2M11.3035.3

Université de Montréal

A Study of the Role Bmp Growth Factors Play in Pituitary *POMC* Gene Expression

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Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de Maître ès sciences (M.Sc.)
en biologie moléculaire

Avril, 2002

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Identification du jury

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Ce mémoire intitulé:

A Study of the Role Bmp Growth Factors Play in Pituitary *POMC* Gene Expression

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RÉSUMÉ

Les corticotropes sont les premières cellules à se différencier dans l'hypophyse embryonaire. Des expériences d'explants suggèrent un role négatif pour les signaux Bmps dans la différenciation corticotropique, tandis que des études de gain-de-function suggèrent le contraire. POMC étant un marqueur corticotropique, mon projet de maîtrise a porté sur le rôle des signaux Bmp dans l'expression de POMC. Je démontre que l'expression de POMC décroit dans les cellules AtT-20, suite à des traitements avec des protéines Bmp-4 recombinantes ou à la surexpression de composantes de la voie des Bmp/Smad, soient les récepteurs Alk-3/-6 et les facteurs de transcription Smad1/4. La surexpression des inhibiteurs Smad6 et Smad7 renversent cette répression. L'effet négatif de Bmp sur le promoter POMC nécessite les éléments de réponse Pitx et Tpit, et interfère avec l'activité synergique des deux protéines. Des interactions *in vitro* entre Pitx1, Tpit et Smad1 appuient un mécanisme d'action des Smads qui passerait directement par Pitx et Tpit.

Mots clés : différenciation cellulaire, signalisation, répression, transcription, hormone et Tgf-β.

SUMMARY

Corticotrophs constitute the first hormone-producing cell type to emerge in the developing pituitary. Tissue explant experiments had suggested a negative role for Bone-morphogenic-protein (Bmp) signals in corticotroph differentiation, but transgenic studies had argued against this. Seeing that proopiomelanocortin (POMC) expression is a hallmark of corticotrophs, my Master's project consisted in studying the role of Bmp signaling on POMC transcription. I found that POMC expression was downregulated in AtT-20 corticotroph cells that either underwent recombinant (r)Bmp-4 treatments or were transiently transfected with Alk-3/-6 constitutively activated Bmp-type I receptors or Smad1/4 effector proteins. Overexpression of Smad6 or Smad7 counteracted this inhibitory Bmp signaling. Corticotroph-specific functions had previously been assigned to Pitx, Tpit and NeuroD1 transcription factors. I show that Bmp action on POMC promoter requires Pitx and Tpit regulatory elements, and appears to be exerted by directly repressing Pitx/Tpit synergistic activities. *In vitro* interactions between Pitx1, Tpit and Smad1 support the latter mechanism.

Key words: cell differentiation, signalisation, repression, transcription, hormone and Tgf-β.

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LIST OF ACRONYMS AND ABBREVIATIONS

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

POMC: proopiomelanocortin

ACTH: adrenocorticotropin hormone

e: embryonary day

RP: Rathke's pouch

α-MSH: α-melanocyte-stimulating hormone

PRL: prolactin

LH: luteinizing hormone

GH: growth hormone

TSH: thyroid-stimulating hormone

FSH: follicule-stimulating hormone

Bmp: Bone morphogenic protein

Fgf: Fibroblast growth factor

SHH: Sonic hedgehog

α-GSU: α-glycoprotein subunit

PC: protein convertase

CRH: corticotropin-releasing hormone

GR: glucocorticoid receptor

GRE: glucocorticoid response element

cAMP: cyclic AMP

PKA: protein kinase A

PKC: protein kinase C

HPA: hypothalamus-pituitary-adrenal

NBRE: Nur-binding response element

LIF: leukemia inhibitory factor

Alk: Activin-like kinase

Tgf-β: transforming growth factor

MH: Mad-homology

SBE: Smad-binding element

HAT: histone acetylase transferase

HDAC: histone deacetylase

CBP: Creb-binding protein

LAP: latency-associated protein

SARA: Smad anchor for receptor activation

MAPK: mitogen-activated protein kinase

JNK: Jun N-terminal kinase

ACKNOWLEDGEMENTS

I would like to thank every member of Drouin's and Nemer's laboratory, past and present, who contributed ideas, advice and support to what has resulted in the very finest experience as a graduate student.

In no special order, I am grateful to:
Frédérique
For taking me under your wing, and introducing me to tissue culture techniques-à ta façon.
Éric
For that talk we had on our first day, and the discussions that would follow.
Gino
For your very opininated view on science, working habits, and life altogether.
Christine
For your generosity, your genuineness and your complicity against "les salops du labo"
Anne-Marie
For the energy you bring into the lab, and for your companionship at meets we attended.
Mario
For being Rio, the man who was born ready to take on the world! Or just a cup of coffee.
Georges
For your sound advice, weather predictions and your general good sense. Bonne Année!
Gwendal
For taking life at your own particular beat, and making the world a better place to do yoga.
Bruno L.
For your advice and ideas, and for keeping me always on alert of that chair of yours.
Steve
For getting me started in the morning with your high-pitched laugh, once I'm off the walls.
Michel, Yves, Lynda, Chantal
For the very best assistance and answers to all of my questions (the good and the bad).
Émilie
For taming those crazy Europeans, and taking on those primary cell cultures when you did.
Pépijn
For all those serious and then not so serious discussions about research and the Dutch.
Jun
For your multiple HI!s on any single day, and for that contagious smile of yours.
Lise
For your work, your devotion and simply being the very best secretary in the universe.
Jean-François
For your work last summer and your enthusiasm for the project.
Aurélio, Alain, Vincent, Mélanie and others collegues that I might be forgeting
For all your contributions, to my project and my experience in the lab.
Everyone in Mona's lab (in general due to lack of space)
For your help, your kindliness and putting up with my intrusions at the 524.

A very special thanks to my professor,
Jacques
For taking me on to your team, and providing me with the tools to do my research but mostly for instilling in me the know hows of critical thinking that should serve me in many journeys.

I am also indebted to Dr. Hoang Trang and Vivianne Jodoin, from the Department of Molecular Biology, for their continued guidance during the course of my Master's, and also to the members of the jury for taking on the task of correcting this thesis. And of course, none of this could have been possible without the support of those that are dearest to my heart.

CHAPTER- 1 INTRODUCTION

1.1 Corticotrophs in the Mature Pituitary

The pituitary gland, also known as the hypophysis, is a specialized neuroendocrine organ that coordinates the control of peripheral physiology in response to stimuli derived from the brain and other endocrine glands. It can be divided morphologically and functionally into an anterior and intermediate lobe, which together constitute the adenohypophysis, and a posterior lobe known as the neurohypophysis. Corticotroph cells are found in the anterior lobe, and they principally produce adrenocorticotropin hormone (ACTH) by proteolytic processing of proopiomelanocortin (POMC). Adrenocortin (ACTH) is well known for its role in the regulation of the stress response by increasing the production of cortisol from the adrenal gland (108). Melanotroph cells, located in the intermediate lobe, also express the *POMC* precursor gene, which is processed in a different manner to generate α-melanocyte-stimulating hormone (α-MSH). The intermediate pituitary is well-defined in rodents, but degenerates after birth in higher animals, including humans wherein α-MSH is essentially produced by extrapituitary cells (115). Melanocortin (α-MSH) was initially characterized as a regulator of skin pigmentation by inducing the production of melanin from keratinocytes. It is now known to be a general modulator of skin biology and pathology (151)and (150).

Four other hormone-producing cells are present in the pituitary anterior lobe; namely, prolactin (PRL)-secreting lactotrophs, thyroid-stimulating hormone (TSH)-secreting thyrotrophs, luteinizing hormone (LH)- and follicle-stimulating hormone (FSH)-secreting gonadotrophs, and growth hormone (GH)-secreting somatotrophs. These hormones regulate such functions as body growth (GH), mammary growth and

development (PRL), as well as thyroid gland (TSH) and gonad (LH and FSH) functions (121). The neuropituitary hormones oxytocin and vasopressin, secreted from nerve endings in the neurohypophysis, serve homeostatic functions in water balance and reproduction, respectively (25).

1.2 Pituitary Organogenesis

1.2.1 Developmental Origin of the Pituitary

The pituitary gland is composed of tissues of two embryologicaly distinct origins: the adenohypophysis consisting of epithelial or glandular cells derives from ectodermal tissue, and the neurohypophysis is of neuroectodermal origin (217). Pituitary development in the mouse occurs in a midline region of oral ectoderm or stomodeum that contacts neuroectoderm (ventral diencephalon) destined to become the floor of the forebrain (Figure 1.1). By embryonic day 9 (e9.0), the oral ectoderm involutes to form the pituitary rudiment, Rathke's pouch. In what is probably the first complete anatomical account of early pituitary development (217), Schwind describes Rathke's pouch of rat as a structure that arises from an invagination of the stomodeal ectoderm. The idea that Rathke's pouch actively folds in to meet up with the neuroepithelium dorsally has since been challenged. Through studies performed in quail/chick chimeras (41), *Bufo* albino/wild-type chimeras (111) and mouse embryos (117), it was demonstrated that contact between oral ectoderm and ventral diencephalon layers in the developing brain is sustained only at the level of the pituitary rudiment. Rather than invaginating, Rathke's pouch merely appears to take an inward fold, because of maintained contact at pituitary level when everywhere else surface ectoderm and neuroepithelium get separated by invading mesenchyme of pre-chordal plate and neural crest origin. At the same time, the overlaying ventral diencephalon grows downward to generate the infundibulum destined to become the neurohypophysis.

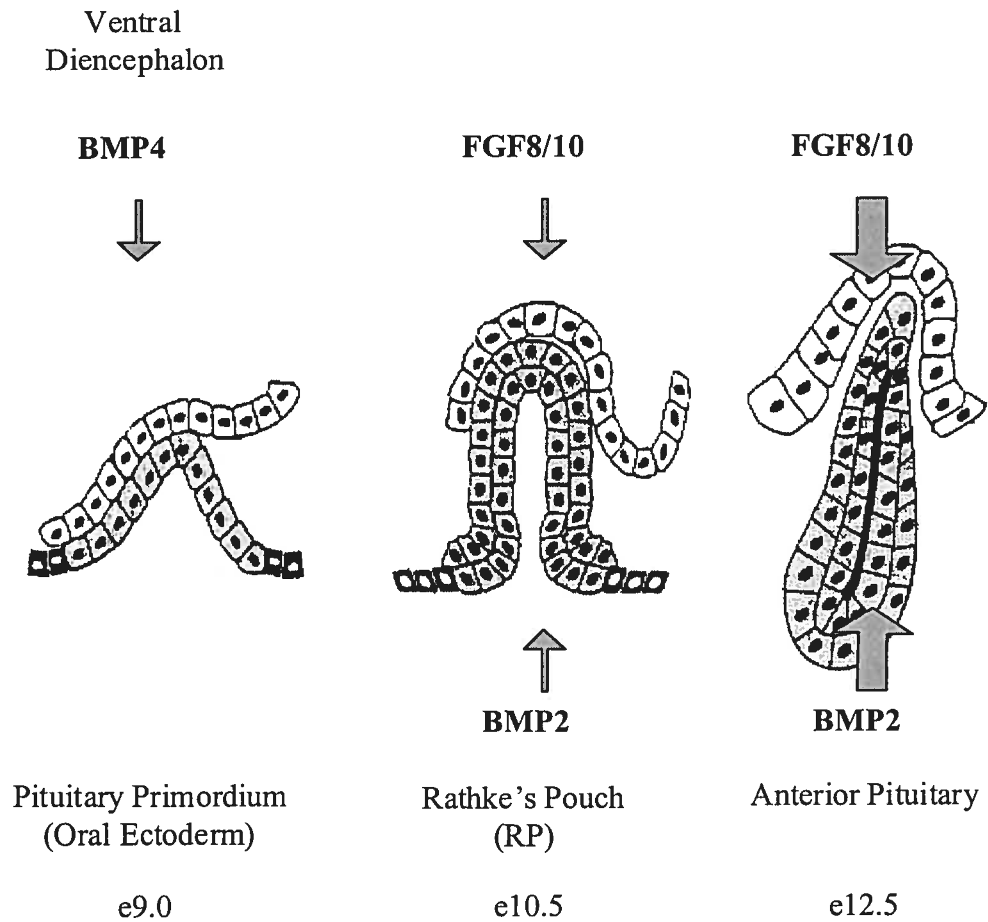


Figure 1.1 Expression Pattern of BMP and FGF Signaling Molecules During Early Pituitary Development (Adapted from Ericson, J. et al., 1998; Treier, M. et al., 1998 and 2001)

1.2.2 Pituitary Primordium Induction

Rathke's pouch formation requires that the pituitary primordium receive and correctly process inductive signals coming from the ventral diencephalon (44). Expression pattern studies have demonstrated Bone morphogenic protein (Bmp)-4, Fibroblast growth factor (Fgf)-8, and Fgf-10 to be expressed in a restricted region of the ventral diencephalon that is in direct contact with the pituitary primordium (244) (67). Bmp-4 expression is detectable in the ventral diencephalon by e8.5, prior to the appearance of Fgf-8 (Figure 1.1), suggestive of a role for Bmp-4 in the earliest phases of pituitary development. The complete lack of pituitary rudiment in a small population of Bmp-4$^{-/-}$ mice that survived to e10, a time at which Rathke's pouch formation is normally well under way, support a role for Bmp-4 signaling in the induction of the pituitary primordium (237). The possibility that conveyance of another inductive signal might have been affected in these *Bmp-4* gene-deleted mice is unlikely since contact between ventral diencephalon and oral ectoderm was maintained in the absence of Bmp-4. Such a penetrating phenotype was however not observed in mice upon ectopic expression of Noggin, a Bmp-specific antagonist (174), in Rathke's pouch and oral ectoderm using the *Pitx1* promoter. Pituitary development in these *Pitx1*-Noggin transgenic mice was abrogated only after the rudimentary pouch had formed (244). A partial blockade of Bmp-4 function by Noggin could be to blame for the discrepancy with *Bmp-4*$^{-/-}$ embryos.

1.2.3 Rathke's Pouch Formation

Formation of Rathke's pouch from the oral ectoderm also appears to be controlled by Fgf and Sonic hedgehog (Shh) signals. Fgf-8 and Fgf-10 have been shown to be expressed in the ventral diencephalon at the time (e9.5) of pouch formation (22). In *Fgf-10* null mice, pituitary development is blocked right after formation of the rudimentary pouch

(187). Fgfr-2(IIIb), a receptor that has been demonstrated to have good binding affinity for Fgf-10 and that has moreover been detected in Rathke's pouch (237), is thought to mediate Fgf signaling within the oral ectoderm. Indeed, *Fgfr-2(IIIb)* gene deleted mice exhibit an arrest in pituitary development that is similar to that observed in *Fgf-10*$^{-/-}$ mice (50). Targeted disruption of the homeobox gene *T/ebp*, expressed during early development in the ventral diencephalon, also resulted in disruption of pituitary development subsequent to a loss of the neuroectodermal region of Fgf-8 expression (237). However, dysmorphogenesis of the vental diencephalon in these *T/ebp* null mice poses a problem in interpreting a direct role for Fgf-8 in pituitary organogenesis.

Two related transcription factors are postulated to mediate Fgf early signals, the Lim homeobox proteins Lhx-3 and Lhx-4 which are both expressed specifically in the pituitary rudiment by the time Rathke's pouch formation begins (e8.5). *In vitro* experiments have shown that Fgf-8 has the ability to maintain Lhx-3 expression (67). In the absence of both Lhx-3 and Lhx-4, mutant mice show no more than a rudiment of Rathke's pouch (219)as do *Fgf-10*$^{-/-}$ and *Fgfr(2IIIb)*$^{-/-}$ mice. Yet, neither *Lhx-3* (220)*or Lhx-4* (219) single mutant mice exhibit such a pronounced phenotype, each single mutant pituitary developing into a glandular structure. Hence, the presence of either Lhx-3 or Lhx-4 seems to be required for the progression of pituitary development beyond the rudimentary stage.

Shh is expressed in surrounding oral ectoderm, mesenchyme and diencephalon, but is specifically excluded from Rathke's pouch (244) (245). Gene deleted mice for *Shh* have not been useful to understand the role of Shh in pituitary development since total loss of hedgehog function altogether interferes with the establishment of ventral diencephalic contacts with the pituitary primordium (245). The significance of Shh signaling was studied using *Pitx1*-Hip transgenic mice; namely, *Pitx1* promoter sequences known to

target gene expression to the oral ectoderm and Rathke's pouch were used to ectopicaly express the hedgehog-specific inhibitor Hip (245). This block in pituitary hedgehog signaling interfered with progression beyond rudimentary pouch development. A direct role for Shh in Rathke's pouch formation was nonetheless suggested by the Pitx1-Hip experiment since the expression domain of Fgf-8 was not disrupted in presence of an intact ventral diencephalon.

1.3 Pituitary Cell Expansion and Differentiation

The six cell types of the anterior pituitary exhibit an ontogenic pattern of hormone gene expression that follows a defined temporal, and somewhat spatial sequence of appearance (100). The first sign of pituitary cell fate commitment comes at e11.5 with the appearance of α-GSU transcripts on the ventral side of the expanding anterior pituitary (Figure 1.2). Corticotroph cell specification, marked by the appearance of POMC/ACTH at e12.5 in the mouse anterior pituitary, appears nonetheless to precede the specification of any other cell lineage in the developing pituitary. Melanotrophs, which constitute the other lineage of POMC-expressing cells in the pituitary, differentiate later as POMC/MSH are detected only at e14.5 in the intermediate pituitary. A transient population of thyrotrophs, identified by expression of the TSH-β subunit, appear on e13 in the rostral end of the anterior pituitary (138). They will disappear after birth. A second cluster of TSHβ-expressing thyrotrophs appears on e14.5, and represents the terminally differentiated thyrotrophs, followed by the contemporaneous expression of GH (somatotrophs) and PRL (lactotrophs) on e16. Gonadotroph-specific LH-β and FSH-β transcripts finally appear at e16.5 and e17.5, respectively.

The specification and expansion of cell fates from a common pituitary primordium has been proposed to be the consequence of overlapping expression patterns of specific sets

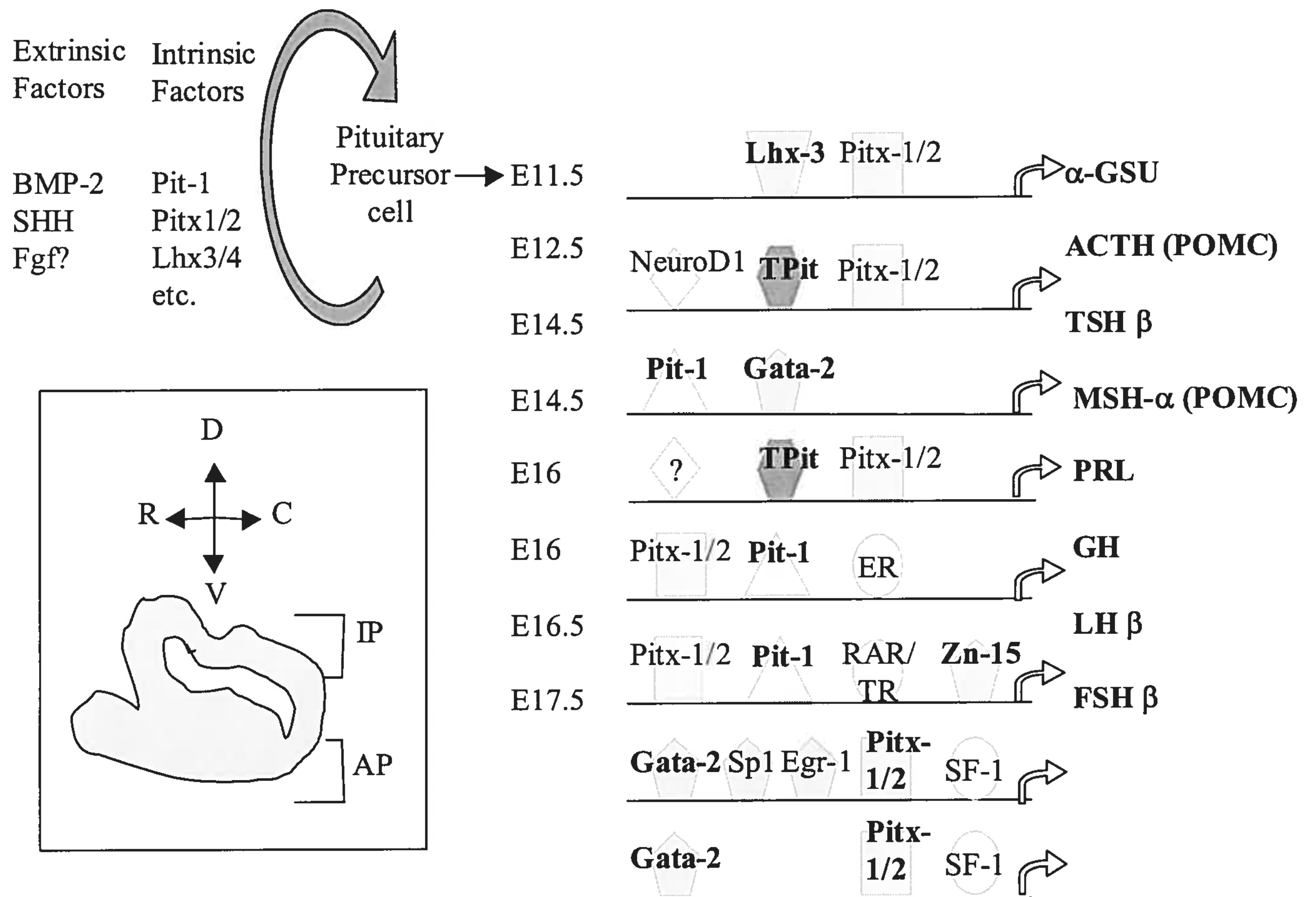


Figure 1.2 Ontogenic Pattern of Pituitary Hormone Expression. (R) rostral, (C) caudal, (D) dorsal, (V) ventral, (IP) intermediate pituitary, (AP) anterior pituitary.

of extrinsic growth and intrinsic transcription factors, expressed in a precise spatiotemporal manner during organogenesis. One approach to understanding the molecular mechanisms that mediate the emergence of pituitary cell types is to study promoter elements implicated in the regulation of hormone marker gene expression (Figure 1.2), as transcription factors conferring cell-specificity are also often implicated in cell differentiation.

Pituitary cells can be classified into three groups according to similarities between components mediating their distinct differentiation pathways. One group is comprised of somatotrophs and lactotrophs, another of thyrotrphs and gonadotrophs, and yet another of corticotrophs and melanotrophs. A parallel can be made between group members with regards to their hormone structure and developmental origin.

1.3.1 Somatotrophs and Lactotrophs

On the basis of primary structure and biological function similarities, GH and PRL have been grouped together with the related placental lactogen (PL) in the PRL/GH/PL family. *GH*, *PRL* and *PL* genes are thought to have arisen from a common ancester by gene duplication and evolutionary divergence (23,33). Cells that secrete both GH and PRL, known as mammosomatotrophs, have been identified in neonate and adult rats by reverse hemolytic plaque assays (27) and immunocytochemistry (29,29). Gene ablation techniques specifically targeting GH-expressing cells in the developing pituitary have shown an almost complete absence of both somatotrophs and lactotrophs in transgenic mice, reinforcing the concept of a stem-somatotroph as common precursor to somatotroph and lactotroph cell populations. Also, somatomammotropic pituitary tumors are quite common (33).

1.3.1.1 Somatomammotropic Pit1- and Prop1-dependent differentiation pathway

The molecular basis of somatotroph and lactotroph cell differentiation implicates the activities of the pituitary-specific homeodomain protein Pit-1. Pit-1 expression is

detected at e13.5 in a region of the pituitary from which sommatotrophs and lactotrophs arise (100). Experiments have shown that Pit-1 mRNA transcripts are actually expressed at the same level in all pituitary cell types, but translated to significant protein levels only in somatotrophs, lactotrophs and thyrotrophs (230). Initially identified as Growth Hormone Factor-1 (GHF-1), Pit-1 was cloned as a transactivator of *PRL* and *GH* gene promoters (97,240).

The importance of Pit-1 as a regulator of somatotroph and lactotroph differentiation was demonstrated by the absence of these two cell types in the pituitary glands of *Snell* (*dw*) and *Jackson* (dw^J) dwarf strains of mice in which the *Pit-1* gene is mutated (6,35,135). Acting upstream in the differentiation pathway of Pit-1 is the pituitary-specific paired-like homeodomain factor Prophet of Pit-1 (PROP-1), detected in Rathke's pouch from e10.5 to e16.0 (225). *Prop-1* mutations were identified in the Ames (df) dwarf mouse, which moreover exhibits defective *Pit-1* gene expression and shares phenotypic defects with *dw* and *dwJ Pit1*-mutant mice. Pituitary hypoplasia in *dw*, dw^J and *df* dwarf strains of mice is in support of a role for PROP-1 and Pit-1 not only in the establishement and maintenance of differentiated phenotypes, but also in the proliferation of precursor cells (135,225). How cell proliferation signals are coupled to cell commitment and differentiation signals during pituitary development remains to be elucidated. In humans, *PROP-1* mutations have been characterized in and related to an absence or low levels of GH, PRL, TSH, LH, FSH and recently ACTH (183) in human combined pituitary hormone deficiency (CPHD) (64).

1.3.1.2 Cooperation between Pit-1 and Nuclear Receptors

Extensive analysis of rat and human *GH* gene regulatory elements have implicated Pit-1 binding and ensuing cooperation with retinoic acid receptor (RAR), thyroid hormone nuclear receptor (TR), and the zinc finger Zn-15 protein in effective somatotroph-specific

expression (40,140). Pit-1 is believed to direct lactotroph-specific *PRL* gene expression by collaborating with estrogen nuclear receptor (ER), as well as Ets and Pitx factors (42,48,248).

1.3.1.3 Pitx Homeobox Proteins Collaborate with Pit1

The homeobox Pitx1 and Pitx2 transcription factors are other general regulators of pituitary-specific transcription, including GH and *PRL* gene expression (Figure 1.2). Pitx1 was identified as a transcriptional regulator of *POMC* gene expression (128), and also as a factor interacting with Pit-1 (235). *Pitx2* was isolated as the causative gene by haploinsufficiency for Rieger's syndrome (218). Pitx1 and Pitx2 expression defines the oral ectoderm as early as e8.0, and is maintained in derivative structures throughout pituitary development (129,137). In addition to their pan-pituitary expression, Pitx1 and Pitx2 are expressed in distinct regions of the embryo. Their complex pattern of expression is consistent with the roles of Pitx1 in such developmental processes as craniofacial and limb development, and the roles of Pitx2 in establishment of laterality, as well as heart, lung, and craniofacial development (129,137,234). These roles will not be discussed further. The last member of the Pitx subfamily, Pitx3, is not expressed in the pituitary (132) which suggests that it does not play a role in pituitary functions.

Pitx1 and Pitx2 have similar transcriptional activities on *POMC*, *α-GSU*, *LH-β*, *FSH-β*, *TSH-β*, *PRL*, and *GH* pituitary-specific promoters (247). Their pan-pituitary expression, and contribution to cell-specific transcription of many pituitary specific genes may reflect the common origin of pituitary cells. Like Pit-1, Pitx factors are thought to confer promoter-specific expression through synergistic interactions with cell-restricted factors. With respect to sommatolactotroph cell differentiation, Pitx1 was shown to cooperate not only physically, but also transcriptionally with Pit-1 on the *GH* and *PRL*

promoters (235,248). *Pitx1*-loss-of-function experiments did not significantly affect the expression levels of either GH or PRL (130,234) as Pitx2 is thought to have compensated for pituitary cell differentiation functions. Such a role for Pitx2 in the differentiation of the sommatolactotroph cell lineage could not be studied in *Pitx2*$^{-/-}$ mice because of premature pituitary developmental arrest (137).

1.3.2 Thyrotrophs and Gonadotrophs

The glycoprotein hormones LH, FSH and TSH are heterodimers composed of a common α-glycoprotein subunit (α-GSU) noncovalently assembled with respective hormone-specific β-subunits LH-β, FSH-β, or TSH-β. The β-subunit confers hormone specificity, while the α-subunit is homologous within a species. Phylogenetic studies using nucleotide and amino acid sequence alignments predict that both α- and β-subunits evolved from a single ancestor through gene duplication (134). During pituitary development, thyrotrophs are thought to derive from a pool of precursor cells that also give rise to sommatotrophs and lactotrophs, but not gonadotrophs. In *α-GSU* gene-deleted mice, hyperplasia and hypertrophy of TSHβ-positive cells as a result of thyroid dysfunction was accompanied by a reduction of GH and PRL cells (49).

1.3.2.1 Interplay between Pit-1 and GATA-2 Activities

Although thyrotrophs and gonadotrophs do not seem to share a common precursor, reciprocal interactions between Pit-1 and the zinc finger protein GATA-2 would be implicated in the specification of both cell types (46). Differential GATA-2 function in these two cell types is defined by extrinsic Bmp2 and Shh signaling (46,245). Bmp2 signals are detected in the ventral juxtapituitary mesenchyme (VJM) as well as in the ventral region of the committed pituitary around e12.5, while Shh is expressed around but not in the pituitary (67,244). Transgenic studies have shown that gonadotroph- and

thyrotroph-specific GATA-2 expression is induced by BMP-2, itself induced by Shh (245). Elevated GATA-2 expression levels have been associated with the inhibition of endogenous Pit-1 gene expression in gonadotrophs (46,244). Expanding GATA-2 expression under the control of Pit-1 regulatory elements in transgenic mice is sufficient to convert all Pit-1 dependent lineages to the gonadotroph fate (46). In thyrotrophs, lower levels of GATA-2 are not believed to interfere with Pit-1 expression; in these cells, Pit-1 and GATA-2 were shown to interact and functionally cooperate to activate the *TSH-β* promoter (73). In thyrotrophs, Pit-1 is moreover thought to function to inhibit GATA-2 binding and activation of *LH* and *FSH* regulatory elements that do not contain adjacent Pit-1 binding sites. The ability of Pit-1 to interfere with GATA-2 function is lost in Snell *dw* mice that have a W48C mutation in the Pit-1 POU homeodomain which disrupts Pit-1/GATA-2 interactions, causing thyrotrophs to assume a gonadotroph fate (46).

1.3.2.2 Cell-Specific Collaboration of Pitx Factors with Egr-1, SF-, Sp1 and Lhx Factors

In pituitaries of *Pitx1*-deleted mice, LH-β, FSH-β and TSH-β levels are severily reduced, suggesting that Pitx1 is required for expression and/or maintenance of gonadotroph and thyrotroph lineages (130,234). The Pitx1 binding site in the *LH-β* promoter was demonstrated to be essential for its activity *in vivo*; transgenic mice harbouring a mutation of the Pitx1 binding site in the *LH-β* promoter lost basal as well as Gonadotropin-Realesing Hormone (GnRH)-stimulated pituitary expression (201). GnRH is a critical hypothalamic peptide that is required for the production and secretion of gonadotropins LH and FSH (54). Along with Pitx1, the zinc finger protein Early response 1 (Egr-1) and Sp1 proteins, as well as nuclear receptor steroidogenic factor-1 (SF-1) have been shown to coordinate the complex control of basal and/or GnRH-stimulated *LH-β* gene

promoter activity (105,246). In both *Egr1*- and *SF1*-deficient mice, LH-β production was compromised (98,153,243). Expression of FSH-β was also absent in *SF-1*$^{-/-}$ pituitaries. It appears that LH and FSH deficiencies in SF1$^{-/-}$ mice may be mediated by defective GnRH expression in hypothalamus (98). The integration of Pitx1 in previously described synergistic functions of GATA-2 and Pit-1 on the *TSH-β* promoter is still poorly understood.

Pitx and Lhx factors, via the actions of Lim-associated cofactor (CLIM), have been implicated in gonadotroph-specific activity of the bovine *α-GSU* promoter (9,109). Distinct *cis*-acting elements, still undefined, are thought to regulate *α-GSU* expression in thyrotrophs (49).

1.3.3 Corticotrophs and Melanotrophs

Both anterior lobe corticotrophs and intermediate lobe melanotrophs express and transcribe the *POMC* gene, beginning in mice at e12.5 in corticotrophs and at e14.5 in melanotrophs (100). A *POMC* cDNA was originally obtained from the intermediate lobe of bovine pituitaries (175), and since then the *POMC* gene has been cloned in rat (59), human (236), and mouse (185) among other species. Two introns and three exons make up the rat *POMC* gene (Figure 1.3), which measures approximately 6.5 kilobases (kbs). A mature 1200 nucleotide POMC mRNA transcript is found in the pituitary as a result of gene splicing (62). It is essentially the same transcript that is translated in corticotrophs and melanotrophs, but the resulting POMC peptide prohormone is subsequently processed in a different manner in each cell type by distinct cohorts of protein convertases (PC). ACTH results from corticotroph PC1 proteolytic activity, and the additional PC2 in melanotrophs processes ACTH further into α-MSH and CLIP (11). ACTH, β-lipotropin, and β-endorphin are the principal hormone end-products of POMC generated in corticotrophs,

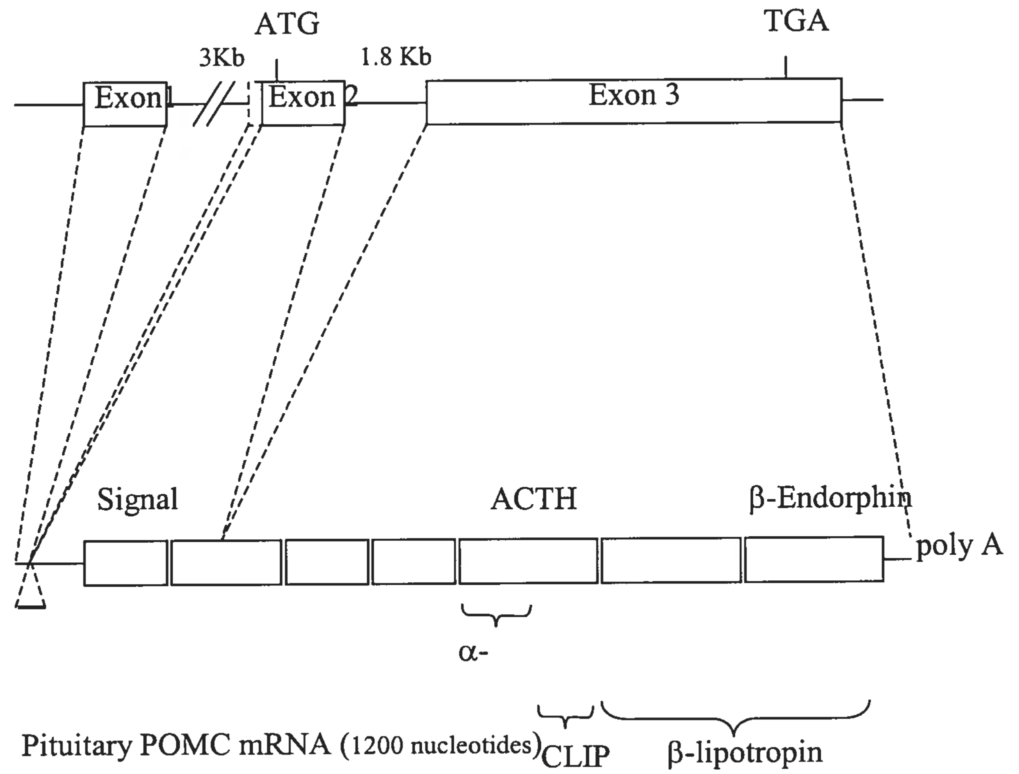


Figure 1.3 Stucture of the *POMC* Gene and mRNA

while POMC is processed further into α-MSH, CLIP, and β-endorphin in melanotrophs (152). Processing into ACTH depends on the PC1 proteolytic activity; the additional activity of PC2 in melanotrophs processes ACTH further into α-MSH and CLIP (11).

1.3.3.1 Contribution of Pitx Factors to POMC Lineages

In the end, single *Pitx* gene ablation experiments have not proven to be useful in assessing the actual need for Pitx activity for corticotroph and melanotroph cell function. In neither *Pitx1*$^{-/-}$ nor *Pitx2*$^{-/-}$ mice was POMC expression affected (130,137,234), suggesting that POMC expression *in vivo* would require either Pitx gene activity. *Pitx1/Pit2* double knock-outs could have been more informative in this case. However, the premature block in pituitary development observed in the latter double null mice has made the study of pituitary cell differentiation impossible (229).

Clues on the contribution of Pitx factors to the specification of corticotroph, and possibly melanotroph cell fates, have been provided by studies performed on the *POMC* promoter in AtT-20 corticotroph cell model. An account of *POMC* gene transcription studies will be given below. It is worth mentioning at this point the central position that Pitx factors hold in mediating protein interactions with two major determinants of the corticotroph cell fate; that is, the bHLH factor NeuroD1and the newly characterized T-box factor Tpit.

1.3.3.2 NeuroD1, an Exclusive bHLH Factor for Corticotrophs

The bHLH class of transcription factors has been studied extensively for their role in myogenic and neurogenic processes, as tissue-specific regulators of cell specification. NeuroD1 is a neuro- and pancreatic-islet-specific bHLH transcription factor implicated in neuronal precursor differentiation and tissue-specific expression of the *insulin* gene, respectively (133,178). The expression of NeuroD1 is transient in the developing mouse

pituitary, present between e12 and e15, but not after e16 (198); moreover, its expression is restricted to corticotrophs. NeuroD1 mRNA but not protein can be detected in the adult mouse (199). NeuroD1 transcripts have also been detected in normal human pituitaries and in ACTH-secreting pituitary adenomas (191). NeuroD1 expression preceedes that of POMC in differentiating corticotrophs, inferring a role for NeuroD1 in the induction of corticotroph differentiation. *NeuroD1* null mice did not exhibit a significant loss of pituitary POMC expression when analyzed at e17.5. However, a delay in the appearance of POMC expression in the anterior pituitary of *NeuroD1*$^{-/-}$ mice was recorded at earlier times (e14.5), suggesting that NeuroD1 participates but is not essential for the onset of corticotroph differentiation (Lamolet B, unpublished). NeuroD1 has been demonstrated to confer corticotroph-specific activity to the *POMC* promoter through synergistic interactions with Pitx1, the details of which are discussed below.

1.3.3.3 Conversion of POMC$^-$ Cells into POMC$^+$ Cells by the T-box Factor Tpit

T-box factors are defined by a conserved DNA-binding motif known as the T-box, named after the first-discovered T-box gene *T* or *Brachyury* (255). Members of this family of transcription factors have been identified in both vertebrates and invertebrates, where they have been implicated in developmental decisions concerning patterning (224)and recently cell differentiation (127,233). Tpit expression studies have shown that it is restricted to corticotroph and melanotroph POMC-expressing cells in the developing pituitary and precedes POMC expression in each cell type by 0.5 days, suggesting a role for this factor in corticotroph and melanotroph differentiation. Transgenic expression of Tpit under the control of the *α-GSU* promoter was sufficient to induce expression of POMC in cells of the rostral tip of the developing pituitary (127). This particular structure contains a population of transient, proliferating, uncommitted cells (179) that do not normally express

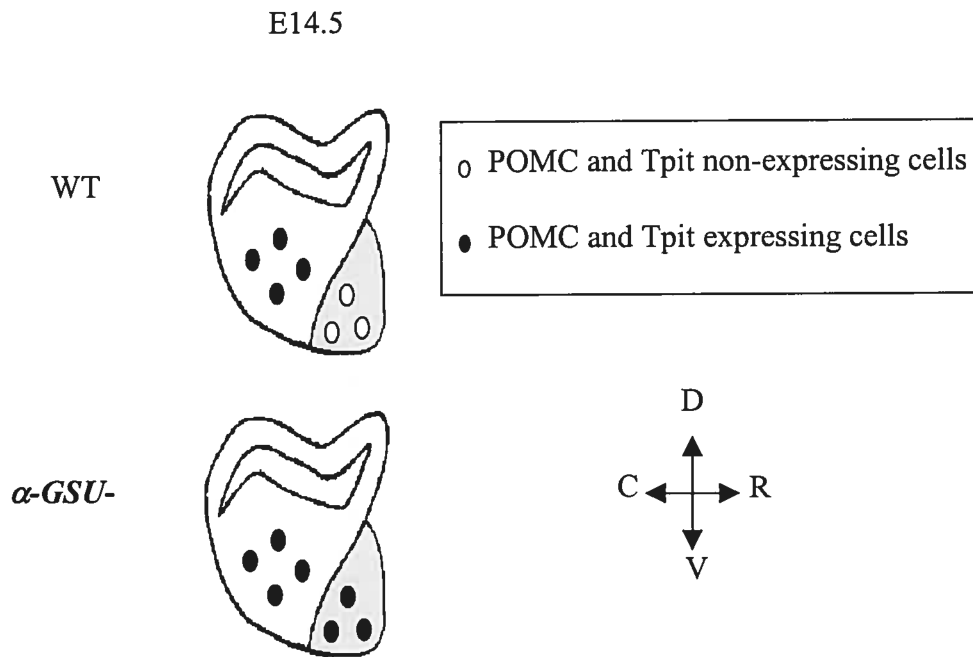
POMC but express high levels of Pitx1 (129). Hence, Tpit appears sufficient to induce *POMC* transcription in Pitx-1-expressing uncommitted pituitary cells (Figure 1.4). The expression of the corticotroph marker NeuroD1 was not induced in these ectopic POMC-expressing cells, indicating that additional information is required for terminal differentiation into corticotrophs.

1.3.3.3.1. Isolated ACTH Deficiency

In humans, POMC is expressed in three tissues: in the anterior pituitary to stimulate cortisol production by the adrenal gland (the intermediate pituitary degenerates after birth in humans), in the hypothalamus to regulate appetite via the leptin pathway, and in skin where it plays a role in pigmentation and cutaneous inflammation. Genetic defects in the *POMC* gene have been identified (122). Consistent with the expression pattern of POMC, these patients suffer from adrenal insufficiency, early onset obesity and red hair pigmentation. The lack of α-MSH peptide in *POMC*-deficient patients is likely to blame for the weight and pigmentation abnormalities. Hypocorticolism and hypoglycemia on the other hand are thought to reflect ACTH insufficiency, a phenotype that has moreover been documented separately in patients with an isolated deficiency of pituitary ACTH (26). Our findings on Tpit, and its particular expression and role in ACTH-expressing corticotrophs

in mouse, led us to investigate whether mutations in the *TPIT* human gene should produce an isolated deficiency of pituitary ACTH.

Two out of three analyzed cases of children born with an isolated deficiency in ACTH turned up with mutations in the *TPIT* gene (127). In one case, a homozygous point mutation was identified that introduces a premature translation termination codon in the open reading frame of *TPIT*. As a result, the TPIT protein is either translated in a truncated



1.4 Ectopic Expression of Tpit in α*GSU*-Tpit Rostral Cells Induces the Expression of POMC (adapted from Lamolet B, 2001). (R) rostral, (C) caudal, (D) dorsal, (V) ventral.

form that is inactive, or more likely the faulty transcript is eliminated by a surveillance mechanism known as non-sense mediated mRNA decay (NMD) (86). This child's parents, and one grandmother, were found to be heterozygotes for the mutation but free of the ACTH deficiency, making inheritance recessive. The second child studied was found to be heterozygous for a point mutation that changes serine residue 128, a residue conserved in the T-box of all known family members, to phenylalanine. The ensuing mutant TPIT protein in this heterozygote patient might be acting in a dominant negative manner to inhibit normal TPIT function coming from the *TPIT* allele that is not mutated.

1.3.3.4 Growth Signaling Factors

How the activities of Pitx, NeuroD1 and Tpit transcription factors are regulated by outside growth signaling factors is still misunderstood. Signaling factors intrinsically expressed in Rathke's pouch have been shown to suffice for corticotroph cell specification from e9.5 and on (67,244). Prior to this, Shh signals seem to be required either for induction or maintenance of ACTH expression, which is lacking in *Pitx1*-Hip transgenic mice (245). No other gene deleted or mutant animals for pituitary restricted signaling or transcription factors have shown a complete absence of POMC/ACTH expression. The early arrest of pituitary development caused by the absence of such pleiotropic molecules as Bmps and Fgfs, or the possible incomplete penetrance of transgenic experimental methods render the *in vivo* study of the specific roles of signaling factors in the differentiation of POMC-expressing corticotrophs and melanotophs difficult. My Master's project stems from contradictory results that had previously been obtained by two groups of investigators in their attempt to define the role of Bmp-4 signaling on embryonic corticotroph specification. Indeed, ACTH expression was downregulated in *ex vivo* e9.5 Rathke's pouch explants cultured with Bmp-coated beads (67), while ACTH expression did

not appear to be affected in gain-of-function *αGSU*-BMP4 transgenic embryos (244). By means of the extensive characterization of the rat *POMC* gene promoter in Dr. Drouin's laboratory and given that POMC expression is a marker of corticotroph cells in the anterior pituitary, my approach in elucidating the contribution of Bmp factors to corticotroph cell differentiation has consisted in studying the role that Bmp signaling plays in the control of *POMC* promoter activity.

1.4 POMC Transcription

POMC promoter studies in our laboratory have been executed in the mouse POMC/ACTH-expressing AtT-20 cell line, which has its origins in pituitary tumorigenic tissues (24). Seeing that these cells express such early protein markers as NeuroD1, Pitx and Tpit, AtT-20 cells are used as a model of differentiating corticotrophs (127,128,199). The same mechanisms driving POMC promoter activity in AtT-20 cells would hence be expected to induce POMC expression in corticotrophs, but not necesarily in melanotrophs which seem to differ. NeuroD1 for example is not expressed in melanotrophs (199). POMC promoter studies in our laboratory have typically led to supporting *in vivo* evidence in mouse models, and hence constitute an important tool to study the molecular basis of corticotroph differentiation.

1.4.1 Corticotroph-Specific Regulation of POMC Expression

1.4.1.1 *POMC* Promoter

POMC promoter sequences from –480 to +63 bp were shown to have corticotroph-specific activity in AtT-20 cells, as well as in transgenic mice (141,249). The –480/+63 bp promoter, hereafter referred to as the full length promoter, was only expressed in transgenic anterior and intermediate pituitary but not in hypothalamic regions also known to express POMC. Other regulatory sequences would be involved in hypothalamic expression of POMC (272). Deletion studies that made use of different regions of the pituitary-specific

POMC promoter, fused either alone or in different combinations to the *luciferase* (*luc*) reporter gene and transfected into AtT-20 cells, were performed to determine the contribution to promoter activity made by the distal (-480/-324), central (-323/-166) and proximal regions (-165/-34) (241). In doing so, the distal together with the central domains of the promoter were demonstrated to confer specificity to *POMC* promoter activity. Out of the three latter regions, only the central one was shown to possess some activity on its own.

Through footprint and mutation analyses, it was shown that individual regulatory elements within the *POMC* promoter contribute to its transcriptional activity (241). Indeed, the loss of any of these elements decreased *POMC* promoter activity significantly. Among the many transcription factors that have been identified to mediate transcriptional control of *POMC* at such binding sites (Figure 1.5), only a few have been retained as having corticotroph-specific roles. Namely, the bHLH factor NeuroD1 acting in the distal region, and the homeodomain-containing transcription factor Pitx1 with its obligate T-box partner, Tpit, both acting in the central region of the promoter. Not only would these two regulatory elements, namely the distal NeuroD1 Ebox ($Ebox_{neuro}$) and central Pitx/Tpit binding sites, be responsible for corticotroph specificity but they would moreover be mediating transcriptional synergy between the distal and central domains of the *POMC* promoter.

1.4.1.2 NeuroD1 and Pitx Synergistic Interactions

Analysis of the regulatory elements contributing to AtT-20 cell-specific transcription within the central domain of the *POMC* promoter led to the cloning of the homeobox transcription factor Pitx1 (Ptx1) (128). *Drosophila* bicoid-related Pitx1 is a member of a subfamily that also includes mammalian Pitx2, Pitx3, Otx1, Otx2, and goosecoid (60). The Pitx subfamily of transcription factors is characterized by a paired-like DNA-binding homeodomain in which residue 50 is a lysine. Pitx factors bind as monomers

to a single site in the *POMC* promoter, where they act as transcriptional activators through their C-terminal transactivation domain (128). Besides its pituitary actions, Pitx1 has also been implicated in *POMC* expression in human small cell lung carcinomas (SCLC) (180).

Pitx factors have been shown to be the basis for synergistic activities between the distal and central domains of the *POMC* promoter (Figure 1.5), Pitx bound to the central domain interacting with NeuroD1-containing bHLH heterodimers bound to the distal domain (198,199,242). NeuroD1, and related tissue-specific bHLH factors like MyoD in muscle, dimerize through their HLH motif with ubiquitously expressed bHLH factors E12 and E47 (72). Only in the heteromeric form may NeuroD1, through its basic motif, bind to $Ebox_{neuro}$ and moreover collaborate with Pitx factors. Indeed, *in vitro* binding as well as *in vivo* co-immunoprecipitation and transfection studies have shown that it is the ubiquitous bHLH partner of NeuroD1 that directly interacts with Pitx factors (198).

In the context of the full length promoter, both Pitx and NeuroD1 binding sites are required for transcriptional synergism (198). In transfection assays however, transcriptional synergism can be reconstituted in the absence of Pitx1 DNA binding activity but not independently of DNA binding by bHLH factors, highlighting the importance of protein:protein interaction for synergism (198). Deletion of the distal domain resulted in a thousand-fold loss of *POMC* promoter activity in transgenic mice (198), and mutation of $Ebox_{neuro}$ significantly compromised the activity of the full length *POMC* promoter in transfected AtT-20 cells (199). Although the $Ebox_{neuro}$ binding site for NeuroD1 bHLH transcription factors seems to be at the foundations of corticotroph-specific synergistic activation of the *POMC* promoter, NeuroD1 itself is not required *sensu strictu* for *POMC* transcriptional activation. Indeed, substituting the $Ebox_{neuro}$ for an Ebox that binds ubiquitous bHLH homodimers ($Ebox_{ubi}$) restored promoter activity (199). Still, the $Ebox_{neuro}$ of the distal region of the *POMC* promoter is incapable of binding ubiquitous

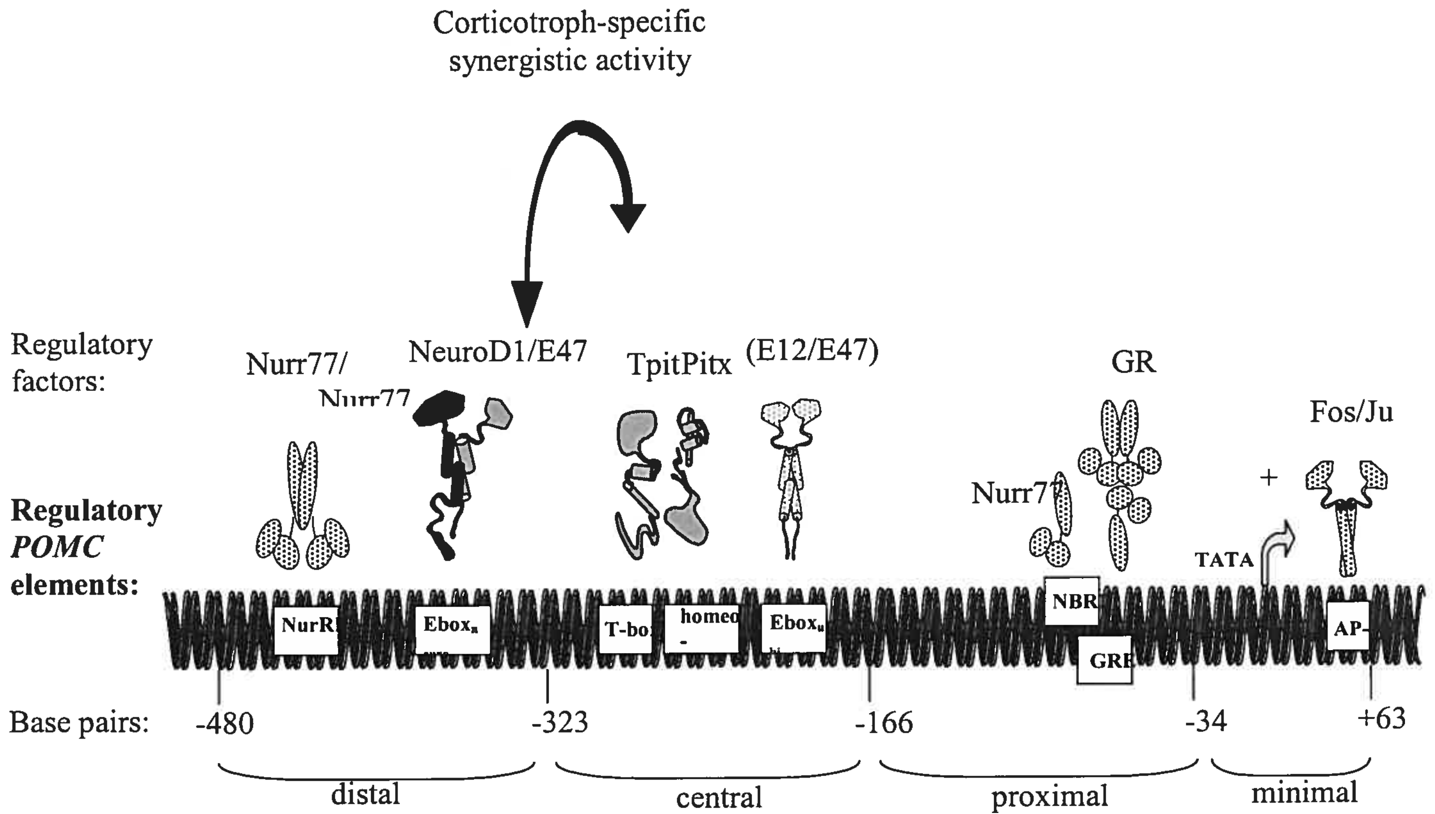


Figure 1.5 *POMC* Promoter Regulatory Elements

bHLH homodimers, hence the requirement for NeuroD1 in corticotrophs for sequence-specific recognition and activation of the $Ebox_{neuro}$.

1.4.1.3 Tpit, an Obligate Partner of Pitx Factors

Pitx activity on the central domain of the *POMC* promoter is dependent on synergistic interactions with Tpit. From a mutational analysis of the regulatory elements surrounding the Pitx binding site, Tpit was recently cloned in our laboratory as an obligate Pitx partner (127). The Tpit binding site in the *POMC* promoter actually corresponds to a half site of the palindromic Brachyury (T)-binding element (170). Tpit activity on *POMC* can actually be replaced by T, to which it is the most related, but not by Tbx1 which is another T-box factor that is expressed in AtT-20 cells (127).

Pitx and Tpit can each weakly activate *POMC* transcription through their respective binding sites, which are 5 bp away from each other. Together, they collaborate in a synergistic interaction that would originate from their ability to cooperatively bind DNA, as was evidenced in *in vitro* binding studies (127). The loss of either binding site obliterates the synergistic activity between Pitx and Tpit. Protein:protein interactions between Pitx1 and Tpit factors are thought to be mediated by the Pitx1 homeodomain. Synergistic interactions between T-box factors and Pitx family members has been shown in cell culture for Pitx1, Pitx2 and Pitx3, but not for the closely related subfamily of Otx transcription factors (127).

1.4.2 Hormonal Regulation of *POMC* Expression

Once the terminal differentiation of corticotrophs is completed, the regulation of *POMC* expression mainly falls under a balance of stimulatory signals such as hypothalamic corticotropin-releasing-hormone (CRH) and negative feedback signals such as glucocorticoids (Gc) from the adrenal gland (Figure 1.6). These two modes of regulation

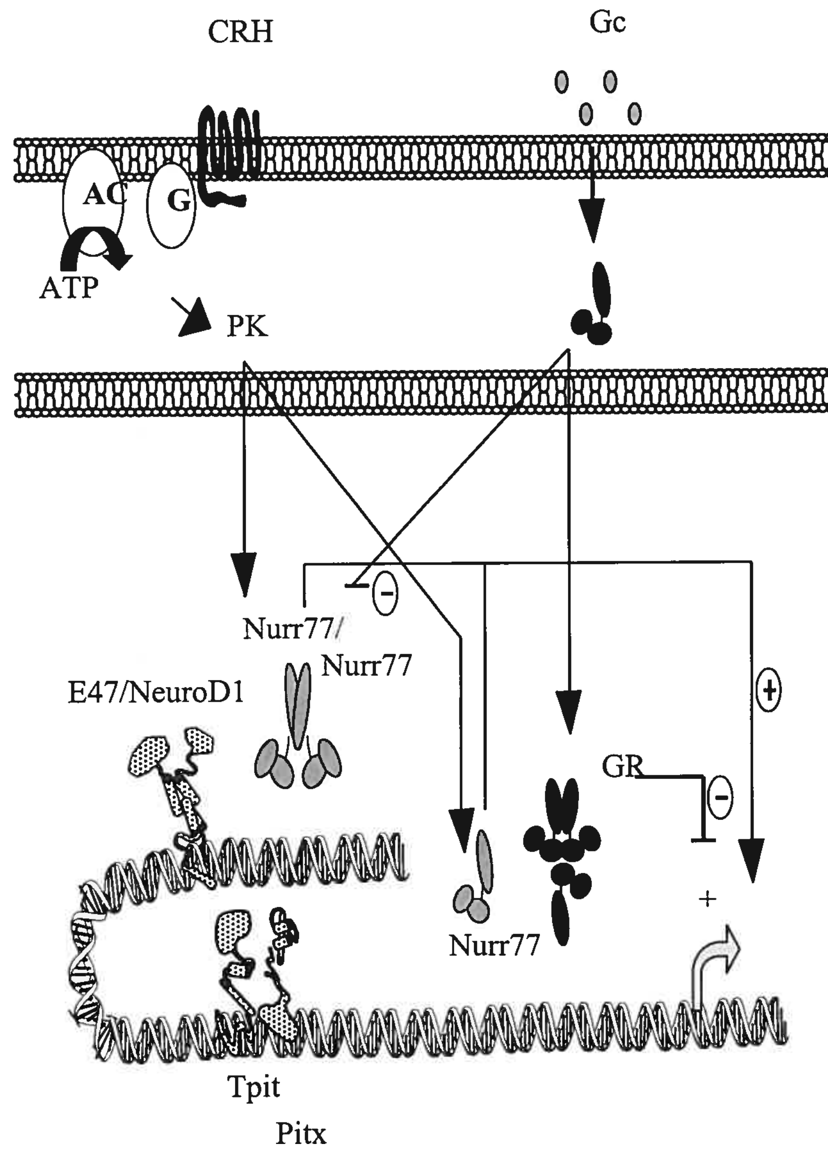


Figure 1.6 Hormonal Regulation of *POMC* Expression

converge in corticotrophs to mainly control the secretion of pre-stored ACTH hormone in an immediate response, or in a long-term response to adjust the transcription rate of the *POMC* gene.

1.4.2.1 CRH

The biological actions of CRH are mediated through the G-protein coupled transmembrane CRH receptor (193). Receptor activation subsequently turns on the adenylate cyclase second messenger system, leading to the production of cyclic AMP (cAMP) and activation of cAMP-dependent protein kinase A (PKA). PKA is known to control cellular functions through the phosphorylation of such transcription modulators as the cAMP response element binding protein (CREB), cAMP response element modulator (CREM), activation transcription factor (ATF)1/2 and general coactivators CBP/p300 (166). No such regulatory elements are present with the –480/+63 *POMC* promoter, yet CRH stimulation of AtT-20 cells does result in the increase of *POMC* transcription (70,71). These CRH effects are mimicked by cAMP analogs or by forskolin, and do not appear to require *de no*vo protein synthesis since POMC levels increase even in the presence of cyclohexamide, an inhibitor of translation.

Several pituitary-specific *POMC* promoter sequences have been implicated in CRH responses (8,15,102,136), but not much is known of their mechanism of action. We and others have shown that CRH inductive regulation of *POMC* expression in AtT-20 corticotroph cells seems to be the function of promoter regulatory elements for members of the Nur77 subfamily of orphan nuclear receptors (154,172,194). This subfamily of transcription factors includes Nur77, also known as NGFI-B (164), Nur-related factor 1 (Nurr1) (131)and neuron-derived orphan receptor 1 (NOR-1) (186). Evidence that Nur77-related activity is the principal mediator of CRH inductive effects on *POMC* comes from experiments wherein the overexpression of a dominant negative Nur77 mutant blocks both

CRH and forskolin actions (194). The binding site for Nur77 subfamily members is located in the distal region (-395bp) of the *POMC* promoter (194). This Nur response element (NurRE) is a palindrome that binds Nur factor homodimers and heterodimers (154). The two half sites share a partial homology with the monomeric Nur-binding response element (NBRE) first characterized in yeast (256). Such a NBRE is moreover found in the proximal region (-63bp) of the *POMC* promoter, but as the NurRE has been shown to be much more responsive to CRH treatments, corticotroph-specific CRH actions on the *POMC* promoter would appear to be primarily mediated through the NurRE rather than the NBRE. Unfortunately, functional redundancy between members of the Nur subfamily have interferred with *in vivo* analysis, through gene deletion techniques, of their individual pituitary or hypothalamo-pituitary functions (38,43).(38)

1.4.2.2 GR

Glucocorticoid (Gc) negative regulation of ACTH release and *POMC* transcription has emerged as a concept of feedback regulation operating to adjust activity of the hypothalamo-pituitary-adrenal (HPA) axis with physiological homeostasis. Gc acts at two levels in the HPA, basically on CRH neurons in the hypothalamus and on corticotrophs in the pituitary. Gc effects are mediated through the glucocorticoid receptor (GR) (155). To date, two different mechanisms have been proposed to account for GR-mediated repression of *POMC* promoter activity. Whether one or both of these mechanisms interfere with *POMC* expression during development, HPA homeostasis or pathology is not well understood.

In one case, GR would mediate Gc-induced repression of *POMC* through direct DNA contacts with a negative Gc response element (nGRE) located in the proximal domain of the promoter (63). Deletion analysis has demonstrated that the nGRE is required for Gc repression of *POMC* in AtT-20 cells (63,173) and in transgenic mice (79,249). *In vitro*,

three molecules of GR bind to the nGRE (61); however, the exact molecular basis of transcriptional repression by the GR complex at this site is still elusive. *In* vivo experiments are moreover in support of a nGRE-mediated mechanism for GR repression of *POMC* expression. GR-deficient mice are characterized by an upregulation of CRH and POMC at both levels in the HPA axis (37). Mice that express, using knock-in technologies, a mutant GR deficient in dimerization do not exhibit the same phenotype. GR molecules that no longer dimerize would still be able to participate in transrepression mechanisms of gene control, but not in GRE/nGRE binding. Mice expressing this form of GR showed upregulated POMC expression in the pituitary, while their CRH levels in the hypothalamus were under negative control presumably by monomers of GR (205). GR dimerization hence seems to be required for the negative regulation of pituitary POMC levels. Nevertheless, other mutant mice models are in support of alternative mechanisms of negative Gc feedback on the *POMC* promoter (168,169).

Centered at –63bp, the nGRE overlaps with the NBRE, which suggests that GR may likewise participate in a competitive mode of action to interfere with CRH/Nur-mediated activation of the *POMC* promoter. However, through AtT-20 transfection experiments, the distal NurRE appeared to be a target for GR. Using *luc* reporters containing three copies of the NurRE, GR was shown to be able to block Nur-dependent activity (195). Direct protein interactions between GR and Nur77 are possible *in vivo* (156), suggesting that the mechanism of GR transrepression of *POMC* would be one that is similar to the one characterized between AP-1 and GR on the *collagenase* gene promoter. Control of transcription on this latter promoter, which is devoid of GR-binding sites, appears to rest on antagonistic protein:protein interactions between AP-1 activators and GR (10,103,269).

1.4.3 Growth Factor Regulation of POMC Expression

1.4.3.1 LIF

Leukemia Inhibitory Factor (LIF) is a member of the interleukin (IL)-6 family of cytokines and is expressed in normal fetal and adult corticotrophs, as well as in ACTH-secreting adenomas (1). *In vivo*, LIF has been shown to favor the differentiation of the POMC-expressing cells at the expense of other pituitary cells. That is, the pituitary glands of *αGSU*-LIF transgenic mice exhibited a hyperplasia of ACTH-expressing cells, which accounted for 65% of the population of anterior pituitary cells in comparison to 13% in the wild-type (270). Although a potential role in the development and maintenance of corticotroph cell biology is suggested by these results, an understanding of the precise actions of LIF on either proliferation or differentiation processes in the pituitary is lacking. While LIF has been attributed a role in the regulation of corticotroph differentiation in the pituitary, it has also been shown to inhibit proliferation of AtT-20 cells (227). LIF does not seem to be required for the establishment of the corticotroph cell lineage seeing that the pituitaries of *LIF* knockout mice exhibit reduced, but still easily detectable POMC mRNA levels (34).

In the context of *POMC* promoter activity, LIF has been demonstrated to enhance CRH effects (204). LIF signaling is mediated by STAT transcription factors, and in particular by STAT3 in pituitary cells. A few LIF/STAT3 response elements have been identified on the *POMC* promoter (19), including one that overlaps the NurRE (20). Not much more is known of the LIF-induced STAT3 mechanism of action on the *POMC* promoter.

1.4.3.2 TGF-β

Studies had previously looked at the direct modulation of *POMC* expression by Transforming growth factor (Tgf)-β and Activin members of the Tgf-β superfamily. Activin-A has been reported to suppress basal ACTH secretion and POMC mRNA accumulation from AtT-20 cells (14). Activins were initially isolated and characterized based on their ability to promote FSH secretion from pituitary gonadotropes (139). In Bilezikjian's work, recombinant (r) human Activin-A ($\beta_A\beta_A$) treatments of AtT-20 cells for 48 hrs were shown to suppress ACTH secretion and POMC mRNA expression by about 50%; this same treatment was however shown to inhibit by 25% the growth rate of AtT-20 cells (14). While the latter study reported that rTgf-β_1 had no effect on POMC transcript levels in AtT-20 cells; more recent work suggests otherwise (18). Tgf-β is secreted by hypothalamic astrocytes and the presence of TgfβR-I receptors transcripts in POMC-expressing neurons had suggested that Tgf-β might be implicated in the modulation of POMC neuronal activity. Indeed, rTgf-β_1 treatments of mediobasal hypothalamic fragments lead to an average 50% decrease of POMC mRNA levels detected by *in situ* hybridization (18).

A relationship between members of the Tgf-β superfamily of signaling molecules and *POMC* expression had already been established in the aforementioned studies. The basis of this relationship seems to rest on negative modulation of *POMC* expression by members of the Tgf-β family. Whether Bmp regulatory pathways also lead to the decline of *POMC* expression has yet to be determined.

1.5 Bmp Signaling

1.5.1 Tgf-β Superfamily Functions in Embryos

Bmps are a subfamily of the large Tgf-β superfamily of polypeptide growth factors characterized by three conserved pairs of disulfide bonds and that moreover includes Tgf-βs, Activins, Inhibins, Growth differentiation factors (GDFs) Nodals and Mullerian-inhibiting substance or MIS (158). Tgfβ-related factors are secreted factors that mediate a diverse set of cellular responses in species ranging from worms to mammals. Contributing to our current knowledge of the developmental aspects of Tgf-β signaling are three general types of genetic experiments: genetic loss-of-function experiments in *Drosophila*, ectopic expression (mRNA injection into embryos or factor addition to tissues) in *Xenopus*, and genetic loss of function in mice by homologous recombination. Through such work, the Bmp homologue Decapentaplegic (Dpp) in *Drosophila*, initially identified as a dorsalizing agent, has been shown to mediate among other developmental events the dorsoventral patterning of the embryonic ectoderm and midgut morphogenesis (113). *Xenopus* Bmp2/4 and Activin have been implicated in the formation of ventral and dorsal mesoderm, respectively (45,77); while mammalian Activin and Tgf-β have been found to be modulators of cell-cycle arrest, adhesion, and death (213). Bmp factors have been designated as regulators of embryonal cell specification and morphogenic processes, such as bone formation for which they were first identified (87).

The basic Tgf-β signaling system involves ligand-induced assembly of a transmembrane receptor complex, direct activation of Smad receptor substrates, nuclear translocation and formation of a Smad multisubunit transcriptional complex (Figure 1.7). An important way in which diversity is achieved in Tgf-β responses is through specific ligand-receptor and receptor-Smad interactions.

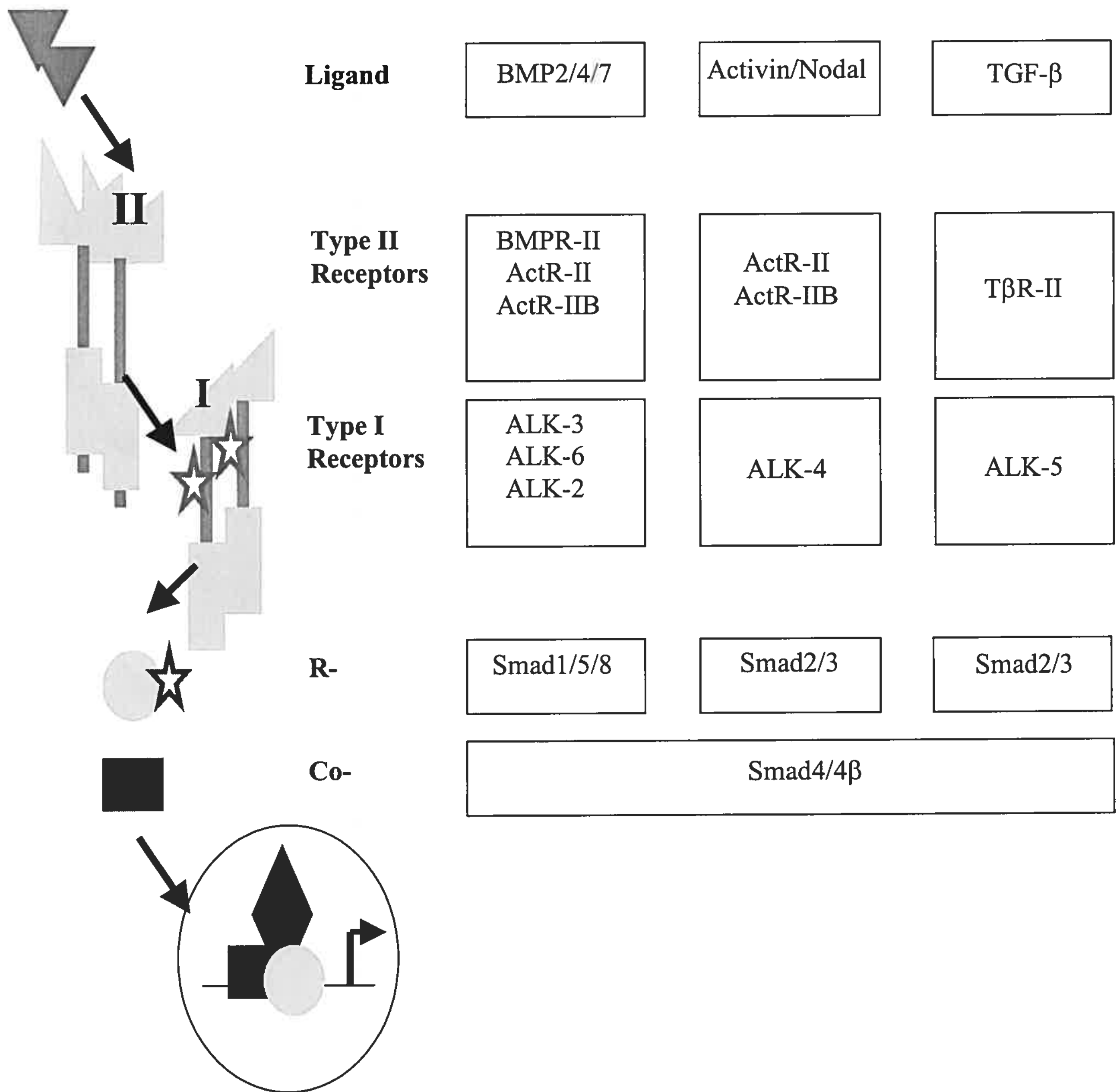


Figure 1.7 TGF-β Superfamily Signalling Pathways

1.5.2 Ligand and Receptor Families

All Tgfβ-related ligands are synthesized as inactive precursors that are proteolytically activated by cleavage to yield mature C-terminal peptides that subsequently assemble into dimers. This processing event is thought to occur within the *trans*-Golgi network and would regulate the rate of Tgf-β peptide secretion and hence signal production during embryonic development. Subtilisin-like proprotein convertases (SPCs) have been implicated in the latter maturation process. The function of these SPCs has been evaluated *in vivo* and determined to be essential for Tgf-β activity since SPC-deficient mice (39,209)develop defects as severe as the ones observed in mice deficient in ligand, receptor or Smad constituents of the Tgf-β signaling pathway.

Tgf-β ligands bind to two different types, termed typeI and II, of cell membrane receptors with intrinsic serine/threonine kinase activity. Ligand binding experiments suggest that the receptor complex is a heterotetramer of two type I and type II molecules (190). The type I receptor is inactive because a wedge-shaped GS (glycine-serine rich) region is inserted into the kinase domain, disrupting the catalytic center (92). This GS region is subject to phosphorylation by the ligand-activated typeII receptor, which is itself constitutively active but requires ligand binding to trigger receptor complex formation and activation of receptor kinase I (7). In the activated receptor complex, it is the type I receptor that is the primary transducer for specific intracellular responses. Mutating GS domain serines and threonines arrests both phosphorylation and signal propagation (7). Mutations within the GS domain can also lead to the constitutive activation of type I receptors. Replacing glutamine (Q) residues 233 in the type I Activin-like kinase (Alk)-3 receptor (BmpR-IA) and 203 in Alk-6 (BmpR-IB) by an aspartic acid residue generates constitutively activated Alk-3 (Q223D) and Alk-6 (Q203D), which activate the Bmp-

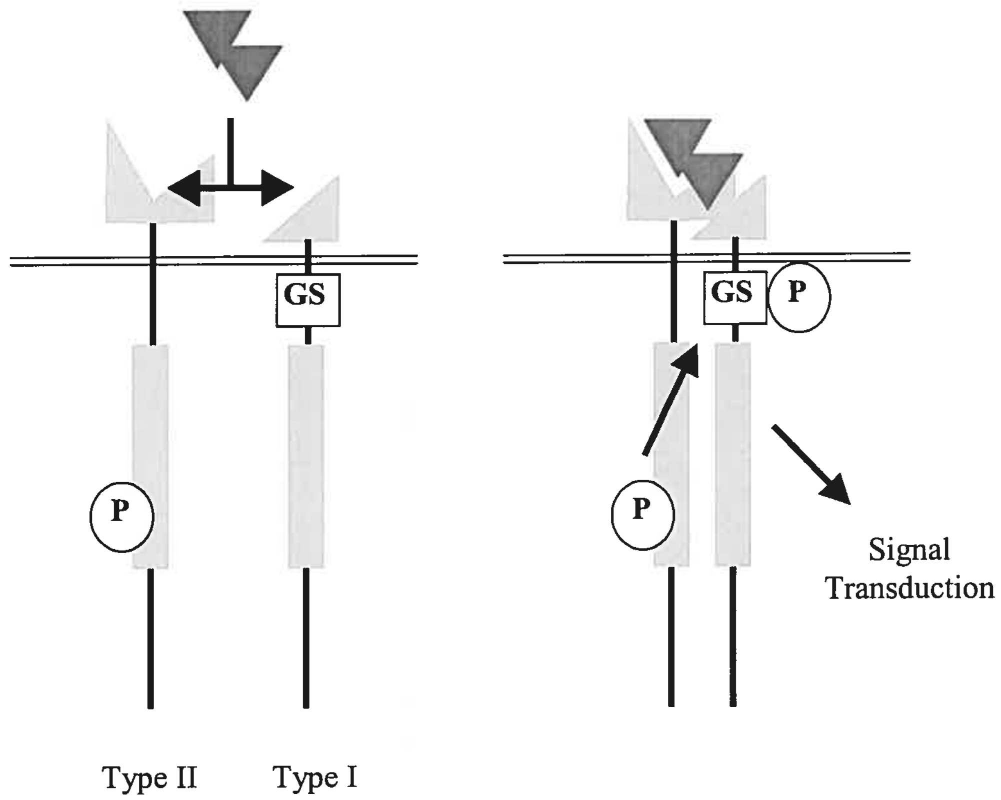


Figure 1.8 Cooperative Binding of BMP Ligand to Type I and Type II Receptors

specific signaling pathway in a ligand-independent fashion (2). In general, Tgfβ-related ligands are thought to first bind to type II receptors to then recruit type I receptors (Figure 1.7). However, Bmp ligand binding ability does not appear to be restricted to receptor type and implicates a cooperative model (Figure 1.8) of receptor interactions with Bmp ligands (87,145). Bmps can directly bind to type I receptors overexpressed in COS cells (239), but require type II receptor phosphorylation for activation of the signaling pathway. The latter requirement can be bypassed through the overexpression of constitutively active forms of Bmp-specific type I receptors Alk-3 (Q223D) and Alk-6 (Q203D), which exert Bmp effects even in the absence of ligand stimulation and type II receptors (2,238).

1.5.3 Smad Signaling

The first mediator of Tgf-β signals to be characterized was the *Drosophila* Mothers Against Decapentaplegic (Mad) protein, identified in a genetic screen for genes required to maximize Dpp signaling (124,203). Mad homologues Sma-2, Sma-3 and Sma-4 were then identified in *C. elegans* (215), while the first reported vertebrate homologue was the tumor supressor DPC4/Smad4 (78). The general term Smad, derived from Mad and Sma, has been adopted to designate members of this family of proteins which can be classified into three groups according to structural and functional similarities. That is; receptor-regulated Smads (R-Smads) Smad1, -2, -3, -5, and -8 involved in ligand-specific signaling; co-mediator Smads (Co-Smads) Smad4 and Smad4β participating in signaling by diverse Tgf-β family members; and inhibitory Smads (I-Smads) Smad 6 and Smad7 that negatively regulate these pathways by preventing R-Smad phosphorylation or formation of R-/Co-Smad functional complexes (36).

Smad proteins are the only known intracellular mediators of Tgf-β responses with an established capacity to transmit signal directly from the cell membrane into the nucleus.

Overexpression of R-Smads with Co-Smads in cell culture systems mimicks Tgf-β effects, which are counteracted by specific Smad signaling inhibitors (85). Be that as it may, Bmp signaling has also been shown to take effect in a Smad-independent fashion. The Tgfβ-mediated decrease in IGFBP-5 transcript and protein synthesis, ultimately resulting in a block of muscle differentiation, are thought to implicate the c-Jun N-terminalinal kinase (JNK) signaling pathway rather than the Smad pathway (210). Inhibitors of MAP (Mitogen-activated protein) kinase kinase-4 (MKK4), an upstream JNK activator, but not of Smad signaling blocked Tgf-β effects on *IGRBP-5* expression. It is however unlikely that JNK is the primary mediator of Tgf-β signals seeing that the JNK-kinase takes several hours to respond. The activity of a particular member of the Extracellular signal-regulated kinase (Erk) subfamily of MAPKK kinases (MAPKKKs), known as TGFβ-activated kinase I (TAK1), had been shown to be rapidly increased by Tgf-β and Bmp-4 (267). The indispensable role that Bmp factors seem to have in cardiomyocyte differentiation for example, as inducers of cardiac transcription factors, appears to be mediated by TAK1 (167). Tgf-β activation of TAK1 has been described to occur through Bmp receptors as well as the upstream regulator TAB1 (TAK1 binding protein) (266). TAK1 in turn has been shown to stimulate the stress activated kinase p38 pathway, which subsequently induces the nuclear activity of Activating transcription factor 2 (ATF2) (80). Smad and the TAK1/p38 pathways were moreover found to act together in synergistically enhancing the activity of the ATF-2 (214). Such work undelines the important role that Smad-independent pathways play as mediators of Tgf-β signaling.

1.5.3.1 Smad Structure

Smad proteins (Figure 1.9), about 400-500 amino acids in length, are made up of two highly conserved N-terminal and C-terminal domains of globular structure, separated

by a more variable proline-rich linker (222,223). The N-terminal or Mad Homology-1 (MH1) domain typically corresponds to the DNA-binding domain of Smads (223). Once recruited to DNA, Smads can independently regulate transcriptional processes through their MH2 C-terminal (C-terminal) transactivation domain. Indeed, a construct composed of the Smad MH2 domain fused to the heterologous GAL4 DNA-binding domain was able to transactivate transcription of a reporter construct containing a GAL4-binding site (143). A construct containing full length Smad was however inactive in this assay. This speaks of the tight regulation of MH2 transactivating capacity by the MH1 (transactivation repressor) domain in the absence of ligand. The MH2 domain furthermore functions in receptor interactions, Smad oligomer formation, and negative regulation of MH1 DNA-binding activity (161).

1.5.3.2 Specificity in Recruitment and Activation of R-Smads by Receptor/Ligand Complexes

In the absence of ligand stimulation, R-Smads as well as Co-smads are mainly found in the cytoplasm as oligomers in a closed conformation. Upon ligand-induced Tgfβ-receptor complex formation, cytoplasmic R-Smads are recruited and phosphorylated by activated type I receptors thereby alleviating the mutually inhibitory interactions between the MH1 and MH2 domains (161). Receptor phosphorylation of R-Smads occurs in a ligand-specific manner on a serine-rich SSXS motif found exclusively in the C-terminal domain of R-Smads (120): thus, the Tgf-β and Activin type I receptors activate Smad2 and Smad3, whereas the Bmp type I receptors target Smad1,-5, and -8. Smad-receptor interactions are prompted by a basic pocket in the C-terminal domain of R-Smads that docks to the phosphorylated GS receptor region. Specificity in the latter interaction is mediated by loop 3 and α-helix 1 (αH-1) of R-Smads (Figure 1.9) and loop 45 of the type I

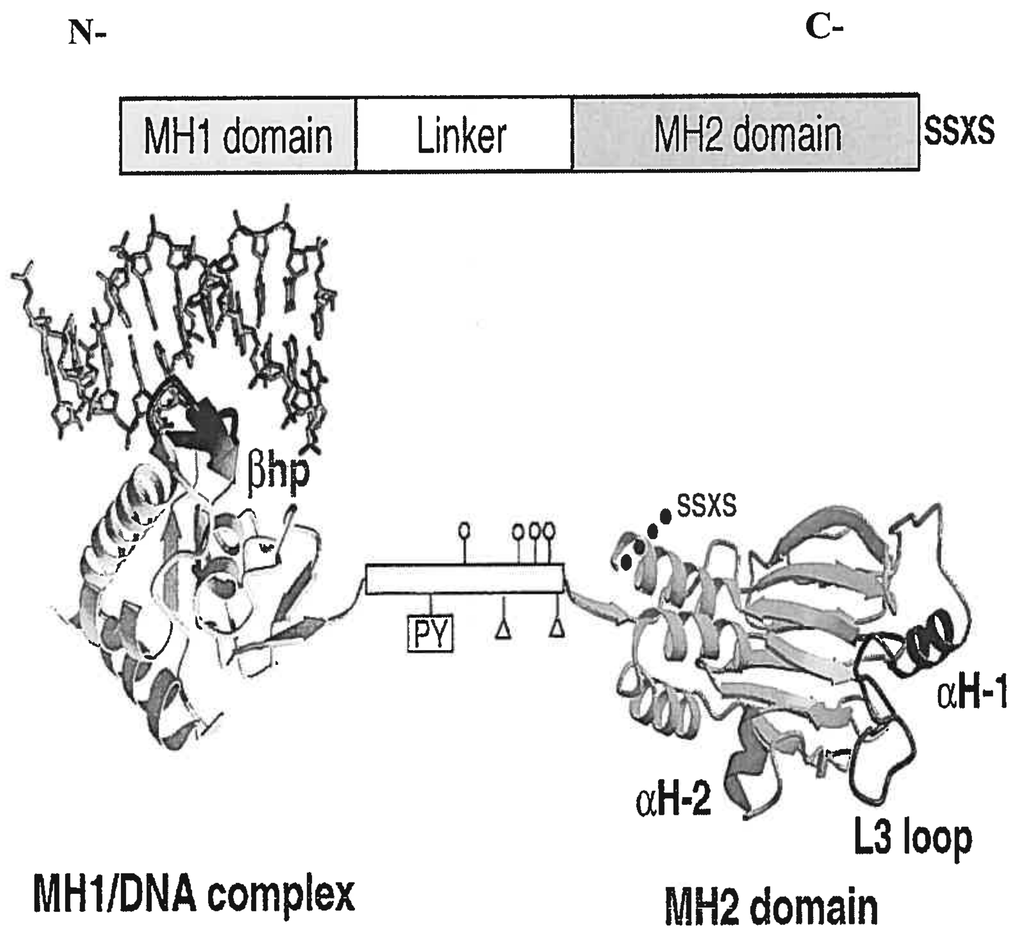


Figure 1.9 Crystallographic Representation of Smad3 MH1 Domain Bound to 5'-AGAC-3' DNA Sequence and Smad2 MH2 Domain (Massagué J. 2000)

receptor kinase (158). Activated R-Smads then dissociate from the receptor and assemble into complexes with Co-Smad4, subsequently translocating into the nucleus where they either activate or repress gene transcription in collaboration with specific DNA-binding and co-regulator proteins.

1.5.3.3 R-Smad/Co-Smad Complex Formation and Translocation into the Nucleus

Transcriptional activity of R-Smads requires the participation of Smad4 in the activated Smad nuclear complex, as demonstrated for Gal4-Smad1 and -Smad2 fusion proteins in Smad4-deficient cells (144). Structurally, Smad4 is very similar to R-Smads minus the C-terminal phosphorylation SSXS motif (158). Smad4 was identified as the tumor supressor product of the deleted in pancreatic carcinoma (DPC) locus 4 (58); (126), mutated in nearly half of pancreatic cancers (216). The requirement for Smad4 in Tgf-β signaling is suggested by its partnership with Tgfβ-, as well as Activin- and Bmp-activated R-Smads (126,276,277). Interactions between R- and Co-Smads are mediated by respective MH2 domains; Smad-mediated transcription is suppressed by Smad4 MH2 mutations disrupting Smad hetero-oligomerization (161). Smad4 is not required for nuclear translocation of the Smad complex, nor for the association of R-Smads with DNA-binding partners. Rather, Smad4 promotes binding to DNA and stability to the transcriptional Smad complex through its MH1domain, while its MH2 domain provides Smad4 with the capacity to act as a transcription co-activator (82). Smad transcriptional activity is abrogated in Smad4-defective cells (144). *In vivo*, interactions between Smad and general co-activators on promoters actually requires intact Smad4 activity, as is the case for efficient co-activation of Smad3 by CREB-binding protein (CBP) (68).

Tgfβ-mediated regulation of gene responses depends on the translocation of the activated Smad complex to the nucleus. One set of data suggests that this nuclear import

mechanism would rely on a basic nuclear localization sequence (NLS)-like motif located in the MH1 domain of R-Smads. Mutating this motif in Smad3 eliminated its Tgfβ-induced capacity for nuclear translocation, without interfering with its ligand-induced phosphorylation, DNA-binding activity or heteroligomeric association with Smad4 (208). Another mechanism which diverges from the classical NLS-directed nuclear translocation implicates a ligand-independent activity found in the MH2 domain of Smad2 in particular (262).

1.5.4 Smads as Transcription Factors

Smad proteins fulfill their role as direct mediators of Tgf-β gene responses through their sequence-specific DNA binding and transactivation or transrepression activities. Smad recruitment to DNA is essential for Smad control of transcription. The Smad MH2 domain, when fused to the heterologous GAL4 DNA-binding domain, was able to activate transcription of a reporter construct containing a GAL4-binding site (143). Smad proteins therefore possess an intrinsic transactivating activity that is present in their MH2 domain and that is independent of any other protein collaboration, but requires Smad binding to DNA.

1.5.4.1 Smad Binding to DNA

There are currently two prevailing views regarding the tethering of Smad proteins to target gene promoters. One view is that Smad proteins regulate transcriptional processes by directly binding to DNA. Both R-Smads and Co-Smads can bind DNA through a β-hairpin motif in their MH1 domain (Figure 1.9). The DNA-binding activity of the MH1 domain is under negative control by the MH2 domain in the absence of ligand, the latter mechanism contributing to Smad inactivation in the absence of ligand. Commonly accepted as a Smad-binding element (SBE) or Smad box is the 5'-AGAC-3' sequence

found in one or multiple copies in such TGF-β/Smad-responsive promoters as those of the *c-jun, collagenase I, Immunoglobulin-A (IgA) and Plasminogen activation inhibitor* (*PAI-1*) genes (55,90,192,275). Oligonucleotide selection experiments using recombinant Smad3 and Smad4 (273) had originally identified an 8bp 5'-GTCTAGAC-3' sequence as a putative SBE. Direct binding of the Smad3 MH1 domain to this sequence has been characterized through crystallographic analysis (223). Smad3 was shown to bind each half of the palindromic SBE through an 11-amino acid β-hairpin that established contacts with the 5'-AGAC-3' sequence in the major groove of the DNA. GC-rich sequences with a 5'-GCCGnCG-3' consensus motif as found in the *tinman* promoter (263) have been demonstrated to interact directly with *Drosophila* Smad1-related Mad. Smads have been shown to be able to activate Tgf-β inducible transcription from certain promoters in the absence of other transcription factors (275), but DNA-binding properties of Smads considered, it is very unlikely that Tgf-β responses can be mediated solely by SBEs. Indeed, *in vitro* Smad1, Smad3 and Smad4 can bind equally well to the SBE (223). That is not surprising considering that the sequences that make up the DNA binding β-hairpin of the three Smad proteins are the same. The β-hairpin loop of Smad2 on the other hand bears an extra 30 amino acids that interfere with its DNA binding activity (223). In addition to the apparent lack of DNA binding specificity, there is the question of low binding affinity of Smad proteins which has been estimated to be 2.6-4.9 X 10^{-7} M (223). Greater affinity is obtained by multiple copies of the SBE, which have been found in the PAI-1 promoter (52) for example.

Compelling evidence supports the view that Smad heterodimers associate to promoter sequences through protein:protein interactions with specific DNA-binding partners, allowing for greater affinity and specificity in Tgf-β responses. The most

common splice variant of Smad2 actually lacks DNA-binding activity because of an amino acid insert located next to the β-hairpin loop (223,265). Whether or not a particular Smad can bind to DNA, specificity in their recruitment to promoters would for the most part occur through interations with DNA-binding partners. Efficient activation of the *Xenopus Mix.2* promoter for example (31) requires that Smad binding sites lie in close proximity to binding sites for the winged helix Forkhead activin signal transducer (FAST)-1 transcription factor, also known as FoxH1.

1.5.4.2 Tgfβ-Responsive Genes

There are many examples of Smad cooperativity with DNA-binding partners in the regulation of Tgf-β/Activin signaling pathways. The first natural Smad transcriptional complex was described on the *Xenopus* homeobox *Mix.2* gene, an immediate-early activin-response gene (31). FAST-1 was identified as the Smad2/4 DNA-binding partner in the ternary complex bound to the Activin response element (ARE) of the activated *Mix.2* gene (31). Smad2-FAST specific interactions have been shown to be mediated by α-helix 2 (α-H2) of the Smad MH2 domain (32). The mouse homologue FAST-2 was soon after implicated in mouse *goosecoid* (*gsc*) gene regulation by Activin (125). FAST-2 transactivation of the *gsc* promoter requires Smad2 protein interactions and subsequent Smad4 recruitment into a ternary complex. Smad2 activation of the *gsc* promoter was shown to be antagonized by Smad3, which competes for DNA contacts with the Smad4 MH1 domain (125). On 12-O-tetradecanoyl-13-acetate (TPA)-responsive gene promoter elements (TREs), which were shown to mediate Tgf-β transcriptional responses on such genes as *PAI-1* and *clusterin* (101,107), Smad3 has the capacity to enhance the activity of c-Jun at AP-1 binding sites. Such protein associations to TPA-containing Tgfβ-responsive elements (TREs) depend on ligand- stimulated receptor activation, which correlates with

the Smad complex marching into the nucleus as a result of receptor activation/phosphorylation.

Although physical interactions between Smads and binding partners have been detected in almost all cases, and would be at the basis of target gene selectivity, they do not seem to be required to carry out some Tgf-β gene responses. Indeed, no interactions between the bHLH Transcription factor E3 (TFE3) and Smad3/4 proteins was shown (89) yet all three proteins interact with both *PAI-1* and *Smad7* promoter sequences through a TFE3 Ebox and a SBE found in proximity to each other.

Less is known of the mechanisms through which Tgf-β signaling pathways directly inhibit gene transcription. Tgf-β is a prominent inhibitor of skeletal muscle cell differentiation (159,188) that has recently been demonstrated to block the activity of MyoD in initiating the myogenic program (142). Downstream of Tgf-β, Smad3 was shown to repress the transcriptional activity of MyoD on Ebox motifs of the *muscle creatine kinase* (*MCK*) promoter. By physically interacting with MyoD, Smad3 interferes with the formation of MyoD/E protein dimers required for efficient activation of E-box regulatory elements such as those found in the *MCK* enhancer. Although Smad3 has also been implicated in the downregulation of androgen receptor- (84) and osteoblast transcription factor CBFA1- (4) activated transcription, respective mechanisms of transcriptional repression have yet to be elucidated.

1.5.4.3 Bmp-responsive genes

Transcriptional responses to Bmp signals are less understood due to the smaller number of Bmp target genes identified so far. Despite this, unique insights on Smad function have been provided by the study of molecular mechanisms underlying Bmp signaling pathways. Bmp/Smad1-mediated induction of *osteopontin* gene expression

occurs through Smad1 displacement of homeodomain-containing Hoxc-8 inhibitory activity (221). In this case, direct interactions between Smad1 and Hocx-8, which do not depend on Smad contacts with DNA, prevent Hoxc-8 repressor from binding to *osteopontin* promoter elements. Bmp-mediated transcriptional activation has moreover been shown to occur through cooperative mechanisms similar to those characterized in Tgfβ/Activin pathways, wherein DNA binding sites for both Smad and partner proteins contribute to the response. Bmp induction of *Xvent2* expression requires both SBE and (Ornithine decarboxylase antizyme) OAZ binding elements (83). Similar to the Smad2 partner FAST, efficient binding of OAZ to the Bmp response element (BRE) in the mammalian *Vent-2* promoter requires association with Smad1 and an intact SBE (83). The lack of a consensus Smad1/5/8 DNA binding site renders the study of Bmp gene responses at the molecular level all the more difficult. Smad1 has been demonstrated to act through a Mad-like GC-rich site to activate the *Smad6* gene (99,123) and through both Smad box (AGAC) and Mad consensus sites to induce *Id* expression (116). The Bmp consensus may perhaps be a reflection of the cofactor involved.

There are also examples of Smads cooperating with DNA-binding partners in the regulation of *Drosophila* Dpp-target genes. Dpp-dependent activation of *Tinman* expression depends on the cooperativity of the Medea (Smad4 homologue)/Mad complex with Tinman, which participates in its own autoregulation (263). For other genes, the Tgf-β pathway that mediates their transcription is not a direct one. Activation of *vestigial* (*vg*) for example requires prior transcriptional downregulation of *brinker* (*brk*), coding a homeodomain-containing repressor of Dpp target genes that acts by binding to Mad sites (114,157,211). Repression of *brk* is mediated by Mad and the zinc finger Mad cofactor

Schnurri (Shn). In the end, expression of *vg* is determined by competitive positive and negative Mad and Brk signals, respectively.

1.5.4.4 Co-Activators and Co-Repressors

R-Smad and Co-Smad recruitment of general co-activators, such as p300 and Creb-binding protein (CBP) (68,200) has been implicated in Smad-mediated transcriptional activation (68). Through their ability to position histone acetyl transferases (HATs) near nucleosomes and hence remodel chromatin, or to interact with components of the general transcriptional machinery, co-activators such as p300 and CBP increase transcription of their target genes (253). Melanocyte-specific gene (MSG1), an orphan transcriptional activator with strong transactivating activity but lacking DNA binding activity, associates with Smad4 and contributes to Smad-mediated transcription (161).

Cooperative effects between Smads and other transcription factors may occur at the level of co-activator recruitment. The cooperative signaling of Bmp-2 and the cytokine LIF in astrocyte formation is mediated by a complex formed of Smad1 and Stat3 proteins that do not interact directly, but are bridged by contacts with p300 (96). The vitamin D receptor (VDR) has been shown to physically interact with Smad3 in transcriptional assays and this interaction has been shown to involve steroid receptor co-activator (SCR)-1, another protein with associated HAT activity (268).

In some cases of genes repressed by Tgf-β, downstream Smad proteins serve to disrupt specific transcription factor associations with coactivators. Smad repression of the *c-myc* gene for example has been described to occur through a Transcription inhibitory element (TIE)/Elongation factor 2 (E2F) binding element (264), to which both Smad3 and E2F-4 bind. Tgf-β induction leads to the binding of Smad3, without disrupting E2F-4 DNA tethering, and interferes with the recruitment of co-activators by E2F-4.

The transcriptional shut down of other Tgf-β target genes, such as that of *osteocalcin* (21,182,196), *transin/stromelysin* (110) and *Cdc25A* (94), has been attributed to Smad recruitment of histone deacetylase (HDAC) activity that is associated with such co-repressors as c-ski (3,231,232), SnoN (228,232)and TG-interacting protein (TGIF) (258). Co-repressor associations with DNA typically result in the deacetylation of chromatin and the packing of nucleosome, hindering further transcriptional processes (47). SnoN and c-ski are two closely related members of the Ski family of nuclear proto-oncoproteins recently associated with the negative control of Tgfβ-/Activin-induced gene responses, although c-ski has also been shown to antagonize Bmp signaling (254). These two repressors are thought to maintain Tgf-β/Activin target genes in a repressed state in the absence of ligand and to participate in negative feedback control of Tgf-β signaling (228,232); in response to ligand activation, SnoN and c-ski would rapidly be degraded and would hence allow target gene expression to proceed. c-ski, in addition to its HDAC-associated activity, has been shown to repress transcription by competing with Smads for co-activators.

Homeodomain transcriptional repressors of the TGIF family have been demonstrated to negatively regulate transcriptional processes through their ability to directly bind DNA (Bertolino E 1995; Yang Y 2000), and recently through interactions with Smad2 as well as the pleiotropic Sin3 co-repressor complex to block Tgfβ-induced gene expression (258,259) (163). Smad transcriptional activity can also be blocked by binding of the zinc finger nuclear protein Ecotropic virus integration (Evi)-1 or adenoviral oncoprotein E region 1A (E1A), which interfere with Smad associations to co-activators (53,181). Competition between Smads and limitng amounts of co-activators and co-repressors in a cell may determine the outcome of specific genetic responses to Tgf-β.

1.5.5 Regulation of the TGF-β Signaling Pathway

Controlling the level of Tgf-β/Smad signaling in a cell is believed to determine the biological activity of the pathway. Regulators have been identified at several steps.

1.5.5.1 Ligand/Receptor Interactions

Positive regulators of the Tgf-β ligand subfamily, in particular, are betaglycan and endoglin accessory proteins. Initially detected through ligand cross-liking methods (30), betaglycan or endoglin have come to be known as the type III Tgf-β receptors. These membrane-anchored proteoglycans stabilize Tgf-β molecules in a conformation optimal for binding to the signaling receptors and hence facilitate ligand interactions with type II receptors. The extracellular component of type III receptors may also be released; in this case, the soluble form of the receptor may antagonize Tgf-β signaling by sequestering ligand (147). The outcome of type III receptor regulation of Tgf-β signaling appears to be a function of glycosaminoglycan modifications (66). The Tgf-β receptor activition process itself is maintained in check by immunophilin FKBP12 binding to the GS domain of type I receptors, which prevents the occurrence of ligand-independent receptor phosphorylation (160). Phosphorylation actually activates the type I receptor by switching its GS region from a FKBP12 inhibitor- to a Smad substrate-binding site (93). The pseudoreceptor BAMBI, which lacks an intracellular kinase domain, is another inhibitor of Tgfβ type I receptors that functions through the formation of inactive dimers with type I receptors (189).

Various families of diffusible Tgf-β ligand-binding proteins have been characterized in several species as negative regulators of respective signaling pathways. Through ligand sequestration methods, these extracellular antagonists are thought to function in vivo to further delineate the areas of Tgf-β activity in tissues. One such

negative regulator of Tgf-β activity is the N-terminal propeptide that is cleaved off of the prohormone, but remains non-covalently bound to the secreted C-terminal mature Tgf-β molecule. The N-terminal propeptide, known as Latency-associated protein (Lap), interferes with receptor recognition of the mature Tgf-β peptide (226). The physiological mechanism of latent Tgf-β activation remains undefined.

Follistatin on the other hand is a soluble secretory polypeptide that specifically blocks receptor recognition by Activin (51). Its contacts with Activin molecules are made through a cysteine-rich protein module that is found in many growth factor binding proteins. *In vivo* evidence supporting a key role for follistatin in the regulation of Activin-mediated developmental processes comes from null mice, which exhibit developmental defects similar to those of *Activin*-deficient mice (162).

Many proteins across several species have been characterized to form inhibitory complexes with ligands of the Bmp family. Chordin and Noggin, initially identified for their ability to counteract Bmp ventralizing activity in *Xenopus*, also exert their negative control over mammalian Bmps in such processes as neuralization, bone and tooth morphogenesis (74). Both specifically bind to Bmps, but not to Activin or Tgf-β. The *Drosophila* Short gastrulation (sog) is a homologue of Chordin and blocks Dpp signaling (13). The DAN family of Bmp antagonists has very recently been implicated in a variety of developmental processes governed by Bmp and Nodal factors, and includes: *Xenopus* Cerberus (Cer); chick Caronte; as well as mammalian DAN, Dante, Drm/Gremlin, Cer 1 and protein related to DAN and Cerberus (PRDC) (88,207,271).

There are also antagonists of the aforementioned antagonists shaping Tgf-β responses. Tolloid, Xolloid, Twisted gastrulation (Tsg) and orthologues identified in flies, amphibians and mammals are all metalloproteases that interact and cleave BMP antagonists

thereby releasing biologically active Dpp/Bmp (171). *In vivo*, these metalloproteases are thought to counteract the activity of Tgf-β antagonists and hence provide surrounding tissues with high Dpp/Bmp signaling.

1.5.5.2 Smad Signaling

1.5.5.2.1 R-Smad Availability

R-Smad availability to Tgf-β receptors is regulated by Smad anchor for receptor activation (SARA), which binds and escorts unphosphorylated R-Smads to the transmembrane receptor kinase (250). R-Smad activation appears to depend on its association with SARA since interfering with Smad2-SARA interactions inhibited Smad2 signaling (261). Moreover sequestering R-Smads in the cytoplasm prior to receptor activation is the microtubule network (56). Unphosphorylated Smad 2 associates with β-tubulin, a constituent of the microtubule network; activation of the signaling pathway results in Smad2 dissociation from β-tubulin. Microtubules are hence thought to impose a negative regulation on Tgf-β signaling in the absence of ligand stimulation. Once signaling is turned on, receptor access to microtubule-bound Smads would be facilitated by SARA.

1.5.5.2.2 Ubiquitin-Proteosome Ubiquitination

Actively controlling Smad turnover in Tgf-β cellular responses is the ubiquitin-proteasome pathway (260). Essentially, ubiquitin residues are first activated by an E1 ubiquitin-activating enzyme, then transferred to a ubiquitin-conjugating (E2) enzyme and attached to the substrate in the presence of an E3 ubiquitin ligase. The Smurf family of E3 ubiquitin ligases specifically regulates Smad activity (17,274,278). Smurfl preferentially binds and inactivates Bmp-regulated Smads; overexpressed in *Xenopus* embryos, Smurfl is capable of antagonizing Bmp signals involved in pattern formation (278). Smufr2 on the other hand antagonizes Smad-2 dependent signals, recognizing Smad2 over Smad3 (17).

Such interactions between Smurfs and Smads are ligand-induced, and would hence be implicated in a loop of negative feedback regulation. But then again, studies showing that the ubiquitin-proteasome system is implicated in the degradation of co-repressor SnoN as a result of Tgf-β signaling activation would argue that proteolysis allows signaling to begin (228,232). Phophorylated Smad2 and Smad3, once translocated into the nucleus, have been shown to induce proteolytic degradation of SnoN by collaborating with the anaphase-promoting complex (APC) (17). Another study suggests that a Smad2-Smurf2 ubiquitin ligase complex targets SnoN for degradation by the proteasome (17). In *Drosophila*, Smurfl/2 homologue Dsmurf has been identified as an important negative regulator of Dpp signaling *in vivo* (197).

1.5.5.2.3 Inhibitory Smads

Smad6 (95) and Smad7 (176) are Smad proteins that antagonize rather than transduce Tgf-β signals. Although structurally different from other members of the Smad family, inhibitory Smads are evolutionarily conserved; Smad6 and Smad7 were also identified in *Xenopus* (176) and Daughters against Dpp (Dad) in *Drosophila* (251). Smad6 specifically blocks Bmp signaling pathways by competing with Smad4 for binding to receptor-activated Smad1 (81). Smad7 on the other hand acts as a negative regulator of signals by Tgf-β, Activin as well as Bmp. By interacting with activated type I receptors, Smad7 interferes with the phosphorylation of R-Smads by a mechanism that implicates ubiquitin-proteasome degradation (65). That is, Smad7 promotes the association of ubiquitin ligases Smurfl and Smurf2 to Tgf-β type I receptors and thereby induces their turnover (65,106).

Transcription of Smad6 and Smad7 is induced by the lignads they inhibit (176,177)), suggesting that inhibitory Smads function in a feedback loop to control the

extent of the Tgf-β response. In developmental processes, signal regulation by inhibitory Smads may serve to attenuate tissue sensitivity to Tgf-β signals (177). Smad6 and Smad7 autoregulatory circuits appear to fine-tune Bmp-4 activities implicated in *Xenopus* ectodermal patterning (177). Seeing that inhibitory Smads have different specificities for Bmp and Tgf-β signaling pathways, the relative strength of such distinct signaling pathways could be detemined by Smad6 and Smad7 expression levels in a cell.

1.5.5.2.4 Crosstalk

In vivo, a cell is likely to be simultaneously exposed to multiple cytokines. It seems the case therefore that two or more signaling pathways could be activated in one same cell, either participating in the synergistic or antagonistic control of gene responses. Bmp and Activin factors of the Tgf-β superfamily have been shown to antagonize themselves in specifying the ventraldorsal polarity of mesoderm in *Xenopus*; Bmp/Smad1 signals ventralizing and Activin/Smad2 dorsalizing embryonic mesoderm (75). Inhibition of Bmp signaling in early embryos results in ectopic formation of dorsal mesoderm, suggesting that endogenous Bmp signals are able to antagonize Activin dorsalizing effects *in vivo* (75). It has been suggested that the two Smad–mediated signaling pathways might cross-interfere through competition for Smad4 (28). Both positive and negative MAPK regulation of Smad activity has been documented. Epidermal growth factor (Egf) activation of MAPK or Erk, mediated by the Ras pathway, would interfere with Smad signaling by phophorylating R-Smads in the linker region and in doing so inhibit Smad nuclear translocation (118,119). Inhibitory crosstalk between Egf and Tgf-β signaling pathways has moreover been demonstrated to occur at the level of TGIF Smad co-repressor activity. Indeed, Egf signaling via the Ras-pathway can cause the phosphorylation of TGIF, leading to its stabilization and favouring the formation of Smad-TGIF inhibitory

complexes (146). Oncogenic Ras has moreover been shown to negatively modulate Tgf-β signaling by increasing the turnover of tumor supressor Smad4 (212); whereas cells treated with interleukin-1 and interferon-γ become resistant to Tgf-β growth inhibition effects through the induction of Smad7 expression mediated by activated Nuclear factor kappa B (NF-Kb) and Jun-activated kinase (Jak)/Stat-1, respectively (16,252).

Different signaling pathways have also been shown to converge to enhance Smad-mediated gene responses. Functional and physical interactions between Smad3/4 and c-Jun/c-Fos suggest that Smad and MAPK/JNK signaling can converge at AP1-binding promoter sites (275). Bmp-2 and LIF act in synergy to promote astrocyte development through transcriptional complexes containing Smad1 and STAT3, bridged together by p300. Many other Smad modulatory signaling pathways, positive and negative, have been described (160) and would certainly account for the diversity in biological Tgf-β responses.

1.5.5.3 Aberrant Regulation of Tgf-β Signaling Leading to Cancer and Human Diseases

In the last years, the interest in studying the extent to which Tgf-β growth factors exercise control over growth and differentiation during normal development stems from the recognition that disregulation of these pathways can result in malignant transformation and human disorders. Tgf-β is well known for its inhibitory effects on cell proliferation, through the induction of G1 arrest or activation of cell death mechanisms for example (213); loss of such a signal hence thought to predispose or cause cancer. Mutations in genes encoding components of the Tgf-β signaling pathway have been detected in different forms of cancer (78,206). Tgf-β type II receptor mutations have been found in gastrointestinal cancer, as well as in colon or gastric tumors of individuals with hereditary non-polyposis colon cancer (HNPCC) (149). Smad2 and Smad4 have been shown to be

inactivated in a significant portion of pancreatic and colon cancers (257). Elevated levels of Tgf-β negative regulators can result in oncogenesis owing to their ability to render Tgfβ-responsive cells resistant to Tfg-β signals. Indeed, abnormally high expression of Ski and SnoN has been detected in human tumors (69,184). Developmental processes are moreover profoundly affected by physiologically abnormal levels of Smad signaling in a cell. Individuals carrying a single mutant *TGIF* allele have been shown to suffer from holoprosencephaly, the most common structural defect of the developing forebrain in humans (76). In these patients, Nodal signaling pathways implicated in neural axis formation are believed to be affected. Perturbations of the Tgf-β superfamily may also lead to bone and vascular diseases; mutations in the human *BMPR-II* gene have been shown to be responsible for the pathogenesis of primary pulmonary hypertension (PPH), while mutations in endoglin and Alk-1 have been associated with the pathogenesis of hereditary hemorrhagic telangiectasia (HHT) type I and type II, respectively (165).

It is through the identification of mutations within genes encoding components of the Tgf-β signaling pathway, and the study of genetic defects that are brought about by such mutations that one may fully comprehend the many parameters of normal Tgfβ-mediated cell growth and development. In the mean time, one would hope to understand the molecular basis of such genetic responses in more accessible but still relevant systems such as cell culture to ultimately be able to correctly process the available genetic information.

CHAPTER2- PROJECT DESCRIPTION

Conflicting results in the litterature initially led me to question the particular role that Bmp signals, expressed in the developing pituitary during the period of cellular differentiation, play in the determination of the POMC-expressing corticotroph cell lineage. As an initial step towards this question, my approach consisted in studying the regulation of POMC expression by Bmp signaling pathways in the AtT-20 corticotroph cell line. An action of Bmp signaling on POMC transcription would constitute a strong argument in favor of a role of this pathway in cell differentiation since expression of the POMC gene constitutes the "raison d'être" of corticotrophs. The finding that Bmp signals repress POMC expression and POMC promoter activity is interesting in itself because no other mammalian target gene has been reported to be negatively regulated by Bmps. Hence, I proceeded to investigate the molecular basis of this Bmp negative gene regulation. The role of Bmp-specific Alk receptors, Alk-3 and Alk-6, as well as the role of the Smad1 signal transducer in POMC regulation was investigated. Specificity of Bmp action was moreover an important issue in understanding the mechanism of Smad action on the POMC promoter. With the understanding that specificity in Smad transcriptional responses relies on the ability of Smads to bind DNA at consensus sites and moreover make contacts with distinct DNA-binding partners, I defined Smad interactions with POMC transcription factors as well as with POMC promoter sequences. Crucial to this course of study were previous findings that activation of POMC transcription requires the combinatorial activity of different trans-acting transcription factors, including NeuroD1, Pitx, and Tpit.

CHAPTER3- ARTICLE:

"Bmp (Smad)-Mediated Repression of POMC Transcription by Interference with Pitx/Tpit Activity"

NUDI Maria, Jean-François Ouimette and Jacques Drouin

3.1 Summary

The mature anterior pituitary gland harbours six distinct hormone-producing cell types. The emergence of these different cell populations early on in development is thought to depend on specific sets of transcription factors, the activity of which would be coordinated by growth factors expressed within and around the developing pituitary. Previous studies do not agree on the role Bone morphogenic proteins (Bmps) play in the differentiation of corticotroph cells, which are characterized by the expression of proopiomelanocortin (POMC) prohormone and adrenocorticotropin hormone (ACTH). We show that activation of Bmp signaling pathways in AtT-20 corticotroph cells, whether it be through recombinant (r)Bmp-4 treatments and/or overexpression of constitutively activated Alk-3/-6 receptors or Smad1/4 intracellular mediators, downregulates *POMC* expression and promoter activity. Overexpression of Smad6 or Smad7 inhibitors counteracts these negative Bmp effects. *POMC* promoter activity relies on the corticotroph-specific functions of Pitx, Tpit and NeuroD1 transcription factors. We find that intact Pitx and Tpit regulatory promoter elements are required for Bmp-mediated repression of *POMC* activity. In showing that Bmp signaling can directly interfere with synergistic interactions between Pitx1 and Tpit, and moreover that Smad1 binds Pitx1 and Tpit *in vitro*, we propose a Smad-mediated mechanism of *POMC* promoter regulation by Bmp that directly targets Pitx and/or Tpit.

3.2 Introduction

Bone morphogenic protein (Bmp) factors belong to the Transforming growth factor-β (Tgf-β) superfamily of multifunctional secretory peptides that regulate such diverse cellular responses as cell migration, adhesion, proliferation, differentiation and death. Recently, transgenic and gene-deleted mice experiments have implicated Bmp-4 in the initial inductive phase of pituitary morphogenesis. The pituitary develops out of a layer of competent oral ectoderm, which upon contact with inducing neuroectoderm or ventral diencephalon folds into a structure known as Rathke's pouch (RP). Bmp-4 is detected in the ventral diencephalon directly overlying the pituitary primordium; abrogation of this activity in *Bmp-4* null (1) or *Pitx1*-Noggin transgenic mice (2) compromised initiation of RP formation. Neuroectodermal Bmp-4 signals are gradually lost as cellular proliferation and differentiation events are initiated in the glandular pituitary structure derived from RP. In parallel, Bmp-2 signals appear ventrally in the oral ectoderm as well as mesenchyme, and together with dorsal fibroblast growth factor (Fgf)-8 signals, they have been proposed to establish pituitary cell fate-defining patterns of gene expression (2;3).

Six distinct hormone-producing cell types arise in the developing pituitary: corticotrophs, gonadotrophs, lactotrophs, somatotrophs and thyrotrophs within the anterior lobe, and melanotrophs within the intermediate lobe (4). The emergence of these distinct cell fates from a common primordium is coordinated by specific sets of transcription factors expressed in a precise spatiotemporal manner during pituitary organogenesis. The expression and activities of these transcription factors are thought to be specified early on by extracellular signaling molecules such as Bmp factors. In the case of gonadotroph differentiation, Bmp-2 has been shown to directly induce the expression of the GATA-2 zinc finger transcriptional activator of gonadotroph-specific *luteinizing hormone* (*LH*) and *follicle-stimulating hormone* (*FSH*) gene expression (5). The role that Bmp signaling might

be playing in the establishement of corticotroph cell identity is presently unclear. Corticotroph cells distinguish themselves from other anterior pituitary cells through their expression of proopiomelanocortin (POMC) and adrenocorticotrophin hormone (ACTH) by e12.5, ACTH arising from the proteolytic maturation of POMC (6). ACTH expression in RP explants cultured in the presence of Bmp-2 expressing COS cells was significantly downregulated (3); however, unaffected ACTH protein levels in the pituitaries of *αGSU-*Bmp4 transgenic mice (2) have argued against a negative role for Bmp signals in the differentiation of corticotrophs.

As an initial approach towards elucidation of the role of Bmp signaling in corticotroph differentiation, we studied regulation of proopiomelanocortin (POMC) gene expression by Bmp signaling pathways in the corticotroph AtT-20 cell line model. Previous analyses of the POMC (-480/+63bp) gene promoter known to confer corticotroph-specific activity (7;8) had implicated distal and central regulatory elements in the maintenance of promoter activity (9). In particular, cell-specificity of POMC promoter activity has been attributed to an interaction between bHLH transcription factors bound to the distal domain, and Pitx homeodomain and Tpit T-box transcription factors bound to the central domain (10-12). Tethered to the distal Ebox are bHLH heterodimers containing NeuroD1/BETA2 factors that have $Ebox_{neuro}$-specific binding activity. NeuroD1 is expressed in the developing pituitary exclusively in corticotrophs at a time (e12-e15) when ACTH begins to be expressed. Its role in the onset of corticotroph differentiation has been recognized in *NeuroD1* null mice wherein the emergence of $ACTH^{+}$ cells is delayed (Lamolet B, unpublished). The dimerization partner of NeuroD1, either the Pan1 (E12) or the Pan2 (E47) ubiquitous bHLH factor, has been shown to participate in synergistic protein:protein interactions with Pitx factors (11). The pan-pituitary Pitx factors have indeed been assigned a central role in the combinatorial program that coordinates

corticotrophic POMC expression, collaborating not only with distal bHLH factors but also with the newly identified Tpit (11) (12). Obligate partners of one another, both Tpit and Pitx factors are required for significant transcriptional activation of POMC promoter activity in AtT-20 cells. Unlike Pitx factors, Tpit stands out in its contribution to corticotroph cell fate decisions because it is expressed solely in the pituitary POMC-lineage from the time corticotrophs start to differentiate (12).

POMC expression in terminally differentiated corticotrophs is regulated positively by hypothalamic Corticotropin releasing hormone (CRH) and negatively by glucocorticoids (Gc) (13-15). Signaling events that would coordinate the activities of POMC promoter regulators, such as Pitx, Tpit and NeuroD1 implicated in early pituitary developmental decisions have not yet been identified.

Tgf-β cellular responses occur through changes in gene expression mediated for the most part by the Smad family of transcription factors. Specific ligand-induced Tgf-β serine/threonine receptor complexes recruit and phosphorylate receptor-regulated Smads (R-Smads): Smad-1/5/8 in response to Bmp and Smad2/3 in response to Tgf-β/Activin stimulation (13-15). Activated R-Smads subsequently associate with the common-mediator Smad4 (Co-Smad4) and translocate into the nucleus where they enable target gene selection and either positive or negative gene regulation through tight collaborations with cell-type specific transcriptional partners. The list of Smad DNA-binding partners lengthens with every new target gene characterized; some of the first characterized include the Xenopus FAST-1 protein in the activation of Nodal-responsive *Xenopus Mix.2* (16), the mouse FAST-2 protein mediating activin-induced activation of the *goosecoid* gene (17) and the OAZ protein in Bmp-mediated positive control of *Xenopus Xvent-2* activity (18). Tgf-β induction of transcription has been reported to occur either through the recruitment by

Smads and/or associated proteins of co-activators like p300, or through a derepression mechanism that involves Smad-directed removal of negative regulators from their binding sites (19). Tgfβ-induced repression of transcription is less understood. Recently, Smad3 was shown to inhibit myogenic processes by directly interfering with the transcriptional activity of MyoD (20). HDAC-recruiting repressors such as Tumor growth inhibitory factor (TGIF) (21), c-ski (22-24) and SnoN (25;26) have been implicated in the attenuation of Smad activity. Other negative regulators of Smad signaling are Smad6 and Smad7 inhibitors that interfere with phosphorylation and/or nuclear translocation of R-Smads (27).

An understanding of the molecular events underlying Bmp-specific Smad gene responses is limited to the few natural Bmp target genes identified so far (28). In *Drosophila*, the Smad1/5/8 homologue Mothers against decapentaplegic (Mad) mediates the induction of *spalt*, *optomotor blind*, *vestigial*, and *Dad* genes in collaboration with the zinc finger transcription factor Schnurri (Shn) (29), and synergizes with Tinman to induce the *Tinman* promoter (30). In mammals, Smad1 and Smad4 together are able to confer Bmp2-responsiveness to the human *Id* gene promoter independently of other transcription factors (31), while OAZ is required to direct the same Bmp-activated Smad complex to the Xenopus *Vent-2* promoter (18). Of the Bmp responsive genes identified to date in mammals, all are positively regulated by Smad factors.

We show for the first time that Bmp-4 signaling negatively regulates endogenous as well as luciferase (luc)-reporter POMC expression in AtT-20 as well as in P19 cells. The negative regulation of *POMC* promoter activity by Bmp-4 is mediated by the classical Smad signaling pathway since Bmp effects are mimicked and/or increased by the overexpression of specific Activin-like kinase (Alk)-3/6 receptors and Smad1 mediator signaling components (32) and counteracted by the overexpression of the specific Bmp-

inhibitor Smad6, and the general Tgf-β inhibitor Smad7. Our studies moreover identify Pitx homeobox and Tpit T-box transcription factors as targets of negative Bmp/Smad activity. Direct *in vitro* binding of Smad1 with Tpit and/or Pitx1 suggest that protein interactions would be the basis of Smad-directed interferences with Pitx- and Tpit- induced transcription of POMC.

3.3 Experimental Procedures

Material

Recombinant (r) human Tgf-β_1, Activin-A, and Bmp-4 were purchased from R&D Systems. Anti-Smad1/5/8 (N-18) and anti-phopho-Smad1 (Ser 463 and 465) antibodies were purchased from Santa Cruz Biotechnology and Upstate Biotechnology, respectively. Anti-Pitx1 and anti-Tpit antibodies were prepared in rabbits as described in (33) and (12), respectively.

Plasmids and Oligonucleotides

The different POMC reporter plasmids were constructed in the vector pXP1-luciferase (luc) as described previously (34). Deleted versions of the –480/+63bp POMC promoter construct were generated as described previously (9). Ponctual mutations and replacement of NurRE, $Ebox_{neuro}$, Pitx, Tpit and $Ebox_{ubi}$ POMC regulatory elements were described in the work of (9). The simplified luciferase reporter plasmid with three copies of the 40-bp *POMC* fragment oligo containing Pitx and Tpit regulatory elements was constructed as described in (35). For Pitx1 and Tpit expression vectors, Pitx1 and Tpit coding sequences were inserted in a RSV-driven vector as described in (12;36), and further modified with the HindIII/KpnI insertion of a double stranded oligonucleotide corresponding to the T3 promoter to allow the *in vitro* synthesis of Pitx1 and Tpit. Expression vectors for *3TP*-lux Tgf-β and *Tlx2*-lux Bmp-responsive target gene reporters, constitutively active Bmp type I

receptors and Smad mediators were a gift from Wrana J and Attisano L (Hospital for Sick Children, Toronto, Canada), and have been described before: *Tlx2*-lux (37); *3TP*-lux (38); pCMV5B/Alk3-HA (Q233D) and Alk6-HA (Q203D) (37;39); pCMV5B/Flag-Smad1, Flag-Smad2, Flag-Smad3, Smad4-HA and $pGEX_1$4T-1/Smad1 (37;40-42).

Cell Culture and Transfection Assays

AtT-20 (9) and CV-1 (43) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin/streptavidin antibiotics, and maintained at 37°C and 5% CO_2. CV-1 cells were transfected by the calcium phosphate coprecipitation method. 40,000 cells were planted in 12-well-plates. A total of 6 ug of total DNA (3 ug of reporter plasmid, 0-1.5 ug of effector plasmid, 50 ng of CMV-β-galactosidase as internal control), was used for each transfection, performed in duplicate. Control experiments contained equivalent amounts of empty expression vector and carrier DNA psp64. 16 hrs after transfection, medium was changed, and cells were harvested 24 hrs later. AtT-20 cells were transfected using Lipofectamine (Pharmacia) as described previously (11). Briefly, 250,000 cells/well were plated into 12-well plates; 1.5 ug total DNA was used for each transfection, performed in duplicate (0.5 ug reporter, 0-1 ug effector plasmid, 20 ng of CMV-β-galactosidase as internal control and psp64) together with 5 uL of Lipofectamine in a final volume of 400 uL without serum. After a 20 minute incubation at room temperature (RT), the volume was made up to 1mL with DMEM without serum and left for 4 hrs on the cells previously rinsed with DMEM without serum. 500 uL of DMEM with 20% FBS was then added to each well, and the cells were recovered 20 hrs later, using transfection lysis buffer (0.1M Tris pH 8.0, 0.5 % NP-40, 1 mM DTT). Luciferase activity was assayed as described previously. β-gal activity was determined using the β-gal reporter gene Galacto-Star™ (TROPIX) assay system.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated by the guanidium Thiocyanate-Phenol-Chloroform extraction method (44). 10 ug RNA was analyzed by electrophoresis on a 1.2 % agarose gel by the RNA-glyoxal method (45). Transfer was performed on a Hybond-N (Amersham) membrane. The RNA was crosslinked on the membrane, which was incubated overnight in a pre-hybridization solution (4 X SET, 0.1 % Na-pyrophosphate, 0.2 % SDS and 100 ug/mL of Heparin) at 65 °C. A 923-bp mouse (m)POMC cDNA fragment was ^{32}P-labelled as described in (43) and used to reveal endogenous mPOMC mRNA in AtT-20 cells. Hybridization and washes were performed as described in (43). β-Actin mRNA was revealed on the same Northern Blot using a ^{32}P-labelled β-actin cDNA fragment that is described in (46).

RT-PCR

AtT-20 cell RNA (2 ug) was used for cDNA synthesis with AMV reverse transcriptase (Promega) according to manufacture's intructions. RNA extracted from e13.5 embryo forelimbs was similarly processed to obtain cDNA that was used as a positive control for Alk receptor and Bmp ligand expression. Each PCR reaction was performed for the detection of Bmp-2, Bmp-4, Bmp-7 or Alk-2 transcripts as described in (12), whereas an annealing temperature of 47 °C was used for Alk-6. The primers used are the following: Bmp-2 sens: AGACGTCCTCAGCGAATTTG Bmp-2 antisens: GTTTGTGTTTGGCTTGACGC Bmp-4 sens: CGCCGTCATTCCGGATTACAT Bmp-4 antisens: GGCCCAATCTCCACTCCCTT Bmp-7 sens: GACATGGTCATGAGCTTCGT Bmp-7 antisens: GTCGAAGTAGAGGACAGAGA Alk-2 sens: GAGTGATGATTCTTCCTGTGC Alk-2 antisens: TTGGTGGTGATGAGCCCTTCG

Alk-6 sens: TGGAGCAGTGATGAGTGTCT Alk-6 antisens: TCTGGGTTCCTCTGTGTCTG.

Nuclear Extracts and Western Blot Analysis.

AtT-20 nuclear extracts were prepared by resuspending the cellular pellet in 400ul cold buffer A (10 mM KCl, 10 mM Tris pH7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM PMSF, 1 mM DTT and protease inhibitors) and the suspension of cells left to swell on ice for 15 min. NP-40 (0.01 %) was added and the suspension was vortexed vigourously for 30 seconds. The suspension was gently spun down, the supernatant discarded, and the nuclear pellet resuspended in 50 uL of cold Buffer C also containing protease inhibitors (20 mM Tris pH7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM PMSF, 1 mM DTT). The nuclear suspension was shaken vigourously at 4 °C for 1 hr, then spun and the supernatant assayed for protein content using the Bradford assay.

Western Blot analysis was performed as follows: 25 ug of AtT-20 nuclear extracts/sample was resolved on 10% SDS-polyacrylamide gel, transferred to PVDF membrane and immunoblotted with either 1:2000 dilution of anti-Pitx1 antibody, 1:1000 dilution of anti-Tpit, or 1:1000 dilution of anti-phopho-Smad1 antibody. Immunodetection was possible with the use of horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:20000), followed by incubation with ECL Plus detection reagents (Amersham Pharmacia biotech).

GST protein purification and Pull-Down Assay

GST and GST-Smad1 proteins were purified from BL21 bacterial cell cultures following GST Gene Fusion System (Pharmacia Biotech) instructions. The yield of GST proteins was assayed by Bradford and SDS-PAGE analysis. ^{35}S Methionine-labelled Pitx1 and Tpit proteins were synthesized using the TNT-coupled transcription and translation system (Promega) to manufacturer's instructions, and assayed by SDS-polyacrylamide gel. 500 ng

of purified GST and GST-Smad1 fusion protein coupled to Glutathione Sepharose 4B beads was incubated with 5 ul of radiolabelled Pitx1 and/or Tpit proteins in 500 uL final volume of a buffer solution made up of 50mM NaCl, 50mM Tris pH 7.9, 1mM EDTA and 0.1mM of NP-40, at 4° for 2 hours. The sepharose beads were then washed twice in 125nM NaCl, 50mM Tris pH 7.9, 1mM EDTA and 0.1mM of NP-40 buffer; and twice in 200mM NaCl 50mM Tris pH 7.9, 1mM EDTA and 0.1mM of NP-40 buffer. Bound protein complexes were eluted before being loaded on a 10% SDS-polyacrylamide gel.

3.4 Results

Bmp-4 Downregulates POMC Expression in AtT-20 Cells – AtT-20 cells are a corticotroph model cell line that endogenously expresses the *POMC* gene. To investigate the role of Bmp signaling in regulation of *POMC* expression, we first determined whether POMC mRNA levels are affected by Bmp signals in AtT-20 cells. AtT-20 cells were incubated in presence of recombinant (r)Bmp-4 for 24 and 48 hours and *POMC* expression was assessed by Northen blot hybridization. As shown in Figure 3.1A, rBmp-4 reduced POMC mRNA levels reaching near 60% repression within a 48hr period and without affecting actin mRNA. These results are consistent with previous observations that described downregulation of ACTH expression in e9.5 RP explants cultured in the presence of Bmp2-coated beads (3).

To determine whether Bmp signaling negatively regulates *POMC* expression at the transcriptional level, a *POMC* (-480/+63) promoter-luciferase (luc) reporter construct was transiently transfected into AtT-20 cells and the response to rBmp-4 was assayed. rBmp-4 treatments downregulated *POMC* promoter activity in AtT-20 cells in a time-dependent (Fig. 3.1B) and in a concentration-dependent manner (Fig. 3.1C). We observed a twofold repression of *POMC*-luc basal activity in AtT-20 cells following 8hrs of 1nM rBmp-4 stimulation, and an almost maximal 80% loss of activity after a 24hr treatment. When tested for 24hrs, repression was nearly maximal (80%) with 1nM Bmp-4.

Bmp-4 repression is mediated through Bmp-specific receptors and Smad1/4 transcription factors – Bmp signals from the membrane to the nucleus are mediated through Bmp-specific receptors that activate R-Smads (Smad1,5,8). Bmp receptor type-I ligand activation can be mimicked by mutations within the GS domain of Activin-like kinase receptors (Alk)-3 and Alk-6 (39), so that Alk-3(Q223D) and Alk-6(Q203D) constitutively activated mutants can signal in the absence of ligand. To assay whether Bmp

repression of *POMC* activity in AtT-20 cells is mediated by the activation of Bmp-specific receptors, expression vectors encoding Alk-3(Q223D) and Alk-6(Q203D) were used in transfection assays. Overexpressed Alk-3 and Alk-6 decreased *POMC*-luc activity to levels similar to those generated through rBmp-4 stimulation (Fig. 3.2A). In subsequent assays, 250 ng of Alk-3(Q223D) was used as an alternative for 1nM rBmp-4 treatments.

If Smad1 participates in Bmp-mediated repression of POMC in AtT-20 cells, then its overexpression would be expected to increase the sensitivity of AtT-20 cells to exogenous Bmp signals and hence enhance Bmp-4 inhibitory effects on POMC. When Flag-Smad1 and/or Smad4-HA were transiently overexpressed in AtT-20 cells, Bmp4-mediated repression of *POMC*-luc activity was significantly enhanced (Fig. 3.2B). The fourfold inhibition of *POMC* promoter activity encountered upon the overexpression of Smad1/4 in the absence of Bmp treatment is an indication that endogenous Bmp signals might already be present in cultured AtT-20 cells to activate Smad proteins. It moreover seems that a limiting amount of free Smad1 and Smad4 are available in AtT-20 cells to participate in Bmp signaling since the addition of Smads in these cells significantly enhances Bmp reponses (Fig. 3.2B). In unstimulated P19 cells, overexpression of Smad1 and Smad4 only slightly repressed *POMC* promoter activity, and this effect was not observed in Bmp stimulated conditions. Saturating levels of Smad1 and Smad4 may be present in P19 cells or alternatively, the cellular context of AtT-20 cells is particularly suited for Bmp repressor effects on *POMC* transcription.

Smad1 is the best-characterized intracellular transducer of Bmp signals. Activation of cytoplasmic Smad1 by Bmp receptors is characterized by the phosphorylation of carboxy-terminal serines 462, 463 and 465, and subsequently translocation into the nucleus. To determine whether endogenous Smad1 is indeed activated in AtT-20 cells following rBmp-4 treatments, nuclear protein extracts of rBmp4-stimulated and non-stimulated cells

were immunoblotted for the presence of phosphorylated Smad1 using an Upstate Biotechnology antibody specific for serines 463/465 phosphorylated Smad1. As shown in Fig. 3.2C, small amounts of phosphorylated Smad1 are present in unstimulated AtT-20 cells, constitent with constitutive Bmp signaling in these cells. Upon rBmp-4 stimulation, nuclear phosphorylated Smad1 was increased, peaking 24 hrs after rBmp-4 addition. When cultured AtT-20 cells were assayed for Bmp ligand and/or receptor expression using RT-PCR techniques, they were found to have transcripts for Bmp-7, as well as for Alk-2 and Alk-6 Bmp-specific type-I receptors (data not shown). Alk-2 and Alk-6 type I receptors have been reported to function in the activation of Bmp-specific R-Smads following ligand stimulation (47), thus we have shown that AtT-20 cells are Bmp-responsive and that they possess the appropriate machinery to convey Bmp signals to the nucleus through Smad1 activation. Bmp-7 appears to be the active endogenous ligand in AtT-20 cells.

BMP/Smad1 signaling specifically represses POMC promoter activity – Bmp/Smad1 signaling is known to activate transcription from the mouse *Tlx-2* promoter in P19 cells (37). We first asked whether the same Bmp- signaling pathway may repress *POMC* and activate *Tlx2* promoter activity. In AtT-20 cells, we show that whereas Alk-3 and Smad1/4 overexpression represses *POMC*-luc activity by at least twofold, it does not repress *Tlx2*-lux activity but induces it weakly (Fig. 3.3A). *POMC*-luc and *Tlx2*-lux promoter activities responded in a similar fashion to Bmp signaling in P19 cells, but with a noticeable difference in response sensitivity of *Tlx2*-lux (Fig. 3.3B). The relatively weak induction of *Tlx2* in AtT-20 cells may be due to the lack of a Smad1 partner in these cells compared to P19 cells. Clearly however, the inhibitory effects of the Bmp pathway on the POMC promoter are promoter-specific and do not reflect a general cellular response.

Different members of the Tgf-β superfamily of growth factors were tested for the specificity of their effects on *POMC* promoter activity in both AtT-20 and P19 cells. The

Tlx2-lux and *3TP*-lux reporters were used, respectively, as controls of Bmp- and Tgfβ-dependent signals (37;48). By showing that *3TP*-lux activity is induced in AtT-20 cells upon treatment with rActivin or rTgf-β, we verified that AtT-20 cells are responsive to different members of the Tgf-β family of growth factors (Fig 3.3C-D). *POMC* promoter activity was repressed by 75% in AtT-20 cells and by 50% in P19 cells treated with rBmp-4 (1nM) or rActivin-A (500pM), but was not affected in either cell type challenged with Tgf-β (100pM) (Fig 3.3C-D). The Activin-mediated repression of *POMC* promoter activity observed in AtT-20 cells is in support of previous work showing reduced accumulation of secreted ACTH upon Activin-A treatment (49). Also highlighting their difference in signaling, rActivin, but not rBmp-4 treatment, repressed *Tlx2* promoter activity in AtT-20 cells (Fig. 3.3C).

While the intracellular activity of Smad1 is specific to Bmp responses, Smad2 and Smad3 activities have been assigned to Activin and Tgf-β signaling pathways (50). To determine whether Activin inhibitory effects on POMC expression may be mediated by Smad2 and/or Smad3 in AtT-20 cells, increasing concentrations of Flag-Smad1, Flag-Smad2 and Flag-Smad3 were overexpressed in these cells and the activity of *POMC*-luc assayed. Only overexpressed Smad1 was efficient in blocking transcription from the *POMC* promoter (Fig. 3.3E). The inability of Smad2 or Smad3 overexpression alone at mimicking inhibitory Activin effects may suggest that Smad-independent pathways are acting downstream of Activin to repress *POMC* in AtT-20 cells. Ultimately, the role Smad2 and/or Smad3 play in activin-induced repression of POMC activity will be addressed through the use of Smad dominant negative forms. Notably, *POMC*-luc activity was slightly decreased with higher concentrations of Flag-Smad3 (0.5-1ug), however the

loss of *POMC* activity was accompanied by a similar reduction in the activity of *CMV*-βgal used as internal control (data not shown).

Smad6 and Smad7 counteract Bmp-mediated repression of POMC – Smad6 and Smad7 are known inhibitors of Tgfβ-induced cellular responses. The downregulation of POMC promoter activity following Bmp-4 treatment or Smad1/4 overexpression was completely blocked by overexpression of the Bmp-specific inhibitor Smad6 and the general Tgf-β inhibitor Smad7 (Fig. 3.4). Notably, basal levels of POMC expression in AtT-20 cells were augmented by the overexpression of Smad6 and Smad7, indicating once more that endogenous Bmp signaling pathways negatively control POMC promoter activity in these cells.

Negative Bmp/Smad signals target Pitx/Tpit activities on the POMC promoter – The *POMC* promoter has been divived into three regions; namely, distal (-480/-324bp), central (-323/-166bp) and proximal (-165/-34bp) regions (9). In an attempt to identify *POMC* promoter sequences that are responsive to Bmp/Smad signaling, we tested the Bmp-4 response of constructs containing these promoter regions, alone or in combination. Previous studies in our laboratory had shown that central and distal domains act in synergy and that this synergism is cell-specific (9;51). Only the reporter construct containing both distal and central *POMC* promoter regions responded to rBmp-4 stimulations to the same extent as did the full-length promoter (Fig 3.5A). These results suggest that Bmp signaling negatively targets synergistic activities acting on the distal and central domains of the POMC promoter.

The distal and central domains of the *POMC* promoter contain most of the critical regulatory elements for cell-specific recognition, for synergism between the two domains, as well as for hormone response (10;12;52). We used element-specific mutants to

determine which is/are required for Bmp inhibitory signaling. Mutagenesis of either NurRE element that confers hormone responsiveness or of the $Ebox_{neuro}$ that confers cell-specific recognition by neurogenic bHLH factors and synergism with the central promoter domain did not affect Bmp4 responsiveness (Fig. 3.5B). However, Bmp-4 sensitivity was lost upon mutagenesis of either Pitx1 or Tpit binding sites.

Tpit, a newly identified member of the T-box transcription factor family (12), synergizes with Pitx on respective binding sites which are only 5 bp away from each other. Together, Pix and Tpit binding sites comprise the central regulatory element that participates in synergistic interactions with NeuroD1-containing bHLH heterodimers acting on the distal promoter E box_{neuro} (10;11). The restricted action of Bmp-4 on Tpit/Pitx1 indicates that it is their activity itself rather than their synergism with NeuroD1 dimers that is targeted by Smad action. In order to verify this, a reporter construct driven by oligotrimers containing Pitx/Tpit response elements (12) was transfected in CV-1 cells and found to be repressed by Alk-3 (Q223D) (Fig. 3.5C). Their repression was most evident in the presence of both Pitx1 and Tpit, but a similar tendency was also observed on Tpit-dependent activity. Since Bmp4-expressing epithelia have been documented to repress Pitx1 expression in mandibular mesenchyme (53), we analyzed whether activation of the Bmp-4 signaling pathway in AtT-20 cells affected the expression of Pitx1 and/or Tpit. Nuclear protein extracts from AtT-20 cells treated or not with rBmp-4 were assayed by Western Blot for Pitx1 and Tpit protein levels. No change in Pitx1 or Tpit protein expression was detected (Fig 3.5D), in agreement with data from Figure 3.5C.

Smad1, Pitx1 and Tpit proteins interact in vitro – One manner in which Bmp-activated Smad1 could interfere with the transcriptional activities of Pitx and Tpit in AtT-20 cells would be through protein:protein interactions. With this in mind, purified GST and GST-Smad1 fusion proteins were assayed for binding to ^{35}S-labelled, individually- or co-

translated Pitx1 and Tpit proteins. GST-Smad1 bound to resin was able to pull-down Pitx1, Tpit, or both protein, whereas the GST moiety alone could not (Fig. 3.6). In vitro translated luciferase did not interact with either GST or GST-Smad1. Bmp signaling might hence interfere with transcriptional activation of the POMC gene through the recruitment of Smad1 by Pitx and Tpit factors.

3.5 Discussion

The identity of a corticotroph cell is determined by a developmental program that implicates the activities of Pitx and Tpit factors. Pitx and Tpit participate in synergistic interactions that are the basis of cell-specific POMC expression. We have shown that Bmp- and Smad1-specific signaling pathways repress *POMC* expression in AtT-20 cells, and we propose that Smad1 antagonistic interactions with Pitx and Tpit transcription factors form the basis of this repression.

Bmps had to date only been characterized as inductive signaling molecules in mammals, activating the expression of the *Tlx2* homeobox gene for example (37). We show for the first time that Bmp-4 signaling represses endogenous *POMC* expression in AtT-20 cells as well as *POMC* (-480/+43) promoter-luciferase reporter activity. We also show that the classical cognate receptor/Smad signaling pathway conveys Bmp signals to the nucleus in AtT-20 cells to repress *POMC* expression. Indeed, Bmp inhibitory effects on *POMC* promoter activity observed in rBmp4-treated conditions could be mimicked with the overexpression of the Bmp-specific intracellular mediator Smad1 and with constitutively-activated forms of either Alk-3 or Alk-6 Bmp receptors. Recently, other signaling pathways such as the cascades that implicate extracellular signal-regulated kinase (ERK), protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), and Tgf-activated kinase-1 (TAK1) activities have been implicated in Bmp responses (54-57). In light of this, there was a possibility that Smad-independent pathways might be involved in Bmp-induced effects on *POMC* expression is not excluded. However, Smad6 and Smad7 overexpression studies suggest that Bmp-induced inhibitory *POMC* responses are principally mediated by the Smad signaling pathway.

It appears that different members of the Tgf-β superfamily may repress *POMC* expression. We observed that rActivin-A, but not rTgf-β treatments of AtT-20 cells repressed *POMC* expression to a similar extent as did rBmp-4 treatments. Others had implicated Activin-A in the inhibition of POMC mRNA accumulation and ACTH secretion from AtT-20 cells (49) and also Tgf-β was shown to inhibit POMC mRNA in hypothalamic neurons (58). Our failure to observe Tgf-β downregulation of *POMC* expression in AtT-20 cells may be due to the use of the *POMC* (–480/+63) promoter region in our assays, which does not contain regulatory sequences regulating hypothalamic expression of *POMC* (59). Although we did detect transcripts encoding for the Alk-2 type I Tgf-β receptor in AtT-20 cells which is known to mediate common responses to Activin and Bmp-7 (47) and although Tgf-β and Activin responsiveness was shown in AtT-20 cells (Fig 3.3C), we could not detect activation of the Smad1 signaling pathway in rActivin-treated AtT-20 cells. We were moreover unable to detect any effects on *POMC* promoter activity following the overexpression of Activin-specific Smad2 and Smad3 at concentrations for which Smad1 inhibited *POMC* expression, suggestive of a Smad-independent effect of Activin and Tgf-β on *POMC* expression in AtT-20 cells. In muscle cells for example, Tgfβ-mediated repression of *IGFBP-5* expression has been described to occur through c-Jun N-terminal kinase (JNK) signaling pathways (60).

Smad translocation into the nucleus is known to require ligand stimulation, suggesting that the mere presence of supplementary Smad mediator proteins might not suffice to affect gene responses in cells. However, some groups have demonstrated that transiently overexpressed Smad proteins are able to transactivate target genes in a ligand-independent manner (61;62). We found that overexpression of Smad1 alone or in combination with Smad4 could repress *POMC* expression up to fourfold in AtT-20 cells

even in the absence of exogenous Bmp stimulation. However, overexpressed Smad1 could be acting in a ligand-independent manner if supplementary amounts of Smad1 were to overcome some mechanism of negative signaling regulation established in AtT-20 cells, such as inhibitory Smad6 or Smad7 expression or simply microtubule Smad sequestration (27). Still, evidence that Bmp7 signaling pathways may endogenously be activated in AtT-20 cells is in favor of a ligand-dependent activity of overexpressed Smad1 in these cells. Our findings that overexpression of Smad6 or Smad7 could reverse *POMC* repression by Bmp signals, whether they are endogenous or exogenous, furthermore suggests that similar Bmp antagonists might be working against Bmp/Smad-mediated repression of *POMC* expression.

Smad1 and Alk-3/-6 signaling components shown to repress *POMC* expression when overexpresssed, also induce transcription from the *Tlx2* promoter (37). Smad interactions with different *POMC* and *Tlx2* promoter-specific transcription factors are likely responsible for the opposite Bmp responses observed. Indeed, Smad binding to DNA is not selective (63), and gene recognition by the Smad complex has been recognized to occur by way of interactions with specific transcription cofactors. Smad1-mediated induction of *Xvent-2* expression for example requires cooperation with the Ornithine decarboxylase antizyme (OAZ) transcription factor (18). Differential Smad interactions with *POMC* and *Tlx2* promoter-specific transcription factors are the likely explanation for the difference in Bmp responses we observe, which presumably originates from promoter-specific differences in Smad activity. A Smad DNA-binding partner that would make possible the activation of *Tlx2* transcription has yet to be elucidated. Our studies support an essential role for Pitx and Tpit transcription factors in Smad-mediated repression of POMC expression. Not only are Pitx and Tpit regulatory elements important for induction of

POMC transcription (12), but we show that loss of either activity abolishes repression of the POMC promoter by Bmp (Fig. 3.6B). These findings suggest that Pitx and Tpit transcription factors are the principal coordinators of negative Smad action on the POMC promoter. The *in vitro* interaction of Smad1 with Pitx1 and Tpit is consistent with a role for these homeobox and T-box factors in POMC repression. Smad2 has been shown to interact with paired-like homeodomain proteins of the Mix family, Mixer and Milk, through a smad-interacting motif that is also found in members of the FAST family of winged-helix transcription factors (64). We could not locate such motif in bicoid-related Pitx homeodomain proteins. There are no precedents for interactions between members of the T-box and Smad families.

Pitx and Tpit factors are obligate partners of one another for POMC activation (12). Bmp signaling in heterologous CV-1 cells being able to block Pitx/Tpit synergistic interactions that relie exclusively on their respective binding sites suggests that Pitx and Tpit regulatory elements are after all direct targets of Bmp signals in corticotrophs. How exactly Bmp-activated Smads antagonize with Pitx and Tpit-dependent *POMC* transcription remains to be clearly explained. Smad interactions with DNA-bound Pitx and Tpit factors could interfere with their transcriptional activity by acting directly on the transactivation domains of these activators or by interfering with the recruitement by Pitx or Tpit of yet undefined co-activators. Another way the Smad complex could remodel the chromatin template into a closed conformation would be through the recruitment of HDACs. For example, Smad2 has been shown to act as a repressor of transcription by associating with TGIF in the repression of the *Cdc25A* promoter for example (65). Bmp-activated Smad proteins could also be competing with Pitx and/or Tpit binding to DNA.

Also, activated Smad1 induces the expression from the *osteopontin* promoter through its ability to sequester the Hoxc-8 repressor away from DNA.

Repression of gene activity plays an important role in the restriction of many regulatory genes during embryonic development. Tgf-β was recently implicated in the inhibition of myogenic differentiation through Smad3-mediated repression of MyoD transcriptional activity (20). In a similar fashion, Smad1/4 antagonistic interactions with Pitx1 and/or Tpit could act to negatively modulate *POMC* promoter activity in corticotrophs, either for appropriate timing of differentiation during development or for coordination of POMC expression in response to signals elicited by other cells involved in pituitary function. The use of AtT-20 cells is limited subsequently the relevance of Bmp signaling in the timing or maintenance of pituitary corticotroph differentiation will require conditional gene-targeting studies in mice. Investigating the molecular basis of Bmp participation in corticotroph cell phenotype determination and differentiation should give us a clue of the implications of these pathways not only in developmental processes, but also in pituitary function and possibly in tumorigenesis.

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3.7 Figure Legends

Fig. 3.1 **Bmp-4 represses POMC expression and promoter activity.** (A) Northern blot analysis of POMC mRNA in AtT-20 cells treated with 1nM Bmp-4 for 24 and 48 hrs, compared to non-treated cells. Bands corresponding to POMC mRNA were quantified by densitometry and compared to β-actin mRNA used as internal control. (B and C) Effect of rBmp-4 on AtT-20 cells transfected with a luciferase reporter gene driven by the *POMC* promoter (-480/+63). (B) rBmp4 (1nM) represses *POMC*-luc activity in a time-dependent manner. (C) Dose response of *POMC* promoter repression by rBmp-4 measured after 24hr treatment. Results in (B) and in (C) are for representative experiments in which luciferase values were standardized relative to CMV-βgal reporter activity as internal control.

Fig. 3.2 **Bmp-4 repression is mediated through Bmp-specific receptors and Smad1/4 transcription factors.** (A) Overexpression of increasing amounts of constitutively active Alk-3 (Q223D) and Alk-6 (Q203D) receptors downregulates *POMC*-luc reporter activity in AtT-20 cells to similar or greater levels than those observed in cells stimulated with rBmp-4. pcDNA 1 was used as control vector. (B) Flag-Smad1 (S1) overexpressed alone, or together with Smad4-HA (S4), represses *POMC*-luc activity when transfected in AtT-20 and P19 cells, and furthermore enhances Bmp inhibitory effects in AtT-20- cells. (C) Following 4, 24 and 48 hr 1nM Bmp-4 treatment of AtT-20 cells, nuclear extracts (25ug) were assayed for content of phosphorylated Smad1 protein using an Upstate Biotechnology antibody against Smad1 phosphorylated on Serine 463 and 465 of the C-term SSXS motif. The 65 kb band may correspond to phosphorylated Smad1 (arrow), while the slower migrating band may be another Bmp-specific Smad, such as Smad5 or Smad8. Results are the average (± standard error of the mean [SEM]) from at least three sets of experiments performed in duplicate.

Fig. 3.3 **Bmp/Smad1 signaling specifically represses *POMC*.** In both AtT-20 and P19, overexpression of Alk-3 and Smad1 (A,B) as well as rBmp-4 treatments (C,D) repress *POMC*-luc but not *Tlx*2-luc or *3TP*-lux reporter activities. Noteworthy is the greater sensitivity of *POMC* promoter activity to Bmp/Smad-mediated repression in AtT-20 cells than in P19 cells. *POMC*-luc activity is also repressed by rActivin-A, but not rTgf-β treatments (C, D). *Tlx2*-lux activity is induced by Smad1/4, Alk-3 and Bmp-4 in P19 cells (B,D), but only by Smad1/4 and Alk-3 overexpression in AtT-20 cells (A). The Tgfβ-specific *3TP*-lux reporter is activated in both P19 and AtT-20 cells by rActivin and rTgf-β, but not by rBmp-4. (E) Increasing concentrations of Flag-Smad1, but not Flag-Smad2 or Flag-Smad3 repress the activity of *POMC*-luc transfected in AtT-20 cells. Results are the average (± standard error of the mean [SEM] from at least three sets of experiments in duplicate).

Fig. 3.4 **Inhibitory Smad6 and Smad7 reverse Bmp/Smad-dependent *POMC* repression.** Increasing concentrations of the Bmp-specific inhibitor Smad6 (A) and the general Tgf-β inhibitor Smad7 (B) counteract the repressive effects of Smad1/4 overexpression and rBmp-4 treatment on *POMC*-luc activity in AtT-20 cells. Results are the average (± standard error of the mean [SEM] from at least three sets of experiments in duplicate).

Fig. 3.5 **Bmp signals target Pitx1 and Tpit regulatory elements on *POMC*.** (A) Bmp4-responsiveness of distal, central and proximal regions of the -480/-34 *POMC* promoter, alone or in combination, and upstream of the minimal (-34/+63) *POMC* promoter were tested in Bmp4-treated AtT-20 cells, and are shown relative to their respective basal activities in non-treated cells. Only the combined activity of distal and central promoter regions, in a similar fashion to the (–480/+63) full-lenght promoter, is repressed by Bmp

signaling. (B) Relative activities (Bmp4-treated vs. –nontreated) of replacement mutants of either NurRE, $Ebox_{neuro}$, Tpit, Pitx1, and $Ebox_{ubi}$ regulatory elements within the r*POMC* promoter. The loss of Tpit or Pitx regulatory elements abolishes Bmp-4 repression (C) Overexpression of constitutively active Alk-3 (Q223D) receptor in heterologous CV-1 cells represses Tpit-dependent, as well as Pitx1 and Tpit-dependent activities. Results are the average (± standard error of the mean [SEM]) from at least three sets of experiments in duplicate. (D) Phosphorylated Smad1, Pitx1 and Tpit protein levels were assayed by Western Blot in nuclear extracts of control AtT-20 cells, and treated with 1nM rBmp-4 for 4, 24 and 48 hrs. The amount of nuclear Pitx1 and Tpit transcription factors is not downregulated with increased nuclear Smad1 activity in AtT-20 cells.

Fig. 3.6 **Smad1 interacts with Pitx1 and Tpit *in vitro*.** In pull-down assays, GST resin-bound Smad1 (GST-S1) but not resin control (GST) pulled down ^{35}S-labelled Pitx1 and Tpit proteins synthesized seperately or cosynthesized *in vitro*. *In vitro* translated ^{35}S-labeled luciferase did not bind to either GST or GST-S1.

Fig. 3.7 **Model for Bmp-induced transcriptional repression of *POMC*.** A Smad1/4 complex translocates to the nucleus upon Bmp-stimulation, is recruited to the POMC promoter by Pitx and Tpit and subsequently disrupts transcriptional synergism between Pitx/Tpit factors bound to the POMC promoter in the central region.

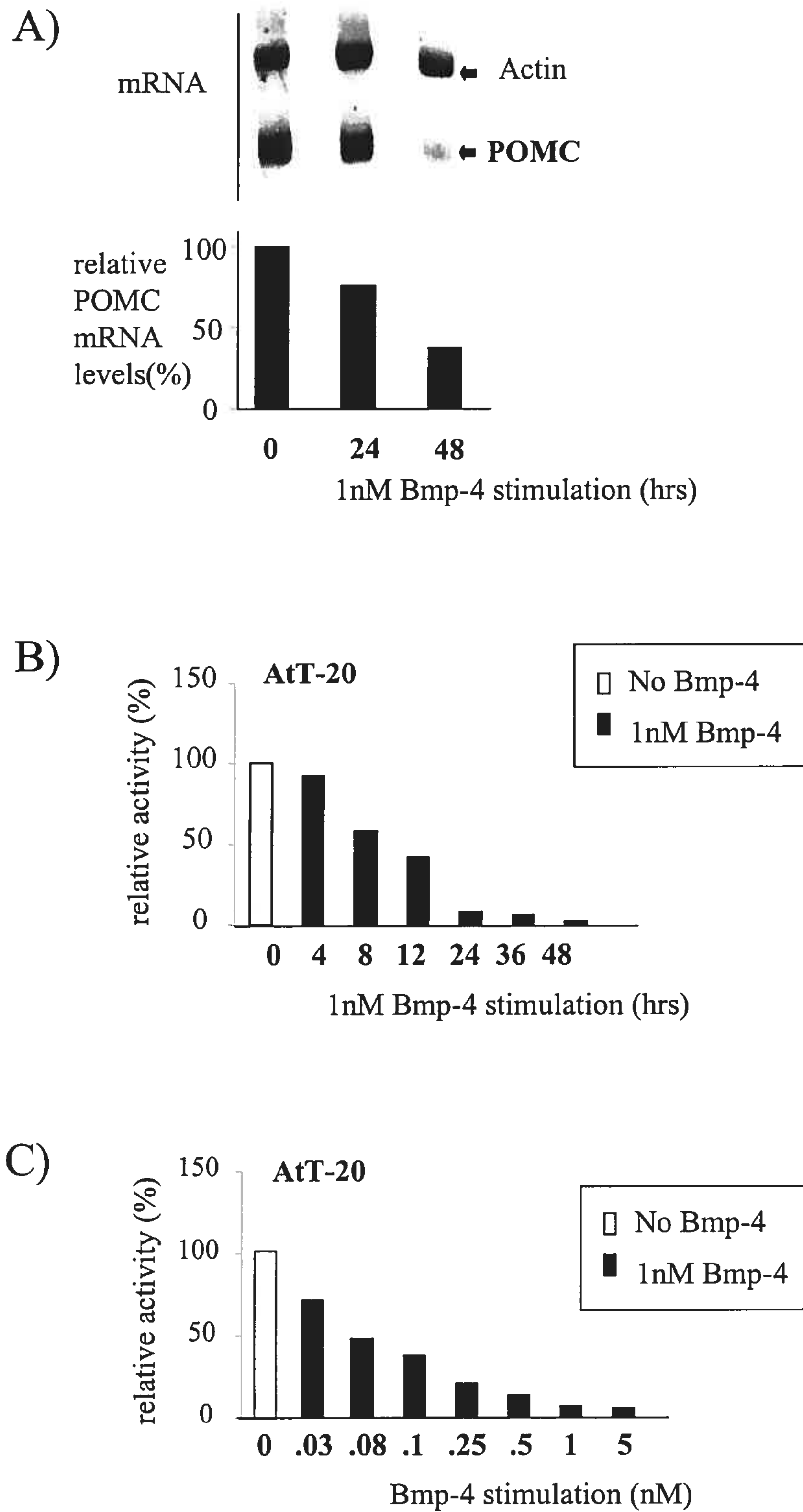
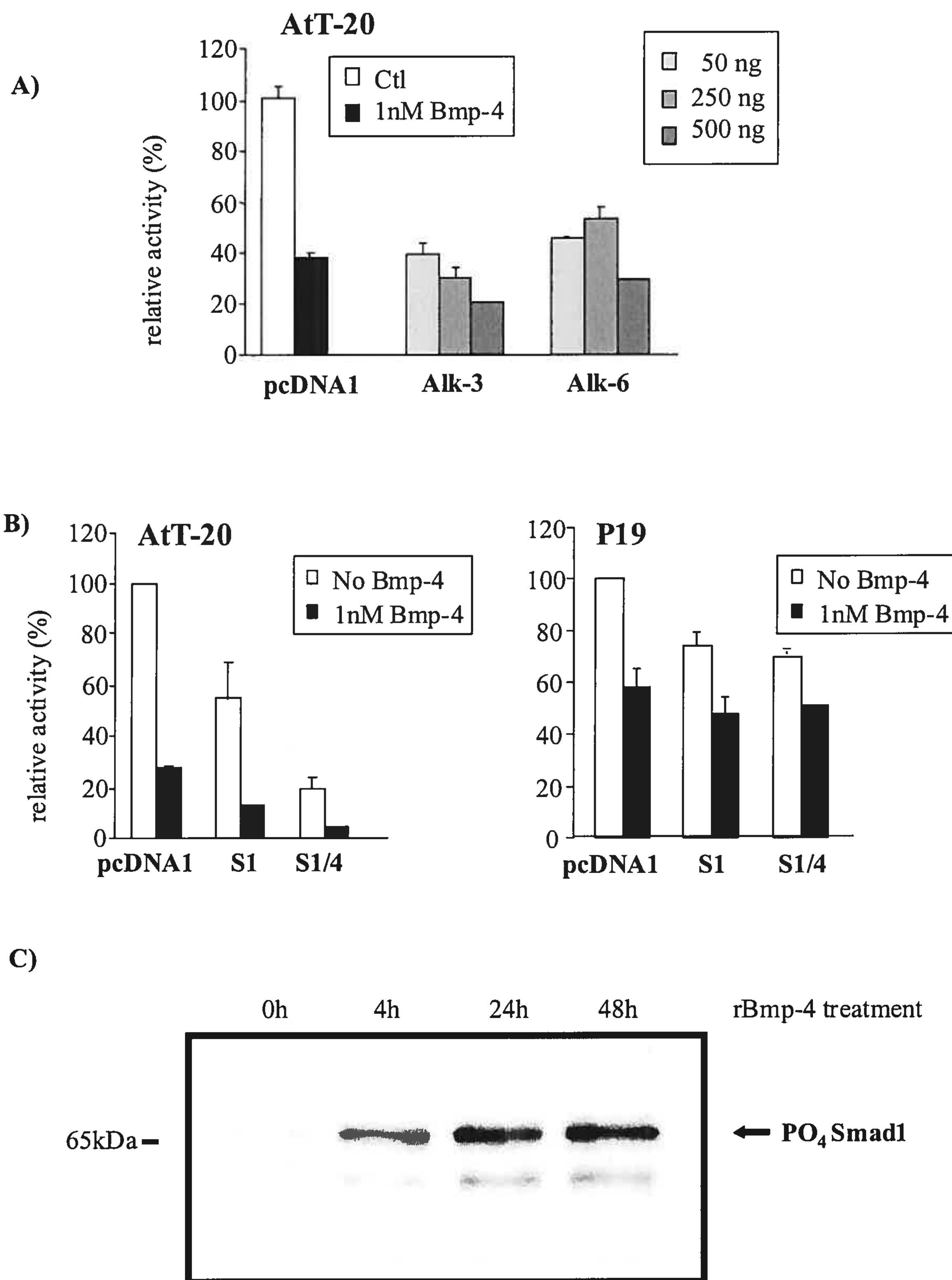


Fig. 1.



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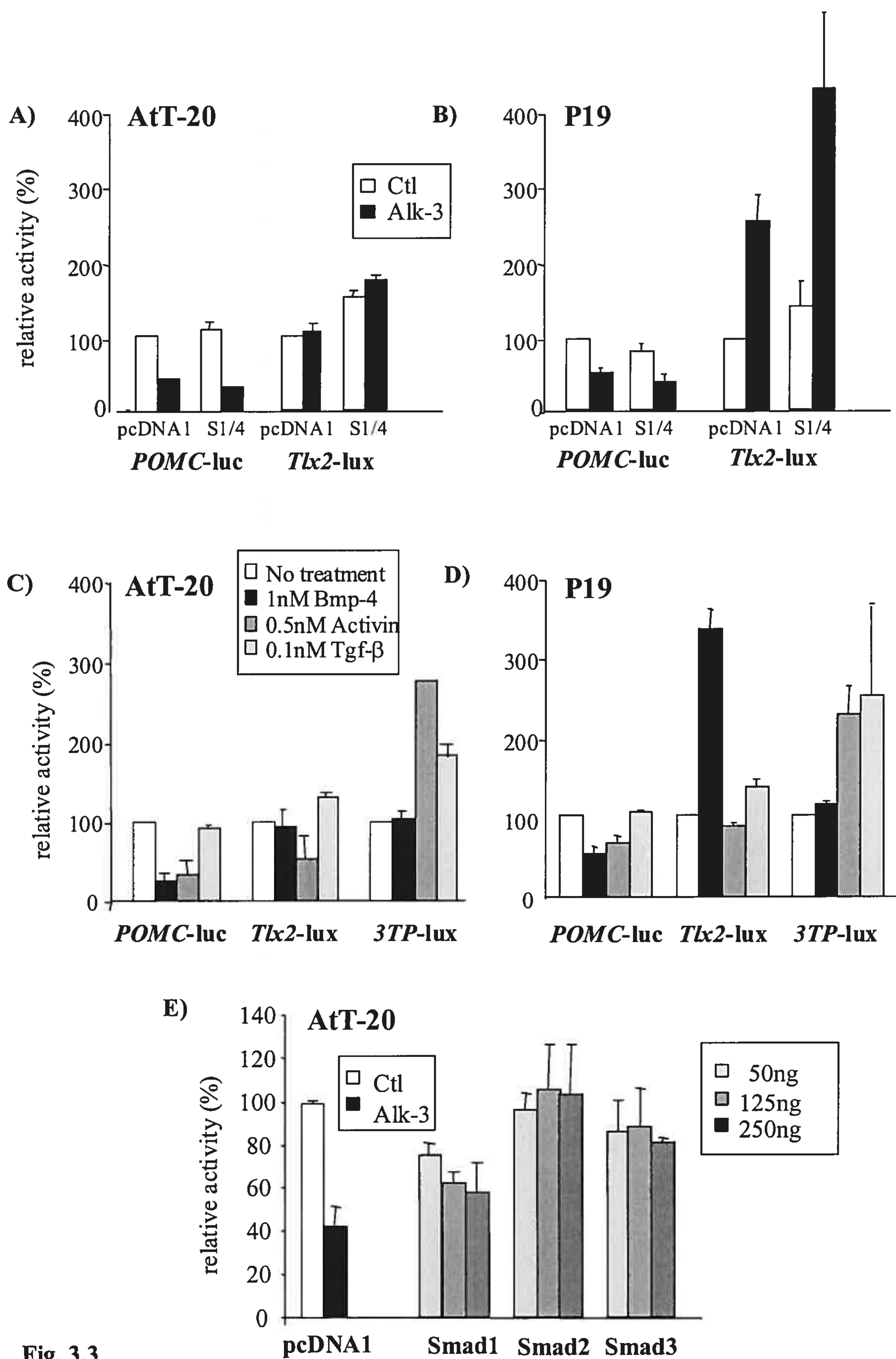

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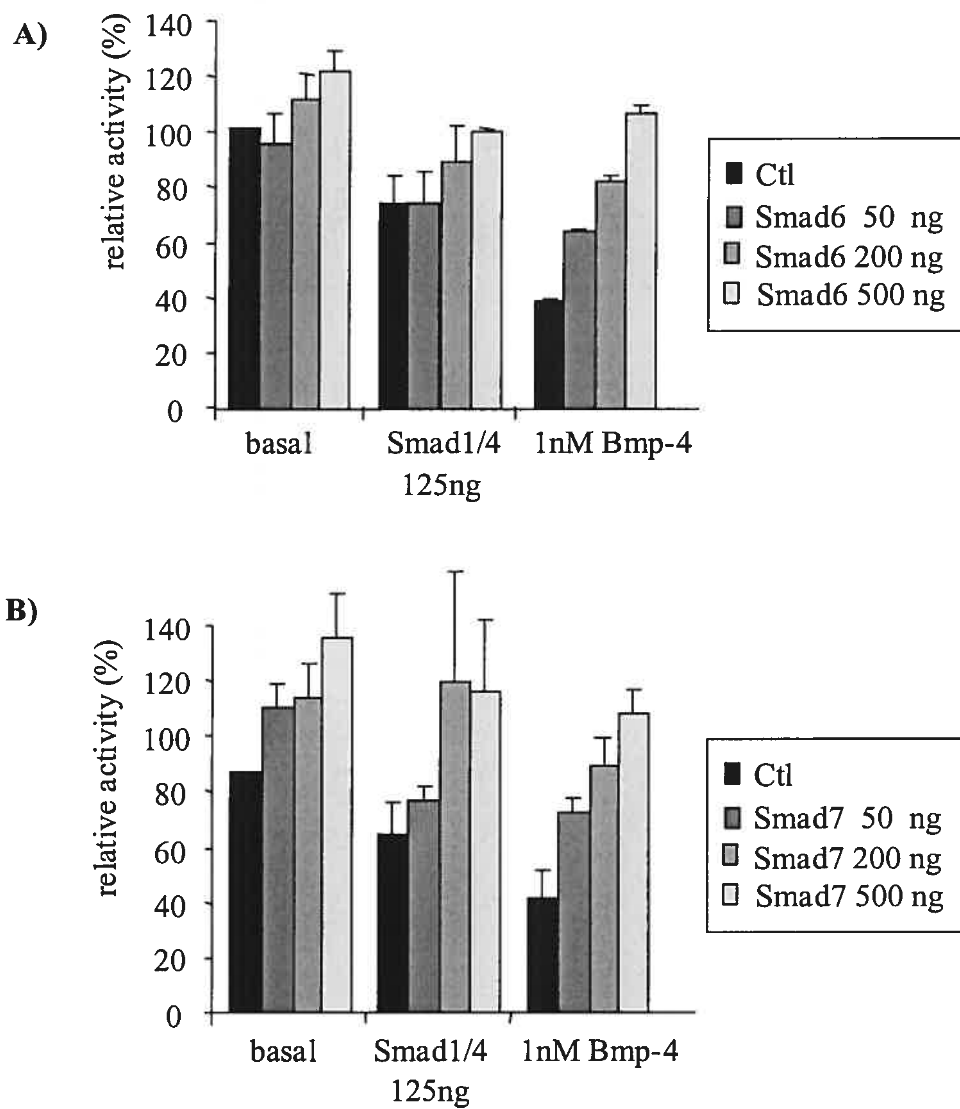

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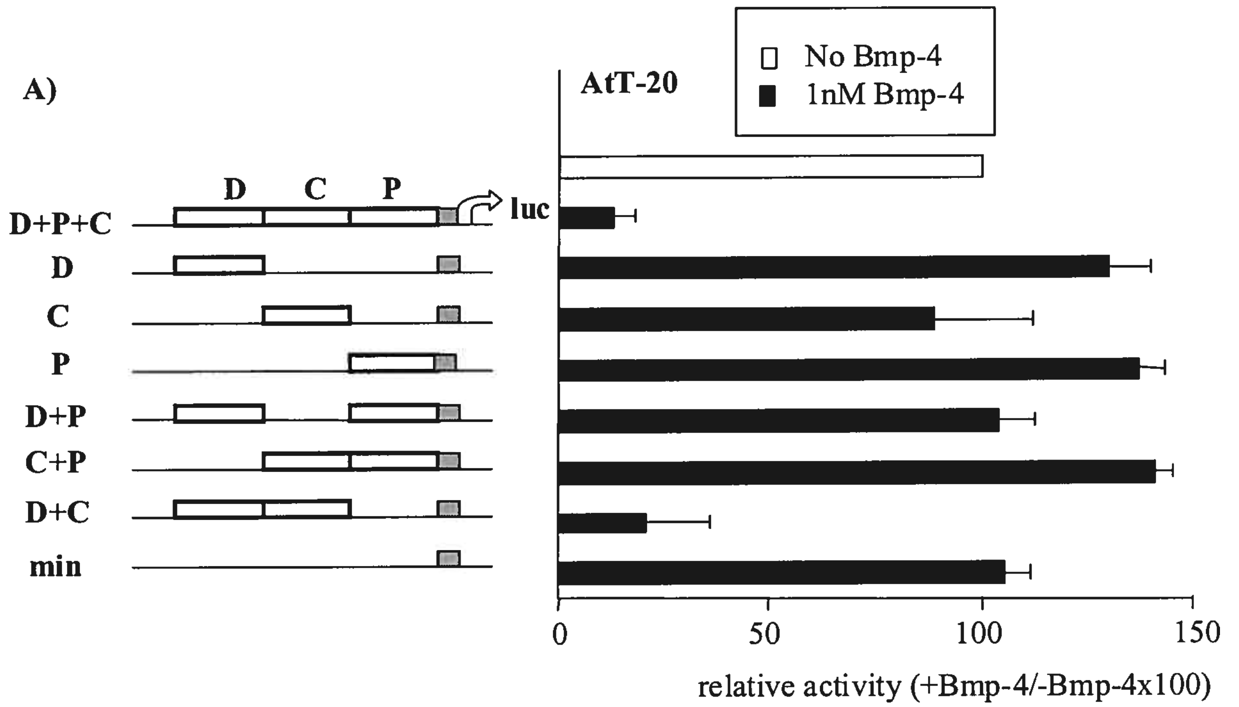

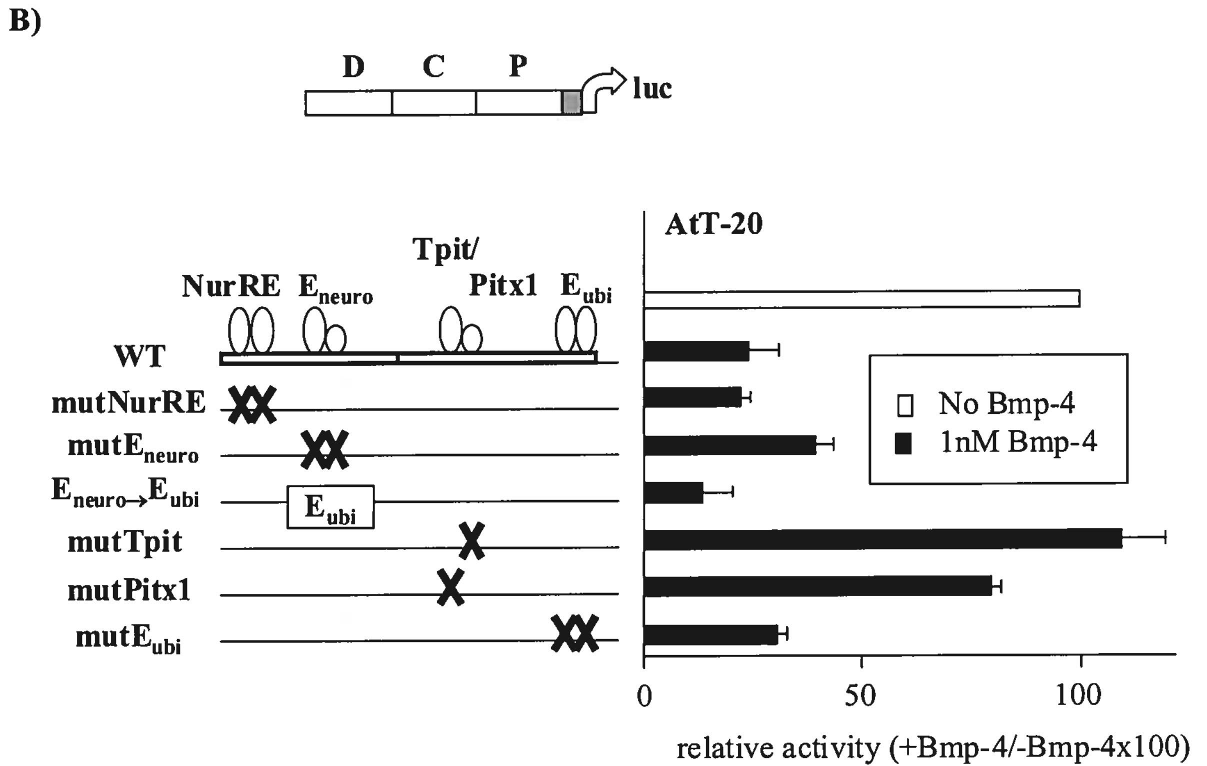

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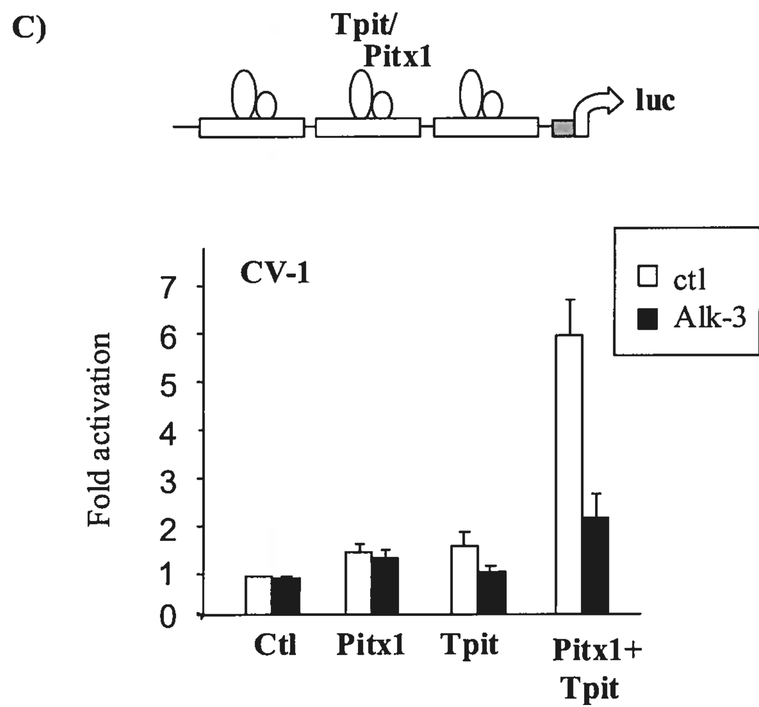

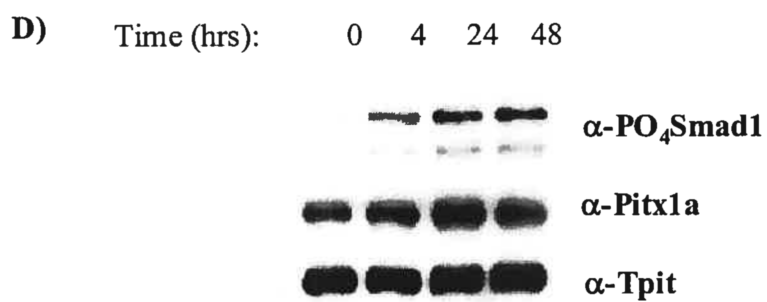

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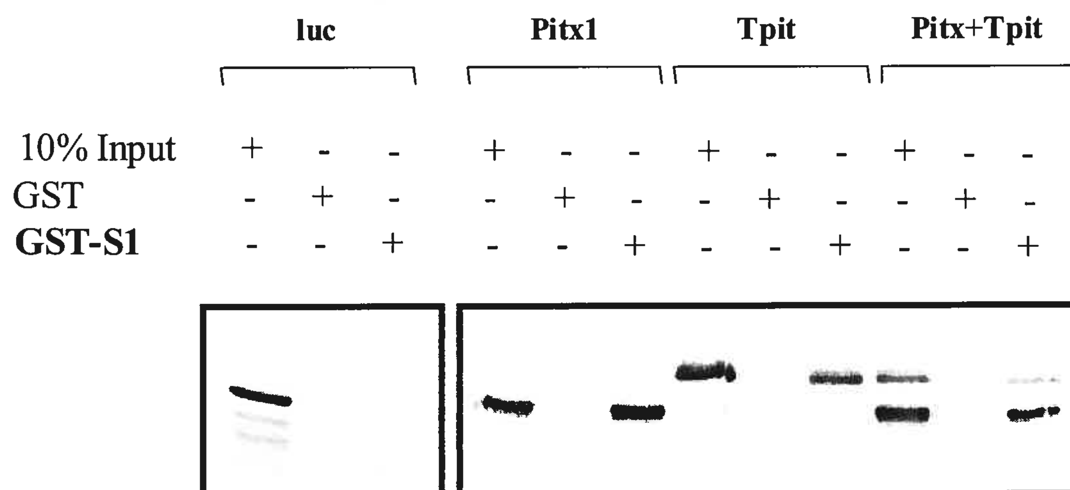

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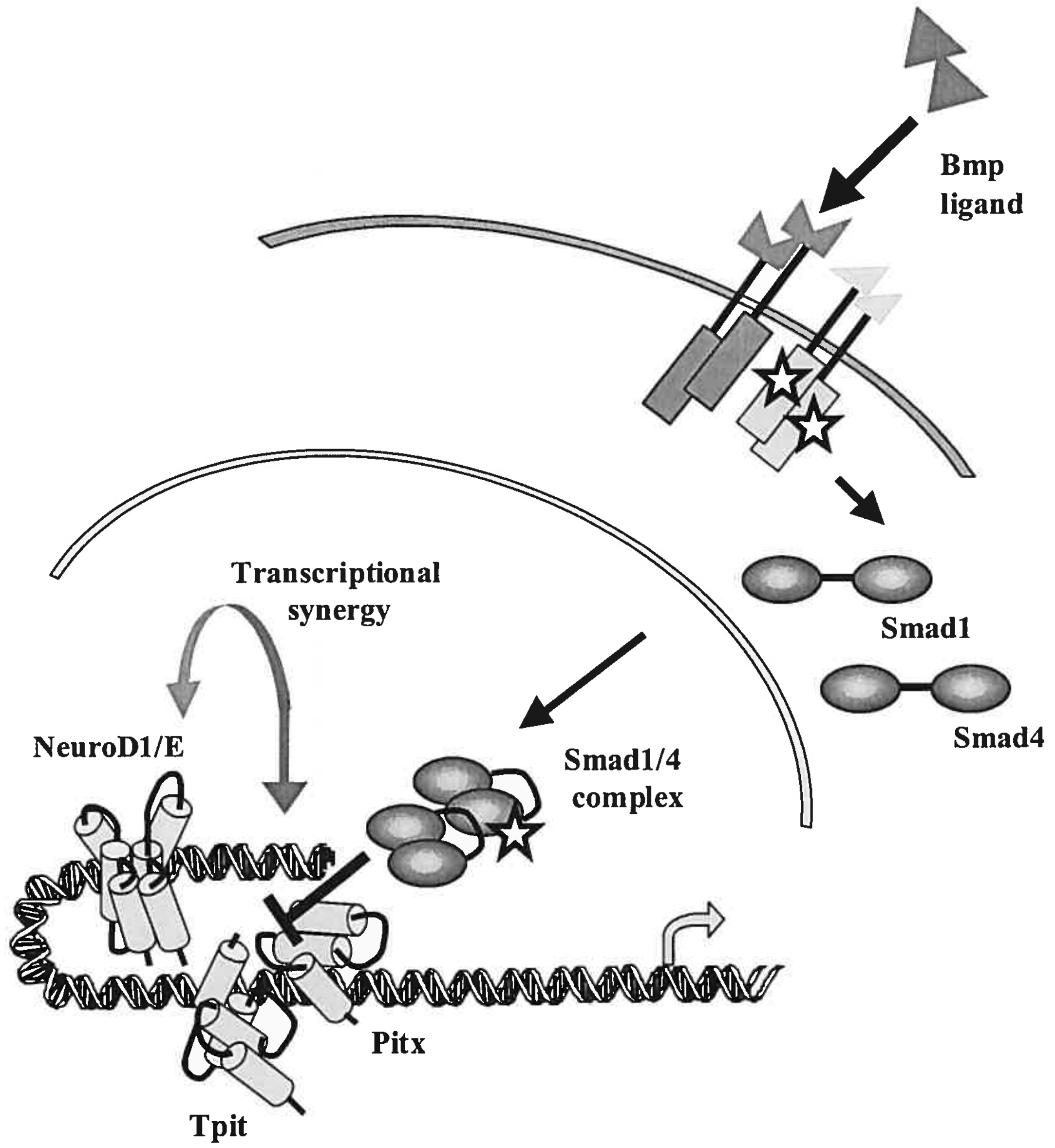


Fig. 3.7

CHAPTER4- DEFINING THE MECHANISM OF ACTION OF BMP-SPECIFIC SMADS ON THE *POMC* PROMOTER

During my Master's project, I was able to show that *POMC* expression is repressed by a Smad-mediated Bmp signaling pathway that appears to target Pitx and Tpit transcriptional activities in the central promoter region. Work to determine how Bmp-activated Smads act, directly or indirectly, on Pitx and Tpit regulatory elements to subsequently repress *POMC* transcription is ongoing, and for this reason I omitted it from my article preferring to present this incomplete portion of my graduate studies as a separate chapter in my thesis. Any relevant information this work should provide to elucidate the mechanism of Bmp-specific Smad action on the *POMC* promoter is to be included in my article to allow for a more thorough understanding of Smad-mediated repression of *POMC* transcription. Such findings may open the way to understanding the so far unresolved role that Smad proteins, and especially Bmp-restricted Smads play in transcriptionally repressive complexes.

4.1 DNA-Binding Activity of Smad Proteins

Smad proteins are known as independent and sequence-specific DNA binding proteins that regulate transcription mostly, if not solely with the collaboration of DNA-binding partners. As mentionned in the introduction, the consensus sequence 5'-AGAC-3' that has come to be known as the Smad-binding-element (SBE) has been demonstrated to be critical for Smad binding and activity on target promoters. Unfortunately, most of the early work that looked at the regulative role of the SBE in transcription was performed on Tgfβ- and Activin-inducible genes (55,91,275). As much as some studies have characterized SBEs as functional binding sites in Bmp-responsive promoters such as in the

Id promoter (116,148), certain studies have demonstrated that SBE sequences are not high affinity binding sites for Bmp-regulated Smads, as is the case in the *JunB* (104) and *PAI-1* (52) promoters. Another Smad1-binding element was identified in Bmp-responsive *Id* (116,148) and *Msx1* (5) promoters, and consists of a GC-rich sequence that resembles the *Drosophila* Mad recognition site (112). Indeed, Dpp responses in *Drosophila* are elicited by the binding of Smad1-related Mad to GCCGnCG promoter sequences, and cooperation with sequence-specific transcription factors (112,202).

Still not clearly defined in the field of Bmp and moreover Tgf-β signaling is whether Smad binding to DNA is required for Smad action on nuclear targets. The *osteopontin*-inducing activity of Bmp for example appears to relie solely on Smad1 protein interactions with the Hoxc-8 transcriptional repressor, for which access to promoter binding elements is subsequently denied.

4.2 SBEs in the POMC Promoter: Preliminary Results

The difficulty in identifying Bmp-responsive sequences in the *POMC* promoter is that nothing is known of how Bmp-specific Smads actively repress genes, and only a few cases have been described of Smad3-mediated Tgf-β inhibition of gene transcription (4,84,142). The mechanism of Smad transcriptional repression that is the most clearly defined in these studies describes the ability of Smad3 to inhibit *muscle creatine kinase* transcription by directly interfering with the transcriptional activity of MyoD-containing bHLH heterodimers (142). In this context, Smad3 does not appear to require binding to DNA to repress the activity of MyoD, which raises the question of whether a Smad-binding site is really mediating Bmp responses on the *POMC* promoter. Other studies have reported the recruitment of Smad-interacting repressor molecules (Sno, Ski, TGIF) and HDACs to Tgfβ-responsive promoter SBEs, but in this case to turn off Smad activity (279).

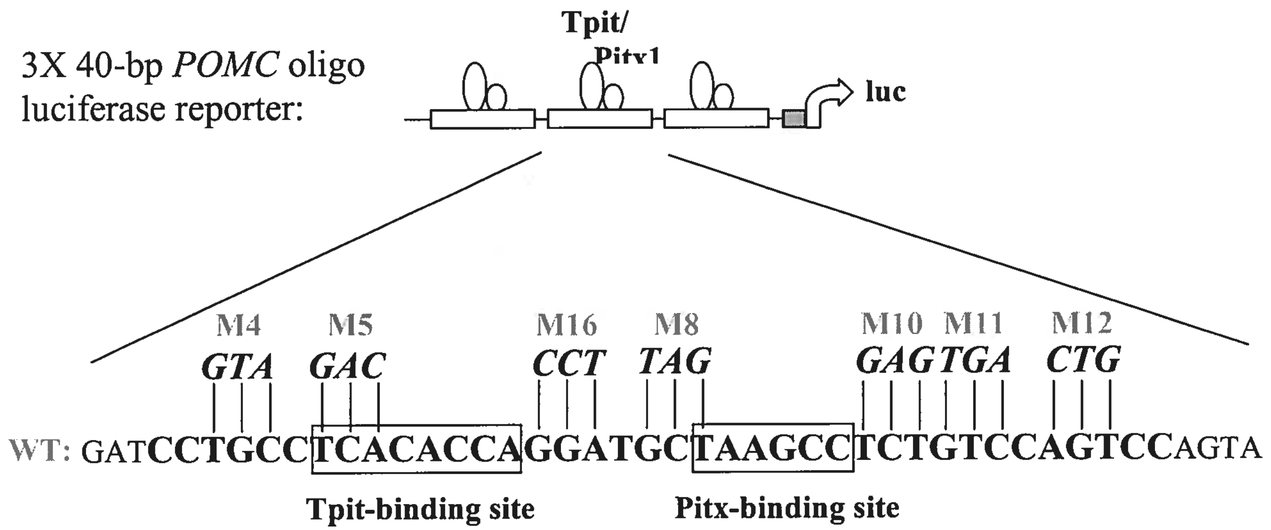
Interestingly, unpublished data proposes that a binding site that differs from the characterized SBE, which has been associated with transcription activation, would be mediating transcriptional repression by Tgf-β (Wang X.-F., unpublished).

My search for a Smad-binding element in the *POMC* promoter was focused on the 40-bp promoter element that includes Pitx and Tpit binding sites since this POMC sequence was sufficient to mediate Bmp inhibitory effects on Pitx and Tpit-dependent activity in heterologous cells (Figure 3.5C). As shown in figure 4.1, the analysis of *POMC* promoter sequences located within and around Pitx and Tpit binding sites for putative SBEs resulted in no perfect match for the 5'-AGAC-3' consensus, but quite a few sequences (shown in boxes) harbouring one mismatched nucleotide were identified. Although the same POMC fragment contains some scattered GC-rich clusters of nucleotides, none of these correspond to the characterized GCCGnCG Mad-like consensus binding site (263). One of the experimental approaches used to assay whether any one, or more likely a combination of these sites mediates the Bmp response on *POMC*, was to resort to site-directed mutagenesis. I made use of available reporter plasmids driven by three copies of a wild-type (WT) or 3-bp mutant oligonucleotide corresponding to the 40-bp *POMC* fragment, and analyzed their transcriptional activity in Alk3-transfected and non-transfected AtT-20 cells. Results from this analysis are summarized in Figure 4.2. The wild-type construct was consistently observed to loose 50% of its activity in Alk3-overexpressing AtT-20 cells, confirming that the 40-bp POMC promoter fragment containing Pitx and Tpit regulatory elements is a direct target of Bmp signals in these cells. Noteworthy in the latter experiment is: firstly, that the M5 *POMC*-luc mutant reporter that contains mutations within the Tpit binding site was less repressed than the WT construct in AtT-20 cells transfected with Alk-3; and secondly, that the M16 *POMC*-luc mutant containing mutant

SBE: 5' -AGAC- 3'
or
5' -GTCT- 3'

Drosophila Mad-related SBE: GC-rich sequence (GCCGnCG)

Figure 4.1 Putative Smad-binding elements in POMC



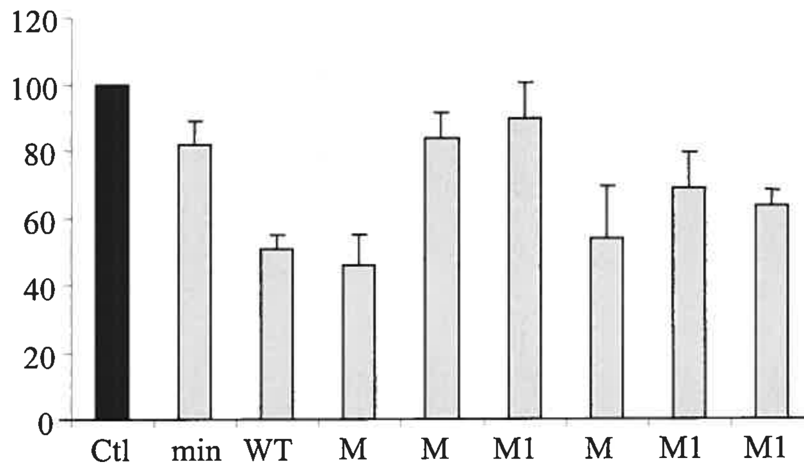


Figure 4.2 Nucleotides within Tpit regulatory sequences and nucleotides lying between Tpit and Pitx regulatory sites contribute to Bmp-mediated POMC expression (only the nucleotides in bold in the oligo are *POMC*-specific)

nucleotides between the Pitx and Tpit regulatory elements also exhibited decreased repression. The observation that nucleotides located between Pitx and Tpit binding sites are partially implicated in *POMC* repression by Bmp signaling suggest that these nucleotides might be mediating Smad action through Smad binding to DNA.

Loss of Bmp-response from the M5 *POMC* oligo construct bearing mutations within the Tpit binding site was predicted by previous findings that showed a requirement for this site in the ability of the 480-bp (full-length) *POMC* promoter fragment to negatively respond to Bmp treatments (Figure 3.5B). However, the two different mutations of the Pitx binding site (in which different nucleotides are targeted) have different outcomes. In the context of the full-length promoter, Pitx binding site mutation abolished almost entirely *POMC* response to Bmp (Figure 3.5B). On the other hand, the M8 40bp-*POMC* oligo trimer construct containing mutated Pitx sequences previously demonstrated to play an important role in Pitx/Tpit synergistic activities (127), was repressed as much as the WT construct in Alk3-overexpressing AtT-20 cells. Is the Pitx binding site hence really important for Smad-mediated transcriptional repression of *POMC* activity? Other Pitx mutations will be analyzed to answer this question.

To determine whether *POMC* promoter nucleotides that span or surround Pitx and Tpit regulatory elements can bind Smad proteins, I turned to the EMSA technique. I assayed whether bacterially-expressed GST-Smad1 moieties could directly bind to a 40-bp DNA probe corresponding to the same oligo *POMC* sequences mentionned above and shown in Figure 4.2. I show that although both 1 ug of GST-Smad1 and 1ug of GST-Smad4 are able to bind a probe containing four repeats of the *JunB* promoter SBE (4XSBE), only GST-Smad1 binds to the 40-bp *POMC* probe (Figure 4.3). The binding affinity of GST-Smad1 to the *POMC* probe is much lower than that observed for the 4XSBE probe, suggesting that the putative SBE in the *POMC* sequence fragment is of low

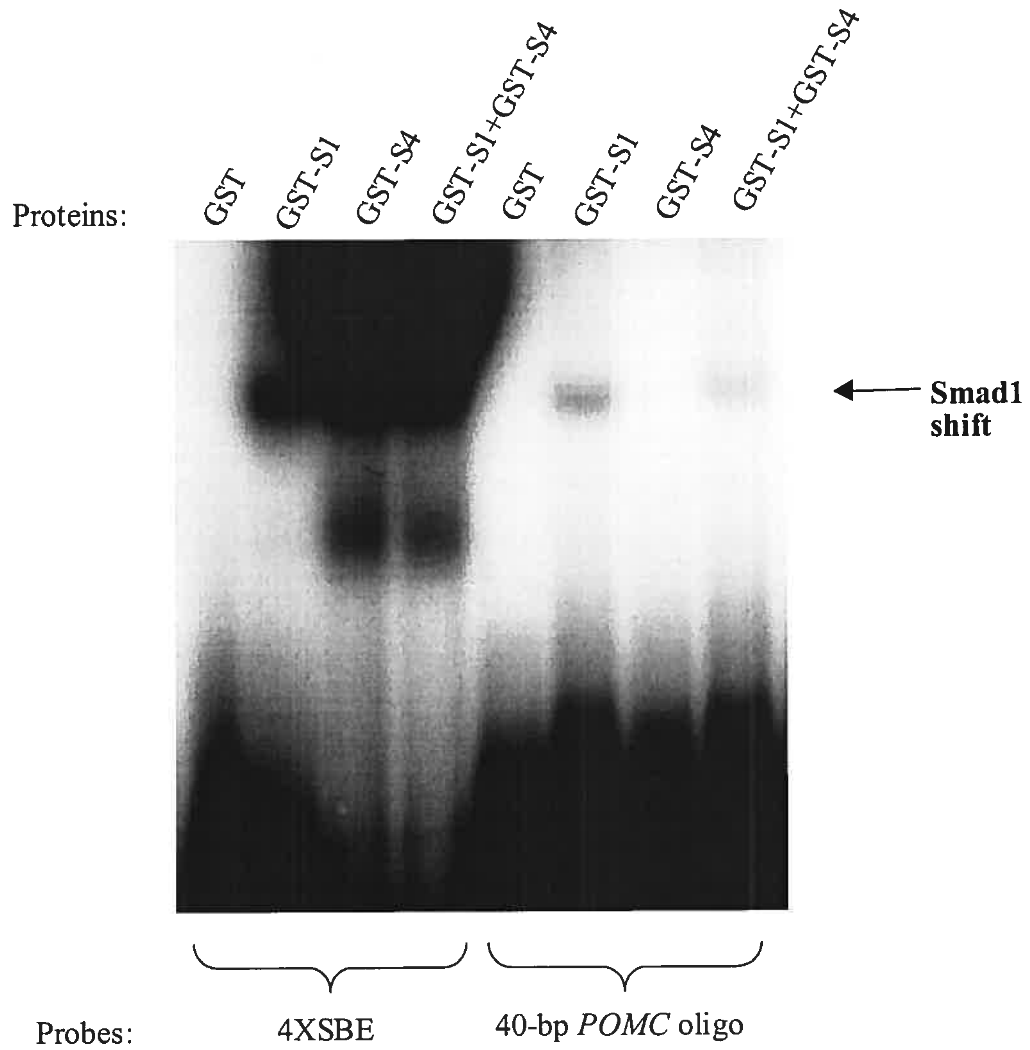


Figure 4.3 GST-Smad1 binds to *POMC* promoter nucleotides located in proximity to Tpit- and Pitx-regulatory elements in the central region.

affinity and is probably not present in multiple copies. The 4XSBE probe should hence, for comparison purposes, be replaced by a lower-affinity 2XSBE or 1XSBE probe when a positive control is to be used. Seeing that GST-Smad1 is not phosphorylated, it should be mentioned that the ability of Smad1 to interact with DNA in my EMSA essays may not necessarily reflect the DNA binding properties of phosphorylated Smad1 in the Bmp-activated cell.

I verified that the GST-Smad1 shift that I observe with the *POMC* probe could be supershifted with an antibody that recognizes Smad1, Smad5 or Smad8 (Santa Cruz N-18). I found that a Smad-1 supershift worked best following a 20 minute incubation on ice of the antibody with the protein:DNA mixture, itself previously incubated on ice for 40 minutes (figure 4.4). The antibody on its own did not yield any similar migrating band in the same gel (data not shown).

4.3 SBEs in the POMC Promoter: Perspectives for the Future

A good deal of work is yet to be done to properly understand how Bmp-activated Smad proteins interfere with the activity of Pitx and Tpit transcription factors on the *POMC* promoter in corticotroph cells. Through the genetic analysis of different mutant forms of *POMC* fragments, it appears that nucleotides lying between Pitx and Tpit binding sites play an important role in mediating Bmp-inhibitory effects on the promoter. Upon showing that GST-Smad1, and not GST-Smad4, binds to this *POMC* promoter fragment in EMSA assays, I immediately tested the role that nucleotides between Pitx and Tpit regulatory elements might be playing in Smad DNA binding. I observed that GST-Smad1 moieties bound to the M16 probe as well as to the WT probe (data not shown), implying that the aforementionned nucleotides are not required for Smad binding. It is possible that these inner nucleotides might coordinate the anchoring of some repressor protein that is recruited by the Pitx/Tpit-bound Smad complex to negatively regulate transcription from the *POMC*

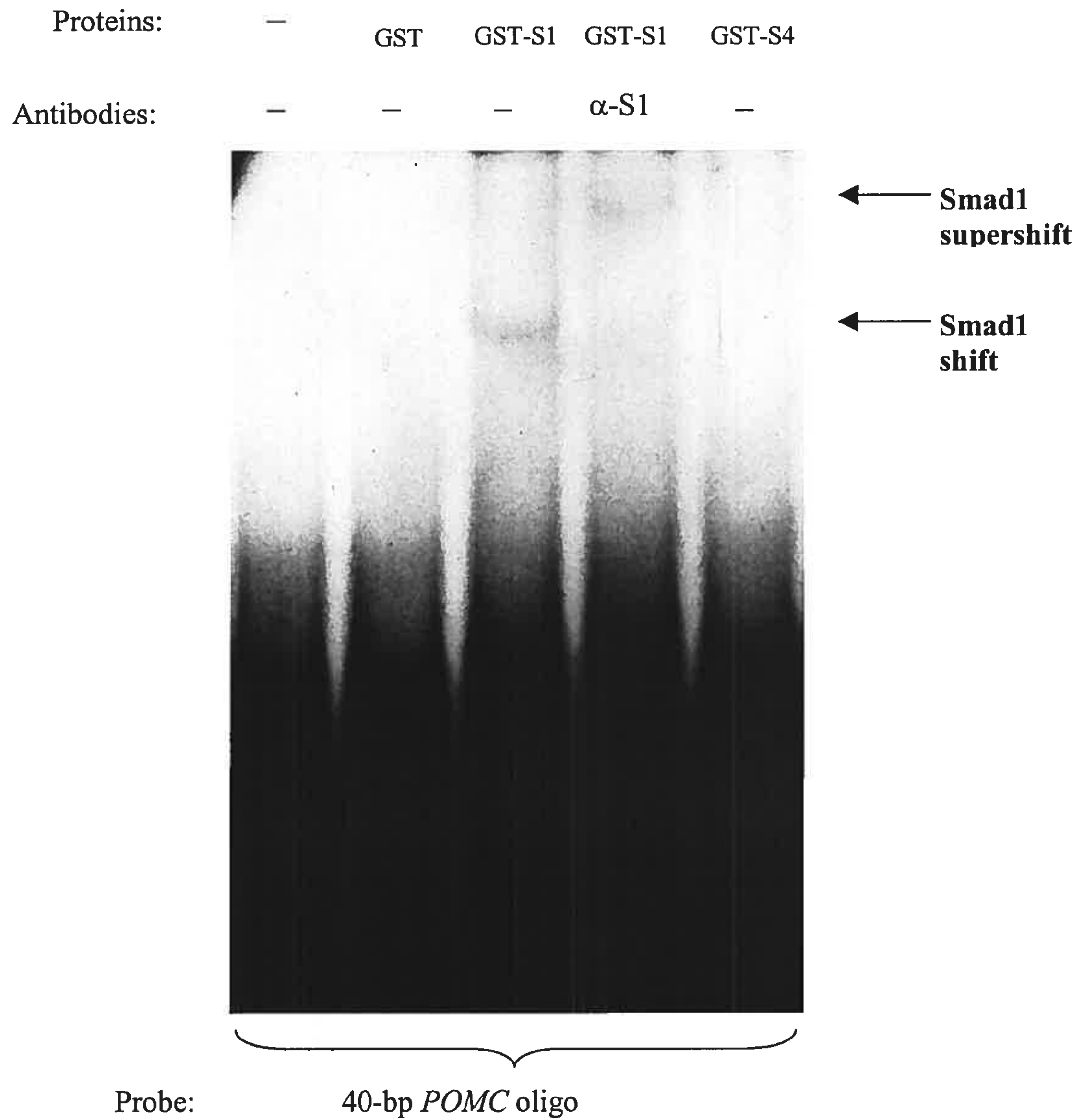


Figure 4.4 GST-Smad1 binding to POMC is specifically supershifted by an α-Smad1/5/8 antibody (N-18) from Santa Cruz

promoter. Such a repressor protein underlying *POMC* negative responses to Bmp challenges could be the TGIF transcription factor, recently implicated in the negative regulation of Tgf-β responses (258). Although an optimal DNA binding sequence for TGIF has been identified *in vitro* (12), it is not yet known whether TGIF binding to DNA plays an important role in modulating Tgf-β responses. Some simple ways of determining whether TGIF is recruited to the *POMC* promoter would consist in using the EMSA technique to determine in one case whether GST-TGIF fusion proteins are able to bind to a *POMC* probe *in vitro*, and in the other case to show *in vivo* TGIF-binding activity in AtT-20.

Much work is still required to understand the role that Smad binding to *POMC* plays in corticotrophs to repress transcription in response to Bmp signals. The EMSA technique should be helpful in identifying Smad1-binding element(s) within the Bmp-responsive 40-bp *POMC* promoter fragment. That is, different mutant *POMC* probes will be tested for their ability to bind GST-Smad1 in comparison to the WT *POMC* probe. In parallel, it would be nice to show binding of a Smad1-, Smad5- or Smad8-containing complex in nuclear extracts from Bmp-treated AtT-20 cells to corroborate *in vitro* results obtained with GST-Smad proteins. The latter information can be obtained through the use of EMSA as well as chromatin immunoprecipitation (ChIP) techniques.

Another question that should be dealt with concerns the effect that Smad binding to DNA might have on the DNA-binding activity of Pitx and Tpit. Again, an *in vitro* approach is probably the quickest in providing us with some answers. The idea is to assay any changes that might occur in the ability of MBP-Pitx1 and/or in MBP-Tpit to bind the 40-bp *POMC* probe (127) upon the addition of GST-Smad1 moieties. If Smad1 blocks Pitx1 or Tpit binding to *POMC*, respective shifts are expected to disappear completely or

partially in presence of GST-Smad1. Alternatively, Smad1-Pitx1 or Smad1-Tpit might form *POMC*-binding complexes that would be transcriptionally-impaired; such complexes would be expected to be supershifted by an α-Smad1 antibody, but also by either the α-Pitx1 or the α-Tpit antibody. ChIP assays could moreover be used to compare the ability of Pitx and Tpit as well as Smad proteins to tether on POMC promoter sequences in Bmp-treated and non-treated AtT-20 cells.

CHAPTER5- CONCLUSION

As a first step towards the development of a coherent model for the role of Bmp/Tgf-β signals in corticotroph cell differentiation, my Master's project investigated the actions of Bmp signaling on POMC expression and promoter activity in the AtT-20 corticotroph cell line model. I was able to show that rBmp-4 stimulation of AtT-20 cells represses endogenous POMC mRNA as well as *POMC*-luc reporter activity. The most prevalent Bmp signal transduction mechanism implicates ligand binding to Alk-3 and -6 receptors, and activation of Smad1/5/8 intracellular mediators. In agreement with this model, I showed that inhibitory Bmp effects on POMC promoter activity are either reconstituted or enhanced, respectively, by co-expression of constitutively active forms of the Bmp receptors, Alk-3 (Q223D) and Alk-6 (Q203D), or by co-expression of Smad1 and Smad4. In addition, Bmp-dependent repression of POMC could be reversed by the expression of inhibitory Smad6 or Smad7 factors.

The search for Bmp-responsive elements within the mouse POMC promoter proved to be a complex task. When the basal activities of the distal, central or proximal POMC promoter regions were examined separately for their response to rBmp-4 treatments in AtT-20 cells, none were significantly repressed. These results suggest that Bmp signals might be targeting a transcriptional complex on the POMC promoter that is made up of multiple regulatory elements. Previous work in our laboratory on *POMC* promoter organization demonstrated that most of its activity is generated by synergistic interactions between distal and central regions. Interestingly, only the combined activity of distal and central promoter regions was significantly repressed in AtT-20 cells following rBmp4-treatments.

The regulatory mechanism for cell-specific transcription of the POMC gene implicates synergistic interactions between distal NeuroD1/BETA2 bHLH heterodimers,

and central Pitx1 homeobox and Tpit T-box factors. Findings from the latter part of my work extend these studies to show that Smad-mediated Bmp signaling pathways might be acting through Pitx and Tpit transcription factors to block or downregulate POMC expression in AtT-20 corticotroph cells. How Bmp-activated Smads interfere with the activity of Pitx and Tpit and hence POMC expression remains to be clearly understood. In heterologous cells, I show that Bmp signals can block transcriptional synergy between Pitx and Tpit, suggesting that the activity of Pitx and Tpit on their respective binding sites in the central region of the POMC promoter would directly be targeted by Bmp signals. *In vitro* binding studies that I performed furthermore support a mechanism of Bmp action that would see the recruitment of Bmp-activated Smads to the promoter through protein:protein interactions with Pitx and Tpit factors. Preliminary EMSA results indicate that Smad1 but not Smad4 binds to yet undefined POMC promoter sequences in proximity to Pitx and Tpit regulatory elements. Smad1-tethering to *POMC* might enable the Smad complex to negatively regulate Pitx/Tpit synergistic activities by either interfering with their respective DNA binding activities, or rather by modulating their association with other POMC-specific or general co-factors. The observation that maximal Bmp inhibition of POMC promoter activity requires that both distal and central domains be present and intact suggests that Bmp signaling, through Pitx and/or Tpit recruitment, might interfere with the activity of a bigger transcriptional complex composed of both central and distal regulatory elements. Preliminary work in our laboratory suggests that Tpit activity in the central region of the promoter is greatly enhanced by co-activators previously thought to increase distal-specific transcriptional activity. A combination of EMSA and chromatin immunoprecipitation techniques should be helpful in further delineating the molecular basis of Smad-mediated Bmp repression of *POMC* expression, and the role both central and distal parts of the POMC promoter play in the mediation of Bmp inhibitory effects.

A useful tool in my analysis of the role Bmp signals play in the regulation of *POMC* expression turned out to be the AtT-20 corticotroph cell line, seeing that it endogenously expresses NeuroD1, Tpit and Pitx cell-specific regulators of POMC activity and moreover responds to Bmp signaling. RT-PCR studies revealed the incidence of Bmp-specific type I receptor as well as Bmp-7 expression in AtT-20 cells, suggesting that these cells might endogenously harbour some Bmp-7 signaling activity that would maintain POMC expression in a slightly repressed state. Evidence to support the latter idea came with the overexpression of Smad6 or Smad7 inhibitors in AtT-20 cells, which in itself led to the induction of POMC promoter activity. As appropriate as AtT-20 cells have been to study the molecular basis of POMC repression by Bmp/Smad1 signaling, their worth as a model to study corticotroph differentiation is limited.

Still, in showing that the Bmp/Smad1 signaling pathway represses the expression of POMC in AtT-20 cells, which is in support of the previously reported downregulation of ACTH expression in Rathke's Pouch explants cultured in the presence of Bmp2-coated beads, it would appear that Bmp-2 and/or Bmp-4 signals expressed early on during pituitary organogenesis would be acting to negatively regulate POMC expression. Indeed, Bmp signals could be set in place in the developing pituitary to correctly turn on the corticotroph differentiation program and/or to downregulate POMC expression in differentiated corticotrophs. Relevant evidence for such biological functions of Bmp signals in corticotroph differentiation could come from studying the spatiotemporal activity of Bmp signaling pathways in the developing pituitary. Bmp activity in mice pituitaries of different developmental stages could be assayed by immunohistochemical analyses using antibodies that are specific for the phosphorylated form of Smad1, Smad5 and/or Smad8. I attempted such studies but could detect no activity of phosphorylated Smad1 in the pituitary, while signal was obtained in some tissues in e10.5, e11.5 e12.5 and e14.5 mice.

These negative results could mean that the less characterized Smad5 or Smad8 proteins act downstream of Bmp signals in the early developing pituitary to negatively regulate *POMC* expression, or that Smad-mediated Bmp signaling pathways play a regulatory role in *POMC* expression only later on in the developing embryo or in the adult stage. If Smad activity were to be detected in the pituitary, it would be interesting to determine through co-staining techniques and hormone markers which population of pituitary cells harbors active Bmp signaling pathways. Phosphorylated-Smad and ACTH co-staining for example would be a strong indication that Bmp signals are indeed working in corticotroph cells to repress POMC expression. A peak of Bmp/Smad activity in corticotrophs at e12 or earlier, would be an indication that Bmp plays a role in timing the onset of corticotroph differentiation, while the detection of Bmp signaling activity at later stages of pituitary development would support a role for Bmp in the maintenance of the corticotroph differentiated phenotype. The possibility that Bmp signals do not function in corticotrophs, but rather act in the rest of pituitary cells to altogether block the expression of *POMC*, should not be excluded.

Bmp gain-of-function or gene-inactivation studies that specifically target the pituitary would constitute a problematic approach to understanding the biological effects of Bmp signaling on pituitary cell differentiation since Bmp signals are required for proper pituitary organogenesis. More informative perhaps is studying the biological role of distinct Bmp-specific Smads in the pituitary, supposing that different Smads are implicated in organogenesis and cell differentiations decisions.

Tgf-β signaling appears to be highly regulated in developmental processes. Deficient expression of Smad7 for example has recently been implicated in the pathogenesis of scleroderma, which is moreover associated with high levels of Smad3 activity (57). In my work, Bmp action in AtT-20 cells was efficiently counteracted by the transient overexpression of Samd6 or Smad7. It would be interesting to assess the

expression pattern and biological functions of such Bmp or Smad inhibitors in the pituitary. Bmp/Smad inhibitors would function in corticotroph cells to counteract Bmp inhibitory effects on POMC expression and hence allow corticotroph differentiation to begin, or simply permit the upregulation of *POMC* expression in response to some physiological cue. An interplay of positive and negative inputs into Bmp signaling pathways might serve to regulate *POMC* expression all along the life of a corticotroph cell. It is also possible that the normal control Bmp pathways exercise on *POMC* transcriptional processes could become disregulated and subsequently result in malignant transformations. Investigating the molecular basis of Bmp participation in corticotroph cell phenotype determination and differentiation could give us clues as to the implications of these same pathways in tumorigenesis.

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