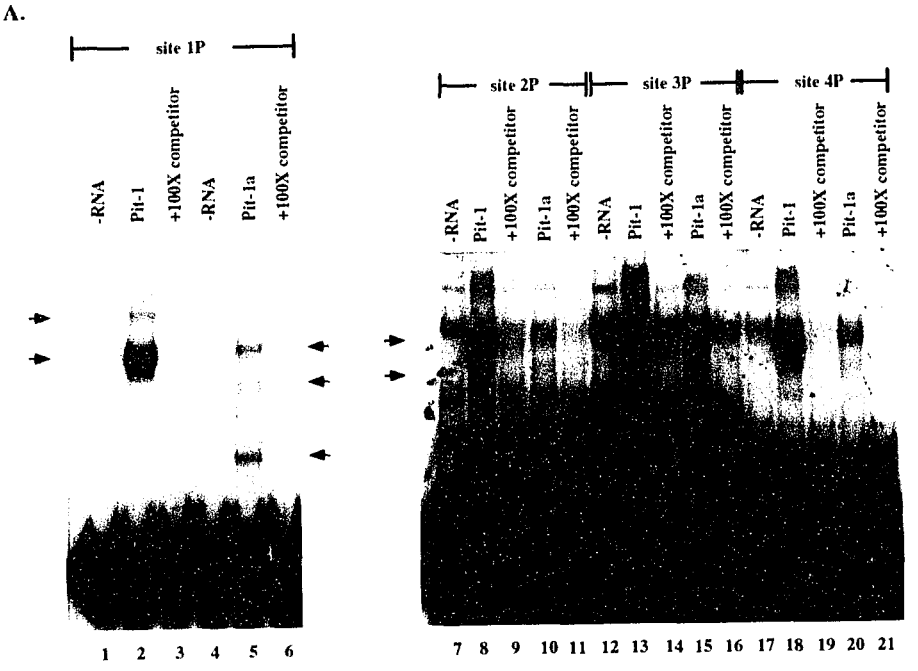


Figure 7. DNA binding analysis of Pit-1 and Pit-1a. The ability of Pit-1 and Pit-1a to bind to sites in the rat prolactin gene proximal promoter was examined by gel-shift analysis. (A) Approximately equal quantities of unlabelled, in vitro translated Pit-1 and Pit-1a were combined with approximately 50fmol of each of the indicated radiolabelled probes and the protein-DNA complexes were separated from unbound probe by electrophoresis through 10% acrylamide/0.25X TBE non-denaturing gels as described in Materials and Methods. (B) Approximately four micrograms of nuclear extract prepared from each of the indicated cell lines was analyzed by gel-shift analysis using a radiolabelled, site 1P-containing probe as in A. 1P* contains a mutated Pit-1 binding site and has previously been shown not to be bound by Pit-1 (Yan et. al. 1991). Probes used for gel-shift analysis were as follows: 1P (CB3/CB4), 2P (CB24/CB25), 3P (CB10/CB11), 4P (CB1/CB2) and 1P* (CB47/CB48). For competition analysis a 100-fold molar excess of unlabelled probe was added to each reaction prior to the addition of the radiolabelled probe. Protein-DNA complexes were visualized by autoradiography. Sequence-specific protein-DNA complexes are indicated by arrows.

B.

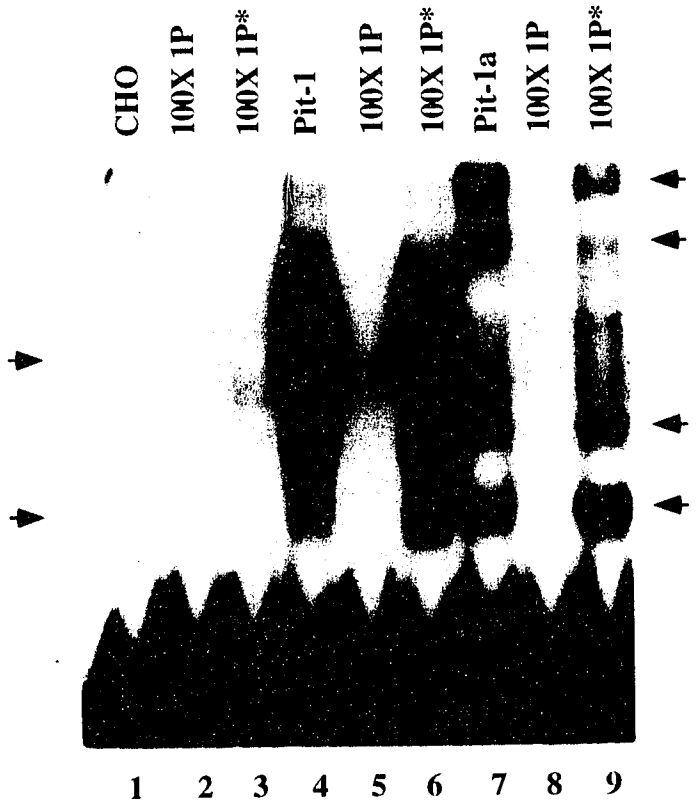


Figure 7 (continued). See legend on preceding page.

molar excess of unlabelled probe (**Figure 7b**, lanes 5 and 8), but is not be competed by the addition of a 100-fold molar excess of 1P*, a double-stranded oligonucleotide containing a mutated 1P site (**Figure 7b**, lanes 6 and 9), previously shown not to be bound by Pit-1 (Yan et. al. 1991), indicating that these complexes are sequence specific. Interestingly, the Pit-1a-DNA complexes formed by the in vitro translated Pit-1a protein and those formed by extracts prepared from stably transfected CHO cells are quite different (compare **Figure 7a**, lane 5 with **Figure 7b**, lane 7). This may be due to the ability of Pit-1a to interact with other nuclear proteins to form heteromeric complexes on DNA (see Discussion).

Summary

Pit-1 and Pit-1a transcripts are both detected in total RNA prepared from several rat anterior pituitary cell lines and from adult rat pituitaries and Pit-1a is capable of binding the same sites of the rat prolactin gene proximal promoter as Pit-1. However, Pit-1a is unable to trans-activate the rat prolactin gene (Morris et. al. 1992), leading one to speculate what the biochemical role of the Pit-1a isoform may be (see Discussion).

Chapter II. Detection and functional analysis of Δ4Pit-1

Background

Following RT-PCR amplification of GH_3 cell total RNA, using Pit-1-specific oligonucleotide primers, an amplified product smaller than that expected for Pit-1 was detected. The amplified product was subcloned, sequenced and found to be identical in sequence to Pit-1, except for a deletion of 165 base pairs (A. E. Jackson and C. Bancroft, unpublished data). The result is in an in-frame, 54 amino acid deletion of a majority of the $POU_{specific}$ domain of Pit-1, shown to be necessary for high-affinity, sequence-specific DNA binding as well as homo- and heterodimer formation (**Figure 3c**, Ingraham et. al. 1990, Voss et. al. 1991a, Verrijzer et. al. 1992). This isoform has been termed Δ4Pit-1 (Voss et. al. 1993), since the excised region precisely matches exon IV of the mouse Pit-1 gene (Lin et. al. 1993). Therefore, like Pit-1a, Δ4Pit-1 is thought to arise as a result of alternate splicing of the Pit-1 precursor mRNA.

Detection of Δ4Pit-1 mRNA and protein in various cell types

Δ4Pit-1-specific transcripts can be detected by RT-PCR/Southern hybridization analysis under the same conditions as those used to detect Pit-1 mRNA (**Figure 5b**). In vitro transcribed Δ4Pit-1 mRNA served as a control for RT-PCR (**Figure 5b**, lane 2). As expected, the Δ4Pit-1 mRNA is detected in total RNA prepared from GH_3 cells (**Figure 5b**, lane 6). Interestingly, the Δ4Pit-1 transcript could not be detected in total RNA prepared from the 235-1 and MMQ mammotropic cell lines (**Figure 5b**, lanes 4 and 5), nor from adult rat pituitaries (**Figure 5b**, lanes 7-9). The inability to detect Δ4Pit-1 mRNA in the pituitary RNA preparation was surprising. However, even when five micrograms of total RNA was used as template for first strand cDNA synthesis (**Figure 5b**, lane 9), or following over-exposure of the film (**Figure 5b**, lanes 10-13), the Δ4Pit-1 transcript is not detected in the adult rat pituitary total RNA preparation (see Discussion).

The largest open reading frame of the Δ4Pit-1 cDNA is predicted to encode a protein of 27.5KDa. Western analysis was performed to determine which Pit-1 isoform proteins are present in an extract prepared from the GH_3 rat anterior pituitary cell line (**Figure 8**). Equal quantities of either Rat 6 or GH_3 whole cell extract protein, as determined by the method of Bradford (Bradford 1976), were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. The immobilized proteins were then probed using a Pit-1 antibody, kindly provided by Dr. Richard Maurer (Oregon Health Sciences University) and the immunoreactive proteins detected by enhanced chemiluminescence (ECL). No Pit-1 immunoreactive proteins are detected in the extract prepared from the Rat 6 fibroblast cell line (**Figure 8**, lane 1). However, the 31- and 33KDa Pit-1 doublet (Voss et. al. 1991b), as well as an immunoreactive protein identical in molecular weight to that predicted for Δ4Pit-1, is detected in the GH_3 whole cell extract (**Figure 8**, lane 2). An immunoreactive protein of approximately 45KDa, is routinely seen in whole cell extracts prepared from GH_3 cells (**Figure 8**, lane 2). This immunoreactive protein has been observed by others (Fox et. al. 1990) and could potentially represent an as of yet un-characterized, Pit-1-related protein.

Ability of Δ4Pit-1 to bind DNA

To provide a more convenient source of the Pit-1 isoform proteins, each of the Pit-1 isoform cDNAs was subcloned into a prokaryotic expression vector. The Pit-1 isoforms were then expressed in- and partially purified from E. Coli. Several different mass quantities of each partially purified preparation were separated by SDS-PAGE and the recombinant Pit-1 isoforms detected by Western analysis (data not shown). The resulting films were scanned densitometrically and the relative amount of recombinant protein present in each extract determined in arbitrary units. This allowed equal immunoreactive quantities of each isoform to be used for subsequent gel-shift analysis and the relative DNA binding affinities of each isoform to be directly compared. Equal immunoreactive

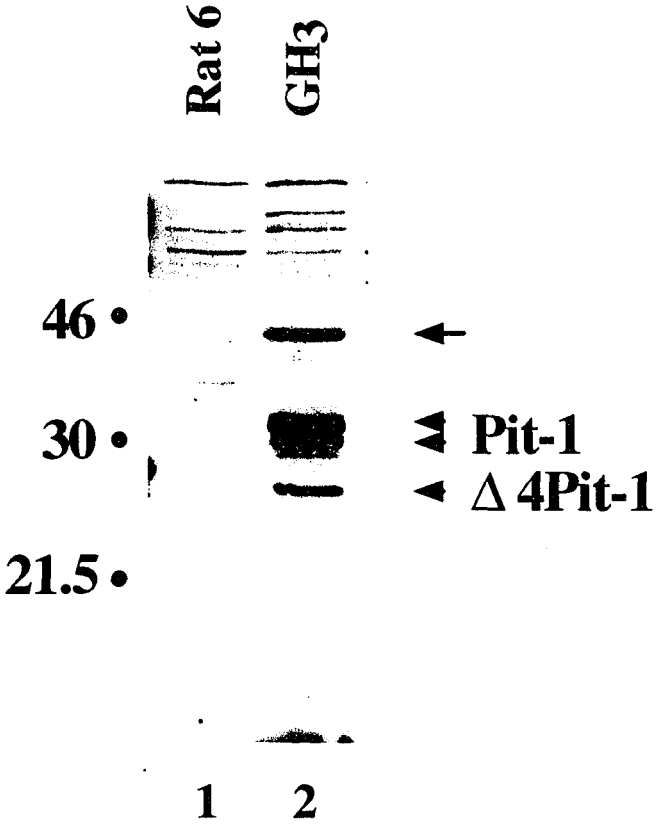


Figure 8. Detection of the Δ4Pit-1 protein. Pit-1 isoforms were detected by Western analysis as described in Materials and Methods. Approximately 40 micrograms of whole cell protein, prepared from Rat 6 or GH_3 cells, was separated through denaturing gels containing 15% acrylamide, transferred to nitrocellulose and probed with a Pit-1 antibody. Immunoreactive proteins were detected by ECL. The positions of molecular weight standards are indicated. Immunoreactive proteins are indicated by arrows.

quantities of each partially purified Pit-1 isoform were separated by SDS-PAGE and detected by Western analysis (**Figure 9**). All three of the Pit-1 isoforms are seen as 'doublets' which differ by approximately 3KDa, presumably due to alternate translation initiation site usage (Voss et. al. 1991b). In the partially purified Pit-1a preparation (**Figure 9**, lane 2), several more rapidly migrating immunoreactive proteins are detected and are presumed to be Pit-1a degradation products.

Gel-shift analysis using a radiolabelled site 1P of the rat prolactin gene proximal promoter (**Figure 10a**), or site GH-1 of the rat growth hormone gene proximal promoter (**Figure 10b**), was employed in order to test the ability of Δ4Pit-1 to bind DNA. Pit-1 and Pit-1a both form multiple protein-DNA complexes when either probe is used (**Figures 10a and 10b**, lanes 2 and 5). These complexes are competed by the addition of a 100-fold molar excess of unlabelled probe (**Figures 10a and 10b**, lanes 3 and 6), but not by the addition of a 100-fold molar excess of a probe containing a mutated Pit-1 binding site (**Figures 10a and 10b**, lanes 4 and 7). By contrast, Δ4Pit-1 forms no detectable complexes with either probe (**Figures 10a and 10b**, lane 8), most likely due to the absence of the $POU_{specific}$ domain. Interestingly, in these experiments it appears that Pit-1a binds to the prolactin 1P- and growth hormone GH-1 sites with a higher affinity than Pit-1, in apparent disagreement with the previously described gel-shift experiments which employed the in vitro translated Pit-1 and Pit-1a proteins (**Figure 7a**). The reasons for this discrepancy are unclear.

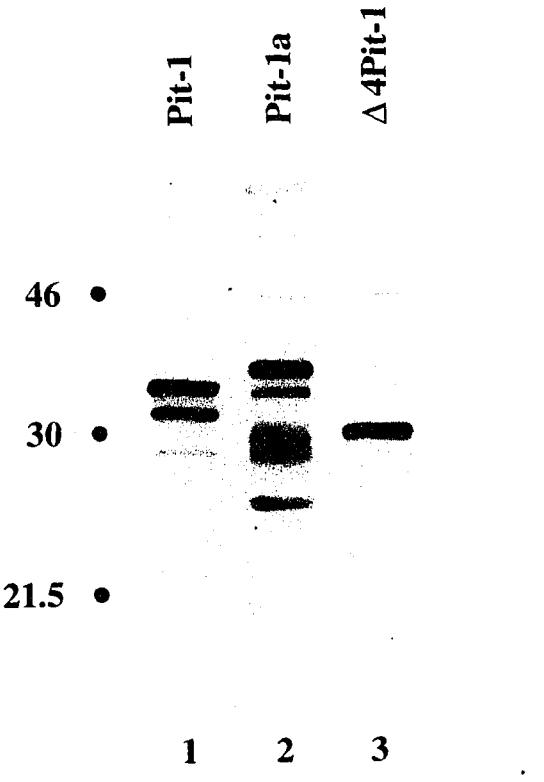
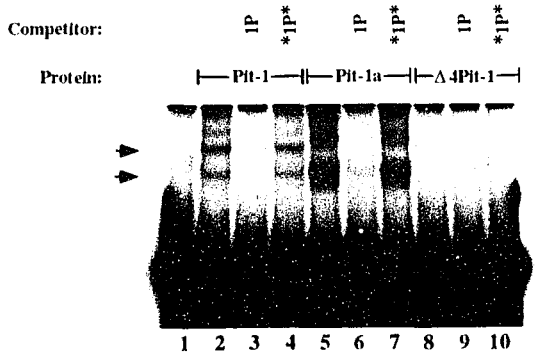


Figure 9. Expression of the Pit-1 isoforms in E. Coli. Each of the Pit-1 isoform cDNAs was amplified by PCR, using oligonucleotide primers CB80 and CB81. Each amplified cDNA was then subcloned into a prokaryotic expression vector and used to transform the host strain BL21(DE3)pLysS. Expression of the Pit-1 isoforms was induced by the addition of IPTG and the recombinant proteins were partially purified as described in Materials and Methods. Approximately equal immunoreactive quantities of each isoform were separated by SDS-PAGE, transferred to nitrocellulose and detected by Western analysis as described above. The 21.5-, 30- and 46KDa molecular weight standards are indicated.

A. Prolactin site 1P



B. Growth Hormone site GH-1

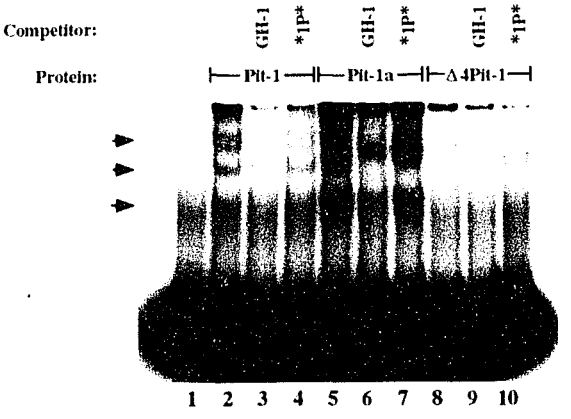


Figure 10. DNA binding analysis of the Pit-1 isoforms. Approximately equal immunoreative quantities of each of the recombinant Pit-1 isoforms was combined with a radiolabelled site 1P-containing probe (CB3/CB4) from the rat prolactin gene proximal promoter (A), or a radiolabelled site GH-1 containing probe (CB19/CB20) from the rat growth hormone gene proximal promoter (B). In both A and B, protein-DNA complexes were separated from unbound probe by electrophoresis through 10% acrylamide/0.5X TBE non-denaturing gels. Protein-DNA complexes were visualized by autoradiography. For competition analysis, a 100-fold molar excess of unlabelled probe was added prior to the addition of radiolabelled probe. *1P* (CB52/CB53) contains a mutated Pit-1 binding site that has been previously shown not to be bound by Pit-1 (Yan et. al. 1991). Sequence-specific protein-DNA complexes are indicated by arrows.

Summary

Δ4Pit-1, an isoform of the Pit-1 protein which lacks a majority of the $POU_{specific}$ domain, has been identified. The Δ4Pit-1 isoform mRNA and protein are detected only in the GH_3 somatomammotropic cell line. Δ4Pit-1 is unable to bind to two previously described Pit-1 binding sites in the rat prolactin and growth hormone gene proximal promoters. Furthermore, Δ4Pit-1 is unable to trans-activate either the prolactin or growth hormone genes (Fischberg 1994). No function for this isoform has yet been determined (see Discussion).

Chapter III. Identification of two absolute mammotropic cell lines

Background

The rat somatomammotropic GH_3 (Bancroft 1981) and mammotropic 235-1 (Reymond et. al. 1984) and MMQ (Judd et. al. 1988) cell lines have already proven extremely useful for studies of the molecular mechanisms employed by cells of the anterior pituitary to restrict expression of the prolactin and growth hormone genes to the appropriate cell types. Here, these cell lines were re-examined, to determine if expression of the prolactin or growth hormone (or both) genes correlates with a difference in the pattern of expression of the Pit-1 isoforms.

Detection of the prolactin and growth hormone mRNAs

RT-PCR/Southern hybridization analysis was employed to detect the prolactin and growth hormone gene transcripts in total RNA prepared from several rat cell lines (**Figure 11**). Using oligonucleotide primers complementary to sequences of the rat prolactin mRNA, prolactin transcripts are detected in total RNA preparations from the GH_3, 235-1 and MMQ cell lines (**Figure 11a**, lanes 2-4), while prolactin mRNA is not detected in the Rat 6 fibroblast cell line (**Figure 11a**, lane 1). Total RNA prepared from these same cell lines was then used as template for the RT-PCR/Southern hybridization assay, using rat growth hormone mRNA-specific oligonucleotide primers (**Figure 11b**). Growth hormone mRNA is not detected in the Rat 6 fibroblast or 235-1 and MMQ rat anterior pituitary cell line total RNA preparations (**Figure 11b**, lanes 1, 3 and 4). Only total RNA prepared from the GH_3 cell line contains any detectable growth hormone mRNA (**Figure 11b**, lane 2).

To place a lower limit on the ability to detect the growth hormone message and to demonstrate the sensitivity of the RT-PCR/Southern hybridization assay, ten-fold serial dilutions of total RNA prepared from GH_3 cells was used as template to detect the growth

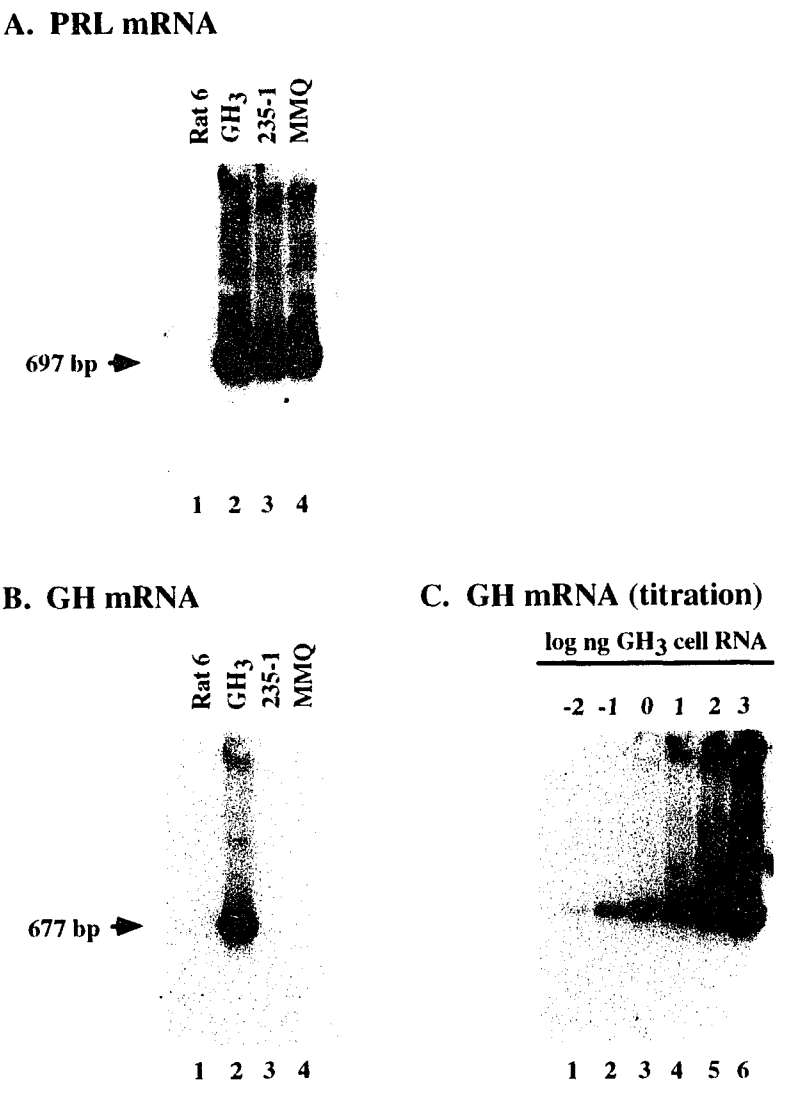


Figure 11. Distribution of the prolactin and growth hormone mRNAs. One microgram of total RNA prepared from each of the indicated cell lines was used as template to amplify the prolactin (A) or growth hormone (B and C) mRNAs. The amplified products were separated through 1.4% agarose gels and detected by Southern hybridization as described in Materials and Methods. (A) Prolactin mRNA was amplified using oligonucleotides CB104 and CB105 and the amplified products detected by Southern hybridization using CB106. (B) Growth hormone mRNA was amplified using oligonucleotides CB101 and CB102 and the amplified products detected by Southern hybridization using CB103. (C) The indicated quantities of total RNA prepared from GH_3 cells were used as template to detect the growth hormone mRNA as in B. The sizes of the amplified products are indicated.

hormone mRNA. By this approach, it is estimated that the growth hormone mRNA is greater than 10^5 times as abundant in total RNA prepared from GH_3 cells than in the 235-1 or MMQ total RNA preparations (**Figure 11c**). Together, these data demonstrate that in the 235-1 and MMQ cell lines, transcription of the growth hormone gene is undetectable and therefore, these cell lines are true representatives of the mammotroph cell lineage.

Investigation of the molecular basis for the mammotropic phenotype

Since two true mammotropic cell lines have been identified, studies were initiated to identify the molecular mechanism(s) responsible for the apparent absence of growth hormone gene expression in these cells. Whole cell extracts were prepared from the GH_3, 235-1 and MMQ cell lines and used for gel-shift analysis (**Figure 12**). Each whole cell extract was combined with a radiolabelled, double-stranded oligonucleotide probe, containing site GH-1 of the rat growth hormone gene promoter and protein-DNA complexes identified as described above. Both the GH_3 (**Figure 12**, lanes 2 and 3) and MMQ (**Figure 12**, lanes 6 and 7) whole cell extracts form protein-DNA complexes that electrophoretically co-migrate with those formed by recombinant Pit-1 (**Figure 12**, lane 1). The Pit-1-site GH-1 complexes are competed by the addition of a 100-fold molar excess of unlabelled probe (**Figure 12**, lanes 8-10, 13 and 14). These same Pit-1-DNA complexes are not competed by the addition of a 100-fold molar excess of two different unlabelled probes containing mutated Pit-1 binding sites (**Figure 12**, lanes 15-17, 20-24, 27 and 28). Intriguingly, whole cell extracts prepared from the 235-1 cell line contain no detectable Pit-1 binding activity (**Figure 12**, lanes 4 and 5), suggesting that although these cells contain the Pit-1 mRNA (**Figure 5b**), they do not contain any Pit-1 protein.

Whole cell extracts prepared from the Rat 6, GH_3, 235-1 and MMQ cell lines were subjected to Western analysis using a Pit-1 antibody, as described above. Each of the recombinant Pit-1 isoforms (**Figure 13**, lanes 1-3) were separated alongside the whole

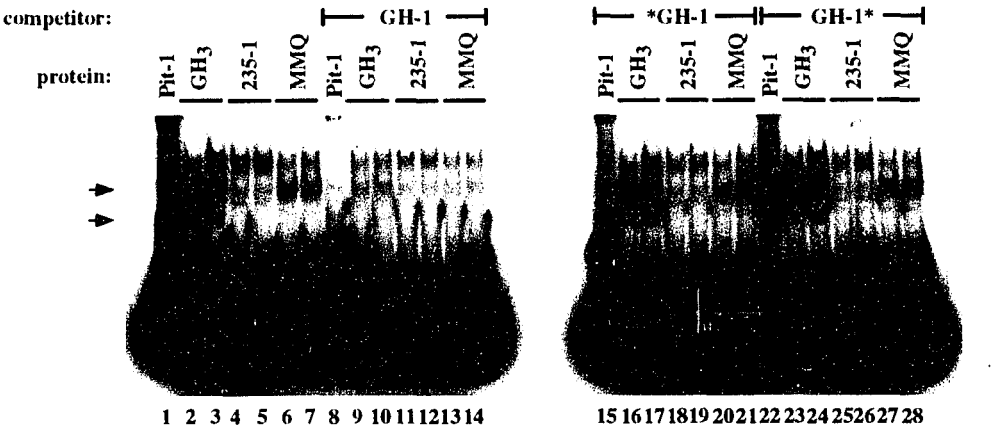


Figure 12. DNA binding analysis of whole cell extracts. Two or four micrograms of whole cell protein from each of the indicated cell lines was used for gel-shift analysis using a radiolabelled probe containing site GH-1 (CB19/CB20) of the rat growth hormone gene proximal promoter. Protein-DNA complexes were separated from unbound probe by electrophoresis through 10% acrylamide/0.5X TBE non-denaturing gels. Protein-DNA complexes were visualized by autoradiography. Recombinant Pit-1 served as a reference to identify Pit-1-specific complexes formed by the whole cell extracts. For competition analysis, each of the indicated unlabelled oligonucleotides were added at a 100-fold molar excess prior to the addition of radiolabelled probe. *GH-1 (CB58/CB59) and GH-1* (CB62/CB63) are oligonucleotides that contain a mutated Pit-1 binding site. Pit-1-DNA complexes are indicated by arrows.

cell extracts and were used to identify each of the isoforms in the cell line samples. The 31-33KDa Pit-1 doublet is detected in whole cell extracts prepared from the GH_3 and MMQ rat anterior pituitary cell lines (**Figure 13**, upper panel, lanes 5 and 7, respectively), while no Pit-1 immunoreactive proteins are detected in the control Rat 6 fibroblast whole cell extract (**Figure 13**, upper panel, lane 3). Consistent with the results of RT-PCR analysis, the Δ4Pit-1 protein is detected only in the GH_3 whole cell extract (**Figure 13**, upper panel, lane 5). Surprisingly, no immunoreactive Pit-1 proteins are detected in the whole cell extract prepared from the 235-1 cell line (**Figure 13**, upper panel, lane 6). Identical samples were separated by electrophoresis and transferred to nitrocellulose in parallel, then subjected to Western analysis using an actin antibody (**Figure 13**, lower panel), demonstrating that approximately equal quantities of protein were loaded in each lane. Furthermore, this demonstrates that the absence of any detectable Pit-1 protein in the 235-1 cell whole cell extract was not the result of proteolytic activity.

To examine this phenomenon more closely, a second 235-1 cell line was obtained from Dr. Daniel Catanzaro (Cornell University Medical School). These cells have been shown to be capable of expressing a transfected growth hormone gene promoter-reporter construct (Fox et. al. 1990, Jones and Catanzaro 1991) and therefore, have been termed 235-1(GH+) cells. Total RNA was isolated from these cells and used as template for RT-PCR analysis, to detect the prolactin, growth hormone and pit-1 transcripts (**Figure 14**). Prolactin mRNA is not detected in the Rat 6 fibroblast total RNA preparation (**Figure 14a**, lane 1), but prolactin transcripts are readily detected in the GH_3, 235-1, 235-1(GH+) and MMQ RNA preparations (**Figure 14a**, lanes 2-5, respectively). These same total RNA preparations were used as template to detect the growth hormone mRNA by RT-PCR (**Figure 14b**). Growth hormone transcripts are detected in the GH_3 cell total RNA (**Figure 14b**, lane 2), but not in total RNA prepared from the Rat 6, 235-1, 235-1(GH+)

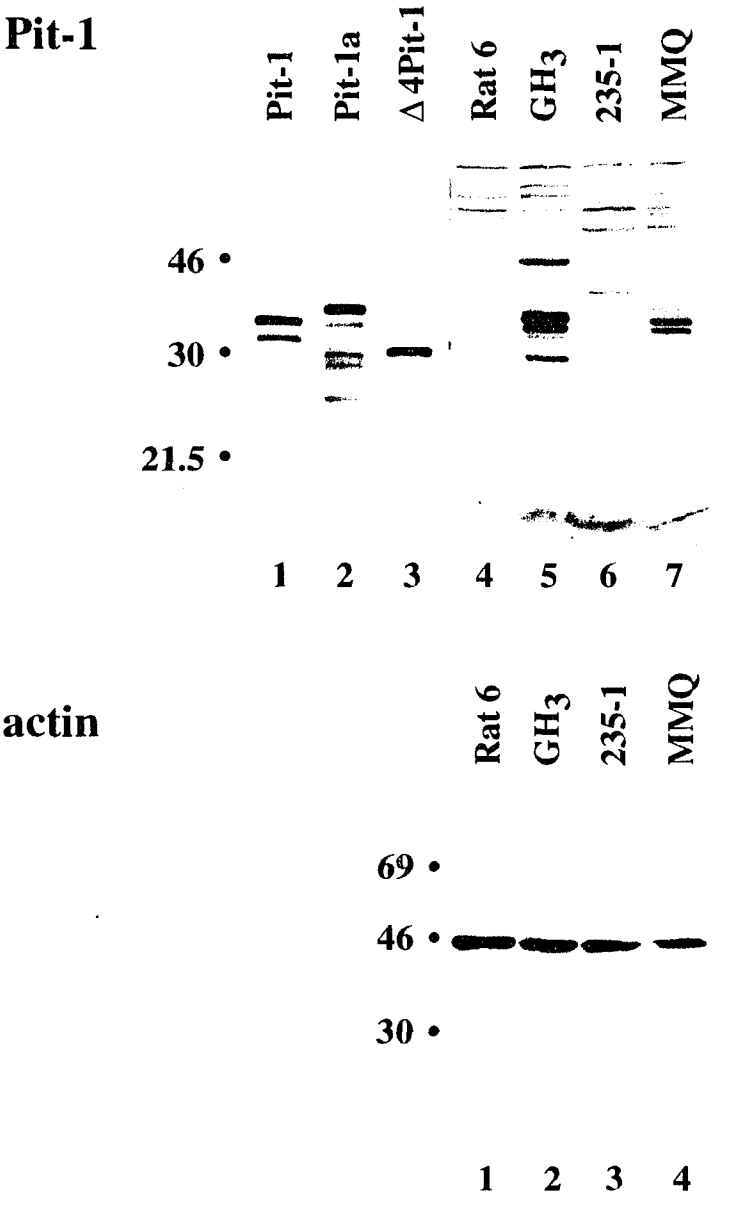


Figure 13. Western analysis of whole cell extracts. Forty micrograms of whole cell protein from each of the indicated cell lines was separated by electrophoresis, transferred to nitrocellulose and Pit-1 (upper panel) or actin lower panel) was detected by Western analysis as described in Materials and Methods. In the upper panel, each of the recombinant Pit-1 isoforms were separated alongside the whole cell extracts to aid in the identification of each Pit-1 isoform from the whole cell extracts. Relevant molecular weight standards are indicated.

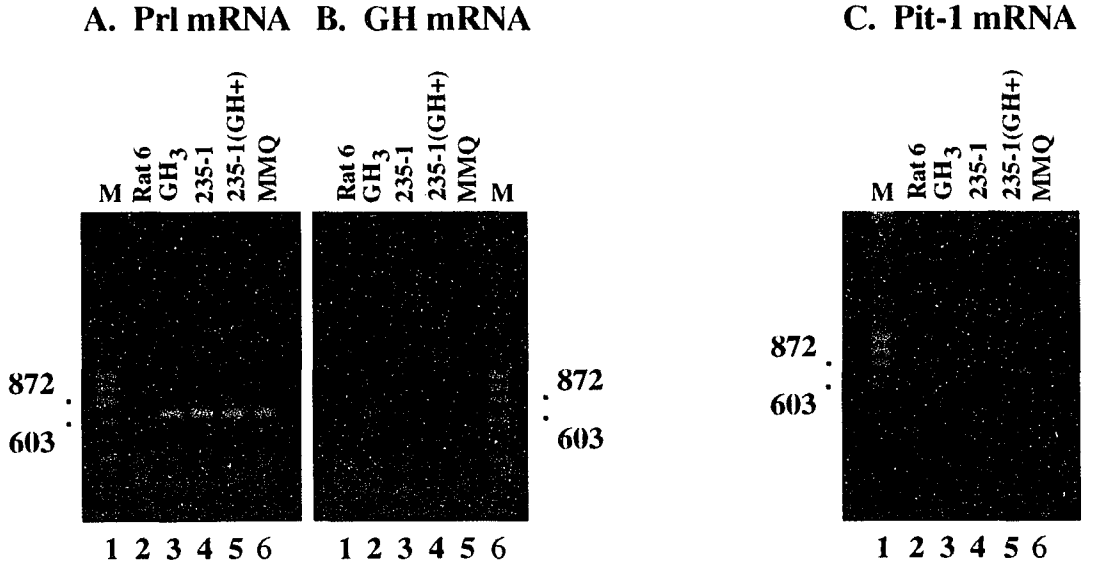


Figure 14. Detection of the prolactin, growth hormone and Pit-1 isoform mRNAs. One microgram of total RNA prepared from each of the indicated cell lines was used as template for RT-PCR analysis to detect the prolactin (A), growth hormone (B) or Pit-1 isoform (C) mRNAs. The amplified products were separated through 0.9% agarose gels and visualized by ethidium bromide staining. The oligonucleotides used for amplification were as follows: prolactin (A) CB104 and CB105; growth hormone (B) CB101 and CB102 and the Pit-1 isoforms (C) CB74 and 78. The sizes of the relevant DNA standards (HaeIII-digested ϕX174 DNA) are indicated.

and MMQ cell lines (**Figure 14b**, lanes 1, 3-5, respectively). These same total RNA preparations were subjected to RT-PCR analysis to detect Pit-1 transcripts (**Figure 14c**), the Pit-1 mRNA is detected in all of the anterior pituitary cell lines examined (**Figure 14c**, lanes 2-5). No Pit-1-specific amplified products are detected when total RNA from Rat 6 fibroblasts is used as template (**Figure 14c**, lane 1). Δ4Pit-1 transcripts are detected only in the GH_3 cell total RNA preparation (**Figure 14c**, lane 2).

Nuclear extracts were prepared from the GH_3 and the two 235-1 cell lines. Nuclear proteins were separated by electrophoresis, transferred to nitrocellulose and probed with a Pit-1 antibody (**Figure 15**, upper panel). Each of the recombinant Pit-1 isoforms was separated alongside the nuclear extracts and served as a control to identify each of the Pit-1 isoforms (**Figure 15**, upper panel, lanes 1-3). No detectable Pit-1 protein is observed in the nuclear extract prepared from the 235-1 cells (**Figure 15**, upper panel, lane 5), but is detected in the GH_3 and 235-1(GH+) cell nuclear extracts (**Figure 15**, upper panel, lanes 4 and 6, respectively). As a control, the Pit-1 antibody was removed and the filters reprobed with an actin antibody (**Figure 15**, lower panel), as described above.

It has been observed that sequences separating the Pit-1 binding sites GH-1 and GH-2 are extremely well conserved between species and it has been demonstrated that this region is required for maximal expression of the growth hormone gene (Lipkin et. al. 1993). This region has been termed the 'Z box' and has been shown to bind an unusual, widely-expressed protein, Zn-15, which contains 15 putative zinc fingers (Lipkin et. al. 1993). To determine if the Zn-15 transcript is present in the rat anterior pituitary cell lines described above, RT-PCR/Southern hybridization analysis was employed, using oligonucleotide primers specific for the Zn-15 mRNA (**Figure 16**). Surprisingly, the Zn-15 transcript is detected in total RNA prepared from all rat cell lines tested (**Figure 16**, lanes 2-5), including the Rat 6 fibroblast and 235-1 and MMQ mammotropic cell

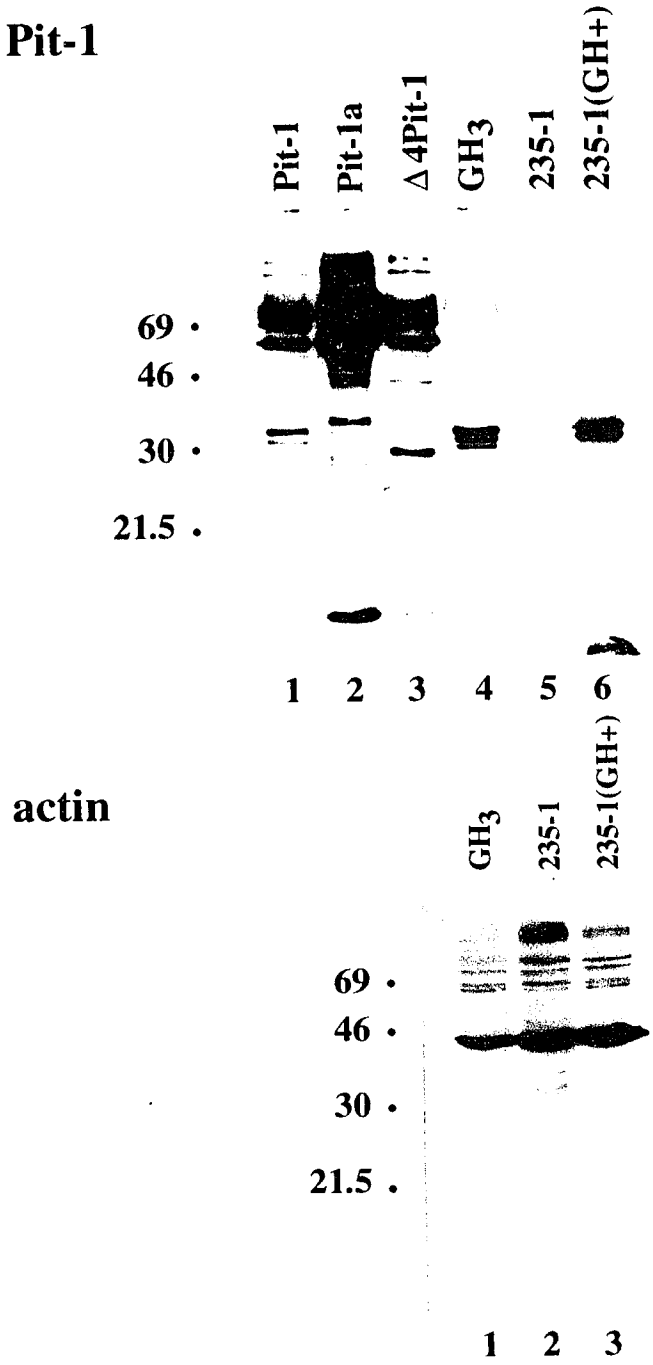


Figure 15. Western analysis of nuclear extracts. Forty micrograms of nuclear protein, isolated from each of the indicated cell lines, was separated by SDS-PAGE, transferred to nitrocellulose and Pit-1 (upper panel) was detected by Western analysis as described in Materials and Methods. Following detection of the Pit-1 isoforms by Western analysis (upper panel), the filters were stripped and reprobed to detect actin lower panel). In the upper panel, each of the recombinant Pit-1 isoforms were separated alongside the nuclear extracts to aid in the identification of the Pit-1 isoforms in the nuclear extract preparations. The sizes of molecular weight standards are indicated.

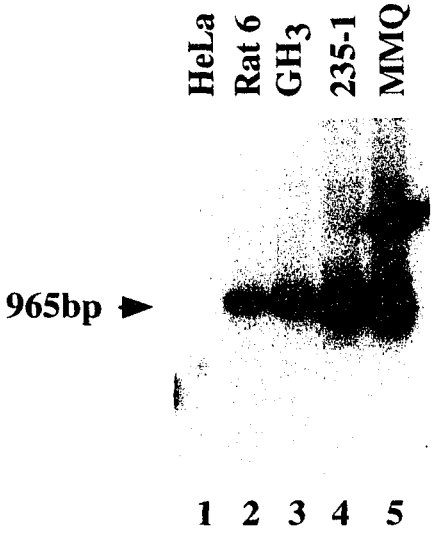


Figure 16. Detection of the Zn-15 mRNA. One microgram of total RNA prepared from each of the indicated cell lines was used as template for RT-PCR/Southern hybridization analysis to detect the Zn-15 mRNA. Zn-15 transcripts were amplified using oligonucleotides CB111 and CB112. CB113 was used as a probe for Southern analysis to detect the Zn-15-specific amplified products. The size of the Zn-15-specific amplified product is indicated.

lines, which do not express the growth hormone gene. The Zn-15 transcript is not detected in total RNA prepared from the human HeLa cell line (**Figure 16**, lane 1).

Summary

Two absolute mammotropic cell lines, 235-1 and MMQ, have been identified. These two cell lines contain at least 10^5 times less growth hormone gene mRNA than the somatomammotropic GH_3 cell line, suggesting that the growth hormone gene is transcriptionally silent in the 235-1 and MMQ cell lines. These results imply that, in the 235-1 cell line, the prolactin gene is expressed through Pit-1-independent mechanisms and expression of the pit-1 gene is regulated post-transcriptionally. Furthermore, detection of the Pit-1 protein and Zn-15 transcript in the MMQ cell line, in the absence of any detectable growth hormone mRNA, suggests that in addition to these two transcription factors, an as of yet unidentified factor(s) is required for cell type-specific expression of growth hormone gene. Consequently, further study of these cell lines will lead to a greater understanding of the molecular mechanisms responsible for cell type-specific expression of the prolactin and growth hormone genes. The results of mRNA and protein analysis of the cell lines described above are summarized in **Table II**.

cell line or tissue	Pit-1 mRNA	Pit-1 protein	Pit-1a mRNA	Pit-1a protein	Δ4Pit-1 mRNA	Δ4Pit-1 protein	Prl mRNA	GH mRNA	Zn-15 mRNA
HeLa	ND	ND	ND	ND	ND	ND	ND	ND	no
Rat 6	no	no	no	unlikely	no	no	no	no	yes
GH_3	yes	yes	yes	?	yes	yes	yes	yes	yes
235-1	yes	no	yes	?	no	no	yes	no	yes
235-1 (GH+)	yes	yes	ND	?	no	no	yes	no	ND
MMQ	yes	yes	yes	?	no	no	yes	no	yes
rat pituitary	yes	ND	yes	ND	no	ND	ND	ND	ND

Table II. Summary of RNA and protein analysis of cell lines and tissue. The results of mRNA and protein analysis of the cell lines described in the text are summarized here. ND (not determined. Detection of the Pit-1a protein in the anterior pituitary cell lines is indicated by a question mark because the Pit-1a protein is not reproducibly detected (see text).

Chapter IV. Construction, expression and immunoprecipitation of epitope-tagged Pit-1

Background

In order to better understand the role Pit-1 plays in the cell type-specific and hormonally regulated expression of the prolactin and growth hormone genes, it is desirable to readily immunoprecipitate the Pit-1 protein from solution. In this laboratory, previous attempts at immunoprecipitation of Pit-1 from crude extracts have been unsuccessful (data not shown). Recently, the use of an epitope-tagging technique has proven useful for the identification of novel protein-protein interactions (Kolodziej and Young 1991). Such an approach has several advantages: (a) antibodies recognizing the commonly used epitopes are very specific and easily obtainable. (b) because of the ease in recovering the tagged proteins from solution, transient protein-protein interactions and post-translational modification of the tagged proteins can be observed. (c) epitope-tagged versions of any protein of interest can easily be generated.

Synthesis and immunoprecipitation of epitope-tagged Pit-1

An eight amino acid, epitope 'tag' from the influenza hemagglutinin protein (HA), was added to the NH_2-terminus of the Pit-1 protein by PCR (Kolodziej and Young 1991). Preliminary experiments were performed using in vitro translated, epitope-tagged Pit-1 protein HAPit-1). mRNAs encoding HAPit-1 or CREB were prepared by in vitro transcription and a portion of each transcription reaction was separated on a denaturing agarose gel to demonstrate that full length transcripts had been generated (**Figure 17a**). The HAPit-1 and CREB mRNAs were translated in vitro, using a wheat germ lysate system, in the presence of [^{35}S]-methionine. The HAPit-1 and CREB in vitro translation products were combined with normal rabbit serum or the monoclonal antibody 12CA5, which recognizes the hemagglutinin epitope and immune complexes collected following

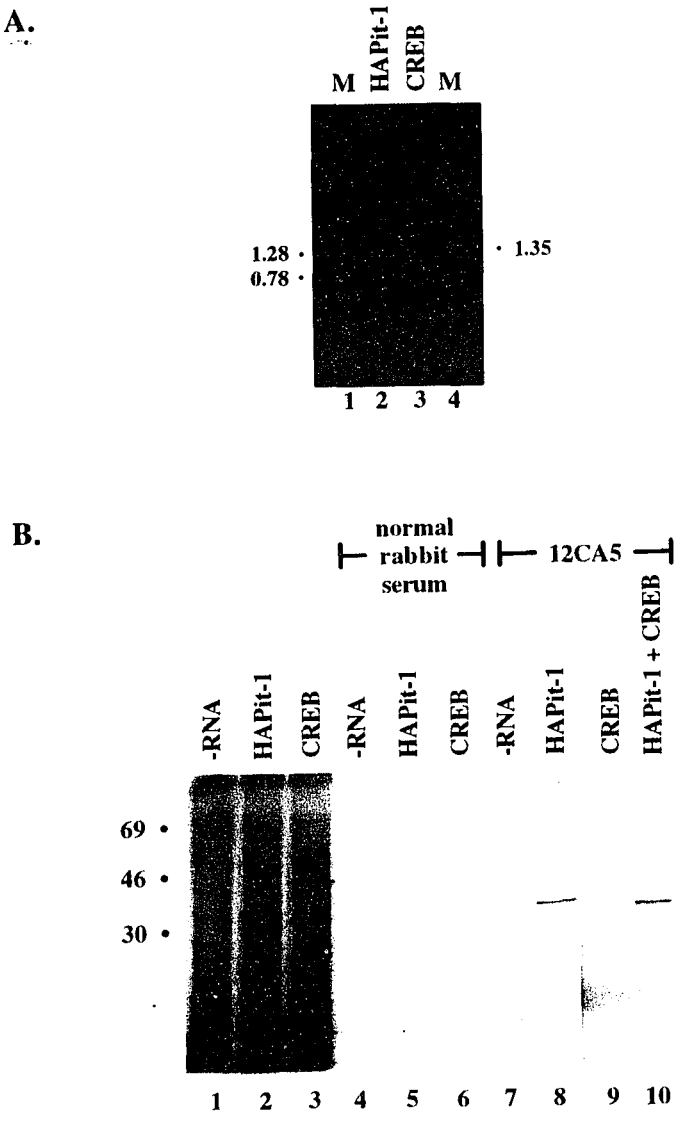


Figure 17. Immunoprecipitation of epitope-tagged Pit-1. HAPit-1 and CREB were transcribed and translated in vitro and directly used for immunoprecipitation with normal rabbit serum or monoclonal antibody 12CA5. (A) One microgram of the in vitro transcribed HAPit-1 and CREB mRNAs were separated through denaturing, 1.4% agarose gels, alongside RNA size standards and visualized by ethidium bromide staining. The sizes of each of the relevant RNA standards are indicated. (B) The [^{35}S]methionine-labelled, in vitro translated HAPit-1 and CREB proteins were analyzed directly, or used for immunoprecipitation with normal rabbit serum or monoclonal antibody 12CA5 as described in Materials and Methods. Immune complexes were collected and separated by SDS-PAGE. Immunoprecipitated proteins were visualized by fluorography. The sizes of molecular weight standards are indicated.

the addition of protein A Sepharose beads. The in vitro translation products and immunoprecipitates were separated by SDS-PAGE and visualized by fluorography (**Figure 17b**). While there are a considerable number of translation products from control reactions to which no exogenous mRNA has been added (**Figure 17b**, lane 1), the addition of either the in vitro transcribed HAPit-1 or CREB mRNAs generates protein products of the expected sizes (**Figure 17b**, lanes 2 and 3, respectively). Neither the epitope-tagged Pit-1, nor CREB, is immunoprecipitated by the addition of rabbit pre-immune serum (**Figure 17b**, lanes 5 and 6, respectively). 12CA5 is unable to precipitate in vitro translated CREB from solution (**Figure 17b**, lane 9), but HAPit-1 is precipitated by the addition of the monoclonal antibody 12CA5 when translated alone (**Figure 17b**, lane 8), or when co-translated with CREB (**Figure 17b**, lane 10).

Summary

An eight amino acid 'tag' has been added to the NH_2-terminus of Pit-1 and the in vitro translated HAPit-1 can be recovered by immunoprecipitation. Epitope-tagged versions of each of the Pit-1 isoforms were generated by the same PCR strategy as above. The cDNAs encoding each epitope-tagged isoform were subcloned into a eukaryotic expression vector and will be stably transfected into several pituitary and non-pituitary cell lines. MMQ and HeLa cell lines, stably transfected with pRcRSV-Pit-1, have already been generated. These cell lines are intended to be used for detailed study of Pit-1 function. However, immunoprecipitation of HAPit-1 from extracts prepared from these cell lines has not yet been attempted. This technique will be useful for two important applications: (a) the study of post-translational modification of the Pit-1 isoforms in response to hormones, growth factors and other extracellular cues. (b) the identification of any novel protein-protein interactions between Pit-1 and other cellular factors. Should any Pit-1-protein interaction(s) be identified, the approach may be scaled-up to facilitate purification and cloning of the protein (see Discussion).

DISCUSSION

Summary

To date, the single most important regulator of the prolactin and growth hormone genes to have been identified is Pit-1 (Bodner et. al. 1988, Ingraham et. al. 1988, McCormick et. al. 1988, Mangalam et. al. 1989, Fox et. al. 1990). This pituitary-specific transcription factor is required for expression of both genes during development (Li et. al. 1990, Radovick et. al. 1992, Prager et. al. 1993), regulates expression of its own gene (Chen et. al. 1990, McCormick et. al. 1990, Rhodes et. al. 1993), is involved in regulation of the prolactin gene by TRH (Yan et. al. 1991) and is necessary, along with a thyrotrope-specific isoform of Pit-1, for expression of the TSHβ gene (Haugen et. al. 1993, Haugen et. al. 1994, Lin et. al. 1994). The apparent functional versatility of the Pit-1 protein is remarkable and has prompted a search for transcription factors, in addition to Pit-1, which are required for cell type-specific and hormonal regulation of the prolactin and growth hormone genes. Such efforts have led to the identification of the Pit-1a and Δ4Pit-1 isoforms.

Pit-1a is identical to Pit-1 except for a 26 amino acid, in-frame insertion in the trans-activation domain and like the remainder of the trans-activation domain of Pit-1, the region unique to Pit-1a is serine and threonine rich (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Pit-1a has been shown to be encoded by the same gene as Pit-1 and therefore, Pit-1a is thought to arise as a result of alternate splicing of the pit-1 gene primary transcript (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Pit-1a transcripts are detected in all rat anterior pituitary cell lines examined, as well as in the adult rat pituitary (see Results, Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Although Pit-1a is capable of binding to the same sites of the rat prolactin and growth hormone gene proximal promoter regions as Pit-1, Pit-1a is unable to trans-activate the prolactin gene (see Results, Konzak and Moore 1992, Morris et. al. 1992,

Theill et. al. 1992). However, Pit-1a has been shown to trans-activate the growth hormone gene at least as well as Pit-1 (Konzak and Moore 1992, Theill et. al. 1992).

Δ4Pit-1 is identical to Pit-1 except for the in-frame deletion of a 54 amino acid region of the $POU_{specific}$ domain (Voss et. al. 1993, Day and Day 1994a). This region is required for sequence-specific, high affinity DNA binding, as well as homo- and heterodimerization (Ingraham et. al. 1990, Voss et. al. 1991a, Shupnik et. al. 1992). This region is encoded by exon IV of the mouse pit-1 gene (Lin et. al. 1993) and therefore, Δ4Pit-1, like Pit-1a, is thought to arise as a result of alternative splicing of the pit-1 gene primary transcript (Voss et. al. 1993, Day and Day 1994b, Day and Day 1994a). As might be expected, Δ4Pit-1 is unable to bind DNA or trans-activate the prolactin and growth hormone genes (see Results, Voss et. al. 1993, Fischberg 1994). Data presented here demonstrates that the Δ4Pit-1 transcript and protein are detected only in the GH_3 rat anterior pituitary cell line.

The phenotypes of the somatomammotropic (GH_3) and mammotropic (235-1 and MMQ) cell lines described above were originally determined by the ability of these cell lines to secrete prolactin or growth hormone (Bancroft 1981, Reymond et. al. 1984, Judd et. al. 1988). Recently, an extremely sensitive technique of reverse transcription and PCR amplification (RT/PCR) has been described (Kawasaki et. al. 1988). Here, the RT-PCR technique was employed in order to study transcription of the prolactin and growth hormone genes rather than secretion of their protein products. To further increase the sensitivity and specificity of this technique, RT-PCR analysis was combined with Southern hybridization. Total RNA preparations from several rat anterior pituitary cell lines were examined by RT-PCR/Southern hybridization analysis to detect the prolactin and growth hormone gene transcripts. RNA from three rat anterior pituitary cell lines, GH_3, 235-1 and MMQ, contain readily detectable prolactin gene transcripts. However, only RNA from the GH_3 cell line contains any growth hormone mRNA. Dilution experiments have demonstrated that there is at least 10^5 times as much growth hormone

mRNA in the GH_3 cell total RNA preparation than in the 235-1 and MMQ cell RNA preparations, suggesting that in these two cell lines, the growth hormone gene is transcriptionally silent. However, the molecular mechanisms responsible for silencing this gene in the 235-1 and MMQ cells, or for activating the gene in the GH_3 cells, have not yet been determined (see below).

Pit-1 is not sufficient for expression of the prolactin and growth hormone genes

Several lines of evidence suggest that, in addition to Pit-1, other transcription factors are necessary for limiting expression of the prolactin and growth hormone genes to the appropriate cell types during development and in the mature pituitary. First, during murine development, pit-1 transcripts are first detected on day e15.5-16 (Simmons et. al. 1990). Activation of the pit-1 gene is sufficient to lead to the expression of the growth hormone gene, whose transcripts are first detected on day e18 (Simmons et. al. 1990). Although prolactin gene transcripts are also first detected on day e18, they do not reach maximal levels until after birth (Simmons et. al. 1990), suggesting that a factor, in addition to Pit-1, is required for maximal, postnatal expression of the prolactin gene.

Second, the Pit-1 mRNA and protein are detected in cell types that express either the prolactin gene alone, or both the prolactin and growth hormone genes (see Results). While it has been convincingly demonstrated that Pit-1 is necessary for expression of the prolactin and growth hormone genes, it is not sufficient for expression of the two genes. Otherwise, one would expect all cells that contain the Pit-1 protein to also contain both prolactin and growth hormone transcripts. These results are consistent with a previously proposed growth hormone gene-specific repressor (Larson et. al. 1986, Pan et. al. 1990).

Third, several spontaneously occurring dwarf mouse lines have been identified (ie. (Snell 1929). These mice suffer from a combined pituitary hormone deficiency and a pituitary hypoplasia due to a mutation in the pit-1 gene, subsequently producing a functionally altered Pit-1 protein (Li et. al. 1990, Radovick et. al. 1992, Shibayama et. al.

1993). Interestingly, hereditary dwarfism and combined hormone deficiency in humans has also been attributed to a mutation in the pit-1 gene, yet these patients do not suffer from a pituitary hypoplasia (Pfäffle et. al. 1992, Tatsumi et. al. 1992). Such observations lead to two possibilities: (a) Regions important for Pit-1 protein function are precisely defined, since the mutation of the Pit-1 protein does not affect its ability to maintain expression of its own gene, but it is no longer capable of directing expression of the prolactin or growth hormone genes. Regions outside of the previously defined serine/threonine-rich domain may also be important for trans-activation activity by properly aligning Pit-1 on its DNA binding site. (b) Alternatively, mutations in the Pit-1 protein may interfere with its ability to interact with other proteins thereby preventing Pit-1-dependent, developmental activation of the prolactin and growth hormone genes.

Perhaps the most elegant demonstration of the requirement for additional factors necessary for expression of the prolactin and growth hormone genes, was carried out in transgenic mice (Lew et. al. 1993). These mice develop pituitary tumors by Pit-1 gene promoter-directed expression of the SV40 T-antigen, resulting in dwarfism and pituitary hypoplasia (Lew et. al. 1993). The pituitaries of these animals express both the pit-1 gene transcript and protein product, however, they fail to express either the prolactin or growth hormone genes (Lew et. al. 1993). Moreover, a cell line derived from one of the pituitary tumors does not express a transiently transfected prolactin or growth hormone gene promoter-luciferase reporter construct (Lew et. al. 1993). However, extracts prepared from this cell line are still capable of protecting sites GH-1 and GH-2 of the rat growth hormone gene promoter in a DNAse I footprinting assay (Lew et. al. 1993). Therefore, while the Pit-1 protein is detected in these tumors and it is still capable of binding to sites of the growth hormone gene promoter, additional signals are required in order to direct further differentiation of the pituitary and transcription of the pituitary-specific genes.

Lastly, it has been demonstrated that Pit-1-independent mechanisms are responsible for expression of the prolactin gene in 235-1 cells and expression of the pit-1 gene in these cells is regulated post-transcriptionally (see Results). Interestingly, prolactin gene transcripts have been detected in lymphocytes (Pellegrini et. al. 1992, Gellerson et. al. 1994) and there is increasing evidence that prolactin plays an important role in the immune response (reviewed in Reber 1993). Expression of the prolactin gene in lymphocytes occurs through Pit-1 independent mechanisms and transcription of the prolactin gene in these cells is initiated at a site 5'- of the pituitary prolactin transcription start site (Pellegrini et. al. 1992, Berwaer et. al. 1994, Gellerson et. al. 1994). Whether transcription in 235-1 cells is initiated from the lymphoid- or pituitary prolactin-specific transcription start site cannot be resolved with the oligonucleotides used for RT-PCR analysis studies described above (see Results). Although unlikely, there thus remains the possibility that the 235-1 cells are actually of lymphoid origin and not pituitary-derived, as previously thought. Studies demonstrating that these 235-1 cells are truly of pituitary origin have not yet been performed.

What then, are the molecular mechanisms responsible for the maximal, cell type-specific expression of these two genes? Several prolactin and growth hormone gene-specific transcriptional activators (other than Pit-1) and repressors have been proposed (Larson et. al. 1986, Pan et. al. 1990, Jackson et. al. 1992). However, to date, none have been purified or cloned. Zn-15, a unique transcription factor with 15 putative zinc fingers, has been shown to be necessary for maximal expression of the growth hormone gene (Lipkin et. al. 1993). While this may be true, maximal expression of a gene and restriction of its expression to the appropriate cell types, are mutually exclusive events. Moreover, the Zn-15 gene is widely expressed (see Results, Lipkin et. al. 1993), while one would predict that a transcription factor responsible for the cell type-specific expression of its target gene, would have its expression restricted to those same cell types. Therefore, while Zn-15 unquestionably is required for maximal expression of the

growth hormone gene, it is not likely to represent the single factor responsible for directing cell type-specific expression of the growth hormone gene.

Unresolved issues

Pit-1a has been shown to arise as a result of alternative splicing of the Pit-1 primary transcript (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). The protein product contains an in-frame 26 amino acid insert in the trans-activation domain (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). This insert, like the remainder of the trans-activation domain of Pit-1, is serine and threonine-rich (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992) and contains a potential Protein Kinase C phosphorylation site (Konzak and Moore 1992, Morris et. al. 1992), raising the possibility that Pit-1a activity may be regulated differently than Pit-1 (**Figure 18a**). The Pit-1a mRNA is approximately $1/7^{th}$-$1/5^{th}$ as abundant as the Pit-1 mRNA in the GH_3 somatomammotropic cell line (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). However, the Pit-1a protein is not consistently detected by Western analysis, suggesting that the 26 amino acid insert may somehow affect its translation efficiency or stability (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992). Pit-1a binds to the same regions of the rat prolactin and growth hormone gene proximal promoters as Pit-1 (see Results, Morris et. al. 1992, Theill et. al. 1992). Pit-1a is unable to trans-activate the rat prolactin gene (Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992), but is capable of trans-activating the rat growth hormone gene (Konzak and Moore 1992, Theill et. al. 1992).

The biochemical basis of the ability of isoforms of Pit-1 to differentially regulate the rat prolactin and growth hormone genes has not been determined. However, there are several intriguing possibilities. Although Pit-1a is able to trans-activate the rat growth hormone gene, but not the rat prolactin gene, it is unlikely that Pit-1a could in some way be directly involved in the cell-type specific expression of the rat growth hormone gene.

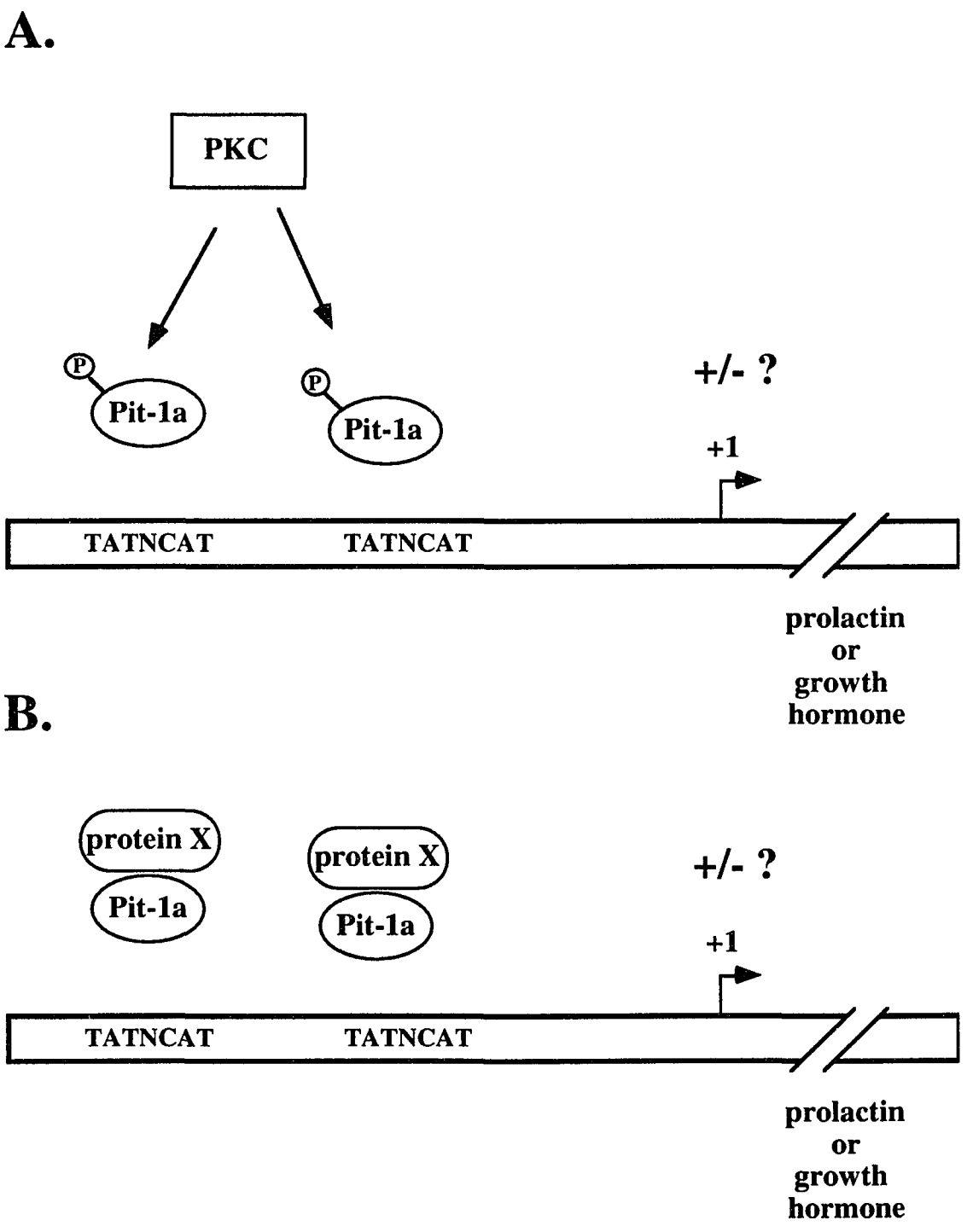


Figure 18. Model of possible Pit-1a function. (A) Since the Pit-1a insert contains a potential Protein Kinase C phosphorylation site, its activity may be regulated differently than that of Pit-1. (B) The region unique to Pit-1a may direct specific protein-protein interactions (see text).

The Pit-1a mRNA is detected in the same cell types that contain the Pit-1 mRNA (see Results, Konzak and Moore 1992, Morris et. al. 1992, Theill et. al. 1992) and Pit-1a mRNA has also been detected in a mouse thyrotropic tumor (Morris et. al. 1992). However, not all of these cell types express the growth hormone gene (see Results). Since the Pit-1a protein is not reproducibly detected by Western analysis, it is possible that these same cell lines which have been shown to contain Pit-1a transcripts never synthesize the Pit-1a protein.

Alternatively, it may not be Pit-1a alone which directs cell type-specific expression of the growth hormone gene, but an additional factor which exerts its effects through interactions with the Pit-1a-specific region (**Figure 18b**). This factor need not bind DNA, but instead act as a 'bridge' between Pit-1a and the basal transcription machinery, like CBP's ability to direct increased transcription of cAMP-responsive genes through interactions between phosphorylated CREB and TFIIB (Chrivia et. al. 1993, Arias et. al. 1994, Kwok et. al. 1994). The expression of the gene encoding this putative trans-acting factor could be restricted to cells of the anterior pituitary determined to express the growth hormone gene. The identification of a potential Protein Kinase C phosphorylation site within the 26 amino acid insert of Pit-1a suggests that Pit-1a function may be regulated differently than Pit-1 (Konzak and Moore 1992, Morris et. al. 1992). Examination of the ability of Pit-1a to interact with other nuclear proteins and post-translational modification studies of Pit-1a both can be performed using the epitope-tagging technique described below.

While the studies described in this thesis were being carried out, several reports were published describing the detection and cloning of the Δ4Pit-1 isoform (Voss et. al. 1993, Day and Day 1994b, Day and Day 1994a). Many of the results obtained from the distribution and functional studies described here and in the published reports are in agreement, while some observations are at odds. This is an attempt to reconcile these discrepancies. Δ4Pit-1 is identical to Pit-1 except for an in-frame, 54 amino acid deletion

of a majority of the POUspecific domain. Since the excised region exactly matches exon IV of the mouse pit-1 gene (Li et. al. 1990, Voss et. al. 1993), Δ4Pit-1 is proposed to arise as a result of alternative splicing of the Pit-1 primary transcript (Voss et. al. 1993, Day and Day 1994a). Δ4Pit-1 is unable to bind to several previously characterized Pit-1 binding sites in the rat prolactin or growth hormone gene proximal promoters (see Results, Voss et. al. 1993). However, using a method of random oligonucleotide selection, an artificial Δ4Pit-1 consensus binding site has been identified (Voss et. al. 1993). Like the binding sites of many other POU family members, the Δ4Pit-1 binding site identified by random oligonucleotide selection is AT-rich (Voss et. al. 1993), however, it does not resemble the canonical TATNCAT Pit-1 DNA binding site motif (Nelson et. al. 1988, Voss et. al. 1993), nor has any target gene containing a Δ4Pit-1 binding site been identified. While Δ4Pit-1 lacks the $POU_{specific}$ domain, the POU_{homeo} domain remains intact and previous studies have shown that the Pit-1 POU_{homeo} domain is capable of binding DNA, albeit with lower affinity and different specificity than the entire POU domain (Theill et. al. 1989, Ingraham et. al. 1990, Verrijzer et. al. 1990, Verrijzer et. al. 1992). Clearly, it is the POU_{homeo} domain of Δ4Pit-1 that is directing Δ4Pit-1-DNA interactions, but the lack of a $POU_{specific}$ domain causes the sequence specificity of Δ4Pit-1 to differ from that of Pit-1 (Voss et. al. 1993). Interestingly, Δ4Pit-1 is unable to trans-activate a reporter gene containing three copies of the Δ4Pit-1 consensus binding site, suggesting that the $POU_{specific}$ domain may play some role in the trans-activation function of Pit-1 (Voss et. al. 1993).

It has been demonstrated that GH_3 cells, maintained as a tumor in rats, lose their ability to express the prolactin gene, but prolactin gene transcripts are detected once the tumor has been removed and returned to cell culture (Day and Day 1994b). The loss of prolactin gene expression temporally coincides with the appearance of Δ4Pit-1 mRNA and protein following transplantation of the GH_3 cells into rats (Day and Day 1994a). Likewise, the loss of detectable Δ4Pit-1 mRNA and protein temporally coincides with the

reappearance of the prolactin gene transcripts following return of the GH_3 cell-derived tumors to culture (Day and Day 1994a). Transient co-transfection analysis has demonstrated that Δ4Pit-1 can interfere with Pit-1-dependent activation of a rat prolactin gene promoter-containing reporter gene in non-pituitary cells, presumably by forming heterodimers with Pit-1 and preventing it from binding DNA (**Figure 19b**, Day and Day 1994a).

It has been suggested that expression of the Δ4Pit-1 mRNA is restricted to GH_3 cells (see Results), or to GH_3 cells maintained as a tumor in rats (Day and Day 1994a). In contrast, one group has reported detecting the Δ4Pit-1 mRNA in total RNA prepared from three rat anterior pituitary cell lines and from the adult rat pituitary, although no negative control was included (Voss et. al. 1993). The Δ4Pit-1 protein has been detected in extracts prepared from GC cells (Voss et. al. 1993), GH_3 cells (see Results) or from GH_3 cells maintained as a tumor in rats (Day and Day 1994b, Day and Day 1994a). The underlying reason(s) for the discrepancy between the Δ4Pit-1 distribution studies is unclear. However, it is possible that the Δ4Pit-1 isoform may be generated by a cell line only under certain culture conditions.

It is puzzling that the Δ4Pit-1 isoform is not detected in the adult rat pituitary by two of the three groups who have identified it (see Results, Day and Day 1994b). The detection of the Δ4Pit-1 mRNA in the pituitary by the third group, is dubious for the same lack of a negative control as described above (Voss et. al. 1993). The results of experiments described here demonstrate that the Δ4Pit-1 isoform is detected only in the somatomammotropic GH_3 cell line (see Results). It has been proposed that the number of somatomammotropes is greatest in the developing pituitary, but in the adult there are relatively few (reviewed in Frawley and Boockfor 1991), perhaps explaining why, even when using extremely sensitive techniques, the Δ4Pit-1 transcript cannot be detected in total RNA prepared from adult rat pituitaries.

As described above, transient transfection analysis of non-pituitary cell lines has

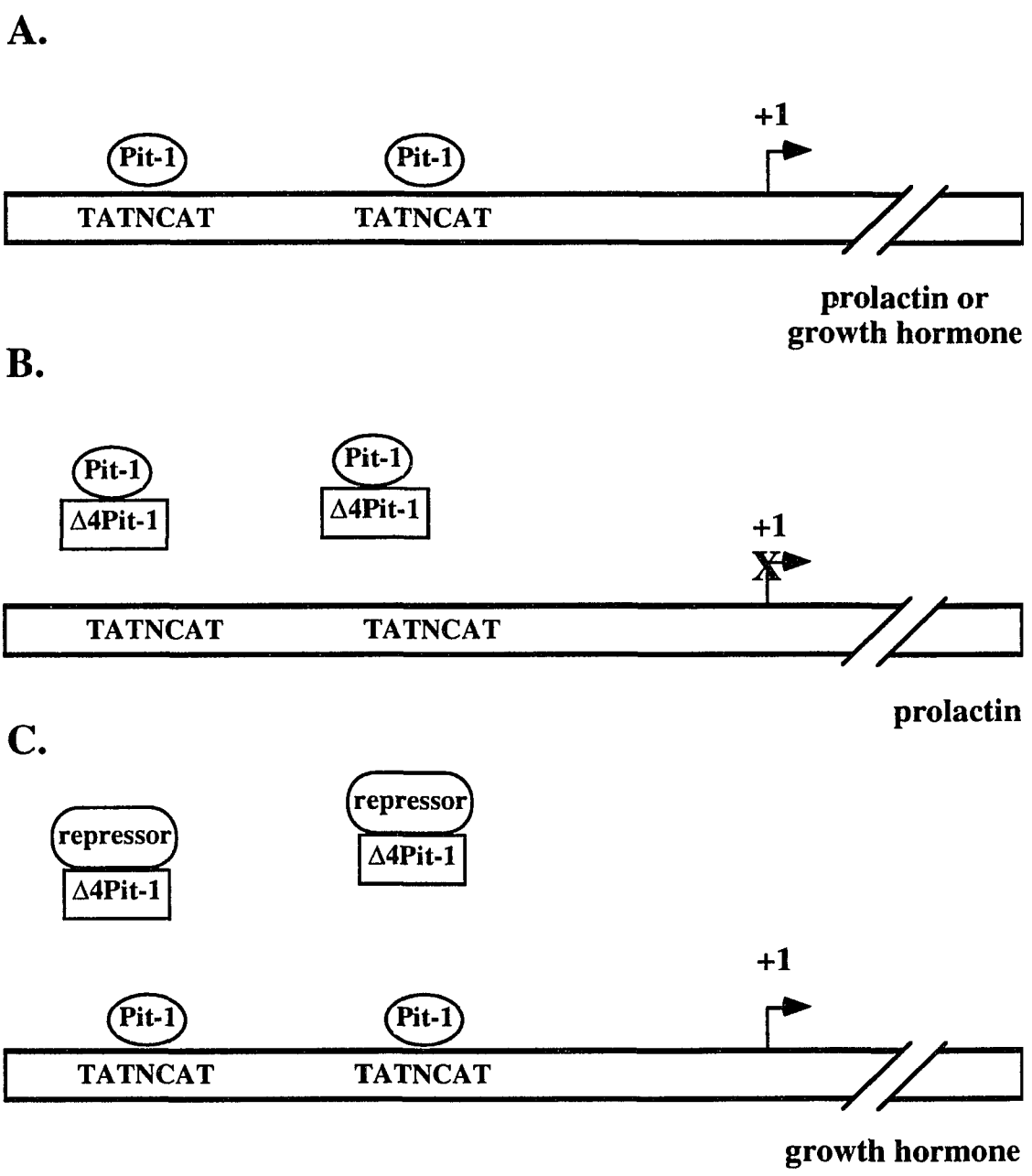


Figure 19. Model of possible Δ4Pit-1 function. (A) Pit-1 binding to the prolactin and growth hormone gene promoters leads to expression of these two genes. (B) Δ4Pit-1 forms heterodimers with Pit-1 to inhibit Pit-1 binding to sites within the rat prolactin gene, repressing prolactin gene transcription. (C) Δ4Pit-1 interacts with an as of yet unidentified growth hormone gene-specific repressor, thereby inhibiting its repressive activity. Pit-1 is now able to bind to sites within the growth hormone gene promoter, resulting in expression of the growth hormone gene.

demonstrated that Δ4Pit-1 can inhibit Pit-1-dependent activation of the rat prolactin gene by approximately 50% (Day and Day 1994a). Data presented here suggests that Δ4Pit-1 may be involved in rat growth hormone gene activation, because the Δ4Pit-1 isoform is only detected in a cell line which expresses both the prolactin and growth hormone genes (see Results). Although experiments performed by others, demonstrating the ability of Δ4Pit-1 to inhibit Pit-1 activity (Day and Day 1994a), have been repeated in this laboratory (Fischberg 1994), the result of a preliminary experiment has shown that Δ4Pit-1 is capable of activating transcription of a growth hormone-CAT reporter gene in a cell line that contains Pit-1, but does not normally express either the Δ4Pit-1 isoform, or the growth hormone gene (Fischberg 1994). The apparent contradictory roles of the Δ4Pit-1 protein as both a repressor of prolactin gene transcription and a growth hormone gene-specific activator are not necessarily so.

It has been proposed that Δ4Pit-1 interferes with Pit-1-dependent activation of the rat prolactin gene by forming heterodimers with Pit-1 and the Δ4Pit-1-Pit-1 heterodimers are not likely to bind DNA as well as a Pit-1 homodimer (Day and Day 1994a). Results presented here are more consistent with a previously proposed growth hormone gene-specific repressor (Larson et. al. 1986, Pan et. al. 1990). This putative repressor could be present in cell types that express only the prolactin gene (see Results). Such a repressor could interfere with Pit-1-dependent activation of the growth hormone gene by binding to sites within the growth hormone promoter, in close proximity to the Pit-1 binding sites, or perhaps even in direct contact with Pit-1, thus interfering with Pit-1-DNA binding. Pit-1-dependent activation of the prolactin gene would be unaffected. However, in a cell line which expresses both the prolactin and growth hormone genes (see Results), expression of the Δ4Pit-1 isoform could repress the activity of the putative repressor, allowing Pit-1 to bind to its target sites in the growth hormone gene promoter and as a consequence, activate transcription of the gene (**Figure 19c**).

Future studies

The Pit-1 protein is routinely seen as a 31-33KDa 'doublet' by Western analysis as a result of alternate translation initiation site usage (Voss et. al. 1991b) and these two species could be considered additional isoforms of the Pit-1 protein. However, no unique functional properties have been attributed to these two isoforms, as have been demonstrated for the liver-enriched transcription factors, LAP and LIP (Descombes and Schibler 1991). Two isoforms of Pit-1, Pit-1a and Δ4Pit-1, which arise by alternative splicing of the Pit-1 precursor RNA have been identified (see Results), but no unique functional properties have been attributed to them. One possibility is that the isoforms of Pit-1, particularly Pit-1a, may be capable of interacting with cellular proteins with which Pit-1 is unable to interact, resulting in a unique heteromeric activity. Alternatively, one or both of the isoforms may interact with a cellular protein which normally inhibits Pit-1 activity. By interacting with the putative Pit-1 inhibitor, the alternatively spliced Pit-1 isoform could allow Pit-1 to carry out its function.

In order to investigate such intriguing possibilities, epitope-tagged versions of each of the Pit-1 isoforms have been developed (see Results). This 'tag' will allow the Pit-1 isoforms to be recovered from solution by immunoprecipitation (see Results) and since the tag is small and has been placed at the NH_2-terminus of each of the isoforms, it should not interfere with normal Pit-1 isoform function. Once cell lines stably transfected with the Pit-1 isoforms have been generated, extracts can then be prepared from these cell lines. Subsequently, the Pit-1 isoforms can be immunoprecipitated, under non-stringent conditions (ie. physiological salt concentrations, no detergents, etc.), or in the presence of a reversible chemical cross-linker, both intended to preserve protein-proteins interactions. Following separation of the immunoprecipitates by SDS-PAGE and silver staining, proteins which co-precipitate with Pit-1 and its isoforms may be identified. Should any unique protein-protein interactions be observed, the procedure could be scaled up to facilitate the purification and subsequent cloning of the Pit-1

isoform protein partner(s). Such an approach could also be utilized for studies of the post-translational modification of the Pit-1 isoforms in response to hormones. So much has been learned about the important role Pit-1 plays in the developmental and homeostatic regulation of the prolactin and growth hormone genes since it was first cloned (Bodner et. al. 1988, Ingraham et. al. 1988), but it is apparent that there still is much to be gained from continued study of Pit-1 and its isoforms.

Literature Cited

Arias, J., A. S. Alberts, P. Brindle, F. X. Claret, T. Smeal, M. Karin, J. Feramisco and M. Montminy (1994). "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor." Nature **370**: 226-229.

Assa-Munt, N., R. J. Mortishire-Smith, R. Aurora, W. Herr and P. E. Wright (1993). "The Solution Structure of the Oct-1 POU-Specific Domain Reveals a Striking Similarity to the Bacteriophage Lambda Repressor DNA-Binding Domain." Cell **73**: 193-205.

Aurora, R. and W. Herr (1992). "Segments of the POU Domain Influence One Another's DNA-Binding Specificity." Molecular and Cellular Biology **12**: 455-467.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Siedman, J. A. Smith and K. Struhl (1992). Short Protocols in Molecular Biology. New York, John Wiley and Sons.

Bancroft, F. C. (1981). GH Cells: Functional Clonal Lines of Rat Pituitary Tumor Cells. Functionally Differentiated Cell Lines. New York, Alan R. Liss, Inc. 47-59.

Barinaga, M., G. Yamonoto, C. Rivier, W. Vale, R. Evans and M. G. Rosenfeld (1983). "Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor." Nature **306**: 84-85.

Bedo, G., P. Santisteban and A. Aranda (1989). "Retinoic acid regulates growth hormone gene expression." Nature **339**: 231-234.

Berwaer, M., J. A. Martial and J. R. E. Davis (1994). "Characterization of an Up-Stream Promoter Directing Extrapituitary Expression of the Human Prolactin Gene." Molecular Endocrinology **8**: 635-642.

Bodner, M., J.-L. Castrillo, L. E. Theill, T. Deernick, M. Ellisman and M. Karin (1988). "The Pituitary-Specific Transcription Factor GHF-1 Is a Homeobox-Containing Protein." Cell **55**: 505-518.

Bradford, M. M. (1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." Analytical Biochemistry **72**: 248-254.

Brent, G. A., P. R. Larsen, J. W. Harney, R. J. Koenig and D. D. Moore (1989). "Functional Characterization of the Rat Growth Hormone Promoter Elements Required for Induction by Thyroid Hormone with and without a Co-transfected ß-Type Thyroid Hormone Receptor." The Journal of Biological Chemistry **254**: 178-192.

Chen, R., H. A. Ingraham, M. N. Treacy, V. R. Albert, L. Wilson and M. G. Rosenfeld (1990). "Autoregulation of pit-1 gene expression mediated by two cis-active promoter elements." Nature **346**: 583-586.

Chrivia, J. C., R. P. S. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy and R. H. Goodman (1993). "Phosphorylated CREB binds specifically to the nuclear protein CBP." Nature **365**: 855-859.

Clerc, R. G., L. M. Corcoran, J. H. LeBowitz, D. Baltimore and P. A. Sharp (1988). "The B-cell-specific Oct-2 protein contains POU box- and homeo box-type domains." Genes and Development **2**: 1570-1581.

Coleman, D. T. and C. Bancroft (1993). "Pituitary Adenylate Cyclase-Activating Poypeptide Stimulates Prolactin Gene Expression in a Rat Pituitary Cell Line." Endocrinology **133**: 2736-2742.

Day, R. N. and K. H. Day (1994a). "An Alternatively Spliced Form of Pit-1 Represses Prolactin Gene Expression." Molecular Endocrinology **8**: 374-381.

Day, R. N. and K. H. Day (1994b). "Specific Repression of Rat Prolactin Gene Expression in Transplanted Tumor Cells." Molecular Endocrinology **8**: 12-20.

Day, R. N., S. Koike, M. Sakai, M. Muramatsu and R. A. Maurer (1990). "Both Pit-1 and the Estrogen Receptor Are Required for Estrogen Responiveness of the Rat Prolactin Gene." Molecular Endocrinology **4**: 1964-1971.

Delhase, M., P. Vergani, A. Malur, E. L. Hooghe-Peters and R. J. Hooghe (1993). "The transcription factor Pit-1/GHF-1 is expressed in hemopoietic and lymphoid tissues." European Journal of Immunology **23**: 951-955.

Delidow, B. C., W. M. Billis, P. Agarwal and B. A. White (1991). "Inhibition of Prolactin Gene Transcription by Transforming Growth Factor-β in GH_3 Cells." Molecular Endocrinology **5**: 1716-1722.

Descombes, P. and U. Schibler (1991). "A Liver-Enriched Transcriptional Activator protein, LAP, and a Transcriptional Inhibitory Protein, LIP, Are Translated from the Same mRNA." Cell **67**: 569-579.

Dignam, J. D., R. M. Lebovitz and R. G. Roeder (1983). "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nucleic Acids Research **11**: 1476-1489.

Elsholtz, H. P., V. R. Albert, M. N. Treacy and M. G. Rosenfeld (1990). "A two-base change in a POU factor-binding site switches pituitary-specific to lymphoid-specific gene expression." Genes and Development **4**: 43-51.

Elsholtz, H. P., A. M. Lew, P. R. Albert and V. C. Sundmark (1991). "Inhibitory Control of Prolactin and Pit-1 Gene Promoters by Dopamine." The Journal of Biological Chemistry **266**: 22919-22925.

Emanuele, N. V., J. K. Jurgens, M. M. Halloran, J. J. Tentler, A. M. Lawrence and M. R. Kelley (1992). "The Rat Prolactin Gene Is Expressed in Brain Tissue: Detection of Normal and Alternatively Spliced Prolactin Messenger RNA." Molecular Endocrinology **6**: 35-42.

Erickson, A. H. and G. Blobel (1983). "Cell-Free Translation of Messenger RNA in a Wheat Germ System." Methods in Enzymology **96**: 38-50.

Ezzat, S., C. Ezrin, S. Yamashita and S. Melmed (1993). "Recurrent Acromegaly Resulting from Ectopic Growth Hormone Gene Expression by a Metastatic Pancreatic Tumor." Cancer **71**: 66-70.

Finney, M., G. Ruvkun and H. R. Horvitz (1988). "The C. elegans Cell Lineage and Differentiation Gene unc-86 Encodes a Protein with a Homeodomain and Extended Similarity to Transcription Factors." Cell **55**: 757-769.

Fischberg, D. J. (1994). Transcription of the Rat Prolactin Gene: Regulation by Dopamine and the Pituitary-specific Protein, Pit-1. The Mount Sinai School of Medicine of the City University of New York.

Fischberg, D. J., X.-h. Chen and C. Bancroft (1994). "A Pit-1 Phosphorylation Mutant Can Mediate Both Basal and Induced Prolactin and Growth Hormone Promoter Activity." Molecular Endocrinology **8**: 1566-1573.

Foulkes, N. S. and P. Sassone-Corsi (1992). "More Is Better: Activators and Repressors from the Same Gene." Cell **68**: 411-414.

Fourney, R. M., J. Miyakoshi, R. S. Day III and M. C. Paterson (1988). "Northern Blotting: Efficient RNA Staining and Transfer." Focus **10**: 5-7.

Fox, S. R., M. T. C. Jong, J. Casanova, Z.-S. Ye, F. Stanley and H. H. Samuels (1990). "The Homeodomain Protein, Pit-1/GHF-1, Is Capable of Binding to and Activating Cell-Specific Elements of Both the Growth Hormone and Prolactin Gene Promoters." Molecular Endocrinology **4**: 1069-1080.

Frawley, L. S. and F. R. Boockfor (1991). "Mammosomatotropes: Presence and Functions in Normal and Neoplastic Pituitary Tissue." Endocrine Reviews **12**: 337-355.

Gellerson, B., R. Kempf, R. Telgmann and G. E. DiMattia (1994). "Nonpituitary Human Prolactin Gene Transcription is Independent of Pit-1 and Differentially Controlled in Lymphocytes and in Endometrial Stroma." Molecular Endocrinology **8**: 356-373.

Gourdji, D. and J.-N. Laverriére (1994). "The rat prolactin gene: a target for tissue-specific and hormone-dependent transcription factors." Molecular and Cellular Endocrinology **100**: 133-142.

Hatzopoulos, A. K., A. s. Stoykova, J. R. Erselius, M. Goulding, T. Neuman and P. Gruss (1990). "Structure and expression of the mouse Oct2a and Oct2b, two differentially spliced products of the same gene." Development **109**: 349-362.

Haugen, B. R., D. F. Gordon, A. R. Nelson, W. M. Wood and E. C. Ridgway (1994). "The Combination of Pit-1 and Pit-1T Have a Synergistic Stimulatory Effect on the Thyrotropin β-Subunit Promoter but not the Growth Hormone or Prolactin Promoters." Molecular Endocrinology **8**: 1574-1582.

Haugen, B. R., W. M. Wood, D. F. Gordon and E. C. Ridgway (1993). "A Thyrotrope-specific Variant of Pit-1 Transactivates the Thyrotropin ß Promoter." The Journal of Biological Chemistry **268**: 20818-20824.

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 and Development **2**: 1513-1516.

Howard, P. W. and R. A. Maurer (1994). "Thyrotropin Releasing Hormone Stimulates Transient Phosphorylation of the Tissue-specific Transcription Factor, Pit-1." The Journal of Biological Chemistry **269**: 28662-28669.

Hsu, T., J. A. Gogos, S. A. Kirsh and F. C. Kafatos (1992). "Multiple Zinc Finger Forms Resulting from Developmentally Regulated Alternative Splicing of a Transcription Factor Gene." Science **257**: 1946-1950.

Hunter, T. and M. Karin (1992). "The Regulation of Transcription by Phosphorylation." Cell **70**: 375-387.

Ingraham, H. A., V. R. Albert, R. Chen, E. B. Crenshaw III, H. P. Elsholtz, X. He, M. S. Kapiloff, H. J. Mangalam, L. W. Swanson, M. N. Treacy and M. G. Rosenfeld (1990). "A family of POU-domain and Pit-1 tissue-specifc transcription factors in pituitary and neuroendocrine development." Annual Review of Physiology **52**: 773-791.

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 Homeodomain Specifies a Pituitary Phenotype." Cell **55**: 519-529.

Ingraham, H. A., S. E. Flynn, J. W. Voss, V. R. Albert, M. S. Kapiloff, L. Wilson and M. G. Rosenfeld (1990). "The POU-Specific Domain of Pit-1 Is Essential for Sequence-Specific, High Affinity DNA Binding and DNA-Dependent Pit-1-Pit-1 Interactions." Cell **61**: 1021-1033.

Jackson, A. E., S. K. Bandyopadhyay and C. Bancroft (1990). "Epidermal growth factor and phorbol ester regulate prolactin gene expression via distinct pathways." Molecular and Cellular Endocrinology **69**: R7-R11.

Jackson, S. M., C. A. Keech, D. J. Williamson and A. Gutierrez-Hartmann (1992). "Interaction of Basal Positive and Negative Transcription Elements Controls Repression of the Proximal Rat Prolactin Promoter in Nonpituitary Cells." Molecular and Cellular Biology **12**: 2708-2719.

Jones, G. J. and D. F. Catanzaro (1991). "Interactions between Rat Prolactin Gene Promoter and Enhancer Regions in Mammosomatotrope and Lactotrope Cell Lines." Molecular Endocrinology **5**: 1836-1991.

Judd, A. M., I. S. Login, K. Kovacs, P. C. Ross, B. L. Spangelo, W. D. Jarvis and R. M. MacLeod (1988). "Characterization of the MMQ Cell, A Prolactin-Secreting Clonal Cell Line That Is Responsive to Dopamine." Endocrinology **123**: 2341-2350.

Kapiloff, M. S., Y. Farkash, M. Wegner and M. G. Rosenfeld (1991). "Variable Effects of Phosphorylation of Pit-1 Dicatated by the DNA Response Elements." Science **253**: 786-789.

Karin, M. (1992). "Signal transduction from cell surface to nucleus in development and disease." FASEB J. **6**: 2581-2590.

Karin, M., J.-L. Castrillo and L. E. Theill (1990). "Growth hormone gene regulation: a paradigm for cell-type-specific gene activation." Trends in Genetics **6**: 92-96.

Kawasaki, E. S., S. S. Clark, M. Y. Coyne, S. D. Smith, R. Champlin, O. N. Witte and F. P. McCormick (1988). "Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro." Proceedings of the National Academy of Sciences, USA **85**: 5698-5702.

Koenig, R. J., M. A. Lazar, R. A. Hodin, G. A. Brent, P. R. Larsen, W. W. Chin and D. D. Moore (1989). "Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing." Nature **337**: 659-661.

Kolodziej, P. A. and R. A. Young (1991). "Epitope Tagging and Protein Surveillance." Methods in Enzymology **194**: 508-519.

Konzak, K. E. and D. D. Moore (1992). "Functional Isoforms of Pit-1 Generated by Alternative Messenger RNA Splicing." Molecular Endocrinology **6**: 241-247.

Kozak, M. (1991). "An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control." The Journal of Cell Biology **115**: 887-903.

Kwok, R. P. S., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bächiner, R. G. Brennan, S. G. E. Roberts, M. R. Green and R. H. Goodman (1994). "Nuclear protein CBP is a coactivator for the transcription factor CREB." Nature **370**: 223-226.

Laemlli, U. K. (1970). "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4." Nature **227**: 680-685.

Larson, P. R., J. W. Harney and D. D. Moore (1986). "Repression mediates cell-type-specific expression of the rat growth hormone gene." Proceedings of the National Academy of Sciences, USA **83**: 8283-8287.

Lew, D., H. Brady, K. Klausing, K. Yaginuma, L. E. Theill, C. Stauber, M. Karin and P. L. Mellon (1993). "GHF-1-promoter-targeted immortalization of a somatotropic progenitor cell results in dwarfism in transgenic mice." Genes and Development **7**: 683-693.

Li, S., E. B. Crenshaw III, E. J. Rawson, D. M. Simmons, L. W. Swanson and M. G. Rosenfeld (1990). "Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1." Nature **347**: 528-533.

Lin, C., S.-C. Lin, C.-P. Chang and M. G. Rosenfeld (1992). "Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth." Nature **360**: 765-768.

Lin, S.-C., S. Li, D. W. Drolet and M. G. Rosenfeld (1994). "Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope." Development **120**: 515-522.

Lin, S.-C., C. R. Lin, I. Judovsky, A. J. Lusis, P. E. Sawchenko and M. G. Rosenfeld (1993). "Molecular basis of the little mouse phenotype and implications for cell type-specific growth." Nature **364**: 208-213.

Lipkin, S. M., A. M. Naar, K. A. Kalla, R. A. Sack and M. G. Rosenfeld (1993). "Identification of a novel zinc finger protein binding a conserved element critical for

Pit-1-dependent growth hormone gene expression." Genes and Development **7**: 1674-1687.

Lira, S. A., K. A. Kalla, C. K. Glass, D. W. Drolet and M. G. Rosenfeld (1993). "Synergistic Interactions Between Pit-1 and Other Elements Are Required for Effective Somatotroph Rat Growth Hormone Gene Expression in Transgenic Mice." Molecular Endocrinology **7**: 694-701.

Lufkin, T., A. E. Jackson, W. T. Pan and C. Bancroft (1989). "Proximal Rat Prolactin Promoter Sequences Direct Optimal, Pituitary Cell-Specific Transcription." Molecular Endocrinology **3**: 559-566.

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 and Development **3**: 946-958.

McCormick, A., H. Brady, L. E. Theill and M. Karin (1990). "Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues." Nature **345**: 829-832.

McCormick, A., D. Wu, J.-L. Castrillo, S. Dana, J. Strobl, E. B. Thompson and M. Karin (1988). "Extinction of Growth Hormone Expression in Somatic Cell Hybrids Involves Repression of the Specific Trans-Activator GHF-1." Cell **55**: 379-389.

Morris, A. E., B. Kloss, R. E. McChesney, C. Bancroft and L. A. Chasin (1992). "An alternatively spliced Pit-1 isoform altered in its ability to trans-activate." Nucleic Acids Research **20**: 1355-1361.

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-acitve genomic sequences dictate the pititary cell type-specific expression of rat prolactin and growth hormone genes." Nature **322**: 557-562.

Nowak, R. A., M. S. Rein, L. J. Heffner, A. J. Friedman and A. H. Tashjian Jr. (1993). "Production of Prolactin by Smooth Muscle Cells Cultured from Human Uterine Fibroid Tumors." Jounal of Clinical Endocrinology and Metabolism **76**: 1308-1313.

Okimura, Y., P. W. Howard and R. A. Maurer (1994). "Pit-1 Binding Sites Mediate Transcriptional Responses to Cyclic Adenosine 3',5'-Monophosphate Through a Mechanism That Does Not Require Inducible Phosphorylation of Pit-1." Molecular Endocrinology **8**: 1559-1565.

Pan, W. T., Q. Liu and C. Bancroft (1990). "Identification of a Growth Hormone Gene Promoter Repressor Element and Its Cognate Double- and Single-stranded DNA-binding Proteins." The Journal of Biological Chemistry **265**: 7022-7028.

Pellegrini, I., J.-J. Lebrun, S. Ali and P. A. Kelly (1992). "Expression of Prolactin and Its Receptor in Human Lymphoid Cells." Molecular Endocrinology **6**: 1023-1031.

Pfäffle, R. W., G. E. DiMattia, J. S. Parks, M. R. Brown, J. M. Wit, M. Jansen, H. Van der Nat, J. L. Van den Brande, M. G. Rosenfeld and H. A. Ingraham (1992). "Mutation of the POU-Specific Domain of Pit-1 and Hypopituitarism Without Pituitary Hypoplasia." Science **257**: 1118-1121.

Prager, D., M. M. Weber, S. Gebremedhin and S. Melmed (1993). "Interaction between insulin and thyroid hormone in rat pituitary tumour cells: insulin attenuates triiodothyronine-induced growth hormone mRNA levels." Journal of Endocrinology **137**: 107-114.

Radovick, S., M. Nations, Y. Du, L. a. Berg, B. D. Weintraub and F. E. Wondisford (1992). "A Mutation in the POU-Homeodomain of Pit-1 Responsible for Combined Pituitary Hormone Deficiency." Science **257**: 1115-1118.

Reber, P. M. (1993). "Prolactin and Immunomodulation." The American Journal of Medicine **95**: 637-644.

Reymond, M. J., D. D. Nansel, G. H. Burrows, W. B. Neaves and J. C. Porter (1984). "A new clonal strain of rat pituitary tumour cells: a model for non-regulated secretion of prolactin." Acta Endocrinologica **106**: 459-470.

Rhodes, S. J., R. Chen, G. E. DiMattia, K. M. Scully, K. A. Kalla, S.-C. Lin, V. C. Yu and M. Rosenfeld (1993). "A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation of the Pit-1 gene." Genes and Development **7**: 913-932.

Rosenfeld, M. G. (1991). "POU-domain transcription factors: pou-er-ful developmental regulators." Genes and Development **5**: 897-907.

Sanger, F., S. Nicklen and A. R. Coulson (1977). "DNA sequencing with chain-terminating inhibitors." Proceedings of the National Academy of Sciences, USA **74**: 5463-5467.

Schwind, J. L. (1928). "The development of the hypophysis cerebri of the albino rat." The American Journal of Anatomy **41**: 295-315.

Shibayama, K., Y. Ohyama, M. Ono and S. Furudate (1993). "Expression of mRNA coding for pituitary hormones and pituitary-specific transcription factor in the pituitary gland of the rdw rat with hereditary dwarfism." Journal of Endocrinology **138**: 307-313.

Shupnik, M. A., B. A. Rosenzweig, D. E. Friend and M. E. Mason (1992). "Thyrotropin TSH)-Releasing Hormone-Responsive Elements in the Rat TSHβ Gene Have Distinct Biological and Nuclear Protein-Binding Properties." Molecular Endocrinology **5**: 43-52.

Simmons, D. M., J. W. Voss, H. A. Ingraham, J. M. Holloway, R. S. Broide, M. G. Rosenfeld and L. W. Swanson (1990). "Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors." Genes and Development **4**: 695-711.

Skipper, J. K., L. J. Young, J. M. Bergeron, M. T. Tetzlaff, C. T. Osborn and D. Crews (1993). "Identification of an isoform of the estrogen receptor messenger RNA lacking

exon four and present in the brain." Proceedings of the National Academy of Sciences, USA **90**: 7172-7175.

Snell, G. D. (1929). "Dwarf, A New Mendelian Recessive Character of the House Mouse." Proceedings of the National Academy of Sciences, USA **15**: 733-734.

Sohn, K.-Y., S. N. Maity and B. de Crombrugghe (1994). "Studies on the structure of the mouse CBF-A gene and properties of a truncated CBF-A isoform generated from and alternatively spliced RNA." Gene **139**: 147-153.

Stanley, F. M. (1992). "An Element in the Prolactin Promoter Mediates the Stimulatory Effect of Insulin on Transcription of the Prolactin Gene." The Journal of Biological Chemistry **267**: 16719-16726.

Stehle, J. H., N. S. Foulkes, C. A. Molina, V. Simonneaux, P. Pévet and P. Sassone-Corsi (1993). "Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland." Nature **365**: 314-320.

Steinfelder, H. J., S. Radovick and F. E. Wondisford (1992). "Hormonal regulation of the thyrotropin β-subunit gene by phosphorylation of the pituitary-specific transcription factor Pit-1." Proceeding of the National Academy of Sciences USA **89**: 5942-5945.

Struthers, R. S., D. Gaddy-Kurten and W. W. Vale (1992). "Activin inhibits binding of transcription factor Pit-1 to the growth hormone promoter." Proceedings of the National Academy of Sciences USA **89**: 11451-11455.

Sturm, R. A., G. Das and W. Herr (1988). "The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain." Genes and Development **2**: 1582-1599.

Sugihara, H., S. Minami, K. Okada, J. Kamegal, O. Hasegawa and I. Wakabayashi (1993). "Somatostatin Reduces Transcription of the Growth Hormone Gene in Rats." Endocrinology **132**: 1225-1229.

Tatsumi, K.-i., K. Miyai, T. Notomi, K. Kaibe, N. Amino, Y. Mizuno and H. Kohno (1992). "Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene." Nature Genetics **1**: 56-58.

Theill, L. E., J.-L. Castrillo, D. Wu and M. Karin (1989). "Dissection of functional domains of the pituitary-specific transcription factor GHF-1." Nature **342**: 945-948.

Theill, L. E., K. Hottori, D. Lazzaro, J.-L. Castrillo and M. Karin (1992). "Differential splicing of the GHF1 primary transcript gives rise to two functionally distinct homeodomain proteins." The EMBO Journal **11**: 2261-2269.

Theill, L. E. and M. Karin (1993). "Transcriptional Control of GH Expression and Anterior Pituitary Development." Endocrine Reviews **14**: 670-689.

Towbin, H., T. Staehelin and J. Gordon (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications." Proceedings of the National Academy of Sciences USA **76**: 4350-4354.

Treacy, M. N., X. He and M. G. Rosenfeld (1991). "I-POU: a POU-domain protein that inhibits neuron-specific gene activation." Nature **350**: 577-584.

Treacy, M. N., L. I. Neilson, E. E. Turner, X. He and M. G. Rosenfeld (1992). "Twin of I-POU: A Two Amino Acid Difference in the I-POU Homeodomain Distinguishes an Activator from an Inhibitor of Transcription." Cell **68**: 491-505.

Treacy, M. N., F. Ryan and F. Martin (1991). "Functional Glucocorticoid Inducible Enhancer Activity in the 5'-flanking Sequences of the Rat Growth Hormone Gene." Journal of Steroid Biochemistry and Molecular Biology **38**: 1-15.

Verrijzer, C. P., M. J. Alkema, W. W. van Weperen, H. C. Van Leeuwen, M. J. J. Strating and P. C. van der Vliet (1992). "The DNA binding specificity of the bipartite POU domain and its subdomains." The EMBO Journal **11**: 4993-5003.

Verrijzer, C. P., A. J. Kal and P. C. van der Vliet (1990). "The oct-1 homeo domain contacts only part of the octamer sequence and full oct-1 DNA-binding activity requires the POU-specific domain." Genes and Development **4**: 1964-1974.

Verrijzer, C. P., J. A. W. M. van Oosterhout and P. C. van der Vliet (1992). "The Oct-1 POU Domain Mediates Interations between Oct-1 and Other POU Proteins." Molecular and Cellular Endocrinology **12**: 542-551.

Voss, J. W., L. Wilson, S. J. Rhodes and M. G. Rosenfeld (1993). "An Alternative Pit-1 RNA Splicing Product Reveals Modular Binding and Nonmodular Transcriptional Acivities of the POU-Specific Domain." Molecular Endocrinology **7**: 1551-1560.

Voss, J. W., L. Wilson and M. G. Rosenfeld (1991a). "POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter." Genes and Development **5**: 1309-1320.

Voss, J. W., T.-P. Yao and M. G. Rosenfeld (1991b). "Alternative Translation Initiation Site Usage Results in Two Structurally Distinct Forms of Pit-1." The Journal of Biological Chemistry **266**: 12832-12835.

Wegner, M., D. W. Drolet and M. G. Rosenfeld (1993). "POU-domain proteins: structure and function of developmental regulators." Current Opinion in Cell Biology **5**: 488-498.

Yan, G.-z., W. T. Pan and C. Bancroft (1991). "Thyrotropin-Releasing Hormone Action on the Prolactin Promoter Is Mediated by the POU Protein Pit-1." Molecular Endocrinology **5**: 535-541.