REGULATION OF PITUITARY-SPECIFIC GENE EXPRESSION BY CONSTITUTIVELY ACTIVE G PROTEINS

by

JUN TIAN

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

1995

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

3/21/95
Date

F. Carter Bancroft
Dr. Carter Bancroft
Chair of Examining Committee

3/21/95
Date

Dr. Terry Krulwich
Executive Officer

Dr. James Bieker

Dr. Ravi Iyengar

Dr. William Thornhill

Dr. Gregory Gick

Supervisory Committee

Abstract

REGULATION OF PITUITARY-SPECIFIC GENE EXPRESSION BY CONSTITUTIVELY ACTIVE G PROTEINS

by

JUN TIAN

Adviser: Profssor Carter bancroft

The G proteins are a family of important intracellular signal transducers that play an essential role in linking diverse cell surface receptors to different intracellular signaling systems. Although the functions of G proteins in regulating many cellular functions has been intensely studied, virtually nothing is known about the role of G proteins in gene transcriptional regulation.

Transfection experiments with rat pituitary GH3 cells were employed to show that expression of a constitutively active mutant of an αs subunit (αs*) specifically stimulates expression of a co-transfected prolactin (PRL) gene promoter. Two copies of site 1P (Pit-1 binding site in prolactin promoter) direct a response to αs* stimulation, implying that site 1P can serve as an independent cis response element for αs* and that Pit-1 may be the transcription factor that mediates the αs* action on the PRL promoter. Further studies of the intracellular signaling pathways through which αs stimulated PRL gene expression implicated protein kinase A (PKA), since co-expression of a dominant negative inhibitor of PKA blocked the αs* stimulation of the PRL promoter activity. Similarly, a dominant negative inhibitor of CREB, k-CREB, also inhibited αs* induction of the PRL promoter. In addition, CREB bound with low affinity to the CLE, a CRE-like element in the PRL proximal promoter which was shown

previously to be required for basal expression and cAMP induction of the PRL promoter, implying that PKA action on the PRL promoter may be mediated by CREB.

Gq is generally thought to couple to TRH receptor whose activation stimulates PRL gene expression. To investigate the function of Gq in regulating PRL gene expression, a constitutively active mutant of an αq subunit (αq*) was used as described above. G-αq* specifically stimulated the expression of a co-transfected prolactin or growth hormone gene promoter. Site 1P was observed to function as a heterlogous αq response element. G-αq stimulation of PRL promoter activity was strongly inhibited by co-expression of a dominant negative Raf mutant, Raf-C4, indicating that Raf may be involved in mediating the stimulation by αq of the PRL gene.

To expand our understanding of the G-αs regulation of gene expression, the action of constitutively active αs on Pit-1 gene promoter was investigated. G-αs* stimulated Pit-1 gene promoter activity. Mutation of the Pit-1 binding site in the Pit-1 gene promoter reduced the stimulation. Additionally, both the dominant negative PKA and CREB inhibitors described above inhibited αs* stimulation of the Pit-1 gene promoter activity. These data suggest that the activated αs subunit may employ PKA, CREB, and the Pit-1 binding site to stimulate Pit-1 gene promoter activity.

In addition to G protein α subunits, the βγ dimer complex was recently recognized to be able to regulate cellular functions in higher eukaryotes. I found that γ2, but not β2 or β2 + γ2, regulated the prolactin gene promoter activity. This study represents both the first study of transcriptional action of βγ in higher organisms, and the first indication of a independent function of the G protein γ subunit. In addition, a non-prenylated γ2 mutant that was incapable of

membrane targeting did not stimulate PRL promoter activity, indicating that the prenylated site of γ2 is necessary for its transcriptional action.

Acknowledgements

I would like to thank my mentor, Dr. Carter Bancroft, for his support and encouragement throughout my graduate studies. I have learned from him what it means to be a good scientist, my training in his laboratory has given me a solid groundwork for future successes.

It has been my pleasure to work with talented members, past and present, of the Bancroft laboratory. Many thanks to them for their support thoughout my study at the Mount Sinai School of Medicine.

My special thanks to my wife, Beidong Su, for her support, assistant, and personal sacrifices that made all thing possible.

Table of contents

List of figures

Table 1

Sources of Recombinant Plamids Constructed by Other Investigators

Name of contruct	Source
CMV-αs	Dr. Ravi Iyengar
CMV-Q227L-αs	Dr. Ravi Iyengar
(-1957)PRL-CAT	Dr. Carter Bancroft
(-187)PRL-CAT	Dr. Carter Bancroft
(-224)GH-CAT	Dr. Carter Bancroft
(-39)mMT-CAT	Dr. Carter Bancroft
$(1P)^2$(-39)mMT-CAT	Dr. Carter Bancroft
RSV-CREB	Dr. Richard Goodman
HL-REV_{WT}neo (R_{WT})	Dr. Stanley McKnight
HL-REV_{ab}neo (R_{ab})	Dr. Stanley McKnight
CMV-αq	Dr. Ravi Iyengar
CMV-Q209L-αq	Dr. Ravi Iyengar
RSV-C4B	Dr. Ulf Rapp
RSV-C4m17B	Dr. Ulf Rapp
RSV-BXB	Dr. Ulf Rapp
Δ5'-200GHF1-CAT	Dr. Michael Karin
Δ5'-200.1GHF1-CAT	Dr. Michael Karin
CMV-γ2	Dr. Ravi Iyengar
CMV-β2	Dr. Ravi Iyengar
pCDM-γ2m	Dr. William Simonds
CMV-Q205L-αo	Dr. Ravi Iyengar

Chapter 1

Introduction

A. Prolactin and Prolactin Gene Regulation

1. Introduction

Prolactin is a polypeptide hormone produced by the lactotrophic cells of the anterior pituitary. It is involved in a broad spectrum of functions including metabolism, growth and reproduction. Prolactin gene expression is developmentally, cell-specifically, and hormonally regulated. Most of the known signal transduction pathways (PKA, PKC, and intracellular Ca^{2+}) are involved in hormonal regulation of prolactin gene expression (Maurer 1981; Laverriere et al. 1988; Day et al. 1989; Yan et al. 1991; Yan et al. 1991). Therefore, prolactin gene expression has provided an excellent model with which to define the molecular mechanisms of transcriptional regulation by different hormones. Our present knowledge of developmental and homeostatic regulation of the prolactin gene is summarized below.

2. Developmental regulation of prolactin gene expression

The developing anterior pituitary gland provides an excellent model system to study cell specific gene expression and the processes by which distinct cell types develop within an organ. The mammalian anterior pituitary originates from Rathke's Pouch during the early stages of embryonic development (Sharp et al. 1990). The primordial anterior pituitary stem cell differentiates into five phenotypically distinct cell types defined by the trophic factors that they synthesize and secrete Corticotrophs produce adrenocorticotrophin (ACTH) by proteolytic processing of proopiomelanocortin (POMC); thyrotrophs synthesize thyroid-stimulating hormone (TSH); gonadotrophs produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH); somatotrophs produce growth hormone (GH); and lactotrophs

synthesize prolactin (Frohman 1981). Lactotrophs and somatotrophs arise from a common stem cell (acidophils) which at a later stage gives rise to presomatotrophs able to express both the growth hormone and prolactin genes (Karin et al. 1990; Simmons et al. 1990). It is within these presomatotrophic cells that critical events in cell-specific determination occurs, causing some cells to differentiate into somatotrophs expressing only the growth hormone gene while others differentiate into lactotrophs expressing only the prolactin gene.

The molecular events that determine specific expression of prolactin gene in lactotrophs are poorly understood. A large amount of evidence shows that the transcription factor Pit-1 is responsible for turning on both growth hormone and prolactin gene in presomatotrophic cells (Crenshaw III et al. 1989; Simmons, Voss et al. 1990). The temporal pattern of Pit-1 protein is correlated with the activation of both prolactin and growth hormone gene expression. Also quite interestingly, Pit-1 mRNA is made by all five types of anterior pituitary cells, but only in thyrotrophs, somatotrophs, and lactotrophs is the Pit-1 message translated into the functional protein (Simmons, Voss et al. 1990). Natural mutations in the Pit-1 gene or antisense blockade of Pit-1 expression in stem cells leads to a dramatic decrease in prolactin and growth hormone gene expression as well as a severe deficiency of lactotrophic and somatotrophic cells, indicating that Pit-1 not only mediates transcription of the differentiated products of these cells, but also is necessary for the formation of the anterior pituitary cells in Rathke's pouch (Li et al. 1990; Castrillo et al. 1991; Tatsumi et al. 1992). The growth hormone gene appears to respond directly to the presence of Pit-1 while the prolactin gene is maximally stimulated by Pit-1 in the presence of estrogen (Simmons, Voss et al. 1990). What is still not known is the mechanism(s) that cause lactotrophs and somatotrophs to specifically express, respectively, the prolactin gene and the growth hormone gene (see below).

3. Cell-type-specific expression of the prolactin gene

As a consequence of cell differentiation, the prolactin gene is specifically expressed in lactotrophs of the anterior pituitary. A critical question is then raised about mechanisms that are involved in regulating this cell-type-specific expression of the prolactin gene. Although the answer to this question is still unclear, impressive progress has recently been made toward a fundamental understanding of the process involved.

3a. Cis-acting regulatory elements responsible for cell type specific expression of the rat prolactin gene

Pituitary cell-specific expression of the rat prolactin gene depends on synergistic interactions between cis-acting elements (Nelson et al. 1986; Lufkin et al. 1987; Bodner et al. 1988; Cao et al. 1988; Nelson et al. 1988; Crenshaw III, Kalla et al. 1989). The cis-acting elements of the rat prolactin gene have been studied by transfection of various gene constructs into pituitary-derived cells and non-pituitary cells. Cell-type specific expression of the rat prolactin gene is dictated by two upstream regions, a distal enhancer (-1831 to -1530) region and a proximal promoter region (-422 to +33) (Nelson, Crenshaw III et al. 1986; Crenshaw III, Kalla et al. 1989; Davis 1990). However, it is not clear whether it is the distal or proximal region that dominantly drives expression of the prolactin gene in rat pituitary cells. Cell transfection analysis from Rosenfeld's laboratory indicated that the distal enhancer is predominantly responsible for pituitary cell-specific expression of the rat prolactin gene (Crenshaw III, Kalla et al. 1989), but similar experiments by other groups showed slight to no decreases in prolactin gene expression upon deletion of the distal enhancer region (Sharp 1990 ; Lufkin et al. 1989; Lufkin et al. 1987). Also, by transfection-cell fusion, transient

transfection, and in vitro transcription using GH3 nuclear extracts, Bancroft's laboratory provided evidence that the proximal regions play a dominant role in directing cell-specific expression of the prolactin gene in pituitary cell lines (Sharp, 1990; Lufkin et al. 1989; Lufkin et al. 1987) In addition to these transfection experiments, observations with transgenic mice indicated that either region is sufficient to direct expression of reporter genes to pituitary cells of transgenic mice, but both regions are necessary for full level expression of the rat prolactin gene in lactotrophs (Crenshaw III, Kalla et al. 1989). This implies that synergistic interaction between the proximal and distal elements is necessary for determining cell specific expression of the rat prolactin gene at physiological levels (Simmons, Voss et al. 1990). By using DNase I footprinting and further mutagenesis analysis, it has been shown that the distal rat prolactin enhancer region contains four Pit-1 binding sites (designated 1D-4D) as well as one estrogen receptor binding site (designated ERE), and the proximal rat prolactin promoter region also contains four Pit-1 binding sites (designated 1p-4p) (**see Fig. 1**) (Gutierrez-Hartmann et al. 1987; Cao, Barron et al. 1988; Kim et al. 1988; Nelson, Albert et al. 1988; Schuster et al. 1988; Sharp et al. 1989; Harvey et al. 1991; Yan, Pan et al. 1991; Yan and Bancroft 1991). The proximal promoter also contains an element that may be capable of conferring cAMP responsiveness, termed the CRE-like element, which can bind CREB with a lower affinity than the classic CRE sequence (see Chapter 2).

3b. Pit-1 is the major trans-acting factor responsible for cell type specific expression of the rat prolactin gene

The multiple related cis-acting elements required for cell-specific activation of the rat prolactin gene appear to bind a pituitary-specific positive transcription factor Pit-1. Pit-1 was cloned independently by two groups in 1988,

and referred to either as GHF-1 (Bodner, Castrillo et al. 1988) or as Pit-1 (Ingraham et al. 1988). Pit-1 is a member of the POU family of transcriptional regulators (**P**it-1, **O**ct-1/Oct-2, and **u**nc-86) sharing two regions of high homology: a highly conserved POU-specific (POUs) domain and a more divergent homeodomain (POU$_{HD}$). The POUs domain appears to be required for high affinity, site-specific DNA binding as well as DNA-dependent protein-protein interaction, while POU$_{HD}$ is critical for DNA binding (Treacy et al. 1992). The trans-activation domain of Pit-1 is contained in the N-terminal region (**see Fig. 2**).

Pit-1 is capable of activating the rat prolactin gene promoter in non-pituitary cells. Expression of Pit-1 in heterologous cell types including Hela, CV-1, and RAT-1 activated the prolactin gene promoter, even when Pit-1 was expressed at levels much lower than those present in pituitary cells. (Ingraham, Chen et al. 1988; Mangalam et al. 1989; Fox et al. 1990). In addition, by using antisense oligonucleotide technology, it was shown that inhibition of Pit-1 synthesis leads to dramatically reduced prolactin gene expression (Castrillo, Theill et al. 1991). Furthermore the POU-domain mutations of Pit-1 in the pituitary of dwarf mouse indicates that Pit-1 is required for specification of the lactotrophic phenotype, which includes the activation of prolactin gene expression (Li, Crenshaw et al. 1990; Tatsumi, Miyai et al. 1992).

It is now quite clear that Pit-1 is necessary for cell-specific expression of the prolactin gene, but it is not surprising that other factors may also be involved. The estrogen receptor is such a factor. Its binding site appears to be important for the activity of the distal enhancer (Maurer et al. 1987; Kim, Day et al. 1988; Day et al. 1990). Co-expression of estrogen receptor in transient transfection experiments with Pit-1 synergically activates the prolactin gene to maximal expression (Day, Koike et al. 1990). Furthermore, Martial's group has

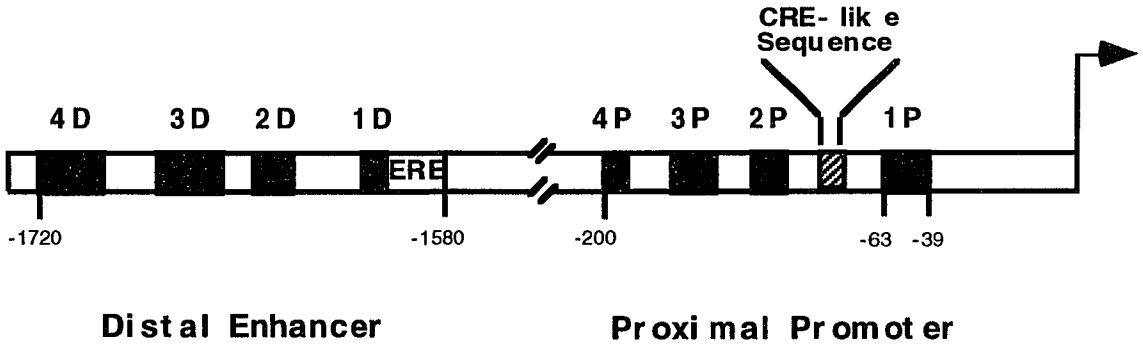

Figure 1. **Cis-acting regions of the prolactin gene 5'-flanking sequence.** 1P-4P indicate Pit-1 binding sites in the proximal prolactin gene promoter. 1D-4D indicate Pit-1 binding sites in the distal prolactin gene enhancer region. ERE, estrogen response element. CRE, cAMP response element.

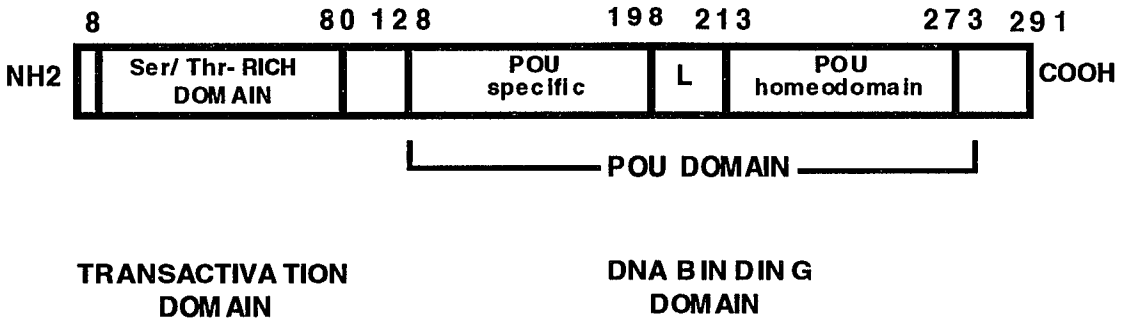

Figure 2. **Functional domains of Pit-1 protein.**

recently identified a 100kd protein which can specifically bind the CRE-like element of the human prolactin gene and is required for full basal and hormonal regulation of human prolactin promoter activity (Peers et al. 1992). Moreover, Pit-1 and the ubiquitous POU protein Oct-1 co-expressed in non-pituitary cell lines can bind as a heterodimer to Pit-1 binding sites in rat prolactin gene, suggesting that a combinatorial pattern of heterodimeric and homodimeric interactions between different members of the POU-domain family may be important for regulating cell-type specific expression of the rat prolactin gene (Voss et al. 1991). Likewise, other unidentified negative trans-acting factors might be important for repressing prolactin gene expression in other cell types. Hence, a complex interaction among multiple trans-acting factors might confer cell-specific expression on the prolactin gene.

4. Peptide hormone regulation of prolactin gene expression

Although the prolactin gene is specifically expressed in lactotrophs, homeostatic regulation of its expression in mature mammotrophs is under complex regulation by both stimulatory and inhibitory hormones. These hormones are mainly synthesized in the hypothalamus and target on specific receptors on lactotrophic cells via a portal blood system. Upon binding to their receptors, these hormones initiate a series of gene-distal and gene-proximal signaling events which result in the regulation of the prolactin gene. Hence, the prolactin gene provides us an excellent model with which to define the molecular mechanism of transcriptional regulation by peptide hormones. Dopamine is the major hormone that negatively regulate prolactin gene expression (Hinkle 1988; Escalera et al. 1992), probably via Gαi2 (Albert et al. 1990; Lew et al. 1994). On the other hand, TRH and VIP are two well-studied stimulatory peptide hormones, which may exert their regulation of prolactin gene

expression through different signal transduction pathways involving different G proteins (see below).

4a. TRH regulation of prolactin gene expression

Thyrotropin-releasing hormone (TRH) is a tripeptide hormone. It was the first hypothalamic hormone isolated (Aizawa et al. 1985; Aizawa et al. 1985), and was found to function as an activator of prolactin production (Tashjian et al. 1990) and PRL gene transcription (Murdoch et al. 1983). An intensive effort has been directed toward understanding the signaling mechanism through which TRH regulates prolactin secretion and gene expression in lactotrophs. Most of these studies have used GH cells, which are rat pituitary tumor cells.

TRH specifically binds to high affinity TRH receptors present in pituitary and brain. The TRH receptor was first cloned by Gershengorn's group in 1990, and found to be a seven transmembrane protein characteristic of G-protein coupled receptors (Straub et al. 1990). Using GH3 cells, recent studies have shown that the TRH receptor activates cytoplasmic signals through coupling to Gq and G11 (Aragay et al. 1992; Hsieh et al. 1992). Gq and G_{11} are novel a-subunit proteins of the Gq subfamily which regulate the activity of the β1 isoenzyme of PLC (Smrcka et al. 1991). Previous studies with GH cells showed that TRH stimulation results in rapid hydrolysis of phosphatidylinositol bisphosphate (PIP2) into IP3 and DAG (Martin 1983; Rebecchi et al. 1983; Rebecchi et al. 1983; Martin et al. 1984), and activation of protein kinase C (Drust et al. 1984). Furthermore, TRH stimulates a rapid, biphasic elevation of intracellular Ca^{2+}. The early phase arises from mobilization of intracellular Ca^{2+} stores by IP3 , and the sustained second phase of Ca^{2+} elevation is caused by influx of extracellular Ca^{2+} (Albert et al. 1984; Snowdowne et al. 1984; Gershengorn et al. 1985) through voltage-dependent Ca^{2+} channels (Hagiwara

et al. 1983; Tan et al. 1984). A rapid translocation of protein kinase C to the membrane has also been reported in response to TRH (Fearon et al. 1985; Fearon et al. 1987). In addition, TRH can stimulate the phosphorylation of a series of cytoplasmic proteins (Drust et al. 1982; Drust et al. 1982; Sobel et al. 1983; Drust and Martin 1984) in a Ca^{2+}-(Drust and Martin 1982) and protein kinase C- (Drust and Martin 1984) dependent manner.

The nuclear events involved in prolactin gene regulation by TRH are just starting to be clarified. A TRH response can be directed by either the proximal promoter region or the distal enhancer region of the prolactin gene, implying that each region contains at least one TRH response element (Day et al. 1989). Recently, Yan and Bancroft (Yan and Bancroft 1991) have shown that the 1p site in the proximal region of PRL gene (**see Fig. 1**) is both a Ca^{2+} response element and a TRH response element, implying that Pit-1, by its interaction with 1P site represents the gene-proximal protein in a TRH-Ca^{2+} signaling pathway (Yan et al. 1991).

4b. VIP regulation of prolactin gene expression

Vasoactive intestinal peptide (VIP) is another peptide hormone that can increase PRL mRNA levels in GH_3 cells (Carrillo et al. 1985). VIP is a highly basic 28-amino acid peptide which was originally isolated from the porcine duodenum (Said et al. 1970) and has a wide distribution. The effects of VIP are mediated by specific binding to its receptor which has recently been cloned (Ishihara et al. 1992) and found to exist in pituitary cells (Agui et al. 1990). Upon binding to its receptor, VIP stimulates the activity of adenylate cyclase (Robberecht et al. 1979; Ishihara, Shigemoto et al. 1992) which leads to increased levels of intracellular cAMP (Gozes et al. 1989). The structure of the VIP receptor (Ishihara, Shigemoto et al. 1992) suggests that its action on

adenylate cyclase is mediated by Gs. Although VIP has no effect on the production of inositol phosphates (Sutton et al. 1982), it can increase intracellular Ca^{2+} in GH3 cells (Prysor-Jones et al. 1987), probably via a cAMP-dependent mechanism. cAMP activates protein kinase A (PKA), which stimulates the phosphorylation of several intracellular proteins. Agents that increase intracellular cAMP such as forskolin or cAMP derivatives can stimulate prolactin gene transcription (Maurer 1981). However, the detailed mechanism by which VIP regulates prolactin gene expression still remains to be determined.

B. Heterotrimeric G proteins

1. Introduction

G proteins are a family of membrane-bound, heterotrimeric GTP-binding and hydrolyzing proteins (Hepler et al. 1992). They are present in all eukaryotic cells and play an essential role in linking diverse cell-surface receptors to different intracellular signaling systems at the plasma membrane, including adenylate cyclase (Northup et al. 1983a; Northup et al. 1983b), ion channels (Codina et al. 1987; Mattera et al. 1989; Schubert et al. 1989), phospholipid breakdown through phospholipase C (PLC) (Smrcka, Hepler et al. 1991; Taylor et al. 1991), phospholipase A2 (PLA2) (Axelrod et al. 1988; Kim et al. 1989), phosphoinositide 3 kinase (Stephens et al. 1994; Thomason et al. 1994), receptor kinase (Kameyama et al. 1993; Koch et al. 1993) and mitogen-activated protein kinase (Faure et al. 1994). These G-protein coupled effectors, in turn, generate regulatory molecules or second messengers (i.e. cAMP or IP3) which lead to intracellular events such as selective protein phosphorylation-that initiates the appropriate biochemical response. For example, the pheromone response in yeast starts with extracellular peptide mating factors binding to membrane receptors which activate G protein, then activates downstream components of the signaling pathway, ultimately leading to increased transcription of several genes including those encoding cell-surface proteins involved in cell-cell interaction and fusion. At same time, G1 cyclins are inactivated in this process, leading to cell cycle arrest (Marsh 1991). Therefore, G proteins are very important signal transducers that control biological functions. Here I provide a brief summary of knowledge of G protein structure, function, and mechanism of action.

2. General structure and function

G proteins are heterotrimers, composed of three distinct subunits: α, β and γ. The β and γ subunits exist as a tightly associated complex that is believed to function as a unit. The α subunit has a single, high affinity binding site for either GDP or GTP. The GDP-bound form of α binds tightly to βγ and is inactive. Upon binding to activated receptors which catalyze the exchange of GDP to GTP, the α subunit in its activated GTP-bound form dissociates from βγ, and both the α and βγ subunits can then serve as regulators that associate with and stimulate the effector proteins at the plasma membrane. G protein activation is terminated by the GTPase activity intrinsic to the α subunit, which hydrolyses GTP to GDP and Pi and leads to subunit reassociation. By switching between the "on" and "off" state governed by the GTPase cycle (Spiegel et al. 1992) (**see Fig. 3**), G proteins function as signal transducers linking diverse receptors to a variety of effector proteins at the plasma membrane.

G proteins are divided into four major subfamilies (**G**s, **G**i, **G**q and **G**12) according to the amino acid sequence of the α subunit (Hepler and Gilman 1992). The members of the Gs family are known to specifically couple the hormone receptors to adenylate cyclase, resulting in elevated level of intracellular cAMP, which in turn simulates PKA activity (Hepler and Gilman 1992; Seuwen et al. 1992). In addition to activation of adenylate cyclase, the Gs α-subunit regulates at least two ion channels , stimulating voltage-gated Ca^{2+} channels in excised skeletal muscle (Mattera, Graziano et al. 1989) and inhibiting cardiac Na^{+} channels (Schubert, VanDongen et al. 1989), indicating that a single Gs α-subunit can regulate more than one effector. Members of the Gq family have recently been identified as regulators of the PLC pathway which can be activated by a broad class of hormones, neurotransmitters and growth factors (Hepler and Gilman 1992). Upon activation by the Gq α-subunit, PLC

catalyzes hydrolysis of PIP2 to generate two second messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol. Diacylglycerol can stimulate PKC activity, whereas IP3 mobilizes intracellular Ca^{2+} from the ER to cytosol, where the Ca^{2+} can then function as an activator of numerous effector proteins including PKC and Ca2+/calmodulin-dependent kinase (Seuwen and Pouyssegur 1992).

Many of the G protein-mediated signaling pathways involved in hormonal regulation are still poorly understood. Different approaches have been developed to investigate the identify of the G protein involved in a specific pathway (Spiegel, Shenker et al. 1992). Pharmacological studies are often used for this purpose. Cholera toxin can covalently modify as subunits, leading to the inhibition of their GTPase activity and constitutive activation of these Gs proteins, whereas modification of αi subunits by pertussis toxin prevents receptor-mediated activation of Gi proteins (Birnbaumer et al. 1990). By contrast, Gq proteins are insensitive to both pertussis toxin and cholera toxin (Hinkle et al. 1986; Martin et al. 1986; Martin et al. 1986). Mutational analysis of G proteins provides another powerful tool to understand their cellular functions. Specific point mutations within the GTP binding domain of the α subunit can inhibit its GTPase activity, thereby locking the G protein in a constitutively active state in the absence of receptor agonists (Graziano et al. 1989; Lowndes et al. 1991; De Vivo et al. 1992). Finally, transfecting cells with selective antisense vectors for specific α subunit can also be used to define the functions of particular G proteins in different cellular signaling pathways (Kleuss et al. 1991; Spiegel, Shenker et al. 1992; Wang et al. 1992).

3. Gq and Gs as signal transducers in GH3 pituitary cells

Gq and Gs are both expressed in the rat pituitary GH3 cells (Paulssen et al. 1991). Each of them functions as an important signal transducer which

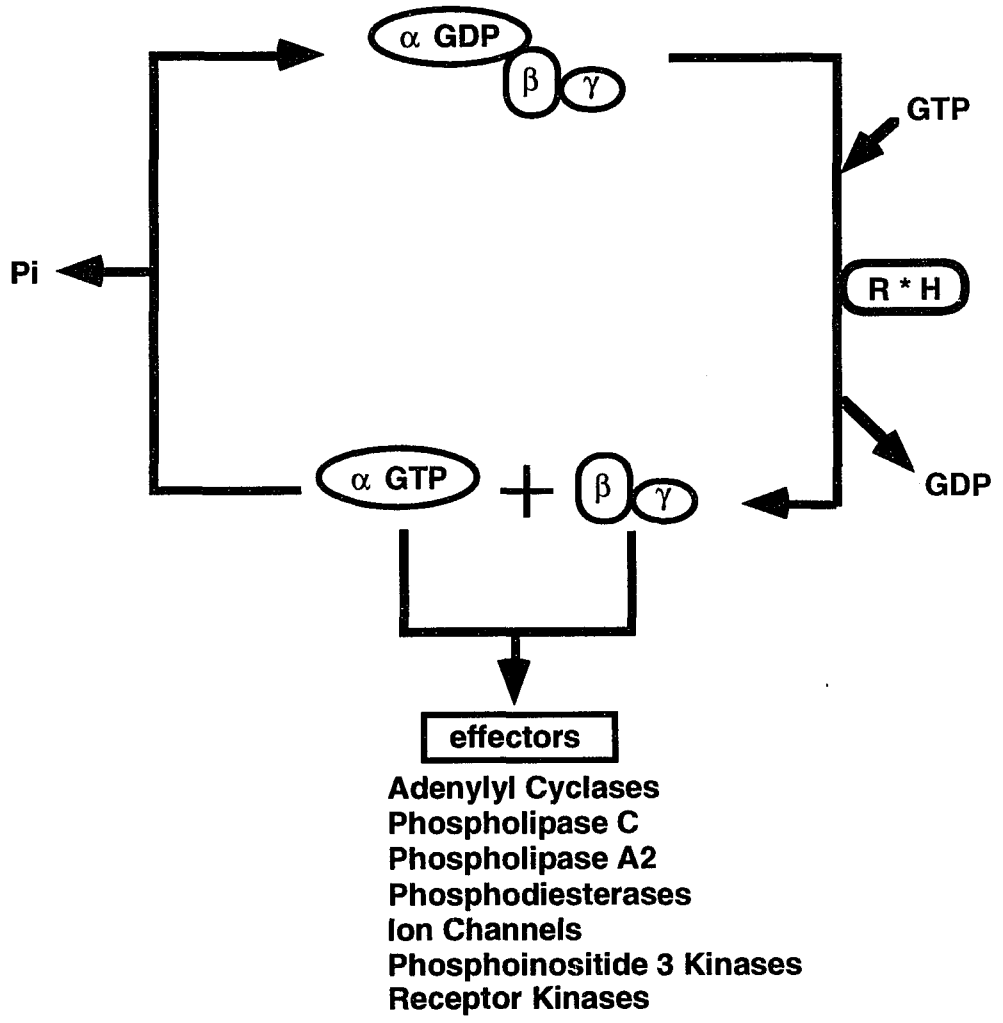

Figure 3. **GTPase cycle of G protein.** α, G protein α subunit; βγ, G protein βγ complex; R * H, hormone-receptor complex.

couples different receptor respectively to intracellular effectors, resulting in the regulation of biological functions. Previous studies have demonstrated that TRH causes a rapid increase in the levels of IP3 and DAG (Martin 1983; Rebecchi and Gershengorn 1983; Rebecchi, Kolesnick et al. 1983; Martin and Kowalchyk 1984), employing a G protein as an intermediary (Hinkle et al. 1984; Hinkle et al. 1984; Martin, Lucas et al. 1986). Since treatment of pituitary cells with either cholera toxin or pertussis toxin had no effect on either membrane PLC activity or membrane GTPase activity mediated by TRH (Hinkle et al. 1986; Martin et al. 1986; Martin, Lucas et al. 1986; Hsieh and Martin 1992), a novel G protein was suggested to mediate TRH receptor action. Recent molecular cloning and biochemical studies have characterized two novel, pertussis toxin-insensitive a subunit proteins of the Gq family (αq and α11) (Smrcka, Hepler et al. 1991; Waldo et al. 1991; Hsieh and Martin 1992). More recently, two groups have shown that the TRH receptor can specifically activate Gq which stimulates PLC, resulting in the hydrolysis of PIP2 into IP3 and DAG (Martin 1983; Rebecchi and Gershengorn 1983; Rebecchi, Kolesnick et al. 1983; Martin and Kowalchyk 1984). Although the gene-distal events involved in the Gq mediation of TRH receptor signaling seems well established, whether Gq is directly involved in the transcriptional effects of TRH remains to be determined.

Gs is believed to another very important signal transducer in GH3 cells. It was postulated that the VIP receptor is coupled to the α subunit of Gs protein based on the following observations: First, the third inner loop of the VIP receptor contains the conserved sequences which are important for interaction with the Gs protein (Ishihara, Shigemoto et al. 1992). Second, VIP stimulates the activity of adenylate cyclase (Robberecht, Deschodt-Lanckman et al. 1979) and cellular accumulation of cAMP (Gozes and Brenneman 1989), which are two characteristic response to Gs stimulation. In addition, GTP and GTP analogs

potentiate the stimulatory effects of VIP on adenylate cyclase (Robberecht, Deschodt-Lanckman et al. 1979), which further supports the idea that Gs protein is an important signal transducer involved in VIP action in GH3 cells. However, the detailed mechanism of the signal transduction mediated by the VIP receptor through Gs is completely unknown.

As described above, both Gq and Gs are thought to be involved in hormonal effects on pituitary cells. However, nothing is known about the ability of either G protein to regulate specific gene expression. In Chapter 2 and 3, I will describe my work on regulation by constitutively active forms of Gs and Gq, respectively, of prolactin and growth hormone gene expression.

4. βγ as signal transducers in eukaryotic cells

As mentioned above, a heterotrimeric G protein is composed of three subunits: α, β, and γ. It had been long thought that the biological functions of a G protein reside entirely in α subunit and that βγ dimers only play a role in modulating subunit. However, recently, Logothetis et al. demonstrated that G protein βγ dimers could activate the K^+ channel which is normally regulated by cholinergic muscarinic receptors in a GTP-dependent fashion in the membranes of atrial myocytes (Logothetis et al. 1987). This was the first indication that Gβγ could regulate cellular effectors through their independent action. Subsequently, βγ subunits were also found to mediate the mating factor signaling pathway in yeast (Cole et al. 1990), to activate the β-adrenergic and muscarinic receptor kinase (Kameyama, Haga et al. 1993; Koch, Inglese et al. 1993), and to regulate adenylyl cyclase (Federman et al. 1992; Taussig et al. 1993), phospholipase C (Camps et al. 1992), phosphoinositide 3 kinase (Stephens, Smrcka et al. 1994; Thomason, James et al. 1994), and mitogen-activated protein kinase (Faure, Voyno Yasenetskaya et al. 1994) in different cell systems.

Following these studies, a more comprehensive perspective on the intracellular mechanisms of $G\beta\gamma$ protein function has emerged. In addition to its previously ascribed role in controlling the interaction of $G\alpha$ subunit with the receptor, the $\beta\gamma$ subunit complex is known to also mediates cellular signaling independently following its dissociation from the $G\alpha$ subunit. The two signaling pathways through $G\alpha$ and $G\beta\gamma$ respectively, may act separately or synergistically (Iniguez-Lluhi et al. 1993). Therefore, the signal transduction pathway from receptor to effector is not dictated exclusively by the $G\alpha$ subunits.

Although the $G\beta$ and $G\gamma$ subunits are not covalently linked to each other, the native proteins cannot be dissociated without denaturation and are thus thought to form a single functional unit. To date the only report of isolation of the native β subunit alone is from Yamazaki et al (Yamazaki et al. 1987). But until now, it has not been known whether functions attributed to the $\beta\gamma$ subunit are due to β, to γ, or both proteins.

My recent observation, described in Chapter 4, that γ_2 but not β_2 is able to regulate the prolactin gene promoter in GH3 cells gives the first evidence that the γ subunit alone is sufficient to signal cellular events in higher organisms.

C. Regulation of Pit-1 Gene Expression

The Pit-1 gene is a member of a large gene family whose protein products are transcription factors containing a highly homologous domain, referred to as POU domain (Herr et al. 1988; Rosenfeld 1991) and that are typically involved in development. The Pit-1 gene is expressed exclusively in anterior lobe of the pituitary gland during embryonic development just before the activation of the prolactin and growth hormone genes (He et al. 1989; Simmons, Voss et al. 1990). Its protein product Pit-1 - a pituitary specific transcription factor that can activate the prolactin and growth hormone gene promoter - is expressed in

mature thyrotroph, somatotroph and lactotroph cell types of the anterior pituitary (Crenshaw III, Kalla et al. 1989; Simmons, Voss et al. 1990) and is critical for the sequential appearance of these three cell types during development (Cooke et al. 1985). Hence, regulation of Pit-1 gene expression is very important in determining the fate of anterior pituitary cells.

The Pit-1 gene promoter contains a Pit-1 binding site and two CREB binding sites (**see Fig. 4**) (Chen et al. 1990; McCormick et al. 1990). Basal expression of the Pit-1 gene in GH cells depends on the Pit-1 binding site that is autoregulatory, while activation of the Pit-1 gene by forskolin is conferred by the two CREB binding sites (McCormick, Brady et al. 1990). In addition, Pit-1 promoter is negatively regulated by dopamine and this regulation requires the Pit-1 binding site and TATA box (Elsholtz et al. 1991). These Pit-1 gene proximal events have been better understood than the gene distal events. The intracellular signaling pathways from membrane receptors to the nucleus which control Pit-1 gene expression are still not identified. In chapter 4, I will provide evidence that expression of the Pit-1 gene is regulated by constitutively active Gs α subunit.

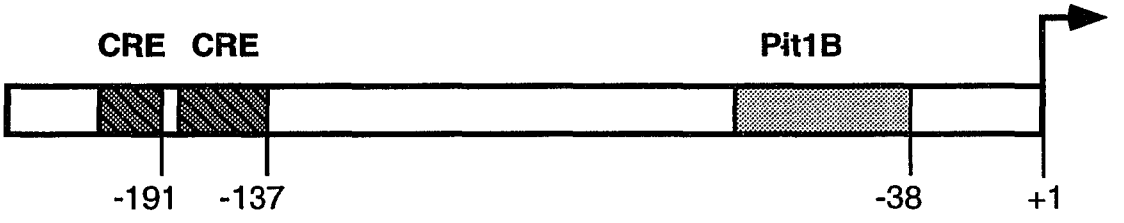

Figure 4. **Pit-1 gene promoter.** CRE, cAMP response element; Pit1B, Pit-1 binding site.

Chapter 2

Expression of Constitutively Active Gs α-Subunits in GH3 Pituitary Cells Stimulates Prolactin Promoter Activity

*** All of the material in this chapter, except for Fig. 8, Fig. 9, and Fig. 10 has been published: Jun Tian, Jianghao Chen, and Carter Bancroft. (1994) J. Biol. Chem. 269:33-36**

A. Summary

Although G protein α subunits are known to regulate such cellular functions as growth and enzymatic activity, the ability of these proteins to regulate target gene expression has not yet been directly investigated. Transient expression in GH3 pituitary cells of a target rat prolactin promoter-chloramphenicol acetyltransferase construct, (-1957)PRL-CAT, was increased by coexpressed constitutively active as mutant Q227L-αs but not by wild-type αs. Thus activated αs but not basal state αs can stimulate prolactin promoter activity. Q227L-αs also stimulated expression of construct (-187)PRL-CAT, showing that only the proximal prolactin promoter region is required for a response to activated αs. The promoter specificity of the transcriptional influence of activated αs was demonstrated by the inability of either Q227L-αs or wild-type αs to stimulate expression of control target constructs containing either the rat growth hormone promoter or the thymidine kinase promoter. Previous studies have shown that the most proximal prolactin promoter binding site for the pituitary-specific transcription factor pit-1, site 1P, can act as an independent response element for either thyrotropin-releasing hormone (TRH) or Ca2+. Two copies of site 1P conferred upon a heterologous metallothionein promoter a response to Q227L-αs. This implies that site 1P can also serve as an independent response element for αs and suggests that pit-1 may be a mediator of the cellular regulation by αs of the prolactin promoter.

To further understand the intracellular signaling pathways through which αs stimulates prolactin gene expression, a mutant regulatory subunit of protein kinase A (PKA), which contains an inactive cAMP binding domain and thus suppresses cAMP dependent PKA activation, was co-expressed with (-187)PRL-CAT construct and shown to block the Q227L-αs stimulation of the prolactin

gene promoter. This indicates that PKA may be involved in mediating αs action on prolactin gene promoter. I also found that K-CREB, a dominant negative inhibitor of CREB, inhibited αs induction of the PRL promoter, implying that PKA action on the PRL promoter is mediated by CREB. I then investigated whether CREB binds to a "CRE-like" element (CLE) in the PRL promoter. I found that CREB binds to the CLE, although with an affinity about 10-fold lower than its affinity for a canonical CRE.

B. Introduction

A number of cell surface ligand receptors have been shown to couple via specific heterotrimeric G proteins to various intracellular "second messenger" molecules (Probst et al. 1992; Graeme 1993). Several ligands in this class have been shown to regulate pituitary prolactin gene expression. These include vasoactive intestinal peptide (Carillo et al. 1985), pituitary adenylyl cyclase-activating polypeptide (Coleman et al. 1993), dopamine, and thyrotropin-releasing hormone (TRH) (Murdoch, Franco et al. 1983), whose receptors have been suggested or shown to couple to, respectively, G_S (Ishihara, Shigemoto et al. 1992), G_S (Spengler et al. 1993), G_i (Montmayeur et al. 1993) (for the prolactin regulatory receptor subtype D2R), and Gq and/or G11 (Aragay, Katz et al. 1992; Hsieh and Martin 1992).

Many of the ligands that interact with G protein-linked receptors, including those listed above, are known to regulate transcription of specific cellular genes, leading to the prediction that G proteins should play significant roles in the pathways of action of these ligands on expression of their target genes. However, investigations to date of this important question have relied on the use of pharmacological inhibitors. For example, pertussis toxin, which inhibits the actions of a variety of G proteins, has been shown to block the induction of c-jun gene expression by insulin (Wang et al. 1992) and of somatostatin inhibition of epidermal growth factor stimulation of gastrin gene expression (Bachwich et al. 1992).

To investigate directly whether a specific receptor-linked G protein can regulate a specific transcriptional pathway, it would be useful to employ an approach that is not dependent upon an interaction of a receptor with either its ligand or the G protein in question. Such an approach has been provided by the

generation of constitutively active G protein α subunit mutants, which have proven very useful in examining the ability of various G proteins to regulate such cellular functions as growth and enzymatic activity (Masters et al. 1989; Zachary et al. 1990; De Vivo, Chen et al. 1992; Kroll et al. 1992). However, constitutively active G protein α subunit mutants have apparently not yet been employed to examine whether a specific G protein can regulate transcription of a target gene.

Cellular expression of one of these constitutively active α subunit mutants, Q227L-α_S, has been shown to constitutively activate adenylyl cyclase and reduce the k_{cat} for GTP hydrolysis by more than 100-fold (Masters, Miller et al. 1989), and to increase the mitogenic response of Swiss 3T3 cells (Zachary, Masters et al. 1990). Furthermore, a similar mutation in the α subunit, Q227R, has been found in a subset of human pituitary tumors.

In the first part of my studies I have investigated whether Q227L-αs can regulate expression of rat prolactin gene. I show that transient expression in GH3 cells of Q227L-αs yields a promoter-specific stimulation of expression of cotransfected prolactin-chloramphenicol acetyltransferase (CAT) constructs, and I provide evidence that a prolactin promoter binding site for the pituitary cell-specific transcription factor pit-1, site 1P, can act as a heterologous α_S response element.

I then investigated the mechanism of αs stimulation of prolactin gene expression. Q227L-αs has been shown to stimulate cellular adenylyl cyclase activity (Masters, Miller et al. 1989) and is believed to exert many of its cellular actions via increasing intracellular cyclic AMP levels (Graeme 1993). Exposure of GH3 cells to cAMP analogs (Iverson et al. 1990), forskolin (FSK) (Keech et al. 1989) or a PKA expression vector (Day and Maurer 1989), all of which increase PKA activity, results in the stimulation of rat PRL gene transcription. By contrast, lowering of intracellular cAMP by dopamine-D2 receptor that couples to an

inhibitory nucleotide-binding (Gi) protein negatively regulates prolactin gene transcription. Furthermore, PRL gene expression is positively regulated by activation of the protein kinase C (PKC) pathway via phorbol esters (Osborne et al. 1981; Elsholtz et al. 1986; Iverson, Day et al. 1990) and the protein kinase pathways (White et al. 1983; Keech et al. 1991).

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 cell-specific and hormonal regulation of the prolactin gene. The CLE has been implicated in optimal cAMP regulation of both the human (Iverson, Day et al. 1990; Peers et al. 1991). and rat PRL promoter (Liang et al. 1992). The observation that mutation of the CRE-like element (referred to here as the CLE) (d'Emden et al. 1992) or deletion of it in the human PRL promoter (Peers, Nalda et al. 1992). strongly reduces expression of a transfected PRL promoter in GH3 cells further demonstrated the significance of this element for basal expression of the PRL gene. The PRL CLE (TGACGGAA) bears a strong resemblance to the classical cAMP response elements previously identified in the promoters of a number of genes, including somatostatin (TGACGTCA) (Montminy et al. 1986; Yamamoto et al. 1988) and fos (TGACGTTT) (Sheng et al. 1990), that have been shown to interact functionally with the CRE-binding protein CREB. More recently, Martial's group has identified a 100kd protein which can specifically bind the CRE-like element of the human prolactin gene and is required for full basal and hormonal regulation of human prolactin promoter activity (Peers, Nalda et al. 1992). Hence, transcriptional regulation of PRL gene expression does not seem to be controlled solely via Pit-1. CREB, which binds CRE and confers cAMP response on the expression of the genes, may also be involved in as stimulation of prolactin gene expression. In the second part of my studies on the mechanisms of αs stimulation of PRL gene promoter activity, I have examined

the role of PKA and CREB in mediating αs action on Q227L-αs stimulation of the PRL proximal promoter.

C. Material and Methods

1.Sources of Recombinant Plasmids:

Plasmids CMV-α_S and CMV-Q227L-α_S were constructed as follows. Gα_S cDNA in plasmid pT7-7 (Mattera, Graziano et al. 1989) was mutated by site-directed mutagenesis using polymerase chain reaction. The primers 5'-GCAGATGAGGATCCTGCATGTTAATG-3' and 5'-CGTTGAAGCACTGGATCCACTTGCGGCGTTCATCGCG**AA**GGCCACCCACG-3' were used to generate the mutant cDNA from wild-type Gα_S cDNA. Two changes (CÆA and TÆA), at the underlined bold positions, result in changing Gln-227 to Leu and in the appearance of an new Nru I site in the mutant form. Plasmid pT7-7-α_S was digested with Bam HI and the wild-type region was replaced with the Bam HI-digested polymerase chain reaction product. Full-length Q227L-α_S cDNA was then isolated and the presence of the mutation confirmed by DNA sequencing of the α_S coding region. To transfer the α_S cDNAs into mammalian expression vector pRc/CMV (pCMV), containing a cytomegalovirus promoter, the wild-type or mutant α_S cDNAs were excised from pT7-7-α_S or pT7-7-Q227L-α_S by Ncol and Hind III digestion and were inserted into pCMV by blund end ligation. Constructs containing the growth hormone (GH) of prolactin promoter are identified by the 5' terminus of the respective promoter sequence. We have described previously the construction of (-1957)PRL-CAT, (-187)PRL-CAT (Lufkin et al. 1989), (-224)GH-CAT (Pan et al. 1990), and (-39)mMT-CAT and $(1P)^2$(-39)mMT-CAT (Yan, Pan et al. 1991), and the source of TK-CAT (Jackson et al. 1988). Plasmid pRc/CMV, employed to equalize overall amounts DNA transfected, was purchased from Invitrogen Corp. Plasmid RSV-K-CREB was provided by Dr. Richard H. Goodman (Walton et al. 1992) , plasmid RSV-CREB was provided by Dr. Marc R. Montminy (Gonzalez et

al. 1989), plasmid HL-REVwtneo. (expressing the mouse PKA regulatory subunit), and HL-REVabneo. (expressing the mutant PKA regulatory subunit) were provided by Dr. Stanley G. McKnight (Uhler et al. 1987; Schecterson et al. 1991).

2.Transfection and Culture of GH3 Cells:

Cells growing in suspension culture as described (Coleman and Bancroft 1993) were seeded into 60-mm tissue culture dishes (1.5 x 10^6/dish). One day later they were transfected by one of the following two protocols. For the experiments depicted in **Fig. 5** and **Fig. 6**, DEAE-dextran-mediated transfection was performed as described (Jackson and Bancroft 1988; Coleman and Bancroft 1993). For the experiments depicted in **Fig. 7**, Lipofectin-mediated transfection was employed instead so as to obtain optimal transfection efficiency, as follows; after rinsing with phosphate-buffered saline, each dish was incubated 6h at 37 °C with 3 ml of Opti-MEM (Life Technologies, Inc.) containing 20mg of lipofectin (Life Technologies, Inc.) plus the indicated plasmid DNAs. Following transfection by either protocol, transfection medium was replaced by Dulbecco's modified Eagle's medium plus 5% fetal calf serum, and the cells were incubated for 2 days, then harvested for CAT assay. For the experiments described in **Fig. 8**, and **Fig. 9**, Transfection via electroporation was performed as follows. Approximately 5x10^6 cells were resuspended with DNA's for transfection in 0.5 ml of DMEM/5% FCS and electroporated (960μF, 200V) in Gene Pulser™ cuvette (Bio-Rad) with a 0.4 cm pathlengh in a Gene Pulser™ (Bio-RAD). Immediately after transfection, the cells were diluted into the appropriate growth medium and aliquoted in tissue culture dishes and the cells were incubated for 1 day, then harvested for CAT assay. For each experiment, triplicate dishes were employed for each condition, and each experiment except **Fig. 8** and **Fig. 10**

performed 3-11 separate times. By using triplicate dishes for each experimental condition and performing statistical analysis of the results of multiple independent experiments, it was possible to obtain reliable data without the use of internal control reporters. Results of the multiple experiments are presented as mean ± SE. of the -fold stimulation relative to cells receiving only plasmid Rc-RSV. Significance of differences were determined using Student's unpaired t test. Experimental conditions that yielded results significantly different from controls are indicated by an asterisk

3. Measurement of CAT activity

CAT Enzymatic Assays-CAT activity was assayed by thin layer chromatography without autoradiography (except **Fig. 5**) plus scintillation counting as described (Jackson and Bancroft 1988). Half the contents of each dish were assayed, employing incubation times of 10-12 or 2-4 h for transfection mediated by, respectively, DEAE-Dextran or Lipofectin. No correction was necessary for effects of a_S expression vectors on cell growth, since assays of triplicate dishes yielded the following cell counts ($x10^6$)/dish for cells transfected by DEAE-Dextran with the indicated plasmids: control, 1.01; CMV-α_S, 1.05; CMV-Q227L-α_S, 1.05. For Lipofectin-mediated transfection, the corresponding cell counts were, respectively, 1.95, 2.11, and 2.20.

4.Analysis by DNA mobility shift of oligonucleotide binding to CREB:

Oligodexynucleotides were synthesized by the Mt. Sinai DNA Core Facility, and were gel-purified and annealed as described previously (Pan, Liu et al. 1990). The CRE oligonucleotides contain the CREB binding site of the rat somatostatin gene (SS CRE) (Montminy, Low et al. 1986), with a XhoI or SalI site at the 5' end of, respectively, the sense and antisense strands. The rat PRL

CRE-like oligonucleotides contain positions -116 to -88 of the rat PRL promoter, with a SalI or XhoI site at the 5' end of, respectively, the sense and antisense strands. The sequence of each of the sense strands was:

SS CRE: 5'-TCGAGGCCTCCTTGGCTGACGTCAGAGAGAGAGTTTG-3'.

Rat PRL CRE-like: 5'-TCGACGGGGCTATCTTAATGACGGAAATAGATGAC-3'.

The pure CREB protein employed had been prepared from bacterial lysates, and was kindly supplied by Richard Goodman and Jane Roberts. Aliquots of this CREB preparation (250 ng) were employed for mobility shift with the indicated [^{32}P]-labeled double stranded oligonucleotides, as described previously (Pan, Liu et al. 1990), except that the diothiothreitol concentration was increased to 5 mM to maintain the CREB protein in a reduced form (Jane Roberts, personal communication), and the gels were dried prior to analysis. Radioactivity in shifted and free bands was analyzed by storage phosphor imaging autoradiography, employing a Molecular Dynamics Phosphorimager.

D. Results

Activity of the prolactin promoter has been shown to be influenced by both a distal enhancer region, ~1500 base pairs upstream of the cap site, and the proximal promoter region containing the first 200 base pairs of the promoter (d'Emden, Okimura et al. 1992). We thus began the present studies by examining the ability of expression vectors for wild-type or mutant α_s to regulate transient expression in GH3 cells of cotransfected construct (-1957)PRL-CAT, containing the first 1957 base pairs of the prolactin promoter cloned in front of the CAT gene. It is seen in **Fig. 5** that expression of wild-type α_s did not increase CAT enzymatic activity over that seen in control cells cotransfected with a control plasmid, while cells expressing mutant Q227L-α_s yielded greater CAT activity than did control cells, suggesting that Q227L-α_s can specifically stimulate (-1957)PRL-CAT activity. This was confirmed by the results of multiple experiments identical in design to that depicted in **Fig. 5** which, in the number of independent experiments indicated in parentheses, yielded the following values for the mean-fold increases ± S.E. of CAT activity over control cells for cells cotransfected with an expression vector for the indicated form of αs: wild-type αs, 1.26 ± 0.09 n=6); Q227L-αs, 2.54 ± 0.41 (n=12). The -fold stimulation observed with Q227L-αs was statistically significant ($p < 0.01$ versus control cells). Thus, a constitutively active αs mutant can specifically stimulate transcription from a cotransfected prolactin promoter construct.Previous similar studies with other agents that stimulate prolactin gene expression have shown that, although both the distal and proximal prolactin promoter regions described above contain regulatory elements for several hormones (Day and Maurer 1989), the proximal prolactin promoter alone (diagrammed in **Fig. 6A**) can yield a complete response to TRH (Yan, Pan et al. 1991), Ca2+

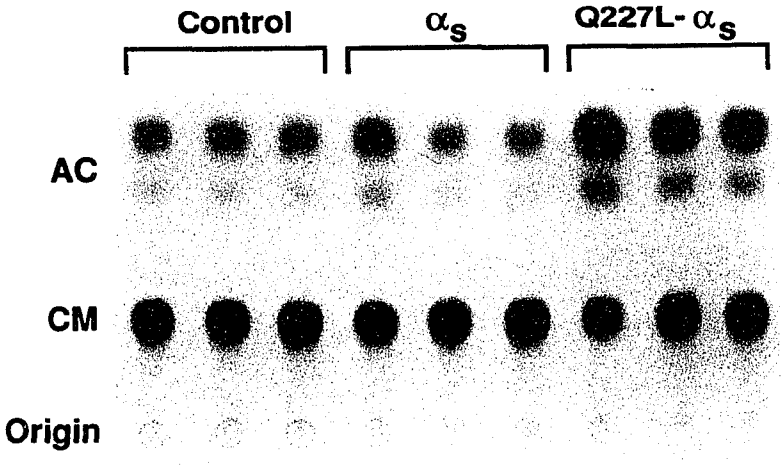

Figure 5. **Effect of wild-type or mutant αs on expression of a PRL-CAT construct.** GH3 cells were transfected with 5 μg of (-1957)PRL-CAT plus 5 μg of pRc/CMV, CMV-αs, or CMV-Q227L-αs, incubated 2 days, and assayed for CAT activity. Shown is an autoradiogram of chromatographed extracts from cells transfected in triplicate dishes with either pRc/CMV (control), or expression vectors for the indicated proteins. CM and AC represent, respectively, unacetylated and acetylated forms of [^{14}C]chloramphenicol.

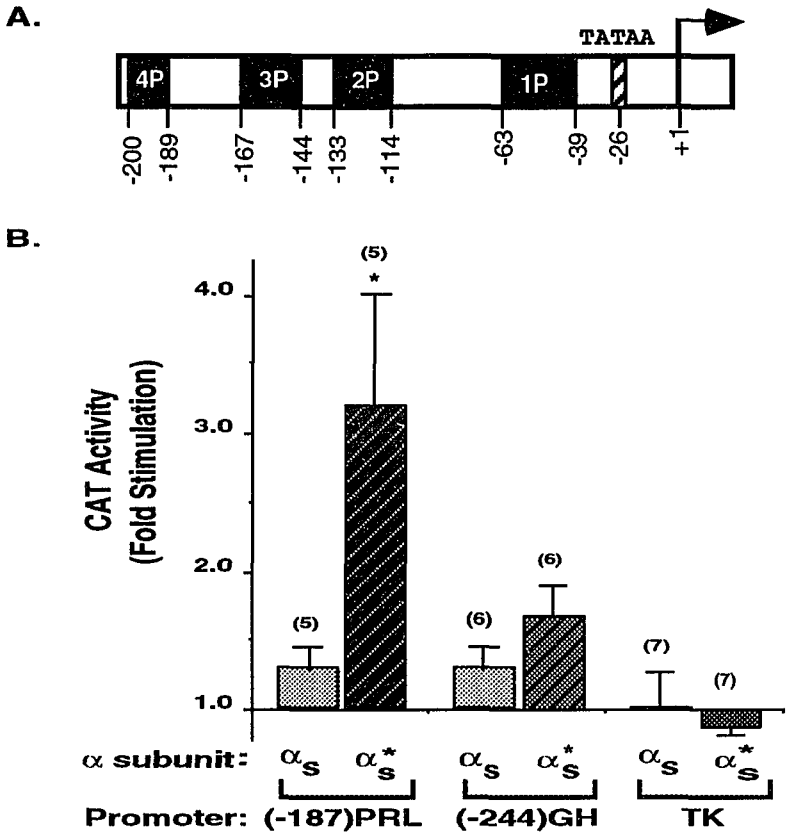

Figure 6. **Regulation by wild-type or mutant αs of transfected promoter-CAT constructs.** A, diagram of the proximal rat prolactin promoter. Location of binding sites 1P-4P for the pituitary-specific factor pit-1 are indicated. Site 2P has also been reported to bind a ubiquitous factor. B, GH3 cells were transfected with 5 μg of (-187)PRL-CAT, (-244)GH-CAT, or TK-CAT, plus 2 μg of pRc/CMV (control), CMV-αs, or CMV-Q227L-αs (αs*), incubated 2 days, and assayed for CAT activity. Shown is the mean ± S.E. of the -fold stimulation of CAT activity relative to controls observed in the indicated number (in parentheses) of independent experiments. For cells transfected with CMV-Q227L-αs plus (-187)PRL-CAT, (-244)GH-CAT, or TK-CAT, the mean conversion of chloramphenicol to acetylated products was, respectively, 33, 21, and 6%. (*, $p < 0.03$ versus control level).

(Yan and Bancroft 1991), cyclic AMP (Keech et al. 1992; Liang, Kim et al. 1992), or pituitary adenylate cyclase-activating polypeptide (Coleman and Bancroft 1993). We therefore next investigated whether the proximal prolactin promoter can yield a specific response to Q227L-αs (**Fig. 6B**). Q227L-αs (αs*) yielded an ~3-fold stimulation of expression of (-187)PRL-CAT, while wild-type αs yielded only an ~30% increase in expression of this CAT construct, demonstrating that the proximal prolactin promoter can direct a complete response to Q227L-αs. It is also seen in that parallel experiments with two other promoter-CAT constructs showed that the response to activated αs is promoter-specific. Expression of a construct containing the proximal rat growth hormone promoter, (-224)GH-CAT, was only slightly stimulated by Q227L-αs (~70% increase), which was not significantly greater than that in response to wild-type αs (~30% increase). Furthermore, expression of another construct containing the thymidine kinase promoter, TK-CAT, was unaffected by expression of wild-type αs, and was actually slightly decreased (by ~30%) by expression of Q227L-αs. Thus, the proximal prolactin promoter region can direct a stimulatory response to αs that is specific both for an αs mutant in a constitutively active state and for the prolactin promoter.

The proximal prolactin promoter contains four binding sites for the pituitary-specific transcription factor pit-1 (Nelson, Albert et al. 1988) (see also **Fig. 6A**). We have shown previously that the most proximal of these, site 1P, can serve as a heterologous response element for both TRH and Ca^{2+} (Yan and Bancroft 1991). It was therefore of interest to investigate whether the ability of the proximal prolactin promoter element to respond to Q227L-αs might be due, at least in part, to an ability of site 1P to serve as an αs response element. To investigate this question, we examined the ability of 1P oligomers to transfer a response to Q227L-αs to the minimal mouse metallothinoein construct (-

39)mMT-CAT. (Since we have found previously that at least two copies of site 1P are required to transfer a TRH response (Yan, Pan et al. 1991), two tandem copies of site 1P were employed for these studies.) As shown in **Fig. 7** neither wild-type αs nor Q227L-αs (αs*) stimulated expression of the control minimal (-39)mMT-CAT construct. Control wild-type as yielded a small (~50% increase) but significant ($p < 0.02$) stimulation of expression of $(1P)^2$(-39)mMT-CAT. By contrast, Q227L-αs yielded a significantly larger, ~3-fold stimulation of expression of $(1P)^2$(-39)mMT-CAT. Thus, prolactin promoter site 1P can serve as an independent response element response for activated αs.

I then studied the mechanism of αs action on the PRL promoter. Earlier studies have shown that Q227L-αs can activate adenylyl cyclase (Masters, Miller et al. 1989), and PKA can activate the PRL promoter in GH4 cells (Conrad et al. 1992). I therefore investigated whether PKA lies on the signaling pathway of αs action on the PRL gene transcription (**Fig. 8**). For those studies I used an expression vector for a mutant PKA regulatory subunit, Rab, which cannot bind cAMP, and is thus a dominant negative inhibitor of PKA (Schecterson and McKnight 1991). Transient expression of Rab resulted in about 4-fold reduction in the αs stimulation of expression of (-187)PRL-CAT construct, whereas the wild type PKA regulatory subunit (Rwt) yielded no inhibitory effect on the stimulation by αs of this construct. Neither Rab nor Rwt affected the basal expression level of PRL-CAT, indicating the blockade effect of Rab is specific to the αs action. These results implies that PKA is involved in the signaling pathway of αs on prolactin gene expression.

Since CREB is the gene-proximal target of PKA in many transcription systems, I therefore examined the role of CREB in mediating αs action on prolactin gene transcription (**Fig.9**). Co-expression of (-187)PRL-CAT with the dominant negative CREB mutant, K-CREB

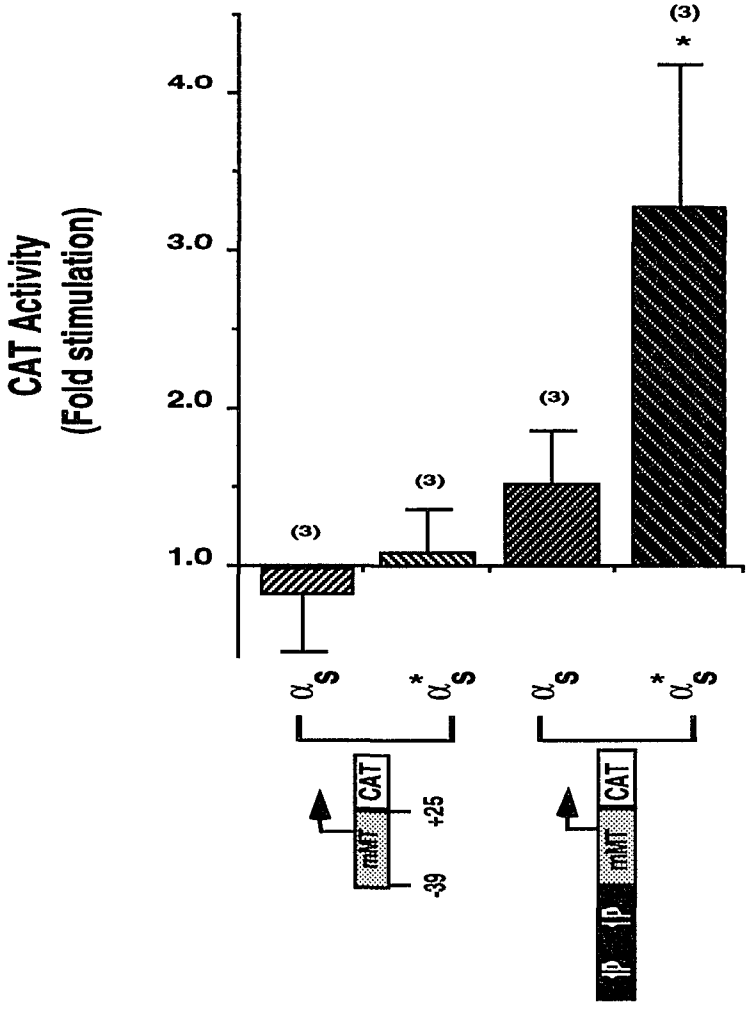

Figure 7. **Site 1P can act as an independent response element for activated αs.** GH3 cells were transfected with 5 μg of (-39)mMT-CAT or ($1P^2$)(-39)mMT-CAT plus 2 μg of pRc/CMV (control), CMV-αs, or CMV-Q227L-αs (αs*), incubated 2 days, and assayed for CAT activity. Results are presented as in Fig. 6. For cells transfected with CMV-Q227L-αs plus (-39)mMT-CAT or ($1P^2$)(-39)mMT-CAT, the mean conversion of chloramphenicol to acetylated products was, respectively, 11 and 23%. *, $p < 0.01$ versus control level, <0.03 versus (-39)mMT-CAT plus αs*, and <0.03 versus ($1P^2$)(-39)mMT-CAT plus αs.

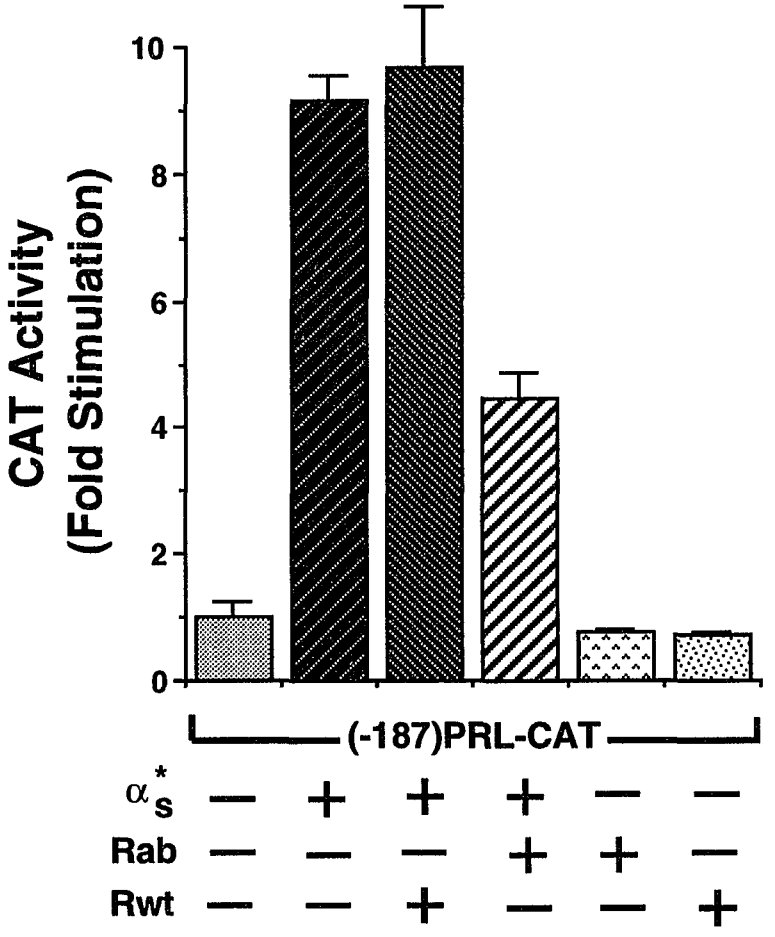

Figure 8. **A mutant PKA regulatory subunit (Rab) blocks stimulation of αs on the expression of PRL proximal promoter.** GH3 cells were transfected with 10 μg of (-187)PRL-CAT, plus 10 μg of pRc/CMV-Q227L-αs (αs*), plus 10 μg of either HL-REV$_{wt}$neo (Rwt) or HL-REV$_{ab}$neo (Rab), incubated for 1 day, and assayed for CAT activity. Results represent the mean ± SD of results with triplicate dishes.

(Walton, Rehfuss et al. 1992) resulted in a strong reduction of αs stimulation of the PRL proximal promoter, as compared to the slight reduction yielded by wild type CREB (w-CREB). Neither w-CREB nor k-CREB affected basal expression level of PRL-CAT, indicating that the inhibitory function of k-CREB on αs action is specific. These results implies that CREB may mediate αs action on prolactin gene expression.

The next obvious question I wanted to investigate is whether CREB has a possible biochemical interaction with the PRL CRE-like sequence (CLE). DNA mobility shift was employed to examine interaction of purified CREB with on oligonucleotide corresponding to either the PRL CLE (TGACGGAA) or the classical CRE octanucleotide motif (TGACGTCA) found in the somatostatin (SS) promoter. As shown in **Fig. 10**, incubation of CREB (250 ng) with high concentrations (≥1.5 nM) of the PRL CLE yielded a shifted band doublet (Fig.10A, arrows). Incubation of CREB with the SS CRE yielded a shifted band doublet with a similar mobility, but detectable at lower oligonucleotide concentrations (≥0.15 nM) (Fig. 10A). The observation of a doublet with each probe presumably corresponds to binding of CREB as either a monomer or a dimer. Quantitation of these results (Fig, 10B) indicated that CREB binds to the PRL CRE-like sequence with an affinity approximately 10% of the affinity of this protein for the SS CRE.

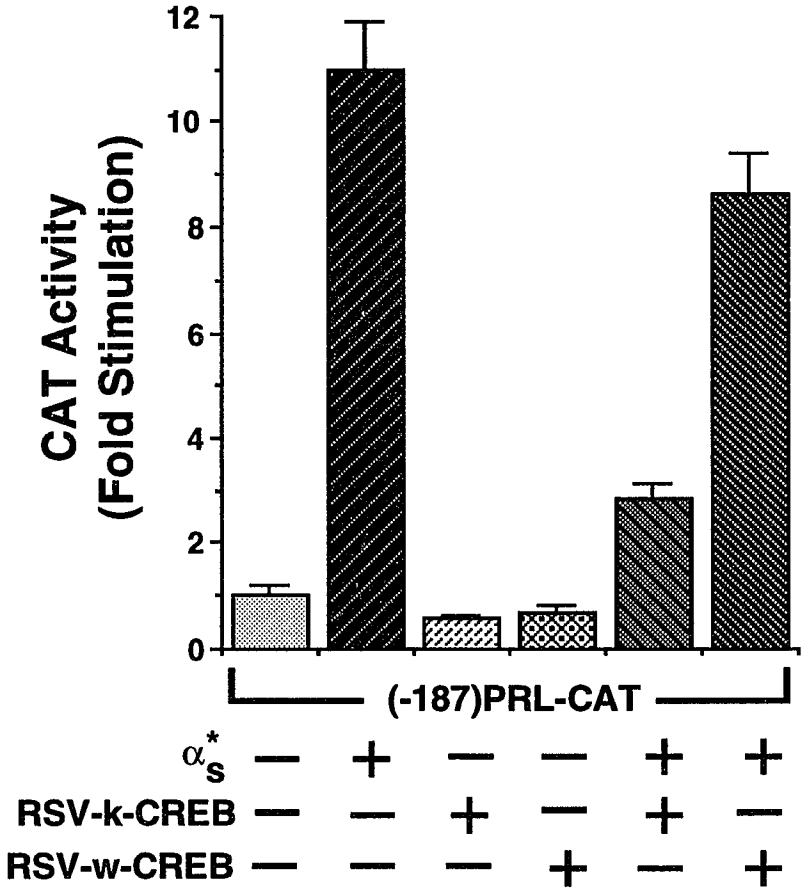

Figure 9. **A mutant CREB (K-CREB) blocks αs stimulation of the prolactin proximal promoter**. GH3 cells were transfected with 10 μg of (-187)PRL-CAT, plus 10 μg of either pRc/CMV-Q227L-αs (αs*), plus10 μg of either pRc/RSV-k-CREB or ± 10 μg pRc/RSV-w-CREB, incubated for 1 day, and assayed for CAT activity. Results represent the mean from triplicate dishes.

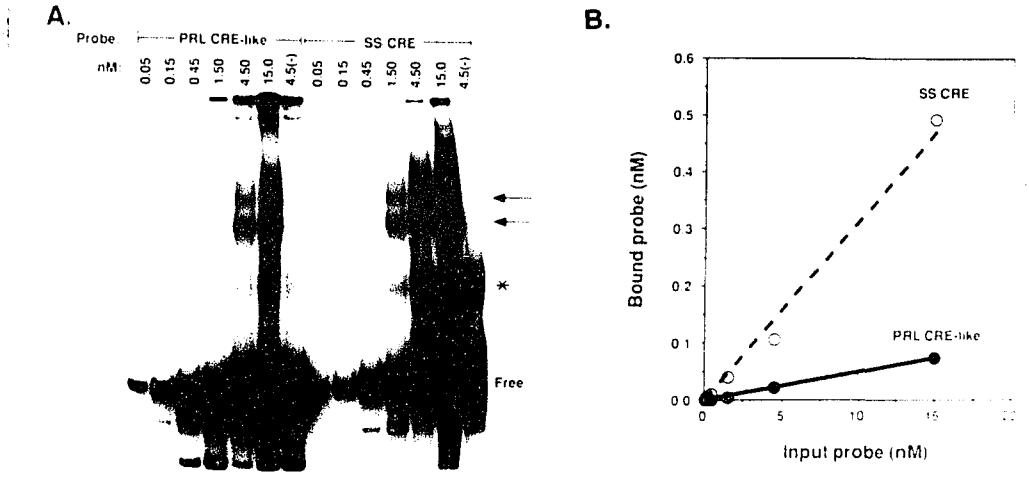

Figure 10. **Binding of CREB to the PRL CRE-like element and the somatostatin CRE.** **A.** DNA shifts observed with the indicated concentrations of either the PRL CRE-like element (PRL CRE-like) or the somatostatin CRE (SS CRE) ^{32}P-labeled probes incubated with purified CREB protein. The specific activities of the two probes employed were approximately equal. Samples in all lanes were incubated with 250 ng CREB, except for the lanes labeled 4.5(-), which contained probe alone. The two CREB-shifted bands observed with either probe are indicated with arrows. An impurity of unknown origin present in the SS CRE probe in the absence of added protein is indicated by an asterisk. B, quantitation of the data shown in **A.** The radioactivity in the two bands indicated by arrows and in the free probes in **A** were quantitated by storage phosphor imaging autoradiography.

E. Discussion

The identification of the signal transduction pathways employed by peptide hormones to regulate cellular expression of target genes is presently an area of intensive investigation. A number of transcriptionally active peptide hormones interact with cell surface receptors that are linked with specific heterotrimeric G proteins, leading to the prediction that G proteins should reside on the pathways of action on gene expression of this class of peptide hormones. For a given G protein, a prerequisite for this proposed role is a demonstration that it can specifically regulate transcription of its putative target gene(s). We have initiated studies in this area by investigating whether a constitutively active form of one class of G protein α subunit, αs, can stimulate transcription directed by the prolactin promoter.

The observation that transfection into GH3 cells of an expression vector for the constitutively active form of αs, Q227L-αs, stimulated transient expression of cotransfected construct (-1957)PRL-CAT (**Fig.5**) shows that activated αs can stimulate activity directed by the prolactin promoter region. This effect was specific for the activated form of αs, since expression of (-1957)PRL-CAT was essentially unaffected by cotransfection of an expression vector for wild-type αs. The latter negative result implies that in these experiments the exogenous wild-type αs remained inactive following introduction into the GH3 cells, probably because the medium employed does not contain effective concentrations of ligands capable of activating αs-linked receptors present on the cells. This would also explain how effects of the exogenous constitutively active αs were observed in the presence of the wild-type αs subunits known to be present in GH3 cells (Paulssen et al. 1992).

The observation that Q227L-αs stimulated expression of construct (-187)PRL-CAT (**Fig. 6B**) shows that the proximal prolactin promoter region can direct a response to αs. The prolactin promoter specificity of the stimulatory effects of constitutively active αs was also demonstrated in the same experiment by the ability of Q227L-αs to significantly increase expression of (-187)PRL-CAT but not of similar constructs containing either the first 244 base pairs of the rat growth hormone promoter or thymidine kinase promoter. Since activating mutations in the gene for αs have been identified in human growth hormone-secreting pituitary tumors (Landis et al. 1989), it is interesting that in the present experiments Q227L-αs was observed to yield a significant stimulation of rat prolactin activity but only a slight, statistically insignificant stimulation of rat growth hormone promoter activity (**Fig. 6B**). However, Bourne's group (Landis et al. 1990) has found that patients whose pituitary tumors contain mutant constitutively active αs protein actually secrete less growth hormone (due to a smaller tumor size) than do patients whose pituitary tumors contain wild-type αs. More significantly, the same studies showed that some patients whose tumors contain mutant αs protein also display hyperprolactinemia, consistent with the possibility that some naturally occurring αs mutants might have effects on the human prolactin promoter similar to those observed in the present studies with the rat prolactin promoter.

The action of TRH on the rat prolactin promoter has been shown previously to involve an interaction of the pituitary-specific transcription factor pit-1 with its most proximal prolactin promoter binding site, 1P (Yan, Pan et al. 1991). The present experiments with a heterologous promoter showed that site 1P is also a response element for αs (**Fig. 7**), suggesting that pit-1 may also act as a gene-proximal mediator of the action of αs on the prolactin promoter.

To regulate the PRL gene transcription in the nucleus, Q227L-αs must employ cytosolic and nuclear effectors to mediate its action. Among the possible effectors, adenylyl cyclase has been shown to be stimulated by Q227L-αs (Masters, Miller et al. 1989), and many cellular actions of αs are believed to be mediated by increasing intracellular cAMP levels (Graeme 1993). PKA is the major direct downstream target of cAMP and has been shown to stimulate PRL gene expression (Maurer 1981; Day, Walder et al. 1989). My results (**Fig. 8**) shows that expression of a mutant of the regulatory subunit of PKA (Rab), a dominant inhibitor of PKA activity (Schecterson and McKnight 1991), inhibits Q227L-αs stimulation of expression of the PRL proximal promoter, implying that PKA may mediate the stimulation by Q227L-αs of the PRL gene expression.

CREB represents the best downstream candidate for PKA-mediated stimulation by αs of PRL gene promoter. CREB is a leucine-zipper transcription factor that can bind to a CRE consensus DNA element, and confer cAMP and PKA response to gene expression in many cell systems. Recent studies have also 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 promoter activity (Peers, Monget et al. 1991; d'Emden, Okimura et al. 1992; Liang, Kim et al. 1992). These clues therefore encouraged us to investigate the role of CREB in mediating αs regulation of PRL gene transcription and in binding the CLE in PRL proximal promoter. Our results (**Fig.9**) show that K-CREB, which is a dominant negative form of wild type CREB, yielded a dramatic inhibition of αs stimulation of PRL gene expression, indicating that αs may employ CREB as the nuclear target to stimulate PRL gene expression.

The results from DNA mobility shift assays (**Fig. 10A**) shows that CREB can bind to the PRL CLE. However, since the PRL CLE (TGACGAA) contains only the left half of the palindromic sequence (TGACGTCA) found in the

canonical CRE, it exhibits a correspondingly lower affinity (10% of that of standard CRE) for CREB (**Fig.10B**) as expected. This observation is consistent with a conclusion drawn from DNAse footprinting that CREB "appears to have a rather low affinity" for the PRL CLE (Liang, Kim et al. 1992).

My results indicate that both pit-1 and CREB may be involved in mediating as stimulation of PRL gene transcription. Both the CLE and pit-1 binding sites are localized in PRL proximal promoter, which is consistent with my observation that the PRL proximal promoter alone can fully respond to as stimulation. The CLE is located between 1p and 2p, with at least 15bp separating it from either site, suggesting that CREB and pit-1 may interact with each other and together exert positive regulation of the PRL proximal promoter.

In summary, my studies demonstrate that a constitutively active G protein α subunit can specifically stimulate the activity of a target gene promoter, and that Pit-1, PKA, and CREB may all be involved in mediating αs action on PRL gene transcription. My work provides a conceptual framework for investigation of the role of G proteins in regulation of transcription via G protein-linked receptors.

Chapter 3

Constitutively Active Gq-α Stimulates Prolactin Promoter Activity via Raf

(A manuscript describing the results described in this chapter has been submitted for publication by Jun Tian, Hai-Wen Ma, and Carter Bancroft)

A. Summary

We have investigated the ability of a constitutively active Gq-α mutatnt, Q209L-αq, to regulate target gene expression. Transient expression in GH3 pituitary cells of a rat proximal prolactin promoter-chloramphenicol acetyltransferase construct, (-187)PRL-CAT, was stimulated by co-expression Q209L-αq, but not by wild-type αq. Q209L-αq did not stimulate expression of control constructs containing the thymidine kinase promoter. Thus, transcriptional effects of αq are specific both for the activated state of this G-α subunit and the promoter examined. Q209L-αq stimulated expression of constructs driven by the proximal promoters for either rat prolactin or growth hormone, both of which are known to be activated by the pituitary cell-specific transcription factor pit-1. Two copies of a prolactin promoter pit-1 binding site, site 1p, conferred upon a heterologous metallothionein promoter a response to Q209L-αq, implying an involvement of a pit-1 binding site in the transcriptional action of Q209L-αq. The phorbol ester activator of protein kinase C, 12-O-tetradecanoylphorbol-13-acetate, stimulated (-187)PRL-CAT activity, but opposed the action of Q209L-αq on activity of this PRL-CAT construct. Q209L-αq stimulation of (-187)PRL-CAT activity was strongly inhibited by co-expression of a dominant negative Raf mutant, Raf-C4, but not by a point mutant of Raf-C4 with reduced inhibitory properties. These results imply that activated αq subunits can stimulate prolactin promoter activity via a pathway that involves a pit-1 DNA binding site(s), is opposed by protein kinase C, and is mediated by Raf.

B. Introduction

A great deal of information has recently been obtained about the mechanisms whereby heterotrimeric G proteins mediate transduction of extracellular signals, via hormone receptors, into regulation of such extranuclear cellular activities as kinases and other effectors such as enzymes and ion channels (reviewed in Graeme 1993; Watson et al. 1994) .Constitutively active G protein subunit mutants have been very usefull in these studies, since activity of such a mutant is independent of interaction of a particular G protein-coupled receptor with either its ligand or the G protein under study. Although numerous G protein-coupled receptors are also known to regulate transcription of target genes, there has been little corresponding direct evidence concerning the ability of G protein subunits to regulate specific gene expression. Very recently, however, constitutively active G protein subunit mutants have begun to be employed to obtain information in this area. In the first such study, a constitutively active mutant αs subunit was shown to stimulate prolactin promoter activity in the rat pituitary GH3 cells (Tian et al. 1994). It was subsequently reported that activity of this promoter can be inhibited by constitutively active mutants of αi2 or αo (Lew, Yao et al. 1994).

One type of G-α subunit, αq, has been shown to activate phospholipase C enzymatic activity in vitro (Sternweis et al. 1992; Hepler et al. 1993). In addition, cellular expression of constitutively active αq mutants has been found to stimulate phospholipase C activity and to induce malignant transformation of cultured cells (Conklin et al. 1992; De Vivo, Chen et al. 1992; Kalinec et al. 1992). Previous observations that a member(s) of the αq family couple the thyrotropin-releasing hormone (TRH) receptor to extranuclear events in the GH3 cells, including phosphoinositide hydrosis (Aragay, Katz et al. 1992; Hsieh and

Martin 1992) and Ca^{2+} channel activity (Gollasch et al. 1993) suggest that the ability of TRH to stimulate prolactin promoter activity in these cells (Murdoch, Franco et al. 1983; Yan, Pan et al. 1991) might also be mediated by αq. However, nothing is presently known about either the ability of activated αq subunits to regulate specific gene expression, or the possible pathways involved in such an action. In the present studies, we have investigated whether a constitutively active αq mutant, Q209L-αq, can regulate pituitary-specific gene expression. We report here that transient expression in GH3 cells of Q209L-αq stimulates expression of co-transfected prolactin or growth hormone promoter constructs, and that a binding site for the pituitary-specific transcription factor pit-1 can act as an αq response element. Our findings further imply that stimulation by Q209L-αq of the prolactin promoter is mediated by a cellular pathway that is opposed by protein kinase C and mediated by Raf.

C. Material and Methods

1. Sources of Recombinant Plasmids

Cytomegalovirus (CMV)-based vectors containing G-α coding sequences were constructed as follows. The coding sequence for Q205L-α_o in plasmid pAGA (obtained from Dr. Juan Codina, Baylor College of Medicine) was excised with Eco R1 plus Hind III. Following addition of a Xba I adapter to the Hind III site and digestion with Xba I, the excised sequence was ligated by cohesive end ligation into the multiple cloning site of plasmid pRc/CMV (Invitrogen Corp.) to yield plasmid CMV-Q205L-α_o. The coding sequence for αq was excised from plasmid PMN-αq (De Vivo, Chen et al. 1992) with Hind III and Apa I, and cloned into pRc/CMV as above to yield plasmid CMV-αq. The coding sequence for Q209L-αq in plasmid pAGA (also obtained from Dr. Juan Codina) was excised in two pieces by cuts with Eco R1 and Xho I, or Xho I and Xba I, followed by triple cohesive end ligation of the two pieces plus pRc/CMV, to yield plasmid CMV-Q209L-αq. The correct sequences of all of the resultant plasmids were ascertained by sequencing of the splice junctions. Empty plasmid pRc/CMV was employed to equalize overall amounts of DNA transfected. We have described previously the construction of (-187)PRL-CAT (Lufkin, Jackson et al. 1989), (-244)GH-CAT (Pan, Liu et al. 1990), and (-39)mMT-CAT and $(1P)^2$mMT-CAT (Yan, Pan et al. 1991), and the source of TK-CAT (Jackson and Bancroft 1988). Plasmid RSV-C4B, RSV-C4m17B, and RSV-BXB were kindly supplied by Dr. Ulf R. Rapp (National Cancer Institute) (Bruder et al. 1992). The former two constructs are identical to the constructs RSV-C4 and RSV-C4pm17 described in (Bruder, Heidecker et al. 1992), except for the inclusion of a C-terminal B-Raf antigen tag, which does not affect their gene regulatory properties (Tom Beck and Ulf Rapp, personal communication).

2. Transfection and Culture of GH3 Cells

Cells growing in suspension culture as described (Coleman and Bancroft 1993) were pelleted and resuspended in Dulbecco's Modified Eagle's Medium plus 5% fetal calf serum (DMEM/5%FCS) ($5x10^6$ cells/0.5 ml) in the presence of DNA to be transfected, and pulsed in a 0.4 cm path-length cuvette at 960μF/350 V. The cells were then promptly diluted into 15ml DMEM/5%FCS, distributed into three 60 mm tissue culture dishes, and incubated one day at 37^0/5% CO_2.

3. Measurement of CAT Activity and Correction for Transfection Efficiency

CAT enzymatic activity was measured by the phase-extraction assay as described (Seed et al. 1988), except that cold chloramphenicol was omitted from the assay in order to yield sufficient specific activity of the [^{3}H]-chloramphenicol substrate. All assays were performed under predetermined linear conditions. The internal control plasmid pTKGH (10mg) was included in all transfections. Human growth hormone (hGH) in the medium was assayed as recommended by the supplier (Nichols Institute, San Juan Capistrano, CA), and the result employed to correct the CAT activity observed in each dish for any variations in transfection efficiency.

D. Results

Activity of the prolactin promoter is influenced by two regions upstream of the cap site: a distal enhancer ~500 base pairs upstream of this site, and the proximal region encompassing the first ~200 bases of the promoter (d'Emden, Okimura et al. 1992), and references therein). Since we have found that the proximal promoter can direct a complete response to either TRH (Yan, Pan et al. 1991) or Gs-α (Tian, Chen et al. 1994), we began the present studies by investigating the ability of transiently expressed wild type or mutant αq subunits to regulate co-transfected construct (-187)PRL-CAT, containing the proximal prolactin promoter region cloned in front of the CAT gene. As illustrated in **Fig. 11A**, expression of wild-type αq yielded no increase in CAT enzymatic activity over that seen in control cells. However, expression of mutant Q209L-αq (αq*) yielded a sizable stimulation of expression of (-187)PRL-CAT. This stimulation exhibited promoter specificity, since Q209L-αq did not affect expression of a TK-CAT control contruct (**Fig. 11B**). The inability of Q209-αq to stimulate the thymidine kinase (TK) promoter was also demonstrated by the activity in multiple experiments of plasmid pTKGH, routinely employed to normalize transfection efficiency, since the ration of hGH released from cells transfected with Q209L-αq to hGH released from control cells was 0.91 ± SE 0.27 (n=10). Thus, expression in GH3 cells of a constitutively active form of αq yields a stimulation of prolactin promoter activity that is both specific for the activated form of this G-α subunit and promoter-specific.

Previous studies have implicated a binding site for the pituitary-specific transcription factor pit-1 in transcription stimulation by another G-α subunut, αs (Tian, Chen et al. 1994). To begin to investigate whether this is also the case for αq, we compared the ability of Q209L-αq to regulate expression of CAT

constructs driven by the prolactin and growth hormone promoters, each of which is trans-activated by pit-1 (Mangalam, Albert et al. 1989; Fox, Jong et al. 1990) and contains multiple pit-1 binding sites (Nelson, Albert et al. 1988; Castrillo et al. 1989), with the results shown in **Fig. 12**. As before, Q209L-αq (αq*) yielded a strong stimulation of expression of (-187)PRL-CAT. This constitutively active form of αq also yielded a strong but somewhat reduced stimulation of expression of (-244)GH-CAT. In multiple experiments of this type we have consistently found that Q209L-αq stimulates (-244)GH-CAT expression, but less strongly than its stimulation of (-187)PRL-CAT expression (data not shown). It also seen in **Fig. 12** that expression of either promoter-CAT construct was not stimulated (and was in fact slightly decreased) by another type of constitutively active a subunit, Q209L-α_o (Kroll, Chen et al. 1992), implying that the results with Q209L-αq are not due simply to a non-specific effect of expression in the GH3 cells of a constitutively active α subunit.

The observation that Q209L-αq can stimulate activity of either the prolactin or growth hormone promoter is consistent with an involvement of a pit-1 binding site in a transcriptional response to active αq. The proximal prolactin promoter contains four pit-1 binding sites (Nelson, Albert et al. 1988). It has been shown that the most proximal of these DNA elements, termed site 1P, can mediate a response to a number of agents, including thyrotropin-releasing hormone (TRH) and Ca^{2+} (Yan and Bancroft 1991), αs (Tian, Chen et al. 1994), and epidermal growth factor and phorbol esters (Elsholtz, Mangalam et al. 1986), As noted above, it has also been demonstrated that a member(s) of the Gq-α family couples the TRH receptor to extranuclear events in the GH3 cells, including phosphoinositide hydrolysis (Aragay, Katz et al. 1992; Hsieh and Martin 1992) and Ca^{2+} channel activity (Gollasch, Kleuss et al. 1993). Taken together, these observations suggested that site 1P might also act as a

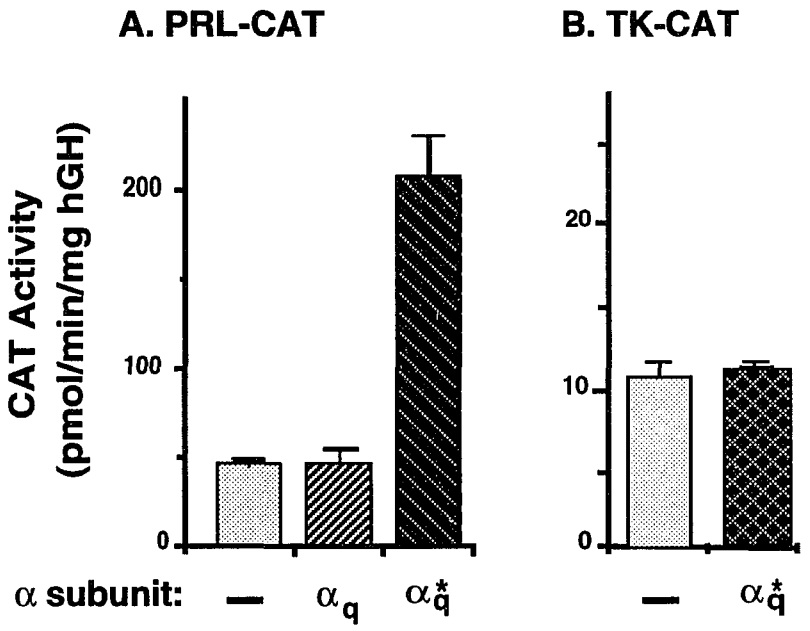

Figure 11. **Effect of wild-type or mutant αq on expression of co-transfected promoter-CAT constructs.** GH3 cells were transfected with either 20μg (-187)PRL-CAT(A) or 10μg TK-CAT (B), plus 10μg pTKGH, plus 10μg either pRc/CMV (-), CMV-αq (αq), or CMV-Q209L-αq (αq*), incubated one day, and cell extracts and media were assayed for, respectively, CAT activity and hGH levels. Results represent the mean ± SD of results with triplicate dishes, and are representative of either eight (A) or two (B) independent experiments.

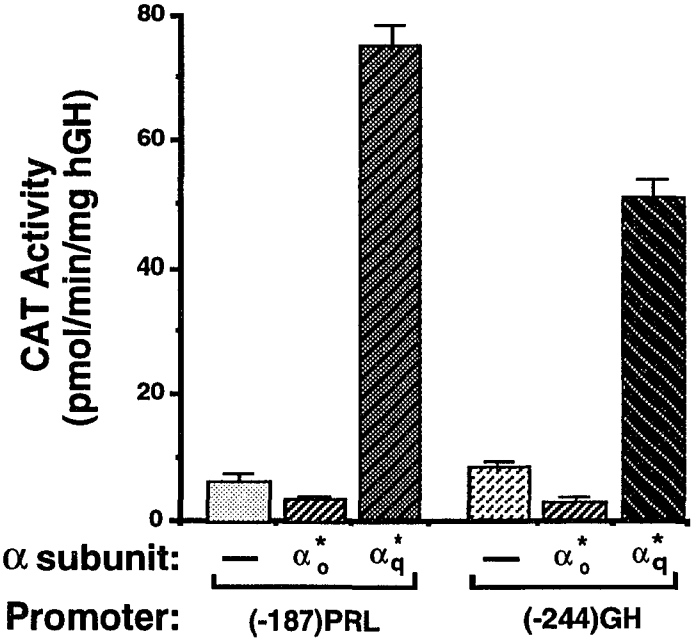

Figure 12. **Regulation by wild-type or mutant G-α subunits of co-transfected PRL-CAT or GH-CAT constructs.** GH3 cells were transfected with 10 μg either (-187)PRL-CAT or (-244)GH-CAT, plus 10μg pTKGH, plus 10 μg either pRc/CMV (-), CMV-Q205L-α_o (α_q*), or CMV-Q209L-αq (α_q*). Following a one day incubation, assays were performed as in Fig. 11. Results present the mean ± SD of results with triplicate dishes, and are representative of three independent experiments.

response element for αq. To investigate this possibility, we examined the ability of site 1P to transfer a TRH response to a heterologous mouse metallothionein (mMT) promoter. (Two tandem copies of site 1P were employed, yielding construct $(1P)^2$(-39)mMT-CAT, since at least this number is required to transfer a TRH response (Yan, Pan et al. 1991). It is seen in **Fig. 13** that neither wild-type αq nor Q205L-α_o (αo*) stimulated expression of either the parental (-39)mMT construct or of $(1P)^2$(-39)mMT-CAT. By contrast, while Q209L-αq (αq*) also yielded only a very slight stimulation of (-39)mMT-CAT expression, this constitutively active αq mutant yielded a strong -3-fold stimulation of expression of $(1P)^2$(-39)mMT-CAT. These results thus imply that prolactin promoter site 1P canserve as a heterologous response element for activated αq.

Constitutively active αq mutants have been shown to activate an intermediate in cellular activation of protein kinase C, phospholipase C (Conklin, Chabre et al. 1992; De Vivo, Chen et al. 1992), suggesting the possible involvement of protein kinase C in the action of activated αq on the prolactin promoter. However, in preliminary experiments of the type described above we found that a protein kinase C inhibitor, H-7 (Hidaka et al. 1992), did not significantly inhibit Q209L-αq stimulation of (-187)PRL-CAT activity (data not shown), suggesting that the action of activated αq on the prolactin promoter may not be mediated by protein kinase C. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is known to activate either cellular prolactin mRNA levels (Jackson et al. 1990) or prolactin promoter activity (Oberwetter et al. 1993) via activation of protein kinase C. Therefore, in order to investigate further a possible involvement of protein kinase C in the action of activated αq, we examined the actions of TPA and αq*, separately or together, on activity of the prolactin promoter. In agreement with previous studies

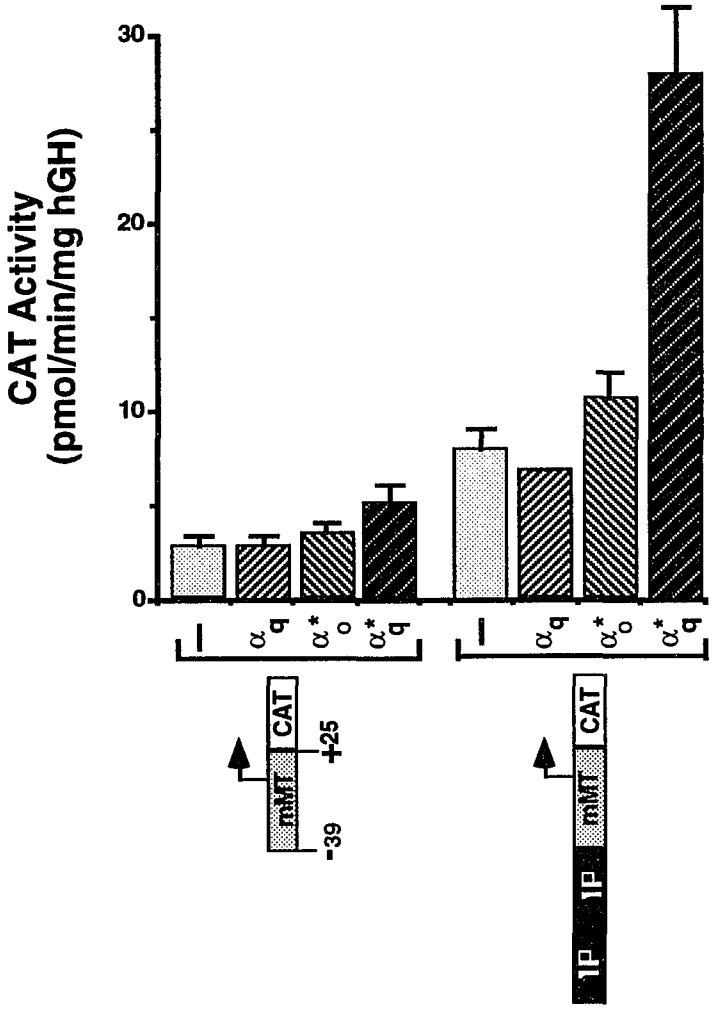

Figure 13. **Ability of site 1P to direct a response to activated α_q.** GH3 cells were transfected with 20 μg mMT-CAT or $(1P)^2$mMT-CAT, plus 10 μg pTKGH, plus 10 μg either pRc/CMV (-), CMV-α_q (α_q), CMV-Q205L-α_o (α_o*), or CMV-Q209L-α_q (α_q*). Following one day incubation, assays were performed as in Fig. 11. Results represent the mean ± SD of results with triplicate dishes, and are representative of two independent experiments.

(Oberwetter, Conrad et al. 1993), TPA alone yielded a dose-dependent stimulation of (-187)PRL-CAT activity, reaching maximal stimulation at approximately 200nM TPA (**Fig. 14, upper panel**). However, it is seen in **Fig. 14 (lower panel)** that TPA yielded a dose-dependent inhibition of the fold stimulation by Q209L-αq of (-187)PRL-CAT activity, such that at concentrations where TPA alone yielded maximal stimulation of CAT activity (≥200nM), the stimulation by Q209L-αq was completely abolished. These observations imply not only that C kinase activity is unlikely to be a mediator of the action of activated αq on the prolactin promoter, but also that activation of C kinase can actually oppose this αq transcriptional pathway.

Recent studies have shown that activated αq can stimulate activity of mitogen-activated protein kinase (MAPK) activity in COS cells (Qian et al. 1993; Faure, Voyno Yasenetskaya et al. 1994). In addition, it has recently been demonstrated that oncogenic Ras can stimulate prolactin promoter activity in GH cells via a pathway that involves activation of Raf, and that is antagonized by TPA (Oberwetter, Conrad et al. 1993; Conrad et al. 1994). These observations, plus the results presented above, suggested the possible involvement of such a pathway in stimulation by activated αq of prolactin promoter activity. To investigate this concept, we examined whether co-expression of a dominant negative Raf mutant, Raf-C4 (Bruder, Heidecker et al. 1992) can inhibit stimulation by Q209L-αq (αq*) of (-187)PRL-CAT activity (**Fig. 15A**). As before, wild-type αq and Q209L-αq (αq*) exhibited, respectively, little effect on or a strong stimulation of CAT activity. Raf-C4 alone slightly inhibited CAT activity. However, Raf-C4 was observed to strongly inhibit the stimulation by Q209L-αq of CAT activity. In addition, while a constitutively active form of Raf, Raf-BXB (Bruder, Heidecker et al. 1992) stimulated CAT activity, in agreement with previous observations(Conrad, Oberwetter et al. 1994), stimulation by

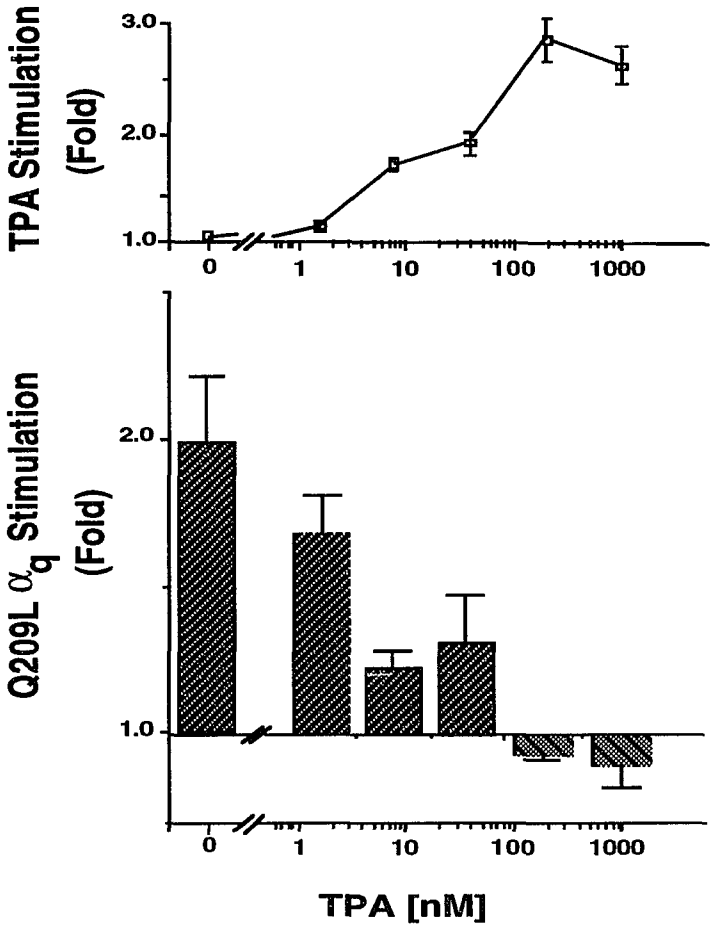

Figure 14. **TPA stimulate prolactin promoter activity but opposes the stimulation by Q209L-α_q.** GH3 cells were transfected with 10 μg (-187)PRL-CAT plus 10 μg pTKGH, plus 10 μg either pRc/CMV or CMV-Q209L-α_q. After one hour, some dishes received the indicated final concentrations of TPA. Following a one day incubation, assays were performed as in Fig. 11. Results represent the mean ± SD of fold stimulation observed with triplicate dishes, and are representative of five independent experiments. (A) Shown is the fold stimulation by TPA relative to control cells incubated without TPA, of CAT activity corrected for medium hGH, all with cells transfected with pRc/CMV. (B) Shown is the ratio, at the indicated TPA concentration, of CAT activity corrected for medium hGH in cells transfected with CMV-Q209L-α_q relative to cells transfected with pRc/CMV.

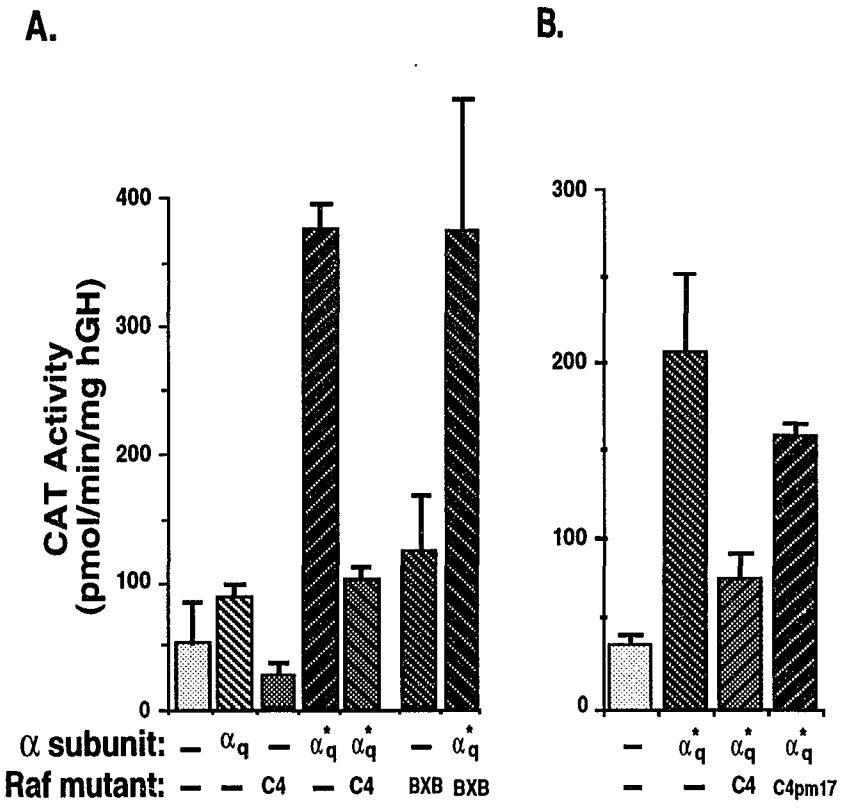

Figure 15. **A dominant negative Raf mutant can block stimulation by activated α_q of the prolactin promoter.** GH3 cells were transfected with 20 μg (-187)PRL-CAT plus 10 μg pTKGH, plus 10 μg either pRc/CMV (upper-), CMV-α_q (α_q), or CMV-Q209L-α_q (α_q*), plus 10 μg either pRc/CMV (lower-), RSV-C4B (C4), RSV-BXB (BXB), or RSV-C4pm17B (C4pm17), Following a one day incubation, assays were performed as in Fig. 11. Results represent the mean ± SD of fold stimulation observed with triplicate dishes, and are representative of three independent experiments.

Q209L-αq plus BXB was no higher than by activated αq alone, suggesting a shared mechanism of action for these agents, The specificity for Raf of the results with the dominant negative Raf inhibitor Raf-C4 was investigated with a Raf-C4 point mutant, C4pm17, which exhibits strongly reduced inhibition of Raf-mediated stimulation of reporter constructs compared to RafC4 (Bruder, Heidecker et al. 1992) (**Fig. 15B**). while, as above, Raf-C4 strongly inhibited Q209L-aq stimulation of (-187)PRL-CAT, this stimulation was only slightly inhibited by Raf-C4pm17, indicating the specificity of the effects observed with Raf-C4. These results thus imply that Raf is an intermediate in the action of activated αq on the prolactin promoter.

E. Discussion

In the present studies we have found that transient expression in GH3 rat pituitary cells of the constitutively active αq mutant Q209L-αq can stimulate expression directed by either the prolactin promoter or, to a lesser extent, the growth hormone promoter. Stimulation of prolactin promoter activity was specific for the active form of αq, since this promoter was not stimulated by either wild-type αq (**Fig. 11 and 15**) or a constitutively active mutant of another α subunit, Q205L-α_O (**Fig. 12**). The exogenously expressed control wild-type αq subunits apparently remained inactive under the conditions employed here, either because the medium employed contains ineffective concentrations of ligands for αq-coupled receptors on the GH3 cells, or because of an inability of the exogenously expressed αq subunits to couple to these receptors. The transcriptional stimulation by Q209L-αq also exhibited promoter specificity, since this mutant did not affect expression directed by the thymidine kinase promoter (**Fig. 11** and text). The observation that constitutively active but not wild-type αq can stimulate activity of specific pituitary-specific promoters implies the existence of a cellular pathway(s) for transduction by αq subunits of a signal leading from a Gq-coupled receptor to its target gene(s).

The observation that activated αq can stimulate both the prolactin and growth hormone promoters, each of which contain pit-1 binding sites, led to the prediction that a Gq-α response element might involve a binding site for this transcription factor. The observation that the most proximal pit-1 binding site in the prolactin promoter, site 1P, can direct a response to activated αq (**Fig.13**) supports this concept. Taken together with previous investigations showing that site 1P can also act as a TRH response element (Yan, Pan et al. 1991), these observations are consistent with a role for αq in the transcriptional action of TRH

on the prolactin promoter. It is interesting to note that site 1P has also been found to direct a response to a number of other activated G-α subunits, including α_s (Tian, Chen et al. 1994) and α_{i2} or α_o (Lew, Yao et al. 1994), suggesting that the transcriptional pathways mediated by a number of different classes of α subunits can converge on the same regulatory DNA element.

Gq-α exerts a number of its cellular actions via activation of phospholipase C (Aragay, Katz et al. 1992; Hsieh and Martin 1992; Sternweis, Smrcka et al. 1992). In GH3 cells, activation of phospholipase C following interaction of TRH with its αq-coupled receptor results in transient activation of protein kinase C and of Ca2+ mobilization, resulting in stimulated secretion of prolactin (Gershengorn 1982; Martin et al. 1990). In addition, the phorbol ester TPA has been found previously to stimulate either cellular prolactin mRNA levels (Jackson, Bandyopadhyay et al. 1990) or prolactin promoter activity (Oberwetter, Conrad et al. 1993) via activation of protein kinase C. In agreement with the latter observations, we found in the present studies that TPA alone stimulates prolactin promoter activity in the GH3 cells (**Fig. 14A**). However, we also found that TPA yielded a dose-dependent inhibition of the stimulation by Q209L-αq of prolactin promoter activity in these cells (Fig. 14B). This observation implies that activation of protein kinase C can inhibit some step(s) in the transcriptional pathway employed by αq to stimulate the prolactin promoter.

It has been found that cellular activity of mitogen-activated protein kinase (MAPK) can be stimulated by constitutively activated mutants of a number of different types of G protein subunits, including αi (Gupta et al. 1992; Faure, Voyno Yasenetskaya et al. 1994) and αq and βγ dimers (Faure, Voyno Yasenetskaya et al. 1994). It has also been observed that either the α_i-coupled acetylcholine muscarinic m2 receptor (Winitz et al. 1993) or the G protein-coupled lysophosphatidic acid receptor (Howe et al. 1993) can activate MAPK

activity via pathways involving both Ras and Raf. The present observation that expression of a dominant negative Raf mutant inhibits stimulation by Q209L-αq of prolactin promoter activity (**Fig. 15**) implies that a similar pathway, involving Raf, is involved in transcriptional regulation by active αq subunits of prolactin gene expression. A pathway of action of Ras on the prolactin promoter in pituitary cells has very recently been described, and shown to involve sequential activation of Raf, MAPK, and a transcription factor in the ets family (Conrad, Oberwetter et al. 1994). The results presented here suggest that the transcriptional action of active αq in pituitary cells may involve a similar pathway, in which an interaction of pit-1 with one or more of its binding sites plays an important role.

In summary, the present studies demonstrate that a constitutively active form of Gq-α can stimulate specific target gene transcription. Our results also imply that the cellular pathway employed by the activated form of this G protein subunit to regulate prolactin promoter activity is inhibited by protein kinase C, is mediated by Raf, and involves a DNA binding site(s) for pit-1. It will be of interest to determine whether a Raf-mediated pathway is employed in pituitary cells for transduction of signals from a Gq-α-coupled receptor such as the TRH receptor to its specific nuclear target genes.

Chapter 4

Expression of the Constitutively Active Gs α-subunit in GH3 Pituitary Cells Stimulates Pit-1 Gene Promoter Activity

A. Summary

The pituitary-specific transcription factor Pit-1 is required for expression of both the rat prolactin and growth hormone genes. In addition, the Pit-1 gene is necessary for growth as well as differentiation of pitutary cells. However, much less is known about the regulation of Pit-1 gene expression, especially by G proteins. In this chapter, I report my studies of the action of constitutively active αs on the Pit-1 gene promoter. The constitutively active αs stimulated expression of the Δ5'-200GHF1-CAT construct, but this stimulation was reduced by the mutation of the Pit-1 binding site in the Pit-1 gene promoter. This result is consistent with my previous observation that site 1P can direct αs action on the prolactin gene promoter (Chapter **2**), and implies that a Pit-1 binding site can serve as a cis response element and Pit-1 as a trans mediator of αs action. To further understand the intracellular mechanism through which αs stimulates Pit-1 gene expression, a mutant regulatory subuit of protein kinase A (PKA), which contains an inactive cAMP binding domain and thus suppresses cAMP dependent PKA activation, was co-expressed with Δ5'-200GHF1-CAT construct. This dominant negative PKA subunit inhibited the Q227L-αs stimulation of the Pit-1 gene promoter, indicating that PKA may be involved in the signal transduction pathway of αs action on the Pit-1 gene promoter. In addition, k-CREB, a dominant negative inhibitor of CREB, inhibited αs stimulation of the Pit-1 gene promoter, suggesting that the PKA action was mediated by a member of the CREB/ATF family.

B. Introduction

The Pit-1 gene is a member of a large family of genes that encode proteins which contain a highly homologous region, referred to as the POU domain (Ruvkun et al. 1991) and thus are involved in the regulation of development. The Pit-1 gene encodes a protein of 291 amino acids that is required for generation of somatotropes and lactotropes during the pituitary devlopment. Pit-1 is very abundant in the nuclei of mature somatotrophs, lactotrophs and thyrotrophs and is responsible for the expression of both the growth hormone (GH) and prolactin (PRL) gene. Mice with naturally occuring mutations or deletions of the Pit-1 gene fail to develop somatotropes and lactotropes and exhibit anterior pituitary hypoplasia and dwarfism (Li, Crenshaw et al. 1990; Castrillo, Theill et al. 1991). In addition, antisense oligonucleotide of the pit-1 gene, which specifically inhibited the Pit-1 protein synthesis, resulted in a marked decrease in growth and GH and PRL expression in tumor cells (Castrillo, Theill et al. 1991).

Previous studies of Pit-1 gene regulation were 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 own expression by binding to the Pit-1 binding element in its promoter. Mutation of the Pit-1 binding site abolished the positive autoregulation (Chen, Ingraham et al. 1990; McCormick, Brady et al. 1990). The Pit-1 promoter also contains two binding sites for the ubiquitous CREB protein, which is responsible for induction of pit-1 gene transcription in response to cAMP (Chen, Ingraham et al. 1990; McCormick, Brady et al. 1990). CREB is thought to be involved in stimulating expression of Pit-1 in response to the hypothalamic growth hormone releasing factor. On the other hand, the Pit-1 gene promoter is

negatively regulated by dopamine. Deletion of both CRE elements in the pit-1 promoter only paritially reduced the response to dopamine (Elsholtz, Lew et al. 1991).

However, the intracellular signaling pathways that mediate hormonal regulation of Pit-1 gene expression is still unclear. Cellular expression of a constitutively active α subunit mutants, Q227L-αs, has been shown to continuously activate adenylyl cyclase (Masters, Miller et al. 1989), Furthermore, a similar mutation in the α subunit, Q227R, has been found in a subset of human pituitary tumors (Landis, Masters et al. 1989), suggesting the importance of G protein in regulating Pit-1 gene expression. To investigate whether constitutively active αs stimulates the Pit-1 gene promoter, I used the approaches similar to those employed in Chapter **2**. These studies were carried out in collaboration with Christian Gaiddon, a graduate student visiting the Bancroft laboratory from Dr. Jean-Philippe Loeffler's laboratory in France.

C. Material and methods

1.Sources of Recombinant Plasmids

Plasmids CMV-α_s and CMV-Q227L-α_s were constructed by Jianghao Chen in the laboratory of Dr. Ravi Iyengar. Plasmid pSV2-G226A-αs was provided by Dr. Jean-Philippe Loeffler (Gaiddon et al. 1995), Δ5'-200GHF1-CAT and Δ5'-200.1GHF1-CAT were provided by Dr. Michael Karin (McCormick, Brady et al. 1990), RSV-K-CREB was provided by Dr. Richard H. Goodman (Walton, Rehfuss et al. 1992), RSV-CREB was provided by Dr. Marc R. Montminy (Gonzalez and Montminy 1989), HL-REVwtneo. (expressing the mouse PKA regulatory subunit) and HL-REVabneo. (expressing the mutant PKA regulatory subunit) were provided by Dr. Stanley G. McKnight (Uhler and McKnight 1987; Schecterson and McKnight 1991), and pRc/CMV, which was used to equalize the overall amount of DNA transfected, from Invitrogen Corp.

2.Transfection and Culture of GH3 Cells

Cells growing in suspension culture as described before (Coleman and Bancroft 1993) were seeded into 6 well tissue culture dishes (0.75 x 10^6/dish) one day before they were transfected. Cells were then serum-deprived for 12 h. DNA (1 or 2 μg/well) in 50 ml of NaCl 150 mM was mixed with (6 or 8 ml respectively) a 2mM ethanolic solution of dioctadecylaminoglycylspermine (Transfectam™) in 50 ml of 150 mM NaCl. After 10 min, the mixture was brought to a 1 x final concentration of DMEM, and the transfectant solution was applied overnight to the cells. Cells were rinsed, cultured in DMEM/10%FCS for 12 h, and treated or not for another 12 h (Gaiddon, Boutillier et al. 1995), and then harvested for CAT assay.

3. Measurement of CAT Activity

CAT enzymatic activity was measured by the phase-extraction assay as described (Seed and Sheen 1988), except that cold chloramphenicol was omitted from the assay in order to yield sufficient specific activity of the [3H]-chloramphenicol substrate. All assays were performed under predetermined linear conditions.

D. Results

I first examined whether the Pit-1 gene promoter is subject to regulation by the Gs α subunit. Transient transfection assays were performed to test the action of the wild type or mutant αs subunits on the co-transfected construct Δ5'-200GHF1-CAT which contains the first 200 bp of the proximal Pit-1 gene promoter region cloned in front of the CAT gene. As shown in **Fig. 16**, expression of wild-type αs generated a slight increase in CAT enzymatic activity over that seen in control cells. However, expression of mutant Q227L-αs (αs*) yielded about 12 fold stimulation of Δ5'-200GHF1-CAT expression. Thus, the expression in GH3 cells of a constitutively active αs mutant can stimulate the transcription of the Pit-1 gene proximal promoter.

Previous studies have shown that the Pit-1 binding site is important for the transcription of its own gene. Pit-1 autoregulates Pit-1 gene expression and mutation of the 5' Pit-1 binding site abolished this positive autoregulation (Chen, Ingraham et al. 1990; McCormick, Brady et al. 1990). In addition, a Pit-1 binding site was shown to be important in mediating both Gsα and Gqα subunit stimulation of prolactin gene promoter activity (Tian, Chen et al. 1994; Tian et al. 1995). To investigate whether a Pit-1 binding site also mediates the Gαs action on the Pit-1 gene promoter, Q227L-αs stimulation of Δ5'-200GHF1-CAT was compared with Δ5'-200.1GHF1-CAT that contains a mutated Pit-1 binding site. Plasmid pSV2-G226A-αs which encodes an inactive Gs α subunit was used (Gaiddon, Boutillier et al. 1995) as a control for any unspecific effects of protein overexpression. As expected, **Fig. 17** showed that the inactive mutant G226A-αs stimulated neither Δ5'-200GHF1-CAT nor Δ5'-200.1GHF1-CAT expression when compared to control cells. In addition, Q227L-αs (αs*) displayed a 11 fold stimulation expression of Δ5'-200.1GHF1-CAT, while Q227L-αs (αs*) yielded a

35 fold stimulation of Δ5'-200GHF1-CAT, implying that the Pit-1 binding site of the Pit-1 gene promoter is partially required for a response to αs.

Next, I investigated the molecular mechanisms of the αs action on the Pit-1 gene promoter. Earlier studies have shown that Q227L-αs can activate adenylyl cyclase (Masters, Miller et al. 1989) and that the Pit-1 gene promoter is induced by forskolin (McCormick, Brady et al. 1990). Since PKA is generally thought to be the downstream target of adenylyl cyclase and forskolin in many transcription systems, I therefore investigated whether PKA lies in the signaling pathway of αs action on Pit-1 gene transcription (**Fig. 18**). In these studies I used an expression vector that encodes a mutant PKA regulatory subunit, Rab, which cannot bind cAMP and is thus a dominant negative inhibitor of PKA (Schecterson and McKnight 1991). Expression of Rab strongly reduced the αs stimulation of expression of the Δ5'-200GHF1-CAT construct. However, Rab did not chanage the basal level expression of the Δ5'-200GHF1-CAT construct (data not shown), indicating that the blockade effect of Rab is specific to the αs action. These results suggested that PKA is involved in the signaling pathway of αs on Pit-1 gene expression.

Since the cAMP-regulated transcription factor CREB is the gene-proximal target of PKA in many transcription systems, and DNase footprinting has demonstrated the binding of CREB at two separate sites in the Pit-1 gene promoter (Chen, Ingraham et al. 1990; McCormick, Brady et al. 1990) which respond to forskolin induction (McCormick, Brady et al. 1990), I therefore examined the role of CREB in mediating as action on Pit-1 gene transcription. I employed the dominant negative CREB mutant (k-CREB) which carries a single point mutation that changes leucine-287 to arginine-287 within the DNA binding domain. This k-CREB does not bind to DNA as a homodimer, but competes with CREB for binding to the CRE site within the somatostatin promoter

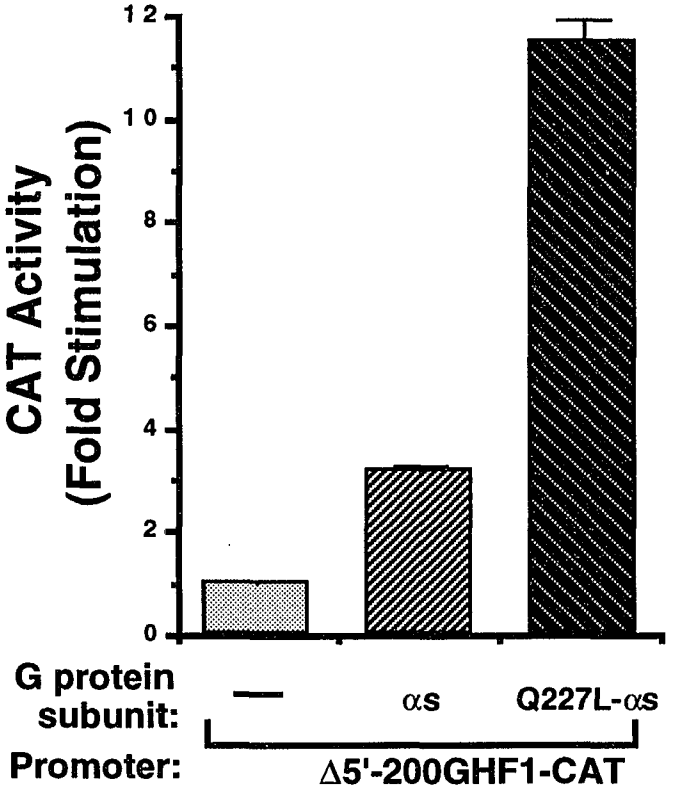

Figure 16. **Wild-type or mutant αs regulation of the expression of the Pit-1 promoter.** GH3 cells were transfected via Transfectam with 1.5 μg of Δ5'-200GHF1-CAT, plus 1.5 μg of pRc/CMV (control), CMV-αs, or CMV-Q227L-αs. Following 1 day incubation, cell were harvested for the assay of CAT activity. Data represented the mean ± SD from the results of triplicate dishes.

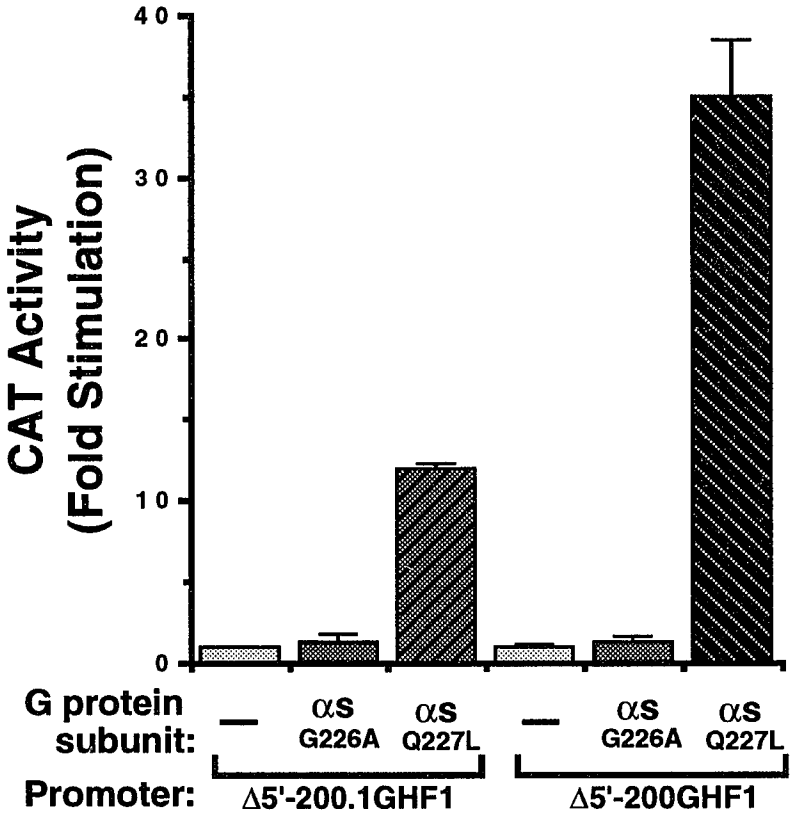

Figure 17. **Stimulation by constitutively active αs of wild type or mutant Pit-1 promoters.** GH3 cells were transfected via Transfectam with 1.5 μg of either Δ5'-200GHF1-CAT or Δ5'-200.1GHF1-CAT plus 1.5 μg of pRc/CMV (control), pSV2-G226A-αs, or CMV-Q227L-αs. Following 1 day incubation, cells were harvested for the assay of the CAT activity. Data represents the mean ± SD of the results with triplicate dishes.

(Walton, Rehfuss et al. 1992). As shown in **Fig. 19**, co-expression of Δ5'-200GHF1-CAT with the k-CREB resulted in a strong reduction of the αs stimulation of Δ5'-200GHF1-CAT. k-CREB did not affect basal expression level of Δ5'-200GHF1-CAT (data not shown), indicating that the inhibitory function of k-CREB is specific for αs stimulation.. These results implies that CREB may mediate αs action on Pit-1 gene expression.

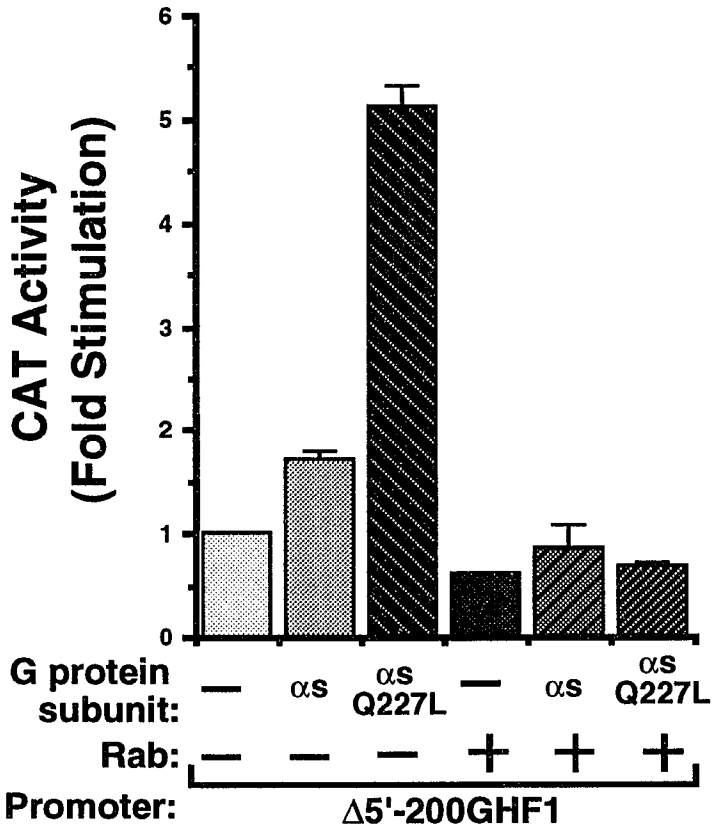

Figure 18. **A mutant PKA regulatory subunit (Rab) blocks the stimulation by αs of expression of the Pit-1 gene proximal promoter.** GH3 cells were transfected via Transfectam with 1.5 μg of Δ5'-200GHF1-CAT, plus 1.5 μg of pRc/CMV (control), CMV-αs, or with CMV-Q227L-αs, plus 1 μg of either HL-REV$_{wt}$neo (Rwt) or HL-REV$_{ab}$neo (Rab). Following 1 day incubation, cells were collected and assayed for CAT activity. Data represent the mean ± SD of results with triplicate dishes.

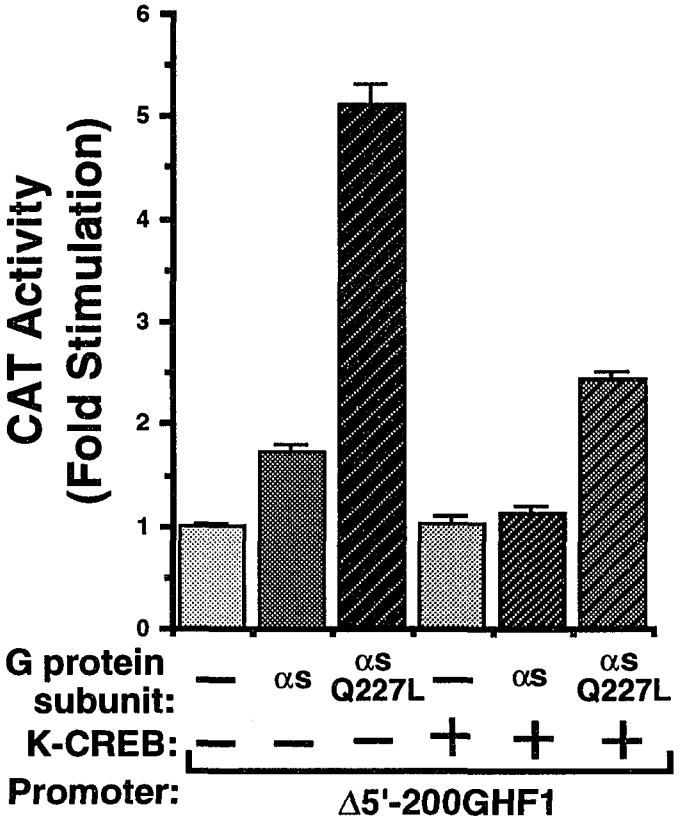

Figure 19. **A mutant CREB (K-CREB) blocks αs stimulation of the Pit-1 gene proximal promoter.** GH3 cells were transfected via Transfectam™ with 1.5 μg of Δ5'-200GHF1-CAT, plus 1.5 μg of pRc/CMV (control), CMV-αs, or with CMV-Q227L-αs, plus 1μg of pRc/RSV-k-CREB. Following 1 day incubation, cell were collected and assayed for CAT activity. Data represent the mean from triplicate dishes.

E. Discusion

Activation of the Pit-1 gene is one of the key regulatory steps in anterior pituitary develpoment. Pit-1 protein not only participates in the determination of pituitary cell fate but also regulates expression of the growth hormone and prolactin genes in differentiated pituitary cells (Li, Crenshaw et al. 1990; Castrillo, Theill et al. 1991; Andersen et al. 1994). Therefore, the mechanisms of Pit-1 gene activation are of considerable interest. But by now, few studies have been done on the regulation of Pit-1 gene expression, and serveral important questions still remain to be answered, such as what are the intracellular molecular mechanisms that relay the signals from the membrane to the nucleus to control Pit-1 gene expression, and how Pit-1 gene expression is selectively modulated.

In my study, a constitutively active Gs α subunit was used to investigate whether it can regulate Pit-1 gene expression and which signaling pathways are involved. I found that transient expression in GH3 pituitary cells of the constitutively active αs mutant Q227L-αs can stimulate expression of Δ5'-200GHF1-CAT (**Fig. 16**), and this stimulation is specific since only the constitutively active but not the wild-type αs strongly stimulates the activity of the Pit-1 gene promoter.

My previous data has shown that activated αs and αq can stimulate the prolactin gene promoter through a Pit-1 binding site, and this led to the prediction that a Pit-1 binding site may also serve as a response element for αs action on the Pit-1 gene promoter. I hence examined whether Pit-1 binding site is involved in αs action on the Pit-1 gene promoter (**Fig.17**). The observation that mutation of Pit-1 binding site in the Pit-1 gene promoter dramatically decreased αs stimulation of Pit-1 gene promoter activity confirms the critical role

of Pit-1 and its binding site. However, mutation of Pit-1 binding site still yieled some stimulation of Pit-1 gene expression in response to αs. This result is consistent with the previous studies that, although Pit-1 binding is important for optimal Pit-1 promoter activity, the basal cell-type specific activity of the Pit-1 promoter is probably due to binding of at least one other pituitary-specific factor distinct from Pit-1 (McCormick, Brady et al. 1990).

I next studied which signaling pathway is involved in αs action on the Pit-1 gene promoter. To regulate Pit-1 gene transcription in the nucleus, Q227L-αs must employ cytosolic and nuclear effectors to mediate its action. Among the possible effectors, adenylyl cyclase has been shown to be stimulated by Q227L-αs (Masters, Miller et al. 1989), and many cellular actions of αs are believed to be mediated by increasing intracellular cAMP level (Graeme 1993). Since PKA is the major direct downstream target of cAMP (Maurer 1981; Day, Walder et al. 1989), its function in mediating αs action on Pit-1 gene expression was futher studied. My results (**Fig. 18**) show that expression of a mutant regulatory subunit of PKA (Rab), which dominantly inhibits PKA activity (Schecterson and McKnight 1991), blocks the Q227L-αs stimulation of the Pit-1 proximal promoter activity, implying that PKA mediates the action of Q227L-αs on Pit-1 gene expression.

CREB is the major direct downstream target of PKA. It is a leucine-zipper transcription factor that can bind to a CRE consensus DNA element, and confer a cAMP and PKA response to gene expression in many cell systems (Habener 1990; Herschman 1991). Previous studies have also shown that, in addition to pit-1 binding sites, the Pit-1 proximal promoter contains two CRE sites. Following these clues, I investigated the role of CREB in mediating αs regulation of Pit-1 gene transcription. My results (**Fig.19**) show that K-CREB, a dominant negative CREB, yielded a dramatic inhibition of αs stimulation of Pit-1 gene expression,

indicating that αs may employ CREB as nuclear target to stimulate Pit-1 gene expression.

In summary, my studies demonstrate that a constitutively active G protein α subunit can specifically stimulate the activity of a target gene promoter, and that PKA, Pit-1, and CREB may all be involved in mediating αs action on pit-1 gene expression.

Chapter 5

Actions of Gγ and Gβ subunits on the prolactin promoter

A. Summary

The βγ subunit complex of G protein was only recently recognized to be able to directly regulate cellular functions in higher eukaryotes. My finding that γ2, but not β2 or β2 + γ2, regulates the prolactin gene promoter represents both the first study of transcriptional action of βγ in higher organisms, and the first observation of an effect of the G protein γ subunit. In addition, I found that a mutant γ2 (γ2m) that is incapable of membrane targeting does not stimulate PRL-CAT activity, indicating that the prenylated site of γ2 is necessary for its transcriptional action. The γ2 stimulation of prolactin gene expression also exhibited promoter specificity, since neither the rat growth hormone nor the thymidine kinase promoter could be activated by Gγ.

B. Introduction

A heterotrimeric G protein is composed of three distinct subunits: α, β, and γ. Studies of G proteins initially focused on the Gα subunit, which is responsible for GTP hydrolysis and downstream effector signaling. It was long thought that the biological properties of a G protein resided entirely in the α subunit following its dissociation from βγ. However, this common view was challenged in 1987 by the report that the K^+ channel could be activated by Gβγ (Logothetis, Kurachi et al. 1987). Since then, the list of Gβγ targets has steadily grown. For example, genetic analysis of the pheromone signaling pathway in budding yeast showed that Gβγ, rather than Gα, is the primary regulator of downstream events (Cole, Stone et al. 1990). Gβγ was also shown to stimulate PLC activity (Camps, Carozzi et al. 1992), members of the β-adrenergic receptor kinase (bARK) (Kameyama, Haga et al. 1993; Koch, Inglese et al. 1993), and stimulate or inhibit adenylate cyclase activity (Federman, Conklin et al. 1992; Taussig, Quarmby et al. 1993) in higher eukaryotic cells. More recently, Lefkowitz's group found that Gβγ is the primary mediator of Ras activation and subsequent signaling via MAP kinase in response to stimulation of Gi-coupled receptors (Koch et al. 1994). With these intensive studies, by now Gβγ is known to regulate as many cellular effectors as Gα (Clapham et al. 1993).

However, the Gβγ subunits are thought to exert their function as a dimer complex, and nothing is presently known about whether Gβ or Gγ can independently regulate specific gene expression in higher organisms. In this chapter, I report that γ_2, but not β2 or γ2 + β2 subunits, can stimulate transfected PRL promoter activity, and that γ2 stimulation of PRL promoter activity is promoter-specific. Furthermore, I report that an expression vector encoding a mutant γ2 that is incapable of membrane targeting does not stimulate PRL

promoter activity. These observations represent both the first study of transcriptional actions of βγ subunits, and the first observation of a possibly independent biological activity of one of these subunits in a higher organism.

C. Materials and Methods

1. Sources of recombinant Plasmids

Plasmids CMV-γ2, CMV-β2, pCDM-γ2m, and CMV-Q205L-αo were constructed as follows. The coding sequences of γ2 and β2 in plasmid pEV (obtained from Dr. Narasimhan Gautam, Washington University School of Medicine, St. Louis, Missouri) were excised with Hind III and Xba I, and the excised sequences were ligated by cohesive end ligation into the Hind III and Xba I sites of plasmid pRc/CMV to generate the plasmid CMV-γ2 and CMV-β2. The cDNA corresponding to mutant γ2 in the expression vector pCDM was obtained from Dr. William F. Simonds, National Institutes of Health (Simonds et al. 1991). The coding sequence of Q205L-α_o in plasmid pAGA (obtained from Dr. Juan Codina, Baylor College of Medicine) was excised with Eco R1 and Hind III. Following addition of a Xba I adapter to the Hind III site and digestion with Xba I, the excised sequence was ligated by cohesive end ligation into the multiple cloning site of plasmid pRc/CMV to make the plasmid CMV-Q205L-α_o. The preparation of plasmids (-187)PRL-CAT (Lufkin, Jackson et al. 1989), (-244)GH-CAT (Pan, Liu et al. 1990), and TK-CAT (Jackson and Bancroft 1988) was described previously. Plasmids pRc/CMV, which was used to equalize the overall amount of the transfected DNA, was purchased from Invitrogen Corp.

2. Transfection and Culture of GH3 Cells

Cells growing in suspension culture as described (Coleman and Bancroft 1993) were pelleted and resuspended in Dulbecco's Modified Eagle's Medium plus 5% fetal calf serum (DMEM/5%FCS) ($5x10^6$ cells/0.5 ml) in the presence of DNA to be transfected, and pulsed in a 0.4 cm path-length cuvette at 960 μF/350 V. The cells were then promptly diluted into 15 ml DMEM/5%FCS, distributed

into three 60 mm tissue culture dishes, and incubated one day at 37^0C with 5% CO_2.

3. Measurement of CAT Activity

CAT enzymatic activity was measured by the phase-extraction assay as described (Seed and Sheen 1988), except that cold chloramphenicol was omitted from the assay in order to yield sufficient specific activity of the [3H]-chloramphenicol substrate. All assays were performed under predetermined linear conditions.

D. Results

The major activity of the prolactin (PRL) promoter is determined by two regions upstream of the cap site: a distal enhancer, including ~1500 base pairs upstream of this site, and the proximal region encompassing the first ~200 base pairs (d'Emden, Okimura et al. 1992). Since our previous finding shows that the proximal promoter can direct a complete response to either TRH (Yan, Pan et al. 1991), Gs-α (Tian, Chen et al. 1994), or Gq (Tian, Ma et al. 1995), I began the investigation of G protein β or γ subunit regulation of the PRL gene transcription using the proximal promoter region of PRL. Expression in GH3 cells of (-187)PRL-CAT was stimulated about 5-fold by co-expressed γ2, but not by a constitutively active αo mutant (αo*) or by β2 (**Fig 20**), demonstrating the G protein specificity effect of γ2. Interestingly, co-expression of γ2 plus β2 did not stimulate (-187)PRL-CAT activity, suggesting that β2 may inhibit the action of γ2. Similar effects of γ2 and β2 were observed in four other independent experiments.

With this novel finding with the Gγ2 subunit, I explored further the mechanism of γ2 action on prolactin gene expression. Attachment of heterotrimeric G proteins to the inner face of the plasma membrane was previously shown to be fundamental to their role as signal transducers by allowing interaction with both receptors and effectors. Certain G protein α subunits are anchored to the membrane by covalent myristolyation (Wedegaertner et al. 1995). A series of carboxyl-terminal modifications of G protein γ subunits has been identified recently (Wedegaertner, Wilson et al. 1995). Different groups have found that mutation of prenylated sites of either yeast G protein γ subunits or Ras affected their both membrane association and biological activity (Finegold et al. 1990). In order to investigate whether the

prenylated site of γ subunit is involved in γ subunit stimulation of PRL promoter activity, I employed a mutant non-prenylated γ which is incapable of membrane targeting. As illustrated in **Fig.21**, expression of (-187)PRL-CAT was stimulated about 2-fold by co-expressed γ2, but not by either γ2m, or β2, implying that prenylation of γ is necessary for the transcriptional action of the G protein subunit.

The promoter-specificity of γ subunit stimulation was investigated by using the prolactin promoter-CAT together with two other promoter-CAT constructs (**Fig. 22**). As expected, the γ subunit generated a 3.5 fold stimulation of (-187)PRL-CAT expression, whereas the mutant γ in this case actually inhibited expression. Expression of a construct containing the proximal rat growth hormone promoter, (-244)GH-CAT, was not stimulated by either γ2 or mutant γ2. Furthermore, expression of TK-CAT was also unaffected by expression of γ2, and was very slightly increased by expression of mutant γ2. Thus, the γ2 stimulation of the proximal prolactin promoter region is promoter-specific.

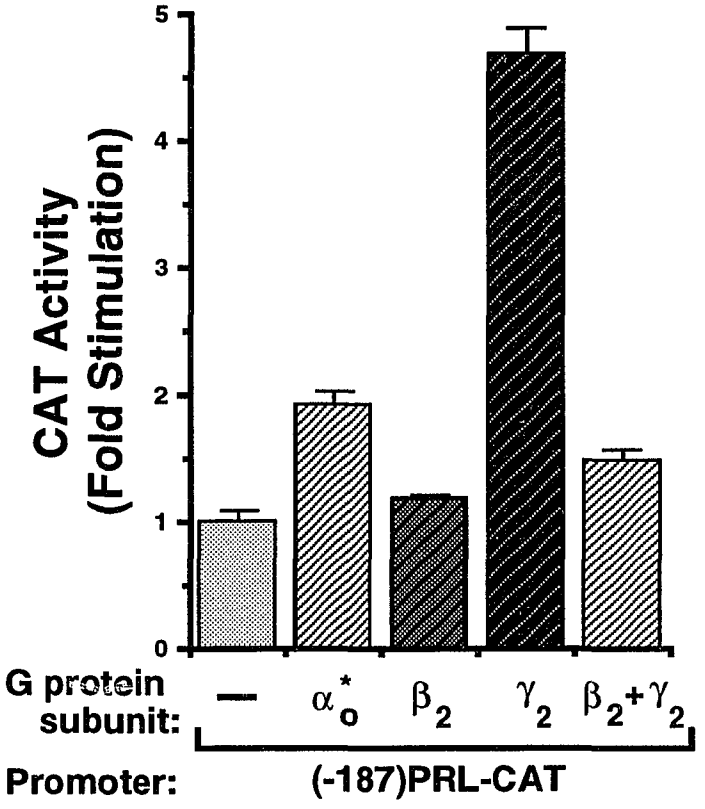

Figure 20. **Regulation of PRL promoter activity by G-β2 and G-γ2.** GH3 cells were transfected with 20 μg (-187)PRL-CAT plus 5 μg of either pRc/CMV (-), CMV-Q205L-αo (αo*), CMV-β2, CMV-γ2, or 2.5 μg CMV-β2 + 2.5 μg CMV-γ2. Following one day incubation, cell extracts were prepared and assayed for CAT activity. Data represent the mean ± SD of results with triplicate dishes.

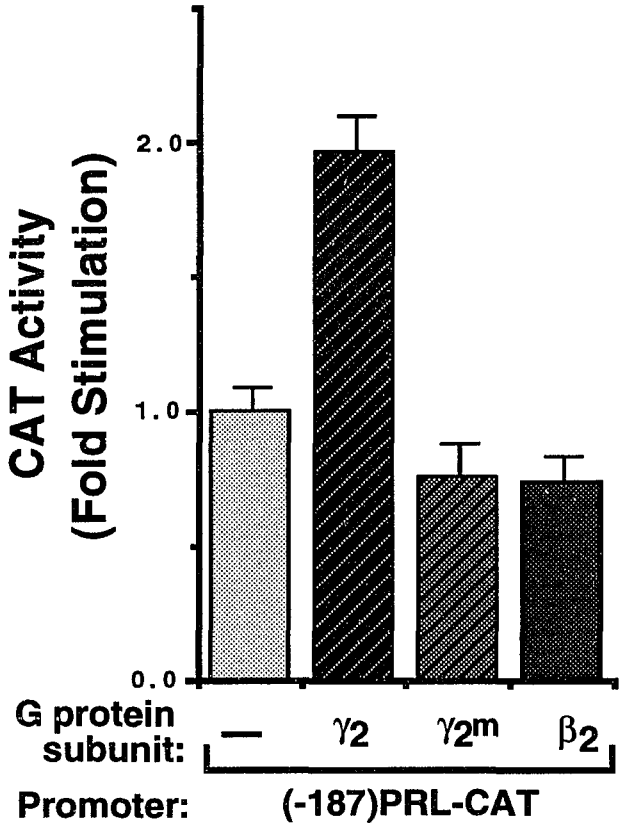

Figure 21. **Effect of wild-type, mutant γ2, and wild-type β2 on the expression of co-transfected PRL promoter-CAT constructs**. GH3 cells were transfected with 10 μg (-187)PRL-CAT plus 5 μg of either pRc/CMV (-), CMV-γ2, pCDM-γ2m, or CMV-β2. Following one day incubation, cell extracts were collected and assayed for CAT activity. Data represent the mean ± SD of results with triplicate dishes.

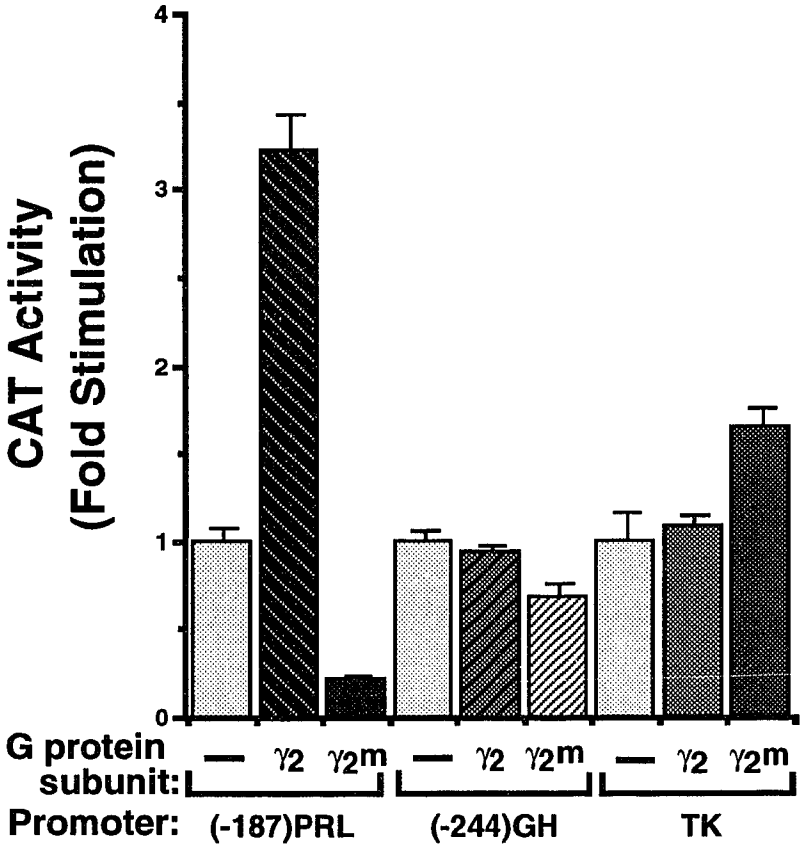

Figure 22. **Regulation of transfected promoter-CAT constructs by wild-type or mutant γ2.** GH3 cells were transfected with 10 μg of (-187)PRL-CAT, (-244)GH-CAT, or TK-CAT, plus 5 μg of pRc/CMV (-), CMV-γ2, or pCDM-γ2m. Following one day incubation, cell extracts were prepared and assayed for CAT activity. Data represent the mean ± SD of results with triplicate dishes.

E. Discussion

The signaling transduction of heterotrimeric G proteins, which couple the cell membrane receptors to intracellular effectors, is not only dictated by their a subunits but also by the βγ subunit complex. Upon dissociation from each other, the Gα and Gβγ may trigger two signaling pathways which can act independently or synergistically (Iniguez-Lluhi, Kleuss et al. 1993). The function of different G protein subunits in regulating cellular effectors may render the cells greater flexibility and fine adjustment of cellular response.

In the above studies, I discovered that transient expression in GH3 rat pituitary cells of γ, but not of β or γ + β, stimulated PRL gene expression. Stimulation of PRL promoter activity was specific for the γ subunit, since this promoter was not stimulated by either the β subunit or a constitutively active mutant α subunit, Q205L-αo (**Fig. 20**). When co-expressed with the β2, the γ2 lost its function of stimulating PRL gene transcription, suggesting that β2 can inhibit the action of γ2. One possible explanation for this observation is that exogenous β2 does not accumulate in GH3 cells, or properly attach to the membranes. This seems to be unlikely because the ability of β2 to inhibit the action of γ2 implies the former does accumulate. To test this, I attempted to examine the accumulation of γ2 and β2 subunits in the membrane fraction of my transfected cells by Western blot, using antisera provided by Dr. N. Gautam. I found that accumulation of γ2 and β2 cannot be detected under the condition I used in my experiment system (data not shown). It should be noted, however, that it may be difficult to detect the accumulation of the transiently expressed exogenous β and Y subunits against the high background of the endogenous ones in GH3 cells.

The observation that γ2m did not stimulate expression of construct (-187)PRL-CAT (**Fig. 21**) showed that the prenylated site of Gγ subunit is necessary for its transcriptional action. This result is consistent with previous studies with both the yeast Gβγ subunit complex and Ras, which were shown to be rendered nonfunctional by mutating the prenylated site of the Gγ subunit or ras respectively (Finegold, Schafer et al. 1990). This result is an important negative control for my observation of the function of wild-type γ2 and also implies that Gγ2 may employ membrane effector(s) to transduce its action on PRL gene expression.

The promoter specificity of the stimulatory effects of γ2 was demonstrated in **Fig.22** by the ability of γ2 to increase expression of (-187)PRL-CAT but not of similar constructs containing either rat growth hormone promoter or the thymidine kinase promoter. This experiment also indicates that there must be a defined specific intracellular pathway(s) that leads from Gγ to PRL promoter.

In summary, my observation that γ2, but not β2 or β2 + γ2, is able to regulate the PRL gene promoter in GH3 cells provides the first evidence that the γ subunit can signal cellular events in higher organisms.

Chapter 6

Summary and Perspectives

Summary and Perspectives

G proteins are important mediators of the actions of a large class of G protein-coupled receptors that are activated by hormone binding. The findings in my thesis represent the first direct demonstration a function of G proteins in gene regulation in higher organisms. In addition, the results in Chapter 5 that γ2, but not β2 or β2 + γ2, regulates the prolactin gene promoter activity represents both the first study of transcriptional action of βγ in higher organisms, and the first observation of an effect of the G protein γ subunit.

Based on the results in Chapter **2, 3, 4**, which imply that Pit-1 may be involved in αs and αq action on the PRL gene promoter as well as in αs action on the Pit-1 gene promoter, several additional experiments need to be carried out to further confirm the important gene regulatory function of Pit-1 in response to αs or αq. To this end, various approaches can be used to examine this question. First, the mutant Pit-1 binding site in the prolactin gene promoter which dramatically decreases the Pit-1 binding should be examined for its ability to mediate the response to αs and αq. Second, the ability of a dominant inhibitor of Pit-1, DPit-1 (Yan and Bancroft 1991), to modulate the αs* stimulation of the Pit-1 and PRL promoter, and αq* stimulation of the PRL promoter, should be further investigated. Third, the gene-distal mechanism of the Pit-1 regulation of the PRL and Pit-1 gene promoter activity in response to αs and αq should be investigated. My data imply that PKA is involved in mediating αs stimulation on PRL and Pit-1 gene expression and that raf-1 is responsible for αq action. The involvement of protein kinases in αs and αq action implies that Pit-1 may be the downstream target of these kinases, and phosphorylated in response to αs and αq activation. It is true that Pit-1 protein contains putative phosphorylation sites for various kinases. Therefore, analysis of Pit-1 phosphorylation is important for

understanding its mechanism of action. Pit-1 is known to be phosphorylated by PKA, however, a mutant Pit-1 that lacks the PKA phosphorylation site is as effective as wild-type Pit-1 in mediating induction of the rat PRL promoter by either forskolin or phorbol ester (Fischberg et al. 1994), suggesting that phosphorylation of Pit-1 by PKA may not be required for either αs or αq action on the PRL promoter or αs action on the Pit-1 promoter. However, this does not exclude the possibility that other protein kinases may phosphorylate Pit-1.

Besides Pit-1, my results presented in Chapter **2, 4** suggests that CREB is involved in αs action on the PRL and Pit-1 promoter and that CREB binds the CLE in the prolactin proximal promoter with low affinity. Additionally, previous experiments have shown that CREB binds the CRE in the Pit-1 gene promoter. To further investigate the role of CREB in mediating G protein action, either the CLE in the PRL promoter or the CRE in the Pit-1 promoter should be mutated. Furthermore, since the phosphorylation of CREB by PKA activates gene expression in many transcription systems, and my data shows that PKA is required for G protein action, it is highly possible that PKA phosphorylates CREB to regulate its activity in stimulating PRL and Pit-1 gene expression. To address these questions, CREB-m1, which is mutated at the PKA phosphorylation site by conversion of Ser-133 to Ala-133 (Gonzalez and Montminy 1989) should be used in competition with the wild type CREB to bind the CLE in PRL promoter or the CRE in the Pit-1 promoter to examine the effect of PKA phosphorylation of CREB in transcriptional activation of PRL and Pit-1 gene expression in response to αs stimulation. In addition, the potential functional role of phosphorylation of CREB by other kinases needs to be examined.

Since both Pit-1 and CREB are involved in stimulating PRL and Pit-1 gene expression, further investigations should be done to investigate whether CREB interacts with Pit-1. This can be tested by supershift in a gel-shift assay

using Pit-1 or CREB antibody. In addition, besides Pit-1 and CREB, there may be other transcriptional factors involved in regulating PRL and Pit-1 gene expression. A Pit-1 binding site (site 1P) can act as a response element for TRH and Ca^{2+} (Yan, Pan et al. 1991; Yan and Bancroft 1991), αi2 and αo (Lew, Yao et al. 1994), and αs and αq (chapter 2 and 3). The converging of different signaling pathways on site 1P raises the possibility that there may be other transcription factor(s) that bind(s) site 1P to direct different effects of hormones and G proteins.

Besides gene proximal regulation, the gene distal events that mediates the G protein action on PRL and Pit-1 gene expression should be studied in detail. My results in Chapter **2, 3**, and **4** showed that αs and αq can stimulate the PRL promoter through pit-1 binding site. However, αs also stimulates the Pit-1 promoter to induce the expression of Pit-1 mRNA, since it is known that Pit-1 can stimulate the PRL promoter activity. These results raise two possibilities: either αs may first stimulate Pit-1 gene expression in GH3 cells, and the increase in Pit-1 in turn stimulates PRL gene expression, or αs may employ different signaling pathways to stimulate the PRL promoter and the Pit-1 promoter respectively. One approach to resolve this is to co-express the PRL promoter with αs* or αq* in a Pit-1-deficient pituitary cell line such as the AtT20. Clearly, further studies are required to answer these questions.

On the other hand, the Gq α subunit regulates the Pit-1 gene promoter activity through a Raf pathway (Chapter **3**). Previous studies showed that Gq can stimulate MAPK activity (Faure, Voyno Yasenetskaya et al. 1994) and Ras can stimulate PRL promoter activity (Conrad and Gutierrez-Hartmann 1992; Conrad, Oberwetter et al. 1994). In addition, since both Grb2-sos and Ras are upstream effectors of Raf and MAPK is downstream effector of Raf, it will be interesting to know whether Gq associate with SOS or Ras. Ras and MAPK activity should be

measured following transfection of Gq α subunit. In addition, downstream targets of MAPK should be further examined.

Previous studies have shown that the PKA pathway and the Raf, MAPK pathway generally antagonize each other in downstream signaling. However, PRL gene expression can be activated by both Gαs that activates the PKA pathway and Gαq that activates the Raf pathway. It is of great interest to see if in GH3 cells, the PKA and MAPK pathways synerginize to stimulate PRL gene transcription. To test this, the PKA catalytic subunit should be co-transfected with αq*, or else the constitutively active Raf (Raf BXB) should be co-transfected with αs*, to test activation of the PRL gene promoter.

In Chapter **5**, the results show that Gγ alone can activate PRL gene expression, while β2 alone does not stimulate PRL promoter activity, co-expression of it with γ2 appears to inhibit the stimulation of the PRL promoter by γ2. In addition, the results also show that the prenylated site of the Gγ subunit is necessary for its transcriptional action on the PRL promoter. These results imply that exogenous γ2 is not acting simply by interacting with endogenous β2, but probably by interacting with a different membrane effector(s) to transduce its action on PRL gene expression. The two hybrid system as well as other genetic and biochemical experiments should be used to identify this hypothetical γ2-proximal effector protein. Since the finding that γ2 alone stimulates PRL gene is a very novel phenomenon, more investigation needs to be done, such as the intracellular signaling pathways involved in Gγ signaling of PRL gene expression. Dominant negative inhibitors of Raf, MEK, PKA, CREB can be co-transfeced with the PRL promoter construct to examine their role in mediating Gγ signaling.

Since both Gα and Gγ activate PRL gene expression, the two pathways involved may act separately or synergistically. Co-expression of αs or αq and g2 in GH3 cells with the PRL promoter will provide some answers to this question.

Bibliography

Agui, T. and K. Matsumoto (1990). "Direct demonstration of guanine nucleotide sensitive receptor for vasoactive intestinal peptide in the anterior lobe of the rat pituitary gland." peptides **11**: 609-611.

Aizawa, T. and P. M. Hinkle (1985). "Differential effects of thyrotropin-releasing hormone, vasoactive intestinal peptide phorbol ester, and depolarization in GH4C1 rat pituitary cells." Endocrinology **116**: 909-919.

Aizawa, T. and P. M. Hinkle (1985). "Thyrotropin-releasing hormone rapidly stimulates a biphasic secretion of prolactin and growth hormone in GH4C1 rat pituitary tumor cells." Endocrinology **116**: 73-82.

Albert, P. R., K. A. Neve, et al. (1990). "Coupling of a cloned rat dopamine-D2 receptor to inhibition of adenylyl cyclase and prolactin secretion." J. Biol. Chem. **265**: 2098-2104.

Albert, P. R. and A. H. Tashjian (1984). "TRH-induced spike and plateau in cytosolic free Ca^{2+} concentrations in pituitary cells." J. Biol. Chem. **259**: 5827-5832.

Andersen, B. and R. M. G. (1994). "Pit-1 determines cell types during development of the anterior pituitary gland." J. Biol. Chem. **269**: 29335-29338.

Aragay, A. M., A. Katz, et al. (1992). "The Gαq and Gα11 proteins couple the thyrotropin-releasing hormone receptor to phospholipase C in GH3 rat pituitary cells." J. Biol. Chem. **267**(35): 24983-24988.

Axelrod, J., R. M. Burch, et al. (1988). "Receptor-mediated activation of phospholipase A2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers." Trends Neurosci. **11**: 117-123.

Bachwich, D., J. Merchant, et al. (1992). "Identification of a cis-regulatory element mediating somatostatin inhibition of epidermal growth factor-stimulated gastrin gene transcription." Mol. Endocrinol. **6**: 1175-1184.

Birnbaumer, L., J. Abramowitz, et al. (1990). "Receptor--effector coupling by G proteins." Biochim. Biophys. Acta. **1031**: 163.

Bodner, M., J. L. Castrillo, et al. (1988). "The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein." Cell **55**: 505-518.

Bruder, J. T., G. Heidecker, et al. (1992). "Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase." Genes Dev. **6**: 545-556.

Camps, M., A. Carozzi, et al. (1992). "Isozyme-selective stimulation of phospholipase C-β2 by G proein βγ-subunits." Nature **360**: 684-692.

Cao, Z., E. A. Barron, et al. (1988). "Prolactin upstream factor I mediates cell-specific transcription." Mol. Cell. Biol. **8**: 5432-5438.

Carillo, A. J., T. B. Pool, et al. (1985). "Vasoactive intestinal peptide increases prolactin messenger ribonucleic acid content in GH_3 cells." Endocrinology (116): 202-206.

Carrillo, A. J., T. B. Pool, et al. (1985). "Vasoactive intestinal peptide increases prolactin messenger robonucleic acid content in GH3 cells." Endocrinology **116**: 202-206.

Castrillo, J. L., M. Bodner, et al. (1989). "Purification of growth hormone-specific transcription factor GHF-1 containing homeobox." Science **243**: 814-817.

Castrillo, J. L., L. E. Theill, et al. (1991). "Function of the homeodomain protein GHF1 in pituitary cell proliferation." Science **253**: 197-199.

Chen, R., H. A. Ingraham, et al. (1990). "Autoregulation of pit-1 gene expression mediated by two cis-active promoter elements." Nature **346**: 583-586.

Clapham, D. E. and E. J. Neer (1993). "New roles for G-protein βγ-dimers in transmembrane signalling." Nature **365**: 403-406.

Codina, J., A. Yatani, et al. (1987). "The Alpha-subunit of the GTP binding protein Gk opens atrial potassium channels." Science **236**: 442-445.

Cole, G. M., D. E. Stone, et al. (1990). Mol. Cell Biol. **10**: 510-517.

Coleman, D. T. and C. Bancroft (1993). "Pituitary adenylate cyclase-activating polypeptide stimulates prolactin gene expression in a rat pituitary cell line." Endocrinology **133**: 2736-2742.

Conklin, B. R., O. Chabre, et al. (1992). "Recombinant $G_q\alpha$." J. Biol. Chem. **267**: 31-34.

Conrad, K. E. and A. Gutierrez-Hartmann (1992). "The ras and protein kinase A pathways are mutally antagonistic in regulating rat prolactin promoter activity." Oncogene **7**: 1279-1286.

Conrad, K. E., J. M. Oberwetter, et al. (1994). "Identification of the functional components of the Ras signaling pathway regulating pituitary cell-specific gene expression." Mol. Cell Biol. **14**: 1553-1565.

Cooke, N. E., F. R. Brook, et al. (1985). Endocrinology **117**: 187.

Crenshaw III, E. B., K. Kalla, et al. (1989). "Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements." Genes & Dev. **3**: 959-972.

d'Emden, M. C., Y. Okimura, et al. (1992). "Analysis of functional cooperativity between individual transcription-stimulating elements in the proximal region of the rat prolactin gene." Mol. Endocrinol. **6**: 581-588.

Davis, J. R. E. (1990). "Tissue-specific regulation of prolactin gene expression." J. Endocrinol. **125**: 171-173.

Day, R. N., S. Koike, et al. (1990). "Both Pit-1 and the Estrogen Receptor Are Required for Estrogen Responsiveness of the Rat Prolactin Gene." Mol. Endocrinol. **4**(12): 1964-1971.

Day, R. N. and R. A. Maurer (1989). "The distal enhancer region of the rat prolactin gene contains elements conferring response to multiple hormones." Mol. Endocrinol. **3**: 3-9.

Day, R. N., J. A. Walder, et al. (1989). "A protein kinase inhibitor gene reduces both basal and multihormone-stimulated prolactin gene transcription." J. Biol. Chem. **264**: 431-436.

De Vivo, M., J. Chen, et al. (1992). "Enhanced phospholipase C stimulation and transformation in NIH-3T3 cells expressing Q209LGq-α-subunits." J. Biol. Chem. **267**: 18263-18266.

Drust, D. S. and T. F. J. Martin (1982). "TRH rapidly and transiently stimulates cytosolic calcium-dependent protein phosphorylation in GH3 pituitary cells." J. Biol. Chem. **257**: 7566-7573.

Drust, D. S. and T. F. J. Martin (1984). "TRH rapidly activates protein phosphorylation in GH3 pituitary cells by a lipid linked, protein kinase C-mediated pathway." J. Biol. Chem. **259**: 14520-14530.

Drust, D. S., C. A. Sutton, et al. (1982). "TRH and cAMP activates distinctive pathway s of protein phosphorylation in GH3 pituitary cells." J. Biol. Chem. **257**: 3306-3312.

Elsholtz, H. P., A. M. Lew, et al. (1991). "Inhibitory control of prolactin and Pit-1 gene promoters by dopamine." J. Biol. Chem. **266**(34): 22919-22925.

Elsholtz, H. P., H. J. Mangalam, et al. (1986). "Two Different cis-Active Elements Transfer the Transcriptional Effects of Both EGF and Phorbol Esters." Science **234**: 1552-1557.

Escalera, G. M. D. L. and R. I. Weiner (1992). "Dissociation of dopamine from its receptor as a signal in the pleiotropic hypothalamic regulation of prolactin secretion." Endocrine Reviews **13**(2): 241-255.

Faure, M., T. A. Voyno Yasenetskaya, et al. (1994). "CAMP and βγ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells." J. Biol. Chem. **269**: 7851-7854.

Fearon, C. W. and A. H. Tashjian (1985). "Thyrotropin-releasing hormone induces redistribution of protein kinase C in GH4C1 rat pituitary cells.".J. Biol. Chem. **260**: 8366-8371.

Fearon, C. W. and A. H. Tashjian (1987). "Ionomycin inhibits tyrotropin-releasing hormone-induced translocation of protein kinase C in GH4C1 pituitary.".J. Biol. Chem. **262**: 9515-9520.

Federman, A. D., B. R. Conklin, et al. (1992). "Hormonal stimulation of adenylyl cyclase through G_i-protein βγ subunits." Nature **356**: 159-161.

Finegold, A. A., W. R. Schafer, et al. (1990). "Common modifications of trimeric G proteins and ras protein: Involvement of polyisorenylation." Science **249**: 165-169.

Fischberg, D. J., X. H. Chen, et al. (1994). "A Pit-1 phosphorylation mutant can mediate both basal and induced prolactin and growth hormone promoter activity." Mol. Endocrinol. **8**: 1566-1573.

Fox, S. R., M. T. C. Jong, et al. (1990). "The Homeodomain Protein, Pit-1/GHF-1, Is Capable of Binding to and Activating Cell-Specific Elements of Both the Growth Hormone and Prolactin Gene Promoters." Mol. Endocrinol. **4**(7): 1069-1080.

Frohman, L. A. (1981). " Endocrinology and Metablism ." McGraw-Hill, New York. : 151-231.

Gaiddon, C., A. L. Boutillier, et al. (1995). "Genomic effects of the putative oncogene Gαs: chronic transcriptional activation of the c-fos proto-oncogene in endocrine cells." J. Biol. Chem.

Gershengorn, M. C. (1982). "Thyrotropin releasing hormone." Mol. Cell. Biochem. **45**: 163-179.

Gershengorn, M. C. and C. Thaw (1985). "TRH stimulates biphasic elevation of cytoplasmic free calcium in GH3 cells. Further evidence that TRH mobilizes cellular and extracellular Ca^{2+}." Endocrinology **116**: 591-596.

Gollasch, M., C. Kleuss, et al. (1993). "G_{i2} and protein kinase C are required for thyrotropin-releasing hormone-induced stimulation of voltage-dependent Ca^{2+} channels in rat pituitary GH3 cells." Proc. Natl. Acad. Sci. U. S. A. **90**: 6265-6269.

Gonzalez, G. A. and M. R. Montminy (1989). "Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at Serine 133." Cell **59**: 675-680.

Gozes, I. and D. E. Brenneman (1989). "VIP: molecular biology and neurobiologicalfunction." Mol. Neurobiol. **3**: 201-236.

Graeme, M. (1993). "Mechanisms of multifuctional signalling by G protein-linked receptors." Trends Pharmacol. Sci. **14**: 239-244.

Graziano, M. P. and A. G. Gilman (1989). "Synthesis in *Escherichia coli* of GTPase-deficient mutants of Gsα." J. Biol. Chem. **264**: 15475-15482.

Gupta, S. K., C. Gallego, et al. (1992). "MAP kinase is constitutively activated in gip2 and src transformed Rat 1a fibroblasts." J. Biol. Chem. **267**: 7987-7990.

Gutierrez-Hartmann, A., S. Siddiqui, et al. (1987). "Selective transcription and DNase I protection of the rat prolactin gene by GH3 pituitary cell-free extracts." Proc. Natl. Acad. Sci. USA **84**: 5211-5215.

Habener, J. F. (1990). "Cyclic AMP response element binding proteins: a cornucopia of transcription factors." Mol. Endocrinol. **4**: 1087-1094.

Hagiwara, S. and H. Ohmori (1983). "Studies of single calcium channel currents in rat clonal pituitary cells." J. Physiol. **336**: 649-661.

Harvey, C., S. M. Jackson, et al. (1991). "Structure-Function Analysis of the Rat Prolactin Promoter: Phasing Requirements of Proximal Cell-Specific Elements." Mol. Endocrinol. **5**(6): 836-843.

He, X., M. N. Treacy, et al. (1989). "Expression of a large family of POU-domain regulatory genes in mammalian brain development." Nature **340**: 35-42.

Hepler, J. R. and A. G. Gilman (1992). "G proteins." TIBS **17**: 383-387.

Hepler, J. R., T. S. Kozasa A. V., et al. (1993). "Purification from Sf9 cells and characterization of recombinant $G_q\alpha$." J. Biol. Chem. **268**: 14367-14375.

Herr, W., R. A. Sturm, et al. (1988). "The POU domain: a large conserved region in the mammalian pit-1, oct-1, and oct-2 and Caenorhabditis elegans unc-86 gene products." Genes Dev. **2**: 1513-1516.

Herschman, H. R. (1991). "Primary response genes induced by growth factors and tumor promoters." Ann. Rev. Biochem. **60**: 281-319.

Hidaka, H. and R. Kobayashi (1992). "Pharmacology of protein kinase inhibitors." Annu. Rev. Pharmacol. Toxicol. **32**: 377-397.

Hinkle, P. (1988). Regulation of prolactin synthesis and secretion by peptide hormones. Peptide Hormones: Effects and Mechanisms of Action. Boca Raton, FL, CRC Press. 143-166.

Hinkle, P. M., E. L. Hewlett, et al. (1986). "Thyroliberin action in pituitary cells is not inhibited by pertussis toxin." Biochem. J. **237**: 181-186.

Hinkle, P. M. and P. A. Kinsella (1984). "Regulation of TRH binding by monovalent cations and guanyl nucleotides." J. Biol. Chem. **259**: 3445-3456.

Hinkle, P. M. and W. J. Phillips (1984). "TRH stimulates GTP hydrolysis by membranes from GH4C1 rat pituitary tumour cells." Proc. Natl. Acad. Sci. **81**: 6183-6187.

Howe, L. R. and C. J. Marshall (1993). J. Biol. Chem. **268**: 20717-20720.

Hsieh, K.-P. and T. F. J. Martin (1992). "Thyrotropin-releasing hormone and gonadotropin-releasing hormone receptors activate phospholipase C by coupling to the guanosine triphosphate-binding proteins Gq and G11." Mol. Endocrinol. **6**: 1673-1681.

Ingraham, H. A., R. Chen, et al. (1988). "A Tissue-Specific Transcription Factor Containing a Homeodomain Specifies a Pituitary Phenotype." Cell **55**: 519-529.

Iniguez-Lluhi, J., C. Kleuss, et al. (1993). "The importance of G-protein βγ subunits." Trends in Cell Biol**3**: 230-236.

Ishihara, T., R. Shigemoto, et al. (1992). "Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide." Neuron **8**: 811-819.

Iverson, R. A., K. H. Day, et al. (1990). "Clustered point mutation analysis of the rat prolactin promoter." Mol. Endocrinol. **4**: 1564-1571.

Jackson, A. E. and C. Bancroft (1988). "Proximal upstream flanking sequences direct calcium regulation of the rat prolactin gene." Mol Endocrinol **2**(11): 1139-44.

Jackson, A. E., S. K. Bandyopadhyay, et al. (1990). "Epidermal growth factor and phorbol ester regulate prolactin gene exression via distinct pathways." Mol. Cell Endocrin **69**: R7-R11.

Kalinec, G., A. J. Nazarali, et al. (1992). "Mutated α subunit of the G_q protein induces malignant transformation in NIH 3T3 cells." Mol. Cell Biol. **12**: 4687-4693.

Kameyama, K., K. Haga, et al. (1993). "Activation by G protein βγ subunits of β-adrenergic and muscarinic receptor kinase." **268**: 7753-7758.

Karin, M., J. L. Castrillo, et al. (1990). "Growth hormone gene regulation: a paradigm for cell-type-specific gene activation." Trends Genet. **6**: 92-96.

Keech, C. A. and H. A. Gutierrez (1991). "Insulin activation of rat prolactin promoter activity." Mol Cell Endocrinol **78**(1-2): 55-60.

Keech, C. A. and A. Gutierrez-Hartmann (1989). "Analysis of rat prolactin promoter sequences that mediate pituitary-specific and 3', 5'-cyclic adenosine monophosphate-regulated gene expression in vivo." Mol. Endocrinol. **3**(5): 832-839.

Keech, C. A., S. M. Jackson, et al. (1992). "Cyclic adenosine 3', 5'-monophosphate activation of the rat prolactin promoter is restricted to the pituitary-specific cells type." Mol. Endocrinol. **6**: 2059-2070.

Kim, D., D. L. Lewis, et al. (1989). "G-protein βγ-subunits activate the cardiac muscarinic K^+-channel via phospholipase A2." Nature **337**: 557-560.

Kim, K., R. N. Day, et al. (1988). "Functional analysis of the interaction of a tissue-specific factor with an up-stream enhancer element of the rat prolactin gene." Mol. Endo. **2**: 1374-1381.

Kleuss, C., J. Hescheler, et al. (1991). "Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents." Nature **353**: 43-48.

Koch, W. J., B. E. Hawes, et al. (1994). "Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by Gβγ activation of p^{21ras}." Proc. Natal. Acad. Sci. USA **91**: 12706-12710.

Koch, W. J., J. Inglese, et al. (1993). "The binding site for the βγ subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase." J. Biol. Chem. **268**: 8256-8260.

Kroll, S. D., J. Chen, et al. (1992). "The Q205LG_o-α subunit expressed in NIH-3T3 cells induces transformation." J. Biol. Chem. **267**: 23183-23188.

Landis, C. A., G. Harsh, et al. (1990). "Clinical characteristics of acromegalic patients whose pituitary tumores contain utant G_s protein." J. Clin. Endocrinol. Metab. **71**: 1416-1420.

Landis, C. A., S. B. Masters, et al. (1989). "GTPase inhibiting mutations activate the a chain of G_S and stimulate adenylyl cyclase in human pituitary tumours." Nature **340**(August 31): 692-696.

Laverriere, J. N., A. Tixier-Vidal, et al. (1988). "Preferential role of calcium in the regulation of prolactin gene transcrioption by thyrotropin-releasing hormone in GH3 pituitary cells." Endocrinology **122**: 333-340.

Lew, A. M., H. Yao, et al. (1994). "Giα2- and Goα-mediated signaling in the Pit-1-dependent inhibition of the prolactin gene promoter." J. Biol. Chem. **269**: 12007-12013.

Li, S., E. B. Crenshaw III, et al. (1990). "Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1." Nature **347**(October 11): 528-533.

Liang, J., K. E. Kim, et al. (1992). "Characterization of a nontissue-specific, 3',5'-cyclic adenosine monophosphate-responsive element in the proximal region of the rat prolactin gene." Mol. Endocrinol. **6**: 885-892.

Logothetis, D. E., Y. Kurachi, et al. (1987). "The βγ subunits of GTP-binding proteins activate the muscarinic K+ channel in heart."Nature **325**: 321-326.

Lowndes, J. M., S. K. Gupta, et al. (1991). "GTPase-deficient Gαi2 oncogene gip2 inhibits adenylylcyclase and attenuates receptor-stimulated phospholipase A2 activity." J. Biol. Chem. **266**: 14193-14197.

Lufkin, T. and C. Bancroft (1987). "Identification by cell fusion of gene sequences that interact with positive trans-acting factors." Science **237**(4812): 283-286.

Lufkin, T., A. E. Jackson, et al. (1989). "Proximal rat prolactin promoter sequences direct optimal, pituitary cell-specific transcription." Mol. Endocrinol. **3**: 559-566.

Mangalam, H. J., V. R. Albert, et al. (1989). "A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally." Genes Dev. **3**: 946-958.

Marsh, L. (1991). "signal transdution during pheromoe response in yeast " Annu. Rev. Cell Biol. **7**: 699-728.

Martin, T. F., K. P. Hsieh, et al. (1990). "The sustained second phase of hormone-stimulated diacylglycerol accumulation does not activate protein kinase C in GH3 cells." J. Biol. Chem. **256**: 7623-7631.

Martin, T. F. J. (1983). "Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH_3 pituitary cells." J. Biol. Chem. **258**: 14816-14822.

Martin, T. F. J., S. M. Bajjalieh, et al. (1986). "Thyrotropin-releasing hormone stimulation of polyphosphoinositide hydrolysis in GH3 cells membranes is GTP dependent but insensitive to cholera or pertussis toxin." J. Biol. Chem. **261**: 10141-10149.

Martin, T. F. J. and J. A. Kowalchyk (1984). "Evidence for the role of calcium and diacylglycerol as dual second messengers in thyrotropin-releasing hormone action: involvement of Ca^{2+}." Endocrinology **115**: 1527-1536.

Martin, T. F. J., D. O. Lucas, et al. (1986). "Thyrotropin-releasing hormone activates a Ca^{2+}-dependent polyphosphoinositide phosphodiesterase in permeable GH3 cells." J. Biol. Chem. **261**: 2918-2927.

Masters, S. B., R. T. Miller, et al. (1989). "Mutations in the GTP-binding site of $G_{s\alpha}$ alter stimulation of adenylyl cyclase." J. Biol. Chem. **264**: 15467-15474.

Mattera, R. R., M. P. Graziano, et al. (1989). "Splice variants of the α subunit of the G protein Gs activate both adenylyl cyclase and calcium channels." Science **243**: 804-806.

Maurer, R. (1981). "Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP." Nature **295**(November 5): 94-97.

Maurer, R. A. and A. C. Notides (1987). "Identification of an estrogen-responsive element from the 5'-flanking region of the rat prolactin gene." Mol. Cell. Biol. **7**(4247-4254):

McCormick, A., H. Brady, et al. (1990). "Regulation of the pituitary-specific homeobox gene GHF-1 by cell-autonomous and environmental cues." Nature **345**(June 28): 829-832.

Montmayeur, J. P., J. Guiramand, et al. (1993). "Preferenial coupling between dopamine D2 receptors and G-protens." Mol. Endocrinol. **7**: 161-170.

Montminy, M. R., M. J. Low, et al. (1986). "Cyclic AMP regulates somatostatin mRNA accumulation in primary diencephalic cultures and in transfected fibroblast cells." J. Neurosci. **6**(4): 171-176.

Murdoch, G. H., R. Franco, et al. (1983). "Polypeptide hormone regulation of gene expression." J. Biol. Chem. **258**: 15329-15335.

Nelson, C., V. R. Albert, et al. (1988). "Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor." Science **239**: 1400-1405.

Nelson, C. E., B. Crenshaw III, et al. (1986). "Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression in rat prolactin and growth hormone genes." Nature **322**: 557-562.

Northup, J. K., H. Smigel, et al. (1983b). "The subunits of the stimulatory regulatory component of adenyly cyclase: Resolution of the activated 45,000 Dalton (Alpha) subunit." J. Biol. Chem. **258**: 11369-11376.

Northup, J. K., P. C. Sternweis, et al. (1983a). "The subunits of the stimulatory regulatory component of adenylyl cyclase." J. Biol. Chem. **258**: 11361-11368.

Oberwetter, J. M., K. E. Conrad, et al. (1993). "The Ras and protein kinase C signaling pathways are functionally antagonistic in GH4 neuroendocrine cells." Mol. Endocrinol. **7**: 915-923.

Osborne, R. and A. H. Tashjian Jr. (1981). "Tumor-promoting phorbol esters affect production of prolactin and growth hormone by rat pituitary cells." Endocrinology **108**(4): 1164-1170.

Pan, W. T., Q. Liu, et al. (1990). "Identification of a growth hormone gene promoter repressor element and its cognate double- and single-stranded DNA-binding proteins." J. Biol. Chem. **265**(12): 7022-7028.

Paulssen, E. J., R. H. Paulssen, et al. (1991). "Cell specific distribution of guanine nucleotide-binding regulatory proteins in rat pituitary tumour cell lines." Mol. Cell. Endocrinol. **76**: 45-53.

Paulssen, R. H., E. J. Paulssen, et al. (1992). "The thyroliberin receptor interacts directly with a stimulatory guanine-nucleotide-binding protein in the activation of adenylyl cyclase in GH3 rat pituitary tumour cells." Eur. J. Biochem. **204**: 413-418.

Peers, B., P. Monget, et al. (1991). "Transcriptional induction of the human prolactin gene by cAMP requires two cis-acting elements and at least the pituitary-specific factor Pit-1." J. Biol. Chem. **266**(27): 18127-18134.

Peers, B., A. M. Nalda, et al. (1992). "Binding of a 100-kDa ubiquitous factor to the human prolactin promoter is required for its basal and hormone-regulated activity." Eur. J. Biochem. **210**: 53-58.

Probst, W. C., L. A. Snyder, et al. (1992). "Sequence Alignment of the G-protein Coupled Receptor Superfamily." DNA Cell Biol. (11): 1-20.

Prysor-Jones, R. A., J. J. Silverlight, et al. (1987). "VIP increases intracellular free calcium in rat and human pituitary tumour cells in vitro." J. Endocrinol. **114**: 119-123.

Qian, N. X., S. Wintz, et al. (1993). "Epitope-tagged G_q α subunits: Expression of GTPase-deficient α subunits persistently stimulates phosphatidylinositol-specific phospholipase C but not mitogen-activated protein kinase activity regulated by the M_1 muscarinic acetylcholine receptor." Proc. Natl. Acad. Sci. U. S. A. **90**: 4077-4081.

Rebecchi, M. J. and M. C. Gershengorn (1983). "Thyroliberin stimulates rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phosphodiesterase in rat mammotropic pituitary cells." Biochem. J. **216**: 287-294.

Rebecchi, M. J., R. N. Kolesnick, et al. (1983). "TRH stimulates rapid loss of phosphatidylinositol and its conversion to 1,2 diacylglycerol and phosphatidic acid in rat mammotropic pituitary cells." J. Biol. Chem. **258**: 227.

Robberecht, P., M. Deschodt-Lanckman, et al. (1979). "VIP activation of rat anterior pituitary adenylate cyclase." FFBS Lett. **103**: 229-233.

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

Ruvkun, G. and M. Finney (1991). "Regulation of transcription and cell identity by POU domain proteins." Cell **64**: 475-478.

Said, S. I. and A. Mutt (1970). "Polypeptide with broad biological activity: isolation from small intestine." Science **169**: 1217-1218.

Schecterson, L. C. and G. S. McKnight (1991). "Role of cyclic adenosine 3',5'-monophosphate-dependent protein kinase in hormone-stimulated β-endorphin secretion in AtT20 cells." Mol. Endocrinol. **5**: 170-178.

Schubert, B., A. M. J. VanDongen, et al. (1989). "β-adrenergic inhibition of cardiac sodium channels by dual G-protein pathways." Science **245**: 516-519.

Schuster, W. A., M. N. Treacy, et al. (1988). "Tissure specific *trans*-acting factor interaction with proximal rat prolactin gene promoter sequences." EMBO J. **7**: 1721-1733.

Seed, B. and J. Y. Sheen (1988). "A simple phase-extraction assay for chloramphenicol acyltransferase activity." Gene **67**: 271-277.

Seuwen, K. and J. Pouyssegur (1992). "G protein-controlled signal transduction pathways and the regulation of cell proliferation." Adv. Cancer Res. **58**: 75-94.

Sharp, Z. D. and Z. Cao (1990). "Regulation of Cell-type-specific Transcription and Differentiation of the Pituitary." BioEssays **12**(2): 80-85.

Sharp, Z. D., S. Helsel, et al. (1989). "DNA recognition element required for PUF-1 mediated cell-type-specific transcription of the rat prolactin gene." Nucleic Acids Res. **17**: 2705-2722.

Sheng, M., G. McFadden, et al. (1990). "Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB." Neuron **4**: 571-582.

Simmons, D. M., J. W. Voss, et al. (1990). "Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors." Genes & Dev. **4**: 695-711.

Simonds, W. F., J. E. Butrynski, et al. (1991). "G-protein βγ dimers." J. Biol. Chem. **266**: 5363-5366.

Smrcka, A. V., J. R. Hepler, et al. (1991). "Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq." Science **251**: 804-807.

Snowdowne, K. W. and A. B. Borle (1984). "Changes in cytosolic ionized calcium induced by activators of secretion in GH3 cells." Am.J. Physiol. **246**: E198-E201.

Sobel, A. and A. H. Tashjian (1983). "Distinct patterns of cytoplasmic protein phosphorylation related to regulation of synthesis and release of prolactin by GH cells." J. Biol. Chem. **258**: 10312-10314.

Spengler, D., C. Wseber, et al. (1993). "Differential signal transduction by five splice variants of the PACAP receptor." Nature **365**: 170-175.

Spiegel, A. M., A. Shenker, et al. (1992). "Receptor-effector coupling by G proteins: Implications for normal and abnormal signal transduction." Endocrine Reviews **13**(3): 536-565.

Stephens, L., A. Smrcka, et al. (1994). "A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits." Cell **77**: 83-93.

Sternweis, P. C., A. V. Smrcka, et al. (1992). "Hormone signalling via G-protein: regulation of phosphatidylinositol 4,5-bisphosphate hydrolysis by G_q." Philos. Trans. R. Soc. Lond. Biol. **336**: 35-41.

Straub, R. E., G. C. Frech, et al. (1990). "Expression cloning of a cDNA encoding the mouse pituitary thyrotropin-releasing hormone receptor." Proc. Natl. Acad. Sci. USA **87**: 9514-9518.

Sutton, C. A. and T. F. J. Martin (1982). "TRH selectively and rapidly stimulates phosphatidylinositol turnover in GH pituitayr cells: a possible second step of TRH action ." Endocrinology **110**: 1273-1280.

Tan, K. and A. H. Tashjian (1984). "Voltage-dependent calcium channels in pituitary cells in culture.I. Characterization of $45Ca^{2+}$ fluxes." J. Biol. Chem. **259**: 418-426.

Tashjian, J. A. H., N. J. Barowsky, et al. (1990). "Thyrotropin-releasing hormone: direct evidence for the stimulation of prolactin production by pituitary cells in culture." Biochem. Biophys. Res. Commun. **43**: 516-523.

Tatsumi, K., K. Miyai, et al. (1992). "Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene." nature Genetics **1**: 56-58.

Taussig, R., L. M. Quarmby, et al. (1993). "Regulation of purified type I and type II adenylylcyclase by G protein βγ subunits." J. Biol. Chem. **268**: 9-12.

Taylor, S. J., H. Z. Chae, et al. (1991). "Activation of the β1 isozyme of phospholipase C by α subunits of the Gq class of G proteins." Nature **350**: 517-519.

Thomason, P. A., S. R. James, et al. (1994). "A G-protein βγ-subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol." J. Biol. Chem. **269**: 16525-16528.

Tian, J., J. Chen, et al. (1994). "Expressionof Constitutively Active G_S α-Subunits in GH3 Pituitary Cells Stimulates Prolactin Promoter Activity." J. Biol. Chem. **269**(1): 33-36.

Tian, J., H. W. Ma, et al. (1995). "Constitutively Active G_q-α Stimulates Prolactin Promoter Activity via Raf." submitted

Treacy, M. N., L. I. Neilson, et al. (1992). "Twin of I-POU: A two amino acid difference in the I-POU homeodomain distinguishes an activator from an inhibitor of transcription." Cell **68**: 491-505.

Uhler, M. D. and G. S. McKnight (1987). "Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase." J. Biol. Chem. **262**(31): 15202-15207.

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

Waldo, G. L., J. L. Boyer, et al. (1991). "Purification of an Al F_4^- and G-protein βγ-subunit-regulated phospholipase C-activaing protein." J. Biol. Chem. **266**: 14217-14225.

Walton, K. M., R. P. Rehfuss, et al. (1992). "A dominant repressor of cyclic adenosine 3',5'-monophosphate (cAMP)-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter *in vivo*." Mol. Endocrinol. **6**: 647-655.

Wang, H. and R. E. Scott (1992). "Induction of c-jun independent of PKC, pertussis toxin-sensitive G protein, and polyamines in quiescent SV40-transformed 3T3 T cells." Exp. Cell Res. **203**: 47-55.

Wang, H. Y., D. C. Watkins, et al. (1992). "Antisense oligodeoxynucleotides to G_s protein α-subunit sequence accelerate differentiation of fibroblasts to adipocytes." Nature **358**: 334-337.

Watson, S. and S. Arkinstall (1994). The G-Protein Linked Receptor Facts Book. Academic Press, London.

Wedegaertner, P. B., P. T. Wilson, et al. (1995). "Lipid modifications of trimeric G proteins." J. Biol. Chem. **270**: 503-506.

White, B. A. and F. C. Bancroft (1983). "Epidermal growth factor and thyrotropin-releasing hormone interact synergistically with calcium to regulate prolactin mRNA levels." J. Biol. Chem. **258**: 4618-4622.

Winitz, S., M. Russell, et al. (1993). "Involvement of Ras and Raf in the G_i-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase." J. Biol. Chem. **268**: 19196-19199.

Yamamoto, K. K., G. A. Gonzalez, et al. (1988). "Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB." Nature **334**: 494-498.

Yamazaki, A., M. Tatsumi, et al. (1987). "The GTP-binding protein of rod outer segments. I. Role of each subunit in the GTP hydrolytic cycle." J. Biol. Chem. **262**: 9316-9323.

Yan, G., W. T. Pan, et al. (1991). "Thyrotropin-releasing hormone action on the prolactin promoter is mediated by the POU protein Pit-1." Mol. Endocrinol. **5**: 535-541.

Yan, G. Z. and C. Bancroft (1991). "Mediation by calcium of thyrotropin-releasing hormone action on the prolactin promoter via transcription factor Pit-1." Mol. Endocrinol. **5**(10): 1488-1497.

Zachary, I., S. B. Masters, et al. (1990). "Increased mitogenic responsiveness of swiss 3T3 cells expressing constitutively activity $G_{s\alpha}$." Biochem. Biophys. Res. Commun. **186**: 1184-1193.