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Peptide hormone regulation of prolactin gene expression

Yan, Guo-Zai, Ph.D.

City University of New York, 1993

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A

PEPTIDE HORMONE REGULATION OF PROLACTIN GENE EXPRESSION

by

GUO-ZAI YAN

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

1993

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Abstract

PEPTIDE HORMONE REGULATION OF PROLACTIN GENE EXPRESSION

by

GUO-ZAI YAN

Adviser: Professor Carter Bancroft

Expression in pituitary cells of the prolactin (PRL) gene is subject to regulation by a number of peptide hormones. TRH is one of the major positive regulators of PRL gene expression. Transfection experiments with rat pituitary GH₃ cells were employed to show that two TRH response elements (TRHREs) in the PRL proximal promoter correspond to binding sites for the pituitary cell-specific transcription factor pit-1. Either mutation of the strongest TRHRE, site 1P, or coexpression of a dominant mutant pit-1 possessing reduced *trans*-activational activity, strongly inhibited TRH regulation of PRL promoter constructs, implying that the cell-specific activator pit-1 is a gene-proximal mediator of TRH action on the PRL gene. Similar experiments showed that site 1P is also a Ca²⁺ response element, and that pit-1 also represents the gene-proximal protein that transduces the Ca²⁺ signal to the PRL promoter. Pharmacological experiments implied that Ca²⁺ but not protein kinase C (PKC) activity is required for TRH action on the PRL promoter. Thus, Ca²⁺ is apparently a mediator of TRH regulation of PRL gene expression, but not via a pathway involving PKC.

To examine the generality of these results, regulation of transfected PRL promoter constructs by another significant PRL gene activator,

vasoactive intestinal peptide (VIP), was also examined. 5'-deletion analysis showed that the first 174 base pairs of the PRL promoter is required for induction by VIP. However, none of the pit-1 binding sites in this region, 1P, 2P, or 3P conferred a strong VIP response to a reporter construct. In addition, studies with the dominant pit-1 mutant described above implied that VIP action on the PRL promoter is not mediated by pit-1. These observations imply that different peptide hormones employ different mechanisms to regulate PRL gene expression. Pharmacological studies of VIP regulation of the PRL promoter yielded the following results: The use of cellular Ca^{2+} blockers implied that Ca^{2+} is a mediator of VIP stimulation of the PRL promoter. Down-regulation of PKC did not inhibit induction of PRL promoter expression by VIP, implying that regulation of PRL gene expression by VIP is also independent of PKC activity. Inhibition of cAMP-dependent protein kinase A (PKA) also did not block VIP induction of PRL promoter expression, suggesting that PKA also does not play a major role in this action of VIP. However, a calmodulin inhibitor could block VIP induction of PRL promoter expression, implying that VIP action on the PRL promoter may be mediated by Ca^{2+} /calmodulin-dependent kinase.

It has been shown previously that a classical cAMP response element (CRE)-like element (CLE) in the PRL proximal promoter is required for basal expression and cAMP induction of the PRL promoter in GH-cells, and that CREB binds to the PRL CLE with a low affinity. The present studies showed that CREB can act as a specific repressor of PRL promoter activity in GH₃ cells, and can repress pit-1 activation of the PRL promoter in the non-pituitary C6 rat glial cells. In either pituitary or non-pituitary cells, this repression can be reversed by co-expression of PKA. These results suggest that CREB may act by

competing for binding to the PRL CLE with a factor which is required for pit-1 activation of the PRL promoter.

Prior to the present studies, the pituitary-specific transcription factor pit-1 was the only factor known to be capable of *transactivating* the PRL promoter in non-pituitary cells. The present studies showed that either PKA or a constitutively active form of CREB (CREB-VP16) are capable of *transactivating* the PRL promoter through the PRL CLE site in C6 rat glial cells. These findings raise the possibility that expression of the PRL promoter in the absence of pit-1 (for example in the brain) may involve binding to the CLE of a factor similar to CREB.

**To my parents
Changjing Yan and Baoxiou Shao
and my brother
He Yan
and my sisters
Jiawei Yan and Lilai Yan
for all that they have done for me.**

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INTRODUCTION

The prolactin (PRL) gene is an attractive model for the study of tissue specific gene control since the PRL gene is cell-specifically, developmentally and hormonally regulated. Prolactin, a polypeptide hormone, is restricted in its expression to pituitary somatomammotropes cells where it is produced in conjunction with growth hormone, and pituitary mammotropes cells where it is uniquely expressed (Miller and Eberhardt, 1983). Prolactin gene transcription is also subject to regulation by a number of hormones including positive regulators: estradiol (Maurer et al., 1987; Waterman et al., 1988), epidermal growth factor (EGF) (Supowit et al., 1984; Elsholtz et al., 1986; Day and Maurer, 1989), thyrotropin-releasing hormone (TRH) (Murdoch et al., 1982; 1983; Day and Maurer, 1989; Yan et al., 1991), and vasoactive intestinal polypeptide (VIP) (Carrillo et al., 1985), as well as negative regulators: the catecholamine dopamine (Maurer, 1981; Elsholtz et al., 1991; McChesnery et al., 1991) and glucocorticoids (Somasekhar and Gorski, 1988).

Both tissue-specific expression (Miller and Eberhardt, 1983; Lufkin and Bancroft, 1987; Nelson et al., 1988) and the complex hormonal regulation (Camper et al., 1985; Elsholtz et al., 1986) of the prolactin gene involve the interaction of multiple trans-activating factors with DNA elements of the prolactin gene. It has been previously shown that there are specific DNA regions in the 5' flanking region of the PRL gene which are sufficient for transcriptional regulation in response to TRH, EGF, cAMP and phorbol esters (Day and Maurer, 1989). A recently cloned pituitary-specific transcriptional factor, termed pit-1, has been demonstrated to regulate cell-specific PRL and

growth hormone gene expression by interacting with specific regulatory sequences (Ingraham et al., 1988; Bodner et al., 1988). Pit-1 has also been found to be required for the normal development (Crenshaw et. al., 1990) and proliferation (Castrillo et. al., 1991) of specific anterior pituitary cell types. Therefore, there is considerable interest in understanding the mechanisms of hormonal regulation of prolactin gene expression and identifying other factors involved in cell-specific expression of the PRL gene.

Developmental Regulation of Rat Prolactin Gene Expression.

The development of the anterior pituitary gland provides an excellent model system to study cell-specific gene activation and the processes by which distinct cell types develop within an organ. The anterior pituitary gland, which is derived from a common primordium originating in Rathke's pouch, consists of five phenotypically distinct cell types (review in Frohman 1981), each of which expresses discrete trophic hormones: corticotrophs, producing pro-opiomelanocortin (POMC); thyrotrophs, producing the common α -subunit and the β -subunit of thyroid-stimulating hormone (α -TSH; β -TSH); gonadotrophs, producing the common α -subunit and the β -subunit of follicle-stimulating and luteinizing hormone (α -FSH and β -FSH; α -LH and β -LH); somatotrophs, producing growth hormone (GH); and lactotrophs, producing prolactin (PRL). Among the lactotrophs and somatotrophs are a subpopulation of cells which produce both growth hormone and prolactin, known as somatomammotrophs. It is within these cells that a critical event in cell-specific determination occurs, causing either the growth hormone or prolactin gene to be expressed.

The pituitary hormones exhibit a characteristic temporal pattern of developmental expression, with growth hormone and prolactin characteristically being the last hormones to be expressed in the developing rat embryo (Gash et al., 1982; Hoeffler et al., 1985). Rat growth hormone expression is first observed in late fetal development (days 17-18), while initial expression of rat prolactin is variably reported between fetal day 17 and 2-4 days after birth (Gash et al., 1982), with the percentage of lactotrophs rising dramatically 3-5 days post-partum to constitute above 10% of total pituitary cells. The possible co-expression of these two structurally related neuroendocrine genes (PRL and GH) within a single cell type prior to the appearance of lactotrophs raises the possibility that at least most lactotrophs are derived from a presomatotrophic lineage (Behringer et al., 1988; Borrelli et al., 1989).

Appearance of the pit-1 transcripts is correlated with the activation of the prolactin and growth hormone gene expression. The pit-1 gene transcripts are initially detected on embryonic day 15 in most cells of the anterior pituitary, with pit-1 protein appearing at very low levels at essentially the same time, preceding PRL and GH activation. Although the pit-1 gene transcripts are expressed in all five phenotypically distinct pituitary cell types, pit-1 protein is detected only in the nuclei of thyrotrophs, lactotrophs and somatotrophs (Simmons et al., 1990.). The expression of pit-1 protein in thyrotrophs, in concert with previous analysis in transgenic mice of strict sommatotrophic and lactotrophic GH and PRL gene expression (Crenshaw et al., 1989), suggests that specific restrictive mechanisms prevent the expression of GH and PRL genes in this cell type. There is evidence that full prolactin activation requires additional factors which may not appear until after birth. In the case of the PRL gene, the progressive increase in estrogen receptor gene

expression after birth may account, at least in part, for the progressive postnatal increase in PRL gene expression characteristic of phenotypically mature lactotrophs (Simmons et al., 1990).

Naturally-occurring animal models of pituitary dwarfism provides the direct evidence that links a transcription factor to commitment and progression events in mammalian organogenesis (Li et al., 1990). Analysis of natural mutants at the mouse dwarf (dw) locus has shown that the Jackson and Snell mutations most likely arise from a disruption of the pit-1 gene (Li et al., 1990). Since these mice lack lactotrophs, thyrotrophs as well as somatotrophs, it appears that pit-1 function is necessary for the specification of the phenotype of three cell types in the anterior pituitary. The involvement of pit-1 in determining cell fates is further supported by a cell culture model of the genetic defect with antisense oligonucleotide technology (Castrillo et al., 1991), where it was found that specific inhibition of pit-1 synthesis by complementary oligonucleotides leads to a marked decrease in GH and PRL expression and to a marked decrease in proliferation of somatotrophic cell lines. These results provide direct evidence that pit-1 is required not only for the establishment and maintenance of the differentiated phenotype but also for cell proliferation as well .

The Pituitary-Specific Transcription Factor Pit-1.

The characterization and purification of a factor that binds to particular cell-specific cis-acting elements in rat PRL and GH promoters permitted the cloning of a pituitary-specific factor, referred to as pit-1 (Bodner et al., 1988); or GHF-1 (Ingraham et al., 1988) (referred to here as pit-1). The coding sequence of pit-1 cDNA predicts a 291- amino acid protein (see Fig. I-1). Western

immunoblot analysis using an affinity-purified antiserum directed against a pit-1 peptide (amino acid residues 136-150) has detected a protein doublet (major band at 33 kD and minor band at 31 kD) in nuclear extracts from rat sommatomammotrophic cell lines as well as from adult rat pituitaries (Fox et al., 1990). Moreover, labeled pit-1 DNA binding elements recognizes species of 31 and 33 kD proteins on a southwestern blot using extracts from rat pituitary derived cell lines (Ingraham et al., 1988 and Simmons et al., 1990). These two forms of pit-1 have different abilities of DNA binding and transactivation (Voss et al., 1991). It has been showed that the alternative forms of pit-1 results from utilizing alternative translation of initiation site (Voss et al., 1991).

Pit-1 is a member of a novel family of transcription factors sharing two regions of homology: a highly conserved POU-specific (POU_S) domain and a more divergent homeodomain (POU_{HD}) (see Fig. I-1) (Herr et al., 1988). Studies of mutant pit-1 proteins have shown that the POU_S domain is necessary for high affinity binding and accurate recognition of natural pit-1 response elements, while the POU_{HD} is required and sufficient for low affinity DNA binding (Ingraham et al., 1990). Using cross-linking techniques, it has been shown that pit-1 is monomeric in solution but associates as a dimer on its DNA binding element (Ingraham et al., 1990). The DNA-dependent pit-1 protein-protein interactions requires the POU_S domain. Transcriptional activity of pit-1 on enhancer elements is conferred primarily by a novel serine and threonine rich (21%) N-terminal region, unrelated to other known transcription activating motifs. It has been reported that amino acid sequence 10-40 is the major *trans*-activation domain and amino acid sequence 40-80 is the minor *trans*-activation domain (see Fig. I-1) (Ingraham et al., 1990).

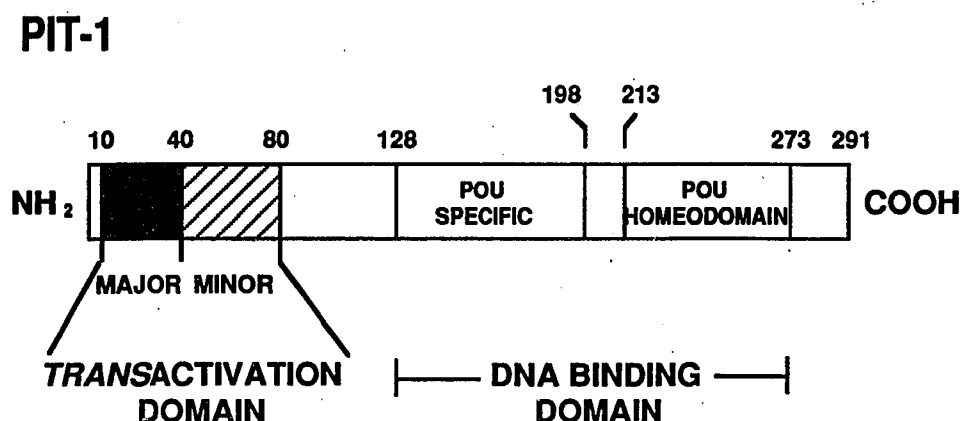


Fig. I-1 Schematic Representation of Functional Domains of Pit-1 Protein.

Expression of pit-1 in heterologous cell types including Hela, CV-1, and RAT-1 is capable of activating both PRL and GH promoters, even when pit-1 is expressed at levels much lower than those in pituitary cells (Ingraham et al., 1988, 1990; Mangalam et al., 1989; Fox et al., 1990). Furthermore, pit-1 can trans-activate gene expression in yeast. Though several aspects of pit-1-dependent transcriptional activation have been found to be similar between mammalian cells and yeast, a difference in the prolactin DNA sequences for pit-1 binding that can confer activation in the two systems has been observed (Ding et al., 1991). These studies establish the ability of pit-1 to stimulate

transcription in the absence of other tissue-specific factors and provide a system for further genetic studies of pit-1 structure/function relationships as well as evaluation of target sequences necessary for pit-1 action.

Studies aimed at elucidating the mechanisms of pit-1 gene activation and regulation of its expression, have been carried out by *in vitro* transcription and transfection analysis using wild-type and mutated pit-1 promoters. These experiments demonstrated that pit-1 can positively autoregulate its expression by binding to two pit-1 binding elements in its gene promoter. Mutation of the 5' pit-1 binding site abolished positive autoregulation, whereas mutation of the element located immediately 3' of the cap site markedly increased expression of the pit-1 promoter. These data are consistent with a positive, attenuated autoregulatory loop that seems to function in maintaining pit-1 gene expression (Chen et al., 1990; McCormick et al., 1990).

Further studies have demonstrated that pit-1 is phosphorylated in pituitary cells at two distinct sites in response to phorbol esters and cAMP. Phosphorylation of pit-1 results in changes of DNA binding activity. One of these sites Thr²²⁰, located in the POU homeodomain within a sequence conserved throughout the POU-domain family, confers these responses (Kapiloff et al., 1991).

Cell-Specific Regulation of the Rat PRL Promoter.

The GH cells are derived from rat pituitary tumor cells. Under the proper growth conditions, growth hormone or prolactin synthesis by the GH cells can consist of a large fraction (2-15%) of total protein synthesis (Bancroft, 1981). In addition, synthesis of the specifically expressed proteins in the GH

cells can be strongly regulated by addition to growth medium of a variety of hormones which regulate pituitary function in animals (Bancroft, 1981). Furthermore, the GH cells have been shown to contain specific receptors for these hormones (Bancroft, 1981). Therefore, GH cell lines are a suitable model system for studies of the cell-specific and the hormonal regulation of PRL gene expression.

Cell-specific expression of the rat prolactin gene is dictated by two separate regions, a distal enhancer (-1831 to -1530) and a proximal region (-422 to +33) (Nelson et al., 1986,1988). The known anatomy of the PRL gene 5'-flanking sequence is summarized in Fig. I-2. By using DNase protection analysis, it has been shown that the distal rat promoter region contains four pit-1 binding sites (designated 1D-4D) and one estrogen receptor binding site (see Fig. I-2); the proximal rat PRL promoter region contains four pit-1 binding sites (designated 1P-4P; see Fig. I-2) (Gutierrez-Hartmann et al.,1987; Nelson et al., 1988; Cao et al., 1988; Kim et al., 1988; Harvey et al., 1991; Schuster et al., 1988; Sharp et al., 1989; Yan et al., 1991). However, it is not yet clear whether it is the proximal or distal DNA sequences that primarily drive expression of the PRL gene in rat pituitary (GH) cell lines. Rosenfeld's group reported that removal of a distal 300 bp region from the rPRL promoter, residing 1500 bp upstream of the cap site, causes a dramatic (about 100-fold) decrease in transient expression in GH cells of either PRL-CAT constructs or PRL-luciferase constructs (Elsholtz et al., 1986; Nelson et al., 1986,1988). These experiments suggest that the distal enhancer is predominantly responsible for the tissue-specific expression of the PRL gene. By contrast, other groups, including ours, have shown that tissue-specific expression of the PRL gene in cell culture requires only the proximal region. Rottman's group demonstrated that deletion of all but 250 bp of the bovine PRL 5'-flanking

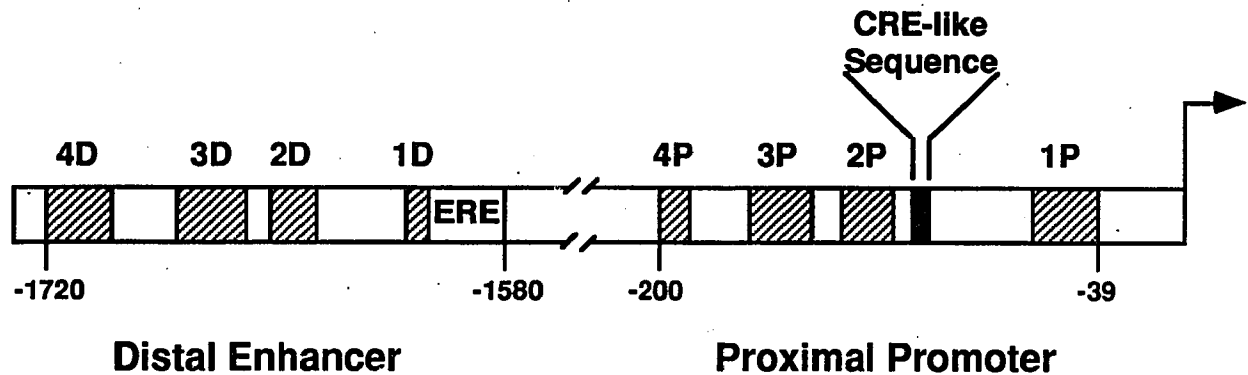


Fig. I-2 Cis-Acting Regions of the Rat PRL Gene 5'-Flanking Sequence. 1P-4P indicate pit-1 binding sites in the proximal prolactin promoter. 1D-4D indicate pit-1 binding sites in the distal prolactin enhancer region. ERE, estrogen response element. CRE, cAMP response element.

DNA had no effect on the transient expression in rat pituitary GH₃ cells (Camper et al., 1985). By transfection cell fusion, transient transfection, and *in vitro* transcription using GH₃ nuclear extracts, Bancroft's group has also found that only proximal sequences are required for expression of rat PRL-CAT constructs (Lufkin and Bancroft, 1987; Lufkin et al., 1989). Results of analysis of transgenic mice indicated that either region is sufficient to direct the expression of reporter genes to pituitary cells of transgenic animals. Both regions are required for high level expression of the rPRL gene in lactotropes

(Crenshaw et al., 1989), implying that synergistic interactions between proximal and distal elements are required for determining cell-specific expression of the rPRL gene at physiological levels.

Though it is clear that pit-1 is very important for cell-specific expression of the GH and PRL gene, it would not be surprising that other factors may be involved in cell-specific expression of these genes. Indeed, the estrogen receptor is such a factor. An estrogen receptor binding site appears to be important for the activity of the distal enhancer (Maurer and Notides, 1987; Kim et al., 1988 and Day et al., 1990). Co-expression of estrogen receptor in transient transfection experiments with pit-1 synergically activated the prolactin gene, and estradiol induction requires the presence of pit-1 (Day et al., 1990).

By using linker scanning studies, Maurer's group has recently demonstrated that one DNA element that is different from the known pit-1 binding sites is present within the proximal region of the rPRL gene (Iverson et al., 1990). It is located at positions -99 to -92, termed either the CRE-like sequence (Iverson et al., 1990; d'Emden et al., 1992) or the "A" box (Peers et al., 1992), and shares some similarity to a consensus cAMP-responsive element (CRE). Mutating either pit-1-binding sites, or the CRE-like sequence greatly decreased expression of the PRL promoter in GH₃ cells (d'Emden et al. 1992). These findings suggest that the normal transcription of the PRL gene involves strong cooperative interactions between individual DNA elements in the proximal region.

By using *in vitro* DNase I protection analysis, four pituitary-specific factor binding sites, 1P, 2P, 3P and 4P (or FPI, FPII, FPIII, and FPIV) have been localized to the proximal 210 bp of the proximal rat prolactin promoter (Gutierrez-Hartmann et al., 1987; Harvey et al., 1991; Lufkin and Bancroft

1987; Nerson et al., 1988; Schuster et al., 1988; Sharp et al., 1989; Yan et al., 1991). More recently, Gutierrez-Hartmann's group has reported that 2P (FP11) which is located at position of -120 to -130, binds a ubiquitous factor (F2F) that is present in pituitary and non-pituitary cells. By gene transfer analysis, they have showed that 2P (FP11) functions as a dominant repressor element in non-pituitary cells, whereas the inhibitory activity by 2P (FP11) site is masked by the positive effects of the redundant cell-specific elements in pituitary cells (Jackson et al., 1992).

The Mechanisms of Activation of Gene Transcription by Steroid and Polypeptide Hormones.

Studies of regulation of gene transcription in response to intercellular signals, such as hormones, reveals a crucial role of trans-activating regulators. Regulation by steroid hormones is accomplished by their binding to specific high affinity intracellular binding proteins, referred to as receptors (Gorski et al., 1976; Hayward et al., 1982); whereas regulation of peptide hormones is mediated by their interaction with specific high-affinity receptors located on the plasma membrane.

There are wide variations in our understanding of the pathways of action of different classes of hormones. Members of the steroid receptor super-family, including receptors for steroids, T_3 and retinoic acid, are DNA-binding transcription factors, and all directly transmit a hormonal signal to their target gene. For steroid hormone receptors, it is postulated that the binding of the ligand induces an allosteric modification which permits binding by a specific domain to a short consensus enhancer sequence present in the regulated transcription unit. Additional protein-protein interactions

may be of importance for implementing the full quantitative transcriptional effect of the binding of the steroid receptor to its cognate DNA site (reviewed in Rosenfeld et al., 1987). Polypeptide hormones can exert rapid effects on the transcription of specific genes, comparable to steroid hormone effects on gene transcription (Murdoch et al., 1982; Granner et al., 1983). Peptide hormone receptors do not bind to DNA, but instead employ intermediate gene-distal proteins that probably engage only in protein-protein interactions. For peptide hormone regulation, it is postulated that transacting factors might be associated with specific DNA sequences even in the absence of hormonal stimulation. Allosteric modification of the ability of these proteins to activate transcription, rather than regulation of their DNA binding, might be the critical event controlled by binding of peptide hormones to their surface receptors (reviewed in Rosenfeld et al., 1987). Unlike steroid hormones, peptide hormones do not share a single regulatory theme. Further understanding of the mechanisms by which polypeptide hormones regulate gene expression is central to addressing this critical biological question.

Since rat prolactin gene transcription is regulated by a number of different polypeptide and steroid hormones including positive and negative regulators, it is therefore a very valuable model for studies of the mechanism of hormonal regulation. These hormones mediate the expression of the PRL gene by acting through two separate DNA regions located in its 5'-flanking sequences (proximal regulatory region, distal region). The distal element contains a tissue-specific enhancer (Nelson et al., 1986) and elements that confers a positive transcriptional response to cAMP, EGF, and TRH (Day and Maurer, 1989). Thyroid hormone (T_3) also influences the transcription rate of the PRL gene through the distal element (Forman et al, 1988; Day and Maurer, 1989). Induction of PRL transcription by estrogen has been shown to be

mediated through an estrogen response element (ERE) located in the distal element (Maurer et al., 1987; and Waterman et al., 1988). The proximal region directs a positive response to cAMP, phorbol esters, EGF, TRH and Ca^{2+} (Supowit et al., 1984; Elsholtz et al., 1986; Day and Maurer, 1989; White and Bancroft, 1983; Jackson and Bancroft, 1988; Yan et al., 1991; Yan and Bancroft, 1991) and a negative response to glucocorticoids (Somasekhar and Gorski, 1988) and dopamine (Maurer 1981; Elsholtz et al., 1991; Fischberg's unpublished observation in the Bancroft laboratory). However, there is little known about the signal transduction pathway employed by peptide hormones on PRL gene expression. Fig. I-3 summarizes the possible pathways involved in regulation of the PRL gene by polypeptide hormones.

We have focussed our studies on the PRL proximal promoter because our laboratory has shown that this region alone directs optimal, pituitary cell-specific expression (Lufkin and Bancroft, 1987; Lufkin et al., 1989), as well as optimal response to Ca^{2+} , TRH, EGF, and TPA (Jackson and Bancroft, 1988; Jackson et al., 1990; Yan et al., 1991; Yan and Bancroft, 1991).

Pathway of TRH Action on Prolactin Gene.

Thyrotropin-releasing hormone (TRH) is a tripeptide hormone synthesized in the hypothalamus. TRH is a major physiological activator of prolactin production. High affinity receptors for TRH exist on the cytoplasmic membrane in the anterior pituitary where prolactin is synthesized (Hinkle, 1989). The transduction pathways linking the interaction of TRH with its specific plasma membrane receptor to the subsequent secretion of PRL by GH cells have been extensively investigated, and shown to involve activation of

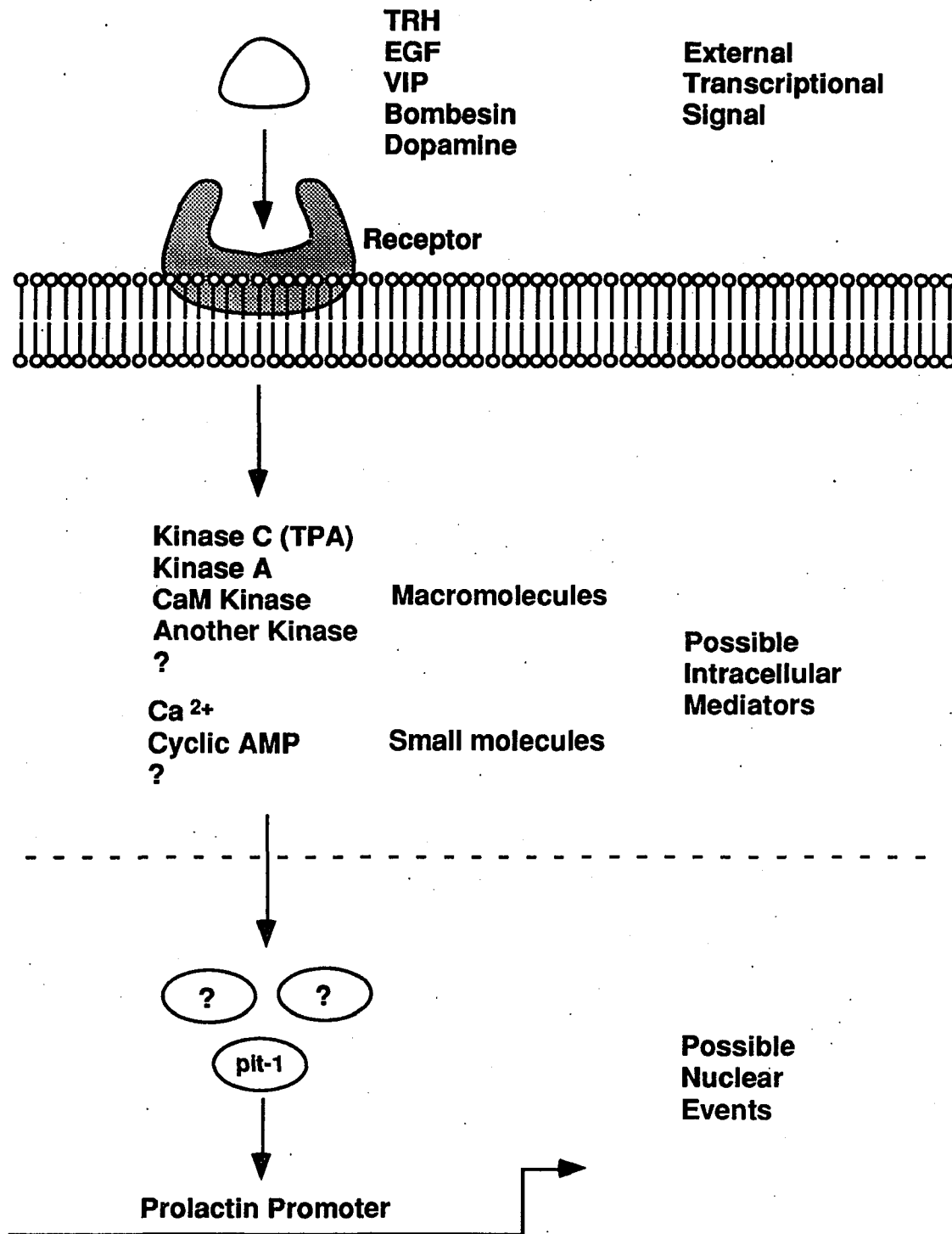


Fig. I-3. The Possible Pathways of Regulation of the PRL Gene by Polypeptide Hormones.

phosphoinositide metabolism (Hinkle et al., 1988) and protein kinase C (Gershengorn, 1982), as well as intracellular calcium levels (Gershengorn, 1982). Indirect evidence (Hinkle et al., 1984) is consistent with the hypothesis that a G protein couples the TRH receptor (TRH-R) to a phospholipase C that hydrolyzes phosphatidylinositol 4, 5-bisphosphate. Therefore, it has been assumed that the TRH-R is a member of the large family of G protein-coupled receptors. A cDNA for mouse TRH-R has been isolated. Sequence analysis shows that it encodes a protein of 393 amino acids, and that it shares homology with other guanine nucleotide-binding regulatory protein-coupled receptors (Richard et al., 1990).

Early studies with the hormone-responsive rat pituitary GH cell lines showed that TRH stimulates both the release and synthesis of prolactin from pituitary cells and increases prolactin mRNA levels in these cells (Martin et al., 1983; and Murdoch et al., 1982). It has been reported that TRH induces the phosphorylation of a number of cytoplasmic proteins in cultured GH₃ and GH₄C₁ cells (Drust et al., 1982; Drust and Martin, 1982, 1984; Sobel and Tashjian, 1983), and the phosphorylation of certain cytoplasmic proteins appears to be correlated with PRL release from cells while phosphorylation of others appears to be correlated with PRL synthesis (Sobel and Tashjian, 1983). Murdoch et al, (1983) have found that TRH at 3×10^{-7} M stimulated the transcription of the PRL gene 7- to 12-fold within minutes of its addition to cultured GH₄ cells, and a comparable increase in phosphorylation of a novel nuclear protein, basic regulated phosphoprotein (BRP). After 1 hour, the stimulation of PRL gene transcription began to decline. PRL gene transcription remained approximately two-fold stimulated at 36 hours. This markedly diminished effect exhibits a close quantitative correlation between the prolactin gene transcription and BRP phosphorylation (Murdoch et al.,

1983). The similarity in the rapid kinetic responses of TRH-stimulated BRP phosphorylation and increased prolactin gene transcription is consistent with the possibility that both events represent primary effects of the TRH-receptor interaction. It has been shown by another group that TRH increased PRL mRNA by both transcriptional stimulation and specific stabilization of PRL mRNA (Laverriere et al., 1983). However, little is currently known about the nuclear events involved in PRL gene regulation by TRH.

An intensive effort is being directed toward understanding the mechanism through which TRH regulates PRL gene expression. By using transient transfection analysis, it was shown that the distal enhance element (Day and Maurer, 1989) and the proximal region (Day and Maurer, 1989; Camper et al., 1985) of the PRL promoter each contain TRH response elements. Recently, it has been reported that a series of clustered point mutations of the PRL proximal promoter region did not produce substantial decreases in TRH-stimulated expression of PRL gene implying that there are at least two TRH response elements (TRHRE) in the PRL proximal promoter (Iverson et al., 1990). These findings suggested a role for pit-1 as well as other transcription factors in mediating TRH stimulation of PRL gene expression. However, no TRHRE has yet been precisely identified, and there is no any information about a gene-proximal protein(s) that can bind to a TRHRE and thus mediate the TRH induction. Maurer's group has shown that down-regulation of protein kinase A (PKA) by cotransfection of a PKA inhibitor construct inhibits the induction of PRL-CAT constructs by all hormones examined, including TRH (Day et al 1989). These findings imply that inhibition of protein kinase A in GH cells results in some general, non-specific effect, perhaps on basal PRL promoter activity. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which can activate protein kinase C,

was shown to activate the PRL gene transcription to a lesser extent than TRH, suggesting a limiting role for protein kinase C in the neuropeptide-stimulating action at the gene transcription level (Laverriere, et al., 1988). Although evidence has been presented that calcium may be also involved in the regulation by TRH of GH-cell expression of the PRL gene (White and Bancroft, 1983; Laveriere et al., 1988; and Day and Maurer, 1990.), the gene-proximal mechanism of TRH action on PRL gene expression is unknown.

The Role of Calcium in Regulation of PRL Gene Expression.

There are a number of mechanisms by which various types of extracellular stimuli can result in a rise in cytosolic Ca^{2+} . Receptor regulation of cytosolic Ca^{2+} generally involves two types of receptors: phospholipase C (PLC)-linked receptors, such as TRH, catecholamine, and arginine vasopressin receptors or PKA-linked receptors, such as the CRH, Dopamine receptor. The activation of PLC-linked receptors elicits a rapid elevation of cytosolic Ca^{2+} mobilized from intracellular and extracellular pools. PLC catalyzes hydrolysis of membrane phosphoinositides, resulting in a GTP-binding protein (Gp)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) which yields two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (Berridge & Irvine, 1984). A rise in cytosolic Ca^{2+} and diacylglycerol synergistically activates protein kinase C (PKC). A number of proteins can interact with Ca^{2+} . One of the Ca^{2+} -binding protein is Calmodulin. Calmodulin has been implicated in a variety of regulatory functions (Cheung, 1980). There are also two types of voltage-gate calcium channels (VGCC) involved in regulation of cytosolic Ca^{2+} . Cells display spontaneous action potential activity as a result of the activation of plasma

membrane VGCC. VGCCs include fast deactivating or high threshold- L channels which are target for dihydropyridine (DHP) pharmacological agents, and low threshold or transient-T and N channels.

Ca^{2+} is a well known intracellular messenger, which mediates stimulated secretion of hormones in a variety of cell types. The role of calcium in the regulation of PRL gene expression has been investigated. It has been reported that addition of extracellular calcium to GH_3 cells grown in a calcium-depleted medium causes an increase PRL mRNA and PRL synthesis by GH_3 cells (White et al., 1981; Bancroft et al., 1985). Interestingly, it has been reported that Ca^{2+} has a small effect on the transcriptional rate of the prolactin gene, but produces a significant increase in prolactin mRNA levels by acting at a post-transcriptional level (Preston et al., 1990). Further studies showed that Ca^{2+} appears to stabilizing prolactin mRNA through a mechanism that is independent of ongoing protein synthesis (Preston et al., 1990). Another experimental approach to the study of Ca^{2+} regulation of PRL gene expression has been the use of drugs which alter intracellular Ca^{2+} levels. For example, TMB-8, which lowers intracellular Ca^{2+} by promoting sequestration or membrane binding of Ca^{2+} , specifically inhibits PRL mRNA levels (Preston and White, 1987). The dihydropyridine drugs selectively enhance or block calcium entry through voltage-gate calcium channels (Hess et al., 1984). Dihydropyridine calcium channel antagonists verapamil, nimodipine, and agonist Bay K8644 have been shown to inhibit and stimulate, respectively, prolactin gene expression. Another class of drugs which has been used is the Ca^{2+} ionophores, including A23187 and ionomycin. These agents are presumed to increase intracellular Ca^{2+} levels in all compartments by permeabilizing cellular membranes to Ca^{2+} . A23187 has been reported to increase the rate of PRL transcription measured after 4 h in the GH_3B_6 cells

(Laverriere et al., 1988). Moreover, the effect of Ca^{2+} on the basal prolactin gene expression appears to be mediated through a Ca^{2+} -calmodulin complex, as the calmodulin inhibitors, W7, W13 and calmidazolium block the ability of Ca^{2+} to enhance rat prolactin mRNA level (Bancroft et al., 1985; White, 1985), and human PRL-CAT (hPRL-CAT) expression in transfected GH₃ cells (Davis et al., 1991). This Ca^{2+} -dependent enhancement of prolactin gene expression is highly specific, since Ca^{2+} has little or no effect on the mRNA levels for growth hormone, metallothionein, histon 3, or glucose-regulated protein 78 (GRP78) (Gick and Bancroft, 1985; White et al., 1981, White et al., 1989).

In studies of the Ca^{2+} response, Jackson and Bancroft have shown that the first 174 bp of the PRL promoter can direct a Ca^{2+} induction (Jackson and Bancroft, 1988), and Day and Maurer have also shown that the distal and proximal region can each confer a Ca^{2+} response (Day and Maurer, 1990). More recently, Hoggard et al., have shown that the hPRL gene promoter is responsive to raised intracellular calcium. This response involves pit-1 binding sites and appears to require the presence of pit-1 protein (Hoggard et al., 1991). Studies by a number of groups have implicated Ca^{2+} in the stimulation by TRH of the PRL gene expression. Bancroft's group has found that, in GH₃ cells incubated with the Ca^{2+} chelator EGTA, TRH alone yielded no detectable stimulation of PRL mRNA levels, while Ca^{2+} alone or Ca^{2+} plus TRH strongly stimulated PRL mRNA levels (White et al., 1983). Studies by other groups have found that incubation of GH₃ cells with TRH alone or TRH plus the Ca^{2+} channel modulator Bay K 8644 or Ca^{2+} ionophores yielded equal stimulatory effects on either accumulation of PRL mRNA (Laverrier et al., 1989) or transient expression of the PRL gene (Day and Maurer, 1990). Therefore, there is a possible role for Ca^{2+} as a mediator of TRH induction of PRL gene transcription.

Stimulation of PRL Secretion and PRL mRNA by VIP.

Vasoactive intestinal peptide (VIP) is a highly basic 28-amino acid peptide which was originally isolated from the porcine duodenum (Said and Mutt, 1970) and is structurally and biologically related to glucagon and secretin (Mutt, 1982). VIP has been shown to be widely distributed in the central and peripheral nervous system, the gastrointestinal, respiratory and urogenital tracts, various exocrine glands, and endocrine glands including the thyroid, ovary (Ahren et al., 1982; Larsson et al., 1977; 1982; Hokfelt et al., 1982). Recently, Lam has shown that VIP is synthesized and secreted by the hypothalamus and anterior pituitary and that it participates in the regulation of pituitary functions (Lam 1991).

The effects of VIP are mediated by specific high affinity receptors, which have been identified in many tissues including neural, respiratory, lung, liver, gastrointestinal, and immune cells (Robberecht et al., 1981; 1990; Rorstad et al., 1990; Carstair et al., 1986). There is compelling evidence that signal transduction from the VIP receptor occurs by way of the stimulatory guanine nucleotide-binding protein (stimulatory G-protein, G_s) and adenylyate cyclase (Robberecht et al., 1981; Christophe et al., 1986; Couvineau et al., 1990; Paul et al., 1988; Agui et al., 1990). The mechanism of signal transduction from the VIP receptor to adenylyate cyclase is presumed to parallel that for other G-protein-coupled receptors (Gilman 1987; Brinbaumer 1990; Taylor 1990; Simon et al., 1991).

It is well known that pituitary PRL secretion is stimulated by TRH, estrogen and VIP, and inhibited by dopamine and Dexamethasone (Dannies and Tam, 1982; Rotsztejn et al., 1980; Enjalbert et al., 1980) as well as on

tumoral PRL-secreting cells (Gourdji et al., 1979). Several lines of evidence indicate that the stimulatory effect of VIP on PRL release is mediated by cAMP. VIP has been shown to increase cAMP accumulation in pituitary cells (Gourdji et al., 1979; Rotsztein et al., 1981) and to stimulate adenylate cyclase activity in anterior pituitary membrane (Pizzi et al., 1990). Moreover, it has been demonstrated that in pituitary cells as well as in the brain, VIP stimulates the accumulation of cAMP in a Ca^{2+} -dependent manner (Brostrom et al., 1982; Etgen et al., 1987; Pizzi et al., 1990). Recently, Schecterson and McKnight have reported that VIP can act by a different mechanism that does not involve cAMP-dependent protein kinase or protein kinase-C on β -endorphin secretion in AtT20 cells. These studies employed expression of the inhibitory mutant regulatory subunit of cAMP-dependent protein kinase in stable transformants or down-regulation of protein kinase C (Schecterson and McKnight, 1991). However, the pathways of PRL secretion by VIP stimulation clearly is different from β -endorphin secretion.

VIP stimulation of PRL mRNA was investigated in the PRL-producing GH₃ cells using cytoplasmic dot hybridization and RNA blot analysis by Carrillo et al. (1985), who showed that incubation of GH₃ cells with VIP increases the levels of cytoplasmic PRL mRNA. VIP can also suppress the inhibitory effect of dexamethasone (Dex) on cytoplasmic PRL mRNA. The mechanism by which VIP regulates PRL gene expression remains to be determined.

Regulation of Gene Expression by cAMP via Phosphorylation of CREB.

Regulation of eukaryotic gene expression is mediated through short DNA sequences that provide binding sites for sequence-specific transcription

factors (Dyran, 1989.). One of the major classes of regulatory elements that contribute to transcriptional regulation by extracellular signals is the CRE/ATF sequence motifs (Montminy et al., 1986; Lee et al., 1987; Tan et al., 1989; Jalinol et al., 1988; and Deutsch et al., 1988). The CRE/ATF element, which consists of eight-base palindromic sequence, (TGACGTCA), was defined as the cAMP responsive element (CRE) or the activating transcription factor (ATF) binding site. The CRE/ATF site can be recognized by a family of proteins including CREB and the ATF family (Hai et al., 1988; Hurrst and Jones, 1987; Montminy and Bilezikjian, 1987; and Andrisani et al., 1989, 1990). This family of CRE-binding proteins has been implicated in cAMP, calcium, and virus-induced alterations in transcription (Lee and Green, 1987; Roesler et al., 1988; Sheng et al., 1990).

CREB is a 43 kD protein containing a transactivation domain and a DNA binding domain (see Fig. I-4). It has a leucine zipper motif which consists of a heptad repeat of either four or five leucines. Adjacent to the leucine repeat is a basic region, which, along with the leucine zipper, has been shown to play a role in binding to DNA (Dwarki et al., 1990; Hai et al., 1989; Yun et al., 1990). Members of this family form homodimer or heterodimer through interactions involving the leucine repeats (Hai et al., 1989; Kara et al., 1990). A single point mutation of CREB (K-CREB) resulting in an amino acid change at position of leucine-287 to arginine-287 within the DNA binding domain does not bind to DNA as a homodimer, and competes the binding of wild-type CREB for the CRE site of the somatostatin promoter (Walton et al., 1992).

The molecular mechanisms by which transcription factors mediate cellular responsiveness to second-messenger pathways are in most cases not well understood. However, it seems clear that many cAMP-mediated

CREB

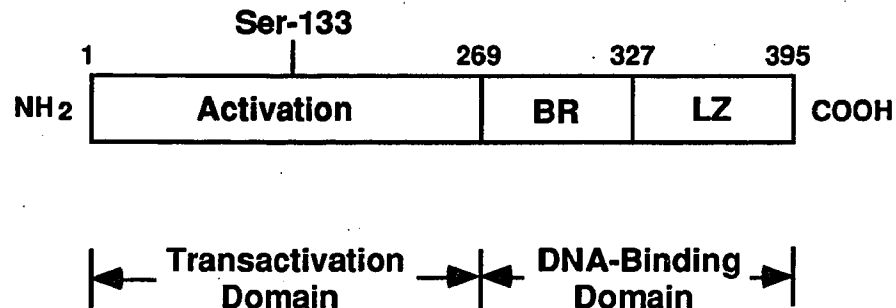


Fig. I-4 Schematic Representation of Functional Domain of CREB. BR, basic region. LZ, leucine zipper.

responses are mediated by the CRE-binding protein CREB. Transcriptional induction by cAMP is rapid, peaking at 30 min and decreasing gradually over 24 hr (Sasaki et al., 1984; Lewis et al., 1987). This stimulation in transcription is insensitive to inhibitors of protein synthesis, suggesting that cAMP may stimulate gene expression by inducing the covalent modification rather than *de novo* synthesis of specific nuclear factors. Since all of the known cellular effects of cAMP occur via a catalytic subunit (C subunit) of cAMP-dependent protein kinase (kinase A), it appears likely that this enzyme mediates the phosphorylation of factors that are critical for the transcriptional response. It has shown that kinase A-deficient cell lines are unable to stimulate

somatostatin gene transcription in response to forskolin (Montminy et al., 1986), and microinjection of the C-subunit into cells can directly activate CRE-dependent transcription without simultaneous addition of cAMP (Riabowol et al., 1988). The activated catalytic subunit of the cAMP-dependent protein kinase (protein kinase A, PKA) appears to translocate to the nucleus (Meinkoth, et al., 1990). PKA phosphorylates a single residue of the CRE-binding protein (CREB) at Ser-133 (Gonzalez and Montminy, 1989), leading to cAMP-dependent activation of somatostatin gene transcription at the CRE (Montminy and Bilezikjian, 1987). Mutation of the CREB protein by substitution of Ser-133 with Ala-133, which resides within the transactivation domain of the protein, completely abolished CREB transcriptional activity (Gonzalez and Montminy, 1989). In addition, phosphorylation by other kinases at sites adjacent to the PKA site of CREB protein also plays a role in its action (Lee et al., 1990).

Depolarization of PC12 pheochromocytoma cells is correlated with rapid phosphorylation of CREB on amino acid residue Ser-133 (Gonzalez and Montminy, 1989). This finding suggests that CREB may mediate transcriptional induction by Ca^{2+} . According to a recent report by Greenberg's group, CREB activity is induced not only by cAMP but also by elevated intracellular Ca^{2+} , which leads to phosphorylation of CREB by Ca^{2+} -calmodulin-dependent protein kinases (CaM kinases) on a site identical to the one recognized by PKA (Sheng et al., 1991). These results suggest that CaM kinases may transduce electrical signals to the nucleus and that CREB functions to integrate Ca^{2+} and cAMP signals. The Ca^{2+} activation of CREB could be indirect and involve activation of a calmodulin-sensitive adenylate cyclase that leads to activation of PKA (reviewed in Karin 1992).

In contrast to the somatostatin gene, where a single element is sufficient for cAMP regulation (Montminy and Bilezikjian, 1987), analysis of proenkephalin gene indicated that two closely related elements are necessary for cAMP regulation (Comb et al., 1988). One of the CRE elements contains a TPA response element (TRE) which is an AP-1 binding site. It has been reported that CREB can bind to the AP-1 site present in the promoter of the c-Jun gene, and activate this promoter upon its phosphorylation by catalytic subunit of cAMP-dependent protein kinase A. Interestingly, CREB in the absence of PKA activity is a repressor of the c-Jun promoter. The ability of CREB to repress transcription from the c-Jun promoter extends to both serum and phorbol 12-myristate 13-acetate (PMA) induction (Lamph et al., 1990). This finding suggests that the transcription factor CREB may have a pleiotropic function, acting as a potent repressor of transcription, but reversing its role upon phosphorylation. Recently, Comb's group has found by co-transfection analysis that in the presence of the catalytic subunit of PKA, AP-1 complexes involving JunD and JunB may act to induce or repress transcription of the proenkephalin gene in response to cAMP (Kobierski et al., 1991). These data suggested that cAMP can regulate gene expression through at least two pathways: one involving the previously described CREB protein acting on classic CRE sequences, and a second pathway involving AP-1 proteins acting at both CRE- and TRE-like sequences.

The "CRE"-like sequence (TGACGGA) of the prolactin proximal promoter region bears a strong resemblance to cyclic AMP response elements (TGACGTCA) that have been identified in the promoters of a number of genes, including somatostatin and tyrosine hydroxylase (two overlapping CRE sequences) (Montminy et al., 1986; Lee et al., 1987); *c-fos* (one CRE sequence) (Treisman, 1986); VIP (two non-overlapping CRE sequences)

(Tsukada et al., 1987) and proenkephalin (one CRE sequence) (Comb et al., 1986). DNase footprint studies have shown that the proximal region of the PRL gene does not contain a high affinity CREB binding site (Liang et al., 1992). DNA gel shift studies have shown that the CRE-like sequence present in the PRL promoter binds to CREB with a lower affinity than to the classic CRE sequence (Yan, G-z., Tian J., and Bancroft, C. in preparation). Martial's group reported that transcriptional induction of the human prolactin gene by cAMP requires two cis-acting elements, including a pit-1 binding site and the CRE-like sequence (Jackson et al., 1992). However, the identify of the protein(s) mediating the cAMP response of the PRL gene, and the role of CREB in regulating this gene, remain to be determined.

CHAPTER 1

Thyrotropin-Releasing Hormone Action on the Prolactin Promoter is Mediated by the POU Protein Pit-1 *

*** All of the material in this chapter, except for Fig. 1-5, has been published:**

Yan, G-Z, W. Pan, and C. Bancroft. (1991) Mol. Endocrin. 5: 535-541.

Abstract

TRH is known to regulate transcription of the PRL gene in pituitary cells, but little is known about the mechanism involved. We have characterized TRH response elements (TRHREs) in the promoter region of the rat PRL gene and the gene-proximal protein that transmits the TRH signal to these elements. Exposure of GH₃ rat pituitary cells to TRH yielded a large specific stimulation of transient expression of a PRL-chloramphenicol acetyltransferase (PRL-CAT) construct containing the PRL promoter region [(-204)PRL-CAT]. Analysis of 5' deletions of this construct implied that regions -174/-113 and -75/+38 each contain a TRHRE. GH₃ cell nuclear extracts are known to footprint four sites, termed, respectively, 1P-4P, on the PRL promoter region. The TRHRE between positions -75/+38 was identified as element 1P, residing at -63/-39, since two copies of a 1P oligodeoxynucleotide transferred a TRH response to either (-39)PRL-CAT or mouse metallothionine-CAT construct (-39)mMT-CAT. Similarly, the more proximal TRHRE may be element 3P, residing at -167/-144, since two copies of this element also transferred a TRH response to (-39)PRL-CAT. Binding of pit-1 to site 1P is known to be capable of activating pituitary cell-specific PRL gene expression. To investigate whether pit-1 can also transduce a TRH signal to this site, oligodeoxynucleotides were prepared corresponding to mutations in either or both of two consensus sequences in site 1P. A strong correlation was observed between the ability of each oligodeoxynucleotide to bind pit-1 and transfer a TRH response to (-39)PRL-CAT, directly implicating pit-1 as the gene-proximal protein that transduces the TRH signal to site 1P. Pit-1 also bound avidly to element 3P (and more weakly to elements 2P and 4P),

suggesting that pit-1 may also transduce a TRH signal to element 3P. These results show that the cell-specific activator pit-1 is also a mediator of peptide hormone regulation of the PRL gene.

Introduction

Early studies with the GH lines of rat pituitary cells showed that the hypothalamic tripeptide TRH is an activator of both secretion of PRL from pituitary cells (Tashjian et al., 1990) and PRL mRNA levels in these cells (Dannies and Tashjian, 1976; Evans and Rosenfeld, 1976). Subsequent studies showed that the stimulation by TRH of PRL gene expression is exerted at the level of transcription (Murdon et al., 1983). TRH response can be directed by either the proximal promoter region (Bay and Maurer, 1989; Camper et al., 1985) or the distal enhancer (Bay and Maurer, 1989). However, TRH response elements in the proximal promoter region of the PRL gene and the functional interaction of pit-1 with these elements have not yet been defined.

Results

The Proximal Prolactin Promoter Contains at Least Two TRH Response Elements.

First, we investigated TRH regulation of transfected proximal PRL promoter-CAT constructs or control Rous sarcoma virus-CAT (RSV-CAT). A typical result employing plasmid (-204)PRL-CAT, containing the first 204 base pairs of DNA upstream of the PRL cap site, is shown in Fig. 1-1, TRH very strongly stimulated (-204)PRL-CAT activity, but only slightly stimulated RSV-CAT activity. GH₃ cells nuclear extracts have been shown previously to footprint four sites on the proximal PRL promoter (Fig. 1-2A). To test whether any of these sites corresponds to a TRH response element (TRHRE), we examined the ability of TRH to stimulate expression of (-204)PRL-CAT and 5'

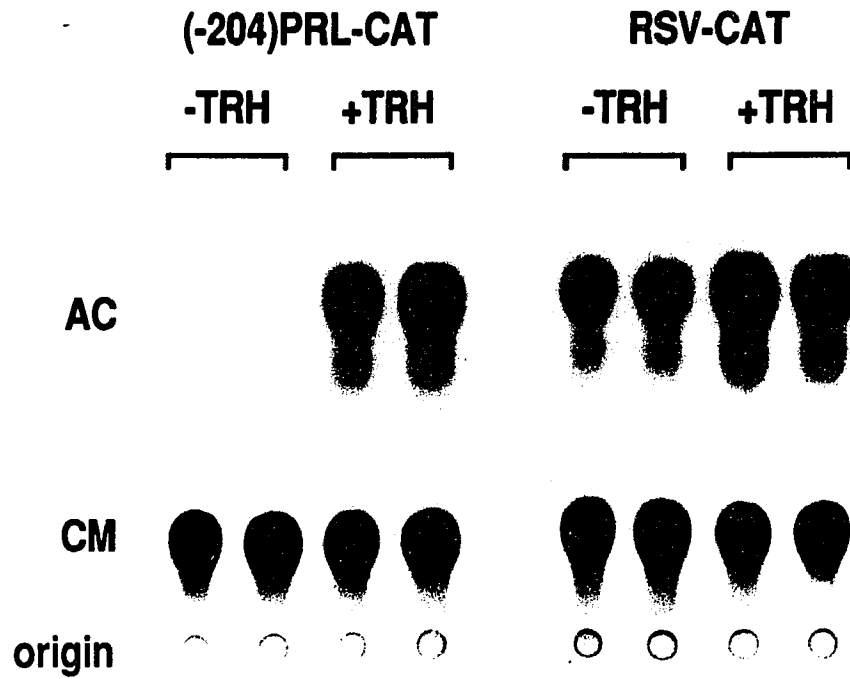


Fig. 1-1. TRH regulation of transfected promoter-CAT constructs. GH₃ cells were transfected with the indicated constructs, preincubated two days, then incubated one day $\pm$ TRH (100nM), and harvested for assay of CAT enzymatic activity. Autoradiographic exposure time was 12 hours. RSV, Rous sarcoma virus promoter; CM, [¹⁴C]Chloramphenicol; AC, acetylated forms of [¹⁴C]Chloramphenicol.

deletion mutants thereof. It is shown in Fig. 1-2B that TRH again yielded a sizable (60 fold) stimulation of expression of (-204)PRL-CAT. This stimulation was not decreased by deletion of PRL promoter sequences between positions -204 to -187 (relative to the cap site). Further deletion from -174 to -75 strongly decreases but does not abolish the response of TRH. However, the first 75 bp of PRL promoter, containing only site 1P plus a TATAA consensus yields a 10-fold TRH response, compared to the non-specific 2-fold response of DHFR-CAT. In numerous independent experiments, TRH was found to consistently yield an approximately 5-fold greater stimulation of (-75)PRL-CAT than of DHFR-CAT (Fig. 1-2C). Thus, 5'-deletion analysis implied that both -174/-113 and -75/+37 contain TRH response elements (TRHRE).

Prolactin Promoter Element 1P is An Independent TRHRE.

It seemed likely that TRH regulation of (-75)PRL-CAT is mediated by PRL promoter element 1P residing at -63/-39, rather than by the other PRL gene sequences present in this construct. To examine this, we have employed an expressible (-39)PRL-CAT(RSVenh.) construct that consists of a further 5' deleted construct (-39)PRL-CAT with the RSV enhancer cloned downstream of the CAT gene. The TRH response of this construct was then compared to constructs containing single or multiple copies of element 1P, that were cloned in the proper orientation in front of (-39)PRL-CAT (Fig. 1-3). TRH yielded a 3.7-fold stimulation of control (-39)PRL-CAT(RSVenh.), close to the 2-fold non-specific stimulation yielded by RSV-CAT. However, two or three copies of 1P conferred upon (-39)PRL-CAT a large, 15-25 fold response to TRH. Primer extension analysis showed that transcription of (1P)²(-39)PRL-CAT

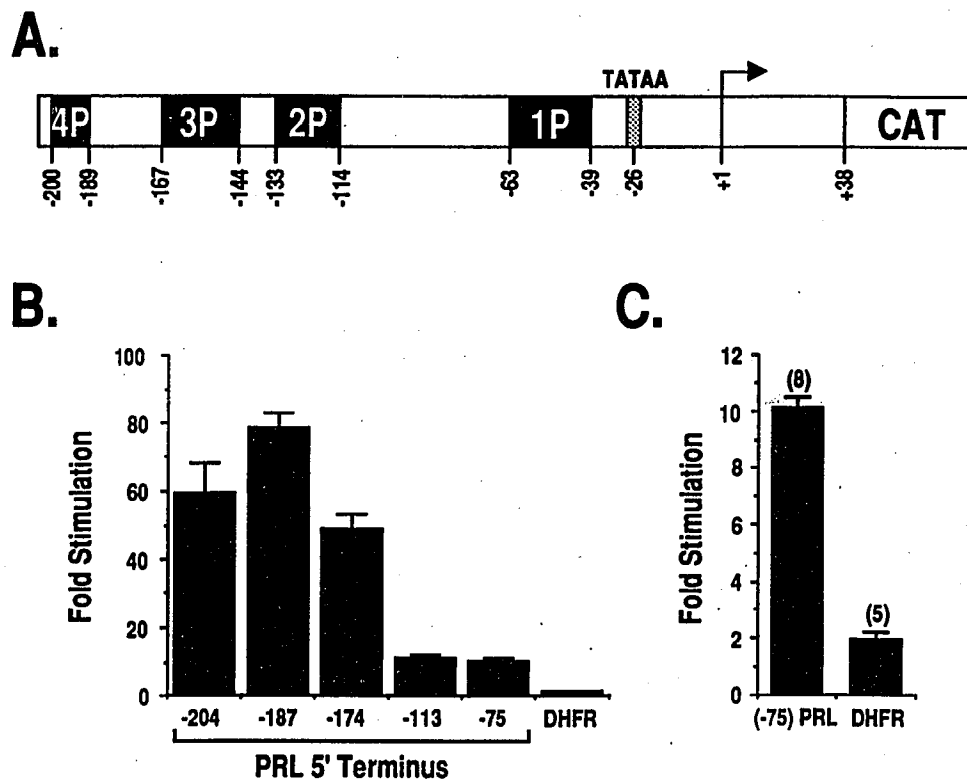


Figure 2

Fig. 1-2. 5'-deletion analysis of PRL promoter TRH response regions. (A). Map of CAT constructs employed and of GH₃ nuclear footprint sites, 1P-4P in proximal PRL promoter region. (B). TRH regulation of (-204)PRL-CAT and its 5'-deletion mutants, and of DHFR-CAT (DHFR). The experiment procedures described as in Fig.1-1. Values shown are the mean $\pm$ SE of the fold stimulation observed with triplicate dishes. Mean basal (without TRH) expression for the PRL-CAT constructs (percent conversion of chloramphenicol) was: (-204), 0.58; (-187), 0.5 (0.5-h enzymatic incubation time); (-174), 0.14; (-113), 0.53; and (-75), 0.40; that for DHFR-CAT was 19. (C). Mean $\pm$ SE of the fold TRH stimulation of CAT enzymatic activity directed in GH₃ cells by (-75)PRL-CAT or DHFR-CAT in the indicated number (in parentheses) of independent experiments. DHFR, Chinese hamster dihydrofolate reductase promoter.

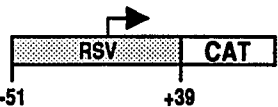
	CAT Activity (% conversion)		Fold Stimulation
	- TRH	+TRH	
	24 ± 0.6	51 ± 4	2.1 ± 0.1
	0.7 ± 0.03	2.6 ± 0.1	3.7 ± 0.04
	1.4 ± 0.2	6.2 ± 0.7	4.3 ± 0.04
	0.4 ± 0.02	9.6 ± 1.3	25 ± 2
	0.6 ± 0.05	9.2 ± 1.5	15 ± 1
	1.1 ± 0.1	3.3 ± 0.3	3.0 ± 0.1
	0.73 ± 0.03	5.9 ± 0.6	8.1 ± 0.4
	0.39 ± 0.02	2.0 ± 0.2	5.1 ± 0.3

Fig. 1-3. Ability of proximal prolactin promoter DNA elements to confer TRH responsiveness to a minimal PRL-CAT construct. GH₃ cells transfected with the indicated constructs were incubated ± TRH as in Fig. 1-1. Values shown are the mean ± SE of results with triplicate dishes. Enh., RSV enhancer.

initiated at the appropriate site (data not shown). Also we have examined the TRH response of two copies of site 2P, 3P, or 4P, cloned in the proper orientation in front of (-39)PRL-CAT. Site 2P yielded a non-specific 3-fold response, while 3P directed a sizable 8-fold response and 4P yielded a more modest 5-fold response.

To determine whether element 1P can direct a TRH response in the absence of any other PRL gene elements, we examined the ability of site 1P to transfer a TRH response to a synthetic mouse metallothionein promoter construct, (-39)mMT-CAT, which was designed to position the inserted 1P element at its natural distance from the cap site (Fig. 1-4). Interestingly, a construct containing two copies of 1P in the proper orientation, (1P)²(-39)mMT-CAT, implying that, in this context, element 1P can act as a gene repressor. More significantly, construct (1P)²(-39)mMT-CAT yielded an 8.4-fold TRH response, compared to the 1.3-fold response of (-39)mMT-CAT. This is not likely to be due to a nonspecific enhancer effect, since, as shown in Fig. 1-3, TRH yields only a 2-fold stimulation of the strong enhancer-containing construct RSV-CAT. Thus, PRL promoter element 1P can function as an independent TRHRE.

Transcription Factor Pit-1 Transduces the TRH Transcription Signal to the Prolactin Promoter.

The results presented above implicated site 1P (as well as site 3P) as a TRH response element, but do not yield information about DNA-binding transcription factor(s), such as pit-1, that might mediated the TRH response. It has been reported that binding to site 1P of pit-1 transcription factor can activate pituitary cell-specific PRL gene expression (Mangalam et al., 1989).

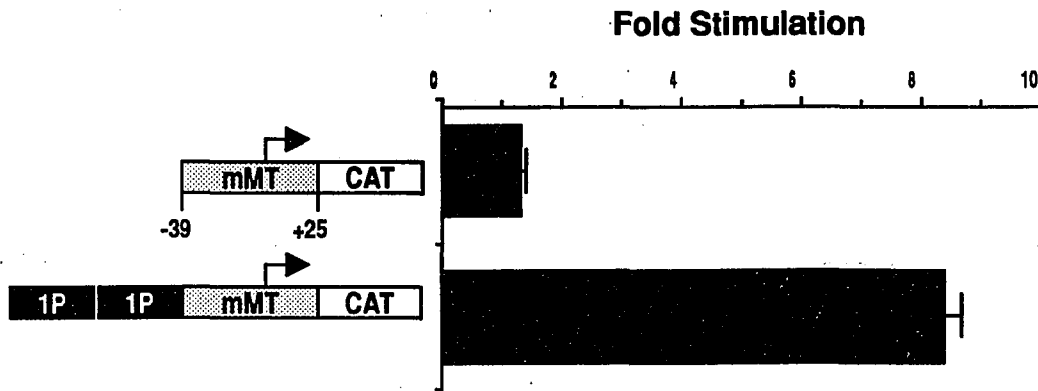


Fig. 1-4. Ability of element 1P to confer TRH responsiveness to a minimal mMT-CAT constructs. GH₃ cells transfected with the indicated constructs were incubated $\pm$ TRH as in Fig. 1-1. Values shown are the mean $\pm$ SE of results with triplicate dishes. Mean basal (without TRH) expression for the mMT-CAT constructs (percent conversion of chloramphenicol) was: -39mMT, 16; (1P)²(-39)mMT, 4.6. mMT, mouse metallothionein promoter.

The time course of TRH-stimulated prolactin transcription showed that the signal generated by the binding of TRH to its plasma membrane receptor rapidly reaches the cell nucleus (Murdoch et al., 1983). To investigate whether interaction of pit-1 with site 1P also plays a role in the TRH response of the prolactin gene, we first examined the acute effect of TRH addition to GH₃ cells on the ability of pit-1 to bind to site 1P. Fig. 1-5A shows that there is a remarkably rapid onset of the TRH-stimulated DNA binding activity of GH₃ nuclear extracts. DNA binding activity was significantly increased after 2 min of TRH treatment and reached maximum levels at 5 min. With continuing exposure to TRH, DNA binding activity gradually decreased after 30 min (Fig. 1-5A), reaching levels only slightly above the control by 24 hours (data not shown). These DNA binding shift bands can be supershifted by using pit-1 antiserum (Fig. 1-5B lane 2) and competed by using 1P oligodeoxynucleotide (Fig. 1-5B lane 3), showing that the shifts in Fig. 1-5A are due to pit-1.

The four PRL promoter proximal footprints plus two pit-1-binding sites in the GH promoter yielded the consensus sequence (A/T)TA(A/T)TCA (Fig. 1-6). It is seen in Fig. 1-6 that site 1P contains an inverted repeat of this sequence. To test the importance of each consensus for pit-1 binding and TRH induction, 1P mutant oligomers were prepared corresponding to a four-base non-conservative transverse mutation in either the left-hand (*1P), the right-hand (1P*), or both (*1P*) consensus sequences (Fig. 1-6). Oligomers corresponding to footprint sites 2P, 3P, and 4P (see Fig. 1-6) were also prepared. First, the ability of pit-1 synthesized *in vitro* to bind to 1P and mutants as well as the other three prolactin promoter elements 2P-4P (Fig. 1-2A) was investigated by Wayne Pan of the Bancroft laboratory, yielding results described in Yan et al. (1991). We then examined the ability of double copies of

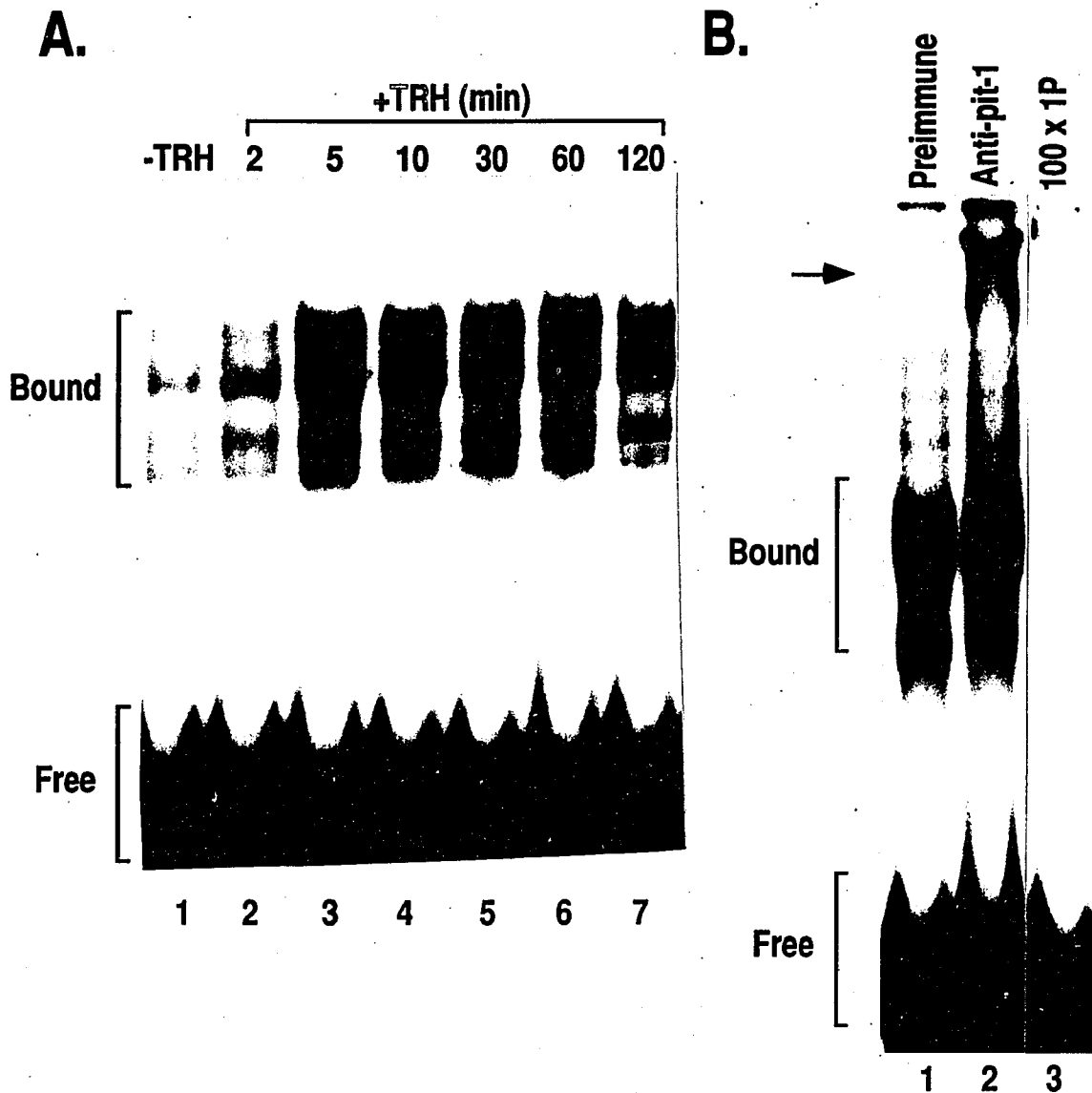


Fig. 1-5. Binding of TRH-stimulated GH₃ cell nuclear extracts to 1P oligodeoxynucleotide. (A). DNA shifts observed with a 1P [³²P]-labeled probe incubated with nuclear extracts of TRH-stimulated GH₃ cells. GH₃ cells were preincubated with SFM+Ca²⁺ for two days, then incubated with SFM+Ca²⁺+TRH (200nM) for 2, 5, 10, 30, 60, and 120 minutes respectively, (lane 2-7), or without TRH for 120 minutes (lane 1). **(B).** DNA shifts observed with 1P[³²P]-labeled probes incubated with nuclear extracts of TRH-stimulated GH₃ cells for 120 min in presence or absence of antisera to pit-1 (lane 1-2), and in presence of 100 x 1P oligodeoxynucleotide as a competitor (lane 3). Autoradiographic exposure time (-70 °C with screen) was 12 hours.

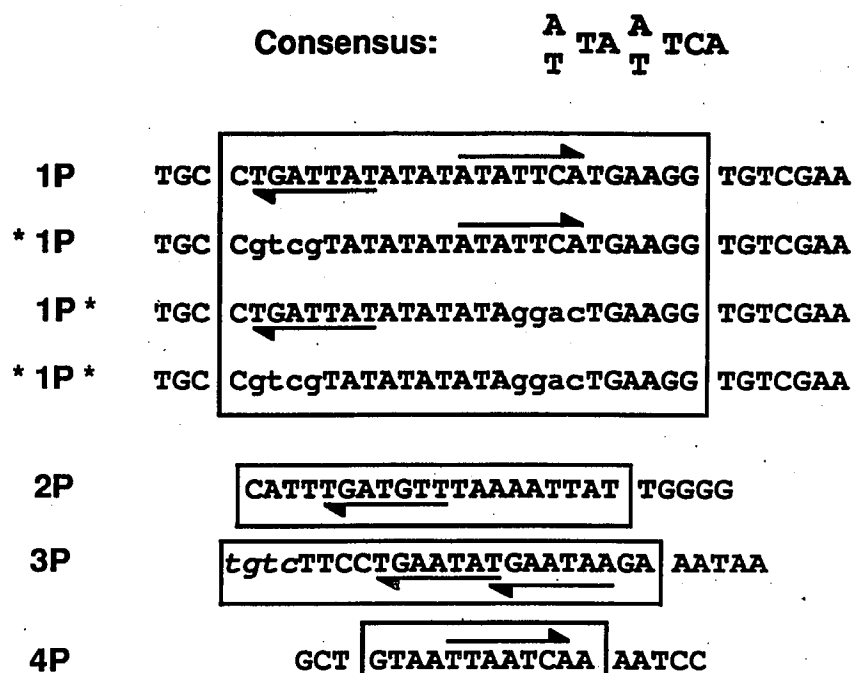


Fig. 1-6 Sequences of oligodeoxynucleotides of pit-1 binding sites. For 1P-4P, each sequence corresponds to a region of the rat PRL promoter; *boxes* correspond to the footprints detected with GH₃ cell extracts (diagrammed in Fig. 1-2A). *Right or left arrows* indicate consensus sequences in, respectively, the top or bottom strand. Mutated 1P oligodeoxynucleotides are indicated by *asterisks*. *Italicized letters* in the 3P sequence indicate footprinted bases not included in the oligodeoxynucleotide. In addition to the sequence shown, oligodeoxynucleotides contained short cloning linker overhangs (see *Materials and Methods*).

either site 1P or its mutants to direct a TRH response. To reproduce as closely as possible the natural context of these oligomers, they were cloned, in the proper orientation, in front of the minimal (-39)PRL-CAT construct.

However, as expected, construct (*1P*)²(-39)PRL-CAT was not detectably expressed in GH₃ cells. Therefore, the TRH response of *1P* was examined by inserting the RSV enhancer downstream of (*1P*)²(-39)PRL-CAT to produce (*1P*)²(-39)PRL-CAT(enh.). It is seen in Fig. 1-7 that either 1P or *1P directed a strong, 16-18 fold TRH response. By contrast, 1P* directed a greatly reduced 5-fold response, while *1P* yielded only a non-specific 2.6-fold response.

We then compared, for 1P and each of its mutants, the ability to bind pit-1 with the ability to direct a TRH response. To do this, the pit-1 binding data (Yan et al., 1991.) were quantitated and plotted against the TRH responses shown in Fig. 1-7. It is seen in Fig. 1-8 that there is a very good linear correlation between pit-1 binding and TRH induction, implying that the cell-specific protein pit-1 transduces the TRH transcriptional signal to element 1P of the prolactin promoter.

Discussion

In this study, we have characterized TRH response elements in the proximal promoter region of the PRL gene and the functional interaction of pit-1 with these elements. Transient expression in GH₃ cells was employed to analyze TRHREs in the proximal PRL gene promoter region. Construct (-204)PRL-CAT exhibited a large (60-fold) TRH response, which was not significantly affected by deletion to position -174 (Fig. 1-2B). Further 5' deletion analysis showed that the first 174 bp of the PRL promoter contain at least two TRHREs, respectively above and below position -113.

	CAT Activity (% conversion)		Fold Stimulation
	- TRH	+TRH	
	0.80 ± 0.05	2.1 ± 0.1	2.6 ± 0.1
	0.42 ± 0.02	7.6 ± 0.3	18 ± 0.2
	0.38 ± 0.02	6.0 ± 0.2	16 ± 0.3
	0.63 ± 0.05	3.2 ± 0.1	5.1 ± 0.3
	2.6 ± 0.1	6.6 ± 0.3	2.6 ± 0.02

Fig. 1-7. Ability of wild-type and mutant 1P elements to confer TRH responsiveness to a minimal PRL-CAT constructs. GH3 cells transfected with the indicated constructs were incubated $\pm$ TRH as in Fig. 1-1. Values shown are the mean $\pm$ SE of results with triplicate dishes. enh., RSV enhancer.

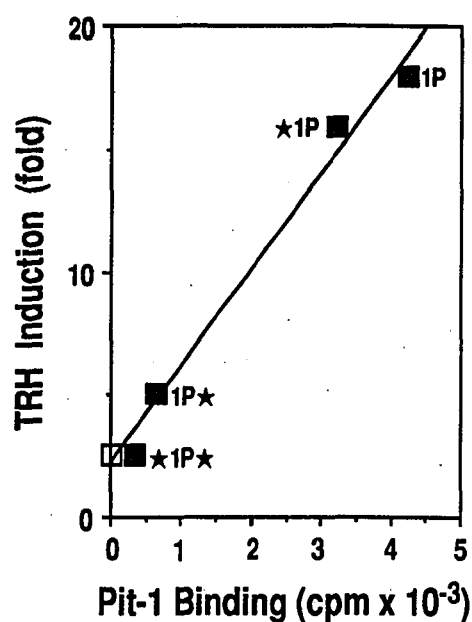


Fig. 1- 8. Correlation for wild-type and mutant 1P elements between ability to bind pit-1 and ability to direct a TRH response. The fold TRH stimulations shown in Fig. 1-6 were plotted against the radioactivity in the shifted bands which data produces from Yan et al., 1991. The open box corresponds to the fold TRH stimulation obtained with construct (-39)PRL-CAT(enh.). Linear regression analysis yielded a correlation coefficient of 0.98.

Analysis of the extensively deleted construct (-75)PRL-CAT suggested that the more proximal TRHRE corresponds to element 1P, centered at position -51 (Fig. 1-2C). The identity of this element as an independent TRHRE was confirmed by the ability of multiple copies of 1P to confer a TRH response to either a homologous (Fig. 1-3) or a heterologous (Fig. 1-4) promoter. It has been shown previously that interaction of the transcription factor pit-1 with element 1P can direct cell-specific PRL gene expression (Mangalam et al., 1989). To investigate whether pit-1 might also mediate the TRH response of this gene, mutations were introduced into either of two consensus sequences in site 1P; their abilities to bind pit-1 and direct a TRH response were then compared. The excellent correlation observed between these two parameters (Fig. 1-7) strongly implicates pit-1 as the gene-proximal protein that transduces the TRH transcriptional signal to element 1P. This represents the first demonstration of a role for pit-1 in hormonal regulation, and thus shows that pathways for cell-specific and peptide hormone regulation of the PRL gene intersect at or before the level of interaction of this transcription factor with site 1P.

In the present experiments, we have found that TRH treatment of GH₃ cells increases pit-1 binding to site 1P within 2 minutes (Fig. 1-5A). This result is correlated to the acute effect of TRH on prolactin transcription (Murdoch et al., 1983). Kapiloff et al. have shown that activators of PKA or PKC are able to stimulate pit-1 phosphorylation in GH₃ cells (Kapiloff et al., 1991). These findings suggest that regulation of PRL transcription may be mediated by changes in pit-1 phosphorylation. Recently, Maurer has found that pit-1 can be phosphorylated by acute TRH stimulation. The TRH-induced phosphorylation of pit-1 reaches a peak in about 5 min, but it rapidly returns to near basal level after 30 minutes of treatment (Dr. R. Maurer, personal

communication). These findings imply that TRH-induced pit-1 phosphorylation is neither sufficient nor necessary for changes in PRL gene expression. Hence, further analysis will be required to elucidate both the biochemical basis of the transduction by pit-1 of the TRH signal to element 1P and the identity of protein(s) that conducts the TRH signal from its plasma membrane receptor to pit-1.

Previous experiments employing GH₃ cell nuclear extracts fractionated on either a phosphocellulose column or a DNA affinity column have yielded footprints over sites 1P-4P (Mangalam et al., 1989). However, the present DNA shift experiments demonstrate for the first time that cloned pit-1 can bind to all four of these sites in the PRL promoter, albeit with varying affinities (Yan et al., 1991). The observation that site 2P can bind pit-1, which has been detected only in pituitary cells (Bodner et al., 1988; Ingraham et al., 1988), conflicts with a previous report of a ubiquitous footprint in this region (Gutierrez-Hartmann et al., 1987). The reason for this discrepancy is not clear; however, it should be noted that of the four DNA sites examined, site 2P exhibited the lowest affinity for pit-1 (Yan et al., 1991).

The results of 5' deletion analysis (Fig. 1-2B) implied that the PRL proximal region contains at least one TRHRE in addition to element 1P, residing between positions -174/-113. The observation that element 3P, but not 2P, can direct a significant TRH response (Fig. 1-3) suggests that 3P may represent the additional up-stream TRHRE detected by 5' deletion analysis. Mutational studies of 3P, similar to those described here, should reveal whether pit-1 can also transduce a TRH transcriptional signal to this element. The fact that 4P also yields a weak TRH stimulation (Fig. 1-3) implies that the proximal PRL promoter in fact contains multiple TRHREs. This conclusion is consistent with the recent finding by Maurer's group that the TRH response

of the PRL proximal promoter is unchanged by any of 19 clustered point mutations covering positions -193/-30 of the PRL promoter (Iverson et al., 1990). The observation that the distal PRL enhancer can also direct a TRH response (Day and Maurer, 1989) implies that there is an abundance of TRH regulatory motifs in the DNA region up-stream of the PRL gene cap site. The regulatory advantage of having multiple (and thus possibly redundant) TRHREs is not presently known, but it seems possible that this pattern plays a role in the complex, multihormonal regulation of the PRL gene (Hinkle, 1988; Bancroft, 1981).

CHAPTER 2

Mediation by Calcium of Thyrotropin-Releasing Hormone Action on the Prolactin Promoter via Transcription Factor Pit-1 *

*** All of the material in this chapter has been published: Yan, G-Z and C.
Bancroft. (1991) Mol. Endocrin. 5: 1488-1497.**

Abstract

Mediation by Ca^{2+} of TRH action on the PRL promoter was investigated by both additivity and pharmacological studies and by techniques that probe more gene-proximal events. TRH required the presence of Ca^{2+} in the medium for stimulation of transient expression in GH₃ cells of a PRL-chloramphenicol acetyltransferase (PRL-CAT) construct containing proximal PRL promoter sequences [(-187)PRL-CAT]. Chronic 12-O-tetradecanoyl phorbol-13-acetate down-regulation of cellular protein kinase C did not block induction of expression of (-187)PRL-CAT by either Ca^{2+} or TRH. In studies with Ca^{2+} blockers, the Ca^{2+} flux inhibitors cobalt ion and nimodipine blocked induction of (-187)PRL-CAT expression by either Ca^{2+} or TRH. On the other hand, the Ca^{2+} immobilizers 1,2-bis(O-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyltetraester and 8-(*N,N*-diethylamino)octyl 3, 4, 5-trimethoxybenzoate blocked induction of expression of this construct by Ca^{2+} but not by TRH, suggesting that TRH regulation of the PRL promoter may dependent on Ca^{2+} immobilization. We have shown previously that the PRL promoter pit-1 binding site 1P is a TRH response element. In the present studies, Ca^{2+} regulation studies with 5'-deletion mutants of (-204)PRL-CAT showed that (-75)PRL-CAT, containing the single pit-1 binding site 1P, also contains a Ca^{2+} response element. The observation that two copies of site 1P oligomer transferred a Ca^{2+} response to either of the two minimal constructs (-39)PRL-CAT or (-39)mouse metallothionine-CAT showed that site 1P is an independent Ca^{2+} response element. Analysis of site 1P mutants yielded a strong correlation between the ability to bind pit-1 and to transfer a Ca^{2+} response. In addition, coexpression of a mutant pit-1 possessing reduced

trans-activational activity strongly inhibited TRH regulation of (-187)PRL-CAT and partially blocked Ca^{2+} regulation of this construct. We conclude that Ca^{2+} mediates TRH action on the PRL promoter, and that pit-1 represents a gene-proximal mediator in this signaling pathway.

Introduction

Evidence has been presented previously for a possible involvement of Ca^{2+} in regulation by TRH of expression of the PRL gene in GH-cells (Laverriere et al., 1989; White and Bancroft, 1983). Ca^{2+} itself has been shown to specifically and strongly stimulate PRL mRNA accumulation in cultured pituitary cells (White et al., 1981). In Chapter 1, I have localized two TRHREs in the proximal promoter region to known binding sites for the cell-specific activator pit-1, and provided evidence that TRH action via the most proximal site, 1P, is mediated by the pit-1 protein. In studies of the Ca^{2+} response, Arthur Jackson in our laboratory showed that the first 174 bp of the PRL promoter can direct a Ca^{2+} induction (Jackson and Bancroft, 1988), and Day and Maurer have shown that the distal and the proximal regions can each confer a Ca^{2+} response (Day and Maure, 1990). In this study, I have employed additivity and pharmacological studies, as well as 5'-deletion analysis and mutation of site 1P and of pit-1, to investigate further the involvement of Ca^{2+} in regulation by TRH of transfected constructs containing the PRL promoter or its fragments.

Results

Interaction of Ca^{2+} and TRH in Regulation of PRL-CAT Expression.

In our previous studies of Ca^{2+} and TRH regulation of endogenous PRL gene, we found that, in Ca^{2+} -deprived GH₃ cells, Ca^{2+} alone yields a large stimulation of PRL mRNA levels, while TRH can stimulate PRL mRNA levels only in the presence of added Ca^{2+} (White and Bancroft, 1983). Since the

present studies were conducted with transfected PRL-CAT constructs, we first investigated whether regulation of such a construct by Ca^{2+} and TRH would be similar to regulation of endogenous PRL mRNA. Because previous studies have shown that the first 200 bp of the PRL promoter yield a large response to either Ca^{2+} (Jackson and Bancroft, 1988) and TRH (Yan et al., 1991), plasmid (-187)PRL-CAT, containing the first 187 bp of DNA upstream of the cap site, was employed. Furthermore, since we have found (Jackson and Bancroft, 1988) that the 7.8-fold Ca^{2+} induction of (-231)PRL-CAT obtained by treating Ca^{2+} -deprived cells with Ca^{2+} (0.5mM) can be increased to about 45-fold by also including the voltage-dependent Ca^{2+} channel modulator Bay K8644 (300nM), this Ca^{2+} /Bay K8644 protocol was employed in the present experiments.

results with (-187)PRL-CAT or control construct DHFR-CAT, containing the dihydrofolate reductase promoter, are shown in Fig. 2-1 with (-187)PRL-CAT. While Ca^{2+} / BayK alone yielded an 11-fold stimulation of CAT activity, TRH alone yielded only a 2.5-fold increase in CAT activity. By contrast, Ca^{2+} /BayK plus TRH yielded a 36-fold stimulation of CAT activity. These effects are promoter-specific, since Ca^{2+} / BayK and TRH, alone or in combination, had no significant effect on expression of the control DHFR-CAT construct. Thus, in agreement with our previous studies of endogenous PRL mRNA levels (White and Bancroft, 1983), TRH acts synergistically with Ca^{2+} in regulating PRL-CAT.

Pharmacological Studies of Regulation by Ca^{2+} and TRH of PRL-CAT Expression.

We previously have employed chronic exposure of GH₃ cells to 12-O-tetradecanoyl phorbol-13-acetate (TPA) to investigate a possible role of protein

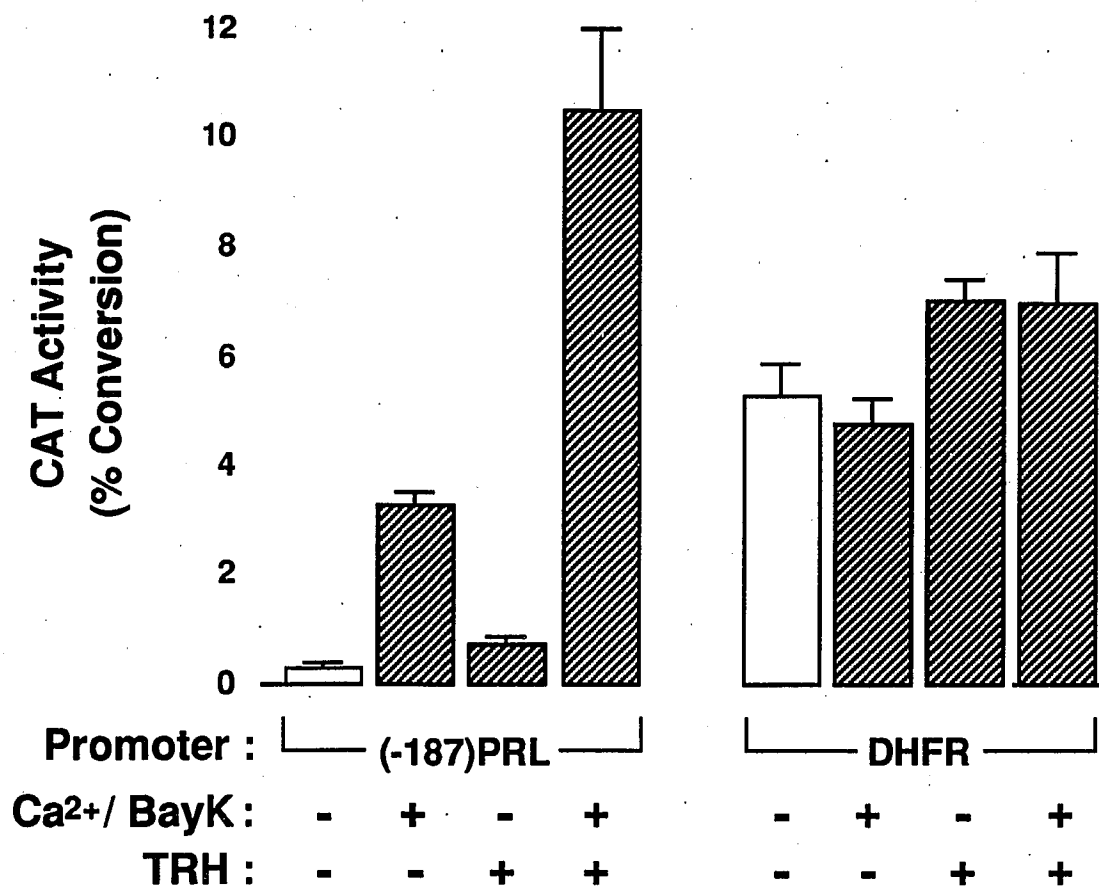


Fig. 2-1. Regulation of transfected promoter-CAT constructs by TRH in the presence or absence of added Ca²⁺. GH₃ cells were transfected with the indicated constructs, then preincubated in SFM and incubated with or without Ca²⁺/Bay K (0.5mM/300nM) or TRH (100nM), as described in Materials and Methods, and assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of the percent conversion of [¹⁴C]chloramphenicol observed with triplicate dishes. (-187)PRL, (-187)PRL-CAT; DHFR (Chinese hamster dihydrofolate reductase promoter), DHFR-CAT; Bay K, Bay K8644.

kinase C in regulation of the endogenous PRL gene. We have found that chronic exposure of GH₃ cells to TPA under culture conditions employed in the present experiments reduced protein kinase C activity by 7- to 15-fold and blocked the ability of platelet-derived growth factor to stimulate cellular *c-fos* mRNA levels (Bandyopadhyay and Bancroft, 1989), or of epidermal growth factor to stimulate either PRL mRNA levels or expression of a PRL-CAT construct (Jackson et al., 1990).

If Ca²⁺ lies on the pathway of TRH action, it would be predicted from the above results that neither Ca²⁺ nor TRH stimulation of the PRL promoter would require protein kinase C activity. To investigate this question, we examined the effect of chronic TPA treatment of GH₃ cells on ability of Ca²⁺ or TRH to stimulate (-187)PRL-CAT activity (Fig. 2-2). As observed previously (Jackson et al., 1990), chronic pretreatment with TPA (648nM) had little effect on control levels of CAT activity, implying that basal PRL promoter expression is not influenced by C kinase activity. Furthermore, as expected, the stimulation of CAT activity by acute TPA (100nM) was almost entirely blocked by chronic pretreatment with TPA. It is seen in Fig. 2-2A that chronic TPA pretreatment had no effect on the stimulation by Ca²⁺ of CAT activity, in agreement with our previous studies of Ca²⁺ regulation of endogenous PRL mRNA levels (Bandyopadhyay and Bancroft, 1989). Chronic TPA pretreatment also had no effect on the stimulation by TRH of CAT activity (Fig. 2-2B), implying that TRH stimulation of PRL promoter activity is also not blocked by down-regulation of protein kinase C. These results imply that protein kinase C does not lie on the pathway of action of either Ca²⁺ or TRH on PRL gene expression.

To investigate further whether Ca²⁺ lie on the pathway of action of TRH on PRL promoter, we examined the effects of types of cellular Ca²⁺

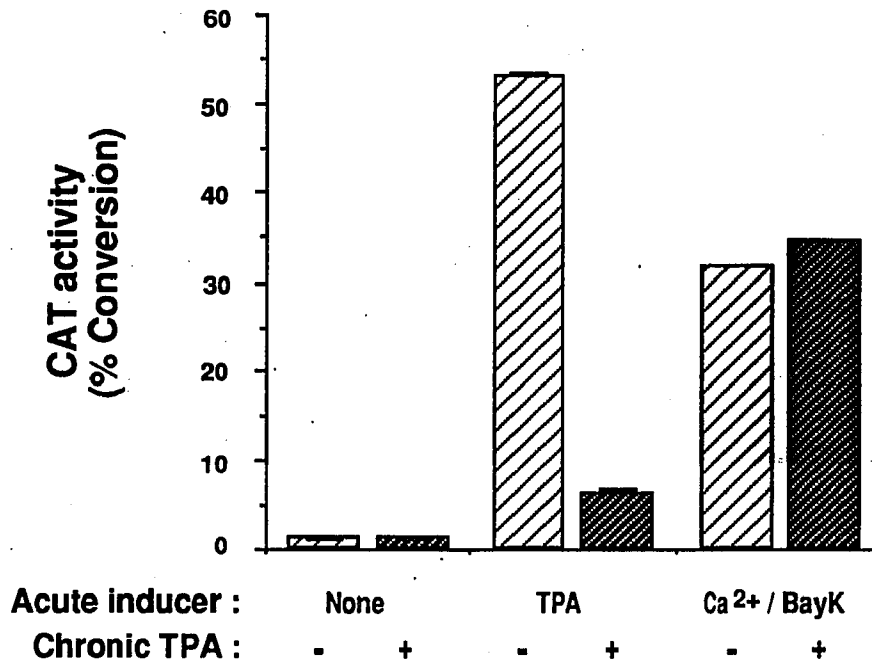
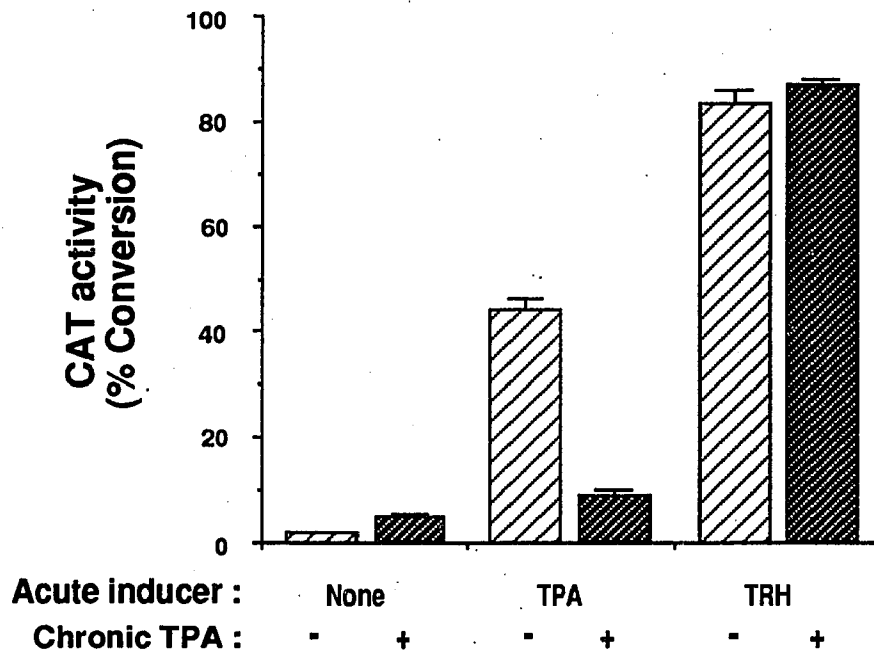
A. Ca^{2+} **B. TRH**

Fig. 2-2. Effect of chronic TPA treatment on induction by Ca^{2+} or TRH of transient expression of a PRL-CAT construct. GH₃ cells were transfected with plasmid (-187)PRL-CAT, then preincubated 2 days with or without TPA (648nM) (Chronic TPA), after which some cultures additionally received one of the following (Acute inducer): None, TPA (100nM), Ca^{2+} /Bay K (0.5mM/300nM), or TRH (100nM). After additional incubation, cultures were assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of results with triplicate dishes.

inhibitors on regulation of (-187)PRL-CAT by either Ca^{2+} or TRH. The first type consisted of two inhibitors of Ca^{2+} fluxes, cobalt inhibits Ca^{2+} -mediated effect on GH₃ cells both at the level of influx of extracellular Ca^{2+} and at an intracellular locus, and also inhibits TRH stimulation of GH₃ cell secretion of PRL (Thaw et al., 1984). the voltage-dependent Ca^{2+} channel inhibitor nimodipine blocks Ca^{2+} fluxes during depolarization (Lee and Tsien, 1983 ; Janis et al., 1987), which occurs spontaneously in GH₃ due to Ca^{2+} action potentials (Taraskevitch and Douglas, 1980). Nimodipine has been shown previously to inhibit both endogenous PRL mRNA levels (Hinkle et al., 1988) and expression of (-231)PRL-CAT (Jackson and Bancroft, 1988) in GH₃ cells. The second type, immobilizers of intracellular Ca^{2+} , consisted of 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid acetoxymethyltetraester (BAPTA/AM), which selectively chelates intracellular Ca^{2+} (Tsien, 1980; Lew et al., 1982), and 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8), which immobilizes Ca^{2+} present in intracellular sequestering site (Chiou and Malagodi, 1975).

Results with these Ca^{2+} inhibitors are shown in Fig. 2-3. Exposure of GH₃ cells to either Ca^{2+} /BayK (Fig. 2-3A) or TRH (Fig. 2-3B) alone yielded a sizable stimulation of expression of CAT activity by (-187)PRL-CAT. Each of the two Ca^{2+} flux inhibitors exhibited similar inhibitory effects on the stimulatory actions of Ca^{2+} and TRH. Thus, the stimulation of CAT activity by Ca^{2+} was entirely blocked by either cobalt or nimodipine. Similarly, stimulation by TRH of CAT activity was totally blocked by cobalt and almost totally inhibited by nimodipine. By contrast, the Ca^{2+} immobilizers distinguished between the regulatory effects of Ca^{2+} and TRH. While BAPTA/AM and TMB-8 each totally blocked Ca^{2+} stimulation of CAT activity,

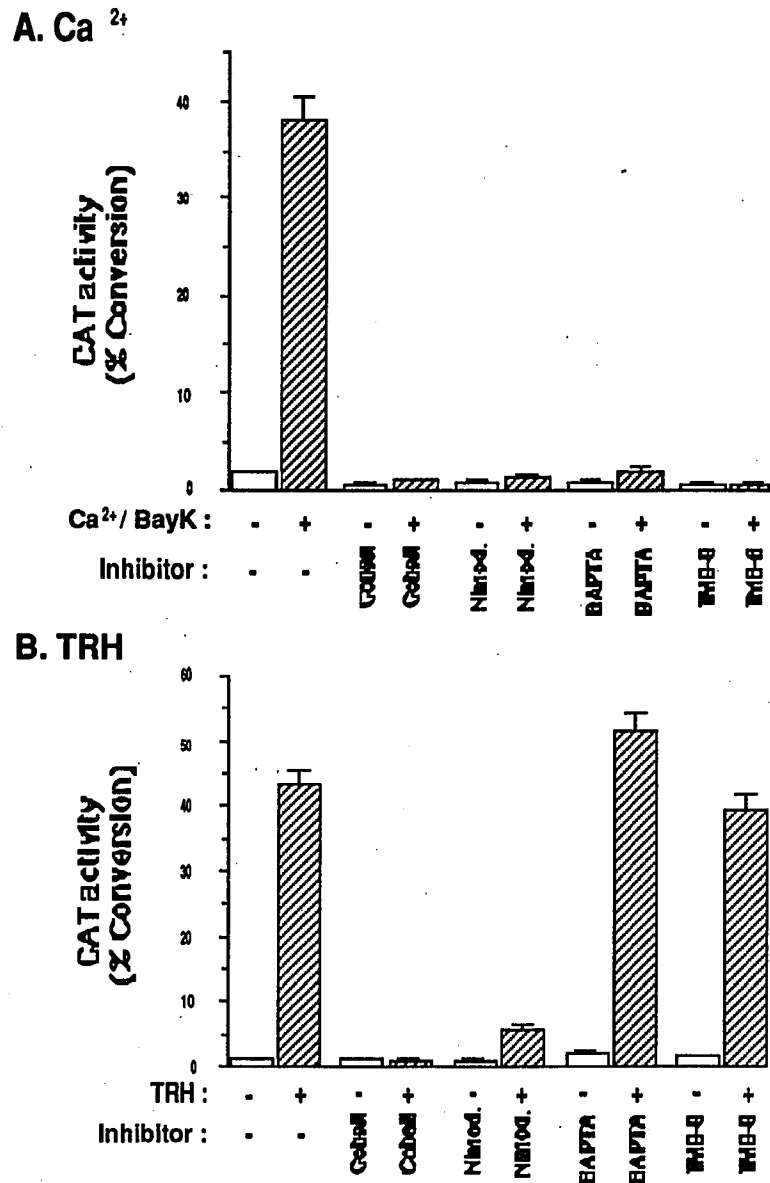


Fig. 2-3. Effect of cellular Ca^{2+} inhibitors on induction by Ca^{2+} or TRH of transient expression of a PRL-CAT construct. GH₃ cells were transfected with plasmid (-187)PRL-CAT, then incubated with or without either Ca^{2+} /Bay K (A) or TRH (B) as in Fig. 2-1. Where indicated, at 3h before the addition to some cultures of Ca^{2+} /Bay K or TRH, some cultures received either CoCl_2 (Cobalt, 1mM), nimodipine (Nimod., 200nM), BAPTA/AM (BAPTA, 10 μ M), or TMB-8 (10 μ M). After additional incubation with these additions, cells were assayed for CAT activity. Shown is the mean $\pm$ SE of results with triplicate dishes.

neither of these inhibitors significantly affected the ability of TRH to stimulate CAT activity. Although it is difficult to draw mechanistic conclusions from these pharmacological studies, these results support the concept that TRH regulation of PRL promoter activity is dependent upon cellular Ca^{2+} fluxes, but is not sensitive to chelation or immobilization of intracellular Ca^{2+} .

Pit-1 Interaction with Its Proximal Binding Site in the PRL Promoter Can Mediate the Action of Both Ca^{2+} and TRH.

The results of the combinatorial and pharmacological experiments described above imply that Ca^{2+} is a mediator of TRH stimulation of PRL gene expression. If Ca^{2+} does mediate this transcriptional action of TRH, then at the DNA level one would expect: 1) a TRHRE to colocalize with a Ca^{2+} response element (CaRE); and 2) the same gene-proximal transcriptional factor to mediate the response of each of these factors. As illustrated in Fig. 2-4A, the proximal PRL promoter has been found to contain four binding sites for the cell-specific transcription factor pit-1 (Lufkin et al., 1989; Nelson et al., 1989). We previously have provided evidence that the proximal PRL promoter contains at least two TRHREs, corresponding to sites 1P and 3P, and that pit-1 is the gene-proximal mediator of TRH action on site 1P (Yan et al., 1991).

We have shown previously that PRL-CAT constructs containing progressive 5' deletions of the PRL promoter from position -958 to -174 yielded a constant Ca^{2+} stimulation, implying that the first 174 bp of the PRL promoter can direct a full Ca^{2+} response (Jackson and Bancroft, 1988). To

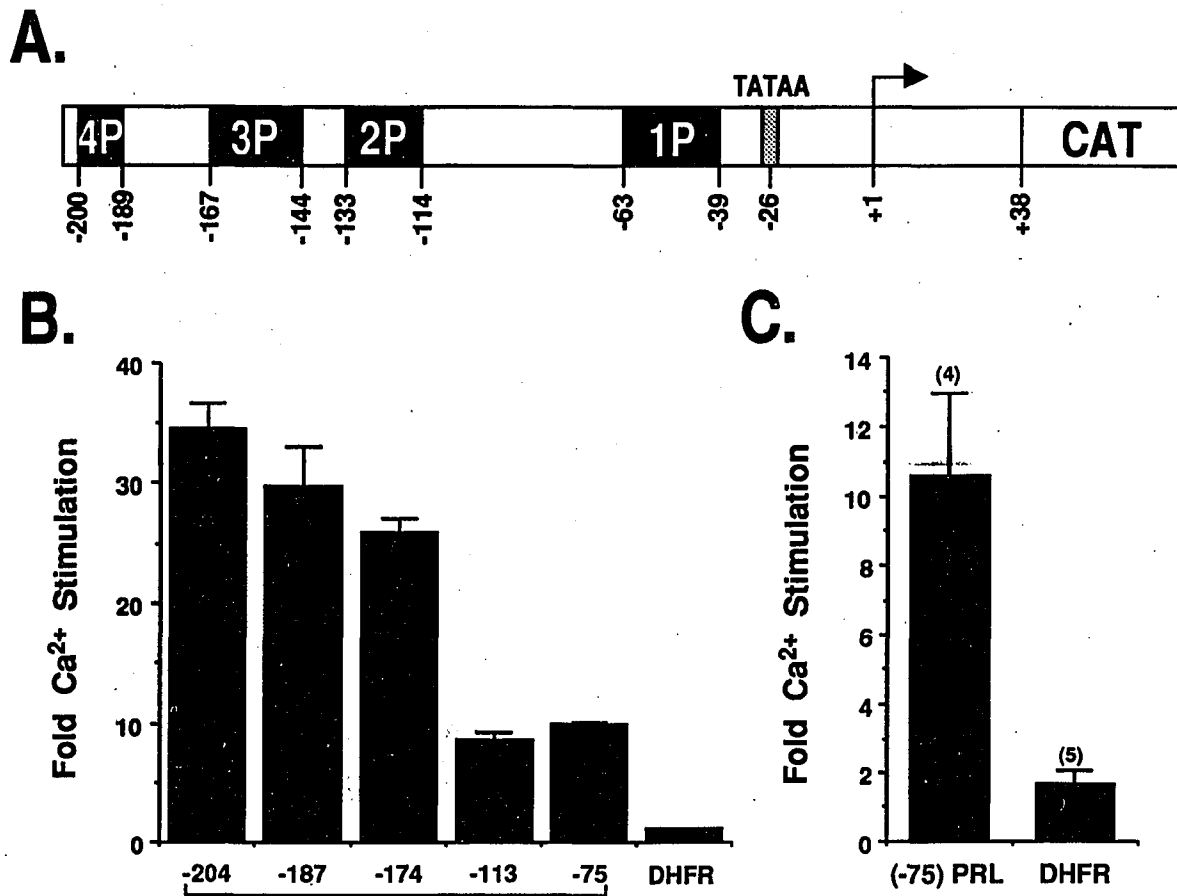


Fig. 2-4. Analysis by 5'-deletion of PRL promoter Ca²⁺ response region. A, Map of construct (-204)PRL-CAT, containing rat PRL gene sequences from positions -204/+38 and of pit-1 binding site 1P-4P in the PRL promoter. **B,** Ca²⁺ regulation of (-204)PRL-CAT and its 5'-deletion mutants and of DHFR-CAT (DHFR). PRL-CAT constructs are indicated by their 5'-terminus. GH₃ cell transfected with the indicated constructs were incubated with or without Ca²⁺/Bay K and assayed for CAT activities. Values shown are the mean $\pm$ SE of the fold stimulation observed with triplicate dishes. Mean basal (without Ca²⁺/Bay K) percent conversion of [¹⁴C]chloramphenicol for the PRL constructs was: (-204), 1.2; (-187), 1.6; (-174), 0.73; (-113), 0.48; (-75), 0.40; that for DHFR-CAT was 4.7. **C,** Mean $\pm$ SE fold Ca²⁺ stimulation of CAT activity directed in GH₃ cells by (-75)PRL-CAT in the indicated number (in parentheses) of independent experiments.

further localize Ca^{2+} response elements in the proximal PRL promoter, we examined the ability of Ca^{2+} to stimulate expression of (-204)PRL-CAT and its 5'-deletion mutants. It is seen in Fig. 2-4B that transient expression in GH₃ cells of (-204)PRL-CAT was strongly (35-fold) stimulated by exposure to Ca^{2+} . In agreement with our previous results (Jackson and Bancroft, 1988), deletion to position -174 did not greatly reduce this stimulation. However, further deletion to position -113 resulted in a strong reduction in the Ca^{2+} stimulation to about 8.5-fold, implying that the region between -174 and -113 contains at least one CaRE. Further deletion yielded the extensively deleted construct (-75)PRL-CAT, which still exhibited a 10-fold response to Ca^{2+} , compared to the total lack of response by the control construct DHFR-CAT. As shown in Fig. 2-4C, in a number of independent experiments Ca^{2+} was found to consistently yield an approximately 5-fold greater stimulation of (-75)PRL-CAT than of DHFR-CAT. Thus, the PRL gene sequence in (-75)PRL-CAT contains a CaRE.

Since the only known elements in construct (-75)PRL-CAT are site 1P and a TATAA consensus, and site 1P corresponds to a TRHRE (Yan et al., 1991), it seemed likely that the CaRE in (-75)PRL-CAT is site 1P. To begin to examine this question, we cloned copies of 1P in front of construct (-39)PRL-CAT(Enh.), from which site 1P has been deleted, and a Rous sarcoma virus (RSV) enhancer (Enh.) added to permit expression in GH₃ cells (Yan et al., 1991). (Since we have found previously that multiple copies of site 1P are required to transfer a TRH response (Yan et al., 1991), all constructs employed in the present studies contained two copies of each element examined). It is seen in Fig. 2-5 that basal construct (-39)PRL-CAT(Enh.) yielded a 3-fold Ca^{2+} stimulation, only slightly above that yielded by a control RSV-CAT construct. By contrast, construct (1P)²(-39)PRL-CAT(Enh.), containing two copies of site

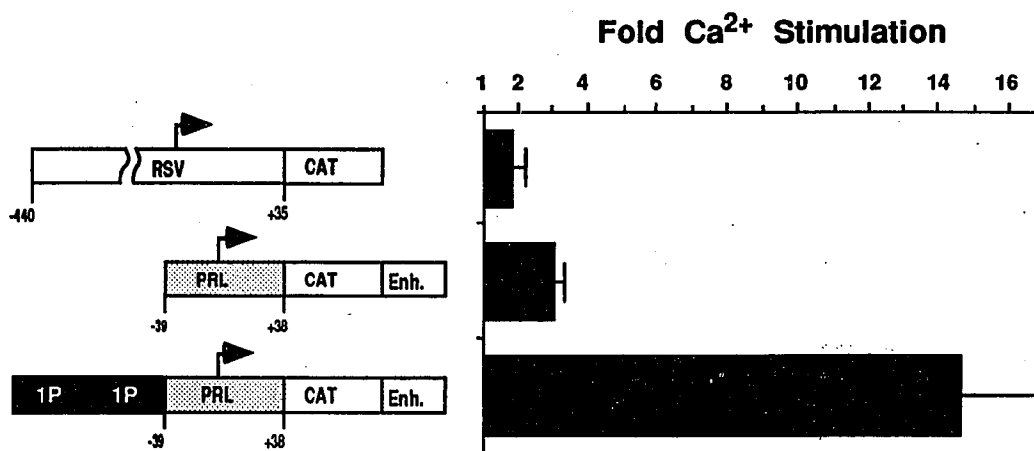
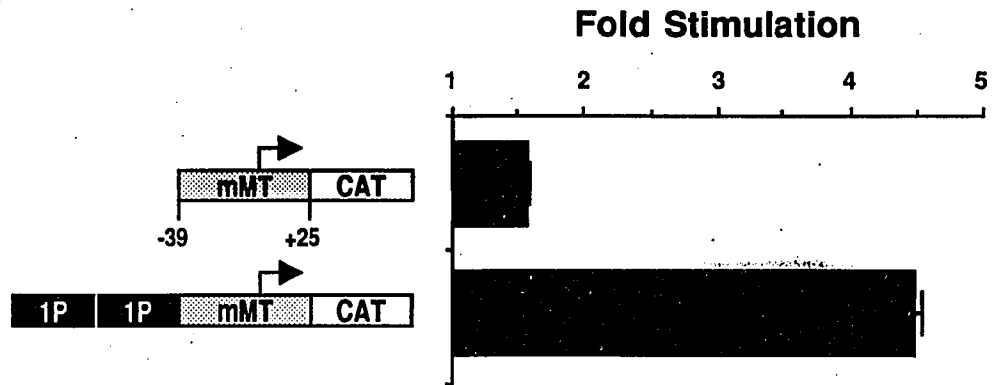


Fig. 2-5. Ability of element 1P to confer Ca^{2+} responsiveness to a minimal PRL-CAT construct. GH₃ cells transfected with the indicated constructs were incubated with or without Ca^{2+} /Bay K. Shown is the mean $\pm$ SE of the fold stimulation of CAT activity observed with triplicate dishes. Mean basal (- Ca^{2+} /Bay K) percent conversion of [^{14}C]chloramphenicol for RSV-CAT, (-39)PRL-CAT(Enh.), and (1P)²(-39)PRL-CAT(Enh.) was, respectively, 12.5, 1.3, and 3.0.

1P in the proper orientation, yielded a 15-fold response to Ca^{2+} . To investigate whether site 1P is an independent CaRE, we examined the ability of 1P oligomers to transfer a Ca^{2+} response to the minimal mouse metallothionein construct (-39)mMT-CAT. As shown in Fig. 2-6A, (-39)mMT-CAT yielded a 1.5-fold response to Ca^{2+} , while (1P)²(-39)mMT-CAT yielded a 4.5-fold Ca^{2+} stimulation. In addition, in confirmation of our previous results, the TRH response of (-39)mMT-CAT and (1P)²(-39)mMT-CAT was, respectively, 1.2-fold and 8-fold. Thus, site 1P can serve as an independent response element for both Ca^{2+} and TRH.

The only protein presently known to bind to site 1P is the pituitary-specific transcription factor pit-1 (Mangalam et al., 1989). In our previous studies of TRH action via site 1P, we have compared the ability of 1P and its mutants to bind pit-1, and to direct a TRH response. The strong correlation observed between these quantities strongly implicated pit-1 in transduction of the TRH transcriptional signal to site 1P (Yan et al., 1991). In the present studies, we compared the ability of 1P and its mutants to direct a Ca^{2+} response and to bind pit-1. The 1P mutants examined contained a four-base mutation either in the lefthand (*1P), the righthand (1P*), or both (*1P*) consensus sequence (Yan et al., 1991) and were cloned in front of the construct (-39)PRL-CAT(Enh.) described above. It is seen in Fig. 2-7 (left) that, similar to previous observations with TRH (Yan et al., 1991), 1P yielded a strong 15-fold Ca^{2+} response, *1P yielded a moderately reduced 8-fold response, while 1P* and *1P* yielded greatly reduced responses of, respectively, 2.5- and 1.5-fold. Comparison with Fig. 2-7 (right), showing our previously determined pit-1 binding abilities of pit-1 and its mutants, yields a good correlation between Ca^{2+} induction and pit-1 binding, implying that, as with TRH, pit-1 transduces the Ca^{2+} transcriptional signal to site 1P.

A. Ca^{2+} 

B. TRH

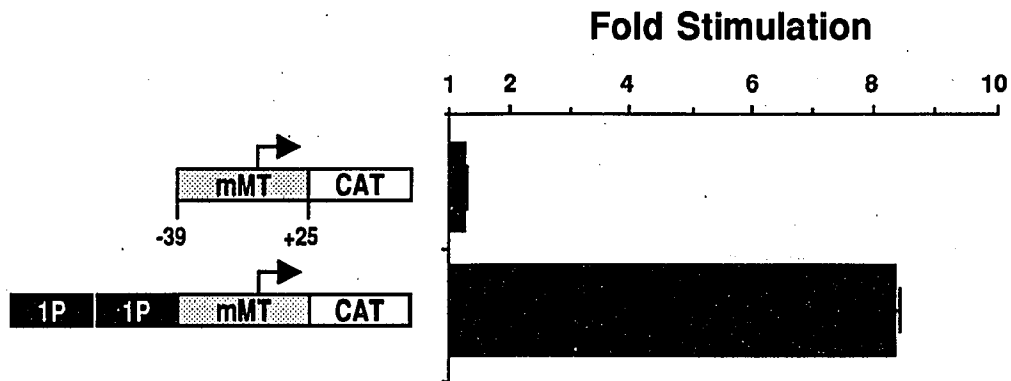


Fig.2-6. Ability of element 1P to confer Ca^{2+} or TRH responsiveness to a minimal mMT-CAT construct. GH₃ cells transfected with the indicated constructs were incubated with or without Ca^{2+} /Bay K (A) or TRH (B). Shown is the mean $\pm$ SE of the fold stimulation of CAT activity observed with triplicate dishes. Mean basal percent conversion of [¹⁴C] chloramphenicol in cells incubated, respectively, - Ca^{2+} /Bay K or -TRH was: (-39)mMT-CAT, 2.0, 13.4; (1P)²(-39)mMT-CAT, 4.5, 3.4.

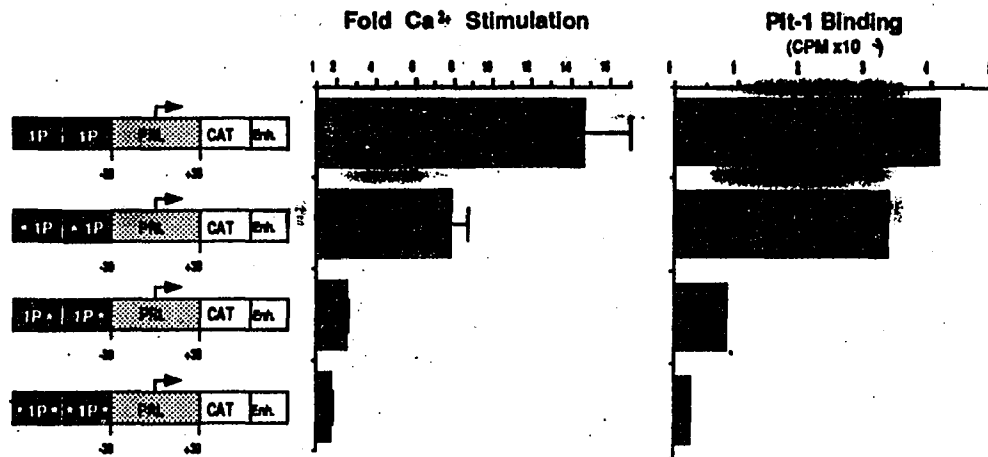
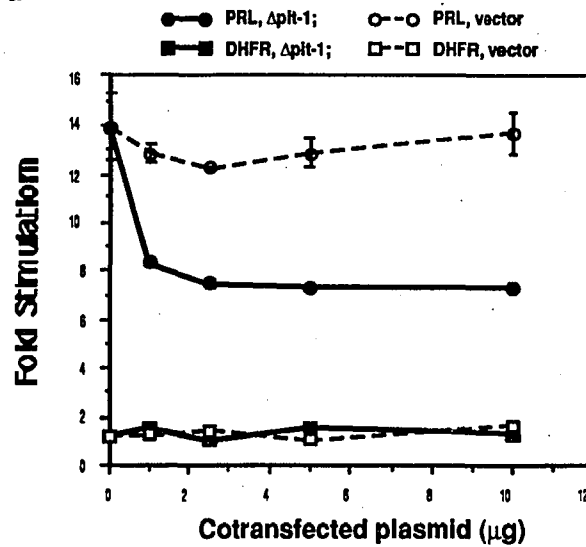


Fig. 2-7. Ability of wild-type and mutant 1P elements to confer Ca^{2+} responsiveness to a minimal PRL-CAT construct. Left, GH3 transfected with the indicated constructs were incubated with or without Ca^{2+} / Bay K. Shown is the mean $\pm$ SE of the fold stimulation of CAT activity observed with triplicate dishes. Mean basal ($-\text{Ca}^{2+}$ / Bay K) percent conversion of [^{14}C] chloramphenicol for the constructs containing 1P, *1P, 1P*, or *1P* were, respectively, 3.0, 1.7, 0.95, 0.99. Right, for comparison, the ability of wild-type and mutant 1P elements to bind pit-1 that we have reported previously is reproduced from Yan et al., 1991.

The above experiments implicating pit-1 in the action of Ca^{2+} on the PRL promoter, as well as our similar previous results with TRH (Yan et al., 1991), are correlative in nature. To investigate more directly whether pit-1 transduces both a TRH and a Ca^{2+} signal to the proximal PRL promoter, we examined whether expression in GH₃ cells of a pit-1 antagonist could interfere with induction by either Ca^{2+} or TRH of a cotransfected PRL-CAT construct. The antagonist employed was a pit-1 mutant, (Δ 2-45)pit-1, which has been shown to inhibit TRH regulation in GH₃ cells of a transfected thyrotropin promoter (Steinfeldt et al., 1991). (Δ 2-45)pit-1 contains a deletion of the major trans-activation domain (amino acid 10-40), yielding a protein that exhibits reduced trans-activation but retains high affinity DNA binding (Ingraham et al., 1990). This pit-1 mutant thus would be expected to bind to pit-1 binding sites and competitively inhibit *trans*-activation by pit-1.

It is seen in Fig. 2-8A that coexpression with (-187)PRL-CAT of increasing amounts of an RSV-(Δ 2-45)pit-1 expression vector led to progressive decrease in Ca^{2+} induction of CAT activity. At the highest concentration of RSV-(Δ 2-45)pit-1 (10 ug) employed, Ca^{2+} induction was inhibited by about 47% relative to cells receiving vector DNA. This effect was promoter specific, since expression of the control construct DHFR-CAT was not detectably affected by Ca^{2+} in the presence or absence of RSV-(Δ 2-45)pit-1. As shown in Fig. 2-8B, coexpression with (-187)PRL-CAT of increasing amounts of RSV-(Δ 2-45)pit-1 also led to a progressive decrease in TRH induction of CAT activity. The greatest inhibition of TRH induction, observed at 2.5 ug RSV-(Δ 2-45)pit-1, was 79% relative to cells receiving vector DNA. This effect was also promoter specific, since the approximately 2-fold TRH stimulation of DHFR-CAT expression was not affected by the presence of RSV-(Δ 2-45)pit-1 interferes with the induction by either Ca^{2+} or

A. Ca^{2+} 

B. TRH

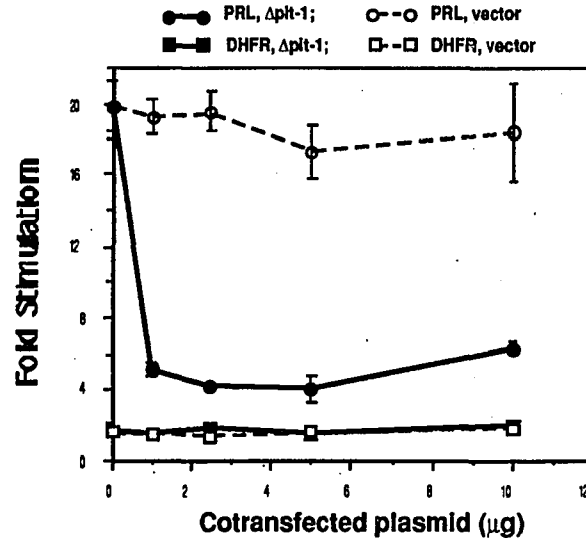


Fig. 2-8. Ability of a *pit-1* *trans*-activation mutant to block induction by Ca^{2+} or TRH of a PRL-CAT construct. GH₃ cells were transfected with 5 μg of either (-187)PRL-CAT (PRL) or DHFR-CAT (DHFR), plus the indicated amount of either (Δ 2-45)*pit-1* ($\Delta\text{pit-1}$), or an equal amount of plasmid pRSV-*pit-1*/GHF-1 from which the *pit-1*/D+GHF-1 sequences had been deleted, leaving only the RSV promoter (vector), plus sufficient pUC19 DNA to bring the total mass of transfected DNA to 15 μg , then incubated with or without Ca^{2+} /Bay K (A) or TRH (B). Shown is the mean $\pm$ SE of the fold stimulation of CAT activity observed with triplicate dishes. Mean basal percent conversion of [^{14}C] chloramphenicol in stimulated cells incubated with (-187)PRL-CAT plus 10 μg of, respectively, vector or (Δ 2-45)*pit-1*, was: A, 7.1, 3.8; B, 4.7, 2.1.

(-187)PRL-CAT expression, implying that pit-1 lies on the pathway of action of both Ca^{2+} and TRH on the proximal PRL promoter.

Discussion

In the present studies, we have employed techniques focusing on both gene-distal and gene-proximal events to investigate the possible involvement of Ca^{2+} in TRH stimulation of the PRL promoter. The techniques in the former category included both additivity and pharmacological studies. The results of additivity studies (Fig. 2-1), showing that TRH can regulate (-187)PRL-CAT expression only in the presence of added Ca^{2+} , supports a role for Ca^{2+} in this action of TRH. Further support for this concept was obtained from the pharmacological experiments. We previously have provided evidence that Ca^{2+} regulation of PRL mRNA levels in GH₃ cells is unaffected when protein kinase C activity is down-regulated by chronic exposure to TPA (Bandyopadhyay et al., 1989). The observation in the present studies that regulation by either Ca^{2+} or TRH of expression of (-187)PRL-CAT is independent of kinase C activity (Fig. 2-2) implies that neither Ca^{2+} nor TRH depend upon protein kinase C for their action on the PRL gene, consistent with an overlap of the pathways of action of these two factors. It is interesting that TRH is known to activate kinase C in GH₃, and that this activation has been implicated in the regulation by TRH of PRL secretion from these cells (White, 1985). Thus, the apparent absence of this enzyme from the pathway of action of TRH on PRL gene expression implies a divergence of the actions of TRH on PRL secretion and gene activity before the level of protein kinase C activity. Results with cellular Ca^{2+} inhibitors (Fig. 2-3) also supported a role for Ca^{2+} in TRH action on the PRL promoter, since the two Ca^{2+} flux

inhibitors cobalt ion and nimodipine were each able to block stimulation by either Ca^{2+} or TRH of expression of (-187)PRL-CAT. As discussed in results, the observation that either of two cellular Ca^{2+} immobilizers, BAPTA/AM or TMB-8, blocked stimulation of (-187)PRL-CAT expression by Ca^{2+} but not by TRH suggests that the TRH/ Ca^{2+} transcriptional signaling pathway may not be sensitive to Ca^{2+} immobilization.

The gene-distal macromolecular mediators in this pathway have yet to be identified. Previous pharmacological studies have suggested that the Ca^{2+} -activated enzyme calmodulin is involved in regulation of PRL gene activity (White, 1985; Davis et al., 1991). However, it has been recently reported that constitutive expression in GC cells of type II Ca^{2+} /calmodulin-dependent protein kinase yields a much stronger stimulation of expression of the RSV long terminal repeat than of the PRL promoter (Kapiloff et al., 1991). Our finding in the present experiments (Fig. 2-5) that Ca^{2+} stimulates PRL promoter fragments much more strongly than the RSV long terminal repeat thus suggests that the Ca^{2+} response of the PRL promoter may not be mediated by type II Ca^{2+} /calmodulin-dependent protein kinase.

Another approach to investigating whether Ca^{2+} lies on the pathway of TRH on PRL gene expression was taken by comparing gene-proximal events in the action of Ca^{2+} on the PRL promoter with TRH (Yan et al., 1991). 5'-deletion analysis (Fig. 2-4B) showed that the first 174 bp of the PRL promoter contain at least two CaREs, respectively, above and below positions -113, in agreement with our previous results with TRH (Yan et al., 1991). The observation that the extensively deleted construct (-75)PRL-CAT yielded a promoter-specific Ca^{2+} stimulation (Fig. 2-4C) suggested that site 1P is a CaRE, and this was confirmed by the ability of site 1P to transfer a Ca^{2+} response to a basal PRL promoter (Fig. 2-5). The observation that site 1P could transfer a

response to either Ca^{2+} or TRH to a heterologous promoter (Fig. 2-6) demonstrate that site 1P is both a TRHRE and a CaRE, thus providing independent support for an overlap of the pathways of action of Ca^{2+} and TRH on the PRL promoter.

We previously have employed mutants of site 1P to obtain evidence that pit-1 is the gene-proximal protein that transduces a TRH signal to site 1P (Yan, et al., 1991). The present observation of a good correlation between the ability of 1P and its mutants to bind pit-1 and to direct a Ca^{2+} response (Fig. 2-7) implies that pit-1 also transduces a Ca^{2+} response to site 1P. To seek independent evidence that pit-1 plays this role, we investigated whether expression of (Δ 2-45)pit-1, containing a deletion in the pit-1 *trans*-activation domain (Ingraham et al., 1990), could block either the Ca^{2+} or TRH induction of (-187)PRL-CAT expression. Expression of (Δ 2-45)pit-1 was observed to inhibit the induction of (-187)PRL-CAT expression by either Ca^{2+} or TRH (Fig. 2-8), implying that pit-1 is indeed the gene-proximal protein transducing either of these signals to site 1P. It is worth noting that, since pit-1 also regulates pituitary-specific expression of the PRL gene (Mangalam et al., 1989), expression in GH₃ cells of construct (Δ 2-45)pit-1 might be expected to lower basal levels of PRL-CAT expression. However, because the basal levels of CAT activity in the experiments shown in Fig. 2-8 were quite low even in the absence of this construct (two to three times background), we were unable to investigate this question in the present experiments. It is also notable that (Δ 2-45)pit-1 yielded a greater maximal inhibition of induction of (-187)PRL-CAT by TRH than of Ca^{2+} (79% *vs.* 47%; Fig. 2-8). the reason for this difference is not presently clear. However, it should be noted that the *trans*-activation domain of pit-1 has not yet been mapped in detail, and also that (Δ 2-45)pit-1 still retains approximately 50% of the transcriptional activity of

native pit-1 (Ingraham et al., 1990). It is therefore possible that (Δ 2-45)pit-1 contains only a partial deletion of the region required for transduction of a Ca^{2+} response, thus yielding only a partial inhibition of Ca^{2+} induction of (-187)PRL-CAT.

In summary, the additivity and pharmacological results presented here imply that Ca^{2+} is a mediator of the induction by TRH of PRL gene expression. This conclusion was independently supported by the virtually identical results obtained with TRH and Ca^{2+} in regulation studies employing 5'-deleted PRL-CAT constructs, transfer of site 1P and its mutants to indicator promoter constructs, and analysis with pit-1 and its *trans*-activation mutant (Δ 2-45)pit-1. The latter series of investigations also demonstrated that PRL promoter site 1P is both a CaRE and TRHRE, and that pit-1 represents the gene-proximal protein that transduces the TRH/ Ca^{2+} signal to the PRL promoter.

CHAPTER 3

CREB Can Act as a Conditional Repressor of the Rat Prolactin Promoter

Abstract

The proximal PRL promoter contains a "CRE-like" element (CLE) (TGACGGAA) that resembles the classical symmetric cyclic AMP (cAMP) response element (TGACGTCA) found in the promoters of a number of cAMP-responsive genes, including somatostatin. The action of cAMP on the promoters containing the classical cAMP response element is mediated by the transcription factor CREB. We have therefore investigated whether CREB can interact, biochemically and/or functionally, with the CLE in the PRL promoter. We reported that CREB does bind to the PRL CLE, although with an affinity about 10-fold lower than its interaction with the somatostatin CRE. Furthermore, CREB can strongly repress expression in GH₃ cells of co-transfected PRL promoter, since β -galactosidase did not repress a co-transfected PRL-CAT construct, and CREB did not repress a co-transfected TK-CAT construct. A CREB mutant (CREB-M1), in which the major phosphorylation site (Ser-133) is mutated, was as effective as CREB at repressing PRL-CAT activity, while a DNA-binding mutant of CREB (K-CREB) was impaired in the ability to repress PRL-CAT expression, implying that CREB repression of the PRL promoter requires the ability to bind to DNA, but not the major phosphorylation site on CREB. CREB repression of the PRL promoter was relieved by coexpression in GH₃ cells of the catalytic subunit of protein kinase A (PKA). Relief of repression by PKA was incomplete with CREB-M1 mutant, implying that relief requires phosphorylation of CREB by PKA. Thus, CREB can act as a specific repressor of PRL promoter activity in pituitary cells, but this repression can be reversed by PKA phosphorylation of CREB.

Introduction

In recent years, it has been shown that interaction of the pituitary cell-specific protein pit-1 with multiple binding sites in the proximal PRL promoter plays an important role in both cell-specific (Mangalam et al., 1989; Fox et al., 1990; Castrillo et al., 1991) and hormonal (Yan et al., 1991; Yan and Bancroft, 1991; Peer et al., 1991) regulation. More recent studies by a number of groups have revealed the importance of an additional PRL promoter sequence, termed either the CRE-like element or the A box, in both of these types of regulation. The observation that mutation of the CRE-like element (referred as the CLE) strongly reduces expression in GH₃ of a transfected PRL promoter (d'Emden et al., 1992) demonstrated the significance of this element for basal expression of the PRL gene. In addition, the CLE has been implicated in regulation by cyclic AMP of either the human (d'Emden et al., 1992) or rat (Liang et al., 1992) PRL promoter.

The identity of the DNA-binding protein(s) that interact functionally with the PRL CLE has not yet to be determined. However, our recent studies has shown that the CRE-binding protein CREB can interact biochemically with the PRL CLE, although with an affinity about 10-fold lower than its interaction with the symmetric somatostatin CRE. We have therefore investigated whether CREB can interact functionally with the CLE in the PRL promoter.

Results

GH₃ Cells Contain Levels of CREB Similar to Those Found in Other Cell Lines.

Nuclear extracts were prepared from a number of cell lines, and analyzed for CREB by Western blot analysis using a antipeptide antibody against the P-box region of CREB (**Fig. 3-1**). The results demonstrated that GH₃ cell nuclei contain CREB, at levels comparable to those present in the nuclei of rat glial (C6), human epithelioid carcinoma (Hela), mouse mammary tumor (C127), rat carcinoma (LLC-WRC), and Monkey kidney (CV1) cells.

CREB Specifically Represses Expression of the PRL Promoter

Recently, it has been shown that PRL CRE-like element (CLE) binds to CREB, but lower binding affinity than classic CRE element by DNase footprint and DNA gel shift studies (liang, et al., 1992; Yan, Tian and Bancroft in preparation). Together these results with the result presented in **Fig. 3-1** imply that CREB could play a role in regulating pituitary PRL gene expression by binding to the PRL CLE. To investigate this possibility, we examined the effect of co-expression of CREB on the expression in GH₃ cells of PRL-CAT constructs that either contain or lack the PRL CLE (**Fig. 3-2**). Expression of (-187)PRL-CAT, which contains a CLE plus the three most proximal pit-1 binding sites 1P, 2P, and 3P, was strongly repressed by co-expression of CREB. CREB exhibited a similar repression of the further 5'-deleted construct



Fig. 3-1. Analysis by western immunoblot of CREB in various cell lines. Nuclear extracts of the indicated cell lines were analyzed by western immunoblot analysis with antiserum to CREB, as described in Materials and Methods.

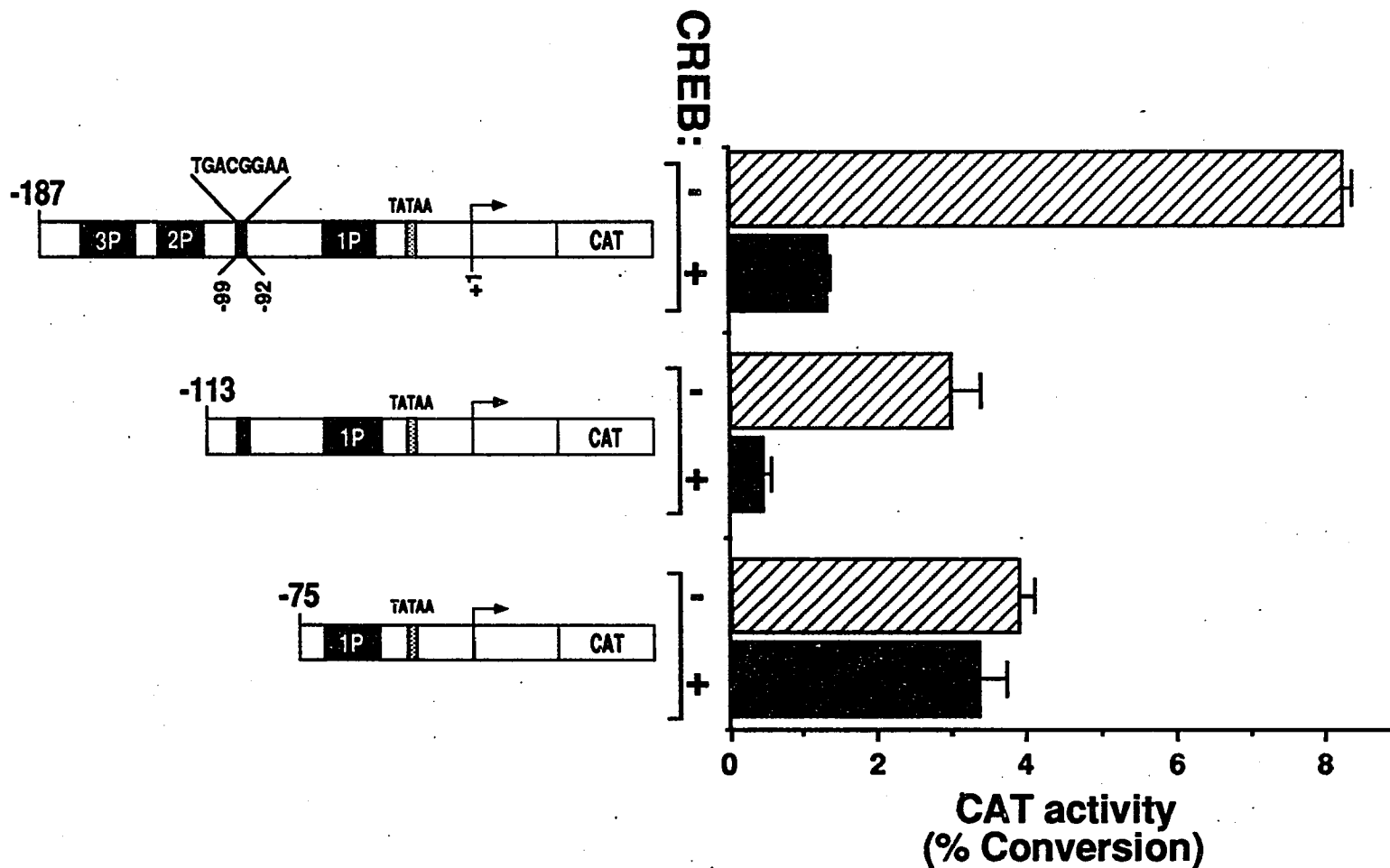


Fig. 3-2. Effect of exogenous CREB on expression of co-transfected PRL-CAT constructs. GH3 cells were transfected with 5 ug of the indicated PRL-CAT constructs (identified by their 5'-termini), plus 5 ug of either an RSV-CREB expression vector (+CREB) or control plasmid pUC19 (-CREB), and assayed for CAT enzymatic activity. The three pit-1 binding sites 1P-3P and the CRE-like element at positions -99/-92 in the PRL promoter are indicated. Shown is the mean $\pm$ SE of the percent conversion of [^{14} C]chloramphenicol observed with triplicate dishes.

(-113)PRL-CAT, from which sites 2P-3P have been removed. However, removal of the PRL CLE by further 5'-deletion yielded a construct, (-75)PRL-CAT, which was expressed equally well in the presence or absence of co-expressed CREB, implicating the PRL CLE in the observed repressive effects of CREB on the PRL promoter.

Repression is Specific Both for CREB and for the PRL Promoter.

To examine the specificity of the effects described above, we examined the effect of co-expression of either CREB or the non-specific protein β -galactosidase on either the PRL promoter or a control thymidine kinase (TK) promoter (Fig. 3-3). Expression of β -galactosidase had no effect on expression of (-187)PRL-CAT, while, as before, expression of CREB strongly repressed expression of this construct. Hence, repression is not just a general effect of protein overexpression, but is specific for CREB. Furthermore, neither β -galactosidase nor CREB affected expression of control construct TK-CAT, showing that CREB repression exhibits specificity for the PRL promoter.

CREB Structural Requirement for Repression of the PRL Promoter.

Previous experiments have shown that transactivation of genes via the classic cyclic AMP response element (CRE) involves both DNA binding and phosphorylation of CREB (Yamamoto et al., 1988; Struther et al., 1991; Walton et al., 1992; Gonzalez and Montminy, 1989; and Lee et al., 1990). To investigate the CREB structural requirements for repression of the PRL promoter, we employed two previously described CREB mutants. The first was CREB-M1,

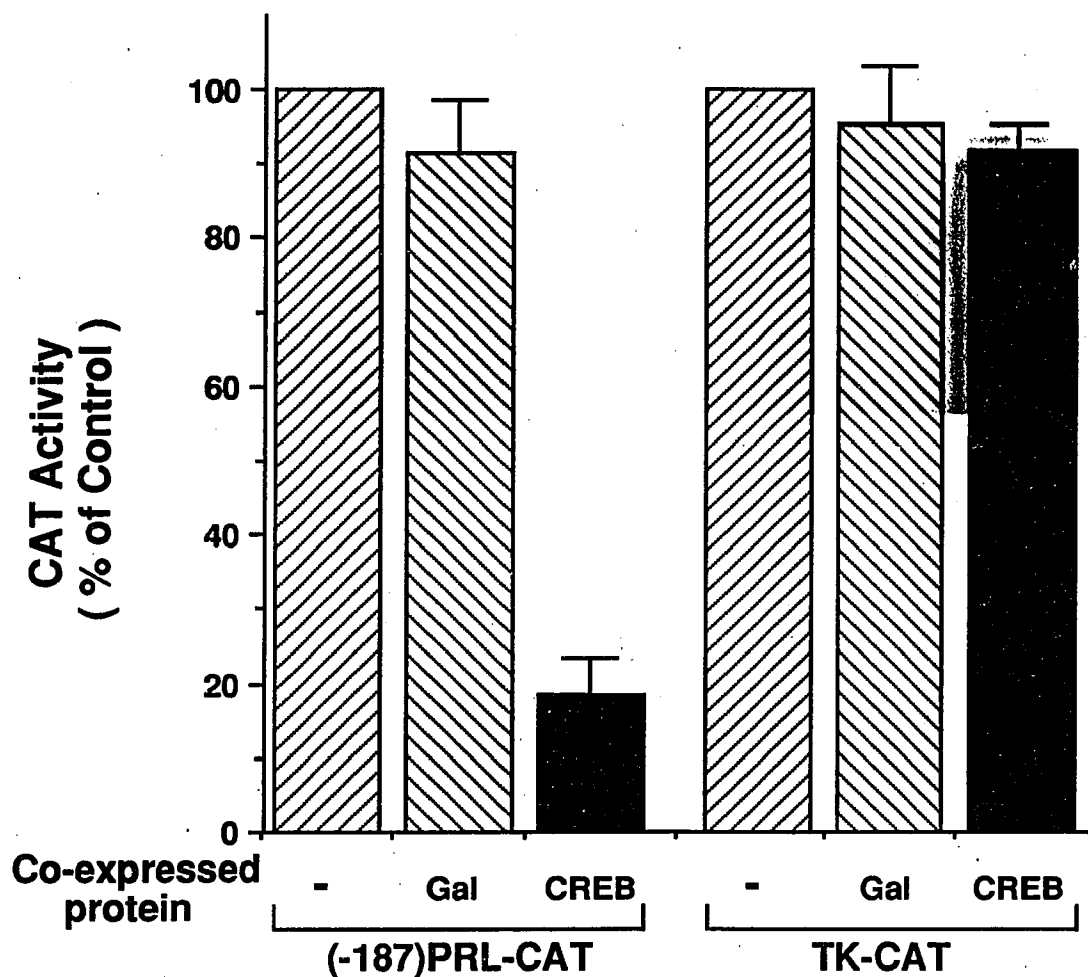


Fig. 3-3. Ability of exogenous β -galactosidase or CREB to block expression of co-transfected promoter-CAT constructs. GH₃ cells were transfected with 5 ug of either (-187)PRL-CAT or control construct TK-CAT, plus 5 ug of either pUC19 (-), RSV- β gal (Gal), or RSV-CREB (CREB), and assayed for CAT enzymatic activity. Shown is the mean $\pm$ SD of results with duplicate dishes. Mean basal expression for (-187)PRL-CAT constructs (percent conversion of Chloramphenicol) was: -, 8.97; Gal, 8.28; CREB, 1.66. for TK-CAT constructs (percent conversion of Chloramphenicol) was: -, 21.5; Gal, 20.4; CREB, 19.7.

containing a mutation in the major A kinase phosphoacceptor site serine-133 which permits binding to a CRE, but abolishes the ability to transactivate the somatostatin gene (Gonzalez and Montminy, 1989). The second was K-CREB, in which arginine-287 is mutated to leucine, resulting in an inability to bind to the somatostatin gene CRE (Walton et al., 1992). The results are shown in Fig. 3-4 as before, expression of CREB yielded a sharp repression of (-187)PRL-CAT relative to cells expressing the control protein aminoglycoside phosphotransferase (Neo). CREB-M1 was equally effective as CREB in repressing PRL-CAT expression, implying that the Phosphoacceptor site serine-133 is not required for the action of CREB on the PRL promoter. By contrast, K-CREB was much less effective than either CREB or CREB-M1 in repressing PRL-CAT expression, implying that CREB must bind to the PRL promoter to exert its repressive effects.

CREB Repression of the PRL Promoter is Reversed by Co-expression of the Protein Kinase A Catalytic Subunit.

As described above, full repression by CREB of the PRL promoter apparently requires binding to the promoter. It is thus probable that repression by CREB arises from competitive inhibition by this protein of binding to the PRL CRE of a positive transcription factor endogenous to the GH₃ cells. Since phosphorylation of CREB by the protein kinase A catalytic subunit (PKA) has been shown previously to transactivate promoters containing a classical CRE, e.g., the somatostatin promoter (Gonzalez and Montminy, 1989), we asked whether co-expression with CREB of PKA would reverse the CREB inhibition of the PRL promoter. It is seen in Fig. 3-5 that CREB alone again inhibited (-187)PRL-CAT expression (bar 2 *vs* bar 1), and

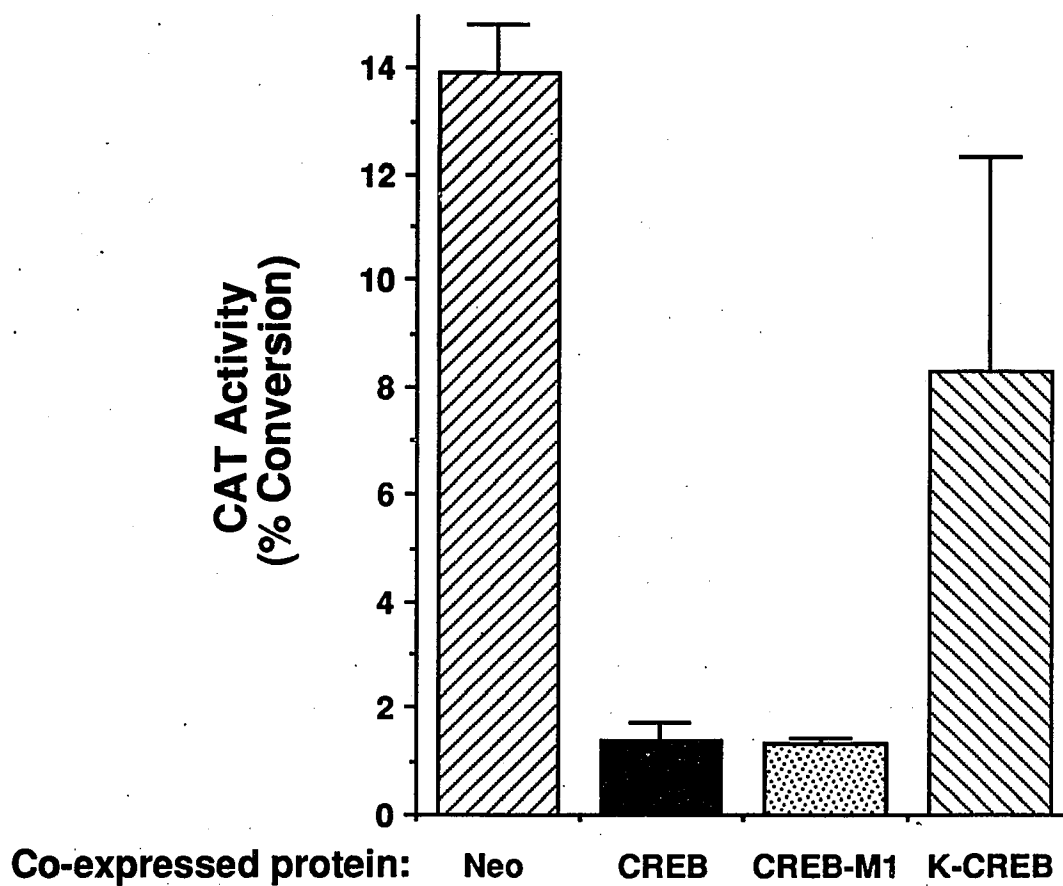


Fig. 3-4. Ability of exogenous CREB or its mutants to block expression of a co-transfected PRL-CAT construct. GH₃ cells were transfected with 5 ug of (-187)PRL-CAT, plus 5 ug of either RSV-Neo, RSV-CREB, RSV-CREB-M1, or RSV-K-CREB, and assayed for CAT activity. Shown is the mean $\pm$ SD of results with duplicate dishes.

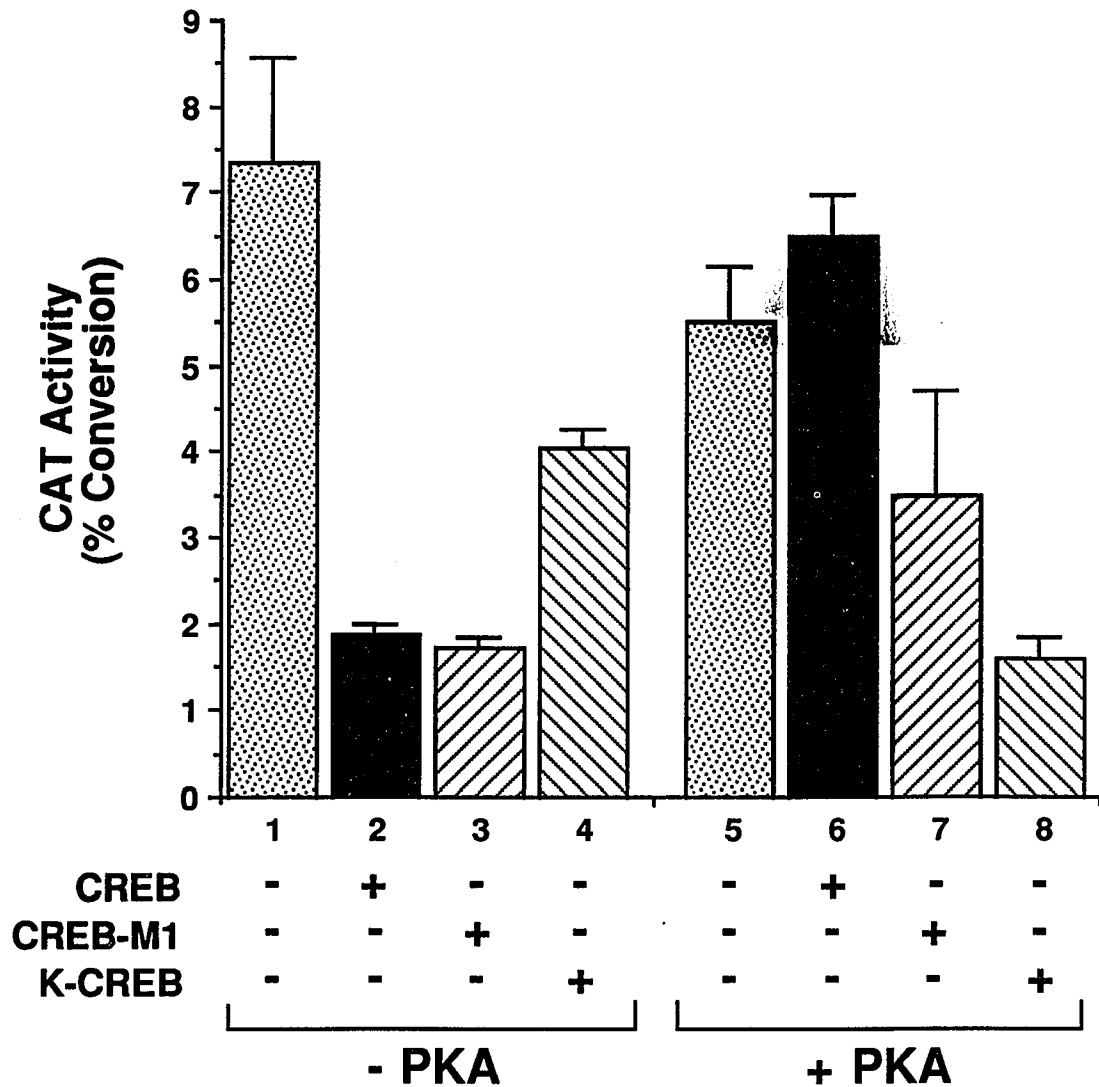


Fig. 3-5. Effect of exogenous PKA catalytic subunit on expression of a PRL-CAT construct co-transfected with vectors expressing CREB or its mutants. GH₃ cells were transfected with 5 ug of (-187)PRL-CAT, plus 5 ug of either RSV-Neo (-), RSV-CREB, RSV-CREB-M1, or RSV-K-CREB, plus 5 ug of either pUC19 (-PKA) or MtC (+PKA), and assayed for CAT activity. Shown is the mean $\pm$ SD of results with duplicate dishes.

that this repression was completely reversed by coexpression of both CREB and PKA (**bar 6 *vs* bar 1**). The PKA activation observed is probably not due to the ability of this kinase to phosphorylate either pit-1 (Kapiloff, et al., 1991) or the endogenous CREB in GH₃ cells, since in the absence of exogenous CREB, PKA did not stimulate PRL-CAT activity (**bar 5 *vs* bar 1**). These results thus imply that the ability of CREB to repress the PRL promoter is completely reversed by PKA-mediated phosphorylation of CREB.

It is also seen in **Fig. 3-5** that, as before, CREB-M1 was equally effective as CREB in repressing PRL-CAT activity (**bar 3 *vs* bar 2**). Co-expression of PKA yielded a partial reversal of the repression by CREB-M1 (**bar 7 *vs* bar 3**), possibly arising from phosphorylation of other phosphoacceptor sites still present in CREB-M1 (Lee et al., 1990). Finally, K-CREB alone was again less effective than either CREB or CREB-M1 in repressing PRL-CAT activity (**bar 4 *vs* bars 2 and 3**). Rather surprisingly, co-expression of K-CREB and PKA yielded strong repression of PRL-CAT, equivalent to levels seen with either CREB or CREB-M1 in the absence of exogenous PKA (**bar 8 *vs* bars 2 and 3**).

Discussion

Recently studies have shown that, in addition to pit-1 binding sites, the PRL proximal promoter contains a "CRE-like" element (CLE) that is important for regulation of PRL gene expression (Peers et al., 1991; d'Emden et al., 1992; Liang et al., 1992). DNA mobility shift assays have been shown that CREB can bind to the PRL CLE (Yan et al., 1992). However, the PRL CLE (TGACGGAA) contains only the left half of the palindromic sequence (TGACGTCA) found in the somatostatin (SS) CRE, and thus might be expected to exhibit a corresponding lower affinity for CREB. The results of the

mobility shift assay has confirmed this expectation, showing that the affinity of CREB for the PRL CLE is about 10% of its affinity for the canonical SS CRE (Yan et al., 1992). This observation is consistent with a report in which results of DNase footprinting led to the conclusion that CREB "appears to have a rather low affinity" for the PRL CLE (Liang et al., 1992).

The ability of the PRL CLE to bind the CRE-binding protein CREB, together with the detection in GH₃ cell nuclei of significant levels of CREB (Fig. 3-1), suggests that CREB might be capable of regulating expression of the PRL promoter. Experiments involving co-transfection into GH₃ cells of PRL-CAT constructs plus a CREB expression vector showed that over-expression of CREB resulted in a 7-fold repression of PRL-CAT activity (Fig. 3-2), which was specific for both CREB protein and the PRL promoter (Fig. 3-3). The observation that only PRL-CAT constructs containing the PRL CLE were repressed by CREB (Fig. 3-2) implies that repression requires the PRL CLE. Furthermore, the sizable repression of PRL-CAT observed in these experiments confirms by another method in a previous report (d'Emden et al., 1992) that the CLE is required for strong basal expression of the PRL promoter.

Gene *activation* by CREB via the canonical cyclic AMP response element has been shown previously to require both binding to DNA and CREB phosphorylation (Yamamoto et al., 1988; Struther et al., 1991; Walton et al., 1992; Gonzalez and Montminy, 1989; and Lee et al., 1990). However, the *inhibition* by CREB of the PRL promoter might involve either direct binding to a specific DNA sequence, or interaction with a positive transcription factor. In the first type of mechanism, repression could be exerted either by direct competition with the binding of a positive transcription factor to the same DNA site, or by steric hindrance of binding of a positive factor such as pit-1 to

an adjacent site. However, the latter scenario seems less likely than the former, since at least 15 bp separate the two flanking pit-1 binding sites 1P and 2P from the PRL CLE (**Fig. 3-2**). In second type of mechanism, CREB could act by protein-protein interaction with a positive transcription factor such as pit-1, thereby preventing or reversing binding of the positive factor to its DNA binding site. Mutants of CREB were employed to attempt to differentiate among these possible mechanisms. The observation that CREB-M1, which cannot be phosphorylated at the major PKA phosphoacceptor site, was as effective as CREB at repressing PRL-CAT expression (**Fig. 3-4**) shows that repression does not require phosphorylation of CREB at this site. Furthermore, the observation that K-CREB, which is incapable of binding to the SS CRE, yielded a greatly reduced ability to repress PRL-CAT activity (**Fig. 3-4**) implies that CREB repression of the PRL promoter requires the ability of CREB to bind to the PRL CRE.

In light of the above results, it seems probable that exogenously expressed CREB represses the PRL promoter by directly competing for binding to the CLE with a positive transcription factor endogenous to the GH₃ cells. Since phosphorylation of CREB bound to the SS CRE is known to activate the SS promoter (Yamamoto et al., 1988), it seemed possible that CREB phosphorylation might similarly activate the PRL promoter. The observation (**Fig. 3-5**) that co-expression of PKA completely reversed the CREB inhibition of the PRL promoter implies that CREB can in fact act as a conditional repressor of the PRL promoter, with repression depending entirely upon phosphorylation of CREB. It should be noted finally that interpretation of the results of similar experiments with the CREB mutants (**Fig. 3-5**) is not as straightforward. However, it seems possible that the observation that co-expressed PKA partially relieved PRL-CAT repression by the phosphorylation

mutant CREB-M1 is a consequence of phosphorylation of the other phosphorylation sites still present in CREB-M1 (Lee et al., 1990). Furthermore, the observation that co-expressed PKA increased the repression by the DNA-binding mutant K-CREB could be explained if phosphorylation of K-CREB increased its ability to dimerize with and thus inactivate the endogenous GH₃ cell transcription factor that binds to the PRL CLE.

It might appear that CREB itself would be a logical candidate for the endogenous cellular factor whose binding to the CLE is competed by exogenous unphosphorylated CREB. However, this seems unlikely to be the case, at least for the basal cells employed in the present experiments, for the following reasons. 1. If the endogenous factor were CREB, expression of PKA alone would have been expected to stimulate PRL-CAT activity in the GH₃ cells. However, this was not observed (Fig. 3-5). 2. Maurer's group has very recently reported the use of oligomer competition and anti-CREB antiserum in DNA mobility shifts to show that the major CLE-shifted band detected with nuclear extracts of basal GH₃ cells does not contain CREB (Liang et al., 1992). We have performed similar experiments that agree with this conclusion (unpublished observations). Martial's group has also reported similar observation with the human analogue of the PRL CLE, and has additionally reported detection of a CLE-binding protein that is not pituitary cell-specific (Peers et al., 1991). Thus, the identity of the endogenous GH₃ cell protein(s) that binds to the PRL CLE remains to be determined.

CREB (as well as other members of the CREB/ATF family) has been implicated as the gene-proximal step in hormonal regulation via cyclic AMP of a number of genes (Habener, 1990). This protein has also been implicated in a number of developmental processes, including developmental expression and repression of liver-specific genes (Boshart et al., 1991; Jones et al., 1991),

and population of the pituitary by somatotrophic cells (Struthers et al., 1991). Furthermore, we have recently found that expression of a PRL-CAT construct in non-pituitary cells can be activated as strongly by co-expression of a constitutively active CREB protein as by co-expression of the pituitary-specific factor pit-1 (Chapter 4). Thus, although interaction between CREB and the PRL CLE has not yet been detected in DNA mobility shift experiments with GH₃ cell nuclear extracts, it seems quite possible that interaction of this PRL DNA element with CREB or a CREB-like protein will prove to play a role in the hormonal and/or developmental regulation of the PRL gene.

CHAPTER 4

**Either Protein Kinase A or A Dominant Activator CREB-VP16 can
Activate PRL-CAT in Non-pituitary Cells Independently of Pit-1**

Abstract

The pituitary-specific transcription factor pit-1 is the only known protein which can *transactivate* the PRL promoter in non-pituitary cells. Therefore, we investigated whether the PRL promoter in non-pituitary cells can be activated by factors other than pit-1. We report here that co-expression of the catalytic subunit of cAMP-dependent protein kinase (PKA-C) stimulated about 10-fold expression in C6 cells of (-113)PRL-CAT, which contains most of the PRL proximal promoter. A 5'-deleted construct, (-75)PRL-CAT which lacks the PRL cAMP-like element (CLE) was not activated by co-expression of PKA-C, implying that the PRL CLE was required for PKA-C activation of PRL-CAT expression in C6 cells. The possibility that a dominant positive CREB protein CREB-VP16 could activate expression of PRL-CAT constructs was also investigated, since recent studies by Jun Tian in our laboratory have found CREB does bind to the PRL CLE. Expression of a constitutively active CREB protein CREB-VP16 specifically and strongly activated PRL-CAT constructs via the PRL CLE in C6 cells. *Transactivation* of (-187)PRL-CAT expression by CREB-VP16 was as strong as by the pituitary specific transcription factor pit-1. These findings demonstrate that either PKA or a dominant positive CREB can activate the PRL promoter in non-pituitary cells. On the other hand, co-expression of wild-type CREB completely inhibited pit-1 induction of PRL-CAT activity in C6 cells. A CREB mutant (CREB-M1), which carries a mutation in the major phosphorylation site (Ser-133), was as effective as CREB at inhibiting pit-1 induction of PRL-CAT activity; while a DNA-binding site mutant of CREB (K-CREB) only slightly inhibited pit-1 induction, implying that CREB inhibition of pit-1 induction of the PRL promoter

requires its ability to bind to DNA, but not the major phosphorylation site on CREB. Co-expression of PKA-C relieved CREB inhibition of pit-1 induction of the PRL promoter, and partially relieved the inhibition resulting from the CREB-M1 mutant. These results imply that CREB can act as a specific inhibitor of PRL promoter activity by interfering with some endogenous factor(s) which is required for pit-1 *trans*activation of the PRL promoter, and that this inhibition can be reversed by PKA phosphorylation of CREB.

Introduction

Pit-1, a POU protein, which is important for cell-specific expression of PRL, is only factor known that can *transactivate* the PRL promoter in non-pituitary cells (Mangalam et al., 1989; Fox et al., 1990). In Chapter 3, we showed that CREB can interact functionally with the CLE in the PRL promoter as a conditional repressor, implying that inhibition by CREB of the PRL promoter might involve competition with binding of a positive transcription factor to the PRL CLE. Moreover, a recent report has shown that rat PRL gene is expressed in brain, including hypothalamus and extrahypothalamic brains, without detectable co-expression of pit-1 (Emanucele et al., 1992), implying that PRL gene expression can occur independently of pit-1. Together with the results in chapter 3, it suggested that other proteins might activate the PRL promoter. Therefore, a study was carried out to attempt to induce the PRL promoter without pit-1, using C6 cells which do not express pit-1.

Results

The PKA Catalytic Subunit can Activate the PRL Promoter in Non-pituitary Cells.

The PRL promoter is known to be inactive in non-pituitary cells (Ingraham et al., 1988). This could be partially due to the presence of a cell-specific repressor in non-pituitary cells. Recent reports have shown that the tissue-specific extinguisher of the liver-specific gene TAT is one of the regulatory subunits of PKA, and extinction can be reversed by increasing activity of the PKA-C (Boshart et al., 1991). We therefore investigated whether

co-expression of the catalytic subunit of cAMP-dependent protein kinase (PKA-C) would permit expression of the PRL promoter in the rat glial C6 cells which do not express pit-1 (data not shown). As shown in Fig. 4-1, a PRL proximal promoter construct, (-187)PRL-CAT which contains the PRL "CRE-like" element (CLE) plus the three most proximal pit-1 binding sites, 1P, 2P, and 3P, was inactive in the C6 rat glial cells. Co-expression of PKA-C had no effect on expression of this construct. A 5'-deleted construct (-113)PRL-CAT, containing the PRL CLE and 1P, yielded a significantly higher level of basal expression in the C6 cells. This finding is consistent with the recent observation that deletion from the PRL promoter of a repressor sequence (FP1) at position -127/-116 permits expression in Hela cells (Jackson et al., 1992). Co-expression of PKA-C resulted in a 10-fold stimulation of expression of (-113)PRL-CAT, indicating that PKA-C can strongly *transactivate* expression of the PRL promoter in non-pituitary cells. However, expression of a further 5'-deleted construct, (-75)PRL-CAT which lacks the PRL CLE was only slightly stimulated by co-expression of PKA-C, indicating that the sequences involved in PKA-C activation of the PRL promoter lies between positions -113 and -75, which contains the PRL CLE located between positions -99 and -92. Thus, activation by PKA of the PRL promoter in absence of pit-1 is likely due to phosphorylation of an endogenous factor by PKA which probably acts through the PRL CLE.

A Dominant Positive Form of CREB Can Activate the PRL Promoter in Non-Pituitary Cells.

We have recently shown that CREB binds to the PRL CLE with an

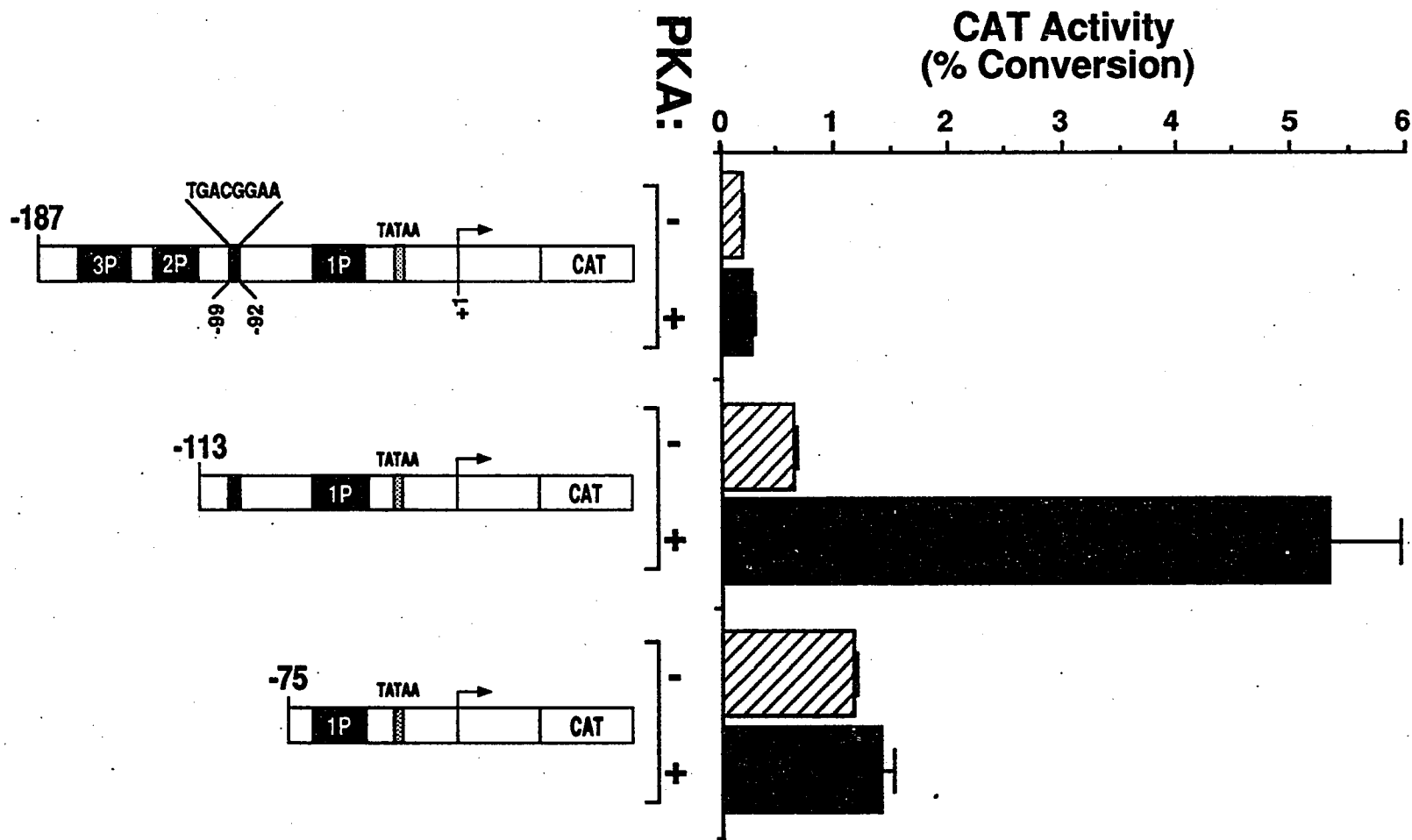


Fig. 4-1. Effect of exogenous PKA on expression of co-transfected PRL-CAT constructs. C6 cells were transfected with 5 ug of the indicated PRL-CAT constructs (identified by their 5'-termini), plus 5 ug of either MtC expression vector (+PKA) or control plasmid pUC19 (-PKA), and assayed for CAT enzymatic activity. The three pit-1 binding sites 1P-3P and the CRE-like element at positions -99/-92 in the PRL promoter are indicated. Shown is the mean $\pm$ SE of the percent conversion of [^{14} C]chloramphenicol observed with triplicate dishes.

affinity about ~10-fold lower than to the canonical somatostatin CRE (Yan, Tian and Bancroft in preparation). In light of the above results, we tested whether a CREB-like protein can activate the PRL promoter by binding the PRL CLE. To do this, we investigated the effect of expression of two fusion proteins, ATF-2-VP16 or CREB-VP16 on the PRL proximal promoter containing or lacking the PRL CLE. ATF-2-VP16 (Liu and Green, 1990) or CREB-VP16 (Liu and Green, personal communication) were generated by adding the potent VP16 acidic activating region to ATF-2 or CREB, respectively, resulting in the corresponding permanently activated ATF-2 or CREB. As shown in Fig. 4-2, expression of CREB-VP16 dramatically activated (-187)PRL-CAT and (-113)PRL-CAT in C6 cells. The activation of (-113)PRL-CAT was 4-fold stronger than the activation of (-187)PRL-CAT. As described above, this is probably due to the presence of a repressor sequence (FP11) in the (-187)PRL-CAT construct. However, a further 5'-deleted construct (-75)PRL-CAT which lacks the PRL CLE was not activated by CREB-VP16. ATF-2-VP16 had little effect on the induction of expression of these PRL-CAT constructs. The failure to *transactivate* the PRL promoter by ATF-2-VP16 could be due to an inability of ATF-2-VP16 to bind to the PRL CLE. In any case, negative result with this construct shows that the stimulation by CREB-VP16 is not just a non-specific effect of the VP16 activator protein.

To investigate the promoter specificity of the activation by CREB-VP16 of the PRL promoter, we examined the effect of expression of CREB-VP16 on a number of gene promoters. These include the Rous sarcoma virus (RSV) promoter, the Chinese hamster dihydrofolate reductase (DHFR) promoter and the mouse metallothionein (mMT) promoter. As shown in Fig. 4-3, expression of CREB-VP16 strongly activated expression of (-113)PRL-CAT,

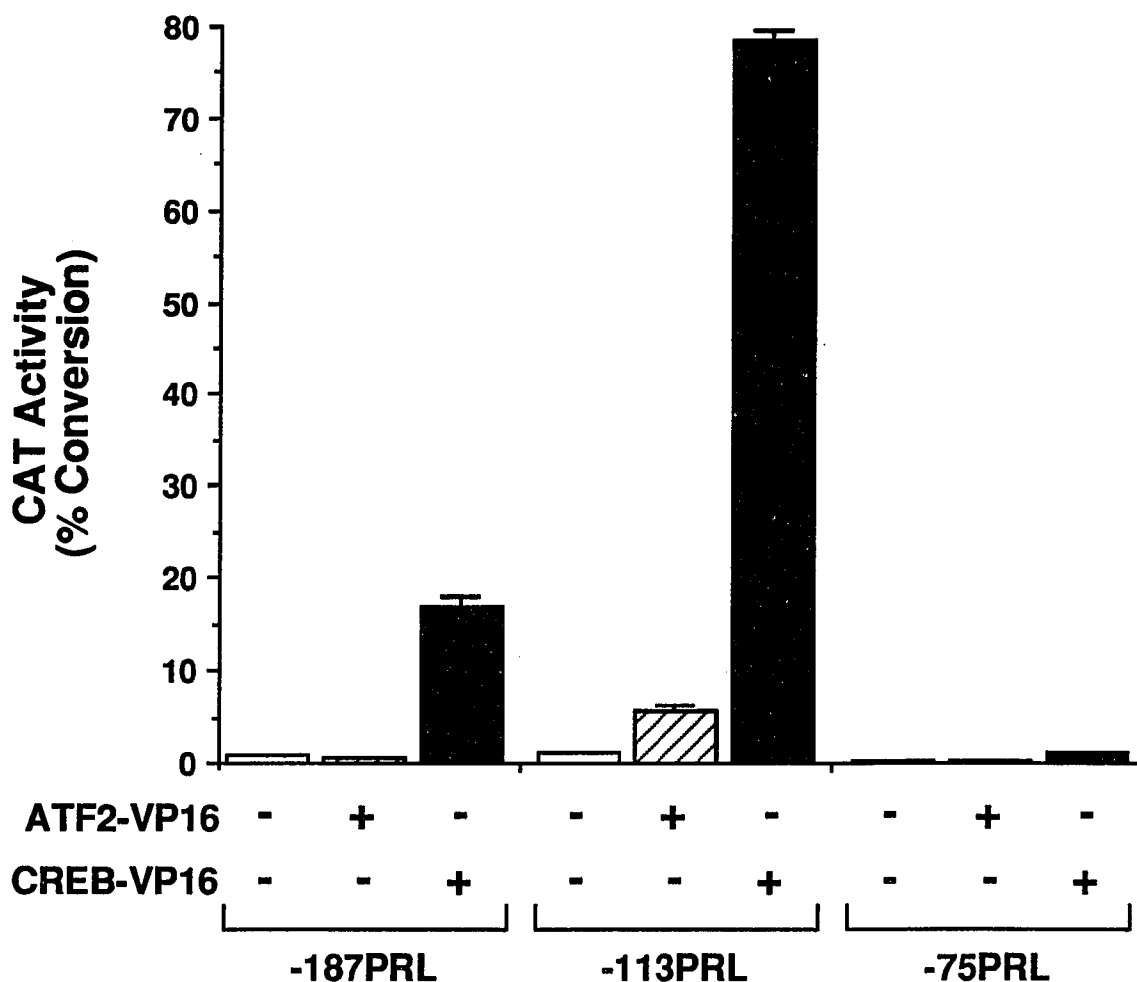


Fig. 4-2. Ability of exogenous CREB-VP16 or ATF-2-VP16 to activate co-transfected PRL-CAT constructs. C6 cells were transfected with 5 ug of the indicated PRL-CAT constructs (identified by their 5'-termini), plus 5 ug of either a SV40-CREB-VP16 expression vector (CREB-VP16), or a SV40-ATF-2-VP16 expression vector (ATF-2-VP16), and assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of the percent conversion of [^{14} C]choamphetamine observed with triplicate dishes.

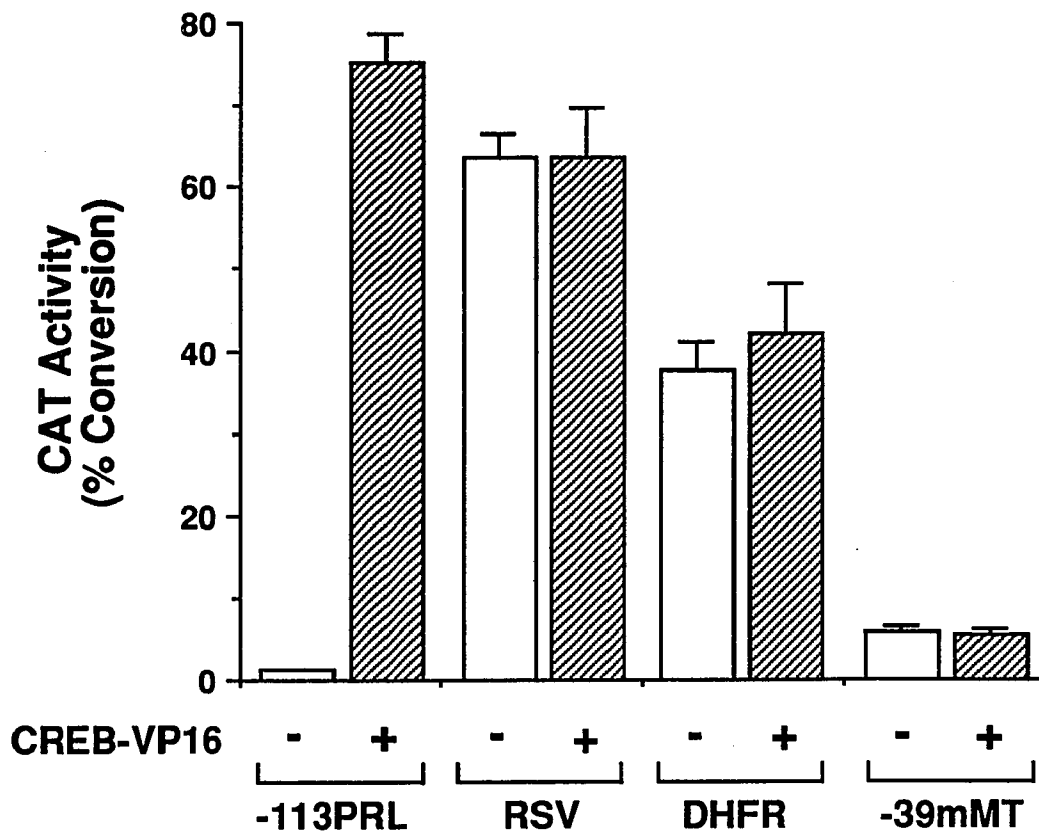


Fig. 4-3. Ability of exogenous CREB-VP16 to activate co-transfected promoter-CAT constructs. C6 cells were transfected with 5 ug of either (-113)PRL-CAT (-113PRL) or control constructs RSV-CAT (RSV), DHFR-CAT (DHFR), (-39)mMT-CAT (-39mMT), plus 5 ug of SV40-CREB-VP16 (CREB-VP16), and assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of the percent conversion of [^{14} C]chloramphenicol observed with triplicate dishes.

while CREB-VP16 had no effect on expression of the control constructs RSV-CAT, DHFR-CAT, or mMT-CAT. These results indicated that the constitutively active CREB protein CREB-VP16 can specifically activate expression of the PRL promoter, probably by acting through the PRL CLE.

CREB-VP16 Activates (-187)PRL-CAT Expression in Non-pituitary Cells as Strongly as the Pituitary-Specific Factor Pit-1.

The pituitary-specific transcription factor pit-1 has shown previously to *trans*-activate expression of PRL-CAT in non-pituitary cells (Mangalam et al., 1989; Fox et al., 1990). As described above, a constitutively active CREB protein CREB-VP16 could also activate expression of a PRL-CAT construct in non-pituitary cells. It was thus interesting to see if CREB-VP16 and pit-1 could interact with each other in activating expression of PRL-CAT in C6 cells. The (-187)PRL-CAT construct that contains three binding sites for pit-1 plus a PRL CLE was cotransfected in C6 cells along with expression plasmids that produce CREB-VP16 and/or pit-1. The results are shown in Fig. 4-4, CREB-VP16 could activate expression of (-187)PRL-CAT, but ATF-2-VP16 could not (**bar 4 vs bar 2**). The (-187)PRL-CAT could be activated in C6 cells by co-expression of CREB-VP16 as strongly as by co-expression of pit-1 (**bar 4 vs bar 5**), indicating CREB-VP16 can be a powerful *trans*-activator for expression of PRL-CAT in non-pituitary cells. Moreover, co-expression of pit-1 and CREB-VP16 synergistically activated expression of (-187)PRL-CAT, suggesting that they may function cooperatively in regulating the expression of the PRL promoter. The results with CREB in Fig. 4-4 are discussed in the next section.

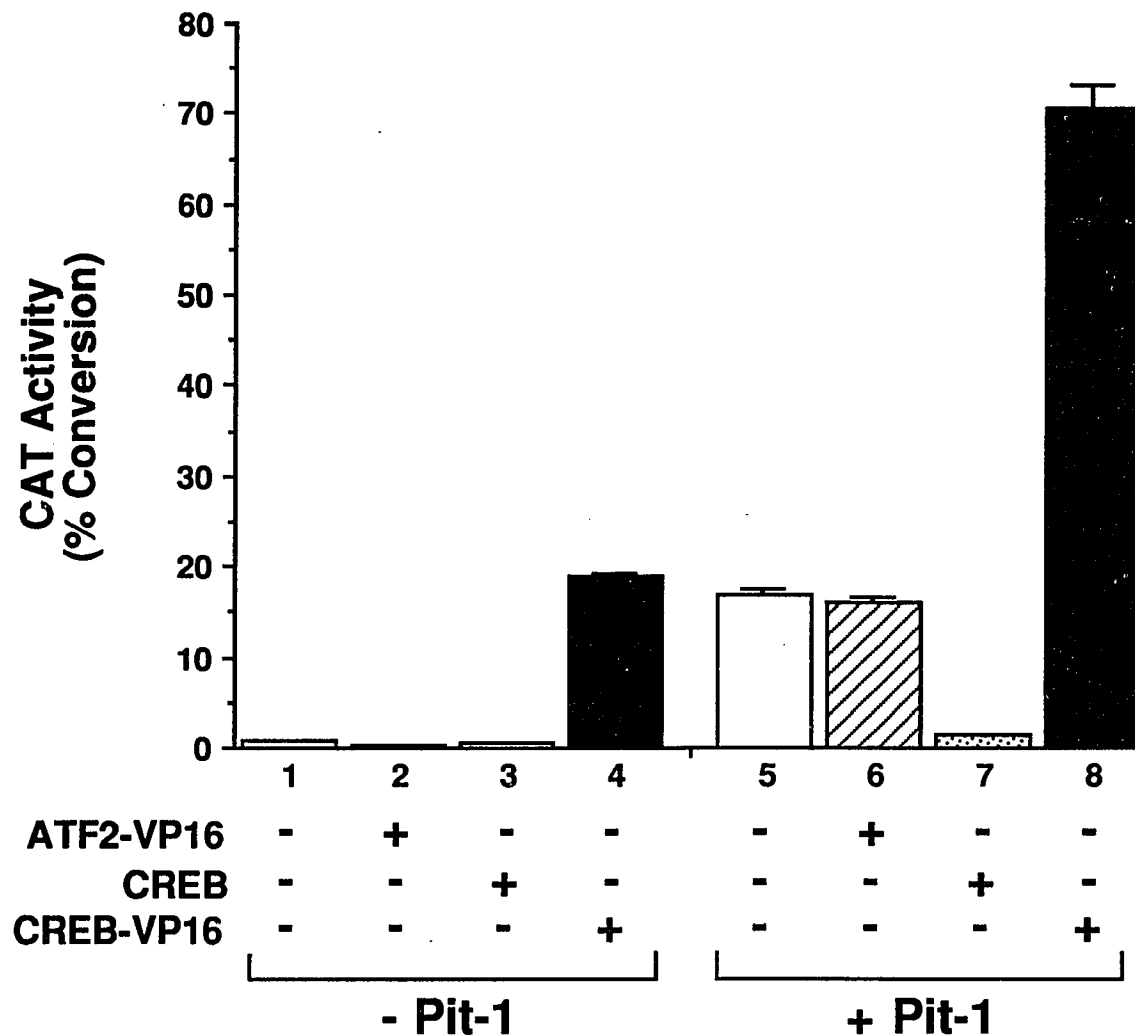


Fig. 4-4. Ability of CREB-VP16, ATF-2-VP16 or pit-1 to activate (-187)PRL-CAT and CREB to block pit-1 induction of (-187)PRL-CAT. C6 cells were transfected with 5 ug of (-187)PRL-CAT, plus 5 ug of either a SV40-CREB-VP16 expression vector (CREB-VP16), or a SV40-ATF-2-VP16 expression vector (ATF-2-VP16), or RSV-CREB (CREB), or pUC19, plus 5 ug of either pUC19 (-Pit-1) or RSV-pit-1 (+Pit-1), and assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of the percent conversion of [^{14} C]choamphenicol observed with triplicate dishes.

CREB Structural requirement for Inhibition of Pit-1 *Trans*-activation of the PRL Promoter, and Reversal of CREB Inhibition by Co-expression of PKA-C.

The PRL CLE is required for basal expression of the PRL promoter in pituitary and non-pituitary cells (Jackson et al., 1992). I have shown that CREB can act as a conditional repressor of the rat prolactin promoter through the PRL CLE in GH₃ cells (Chapter 3). To test whether CREB can repress the pit-1 activation of PRL-CAT in non-pituitary cells, we employed co-transfection experiments in which pit-1 and CREB-expression vectors were transfected with (-187)PRL-CAT into C6 cells. The results are shown in Fig. 4-4.

Expression of CREB alone had little effect on expression of (-187)PRL-CAT (Fig. 4-4, bar 3), whereas expression of CREB inhibited the pit-1 induction of PRL-CAT (Fig. 4-4, bar 7 *vs* bar 5), implying that CREB may interfere with a factor which is required for pit-1 activation of PRL-CAT by competing binding to the PRL CLE in the non-pituitary cells.

The ability of CREB and its mutants to block pit-1 *trans*-activation of the PRL promoter was further studied. Two previously described CREB mutants were used in this study. The first one was CREB-M1 which contains a mutation in the major A kinase phosphoacceptor site serine-133. This mutant permits binding to a CRE, but abolishes the ability to transactivate the somatostatin gene (Gonzalez and Montminy, 1989). The second one was K-CREB which carries a mutation in the DNA binding domain and is thus deficient in binding to the somatostatin CRE (Walton et al., 1992). The results are shown in Fig. 4-5. As before, CREB dramatically reduced pit-1 activation of (-187)PRL-CAT in C6 cells (bar 1 *vs* bar 2). CREB-M1 was equally effective as CREB in inhibiting pit-1 induction of PRL-CAT expression (bars 3 and 2 *vs* bar

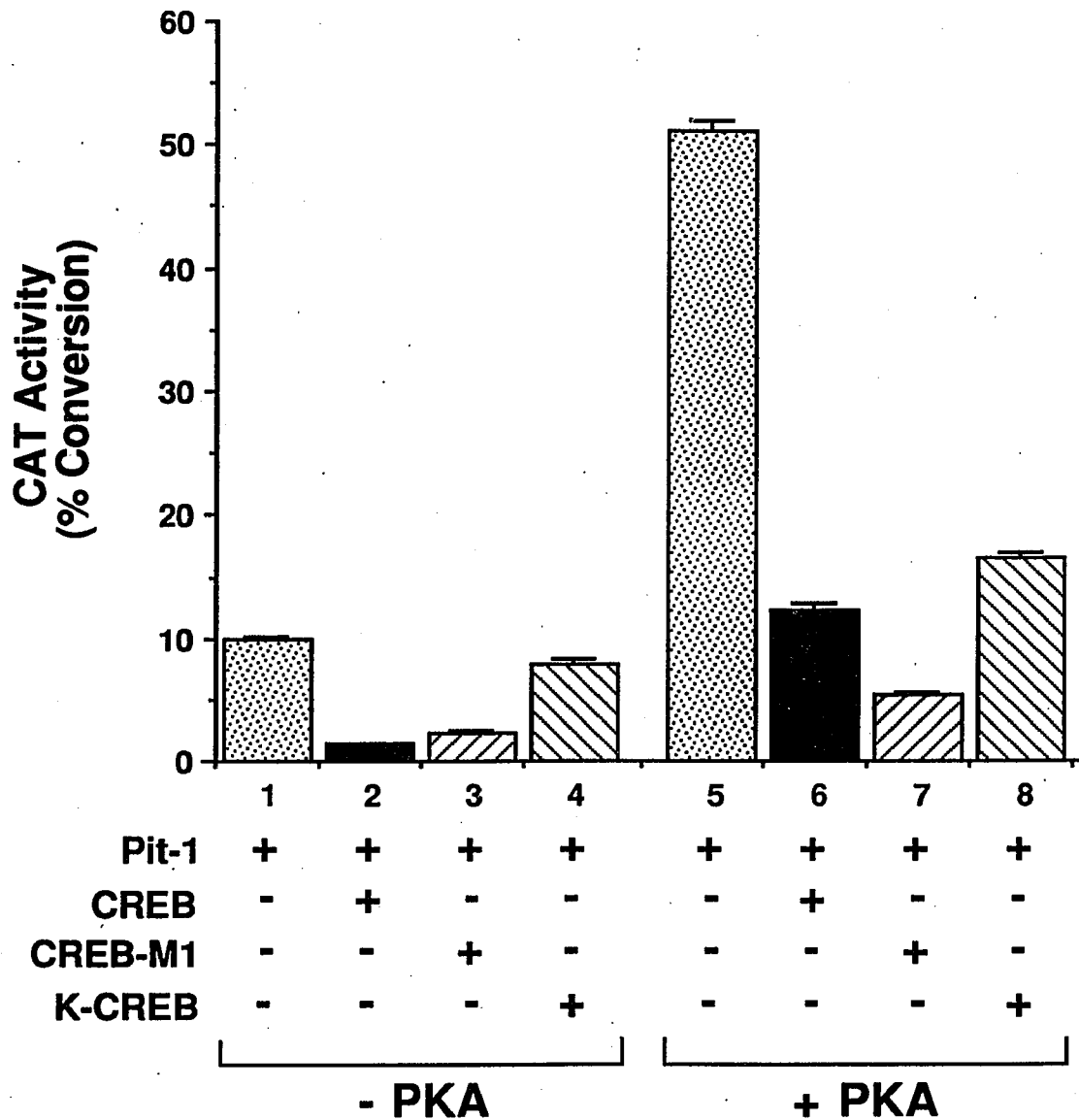


Fig. 4-5. Effect of exogenous PKA catalytic subunit on pit-1 induction of expression of a PRL-CAT construct co-transfected with vectors expressing CREB or its mutants. C6 cells were transfected with 5 ug of a (-187)PRL-CAT construct, plus 5 ug of RSV-pit-1, plus 5 ug of either a RSV-CREB (CREB), or a RSV-CREB-M1(CREB-M1), or RSV-K-CREB (K-CREB), and plus 5 ug of either pUC19 (-PKA) or MtC (+PKA), and assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of the percent conversion of [^{14} C]chloamphenicol observed with triplicate dishes.

1), implying that the phosphoacceptor site serine-133 is not required for the inhibitory action of CREB on the PRL promoter. By contrast, K-CREB was much less effective than either CREB or CREB-M1 in inhibiting pit-1 induction of PRL-CAT expression (bar 4 *vs* bar 1, bar 4 *vs* bars 2 and 3), indicating that CREB exerts its repressive effect on the induction of the PRL promoter by pit-1 in non-pituitary cells by binding to DNA. This effect is reminiscent of the repression of expression of PRL-CAT by CREB in GH₃ cells where CREB must bind to the PRL promoter to exert its repressive effects.

Phosphorylation of CREB by PKA-C has been shown previously to *transactivate* a classical CRE in SS promoter (Gorzalez and Montminy, 1989). Our recent finding has indicated that CREB repression of the PRL promoter can be reversed by coexpression of PKA-C in GH₃ (Chapter 3). Therefore, we tested whether coexpression of CREB with PKA-C would reverse the CREB inhibition of pit-1 activation of the PRL promoter. I have already shown in Fig. 4-1 that PKA-C alone can activate expression of PRL-CAT in C6 cells, implying that the phosphorylation of some endogenous factor by PKA-C can activate the PRL promoter in non-pituitary cells. Co-expression of pit-1 with PKA-C dramatically increased expression of (-187)PRL-CAT in C6 cells (Fig. 4-5, bar 5 *vs* bar 1). Interestingly, CREB inhibition of pit-1 induction of (-187)PRL-CAT expression was completely relieved by coexpression of PKA (Fig. 4-5, bar 6 *vs* bar 2). The PKA activation observed is probably due to the ability of this kinase to phosphorylate CREB resulting in converting inactive CREB to activated CREB. However, co-expression of PKA with CREB-M1 yielded a partial reversal of the inhibition of pit-1 induction of PRL-CAT (Fig. 4-5, bar 7 *vs* bar 3), possibly arising from phosphorylation of other phosphoacceptor sites present in CREB-M1 (Lee et al., 1990). Finally, it is not

clear why co-expression of PKA with K-CREB slightly (2-fold) increased expression of PRL-CAT by pit-1 (Fig. 4-5, bar 8 *vs* bar 4).

Discussion

The pituitary-specific transcription factor pit-1 is clearly important for cell-specific expression of PRL and growth hormone. However, there is evidence that full activation of the PRL gene requires additional factors. The estrogen receptor is a candidate for such a co-factor. Co-expression of the estrogen receptor in transient transfection experiments with pit-1 synergistically activated the prolactin gene (Maurer and Notides, 1987; Kim et al., 1988; Waterman et al., 1988). Moreover, it has been shown recently that the rat PRL gene is expressed in brain, including hypothalamus and extrahypothalamic brain, without detectable co-expression of pit-1 (Emanuele et al., 1992). This finding implies that PRL gene expression can occur independently from pit-1. In this report, a model study was carried out to test if prolactin expression could be induced without pit-1, using C6 glial cells which do not contain detectable pit-1.

We here showed that sequences between -187 and -113 contained a basal repression element for expression of the PRL promoter in non-pituitary cells, and that sequences between -113 and -75 in the PRL proximal promoter are required for basal expression in C6 cells (Fig. 4-1), in accordance with recent observation that deletion from the PRL promoter of a repressive sequence (FP11) at position -127/-116 permits expression in Hela cells and further deletion from -112/-80 loses expression in Hela cells (Jackson et al., 1992). On the other hand, PKA-C could increase about 10-fold basal expression of

(-113)PRL-CAT in C6 cells, but it only had slight effect on (-75)PRL-CAT (Fig. 4-1), implying that sequences between -113/-75 are required for activation of the PRL promoter by PKA. This finding implied that repression of expression of the PRL promoter in non-pituitary cells may involve a basal repression element, which binds a basal repressor whose activity can be relieved by phosphorylation by PKA.

The PRL CLE located between positions -99 and -92 is involved in basal expression and cAMP induction of the PRL promoter (d'Ement et al., 1992; Peers et al., 1991; Jackson et al., 1992). CREB is the only known protein to bind to the PRL CLE, although it does that with a low affinity. The major complex detected with the PRL CLE as a probe in pituitary (GH₃) cell (Liang et al., 1992) and in non-pituitary (C6) cell (Yan, Tian, and Bancroft, in preparation) nuclear extracts does not contain CREB, since a CRE could not compete these complexes, and addition of antiserum to CREB to the reaction mixture had little effect on the formation of the complex. Therefore, it could be that the PRL CLE interacts with some CREB-like protein or a CREB-heterodimer associated with another protein. Co-transfection experiments revealed that a constitutively active CREB/ATF-2-VP16 protein can activate the PRL promoter in non-pituitary cells. Expression of CREB-VP16 dramatically activated (-187)PRL-CAT and (-113)PRL-CAT containing the PRL CLE in C6 cells (Fig. 4-2). This activation effect of CREB-VP16 was dependent upon the presence of the PRL CLE, since a further 5'-deleted construct lacking the PRL CLE was not activated by CREB-VP16. These observations indicate that activation of PRL gene expression by a dominant positive CREB protein CREB-VP16 may mimic some endogenous protein in activating the PRL promoter in non-pituitary cells by binding to the PRL CLE.

ATF-2-VP16 is also a CREB/ATF family member. The failure of ATF-2-VP16 to transactivate the PRL promoter (Fig. 4-2) may be due to a lack of its capacity to bind to the PRL CLE. Experiments to further determine whether ATF-2 can bind to the PRL CLE, as well as site-mutagenesis study of the PRL CLE, are needed to see if binding to the PRL CLE is required for activation of PRL-CAT by CREB-VP16.

(-187)PRL-CAT contains three pit-1 binding sites and only one PRL CLE. However, the ability to transactivate this construct by CREB-VP16 is comparable to pit-1 (Fig. 4-3), indicating that CREB-VP16 is a powerful *trans*-activator of expression of PRL-CAT in non-pituitary cells. Co-expression of pit-1 and CREB-VP16 are additive in activating expression of (-187)PRL-CAT (Fig. 4-3), suggesting that a factor similar to CREB may cooperate with pit-1 in regulating PRL gene expression in pituitary cells.

Our recent studies have shown that overexpression of CREB in GH₃ cells can repress expression of the PRL promoter (Chapter 3). It is not known what mechanisms is involved in the repression of PRL gene expression by CREB. In this report, we have also tested whether CREB can repress the pit-1 induction of (-187)PRL-CAT in non-pituitary cells. Co-transfection experiments revealed that CREB inhibited the pit-1 induction of (-187)PRL-CAT in rat C6 glial cells (Fig. 4-4). Furthermore, the CREB-M1 mutant, which cannot be phosphorylated at the major PKA phosphoacceptor site, was equally strong as CREB at inhibiting pit-1 induction of PRL-CAT (Fig. 4-4), revealing that this inhibition does not require phosphorylation of CREB at this site. By contrast, a CREB binding site mutant K-CREB had only a slight effect on this induction (Fig. 4-4), implying that inhibition of pit-1 activation of the PRL promoter required CREB binding to the PRL CLE. Taken together, the results presented here suggest that CREB may interfere with a factor which is

required for pit-1 activation of PRL-CAT by competing binding of the factor to the PRL CLE .

Phosphorylation of CREB by PKA has previously been shown to *transactivate* a classical CRE in the somatostatin promoter (Gonzalez and Montminy, 1989). CREB repression of the PRL promoter can be reversed by co-expression of PKA in GH₃ (Chapter 3). The present studies showed that CREB inhibition of pit-1 induction of (-187)PRL-CAT in C6 cells was completely relieved by PKA (Fig. 4-5). CREB-M1 yielded a partial reversal of the inhibition of pit-1 induction of PRL-CAT (Fig. 4-5), probably due to the consequence of phosphorylation of other phosphorylation sites still present in CREB-M1 (Lee et al., 1990). These results thus indicate that the ability of CREB to block pit-1 induction of PRL-CAT expression may be completely reversed by PKA-mediated phosphorylation of CREB. We could not explain why PKA yielded only a slight increase in the inhibition of pit-1 induction of PRL-CAT by K-CREB (Fig. 4-5). On the other hand, PKA alone can activate expression of PRL-CAT in C6 cells (Fig. 4-1). This suggests that phosphorylation of a factor by PKA can transactivate the PRL promoter in a way similar to phosphorylation of CREB by PKA in activating the somatostatin promoter. Moreover, co-expression of pit-1 with PKA dramatically increase expression of (-187)PRL-CAT in C6 cells (Fig. 4-5), suggesting that this factor can act in concert with pit-1 in activating the PRL promoter in C6 cells.

The results presented in this study may be significant in light of the observation that the rat PRL gene is expressed in brain where pit-1 is absent. The observation that a constitutively active CREB is capable of transactivating the PRL promoter through the PRL CLE site raises the possibility that a factor similar to CREB may bind to this site in non-pituitary tissue such as the brain,

and thus activate the PRL promoter in the absence of pit-1. It will be possible to establish whether expression of the PRL gene can occur independently from pit-1 by studying the *in vitro* expression of PRL-CAT constructs using nuclear extracts from brain where the PRL gene is expressed in absence of pit-1, and by identifying the factor that binds to the PRL CLE.

CHAPTER 5

Vasoactive Intestinal Peptide (VIP) Stimulation of Prolactin Promoter Activity: Evidence for Mediation by Calmodulin

Abstract

It has been shown that PRL secretion induced by vasoactive intestinal peptide (VIP) is accompanied by an increase in PRL mRNA content in GH₃ cells. However, little is known about the mechanisms involved in regulation of transcription of the PRL gene by VIP. In the present studies, VIP action on the PRL promoter was investigated by studying the gene-proximal events and gene-distal mediators. Exposure of GH₃ rat pituitary cells to VIP yielded a large specific stimulation of transient expression of a (-204)PRL-CAT construct that contains the first 204 base pairs of the PRL proximal promoter, implying that the proximal PRL promoter region is sufficient for VIP induction. Analysis by 5'-deletion of the (-204)PRL-CAT construct showed that the region between -174/-113, which contains two pit-1 binding sites, is required for VIP stimulation. Binding of pit-1 to either 1P or 3P is known to be capable of activating pituitary cell-specific PRL gene expression. However, pit-1 is apparently not involved in transducing the VIP signal to the PRL promoter via pit-1 binding sites since: 1. two copies of each of the four pit-1 binding sites (1P-4P) transferred only a modest VIP response to a (-39)PRL-CAT construct; 2. expression of a mutant pit-1 possessing reduced transactivational activity did not inhibit induction of (-187)PRL-CAT by VIP, whereas the TRH induction of this construct was inhibited by this pit-1 mutant. Expression of a mutant CREB (K-CREB) which cannot bind to the somatostatin gene CRE also did not inhibit VIP stimulation of (-187)PRL-CAT expression. These results imply that neither pit-1 nor CREB can mediate VIP action on the PRL proximal promoter. Investigation with kinase or Ca²⁺ inhibitors yielded the following results. Expression of a regulatory subunit mutant of cAMP-dependent

protein kinase (PKA) inhibited induction of (-187)PRL-CAT by cAMP, but not by VIP. Chronic 12-O-tetradecanoyl phorbol-13-acetate (TPA) down-regulation of cellular protein kinase C or use of a protein kinase inhibitor H7, which is most potent in inhibiting protein kinase C activity, did not block induction of expression of (-187)PRL-CAT by VIP. Ca^{2+} flux inhibitors, such as cobalt ion and nimodipine, blocked induction of (-187)PRL-CAT expression by VIP. However, the Ca^{2+} immobilizer 8-(*N,N*-diethylamino)octyl 3, 4, 5-trimethoxybenzoate (TMB-8) did not block induction of expression of this construct by VIP, suggesting that VIP regulation of the PRL promoter may be dependent on Ca^{2+} fluxes but insensitive to Ca^{2+} immobilization. Lastly, the calmodulin inhibitor W13 dramatically inhibited induction of (-187)PRL-CAT expression, while VIP induction of (-187)PRL-CAT was not inhibited by the relatively inactive Ca^{2+} calmodulin inhibitor W12, suggesting that VIP action on the PRL promoter may be mediated by Ca^{2+} /calmodulin-dependent protein kinase.

Introduction

It has been shown previously that PRL secretion induced by vasoactive intestinal peptide (VIP) is accompanied by an increase in PRL mRNA content in GH₃ cells (Alberto et al., 1985), and that the stimulatory effect of VIP on PRL secretion is accompanied by increased cAMP in anterior pituitary cells (Gourdji et al., 1979; Rotsztejn et al., 1981). However, little is known about the mechanisms involved in regulation of transcription of the PRL gene by VIP. From the results presented in Chapter 1 and 2, I concluded that Ca²⁺ mediates TRH action on the PRL promoter, and that pit-1 represents a gene-proximal mediator in this signaling pathway. To further understand whether the TRH signal pathway of induction of the PRL promoter is a general model for regulation of PRL gene expression by peptide hormones, we examined the mechanism of regulation of the PRL promoter by another positive regulator, VIP.

Results

The Proximal Prolactin Promoter Contains At Least One VIP Response Element.

We first examined the ability of VIP to stimulate transient expression in GH₃ cells of a PRL-chloramphenicol acetyltransferase (PRL-CAT) construct containing a PRL gene promoter fragment cloned upstream of the bacterial CAT gene. Chinese hamster dihydrofolate reductase (DHFR-CAT) construct was used as a control. A typical result employing plasmid (-204)PRL-CAT

which contains the first 204 base pairs of DNA upstream of the PRL cap site is shown in Fig. 5-1. VIP strongly stimulated CAT enzymatic expression of (-204)PRL-CAT, but it had little effect on expression of DHFR-CAT. A larger PRL-CAT construct which contains the first 1957 base pairs of PRL sequences yielded only half stimulation by VIP as compared to (-204)PRL-CAT (data not shown), implying that the first 204 base pairs of the PRL proximal promoter is sufficient for VIP stimulation.

GH₃ cell nuclear extracts have previously been shown to footprint four sites in the PRL proximal promoter (Fig. 5-2A), due in large part to pit-1 binding site (Gutierrez-Hartmann et al., 1987; Nelson et al., 1988; Kim et al., 1988; Yan et al., 1991). Recently, a CRE-like element (CLE) (Fig. 5-2A), which binds to CREB with lower affinity, has been found in the PRL proximal promoter (Peers et al., 1991; d'Emden et al., 1992; Liang et al., 1992; Yan, Tian, and Bancroft, in preparation). To further determine the sequences responsible for VIP stimulation in the PRL proximal promoter, we examined the ability of VIP to stimulate expression of (-204)PRL-CAT and its 5' deletion mutants. As shown in Fig. 5-2B, VIP yielded a strong (16 fold) stimulation of expression of (-204)PRL-CAT. This stimulation was not reduced by removal of the PRL promoter sequences between positions -204 to -187 or -174 (relative to the cap site). However, deletion from -174 to -113 strongly reduced the stimulation by VIP, implying that sequences between -174 and -113 are required for VIP stimulation. Further deletion from -174 to -75 yielded a farther 2-fold decrease in the VIP stimulation, close to that observed with the control construct DHFR-CAT.

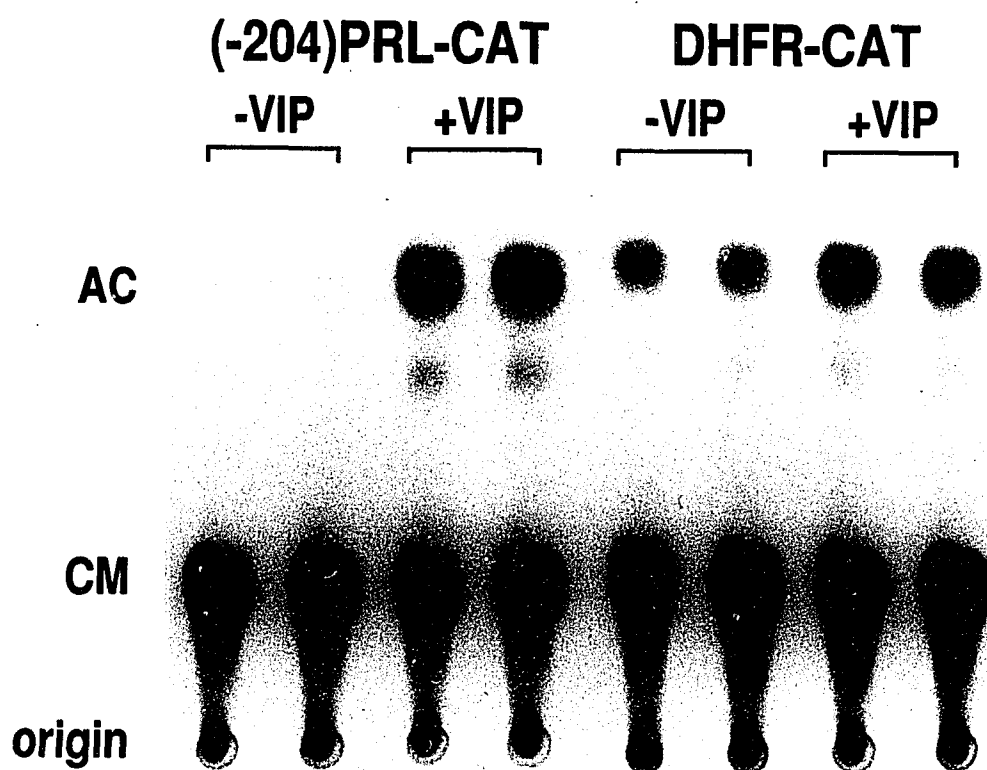


Fig. 5-1. VIP regulation of transfected promoter-CAT constructs. GH₃ cells were transfected with the indicated constructs, preincubated two days, then incubated one day $\pm$ VIP (200nM), and harvested for assay of CAT enzymatic activity. Autoradiographic exposure time was 12 hours. DHFR, Chinese hamster dihydrofolate reductase promoter; CM, [¹⁴C]Chloramphenicol; AC, acetylated forms of [¹⁴C]Chloramphenicol.

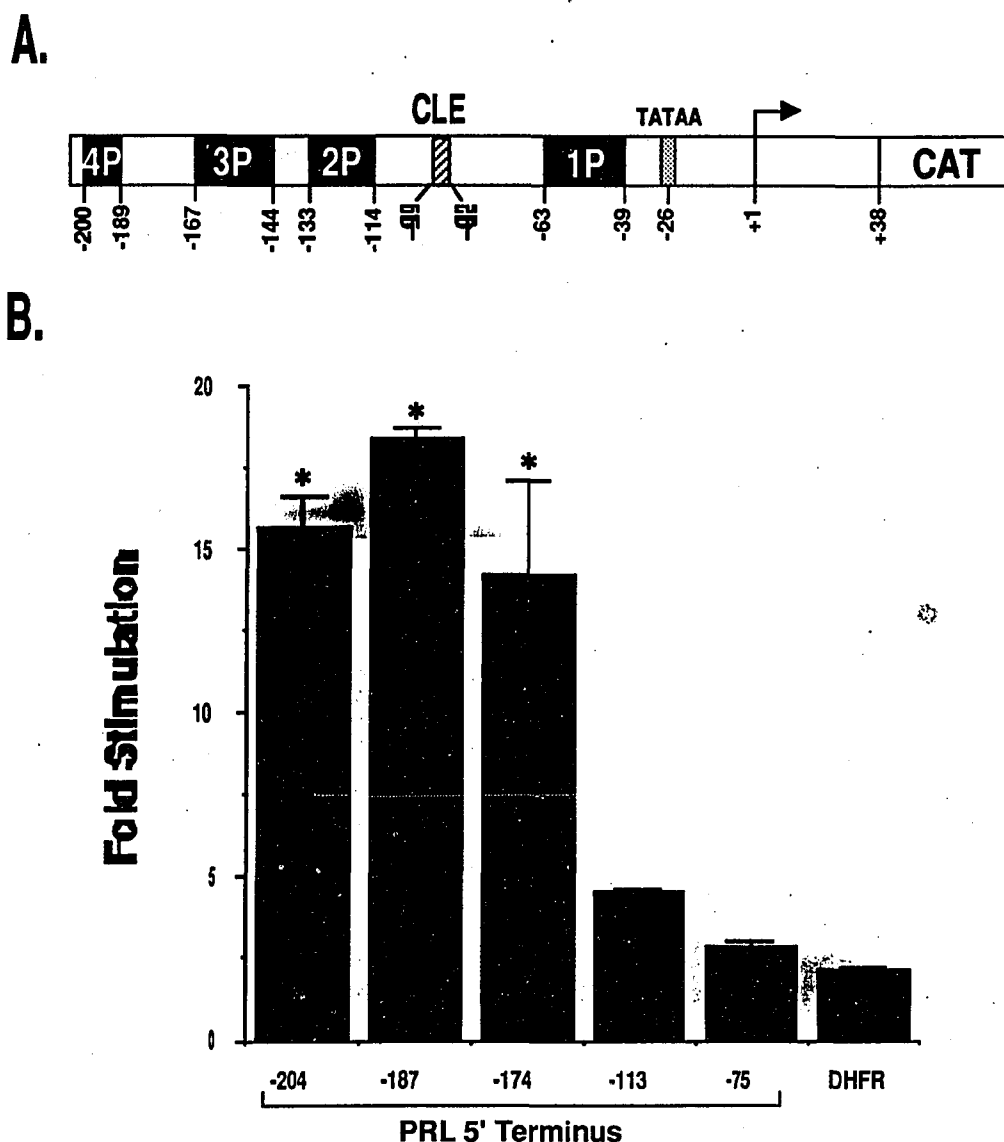


Fig. 5-2. 5'-Deletion analysis of PRLpromoter VIP response regions. (A). Map of construct (-204)PRL-CAT, containing rat PRL gene sequences from positions -204/+38 and of GH₃ nuclear footprint sites 1P-4P and CLE in the PRL promoter region. **(B).** VIP regulation of (-204)PRL-CAT and its 5'-deletion mutants, and of DHFR-CAT (DHFR). The experiment procedures described as in Fig.3-1. Values shown are the mean $\pm$ SE of the fold stimulation observed with triplicate dishes. Mean basal (without VIP) expression for the PRL-CAT constructs (percent conversion of Chloramphenicol) was: (-204), 0.79; (-187), 1.17; (-174), 0.58; (-113), 0.43; (-75), 0.99; that for DHFR-CAT was 6.65. *, $P < 0.01$ vs. control group (DHFR-CAT).

VIP Action on the Prolactin Promoter is Apparently Not Mediated by Pit-1.

The results of 5'-deletion analysis described above suggest that VIP regulation of (-174)PRL-CAT could involve a pit-1 binding site since region -174/-113 contains two such sites (2P and 3P). To examine whether a pit-1 binding site can function as a VIP response element, we cloned two copies of 1P, or 2P, or 3P, or 4P sites in front of (-39)PRL-CAT which lacks a cell-specific element and is not detectably expressed in GH₃ cells (data not shown). The ability of VIP to regulate these constructs is shown in Fig. 5-3. The parental (-187)PRL-CAT construct yielded a strong 24-fold VIP stimulation. (-39)PRL-CAT with either site 1P or site 2P yielded a moderate stimulation (7 or 4.5 fold, respectively), while (-39)PRL-CAT with site 3P or 4P yielded a 2-fold stimulation, similar to the 2-fold non-specific stimulation of the control construct DHFR-CAT. These results imply that sites 1P and 2P, but not 3P and 4P can serve as weak VIP response elements.

To investigate further whether pit-1 can transduce the VIP signal to the proximal PRL promoter, we examined if expression in GH₃ cells of a pit-1 antagonist could interfere with induction by VIP of a (-187)PRL-CAT construct. The antagonist employed was the pit-1 mutant, (Δ 2-45)pit-1, which can bind to pit-1 binding site and combatively inhibit *trans*-activation by pit-1 (Steinfeld et al., 1991; Yan et al., 1991). I have previously shown that this pit-1 mutant inhibits TRH and Ca²⁺ induction in GH₃ cells of a transfected PRL promoter (Chapter 2). As shown in Fig. 5-4 (bottom), coexpression with (-187)PRL-CAT of increasing amounts of an RSV-pit-1 expression vector resulted in a slight decrease in TRH induction of CAT activity, which was only observed after 2 ug RSV-pit-1. The slight decrease in TRH induction may

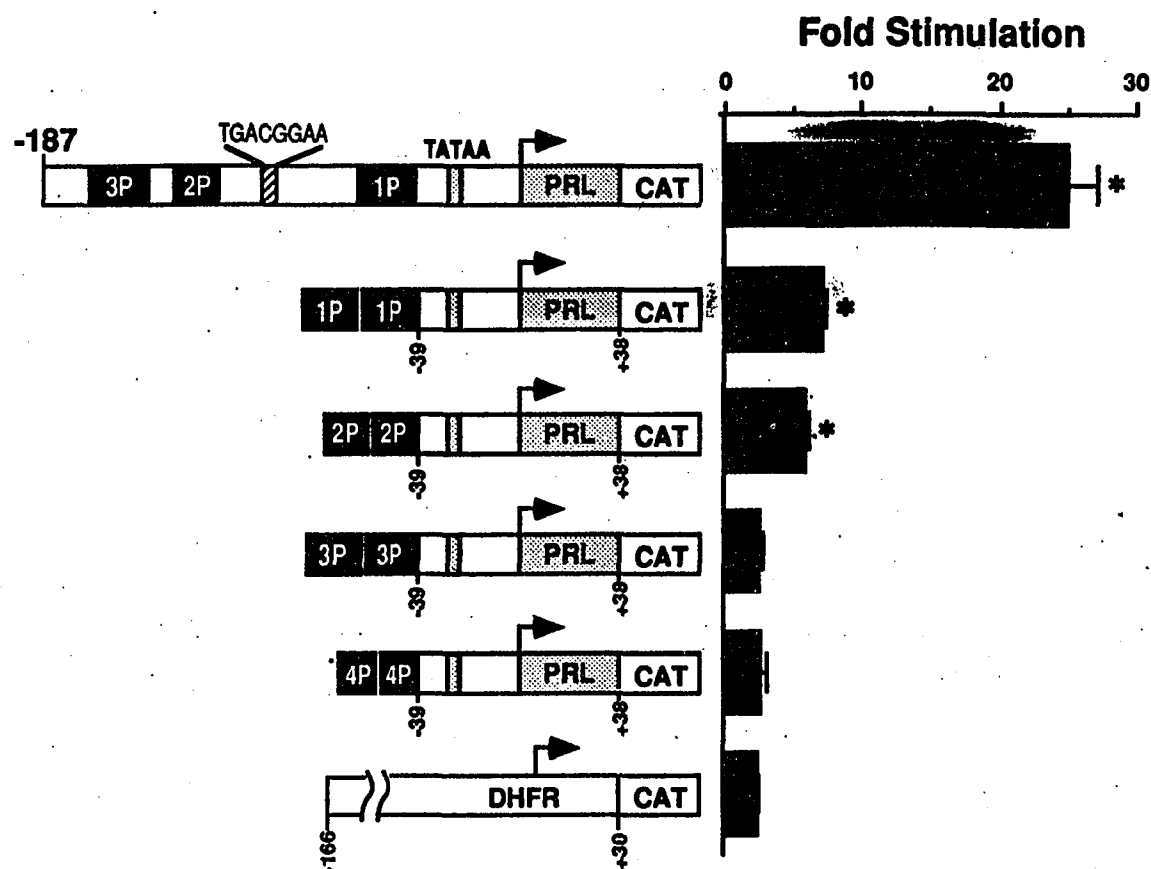


Fig. 5-3. Ability of proximal PRL promoter DNA elements to confer VIP responsiveness to a minimal PRL-CAT construct. GH3 cells transfected with the indicated constructs were incubated with or without VIP as described in Fig. 5-1. Values shown are the mean $\pm$ SE of the fold stimulation observed with triplicate dishes. Mean basal (without VIP) percent conversion of [^{14} C] chloramphenicol for (-187)PRL-CAT was 0.97; for the constructs containing duplicate copies of 1P, 2P, 3P, and 4P was respectively, 0.98; 0.79; 1.77; and 0.55; and for DHFR-CAT was 10.3. *, $P < 0.01$ vs. control group (DHFR-CAT).

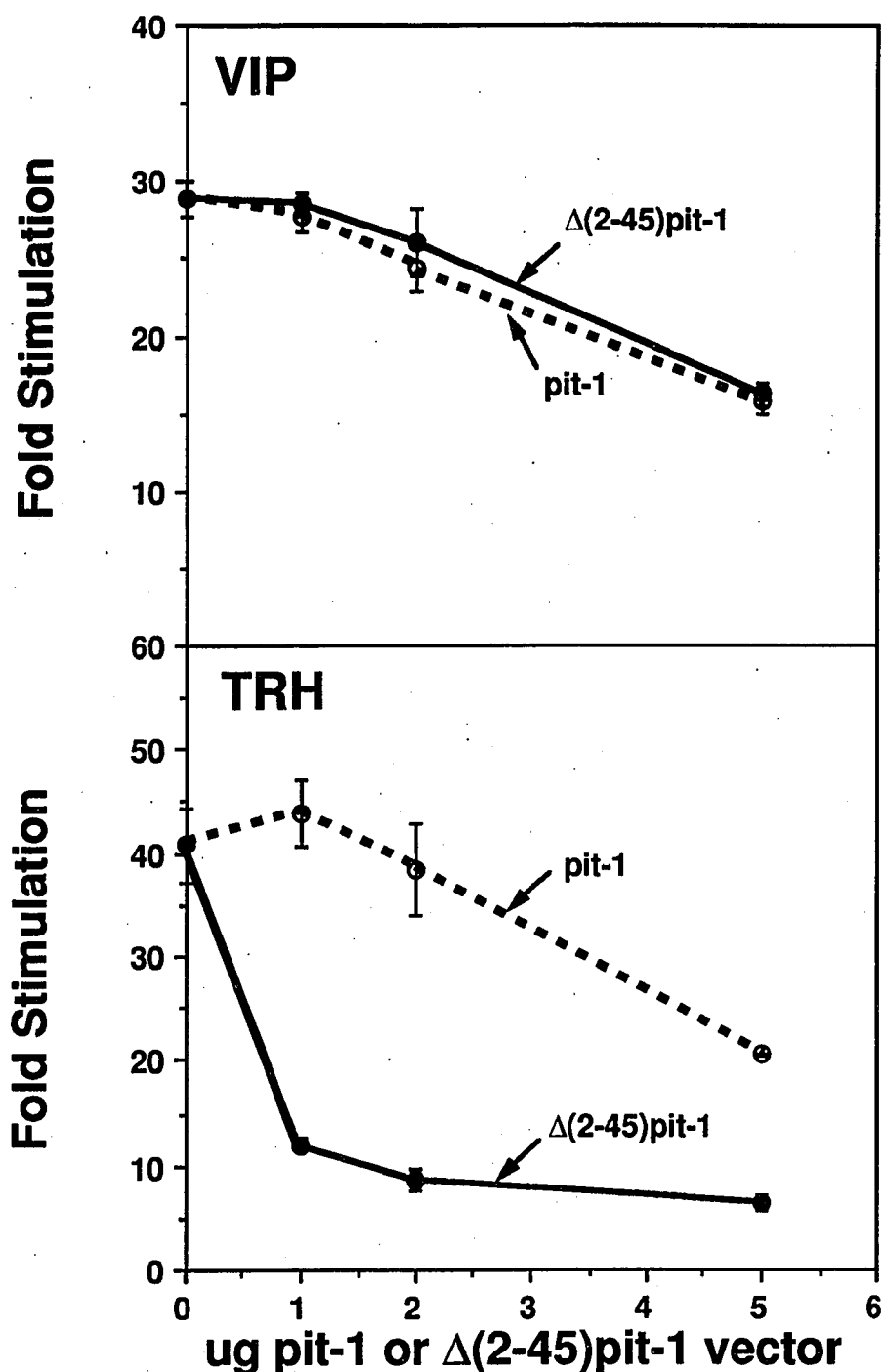


Fig. 5-4. Ability of a *pit-1* *trans*-activation mutant to block induction by TRH or VIP of a PRL-CAT construct. GH₃ cells were transfected with 5 ug of (-187)PRL-CAT, plus 1 or 2 ug of either (Δ 2-45)*pit-1* or *pit-1*, and pUC19 to bring the total mass of transfected DNA to 7 ug, or plus 5 ug either (Δ 2-45)*pit-1* or *pit-1*, then incubated $\pm$ TRH (100nM) or VIP as described in Fig. 5-1. Values shown are the mean $\pm$ SD of the fold stimulation observed with duplicate dishes. Mean percent conversion of [¹⁴C] chloramphenicol in stimulated cells with (-187)PRL-CAT plus 5 μ g of, respectively, (Δ *pit-1*) or *pit-1*, was: TRH, 3.84 or 11.7; VIP, 9.64 or 8.86.

be due to overexpression of pit-1 protein which quenches the TRH induction. However, coexpression with (-187)PRL-CAT of increasing amounts of an RSV-(Δ 2-45)pit-1 expression vector led to a progressive decrease in TRH induction of CAT activity. The greatest inhibition of TRH induction was observed at 2 ug RSV-(Δ 2-45)pit-1, which was 76% relative to cells receiving RSV-pit-1 DNA. Thus, in agreement with the results shown in Chapter 2, (Δ 2-45)pit-1 can inhibit TRH stimulation of the PRL promoter. By contrast, coexpression with (-187)PRL-CAT of increasing amounts of either RSV-pit-1 or RSV-(Δ 2-45)pit-1 expression vectors had identical effects on VIP induction (Fig. 5-4, top). Thus, expression of RSV-(Δ 2-45)pit-1 did not interfere with induction of (-187)PRL-CAT expression by VIP, implying that pit-1 does not lie on the pathway of action of VIP on the proximal PRL promoter.

Although 5'-deletion analysis did not strongly implicate the first 113 bp of the PRL promoter, which contains the PRL CLE, in the VIP response, the PRL CLE is important for the basal expression and cAMP induction (Peer et al., 1991; d'Emden et al., 1992; Liang et al., 1992). It has been shown that CREB protein can bind to the PRL CLE (Liang et al., 1992; Yan, Tian and Bancroft, in preparation). To investigate whether CREB protein might function as a gene proximal protein to transduce VIP signal to the PRL CLE, co-transfection of (-187)PRL-CAT with a CREB mutant (RSV-K-CREB) was carried out to see if this mutant would block VIP stimulation on the PRL proximal promoter. This CREB mutant has a leucine in place of arginine-287 and fails to bind to the somatostatin gene CRE (Walton et al., 1992). as shown in Fig. 5-5, expression of K-CREB as CREB had little or no effect on the stimulation of (-187)PRL-CAT by VIP, even when a higher concentration of CREB or K-CREB (10 ug) was employed (data not shown). The results indicate that VIP

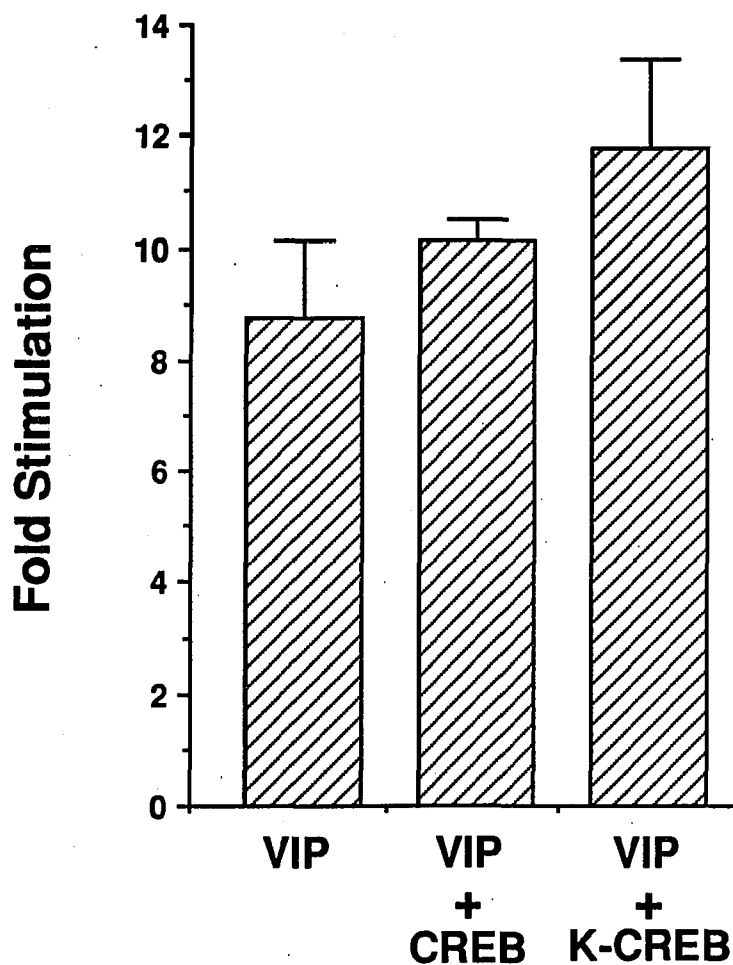


Fig. 5-5. Ability of CREB or K-CREB to block induction by VIP of a PRL-CAT construct. GH₃ cells were transfected with 5 ug of (-187)PRL-CAT, plus the 5 ug of either RSV-CREB, RSV-K-CREB or only RSV promoter (vector), then incubated with VIP and harvested for assay of CAT enzymatic activity. Values shown are the mean $\pm$ SD of the fold stimulation observed with duplicate dishes. Mean basal (without VIP) percent conversion of [¹⁴C] chloramphenicol for VIP, 2.96; VIP+CREB, 0.29; VIP+K-CREB, 1.92.

stimulation of PRL gene expression may not require CREB binding to the PRL CLE.

Pharmacological Studies of Regulation of VIP-Stimulated PRL Gene Expression by Potential Intracellular mediators.

It has been suggested that the stimulatory effect of VIP on PRL secretion is mediated by cAMP in anterior pituitary cells (Gourdji et al., 1979; Rotsztein et al., 1981). However, little is presently known about the gene distal mediators involved in the VIP action on PRL gene expression. To investigate whether cAMP-protein kinase A lies on the pathway of VIP action on the PRL gene expression, we examined whether transient expression in GH₃ cells of a cAMP-dependent protein kinase (PKA) antagonist could inhibit the stimulation of a co-transfected PRL-CAT construct by VIP. The antagonist employed was a mutant of regulatory subunit (RWT) of PKA. This mutant (RAB) contains inactivated cAMP binding domains, and can thus suppress cAMP stimulation of cAMP-dependent kinase activity (Leslayann and McKnight, 1991). As shown in Fig. 5-6 (top), coexpression with (-187)PRL-CAT of increasing amounts of a RAB expression vector resulted in a progressive decrease in cAMP induction of CAT activity. At the highest concentration of RAB (10 ug) employed, cAMP induction was inhibited by about 78% relative to cells receiving RWT DNA, showing that expression of RAB in GH₃ cells can block a protein kinase A-mediated stimulation. However, as seen in Fig. 5-6 (bottom), coexpression with (-187)PRL-CAT of increasing amounts of the RAB expression vector had only a slight effect on VIP stimulation of CAT activity relative to RWT. At the highest concentration of RAB (10 ug) employed, VIP

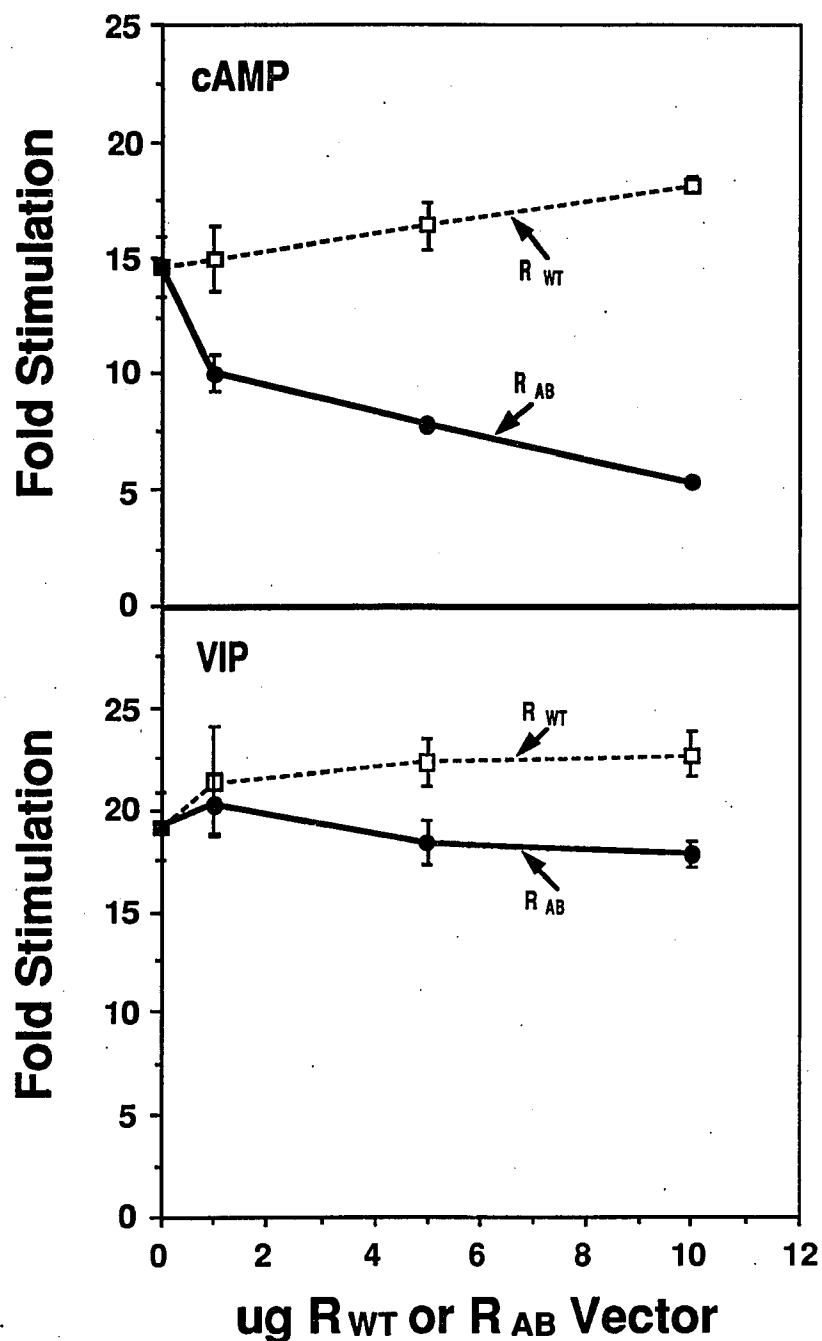


Fig. 5-6. Ability of a regulatory subunit mutant of PKA to block induction by cAMP or VIP of a PRL-CAT construct. GH₃ cells were transfected with 5 μ g of (-187)PRL-CAT, plus the indicated amount of either RAB, or an equal amount of RWT, plus sufficient pUC19 to bring the total mass of transfected DNA to 15 μ g, then incubated with or without cAMP (0.5mM) or VIP as described in Fig. 5-1. Values shown are the mean $\pm$ SE of the fold stimulation observed with triplicate dishes. Mean percent conversion of [¹⁴C] chloramphenicol in stimulated cells incubated with (-187)PRL-CAT plus 10 μ g of, respectively, RAB or RWT, was: cAMP, 3.33 or 7.22; VIP, 11.2 or 9.95.

induction was slightly reduced by about 23% relative to cells receiving Rwt DNA. Since a mutant regulatory subunit of PKA only slightly affected VIP action on the PRL promoter, we conclude that PKA does not play a major role in the stimulation of PRL gene expression by VIP.

To investigate whether protein kinase C (PKC) has a role in VIP action on the PRL promoter, we used two inhibitors: degradation of endogenous PKC by prolonged exposure of GH₃ cells to 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Ballester and Rosen, 1985; Bandyopadhyay and Bancroft, 1989); and a protein kinase inhibitor 1-(5-isoquinolinesulfonyl)2-methylpiperazine dihydrochloride (H7), which is most potent in inhibiting PKC (Hidaka et al., 1984). As shown in Fig. 5-7A, Chronic exposure to TPA (648 nM), which strongly down-regulates PKC activity (Bandyopadhyay and Bancroft, 1989), did not lower control levels of (-187)PRL-CAT activity, implying that PKC activity is not required for basal PRL promoter expression. Furthermore, as expected, the stimulation of CAT activity by acute TPA (100nM) was totally inhibited by chronic TPA pretreatment, consistent with the ability TPA to stimulate PRL gene transcription via its ability to activate PKC. By contrast, chronic TPA pretreatment had no effect on the stimulation of PRL promoter activity by VIP. Moreover, TPA-stimulated PRL gene transcription was entirely blocked by a more general protein kinase inhibitor, H7, while VIP-stimulated PRL gene transcription was not affected by this inhibitor (Fig. 5-7B). These results imply that VIP stimulation of PRL promoter activity does not require PKC activity.

Previous studies have demonstrated that Ca²⁺ itself specifically and strongly stimulate PRL gene expression in cultured pituitary cells (White et al., 1981). It has also been shown that Ca²⁺ mediates TRH action on PRL gene expression (White et al., 1983; Laverriere et al., 1989; Day et al., 1990; Chapter

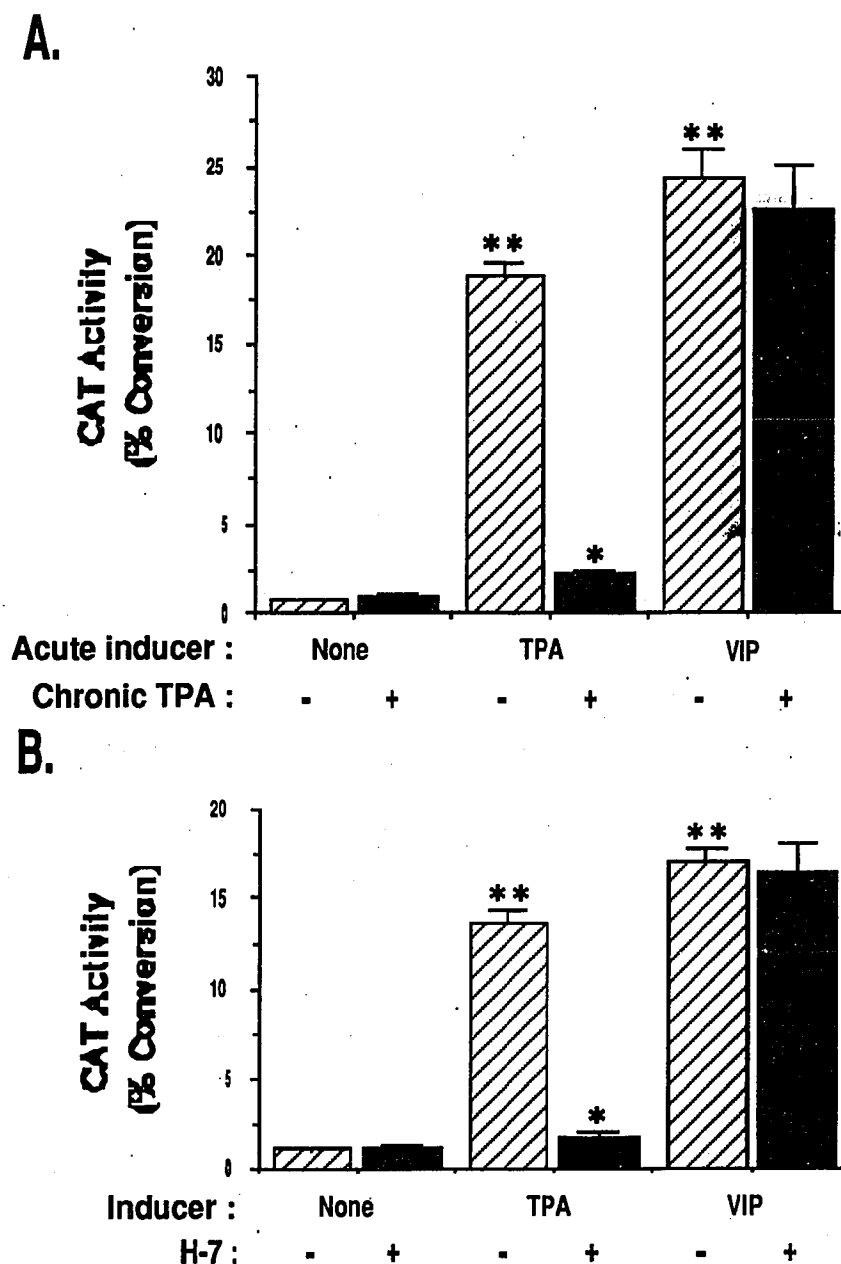


Fig. 5-7. Effect of down-regulation protein kinase C on induction by VIP of transient expression of a PRL-CAT construct. GH₃ cells were transfected with plasmid (-187)PRL-CAT, then (A). preincubated 2 days with or without TPA (648nM) (Chronic TPA). (B). preincubated 3 hours with or without H7 (20 uM), after which some cultures additionally received one of the following (Acute inducer): None, TPA (100nM), VIP (200nM). After additional incubation, cultures were assayed for CAT enzymatic activity. Shown is the mean $\pm$ SE of results with triplicate dishes. *, $P < 0.01$ vs. acute TPA without chronic TPA treatment or H-7. **, $P < 0.01$ vs. no inducer without chronic TPA treatment or H-7.

2). To determine whether Ca^{2+} also plays a role in VIP stimulation of PRL gene expression, we examined the effects of three types of cellular Ca^{2+} inhibitors on regulation of (-187)PRL-CAT by VIP (Fig. 5-8). Exposure of GH₃ cells to VIP again yielded a sizable stimulation of expression of (-187)PRL-CAT. The stimulation of CAT activity by VIP was completely blocked by either cobalt or nimodipine, both of which are Ca^{2+} flux inhibitors (Thaw et al., 1984; Lee and Tsien, 1983; Janis et al., 1987), or by the intracellular Ca^{2+} chelator BAPTA/AM (Tsien, 1980; Lew et al., 1982). By contrast, an immobilizer of intracellular Ca^{2+} (TMB-8) (Chiou and Malagodi, 1975) had no effect on the ability of VIP to stimulate CAT activity.

The possibility that the Ca^{2+} mediator calmodulin is involved in VIP action on the PRL promoter was examined by using the structurally similar naphthalene-sulfonamides W12 and W13 (Chafouleas et al., 1982). As shown in Fig. 5-8, W12 which is relatively inactive against calmodulin (Chafouleas et al., 1982) did not inhibit the induction of (-187)PRL-CAT by VIP. However, VIP induction of (-187)PRL-CAT was dramatically inhibited by the active compound, W13, implying that this action of VIP does require calmodulin activity. These results suggest that VIP regulation of PRL promoter activity is dependent upon cellular Ca^{2+} fluxes and Ca^{2+} calmodulin, but is insensitive to immobilization of intracellular Ca^{2+} .

Discussion

In the present studies, we have used transient expression in GH₃ cells to study both gene-proximal and gene-distal events involved in VIP stimulation of the PRL promoter. Construct (-204)PRL-CAT, containing the proximal PRL promoter, exhibited a 16-fold VIP response (Fig. 5-1; Fig. 5-2B).

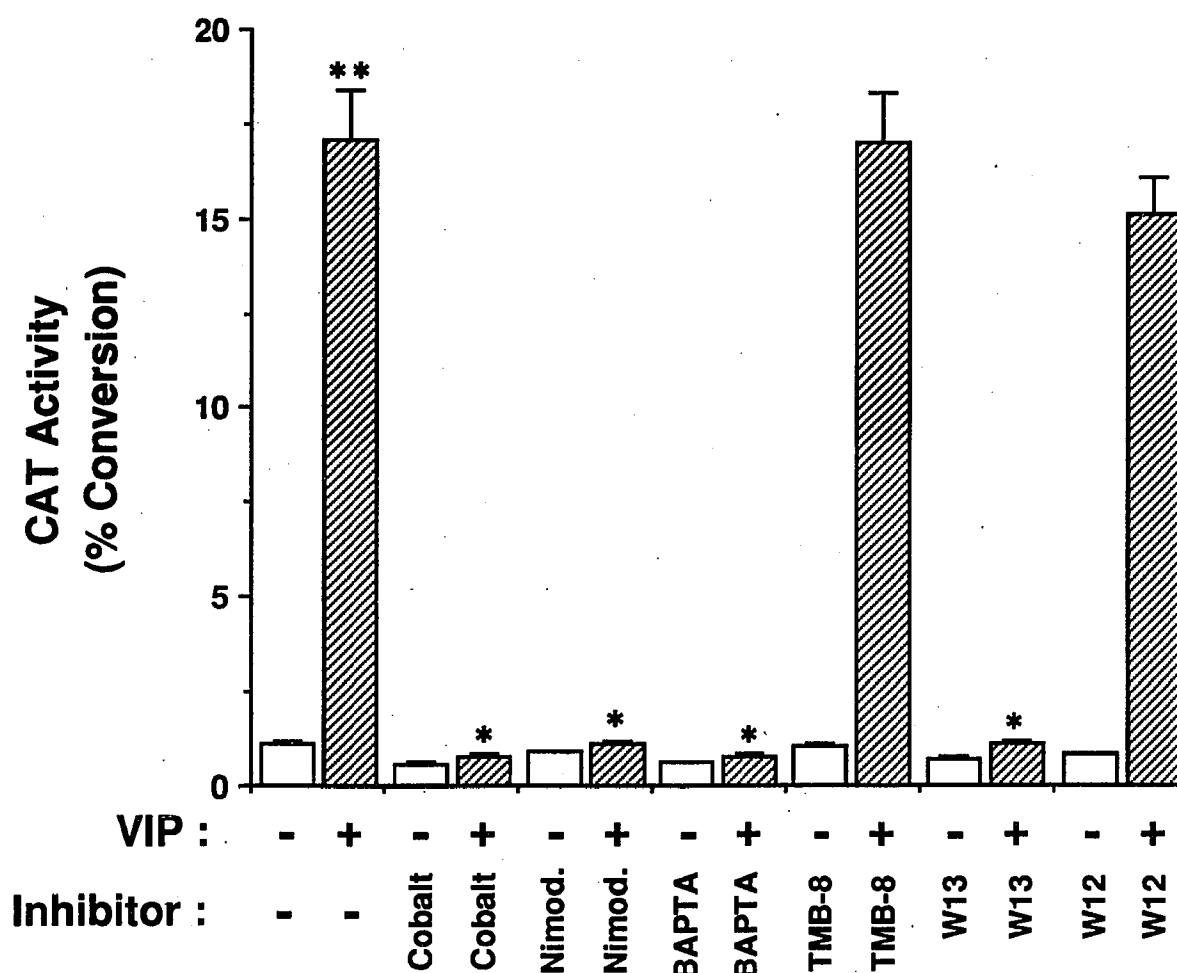


Fig. 5-8. Effect of cellular Ca^{2+} inhibitors on induction by VIP of transient expression of a PRL-CAT construct. GH₃ cells were transfected with plasmid (-187)PRL-CAT, then incubated with or without VIP as in Fig. 5-1. Where indicated, at 3 hours before the addition to some cultures of VIP, some cultures received either CoCl_2 (Cobalt, 1mM), nimodipine (Nimod., 200nM), TMB-8 (10 μ M), W12 (10 μ M), or W13 (10 μ M). After additional incubation with these additions, cells were assayed for CAT activity. Shown is the mean $\pm$ SE of results with triplicate dishes. *, $P < 0.01$ vs. VIP without inhibitor; **, $P < 0.01$ vs. control group (without VIP and/or inhibitor).

Analysis of further 5'-deleted PRL-CAT constructs showed that the regulatory DNA sequences required for a full response of the PRL gene to VIP are contained within the first 174 base pairs upstream of DNA flanking sequence, and that sequences between -174/-113 are required for a strong response (**Fig. 5-2B**). The VIP regulatory region identified here contains both binding sites for pit-1 and a CLE element. The most proximal pit-1 binding site at -63 to -39 has been shown in Chapters 1 and 2 to direct the response of the PRL gene to TRH/Ca²⁺, while the CLE is required for basal expression and cAMP induction of the PRL gene (Peer et al., 1991; d'Emden et al., 1992; Liang et al., 1992). Analysis of the ability of two copies of each of pit-1 binding sites, 1P, 2P, 3P and 4P to confer a VIP response to a minimal PRL promoter [(-39)PRL-CAT] showed that sites 1P or 2P, but not 3P or 4P can serve as weak VIP response elements (**Fig. 5-3**). Since VIP can strongly stimulate the first 174 bp of the PRL promoter, containing sites 1P, 2P, and 3P, these pit-1 binding sites individually do not seem to be sufficient for the VIP action.

In Chapters 1 and 2, I have shown that pit-1 is a gene-proximal protein that transduce a TRH signal to site 1P. To determine if pit-1 plays a similar role in the VIP response, I examined whether expression of (Δ 2-45)pit-1, containing a deletion in the pit-1 *transactivation* domain (Ingraham et al., 1990), could block VIP induction of (-187)PRL-CAT expression. Expression of (Δ 2-45)pit-1 was observed to inhibit the induction of (-187)PRL-CAT expression by TRH, but not by VIP (**Fig. 5-4**), implying that VIP action on the PRL proximal promoter is not mediated by pit-1. It is also notable that coexpression with (-187)PRL-CAT of a large amounts of RSV-pit-1 (>2 ug) yielded decreases in TRH and VIP induction of CAT activity (**Fig. 5-4**). The reason for this decrease is not clear, but is probably due to a quenching effect of pit-1.

Given the recently documented binding capacity of CREB to the PRL CLE (Liang et al., 1992; Yan, Tian and Bancroft, in preparation), we investigated the effect of CREB on VIP induction of (-187)PRL-CAT by co-expressing CREB or its non-DNA-binding mutant K-CREB (Fig. 5-5). The observation that neither CREB nor its mutant can repress the stimulation of expression of (-187)PRL-CAT by VIP suggests that VIP stimulation of PRL gene expression requires neither CREB binding to the PRL CLE nor CREB to transduce the VIP signal to the PRL proximal promoter.

The gene-distal mediators in the pathway of VIP action on the PRL gene expression are yet to be identified. As a first step, we examined the possibility that cAMP lies on the pathway of VIP action on the PRL gene expression. Expression of a cAMP-dependent protein kinase (PKA) antagonist, which is a mutant regulatory subunit of PKA (RAB) (Leslayann and McKnight, 1991), resulted in a large decrease in cAMP induction of CAT activity (Fig. 5-6). However, expression of this mutant resulted in only a slight effect on VIP stimulation of CAT activity (Fig. 5-6). This observation suggests that PKA does not play a major role in stimulation of PRL gene expression by VIP. It is interesting that VIP is known to increase cAMP accumulation (Gourdji et al., 1979; Rotsztejn et al., 1981) and stimulate adenylate cyclase activity (Pizzi et al., 1990) in GH₃, and this response has been implicated in the regulation by VIP of PRL release from these cells (Pizzi et al., 1990). Thus, the apparent absence of PKA from the pathway of action of VIP on PRL gene expression implies a divergence of the actions of VIP on PRL release and gene activity before the level of PKA activity.

We previously have provided evidence that Ca²⁺ and TRH regulation of PRL in GH₃ cells is unaffected when protein kinase C activity is down-regulated by chronic exposure to TPA (Bandyopadhyay et al., 1989; Chapter 2).

The observation in the present studies that regulation by VIP of expression of (-187)PRL-CAT is also unaffected when protein kinase C activity is down-regulated either by chronic exposure to TPA (Fig. 5-7A) or by the protein kinase inhibitor H7 (Fig. 5-7B) implies that VIP regulation of PRL is also independent of kinase C activity. These results suggest that PKC also may not lie on the pathway of VIP action on PRL gene expression.

Ca^{2+} is also a significant candidate to be a mediator of VIP action, since VIP stimulates the accumulation of cAMP and adenylate cyclase activity in Ca^{2+} -dependent manner (Brostrom et al., 1982; Etgen et al., 1987), although Ca^{2+} did not affect the ability of VIP to stimulate PRL release from rat pituitary cells (Pizzi et al., 1990). Additivity and pharmacological studies have indicated that Ca^{2+} mediates TRH action on PRL gene expression (White et al., 1983; Laverriere et al., 1989; Day et al., 1990; Chapter 2). Results with cellular Ca^{2+} inhibitors (Fig. 5-8) supported a role for Ca^{2+} in VIP action on the PRL promoter, since the two Ca^{2+} flux inhibitors cobalt ion and nimodipine and the Ca^{2+} chelator BAPTA-AM were each able to block stimulation by VIP of expression of (-187)PRL-CAT. The observation that the cellular Ca^{2+} immobilizer, TMB-8, did not block stimulation of (-187)PRL-CAT expression by VIP, suggests that the VIP/ Ca^{2+} transcriptional signaling pathway may not be sensitive to Ca^{2+} immobilization in intracellular stores.

Previous pharmacological studies have suggested that the Ca^{2+} -activated enzyme calmodulin is involved in regulation of PRL gene activity (White, 1985; Davis et al., 1991). It has been recently reported that constitutive expression in GC cells of type II Ca^{2+} /calmodulin-dependent protein kinase yields a weak (2.7-fold) stimulation of expression of the PRL promoter (Kapiloff et al., 1991). Our findings (Fig. 5-8) that the calmodulin inhibitor W13 can repress the VIP stimulatory effects, but not W12, which is relatively

inactive against calmodulin, suggests that the VIP response of the PRL promoter may be mediated by a Ca^{2+} /calmodulin-dependent protein kinase.

SUMMARY AND PERSPECTIVES

Expression of the PRL gene is subject to both positive and negative regulation by hormones in the pituitary cells. To further our understanding of the mechanisms involved in positive regulation, I first focused my investigation on TRH, which is one of the major positive regulators of expression of the PRL gene. In Chapter 1, I identified two TRH response elements (TRHREs) in the PRL proximal promoter which are also pit-1 binding sites. This finding is in agreement with previous findings that the TRH response of the PRL proximal promoter is unchanged by any of 19 clustered point mutation covering positions -193/-30 of the PRL promoter (Iverson et al., 1990). Either mutation of the strongest TRHRE, site 1P (Chapter 1), or coexpression of a mutant pit-1 possessing reduced trans-activational activity (Chapter 2), strongly inhibited TRH regulation of PRL promoter constructs, implying that the cell-specific activator pit-1 is a gene-proximal mediator of TRH regulation of the PRL gene. Ca^{2+} is another positive regulator of PRL gene expression, and 5'-deletion analysis of the PRL promoter and mutation of site 1P studies showed that site 1P is also a Ca^{2+} response element (CaRE), and that pit-1 represents the gene-proximal protein in transducing Ca^{2+} signal to the PRL promoter (Chapter 2). In addition, pharmacological experiments implied that Ca^{2+} is a mediator of TRH stimulation of the PRL promoter (Chapter 2).

Although the TRH-induced stimulation of PRL transcription persists for at least 24 hours (Murdoch et al., 1983), experiments using nuclear extracts of GH₃ cells incubated with TRH for 24 hours failed to detect any difference in the binding of pit-1 to site 1P or any gross TRH-induced covalent modifications of pit-1 (Chapter 1). However, acute exposure of GH₃ cells to

TRH increases pit-1 binding to site 1P (Chapter 1). This result is correlated to the acute effect of TRH on prolactin transcription by GH₃ cells (Murdoch et al., 1983). Recently it was found that pit-1 can be phosphorylated by acute TRH stimulation, with phosphorylation reaching a peak in about 5 min and rapidly returning to near basal levels after 30 minutes (Dr. R. Maurer personal communication). Moreover, I have found that pit-1 mutated at its three major phosphorylation sites failed to inhibit TRH induction of the PRL promoter in GH₃ cells (data not shown). These findings imply that TRH-induced pit-1 phosphorylation is neither sufficient nor necessary for induction of the PRL gene expression. Other TRH-induced modifications of pit-1, such as glycosylation, and any TRH-induced changes of pit-1 binding by employing *in vivo* footprint technique (Mueller and Wold, 1989), should now be employed to investigate TRH-induced modification of pit-1.

Recently, an alternatively spliced pit-1 isoform, termed pit-1a, was characterized which differs from pit-1 by having 26 amino acids inserted into transactivation domain (Morris et al., 1992; Kozak and Moore, 1992; Theill et al., 1992). Pit-1a retains the DNA binding activity of pit-1 and can activate the GH promoter but has lost the ability to activate the PRL and pit-1 promoters (Kozak and Moore, 1992; Theill et al., 1992). since as shown in Chapter 1, TRH/Ca²⁺ action on the PRL promoter is mediated by pit-1, it would be interesting to test whether TRH/Ca²⁺ affects pit-1 and/or pit-1a mRNA levels in pituitary GH₃ cells resulting in stimulation of the PRL gene expression, and whether coexpression of pit-1a will affect the TRH/Ca²⁺ induction of the PRL promoter. It has been shown that pit-1 can form a DNA-dependent homodimer (Voss et al., 1991), and that pit-1 and Oct-1 can form a heterodimer that cooperatively activates the PRL gene (Ingraham et al., 1990). The possibility that interaction of pit-1 and pit-1a, or pit-1 and Oct-1, or other

factors may contribute to hormonal regulation of PRL gene expression remains to be determined.

To further understand whether the signal pathway of TRH induction of the PRL promoter involving pit-1 as a gene-proximal mediator is employed by other peptide hormones for regulation of PRL gene expression, we examined another positive regulator, VIP. In Chapter 5, 5'-deletion analysis showed that the first 174 base pairs of DNA upstream of the PRL promoter transcription start site, containing three pit-1 binding sites and the PRL CLE, is required for induction of the PRL promoter by VIP. However, it is unlikely that either pit-1 binding site 1P, or 2P, or 3P, or 4P alone is a strong VIP response element (VIPRE). Furthermore, studies with a pit-1 mutant indicate that VIP action on the PRL promoter is not mediated by pit-1. These observations imply that there are different mechanisms of regulation of the PRL gene expression by peptide hormones. Further experiments, involving site-mutagenesis and clustered point mutation, are needed to identify the PRL promoter VIPRE(s), and to determine whether cooperative interaction of these pit-1 binding sites and the PRL CLE as well as the distance between these pit-1 binding sites and/or the PRL CLE is important for VIP action on the PRL promoter. In addition, it may be possible to identify specific proteins in GH₃ cells required for VIP action on the PRL promoter by *in vitro* or *in vivo* footprinting.

Common gene-distal candidates for signal transduction pathways leading to gene expression include protein kinase A (PKA), protein kinase C (PKC), Ca²⁺, and cAMP. Previous studies have shown that Ca²⁺ is probably involved in transcriptional regulation of the PRL promoter by both positive and negative regulators. An important role for Ca²⁺ in the action of TRH (Chapter 2) and VIP (Chapter 5) on the PRL promoter was implied by the

results of pharmacological studies. Down-regulation of PKC by chronic TPA treatment did not inhibit induction of the PRL promoter by either TRH or Ca^{2+} or VIP (Chapter 2 and 5), suggesting that regulation of PRL gene expression by these agents is independent of kinase C activity. More interestingly, VIP apparently stimulates PRL secretion by increasing cAMP accumulation, but inhibition of cAMP-dependent PKA using a regulatory subunit mutant (RAB) did not block the VIP induction of PRL gene expression (Chapter 5), suggesting that PKA may not play a major role in this action of VIP. However, a calmodulin inhibitor could block VIP induction of PRL-CAT (Chapter 5), implying that VIP action on the PRL promoter may be mediated by Ca^{2+} /calmodulin-dependent kinase. To investigate this further, one could examine whether VIP affects Ca^{2+} /calmodulin-dependent kinase activity in GH₃ cells, and the targets for Ca^{2+} /calmodulin-dependent kinase in VIP action on the PRL gene.

Recent studies by a number of groups have revealed the importance of an additional PRL proximal promoter sequence, the CRE-like element (CLE), which is required for basal expression and cAMP induction of the PRL promoter. The PRL CLE resembles the classical CRE found in the promoters of a number of cAMP-responsive genes. The action of cAMP on the promoters containing the classical CRE is mediated by the transcription factor CREB. Recently Maurer's group and our group have found that CREB binds to the PRL CLE with a low affinity. In Chapter 3, I showed that CREB or its phosphoacceptor site Ser-133 mutant CREB-M1 can act as a specific repressor of PRL promoter activity in pituitary GH₃ cells, but that the DNA binding site mutant K-CREB cannot. Further, this repression can be reversed by PKA phosphorylation of CREB, but not of CREB-M1. These findings suggest that

the inhibition by CREB of the PRL promoter might arise by competition with a positive transcription factor for binding to the PRL CLE.

I further tested whether CREB can repress the pit-1 activation of PRL-CAT in non-pituitary cells by employing co-transfection of pit-1 and CREB or its mutants, with (-187)PRL-CAT in C6 cells. As shown in Chapter 4, the inhibition of pit-1 activation of PRL-CAT by CREB and the relief of this inhibition by phosphorylation of CREB by PKA in C6 cells mimic the repression of PRL-CAT by CREB and the relief of this repression by PKA phosphorylation of CREB in GH₃ cells (Chapter 3). These findings suggest that CREB may interfere with a factor which is required for pit-1 activation of PRL-CAT by competing for binding to the PRL CLE.

The pituitary-specific transcription factor pit-1 is clearly important for cell-specific expression of the PRL gene. However, there is evidence that full activation of the PRL gene requires additional factors, such as the estrogen receptor which binds to the estrogen response element (ERE) in the PRL distal enhancer (Maurer and Notides, 1987; Kim et al., 1988; Day et al., 1990). A recent report has shown that the PRL gene is expressed in rat brain, including the hypothalamus and extrahypothalamic tissue, without detectable expression of pit-1 (Emanuele et al., 1992). This finding indicates that PRL gene expression can occur independently of pit-1. The results presented in Chapter 4 may therefore be significant in light of this observation. The major complex detected with the PRL CLE as a probe in pituitary (GH₃) cells (Liang et al., 1992) and in non-pituitary (C6) cell (Yan, Tian, and Bancroft, In preparation) nuclear extracts does not contain CREB. Moreover, the observation that either PKA or a constitutively active CREB (CREB-VP16) are capable of *transactivating* the PRL promoter through the PRL CLE site in C6 cells which lack pit-1 raises the possibility that a factor similar to CREB may

bind to the CLE and thus activate the PRL promoter in the absence of pit-1. In the future, it may be possible to further establish that expression of the PRL gene can occur independently from pit-1 by studying *in vitro* transcription of PRL-CAT constructs using nuclear extracts from brain, and/or by identifying the cellular factor that binds to the PRL CLE.

MATERIALS AND METHODS

Construction of Promoter-CAT Constructs and Mutant Pit-1 Constructs

Preparation of (-204)PRL-CAT and its 5'-deletion mutants were by Thomas Lufkin (Lufkin and Bancroft, 1987). These constructions are designated by the 5'-terminus of the PRL gene promoter insert.

Plasmid (-39)mMT-CAT was constructed as follows. Prepared oligodeoxynucleotides corresponding to the coding and noncoding strands from position -39 to +25 of mouse metallothionein (mMT) promoter (Stuart et al, 1984), plus, respectively SalI and HindIII 5' extensions, were directionally cloned into a PUC19 based vector containing the CAT gene isolated from plasmid (-39)PRL-CAT. (-39)PRL-CAT(enh), (1P)²(-39) PRL-CAT(enh), (*1P)²(-39)PRL-CAT(enh), (1P*)²(-39)PRL-CAT(enh), and (*1P*)²(-39)PRL-CAT(enh) were constructed by excising the NdeI-EcoRI fragment (~500bp), containing the RSV enhancer, from plasmid RSV-CAT, blunt-ending the gel-purified fragment, and inserting it into the blunt-ended BamHI site of, respectively, (-39)PRL-CAT, or (1P)²(-39)PRL-CAT, or (*1P)²(-39)PRL-CAT, or (1P*)²(-39)PRL-CAT, or (*1P*)²(-39)PRL-CAT, which were constructed as described below.

The coding and non-coding oligodeoxynucleotides of 1P-4P shown in Fig.5B were synthesized with the following additional sequences. At the 5' end, the 1P mutant series all have a XhoI site, and all other have a SalI site. At the 3' end, 1P and its mutants, and 4P, contain a XhoI site; 2P, a SalI site; and 3P, a ClaI site. Single or multiple copies of each double-stranded oligodeoxynucleotide were then cloned into the SalI site of either

(-39)PRL-CAT, (-39)PRL-CAT(enh), or (-39)mMT-CAT. Dideoxy sequencing (Sanger et al., 1977) was employed both to determine the orientation and copy number of the inserted oligodeoxynucleotides, and to confirm the structures of all plasmids constructed for these studies.

Construction of plasmid $\Delta(2-45)$ pit-1 essentially involved removal of the HindIII-BsmI pit-1 cDNA fragment from plasmid pRSV-Pit-1/GHF-1 (Fox et al., 1990), yielding a construct that employed the methionine at position 46 for initiation of translation. To do this, the 3'-extension of a BsmI fragment removed from pRSV-pit-1/GHF-1 was digested with Mung bean nuclease, followed by digestion with XhoI. This fragment was then directionally cloned into pRSV-Pit-1/GHF-1 that had previously been digested with HindIII, filled in with Klenow fragment, and then digested with XhoI.

Plasmid RSV-KCREB was provided by Dr. Richard H. Goodman (Walton et al., 1992), plasmid RSV-CREB and RSV-MICREB were provided by Dr. Marc R. Montminy (Gonzalez and Montminy, 1987), and plasmid MtC (expressing the α isoform of the mouse PKA catalytic subunit), HL-REV_{WT}neo. (expressing the mouse PKA regulatory subunit), and HL-REV_{AB}neo. (expressing the mutant PAK regulatory subunit) were provided by Dr. Stanley G. McKnight (Uhler and McKnight, 1991; Schecterson and McKnight, 1991), and plasmids CREB-VP16 and ATF-2-CREB were provided by Dr. Michael R. Green (Liu and Green, 1990).

Transient and Induction of Eukaryotic Cells.

GH3 cells growing in suspension culture were seeded onto poly-L-lysine-treated tissue culture dishes (7×10^5 cells/35mm dish or 1.5×10^6 cells/60mm dish) in Dulbecco's Modified Eagle's Medium (DMEM) +5% Fetal

Calf Serum (FCS). One day later, the following modification of a DEAE-dextran protocol (Lopata et al 1984) was employed to transfect GH3 cells with promoter-CAT constructs. After a rinse with PBS, each dish received 0.5 ml or 1 ml transfection medium containing plasmid DNA (5-10 ug) plus DEAE-dextran (200ug/ml) in serum-free DMEM. The dishes were then incubated for 30 min at 4°C. After rinsing the dishes once with PBS, the cells were incubated in DMEM + 5% FCS overnight. For TRH or VIP induction, following preincubation for 2 days (with a medium change after one day) in SFM/Ca²⁺ medium containing Joklik's MEM plus a serum substitute (Yan et al., 1991), plus 0.5 mM Ca²⁺, the cells were further incubated in SFM/Ca²⁺ ± 100 nM TRH or 200 nM VIP for one day, then harvested for assay of CAT activity or preparation of nuclear extracts. For Ca²⁺ induction, following preincubation for 2 days (with a medium change after one day) in SFM medium without Ca²⁺, the cells were further incubated in SFM ± 0.5 mM Ca²⁺ for two day, then harvested for assay of CAT activity.

C6 cells growing in monolayer cultures were transfected by the standard calcium phosphate technique (Ausubel et al., 1989). Cells were plated at a density of 2×10^6 cells/60mm dish. DMEM medium with 10% fetal calf serum was changed 2 hours prior to transfection. DNA-calcium solution was slowly dripped into an equal volume of bubbling 2x HBS. Samples were incubated at room temperature for 20 minutes. Calcium-phosphate DNA solution was added directly to medium, and incubated at 37°C for 6 hours. The medium-DNA solution was then aspirated and 20% glycerol in PBS was added for 2 minutes, then cell monolayers were washed twice with PBS. Cells continued to be incubated at 37°C for a total of 48 hours in medium with 10% FCS with daily medium changes. 15-20 ug/60mm dish of the indicated plasmid DNA's was used for transfection. When less than the indicated

amount of specific DNA was used per 60mm dish, PUC19 plasmid DNA was added to give a constant total amount of DNA. Triplicate were used in each experiment.

All plasmid DNA used in transfection experiments was prepared from bacterial culture grown in TB broth plus ampicillin, using a modified Birnboim protocol involving additional NH_4OAc treatment, polyethylene glycol precipitation, RNase treatment, phenol/chloroform extraction 2x, ether extraction, and ethanol precipitation (Birnboim et al., 1979). Greater than 80% of DNA was supercoiled which analyzed by agarose gel electrophoresis.

CAT enzymatic assays

Cells were harvested 48-72 hr after transfection and assayed for CAT activity as previously described (Gorman et al, 1982), with modifications as described below. Medium was aspirated from the cells and monolayers were washed once with PBS. Cells were scraped in 1 ml 1x PBS and transferred to a microfuge tube. Cells were disrupted by sonication, two cycles of 4 minutes, on ice, and pelleted by 30 seconds centrifugation in a microfuge. Supernatants were aspirated off and the pellet was resuspended in 100 μl CAT Buffer (0.25 mM Tris-HCl (pH 7.8) and 10 mM EDTA). Extracts were heated at 65°C for 5 min to inactivate an endogenous deacetylase. Cellular debris were pelleted by centrifugation and supernatants were transferred to fresh microfuge tubes. For CAT assay, 5 μl of [^{14}C]-chloramphenicol diluted 1:10 in 95% ethanol was dried down in an eppendorf tube. 5 μl 50mM acetyl CoA was added to each tube, followed by 50 μl of cell extracts, and incubated at 37°C for 4-12 hours. The reaction was stopped by extracting with 200 μl ethyl acetate. The supernatant (ethyl acetate) was transferred to another eppendorf tube and

dried in a Speed-vac. The pellet was suspended in 20 ml ethyl acetate with cold acetylated chloramphenicol markers (diluted 1:500) and spotted on to a silica gel TLC plate. The TLC plate was developed in 95:5 chloroform: methanol until the solvent front reached the top. Acetylated and unacetylated chloramphenicol spots were visualized by UV illumination, the chloramphenicol spots were cut out separately and counted in a scintillation counter. Percent conversion was cpm of acetylated divided by total cpm (cpm of acetylated plus cpm of unacetylated). Each assay represents the percent conversion of ^{14}C -labeled chloramphenicol by one half of the contents of a dish.

Nuclear Extract Preparation

GH3 cells growing in suspension culture were seeded onto poly-L-lysine-treated tissue culture dishes (3×10^6 cells/100mm dish), other cell lines growing in monolayer culture were plated in tissue culture dishes (4×10^6 cells/100mm dish). Nuclear extracts were prepared according to the small scale procedure described below (Schreiber, personal communication). For hormonally stimulated-GH₃ cell nuclear extracts, cells are incubated in SFM/ Ca^{2+} for two days, then treated with hormone for vary times. Cells on monolayer were washed once with PBS and scraped in 2ml PBS. Cells in suspension were harvested by centrifugation (1000x g) at 4°C. After pelleting the cells, they were washed once with ice-cold PBS and repelleted by centrifugation (1000x g) at 4°C. Washed cells were incubated 15 min on ice in buffer A (10mM HEPES, pH 7.8, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA) with freshly added DTT (1mM) and PMSF (50 ug/ml). After addition of NP-40 to 0.6% and vortexing 10 seconds, nuclei were pelleted and then extracted in

Buffer C (20 mM HEPES, PH 7.9, 25% glycerol, 0.4M NaCl, 1mM EDTA and 1mM EGTA) with freshly added DTT (1mM) and PMSF (50ug/ml), and with vigorously rocking on an Eppendof t shaker for 30 min at 4°C. Samples were then spun 5 minutes in a microfuge at 4°C. Supernatants were aliquoted into eppendof t tubes, and frozen, and stored at -70°C. Protein concentration was determined by method of Lowry et al.

Western Blot Analysis

SDS gel electrophoresis separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. After solubilizing nuclear extracts by boiling in presence of SDS sample buffer containing DTT, an aliquot of the protein solution is applied to a 10% SDS-PAGE gel lane, and the individual proteins or their subunit are separated electrophoretically at 25mA, constant current.

A specific antibody directed against Pit-1/GHF-1 was produced at Pocono Rabbit Farms by immunizing rabbits with a synthetic peptide containing amino acid 136-150 of the POU-specific domain of Pit-1. CREB Antiserum made in rabbit against the P-box region was provided by Dr. Joel Habener and Heather Hermann. 50-100ug of nuclear proteins from cultured cells were separated on 0.1% SDS-10% PAGE gels and then transferred to nitrocellulose by semi-dry electroblotting. Non-specific protein-binding sites were blocked by soaking the blot in 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blot was then incubated 2 hours at 25°C or overnight at 4°C with an affinity-purified antiserum against Pit-1 at 1:1000 dilution or CREB antiserum at 1:5000. After washes in TBST, using the Amersham ECL kit, the blots were incubated for 1

hr at 25°C with secondary Ab-HRP (Rabbit Ig, horseradish peroxidase-linked whole antibody) 1:1000 diluted in TBST buffer. Following washing and air drying of blotted membrane strips on filter paper, the strips were placed in equal volumes of detection reagent 1 and detection reagent 2 for 1 minute, air-dried for 20-30 minutes, then exposed to the X-ray film for 10 seconds to 1 minute.

Analysis by DNA Mobility Shift of Oligodeoxynucleotide Binding to Nuclear Extracts

Oligodeoxynucleotides were prepared by the Mt. Sinai DNA Core Facility. Following synthesis, oligodeoxynucleotides were purified on an 8 M urea, 12.5% polyacrylamide gel. Complementary strands were annealed in 40 mM Tris, PH 7.5, 20 mM MgCl₂, 50 mM NaCl by heating at 65°C for 15 min and then cooling for 15 min each at 37, 25, and 4°C. Double-stranded probes were end-filled with [³²P]dCTP plus the other three cold dNTPs, employing Sequenase (United States Biochemical). Free deoxynucleotides were removed by centrifugation through a small Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) spin column. Final concentration of labeled oligodeoxynucleotides was 1 ng/ml.

DNA-protein binding analysis was performed as follows. Each 25 ml reaction contained 10 mM Tris, PH 8.0, 5% glycerol, 50 mM KCl, 1 mM each EDTA and dithiothreitol, 4 mg of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.), 4 mg of extracted nuclear proteins. Following addition of any other competitor employed, and incubation for 20 min at room temperature, 1 ng of labeled probe (5-10 × 10⁴ cpm) was added and incubation continued for an additional 10 min at room temperature. Each sample then received 5 ml of 6 ×

agarose gel loading buffer (Maniatis et al., 1982) and was applied to a pre-run 1.5-mm 0.25x TBE (1x TBE is 89 mM Tris, 89 mM borate, 2.5 mM EDTA), 10% polyacrylamide gel and run at 30 V/cm at 4°C. The gel was dried, then wrapped in Saran-Wrap and exposed to X-ray film with intensifying screen at -70°C.

Statistical Analysis

Statistical significance was determined for data obtained from six samples (three experimental samples were directly compared with three control samples), using the SAS proc ttest [SAS (r) Proprietary Software Release 6.04; SAS Institute Inc., Cary, NC 27512-8000, U.S.A.]. A statistically significant difference was taken to correspond to $P < 0.01$.

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