Transcriptional Regulation of the Rat Growth Hormone and Prolactin Promoters: *In vitro* and *in vivo* studies

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To my Auntie Lou.

This dissertation is dedicated to the memory of Louana Howling, whose unending love, persevering encouragement, and constant support enabled me to complete this project. We love her and all miss her dearly.

Abstract

Transcriptional Regulation of the Rat Growth Hormone and Prolactin Promoters: *In vitro* and *in vivo* studies

by Wayne T. Pan

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The rat growth hormone (GH) and prolactin (PRL) genes are evolutionarily and ontogenically related. Therefore, it is not surprising that their promoters would share common elements. Indeed, a single transcription factor, pit-1, has been identified which activates both promoters. However, under physiologic conditions, these two pituitary genes are expressed divergently. The question we attempt to address in this dissertation is how these two promoters are differentially regulated.

It had been previously demonstrated that the expression of a PRL promoter construct stably transfected into a non-pituitary cell line could be activated on cell fusion to a pituitary cell line, whereas a GH promoter construct could not. Studies described herein using other non-pituitary host cell lines, a GH promoter construct containing additional upstream promoter sequences, and a more efficient cell fusion protocol confirmed and extended those results.

Various *in vitro* methods were used to identify and characterize the pit-1-binding sites on both promoters. Using DNase footprinting techniques, four sites were identified on the proximal PRL promoter and two on the GH promoter. Sequence analysis of the binding sites revealed a consensus sequence, (A/T)TA(A/T)TCA. Gel-shift experiments using site-specific mutants of the consensus sequence identified nucleotide base-pairs critical for pit-1 binding.

Functional *in vitro* transcription experiments demonstrated the ability of a minimal PRL or GH promoter to direct cell-specific transcription. In addition,

co-transfection experiments demonstrated the ability of pit-1 to activate both promoters *in vivo*.

Deletion analysis of the GH promoter identified two repressor elements. We characterized two DNA-binding proteins which interact specifically with the proximal repressor element (PRE). ssPREB is a ubiquitous, single-strand-specific DNA-binding protein. PREB is a double-stranded DNA-binding protein found in non-pituitary cell lines. When the PRE was cloned in front of a heterologous promoter, herpesvirus thymidine kinase (HSV-tk), each class of DNA-binding protein had a unique effect on expression of the HSV-tk promoter. In a ssPREB-rich environment, the PRE is an activator, whereas in a PREB-rich environment, the PRE acts as a repressor.

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"It is often said or implied that, in our profession, a man cannot be both practical and scientific; science and practice seem to some people to be incompatible. Each man, they say, must devote himself to the one or the other. The like of this has long been said, and it is sheer nonsense."

- Sir James Paget, 1894

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Introduction

The study of mechanisms involved in cell-type specific gene expression attempts to answer a fundamental question in development: what are the controlling factors that determine the expression of a restricted subset of highly specialized genes? In the face of identical genetic material in each somatic cell, the solution to this problem may be found in three basic areas:

(1) In a purely positive or negative direct control system, there is a factor, which by its presence, either allows or inhibits the expression of those genes with which it interacts. This is a simple solution but it fails to solve the basic question. It merely shifts the answer to a secondary level: how is (are) the regulator(s) turned on?

(2) Genes can also be regulated indirectly through modification of the direct regulators or by modifying their chromosome structures allowing or preventing direct interaction by direct regulators. This too, shifts the answer to a secondary level.

(3) Through the expression of "master genes," a subset of regulators are switched on, the unique combination and levels of which determine a particular phenotype. While this is a more complex solution to the basic question, it still fails to explain the mechanism.

Thus, these three solutions each fail to specifically address the question of mechanism. Therefore, the questions that one can address at this point are limited to the identification of fundamental tools used in a particular gene expression program, which can suggest a possible mechanism for cell-specific gene expression.

In selecting a system in which to study this question, it is important not only to have a clear-cut pattern of expression, but also to have an experimental system that can be easily manipulated. These two attributes are found in the pituitary gene expression system involving the growth hormone and prolactin genes. Both of these genes are only expressed in cells of the anterior pituitary. In addition, there are established pituitary tissue culture cell lines that still retain

characteristics of their differentiated phenotype, providing a readily accessible biological model (Bancroft, 1981; Chomczynski *et al.*, 1988; Inoue and Sakai, 1991). Thus, the study of pituitary-specific genes offers investigators an ideal system in which to explore the basic mechanisms of gene regulation.

The anterior lobe of the pituitary arises during embryonic development from a placode in Rathke's pouch, an invagination of the somatic ectoderm (Schwind, 1928) (Figure 0.1). The anterior pituitary is made up of five basic cell types: corticotropes, thyrotropes, gonadotropes, somatotropes, and lactotropes (Nakane, 1970). Among the somatotropes and lactotropes are a subpopulation of cells known as somatomammotropes, that produce both growth hormone and prolactin. Within these cells, a critical event in cell-specific determination occurs, causing either the growth hormone or prolactin gene to be expressed. Before we discuss a possible mechanism by which this decision is carried out, let us examine the known facts of this unique cell type, which will shed some light on the problem at hand.

Evidence for the existence of this subpopulation of cells comes from developmental studies, as well as from studies of adenomas and normal adult pituitaries, using immunocytochemical and *in situ* hybridization methods. Prior to these studies, it was believed that an acidophilic pituitary cell expressed either growth hormone or prolactin but not both. An ontologic immunohistochemical study of rat fetal pituitary cells by Chatelain *et al.* (1979) showed the first evidence of cells containing both prolactin and growth hormone. It also demonstrated that the development of growth hormone-containing cells preceded the development of prolactin-containing cells in rats. This pattern is similar to that found in other mammalian species studied (Slabaugh *et al.*, 1982). Using immunogold electron microscopy, Asa *et al.* (1988) established the presence of bihormonal somatomammotrope cells among acidophilic cells in

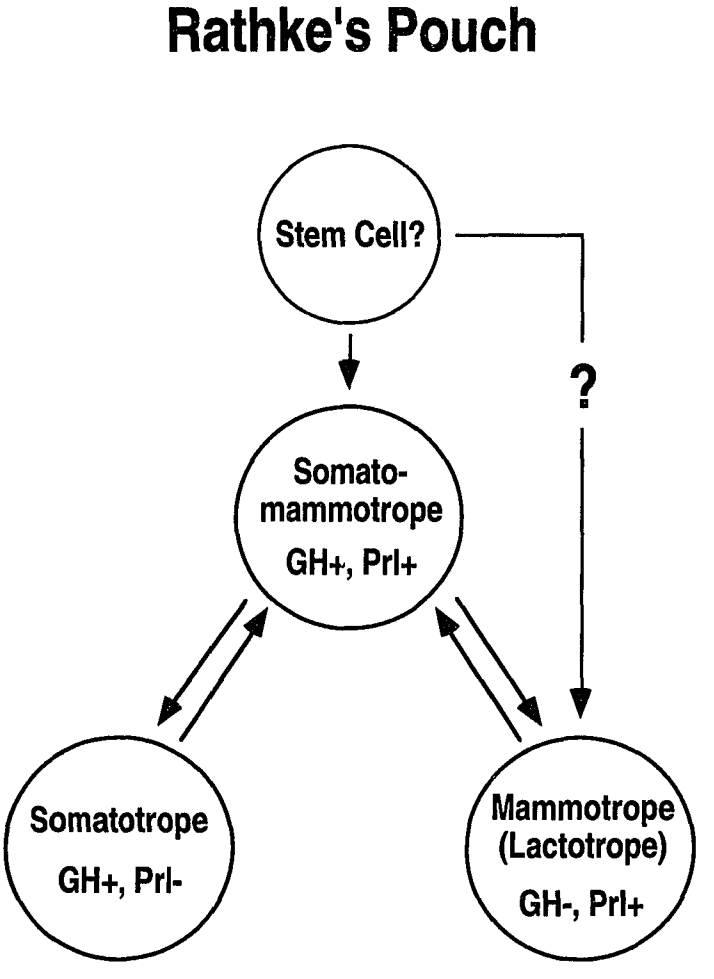

Figure 0.1. Schematic diagram of pituitary development.

human fetal pituitaries at twelve weeks of gestation, four weeks after the appearance of somatotropes, but, again, prior to the detection of pure lactotropes. *In situ* hybridization studies reported by Nogami *et al.* (1989), who examined the development of fetal and neonatal rat pituitaries, confirmed the previous ontogenic immunohistochemical results, although this study did not directly examine the cellular co-localization of growth hormone and prolactin messenger RNAs (mRNA). Studies of adult rat pituitaries, however, have further substantiated the presence of somatomammotropes. Using a reverse hemolytic plaque assay, Frawley and co-workers demonstrated these dual secretory cells made up 12-15% of the pituitary cell population isolated from normal adult rats (Frawley, Boockfor and Hoeffler, 1985; Frawley, 1989). Whether these cells represent the progenitor cells of the lactotropes and somatotropes is unclear. However, the emergence of somatotropes prior to lactotropes suggests that somatomammotropes may represent a transitional intermediate population in lactotrope development (Asa *et al.*, 1988; Hoeffler, Boockfor and Frawley, 1985).

The ratio of somatomammotropes to somatotropes and lactotropes in the adult pituitary fluctuates depending on the animal's hormonal status. The relationship of the ovarian hormonal cycle to the ratio of the various pituitary acidophillic sub-types strongly suggests that sex hormones play an important role in the regulation of this phenomenon (Kineman *et al.*, 1991). Somatomammotropes and somatotropes appear to be most affected while the proportion of lactotropes remains relatively stable. Estrogen decreases the amount of somatotropes while increasing the amount of dual secretors. Other hormones have also demonstrated an effect on somatotrope/lactotrope cell ratios. Inoue and Sakai (1991) showed convertibility of growth hormone-secreting cells to prolactin-secreting cells in the presence of insulin and insulin-like growth factor-1 in a newly established rat pituitary cell line. The

presence of insulin or insulin-like growth factor-1 increased the amount of prolactin-secreting cells. Hypothalamic factors can also affect the relative proportion of growth hormone- and prolactin-secreting cells in culture (Hoeffler and Frawley, 1987). Growth hormone-releasing hormone (GHRH) and luteinizing hormone-releasing hormone (LHRH) caused an increase in prolactin-secreting cells with a concomitant decrease in growth hormone-secreting cells. Corticotropin-releasing factor (CRF) also increased lactotropes, but without affecting the number of somatotropes. Thyrotropin-releasing hormone (TRH) reduced both populations, while vasoactive intestinal polypeptide (VIP) had no effect. Of all these hypothalamic factors, only LHRH could affect these changes by a proliferation-independent mechanism (Frawley and Hoeffler, 1988). These lines of evidence demonstrate the plasticity of the acidophillic cells of the anterior pituitary, which may depend in large part to the presence of somatomammotropes.

It has been shown that patients with acromegaly associated with pituitary tumors often develop hyperprolactinemia (Lamberts *et al.*, 1985). *In situ* hybridization analysis of some of these acidophillic pituitary adenomas has demonstrated the presence of both prolactin and growth hormone mRNAs. This is in contrast to prolactinomas, which rarely have growth hormone mRNA detectable by *in situ* hybridization analysis. These data suggest that lactotrophic tumors either develop from a more terminally differentiated cell, no longer able to express growth hormone (Lloyd *et al.*, 1989; Nagaya *et al.*, 1990) or somehow arise from a somatotrope-independent lineage (Behringer *et al.*, 1988).

Transgenic studies have also alluded to the embryologic association of prolactin- and growth hormone-producing cells. Behringer *et al.* (1988) created a transgenic dwarf mouse by introducing a growth hormone promoter-driven diphtheria toxin construct into mouse embryos. These dwarf mice lack nearly all

somatotropes and also have a sharply reduced amount of lactotropes. The presence of some lactotropes in these dwarf mice, however, points to a lactotrope developmental pathway that apparently does not include a growth hormone-expressing stage (dual lineage pathway). Borrelli *et al.* (1989) developed a strain of transgenic mice that carried the herpes virus 1 thymidine kinase (HSV1-TK) gene linked to either a rat growth hormone or rat prolactin promoter. Upon treatment with 1-(2-deoxy-2-fluoro-b-d-arabinofuranosyl)-5-iodouracil (FIAU), dividing cells expressing the HSV1-TK gene died due to interference with nucleotide metabolism. Mice with the growth hormone promoter driven HSV1-TK transgene were virtually devoid of both somatotropes and lactotropes when treated with FIAU. Mice carrying the prolactin promoter transgene however, had histologically normal-appearing pituitaries. Surprisingly, withdrawal of FIAU from FIAU-treated growth hormone-HSV1-TK mice resulted in the reappearance of mature somatotropes with partial recovery of lactotropes, presumably from proliferating, growth hormone-deficient pituitary stem cells. Another possibility could be the presence of quiescent somatomammotropes, which would not be destroyed with FIAU treatment. The authors of this study claim that these results strongly suggest a single direct line of descent of lactotropes from somatotropes, rather than a binary developmental pathway as suggested by Behringer *et al.* (1988).

Naturally-occurring animal models of pituitary dwarfism, however, exhibit primarily dual lineage patterns. Classical murine dwarf strains, including the Little (*lit/lit*), Ames (*df/df*), and Snell (*dw/dw*), all display a defect in growth hormone production (Eicher and Beamer, 1976; Schaible and Gowen, 1961; Eicher and Beamer, 1980). Only the Ames and Snell dwarves also demonstrate a prolactin deficiency, which is part of a panhypopituitarism syndrome (Phillips *et al.*, 1982). The Little mouse strain has normal prolactin production and does

possess some somatotropes, although with markedly reduced levels of growth hormone mRNA (Cheng *et al.*, 1983). While the genomic growth hormone gene is present and grossly intact in each of these dwarf mice strains, the autosomal recessive mutant alleles associated with the Little, Ames, and Snell strains have been localized to different chromosomes: 6, 11, and 16, respectively (Snell, 1929; Schaible and Gowen, 1961; Eicher and Beamer, 1976). The Snell dwarf genotype is associated with a mutation in pit-1, a pituitary-specific transcription factor (see below) (Camper *et al.*, 1990; Li *et al.*, 1990). In rats, there are two dwarf strains, dwarf rat (*Dw/Dw*) and spontaneous dwarf rat (SDR), which have been described as exclusively deficient in growth hormone production (Charlton *et al.*, 1988; Nogami *et al.*, 1989b). The dwarf rat strain has decreased growth hormone mRNA and serum levels, while the serum concentration of prolactin and other anterior pituitary hormones were comparable to that found in normal rats (Charlton *et al.*, 1988). The SDR strain was shown by immunoelectron microscopic analysis to be entirely devoid of somatotropes while still possessing normal-appearing lactotropes (Nogami *et al.*, 1989b). The combined data from naturally-occurring animal models of pituitary dwarfism tend to support the dual, binary developmental pathway proposed by Behringer *et al.* (1988) as evidenced by the presence of lactotropes despite little or no detectable somatotropes. This data does not exclude a role for somatomammotropes nor does it rule out a somatotropic pathway for lactotrope development.

The existence of a pituitary cell sub-type that expresses both growth hormone and prolactin establishes an ontologic linkage in the expression of these two genes. Sequence analysis of the growth hormone and prolactin genes has also revealed an evolutionary relationship.

These two pituitary-specific genes, along with the syncytiotrophoblast-specific placental lactogen/chorionic somatomammotropin

(PL/CS) gene, are members of a gene family that apparently evolved from a single evolutionary ancestral gene following a series of gene duplication events. Comparative analysis of amino acid sequences demonstrated over 80% amino acid homology between human growth hormone and PL/CS proteins (Niall *et al.*, 1971). Sequence data at the nucleotide level from cDNA clones demonstrated a greater conservation of nucleotide sequences than amino acid sequences between growth hormone, prolactin and PL/CS genes, with very few silent codon changes between rat growth hormone and human PL/CS (Seeburg *et al.*, 1977; Shine *et al.*, 1977; Cooke *et al.*, 1981). This data provided further evidence for a common evolutionary origin of these genes. With the addition of prolactin cDNA sequence data, it can be predicted that the origin of this gene family was the result of a gene duplication event that probably occurred some 340-390 million years ago (Cooke *et al.*, 1980; Miller and Eberhardt, 1983) separating the original ancestral gene into two branches, growth hormone and prolactin. The PL/CS gene evolved after the mammalian radiation within the last 10-60 million years, with the PL/CS gene derived from the growth hormone branch in humans (Cooke *et al.*, 1981; Miller and Eberhardt, 1983) and the prolactin branch in rats (Kawauchi *et al.*, 1990). Examination of the genomic organization of the individual genes in this gene family has provided additional support to the hypothesis of common ancestry. The coding region of each gene is interrupted by four introns, whose exonic junctions occur at identical locations, when the regions are aligned to maximize amino acid homology (Chien and Thompson, 1980; Barta *et al.*, 1981).

Besides having a relatively high degree of homology within their coding regions and similarity in genomic organization, analysis of the sequences 5' to the transcriptional start site has also revealed interesting patterns of conserved elements. It is thought that the first "exon" contains primordial promoter

elements directing expression of the ancestral gene. Pairs of direct repeats have been found in the sequences flanking exon 1 of various members of this gene family, suggesting that these are sites where insertional events have occurred. Direct repeats are generated by duplication of DNA segments at the sites of insertion or transposition. These structures are highly statistically significant and are not likely to have arisen randomly (Selby *et al.*, 1984; Slater *et al.*, 1986). Furthermore, based on evidence of direct repeats located downstream from a conserved "promoter" sequence [(A/C)ATA(A/G)A], it is believed that novel promoter elements were inserted subsequent to the divergence of the genes; this would account for their differential expression (Selby *et al.*, 1984; Slater *et al.*, 1986).

Despite these common evolutionary and ontologic origins, the growth hormone and prolactin genes have separate and distinct physiologic functions and are differentially regulated by a variety of hormones. Thyroid hormone, for example, is a major inducer of growth hormone synthesis (Ivarie *et al.*, 1981). It up-regulates the expression of the growth hormone gene at the transcriptional level (Diamond and Goodman, 1985). Experimental evidence indicates that these effects are mediated via a cellular receptor located in the cell nucleus (Oppenheimer, 1985). Through direct receptor-binding to *cis*-acting sequences in the growth hormone promoter, the hormone-receptor complex potentiates transcriptional activation resulting in increased expression of growth hormone mRNA (Casanova *et al.*, 1985; Flug *et al.*, 1987; Glass *et al.*, 1987; Lavin *et al.*, 1988; Wight *et al.*, 1988; Samuels *et al.*, 1988; Brent *et al.*, 1989; Norman *et al.*, 1989). Glucocorticoids also stimulate growth hormone production. While this induced expression may be partially the result of stabilization of growth hormone mRNA (Diamond and Goodman, 1985; Paek and Axel, 1987), there are data which demonstrate that it also involves a significant increase in transcription (Treacy *et*

al., 1991). Retinoic acid, another member of the steroid hormone superfamily (Carson-Jurica *et al.*, 1990; O'Malley, 1990; Beato, 1991), also has been shown to induce growth hormone gene expression at the transcriptional level (Umesono *et al.*, 1988; Bedo *et al.*, 1989; Morita *et al.*, 1989). Various other non-steroidal hormones also regulate growth hormone gene expression. These include insulin, insulin-like growth factor-1, and growth hormone releasing factor/cyclic AMP. Isaacs *et al.* (1987) demonstrated that insulin can negatively or positively regulate the expression of growth hormone mRNA depending on the metabolic state of the cultured cells, whereas other investigators have shown that insulin down-regulates growth hormone gene expression at the transcriptional level (Melmed *et al.*, 1985; Yamashita and Melmed, 1986a). Insulin treatment of cultured CHO cells induces a nuclear factor which binds to the promoter region of the human growth hormone gene (Prager *et al.*, 1990). Insulin-like growth factor-1 has been shown to negatively regulate growth hormone gene expression as well (Yamashita and Melmed, 1986b; Yamashita *et al.*, 1986a; Yamashita *et al.*, 1986b). Growth hormone releasing factor, another peptide hormone, causes an increase in growth hormone gene transcription (Gick *et al.*, 1984). This has been shown to be mediated by an increase in intracellular cyclic AMP levels (Barinaga *et al.*, 1985; Simard *et al.*, 1986). A cyclic AMP response element has been identified in the growth hormone promoter (Copp and Samuels, 1989; Dana and Karin, 1989) and a cyclic AMP response element binding protein (CREB) presumably acts through this site. Creation of a transgenic mouse using the rat growth hormone promoter linked to an intracellular form of the cholera toxin gene, designed to induce supraphysiologic levels of cyclic AMP in somatotropes, confirms *in vivo* that the growth hormone promoter is stimulated by cyclic AMP (Burton *et al.*, 1991). These transgenic mice developed somatotrope hyperplasia and had elevated serum levels of growth hormone, but not prolactin.

Incidentally, interference with CREB activation has been associated with abnormal development of somatotropes, not lactotropes (Struthers *et al.*, 1991).

Like growth hormone, prolactin gene transcription is also subject to both positive and negative regulation. Thyrotropin-releasing hormone (TRH) is a tri-peptide hypothalamic hormone which has been shown to stimulate the synthesis of prolactin mRNA (Murdoch *et al.*, 1983). Its actions are mediated by calcium, but are independent of protein kinase C activity (Yan and Bancroft, 1992, Bandyopadhyay and Bancroft, 1989). Both TRH and calcium act via *cis*-acting sequences located in the proximal promoter region of the prolactin gene (see below). Cyclic AMP regulates the expression of prolactin mRNAs by increasing the rate of transcription initiation (Maurer, 1981). This pathway is also the mechanism by which dopamine down-regulates prolactin gene expression, by inhibiting adenylate cyclase activity (DeCamilli and Macconi, 1979). Epidermal growth factor (EGF), another peptide hormone, has also been shown to up-regulate prolactin mRNA production (White and Bancroft, 1983). Estrogen has been shown to augment prolactin gene expression as well (Maurer, 1982, Shull and Gorski, 1984). The mechanism appears to be the direct binding of activated estrogen receptors to specific DNA sequences in the distal prolactin promoter (Day and Maurer, 1989a), though there may be another indirect method as well (Shull and Gorski, 1990). Thyroid hormone, a member of the steroid superfamily, can cause an increase of prolactin mRNA expression *in vitro*, though physiologically it is probably a suppressor of prolactin mRNA synthesis (Day and Maurer, 1989b, Stanley, 1989). Prolactin gene expression is also regulated by vasoactive intestinal peptide (Carillo *et al.*, 1985) and vitamin D_3 (Murdoch and Rosenfeld, 1981; Wark and Tashjian, 1982, 1983).

As one can see from this brief overview of the "anatomy and physiology" of the growth hormone and prolactin genes, there are many questions that need

to be addressed at the molecular level to sort out the complex transcriptional regulation of these two genes. The first of these questions is the characterization of the nuclear proteins (transcriptional factors) that are responsible for expression of these two genes. White *et al.* (1985) identified two chromatin proteins, concentrated in pituitary nuclear extracts, that specifically bound to the 5'-flanking region of the prolactin gene. Using a "South-Western" blotting technique (probing a gel blot of nuclear proteins with a ^{32}P-labeled DNA fragment), these authors detected a 44 kD and a 48 kD protein band. While these proteins, designated as NP44 and NP48, were found predominantly in pituitary derived extracts, they were also present in much lower quantities in non-pituitary extracts. NP44 and NP48, however, did not bind to other DNA probes used, exhibiting an exclusive prolactin flanking DNA sequence specificity. The authors postulate that these proteins may play a role in the pituitary-specific expression of the prolactin gene.

Another technique used to identify cell-specific DNA-protein interaction sites is by DNase I footprinting. When comparing pituitary and non-pituitary nuclear extracts, these experiments have revealed cell-specific footprints in the promoter region of both the growth hormone and prolactin genes (Nelson *et al.*, 1986; Catanzaro *et al.*, 1987, West *et al.*, 1987). These regions are highly conserved among mammalian species and are believed to be important in cell-specific and/or hormonal regulation of gene transcription.

Given the diversity of hormones and cellular factors which affect these two genes and the distinct physiologic roles they play in the body, it would be expected that these two genes would have different transcriptional factors regulating their expression. However, the co-expression of growth hormone and prolactin in pituitary cells during development and in somatomammotropes, along with their shared evolutionary origin, raises the possibility that these two

genes may share a common transcriptional machinery. Comparison of the DNA sequences of these cell-specific footprints from both promoters has revealed a consensus sequence, (A/T)TA(A/T)TCA. Other experimental evidence for a single common transcription factor comes from oligonucleotide competition experiments. Cell-specific footprints of one promoter can be blocked by competition with oligonucleotides probes corresponding to footprint sequences from the other promoter. It was not until after a pituitary-specific nuclear protein, pit-1, had been isolated and cloned that it became clear that there was indeed a common transcription factor which was responsible for the transcriptional activation of both these genes. These results were confirmed by co-transfection experiments, utilizing a pit-1 expression vector and hybrid plasmid constructs containing these promoter regions linked to a reporter gene.

The problem now facing investigators is how are these two genes differentially regulated given that a single common transcription factor can activate both genes? This is the main question to be addressed in this dissertation. Are there other *cis*- and/or *trans*-acting factors involved in their regulation? If not, how can a single transcription factor separately regulate two genes? What implications do these studies have for other cell-specific gene expression issues in development?

Chapter One: Extinction phenomenon and cell fusion experiments

Introduction

One approach to this problem of two genes and one *trans*-activator is to identify additional proteins which can modify the *trans*-activator's actions, for example a gene-specific repressor protein. Evidence for negative regulation of the growth hormone gene can be seen in the extinction phenomenon exhibited by growth hormone in somatic cell hybrids.

One of the early systems used to investigate the underlying mechanisms governing gene expression was the technique of somatic cell hybridization (Davidson, 1973; Davidson and de la Cruz, 1974). These studies, begun in the late 1960's, examined the properties of hybrids generated by the fusion of two cells which exhibited phenotypic differences. Cell hybrids allowed investigators to examine the result of interactions between genomes from cells in different functional states. The main assumption here was that the difference in exhibited phenotypes was caused by a change in gene expression, not a change in genetic information (*i.e.* mutation) and that this change in expression was mediated by soluble regulatory factors (Davidson, 1974; Weiss, 1981).

Experiments using hybrids between pigmented Syrian hamster melanoma cells and unpigmented mouse fibroblasts illustrated the dramatic changes in gene expression that occur upon fusion (Davidson *et al.*, 1966; 1968). Of the over 100 hybrids examined, all were unpigmented and lacked dopa oxidase activity (the enzyme required to synthesize melanin from tyrosine). Upon further investigation, this apparent shut-off of pigment synthesis was not caused by a specific inhibitor of dopa oxidase, nor a general repression of all melanoma cell-associated genes, nor the loss of structural genes required for pigment synthesis, nor the selective fusion of fibroblasts with unpigmented melanoma cell variants. In addition, growth of these hybrids under low density culture

conditions did not induce pigment expression. Examination of early hybrids (colonies which had undergone less than ten generations) showed that repression of pigment synthesis occurred soon after fusion. These studies suggested that the genome of unpigmented fibroblasts produced a substance(s) which specifically repressed the expression of melanin synthesizing genes of the melanoma genome in stable cell hybrids. This was the first indirect evidence for negative regulation of gene expression in eukaryotes.

Studies of the expression of liver-specific traits in cell fusion experiments using a variety of hepatoma cells further characterized the extinction phenomenon. Examination of tyrosine aminotransferase (TAT) expression in heterokaryons formed by the fusion of Buffalo rat liver cells that had high levels of TAT expression (HTC) to Buffalo rat liver cells that had very low levels of TAT (BRL-62) demonstrated that extinction of TAT expression occurred within 24 hours after cell fusion, before nuclear fusion took place (Thompson and Gelehrter, 1971). Experiments involving cybrids (enucleated mouse fibroblasts fused to differentiated rat hepatoma cells) suggested the existence of short-lived cytoplasmic factors from fibroblasts which were responsible for extinction of albumin gene expression (Kahn *et al.*, 1981). Re-expression following extinction could often be correlated to the loss of chromosomes from stable hybrids (presumably the chromosome which expressed the repressor) (Bertoloti and Weiss, 1972). Microcell hybrid studies utilizing hepatoma cells containing single fibroblast chromosomes provided strong evidence that the extinction phenomenon has a genetic basis that could be correlated to a specific genetic locus (Killary and Fournier, 1984; Petit *et al.*, 1986).

Extinction of the rat growth hormone gene expression in rat x mouse somatic cell hybrids was first demonstrated by Sonnenschein *et al.* (1971). Subsequently, Strobl *et al.* (1982) showed that it was the lack of transcription, not

the loss of the gene itself, that resulted in the extinction phenomenon, and therefore raised the possibility of a transcriptional control mechanism. Stable somatic cell hybrid cloned lines, which extinguished endogenous rat growth hormone gene expression, however, were able to express transiently transfected rat growth hormone promoter constructs, thus demonstrating the continued presence of *trans*-activating factors in the cell hybrids (Strobl *et al.*, 1984). These results suggest that the control mechanism acts at the chromosomal level, somehow inhibiting the access of transcriptional activators to the promoter site of the endogenous genes. On the other hand, Tripputi *et al.* (1988) and McCormick *et al.* (1988) both argue that extinction involves the repression of the *trans*-activators. In the Triputti paper, the authors present data in which they show the disappearance of one of the two cell-specific DNase I footprints (GC-1) when using nuclear extracts prepared from stable rat x mouse cell hybrid clones. From this result, they conclude that extinction of rat growth hormone gene expression is the consequence of the absence of pituitary-specific proteins that bind to the rat growth hormone promoter. However, they do not adequately address the issue of the presence of the GC-2 "cell-specific" site in the experiments using nuclear extracts from the non-pituitary cell as well as the stable cell hybrid clones. The authors explain away this anomaly by referring to papers describing studies on the <u>human</u> growth hormone promoter, a highly homologous but not identical promoter. In addition, their transient transfection data is suspect in that they report the expression of a rat growth hormone promoter construct in the non-pituitary mouse L cell (6.1% CAT conversion) almost as high as that in the pituitary GH_4 cells (9.7% CAT conversion), with the stable cell hybrid clone expression at only 2% CAT conversion. [Besides, the figures are reported without standard error and are not in a statistically significant range to make any definitive statements.] Another interpretation of

these results might be that the stable cell hybrid clone used in these experiments simply lost the chromosome which carried the gene(s) encoding the transcription factor. Given the results reported in the paper by Strobl *et al.* (1984) [a very relevant article to which they did not refer], the loss of the transcription factor, therefore, is ***not*** a causal factor in the extinction of growth hormone gene expression in stable cell hybrids.

McCormick *et al.* (1988) also arrived at the same conclusion as Triputti *et al.* (1988), using human rather than rat growth hormone promoter constructs. Their experiments on the stable cell hybrid clones, originally characterized by Thompson *et al.* (1980), basically confirmed earlier studies and explored additional *in vitro* studies, including *in vitro* transcription using whole cell and nuclear extracts prepared from cell hybrid clones. However, their basic tenet that extinction is the result of the loss of the transcription factor, GHF-1, remains unsupported. The definitive study to substantiate their claim would be an experiment showing that an autonomously regulated GHF-1 expressing vector introduced into a cell hybrid clone would cause the re-expression of an extinguished rat growth hormone gene. This experiment has yet to be done.

The experiments discussed thus far have only suggested possible theories as to the mechanism behind the extinction phenomenon. In 1987, Lufkin and Bancroft published a series of experiments which in retrospect, indirectly addresses the extinction phenomenon. Using stably transfected mouse fibroblasts (C127) containing either a prolactin or a growth hormone promoter construct linked to a CAT gene, they were able to show that fusion of these cells to a rat pituitary cell line (GH_3) caused the re-activation of only the prolactin promoter constructs (Figure 1.1). Neither the endogenous mouse genes nor the rat growth hormone promoter construct were activated by cell fusion. This demonstrates a significant difference in the regulation of these two gene

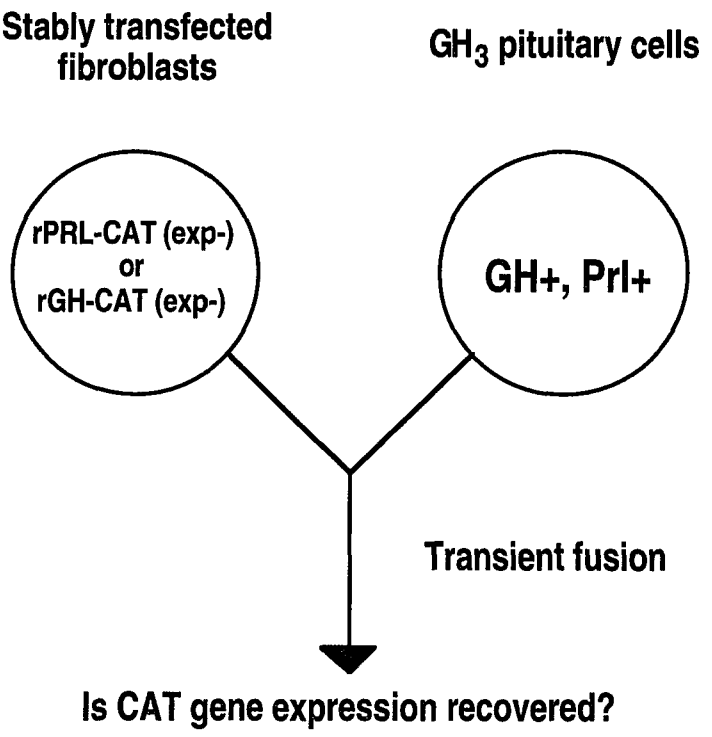

Figure 1.1. Schematic diagram of the transfection-cell fusion assay.

promoters. Furthermore, it shows that the mere presence of the transcription factor, contributed by fusion of the pituitary cell, is not sufficient for the expression of an "extinguished" growth hormone gene and therefore its absence is probably not primarily responsible for extinction. These results lend further evidence for an additional factor(s) (*i.e.* repressor) to be involved in the regulation of the growth hormone gene promoter. However, it is possible that additional upstream flanking sequences of the growth hormone promoter are required for its activation and/or these results are only specific to the non-pituitary host cell used. Therefore, the following experiments were performed to investigate these possibilities: (1) Can fusion of GH_3 cells to other cell types stably transfected with GH-CAT lead to re-activation? (2) Are additional upstream flanking sequences required for activation of the GH-CAT by transfection-cell fusion? (3) Can GH-CAT be activated by increasing the efficiency of cell fusion?

Results

A stably transfected rat growth hormone promoter construct, containing 580 bp of the upstream promoter region, remains inactive in the cell-fusion assay in all non-pituitary cell lines tested — The ability of rat growth hormone-CAT constructs to be reactivated in a number of different non-pituitary cell lines was examined. Among the different stably transfected cell lines tested were rat glioma (C6), rat hepatoma (MH_1C_1), human embryonic kidney (293), and mouse mammary tumor (C127) cell lines. Fusion of rat pituitary tumor cells (GH_3) with each of these cell lines stably transfected with (-580)rGH-CAT failed to yield CAT enzymatic activity above background (0.3% conversion of chloramphenicol). Evidence of successful fusion was obtained by direct microscopic visualization prior to harvesting of the cells. Greater than 50% of all cells per high powered

field in three randomly selected, noncontiguous areas, contained at least two nuclei. In addition, parallel control cell-fusion experiments using a stably transfected rat prolactin-CAT construct in C127 cells were used to demonstrate the ability of CAT activity to be detected after processing of the cell extracts. The results of these experiments demonstrate that the inability of a rat growth hormone-CAT construct to be reactivated in the cell-fusion assay is not specific to the non-pituitary cell line tested.

Effect of additional upstream promoter sequences on re-activation of a stably transfected growth hormone promoter-CAT construct in the cell-fusion assay — In an effort to determine whether additional upstream flanking sequences might be needed for activation by cell fusion, a rat growth hormone-CAT construct was obtained, kindly provided by Dr. H. Samuels (NYU School of Medicine), which contained the first 1800 base-pairs of the rat growth hormone gene promoter (Flug *et al.*, 1987). Stably transfected C127 mouse fibroblasts containing this rat growth hormone-CAT construct were also refractory to activation by pituitary cell fusion. The extent of cell fusion was determined by direct microscopic visualization with greater than 50% of cells per high powered field, in three randomly selected, noncontiguous areas, containing at least two nuclei. Parallel cell fusion experiments using rat prolactin promoter-CAT constructs were used to control for processing of the cell extracts for CAT activity. Therefore, these results suggest that the inability of the rat growth hormone promoter-CAT construct to be reactivated in the cell fusion assay is probably not due to the lack of upstream flanking sequences.

Effect of increasing the efficiency of cell fusion on the re-activation of a stably transfected rat growth hormone promoter-CAT construct in the cell fusion assay — It was reported in the literature that addition of polyunsaturated fatty acids like linolenic acid, into the cell culture medium transiently modified cell membranes

such that they would be more susceptible to cell fusion by polyethylene glycol (PEG) (Roos and Choppin, 1985). The addition of linolenic acid into the culture medium 18 hours prior to PEG-induced cell fusion enabled us to boost CAT activity by 2.5-fold when tested in experiments using stably transfected rat prolactin-CAT in C127 cells (Figure 1.2). Despite a more efficient cell fusion protocol, transfected growth hormone-CAT constructs in all of the different cell lines tested remained silent after fusion with rat pituitary tumor cells. Therefore, enhancing the efficiency of cell fusion has no effect on the ability of the growth hormone promoter-CAT construct to be expressed in the cell fusion assay.

Discussion

The results of these additional experiments extend and support the previous observations made by Lufkin and Bancroft (1987). There appears to be two different levels of repression occurring within the non-expressing cell type. The first is at the chromosomal level as demonstrated by the continued repression of the endogenous mouse prolactin gene upon cell fusion. This may represent a more permanent state which is established during development and cellular differentiation. Such processes as methylation, chromosomal packing may affect the accessibility of *trans*-activators to the endogenous gene promoters (Strobl *et al.*, 1986; Laverriere *et al.*, 1986; Gaido and Strobl, 1989; for review see Selker, 1990). Conversely, the absence of transcriptional activators during development may lead to repression mediated by nucleosome formation (Workman *et al.*, 1990). The second is at the level of stably transfected genes. While these constructs are chromatinized and integrated into the host genome, apparently the host's developmental information does not become encoded onto the plasmid DNA, therefore, allowing interaction with the transcription factors as seen with the prolactin promoter construct. Perhaps the presence of basal

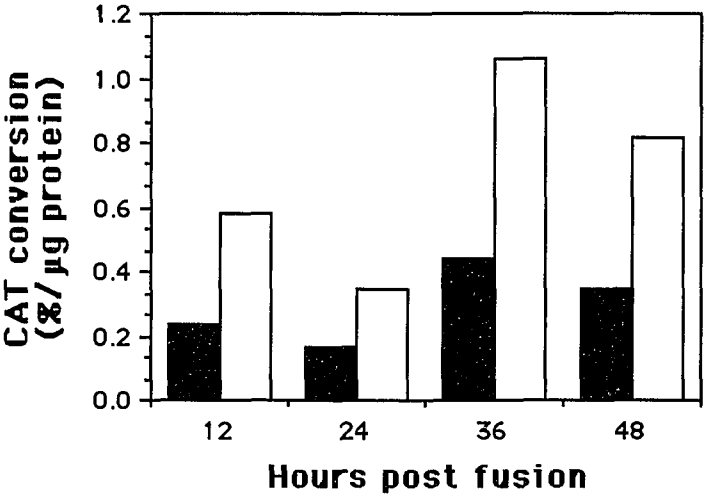

Figure 1.2. Enhancement of cell fusion signal by linolenic acid treatment. GH_3 rat pituitary cells were fused to C127 mouse mammary tumor cells, stably transfected with (-204)Prl-CAT, using the protocol described in Lufkin and Bancroft (1987) with the following modification. Four hours after the stably transfected cells were plated, linolenic acid was added to the medium of some cells to a final concentration of 100 mM and the cells were incubated in this medium for 18 hours prior to fusion by polyethylene glycol. Black bars indicate CAT conversion without linolenic acid. Clear bars indicate CAT conversion using linolenic acid.

transcriptional factors during the integration of plasmid DNA prevents the formation of non-specific repression complexes (Croston *et al.*, 1991) on transfected promoters, thereby allowing *trans*-activators to activate their respective transfected genes. With respect to developmental issues in gene expression, it would be interesting to investigate any differences in plasticity of the cell-fusion induced activation of a rat prolactin promoter-construct stably transfected into an undifferentiated cell line, as compared to a "differentiated-in-situ" cell line (*i.e.* stably transfected into an undifferentiated cell then stimulated to differentiate), or a differentiated cell line. Would then, a rat growth hormone promoter be activated by cell fusion if it were stably transfected into an undifferentiated cell line?

The interpretation of these results is not that simple, however. Why is it then that the transfected prolactin promoter can be activated while the transfected growth hormone promoter remains silent? Is it related to the fact that the expression of growth hormone is eight orders of magnitude less in non-pituitary cells than in anterior pituitary cells (Ivarie *et al.*, 1983)? Perhaps while there is "illegitimate transcription" occurring in all cell types (Chelly *et al.*, 1989), presumably due to the interaction of basal transcriptional factors with basic promoter elements (*e.g.* TATA), the growth hormone promoter may be subject to even more rigid controls. Or another possibility is the presence of a specific repressor(s) of the growth hormone promoter in non-expressing cell types which prevents even "illegitimate" transcription from occurring. Further experiments delineating the transcriptional machinery acting on each of these promoters will provide greater insight into this complex question.

Chapter Two: Identification of *cis*-acting elements in the rat prolactin and rat growth hormone promoters and characterization of nuclear protein binding

Introduction

Investigations into the mechanism of cell-specific gene transcription often begin with the identification of *cis*-acting promoter elements, generally found in the 5'-upstream flanking sequences. The transcriptional factors which bind to these *cis*-acting sites presumably direct the cell-specific expression of the promoter. Transfection of chimeric promoter-CAT constructs into differentiated tissue culture cells is then used to determine which of these *cis*-acting sites actually function in determining cell-specific gene expression. Following the identification of these sites, the experiments next focus on the characterization of the nuclear protein(s) which interact at the various cell-specific elements.

Before the discovery of pit-1 as a pituitary-specific transcription factor, there were many lines of evidence which suggested that the prolactin and growth hormone promoters shared a single *trans*-activating factor, as outlined in the Introduction. The experiments described in this chapter were conducted prior to the discovery of the transcription factor pit-1 to determine: (1) What are the cell-specific elements in the prolactin and growth hormone promoters? (2) What are the nuclear protein binding characteristics of these sites? (3) Is there a consensus sequence among these sites?

Results

Identification of pituitary-specific nuclear protein binding sites in the rat growth hormone and prolactin promoters — DNase I footprinting studies using GH_3 pituitary cell nuclear extracts have shown pituitary-specific protein binding sites in the rat growth hormone and prolactin promoters (Figure 2.1). These sites are located at -62 to -92 bp (GH-1) and -106 to -134 bp (GH-2) relative to the

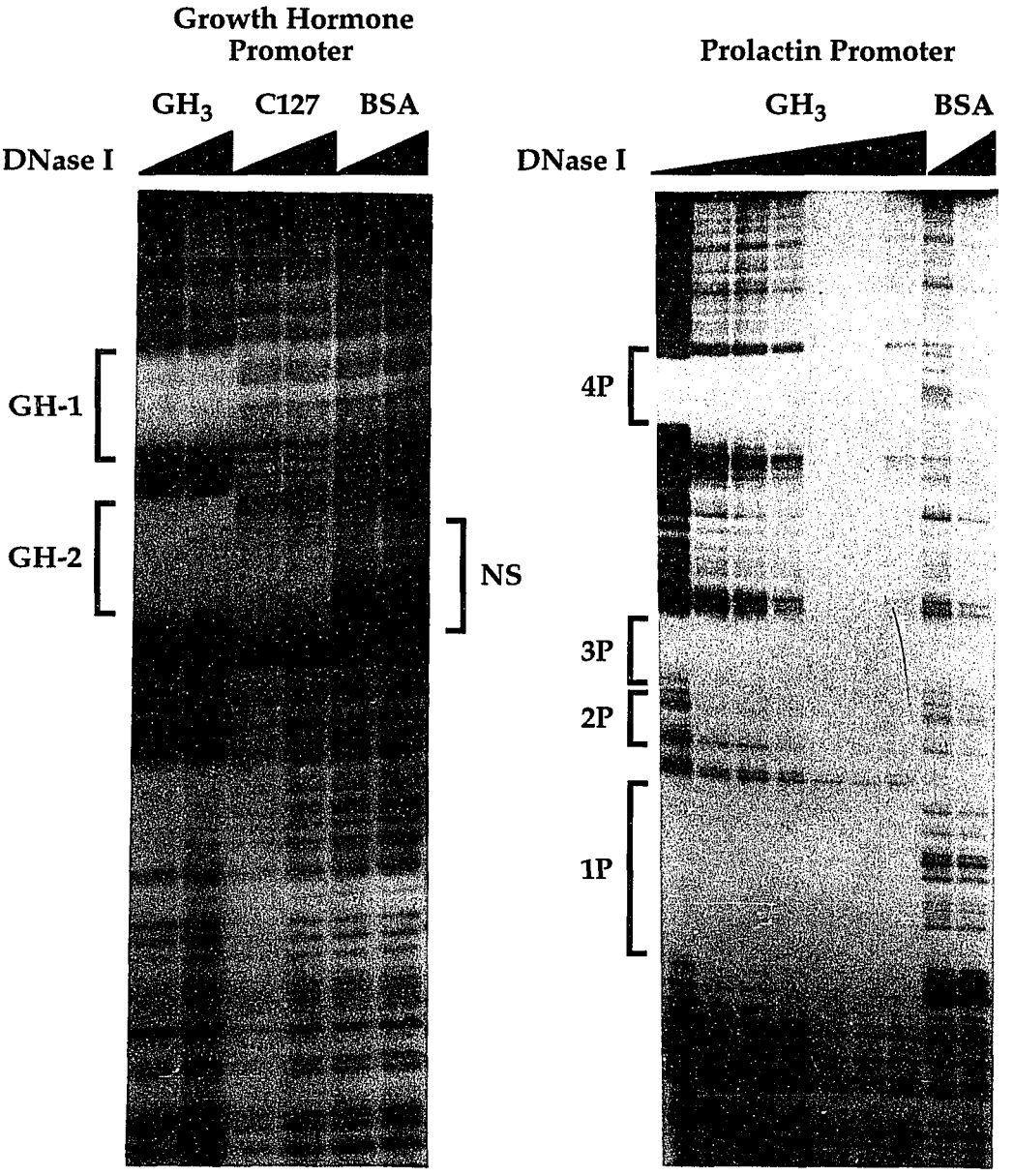

Figure 2.1. DNase I footprinting of the growth hormone and prolactin promoters. Gel-purified restriction fragments of the respective promoters were specifically end-labeled and treated with increasing amounts of DNase I in the presence of the indicated nuclear extracts or BSA. Treated DNA fragments were phenol-chloroform extracted, ethanol precipitated, and then separated on an 8% polyacrylamide - 8M urea sequencing gel. DNA bands were visualized by autoradiography at -70°C with an intensifying screen. Footprints labeled GH-1, GH-2, 4P, 3P, 2P, 1P are pituitary-specific. Footprint labeled NS is non-pituitary specific.

transcription start site in the rat growth hormone gene and at -39 to -63 bp (1P), -114 to -133 bp (2P), -144 to -167 bp (3P), and -189 to -200 bp (4P) relative to the transcription start site in the prolactin gene. The footprint sites are diagrammed schematically in Figure 2.2 along with two other additional functional binding sites, T_3RE and PRE, the thyroid hormone response element (Ye *et al.*, 1988) and the proximal repressor element (see Chapter Four). These sites are in agreement with other published studies on pituitary-specific protein binding sites in the rat growth hormone and prolactin promoters (West *et al.*, 1987; Catanzaro *et al.*, 1987; Ye and Samuels, 1987; Gutierrez-Hartmann *et al.*, 1987).

The question of whether all these footprints are created by a single pituitary-specific nuclear protein or multiple factors was addressed in competition experiments, using oligonucleotide probes corresponding to either the 1P or GH-1 footprint sites. These footprinting experiments demonstrated cross-competition between the promoters, suggesting that a single pituitary-specific nuclear protein binds to all the footprint sites (data not shown). Further evidence for a single factor responsible for binding to all the footprint sites was obtained in the gel-shift experiments described below.

To confirm the cell-type specificity of these footprints, non-pituitary nuclear extracts were used to determine whether there were non-cell-type specific footprints located on these two promoters. Using C127 mouse mammary tumor cell nuclear extracts, an additional binding site was detected on the rat growth hormone promoter (Figure 2.1). Its footprint overlaps the 5'-end of the GH-2 site. Given its proximity to the cell-specific GH-2 site, we were interested in determining what effect protein-binding to this site had on the binding of the GH-2 site. Could this footprint represent a "repressor" protein binding site, which may explain the inability of the growth hormone promoter from activating in the cell-fusion assay?

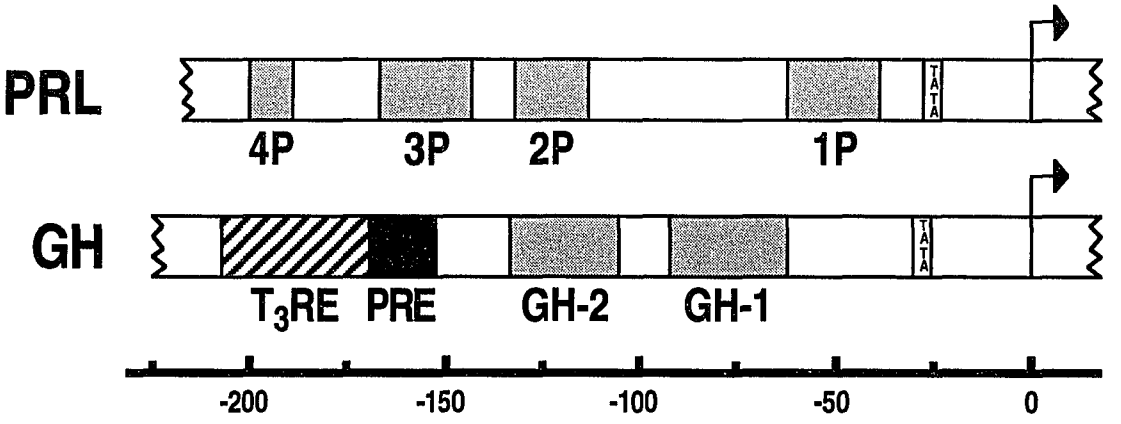

Figure 2.2. Rat growth hormone and prolactin proximal promoter organization. *In vitro* DNase I experiments using pituitary cell nuclear extracts have identified pituitary-specific footprints in the prolactin and growth hormone proximal promoter regions (1P, 2P, 3P, 4P, GH-1, GH-2, respectively) (Nelson *et al.*, 1988; Catanzaro *et al.*, 1987; West *et al.*, 1987). Sequence analysis revealed a "TATA"-box at approximately -30 bp from the transcription start site of each gene. Additional sites identified in the growth hormone gene correspond to the proximal repressor element (PRE) (see Chapter Four) and the T_3 receptor binding site (T_3RE) (Ye *et al.*, 1988).

Mixing experiments using GH_3 pituitary and C127 non-pituitary nuclear extracts however do not demonstrate any interaction between these two adjacent binding sites thus precluding a possible "repressor" role for the non-cell-type specific binding site (data not shown). This is in agreement with other published studies (Bodner and Karin, 1987).

It has also been reported in the literature that the 2P binding site is a non-cell-type specific nuclear protein binding site (Gutierrez-Hartmann *et al.*, 1987). Again, mixing experiments using pituitary and non-pituitary nuclear extracts do not show any enhancing or inhibitory activity, at least at the level of protein-DNA binding (data not shown). These experiments suggest that pituitary-specific nuclear protein binding to cell-specific sites on the rat growth hormone and prolactin promoters is not affected by non-pituitary nuclear protein binding. Therefore, if there are specific repressors of pituitary gene promoters present in non-pituitary cell nuclear extracts, they do not act by blocking the binding of pituitary-specific nuclear proteins.

Characteristics of pituitary-specific nuclear protein binding sites in the rat growth hormone and rat prolactin promoters — Using oligonucleotide probes corresponding to the identified cell-type specific footprint sites, gel-shift experiments were performed. The purpose of these experiments was to characterize each binding site with respect to gel-shift mobility patterns and the ability of each site to cross-compete with one another. Figure 2.3 shows the results of these experiments. Each of the prolactin footprints (1P - 4P) and the growth hormone footprints (GH-1 and GH-2) creates a single major gel-shifted band, with the presence of a minor, more slowly moving, band appearing somewhat less consistently (see below). Each of these pituitary-specific gel-shifted bands can be competed away with a 100-fold molar excess of cold oligonucleotides corresponding to either the 1P or GH-1 footprints, but not by a

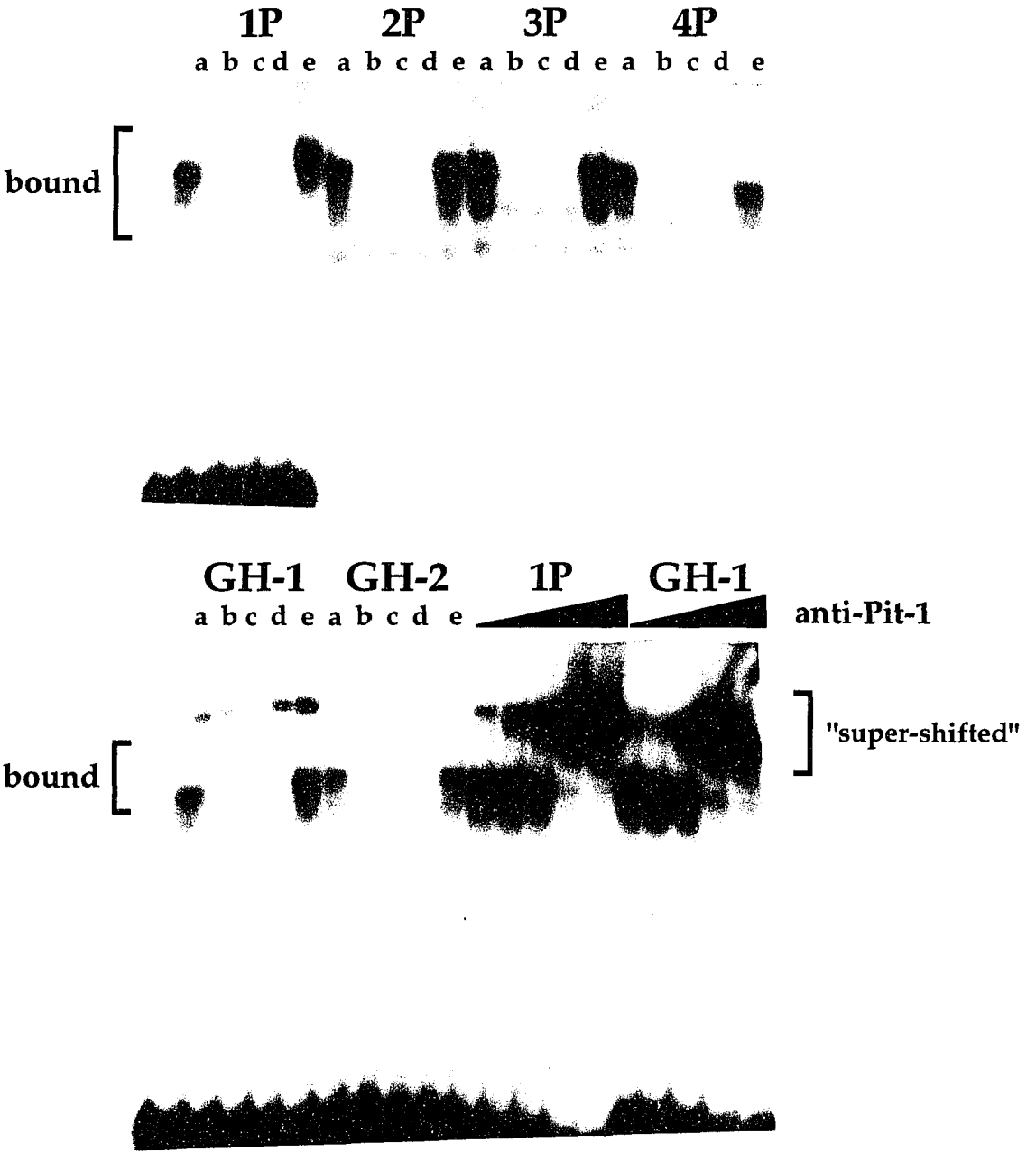

Figure 2.3. Gel-shift experiments using pituitary cell nuclear extracts: competitive binding of labeled 1P-4P, GH-1 and GH-2 probes in the presence of 1P, *1P, GH-1, and *c-fos* calcium response region and the effect of the addition of anti-pit-1 antibody. The indicated probes were end-labeled and added to a GH_3 nuclear extract mixture as described in *Experimental Methods* with the following additions: lane *a*, no additions; *b*, 1P; *c*, *1P; *d*, GH-1; *e*, *c-fos* calcium response region. Increasing amounts of anti-pit-1 antibody was added to gel-shift reactions containing the indicated probe. Samples were run on 0.25X TBE-10% polyacrylamide gel at 4°C. Bands were visualized by autoradiography at -70°C using an intensifying screen.

non-specific oligonucleotide derived from the *c-fos* promoter region (Figure 2.3). These results suggest that there is a pituitary-specific nuclear protein which is capable of binding to both promoters.

An antibody directed against a peptide fragment, synthesized *in vitro* based on the amino acid sequence of the putative pituitary-specific transcription factor, pit-1, was added in gel-shift experiments to see if the same transcription factor could bind to both promoters. Using the 1P probe and the GH-1 probe as representative prolactin and growth hormone promoter binding sites, respectively, we showed that as the concentration of antibody was increased in the gel-shift mixture, more of the shifted band was "super-shifted," suggesting that indeed the same protein, pit-1, did bind to both footprint sites (Figure 2.3).

The more slowly moving gel-shift band identified in the original gel-shift experiment probably represents a "dimer" form of the pituitary-specific nuclear protein binding to the oligonucleotide (Figure 2.4). Using a labeled 1P probe, increasing concentrations (10-fold, 25-fold, 50-fold, or 100-fold) of cold oligonucleotides corresponding to either the 1P or GH-1 footprints were added to a standard gel-shift assay mixture. The results of this experiment are shown in Figure 2.4. As can be seen, there is a difference in the ability of 1P and GH-1 to compete away the dimer form. It appears that the dimer form may bind preferentially to the 1P site. This may be a mechanism by which a single trans-acting factor can regulate two different gene promoters with similar binding properties.

Comparison of all the pituitary-specific nuclear protein binding sites reveals a consensus sequence — Sequence analysis of the binding sites revealed a seven base-pair consensus sequence: (A/T)TA(A/T)TCA (Gutierrez-Hartmann *et al.*, 1987; Nelson *et al.*, 1988). It was noted that this sequence was found twice in the prolactin promoter at the 1P site, as an almost perfect palindrome, once in 2P, on

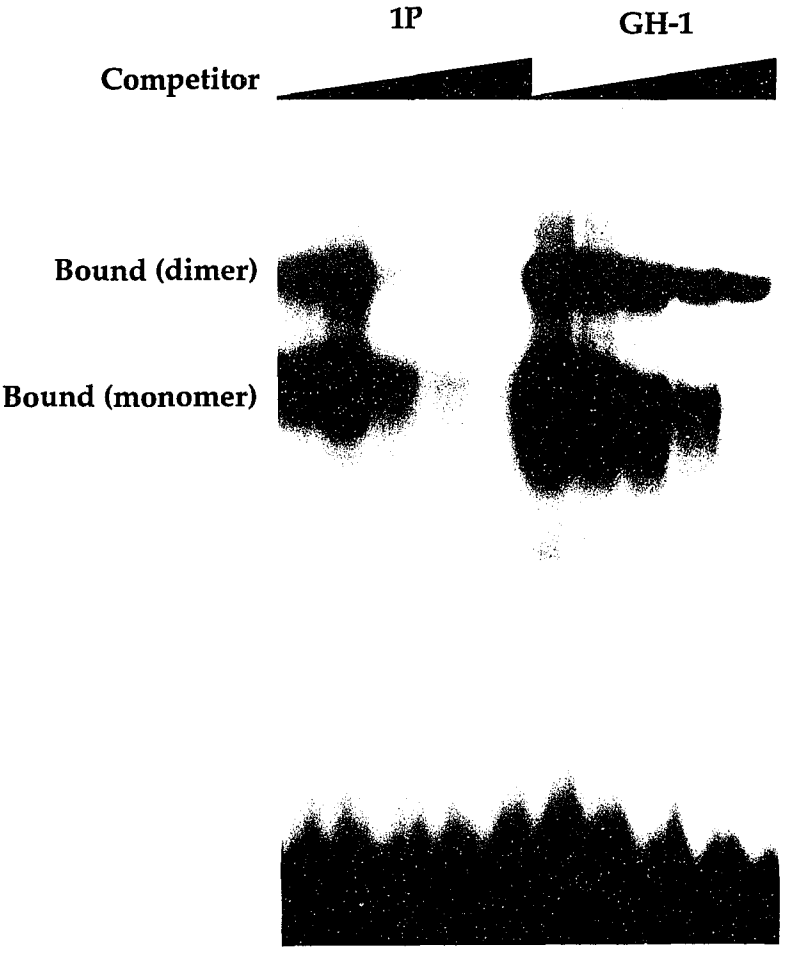

Figure 2.4. Gel-shift experiments using pituitary cell nuclear extracts: competitive binding characteristics of 1P versus GH-1. Using labeled 1P probe, increasing amounts of the indicated cold competitor were added to a GH_3 nuclear extract mixture as described in *Experimental Methods*. Samples were run on 0.25X TBE-10% polyacrylamide gel at 4°C. Bands were visualized by autoradiography at -70°C using an intensifying screen.

the non-coding strand with two out of seven mismatches, twice in 3P, overlapping on the non-coding strand, and once in 4P, on the coding strand (Figure 2.5). In the growth hormone gene, this consensus sequence is found once in GH-1, on the non-coding strand and once in GH-2, on the coding strand with one out of seven mismatches (Figure 2.5). It is unclear as to the significance of the arrangement of this consensus sequence with respect to orientation and its effect on the binding of the pituitary-specific nuclear protein.

Cross-species comparisons of the growth hormone promoter revealed an additional growth hormone promoter-specific consensus sequence which is centered around the sequence: TAAATNNNN$(G/A)_8$ (Figure 2.6). Given that the growth hormone promoter footprints displayed a somewhat different dimer-binding capability, perhaps this secondary consensus sequence plays a role in the differential regulation of these two promoters. Therefore, we set out to examine the sequences necessary for binding. Since from previous experiments it was shown that 1P appears to bind the pituitary-specific nuclear protein with the most affinity (Nelson *et al.*, 1988), the 1P oligonucleotide selected as the model. It was mutated in each of its two consensus sequences, each separately (*1P, 1P*) and then, both together (*1P*), using non-conservative transverse mutations of the last four bases of the consensus sequence (Figure 2.5). Mutation of the distal consensus sequence (*1P) does not appear to alter binding much as compared to wildtype (1P), whereas mutation of the proximal site (1P*) has a greater effect and mutation of both sites (*1P*) has the greatest effect (Figure 2.7). These results suggest that though the 1P site appears to be a nearly perfect palindrome, formation of this palindromic structure is not absolutely necessary for the pituitary-specific nuclear protein to bind. Furthermore, the proximal consensus sequence, located on the coding strand *in situ*, appears to be

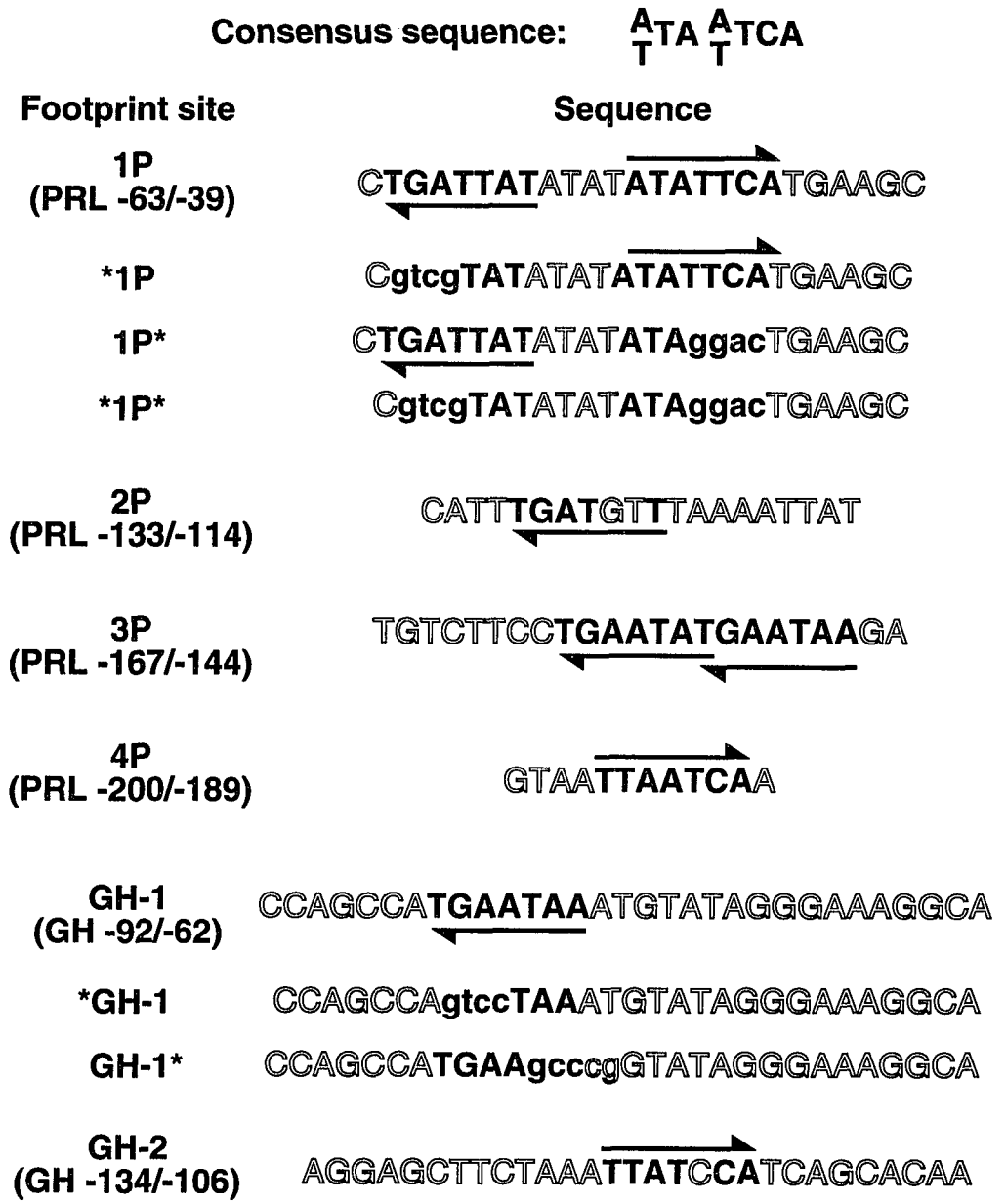

Figure 2.5. Consensus sequence analysis of prolactin and growth hormone footprints. Consensus sequence on coding strand highlighted by right arrow. Consensus sequence on the non-coding strand highlighted by left arrow.

```
          -200          -190          -180          -170          -160
Bovine    GGaTNNNATG    ACaAGCCTGG    GGGACATGAC    CCCAGAGaAG    GAACGGGAAC
Ovine     GGGGatGATG    ACgAGCCTGG    GGGACATGAC    CCCAGAGaAG    GAACGGGAAC
Porcine               CCcGG         GGGACATGAC    CCCAGAGGAG    GAgCGGGAAC
Rat       GcGGtGGAAa    GGTAagaTca    GGGACgTGAC    CgCAGgaGAG    cAgtGGGgAC
Human     cacTgGtgAc    GGTgGgaaGG    GaaAgATGAC    aagccAGGgG    GcAtGatccC
                                      *  *  ****          *       *    *
                                -T3RE-]       [-      --- PRE --      ----]

          -150          -140          -130          -120          -110
Bovine    AGGATGAGTG    aGAGGAGGTT    CTAAATTATC    CATTAGCACA    GGC TGCCAGT
Ovine     AGGATGAGTG    aGAGGAGGTT    CTAAATTATC    CATTAGCACA    GGC TGCCAGT
Porcine   AGGATGAGTG    GGAGGAGGTT    CTAAATTATC    CATTAGCACA    tGCCTGCCAGT
Rat       gcGATGtGTG    GGAGGAGcTT    CTAAATTATC    CATcAGCACA    AGC TGtCAGT
Human     AGcATGtGTG    GGAGGAGcTT    CTAAATTATC    CATTAGCACA    AGCCcGtCAGT
             *** ***     ****** **    **********    *** ******     **   * ****
                         [-------     ----- GH-2    ----------     ]

          -100          -90           -80           -70           -60
Bovine    GG T     CC   tTGCATAAAT    GTATAGAGcA    cA CAGGTGG    GGGgAAAGGG
Ovine     GG T     CC   tTGCATAAAT    GTATAGAGcA    cA CAGGTGG    GGGgAAAGGG
Porcine   GG       GCC  ATGCATAAAT    GTATAGAGAA    AA tAGGTGG    GGGCAgAGGG
Rat       GGCTCCaGCC    ATGaATAAAT    GTATAGgGAA    AgGCAGGaG       cCtt GGG
Human     GG     ccCC   ATGCATAAAT    GTAcAcAGAA    A  CAGGTGG    GGGCAAcaGt
          **       **    ** ******    *** *  * *       *** *              *
              [-----      ------- GH-1  ----------      ----]

          -50           -40           -30           -20           -10
Bovine    AGAGAGAGAA    GAAGCCAGGG    TATAAAAAtG    GCCCAGcAGG    GACCA TTCC
Ovine     AGAGAG  AA    GAAGCCAGGG    TATAAAAAGG    GCCCAGcAGa    GACCAATTCC
Porcine   AGAGAG  AA    GAgGCCAGGG    TATAAAAAGG    GCCCAaAAGG    GACCAATTCC
Rat       gtcGAGgAAA    acAGgtAGGG    TATAAAAAGG    GCatgCAAGG    GACCAAgTCC
Human     gG GAGAGAA    GggGCCAGGG    TATAAAAAGG    GCCCACAAGa    GACCAgcTCa
             ***  **       *  ****    **********    **     **    *****  **
                                      TATAA

          +1
Bovine    AGGATCCCAG    GAC
Ovine     AGGATCCCAG    GAC
Porcine   AGaATCCCAG    GAC
Rat       AGcAcCCtcG    agC
Human     AGGATC
          ** * *
```

Figure 2.6. Cross-species comparison of mammalian growth hormone promoters. Promoter sequences obtained from Woychik *et al.* (1982) (bovine), Orian *et al.* (1988) (ovine), Vize and Wells (1987) (porcine), Barta *et al.* (1981) (rat), DeNoto *et al.* (1981) (human). Asterix indicates conserved sequences among species. Underlined sequence indicates mutations created in the rat promoter which caused increase in promoter activity (see Chapter Four). Sequence alignment performed by direct observation.

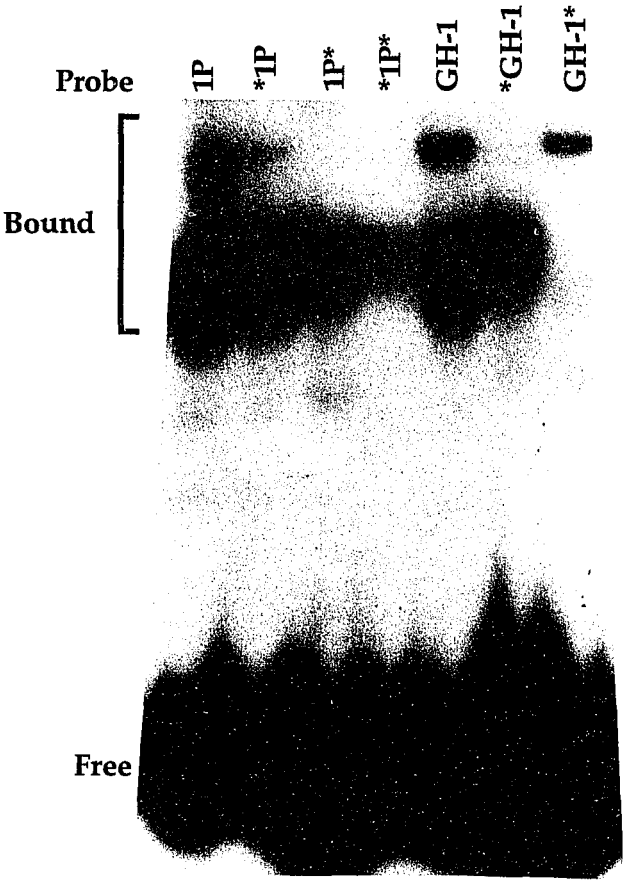

Figure 2.7. Gel-shift experiments using pituitary cell nuclear extracts: the binding of 1P and its mutants and GH-1 and its mutants. The indicated probes were end-labeled and added to a GH_3 nuclear extract mixture as described in *Experimental Methods*. Samples were run on 0.25X TBE -10% polyacrylamide gel at 4°C. Bands were visualized by autoradiography at -70°C using an intensifying screen.

responsible for slower moving band, as disruption of this sequence causes the loss of the upper band (Figure 2.7).

Similar mutation of the primary consensus sequence in the growth hormone GH-1 footprint (GH-1*) completely abolishes the faster moving of the two bands, however the slower moving band does not appear to be affected (Figure 2.7). Conversely, when the growth hormone promoter-specific consensus sequence is mutated at the "TAAAT" motif, using non-conservative transverse mutations (*GH-1), only the slower moving band is affected (Figure 2.7). These results suggest that either these two bands may be the result of two different pituitary-specific binding proteins which bind to the growth hormone footprint, or there may be two different DNA-binding sites on the same nuclear protein, one for monomeric binding, and one for dimeric binding, each with a different DNA sequence specificity. These results warrant further investigation.

Discussion

The elucidation of pituitary-specific nuclear protein binding sites on the prolactin and growth hormone promoters is a necessary first step towards deciphering the complex regulatory mechanisms controlling the expression of these two genes. DNase I footprinting is a simple method of surveying a promoter for such specific nuclear protein binding sites (Galas and Schmitz, 1978). However, interpretation of results with this technique and extrapolation to what is occurring in the nucleus requires an understanding of its limitations. This method probably over-estimates the number of *in vivo* binding sites since the DNA template used *in vitro* is a naked, linearized piece of DNA. Therefore, all potential sites should be detectable. On the other hand, the nuclear proteins which require interaction with the complex protein scaffolding surrounding a quaternary supercoiled DNA structure may not be detectable by this method.

Genomic footprinting would be another alternative method by which to detect regions on DNA which bind nuclear proteins (Church and Gilbert, 1984; for review, see Saluz and Jost, 1993). Besides being able to detect more labile binding proteins, it has an additional advantage in that one can compare *in situ* differences in protein-DNA binding during different states of hormonal stimulation or development. It would be interesting to examine the protein-DNA binding on stably transfected promoters, before and after cell-fusion. While this would be technically difficult, given the percentage of fused cells in any given experiment does not approach 100%, even with the linoleic acid protocol, it would provide greater insight into what is happening to the promoters during cell fusion.

The results of the binding studies suggest that a single pituitary-specific nuclear protein is responsible for binding to both promoters. Cross-competition between the footprint sites in the DNase footprinting and the gel-shift experiments and identification of a consensus sequence among the footprint sites demonstrate only similar binding characteristics and do not prove definitively that a single protein binds to both promoters. Further proof will lie in the identification and cloning of the protein.

It is interesting that these two cell-type specific promoters share a single cell-type specific *trans*-activator, whereas other cell-type specific promoters that have been identified are associated with many different DNA-binding proteins, both cell-type specific, as well as ubiquitous specific DNA-binding proteins (Maire *et al.*, 1989; Jaynes *et al.*, 1988; for review, see Müller *et al.*, 1988; Wasylyk, 1988). Perhaps the ability of this pituitary-specific nuclear protein to dimerize may allow it to associate with other non-specific nuclear proteins which may allow it to change its promoter-binding specificity. Further studies on the dimer form are needed to address these issues.

The next chapter will discuss experiments involved in demonstrating that a cloned protein, pit-1 is indeed the pituitary-specific factor which not only binds to but also activates both the growth hormone and prolactin promoters.

Chapter Three: Analysis of pit-1 interactions with the rat growth hormone and prolactin promoters

Introduction

Using biochemical purification methods and advanced cloning techniques, a single cDNA clone was isolated whose expressed protein possessed the same binding characteristics as that seen in the pituitary nuclear extract (Bodner *et al.*, 1988; Ingraham *et al.*, 1988). This cloned protein was named pit-1/GHF-1. While it would seem that the story would end here, however, a raging controversy remained. One group (Ingraham *et al.*, 1988) claimed that their cloned protein, pit-1, was capable of activating both growth hormone and prolactin promoters, while the other group (Bodner *et al.*, 1988) insisted that their protein, GHF-1, identical in amino acid sequence to pit-1, activated only the growth hormone promoter. Obviously, they both cannot be correct. If, as Rosenfeld's group proposes, pit-1 alone activates both promoters, what is the mechanism for the differential expression of prolactin and growth hormone in the anterior pituitary cells? On the other hand, if GHF-1 only activates the growth hormone promoter, how does one explain Rosenfeld's data, given that the two proteins are identical in amino acid sequence?

Therefore, we set out to examine the following questions: (1) Is the cloned pit-1 protein alone sufficient to bind to all the pituitary cell-specific footprint sites?; (2) What is the minimum promoter sequence necessary for directing cell-specific expression *in vitro*?; and (3) Can a cloned pit-1 gene activate both the growth hormone and prolactin promoters *in vivo*?.

Results

The cloned pit-1 protein is sufficient to bind all the pituitary cell-specific footprint sites — Using *in vitro* translated pit-1, gel-shift experiments were performed on each of the mutated 1P oligonucleotides (see Figure 2.5 for structures) (Figure 3.1). It is seen that the oligonucleotides bound pit-1, from greatest affinity to lowest affinity: 1P> *1P>> 1P*> *1P* (Figure 3.2). These results suggest that though the 1P site appears to be a nearly perfect palindrome, formation of this palindromic structure is not absolutely necessary for pit-1 to bind. Furthermore, the proximal consensus sequence, located on the coding strand *in situ*, appears to determine pit-1 binding, as disruption of this site virtually abolishes binding. This finding, which has been published (Yan *et al.*, 1991) agrees with other published studies (Nelson *et al.*, 1988, Sharp *et al.*, 1989).

In a related experiment, the binding of pit-1 to each of the other pituitary-specific footprints was studied (Figure 3.3). The order of pit-1 binding, from greatest affinity to lowest affinity, was as follows: 1P=3P>2P>4P>GH-1>GH-2. In light of this result, it is interesting to note that *in vivo*, the growth hormone promoter constructs are generally expressed at a lower level than the prolactin promoter constructs (18.2% vs. 4.3%, Nelson *et al.*, 1986).

The minimal promoter that is needed for cell-type specific transcription in vitro contains either just the 1P site or the GH-1 site — Whereas footprinting and gel-shift studies are limited to examination of the qualitative and physical aspects of *in vitro* pit-1 binding, *in vitro* transcription experiments can provide data on the functional aspects of pit-1 binding. *In vitro* transcription using nuclear extracts prepared from GH_3 cells demonstrated the ability of the 1P site to direct cell-specific transcription (Figure 3.4). In previous studies, deletion analysis of the prolactin promoter had shown that expression of the prolactin promoter *in vitro* required a minimum of the 1P site (Lufkin *et al.*, 1989). Transcription of this

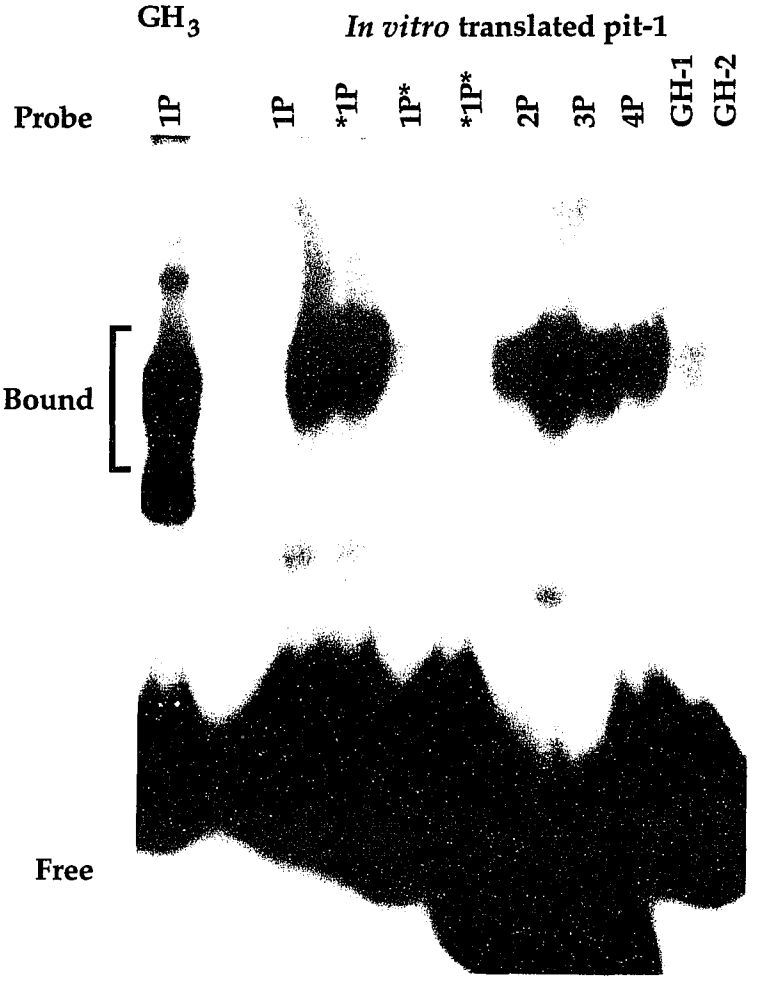

Figure 3.1. Gel-shift experiments using *in vitro* translated pit-1. Pit-1 messenger RNA generated from a cloned pit-1 *in vitro* transcription vector was used to prime a rabbit reticulocyte lysate *in vitro* translation system using cold amino acids. Immediately after translation, 4 µl of the reaction were employed in a gel-shift assay as described in *Experimental Methods*. Labeled probes were used as indicated. Leftmost lane contains a standard GH_3 nuclear extract gel-shift reaction mixture with 1P as the labeled probe. Samples were run on 0.25X TBE-10% polyacrylamide gel at 4°C. Bands were visualized by autoradiography at -70°C using an intensifying screen.

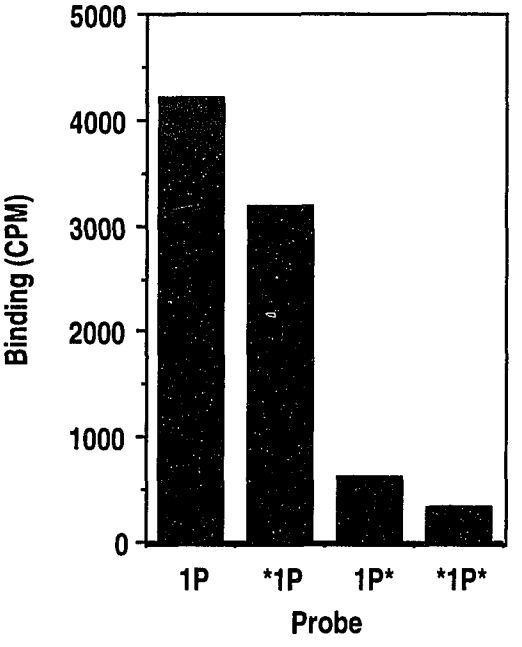

Figure 3.2. Binding of *in vitro* translated pit-1 to 1P and mutated 1P oligonucleotide probes. Pit-1 mRNA was synthesized from *StyI* linearized pSP6-pit-1 transcription vector using SP6 RNA polymerase. The transcribed RNA was extracted with phenol-chloroform, ether extracted, and ethanol precipitated. The RNA was then resuspended in RNAse-free distilled water at 5 mg/ml and translated in a rabbit reticulocyte lysate system using 1 mM methionine. Immediately after translation, 4 µl of the reaction mix was used for each gel-shift assay which included 1 ng of a [^{32}P]-labeled double stranded oligonucleotide. After localization of the shifted bands by autoradiography at -70°C, radioactivity in the excised bands was measured by liquid scintillation counting.

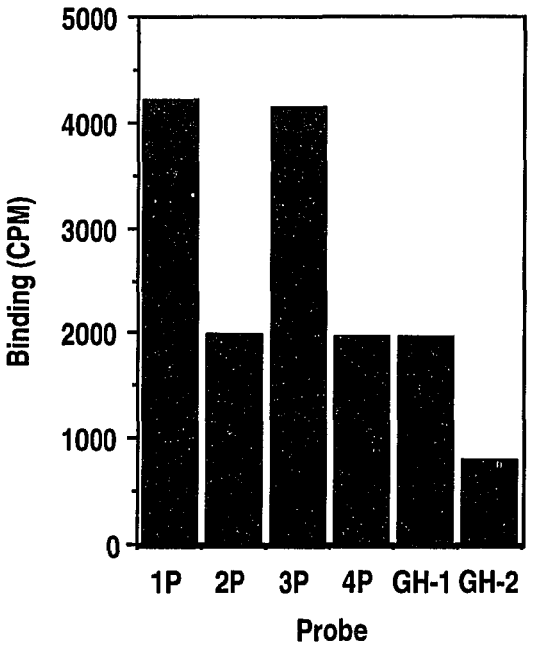

Figure 3.3. Binding of *in vitro* translated pit-1 to oligonucleotides corresponding to prolactin and growth hormone promoter cell-specific footprint sites. Please see legend for Figure 3.2.

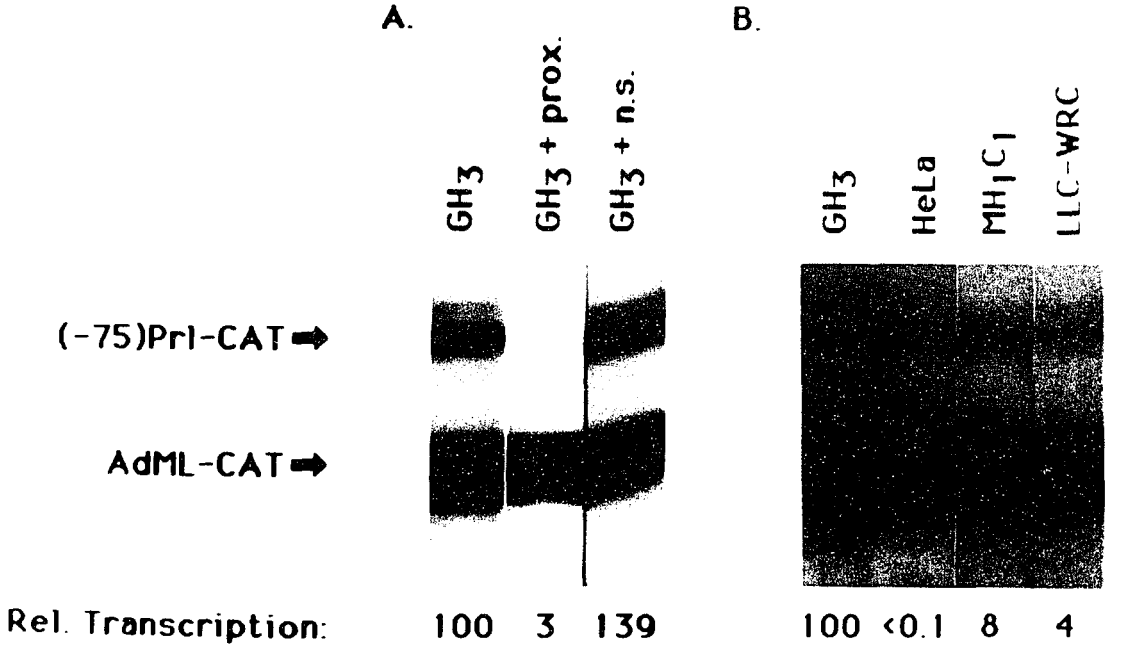

Figure 3.4. ***In vitro*** **transcription of a minimal prolactin promoter directs cell-specific transcription.** Plasmids p(-75)PRL-CAT and pAdML-CAT were transcribed together, in a 4:1 ratio, in nuclear extracts of the indicated cell lines. Arrows indicate the expected positions of the indicated transcripts. Conditions were as in the standard *in vitro* transcription reaction as described in *Experimental Methods*. In B, the GH_3 reaction contained half the usual DNA and protein concentrations, the MH1C1 extract contained 250 µg extract/50 µl reaction, and the HeLa, MH_1C_1, and LLC-WRC lanes received the product of twice as much reaction as did the GH_3 lane (to yield approximately equivalent AdML-CAT transcript levels). In A, lanes 2 and 3, transcription was in the presence of an 80-fold molar excess of 1P and a non-pituitary specific oligonucleotide, respectively. Appropriate exposures of the gels shown were quantitated by densitometric scanning. Shown *below* each lane is the value of relative (-75)PRL-CAT expression (PRL-CAT band intensity/AdML-CAT band intensity normalized to a value of 100% of GH_3 cells). Different GH_3 cell extracts were employed for the experiments shown in A and B; relative (-75)PRL-CAT expression in these extracts was, respectively, 0.31 and 0.60. Prox., 1P oligomer; n.s., non-specific oligomer.

highly truncated construct was specifically inhibited (~33 fold) by an 80-fold molar excess of double-stranded oligonucleotide corresponding to the 1P site, but not by a non-specific oligomer. This demonstrates that the *trans*-acting factor which binds to the 1P site is clearly critical for *in vitro* transcription of the prolactin promoter. Therefore, the (-75)Prl-CAT construct, containing the 1P site, was used in studies to determine whether the 1P site alone was sufficient to direct cell-specific transcription *in vitro*.

Comparing the transcriptional efficiency of this minimal prolactin promoter construct in pituitary nuclear extracts relative to various non-pituitary nuclear extracts, we showed that transcription of the (-75)PRL-CAT construct was more than 1000-fold higher using extracts of rat GH_3 cells than extracts of human HeLa cells. To determine whether or not this was a species-specific effect rather than a cell-type specific effect, the transcriptional efficiency of the minimal prolactin construct was also examined in two rat non-pituitary cell lines: MH_1C_1, rat hepatoma cell line and LLC-WRC, rat carcinoma cells. Each of these extracts yielded low but detectable levels of CAT message, comparable to the level seen in the transcription in GH_3 extracts in the presence of excess 1P oligomers. The transcription level of the (-75)PRL-CAT construct in GH_3 extracts was 12- and 25-fold higher than in MH_1C_1 and LLC-WRC extracts, respectively. The low level of transcription seen could be due to basal "illegitimate" transcription activity directed by the TATA box (Chelly et al., 1989). In any case, the minimal prolactin construct, (-75)PRL-CAT, containing only the 1P site, was able to direct cell-specific transcription *in vitro*.

In related experiments, deletion constructs of the growth hormone promoter were also analyzed using *in vitro* transcription. These studies showed that sequential deletion of the growth hormone promoter down to -96 bp (relative to the transcriptional start site) had no effect on the transcriptional

efficiency of the promoter *in vitro* (Figure 3.5). Furthermore, pit-1 binding to the GH-1 site is necessary for transcription of this minimal growth hormone promoter construct, as evidenced by competition studies using GH-1 or 1P oligonucleotides. Cell-type specific transcription can be directed using this minimal growth hormone construct (Figure 3.6).

Mixing nuclear extracts prepared from C127 mouse fibroblasts with pituitary nuclear extracts did not show any inhibitory effects with respect to the efficiency of *in vitro* transcription of either growth hormone or prolactin promoter constructs (data not shown).

The cloned pit-1 protein can activate both the growth hormone and prolactin genes in vivo in most cultured cell lines — The cloning of the pit-1 cDNA has enabled *in vivo* experiments to be conducted in non-pituitary cell lines. This approach has an obvious advantage over *in vitro* studies in that it provides a physiologic environment in which to study the interaction of the promoters with pit-1. In intact cells, other cell-specific modifications can occur, which may impact on the ability of pit-1 to *trans*-activate one or both promoters. Another advantage this system provides is the potential for pit-1 to form heterogeneous heteromeric or homomeric protein-protein interactions (Ingraham *et al.*, 1990). As has been shown with other transcription factors, heterogeneous protein-protein interactions can determine the functional specificity of a transcription factor (Diamond *et al.*, 1990; Glass *et al.*, 1990; Nasmyth and Shore, 1987; Vinson *et al.*, 1989; Brennan and Olson, 1990; Abate *et al.*, 1991).

We have examined this question in co-transfection experiments using three non-pituitary cell lines: HeLa (human carcinoma), CV-1 (monkey kidney), and F9 (mouse embryonal). Our results show that both growth hormone and prolactin promoter constructs can be activated in HeLa and CV-1 cell lines by co-transfection of a pit-1 expression vector (Figure 3.7). However, in F9 cells,

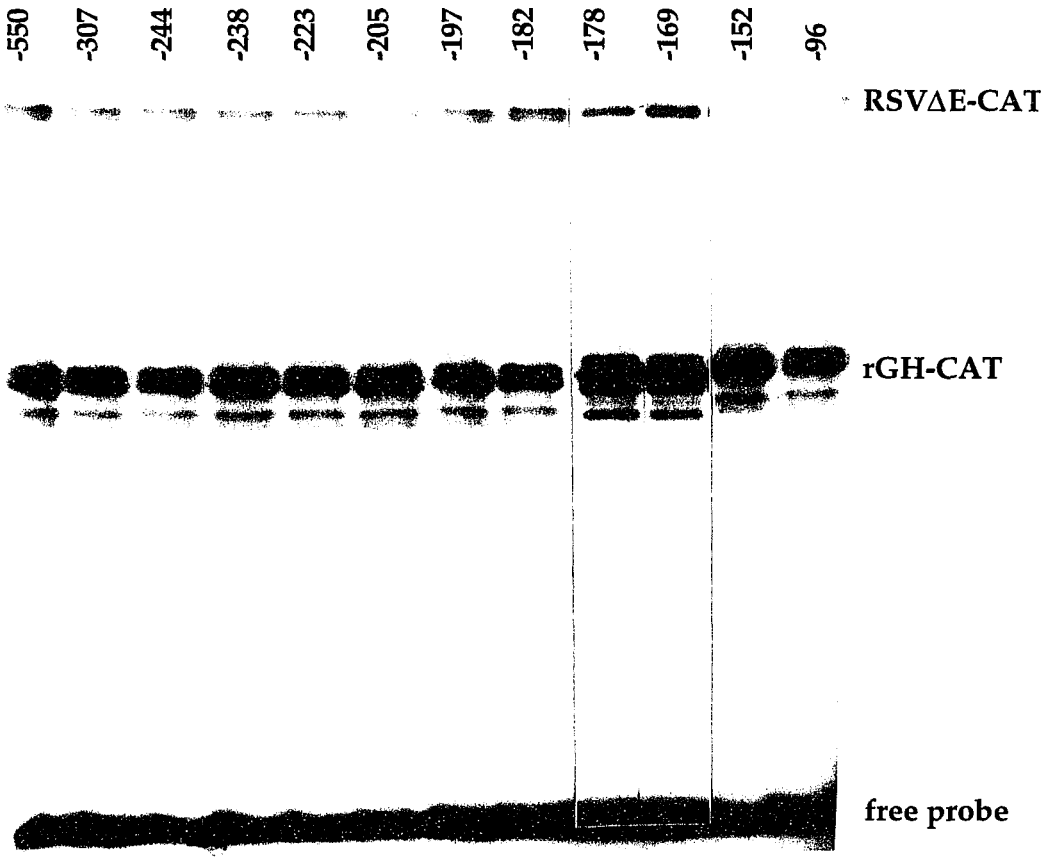

Figure 3.5. ***In vitro*** **transcription analysis of growth hormone promoter deletion constructs.** Plasmid p(-550)GH-CAT or its indicated 5' deletion mutant (400 ng) was transcribed together with 100 ng internal control pRSVΔE-CAT in the presence of GH_3 nuclear extracts as described in *Experimental Methods*. The transcription products were analyzed by primer extension and separated on an 8% polyacrylamide - 8M urea gel. Bands were visualized by autoradiography at -70°C using an intensifying screen. Lanes containing transcription products from p(-178)GH-CAT and p(-169)GH-CAT were from a another experiment and run on a separate gel.

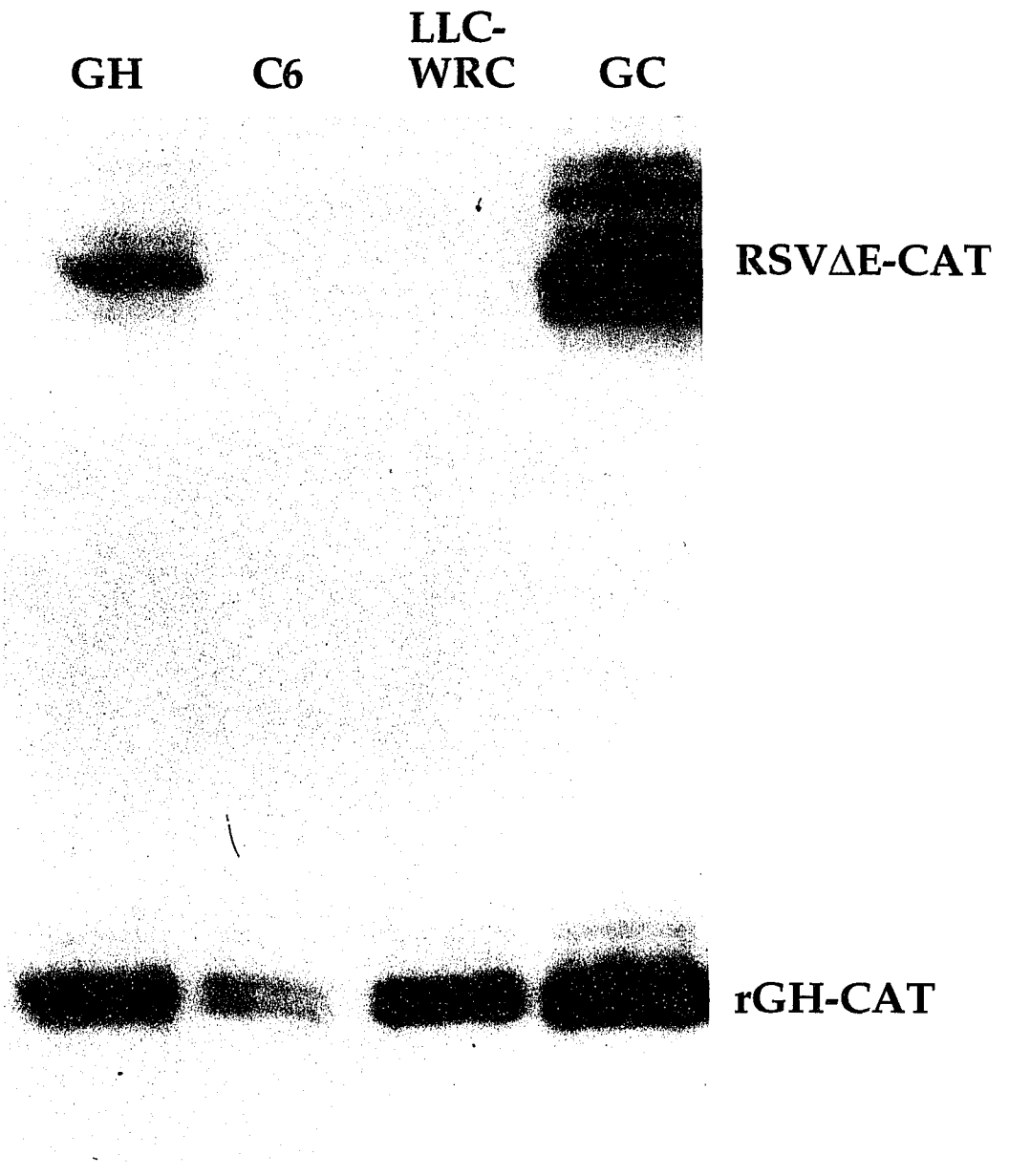

Figure 3.6. ***In vitro*** **transcription of a minimal growth hormone promoter directs cell-specific transcription.** Plasmids p(-96)GH-CAT and pRSVΔE-CAT were transcribed together in a 4:1 ratio, in nuclear extracts of the indicated cell lines. Arrows indicate the expected positions of the growth hormone and RSVΔE promoter derived transcripts. Conditions were as in the standard *in vitro* transcription reaction as described in *Experimental Methods*.

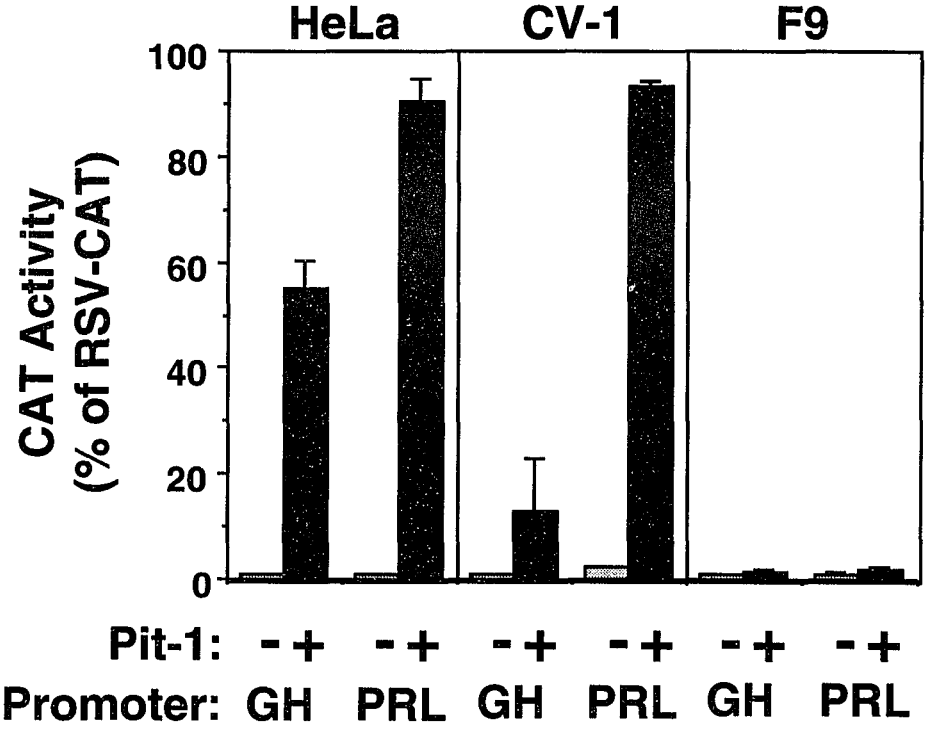

Figure 3.7. Cell-type specific activation by pit-1 of the GH and prolactin promoters. The indicated cell lines were transfected with 10 μg of either pUC19 (-) or an RSV-driven pit-1 expression vector (+). Parallel cultures received 10 μg of RSV-CAT. After a three day incubation, CAT activity was assayed, employing 8 hour incubation time. GH-CAT and Prl-CAT are expressed relative to RSV-CAT activity, which yielded the following % conversion of [^{14}C]-chloramphenicol: HeLa, 90%; CV-1, 94%; F9, 37%. Shown is the mean ± SE of results with triplicate dishes.

neither promoter can be activated by pit-1 alone (Figure 3.7). In these experiments, CAT activity as a result of the expression of RSV-CAT transfected into parallel cultures, was used as a relative indicator of the level of RSV-pit-1 expression. In F9 cells, CAT activity was about 40% as compared to that in HeLa and CV-1 cells. Therefore, the lack of expression of GH-CAT and Prl-CAT in F9 cells in these experiments may simply reflect an insufficient concentration of pit-1.

Discussion

We have shown in these experiments that (1) a protein expressed from a cloned pit-1 cDNA can bind to all pituitary cell-specific footprint sites *in vitro;* (2) promoter constructs containing just the most proximal pituitary cell-specific footprint site are capable of directing cell-specific expression *in vitro;* and (3) expression of a cloned pit-1 cDNA construct *in vivo* can activate both co-transfected growth hormone and prolactin promoter constructs in HeLa and CV-1 cells but not F9 cells. All this evidence supports pit-1 as solely responsible for the cell-specific expression of both promoters. Given that a single protein, pit-1, is responsible for all the cell-specific binding sites of both promoters and that a single proximal binding site is all that is required for cell-specific transcription for each promoter, at least *in vitro,* it also suggests that there must be another mechanism by which these two promoters are differentially regulated. Indeed, the experiments by Lufkin and Bancroft (1987), implied that the growth hormone promoter *in vivo* must either require something additional in order to be reactivated or have a gene-specific repressor which prevents its expression in that system.

While *in vitro* studies do not reflect the physiologic state within the cell, they can provide some clues as to the possible mechanisms used in cell-specific

gene regulation. Up to this point, the only experimental demonstration of differential regulation of the growth hormone and prolactin gene promoters is the Lufkin and Bancroft study (1987). All of the other studies show the coordinate expression of these two promoters in the presence of pit-1. Clearly, this does not reflect the situation in pure lactotropes or somatotropes, which express only one of the two promoters. Therefore, *in vitro* studies which more accurately model the physiologic state should be pursued. For example, *in vitro* chromatinized prolactin and growth hormone promoters could be used in transcription experiments to demonstrate any effect of chromatin on the expression of these two promoters. In addition, *in vivo* footprinting of the quiescent promoter in lactotrope or somatotrope cell lines may lead to discovery of new footprints which may be candidates for a repressor factor binding site. Another possible consideration would be to study protein-protein interactions between pit-1 and other nuclear and/or cytoplasmic factors. Labeled pit-1 could be used to probe a protein blot, similar to a Western blot to detect specific protein bands. Alternatively, pit-1 could be covalently attached to a column matrix and be used to fish out interactive proteins. These proposed studies would help to elucidate the mechanism(s) behind the differential regulation of the growth hormone and prolactin genes.

Thus far, the majority of studies on these two promoters have focused on the identification of *trans*-activators, culminating in the cloning and characterization of a single, pituitary-specific transcription factor, pit-1. Further studies have identified a myriad of potential co-activators which appear to augment pit-1-induced transcriptional activation. Given that in the physiologic state, there are cells in the pituitary which contain pit-1 yet do not express either growth hormone or prolactin, there remain factor(s) yet to be identified which modulate the expression of these two promoters.

The question of whether other transcription factors are involved in the expression of these genes has been addressed by published experiments from Reudelhuber's group (Schaufele *et al.*, 1990a,b,c). Using *in vitro* and *in vivo* techniques they have characterized six other "factors" which interact with the rat growth hormone promoter (GHF-2 - GHF-7). In Schaufele *et al.* (1990b), the authors present data which demonstrate Sp1 binding to the rat growth hormone promoter at the GHF-2 site (between -147 and -129 basepairs upstream of the transcription start site). However, it is not clear from the experiments described that Sp1 is necessary for the physiologic expression of the rat growth hormone promoter. Mutation of the GHF-2 site causes partial disruption of the "dGHF-1" site ("GH-2" site, see Figure 2.1) and therefore, one cannot exclude the possibility that the decrease in transcriptional efficiency seen by mutation of the GHF-2 site is simply due to interference of pit-1 binding to the "dGHF-1" site. Further studies involving transfection experiments in Sp1-deficient cells, for example Schneider drosophila cells, would be needed to address this issue adequately. Competitive binding of Sp1 to the distal pit-1 binding site, however, has been implicated in the mechanism which differentially modulates the expression of the human growth hormone and chorionic somatomammotropin genes (Tansey and Catanzaro, 1991). The GHF-3 site is located just upstream of the thyroid response element in the rat growth hormone promoter (-239 to -219) (Schaufele *et al.*, 1990a). GHF-3 is an ubiquitous 43kDa protein which appears to interact with other "nuclear factors" *in vitro*. Mutation of the GHF-3 site reduces expression from the growth hormone promoter suggesting that the GHF-3 complex provides an activating influence on the growth hormone promoter. The GHF-4 site is located just proximal to the GHF-3 site (-218 to -198). It appears to be a single, pituitary-specific DNA-binding protein, though mutations affecting its binding to the growth hormone promoter do not affect promoter activity (Schaufele *et al.*,

1990a). GHF-7 has been found only in cells expressing growth hormone and is not present in pure lactotrophs (Schaufele *et al.*, 1990c). It's binding to the proximal growth hormone promoter requires both pit-1 binding sites to be intact. Mutations to either site abolishes binding of the GHF-7 factor. The same is true for GHF-5. However, expression of GHF-5 is not solely restricted to pituitary cells, as it is found in abundance in HeLa and in CHO cells. GHF-6 binds to a site immediately downstream from the proximal pit-1 binding site. It is found in greater abundance in the lactotrope cell line, 235-1 as compared to the somatotrope cell line, GC.

Rousseau's group has also characterized other non-cell-type specific transcription factors (Sp-1, USF/MLTF, NF-1, AP-2) acting on the human growth hormone promoter (Lemaigre *et al.*, 1989; Lemaigre *et al.*, 1990; Courtois *et al.*, 1990). These studies implicate but do not provide conclusive evidence that other non-cell-type specific transcription factors act on the growth hormone promoter. Additional *in vivo* experiments are needed to clarify and confirm these results. For example, co-transfection experiments using HepG2, which lack AP-2 transcription factor, could be used to demonstrate the role AP-2 plays in growth hormone promoter regulation. Voss *et al.* (1991) has presented data which describes the direct interaction of pit-1 with other "ubiquitous" transcription factors (Oct-1). Heterodimerization of pit-1 with other homeo/POU-domain-containing transcription factors provides another mechanism by which a single transcription factor can differentially regulate two different promoters.

Direct promoter interaction by hormone receptors also plays a role in the differential regulation of both promoters. The well-characterized estrogen receptor binding site in the distal prolactin promoter has been shown to be an up-regulator of prolactin promoter expression (Ryan *et al.*, 1979; Maurer, 1982,

1985; Shull and Gorski, 1984; Maurer and Notides, 1987; Stone *et al.*, 1977; Waterman *et al.*, 1988; Day and Maurer, 1989a). Whereas the thyroid hormone receptor binding site of the growth hormone promoter, located just upstream from the distal pit-1 binding site, is a well-known positive regulator of growth hormone gene expression (Spindler *et al.*, 1982; Evans *et al.*, 1982; Nyborg *et al.*, 1984; Crew and Spindler, 1986; Larsen *et al.*, 1986; Flug *et al.*, 1987; Glass *et al.*, 1987; Lavin *et al.*, 1988; Wight *et al.*, 1988; Norman *et al.*, 1989; Brent *et al.*, 1989a,b). Steroid hormone receptors can also negatively regulate gene expression, as seen in the negative glucocorticoid response element found in the bovine prolactin promoter (Sakai *et al.*, 1988).

With all these various "factors" acting directly on the promoter region of these two genes, it is easy to see how difficult it can be to sort out the effect of each individual factor. Therefore, other methods of analysis need to be pursued. *In vivo* footprinting has been used to characterize DNA-protein interaction sites (Church and Gilbert, 1984; for review, see Saluz and Jost, 1993). Its main advantage is that it reflects the physiologic state of the cell. These experiments can therefore validate footprints identified *in vitro* as well as identify novel DNA-protein interaction sites. Another powerful tool which can be used to study the mechanisms of transcriptional regulation is genetics. A system has been developed using stably transfected prolactin promoter constructs in CHO cells to identify novel proteins which act on the prolactin promoter (Morris *et al.*, 1990). With this system, proteins can be cloned functionally as the selection method involves direct and/or indirect activation of the promoter. This technique depends on the potential factor to be the result of a single gene-product, and therefore, multimeric factors would go undetected. Another host for genetic analysis of the prolactin promoter has been developed in yeast (Ding *et al.*, 1991). As yeast basic transcription factors have been shown to be

functionally equivalent and interchangeable with mammalian factors, this system has the potential advantage of having a well-characterized genetic map and the ease of genetic manipulation. However, since transcription factors may undergo cell-specific post-translational modification (Jackson and Tjian, 1988; Kapiloff *et al.*, 1991), these reactions may not occur in yeast, thus potentially limiting its usefulness as an experimental model.

Our *in vivo* data, demonstrating activation of both prolactin and growth hormone promoter constructs by pit-1 co-transfection, refute the claim of Karin's group that GHF-1 (a.k.a. pit-1) only activates the growth hormone promoter and not the prolactin promoter. Other investigators have also demonstrated quite convincingly that indeed pit-1 can and does activate both growth hormone and prolactin promoters. Mangalam *et al.* (1989) demonstrated the ability of co-transfected pit-1 to activate both a growth hormone promoter construct and a prolactin promoter construct in HeLa cells. Fox *et al.* (1990) presented similar data in HeLa and Rat-2 cells. If a single protein, pit-1, activates both promoters, then what is happening at the transcriptional level in lactotrophs and somatotropes, which only express prolactin or growth hormone, respectively?

As discussed below, evidence presented by Karin's group showed that their cloned protein, GHF-1, binds to and activates only the growth hormone promoter. They have maintained that claim even though the cDNA sequence of their cloned protein is identical to that described by Rosenfeld's group, who propose that their protein, pit-1, binds to and can activate both promoters. While the *in vitro* data using each of these clones leans towards the single transcription factor theory, the interpretation of the *in vivo* data is not so straightforward.

Karin's initial paper describing the cloning of their pituitary-specific transcription factor, GHF-1, does not provide any definitive data on the ability of GHF-1 to activate either promoter (Bodner *et al.*, 1988). However, some

immunofluorescence data is presented that show cells in which GHF-1 is found in the nucleus, but growth hormone is not expressed. Their explanation of this finding is that perhaps the antibody used in these studies was somehow cross-reacting to a prolactin-specific factor (although in a previous paper from their laboratory, they plainly state that "antibodies to GHF-1 did not react with the Prl binding activity", Castrillo *et al.*, 1989), or that GHF-1 is somehow necessary in the developmental pathway of anterior pituitary cells in becoming lactotrophs. Either way, there is no data in this publication which supports their claim that GHF-1 is growth hormone promoter-specific. This paper does, however, provide good evidence for refuting their own claim that extinction of growth hormone gene expression is due solely to the extinction of GHF-1, as clearly, there are cells in the anterior pituitary which have extinguished growth hormone expression, yet still have GHF-1 present in their nuclei.

Karin's group has also published some co-transfection data which shows activation of a growth hormone promoter construct but not a prolactin promoter construct (Theill *et al.*, 1989). An expression vector with a human metalothionein promoter is used to drive the synthesis of GHF-1 in this experiment. Thus, GHF-1 expression can be activated by the addition of a heavy metal, like cadmium to the tissue culture medium. It had been shown previously that the heavy metal cobalt can inhibit the activation of prolactin gene expression in GC cells (Murdoch *et al.*, 1985). This system using a heavy metal ion to activate transcription is therefore, a somewhat unfortunate choice. Given that they already had a suitable expression vector (RSV-GHF-1) and that they used a cloned stably transfected cell line, it is not clear why this circuitous method was used. It would have been preferable to use the RSV-GHF-1 vector to generate a stably transfected cell line, and then select clones that expressed GHF-1 at the level seen in pituitary-derived cell lines. In fact, they could have generated many

cell lines expressing GHF-1 at different levels to provide solid evidence for their claim that given a high enough level of GHF-1, the prolactin gene can also be expressed, like that seen in the *in vitro* experiments. In addition, they discuss data from other experiments claiming that in both Rat-6 and F9 cells (data not shown), GHF-1 can activate a growth hormone promoter-CAT construct. This publication, again, does not provide any evidence to support their claim.

In Dollé *et al.* (1990), Karin's group attempts to correlate GHF-1 expression with growth hormone expression during mouse embryogenesis. Using *in situ* hybridization techniques, they show that GHF-1 RNA first appears at 13.5 days postcoitum (p.c.). Both growth hormone and prolactin RNA are detectable in the anterior pituitary by p.c. day 15.5 primarily in separate distinct cell populations. The lag between the expression of GHF-1 RNA and the expression of GH and prolactin RNA is explained by a translational delay in the expression of GHF-1, as the antigenic detection of GHF-1 correlates with the expression of GH and prolactin RNA. Regarding the question of the role GHF-1 plays in growth hormone and prolactin gene expression, they present data that places GHF-1 and GH expression in the same cells, but do not show any data which clearly shows the lack of GHF-1 expression in cells expressing prolactin. All references to prolactin are presented as "data not shown." The authors then cite articles and personal communications from which they claim that another factor, "LSF-1" has been characterized, and is responsible for prolactin gene expression. Indeed the articles to which they refer merely speculate that a factor "LSF-1" exists, but again there is no data shown that supports their claim. Therefore, one can only draw conclusions regarding GHF-1's obvious role in growth hormone gene expression.

Developmental studies of pit-1 in rats have shown that pit-1 transcripts can be found in the anterior pituitary of rat embryos by day 15.5 p.c. (Simmons *et al.*, 1990). Unlike in mice, there apparently is no detectable lag between pit-1

gene transcription and protein expression in rats. Based on the observation of coincident expression of pit-1 and the activation of both the growth hormone and prolactin genes by day 17.5 p.c., the authors conclude that the data support the model that pit-1 is responsible for the initial transcriptional activation of both genes. When the authors examined the distribution of expression of pit-1 mRNA in the various cell types of the anterior pituitary, surprisingly, they found pit-1 mRNA in all five cell types of the adult anterior pituitary. However, pit-1 protein is expressed only in lactotrophs, somatotropes, and thyrotrophs. It is regrettable that these experiments were not performed in intact pituitaries, as dispersing pituitary cells and culturing them could have introduced artifacts which may not represent their condition physiologically. However, these observations suggest that there exist mechanisms which (1) prevent the translation of pit-1 mRNA in a cell-type specific manner, and (2) prevent the expression of growth hormone and prolactin in thyrotropes.

While co-transfection of pit-1 can activate both growth hormone and prolactin promoters in HeLa and CV-1 cells, a surprising finding came from experiments involving undifferentiated F9 cells. We were unable to activate either the prolactin or growth hormone promoter constructs by pit-1 co-transfection in this cell line. Whether this is a result of the undifferentiated state of F9 cells, perhaps lacking in a "basic" transcription factor(s) (Müller and Wagner, 1984; Edwards *et al.*, 1988; Sleigh, 1988) or the presence of a "repressor" protein(s) (Gorman *et al.*, 1985; Shivji and La Thangue, 1991), will be determined by further studies involving differentiating F9 cells with retinoic acid (Strickland and Mahdavi, 1978). Other possible explanations of this observation include different post-translational modifications of the pit-1 protein in undifferentiated cells (Kapiloff *et al.*, 1991) or interference with protein translation or nuclear translocation of pit-1.

Another interesting finding is the consistently higher level (up to five-fold) of expression of the prolactin promoter relative to the growth hormone promoter. While this has been seen in all published experiments, it has not directly been addressed. One explanation may lie in the organization of the prolactin promoter, having four pit-1 binding sites as compared to the growth hormone promoter, having only two. Harvey *et al.* (1991) showed that pit-1 binding to these sites induces alteration in promoter structure which is critical in stabilizing the transcription initiation complex. While these experiments were performed *in vitro*, it would be interesting to see if these results are carried through to *in vivo* transfection studies.

In the next chapter, we present evidence for another possible mechanism by which these two cell-specific genes are differentially regulated: negative regulation of the growth hormone promoter.

Chapter Four is reprinted, with permission, from *Journal of Biological Chemistry* Volume 265, pages 7022-7028 (1990), "Identification of a growth hormone gene promoter repressor element and its cognate double- and single-stranded DNA-binding proteins." by Wayne T. Pan, Qiurong Liu, and Carter Bancroft. It is included in this thesis in accordance with the Rules and Regulations governing dissertations set forth by the Executive Committee of the City University of New York. The section entitled "Experimental Procedures" in the original paper is included in the chapter "Experimental Methods" in order to preserve continuity of the thesis format. Additional data is presented at the end of the chapter under "Additional Experiments and Discussion" which were not included in the original publication.

Chapter Four: Identification of a Growth Hormone Gene Promoter Repressor Element and Its Cognate Double- and Single-stranded DNA-binding Proteins

Abstract

In previous investigations, cell fusion was found to silence either the endogenous rat growth hormone (GH) gene or a transfected rat GH gene promoter, implying that repression plays a role in regulation of this gene. To search the rat GH gene promoter for repressor sequences, a series of 5'-deleted GH-CAT constructs was analyzed by transient expression in GH_3 rat pituitary cells. Deletion of either a distal region between positions -307/-244 or a proximal sequence between -169/-152 increased CAT enzymatic activity by 3-4-fold. Since the action of the proximal repressor element (PRE) at -169/-152 was serum-independent, and the element is located between two strong positive elements, the PRE and its cognate binding proteins were further analyzed. A 5-base pair sequence centered at -163 is critical for PRE repressor activity, since mutation of this sequence in GH-CAT constructs yielded 6-11-fold increases in expression in GH_3 cells. Although the PRE is adjacent to the GH thyroid hormone (T_3) response region, they are distinct elements, since the PRE mutation has little effect on T_3 response of GH-CAT constructs. Nuclear extracts of 10 cell lines were searched by DNA mobility shift for protein(s) binding specifically to a double-stranded PRE probe. No such protein was detected in any of four rodent pituitary cell lines or three human cell lines. However, three different rodent non-pituitary cell lines yielded a common shifted band, corresponding to a DNA sequence-specific PRE-binding protein (PREB). Similar analysis with the coding strand of the PRE detected no shifted band in any of these cell lines. However, the PRE non-coding strand yielded a common shifted band in all of the cell lines, corresponding to a ubiquitous, strand-specific, single-stranded PRE-binding protein (ssPREB). Mutation of the PRE permitted ssPREB binding to the coding

strand, implying that the wild-type coding strand somehow excludes ssPREB binding. That PREB and ssPREB are distinct proteins was confirmed by the inability of their DNA binding sites to cross-compete binding of the proteins.

Introduction

The genes for growth hormone (GH) and prolactin exhibit strong tissue-specific regulation, since their expression is virtually exclusive to the pituitary gland. The pituitary gland itself exhibits an interesting sub-glandular distribution of the hormones coded for by these genes, since it contains cells that produce either only GH (somatotropes), only prolactin (mammotropes), or both hormones (somatomammotropes) (Frawley *et al.*, 1985). This pattern implies that both common and distinct mechanisms regulate cell-specific expression of these genes.

Studies of these mechanisms have generally employed either the GH_3 rat pituitary cell line or its variants (collectively termed GH cells (Bancroft, 1981)). The GH_3 cells produce both GH and prolactin and are thus presumably descended from somatomammotropes. Transient expression assays with transfected promoters of the two genes have yielded the same overall pattern of cell-type expression: strong in GH cells and undetectable in non-pituitary cells. Transfection experiments with GH cells have identified positive cell-specific elements in the promoters of the GH gene (West *et al.*, 1987; Lefevre *et al.*, 1987; Ye *et al.*, 1988; Nelson *et al.*, 1988) and the prolactin gene (Lufkin and Bancroft, 1987; Cao *et al.*, 1987; Gutierrez-Hartmann *et al.*, 1987; Nelson *et al.*, 1988; Lufkin *et al.*, 1989). A pituitary-specific factor pit-1/GHF-1 has been cloned recently (Ingraham *et al.*, 1988; Bodner *et al.*, 1988) and reported to bind to and activate the cell-specific elements in either both promoters (Mangalam *et al.*, 1989) or only the GH promoter (Theill *et al.*, 1989). Thus these studies have not yet clearly

elucidated distinct mechanisms for regulating cell-specific expression of the GH and prolactin genes.

By contrast, the results of cell fusion assays have revealed that cell type-specific expression of these two genes differ and have suggested that regulation of the GH gene may involve repression as well as activation. In stable hybrids formed between GH_3 cells and fibroblasts, GH_3 cell expression of the GH gene is repressed (Sonnenschein *et al.*, 1971; Strobl *et al.*, 1982). More recently, results with a transient cell fusion assay have shown that fibroblast expression of a silent stably transfected rat prolactin promoter is strongly activated by fusion to GH_3 cells, whereas a similarly transfected rat GH promoter remains silent (Lufkin and Bancroft, 1987). In addition, the observation that GH is synthesized at a rate $\geq 10^8$-fold higher in rat pituitary cells than in rat non-pituitary (liver) cells (Ivarie *et al.*, 1983) suggests a requirement for the action in these two cell types of, respectively, activator and repressor mechanisms.

To investigate further the role of repression in regulation of the GH gene, we have employed transient expression in GH_3 cells to search the rat GH promoter region for negative regulatory DNA elements. We report here the identification of a proximal repressor element (PRE) residing between two strong positive regulatory elements and the detection of two nuclear proteins (PREB and ssPREB) that bind specifically to, respectively, double-stranded PRE DNA or its non-coding strand.

Results

Effect of successive 5' deletions on growth hormone promoter activity in pituitary cells — As outlined in Fig. 4.1B, previous studies have identified two positive cell-specific elements (dGHF-1 and pGHF-1) and a positive thyroid hormone (T_3) response region in the promoter region of the growth hormone gene. However,

the overall regulatory profile in pituitary cells of this region has not yet been examined systematically. We began the present search for repressor sequences by examining transient expression by the rat pituitary GH_3 cells of a series of 5' deletions of the rat growth hormone promoter, cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. It has been shown previously that transcription by GH cells of similar GH-CAT constructs initiates at the appropriate start site (Nelson *et al.*, 1986; West *et al.*, 1987).

Results are shown in Fig. 4.1A. Deletion of sequences between positions -550 and -307 (relative to the cap site (Barta *et al.*, 1981)) yielded only a moderate decrease in CAT activity. Further deletion from -307 to -244 yielded a 3-fold increase in expression, whereas successive deletions from -244 to -178 yielded a gradual ~3-fold decrease. Deletion from -178 to -169 caused a further three-fold decrease, due most probably to interruption of a thyroid hormone response element. By contrast, deletion of the next 17 base pairs, between -169 and -152 yielded a sizable 4-fold increase in expression. Finally, when the more distal cell-specific activator element dGHF-1 was removed by deletion to -96, CAT activity was reduced to background levels. The growth hormone promoter region thus contains, in addition to the activator elements described above, two negative elements: PRE at -169/-152, and a distal repressor element (DRE) at -307/-244 (Fig. 4.1B).

The repressor activity of the PRE, but not the DRE, is independent of serum — Since the above experiments were performed with a serum-containing incubation medium, the negative elements identified could each represent either a basal repressor element or a negative response element for an unknown serum factor. In cells incubated with serum, deletion of either class of regulatory element should result in increased expression. To distinguish between these possibilities,

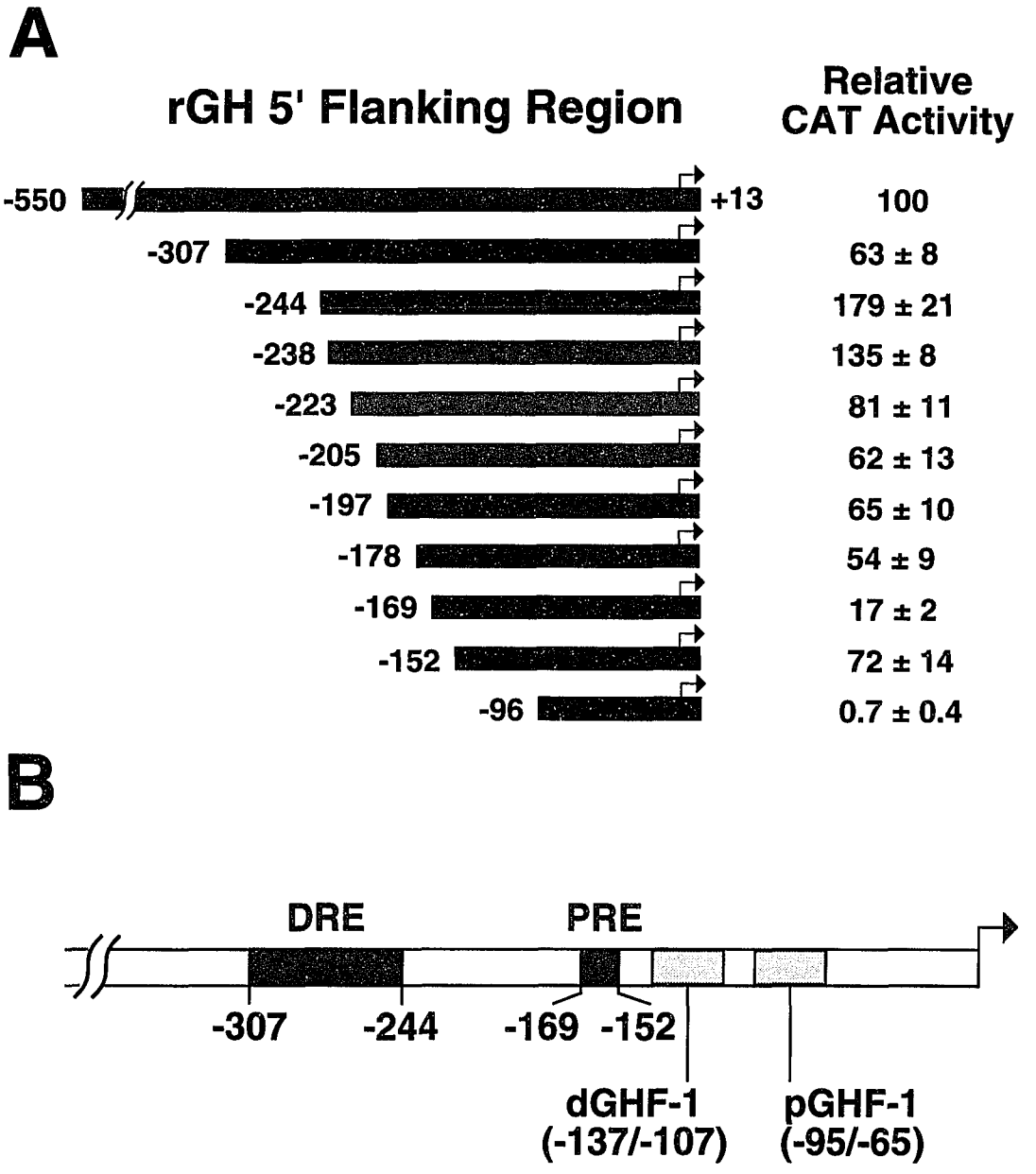

FIG. 4.1. **Analysis by 5' deletion mutation of GH promoter regulatory elements involved in transient CAT gene expression in GH_3 cells.** A, plasmid pGH-CAT (-550) or its 5' deletion mutants (each identified by the 5' terminus of its GH promoter sequence) were transfected into GH_3 cells. Following a 3-day incubation in Dulbecco's modified Eagle's medium plus 5% fetal calf serum, cellular CAT activity was assayed. Results are normalized to a value of 100% for pGH-CAT, which yielded 10-20% conversion of [^{14}C]chloramphenicol to its acetylated products. Shown are the mean ± S.E. of results of three independent transfection experiments (except for -244, representing the mean ± S.D. of two experiments). B, summary of rat GH gene promoter regulatory elements.

the effect of deleting each element was re-examined in cells incubated in presence (Fig. 4.2A) or absence (Fig. 4.2B) of serum. As shown previously in Fig. 4.1, deletion from the growth hormone promoter of either the DRE (-307/-244) or the PRE (-169/-152) increased expression in cells incubated with serum. In cells incubated without serum, the effect of deletion of DRE was lost, whereas deletion of the PRE still increased expression. These results suggest that the DRE may correspond to a negative response element for a serum factor, whereas the negative regulatory property of the PRE is independent of extracellular stimuli. Furthermore, the PRE forms part of an interesting regulatory pattern, since it is bounded by two strong positive regulatory elements, the thyroid hormone response element and the cell-specific element dGHF-1 (see Fig. 4.1B). We thus decided to characterize further the PRE and its cognate binding proteins. Since serum-containing medium yielded considerably higher cellular CAT enzymatic activities than serum-free medium (Fig. 4.2), the former was employed for all subsequent studies.

Mutation of the PRE inhibits its repressor activity — The sequence of the PRE is (-169)GACCGCAGGAGAGCAGTG(-152) (Barta *et al.*, 1981). We sought to produce a minimal mutation in this sequence that would be likely to block binding of a trans-acting factor, thus inhibiting its repressor activity. However, initial experiments with nuclear extracts of either the GH_3 pituitary cells or various non-pituitary cells (C127, C6, HeLa) yielded no consistent DNase footprints over this sequence. The mutation site was therefore chosen as follows. A mutational study of the IgH gene has shown that deletion of GCAG at position +7 of its enhancer stimulates activity in CV1 cells (Kadesch *et al.*, 1986). In addition, one of several experiments with GH_3 cell extracts yielded a faint DNase footprint over the PRE sequence (-165)GCAGG(-161) (data not shown). We thus employed site-directed mutagenesis to introduce into the growth hormone

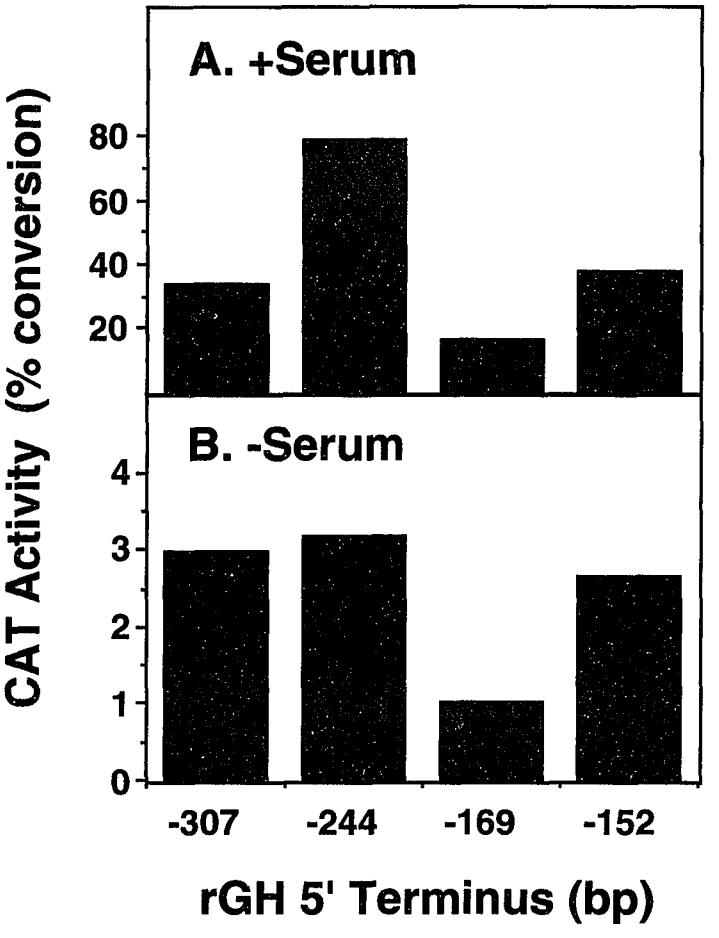

FIG. 4.2. **Effect of serum on GH_3 cell expression of rGH-CAT constructs.** Following transfection, cultures of GH_3 cells were incubated 2 days in the serum-free medium described previously (White *et al.*, 1981), then 2 days in this medium ± 10% fetal calf serum, and then assayed for CAT enzymatic activity.

promoter non-conservative transversions at -165/-161, changing GCAGG to TACTT (Fig. 4.3A).

Mutation of this sequence in GH-CAT constructs containing either 178, 205, or 244 base pairs of growth hormone promoter upstream flanking sequence yielded strong (6-11-fold) increases in their transient expression in GH_3 cells (Fig. 4.3B). Hence, the growth hormone promoter sequence GCAGG at position -163 is critical for the repressor activity of the PRE. The observation that introduction of this mutation into all three constructs yielded similar effects implies, in agreement with the 5' deletion analysis shown in Fig. 4.1, that repression by the PRE does not require interaction with more distal DNA sequences in the growth hormone gene promoter.

Mutation of the PRE does not interfere with thyroid hormone action on the growth hormone promoter — As indicated in Fig. 4.1B, the PRE at positions -169/-152 is adjacent to, and might even partially overlap the T_3 response region. The question of the number, location, and relative strengths of the T_3 response elements in the rat growth hormone promoter has not yet been resolved. A number of reports have indicated that sequences upstream of -173 are largely responsible for the T_3 response (Ye *et al.*, 1988; Wight *et al.*, 1988; Brent *et al.*, 1989). The decreased expression we have observed upon removal of -178/-169 (Fig. 4.1A) is thus probably due to removal of T3 response element(s). However, it has also been reported that a complete response to this hormone requires sequences either between -179 to -164 (Glass *et al.*, 1987) or centered at -160 (Norman *et al.*, 1989).

It seems unlikely that the effects described above are due to action of a T_3 response element, since mutation of the PRE *increases* expression. However, because of the possible overlap suggested by some of the localization studies described above, we investigated whether mutation of the PRE affects the ability

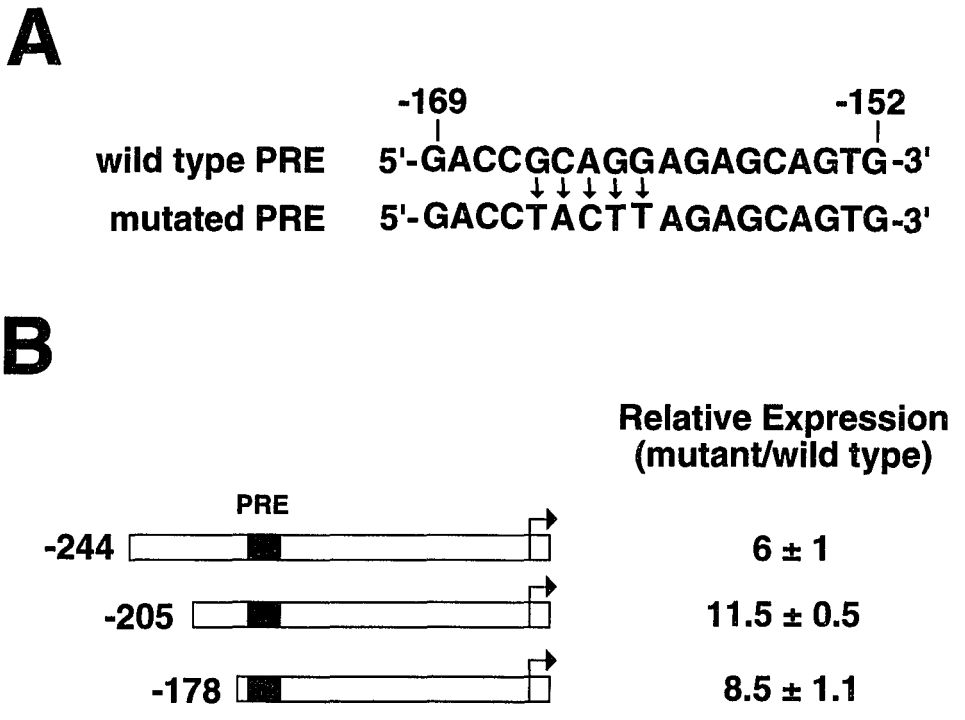

FIG. 4.3. **Effect of mutation of the PRE on transient CAT gene expression in GH_3 cells.** A, sequences of the coding strand of the wild-type and mutated PRE. B, the mutation shown in A was introduced into the indicated pGH-CAT constructs, and transient expression was assayed as in Fig. 4.1. Results are presented for each construct as a ratio of mutant CAT activity to wild-type activity. Shown are the mean ± S.D. of results of two independent experiments.

of T_3 to regulate transient expression of GH-CAT constructs in GH_3 cells (Table I). The control constructs RSV-CAT and TK-CAT yielded stimulations by T_3 of, respectively, 0.9- and 1.6-fold. T_3 yielded a 4-fold stimulation of expression of (-244)GH-CAT, which was not significantly reduced by mutation of the PRE. The T_3 stimulation of expression of wild-type construct (-178)GH-CAT (2-fold) was close to that observed with the control constructs, consistent with the former set of previous localizations described above. This small stimulation was also apparently not significantly reduced by mutating the PRE. The observation that a mutation of the PRE that increase GH-CAT expression in GH_3 cells by 6-11-fold has little effect on the T_3 response of the GH gene promoter implies that the PRE is distinct from a T_3 response element.

A sequence-specific PRE-binding protein is expressed specifically in rodent non-pituitary cell lines — To explore specific interactions of the PRE with nuclear proteins, this element was employed as a probe in DNA mobility shift assays. Nuclear extracts of three types of cell lines were examined. These included: (i) four rodent pituitary lines: the independently isolated, growth hormone-deficient rat 235-1 cells, and the adrenocorticotropic hormone-producing mouse AtT-cells; (ii) three non-pituitary rodent lines: C6 (differentiated rat glial), C127 (mouse mammary tumor), LLC-WRC (rat carcinoma); (iii) three human lines: HeLa (epithelioid carcinoma), HepG2 (hepatoma), and JEG-3 (choriocarcinoma).

Results are shown in Fig. 4.4. No shifted band corresponding to a PRE-binding protein was detected in extracts of any of the pituitary rodent cells lines GH_3, GC, 235-1, or AtT20 (*lanes 1-4*). This is not due to inactivation of DNA-binding proteins during extract preparation, since the same extracts of the first three lines yielded a strong signal with both prolactin promoter probes (data not shown) and a single-stranded DNA probe (see Fig. 4.5, *lanes 11-13*), whereas the AtT20 extract yielded a strong signal with a glucocorticoid receptor

TABLE I

Effect of the PRE mutation on the ability of T_3 to stimulate GH-CAT expression by GH_3 cells

GH_3 cells were transfected with either control constructs (RSV-CAT or TK-CAT), the indicated wild-type GH-CAT constructs, or these GH-CAT constructs containing the PRE mutation shown in Fig. 4.3A. The cells were then incubated 24 h in Dulbecco's modified Eagle's medium plus 5% fetal calf serum, followed by 48 h in the serum-free medium described previously (White *et al.*, 1981), and then an additional 48 h in this medium plus or minus T_3 (5 nM). Shown is the mean ± S.E. of the -fold T_3 stimulation of CAT enzymatic activity observed in the indicated number (in parentheses) of independent experiments.

Construct	-Fold stimulation by T_3	
	Wild type	Mutated PRE
RSV-CAT	0.88 ± 0.11 (3)	
TK-CAT	1.6 ± 0.9 (2)	
(-244)GH-CAT	4.2 ± 1.3 (3)	3.2 ± 0.8 (3)
(-178)GH-CAT	2.1 ± 0.4 (4)	1.6 ± 0.5 (4)

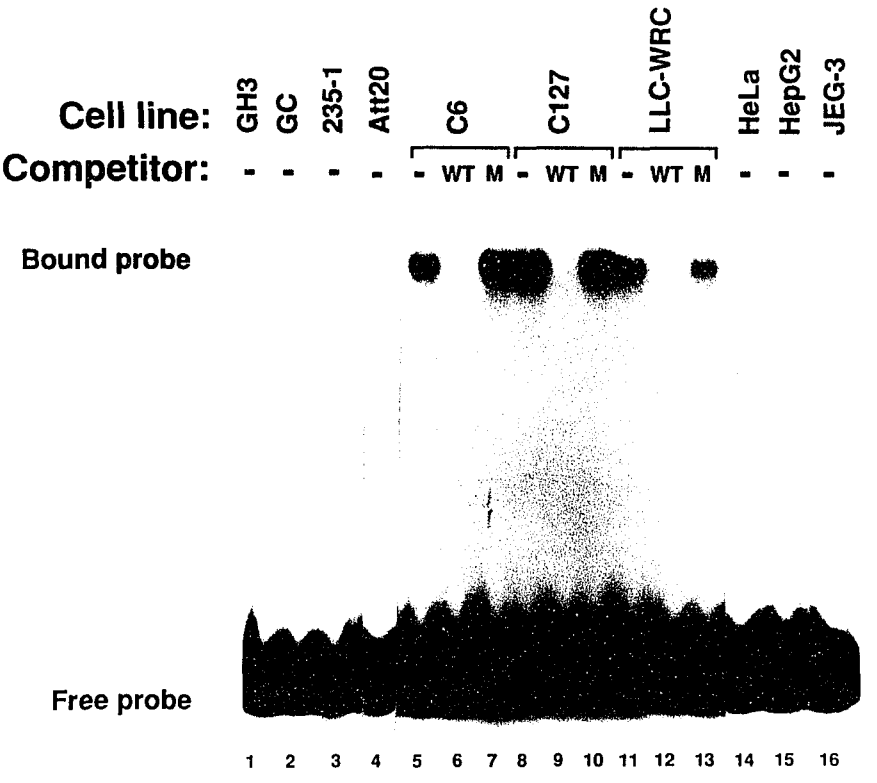

FIG. 4.4. **Binding of double-stranded PRE DNA to nuclear proteins from various cell types.** Nuclear extracts were prepared from the indicated cell lines and analyzed by DNA mobility shift with a 32-base pair double-stranded PRE oligodeoxynucleotide probe, as described under "Experimental Procedures." The indicated lanes received a 100-fold molar excess of either the wild-type (*WT*) or mutated (*M*) PRE oligodeoxynucleotide. The samples in *lanes 4* and *14-16* were analyzed on a separate gel.

consensus DNA binding probe (data not shown) and with a single-stranded DNA probe (see Fig. 4.5, *lane 14*). Surprisingly, all three, non-pituitary rodent cell lines, C6, C127, and LLC-WRC, yielded readily detectable levels of a single shifted band (Fig. 4.4, *lanes 5, 8, and 11*, respectively). The mobility of this band was indistinguishable among these three cell types, implying that they contain a common (presumably single) PRE-binding nuclear protein, designated PREB. Among these rodent cell lines, PREB thus exhibits an unusual cell type-specific distribution, accumulating specifically in non-pituitary cells. PREB binding is DNA sequence-specific in each of these non-pituitary cell lines, since binding of this protein was competed by excess wild-type PRE (lanes labeled *WT*) but not by mutated PRE (lanes labeled *M*). It should be noted that some types of pituitary cells apparently contain trace levels of PREB, since an extended autoradiographic exposure of the gel shown in Fig. 4.4 yielded a detectable band at the appropriate position in the GH_3 and GH cells, but not in the 235-1 or AtT20 cells (data not shown). The PRE probe also yielded no detectable shifted band in any of the human cell lines, HeLa, HepG2, or JEG-2 (*lanes 14-16*), even though the same extracts of these human cells yielded readily detectable levels of single-stranded DNA-binding proteins (see Fig. 4.5, *lanes 18-20*). Thus PREB accumulates specifically in rodent non-pituitary cell types.

A ubiquitous protein binds specifically to the PRE non-coding strand — Most of the characterized DNA-binding eukaryotic regulatory proteins bind to double-stranded DNA (Mitchell and Tjian, 1989). However, recent reports have described binding of nuclear proteins to individual strands of either positive (Lannigan and Notides, 1989) or negative (Spiegelman *et al.*, 1988) regulatory elements. We thus investigated whether any of the nuclear extracts described above contain proteins that can bind to either single strand of the PRE (Fig. 4.5). No protein capable of binding to the PRE coding strand was detected in any of

the ten cell lines examined (*cPRE; lanes 1-10*). However, all 10 cell lines yielded a common shifted band with the non-coding strand of the PRE (*ncPRE; lanes 11-20*). The doublet apparent in the human cell extracts has been observed reproducibly, the ubiquitous (presumably single) protein responsible for the common band detected in all ten cell types with the non-coding single strand of the PRE is designated ssPREB.

The effect of the mutation shown in Fig. 4.3A on binding of GH_3 cell nuclear proteins to individual strands of the PRE was examined (Fig. 4.6). As before, the ncPRE (*lane 3*) but not the cPRE (*lane 1*) yielded a single strong shifted band corresponding to binding of ssPREB. By contrast, *either* strand of the mutated PRE, cPRE(mut) (*lane 2*), or ncPRE(mut) (*lane 4*), yielded a strong shifted band at this position. Thus ssPREB exhibits a degree of sequence specificity in its binding to single-stranded DNA, since it strongly binds the ncPRE sequence 5'-CACTGCTCTCCTGCGGTC-3', but not its complementary cPRE sequence. However, the observation that mutation of the cPRE permits strong ssPREB binding implies that this specificity arises from some ability of the cPRE to specifically *exclude* binding of ssPREB, rather than from sequence -specific binding of this protein to the ncPRE. We have also observed that several single-stranded oligonucleotides lacking evident similarities to the ncPRE can compete binding of ssPREB to the ncPRE (data not shown), further demonstrating that the DNA-binding sequence specificity of ssPREB is limited.

In experiments described above, the evident differences between PREB and ssPREB, in both cell type distribution and DNA binding site secondary structure, implies that they are distinct proteins. This was further demonstrated by the inability of their respective DNA binding sites to cross-compete DNA binding of the proteins (Fig. 4.7). Excess single-stranded ncPRE did not inhibit PREB binding to the double-stranded PRE (*lanes 1 and* 2), nor did excess PRE

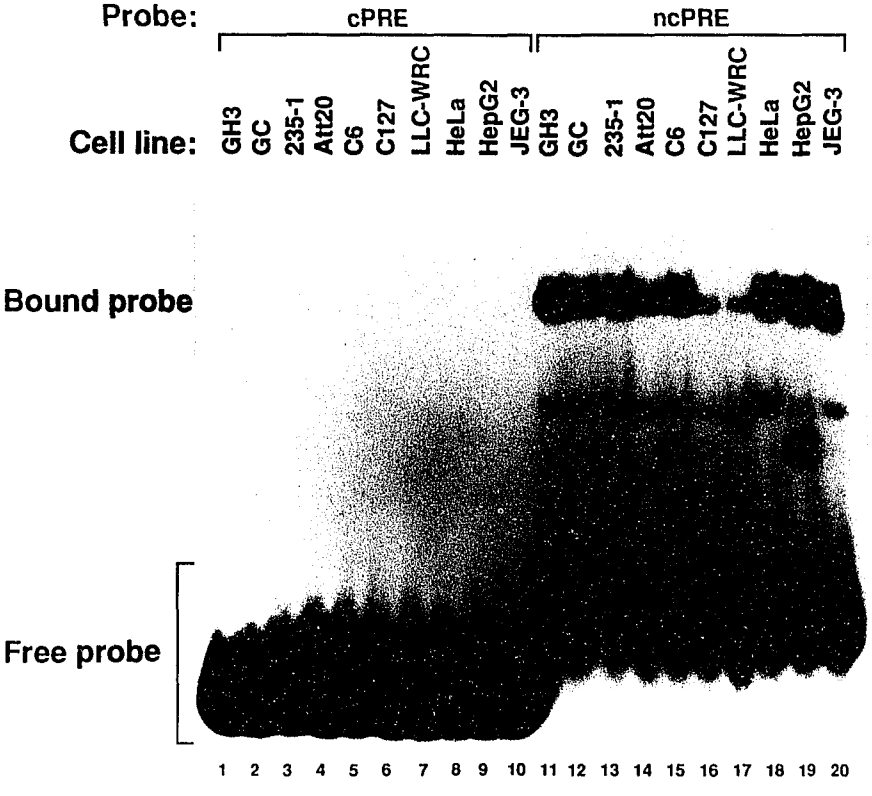

FIG. 4.5. **Binding of individual strands of the PRE to nuclear proteins from various cell types.** Analysis was as in Fig. 4.4, except that the oligodeoxynucleotide probes employed corresponded to either the coding strand (*cPRE*) or the non-coding strand (*ncPRE*) of the PRE. The extracts employed were the same as in Fig. 4.4.

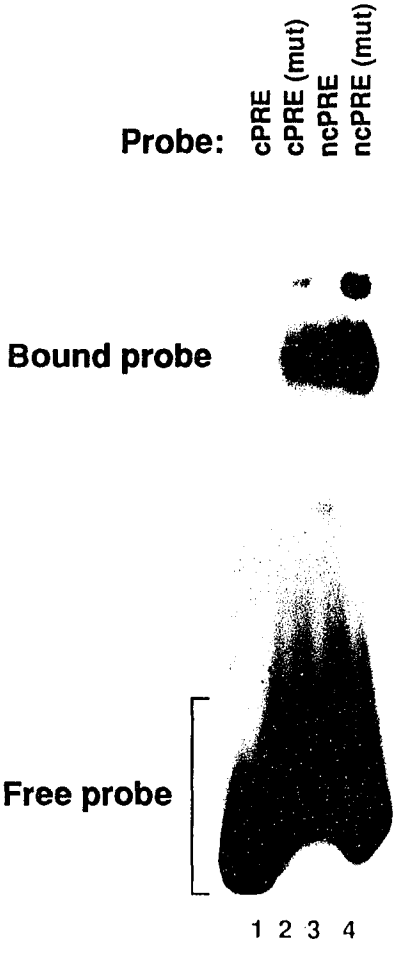

FIG. 4.6. **Effect of mutation on the ability of the individual strands of the PRE to bind to nuclear extracts of GH_3 cells.** Analysis was as in Fig. 4.5, employing the wild-type or mutated (*mut*) cPRE or ncPRE oligodeoxynucleotide probes described under "Experimental Procedures."

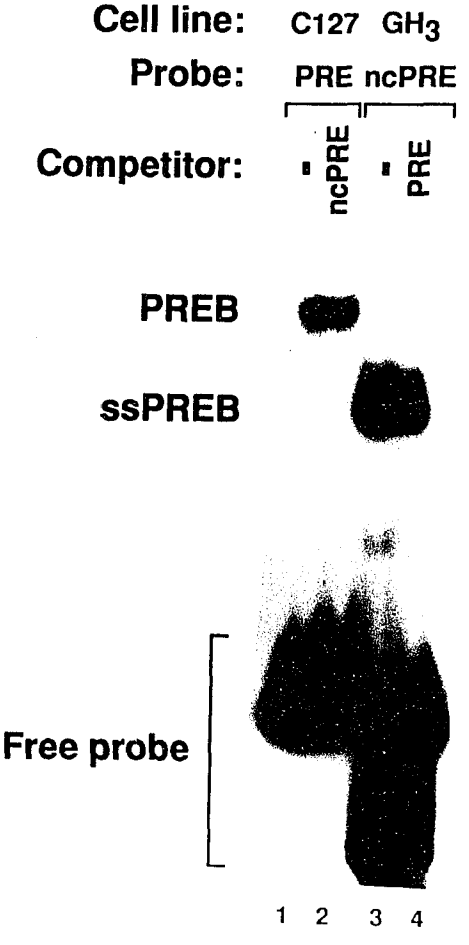

FIG. 4.7. **Inability of the DNA binding sites for PREB and ssPREB to cross-compete binding of the proteins.** Nuclear extracts of the indicated cell lines were analyzed as in Figs. 4.4 and 4.5, employing as probe either the double-stranded PRE or its non-coding strand (*ncPRE*), plus, where indicated, a 100-fold molar excess of the indicated competitor oligodeoxynucleotide.

inhibit ssPREB binding to the ncPRE (*lanes 3 and 4*). These results imply both that PREB and ssPREB are different proteins and that PREB exhibits a considerably higher affinity for double-stranded DNA than for single-stranded DNA, whereas the opposite is true for ssPREB.

Discussion

Analysis by transient expression in rat pituitary GH_3 cells of a 5'-deleted series of GH-CAT constructs has revealed that the proximal rat GH promoter region contains two negative regulatory elements. One of these, the PRE centered at position -161, resides just downstream of a strong positive hormonal regulatory element, the T_3RE, and just upstream of two strong positive cell-specific elements, dGHF-1 and pGHF-1. Thus the PRE represents part of an interesting mosaic of positive and negative regulatory elements contained within the first 200 base pairs of the rat GH promoter. Although 5' deletion analysis identified boundaries for the PRE at positions -169 to -152 (Fig. 4.1), the complete element could extend farther in either direction. To begin to determine which DNA sequences within these minimal boundaries are required for PRE action, we introduced a transverse mutation of bases 5'-GCAGG-3' centered at position -163. The observation that introduction of this mutation into each of three different GH-CAT constructs resulted in strongly increased expression in GH_3 cells (Fig. 4.3) implies that these 5 bases or a subset thereof are critical for activity of the PRE. The DRE detected in the present studies between positions -307 to -244 probably corresponds to the "silencer" element reported by Larsen *et al.* (1986) to reside between positions -237 to -554. However, in contrast to their studies, and in agreement with other reports (Ye *et al.*, 1988; Nelson *et al.*, 1986), we have observed that deletion of the DRE does not activate GH-CAT expression in any non-pituitary cell lines examined (data not shown). Similarly, deletion or

mutation of the PRE does not result in detectable GH-CAT expression in non-pituitary cell lines, either during transient expression or following transient fusion (Lufkin and Bancroft, 1987) to GH_3 cells (data not shown). Hence, ablation of either the DRE or the PRE is not alone sufficient for detectable expression of the GH promoter in non-pituitary cells, presumably because of the absence from these cells of the pituitary cell-specific factor pit-1/GHF-1 recently shown to bind to pGHF-1 and dGHF-1 activator elements shown in Fig. 4.1 (Ingraham *et al.*, 1988; Bodner *et al.*, 1988).

The proximity of the PRE to the T_3 response region of the rat GH promoter (Fig. 4.1A) suggested that these regulatory regions might partially overlap. However, the following observations imply that the PRE is distinct from a T_3 response element: (i) deletion of the PRE increases transient GH-CAT expression in GH_3 cells grown in either the presence or absence of serum (Fig. 4.2), implying that the PRE is not a response element for any serum hormone or factor, including T_3. (ii) Removal or mutation of the PRE *increases* expression in GH_3 cells grown in the presence of serum (which contains sufficient T_3 to yield a near-maximal response to T_3 (Samuels *et al.*, 1979)), whereas removal of a T_3 response element under these conditions would be expected to *decrease* expression. (iii) Mutation of the PRE had little effect on the ability of T_3 to stimulate GH-CAT expression in GH_3 cells (Table I).

Use of the DNA mobility shift assay to search for a nuclear protein that binds specifically to the PRE, and thus might mediate its effects, revealed the existence of a protein(s) (assumed for simplicity to be a single protein) designated PREB that exhibits sequence-specific binding to double-stranded PRE, PREB was readily detectable in three non-pituitary rodent cell lines, but was present at either undetectable or very low levels in four rodent pituitary cell lines and three human cell lines (Fig. 4.4). The virtual absence of PREB from

nuclear extracts of these latter two cell types is apparently not due to the action of an inhibitor of PREB DNA-binding activity, since high levels of PREB detected in C6 cell nuclear extracts (Fig. 4.4) was not decreased by prior incubation (30 min, 37°C, ATP added to 1 mM) with either nuclear or cytoplasmic extracts of the GH_3 pituitary cells or nuclear extracts of the human HeLa cells (data not shown). Preliminary attempts to detect PREB in the cytoplasm of GH_3 cells has also been unsuccessful. PREB thus exhibits an unusual type of cell-type distribution, since it accumulates specifically in rodent *non-pituitary* cells.

Although the mechanism for this anti-pituitary cell specificity of PREB accumulation is unknown, it is most probably mediated by a pituitary cell-specific repressor of some step in expression of the gene for PREB. Western blotting experiments in our laboratory have shown that the three acidophilic (*i.e.* GH- and/or prolactin-producing) pituitary cell lines GH_3, GC, and 235-1 all contain the transcription factor pit-1/GHF-1 discussed above.* However, this pituitary cell-specific factor is probably not a repressor of PREB accumulation, since pit-1/GHF-1 levels are undetectable in the corticotropic AtT20 cells,* but these cells also do not accumulate PREB (Fig. 4.4). The observation that neither acidophils nor corticotropes contain significant levels of PREB also suggests that restriction of PREB accumulation might be a general property of specialized pituitary cells.

In a further search for proteins that might mediate the effects of the PRE, we detected a ubiquitous protein, designated ssPREB, which exhibits strand-specific binding to the non-coding PRE strand (ncPRE) (Fig. 4.5). As discussed above, this strand specificity probably arises from a specific inability of ssPREB to bind to the coding strand of the PRE, rather than from

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* R. McChesney, unpublished observations.

sequence-specific binding of this protein to the ncPRE. The mechanism involved in this "reverse" specificity of ssPREB binding to DNA is not known, but it clearly requires the same pentameric DNA sequence as does PRE activity (Fig. 4.6). It is also worth noting that the limited DNA sequence specificity exhibited by ssPREB is apparently a common property of proteins that bind to single-stranded DNA (Kowalczykowski *et al.*, 1981).

It is interesting that the center of the sequence 5'-GCAGG-3' required for PRE action commences a 12-base pair purine-rich (10/12) sequence in the rGH promoter (Fig. 4.3A), since a polypurine or polypyrimidine stretch of DNA is capable of forming a novel triple-stranded form of DNA ("H-DNA") containing a single-stranded loop (Htun and Dahlberg, 1988). Such a structure could result in exposure of the single-stranded binding site for ssPREB. Furthermore, inspection of repressor elements identified in the promoters of the c-*myc* (Kakkis *et al.*, 1989) and β-interferon (Keller and Maniatis, 1988) genes shows that these elements also contain DNA stretches capable of forming H-DNA. It is thus possible that ssPREB represents a member of a class of single-stranded DNA-binding proteins capable of repressing expression of specific eukaryotic genes.

In summary, the salient properties of the PRE and its binding proteins are (1) a sequence within the PRE critical for its repressor activity is capable of forming single-stranded DNA; (2) the nuclear protein PREB binds specifically to double-stranded PRE DNA, and accumulates specifically in non-pituitary rodent cells; (3) a different nuclear protein ssPREB binds to one strand of PRE DNA, and is ubiquitous. Since mutation of a 5-base pair DNA sequence critical for PRE repressor activity strongly alters binding of PREB or ssPREB to, respectively, the PRE or to its individual strands, either or both of these proteins might mediate the repressor effects of the PRE. Furthermore, each of these proteins has properties consistent with such a regulatory role. Thus, a protein responsible for

repressing GH gene expression in non-pituitary cells would be expected to exhibit the anti-pituitary cell distribution observed for PREB. Our detection of the repressor phenotype in the pituitary GH_3 cells is not inconsistent with such a role for PREB, since as described above the GH_3 cells apparently do contain this protein, albeit at very low levels. It is also possible that the ubiquitous ssPREB mediates the repressor effect of the PRE via a mechanism requiring DNA strand separation plus ssPREB binding specifically to only one strand of the PRE. Purification and characterization of both PREB and ssPREB should help to elucidate the possible roles of each of these proteins in the repressor function of the PRE.

Additional Experiments and Discussion

The two newly identified proteins, PREB and ssPREB, are not the same protein entities. This is evidenced by their difference in tissue distribution (Figures 4.4 and 4.5) as well as the inability of the non-coding single-stranded probe from competing the PREB band and the inability of the double-stranded probe from competing the ssPREB band (Figure 4.7). Size fractionation of C6 nuclear extracts, containing both PREB and ssPREB, showed that ssPREB elutes in earlier fractions (Figure 4.8). This suggests that ssPREB is a larger protein (or protein complex) than PREB. Of note, it also appears that ssPREB binds in multimers, as evidenced by the multiple species seen on the gel-shift assay (Figure 4.8b).

The physiologic role of ssDNA binding proteins in transcription has not been completely defined in eukaryotes, though it has been hypothesized that sequence-specific binding proteins with preferential affinity for single-stranded DNA may play a regulatory role (Gierer, 1966, 1974; Crick, 1971). Numerous examples have recently been reported, though few have been assigned any

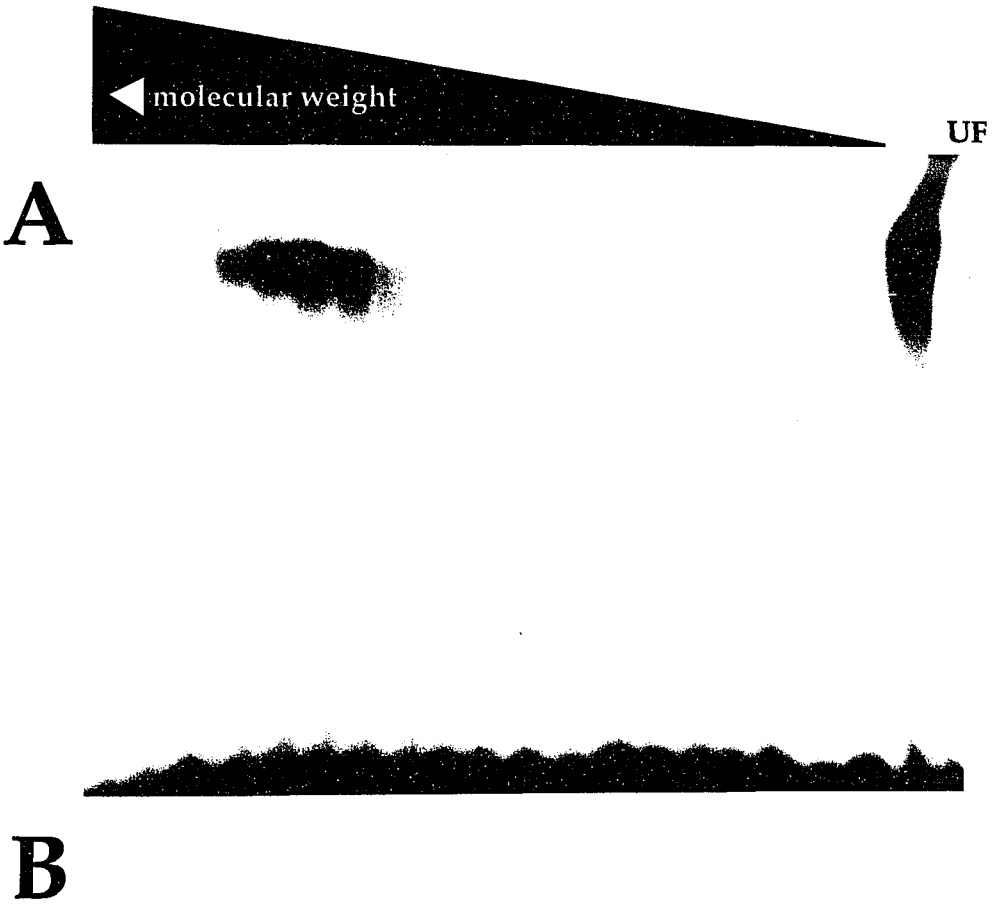


FIG. 4.8. **Separation of PREB and ssPREB by size fractionation.** See following page for legend.

FIG. 4.8. **Separation of PREB and ssPREB by size fractionation.** C6 rat glial cell nuclear extracts were prepared as described in *Experimental Methods*. An 0.5 ml (3.5 mg/ml) aliquot of C6 nuclear extract was first pre-incubated with DE52 (Whatman) equilibrated with Buffer C (Dignam *et al.*, 1983), equal packed bead volume, for 15 minutes on ice with gentle rocking. The treated nuclear extract was then loaded onto a 15mm x 100 cm Sephacryl S-200 HR column which was equilibrated with Buffer D containing 10% glycerol (Dignam *et al.*, 1983). 0.5 ml fractions were collected and stored at 4°C until gel shift analysis was performed. Gel shift analysis was carried out as essentially as described in *Experimental Methods* using 12.5 µl of each fraction and the designated radiolabeled probe. A. Double-stranded probe, PRE; B. Single-stranded probe, ncPRE. *UF*: unfractionated C6 nuclear extract.

specific functional role in the regulation of transcription (Lannigan and Notides, 1989; Feavers *et al.*, 1989; Rajavashisth *et al.*, 1989; Gaillard and Strauss, 1990; Wilkison *et al.*, 1990; Flavin and Strauss, 1991; Brunel *et al.*, 1991; Stigare and Egyhazi, 1991; Santoro *et al.*, 1991; Gilchrist *et al.*, 1992; Stark *et al.*, 1992; Kamada and Miwa, 1992; Jansen-Dürr *et al.*, 1992). Lannigan and Notides (1989) discovered a single-stranded DNA binding site in the estrogen responsive element (ERE) in the far upstream region of the rat prolactin gene promoter (-1784 to -1531). They demonstrated that the estrogen receptor bound the coding strand of this region with 60-fold greater affinity than its double-stranded counterpart. Although no functional experiments were described, they postulate that strand-specific DNA binding may play a role in estrogen-stimulated prolactin gene transcription. Rajavashisth *et al.* (1989) cloned a zinc-finger protein from a human hepatoma cDNA library that binds to a highly conserved octanucleotide sequence in the sterol regulatory element (SRE-1) of the hydroxymethylglutaryl (HMG)-CoA reductase promoter. This 19-kDa protein, which they designated as "cellular NBP" (CNBP), has no demonstrable affinity for the double-stranded DNA sequence, is up-regulated by sterols, and is expressed in a wide variety of tissues. The properties exhibited by this strand-specific DNA binding protein are consistent with a regulatory role in the sterol-mediated control of transcription. Another SRE-1-specific DNA binding protein has been characterized by Stark *et al.* (1992). This 45- to 49-kDa protein, SRE-BF, binds to both the native, double-stranded DNA as well as preferentially to the non-coding strand of the SRE-1 in the LDL receptor promoter. Although the sequence requirements for single- and double-stranded binding of SRE-BF partially overlap, the results of *in vivo* functional point mutation analysis of SRE-1 correlate well with SRE-BF binding of single-stranded SRE-1 *in vitro*. This suggests a possible role for SRE-BF single-stranded binding in the

sterol-regulated transcription of the LDL receptor. Santoro *et al.* (1991) demonstrated that a myocyte-specific nucleoprotein, muscle factor 3 (MF3) as well as MyoD, a myogenic determination protein, both bind specifically to the non-coding strand of single-stranded DNA probes corresponding to the MCAT element. The authors hypothesize that strand-specific DNA binding may play a role in myocyte development. Strand-specific DNA binding proteins have also been implicated in the developmental pathway of adipocytes. Wilkison *et al.* (1990) identified a strand-specific DNA-binding site in the mouse adipsin gene promoter. Unlike the previously described sites, the site in the adipsin promoter correlated with repression of gene expression in non-adipose cell types as well as activation of gene expression in adipocytes. Kamada and Miwa (1992) cloned a CArG box-binding muscle-specific protein, CBF-A, which demonstrated single-stranded DNA-binding characteristics. This protein also functions as a transcriptional repressor *in vivo*.

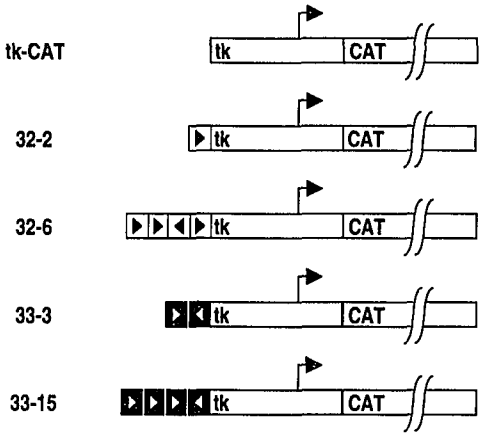
As one can see, strand-specific DNA-binding proteins should be considered a new class of transcriptional regulators, found in a variety of different environments, functioning as both activators as well as repressors of gene transcription. Thermodynamically, a single-stranded DNA-protein complex would be more flexible as compared to its double-stranded counterpart and therefore would allow protein-protein interactions to occur at a lower free energy level. This would be advantageous in situations where multiple regulatory sites are juxtaposed and transient uncoiling, stabilized by strand-specific DNA-binding proteins, could provide promoter access to allow activation or repression of gene transcription to occur.

The family of single- and double-stranded binding proteins found in the growth hormone promoter (ssPREB and PREB) is a unique situation as compared to the other examples previously described. In the growth hormone promoter,

the single-stranded and the double-stranded protein both have the same binding requirements (*i.e.* mutation of the 5-base pair region, abolishes both single-strand specificity as well as double-strand binding). This makes it very difficult to distinguish genetically the role that each plays in the regulation of the growth hormone promoter. However, one can take advantage of the difference in tissue distribution of these two proteins to examine their individual contribution to the transcriptional regulation of the growth hormone promoter.

One method of confirming the function of an *cis*-acting DNA element is to clone the sequence of interest in front of an heterologous promoter and examine its effect. We cloned multiple copies of the PRE or its corresponding mutant PRE in front of a herpesvirus thymidine kinase promoter-CAT construct (tk-CAT) (Figure 4.9). Linkage of the PRE to the tk-CAT construct caused an unexpected increase in CAT expression in a pituitary cell-specific manner. Mutant PRE sequences linked to the tk-CAT construct did not appreciably change the relative CAT expression in either the pituitary or non-pituitary cell lines.

At first examination, these results seem odd. One would expect a decrease in CAT expression with the cloning of a "repressor" element in front of the tk-CAT construct. However, if one examines the tissue distribution of ssPREB and PREB in the cell lines used, one can start to see a pattern emerging. PREB is relatively scarce in GH_3 cells as compared to C6 cells (Figure 4.4). However, ssPREB is abundant in GH_3 and less abundant in C6 cells (Figure 4.5). Therefore, the results in the GH_3 cells reflect the effect of ssPREB and while those in C6 cells demonstrate the relative effect of PREB. The ssPREB appears to activate transcription, perhaps by lowering the energy required for protein-protein interactions on the tk promoter. PREB, taken out of the growth hormone promoter context, appears to have a mild, if any, repressor effect on the tk promoter.



	GH_3 rat pituitary cells		C6 rat glial cells	
	percent conversion	fold induction	percent conversion	fold induction
tk-CAT	4.9 ± 1.1	—	23.9	—
32-2	29.6 ± 1.0	6.0	10.0	0.42
32-6	78.5 ± 9.5	16.0	27.7	1.2
33-3	8.2 ± 2.0	1.7	23.9	1.0
33-15	13.2 ± 3.8	2.7	12.9	0.54

FIG. 4.9. **Effect of PRE sequences cloned in front of tk-CAT.** Oligonucleotides corresponding to the PRE (white boxes) or a mutated version (black boxes) (see text) were cloned in front of p(-109)tk-CAT. Orientation of the of the oligonucleotide(s) were determined by dideoxynucleotide sequencing (Chen and Seeburg, 1980) and are indicated by the direction of the arrowheads. Indicated constructs were transfected into GH_3 rat pituitary cells or C6 rat glial cells. The average CAT activity of each construct is indicated in the table above.

An alternative possibility is that either more than one protein binds to this sequence or a protein with multiple functional domains binds to this sequence. A comparison of the PRE sequence with other known transcription factors has revealed an 100% homology to the 8-nucleotide PEA2 binding site of the polyoma virus enhancer, 5'-GACCGCAG-3' (Veldman *et al.*, 1985; Piette and Yaniv, 1987; Kamachi *et al.*, 1990). A protein, PEBP2 (polyomavirus enhancer-binding protein) has been purified from mouse NIH 3T3 cells which binds to the sequence motif, PEA2 (Kamachi *et al.*, 1990). Interestingly, it is a differentiation-specific factor, detectable by gel-shift assay in fibroblasts (Satake *et al.*, 1988) and retinoic acid-induced F9 cells but not in undifferentiated F9 cells (Furukawa *et al.*, 1990). It also appears to undergo dramatic molecular and functional changes in association with cellular transformation by the Ha-*ras* oncogene. It has been shown to behave as a transcriptional repressor (Wasylyk *et al*, 1988) or as a transcriptional activator (Furukawa *et al.*, 1990) depending on its context. Mutation experiments of the consensus binding sequence show that PEBP2 has similar binding requirements as PREB (Kamachi *et al.*, 1990). Given their similar functional and binding characteristics, it is possible that PREB is PEBP2. Further experiments will be needed to prove this hypothesis.

Summary and Future Directions

We have demonstrated that the rat growth hormone and prolactin promoters are indeed activated by the same transcription factor, pit-1 *in vivo*. Given that a single transcription factor is responsible for the expression of these two differentially expressed genes, we have presented evidence for two *cis*-acting repressor elements located in the growth hormone promoter which may function as part of a molecular switch in the differential regulation of these two genes. We have also characterized two different proteins, PREB and ssPREB, which bind to the growth hormone proximal repressor element. Cell fusion experiments showed that even with a different host cell background, a larger growth hormone promoter, or higher efficiency fusion protocol, the stably transfected growth hormone promoter remained refractory to re-activation.

Additional experiments are needed to clarify the role of pit-1 in the cell-fusion induced-re-activation of a stably transfected prolactin promoter. Is pit-1 alone sufficient to re-activate the prolactin promoter or are there other pituitary cell-specific factors required? In Lufkin and Bancroft (1987), the authors present data from a 5'-deletion experiment which demonstrate the loss of activation on cell fusion with the deletion of sequences corresponding to the "4P" binding site (see Figure 2.2). Does the loss of activation on cell fusion with the deletion of the "4P" binding site reflect a quantitative or a qualitative requirement of the prolactin promoter? Would cloning prolactin sequences in front of a silent growth hormone promoter allow its re-activation in the Lufkin-Bancroft cell-fusion assay? Are there any differences in the activational plasticity of a prolactin promoter construct stably transfected into an undifferentiated cell line, a "differentiated-*in-situ*" cell line (*i.e.* stably transfected into an undifferentiated cell then stimulated to differentiate), and a differentiated cell line? Would a

growth hormone promoter be activated by cell fusion if it were stably transfected into an undifferentiated cell line?

These questions illustrate the complexity of a deceptively simple problem of regulating cell-specific gene expression. The standard paradigm in studying cell-specific gene expression has been to identify cell-specific, double-stranded DNA-binding proteins which activate gene expression when present. This somewhat naïve view, of course, must now be expanded to include factors which negatively regulate cell-specific genes as well. Indeed, repressors of gene expression, both cell-specific and ubiquitous, may play an equal role in the regulation of cell-specific promoters. In addition, we must also look at sequence-specific single-stranded binding proteins as a possible new class of transcriptional regulatory proteins.

Experimental Methods

A. Preparation of nuclear extracts

Cells were grown to subconfluence on monolayer or to 1 x 10^6 cells/ml in suspension. Cells were fed the night prior to harvesting. Cells on monolayer were washed once with 1X PBS and trypsinized. Trypsinization was neutralized by addition of media with fetal calf serum. Cells in suspension were harvested by centrifugation (1000 x g) at 4°C.

Except for the AtT20 cells, nuclear extracts were prepared as described by Dignam *et al.* (1983) (see below). Because of difficulties in growing large numbers of AtT20 cells, extracts of these cells were prepared according to the following protocol (E. Shreiber, personal communication): washed cells were incubated 15 min on ice in Buffer A (10mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM each EDTA, EGTA, dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After addition of Nonidet P-40 to 0.6% and vortexing for 10 seconds, nuclei were pelleted and then extracted (30 min, 4°C) with Buffer A containing 25% glycerol and 0.4 M NaCl.

For other cell lines, the Dignam protocol, with minor modifications, was used to prepare nuclear extracts. Briefly, the procedure is as follows. After pelleting cells, cells were washed once with ice-cold 1X PBS and repelleted by centrifugation (1000 x g) at 4°C. Cells were resuspended in 5 packed cell volumes of ice-cold Buffer A with freshly added DTT (1 mM) and PMSF (50 mg/ml). After 10 minutes on ice, cells were pelleted by centrifugation (1000 x g) at 4°C. The supernatant was carefully aspirated without disturbing the loose cell pellet. Cells were gently resuspended in 1 packed cell volume of ice-cold Buffer A and transferred to an ice-cold dounce with Type B (tight-fitting) pestle. Cell transfer was facilitated with an additional half packed cell volume of ice-cold Buffer A.

Cell membranes were disrupted by ten to twenty strokes of the dounce. Greater than 80% of cell disruption was achieved. Degree of disruption was confirmed by examination under light microscope. Liberated nuclei were pelleted by low-speed centrifugation (1000 x g) for 5 minutes. Supernatant was carefully aspirated and nuclear pellet was subjected to high-speed centrifugation (10,000 x g) for 25 minutes. Isolated nuclei were resuspended in half packed cell volume of ice-cold Buffer C (0.01) with freshly added DTT (1 mM) and PMSF (50 mg/ml). Combined volume of nuclei and Buffer C (0.01) was added to equal volume of ice-cold Buffer C (0.84) with freshly added DTT (1 mM) and PMSF (50 mg/ml). Nuclei were extracted for 30 minutes on ice on a rocking platform.

Nuclear debris was pelleted at 10,000 x g for 30 minutes. Nuclear extract was dialyzed against 200 volumes x 2, of ice-cold Buffer D with freshly added DTT (1 mM) and PMSF (50 mg/ml) for 2 hours x 2. Precipitated material was removed by centrifugation at 10,000 x g. Dialyzed nuclear extract were aliquoted into sterile, ice-cold microfuge tubes and immediately frozen in liquid nitrogen. Protein concentration was assayed by dye binding with bovine serum albumin as standard (Bradford).

B. *In vitro* transcription

Supercoiled DNA templates were diluted to 100 ng/ml. Test DNA composed of 400 ng specific template (containing either the growth hormone or prolactin promoter) and 100 ng reference template (containing either the adenovirus major late promoter or Rous sarcoma virus promoter). Addition of 500 ng of pUC-19 was added to increase transcription efficiency. Total transcription reaction volume of 50 µl consisted of 5 µl 10X IVT Buffer, 100 mM rNTPs, and 30 µl of freshly thawed nuclear extract (protein concentration >3.5 mg/ml). Reactions were carried out at 30°C for 1 hour.

The transcription reactions were stopped with 100 µl STOP I Buffer. Samples were extracted twice with Tris-buffered phenol/chloroform (1:1) and once with ether. RNA was precipitated with the addition of 1 µl of 95% ethanol and centrifugation at 10,000 x g for 30 minutes. Supernatant was carefully aspirated and pellet was resuspended in 10 µl of Hybridization Buffer containing end-labeled CAT primer (50 - 100,000 CPM/ng). Samples were heated to 95°C for 2 minutes, 65°C for 15 minutes and then slowly cooled to room temperature (> one hour). Samples were quickly centrifuged and 25 µl Primer Extension cocktail (containing AMV reverse transcriptase, actinomycin D, dNTPs, ATP, Mg^{2+}, Tris buffer) was added. Primer extension was carried out at 37°C for 45 minutes. Reaction was stopped with STOP II Buffer and extracted once with Tris-buffered phenol/chloroform (1:1). Reaction products were concentrated by ethanol precipitation and analyzed by electrophoresis through an 8M urea, 6% polyacrylamide gel in a Tris-Borate-EDTA buffer. DNA was visualized on X-ray film, exposed at -70°C with an intensifying screen.

C. Gel-shift assay

Oligonucleotides were chemically synthesized by the Mt. Sinai DNA Core Facility. Following synthesis, oligodeoxynucleotides were purified on an 8M urea, 12.5% polyacrylamide gel. Full-length products were then eluted from the gel and further purified by reverse phase column chromatography. Complementary strands were annealed in 40 mM Tris, pH 7.5, 20 mM MgCl2, 50 mM NaCl by heating at 65°C for 15 min and then cooling for 15 min each at 37°C, 25°C, and 4°C. Double-stranded probes were end-filled with [^{32}P]dCTP plus the three other cold dNTPs, employing Sequenase (United States Biochemical) or Klenow, large fragment. Single-stranded probes were end-labeled with [^{32}P]ATP employing T4 polynucleotide kinase (New England Biolabs) (Maniatis *et al.*,

1982). Labeled oligonucleotide was separated from unincorporated label by centrifugation through a 0.75 ml Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) spin-column. Concentration of labeled oligonucleotide was maintained at 1 ng/ml.

Total reaction volume was 25 µl, which consisted of 6.5 mg extracted nuclear protein, 5 µl 5X Gel Shift Buffer, 1 ng labeled probe, 4 mg poly [dI-dC] • poly [dI-dC] (Pharmacia) and stated molar excess concentration of competitor DNA. Binding reaction was incubated at room temperature for a total of 30 minutes. Competitor DNA was added 20 minutes prior to addition of labeled probe. Binding was allowed to continue for an additional 10 minutes. Each sample received 5 µl of 6X agarose gel loading buffer (Maniatis *et al.*, 1982) and was immediately loaded onto a pre-run, pre-cooled, 1.5 mm 0.25X TBE (1X TBE is 89 mM Tris, 89 mM borate, 2.5 mM EDTA), 10% PAGE. The gel was run at 30V/cm at 4°C. DNA bands were visualized on X-ray film, exposed at 70°C with intensifying screen.

D. Transfection of DNA into cells

GH_3 rat pituitary cells were transfected using the DEAE dextran method. GH_3 cells were plated at 2.5 x 10^6 cells/60 mm plate pre-treated with poly-L-lysine. Cells were incubated in DMEM with 10% fetal calf serum and allowed to attach to the plate overnight. Transfected DNA was banded twice in cesium chloride or prepared by PEG precipitation. Greater than 80% of DNA was supercoiled as confirmed by agarose gel electrophoresis. DNA was diluted to <1 mg/ml prior to addition to 400 mg/ml DEAE dextran in DMEM to a final DNA concentration of 10 µg/ml. 0.5 ml DNA-DEAE dextran solution was applied to 1X PBS washed cell monolayers and incubated at 4°C on a rocking platform for 30 minutes. DNA-DEAE dextran solution was aspirated and cell

monolayers were washed twice with 1X PBS. Cells were incubated at 37°C in DMEM with 10% FCS for two days with daily medium changes for cells incubated in serum-containing medium or in serum-free medium (Bancroft *et al.*, 1980) (*i.e.* Figure 3.2).

All other cell lines were transfected using the calcium phosphate method. Cells were plated at a density of 2.5 x 10^6 cells/60 mm plate. Medium with fetal calf serum was changed 2 - 6 hours prior to transfection. DNA was diluted into 0.25 M $CaCl_2$ at a concentration of 20 µg/ml. DNA-calcium solution was slowly dripped into equal volume of bubbling 2X HBS. Samples were incubated at room temperature for 20 minutes. Calcium-phosphate DNA solution was added directly to medium (5 µg/60 mm plate) and incubated at 37°C for 14 - 18 hours. Medium-DNA solution was aspirated and cell monolayers were washed twice with 1X PBS. Cells continued to be incubated at 37°C for a total of 48 hours in medium with fetal calf serum with daily medium changes.

E. Isolation of total RNA

Cells were maintained at sub confluent levels prior to preparation of RNA. Guanidinium isothiocyanate (GuITC) solution was added to cells at a ratio of 3.5 mls/10^8 cells. Genomic DNA was sheared by forcing the gelatinous solution through a 20G needle 15 - 20 times. GuITC-cell mixture was layered on top of a 0.5 ml 5.7 M CsCl cushion in an 5 ml, autoclaved, siliconized, polypropylene ultra-centrifuge tube. Sample was centrifuged at 100,000 x g for 12 - 20 hours at 18°C.

Supernatant was carefully aspirated and the RNA pellet was resuspended in TES Buffer. RNA was precipitated, resuspended in DEPC-ddH_2O and then reprecipitated. RNA was finally resuspended in DEPC-ddH_2O and stored at -70°C.

F. Primer extension

Total RNA was incubated with end-labeled CAT probe at a ratio of 100mg RNA:1 ng probe in a total volume of 50 µl. RNA-probe mixture was incubated at 95°C for two minutes, 65°C for 15 minutes, and allowed to cool to room temperature (> one hour). Samples were quickly centrifuged and 15 µl of Primer Extension cocktail was added. Reactions were carried out at 37°C for 45 minutes. Samples were then phenol/chloroform extracted and ethanol precipitated, prior to loading on an 8M urea, 6% polyacrylamide sequencing gel. Bands were visualized on X-ray film, exposed at -70°C with intensifying screen.

G. Cell-fusion assay

GH_3 cells were plated onto poly-L-lysine (10 mg/ml) pre-treated 60 mm plates at a ratio of 2:1 (GH_3 cells:stable transfectants). GH_3 cells were allowed to attach at least 2-3 hours prior to seeding of stable transfectants. Cells were incubated in DMEM (GIBCO) with 10% fetal calf serum overnight. Cells were fused by addition of PEG Solution (polyethylene glycol 1000 (Baker) pretreated as described in Yoakum *et al.*, 1984) to 1X PBS washed cell monolayers. Total contact time with PEG Solution was limited to two minutes, followed by aspiration of PEG Solution and three washes with 1X PBS. Cells were incubated at 37°C in DMEM with 10% fetal calf serum for two days with daily changes of medium. Evidence of successful fusion was obtained by direct microscopic visualization prior to harvesting of the cells. Greater than 50% of all cells per high powered field in three randomly selected, noncontiguous areas, contained at least two nuclei. In addition, parallel control cell-fusion experiments using a stably transfected rat prolactin-CAT construct in C127 cells were used to

demonstrate the ability of CAT activity to be detected after processing of the cell extracts.

H. CAT assay

Medium was aspirated from cells and monolayers were washed once with 1X PBS. Cells were scraped in 0.75 ml 1X PBS and transferred with an additional 0.75 ml 1X PBS to a microfuge tube. Cells were pelleted by 15 second centrifugation in a microfuge. Supernatant was aspirated off and pellet was resuspended in 100 µl CAT Buffer. Cells were disrupted by sonication, two cycles of 4 minutes, on ice. Extracts were incubated at 65°C for 5 minutes. Cellular debris was pelleted by centrifugation and supernatants were transferred to fresh microfuge tubes.

50 µl of each extract were added to microfuge tubes containing 5 µl 50 mM acetyl CoA and 50 mCi ^{14}C-chloramphenicol. Samples were incubated at 37°C for 2-12 hours. Reaction products were extracted with 200 µl ethyl acetate. Extracted product was dried down, resuspended in 20 µl of ethyl acetate containing acetylated (cold) chloramphenicol markers, and applied to onto mylar-backed silica gel TLC plate containing fluor. Chromatography was carried out in an equilibrated tank containing chloroform:methanol (95:5) until the solvent line reached the top edge of the TLC plate. The plate was dried and then either exposed to X-rat film at -70°C, or acetylated products were visualized under short-wave UV light and excised and counted in a scintillation counter.

I. Sources of promoter-CAT constructs

A map of the parental plasmid pGH-CAT ((-550)GH-CAT) has been published previously (Lufkin and Bancroft, 1987). Details of its construction are as follows. A rat genomic bacteriophage library (Sargent *et al.*, 1979) was used to

isolate a rat GH genomic clone (D. Gerhard and C. Bancroft, unpublished observations). A sequence containing approximately 550 base pairs of GH flanking sequence plus 13 base pairs of the first GH exon was removed from this clone by *Hae*II-*Xho*I cleavage, blunt-ended, *Hind*III linkers added, and subjected to *Hind*III-*Eco*RI digestion. This GH gene sequence was then cloned, upstream of a 1.6-kilobase pair *Hind*III-*BamH*I fragment isolated from pRSV-CAT and containing the bacterial CAT gene plus SV40 splicing and adenylation signals (Gorman *et al.*, 1982), into plasmid pUC19. 5' deletion mutants of pGH-CAT were prepared by *Bal*-31 deletion essentially as described previously for 5' deletion mutants of pPRL-CAT (Lufkin *et al.*, 1989); hence all *Bal*-31 deletions share the same upstream flanking vector sequences. Site-directed mutagenesis of GH-CAT constructs was performed by synthesizing the non-coding oligodeoxynucleotide 5'-ACTGCTCTAAGTAGGTCACGTC-3' (changes from wild-type rGH sequences centered at position -163 are underlined) and then employing a MUTA-GENE kit from BioRad, essentially according to the instructions provided with the kit. Both the deletion end points of the 5' deletion mutants and the DNA sequences resulting from internal site-directed mutagenesis were determined previously by dideoxy sequencing (Chen and Seeburg, 1985).

The sources of plasmids pRSV-CAT and pTK-CAT have been described previously (Jackson and Bancroft, 1988).

J. Sources and culture of cell lines

The following cell lines were employed: GH_3 and GC (rat pituitary) (Bancroft, 1981), 235-1 (rat pituitary) (Reymond *et al.*, 1984), AtT20/D16v (mouse pituitary) (Gumbiner and Kelly, 1982), C6 (rat glial) (Benda *et al.*, 1969), C127 (mouse mammary tumor) (Lowy *et al.*, 1978), LLC-WRC (rat carcinoma) (ATCC

Catalogue, 1988), HeLa (human epitheloid carcinoma) (Gey *et al.*, 1952), HepG2 (human hepatoma) (Aden *et al.*, 1979), and JEG-3 (human choriocarcinoma) (Kohler *et al.*, 1971). GH_3, GC, and HeLa cells were propagated in spinner culture in Joklik's modified Eagle's medium plus 12.5% horse serum, 2.5% fetal calf serum (FCS). All other cells were propagated in monolayer culture in Dulbecco's modified Eagle's medium plus 5% FCS, except for JEG-3, 235-1, and HepG2 cells, for which 10% FCS was employed. For HepG2 cells, dishes were pretreated with poly-L-lysine as described (Jackson and Bancroft, 1988) to promote cell attachment.

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