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Transcription of the rat prolactin gene: Regulation by dopamine and the pituitary-specific protein Pit-1

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TRANSCRIPTION OF THE RAT PROLACTIN GENE: REGULATION BY DOPAMINE AND THE PITUITARY-SPECIFIC PROTEIN PIT-1

by

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Abstract

TRANSCRIPTION OF THE RAT PROLACTIN GENE: REGULATION BY DOPAMINE AND THE PITUITARY-SPECIFIC PROTEIN PIT-1

by

Daniel J. Fischberg

Adviser: Professor Carter Bancroft

While dopamine is known to be the major tonic regulator of prolactin synthesis and secretion, little is known of the mechanism by which dopamine inhibits expression of the prolactin gene. A difficulty in studying dopaminergic repression of the prolactin gene has been the lack of an appropriate model system. Cloning of the D_2 dopamine receptor has allowed the expression of this receptor in rat lactotrope cell lines which do not express the endogenous receptor such as the GH_3 cell line. This system was used to address the mechanism by which dopamine represses transcription of the rat prolactin (rPRL) gene.

The ability of either isoform of the D_2 receptor to mediate repression of the rPRL promoter was first confirmed using expression vectors containing cDNA's for the D_{2A} and D_{2B} receptors. It was then demonstrated that dopaminergic repression of the rPRL promoter in this system is mediated by one or more pertussis toxin-sensitive G proteins and, furthermore, that the proximal 187 base pairs of the prolactin promoter confer a full dopaminergic response. Over-expression of the catalytic subunit of the cAMP-dependent protein kinase (PKA) caused a nearly complete attenuation of dopaminergic repression, suggesting a possible role for PKA in mediating dopaminergic repression of the rPRL promoter.

The implication of both Pit-1 and PKA in the dopaminergic repression of the rPRL promoter led to studies described in Chapter 3 of the functional role of phosphorylation of Pit-1. The abilities of the wild type Pit-1 and a phosphorylation-deficient mutant to activate the rat prolactin and growth hormone (rGH) promoters were compared. The results of these experiments suggest that phosphorylation plays a modulatory role in regulating the activity of Pit-1 upon both the rPRL and rGH promoters. The magnitude of this modulatory role on the rPRL promoter is equivalent with the magnitude of dopaminergic repression of the rPRL promoter, consistent with the model that dopaminergic repression is mediated via inhibition of PKA-dependent phosphorylation of Pit-1.

In Chapter 4, the results of functional studies of isoforms of the pituitary-specific transcription factor Pit-1 are presented. While both Pit-1 and Pit-1a are capable of transactivating the rPRL and rGH promoters, Δ4Pit-1, by itself, activates neither promoter. Additionally, the function of Δ4Pit-1 as a repressor of the rPRL promoter has been confirmed in HeLa cells. Finally, preliminary evidence suggests that Δ4Pit-1 may exert a permissive role in allowing expression of the rGH gene in somatomammotropes.

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Finally, I would like to express my deepest gratitude to my wife, Barbara, for her support throughout this project. Her critical and technical contributions have been major. However, it was her extensive personal sacrifices that made the completion of this project possible. To Barbara, and my son, Benjamin, whose cooperative nature is gratefully acknowledged, I dedicate this work.

Table of contents

List of tables

List of figures

I. Chapter 1: Introduction

A. Prolactin as a model for studies of gene expression

Prolactin is a protein hormone synthesized in the anterior pituitary of all vertebrate animals. Prolactin functions in growth, reproduction and, in mammals, lactation. The synthesis and release of prolactin is subject to both positive and negative regulation by factors originating outside of the anterior pituitary. The goal of this project has been to gain a better understanding of the mechanism of transcriptional regulation of the rat prolactin gene.

Prolactin serves as an excellent model for studies of gene expression. The usefulness of the prolactin gene as a model for the study of gene expression is two-fold. First, it is expressed in a tightly regulated, cell-type-specific pattern. The prolactin gene is expressed only in two cell types of the anterior pituitary (mammotropes and somatomammotropes) and is silent in almost every other cell of the body (Davis, 1990). Second, expression of the prolactin gene by mammotropes and somatomammotropes is subject to regulation by a large number of hormones, growth factors, and neurotransmitters including: thyrotropin releasing hormone (TRH), epidermal growth factor (EGF), estrogen, insulin, somatostatin, and dopamine (Hinkle, 1988).

B. Cell-type-specific expression of prolactin

The anterior pituitary is the source of at least five different protein hormones each of which is expressed in a cell-type-specific manner. Corticotropes produce adrenocorticotropin; thyrotropes produce thyroid-stimulating hormone; gonadotropes produce luteinizing hormone and follicle-stimulating hormone; somatotropes produce growth hormone, and lactotropes produce prolactin. The cell-type-specific expression of the genes encoding these hormones has been the subject of extensive study. In 1988, two groups independently cloned a pituitary-specific transcription factor that they named Pit-1

(Ingraham et al., 1988) or GHF-1 (Bodner et al., 1988). While the discoverers of Pit-1 demonstrated a role for Pit-1 in transcriptional activation of both the prolactin and growth hormone genes, the discoverers of GHF-1 claimed that its transcriptional activity was restricted to the growth hormone gene. The cDNA clones isolated by the two groups were identical and subsequent studies have confirmed the role of Pit-1/GHF-1 (referred to hence as Pit-1) in transcriptional activation of both the prolactin and growth hormone genes (Mangalam et al., 1989; Fox et al., 1990; Simmons et al., 1990). Pit-1 expression is required for the development of somatotropes, lactotropes, and thyrotropes in mice (Li et al., 1990; Castrillo et al., 1991), and artificial expression of Pit-1 in nonpituitary cell lines is sufficient to activate transcription of the prolactin and growth hormone genes (Ingraham et al., 1988; Mangalam et al., 1989; Fox et al., 1990).

Pit-1 has been classified as a member of the POU domain family of transcription factors based upon its deduced amino acid sequence (Herr et al., 1988). The POU domain is a region of amino acid sequence homology found in a subset of homeo domain transcription factors. The POU domain name is an acronym for Pit-1, Oct-1/Oct-2, and *unc-86,* the first four identified members of the family. The POU domain contains both a homeo domain (60 amino acids) and a POU-specific domain (80 amino acids in the case of Pit-1). The homeo domain has been well characterized as a highly conserved DNA binding domain originally identified in Drosophila homeotic gene products (McGinnis et al., 1984; Scott and Weiner, 1984). The POU-specific domain of Pit-1 is required for sequence-specific high affinity DNA-binding as well as protein-protein interactions (Ingraham et al., 1990).

The prolactin gene contains two regions 5' to its transcription start site that are required for high levels of expression: a proximal promoter and a distal enhancer. The promoter extends approximately 200 base pairs 5' to the transcription start site. The enhancer is found in the region between 1500 and 1800 base pairs 5' to the transcription start site (see Figure 1). Each of these regions contains four binding sites for Pit-1

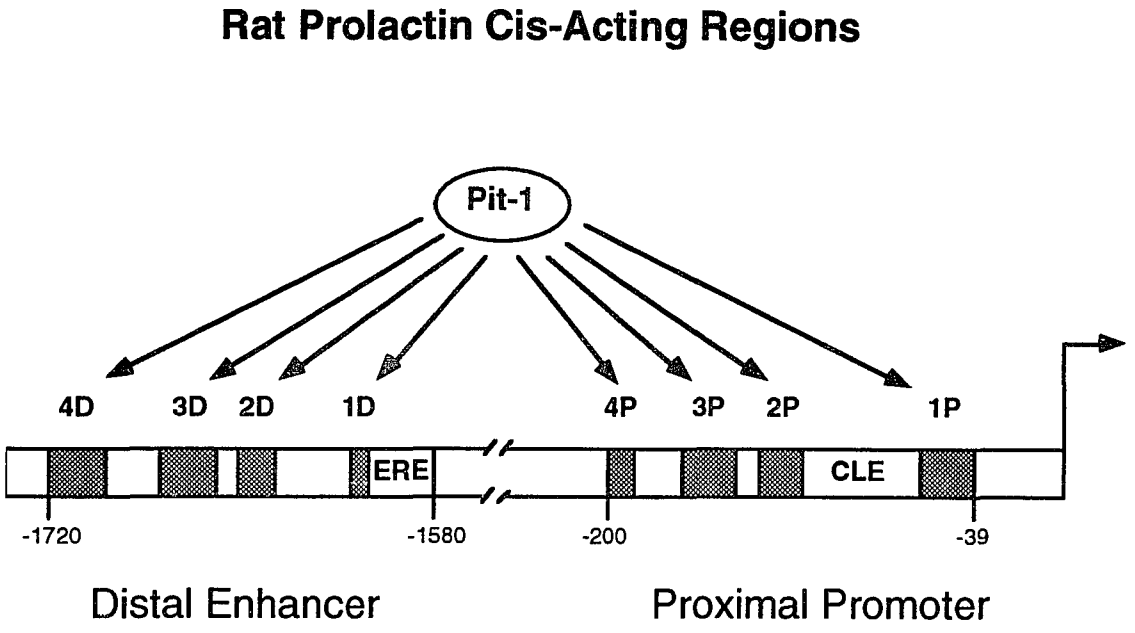


Figure 1: Schematic representation of known elements in prolactin gene 5'-flanking DNA. Shown are the eight Pit-1 binding sites of the promoter and enhancer regions. Also shown are an estrogen response element (ERE) from -1578 to -1550, and a cAMP response element-like element (CLE) from -99 to -92. Not shown, for clarity, is a TATAA box from -28 to -24, This figure is based on similiar figures by Nelson et. al. (1988) and Lufkin et. al. (1989).

(Mangalam et al., 1989). The sites in the distal enhancer are referred to as sites 1D-4D and those in the proximal promoter are 1P-4P, with the lower numbered elements lying more proximal to the transcription start site. While the enhancer region is required for high levels of prolactin expression, the proximal portion of the promoter is sufficient to confer cell-type-specific expression (Lufkin et al., 1989).

C. Hypothalamic regulation of anterior pituitary function

The hypothalamus plays a major role in regulating the activity of the anterior pituitary. This control is exerted not through direct innervation but via humoral factors that are delivered to the anterior pituitary via a portal blood system. The blood supply to the anterior pituitary is supplied by the hypophysial arteries via the long and short hypophysial portal veins. These portal vessels originate in the median eminence and posterior pituitary respectively. In addition to providing a blood supply, these vessels serve as the means by which the brain communicates with the anterior pituitary.

Synthesis and secretion of all of the protein hormones of the anterior pituitary are under the influence of factors released from the hypothalamus and carried to the anterior pituitary via the portal vessels. In the case of prolactin, while several compounds found in portal blood are capable of regulating prolactin synthesis, by far the most important of these compounds is the neurohormone dopamine. Dopamine, originating from perikarya of the arcuate and periventricular nuclei of the hypothalamus (Ben-Jonathan et al., 1989) and conveyed via the portal vessels to the lactotropes of the anterior pituitary, inhibits both the synthesis and release of prolactin. Dopaminergic inhibition of prolactin synthesis is discussed more fully in Chapter 1. Here, induction of prolactin synthesis by other factors from portal blood will be briefly reviewed.

D. Inducers of prolactin synthesis

As mentioned above, a number of factors isolated from portal blood have been found to induce prolactin synthesis. Of these factors, the most thoroughly studied transcriptional activators are estrogen and thyrotropin-releasing hormone (TRH). The distal enhancer of the prolactin gene contains an estrogen response element. Estrogenic induction of prolactin transcription results from binding of the hormone-receptor complex to this element (Maurer and Notides, 1987; Waterman et al., 1988). In addition, estrogenic induction of prolactin requires Pit-1 (Day et al., 1990). While the nature of the requirement for Pit-1 in this induction is unknown, both Pit-1 and Pit-1 binding sites are required (Day et al., 1990).

Induction of prolactin synthesis by TRH was discovered in rat pituitary cell lines (Dannies and Tashjian, 1973) . Murdoch et. al. (1983) demonstrated that the induction of prolactin synthesis in GH_4 cells is mediated at least in part by an increase in the rate of prolactin gene transcription. This induction of transcription begins within minutes of exposure of GH_4 cells to TRH. Yan et. al. (1991) have implicated Pit-1 as the gene-proximal mediator of TRH induction. It has been also been suggested that calcium, another inducer of prolactin transcription (White et al., 1981; Jackson and Bancroft, 1988), may mediate the TRH stimulation of prolactin gene transcription (White and Bancroft, 1983; Day and Maurer, 1990). Most recently, Yan and Bancroft (Yan and Bancroft, 1991) have shown that prolactin promoter site 1P is both a calcium response element and a TRH response element, and that induction by either of these compounds is most likely mediated through Pit-1 interactions with Pit-1 binding sites upstream of the prolactin gene.

In addition to calcium, another intracellular mediator that induces prolactin transcription is adenosine 3',5'-monophosphate (cAMP). Maurer first demonstrated cAMP induction of rPRL transcription in cultured rat pituitary cells (Maurer, 1981). Responsiveness to cAMP has been mapped to both the proximal and distal enhancers of

the rat prolactin promoter (Day and Maurer, 1989). At least two elements have been demonstrated, by promoter mutation studies, to be capable of conferring responsiveness to cAMP upon the rPRL promoter (Iverson et al., 1990; Peers et al., 1991). The first of these elements is actually a group of elements, the Pit-1 binding sites of the proximal promoter, demonstrating that as with TRH and calcium, the cell type-specific promoter elements can also serve as hormone response elements.

A second type of cAMP response element also lies within the proximal promoter region, extending from -97 to -84 bp upstream of the transcription start site (d'Emden et al., 1992). This region contains an element that is similar to the classical cAMP response element (CRE) first described in the rat somatostatin gene (Montminy et al., 1986). While it is becoming clear that this prolactin CRE-like element (CLE) plays a role in mediating cAMP responsiveness of the prolactin gene, it is unclear what factor binds functionally to the CLE in pituitary cells. CREB is capable of binding to the CLE, although with an affinity that is lower than its affinity for a classical CRE (Liang et al., 1992). Most likely, a ubiquitous factor, perhaps a member of the CREB/ATF family, binds to the CLE and, along with the pituitary specific factor Pit-1, is responsible for mediating transcriptional induction of the prolactin gene by cAMP (Peers et al., 1991).

II Chapter 2: Dopaminergic Repression of the Rat Prolactin Promoter

A. Summary

While dopamine is acknowledged as the major tonic regulator of prolactin synthesis and secretion, little is known of the mechanism by which dopamine inhibits expression of the prolactin gene. A difficulty in studying dopaminergic repression of the prolactin gene has been the lack of an appropriate model system. Cloning of the D_2 dopamine receptor has allowed the expression of these receptors in rat lactotrope cells which do not express the endogenous receptor such as the GH_3 cell line. I have used this system to address the mechanism by which dopamine represses transcription of the rat prolactin (rPRL) gene.

The ability of either isoform of the D_2 receptor to mediate repression of the rPRL promoter has been confirmed. It was found that dopaminergic repression of the rPRL promoter in this system is mediated by one or more pertussis toxin-sensitive G proteins. Deletion mapping experiments were used to demonstrate that the proximal 187 base pairs of the prolactin promoter confer a full dopaminergic response, suggesting the possible involvement of one or more of the binding sites for the pituitary-specific transcription factor Pit-1.

Studies of intracellular effectors were performed to attempt to assess their role in the pathway of dopaminergic repression of the rPRL promoter. The induction of the rPRL promoter by two agents that raise intracellular free calcium, TRH and BayK8644, is completely attenuated by dopaminergic stimulation. In addition, over-expression of the catalytic subunit of the cAMP-dependent protein kinase (PKA) causes a nearly complete attenuation of dopaminergic repression, suggesting a possible role for PKA in mediating dopaminergic repression of the rPRL promoter. The possiblity that phosphorylation/dephosphorylation of Pit-1 is involved in the dopamine pathway is discussed and explored further in Chapter 3.

B. Introduction

1. Significance

A fundamental property of all eukaryotic cells is the ability to sense and respond to environmental stimuli. In many cases, these stimuli are soluble factors that are first recognized by the cell via specific cell surface receptors. Activation of these receptors by a ligand must be coupled to an effector system within the cell if the stimulus of ligand binding is to achieve a physiological response. In many cases, the response of a cell to an extracellular stimulus will ultimately involve modulation of gene expression. Signal transduction from a cell surface receptor to a gene is therefore required for response by the cell to many extracellular stimuli. However, with few exceptions, the details of the pathways involved in signal transduction from cell surface receptors to genes are poorly understood. The goal of this project has been to gain a better understanding of the signal transduction pathway from a cell surface receptor (the D_2 dopamine receptor) to a gene (the rat prolactin gene).

As described in Chapter 1, recent studies have shed a great deal of light on the regulatory mechanisms underlying both cell-type-specific expression (Nelson et al., 1988; Keech and Gutierrez-Hartmann, 1989; Lufkin et al., 1989; Supowit et al., 1992) and hormonal activation of the rat prolactin gene (Ramsdell and Tashjian, 1985; Seyfred and Gorski, 1990; Peers et al., 1991; Yan et al., 1991; Liang et al., 1992). However, while the major regulation of prolactin synthesis involves inhibition by dopamine (Ben-Jonathan, 1985), little advance has been made in understanding the mode of this inhibition.

Even beyond its role as a model for studies of transcriptional repression, dopaminergic inhibition of prolactin synthesis is a subject of immediate clinical interest in medicine. Hyperprolactinemia due to a prolactin secreting pituitary adenoma is a significant cause of infertility in women and impotence in men. In addition to the endocrine sequelae of these tumors, their uncontrolled growth can, in some cases, lead to

mass effects and subsequent neurological sequelae. While treatment of aggressive tumors often requires microsurgery, many of these tumors respond to treatment with the dopamine agonist bromocriptine. Tumors may respond with reduced secretion and even cessation of growth. There is a clear need to improve our understanding of the mechanism by which dopaminergic activation can suppress these neoplasms. One step toward this greater understanding will be to map the pathway by which dopamine inhibits prolactin gene transcription.

2. The D_2 dopamine receptor

Kebabian and Calne (1979) first postulated the existence of multiple receptor subtypes for dopamine. Their classification of two subtypes, D-1 and D-2, was based upon observed differences in anatomical distribution, pharmacological profile and effects on adenylyl cyclase activity. More recently, this classification has yielded a further division of subtypes, now numbering six, based on the cloning of the human and rat genes for the individual receptors. The receptors are denoted D_1, D_{2A}, D_{2B}, D_3, D_4 and D_5.

All six cloned dopamine receptor subtypes belong to the superfamily of G protein-coupled receptors. The members of this superfamily transduce their physiological effects through interactions with guanine nucleotide binding proteins (G proteins). Structurally, members of the superfamily are characterized by the presence of seven hydrophobic regions which are putative membrane-spanning domains.

The dopamine receptors expressed by the lactotropes of the anterior pituitary appear to be the two isoforms of the D_2 receptor subtype, D_{2A} and D_{2B}. This conclusion is based on *in situ* hybridization data (Dal Toso et al., 1989; Monsma et al., 1989b; Sokoloff et al., 1990; Sunahara et al., 1991) as well as affinity purification studies (Strange, 1992), although the D_{2A} receptors outnumber the D_{2B} receptors by at least 5:1

(Dal Toso et al., 1989; McChesney et al., 1991). These two isoforms are thus the logical candidates to use in my attempt to reconstitute dopaminergic repression.

Classically, receptors of the D_2 subtype are linked to inhibition of the enzyme adenylyl cyclase (Frey et al., 1982; Enjalbert and Bockaert, 1983). More recently, D_2 receptor activation has been shown to hyperpolarize rat lactotropes through an increase in K^+ conductance (Israel et al., 1987). In addition, D_2 receptor activation has been shown to decrease cytosolic free calcium concentration (Malgaroli et al., 1987) and inhibit the activation of voltage-gated calcium channels (Lledo et al., 1990). However, the effect of dopamine on intracellular calcium concentration may not be mediated entirely by the alteration in voltage-gated calcium channel activity, since it has been proposed that D_2 receptor stimulation is coupled to the inhibition of phosphatidylinositol turnover (Canonico et al., 1983). It has also been argued (Vallar and Meldolesi, 1989), however, that this inhibition is indirect and secondary to the inhibition of calcium influx. Most recently, the D_2 receptor has been shown to be coupled to a phosphotyrosine phosphatase activity in the GH_4C_1 pituitary tumor cell line (Florio et al., 1992). All of these signal transduction pathways are thus candidates for the pathway that mediates dopaminergic repression from the cell surface to the nucleus.

3. Dopaminergic repression of prolactin synthesis

There is convincing evidence that dopamine, acting through the D_2 receptor, is the major tonic regulator of prolactin synthesis and release. Removal of the rat pituitary from the influence of the hypothalamus by transplantation, hypothalamic lesion (Chen et al., 1970) or pituitary stalk transection (Ben-Jonathan et al., 1989) leads to elevated prolactin serum levels, suggesting that prolactin levels are under tonic inhibition by some hypothalamic inhibitory factor. It has been concluded that this prolactin inhibitory factor (PIF) is dopamine, based upon the following observations. Dopamine is secreted into rat portal blood (Ben-Jonathan et al., 1977). Cyclical changes in the concentration of

dopamine in the portal blood of female rats are paralleled by reciprocal changes in the concentration of prolactin in systemic blood (Ben-Jonathan, 1985). Dopamine D_2 receptors have been characterized on anterior pituicytes of a number of species (Caron et al., 1983). Dopamine specifically inhibits prolactin release by rat anterior pituitary cells *in vitro* (Caron et al., 1978) and administration of dopamine D_2-selective receptor antagonists to rats specifically elevates prolactin synthesis *in vivo* (Maurer and Gorski, 1977).

Dopaminergic inhibition of prolactin production has been shown to occur on at least two levels. Dopamine agonists induce degradation of prolactin stores in cultured pituitary cells (Dannies and Rudnick, 1980; Maurer, 1980). More relevant to this project, dopamine agonists have been shown to inhibit transcription of prolactin in cultured rat pituitary cells (Maurer, 1981).

4. Mechanisms of dopaminergic repression of prolactin transcription

Since the demonstration of dopaminergic repression of prolactin transcription by Maurer, very little progress has been made in identifying the pathway of this repression. This has been largely due to the lack of a suitable system for studying the mechanism of repression, that is, a homogeneous lactotrope cultured cell line that expresses functional D_2 receptors. While a number of lactotrope cell lines exist, none of these expresses functional D_2 receptors. The number of cells needed for such studies renders the use of primary pituitary cultures impractical. In addition, primary pituitary cultures represent too heterogeneous a population of cells to be useful for such studies.

However, the cloning of the D_2 dopamine receptor in 1988 (Bunzow et al., 1988) and a second, longer, isoform in 1989 (Giros et al., 1989; Monsma et al., 1989b) has allowed the transfection of these two receptor subtypes (D_{2B} and D_{2A} respectively) into a lactotrope cell line in order to reconstitute the pathway of dopaminergic repression of prolactin transcription. Two groups have used this approach to successfully reconstitute

dopaminergic inhibition of prolactin transcription. McChesney et. al. (1991) have shown that either isoform of the D_2 receptor can mediate dopaminergic repression of prolactin transcription when transfected into the lactotrophic GH_3 cell line. GH_3 cells were cotransfected with both a D_2 expression vector (RSV-D_{2L} or RSV-D_{2S}) and a PRL-CAT reporter construct that contains the coding region of the chloramphenicol acetyltransferase (CAT) gene under the control of the proximal 1957 base pairs of prolactin gene 5'-flanking DNA. When these transiently transfected cells were assayed for CAT activity, a 4- to 5-fold decrease in activity was seen in the presence of D_2-selective agonists. The repression of transcription was specific to the prolactin reporter construct, required the cotransfected D_2 expression vector, and was blocked by the D_2-selective antagonist spiperone (McChesney et al., 1991).

During the course of the studies descibed in this thesis, Elsholtz et. al. (1991) using an expression vector of the short isoform, D_{2B}, stably transfected into GH_4C_1 cells, reported that sequences within the proximal region of the rat prolactin gene can confer dopaminergic repression of prolactin transcription. Additionally, this group has suggested that dopaminergic repression may be mediated by two distinct signaling pathways: inhibition of adenylyl cyclase and membrane hyperpolarization. Dopaminergic repression of transcription was blocked under conditions that prevented either dopamine-dependent reductions in cAMP or dopamine-dependent hyperpolarization. Most recently, this group has shown that a concatamer of Pit-1 binding sites is capable of transfering dopaminergic repression to a heterologous promoter (Lew et al., 1994). While the work of this group closely parallels that presented here, their results do not resolve the identity of the *cis*-active elements, the *trans*-acting factors, or the intracellular mediators involved in mediating dopaminergic repression of prolactin gene transcription.

C. Materials and Methods

1. Sources and/or construction of recombinant plasmids

The RSV-D_{2A} and RSV-D_{2B} expression vectors were constructed essentially as described (McChesney et al., 1991) except that polymerase chain reaction amplification of rat brain mRNA (performed by Dr. S.C. Sealfon, Mount Sinai School of Medicine, New York, NY) was carried out using Vent™ polymerase (Promega). The amplified products were digested with Pst I/Kpn I and cloned into the Rous sarcoma virus expression vector RSVi$^-$ (Forman et al., 1988). The cloned fragments were sequenced and found to be in complete agreement with the published sequence of both isoforms of the D_2 receptor (Monsma et al., 1989b).

The source of the expression vectors pRSV-neo and RSV-Pit-1 as well as the reporter constructs (-1957)PRL-CAT (referred to here as PRL-CAT), (-204)PRL-CAT, (-187)PRL-CAT, (-113)PRL-CAT, (-75)PRL-CAT, $(1P)^2$(-39)PRL-CATEnh, and RSV-CAT have all been described previously (Lufkin and Bancroft, 1987; Yan et al., 1991; Yan and Bancroft, 1991; Yan et al., 1994). The expression vector pSV_2-CREB-VP16, which contains a DNA fragment encoding the N-terminal 341 amino acids of CREB fused in-frame to the VP16 activating domain, was obtained from Drs. F. Liu, F. Lemaigre, and M.R. Green (University of Massachusetts, Worcester, MA) and has been previously described (Yan et al., 1994). The hamster dihydrofolate reductase minigene pDCHIP was obtained from Dr. L.A. Chasin (Columbia University, New York, NY) and has been described previously (Ciudad et al., 1988). The Rous sarcoma virus expression vector pRcRSV was obtained from Stratagene. The expression vector pCMV-$G_{\alpha i2}$ was constructed by subcloning the mouse $G_{\alpha i2}$ subunit cDNA (Simonds et al., 1989) into the Stratagene RcCMV expression vector (J. Tian and C. Bancroft, unpublished data). The mMT-PKA expression vector has been described previously (Uhler and McKnight, 1987).

2. Culture and transfection of cells

GH_3 cells were grown in suspension culture at 37° C. in Joklik-modified minimal essential medium supplemented with 25 mM $NaHCO_3$, 10 mM Hepes pH 7.4, 15% horse serum, 2.5% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. For most transfections, lipofectin (GibcoBRL) was used as follows. GH_3 cells were switched to monolayer culture at 1.5 x 10^6 cells/60mm plate one day prior to tranfection. DNA for tranfection was brought to 1.5 ml with Optimem (GibcoBRL) and combined with 20 μg of lipofectin in 1.5 ml Optimem. The 3 ml DNA/lipofectin mixture was allowed to sit at room temperature for 15 minutes. GH_3 cells were rinsed once with phosphate-buffered saline and the DNA/lipofectin mixture was added to each plate. Cells were incubated with the transfection solution for six hours at 37° C. after which the transfection solution was aspirated and the cells were fed with Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (DMEM/5% FCS) and drugs or vehicle as indicated.

Transfection via electroporation, where indicated, was performed as follows. Approximately 1-2 x 10^7 cells were resuspended in 0.5 ml of DMEM/5% FCS and electroporated at the indicated capacitance and voltage in a Gene Pulser™ cuvette (Bio-Rad) with a 0.4 cm pathlength in a Gene Pulser (Bio-Rad). Immediatedly after transfection, the cells were diluted into 30 ml of DMEM/5% FCS ± quinpirole and evenly distributed over six 60mm tissue culture dishes.

Transfection via the diethylaminoethyl-dextran (DEAE-dextran) technique and hormonal induction with TRH, where indicated, were both performed as described (Yan and Bancroft, 1991).

Transfection of C6 glioma cells was performed via calcium phosphate precipitation according to the basic protocol of Kingston (1990).

3. Measurement of CAT activity

Except where otherwise indicated, cells were harvested two days following transfection and cellular extracts were prepared and assayed for CAT activity as described (Jackson and Bancroft, 1988). During the course of this work, a phase extraction assay was adopted, where indicated, due to its rapidity and decreased expense. It was determined that equivalent results are obtained by either assay method. The phase extraction assay was performed essentially as described by Seed and Sheen (1988) but with the omission of unlabeled chloramphenicol in order to maximize specific activity. In this assay, CAT activity is measured not as % conversion of [^{14}C]-chloramphenicol to acetylated products but as the rate of conversion of [^{3}H]-chloramphenicol to acylated products. Phase extraction assays were performed for 1 hour at 37° C., under predetermined linear conditions.

4. Construction of cell lines stably transfected with RSV-D_{2A}

GH_3 cells were transfected via lipofectin as described above with 10 μg RSV-D_{2A}, 10 μg of pDCHIP, and 1 μg of pRSV-neo. One day following transfection, cells were treated with 800 μg/ml of the neomycin analogue G418 (GibcoBRL). Cells were maintained in G418 to promote selection of stable transfectants. After two weeks, 18 colonies were selected for expansion. Ten clones were successfully expanded and assayed for specific binding of [^{3}H]-spiperone as described below. Two clones, GH_3D_2-1 and GH_3D_2-10 were selected for further expansion based upon the results of [^{3}H]-spiperone binding assays (estimated [^{3}H]-spiperone binding sites of 600 and 400 fmol/mg protein, respectively). I attempted to make use of the cotransfected DHFR minigene (pDCHIP) to amplify the copy number of the stably transfected RSV-D_{2A} construct. To this end, GH_3D_2-1 and GH_3D_2-10 were exposed to increasing concentrations of methotrexate (Schimke et al., 1987). However, after achieving resistance to levels of

methotrexate above 1 μM, no significant difference in the level of D_{2A} expression was detected in either clonal line.

5. Meaurement of specific binding of [^{3}H]-spiperone to cellular membranes

Membrane homogenates were prepared from GH_3D_2-1 and GH_3D_2-10 cells or striatum from male (150-250 g) Sprague-Dawley rats and assayed for specific binding of [^{3}H]-spiperone as described (Neve et al., 1989). Briefly, 50-100 μg of protein from a crude membrane homogenate was incubated in the presence of a single, theoretically saturating, concentration of [^{3}H]-spiperone (500 pM). Because of the scarcity of the stably transfected cells, it was not practical to perform full Scatchard analyses. The K_d of [^{3}H]-spiperone for the D_2 receptor is approximately 40 pM (Bunzow et al., 1988). Therefore, assuming a similar K_d under these conditions, the concentration of [^{3}H]-spiperone used in these assays would be expected to label in excess of 90% of the D_2 receptors present. Specific binding was defined using 2 μM (+)butaclamol. The labeled membranes were collected via vacuum filtration through Millipore GF/C filters. Filters were counted in a Beckman LS1801 scintillation counter using Ecolite(+) scintillation fluid (ICN). Homogenates were assayed for protein using the Bio-Rad protein assay. My estimated specific binding of [^{3}H]-spiperone to a homogenate of rat striatum (500 fmol/mg protein) compares with a previously reported estimate of 547 fmol/mg protein (Bunzow et al., 1988) suggesting a reasonable accuracy for these estimates of D_2 receptor number.

6. Nuclear run-off transcription assays

Nuclear run-off transcription assays were performed according to the method of Greenberg (1990). Briefly, nuclei were isolated from approximately 5 x 10^7 GH_3 or GH_3D_2-10 cells using the dounce homogenization protocol of Bender (1990). Nascent transcripts were allowed to run off in the presence of [α^{32}P]-uridine 5'-triphosphate. The

labeled run-off products were hybridized to nitrocellulose-bound cDNA's for Pit-1 and D_{2A} receptor. The cDNA's for hybridization were obtained by linearization, with Hind III, of the plasmids RSV-Pit-1 and RSV-D_{2A} described above. The blots were exposed overnight and imaged using Image-Quant 3.3 on a Molecular Dynamics Phosphor-Imager.

7. Assay of cellular cAMP levels

GH_3D_2-1 cells were assayed for levels of intracellular cAMP as follows. Monolayers of cells were seeded at 6 x 10^4 cells/well on 24 well plates one day prior to assay. Cells were washed twice with Hank's buffered salt solution (GibcoBRL) that contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH = 7.55 at room temperature (HBSS). Cells were preincubated for 15 minutes at 37° C. in HBSS plus 100 μM of the phosphodiesterase inhibitor rolipram. Forskolin (1 μM final concentration) and quinpirole (200 nM final concentration) were added for a final volume of 500 μl, and the cells were incubated for 10 minutes at 37° C. The incubation was terminated by aspiration of the medium and addition of 500 μl of ice-cold absolute ethanol to each well. After a 30 minute extraction, the supernatants from the wells were transferred to tubes, and the wells were rinsed gently with an additional 250 μl of ethanol which was added to the first extract. The ethanol extracts were dried and resuspended in 500 μl of 50 mM sodium acetate, pH = 6.2. The cAMP content was determined by radioimmunoassay of duplicate aliquots from each well with anti-cAMP antibody (ICN Immunobiologicals) and cAMP ^{125}I-tracer (New England Nuclear). Bound antibody complex was separated by precipitation with ethanol (-20° C) in the presence of 10 mg bovine serum albumin per tube and centrifugation for 10 minutes at 2,000 g. Precipitated antibody complexes were counted in a LKB RIA 1275 Minigamma counter.

D. Results

1. Transient transfection of a D_2 dopamine receptor expression vector into GH_3 cells confers dopaminergic repression of PRL-CAT.

The first step in characterizing the pathway of dopaminergic repression of prolactin gene transcription was to develop an appropriate experimental system. As mentioned above, recent work from a collaborative effort between our laboratory and the laboratories of Dr. J.L. Roberts and Dr. S.C. Sealfon (Mount Sinai School of Medicine, New York, NY) has shown that transfection of the constructs RSV-D_{2S} or RSV-D_{2L} into the GH_3 cell line can confer dopaminergic repression of a PRL-CAT reporter construct (McChesney et al., 1991). However, each of the two cloned D_2 cDNAs used in that work contained a single base pair substitution which leads to an amino acid substitution in the final protein. In D_{2L}, serine^{25} was changed to threonine. This substitution occurs in the amino terminal domain of the receptor which is proposed to be extracellular. In D_{2S}, glutamate^{181} was changed to valine. This substitution occurs in the proposed second extracellular loop. Despite the demonstration of dopaminergic repression mediated through these two clones, the amino acid substitutions left some question as to the validity of their continued use. Therefore, again in collaboration with members of the laboratories of Dr. J.L. Roberts and Dr. S.C. Sealfon, both D_2 receptor isoforms were recloned from rat brain mRNA. Primers identical to those used previously were used for PCR amplification but Vent^{TM} polymerase was used because of its reported lower error rate (Cariello et al., 1991). The PCR amplified products provided by Dr. Sealfon were subcloned into a eukaryotic expression vector creating the constructs RSV-D_{2A} (long isoform) and RSV-D_{2B} (short isoform). In these constructs, expression of the D_2 receptor isoform is under the control of the Rous sarcoma virus long terminal repeat (RSV LTR). Both constructs were sequenced and found to be in complete agreement with the

published sequence of the two isoforms (Bunzow et al., 1988; Giros et al., 1989; Monsma et al., 1989b).

Transfection of the newly constructed RSV-D_{2A} construct into GH_3 cells was performed in order to confirm that this construct would give the same results as the original RSV-D_{2L} construct containing the single base pair substitution. In these experiments, RSV-D_{2A} was transiently cotransfected into GH_3 cells along with the PRL-CAT reporter construct. After transfection, cells were incubated in the presence or absence of the D_2-selective agonist quinpirole. Two days later, the cells were harvested and cellular extracts were assayed for CAT reporter activity. In the experiment shown in Figure 2 cotransfection of the RSV-D_{2A} construct confers a four- to five-fold repression of PRL-CAT activity in the presence of quinpirole. This repression is specific in that it occurs only in the presence of quinpirole and is blocked by the D_2-selective antagonist spiperone (Figure 3).

The repression seen in this system exhibits promoter specificity and clearly occurs at the level of prolactin gene transcription, since expression of RSV-CAT and DHFR-CAT are not inhibited or are only partially inhibited, respectively (McChesney et al., 1991, Figure 4, and data not shown). In a similarly reconstituted system of lactrotrophic GH_4C_1 cells stably transfected with an expression vector encoding the D_{2B} receptor, Elsholtz et. al. have also demonstrated promoter specificity: a prolactin-luciferase construct was inhibited by the D_2-selective agonist bromocriptine but neither RSV-luciferase nor TGF-α-luciferase constructs were affected (Elsholtz et al., 1991).

To attempt to confirm the findings of McChesney et. al. (1991) using the correct D_2 cDNA's, the experiment illustrated in Figure 5 was performed. The results shown in this figure confirm the initial findings of McChesney et. al. that either isoform of the D2 receptor is capable of mediating dopaminergic repression of the prolactin promoter.

The long isoform of the D_2 receptor (D_{2A}) is the predominant isoform in the anterior pituitary (Dal Toso et al., 1989; Monsma et al., 1989b). The ratio of D_{2A}:D_{2B} in

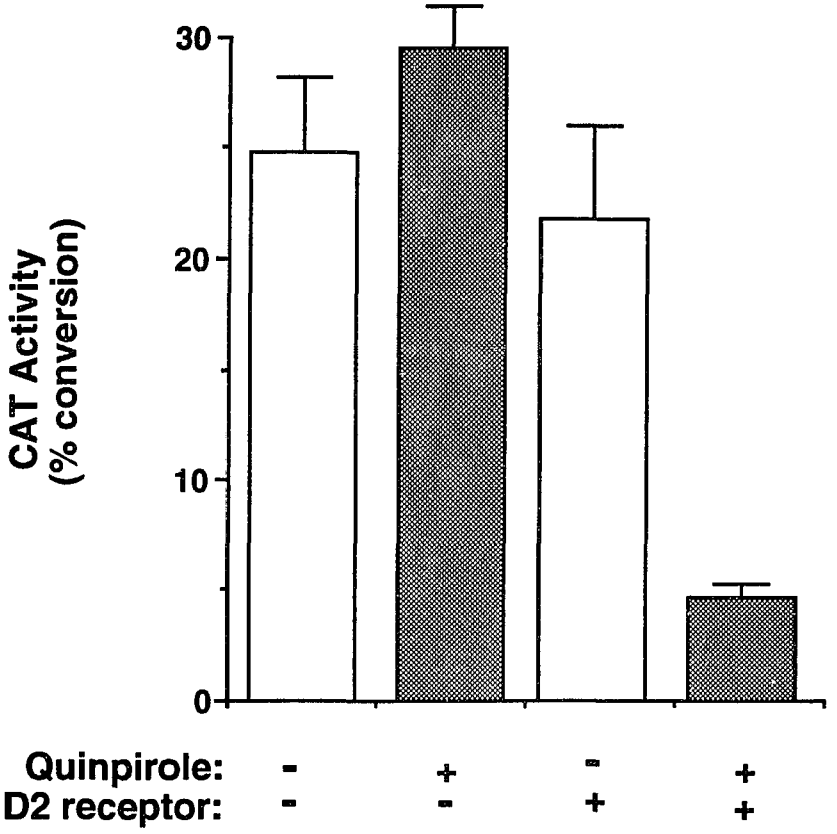


Figure 2: Dopaminergic repression of PRL-CAT expression in GH_3 cells transiently transfected with RSV-D_{2A}. GH_3 cells were transiently transfected via lipofectin with 10 μg of PRL-CAT and 5μg of RcRSV (-) or RSV-D_{2A} (+). Transfected cells were incubated ± 200 nM quinpirole as indicated for two days prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

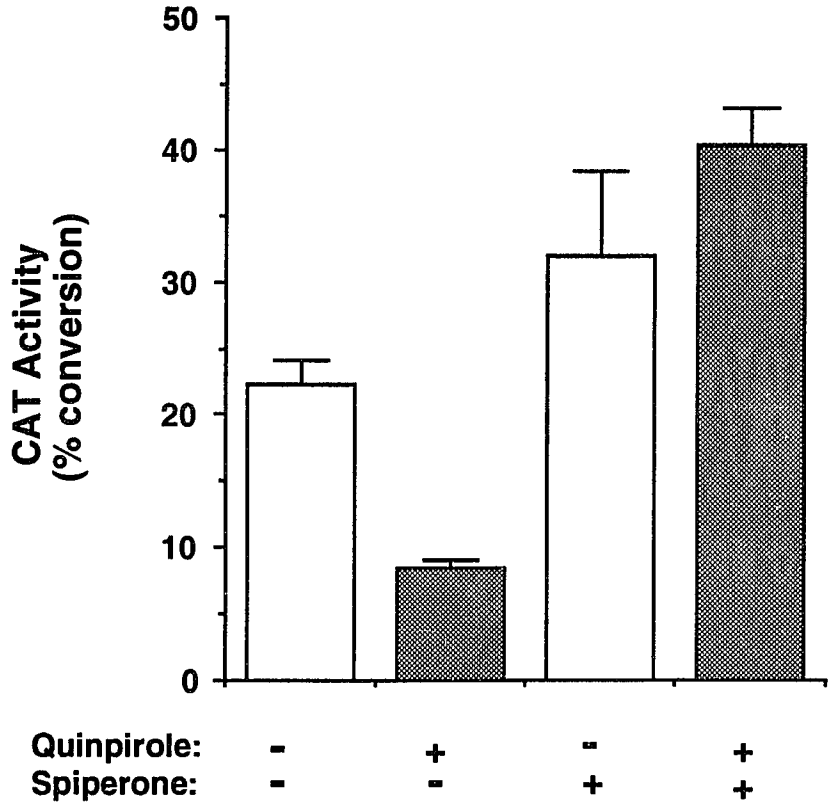


Figure 3: Dopaminergic repression of PRL-CAT expression in GH3 cells is blocked by the D_2-selective antagonist spiperone. GH3 cells were transiently transfected via lipofectin with 10 μg of PRL-CAT and 5μg of RSV-D_{2A}. Transfected cells were incubated ± 200 nM quinpirole and/or 1 μM spiperone as indicated for two days prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

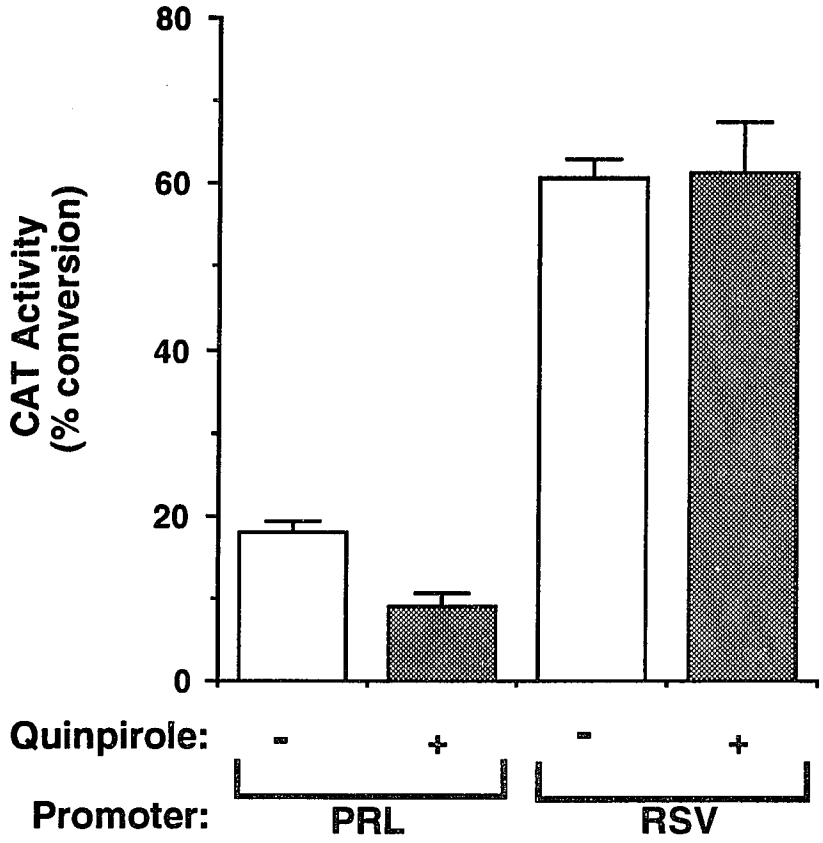


Figure 4: Dopaminergic repression of PRL-CAT in GH_3 cells demonstrates promoter specificity. GH_3 cells were transiently transfected via lipofectin with 20 μg of PRL-CAT (PRL) or RSV-CAT (RSV) and 5μg of RSV-D_{2A}. Transfected cells were incubated ± 200 nM quinpirole as indicated for two days prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

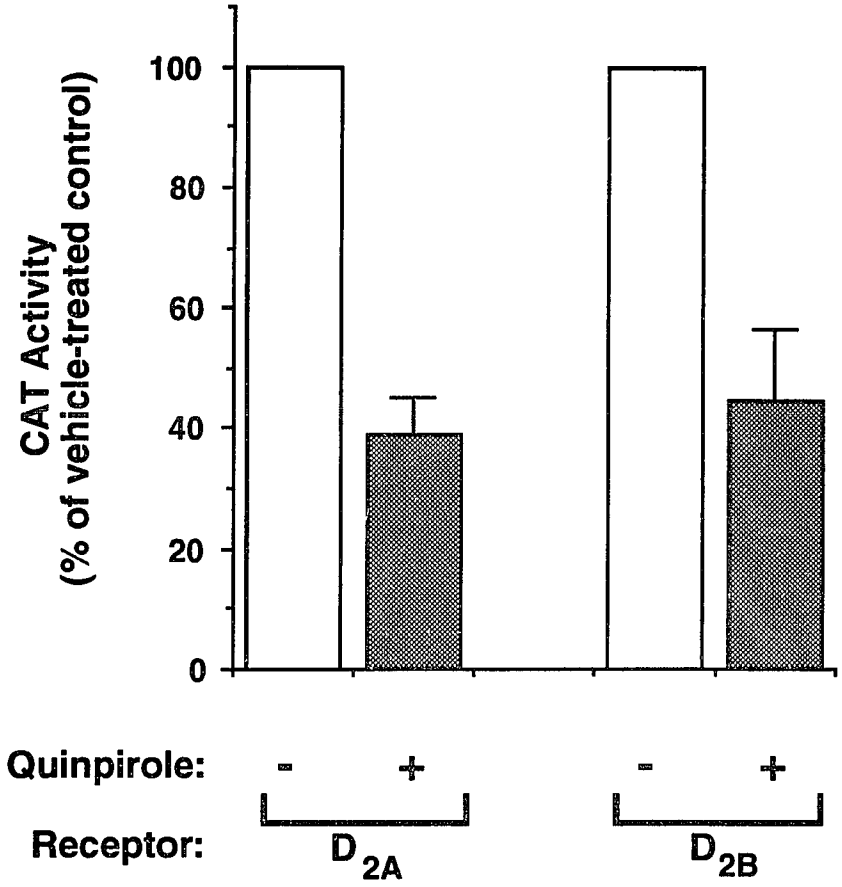


Figure 5: Dopaminergic repression of PRL-CAT expression can be mediated by either isoform of the D2 receptor. GH3 cells were transiently transfected via lipofectin with 10 μg of PRL-CAT and 5μg of RSV-D_{2A} or RSV-D_{2B} as indicated. Transfected cells were incubated ± 200 nM quinpirole as indicated for two days prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

the anterior pituitary has been estimated at 6:1 (McChesney et al., 1991) and 5-10:1 (Dal Toso et al., 1989). Because of the apparent excess of the D_{2A} isoform in the anterior pituitary, I used the RSV-D_{2A} construct exclusively in the majority of my further experiments.

2. Repression of PRL-CAT expression in this system is mediated by one or more pertussis toxin-sensitive G proteins.

As discussed earlier, the D_2 receptor has been shown to be coupled to a number of different signal transduction pathways. All of these pathways appear to involve interactions with pertussis toxin-sensitive GTP-binding proteins (i.e. G_i or G_o). Pertussis toxin ADP-ribosylates and inactivates members of the G_i and G_o families of G proteins (Gierschik, 1992). It was, therefore, expected that dopaminergic repression of PRL-CAT in the present system might be mediated via a pertussis toxin-sensitive G protein. In order to investigate this hypothesis, GH_3 cells were again cotransfected with RSV-D_{2A} and PRL-CAT in the presence or absence of the D_2-selective agonist quinpirole. In addition, some of the cells were pretreated with pertussis toxin. As shown in Figure 6, treatment with pertussis toxin completely attenuates dopaminergic repression of PRL-CAT. This demonstrates that, in this model system, the repression mediated via the D_{2A} receptor is indeed transduced via a pertussis toxin-sensitive G protein. This G protein is most likely one or more of the G_i/G_o proteins that have been demonstrated to be present in GH_3 cells: $G_{o\alpha}$, $G_{i\alpha 2}$, or $G_{i\alpha 3}$. The $G_{i\alpha 1}$ subunit has not been detected in GH_3 cells (Paulssen et al., 1991).

In an attempt to both increase the magnitude of the dopaminergic repression of the rPRL promoter and implicate a member of the G_i/G_o family in the dopminergic repression pathway, an expression vector encoding the $G_{i\alpha 2}$ subunit (CMV-$G_{i\alpha 2}$) was transiently transfected into GH3 cells. The results, shown in Figure 7 demonstrate that CMV-$G_{i\alpha 2}$ alone caused a dose-dependent repression of the rPRL promoter in the

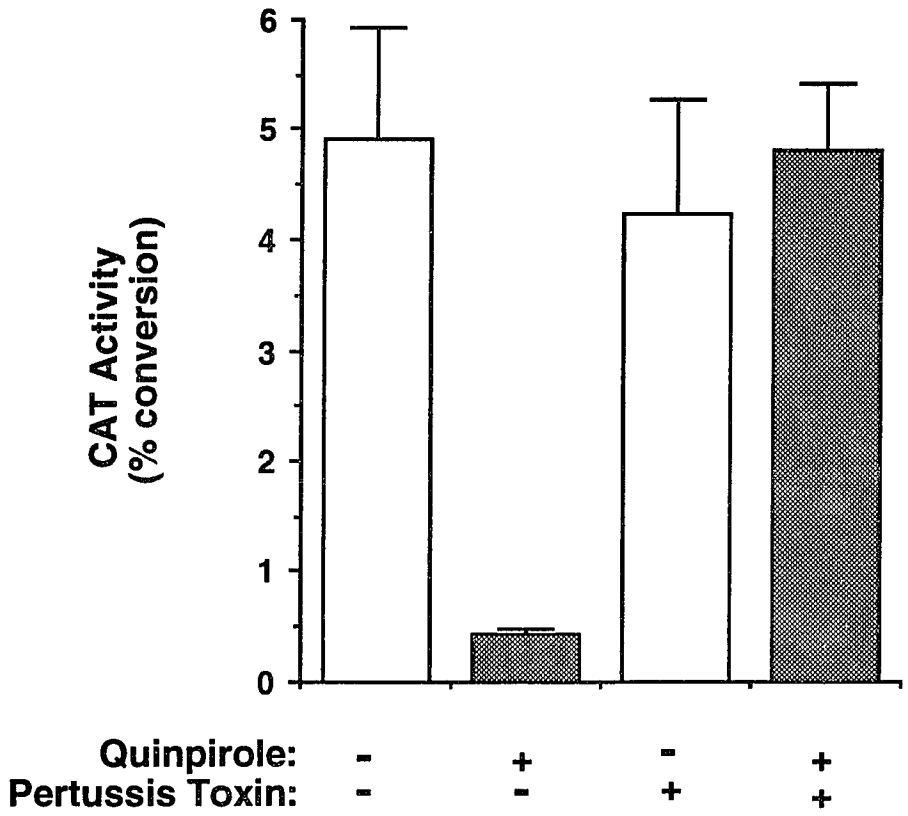


Figure 6: Dopaminergic repression of PRL-CAT in GH3 cells is mediated by one or more pertussis toxin-sensitive G proteins. GH3 cells were preincubated for 16 hours ± 200 ng/ml pertussis toxin as indicated prior to transfection. Cells were transiently transfected via lipofectin with 20 μg of PRL-CAT and 5μg of RSV-D_{2A}. Transfected cells were incubated ± 2 μM quinpirole and 200 ng/ml pertussis toxin as indicated for two days prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

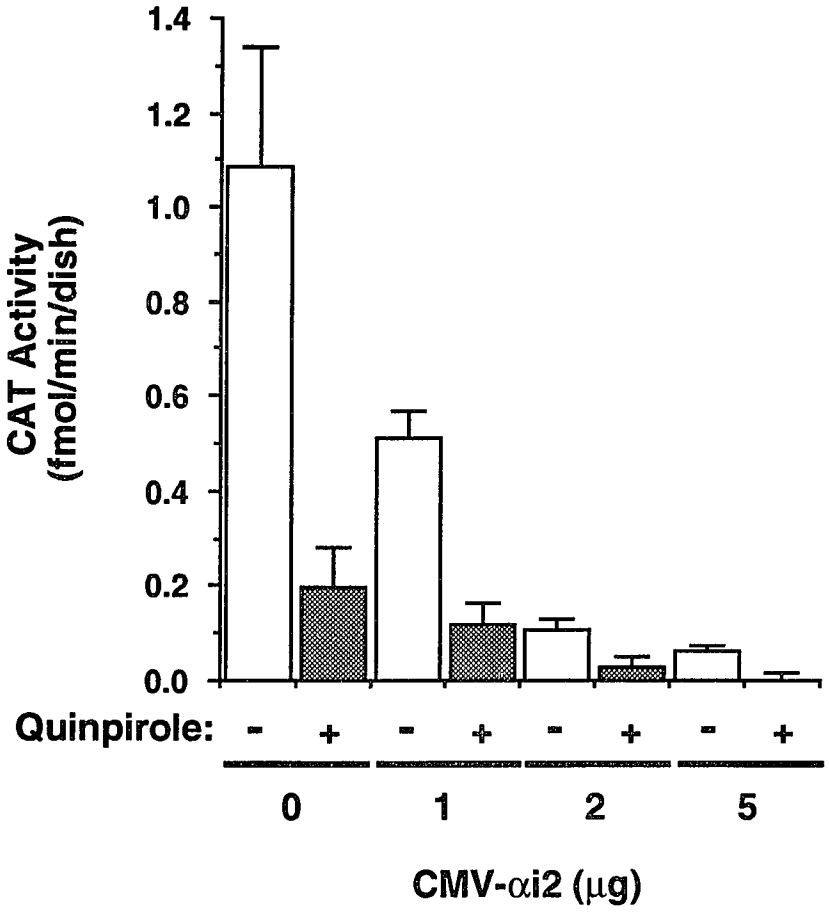


Figure 7: $G_{\alpha i2}$ represses PRL-CAT in the absence of dopamine agonist. Approximately 1 x 10^7 GH_3 cells were transiently transfected via electroporation (960 μF, 200 V) with 10 μg of PRL-CAT, 10 μg of RSV-D_{2A}, and the indicated amounts of CMV-αi2. Transfected cells were incubated ± 200 nM quinpirole as indicated for one day prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion rate of [^{3}H]-chloramphenicol to acylated products observed with triplicate plates.

absence of quinpirole. Additionally, the fold-repression was not increased by co-expression of $G_{i\alpha 2}$. While these results suggest that the $G_{i\alpha 2}$ subunit is capable of mediating repression of the rPRL promoter, they provide no evidence for such a role in the dopaminergic pathway. Since repression by CMV-$G_{i\alpha 2}$ is not seen in the absence of cotransfected D_2 receptor (J. Tian and C. Bancroft, unpublished data), it may be that there are sufficient catecholamines in the serum-containing medium to cause an interaction between over-expressed D_2 receptor and over-expressed $G_{i\alpha 2}$. Alternatively, the over-expressed unliganded receptor may be capable of achieving some level of activation of the over-expressed $G_{i\alpha 2}$ subunit (Schütz and Freissmuth, 1992).

3. The proximal 187 bp of the prolactin promoter is sufficient to confer full dopaminergic repression.

After having evaluated the experimental system as described above, I proceeded to attempt to identify a dopaminergic response element in the rat prolactin promoter. To begin this process, transient cotransfection experiments were performed as outlined above but using a series of 5'-deletion mutants of PRL-CAT. The constructs (-1957)PRL-CAT (PRL-CAT), (-204)PRL-CAT, (-187)PRL-CAT, and (-75)PRL-CAT contain 5' endpoints of the rat prolactin gene 5'-flanking DNA of -1957 base pair (bp), -204 bp, -187 bp, and -75 bp as measured from the transcription start site (Lufkin and Bancroft, 1987). The largest construct, PRL-CAT, contains the entire region of prolactin gene 5'-flanking DNA known to be important for transcriptional regulation of the gene in the pituitary, since it contains both the distal enhancer and the proximal promoter (see Figure 1). The other constructs all lack the distal enhancer and therefore exhibit markedly reduced basal levels of expression. However, as seen in Figure 8, PRL-CAT, (-204)PRL-CAT, and (-187)PRL-CAT all exhibit comparable levels of transcriptional repression in the presence of the D_2-selective agonist quinpirole. These results indicate that one or more

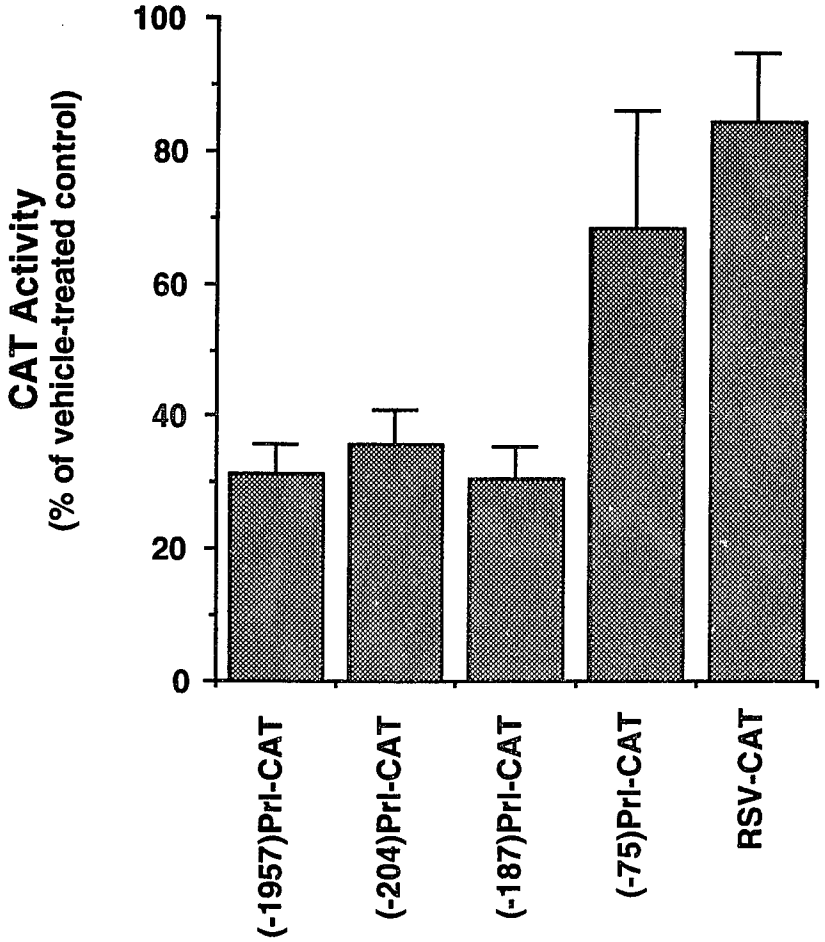


Figure 8: The proximal 187 bp of the prolactin promoter can confer full dopaminergic repression. GH_3 cells were transiently transfected via lipofectin with 20 μg either PRL-CAT, (-204)PRL-CAT, (-187)PRL-CAT, (-75)PRL-CAT, or RSV-CAT as indicated and 5μg of RSV-D_{2A}. Transfected cells were incubated ± 200 nM quinpirole as indicated for two days prior to harvesting of cells for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products as % of vehicle-treated cells observed with triplicate plates.

dopamine response elements lie within 187 base pairs of the transcription start site of theprolactin gene, and that this element(s) is capable of mediating the full effect of dopamine on transcription of the prolactin gene.

Contained within this 187 base pair region are three binding sites for the pituitary-specific transcription factor Pit-1 (Mangalam et. al. 1989 and Figure 1). In addition, recent attention has been focused on a novel, CRE-like element (CLE) extending from -99 to -92 base pairs upstream of the transcription start site (Iverson et al., 1990; Peers et al., 1991). In order to determine if a single Pit-1 binding site or the CLE might be able to mediate dopaminergic repression, I used the reporter constructs (-113)Prl-CAT and (-75)PRL-CAT. The construct (-113)Prl-CAT contains both the CLE and 1P (the most 3' of the Pit-1 binding sites of the rPRL promoter (Figure 1). The construct (-75)PRL-CAT has had the CLE deleted. Unfortunately, (-113)Prl-CAT and (-75)PRL-CAT yielded such low levels of basal expression that measuring any subsequent dopaminergic repression proved impossible (data not shown).

In a further attempt to determine if a Pit-1 binding site is capable of serving as a dopamine response element, transfection experiments were performed as before but using the construct $(1P)^2$(-39)PRL-CAT(RSV Enh). This construct contains two concatameric Pit-1 binding sites upstream of a minimal prolactin promoter (Yan and Bancroft, 1991). Basal expression of this construct is raised by the presence of the Rous sarcoma viral enhancer. However, I was unable to demonstrate any reliable dopaminergic repression of this reporter (data not shown).

Recently Lew et. al. have demonstrated that three concatameric, high-affinity Pit-1 binding sites can confer dopaminergic repression to a heterologous promoter (Lew et al., 1994). It is unclear whether the success of this group in demonstrating dopaminergic repression through concatameric Pit-1 binding sites is due to the higher number of concatamers used, three versus two, or perhaps due to the choice of Pit-1 binding site used. Lew et. al. used a concatameric artificial Pit-1 binding site while I used a

concatamer of the 1P site from the proximal promoter. What is clear is that three Pit-1 binding sites, whether alone, as seen in the results of Lew et. al., or in the natural context of the rPRL promoter as shown here with (-187)Prl-CAT, yield comparable repression to that seen with the entire rPRL promoter. It thus seems likely that Pit-1 binding sites can serve as dopamine response elements.

4. Generation of GH_3 cells stably transfected with RSV-D_{2A}

A difficulty encountered in attempting to map more finely the DARE(s) of the rPRL promoter was that, on average, only a two- to three-fold repression was seen in the transiently transfected GH_3 cells. The transient transfection assay requires two days for the transfected cells to reach maximal levels of dopaminergic repression (data not shown). It seemed likely that this lag in repression could be due to the time required to transcribe the RSV-D_{2A} construct, translate the D_{2A} message into protein, and for receptor protein to be translocated and accumulate in the cytoplasmic membrane. Furthermore, it seemed possible that the continued presence of dopamine agonists during this time would lead to desensitization and down-regulation of the newly synthesized D_{2A} receptors. Therefore, I concluded that it might be possible to increase dopaminergic repression in this system by generating a line of GH_3 cells stably expressing high numbers of functional D_{2A} receptors, and examining dopaminergic repression of PRL-CAT in these cells at the earliest possible time points.

After transfection of GH_3 cells with RSV-D_{2A} plus RSV-neo, and selection with the neomycin analog G418, clones of stable transfectants were selected, and screened for expression of the D_2 receptor by binding to the D_2-selective radioligand [^{3}H]-spiperone. The clones demonstrating the greatest amount of specific binding to [^{3}H]-spiperone, clone GH_3D_2-1 and clone GH_3D_2-10, were selected for further characterization.

While cells of the GH_3 line fail to express the endogenous D_2 receptor gene, it is unknown at what level the gene is repressed. Therefore, in an attempt to determine if the

D_2 receptor gene is actively transcribed in the GH_3 cell line as well as the stably transfected cells, a nuclear runoff transcription assay of wild-type GH_3 cells and clone GH_3D_2-10 cells was performed to compare the relative transcription rates of the D_2 receptor gene in the two cell lines. As can be seen in Figure 9, while a positive signal can be detected in both cell lines for the pituitary-specific transcription factor Pit-1, only the stable transfectant displays a positive signal for the D_2 receptor. This result suggests that the block in D_2 receptor expression in GH_3 cells is at, or before, the level of transcription and that in cells stably transfected with RSV-D_{2A}, this blockade is corrected.

After demonstrating that the stable transfectants could actively transcibe the stably transfected D_{2A} construct, I proceeded to determine if the transfectants were expressing significant numbers of functional D_{2A} receptors. The expression of D_2 receptors was assayed for by performing a radioligand binding assay at a single, theoretically saturating, concentration of [^{3}H]-spiperone. The data in Figure 10 compare the levels of specific binding of [^{3}H]-spiperone by membrane preparations from clones GH_3D_2-1 and GH_3D_2-10 cells and wild-type GH_3 cells to the level of binding in a homogenate of rat striatum. While the wild-type GH_3 cells show no detectable specific binding of [^{3}H]-spiperone, both of the stable transfectants demonstrate a significant number of [^{3}H]-spiperone binding sites. I have not calculated an estimate of the number of receptor sites expressed per cell because such an estimate would be of dubious value for the following reasons: the membrane preparations used in these experiments were limited in quantity and there is no commercially available D_2-selective radioligand with a high specific activity. These two limitations prevented me from performing full Scatchard analyses of these clones and thereby calculating an accurate value for Bmax. The binding data obtained, however, served its purpose; to identify those clones expressing the highest number of D_2 receptor sites.

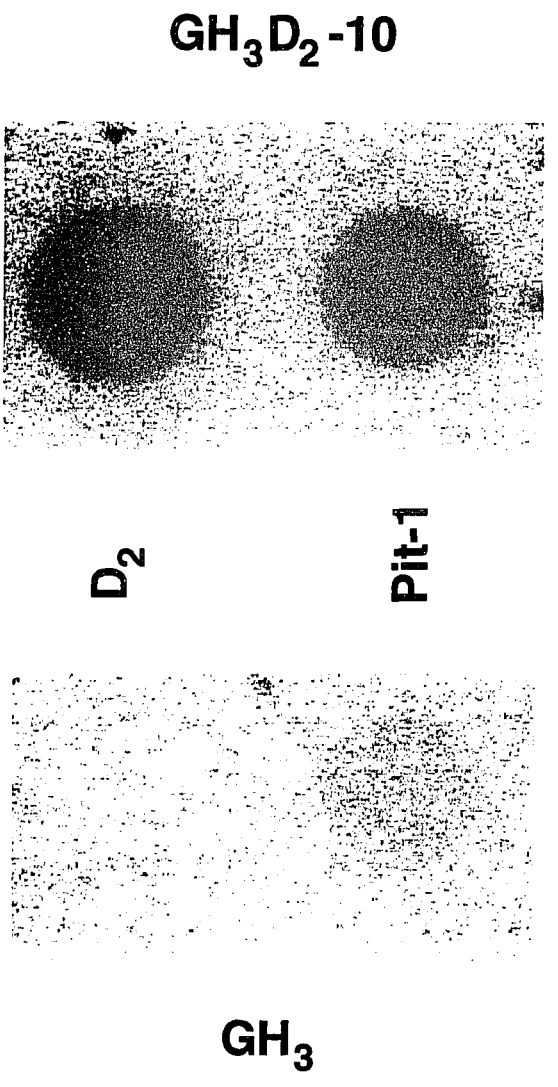


Figure 9: GH_3D_2-10 cells transcribe the stably transfected RSV-D_{2A} construct. Nuclei were isolated from wild-type GH_3 cells and GH_3D_2-10 stable transfectants. Nascent transcripts from the isolated nuclei were run-off in the presence of [^{32}P]-UTP and hybridized to cDNA's of Pit-1 and D_{2A} as indicated.

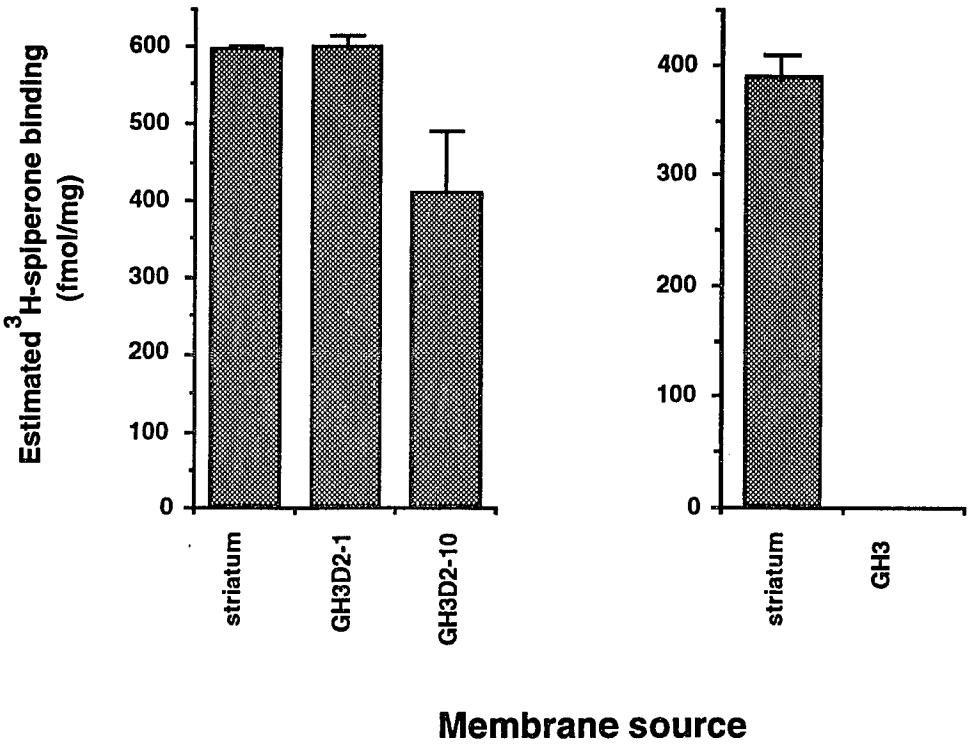


Figure 10: Stable transfectants GH_3D_2-1 and GH_3D_2-10 express binding sites for the D_2-selective radioligand [^{3}H]-spiperone. Membrane homogenates were prepared from wild-type GH_3, GH_3D_2-1, and GH_3D_2-10 cells as well as rat striatum. Membrane homogenates were assayed for specific binding of [^{3}H]-spiperone. Results represent the mean (± SE) fmol [^{3}H]-spiperone specifically bound per mg of protein from triplicate assays.

After determining that the stable transfectants were expressing high numbers of D_{2A} receptors, I next attempted to assess the functional state of these receptors. The D_{2A} receptor is classically coupled to the inhibition of adenylyl cyclase. Therefore, to assess the functional state of the receptors expressed in the stable transfectants, I chose to measure the intracellular accumulation of cAMP in the presence or absence of the dopamine agonist quinpirole. Using a radio-immunoassay, a variable amount of repression of cAMP accumulation was observed in the stable transfectants in different experiments. Figure 11 shows the result of one experiment with the GH_3D_2-1 cells, in which quinpirole yielded a 70% repression of the basal level of cAMP and a 40% repression of the forskolin-stimulated level of cAMP.

The ultimate test of the function of the receptors expressed in the stable transfectants would be their ability to repress expression of transiently transfected PRL-CAT by the D_2-selective agonist quinpirole. Again, variable results were observed in different experiments. The data in Figure 12 show the results of one experiment in which PRL-CAT activity in GH_3D_2-1 cells is repressed in the presence of quinpirole. The level of the repression observed, however, fails to exceed that obtained previously in transiently transfected cells. This observation agrees with the finding of Elsholtz et. al. that transiently transfected D_2 receptors yield a larger repression of the rPRL promoter than stably transfected receptors (Elsholtz et al., 1991). I was, therefore, unable to achieve my original goal of using the stable transfectants to map more finely the pathway of dopaminergic repression.

5. Dopaminergic effects on the rPRL promoter in nonpituitary cell lines

While the 5' deletion analysis presented above is consistent with a role for Pit-1 in transducing the dopaminergic repression to the rPRL promoter, it provides no direct evidence for such a role. In an attempt to provide such evidence, I attempted to reconstitute the dopaminergic repression of the rPRL promoter in a nonpituitary cell line.

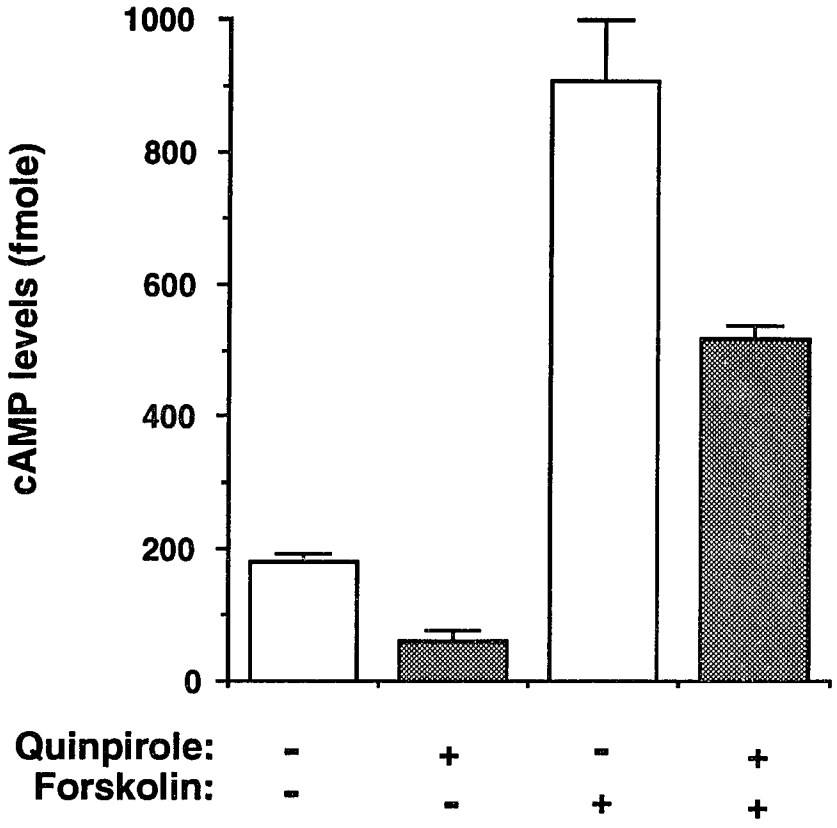


Figure 11: Dopaminergic inhibition of both basal and forskolin-stimulated levels of intracellular cAMP in GH_3D_2-1 stable transfectants. GH_3D_2-1 cells were incubated for 15 minutes ± 2 μM quinpirole and 1 μM forskolin as indicated. Cells were extracted with absolute ethanol and extracts were assayed by radio-immunoassay for levels of cAMP. Results represent the mean (± SE) intracellular cAMP level from cellular extracts of triplicate wells of cells.

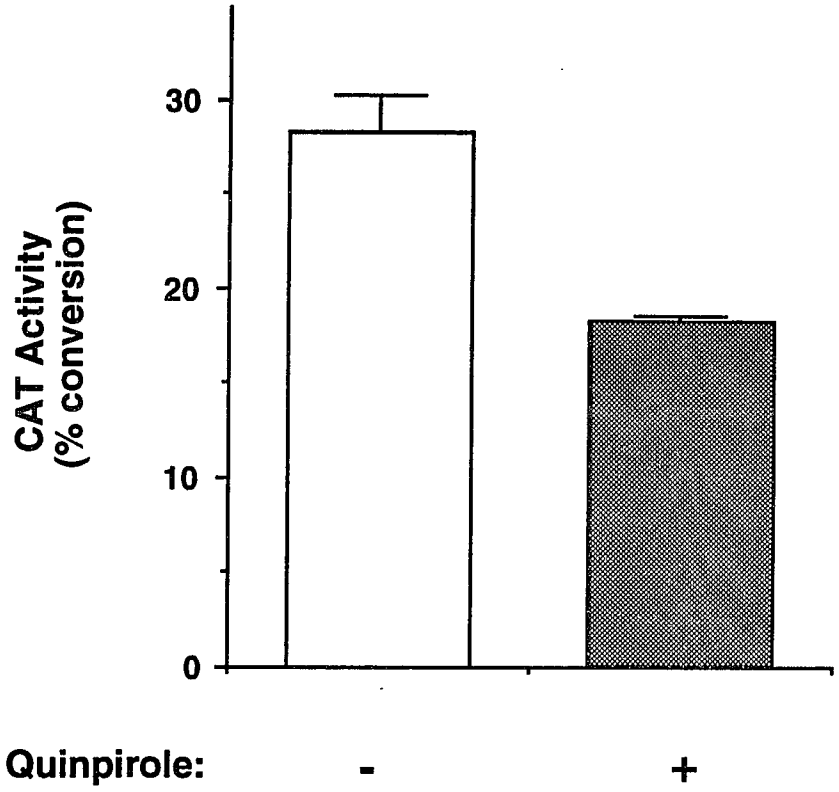


Figure 12: Dopaminergic repression of PRL-CAT in GH_3D_2-1 stable transfectants. Approximately 2 x 10^7 GH_3D_2-1 cells were transfected via electroporation (250 μF, 200 V) with 60 μg of PRL-CAT. Cells were then incubated ± 2 μM quinpirole as indicated for two days prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

I hoped to show that, in nonpituitary cells, repression of a PRL-CAT reporter is dependent on the presence of Pit-1.

In order to obtain basal levels of expression of PRL-CAT constructs in nonpituitary cells, I employed an expression vector encoding a fusion protein, CREB-VP16 which contains the DNA-binding domain of the cAMP regulatory element binding protein (CREB) fused to the *trans*-activating domain of the viral transcriptional activator VP16. It has been shown that expression of this fusion protein permits expression of the rPRL promoter in nonpituitary cell lines (Yan et al., 1994).

The results of a reconstitution assay in C6 glioma cells transfected with (-187)PRL-CAT and RSV-D_{2A} can be seen in Figure 13. While basal (-187)PRL-CAT expression was successfully induced by cotransfection with either the CREB-VP16 expression vector or a Pit-1 expression vector, the presence of the dopamine agonist quinpirole caused an induction of (-187)PRL-CAT activity rather than a repression. This induction of (-187)PRL-CAT probably represents paradoxical coupling of the D_{2A} receptor to one or more of its signal transduction pathways. Such paradoxical coupling by transfected D_2 receptors was first observed by Neve et. al. (1989). Most recently, Lew et. al. (1994) have reported a similar dopaminergic induction of the rPRL promoter in Ltk^- cells. While the paradoxical induction of the rPRL promoter appears to be in part Pit-1-dependent, the significance of this finding in terms of the role of Pit-1 in repression of the rPRL promoter remains unclear.

6. The role of calcium in dopaminergic repression of the rPRL promoter

Intracellular free calcium levels regulate the rPRL gene (White et al., 1981; Jackson and Bancroft, 1988) and are themselves negatively regulated by the D_2 receptor in lactotrope cells (Malgaroli et al., 1987). It was therefore of interest to investigate the role, if any, that regulation of the intracellular free calcium concentration might play in mediating dopaminergic repression of the rPRL promoter. Two agents that would be

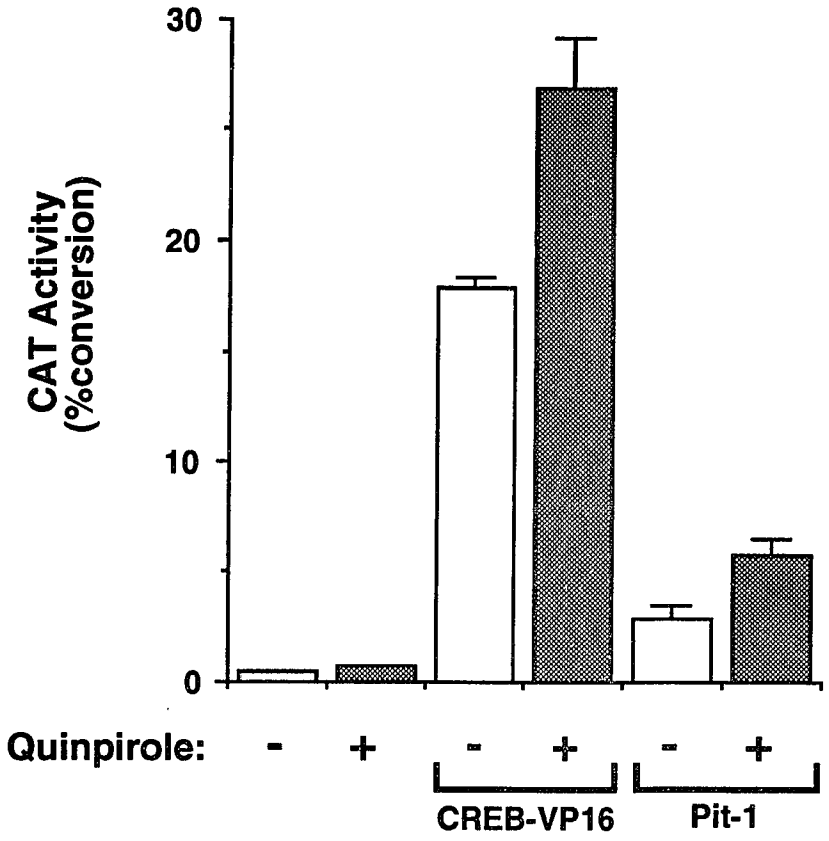


Figure 13: Dopaminergic induction of (-187)PRL-CAT activity in C6 cells. C6 cells were transfected via calcium phosphate with 5 μg of (-187)PRL-CAT, 5 μg RSV-D_{2A} and 5 μg of RSV-Pit-1 or pSV$_2$-CREB-VP16 as indicated. After transfection, cells were incubated for two days ± 2 μM quinpirole as indicated prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

expected to raise intracellular levels of free calcium were employed: BayK8644 and thyrotropin releasing hormone (TRH). BayK8644 is a dihydropyridine that acts as an agonist on voltage-gated calcium channels. TRH is a peptide hormone that binds to a G protein-coupled receptor and effects increases in intracellular free calcium via release from intracellular pools and via activation of voltage-gated calcium channels (Gershengorn and Thaw, 1985). Both BayK8644 and TRH have been shown to induce prolactin promoter activity (Murdoch et al., 1983; Jackson and Bancroft, 1988). As expected, treatment of transiently transfected GH_3 cells with either BayK8644 (Figure 14) or TRH (Figure 15) causes an induction of PRL-CAT activity. However, the D_2-selective agonist quinpirole completely blocks the induction mediated by either BayK8644 (Figure 14) or TRH (Figure 15). Because these experiments were performed in transiently transfected cells, it was not possible to measure directly the effects of BayK8644, TRH, or quinpirole on intracellular free calcium concentrations. It is, therefore, possible that quinpirole acts to attenuate the induction by BayK8644 or TRH at a point distal to alterations of the levels of intracellular free calcium. Nonetheless, the results of these experiments suggest that dopamine agonists can exert their influence upon the rPRL promoter via a pathway common to both TRH and BayK8644.

7. Over-expression of cAMP-dependent protein kinase catalytic subunit attenuates the dopaminergic repression of PRL-CAT.

Inhibition of adenylyl cyclase was the first effector shown to be coupled to the D_2 receptor (De Camilli et al., 1979; Frey et al., 1982). Since cAMP has been shown to be an inducer of prolactin gene transcription (Maurer, 1981), I wished to investigate whether dopaminergic inhibition of prolactin transcription was mediated through a cAMP-dependent pathway. Indeed, Maurer (1981) has shown that elevation of intracellular levels of cAMP can attenuate dopaminergic repression of the prolactin promoter.

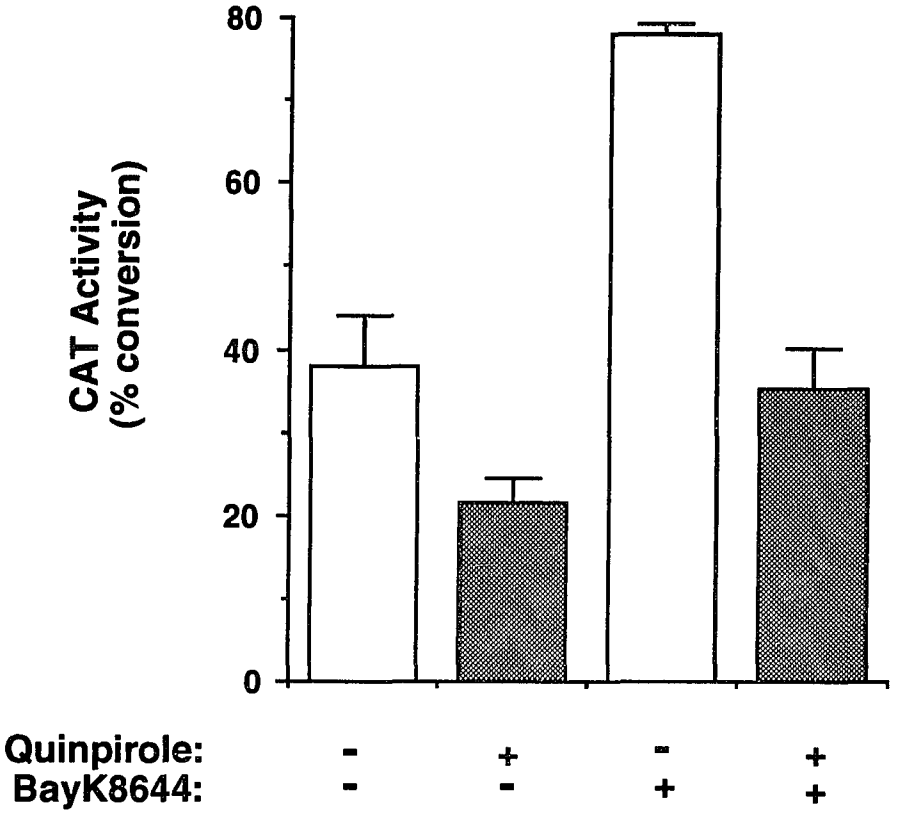


Figure 14: Dopaminergic repression of PRL-CAT in GH_3 cells is not altered by treatment with the calcium channel agonist BayK8644. GH_3 cells were transfected via lipofectin with 20 μg of PRL-CAT and 5 μg of RSV-D_{2A}. After transfection cells were incubated ± 2 μM quinpirole and 1 μM BayK8644 as indicated for two days prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

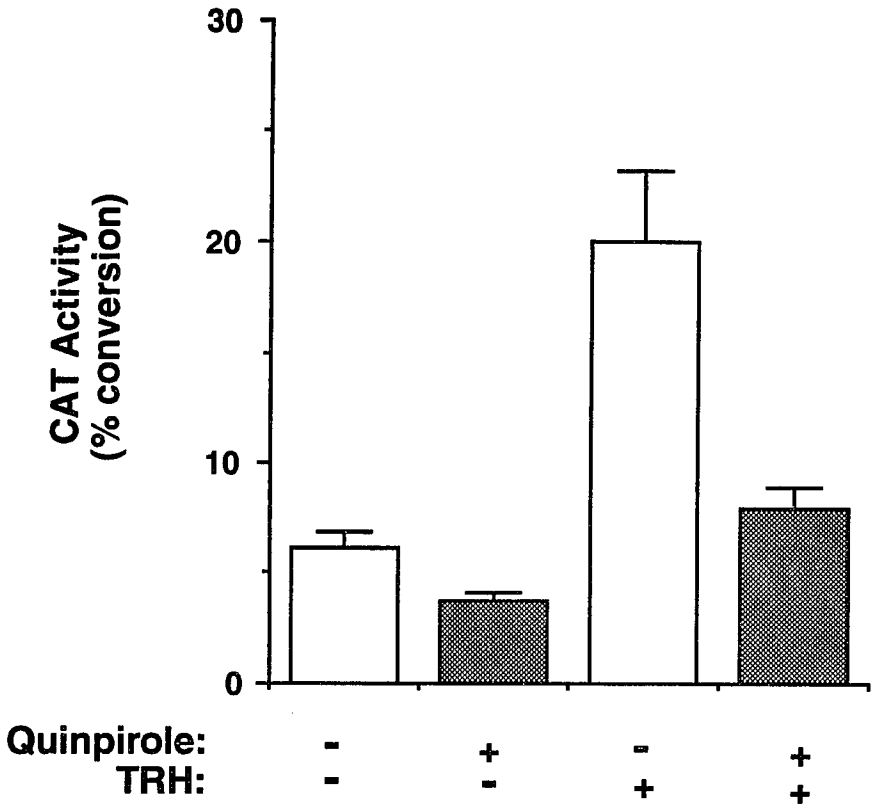


Figure 15: Dopaminergic repression of PRL-CAT in GH_3 cells is not altered by treatment with the peptide hormone TRH. GH_3 cells were transfected via DEAE-dextran with 10 μg of (-187)PRL-CAT and 5 μg of RSV-D_{2A}. On the second day after transfection, cells were switch to serum-free medium plus calcium (0.5 mM) for two days. After deinduction, cells were incubated ± 200 nM quinpirole and 100 nM TRH as indicated for one day prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

Because all of the transcriptional actions of cAMP in higher eukaryotes are thought to be mediated by cAMP-dependent protein kinase (PKA), dopamine may repress transcription by reducing cellular levels of cAMP and subsequently reducing PKA activity. To test this hypothesis, I attempted to block the effect of quinpirole by preventing repression of PKA.

Inactive protein kinase A exists as a tetramer of two catalytic and two regulatory subunits (Edelman et al., 1987). The regulatory subunits each contain two binding sites for cAMP. When cAMP binds to the sites on the regulatory subunits, the tetramer disassociates and the catalytic subunits are activated. Dopaminergic inhibition of adenylyl cyclase leads to reduced levels of intracellular cAMP which shifts the equilibrium of the disassociation equation back to the inactive tetramer state. I attempted to prevent the repression of PKA activity by over-expression of the catalytic subunit. I reasoned that if the levels of catalytic subunit in the cell were raised sufficiently, there would be inadequate levels of regulatory subunit to inactivate them thereby making the activity of PKA independent of the activity of adenylyl cyclase.

The transient transfection protocol was repeated as before, using PRL-CAT as a reporter construct. In addition, however, some of the cells were transfected with an expression vector containing the cDNA for a PKA catalytic subunit under the control of the mouse metallothionein promoter (Uhler and McKnight, 1987). As shown in Figure 16, over-expression of the PKA catalytic subunit had minor effects on the level of basal expression of PRL-CAT but greatly attenuated the dopaminergic repression of PRL-CAT.

This result suggests that dopaminergic repression of prolactin gene transcription is mediated through inhibition of PKA. However, it is possible that PKA is capable of overcoming repression mediated through an independent, but converging, pathway. For example, Pit-1 is a substrate for phosphorylation *in vitro* by both protein kinase A and protein kinase C (Kapiloff et al., 1991). The natural pathway of dopaminergic inhibition could be mediated through inhibition of PKC-mediated phosphorylation of Pit-1 but

artificial elevation of PKA attenuates this inhibition. Had the overexpression of PKA not resulted in the attenuation of repression, then it could be argued that PKA may not play a role in the dopamine pathway. The results obtained, therefore, while subject to various interpretations, are consistent with a role for regulation of the activity of PKA in the dopaminergic repression of prolactin transcription.

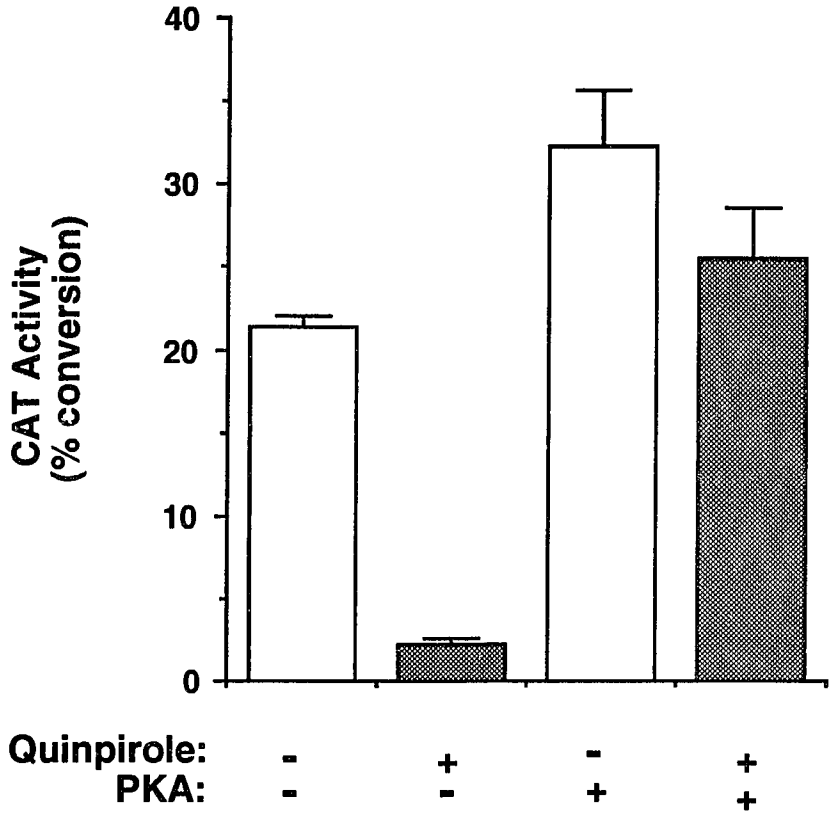


Figure 16: Dopaminergic repression of PRL-CAT in GH_3 cells is attenuated by expression of the catalytic subunit of the cAMP-dependent protein kinase (PKA). GH_3 cells were transfected via DEAE-dextran with 2 μg of PRL-CAT, 1μg of RSV-D_{2A}, and 5 μg of pUC19 (-) or mMT-PKA (+). After transfection cells were incubated ± 2 μM quinpirole as indicated for two days prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

E. Discussion

In the present work. the correct cDNA clones of the D_2 dopamine receptor have been used to confirm the observation of McChesney et. al. that either isoform of the D_2 receptor can mediate repression of the rat prolactin promoter. Furthermore, this finding has been extended to demonstrate that repression is mediated through one or more pertussis toxin-sensitive G proteins. The identity of the G protein(s) involved is unresolved. While it has been demonstrated here that the $G_{i\alpha 2}$ subunit is capable of repressing the prolactin promoter in transiently transfected GH3 cells, this in no way implicates $G_{i\alpha 2}$ as a mediator of the dopaminergic repression of the prolactin promoter in the pituitary. Similarly, Lew et. al. have shown that constitutively active mutants of both $G_{i\alpha 2}$ and $G_{o\alpha 1}$ are capable of repressing the rat prolactin promoter (Lew et al., 1994). These findings, which again provide no evidence for a role for these G proteins in repression of prolactin, are of interest because $G_{i\alpha 2}$ and $G_{o\alpha 1}$ have been associated with different signal transduction pathways in lactotrope cell lines. Recently, Liu et. al. have applied the powerful antisense technique to selectively knockout either $G_{\alpha i2}$ or $G_{\alpha o}$ in GH_4C_1 cells stably transfected with either isoform of the D_2 receptor (Liu et al., 1994). Using this system, they were able to demonstrate that depletion of $G_{\alpha i2}$ is associated with loss of dopaminergic inhibition of adenylyl cyclase while depletion of $G_{\alpha o}$ is associated with loss of inhibition of dihydropyridine-sensitive calcium channels (Liu et al., 1994). Thus it is possible that two or more independent signal transduction pathways may link D_2 receptors to inhibition of the rat prolactin gene. The development of this knockout system in GH_4C_1 cells, a pituitary-derived cell line closely related to the GH_3 cell line used in the current work, should very shortly prove useful in resolving the question of which G proteins, as well as which signal transduction pathways, are involved in mediating dopaminergic repression of the prolactin gene.

The dopaminergic repression of (-187)Prl-CAT demonstrated here is consistent with the recent findings of Lew et. al. that three Pit-1 binding sites are sufficient to confer

dopaminergic responsiveness to a heterologous promoter (Lew et al., 1994). Moreover, Lew et. al. have presented further evidence to implicate Pit-1 in dopaminergic regulation of prolactin transcription. In an experiment analagous to that shown in Figure 13, dopaminergic regulation of a prolactin reporter construct was partially reconstituted in a nonpituitary cell line. Lew et. al. found, as has been shown here, that in a nonpituitary cell line, D_2 activation induces rather than represses the prolactin reporter. More importantly, the induction seen by Lew et. al. was Pit-1-dependent. My results in the C6 glioma cell line differ somewhat, however, from those obtained by Lew et. al. in stably transfected Ltk^- cells. Lew et. al. have reported that the latter cell line, while not of pituitary origin, does allow a low level of basal expression of the prolactin promoter. Using this cell line, they were able to compare directly the effects of dopamine in the presence or absence of cotransfected Pit-1. In the C6 cell line, I have observed no such low level of basal expression and therefore chose to make use of the CREB-VP16 expression vector to induce prolactin promoter expression in the absence of Pit-1. While an induction was observed in the presence of CREB-VP16 and the absence of Pit-1, this induction was only 50% compared with the 100% induction seen in the presence of Pit-1. Because of the paradoxical response of the (-187)PRL-CAT reporter in this system, I chose not to continue this line of investigation. Therefore, while these results were obtained in multiple cell lines (data not shown), no statistical analysis of this effect has been performed. In comparison, the Pit-1-dependent induction seen by Lew et. al. (1994) was approximately 40%. Therefore the Pit-1-dependent component of the induction shown here is comparable to that reported by Lew et. al. Nonetheless, the relevance of a Pit-1-dependent induction in nonpituitary cells to the actual mechanism of repression in cells of the anterior pituitary remains unclear.

As discussed earlier, it is possible that the dopaminergic induction of the prolactin promoter seen in Ltk^-, C6 and HeLa (data not shown) cells is the result of paradoxical coupling of the D_2 receptor to one or more signal transduction pathways. Lew et. al.

(1994) have proposed a calcium-dependent mechanism for this induction. Indeed, they find that EGTA can specifically attenuate the dopaminergic induction. A calcium-dependent mechanism for induction could also explain the induction observed here with the CREB-VP16 construct, since CREB has been shown to be activated by calmodulin-dependent kinases (Sheng et al., 1991). However, while calcium may be playing a role in the nonphysiological dopaminergic induction of the prolactin promoter in nonpituitary cell lines, it remains to be shown that calcium is involved in the more physiologically representative repression observed in pituitary cell lines. A physiologic role for dopamine-mediated changes in intracellular calcium has been suggested, but not proven, by the experiments presented here in which quinpirole blocked induction of the rPRL promoter by BayK8644 or TRH .

An analogous approach has proved successful, however, in implicating another intracellular mediator, cAMP, in this pathway. Maurer found that treatment of cultures of primary pituicytes with cAMP blocked dopaminergic repression of transcription of the endogenous prolactin gene (Maurer, 1981). Elsholtz et. al. (1991), using forskolin to raise intracellular levels of cAMP, abolished dopaminergic repression of a transfected prolactin reporter construct in GH_4C_1 cells stably transfected with D_{2B} receptors. Extending this finding, I have found that over-expression of the catalytic subunit of PKA can also abolish dopaminergic repression in transiently transfected GH_3 cells. Caution is advised in interpreting these findings, however, because of the possibility of cross-talk between signal transduction pathways within the cell. For example, both cAMP and PKA can, in some systems, modify the activity of ion channels (Belardetti and Siegelbaum, 1988; Goulding et al., 1992). Therefore, the experimental observation of a role for cAMP and PKA in dopaminergic repression could merely reflect these actions and not a direct role in the dopaminergic pathway.

The observation that the *pit-1* gene promoter contains two binding sites for the cAMP response element binding protein (CREB) (Chen et al., 1990) suggests a plausible

mechanism for dopaminergic repression of the prolactin gene that is mediated via PKA. Activation of CREB has been shown to be mediated in F9 cells by phosphorylation of serine^{133} by PKA (Gonzalez and Montminy, 1989). However, while dopaminergic repression of the *pit-1* promoter has been reported, the repression does not require the CREB binding sites of the *pit-1* promoter (Elsholtz et al., 1991). Instead, the dopaminergic repression appears to be mediated by a short stretch of the *pit-1* promoter that contains a single autoregulatory Pit-1 binding site (Elsholtz et al., 1991). Therefore, dopaminergic repression of the prolactin promoter could be mediated, at least in part, through a reduction in levels of Pit-1. This would be consistent with the mapping of dopaminergic response elements of the prolactin promoter to Pit-1 binding sites. However, Lew et. al. have reported a failure to detect changes in levels of Pit-1 binding activity in gel mobility shift assays performed on control and dopamine-treated GH_4C_1 cells stably transfected with D_{2B} receptors (Lew et al., 1994).

On the other hand, the demonstration that Pit-1 is a substrate for phosphorylation by PKA (Kapiloff et al., 1991) suggests another plausible mechanism by which dopaminergic activation could transduce a signal to the prolactin gene (Figure 17). Activation of D_2 receptors on the surface of lactotropes could initiate a signal transduction pathway through $G_{i\alpha 2}$ leading to inhibition of adenylyl cyclase, reduced levels of intracellular cAMP, inhibition of PKA activity, and a subsequent hypophosphorylation of Pit-1. I have included in this model a cAMP-independent pathway that involves dopaminergic effects on membrane potential to agree with the observations of others (Elsholtz et al., 1991; Lew et al., 1994). While this model is consistent with the results presented here as well as those of others, it has a major deficiency: nothing is known about the effects of phosphorylation of Pit-1 upon its ability to transactivate the rPRL promoter. In order to address this question, I have undertaken a study of the functional significance of phosphorylation of Pit-1. These studies are the subject of Chapter 3.

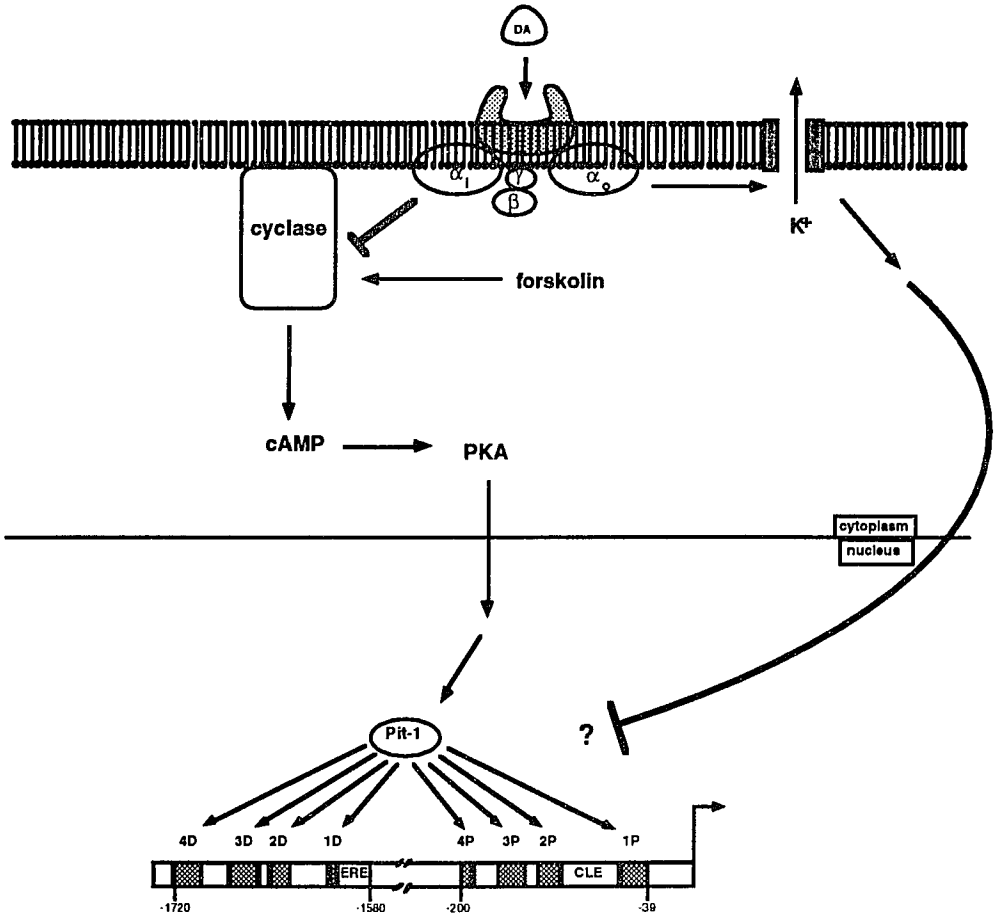


Figure 17: Model of the putative pathways of dopaminergic repression of the rPRL promoter.

III. Chapter 3: Studies of the Functional Role of Phosphorylation of Pit-1

A. Summary

The pituitary-specific transcription factor Pit-1 is known to be required for basal expression of both the rat prolactin (rPRL) and rat growth hormone (rGH) genes. In addition, regulation of the rPRL and rGH promoters by various hormones and intracellular effectors has been shown to be mediated through Pit-1. However, nothing is known about the mechanism by which Pit-1 mediates this regulation. Because Pit-1 is a substrate for both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), and because regulation of transcription factor activity so often involves changes in phosphorylation state, I have attempted to determine the functional role, if any, of phosphorylation of Pit-1. To this end, the wild type Pit-1 (Pit-1(WT)) was compared to a phosphorylation-deficient mutant (Pit-1(A3)) in the ability to activate the rPRL and rGH promoters in HeLa cells. The results presented here demonstrate that while Pit-1(A3) retains substantial ability to transactivate both the rPRL and rGH promoters, this activity is approximately 50% and 35%, respectively, less than that of Pit-1(WT). In addition, the Pit-1(A3) mutant retains the full activity of Pit-1(WT) to fulfill the requirement for Pit-1 for induction of the rPRL promoter by the estrogen receptor. Therefore, while I have found no evidence for a critical role for phosphorylation of Pit-1 at any of its known sites, it is possible that phosphorylation plays a modulatory role in regulating the activity of Pit-1 upon both the rPRL and rGH promoters.

B. Introduction

Changes in phosphorylation state have been shown to be critical in regulating the activity of a growing number of transcription factors (Hunter and Karin, 1992). Modulation of transcription factor activity by these changes can occur in a number of ways. An inactive transcription factor may be sequestered in the cytoplasm and a change in the phosphorylation state of the transcription factor (or an anchoring protein) allows the transcription factor to relocate to the nucleus where it can exert its action. Alternatively, the DNA-binding activity of a transcription factor may change as the result of a change in phosphorylation state. Finally, the transactivational activity of a transcription factor may be altered upon a change in phosphorylation state.

The regulation of the Rel-family of transcription factors is an example of the first mode of regulation. In most cell types, inactive NF-κB exists as a complex with IκB in the cytoplasm. Stimulation of these cells (by mitogens, cytokines, and other stimuli) leads to a dissociation of NF-κB from this complex and a translocation to the nucleus where NF-κB is capable of binding to its DNA recognitition elements and stimulating transcription of target genes (Blank et al., 1992). Phosphorylation of IκB has been shown, *in vitro*, to activate NF-κB DNA-binding activity (Ghosh and Baltimore, 1990).

Regulation of the activity of the c-Jun proto-oncogene displays two of the other modes of regulation. The DNA-binding activity of c-Jun is increased upon dephosphorylation of sites proximal to its DNA-binding domain (Boyle et al., 1991). In addition, phosphorylation of sites within the activation domain of c-Jun increase its transactivational ability (Pulverer et al., 1991; Smeal et al., 1991).

The critical role of the pituitary-specific transcription factor Pit-1 in the hormonal regulation of the prolactin gene has been discussed earlier. Perhaps not surprisingly, Pit-1 also appears to be involved in mediating regulation of the growth hormone gene (Shepard et al., 1994). The mechanism by which Pit-1 mediates regulation of the prolactin and growth hormone genes remains unknown.

Many of the hormones and intracellular mediators that regulate prolactin or growth hormone transcription also regulate specific cellular protein kinases. Pit-1 itself has been shown to be phosphorylated in GC cells treated with cAMP or the phorbol ester tetradecanoylphorbol acetate (TPA) and to serve as a substrate for phosphorylation by PKA and PKC *in vitro* (Kapiloff et al., 1991) Phosphorylation has been shown to have variable effects upon the ability of Pit-1 to bind to its DNA recognition elements. Phosphorylation decreased the affinity of Pit-1 to the GH-1 element within the growth hormone promoter by 10-20 fold in one study (Kapiloff et al., 1991) and by four-fold in another (Steinfelder et al., 1992). Phosphorylation of Pit-1 had only modest effects on its ability to bind to elements of the prolactin promoter, increasing the affinity one- to three-fold for proximal promoter elements 1P and 3P and decreasing the affinity for distal enhancer elements 1D, 2D, and 4D (Kapiloff et al., 1991).

Given these findings and those in Chapter 2 which suggest that dopaminergic repression of the prolactin gene can be mediated through both PKA and Pit-1, I attempted to test the functional role of phosphorylation of Pit-1 on regulation of the prolactin and growth hormone genes. In order to test the functional role of phosphorylation of Pit-1, experiments carried out on cells lacking endogenous Pit-1 were employed to compare the abilities of a phosphorylation-deficient mutant and wild-type Pit-1 to transactivate co-transfected prolactin and growth hormone promoter constructs. The results of these studies are consistent with the model of dopaminergic repression of the prolactin gene: the phosphorylation-deficient Pit-1 exhibits approximately half of the activity of the wild-type Pit-1. Despite this difference, the phosphorylation-deficient Pit-1 retains substantial activity to stimulate basal expression of the prolactin and growth hormone promoters and the full activity of the wild-type Pit-1 to mediate estrogen receptor-mediated induction of the rat prolactin gene.

C. Materials and Methods

1. Sources and/or construction of recombinant plasmids

The preparation of plasmids (-1957)PRL-CAT and (-244)GH-CAT, referred to here as PRL-CAT and GH-CAT, have been described (Lufkin and Bancroft, 1987). The human estrogen receptor expression vector HEGO (Tora et al., 1989) was generously provided by Dr. P. Chambon. The internal control plasmid pTKGH was obtained from Nichols Institute.

Plasmids RSV-Pit-1(WT) and RSV-Pit-1(A3) were constructed using the corresponding constructs CMV-Pit-1 and CMV-Pit-1(A3), kindly supplied by Dr. M.G. Rosenfeld (Kapiloff et al., 1991). Two oligonucleotide primers were synthesized with the sequence 5'-TTCTCTACTCACTAGTGGGA-3' and 5'CTTCTGATTTTTCTAGAAAT-CAG-3', corresponding to positions -20/-1 and 943/921, respectively, of the original published Pit-1 cDNA sequence (Ingraham et al., 1988) except for the underlined nucleotides which represent alterations from the cDNA sequence designed to create restriction sites. Using these oligonucleotides as primers and CMV-Pit-1(WT) or CMV-Pit-1(A3) as template, 35 cycles of polymerase chain reaction (PCR) were performed using Vent™ polymerase (Promega) under conditions described by the manufacturer. The products from the PCR reactions were digested with Xba I and Spe I. The digested PCR products were isolated via gel eletrophoresis and ligated into the RcRSV eukaryotic expression vector (Stratagene). The Pit-1 inserts of RSV-Pit-1(WT) and RSV-Pit-1(A3) were sequenced in the Mount Sinai DNA Core facility to confirm that RSV-Pit-1(WT) codes for the correct Pit-1 amino acid sequence, and that RSV-Pit-1(A3) codes for this sequence except at positions 115, 219, and 220 where alanine substitutions have been made.

2. Culture and transfection of cells

HeLa cells were maintained in monolayer culture in a humidified/5% CO_2 atmosphere at 37°C. Except where noted otherwise, cells were grown in Dulbecco's Modified Eagle's Medium (Bio-Whittaker) supplemented with 5% fetal calf serum, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate (DMEM/5% FCS). For transfection, approximately 5 x 10^6 HeLa cells were trypsinized briefly and resuspended in 0.5 ml DMEM/5% FCS. The resuspended cells, along with DNA's for transfection, were placed in a Gene Pulser™ cuvette (Bio-Rad) with a 0.4 cm pathlength. Cells were pulsed in a Gene Pulser™ at 960 µF and 200 V and promptly diluted into 15 ml of DMEM/5% FCS. The transfected cells were distributed onto three 60 mm tissue culture dishes and harvested two days later.

In experiments with the HEGO human estrogen receptor expression vector, no estrogen was added to the medium. This was because preliminary experiments showed that estrogen addition did not further increase the activity of PRL-CAT, indicating that the DMEM/5% FCS medium contains sufficient estrogen to fully activate the estrogen receptor. Alternatively, the estrogen receptor-mediated induction of the rPRL promoter may be caused by action of the unliganded receptor (Ignar-Trowbridge et al., 1993). Cells that were used in experiments involving treatment with the estrogen antagonist tamoxifen were grown and transfected in DMEM without phenol red (Gibco/BRL). In addition, in these experiments the fetal calf serum used was treated for 30 minutes at room temperature with 0.5% (w/v) Norit and 0.05% (w/v) dextran T-70 (Sigma Chemical) to reduce the levels of estrogen. The charcoal was removed by centrifugation for 30 minutes at 10,000 x g.

3. Measurement of CAT activity and correction for transfection efficiency.

Plates of cells to be harvested were washed once with ice-cold PBS prior to collection of cells with a cell scraper in 0.75 ml ice-cold PBS. Cells were collected into a microfuge tube. The plates were then rinsed with an additional 0.75 ml cold PBS and the

rinsings added to the microfuge tube. Cells were pelleted in a microfuge (10 seconds at 16,000 x g) and resuspended in 100 µl of CAT buffer (250mM Tris-HCl pH 7.8, 10 mM EDTA). The cell suspension was disrupted by sonication on ice for eight minutes in a Fisher Sonic Dismembrator at 90% power. The cellular extracts were then incubated at 65° C. for five minutes to deactivate cellular deacetylases. The extracts were spun in a micofuge (five minutes at 16,000 x g) and the supernatant was removed and stored at -20° C.

Assays of CAT activity were performed essentially as described by Seed and Sheen (1988). Briefly, extracts were incubated in a 100 µl volume with 0.2 µCi [^{3}H]chloramphenicol (New England Nuclear), 25 µg butyryl Coenzyme A (Sigma Chemical), and 100 mM Tris-HCl pH 8.0. Assays were performed under predetermined linear conditions, with incubations from 30 minutes to six hours at 37° C. The reaction mixture was extracted once with 200 µl of 2:1 tetramethylpentadecane:xylenes and the organic phase containing the acylated product was removed to a scintillation vial. Scintillation counting was performed on a Beckman LS-1801 counter using Ecolite(+) scintillation fluid (ICN).

The plasmid pTKGH was included in all transfections as an internal control. Where indicated, samples of medium were assayed for human growth hormone using the Transient Gene Expression System (Nichols Institute). It was found, however, that RSV-Pit-1(WT) and RSV-Pit-1(A3) caused an unequal induction of the pTKGH contruct. This statistically significant induction was observed at levels of input vector $\geq$ 2 µg. Therefore, in experiments requiring the use of $\geq$ 2 µg of RSV-Pit-1(WT) or RSV-Pit-1(A3) (Figures 18 and 19), pTKGH activity was not used to correct for transfection efficiency. Instead, pooled results from four independent experiments are reported.

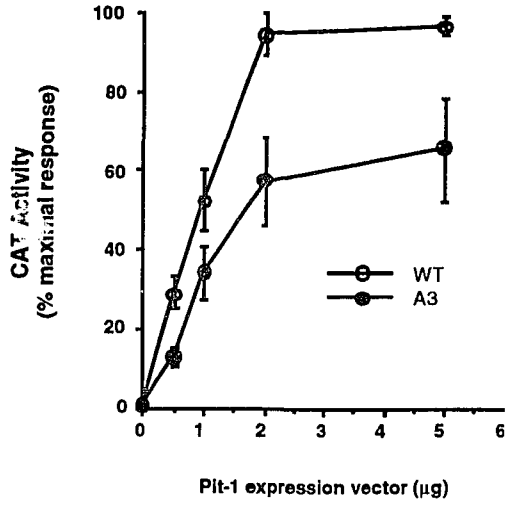
D. Results

1. A phosphorylation-dependent component of basal activation of the rPRL promoter by Pit-1

In order to determine if phosphorylation of Pit-1 in any way regulates its biological activity, a phosphorylation-deficient Pit-1 expression vector was created. Rosenfeld's group has already created such a mutant, termed Pit-1(A3), and characterized its DNA-binding ability (Kapiloff et al., 1991). This construct was obtained (as a generous gift from Dr. Rosenfeld) and subcloned into the RcRSV eukaryotic expression vector. The resulting RSV-Pit-1(A3) encodes a Pit-1 protein mutated at three putative serine/threonine kinase phosphorylation sites that, unlike the wild-type Pit-1, is no longer a substrate for phosphorylation by PKA (Kapiloff et al., 1991). A construct containing the cDNA for the wild-type Pit-1 was also cloned into the RcRSV vector to create the construct RSV-Pit-1(WT).

To determine if Pit-1(A3) is altered in its ability to transactivate the rPRL promoter, RSV-Pit-1(WT) or RSV-Pit-1(A3) were cotransfected into HeLa cells along with the PRL-CAT reporter gene. The results shown in Figure 18A represent pooled data from four such experiments. While both Pit-1(WT) and Pit-1(A3) activate PRL-CAT in a dose-dependent manner, the maximal level of activation achieved by Pit-1(A3) is less than that achieved by Pit-1(WT). However, a paired Student's t-test performed on this data failed to detect a statistically significant difference between the levels of PRL-CAT activation achieved by Pit-1(WT) and Pit-1(A3) at most concentrations. While the results of the statistical analysis were not significant ($p > 0.05$), the results did show a trend toward significance. For this reason, further transfections were performed at the saturating concentration of 5 μg of Pit-1 expression vector. The results of these further transfections are shown in Figure 18B. As before, the levels of PRL-CAT transactivation achieved by Pit-1(A3) are approximately half of those achieved by Pit-1(WT). However, a paired Student's t-test performed on these data revealed a statistically significant

A.



B.

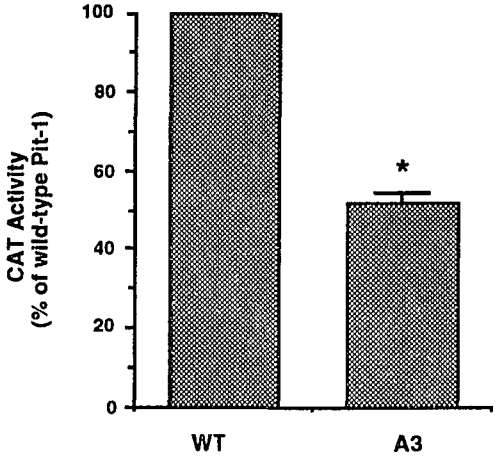


Figure 18: Ability of Pit-1(WT) and Pit-1(A3) to transactivate basal rPRL promoter activity. HeLa cells were transected via electroporation with 10 µg of PRL-CAT, 10 µg pTKGH, and either (A) the indicated amounts, or (B) 5 µg, of either RSV-Pit-1(WT) or RSV-Pit-1(A3). Cells were incubated for two days prior to harvesting for assay of CAT activity. Results in (A) represent the mean (± SE) of four independent experiments; and those in (B) the mean (± SE) of results of eight independent transfections. *, $p < 0.001$.

difference between Pit-1(WT) and Pit-1(A3) ($p < 0.001$). Therefore, while the phosphorylation-deficient mutant retains substantial activity to transactivate the rPRL promoter, the greater activity of the wild-type Pit-1 suggests the existence of a phosphorylation-dependent component to this activity.

2. A phosphorylation-dependent component of basal activation of the rGH promoter by Pit-1

Given the finding that rPRL transcription is in part dependent on the phosphorylation state of Pit-1, I wished to see if these findings extended to the rGH promoter. Therefore, analagous experiments were carried out cotransfecting a GH-CAT reporter construct along with either RSV-Pit-1(WT) or RSV-Pit-1(A3) into HeLa cells. The results shown in Figure 19A represent pooled data from four such experiments. As was seen above for the rPRL promoter, these concentration-response curves reveal a modest difference in the ability between Pit-1(WT) and Pit-1(A3) in the ability to transactivate the rGH promoter. Also as before, statistical analysis of these curves did not reveal a statistically significant difference between Pit-1(WT) and Pit-1(A3) ($p > 0.05$). Further transfections performed with 5 μg of Pit-1 expression vector yielded the data shown in Figure 19B. A paired Student's t-test of these data revealed a statistically significant difference between Pit-1(WT) and Pit-1(A3) ($p < 0.01$). Therefore, as seen with the rPRL promoter, a component of the activity of Pit-1 to transactivate the rGH promoter appears to be phosphorylation-dependent.

3. The Pit-1-dependent induction of the rPRL promoter mediated by the estrogen receptor is independent of phosphorylation of Pit-1 at its known phosphorylation sites.

Given that there is only a modest, though statistically significant, difference between Pit-1(WT) and Pit-1(A3) under conditions of basal expression of the rPRL and

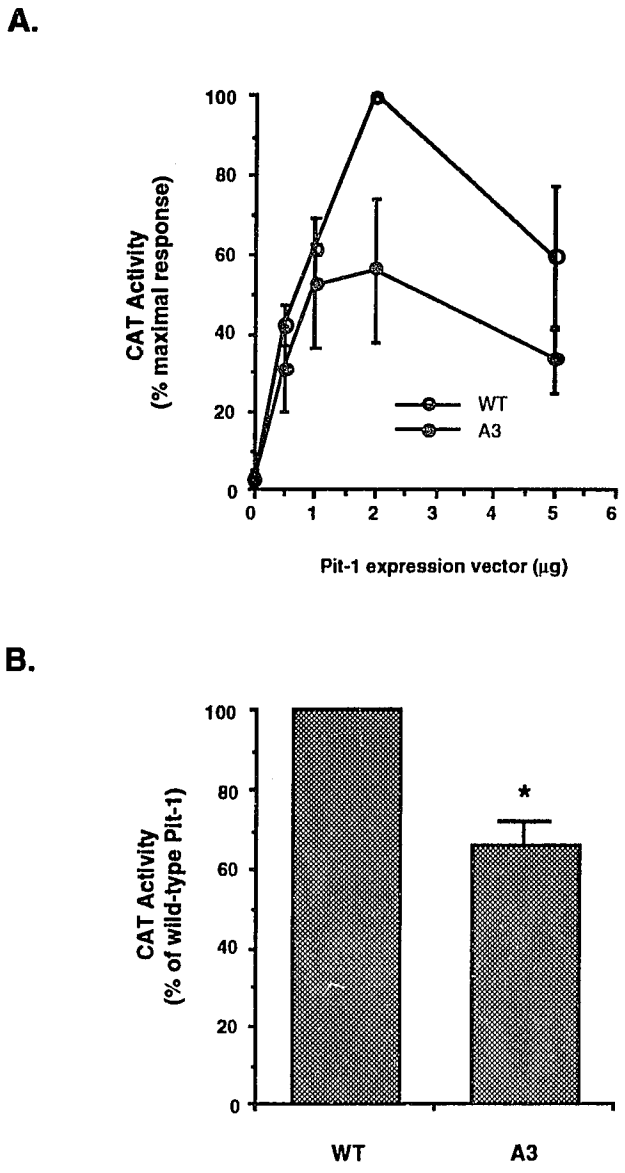


Figure 19: Ability of Pit-1(WT) and Pit-1(A3) to transactivate basal rGH promoter activity. HeLa cells were transfected via electroporation with 10 μg of GH-CAT, 10 μg pTKGH, and either (A) the indicated amounts, or (B) 5 μg, of either RSV-Pit-1(WT) or RSV-Pit-1(A3). Cells were incubated for two days prior to harvesting for assay of CAT activity. Results in (A) represent the mean (±SE) of four independent experiments; and those in (B) the mean (± SE) of results of four independent transfections. *, $p < 0.01$.

rGH promoters, I asked whether the PKA phosphorylation sites of Pit-1 might play a greater role in hormonal regulation of the rPRL promoter. The prolactin-regulating hormone chosen for study was estrogen. Estrogenic induction of the rPRL promoter has been shown to require both Pit-1 and the estrogen receptor (Day et al., 1990).

For these studies of estrogenic induction of the rPRL promoter, HEGO, an expression vector of the human estrogen receptor (Tora et al., 1989) was employed. When cotransfected into HeLa cells, which are naturally estrogen receptor-deficient, along with PRL-CAT and Pit-1(WT), this construct yields a greater than twenty-fold induction of PRL-CAT in estrogen-containing medium (Figure 20A). Of note, there is no detectable difference in the levels of induction between cells transfected with Pit-1(WT) and Pit-1(A3) . From these data, it appears that the mutated phosphorylation sites in Pit-1(A3) play no role in mediating the Pit-1-dependent induction of the rPRL promoter by the estrogen receptor.

It is possible, however, that I failed to detect any difference between Pit-1(WT) and Pit-1(A3) in estrogenic induction of PRL-CAT merely because the levels of estrogen receptor achieved by transient transfection were so great as to overwhelm any putative differences between Pit-1(WT) and Pit-1(A3). In order to address this question, concentration-response curves of HEGO transfected into HeLa cells along with PRL-CAT and a submaximal amount (Figure 18A) of either RSV-Pit-1(WT) or RSV-Pit-1(A3) were performed. The results shown in Figure 20B reveal that even at submaximal amounts of HEGO, there is no detectable difference between the estrogen receptor-mediated inductions seen with Pit-1(WT) and Pit-1(A3).

In further characterizing the estrogen receptor-mediated induction of the rPRL promoter in this system, I sought to determine that the induction exhibited the characteristics expected of a classical estrogenic induction. To attempt to demonstrate that the estrogen antagonist tamoxifen could block the induction, transfection experiments were performed in medium containing serum that had been stripped of

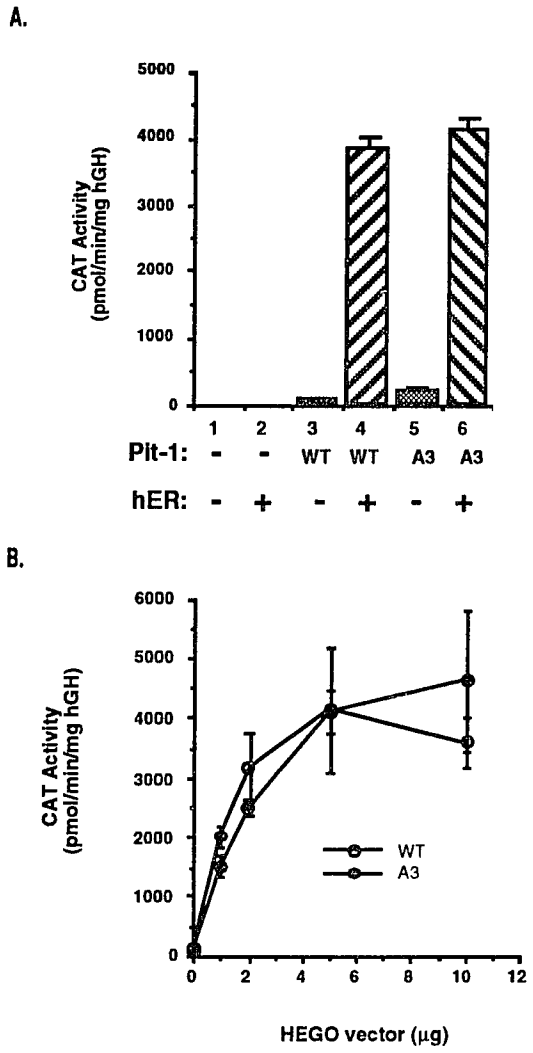


Figure 20: Ability of Pit-1(WT) and Pit-1(A3) to mediate estrogen receptor induction of rPRL promoter activity. HeLa cells were transfected via electroporation with 10 μg of PRL-CAT, 10 μg of pTKGH, and either (A) ± 1 μg of either RSV-Pit-1(WT) or RSV-Pit-1(A3), ± 10 μg HEGO (hER), or (B) 1 μg of either RSV-Pit-1(WT) or RSV-Pit-1(A3) plus the indicated amounts of HEGO. Following a two day incubation, cell extracts and media were assayed for, respectively, CAT activity and hGH levels. Results represent the mean ± SE of results with triplicate dishes.

steroids using a charcoal and dextran treatment. However, as has been reported by Tora et. al. (Tora et al., 1989), this treatment fails to completely remove all estrogens. This is suggested by the data shown in Figure 21 in which cells incubated in medium containing charcoal/dextran treated serum were transfected with PRL-CAT, HEGO, and RSV-Pit-1(WT) or RSV-Pit-1(A3). Under these conditions, the presence of the estrogen receptor and either Pit-1 isoform causes an induction of PRL-CAT in the absence of added estrogen. This induction is blocked to a great extent by the estrogen antagonist tamoxifen. Of note, these results do not rule out the possibility that the induction seen here is mediated by an unliganded estrogen receptor. Nonetheless, the data in Figure 21 again demonstrate that there is no difference in the ability of Pit-1(WT) and Pit-1(A3) to participate in the estrogen receptor-mediated induction of PRL-CAT.

Recent attention has been drawn to the phenomenon of transcriptional induction by liganded estrogen receptor that is mediated via a cryptic AP-1 site within the pUC series of promoters (Kushner et al., 1994). For this reason, I wished to determine whether the estrogen receptor-mediated induction seen with Pit-1 and Pit-1(A3) was dependent on the presence of the well characterized estrogen-response element (ERE) of the prolactin distal enhancer. Dependence of the induction on the presence of a DNA region containing the ERE is demonstrated in Figure 22 where it can be seen that PRL-CAT is induced by HEGO but a shortened prolactin reporter construct lacking the ERE-containing enhancer region, (-187)PRL-CAT, is not induced. The lack of estrogenic induction of (-187)PRL-CAT is not due to a failure of this construct to be expressed. Basal levels of expression of (-187)PRL-CAT, while substantially lower than those of the enhancer-containing contruct PRL-CAT, were two- to three-fold above background in this experiment. From these data I conclude that the estrogen receptor-mediated induction observed in this series of experiments is mediated via an element within the rPRL distal enhancer region (most likely the classical ERE of the distal enhancer) and not via any pUC12 vector sequences as has been described elsewhere (Kushner et al., 1994).

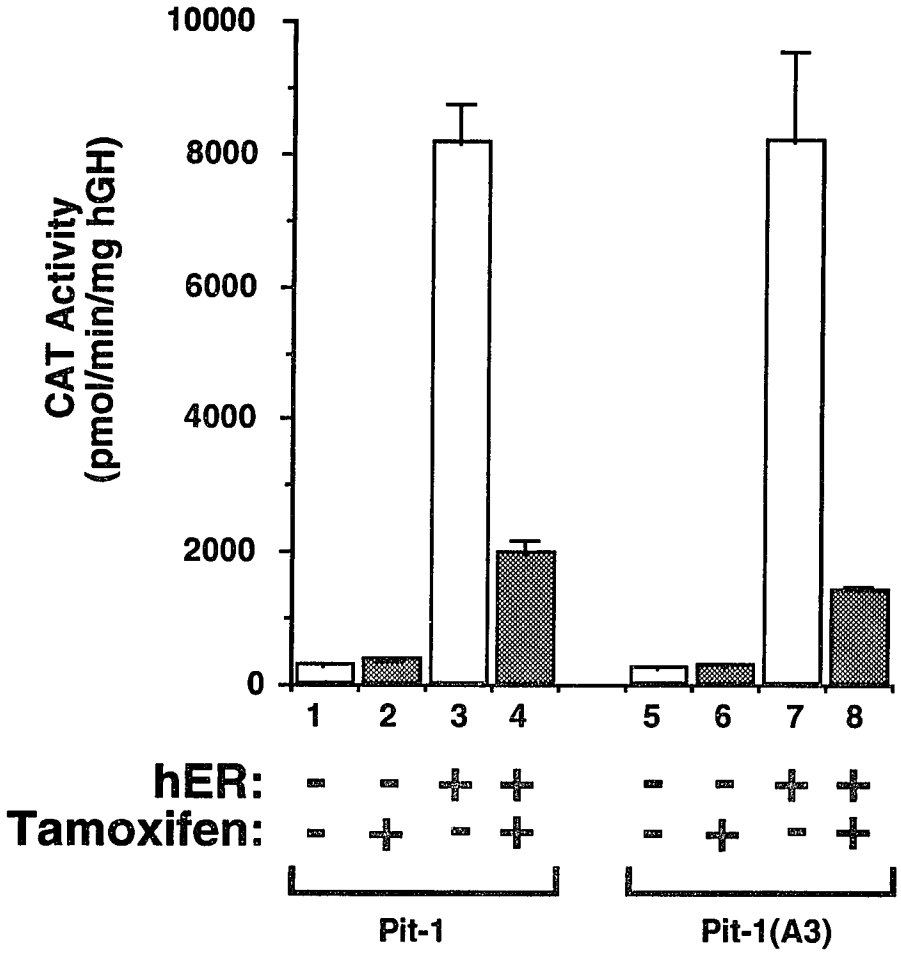


Figure 21: Ability of tamoxifen to inhibit the estrogen receptor induction of rPRL promoter activity mediated by Pit-1(WT) or Pit-1(A3). HeLa cells were transfected via electroporation with 10 μg of PRL-CAT, 10 μg of pTKGH, plus 1 μg of either RSV-Pit-1(WT) or RSV-Pit-1(A3), ± 10 μg HEGO (hER). Following a two day incubation ± 1 μM tamoxifen, cell extracts and media were assayed for, respectively, CAT activity and hGH levels. Results represent the mean ± SE of results with triplicate dishes.

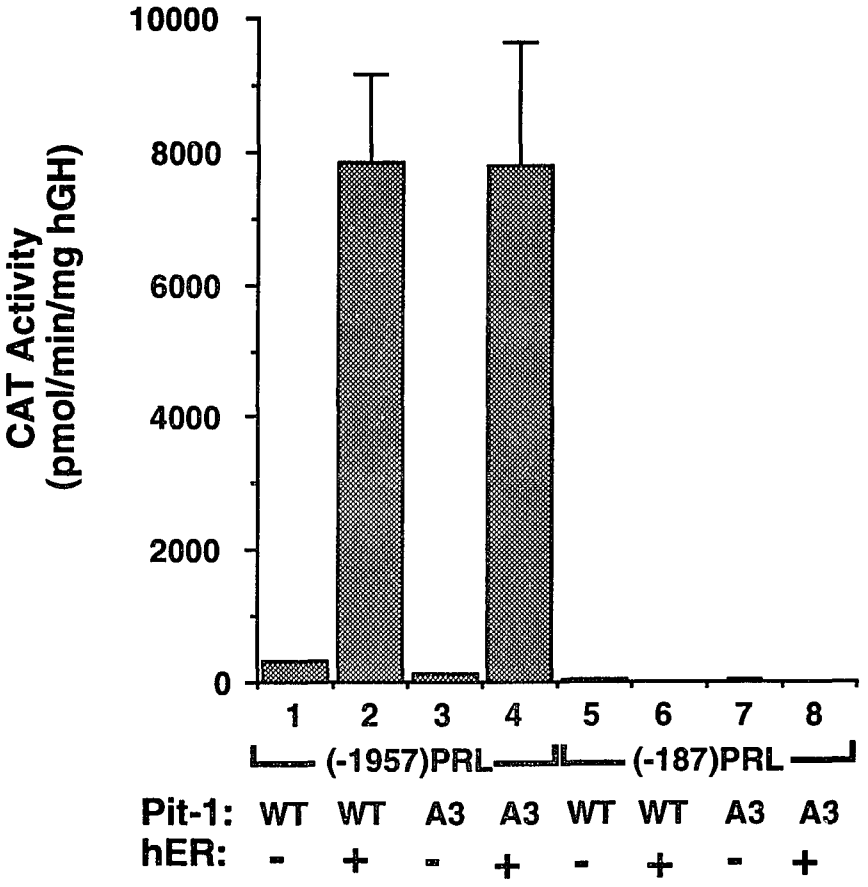


Figure 22: Requirement for the rPRL gene distal enhancer for a response to the estrogen receptor mediated by Pit-1(WT) or Pit-1(A3). HeLa cells were transfected with 10 μg pTKGH, plus 10 μg of either PRL-CAT or GH-CAT, plus 1 μg of either RSV-Pit-1(WT) or RSV-Pit-1(A3) ± 10 μg HEGO (hER). Following a two day incubation, cell extracts and media were assayed for, respectively, CAT activity and hGH levels. Results represent the mean ± SE of results with triplicate dishes.

E. Discussion

In this chapter I have addressed the question of what effect, if any, phosphorylation of Pit-1 has upon its ability to activate target genes. This question is raised by the model of dopaminergic repression of the prolactin promoter presented in Chapter 2. The model proposes that dopaminergic repression of prolactin transcription is mediated through both PKA and Pit-1. To date, however, no functional role has been established for phosphorylation of Pit-1.

The question of what effect phosphorylation has on Pit-1 activity has also been raised by a number of observations in the literature suggesting, but not demonstrating, a role for phosphorylation in the transactivational activity of Pit-1. First, calcium (Hoggard et al., 1991; Yan and Bancroft, 1991), cAMP (Iverson et al., 1990), and phorbol esters (TPA) (Supowit et al., 1984; Iverson et al., 1990) as well as the hormones TRH (Yan et al., 1991), EGF (Supowit et al., 1984), and dopamine (Lew et al., 1994) have been shown to mediate their effects on prolactin transcription at least partially through Pit-1 binding sites. All of these effectors are capable of regulating the activity of specific cellular kinases. Second, Pit-1 has been shown to be phosphorylated in cells treated with either cAMP or TPA as well as serve as a substrate for phosphorylation *in vitro* by PKA or PKC (Kapiloff et al., 1991). Third, phosphorylation has been reported to alter the DNA-binding ability of Pit-1 (Kapiloff et al., 1991; Steinfelder et al., 1992). And fourth, inhibition of PKA in GH_3 cells has been shown to reduce both unstimulated and hormone stimulated prolactin transcription (Day et al., 1989). Taken together, these findings suggest that phosphorylation of Pit-1 might play a role in regulating its activity. However, the results presented in this chapter represent the first study of the functional role of phosphorylation of Pit-1, that is, of its ability to transactivate target genes.

In initiating these studies I have relied on the identification by Rosenfeld's group of the major phosphorylation sites of Pit-1 (Kapiloff et al., 1991). This group identified three residues (alanine^{115}, threonine^{219}, and threonine^{220}) that were observed to be

phosphorylated by PKA *in vitro*. Of note, these sites were also observed to be phosphorylated in basal GC cells (Kapiloff et al., 1991). The Pit-1(A3) mutant used in these studies has had all three of these residues changed to alanine, and is no longer a substrate for phosphorylation by PKA (Kapiloff et al., 1991).

Despite the fact that the Pit-1(A3) mutant was phosphorylation-deficient, it was found to exhibit significant activity in its ability to activate both PRL-CAT and GH-CAT basal expression. The greater activity of the wild-type Pit-1, however, does suggest the existence of a phosphorylation-dependent component to this activity. This component is estimated from these results to be approximately 50% and 35% for the activation of the prolactin and growth hormone promoters, respectively. Interestingly, the 50% phosphorylation-dependent component of the activity of Pit-1 in transactivation of the prolactin promoter correlates well with the 50-70% dopaminergic repression of prolactin transcription reported in Chapter 2 and by others (Maurer, 1981; Elsholtz et al., 1991). The observation of this phosphorylation-dependent component of the activity of Pit-1 on the prolactin promoter is consistent with the model proposed in Chapter 2 (Figure 17). This model proposes that dopaminergic repression can be mediated via inhibition of adenylyl cyclase, reduction of intracellular levels of cAMP, reduced PKA activity, and a decrease in the phosphorylated form of Pit-1, leading to a 50-70% reduction in prolactin gene transcription. Of course, these results do not rule out the possibility that dopaminergic repression of the prolactin gene is mediated at least partially through another pathway. As mentioned in Chapter 2, it is possible, although it has not been demonstrated, that dopamine may regulate overall Pit-1 levels in lactotrope cells.

While the findings in this chapter are consistent with the model proposed for dopaminergic repression of prolactin transcription, they leave largely unexplained the effects of many of the activators of prolactin transcription, the effects of which are often of much greater than two- to three-fold in magnitude. Clearly, inductions of prolactin transcription of large magnitude cannot be accounted for entirely by the modest

phosphorylation-dependent component of Pit-1 transactivational activity demonstrated here. One possible explanation could be that Pit-1 is phosphorylated at sites other than those mutated in Pit-1(A3). However, Kapiloff et. al. (1991) have reported that Pit-1 is not phosphorylated *in vitro* by either casein kinase II or calcium-calmodulin-dependent protein kinase II. In addition, tryptic phosphopeptide analysis by Kapiloff et. al. (1991) strongly suggests that the sites mutated in Pit-1(A3) are also sites for phosphorylation by PKC. It remains to be shown, however, that Pit-1(A3) is no longer a substrate for PKC. It is still possible, although it has not been shown, that Pit-1 is phosphorylated at sites other than those mutated in Pit-1(A3) by some as yet unidentified kinase.

A second possibility to explain large magnitude inductions is that while phosphorylation of Pit-1 greatly increases its ability to transactivate the prolactin promoter, in unstimulated HeLa cells, Pit-1 exists primarily in a dephosphorylated form. According to this model, stimulation of HeLa cells by activators of protein kinases would be expected to unmask differences between Pit-1(WT) and Pit-1(A3). Results obtained by X. Chen, another student in the Bancroft laboratory, challenge this notion. He has found that Pit-1(WT) and Pit-1(A3) are equally effective in mediating induction of PRL-CAT by either forskolin or TPA in AtT20 cells (Fischberg et al., 1994). This observation shows that even under conditions where PRL-CAT is induced by agents known to activate PKA or PKC, the induction does not rely upon phosphorylation of Pit-1 at any of its known phosphorylation sites.

A third possibility is that large magnitude inductions of prolactin transcription are mediated in part via a factor other than Pit-1. This putative factor may interact, functionally and possibly physically, with Pit-1 to increase transcription. An interaction with Pit-1 would explain why the response elements of the inducing agents map to Pit-1 binding sites. An example of such a situation is the cooperation between the estrogen receptor and Pit-1 in mediating estrogenic induction of prolactin transcription. While no direct physical interaction between Pit-1 and the estrogen receptor has been demonstrated

to date, estrogenic induction of prolactin transcription requires both the estrogen receptor and Pit-1 as well as DNA-binding sites for both (Day et al., 1990). Perhaps a pituitary-specific or ubiquitous factor(s) similarly cooperates with Pit-1 in mediating other hormonal inductions of the prolactin gene.

Finally, as mentioned above, the exact nature of the requirement for Pit-1 for estrogen receptor-mediated activation of the prolactin promoter is unknown. Nonetheless, the data presented here demonstrate that this requirement for Pit-1 does not rely on Pit-1 being phosphorylated at any of its known phosphorylation sites.

IV. Chapter 4: Functional Studies of Pit-1 Isoforms

A. Summary

In this chapter, the results of functional studies of isoforms of the pituitary-specific transcription factor Pit-1 are presented. The *pit-1* gene has been shown to produce at least four products: Pit-1, Pit-1a, Pit-1T, and Δ4Pit-1. My results suggest that while both Pit-1 and Pit-1a are capable of transactivating either the rat prolactin (rPRL) or rat growth hormone (rGH) promoters, Δ4Pit-1, by itself, activates neither promoter. Additionally, I have confirmed the reported function of Δ4Pit-1 as a repressor of the rPRL promoter. In addition, preliminary evidence suggests that Δ4Pit-1 may exert a permissive role in allowing expression of the rGH gene in somatomammotropes.

B. Introduction

A growing number of transcription factors have been found to be expressed as functionally distinct isoforms. The cAMP regulatory element modulator protein (CREM) is an example of such a transcription factor. Through alternative RNA splicing of two transactivation domains, various isoforms are created that may activate (CREMτ) or repress (e.g. CREMα, CREMβ, or CREMγ) target genes (de Groot and Sassone-Corsi, 1993).

The transcript of the gene for the pituitary-specific transcription factor Pit-1 is also subject to alternative splicing. As mentioned in Chapter 1, the rat pituitary-specific transcription factor Pit-1 was originally identified in 1988 (Bodner et al., 1988; Ingraham et al., 1988). Four years passed before the Pit-1a isoform was identified (Konzak and Moore, 1992; Morris et al., 1992; Theill et al., 1992). The Pit-1a isoform contains an in-frame insert encoding an additional 26 amino acids at amino acid 48. This insert occurs in the region of Pit-1 previously identified as a modular transactivation domain (Theill et al., 1989; Ingraham et al., 1990). Shortly after the identification of Pit-1a, a murine thyrotrope-specific isoform, Pit-1T, was reported (Haugen et al., 1993). Pit-1T contains a 14 amino acid insert which is identical to the carboxy terminal 14 amino acids of the Pit-1a insert. Haugen et. al. have reported that this thyrotrope-specific isoform can cooperate with Pit-1 to induce the murine thyroid-stimulating hormone β-subunit promoter (Haugen et al., 1993). Most recently, several groups have identified a rat Pit-1 isoform, Δ4Pit-1, that has an in-frame deletion of 54 amino acids in the POU-specific domain (Voss et al., 1993; Day and Day, 1994; Kloss et al., in prep). Transcipts for the Pit-1a, Pit-1T, and Δ4Pit-1 isoforms have been identified in both rodent pituitary and pituitary-derived cell lines (Konzak and Moore, 1992; Morris et al., 1992; Theill et al., 1992; Haugen et al., 1993; Voss et al., 1993; Day and Day, 1994; Kloss et al., in prep). Protein expression of Pit-1a, Pit-1T, and Δ4Pit-1 has, thus far, been reported in pituitary-derived cell lines only (Konzak and Moore, 1992; Haugen et al., 1993; Day and Day, 1994; Kloss et al., in

prep). It remains to be shown, therefore, that the protein of any of these isoforms is expressed in the anterior pituitary.

I report here the results of studies on the function of two of these isoforms, Pit-1a and Δ4Pit-1. These studies were carried out in close collaboration with B. Kloss, a fellow student in the Bancroft laboratory. We find that while Pit-1a retains some activity to transactivate the rPRL and rGH promoters, Δ4Pit-1, by itself, does not transactivate either promoter. Further studies on the function of Δ4Pit-1 suggest it can act both as a repressor of the rPRL promoter and, possibly, can exert a permissive effect on the rGH promoter.

C. Materials and Methods

1. Source of recombinant plasmids

The (-1957)PRL-CAT and (-244)GH-CAT reporter constructs (referred to here as PRL-CAT and GH-CAT) and RSV-CAT have been described (Gorman et al., 1982; Lufkin and Bancroft, 1987). The RSV-Pit-1 expression vector was a generous gift of Dr. H.H. Samuels (New York University School of Medicine, New York, NY) and has been described (Fox et al., 1990). Briefly, this construct contains the complete Pit-1 cDNA cloned into the Hind III/Kpn I sites of the Rous sarcoma virus expression vector RSVi$^-$ (Forman et al., 1988). The RSV-Pit-1a and RSV-Δ4Pit-1 expression vectors were obtained from B. Kloss in the Bancroft laboratory. The RSV-Pit-1a construct contains the previously described Pit-1a cDNA (Morris et al., 1992) cloned into the Hind III/Kpn I sites of RSVi$^-$. The Δ4Pit-1 cDNA was originally cloned by Dr. A.E. Jackson of the Bancroft laboratory using Pit-1-specific primers and polymerase chain reaction amplification of GH_3 cell mRNA. The Δ4Pit-1 cDNA was found to encode a peptide identical to Pit-1 with the exception of a 54 amino acid deletion in the POU-specific domain (Kloss et al., in prep). This cDNA was also cloned into the Hind III/Kpn I sites of RSVi$^-$. The control vectors RcRSV and pTKGH were obtained from Stratagene and Nichols Institute, respectively.

2. Culture and transfection of cells

All cells were grown at 37° C. in a humidified 5% CO_2 atmosphere. GH_3 and HeLa cells were grown in monolayer in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (DMEM/5% FCS). MMQ cells were grown in T-flasks in RPMI/1640 medium supplemented with 12.5% horse serum, 2.5% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (RPMI/complete).

Transfection via electroporation, where indicated, was performed as follows. Approximately 1-2 x 10^7 cells were resuspended with DNA's for transfection in 0.5 ml of DMEM/5% FCS and electroporated (960 μF, 200 V) in a Gene Pulser™ cuvette (Bio-Rad) with a 0.4 cm pathlength in a Gene Pulser™ (Bio-Rad). Immediatedly after transfection, the cells were diluted into the appropriate growth medium and aliquoted into tissue culture dishes.

Transfection of GH_3 cells via the diethylaminoethyl-dextran (DEAE-dextran) technique, where indicated, was performed as described (Yan and Bancroft, 1991). Transfection of HeLa cells via calcium phosphate precipitation, where indicated, was performed according to the basic protocol of Kingston (1990).

3. Measurement of CAT activity

Except where otherwise indicated, cells were harvested two days following transfection and cellular extracts were prepared and assayed for CAT activity as described (Jackson and Bancroft, 1988). During the course of this work, a phase extraction assay was adopted, where indicated, due to its rapidity and decreased expense. It was determined that equivalent results are obtained by either assay method. The phase extraction assay was performed essentially as described by Seed and Sheen but with the omission of unlabeled chloramphenicol in order to maximize specific activity (Seed and Sheen, 1988). In this assay, CAT activity is measured not as % conversion of [^{14}C]-chloramphenicol to acetylated products but as the rate of conversion of [^{3}H]-chloramphenicol to acylated products. Phase extraction assays were performed for 0.5-6 hours at 37° C, under predetermined linear conditions. Where indicated, the internal control plasmid pTKGH was included in transfections and human growth hormone activity was assayed using a Transient Gene Expression System (Nichols Institute) to correct for transfection efficiency.

D. Results

1. Relative activities of three Pit-1 isoforms in transactivation of the rPRL and rGH promoters

A collaborative effort with B. Kloss, a fellow student in the Bancroft laboratory, was undertaken to study the function of the three Pit-1 isoforms, Pit-1, Pit-1a and Δ4Pit-1. I first examined the ability of each of these isoforms to transactivate the rPRL and rGH promoters. Constructs containing cDNA's encoding each isoform cloned into the identical background vector under the control of the RSV promoter were obtained from B. Kloss (Kloss et al., in prep). These three constructs, RSV-Pit-1, RSV-Pit-1a, and RSV-Δ4Pit-1, were then individually transfected into HeLa cells along with either GH-CAT or PRL-CAT reporter constructs. As shown in Figure 23, Pit-1 is much more effective in transactivating both PRL-CAT and GH-CAT than either of the other two isoforms. However, the Pit-1a isoform posseses some activity in that expression of GH-CAT and PRL-CAT is approximately two-fold above background. While similar results have been obtained in one (GH-CAT) and two (PRL-CAT) other experiments (data not shown), the small, but consistent, activation by the Pit-1a isoform failed to achieve statistical significance ($p > 0.05$). The consistency of the finding, however, suggests that further experiments would provide a large enough sampling to achieve statistical significance. Indeed, Konzak and Moore (1992) have reported that Pit-1a is transcriptionally active on both the rGH and rPRL promoters. In addition, Theill et. al. (1992), while unable to demonstrate transactivation by Pit-1a of the rPRL promoter, also found Pit-1a could transactivate the rGH promoter. From my preliminary results, the rank order potency for transactivation of both the rGH and rPRL promoters appears to be Pit-1 >> Pit-1a > Δ4Pit-1 = inactive.

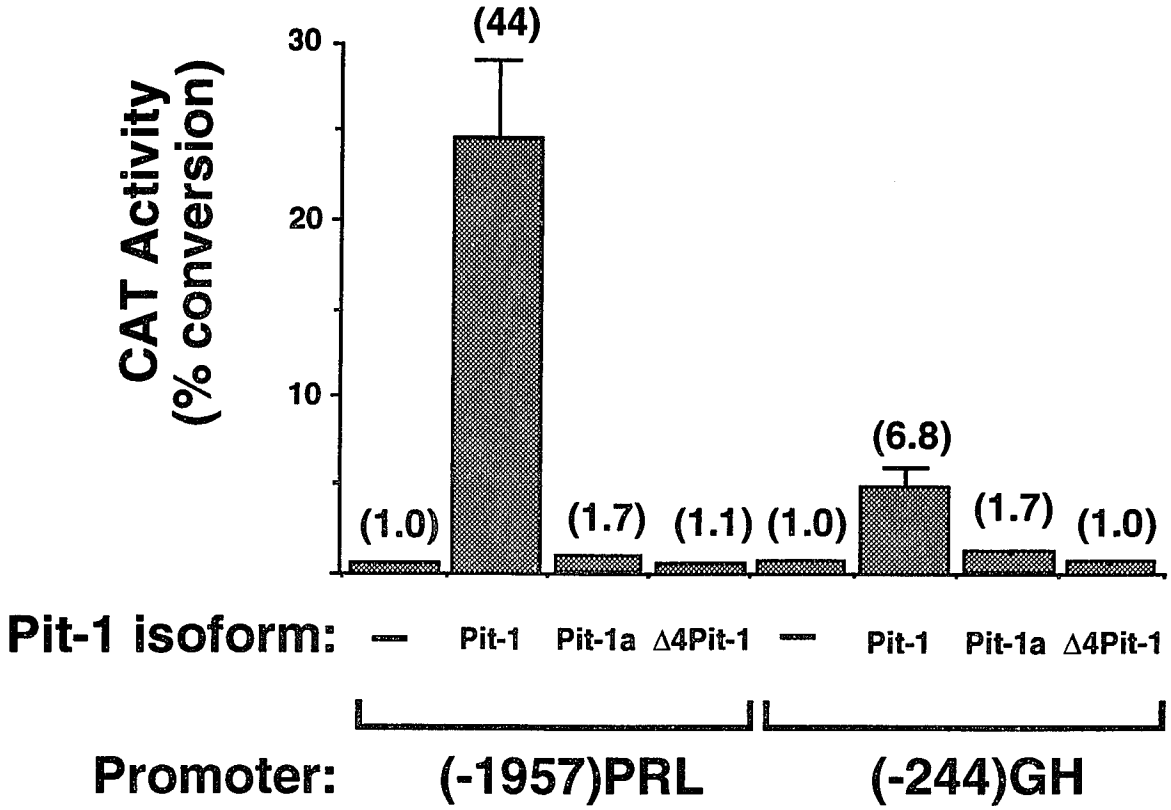


Figure 23: Pit-1 and Pit-1a transactivate both the rPRL and rGH promoters in HeLa cells while Δ4Pit-1 transactivates neither promoter. HeLa cells were transfected via calcium phosphate with 5 μg of either PRL-CAT or GH-CAT and 5 μg of either RSV-Pit-1, RSV-Pit1a, RSV-Δ4Pit-1, or empty vector as indicated. Cells were incubated for one day prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates. The numbers in parentheses represent the fold-activation relative to that seen with the empty vector.

2. Studies of the possible role of Δ4Pit-1 as a repressor of the rPRL promoter

The inability of Δ4Pit-1 to transactivate either the rPRL or rGH promoters suggests that if Δ4Pit-1 plays a role in regulation of either of these promoters, that role may be negative. That is, Δ4Pit-1 might act as a repressor of the rPRL or rGH genes. In an attempt to discover such a role, GH_3 cells were transfected, via DEAE-dextran, with the PRL-CAT reporter and increasing amounts of either RSV-Pit-1 or RSV-Δ4Pit-1. The results shown in Figure 24 demonstrate that while increasing amounts of RSV-Δ4Pit-1 caused a reduction in the activity of the PRL-CAT reporter, this effect was also seen with RSV-Pit-1. The inhibitory effect of the Pit-1 expression vectors may represent a squelching effect on PRL-CAT but could also merely represent an inhibitory effect of bulk DNA which has been observed with the DEAE-dextran transfection procedure (data not shown). Nonetheless, the data in Figure 24 fail to support a role for Δ4Pit-1 as a specific repressor of the rPRL promoter.

Recently, Day and Day have reported that Δ4Pit-1 can act as a repressor of the rPRL promoter (Day and Day, 1994). They have shown that Δ4Pit-1 is capable of repressing, by approximately 50%, the activity of a prolactin reporter construct in both GH_3 cells and Rat1 cells. In reporting this repression, Day and Day reported, but did not show, that no repression was seen with a Pit-1 expression vector (Day and Day, 1994). Because this report contradicted the data shown in Figure 24, I attempted to repeat the findings of Day and Day in HeLa cells. Because I suspected a bulk DNA effect as being responsible for the repression mediated by Pit-1, I chose to use electroporation, which is less sensitive to these effects, to transfect HeLa cells with PRL-CAT and increasing concentrations of RSV-Pit-1 and RSV-Δ4Pit-1. The results shown in Figure 25 show that PRL-CAT activity is repressed approximately 75% by increasing amounts of RSV-Δ4Pit-1 yet induced by increasing amounts of RSV-Pit-1. These data support the findings of Day and Day (1994) that Δ4Pit-1 can act as a repressor of the prolactin promoter.

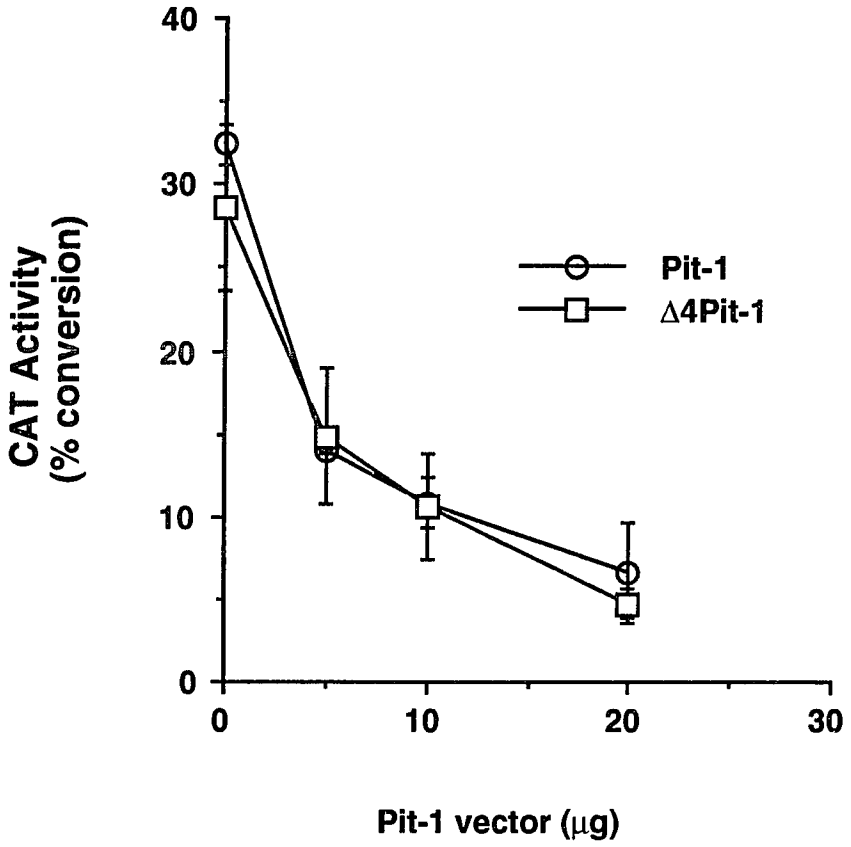


Figure 24: Δ4Pit-1 fails to repress specifically rPRL promoter activity. GH_3 cells were transfected via DEAE-dextran with 5 μg of PRL-CAT and the indicated amounts of either RSV-Pit-1 or RSV-Δ4Pit-1. Cells were incubated for two days prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion of [^{14}C]-chloramphenicol to acetylated products observed with triplicate plates.

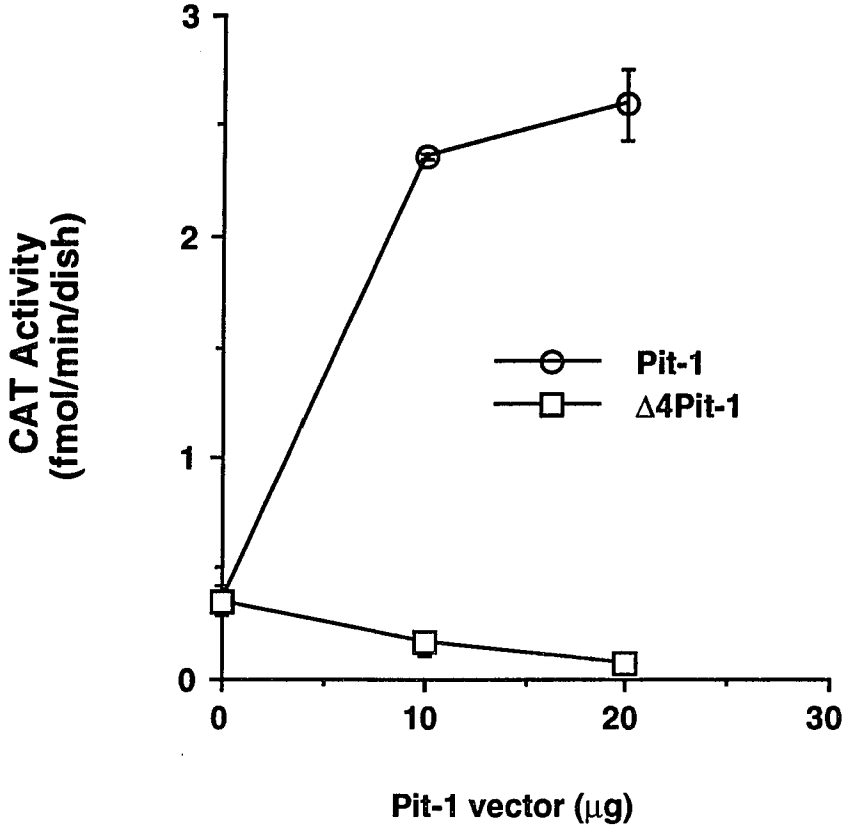


Figure 25: Δ4Pit-1 represses rPRL promoter activity in HeLa cells. HeLa cells were transfected via electroporation with 10 μg of PRL-CAT, 10 μg of pTKGH, 2 μg of RSV-Pit-1 and the indicated amounts of RSV-Δ4Pit-1 or additional RSV-Pit-1. Cells were incubated for two days prior to harvesting for assay of CAT activity. Results represent the mean (± SE) conversion rate of [^{3}H]-chloramphenicol to acylated products observed with triplicate plates.

3. A possible role for Δ4Pit-1 in activation of the rGH promoter

In further studies of the distribution of the Pit-1 isoforms, it was observed by B. Kloss that while Pit-1 and Pit-1a are both expressed in GH_3, 235-1, and MMQ cells, Δ4Pit-1 is not expressed in the MMQ and 235-1 cell lines (Kloss et al., in prep). This differential expression lead us to look for potential differences in the expression of the prolactin and growth hormone genes among these cell lines. B. Kloss has demonstrated that cells of the GH_3 cell line contain transcripts for both prolactin and growth hormone, and are therefore accurately characterized as somatomammotropes (Kloss et al., in prep). However, the 235-1 and MMQ cell lines, which synthesize and secrete prolactin, express no growth hormone transcripts, and are therefore absolute lactotropes (Kloss et al., in prep). That is, in these cell lines, expression of the growth hormone gene is correlated with the presence of the Δ4Pit-1 isoform.

I thus investigated whether the differences in growth hormone mRNA levels between somatomammotropes and absolute lactotropes were the result of differential levels of transcription of the growth hormone gene or post-transcriptional effects. To answer this question, transient transfections were performed comparing the expression of GH-CAT and PRL-CAT reporter constructs in MMQ and GH_3 cells. The 235-1 cell line proved resistant to transfection by multiple protocols (data not shown). The results shown in Table 1 demonstrate that expression of GH-CAT is considerably weaker in MMQ cells than in GH_3 cells. The ratio of expression of PRL-CAT:GH-CAT was 9.4:1 in GH_3 cells and 55:1 in MMQ cells.. The minimal expression seen with the transfected GH-CAT construct in MMQ cells suggests that the failure to observe transcripts of the endogenous growth hormone gene in these cells is due, at least in part, to a transcriptional effect.

The observation that expression of growth hormone is correlated with the expression of the Δ4Pit-1 isoform led me to attempt to reconstitute growth hormone gene expression in an absolute lactotrope cell line by artificial expression of Δ4Pit-1. MMQ

Promoter	Activity		Ratio
	(pmol/min/mg hGH) ($x10^{-2}$)	(relative to RSV)	(PRL/GH)
GH3 cells			
PRL	283 ± 21.6	1.5	
GH	29.8 ± 2.5	0.16	9.4
MMQ cells			
PRL	43.5 ± 3.6	1.1	
GH	0.94 ± 0.26	0.02	55

Table 1: Activation of the rGH promoter is minimal in MMQ cells compared to activation in GH_3 cells. GH_3 and MMQ cells were transfected via electroporation with 40 μg of either PRL-CAT, GH-CAT, or RSV-CAT and 10 μg of pTKGH. Following a one day incubation, medium and cells were harvested for assay of human growth hormone and CAT activity, respectively. Results represent the mean (± SE) rate of conversion of [^{3}H]-chloramphenicol to acylated products from triplicate dishes of cells corrected for transfection efficiency.

cells were transfected with the GH-CAT reporter construct alone or with RSV-Pit-1 or RSV-Δ4Pit-1. The results of a single, preliminary experiment are shown in Figure 26. While the level of activation of the GH-CAT reporter is quite low in this experiment, it is increased approximately five-fold by cotransfection with RSV-Δ4Pit-1. While these data are consistent with a role for Δ4Pit-1 in permitting expression of the rGH promoter, further experiments are required to establish such a role.

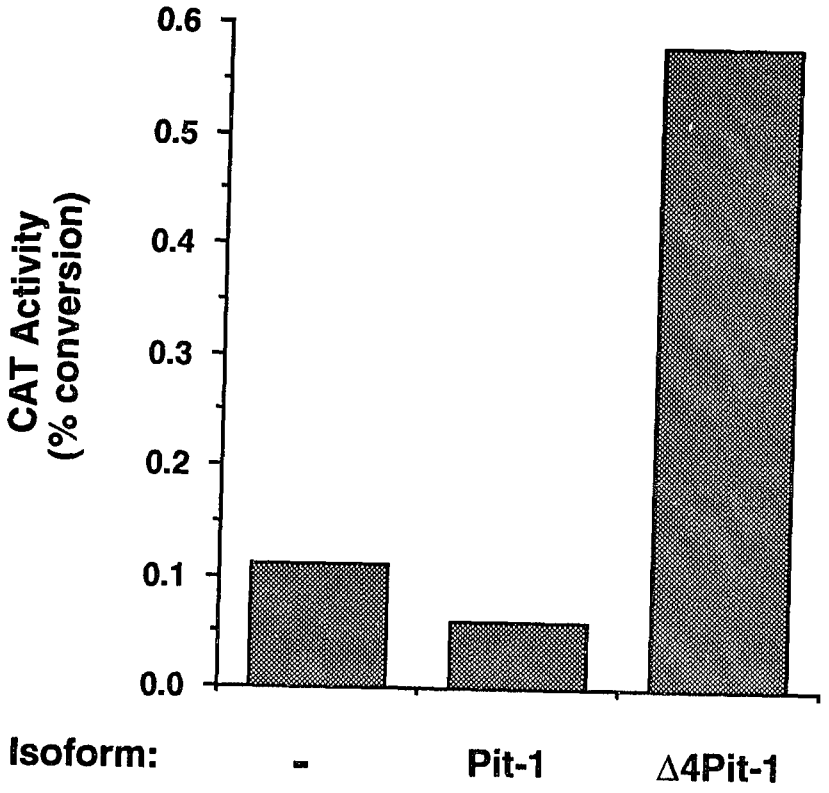


Figure 26: GH-CAT activity is induced by expression of Δ4Pit-1 in MMQ cells. Approximately 2 x 10^7 MMQ cells were transfected via electroporation with 60 μg of GH-CAT ± 30 μg of RSV-Pit-1 or RSV-Δ4Pit-1 as indicated. Cells were incubated for two days prior to harvesting for assay of CAT activity. Results represent the conversion of [^{14}C]-chloramphenicol to acetylated products observed with individual transfections.

E. Discussion

The results presented in this chapter suggest that while both Pit-1 and Pit-1a are able to transactivate the rPRL and rGH promoters, Δ4Pit-1, while possibly exerting a permissive effect on GH expression in MMQ cells, cannot transactivate either promoter and can act as a repressor on the rPRL promoter. However, a difficulty arises in trying to compare directly the activities of the three isoforms. We were unable to measure the levels of any of the isoforms in our transfected cells by immunoblot and therefore could not take into account any putative differences in the level of expression of the three isoforms in our analysis (Kloss et al., in prep). While identical background vectors were used to express each isoform, this by no means ensures that equivalent levels of protein are expressed. Indeed, Morris et. al. have reported that the Pit-1a isoform is substantially less stable than the Pit-1 isoform in CHO cells (Morris et al., 1992). However, Konzak and Moore (1992) have concluded, based on transfection analysis and quantitation of isoform protein levels by immunoblot, that Pit-1a exhibits the full activity of Pit-1 in transactivation of the rPRL promoter, and that Pit-1a is in fact more potent than Pit-1 in activation of the rGH promoter in Ltk$^-$ and HeLa cells. Theill et. al. (1992) detected no differences in the stability of Pit-1 and Pit-1a in RQ6 cells. In their analysis, Pit-1a was as active as Pit-1 in activation of the rGH promoter in Rat6 cells (Theill et al., 1992). However, this group was unable to demonstrate activation of the rPRL promoter by Pit-1a (Theill et al., 1992).

There is still not a complete consensus, then, on the transactivational activity of the Pit-1a isoform. While we agree with both Theill et. al. and Konzak and Moore that Pit-1a can transactivate the rGH promoter, Theill et. al. find Pit-1a to be equivalent to Pit-1 in transactivation of the rGH promoter, while Konzak and Moore have concluded that Pit-1a is the more active isoform. This conclusion is based on equivalent activation of the rGH promoter by the two isoforms in the face of much lower levels of Pit-1a protein. We reserve judgement due to our inability to meaure levels of isoform

expression. With regard to the activation of the rPRL promoter, we agree with the conclusion of Konzak and Moore that Pit-1a is capable of transactivaiton of the rPRL promoter while Theill et. al. find no such activity. Given that the studies by these three groups have been carried out in different cell lines, it is possible that some, if not all, of the differences observed may reflect cell-specific differences.

The evaluation of the activity of the Δ4Pit-1 isoform is less ambiguous. The observation that Δ4Pit-1 is able to repress expression of PRL-CAT in HeLa cells (Figure 25) demonstrates that the transfected cDNA for this isoform is expressed in HeLa cells. Therefore, the lack of activation of PRL-CAT and GH-CAT in HeLa cells transfected with RSV-Δ4Pit-1 strongly suggests that Δ4Pit-1 cannot activate either the rPRL or rGH promoter. Voss et. al. have also reported Δ4Pit-1 to be incapable of transactivation of a target gene (Voss et al., 1993). This group has defined a synthetic element that Δ4Pit-1 can bind to efficiently (Voss et al., 1993). Interestingly, Δ4Pit-1 cannot activate a reporter construct containing this element (Voss et al., 1993). The authors conclude that the region of the POU-specific domain deleted in Δ4Pit-1 contains a nonmodular transactivation activity.

While Δ4Pit-1 cannot activate either the rPRL or rGH promoter in HeLa cells, the isoform is not without function. Day and Day have demonstrated a role for Δ4Pit-1 in Rat-1 cells as a repressor of the rPRL promoter (Day and Day, 1994). I have confirmed this finding in HeLa cells using the electroporation transfection technique to overcome the presumably nonspecific inhibitory effects of bulk plasmid DNA. In addition, the finding that Δ4Pit-1 expression parallels GH expression in the GH_3, MMQ, and 235-1 cell lines suggested that while Δ4Pit-1 cannot stimulate transcription, it may exert a permissive effect on the expression of the rGH gene. This effect might involve a pituitary-specific repressor of the GH gene, the repressor activity of which is relieved by Δ4Pit-1. The results of a preliminary experiment that support this model have been presented. A thorough test of this model awaits the results of further investigation.

V. Chapter 5: Summary and Future Directions

The results presented in this thesis confirm that either isoform of the D_2 dopamine receptor can mediate the dopaminergic inhibition of rat prolactin (rPRL) gene transcription first described by Maurer (1981). Additionally, it has been demonstrated that repression mediated by the D_{2A} receptor, the predominant form in the rat anterior pituitary, requires one or more pertussis toxin-sensitive G proteins. The pertussis toxin-sensitive G proteins found in GH_3 cells include $G_{o\alpha}$, $G_{i\alpha 2}$, and $G_{i\alpha 3}$ (Paulssen et al., 1991). All of these isoforms have also been identified in rat pituitary which, in addition, expresses $G_{i\alpha 1}$ (Paulssen et al., 1991). Recently, Liu et. al. have used the powerful antisense technique to selectively knock-out expression of $G_{o\alpha}$ or $G_{i\alpha 2}$ in GH_4C_1 cells stably transfected with D_2 receptors (Liu et al., 1994). These cell lines have been used to demonstrate that in GH_4C_1 cells, $G_{o\alpha}$ and $G_{i\alpha 2}$ expression are required for coupling of the D_2 receptor to inhibition of voltage-gated calcium channels and adenylyl cyclase, respectively (Liu et al., 1994). Clearly, lines of cells such as these will prove invaluable in further delineation of the G proteins, and signal transduction pathways, involved in dopaminergic repression of the rPRL gene. The implication of the cAMP-dependent protein kinase in dopaminergic repression of the rPRL promoter suggests that cells in which $G_{i\alpha 2}$ expression has been abolished, may fail to exhibit dopaminergic repression of the rPRL promoter. The demonstration of dopaminergic repression in such cells would support the model of Elsholtz et. al. (1991) that a cAMP-independent pathway, involving changes in membrane potential, exists (see Figure 17). Further support for this model would be provided by the loss of dopaminergic repression of the rPRL promoter in a cell in which expression of both $G_{o\alpha}$ and $G_{i\alpha 2}$ has been suppressed.

In analyzing the gene proximal events mediating dopaminergic repression of the rPRL promoter, it was shown that the proximal 187 base pairs of the rat prolactin promoter confer a full dopaminergic repression. This region contains three binding sites

for the pituitary-specific transcription factor Pit-1, which has been implicated in mediating regulation of both the prolactin and growth hormone genes by hormones and intracellular mediators. Most recently, it has been shown that three concatameric Pit-1 binding sites can confer dopaminergic repression to a heterologous promoter (Lew et al., 1994). Attempts at more directly implicating Pit-1 in the pathway of dopaminergic repression of the rPRL promoter have been less successful. It has been shown here, as well as by others (Lew et al., 1994), that reconstitution of dopaminergic regulation of the rPRL promoter in nonpituitary cell lines can result in a nonphysiological dopaminergic induction of rPRL promoter activity. Lew et al. (1994), have shown this induction to be Pit-1 dependent. However, the paradoxical nature of the Pit-1-dependent induction of the rPRL promoter questions the relevance of these findings for physiological repression of prolactin. A more satisfying proof that Pit-1 mediates dopaminergic repression of the rPRL gene awaits reconstitution of the appropriate regulation, perhaps in a Pit-1-deficient pituitary cell line such as the AtT20 or α-TSH cell lines (B. Kloss and C. Bancroft, unpublished data and Haugen et al., 1993).

Given the available evidence, implicating both Pit-1 and cAMP-dependent protein kinase (PKA) in dopaminergic repression of the rPRL gene, an investigation of the functional consequences of phosphorylation of Pit-1 was undertaken. The Pit-1(A3) mutant, which no longer serves as a substrate for phosphorylation by PKA, was compared to the wild-type Pit-1 in the ability to activate the rPRL and rGH promoters. While significant promoter activity is seen with the phosphorylation-deficient mutant, the maximal prolactin promoter activity achieved by Pit-1(A3) is half that achieved by the wild-type Pit-1. This finding is consistent with the proposed model for dopaminergic repression of the rPRL promoter being mediated, in part, through inhibition of PKA-dependent phosphorylation of Pit-1. A further test of this model again relies on reconstitution of the appropriate dopaminergic regulation of the rPRL promoter in Pit-1-deficient cell lines. Such lines could be used to express D_2 receptors, a rPRL reporter,

and either Pit(A3) or wild-type Pit-1. The model predicts that cells expressing wild-type Pit-1 would exhibit dopaminergic repression of the rPRL reporter while cells expressing Pit-1(A3) would exhibit reduced or no repression. While these experiments are currently feasible in the cell lines which have been used for such reconstitution assays (HeLa, C6, and Ltk$^-$), the paradoxical dopaminergic induction observed limits interpretation of the results from any such experiments.

While a possible modulatory role for phosporylation of Pit-1 has been demonstated for basal expression of the rat prolactin and growth hormone (rGH) promoters, no such role has been found for phosphorylation of Pit-1 for induced promoter expression. It has been shown here that phosphorylation of Pit-1 at any of its known phosphorylation sites is not required for the role of Pit-1 in participating in induction of the rPRL promoter by the estrogen receptor. Similarly, it has been found that Pit-1(A3) is as effective as wild-type Pit-1 in mediating induction of the rPRL promoter by either forskolin or phorbol ester (Fischberg et al., 1994). These findings raise the question, if phosphorylation of Pit-1 is not required for induction of the rPRL promoter, how is induction mediated? Several possibilities have been discussed including the existence of other, as yet undetected, sites of phosphorylation of Pit-1. Additionally, it has been proposed that another factor, as yet undiscovered, may cooperate with Pit-1 to mediate induction of the rPRL promoter in a fashion analagous to the estrogen receptor. Further biochemical studies of Pit-1 may provide support for one or both of these models.

The final section of this thesis has presented functional studies of the Pit-1 isoforms Pit-1a and Δ4Pit-1. Preliminary studies have indicated that Pit-1a exhibits activity in activation of both the rPRL and rGH promoters in HeLa cells. This finding agrees with the observations of Konzak and Moore (1992) in HeLa and Ltk$^-$ cells but contradicts the findings of Theill et. al. (1992) in Rat6 cells. Further studies are required to determine if this disagreement is a result of cell-specific differences.

Studies of the function of the Δ4Pit-1 isoform indicated that while, by itself, it is unable to activate either the rPRL or rGH promoter in HeLa cells, it can act as a repressor of the rPRL promoter in HeLa cells. Preliminary results have also suggested that Δ4Pit-1 may play a role in allowing expression of the rGH gene in somatomammotropes. A working model for this phenomenon proposes the existence of a pituitary-specific repressor of the rGH gene, the action of which is relieved by the Δ4Pit-1 isoform. This model is consistent with the findings that Pit-1 or Pit-1a can activate the rGH promoter in the nonpituitary HeLa cell line but that strong expression of the rGH promoter in pituitary cell lines is correlated with expression of the Δ4Pit-1 isoform. Clearly, further studies are required to document this permissive effect. Finally, before a physiological role for Δ4Pit-1 can be confirmed, either as a repressor of the rPRL gene or as a permissive activator of the rGH gene, it remains to be determined that the Δ4Pit-1 isoform is expressed in the normal rat pituitary.

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