Characterization of Human Growth Hormone Receptor (hGHR) Gene Expression in Human Adipocytes

by

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当一切辉煌散尽，唯有坚强！

The beauty of soul shines out when a man bears with composure on heavy chance after another. Not because he does not feel them, but because he is a man of high and heroic temper.

-Aristotle

This thesis is dedicated to my beloved father, who gave me the dream to pursue a PhD degree and the courage to accomplish it.

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Today, when I close this chapter, when I envision the new life ahead of me, when I think back over all I have been through, I ask myself, is this journey worthy? The answer is, **Yes**. I can only hope that the final product justifies the time everyone so graciously gave me.

ABSTRACT

Human growth hormone (hGH) is a key regulator of postnatal growth and metabolism, exerting its effects through a specific cell-surface receptor, hGHR. Eight hGHR mRNAs are produced by the hGHR gene due to transcribing from different 5'non-coding exons. Four (V2,V3,V5,V9) are ubiquitously expressed, whereas the others (V1,V4,V7,V8) are present only in normal postnatal liver, suggesting that different promoter usage is a mechanism for developmental and tissue-specific regulation of the hGHR gene.

hGH influences adipose tissue development and function. By 5'RACE screening of a human adipocyte cDNA library, I determined that the V2 transcript is the predominant (~90%) hGHR mRNA expressed in fat cells. Although five novel mRNA variants (VA-VE) were isolated, they were demonstrated to be widely expressed minor hGHR transcripts, instead of adipocyte-specific. Using the human SGBS preadipocyte cell line, I showed that total hGHR mRNAs increase significantly during adipocyte differentiation, primarily due to increases in the V2 transcript, reaching a maximum with maturity.

The predominance of the V2 variant in all cells examined to date led us to carry out an in-depth study of the V2 promoter. Two major transcriptional start sites were identified that were identical for SGBS preadipocytes, liver and kidney. Transient transfection analysis of the 5'flanking region of V2 confirmed its promoter activity in multiple human cell lines. Similar transcriptional profiles were observed in SGBS pre- and mature adipocytes, with much higher V2 promoter activity seen in mature adipocytes, suggesting that increases in adipocyte factor(s) play a role in V2 promoter regulation. The V2 proximal promoter is TATA-less, with several characteristics of a housekeeping gene promoter. Cis-elements for Ets1, CHOP and Hes1 were found in the proximal promoter and within the V2 exon. They exert either positive (Ets1, CHOP) or negative (Hes1) regulatory effects on V2 transcription when their respective transcription factors are over-expressed, via direct binding (Ets1, Hes1) or interaction

with other proteins (CHOP). These sites likely regulate hGHR expression in response to extra-cellular (developmental, growth, stress) signals.

Thus, transcriptional regulation of hGHR V2 in human cells, including adipocytes, is the result of a complex interplay by multiple factors, to ensure sufficient local production of hGHR throughout life.

RÉSUMÉ

L'hormone de croissance humaine (hGH) est un régulateur clé de la croissance et du métabolisme postnatal. Elle exerce ses fonctions en utilisant un récepteur spécifique à la surface de la cellule (hGHR). Huit ARN messagers sont produits à partir du gène du récepteur de l'hormone de croissance humaine (hGHR) grâce à la transcription de différents exons non codants dans la partie 5' du gène. Quatre (V2, V3, V5 et V9) sont exprimés largement alors que les autres (V1, V4, V7 et V8) sont présents seulement dans le foie après la naissance. Cela suggère que l'usage de différents promoteurs soit un mécanisme impliqué dans le développement et la régulation spécifique des tissus du gène du récepteur de l'hormone de croissance humaine.

hGH influence le développement des tissus adipeux et la fonction de ceux-ci. Un criblage en utilisant la technique de 5' RACE PCR a été effectué et le transcrit V2 du récepteur de hGH a été déterminé comme étant celui dont l'expression prédomine dans les cellules adipeuses. Bien que 5 nouveaux variants d'ARN messager (VA-VE) ont été isolés, ils sont exprimés abondamment et sont des transcrits de moindre importance des récepteurs de hGH à défaut d'être spécifique aux adipocytes. En utilisant la lignée cellulaire pré-adipocytaire humaine SGBS, j'ai pu démontrer que la quantité totale d'ARN messager des hGHR augmente significativement durant la différentiation des adipocytes et atteint son maximum avec la maturité des adipocytes, principalement grâce à l'augmentation de la quantité du transcrit V2.

La prédominance du variant V2 chez toutes les cellules examinées jusqu'à maintenant nous a conduit à une recherche en profondeur du promoteur de V2. Deux sites transcriptionels majeurs de départ ont été identifiés chez la lignée cellulaire pré-adipocytaire humaine SGBS, le foie et les reins ; dans les trois cas, les sites sont identiques. L'analyse par transfections transientes de la région 5' flanquante de V2 a confirmé l'activité de son promoteur chez plusieurs lignées cellulaires humaines. Plusieurs profils similaires de transcription ont été observés chez les pré-adipocytes et chez les adipocytes matures dans la lignée cellulaire SGBS, les adipocytes matures

révélant une activité plus grande du promoteur de V2 suggérant ainsi que des facteurs adipocytes aient un rôle à jouer dans la régulation du promoteur de V2. Le promoteur proximal de V2 est sans TATA et possède plusieurs caractéristiques des promoteurs de gènes constitutifs. Des éléments cis-régulateurs de Ets1, CHOP et Hes1 sont présents dans la partie proximale du promoteur ainsi que dans l'exon de V2. Ils exercent des effets régulateurs positifs (Ets1, CHOP) ou négatifs (Hes1) sur la transcription de V2 lorsque leurs facteurs de transcriptions respectifs sont surexprimés, par l'intermédiaire d'un attachement direct (Est1, Hes1) ou par l'interaction avec d'autres protéines (CHOP). Ces sites sont vraisemblablement des régulateurs de l'expression du récepteur de hGH en réponse aux signaux extracellulaires (croissance, stress, développement).

Ainsi, la régulation transcriptionelle du variant V2 de hGHR chez les cellules humaines, incluant les adipocytes, est le résultat d'effets complexes de facteurs multiples qui assurent la production suffisante de hGHR au cours de la vie.

FOREWARD

Chapter II of this thesis is the text of a published original paper. **Chapter III** is a manuscript in preparation. The texts of these manuscripts have been included in this thesis in compliance with the Faculty of Graduate Studies and Research "Thesis preparation and submission guidelines", the text of section I, part C entitled "Manuscript based thesis" is cited below:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

1. a table of contents;
2. a brief abstract in both English and French;

3. an introduction which clearly states the rational and objectives of the research;
4. a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
5. a final conclusion and summary;
6. a thorough bibliography;
7. Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

6. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

7. Irrespective of the internal and external examiners reports, if the oral defence committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis. See the

"Guidelines for Doctoral Oral Examinations," which can be obtained from the web (http://www.mcgill.ca/fgsr), Graduate Secretaries of departments or from the Graduate and Postdoctoral Studies Office, James Administration Building, Room 400, 398-3990, ext. 00711 or 094220.

8. In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis."

The format specified in this thesis has been approved by the Division of Experimental Medicine, McGill University. The figures and tables in each chapter are numbered relative to the chapter. Minimal formatting of sections within the text of the chapters and of references has been performed to maintain stylistic uniformity throughout the thesis.

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS

Publications

"Characterization of Growth Hormone Receptor Messenger Ribonucleic Acid Variants in Human Adipocytes" by **Yuhong Wei**, Zakaria Rhani, and Cynthia G. Goodyer in J. Clin. Endo. Metab. (2006) 91(5): 1901-1908.

"Transcriptional Regulation of Human Growth Hormone Receptor (hGHR) Gene by its V2 Promoter" by **Yuhong Wei** and Cynthia G. Goodyer (manuscript in preparation).

Abstracts

"Transcriptional regulation of the human growth hormone receptor (hGHR) gene by its V2 promoter in human adipocytes" by **Yuhong Wei** and Cynthia G. Goodyer, FASEB Conference on "Energy Balance, Body Fat and Disease", Indian River, California, USA, August 2007 (poster). *(CIHR Institute of Human Development and Child and Youth Health Trainee Travel Award)*

"Transcriptional regulation of the human growth hormone receptor gene from V2 promoter" by **Yuhong Wei** and Cynthia G. Goodyer , Canadian Diabetes Association/Canadian Society of Endocrinology and Metabolism Annual Meeting, Toronto, ON, Canada, October 2006 (poster). *(CSEM/CDA Trainee Travel Award)*

"Growth hormone receptor (GHR) gene expression in the human adipocyte" by **Yuhong Wei** and Cynthia G. Goodyer, 87th Annual Meeting of the Endocrine Society, San Diego, CA, USA June 2005 (poster). *(Women in Endocrinology Trainee Travel Award)*

"Characterization of human growth hormone receptor gene expression in adipocytes" by **Yuhong Wei**, Zakaria Rhani and Cynthia G. Goodyer, 86th Annual Meeting of the Endocrine Society, New Orleans, LA, USA, June 2004 (poster). *(Endocrine Society Trainee Travel Award)*

"Characterization of human growth hormone receptor (hGHR) 5'untranslated region (5'UTR) mRNA variants in adipocytes" by **Yuhong Wei**, Zakaria Rhani and Cynthia G. Goodyer, Canadian Diabetes Association/Canadian Society of Endocrinology and Metabolism Annual Meeting, Ottawa, ON, Canada, October 2003 (oral). *(Canadian Society of Endocrinology and Metabolism D. Harold Copp Trainee Travel Award)*

CONTRIBUTIONS OF AUTHORS

All of the work published, in preparation or presented was conducted by the candidate with one exception: most of the 5'-rapid amplification of cDNA ends (5'RACE) assays were done by a postdoctoral fellow in the lab, Dr. Zakaria Rhani.

ABBREVIATIONS

µg:	microgram
3'UTR:	3'-untranslated region
5'UTR:	5'-untranslated region
aa:	amino acids
AARE:	Amino Acid response element
ADD1:	adipocyte-determination and differentiation factor-1
ALS:	acid labile subunit
aP2:	fatty acid binding protein
ARG:	arginine
ASH1:	achaete-scute homolog-1
ATF:	activating transcription factor
b1A/B:	bovine 1A/B exons
bHLH:	basic Helix-Loop-Helix
bp:	basepair
BRE:	TFIIB response element
BRET:	bioluminescence resonance energy transfer
BTEB-1:	basic transcription element binding protein
bZip:	basic leucine zipper
C/EBP:	CCAAT/enhancer binding protein
cAMP:	cyclic AMP
CBP:	CREB binding protein
CDK:	cyclin-dependent kinase
ChIP:	chromatin immunoprecipitation
CHOP:	C/EBP homologous protein
CIS:	cytokine-inducible SH2-domain protein
CLAs:	conjugated linoleic acids
CNTFR:	ciliary neurotropic factor receptor
CPHD:	combined pituitary hormone deficiency
CRE:	cAMP response element
CRF:	chronic renal failure
DAG:	diacylglycerol
DBD:	DNA-binding domain
DEX:	dexamethasone
DHFR:	dihydrofolate reductase
DN:	dominant negative
DOC:	downstream of CHOP
DPE:	downstream promoter element
E1:	ubiquitin-activating enzyme
EBS:	Ets binding site
ECD:	extracellular domain
ECM:	extracellular matrix
EGFR:	early growth factor receptor
Egr-1:	early growth related factor-1
Elk-1:	Ets-like gene 1

EMSA: electrophoretic mobility shift assay
EMSSA: electrophoretic mobility supershift assay
EPO: erythropoietin
ER: endoplastic reticulum
ERK: extracellular signal-regulated kinase
ERSE: ER stress responsive element
Ets: E26 (E-twenty-six) transformation-specific
FAK: focal adhesion kinase
FAS: fatty acid synthase
FFA: free fatty acid
FGF-1: fibroblast growth factor-1
FRET: fluorescence resonance energy transfer
Gαi: inhibitory Gα subunit
Gαs: stimulatory Gα subunit
GAA: acid α-glucosidase
GAS: IFN γ-activated sequence
G-CSFR: granulocyte-colony stimulating factor receptor
GH: growth hormone
GHBP: growth hormone binding protein
GHD: growth hormone deficiency
GHR: growth hormone receptor
GHRE: growth hormone response element
GHRH: growth hormone releasing hormone
GHRHR: growth hormone releasing hormone receptor
GHRP: growth hormone releasing peptide
GHS: growth hormone secretagogues
GLE: GAS like element
GLUT4: glucose transporter-4
GM-CSF: granulocyte macrophage-colony stimulating factor
GM-CSFR: granulocyte macrophage-colony stimulating factor receptor
GPDH: glycerol-3-phosphate dehydrogenase
Grb2: growth factor receptor-bound protein 2
HERP: Hes-related repressor protein
Hes: Hairy and Enhancer of Split
HGF: hepatocyte growth factor
hGH : human growth hormone
HMG-Y/I: high mobility group I/Y
HNF-4: hepatic nuclear factor-4
HSL: hormone sensitive lipase
IBMX: isobutylmethylxanthine
IGFBP: insulin-like growth factor binding protein
IGF-I: insulin-like growth factor I
IGHD: isolated growth hormone deficiency
IL: interleukin
ICD: intracellular domain
INF-γ: interferon γ

Inr:	Initiator
IP:	immunoprecipitation
IP_3:	phosphatidylinositol-3,4,5-triphosphate
IRS:	insulin receptor substrate
JAK:	Janus kinase or Just another kinase
JH1-7:	JAK homology domains 1-7
JNK:	c-Jun N-terminal kinase
KO:	knockout
KIR:	kinase inhibitory region
LCR:	locus control region
LETF:	liver-enriched transcription factors
LIFR:	leukocyte inhibitory factor receptor
LPL:	lipoprotein lipase
LXR:	liver X receptor
M:	mean
MAP2:	microtubule-associated protein 2
MAPK:	mitogen activated protein kinase
MEK:	MAP/ERK kinase
NF-Y:	nuclear factor Y
ng:	nanogram
NRR:	negative regulatory region
nt:	nucleotide
o1A/B:	ovine 1A/B exons
obR:	leptin receptor
OM:	Oncostatin M
PD:	pyridostigmine
PDGF:	platelet drived growth factor
PF4:	platelet factor 4
PI-3K:	phosphatidylinositol-4,5-biphosphate 3 kinase
PIAS:	protein inhibitors of activated STATs
PKA:	protein kinase A
PKC:	protein kinase C
PLC:	phospholipase C
PMA:	phorbol ester 13-acetate
PPARγ:	peroxisome proliferators activated receptor γ
Pref-1:	preadipocyte factor-1
PRL:	prolactin
PRLR:	prolactin receptor
PRR:	positive regulatory region
PTB:	phosphotyrosine binding
PTP:	protein tyrosine phosphatase
RTK:	receptor tyrosine kinase
SAP-1	stomach-cancer associated PTP1
SAPK:	stress activated protein kinase
SE:	standard error
SGBS:	Simpson-Golabi-Behmel Syndrome

SH2:	Src-homology 2
Shc:	Src-homology 2 domain-containing transforming protein C
SHP:	SH2 domain-containing protein tyrosine phosphatase
SIE:	Sis-Inducible Element
SIRP:	signal regulatory protein
SOCS:	suppressor of cytokine receptor signaling
SOS:	son of sevenless
Spi2.1	serine protease inhibitor 2.1
SRC:	steroid receptor coactivator
SRE:	serum response element
SREBP:	sterol-regulatory element binding protein
SRF:	serum response factor
SS:	Somatostatin
STAT:	signal transducer and activator of transcription
SUMO:	small ubiquitin-like modifier
T3:	triiodothyronine
T4:	thyroxine
TACE:	tumor necrosis factor (TNF) -α converting enzyme
TAD:	transactivation domain
TBP:	TATA-binding protein
TCF:	ternary complex factor
TC-PTP:	T-cell-protein tyrosine phosphatase
TF:	transcription factor
Tg:	transgenic
TG:	triglycerides
TLE:	transducin-like enhancer of split
TM:	transmembrane
TNFα:	tumor necrosis factor α
TPO:	thrombopoietin
TSH:	thyroid stimulating hormone
TSHR:	TSH receptor
TSS:	transcription start site
UbE:	ubiquitin-dependent endocytosis motif
uORF:	upstream open reading frame
USF:	upstream stimulating factor
VDR:	Vitamine D receptor
VLDL:	very low density lipoprotein
ZAG:	Zinc-alpha2-glycoprotein
ZBP-89:	Zinc finger binding protein-89

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CHAPTER I

INTRODUCTION & LITERATURE REVIEW

INTRODUCTION

The endocrine system is responsible for the maintenance of whole body homeostasis in mammals. Normal functioning of an endocrine system depends on adequate amounts of the hormone as well as the target cells' response to the hormone. In the case of peptide hormones, the biological responses are generated by binding of the ligand to its specific cell surface receptor and subsequent triggering of multiple tightly-regulated intracellular signaling cascades. One of the best studied examples is growth hormone (GH) and its high-affinity receptor (GHR). Pleiotropic actions of GH have been identified in various target tissues. Our understanding of the signaling pathways activated following GH-GHR binding has rapidly advanced in the past two decades and disorders associated with receptor dysfunctions have been delineated. All of these findings point to the central importance of GHR to GH actions. At the cellular level, the receptor number on the cell surface is the initial factor regulating the cellular response to GH. Transcriptional regulation of GHR gene expression is one of major mechanisms controlling GHR biosynthesis.

Human GHR (hGHR) is encoded by a single gene located on chromosome 5p13.1-p12. This gene contains multiple 5'untranslated exons under the control of different promoters. Multiple mRNA variants are produced through transcribing from the different 5'UTR non-coding exons, although they all code for the same GHR protein. Some mRNA variants are developmentally regulated and are exclusively expressed in postnatal liver, whereas others are widely expressed. These expression patterns have been observed in several species, suggesting that different promoter usage together with alternative splicing provide a common regulatory mechanism for controlling GHR gene expression.

Due to the prevalence and severity of obesity worldwide, more and more research efforts have focused on characterizing the ontogeny and function of adipose tissue. It is now known that adipose tissue is an active endocrine organ instead of just a reservoir for energy storage. Adipose tissue is one of the major target organs for GH. Various clinical observations and scientific research have indicated several important roles of GH in adipose tissue, including regulation of the number of adipocytes as well as lipolysis.

Adipocytes express high levels of GHR, which mediate the direct effects of GH on regulating adipocyte differentiation and metabolism. However, our current knowledge of how *GHR* gene expression is regulated in adipocytes remains very limited. This thesis focuses on human *GHR* gene expression and its related regulatory mechanisms in human adipocytes.

LITERATURE REVIEW

1. GROWTH HORMONE (GH)

GH, also know as somatotropin, is a polypeptide hormone that is mainly secreted from the anterior pituitary (Tannenbaum 1991). Since it was first isolated in 1944 (Li & Evans 1944), extensive studies of GH have demonstrated its central role in postnatal linear growth (Isaksson *et al.* 1985) and its various important physiological effects on metabolism and body composition (Casanueva 1992). The availability of recombinant human GH (rhGH) has led to a widespread clinical interest in the administration of rhGH for GH-deficient children and adults, patients with HIV wasting, obesity, and elderly adults, in whom normalizing GH status has demonstrated beneficial effects by changing body conformation (increasing lean body mass and decreasing body fat), enhancing bone mineral density, improving physical strength and mobility as well as quality of life (Hull & Harvey 2003).

1.1. Growth Hormone Biosynthesis

Human growth hormone (hGH) is produced primarily as a single-chain, 191 amino acids (aa), 22kD protein by the somatotroph cells in the anterior pituitary (Reiter & Rosenfeld 2002). The crystal structure, solved by Bdel-Meguid and colleagues, revealed that hGH contains two disulfide bonds and four α-helices that are necessary for functional interaction with the GH receptor (Bdel-Meguid *et al.* 1987). hGH is structurally and evolutionarily homologous to prolactin and chorionic somatomammotropin (CS, also known as placental lactogen), whose genes are believed to have arisen by duplication of a common precursor more than 350 million years ago (Miller & Eberhardt 1983). The hGH

gene cluster is located on the long arm of human chromosome 17q22-24 and contains five highly-conserved, tandemly-linked genes spanning approximately 66 kb (Ho *et al.* 2004; Cooke *et al.* 1988). There are two GH genes interspersed with three CS genes, lined up from 5' to 3' as: *hGH-N (*or *GH-1), hCS-L, hCS-A, hGH-V (*or *GH-2)* and *hCS-B* (Cattini *et al.* 2006; Ho *et al.* 2006). The *hGH-N* gene is primarily transcribed in pituitary somatotrophs, whereas the *hCS-A*, *hCS-B* and *hGH-V* genes are expressed in placental syncytiotrophoblasts (Cattini *et al.* 2006; Ho *et al.* 2004). The *hGH-V* gene encodes a 22kD protein similar to hGH-N and can be detected in the maternal circulation from midgestation. As maternal hGH-V levels increase, they regulate the maternal hypothalamic-pituitary axis and inhibit maternal hGH-N production. After birth, circulating hGH-V levels quickly drop and become undetectable after 1 hour (Melmed & Kleinberg 2002).

The onset of pituitary hGH gene expression is regulated by a complex upstream locus control region (LCR), which involves regulation of chromatin domains and determines somatotroph-specific hGH expression (Cattini *et al.* 2006; Ho *et al.* 2004; Shewchuk *et al.* 2001). In addition, the proximal promoter region of the *hGH-N* gene contains *cis*-elements mediating tissue-specific and hormone-specific controls: for example, the Pit1 response element confers pituitary specific expression of hGH genes, while the cAMP and glucocorticoid response elements mediate hGH transcription in response to differential hormonal signals (Cattini *et al.* 2006; Melmed & Kleinberg 2002; Casanueva 1992).

The pituitary *hGH-N* gene contains five exons separated by four introns. Alternative splicing of exon 3 leads to the formation of two different mRNAs which translate into two hGH isoforms secreted from the somatotroph cells (Boguszewski 2003). The full length 22kD form, composed of 191aa, is considered to be the classical hGH and is the most abundant isoform in the circulation (~85- 90% of total hGH). This predominant GH form is growth-promoting and influences energetic and anabolic lipolysis as well as other metabolic processes (Melmed & Kleinberg 2002). The 20kD form, which has 15 aa deleted (from 32 to 46), accounts for ~10-15% of pituitary GH. It binds equally well to the GH receptor as the 22kD GH and exerts lipolytic activities, but with less of an acute

insulin-like effect as well as less agonistic activity at the prolactin receptor (PRLR) (Boguszewski 2003).

In addition to the established production of hGH in the anterior pituitary, it has become apparent within the last two decades that synthesis of hGH can also occur at a number of extrapituitary sites, including the brain, immune system, placenta and mammary gland. This supports the newly accepted concept that, besides its classical endocrine actions, hGH also exerts important autocrine/paracrine effects (Perry *et al.* 2006; Harvey & Hull 1997; Liu *et al.* 1997).

1.2. Growth Hormone Secretion

Pituitary GH is secreted in a pulsatile nature characterized by intermittent bursts of secretory episodes throughout the day. The periodic pattern of GH release plays an important role in transmitting the GH message in a tissue-specific manner (Goldenberg & Barkan 2007). The number of spontaneous pulses ranges from 6 to 11 per day, with the majority of GH secreted at night, particularly after the onset of deep slow wave sleep (Casanueva 1992). The pulsatile pattern of GH secretion is regulated primarily by the two hypothalamic neuropeptides, GH-releasing hormone (GHRH) and Somatostatin (SS) (Goldenberg & Barkan 2007; Root & Diamond, Jr. 2000). The recently discovered endogenous GH secretagogue (GHS), ghrelin, may also play a minor role (Goldenberg & Barkan 2007). GH itself and the end product of GH actions, Insulin-like growth factor (IGF)-I, exert negative feedback effects on GH secretion. Additionally, the amount of GH secreted and its releasing pattern is subject to both hormonal and nutritional regulations (Goldenberg & Barkan 2007; Meinhardt & Ho 2006; Root & Diamond, Jr. 2000). All of these factors interact with each other in a precise and coordinated manner and the interplay between them is complex (Goldenberg & Barkan 2007) (**Figure I-1**).

Figure I-1: Schematic of the physiological regulation of growth hormone (GH) secretion.

Pituitary GH secretion is regulated primarily by the two hypothalamic neuropeptides, GH-releasing hormone (GHRH) and somatostatin (SS). The GH secretagogues (GHSs), including the recently discovered endogenous ligand, ghrelin, may also play a role via action on the pituitary somatotrope and within the hypothalamus. GH itself and the end product of GH actions, Insulin-like growth factor (IGF)-I, exert negative feedback effects on GH secretion. Additionally, the amount of GH secreted and its releasing pattern is subject to both stimulatory and inhibitory regulations by hormonal, neural and nutritional signals. All of these factors interact with each other in a precise, coordinated and complex manner (Goldenberg & Barkan 2007; Melmed & Kleinberg 2002; Root & Diamond, Jr. 2000).

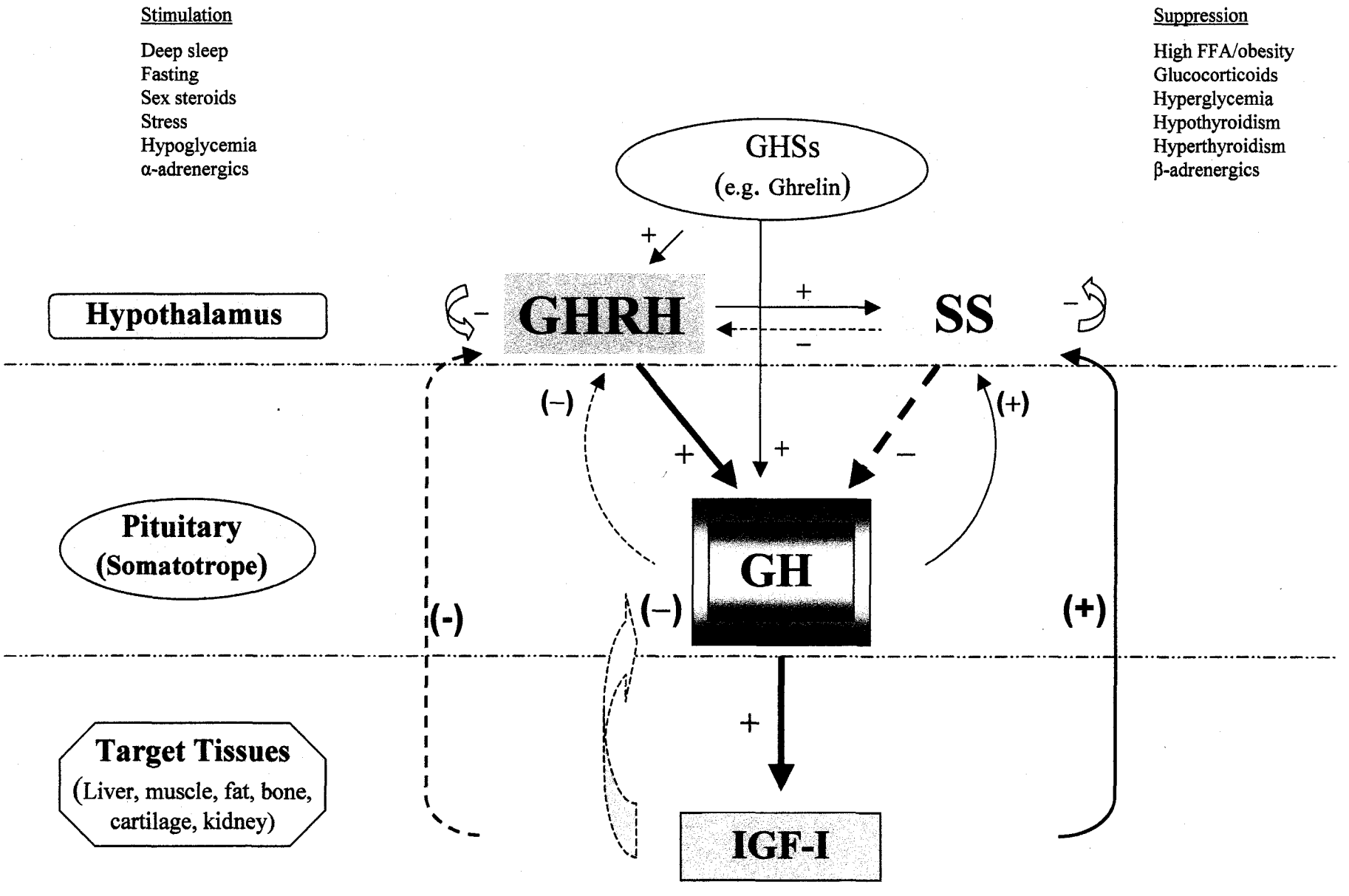

Stimulation
Deep sleep
Fasting
Sex steroids
Stress
Hypoglycemia
α-adrenergics
Suppression
High FFA/obesity
Glucocorticoids
Hyperglycemia
Hypothyroidism
Hyperthyroidism
β-adrenergics
GHSs
(e.g. Ghrelin)
Hypothalamus
GHRH
SS
Pituitary
(Somatotrope)
GH
Target Tissues
(Liver, muscle, fat, bone, cartilage, kidney)
IGF-I

Figure I-1

1.2.1. Role of GHRH and Somatostatin in the Integrated Control of GH Secretion

GHRH, produced from the hypothalamic arcuate and ventromedial nuclei, reaches the anterior pituitary via the long portal vessels. Through interaction with its specific Gs-coupled receptors expressed on the surface of somatotroph cells, GHRH activates the adenylyl cyclase-cAMP-protein kinase A (PKA) signaling pathway to induce GH gene transcription and increase GH synthesis. In addition, GHRH increases somatotroph cytosolic Ca^{++} levels by extracellular Ca^{++} influx and, thus, stimulates GH secretion as well (Root & Diamond, Jr. 2000). GHRH is crucial for the generation of acute responses to a variety of pharmacologic stimuli, including hypoglycemia, arginine, clonidine, pyridostigmine and levadopa (Goldenberg & Barkan 2007). Chronic GHRH stimulation, by either continuous infusion or repeated bolus administration, results in desensitization of GH release both *in vitro* and *in vivo*, possibly by decreasing GHRH receptor (GHRHR) expression on the surface of somatotroph cells (Melmed & Kleinberg 2002).

Somatostatin, a 14-amino acid peptide, is the major negative regulator of GH secretion (Goldenberg & Barkan 2007). It is synthesized in the hypothalamic arcuate and periventricular nuclei and acts through a family of Gαi-coupled G-protein receptors, mainly the subtype 2 and 5 receptors, to mediate inhibition of pituitary GH secretion. The interaction of somatostatin with its receptor inhibits adenylyl cyclase activity and "opens" K+ channels, thus increasing cytosolic K+ levels and inhibiting Ca+ inflow, which consequently reduces GH release from the somatotroph cells (Root & Diamond, Jr. 2000). Somatostatin powerfully antagonizes the mitogenic effect of GHRH on somatotrophs, but does not inhibit GH synthesis. It suppresses the frequency and amplitude of spontaneous GH release and GH responses to all stimuli tested: GHRH, hypoglycemia, arginine, exercise, etc. (Goldenberg & Barkan 2007).

Although GHRH and Somatostatin autoregulate their own and each other's secretion, their secretion is primarily regulated by multiple neurotransmitters and neuromodulators, and by feedback from GH and IGF-I as well as by other peripheral signals, such as hormones and metabolic status of the subject (**Figure I-1**) (Goldenberg & Barkan 2007; Root & Diamond, Jr. 2000). It was hypothesized that the periodic, pulsatile GH release *in vivo* is a coordinated result of simultaneous decrease of hypothalamic somatostatin

release and an increase of GHRH release (Hartman *et al.* 1993; Tannenbaum 1993; Hartman *et al.* 1991). However, direct measurements of SS in the pituitary-portal circulation in conscious sheep failed to disclose any relations between SS and GH pulses (Cataldi *et al.* 1994). Therefore, endogenous GHRH is viewed as the principal regulator of pulsatile GH secretion in humans (Goldenberg & Barkan 2007).

1.2.2. Regulation of GH Secretion by GH Secretagogues (GHS) and Ghrelin

Synthetic hexapeptides with potent and relatively specific GH-releasing activity are termed GH secretagogues (GHSs) (Reiter & Rosenfeld 2002). These GH releasing peptides (GHRP) stimulate GH release directly from the pituitary and indirectly by enhancing GHRH release from the hypothalamus. Their GH-releasing action appears to be mediated through binding to Gq (Gα11) -coupled cell membrane receptors and the activation of phospholipase Cβ to produce the second messenger, phosphoinositide triphosphate (IP_3). Increased cytoplasmic levels of IP3 lead to mobilization of Ca^{++} from the endoplasmic reticulum (ER) and to inhibition of K^+ influx, ultimately raising cytosolic Ca^{++} concentrations and facilitating pituitary GH secretion (Root & Diamond, Jr. 2000; Casanueva & Dieguez 1999). GHS appear to act in concert with GHRH in stimulating of GH secretion, as co-administration of GHS and GHRH results in a GH rise that is greater than the effect of either peptide administered alone. GHS act directly at the pituitary level, but are more powerful when applied to combined hypothalamic-pituitary segments *in vitro* or in intact animals, suggesting that an intact GHRH signaling system is essential for their full action (Goldenberg & Barkan 2007; Pandya *et al.* 1998).

Ghrelin was identified as the natural ligand for the GHS receptor by Kojima and co-workers (Kojima *et al.* 1999). It is a 28-aa peptide of a primarily gastric origin (Date *et al.* 2000), although *ghrelin* mRNA is also found in the hypothalamus, heart, lung and adipose tissue (Reiter & Rosenfeld 2002). It circulates in two forms; the biologically active octanoylated one and the inactive deoctanoylated form. Administration of ghrelin not only raises plasma GH concentrations but also stimulates food intake and induces obesity. Most of the information regarding the GH-promoting effects of ghrelin has been obtained with its synthetic analog. It has not been proven to be a major regulator of GH synthesis or secretion in humans (Goldenberg & Barkan 2007).

1.2.3. Feedback Regulation of GH Secretion

GH secretion is negatively regulated by itself and its target tissue mediator, IGF-I, both of which participate in a hypothalamic-pituitary feedback regulatory system (Goldenberg & Barkan 2007; Meinhardt & Ho 2006; Melmed & Kleinberg 2002). In normal subjects, a GH injection reduces the subsequent GH secretory response to a GHRH stimulus. Clinical experiments suggest that GH autonegative feedback is mainly mediated through stimulating hypothalamic somatostatin release (Giustina & Veldhuis 1998). GH stimulates IGF-I production in target tissues, especially the liver. IGF-I then exerts a negative feedback effect on GH secretion by stimulating hypothalamic somatostatin release and by suppressing pituitary GH gene transcription and secretion (Meinhardt & Ho 2006; Melmed & Kleinberg 2002).

1.2.4 Regulation of GH Secretion by Peripheral Signals

1.2.4.1 Hormones

A variety of nonpeptide hormones, including thyroid hormone, glucocorticoids and sex steroids, also influence GH secretion. The mechanisms by which these hormones regulate GH secretion may take place at both hypothalamic and pituitary sites (Reiter & Rosenfeld 2002). In humans, hypothyroidism is associated with low GH basal levels and decreased GHRH-stimulated GH secretion, and these defects can be normalized by thyroxine (T4) replacement (Giustina & Veldhuis 1998; Casanueva 1992). However, human GH gene transcription is not induced by thyroid hormone in *in vitro* studies, and is even downregulated by the addition of triiodothyronine (T3). On the other hand, blunted GH release in response to both hypothalamic and pituitary stimuli has also been reported for hyperthyroidism. Therefore, both hypo- and hyper-thyroidism can lead to a reduced GH secretion, but may be working through different mechanisms (Casanueva 1992).

Glucocorticoids also exert opposing effects on GH secretion (Root & Diamond, Jr. 2000). Administration of an acute, low dose of glucocorticoids stimulates GH secretion. This effect takes 3 hours to become evident and GH levels remain elevated for up to 2 hours (Casanueva 1992). In contrast, chronic administration of supraphysiologic amounts of glucocorticoids inhibits GH secretion (Root & Diamond, Jr. 2000). Human subjects

undergoing long-term steroid treatment or exposure show blunted GH secretion in the face of classic GH stimuli. One example is Cushing's disease, which is associated with growth retardation, reduced serum GH and reduced pituitary GH content. Such inhibitory effects appear to be dependent on an increase of hypothalamic somatostatin discharge (Root & Diamond, Jr. 2000; Casanueva 1992).

Gonadal sex hormones play an important role in the neuroregulation of GH secretion. Both estrogens and testosterone drive GH secretion during puberty in boys and girls. Hypogonadal children have reduced GH secretion, while precocious purberty is linked to increased GH secretion, and successful therapy of either normalizes GH secretion (Meinhardt & Ho 2006). The stimulatory effects of sex hormones appear to result from action at both the hypothalamic and pituitary levels to increase the transcription rates for GHRH and GH, respectively (Root & Diamond, Jr. 2000). Estrogen stimulates GH secretory rates, while testosterone increases GH secretory mass per pulse (Veldhuis *et al.* 2005a; Giustina & Veldhuis 1998).

1.2.4.2 Nutritional and metabolic statuses

GH is an important regulator of metabolism. In turn, GH secretion is profoundly altered in various metabolic disease states such as malnutrition, fasting, obesity and diabetes mellitus (Goldenberg & Barkan 2007). Prolonged fasting and malnutrition are associated with elevated GH secretion frequency and amplitude. In contrast, obesity results in reduced basal and stimulated GH secretion (Melmed & Kleinberg 2002). Hypoglycemia is one of the most potent stimuli of GH secretion in humans (Casanueva 1992), while hyperglycemia inhibits GH secretion. However, chronic hyperglycemia does not appear to link to low GH levels, and increased basal and exercise-induced GH secretion have been seen in poorly controlled diabetics (Melmed & Kleinberg 2002). High-protein meals or intravenous infusions, which lead to an increase in the blood level of amino acids, can cause a GH discharge (Melmed & Kleinberg 2002). Free fatty acids (FFA) may well participate in the regulation of GH secretion (Giustina & Veldhuis 1998). Increased serum FFA blunt GH secretion provoked by virtually all stimuli; conversely, GH secretion is increased when FFA levels are reduced. The drug, acipimox, which

suppresses plasma concentration of FFA by blocking their release from adipose tissue, concomitantly enhances both basal and GHRH-stimulated GH secretion in both normal and obese subjects (Cordido *et al.* 1996; Peino *et al.* 1996; Pontiroli *et al.* 1990). The FFA inhibitory effect on GH secretion may reflect a direct feedback on the pituitary (Goldenberg & Barkan 2007).

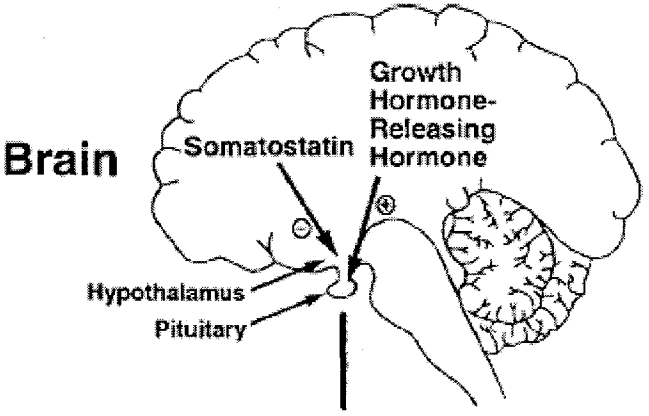
1.3. Physiologic Effects of GH

1.3.1. Pleiotropic Actions of GH at Multiple Sites

GH is the major regulator of postnatal body growth. It is also an important metabolic hormone with a variety of metabolic actions that persist throughout adult life (Reiter & Rosenfeld 2002; Waters *et al.* 1999).The pleiotropic actions of GH involve multiple target tissues and physiological systems (**Figure I-2**) (Le Roith *et al.* 2001; Carrel & Allen 2000). Briefly, in skeletal bone, GH promotes epiphyseal growth and stimulates longitudinal bone growth. In addition, GH influences bone metabolism and remodeling through stimulating osteoclast differentiation and activity, promoting osteoblast activity and increasing bone mass by endochondral bone formation (Reiter & Rosenfeld 2002; Carrel & Allen 2000). In liver, GH stimulates glucose transport and increases hepatic gluconeogenesis and glycogenolysis to enhance glucose output (Holt *et al.* 2003). In skeletal muscle, GH enhances amino acid uptake, inducing positive nitrogen balance and protein synthesis, thus increasing lean body mass and energy expenditure (Le Roith *et al.* 2001; Carrel & Allen 2000). In adipose tissue, GH decreases fat deposition and increases fat mobilization by increasing lipolysis through the stimulation of triglyceride into free fatty acids and glycerol as well as by decreasing fatty acid reesterification (Reiter & Rosenfeld 2002; Melmed & Kleinberg 2002; Carrel & Allen 2000). Sections 1.3.3 and 1.3.4 will describe in detail the growth promoting effect of GH and the metabolic actions of GH on adipose tissue, respectively.

Figure I-2: Major sites of GH action.

GH, secreted from anterior pituitary, exerts pleiotropic actions on multiple target tissues. Figure modified from Carrel A.L. and Allen D.B. (2000) *Endocrine* **12**, 163-172.



Bone

•Promotes epiphyseal growth

• Stimulates longitudinal bone growth

• Increases osteoclast differentiation and activity

• Increases osteoblast activity

• Increases bone mass by endochondral bone formation

Liver

•Stimulates glucose transport

• Increases gluconeogenesis & glycogenolysis

• Enhances glucose output

Adipose Tissue

• Increases lipolysis by stimulating hormone sensitive lipase (HSL)

• Decreases lipogenesis by inhibiting lipoprotein lipase (LPL)

• Decreases fatty acid reesterificaiton

• Decreases glucose transport

Muscle

• Increases amino acid uptake

• Increases nitrogen retention

• Increases protein synthesis

• Increases lean body mass and energy expenditure

Figure I-2

1.3.2. GH Actions: Direct and Indirect Mechanisms

The physiologic effects of GH occur through both direct and indirect mechanisms (Reiter & Rosenfeld 2002; Le Roith *et al.* 2001; Carrel & Allen 2000).

(A) *Indirect mechanisms*: In general, the growth-promoting effects of GH appear to be mediated through the generation of the target tissue growth factor, IGF-I (Le Roith *et al.* 2001). IGF-I, also known as somatomedin C, is a member of the IGF growth factor family that includes IGF-I and IGF-II (Le Roith *et al.* 2001). IGF-I shares structural homology with insulin and functions through a specific cell-surface receptor that is structurally very similar to the insulin receptor. Therefore, IGF-I shares many biological properties with insulin and elicits both growth-promoting and insulin-like effects (Corpas *et al.* 1993). IGF-I is generated in response to GH and mediates many of the mitogenic and anabolic effects of GH. Initially, the liver was thought to be the only site for GH-induced IGF-I production (Salmon, Jr. & Ughday 1957), but later studies demonstrated that many tissues in the body are able to synthesize IGF-I in response to GH exposure (D'Ercole *et al.* 1984). It is now accepted that IGF-I mediates GH actions through a dual system: (1) circulating IGF-I mainly produced by the liver acts by an endocrine mechanism, and (2) locally produced IGF-I acts through autocrine or paracrine mechanisms (Le Roith *et al.* 2001; Casanueva 1992). Circulating IGF-I is bound by a number of high affinity binding proteins, IGF-binding proteins (IGFBPs)-1 through 6. These binding proteins transport IGF-I in the circulation to target tissues and protect IGF-I from degradation, thus prolonging its half-life. IGFBP-3 is the predominant circulating binding protein in adult serum and functions as a reservoir for IGF-I through the formation of a 150kD protein complex which contains 70-95% of the circulating IGF-I in human serum. IGFBP-1 and IGFBP-2 carry a minor fraction of IGF-I, and appear to inhibit IGF-I action (Le Roith *et al.* 2001; Spagnoli & Rosenfeld 1996; Corpas *et al.* 1993; Casanueva 1992).

(B) *Direct mechanism*: Studies of GH reveal that some important metabolic actions of GH occur independently of IGF-I production (for example: enhancement of lipolysis, stimulation of amino acid transport in diaphragm and heart, and increase of liver protein synthesis) (Reiter & Rosenfeld 2002). These actions are sometimes even opposing to

IGF-I activity, such as when GH increases glucose levels and causes diabetogenic effects, while IGF-I reduces glucose levels like insulin (Reiter & Rosenfeld 2002; Carrel & Allen 2000).

1.3.3. GH Actions on Postnatal Body Growth

Longitudinal bone growth is achieved by a process termed "endochondral ossification", which is a complex cellular process involving the differentiation and proliferation of chondrocytes at the growth plate (Spagnoli & Rosenfeld 1996). The epiphyseal growth plate is a transverse band that separates the epiphyseal and the diaphyseal portions of bone. It can be divided into three zones: the germinal zone, the proliferative zone and the hypertrophic zone (Ohlsson *et al.* 1998). In the process of bone growth, fibroblast-like precursor cells (prechondrocytes) in the germinal cell layer first differentiate into chondrocytes, which then enter into the proliferating layer and undergo several rounds of clonal expansion. Subsequently, cells enter into the hypertrophic cell layer, mature and degenerate, and eventually become ossified (Ohlsson *et al.* 1998; Casanueva 1992). Several different hormones and growth factors are involved in the regulation of this ordered proliferation and maturation process; however, it is generally accepted that GH plays a very important role (Ohlsson *et al.* 1998).

Although the growth-promoting effect of GH is beyond doubt, the mechanisms and sites of action have been a matter of debate. For many years, the central dogma of GH's growth-promoting action was based on the somatomedin hypothesis, which asserted that pituitary GH activated the growth hormone receptor (GHR) in the liver and stimulated liver production of somatomedin C, now known as IGF-I. IGF-I was then transported to the growth plate and stimulated longitudinal bone growth in an endocrine manner (Daughaday & Rotwein 1989; Daughaday *et al.* 1972; Daughaday & Reeder 1966). In the early 1980s, the somatomedin hypothesis was challenged by Isaksson and coworkers, whose studies demonstrated that direct injection of GH into the cartilage growth plate of hypophysectomized rats accelerated longitudinal bone growth at the site of injection (Isaksson *et al.* 1982). They concluded that GH stimulates the epiphyseal growth plate directly and that the circulating IGF-I is not required for stimulation of longitudinal bone

growth. Their theory was modified later by Russel and Spencer (Russell & Spencer 1985), who demonstrated that both GH and IGF-I have direct growth-promoting effects on cartilage *in vivo* and suggested that GH may act by stimulating local IGF-I synthesis. This concept was further supported by the observations that GH administration could induce IGF-I production at the cartilage level (Nilsson *et al.* 1986) and that the direct stimulatory effects on bone growth by a local injection of GH were completely abolished with concurrent administration of IGF-I antiserum (Schlechter *et al.* 1986). It is now accepted that GH stimulates long bone growth by inducing local production of IGF-I at the epiphyseal level, IGF-I then in turn stimulates cell proliferation in an autocrine or paracrine fashion (Butler & Le Roith 2001; Le Roith *et al.* 2001; Spagnoli & Rosenfeld 1996), though the circulating IGF-I may also play a role. Using *in vitro* cultured growth plate chondrocytes, both GH and IGF-I have been shown to stimulate chondrocyte colony formations. However, GH selectively promotes the formation of large chondrocyte colonies, whereas IGF-I preferentially stimulates the formation of small and middle-size colonies (Lindahl *et al.* 1987a; Lindahl *et al.* 1987b; Lindahl *et al.* 1987c; Lindahl *et al.* 1986). Similar phenomena were observed in *in vivo* experiments (Ohlsson *et al.* 1992).

These data led both the Green and Isaksson groups to develop the "dual effector theory" (Isaksson *et al.* 1987; Green *et al.* 1985),which proposes dual actions for GH: (1) GH acts directly at the growth plate to induce differentiation of prechondrocytes to chondrocytes; and (2) GH acts to stimulate local synthesis and secretion of IGF-I, which then promotes clonal expansion of differentiated chondrocytes via autocrine/paracrine mechanisms and, consequently, leads to long bone growth. However, the validity of the dual effector theory has also been questioned, as recent experimental evidence and clinical observations show different findings (Le Roith *et al.* 2001; Spagnoli & Rosenfeld 1996). Current data suggest that the situation is more complex than any of the previous hypotheses envisioned. In addition to stimulating liver IGF-I synthesis, GH also stimulates hepatic IGFBP-3 production and promotes the formation of the ternary IGF binding complex that stabilizes IGF-I in circulation, thus regulating the bioavailability of IGF-I. Although it is hard to precisely distinguish the endocrine effects vs. local paracrine/autocrine effects on somatic growth, there is sufficient evidence to support a

role for both systems in normal postnatal linear growth (Le Roith *et al.* 2001) (**Figure I-3**).

Figure I-3: The stages of evolution of the somatomedin hypothesis.

A) The original somatomedin hypothesis postulated that somatic growth was regulated by GH-dependent liver IGF-I production, which acted in an endocrine fashion on skeletal bone to promote linear growth. B) The dual effector theory proposed that GH has direct effects (independent of IGF-I) on the epiphyseal growth plate as well as indirect effects through GH-stimulated local IGF-I that acts in an autocrine/paracrine manner. C) Current data suggest that the situation is more complex than either of the two previous hypotheses envisioned. GH, in addition to stimulating hepatic and growth plate IGF-I production, also stimulates the formation of a ternary binding complex, including IGF-I, IGFBP-3 and ALS, which helps to stabilize IGF-I in the serum. While it is not clear the precise contribution to somatic growth from the endocrine and autocrine/paracrine effects of IGF-I, there is sufficient evidence to support a role for both systems in normal postnatal growth (Le Roith *et al.* 2001). Figure is reproduced with permission from Le Roith D. *et al.*, (2001). *Endo Rev.*, **22**, 53-74.

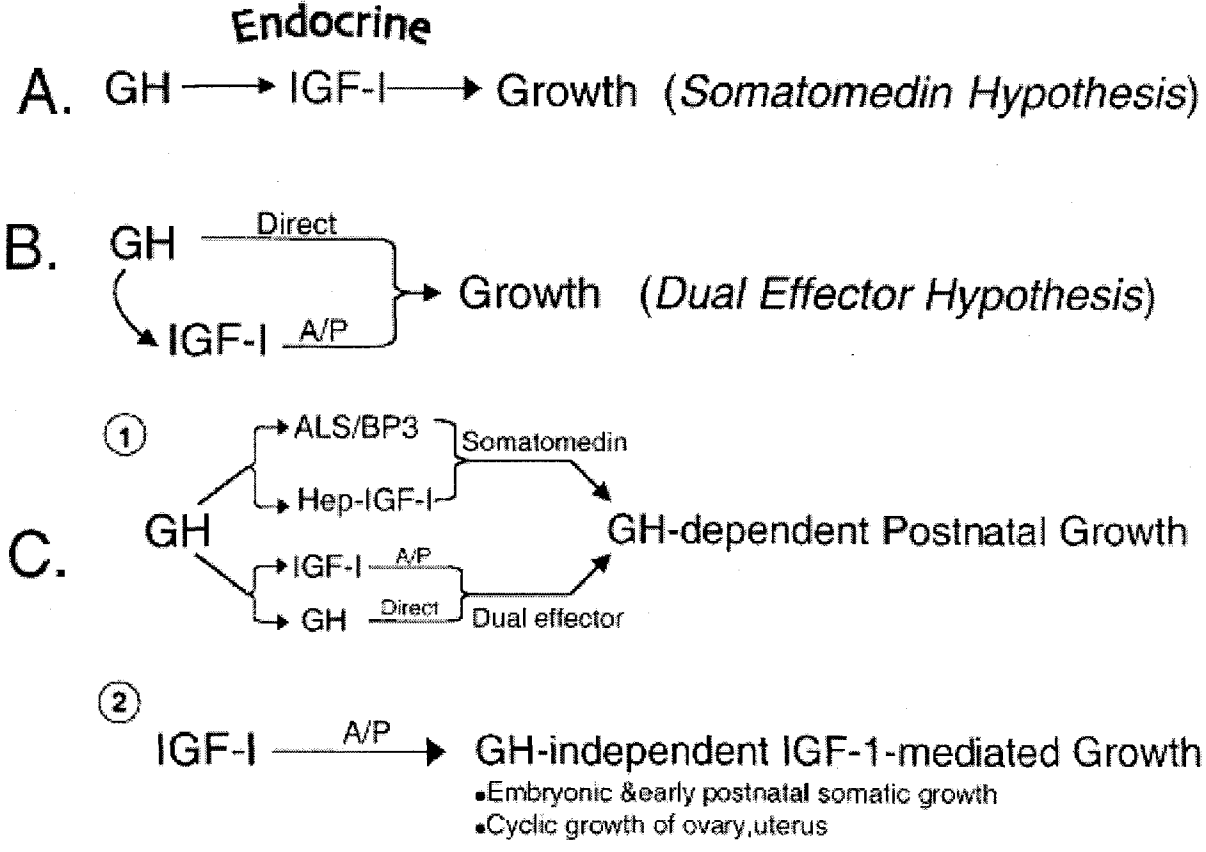

Endocrine
A. GH → IGF-I → Growth (Somatomedin Hypothesis)
B. GH
Direct
IGF-I
A/P
Growth (Dual Effector Hypothesis)
C.
1
ALS/BP3
Hep-IGF-I
Somatomedin
GH
IGF-I
A/P
GH
Direct
Dual effector
GH-dependent Postnatal Growth
2
IGF-I
A/P
GH-independent IGF-1-mediated Growth
•Embryonic &early postnatal somatic growth
•Cyclic growth of ovary,uterus

Figure I-3

1.3.4. GH Actions on Metabolism and Body Composition

GH exerts profound effects on carbohydrate, protein and lipid metabolism in multiple target organs (Carrel & Allen 2000; Davidson 1987), as briefly summarized in section 1.3.1. The combination of anabolic effects on lean body tissue and lipolytic effects in adipose tissue results in a substantial influence of GH on body composition. The following section will discuss the metabolic action of GH on adipose tissue, as the major focus of this PhD project is about growth hormone receptor gene expression in adipocytes.

1.3.4.1. GH effects on fat metabolism and body composition

Since Lee and Schaeffer first suggested lipolytic activity for GH approximately 70 years ago, on the basis of their observation that prolonged treatment of rats with growth-promoting pituitary extracts resulted in a decrease in carcass fat (Lee & Shaffer 1934), it is evident today that GH has several metabolic actions on adipose tissue, including the lipolytic effect (Wabitsch *et al.* 1994). Various experimental observations and clinical studies led to the identification of adipose tissue as a major target organ for GH actions and provided compelling evidence that GH has diverse and incompletely understood effects, influencing both the development and metabolism of adipocytes (Carrel & Allen 2000; Nam & Marcus 2000; Nam & Lobie 2000; Wabitsch *et al.* 1995; Wabitsch *et al.* 1994). Hypophysectomy in rats results in an increase of body fat and a decrease of lean body mass, and such changes can be normalized by GH administration. Similarly, transgenic expression of an inactive GH gene in mice causes an obese phenotype (Flint *et al.* 2003; Nam & Lobie 2000). GH-deficient children are generally mildly obese, with increased fat volume and reduced fat cell numbers as compared to normal children. Treatment with hGH leads to a reduction in body fat and normalization of the fat cell numbers in these children (Bonnet *et al.* 1974). GH deficient adults also exhibit abnormal body composition, with higher body fat mass and lower lean body mass (Rosen *et al.* 1993; Salomon *et al.* 1989), and hGH therapy results in a decrease in body fat and an improvement in body mass index (Bengtsson *et al.* 1993; Zachmann *et al.* 1980). In addition, certain recent reports showed that GH treatment of obese individuals could cause a reduction of body fat mass (Veldhuis *et al.* 2005b; Nam & Lobie 2000). GH

appears to mediate its fat-reducing effects through two major mechanisms: an enhancement of lipolytic activity via stimulation of hormone sensitive lipase (HSL) and a decrease in triglyceride accumulation through inhibition of lipoprotein lipase (LPL) activity (Nam & Marcus 2000; Nam & Lobie 2000; Richelsen 1997). GH administration typically does not cause loss of body weight but rather causes a change in body composition mainly by increasing fat mobilization and oxidation as well as increasing protein synthesis (Mauras & Haymond 2005).

1.3.4.2. Metabolic effects of GH on adipocytes

The metabolic effects of GH on adipose tissue have been extensively studied at the cellular level. Two opposing effects on glucose and lipid metabolism have been documented, the acute insulin-like effect and the chronic anti-insulin or diabetogenic effect (Nam & Marcus 2000; Nam & Lobie 2000; Richelsen 1997; Wabitsch *et al.* 1994).

(A) *Acute metabolic effects*

The acute effects of GH on adipocytes include the stimulation of glucose uptake, increased conversion of glucose to glycogen, increased lipogenesis and inhibition of noradrenaline-induced lipolysis (Nam & Lobie 2000; Wabitsch *et al.* 1994). These insulin-like actions are only observed during *in vitro* incubations of GH with mature fat cells or adipose tissues that have not previously been exposed to GH (e.g. from hypophysectomized rats) or that have been kept under GH-free conditions for 4 hours. It appears immediately after GH administration and has a duration of nearly 2 hours. Once the initial insulin-like effect has dissipated, a second similar response cannot be initiated even with very high concentrations of GH.

The mechanism responsible for the acute effects is not completely understood. It has been suggested that GH may elicit these effects by sharing a common downstream signaling pathway with insulin through phosphorylation of insulin receptor substrate (IRS)-1 or -2 and subsequent activation of PI3-kinase (Ridderstrale & Tornqvist 1996). However, the acute lipogenic effect of GH needs tyrosine residues 333 and 338 on the intracellular domain of GHR (Lobie *et al.* 1995), whereas these two residues are not required for GH-dependent tyrosine phosphorylation of IRS-1 or IRS-2 (Vanderkuur *et al.* 1995).

Therefore, the acute effect of GH in adipocytes may not be mediated by simple phosphorylation of IRS-1 or IRS-2, and GH may use other molecules (such as FAK-tensin or c-Cbl) to activate PI3-kinase, thus promoting glucose uptake and lipid synthesis (Nam & Lobie 2000). The metabolic significance of GH's insulin-like action is not clear, but as this action can be abolished by prior GH administration, it is not considered to be important for normal physiology (Casanueva 1992).

(B) *Chronic Metabolic Effects*

Prolonged *in vitro* incubation of adipose tissue or isolated adipocytes with GH or *in vivo* treatment with GH results in an increased lipolysis and a reduced lipogenesis as well as a marked decrease in insulin-regulated events, such as glucose transport, glucose oxidation and lipogenic enzyme activities (Louveau & Gondret 2004; Nam & Lobie 2000; Wabitsch *et al.* 1994). These anti-insulin-like effects lead to a reduction of the lipid content in the fat cells and a subsequent decrease in body fat mass, although hyperglycemia and certain insulin resistance effects are also seen.

(i) Inhibition of lipogenesis

The primary function of adipose tissue is to store energy by accumulating triglycerides (TG) during excessive energy intake and to release energy during a fasting state (Louveau & Gondret 2004). Because of the limited ability of adipose tissue for *de novo* synthesis of FFA, the accumulation of TG in adipose tissue is dependent on lipoprotein lipase (LPL) (Carrel & Allen 2000). LPL is synthesized by adipocytes, secreted to the intracellular space and attached to the luminal portion of the blood vessels supplying the adipocytes. There it hydrolyzes TG from circulating very-low-density-lipoprotein (VLDL) and chylomicron particles and releases FFA. GH produces a pronounced inhibitory effect on adipose LPL activity (Ottosson *et al.* 2000; Richelsen *et al.* 1994), thus reducing FFA that can be taken up by adipocytes for the generation of lipid and, ultimately, reducing fat accumulation. This inhibitory effect does not seem to happen at the gene expression level, and may be due to post-translational modification of enzyme activity (Richelsen 1999). The GH-induced suppression of LPL activity differs in individual adipose depots, which could be partially responsible for the more pronounced

reduction observed in intra-abdominal fat depots than in subcutaneous fat after GH treatment (Nam & Marcus 2000).

(ii) Stimulation of lipolysis

The lipolytic effect of GH on adipose tissue has been well-established from both *in vivo* and *in vitro* studies (Wabitsch *et al.* 1994). The lipolytic action of GH exhibits a slow onset and a relatively lower magnitude in comparison with the effects of the well-characterized lipolytic agent, catecholamines. For example, epinephrine dramatically increases lipid breakdown within a few seconds (Wabitsch *et al.* 1994), whereas the lipolytic effect of GH is observed after a 1-2 hour delay and can be blocked by puromycin or actinomycin D. This observation led to the suggestion that the lipolytic activity of GH is indirect and is dependent on RNA and protein synthesis (Fain 1967). Indeed, Fain *et al.* observed that no significant lipolytic action of GH was seen in adipose tissue from STAT5a$^{-/-}$b$^{-/-}$female mice and suggested that the lipolytic actions of GH involves STAT5 mediated transcription (Fain *et al.* 1999). These effects appear to be the direct action of GH instead of indirect effects mediated through IGF-I production (Wabitsch *et al.* 1995).

One major mechanism by which GH promotes lipolysis is through enhancing hormone sensitive lipase (HSL) activity (Nam & Marcus 2000; Nam & Lobie 2000). HSL is the rate-limiting enzyme in charge of hydrolysis of adipocyte-stored TG into FFA and glycerol. FFA then can leave the adipocytes and travel in blood to other tissues where they can be used as fuel. HSL is primarily regulated by the cAMP-mediated phosphorylation pathway: for example, catecholamines, via the β-adrenergic receptor, activate and stimulate lipolysis, while other compounds, including prostaglandins, inhibit lipolysis by this mechanism (Carrel & Allen 2000). The mechanism by which GH stimulates HSL activity is not completely clear yet. It appears to be indirect, through up-regulation of cell surface β-adrenergic receptor numbers and/or through suppression of phosphodiesterase activity to prolong cAMP-signaling, thus ultimately amplifying HSL activity (Osafo *et al.* 2005; Nam & Marcus 2000). The other related mechanism by which GH increases lipolysis is by reducing the sensitivity and response of adipocytes to

antilipolytic factors, such as adenosine or prostaglandin E, which act through Gi-coupled receptors to inhibit HSL and lipolysis. GH may alter the actions of these antilipolytic factors via decreasing the abundance of inhibitory G proteins (Gi α-subunit) or via interfering with the interaction of Gi subunits with adenylate cyclase (Carrel & Allen 2000; Nam & Lobie 2000).

(iii) Other anti-insulin like effects

Besides stimulation of lipolysis and inhibition of fat accumulation, GH treatment *in vivo* or longer exposure of GH *in vitro* also induces inhibition of glucose metabolism, characterized by decreased glucose transport and glucose oxidation (Nam & Marcus 2000; Wabitsch *et al.* 1994). Both basal and insulin stimulated glucose transport have been demonstrated to be suppressed after chronic GH treatment (Richelsen 1997). GH may, by this way, limit the availability of substrate (α-glycerol-phosphate) for TG synthesis (Goodman *et al.* 1990). The mechanism by which GH reduces glucose uptake in adipocytes is likely through transcriptional down-regulation of GLUT1, which in turn reduces the cellular level of GLUT1 glucose transporter. In contrast, the RNA and protein levels of the GLUT4 glucose transporter do not seem to be affected by chronic GH exposure (Tai *et al.* 1990).

Although the chronic inhibitory effect of GH on both basal and insulin stimulated lipogenesis has been observed in sheep adipose tissue (Borland *et al.* 1994), this inhibition exhibits species specificity, as no effect was observed on basal or insulin-stimulated lipogenesis after GH treatment of prepubertal severely obese children (Kamel *et al.* 2000).

1.3.5. GH Effects on Preadipocyte Differentiation (Adipogenesis)

Unlike other tissues, adipose tissue mass has considerable capacity to expand. At the cellular level, the development and growth of adipose tissue results from both the proliferation and differentiation of adipocyte precursor cells (increase in fat cell number) and subsequent enlargement of mature adipocytes (increase in fat cell size). These processes are regulated by a variety of hormones and growth factors (Bluher *et al.* 2005; Louveau & Gondret 2004). Studies of GH effects on adipose tissue have indicated that

GH, besides affecting the metabolic function of mature adipocytes (as described above), is also involved in the regulation of adipocyte numbers and differentiation (Bluher *et al.* 2005; Carrel & Allen 2000; Nam & Lobie 2000).

1.3.5.1. Preadipocyte differentiation or adipogenesis

Fat cells are generated by a process called adipogenesis, whereby preadipocytes, fibroblast-like precursor cells that are derived from mesenchymal multipotential precursors but have committed to the adipocyte lineage, undergo a series of morphological and biochemical changes to eventually develop into lipid-filled functioning mature adipocytes (Bluher *et al.* 2005; Louveau & Gondret 2004; Tong & Hotamisligil 2001). Two major differentiation models, such as established murine preadipocyte cell lines and primary cultures of adipose-derived stromal-vascular precursor cells, have been extensively used to study this process. These *in vitro* cellular models for adipogenesis have greatly advanced our understanding of the molecular basis of differentiation (Tong & Hotamisligil 2001). An overview of the stages of adipogenesis is illustrated in **Figure I-4**. This developmental program can be conveniently summarized in four distinct stages that include: (1) preconfluent proliferation; (2) confluent growth arrest; (3) clonal expansion; and (4) permanent growth arrest/terminal differentiation (Cowherd *et al.* 1999). Preadipocytes divide in the growth phase until they reach confluence and become growth arrested at G1/S phase. In order to undergo adipose conversion, committed preadipocytes have to withdraw from the cell cycle. Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals, which are derived *in vitro* from a standard adipogenic "cocktail" (including insulin, dexamethasone, isobuthylmethylxanthine (IBMX)). Post-confluent growth-arrested preadipocytes re-enter the cell cycle and undergo limited rounds of proliferation, leading to clonal expansion of the committed cells. Although the clonal expansion step has been viewed as the essential step for downstream differentiation, this concept has been questioned by recent observations (Gregoire 2001).

Figure I-4: Overview of the major phases in the development of adipocytes. Pluripotent stem cell precursors give rise to a mesenchymal progenitor cell with the potential to differentiate along mesodermal lineages to myoblasts, chondroblasts, osteoblasts and adipocytes. As described in the text, preadipocytes first proliferate to reach confluence. Exposure of growth-arrested, confluent preadipocytes to the appropriate differentiation inducers triggers several rounds of mitotic clonal expansion, activation of a large family of adipocyte genes, and subsequent terminal differentiation (Otto & Lane 2005; Gregoire *et al.* 1998). Selected molecular events and changes in gene expression accompanying this process are indicated to the right, with their approximate duration reflected by the solid line. PPARγ: peroxisome proliferator-activated receptor-γ; C/EBP: CCAAT enhancer binding protein; Pref-1: preadipocyte factor-1; ECM: extracellular matrix; FA: fatty acid.

Figure is reproduced with permission from Gregoire F. M. *et al.* (1998). *Physiol. Rev.* **78**, 783-801.

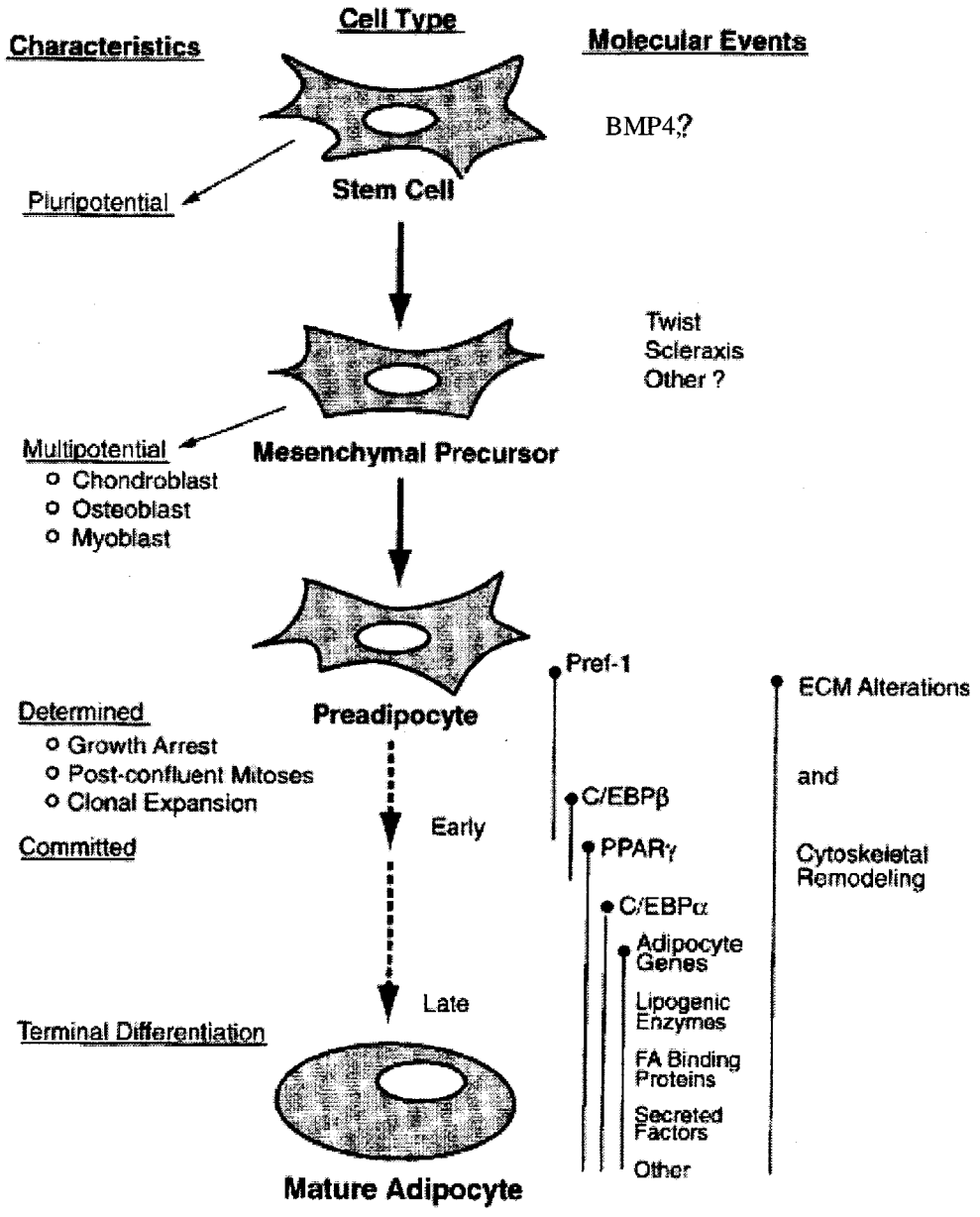

Cell Type
Characteristics
Molecular Events
BMP4?
Stem Cell
Pluripotential
Twist
Scleraxis
Other ?
Mesenchymal Precursor
Multipotential
Chondroblast
Osteoblast
Myoblast
Pref-1
Preadipocyte
Determined
Growth Arrest
Post-confluent Mitoses
Clonal Expansion
Committed
Early
C/EBPβ
PPARγ
C/EBPα
Late
Adipocyte Genes
Lipogenic Enzymes
FA Binding Proteins
Secreted Factors
Other
Terminal Differentiation
Mature Adipocyte
ECM Alterations
and
Cytoskeletal Remodeling

Figure I-4

The first hallmark of the differentiation process is a dramatic change in cell shape as cells convert from a fibroblast-like to a spherical appearance. These morphological modifications result from changes in extracellular matrix (ECM) and cytoskeletal components. Many changes occur at the gene expression level during this differentiation program. Various transcription factors are sequentially expressed and activated to control the adipogenesis process. The master regulator of adipocyte differentiation is peroxisome-proliferator-activated receptor γ (PPARγ). Activated by an agonist ligand, PPARγ stimulates the full differentiation program, including the morphological changes, expression of adipocyte-specific genes, and lipid accumulation. Several CCAAT/enhancer-binding proteins (C/EBPs) also play crucial roles during adipogenesis. C/EBP-β and C/EBP-δ are transiently induced in the early stage of adipocyte differentiation and drive PPARγ and C/EBP-α expression. PPARγ and C/EBPα then mutually activate and remain at high expression levels to activate many target genes to achieve terminal differentiation. Production of the appropriate ligand of PPARγ appears to be the limiting step in this transcriptional regulation cascade. Although the nature of the endogenous ligand for PPARγ and the mechanism for its production have not been completely clarified, another transcription factor, sterol-regulatory element binding protein-1c (SREBP-1c), also known as adipocyte-determination and differentiation factor-1 (ADD1), and is important in the regulation of cholesterol and fatty acids metabolism, has been suggested to play a critical regulatory role in the generation of the PPARγ ligand and early adipogenesis.

In addition to these transcriptional activators, certain signaling molecules are involved in regulation of adipogenesis. Pref-1 is an inhibitor of adipocyte differentiation; it is highly expressed in preadipocytes but is not detectable in mature adipocytes. Wnt signaling also appears to negatively regulate adipocyte differentiation, as constitutive expression of Wnt-1 in preadipocytes results in a failure to differentiate.

In the terminal differentiation phase, the expression levels (both mRNA and protein) and the activities of various enzymes involved in TG synthesis and degradation are enhanced. Glucose transporters and insulin receptor number also increase. Differentiated adipocytes

exhibit a marked increase in lipid accumulation and acquire enhanced insulin sensitivity. In addition, synthesis of adipocyte-specific markers (i.e. fatty acid binding protein, aP2) and adipocyte-secreted factors (e.g. leptin, adiponectin, resistin and angiotensinogen II) occurs as the triglyceride-filled, mature adipocytes finally form (Rosen & Spiegelman 2006; Otto & Lane 2005; Gregoire 2001; Gregoire *et al.* 1998).

1.3.5.2. Contradictory effects of GH on preadipocyte differentiation

GH has been demonstrated to elicit controversial effects on preadipocyte differentiation, depending on whether preadipocyte clonal cell lines or primary preadipocyte cultures are used (Nam & Marcus 2000; Nam & Lobie 2000).

(A) *Stimulatory effects of GH on differentiation of preadipocyte cell lines*

Extensive studies from several murine preadipocyte cell lines (such as 3T3-F442A, 3T3-L1 or OB1771) indicate a stimulatory action of GH on preadipocyte differentiation (Kawai *et al.* 2007; Nanbu-Wakao *et al.* 2002; Xu *et al.* 1995; Hauner & Loffler 1986; Doglio *et al.* 1986; Nixon & Green 1984; Morikawa *et al.* 1982). In 3T3-F442A cells, GH has been documented to promote adipocyte conversion first by inducing an antimitogenic state in the dividing preadipocytes: the cells are partially refractory to mitogenic stimuli and decrease proliferation (Corin *et al.* 1990; Guller *et al.* 1989b), thereby entering into a state of growth arrest which is the initial step for adipogenesis. In agreement with this hypothesis, GH does not induce an antimitogenic state in 3T3-C2 cells, a sister clone of 3T3 cells that lacks the capacity for adipose conversion (Corin *et al.* 1990; Guller *et al.* 1989b).

Subsequently, GH is thought to prime the post-confluent preadipocytes (ready to differentiate) to become more responsive to IGF-I and insulin, which subsequently promote clonal expansion and terminal differentiation (Zezulak & Green 1986). In addition, GH has been shown to modulate the composition of the cytoskeleton (i.e. increase tubulin and vinculin) and the extracellular matrix (i.e. decrease fibronectin), thus triggering morphological changes and promoting adipogenesis (Wabitsch *et al.* 1994; Guller *et al.* 1992; Guller *et al.* 1991; Guller *et al.* 1989a). These morphological modulation effects may be mediated by JAK2-dependent phosphorylation of focal adhesion kinase (FAK), which then functions as a link between GH and extracellular matrix signal transduction (Nam & Lobie 2000). Interestingly, the ability of GH to induce an antimitogenic state and to promote morphological change is specific to GH itself and is not mediated by IGF-I (Guller *et al.* 1992; Corin *et al.* 1990).

The intracellular signaling mechanism by which GH stimulates adipose differentiation in 3T3 preadipocyte cell lines has been the subject of much study. Using a two-phase protocol that allows the GH-priming effect to be separated from terminal differentiation, Yarwood *et al.* (Yarwood *et al.* 1999) demonstrated conclusively that GH-stimulated differentiation of 3T3-F442A preadipocytes depends on the JAK2-STAT5 signaling cascade. In contrast, activation of p42/p44 MAP-kinase and p70 S6 kinase is not necessary for the promotion of differentiation by GH, although these signals are required for GH-independent terminal differentiation. Shang and Waters showed that ectopic expression of a constitutively active STAT5A mutant is sufficient to replace the requirement for GH in adipogenesis of 3T3-F442A preadipocytes; they conclude that STAT5 is a critical factor in GH-induced adipogenesis (Shang & Waters 2003). A recent study in the 3T3-L1 cell model also elucidates a role for STAT5 in the stimulatory effect of GH on adipogenesis. It was shown that, during 3T3-L1 differentiation, GH acts through STAT5A and 5B to stimulate PPARγ expression and to enhance the transcriptional activity of PPARγ, thus promoting PPARγ-induced adipogenesis (Kawai *et al.* 2007).

In addition, GH has been demonstrated to activate and/or induce the expression of a number of other transcription factors including c-Jun, c-fos, Egr-1, C/EBPβ, C/EBPδ, and PPARγ during 3T3-F442A or 3T3-L1 preadipocyte differentiation (Kawai *et al.* 2007; Clarkson *et al.* 1999; Clarkson *et al.* 1995; Gurland *et al.* 1990). Except that C/EBPβ/δ and PPARγ are known to participate in the regulation of adipogenesis, the roles of the other upregulated transcription factors, that are mainly early response genes, in GH-stimulated adipocyte differentiation remain to be characterized (Nam & Lobie 2000). It is possible that GH stimulates adipogenesis in association with modulation of cell growth (Kawai *et al.* 2007).

Studies of adipose conversion of OB1771 preadipocytes, which are derived from epididymal fat of the adult ob/ob mouse, also demonstrate a stimulatory effect of GH on adipocyte differentiation (Doglio *et al.* 1986). It has been observed that exposure to GH increases the expression of late differentiation specific genes such as glycerol-3-

phosphate dehydrogenase and enhances its activity. However, GH does not seem to affect the expression of early differentiation genes such as lipoprotein lipase. These results indicate that the permissive role of growth hormone during OB1771 adipose cell differentiation is related to terminal events only. This finding is somewhat different from what was observed in 3T3 murine preadipocyte cell lines that are derived from the mouse embryo; there the stimulatory effect of GH on adipogenesis is more associated with the early differentiation stage. Similar to 3T3 cells, the effect of GH on adipocyte differentiation of OB1771 cells is independent of IGF-I, since IGF-I can not replace the requirement for GH to promote terminal differentiation of either of these cell lines (Nam & Lobie 2000).

Besides these data from *in vitro* studies, certain *in vivo* observations also support a positive role for GH in the differentiation of preadipocytes to adipocytes. Long-term treatment of neonatal rats with antiserum to GH causes a dramatic reduction (~80%) in the number of differentiated adipocytes in two internal fat depots, clearly demonstrating that GH is required for the differentiation of adipocytes *in vivo* (Flint & Gardner 1993). The finding that the epididymal fat depot is significantly smaller in young GHR-deleted mice before manifestation of the lipolytic actions of GH, supports an *in vivo* role of GH in murine adipogenesis (Shang & Waters 2003). Furthermore, as mentioned in section 1.3.4.1., the clinical finding that GH-deficient children have a reduced adipocyte number but an enlarged fat cell size and that GH substitution results in normalization of both fat cell number and size, also indicates that GH not only has lipolytic actions on adipocytes but also has the effect to promote adipocyte conversion, thus leading to an increase in fat cell number (Wabitsch *et al.* 1995; Wabitsch *et al.* 1994).

(B) *Inhibitory effects of GH on differentiation of primary preadipocytes*

In contrast to preadipocyte clonal cell lines, studies using cultured primary preadipocytes isolated from either rat or human adipose tissues show that GH stimulates preadipocyte proliferation and, consequently, inhibits preadipocyte differentiation (Nam & Marcus 2000; Nam & Lobie 2000; Hansen *et al.* 1998; Wabitsch *et al.* 1996). During differentiation of primary rat preadipocytes, the addition of GH markedly inhibits TG

accumulation and the expression of late markers of adipocyte differentiation such as fatty acid synthase (FAS), aP2, and HSL (Hansen *et al.* 1998). Similarly, during differentiation of cultured human adipocyte precursor cells obtained from children or adults, GH dose-dependently reduces the number of differentiating cells and suppresses the expression of glycerol-3-phosphate dehydrogenase (GPDH) (Wabitsch *et al.* 1996). The molecular mechanism by which GH inhibits the differentiation of primary preadipocytes to adipocytes has not been as well studied as that in the clonal preadipocyte cell lines (Nam & Lobie 2000). Hansen *et al.* demonstrated that the addition of GH does not alter the expression level of ADD1/SREBP-1c, but significantly reduces PPARγ expression and inhibits its DNA-binding activity. They also showed that the antiadipogenic effect of GH is not affected by a MAP kinase inhibitor, indicating that the MAP kinase pathway is not involved. But the authors observed that the expression of preadipocyte factor-1 (Pref-1) is decreased during primary preadipocyte differentiation, whereas GH treatment prevents this (Hansen *et al.* 1998). They therefore have proposed that GH inhibition of adipogenesis in primary preadipocyte cultures is a result of GH-dependent increase of Pref-1 expression which, in turn, acts as a potent inhibitor of adipocyte differentiation (Shang & Waters 2003). Alternatively, Richter *et al.* pointed to STAT5 as the modulator of GH-mediated inhibition in primary preadipocytes; this STAT5-dependent mechanism does not appear to be mediated through its transactivation domain (Richter *et al.* 2003).

The reason for such obvious differences observed between clonal cell lines and primary cells is not known. It may be that different developmental stages of adipocytes have different responses to GH (Osafo *et al.* 2005) and that primary cells, which are most likely harvested in the later stage of adipocyte differentiation, may have already been stimulated by GH *in vivo* (Louveau & Gondret 2004). The various effects of GH seen in preadipocytes and adipocytes are summarized in **Table I-1**.

Table I-1: Effects of GH on preadipocytes and mature adipocytes.

Preadipocytes	Adipocytes
In murine clonal cell lines (e.g. 3T3-L1, 3T3-F442A): 1. promotes preadipocyte differentiation • induces antimitogenic state • primes post-confluent preadipocytes to be more responsive to IGF-I and insulin • alters cellular cytoskeletal composition • induces expression of a number of TFs, including c-fos, C/EBPs and PPARγ • stimulates expression of adipocyte-specific genes 2. stimulates local IGF-I production	**Acute insulin-like effects:** 1. stimulation of glucose uptake 2. increases in lipogenesis 3. inhibition of lipolysis
In cultured primary adipocyte precursor cells (isolated from human or rat adipose tissue): 1. stimulates preadipocyte proliferation 2. inhibits adipocyte differentiation • inhibits TG accumulation • inhibits adipocyte specific gene expression 3. increases IGF-I production	**Chronic anti-insulin effects:** 1. decreases fat accumulation • inhibits LPL activity • reduces lipogenesis • decreases fatty acid reesterification 2. increases fat lipolysis • stimulation of HSL activity • stimulation of breakdown of TGs to FFA and glycerol 3. decreases glucose transport and glucose oxidation 4. causes refractoriness to acute insulin-like effects

1.4. GH and Associated Pathophysiological States

Pathophysiological states associated with GH derive from either an abnormality in GH secretion, such as too much (acromegaly or gigantism) or too little (GH deficiency), or from defects in the target cell's response to GH, such as GH insensitivity.

1.4.1. GH Deficiency

GH deficiency (GHD) is the medical condition of inadequate production of GH and its effects on children and adults. Deficiency of GH results from various causes (Kato *et al.* 2002). It may be idiopathic, when no organic lesion can be identified during life, genetic, due to mutations in specific genes (e.g. *GHRHR*, *GH-N*), congenital, resulting from abnormal formation of the hypothalamus and pituitary before the child is born, or acquired, stemming from hypothalamic-pituitary lesions obtained at any time during life. By far, the most common cause of acquired GH deficiency in children is a tumor, particularly a craniopharyngioma. Others include head trauma, radiation therapy and autoimmune inflammation (Ayuk & Sheppard 2006).

GH deficiency may occur by itself, designated as isolated GH deficiency (IGHD), or in combination with one or more other pituitary hormone deficiencies, known as combined pituitary hormone deficiency (CPHD) (Lopez-Bermejo *et al.* 2000). A number of genetic causes are known in CPHD. For example, mutations of the pituitary transcription factor *PROP1* gene result in thyroid-stimulating hormone (TSH), GH, prolactin (PRL), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) deficiencies. Similarly, mutations in the *POU1F1* (formerly referred to as Pit-1) gene cause patients with GH, PRL and TSH deficiencies.

In contrast, IGHD has been found to result from genetic mutations occurring in the *GH-N* (or *GH-1*) gene or in the GHRH receptor gene (*GHRHR*). GHRH not only regulates GH synthesis and secretion but is also required for somatotrope proliferation. Patients with mutations of the *GHRHR* gene have pituitary hypoplasia and isolated GH deficiency (Kato *et al.* 2002). However, extensive analysis of patients with IGHD suggests that mutations of *GHRHR* are an infrequent cause of GHD. Of note, to date, no mutation of the *GHRH* gene has been described (Lopez-Bermejo *et al.* 2000). The *GH-N* gene is part

of the GH gene cluster and is responsible for pituitary GH. Inactivating mutations of the GH-N gene directly affect GH production (Kato *et al.* 2002); approximately 5-30% of patients with IGHD are associated with *GH-N* gene defects. Four Mendelian types of IGHD have been delineated based on the mode of inheritance and the degree of GH deficiency, as summarized in **Table I-2** (Kato *et al.* 2002; Lopez-Bermejo *et al.* 2000; Perez Jurado & Argente 1994).

GH deficiency causes significantly different problems at various ages. In children, growth failure and short stature are the major manifestations of GH deficiency. Idiopathic GH deficiency is the most common cause of GH deficiency in childhood; it is a poorly defined and often reversible condition, with patients showing blunted, but not absent, GH responses to GH stimuli. In adults, the effects of GH deficiency are more subtle. There is no single sign, but a number of physical and psychological symptoms which lead to the recognition of "GH deficiency syndrome". Adults with GH deficiency are often associated with changed metabolism and body compositions characterized by reduced lean body mass and increased fat mass, particularly distributed in the truncal region (visceral fat). They may also include poor bone density, dyslipidemia, insulin resistance, increased cardiovascular risk, reduced body strength and energy, as well as impaired quality of life (Ayuk & Sheppard 2006; Kato *et al.* 2002).

The diagnosis of GH deficiency involves a multiple-step diagnostic process. For children it is relatively straightforward due to the association with growth retardation, whereas for adult onset it is more challenging because of the lack of subjective features. Routinely, GH stimulation test(s) are performed to see if the patient's pituitary gland responds properly when provoked by various single or combined stimuli. Low plasma concentrations of IGF-I and IGFBP-3, which are both regulated by GH, also are useful in making the diagnosis (Ayuk & Sheppard 2006; Dattani & Preece 2004).

Table I-2: Four Mendelian types of Isolated GH Deficiency (IGHD).

Type	Mode of Inheritance	Endogenous GH	Molecular Causations	Features
Type IA	autosomal recessive	absent	• Large deletions of the *GH-N* gene • Nonsense mutations of *hGH-N* gene • Frameshift mutations of *hGH-N* gene	• Severe clinical phenotypes • Developing anti-GH antibodies to hGH therapy
Type 1B	autosomal recessive	low	• Splicing site mutations within the *hGH-N* gene	• A milder clinical features • Response to hGH therapy
Type II	autosomal dominant	low	• Dominant negative mutations of the *hGH-N* gene	• Affected individuals typically have one affected parent • Response to hGH therapy
Type III	X-linked	low		• Clinical findings differ in different families • Affected individuals usually associated with hypo- or agam-maglobulinemia • Response to hGH therapy

GH deficiency is treated by replacing hGH. All hGH currently in use is recombinant hGH, a biosynthetic version manufactured by DNA technology. In children treated early, GH replacement effectively accelerates growth to obtain catch-up growth velocity and normal final adult height. In adults, GH therapy helps to normalize body composition by reducing body fat and increasing lean body and muscle mass. It is also associated with an increase in the ratio of high-density lipoprotein to total cholesterol. In addition, GH therapy may have beneficial effects on bone mass, cardiovascular diseases and quality of life (Ayuk & Sheppard 2006; Dattani & Preece 2004; Kato *et al.* 2002). Overall, GH treatment is believed to be safe (Dattani & Preece 2004). There is one report suggesting that long-term follow-up of patients on GH treatment has shown a higher than expected incidence and mortality of colonic cancer and Hodgkin's disease (Swerdlow *et al.* 2002). However, these data may result from the generation of excessive IGF-I concentrations, which has been associated with several types of cancer (Dattani & Preece 2004)

1.4.2. GH Excess (Gigantism and Acromegaly)

Overproduction of GH, mostly caused by a GH-secretion pituitary tumor (somatotroph adenoma), results in gigantism in prepubertal children and acromegaly in adults (Kato *et al.* 2002). Because the onset of acromegaly is typically insidious and progresses slowly, this disorder is usually not recognized until striking changes in appearance have occurred, characterized as "coarse features" due to overgrowth of bone and swelling of connective tissues. Although the incidence of acromegaly is quite low and it most commonly affects middle-aged adults, it can result in serious illness and premature death (Ayuk & Sheppard 2006). In acromegaly, GH secretion maintains a pulsatile pattern, but with increased amplitude and the loss of the nadir between pulses (Kato *et al.* 2002). The major aims of treatment of acromegaly are to reverse the disease symptoms and to improve long-term survival. It usually combines the use of surgery, radiotherapy, and/or medical therapy using long-acting somatostatin agonists (e.g. Octreotide) and/or the recently developed GH receptor antagonist (pegvisomant) (Ayuk & Sheppard 2006; Kato *et al.* 2002). It is extremely rare for such a tumor to occur in childhood, but when it does before the epiphyses have fused, the primary effect of GH hypersecretion is to stimulate excessive linear bone growth, thus resulting in gigantism.

1.4.3. GH Insensitivity

GH insensitivity (GHI) describes a broad range of clinical disorders characterized by insensitivity to the physiologic actions of GH, in which there are clinical and biochemical features of resistance to exogenous GH and IGF-I deficiency (David *et al.* 2005; Savage *et al.* 1999). GHI exhibits very similar clinical characteristics to those seen in IGHD, but differs due to its high levels of circulating GH, whereas IGHD presents with absent or low levels of serum GH (see section 1.4.1.).

Since the original report by Laron *et al.* (Laron *et al.* 1966), first describing the clinical phenotype of the congenital GHI syndrome (also known as Laron syndrome), this group of disorders has become larger and more heterogeneous. This has been due to improved diagnostic methods and the broader application of molecular analysis for patients with short stature. In general, GHI can be classified into two groups, primary (or inherited) GHI and secondary (or acquired) GHI (Laron 2004; Laron *et al.* 1993). Three major classes of molecular defects have been identified for primary GHI: (1) defects in the GH receptor (GHR) resulting from mutations within the *GHR* gene; (2) abnormalities in the GH signaling cascades, mainly the JAK2-STAT5b pathway; and (3) genetic defects affecting IGF-I synthesis and actions. The cause of secondary GHI is also heterogeneous; it may be associated with malnutrition, liver disease, uncontrolled diabetes mellitus or other chronic diseases, and conditions associated with increased catabolic rate such as trauma and sepsis, as well as antibodies against GH or the GHR (Mullis *et al.* 2005; Laron 2004; Rosenfeld & Hwa 2004). **Table I-3** summarizes the classification of GHI. A detailed review of the molecular mechanism of primary GHI will be given in section 2.4.5.1, following the review of GHR and its signaling pathways.

Most classic GHI patients have high circulating hGH and low serum levels of IGF-I, IGFBP-3 and GH binding protein (GHBP), but some patients may show normal or even high GHBP levels, depending on where the gene mutations are located (David *et al.* 2005). The primary defect of the GHI patient in childhood is severe growth failure, leading to, if untreated, an extremely short stature in adult life. Because of their resistance to exogenous GH administration, the treatment of GHI patients depends on recombinant human IGF-I (rhIGF-I). Trials with rhIGF-I in GHI syndrome started in the late 1980s

(David *et al.* 2005). Most investigators give IGF-I subcutaneously twice per day, using doses ranging from 40-120μg/kg. It has also been tested as a single daily dose of 150μg/kg (Laron *et al.* 1992). These studies have demonstrated a clear benefit of rhIGF-I therapy in comparison to placebo, showing an increase in growth velocity during the treatment (Ranke *et al.* 1995; Guevara-Aguirre *et al.* 1995). However, a range of adverse effects caused by rhIGF-I treatment have also been reported, such as pain at the injection site, headaches, lipohypertrophy at the injection site, and an increase of the fat mass and body mass index (David *et al.* 2005)

In addition, GH insensitivity can occur in an incomplete form, resulting in partial GHI. Affected children exhibit less severe growth failure, show normal or slightly increased GH secretion, variable but usually reduced GHBP levels and low IGF-I concentrations although not as severe as in GHD or complete GHI. Instead of complete resistance to exogenous GH, patients with partial GHI may respond to supraphysiologic doses of GH. Partial GHI may account for a certain portion of children with idiopathic short stature (ISS), which defines a large group of individuals who seek medical advice about short stature but for whom it has not been possible to diagnose an underlying cause (Rosenbloom 2000; Hull & Harvey 1999; Ranke 1996).

Table I-3: Classification of human growth hormone insensitivity (GHI)

1) Primary GH insensitivity (hereditary)

   a) Defects in human GH receptor
      i) Mutation affecting the ECD
      ii) Mutation affecting the TMD
      iii) Mutation affecting the ICD

   b) Defects in hGH signal transduction (postreceptor defects)
      i) Abnormalities of STAT5b (mutation of *STAT5b* gene)

   c) Primary defects of IGF-I synthesis and actions
      i) IGF-I gene mutations and deletions

2) Secondary GH insensitivity (acquired)

   a) Associated with malnutrition
   b) Caused by liver disease
   c) Caused by uncontrolled diabetes mellitus
   d) Caused by other chronic diseases (e.g. uremia)
   e) Associated with fasting or catabolic states (i.e. trauma or sepsis)
   f) Circulating antibodies of GH that inhibit GH actions
   g) Antibodies to the hGHR (theoretical?)
   h) Other conditions

Adapted from (Laron 2004) with permission.

1.4.4. GH and Obesity

Obesity, defined as an abnormal accumulation of body fat in proportion to body size, is a chronic clinical condition that frequently results in a significant impairment of health (Scacchi *et al.* 1999). It has been shown to predispose individuals to various diseases, including type 2 diabetes mellitus, cardiovascular disease, hypertension and cancer, and is directly related to increased mortality and lower life expectancy. In recent years, the prevalence and severity of obesity, particularly childhood obesity, are increasing rapidly worldwide (Strauss & Pollack 2001), which has led to a growing awareness that obesity in not just an individual clinical condition anymore, but is becoming a serious public health problem (Scacchi *et al.* 1999). Approximately half of the obese schoolchildren remain obese as adults (Serdula *et al.* 1993) and, once established in adulthood, obesity can rarely be resolved through voluntary weight loss (Nam & Marcus 2000). Thus, early intervention is viewed as an attractive strategy to avoid adult obesity complications (Kamel *et al.* 2000). However, so far, the mechanisms responsible for the increased incidence of childhood obesity remain not well understood, which makes treating obese children and adolescents a difficult task (Nemet *et al.* 2002). Among the endocrine changes in obesity, alterations of the GH/IGF-I axis are of particular interest because adipose tissue is a well-documented target tissue for GH and the GH/IGF-I axis is well known to play important roles in the regulation of lipolysis and lipogenesis (Nam & Marcus 2000). Changes in the GH/IGF-I axis with obesity reflect an integration of neuroendocrine and metabolic abnomalities.

1.4.4.1. GH secretion in obesity

In both obese children and adults, circulating GH levels are reduced (Maccario *et al.* 2000). Obese patients, compared to normal weight subjects, display a significantly shorter GH half-life, a decreased frequency of GH secretory episodes, and both the spontaneous 24h GH secretion and the GH release after stimulation are markedly blunted (Nam & Marcus 2000; Scacchi *et al.* 1999; Veldhuis *et al.* 1995; Veldhuis *et al.* 1991). Furthermore, in these patients GH release is impaired in response to all traditional hypothalamic provocative stimuli such as insulin-induced hypoglycemia, infusion of arginine, and administration of L-dopa or glucocorticoids. In addition, a blunted GH rise

has been consistently described in response to direct somatotrope stimulation by exogenous GHRH (Scacchi *et al.* 1999).

Although the pathogenesis of reduced GH secretion in obesity is still undefined, several possible mechanisms have been proposed: (A) hypothalamic alterations, resulting in decreased GHRH or increased somatostatin; (B) a relative insensitivity of pituitary somatotrophs to GHRH; and (C) a perturbation of the peripheral signals, such as elevated plasma FFA levels or enhanced negative IGF-I feedback (Smotkin-Tangorra *et al.* 2006; Nam & Marcus 2000; Maccario *et al.* 2000; Scacchi *et al.* 1999).

(A) *Hypothalamic alteration: GHRH hypoactivity*

Significantly fewer GH secretory bursts are observed in obese subjects leading researchers to speculate that a decrease in GHRH secretion might be the cause of GH hyposecretion in human obesity (Veldhuis *et al.* 1995; Iranmanesh *et al.* 1991). Consistent with this hypothesis, genetically engineered obese rats were observed to have reduced hypothalamic GHRH content and expression as well as reduced pituitary GH stores (Nam & Marcus 2000). In contrast, a finding that repetitive GHRH pretreatment in humans could enhance the low GH response to GHRH in elderly subjects but had no effect in obese patients suggests that a somatotrope defect is responsible for GH insufficiency in obesity (Ghigo *et al.* 1993). However, this finding does not definitively rule out the possibility that endogenous GHRH hypoactivity might play a critical role in GH insufficiency in obesity (Maccario *et al.* 2000).

(B) *Hypothalamic alteration: somatostatinergic hyperactivity*

On the basis of the studies that pharmacological compounds thought to inhibit hypothalamic somatostatin release (i.e. pyridostigmine (PD), arginine (ARG), galanin and β-adrenergic antagonists) consistently improve GHRH-stimulated GH secretion in obese subjects, an augmented somatostatinergic response has been hypothesized as a contributing factor for causing the obesity-related GH secretory dysfunction. However, even though these drugs can suppress the activity of hypothalamic somatostatin, the GH response is still lower in obese patients than that observed in normal weight subjects, indicating that somatostatinergic hyperactivity, if is the reason, is unable to fully explain

the reduced GH secretion in obesity (Nam & Marcus 2000; Procopio *et al.* 1995; Ghigo *et al.* 1992).

(C) *Metabolic alteration: elevated free fatty acids*

GH and FFA function together in a regulatory feedback fashion. GH stimulates lipolysis, resulting in elevated levels of FFA. In turn, FFA acts in a negative feedback manner to inhibit GH secretion (see section 1.2.4.2). Obesity is associated with elevated circulating FFA levels, which are thought to be an important metabolic alteration that is partially responsible for the pathogenesis of reduced GH secretion seen in the condition. Indeed, obese patients treated with acipimox, a lipolysis inhibition drug, for either a short or a more long-term period, demonstrate a restoration of their GH response to several stimuli, including GHRH alone and GHRH in combination with arginine or GH releasing peptides (Maccario *et al.* 2000). Although some FFA actions may be exerted at the hypothalamic level, their major effects seem to take place directly at the pituitary level through inhibition of somatotroph cell membrane depolarization (Alvarez *et al.* 1991).

Whatever its cause, the defect in GH secretion in obesity appears to be a secondary, probably an adaptive, phenomenon, since it can be completely restored by normalization of body weight (Maccario *et al.* 2000; Scacchi *et al.* 1999) and, thus, is considered to be a consequence rather than a cause of the obese state (Nam & Marcus 2000; Maccario *et al.* 2000).

1.4.4.2. IGF-I secretion in obesity

Despite GH hyposecretion in obesity, total serum IGF-I levels are relatively normal in obesity, while the circulating free IGF-I levels are consistently elevated (Bouhours-Nouet *et al.* 2007; Nam & Marcus 2000; Maccario *et al.* 2000). These normal or increased IGF-I levels suggest an increase in responsiveness to GH and may explain why prepubertal obese children maintain normal or even accelerated growth in spite of reduced GH secretion (Bouhours-Nouet *et al.* 2007; Ballerini *et al.* 2004; Nam & Marcus 2000).

The discordance between GH secretion and the circulating IGF-I level is not seen solely in obesity. In those malnourished patients with anorexia nervosa, total IGF-I levels are

markedly decreased regardless of GH secretory profiles (Nam & Marcus 2000; Hochberg *et al.* 1992). Taken together, these findings suggest that, in either overnutrition or extreme undernutrition states, the IGF-I system seems to be mainly regulated by factors other than GH, including nutritional factors.

The elevated free IGF-I levels in obesity despite GH insufficiency can be explained by the following rationales:

1) In the circulation, the majority of IGF-I is bound to IGFBP-3, whereas the remainder of serum IGF-I is believed to bind to other circulating proteins, including IGFBP-1 and IGFBP-2. In obese states, serum levels of IGF-I, IGFBP-3 and the IGF-I/IGFBP-3 ratio were found to be normal; in contrast, both the IGFBP-1 and IGFBP-2 levels are significantly decreased, thus resulting in an increase in the proportion of free, biologically active IGF-I (Ballerini *et al.* 2004; Nam & Marcus 2000).

2) It has been demonstrated that the IGF-I mRNA levels in adipose tissue appear to be similar to those found in the liver (Peter *et al.* 1993), which is known to be the major source of circulating IGF-I. This evidence has led to the hypothesis that adipose tissue could serve as a major contributor of circulating IGF-I in obesity (Nam & Marcus 2000). In obese subjects, although reduced GH secretion may cause a decrease in the production of IGF-I from each individual adipocyte, significantly greater amounts of IGF-I could be obtained from the extremely expanded adipose tissue than from other tissues on a tissue weight basis (Nam & Marcus 2000; D'Ercole *et al.* 1984). This may then contribute to the normal or even increased IGF-I levels, including free IGF-I.

3) IGF-I synthesis and secretion depend on peripheral GH sensitivity that is reflected by GHR and GH-binding protein (GHBP) levels, both of which are thought to be increased in obese patients. The increased responsiveness to GH may contribute to increased IGF-I levels.

4) Elevated free IGF-I, in turn, can act back to suppress GH secretion in a negative feedback manner, which partly accounts for the GH insufficiency in obesity.

1.4.4.3. GHBP and GHR in obesity

Elevated circulating GHBP levels have been consistently observed in obesity, with a strong positive correlation between plasma GHBP levels and body mass index as well as body fat, particularly visceral fat, being reported (Nam & Marcus 2000; Roelen *et al.* 1997; Rasmussen *et al.* 1996; Postel-Vinay *et al.* 1995). Increased GHBP levels in obese subjects seem to be restored to normal by massive weight loss, but are unaffected by short-term hypocaloric feeding, suggesting that GHBP may be regulated by the same or closely related factors that regulate fat mass, in particular abdominal fat mass (Roelen *et al.* 1997). In the human, GHBP is generated from proteolysis of the extracellular domain of the human GHR; therefore, it has been assumed that the changes in circulating GHBP levels reflect the changes in GHR levels in the target tissues (Nam & Marcus 2000; Baumann *et al.* 1994).

To our knowledge, almost all of the studies to date on the GHR status in obese subjects are based on the measurements of GHBP. The precise changes in GHR expression, at both the mRNA and protein levels, in obese patients and their association with different fat depots remain to be studied.

1.4.4.4. Effects of GH administration on body composition in obesity

Dietary restriction has been used as a basic method to treat obesity. However, caloric restriction is usually accompanied by protein catabolism, resulting in a negative nitrogen balance and a loss of both fat mass and lean body mass (Nam & Marcus 2000; Scacchi *et al.* 1999). GH, whose secretion is markedly impaired in obesity, is endowed with both lipolytic and protein anabolic properties which makes GH a potential tool in the treatment of obesity (Scacchi *et al.* 1999; Tagliaferri *et al.* 1998). The availability of recombinant human growth hormone (rhGH), and the consistent finding that rhGH treatment of GH-deficient adults with a characteristic increased body fat mass, especially visceral fat, is able to reduce total fat mass, has encouraged a series of clinical trials to investigate the effect of rhGH treatment in obese subjects. Several groups have demonstrated that rhGH treatment of obese adults, when kept on a normal caloric diet, results in a significant decrease in total fat mass, especially intra-abdominal fat (visceral fat) (Johannsson *et al.*

1997; Richelsen *et al.* 1994; Jorgensen *et al.* 1994; Skaggs & Crist 1991). In contrast, other studies carried out with calorie-restricted obese patients showed that rhGH treatment was unable to enhance diet-induced weight and fat mass reduction (Scacchi *et al.* 1999; Drent *et al.* 1995; Snyder *et al.* 1990). Besides the effect on body fat, rhGH administration has been consistently seen to prevent diet-induced reduction of lean body mass and to enhance the energy metabolism of lean body mass in obese patients, even in those individuals undergoing severe calorie-restriction (Scacchi *et al.* 1999). There are also reports that rhGH treatment of obese adults helps to improve insulin sensitivity and blood lipid profiles, although these changes may be secondary to the reduction of abdominal obesity by GH (Johannsson *et al.* 1997). Kamel *et al.* evaluated the effects of rhGH treatment in obese children, in which they observed that GH treatment for 6 months of obese prepubertal boys reduces the percentage of total body fat, possibly via stimulation of catecholamine-induced lipolysis by GH. Minimal or no effect on insulin sensitivity and glucose homeostasis by GH treatment was found (Kamel *et al.* 2000).

When evaluating GH effects on body composition in obesity, several factors, including the timing and methods used to assess the effects of GH as well as the dosage of GH used, need to be taken into account (Nam & Marcus 2000). In general, administration of rhGH at an adequate dosage appears to have beneficial effects on body composition in obesity, while high doses of GH could induce hyperinsulinemia that may antagonize the GH lipolytic effects in obese subjects (Nam & Marcus 2000; Scacchi *et al.* 1999).

2. GROWTH HORMONE RECEPTOR (GHR)

GH, after secretion from the anterior pituitary, will bind to its specific cell-surface receptors expressed on target tissues, such as liver, muscle, adipose tissue and bone. This interaction activates tightly regulated signaling cascades, which then trigger multiple GH responses. Therefore, the ability of GH to promote its various physiological effects depends initially on the interaction of GH with GHR (Bougneres & Goffin 2007; Kopchick & Andry 2000). GHR defects, due to mutations or deletions, can have severe effects on GH actions and lead to GH insensitivity syndrome.

This section will describe the GHR structure, GHR isoforms and several major GH signaling cascades activated post GH-GHR interactions, as well as the molecular mechanisms for primary GH insensitivity.

2.1. The GHR Is A Class I Cytokine Receptor

The cytokine receptors are a group of single transmembrane polypeptides with characteristic structural motifs in their extracellular ligand-binding domains, but lacking intrinsic protein tyrosine kinase activity in their intracellular domains (Bougneres & Goffin 2007; Moutoussamy *et al.* 1998). They can be divided into four subclasses based on common structural features (Taniguchi 1995).

GHR is a member of the Class I cytokine receptor superfamily, which is also known as the cytokine/hematopoietin receptor superfamily (Bougneres & Goffin 2007). In addition to GHR, this superfamily includes receptors for prolactin (PRL), leptin, erythropoietin (EPO), thrombopoietin (TPO), Oncostatin M (OM), ciliary neurotropic factor (CNTF), leukemia inhibitory factor (LIF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and several interleukins (ILs) (Piwien-Pilipuk *et al.* 2002a). As pictured in **Figure I-5**, members of this family share several homologous structural motifs, which help to define the Class I receptor superfamily (Zhu *et al.* 2001; Herrington & Carter-Su 2001; Waters *et al.* 1999; Argetsinger & Carter-Su 1996b). They all possess a single membrane-spanning domain of 24 amino acids. They have limited amino acid homology (14-44%) in a region spanning ~210 amino acids in the extracellular domain, which corresponds to two fibronectin type III modules. Within these fibronectin subdomains, two highly conserved features are found: the first is conserved pairs of disulfide-linked cysteines in the N-terminal part, and the second is a pentapeptide termed the "WSXWS motif" (Trp-Ser-any amino acid-Trp-Ser) in the C-terminal part, which has been predicted to function as a ligand binding site for the PRL receptor (Rozakis-Adcock & Kelly 1992), the EPO receptor (Quelle *et al.* 1992) and the IL-2 receptor (Miyazaki *et al.* 1991). For mammalian GHR, this motif sequence is YXXFS. Although it does not make direct contact with GH, the YXXFS motif is likely to serve a structural role critical for ligand

binding (Jorge *et al.* 2004; de Vos *et al.* 1992).

No canonical tyrosine kinase consensus sequences have been identified in the intracellular region, but two short homologous domains termed Box-1 and Box-2 are relatively conserved. Box-1 is a membrane-proximal, proline-rich domain present in all members. It is eight residues in length, consisting of a P-X-P consensus sequence and a preceding cluster of hydrophobic amino acids. In mammalian GHR, this sequence reads ILPPVPVP (Zhu *et al.* 2001). Box-1 is critical for GHR signaling (Waters *et al.* 1999) as it is involved in the interaction of GHR with the associated JAK2 kinase, a major mediator of cytokine receptor signaling, and is also required for the activation of MAP- and PI-3 kinase pathways (Bougneres & Goffin 2007). Box-2, the second cytoplasmic motif, is much less conserved than Box-1 despite its presence in most of the family members. It begins with a cluster of hydrophobic, negatively charged residues and ends with one or two positively charged amino acids. In GHR, Box-2 is located ~30 amino acids distal to Box-1 and is comprised of ~15 amino acid residues. The importance of Box-2 in GHR is less well defined, but this region may be involved in ligand-mediated receptor-internalization (Strous *et al.* 2004; Frank 2002; Frank 2001; Strous & Govers 1999), as the ubiquitin-dependent endocytosis (UbE) motif that mediates GHR endocytosis is located in Box-2 .

Figure I-5: Schematic representation of the common structural features of the cytokine/hematopoietin receptor superfamily.

Abbrevations: GHR, growth hormone receptor; PRLR, prolactin receptor; EpoR, erythropoietin receptor; IL-R: interleukin receptor; GM-CSFR: granulocyte macrophage-colony stimulating factor receptor; G-CSFR: granulocyte colony stimulatory factor receptor; LIFR: leukocyte inhibitory factor receptor; obR, leptin receptor; CNTFR, ciliary neurotropic factor receptor; C: cysteine.

Figure is reproduced with permission from Moutoussamy *et al.* (1998). *Eur. J. Biochem.* **255**, 1-11.

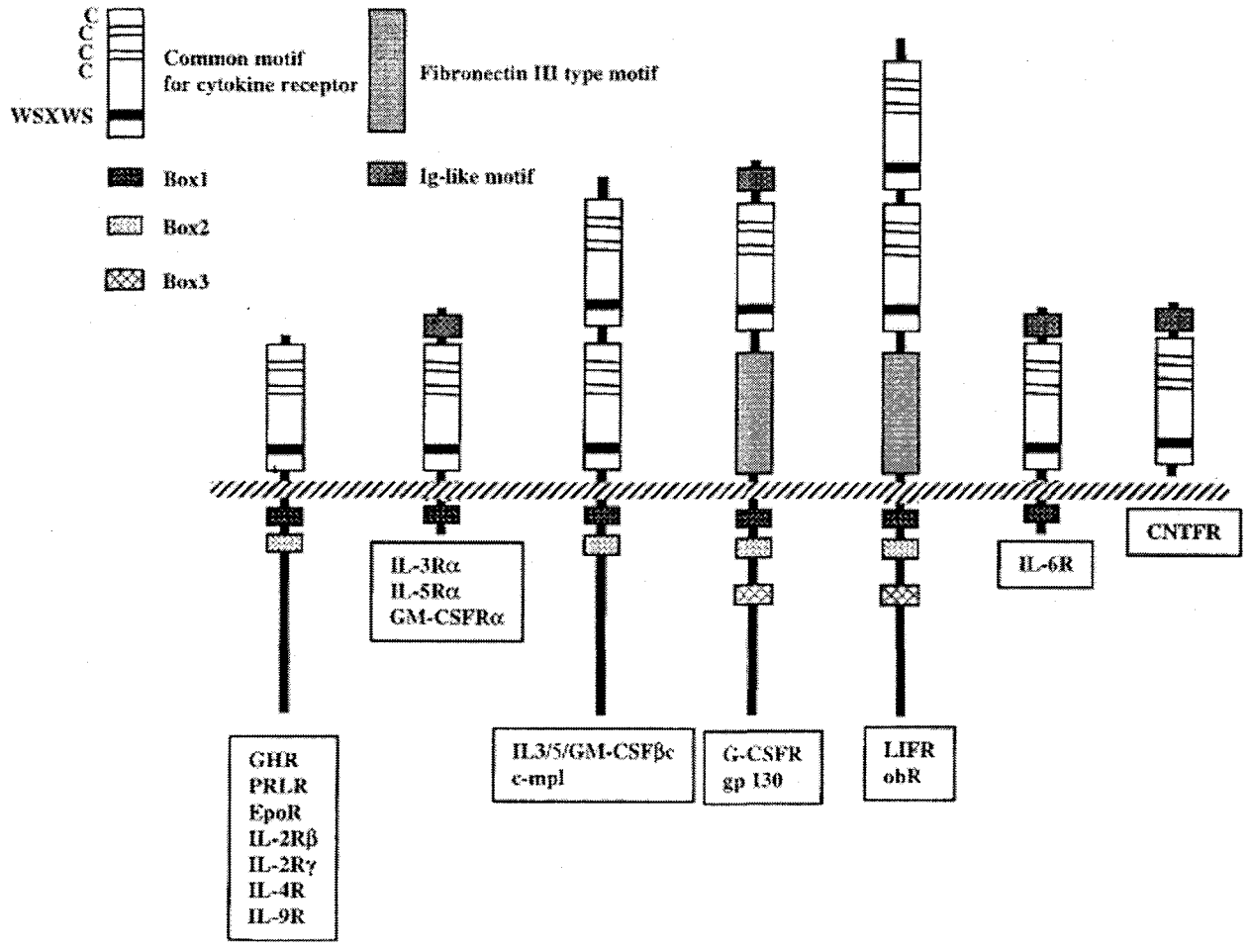

C
C
C
C
WSXWS
Common motif
for cytokine receptor
Fibronectin III type motif
Box1
Box2
Box3
Ig-like motif
IL-3Rα
IL-5Rα
GM-CSFRα
CNTFR
IL-6R
GHR
PRLR
EpoR
IL-2Rβ
IL-2Rγ
IL-4R
IL-9R
IL3/5/GM-CSFβc
c-mpl
G-CSFR
gp 130
LIFR
obR

Figure I-5

2.2. Structure of the Growth Hormone Receptor (GHR)

A full-length, mature form of GHR is ~620 amino acids in length, with the exact number of amino acids varying from species to species. The extracellular, hormone-binding domain contains 246 amino acids, followed by a single transmembrane domain and an intracellular signaling domain of ~350 amino acids (Bougneres & Goffin 2007) (**Figure I- 6**). Structurally, the extracellular portion of the GHR can be divided into two domains linked by 4 residues. Each domain contains seven β-strands arranged in an anti-parallel fashion to form a barrel-like structure. As illustrated in **Figure I-6**, three pairs of disulfide bond-linked cysteines and one free cysteine are found in the GHR extracellular portion (Moutoussamy *et al.* 1998). It appears that the first disulfide-bond is essential for ligand binding (Gobius *et al.* 1992). The YXXFS (WSXWS-like) motif is adjacent to the transmembrane domain (Jorge *et al.* 2004).

In the intracellular region, in addition to the conserved Box-1 and Box-2 motifs, multiple tyrosine residues are recognized (**Figure I-6**). Some of them have been demonstrated to participate in GH effects on gene transcription and metabolism. Rowland et al. have performed an extensive study using knock-in mouse models, for the first time demonstrating *in vivo* that five distal phosphotyrosines (m539, m545, m577, m606 and m639) are essential for full activation of the JAK2/STAT5/IGF cascade. Mice expressing truncations at residue m569 and at residue m391 showed progressive impairment of postnatal growth and obesity as well as hyperglycemia (Rowland *et al.* 2005b).

According to the amino acid sequence, the GHR is predicted to be ~70kD in molecular weight. However, GHR is characterized as a ~120-130kD protein based on its migration on SDS-polyacrylamide gels (Carter-Su *et al.* 1996). With regard to this observed difference, post-translational modifications such as glycosylation, phosphorylation and ubiquitination are possible contributors. Five potential N-linked glycosylation sites are conserved in the extracellular domain of the GHR in all species sequenced to date, several of which are glycosylated (Kopchick & Andry 2000) (**Figure I-6**). In porcine GHR, it has been documented that mutations of all five sites together would result in significantly reduced GH binding (Harding *et al.* 1994). The GHR has 19 potential ubiquitination sites. Its degree of ubiquitination increases upon GH binding and is

Figure I-6: Structure of the growth hormone receptor (GHR).

The key structural features of the GHR important for signal transduction and GH actions are illustrated in this diagram. The transmembrane domain (TMD) is shown in black. The Box-1 (interacting with JAK2) and Box-2 regions are shown in light gray. The extracellular N-linked glycosylation sites (N) and the ubiquitin-dependent endocytosis (UbE) motif are shown on the left. The 10 cytoplasmic tyrosine residues (Y) and the 7 extracellular cysteines (C) are shown on the right, with 3 pairs of cysteines linked by disulfide-bonds. The striped box represents the YGEFS motif, which is a WSXWS-like motif. Intracellular tyrosine residues critical for STAT5 activation and function as well as regions of GHR required for various other functions are indicated. ECD: extracellular domain; ICD: intracellular domain. Figure is adapted and modified from Carter-Su C. *et al.* (1996). *Annu. Rev. Physiol.* **58**, 187-207.

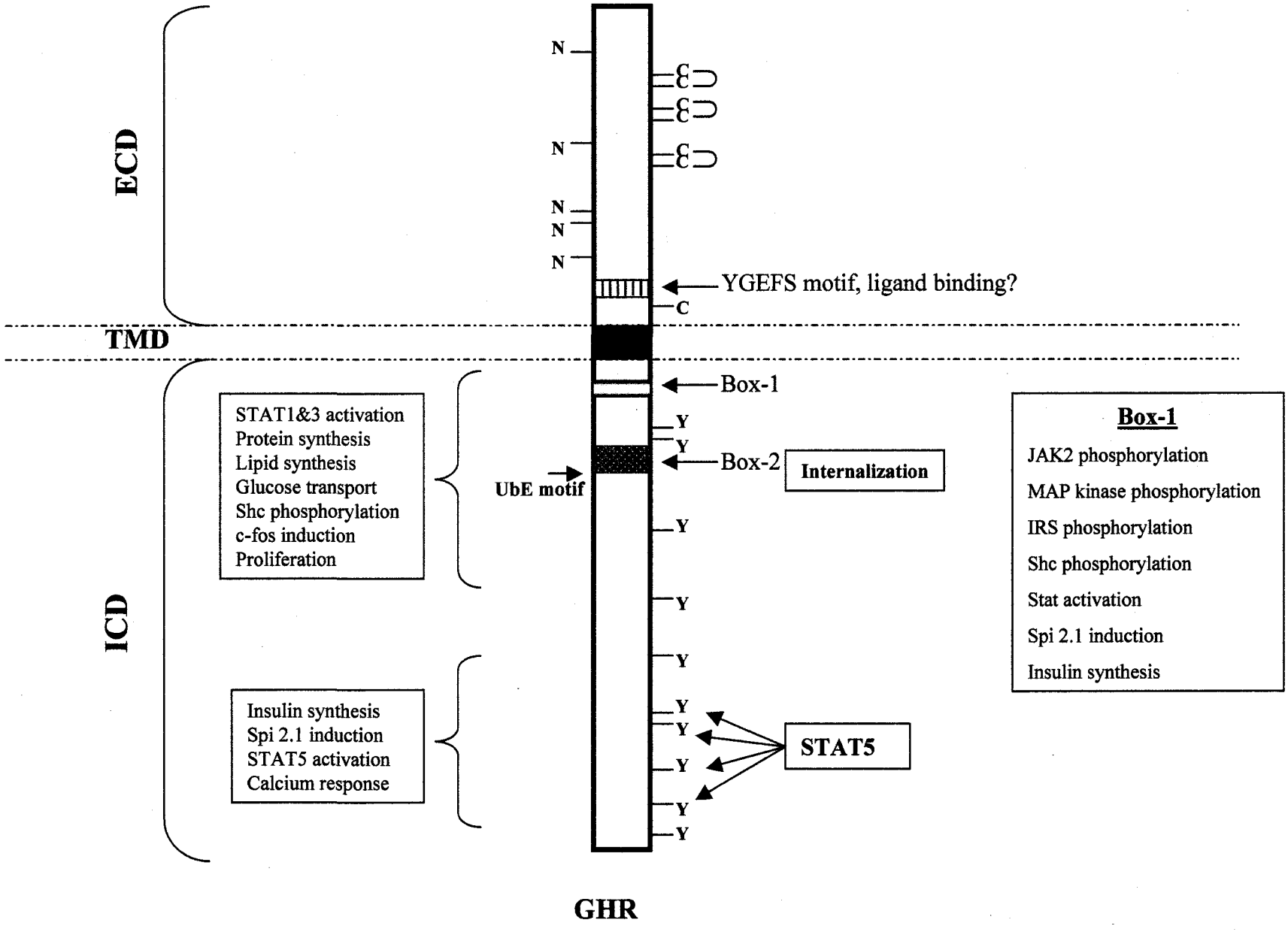

ECD
TMD
ICD
N
N
N
N
N
YGEFS motif, ligand binding?
C
Box-1
Box-2
Internalization
UbE motif
Y
STAT1&3 activation
Protein synthesis
Lipid synthesis
Glucose transport
Shc phosphorylation
c-fos induction
Proliferation
Insulin synthesis
Spi 2.1 induction
STAT5 activation
Calcium response
STAT5
Box-1
JAK2 phosphorylation
MAP kinase phosphorylation
IRS phosphorylation
Shc phosphorylation
Stat activation
Spi 2.1 induction
Insulin synthesis
GHR

Figure I-6

involved in the receptor's internalization (Kopchick & Andry 2000). A specific domain, designated as the UbE motif, has been recognized in the cytoplasmic part of the GHR (Govers *et al.* 1999) (**Figure I-6**). It is a 10-amino-acid-long sequence (DSWVEFIELD) in Box 2 of the cytosolic region of the GHR, and is essential for regulating both GHR ubiquitination and ligand-induced GHR internalization, because a mutation here, for instance F327A, inhibits GHR ubiquitination, internalization and degradation (Gent *et al.* 2002).

2.3. GHR Isoforms

In addition to the full-length, membrane-bound form of GHR, other GHR isoforms, such as the soluble form and several membrane-bound, shorter forms of GHR, have also been identified.

2.3.1. Soluble Form of GHR

Growth hormone binding protein (GHBP) is characterized as a soluble, circulating form of the GHR. It corresponds to the extracellular part of the full-length receptor. Initially isolated from human serum, it is now known that the GHBP is conserved through vertebrate evolution, and is produced in many tissues (Baumann 2001). Two different mechanisms used for the generation of GHBP have been identified, depending on the species analyzed. In mice and rats, GHBP is encoded by a specific mRNA derived from alternative splicing of the primary GHR transcript (Edens *et al.* 1994; Zhou *et al.* 1994). Therefore, the rodent GHBP is composed of the extracellular domain and a short hydrophilic C-terminal extension in place of the trans-membrane and intracellular regions (Edens & Talamantes 1998). In humans, rabbits and other species, the GHBP is generated by proteolytic cleavage of the membrane-bound GHR molecules, a shedding process catalyzed by a metalloprotease, named TACE (tumor necrosis factor (TNF) -α converting enzyme) or ADAM-17 (Zhang *et al.* 2000). In the rhesus monkey, both mechanisms seem to operate, although the relative contribution of each mechanism to GHBP production is not known (Baumann 2001).

GHBP appears to have dual effects on GH actions (Leung *et al.* 2004; Baumann 2001). In plasma, GHBP binds to circulating GH and forms a complex. This GH-GHBP complex

creates a reservoir for circulating GH, protecting it from rapid degradation and clearance, thus prolonging its half-life. These aspects have been proposed to promote GH bioavailability and to enhance GH actions *in vivo*. At the cellular level, GHBP may elicit inhibitory effects on GH actions. *In vitro* studies have shown that GHBP inhibits the binding of GH to GHR in a dose-dependent manner, likely through competition with GHR for ligand binding and sequestration of GH. In addition, a recent study has documented that the GHBP is also intracellularly located, and demonstrated that endogenously produced GHBP can enhance the transcriptional regulatory activity of GH (Graichen *et al.* 2003).

The level of circulating GHBP has been suggested to represent the status of tissue GHR (Baumann 2001), which makes GHBP a useful tool for measuring GHR abundance in plasma. Measuring GHBP has also been clinically useful in detecting mutations in the extracellular region of the receptor, thus helping to diagnose the cause of GH insensitivity (Waters *et al.* 1999).

2.3.2. Truncated Forms of GHR

Two truncated forms of human GHR, named GHR_{1-279} and GHR_{1-277} , have been identified (Ross 1999; Ross *et al.* 1997; Dastot *et al.* 1996). They are encoded by two specific hGHR mRNA variants that result from alternative splicing at exon 9. The GHR_{1-279} is produced by the use of an alternative 3'-acceptor site 26-bp downstream in exon 9, while the GHR_{1-277} results from skipping of the entire exon 9 (Ross *et al.* 1997). These alternative splicing events cause a frame shift and premature terminations, thus giving rise to two truncated receptors of 279 amino acids (GHR_{1-279}) and 277 amino acids (GHR_{1-277}). Both contain the intact extracellular and trans-membrane domains but lack most of the intracellular domains that are required for receptor signaling. As a consequence, these truncated receptor isoforms are still membrane-anchored and can interact with GH similar to full-length hGHR, but are functionally inactive by themselves. By forming nonproductive heterodimers with full-length hGHR, they have been shown to inhibit the function of full-length receptor in a dominant negative manner, which

subsequently modulates the cellular response to GH (Iida *et al.* 1999; Iida *et al.* 1998; Ross *et al.* 1997; Ayling *et al.* 1997).

Both GHR_{1-279} and GHR_{1-277} mRNA variants are readily detectable in a range of human tissues and cell lines, with different tissue expression levels being recognized. For example, the relative abundance of GHR_{1-279} is lowest in postnatal liver and proportionally higher in fat and muscle. In contrast, the relative abundance for GHR_{1-277} is highest in liver, intermediate in muscle and lowest in fat (Ballesteros *et al.* 2000). Generally speaking, the proportion of truncated isoform transcript to full length is <10% for GHR_{1-279} and <1% for GHR_{1-277}, (Ross 1999; Ross *et al.* 1997), but the exact ratio varies between tissues. In liver, fat and muscle, the ratios of full length GHR to GHR_{1-279} and GHR $_{1-277}$ are 27:1:0.1, 14:1:0.002 and 9:1:0.01, respectively. Considering that the truncated receptors act as dominant negative inhibitors of GH signaling, and that the degree of inhibition increases as the ratio of full-length to truncated receptor decreases, the different ratios expressed in individual tissues may have an impact on tissue sensitivity to GH (Ballesteros *et al.* 2000).

The truncated GHR isoforms have been thought to be a major source of the GHBP, as they seem to be proteolyzed more easily than the full-length receptor. It has been proposed that the absence of the intracellular domain that contains the motifs for internalization renders the truncated receptors unable to internalize properly and, thus, exposed externally for long periods to protease attack (Ross *et al.* 1997). Because the relative abundance of the truncated GHR isoform mRNAs is proportionally higher in extrahepatic tissues such as fat and muscle, there may be more GHBP generated locally in these tissues. Indeed, it has been observed that the GHBP level is highly related to fat mass and is reversed upon weight loss (Ross *et al.* 1997). Further studies of the mRNA and protein expression levels of these truncated GHR isoforms in obese patients as well as different fat depot will be desirable.

2.4. GHR Signal Transduction

2.4.1. GH Binding and Receptor Dimerization

The downstream signaling pathways mediated by the GHR are initiated upon binding of GH to the extracellular domain of the GHR (Lanning & Carter-Su 2006). Initially, through high-resolution structural and functional studies of the binding reactions between human GH (hGH) and its receptor (GHBP isoform), it was observed that a single molecule of hGH uses two different sites (site 1 and site 2) to bind two identical receptor molecules, and forms a GH-GHR_2 trimeric complex (de Vos *et al.* 1992; Cunningham *et al.* 1991) (**Figure I-7**). Mutational analysis of each site demonstrated that formation of the ternary complex was sequential. First, hGH binds to one hGHR molecule through its high-affinity site 1, and then the second hGHR molecule was bound to the same GH molecule via the low-affinity site 2 (Wells 1996; Fuh *et al.* 1992). This led to the conclusion that GH binding to the extracellular domain of GHR on target cells promoted receptor dimerization and activated the receptor, presumably by bringing the intracellular domains into close proximity (Wells 1996). The importance of GHR dimerization in GH-dependent signaling has been supported by several studies (Fuh *et al.* 1992; Cunningham *et al.* 1991; Chen *et al.* 1990). For example, an hGH analog, G120R, which is able to interact with the first hGHR via its site 1 but is defective in its site 2 and thus cannot induce dimerization, can not transduce signals and generates antagonist activities both *in vivo* and *in vitro*. Therefore, it was believed that GH-induced receptor dimerization was the initial step for GH signaling (Wells 1996) and that dimerization alone was sufficient for signaling (Waters *et al.* 1999).

However, recent works have challenged this long-believed model. First, Rowlinson et al. showed that receptor dimerization was insufficient for GH-mediated signaling, and that GH-induced change in the receptor conformation is also needed for GH biological responses (Rowlinson *et al.* 1998). Then, the Strous group provided striking evidence to indicate that preformed GHR dimers are present in the endoplastic reticulum (ER) and at the cell surface prior to GH binding (Gent *et al.* 2002) (**Figure I-7**). Furthermore, a recent study done by Brown *et al.* confirmed that unliganded hGHR exists as a dimer using FRET (fluorescence resonance energy transfer), BRET (bioluminescence resonance

energy transfer) and co-immunoprecipitation (co-IP) studies (Brown *et al.* 2005). They also demonstrated that no substantial differences were observed between the crystal structures of liganded and unliganded hGHR extracellular domains. Taken together, these findings have laid the groundwork for a revised model of GHR activation. Indeed, Brown et al. have proposed that GH binds asymmetrically with a preformed GHR dimer via site 1 and site 2; this interaction leads to a relative rotation of the subunits within the dimeric receptors, which activates the tyrosine kinase JAK2 and stabilizes the GHR-JAK2 association, thus initiating intracellular signal transduction (Lanning & Carter-Su 2006; Brown *et al.* 2005).

Figure I-7: Hypothetical model of GHR dimerization.

Dimerization of GHRs occurs in the endoplasmic reticulum (ER) and is maintained during transport to the cell surface. The GHR extracellular domain is composed of an N-terminal GH-binding domain 1 and a membrane-proximal domain 2 which interacts with domain 2 of an adjacent GHR in the GH-$(GHR)_2$ complex. GH binding to GHR induces a conformational change that enables the associated JAK2 tyrosine kinase molecules (regular pentagon) to be close enough to induce cross-phosphorylation. Figure is reproduced with permission from Gent J. *et al.* (2002). *Proc. Natl. Acad. Sci. USA* **99**, 9858-9863.

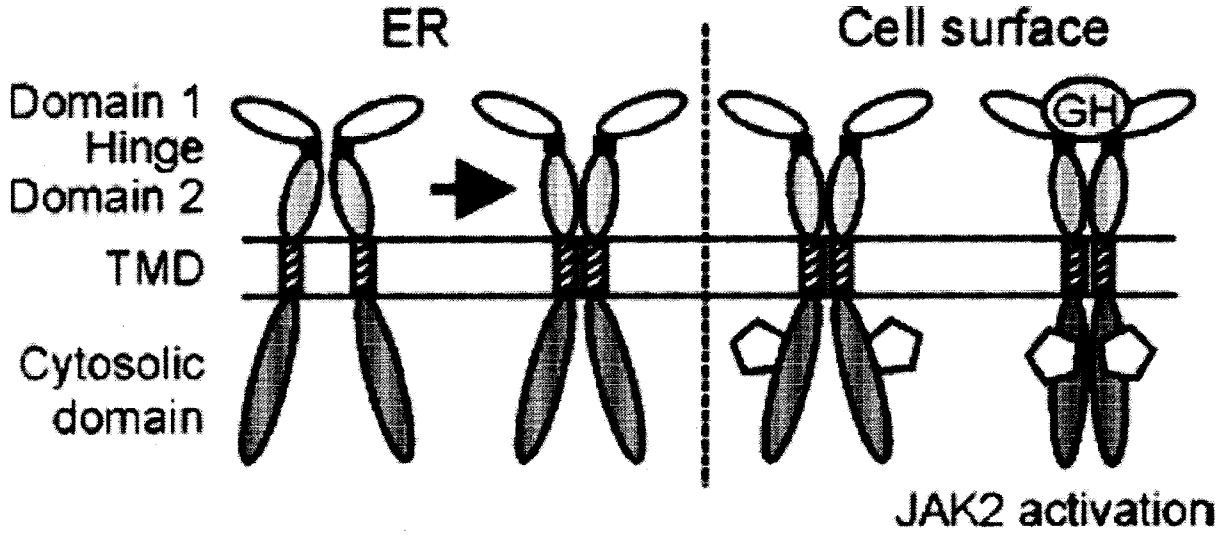

ER
Cell surface
Domain 1
Hinge
Domain 2
TMD
Cytosolic
domain
GH
JAK2 activation

Figure I-7

2.4.2. Activation of JAK2 Tyrosine Kinase

Although the GHR does not possess intrinsic tyrosine kinase activity, GH binding to GHR induces rapid tyrosine phosphorylation of multiple cytoplasmic proteins as a result of the activation of specific receptor-associated tyrosine kinases of the JAK kinase family (Janus kinase or Just another kinase) (Pilecka *et al.* 2007; Bougneres & Goffin 2007).

The JAK kinases represent a distinct family of cytoplasmic protein tyrosine kinases that have been strongly implicated in the signal transduction of many members of the cytokine receptors (Argetsinger & Carter-Su 1996a; Argetsinger & Carter-Su 1996b). Currently, this family includes four members: JAK1, JAK2, JAK3 and Tyk2. They are widely expressed except that JAK3 is expressed primarily in hematopoietic cells. Although JAK kinases do not contain canonical Src-homology 2 (SH2) or 3 (SH3) domains, they possess seven well-conserved JAK Homology domains (JH1-JH7). Of these, the JH1 comprises the typical kinase domain and is important for the enzymatic activity of JAKs, while the JH2 is a pseudokinase domain. A FERM domain is situated at the amino-terminus and has been shown to be responsible for association with cytokine receptors (Gadina *et al.* 2001).

JAK2 is the predominant kinase utilized for GHR signal transduction, and activation of JAK2 is thought to be the key step in initiating GH signaling (Lanning & Carter-Su 2006). In addition, GH has been reported to induce phosphorylation of JAK1 (Smit *et al.* 1996) and JAK3 (Johnston *et al.* 1994), but with much lower activation levels than for JAK2 (Carter-Su *et al.* 1996). Whether GH activates Tyk2 is still unclear, but the association of GHR with Tyk2 has been detected in human liver cells (Zhu *et al.* 2001).

It was initially believed that GH binding promoted the recruitment of JAK2 to GHR (Argetsinger *et al.* 1993). Now, it is accepted that JAK2 constitutively associates with the GHR, but receptor-ligand binding stabilizes the preformed receptor-JAK complex and facilitates juxtapositioning and cross-phosphorylation of the kinase activation loops (Pilecka *et al.* 2007). The interaction between JAK2 and GHR is mediated through the N-terminal FERM domain of JAK2 and the membrane-proximal, proline-rich Box 1 region in the cytoplasmic domain of the GHR (Lanning & Carter-Su 2006; He *et al.* 2003; Frank

et al. 1995; Vanderkuur *et al.* 1994). Mutation or deletion of Box 1 in the GHR abolishes GHR-JAK2 complex formation and GH-dependent activation of JAK2 (Herrington & Carter-Su 2001). Moreover, the Frank group recently demonstrated that JAK2, in addition to being a signal transducer, dramatically increases the half-life of mature GHR, partly by preventing constitutive GHR downregulation (He *et al.* 2005; He *et al.* 2003). The protective effect requires the presence of GHR's Box-1 element and the intact JAK2 FERM domain, but not the JAK2 kinase-like or kinase domains (Deng *et al.* 2007).

Activated JAK2 will then phosphorylate the tyrosine residues in the cytoplasmic domain of the GHR. Phosphorylated tyrosine residues in JAK2 and in GHR serve as docking sites for a variety of intracellular signaling molecules that contain phosphotyrosine binding domains, such as SH2 or PTB domains. Recruitment of these proteins to the GH-GHR-JAK2 complex and their subsequent activation initiates multiple signaling cascades and produces the pleiotropic physiological effects of GH. Among the direct substrates activated by the GH-GHR-JAK2 complex are: 1) STATs, the latent transcription factors known as Signal Transducers and Activators of Transcription; 2) SH2-containing proteins, such as the adaptor proteins Shc or Grb2, which are involved in the Ras and MAP kinase pathway; 3) insulin receptor substrate proteins, IRS-1 and IRS-2 , which initiate the PI-3 kinase pathway; 4) phospholipase C, activating the PKC pathway; and 5) several other non-receptor tyrosine kinases, such as c-Src or Focal Adhesion Kinase (FAK) (Pilecka *et al.* 2007; Lanning & Carter-Su 2006; Leung *et al.* 2004; Zhu *et al.* 2001; Herrington & Carter-Su 2001) (**Figure I-8**).

Although JAK2 activation plays a central role in GHR signal transduction for many GH-induced biological effects, JAK2 is dispensable for some GH-induced processes, such as the induction of calcium entry or receptor internalization (Pilecka *et al.* 2007).

Figure I-8: Schematic representation of the main GHR signaling pathways stimulated by GH.

Upon GH binding, the activated GHR-JAK2 complex triggers the phosphorylation of several intracellular proteins and initiates multiple signaling events (see details in the text). Many of these pathways are shared by other cytokines and growth factors. Figure is adapted with permission from Leung K.C. *et al.* (2004) *Endo. Rev.* **25**, 693-721.

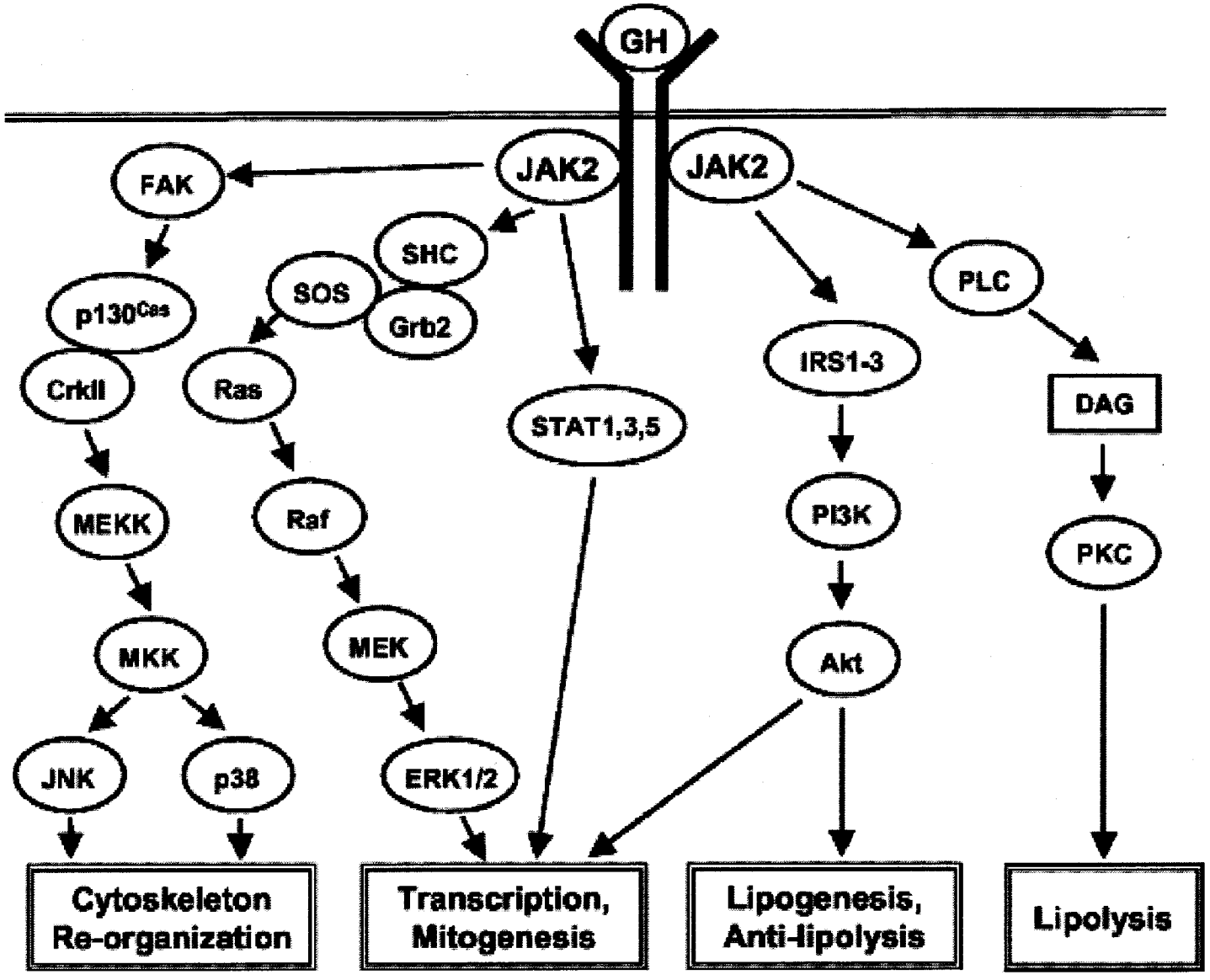

GH
JAK2
JAK2
FAK
SHC
SOS
Grb2
p130Cas
CrkII
Ras
STAT1,3,5
IRS1-3
PLC
DAG
MEKK
Raf
PI3K
PKC
MKK
MEK
Akt
JNK
p38
ERK1/2
Cytoskeleton Re-organization
Transcription, Mitogenesis
Lipogenesis, Anti-lipolysis
Lipolysis

Figure I-8

2.4.3. GHR Signaling Pathways

The main signaling pathways stimulated by GH are shown in Figure 8. Many of these pathways are shared by other cytokines and growth factors.

2.4.3.1. STATs pathway

A number of GH biological effects result from modulations of gene expression and involve various transcription factors (TFs). Among those characterized TFs, members of the STAT family proteins have been shown to be particularly important for JAK2-mediated GH signaling (Lanning & Carter-Su 2006) (**Figure I-8**).

STAT proteins are a group of SH2-domain containing, cytoplasmic transcription factors, which possess the ability to transduce signals from the cell membrane to the nucleus and to activate gene transcription. Originally identified in the IFN signaling pathway, it is now known that STAT proteins are implicated in the signal transduction pathways for a large number of cytokines and growth factors, including GH, and play important roles in activating the transcription of their target genes (Herrington *et al.* 2000). Seven mammalian STATs have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. The two STAT5s are isoforms, encoded by two highly-related genes (~95% homology) (Shuai 1999). All STAT proteins share conserved domain structures: the C-terminal transactivation domain (TAD); the SH2 domain, for binding to phosphorylated tyrosine residues; the central DNA-binding domain; a coiled-coil domain, for potential protein-protein interactions; and the N-terminal domain, involved in dimerization and tetramerization (Lim & Cao 2006).

STAT proteins are thought to be mainly present as latent monomers in the cytoplasmic pool before activation (Levy & Darnell, Jr. 2002; Sehgal 2000). Activation of STATs requires tyrosine phosphorylation-dependent dimerization, a process facilitated by GH or other cytokines following JAK activation. Upon GH binding to GHR and activation of JAK2, cytoplasmic latent STAT proteins are recruited through their SH2 domains to the phosphorylated tyrosine residues in the GHR-JAK2 complex, where they are subsequently phosphorylated by JAK2 on their conserved C-terminal tyrosine. Once STAT proteins are phosphorylated, they dissociate from the GHR-JAK2 complex, form

homo- or heterodimers through their reciprocal SH2 domain-phosphotyrosine interactions, translocate into the nucleus, bind specific DNA sequences (GAS-like elements (GLE) or Sis-inducible elements (SIE), interact with other nuclear factors and activate gene expression (Murray 2007; Herrington *et al.* 2000). STAT1, STAT3, STAT4, STAT5a and STAT5b all form homodimers. STAT1 and STAT2, and STAT1 and STAT3 can also form heterodimers (Levy & Darnell, Jr. 2002).

A given ligand can activate one or more STAT proteins. GH has been shown to induce tyrosine phosphorylation of and to activate STAT1, STAT3, STAT5a and STAT5b in several cell types, including preadipocytes and hepatocytes (Moutoussamy *et al.* 1998). But above all, STAT5 is the predominant STAT utilized by GH (Zhu *et al.* 2001). Many past and present studies have demonstrated the physiological importance of STAT5 in a variety of GH actions (Rowland *et al.* 2005b; Herrington *et al.* 2000). STAT5b deficient ($STAT5b^{-/-}$) male mice display a pronounced impairment in postnatal body growth and they have a major loss of multiple, differentiated responses associated with the sexual-dimorphic pattern of GH secretion (Waxman 2000; Teglund *et al.* 1998; Udy *et al.* 1997). Although the responses are similar to those observed in GH-deficient mice, $STAT5b^{-/-}$ mice are not GH-deficient. Indeed, elevated plasma GH, reduced circulating IGF-I and the development of obesity were seen in $STAT5b^{-/-}$ mice, all characteristics similar to the GH insensitivity syndrome (Udy *et al.* 1997). In addition, Fain *et al.* reported the loss of GH-stimulated lipolysis in adipose tissue of STAT5b knockout female mice, suggesting that the lipolytic action of GH involves the STAT5b protein (Fain *et al.* 1999).

In contrast, STAT5a knockout mice ($STAT5a^{-/-}$) of either sex did not reveal any alteration in body growth, serum IGF-I levels or expression of GH-regulated genes (Teglund *et al.* 1998), but did show defects in mammary gland development and lactogenesis, suggesting that STAT5a is necessary for PRL function but plays a minor role in GH actions, or that the loss of STAT5a can be compensated for by STAT5b (Herrington *et al.* 2000). Indeed, STAT5a and 5b double knockout mice show more severe impairments in body growth and liver gene expression in both sexes (Teglund *et al.* 1998). Therefore, STAT5a and STAT5b possess both redundant and distinct functions in

GH signal transduction, and it is STAT5b that is responsible for the GH-dependent postnatal body growth (Bougneres & Goffin 2007; Herrington *et al.* 2000).

Although activation of STAT1, STAT3 and STAT5 by GH are all mediated by JAK2 phosphorylation, the mechanism varies. For activation of STAT1 and STAT3, JAK2 phosphorylation itself is sufficient. In contrast, to obtain maximum activation of STAT5 by GH, the phosphotyrosine residues in the intracellular domain of the GHR are also required, apart from JAK2 phosphorylation itself (Zhu *et al.* 2001; Moutoussamy *et al.* 1998). The elegant knock-in studies by Rowland and coworkers identified four tyrosine residues as STAT5 recruitment sites in the cytoplasmic regions of the mouse GHR, and showed a strong correlation among STAT5, IGF-I levels and body size (Bougneres & Goffin 2007; Rowland *et al.* 2005b). Thus, JAK2 phosphorylates STAT5 through direct interactions between JAK2, GHR and STAT5.

2.4.3.2. MAP kinase (MAPK) pathway

Mitogen activated protein kinases (MAPKs) are a specific class of serine/threonine protein kinases that mediate intracellular phosphorylation events linking various extracellular signals stimulated by many hormones and growth factors (Roux & Blenis 2004). Signaling through the MAP kinase cascade can lead to a variety of cellular responses including embryogenesis, cell proliferation and differentiation as well as response to various stresses (Mishra *et al.* 2006). To date, more than a dozen mammalian MAPK family members have been discovered (Zhu *et al.* 2001). Among them, the p44/42 MAPK (also known as extracellular signal-regulated kinase 1 (ERK1) and 2 (ERK2)), the JNK/SAPK (C-Jun N-terminal kinase/stress activated protein kinase) and the p38 MAPK have been shown to be phosphorylated and activated in response to GH stimulation in several cell types (Zhu *et al.* 2001; Zhu & Lobie 2000; Zhu *et al.* 1998; Vanderkuur *et al.* 1997; Campbell *et al.* 1992).

The best-characterized MAPK pathway regulated upon GH stimulation is the p44/42 MAPK pathway, which is often involved in GH-induced cell growth and differentiation (Piwien-Pilipuk *et al.* 2002a). GH activates p44/42 MAPK through the sequential activation of Shc, growth factor receptor bound protein (Grb) 2, son of sevenless (SOS),

Ras, Raf, MAP/ERK and MAPK (Pilecka *et al.* 2007; Lanning & Carter-Su 2006; Vanderkuur *et al.* 1997) (**Figure I-8**). GH activation of p44/42 MAPK is mainly linked to JAK2 phosphorylation, but there was one report suggesting that GH might also regulate p44/42 MAPK through a JAK2-independent pathway (Zhu *et al.* 2002). In addition, a Japanese group has documented that GH stimulates tyrosine phosphorylation of the EGF receptor (EGFR) and stimulates MAPK activity at the same time; they propose that GH may activate the MAPK pathway through EGFR signaling (Yamauchi *et al.* 1997).

The substrates regulated by GH-dependent activation of the p44/42 MAPK pathway include downstream kinases such as p70S6K and p90RSK, phospholipase A2, and some transcription factors such as Elk-1, C/EBPβ, c-jun and STATs (Lanning & Carter-Su 2006; Zhu *et al.* 2001). Elk-1, also known as p62 ternary complex factor (TCF), is an Ets-related transcription factor that mediates growth factor stimulation of the c-fos promoter (Price *et al.* 1995). In 3T3-F442A preadipocytes, it has been shown that Elk-1 is serine phosphorylated by p44/42 MAPK in response to GH. Phosphorylated Elk-1 mediates transcription activation together with serum response factor (SRF) via binding to the serum response element (SRE)/Ets sites on the promoter of target genes, including *c-fos*, *egr-1* and *junB* (Piwien-Pilipuk *et al.* 2002a; Clarkson *et al.* 1999; Hodge *et al.* 1998). Using the same cell system, the Schwartz group recently showed that GH stimulates C/EBPβ phosphorylation through p44/42 MAPK and suggests that this phosphorylation contributes to C/EBPβ transcriptional activation and nuclear translocation (Piwien *et al.* 2003; Piwien-Pilipuk *et al.* 2002c). Furthermore, recent results from Yang et al. indicate that, in 3T3-F442A murine preadipocytes, GHR, ERK1/2 and the adaptor protein Grb2 are selectively enriched in caveolae membranes, a cholesterol-rich plasma membrane microdomain. GH stimulation results in accumulation and activation of the p44/42 MAPK signaling in this fraction, but not STAT signaling (Lanning & Carter-Su 2006; Yang *et al.* 2004a). However, GH-dependent IGF-I stimulation in humans does not seem to require p44/42 MAPK (Rosenfeld *et al.* 2007), because GH-stimulated normal p44/42MAPK signaling is insufficient to compensate for the absence of STAT5b-activated IGF-I expression resulting from defects in STAT5b.

Activation of the JNK/SAPK pathway in response to GH has been demonstrated to be mediated by a multi-protein signaling complex centered around the FAK-associated protein p130 (Cas) and the adaptor protein CrkII (Zhu *et al.* 1998) (**Figure I-8**). Overexpression of CrkII enhances GH-stimulated JNK/SAPK activity. Since JNK/SAPK is implicated in many cellular processes, including transcriptional regulation and apoptosis, it is likely that GH uses the JNK/SAPK pathway to produce some of its physiological effects (Zhu *et al.* 2001).

Like JNK/SAPK, p38 MAPK is mainly activated by environmental stress and inflammatory cytokines, but not appreciably by mitogenic stimuli. Several cellular targets have been shown to be phosphorylated by activated p38 MAPK, including phospholipase A2, protein Tau, and transcription factors ATF-1 and -2, NF-κB, p53, Elk-1 and Ets-1, indicating that p38 MAPK activity is critical for a normal immune system and inflammatory responses (Roux & Blenis 2004). The Lobie lab has demonstrated that hGH can phosphorylate and activate p38 MAPK in a JAK2-dependent manner (Zhu & Lobie 2000) (**Figure I-8**). hGH-dependent activation of the p38 MAPK stimulates transcriptional activation of transcription factors ATF-2 (activating transcription factor) and CHOP (C/EBP homologous protein), but not Elk-1. Enhanced CHOP transcription promotes cell survival in response to autocrine GH production, leading to an increase in mammary carcinoma cell numbers (Mertani *et al.* 2001). In addition, p38 MAPK has been demonstrated to be involved in GH-stimulated reorganization of the actin cytoskeleton (Zhu & Lobie 2000) (**Figure I-8**). However, Hodge et al only observed slightly increased p38 MAPK activity in response to GH-stimulation in 3T3-F442A cells (Hodge *et al.* 1998), suggesting that cell-type specificities are present.

2.4.3.3. IRS and the PI-3 kinase pathway

GH has also been shown to activate the PI-3 kinase pathway (Lanning & Carter-Su 2006; Leung *et al.* 2004; Zhu *et al.* 2001) (**Figure I-8**). One possible mechanism is through inducing tyrosine phosphorylation of the adaptor proteins insulin receptor substrate (IRS)-1 and -2 and their association with the p85 regulatory subunit of PI-3 kinase although neither the GHR nor JAK2 contain the NPXY motif that is required for direct

association with the IRSs (Pilecka *et al.* 2007; Zhu *et al.* 2001). Other data suggest that GH might activate PI-3 kinase via interaction with the JAK2-dependent adaptor CrkII or via direct binding of the p85 subunit to the GHR (Lanning & Carter-Su 2006; Goh *et al.* 2000; Moutoussamy *et al.* 1998). In any case, GH and insulin seem to use different mechanisms for stimulation of IRS phosphorylation (Zhu *et al.* 2001).

Activated PI-3 kinase catalyzes the production of phosphatidylinositol-3, 4, 5-triphosphate (IP_3) and triggers several downstream signaling pathways, including regulation of glucose uptake, lipogenesis, cell cycle progression, p70S6K activation, and inhibition of apoptosis via Akt kinase (Lanning & Carter-Su 2006). The ability of GH to stimulate the PI 3'-kinase pathway provides a biochemical basis for several responses shared by insulin and GH observed in a variety of cell types (Piwien-Pilipuk *et al.* 2002a) and is responsible for the acute insulin-like effects of GH stimulation (Pilecka *et al.* 2007) (**Figure I-8**).

2.4.3.4. Protein kinase C (PKC) pathway

Several lines of evidence suggest that PKC plays a role in GH-induced signal transduction (Piwien-Pilipuk *et al.* 2002a; Zhu *et al.* 2001; Nam & Lobie 2000) (**Figure I-8**). Depletion of PKC activity by chronic treatment of cells with phorbol ester 13-acetate (PMA) or by the addition of PKC inhibitors results in a significant decrease in several GH-induced cellular events, such as lipogenesis, induction of c-fos expression, enhanced binding of nuclear proteins to a C/EBP DNA binding site-containing oligonucleotide, increased intracellular Ca^{++} concentrations and activation of p44/42 MAPK, suggesting that these GH actions are mediated by the PKC signaling pathway (Zhu *et al.* 2001; Carter-Su *et al.* 1996). In addition, the Frank lab has demonstrated that PMA-induced activation of PKC causes metalloprotease-mediated GHR proteolysis and GHBP shedding in several cell lines with endogenous or expressed GHR (Guan *et al.* 2001), suggesting that this GHR processing event is PKC-dependent. Interestingly, they also observed that pretreatment with MAPK inhibitors markedly reduced this event and proposed that the MAPK pathway is involved in the regulation of this PKC-dependent GHR modulation process.

PKC is a family of serine/threonine kinases comprising at least 12 isoforms that phosphorylate and activate a variety of target proteins (Piwien-Pilipuk *et al.* 2002a). For most of the PKC isoforms, their activation and translocation from the cytosol to the plasma membrane depend on the second messenger diacylglycerol (DAG), which is produced by phospholipase C (PLC)-catalyzed hydrolysis of the phospholipid PIP2 (Zhu *et al.* 2001; Newton 1997). GH has been documented to use different mechanisms to induce a rapid and transient increase of DAG in several cell types (Carter-Su *et al.* 1996).

Whether GH-dependent activation of PKC depends on JAK2 or includes JAK2-independent mechanisms has not been definitively proven. The finding that the PI-3 kinase inhibitor (wortmannin) inhibits GH-stimulated DAG production in rat adipocytes (He *et al.* 1995) suggests that GH in certain cell types may activate PKC using a PI-3 kinase related pathway (Carter-Su *et al.* 1996).

2.4.3.5. Ca^{++} signaling pathway

An increase in intracellular free calcium concentrations in response to GH treatment has been observed in several cell types, including freshly isolated rat adipocytes, IM-9 lymphocytes and CHO cells expressing rat GHR. This GH-induced Ca^{++} increase appears to depend on activation of the L-type calcium channel through a mechanism involving phospholipid hydrolysis and PKC activation (Piwien-Pilipuk *et al.* 2002a; Zhu *et al.* 2001). Several GH actions, like induction of *Spi 2.1* gene expression and being refractory to insulin-like effects in adipocytes, seem to be mediated by a GH-stimulated increase in Ca^{++}. Mutagenesis studies suggest that GH-induced Ca++ activation requires the C-terminal half of GHR but not the JAK2-associated Box1 region (Carter-Su *et al.* 1996). To date, regulation of intracellular Ca^{++} is the only GHR signaling cascade that is not regulated by JAK2 activation (Pilecka *et al.* 2007).

2.4.4. Negative Regulation of GHR Signaling

GH is secreted in a pulsatile manner and this GH secretion pattern is thought to be an important factor in regulating body growth rate and sex-specific gene expression. Hence, the intensity and kinetics of signals generated from the GH-activated GHR-JAK2

complex is expected to shape GH actions (Herrington & Carter-Su 2001). For example, the JAK-STAT signal transduction pathway activated upon GH binding is both rapid and transient, whereas prolonged JAK2 activation has been linked to several pathogenic consequences, most notably oncogenesis. Therefore, precise regulation of GHR signaling is vitally important for the proper maintenance of GH physiological functions (Lanning & Carter-Su 2006).

To achieve this precise control, limitation of the magnitude and duration of signals through negative regulation is indispensable (Zhu *et al.* 2001). Several intracellular mechanisms for limiting GHR signaling have been identified. They were comprehensively reviewed in a recent publication (Flores-Morales *et al.* 2006). These negative regulatory processes occur at several different levels, including receptor turnover (Strous & Gent 2002), inactivation of intracellular signaling by dephosphorylation (Stofega *et al.* 1998) or actions of the negative regulators (Krebs & Hilton 2001), degradation of signal intermediates, as well as other mechanisms that are not completely understood.

2.4.4.1. GHR turnover

The initial termination of GHR signaling is presumably achieved by GHR turnover, a process of removal of the cell-surface GH-bound receptor (Zhu *et al.* 2001). Two distinct biochemical processes can lead to GHR turnover: proteolytic cleavage and receptor internalization (Flores-Morales *et al.* 2006; Frank 2001).

(A) GHR proteolysis

As previously mentioned in the GHBP section, GHR proteolysis occurs due to a combined action of two proteases. First, a metalloprotease cleaves the receptor at the cell surface near the transmembrane domain, liberating the GHBP into the circulation. The membrane-bound remnant is subsequently digested by the γ-secretase complex and targeted for proteosomal degradation (Schantl *et al.* 2004; Frank 2001). However, this mechanism is unlikely to contribute significantly to down-regulation of GH-induced signaling because GH binding to GHR inhibits GHR proteolysis (Flores-Morales *et al.* 2006; Piwien-Pilipuk *et al.* 2002a; Frank 2001; Zhang *et al.* 2001).

(B) GHR internalization

In contrast, GHR is rapidly internalized in the presence of GH (Piwien-Pilipuk *et al.* 2002a). Removal of cell surface GHR by endocytosis is thought to be an early and essential step in termination of GH-induced signal transduction (Flores-Morales *et al.* 2006; Zhu *et al.* 2001). Although GHR is constitutively (ligand-independently) internalized, GH binding enhances the rate of GHR internalization (He *et al.* 2005; Strous & van 2002; Strous & Govers 1999). Internalization is mediated through the clathrin-coated pits and caveolae (Flores-Morales *et al.* 2006; Strous *et al.* 2004; Zhu *et al.* 2001). Once internalized, most of the GH-GHR complex is degraded either in the lysosome or in the proteosome, and only a few receptors will be recycled from the endosomes to the cell surface (Flores-Morales *et al.* 2006) (**Figure I-9A**).

Ligand-induced GHR internalization is regulated by both the ubiquitin system and the proteasome (Alves dos Santos *et al.* 2001). Inactivation of the ubiquitin conjugation system results in an accumulation of non-ubiquitinated GHRs at the plasma membrane, and the abolition of GH-dependent uptake and degradation of the GHR (Strous *et al.* 1996). Both GHR ubiquitination and ubiquitin-dependent GHR endocytosis rely on the UbE motif (Govers *et al.* 1999) located in Box-2 (Strous *et al.* 2004; Strous & Govers 1999). However, it remains unknown how the UbE motif mediates GHR ubiquitination. It is possible that it recruits the ubiquitin conjugation system to the GHR, enabling ubiquitination of the GHR and some of its associated molecules. Such protein complexes may facilitate the interaction of GHR with the clathrin-coated pits (Flores-Morales *et al.* 2006; Lanning & Carter-Su 2006).

Recent studies have also demonstrated that proteosome inhibition can prolong GH-induced activity of both GHR and JAK2, presumably through extension of GHR and JAK2 phosphorylation. These findings provide further support for the conclusion that the ubiquitin-proteosome system is implicated in the negative regulation of GHR signaling (Alves dos Santos *et al.* 2001).

Figure I-9: Mechanisms of negative regulation of GHR signaling.

A) *Downregulation of GHR signaling via receptor internalization.* Removal of cell surface GHR by internalization is thought to be an early and essential step in termination of GH-induced signal transduction. Internalization is mediated through the clathrin-coated pits and caveolae [not shown]. Once internalized, the GH-GHR complex is degraded either in the lysosome or in the proteosome. GHR internalization requires an intact ubiquitin system. In the absence of GH, GHR ubiquitination and internalization occurs constitutively, while GH binding enhances the rate of GHR internalization. Abbreviations: SHP, SH2 domain-containing protein tyrosine phosphatase; PTP, phosphotyrosine phosphatase; SOCS, suppressor of cytokine receptor signaling; STAT, signal transducer and activator of transcription; SUMO, small ubiquitin-like modifier; CIS, cytokine-inducible SH2-domain protein; E3-UL, E3-ubiquitin ligase.

B) *Termination of GHR signaling by dephosphorylation.* Protein tyrosine phosphatases (purple) specifically recognize and dephosphorylate the receptor, JAK2 or STATs, resulting in signaling termination.

C) *Deactivation of GHR signaling by SOCS proteins.* SOCS 1, 2, 3 and CIS are induced in response to GH and are recruited to the activated GHR and JAK2 to downregulate GHR signaling. Different SOCSs exert suppression on GHR signal transduction via different mechanisms (details in the text). In addition, SOCS proteins are part of the E3 ubiquitin ligase complex and lead to GHR ubiquitination and degradation upon association with the activated receptor.

D) *Deactivation of STAT signaling cascade by PIAS proteins.* PIAS family proteins are SUMO ligases. They are constitutively expressed and specifically block STATs by interfering with their activity as transcription factors and by other mechanisms; each PIAS inhibits its own subset of STATs.

Abbreviations: SHP, SH2 domain-containing protein tyrosine phosphatase; PTP, phosphotyrosine phosphatase; SOCS, suppressor of cytokine receptor signaling; STAT,

signal transducer and activator of transcription; SUMO, small ubiquitin-like modifier; CIS, cytokine-inducible SH2-domain protein; E3-UL, E3-ubiquitin ligase.

Figure I-9A is adapted from Flores-Morales A. *et al.* (2007). *Mol. Endo.* **20**, 241-253. Figures I-9B to I-9D are adapted with permission from Pilecka I. *et al.* (2006) *Trends in Endocrinol. & Metabol.* **18**, 12-18.

A.

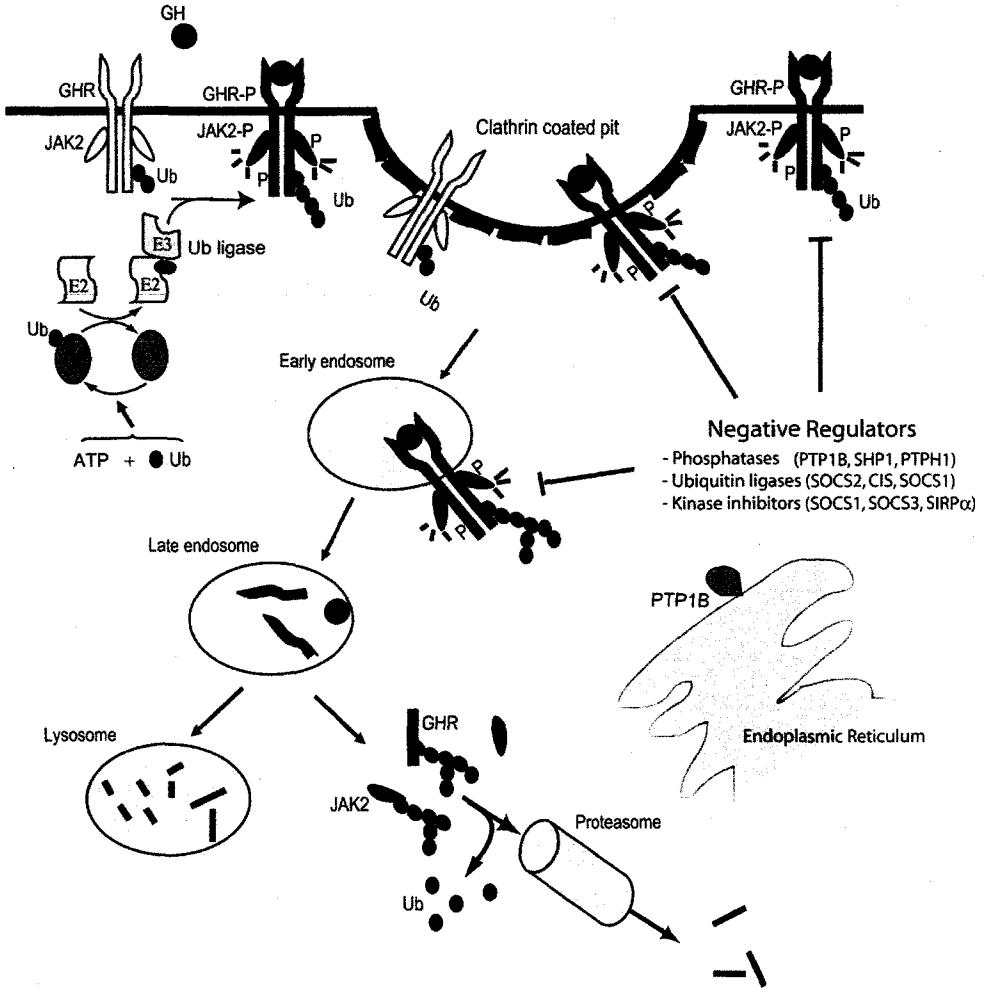
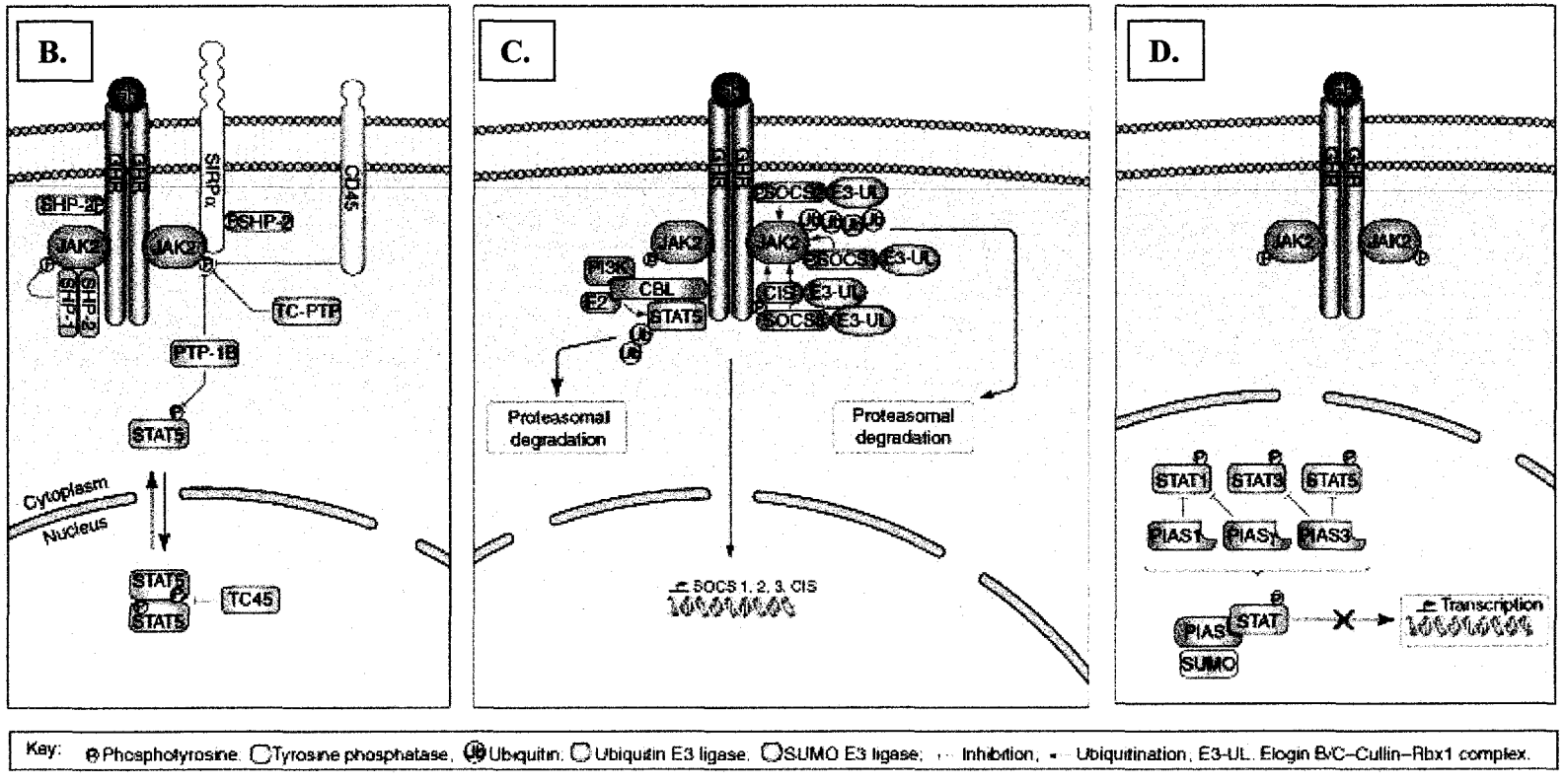

GH
GHR
GHR-P
JAK2
JAK2-P
P
Ub
E3
Ub ligase
E2
E2
Ub
ATP + Ub
Clathrin coated pit
Early endosome
Negative Regulators
- Phosphatases (PTP1B, SHP1, PTPH1)
- Ubiquitin ligases (SOCS2, CIS, SOCS1)
- Kinase inhibitors (SOCS1, SOCS3, SIRPα)
Late endosome
PTP1B
Endoplasmic Reticulum
Lysosome
GHR
JAK2
Proteasome

Figure I-9



Key: Phosphotyrosine, Tyrosine phosphatase, Ubiquitin, Ubiquitin E3 ligase, SUMO E3 ligase, Inhibition, Ubiquitination, E3-UL, Elogin B/C–Cullin–Rbx1 complex.

Figure I-9

2.4.4.2. Termination of GHR signaling by dephosphorylation

Protein phosphatases are major players in down-regulation of GHR signal transduction (Flores-Morales *et al.* 2006; Piwien-Pilipuk *et al.* 2002a). Pre-incubation of phosphatase inhibitors prolongs tyrosine phosphorylation of GH-stimulated JAK2 and STAT5 (Gebert *et al.* 1999). Several protein tyrosine phosphatases (PTPs) have been reported to negatively regulate GHR signaling, including SHP-1, SHP-2, PTP1b and PTP-H1 (Flores-Morales *et al.* 2006). They induce dephosphorylation both at the level of the receptor and its downstream molecules (Pilecka *et al.* 2007).

SHP-1 is primarily expressed in haematopoietic cells (Neel *et al.* 2003). Through binding with its SH2-domain to phosphotyrosine residues of GH-activated JAK2, SHP-1 limits the extent and duration of GH-stimulated JAK2 activation (**Figure I-9B**). GH also can activate SHP-1 and induce its translocation into the nucleus where it binds to phosphorylated STAT5b and results in an attenuation of STAT5 activity (Flores-Morales *et al.* 2006). Consistent with these findings, SHP-1 deficient ("motheaten") mice display prolonged hepatic GH signaling in comparison to normal mice, due to extended JAK2 and STAT5 phosphorylation in response to GH stimulation (Hackett *et al.* 1997).

In contrast to SHP-1, SHP-2 is ubiquitously expressed in mammalian tissues (Salmond & Alexander 2006) and directly associates with the GHR (Stofega *et al.* 2000b) (**Figure I-9B**). SHP-2 appears to exert dual roles in the regulation of GHR signaling. Kim *et al.* proposed a positive role for SHP-2 in GH signaling when they observed that coexpression of a catalytically inactive SHP-2 mutant significantly reduced GH-induced transactivation of a c-fos enhancer driven luciferase reporter (Kim *et al.* 1998). In contrast, Stofega *et al.* concluded that GHR-bound SHP-2 negatively regulates GHR/JAK2 and STAT5b signaling by showing that abrogation of SHP-2 binding to the GHR prolongs the duration of tyrosine phosphorylation of the receptor, JAK2 and STAT5b, as well as markedly increases GH-induced STAT5b transcriptional activity (Stofega *et al.* 2000b).

Four other PTPs bound to phosphorylated GHR in response to GH have recently been identified: PTP1b, PTP-H1, SAP-1 (stomach-cancer associated PTP1) and TC-PTP. Only

PTP1b and PTP-H1 are capable of inducing dephosphorylation of the GHR (Pasquali *et al.* 2003). Interestingly, PTP1b is an enzyme located mainly at the surface of ER where it exerts its phosphatase activity. Given the special cellular location of PTP1b, Flores-Morales et al have proposed that the prolonged hepatic GH-dependent signaling under ER stress could be related to inactivation of this phosphatase (Flores-Morales *et al.* 2006).

2.4.4.3. Deactivation of GHR signaling by negative regulators

In addition to phosphatases, three other groups of negative regulators involved in down-regulation of GH signaling have been identified. They are the Suppressor of Cytokine Receptor Signaling (SOCS) proteins, the Protein Inhibitors of Activated STATs (PIAS) and Signal Regulatory Protein α (SIRP-α) (Pilecka *et al.* 2007; Flores-Morales *et al.* 2006). The SOCS proteins and PIAS proteins are mainly implicated in regulation of the JAK-STAT pathway.

(A) Suppressor of cytokine receptor signaling (SOCS) proteins

SOCS proteins are general inhibitors of cytokine signal transduction. Members of the SOCS family form a classic negative feedback loop with key actions involving inhibition of the JAK-STAT signaling cascade (Rakesh & Agrawal 2005). There are eight members of the SOCS family: SOCS-1 through SOCS-7 and the cytokine-inducible SH2-domain protein, CIS. Each SOCS member contains a central SH2 domain flanked by a variable N-terminal domain and a conserved 40-amino-acid C-terminal domain, the SOCS box (Wormald & Hilton 2004). The SH2-domain mediates the binding of SOCS proteins to phosphotyrosine residues in specific cytokine receptors and JAKs. In addition, the SOCS box has been found to interact with the ubiquitin E3-ligase complex and, thus, mediates the ubiquitination of SOCS-associated proteins, leading to their ubiquitin-linked proteasomal degradation (Alexander 2002).

SOCS proteins are generally present at low levels in unstimulated cells, but their expression levels are rapidly induced in response to signals from various cytokines and growth factors, including GH. However, the pattern and kinetics of induction appear to be both factor- and tissue-specific (Cooney 2002). SOCS-1, SOCS-3 and CIS are rapidly

and transiently induced following GH stimulation, while SOCS-2 steadily increases with time (Flores-Morales *et al.* 2006). Administration of GH to mice primarily induces SOCS-3 and CIS in liver, but preferentially induces SOCS-2 and CIS in the mammary gland (Cooney 2002). In many cases, the transcriptional activation of the SOCS genes is mediated by STAT proteins (Cooney 2002). For example, expression of CIS is modulated by STAT5, with STAT5 binding sites being identified in the CIS promoter (Verdier *et al.* 1998).

All four SOCS members have been shown to exert negative actions on GHR signaling when ectopically expressed in cell lines (Ram & Waxman 1999; Hansen *et al.* 1999; Adams *et al.* 1998). However, the mechanisms by which these four SOCS proteins exert suppression on GHR signal transduction are different (Ram & Waxman 1999). To date, at least three mechanisms are recognized: (i) direct JAK2 inhibition, (ii) competition with positive regulators binding to the GHR, and (iii) mediating ubiquitin-dependent proteasomal degradation (Pilecka *et al.* 2007).

SOCS-1 acts at the level of JAK2 kinase by binding directly to the catalytic loop of JAK2 through its SH2-domain and blocking JAK2 kinase activity by its kinase inhibitory region (KIR) motif, thus leading to reduced JAK2 and STAT5 phosphorylation (Flores-Morales *et al.* 2006; Nicholson *et al.* 1999; Yasukawa *et al.* 1999; Narazaki *et al.* 1998). In addition, SOCS-1 is thought to mediate the ubiquitin- proteasome-dependent degradation of the activated JAK2, through its association with ubiquitin ligase (Ungureanu *et al.* 2002) (**Figure I-9C**).

SOCS-3 binds to both the GHR and JAK2. The sites for SOCS-3 on the GHR have been mapped to phosphotyrosines adjacent to Box-1 (Ram & Waxman 1999). These phosphotyrosine residues are required for GH-stimulated lipogenesis and protein synthesis, but are not obligatory for GH-dependent STAT5b activation (Rowland *et al.* 2005b; Carter-Su *et al.* 1996). Hence, SOCS-3 inhibits JAK-STAT signaling possibly by blocking the interaction of JAK2 and STAT5 (Ram & Waxman 1999) (**Figure I-9C**).

SOCS-2 and CIS only bind to the GHR. The sites for their association with the GHR are mapped to the distal intracellular domain. Within this region, two phosphotyrosine residues (m577 and m606) are crucial docking sites for STAT5 binding in response to GH (Rowland *et al.* 2005b). Therefore, the inhibition of GHR-signaling by SOCS-2 or CIS appears to result from direct competition with STAT5 for GHR binding (Ram & Waxman 1999) (**Figure I-9C**). Alternatively, CIS or SOCS-2 may also exert negative regulatory effects on GH signaling through protein ubiquitination followed by proteasomal degradation of the CIS/SOCS-bound GHR-JAK2 complex (Landsman & Waxman 2005) (**Figure I-9C**).

Studies in genetically engineered mice that either overexpress or have a targeted deletion of specific SOCS protein have provided considerable information regarding the physiological roles of SOCS proteins (Cooney 2002). **Table I-4** summarizes the phenotypes and altered mechanisms associated with the genetic modifications of these SOCSs. Only SOCS-2 knockout mice show enhanced signaling by GH and IGF-I and are significantly larger than their wildtype littermates (Metcalf *et al.* 2000).

Although elevated IGF-I mRNA levels are observed in some organs, hepatic and circulating IGF-I levels do not show major changes. The increased body size and growth observed in SOCS-2$^{-/-}$ mice resemble the phenotypes of GH and IGF-I transgenic mice as well as patients with acromegaly or gigantism (Metcalf *et al.* 2000). These findings provide strong evidence for the importance of SOCS-2 in regulating GH-IGF-I mediated growth and indicate its physiological relevance as a negative regulator of GH signaling. However, using cell models, it has been observed that SOCS-2 exhibits biphasic effects on GH-induced STAT5 activation: inhibition at low levels and stimulation when expressed at high levels (Leung *et al.* 2004). Although the stimulatory effect was also seen in SOCS-2 transgenic mice, where expression of SOCS-2 at high levels caused a 10% increase in body growth instead of expected growth inhibition (Greenhalgh *et al.* 2002), this result has to be interpreted with caution because no such high levels of endogenous SOCS-2 have been seen in physiological situations (Flores-Morales *et al.* 2006)..

Table I-4: Murine phenotypes associated with genetic modifications of the SOCS genes.

SOCS	Phenotype	Reference
SOCS-1	Tg: altered T cell homeostasis and thymocyte development	(Fujimoto *et al.* 2000)
	K/O: stunted postnatal growth, perinatal death, liver fatty degeneration, organ monocytic filtration	(Starr *et al.* 1998)
SOCS-2	Tg: mild excessive growth	(Greenhalgh *et al.* 2002)
	K/O: gigantism; increased weight of visceral organs, muscle and bone, but not adipose tissue; elevated IGF-I in some extra-hepatic tissues	(Metcalf *et al.* 2000)
SOCS-3	Tg: embryonic lethal, anemia	(Marine *et al.* 1999)
	K/O: embryonic lethal due to failure of erythrocytosis, placental insufficiency Liver specific K/O: similar size to wildtype littermates, IL-6 hyperresponsiveness	(Croker *et al.* 2003; Roberts *et al.* 2001)
CIS	Tg: similar to STAT5b-/- mice, growth retardation, defective mammary gland development, altered T cell function	(Matsumoto *et al.* 1999)
	K/O: no obvious phenotypic changes	(Li *et al.* 2000b)

Abbreviations: SOCS: suppressor of cytokine signaling; CIS: cytokine-inducible SH2 domain protein; STAT: signal transducer and activator of transcription; IGF-I: insulin-like growth factor I; IL-6: interleukin 6; IFN-γ: interferon-γ; Tg: transgenic; K/O: knockout

Because SOCS proteins down-regulate GHR signaling, a number of recent studies have investigated whether the GH insensitivity acquired during some pathophysiological states is the consequence of elevated SOCS protein levels. Indeed, SOCS proteins have been implicated in the development of GH resistance caused by fasting and catabolic conditions such as sepsis, inflammation, bacterial infection and chronic renal failure (CRF) (Lanning & Carter-Su 2006; Leung *et al.* 2004). Hepatic mRNA expression of SOCS-1, -3 and CIS have been seen to be transiently increased during abdominal sepsis, and are associated with the development of hepatic GH resistance (Yumet *et al.* 2006). Similarly, fasting has been shown to increase GH-induced expression of SOCS-3 in rat liver, which in turn contributes to GH insensitivity (Beauloye *et al.* 2002).

(B) Protein inhibitors of activated STATS (PIAS) proteins

In contrast to the SOCS proteins, the PIAS proteins are constitutively expressed negative regulators of cytokine signaling, and specifically inhibit STAT-mediated transcription in the nucleus (O'Shea & Watford 2004; Schmidt & Muller 2003; Liu *et al.* 1998; Chung *et al.* 1997) (**Figure I-9D**). It has been discovered recently that PIAS can also act as an E3-like SUMO (small ubiquitin-like modifier) ligase, which subsequently stimulates SUMO attachment to target proteins and thus modifies their transcriptional activities (Rakesh & Agrawal 2005; Schmidt & Muller 2003).

The human family of PIAS proteins consists of at least five members: PIAS1, PIAS3, PIASxα, PIASxβ and PIASy (Rakesh & Agrawal 2005; Schmidt & Muller 2003). They appear to utilize different mechanisms to elicit their inhibitions of STAT-dependent gene regulation (Pilecka *et al.* 2007; Rakesh & Agrawal 2005). PIAS1 blocks STAT1-dependent signaling, PIAS3 inhibits STAT3 and STAT5, and PIASx and PIAXy have been shown to block STAT4 and STAT1 signaling, respectively (**Figure I-9D**). They act by preventing STAT binding to DNA. In addition, because all members of the PIAS family display the SUMO ligase activity, it is very possible that PIAS-mediated SUMO modification of STATs is involved in the inhibition process (O'Shea & Watford 2004; Schmidt & Muller 2003). Although no report directly relates PIAS actions with GH-dependent STATs activation, the STAT proteins induced by GH have all been

demonstrated to be inhibited by individual PIAS. In addition, some other GH-activated transcription factors, including C/EBPα or c-Jun, have recently been identified as potential targets for PIAS proteins (Schmidt & Muller 2003).

(C) Signal regulatory protein α (SIRPα)

SIRPα belongs to a family of ubiquitously expressed transmembrane glycoproteins which currently includes inhibitory SIRPα, activating SIRPβ, non-signaling SIRPγ and soluble SIRPδ (Barclay & Brown 2006). SIRPα was identified as a substrate of activated receptor tyrosine kinase (RTK), with the ability to bind to the SH2 domain of SHP-1, SHP-2 and Grb2 in response to insulin, growth factors and oncogenes (Flores-Morales *et al.* 2006; Kharitonenkov *et al.* 1997).

The Carter-Su group first demonstrated that GH can induce JAK2-dependent tyrosyl phosphorylation of SIRPα and the association of SIRPα with SHP-2 and, thus, proposed that SIRPα is a signaling molecule for GH (Stofega *et al.* 1998). Their later work suggests that SIRPα appears to bind directly to JAK2 by a process that does not require tyrosyl phosphorylation, although it is highly phosphorylated on tyrosines in response to GH (Stofega *et al.* 2000a). Overexpression of SIRPα inhibits GH-induced signaling by decreasing the phosphorylation of JAK2, STAT5b, STAT3 and ERK1/2. In contrast, such inhibitory effects are not observed when SIRP 4YF (a mutant lacking four cytoplasmic tyrosine residues) are overexpressed. The authors thus concluded that SIRPα acts as a negative regulator of GH signaling (Stofega *et al.* 2000a). The exact mechanism whereby SIRPα negatively controls GH signaling is not completely understood. But because the ability of SIRPα mutants to negatively regulate GHR-JAK2 signaling correlates with their ability to bind SHP-2, it has been suggested that phosphorylated SIRPα recruits one or more molecules of the tyrosine phophatase SHP-2, which in turn dephosphorylate SIRPα and most likely JAK2 (Carter-Su *et al.* 2000; Stofega *et al.* 2000a).

2.4.5. Primary Disorders of GHR and GH-GHR Signal Transduction

An intact GHR structure and functional GHR signaling are crucial for GH actions. Defects in either result in loss or reduced cellular response to GH, leading to a clinical disorder known as GH insensitivity (GHI), which includes Laron syndrome (or classical GHI syndrome), atypical GHI syndrome and idiopathic short stature (David *et al.* 2005). In the following section, I will discuss their possible molecular mechanisms.

2.4.5.1. Defects of GH-GHR signaling resulting from mutations of the human GHR gene

(A) Mutations affecting the extracellular domain (ECD) of the GHR

Laron syndrome, originally called Laron-type dwarfism, was first described as a "genetic pituitary dwarfism with high serum concentrations of GH" (Hull & Harvey 1999). Patients with this disorder display severe growth failure, IGF-I deficiency, characteristics of abnormal metabolism such as central (or truncal) obesity and hypoglycemia, as well as delayed puberty. They have unique dimorphic facial features (Hull & Harvey 1999). Because they phenotypically look like GH-deficiency patients, initially it was thought the defect was in the GH molecules that the patients produced. However, Laron syndrome individuals are resistant to exogenous GH but are highly responsive to IGF-I administration, suggesting that the defect is in the GHR (Rosenfeld *et al.* 1994). Direct evidence of the receptor dysfunction was provided by the failure of hepatic microsomes to bind to GH (Rosenfeld *et al.* 2007). With the cloning and sequencing of the human GHR gene, the molecular basis for such disorders was clarified. They are caused by mutations or deletions in the coding exons of the human GHR gene, which result in a dysfunctional hGHR (Godowski *et al.* 1989). Since then, more than 60 different alterations of the GHR have been reported, ranging from exon deletions to a variety of point mutations including frameshift, nonsense and missense mutations.

The majority of the molecular defects of the GHR identified to date have been in the region of the gene encoding the ECD of the hGHR (Rosenfeld *et al.* 2007), although this in part reflects a certain bias, because abnormalities in the extracellular domain often results in reduced serum GHBP concentrations, and testing circulating GHBP levels is a routine procedure in diagnosis of the GHI syndrome. All of these mutations share the

same pathogenetic mechanism, which is abolishing the GH-binding ability of both the GHR and the GHBP (David *et al.* 2005). Patients with such molecular defects usually have extremely low or absent circulating GHBP levels (David *et al.* 2005).

(B) Mutations affecting the transmembrane and intracellular domains of the GHR

A point mutation in either the splice donor site (Woods *et al.* 1996) or the splice acceptor site (Silbergeld *et al.* 1997) of exon 8 of the human GHR gene has been identified in GHI syndrome patients. These mutations result in the skipping of exon 8 and generate a hGHR lacking the transmembrane and intracellular domains but still with a normal GH-binding extracellular domain. These mutant GHRs lack the ability to incorporate into the plasma membrane and are released into the circulation, leading to high GHBP levels and activity despite GH resistance (Hull & Harvey 1999).

Two mutations within the intracellular domain of the hGHR have been reported that result in an autosomal dominant, GHBP-positive form of GHI syndrome (Ayling *et al.* 1999; Iida *et al.* 1998; Ayling *et al.* 1997). In 1997, Ayling et al reported a family history of short stature due to the heterozygous expression of a severely truncated hGHR resulting from mutation of a splice acceptor site of exon 9 (IVS8as-1 G→C) and deletion of exon 9. The truncated $hGHR_{1-277}$ lacks the entire intracellular signaling domain, although the extracellular and transmembrane domains are normal. Therefore, this mutant receptor is devoid of any signaling capacity. However, because its internalization is also impaired, due to absence of the UbE motif, this truncated receptor accumulates at the cell membrane and acts as a dominant-negative toward the wildtype, full-length receptor, and inhibits GH signaling (Bougneres & Goffin 2007; Ayling *et al.* 1997). Iida et al described a second mutation (IVS9ds+1 G→A) that results in the $hGHR_{1-279}$ isoform that is also a dominant negative form (Iida *et al.* 1998).

Milward et al. have demonstrated another type of intracellular domain mutation, in which a homozygous 22 bp deletion occurs in the exon 10 region of the hGHR gene encoding the intracellular domain. This causes premature termination and results in the truncation of hGHR after residue 449 ($hGHR_{1-449}$), creating a mutant receptor lacking the tyrosine residues required for STAT5 binding (Milward *et al.* 2004). In 2005, Tiulpakov *et al.*

reported a patient who was compound heterozygous for a mutation in the ICD, resulting in truncation of the hGHR after residue 581 (nonsense sequence of residues 560-581) (Tiulpakov *et al.* 2005). In both cases, receptor expression, cellular distribution, ligand binding and GH-stimulated phosphorylation of JAK2, STAT3 and p44/42 MAPK were not affected by hGHR truncation, but the ability to activate STAT5 was markedly impaired (Bougneres & Goffin 2007). Such patients exhibit severe growth retardation, marked IGF-I deficiency, low but detectable levels of GHBP. These clinical observations agree well with the experimental results obtained from mouse studies (Rowland *et al.* 2005a; Rowland *et al.* 2005b) and emphasize the critical role of the JAK2-STAT5 pathway in the growth-promoting effects of GH in humans (Rosenfeld *et al.* 2007; Bougneres & Goffin 2007).

GHI patients with the above-mentioned transmembrane or intracellular domain mutations share a common feature: they are GHBP-positive. Their growth failure is usually more modest in comparison to the classical GHI syndrome (Laron syndrome), with normal faces or a softening of the characteristic facial features, and their serum IGF-I concentrations are not as severely affected, although still reduced. Thus, they belong to the "atypical" GHI syndrome.

Although more than 60 different molecular mutations in the hGHR have been described, there are still short-statured patients in whom the cause of growth retardation has not been completely understood, despite the fact that their GH secretion is normal. This group of individuals is known as having idiopathic short stature (ISS). Carriers of heterozygous mutations in the human GHR genes appear to be the reason for a small number (2-5%) of ISS cases. Although single nucleotide polymorphisms in the human GHR gene have been hypothesized to be one possible cause, no definitive data have been reported and further studies are desirable.

2.4.5.2. Defects of GH-GHR signaling resulting from mutations of the STAT5b gene

To date, six cases of GHI patients with severe growth failure have been reported that are associated with homozygous mutations of the STAT5b gene (Rosenfeld *et al.* 2007). These cases provide conclusive evidence of the central role of STAT5b in GH-dependent

postnatal body growth and GH-induced IGF-I gene expression. In these six patients, five different mutations of the human STAT5b gene have been revealed, including insertion, deletion and point mutations (Rosenfeld *et al.* 2007; Vidarsdottir *et al.* 2006; Hwa *et al.* 2005; Kofoed *et al.* 2003). Kofoed et al described the first homozygous point mutation in the STAT5b gene, and demonstrated that such a mutation results in a change of an amino acid residue within the SH2-domain of the protein, producing a mutant $STAT5b^{A630P}$ with a protein folding disorder that can not be activated by GH (Chia *et al.* 2006; Kofoed *et al.* 2003).

Patients with defects in the STAT5b gene display growth patterns, facial features and biochemical indices (serum concentrations of GH, IGF-I and IGFBP-3 and ALS) indistinguishable from patients with severe defects in GHR. Exogenous GH treatment has no effect on acceleration of body growth and has minimal effects on increasing serum IGF-I concentrations. However, different from patients with GHR defects, their circulating GHBP levels are normal, and sequencing of the hGHR gene is consistent with the normal gene. In addition, they all have a history of immunological dysfunction. Interestingly, five of the six studied patients are females, implying that, in humans, STAT5b is crucial for body growth in both sexes, unlike in mice where it mainly functions in males (Rosenfeld *et al.* 2007).

3. GROWTH HORMONE RECEPTOR (GHR) GENE

From the above section, it is obvious that the pleiotropic actions of GH depend on the presence of its specific cell surface receptor. Cell surface levels of GHR are the initial determinant of the magnitude and duration of GH action and, thus, control cellular responsiveness to GH. Various transcriptional, translational and even posttranslational factors can influence GHR synthesis and, thereby, regulate GH sensitivity and actions (Flores-Morales *et al.* 2006). In this section, I will focus primarily on what is known about the transcriptional regulation of the human GHR gene.

3.1. Human GHR (hGHR) Gene Structure

The hGHR is encoded by a single gene located on the proximal short arm of chromosome 5 in region p13.1-p12 (Barton *et al.* 1989), where the genes for PRLR and several other cytokine receptors have also been found [(Arden *et al.* 1990), and NCBI human genome map]. With publication of the human chromosome 5 sequence, it is now known that the hGHR gene spans approximately 300 kb (Orlovskii *et al.* 2004). The coding and 3' untranslated regions (3'UTR) of the receptor are encoded by 9 exons (exons 2-10). Exon 2 encodes the signal sequence and the first 5 amino acids of the extracellular domain. Exons 3-7 encode most of the extracellular ligand-binding domain, whereas exon 8 encodes the transmembrane domain. Exons 9 and 10 together code for the entire intracellular domain and the 3'UTR (Godowski *et al.* 1989). In addition, an intriguing structural feature has been found at the 5'untranslated region (5'UTR) of the *hGHR* gene, characterized by its considerable length (140 kb out of the 300 kb) and complex organization, with the presence of multiple 5'non-coding leader exons (Wei *et al.* 2006; Orlovskii *et al.* 2004; Goodyer *et al.* 2001b). A schematic illustration of the hGHR gene and mRNA is shown in **Figure I-10**.

3.2. hGHR 5'UTR mRNA Variants

Although the hGHR is encoded by a single gene, multiple hGHR mRNA variants have been identified. They are characterized by the presence of different 5'UTR sequences (Goodyer *et al.* 2001b; Schwartzbauer & Menon 1998). This 5'UTR heterogeneity of hGHR transcripts results from transcribing and splicing of different 5'UTR non-coding exons to the common splice acceptor site located 11bp upstream of the translation initating ATG in exon 2. Therefore, they all code for the same hGHR protein (Goodyer *et al.* 2001b; Schwartzbauer & Menon 1998).

Heterogeneity in the 5'UTR of hGHR transcripts was observed when hGHR cDNAs were was first cloned. Of the six human cDNA clones that were sequenced, five had distinct 5'UTRs (Edens & Talamantes 1998; Leung *et al.* 1987). By performing 5'RACE screening of a human liver cDNA library, Pekhletsky et al. then isolated eight different hGHR mRNAs that vary in their 5'UTR regions and numbered them as V1-V8 according

to their relative abundance from the examined clones (Pekhletsky *et al.* 1992). Later, Goodyer et al. identified a new mRNA variant, V9, from a cardiac muscle cDNA library (Goodyer *et al.* 2001b).To date, except for V6, which is now considered to be an artifact and corresponds to chromosome 11, all other 8 variants have been validated by genomic location and expression profiling (Orlovskii *et al.* 2004; Goodyer *et al.* 2001b).

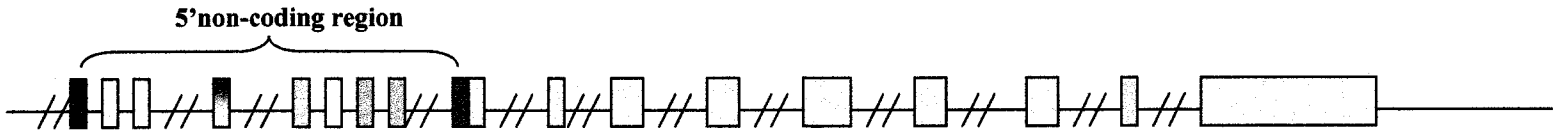
Chromosomal mapping of these 5'non-coding exons demonstrated that seven of the 5'UTR exons are clustered within two separate small regions, each approximately 2 kb in size. The distal cluster (~140 kb upstream of exon 2), also known as Module A, contains exons V2, V9 and V3; whereas the proximal cluster (16-17 kb from exon 2), known as Module B, includes exons V7, V1, V4 and V8 (**Figure I-10**). Through analysis of the expression patterns of all variants in a panel of human fetal and postnatal tissues, it was demonstrated that these two modules are not only physically distant, but exhibit distinct expression patterns (Goodyer *et al.* 2001b) (**Figure I-10**). Module A transcripts are widely expressed and readily detectable at every developmental stage. In contrast, module B-derived mRNA variants are only detectable in normal postnatal liver, suggesting that they are induced after birth in a tissue-specific manner (Goodyer *et al.* 2001b). The exon V5 is situated next to exon 2 and, thus, may represent a partially spliced form of the hGHR transcript instead of a true alternative first exon (Edens & Talamantes 1998).

Of the eight hGHR mRNA variants, the V1 and V2 variants are the predominant mRNA variants expressed in human liver and extrahepatic tissues, respectively (Goodyer *et al.* 2001b). Other mRNA variants are usually expressed at low levels compared to the total hGHR mRNA pool, and their functions are still questions to be solved.

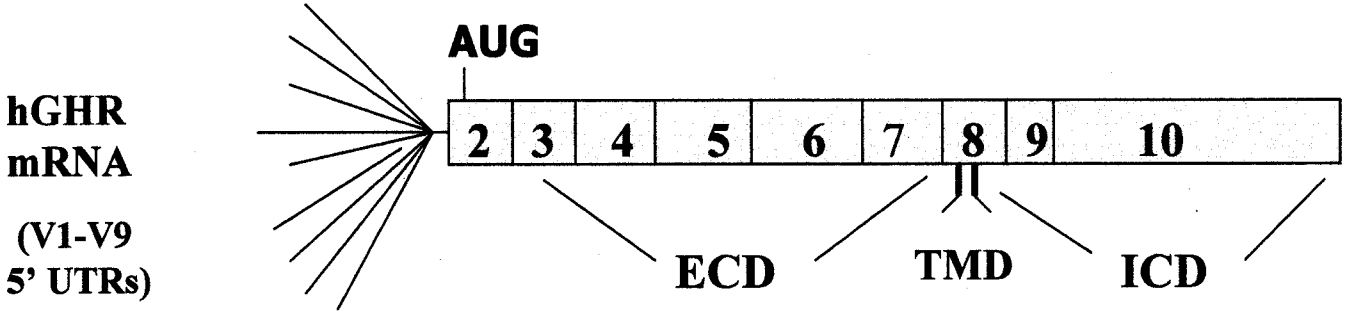
Figure I-10: Schematic representation of the human GHR gene and mRNAs.
A) hGHR is encoded by a single gene located on chromosome 5. It has 9 coding exons (exons 2-10) and several 5' non-coding exons. B) Transcribing from and splicing of different leader exons onto a common acceptor site located 11bp upstream of the AUG codon on exon2 generates multiple hGHR mRNA variants which differ in their 5'UTRs but code for the same protein. C) Eight hGHR 5'UTR mRNA variants have been described prior to the present work (V1-V5, V7-V9; V6 is now known to be an artefact). Chromosomal mapping of seven of these 5'UTR exons showed that they are clustered in two small regions: V2, V3 and V9 form module A and produce ubiquitously expressed hGHR mRNA variants while module B variant mRNAs (from exons V1, V4, V7 and V8) are limited to normal postnatal liver. V5 mRNAs are widely expressed.

Abbreviations: ECD: extracellular domain; TMD: transmembrane domain; and ICD: intracellular domain.

A) hGHR gene [chromosome 5p13.1-12]



B)



C) Genomic localization of different 5'non-coding exons

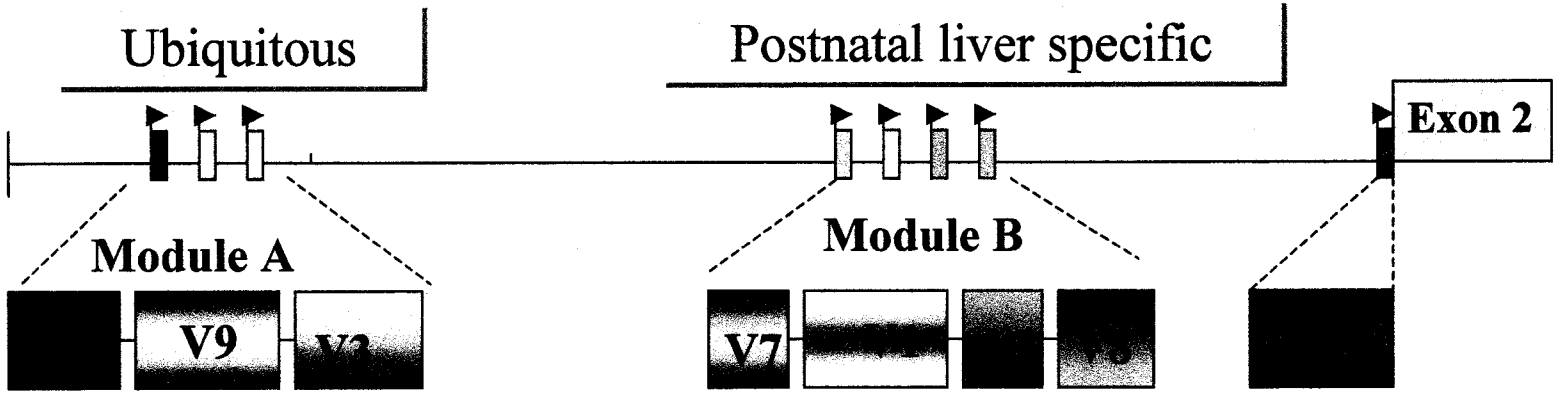


Figure I-10

3.3. 5'-Heterogeneity Is a Common Feature across Species

The presence of multiple alternative 5'UTRs has been found to be a common feature of GHR transcripts from a number of species (Schwartzbauer & Menon 1998). Collectively, five distinct 5'UTRs have been identified in mice (L1-L5) (Moffat *et al.* 2000; Southard *et al.* 1995b) and in rats (GHR1-GHR5) (Domene *et al.* 1995), two in sheep (o1A and o1B) (Adams 1995; Adams *et al.* 1990), three in rabbits (Leung *et al.* 1987) and monkeys (Zogopoulos *et al.* 1999) and nine in cattle (b1A-b1I) (Jiang & Lucy 2001b). The mouse GHR mRNA variant L1, rat GHR1, ovine 1A and bovine 1A are similar in sequence to the human V1 mRNA variant, while the mouse L2, the rat GHR2, as well as the ovine and bovine 1B variants, show significant homologies with the human V2 variant (Goodyer *et al.* 2001b; Edens & Talamantes 1998). In general, V1-like mRNA variants are expressed in a liver-specific manner and predominate within the hepatic GHR mRNA pool, whereas V2-like transcripts are widely expressed and account for the majority of the total GHR transcript in extrahepatic tissues (Goodyer *et al.* 2001b; Edens & Talamantes 1998; Schwartzbauer & Menon 1998). Mice are an exception: their L1 variant is expressed in the liver only during pregnancy. In nonpregnant mice, it is still the L2 variant that accounts for the predominant transcript in liver (Schwartzbauer & Menon 1998).

Taken together, the expression of GHR mRNA variants both in the human and in other species is regulated in a tissue- and developmental-specific nature. Even though 5'-heterogeneity is conserved across species, the causes and significance of this 5'UTR diversity are not completely understood.

3.4. Regulation of GHR Gene Expression

hGHR transcripts have been detected in a variety of tissues including liver, skeletal muscle, kidney, lung, heart, mammary gland, placenta, skin and adipose tissue (Edens & Talamantes 1998). However, the expression levels vary between tissues, with the highest level of expression being in the liver, followed by muscle, fat, kidney and heart (Ballesteros *et al.* 2000; Edens & Talamantes 1998). Numerous factors including

development (ontogeny), hormonal presence and nutritional status, as well as tissue or cell-specific control, can regulate hGHR gene expression.

3.4.1. Ontogeny of GHR Expression

In both man and animals, *GHR* gene expression displays a distinct ontogenic pattern, with low expression during fetal development and a significant increase after birth. The lower levels of GHR during fetal life correlate with the observation that intrauterine growth is, for the most part, GH-independent, due to the presence of many GH-like factors produced by the feto-placental unit (Osafo *et al.* 2005; Schwartzbauer & Menon 1998). The molecular mechanisms governing the increase of *GHR* gene expression after birth remain obscure. It has been proposed that postnatal onset of liver-specific *GHR* mRNA variants could be one contributor in hepatocytes (Goodyer *et al.* 2001b). But what factors are responsible for this onset and which are linked to changes in non-hepatic tissues still await identification. Yu et al. have suggested that alterations in the levels of the Sp family of transcription factors (Sp1 vs. Sp3) may play a role in the increased expression of the murine GHR gene in postnatal life (Yu *et al.* 1999).

3.4.2. Regulation of GHR Expression by Hormones

Hormonal effects on GHR expression have been extensively studied in rodents and other animals, but data regarding regulation of GHR expression in the human remain limited (Waters 1999; Schwartzbauer & Menon 1998). The majority of the studies of GHR regulation in man are clinical studies, mainly through measuring plasma GHBP levels, based on the assumption that circulating GHBP levels reflect tissue GHR levels(Waters 1999).

3.4.2.1. Regulation of GHR expression by GH

GH regulates liver GHR expression but the regulation appears to be complex. Contradictory results are often seen, in part due to the time dependency of GH treatment, whether the experiments are carried out in *vitro* or in *vivo*, and the cell types studied (Kopchick & Andry 2000; Schwartzbauer & Menon 1998). Acute GH treatment in GH deficient rats tends to downregulate hepatic GHR (Maiter *et al.* 1988b), while chronic treatment of GH using either continuous infusion or subcutaneous injection is associated

with an increase in hepatic GHR number as seen in the rat (Maiter *et al.* 1988a), pig (Schwartzbauer & Menon 1998) and sheep (Schwartzbauer & Menon 1998), suggesting that acute GH exposure affects "desensitization" of the GHR while chronic GH action appears to promote liver *GHR* gene expression. Baumbach and Bingham observed that hypophysectomy in rats reduces the expression of liver-specific *GHR* mRNA variant (GHR1), and chronic GH treatment restores its expression (Baumbach & Bingham 1995). Additionally, Iida *et al.* detected a decrease in liver *GHR* mRNA in GH-deficient mice and an increase in GH-overexpressing transgenic mice (Iida *et al.* 2004). A recent study by Jiang and his coworkers showed that injection of GH in a slow-release manner increases bovine hepatic GHR levels both at the mRNA and protein levels, consistent with what Hammon's group had previously reported, that prolonged GH treatment resulted in an increase in hepatic GHR mRNA levels in calves (Hammon *et al.* 2003). Jiang *et al.* also demonstrated that the increase in liver *GHR* mRNA levels is mainly due to the augmentation of the liver-specific *GHR* transcript (b1A) by GH, without affecting the other two variants, b1B and b1C, which are widely expressed. This GH stimulatory effect on the b1A *GHR* mRNA variant appears to be mediated by GH-dependent STAT5 transactivation at the proximal promoter of b1A variant (Jiang *et al.* 2007). However, conflicting observations have been reported: studies from several groups indicate that hypophysectomy and GH treatments do not affect rat hepatic *GHR* mRNA levels (Frick *et al.* 1998; Butler *et al.* 1996; Mathews *et al.* 1989).

Consistent evidence for an effect of GH on *hGHR* gene expression comes from cell culture studies. Using human primary thyroid cells, Mullis et al demonstrated that GH exerts a stimulatory effect on *GHR/GHBP* mRNA expression in a dose-dependent manner, acting at the gene transcriptional level (Mullis *et al.* 2000). Similar effects were seen in Huh7 cells, a human hepatoma cell line (Mullis *et al.* 1991). A recent report examining the effect of GH and a GH antagonist on *hGHR* gene expressin in human mesangial cells shows a similar stimulatory effect of GH on *GHR* gene transcription. Howevere, they also found that supraphysiologic doses of GH treatment will lead to down-regulation of GHR expression (Meinhardt *et al.* 2003).

3.4.2.2. Regulation of GHR expression by insulin

Insulin also seems to be involved in the regulation of GHR (Schwartzbauer & Menon 1998). Both children and adult patients with untreated Type 1 diabetes show reduced circulating GHBP levels, indicating a decrease in hepatic tissue GHR expression. Such abnormalities tend to be normalized after the start of insulin treatment. Using human Huh-7 cells, Leung *et al.* demonstrated that insulin treatment upregulates the abundance of *GHR* mRNA and protein (Leung *et al.* 2000). The regulatory role of insulin in GHR expression has also been observed in rodents, with a tissue-specific effect being consistently reported (Menon *et al.* 1994). For example, Menon *et al.* demonstrated that *GHR* mRNA levels were decreased in liver, but increased in kidney, in streprozocin-induced diabetic rats (Menon *et al.* 1994).

3.4.2.3. Regulation of GHR expression by steroid hormones

Steroid hormones, including glucocorticoids and estrogen, are implicated in the regulation of *GHR* gene expression.

A) Glucocorticoids

High doses of glucocorticoids are potent inhibitors of growth, in part by direct interaction with the somatotropic axis (Newnham 2001); therefore, it would be anticipated that such steroids exert inhibitory effects on *GHR* gene expression (Waters 1999). However, the situation is not straightforward as conflicting *in vivo* as well as *in vitro* results have been reported (Swolin-Eide *et al.* 1998). Dexamethasone increases *GHR* mRNA levels in liver and in the growth plate of rabbits (Heinrichs *et al.* 1994), while it decreases *GHR* gene expression in rat liver (Bennett *et al.* 1996). When used alone, dexamethasone decreases bovine hepatic GHR mRNA levels; but combined with GH, it enhances GH's stimulatory effect on *GHR* gene expression (Hammon *et al.* 2003). Glucocorticoids also exhibit opposite regulatory effects on bovine *GHR* mRNAs, depending on the formula feeding regimen and the gastrointestinal tract section examined (Ontsouka *et al.* 2004). In human osteoblast (hOB)-like cells, cortisol has been shown to increase hGHR mRNA levels in a time-dependent manner (Swolin-Eide *et al.* 1998). A recent study using Affymetrix U133 microarrays identified that the hGHR mRNA levels in both subcutaneous and omental fat

depots were significantly upregulated by cortisol treatment of female patients for 24hr (Bujalska *et al.* 2006). These contradictory findings suggest that, like for GH, glucocorticoid effects on GHR expression are dependent on dose, regimen and species studied.

B) Sex steroids

Sex differences in hepatic GH binding and *GHR* gene expression vary between species. Estrogen appears to exert positive regulatory effects on GHR expression in rodents, as pregnancy and prolonged exposure to estrogen consistently increase *GHR* mRNA and protein levels in rat liver (Schwartzbauer & Menon 1998). A liver specific *GHR* mRNA variant, *GHR1*, is expressed at a greater concentration in female rats than in males and is upregulated by estrogen, which results in increased hepatic GH binding (Gatford *et al.* 1998). Therefore, it has been suggested that the liver-specific transcript is likely to be the major contributor to the hepatic GHR sexual dimorphism observed in rodents (Leung *et al.* 2004). Intriguingly, this estrogen-induced expression of the *GHR1* transcript is GH-dependent, because estrogen fails to affect *GHR* mRNA abundance in GH-deficient rats (Gabrielsson *et al.* 1995). This observation has led to the suggestion that upregulation of GHR expression by estrogen is an indirect effect, depending on changes in the GH secretion pattern (Waters 1999; Schwartzbauer & Menon 1998). Furthermore, despite the fact that estrogen stimulates hepatic GHR expression in rats, it has been shown to reduce *GHR* mRNA levels in the central nervous system (CNS) (Bennett *et al.* 1996), indicating a tissue-specific regulation by estrogen. Similarly, species specificity is also seen for the effects of estrogen on GHR expression. Physiological doses of estrogen decrease GHR expression and GH binding in rabbit liver (Gatford *et al.* 1998). In man, significantly higher circulating GHBP concentrations are seen in women than in men (Leung *et al.* 2004). And estrogen stimulates GH binding activity and *GHR* mRNA abundance in cultured human osteoblasts (Slootweg *et al.* 1997). In contrast, the serum GHBP level does not differ between pre- and postmenopausal women (Ho *et al.* 1993), and some studies observed a reduced GHBP concentration in humans after estrogen treatment, suggesting that estrogen downregulates GHR expression (Gatford *et al.* 1998). Taken

together, the effects of estrogen on *GHR* gene expression appears to be both species- and tissue/cell type-dependent.

3.4.2.4. Regulation of GHR expression by thyroid hormone

Thyroid hormone has important effects on growth. Lack of thyroid hormone in mammals is associated with profound growth failure which, in part, involves interactions with the GH-IGF-I axis (Mullis *et al.* 1999; Schwartzbauer & Menon 1998). In human hepatoma Huh-7 cells, addition of T3 on its own increases *GHR* mRNA levels. Such an increase results from an indirect stimulatory effect of T3 on *GHR* gene transcription (Mullis *et al.* 1999). A recent study also showed that T3 significantly stimulates *GHR* mRNA levels when added at a high concentration to cultures of primary human adult osteoblast-like cells (Pepene *et al.* 2003). Similar positive effects on *GHR* gene expression by thyroid hormones have been observed in several other species, including the sheep (Forhead & Fowden 2002), pig (Duchamp *et al.* 1996) and rats (Hochberg *et al.* 1990). Romero *et al.* demonstrated a distinct sexual dimorphism in the effect of hypothyroidism on expression of the *GHR* gene in rats: the hypothyroid condition is associated with increased hepatic *GHR* mRNA in female rats but decreased *GHR* transcript levels in males. These changes were reversed following amelioration of the thyroid state (Romero *et al.* 1996).

3.4.2.5. Nutritional status and GHR

Nutrition is an important regulator of GH actions. In animals, nutritional deprivation causes a state of GH resistance characterized by reduced hepatic GHR levels and a decline in IGF-I production (Schwartzbauer & Menon 1998). Fasting or diet restriction have been shown to result in reduced *GHR* mRNA levels in rat liver (Umana *et al.* 2003; Schwartzbauer & Menon 1998), suggesting that nutrition is involved in regulation of *GHR* gene transcription. Similar results have been observed in pigs, with one study suggesting that GHR mRNA levels are affected by relatively small changes in energy intake that do not necessarily change body growth (Schwartzbauer & Menon 1998). There are few reports regarding *GHR* gene expression during undernutrition states in humans. Shuto et al. reported one case of a severely malnourished elderly man who had severe GH resistance and a greatly reduced hepatic *GHR* mRNA level (Shuto *et al.* 1999).

Furthermore, Holt *et al.* documented that children with end-stage liver disease have reduced hepatic GHR mRNA levels (Holt *et al.* 1997).

Other catabolic conditions, such as trauma, sepsis, renal failure and critical illness, are also associated with GH resistance characterized by low levels of plasma GHBP (Schwartzbauer & Menon 1998). Therefore, these states may also correlate to reduced *GHR* gene expression. Indeed, Hermansson et al. examined the effect of surgical trauma on GHR expression in skeletal muscle: they observed that *GHR* mRNA levels were reduced in 8 of 9 patients three days after elective surgery (Hermansson *et al.* 1997).

3.5. Molecular Mechanisms Regulating GHR Expression

3.5.1. Transcriptional Regulatory Mechanisms for GHR Gene Expression

The differential expression pattern of Module A and Module B mRNA variants suggests that multiple *hGHR* 5'UTRs are under the control of multiple promoters. Indeed, our lab has shown that Module A promoter constructs for V2 and V3 are highly active in either non-liver or hepatoma cell lines, while Module B promoter constructs are quite repressed in these cell lines (Goodyer *et al.* 2007). Despite the fact that these 5'UTR mRNA variants all encode the same hGHR protein, use of alternate promoters for initiating transcription in conjunction with differential splicing provides a complex mechanism for regulating hGHR gene expression (Orlovskii *et al.* 2004; Goodyer *et al.* 2001b; Schwartzbauer & Menon 1998).

3.5.1.1. Promoters for V1 and V1-like exons

In the human, promoter studies have been performed for the 5'UTR exons V1, V9 and V3, following identification of the transcription start sites (Goodyer *et al.* 2007; Goodyer *et al.* 2001b). Because the V1 exon exhibits liver-specific and developmental-regulated expression, and V1 is the predominant GHR transcript expressed in adult liver, it has been of particular interest to understand its transcriptional regulation. Two transcription start sites (TSS) and two TATA boxes have been identified (Goodyer *et al.* 2001b). The upstream non-consensus TATA/TSS has been defined as the major TSS for human V1 transcription (Goodyer *et al.* 2001b; Rivers & Norman 2000; Zou & Menon 1995),

whereas the downstream consensus TATA/TSS has been shown to be conserved across species and has been described as the functional TATA for V1-like mRNA variants in other species, including ovine 1A, bovine 1A and mouse L1 (Jiang & Lucy 2001a; Jiang *et al.* 1999; Zou *et al.* 1997). Promoter deletion analyses have defined the proximal promoter region for human V1 within ~150-160 bp upstream the TSS (Goodyer *et al.* 2007; Orlovskii *et al.* 2004; Rivers & Norman 2000) Two positive regulatory regions (PRRs) and three negative regulatory regions (NRRs) have been revealed in the area 1.8 kb upstream of exon V1 (Goodyer *et al.* 2007). The NRR1 is proximal to the TSS and is unique to human V1. Two putative binding sites for the transcription repressors, Gfi-1/1b, were identified within this region, adjacent to a GAGA element that binds the transcriptional activator GAF. Thus, it has been hypothesized that both Gfi-1/1b and GAF regulate V1 expression (Osafo 2006).

Several response elements for liver-enriched transcription factors (LETFs) have been detected in the ~2 kb promoter region, including C/EBP (Rivers & Norman 2000) and HNF-4 binding sites (Goodyer *et al.* 2007). The HNF-4 binding sites have been found to be in both positive and negative regulatory regions, and shown to mediate dual effects on GHR V1 transcription upon the binding of hepatocyte nuclear factor (HNF)-4 α isoforms. These data suggest that HNF4α may be the factor responsible for liver specific GHR expression (Goodyer *et al.* 2007)

In other species, the promoters for V1-like exons have also been the subject of intensive studies. The transcription start sites and proximal promoter regions have been mapped for mouse L1, ovine 1A and bovine 1A (Jiang *et al.* 1999; Zou *et al.* 1997; O'Mahoney *et al.* 1994). Many similarities to the promoter structure of human V1 have been observed: 1) a functional TATA box (TATA promoter); 2) similar negative regulatory regions with repressor elements that regulate the liver specific expression; 3) putative binding sites for LETFs in the promoter region, like C/EBP, HNF-4; and 4) involvement of HNF-4 mediated transcriptional activation.

As mentioned earlier, GH stimulates hepatic expression of V1-like transcripts, including rat GHR1 (Baumbach & Bingham 1995) and bovine 1A (Jiang *et al.* 2007). This has led

to the search for GH response elements (GHREs) in the promoter regions of V1-like exons. Jiang et al. recently identified a STAT5 binding site in the proximal promoter of bovine GHR 1A exon and demonstrated that it mediates the induction of GHR 1A expression in response to GH. However, this site seems to be unique for bovine 1A (Jiang *et al.* 2007). No STAT5 binding sites been detected in the human V1, ovine 1A or mouse L1 promoters, but it has been proposed that the purine-rich GAGA element is a putative GHRE for V1 (Osafo 2006).

3.5.1.2. Promoters for V2 and V2-like exons

The hGHR V2 transcript is widely expressed and is the predominant mRNA in extrahepatic tissues and is second only to V1 in liver (Goodyer *et al.* 2001b). Orlovskii *et al.* defined the 165bp region upstream of the putative TSS as having the maximal promoter activity. No TATA box is detected within this region, but it is enriched in GC basepairs and includes potential binding sites for the Sp family of transcription factors. Thus, it is structurally related to promoters of "housekeeping genes" (Orlovskii *et al.* 2004). Apart from these data, our current knowledge of how transcription from the human V2 exon is regulated is limited: the TSS remains to be mapped and the cis-regulatory elements to be characterized.

The promoter structures and regulatory mechanisms of V2 homologues in other species, such as ovine 1B, bovine 1B and mouse L2, have been partially studied (Jiang *et al.* 2000; Adams 1999; Yu *et al.* 1999; Moffat *et al.* 1999b; Adams 1995). Multiple transcription start sites have been observed in all cases. The major transcription start sites for both ovine 1B and bovine 1B were mapped within a few nucleotides, while the mouse L2 initiation site is ~290 bp downstream. The proximal promoter regions in all species are TATA-less, but with high GC content, and the ubiquitously expressed transcription factor Sp1 has been demonstrated to play an important role in regulation of their promoter activities. The similar expression pattern and relatively high DNA sequence homology have led to the proposal that a common regulatory mechanism may be present.

Taken together, these data demonstrate that the liver-specific V1-like exons and the ubiquitously expressed V2-like exons possess distinct promoter structures and mediate their expression by different regulatory mechanisms.

3.5.2. 5'UTR Variants as Translational Regulators of GHR Gene Expression

Translation of mRNA into protein represents the next step in the gene expression pathway. Regulation of translation can modulate gene expression in a wide range of biological situations. The 5'UTR of an mRNA is known to affect its translational efficiency. Structural features within the 5'UTR, including upstream AUGs, upstream open reading frames (uORFs) and secondary or tertiary RNA structures, have been discovered to inhibit efficient translation of mRNAs (Gebauer & Hentze 2004).

Jiang and Lucy tested the effects of various bovine GHR 5'UTR variants on translational efficiency by fusing individual 5'UTR fragments upstream of the translation start codon of the luciferase gene in a reporter construct and examining the synthesis of luciferase via *in vitro* translation (Jiang & Lucy 2001b). Interestingly, they found that the 5'UTRs exhibit differential translational efficiency, with the liver-specific 1A variant and some minor transcripts showing relatively high translational efficiencies, while the 1B variants have considerably lower translational efficiency, despite the fact that they are expressed at high levels in most bovine tissues. The authors hypothesized that the inhibitory effect on translation is due to the high GC content of the 1B exon, which may easily form stable secondary structures (Jiang & Lucy 2001b). Orlovskii et al used a similar method to examine the effects of a uORF within the hGHR 5'UTR V2 exon. They demonstrated that substitutions within the initiating AUG codon of the uORF significantly attenuated the inhibition and enhanced the translation efficiency (Orlovskii *et al.* 2004). These observations suggest that various 5'UTR exons may modulate GHR translation and that the expression levels of mRNAs may not completely reflect the expression levels of GHR proteins in tissues or cells. From this point of view, it would be of great interest to check the influences of different hGHR 5'UTR exons on hGHR translation and to explore the mechanisms responsible for such differences.

In summary, the human GHR gene consists of multiple 5'UTR exons under the control of different promoters, which provides mechanisms for fine-tuning *hGHR* gene expression at both the transcriptional and translational levels. Such a complex regulatory system is appropriate for a protein of essential importance in many physiological processes, including growth, development and metabolism. The more we learn about the details and intricacies of these regulatory processes, the better we will be able to prevent and/or treat such disorders as growth failure and chronic complications from cardiovascular diseases, diabetes mellitus and obesity.

4. AIMS OF THE STUDY

4.1. General

The goal of this PhD project is to investigate the molecular mechanisms regulating human GHR gene expression, focusing on human adipocytes as a cell model.

4.2. Specific

1. To identify and determine the expression profile of hGHR 5'UTR mRNA variants in human adipocytes.

2. To define the expression profile of hGHR and hGHR mRNA variants as well as relevant transcription factors during adipocyte differentiation, using a human preadipocyte cell line, SGBS, as a model.

3. To characterize the transcriptional regulation of the hGHR V2 transcript.

CHAPTER II

CHARACTERIZATION OF GROWTH HORMONE RECEPTOR MESSENGER RIBONUCLEIC ACID VARIANTS IN HUMAN ADIPOCYTES

PREFACE

The following chapter has been published under the title "Characterization of Growth Hormone Receptor Messenger Ribonucleic Acid Variants in Human Adipocytes" by Yuhong Wei, Zakaria Rhani and Cynthia Gates Goodyer in *Journal of Clinical Endocrinology and Metabolism* (2006) **91(5)**: 1901-1908.

All of the work presented was conducted by the candidate except for most of the 5'-rapid amplification of cDNA ends (5'RACE) that were done by Zakaria Rhani.

The references have been compiled in alphabetical order at the end of the thesis.

ABSTRACT

Context: Human GH exerts profound effects on adiposity through its specific receptor, hGHR. Eight *hGHR* mRNAs are produced by the *hGHR* gene due to splicing from alternate 5'-untranslated region first exons into a common acceptor site upstream of the start codon in exon 2. Four transcripts (V2, V3, V5, V9) are ubiquitously expressed, whereas the other four (V1, V4, V7, V8) are expressed only in normal postnatal liver, suggesting that different promoter usage is a mechanism for developmental- and tissue-specific regulation of the *hGHR* gene.

Objective: Because it is unknown whether this occurs in adipocytes, we screened human adipocyte cDNA for *hGHR* mRNAs using 5'-rapid amplification of cDNA ends.

Results: Eighty-nine percent of the clones were V2-like, 3% were V3-like, and 8% were five new mRNA variants (VA-VE). All new 5'-untranslated region sequences mapped within the *hGHR* 5'flanking region. RT-PCR assays showed expression in multiple fetal and adult tissues, and, thus, they are not adipocyte specific. We compared expression of *hGHR* mRNAs in adult liver, adult fat, and the human preadipocyte SGBS cell line, using duplex RT-PCR. In liver, V1 and V2 are the major *hGHR* mRNAs, whereas in adipose, V2 predominates; VA and VC are expressed at similar lower levels in both. In SGBS preadipocytes, approximately 70% of *hGHR* mRNA is V2. During differentiation, total *hGHR* and V2 transcripts are markedly up-regulated [*hGHR*: 2.3 ± 0.2-fold (mean ± SE), $P < 0.01$; V2: 3.0 ± 0.8, $P < 0.03$], whereas other variants also increased but remained relatively minor transcripts.

Conclusions: We have identified five new *hGHR* mRNA variants. Because the V2 transcript is predominant in adipocytes at all developmental stages, the mechanisms regulating its expression should be examined.

INTRODUCTION

GH is a key regulator of postnatal growth and metabolism (Veldhuis *et al.* 2005c; Flint *et al.* 2003; Nam & Lobie 2000). GH exerts these effects by binding to its specific cell surface receptor and triggering various intracellular signaling cascades, resulting in modulation of target cell activity (Piwien-Pilipuk *et al.* 2002a). Thus, the receptor expression level is critical for tissue sensitivity to GH.

The GH receptor (GHR), a single transmembrane protein of the class I cytokine receptor family (Piwien-Pilipuk *et al.* 2002a), is expressed in most cell types, and it is well known that its levels can be affected by development (Goodyer *et al.* 2001a; Li *et al.* 1996; Ymer & Herington 1992; Walker *et al.* 1992; Adams *et al.* 1990), nutritional status (Wang *et al.* 2003; Talamantes & Ortiz 2002), and hormones (Rhoads *et al.* 2004; Meinhardt *et al.* 2003; Vottero *et al.* 2003; Talamantes & Ortiz 2002; Jiang & Lucy 2001b; Leung *et al.* 2000; Mullis *et al.* 1999; Schwartzbauer & Menon 1998). However, our understanding of the molecular mechanisms controlling *GHR* expression is incomplete. Studies in different species have shown a common feature: heterogeneity in the 5'-untranslated region (5'UTR) of *GHR* transcripts (Orlovskii *et al.* 2004; Talamantes & Ortiz 2002; Goodyer *et al.* 2001b; Jiang & Lucy 2001b; Schwartzbauer & Menon 1998; Zogopoulos *et al.* 1996b; Adams 1995; Domene *et al.* 1995; Pekhletsky *et al.* 1992). This is generated by transcribing and splicing from different 5'-noncoding leader exons to a common splice acceptor site 9–11 bp upstream of the start codon in exon 2 of the *GHR* gene; thus, they all code for the same protein. In the human, nine 5'UTR mRNA variants (V1-V9) have been reported from screenings of adult liver and cardiac muscle cDNA libraries (Goodyer *et al.* 2001b; Pekhletsky *et al.* 1992). Although V6 is now considered to be an artifact (Orlovskii *et al.* 2004), the other eight variants have been validated by genomic localization and expression profiling.

Chromosomal mapping of these 5'-noncoding exons determined that seven form two separate clusters, each approximately 2 kb in size: V2, V9, and V3 form module A, 140.8–142.4 kb upstream of exon 2, whereas V7, V1, V4, and V8 form module B, 15.8–17.9 kb upstream of exon 2, and V5 is located adjacent to exon 2 (**Figure II-1A**, revision

of Ref.(Goodyer *et al.* 2001b)). Module A and V5 mRNAs are widely expressed; in contrast, module B transcripts are detected only in normal postnatal liver (Goodyer *et al.* 2001b). Similar tissue-specific and developmentally specific expression patterns have been observed in other species, suggesting that the derivatives of these 5'UTR exons are likely to be the result of common regulatory mechanisms controlling *GHR* expression (Orlovskii *et al.* 2004; Goodyer *et al.* 2001b; Jiang & Lucy 2001b; Moffat *et al.* 2000; Schwartzbauer & Menon 1998). Furthermore, recent studies demonstrated that the 5'UTR variant sequences have differential effects on translation efficiency (Jiang & Lucy 2001b; Moffat *et al.* 1999a). Therefore, to understand what controls human *GHR* (*hGHR*) expression and thus hGH responsiveness, it will be essential to isolate all *hGHR* 5'UTR mRNA variants and to study how they are regulated in different tissues.

Adipose tissue is a major target for hGH. Clinical and experimental observations have demonstrated the importance of hGH in regulating body fat through enhancing lipolysis and inhibiting lipogenesis (Franco *et al.* 2005; Veldhuis *et al.* 2005c; Fisker *et al.* 2001; Nam & Lobie 2000). hGH has also been shown to regulate adipocyte differentiation, although the effects have been controversial between preadipocyte cell lines and primary cultures (Nam & Lobie 2000). hGH actions on adipose tissue are thought to be mainly direct, mediated by its receptor, but our knowledge of how hGHR expression is regulated in adipocytes remains limited. The aim of this investigation was to identify the *hGHR* 5'UTR mRNA variants in adipocytes and determine their expression profiles.

MATERIALS & METHODS

5'-rapid amplification of cDNA ends (5'RACE) 5'RACE PCR amplification was performed on a Marathon-Ready cDNA pool from adipocytes of four lean and obese individuals (Clontech, Palo Alto, CA) using a 5'-anchor primer (**Table II-1**) and an *hGHR*-specific reverse primer (Exon2_R4; **Table II-1**). PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Burlington, Ontario, Canada); inserts were checked by *Eco*RI digestion and sequenced using the ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystem Inc., Foster City, CA).

Tissue collection and cell culture Fetal tissues were obtained after therapeutic abortion [10.5–19.5 wk fetal age (Munsick 1984)]. Postnatal specimens (15–75 yr) were collected at surgery or within 4–10 h after death. McGill University Health Centre Ethics Committees approved the studies, and all patients provided informed consent. Tissues were flash frozen and stored at –70 C until analysis.

Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were cultured and differentiated into mature adipocytes (Wabitsch *et al.* 2001). In brief, SGBS preadipocytes were maintained in DMEM/Ham's F12 (Invitrogen) containing 10% fetal bovine serum (Cansera, Rexdale, Ontario, Canada) and antibiotics at 37 C with 5% CO_2. Differentiation was initiated when preadipocytes became confluent (d 0). Initially, confluent cells were incubated for 4 d in Quickdiff medium [serum-free, containing 20 nM insulin (Sigma, St. Louis, MO), 200 pM T3 (Sigma), and 100 nM cortisol (Sigma), supplemented with 25 nM dexamethasone (Sigma), 500 μM 3-isobutyl-methyl-xanthine (Sigma),and 2 μM rosiglitazone (GlaxoSmithKline, West Sussex UK)]. Subsequently the cells were switched to adipogenic medium (containing insulin, T3 and cortisol) and maintained for up to 16 d. Differentiation was visualized by accumulation of lipid, using Oil Red O staining. Samples were collected every 2–4 d during the time course study.

RNA preparation and RT-PCR assays Total RNA was isolated from frozen tissues or cultured cells using TRIZOL reagent (Invitrogen); concentrations were determined by spectrophotometry and integrity verified by gel electrophoresis. To examine *hGHR* expression, 5 μg total RNA were reverse transcribed in 20 μl containing 1x reverse transcription (RT) buffer, 0.5 μM *hGHR*-specific antisense primer (Exon2_R3; **Table II-1**) and 200 U Superscript II RT. Reactions were incubated at 42 C for 50 min and terminated at 70 C for 15 min. PCR assays were carried out in 25 μl containing 1x PCR buffer, 2 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphates, 0.2 μM 5'UTR *hGHR* variant-specific sense primers (**Table II-1**), 0.2 μM *hGHR* antisense primer (Exon2_R3 or Exon2_R4; **Table II-1**), 2.5 μl RT product, and 5 U Taq DNA polymerase (Invitrogen). After an initial incubation for 3 min at 94 C, the sample was amplified for 35 cycles comprised of 94 C for 30 sec, 66 C for 30 sec, and 72 C for 1 min. H_2O and no RT

controls were always included for assessment of contamination. PCR products were cloned into the pCR2.1-TOPO vector and sequenced to confirm correct amplification.

Semiquantitative RT-PCR Relative expression levels were determined by duplex RT-PCR, using the Quantum RNA 18S internal standards kit (Ambion Inc., Austin, TX): this includes 18S primers and a pair of 18S competimers to adjust for variations in PCR amplification and loading. In brief, 1 μg of total RNA was treated with RNase-free DNase I (Invitrogen) and used in a 20-μl RT reaction with random hexamers (Invitrogen) and Superscript II RT. Duplex PCRs were carried out with 2.3 μl of RT product (limiting the analysis to eight variants) mixed with sense primers specific to the *hGHR* 5'UTR noncoding regions and an antisense primer to coding exon 2, and Quantum RNA 18S internal standards with a predetermined ratio (primer to competimer of 1:4). The PCR profile was 94 C for 30 sec, 63–64 C for 30 sec, and 72 C for 1 min. After 34 cycles (preliminary tests showed to be always an exponential amplification), PCR products were resolved on a 2% agarose gel and densitometric analyses performed using GelDoc software (Sigma). Data are presented as the relative ratio of total *hGHR* mRNA or of each 5'UTR *hGHR* variant normalized to 18S RNA.

Statistical analyses Statistical significance ($P < 0.05$) was determined using the Student's *t* test.

RESULTS

Characterization of adipocyte hGHR mRNA variants

5'RACE of human adipocyte cDNA resulted in 97 *hGHR*-derived inserts; 86 were V2-like (*i.e.* matched the exon V2 sequence), three were V3-like, and eight were new variants (**Table II-2**). Five novel 5'UTR sequences, designated VA-VE, were identified (**Figures II-1A and 1C**). Both VB clones contained a 55 nt sequence at their 5'-end derived from V3, indicating that VB is an alternatively spliced product from exon V3. The VE sequence is an 111-nt 3'extension of exon V3a/b, which, given the *hGHR* genomic sequence, must be generated through the use of different GT splice sites. In contrast, the

VA, VC, and VD clones showed no sequence homology with previously published *hGHR* 5'UTR variants. All three VA clones had an extra 15 nt at their 5'-ends that were the same as the 15 nt at the 3'-end of the VC sequence, suggesting that VA is an alternatively spliced product of exon Vc.

Genomic organization of the new 5'UTR non-coding exons

By comparison with the GenBank human genome database, using the Blast algorithm (National Center for Biotechnology Information, Bethesda, MD), all five new 5'UTR sequences were mapped to discrete regions within the 5'-flanking region of the *hGHR* gene, between modules A and B (GenBank no. NT_006576.15) (**Figure II-1A**). It should be noted that previous mapping of the exons (Goodyer *et al.* 2001b) was undertaken with a BAC clone (hcit. 102E14) that must have had deletions and/or recombinations resulting in the loss of approximately 104 kb to account for the differences observed with the 5'-flanking region in the present GenBank human genome.

To determine whether there are homologs in other species, we compared the new 5'UTR sequences to the mouse genome and known rat, bovine, and ovine *GHR* mRNA variants in the GenBank databases. Only VC exhibited any similarity (87%) with the mouse genome. Interestingly, the genomic location of this VC-like sequence (mLC, **Figure II-1B**) is also approximately midway between mouse L2 (V2-like) and L1 (V1-like).

Expression of the new 5'UTR variants in human tissues

Multiple human adult (liver, fat, kidney) and fetal (liver, fat, kidney, lung, skeletal muscle, placenta) tissues were examined to determine whether expression of the new 5'UTR transcripts were adipocyte specific. With VA, VB, and VE primers, amplicons of expected sizes were detected in all tissues (**Figure II-2**; data not shown); these products were sequenced, confirming the correct amplification. Occasionally a second minor slower migrating band was obtained with VA and VB primers. With the VC primer, two major amplicons were obtained in all tissues, suggesting alternative splice products: sequencing revealed that the lower band (223 bp, **Figure II-2**) is VC itself, whereas the upper band (348 bp) is VC+VA. This finding was consistent with the 5'RACE results, in which all three VA clones contained 15 nucleotides derived from VC at their 5' ends. No

specific amplification from VD was detected in any tissue, even fat, suggesting that the PCR conditions were not optimal or the level of expression is extremely low. Thus, the RT-PCR screening confirmed the existence of four (VA, VB, VC, VE) new *hGHR* transcripts. They were expressed in every tissue tested and were not unique for adipocytes.

Relative expression of hGHR 5'UTR variants in human liver and fat

To determine the relative abundance of hGHR transcripts, we developed duplex RT-PCR assays. Because different primer pairs were used for each variant, the resultant data do not add up to total GHR but give a relative comparative value. Studies of adult liver and fat (**Figures II-3A** and **3B**) showed that whereas V2, V3, V9, VA, VC, and VC+A are expressed in both tissues, V1 was highly expressed only in postnatal liver. These data support previous reports that V1 is a postnatal liver-specific transcript (Goodyer *et al.* 2001b; Zou *et al.* 1997; Zogopoulos *et al.* 1996a). In adult liver, V1 and V2 transcripts form the majority of the hGHR mRNA pool, whereas in adult fat, V2 predominates. In both tissues, VA, VC, and VC+A are expressed at lower levels, similar to V3 and V9. Assays (n = 2) of fetal liver showed similar V2, V9, and V3 expression as in adult liver and fat, with a relatively low level of VA, VC, and VC+A, and no V1 (data not shown). Unfortunately, the fetal fat samples were insufficient for duplex assays.

hGHR mRNA expression in SGBS adipocytes

Zou *et al.* reported previously that total GHR mRNA levels increased significantly during differentiation of mouse 3T3-L1 preadipocytes (Zou *et al.* 1997). However, similar studies during human adipocyte differentiation have not been published. We, therefore, obtained the SGBS human preadipocyte cell line (Wabitsch *et al.* 2001) and assessed the quantitative changes in total hGHR mRNA as well as six of the most highly expressed variant transcripts during maturation, using duplex RT-PCR assays. At the predipocyte stage, the predominant variant (~70%) was V2; other forms were either expressed at low levels (e.g. VA and VC+A: ~10%) or were barely detectable (e.g. V3, V9, VC) (**Figure II-4**). Total hGHR and V2 mRNAs were markedly up-regulated in parallel from d 0–4 postinduction of differentiation, reaching a maximum by d 12 [hGHR: 2.3 ± 0.2-fold

(mean ± SE), $P < 0.01$; V2: 3.0 ± 0.8, $P < 0.03$]. VA and VC+A mRNAs also increased but later, between d 4 and 12, whereas the other 5'UTR transcripts remained minor populations. These data clearly demonstrate that total hGHR gene expression increases significantly as human preadipocytes differentiate and that this is mainly due to the V2 transcript, especially during the earliest developmental stages.

DISCUSSION

GH has many biological effects, all of which depend on the presence of its specific receptor on the surface of target cells. Whereas the hGHR is encoded by a single gene, the complex organization of its 5'-flanking region, which now includes at least 13 noncoding first exons, provides several mechanisms (e.g. alternative promoters, splicing) for tissue-specific as well as developmental-specific regulation of hGHR expression (Orlovskii *et al.* 2004; Goodyer *et al.* 2001b).

First isolated from adult liver and cardiac muscle cDNA libraries, eight hGHR mRNA variants have been well characterized to date, with four showing developmental (postnatal)- and tissue (liver)-specific expression (Orlovskii *et al.* 2004; Goodyer *et al.* 2001b; Pekhletsky *et al.* 1992). We were interested in examining the hGHR transcripts expressed in adipose tissue, another important hGH target, to know whether there might be adipocyte-specific variants. Using 5'RACE, we demonstrated that V2 is the predominant (89%) hGHR transcript in fat cells. This is strikingly different from what was reported for an adult liver cDNA library, in which V1 (47%), V2 (27%), and V3 (11%) were all highly expressed (Pekhletsky *et al.* 1992), or from the study of a cardiac muscle library, in which V2 (37.5%), V9 (25%), and V3 (16.7%) were all well represented (Goodyer *et al.* 2001b). This difference was further confirmed by our semiquantitative RT-PCR comparison of the expression patterns of total hGHR and several of its mRNA variants in adult liver vs. fat. Although no other data from human adipose tissue have been reported, similar findings in the mouse were presented by Moffat et al. (Moffat *et al.* 2000): the L2 GHR variant (V2-like) was present in 88% of the 5'RACE clones from adipose tissue of nonpregnant mice. Therefore, although liver,

muscle, and adipose tissues are all major target organs for hGH and all express high levels of hGHR mRNA (Fisker *et al.* 2001; Goodyer *et al.* 2001a; Ballesteros *et al.* 2000; Hermansson *et al.* 1997), the mechanisms regulating hGHR expression in each tissue appear to be very different.

In our study, five new 5'UTR mRNA variants were isolated. Except for VA, which Wickelgren *et al.* also observed in adipose tissue (unpublished data, GenBank no. AY216680), the other four variants have never been reported. The new 5'UTR sequences all map within the 5'-flanking region of the hGHR gene. In a comparison with the mouse genome, the VC sequence showed high similarity (87%) to a homologous region of the mouse GHR 5'-flanking region. None of the other novel variants had significant similarities, and there were no homologies, with any of the known rat, ovine, or bovine GHR 5'UTR exons.

To determine whether these new variants were adipose-specific transcripts, we screened multiple fetal and adult tissues using RT-PCR. Unfortunately, the answer was no: four of the new variants [VA, VB, VC (and VC+A), VE] could be detected in all of the tissues tested. Although we were unable to amplify a VD transcript, the VD exon was localized to 1.2 kb downstream of module A, suggesting that it may be a low-expressing hGHR variant exon. Duplex RT-PCR assays confirmed these data and demonstrated that variants VA, VC, and VC+A are expressed in both adult liver and fat at levels similar to V3 and V9, whereas a more limited study of fetal liver suggests they may be expressed at lower levels early in gestation.

The possible functions of these widely expressed minor transcripts have long been a puzzle that is not limited to the human: there are multiple GHR mRNA variants expressed at low levels in the mouse and bovine as well [e.g. mL3–5 (Moffat *et al.* 2000), b1C-1I (Jiang & Lucy 2001b)]. They do not appear to contribute significantly to GHR expression under normal physiological conditions. However, it cannot be excluded that, under certain pathophysiological conditions, the expression of one minor transcript will increase markedly in a specific tissue or several tissues, causing a major change in GHR

expression and affecting sensitivity to GH. One other possibility is suggested by previous studies showing that different GHR 5'UTR sequences have profound effects on translational activities of test RNAs. Jiang and Lucy (Jiang & Lucy 2001b) found that, in cattle, the predominant nonhepatic GHR 5'UTR variant b1B (V2-like) had the poorest translational ability, whereas other ubiquitously expressed minor transcripts (e.g. b1H, b1I) were more efficiently translated. Moffat et al.(Moffat *et al.* 1999a) reported analogous observations for mouse GHR mRNA variants.

Seven of the previously identified hGHR 5'UTR exons form two small clusters in the 5'-flanking region of the hGHR gene. Three mechanisms have been suggested for regulation of their transcriptional activity: 1) an upstream common promoter that controls transcription from all exons within the cluster; 2) independent promoters that regulate transcription from individual exons; or 3) a combination of the two possibilities (Goodyer *et al.* 2001b; Jiang & Lucy 2001b; Moffat *et al.* 2000). To determine how the five new mRNA variants may be generated, we located their positions on the human genome sequence relative to the known hGHR coding and 5'non coding exons. VC and VA exons are clustered in a 1.2-kb region, 81.3 kb upstream of hGHR coding exon 2, approximately midway between modules A and B, suggesting that these two are regulated by a new promoter region. Both Wickelgren et al. (GenBank submission) and we (present study) found that the VA sequence had extra nucleotides at the 5'end that are homologous to the 3'end of VC, indicating that VA is an alternatively spliced product of VC. These speculations were confirmed by our RT-PCR tissue screening, in which the VC primer was able to consistently amplify two products (VC, VC+A), and by duplex RT-PCR quantification, in which the level of VA approximates the level of VC+A. Based on these data, we believe that the VC and VA transcripts are generated from a common promoter, located 5' to VC. Both VB and VE appear to arise from the hGHR transcript initiated from exon V3 because 5'RACE clones containing VB or VE all include 40–53 nt of the V3 sequence at their 5' end. However, RT-PCR with the V3 primer could not consistently amplify V3+VB or V3+VE products, suggesting that these transcripts have low expression levels in the tissues tested.

The new variants were not adipose tissue specific. However, fat tissue is a complex mix of mature adipocytes as well as preadipocytes, vascular cells, and pericytes (Ailhaud *et al.* 1992). Therefore, we hypothesized that there might be differential expression of hGHR transcripts at specific developmental stages. Using the human preadipocyte SGBS cells, we found that total hGHR mRNA levels increased significantly during early stages (d 0–4) of SGBS cell differentiation and reached a maximum when the adipocytes matured (d 12). Although differentiation procedures for human and mouse cell lines are quite different, these data are consistent with what Zou et al. (Zou *et al.* 1997) observed during 3T3-L1 preadipocyte differentiation. They found, using RNase protection assays, that GHR mRNA levels began to increase after 3 d of differentiation and reached a maximum in mature adipocytes by d 7; the relative abundance of total GHR mRNA was 28 times greater in the mature adipocyte, compared with the preadipocyte. The difference in fold change levels achieved in each case (28 times in 3T3-L1, 2.4 times in SGBS) may be due to different sensitivity of the assay methods as well as the cells themselves.
We show here for the first time that the significant increase in total hGHR mRNA is primarily due to V2, the predominant hGHR transcript in adipose tissue. More interestingly, we observed specific variant expression patterns in preadipocytes vs. mature adipocytes. In the preadipocyte, the major transcript is V2; we also observed low levels of the VA and VC+A forms but could not consistently detect V3, V9, or VC. In the mature adipocyte, VA and VC+A transcripts represented a significant proportion of the total hGHR pool (~35%) whereas V3, V9, and VC remained at low levels. These data suggest that certain hGHR 5'UTR transcripts may be more prominently expressed at certain developmental stages, perhaps leading to differential expression of hGHR and, ultimately, altered responses to hGH.

The widely expressed hGHR V9 transcript was first isolated from a cardiac muscle cDNA library (Goodyer *et al.* 2001b). Two other V9-related mRNA variants, V9a and V9b, were observed by Wickelgren et al. in skeletal muscle (unpublished data, GenBank no. AF230800 and AF230801). By comparison with the human genome, we found that V9b is the 3' end of V9 and that V9a is an alternative splicing product of V9 (with the V9a exon located about midway between VA and module B). However, in our adipose

5'RACE screen, we did not obtain any V9-containing clones. In addition, semiquantitative RT-PCR assays of adult fat samples showed that V9 mRNAs were expressed at a relatively low level. Furthermore, few V9 transcripts could be detected in SGBS preadipocytes, and even after differentiating into mature adipocytes, V9 expression remained low. Thus, in contrast to muscle, it appears that the V9 hGHR transcript is minimally expressed in adipocytes.

Obesity has become a major epidemic health problem, especially in developed nations. To combat this, it will be important to have a thorough understanding of the mechanisms controlling the ontogeny as well as the mass of adipose tissue. hGH has long been known to be one of the most important negative regulatory hormones: hGH treatments significantly reduce body fat in GH-deficient children and adults (Boguszewski *et al.* 2005; Carroll *et al.* 2004; Leal 2004; Maison *et al.* 2004; Fisker *et al.* 2001; Brook 1973), although similar treatments of morbidly obese individuals who are not hGH deficient have been less successful for unknown reasons (Franco *et al.* 2005; Albert & Mooradian 2004; Murray & Shalet 2002).

Whereas the effects of hGH on adipose tissue are known to be through its specific receptor, our understanding of how hGHR expression is regulated in adipocytes is surprisingly limited. It has been well documented that obese people have low hGH levels, due to the inhibitory effects of excess FFA on hGH secretion as well as increased hGH clearance (Franco *et al.* 2005; Gleeson *et al.* 2005; Miller *et al.* 2005). In contrast, there are no reports on their adipose tissue hGHR. Their relatively low responsiveness to chronic hGH therapy suggests decreased hGHR expression and/or function. However, Gleeson et al. (Gleeson *et al.* 2005) observed an increase in circulating IGF-I, positively correlated with GH binding protein levels after a single bolus of hGH in obese individuals. Whether this increase in hepatic responsiveness reflects conditions within the adipocyte remains to be determined.

Our present study begins to address this issue. However, more in-depth studies of the V2 promoter and primary human adipose tissues are needed to better understand what

regulates hGHR expression in adipocytes and how this may alter adipocyte sensitivity to hGH.

ACKNOWLEDGEMENT

We gratefully acknowledge the gift of SGBS cells from Dr. M. Wabitsch (University of Ulm, Ulm, Germany).

Table II-1. Primers used for 5'RACE and RT-PCR.

Oligonucleotide	Sequence (5'-3')
a) 5'UTR of the hGHR gene	
V1 [S]	AAGCCTGGAGGAAACAATACGA
V2 [S]	GCACCATTGGCCCCAGCG
V3 [S]	CTGTTTGTGCCGCCAGGAGAC
V9 [S]	ATTCACCAAGTGCCGTTCACCTGA
VA [S]	AGGGTCATAGTCAGCCACATGTAAA
VB [S]	CGAAGGGGTCCGATTAGTTGTCA
VC [S]	GCCTGCTGCTCTTTGTCTTCAG
VE [S]	AGAAATCTGCCCAAGGAGCCTGA
b) Coding region of the hGHR gene	
Exon2_R3 [AS]	CCTCACTTCCAGAAAAAGCATCACT
Exon2_R4 [AS]	CTGCCAGAGATCCATACCTGTAGG
Exon4 [S]	ATTCACCAAGTGCCGTTCACCTGA
Exon5B [AS]	AGGTATCCAGATGGAGGTAAACG
c) Miscellaneous	
Ap-1	CCATCCTAATACGACTCACTATAGGGC
M13R	CAGGAAACAGCTATGAC

Ap-1, Anchor primer.

Table II-2: Distribution of *hGHR* 5'UTR mRNA variants among 5'RACE clones.

***hGHR* 5'UTR mRNA variants from human adult adipocyte cDNA**		**Maximum length (nt) of cloned 5'UTR sequences**	**Number of clones obtained**	**% of total *hGHR*-related 5'UTR clones**
hGHR-related 5'UTR clones:	V2	71	86	89
	V3	141	3	3
	VA[a]	125	3	3
	VB[a]	220	2	2
	VC[a]	142	1	1
	VD[a]	45	1	1
	VE[a]	111	1	1
			97	100
hGHR non-related clones[b]:			23	
Total sequenced:			120	

[a] Novel variants with no sequence homology with previously identified hGHR 5'UTR mRNA variants.

[b] Clones that do not contain the hGHR gene-specific antisense primer Exon2_R4 sequence.

Figure II-1.

(A) ***Schematic of the hGHR gene structure.***

The 5'non-coding exons (V1-V5, V7-V9, V3a/b, VA-VE) and the nine coding exons (*open boxes*) are shown. The ATG translation start site is located in exon 2. Seven of the 5'non-coding exons are clustered in ~2kb regions to form two separate modules: Module A (V2-V9-V3) is ~140.8-142.4kb upstream of exon 2, whereas Module B (V7-V1-V4-V8) is ~15.7-17.9kb upstream of exon 2. All of the exons were mapped within the 5'upstream region of *hGHR* gene on chromosome 5 based on contig Hs5_6733 (Genbank no. NT_006576.15). Previous mapping of Module A and B exons (Goodyer *et al.* 2001b) was undertaken with a BAC clone (hcit. 102E14) that must have had deletions and/or recombinations resulting in the loss of approximately 104kb to account for the differences observed with the 5' flanking region in the present GenBank human genome sequence.

(B) ***Schematic representation of the chromosomal location of the VC homologous sequence on the mouse GHR gene.***

The 5' flanking region of the mouse *GHR* gene contains a sequence showing homology to the newly characterized VC exon ("mLC"), located approximately midway between the L2 (V2-like) and L1 (V1-like) exons.

(C) ***5'UTR sequences of the five new hGHR mRNA variants.***

Sequences of the newly identified 5'UTR non-coding exons are shown in *uppercase letters*. The numbers indicate the position and the length of each new 5'UTR based on the Genbank human genome database (#NT_006576.15). Extra 5' nucleotides found in respective clones are illustrated in the *shaded box of lowercase letters* (spliced from VC), *the boxed lowercase letters* (spliced from V3) or *the underlined italics* (spliced from V3a/b) (see details in **Results**). These sequences have been submitted to the GenBank nucleotide database under the following accession numbers: VA: DQ157455, VB: DQ157456, VC: DQ157457, and VE: DQ157458. VD is ineligible for submission (<50nt).

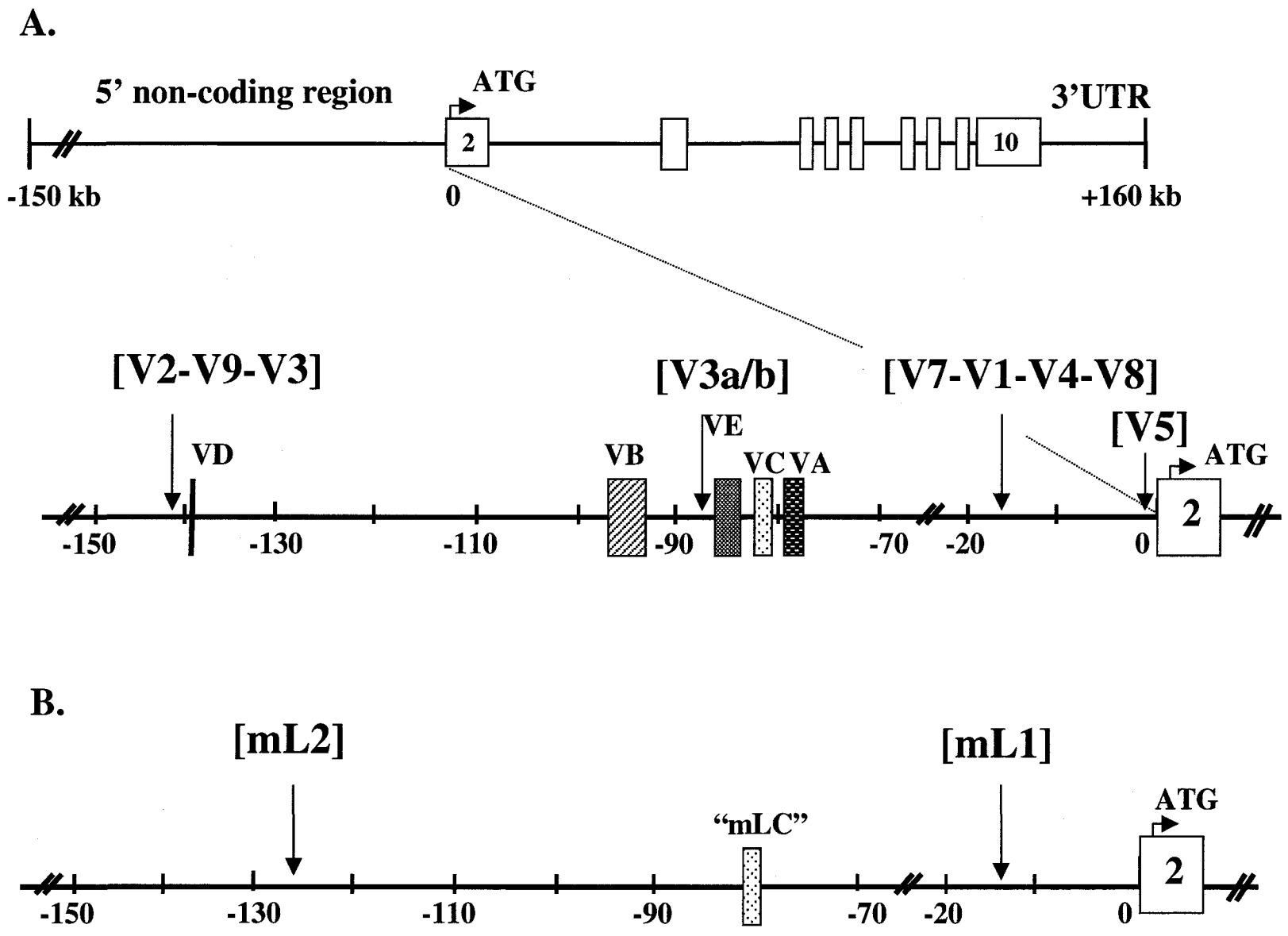

A.
5' non-coding region
ATG
3'UTR
2
10
-150 kb
0
+160 kb
[V2-V9-V3]
[V3a/b]
[V7-V1-V4-V8]
[V5]
VD
VB
VE
VC
VA
ATG
-150
-130
-110
-90
-70
-20
0
2
B.
[mL2]
[mL1]
"mLC"
ATG
-150
-130
-110
-90
-70
-20
0
2

Figure II-1

C.

Exon VA [NT_006576.15: 42458568-42458690]: 123nt

caagacaggaaaaagGGTCATAGTCAGCCACATGTAAATCAGAATGCAGTTTTACATAGATTCATTATTACTGTGCATGGAGAT
TAGAAGCCAGCTCACAAGAAGACTTAATTTGGAAAGATATGTAACCAACTTCAC

Exon VB [NT_006576.15: 42441636-42441855]: 220nt

aggtggtcgcctgtctgtttgtgccgccaggagaccttggaagggacagagaaagCACCTCGAAGGGGTCCGATTAGTTGTCAAATAACTGGTCGAAT
CGGTTGGTGACCAGGCAGCTGAAGCCTTCCTGTAACTGCCCAGGCATGATGGTTGCTCGGCGGCACATTCCTCCACGGA
TTGCAGCGGGCTGCGCCGAGCCAAGAGCGCGTGCTTCAGCTCTTCCCAGAAGATCGAAGAACAATTTTTTTGAAATCC
GCCTGTAAATGTCAGGCAAG

Exon VC [NT_006576.15: 42457397-42457538]: 142nt

AGAAATATGCAGCCTGCTGCTCTTTTGTCTTCAGTGTGACCATGCTGCCCTGAGGAGAGCCCAGAACTGTTTTTAAATT
CAAAAGGACAGCTGCTTTCTGCTCACTCAGGTCCACTGAGGCATGCTCCAAGACAGGAAAAAG

Exon VD [NT_006576.15: 42399138-42399182]: 45nt

CTTGCAGTTGGTCCAACTTGGCTAGCCTTCTTTGGCTATCTCCTG

Exon VE [NT_006576.15: 42456191-42456301]: 111nt

ctgtttgtgccgccaggagaccttggaagggacagagaaag*agctggggttttgccatgttgcccaggcttgatctccaacacctgggatcaagcagtctgcccaccttggcctcccaaagtgc*
*ttgaaatacagggt*ATGAGCCTCCATGGCTGGGCACTTACTTGTCTTTTTAATTATCAGCTTGCAAATGAGAAATCTGCCCAAG
GAGCCTGAGCTTGGTGAATCAAGTTCATTTCAGCCCCATTG

Figure II-1

Figure II-2. ***Expression of new 5'UTR mRNA variants in human tissues.***

The expression patterns of the new mRNA variants (VA-VE) were examined by RT-PCR using total RNAs extracted from several human fetal and adult tissues. The cDNAs were amplified with sense primers specific to the individual non-coding exons and a common antisense primer to *hGHR* exon 2 (Table II-1). The widely expressed V2 and V3 variants were used as positive controls; the two lower V3 bands represent V3 and V3+V3a/b, whereas the third band was minor and invariably expressed. PCR products were run on 2% agarose gels to visualize the specific amplicons, using the100bp DNA ladder as a migration marker (M). Representative gels are shown from studies of samples (n=3) for each tissue. There was occasionally a second VA or VB band that could not be sequenced due to low expression levels. VC primers detected two bands due to alternative splicing (see details in Results). A specific VD product was never visualized, although nonspecific bands did occur occasionally (*e.g.* fetal and adult kidney gels), suggesting either technical problems with the PCR assay conditions or very low levels of expression in these tissues. The VE primers amplified a single amplicon of the expected size in all tissues (V3* shows the results of using a different antisense primer).

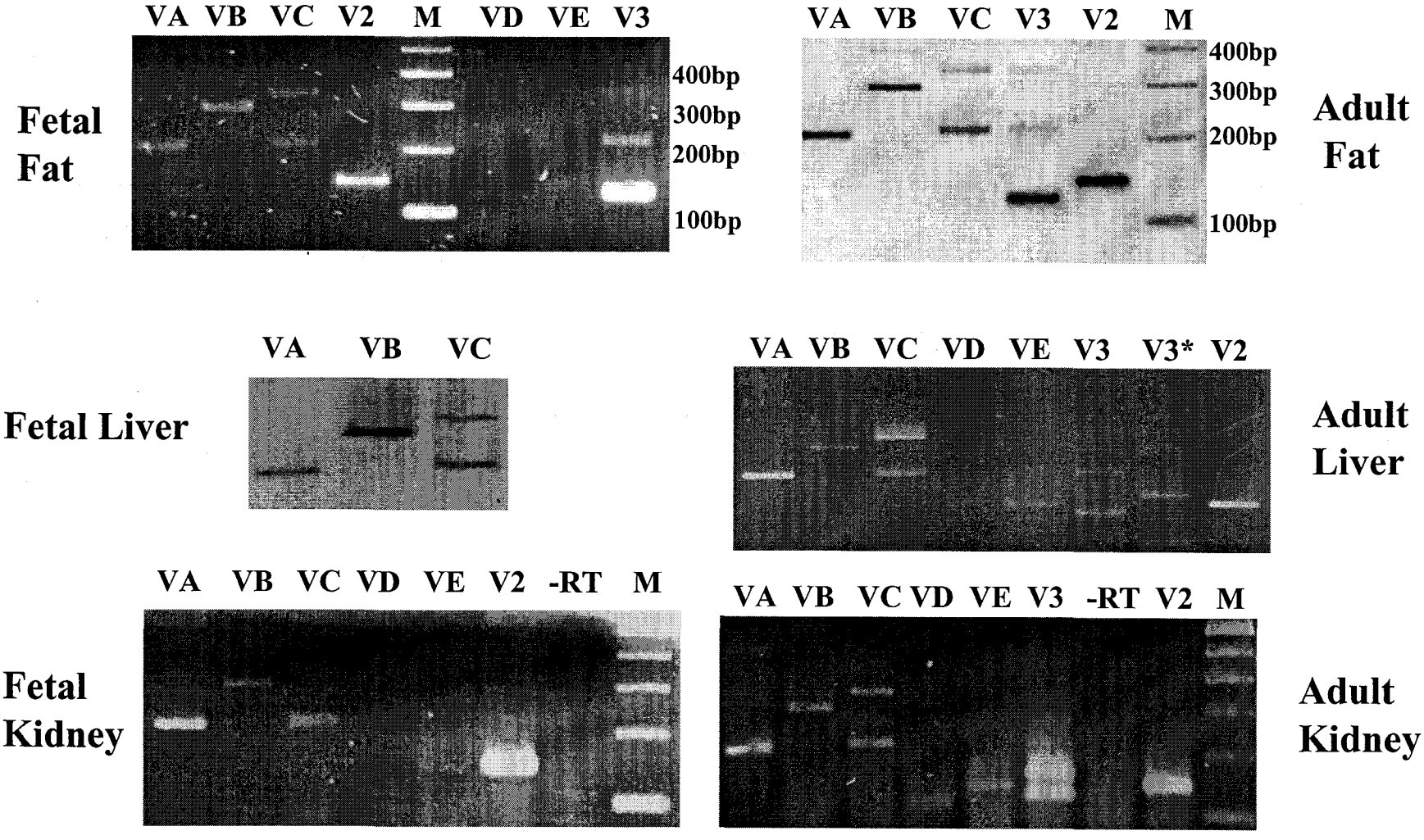

VA VB VC V2 M VD VE V3
400bp
300bp
200bp
100bp
Fetal Fat
VA VB VC V3 V2 M
400bp
300bp
200bp
100bp
Adult Fat
VA VB VC
Fetal Liver
VA VB VC VD VE V3 V3* V2
Adult Liver
VA VB VC VD VE V2 -RT M
Fetal Kidney
VA VB VC VD VE V3 -RT V2 M
Adult Kidney

Figure II-2

Figure II-3. ***Relative expression of total hGHR and seven of its mRNA variants in adult liver and fat.***

The relative expression levels of total *hGHR* and seven of its mRNA variants (V2, V3, V9, V1, VA, VC and VC+A) in human adult liver (hAL) or fat (hAF) tissue were estimated by semi-quantitative duplex RT-PCR.

A) The relative ratios of total *hGHR* or individual mRNA variants were normalized to 18S rRNA (Quantum RNA 18S Internal Standard; Ambion). Each tissue type was analyzed using multiple samples (n=3) and the data are presented as M±SE. *, $p<0.05$; ***, $p<0.001$.

B) Representative gels of the semi-quantitative RT-PCRs. Twelve microliters of PCR products were separated on 2% agarose gels using the 100bp DNA ladder as a marker (M). Arrows indicate the expected 18S amplicon (489bp), which was used as the internal control.

A.

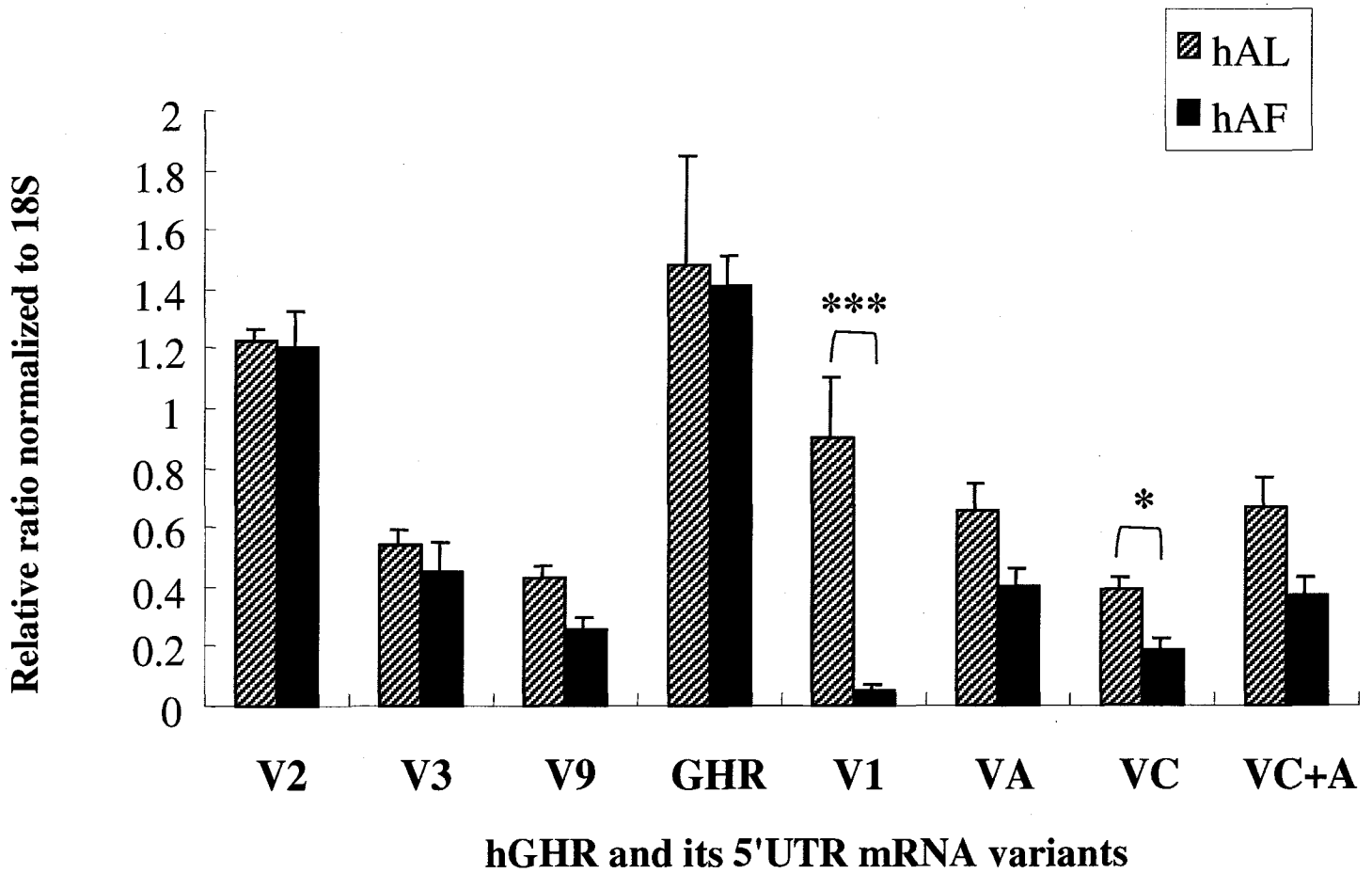

hAL
hAF
Relative ratio normalized to 18S
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
*
V2
V3
V9
GHR
V1
VA
VC
VC+A
hGHR and its 5'UTR mRNA variants

Figure II-3

B.

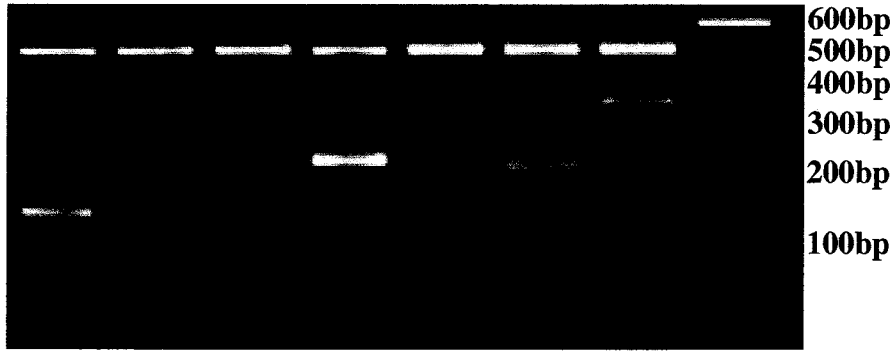
Human Adult Liver

18S (489bp) →

V2 V3 V9 V1 GHR VA VC M

Human Adult Fat

18S (489bp) →



V2 V3 V9 GHR V1 VA VC M

Figure II-3

Figure II-4. ***Relative expression of total hGHR and hGHR mRNA variants during SGBS adipocyte differentiation.***

Human SGBS preadipocytes were cultured to confluence and then differentiated into mature adipocytes (Wabitsch *et al.* 2001). Day 0 represents the confluent preadipocyte stage before the induction of differentiation. Samples were collected at 2 to 4-d intervals during the differentiation process. The relative expression levels of total *hGHR* and six of its mRNA variants (V2, V3, V9, VA, VC and VC+A) were assessed by semi-quantitative RT-PCR using 18S rRNA as internal control (see Results and legend of Fig. 3 for details). Data are presented as the relative ratios of either total *hGHR* or its individual mRNA variants normalized to 18S rRNA. Each time point represents two (M) or three (M±SE) differentiation experiments (n=2 for days 4 and 6; n=3 for days 0, 8 and 12).

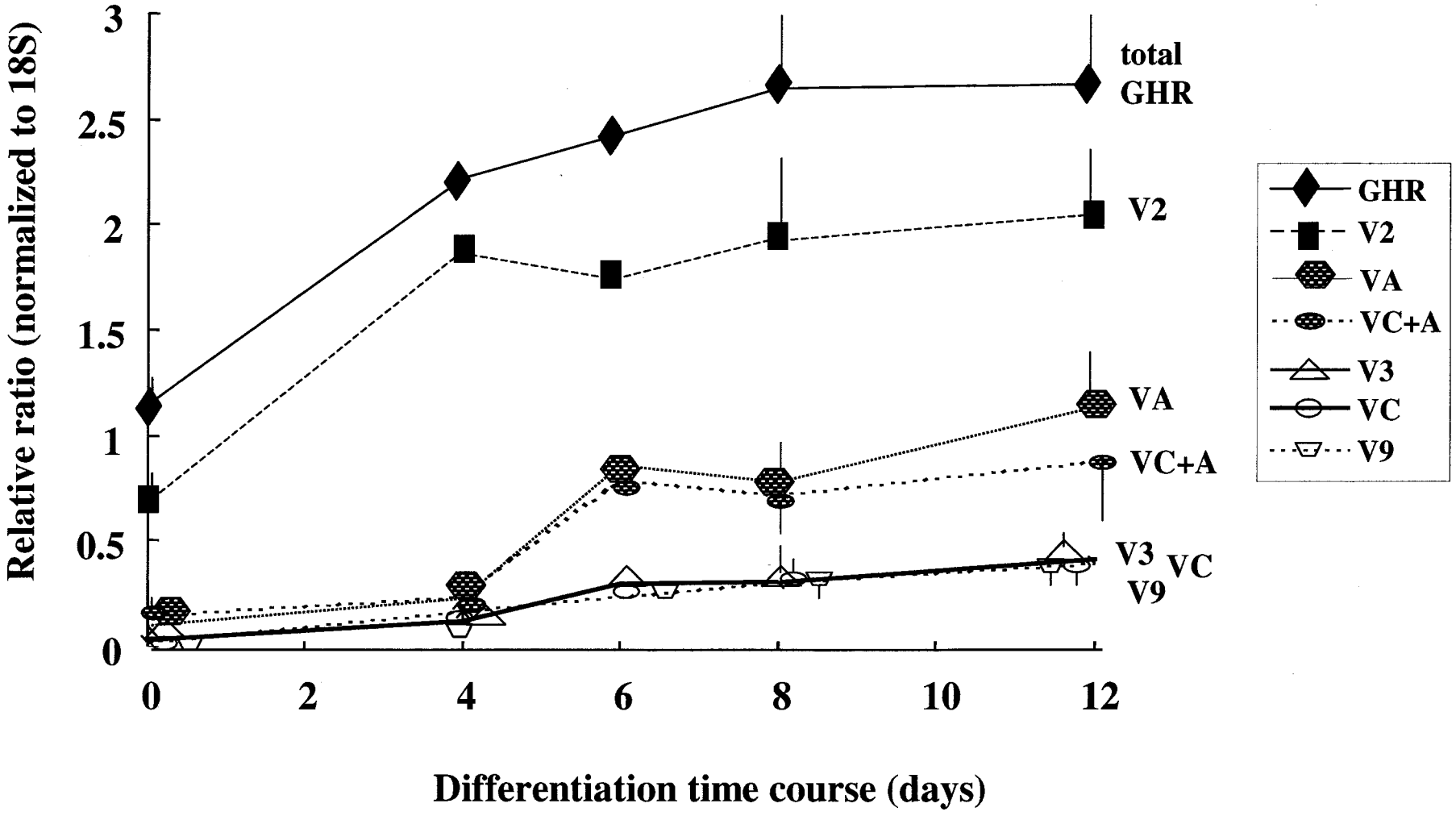

Relative ratio (normalized to 18S)
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
Differentiation time course (days)
total GHR
V2
VA
VC+A
V3
VC
V9
GHR
V2
VA
VC+A
V3
VC
V9

Figure II-4

PREFACE

The predominant expression of the hGHR V2 variant in adipocytes suggests its importance in determining hGHR expression levels and hGH effects on adipose tissue. In addition, the hGHR V2 transcript is known to constitute the majority of the hGHR mRNA pool in almost all non-hepatic tissues and is the second most expressed variant in adult liver. In view of these data, I carried out in-depth studies of the V2 promoter and its regulation in different human cell lines, including human adipocytes.

The following chapter is a manuscript in preparation under the title "Transcriptional Regulation of Human Growth Hormone Receptor (hGHR) Gene by its V2 Promoter" by Yuhong Wei and Cynthia G. Goodyer.

All of the work presented was conducted by the candidate.

CHAPTER III

TRANSCRIPTIONAL REGULATION OF HUMAN GROWTH HORMONE RECEPTOR (hGHR) GENE BY ITS V2 PROMOTER

ABSTRACT

Expression and function of the GHR is critical for the actions of GH. *GHR* gene expression is characterized by the use of multiple 5'non-coding exons and alternative splicing, resulting in the generation of multiple 5'UTR mRNA isoforms. The hGHR V2 transcript is predominant in most tissues, including human fat. However, factors regulating the expression of hGHR V2 have remained unidentified. The present study is aimed at characterizing the mechanisms regulating hGHR V2 expression in different human cell lines, including human adipocytes.

Two major V2 transcriptional start sites were identified by primer extension in human preadipocytes, liver and kidney cells. Transient transfection analysis of the 5'flanking region of V2 confirmed its promoter activity in multiple human cell lines. Similar transcriptional profiles were observed in SGBS pre- and mature adipocytes, with much higher V2 promoter activity seen in mature adipocytes, suggesting that changes in the availability of active forms of positive and/or negative transcription factors play a role in V2 promoter regulation. The V2 proximal promoter is characterized as TATA-less, with several characteristics of a housekeeping gene promoter. Transcription of V2 is determined by several distinct functional domains. Cis-acting elements for Ets1, CHOP and Hes1 were found in the proximal promoter region and within the V2 exon; they exert either positive (Ets1, CHOP) or negative (Hes1) regulatory effects on V2 transcription when their respective transcription factors are over-expressed. These sites are likely responsible for regulating *hGHR* gene expression in response to extra-and intra-cellular signals, including development, growth and stress. Collectively, our data suggest that transcriptional regulation of hGHR V2 in human cells, including adipocytes, is the result of a complex interplay by multiple factors, to ensure sufficient local production of hGHR throughout life.

INTRODUCTION

Human growth hormone (hGH) exerts a number of important biological effects, including regulation of postnatal growth, carbohydrate, protein and fat metabolism, immune function, cell proliferation and differentiation (Perry *et al.* 2006; Herrington & Carter-Su 2001; Waters 1999). These pleiotropic effects result either from direct actions of hGH on target cells or indirect actions by stimulating hepatic- or local tissue production of IGF-I (Le Roith *et al.* 2001). To initiate these actions, hGH has to bind to its specific cell surface receptor (hGHR) and then activate various intracellular signaling cascades (Lanning & Carter-Su 2006; Zhu *et al.* 2001). Therefore, hGHR expression and function in target cells is critical for their responses to hGH. Dysregulation of the GH/GHR axis has been implicated in the pathogenesis of growth retardation, GH insensitivity syndromes and certain types of tumors as well as the progression of chronic complications of diabetes mellitus (DM) (Thimmarayappa *et al.* 2006).

The hGHR is encoded by a single gene (Leung *et al.* 1987). However, multiple hGHR mRNA variants are produced due to transcription from several unique 5'non-coding exons and alternative splicing into a common acceptor site located 11-bp upstream of the ATG translation start codon. Thus, they all code for the same GHR protein but provide a complex regulatory mechanism for *GHR* gene expression through different promoter usage and/or alternative splicing (Edens & Talamantes 1998; Schwartzbauer & Menon 1998). To date, fourteen hGHR mRNA variants have been identified (Wei *et al.* 2006; Orlovskii *et al.* 2004; Goodyer *et al.* 2001b; Pekhletsky *et al.* 1992). Some (V2, V3, V9 and VA-VE) are widely expressed, while others (V1, V7, V4 and V8) are liver and postnatal stage specific (Goodyer *et al.* 2001b). This 5'-UTR heterogeneity is a common feature of *GHR* genes across many species (Edens & Talamantes 1998; Schwartzbauer & Menon 1998).

Several different promoters have been identified (Goodyer *et al.* 2001b; Jiang *et al.* 2000; Yu *et al.* 1999; Jiang *et al.* 1999; Moffat *et al.* 1999b; Zou *et al.* 1997; Adams 1995; O'Mahoney *et al.* 1994). The most well known are: 1) the liver-specific promoter,

also named promoter P1, which is responsible for generating liver-specific transcripts, such as human V1, mouse L1, ovine 1A and bovine 1A; and 2) the promoter for ubiquitously expressed variants, such as human V2, mouse L2, ovine 1B and bovine 1B, which is also called promoter P2.

Originally isolated from a liver cDNA library (Pekhletsky *et al.* 1992), the hGHR V2 mRNA variant is readily detectable in a variety of fetal and adult tissues, including liver, muscle, fat, kidney, lung and heart (Wei *et al.* 2006; Goodyer *et al.* 2001b). Except in adult liver, where the V1 transcript predominates, the V2 transcript accounts for the majority of the hGHR mRNA pool in all other target tissues (Wei *et al.* 2006; Goodyer *et al.* 2001b). Homologues of the hGHR V2, such as ovine 1B (Adams *et al.* 1990), bovine 1B (Heap *et al.* 1996; Heap *et al.* 1995), rat GHR2 (Baumbach & Bingham 1995) and mouse L2 (Southard *et al.* 1995a) also represent the major GHR transcript in their non-hepatic tissues; mouse L2 also predominates in liver of non-pregnant mice (Schwartzbauer & Menon 1998). Such findings indicate that the V2-like transcript is one of the most important amongst all GHR mRNA variants and that its expression is critical to GH actions in a large number of target tissues.

The promoter structures and regulatory mechanisms for ovine 1B, bovine 1B and mouse L2 have been partially studied (Jiang *et al.* 2000; Adams 1999; Yu *et al.* 1999; Moffat *et al.* 1999b; Adams 1995). Multiple transcription start sites have been observed for all three species. They all lack a TATA box in their promoter regions, have a relatively high GC content, and the ubiquitous transcription factor Sp1 appears to play an important role in the regulation of their promoter activities.

Although similar expression patterns and DNA sequences between human V2 and its homologues in other species suggest that a common regulatory mechanism may be present, our knowledge of hGHR V2 promoter structure and its regulation is limited. The transcription start site (s) has not been defined and important *cis*-regulatory elements have not been characterized. Because of the extensive expression of the V2 transcript, filling this gap will be essential for a better understanding of *hGHR* gene expression.

Adipose tissue has long been known to be a major hGH target tissue (Wei *et al.* 2006; Osafo *et al.* 2005). Our previous study demonstrated that hGHR V2 is the predominant GHR transcript in both preadipocytes and mature adipocytes, and that it is the major variant expressed as the total hGHR pool increases during adipocyte differentiation (Wei *et al.* 2006). The purpose of the present study is to characterize the promoter of the hGHR V2 exon and to elucidate the molecular mechanisms underlying the regulation of hGHR V2 in different human cell lines, including human adipocytes.

MATERIALS AND METHODS

Primer Extension Analysis Primer extension analysis was used to map the transcription start sites for hGHR V2. A 28nt oligonucleotide primer (V2PE: 5'-gtcccccagatccagg-ttgcagaagcga-3') complementary to +162 to +135 of the hGHR V2 sequence (Figure 1, relative to designated +1) was end-labelled with [γ-^{32}P] (3000Ci/mmol, Amersham, Piscataway, NS) and T4 polynucleotide kinase (Invitrogen, Burlington, ON, Canada). Total RNAs from multiple cell lines (HEK293, Huh-7, SGBS preadipocytes) and human adult as well as fetal livers were isolated using the QIAGEN RNeasy Mini Kit (Qiagen, Mississauga, ON) or Trizol Reagent (Invitrogen). An aliquot (20μg) of total RNA was mixed with 100fmol of labeled primer (10^6 cpm) and incubated at 65°C for 5min. This primer-RNA mixture was then reverse-transcribed at 60°C for 1hr with ThermoScript reverse transcriptase (Invitrogen). After termination of the reaction, the extension products were ethanol precipitated and size fractionated on a 6% denaturing polyacrylamide gel together with a ^{32}P-labeled ϕX174 *HinfI* DNA marker (Promega, Madison, WI).

Construction of the V2 Promoter Luciferase Reporter Gene Plasmids To characterize the V2 promoter activity, a series of luciferase reporter gene constructs were engineered by ligation of various portions of the hGHR V2 exon and its 5' upstream regulatory regions into the promoterless and enhancerless pGL3-basic vector (Promega). All promoter constructs are identified according to the major ovine transcription start site

(TSS) as +1 throughout this work, because it represents the most farther cDNA 5'end identified for V2 homologues up to date. The largest V2 (-2623/+335) construct was created by three steps: 1) a 3100bp fragment was PCR amplified using forward primer V2F(-2623) and reverse primer V2R(-512) (**Supplementary Table III-1**) with human genomic DNA as a template; 2) this 3100 fragment was cloned into pCR2.1-TOPO vector (Invitrogen); 3) the MluI-BsrBI fragment containing the V2(-2623/+335) piece was excised and ligated into pGL3-basic using MluI and SmaI sites. The V2(-721/+362) construct was engineered by HindIII digestion of a plasmid containing the hGHR V2 exon and approximately 1-kb of 5' upstream region (Goodyer *et al.* 2007) and ligated into the HindIII site of pGL3-basic. The V2(-211/+362) and V2(+11/+362) reporter constructs were prepared by PCR amplification and subcloned into pGL3-basic. Using V2(-211/ +362) as a template, other 5'- or 3'-deletion V2 promoter constructs were generated by amplifying the relevant promoter pieces with nested sense and antisense primers (**Supplementary Table III-1**) and cloning the fragments into pGL3-basic. All of the promoter constructs were sequenced through the vector-insert junctions to ensure nucleotide fidelity.

Mutational Modification of V2 Promoter Sequences The QuickChange II Site-Directed Mutatgenesis kit (Stratagene, La Jolla, CA) was used to introduce mutations into the putative transcription factor binding sites with oligonucleotide primers containing the desired mutations (**Supplementary Table III-1**), as per the manufacturer's instructions. In brief, each 50μl reaction mix contained buffer (1x), dNTPs (200nM each), primers (200nM) and Pfu Turbo DNA polymerase (2.5U). The samples were cycled as follow: 95°C for 30s followed by 16 cycles of 95°C for 30s, 55°C for 1min, and 68°C for 10min. The amplification products were then subjected to DpnI (Roche Diagnostics, Laval, QC, Canada) digestion at 37°C for 1h to digest the non-mutated parental DNA template. All the mutant constructs were sequenced to verify the authenticity of the desired mutations.

Cell Culture HEK293 (human embryonic kidney cells) and COS1 (African green monkey kidney fibroblasts) were obtained from ATCC and grown in standard culture medium (DMEM containing 25mM HEPES, 100IU/ml penicillin, 1.6mg/ml gentamycin

sulphate and 10% fetal bovine serum (FBS). Huh-7, a human hepatoma cell line (kindly provided by Dr. Ken K. Ho, Garvan Institute of Medical Research, Australia), was cultured in Earle's salts MEM with 25mM HEPES, 50IU/ml penicillin and 10% FBS. The human SGBS preadipocytes (kindly provided by Dr. M.Wabitsch, Ulm University, German), derived from the stromal cell fraction of subcutaneous adipose tissue from an infant with Simpson-Golabi-Behmel Syndrome (SGBS) (Wabitsch *et al.* 2001), were cultured and differentiated into mature adipocytes, as previously described (Wei *et al.* 2006).

Transient Transfections, Luciferase and β-Galactosidase Assays Transient transfections of HEK293, COS1 and Huh-7 cell lines were performed using Polyfect transfection reagent (Qiagen), according to the manufacturer's instructions. Briefly, cells were plated in 6- or 12-well tissue culture plates and grown in complete medium for 16-20h to reach 60-80% confluence at the time of transfection. Cells were transfected with a DNA mixture including V2 promoter luciferase reporter constructs (0.5μg), a βgal-control plasmid (0.04-0.2μg), and expression vectors (for Ets1, CHOP, C/EBPβ or Hes1) or their respective empty vectors together with Polyfect reagent at a ratio of 1:3. Transfections were done in triplicate and the empty pGL3-basic vector was used as a negative control. For the human SGBS preadipocytes, transfections were also carried out using the Polyfect method, except that the amount of reporter construct was increased to 1μg for maximal luciferase activity. For differentiated SGBS adipocytes, transfections were performed as previously described (Dalen *et al.* 2003).

Forty eight hours after transfection, cells were harvested in 200μl of 1x passive lysis buffer (Promega) and the lysate supernatant was quantified for luciferase and β-galactosidase activities (Tropix, ABI, Bedford, MA) using an EG&G Berthold Microlumat Plus bioluminometer (LB 96V). The values of luciferase were normalized to the values of β-galactocidase, an internal control for transfection efficiency, and expressed as fold activation, as specified in the figures and legends.

Nuclear Extracts Nuclear proteins were extracted from cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL), as per directions of the manufacturer. The nuclear extract was subjected to dialysis in ice-cold PBS using the Slide-A-Lyzer MINI Dialysis Unit (Pierce) and stored in aliquots at -80°C until use. Protein concentration was estimated using the Bradford assay (Biorad Laboratories Inc., Hercules, CA).

Electrophoretic Mobility Shift and Supershift Assays (EMSA & EMSSA)
Complementary 20- to 35-mer oligonucleotides (**Supplementary Table III-2**) were synthesized, annealed to form double-stranded DNA probes, end-labeled with [γ-^{32}P] and T4 polynucleotide kinase, and cleaned up by passing through G-50 spin columns (Amersham). EMSA were carried out by mixing 50 fmol of labeled probe (~50,000cpm) with nuclear extracts (5-10μg) in 20μl of 10mM Tris-HCl, pH 7.5, 1mM $MgCl_2$, 50mM NaCl, 0.5mM EDTA, 5% glycerol, 1mM DTT and 50μg/ml poly (dI•dC), and incubating at room temperature for 30min. After the incubation, the reactions were loaded onto 5% native polyacrylamide gels and run in 0.5xTBE. The dried gels were exposed to Kodak Biomax-MR film (Kodak, Rochester, NY). For competition assays, a 100-200 fold molar excess of unlabeled probes was co-incubated with the nuclear extracts before the addition of labeled probe. In supershift experiments, 2μg of anti-Ets1 (sc-111, Santa Cruz, Biotechnology, Santa Cruz, CA), or anti-Egr1 (sc-189, Santa Cruz Biotechnology) antibodies were incubated with the nuclear extracts for 1h at 4°C prior to the addition of labeled probes.

Chromatin Immunoprecipitation (ChIP) Assay To analyze the DNA binding of CHOP or Hes1 protein to the hGHR V2 promoter, ChIP assays were performed based on the Upstate protocol (Upstate Biotech, Lake Placid, NY) with certain modifications. Briefly, HEK293 cells were transiently transfected with pcDNA3.1-hygro-CHOP (a gift from Dr. K. Onozaki, Nagoya City University, Japan) or pCMV3.1-Flag-Hes1 (a gift from Dr. S. Stifani, McGill University, Canada). After 48h, the cells were fixed with 1% formaldehyde at room temperature for 10min. Next, cells (10^6) were washed twice with cold PBS and resuspended in 200μl SDS lysis buffer (1%SDS, 10mM EDTA, 50mM

Tris-HCl, pH 8.1, 1x protease inhibitors cocktail) and incubated on ice for 10min. The cell lysates were then sonicated on ice 6x for 10s each (VibraCell Sonicator, Sonics, Betatek Inc., Toronto, ON) to generate genomic fragments between 300-800bp. After sonication, the suspension was centrifuged at 12,000xg for 10min at 4°C. The lysate was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH8.1, 167mM NaCl, 1x protease inhibitors). Equal volumes (1ml) of diluted samples were subsequently precleared with 45µl of protein A/G-agarose beads (sc-2003, Santa Cruz Biotechnology) for 1h at 4°C. Ten percent of the cleared supernatant was kept as input and the remaining volume was immunoprecipitated with 4µg of anti-CHOP/GADD153 (sc-793, Santa Cruz Biotechonology,); anti-Hes1 (sc-13844, Santa Cruz Biotechnology) or normal rabbit IgG (sc-2027) overnight at 4°C with rotation. Protein A/G-agarose beads (45µl) supplemented with 2µg of BSA and 4µg of sonicated herring sperm DNA were then added and further incubated for 2h at 4°C with agitation. The beads were collected and subjected to sequential washes with low-salt buffer, high-salt buffer, LiCl buffer and TE for 10min each at 4°C. The immunoprecipitated protein-DNA complex was eluted in elution buffer (1% SDS, 0.1M $NaHCO_3$). Eluted samples and stored input samples were then reverse cross-linked overnight at 65°C with 200mM NaCl. DNA was extracted using the QIAquick PCR Purification kit (Qiagen) and analyzed by PCR (**Supplementary Table III-2**). PCRs were carried out for a total of 32 cycles and the products were analyzed on 2% agrose gels stained with ethidium bromide.

Real-time quantitative RT-PCR. Quantitative RT-PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen). Total RNAs were extracted from snap-frozen cell pellets collected at different differentiation time points using the RNeasy Mini kit (Qiagen), treated with DNaseI, and reverse transcribed in a 20 µl reaction using random primers and Superscript II reverse transcriptase (Invitrogen). One microliter of each RT reaction was PCR amplified in a 25 µl aliquot containing 2.5 mM MgCl2, 0.4 µM gene specific primers and 1x SYBR Green master mix. Amplification was performed on an Mx4000 QPCR system (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Reactions were carried out in duplicate on three independent sets of samples

for each differentiation time point. Specificity was assessed by melting curve analysis and confirmed on 2% agarose gel after each Q-PCR assay. The abundance of specific mRNAs was determined by comparison with a standard curve generated by serial dilution of the sample and normalized to its corresponding 18S rRNA content. Fold change in expression for a given transcript was calculated relative to the expression of that transcript at Day 0 (calibrator). The relative quantities were expressed as fold (hGHR & V2) or percentage (CHOP & Ets-1) of the calibrator. The sequences of the primers used are provided in **Supplementary Table III-2**.

Statistical Analysis Data are expressed as mean (M) ± Standard Error (SE). The significance of observed differences between groups was determined by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer Multiple Comparison Tests ($p<0.05$ significance) using GraphPad InStat Version 3 (GraphPad Software, Inc.).

RESULTS

Mapping of the Transcription Start Sites

In our previous publications, we have reported the chromosomal location of hGHR V2 (Wei *et al.* 2006; Goodyer *et al.* 2001b). Here, we have mapped the transcription start sites (TSSs) using primer extension analysis with total cellular RNAs isolated from three human cell lines and human liver tissues at different developmental stages. The two longest 5'-cDNA ends that were consistently observed corresponded to approximately 148bp and 135bp in length when analyzed in reference to Promega's *Hinf* I DNA marker (**Figure III-1A**). The 5'-nucleotide of the 148bp product mapped to a position that is 15bp downstream of the major transcription initiation site of ovine 1B (T, **Figure III-1B**) and 2bp downstream of the major transcription site of bovine 1B (G, **Figure III-1B**), both of which are homologues of the hGHR V2 mRNA variant. The 5'-end of the 135bp product corresponded to a site close to the region for minor transcription start sites of ovine 1B and a 2nd major site of bovine 1B (**Figure III-1B**).

Identical multiple start sites for V2 were observed across different human cell lines (HEK293, Huh-7 and SGBS preadipocytes) and human adult and fetal liver, suggesting that these sites are commonly used. As ovine 1B and bovine 1B also show multiple start sites for their transcription initiations and these sites are located in regions similar to the two TSS of human V2 (**Figure III-1B**), we have chosen to designate the ovine 1B major transcription start site (T) as position +1 for our V2 promoter constructs numeration, because it represents the farthest cDNA 5'-end identified amongst all the V2 homologoues.

Functional Analysis of the hGHR V2 Promoter Activity

1) 5'Deletional Analysis: The promoter activity of the –2623 region 5'upstream of the hGHR V2 exon was assessed by its ability to direct the expression of the luciferase reporter gene. DNA fragments having progressively shorter 5'-ends ranging from -2623 to +11 were inserted upstream of the firefly luciferase reporter gene in pGL3-basic, and assayed for luciferase activities following transient transfection into three human cell lines: HEK293 cells, Huh-7 cells and SGBS preadipocytes as well as mature adipocytes. In each cell line, as shown in **Figures III-2B** and **2C**, promoter constructs V2(-2623/ +335), V2(-720/+362), V2(-211/+362) and V2(-29/+301) all showed more than four fold greater activities than did the promoterless control vector pGL3b, with maximal activity consistently seen for the V2(-211/+362) construct. Further 5'-deletion to position +11 resulted in a dramatically decreased promoter activity in HEK293 cells ($p<0.001$, **Figure III-2B**) and mature SGBS adipocytes ($p<0.01$, **Figure III-2C**), and almost background levels of activity in both Huh-7 cells (**Figure III-2B**) and SGBS preadipocytes (**Figure III-2C**), indicating that the fragment between -211 and +11 is essential to direct high level transcription of the hGHR V2. Therefore, we have defined the 211bp region upstream of the TSS as the proximal promoter region of V2 exon. Since the V2(-211/ +362) construct was significantly more active ($P<0.001$) than the V2(-720/+362) construct in all of the tested cells, there appears to be negative regulatory elements in the region between -720 and -211.

2) 3'Deletional Analysis: The initial 3' deletion vector, V2(-211/+71), contained a shorter V2 exon sequence. Intriguingly, the luciferase activity from this plasmid was ~3 fold lower than the activity of its counterpart V2(-211/+362) when transfected into either HEK293 cells or SGBS preadipocytes (**Figure III-3**), suggesting that the presence of a portion of the V2 exon can enhance V2 basal promoter activity. In support of this hypothesis, two other pairs of promoter constructs, which contained different V2 5'-flanking regions, also showed a loss of transcriptional activity with truncation of the 3'end in both cell lines (**Figure III-3**).

We therefore carried out a detailed 3'-serial deletion analysis to study the influence of downstream regions on V2 transcription. A series of reporter plasmids bearing the same 5' proximal promoter element (-211) but varying lengths of the V2 exon were assayed for luciferase activities. As shown in **Figures III-4A** and **4B**, sequential deletions of the 3'end from +362 down to +162 had only minimal effects ($p>0.05$). However, an extended deletion to +103 sharply reduced the activity to ~40% of the maximal values ($p<0.001$), while a further 3'deletion to +71 gave a similar reduced V2 promoter activity. These results demonstrate that: 1) at least 162bp of the V2 exon sequence is required to maintain the maximal V2 promoter activity; and 2) there are positive regulatory elements between +103 and +162.

To more closely define these elements, constructs bearing overlapping deletions within this region were generated and tested in HEK293 cells. A significant ~40% decrease in luciferase activity was observed when the 3' V2 exon sequence was reduced from +162 to +125 ($p<0.001$, **Figure III-4C**), while reduction from +125 to +103 caused a non-significant ~20% loss ($p>0.05$, **Figure III-4C**). These results indicate that the 37bp region between +162 to +125 is an activation domain that is essential for achieving maximal levels of V2 transcription.

To examine if there is specific factor(s) binding within this region, EMSA was performed by incubating nuclear extracts from HEK293 cells with a radiolabeled probe corresponding to this 37bp region. Three protein-DNA complexes were observed (**Figure**

III-4D). All complexes could be competed out in a dose-dependent manner by adding unlabeled probe, suggesting that they are both sequence-specific. Although the MatInspector program (http://www.genomatrix.de) predicted a putative Egr-1 site in this region (**Supplementary Figure III-1**), we did not get a supershift with an Egr-1 antibody, implying that either the binding conditions are not optimal or another, as yet unknown, endogenous protein(s) binds to this site.

3) Identification of the Core Promoter for V2 Transcription. Accurate transcription initiation of eukaryotic genes by the RNA polymerase II machinery requires a minimum strech of contiguous DNA sequences known as the core promoter, which includes the TSS and immediate flanking sequences. The core promoter generally extends from -40 to +40 relative to the TSS (+1) (Yang *et al.* 2007). In order to determine the core promoter for V2 basal transcription (constitutive promoter activity), we carried out a deletion analysis based on the V2(-211/+71) construct, because it includes the 5'proximal promoter region but excludes the influences caused by downstream regulatory elements within exon V2. Removal of the 5'-fragment upstream of the transcription start site from -211 to -29 did not alter luciferase activities (still $p<0.001$ relative to pGL3-basic) (**Figure III-5**). In contrast, fragments (+11/+71) and (-211/-25) gave only background levels of luciferase activities. Thus, the hGHR V2 sequence from -29 to +11 appears to be the core promoter for V2 basal transcription.

In summary, our deletional construct assays revealed several distinct domains in the hGHR V2 promoter that are implicated in the maintenance and modulation of V2 basal transcription: the proximal promoter (211 upstream of the TSS), the core promoter (-29/ +11), a 5'-upstream inhibitory domain (-720/-211) and a 3'-downstream activation domain (+125/+162) (**Figure III-6**).

V2 Transcriptional Activities in SGBS Pre- vs. Mature Adipocytes

The transcriptional regulation of V2 showed a similar pattern in HEK293 and Huh-7 cell lines (**Figures III-2B** and **III-4A**), SGBS pre- and mature adipocytes (**Figures III-2C** and **III-4B**). However, comparison of the promoter profiles of pre- vs. mature adipocytes

suggested several differences. First, 5'-deletion from -211 to -29 in mature adipocytes resulted in a significant 40% drop of the V2 promoter activity ($P<0.05$) while there was no change in activity in preadipocytes (**Figure III-2C**), suggesting that there may be mature adipocyte-specific transcription factor(s) acting through this 5' region to positively regulate V2 basal transcription. Next, the reporter construct V2(+11/+362), which is missing the major transcription start site, showed only background level of luciferase activity in preadipocytes (**Figure III-2C**) but a significant level of luciferase activity in mature adipocytes ($p<0.05$ vs. pGL3b, **Figure III-2C**), implying that mature adipocyte-specific factor(s) may function through the V2 exon to achieve transcription by using the minor transcription start sites. Importantly, the activities of individual 5'- and 3'deletion V2 promoter constructs were much higher (~3-4 fold) in adipocytes than in preadipocytes (**Figures III-2C** and **III-4B**), indicating that V2 transcriptional activity was stronger in the mature adipocyte. This supports our present (**Supplementary Figure III-3**) and previous findings (Wei *et al.* 2006) that differentiation results in a significant increase in V2 mRNA levels.

Putative Transcription Factor Binding Sites

We analyzed a 723bp nucleotide sequence containing the 211bp V2 proximal promoter region and the 512bp complete V2 exon, using computer-based transcription factor binding site programs (MatInspector and Signal Scan). As shown in **Figure III-6** and **Supplementary Figure III-1**, the V2 core promoter region does not contain a canonical TATA box, but does have an Initiator (Inr)-like sequence overlapping the major transcription start site. Immediately upstream of the core promoter, there is a highly conserved CCAAT box followed by an A+T–rich sequence (-28 to -32). Multiple putative binding sites for Ets1, PU.1, CHOP, Hes1, Egr-1, ZBP-89, SREBP and Sp1, as well as several GAGA binding elements, were detected in the promoter region and within the V2 exon itself. Some of them, like the Ets1 site, the PU.1 site, and the V2 exon Sp1 site are conserved across species (ovine, bovine, mouse), while others, such as the CHOP site and Hes1 sites, are only found in the hGHR V2 (**Supplementary Figure III-2**).

Functions of Important Binding Motifs within the Core Promoter on V2 Transcription

Although the promoter region of hGHR V2 lacks canonical TATA elements (TATAWAWR) and other TATA-like sequences, two potentially important binding motifs are detected immediately adjacent to the TSS (+1). They are the TSS-surrounding Initiator (Inr)-like element (TCAGTTG), which matches the Inr consensus sequence very well (Yang *et al.* 2007; Smale 1997), and an adjacent non-consensus Sp1 site in the bottom strand (**Supplementary Figure III-1**). Their importance in regulating V2 basal transcription was examined by mutational analysis in combination with luciferase reporter gene assays in HEK293 cells. Initially, site-directed mutagenesis was carried out on the -29/+71 V2 promoter segment to create a -29/+71 Inr mutant and a -29/+71 Sp1 mutant (**Figure III-7A**). Each mutation caused a moderate but significant decrease in promoter activity, suggesting that each motif contributes to V2 basal transcription. Next, the same mutations were introduced into a larger DNA fragment (-211/+362) to investigate whether the same mutations would have an effect when both the core promoter and upstream as well as downstream regulatory regions are present, which more closely represents the *in vivo* situation. As shown in **Figure III-7B**, mutation of the Sp1 site resulted in the same reduced luciferase activity as above. In contrast, the Inr mutation had little impact on promoter activity ($P>0.05$), implying that initiation of V2 transcription is not solely dictated by the Inr sequence. Collectively, the V2 core promoter appears to function as a null promoter, despite the presence of an Inr-like sequence, and the Sp1 motif within the core promoter plays a minor role in regulating V2 basal transcription.

Immediately 5' to the core promoter region, there is a CCAAT box (-37 to -33) that has been found to be highly conserved across different species (ovine, bovine, mouse). To determine whether this element is required for basal expression, a substitution mutation encompassing the entire CCAAT motif was created in the context of the -211 to +362 promoter fragment. Transfection studies demonstrated that inactivation of this CCAAT box failed to affect V2 basal promoter activities in HEK293 cells (**Figure III-7B**), SGBS preadipocytes and adipocytes (data not shown), indicating that transcription of human V2 does not rely on this CCAAT box.

Dominant Negative Sp1 Does Not Modulate V2 Promoter Activity

Promoter studies of V2 homologues in other species (ovine 1B, bovine 1B and mouse L2) have demonstrated an important role for Sp1 in regulating their promoter activities. However, a sequence search of the V2 fragment spanning from -211 to +362 did not reveal as many Sp1 putative sites as in the other species (**Supplementary Figure III-2**). In addition to the atypical Sp1 site (similar to ovine site IV) within the V2 core promoter, one other Sp1 site (equal to ovine site V) was detected within the V2 exon, which matches the Sp1 consensus sequence and is conserved across species (**Figure III-8**, **Supplementary Figures III-1** and **III-2**). We therefore decided to examine whether Sp1 has regulatory effects on human V2 promoter activity. Because the high level of endogenous Sp1 expression in most cell types generally invalidates the conventional strategy of over-expressing the transcription factor (Yu *et al.* 1999), we chose to test a dominant negative form (Sp1 DN). As seen in **Figure III-8**, the Sp1 DN had little effect on V2 promoter constructs in HEK293 cells whether the putative Sp1 sites were present or absent, although the same DN expression plasmid gave either positive or negative results on promoter constructs of other genes. Thus, it appears that Sp1 is not a critical regulatory factor for V2 transcription. This result is supported by other observations: 1) that there are similar high levels of basal promoter activity when the exon V2 site is present or absent (**Figures III-4A and B**, V2(-211/+162) vs. V2(-211/ +301)), and 2) that mutation of the atypical site near the TSS only led to a moderate decrease in basal promoter activity (**Figures III-7A and B**).

Ets1 Directly Transactivates the hGHR V2 Promoter

Two putative overlapping Ets binding sites lie upstream of the V2 TSS: 1) a c-Ets1 binding site located at -62 to –46, and 2) a binding site for PU.1, an Ets-like transcription factor identified in lymphoid B-cells, situated at -66 to -50 (**Supplementary Figure III-1** and **Figure III-9A**). Both sites are highly conserved across different species (**Supplementary Figure III-2**). Because the Ets family of transcription factors, characterized by a highly conserved DNA binding domain recognizing a core motif of GGAA/T, plays important roles in the regulation of gene expression in response to

multiple developmental and mitogenic signals, we investigated how the Ets factors are involved in the regulation of V2 expression.

We first examined whether these putative Ets binding sites had any function in V2 basal transcription. The V2(-211/+362) promoter reporter construct, which displayed maximal promoter activity, was used as a primary construct and two c-Ets1 mutants and one PU.1 mutant were created (**Figure III-9A**); none of the mutations resulted in a significant change in luciferase activity. This could be because these Ets binding sites do not influence V2 basal transcription or because of the low level of endogenous Ets factor(s) in HEK293 cells. We, therefore, co-transfected an Ets1 expression plasmid together with V2 promoter constructs. Overexpression of Ets1 in HEK293 cells markedly increased the luciferase activities of V2(-211/+362) and V2(-211/+71) promoter constructs in a dose-dependent manner while no effect was observed with the control construct V2(-29/+11) which has lost the Ets binding sites (**Figure III-9B**). Similar effects were observed in the SGBS preadipocytes (**Figure III-9C**). These results demonstrate that Ets1 can stimulate V2 transcription and appears to act through its specific binding sites.

To confirm this, Ets1 was cotransfected with the two Ets1 mutants and the PU.1 mutant. As expected, mutations of the c-Ets1 site significantly decreased ($p<0.001$) the response of the promoter construct to Ets1 stimulation, although it was not completely abolished (**Figure III-9D**). Interestingly, the PU.1 mutant resulted in a similar reduced response to Ets1 (**Figure III-9D**), suggesting that Ets1 could also use this binding site and/or that the flanking sequences next to the Ets1 binding core motif could affect Ets1 binding. Furthermore, we performed EMSA to show direct binding of Ets1 to the Ets binding sites. As seen in **Figure III-9E**, probes containing the c-Ets1+ PU.1 site when incubated with nuclear extracts from HEK293 cells over-expressing Ets1 formed a specific protein-DNA complex (Lane 3), which was clearly supershifted upon addition of an Ets1-specific antibody (Lane 4). When the c-Ets1 site was mutated (Ets1 Mut1 or Mut2 probes, **Supplementary Table III-2**), formation of this specific band was abolished (Lane 7 and data not shown) and no supershift seen (Lane 8). Collectively, our results suggest that Ets1 can activate V2 promoter activity through direct binding to the Ets1 site.

CHOP Upregulates V2 Transcription By Association with a Downstream V2 Exon Region

A putative CHOP-C/EBP heterodimer binding element (CHOP site) was found approximately 42bp upstream of the TSS in the antisense strand (**Supplementary Figure III-1**). This site appears to be unique for the hGHR V2 promoter (**Supplementary Figure III-2**). To address the functional role of this CHOP site in the V2 promoter, we co-transfected serial 5'-deletion reporter gene constructs with a human CHOP expression vector into COS1 cells. Over-expression of CHOP markedly upregulated the luciferase activities of all three V2 promoter constructs that contain the CHOP site (**Figure III-10A**), while the negative controls (pGL3-basic and V2(+11/+362)) did not respond (**Figure III-10A** and data not shown). Similar stimulatory patterns were observed in A549 and HEK293 cells, although the foldactivation s are lower than in COS cells. CHOP effects were further confirmed by a dose-dependent assay on the V2(-211/+362) promoter construct (**Figure III-10B**).

We next performed ChIP assays to determine whether CHOP can physically associate with the V2 promoter through this CHOP site region. HEK293 cells were used for this experiment because they express endogenous hGHR. Proximal promoter fragments surrounding this site could be specifically immunoprecipitated from CHOP transfected HEK293 cells with an anti-CHOP antibody, while no recruitment was observed from non-transfected cells (**Figure III-10C**), suggesting that CHOP can associate with this region but that the endogenous level of CHOP in HEK293 cells is low.

To determine if CHOP acts directly through the putative response element, a mutant reporter was constructed by replacing the core sequence of the CHOP site with a BamHI restriction site that was previously shown to destroy CHOP site function (**Figure III-11A**). Surprisingly, the mutant showed a very similar dose-dependent response to CHOP stimulation as compared to the wildtype construct (**Figure III-11B**), implying that CHOP did not act directly through this CHOP site. This was confirmed by demonstrating that complete deletion of the CHOP site barely affected CHOP stimulation of V2 promoter constructs (**Figure III-11C**). However, when the reporter construct V2(-211/+362) that responded well to CHOP stimulation (**Figures III-10A, 10B, 11B**) had its 3'-sequence

truncated from +362 to +71 (**Figure III-11D**), the dose-related stimulation by CHOP was completely abolished. Thus, the 3'-promoter downstream region appears to be essential for the stimulatory effects of CHOP on V2 promoter activity.

To delineate the 3' critical region, serial 3'-deletion reporter constructs were cotransfected with CHOP expression plasmids. As shown in **Figure III-11D**, deletion from +362 to +162 resulted in a gradual decrease in the dose-stimulatory effects. With a further deletion to +103 there was complete loss of CHOP activation. We, therefore, conclude that the region between +362 nt to +103 nt is important for CHOP to exert its full stimulatory effects on V2 transcription, and that the +162 to +103 fragment appears to be critical for the CHOP transactivational effect. DNA sequence analyses of the above regions did not reveal any potential CHOP binding motifs, suggesting that CHOP may function through interacting with other factor(s).

Interactions Between CHOP and Ets1

Ets proteins have been shown to interact with several other key transcription factors, including from the basic-leucine zipper family (Li *et al.* 2000a) that CHOP belongs to. To characterize whether there are functional interactions between Ets1 and CHOP in regulating the V2 promoter, we co-transfected the V2(-211/+362) construct that contains the Ets sites and the critical 3'-downstream region with a fixed amount of Ets1 and increasing amounts of CHOP in COS1 cells. Overexpression of either Ets1 or CHOP alone enhanced promoter activity approximately 3 fold (**Figure III-12**). The combined overexpression of Ets1 and CHOP at equal amounts gave an ~6 fold activation. Further increasing CHOP levels resulted in similar activities (Figure 12). In contrast, the V2 (-211/+71) construct, which no longer contains the 3'-CHOP interaction region, was only stimulated by Ets1 (**Figure III-12**). Similarly, the Ets1 mutant construct (V2(-211/ +362)_Ets1 Mut2) only responded to CHOP stimulation (data not shown). Taken together, these results suggest that Ets1 and CHOP activate the V2 promoter in independent ways. They can have additive effects, but do not synergistically activate or repress each other.

Stimulation of hGHR V2 Promoter Activity by C/EBPβ

Although no canonical C/EBP sites were predicted within the V2 proximal promoter region by the transcription factor scanning program, both the highly conserved CCAAT box and the unique CHOP-C/EBP heterodimer site could be potential sites for other C/EBP family transcription factors. We chose C/EBPβ in the current study because C/EBPβ has been shown to mediate GH-induced transcriptional regulation (Cesena *et al.* 2007; Huo *et al.* 2006; Liao *et al.* 1999).

The V2(-211/+71) promoter reporter construct was transfected together with a rat C/EBPβ expression plasmid, which shares high homology with human C/EBPβ, and assayed for luciferase activities in HEK293 cells. Overexpression of C/EBPβ markedly stimulated the luciferase activity (**Figure III-13**). Mutation of the CCAAT box did not affect either basal or C/EBPβ induced V2 promoter activity (**Figure III-13**). In contrast, mutation of the CHOP site caused a moderate but significant decrease in response to the C/EBPβ stimulation, despite a similar basal activity in comparison to the wildtype construct. These data suggest that C/EBPβ can stimulate V2 promoter activity, at least in part by using the CHOP-C/EBP heterodimer site. Similar effects for C/EBPβ were seen in COS1 cells (data not shown).

Repression of hGHR V2 Promoter Activity by Hes1

Two putative Hes1 binding sites were detected within the V2 exon: the proximal site (#1) is ~80bp downstream of the TSS (+72/+86), and the distal site (#2) is ~220bp further downstream (+291 to +305) (**Figure III-14A**, **Supplementary Figure III-2**). They are compatible with the Class C site sequence (Murata *et al.* 2005; Kageyama *et al.* 2005; Yan *et al.* 2001). Because Hes1 is a well known transcriptional repressor and both Hes sites are unique to the human V2 promoter (**Supplementary Figure III-2**), we examined whether Hes1 is involved in negative regulation of V2 expression.

Hes1 was co-expressed with V2 promoter constructs containing a single site, both sites or neither site into either HEK293 cells or SGBS preadipocytes. As shown in **Figures**

III-14B and C, reporter construct V2(-211/+103), which contains Hes site 1, displayed a sharp repression (up to 50%) following transfection of a very low amount (10ng) of Hes1 vector. This suppressive effect was amplified when both sites 1 and 2 were present (construct V2(-211/+362)). In contrast, construct V2 (-211/+71), which does not contain either Hes site, was barely affected.

To determine whether Hes1 binds to the putative Hes sites, we first carried out an *in vitro* binding assay (EMSA) using a probe containing Hes site 1 because this element is more similar to the consensus Class C site sequence. Two specific protein-DNA binding complexes were detected with nuclear extracts of HEK293 cells transfected with N-FLAG-tagged Hes1 cDNA (**Figure III-14D**, lane 3); binding was similar but weaker with the endogenous nuclear extract from HEK293 cells (**Figure III-14D**, lane 2). Both bands could be competitively inhibited by excess cold probe in a dose-related manner (**Figure III-14D**, lanes 4 and 5).

We then performed ChIP assays to determine if Hes1 interacts with its putative sites *in vivo*. In HEK293 cells overexpressing Hes1, DNA fragments containing either site 1 or site 2 binding sites could be specifically amplified in the Hes1-immunoprecipitated complexes from formaldehyde-treated cells, indicating Hes1 occupancy at both Hes binding sites (**Figure III-14E**). No specific associations were observed with a control region ~2kb upstream or from non-transfected HEK293 cells (**Figure III-14E**). These results, taken together, indicate that Hes1 can elicit transcriptional repression of the hGHR V2 promoter through association with the V2 exon Hes sites.

DISCUSSION

The GHR gene exhibits a developmental- and tissue-specific expression pattern (Goodyer *et al.* 2001b; Edens & Talamantes 1998). It can also be modulated in response to different hormonal stimuli, nutritional status, and physiological or pathophysiological conditions (Wei *et al.* 2006; Schwartzbauer & Menon 1998). In the past two decades, studies of the molecular mechanisms suggest that different promoter usage in combination with

differential splicing allows for this precise regulation of GHR gene expression (Schwartzbauer & Menon 1998). The current work represents the first extensive characterization of the hGHR V2 promoter and evaluation of transcription factors that may be involved in its regulation.

Regulation of V2 Basal Transcription

From our 5'deletion analysis of a ~2.6kb region upstream of the major transcription start site, the V2 proximal promoter was mapped to 211bp upstream of the major TSS. This result is consistent with what Orlovskii *et al.* reported: they defined the 165bp region upstream of the TSS as having maximal promoter activity for hGHR V2 (Orlovskii *et al.* 2004). Both ovine 1B (o1B) and bovine 1B (b1B), the V2 homologues in sheep and cattle, respectively, were found to have proximal promoters 134bp and 191bp upstream of their respective major TSS; these TSSs are located within only a few nucleotides upstream of the initiation sites we mapped for human V2 in this study (Jiang *et al.* 2000; Adams 1995). The high degree of homology between human V2, ovine 1B (81.5%) and bovine 1B (82.4%) nucleotide sequences within this region highlight the importance of this promoter region in regulating basal expression for V2-like transcripts, and suggest that similar regulatory mechanisms may be present (Jiang *et al.* 2000; Moffat *et al.* 1999b; Edens & Talamantes 1998; Schwartzbauer & Menon 1998).

Apart from species similarity, the V2 proximal promoter was found to be active in all the human cell lines tested in this study, which are distinct cell types derived from different tissues at different developmental stages. These results support the previous studies showing that V2 mRNA exhibits a ubiquitous expression pattern and indicate that ubiquitous transcription factors (TFs) are involved in the regulation of V2 transcription. Sequence analysis of this region reveals the presence of several putative *cis*-elements such as two Ets sites, a CCAAT box, an atypical Sp1 site and an E-box element (CANNTG) that could be bound by ubiquitous transcription factors (e.g. Ets family members, NF-Y, C/EBPs, Sp1 and Upstream Stimulating Factors (USFs)). However, our mutation analyses of either the Ets sites or the CCAAT box showed that the specific mutations barely affected V2 promoter activity. Mutation of the atypical Sp1 site or the

E-box (which is overlapping with the Inr-like sequence) did result in a statistically significant reduction in promoter activity but the decreases are moderate. Although we could not exclude the possibility that these data might be due to relatively low endogenous levels of certain ubiquitous TFs in the cell lines we used (e.g. Ets1, NF-Y), we postulate that the constitutive promoter activity of human V2 exon may not be controlled by a single, specific transcription factor, but is more likely to be the result of input from multiple factors. Indeed, similar perceptions have been obtained from both ovine 1B and bovine 1B studies. Although the authors showed that deletion of the Sp1 site decreases promoter activity in cells, only partial loss of transcriptional activity was observed, suggesting that multiple regulatory elements are required for full basal transcription (Jiang *et al.* 2000; Adams 1999).

The V2 Core Promoter Functions Like A Null Core Promoter

A core promoter is the minimal DNA region required for transcription. Several core promoter elements, including the TATA box, the TFII*B* response element (BRE), the initiator (Inr) and the downstream promoter element (DPE), have been identified in eukaryotes (Yang *et al.* 2007; Butler & Kadonaga 2002). Based on different combinations of these core promoter elements, four types of core promoter are described: the TATA only, the Initiator (Inr) only, the composite ($TATA^{+}Inr^{+}$) and the null ($TATA^{-}Inr^{-}$) (Yang *et al.* 2007; Werner 1999; Novina & Roy 1996).

Our 5'- and 3'-serial deletional analyses suggest that the region -29 to +11 is the core promoter for hGHR V2 transcript. This result is similar to Adam's finding for o1B, that removal of the promoter fragment -39 to -5 caused a complete loss of the promoter activity (Adams 1995). The V2 core promoter is relatively GC rich (~60%), without a TATA box, DPE or BRE, but does contain an Inr-like element that matches well to the 7-mer Inr consensus (6 out of 7) and is located at the TSS position. However, functional analysis demonstrated that mutation of the V2 Inr-like element only causes a marginal decrease in basal promoter activity in the context of a short promoter piece (-29/+71), and showed no effect in the context of a larger fragment (-211/+362), containing both upstream and downstream regulatory regions. These results suggest that initiation of V2

transcription is not dictated by this Inr-element. Instead, the presence of additional promoter elements provides an alternative explanation. First, an A+T rich sequence is found at the -30bp region of the V2 promoter. Such a structure has been shown to have an affinity for TBP and to result in an intermediate effect of an Inr mutation (Smale 1997). Second, an atypical Sp1 binding site is immediately upstream of the Inr-element. Such a motif has been reported to stimulate transcription preferentially through core promoters containing an Inr (Smale 1997). Therefore, despite the presence of an Inr-like sequence within V2 promoter, it is most likely that the V2 core promoter functions like a null core promoter, with multiple elements combining to allow for efficient initiation of V2 transcription, including the Inr-element. Such a feature agrees with the finding that V2 and V2-like transcripts are transcribed at multiple TSSs.

The 3' V2 Exon Region Is Involved in Maximizing V2 Transcriptional Activity

We found that insertion of a portion of the V2 exon sequence can significantly enhance V2 basal promoter activity. This result is consistent with what Orlovskii et al. observed in their study of hGHR V2 expression (Orlovskii *et al.* 2004) but in contrast to the bovine 1B promoter where inclusion of a 300bp 1B exon sequence was not found to be important for its transcriptional activity (Jiang *et al.* 2000). Thus, the stimulatory effects of the first exon appear to be specific for the human V2 promoter. Orlovskii *et al.* hypothesized that this enhancement effect resulted from the additive actions of Sp transcription binding sites located in front of and within the V2 exon (Orlovskii *et al.* 2004). In the present study, we provide data suggesting a different explanation. Deletion of the V2 exon sequence from positions +362 to +162 (**Figures III-4B & III-4C**), which contains multiple TF binding sites, including the Sp1 site, resulted in similar (HEK293 and Huh7 cells) or only slightly reduced (SGBS preadipocytes and adipocytes) promoter activity. Therefore, our results do not suggest that the V2 exon Sp1 site is responsible for achieving maximal V2 promoter activity. Instead, we identified a 37bp activation domain located at position +162 to +125, and demonstrated that this positive regulatory region is required for achieving V2 full promoter activity. Proteins in HEK293 nuclear extract specifically bound to this element, indicating that there are endogenous protein(s) that

can bind to this region. However, the identity of the protein(s) remains to be characterized.

Role of Sp1 in Regulation of V2 Transcription

In contrast to the V1-like exon promoters, the V2-like exon promoters have a high GC content and lack a TATA box. GC rich, TATA-less promoters are often associated with Sp1 binding motifs (Yang *et al.* 2007) and are considered to be a target for regulation by Sp family members (Yu *et al.* 1999). Four Sp binding sites have been delineated in the o1B promoter (Adams 1999) (**Figure III-8** and **Supplementary Figure III-2**): each binds Sp1/3 with different affinities, and sites I, II and IV are functional for o1B promoter activity. However, computer-based TF binding site search programs only predicted one potential Sp1 site within the human V2 proximal promoter. A sequence comparison revealed that the human V2 sequences corresponding to ovine Sp sites I and III have significantly altered, making them unlikely to be functional Sp1 binding elements. The ovine site II (GGGCGG) is a consensus sequence and found to be conserved in the b1B promoter. However, in the human V2 promoter, this sequence is altered to AGGCGG, again not a likely Sp site. Furthermore, deletion of a V2 5'-flanking region including this site did not produce major changes in V2 basal activity. The ovine site IV overlaps with our atypical Sp1 site identified within the V2 core promoter (**Figure III-8**). We observed moderate (~30%) but significant decreases in V2 basal activity when this site was mutated, which is similar to what was seen in the o1B promoter.

The Sp1 binding site within the V2 exon shows high homology to Sp1 consensus, and is conserved in the different species (**Supplementary Figure III-2**). But our 3'-deletional analysis together with dominant negative Sp1 cotransfection assays demonstrated that this site is not crucial for V2 basal promoter activity. Although there are no data from ovine 1B and bovine 1B promoter studies for comparison, the mouse L2 study indicates that this site preferentially binds Sp3, and to a lesser extent, Sp1 (Yu *et al.* 1999). Taken together, we conclude that Sp1 can influence V2 basal transcription, but does not exhibit critical effects as in other species, at least in part due to the sequence alterations.

Transactivational Effects of Ets1 on the V2 Promoter

Ets1 is the founding member of the large Ets transcription factor family, who share an evolutionarily conserved DNA binding region called the Ets domain. As a transcriptional activator, Ets1 regulates target gene expression through binding as a monomer to its specific response element, a purine-rich DNA sequence centered by a GGAA/T motif (Gutierrez-Hartmann *et al.* 2007; Oikawa 2004).

A pair of overlapping putative Ets binding sites (c-Ets1, PU.1) was revealed adjacent to the major TSS in the V2 promoter. Their high conservation across species suggests that Ets factors may play an important role in regulating V2 gene expression. Indeed, by overexpression of Ets1, we observed a marked stimulation of V2 promoter activity in both HEK293 cells and SGBS preadipocytes. Site-directed mutation analysis and EMSA demonstrated that Ets1 stimulates V2 promoter activity via direct binding to the Ets1 site. On the basis of these results, we have, therefore, concluded that hGHR V2 is a direct target for Ets1. As far as we know, this is the first report identifying hGHR as a target gene for the Ets transcription factors. The >200 genes identified to date as Ets targets are involved in many biological effects, including cell proliferation, differentiation, development, hematopoiesis, angiogenesis, remodeling and transformation (Gutierrez-Hartmann *et al.* 2007; Sementchenko & Watson 2000). It is reasonable that hGHR falls into this group, given the many parallel effects of the hGH/hGHR axis.

The precise mechanisms by which Ets1 transactivates the V2 promoter remain to be characterized, but there are several possibilities based on studies of other Ets1 target genes: 1) by interactions with chromatin-remodeling cofactors, such as coactivator CBP/P300 or the SRC family members (SRC, TIF2 and RAC3) (Petit *et al.* 2004; Jayaraman *et al.* 1999; Yang *et al.* 1998); or 2) by cooperative interactions with other transcription factors binding at adjacent sites, for example USF (Sun & Loh 2001) and Sp1 (Kavurma *et al.* 2002). In the present study, we examined the interaction between Ets1 and CHOP, a member of the C/EBP family, since the two binding sites are next to each other. We observed only additive but not synergistic effects on V2 regulation when both factors were overexpressed simultaneously. This suggests that they act

independently on the V2 promoter. This lack of interaction is supported by our later finding that CHOP functions by association with a downstream region instead of binding directly to the proximal promoter CHOP site. However, another C/EBP family member C/EBPβ seems to be able to use this CHOP site to activate the V2 promoter. Therefore, it would be of great interest in the future to examine the interaction between Ets1 and C/EBPβ.

Many Ets transcription factors are known to act as downstream effectors of multiple signaling pathways such as MAP kinase (ERK, JNK, and P38 MAPK), PI-3 kinase and Ca^{2+}, triggered by growth factors or cellular stresses (Gutierrez-Hartmann *et al.* 2007; Yordy & Muise-Helmericks 2000). Phosphorylation of Ets factors by different cascades controls their protein partnerships, target specificity and transactivational activities (Oikawa 2004; Galang *et al.* 2004; Yordy & Muise-Helmericks 2000; Yang *et al.* 1998). GH can initiate all of the aforementioned signaling, making it likely that Ets factors could act as an intermediate in GH regulation of hGHR V2 expression. Indeed, on the basis of our present data, showing that Ets1 can transactivate hGHR V2 promoter via direct binding to the Ets1 binding site in V2 proximal promoter, we have proposed that Ets1 can serve as a downstream effector of MAP kinase and mediates the regulation of hGHR V2 expression by hGH and other growth factors (**Figure III-15A**).

Ets-1 has been observed in many types of human tumors (Seth & Watson 2005) and its level of expression has been associated with the grade of malignancy and prognosis in several tumors, including breast cancer and colorectal cancer (Oikawa 2004). Interestingly, hGHR has also been shown to be frequently overexpressed in colorectal cancer (Wu *et al.* 2006; Yang *et al.* 2004b) and its expression levels predict response to radiotherapy treatment (Wu *et al.* 2006). According to our present finding, the increased hGHR expression in cancer cells may result from transactivation of the hGHR V2 promoter by Ets-1.

Transcriptional Activation of V2 Promoter by C/EBP Family Transcription Factors

In the present work, we characterized the transcriptional regulation of human V2 promoter by two members of the C/EBP transcription factor family.

CHOP, also named as C/EBPζ, is a nuclear, proapoptotic, bZip transcription factor of the C/EBP family (Oyadomari & Mori 2004; Gery *et al.* 2004; Ubeda & Habener 2003; Ron & Habener 1992). Initially identified as an interacting partner with C/EBP proteins and serving as a dominant negative inhibitor of the actions of C/EBP at conventional C/EBP-binding sites of gene promoters (Ron & Habener 1992), it was later recognized that CHOP can transactivate a distinct set of genes by dimerization with C/EBP factors and binding to a new set of promoter regulatory sites, referred to as the CHOP-C/EBP heterodimer site or the CHOP site (Sok *et al.* 1999; Ubeda *et al.* 1996). Intriguingly, a putative CHOP binding element that matches very well to the in vitro PCR-selected CHOP consensus sequence was revealed in the V2 proximal promoter. This led us to investigate the functional role of CHOP on V2 expression.

Overexpression of CHOP resulted in its physical association with the V2 promoter and its transactivation of V2 promoter activity. However, mutation and deletion analysis of the specific CHOP site demonstrated that CHOP does not function by direct binding to this site. Instead, the stimulatory effects of CHOP appear to rely on interacting with a 3'-V2 exon region where no putative CHOP binding motifs are detected. Thus, we have proposed that CHOP activation of V2 transcription is through a protein tethering mechanism. This is the second report: Ubeda *et al.* first reported such a mechanism by demonstrating that CHOP tethers to the DNA bound AP-1 complex via direct interaction with complex members c-Jun and c-Fos and, in doing so, activates promoters of selected genes such as somatostatin, JunD and collagenase (Ubeda *et al.* 1999). Based on their findings, they proposed a model in which CHOP could act through this tethering mechanism to mediate ER stress induced gene expression. The same scenario has not been reported for other C/EBPs, but it has been observed for ATF6, a member from a different bZip subfamily (Ubeda *et al.* 1999). Through tethering to the serum response factor (SRF) complex bound to its specific element on the individual promoters, ATF6

mediates transcriptional activation of the c-Fos promoter (Zhu *et al.* 1997) and the atrial natriuretic factor (ANF) promoter (Thuerauf *et al.* 1998).

In addition to C/EBP family members, CHOP can dimerize or tether with other bZIP proteins including AP1 (Ubeda *et al.* 1999), ATF3 (Chen *et al.* 1996), ATF4 (Ohoka *et al.* 2005) and CREB-2 (Gachon *et al.* 2001) or even with a ribosomal protein, FTE/S3a (Cui *et al.* 2000). To facilitate finding the possible interaction partner for CHOP, we first narrowed down the potential region to which CHOP might attach. The +362 to +162 region appeared to be required for maximal response to CHOP stimulation. We did not find putative binding sites for either Ap1 or SRF within this sequence. But omitting a putative, overlapped SREBP/Sp1 element led to a reduced dose-dependent response to CHOP similar to deletion of this domain (data not shown), suggesting it could be a potential candidate. The +162 to +103 region was crucial for CHOP tethering, since its deletion resulted in loss of the CHOP effect. There are only two putative binding sites for two transcription factors within this sequence: Egr-1 and VDR. There is one report showing that the N-terminal transactivation domain of Egr-1 can interact with C/EBPβ via direct protein to protein contact (Zhang *et al.* 2003). Although our EMSSA studies of the +162 to +125 region did not confirm EGR1 binding, it may well be that the endogenous levels of EGR1 in the HEK293 nuclear extracts were too low to be detectable. Thus, whether it is Egr-1 or other factors with which CHOP might complex so as to exert its stimulatory effects on V2 promoter remains to be determined.

Ubiquitously expressed at low levels in proliferating cells, CHOP is markedly induced when cells are exposed to stressful stimuli, including ER stress, DNA alkylating reagents, increased nitric oxide levels, hypoxia and two types of nutrient deprivation (Oyadomari & Mori 2004; Gery *et al.* 2004; Ubeda & Habener 2003; Bruhat *et al.* 1999; Jousse *et al.* 1999; Carlson *et al.* 1993). During glucose deprivation, CHOP expression is increased inversely to the glucose level in the medium and decreased upon addition of D(+)-glucose (Carlson *et al.* 1993). Amino acid limitation, due to deficiency of one or more of the essential amino acids or an insufficient intake of protein, can transcriptionally activate CHOP expression through interaction between ATF4 and ATF2 (Averous *et al.* 2004; Bruhat *et al.* 2000; Bruhat *et al.* 1997). Because hGH is such an important metabolic

hormone involved in glucose and protein metabolism, it makes sense that hGHR gene expression would be regulated in response to changes in nutrient status. Combined with the present data showing that CHOP is involved in regulation of hGHR V2 transcription, it is very likely that CHOP acts as a mediator link between regulation of nutrient status and hGHR gene expression.

Flores-Morales *et al.* showed that cellular stress prolongs the duration of the JAK2/STAT5 signaling pathway activated by GH (Flores-Morales *et al.* 2001). They suggested that this effect was related to inhibition of the GH-induced transcriptional activation of SOCS genes, since giving transcription inhibitors led to a prolongation of GH-induced STAT5 DNA binding activity (Fernandez *et al.* 1998). However, they could not correlate the stress-induced effects to GH-dependent expression of SOCS mRNA and protein (Flores-Morales *et al.* 2001). Our finding that CHOP can upregulate hGHR V2 transcription provides an alternate explanation: ER stress induces CHOP expression, which in turn will activate hGHR gene expression leading to increased hGHR levels expressed on the cell surface that would amplify the GH signaling magnitude and extend the duration.

Several studies from Lobie's laboratory indicate that hGH, particularly autocrine hGH, can upregulate CHOP expression and enhance its transcriptional activity in a p38 MAPK-dependent manner, which results in an enhanced protection from apoptosis and promotes mammary carcinoma cell survival (Mertani *et al.* 2001; Zhu & Lobie 2000). These effects were confirmed to be generated via the hGHR on the mammary carcinoma cells (Kaulsay *et al.* 2001). Based on our finding that CHOP can upregulate hGHR transcription, we hypothesize that a positive feedback loop can be created through CHOP: autocrine hGH production will enhance CHOP levels and transcriptional activation, including upregulation of the hGHR gene expression, and the increased hGHR levels will produce increased cellular response to autocrine hGH.

Collectively, our present data that CHOP can upregulate V2 promoter activity creates a bridge linking the regulation of hGHR V2 expression by ER stress or nutrient deprivation.

Moreover, CHOP can be utilized as a mediator by hGH in regulating hGHR V2 transcription (**Figure III-15A**).

In addition to CHOP, we have identified that C/EBPβ, a conserved member of the C/EBP family, could activate V2 transcription. Since relatively high levels were used in the cotransfection assay, this transcriptional activation should mainly be generated by C/EBPβ homodimers. Our mutational analysis data suggest that transcriptional activation of V2 by C/EBPβ is mediated via the CHOP-C/EBP heterodimer site. Although the consensus binding site for CHOP-C/EBP heterodimers (RRR-TGCAAT), differs from that of C/EBP cognate sequence (TTNNNGCAAT) (Ubeda *et al.* 1996), it has been demonstrated that C/EBPα and -β homodimers are capable of binding to the CHOP-C/EBP site by both EMSA (Ubeda *et al.* 1996) and DNase I footprint analysis (Sok *et al.* 1999). However, mutation of the CHOP-C/EBP site did not cause a complete loss of C/EBPβ stimulation on V2, suggesting that there might be supplementary site(s) used together with the CHOP-C/EBP site for C/EBPβ transactivation.

A number of studies have reported that C/EBPβ acts as a critical mediator of GH-regulated gene transcription (Cesena *et al.* 2007; Huo *et al.* 2006; Cui *et al.* 2005; Piwien-Pilipuk *et al.* 2002b; Piwien-Pilipuk *et al.* 2002c). For example, in 3T3-F442A preadipocytes, knocking down of C/EBPβ by RNA interference prevents GH-stimulation of c-fos mRNA. GH treatment, through MAPK and PI3-K mediated signaling cascades, leads to an increase in phosphorylated C/EBPβ bound to c-fos promoter and simultaneous recruitment of p300, which then forms a complex and mediates GH-stimulated transcriptional activation of the *c-fos* gene promoter (Cui *et al.* 2005). Our finding that C/EBPβ can stimulate V2 promoter activity provides a mechanism by which GH can regulate hGHR V2 expression. Except for Adams *et al.*, who mentioned that they also observed that C/EBP members, in particular C/EBPδ, could stimulate o1B promoter in their preliminary experiments (Adams 1999), no other studies of C/EBPs on V2 homologous promoters have been reported. However, as C/EBP proteins regulate a variety of physiological processes including cell proliferation, differentiation, energy metabolism and inflammation (Ramji & Foka 2002; Lekstrom-Himes & Xanthopoulos

1998), it is not surprising that C/EBP members contribute to hGHR V2 transcriptional activation.

Transcriptional Repression of V2 promoter Activity by Hes1

Hes1 (*Hairy and Enhancer of Split* homolog-1) is a member of the basic-helix-loop-helix (bHLH) protein family and acts as a transcriptional repressor by binding to two specific DNA sequences, the N box (CACNAG) or the class C type E box (CACGCG), in the promoter regions of target genes (Kageyama *et al.* 2005; Iso *et al.* 2003; Yan *et al.* 2002; Yan *et al.* 2001). In the present study, we demonstrated that human V2 promoter activities are markedly repressed by overexpression of Hes1 in both HEK293 cells and SGBS preadipocytes. This suppression was identified to be mediated through association of Hes1 protein to two Class C-like Hes binding sites located within the V2 exon region, which are unique for the human V2. Thus, this is the first proof that the hGHR gene is a target gene regulated by Hes family members.

To our knowledge, only a few human target genes have been identified so far to be repressed by direct binding of Hes1 to their respective sites (Bhat *et al.* 2006; Abderrahmani *et al.* 2005; Murata *et al.* 2005; Lee *et al.* 2001; Chen *et al.* 1997). They either have the Hes binding sites in their promoter regions (Bhat *et al.* 2006; Abderrahmani *et al.* 2005; Murata *et al.* 2005; Lee *et al.* 2001; Chen *et al.* 1997) or in a gene intron region ~1.7-kb downstream from the exon 1/intron 1 boundary (Yan *et al.* 2002; Yan *et al.* 2001). Therefore, hGHR V2 is the first example that Hes1 binds to sites within the first exon to mediate transcriptional repression.

Hes1 and its homolog are immediate downstream targets of Notch signaling (Ohtsuka *et al.* 1999; Jarriault *et al.* 1995). The Notch-Hes pathway is important for the determination of cell fate and/or the embryonic development in a variety of cells (Kageyama *et al.* 2000). Whether or not the hGHR gene is involved in the Notch-Hes signaling cascade needs to be further investigated, as this is the first report linking the two. However, recent data suggest that Hes1 can also be regulated by other Notch-independent signaling cascades, such as the MAPK/ERK (Stockhausen *et al.* 2005) and the JNK signaling pathways (Curry *et al.* 2006). In light of these findings, it is also possible that Hes1

mediates the modulation of hGHR gene expression in response to MAP kinase signaling cascades generated during different developmental steps and stimulated by various extracellular stimuli.

The presence of HES sites in the human V2 exon is unique amongst all the species examined to date. Thus, although the hGHR V2 promoter shares a lot of similarity with V2-like promoters in other species, this provides a mechanism for species-specific regulation of hGHR. Regulation of human V2 through Hes1 could account for the quantitative differences of widely expressed V2 during development or differentiation, as well as in response to various stimuli, including hGH (**Figure III-15A**).

Transcriptional regulation of hGHR V2 in SGBS preadipocytes and adipocytes

GH exerts profound physiological effects on fat tissue, regulating both preadipocyte differentiation and adipocyte metabolism (Nam & Lobie 2000). All of these effects are mediated through the GHRs expressed on the surface of the adipocyte precursor and mature cells (Wei *et al.* 2006; Carrel & Allen 2000). We have recently demonstrated that hGHR V2 is the predominant hGHR mRNA variant expressed in human adipocytes, and is the key contributor for the increase of hGHR mRNA level during SGBS preadipocyte differentiation (Wei *et al.* 2006). To understand the underlying mechanisms involved, we investigated the transcriptional regulation of human V2 in SGBS pre- and mature adipocytes.

Primer extension studies of SGBS preadipocyte RNA showed that V2 is initiated at identical sites in preadipocytes as in liver or kidney cells. Serial 5'- or 3'- deletion analyses of V2 promoter activities demonstrated similar transcription profiles in both SGBS preadipocytes and mature adipocytes. These results agree with our *in vivo* observations that V2 is highly expressed at both developmental stages. However, we also witnessed certain differences in V2 basal transcription between preadipocytes and adipocytes. For example, mature adipocytes showed ~3-4 fold higher promoter activity than preadipocytes despite similar regulatory trends, suggesting that changes in the availability of active forms of positive and/or negative transcription factor(s) play a role in V2 promoter regulation. This enhanced transcriptional ability during differentiation

agrees with our previous (Wei *et al.* 2006) and present (**Supplementary Figure III-3B**) *in vivo* finding that V2 mRNA levels are markedly upregulated during SGBS preadipocyte differentiation and reach maximum with maturation.

To characterize the specific *trans*-factors or *cis*-elements involved in upregulation of V2 promoter activity during differentiation, we have compared the expression and function of individual transcription factors at the preadipocyte stage and during differentiation. Although we saw that overexpression of Ets1 markedly upregulated V2 promoter activity (**Figure III-9C**), our qPCR data show that Ets1 expression is highest in confluent SGBS preadipocytes, followed by a marked decrease during the initial stage of differentiation, and is maintained at relatively low levels in later differentiation stages (**Supplementary Figure III-3C**). Very few studies have been done with regard to Ets factors and adipocyte differentiation. It is possible that other Ets family members may function in this process or that Ets1 may be implicated in "priming" the cells ready to differentiate and to increase hGHR expression.

Studies of murine clonal cell lines (3T3-L1 or 3T3-F442A) have highlighted the pivotal roles of C/EBP family proteins in adipocyte differentiation. Different C/EBP members (CHOP, C/EBPβ/δ & C/EBPα) are expressed in a highly regulated cascade to allow for successful differentiation (Farmer 2006; Otto & Lane 2005). CHOP is usually transiently induced in growth-arrested confluent preadipocytes, sequesters C/EBPβ by heterodimerization and thus makes C/EBPβ fail to localize to centromeres until preadipocytes traverse the G(1)-S checkpoint of mitotic clonal expansion. As preadipocytes reach S phase and differentiation begins, CHOP expression is markedly downregulated so as to release C/EBPβ from inhibitory constraint (Tang & Lane 2000). Our qPCR analysis of CHOP mRNA levels during human preadipocyte differentiation also revealed that CHOP expression dropped when differentiation was induced, was maintained at a low level during the differentiation stages, and increased gradually when the cells reached maturity (**Supplementary Figure III-3C**). In view of these data, CHOP does not appear to be a suitable candidate for increasing V2 activity during differentiation, despite the fact that CHOP can stimulate V2 promoter activity when overexpressed. However, we can not exclude the possibility that CHOP may function at

the preadipocyte stage to prepare the cells for an increase in hGHR V2 mRNA during differentiation (**Figure III-15B**).

C/EBPβ and δ are induced at the early stages of adipocyte differentiation (Tang & Lane 2000). Exogenous hormonal inducers such as insulin, dexamethasone and isobutyl-methylxanthine have all been shown to stimulate C/EBPβ- and δ expression and to enhance their DNA binding (Lekstrom-Himes & Xanthopoulos 1998; Darlington *et al.* 1998). Induced C/EBPβ/δ activate several target genes, including C/EBPα and PPARγ, to promote differentiation (Otto & Lane 2005). Although the proximal promoter region of hGHR V2 contains no canonical C/EBP binding motif, we were able to demonstrate that overexpression of C/EBPβ can significantly upregulate V2 transcription via the CHOP-C/EBP heterodimer site. Thus, C/EBPβ may be, at least in part, responsible for the differentiation-induced increase of V2 transcription (**Figure III-15B**).

After the early stages of differentiation, the levels of C/EBPβ/δ quickly drop and expression of C/EBPα begins and is maintained at a high level until terminal differentiation (Otto & Lane 2005; Ottosson *et al.* 2000; Tang & Lane 2000). C/EBPα has also been shown to be able to bind to the CHOP-C/EBP heterodimer site (Ubeda *et al.* 1996). Although when we cotransfected of C/EBPα at a low dose (100ng/well) we did not observe any stimulatory effects on V2 transcription, we have not tested C/EBPα at a high dose similar to the amount required to show a stimulatory effect of C/EBPβ. If a similar response occurs, C/EBPα would be another potential candidate contributing to the increase of hGHR V2 expression during differentiation (**Figure III-15B**).

Collectively, our findings suggest that transcription of hGHR V2 in most human cells, including adipocytes, is the result of a complex interplay by multiple factors. On the basis of our results, we propose two regulatory models governing hGHR V2 transcription.

Model 1: In most cells, hGH and growth factors can act through the MAP kinase (including ERK1/2, JNK and p38 MAPK) pathways to activate specific transcription factors which may then exert positive (e.g. CHOP, Ets1) or negative (e.g. Hes1)

regulation of the hGHR V2 promoter. ER stress or nutrient depletion can induce the expression of the downstream factor CHOP and enhance its transcriptional activation through the p38MAP kinase cascade and, thus, modulate V2 expression. Moreover, various developmental stimuli can use the Notch-signaling pathway and act through its downstream target, Hes1, to specifically affect hGHR V2 expression at different developmental stages (**Figure III-15A**).

Model 2: Ets1, CHOP and Hes1 are important regulators of hGHR V2 in preadipocytes. During adipocyte differentiation, they are expressed at relatively high levels in the growth-arrested confluent preadipocytes and may be implicated in preparing the cells for differentiation. Once differentiation starts, their expression levels decrease followed by an increase in the expression of two C/EBP family members, C/EBPβ (at an early stage) and C/EBPα (at a later stage). These C/EBPs may transactivate hGHR V2 at early and late differentiation stages, respectively, and, thus, lead to an increase in V2 mRNA during adipocyte maturation (**Figure III-15B**).

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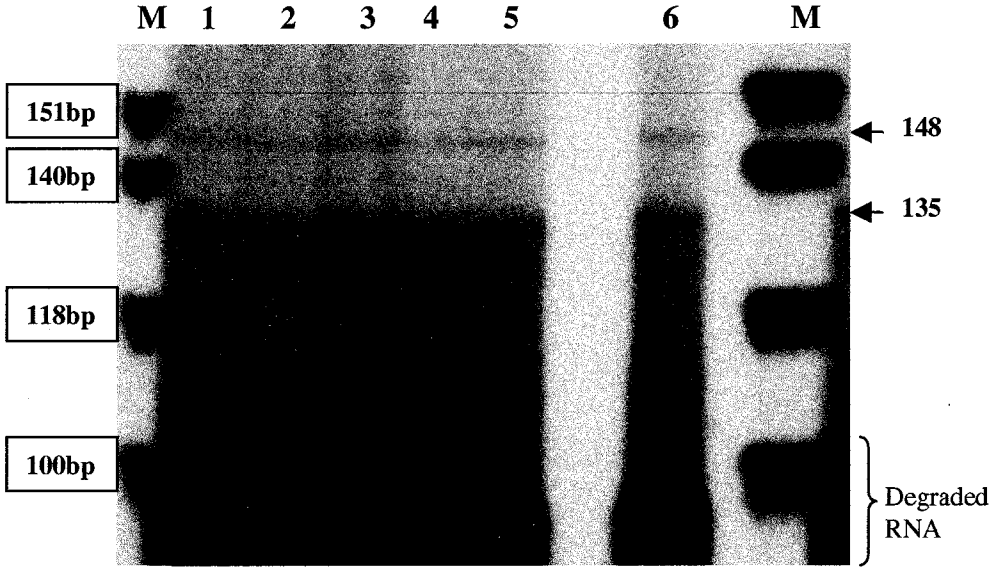
Figure III-1: Identification of transcription start sites for hGHR V2 in multiple human cell lines and human liver tissues.

(A) Representative autoradiograph of size-fractionated products of a primer extension reaction carried out with a primer (V2PE) complementary to the V2 exon sequence and 20μg of total RNA extracted from multiple human cell lines and liver tissues. The sizes of two specific products (arrows) were determined by concurrently electrophoresed ^{32}P-labeled ϕX174 *Hinf*I DNA ladders (lane M). Lane 1: HEK293 cells, Lane 2: HEK293 cells transfected with V2(-2623/+331), Lane 3: Huh-7 cells, Lane 4: SGBS preadipocytes, Lane 5: human fetal liver, Lane 6: human adult liver.

(B) Positions corresponding to the most 5'-ends of two longest, consistently observed primer extension products are shown in bold italic and underlined. The major (**T**, bold, boxed) and minor transcription start sites for ovine 1B are indicated by solid circles. The major (**G**, bold) and minor transcription start sites identified for bovine 1B are marked by empty circles. The oligonucleotide primer (V2PE) used for primer extension is underlined by an arrow. The ovine 1B major transcription initiation site (**T**, bold, boxed) has been designated as position +1 for our hGHR V2 promoter study because it represents the farthest cDNA 5'end identified amongst the homologous V2-like transcripts (ovine 1B, bovine 1B and human V2).

A.



B.

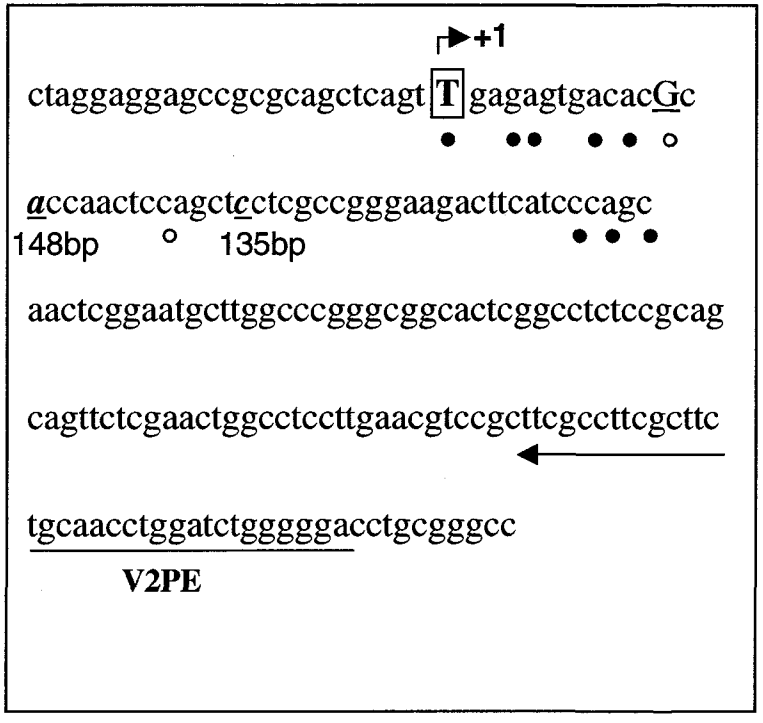


Figure III-1

Figure III-2: 5'-deletion analysis of the promoter activity of hGHR V2.

(A) A schematic diagram representing the first 400nt of the hGHR V2 exon and its 5'-flanking region. The numbering is relative to the designated major transcription start site (+1, indicated by arrow).

(B) 5'-deletion promoter reporter constructs were prepared by inserting different portions of the 5'-flanking sequence of hGHR V2 into the promoterless luciferase plasmid pGL3-Basic (pGL3b). These expression plasmids were transiently transfected into HEK293 and Huh-7 cells.

(C) Similar 5'deletion promoter reporter constructs were transiently transfected into SGBS preadipocytes and mature adipocytes. Luciferase activity of the transfectants was normalized to β-galactosidase activity and then expressed as relative fold activation to the empty pGL3-basic vector. The data are expressed as M±SE, n= 3-9 experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

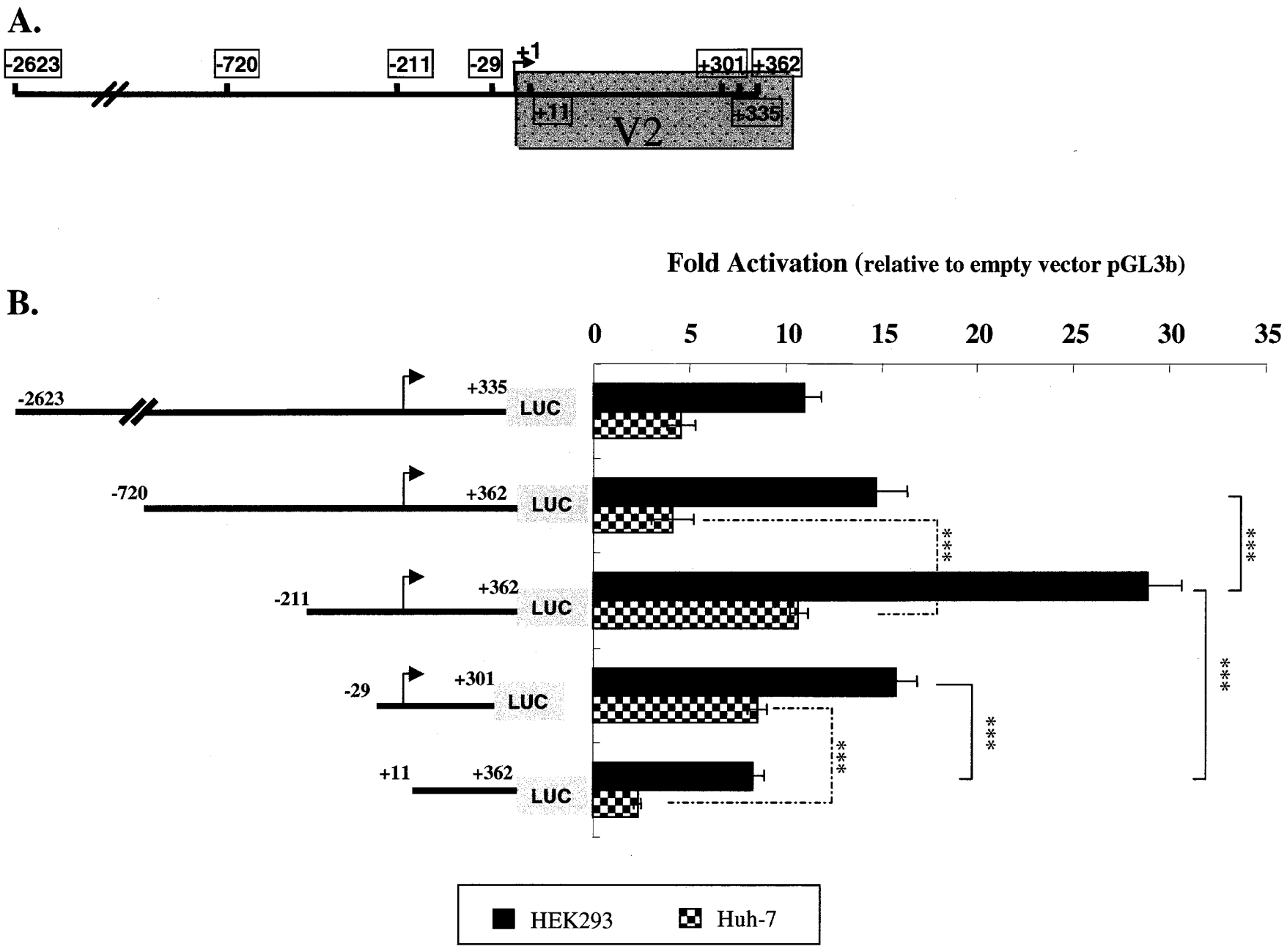

A.
-2623
-720
-211
-29
+1
+301
+362
+11
V2
+335
B.
Fold Activation (relative to empty vector pGL3b)
0
5
10
15
20
25
30
35
-2623
+335
LUC
-720
+362
LUC
-211
+362
LUC
-29
+301
LUC
+11
+362
LUC
***
***
***
***
***
HEK293
Huh-7

Figure III-2

C.

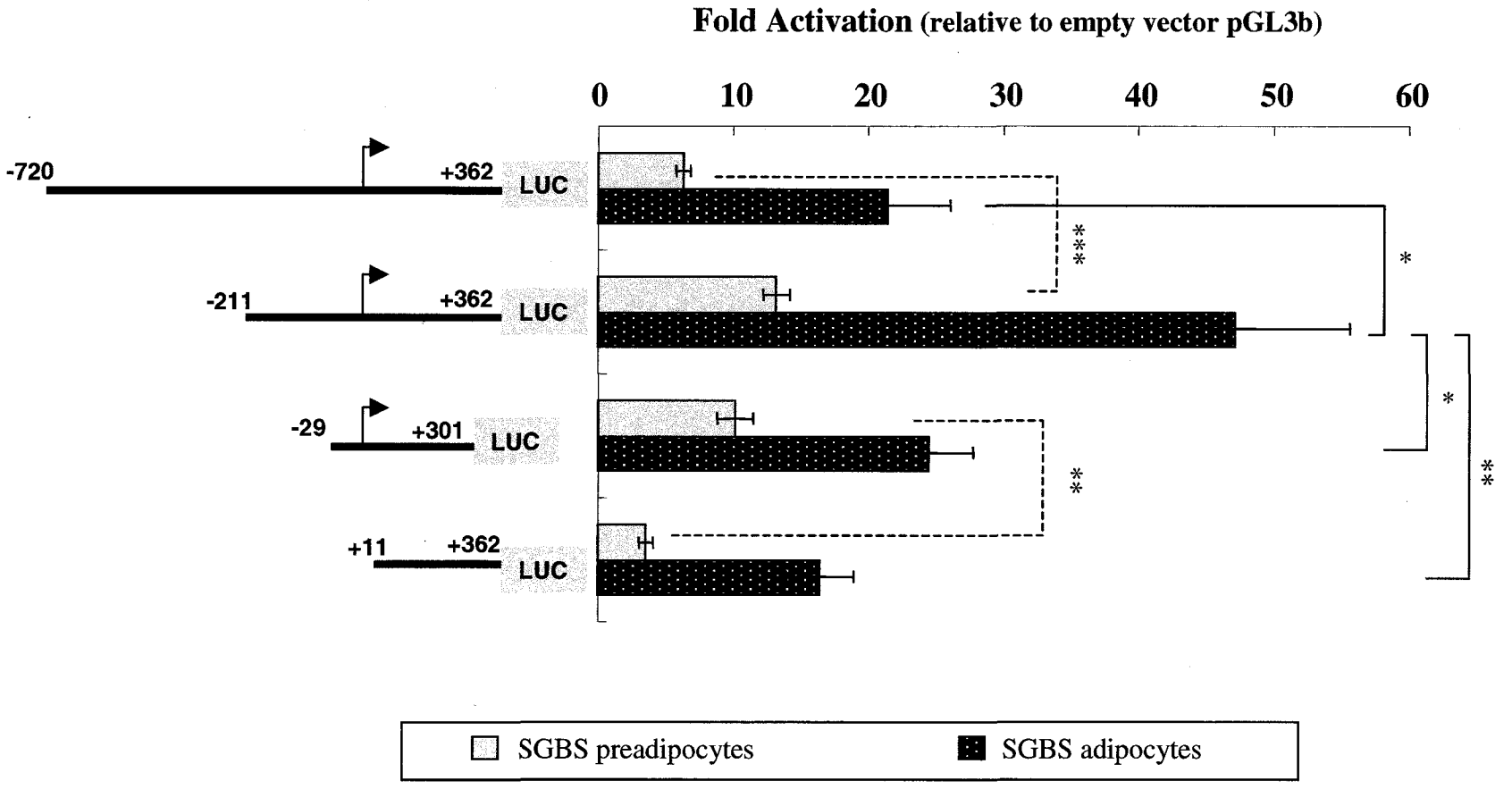
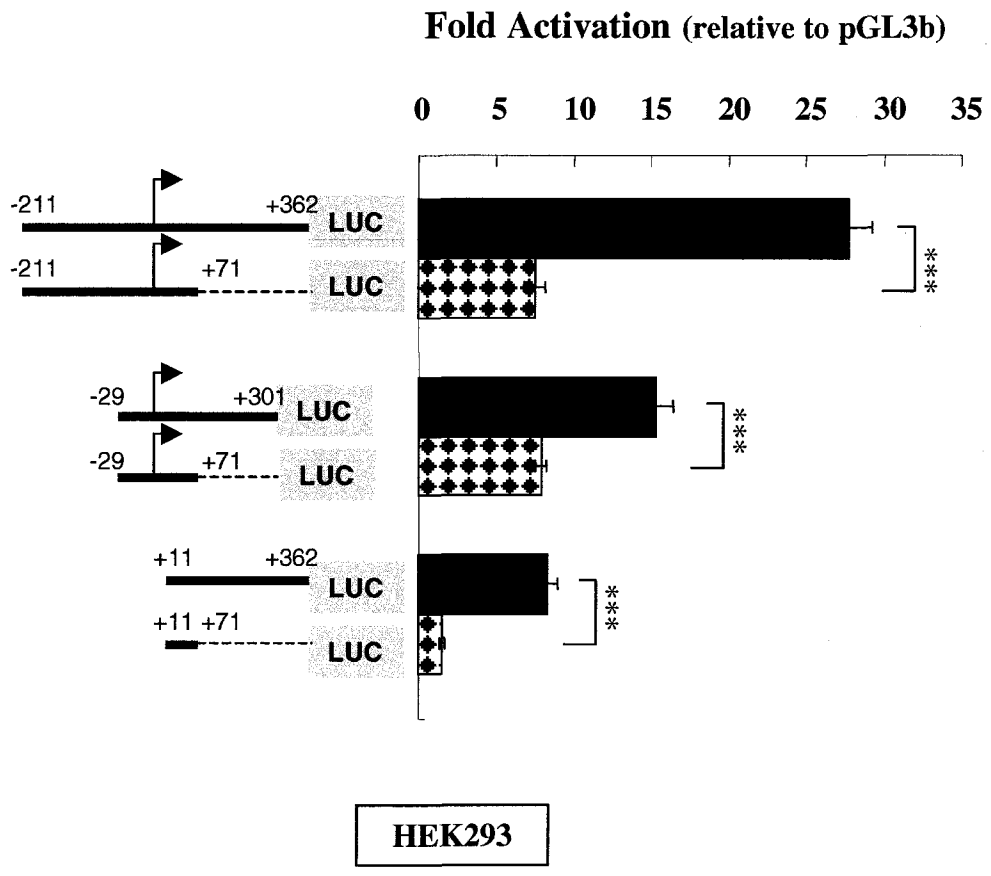


Figure III-2

Figure III-3: Presence of a portion of the V2 exon sequence enhances promoter activity.

Pairs of V2 promoter reporter constructs that contain the same 5'-upstream regions but different 3'-downstream regions were transiently transfected into HEK293 cells or SGBS preadipocytes and compared for fold activation relative to the empty vector, pGL3b. The data are presented as M±SE of n=3-9 independent experiments. Significantly (*$p<0.05$, ***$p<0.001$) higher luciferase activity was noted for V2 reporter constructs that contain longer 3'-downstream region sequences.


Fold Activation (relative to pGL3b)
0 5 10 15 20 25 30 35
-211 +362 LUC
-211 +71 LUC
-29 +301 LUC
-29 +71 LUC
+11 +362 LUC
+11 +71 LUC
***
***
***
HEK293

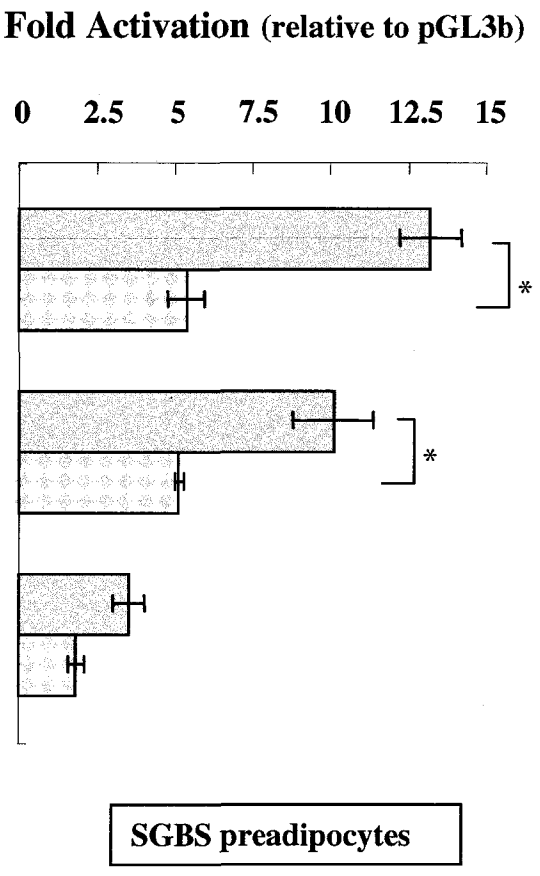

Fold Activation (relative to pGL3b)
0 2.5 5 7.5 10 12.5 15
*
*
SGBS preadipocytes

Figure III-3

Figure III-4: Characterization of the V2 exon regions required for full promoter activity.

V2 promoter reporter constructs with progressive deletions of the 3' V2 exon region were prepared and transfected into **(A)** HEK293 and Huh-7 cells and **(B)** SGBS preadipocytes and mature adipocytes. Data are presented as M±SE from n=3-11 experiments. A significant decrease in promoter activity was observed from +162 to +103 for all four cell types. *p<0.05, **p<0.01, ***p<0.001.

(C) 3'-deletion constructs covering the region from +162 to +103 were generated and transfected into HEK293 cells. Deletion of the region from +162 to +125 in the V2 exon caused the reporter activity to decrease significantly (***p<0.001). Data are expressed as M±SE of n=10 independent experiments.

(D) A double-stranded oligonucleotide probe containing the +162 to +125 sequence was end-labeled with [γ-^{32}P] ATP and incubated with nuclear extracts prepared from HEK293 cells for EMSA. For oligonucleotide competition experiments (lanes 3-4), a 100- or 200-fold excess of unlabeled probe was added before the addition of the labeled probe. The three specific DNA-protein complexes (a, b and c) are indicated by arrows.

A.

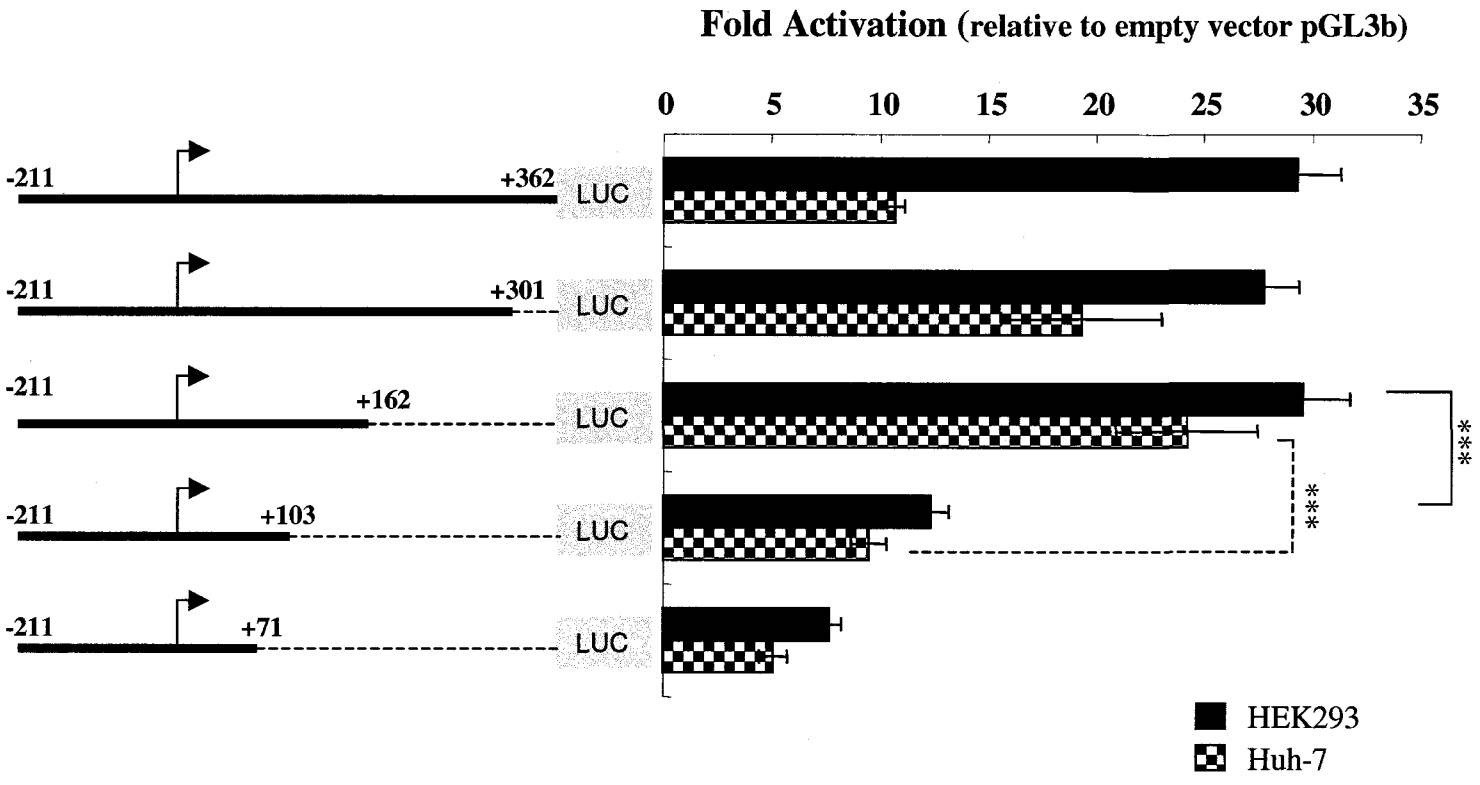


Figure III-4

B.

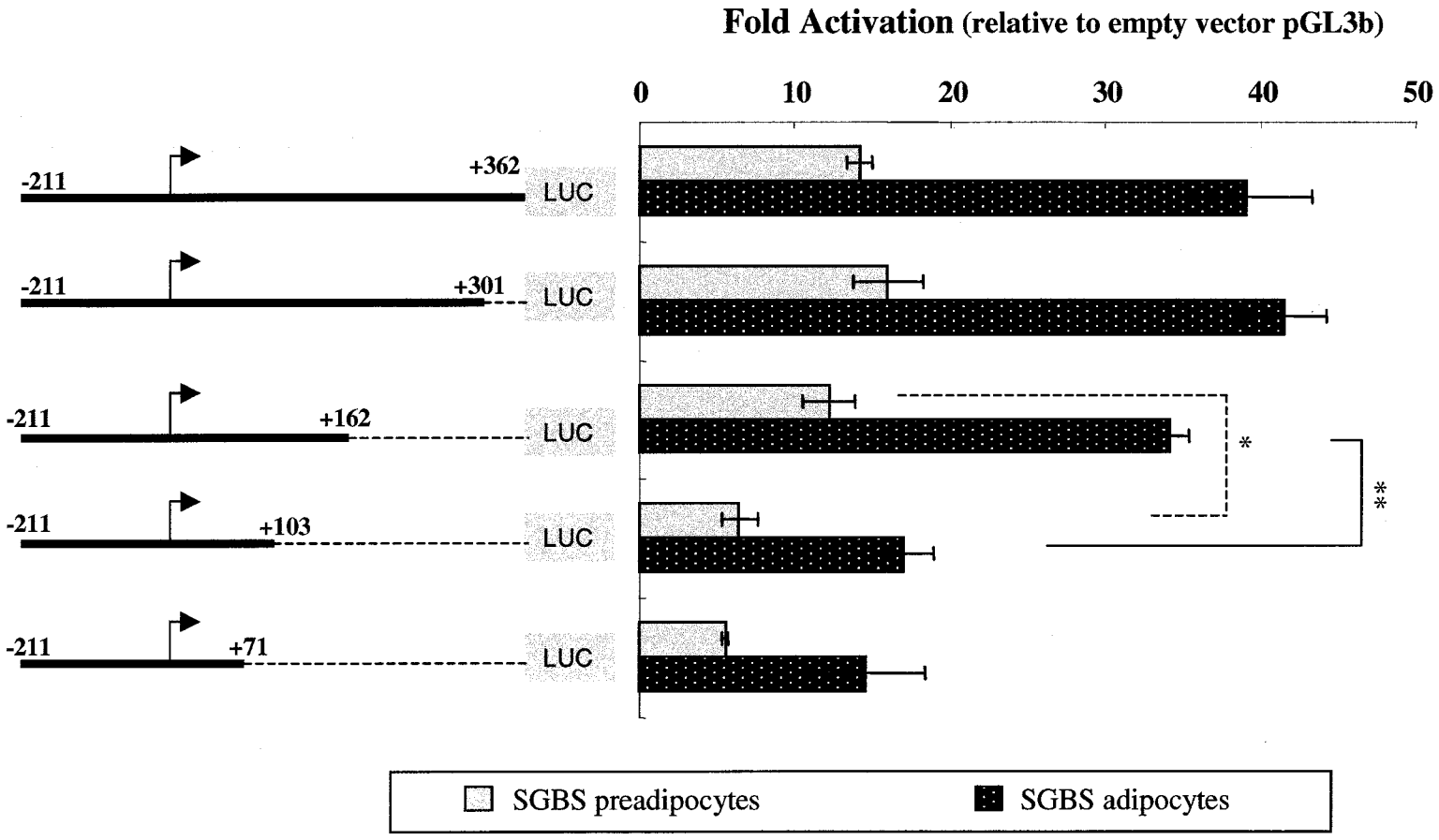
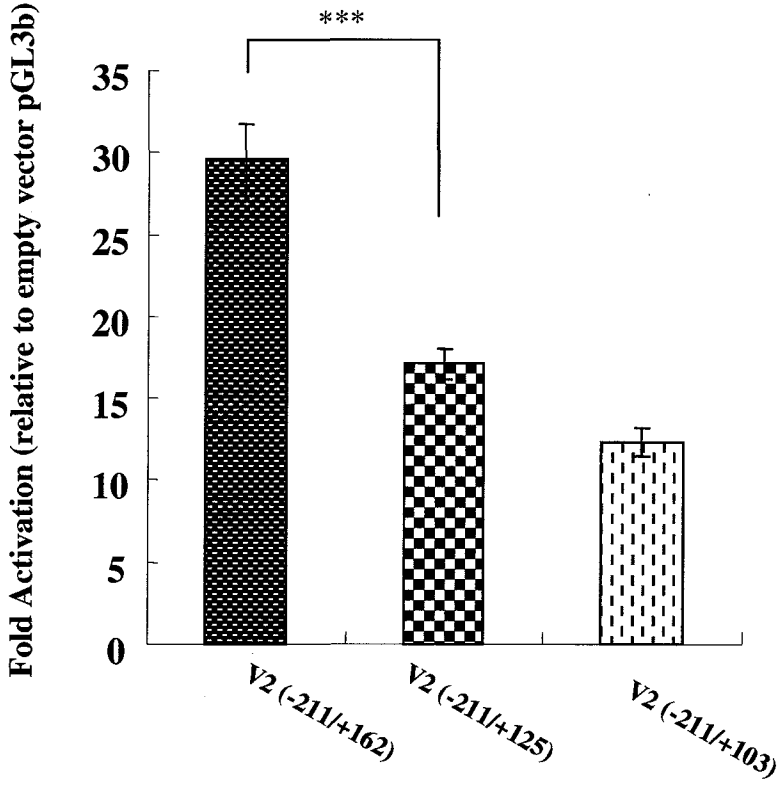


Figure III-4

C.



D.

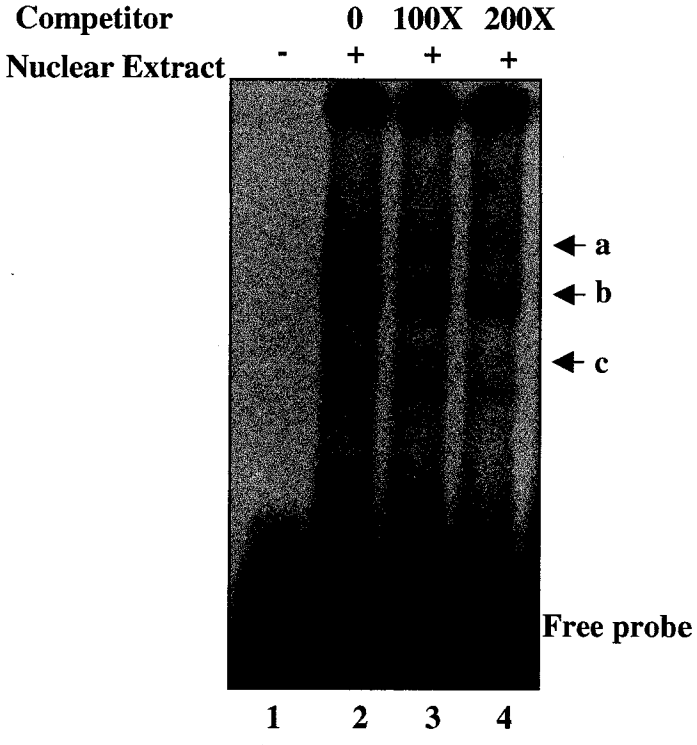


Figure III-4

Figure III-5: Identification of the core promoter for V2 basal transcription.
Using the reporter construct V2(-211/+71) as a primary template, which contains the proximal promoter region but is not influenced by the 3'-downstream activation region, a set of further 5'-& 3'-deletion constructs were tested in both HEK293 cells and SGBS preadipocytes to define the minimal promoter for V2 basal transcription. Data are presented as M±SE of n=3-9 independent experiments. ***$p<0.001$ for the comparison of V2(-29/+71) with V2(+11/+71) and V2(-211/-25).

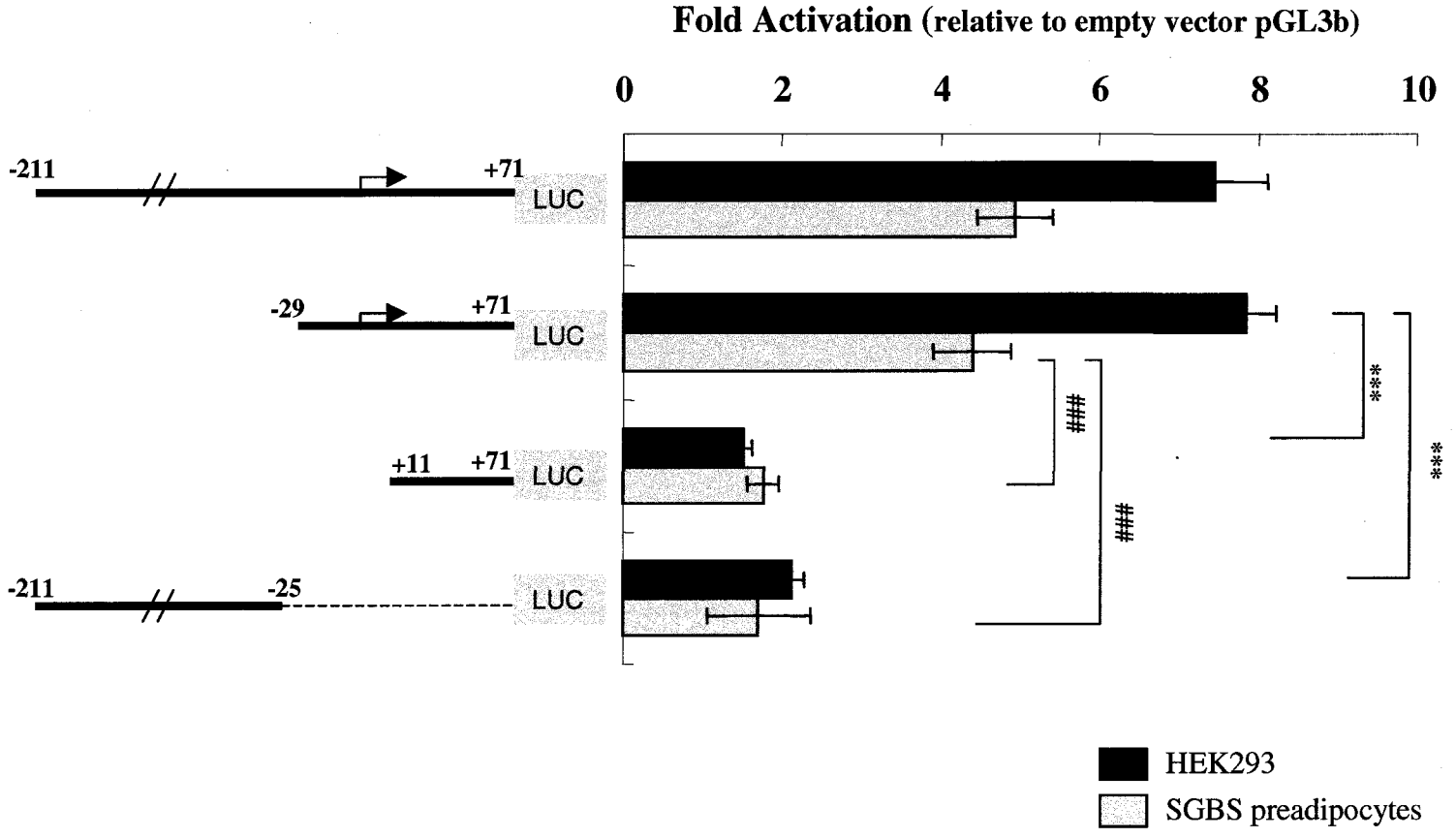

Fold Activation (relative to empty vector pGL3b)
0
2
4
6
8
10
-211
+71
LUC
-29
+71
LUC
+11
+71
LUC
-211
-25
LUC
###
###
***
***
HEK293
SGBS preadipocytes

Figure III-5

Figure III-6: Schematic representation of the hGHR V2 exon and its 5'-upstream promoter.

This diagram shows the hGHR V2 region spanning from 720bp 5'-upstream to 362bp 3'-downstream, with the identified proximal promoter, core promoter, and activation and inhibitory domains. Positions are numbered relative to the major TSS (+1). No TATA box was found within the promoter region; whereas an Initiator-like element (Inr) is present surrounding the TSS. Putative *cis*-elements, including the CCAAT box, CHOP, c- Ets1 and PU.1 binding sites, were found within a 40-bp region approximately 50bp upstream of the TSS. Two putative Hes1 binding sites are present within the V2 exon as well as the putative binding sites for Sp1, SREBP, ZBP-89 and Egr-1 that are located as a cluster within a ~20-bp region.

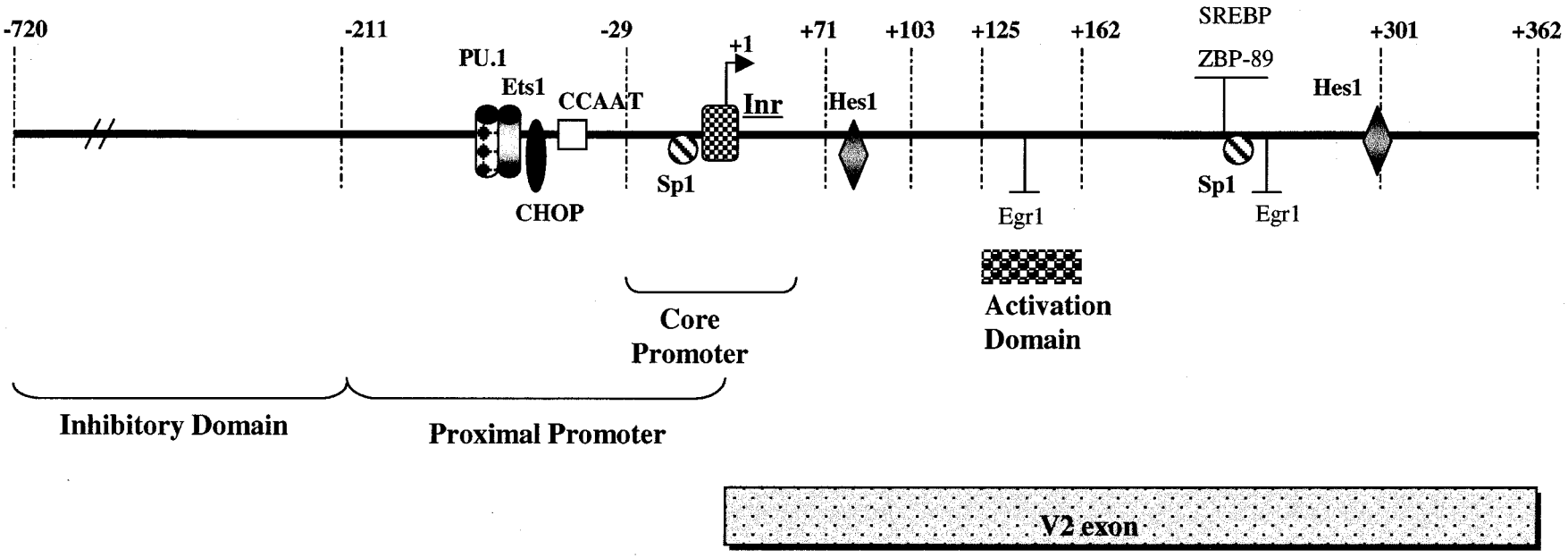

-720
-211
-29
+1
+71
+103
+125
+162
SREBP
ZBP-89
+301
+362
PU.1
Ets1
CCAAT
Inr
Hes1
Hes1
Sp1
CHOP
Egr1
Sp1
Egr1
Core
Promoter
Activation
Domain
Inhibitory Domain
Proximal Promoter
V2 exon

Figure III-6

Figure III-7: Impact of important response elements in the core promoter on V2 basal transcription.

(A) The reporter construct V2(-29/+71), that includes only the core promoter region, was tested in HEK293 cells. Activity of the wildtype constructs was compared with constructs containing mutations in the Inr-like or Sp1 elements. The consensus sequences for the Initiator (Inr) and Sp1 elements are provided at the top and mutated sequences are indicated by italic uppercase. Arrow indicates the major TSS (+1). Horizontal bars demonstrate activities of mutated constructs relative to wildtype, which is arbitrarily set as 100%. Data are expressed as M±SE of n=4 separate assays.

(B) Mutations of the Inr-like, Sp1 or CCAAT box elements were introduced into the larger promoter construct V2(-211/+362), which contains both the core promoter and upstream as well as downstream regulatory elements. Bar graphs on the right demonstrate activities of the mutated constructs in HEK293 cells relative to wildtype vector, arbitrarily set at 100%. Data are presented as M±SE of n=6 experiments. **$p<0.01$ and ***$p<0.001$.

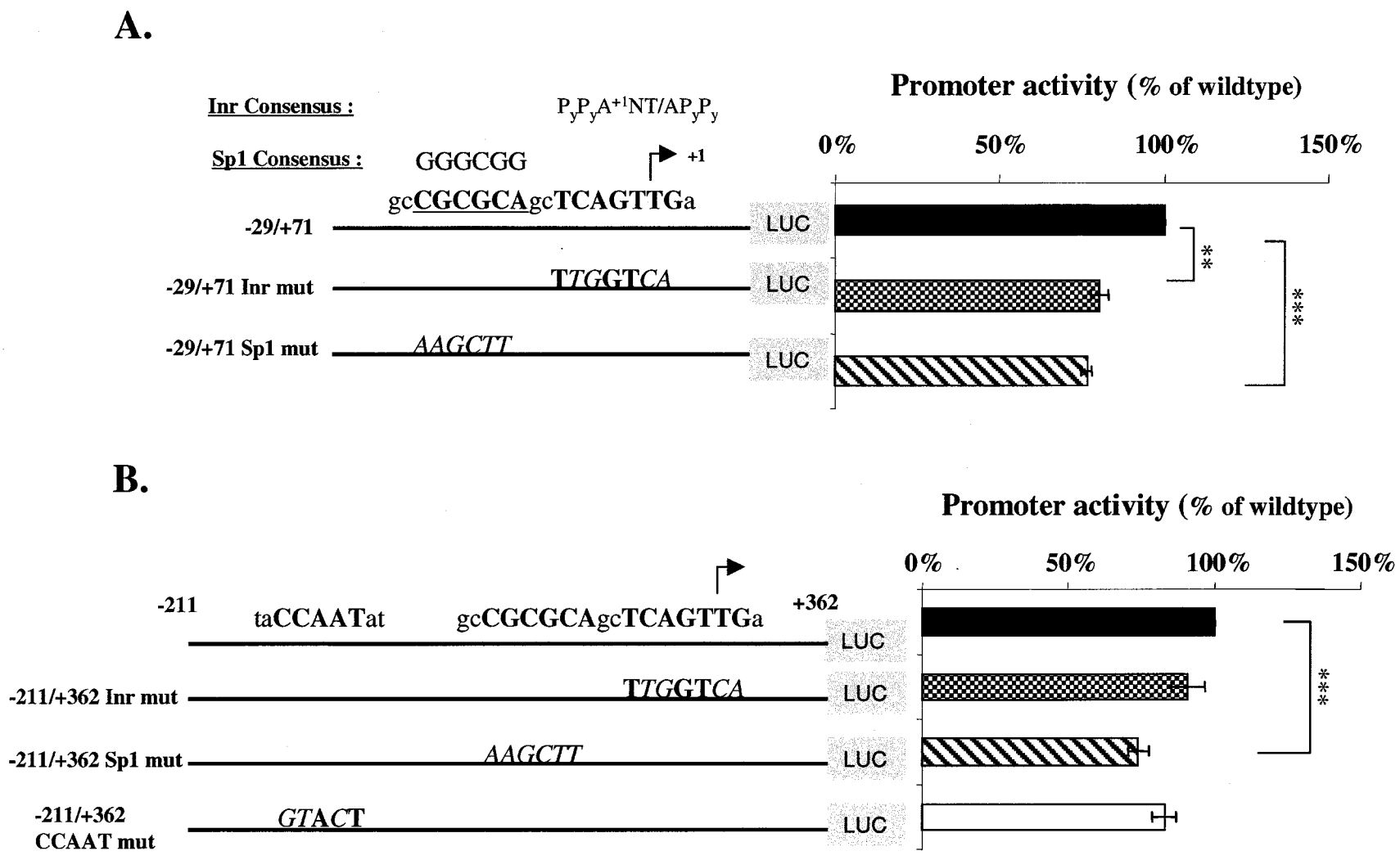

A.
Inr Consensus :
PyPyA+1NT/APyPy
Sp1 Consensus :
GGGCGG
+1
gcCGCGCAgcTCAGTTGa
-29/+71
LUC
-29/+71 Inr mut
TTGGTCA
LUC
-29/+71 Sp1 mut
AAGCTT
LUC
Promoter activity (% of wildtype)
0%
50%
100%
150%
**
***
B.
-211
taCCAATat
gcCGCGCAgcTCAGTTGa
+362
LUC
-211/+362 Inr mut
TTGGTCA
LUC
-211/+362 Sp1 mut
AAGCTT
LUC
-211/+362
CCAAT mut
GTACT
LUC
Promoter activity (% of wildtype)
0%
50%
100%
150%
***

Figure III-7

Figure III-8: Overexpression of dominant negative Sp1 (Sp1 DN) does not affect V2 promoter activity.

(A) Putative Sp1 binding sites identified in ovine 1B (V2 homologue) and human V2 5'-upstream and 3'-downstream regions.

(B) V2 promoter constructs containing 5'- or 3'-deletions or the V2 exon Sp1 mutation were transfected into HEK293 cells together with 400ng Sp1 DN expression plasmid or the same amount of empty control plasmid (0ng Sp1 DN). Data are expressed as fold activation relative to Sp1 DN at 0ng, which is arbitrarily set as 1 and presented as M±SE of n=3 experiments. No significant differences in V2 promoter activities ($p>0.05$) were revealed.

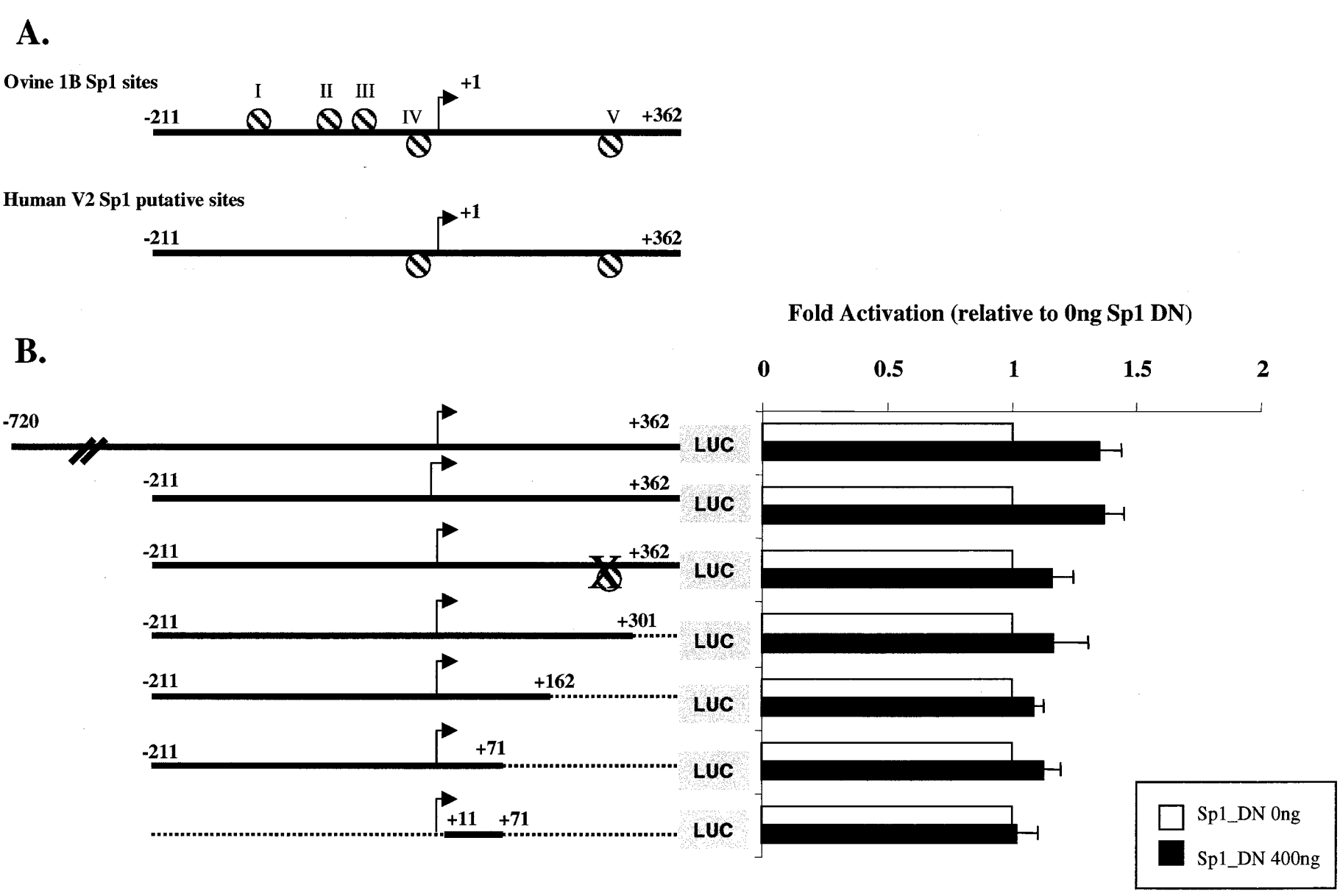

A.
Ovine 1B Sp1 sites
I
II
III
IV
V
+1
-211
+362
Human V2 Sp1 putative sites
+1
-211
+362
B.
Fold Activation (relative to 0ng Sp1 DN)
0
0.5
1
1.5
2
-720
+362
LUC
-211
+362
LUC
-211
+362
LUC
-211
+301
LUC
-211
+162
LUC
-211
+71
LUC
+11
+71
LUC
Sp1_DN 0ng
Sp1_DN 400ng

Figure III-8

Figure III-9: Functional analysis of the Ets binding sites in the hGHR V2 promoter.
(A) *Effects of Ets binding site mutations on V2 basal transcriptional activity.* The luciferase reporter construct V2(-211/+362) was mutated in three regions of the putative Ets binding sites. The core binding sequences for the PU.1 site and the c-Ets1 site are underlined and the mutant sequences are shown in bold lowercase. Data from n=4-8 transient transfection assays in HEK293 cells are presented as M±SE relative to the activity of the V2 (-211 /+362) wildtype vector, which is arbitrarily set as 100%.
(B) *Overexpression of Ets1 stimulates V2 promoter activity in a dose-dependent manner in HEK293 cells.* V2 promoter constructs with or without the putative Ets binding sites were cotransfected with increasing amounts of Ets1 expression plasmid (pEVRF-Ets1) in HEK293 cells. Empty control vector (pEVRF) was added to make equal amounts of total DNA. Data are expressed as fold activation relative to Ets1 at 0ng, which is arbitrarily set as 1. Results are expressed as M±SE of n=3 experiments. ***$p<0.001$ relative to Ets1 at 0ng.
(C) *Overexpression of Ets1 stimulates V2 transcriptional activity in SGBS preadipocytes.* 0.5μg of various V2 promoter constructs that either contain the putative Ets binding sites or not were cotransfected with 0.1μg of Ets1 expression vector (pEVRF-Ets1) or empty expression vector (pEVRF) into SGBS preadipocytes. Transcriptional activities are reported as fold induction compared to empty control vector, which is arbitrarily set as 1. Data are presented as M±SE of n=3 experiments. ***$p<0.001$.
(D) *Impact of mutations in Ets binding sites on transcriptional activation of V2 by Ets1.* The wildtype V2 promoter reporter construct -211/+362 containing the c-Ets1 binding motif (dotted line) and the PU.1 binding motif (dash line) is illustrated at the top. Site-directed mutagenesis was performed to generate individual Ets binding site mutants by changing the core motif sequences (shown in bold lowercases). Wildtype or mutant constructs were co-transfected with 0.1μg pEVRF-Ets1 expression vector (Ets1 100ng) or 0.1μg pEVRF control vector (Ets1 0ng) in HEK293 cells. All values are expressed as fold induction relative to wildtype construct without Ets1 overexpression, arbitrarily set as 1. Data are presented as M±SE of n=3-7 experiments. **$p<0.01$ and ***$p<0.001$ refer to the differences between wildtype and Ets mutants in response to Ets1 overexpression at 100ng.

A.

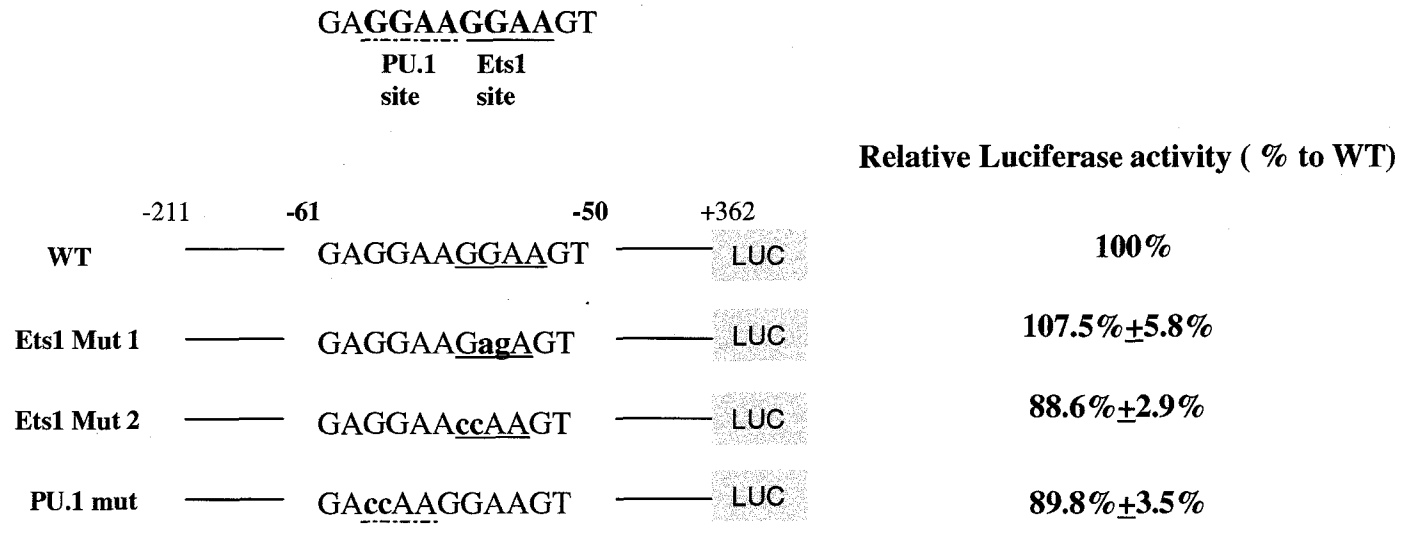


Figure III-9

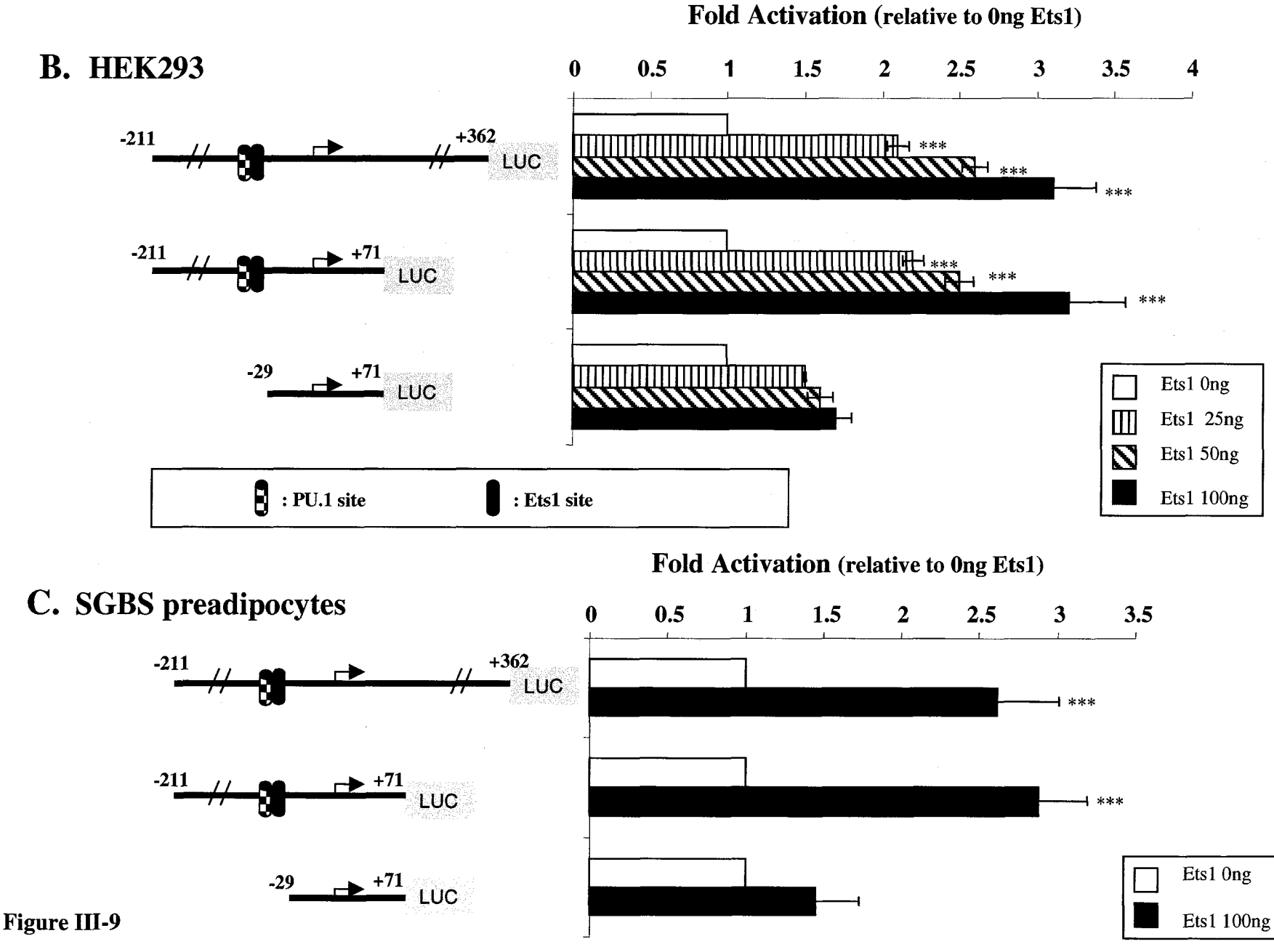

B. HEK293
Fold Activation (relative to 0ng Ets1)
0
0.5
1
1.5
2
2.5
3
3.5
4
-211
+362
LUC
-211
+71
LUC
-29
+71
LUC
***
***
***
***
***
***
Ets1 0ng
Ets1 25ng
Ets1 50ng
Ets1 100ng
: PU.1 site
: Ets1 site
C. SGBS preadipocytes
Fold Activation (relative to 0ng Ets1)
0
0.5
1
1.5
2
2.5
3
3.5
-211
+362
LUC
-211
+71
LUC
-29
+71
LUC
***
***
Ets1 0ng
Ets1 100ng

Figure III-9

D.

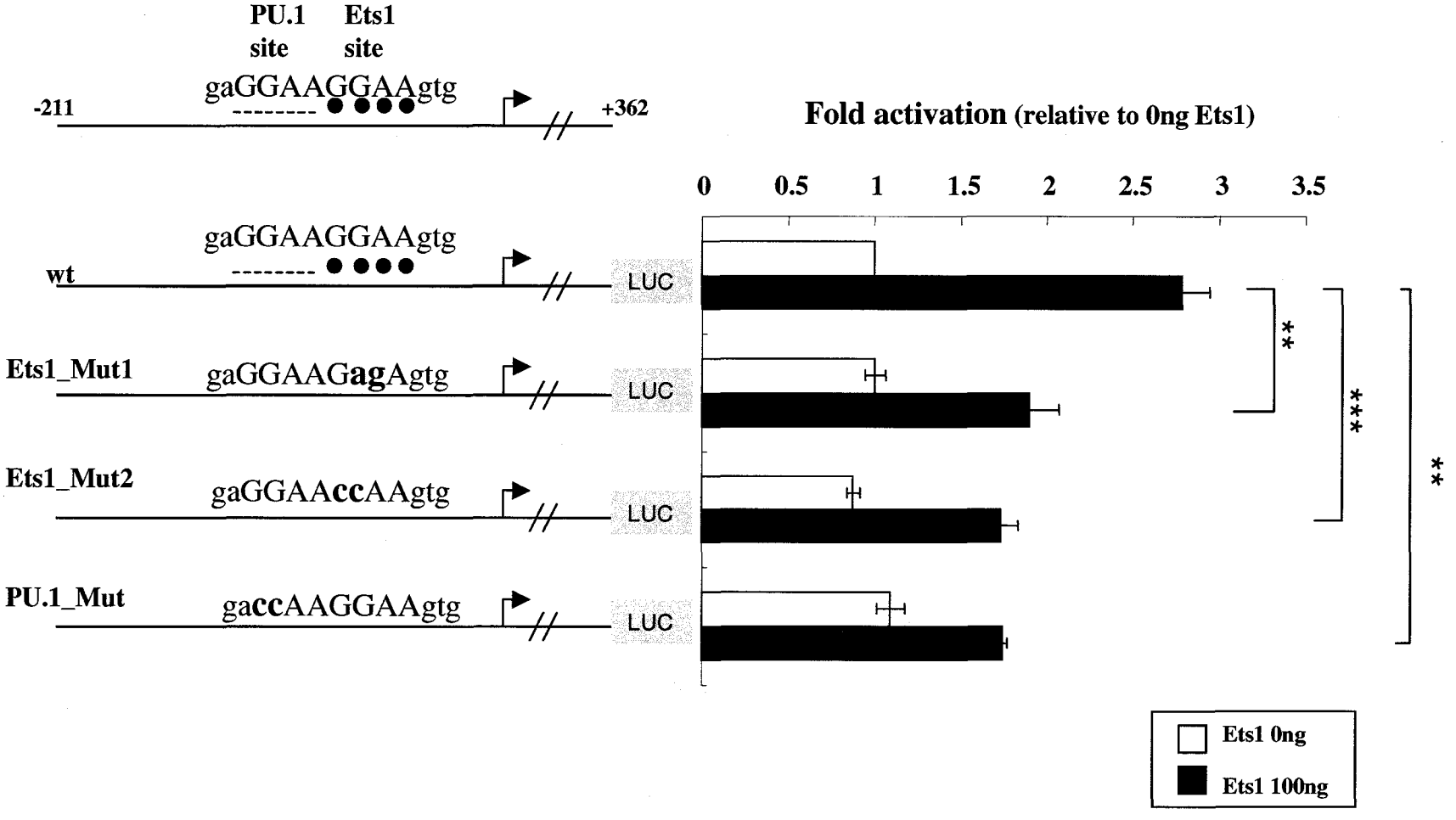


Figure III-9

(E) *Ets1 binds directly to the hGHR V2 promoter via the c-Ets1 binding site.*
Nuclear extracts prepared from HEK293 cells or HEK293 cells cotransfected with pEVRF-Ets1 expression plasmid were incubated with wildtype (PU.1^{wt} Ets1^{wt}) or Ets1 Mut2 (PU.1^{wt} Ets1$^{mut\,2}$) oligonucleotide probes, and then subjected to EMSA. The specific shifted band is indicated by "S" and "NS" represents the non-specific bands. Supershift assay was performed by using an antibody specific for Ets1 (Ets1 (N276), sc-111), and the supershifted signal is indicated by "SS".

E.

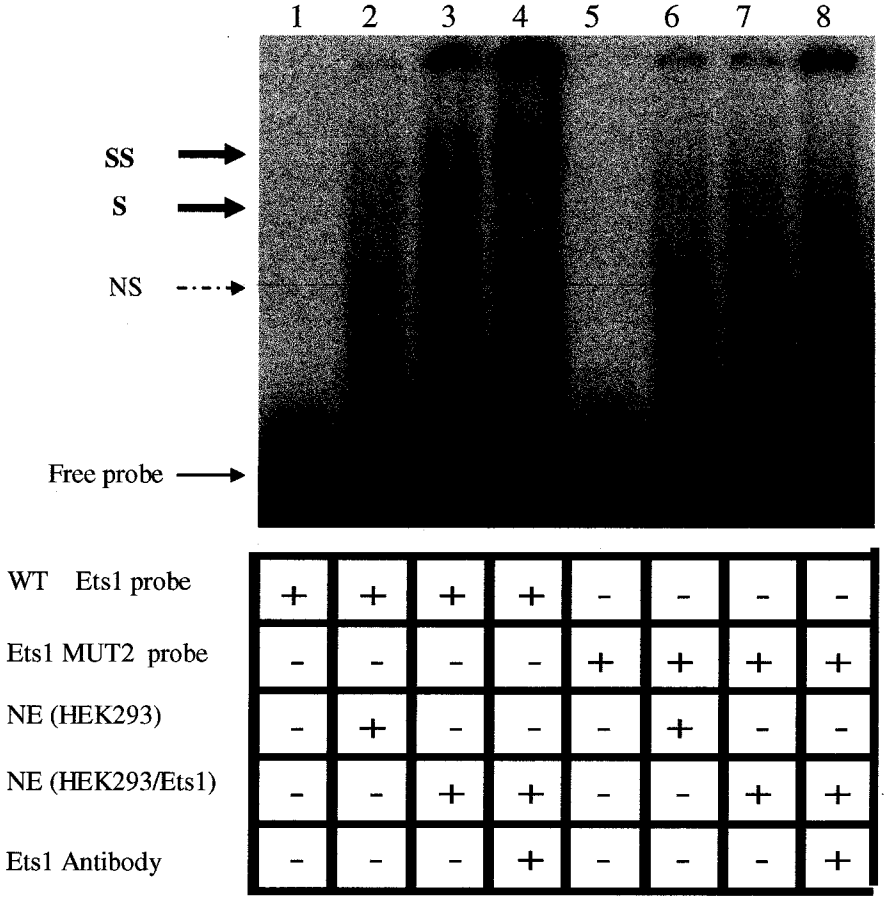


Figure III-9

Figure III-10: Overexpression of CHOP upregulates V2 promoter activity.

(A) 5'-deletion V2 promoter constructs were transfected into COS1 cells together with 0.2μg of expression vector for CHOP (pcDNA3.1-hygro-CHOP) or 0.2μg empty vector (pcDNA3.1-hygro). The fold induction indicates the ratios between CHOP overexpression and CHOP at 0ng, which is arbitrarily set as 1. Data are expressed as M±SE, n=3. ***p<0.001 vs. control. Similar stimulation patterns were observed in A549 cells and HEK293 cells (data not shown). Because COS1 cells gave the highest stimulation, it was chosen for studying CHOP effects on the V2 promoter.

(B) *Dose-dependent stimulatory effect of CHOP on V2 promoter activity.* COS1 cells were transiently co-transfected with the reporter construct V2 (-211/+362) and increasing amounts of the pcDNA3.1-hygro-CHOP expression plasmid. Empty vector (pcDNA3.1-hygro) was added to make equal amounts of total DNA. The fold activation is relative to CHOP at 0ng, arbitrarily set as 1. Data are presented as M±SE of n=6 experiments. *p<0.05 and ***p<0.001.

(C) *In vivo* ChIP *assay of the association of CHOP with the V2 promoter region.* Cross-linked chromatin isolated from HEK293 cells or HEK293 cells transfected with pcDNA3.1-hygro-CHOP (HEK293/CHOP) was immunoprecipitated with an anti-CHOP antibody. Immunoprecipitates were analyzed by PCR using primer pairs encompassing the putative proximal promoter CHOP site, as illustrated on the top panel. M indicates the 100bp DNA ladder (Invitrogen).

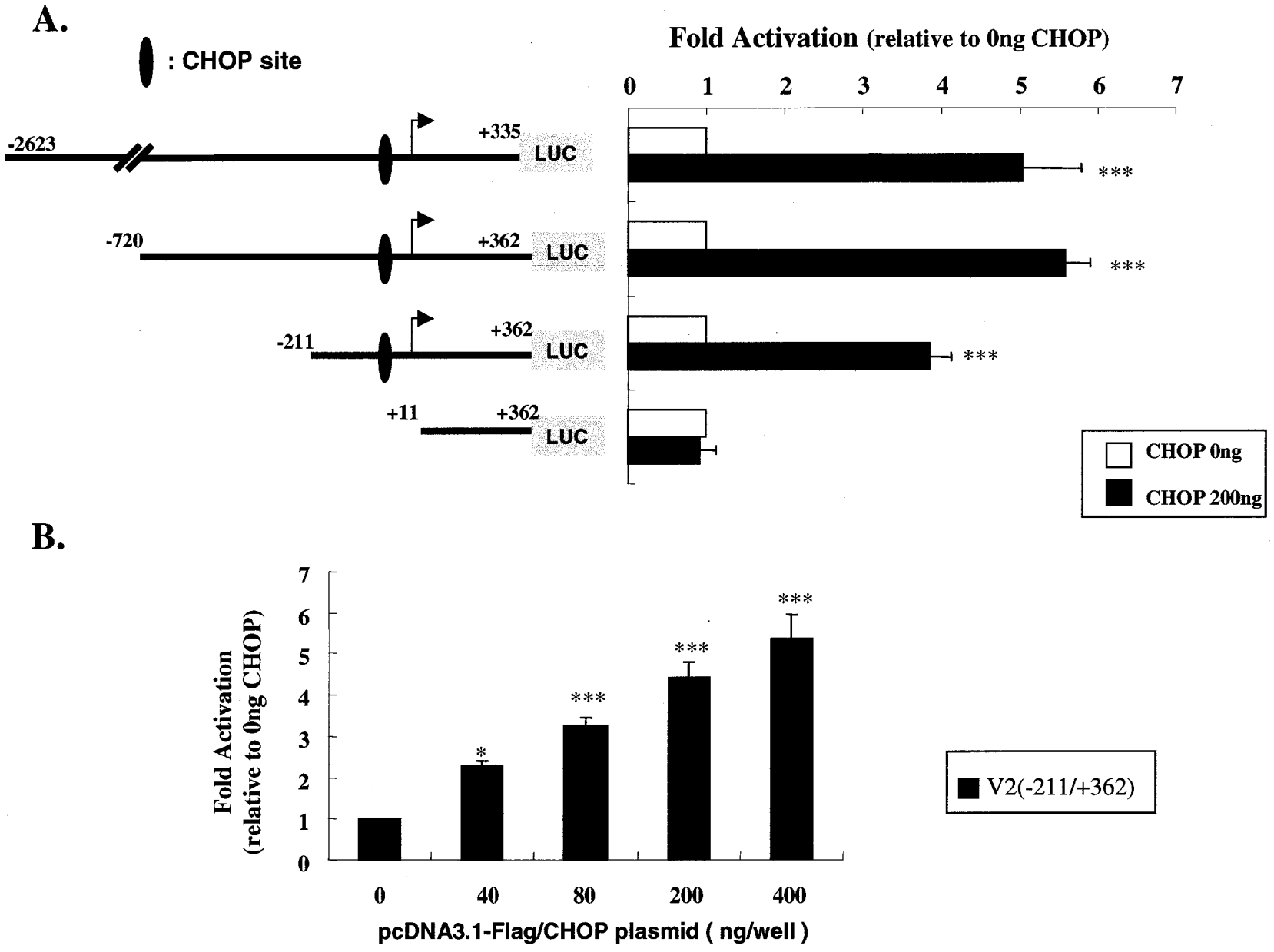
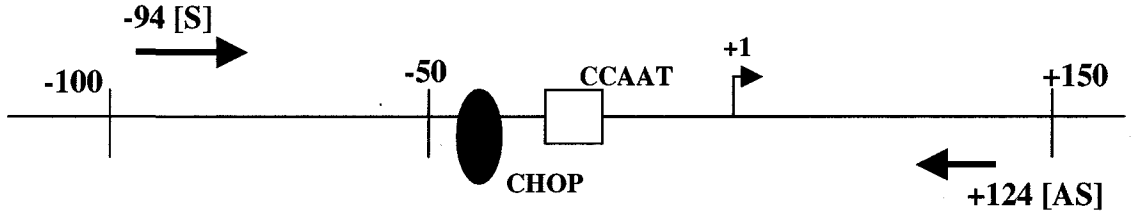


Figure III-10

C.



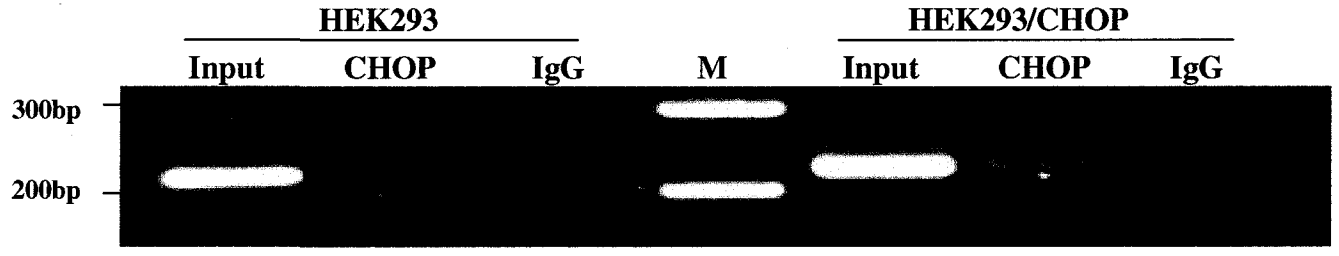


Figure III-10

Figure III-11: Analysis of the functional role of the CHOP site in transcriptional activation of the V2 promoter by CHOP.

(A) The consensus CHOP binding site, hGHR V2 CHOP site and mutated (lowercase) CHOP site sequences.

(B) V2 promoter reporter constructs containing the wildtype or the mutated CHOP site were transfected into COS1 cells along with CHOP expression vector (pcDNA3.1-hybro-CHOP) at increasing amounts.

(C) V2 promoter constructs including the CHOP site or not were transiently transfected into COS1 cells along with CHOP expression vector (pcDNA3.1-hybro-CHOP) at increasing amounts. Data are expressed as fold activation relative to CHOP at 0ng, which is arbitrarily set as 1.

Data are expressed as M±SE of n=4-8 separate experiments. **$p<0.01$ and ***$p<0.001$ indicate differences compared to CHOP at 0ng, while **ns** ($p>0.05$) describes the difference between paired values (wildtype vs. mutant) in response to the same dose of CHOP expression.

(D) A series of promoter reporter constructs with sequential deletion of the 3'-V2 exon region were cotransfected into COS1 cells with increasing amounts of CHOP expression vector. The fold activation is relative to CHOP at 0ng (set as 1). a: $p<0.05$ compared to CHOP at 0ng. b: $p<0.05$ compared to CHOP at 40ng. Data are presented as M±SE of n=3-10 experiments.

A.

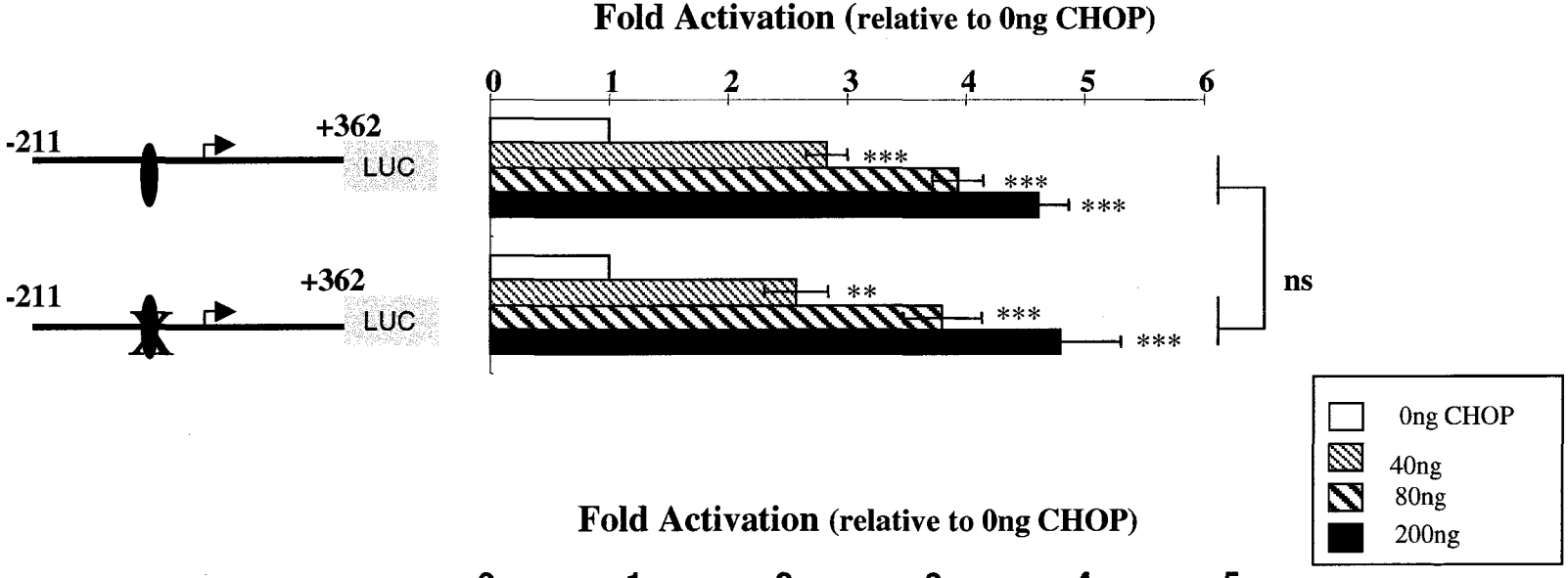
5'-R R R **T G C A A T** A/C C C C -3' **Consensus CHOP**

5'-A G T **T G C A A T** A C A C-3' **hGHR V2 CHOP (11/13)**

5'-A G T **g g a t c c** A C A C-3' **CHOP mut-Luc (5/13)**

: CHOP site

B.



C.

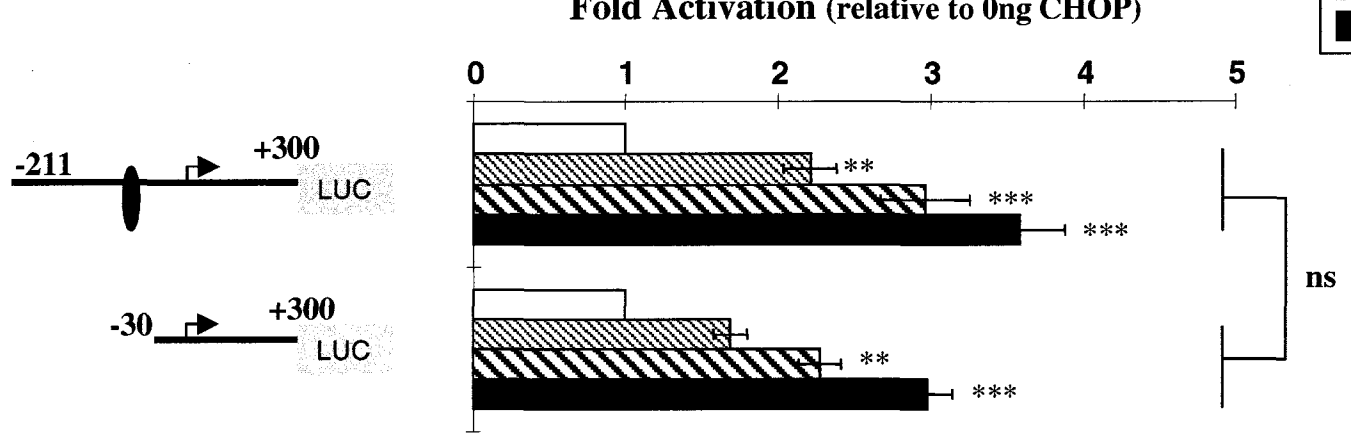


Figure III-11

D.

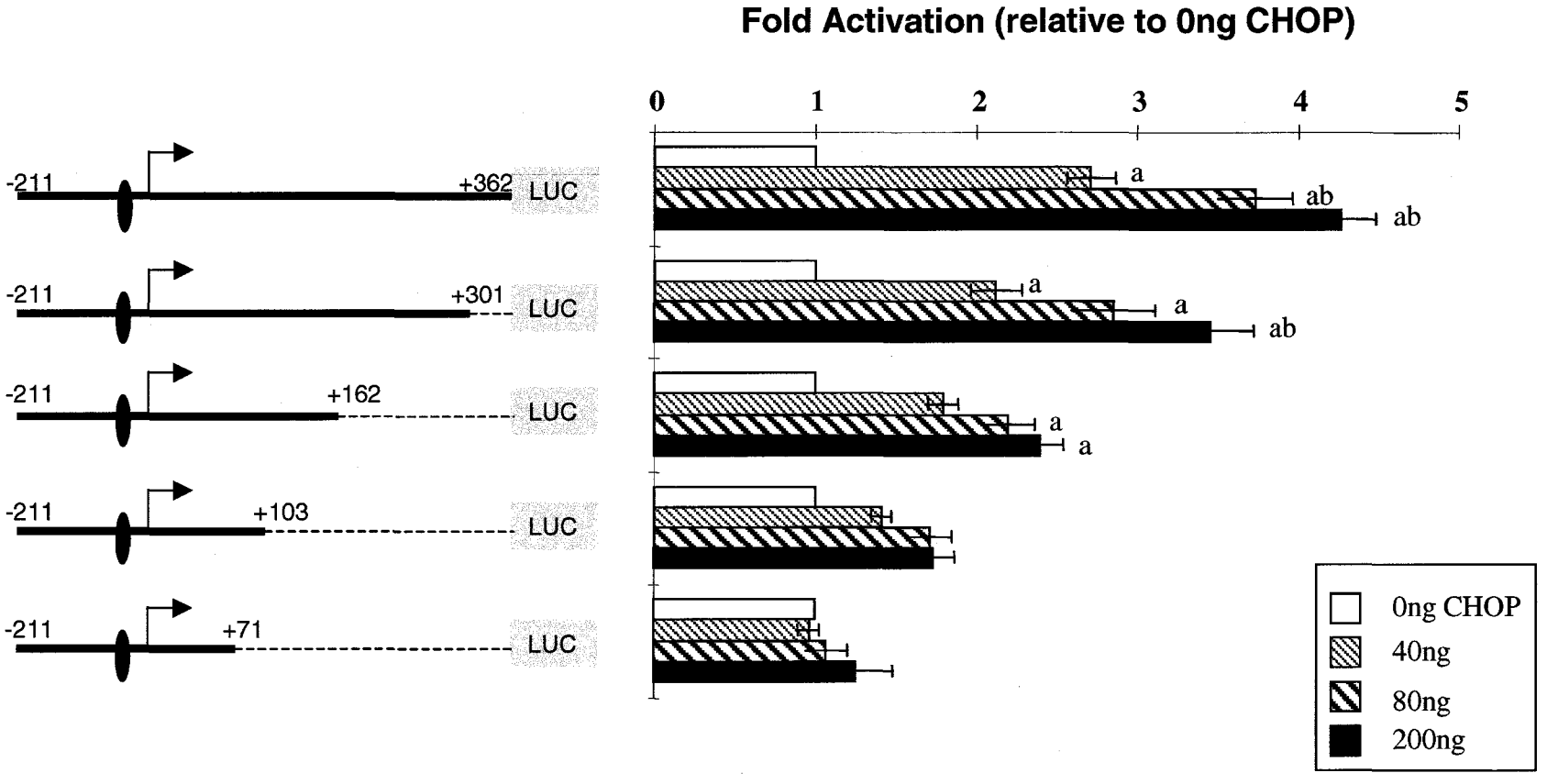


Figure III-11

Figure III-12: Interactions between CHOP and Ets1 in regulating V2 promoter activity.

COS1 cells were cotransfected with V2 (-211/+362) or V2 (-211/+71) and 0.05μg of Ets1 plus increasing amounts (0.05, 0.1 and 0.2μg) of CHOP, and assayed for V2 transcriptional activities. The total amount of plasmid was adjusted to 1μg /transfection using an empty vector. Data are expressed as fold activation relative to no CHOP+Ets1 overexpression (CHOP 0ng + Ets1 0ng), which is set as 1. Significant differences between paired values are shown: *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ns: $p>0.05$. Data are presented as M±SE from n=3-5 different experiments.

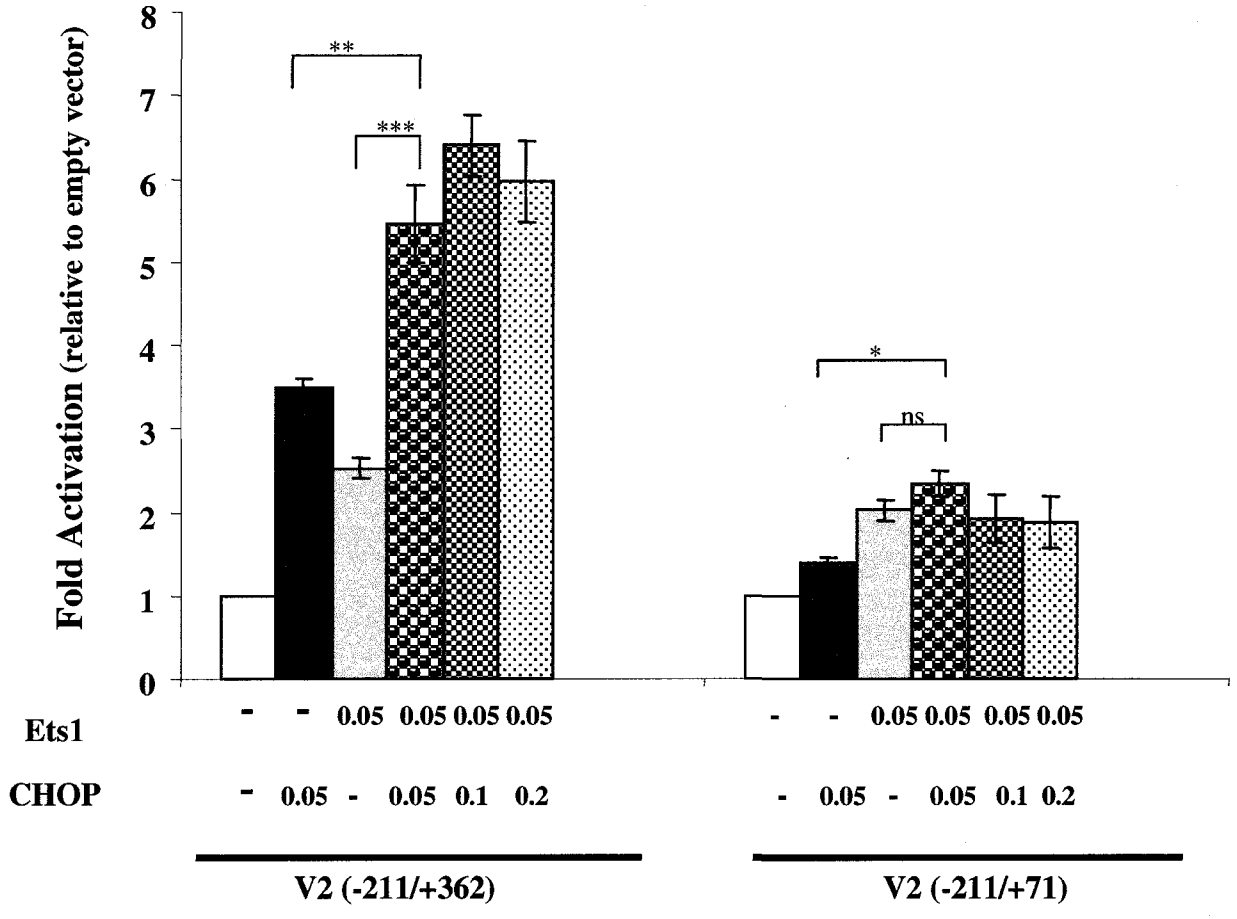


Figure III-12

Figure III-13: Functional analysis of transcriptional activation of V2 by C/EBPβ. The V2 promoter construct V2(-211/+71), its CCAAT box mutant or its CHOP site mutant were transfected into HEK293 cells together with 1μg C/EBPβ expression plasmid (pSG5-C/EBP LAP) or 1μg empty vector, and assayed for transcriptional activities in response to C/EBPβ overexpression. Data are expressed as fold activations relative to the value of V2 (-211/+71) cotransfected with empty vector, which is set as 1. ***$p< 0.001$ indicates the statistical significant difference observed with C/EBPβ overexpression. ns: indicates no statistical difference ($p>0.05$) between wildtype and CCAAT box mutant constructs in response to C/EBPβ overexpression. #: indicates the presence of a statistical difference ($p<0.05$) between wildtype and CHOP site mutant reporter constructs in response to C/EBPβ overexpression. All data are presented as M±SE of n=3 experiments.

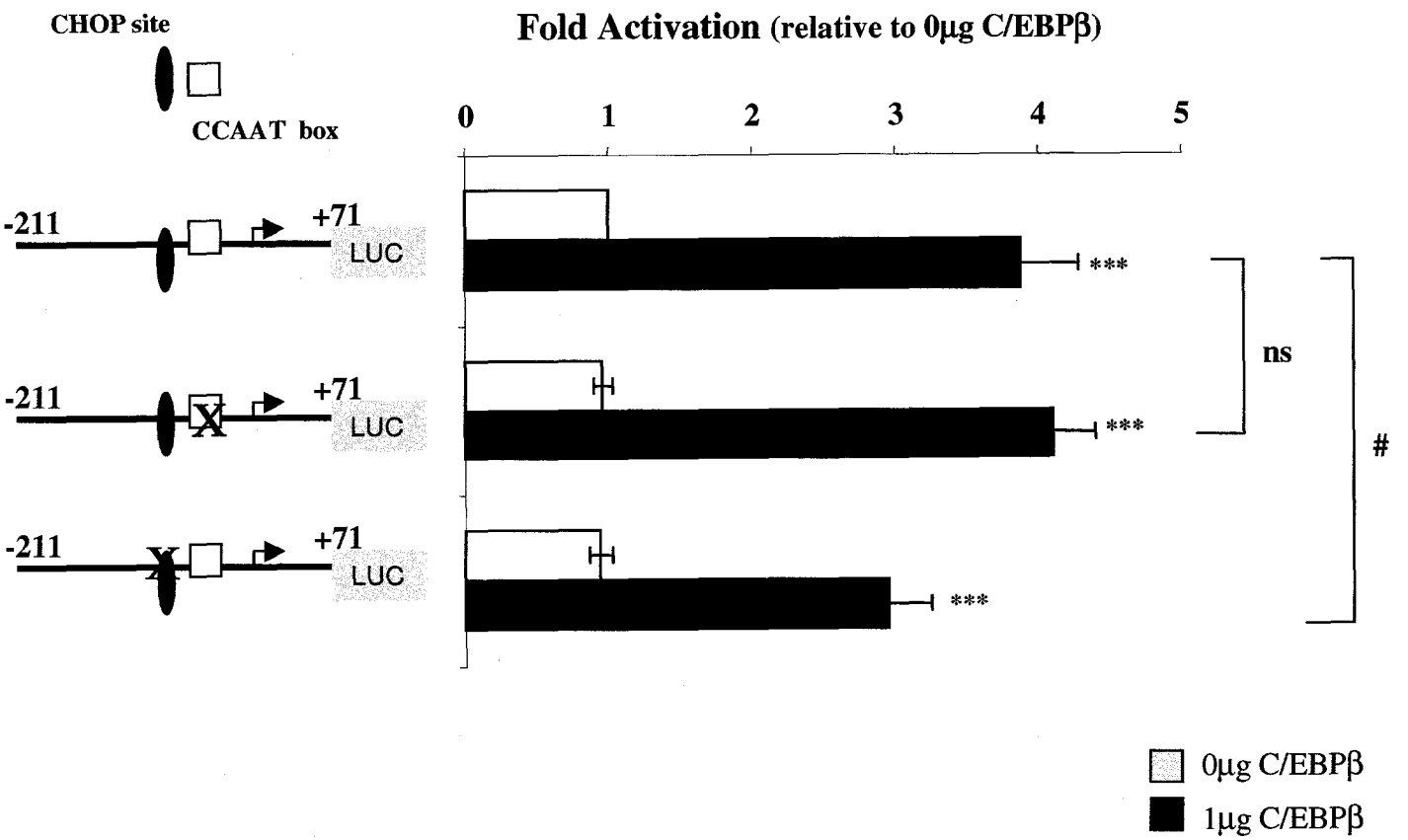


Figure III-13

Figure III-14: Hes1 expression represses hGHR V2 promoter activity via the HES binding sites.

(A) A schematic representation of a 362 bp 3'-region downstream of the transcription start site (+1) in which two putative HES binding sites were detected.

(B) *Hes1 expression inhibits V2 promoter activity in HEK293 cells.* V2 promoter constructs, which contain no Hes site (V2(-211/+71)) or HES site 1 (V2(-211/+103)) or Hes site 1& 2 (V2(-211/+362)), were transfected into HEK293 cells together with increasing amounts of Hes1 expression plasmid. Data are expressed as percentages relative to Hes1 expression at 0ng, which is set as 100%. ***$p<0.001$ indicates the significant difference between Hes1 overexpression (10ng, 25ng, 50ng & 100ng) vs. no Hes1expression (0ng). ##$p<0.01$ indicates the significant difference between paired values at the same dose.

(C) *Hes1 suppresses V2 transcriptional activity in SGBS preadipocytes.* The V2 promoter constructs V2(-211/+71) , V2(-211/+103) and V2(-211 /+362) were transfected into SGBS preadipocytes with 100ng of Hes1 expression vector or 100ng of empty vector (Hes1 0ng). Data are expressed as percentages relative to Hes1 at 0ng, arbitrarily set as 100%. ***$p<0.001$ and #$p<0.05$ indicate the statistically significant differences between paired values.

(D) *In vitro binding of Hes1 protein to HES site 1 of hGHR V2.* The ^{32}P labeled double-stranded hGHR V2 HES site1-containing oligonucleotide probe (Supplementary Table 2) was incubated with nuclear extracts prepared from HEK293 cells (lane 2) or HEK293 cells transfected with N-terminally Flag-tagged Hes1 cDNA (lane3) for EMSA. For competition experiments, a 100- and 200-fold excess of unlabeled Hes site 1-containing probe was added as a competitor before addition of the labeled probe (lanes 4 and 5). The specific DNA-protein bands are indicated by arrows.

(E) *In vivo binding of Hes1 proteins to the HES sites of hGHR V2.* HEK293 cells were transfected with N-terminally Flag-tagged Hes1 cDNA, cross-linked with formaldehyde, and immunoprecipitated with anti-Hes1 antibody or control rabbit IgG. DNA was extracted from immunoprecipitates and PCR amplified by the primer sets surrounding Hes site 1, Hes site 2 or a control region that is ~2-kb 5'-upstream of Hes site 1. Products were resolved on a 2% agarose gel.

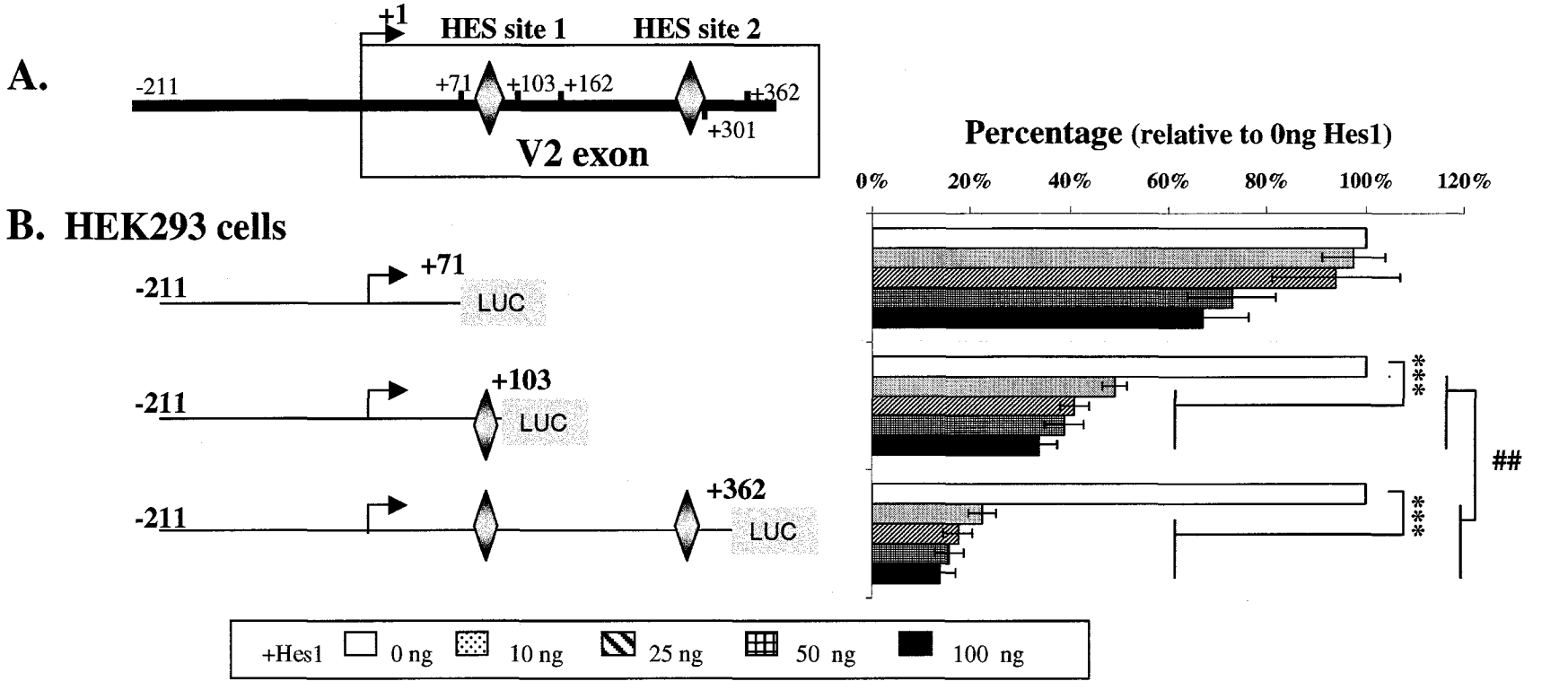
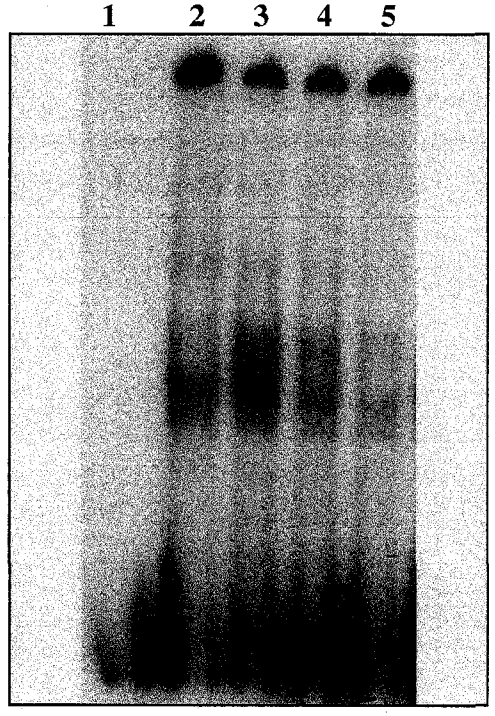


Figure III-14

D.



^{32}P HES site1 probe	+	+	+	+	+
NE (HEK293)	-	+	-	-	-
NE (HEK293/Hes1)	-	-	+	+	+
Cold competitor	-	-	-	100x	200x

E.

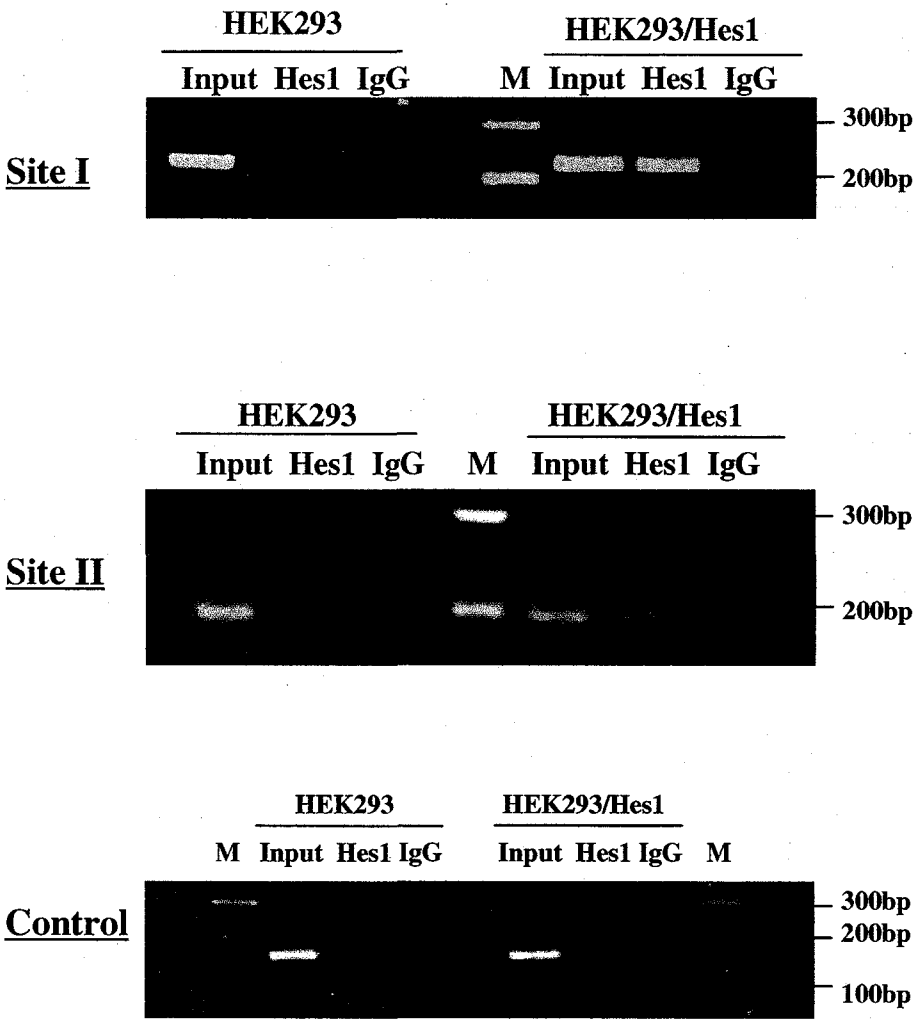


Figure III-14

Figure III-15: Schematic models of our proposed regulatory mechanism governing hGHR V2 transcription in different human cell types (Model 1) and during adipocyte differentiation (Model 2).

(A) Model 1: In different human cell types, Ets1, CHOP and Hes1 can mediate positive (Ets1, CHOP) and negative (Hes1) regulation of hGHR V2 expression in response to extracellular (developmental, growth and stress) signals. (+) indicates: stimulatory effects, (−) indicates: suppressive effects.

(B) Model 2: In preadipocytes, Ets1, CHOP and Hes1 are important regulators of hGHR V2 transcription. They are expressed at a relatively high level by the growth-arrest, confluent preadipocytes and are markedly downregulated when differentiation starts. In contrast, expressions of C/EBPβ and C/EBPα are significantly increased at the early and late differentiation stages, respectively, and become major transactivators of hGHR V2 expression, leading to an increase in hGHR V2 mRNA during human adipocyte differentiation. The light density in thetriangles represents the changes in expression of relevant transcription factor(s) across differentiation stages. (+) and /-): positive (Ets1, CHOP) and negative (Hes1) regulation of V2 transcription. (+):transactivation of V2 expression. Arrow indicates the major TSS of V2.

A.

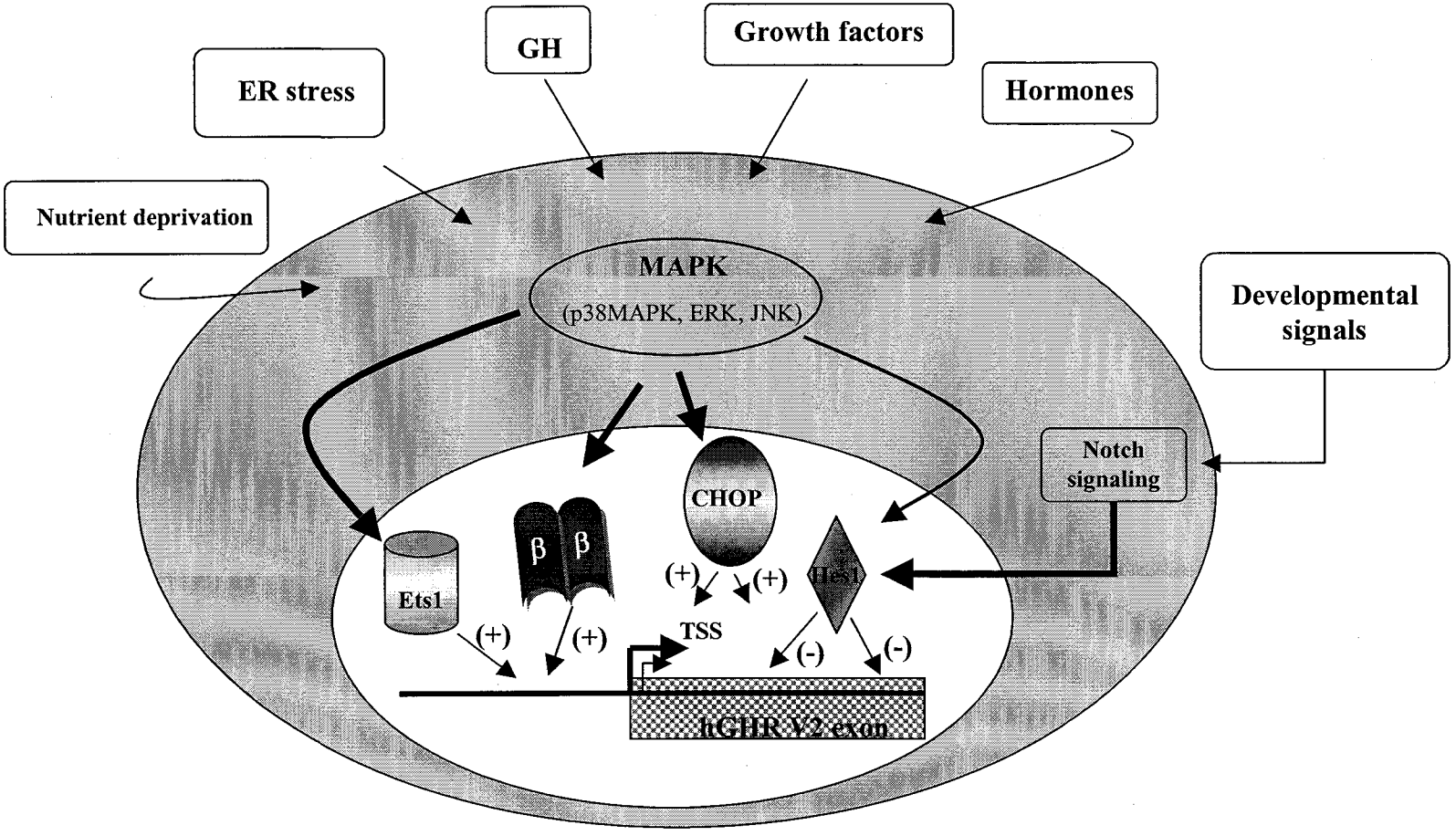


Figure III-15

B.

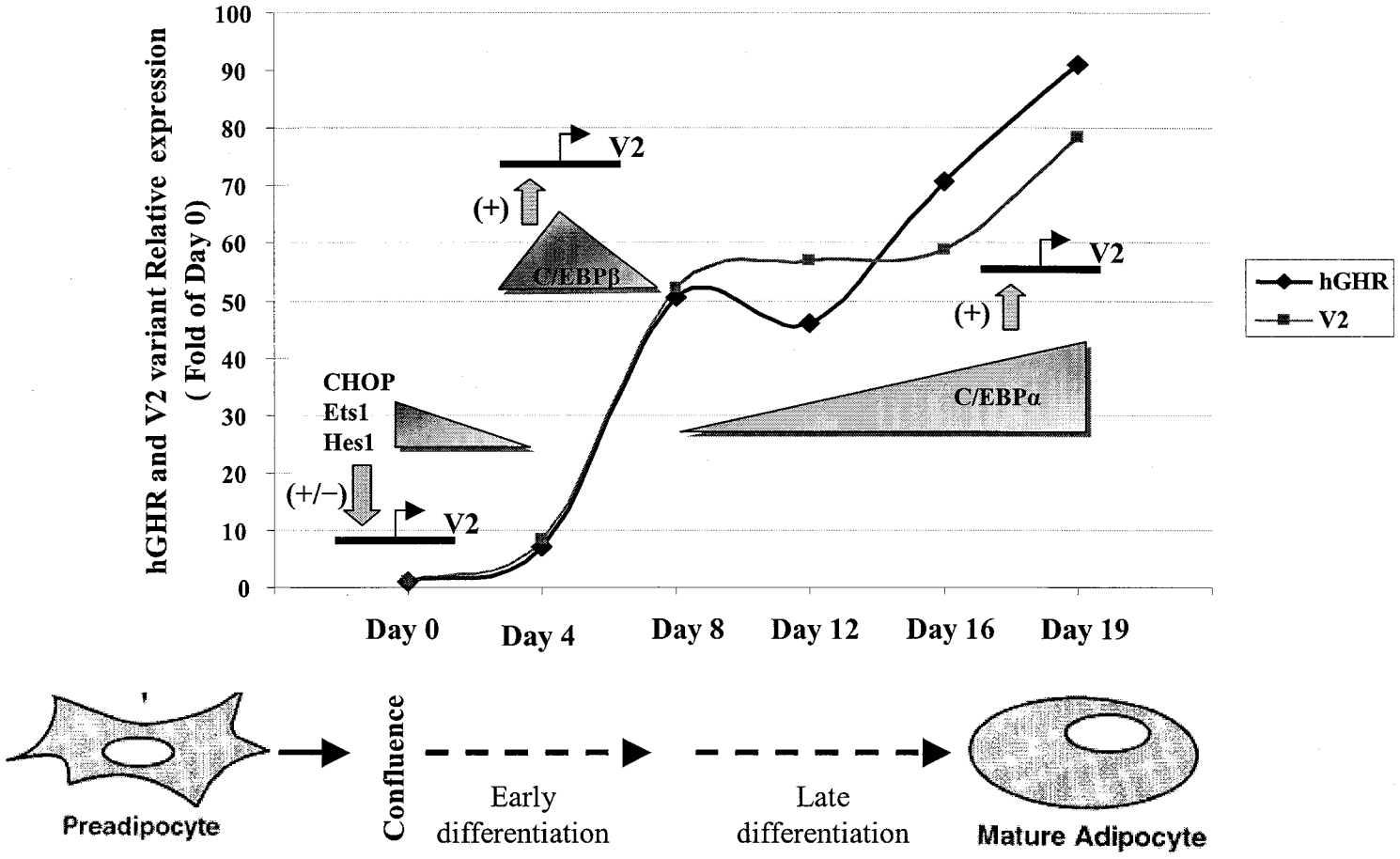
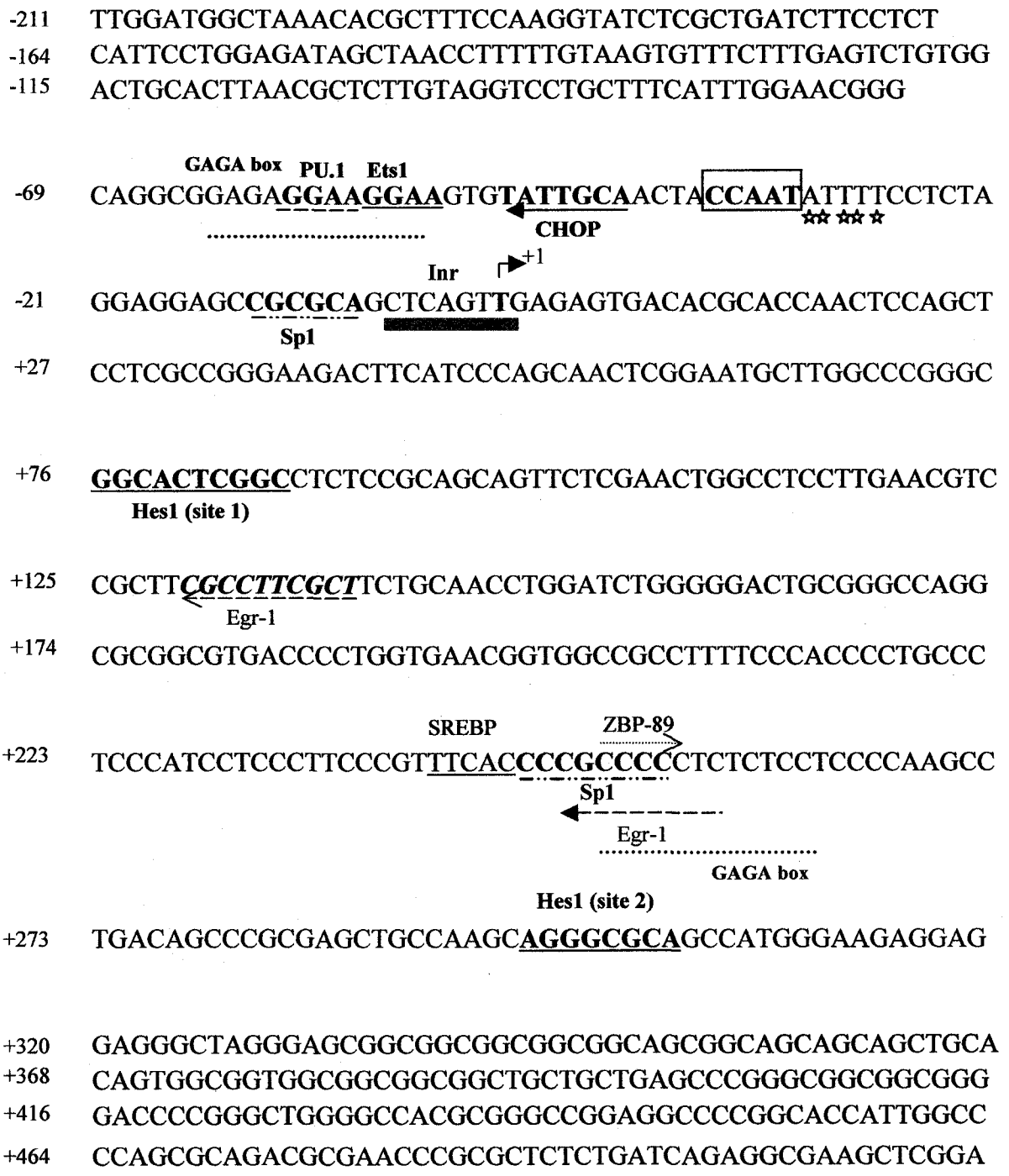


Figure III-15

Supplementary Figure III-1: Nucleotide sequence of the hGHR V2 exon and 211bp of its 5'-proximal promoter.

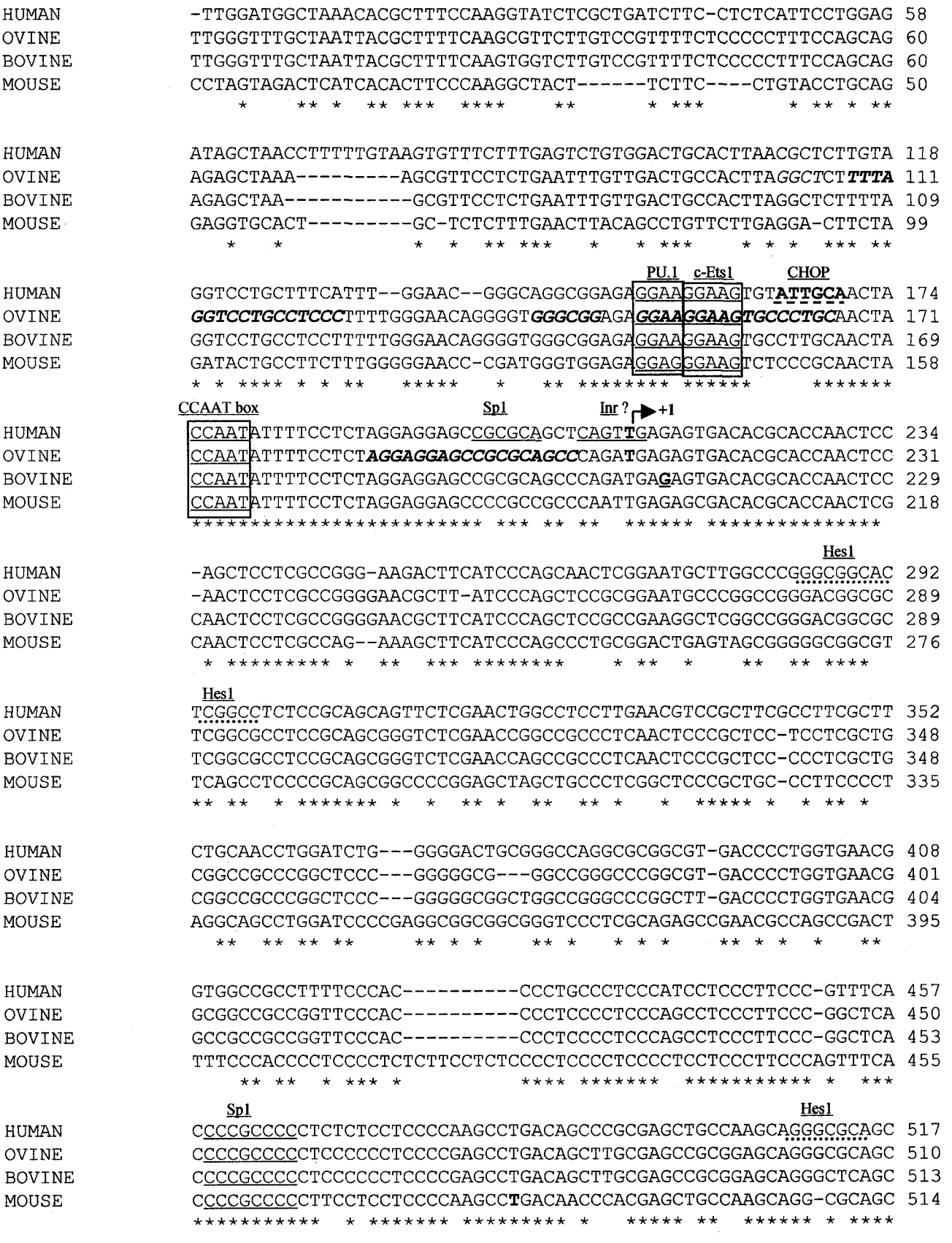
The nucleotide corresponding to the major transcription initiation site is in bold and indicated with an arrow ($\rightarrow$ +1). An Initiator (Inr)-like element is underlined with a solid rectangle. A conserved CCAAT box is boxed, immediately followed by an A+T rich sequence labeled with stars. Putative binding sites of transcription factors are underlined and named either above (sense strand) or below (antisense strand). Identification of these putative binding sites was performed using MatInspector (Cartharius *et al.* 2005) and SignalScan (Prestridge 1991) software programs.



Supplementary Figure III-1

Supplementary Figure III-2: Sequence comparison of exon and 5'-flanking sequences of human V2, ovine 1B, bovine 1B and mouse L2.

The human V2 sequence (AF322014) containing 211bp of the 5'upstream promoter region and ~303bp of V2 exon is aligned with its homologues, including ovine 1B (S78252), bovine 1B (AF046861) and mouse L2 (AF120480). Sequence identity is indicated by asterisks, while dashes indicate gaps introduced to maximize alignment. The major transcription start site of the ovine 1B is indicated by +1 with arrows, which is also used in this study as the major transcription start site (TSS) for human V2 (see text). The TSSs for bovine 1B (G) and mouse L2 (T) are bolded in their respective lanes. Motifs of potential binding sites for transcription factors are underlined and named. The highly conserved CCAAT box and Ets binding sites (c-Ets1 binding site and PU.1 binding site) are boxed. Human specific CHOP site and Hes1 sites are marked with a dash line and round dots, respectively. Sp1 sites identified in ovine 1B studies are indicated (bold and italicized).



Supplementary Figure III-2

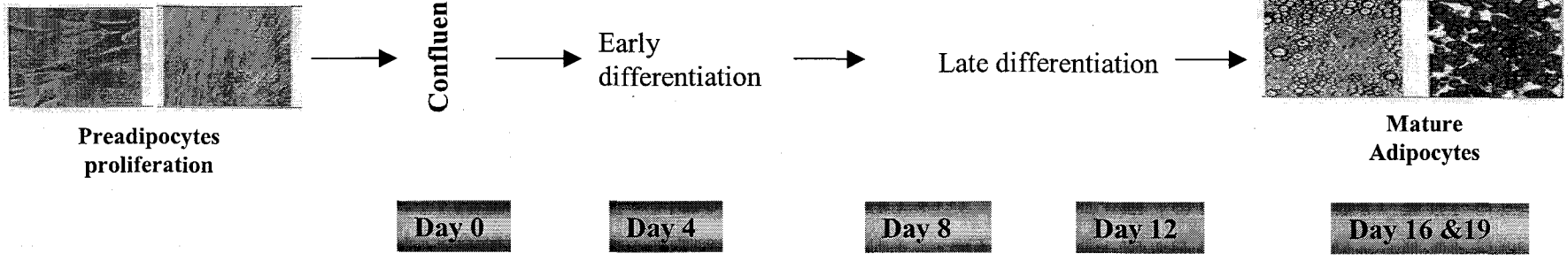
Supplementary Figure III-3: Differential expression of total hGHR, the hGHR V2 variant and the transcription factors CHOP and Ets1 during human SGBS preadipocyte differentiation.

(A) A schematic diagram showing the differentiation process and the time points when RNA samples were collected for Q-PCR analysis. Day 0 stands for the growth-arrested confluent preadipocyte stage before induction of differentiation. Mature adipocyte stages were identified by morphological change (i.e. lipid-filling) and Oil Red O staining.

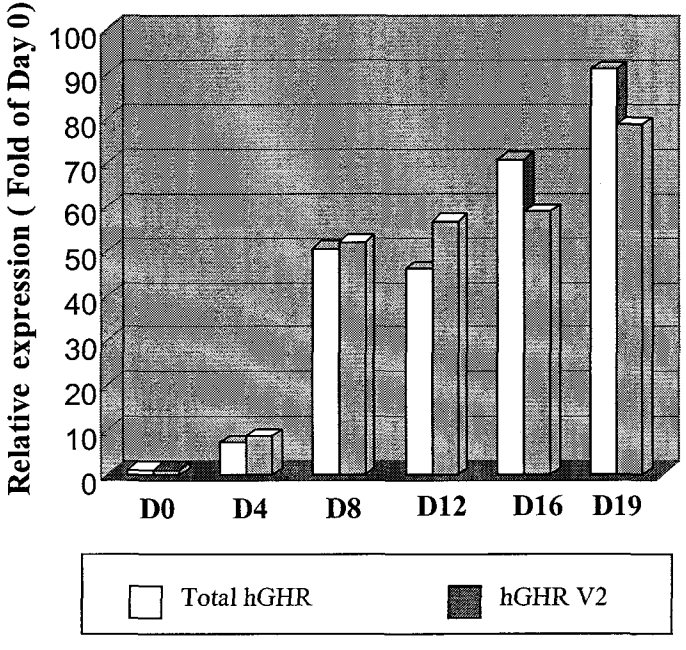
(B) Relative expression of total hGHR and V2 transcripts during SGBS differentiation. Q-PCRs were carried out to estimate the relative expression levels of total hGHR and the V2 variant during the SGBS differentiation process, with 18S rRNA as an internal control for normalization. Data are presented as fold difference relative to the calibrator, which is the same transcript expressed at Day 0 and is arbitrarily set as 1.

(C) Relative expression of CHOP and Ets1 during SGBS differentiation. The relative expression levels of either CHOP or Ets1 at different differentiation time points were estimated using Q-PCR with 18S rRNA as an internal control. Data are expressed as percentage of the calibrator, which is the same transcript at Day 0 and arbitrarily set as 100%.

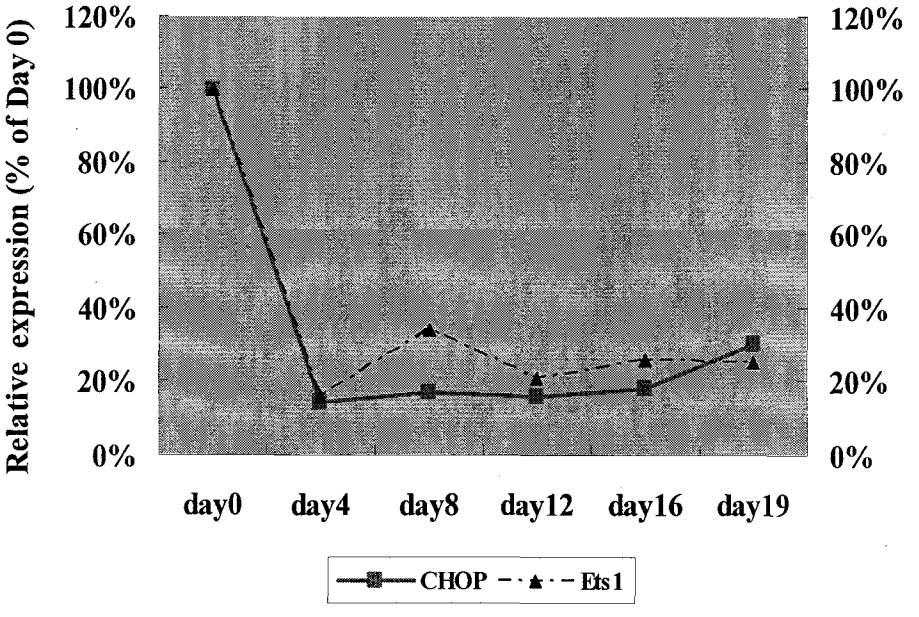
A.



B.



C.



Supplementary Figure III-3

Supplementary Table III-1. Oligonucleotide primers used for V2 promoter reporter constructs and site-directed mutagenesis.

Primer	Sequence	Location	Strand
V2F (-2623)	5'-cgacgcgtcgAATGTGGAATGTGAT-3'	-2623 to -2609	**[s]**
V2F (-211)	5'-cgacgcgtcgTTGGATGGCTAAAC-3'	-211 to -198	**[s]**
V2F (-29)	5'-cgacgcgtTTCCTCTAGGAG-3'	-29 to -18	**[s]**
V2F (+11)	5'-cgacgcgtCGCACCAACTCCAG-3'	+11 to +24	**[s]**
V2R (-25)	5'-AGAGGAAAATATTGGTAGTTGCA-3'	-25 to -46	**[as]**
V2R (+71)	5'-GGGCCAAGCATTCCGAGT-3'	+71 to +54	**[as]**
V2R (+103)	5'-GAGAACTGCTGCGGAGAGGC-3'	+103 to +84	**[as]**
V2R (+125)	5'-CGGACGTTCAAGGAGGCCAGTTCGAGA-3'	+125 to +100	**[as]**
V2R (+162)	5'-GTCCCCCAGATCCAGGTTGCAGAAGCGA-3'	+162 to +135	**[as]**
V2R (+301)	5'-CGCCCTGCTTGGCAGCTC-3'	+301 to +284	**[as]**
V2R (+362)	5'-TGCTGCTGCCGCTGCCGCCG-3'	+362 to +343	**[as]**
V2R (+512)	5'-TCCGAGCTTCGCCTCTGATCAGAG-3'	+512 to +489	**[as]**
Inr_mut [s]	5'-CTAGGAGGAGCCGCGCAG CT**tg**GT**ca**AGAGTGAC-3'		**[s]**
Inr_mut [as]	5'-GTCACTCT**tg**AC**ca**AG CTGCGCGGCTCCTCCTAG-3'		**[as]**
Sp1_mut [s]	5'-CTAGGAGGAG**Caagctt**GCTCAG TTGAGAGTGACAC-3'		**[s]**
Sp1_mut [as]	5'-GTGTCACTCTCAA CTGAG**Caagctt**GCTCCTCCTAG-3'		**[as]**
CAT_mut [s]	5'-GGCGGAGAGGAAGGAAGTGTATT GCAACT**agtact**ATTTTCCTCTAGG-3'		**[s]**
CAT_mut [as]	5'-CCTAGAGGAAAAT**agtact**AGTTGCAA TACACTTCCTTCCTCTCCGCC-3'		**[as]**
Ets1_mut1 [s]	5'-CAGGCGGAGAGGAAG**ag**AG TGTATTGCAACTAC-3'		**[s]**
Ets1_mut1 [as]	5'-GTAGTTGCAATACA CT**ct**CTTCCTCTCCGCCTG-3'		**[as]**
Ets1_mut2 [s]	5'-GGCAGGCGGAGAGGAA**cc**AA GTGTATTGCAACTAC-3'		**[s]**
Ets1_mut2 [as]	5'-GTAGTTGCAATACACTT**gg**TT CCTCTCCGCCTGCC-3'		**[as]**

PU.1_mut [s]	5'-GAACGGGCAGGCGGAG**Acc**AA GGAAGTGTATTGCAAC-3'		**[s]**
PU.1_mut [as]	5'-GTTGCAATACACTTCC TT**gg**TCTCCGCCTGCCCGTTC-3'		**[as]**
CHOP_mut [s]	5'-GGCGGAGAGGAAGGAAGT GT**ggatcc**ACTACCAATATTTTCCTCTAGG-3'		**[s]**
CHOP_mut [as]	5'-CCTAGAGGAAAATATTGGTAGT**ggatcc**AC ACTTCCTTCCTCTCCGCC-3'		**[as]**

1) The underlined lowercase letters represent the restriction site added at the 5'-end of the primer for PCR amplification.
2) Mutated nucleotides within the core sequence of individual transcription factor putative binding sites are shown in bold and lowercase letters.
3) [s]: sense primer; [as]: antisense primer.

Supplementary Table III-2. Oligonucleotide probes for EMSA and oligonucleotide primers for ChIP assays.

Name	Sequence
EMSA probes:	
Ets1 *[s]*	5'-GGCAGGCGGAGAGGAAGGAAGTGTATTGCAACTAC-3'
Ets1 *[as]*	5'-GTAGTTGCAATACACTTCCTTCCTCTCCGCCTGCC-3'
Ets1 mut.2 *[s]*[a]	5'-GGCAGGCGGAGAGGAA**cc**AAGTGTATTGCAACTAC -3'
Ets1 mut.2 *[as]*[a]	5'-GTAGTTGCAATACACTT**gg**TTCCTCTCCGCCTGCC-3'
V2 AD[b] *[s]*	5'-GTCCGCTTCGCCTTCGCTTCTG-3'
V2 AD[b] *[as]*	5'-CAGAAGCGAAGGCGAAGCGGAC-3'
Hes1 site I *[s]*	5'-GGCGGCACTCGGCCTCTCCGC-3'
Hes1 site I *[as]*	5'-GCGGAGAGGCCGAGTGCCGCC-3'
Oligo primers for ChIP assays:	
CHOP primer *[s]*	5'-AGGTCCTGCTTTCATTTGGA-3'
CHOP primer *[as]*	5'-GACGTTCAAGGAGGCCAGT-3'
Hes1 site 1 primer *[s]*	5'-AGGTCCTGCTTTCATTTGGA-3'
Hes1 site 1 primer *[as]*	5'-GACGTTCAAGGAGGCCAGT-3'
Hes1 site 2 primer *[s]*	5'-CTTCTGCAACCTGGATCTGG-3'
Hes1 site 2 primer *[as]*	5'-CCCTAGCCCTCCTCCTCTT-3'
Hes1 CTL primer *[s]*	5'-GGGCACTAACCTAGACATTGC-3'
Hes1 CTL primer *[as]*	5'-TTCTCCCTGTATCAGCACCTTT-3'
Oligo primers for real-time PCRs	
hGHR [s]	5'-ATTCACCAAGTGCCGTTCACCTGA-3'
hGHR [as]	5'-AGGTATCCAGATGGAGGTAAACG-3'
V2 [s]	5'-GCACCATTGGCCCCAGCG-3'
V2 [as]	5'-CCTCACTTCCAGAAAAAGCATCACT-3'
18S [s]	5'-GCCCTGTAATTGGAATGAGTCCACTT-3'
18S [as]	5'-GTCCCCAAGATCCAACTACGAGCTTT-3'
CHOP [s]	5'-CAGAACCAGCAGAGGTCACA-3'
CHOP [as]	5'-AGCTGTGCCACTTTCCTTTC-3'
Ets1 [s]	5'-GATGTCCCACTATTAACTCCAAGCAGC-3'
Ets1 [as]	5'-CGTCTGATAGGACTCTGTGATGAAGC-3'

a: the underlined sequences indicate the core binding sequence of Ets1 binding site. Mutated nucleotides are in bold and lowercase letters.
b: V2AD, V2 activation domain (+125/+162) EMSA probes.
[s]: sense primer. *[as]*: antisense primer.

CHAPTER IV

GENERAL DISCUSSION & SUMMARY

GENERAL DISCUSSION

GH is a key regulator of postnatal growth as well as the metabolism of fat, carbohydrate and protein (Jorgensen *et al.* 2007; Wei *et al.* 2006; Herrington & Carter-Su 2001). At the cellular level, the ability of target cells to respond to GH depends on expression of its specific receptor at their plasma membrane surfaces. Adequate amounts and normal functioning of the GHR is essential for maintaining physiological homeostasis: dysregulation of the GH/GHR axis has been implicated in several forms of short stature, obesity, the pathogenesis of certain tumors and the progression of chronic diabetic complications, such as retinopathy and nephropathy (Thimmarayappa *et al.* 2006).

The expression of GHR is tightly regulated at three levels: transcription, translation and posttranslation (Flores-Morales *et al.* 2006). Our understanding of its posttranslational regulation has advanced quite a lot in the past several decades, while our knowledge of its transcriptional or translational regulation remains limited. Present studies examined the transcriptional control (see below) and I have discussed translational regulation studies in the section of future directions.

Studies of the mechanisms regulating GHR gene transcription in humans and several animal species have revealed a common characteristic: alternative use of different 5'non-coding exons and the generation of multiple mRNA isoforms, all coding for the same protein. Such a feature is not rare. It has been known for more than 20 years that the 5'UTRs of mRNAs transcribed from single genes are often heterogenous (Reynolds *et al.* 1984). Moreover, a recent comprehensive analysis of the mammalian genome indicates that, in fact, most genes contain alternative 5'UTR exons (Carninci *et al.* 2005). Several of these genes exhibit cell-type specific expression patterns. Indeed, this is what has been found for GHR mRNA variants. For example, eight hGHR mRNA variants have been identified prior to the present study, of which four (V2, V3, V5 and V9) are widely expressed while the others (V1, V4, V7 and V8) are liver-specific and developmentally regulated.

Because liver, one of the major target organs for hGH, has four tissue-specific variants, we hypothesized that adipose tissue, another important hGH target organ may also produce adipocyte-specific transcripts, which would mediate tissue-specific regulation of hGHR expression.

1. hGHR mRNA Variants and Expression Profile in Human Adipocytes

A human adipocyte cDNA library created from a pool of RNA from both lean and obese subjects was screened using 5'RACE and five new hGHR mRNA variants (VA-VE) were identified. They account for 8% of the 100 hGHR clones examined. Except for VA, which has also been observed by Wickelgren et al. in adipose tissue (Orlovskii *et al.* 2004), the other 4 variants have never been reported. All five new 5'UTRs mapped within the 5'flanking region of the hGHR gene. However, after screening multiple human fetal and adult tissues, it turned out that none of these new variants are adipocyte-specific. Instead, they are widely expressed, minor hGHR transcripts. What are the possible functions of these widely expressed minor transcripts?

Jiang and Lucy demonstrated that, in cattle, some ubiquitously expressed minor transcripts of bovine GHR (e.g. b1H, b1I) were much more efficiently translated than the predominant variant b1B (Jiang & Lucy 2001b). Many studies have underscored the important roles of 5'UTRs in influencing translation and thus modulating protein expression levels: at least 10% of human 5'UTRs contain upstream open reading frames (uORFs) while an even higher percentage contain secondary structures that can influence translation (Pesole *et al.* 2001). Different promoters also provide opportunities for differential regulation. For example, it has been shown that activation of an alternate promoter of the oncogene *mdm2* gene enables expression of a short 5'UTR form that lacks the inhibitory uORFs present in the longer, constitutively-expressed 5'UTR form. This resulted in enhanced translation of the mdm2 mRNA, leading to overexpression of the MDM2 oncoprotein in a significant number of human soft tissue tumors (Brown *et al.* 1999). Cancer associated changes in expression of 5'UTR isoforms of other genes have also been noted although their contributions to disease progression remain unknown (Hughes 2006). Therefore, although the newly isolated VA-VE hGHR minor transcripts

do not appear to contribute significantly to hGHR expression under normal physiological conditions, they could be more efficiently translated or their expression could increase markedly under certain pathophysiological situations and in specific tissues, which could cause a major change of hGHR expression and affect cellular responses to hGH.

The eight previously identified hGHR 5'UTR variants result from using individual promoters. The TSSs for ubiquitously expressed V2 (Chapter III), V3 and V9 (Goodyer *et al.* 2001b) as well as liver-specific V1 (Goodyer *et al.* 2001b) have been defined. Among the new variants isolated in this study, VB and VE are alternative splicing variants of V3, while VA turns out to be an alternatively spliced product of VC. They appear to transcribe from a novel promoter located 5'of the VC exon. Thus, at least two mechanisms are involved in regulating the expression of multiple 5'-untranslated regions from a single hGHR gene. They are: i) promoters that induce transcription initiation from individual first exons (**Figure IV-1A**), and ii) alternative splicing that includes or excludes non-coding exons (**Figure IV-1B**). It has been estimated that 10-18% of genes express alternative 5'UTRs by using multiple promoters, and that transcripts from ~12% of genes are alternatively spliced within their 5'UTRs (Hughes 2006).

Figure IV-1: Mechanisms that are involved in regulating the expression of multiple 5'UTR regions from a single hGHR gene.

A) Separate promoters induce transcription from alternative first exons (Hughes 2006; Goodyer *et al.* 2001b); B) Alternative splicing includes or excludes non-coding exons (Wei *et al.* 2006; Hughes 2006). The horizontal line represents genomic DNA, with exons shown as boxes. The transcription start sites are illustrated by arrows and ATG indicates the translation start codon

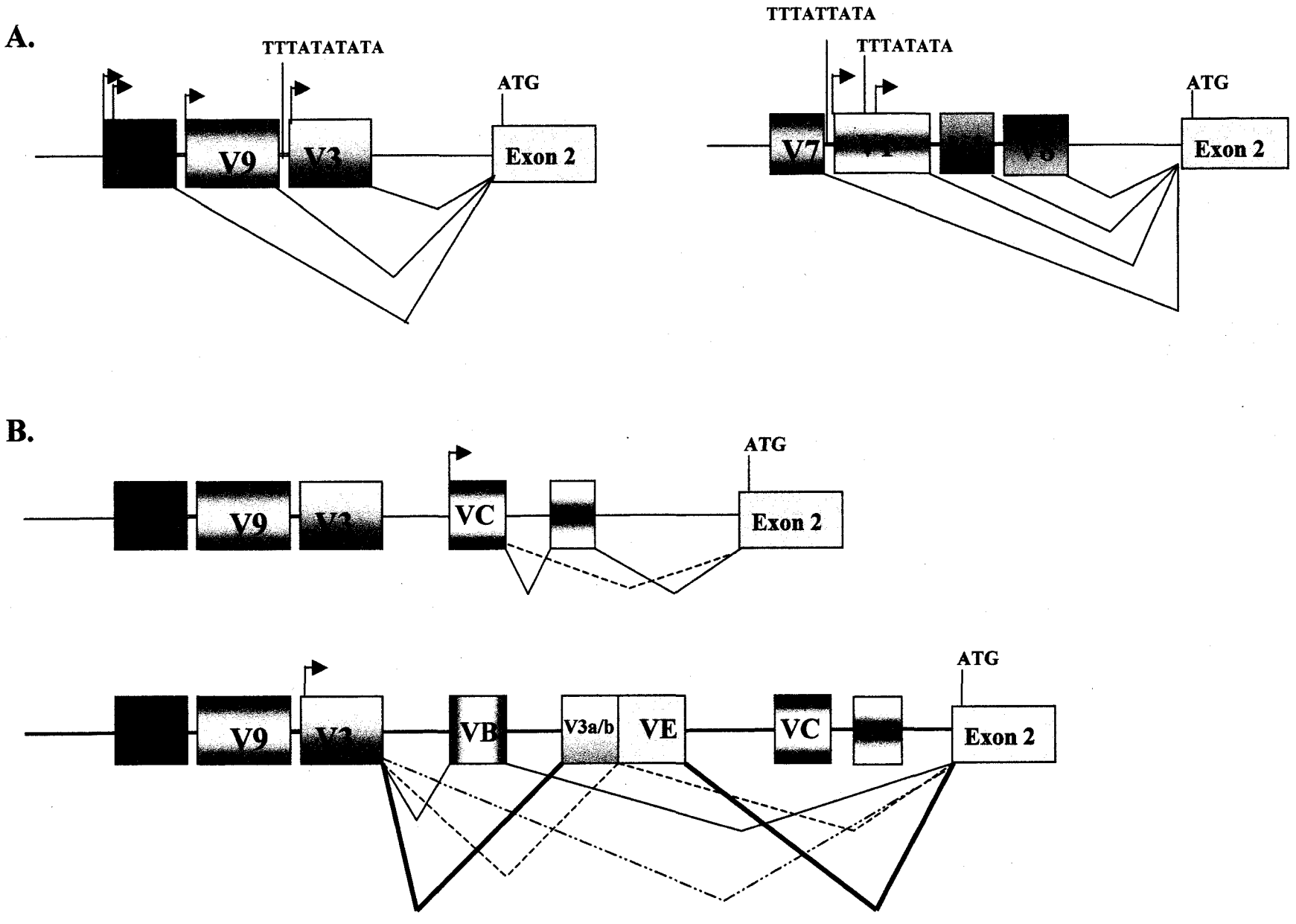

A.
TTTATATATA
ATG
V9
V3
Exon 2
TTTATTATA
TTTATATA
ATG
V7
Exon 2
B.
ATG
V9
VC
Exon 2
ATG
V9
VB
V3a/b
VE
VC
Exon 2

Figure IV -1

The most striking finding from this 5'RACE screening is that the V2 variant is such a predominant hGHR transcript in fat cells (Wei *et al.* 2006). Although we expected V2 to be the major variant, because V2 and V2 homologues in other species (o1B, b1B, mL2, rat GHR2) correspond to the majority of hGHR mRNAs in all non-hepatic tissues examined to date, we were surprised to see that V2-containing clones accounted for almost 90% of the 100 hGHR-related 5'UTR clones, while V3 represented only ~3% clones and no V9 clones were detected. This is not due to experimental artifact, because our semiquantitative RT-PCR comparison of the expression pattern of total hGHR and several of its mRNA variants in adult human liver and fat confirmed this difference (Wei *et al.* 2006). Consistent with what we found, it has been reported that the V2 homologue in mice (L2) was present in 88% of the 5'RACE clones from adipose tissue of nonpregnant mice (Moffat *et al.* 2000). Although liver, muscle, fat and heart are all important target organs regulated by hGH and have high levels of hGHR, the expression pattern of hGHR 5'UTRs appears to be quite different. In liver, V1 (47%), V2 (27%) and V3 (11%) were the most highly expressed (Pekhletsky *et al.* 1992); in cardiac muscle, V2 (37.5%), V9 (25%) and V3 (16.7%) were all well represented. Therefore, such a predominance of the V2 transcript appears to be a specific feature for GHR expression in adipose tissue.

This tissue-specific expression of alternative 5'UTRs provides the potential for complex regulation of the hGHR gene expression, to meet the different biological functions of hGH in different target tissues: e.g. in liver, GH is mainly responsible for carbohydrate metabolism, in muscle protein synthesis, and in adipose tissue lipid metabolism.

2. SGBS Preadipocyte Cell Line: A Model for Studying Human Adipocytes *In Vitro*

The new variants were not adipose tissue specific. However, adipose tissue is a complex mix of different cell types, including mature adipocytes, preadipocytes and stromal-vascular cells as well as pericytes (Ailhaud *et al.* 1992). Hence, to examine a gene expression pattern in a specific cell type, it is better to use cells derived from a single population.

Most of the work related to adipocytes use two types of cell models: the established murine clonal cell lines (e.g. 3T3-L1, 3T3-F442A) or primary cultures from human or rat adipose tissues (Nam & Lobie 2000). Our knowledge of adipocyte differentiation and functions has advanced rapidly and substantially by using these models. However, there are also several shortcomings, including their short life span, the tediousness of preparing primary culture and the variability from one fat sample to another as well as that mouse cell lines may not be suitable for studying certain human genes, of which hGHR is a good example. For example, hGHR is encoded by nine exons (exon 2-10), while mGHR is encoded by eleven exons, with two extra exons 4B and 8A (Edens & Talamantes 1998). Both GH and PRL can bind to mGHR, but only hGH can bind to hGHR.

The SGBS preadipocyte cell line is a non-immortalized and non-transformed cell line derived from the stromal cell fraction of subcutaneous adipose tissue from an infant with Simpson-Golabi-Behmel Syndrome (SGBS), characterized by overgrowth in both weight and height. These cells can differentiate into mature adipocytes *in vitro* when induced under serum-free culture conditions with supplementation of insulin, T3, cortisol and a PPARγ agonist. During the differentiation process, SGBS cells develop a gene expression profile similar to that found in differentiating human preadipocytes, thus making it a good model for studying human adipocyte development, metabolism, and gene regulation *in vitro* (Fischer-Posovszky *et al.* 2007b; Wabitsch *et al.* 2001).

Several studies have been carried out using this cell system. Dalen *et al.* demonstrated that the expression of insulin-responsive glucose transporter GLUT4 was correlated to the induction of liver X receptors (LXRs) alpha during human SGBS adipocyte differentiation (Dalen *et al.* 2003). Bao *et al.* examined the regulation of the expression of Zinc-alpha2-glycoprotein (ZAG), a lipid mobilizing factor using SGBS cells, and showed that ZAG mRNA was detected in SGBS cells post-, but not pre-, differentiation to adipocytes. Relative ZAG mRNA levels increased rapidly after differentiation of SGBS cells, peaking at day 8 post-induction (Bao *et al.* 2005). Newell *et al.* characterized the transcriptional and functional effects of fibroblast growth factor-1 (FGF-1) on human preadipocyte differentiation and observed that FGF-1 treatment of SGBS preadipocytes

further enhanced differentiation (Newell *et al.* 2006). Furthermore, Fisher-Posovszky *et al.* in their recent work demonstrated that treatment of human SGBS preadipocytes and adipocytes with physiological relevant concentrations of conjugated linoleic acids (CLAs) resulted in an impairment of proliferation and differentiation and induction of apoptosis (Fischer-Posovszky *et al.* 2007a). In this PhD project, we chose to use this cell model for initial studies of hGHR gene expression and regulation in human adipocytes.

3. Expression Profile of hGHR and its Variants during SGBS Human Adipocyte Differentiation

Using the human SGBS cell line and duplex RT-PCR assays, I demonstrated that total hGHR mRNA levels increase significantly at early differentiation stages and reach maximum with maturity. This differentiation-induced increase in hGHR gene expression was confirmed by real-time quantitative RT-PCR, although the fold difference is much higher than seen with the duplex RT-PCR assays, which I assume is due to the sensitivity of the two experimental methods. Zou *et al.* also observed ~28 fold upregulation of mouse GHR mRNAs during murine 3T3-L1 preadipocyte differentiation (Zou *et al.* 1997)

The differentiation-dependent increase in total hGHR expression that I observed resulted mainly from an increase in the V2 mRNA variant, which occurs in parallel with total hGHR during differentiation and predominates at each developmental stage. Studies of V2 promoter regulation suggest that the increase in V2 expression is primarily taking place at the transcriptional level, as the promoter activity of V2 is ~3-4 fold higher in adipocytes than in preadipocytes, which we think is due, at least in part, to the actions of adipocyte-specific factors on the V2 promoter.

In addition to the differentiation process, differential expression of GHR has also been observed depending on the location of the adipose tissue depots. In the rat, for example, the highest level of GHR expression is found in the epididymal fat pad, followed by subcutaneous fat, and the lowest in retroperitoneal fat (Flint *et al.* 2003). In humans, although no similar data are available yet, it has been recognized from clinical studies

that hGH treatment has a more obvious lipolytic effect on abdominal fat than on subcutaneous fat (Nam & Lobie 2000). Moreover, under absolute or relative hyposomatotropic conditions, such as hypophysectomy in rats or Prader-Willi's Syndrome in humans, decreased GHR gene expression in adipose tissue has been demonstrated, and GH treatment results in an increase in GHR gene expression (Nam & Lobie 2000). These different levels of GHR expression may explain the differential cellular sensitivity to GH, which eventually results in different biological consequences.

Collectively, the predominant expression of the hGHR V2 variant in adipocytes suggests its importance in determining hGHR expression levels and hGH effects on adipose tissue. In addition, the hGHR V2 transcript is known to constitute the majority of the hGHR mRNA pool in almost all non-hepatic tissues and is the second most expressed variant in adult liver. In view of these data, I carried out in-depth studies of V2 promoter regulation.

4. Determination of V2 Basal Promoter Activity

4.1. The Promoter of the hGHR V2 Exon Functions Like a Housekeeping Gene Promoter

Using RNA primer extension, I identified two initiation sites for V2 transcription. Multiple TSSs appear to be a common feature for V2-like transcripts in other species: two major start sites were mapped for bovine 1B (Jiang *et al.* 2000) and multiple start sites were identified for ovine 1B (Adams 1995) and mouse L2 (Yu *et al.* 1999; Moffat *et al.* 1999b). Although the mouse L2 start site is located approximately three hundred nucleotides further downstream, the initiation sites we mapped for human V2 are located within only a few nucleotides of the transcription start sites of ovine 1B and bovine 1B.

The 5'-flanking region upstream of the most upstream (ovine) TSS was evaluated for promoter activity by fusion into a luciferase reporter gene for testing in transient transfection assays. Significant luciferase activity was observed in several cell lines derived from different tissues at different developmental stages, including human fetal kidney (HEK293) cells, human hepatoma (Huh-7) cells, human (SGBS) preadipocytes and differentiated adipocytes, human cervical cancer (HeLa) cells (data not shown), and human lung adenocarcinoma (A549) cells (data not shown), as well as African green

monkey kidney (COS1) cells. These results support the previous *in vivo* studies showing that V2 mRNA exhibits a ubiquitous expression pattern, and indicates that the V2 promoter is constitutively active in different cellular situations. Similar data were obtained for V2 homologues in other species (Jiang *et al.* 2000; Adams 1999; Yu *et al.* 1999; Adams 1995).

From our 5'deletion series analysis, the V2 proximal promoter was mapped to 211 bp upstream of the major TSS, consistent with the findings of Orlovskii et al (Orlovskii *et al.* 2004). Both ovine 1B and bovine 1B were found to have proximal promoters 134 bp and 191 bp upstream of their individual major initiation site, respectively.

Sequence analysis of the V2 proximal promoter region revealed several interesting features, including the lack of a canonical TATA box or TATA-like elements, a relatively high GC content (~60% within the -100 to +100 promoter region), and the presence of several important putative binding motifs, including a CCAAT box, an Initiator-like element possessing an E box feature (TCAGTTG), an atypical Sp1 site and two Ets binding sites. These features are common to many housekeeping gene promoters (Yang *et al.* 2007; Rouleau *et al.* 1992; Melton 1987; Dynan *et al.* 1986). Not surprisingly, many of these structural features are also found in the proximal promoter regions of the ovine 1B and bovine 1B as well as the mouse L2 exons (Jiang *et al.* 2000; Yu *et al.* 1999; Moffat *et al.* 1999b; Adams 1995). The high degree of similarities in promoter structures and promoter sequences among human V2 and its homologues in other species suggests the importance of this region in maintenance of V2 basal transcriptional activity and implies that regulatory mechanisms may be conserved.

Our site-directed mutation analysis of several potential binding sites (Ets, CHOP, CCAAT box, Sp1) within the V2 proximal promoter region showed that loss of each individual site did not cause a dramatic change in V2 basal promoter activity, suggesting that V2 basal transcription may not be controlled by a single, specific transcription factor, but is more like to be the result of regulation by multiple factors. Although both o1B and b1B promoter studies were limited to the effects of Sp1 on their promoter regulation, the

authors from both studies did observe that deletion of the Sp1 site only resulted in partial loss of the promoter activities and, thus, suggested that multiple regulatory elements are required for full basal transcription (Jiang *et al.* 2000; Adams 1999). Studies of the transcriptional regulation of the mouse L2 exon were also primarily focused on the role of the Sp family of TFs (Yu *et al.* 1999). In addition, Menon's group identified that the transcription factors NF-Y, BTEB1 and HMG-Y/I, in conjunction with the corepressor mSin3b, could form a repressosome complex, bind to a *cis*-element in the promoter of the murine GHR L2 exon which is homologous to the TSS region for human V2, ovine 1B and bovine 1B, and suppress L2 transcription (Gowri *et al.* 2003).

Taken together, our data showing the TATA-less V2 promoter structure, the use of multiple transcription initiation sites, the constitutively active promoter activity in different tissues or cell types, as well as the high conservation of similar transcriptional regulatory elements amongst different mammalian species support our proposal that the V2 promoter functions like a housekeeping gene promoter, to ensure that there is sufficient basal production of hGHR throughout all the tissues in the body.

4.2. The V2 Core Promoter Functions like a Null-Core Promoter

Accurate transcription initiation of eukaryotic genes requires a DNA region, referred to as the core promoter, which is a DNA element generally extending from ~40-bp upstream to ~40-bp downstream of the TSS (+1). It contains several types of core promoter elements such as the TATA box, the Initiator (Inr) or the downstream promoter element (DPE), which are functional DNA motifs directing recruitment and interaction with basal transcriptional machinery to allow accurate initiation of transcription by RNA polymerase II. Thus, the core promoter determines the intrinsic "basal' (constitutive) transcriptional activity (Yang *et al.* 2007; Butler & Kadonaga 2002).

By serial 5'- and 3'-deletion analysis, we defined the region between position -29 and +11 (relative to the major TSS) as the core promoter for hGHR V2. This result is similar to Adam's finding for ovine 1B, that removal of the promoter fragment -39 to -5 caused a complete loss of the promoter activity (Adams 1995).

The V2 core promoter contains no TATA-like sequence and, thus, is a TATA-less promoter. Two types of promoters lacking a TATA element have been described (Yang *et al.* 2007; Werner 1999). One is a so called null-promoter, which frequently leads to multiple transcription start sites, since it has neither a TATA box nor an Inr region. The second type contains an Inr, which functions as an analog of the TATA box. Within the V2 core promoter region, there is an Inr-like element positioned at the major TSS (+1). However, functional analysis demonstrated that mutation of this Inr-like element resulted in either minimal or no change in basal promoter activity. This finding may be due to the presence of other features, such as an A+T rich sequence within the -30-bp region, which can have an affinity for TBP, and the presence of an Sp1 site immediately upstream of the Inr-like element (Smale 1997). It also suggests that the initiation of V2 transcription is not likely to be dictated by this Inr-element. Thus, the V2 core promoter appears to function like a null-promoter. Indeed, it has been documented that the "Inr only" category is predominantly enriched for genes involved in basic biological processes such as protein synthesis and mRNA processing, while the null promoter category includes primarily genes involved in other basic processes such as cell growth and maintenance, and protein metabolism (Yang *et al.* 2007), major biological consequences initiated after GH/GHR interactions.

5. Transcriptional Regulation of hGHR V2 Promoter Activity

Housekeeping gene promoters are not unregulated. For example, the promoter of the housekeeping gene, HMGCoA reductase, responds to cholesterol levels, and the transcription of mouse dihyrofolate reductase (DHFR), a gene with a typical housekeeping promoter, increases during periods of DNA synthesis (Dynan *et al.* 1986). In the present work, I have identified that the V2 basal promoter is under the regulation of two functionally discrete domains, a 5'-inhibitory domain upstream of the proximal promoter, and a 3'-activation domain located within the V2 exon at positions +125 to + 162, which is required for maximizing V2 basal promoter activity. Moreover, I have partially characterized several transactivating factors, and shown that V2 transcription is subject to either positive or negative regulation by these transcription factors. These data

provide mechanisms for the regulation of hGHR V2 expression in response to different extra- and intra-cellular signals.

5.1. Role of Sp1 in V2 Transcription Regulation

The proximal promoter of V2-like transcripts (V2, ovine 1B, bovine 1B and mouse L2) all lack a TATA box, but have a relatively high G+C content. This type of promoter is usually considered to be a target for regulation by the Sp family of factors (Yu *et al.* 1999). Indeed, Adams demonstrated an important role for the Sp transcription factor family in regulation of the ovine 1B promoter (Adams 1999). Four Sp1/Sp3 cis-acting elements were identified within the proximal promoter between positions -99/+87. Site I (TTTAG-GTCCTGCCTCCC) was mapped between positions -99 to -85. Sp1/Sp3 bound to this site, and its deletion caused ~30% reduced promoter activity. Mutations within the last 6 nucleotides significantly affected the binding of Sp factors. Interestingly, the sequence of the same region in the human V2 promoter is TGTAGGTCCTGCTTTCA, with the last six nucleotides changed from GC-rich to AT-rich. We have excluded this region as a functional Sp1 *cis*-element for V2. The ovine site II Sp1/3 binding site (GGGCGG), found at -68/-63 is a consensus Sp1 site and showed the highest binding affinity; it is also conserved in the bovine 1B promoter. Deletion of this site reduced the ovine 1B and bovine 1B luciferase activity to ~60% of the wildtype (Jiang *et al.* 2000; Adams 1999). In the human V2 promoter, this sequence is altered to AGGCGG, again not a likely Sp site. This is supported by the fact that deletion of the V2 5'flanking region including this site did not produce major changes in V2 basal promoter activity. The ovine site III element (GAAGGAAGTGCCCTGCAA) showed the least binding affinity for Sp1/3 (Adams 1999). This element sequence in the human V2 promoter has altered significantly (GAAGGAA-GTGTATTGCAA), again making it unlikely to be a Sp1 element. The ovine site IV binding element, located at position -27 to -5, has an intermediate binding affinity for Sp1/3. A single site mutation caused a 40% drop in ovine 1B promoter activity (Adams 1999). This element sequence overlaps with our atypical Sp1 site identified within the V2 core promoter region. In our hands, mutation of this Sp1 site resulted in a moderate (~30%) but significant decrease of V2 promoter activity, similar to the effect observed in the ovine 1B promoter. The Sp1 binding site

within the V2 exon has a consensus sequence that is conserved across four species. But our 3'-deletional analysis, together with dominant negative Sp1 cotransfection assays, demonstrated that this site is not crucial for V2 basal promoter activity. Although there are no data from ovine 1B and bovine 1B promoter studies for comparison, the mouse L2 study indicates that this site preferentially binds Sp3 and, to a lesser extent, Sp1 (Yu *et al.* 1999). Taken together, these data suggest that Sp1 may be involved in V2 promoter regulation, but that it does not have as determinative a role as in other species.

5.2. Transactivation of the V2 Promoter by Ets1

E26 transformation-specific (Ets) transcription factors are trans-acting phosphoproteins that have become increasingly recognized as key regulators of differentiation, hormone responses and tumorigenesis in endocrine organs and other target tissues (Gutierrez-Hartmann *et al.* 2007). The Ets family is defined by the Ets domain, which is a highly conserved DNA-binding domain (DBD) consisting of 85 amino acids that fold into a winged helix-turn-helix structure to bind a 5'-GGAA/T-3'DNA core motif (Sharrocks 2001).

I demonstrated in the present studies that Ets1 can transcriptionally activate the V2 promoter in both HEK293 cells and SGBS preadipocytes when overexpressed. This stimulatory effect results from direct binding of Ets1 to the c-Ets1 binding site present in the proximal promoter region of the hGHR V2 exon, thus identifying hGHR V2 as a direct target for Ets1.

The mechanism whereby Ets1 upregulates V2 transcription could be related to its own transactivational ability or the functional interactions between Ets1 and other factors, mediated via its specific structural domains. In addition to the conserved Ets DNA binding domain, Ets1 contains an N-terminal transactivation domain, a Pointed domain serving as a protein-protein interaction motif and two autoinhibitory domains flanking the DNA binding region. A number of studies indicate that Ets1 transactivates target gene promoters via at least two distinct mechanisms: 1) by interactions with chromatin-remodelling cofactors, such as coactivators CBP/P300 or the SRC family (SRC, TIF2, and RAC3), to form a stable nuclear complex; and 2) by cooperative interactions with

other TFs binding at adjacent sites. Such functional interactions can enhance Ets1 DNA binding affinity by relieving its own auto-inhibition (Sun & Loh 2001). In addition, Ets1 binding to DNA can induce a distinct DNA conformational change and form a DNase I hypersensitive area. This change in chromatin structure allows its protein partners to make better contacts with their associated cofactors and/or basal transcriptional machinery and, thus, initiate transcription more efficiently (Lu *et al.* 2004; Sun & Loh 2001).

We investigated the interaction of Ets1 and CHOP, a C/EBP family member, in the present study and showed that both factors act independently on the V2 promoter, with additive but not synergistic effects on V2 regulation. This result can be explained by our later finding that CHOP does not function through the putative site which is next to the Ets binding sites. However, another C/EBP protein, C/EBPβ, appears to be able to use this CHOP site. Therefore, it would be of great interest in the future to examine the interaction between Ets1 and C/EBPβ on the V2 promoter.

Ets family members are classical activators that stimulate transcription from both enhancer and proximal promoter regions (Wasylyk *et al.* 1993). They have been proposed to have a primary role in the formation of the initiation complex on a minimal core promoter, either by assisting its assembly or as components of the complex itself (Petit *et al.* 2004; Lambert *et al.* 1997; Wasylyk *et al.* 1993). The *ets* motifs in a number of core promoters have been shown to be important for transcription. I did not observe a significant change in V2 basal promoter activity when the Ets sites were mutated, most likely because of a low level of endogenous Ets1 in the cell lines used (HEK293 and SGBS preadipocytes). Consistent with this, we and others have observed that the Ets1 expression level in HEK293 cells is low (Cederberg *et al.* 1999). Although Ets1 is ubiquitously expressed (Gutierrez-Hartmann *et al.* 2007), high levels of Ets1 expression are mainly seen in lymphoid organ and vascular endothelium (Oikawa 2004). At a low level, the binding of Ets1 to its Ets site appears to be weak due to its specific autoinhibition domain, while a high concentration of Ets1 recombinant protein is required to achieve efficient protection in DNaseI footprinting (Sun & Loh 2001). This would

explain why we see significant stimulatory effect on V2 promoter constructs only when we overexpress Ets1 in HEK293 cells. Given this, it would be of great interest to investigate the effects of Ets site mutations on V2 basal transcriptional activity in hematopoietic cell lines, where Ets factors, like Ets1 and PU.1, are more abundant. These studies would help us to gain insight into V2 transcriptional regulation in the immune system, where hGH is known to modulate immune function.

The Ets family in mammals consists of ~30 proteins homologous to Ets-1 (Oikawa 2004). All members bind to the GGAA/T core motif because of the evolutionally conserved Ets DNA binding domain. But their ultimate effects can vary depending on the adjacent DNA sequence, their differential transactivational abilities, their interactions with transcriptional partners, their pattern of expression, and their modifications by signalling pathways (Oikawa 2004; Galang *et al.* 2004). Therefore, it would be desirable to examine the regulatory effects of other Ets factors on V2 transcription in future studies.

5.3. C/EBP proteins (CHOP and C/EBPβ) Transcriptionally Activate the V2 Promoter via Different Pathways

5.3.1 Effect of CHOP on the V2 Promoter

The CCAAT enhancer binding proteins (C/EBPs) are a family of transcription factors characterized by a conserved C-terminal bZIP domain that consists of a basic-region involved in DNA recognition and an adjacent leucine zipper motif mediating subunit dimerization (Ramji & Foka 2002). They include C/EBPα, β, δ, γ, ε, and ζ , also known as C/EBP homologous protein (CHOP) (Ramji & Foka 2002; Lekstrom-Himes & Xanthopoulos 1998). Because of the high conservation in the bZIP domain, C/EBP members have been documented to be able to form either homodimers or heterodimers and to bind similar DNA sequence to activate transcription, with the exception of CHOP (Ramji & Foka 2002; Ron & Habener 1992). Although CHOP forms stable heterodimers with other C/EBP proteins, the presence of the proline and glycine substitutions in its basic region disrupts its helical structure and DNA-binding activity, preventing dimer binding to the classical C/EBP consensus sequence, 5'-(A/G) TTGCG(C/T)AA(C/T)-3'(Oyadomari & Mori 2004; Gery *et al.* 2004; Fawcett *et al.* 1996; Ron & Habener

1992). Thus, CHOP inhibits the DNA binding and transcriptional activation of C/EBPs on their target genes and acts as a dominant negative inhibitor of C/EBP proteins (Ron & Habener 1992). However, CHOP-C/EBPα or β heterodimers have also been reported to recognize a unique DNA sequence 5'-(A/G)(A/G)(A/G)TGCAAT(A/C)CCC-3' that differs from the canonical C/EBP site, and to activate expression of a subset of distinct target genes, such as Downstream of CHOP (DOC) genes (Sok *et al.* 1999; Wang *et al.* 1998; Ubeda *et al.* 1996).

The unique, putative CHOP binding site found in the hGHR V2 promoter has led us to investigate the functional role of CHOP on V2 expression. I found that overexpression of CHOP resulted in a physical association of CHOP with the V2 promoter (ChIP) and stimulation of V2 transcription (transfection). However, CHOP appears to elicit stimulatory effects by tethering to the 3'-downstream region within the V2 exon instead of functioning directly through the proximal promoter CHOP site. A similar mechanism for CHOP has been reported by Ubeda et al. who demonstrated that CHOP tethers to the DNA bound AP-1 complex by directly interacting with complex members Jun and Fos and, in doing so, activates promoters of selected genes such as somatostatin, JunD and collagenase (Ubeda *et al.* 1999). Based on their findings, they proposed a model in which CHOP could act through this tethering mechanism to mediate ER stress induced gene expression.

To facilitate finding the possible interaction partners for CHOP, we first narrowed down the potential region to which CHOP might tether. From 3'deletional analyses, two regions were identified as being important for CHOP effects. The region from +362 to +162 appeared to be required for maximal response to CHOP stimulation. Region +162 to +103 was also crucial for CHOP tethering, since deletion of this region resulted in loss of the CHOP effect. Several transcription factors, including SREBP, Sp1 and Egr-1, within those two regions could be potential candidates. Future studies will focus on defining the complex with which CHOP interacts.

We predict that the stimulatory effect of CHOP on the V2 promoter is mainly due to its N-terminal transactivation domain because of previous data showing that 1) fusion of the

amino-terminal sequence of CHOP to a GAL4 DNA-binding domain can strongly activate the reporter construct containing the GAL4-binding sites (Zhu & Lobie 2000; Ubeda *et al.* 1996); and 2) that an interaction of TRB3 (an ER stress inducible protein) with the CHOP N-terminal activation domain blocks CHOP transactivational activity (Ohoka *et al.* 2005). Thus, V2 activation is likely to result from protein-protein interactions between the N-terminus of CHOP and other transcription activators bound to the V2 promoter or V2 exon.

CHOP is expressed ubiquitously in many cell types at low levels but is markedly induced by a variety of stressors including ER stress, DNA-damaging reagents, oxidative conditions and nutrient depletion (Oyadomari & Mori 2004; Gery *et al.* 2004; Ubeda & Habener 2003). The expression of CHOP is mainly regulated at the transcriptional level, which is mediated by multiple defined DNA elements on the promoter of the CHOP gene, including an ER stress-responsive element (ERSE), an AP-1 site and an amino acid response element (AARE) (Oyadomari & Mori 2004; van der Sanden *et al.* 2004).

Two types of nutrient deprivations are linked to CHOP induction. One is glucose deprivation, which is believed to induce CHOP expression through the ER stress pathway (Oyadomari & Mori 2004; Carlson *et al.* 1993). The other is amino acid limitation, which happens due to the deficiency of one or more of the essential amino acids or an insufficient intake of protein. This is not triggered by the ER stress signaling pathway but by the binding of bZIP proteins, ATF4 and ATF2, to the AARE, a C/EBP-ATF composite binding site located on the proximal promoter of the CHOP gene. The transactivation requires both ATF4 expression and ATF2 phosphorylation (Averous *et al.* 2004; Bruhat *et al.* 2000; Bruhat *et al.* 1999; Jousse *et al.* 1999; Bruhat *et al.* 1997). Because hGH is such an important metabolic hormone involved in glucose and protein metabolism, it makes sense that hGHR gene expression would be regulated in response to changes in nutrient status in order to achieve efficient effects of hGH in different target organs. Wickelgren once reported that hGHR V2-related mRNA was markedly increased in adipose tissue but decreased in skeletal muscle after surgical trauma that induces catabolism (Wickelgren 2000) . These different changes in hGHR mRNA levels resulted

in different hGH sensitivities in the two tissues, which led to different consequences (deleterious effects in adipose tissue while beneficial effects in skeletal muscle) of a high dose hGH treatment after surgery. Combined with the present data showing that CHOP is involved in regulation of hGHR V2 transcription, it is very likely that CHOP acts as a mediator link between regulation of hGHR gene expression and nutrient status.

Flores-Morales *et al.* showed that cellular stress prolongs the duration of the JAK2/STAT5 signaling pathway activated by GH (Flores-Morales *et al.* 2001). They suggested that this effect was related to inhibition of the GH-induced transcriptional activation of SOCS genes, since giving transcription inhibitors led to a prolongation of GH-induced STAT5 DNA binding activity (Fernandez *et al.* 1998). However, they could not correlate the stress-induced effects to GH-dependent expression of SOCS mRNA and protein, thus suggesting that ER stress modulates the duration of JAK2/STAT5 activation through mechanisms other than inhibition of SOCS expression (Flores-Morales *et al.* 2001). Our finding that CHOP can upregulate hGHR V2 transcription may provide a possible explanation: ER stress induces CHOP expression, which in turn will activate hGHR gene expression, leading to increased hGHR levels expressed on the cell surface that would amplify the GH signaling.

5.3.2. Effect of C/EBPβ on the V2 Promoter

To our surprise, we observed that C/EBPβ, a conserved member of the C/EBP family, could elicit stimulatory effects on V2 transcription by using the putative CHOP site together with other supplementary sites that need to be defined. In light of the fact that high levels of C/EBPβ were used in the cotransfection assays, we postulate that this transcriptional activation is mainly due to C/EBPβ homodimers.

Although no canonical C/EBP binding sequence (TTNNGCAAT) is present in the V2 proximal promoter region, it has been demonstrated that C/EBPα and -β homodimers are capable of binding to the CHOP-C/EBP heterodimer site (also known as CHOP site, (A/G)(A/G)(A/G)TGCAAT) by both EMSA (Ubeda *et al.* 1996) and DNase I footprint

analysis (Sok *et al.* 1999), since the CHOP site may be thought of as containing a high-affinity C/EBP half-site in a context that is permissive for binding.

C/EBPβ activates transcription, at least in part, by recruiting nuclear coactivators p300 or CBP to the target gene promoters (Cui *et al.* 2005; Kovacs *et al.* 2003; Mink *et al.* 1997). Association of C/EBPβ with p300/CBP, which contains intrinsic acetyltransferase activity, results in acetylation of multiple lysine residues within C/EBPβ and contributes to C/EBPβ transactivational ability (Cesena *et al.* 2007). In addition, interaction of C/EBPβ with p300/CBP promotes phosphorylation of the coactivators, which enhances their ability to coactivate C/EBPβ-dependent transcription (Cui *et al.* 2005; Kovacs *et al.* 2003; Schwartz *et al.* 2002). More in depth studies are required to determine the exact mechanism by which C/EBPβ activates the V2 promoter.

5.4. Hes1 Exerts Repressive Effects on V2 Transcription

In addition to being subject to positive regulation by either the helix-turn-helix Ets transcription factors or the leucine-zipper C/EBP proteins, I have demonstrated that the V2 promoter is also subject to negative regulation by a well-known transcription repressor, Hes1. This TF exerts its inhibitory effects through association with the two class C-like Hes binding sites positioned within the V2 exon. Our data provide the first evidence that hGHR gene is transcriptionally regulated by Hes family members.

Hes1 is a mammalian basic-helix-loop-helix (bHLH) transcription factor related to the Drosophila *Hairy and Enhancer of Split* protein. It belongs to a repressor-type *Hes* gene family and negatively regulates target gene expression by binding as a homodimer or heterodimer (with HES-related repressor protein (HERP)) to the N box sequence (CACNAG) or to the nonclassical C type E-box sequence (CACGCG) (Kageyama *et al.* 2005; Yan *et al.* 2002; Yan *et al.* 2001; Iso *et al.* 2001; Lee *et al.* 2001). To our knowledge, only a few human target genes have been identified so far in which Hes1 represses through direct binding to the respective sites, such as the human achaete-scute homolog-1 (ASH1) gene (Chen *et al.* 1997), the human microtubule-associated protein 2 (MAP2) gene (Bhat *et al.* 2006), the human acid α-glucosidase (GAA) gene (Yan *et al.*

2001), the human REST (a transcriptional repressor) gene (Abderrahmani *et al.* 2005) and the human $p27^{Kip1}$ (a cyclin-dependent kinase (CDK) inhibitor) gene (Murata *et al.* 2005).

Three distinct mechanisms for transcriptional repression by Hes1 have been proposed (Iso *et al.* 2003). The first is active repression by recruitment of the TLE/Groucho family of corepressors through the C-terminal WPRW motif when Hes1 binds to class C or N box consensus DNA elements (Iso *et al.* 2003; Fisher *et al.* 1996; Grbavec & Stifani 1996) . The second mechanism is passive repression by interference with activator-type bHLH transcription factors via the formation of non-functional heterodimers (Iso *et al.* 2003; Sasai *et al.* 1992). The third mechanism is through protein-protein interactions mediated by domains other than the WPRW motif: Castella et al demonstrated that the helix 3-helix 4 domain of Hes1 can mediate DNA binding-dependent transcriptional repression in the absence of corepressor recruitment by the WPRW motif (Castella *et al.* 2000). Murata et al. also showed that Hes1 deleted of the WPRW domain could repress the promoter activity of $p21^{Kip1}$ similar to the wildtype Hes1 after binding to the class C site sequence (Murata *et al.* 2005). For the hGHR V2 promoter, since the Hes1 effects depend on the Hes binding sites, it is more likely that Hes1 exerts its repression through the first and/or the third mechanisms.

The inhibitory effect was more pronounced in constructs bearing both Site 1 and Site 2 Hes sites in comparison with the construct only containing Site 1. Whether they act independently or cooperate in some fashion will require further characterization.

Hes1 is the primary downstream target of Notch signaling (Iso *et al.* 2003; Ohtsuka *et al.* 1999; Jarriault *et al.* 1998; Jarriault *et al.* 1995). The Notch-Hes signaling pathway has been shown to play a prominent role in determination of cell fate during proliferation and differentiation, and is essential for several developmental processes such as neurogenesis, myogenesis, hematopoiesis and T cell development (Kageyama *et al.* 2000). Whether the hGHR gene is a target of the Notch-Hes signaling cascade needs to be further investigated, as this is the first report linking the two.

The presence of Hes sites in the human V2 exon is unique amongst all the species examined to date. Thus, although the hGHR V2 promoter shares a lot of similarity with V2-like promoters in other species, this provides a mechanism for species-specific regulation of hGHR V2. Moreover, regulation of the V2 promoter by Hes1 could account for the quantitative differences of widely expressed V2 during development or differentiation.

6. Transcription of hGHR V2 is under the Control of MAP Kinase Signaling

Our characterizations of different *cis*-elements and *trans*-factors suggest a central role for the MAP kinase signaling pathway in regulating V2 transcription (Figure **III-15A**).

In mammalian cells, many Ets transcription factors serve as key nuclear targets of the MAPK signaling pathway (ERK, JNK and p38/RK) in response to signals triggered by growth factors and cellular stress (Gutierrez-Hartmann *et al.* 2007; Yordy & Muise-Helmericks 2000). Extensive studies of the Ras-Raf-MAPK cascade have revealed that members of at least six subfamilies of Ets proteins (Ets, YAN, ELG, PEA3, ERF and the ternary complex factor (TCF)) are nuclear targets of this pathway (Wasylyk *et al.* 1998). In many cases, specific phosphorylation of Ets proteins greatly enhances their ability to activate transcription. For example, phosphorylation by Ras-Raf-MAPK pathway at a conserved threonine (Thr-38) residue within the Pointed domain results in activation of Ets1, which subsequently regulates the expression of multiple growth- and cell cycle-related genes (Oikawa 2004). Ets1 synthesis is also induced by hepatocyte growth factor (HGF), platelet derived growth factor (PDGF) or tumor necrosis factor (TNF) α via activation of the Ras/Raf/MAPK pathway (Dittmer 2003). Our identification that Ets1 transcriptionally activates the hGHR V2 promoter via direct binding to an Ets site makes it likely that Ets1 or even other Ets factors could act as an intermediate linking the MAP kinase signaling pathway to hGHR expression in response to different intra- and extra-cellular stimuli.

Although Hes1 is most known as a downstream target of the Notch signaling pathway, recent data suggest that Hes1 can be regulated by the MAP kinase pathway as well. Stockhausen *et al.* showed that TGFα, a known activator of Ras signaling, could

upregulate Hes1 both at the transcriptional and protein levels in the neuroblastoma cell line SK-N-BE(2)C via activation of the MAP kinase ERK signaling cascade (Stockhausen *et al.* 2005). In addition, Curry et al. have provided evidence that Hes1 expression can be induced in confluent endothelial cells by the JNK signaling pathway in a Notch-independent manner (Curry *et al.* 2006). Thus, Hes1 may also mediate regulation of hGHR gene expression by MAP kinase signaling cascades during different developmental stages and in response to different stimuli.

Both CHOP and C/EBPβ are also regulated by MAP Kinase. Phosphorylation of CHOP at serine 79 and 81 by p38-MAP kinase enhances its transcriptional activity (Ubeda & Habener 2003), while a MAPK substrate site at Thr235 in human C/EBPβ is rapidly and transiently phosphorylated in response to GH in an ERK 1/2-dependent manner (Piwien-Pilipuk *et al.* 2002c). Mutation at the MAPK phosphorylation site of C/EBPβ almost completely abrogates the GH-induced stimulation of *c-fos* (Cesena *et al.* 2007). The involvement of both CHOP and C/EBPβ in the transcriptional regulation of human V2 promoter activity provides two more pathways for modulating hGHR expression in response to MAPK signaling cascade induced by various stimuli.

7. hGH and the Regulation of hGHR V2 expression

Although it is well known that hGH influences expression of its own receptor (Schwartzbauer & Menon 1998), whether the transcription of hGHR V2 is regulated by hGH has remained an issue, since no good binding sites for STATs, the major downstream signaling factor for hGH target genes, are found in the promoter regions.

A number of recent studies have demonstrated that C/EBPβ acts as a critical mediator of GH-regulated transcriptional activation of target genes (Huo *et al.* 2006; Cui *et al.* 2005). In 3T3-F442A preadipocytes, knocking down of C/EBPβ by RNA interference prevents GH-stimulation of c-fos mRNA (Cui *et al.* 2005). GH treatment, through activation of MAPK- and PI3K- mediated signaling cascades, leads to an increase in phosphorylated C/EBPβ bound to the c-fos promoter and simultaneous recruitment of p300, which then forms a complex and mediates GH-stimulated transcription (Cui *et al.* 2005). Thus, our

finding that C/EBPβ can transactivate V2 promoter activity provides a way that GH can take part in regulation of hGHR V2 expression. Furthermore, Ets1 or Hes1, which also act as downstream effectors of MAPK signaling pathway, may also serve as intermediates in hGH regulation of hGHR V2 expression.

Several studies have also shown that hGH can regulate CHOP expression and its transcriptional activity. Flores-Morales et al. observed that GH as well as BAPTA-AM (an ER stress inducer) caused a rapid increase in CHOP mRNA, and an additive effect was observed at the 30- and 45-min time points with combined treatments (Flores-Morales *et al.* 2001). Lobie's lab demonstrated that hGH stimulation of CHO cells stably transfected with GHR cDNA resulted in CHOP transcriptional activation in a p38 MAP kinase-dependent manner (Zhu & Lobie 2000). They also found that CHOP expression is upregulated in response to autocrine hGH production in MCF-7 mammary carcinoma cells stably transfected with the hGH gene. This autocrine hGH stimulation resulted in an increase in both CHOP mRNA and protein levels and, subsequently, increased CHOP-mediated transcriptional activation; ultimately, this led to enhanced protection from apoptosis and increased mammary carcinoma cell numbers (Mertani *et al.* 2001). These effects were confirmed to be generated via the hGHR on the mammary carcinoma cells (Kaulsay *et al.* 2001). Surprisingly, exogenous hGH only exhibits minimal effects on transcription of the CHOP gene. The authors suggested two reasons for the difference: either because the autocrine produced hGH is secreted in low continuous quantities or because the autocrine hGH may interact intracellularly directly with hGHR after synthesis. Our finding that CHOP can upregulate hGHR transcription suggests that a positive feedback loop can be created through CHOP: autocrine hGH production will continuously enhance CHOP levels and transcriptional activation, including upregulation of the hGHR expression, and the increased hGHR levels will result in increased sensitivity to the autocrine hGH.

8. Impact of Different Transcription Factors on Upregulation of hGHR V2 Expression during SGBS Adipocyte Differentiation

We demonstrated that hGHR V2 expression is upregulated during SGBS differentiation, and that the V2 promoter exhibited much higher activity in mature adipocytes than in preadipocytes. In order to characterize the specific trans-factors involved in upregulation of hGHR V2 transcription during SGBS adipocyte differentiation, we have compared the expression and function of several transcription factors in preadipocytes and during differentiation.

Ets1 is the prototypic member of the Ets transcription factor family and plays important roles in development. We showed that overexpression of Ets1 causes marked upregulation of V2 promoter activity. However, our qPCR data indicated that Ets1 expression is highest in confluent SGBS preadipocytes, followed by a marked decrease as differentiation starts; relatively low levels are maintained in later differentiation stages. The opposite correlation between Ets1 expression and V2 transcription does not support Ets1 being the candidate for increasing V2 promoter activity during SGBS adipocytes differentiation.

Very few studies have been done with regard to Ets factors and adipocyte differentiation; therefore, we can not predict whether other Ets family members may function in this process. A Japanese group, who studied TSH receptor (TSHR) gene expression in 3T3-L1 adipocytes, suggested that an Ets binding site might participate in the differentiation-induced increase of TSHR gene promoter activity (Shimura *et al.* 1998). They demonstrated that adipocyte differentiation induces a diminution of nuclear suppressor(s) binding to the putative Ets site, which then allows the adjacent cAMP response element (CRE) to synergistically activate TSHR transcription. Therefore, whether Ets factors other than Ets1 might participate in V2 transcriptional regulation during differentiation is worth further studies.

Evidence from mouse adipocyte cell culture models (3T3-L1 or 3T3-F442A) have revealed that C/EBP family transcription factors act as pivotal regulators of adipocyte differentiation. Different C/EBP members (CHOP, C/EBPβ/δ and C/EBPα) are

expressed in a highly regulated cascade to achieve successful differentiation (Farmer 2006; Otto & Lane 2005). CHOP is transiently expressed by growth-arrested 3T3-L1 confluent preadipocytes; it forms heterodimer with C/EBPβ and prevents C/EBPβ binding to C/EBP cis-regulatory elements (Tang & Lane 2000). After preadipocytes transverse the G1-S checkpoint of mitotic clonal expansion, CHOP expression falls, releasing C/EBPβ from inhibitory constraint and allowing terminal differentiation to begin (Tang & Lane 2000). Our qPCR analysis of CHOP mRNA levels during human SGBS adipocyte differentiation also revealed that the CHOP expression level drops when differentiation is induced, is maintained at a low level during the differentiation stages and increases slowly when the cells reached maturity. In view of these data, CHOP does not appear to be a suitable candidate for increasing V2 activity during differentiation, despite the fact that CHOP can stimulate V2 promoter activity when overexpressed.

C/EBPβ and δ are transiently induced simultaneously when CHOP expression falls. Exogenous hormonal inducers such as insulin, dexamethasone and IBMX have been shown to stimulate C/EBPβ- and δ expression and to enhance their DNA binding (Lekstrom-Himes & Xanthopoulos 1998; Darlington *et al.* 1998). Induced C/EBPβ/δ then activate target genes, including C/EBPα and PPARγ, and promote differentiation. C/EBPα is expressed during the later stages of the differentiation program and is maintained at a high level until terminal differentiation; it transactivates various target genes that produce the mature adipocyte phenotype (e.g. the aP2 gene, the GLUT4 gene and the Ob gene) (Otto & Lane 2005).

Although the proximal promoter region of hGHR V2 contains no canonical C/EBP (α or β) binding motifs, I have been able to demonstrate that overexpression of C/EBPβ can significantly enhance V2 transcription via the CHOP-C/EBP heterodimer site. Therefore, it is possible that differentiation-induced C/EBPβ transactivates V2 expression via this heterodimer site and, thus, is a good candidate for the differentiation-induced increase in V2 transcription.

C/EBPα has also been shown to be able to bind to the CHOP-C/EBP heterodimer site (Ubeda *et al.* 1996). When we cotransfected C/EBPα at a low dose (100ng/well), we did not observe any stimulatory effects on V2 transcription. However, we have not tested C/EBPα at a high dose, similar to the amount required to show a stimulatory effect of C/EBPβ. If a similar response occurs, C/EBPα would be another potential candidate contributing to the increase of hGHR V2 expression during differentiation, since C/EBPα is maintained at a high level throughout differentiation once it has been induced.

Two Hes binding sites were identified within the V2 exon region. We showed that Hes1 can bind and strongly repress V2 promoter activity when overexpressed. Hes1 expression has been shown to be down-regulated during murine adipogenesis *in vitro* and *in vivo* (Soukas *et al.* 2001). These data suggest that a differentiation-induced loss of Hes1 suppressive activity might also cause higher V2 promoter activity in human adipocytes.

SUMMARY

The studies presented in this thesis demonstrate that hGHR V2 mRNA is the predominant transcript expressed in most human cells and during human adipocyte differentiation. Basal transcription of the hGHR V2 exon is regulated by a TATA-less promoter, with characteristics of a housekeeping gene promoter. On the basis of our results, we propose two models regarding to the regulation of hGHR V2 expression in response to various cellular stimuli:

Model 1 (Figure III-15A): In most cells, hGH can act through the MAP kinase (including ERK1/2, JNK and p38 MAPK) pathway to activate specific transcription factors which may then exert positive (e.g. CHOP, Ets1)or negative (e.g. Hes1) regulation of the hGHR V2 promoter. Many growth factors and other hormones can also activate the MAP kinase cascade and may act through these transcription factors to regulate hGHR V2 expression. ER stress conditions derived from either the unfolded protein response or nutrient depletion can induce the expression of the downstream factor CHOP and enhance its transcriptional activation through the p38MAP kinase cascade

and, thus, modulate V2 expression. Moreover, various developmental stimuli can use the Notch-signaling pathway and act through its downstream target, Hes1, to specifically affect hGHR V2 expression at different developmental stages.

Model 2 (Figure III-15B): During adipocyte differentiation, Ets1, CHOP and Hes1 are expressed at relatively high levels by the growth-arrested confluent preadipocytes and are important regulators of V2 and thus hGHR expression in the preadipocytes, which may play an important role in preparing the cells for differentiation. Once differentiation starts, their expression levels decrease followed by an increase in the expression of the C/EBP family members, C/EBPβ and α, which then become major transactivators of hGHR gene expression in the differentiating and maturing adipocytes.

CHAPTER V

FUTURE STUDIES

The results from the present studies provide a basis for future investigations aimed at a more complete understanding of how hGHR gene expression is controlled in adipose as well as other tissues. They have also opened up several interesting routes to explore. In this section I will describe three major future directions: 1) to determine the expression profile of hGHR in primary human adipose tissues; 2) to characterize more in depth the molecular mechanisms regulating hGHR V2 transcription; and 3) to delineate the role of different 5'UTR exons in regulating translation of hGHR protein.

1. To Examine the Expression Profile of hGHR in Primary Adipose Tissues

Our results from human adipocyte cDNA library screening and from human SGBS adipocyte differentiation provided valuable information about hGHR gene expression in adipose tissue. However, there are certain limitations, including: 1) the cDNA library was created from adipose tissues from 4 lean and 4 obese persons and thus is not specific for degree of obesity or fat depot; and 2) the SGBS adipocyte is an established clonal cell line and, although what we know of its physiology and molecular biology suggest that it offers an excellent tool for studies of human adipocyte biology (Fischer-Posovszky *et al.* 2007b), there may be certain differences from primary cells. For one thing, it represents subcutaneous fat cells but not abdominal adipocytes. In addition, Soukas et al reported that the gene expression changes associated with adipocyte development *in vivo* and *in vitro*, while overlapping, are in some respects quite different (Soukas *et al.* 2001). For example, the authors observed that the mouse GHBP mRNA is highly expressed in both the *in vivo* and *in vitro* adipocyte, whereas full-length mouse GHR mRNA is predominant *in vivo*; C/EBPα is highly expressed in both the *in vivo* and *in vitro* adipocyte, while C/EBPβ is primarily expressed *in vitro*. Therefore, it is important and desirable to study the hGHR gene expression profile in parallel in the primary human adipose tissue, investigating both physiological and pathophysiological conditions. Indeed, this is our next step.

We have initiated a collaboration with Dr. Andre Tchernof of Laval University to investigate the relative expression of total hGHR, the V2 variant, the new variants (VC and VC+A) as well as the truncated hGHR isoform ($GHR_{1\text{-}279}$) in adipose tissues from

different fat depots (omental vs. subcutaneous fat) from a range of lean to morbidly obese males and females who have been extensively characterized for biochemical and physical characteristics. Quantitative real-time RT-PCR assays will be used for such purposes. These studies will allow us to characterize hGHR expression by fat depot, and the changes in hGHR expression (due to different levels of specific mRNA variants) or signaling (due to changes in the ratio between truncated/full-length) induced by obesity. We will also be able to correlate hGHR expression and the degree of obesity as well as the lipid profiles. Future studies will also include examining hGHR and variant mRNA expression in the preadipocytes vs. mature adipocytes separated from omental and subcutaneous fat. This will allow us to compare the similarities and differences between *in vivo* and in vitro (SGBS differentiation) adipocyte development and to examine directly the effects of hGH on adipocyte proliferation, differentiation and function. The information obtained from these studies should help in the development of a more appropriate clinical use for hGH as a therapeutic tool, for treatment of obesity in GH-deficient individuals and obese subjects in general.

2. An In Depth Characterization of the Molecular Mechanisms Regulating hGHR V2 Transcription

2.1. Ets Family Transcription Factors

Study 1: to determine the roles of Ets1 and/or PU.1 on V2 transcription in lymphoid cells or other hematopoietic cell lines. Although Ets1 is ubiquitously expressed, it is most enriched in lymphoid organs, especially the quiescent T cells, while PU.1 is specifically expressed in hematopoietic cells (B cells, macrophages and neutrophils) (Oikawa 2004). Thus, it would be of interest to examine the effects of Ets1 and PU.1 on V2 promoter constructs in hematopoietic cell lines (e.g. Jurkat cells or the IM-9 lymphocyte cell line), when individual response elements are mutated or with overexpression of dominant negative forms. Such studies would help us to understand the regulation of hGHR in the immune system, an important issue as there is good evidence that hGH influences immune function (Perry *et al.* 2006; Harvey & Hull 1997; Liu *et al.* 1997).

Study 2: to examine the molecular mechanism whereby Ets1 transactivates the V2 promoter Lu et al. did an elegant study examining the properties of Ets1 binding to chromatin and its effect on platelet factor 4 (PF4) gene expression (Lu *et al.* 2004). They demonstrated that Ets1 binds to chromatin and to naked DNA with a similar affinity. Binding of Ets1 to the PF4 promoter in the context of chromatin is independent of cell-type specific factors or ATP-dependent chromatin remodeling. However, Ets1 binding itself is not sufficient for full activation of transcription, despite the fact that Ets1 binding induces chromatin changes and results in an increased accessibility of DNA. They proposed a model that Ets1 either recruits basal transcriptional machinery and thus increases PF4 promoter activity or, alternatively, Ets1 helps to recruit other transcription factors to bind to chromatin and acts as an enhancer of transcription. It would be of great interest to examine whether any of these mechanisms are involved in activation of V2 transcription.

Study 3: to investigate the regulatory effects of other ubiquitously expressed Ets family members, such as Ets2 or ELK-1, on the V2 promoter

Ets2 and Ets1 are in the same subfamily of the large Ets transcription factor superfamily, which has 11 subgroups. They have a similar structure and binding specificity (Gutierrez-Hartmann *et al.* 2007; Oikawa 2004; Oikawa & Yamada 2003). Although Ets1 is widely expressed (Gutierrez-Hartmann *et al.* 2007), its highest expression is found in lymphoid tissues and vascular endothelium (Oikawa 2004). In contrast, Ets2 is known to be ubiquitously expressed (Oikawa 2004; Oikawa & Yamada 2003). Thus, future studies should examine the regulatory effects of Ets2 on the human V2 promoter.

ELK-1 is from a different Ets subgroup and is also ubiquitously expressed. It has been documented that the ELK-1 binding motif is preferentially found in promoters that lack TATA and Inr. Furthermore, Clarson et al. reported that GH stimulates transcription of the immediate early gene, Egr-1, primarily through activation of ELK-1 and Sap-1a (an Ets factor in the same subgroup of ELK-1) at the SRE/Ets pair sites on the *egr-1* gene promoter (Clarkson *et al.* 1999). Hodge et al. demonstrated that GH-dependent activation of the Ras/MEK/ERK pathway, and subsequent serine phosphorylation of ELK-1,

contributes to GH-stimulated *c-fos* gene expression through the SRE in 3T3-F442A preadipocytes (Hodge *et al.* 1998). Given our finding that the V2 promoter is TATA-less, and is not dictated by the Inr-like element, as well as the presence of two Ets sites in the promoter region, it would be interest to investigate the regulatory role of ELK-1 on V2 transcription, both at the basal transcription level and the modulation of V2 expression in response to GH or other growth factors.

2.2. Role of CHOP on hGHR V2 Expression

Study 1: to identify the protein partner CHOP might interact with to elicit its stimulatory effect. We have narrowed down the potential region in the V2 exon where CHOP associates. However, because the CHOP stimulatory effect gradually reduced when I progressively deleted the 3'-V2 exon region, we could not localize a specific small DNA sequence; this makes it more difficult to identify the binding partner(s). We could start with the potential candidates we predicted (SREBP, Sp1, Egr-1) to see whether CHOP could co-immunoprecipitate with these factors when overexpressed simultaneously with CHOP. We could also perform ChIP assays to locate a smaller V2 exon region with which CHOP associates and then perform reChIP to determine the putative interacting partrner(s). Furthermore, we could mutate the candidate sites individually and in combinations (since multiple sites are likely to be involved), to see whether these mutations affect CHOP's stimulatory effect on V2 transcription. If none of these candidates appears to be the partner, then we might carry out yeast two-hybrid assays or proteomic screening of the nuclear extracts isolated from CHOP overexpressing cells.

Study 2: to investigate whether CHOP can mediate changes in hGHR gene expression in response to ER stress or nutrient deprivation. CHOP is a direct downstream target of ER-stress and an inducible protein in response to nutrient disturbances, including glucose deprivation and amino acid (methonine, leucine) limitation. It would be very interesting to see, during these pathophysiological conditions, how the hGHR mRNA level, especially V2, changes. Do they positively correlate with the change in CHOP level? For example, we could create a glucose depletion status by growing cells in non-glucose or very low glucose media, add glucose at different concentrations and then compare the

level of total hGHR, hGHR V2 and CHOP mRNAs using quantitative real-time RT-PCR, as well as hGHR and CHOP proteins by western blot. Such studies would provide us more definitive proof that CHOP is involved in regulating the expression of hGHR in response to stress or nutrient status.

2.3. Hes1 and hGHR

Notch signaling is generally thought to control cell fate decisions by inhibiting the development of certain lineages and/or by promoting the development of others (Ross *et al.* 2004; Ross & Kadesch 2004). It is well known that hGHR expression is regulated during development (Goodyer *et al.* 2001b; Schwartzbauer & Menon 1998); however, the factors and pathways responsible have not been identified. Our finding that Hes1 exerts a strong suppressive effect on the V2 promoter when overexpressed suggests a link between Notch-signaling and hGHR gene expression. To prove this connection would be an important future study. For this purpose, we could transfect the Notch receptor (e.g. Notch-1 or -2) into our usual test cells, activate with appropriate ligands (e.g. Jagged or Delta), and then measure the changes in endogenous hGHR mRNA and protein using real-time RT-PCR assays and western blots, to see if there are correlations. We could also transfect V2 promoter constructs containing the wildtype or mutated Hes binding sites into endothelial cells, which have been shown to possess Notch receptors (Curry *et al.* 2006), and activate with appropriate ligands, to examine the regulatory effects of Notch signaling on the V2 promoter. These studies would help us to gain greater insight into whether the hGHR gene is regulated by the Notch signaling cascade.

2.4. Interactions Between Different Transcription Factors

Our studies indicate that the regulation of V2 transcription is controlled by multiple transcription factors. Therefore, it will be important to investigate further the interactions between the different trans-acting factors, including Ets1 or other Ets factors, C/EBPβ and α, Sp1, CHOP and Hes1, using methods such as cotransfection, coimmunoprecipitation, EMSSAs and re-ChIP to characterize their interactions and combinatorial effects (additive, synergistic or antagonistic) on V2 transcriptional regulation.

2.5. Role of C/EBPβ and C/EBPα in the Adipocyte Differentiation-Induced Increase in hGHR and hGHR V2 mRNAs.

Our finding that C/EBPβ can stimulate V2 promoter activity by using, at least in part, the CHOP-C/EBP heterodimer site suggests an important direction for future studies of the factors that are responsible for upregulating hGHR V2 mRNA levels during adipocyte differentiation. First, we should confirm the positive correlation between the expression of C/EBPβ and total hGHR as well as V2 mRNAs during SGBS differentiation by real-time RT-PCR. We should also confirm the binding of C/EBPβ to the V2 promoter (via the CHOP site) by EMSA and ChIP. Next, we should examine if overexpression of C/EBPβ in SGBS preadipocytes and in HEK293 cells increases the mRNA level of hGHR V2. Third, we can induce differentiation of SGBS preadipocytes and collect nuclear extracts at different time points during SGBS differentiation and examine by ChIP to determine the kinetics of C/EBPβ association with the V2 promoter.

As C/EBPα is the major transcription factor controlling adipocyte differentiation in the late stages (Otto & Lane 2005) and C/EBPα homodimers have also been shown to bind to the CHOP-C/EBP heterodimer site (Ubeda *et al.* 1996), similar studies should be carried out with C/EBPα. Such studies will help us to define whether C/EBPβ and/or C/EBPα are the factors responsible for the increase in total hGHR and V2 expression during SGBS adipocyte differentiation and maturation.

3. Characterizing the translational regulatory effects of different 5'UTR exons on hGHR protein expression

It is well known that the 5'UTR regions of mRNAs can determine protein expression levels by influencing translational efficiency (Hughes 2006). Jiang and Lucy demonstrated that the 5'UTR sequences of the bovine GHR variant mRNAs had differential effects on translation efficiency in an in vitro transcription-translation assay: 5'UTR sequences from some of the minor transcripts (b1H, b1I) showed much higher translational efficiencies than that from the major variant, b1B (a V2 homologue in cattle) (Jiang & Lucy 2001b). Moffat et al. reported analogous observations for the 5'UTRs of

mouse GHR mRNA variants (Moffat *et al.* 1999a). Therefore, it would be worth studying how the different human 5'UTR exon sequences affect hGHR protein expression, using similar methodology.

In summary, these potential future directions would help us gain more insight into the molecular mechanisms whereby hGHR is regulated and prove or disprove certain hypotheses I have proposed as a result of the present thesis investigations.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

This thesis describes studies carried out to understand transcriptional regulation of human GHR gene expression, focusing on human adipocytes.

1. I characterized the human GHR mRNA variants expressed in a human adipocyte cDNA library and identified that the hGHR V2 transcript is the predominant variant.

2. I isolated five novel hGHR mRNA variants from the same human adipocyte cDNA library and demonstrated that they are widely expressed in different tissues, including human fetal and postnatal liver, kidney and fat. They are present at low levels relative to the total pools of hGHR mRNA in each of these tissues.

3. I compared the relative expression of hGHR 5'UTR mRNA variants in human adult liver and adult fat, and confirmed different expression patterns in the two tissues: in adult liver, V1 and V2 transcripts form the majority of the hGHR mRNA pool, whereas in adult fat, V2 predominates.

4. I demonstrated that hGHR is expressed in both human preadipocytes and mature adipocytes using the SGBS human adipocyte cell line.

5. I assessed the quantitative changes in total hGHR mRNA as well as six of its 5'UTR variants during SGBS adipocyte maturation and provided evidence that:

a) at the preadipocyte stage, the predominant variant is V2; other mRNA isoforms are either expressed at low levels (e.g. VC+A: ~10%) or are barely detectable (e.g. V3, V9 and VC);
b) total hGHR and V2 mRNAs are markedly up-regulated in parallel at the early differentiation stage and reach a maximum with maturity; and
c) the increase in V2 transcript is the major contributor to the increase in total hGHR mRNA during SGBS adipocyte differentiation.

6. I mapped the transcription start sites (TSSs) of the human GHR V2 exon and demonstrated that hGHR V2 mRNA is transcribed from identical TSS in SGBS preadipocytes, liver or kidney cells.

7. I demonstrated that the 5'-flanking region of the hGHR V2 exon possesses promoter activity and delineated several functionally distinct domains that are implicated in the maintenance and modulation of V2 basal transcriptional activity:

a) the proximal promoter for hGHR V2 exon was mapped to 211 bp upstream of the ovine major TSS. It is a TATA-less promoter, having characteristics of a housekeeping gene promoter;
b) the region -29 to +11 appears to be the core promoter of hGHR V2. It functions like a null core promoter, despite the presence of an Inr-like sequence;
c) V2 basal transcription is regulated by two functionally discrete domains: a 5'upstream inhibitory domain and a 3'downstream activation domain; and
d) approximately 160 bp downstream of the V2 major TSS is required for maximal basal promoter activity. Within this region, a 37 bp element from positions +125 to +162 contains positive regulatory elements for maximizing V2 basal promoter activity.

8. I showed that the Sp1 transcription factor can modulate hGHR V2 basal transcription, but that it does not appear to be the determinative regulator as in other species. Instead, data from the present work suggests that V2 basal transcription is more likely to be the result of imput from multiple factors.

9. I provided the first report identifying hGHR as a direct target gene for the Ets family of transcription factors by demonstrating that Ets1 can transactivate the V2 promoter through direct binding to the Ets1 response element located in the proximal promoter of the hGHR V2 exon.

10. I demonstrated that CHOP, a small nuclear, bZiP protein and the major downstream effector of ER stress, upregulates V2 transcription via a potential protein tethering mechanism by complexing with factors bound to the 3' V2 exon region from +103 to +362.

11. I showed that C/EBPβ can stimulate V2 promoter activity, at least in part by using the CHOP-C/EBP heterodimer site in the V2 promoter region.

12. I provided the first evidence that the Notch-signaling downstream target, Hes1, can potently repress V2 promoter activity via association with two Hes binding sites located within the V2 exon. These data also suggest a mechanism for species-specific regulation of hGHR V2.

13. For the first time, I delineated the transcriptional regulation of hGHR V2 expression in human preadipocytes and mature adipocytes. I demonstrated that similar basal transcriptional profiles are present in both cell types, but that the mature adipocytes show much higher V2 promoter activity than the preadipocytes.

14. I provided the first evidence that expression of the transcription factors CHOP and Ets1 is high in the confluent preadipocytes and then decreases during SGBS adipocyte differentiation, suggesting their importance in preparing the cells for differentiation.

In summary, the results provided in this PhD work provide a molecular basis for future studies aimed at better understanding how hGHR gene expression is regulated in human adipose tissue. These investigations will help to develop more efficient therapeutic strategies for using hGH to treat obesity in general and the obese state that accompanies the GH deficiency syndrome.

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Characterization of Growth Hormone Receptor Messenger Ribonucleic Acid Variants in Human Adipocytes

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Context: Human GH exerts profound effects on adiposity through its specific receptor, hGHR. Eight *hGHR* mRNAs are produced by the *hGHR* gene due to splicing from alternate 5′-untranslated region first exons into a common acceptor site upstream of the start codon in exon 2. Four transcripts (V2, V3, V5, V9) are ubiquitously expressed, whereas the other four (V1, V4, V7, V8) are expressed only in normal postnatal liver, suggesting that different promoter usage is a mechanism for developmental- and tissue-specific regulation of the *hGHR* gene.

Objective: Because it is unknown whether this occurs in adipocytes, we screened human adipocyte cDNA for *hGHR* mRNAs using 5′-rapid amplification of cDNA ends.

Results: Eighty-nine percent of the clones were V2-like, 3% were V3-like, and 8% were five new mRNA variants (VA-VE). All new 5′-untranslated region sequences mapped within the *hGHR* 5′flanking region. RT-PCR assays showed expression in multiple fetal and adult tissues, and, thus, they are not adipocyte specific. We compared expression of *hGHR* mRNAs in adult liver, adult fat, and the human preadipocyte SGBS cell line, using duplex RT-PCR. In liver, V1 and V2 are the major *hGHR* mRNAs, whereas in adipose, V2 predominates; VA and VC are expressed at similar lower levels in both. In SGBS preadipocytes, approximately 70% of *hGHR* mRNA is V2. During differentiation, total *hGHR* and V2 transcripts are markedly up-regulated [*hGHR*: 2.3 ± 0.2-fold (mean ± SE), $P < 0.01$; V2: 3.0 ± 0.8, $P < 0.03$], whereas other variants also increased but remained relatively minor transcripts.

Conclusions: We have identified five new *hGHR* mRNA variants. Because the V2 transcript is predominant in adipocytes at all developmental stages, the mechanisms regulating its expression should be examined. (***J Clin Endocrinol Metab* 91: 1901–1908, 2006**)

GH IS A key regulator of postnatal growth and metabolism (1–3). GH exerts these effects by binding to its specific cell surface receptor and triggering various intracellular signaling cascades, resulting in modulation of target cell activity (4). Thus, the receptor expression level is critical for tissue sensitivity to GH.

The GH receptor (GHR), a single transmembrane protein of the class I cytokine receptor family (4), is expressed in most cell types, and it is well known that its levels can be affected by development (5–9), nutritional status (10, 11), and hormones (10, 12–18). However, our understanding of the molecular mechanisms controlling *GHR* expression is incomplete. Studies in different species have shown a common feature: heterogeneity in the 5′-untranslated region (5′UTR) of *GHR* transcripts (10, 12, 13, 19–24). This is generated by transcribing and splicing from different 5′-noncoding leader exons to a common splice acceptor site 9–11 bp upstream of the start codon in exon 2 of the *GHR* gene; thus, they all code for the same protein. In the human, nine 5′UTR mRNA variants (V1-V9) have been reported from screenings of adult liver and cardiac muscle cDNA libraries (19, 20). Although V6 is now considered to be an artifact (21), the other eight variants have been validated by genomic localization and expression profiling.

Chromosomal mapping of these 5′-noncoding exons determined that seven form two separate clusters, each approximately 2 kb in size: V2, V9, and V3 form module A, 140.8–142.4 kb upstream of exon 2, whereas V7, V1, V4, and V8 form module B, 15.8–17.9 kb upstream of exon 2, and V5 is located adjacent to exon 2 (Fig. 1A, revision of Ref. 19) (21). Module A and V5 mRNAs are widely expressed; in contrast, module B transcripts are detected only in normal postnatal liver (19). Similar tissue-specific and developmentally specific expression patterns have been observed in other species, suggesting that the derivatives of these 5′UTR exons are likely to be the result of common regulatory mechanisms controlling *GHR* expression (12, 13, 19, 21, 25). Furthermore, recent studies demonstrated that the 5′UTR variant sequences have differential effects on translation efficiency (12, 26). Therefore, to understand what controls human *GHR* (*hGHR*) expression and thus hGH responsiveness, it will be essential to isolate all *hGHR* 5′UTR mRNA variants and to study how they are regulated in different tissues.

Adipose tissue is a major target for hGH. Clinical and experimental observations have demonstrated the importance of hGH in regulating body fat through enhancing lipolysis and inhibiting lipogenesis (2, 3, 27, 28). hGH has also been shown to regulate adipocyte differentiation, although the effects have been controversial between

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Abbreviations: GHR, GH receptor; hGHR, human GHR; 5′RACE, 5′-rapid amplification of cDNA ends; RT, reverse transcription; SGBS, Simpson-Golabi-Behmel syndrome; 5′UTR, 5′-untranslated region.

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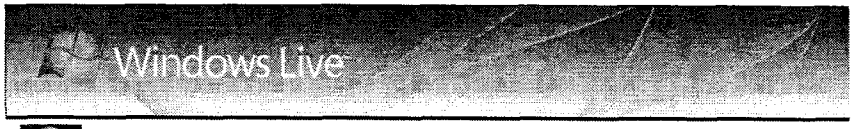

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