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REGULATION OF GROWTH HORMONE RECEPTOR GENE EXPRESSION DURING DEVELOPMENT

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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"What is not clear should be cleared up. What is not easy to do should be done with great persistence."

.....Confucius (551?-479? B.C.)

ABSTRACT

To improve our understanding of the potential function of the human growth hormone receptor (hGHR) during fetal development, the ontogeny of hGHR mRNA expression in human tissues was examined. In addition, portions of the *hGHR* gene regulatory regions were cloned and characterized.

Transcription of the *hGHR* gene was observed in multiple tissues from as early as the first trimester of human fetal life. Two isoforms of the mRNA coding region were identified, exon 3-retained or -deleted, and it was shown that expression of these two transcripts is individual-, but not tissue-, specific. In tissues from 117 individuals (4 weeks of fetal age to 64 years postnatal), predominant expression of the exon 3-deleted isoform occurred prior to 20 weeks of fetal life, suggesting that exon 3-deleted transcripts may be developmentally regulated. A six-fold increase in hepatic hGHR mRNA was observed postnatally, while the levels in kidney and intestine showed no significant developmental change and those in lung decreased six-fold postnatally.

This suggested that multiple gene promoters might be directing tissue-specific developmental changes in hGHR mRNA levels. Since heterogeneity in the 5' untranslated regions (5'UTR) of mRNAs can result from differential gene promoter usage, the expression patterns of three of the eight known 5'UTR variants (V1 to V8, numbered according to their relative abundance in liver) were then investigated. Expression of V1, V3 and V4 was compared in fetal versus postnatal tissues, including hepatoblastomas and hepatocellular carcinomas. While V3 was detected in all tissues examined, the V1 and V4 variants were only present in normal postnatal liver specimens; they were not

expressed in hepatic tumours. It was hypothesized that V1 and V4 are derived by alternative splicing of a common gene transcript, while V3 synthesis is regulated by a distant and more widely transcribed gene promoter.

To identify DNA sequences regulating synthesis of the V1 and V4 5'UTR variants, portions of the 5' flanking region of the *hGHR* gene were cloned. Four of the 5'UTR variant sequences were precisely placed in series (5' V7-V1-V4-V8 3') on a 3.8 kb XbaI-BsaAI genomic fragment. A transcriptional start site was identified upstream of the V1 sequence and found to generate, in postnatal liver, a long 5'UTR exon containing V1, V4 and V8 sequences. Evidence for a second liver-specific transcriptional start site, located upstream of V7, was also obtained. Alternative splicing of these gene products can result in several mRNA species, including the previously isolated V1, V4, V7 and V8 mRNA isoforms. Preliminary characterization of a 4.3 kb HindIII-XbaI genomic clone has revealed that the V3 as well as V2 sequences are located in series (5' V2-V3 3') in a distant genomic region, and computer analysis has suggested that there are transcriptional start sites upstream of both the V2 and V3 sequences. To construct a complete and continuous physical map of the *hGHR* gene, a Bacterial Artificial Chromosome (BAC) recombinant clone, encompassing more than 100 kb human genomic DNA and containing both V1- and V3-variant clusters, was isolated. Future characterization of the BAC clone will produce a complete map of the *hGHR* gene regulatory regions.

In summary, developmental changes in *hGHR* gene expression most likely reflect the maturation of an endocrine system but may also confer fetal-specific hGHR biological activity.

RESUMÉ

Pour approfondir notre connaissance des fonctions potentielles du récepteur de l'hormone de croissance humaine (hGHR) pendant le développement foetal, nous avons étudié l'ontogénèse de l'expression de l'ARNm de l'hGHR dans les tissus humains, de même que nous avons cloné et charactérisé certaines portions des séquances régulatrices du gène *hGHR*.

La transcription du gène *hGHR* a été observée dans différents tissus dès le premier trimestre de la vie foetale humaine. Deux isoformes de la région de codage de l'ARNm ont été identifiées, avec conservation ou suppression de l'exon 3, ce qui a permis de démontrer que l'expression de ces deux transcrits variait entre individues mais pas entre tissus. Dans les tissus de 117 sujets (à 4 semaines de vie foetale jusqu'à 64 ans postnatal), l'expression prédominante de l'isoforme privée de l'exon 3 est observé avant 20 semaines de vie foetale, ce qui donne à penser que les transcrits privés de l'exon 3 font peut-être l'objet d'une régulation développementale. Nous avons observé une augmentation, par un facteur de six, des ARNm de l'hGHR hépatique postnatal, alors que les concentrations dans le rein et les intestins ne subissent aucun changement développementale significative et que celles des poumons sont, après la naissance, six fois inférieures a celles du foetus.

Cela donne à penser que plusieurs promoteurs modulent les changements développementaux spécifiques de tissus dans les ARNm de l'hGHR. Puisque l'hétérogenéité des régions non traduites en 5' (5'UTR) des ARNm peuvent être due à une utilisation différentielle du promoteur du gène, les modes d'expression de trois des

huit variants 5'UTR connus (V1 à V8, numérotés selon leur abondance relative dans le foie) ont été étudiés. L'expression de V1, V3 et V4 a été comparée dans les tissus du foetus par rapport aux tissus postnatals, y compris dans les hépatoblastomes et les carcinomes hépatocellulaires. Alors que V3 a été décelé dans tous les tissus examinés, les variants V1 et V4 n'étaient présents que dans les échantillons de foie postnatal normaux; ceux-ci n'étaient pas exprimés dans les tumeurs hépatiques. Nous avons donc émis l'hypothèse que V1 et V4 dérivent de l'épissage alternatif d'un transcrit commun, alors que la synthèse de V3 est régulée par un promoteur plus distant et de distribution tissulaire plus universelle.

Pour identifier les séquences d'ADN qui régulent la synthèse des variants 5'UTR de V1 et V4, nous avons cloné des parties de l'extrémité en 5' du gène *hGHR*. Quatre des séquences du variant 5'UTR ont été précisément placées en série (5' V7-V1-V4-V8 3') sur un fragment génomique 3,8 kb XbaI-BsaAI. Un site de départ transcriptionnel a été identifié en amont de la séquence V1 et l'on a découvert qu'elle produisait dans le foie postnatal un long exon 5'UTR contenant les séquences V1, V4 et V8. La preuve de l'existence d'un deuxième site de départ transcriptionnel spécifique au foie, situé en amont de V7, a également été obtenue. L'épissage alternatif de ces produits peut produire à plusieurs espèces d'ARNm, parmi lesquelles les isoformes V1, V4, V7 et V8 déjà isolées. La caractérisation préliminaire du clone génomique 4,3 kb HindIII-XbaI révèle que les séquences V3 et V2 sont placées en série (5' V2-V3 3') dans une région génomique distincte, et l'analyse informatique donne à penser qu'il existe des sites de départ transcriptionnel en amont des séquences V2 et V3. Pour élaborer une carte complète et continue du gène *hGHR*, un clone recombinant d'un chromosome artificiel

bactérien (BAC), englobant plus de 100 kb d'ADN génomique humain et contenant les aggrégation des variants V3 et V1, a été isolé. La future caractérisation du clone BAC donnera une carte complète des zones régulatoires du gène *hGHR*.

En résumé, les changements développementaux qui interviennent dans l'expression du gène *hGHR* reflètent vraisemblablement la maturation du système endocrinien, mais révèlent peut-être aussi l'existence de l'activité biologique de hGHR spécifique du foetus.

FOREWORD

This dissertation is written in accordance with the Faculty of Graduate Studies and Research guidelines for thesis preparation in the manuscript-based format. The thesis is divided into four parts, consisting of seven chapters and two appendices in total. Part I provides a general introduction with a literature review and consists of a single chapter. The preface to Chapter 1 places the scope of this thesis into an historical perspective. Chapters 2 through 5, as well as Appendices 1 and 2, form Part II, the experimental section. These chapters are presented in manuscript format, each containing its own abstract, introduction, materials and methods, results, discussion and references sections. The prefaces to the experimental section chapters serve as "bridges" from one manuscript to another. Studies that have not yet been submitted for publication are presented in Appendices 1 and 2. Part III consists of the last two chapters. Chapter 6 discusses the findings reported in the dissertation as they relate to our current understanding of the growth hormone receptor and provides future directions of study. The second chapter of Part III, Chapter 7, lists the claims to original research. A reference list for Chapters 1 and 6 as well as the two appendices is provided in Part IV.

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS AND CONTRIBUTIONS MADE BY CO-AUTHORS

Chapter 2.

George Zogopoulos, Rilene Figueiredo, Arvin Jenab, Ziad Ali, Yves Lefèbvre and Cynthia G. Goodyer. "Expression of the exon 3 retaining and deleted human growth hormone receptor messenger ribonucleic acid isoforms during development." J. Clin. Endo. Metab. 81: 775-782, 1996.

The candidate was responsible for all aspects of the study, which was carried out in the laboratory of Dr. Goodyer. Arvin Jenab and Ziad Ali were undergraduate students in the Department of Anatomy at McGill University that trained with the candidate during two summers. Arvin and Ziad helped with RNA extractions, RT-PCR reactions and Southern blots. The cultured fibroblasts were maintained by Dr. Rilene Figueiredo. Tissue samples were collected by Dr. Goodyer with the support of Dr. Lefèbvre. The manuscript was prepared and revised by the candidate in consultation with Dr. Goodyer.

Appendix 1.

"Quantitative analysis of growth hormone receptor mRNA tissue levels during human development." (Unpublished)

The candidate was responsible for the quantitative reverse transcriptase-PCR assays, the final data analysis and graphic interpretation. The growth

hormone binding experiments reported in the appendix were performed by Dr. Figueiredo. These studies were carried out in the laboratory of Dr. Goodyer.

Chapter 3.

George Zogopoulos, Steffen Albrecht, Torsten Pietsch, Lesley Alpert, Dietrich von Schweinitz, Yves Lefèbvre and Cynthia G. Goodyer. "Fetal- and tumour-specific regulation of growth hormone receptor messenger ribonucleic acid in human liver." Cancer Res. 56: 2949-2953, 1996.

The candidate was responsible for all aspects of the study, which was carried out in the laboratory of Dr. Goodyer. Tissue samples were collected by Drs. Goodyer, Albrecht, Pietsch, Alpert, von Schweinitz and Lefèbvre. The manuscript was prepared and revised by the candidate in consultation with all co-authors.

Chapter 4.

George Zogopoulos, Cynthia G. Goodyer and Geoffrey N. Hendy. "Cloning and characterization of promoter regions in the human growth hormone receptor gene." (Submitted)

The candidate was responsible for all aspects of the study, which was carried out in the laboratories of Drs. Goodyer and Hendy. The manuscript was prepared and revised by the candidate in consultation with Drs. Goodyer and Hendy.

Appendix 2.

"Additional results from the cloning of the 5' regulatory regions of the human growth hormone receptor gene." (Unpublished)

The candidate was responsible for all aspects of the study, which was carried out in the laboratories of Drs. Goodyer, Hendy and Hudson.

Chapter 5.

George Zogopoulos, Peter Nathanielsz, Geoffrey N. Hendy and Cynthia G. Goodyer. "The baboon: a model for the study of primate growth hormone receptor gene expression during development." (Submitted)

The candidate was responsible for all aspects of the study, which was carried out in the laboratories of Drs. Goodyer and Hendy. Baboon tissues were obtained from Dr. Nathanielsz's laboratory at Cornell University. The manuscript was prepared and revised by the candidate in consultation with all co-authors.

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During the course of my studies, I was also fortunate to have had the opportunity to serve as founding President of the Experimental Medicine Graduate Students' Society of McGill University. I would like to thank all of my fellow Council members, past and present, who have shared my enthusiasm and worked hard to ensure the success of the Society. Thanks also to Dominique Besso, Student Affairs Coordinator, for her help, continued support and friendship. I am also greatly indebted to Dr. Gerald Price, "my self-appointed mentor", who has taught me many valuable lessons. His genuine interest

and support for student initiatives has helped the Council reach its goals.

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Many thanks to my colleagues and friends at the Montreal Children's and Royal Victoria Hospitals for their help over the years. I am particularly thankful to Dr. Paul Goodyer for his suggestions and for making his lab available to me at all times, as well as to Drs. Constantin Polychronakos, Celia Rodd and Shayne Taback for their helpful suggestions. A special thank you to Petros Vafiadis and Elena Torban for their friendship and support. I would also like to thank our collaborators, Drs. Lesley Alpert, Steffen Albrecht, Tom Hudson, Torsten Pietsch, Peter Nathanielsz, Yves Lefèbvre, Jean-Martin Laberge and Dietrich von Schweinitz for their support. A very special thank you to Carmela DeLuca for her help with the preparation of this thesis.

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mom, dad and Mary,

and Carmela,

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ALS	Acid labile subunit
BAC	Bacterial artificial chromosome
cAMP	Cyclic adenosine monophosphate
c/EBP	CCAAT/enhancer binding protein
CHO	Chinese hamster ovary
CNTF	Ciliary neurotrophic factor
DBP	D-element binding protein
DNA	Deoxyribonucleic acid
DNase	DNA nuclease
EGF	Epidermal growth factor
EPO	Erythropoietin
ERK	Extracellular regulated kinase
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
GHBP	Growth hormone binding protein
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHRHR	Growth hormone releasing hormone receptor
GHRP	Growth hormone releasing peptide

GHS	Growth hormone secretagogue
GHSR	Growth hormone secretagogue receptor
GH-N	Growth hormone (pituitary-derived)
GH-V	Growth hormone variant (placental derived)
GM-CSF	Granulocyte/macrophage colony stimulating factor
Grb2	Growth factor receptor bound protein 2
GRE	Glucocorticoid response element
HB	Hepatoblastoma
HCC	Hepatocellular carcinoma
HNF	Hepatocyte nuclear factor
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IP_3	Inositol triphosphate
IRES	Internal ribosomal entry site
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
LAP	Liver activating protein
LBM	Lean body mass
LH	Lutenizing hormone
LIF	Leukemia inhibitory factor
LOH	Loss of heterozygosity

Mab	Monoclonal antibody
MAPK	Mitogen activated protein kinase
mdm2	Murine double minute 2 oncogene
MEK	Mitogen activated protein kinase kinase
mRNA	Messenger ribonucleic acid
NF	Nuclear factor
NGF	Nerve growth factor
OSM	Oncostatin
PAC	P1 artificial chromosome
PDGF	Platelet derived growth factor
PI	Phosphatidyl inositol
PI3'K	Phosphatidyl inositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PL	Placental lactogen
PLF	Proliferin
PLP	Prolactin-like protein
PRL	Prolactin
PRP	Prolactin related protein
PTK	Protein tyrosine kinase
PTPs	Phosphotyrosine phosphatases
5' RACE	5' Rapid amplification of cDNA ends
Raf	Serine-threonine kinase

RNA	Ribonucleic acid
RNAsin	RNA nuclease inhibitor
RT-PCR	Reverse transcription-polymerase chain reaction
SH	Src homology
Shc	Src homology 2 docking protein
snRNA	Small nuclear RNA
SOS	Son of sevenless nucleotide exchanger
SRE	Serum response element
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
Taq	*Thermus aquaticus* DNA polymerase
T_h	Hybridization temperature
TSH	Thyroid-stimulating hormone
5' UTR	5' Untranslated region
wks FA	Weeks fetal age

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PART I.

GENERAL INTRODUCTION

AND

LITERATURE REVIEW

CHAPTER 1

CHAPTER 1 - *GENERAL INTRODUCTION AND LITERATURE REVIEW*

1.1. Preface.

The first growth promoting factor was identified in 1933 as an impurity in prolactin (PRL) preparations of rat pituitary extracts (1). The distinct identities of growth hormone (GH) and PRL were clearly delineated in the early 1940's when GH was purified and all somatogenic activity of the extracts was attributed to GH (2,3). Initial experiments aimed at demonstrating the efficacy of subprimate GH preparations in humans and monkeys failed to show significant biochemical or metabolic activity (4). Because of these findings, scientists shifted their emphasis to isolating primate GH and, by 1957, Li and Papkoff had isolated both the human and monkey forms (5). Knobil et al then used primate GH to demonstrate that the effects of GH are species-specific: an anabolic response was observed when monkey GH was administered to hypophysectomized rhesus monkeys, whereas bovine GH had no effect (6). For the next three years, internists, endocrinologists and pediatricians tested the effects of both human (h) and monkey GH in human subjects (7). By 1960 it was clear that GH-deficient children would benefit from pituitary hGH (8,9).

Pituitary hGH was used to treat hGH deficiency until 1985 when several patients who had received the extracted form of the hormone were diagnosed with Creutzfeldt-Jakob disease (10). This tragedy resulted in the withdrawal of pituitary hGH from distribution in North America, causing a halt in an exciting era of pediatric

endocrinology. However, the development of pure 22 kD hGH using recombinant DNA technology by Genentech in the early 1980's, and its approval by the mid-1980's, has revolutionized the therapeutic treatment of hGH deficiency (reviewed in 11).

The availability of pure hGH has also been crucial for a number of subsequent basic science discoveries. Large amounts of hGH were necessary for the isolation and cloning of the human and rabbit growth hormone receptor (GHR) cDNAs (12). The cloning of the receptor made it possible to determine the crystal structure and formal binding biophysics of the hormone (13) and this, in turn, led to a rational design of a human (h) GHR antagonist (14). Our understanding of GH physiology has also been furthered with the discovery of GH binding proteins (GHBPs) (15) and with the application of molecular biology approaches to study *GH* and *GHR* gene expression (16), GHR intracellular signalling cascades and biologic responses to GH stimulus (17).

GH binds to its receptor and triggers growth-promoting effects as well as a wide range of metabolic activities (18). It is well accepted that these are crucial and necessary events for the normal development of a child and for the maintenance of metabolic homeostasis in the adult. However, the role of GH and its receptor during mammalian fetal life has been controversial (19,20).

To advance our understanding of the role of hGH in utero, my doctoral research has examined *hGHR* gene expression during human development. My initial findings, demonstrating that hGHR mRNA levels as well as specific isoforms (Chapters 2, 3 and 4, and Appendix 1) are under tissue and ontogenic regulation, led me to clone specific promoter regions in the *hGHR* gene (Chapter 4 and Appendix 2). These gene regulatory

DNA sequences may play crucial roles in differentiating hGHR function in fetal versus postnatal tissues. In addition, the cloning of these regulatory regions is the first step in evaluating whether genetic mutations within the *hGHR* gene promoters may play a role in the clinical condition of individuals with chronic hGH insensitivity. In a final series of experiments, I cloned the baboon GHR cDNA and provided evidence to suggest that the baboon is an appropriate in vivo model in which to characterize the mechanisms regulating primate *GHR* gene expression during development and under pathophysiological conditions (Chapter 5). Before presenting these chapters and discussing my findings, Chapter 1 gives an overview of GH as well as its receptor and binding proteins. The determinants of fetal and postnatal growth as well as human growth disorders are examined, to critically evaluate the importance of GH and its receptor during the different stages of mammalian development. Throughout these reviews the emphasis is on the human GH/GHR/insulin-like growth factor-1 (IGF-I) axis, but other species are often examined to point out interesting differences and similarities between animal models and humans.

1.2. Growth Hormone.

1.2.1. The GH gene cluster.

GH, PRL and placental lactogen (PL) are homologous hormones that are thought to have arisen from a common ancestral gene by two successive tandem duplications (21). GH and PRL genes are present in all vertebrates and originated from a common progenitor that underwent duplication approximately 400 million years ago. In the

human, the *hGH* gene is located on chromosome 17 (22) whereas the *hPRL* gene has been mapped to chromosome 6 (23). PL is only present in mammals and comparison of the cDNA sequences of the different PLs has suggested that primate PLs evolved from the GH lineage, while rodent and ruminant PLs arose from the PRL lineage (reviewed in 21,24).

The human *GH* and *PL* genes exist as a cluster of five closely related genes (91-99% identity) that are thought to have arisen through relatively recent duplication events (Figure 1.1) (reviewed in 21). It is suggested that a single primitive *hGH* gene duplicated and evolved to form the precursor *hPL* gene. Subsequently, the two gene array duplicated to form a four gene array and, approximately five million years ago, one of these genes duplicated to give rise to the *hPL-L* gene. This gene cluster spans 48 kb (23) and has been mapped to chromosome 17q22-24 (25). Sequencing analysis of the locus revealed that all five genes are in the same transcriptional orientation and that they are ordered, from 5' to 3', *hGH-N*, *hPL-L*, *hPL-A*, *hGH-V* and *hPL-B* (26). Two of the five related genes code for two hGH isoforms: the pituitary-specific hGH-N (for normal) (27) and the placental-specific hGH-V (for variant) (28). The corresponding proteins are highly homologous, with only 13 amino acid differences dispersed throughout the peptide chain (Figure 1.2) (29). The *hPL-A* and *hPL-B* genes are co-expressed in syncytiotrophoblast cells of the placenta (30): while the *hPL-A* and *hPL-B* genes code for the same protein (31), *hPL-L* is thought to be a pseudogene (32). Interestingly, gene transcripts sharing strong similarity to the four human placentally expressed hPL-A, hPL-B, hPL-L and hGH-V mRNAs as well as the pituitary-specific hGH-N mRNA isoform have been

Fig. 1.1. The five genes of the *hGH/hPL* gene cluster. Each gene is represented by a box and only those genes that are expressed in the indicated tissue are shaded. The sizes of the encoded proteins are given (kD) below their respective genes. Protein products that are still hypothetical are in parentheses. [Adapted from Cooke, N.E. and Liebhaber, S.A. In: Vitamins and Hormones, Ed. Litwack, G., Academic Press, San Diego, p. 385-457, 1995 (16).]

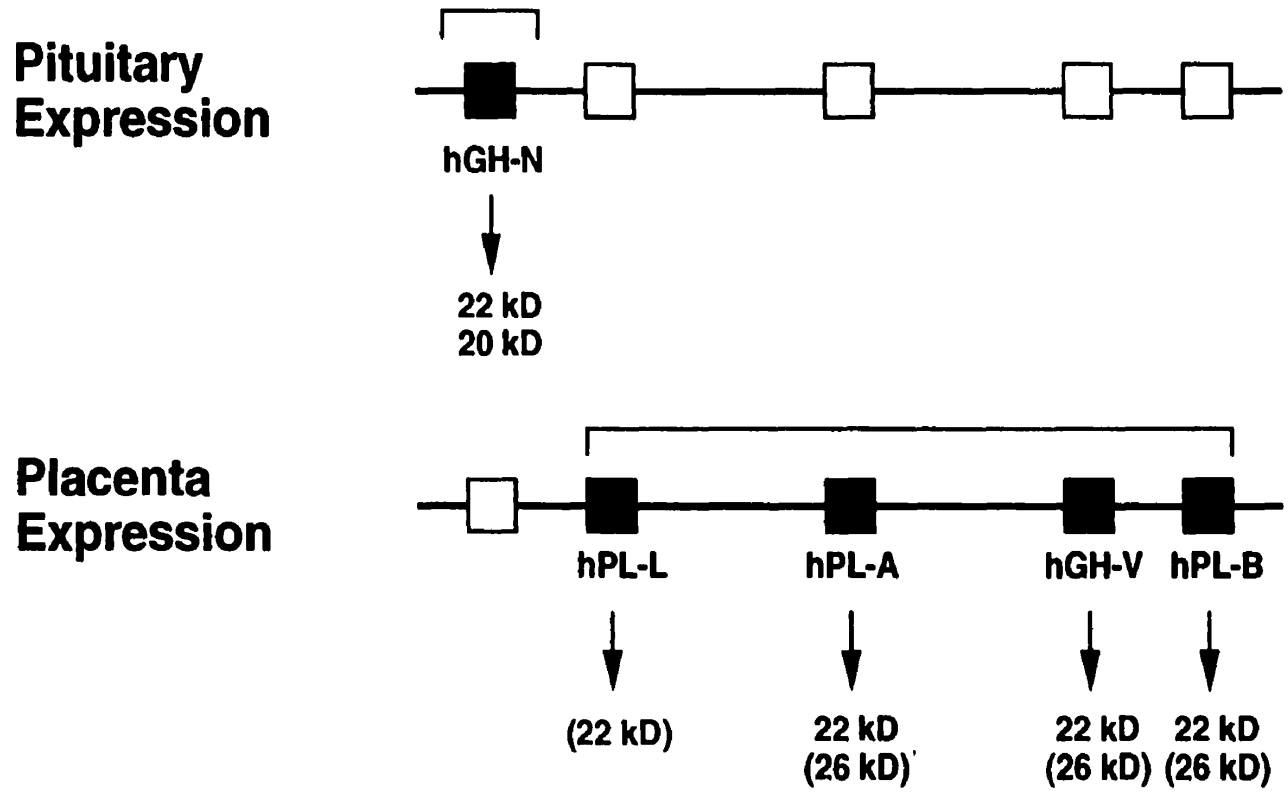
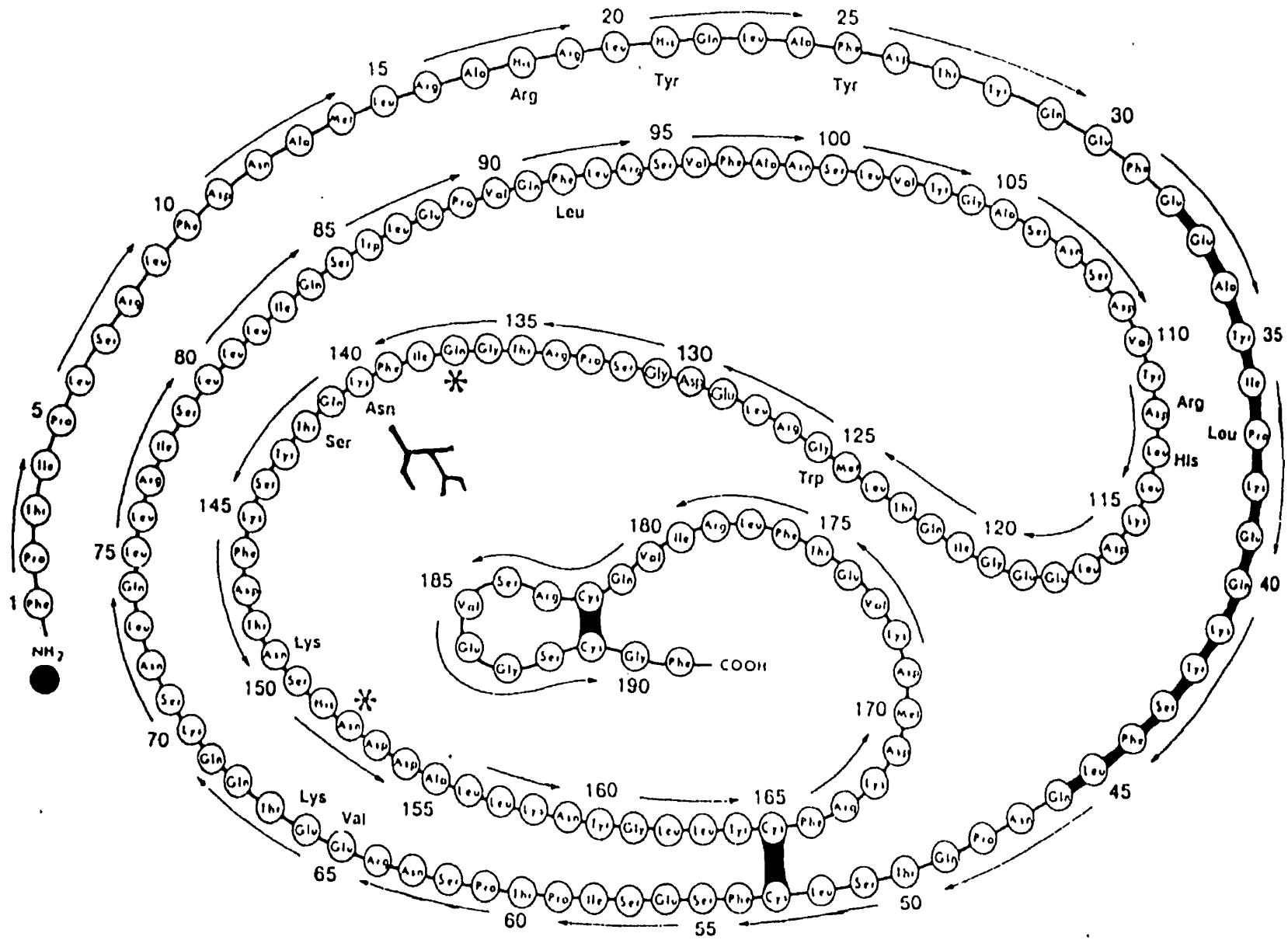

Pituitary Expression
hGH-N
22 kD
20 kD
Placenta Expression
hPL-L
hPL-A
hGH-V
hPL-B
(22 kD)
22 kD (26 kD)
22 kD (26 kD)
22 kD (26 kD)

Fig. 1.2. Primary structure of the different hGH forms. The 22 kD hGH-N polypeptide chain is shown. Amino acid changes in hGH-V are indicated next to the substituted residues. The sequence connected by the heavy lines (amino acids 32-46) is deleted in 20 kD hGH. Tree structure indicates the position of N-glycosylation in hGH-V. Asterisks denote sites of natural deamination, and the dot at the amino terminus depicts an acyl group. [Reprinted from Baumann, G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endo. Reviews 12: 424-49, 1988 (29).]


NH2
COOH
Arg
Tyr
Tyr
Leu
Asn
Ser
Trp
Arg
His
Leu
Lys
Lys
Val

identified in the Rhesus monkey (33). Although the genomic organization and linkage of the Rhesus monkey GH-related genes has not yet been delineated, it is likely to correspond closely to that in the human.

In contrast to the primate cluster of related *GH* genes, a *PRL* gene family exists in rodents. The rat *PRL* gene cluster is located on chromosome 17 (34) and has nine known members: PRL, PL-I, PL-$I_{variant}$, PL-II, PRL-like protein A (PLP-A), PLP-B, PLP-C, PLP-$C_{variant}$, and the decidual/trophoblast PRL-related protein (PRP) (34-42). Studies in the mouse have also identified several PRL-related placental-specific mRNA and/or proteins: PL-I, PL-II, PLP-A, PLP-B, PLP-E, PLP-F, proliferin (PLF) and PLF-related protein and decidual PRP (43-48). All of these genes, with the exception of *PLP-A* and the multicopy *PLF* genes, have been mapped to a 700 kb region on mouse chromosome 13 (47,49,50). Although present on chromosome 13, the *PLP-A* (47) and *PLF* (51) genes lie outside the 700 kb cluster. PL hormones have also been detected in several ruminant placentae (reviewed in 52). In addition, a PL-I homologue cDNA has been isolated from hamster placenta (53).

PLs expressed in ovine and bovine placentae were amongst the earliest purified to homogeneity (reviewed in 52). Structural genes coding for the bovine PL (54) and PRP-1 (55) as well as ovine PL (56) have been cloned and chracterized. Like the rodent *PRL* gene families, these gene products share a higher degree of amino acid sequence identity with PRL than GH. Although the chromosomal localization of ovine *PL* gene has not yet been determined, both placental-expressed members of the bovine *PRL* gene family map to chromosome 23 (57). Gene copy number has not yet been characterized

for the bovine *PL* and *PRP-1* genes, although ovine PL is known to be encoded by a single gene (52). In addition, alternative splicing of the bovine PL mRNA and allelic variants of the bovine *PL* gene have been reported, suggesting that there are multiple isoforms of bovine PL (55). In fact, five additional bovine PRP cDNAs have been isolated from placental mRNA: PRL-II, -III, -IV, -V, and -VI (58-60).

1.2.2. Pituitary hGH-N.

1.2.2.1. *hGH-N* gene expression and biosynthesis.

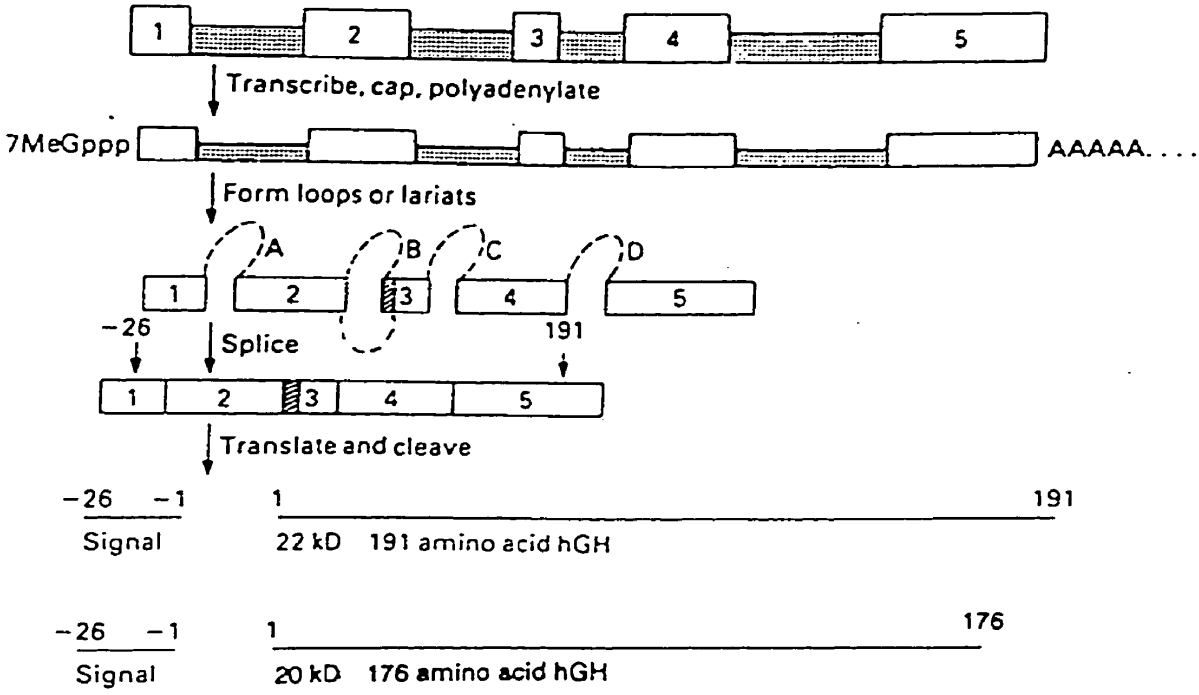
Five different trophic hormone-producing cell types are present within the adult mammalian anterior pituitary. Gonadotropes produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH), whereas corticotropes, thyrotropes, somatotropes and lactotropes synthesize adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), GH and PRL, respectively (reviewed in 61). During anterior pituitary development, there is a highly ordered sequence of cell-type differentiation, and induction of a specific cell-type is marked by the onset of gene transcription for one of the anterior pituitary hormones. In the primate and rodent, somatotrope-specific expression of the *GH* gene requires the binding of the transcription factor, Pit-1, to cis-acting elements within the gene promoter (reviewed in 46,62). Pit-1 is a member of the POU (Pit, Oct, Unc)-domain family of transactivators that is characterized by the conservation of a 60 amino acid homeobox DNA binding domain, as well as the POU-specific region that potentiates the strength and specificity of homeodomain DNA binding. This latter region consists of approximately 75 residues that are located N-terminally to the homeodomain.

The *hGH-N* gene transcript undergoes differential RNA splicing to contribute to hGH heterogeneity (Figures 1.1 and 1.3). In addition to the preferential splice site at the transition between intron B and exon 3, that is used to produce mRNA encoding for the 22 kD form, precursor hGH-N RNA can undergo alternative splicing in which a splice acceptor site within exon 3 is used to yield transcripts encoding for the 20 kD hGH-N (27,63). Approximately, 70-75% of hGH produced by the normal human somatotrope is 22 kD. The 20 kD form differs from the 22 kD protein by an internal deletion of residues 32 to 46 that results in a 176 instead of a 191 amino acid protein (Figure 1.2). Two additional alternatively spliced products of the *hGH-N* gene transcript have been described in pituitary tumor cells (64). The first is an exon 3 deficient form predicting a 17.5 kD protein. The other mRNA contains a frame shift that arises from alternative splicing of a distinct site within exon 3 from that used to generate the 20 kD isoform. However, it is not known whether these mRNAs are, in fact, translated.

The 22 kD form has both somatogenic as well as insulin-like and anti-insulin like properties (reviewed in 18,29,65,66). Comparative bioactivity studies of the two hGH hormones have shown that 20 kD hGH exhibits primarily growth-promoting actions and has its insulin-like properties reduced to about 20% of the potency of 22 kD hGH. In addition, the relative binding affinities of these two hGH forms can vary several-fold depending on the cell or tissue type being studied.

The 22 and 20 kD hGHs are synthesized as prehormones with a 26 amino acid hydrophobic signal sequence (reviewed in 29). The peptide is cotranslationally cleaved and the prehormone is short lived. hGH forms have been shown to undergo a number

Fig. 1.3. hGH-N mRNA alternative splicing patterns. Numbered boxes represent exons, whereas intervening introns are shaded. The portion of exon 3 that is alternatively spliced out is crosshatched. [Reprinted from Baumann, G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endo. Reviews 12: 424-49, 1988 (29).]


1
2
3
4
5
Transcribe, cap, polyadenylate
7MeGppp
AAAAA. . . .
Form loops or lariats
A
B
C
D
1
2
3
4
5
−26
191
Splice
1
2
3
4
5
Translate and cleave
−26 −1
1
191
Signal
22 kD 191 amino acid hGH
−26 −1
1
176
Signal
20 kD 176 amino acid hGH

of post-translational modifications, including deamination, acylation, glycosylation, and aggregation (oligomers) (Table 1.1). Phosphorylation of the polypeptide has been described in rats, sheep and chicken, but not in humans (reviewed in 29). There is also recent evidence that significant proteolytic cleavage of 22 kD occurs within the somatotrope to yield two fragments of 17 and 5 kD (reviewed in 66). Both products are released into the circulation. Although it remains controversial as to whether the 5 kD protein has bioactivity, the 17 kD protein (consisting of residues 44 to 191 of the native 22 kD form) has been described to lack the somatogenic and insulin-like properties of the 22 kD form while its hyperglycemic activity is 10-20 fold greater than that of 22 kD hGH.

Once secreted, the hormone circulates both free and bound to two recently characterized binding proteins (hGHBPs, see section 1.3.2) (reviewed in 29). The "big" hGH form in plasma is derived from pituitary oligomers (Table 1.1). hGH is degraded primarily in the kidney, where it is taken up and lysosomally degraded by tubular cells. Some of the fragments re-circulate and contribute to the heterogeneity. The possibility that bioactive fragments are metabolically generated remains to be assessed.

1.2.2.2. Control of hGH-N synthesis and release.

As much as 10% of the mammalian pituitary's dry weight is comprised of GH (reviewed in 61,67-69). Deep sleep (stages III and IV) and external stimuli such as exercise, stress as well as a high intake of protein potentiate GH secretion by the somatotrophs of the anterior pituitary. The synthesis and secretion of pituitary GH is

Table 1.1. Pituitary hGH variants [Reprinted from Baumann, G. Growth hormone heterogeneity: genes, isohormones, variants and binding proteins. Endocrinol. Rev. 12: 424-449, 1991 (29)].

Monomeric	
Native forms	
22,000 dalton hGH (22K)	70–75%
20,000 dalton hGH (20K)	5–10%
Desamido-hGH (22K-Asp^{152})	5–10%
Desamido-hGH (22K-Glu^{137})	3%
N_{α}-acylated hGH-22K	5%
Possibly native forms	
hGH_{1-43}	1%
hGH_{44-191}	?%
Nonnative forms	
Cleaved forms	
GH-C or $GH\text{-}_{\alpha 1}$	Variable
GH-D or $GH\text{-}_{\alpha 2}$	Variable
GH-E or $GH\text{-}_{\alpha 3}$	Variable
"Slow GH"	Variable
"Slow-slow GH"	Variable
Oligomeric forms (composed of the above)	
Native forms	
Noncovalent oligomers	
Dimers	10%
22K homodimers	
20K homodimers	
22K-20K heterodimers	
Higher oligomers	5%
Covalent oligomers	
Disulfide-linked oligomers	7%
22K-dimer	
22K/20K heterodimers	
Higher oligomers	
Linkage unknown	2%
Dimers	
Higher oligomers	
Nonnative forms	Variable
Aggregates of varying sizes depending on extraction	

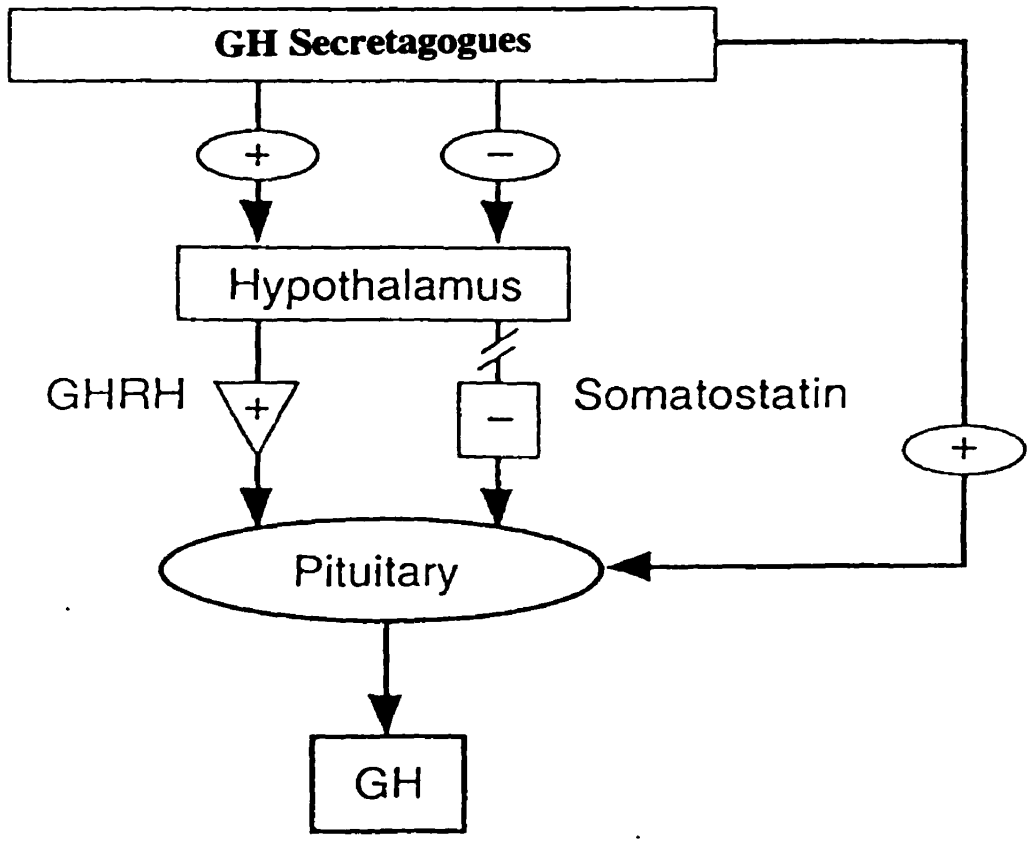
Values represent averages.

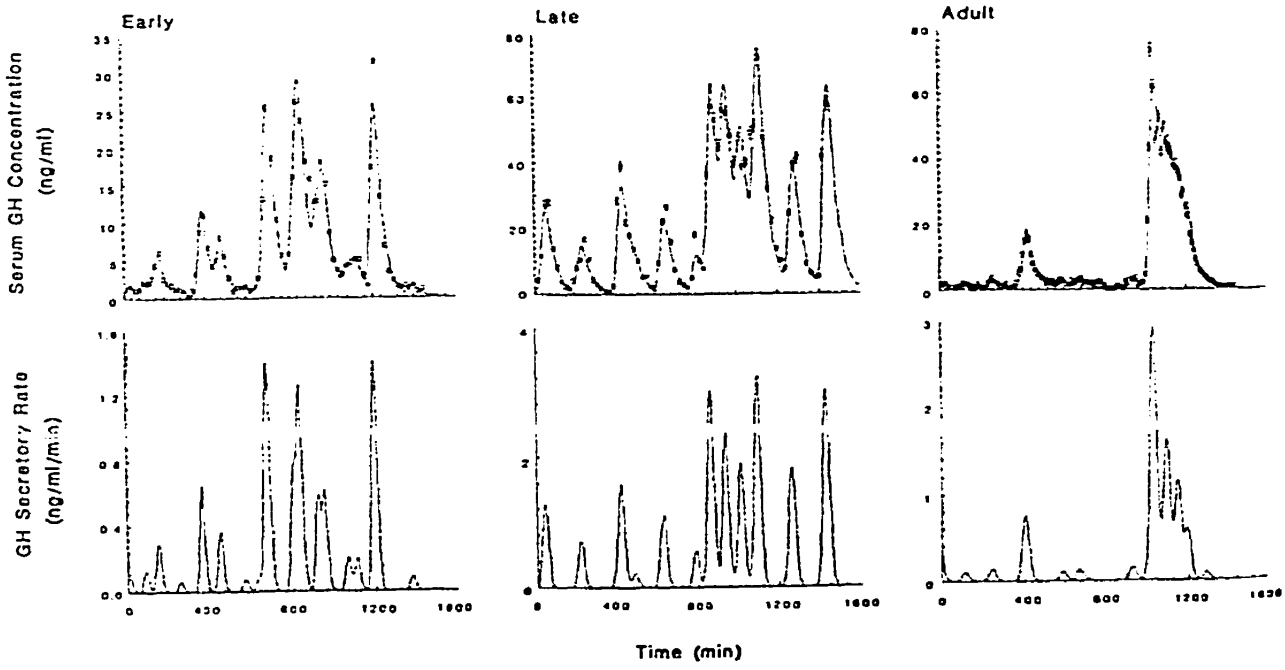
tightly regulated by a number of specific hypothalamic and systemic factors. Two known hypothalamic hormones, growth hormone-releasing hormone (GHRH) and somatostatin, are key regulators of GH release: GHRH stimulates production and secretion of GH, whereas somatostatin inhibits GH release without altering its biosynthesis (Figure 1.4). The somatostatin-containing nerve fibers arise primarily from the anterior hypothalamic periventricular system, whereas the GHRH-containing nerve fibers are mainly located in the arcuate nucleus and to a lesser extent in the ventromedial nucleus. Both GHRH and somatostatin are released into the capillary system within the median eminence and are carried to the anterior pituitary by the hypothalamo-hypophyseal portal system.

In addition to GHRH, there is also a family of synthetic GH-releasing peptides (GHRPs) (Figure 1.4) (reviewed in 70). GHRP-6, a hexapeptide, was the first member of this family to be developed that had biological activity in vivo. The relatively weak metabolic stability of GHRP-6 led to the development of two additional hexapeptides, GHRP-2 and hexarelin, with a longer half-life. More recently, several peptidomimetic GH secretagogues (eg. MK-677 and L-692,429) have been synthesized: these have the advantage of being able to be administered orally with high biological potency (reviewed in 71). Specific receptors for these GH secretagogues have been cloned (GHSRs) (72). Since GHRP-6 and its analogues were derived from met-enkephalin, the natural ligands for the GHSRs are thought to be "opioid-like".

Although there are some species-specific differences, thyroid hormone, estrogens, androgens, glucocorticoids, glucagon and retinoic acid generally stimulate GH production, whereas high levels of glucose, fatty acids and glucocorticoids decrease GH

Fig. 1.4. A model for control of pulsatile GH secretion by the somatotropes of the anterior pituitary. Upper panel, GHRH and GH secretagogues stimulate (+) GH synthesis and release, whereas somatostatin is inhibitory (-). Potential sites of action for these factors are shown. Lower panels, graphic representation of pulsatile hGH secretion in early puberty (left), late puberty (middle) and young adult (right) males. [Adapted from Smith, R.G. et al Peptidomimetic regulation of growth hormone secretion. Endo. Reviews 18: 621-645, 1997 (71); Martha, P.M.Jr. et al Endogenous growth hormone secretion and clearance rates in normal boys, as determined by deconvolution analysis: relationship to age, pubertal status, and body mass. J. Clin. Endo. Metab. 74: 336-344, 1992 (73).]


GH Secretagogues
+
−
Hypothalamus
GHRH
+
−
Somatostatin
+
Pituitary
GH


Early
Late
Adult
Serum GH Concentration (ng/ml)
GH Secretory Rate (ng/ml/min)
Time (min)

release (reviewed in 62,67,68). GH itself and IGF-I, whose synthesis is enhanced by increasing levels of GH, have a negative feedback effect on pituitary GH secretion both directly, at the level of the pituitary, and indirectly, by stimulating hypothalamic somatostatin release. However, it remains to be determined if GH and IGF-I feed back directly on somatostatin-containing neurons or whether the effect is indirectly mediated by the release of an intermediate factor.

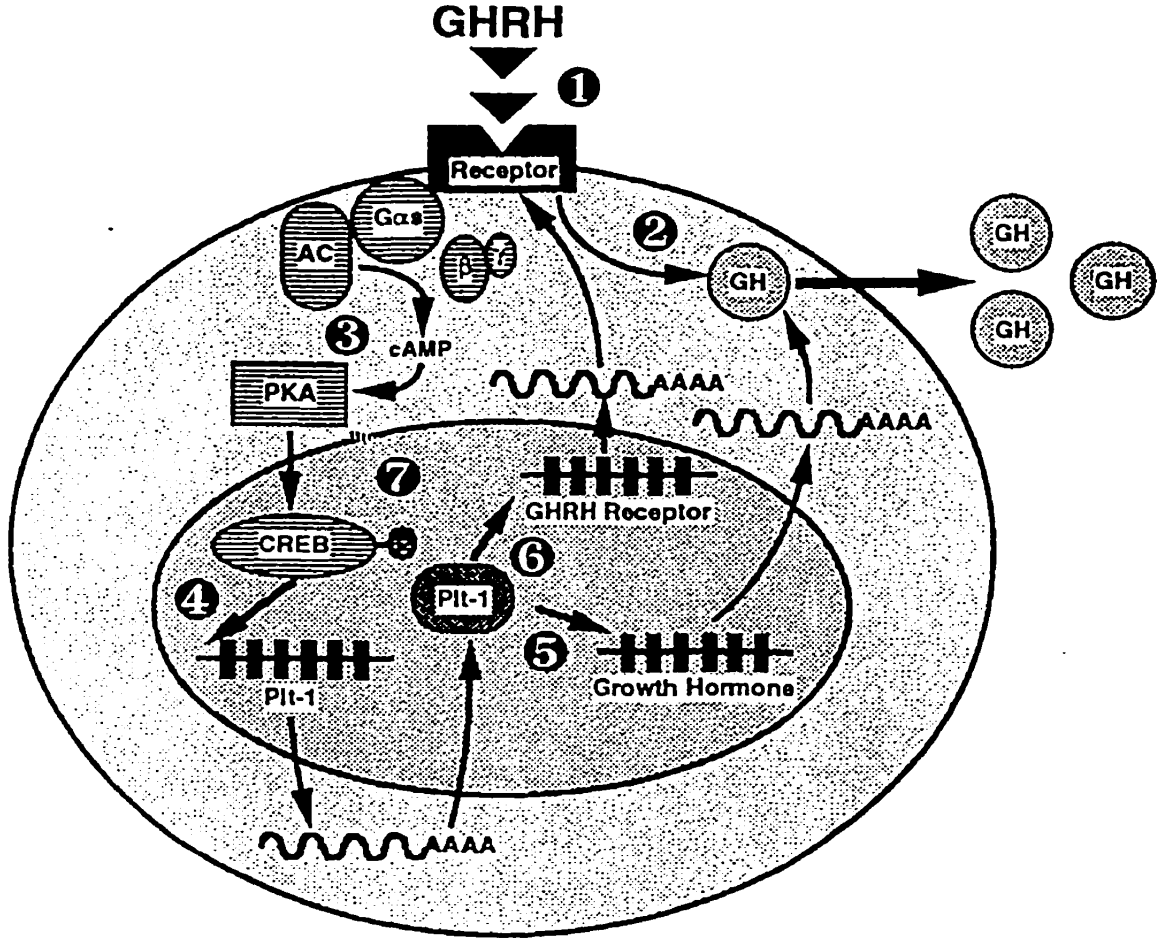
GH secretion follows an ultradian rhythm in all species that have been studied to date. In the rat and human, GH follows a pulsatile pattern with bursts of GH release approximately every three hours (Figure 1.4) (67,73). The amplitude of the peaks in the human are of lower magnitude than in the rat (74). In addition, there are night-time bursts of GH secretion in humans but not in rodents. Another difference between the two species is that the levels of rat GH peaks and troughs are markedly sexually dimorphic: in female rodents, GH troughs are higher and the peaks are lower than in males. This sex-specific distinction is not as striking in humans. The physiological significance of pulsatile GH secretion was elegantly demonstrated in GH-deficient animals in which GH replacement was shown to be more efficient in improving growth when administered in a pulsatile pattern rather than by constant infusion (75).

The discovery of the GHSR led to a re-evaluation of how GH pulsatility is controlled (Figure 1.4). Most recent data suggest that coupling of the biological oscillations of GHRH, somatostatin and the natural ligand for the GHSR sustains the characteristic pattern of GH secretion. Administration of exogenous GHSR ligand has shown that the secretagogue acts predominantly to antagonize hypothalamic somatostatin

release and to stimulate GHRH (reviewed in 71). This may result in a synergy between GHRH and the GHSR ligand at the level of the pituitary somatotrope to upregulate GH release. GH then feeds back on the hypothalamus to start a new cycle by increasing somatostatin inhibitory activity on GHRH-containing neurons and thereby decreasing GHRH and GH secretion. Smith et al have postulated that, although administration of a GHSR ligand can cause a resetting of the coupled oscillations, the secretagogue cannot overcome the inhibitory effects of somatostatin until somatostatin levels drop to a critical concentration, suggesting that somatostatin receptors are present in excess over GHSRs on the pituitary somatotropes (71).

Hypothalamic GHRH (44 and 43 amino acids in human and rat, respectively) acts on the anterior pituitary to induce GH gene transcription and secretion in both human and rat, as well as proliferation of somatotropes in rat (reviewed in 69). The GHRH receptor is a member of the G-protein coupled receptor family containing seven-transmembrane spanning domains. Once stimulated by an agonist, the GHRH receptor activates $G_{\alpha s}$ which in turn adenylate cyclase to produce cyclic adenosine monophosphate (cAMP) which, in turn, activates protein kinase A (PKA) (Figure 1.5). Patch-clamp experiments using rat somatotropes have demonstrated that GHRH activates, in a cAMP-dependent manner, a Na^+ cationic current, which depolarizes the plasma membrane to the threshold potential of L-type calcium channels. Phosphorylation of the calcium channels by PKA acts synergistically with Na^+ depolarization to further activate L-type calcium channels (reviewed in 76). Studies of human fetal pituitary explant cultures and adult GH-secreting adenomas have shown that in human, as in rat, GHRH-activation of adenylate cyclase

Fig. 1.5. A model for GHRH receptor signalling in somatotropes. (1) GHRH binding to its receptor leads to somatotrope GH release (2), possibly through the activation of L-type calcium channels. Stimulation of adenylate cyclase (AC) by the G_s protein (composed of three subunits: α, ß and γ) causes an increase in intracellular cAMP levels (3). cAMP then binds and activates PKA which, in turn, phosphorylates and activates the transcription factor CREB (7). Subsequently, transcription of the *Pit-1* gene is enhanced by CREB (4). Pit-1, in turn, activates transcription of the *GH* gene (5) which increases cellular stores of GH. Pit-1 also stimulates transcription of the *GHRH receptor* gene (6), which most likely leads to an increased number of GHRH receptors on the cell surface of the somatotrope. [Adapted from Mayo, K.E et al Growth hormone-releasing hormone: synthesis and signalling. Recent Prog. Hormone Res. 50: 35-73, 1995 (69).]

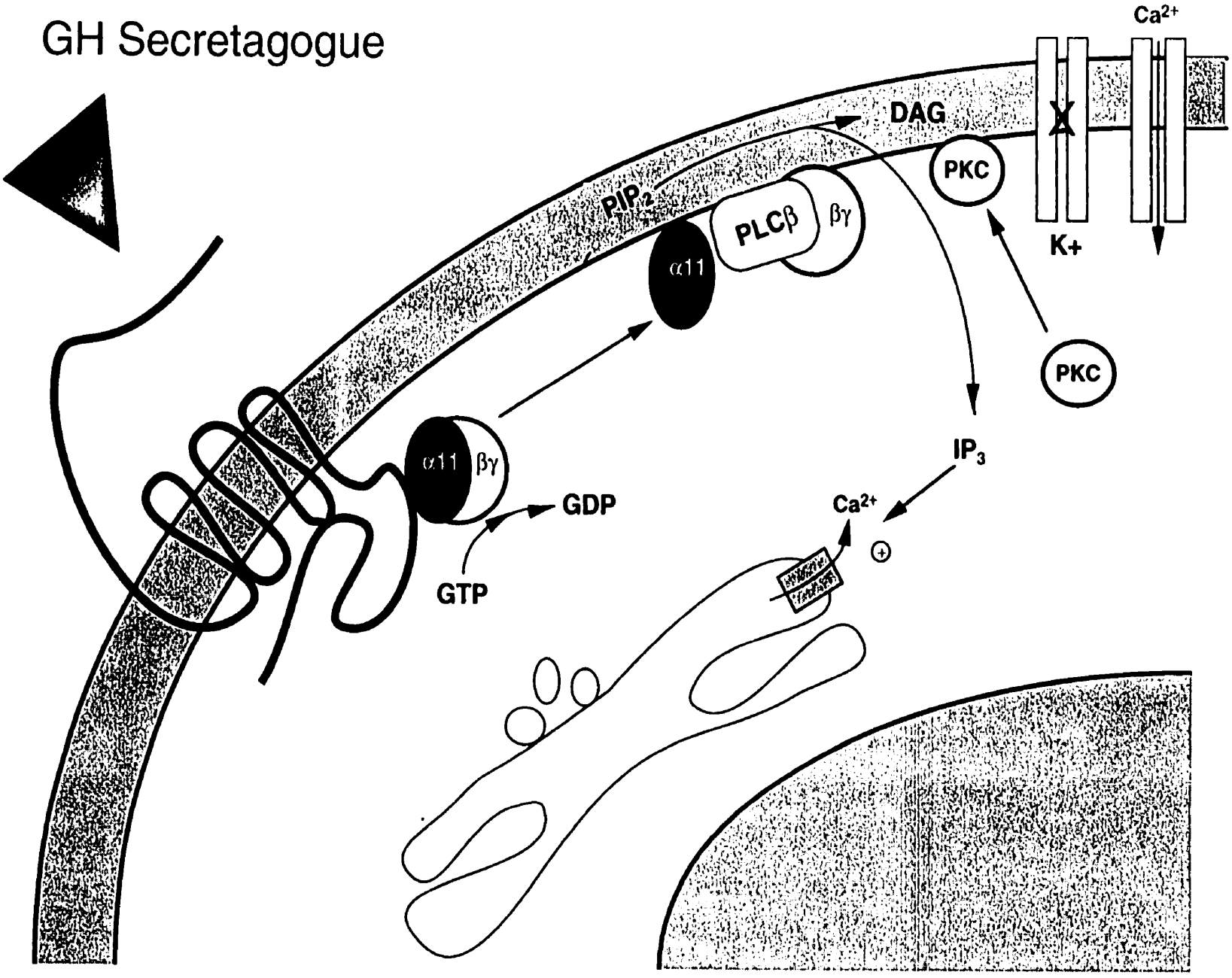

GHRH
1
Receptor
Gαs
AC
βγ
2
GH
GH
GH
GH
3
cAMP
PKA
AAAA
AAAA
7
GHRH Receptor
CREB
6
4
Pit-1
Pit-1
5
Growth Hormone
AAAA

and induction of calcium influxes are associated with increases in GH mRNA synthesis and release (Figure 1.5) (77). Interestingly, the human and rodent *GHRH receptor* genes are under transcriptional control of Pit-1 (78), the same transcription factor that is primarily responsible for cell-specific expression of GH, and Pit-1 is itself activated by the cAMP/PKA signalling cascade (79).

The GHSR is also a G-protein coupled receptor, but it does not share significant amino acid sequence identity with any other member of the G-protein coupled receptor family (72). Two GHSR cDNA isoforms were isolated from human and porcine pituitary: GHSR-1a cDNA sequence predicts a 366 amino acid receptor with seven transmembrane domains, whereas the GHSR-1b cDNA sequence predicts a truncated 289 residue GHSR that lacks transmembrane domains 6 and 7 (72). Transient expression studies have demonstrated that the GHSR-1a is able to bind GHRP-6, GHRP-2 and MK-0677 with high affinity as well as initiate cellular responses following ligand binding, whereas GHSR-1b displays no affinity for these ligands and, thus, no biological function of this isoform has yet been identified (72).

Several signalling cascades of the GHSR have been elucidated by treating subprimate somatotropes with the GHRP-6, GHRP-2, MK-0677 and MK-0751 synthetic GH secretagogues (Figure 1.6) (reviewed in 71). Induction of GHSR by GHRP-6, MK-0677 and MK-0751 stimulates phospholipase C activity which increases inositol trisphosphate (IP_3) turnover and causes both an increase of free intracellular calcium through an IP_3-dependent cascade as well as the translocation of protein kinase C (PKC). In addition, like GHRH, GHRP-6, MK-0677 and MK-0751 depolarize GH-secreting cells

Fig. 1.6. A model for GHSR signalling in somatotropes. For details, see section 1.2.2.2, pages 23 and 26. GTP, guanosyl triphosphate; GDP, guanosyl diphosphate; α_{11}, ß and γ, G-protein subunits; PLCß, phospholipase Cß; PIP_2, phosphatidylinositol diphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol; AC, adenylate cyclase. [Adapted from Smith, R.G. et al Peptidomimetic regulation of growth hormone secretion. Endo. Reviews 18: 621-645, 1997 (71).]


GH Secretagogue
Ca2+
DAG
PKC
PIP2
PLCβ
βγ
α11
K+
PKC
IP3
α11
βγ
GDP
Ca2+
GTP

by potentiating calcium entry through voltage-gated channels. The redistribution of intracellular calcium by IP_3 probably enhances GH release by synchronizing docking of GH-secretory granules to the plasma membrane and making them "readily releasable". Interestingly, pharmacological studies have suggested that there may be different pituitary GHSR subtypes since GHRP-2, in contrast to GHRP-6, MK-0677 and MK-0751, was found to increase cAMP instead of PKC activity in rat and ovine somatotropes (80). However, this finding may be the consequence of the same receptor coupling to different G-proteins when induced by different agonists.

Specific binding of the synthetic GH secretagogues has also been demonstrated in hypothalamic rat membranes and in situ hybridization studies have identified GHSR-1a expression in rat and rhesus monkey arcuate nuclei (70). GH secretagogue activation of *fos* gene transcription in GHRH-containing neurons of the rat arcuate nucleus has been observed (81). Moreover, intravenous administration of GHRP-6 and MK-0751 to anesthetized rats revealed electrical activity in secretory neurons projecting to the median eminence (81). Based on these data, it can be hypothesized that the activated hypothalamic GHSR may be potentiating GHRH secretion by the arcuate neurons into the median eminence.

Hypothalamic somatostatin is found as two bioactive peptides of 14 and 28 amino acids, with the former being a peptide cleavage product having a shortened N-terminus (reviewed in 82). These peptides were initially identified as inhibitors of GH but, since then, numerous other physiological activities have been described for the two peptides in non-hypothalamic tissues, such as pancreas and intestine. Five somatostatin receptor

genes with separate chromosomal localizations have been characterized (reviewed in 83,84). All receptor subtypes consist of seven transmembrane spanning domains and are coupled with G-proteins. Interestingly, multiple receptor subtypes have been detected in almost all tissues and cell lines studied, including the pituitary somatotroph (Table 1.2). Receptor subtypes 1 through 4 have similar high affinity for both somatostatin isoforms, while receptor subtype 5 has a preference for the 28 amino acid form. Each somatostatin receptor subtype activates an array of different intracellular cascades (Figure 1.7). Together, these findings explain the multitude of signalling pathways that are triggered by somatostatin treatment of somatotropes. For example, somatostatin reduces both basal and stimulated cAMP production, by activating G_i-proteins to reduce adenylate cyclase activity, while it lowers intracellular calcium levels through a G_o-protein-dependent inhibition of L-type voltage-gated calcium channels. Specific biological effects of the five different somatostatin receptor subtypes as well as their intracellular signal transducing mechanisms are summarized in Table 1.3.

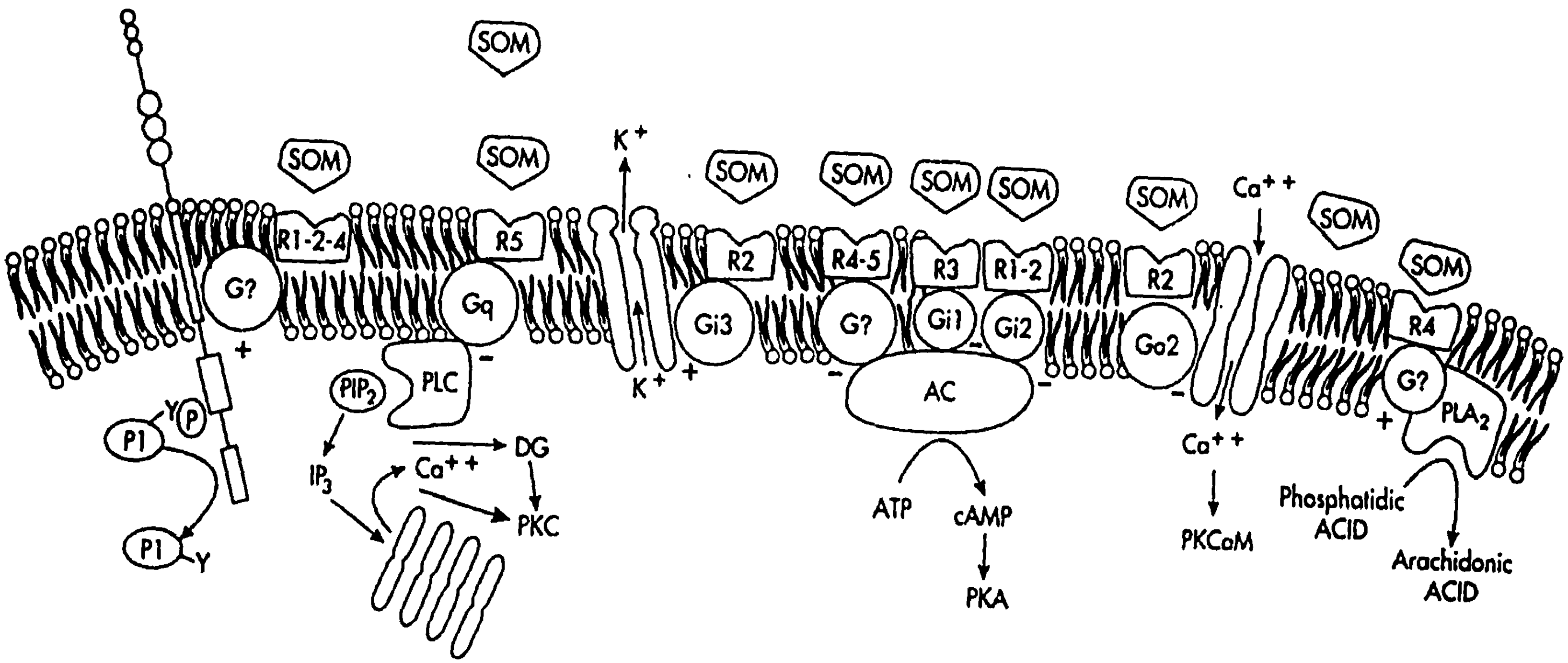
Receptor subtype 5 has been shown to mediate somatostatin inhibition of GH-release in rat pituitaries. Quantitative fluorescence immunocytochemistry and confocal microscopy studies have revealed that somatostatin receptor subtype 5 is the predominant isoform in rat somatotropes, followed by subtype 2, whereas subtypes 3 and 4 are moderately expressed and subtype 1 is the least expressed (85). These findings account for the ability of somatostatin-28, which has a higher affinity for receptor subtype 5, to inhibit human and rat somatotrope GH release with greater potency than somatostatin-14 (reviewed in 83,84). In the human, several studies have identified receptor subtypes 1,

Table 1.2. Expression of the somatostatin receptor subtypes (SSTRs) in the rat brain. [Reprinted from Florio, T. and Schettini, G. Multiple intracellular effectors modulate physiological functions of the cloned somatostatin receptors. J. Mol. Endo. 17: 89-100, 1996 (83).]

	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Brain area					
Cerebral cortex	++++	++++	++	++	+
Cerebellum	++	+	+++++	ND	ND
Hypothalamus	+++	+++	++	+	+
Hippocampus	+++	+++	++	+++++	+
Striatum	+	++	++	++	+
Amygdala	+++++	+++	++	++	+
Thalamus	++	++	+	+	ND
Olfactory bulb	+++	+++	+++	+++	+
Nucleus accumbens	++	+	+	++	ND

+<++<+++<++++<+++++, relative receptor concentration.
ND, not detected.

Fig. 1.7. Intracellular cascades induced by the five different somatostatin receptor subtypes. For details, see section 1.2.2.2, pages 26-32. SOM, somatostatin; R1,-2,-3,-4, and -5, somatostatin receptor subtypes. G, G-proteins; P1-YP, tyrosine phosphorylated protein; P1-Y, non-phosphorylated protein; PLC, phospholipase C; PIP_2, phosphatidylinositol diphosphate; IP_3, inositol triphosphate; DG, diacylglycerol; AC, adenylate cyclase; PKCaM, calmodulin-dependent protein kinase; PLA_2, phospholypase A_2. [Reprinted from Florio, T. and Schettini, G. Multiple intracellular effectors modulate physiological functions of the cloned somatostatin receptors. J. Mol. Endo. 17: 89-100, 1996 (83).]



SOM
SOM
SOM
K+
SOM
SOM
SOM
SOM
SOM
Ca++
SOM
SOM
R1-2-4
R5
R2
R4-5
R3
R1-2
R2
R4
G?
Gq
Gi3
G?
Gi1
Gi2
Go2
G?
PLC
PLA2
K+
AC
PIP2
P1
P
Y
P1
Y
IP3
Ca++
DG
PKC
ATP
cAMP
PKA
Ca++
PKCaM
Phosphatidic ACID
Arachidonic ACID

Table 1.3. Specific biological effects and signalling cascades of the somatostatin receptor subtypes (SSTRs). Only physiological activities of somatostatin reported to be mediated by specific SSTR subtypes are given. AC, adenylate cyclase; PL, phospholipase C; PA_2, phospholipase A_2; MAP kinase, mitogen activated kinases; PTPases, phosphotases. [Reprinted from Florio, T. and Schettini, G. Multiple intracellular effectors modulate physiological functions of the cloned somatostatin receptors. J. Mol. Endo. 17: 89-100, 1996 (83).]

	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Effect					
GH release		↓			
Insulin release					↓
Cell proliferation	↓	↓	=	↓	↓

↓, Inhibition; =, no effect.

	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
AC	↓	↓	↓	↓	↓
Ca^{2+} channels		↓			
K^+ channels		↑		↑*	
Na^+/H^+ exchanger	↓				
PA_2/MAP kinase				↑	
PLC	↑†	↑†	↑†	↑†/=	↑†/↓‡
PTPases	↑	↑/=		↑	

↑, Activation, ↓, inhibition; =, no effects.
*Indirectly through AA production.
†Only in transfected COS-7 cells.
‡Inhibition of CCK-stimulated PLC activity.

2 and 5 in fetal and postmortem pituitaries, but no detectable levels of subtypes 3 and 4 (86,87). However, this finding may only reflect lower expression levels of these latter two receptor subtypes as opposed to their complete absence.

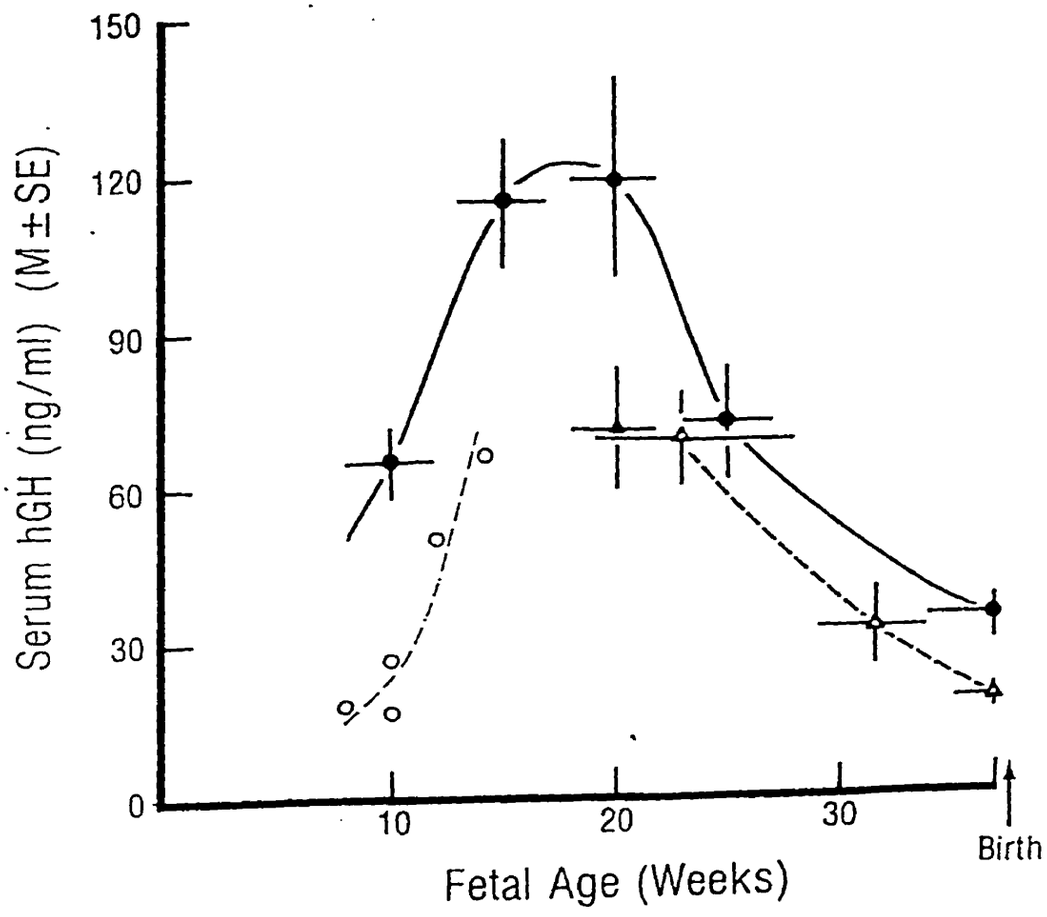
1.2.2.3. Ontogeny of hGH-N.

In the human fetal anterior pituitary, somatotrophic cells have been identified, by immunohistochemical methods, from as early as 6 weeks of fetal age (88-90). The content of hGH in the fetal pituitary increases steadily with gestational age, rising from less than 1 μg/gland at 10 weeks to greater than 60 μg/gland at term. Between the end of the first year of life and the beginning of puberty, there is a further 10-fold increase.

Human GH serum levels have been detected at approximately 8 weeks of fetal age (Figure 1.8) (91,92). They rise rapidly to a peak of $\sim$130 ng/ml at 20 to 24 weeks and then decrease until birth (91,93). However, circulating levels remain high even at term ($\sim$50 ng/ml) relative to the adult (1-10 ng/ml). Serum hGH continues to decline postnatally, reaching basal concentrations at three months of life.

The early high rate of hGH production is thought to be either an autonomous function of the fetal gland or is occurring in response to GHRH. The latter has been suggested by in vitro studies of 9-19 week human fetal pituitary cells which demonstrated a predominant response of somatotrophs to GHRH and a relatively limited inhibitory effect of somatostatin (94-96). There appears to be a progressive maturation of the mechanisms controlling hGH secretion during the second half of gestation and the early postnatal period. Although direct biological studies have not been done during this

Fig. 1.8. Schematic representation of serum hGH levels during human fetal life. Fetal plasma immunoreactive hGH levels rise to a peak between 20 to 24 weeks and then decrease until birth. [Data derived from (●) Kaplan, S.L. et al The ontogenesis of pituitary hormones and hypothalamic factors in the human fetus: growth hormone and insulin. J. Clin. Invest. 51: 3080-3093, 1972 (91); (○) Matsuzaki, F. et al Growth hormone in the fetal pituitary glands and cord blood. J. Clin. Endocrinol. Metab. 33: 908-911, 1971 (92); (△) Furuhashi, N. et al Plasma somatostatin and growth hormone in the human fetus and its mother at delivery. Gynecol. Obstet. Invest. 16: 59-62, 1983 (93).]


150
120
90
60
30
0
Serum hGH (ng/ml) (M±SE)
10
20
30
Birth
Fetal Age (Weeks)

developmental period, it is hypothesized that the hypothalamus continues to differentiate and that either secretion of GHRH decreases or certain inhibitory influences (ie. somatostatin) mature to overcome the actions of GHRH, resulting in the gradual fall in serum hGH. Recent bioassays using synthetic agonists with specific affinities for either somatostatin type 1 or 2 receptors have shown that human fetal pituitaries of 23 to 25 weeks of gestation respond to somatostatin inhibition of hGH release (97). However, this study did not provide developmental quantitative data to compare somatostatin receptor levels in the fetal pituitaries tested versus those found in adult somatotropes. Whether the GHSR and its natural ligand contribute to the high circulating levels of fetal hGH is as yet unknown.

Markedly elevated fetal plasma levels of immunoreactive GH is a characteristic of all mammals. However, the identity of either pituitary or circulating GH forms in the fetus has not been well investigated in any species. The only studies carried out in the human were in the early 1970's, using limited separation and detection methods available at the time (91,98-102). Critical evaluation of these data revealed the presence of unknown hGH isoforms in fetal sera that were significantly decreased in postnatal plasma, suggesting developmentally-related changes in the production of hGH. In support of this, our laboratory (C.G. Goodyer, unpublished data) and others (102a) have recently demonstrated that, unlike in the adult, at least 50% of the immunoreactive hGH found in fetal sera is not the 22 kD protein.

1.2.2.4. Biological effects of hGH-N on postnatal target tissues.

Pituitary-derived 22 kD GH exerts its postnatal growth-promoting and metabolic effects by binding to its widely expressed cell surface receptor (GHR). The insulin-like effects of GH on protein, glucose and fat metabolism are considered to be the result of acute events, whereas cholesterol lowering as well as increases in organ growth and muscle mass (protein synthesis), lipolysis and diabetogenic activities are due to chronic exposure (reviewed in 103,104). In contrast to the subprimate forms, human and monkey 22 kD GHs are also lactogenic and possess all the effects of PRL on the mammary gland (i.e., enhanced DNA synthesis, cell proliferation as well as milk protein, fatty acid and lactose synthesis).

Studies in the human and animal models have revealed that GH has effects on many organs; the majority of these effects are indirect and mediated by IGF-I (reviewed in 103-105). GHR mRNA analysis and binding experiments have suggested that, amongst postnatal tissues, liver is the richest source of GHRs (reviewed in 106). GH acts on hepatocytes to directly stimulate protein synthesis, amino acid uptake and glucose release from glycogen (reviewed in 104). GH exerts at least some of its pleiotropic effects by upregulating transcription of several genes in liver. For example, GH is responsible for regulating a large portion of the circulating IGF-I levels, by stimulating hepatic production of IGF-I (107). In addition, genes encoding the acid labile subunit (ALS) protein of the IGF binding protein-3 (IGFBP-3) complex, IGFBP-3, serine protease inhibitors 2.1 and 2.3, albumin and alcohol dehydrogenase as well as the transactivators c-fos, c-jun, c-myc, hepatocyte nuclear factor-6 (HNF-6) and CCAAT/enhancer binding

protein δ (C/EBPδ) are all activated following GH treatment of rat hepatocytes (108-115). These GH-induced transcription factors can then mediate secondary effects of the hormone by binding to their cis-acting elements in "downstream" target genes. An example of this is the mechanism by which GH exerts sexually dimorphic effects on rat liver gene transcription. Expression of the rat *CYP2C12* gene coding for cytochrome 450 2C12 protein is dependent on continuous exposure to GH (114). Although both males and females release GH in episodic pulses, the males have exaggerated peak and trough release patterns, while females have flatter oscillations (74). Thus, GH preferentially induces transcription of HNF-6 in the female liver; HNF-6, in turn, binds to cis-elements in the *CYP2C12* gene to enhance CYP2C12 mRNA levels in female rat hepatocytes (114).

Studies of GH action in human and rodent have shown that acute GH effects are most likely the result of direct GH action on target tissues (reviewed in 103,104). In contrast, chronic administration of GH results in elevated plasma levels of IGF-I and insulin, which may contribute to the effects of GH on target tissues. Most of the indirect biologic responses to GH are mediated by IGF-I (reviewed in 116). IGF-I, a 70 residue polypeptide in the human, is produced mainly by the liver but also by a variety of other target tissues following a GH stimulus; although GH is the key promoter of IGF-I synthesis in postnatal life, insulin and nutrition also have regulatory effects. In addition to IGF-I there is also IGF-II, a 67 amino acid protein in the human that is believed to be primarily regulated by insulin and nutrition, and only to a minor extent by GH, during postnatal life (reviewed in 116). Both IGFs (also known as somatomedins) can exert their

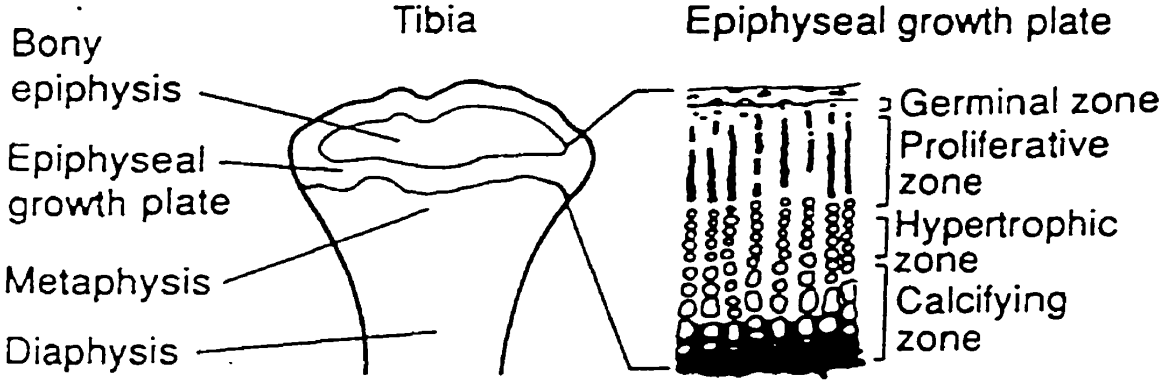
mitogenic and insulin-like responses in an endocrine, autocrine and/or paracrine fashion, by binding to the IGF Type I cell surface receptor (reviewed in 116,117). This receptor is analogous to the insulin receptor in that both receptors are composed of dimers of two α and two ß subunits held together covalently by inter- and intra-subunit disulfide bridges. The α subunits contain the hormone binding domains, whereas the ß subunits possess intrinsic tyrosine kinase activity that is induced with agonist binding. Interestingly, the Type I receptor has almost equal affinity for both IGFs, while the mannose-6-phosphate (IGF Type II) receptor only binds IGF-II (reviewed in 115). The Type II receptor is a single chain transmembrane protein and is thought to downregulate serum IGF-II levels by binding, internalizing and targeting IGF-II for cellular lysosomal degradation. The two IGFs bear a marked structural relationship to insulin and exhibit some degree of affinity for the insulin receptor while the converse is also true: insulin will bind to and activate the IGF Type I receptor when present at very high (μg) concentrations.

Direct as well as IGF-I mediated effects of GH reduce adipose tissue mass by inhibiting adipocyte differentiation, lowering triglyceride accumulation and increasing lipolysis (reviewed in 118). In the heart, GH probably acts through locally synthesized IGF-I to stimulate myocardial hypertrophy and to increase myocyte contractility (reviewed in 119). The actions of GH in increasing kidney size, glomerular filtration rate and renal plasma flow are also thought to be primarily mediated by IGF-I. In muscle, GH has anabolic effects (increased protein synthesis) and antagonizes insulin activity (reviewed in 120).

The lean body mass (LBM) of GH-deficient patients is significantly reduced as compared to matched control subjects (reviewed in 104). GH replacement therapy was found to increase and, subsequently, maintain the normalized LBM in these patients. In contrast to insulin, the anabolic effects of recombinant 22 kD GH on GH-deficient adults potentiate protein synthesis without altering proteolysis. The anabolic effects of GH on protein metabolism also provide therapeutic benefits to individuals suffering from catabolic diseases. A positive nitrogen balance has been observed with GH treatment in post-operative, burn, trauma and AIDS patients.

GH is probably best known for its ability to promote statural growth by its influence on endochondral ossification in the prepubertal and pubertal child. Extension of long bones is the result of cartilage being replaced by bone in the epiphyseal growth plate (Figure 1.9) (reviewed in 121). Chondrocytes in the growth plate have a longitudinal columnar organization. Prechondrocytes from the germinal zone near the bony epiphysis differentiate into chondrocytes and enter the proliferative zone where they continue to replicate and secrete cartilage-specific matrix components. The cytoplasmic volume of the cells increases as they migrate into the hypertrophic zone, which is accompaned by a change in gene expression. The hypertrophic cells calcify their surrounding matrix by mediating deposition of crystalline hydroxyapatite. At the zone of vascular invasion, blood vessels deliver chondroclasts that resorb some of the calcified cartilage; osteoblasts then deposit bone on the remaining cartilage remnants. The formation of bone in the diaphyseal region is the mechanism by which the bone is elongated.

Fig. 1.9. The proximal tibial epiphyseal growth plate and its cellular organization. For details, see section 1.2.2.4, page 39. [Reprinted from Ohlsson, C. et al Endocrine regulation of longitudinal bone growth. Acta Paediatr. Suppl. 391: 33-40, 1993 (121).]


Tibia
Epiphyseal growth plate
Bony epiphysis
Epiphyseal growth plate
Metaphysis
Diaphysis
Germinal zone
Proliferative zone
Hypertrophic zone
Calcifying zone

The exact mechanism by which GH promotes endochondrial ossification has not yet been clearly delineated, although two hypotheses are presently being tested. The somatomedin hypothesis postulates that all growth promoting action of GH on long bones is mediated by IGF-I, which is produced in response to GH stimulation of the liver and other tissues, including the growth plate (reviewed in 105,122). This hypothesis was based on the finding that IGF-I increased tibial epiphyseal cartilage plate width, body weight and DNA synthesis in hypophysectomized rats following ten days of continuous IGF-I infusion (123). However, subsequent studies reported an increase in body weight but only moderate effects on bone length after IGF-I administration in normal growing and hypophysectomized rats (124) as well as Snell dwarf mice (125). Similarly, a third investigation showed an acceleration in longitudinal bone growth of hypophysectomized rats only after pharmacological concentrations of IGF-I, whereas GH produced more effective results with a lower pharmacological dose (126). These IGF-I effects are analogous to the responses observed with GH administration in hGH-deficient patients. On the other hand, the ability of IGF-I to induce growth in patients with a mutated *hGHR* gene supports the somatomedin hypothesis (127,128). However, these latter studies have been limited to approximately 3 years and IGF-I effects on long bone growth decrease with time.

The somatomedin hypothesis has been further challenged by the observations of Isaksson et al suggesting that GH has a direct action in stimulating chondrocyte proliferation in the growth plate (129). These investigators found that GH infusion into the cartilage of hypophysectomized rats results in increased bone growth. More recent

studies in the rat have identified GHR and specific GH binding in the germinal layer of prechondrocytes, whereas IGF-I specific binding and receptors were observed in the proliferative zone of the growth plate (130). Further in vitro studies have shown that GH induces proliferation of rat stem chondrocytes, while IGF-I stimulates the formation of colonies from the intermediate zone (131,132). Based on these findings, the dual effector hypothesis was proposed: GH stimulates a clonal expansion of stem chondrocytes, which then differentiate to form IGF-I producing chondrocytes. IGF-I then acts in an autocrine/paracrine fashion to induce proliferation of the committed chondrocytes in the proliferative zone.

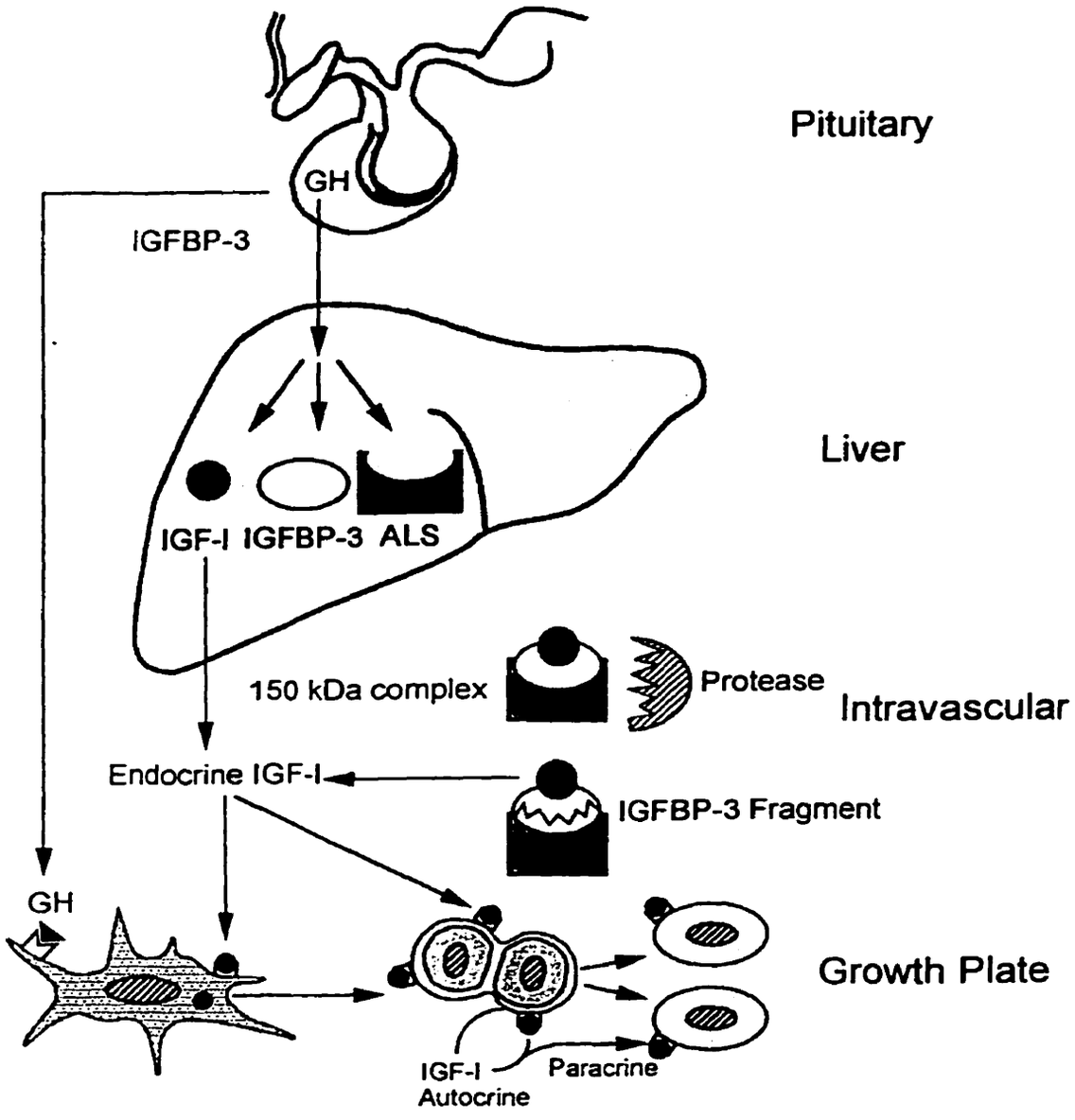
The biological roles of GH and IGFs are markedly affected by the presence of circulating IGF binding proteins (named IGFBP 1, 2, 3, 4, 5, 6 and 7) (reviewed in 105,133). These carrier proteins prolong IGF half-life in serum from 30 minutes to as much as 12-15 hours, transport IGFs across capillary barriers, and enhance or inhibit the presentation of IGFs to cell surface receptors. IGFBP-3 is the major somatomedin carrier protein in postnatal serum, representing 75% of all IGFBP isoforms. It transports between 70-90% of the circulating somatomedins. IGFBP-3 forms a 150 kD complex that consists of IGF-I or IGF-II, IGFBP-3 (a 40-60 kD glycosylated protein) and an acid labile subunit (ALS; an 85 kD glycosylated protein): IGFs first bind to IGFBP-3 and then ALS associates with the complex. Both IGFBP-3 and ALS are synthesized predominantly in the liver. In humans, IGFBP-3 synthesis is regulated directly by GH. GH-deficient patients have low IGFBP-3 serum levels that are normalized by GH administration (134), while acromegalics (individuals with pituitary tumours secreting high levels of hGH) have

increased amounts of this carrier protein in their plasma (135). In contrast to human IGFBP-3, rodent IGFBP-3 is positively regulated by IGF-I, not GH: administration of IGF-I causes an increase of serum IGFBP-3 levels of hypophysectomized rats, while GH has no effect (136). In both humans and rodents, production of ALS is GH-dependent: patients with GH insensitivity have reduced levels of plasma ALS (137), while mice overexpressing IGF-I, but which are GH-deficient, have low levels of the 150 kD ALS-containing IGFBP-3 complex in their serum (138).

Somatomedin bioavailability is also regulated by IGFBP proteases (139). These enzymes degrade IGFBPs and reduce the carrier molecules' affinities for IGFs, liberating the hormones for binding to their cell surface receptors. Together, IGFBPs, ALS and these proteases help to coordinate GH and IGF-I actions by controlling the delivery of IGF-I from the circulation to target cells. Treatment of patients with GH insensitivity with IGF-I results in 80% of the growth response obtained when GH-deficient patients are administered GH (127). This difference is thought to occur because of the direct effects of GH on prechondrocyte differentiation as well as on IGFBP-3 and ALS production. IGF-I administration to patients with GH insensitivity does not increase serum IGFBP-3 and ALS concentrations, keeping the half-life of the infused IGF-I low. In contrast, GH treatment of GH-deficient individuals (with functional GHRs) stimulates both IGFBP-3 and ALS production and release into plasma, helping to prolong the plasma half-life of GH-induced IGF-I bioactivity (reviewed in 105).

Based on the key observations summarized above, Spagnoli and Rosenfeld have proposed an appealing model for postnatal skeletal growth that combines the

Fig. 1.10. An integrated model for the long-bone growth process, showing the synergistic roles of GH, IGFBP-3 and IGF-1. For details, see section 1.2.2.4, pages 44 and 47. [Reprinted from Spagnoli, A. and Rosenfeld, R.G. In: Growth and Growth Disorders, Endocrinology and Metabolism Clinics of North America, Ed. Rosenfield, R.L., W.B. Saunders Co., p.615-631, 1996 (105).]


Pituitary
GH
IGFBP-3
Liver
IGF-I IGFBP-3 ALS
150 kDa complex
Protease
Intravascular
Endocrine IGF-I
IGFBP-3 Fragment
GH
Growth Plate
IGF-I
Autocrine
Paracrine

somatomedin and dual effector hypotheses as well as IGFBP-3 biological actions (Figure 1.10) (105). They suggest that the majority of bone growth is controlled by endocrine actions of IGF-I and that GH-induced IGF-production by the liver and other tissues is critical. IGF-I travels to the growth plate where it exerts its somatogenic actions. GH also stimulates IGFBP-3 and ALS production by the liver. These two molecules associate with IGF-I to form a 150 kD complex that increases the half-life of IGF-I. IGFBP-3 is degraded by a family of proteases to liberate IGF-I and enhance its availability for binding to Type I IGF receptors on the growth plate. In addition to its endocrine effect, IGF-I acts in an autocrine/paracrine manner within the growth plate. IGF-I production at the growth plate is regulated by GH action on prechondrocytes that causes them to differentiate into IGF-I producing and responsive chondrocytes.

1.2.2.5. Biological effects of hGH-N on fetal target tissues.

Fetal hypophysectomy and decapitation experiments in several animal species have suggested that GH does not have major effects during fetal life (140). However, responses to GH treatment, using doses comparable to the immunoreactive GH levels in the fetal circulation, have been observed in fetal cell culture studies. Ovine as well as rat GH can stimulate glucose incorporation into glycogen in hepatocytes obtained from fetal rats of 20 days of gestation (141). Organ culture studies of 17 day fetal rat tibiae and 18-19 day fetal rat metatarsal bone (composed entirely or mainly of chrondrocytes) have provided evidence of a direct GH effect on long bone growth through the stimulation of local IGF-I production within the growth plate (142). GH has recently been shown to

stimulate glucose transport and protein synthesis in mouse blastocysts (143). Moreover, Fukaya et al have demonstrated that GH promotes mouse embryo development: when two-cell stage mouse embryos were cultured with GH, the rate of blastocyst formation was significantly higher than in controls (144). Finally, the localization of GH, its receptor and binding protein in rat fetal odontogenic cells has suggested a role for GH in embryonic tooth development (145).

There are also several in vitro studies demonstrating responsiveness of human fetal tissues to 22 kD hGH-N. Fetal hepatocytes undergo DNA synthesis and begin to release IGF-I in response to hGH treatment (25-250 ng/ml) from as early as 8 weeks of fetal life (146). Interestingly, the presence of antibodies against IGF-I reduces the hGH-induced effect on DNA synthesis, suggesting that IGF-I partially mediates this hGH mitogenic action. More recently, work in our laboratory has demonstrated that first trimester hepatocytes take up [^{14}C]glucose in response to hGH in a dose-dependent manner (147). In the fetal pancreas, hGH treatment of islet cells (12-21 weeks of gestation) stimulates insulin gene transcription (148-150). Finally, in vivo data for an in utero hGH biological effect is provided by the presence of the GH-dependent IGFBP-3 complex in fetal serum from as early as midgestation (151).

1.2.3. Placental hGH-V.

1.2.3.1. *hGH-V* gene expression and biosynthesis.

The *hGH-V* gene is transcribed in the syncytiotrophoblast cells of the placenta to produce a prohormone which is quickly cleaved to give rise to a soluble 22 kD protein;

this form of hGH-V can also be N-glycosylated to attain a molecular weight of 25 kD (Figure 1.2) (28,152). The *hGH-V* gene transcript also undergoes alternative RNA splicing to generate an mRNA in which intron D is retained, causing a frameshift. The resultant mRNA transcript predicts a 26 kD protein with a carboxy-terminus that is entirely different from the soluble 22 kD isoform and includes a hydrophobic region (hGH-V2) (153). The rate of alternative splicing of the hGH-V gene transcript increases with gestation: expression of hGH-V2 mRNA rises from 5 to 15% of total hGH-V mRNA levels from the first trimester to term (154). Injection of this alternatively spliced hGH-V2 mRNA isoform into Xenopus oocytes results in the expression of a membrane-associated nonsecretory hGH-V protein (155). Although hGH-V2 mRNA is readily detectable in the placenta and can be expressed in artificial test systems, the existence of a hGH-V2 protein in placenta has not yet been established and its biological significance remains unknown. In contrast to hGH-N expression, a 20 kD hGH-V isoform does not exist because of minor nucleotide differences that prevent this particular alternative splicing of the hGH-V gene transcript. Interestingly, there has not been any definitive identification of a placental-specific GH-V in subprimates to date.

1.2.3.2. Control of hGH-V synthesis and release.

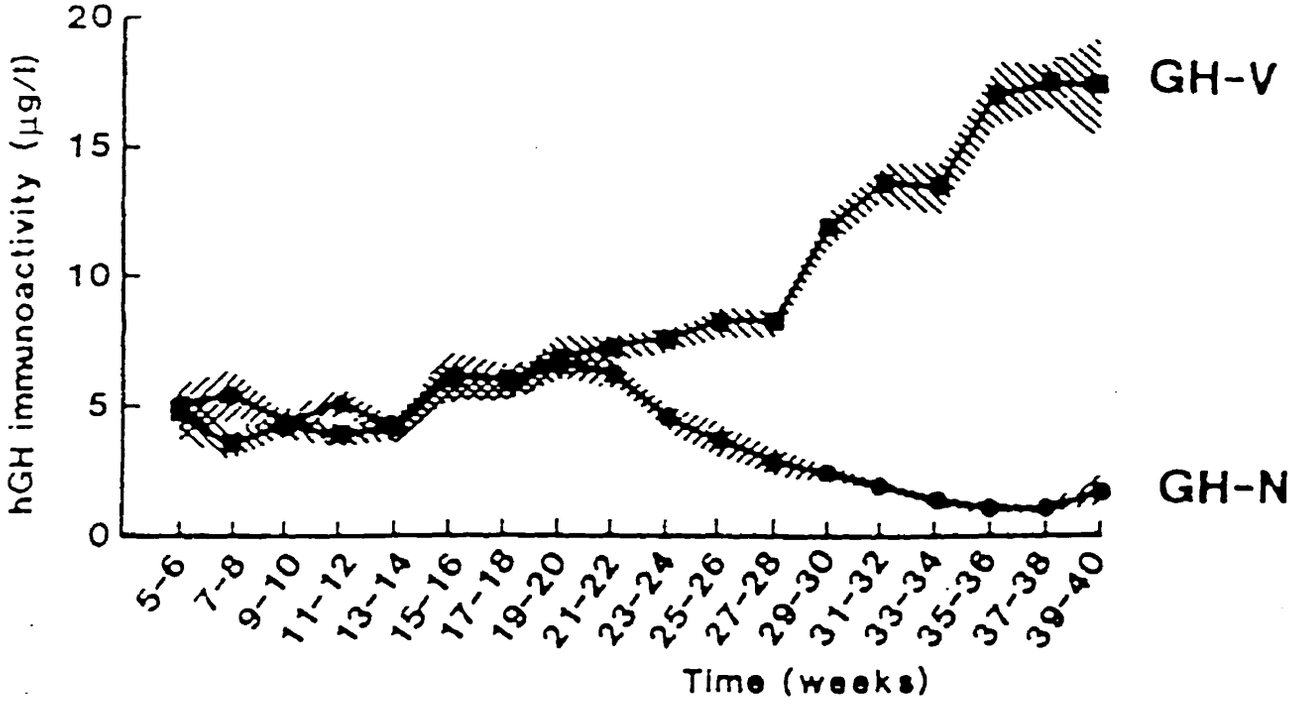
Unlike pituitary hGH-N secretion, placental hGH-V is apparently not under acute GHRH or somatostatin control (156), nor does it follow a pulsatile pattern of release (157). However, recent investigations in our laboratory have revealed that both the *hGHRH receptor* and *hGHSR* gene are expressed in human placental villous tissue,

suggesting that hGHRH and/or the natural ligand of GHSR may be implicated in regulating hGH-V release through a more chronic type of regulatory mechanism (158). Significant levels of hGH-V peptide have been found in maternal but not fetal blood (159). The maternal plasma concentrations of hGH-V climb during the course of pregnancy and, by the second half of pregnancy, this hGH variant suppresses maternal somatotrope activity and becomes the dominant hGH peptide in maternal circulation (Figure 1.11) (160).

1.2.3.2. Biological effects of hGH-V on target tissues.

The physiological roles of hGH-V are, as yet, unknown. In vitro studies suggest that the soluble 22 kD form has both growth-promoting and lactogenic properties, with its somatogenic activity being equivalent to that of hGH-N whereas its lactogenic bioactivity is significantly lower (160,161). Although hGH-N and hGH-V bind with equal affinity to the hGHR, hGH-V has relatively greater somatogenic effects as compared to hGH-N. Overexpression of hGH-V in transgenic mice confirmed the growth potentiating effect of placental hGH-V to be equivalent to that of hGH-N (162). The ability of hGH-V to activate certain metabolic pathways has also been demonstrated using rat adipocytes: the placental and pituitary hGH forms were found to be equally potent in increasing glucose oxidation, inducing lipolysis and stimulating refractoriness of the cells to hGH's insulin-like activity (160). It has been speculated that hGH-V may have important placental growth-promoting and nutrient transfer roles in utero. The replacement of hGH-N by hGH-V in maternal sera during pregnancy suggests that hGH-V may also have a

Fig. 1.11. Maternal plasma patterns of hGH-N and hGH-V during pregnancy. hGH-V becomes the predominant hGH in the second half of pregnancy. Most of all hGH immunoreactivity before week 22 is due to hGH-N. [Reprinted from Frankenne, F. et al The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant. J. Clin. Endo. Metab. 66: 1069-1072, 1988 (159).]


20
15
10
5
0
hGH immunoactivity (µg/l)
GH-V
GH-N
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
23-24
25-26
27-28
29-30
31-32
33-34
35-36
37-38
39-40
Time (weeks)

role in maintaining the metabolic demands of pregnancy (160). In addition, a positive correlation exists between the maternal plasma levels of hGH-V and hIGF-I, suggesting that, like hGH-N, hGH-V potentiates the production of IGF-I by human target cells (163-165).

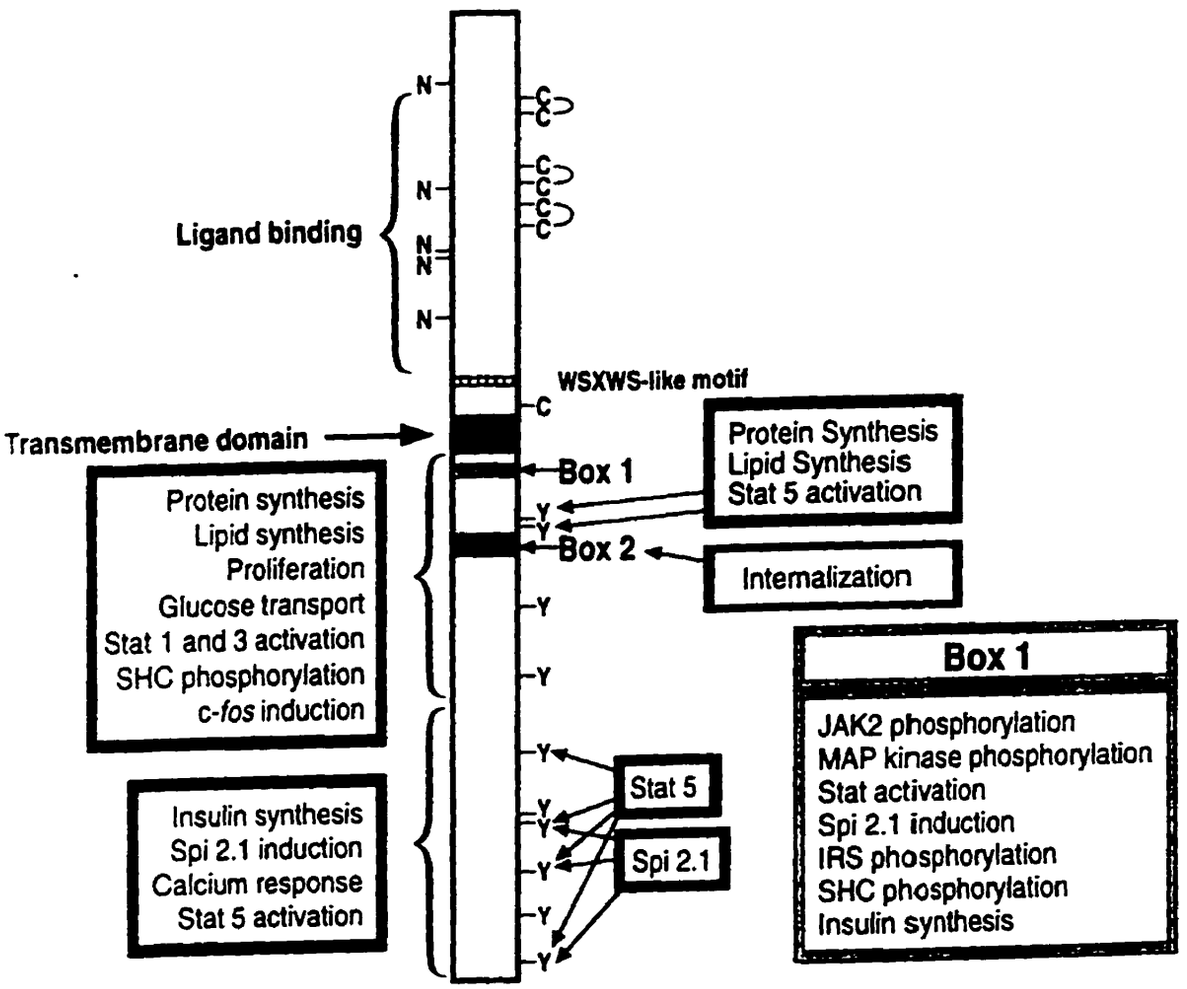
1.3. The Growth Hormone Receptor and Binding Proteins.

1.3.1. GHR protein structure.

The GHR was first purified to homogeneity from rabbit liver in 1987 by Leung et al, permitting the subsequent cloning of the human and rabbit GHR cDNAs (12). Since then, several other mammalian GHR cDNAs have been cloned (rhesus monkey, rat, mouse, bovine, sheep and pig), revealing that there is approximately 70% GHR amino acid sequence identity among these species (reviewed in 17). The primary structure of the hGHR consists of a single chain of 620 amino acids, containing a 246 amino acid extracellular domain, a short 24 amino acid transmembrane domain, and a cytoplasmic domain of 350 residues (Figure 1.12). The GHR is approximately 130 kD and its extracellular domain contains five potential N-glycosylation sites that are conserved in all species examined to date. The electrophoretic mobility of the GHR suggests the protein is indeed glycosylated at several of these sites.

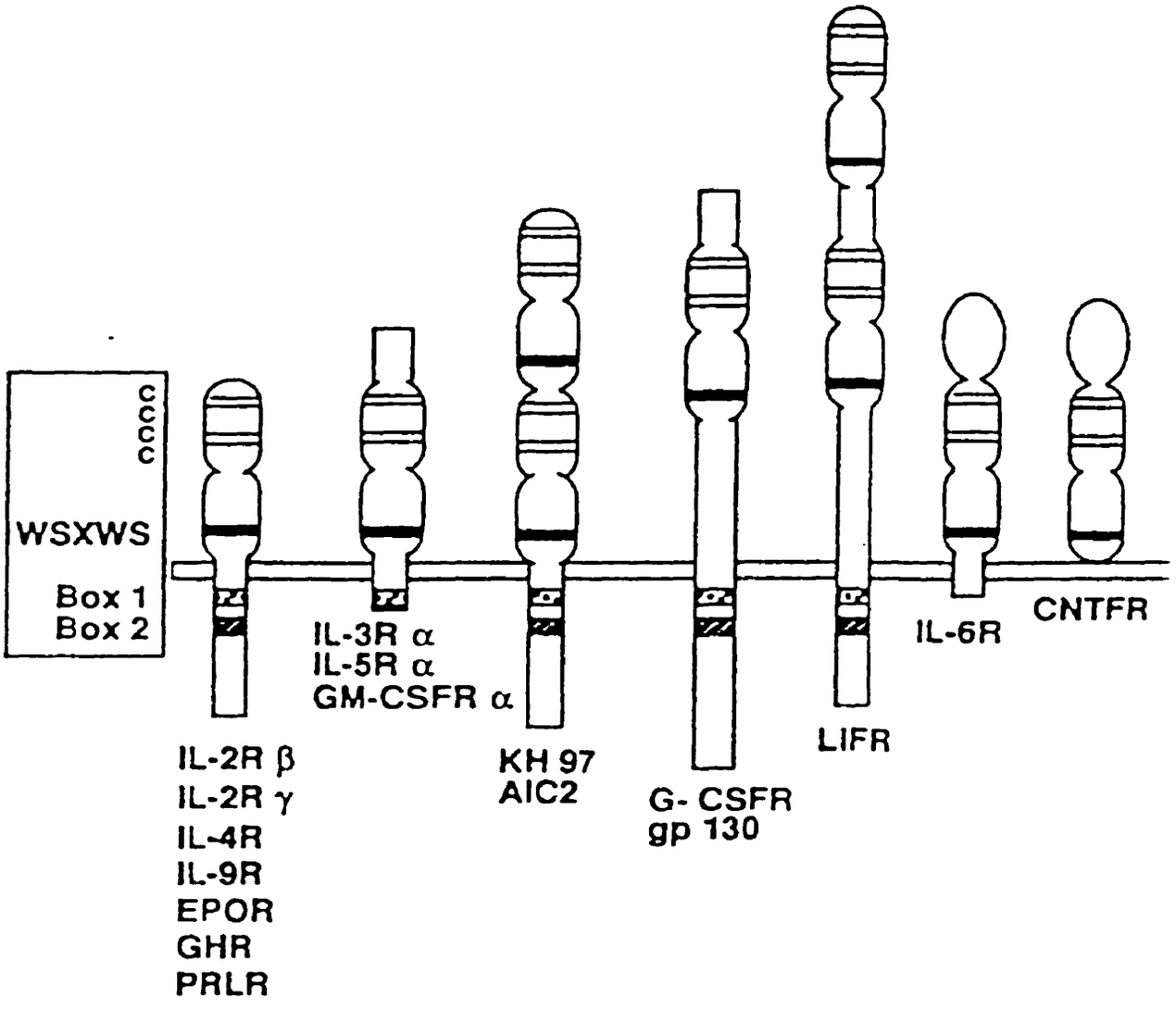
There are considerable similarities in the amino acid sequence of the GH and PRL receptors, and a detailed comparison classes the GHR as a Class I member of the cytokine/GH/PRL receptor superfamily (Figure 1.13) (reviewed in 166). Class I includes receptors for most of the interleukins (IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11,

Fig. 1.12. Structure of the GHR. Potential N-linked glycosylation sites (N) and the extracellular cysteines (C) with three pairs linked by disfulfide bridges are shown. The WSXWS-like motif is noted by a striped box, whereas solid boxes indicate the transmembrane domain as well as the cytoplasmic Box 1 and 2 regions. Intracellular regions of the GHR required for various receptor functions are noted. The ten tyrosine (Y) residues that are present in the cytoplasmic tail of the rat GHR are shown. [Reprinted from Argetsinger, L.S. and Carter-Su, C. Mechanism of signalling by growth hormone receptor. Physiological Rev. 76: 1089-1107, 1996 (17).]


Ligand binding
N
N
N
N
C
C
C
C
C
C
WSXWS-like motif
C
Transmembrane domain
Box 1
Box 2
Y
Y
Y
Y
Y
Y
Y
Y
Y
Protein Synthesis
Lipid Synthesis
Stat 5 activation
Internalization
Protein synthesis
Lipid synthesis
Proliferation
Glucose transport
Stat 1 and 3 activation
SHC phosphorylation
c-fos induction
Insulin synthesis
Spi 2.1 induction
Calcium response
Stat 5 activation
Stat 5
Spi 2.1
Box 1
JAK2 phosphorylation
MAP kinase phosphorylation
Stat activation
Spi 2.1 induction
IRS phosphorylation
SHC phosphorylation
Insulin synthesis

GH receptor

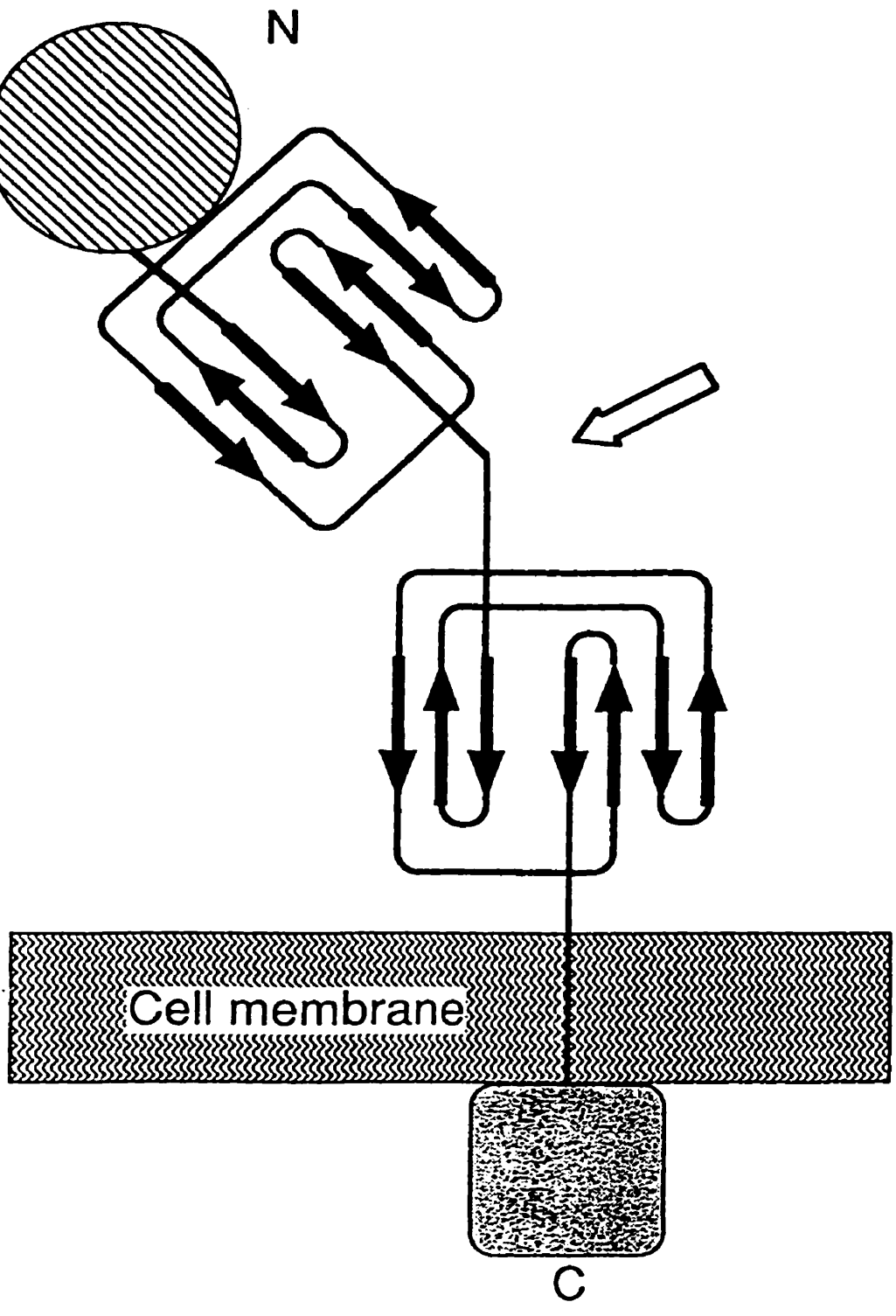
Fig. 1.13. Class I members of the GH/PRL receptor superfamily. Structural features of Class I receptors (R) are shown. Some members of the family have multiple receptor subunits (eg. IL-2 and IL-3). In addition, these receptors often share a common subunit: IL-3, IL-5 and GM-CSF receptors all share a common subunit called KH97 (human) or AIC2 (mouse); the gp130 protein is shared by the IL-6, LIF, OSM, CNTF and IL-11 receptors; the γ subunit of the IL-2R subunit is shared with IL-4R and IL-7R, while the multimeric IL-15 receptor contains both the ß and γ IL-2R subunits. [Reprinted from Finidori, J. and Kelly, P.A. Cytokine receptor signalling through two novel families of transducer molecules: Janus kinases and signal transducers and activators of transcription. J. Endocrinol. 147: 11-23, 1995 (166).]


C
C
C
C
WSXWS
Box 1
Box 2
IL-2R β
IL-2R γ
IL-4R
IL-9R
EPOR
GHR
PRLR
IL-3R α
IL-5R α
GM-CSFR α
KH 97
AIC2
G- CSFR
gp 130
LIFR
IL-6R
CNTFR

IL-12, IL-13 and IL-15), granulocyte/ macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), GH and PRL. The extracellular domain of these single membrane-spanning receptors contains a common sequence motif of approximately 200 amino acids that form 14 antiparallel ß-sheets. The amino-terminal and carboxyl-terminal seven ß-sheets each form a barrel-like structure that is similar to the type III domain of fibronectin (Figure 1.14) (167). The amino-terminal barrel has four conserved cysteines, whereas the carboxyl-terminal barrel contains the sequence motif WSXWS (tryptophan, serine, any amino acid, tryptophan, serine) at the membrane proximal region; the ligand binding pocket is believed to lie between these two barrel-like structures. Interestingly, the GH receptor has a YGEFS (tyrosine, glycine, glutamine, phenylalanine, serine) motif rather than a WSXWS consensus sequence. Mutational analysis of the rat GHR has revealed that the YGEFS motif directs important conformational characteristics of the receptor that contribute to ligand binding and intracellular signalling (168).

The cytoplasmic domains of Class I receptors do not have any intrinsic enzymatic activity and have very little conservation of primary sequence except for a small proline-rich juxtamembrane region (Box 1) (reviewed in 12,166). Box 1 is generally located within 10-20 residues from the transmembrane domain and contains the sequence Al-Ar-P-X-Al-P-X-P (aliphatic, aromatic, proline, any amino acid, aliphatic, proline, any amino acid, proline). The mammalian GHR Box 1 sequence is I-L-P-P-V-P-V-P (isoleucine, leucine, proline, proline, valine, proline, valine, proline). Mutational and deletional

Fig. 1.14. The extracellular domain structure of Class I receptors. Two sets of seven-antiparallel strands form two barrel-like structures. The intervening space between the two barrel-like structures constitutes the ligand-binding pocket (arrow). N = NH_2-terminus, C = COOH-terminus. [Reprinted from Kitamura, T. et al Multimeric Cytokine Receptors. Trends Endocrinol. Metab. 5: 8-14, 1994 (167).]


N
Cell membrane
C

analysis of Box 1 in the GHR has revealed that this proline-rich region is responsible for the cell proliferation effects of GH (169). However, although it is necessary, Box 1 is insufficient to stimulate GH-induced gene transcription. There is also a second cytoplasmic motif, Box 2, that is conserved in approximately half of the Class I members (reviewed in 12,166). Box 2 begins with a cluster of interspersed hydrophobic residues and ends with one or two positively-charged residues. In the GHR, Box 2 begins approximately 30 residues from the carboxy terminus of Box 1 and spans approximately 15 amino acids (170). GHR mutations in Box 2 also result in defective signalling pathways. The biologically active forms of many Class I receptors consist of multiple subunits (IL-2, IL-3, IL-5, IL-6, GMCSF, and probably LIF and CNTFR) or homodimers (GH, PRL and EPO) (reviewed in 166).

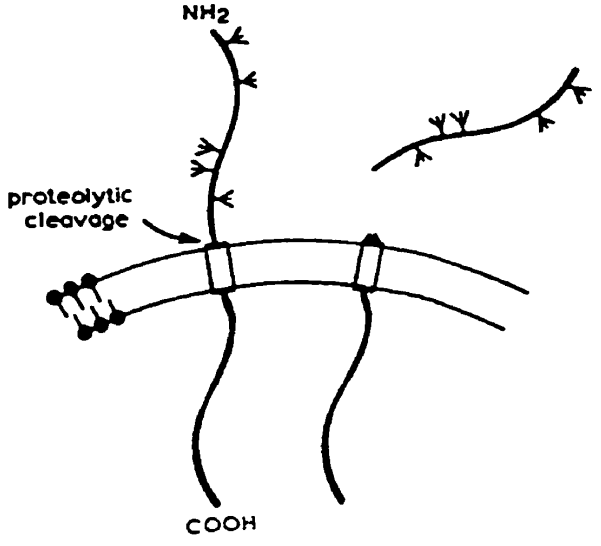
1.3.2. Circulating GHBPs.

A 51 kD glycoprotein that binds 22 kD hGH-N with high affinity and a limited capacity has been isolated from human serum using affinity chromatography (reviewed in 15,29). This hGHBP binds both 22 kD hGH-N and hGH-V equally well, while its preference for 20 kD hGH-N is significantly lower. Studies have yet to assess the binding characteristics of the other hGH isoforms to the carrier protein.

All high affinity GHBPs that have been identified to date represent, regardless of the species they originate from, soluble forms of their GHR's extracellular domain (reviewed in 15,29). The human, rabbit, and cow high affinity GHBPs are proteolytic cleavage products of the plasma membrane bound GHR (Figure 1.15), and are thought

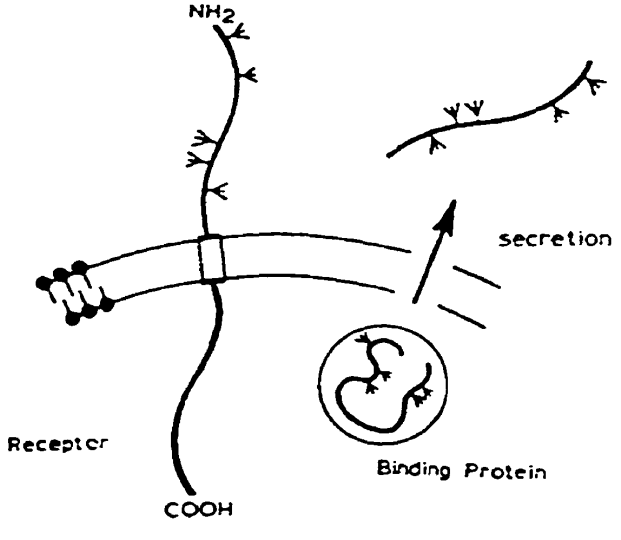
Fig. 1.15. Generation of GHBPs. Depending on the species, GHBP is generated by (A) proteolytic cleavage of the GHR receptor near the transmembrane domain and/or (B) by alternative splicing of the GHR mRNA and de novo protein synthesis. [Reprinted from Baumann, G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endo. Reviews 12: 424-49, 1988 (29).]

A.



Receptor mRNA

B.



Receptor mRNA

BP mRNA

to reflect the relative levels of GHR tissue expression in these species. In contrast, rodent GHBP is generated by an entirely different mechanism: rat and mouse GHR mRNA undergoes alternative splicing to generate a mature transcript coding specifically for the GHBP. In the rodent, the relative proportion of GHR and GHBP mRNA has been shown to differ between tissues, indicating that alternative splicing to yield either GHR or GHBP transcripts is a regulated cellular process (172). Interestingly, recent studies have revealed that, in the rhesus monkey, high affinity GHBP is produced by proteolytic cleavage of the cell surface GHR near the transmembrane domain as well as by differential splicing of the GHR gene transcript to produce an mRNA isoform encoding for a soluble GHR protein (173).

The broad range of hGHR distribution in human tissues has suggested that the high affinity hGHBP is probably produced by many tissues, with the liver as the predominant source as liver is thought to have the highest hGHR expression (reviewed in 15,29). Rodent mRNA encoding for the GHBP is also ubiquitous, whereas there is tissue-specific expression of the rhesus monkey GHBP mRNA: the monkey GHBP transcript was detected in liver, heart, kidney and stomach but not in intestine or pancreas, while GHR mRNA is readily observed in all of these tissues (173). Therefore, even though rhesus monkey GHBP synthesis may occur to some degree in all GHR expressing tissues by shedding of the plasma membrane bound GHRs, it is probably enhanced in tissues that are also capable of generating the alternative mRNA isoform encoding for the soluble GHBP.

Under basal conditions, 40-45% of circulating 22 kD hGH-N is complexed by the

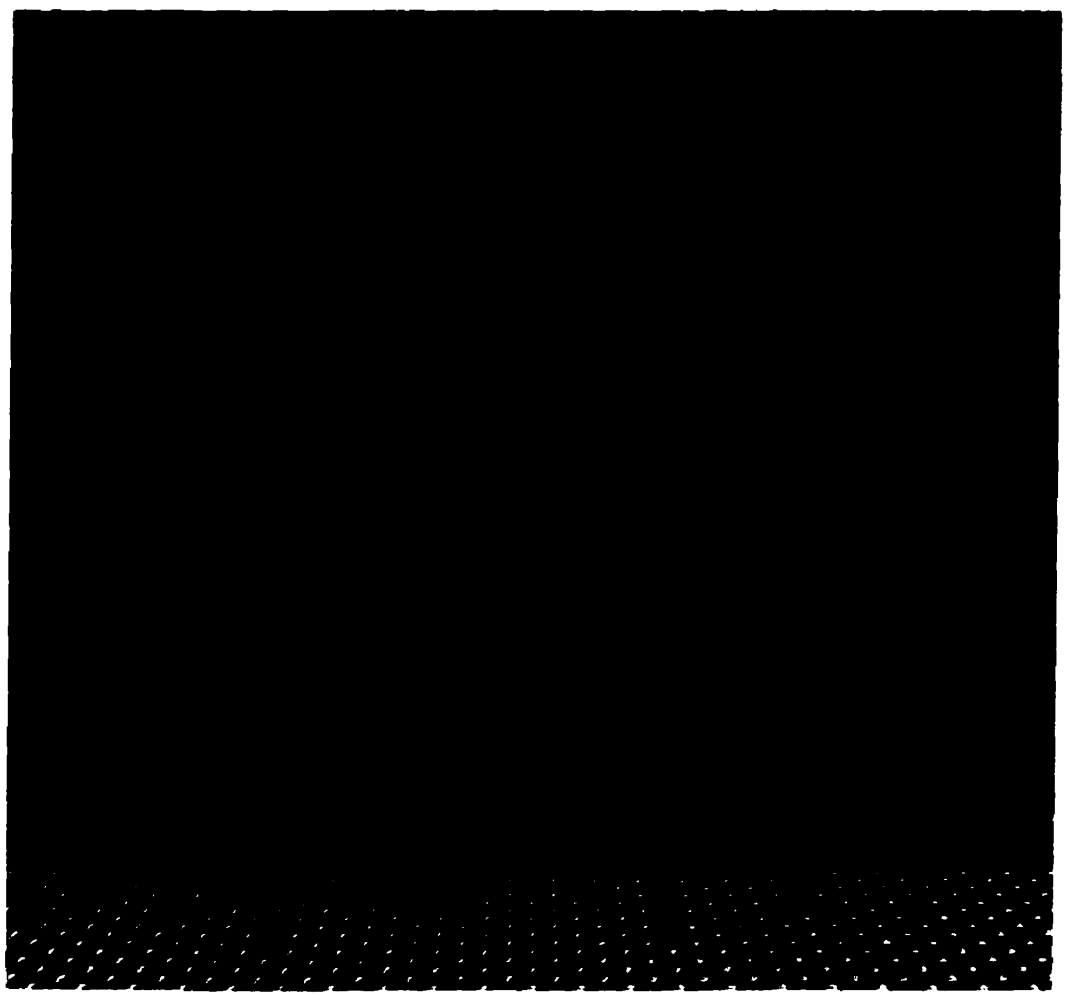
high affinity hGHBP (reviewed in 15,29). The bound hGH fraction decreases with high circulating hGH levels due to partial saturation of the carrier: each GHBP binds only one molecule of hGH. The metabolic clearance and degradation rates of bound hGH are 10-fold lower than the free hormone, suggesting that the complexed hGH serves as a reservoir. In addition, receptor cleavage to yield hGHBPs may be a form of receptor downregulation.

Affinity chromatography studies have also shown that human plasma contains a second hGHBP that has a lower affinity but higher capacity for hGH (reviewed in 15,29). This carrier protein has equal or slightly higher affinity for the 20 kD hGH-N as compared to the 22 kD pituitary form, complexing 7-8% of circulating 22 kD and 25% of 20 kD. It is regulated independently of the high affinity hGHBP and its mechanism of generation as well as its source remain unknown.

1.3.3. GH binding and dimerization.

Scatchard analysis of 22 kD hGH-N binding to human adult hepatic membranes revealed high affinity hGHR binding sites (174). More recent physicochemical, crystallographic and mutational investigations have further characterized the binding of hGH to its binding protein and receptor (175-178). These studies have revealed that there are two distinct binding sites for the binding domain of the receptor on the 22 kD hGH-N, and that 22 kD hGH-N forms a 1:2 complex with hGHBP (Figure 1.16). In the proposed scheme, the first hGHBP binds to a hGH molecule at site 1, then a second hGHBP binds the same hGH molecule at site 2 (175). Within the complex, both binding

Fig. 1.16. Ribbon diagram of hGH (in red) in complex with two molecules of hGHBP (in green and blue). hGH is a four-helix bundle with the amino and carboxyl termini facing left and right, respectively. The hGHBPs contain two ß-sandwich domains with amino-termini in the top domain and the carboxyl-termini next to each other in the bottom domain, where the receptors would enter the membrane. [Reprinted from Wells, J.A. Binding in the growth hormone receptor complex. Proc. Nat. Acad. Sci. USA 93: 1-6, 1996 (13).]



proteins donate essentially the same residues to the hormone even though the two binding sites on the hormone are unique. The complex binding sites on hGHBP consist of several amino acids beginning at residue 31 and ending at amino acid 134 (176). Alanine substitution analysis has suggested that residues 42, 44, 78, 101-107, 125-128 and 132 of the receptor contribute to the hGH/hGHR interface (177). Subsequent crystallographic studies have demonstrated that residues 24, 39-42, 167 and 168 also make contact with hGH, even though substitution of alanine at these positions does not alter hGH binding (178).

Crosslinking of [^{125}I]hGH to GHRs in intact cells has suggested that the membrane-anchored hGHR also forms hGH/hGHR$_2$ dimer complexes (17). The proposed hGH binding scheme results in substantial contact at the surface between the extracellular carboxy-terminal domains of the two receptors. Hormone-induced GHR dimerization has been shown to be an important first step for activating cellular signalling cascades. For example, if cultured cells are treated with a mutant hGH possessing a disrupted binding site 2, which can only bind a single receptor molecule, phosphorylation and activation of intracellular effector proteins is not observed (14).

Interestingly, Wada et al have recently shown that, like 22 kD hGH-N, 20 kD hGH-N can also induce hGHBP and hGHR dimerization (178a). Further studies need to be undertaken to determine whether 17 kD hGH-N, hGH-V and hPL can also form dimer complexes with GHBP or GHR molecules. In addition, a second isoform of the hGHR, that is missing the 22 amino acids encoded by exon 3, also exists. The ability of the exon 3 deficient receptor to homo- and heterodimerize (with the exon 3 containing form) has

also not been investigated. However, it is known that when either exon 3 retaining or deleted hGHR isoforms are overexpressed in test cells, the two receptors bind 22 kD and 20 kD hGH, hGH-V, hPL as well as ovine PRL with equal affinities (179,180).

Primate GHs are able to bind GHRs of lower species (reviewed in 181). In contrast, subprimate GHs have no affinity for the hGHR, accounting for the inability of non-primate GHs to induce growth-promoting effects when administered to hypophysectomized rhesus monkeys. This species specificity results from the inability of the bulky and postively charged His171 of subprimate GHs to interact with Arg43 of the hGHR: interaction between these two amino acids is crucial for GH binding to the human receptor (182). Primate GHs are able to bind the hGHR because they have an aspartic acid instead of a histidine at position 171: histidine has greater bulk than aspartic acid and at physiological pH aspartic acid is negatively charged whereas histidine is slightly positive.

In addition to somatogenic activity, primate GHs also trigger lactogenic responses, whereas subprimate GHs cannot bind to the PRL receptor of any species (reviewed in 24). Cunningham and Wells have demonstrated that the lactogenic binding of primate 22 kD hGH requires the presence of free Zn^{+2} ions: the absence of zinc cations reduces the 22 kD hGH affinity for the extracellular domain of the PRL receptor by four orders of magnitude (183). The fact that, in subprimate GHs, His18, which has been shown to be important for Zn^{+2} chelation, has been replaced with glutamine provides at least a partial explanation for why GH in lower species cannot bind to the lactogenic receptor. In addition, Peterson et al have shown that Phe44 of primate GHs is required for lactogenic

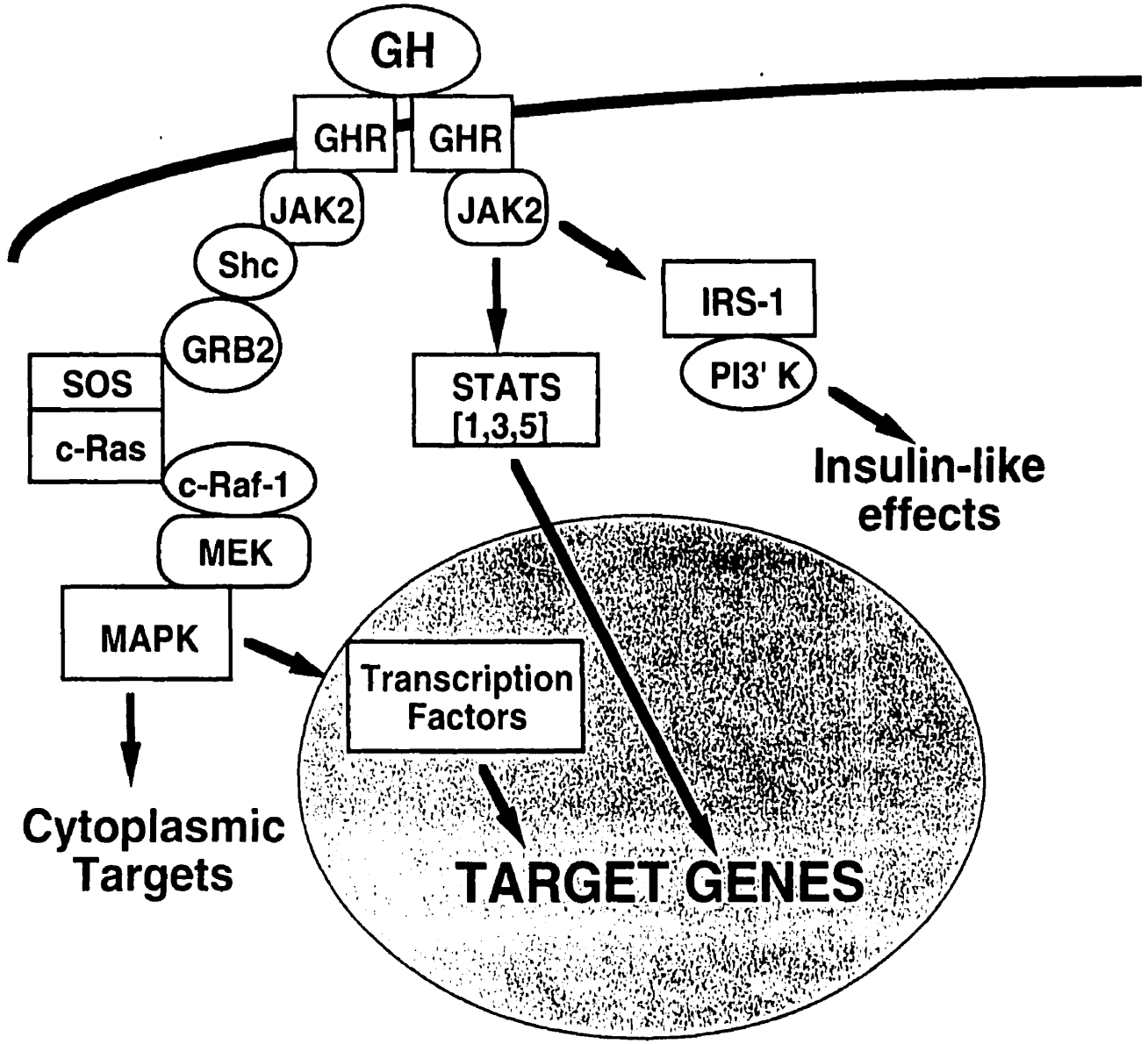
receptor binding and activation (183a). Therefore, the absence of Phe44 in GHs of lower species may be another reason for why subprimate GHs cannot activate the PRL receptor. Interestingly, PRL has very low, if any, affinity for the GHR in all species (reviewed in 24).

1.3.4. GHR intracellular signalling cascades (Figure 1.17).

1.3.4.1. Activation of Janus kinases.

Although all members of the cytokine/GH/PRL receptor superfamily lack intrinsic tyrosine kinase activity, numerous studies have reported that hormone binding to these receptors leads to rapid tyrosine phosphorylation of the receptors as well as many intracellular proteins (reviewed in 17,166). The Janus family (TYK 2, JAK 1, JAK 2 and JAK 3) of nonreceptor protein tyrosine kinases (PTKs) was subsequently shown to play a crucial role in transducing cellular responses of many hormones whose receptors belong to the cytokine/GH/PRL receptor superfamily. These PTKs are approximately 130 kD, have a carboxyl-terminus PTK domain as well as an adjacent kinase-related domain, and share substantial amino acid homology in five other regions extending towards the amino-terminus. Unlike many other PTKs, the Janus family members are characterized by the absence of Src homology 2 (SH2) and 3 (SH3) domains. If present, SH2 and SH3 regions play important roles in protein-protein interactions: SH2 domains recognize phosphotyrosines, whereas SH3 domains associate with proline-rich regions.

Fig. 1.17. Simplified schematic of GHR signalling cascades. For details, see section 1.3.4, pages 70-81. GHR, growth hormone receptor; JAK2, Janus kinase 2; Shc, SH2 domain-containing protein; GRB2, growth factor receptor-bound protein 2; SOS, son-of-sevenless guanine nucleotide exchange factor; RAS, a GTP-binding protein; RAF, a serine/threonine kinase; MEK, a dual-specific threonine/tyrosine kinase; MAPK, mitogen activated protein kinase; STATs, signal transducers and activators of transcription; IRS-1, insulin-substrate-1; PI3'K, phosphatidylinositol 3'kinase.


GH
GHR
GHR
JAK2
JAK2
Shc
GRB2
SOS
c-Ras
c-Raf-1
MEK
MAPK
STATS
[1,3,5]
IRS-1
PI3' K
Insulin-like
effects
Transcription
Factors
Cytoplasmic
Targets
TARGET GENES

The current model for GH-dependent JAK 2 activation is that GH binding to its receptor on target cells triggers GHR dimerization which, in turn, increases the affinity of each receptor molecule for JAK 2 (reviewed in 17). JAK 2 is then auto- and trans-phosphorylated. Several cytoplasmic domain tyrosyl residues of the two GHR molecules are also phosphorylated by two JAK 2 molecules in the complex. The phosphotyrosyl residues on Jak 2 and GHR can then associate with the SH2 domains of downstream effector molecules.

It was initially thought that GH-dependent activation of the Janus kinases in mouse 3T3 fibroblasts and human IM-9 lymphocytes was limited to JAK 2 (184,185). However, more recent investigations, using a higher affinity JAK 1 antibody, have detected low levels of JAK 1 tyrosyl phosphorylation in mouse 3T3 fibroblasts and in COS cells transfected with murine GHR and JAK 1 cDNAs (17, 186). Moreover, GH has also been observed to stimulate some JAK 3 activity in transformed T cells (187). Together, these data imply that GH can activate JAKs 1 and 3 in addition to JAK 2, with JAK 2 having the greater role.

Studies using truncated and mutated receptors have revealed that the Box 1 motif of GHR is required for GH-dependent GHR/JAK 2 interactions and imply direct binding of JAK 2 with the amino acid sequence of Box 1 (169). It is also possible that the secondary structure of the proline-rich Box 1, or changes induced in this secondary structure by ligand binding, are crucial for the association between JAK 2 and GHR (169). Although Box 1 is necessary for GHR/JAK 2 interactions, the membrane proximal one-third region is also required for maximal tyrosyl phosphorylation of the PTK (188).

JAK 2 appears to interact with the GHR through the first one-third of its N-terminal region.

1.3.4.2. STAT pathway.

The signal transducers and activators of transcription (STAT) proteins are a family of latent transcription factors that were first shown, by complementation experiments using interferon (IFN)-resistant cell lines, to be involved in the IFNα and IFNß postreceptor signalling pathways (reviewed in 189). Since then, STATs have been implicated in the intracellular mechanisms induced by many cytokines as well as GH, PRL and several growth factors (e.g., EGF, PDGF and FGF). STATs interact through their SH2 domains with phosphotyrosines of activated receptor/PTK complexes, and are themselves phosphorylated on specific tyrosyl residues by the PTK, permitting the STATs to homo- and heterodimerize. Activated STAT complexes bind specific DNA sequences to stimulate transcription of target genes. To date, STAT 1 (originally identified as p91 in IFN signalling), STAT 3 (originally identified as the acute phase response factor in IL-6 and LIF signalling) and STAT 5 (originally identified as the mammary gland factor in PRL signalling) have been shown to undergo tyrosyl phosphorylation and to form activated DNA-binding sequences in response to GH (reviewed in 17).

These three STATs are thought to be involved in stimulating transcription of several GH-responsive genes, including c-fos, the serine protease inhibitor 2.1, insulin-1, ß-casein and cytochrome P4503A/6ß-hydroxylase (reviewed in 17). Studies using truncated and mutant rat GHRs as well as JAK 2-deficient cell lines have revealed that

JAK 2 activation is necessary for STAT 1, 3 and 5 DNA binding responses (186). For STAT 5 to be tyrosyl phosphorylated and activated, STAT 5 must be bound to the GHR. In constrast, STATs 1 and 3 do not require direct binding to the GHR to be activated, only for maximal activation. The presence of consensus motifs for the SH2 domains of STATs 1 and 3 in JAK 2 has suggested that these two proteins interact directly with JAK 2 (reviewed in 17,189).

1.3.4.3. Shc pathway.

In addition to receptors with intrinsic tyrosine kinase activity (i.e., growth factor and insulin receptors), many members of the cytokine/GH/PRL receptor superfamily also signal through the mitogen activated protein kinase (MAPK) pathway that is thought to be important for cellular differentiation and growth (190). In the case of GH signalling, this is yet another cascade that is stimulated by JAK 2. Following GH treatment of mouse 3T3 or CHO cells expressing the GHR, the 46, 52 and 66 kD splice variants of Shc are rapidly tyrosyl phosphorylated by JAK 2 after binding through their SH2 domains to phosphotyrosine residues on either the GHR or JAK 2 (191).

Once phosphorylated, the Shc variants serve as docking sites for downstream SH2-containing proteins (reviewed in 17,190). One of these proteins is growth factor receptor-bound protein 2 (GRB-2) which associates through its SH2 domain with Shc and through its SH3 region with the son-of-sevenless (SOS) nucleotide exchanger protein. SOS activates c-ras which initiates the MAP kinase cascade, involving c-raf-1 kinase, MAPK kinase (MEK) and the two MAPK isoforms, extracellular regulated kinases

(ERKs) 1 and 2.

ERKs 1 and 2 have been reported to phosphorylate and activate several proteins (phospholipase A_2, cytoskeletal proteins, p70 and p90 ribosomal S6 kinases and c-raf-1) and transcription factors (e.g., c-myc, c-jun, c-fos and the ternary complex factor, $p62^{TCF}$/Elk1) that are implicated in cellular differentiation and proliferation (reviewed in 190). GH has been shown to stimulate the activity of phospholipase A_2 in rat hepatocytes (192) and p90 S6 kinase as well as $p62^{TCF}$/Elk1 in mouse 3T3 fibroblasts (193). Increased phospholipase A_2 has been associated with the calcium dependent sexually dimorphic inhibition of the CYP2C12 gene by GH in rat liver. p90 S6 kinase phosphorylates many cellular proteins, including the serum responsive factor (SRF); SRF binds to to the serum responsive element (SRE) on the c-fos gene to stimulate its transcription (194). $p62^{TCF}$/Elk1 is a second transcription factor that binds the SRE in the c-fos gene promoter (195). Therefore, stimulation of MAPK activity which, in turn, leads to the induction of SRF and $p62^{TCF}$/Elk1 activity, may be one mechanism by which GH increases transcription of the c-fos gene.

1.3.4.4. IRS pathway.

Insulin receptor substrates 1 and 2 (IRS-1 and -2) are two additional docking proteins for SH2-containing GHR signalling intermediates. GH action stimulates tyrosyl phosphorylation of IRS-1 in mouse 3T3 fibroblasts (196) and in CHO cells (197) expressing the rat GHR, as well as IRS-2 tyrosyl phosphorylation in primary rat adipocytes (17). Transfection studies in CHO cells using mutated and truncated rat GHRs

have suggested that GH-dependent tyrosyl phosphorylation of the IRS-1 and 2 docking proteins is dependent on JAK 2 activation by the GHR (17). However, it remains unknown whether the GHR/JAK 2 complex interacts directly or through an intermediate protein with the IRS proteins. The IRS docking proteins have been shown to control the GH-dependent activity of a phosphatidylinositol 3' (PI 3') kinase in several cell types (198). PI 3' kinase phosphorylates phosphoinositol lipids (PI) to yield PI-3-PO_4, PI-3,4-PO_4 and PI-3,4,5-PO_4 (reviewed in 17). These lipids are not hydrolyzed by any known phospholipase and the events that occur downstream remain a mystery. However, PI 3' kinase has been implicated in the insulin-dependent regulation of glucose transport, DNA synthesis and p70 ribosomal S6 kinase (an enzyme involved in cell cycle progession) (199). Therefore, it is possible that this pathway is important in transducing GH-dependent increases in glucose transport activity as well as other insulin-like effects of GH.

1.3.4.5. PKC L-type calcium channels and nitric oxide synthase pathways.

Studies in several cell types have suggested that the GH-dependent stimulation of lipogenesis, c-fos gene transcription, DNA binding of the c/EBP transactivator and increases in intracellular calcium require PKC activity since PKC inhibitors block these responses to GH (reviewed in 17). Further investigations, using reagents that inhibit JAK 2 function, have shown that JAK 2 mediates GH-induced PKC activation. Consistent with this is the ability of other JAK kinase-linked receptors (PRL, IL-2, 3, 6, EPO, IFN-γ, and GM-CSF) to activate PKC (reviewed in 189). Interestingly, there are several PKC

isoforms and some studies have indicated that GH may activate different PKC isoforms in different cell-types.

GH stimulated increases in intracellular calcium are also dependent on the presence of extracellular calcium and on functional L-type plasma membrane calcium channels. Blocking these channels prevents GH from inducing expression of the *serine protease 2.1* (200) as well as the *P4502C12* (192) genes in rat hepatocytes.

GH stimulation of calcium uptake causes rat adipocytes to become refractory to the transient antidiabetogenic effects of GH on glucose metabolism. Preliminary findings suggest that these effects of GH are mediated by the calcium-dependent enzyme nitric oxide synthase, which catalyzes the conversion of L-arginine to L-citrulline and the release of nitric oxide (201).

1.3.4.6. Termination of the GHR-activated intracellular signalling cascades.

Multiple mechanisms are involved in terminating GH signalling pathways, including regulation of the phosphorylation status of cascade members. Activated, and thus phosphorylated, forms of GHR, JAK 2 and cellular substrates of JAK 2 (eg. Shc and IRS proteins) can be rapidly downregulated by tyrosine phosphatases (PTPs) (reviewed in 17). In addition, activation of the MAPK kinase pathway generates a feedback phosphorylation cascade: phosphorylation of SOS causes dissociation of Shc-GRB 2-SOS complexes resulting in rapid termination of this signalling pathway (202). Finally, phospholipase C and newly synthesized proteins have been implicated in the desensitization of the GH-activated JAK2/STAT5 pathway (203).

1.3.4.7. Receptor internalization.

Shortly after GH binding and the initiation of signalling cascades, GHRs undergo endocytosis in a cell-specific fashion. In cultured rat adipocytes there seems to be constitutive internalization of the receptor which is accelerated by GH binding (204). However, in human IM-9 lymphocytes and mouse fibroblasts, GHRs redistribute and aggregate following GH treatment, without any evidence of constitutive internalization (205).

GHR internalization is dependent on the presence of a phenylalanine residue in the proximal one-third of the rat GHR's cytoplasmic domain and an intact cellular ubiquitin-conjugating system that targets cellular proteins for degradation by the proteosome (206). These studies suggest that degradation of both the extracellular and cytoplasmic portions of the GHR occurs within endosomal/lysosomal cellular vesicles. Interestingly, studies of the GHR in human IM-9 lymphocyte cells have demonstrated that internalization may also downregulate the receptor without degrading it: GHRs may be sequestered in cytoplasmic microsomes during the cell's refractory period to GH action and then recycled to the plasma membrane (207).

Receptor internalization appears to play an essential role in the intracellular cascades of several plasma membrane-anchored receptors. Therefore, it is possible that it may also be important for GHR signal transduction pathways. Tyrosyl phosphorylated GHR/JAK 2 complexes extending from the microsomal vesicles into the cytoplasm may serve as SH2 binding domains for cellular signalling substrates and/or may permit further tyrosyl phosphorylation of cytoplasmic proteins by JAK 2. In fact, the ubiquitination

system has been recently implicated in GHR signalling. Using CHO cells transfected with the rat GHR, Strous et al have shown that the GH-induced STAT pathway is completely inhibited by a defective ubiquitination system (208).

Even more intriguing are the findings of Waters et al suggesting that substantial amounts of GH as well as its receptor and binding protein translocate to the nucleus (209,210,210a,210b,210c). Both GHR and GHBP have been detected in the rat nucleus, with microsomal GHRs translocating to the nucleus in response to GH stimuli. Crosslinking, immunological, and Scatchard experiments have revealed high affinity GHBPs in the nuclear membranes, nucleoplasm and chromatin fractions prepared from GH-treated rabbit livers. These observations have suggested a direct action for GH and GHBP on gene transcription. However, Waters and his colleagues have been unable to identify any GHR or GHBP DNA binding activity.

Nuclear localization subsequent to receptor-mediated internalization has been reported for other polypeptide ligands [insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF), IL-1, somatostatin and PRL]. Moreover, as with GH, nuclear localization of IL-1 and NGF is associated with their respective receptors (211). Although these studies are quite intriguing, a nuclear function for polypeptide hormones and their receptors has yet to be identified. Interestingly, JAK 1 and 2 have been detected in the nucleus of CHO cells, suggesting that Janus kinases may play a role in the activation of transcription factors (212). Therefore, one nuclear function for the GHR may be to activate JAKs located in the nucleus.

1.3.4.8. Summary.

Many GHR signalling intermediates have been identified in the past few years (Figure 1.17) and the cytoplasmic regions of the rat GHR responsible for several GH responses have been delineated (Figure 1.12). However, a direct link between intracellular intermediates and the biological responses to GH is still largely a mystery. The fact that GH shares intracellular signalling pathways with other hormones, cytokines and growth factors suggests that similar postreceptor mechanisms may be used to regulate overlapping sets of biologic responses. The timing and duration of activation of the shared cascades may provide a degree of specificity for each signalling pathway. Certain responses may involve a complex mechanism with several pathways converging at a single end point. For example, as discussed in the preceding sections, GH-induced c-fos gene transcription is regulated by at least three transactivator complexes (SIE, SRF, and $p62^{TCF}$/Elk1). In addition, there is always the issue of species- and tissue-specificity in GH post-receptor mechanisms: a recent report demonstrates that while the MAPK pathway is known to be involved in the GH signalling cascades of mouse 3T3 fibroblasts, it is not activated in human IM-9 lymphocytes (213). It is also likely that other GHR signal transduction pathways will be identified or better defined in the future. In fact, a recent report has suggested that some of the GH effects may require crosstalk of the GHR with other receptors: Yamauchi et al demonstrated that GH-induced JAK 2 activity can tyrosine phosphorylate the EGF receptor (214).

1.3.5. GHR and GHBP tissue distribution and regulation.

The GHR is widely expressed in mammalian adult tissues, including liver, adipose tissue, lymphatic and immune cells, intestine, heart, kidney, lung, pancreas, brain, cartilage, muscle, corpus luteum and testis (106,215,216). The lack of GH binding in several tissues that demonstrate responsiveness to GH as well as expression of GHR mRNA has suggested that GHR levels in these tissues are either low or restricted to a small population of specific cell-types. A large proportion of rat and human hepatic receptors are present in microsomal cytoplasmic membranes, most likely a reflection of continuous GHR biosynthesis. Rat studies have demonstrated that the half-life of GHRs in several tissues is very short, with receptors being targeted for lysosomal degradation following internalization. Cleavage of primate, rabbit and sheep GHRs at the plasma membrane to produce soluble GHBPs also contributes to the short half-life of the plasma membrane-anchored receptor (see section 1.3.2).

Homologous regulation of GHR expression by GH varies among species and, within a species, amongst tissues. Absence of pituitary GH causes a decrease in the number of GH binding sites in rabbit and sheep livers as well as in rat adipocytes (reviewed in 216). In contrast to these data, GH-deficiency in rats is linked with an increase in GH binding to hepatic membranes despite decreased levels of GHR mRNA (217), suggesting that there is a reduction in receptor turnover and degradation in the GH-deficient state. Hepatic GH binding sites are also reduced in fasting rats, but are restored following GH replacement therapy while chronic GH treatment results in increased rat liver GHR expression (218). Studies of hypophysectomized mice, rats and

rabbits suggest that elevated levels of GH in pregnant animals are responsible for increases in liver GH binding sites during pregnancy (reviewed in 216). In contrast, exposure of human IM-9 lymphocytes to hGH leads to receptor downregulation (219).

In general, glucocorticoids seem to have stimulatory effects on GHR expression. In sheep, fetal hepatic GHR mRNA levels increase during the last two weeks of gestation in parallel with the normal rise in fetal cortisol levels (220). Glucocorticoids and/or dexamethasone increase the GH binding capacity of rabbit liver as well as rat and porcine hepatocytes, whereas they inhibit GH binding in the mouse 3T3 fibroblast cell line (216,221,222). Cortisol is also able to potentiate hGHR gene transcription in human osteoblast-like cells (223).

Insulin, thyroid hormone, sex hormones and the GHBP have also been implicated in GHR regulation. Insulin treatment enhances liver GHR mRNA synthesis in diabetic rats (224), while thyroid hormone increases GHR mRNA expression in porcine hepatocytes (221). Liver and growth plate GHR mRNA in rabbits is upregulated by testosterone and reduced with estrogen treatment (225). In a recent study, Mullis et al observed that GHBP can stimulate *GHR* gene transcription in a human hepatoma cell line (226).

The above data demonstrate that the mechanisms regulating GHR vary among different species as well as among GH target tissues and cell-types within each species. The recent cloning and characterization of ovine, bovine, mouse and rat *GHR* gene promoters (227-231) provide the basis for a future comparative investigation of subprimate *GHR* gene expression.

1.3.6. Ontogeny of the GHR and GHBP.

Numerous animal studies suggest that there is a significant onset of tissue GHR and GHBP expression beginning after birth. Rat GHR mRNA, barely detectable in fetal and early postnatal livers (< 20 days) by Northern blot analysis, gradually increases to adult levels by 40 days (232,233). Similar developmental increases in mouse hepatic GHR and GHBP mRNAs have been reported (234). In addition, no measurable levels of bovine GH binding to rat hepatic membranes were observed prior to 20 days after birth (232). Northern blot studies have also revealed ontogenic increases in rat kidney, lung and ileum GHR and GHBP mRNA levels (233). The rat GHBP-specific mRNA was detectable at low levels in fetal liver and also increased postnatally, with serum GHBP levels being first detected at 10 days following birth (232).

Immunohistochemical studies of 12 to 18 day rat fetuses identified very low levels of immunoreactive GHR/GHBP until day 18 when prominent staining was observed in all tissues and organs derived from the embryonic endoderm (e.g., hepatic tubules, gonadal tissues and adrenal cortex) (235). Decidual cells of the placenta were also strongly immunoreactive, suggesting that maternal GH may have a function in placental development and nutrient transfer. In support of an in utero role for the rodent GHR during early embryogenesis, Panteleon et al have recently identified functional GHRs in preimplantation mouse embryos (143). Moreover, Ohlsson et al have demonstrated expression of GHR mRNA in mouse embryonic stem cells that can be stimulated in vitro by retinoic acid (236).

The ontogeny of GHR has also been investigated in the rabbit, pig, cow and sheep

(237-240). Low levels of rabbit GHR have been detected in fetal and early postnatal liver, muscle and heart, while fetal kidney samples had relatively high concentrations that remained fairly constant throughout fetal and postnatal life (237). Binding of bovine GH to pig hepatic membranes dramatically increased in fetal versus postnatal specimens; in contrast, no clear age-related differences were observed in bovine GH binding to muscle preparations from 75 days of gestation to the adult stage (238). Porcine GHBP is detected by 75 days of fetal development and increases with age (238). Examination of bovine GH binding to postnatal bull liver membranes revealed a significant increase from neonate (2 day) to adult (365 day) specimens (239). In sheep, there is a dramatic increase in the number of hepatic GH binding sites after the first week of life (240).

In the human, hGHR expression is readily detectable by immunohistochemical techniques by the first trimester of fetal life; by midgestation, the pattern of immunostaining is often identical to that of the adult (215,241). Immunoreactive hGHR is present as early as 8.5 weeks in human hepatic parenchyma, kidney tubular epithelia and dermal fibroblasts. In fetal livers, intense immunoreactivity was localized in hepatocytes surrounding the central veins and portal triad, while the hematopoietic cells within the sinusoids were unstained. Kidney tissues showed predominant staining within the tubular epithelium, with glomerular epithelial immunoreactivity being restricted to the earliest developmental stages, implying a fetal-specific role for hGHR in the developing kidney. In fact, a mixed population of early gestational fetal kidney cells has been shown by our laboratory to produce both IGF-I and IGF-II in response to physiological levels of hGH (241a). hGHR staining in the endocrine pancreatic tissues,

neuronal cell bodies of the cerebral cortex, the germinal layer of the epidermis, sebaceous and sweat glands as well as hair follicles was detected at 12-14 weeks of fetal life. Fetal zone cells of the adrenal displayed only weak immunoreactivity after 13-14 weeks of fetal age, while definitive zone cells of the growth plate showed no evidence of hGHR expression throughout gestation. Fetal growth plates and cultured chondrocytes showed positive staining from as early as the second trimester of gestation. Robust epithelial staining was detected in the crypt region of both small and large intestine but only after 19 weeks of age. Finally, positive staining for the hGHR was present as early as 8.5 weeks in the syncytial layer of the placenta and remained detectable until term. Since the syncytium also releases hGH-V into the maternal circulation, hGH-V may act in an autocrine and/or paracrine fashion on the syncytial layer of the placenta.

Specific binding of hGH has been demonstrated in first trimester fetal liver membranes, chondrocytes and cultured fibroblasts (242-244). Fetal lung membrane preparations, however, did not show any hGH binding activity, consistent with the absence of hGHR immunoreactivity in fetal and postnatal lung. Binding experiments have also failed to detect significant levels of hGHR in fetal skeletal muscle, whereas low levels have been measured in postnatal preparations. Interestingly, the same fetal muscle membrane preparations had considerable hPL binding. Therefore, it is possible that an hPL specific receptor is expressed in fetal muscle or that a fetal form of the muscle hGHR is selective for hPL. In support of the latter hypothesis is the presence of hGHR immunoreactivity in abdominal skeletal muscle from as early as 8.5 weeks of fetal development. There are also limited Northern blotting data in support of fetal hGHR

expression. Werther et al detected significant levels of hGHR mRNA in dermal fibroblasts and chondrocytes obtained from second trimester fetuses (15-20 weeks) (243).

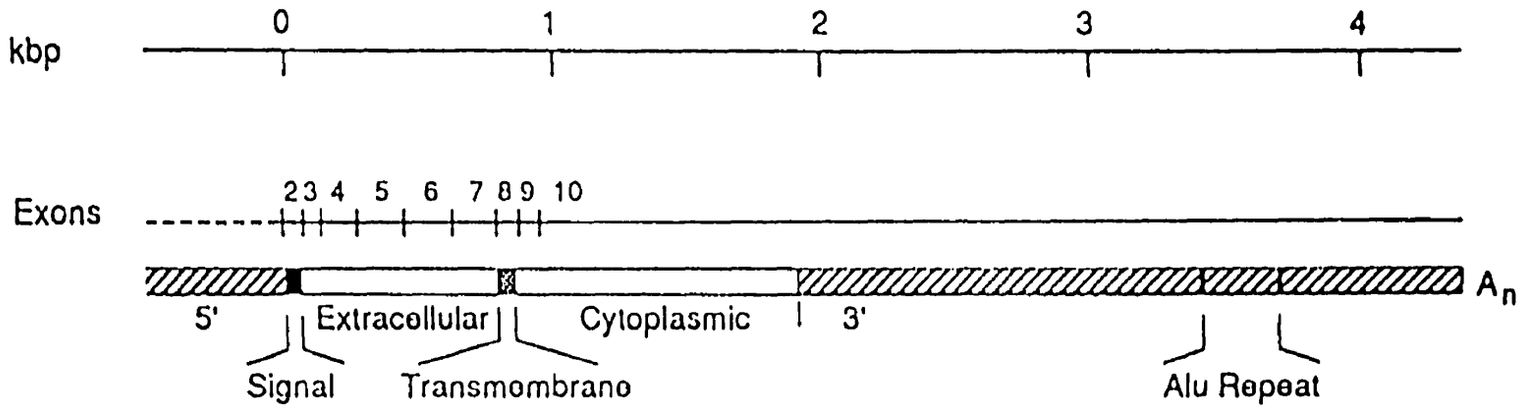
Although the Mab 263 antibody used in all of the previously mentioned immunohistochemical studies cross-reacts with both the hGHR and hGHBP proteins (244a), the absence of hGHBP in human fetal serum until approximately midgestation suggests that a positive cellular stain corresponds primarily to the hGHR. Levels of hGHBP are low in the fetus and newborn and rise throughout childhood to reach maximum levels during puberty.

These mRNA, binding and immunological studies, coupled with in vitro functional data (discussed in section 1.1.4) and clinical observations (described in 1.4) suggest tissue- and cell-specific functions for the hGHR during fetal development.

1.3.7. *GHR* gene expression.

The hGHR and hGHBP are products of a single gene that has been localized to human chromosome 5p13.1-12. Exons 2 to 10 and their corresponding intronic sequences span approximately 87 kb of genomic DNA (245). The signal peptide as well as the first six N-terminal residues of the mature protein are encoded by exon 2. The remaining extracellular domain is encoded by exons 3 to 7, while exon 8 codes for the transmembrane region and exons 9 and 10 for the cytoplasmic tail (Figure 1.18). In contrast to rodents, in which there is a 4.5 kb GHR mRNA as well as a 1.7 kb alternatively spliced product that is specific for the GHBP (246), only hGHR mRNAs of

Fig. 1.18. Schematic representation of the hGHR mRNA. A nucleotide scale (in kb) and the locations of exon boundaries are indicated by the two upper diagrams. The third schematic details the 5' and 3' UTRs (hatched), the Alu-repeat, and the mRNA regions that encode for the signal peptide as well as the extracellular, transmembrane and cytoplasmic domains of the receptor. [Adapted from Godowski, P. et al Characterization of the human growth hormone receptor and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. Proc. Natl. Acad. Sci. USA 86: 8083-8087, 1989 (245).]

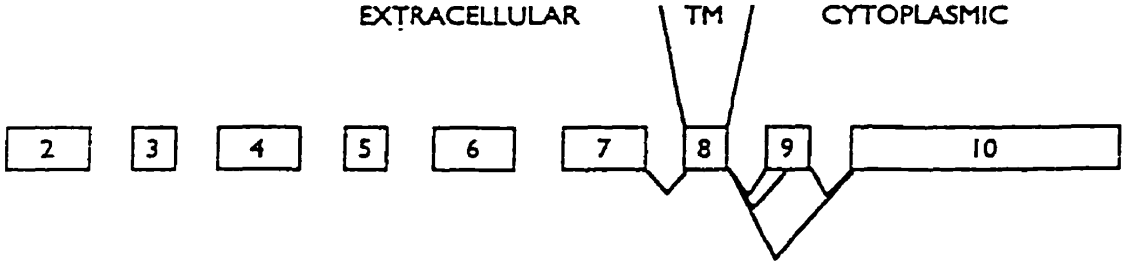

kbp
0
1
2
3
4
Exons
2 3 4 5 6 7 8 9 10
5'
Extracellular
Cytoplasmic
3'
A_n
Signal
Transmembrano
Alu Repeat

4.5-5.1 kb have been detected by Northern blot analysis of human tissues (12). Sequencing of the hGHR cDNA has revealed that the coding exons comprise 1.9 kb, and that there is a large 3' untranslated region (UTR) of approximately 2.4 kb containing an Alu repeat (245).

Different isoforms of the hGHR mRNA, with small size differences indistinguishable by Northern blot, have been identified by RT-PCR. In a 1992 study of placental hGHR expression, Urbanek et al identified an exon 3 deficient GHR mRNA isoform (247). Surprisingly, transient transfection studies have revealed that both transcripts encode hGHR receptors with equal binding affinities for 22 and 20 kD hGH-N, hPL and hPRL (179,180). In addition, a hGH/hGHBP complex was detected in the culture media of cells synthesizing either exon 3 retaining or deficient receptors, as well in the plasma of individuals with exon 3 deficient hGHR mRNA expression (248).

Alternative isoforms of the hGHR that vary in their cytoplasmic tails have also been characterized (Figure 1.19). If an alternative 3' acceptor site that is 26 bp downstream in exon 9 is used, a six residue frameshift occurs and a stop codon is introduced (249-251). The resultant 279 amino acid receptor, hGHR-t1, is truncated. In addition, exon 9 can be spliced out, again creating a frameshift and a stop codon insertion, yielding a second truncated GHR isoform (hGHR-t2) (250). Semi-quantitative reverse transcription (RT)-PCR has revealed that hGHRt1 mRNA is a minor splicing product in liver, fibroblasts and IM-9 lymphocytes, and is only detectable following a second round of amplification in muscle, stomach and lung (249). However, in mammary gland, adipose tissue and EBV-transformed lymphoblasts, both hGHR and hGHR-t1

Fig. 1.19. Schematic representation of alternative splicing patterns within exons 9 and 10 of the hGHR mRNA. Exons are shown by numbered boxes and splicing patterns are depicted by the lower v-shaped lines. Regions of the mRNA encoding for the extracellular, transmembrane (TM) and cytoplasmic domains of the receptor are indicated. [Reprinted from Ross, R.J.M. et al A short isoform of the human growth hormone receptor functions as a dominant negative inhibitor of the full-length receptor and generates large amounts of binding protein. Mol. Endocrinol. 11: 265-273, 1997 (250).]


EXTRACELLULAR
TM
CYTOPLASMIC
2
3
4
5
6
7
8
9
10

mRNA RT-PCR bands are equal in intensity, suggesting that the hGHRt1 has tissue-specific functions (249). The hGHR-t2 has only been studied in liver and IM-9 cells where it represents $< 1\%$ of the total hGHR mRNA (250). Transient transfection studies have demonstrated that, in comparison to the full-length hGHR, the truncated isoforms undergo minimal internalization and are not down-regulated by hGH (251). However, both hGHR-t1 and -t2 have a significantly increased capacity to generate soluble hGHBPs (249-251). Moreover, the finding that these truncated receptors can heterodimerize with full-length hGHRs has suggested that they may alter intracellular signalling cascades in tissues and cell-types where they are expressed in sufficiently high levels (i.e., mammary gland and adipose tissues) (250).

Eight different 5' UTR variants of the hGHR mRNA have been cloned by 5' rapid amplification of cDNA ends (5'RACE) from an adult liver cDNA library (252). All eight variants were found to diverge at -12 bp from the translation initiation codon, and were named V1 to V8 on the basis of their relative abundance. Sequencing analysis of the V3 clones revealed three related cDNA subvariants (V3a, b and c), suggesting that they are alternatively spliced isoforms.

All 5'UTR variants, with the exception of V2, were found to contain ATG codons upstream of exon 2 (252). These upstream open reading frames are either interrupted by stop codons prior to exon 2 or shortly after the main open reading frame of the hGHR mRNA. In addition, none of these upstream ATG sites fall under the C-C-Purine-C-C-A-T-G-G consensus sequence that has been reported by Kozak to be characteristic of strong translation initiation sites in vertebrates (253). However, it has been proposed that the

presence of multiple open reading frames in the 5' UTR of tightly regulated transcripts, such as proto-oncogenes and growth factors, can negatively modulate translation initiation (254). Therefore, the complex 5' UTRs of the hGHR mRNA may regulate expression of the receptor at the level of translation inititiation.

Multiple 5' UTR variants of the GHR mRNA have also been reported in other species (227-231,234,252,255-257). As with the human variants, the different animal 5' UTR transcripts of the GHR mRNA diverge at -12 nucleotides from the AUG start site of translation in exon 2. The ovine and bovine exons 1A (227,229) share 76% identity with human V1 (252), while the sequence similarity between the ovine and bovine exons 1B (228,256) and human V2 (252) is 74%. Ovine exon 1A mRNA isoforms are liver-specific and present only after birth (227), whereas exon 1B expression has been detected in late gestation ovine growth plate and in all postnatal tissues tested, with tissue-specific differences in its levels relative to the total pool of GHR mRNA (228). Human V1 also has 76% sequence homology with the 5'UTR GHR cDNA cloned from postnatal rabbit liver (12).

The mouse L1 (230) and rat GHR1 (231) 5'UTR variants both have 50% sequence homology with the human V7 transcript. In addition, they are both detectable in liver only after birth. Although the tissue distribution of the L1 transcripts has not been reported, the GHR1 transcript is liver-specific (231). Liver L1 (234) and GHR1 (231) mRNAs are upregulated during pregnancy, suggesting that L1 and GHR1 are, at least partially, reponsible for the increases in hepatic GHR mRNA synthesis and GH binding during rodent pregnancy. Further studies have shown that GHR1 exists in much

higher levels in female versus male rats, which may be contributing to the higher levels of GHR in female versus male rat livers (231).

The mouse L2 and rat GHR2 5'UTRs share 77% sequence identity with human V2. L2 mRNAs have been identified in late gestation fetal liver, postnatal liver and placenta (234). GHR2 mRNA is first detectable during late gestation and it is widely expressed in tissues of postnatal male and female rats (257). Three additional GHR mRNA 5'UTR variants have been cloned from rat tissues: the level of expression of each isoform is variable from one tissue to another, with some 5'UTR variants having a preferential association with the GHR or GHBP mRNA transcripts (257).

The diversity of the 5'UTR of the GHR mRNA has suggested that multiple promoters may be regulating *GHR* gene transcription. Genomic mapping of ovine exons 1A and 1B has revealed that exon 1A is located approximately 17 kb upstream of exon 2 and that exon 1B is at least 17 kb 5' to exon 1A (228). Separate transcription start sites and cis regulatory regions have been identified for each ovine 5' UTR variant (227,228). A promoter region has also been identified for the murine L1 transcript, which is at least 8 kb upstream of exon 2 in the mouse *GHR* gene (230).

1.4. Determinants of Normal Mammalian Growth.

1.4.1. Postnatal growth.

Growth is the progressive development by which a fertilized egg attains the size, form and function of an adult. The control of mammalian growth is a complex process, involving the interaction of a number of genetic, hormonal and environmental factors

(reviewed in 258). In postnatal life, the two major hormones are clearly GH and IGF-I. As already discussed, the somatogenic effects of GH on long bone and other organs are both direct and IGF-I mediated. Other growth factors, such as EPO, NGF, EGF, PDGF and fibroblast growth factor (FGF), contribute to the overall development of an organism by regulating growth, proliferation and differentiation of specific cell types. The cell differentiating effects of estrogens, androgens, glucocorticoids, retinoids and thyroid hormones play permissive roles for the growth-promoting actions of polypeptide hormones. In addition, PRL is necessary for mammary gland growth and development during the later stages of pregnancy.

1.4.2. Fetal growth.

1.4.2.1. Insulin-like growth factors.

IGFs have been detected in human fetal tissues as early as 10-12 weeks of gestation and in serum as early as 15 weeks (259). Throughout human fetal life, plasma IGF levels remain low relative to adult basal values (259). After birth, both IGF-I and -2 increase steadily during the childhood years (260). During the pubertal growth spurt, IGF-I rises markedly, while IGF-II remains relatively constant. The changes in IGF-I serum levels during ageing are closely correlated with alterations in plasma hGH concentrations. As with hGH, IGF-I levels begin to decline after puberty, with a more pronounced decrease in males versus females; in females, a rapid fall in both hGH and IGF-I serum levels occurs following menopause (260). In contrast, serum concentrations of IGF-II do not change significantly in the normal postpubertal individual (260).

The ontogeny of IGFs is somewhat different in rodents where serum levels of IGF-II are elevated during fetal life and decrease postnatally as IGF-I synthesis begins (reviewed in 116). Regulatory differences in the *IGF-2* gene promoter regions are at least partially responsible for these developmental differences. The *IGF-2* gene has three promoters (#2-4) that regulate high IGF-II expression in both human and rodent fetal tissues. However, after birth these promoter regions have low activity in human tissues and they are virtually silent in rodents. Only the *hIGF-2* gene has an additional liver-specific promoter (#1) that is activated following birth and stimulates IGF-II synthesis in postnatal human liver, contributing to the rising serum IGF-II levels during childhood (reviewed in 261).

The regulation of fetal expression of the two IGFs is not well understood in either humans or animal models. However, hGH-N can stimulate human fetal cells and tissues to produce both IGF-I and IGF-II [for details see sections 1.2.2.5 and 1.3.6]. In addition maternal IGF-I levels progressively rise during pregnancy (164); the significant correlation of IGF-I and hGH-V concentrations in maternal plasma suggests that hGH-V may have an important stimulatory effect on maternal IGF-I production and subsequent placental transfer of nutrients to the fetus. Studies in fetal sheep have suggested that increased placental glucose transfer stimulates insulin secretion which, in turn, enhances IGF-I release by target tissues (reviewed in 262); fetal IGF-II serum levels are thought to be almost entirely constitutively regulated and only altered by significant nutritional and hormonal changes (reviewed in 116). Infusion of IGF-I into sheep fetal circulation reduces placental lactate production and amino acid uptake by the placenta, enhancing

substrate delivery to the fetus, while maternal IGF-I stimulates nutrient transfer to the fetus (263).

Transgenic animal studies have clearly demonstrated that IGFs are crucial to the overall growth and development of the mammalian fetus (264,265). Ablation of *IGF-2* gene expression in mice causes a 40% growth reduction by midgestation. This finding correlates well with the predominance of IGF-II over IGF-I expression in fetal tissues of all species. The observation that these *IGF-2* mutant mice grow at normal rates following birth is also consistent with the normal absence of IGF-II postnatally in rodents. Although these findings provide considerable insight, they may not be an entirely accurate reflection of IGF-II physiology in humans: the presence of IGF-II in postnatal serum suggests that it may continue to have some growth-promoting activity following birth.

The effects of disrupting *IGF Type II receptor* gene expression in mice was also studied and found to be lethal by midgestation (266). It was concluded that this lethality is probably due to the effects of IGF-II overabundance because the mutant mice are rescued when their *IGF-2* gene is knocked out in parallel (267). Thus, the interpretation of these findings is consistent with the role of the Type II receptor targeting IGF-II to lysosomes where it is degraded.

Efstrafiadis' laboratory has furthered our understanding of fetal IGF physiology by creating mice with a lack of IGF-I and IGF Type I receptor expression (268-270). Transgenic mice homozygous for disruption of the *IGF-1* gene exhibited a 40% weight reduction (268-270), while homozygous mutants for the Type I receptor had a 55% decrease in fetal size and invariably died soon after birth (269,270). Crosses of mice

bearing mutations in their *IGF* and *Type I receptor* genes have provided even more insight. IGF-I and Type I receptor double knockouts (270) had similar phenotypes as mice lacking only the Type I receptor, suggesting that all IGF-I effects are mediated by the Type I receptor. However, transgenic mice with a double knockout of the *IGF-2* and *Type I receptor* genes were even more growth retarded at birth (ie. 70% decrease in size) than mutants for only one of these two genes (269,270). Since the Type II receptor does not appear to mediate any IGF growth-promoting action, these data suggest that IGF-II may have additional somatogenic activity through its limited affinity for the insulin receptor and/or another as yet undefined receptor. Interestingly, this additional IGF-II activity may be necessary for placental growth since placentae of mice with an absence of either IGF-I or Type I receptor expression (or both) are normal, while the placentae are small in mice that do not express IGF-II.

In the human, both fetal and neonatal size correlate with newborn serum levels of IGF-I, but not IGF-II (271). A recent case report has shown that a homozygous molecular defect in the *hIGF-I* gene results in severe perinatal as well as postnatal growth failure (272). The fact that no homozygous mutations in the *hIGF-II, IGF Type I or Type II receptor* genes have been detected to date suggests that, in humans, absence of their expression is either lethal or, although unlikely in the case of the Type I receptor, does not result in any significant pathophysiological phenotype.

Insulin has also been implicated to have fetal growth effects in the human. The fetal pancreas begins to make insulin from as early as 9-10 weeks of fetal age and insulin receptors are expressed on fetal target tissues by 15 weeks of gestation (reviewed in 273).

Children of mothers with uncontrolled or poorly controlled insulin-dependent diabetes mellitus are born large for gestational age. This is generally attributed to fetal hyperinsulinism as the result of the response of the fetal pancreas to hyperglycemia of maternal origin. Fetal macrosomia is thought to occur primarily through the direct adipogenic actions of insulin, although there is a small increase in lean body mass that is most likely promoted by the positive regulatory effect of insulin on IGF-I levels (reviewed in 273).

1.4.2.2. IGF binding proteins.

In addition to their key regulatory roles in postnatal somatomedin physiology, IGFBPs 1, 2, 3 and 5 may also control bioavailability of fetal IGFs. IGFBP-2, which has greater binding affinity for IGF-II as compared to IGF-I, is ubiquitously expressed during the initial stages of ovine and human fetal life, but is restricted to only the liver and kidney in the second half of gestation (274). These findings suggest that, while the autocrine and paracrine actions of IGF-II may decrease during the second half of gestation in the majority of fetal tissues, they continue throughout gestation in the liver and kidney. Transgenic mice studies have demonstrated that IGF-II and IGFBP-2 promote cellular growth in a fashion that is dependent on a balance between both molecules (20). High concentrations of IGFBP-1 are found in amniotic fluid (275), while high levels of IGFBP-5 are detected in multiple human fetal tissues (276).

In the human, rhesus monkey and sheep, plasma concentrations of the IGF/IGFBP-3/ALS 150 kD complex become detectable around midgestation and increase

to term (reviewed in 133). However, fetal rodents lack IGFBP-3 complexes in utero because of their liver's inability to produce the GH-regulated ALS subunit of the carrier protein complex. These observations suggest that GH and its receptor may have species-specific functions during gestation. Indeed, GH binding to fetal hepatocyte GHRs has been observed in both the human and the sheep, but not in fetal rodent liver preparations. Therefore, GH regulation of the 150 kD IGFBP-3 carrier complex synthesis in fetal hepatocytes may have important consequences in the primate and sheep, while the analogous IGF carrier complex in mice and rats may not be necessary until after birth.

1.4.2.3. Placental influences.

The placenta plays a crucial role in nutrient transfer from the mother to the fetus (reviewed in 277,278). In fact, there is a direct relationship between growth of the placenta and fetus. These processes depend on good maternal nutrition, an adequate supply of maternal blood to the placenta, and multiple hormones, growth factors and cytokines produced by the fetus, the mother and the placenta itself (e.g., GH-V [see section 1.2.3], PL, IGF-I, IGF-II, EGF, etc.).

As mentioned in section 1.2.3, placental production of PL has been documented in primates, ungulates and rodents. In the human, hPL can be detected in the maternal circulation by the third week postconception as a 22 kD protein composed of 191 amino acids (reviewed in 278). Peripheral levels of maternal hPL rise throughout gestation and peak at term (1-15 μg/ml). By the third trimester, hPL accounts for $\sim$10% of placental protein production. hPL is also present in fetal serum but its concentration is

approximately 1% that measured in maternal blood throughout gestation. In contrast to hGH-N, but like placental hGH-V, hPL is secreted in a sustained rather than a pulsatile fashion. The regulation of hPL expression by the syncytiotrophoblasts is not yet well understood, but in vitro and transfection studies have shown that retinoids, thyroid hormone and vitamin D stimulate hPL expression (279,280). As mentioned earlier, there are two placental *hPL* genes, *hPL-A* and *hPL-B* that code for the same protein. Further studies are needed to unravel the mechanisms regulating differential expression of these two genes.

Gestational increases in maternal hPL plasma levels correlate well with both placental and fetal size, suggesting that hPL modulates placental growth and nutrient transfer (reviewed in 278). hPL is thought to preferentially increase glucose availability for the fetus by its diabetogenic and lipolytic activities: diabetic mothers have decreased carbohydrate tolerance following hPL treatment, while hPL stimulation of adipocytes results in increased glycerol and nonesterified fatty acid release, glucose uptake and oxidation, as well as glucose incorporation into glycogen, glycerol and fatty acids. When maternal glucose blood levels are high, the hPL-mediated increases in glucose uptake and storage by adipose tissues ensures that there will be sufficient maternal energy supplies, in the form of triglyceride, during fasting periods.

hPL also has direct effects on human fetal tissues. Human fetal liver and muscle membrane preparations bind hPL from as early as 12 weeks of gestation (281). Treatment of cultured fetal (13-19 weeks) fibroblasts and myoblasts with hPL enhances amino acid transport, [^{3}H]thymidine incorporation into DNA, mitogenesis and IGF-I

release (282). hPL also stimulates DNA synthesis and IGF-I production in first trimester fetal hepatocytes (146,282).

There are only a few case reports of low or absent levels of hPL or hGH-V in maternal blood during gestation (reviewed in 16,283). Examination of these rare cases suggests that defects in placental synthesis of either hPL or hGH-V does not alter normal feto-placental development. However, Rygaard et al have recently investigated the outcome of a pregnancy where both hormones were absent and the fetus was severely growth retarded (283). The placenta and fetus were found to have deletions of the *hGH-V* as well as *hPL-A and hPL-B* genes. Therefore, it is possible that both hPL and hGH-V contribute to the normal progression of a pregnancy, and that the presence of one placental hormone can compensate for absence of the other.

A specific receptor for PL has not yet been identified in primates. However, hPL is able to bind the hGHR (reviewed in 24). Interestingly, despite the 87% amino acid sequence identity between 22 kD hGH-N and hPL, the affinity of hPL for the high-affinity hGHBP is 2300-fold less than hGH: this is because four (Met14, Glu56, Arg64 and Ile179) of the 25 binding determinants of hGH are altered in hPL (Val14, Asp56, Met64 and Met179). Given that both 22 kD hGH-N and hPL have only 35% amino acid homology with hPRL, it is not suprising that both hormones have substantially lower affinities for the hPRL receptor; like hGH, hPL requires Zn^{+2} ions for hPRL receptor binding.

It is fascinating that expression of each placental-specific member of the murine *PRL* gene family occurs at a precise phase of pregnancy: giant cells of early to

midpregnancy placentae synthesize PL-I, PL-II, proliferin (PRF) and PRL-like proteins A and E, whereas during mid- to late gestation these cells produce PL-II and proliferin-related hormones (PRPs) (47,48,284-289). These switches may be responsible for first establishing vascularization at the implantation site and then, later in pregnancy, reducing neovascularization to prevent maternal and fetal blood vessels from crossing. Further studies need to be undertaken to determine the synergistic and specific maternal as well as fetal biologic actions of these hormones.

1.4.2.4. The "dogma".

The "dogma" in the literature is that GH has little or no effect on mammalian fetal growth, and that there is a switch to GH-dependent growth sometime following birth. Transgenic mouse studies have, in fact, revealed that GH overexpression only potentiates growth beginning approximately 4 weeks after birth (290), while mice homozygous for a null mutation of the *GHR* gene do not show a decrease in size until 4-6 weeks postnatally (291). Fetal decapitation experiments in rats and rabbits have also implied that GH does not have a growth-promoting effect during fetal life since these animals have a similar body size as their untreated littermates (140). These data are likely due to the fact that there is a significant onset of *GHR* gene expression and GH binding only after birth in all subprimates examined to date.

However, there are findings that challenge this dogma, even in the rodent model. Panteleon et al have recently identified functional GHRs in preimplantation mouse embryos that are capable of mediating GH effects on glucose transport and protein

synthesis in the blastocyst (143). In addition, Zhou et al found that inbreeding of homozygous GHR knockout mice results in significantly smaller litter size and increased perinatal mortality (291). GH infused in a pulsatile manner into fetal sheep increases fetal and placental weight without altering IGF-I or IGFBP-3 plasma levels, suggesting that GH may have direct or IGF-II mediated effects in utero (reviewed in 262). As discussed earlier, the primate and sheep GHR may be mediating both GH as well as PL fetal responses. Furthermore, our laboratory and others have obtained in vitro data demonstrating that hGH treatment directly influences human fetal hepatocyte, pancreas and bone function [for details see section 1.2.2.5].

Critical analysis of these as well as clinical (discussed in the next section) data suggests that, in addition to the necessary actions of IGFs for mammalian growth in utero, GH and its receptor may have species-specific functions during fetal life.

1.5. Human Growth Disorders and the hGH/hGHR/IGF-I Axis.

1.5.1. hGH-N deficiency.

Defects in hGH-N production undoubtedly result in severe growth impairment during childhood (reviewed in 292). The frequency of hGH deficiency is 1:4000 to 1:10000, with most cases being an idiopathic disorder. The critical role of the transcription factor Pit-1 in the generation, proliferation and phenotypic expression of somatotropes make this protein a prime candidate for implication in the etiology of hGH deficiency (see section 1.2.1). In fact, four cases of hGH deficiency being caused by Pit-1 gene mutations have been reported (293-296). In addition, two GH-deficient dwarf

rodent models have Pit-1 defects: the Snell mouse has homozygous rearrangements in its *Pit-1* alleles, and the Jackson mouse has homozygous Pit-1 homeodomain mutations (297).

Approximately half of the autosomal idiopathic hGH deficiency cases have been linked to the *hGH-N* locus. Phillips and Cogan have classified familial hGH deficiency into four groups depending on the mode of inheritance and severity of the disorder (298). Group 1A consists of homozygous *hGH-N* gene deletions, compound mutations consisting of a deletion and a frameshift, as well as homozygous nonsense mutations: all lead to complete absence of hGH-N production, severe dwarfism by six months of age, as well as birth size reduction and hypoglycemia in some infants. The response of children in Group 1A to recombinant hGH is variable and depends on whether they develop antibodies against exogenous hGH. Group 1B, also autosomal recessive, is due to homozygous splice donor mutations at the exon 4 boundary of the *hGH-N* gene or compound *hGH-N* gene alterations consisting of a deletion and a frameshift within exon 3. Group II patients have an autosomal dominant condition that results from heterozygous splice donor site mutations at the exon 3 boundary of the *hGH-N* gene. Both group 1B and II patients have reduced hGH plasma levels with less severe short stature. The last class of pediatric patients with reduced levels of hGH have an X-linked disease that does not have any apparent *hGH-N* gene alteration but, nonetheless, causes short stature and, in some cases, hypogammaglobulinaemia.

Linkage studies have excluded the *hGH-N* locus in at least half of individuals with autosomal isolated hGH deficiency (reviewed in 292). In these patients, the physiological

regulators of GH release are likely candidates for genetic defects. GHRH gene mutations have not been reported in any species; this may be because GHRH is highly expressed in the placenta and may have crucial metabolic functions for fetal survival. The dwarf "little" mouse has an autosomal recessive mutation in its GHRH receptor that is associated with reduced GH plasma levels (299). In addition, a nonsense mutation in the hGHRH receptor has recently been identified in several patients with low serum hGH levels and growth failure (300,301). Mutations in the hGHSR and and its natural ligand as well as genetic alterations that enhance the activity of inhibitory factors (ie. somatostatin and/or its receptors) on hGH release may also prove to be implicated in the pathophysiology of idiopathic hGH-deficiency.

Phillip's and Cogan's findings that neonates with a complete absence of hGH expression can have significant lower birth sizes suggests a role for hGH and its receptor in utero (298). To further assess the potential role of hGH in fetal and infant growth, Gluckman et al analyzed pretreatment data on 52 patients diagnosed with congenital hGH deficiency before 2 years of age (302). In comparison to late-onset organic hGH deficiency and control infants, these patients were found to have reduced birth length standard deviation (SD) scores, an excess birth weight relative to length, and rapid progressive postnatal growth failure. In fact, 12-20% of the neonates were more than 2 SD shorter at birth as compared to control subjects. These clinical data clearly indicate that hGH is necessary for postnatal linear growth, but also suggest that hGH deficiency can cause impaired growth in utero and early infancy.

1.5.2. hGH insensitivity.

Patients with "hGH insensitivity" have normal to elevated circulating levels of hGH levels, low serum IGF-I values and do not respond well to hGH therapy (reviewed in 292a). There is a broad range of hGH insensitivity, ranging from partial to complete (Laron syndrome). In addition to genetic and idiopathic causes, there are also acquired forms of this disease that are thought to cause poor growth in many chronic diseases of childhood, such as cystic fibrosis and inflammatory bowel disease. Acquired forms of hGH insensitivity may be due to a variety of factors, including circulating antibodies to hGH that inhibit the hormone's biological activity.

In its classical inherited form, Laron syndrome is an autosomal recessive disease that is the result of hGHR dysfunction (reviewed in 292a); however, dominant negative mutations in the *hGHR* gene have now been identified in two families with short stature and hGH insensitivity (292b, 292c). Like children with complete hGH deficiency (Group 1A), Laron syndrome patients have hypoglycemia, reduced birth size, abnormal craniofacies and severe childhood growth failure. Large cohorts of patients with Laron syndrome have been reported in Israel, Ecuador and Turkey. These individuals have normal to high plasma hGH levels with extremely low or absent IGF-I values. Laron syndrome is generally accompanied by reduced or absent serum hGHBP, with approximately 20% of affected individuals having normal values. Extremely high hGHBP values in some patients is the result of genetic mutations that cause the *hGHR* gene transcript to be translated into a soluble protein as opposed to a membrane-anchored receptor. The presence or absence of hGHBP does not seem to affect the severity of hGH

insensitivity.

To date, studies have identified deletions as well as missense, nonsense and splice mutations within coding exons of the *hGHR* gene in patients with Laron dwarfism (reviewed in 292a). The absence of hGHR and hGHBP expression in some Laron patients may also be explained by complete silencing of the gene or by the inability of the mature *hGHR* gene transcript to be successfully translated.

There is a large population of idiopathic short stature (ISS) patients who are characterized as having partial hGH insensitivity (reviewed in 292a). These patients are greater than 2 SD below the mean height for their age and sex with a persistently slow growth rate and no evidence of systemic disease, malnutrition, hypothyroidism or hGH deficiency. The cause of growth failure in these children remains poorly understood. Recently, Goddard et al suggested that heterozygous *hGHR* gene mutations may cause partial GH insensitivity syndrome (milder growth retardation as compared to Laron dwarfism); however, only a small group, 8 of 100 ISS children examined, were found to have mutations within *hGHR* gene exons (303). In addition, it is possible that partial silencing of *hGHR* gene transcription contributes to partial hGH insensitivity by lowering the levels of hGHR expression. In support of this hypothesis are studies by Attie et al demonstrating that more than 90% of 500 ISS children had serum hGHBP levels below the normal mean for age and sex, with 18% of the patients having average values greater or equal to 2 SD below the normal mean (304). Similar findings have been obtained by a separate investigation using a more direct radioimmunoassay for hGHBP (305). A complete structural and functional map of the *hGHR* gene regulatory regions must first

be obtained before it will be possible to design experiments aimed at testing whether ISS or Laron children have silencing alterations in their *hGHR* gene promoter regions.

Patients with normal hGHBP activity and no known cause for their hGH insensitivity are good candidates for defects in the activation of post-receptor cascades. In fact, Thanakitcharu et al have recently identified three homozygous missense mutations (422F, L526I and P561T) in the intracellular domain of the hGHR of a hGHBP-positive patient (292d). It is also possible that these patients have an abnormality in either one of the effector proteins involved in intracellular cascades or in the *IGF-1* gene itself. Although defects in signal transducing proteins are unlikely, as these molecules appear to be shared by a large number of receptor signalling pathways, in vitro studies of fibroblasts from a hGHBP-positive patient with no apparent *hGHR* gene mutation have revealed a postreceptor defect in the activation of STAT 5. In addition, another patient with intrauterine as well as postnatal growth failure has been found recently to have an *IGF-1* gene deletion [see section 1.3.2.1].

The finding that both hGH deficiency and insensitivity can affect birth size has suggested a role for hGH and its receptor during development.

1.6. Objectives of the Dissertation Research.

To help clarify the role of hGHR during early human development, several questions were addressed during the thesis project.

First, what is the ontogenic pattern of *hGHR* gene expression in human tissues? (Chapter 2)

Second, are there fetal isoforms of the hGHR mRNA that may give rise to "fetal-specific" receptor biological activity? (Chapters 2, 3, 4 and Appendix 1)

Third, what are the mechanisms regulating transcription of the *hGHR* gene in fetal versus postnatal tissues? (Chapter 4 and Appendix II).

Fourth, what is an appropriate animal model in which to study developmental changes in GHR expression? (Chapter 5)

The long-term goals of the laboratory are to determine whether differential regulation of hGHR mRNA synthesis gives rise to developmental- or tissue-specific hGHR functions and whether abnormal regulatory mechanisms can lead to clinical disorders of growth (i.e., complete or partial hGH insensitivity).

PART II.

EXPERIMENTAL SECTION

CHAPTER 2

CHAPTER 2 - *EXPRESSION OF EXON THREE RETAINING AND DELETED HUMAN GROWTH HORMONE RECEPTOR MESSENGER RIBONUCLEIC ACID ISOFORMS DURING DEVELOPMENT*

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2.1. Preface.

To characterize the onset of *hGHR* gene transcription, several human tissues from as early as the first trimester of fetal life were assayed by reverse transcription-polymerase chain reaction (RT-PCR) for hGHR mRNA expression. The identity of the amplified fragments was confirmed by Southern blot analysis. These studies also investigated the tissue distribution and ontogeny of the exon 3 retaining and deleted hGHR mRNA isoforms. In a final set of experiments, the entire coding region of the hGHR mRNA was "scanned", using a series of overlapping PCR primers, to determine whether there are any additional fetal or postnatal isoforms of the mRNA coding region: one advantage of RT-PCR methodology is that it permits the identification of mRNA isoforms with small size variations, such as the 66 nucleotide difference between the exon 3 retaining and deleted isoforms.

Figure 2.3 has been modified to include further data obtained after publication. In the original study, the ontogeny of the exon 3 retaining and deleted hGHR mRNA isoforms was investigated in 78 subjects. Since then, the sample size has increased to 117. This additional data did not alter the conclusions of the study, but enhanced the statistical significance of the findings from a p value of 0.002 to 0.0002.

2.2. Abstract.

Recent investigations have suggested that human growth hormone (hGH) and its receptor (hGHR) may have specific functions during human fetal life. To improve our understanding of the mechanisms of hGH action during gestation, we have characterised the ontogenic appearance of hGHR mRNA in multiple human fetal and postnatal tissues. Using reverse transcriptase-polymerase chain reaction assays, followed by Southern hybridization to confirm the specificity of the amplified fragments, we scanned the entire coding region of the hGH receptor mRNA.

Transcription of the *hGHR* gene was observed in all fetal tissues studied (liver, kidney, skin, muscle, lung, adrenal, spleen, intestine, CNS, pancreas and placental villi), from the earliest stage that could be examined (4-14.8 weeks fetal age [FA]). Furthermore, we identified only two isoforms of the hGHR mRNA coding region: exon 3 can be retained or deleted. Surprisingly, we found individual-, and not tissue-, specific expression patterns of these two transcripts when we examined multiple tissues (n=2-6) from fifteen individuals (11.5-33 weeks [FA]); this individual-specific pattern of expression is maintained in cultured dermal fibroblasts for at least twelve generations (n=2; 16 and 20 weeks FA). In addition, a cross-sectional study of 117 individuals (4 weeks FA to 64 years postnatal) showed that the exon 3 deleted transcript is predominantly expressed in tissues from fetuses of 4 to 20 wks of FA ($p<0.0002$). Finally, we showed that the absence of exon 3 from the mRNA is not due to genomic deletion of exon 3, by amplifying exon 3 from genomic DNA of three fetuses (13.3-19 weeks FA) expressing only the exon 3 deleted mRNA transcript.

We conclude that: 1) transcription of the *hGHR* gene is occurring in multiple tissues as early as the first trimester of human fetal life; 2) the exon 3 retaining and deleted transcripts are the only two isoforms of the hGHR mRNA coding region during gestation; and 3) the pattern of expression of these transcripts is individual-specific and may be developmentally regulated.

2.3. Introduction.

Despite markedly elevated plasma levels of immunoreactive growth hormone (GH) in the fetus (1), fetal hypophysectomy and decapitation studies in several animal species suggest that GH is not a major determinant of mammalian fetal growth until after birth (2,3). In addition, an excess of GH, as noted in transgenic mice carrying GH fusion genes, accelerates postnatal but not fetal growth (4,5). One possible explanation for GH insensitivity during early development is that fetal target tissues may lack the GH receptor (GH). Indeed, animal studies have shown that there is a significant onset of tissue *GHR* gene expression and binding following birth (6-8).

In contrast, there are both experimental and clinical data to suggest a role for human (h) GH and its receptor in the human fetus. Specific binding of hGH has been demonstrated in early gestation human fibroblasts, chondrocytes and liver membranes (9-11). Immunohistochemical studies have revealed that immunoreactive hGHR peptide appears as early as 8.5 weeks of fetal age (wk FA) in human hepatic parenchyma, kidney tubular epithelia and dermal fibroblasts (12,13). Treatment of human fetal hepatocytes and pancreatic islets with hGH results in stimulation of DNA synthesis and insulin-like growth factor-I (IGF-I) secretion (14-16). Finally, the GH-dependent IGF binding protein, IGF-BP3, is detectable in fetal serum at midgestation with levels rising slowly to term (17).

Clinical data indicate that either hGH deficiency or hGHR dysfunction (Laron dwarfs) may result in impaired growth in utero. The mean birth length and weight of idiopathic hGH deficient children are both significantly below those of control age-

matched neonates; between 12-20% are, in fact, more than 2 SD shorter than normal newborns at birth (18,19). There is also a subpopulation of Laron syndrome infants who are more than 2 SD shorter than normal controls at term (20,21).

Because of the potential role of the hGHR in regulating human fetal growth, we have examined a number of hGH target tissues to determine: 1) the tissue-distribution of the hGHR mRNA during fetal life; and 2) whether there are fetal- (vs. postnatal-) specific isoforms of the hGHR mRNA coding region. To date, two isoforms of the hGHR mRNA coding region have been identified in human tissues and cell lines: exon 3 can be retained or deleted (22-27). The absence of exon 3 from the hGHR mRNA predicts a 22 amino acid deletion in the amino-terminal portion of the receptor's extracellular domain.

Our findings show that: 1) the *hGHR* gene is expressed in multiple tissues as early as the first trimester of human fetal life; 2) the exon 3 retaining and deleted transcripts are the only two isoforms of the hGHR mRNA coding region produced during gestation; and 3) expression of exon 3 is individual-specific and may be developmentally regulated.

2.4. Materials and Methods.

2.4.1. Tissues.

Tissues from human fetuses (4-20 wk FA) were obtained at the time of therapeutic abortion; specimens from premature and term newborns (24-40 wk FA) and postnatal donors (1 wk - 64 yr) were obtained within 12 h following death. All tissues were flash frozen in dry-ice/acetone and immediately stored at -70°C for RNA and DNA extraction. Fetal age was determined by foot length (28). Protocols for obtaining human tissues were approved by local ethics committees.

The premature and term newborns (n=12) had died within 1-7 days following birth due to a variety of pathologies (oligohydramnios, RDS, pneumothorax, polycystic kidney disease, diaphragmatic hernia and sepsis). Postnatal hepatic tissues (n=7) were collected from transplant donors who had died of acute head injuries. Postnatal skin samples were obtained from pediatric patients at the time of surgery (inguinal or abdominal hernia; n=4) or from our local Genetics Cell Bank collection (normal controls; n=2). The postnatal GI tissues (n=4) were biopsy samples subsequently diagnosed as normal.

2.4.2. Cultured cells.

Dermal fibroblasts of various developmental stages (10-38 wk FA; 4 months - 35 yr postnatal) were grown as monolayer cultures in HAM'S F-10/DMEM medium (1:1; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal/newborn calf serum (1:1) (Gibco BRL), 100 UI/ml of penicillin G (Marsam Canada Pharmaceutical, Montréal,

QC), 16 ug/ml of gentamycin sulfate (Schering Canada, Pointe Claire, QC) and 2.5 ug/ml of amphotericin B (Squibb Canada, Montréal, QC). IM-9 human lymphoblast cells were grown in suspension in RPMI-1640 medium (Gibco BRL) with 10% fetal/newborn calf serum (1:1), 100 UI/ml penicillin G, 16 ug/ml gentamycin sulfate and 2.5 ug/ml amphotericin B. Cultured fibroblasts from the 6.5 to 12 generations and IM-9 cell preparations were rinsed in PBS (0.15 M NaCl in 20 mM phosphate buffer pH 7.4), flash frozen in dry-ice/acetone and stored at -70°C for RNA extraction.

2.4.3. Hepatocyte preparations.

Human fetal liver cells were isolated using a modified protocol of Rodd et al. (29). For each experiment, approximately 2 grams of human fetal liver tissue were minced in 10 ml of warm (37°C) solution I (10 mM Hepes pH 7.4, 0.42 M NaCl, 6.7 mM KCl and 1 mM EDTA). 10 ml of warm (37°C) solution II (100 mM Hepes pH 7.4, 67 mM NaCl, 6.7 mM KCl, 4.8 mM CaCl, 20 mg collagenase type IV (Cooper Biomedical, Mississauga, ON) and 2 mg DNAse I (Sigma Chemicals, St. Louis, MO)) were added and the minced tissue was dispersed with a pasteur pipette for 10-15 min at 37°C. The resultant dispersate was filtered through a 50-100 um sterile mesh and the cells collected by centrifuging for 10 min at 500xg. The supernatant was discarded, the pellet resuspended in 20 ml of Minimum Essential Medium (Gibco BRL) containing 4.8 mM EDTA and 3.2 mg DNAse I and the resultant suspension was allowed to settle at unit gravity for 20 min at 37°C. The supernatant was discarded and this step was repeated once more. The final layer of sedimented cells, containing primarily

hepatocytes, was centrifuged at 500xg for 10 min and the resultant pellet was resuspended in HAM'S/DMEM medium supplemented with 10% fetal/newborn calf serum (1:1), 2 uM cortisol, 100 UI/ml penicillin G, 16 ug/ml gentamycin sulfate and 2.5 ug/ml amphotericin B. The isolated hepatocytes were plated on collagen-coated petri dishes. 14-18 h after plating, the hepatocytes were rinsed thoroughly (3-5 times) with HAM'S/DMEM medium to remove all remaining traces of blood cells and then trypsinized. The trysinized cells were collected by centrifuging at 500xg for 10 min. The resultant pellet was rinsed in PBS, flash frozen in dry-ice/acetone and immediately stored at -70°C for RNA extraction. Purity of each preparation (>99%) was monitored using phase microscopy, while viability (>99%) was ascertained using trypan blue uptake.

2.4.4. RNA and DNA isolations.

Total RNA was isolated from approximately 1 gram of frozen tissue or 10^8 cultured cells using the guanidine thiocyanate/CsCl gradient method (30). The acid-guanidium-phenol-chloroform protocol (31) was used to extract total RNA from tissues of 0.5 grams or less and the Nonidet P-40/urea approach (32) was employed to extract total RNA from samples of approximately 10^4-10^6 cultured cells. Genomic DNA was isolated from approximately 1 gram of frozen tissue using the proteinase K/sodium dodecyl sulfate protocol (33).

2.4.5. Reverse transcriptase (RT)-PCR.

For each reaction, approximately 6 ug of total RNA were reverse transcribed for

1 h at 48°C in 2.5 units of Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) (GIBCO BRL), 80 units of RNAsin (Promega, Madison, WI), 71.4 ng/ul random primers (GIBCO BRL), 0.48 mM deoxyribonucleotides (dNTPs) (Pharmacia Biotech, Baie D'Urfe, QC), 10 mM $MgCl_2$, 10 mM DTT, 100 mM Tris-HCl pH 8.3 and 50 mM KCl. Prior to the reverse transcription reaction, the RNA was heated at 70°C for 5 min to disrupt any secondary structure. Six μl of RT product were then amplified for 25 cycles in 2.5 units of Thermus aquaticus (Taq) DNA Polymerase (GIBCO BRL), 0.5 mM dNTPs, 0.25 uM hGHR sense and antisense primers (Figure 2.1, Table 2.1), 3 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4 and 50 mM KCl. Prior to the PCR reaction, the RT product was heated for 5 min at 95-98°C to denature the AMV-RT. The first cycle consisted of 3 min denaturation at 92°C, 1 min annealing at 61°C and 3 min elongation at 72°C. Subsequent cycles consisted of 30 s denaturation at 92°C, 1 min annealing at 61°C and 1.5 min elongation at 72°C. The reaction was terminated with a final elongation of 5 min at 72°C. In each RT-PCR assay, an aliquot of H_2O was amplified in parallel as a negative PCR control.

2.4.6. Southern blotting.

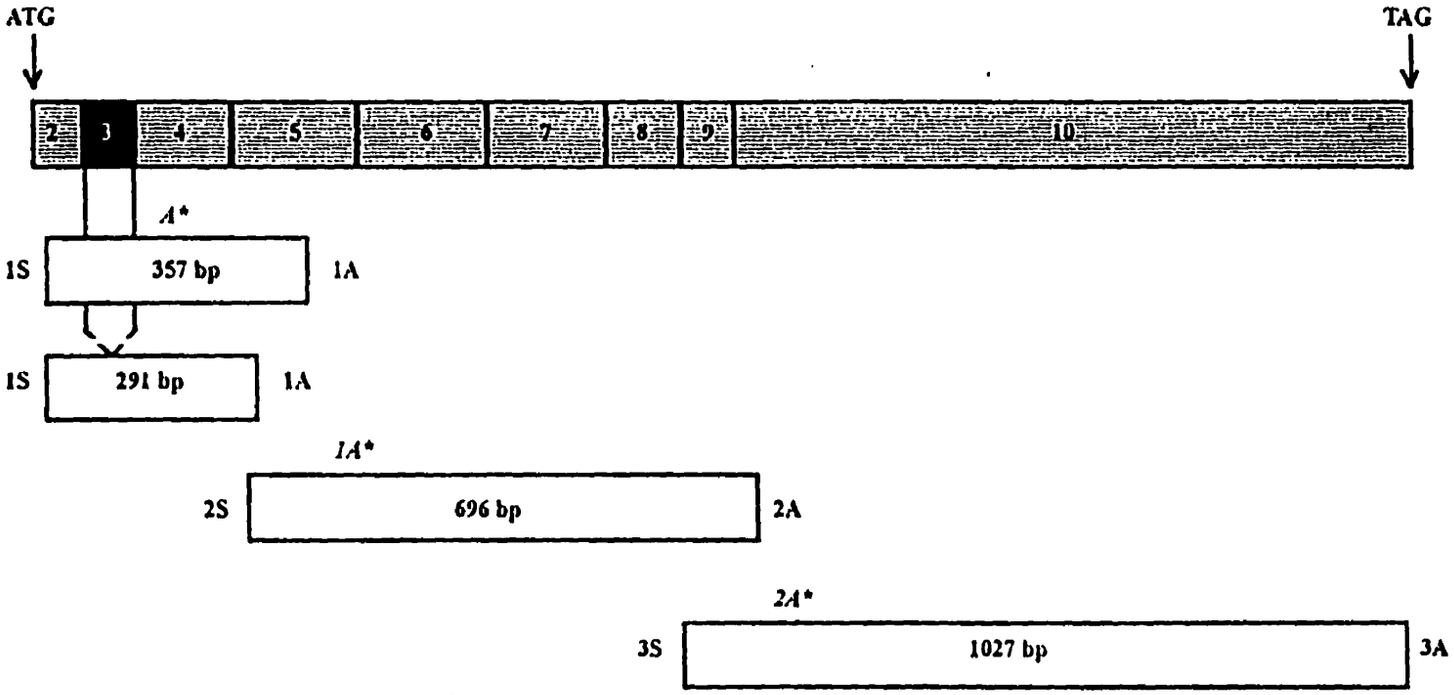
hGH receptor amplified cDNA fragments were electrophoresed through 1% or 2% agarose gels and then transferred to 0.45 um positively charged nylon membranes (Schleicher & Schuell, Keene, NH) by capillary action with 10xSSPE. Prior to blotting, the DNA was denatured by soaking the gels twice for 15 min in 0.5 N NaOH/1.5 M NaCl and then neutralized by soaking twice more for 15 min in 1 M Tris-HCl, pH

Table 2.1. Oligonucleotide sequences of PCR primers and internal probes (*).

Primer	Sequence
1S	5'-CTG CTG TTG ACC TTG GCA CTG GC-3'
1A and 1A*	5'-AGG TAT CCA GAT GGA GGT AAA CG-3'
A*	5'-GAA CCT CAT CTG TCC AGT GGC AT-3'
2S	5'-TCA AGA ATG GAA AGA ATG CCC TG-3'
2A and 2A*	5'-GCT AAG ATT GTG TTC ACC TCC TC-3'
3S	5'-TGA TTC TGC CCC CAG TTC CAG TT-3'
3A	5'-GGC ATG ATT TTG TTC AGT TGG TC-3'

Fig. 2.1. RT-PCR/Southern blot strategy. Shaded boxes represent hGHR mRNA coding exons. Approximate locations of the sense (S) and antisense (A) PCR primers, nested hybridization probes (A^*, $1A^*$ and $2A^*$), and the lengths of the PCR products (non-shaded boxes) are indicated. Oligonucleotides A^*, $1A^*$ and $2A^*$ served as internal hybridization probes for cDNA fragments amplified with PCR primer sets 1S/1A, 2S/2A and 3S/3A, respectively. The precise sequence of each of these oligonucleotides has been detailed in Table 2.1.

hGH Receptor cDNA



7.4/1.5 M NaCl. The nucleic acids were immobilized onto the membranes by exposing the blots to ultraviolet light (254-312 nm) for 5 min.

Blots were prehybridized for 3-5 h at 42°C in 6xSSPE, 1% SDS, 10xDenhardt's and 0.15 mg/ml denatured salmon sperm DNA (Sigma Chemicals). The volumes of the prehybridization and hybridization solutions were 100 μl per cm^2 of nylon membrane. Hybridization occurred overnight at a temperature (T_H) of 5°C less than the melting temperature of the hybrid formed between the probe and its complementary nucleic acid sequence. The hybridization solution contained 6xSSPE, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA and 13.2-26.3 nCi per cm^2 of nylon membrane of probe. Following hybridization, blots were washed twice for 10 min at T_H with 6xSSPE/1%SDS and then once more for 10 min at T_H minus 5°C with 2xSSPE/1%SDS. Bands were visualized by autoradiography. ß-actin cDNA served as a control for specific hybridization on each blot.

Approximately 150 nmol of the nested oligo to be used as the probe (Figure 2.1 and Table 2.1) were end-labelled and then purified through a Sephadex G-50 Medium (Pharmacia Biotech) column. The end-labelling reaction occurred at 37°C for 1 hour in the presence of 2 uCi/ul of γ-^{32}P-ATP (NEN Research Products), 20 units of T_4 polynucleotide kinase (GIBCO BRL), 70 mM Tris-HCl pH 7.6, and 10 mM $MgCl_2$ 100 mM KCl and 1 mM 2-mercaptoethanol.

2.4.7. Sequencing analysis.

Exon 3 retaining and deficient cDNA fragments were sequenced by the dideoxy

chain-termination procedure of Sanger (34) using hGH receptor sense (1S) and antisense (A*) primers (Figure 2.1, Table 2.1). The reaction products were resolved on a 6% polyacrylamide gel and visualized by autoradiography.

2.4.8. Amplification of genomic DNA.

Approximately 100 ng of genomic DNA were amplified for 35 cycles in 2.5 units of Taq DNA Polymerase, 0.2 mM dNTPs, 0.25 uM sense (5'-GAT GGT TTT GCC TTC CTC TT-3') and antisense (5'-AAA TAT CAT TCG AAG GAA GAA A-3') primers, 2.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4 and 50 mM KCl. The first cycle consisted of 3 min denaturation at 95°C, 30 s annealing at 50°C and 25 s elongation at 72°C. Subsequent cycles consisted of 45 s denaturation at 95°C, 30 s annealing at 50°C and 25 s elongation at 72°C. PCR fragments were visualized on a 0.5 ug/ml ethidium bromide, 2% agarose gel.

2.4.9. Statistical analysis.

Statistical evaluation of the exon 3 developmental data (Table 2.3) was carried out using a 2x2 contingency table with Fisher's Exact Test.

2.5. Results.

A combined RT-PCR and Southern blot approach (Figure 2.1) was used to scan the entire coding region of the hGHR mRNA in human fetal and postnatal tissues. As previous studies have shown that human IM-9 lymphoblast cells express both full-length and exon 3 deleted hGHR mRNA isoforms (23), total RNA from IM-9 cells served as a positive control in all of our RT-PCR assays (Figures 2.2-2.6). H_2O and ß-actin cDNA served as negative controls for the PCR reactions and Southern blot hybridizations, respectively (data not shown).

2.5.1. Tissue distribution of hGHR mRNA isoforms.

Using PCR primers 1S and 1A, with oligo A* as the hybridization probe (Figure 2.1 and Table 2.1), the tissue distribution of the exon 3 retaining and deleted hGHR mRNA isoforms was delineated. When we examined multiple tissues (n=2-6) from fifteen different subjects (11.5-19 wk FA, n=14; premature infant, 33 wk FA, n=1) including twins with a common placenta (15 wk FA), we found that, in all fifteen cases, the expression pattern did not vary between different tissues of the same individual, but varied between different subjects (Figure 2.2 and Table 2.2). These data strongly suggest that production of the exon 3 retaining and deficient mRNA transcripts is individual-, and not tissue-, specific during early stages of development.

To determine whether the exon 3 deficient mRNA isoform is due to genomic deletion, PCR primers specific for intronic sequences surrounding exon 3 of the *hGHR* gene were used to amplify exon 3 from genomic DNA of three fetuses (13.3-19 wk FA)

Fig. 2.2. Representative Southern blots illustrating the three types of expression patterns observed in human tissues during gestation. Results from fetuses A (18 wk FA), B (11.5 wk FA) and C (13.3 wk FA) are shown. IM-9 RNA served as a positive RT-PCR control. The sizes (bp) of the PCR fragments are indicated. Abbreviations: exon 3 retaining= 3^+; exon 3 deleted= 3^-; mu= muscle, li= liver, ki= kidney, sk= skin, sp= spleen, pv= placental villi, lu= lung, ad= adrenal and si= small intestine.

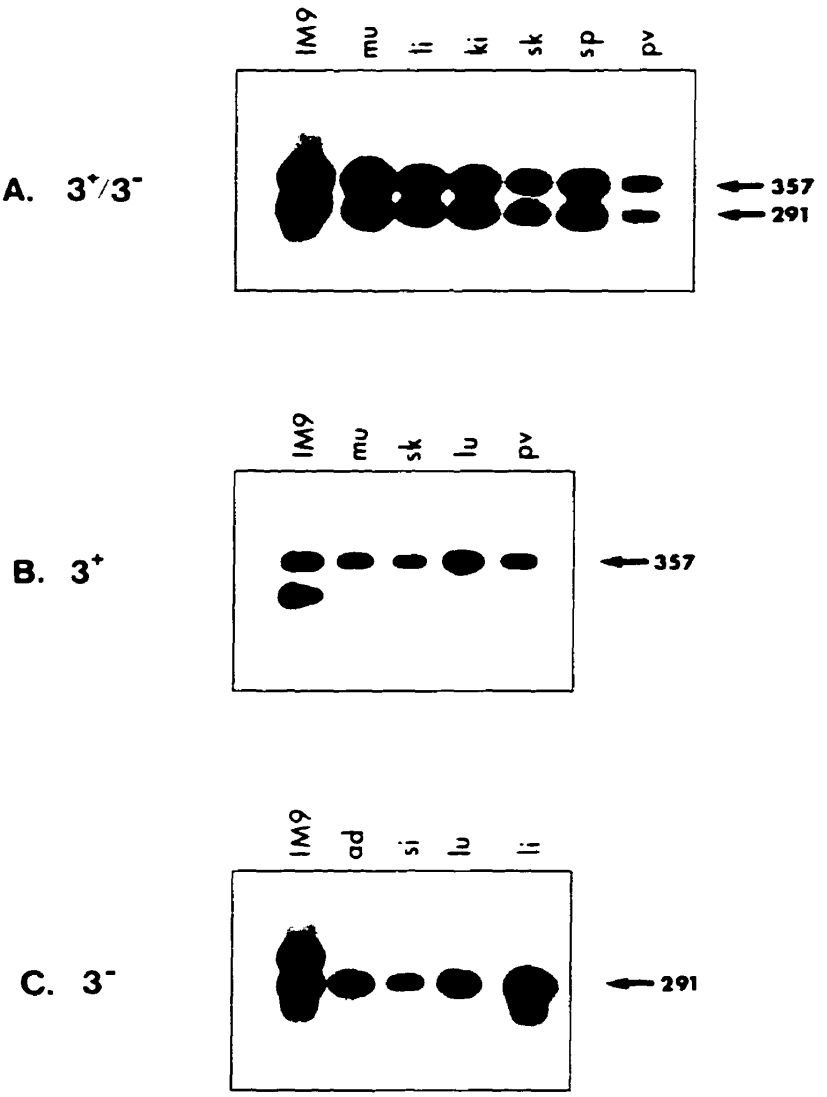

IM9
mu
li
ki
sk
sp
pv
A. 3+/3-
357
291
IM9
mu
sk
lu
pv
B. 3+
357
IM9
ad
si
lu
li
C. 3-
291

Table 2.2. Individual-specific expression of exon 3 retaining (3^+) and deficient (3^-) hGHR mRNA isoforms.

wk FA	Tissues Studied	Exon 3 Expression Pattern
11.5	n=4; lu,mu,sk,pv	3^+
13.3	n=4; ad,lu,li,si	3^-
13.5	n=6; mu,li,ki,sk,sp,pa	3^+
14.8	n=5; CNS,mu,li,ki,sk	$3^+/3^-$
15*	n=4; mu,li,ki,pv	3^+
15*	n=4; mu,li,ki,pv	3^+
15.3	n=5; mu,li,ki,sk,sp	3^+
16	n=6; mu,li,ki,sk,sp,pv	$3^+/3^-$
16.7	n=4; mu,li,ki,sk	$3^+/3^-$
16.7	n=2; mu,li	$3^+/3^-$
16.5	n=4; mu,li,ki,sk	$3^+/3^-$
18	n=6; mu,li,ki,sk,sp,pv	$3^+/3^-$
19	n=4; mu,li,ki,sk	3^+
19	n=2; li,ki	3^-
33	n=2; fi,li	$3^+/3^-$

(Abbreviations: lu= lung, mu= muscle, sk= skin, pv= placental villi, ad= adrenal, si= small intestine, li= liver, ki= kidney, sp= spleen, pa= pancreas, CNS= central nervous system and fi= fibroblasts)
* (These two fetuses were twins with a common placenta.)

expressing only the exon 3 deficient transcript. In all three cases, the expected 136 base pair fragment was obtained (data not shown), suggesting that the exon 3 deleted isoform is not due to genomic deletion of exon 3. Sequencing analysis of randomly chosen exon 3 retaining (liver, 40 yr postnatal) and exon 3 deficient (liver, 13.3 wk FA) RT-PCR products showed that deletion of exon 3 from the mRNA is precise (data not shown).

2.5.2. Ontogenic appearance of hGHR mRNA isoforms.

We then analysed whether there is developmental regulation of the exon 3 retaining and deleted mRNA isoforms. Based on our finding, that synthesis of these two mRNA isoforms is individual-specific in the fetus, and on a recent report (27) that it is also individual-specific in adult subjects, we pooled expression data of one or more tissues from 117 subjects (4 wk FA - 64 yr postnatal) and analyzed them as an ontogenic cross-sectional study. Where there was overlap of tissues from the same individual, only one data point was used. As shown in Figure 2.3, there is a decrease in expression of the exon 3 deleted transcript (either alone or together with the exon 3 retaining) with developmental age. Statistical analyses of these cross-sectional data (Table 2.3) show that predominant expression of the exon 3 deficient transcript occurs during fetal life (groups A and B: 4-20 wk FA). Because of our inability to obtain tissues from fetuses (as opposed to premature newborns) between 21-40 wk FA, we are unable to resolve the question of whether the developmental change suggested by these data occurs because of fetal maturation or the process of birth. In addition, because our RT-PCR methodology is not quantitative, the present data do not resolve the question of whether there is a

Fig. 2.3. Ontogeny of the exon 3 retaining (3^+) and deleted (3^-) hGH receptor mRNA isoforms. The distribution of 3^+ and 3^- expression patterns of 117 subjects as a function of age is shown. Dotted vertical lines separate the data into four age groups: A) early fetal (4-8 wk FA, n=20); B) fetal (9-20 wks FA, n=67); C) premature and term infants (24-40 wks FA, n=12); and D) postnatal (1 week - 64 yr, n=18). Symbols: triangle= 3^- alone; open circle= coexpression of 3^+ and 3^-; closed circle= 3^+ alone.

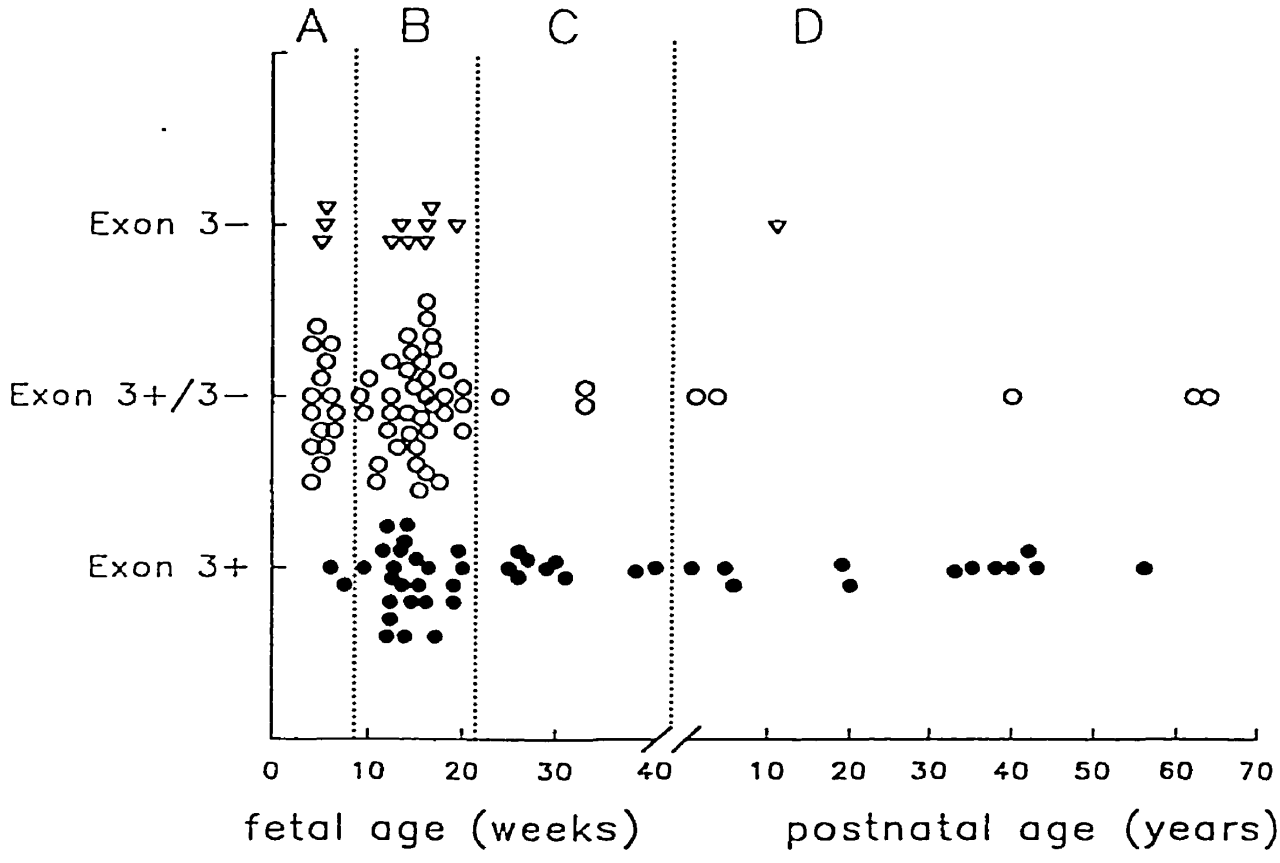

A
B
C
D
Exon 3−
Exon 3+/3−
Exon 3+
0
10
20
30
40
10
20
30
40
50
60
70
fetal age (weeks)
postnatal age (years)

Table 2.3. Statistical analysis of exon 3 retaining (3^+) and deficient (3^-) hGHR mRNA isoform ontogenic data*.

		P	Odds ratio	95% confidence interval
I.	**Groups A + B + C** (4-40 wk FA, n=99) **versus** **Group D** (4 months to 64 yr postnatal, n=18)	0.02	0.26	0.09-0.76
II.	**Groups A + B** (4-20 wk FA, n=87) **versus** **Groups C + D** (24 wk FA to 64 yr postnatal, n=30)	**0.0002**	0.17	0.07-0.43
III.	**Group A** (4-8 wk FA, n=20) **versus** **Groups B + C + D** (24 wk FA to 64 yr postnatal, n=97)	0.003	0.13	0.03-0.61

* 2x2 contingency table analyses, with Fisher's exact test, of 3^+ vs 3^- (either alone or coexpressed with 3^+) ontogenic data. Groups are the same as in Figure 2.3.

developmental change in cellular hGH receptor mRNA levels.

As the cross-sectional developmental study included analysis of fibroblasts harvested after a variable number of generations in culture (8.5-12 generations), the pattern of exon 3 hGHR mRNA transcripts changes in these cells with successive passaging was determined. We found that, in fact, the expression pattern in cultured fibroblasts from 6.5 up to 12 generations was identical to that of the original skin tissue (n=2) (Figure 2.4). In addition, fetal hepatocytes did not change their isoform pattern after 14-18 hours in primary culture (n=3) (Figure 2.5).

Furthermore, liver is a major prenatal hematopoietic organ (35) and, therefore, includes precursor blood cells in addition to several hepatic cell-types. To confirm that our analysis of fetal livers reflected hepatocyte hGHR mRNA expression, hepatocytes from fetal livers were isolated and their exon 3 retaining and deficient hGHR pattern was compared to that of their initial tissue samples. In the three fetal organs tested, the hepatocytes showed the same RT-PCR profile as their parallel tissue and total cell preparations (Figure 2.5).

Transcription of the *hGHR* gene was observed in all fetal tissues studied, from the earliest developmental stage that could be examined: liver (9 wk FA), skin (10 wk FA), kidney (10 wk FA), lung (11 wks FA), intestine (11 wk FA), muscle (11.5 wk FA), adrenal (13.3 wks FA), spleen (13.5 wks FA), pancreas (13.5 wks FA), CNS (14.3 wks FA), and placental villi (4 wk FA). There was no correlation between the expression pattern and the sex of the subject. Under our specific RT-PCR conditions, when both mRNA isoforms were expressed together, amplification of the two transcripts was

Fig. 2.4. Analysis of the effect of culture on hGH receptor mRNA expression patterns. Abdominal skin (sk) tissues from fetuses D (16 wks FA) and E (20 wks FA) were plated as explants and fibroblasts (fi) cultured from the first passage [6.5 generations (6.5g)] up to 12 generations (12g). The expression pattern of the exon 3 retaining and deleted hGHR mRNA transcripts was determined for each passage by RT-PCR/Southern blot analysis. IM-9 RNA served as a positive RT-PCR control. The sizes (bp) of the expected PCR products are indicated.

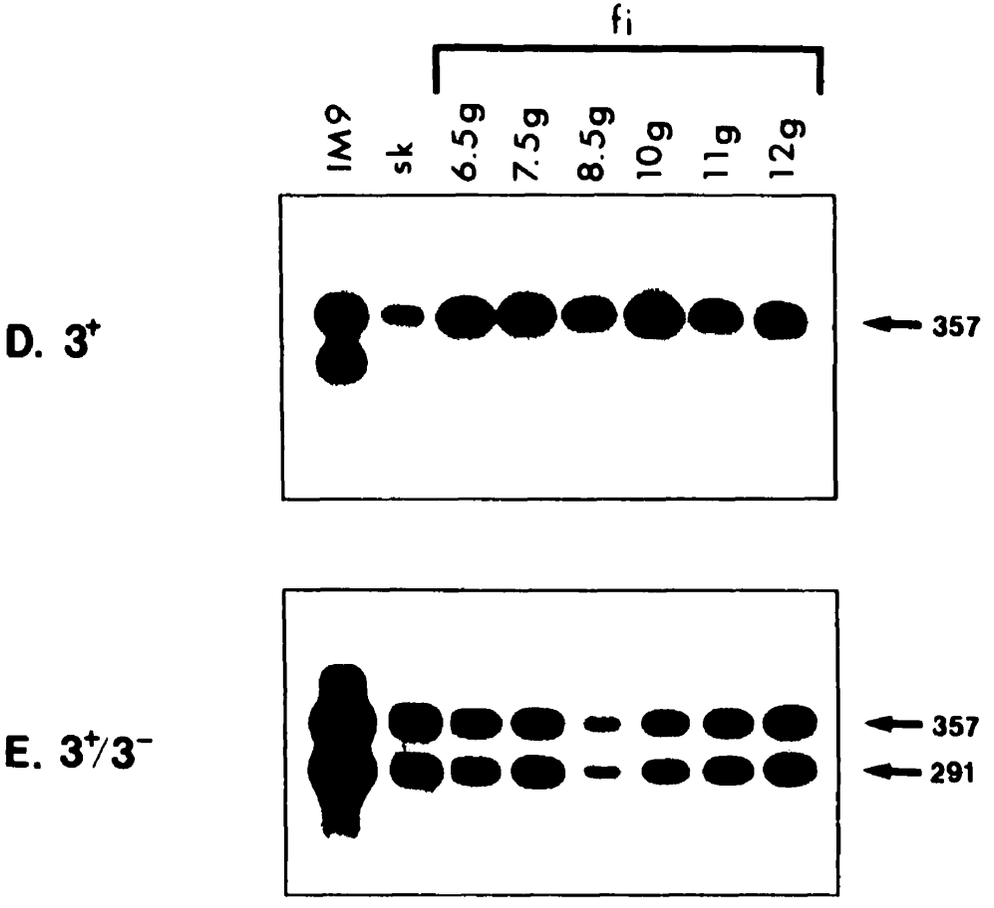
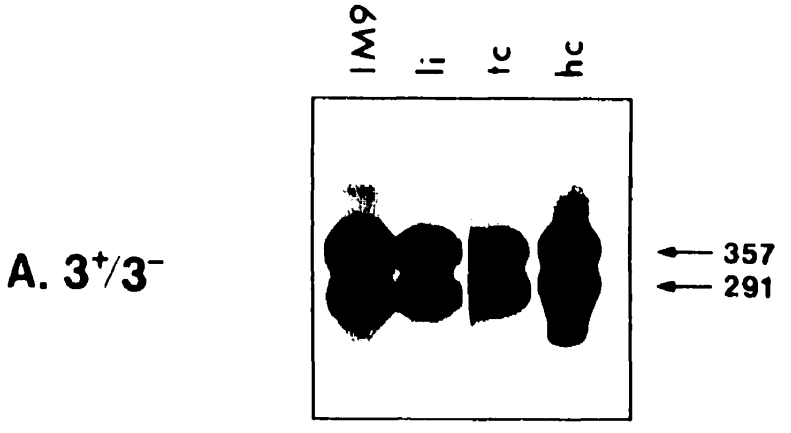

fi
IM9
sk
6.5g
7.5g
8.5g
10g
11g
12g
D. 3+
357
E. 3+/3−
357
291

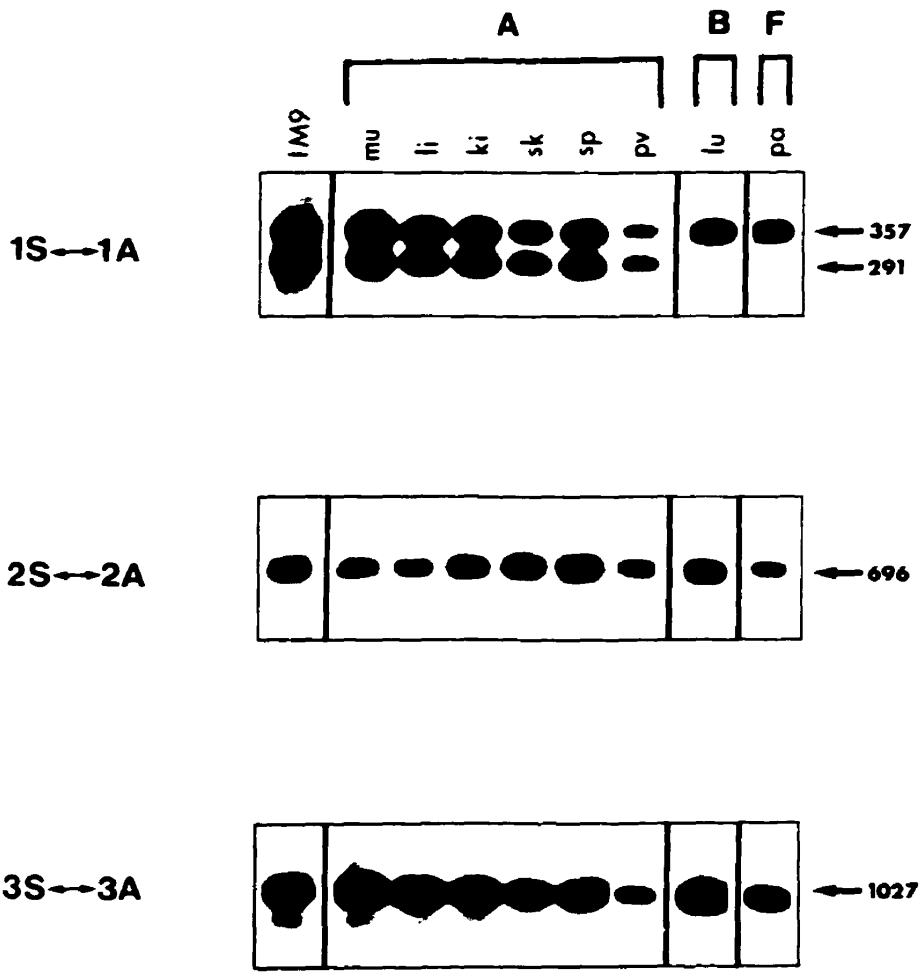
Fig. 2.5. Study of cell-specific expression of hGHR mRNA in fetal liver. Hepatocytes were isolated from whole fetal liver tissue (fetus A, 18 wk FA). The expression patterns of whole fetal liver (li), total liver cells (tc) and hepatocytes (hc) were determined by RT-PCR/Southern blot analysis. IM-9 RNA served as a positive RT-PCR control. The sizes (bp) of the PCR products are indicated. Similar results were obtained with hepatic specimens from 16 (exon $3^-/3^+$) and 19 wk FA (exon 3^+) fetuses.


IM9
li
tc
hc
A. 3+/3−
357
291

similar; only one sample (liver, 18 wk FA) consistently showed a predominance of the exon 3 retaining mRNA transcript.

Finally, using the overlapping PCR primer sets 2S/2A and 3S/3A (Figure 2.1), forty different human tissues of varying developmental stages (9 wks FA - 43 years postnatal) were analyzed for the presence of any additional hGH receptor mRNA transcripts. Only the expected RT-PCR fragments were observed (Figure 2.6 and data not shown), strongly suggesting that the exon 3 retaining and deleted mRNA transcripts are the only two fetal and postnatal isoforms of the hGH receptor mRNA coding region.

Fig. 2.6. Characterization of hGHR mRNA splicing patterns in fetal and postnatal tissues. Southern blots demonstrating hGHR mRNA expression in tissues obtained from fetuses A (18 wk FA), B (11.5 wk FA) and F (13.5 wk FA) are shown. Nested oligos A^*, $1A^*$ and $2A^*$ were hybridized to cDNA fragments amplified with primer sets 1S/1A, 2S/2A and 3S/3A, respectively. IM-9 RNA served as a positive RT-PCR control. The sizes of the expected PCR products are indicated. Similar studies were carried out with additional fetal liver (9-40 wk FA, n=12), kidney (12.7-20 wk FA, n=7) and dermal fibroblast (10- 33 wk FA, n=4) specimens, as well as postnatal liver (8 months - 43 yr postnatal, n=6), kidney (1.5 yr postnatal) and dermal fibroblast (1.5 and 35 yr postnatal) samples. Abbreviations: mu= muscle, li= liver, ki= kidney, sk= skin, sp= spleen, pv= placental villi, lu= lung and pa= pancreas.


A
B
F
IMφ
mu
li
ki
sk
sp
pv
lu
pa
1S←→1A
357
291
2S←→2A
696
3S←→3A
1027

2.6. Discussion.

The *hGHR* gene is ubiquitously transcribed in postnatal (23-25) and, as demonstrated in this study, in fetal tissues. One question that remains is whether postnatal and fetal target cells express comparable hGHR mRNA levels, since our RT-PCR/Southern blot approach gave a qualitative and not a quantitative analysis. We and others have shown, however, that immunoreactive hGHR peptide appears in a tissue- and cell-specific manner during development and that, by midgestation, the pattern of immunostaining is often identical to that found in the adult (12,13). Interestingly, we have observed *hGHR* gene transcription in certain fetal tissues (eg. lung, thigh muscle) that do not exhibit detectable levels of hGHR immunostaining (12) or hGH binding (9,36). These findings suggest that there may be posttranscriptional control of hGHR synthesis in the fetus.

Work in several laboratories has demonstrated that there are two isoforms of the hGHR mRNA coding region: exon 3 can be retained or deleted (22-27). We have confirmed this finding and extended it by showing that these two transcripts are also the only detectable isoforms in a wide variety of fetal tissues. The presence of exon 3 in genomic DNA of three fetuses expressing only the exon 3 deficient mRNA isoform indicates that the mechanism involved here is not genomic deletion of exon 3. Furthermore, our data suggest that expression of these two transcripts is individual-specific and may be developmentally regulated.

Previous studies of postnatal tissues and cell lines have provided contradictory data as to whether differential expression of exon 3 of the *hGHR* gene transcript is a

tissue-specific phenomenon. For example, although both groups agreed that expression of the two mRNA transcripts is related to the tissue-type, Urbanek et al. (23) reported that only the exon 3 retaining transcript is present in liver, whereas Sobrier et al. (24) argued that both mRNA isoforms are coexpressed in this organ. On the other hand, Mercado et al. (25) concluded that synthesis of the two mRNA isoforms is constitutive but that the relative proportions of each transcript depends on the tissue-type. In a more recent study of multiple postnatal liver specimens, Esposito et al. (26) suggested that expression of these two transcripts is a random and not a tissue-specific event.

However, these studies did not address whether the expression pattern of the exon 3 retaining and deleted mRNA transcripts is specific to an individual. Indeed, when we examined multiple tissues (n=2-6) from fifteen fetuses, we found that the pattern did not vary between different tissues of the same fetus, but varied between different fetuses. Wickelgren et al. (27) have reported similar data for adult subjects.

Furthermore, statistical analyses of our cross-sectional ontogenic study suggest that predominant expression of the exon 3 deficient mRNA transcript, either independently or simultaneously with the exon 3 retaining isoform, occurs prior to 20 weeks of fetal age. However, since all tissues studied between 21-40 wks FA were obtained from premature newborns (as opposed to fetuses), our study can not answer the question of whether the developmental change suggested by our cross-sectional data occurs because of fetal maturation or the process of birth. In addition, our present investigation does not resolve whether all fetuses express the exon 3 deleted mRNA isoform at some point during early development (ie. had the fetuses we found to express

only the exon 3 retaining mRNA transcript prior to midgestation already lost their ability to produce the exon 3 deleted mRNA isoform?). Esposito et al. (26) had previously excluded the possibility that the exon 3 deleted isoform has a role in fetal development; however, these investigators examined only four fetal samples, all of which were 21 weeks of fetal age, whereas our study consisted of a greater sample size and covered a wide range of developmental stages (4-40 wks FA, n=99; 1 wk - 64 yr postnatal, n=18).

Based on our results, we hypothesize that there is an ontogenic switch and that the ability to express the exon 3 deleted mRNA isoform is, in general, lost during the second half of gestation; however, the timing of the switch varies from one individual to another and, in fact, some individuals apparently never lose their ability to synthesize the exon 3 deficient mRNA transcript (present data, 23-27). Polymorphisms within the components of the precursor mRNA processing machinery, the spliceosome, and/or the *hGHR* gene could explain both the diversity in the timing of the switch, as well as the three different expression patterns we have found. It is also possible that the 5' untranslated region (5'UTR) of the hGHR mRNA may influence the differential splicing of exon 3. However, in a study of four of the eight known hGHR mRNA 5'UTRs, Esposito et al. (26) observed that both the exon 3 retaining and deleted hGHR mRNA isoforms are present with each one of these four variants. These data suggest that the 5'UTR of the mRNA is not directing whether exon 3 is retained or deleted.

22K hGH, the major pituitary-secreted hGH peptide, forms a 1:2 complex with its receptor (37,38): one receptor binds to site 1 of the 22K hGH after which a second

receptor binds the same hGH molecule at site 2. Even though the two binding sites of the 22K hGH molecule are quite different, both receptors donate essentially the same residues to the receptor-hormone complex. Interestingly, the 22 amino acids [#7-28] encoded by exon 3 of the hGHR mRNA do not directly participate in ligand binding, nor does the alanine #6 at the exon 2-3 junction that is changed to aspartic acid in the exon 3 deficient isoform (39). Recent in vitro studies, using COS cell and Xenopus laevis oocyte transfection systems, have demonstrated that both the exon 3 retaining and deleted mRNA transcripts encode receptors that integrate into the plasma membrane and bind 22K hGH with similar affinity (24,40). Studies in the oocyte system also revealed that the exon 3 deleted receptor is internalized as efficiently as the full-length peptide (40). In addition, Esposito et al. (26) have reported that, in a tumour cell line expressing only the exon 3 deficient hGHR transcript, the extracellular domain of the hGH receptor can be cleaved at the plasma membrane to generate a high-affinity hGH binding protein. However, the intracellular signalling events activated by the two hGHR have not yet been completely investigated. Since hGH-induced receptor dimerization has been demonstrated to be important for activation of signal transduction mechanisms (41), a change in the conformation of the receptor complex ($3^+/3^-$ heterodimer vs 3^+ or 3^- homodimers) may activate alternative intracellular signalling pathways.

In addition, although most hGHR studies are carried out using 22K hGH, there are multiple potential ligands, derived from both the pituitary (22K, 20K) and placenta (hGH-V, placental lactogen [hPL]) (42,43). In fact, Sobrier et al. (24) have reported that the exon 3 retaining and deleted hGHRs, when overexpressed in COS cells, bind hPL

with similar affinity, but neither isoform binds hPRL. In addition, using the Xenopus laevis oocyte expression system, Urbanek et al. (40) have shown that the exon 3 retaining and deficient isoforms have parallel affinities for 20K hGH, hGH-V, hPL and ovine PRL. However, it is not yet known whether the two hGHRs will form monomer or dimer complexes with the various hGH peptides and hPL. This will be important to ascertain since such different receptor complexes may lead to alternate transduction mechanisms and heterogeneous biological functions.

In summary, our data show that: 1) transcription of the *hGHR* gene occurs in a wide variety of tissues from as early as the first trimester of development; 2) exon 3 retaining and deficient transcripts are the only two isoforms of the hGHR mRNA coding region expressed during development; and 3) differential splicing of exon 3 is individual-specific and may be under ontogenic control.

2.7. Acknowledgements.

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2.8. References.

1. Kaplan SL, Grumbach MM, Shepard TH. 1972 The ontogenesis of human fetal hormones: growth hormone and insulin. J Clin Invest. 51: 3080-3093.

2. Gluckman PD, Breier BH, Oliver M, Harding J, Bassett N. 1990 Fetal growth in late gestation - a constrained pattern of growth. Acta Paediatr Scand. 367: 105-110.

3. Chard T. 1989 Hormonal control of growth in the human fetus. J Endocrinol. 123: 3-9.

4. Palmiter RD, Norstedt G, Gelinas RE, Hammer RE, Brinster RL. 1983 Metallothionein-human GH fusion genes stimulate growth of mice. Science 222: 809-814.

5. Stewart TA, Clift S, Pitts-Meek S et al. 1992 An evaluation of the functions of the 22-kilodalton (kDa), the 20-kDA and the N-terminal polypeptide forms of human growth hormone using transgenic mice. Endocrinology 130: 405-414.

6. Ymer SI, Herington AC. 1992 Developmental expression of the growth hormone receptor gene in rabbit tissues. Mol Cell Endocrinol. 83: 39-49.

7. Walker JL, Moats-Staats M, Stiles AD, Underwood LE. 1992 Tissue-specific developmental regulation of the messenger ribonucleic acids encoding the growth

hormone receptor and growth hormone binding protein in rat fetal and postnatal tissues. Pediatric Research 31: 335-339.

8. Tiong TS, Herington AC. 1992 Ontogeny of messenger RNA for the rat growth hormone receptor and serum binding protein. Mol Cell Endocrinol. 83: 1133-141.

9. Hill DJ, Freemark M, Strain AJ, Handwerger S, Milner RDG. 1988 Placental lactogen and growth hormone receptors in fetal tissues: relationship to fetal plasma human placental lactogen concentrations and fetal growth. J Clin Endocrinol Metab. 66: 1283-1290.

10. Werther GA, Haynes KM, Waters MJ. 1993 Growth hormone receptors are expressed on human fetal mesenchymal tissues - identification of messenger RNA and GH binding protein. J Clin Endocrinol Metab. 76: 1638-1646.

11. Figueiredo RMO, Goodyer CG. 1993 Characterisation of the growth hormone receptor in human dermal fibroblasts during development. Prog. 75th Annual Meeting of the Endocrine Soc. 468.

12. Hill DJ, Riley SC, Bassett NS, Waters MJ. 1992 Localization of the growth hormone receptor, identified by immunocytochemistry, in second trimester human fetal tissues and placenta throughout gestation. J Clin Endocrinol Metab. 75: 646-650.

13. Simard M, Manthos H, Giaid A, Lefèbvre Y, Goodyer CG. 1992 Ontogeny of growth hormone receptor in human tissues: an immunohistochemical study. Prog. 74th Annual Meeting of the Endocrine Soc. 684.

14. Strain AJ, Hill DJ, Swenne I, Milner RDG. 1987 Regulation of DNA synthesis in human fetal hepatocytes by placental lactogen, growth hormone, and insulin-like growth factor I/Somatomedin C. J Cell Physiol. 132: 33-40.

15. Swenne I, Hill DJ, Strain AJ, Milner RDG. 1987 Effects of human placental lactogen and growth hormone on the production of insulin and somatomedin C/insulin-like growth factor I by human fetal pancreas in tissue culture. J Endocrinol. 113: 297-303.

16. Otonkoski T, Knip M, Wong I, Simell O. 1988 Effects of growth hormone and insulin-like growth factor I on endocrine function of human fetal islet-like cell clusters during longterm tissue culture. Diabetes 37: 1678-1683.

17. Bang P, Westgren M, Schwander J, Blum WF, Rosenfeld RG, Stangenberg M. 1994 Ontogeny of insulin-like growth factor-binding protein-1, -2, and -3: quantitative measurements by radioimmunoassay in human fetal serum. Pediatric Research 36: 528-536.

18. Albertsson-Wikland K, Niklasson A, Karlberg P. 1990 Birth data for patients who later develop growth hormone deficiency: preliminary analysis of a national register. Acta Paediatr Scand. 370: 115-120.

19. Gluckman PD, Gunn AJ, Wray A et al. 1992 Congenital idiopathic growth hormone deficiency associated with prenatal and early postnatal growth failure. J Pediatrics 121: 920-923.

20. Laron Z. 1973 The role of growth hormone on fetal development in utero. Adv Exp Med Biol. 27: 391-398.

21. Rosenfeld RG, Rosenbloom AL, Guevara-Aguirre J. 1994 Growth hormone (GH) insensitivity due to primary GH receptor deficiency. Endocrine Reviews 15: 369-390.

22. Godowski PJ, Leung DW, Meacham LR et al. 1989 Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. Proc Natl Acad Sci USA 86: 8083-8087.

23. Urbanek M, Macleod JN, Cooke NE, Liebhaber SA. 1992 Expression of a human growth hormone (hGH) receptor isoform is predicted by tissue-specific alternative splicing of exon 3 of the hGH receptor gene transcript. Mol Endocrinol. 6: 279-287.

24. Sobrier ML, Duquesnoy P, Duriez B, Amselem S, Goossens M. 1993 Expression and binding properties of two isoforms of the human growth hormone receptor. FEBS 319: 16-24.

25. Mercado M, Davila N, McLeod JF, Baumann G. 1994 Distribution of growth hormone receptor messenger ribonucleic acid containing and lacking exon 3 in human tissues. J Clin Endocrinol Metab. 78: 731-735

26. Esposito N, Paterlini P, Kelly PA, Postel-Vinay M-C, Finidori J. 1994 Expression of two isoforms of the human growth hormone receptor in normal liver and hepatocarcinoma. Mol Cell Endocrinol 103: 13-20.

27. Wickelgren RB, Landin KLL, Ohlsson C, Carlsson LMS. 1995 Expression of exon 3-retaining and exon 3-excluding isoforms of the human growth hormone (GH)-receptor is regulated in an individual rather than a tissue-specific manner. J Clin Endocrinol Metab. 80: 2154-2157.

28. Munsick RA. 1984 Human fetal extremity lengths in the interval from 9 to 21 menstrual weeks of pregnancy. Am J Obstet Gynecol. 149: 883-887.

29. Rodd C, Schwartz HL, Strait KA, Oppenheimer JH. 1992 Ontogeny of hepatic

nuclear triiodothyronine receptor isoforms in the rat. Endocrinology 131: 2559-2564.

30. Chirgwin JJ, Przbyla AE, MacDonald RJ, Rutter WJ. 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochem. 18: 5294.

31. Chomczynski P, Sacchi N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162: 156-159.

32. Berk AJ, Sharp PA. 1977 Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell 12: 721-732.

33. Gross-Bellard M, Oudet P, Chambon P. 1973 Isolation of high-molecular-weight DNA from mammalian cells. Eur J Biochem. 36:32-38.

34. Sanger F, Nicklen S, Coulson AR. 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467.

35. Hann IM. 1991 Development of blood in the fetus. In: Hann IM, Gibson BES, Letsky EA eds. Fetal and Neonatal Haematology. London, UK: Baillière Tindall; 1-28.

36. Labbé A, Delcros B, Déchelotte P, Nouailles C, Grizard G. 1992 Comparative study of the binding of prolactin and growth hormone by rabbit and human lung cell

membrane fractions. Biol Neonate 61: 179-187.

37. Cunningham BC, Ultsch M, De Vos AM, Mulkerrin MG, Clauser KR, Wells JA. 1991 Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science 254: 821-825.

38. De Vos, Ultsch M, Kossiakoff AA. 1992 Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. Science 255: 306-312.

39. Clackson T, Wells JA. 1995 A hot spot of binding energy in a hormone-receptor interface. Science 267: 383-386.

40. Urbanek M, Russell JE, Cooke NE, Liebhaber SA. 1993 Functional characterization of the alternatively spliced, placental human growth hormone receptor. J Biol Chem. 268: 19025-19032.

41. Finbloom DS, Petricoin III EF, Hackett RH et al. 1994 Growth hormone and erythropoietin differentially activate DNA-binding proteins by tyrosine phosphorylation. Mol Cell Biol. 14: 2113-2118.

42. Baumann G. 1991 Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endocrine Reviews 12: 424-449.

43. Lewis UJ, Sinha YN, Haro LS. 1994 Variant forms and fragments of human growth hormone in serum. Acta Paediatr. 399: 29-31.

APPENDIX 1

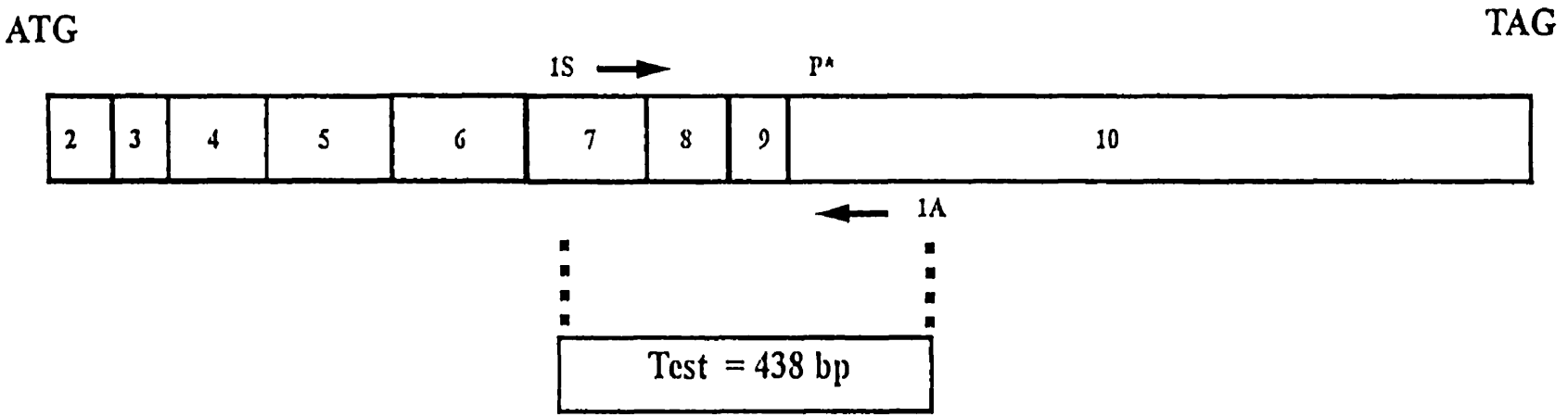
APPENDIX 1 - *QUANTITATIVE ANALYSIS OF GROWTH HORMONE RECEPTOR mRNA TISSUE LEVELS DURING HUMAN DEVELOPMENT.*

APP1.1. Summary.

RT-PCR and immunostaining analyses have identified hGHR mRNA and protein in multiple tissues from as early as the third month of fetal life. In the present study, we used a quantitative RT-PCR approach (Figures A1.1 and A1.2) to determine whether there are tissue-specific developmental changes in total (ie. all isoforms) hGHR mRNA levels. Comparison of fetal versus postnatal tissues showed no significant developmental change in kidney and small intestine, a six-fold decrease in postnatal lung ($p < 0.05$), and a six-fold ontogenic increase in liver ($p < 0.01$) (Figure A1.3) which parallels a four-fold rise in [^{125}I]hGH binding to fetal versus adult hepatic microsomal membranes (Figure A1.4). Therefore, although the hGHR is present in several tissues from early fetal life, there are significant developmental increases in the liver, a major hGH target tissue. We have hypothesized that the turning on of hepatic-specific hGHR gene promoter(s), which are silenced in fetal liver, accounts for these ontogenic increases in liver hGHR mRNA concentrations.

Fig. A1.1. Quantitative RT-PCR/Southern blot strategy for total hGHR mRNA. Upper panel, the hGHR cDNA is shown. Coding exons are represented by numbered boxes. Lower panel, the PCR internal standard was designed using the method of Jin et al (306). The approximate locations of the sense (1S; 5' CCA GTG TAC TCA TTG AAA GTG GAT 3') and antisense (1A; 5' GTC TGA TTC CTC AGT CTT TTC ATC 3') PCR primers and nested hybridization probe (P*; 5' GCT AAG ATT GTG TTC ACC TCC TC 3') are indicated. The lengths of the expected PCR fragments are given below each template. Six μg of total RNA were reverse transcribed with random hexamer primers and then amplified for 23 cycles with varying amounts of internal standard, according to RT-PCR conditions previously used for hGHR mRNA analysis (Chapter 2). The identity of the amplified fragments was confirmed by Southern blotting (Chapter 2).

hGHR cDNA



Internal Standard DNA Construct (907 bp)

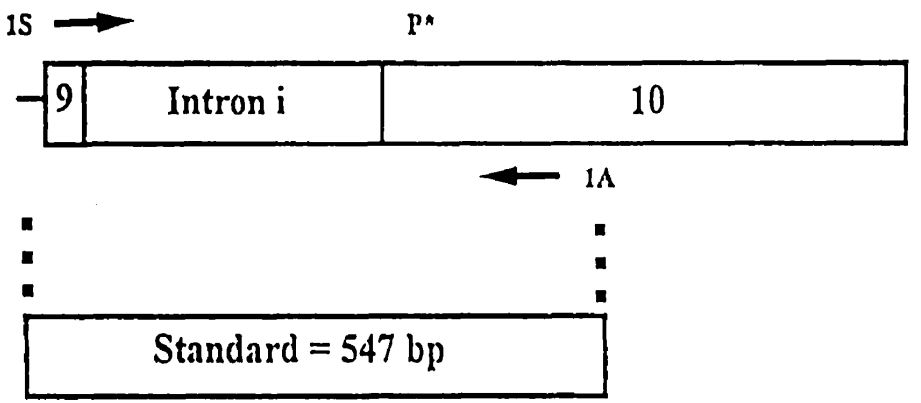
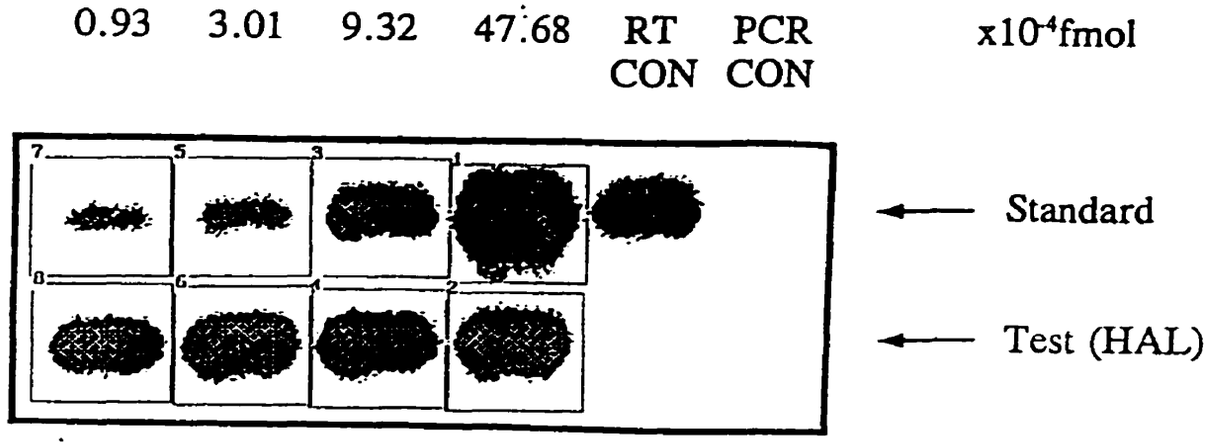


Fig. A1.2. Quantitation of hGHR mRNA by RT-PCR and Southern blotting. Upper panel, representative results from a human adult liver specimen (HAL, 43 yr) are shown. Arrows point to the standard (547 bp) and test (438 bp) PCR products. The amount of standard added is indicated above each lane. Lower panel, quantitation of hGHR mRNAs in liver during development. The $\log_{10}$ initial amount of internal standard template is plotted against the $\log_{10}$ ratio of the radioactivity intensity of the test PCR band over that of the internal standard PCR fragment. The solid line intercepting the y-axis at $\log_{10}$ = 0 corresponds to the ratio at which the concentration of test RNA (as a reverse transcript) is equivalent to that of the internal standard. In total, twelve liver samples were analyzed: 7 fetal (broken lines) (▼10.8 wk, ●14 wk, ■15 wk, △15 wk, ▲15 wk, ▽15.8 wk, □16 wk) and 5 postnatal (solid lines) (●11 yr, □40 yr, ▼40 yr, ▽43 yr, ○62 yr).


0.93
3.01
9.32
47.68
RT CON
PCR CON
x10⁻⁴fmol
Standard
Test (HAL)

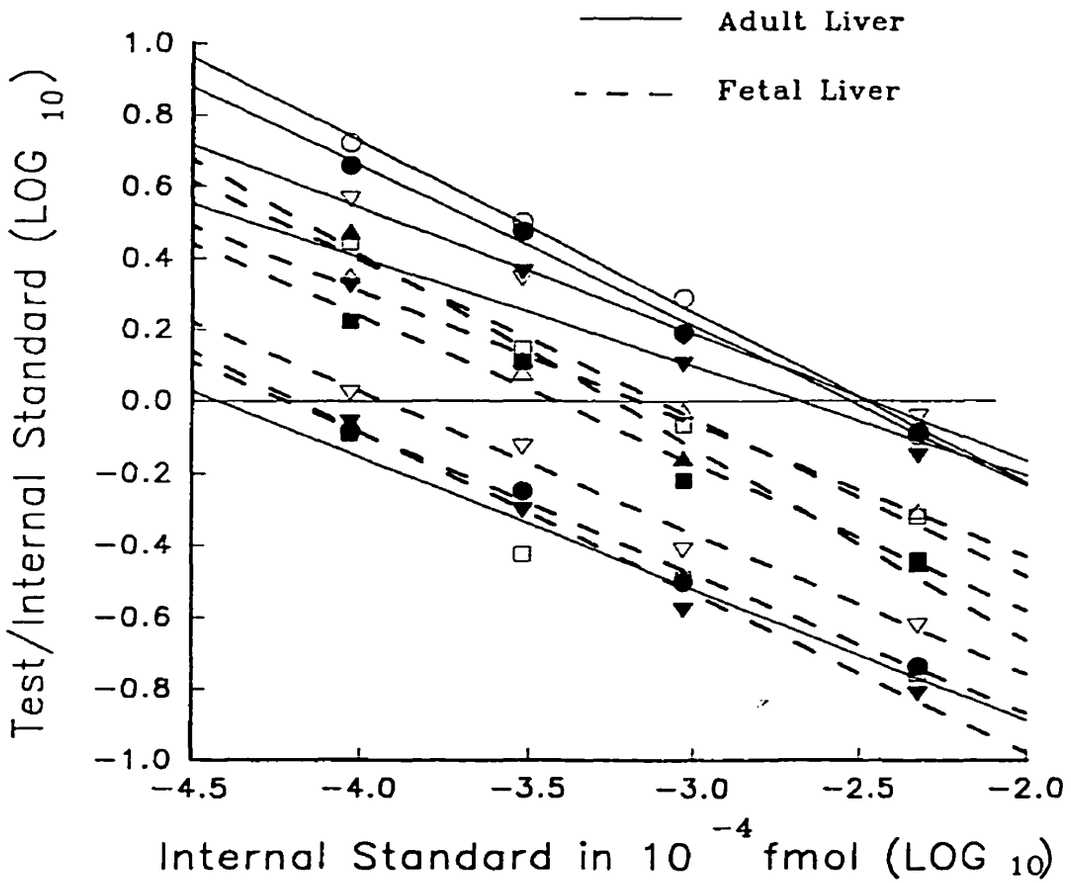

Adult Liver
Fetal Liver
Test/Internal Standard (LOG 10)
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.8
−1.0
−4.5
−4.0
−3.5
−3.0
−2.5
−2.0
Internal Standard in 10 −4 fmol (LOG 10)

Fig. A1.3. Ontogeny of total hGHR mRNA levels in liver, lung, kidney and small intestine. Fetal (11-17 weeks) and postnatal (11-84 yr) tissues were analyzed by quantitative RT-PCR assays. Statistical analysis (t-test) of the mean fetal versus postnatal values revealed a six-fold developmental increase in liver (**$p<0.01$), a six-fold decrease in lung (*$p<0.05$) and a statistically insignificant decrease from fetal to postnatal kidney or small intestine (GI). (M= mean, SE = standard error)

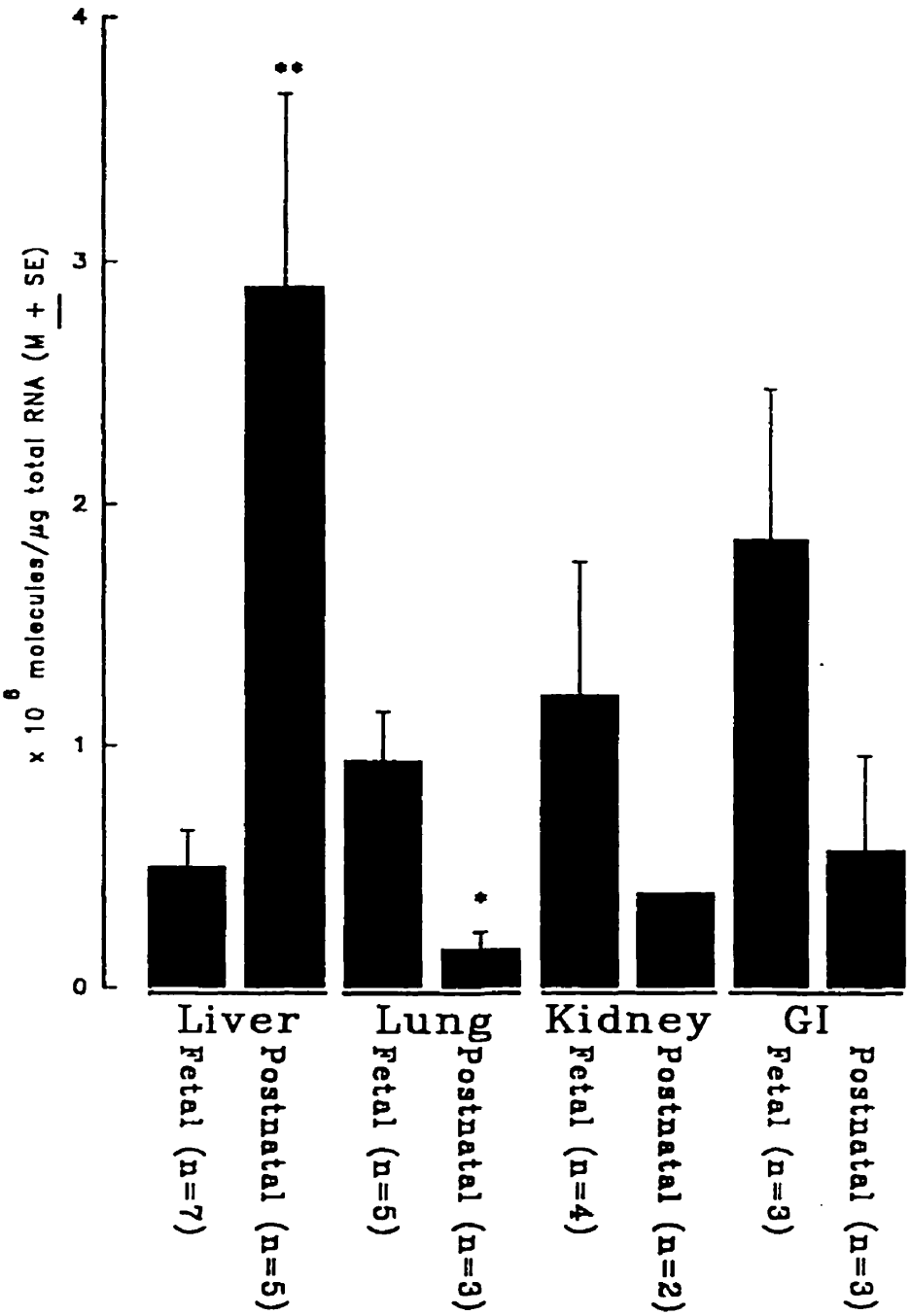
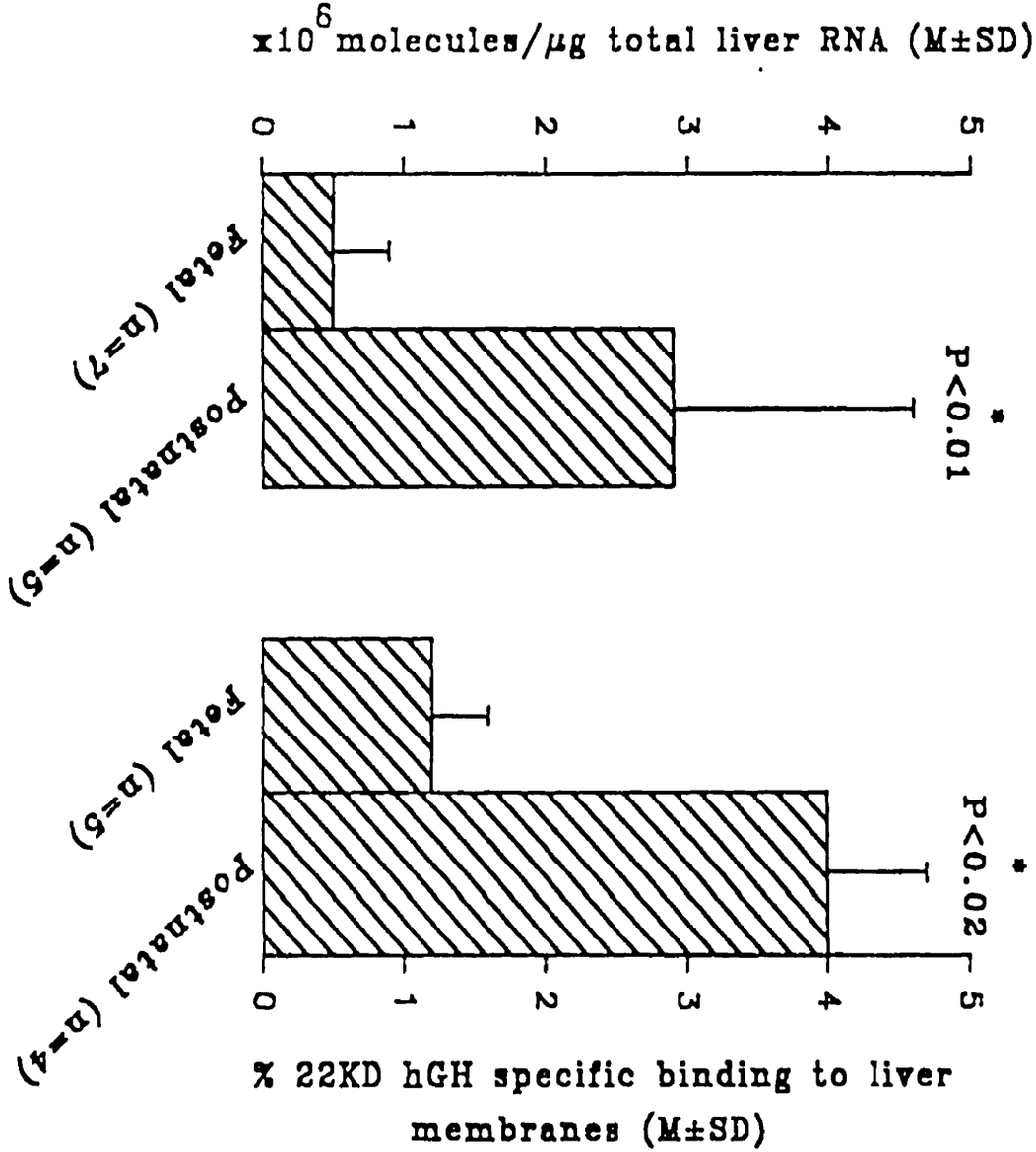

x 10^6 molecules/µg total RNA (M ± SE)
0
1
2
3
4
**
*
Liver
Lung
Kidney
GI
Fetal (n=7)
Postnatal (n=5)
Fetal (n=5)
Postnatal (n=3)
Fetal (n=4)
Postnatal (n=2)
Fetal (n=3)
Postnatal (n=3)

Fig. A1.4. Comparison of the ontogeny of total hGHR mRNA levels in liver and ^{125}I-hGH binding to hepatic microsomal membranes. Fetal (14-20 weeks) and postnatal (20-43 yr) liver samples were analyzed for specific hGH binding. Data were obtained from 1-3 binding assays per liver microsomal preparation and represent the percentage of total radioactivity in the binding buffer/200 μg protein. Statistical analysis (t-test) demonstrated a four-fold developmental increase ($^*p < 0.02$) in hepatic hGH binding. (M= mean, SD = standard deviation)


x10^6 molecules/μg total liver RNA (M±SD)
0
1
2
3
4
5
Fetal (n=7)
Postnatal (n=5)
P<0.01
*
Fetal (n=5)
Postnatal (n=4)
P<0.02
*
0
1
2
3
4
5
% 22KD hGH specific binding to liver membranes (M±SD)

CHAPTER 3

CHAPTER 3 - *FETAL- AND TUMOR-SPECIFIC REGULATION OF GROWTH HORMONE RECEPTOR MESSENGER RIBONUCLEIC ACID EXPRESSION IN HUMAN LIVER*

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3.1. Preface.

The work presented in Chapter 2 demonstrated that *hGHR* gene transcription begins as early as the third month of fetal life in several tissue-types. However, a quantitative analysis (Appendix 1) of hGHR mRNA expression suggested that there are tissue-specific developmental differences in hGHR mRNA levels. Although amounts remain the same or decrease postnatally in intestine, lung and kidney, there is a six-fold increase from fetal to postnatal liver. These findings were especially interesting given that the liver is a major hGH postnatal target tissue and the predominant source of hGHBP. We hypothesized that the activation of hepatic-specific *hGHR* gene regulatory regions accounts for the developmental increases in hepatic hGHR mRNA concentrations.

Because diversity in the 5'UTR of an mRNA may result from the use of multiple promoters, we began testing our hypothesis by characterizing the tissue distribution and ontogeny of two of the eight known 5'UTR variants of the hGHR mRNA, V1 and V3. We used a combined RT-PCR and Southern blot approach to detect with accuracy the relatively short V1 and V3 cDNA sequences that were known at the time. V1 was found to be expressed only in postnatal liver. In contrast, V3 was widely expressed in human tissues throughout development. Since tumours often represent poorly differentiated "fetal-like" cells, the expression profiles of V1 and V3 were also examined in two hepatic tumour-types. Hepatoblastomas (HB) were of special interest because they are classified as "embryonal tumours of childhood", along with Wilms' tumours and medulloblastomas. Indeed, we observed a similar expression profile in HBs as in fetal livers. Hepatocellular carcinomas and Wilms' tumours were then tested to determine if

the fetal-specific hGHR mRNA pattern of expression is restricted to hepatic tumours or whether it is a characteristic of the "embryonal tumours of childhood". These data suggest that the "fetal liver-like" V1 pattern of expression is confined to hepatic tumours.

3.2. Abstract.

Eight different 5'untranslated region (5' UTR) variants of the human growth hormone receptor (GHR) mRNA have been identified in adult liver (V1 to V8). We have compared expression of two of these, V1 and V3, in several human fetal and postnatal tissues, including liver, as well as in hepatoblastomas (HB) and hepatocellular carcinomas (HCC). Using reverse transcriptase-polymerase chain reaction assays, followed by Southern blotting to confirm the specificity of the amplified fragments, we found that V3 was expressed in all fetal (F) and postnatal (P) liver (n= 13F, 5P), kidney (n= 4F, 4P), lung (n= 4F, 2P), intestine (n= 8F, 4P), skeletal muscle (n= 1F, 1P) and adrenal (n= 1F, 1P) samples. In contrast, V1 was expressed only in postnatal liver. We then screened for V1 and V3 in HB (n=17, 6-36 months, including 5 with paired normal liver) and HCC (n=4, 50-75 years, with paired normal liver). V1 was undetectable in 15 of 17 HB, including all HB paired with (V1 expressing) normal liver; the absence of V1 did not correlate with patient age, sex, HB subtype, $\pm$ chemotherapy, exon 3 retaining and deficient hGHR mRNA isoform pattern or loss of heterozygosity at 11p, 1p and 1q. The four HCC showed marked (>20-fold, n=2) or complete (n=2) suppression of V1 as compared to paired normal liver. V3 was expressed in all HB, HCC and paired normal livers. Interestingly, V3, but not V1, was detected in two Wilms' tumor and paired normal kidney specimens. Our findings suggest that, in the human, there is tissue-, fetal- and tumor-specific regulation of V1 hGHR mRNA.

3.3. Introduction.

Hepatoblastomas (HB) are the most frequent hepatic tumor in children, accounting for more than 50% of all pediatric liver malignancies (reviewed in 1,2). Based on histopathological studies, HB is classified as an "embryonal tumor of childhood", along with Wilms' and medulloblastoma. Although most cases are sporadic, HB has been associated with the Beckwith-Wiedemann syndrome and familial adenomatous polyposis (1,2). Molecular genetic analyses have shown an loss of heterozygosity (LOH) at 11p, 1p or 1q in approximately one-third of informative cases (1-4). Hepatocellular carcinomas (HCC) are strongly correlated with liver cirrhosis and the presence of hepatitis B viral antigens, and frequently associated with LOH at the type II insulin-like growth factor (IGF) receptor locus on chromosome 6q (1). However, the etiologies of both HB and HCC remain poorly understood.

The hGHR is encoded by exons 2 to 10 of of the human growth hormone receptor (*hGHR*) gene on chromosome 5 (5). hGHR mRNA has been detected in all fetal and postnatal tissues examined to date (6,7). Two isoforms of the mRNA coding region have been identified: exon 3 can be retained or deleted (6,7). In several species, the GHR mRNA has been characterized by heterogeneity in its 5' utranslated region (5'UTR) (8-10), suggesting complex transcriptional regulation and/or extensive alternative splicing upstream of the translation initiation codon of the precursor mRNA transcripts. In the human, eight different 5'UTR variants (V1 to V8, numbered according to their relative abundance), diverging 12 bp upstream from the translation initiation codon, have been isolated from an adult liver cDNA library (8). Interestingly, the ovine V1 homologue

(exon 1A) appears to be liver-specific and developmentally regulated: exon 1A is expressed in postnatal, but not fetal, sheep liver (9). Given the data from the ovine studies, we hypothesized that the human V1 transcript would be liver-specific and under developmental control. We also speculated that V1 mRNA expression might be absent in the embryonal/fetal HB and altered in adult HCC. Therefore, in the present study, we determined the tissue specificity of the V1 transcript, characterised its ontogeny in human liver and investigated its expression pattern in HB and HCC. V3 was examined in parallel as a positive control since preliminary data had shown that it is widely expressed in human tissues. Our data suggest that, in the human, there is tissue-, fetal- and tumor-specific regulation of V1 mRNA.

3.4. Materials and Methods.

3.4.1. Tissues.

Tissues from human fetuses (n=21; 10-20 weeks of fetal age [wk FA]) were obtained at the time of therapeutic abortion; specimens from premature newborns (n=2; 25,30 wk FA) and postnatal patients (n=38; 1 week - 80 yr) were obtained at the time of surgery or within 4-10 hours following death. All tissues were flash frozen in dry-ice/acetone and immediately stored at -70°C for RNA extraction. Fetal age was determined by foot length (6). Protocols for obtaining human tissues were approved by local ethics committees and informed consent was obtained in all cases.

3.4.2. Reverse transcription (RT)-PCR and Southern Blotting.

Total RNA was isolated using either the acid-guanidium-phenol-chloroform (11) or guanidine thiocyanate/CsCl gradient (12) methods. For each reaction, 5 μg of total RNA were reverse transcribed for 1 hour at 48°C in the presence of 2.5 units of Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) (GIBCO BRL, Gaithersburg, MD), 80 units of RNAsin (Promega, Madison, WI), 0.6 μM of a specific antisense primer (1A; Figure 3.1) (5'-AGG TAT CCA GAT GGA GGT AAA CG-3'), 0.48 mM deoxyribonucleotides (dNTPs) (Pharmacia Biotech, Baie D'Urfe, QC), 10 mM $MgCl_2$, 10 mM DTT, 100 mM Tris-HCl pH 8.3 and 50 mM KCl. Prior to the RT reaction, the RNA was heated at 70°C for 5 minutes to disrupt any secondary structure. 6 μl of RT product were then amplified for 25 cycles in the presence of 2.5 units of Thermus aquaticus (Taq) DNA Polymerase (GIBCO BRL), 0.5 mM dNTPs, 0.25 μM hGH

receptor sense (1S: 5'-AGA TTG AGA ATG ACT GAT TTG GGA G-3' or 3S: 5'-GGA GAC CTT GGA AGG GAC AGA G-3') and antisense (1A) primers (Figure 3.1), 3 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4 and 50 mM KCl. Prior to the PCR reaction, the RT product was heated for 5 minutes at 95-98°C to denature the AMV-RT. The first cycle consisted of 3 minutes denaturation at 92°C, 1 minute annealing at 61°C and 3 minutes elongation at 72°C. Subsequent cycles consisted of 30 seconds denaturation at 92°C, 1 minute annealing at 61°C and 1.5 minutes elongation at 72°C. The reaction was terminated with a final elongation of 5 minutes at 72°C. PCR assays testing for expression of V1 transcripts were carried out in the presence 224 fmol of internal standard. The internal standard was constructed using the method of Jin et al (13).

Amplified hGH receptor cDNA fragments were electrophoresed through 2% agarose gels and then transferred to 0.45 μm positively charged nylon membranes (Schleicher & Schuell, Keene, NH) by capillary action with 10xSSPE. Prior to blotting, gels were denatured by soaking twice for 15 minutes in 0.5 N NaOH/1.5 M NaCl and then neutralized by soaking twice more for 15 minutes in 1 M Tris-HCl, pH 7.4/1.5 M NaCl. The nucleic acids were immobilized onto the membranes by exposing the blots to ultraviolet light (254-312 nm) for 5 minutes.

Blots were prehybridized for 3-5 hours at 42°C in the presence of 6xSSPE, 1% SDS, 10xDenhardt's and 0.15 mg/ml denatured salmon sperm DNA (Sigma Chemicals, St. Louis, MO). The volumes of the prehybridization and hybridization solutions were 100 μl per cm^2 of nylon membrane. Hybridization occurred overnight at 65°C. The hybridization solution contained 6xSSPE, 1% SDS, 0.1 mg/ml denatured salmon sperm

DNA and 26.3 nCi of probe per cm^2 of nylon membrane. Following hybridization, blots were washed twice for 10 minutes at 65°C with 6xSSPE/1%SDS and then once more for 10 minutes at 60°C with 2xSSPE/1%SDS. Bands were visualized by autoradiography (1-5 days exposure) and quantified using the Fuji Bioimager. For quantitative V1 hGHR analysis, a ratio of the radioactivity intensity of each sample PCR band over that of its internal standard PCR fragment was calculated; initial studies had shown that under the specified RT-PCR conditions amplification of the sample and internal standard cDNA fragments was still in an exponential phase. Subsequent comparisons were made for each paired set of normal and tumor liver specimens to determine differences in relative levels of expression.

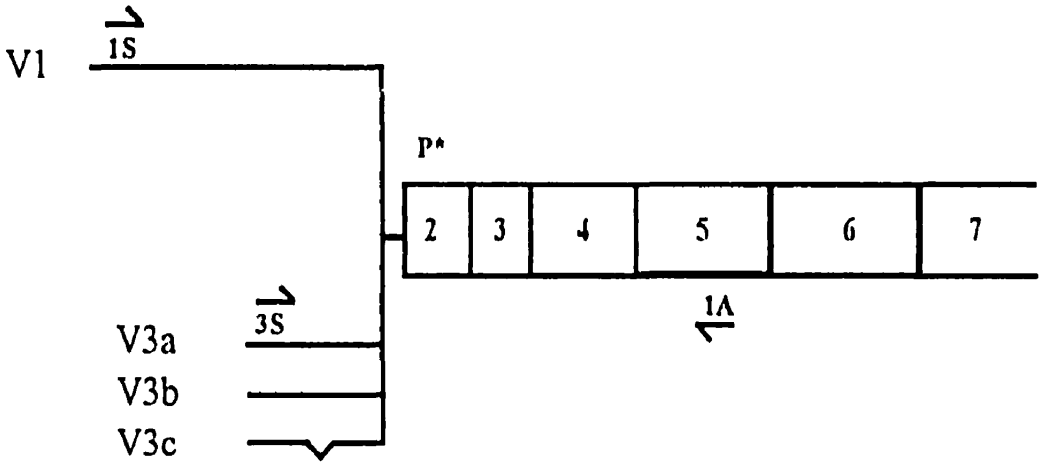
Approximately 150 nmol of the nested oligo (P^*; Figure 3.1) (5'-CTG CTG TTG ACC TTG GCA CTG GC-3') were end-labelled and then purified through a Sephadex G-50 Medium (Pharmacia Biotech) column. The end-labelling reaction occurred at 37°C for 1 hour in the presence of 2.5 μCi/μl of γ-^{32}P-ATP (ICN Pharmaceuticals Canada Ltd., Montreal, QC), 20 units of T_4 polynucleotide kinase (GIBCO BRL), 70 mM Tris-HCl pH 7.6, and 10 mM $MgCl_2$ 100 mM KCl and 1 mM 2-mercaptoethanol.

3.5. Results.

An RT-PCR/Southern blot approach (Figure 3.1) was used to screen fetal and postnatal human tissues for the presence of V1 and V3. As previous studies have shown that human adult liver expresses both V1 and V3 mRNA transcripts (8), total RNA from an adult liver sample served as a positive control in all RT-PCR assays. A water blank served as a negative RT-PCR control. Whenever possible, samples were run in parallel without reverse transcriptase, to rule out sample contamination with cDNA; limited amounts of sample RNA precluded a non-reverse transcriptase test for all specimens. Following each set of RT reactions, two aliquots from each RT product were taken: one was tested for V1, the other for V3. Because we anticipated that V1 transcripts would be undetectable or low in all specimens other than postnatal human normal liver, we synthesized a V1 cDNA construct with an internal deletion and added it to each PCR reaction tube as a positive internal control for amplification. It should also be noted that the antisense primer was designed to permit the detection of both exon 3 retaining and deleted hGHR mRNA isoforms (Figure 3.1).

Previous studies have identified three V3 subvariants (V3a, V3b and V3c), that are thought to arise from alternative splicing of a single precursor mRNA transcript (8). However, our V3 specific sense PCR primer (3S; Figure 3.1) can not differentiate between V3a and V3b cDNA isoforms. All three V3 subvariants are hereafter collectively referred to as V3.

Fig. 3.1. RT-PCR/Southern blot strategy. V1, V3a, V3b and V3c hGHR cDNA transcripts are shown. Exons are represented by numbered boxes, whereas 5'UTRs are depicted by solid lines. The approximate locations of the sense (S) and antisense (A) primers as well as the nested hybridization probe (P*) are indicated. Primer 1A was used in the RT reactions. Single stranded V1 and V3 cDNA transcripts were amplified using primer sets 1S/1A and 3S/1A, respectively. The lengths of the expected V1 and V3 (with or without the exon 3 deletion) PCR fragments are listed. The precise nucleotide sequences of the oligonucleotides are given in the materials and methods section.



PCR Product sizes (base pairs)

Primer Combination	PCR Product	Exon 3 Retaining	Exon 3 Deleted
1S/1A	V1	671	605
3S/1A	V3a/V3b	502	436
3S/1A	V3c	409	343

3.5.1. Tissue distribution of V1 and V3 mRNA isoforms.

V3, but not V1, was detected in fetal (F) and postnatal (P) kidney [n= 4F (14-16 wk FA), 4P (2-62 yr)], lung [n= 4F (13.8-19 wk FA), 2P (69, 80 yr)], intestine [n= 8F (11.7-20 wk FA), 4P (40-64 yr)], skeletal muscle [n= 1F (18 wk FA), 1P (1 week)] and adrenal [n= 1F (13.3 wk FA), 1P (29 yr)] (data not shown). Only the control postnatal liver was V1 positive, suggesting that V1 mRNA expression is liver-specific. The absence of V1 in specimens other than the control adult liver can not be attributed to failed RT or PCR reactions because, in each case, V3 transcripts and V1 internal cDNA controls were readily observed (data not shown).

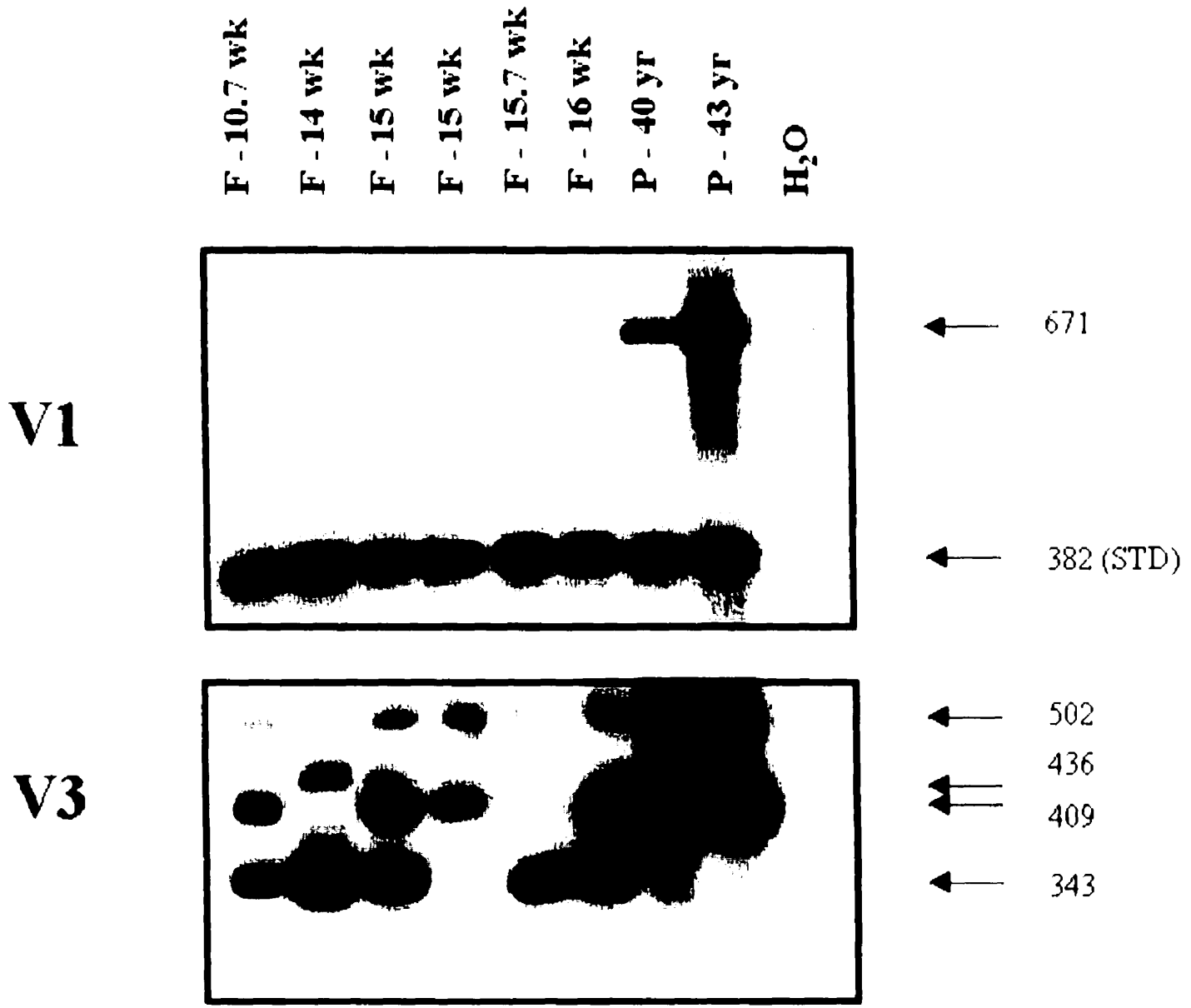
3.5.2. Expression of the V1 and V3 transcripts during liver development.

We then delineated the ontogeny of V1 and V3 in human liver. All thirteen fetal specimens (10.7-30 wk FA) had undetectable levels of V1, whereas V1 was readily observed in transplant donor livers (n=5, 11-62 yr) (Figure 3.2 and data not shown). Both fetal and postnatal samples expressed V3 (Figure 3.2 and data not shown). The V1 internal PCR control was detected in all cases (Figure 3.2 and data not shown). Furthermore, no specific association of V1 or V3 was observed with either exon 3 retaining or deleted mRNA transcripts (Figures 3.2, 3.3 and data not shown).

3.5.3. Characterization of V1 and V3 mRNA expression patterns in hepatic tumours.

Next, we screened for V1 and V3 in a large series of HB (n=17, 6-36 months, including 5 with paired normal liver). Although V3 was present in all samples, V1 was

Figure 3.2. Representative Southern blots illustrating the ontogeny of V1 (upper panel) and V3 (lower panel) hGHR mRNA transcripts in liver. The donor age of fetal (F, in wk FA) and postnatal (P, in yr) samples is given above each lane. H_2O served as a negative RT-PCR control. The sizes (bp) of the expected PCR fragments are indicated. For details on the expected bands, refer to Figure 3.1. Three different V1 and V3 hGHR mRNA expression profiles are illustrated in the upper and lower panels, respectively, based on whether the specimens express the exon 3 retaining isoform (15 wk, 40 yr, 43 yr), the exon 3 deleted isoform (14 wk, 15.7 wk) or a combination of the two isoforms (10.7 wk, 15 wk, 16 wk). Although not all of the expected bands are clearly visible on this autoradiograph (24 hr exposure), they were all apparent following a 72 hr exposure. The 382 base pair band in the upper panel corresponds to the V1 internal PCR control. The band below the expected 671 bp V1 PCR fragment in sample "P-43 yr" is an amplification artifact and was not reproducible.


F - 10.7 wk
F - 14 wk
F - 15 wk
F - 15 wk
F - 15.7 wk
F - 16 wk
P - 40 yr
P - 43 yr
H2O
V1
671
382 (STD)
V3
502
436
409
343

undetectable in 15 of 17 HB, including the five HB paired with V1 expressing normal liver (Figure 3.3 and Table 3.1). The V1 internal PCR control fragment was detected in all V1 assays (Figure 3.3 and data not shown). The absence of V1 did not correlate with patient age, sex, HB subtype, $\pm$ chemotherapy, exon 3 retaining and deficient hGHR mRNA isoform pattern or loss of heterozygosity at 11p, 1p and 1q (Table 3.1). The two HB that expressed V1 were predominantly of an embryonic phenotype; neither showed unique or variant histopathological features.

Four adult HCC (n=4, 50-75 yr, with paired normal liver) were also tested for V1 and V3 expression. Semiquantitative V1 RT-PCR analysis was performed in duplicate. In each case, the ratio of the intensity of the test PCR band over that of the internal standard PCR fragment was calculated and V1 expression compared between tumor and its paired normal specimen. The four HCC showed marked (>20-fold, n=2) or complete (n=2) suppression of V1 as compared to paired normal liver (Figure 3.3 and Table 3.1). The V1 internal standard PCR fragment was detected in all V1 RT-PCR assays (Figure 3.3 and data not shown). V3 was expressed in all HCC and paired normal livers (Figure 3.3 and Table 3.1).

Finally, in order to determine whether altered V1 expression occurred in a second embryonal type of tumor, we tested for V1 and V3 expression in two Wilms' tumor specimens (2 and 4 years) and their paired normal kidney. V3, but not V1, was present in both normal and tumor kidney tissues (data not shown). Amplification of the V1 internal standard was observed in all V1 RT-PCR assays (data not shown).

Fig. 3.3. Representative Southern blots demonstrating the expression profiles of V1 (upper panel) and V3 (lower panel) hGHR mRNA transcripts in HB and HCC. The results from two sets of HB and HCC with their paired normal liver (NL) are shown. H_2O served as a negative RT-PCR control. The sizes (bp) of the expected PCR fragments are indicated. For details on the expected bands, refer to Figure 3.1. Three different V1 and V3 hGHR mRNA expression profiles are illustrated in the upper and lower panels, respectively, based on whether the specimens express the exon 3 retaining isoform (94-268/9), the exon 3 deleted isoform (95-96/7) or a combination of the two isoforms (M11/12, M13/14). Although not all of the expected bands are clearly visible on this autoradiograph (24 hr exposure), they were all apparent following a 72 hr exposure. The 382 base pair band in the upper panel corresponds to the V1 internal PCR control.

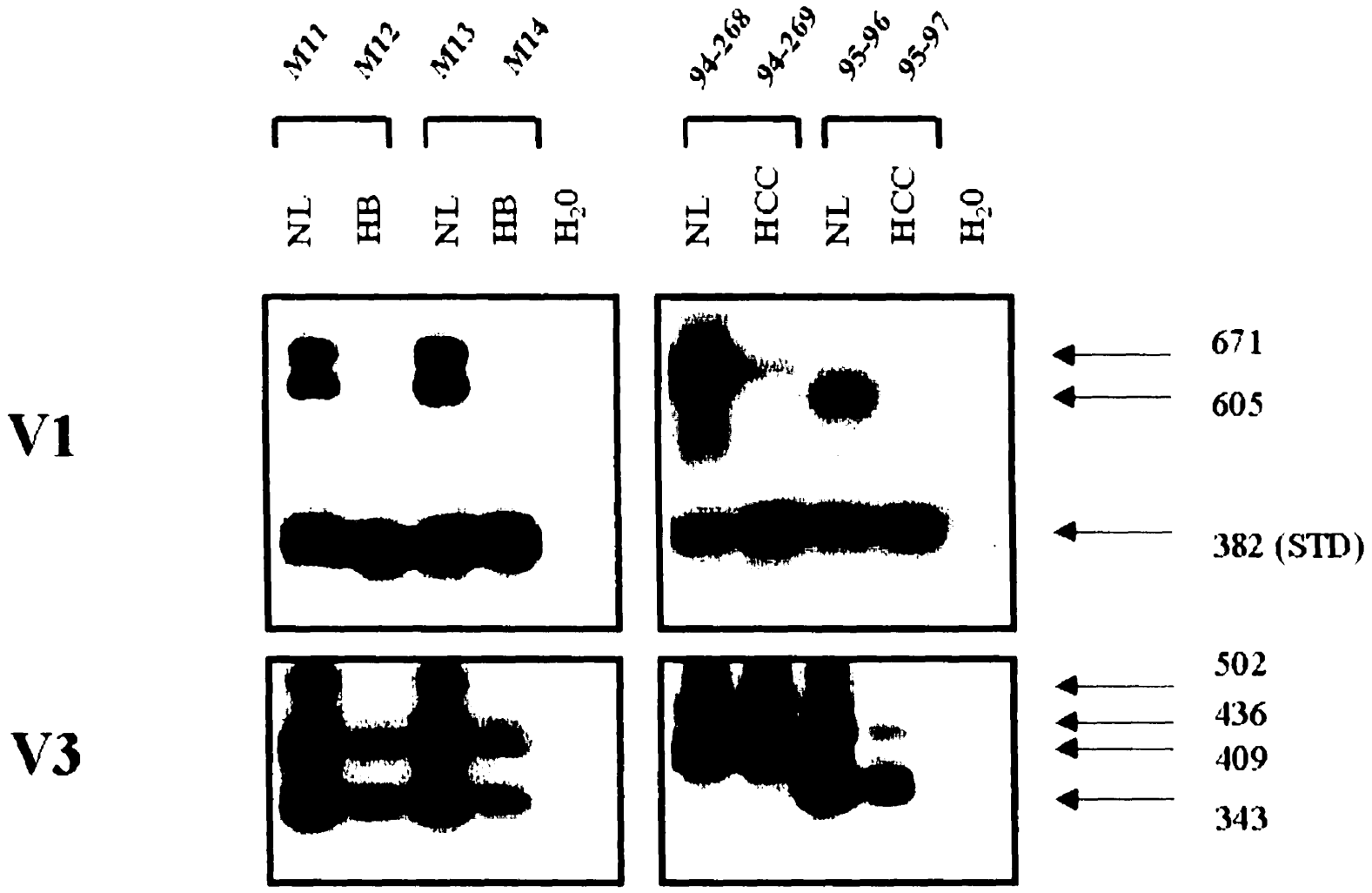

M11
M12
M13
M14
94-268
94-269
95-96
95-97
NL
HB
NL
HB
H2O
NL
HCC
NL
HCC
H2O
V1
V3
671
605
382 (STD)
502
436
409
343

Table 3.1: Summary of clinical and experimental data.

	[1]age	sex	[2]tumor type	± chemo	[3]LOH 11p	[4]LOH 1p	[4]LOH 1q	hGHR exon 3 mRNA	hGHR V1 mRNA	hGHR V3 mRNA
R378	21	M	HB; mixed	+	-	+	+	3+	-	+
R656	11	F	HB; epithelial	-	-	-	-	3+/3-	-	+
R682	8	F	HB; mixed	-	-	-	-	3+	-	+
R683	13	M	HB; epithelial	-	-	-	-	3+/3-	-	+
R684	12	M	HB; epithelial	-	-	-	-	3+	-	+
R685	27	F	HB; epithelial	-	+	+	-	3+/3-	-	+
R704	27	F	HB; epithelial	-	ND	ND	-	3+	-	+
R366	11	F	HB; epithelial	-	-	-	-	3+/3-	-	+
R371	8	F	HB; mixed	-	+	-	-	3+/3-	-	+
U62	6	M	HB; epithelial	-	+	-	-	3+/3-	-	+
R365	6	F	HB; epithelial	+	ND	ND	ND	3+	+	+
R707	34	F	HB; epithelial	-	+	-	-	3+	+	+
R331	9	F	HB; mixed	+	-	+	-	3+	-	+
R332	9	F	NL to R331	+	NA	NA	NA	3+	+	+
R374	12	M	HB; mixed	-	-	-	-	3+	-	+
R375	12	M	NL to R374	-	NA	NA	NA	3+	+	+
M12	13	M	HB; epithelial	-	ND	ND	ND	3+/3-	-	+
M11	13	M	NL to M12	-	NA	NA	NA	3+/3-	+	+
M14	20	M	HB; epithelial	+	ND	ND	ND	3+/3-	-	+
M13	20	M	NL to M14	+	NA	NA	NA	3+/3-	+	+
M16	36	M	HB; epithelial	-	ND	ND	ND	3+	-	+
M15	36	M	NL to M16	-	NA	NA	NA	3+	+	+
94-269	75	M	HCC	-	ND	ND	ND	3+	(+)	+
94-268	75	M	NL to 94-269	-	NA	NA	NA	3+	+	+
95-97	53	M	HCC	-	ND	ND	ND	3-	(+)	+
95-96	53	M	NL to 95-97	-	NA	NA	NA	3-	+	+
CO81P	50	F	HCC	-	ND	ND	ND	3+/3-	-	+
CO82P	50	F	NL to CO81P	-	NA	NA	NA	3+/3-	+	+
1416C	52	F	HCC	-	ND	ND	ND	3+/3-	-	+
1416AB	52	F	NL to 1416C	-	NA	NA	NA	3+/3-	+	+

[1]Donor age of HB specimens is given in months and HCC samples in years; [2]All major HB subtypes are represented [Epithelial (fetal, embryonal, fetal/embryonal, macrotrabecular, anaplastic) and mixed (epithelial/mesenchymal: ± teratoid)]; [3]ref# 4; [4]ref# 3; F= female; M= male; NL= normal liver; NA= not applicable; ND= not done; 3+ = exon 3 retaining hGHR mRNA; 3- = exon 3 deleted mRNA; + = detected; - = not detected; (+) = V1 levels were > 20-fold decreased compared to normal paired liver. All four HCC specimens stained negative for hepatitis B viral antigen.

3.6. Discussion.

In the present study, we found that V3 transcripts are widely expressed in human fetal and postnatal tissues. In contrast, V1 mRNA regulation is tissue- and developmental-specific: as we hypothesized, based on studies in the ovine (9), V1 expression appears to be limited to the postnatal liver. Human V1 and ovine exon 1A GHR transcripts are first detectable around the time of birth. The ovine exon 1A mRNA is 76% identical to the human V1 and shown by ribonuclease protection analysis to be absent in 80 day fetal liver but present in 14 day postnatal hepatocytes (9). Our RT-PCR investigation shows that V1 is absent in two premature (25 and 30 wks FA) newborn hepatic tissues but present in five postnatal infant livers (9-36 months).

We conclude that V1 mRNA is either downregulated in fetal liver or upregulated in postnatal liver. Studies suggest that transcription of the ovine exon 1A transcript is regulated by a liver-specific and developmentally regulated promoter on the *GHR* gene (9). Although we can not exclude the possibility of alternative splicing as the regulatory mechanism for V1 synthesis in the human, we can speculate, based on the sheep model, that V1 expression is controlled by a hepatic-specific promoter on the *hGHR* gene that is under developmental control. If this is the case, then around the time of birth there must be suppression of V1-promoter repressing factor(s) and/or the turning on of transcription activating factor(s). Before exploring these possibilities, the V1 promoter for the *hGHR* gene will have to be mapped and sequenced.

The biological significance of the V1 and V3 leader sequences remains unknown. Complex 5'UTR structures have been shown to modify mRNA translation initiation rates

(14). Interestingly, there is a six-fold increase in total hGHR mRNA concentration and a four-fold increase in hGH binding from the fetal to postnatal liver (Appendix 1, Figure A1.4). One question to pursue, therefore, is whether the turning on of V1 mRNA in postnatal liver accounts for these ontogenic changes.

Interestingly, V1, but not V3, transcripts were absent in 15 of 17 HB, including all five HB specimens with paired normal (V1 expressing) liver. In addition, we tested four adult HCC and observed either marked (n=2, >20-fold) or complete (n=2) suppression of V1, but not V3, as compared to paired normal liver. Thus, we have strong evidence in support of our hypothesis that the lack of V1 mRNA is a marker of human liver differentiation and that absence or suppression of V1 expression reflects the continued "fetal" state of hepatic tumors.

Most striking is the "universality" of V1 regulation in HB. All except two of the seventeen HB tested show lack of V1 expression regardless of patient age, sex, HB subtype (all major subtypes were examined), $\pm$ prior chemotherapy, exon 3 retaining and deficient hGHR mRNA isoform pattern or LOH at 11p, 1p and 1q. These data suggest that the absence of V1 mRNA is a molecular phenotypic marker of HB.

No other HB marker has been described with such a high level of correlation. For example, α-fetoprotein is detected in only 60% of HB patients and ß-human chorionic gonadotropin in 3% (1,2). There is little evidence of a role for any single chromosomal abnormality or p53 mutation (1,2). Thirty-two HBs (some of which were tested for V1 and V3 expression in the present study) were examined for LOH on chromosome 1, since deletions on both 1p and 1q are common in human malignancies: 7 tumors had LOH on

1p, 7 showed LOH on 1q and 3 had LOH on both arms (4). LOH at 11p has been reported for only one-third of all informative HB cases (1,2,3) and loss of imprinting at the IGF II gene locus with one-eighth (15,16). Thus, the lack of V1 expression, that we observed in 88% of HB in the present study, seems to be the most important association reported to date.

We have previously suggested that expression of the exon 3 deleted hGHR mRNA, either alone or together with the exon 3 retaining transcript, is predominant prior to 20 wks FA (6). Therefore, it was interesting to examine whether, like early- to mid-gestational human fetal liver, the fetal/embryonal HB preferentially expresses the exon 3 deficient mRNA. All five HB with paired normal tissue expressed the same exon 3 isoform pattern as their respective adjacent normal liver and 8 of 17 HB specimens tested showed expression of the exon 3 deleted mRNA. Since these results are midway between the early- to mid-gestational (61%) and postnatal (20%) frequency data previously obtained (6), no conclusion can be drawn.

The mechanism for synthesis of the exon 3 retaining and deleted hGHR mRNA isoforms is not understood. Since 5'UTR sequences may potentially influence alternative splicing of the precursor RNA (17), it was of interest to assess whether V1 or V3 preferentially associate with either the exon 3 retaining or deficient transcripts. In agreement with the findings of Esposito et al. (18), our data demonstrate that this is not the case: V1 and V3 did not specifically associate with either the exon 3 retaining or deleted mRNA isoforms.

We conclude that, in the human, there is tissue-, fetal- and tumor-specific

regulation of the V1 hGHR mRNA transcript. We speculate that an inability to activate V1 expression occurs concomitantly with HB tumorigenesis. Furthermore, V1 suppression in HCC may be a measure of the degree of dedifferentiation to the embryonal/fetal stage that the HCC tumor cells have undergone. Whether V1 dysregulation is a cause or effect of the tumor state remains unknown. We hypothesize that, in the human, like in the ovine, transcription of hGHR mRNAs containing the V1 leader sequence is driven by a specific V1 promoter and that, in the hepatic tumor state, there is "fetal" control of the transcription factor(s) regulating this promoter.

3.7. Acknowledgements.

Most of the HB patients (prefixes R & U; Table 3.1) are enrolled in the German Cooperative Pediatric Liver Tumor Study HB-89. Several tissue samples were kindly provided by the Cooperative Human Tissue Network which is funded by the US National Cancer Institute. Studentship support for G.Z. has been awarded by Fonds pour la Formation de Chercheurs et l'Aide à la Recherche and the McGill University-Montreal Children's Hospital Research Institute. This work was supported by the Medical Research Council of Canada (C.G.G), Freundesgesellschaft der Medizinischen Hochschule Hannover (T.P., D.v.S.) and Dr. Mildred Scheel Stiftung fÜr Krebsforschung (T.P., D.v.S.).

3.8. References.

1. Greenberg, M., Filler, R.M. Hepatic tumors. In: P.A. Pizzo, D.G. Poplack (eds), Principles and Practice of Pediatric Oncology, 2nd edition, pp. 697-711. Philadelphia: J.B Lippincott Co., 1993.

2. Stocker, J.T. Hepatoblastoma. Seminars in Diagnostic Pathology 11: 136-143, 1994.

3. Albrecht, S., von Schweinitz, D., Waha, A., Kraus, J.A., von Deimling, A., Pietsch, T. Loss of maternal alleles on chromosome arm 11p in hepatoblastoma. Cancer Res. 54: 5041-5044, 1994.

4. Kraus, J.A., Albrecht, S., Wiestler, O.D., von Schweinitz, D., Pietsch, T. Loss of heterozygosity on chromosome 1 in hepatoblastoma. Int. J. Cancer (in press, 1996).

5. Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J., Wood, W.I. Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330: 530-43, 1987.

6. Zogopoulos, G., Figueiredo, R., Jenab, A., Ali, Z., Lefèbvre, Y., Goodyer, C.G. Expression of the exon 3-retaining and -deleted human growth hormone receptor messenger ribonucleic acid isoforms during development. J. Clin. Endocrinol. Metab. 81: 775-782, 1996.

7. Mercado, M., Davila, N., McLeod, J.F., Baumann, G. Distribution of growth hormone receptor messenger ribonucleic acid containing and lacking exon 3 in human tissues. J. Clin. Endocrinol. Metab. 78: 731-735, 1994.

8. Pekhletsky, R.I., Chernov, B.K., Rubtsov, P.M. Variants of the 5'-untranslated sequence of human growth hormone receptor mRNA. Mol. Cell. Endocrinol. 90: 103-109, 1992.

9. O'Mahoney, J.V., Brandon, M.R., Adams, T.E. Identification of a liver-specific promoter for the ovine growth hormone receptor. Mol. Cell. Endocrinol. 101: 129-139, 1994.

10. Baumbach, W.R., Bingham, B. One class of growth hormone (GH) receptor and binding protein messenger ribonucleic acid in rat liver, GHR1, is sexually dimorphic and regulated by GH. Endocrinology 136: 749-760, 1995.

11. Menon, R.K., Stephan, D.A., Singh, M., Morris, Jr., S.M., Zou, L. Cloning of the promoter-regulatory region of the murine growth hormone receptor gene. J. Biol. Chem. 270: 8851-8859, 1995.

12. Chomczynski, P., Sacchi, N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159,

1987.

13. Chirgwin, J.J., Przbyla, A.E., MacDonald, R.J., Rutter, W.J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochem. 18: 5294, 1979.

14. Jin, C.F., Mata, M., Fink, D.J. Rapid construction of deleted DNA fragments for use as internal standards in competitive PCR. PCR Methods and Applications 3: 252-255, 1994.

15. Darveau, A., Pelletier, J., Sonenberg, N. Differential efficiencies of in vitro translation of mouse c-myc transcripts differing in the 5' untranslated region. Proc. Natl. Acad. Sci. USA 82: 2315-2319, 1985.

16. Li, X., Adam, G., Cui, H., Sandstedt, B., Ohlsson, R., Ekstrom, T.J. Expression, promoter usage and paternal imprinting status of insulin-like growth factor II (IGF2) in human hepatoblastoma: uncoupling of IGF2 and H19 imprinting. Oncogene 11: 221-229, 1995.

17. Montagna, M., Menin, C., Chieco-Bianchi, L., D'Andrea, E. Occasional loss of constitutive heterozygosity at 11p15.5 and imprinting relaxation of the IGFII maternal allele in hepatoblastoma. J. Cancer Res. Clin. Oncol. 120: 732-736, 1994.

18. Green, M.R. Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu. Rev. Cell Biol. 7: 559-599, 1991.

19. Esposito, N., Paterlini, P., Kelly, P.A., Postel-Vinay, M-C., Finidori, J. Expression of two isoforms of the human growth hormone receptor in normal liver and hepatocarcinoma. Mol. Cell. Endocrinol. 103: 13-20, 1994.

CHAPTER 4

CHAPTER 4 - *CLONING AND CHARACTERIZATION OF PROMOTER REGIONS IN THE HUMAN GROWTH HORMONE RECEPTOR GENE*

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(Submitted)

4.1. Preface.

The distinct tissue and developmental expression patterns of the V1 and V3 5'UTR variants suggest that multiple gene promoter regions may be regulating *hGHR* gene transcription. In this study, we used RT-PCR and Southern blot protocols to characterize the expression profile of V4, another 5'UTR variant of the hGHR mRNA. Like V1, V4 was detected only in normal postnatal livers and was repressed in hepatic tumours. Therefore, transcription of V1 and V4 mRNAs may be regulated by common gene regulatory regions, whereas a separate promoter drives the more ubiquitous V3 mRNA synthesis. To test our hypothesis, we isolated portions of the 5' flanking region of the *hGHR* gene, encompassing nucleotide sequences specific for the previously cloned V1 and V4 as well as V7 and V8 cDNAs. Characterization of the genomic fragment provided support for our hypothesis: transcription of V1 and V4 mRNAs is regulated by at least one common gene promoter. We believe that the onset of these developmentally-regulated hGHR gene promoter regions is responsible for the six-fold increase from fetal to postnatal liver that was described in Appendix 1.

4.2. Abstract.

The 5' untranslated region (5'UTR) of growth hormone receptor (GHR) mRNA is heterogeneous in several species. In the human, which is the most complex, eight different 5'UTR variants (V1 to V8, numbered according to their relative abundance) have been cloned from adult liver. We have previously shown that, whereas V1 is not expressed in fetal tissues or hepatic tumours and is only readily detectable in normal postnatal liver, V3 is widely expressed in both the fetus and adult. In this study, we demonstrate that the expression pattern of the V4 5'UTR variant is similar to that of V1 during development and liver tumorigenesis. To identify DNA sequences regulating transcription of V1 and V4, we isolated portions of the 5' flanking region of the human *(h) GHR* gene. Restriction mapping and nucleotide sequencing precisely placed four variant sequences in series (5' V7-V1-V4-V8 3') on a 3.8 kb XbaI-BsaAI genomic fragment. A transcription start site with a proximal TATA motif was identified upstream of the V1 sequence by ribonuclease protection and primer extension analyses of postnatal liver total RNA. Transcription at this position generates a 5'UTR variant containing V1, V4 and V8. Ribonuclease protection analysis and RT-PCR assays provided evidence for additional mRNA isoforms in postnatal liver extending upstream of V1 to include V7 and the 5' flanking region of V7. Differential splicing of these two large gene products can result in several mRNA species, including the V1, V4, V7 and V8 isoforms previously identified. Together, the data imply that 1) certain transcriptional regulatory regions of the *hGHR* gene are homologous to those of the more simple ovine and rodent *GHR* genes; 2) the 5'UTR variants of the hGHR mRNA are due to differential promoter usage

and complex alternative splicing; and 3) the postnatal liver-specific expression pattern of V1 and V4 *versus* the ubiquitous profile of V3 is, at least in part, the result of transcriptional regulation.

4.3. Introduction.

The growth hormone receptor (GHR) mediates multiple metabolic processes as well as growth in mammals (1). Although these pivotal roles of the GHR have been well established in postnatal tissues, the function of the receptor during fetal development remains unclear (2,3). Several animal studies suggest that the onset of significant GHR mRNA expression and GH binding occurs only after birth (4-6). However, in support of a developmental role for the GHR, Pantaleon *et al* (7) have identified a functional GHR in preimplantation mouse embryos, while Zhou *et al* (8) have determined that inbreeding of homozygous *GHR* knockout mice results in significantly smaller litters and increased perinatal mortality.

In addition, we have shown that transcription of the human *(h) GHR* gene begins as early as 4 weeks of gestation in placental villi and that there is ubiquitous tissue expression by the first trimester of human fetal life (9). Immunohistochemical studies have identified the hGHR in human tissues by 8.5 weeks of fetal age and shown that, by midgestation, the pattern of immunostaining is often identical to that found in the adult (10,11). Binding experiments (12-14) and treatment of fetal hepatocytes (15,16) and pancreatic islets (17-19) with hGH have demonstrated that the fetal hGHR is capable of binding hGH and mediating biological effects. Comparative studies of fetal *versus* postnatal tissues have revealed tissue-specific changes in hGHR expression during development: a significant decrease in lung hGHR mRNA levels postnatally, no age-related differences in kidney and small intestine, and a 6-fold increase in postnatal liver (20). The increase in liver *hGHR* gene expression parallels a 4-fold increase in hGH

binding to hepatocyte membranes (14,20). Thus, expression of the *hGHR* gene appears to be regulated by developmental- and tissue-specific mechanisms.

The hGHR is encoded by exons 2 to 10 of the *hGHR* gene on chromosome 5p13.1-p.12 (21,22). Transcriptional regulation of the *hGHR* gene is complex, involving alternative splicing as well as multiple 5' untranslated region (5'UTR) exons. Exon 3 of the gene transcript can be spliced out, resulting in an exon 3 deficient mRNA isoform (23). Expression of exon 3 retaining and deleted transcripts is identical in all tissues of the same individual but varies between different subjects (9,24). The consequences of the lack of exon 3 coding information to hGHR function are not known.

Heterogeneity in the 5'UTR of the GHR mRNA has been demonstrated in several species (25-28). In the human, eight different 5'UTR variants (V1 to V8, numbered according to their relative abundance), diverging at -12 bp from the start site of translation, have been cloned from adult liver by 5' rapid amplification of cDNA ends (5'RACE) (25). Regulation of these different 5'UTR variants may determine fetal- and tissue-specific differences in receptor activity, as has been shown for a number of growth factors and protooncogenes (29). We have recently demonstrated that V1 is, indeed, under tissue-, fetal- and tumour-specific regulation: V1 hGHR mRNA transcripts are only present in postnatal liver, while absent or of markedly lower abundance in hepatoblastomas (HB) and hepatocellular carcinomas (HCC) (30). In contrast, V3 transcripts were detected in all fetal and postnatal tissues examined (30).

The present study further shows that the pattern of V4 expression is identical to that of V1. These observations suggest that V1 and V4 originate from alternative splicing

of a single transcript whose synthesis is driven by a tissue-specific and developmentally-regulated promoter, whereas a second promoter controls ubiquitous expression of the V3 mRNA. To test this hypothesis, we cloned part of the 5' flanking region of the *hGHR* gene and precisely mapped four variant sequences in series (5' V7-V1-V4-V8 3') on a 3.8 kb XbaI-BsaAI genomic fragment. A transcription initiation site upstream of V1 that was utilised in postnatal liver was identified, and evidence was provided for additional mRNA species in postnatal liver extending upstream of V7. Thus, multiple promoters in the *hGHR* gene drive the tissue- and developmental-specific expression of the different 5'UTR mRNA isoforms.

4.4. Results.

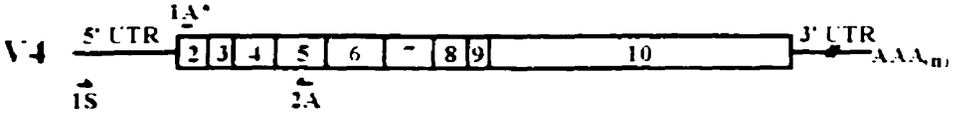
4.4.1. Characterization of V4 mRNA expression during development and liver tumourigenesis.

The RT-PCR/Southern blot strategy used to amplify V4 mRNA is summarized in Figure 4.1A. In each analysis, aliquots from the same RT reaction were taken and tested for V4 and V1. In addition, V3 mRNA was measured since it is ubiquitously expressed (30) and, therefore, served as a positive control for reverse transcription. The V1 and V3 results have been published previously (30). As shown in Figures 4.1B and 4.1C, the V4 5'UTR variant has a similar pattern of expression to V1 transcripts.

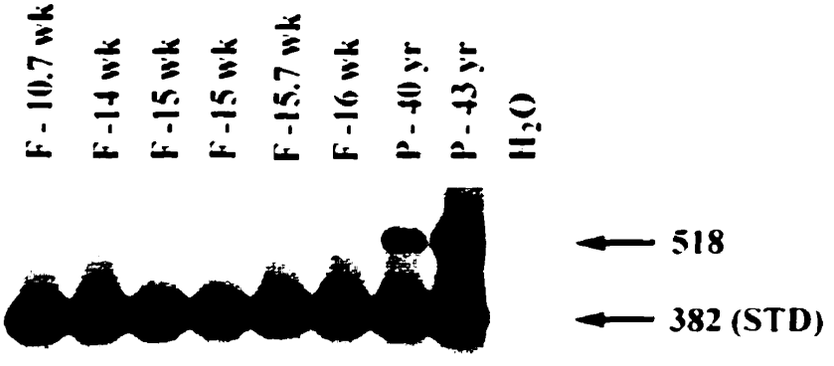
Although V4 was detected in control adult liver (Figure 4.1B), it was not observed in kidney (n=3 fetal, 14-16 weeks of fetal age [wk FA]; n=2 postnatal, 2 & 4 yr), intestine (n=3 fetal, 11.6-16.5 wk FA; n=2 adult, 40 & 84 yr) and lung (n=3 fetal, 14-16 wk FA; 2 adult, 69 & 80 yr) (data not shown). Therefore, V4 expression, like V1, is tissue-specific. Like V1, V4 was suppressed in all fetal livers examined (n=6, 10-16 wk FA), but readily detectable in transplant donor hepatic samples (n=4, 11-43 yr) (Figure 4.1B and data not shown). Since V1 expression is altered in hepatic tumours (30), 12 HB (6-36 months) and two HCC (53 & 75 yr) were analyzed for the presence of V4 (Figure 4.1C and data not shown). V4 was undetectable in the same 11 of 12 HB that had previously tested negative for V1 (30), including the four HB paired with normal (V1 and V4 positive) liver. The single V1- and V4-expressing HB specimen was obtained from a 6 month old pediatric patient; the tumor was predominantly of an embryonic phenotype but did not show unique or variant histopathological features. V4 expression,

Fig. 4.1. Analysis of hGHR V4 mRNA expression. Panel A, Schematic representation of the hGHR V4 cDNA isoform (not drawn to scale). Exons are boxed and the V4 5'UTR and 3'UTR sequences are represented by a solid line. The positions of the sense (1S) and antisense (2A) primers as well as the internal hybridization probe (1A*) are indicated (oligonucleotide sequences are provided in Table 1). Panels B and C, representative Southern blots describing the ontogeny of V4 in liver as well as its expression pattern in HB and HCC. Panel B, expression profile of fetal (F; in wk FA) and postnatal (P; in yr) hepatic samples. Panel C, results from two sets of HB and HCC with their paired normal liver (NL) samples are shown. The sizes of exon 3 retaining and deleted V4 RT-PCR products are 518 and 432 bp, respectively. An internal V4 standard (STD), which generates a PCR product of 382 bp, served as a positive control and H_2O was a negative RT-PCR control. Each amplification reaction was still at an exponential phase at 25 cycles.

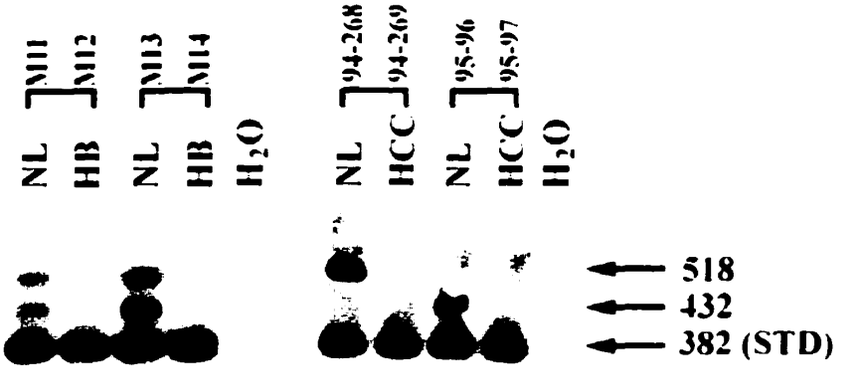
A.


V4
5' UTR
1A*
2
3
4
5
6
7
8
9
10
3' UTR
AAA$_n$
1S
2A

B.


F - 10.7 wk
F -14 wk
F -15 wk
F -15 wk
F -15.7 wk
F -16 wk
P - 40 yr
P - 43 yr
H$_2$O
518
382 (STD)

C.


N11
N12
N13
N14
94-268
94-269
95-96
95-97
NL
HB
NL
HB
H$_2$O
NL
HCC
NL
HCC
H$_2$O
518
432
382 (STD)

like that of V1, was also absent or of very low abundance in both HCC specimens relative to paired normal hepatic tissues (Figure 4.1C).

In contrast, V3 was observed in all fetal, postnatal and tumour tissues examined to date (30). It should be noted that both exon 3 retaining and deleted mRNA transcripts were found to contain V4 leader sequences (Figure 4.1 and data not shown). These findings, together with our previous observations (30), indicate that retention or deletion of exon 3 is not associated with expression of any one of the V1, V3 or V4 5'UTRs.

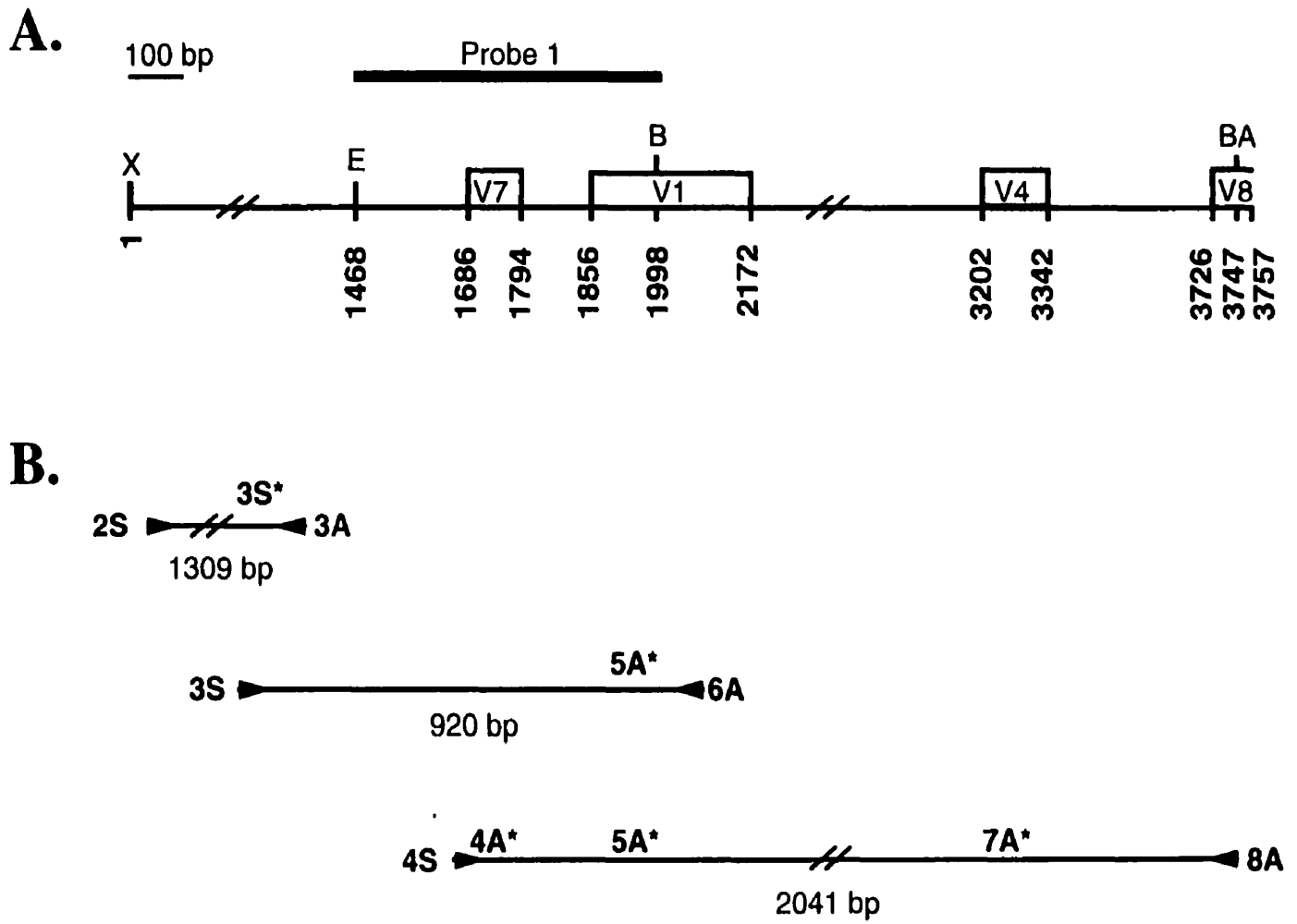
4.4.2. Cloning of genomic DNA containing the V1 hGHR sequence.

Figure 4.2A shows the precise mapping of V7, V1, V4 and V8 sequences in series on a 3.8 kb XbaI-BsaAI genomic DNA fragment. This structure is a composite of data obtained by screening a human genomic λ phage library and by PCR amplification of genomic DNA. The PCR strategy depicted in Figure 4.2B was designed with reference to the sequence of the 2 kb XbaI-BamHI fragment of the recombinant λ phage clone (λ/V1.01) we isolated, a previous report of the V1-V8 5'UTR sequences (25), and a recent preliminary characterisation of a recombinant λ clone of the *hGHR* gene (31).

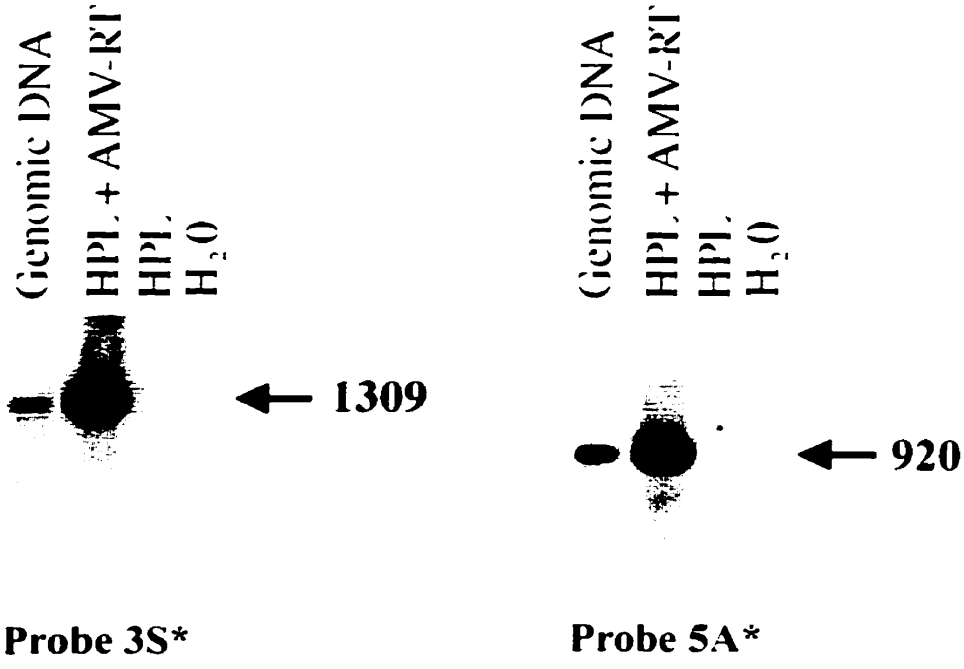
4.4.3. Mapping of promoter regions in the *hGHR* gene.

All transcriptional start site mapping experiments were performed using liver total RNA isolated from transplant donor tissues (11 & 62 yr). Identical results were obtained when the RT-PCR, RNase protection and primer extension experiments were repeated (Figures 4.2-4.5 and data not shown).

Fig. 4.2. Cloning and expression of the V1-containing portion of the *hGHR* gene. Panel A, restriction map of part of the 5' flanking region of the *hGHR* gene. Restriction sites shown are: B, BamHI; BA, BsaAI; E, EcoRI; X, XbaI. The V1, V4, V7 and V8 sequences previously obtained by 5'RACE (25) are boxed. The EcoRI/BamHI DNA fragment (Probe 1, thick line) was used for Southern blot analysis of restriction enzyme digests of bacteriophage recombinant DNA and genomic DNA. Panel B, overlapping PCR fragments corresponding to the 5' end of the *hGHR* gene were obtained by PCR amplification of genomic or λ phage DNA and by RT-PCR analysis of postnatal liver total RNA. The approximate positions of the sense (S) and antisense (A) primers and the internal hybridization probes (*) are indicated. The sequences of these oligonucleotides are detailed in Table 1. Panels C and D, RT-PCR/Southern blot analysis of human postnatal liver (HPL) total RNA. Genomic DNA served as a positive control for amplification, HPL total RNA was tested without AMV-RT to exclude genomic DNA contamination of the RNA, and water was a negative control for amplification. The sizes of the products are indicated in bp. Panel C, HPL total RNA (donor age, 11 yr) was reverse transcribed with primer 3A (left) or with primer 6A (right), the RT products were amplified using primers 2S/3A (left) or 3S/6A (right), and the specificity of the PCR products was confirmed by Southern analysis with internal probes 3S* (left) and 5A* (right). Panel D, HPL total RNA (donor age, 11 yr) was reverse transcribed with primer 8A, the RT product was amplified using primers 4S/8A, and the specificity of the PCR products was confirmed by Southern analysis with internal probes 4A* (left) and 5A* (middle) and 7A* (right).


A.
100 bp
Probe 1
X
E
B
BA
V7
V1
V4
V8
1
1468
1686
1794
1856
1998
2172
3202
3342
3726
3747
3757
B.
3S*
2S
3A
1309 bp
5A*
3S
6A
920 bp
4S
4A*
5A*
7A*
8A
2041 bp

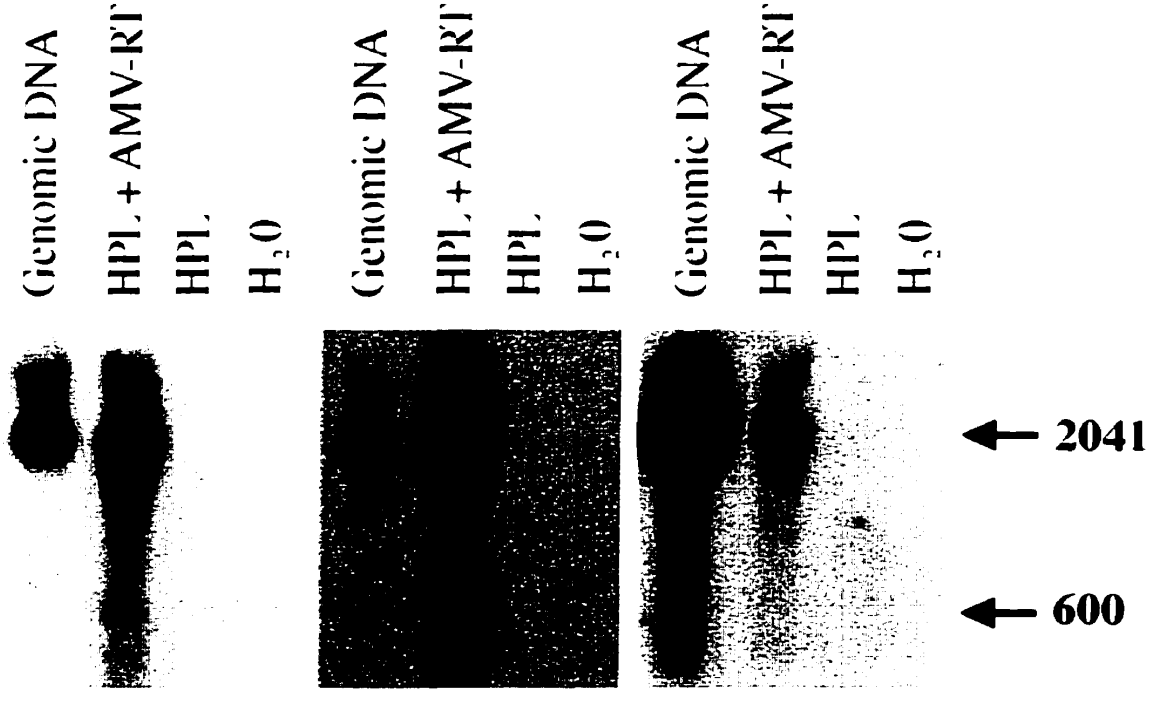
C.



Probe 3S*

Probe 5A*

D.



Probe 4A*

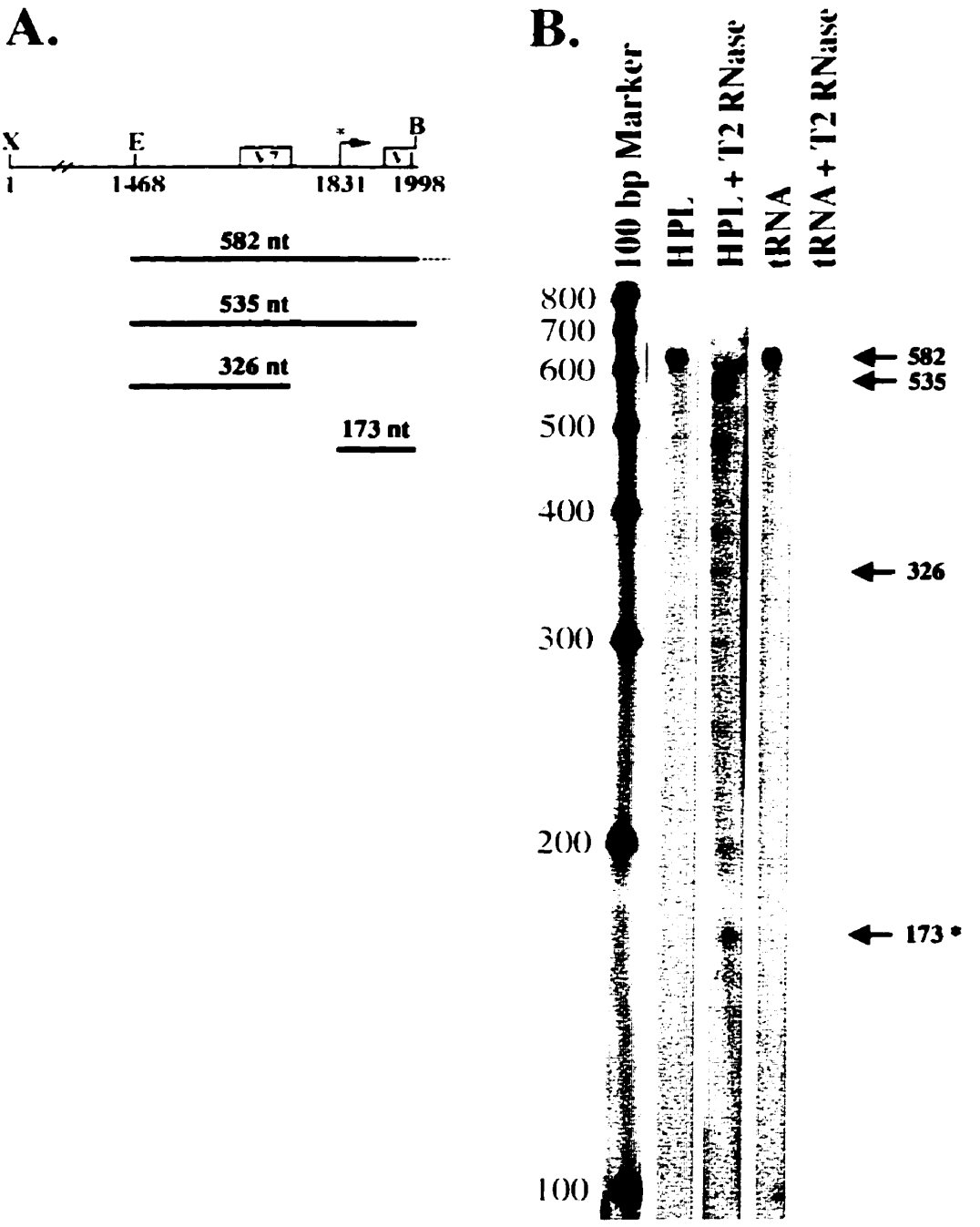
Probe 5A*

Probe 7A*

a) RT-PCR analysis: Using the same PCR strategy depicted in Figure 4.2B (primer combinations 2S/3A and 3S/6A), RT-PCR assays of total RNA from human postnatal liver revealed that there are hGHR mRNA transcripts extending upstream of the XbaI restriction site (Figure 4.2C and data not shown). Interestingly, using the 4S/8A primer set, a 2041 bp RT-PCR band hybridized with the 4A, 5A and 7A probes specific for V7, V1 and V4, respectively (Figure 4.2D). Moreover, this RT-PCR band co-migrated with the PCR product representing the genomic DNA region between primers 4S and 8A. The 600 bp RT-PCR bands seen in Figure 4.2D most likely represent an alternatively spliced mRNA transcript. Together, the RT-PCR data suggest that there is a hGHR mRNA transcript with a 5'UTR of more than 3.7 kb containing the V7, V1, V4 and V8 sequences.

b) RNase protection analysis: RNase protection assays were then employed to determine whether there are start sites of RNA transcription immediately upstream of the V1 sequence. Analysis of total RNA isolated from the two human postnatal liver samples showed that transcription of V1 mRNA species is driven by at least two distinct promoters (Figure 4.3 and data not shown). A protected fragment of 173 nucleotides (nt) identified a start site of transcription at position 1831 bp of the cloned genomic sequence (Figure 4.3); the relative density of this band, given its small size and migration distance, suggests a significant product. A 535 nt protected band indicated that there is at least one additional start site of transcription upstream of the EcoRI site (Figure 4.3). A third fragment of 326 nt was also observed (Figure 4.3). This product is the result of alternative splicing at the 3' end of V7 and its presence was anticipated based on the

Fig. 4.3. RNase protection analysis of human postnatal liver (HPL) total RNA. Panel A, map of genomic DNA flanking the 5' ends of the V7 (closed box) and V1 (open box) sequences previously obtained by 5'RACE (25). Positions of restriction sites (B, BamHI; E, EcoRI; X, XbaI) and the derived transcription start site (*) are given in bp. The 582 nt riboprobe was antisense RNA corresponding to the region between EcoRI and BamHI on the genomic DNA (531 nt, solid line) plus the polylinker vector sequence (51 nt, broken line). Major protected RNA fragments are also depicted (535, 326 and 173 nt; solid lines). Panel B, autoradiograph of a representative RNase protection experiment. The riboprobe was hybridized with HPL total RNA (donor age, 11 yr; lane 3) or yeast tRNA (negative control; lane 5) and digested with T2 RNase. An aliquot of the hybridization solution taken before T2 RNase treatment was run in parallel to show the integrity of the riboprobe (lanes 2, 4). A 100 bp DNA ladder (lane 1) and sequencing reaction (not shown) served as molecular weight markers. Sizes of RNA bands identified by arrows are given in nt. The 173 nt protected fragment (*) corresponds to the transcription start site indicated in panel A. Note that RNA fragments migrate approximately 5% slower than the double-stranded DNA marker fragments on a 5% acrylamide/7 M urea gel.


A.
X
E
B
1
1468
1831
1998
582 nt
535 nt
326 nt
173 nt
B.
100 bp Marker
HPL
HPL + T2 RNase
tRNA
tRNA + T2 RNase
800
700
600
500
400
300
200
100
582
535
326
173 •

sequence of the V7 cDNA cloned by Pekhletsky *et al* (25). The nature of the minor additional protected bands seen in Figure 4.3 remains to be elucidated.

A further RNase protection assay was designed to characterize mRNA species extending upstream of the EcoRI site (Figure 4.4). Analysis of total RNA from the two human postnatal liver specimens revealed a single protected fragment of 558 nt (Figure 4.4 and data not shown). This demonstrated that there is at least one start site of transcription upstream of the AvaI restriction site (Figure 4.4A).

c) Primer extension analysis: Primer extension methodology was used to validate the start site of transcription determined by the initial RNase protection assays (Figure 4.3). Primer 5A (Table 4.1 and Figure 4.5) was extended using total RNA isolated from human postnatal livers as template. A 155 nt primer extended product was obtained which corresponded to the transcription start site at position 1831 bp (Figures 4.5A and 4.5C). There is a TTTATTATA motif in the genomic sequence between -27 and -19 bp from the deduced start site (Figure 4.5A). Interestingly, this TATA motif shares high homology with TATA boxes found in the ovine and mouse *GHR* gene promoter regions (Figure 4.5B) (26,27). Additional primer extension bands of greater length (182, 203 and 219 nt) were also obtained (Figure 4.5C), which predicted transcription start sites that were not observed by the RNase protection assay results. These products are most likely due to the reverse transcriptase failing to extend completely on the longer mRNA species identified by the RNase protection analyses (Figures 4.3 and 4.4).

Fig. 4.4. RNase protection analysis of human postnatal liver (HPL) total RNA. Panel A, map of genomic DNA flanking the 5' end of the V7 (open box) sequences previously cloned by 5'RACE (25). Positions of restriction sites (A, AvaI; E, EcoRI; P, PvuII; X, XbaI) are given in bp. The 645 nt riboprobe was antisense RNA corresponding to the region between AvaI and PvuII on the genomic DNA (558 nt, solid line) and polylinker vector sequence (87 nt, broken lines). The protected RNA fragment is also depicted (solid line). Panel B, autoradiograph of a representative RNase protection experiment. The riboprobe was hybridized with HPL total RNA (donor age, 11 yr; lane 3) or yeast tRNA (negative control; lane 5) and digested with T2 RNase. An aliquot of the hybridization solution taken before T2 RNase treatment was run in parallel to show the integrity of the riboprobe (lanes 2, 4). A 100 bp DNA ladder (lane 1) and sequencing reaction (not shown) served as molecular weight markers. Sizes of RNA bands identified by arrows are given in nt.

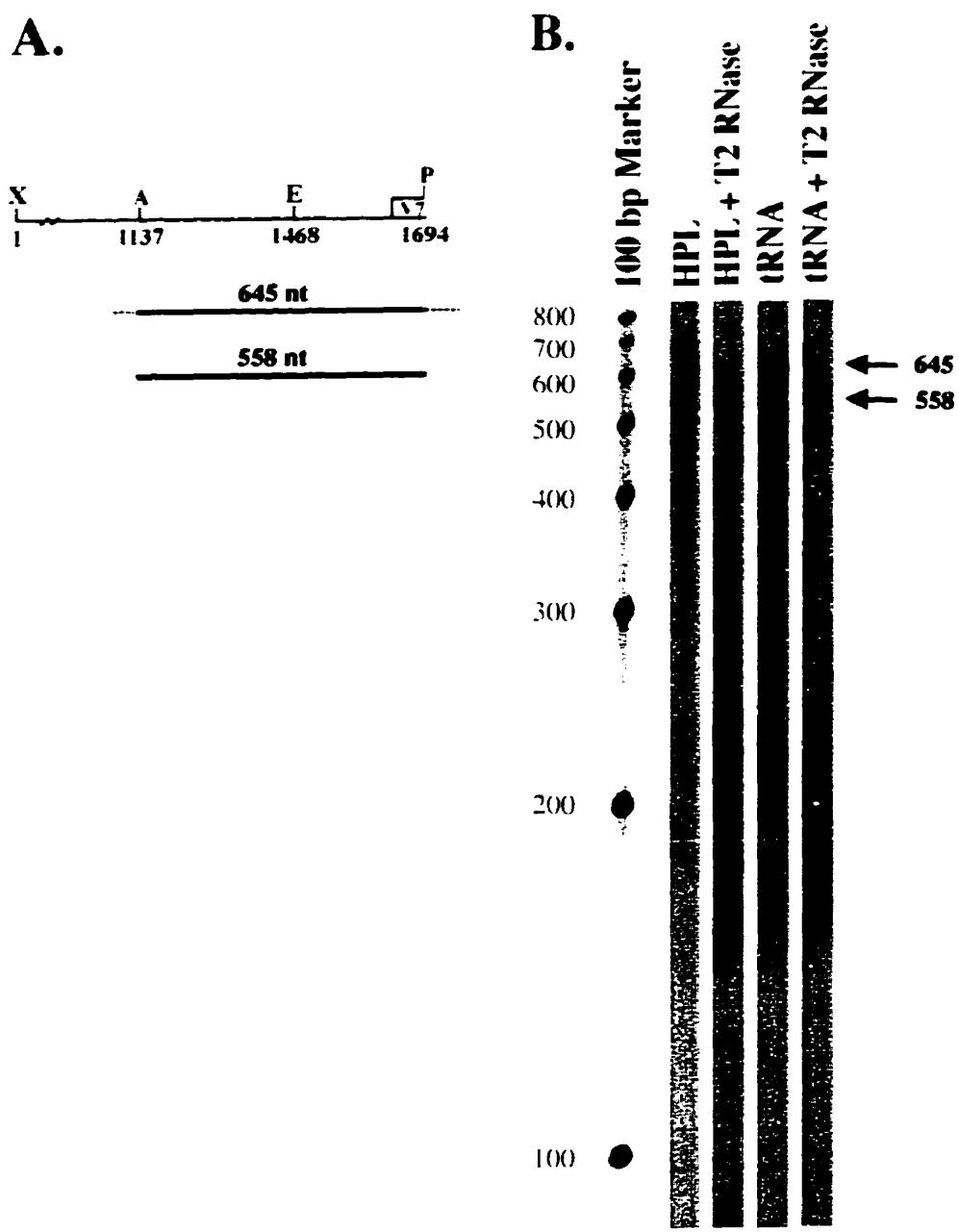

A.
X
A
E
P
1
1137
1468
1694
645 nt
558 nt
B.
100 bp Marker
HPI,
HPI, + T2 RNase
tRNA
tRNA + T2 RNase
800
700
600
500
400
300
200
100
645
558

Table 4.1. Sequences of oligonucleotide primers and internal probes.

Oligonucleotide	Sequence
1S	5' GAG TAG CAA AGA TGG ATT AAG TGA G 3'
2S	5' TCC ATG TGT TCA GTG GTC CAG C 3'
3S	5' GGA GAT CTA TTT CCC TCT ACT AGA 3'
4S	5' GTA ATA GGC CTC ATG AGA CTC CA 3'
1A	5' GAA CCT CAT CTG TCC AGT GGC AT 3'
2A	5' AGG TAT CCA GAT GGA GGT AAA CG 3'
3A	5' CCT TCA AAG TCC TAA TCA ACT AAG T 3'
4A	5' ACA TCT CTG GGC TCT GCC TGC 3'
5A	5' TTG GTT ACC ACT TGG CAT GTA TTA ATC TCT 3'
6A	5' GTT TCC TCC AGG CTT TAT ATC AAG AAC TTT 3'
7A	5' CTC ACT TAA TCC ATC TTT CCT ACT C 3'
8A	5' GCT CAT ACG TGC TGT CAT AGC TA 3'

Fig. 4.5. Primer extension analysis of human postnatal liver (HPL) total RNA. Panel A, nucleotide sequence of the *hGHR* gene promoter with the position of primer 5A (←), the predicted transcriptional start site (*), the upstream TATA motif (boxed), V7 and V1 (underlined). Panel B, alignment of corresponding TATA box consensus sequences of the human, ovine (26) and murine (27) *GHR* genes. Panel C, autoradiograph of a representative primer extension experiment. Primer 5A was annealed to HPL total RNA (donor age, 62 yr; lane 5) or yeast tRNA (control; lane 6) and extended. The 155 nt (*) extended product predicted the transcription initiation site indicated in panel A. The extension products were sized using a sequencing reaction (lanes 1-4) run in parallel.

A.

CAGGCAGAGGAGATGTACTGGGCAAAGGTCA

V7

GGGGA TTTATTATA TGAGACAATGGTCTGAT

*

GTGATTTAATTTACTCCTCTGGTACCAGATA

V1

TGGTGTGTGTGCATGAGAGAGAGAGATTGAG

AATGACTGATTTGGGAGGGATTTTGTGAAGG

TTTATATATCAAAGCAGAAAGACCAAGAATT

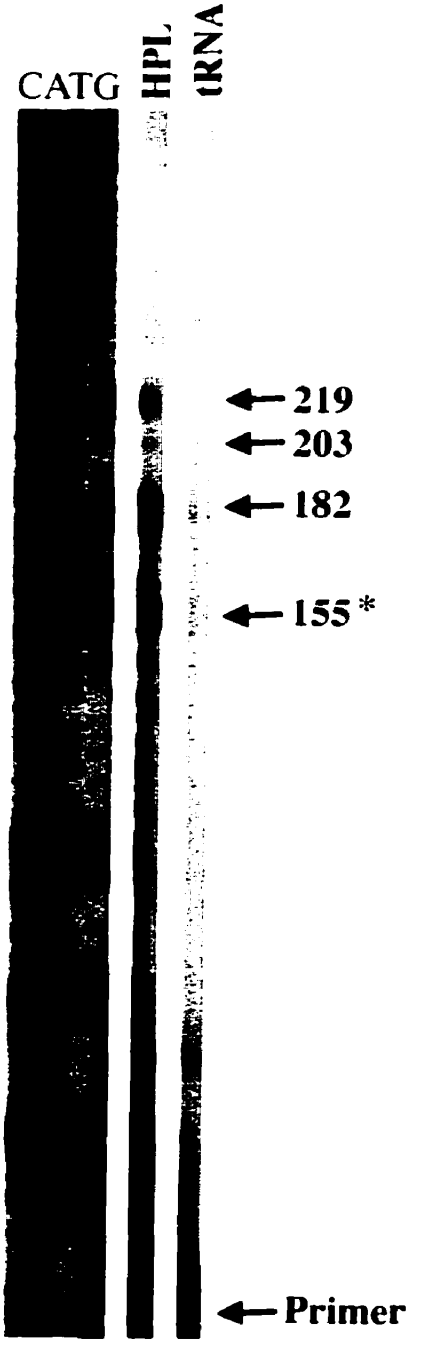
5A Primer

TAGAGATTAATACATGCCAAGTGGTAACCAA

B.

TTTATTATA	**Human**
TTTAT ATA	**Ovine**
TTTAT ATA	**Murine**
ATTATTAAT	**Murine**

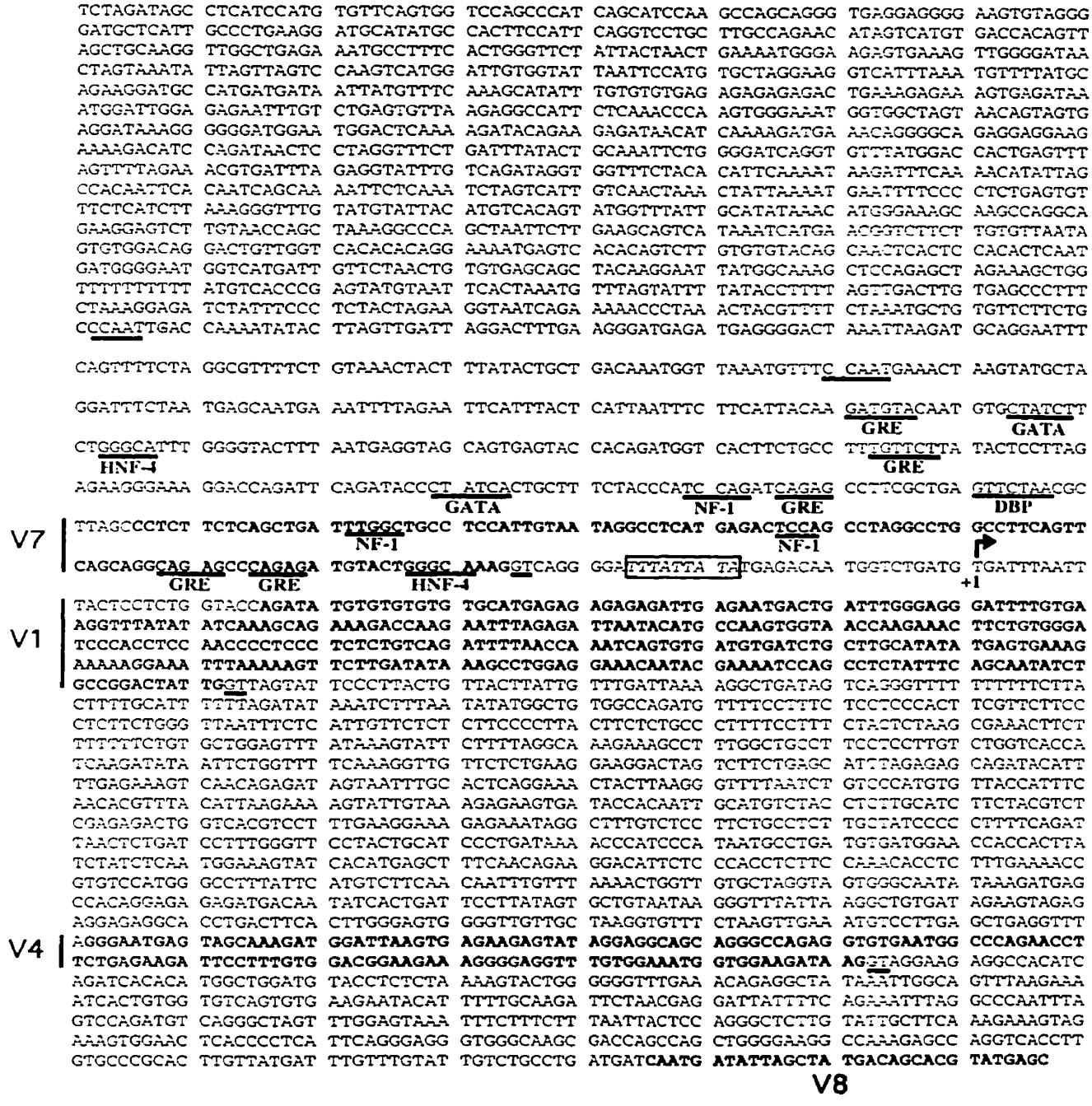
C.



4.4.4. Nucleotide sequence analysis.

The nucleotide sequence of the 3.8 kb XbaI-BsaAI *hGHR* genomic fragment is shown in Figure 4.6. In addition to a conserved TATA motif proximal to the determined transcription initiation site, computer analysis using the Signal Scan program (32) and Transfac database (33) revealed several putative mammalian transactivator recognition sites (DBP [D-element binding protein], CCAAT, GATA, GRE [glucocorticoid response element], HNF-4 [hepatocyte nuclear factor 4] and NF-1 [nuclear factor 1]) in the region immediately upstream of the TATA box. Dinucleotide GT splice donor sites were identified at the 3' ends of the V7, V1 and V4 genomic sequences, consistent with the splicing out of intronic sequences at these positions.

Fig. 4.6. Nucleotide sequence of the 3757 bp genomic DNA fragment encompassing the V7, V1, V4 and V8 sequences (in bold). Splice donor dinucleotide GT sites at the 3' end of the V7, V1 and V4 genomic sequences are underlined. The transcription start site (*) we determined and the proximal upstream TATA motif (boxed) are indicated. Putative transactivator response elements (DBP, CCAAT, GATA, GRE, HNF-4 and NF-1) in the region immediately upstream of the transcription initiation site are underlined.


HNF-4
GATA
NF-1
GRE
DBP
V7
V1
V4
V8
+1

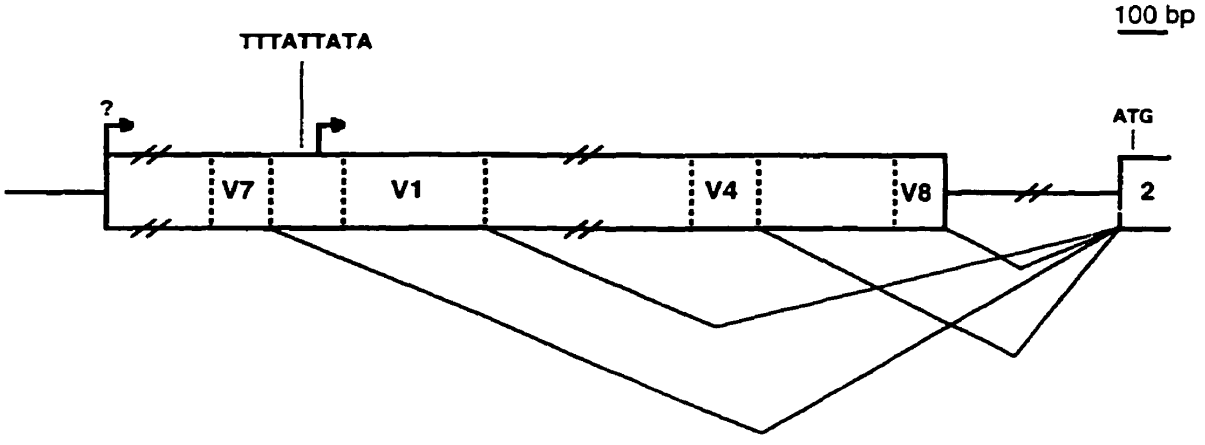
4.5. Discussion.

In the present study, we demonstrate that V4 is not expressed in fetal tissues and hepatic tumours, and is only detectable in normal postnatal liver, similar to the pattern of expression of V1 (30). To delineate the mechanism responsible for this parallel expression of V1 and V4 hGHR mRNAs, we characterized a 3.8 kb XbaI-BsaAI genomic fragment which represents part of the 5' flanking region of the *hGHR* gene and confirms the integrity of the recombinant *hGHR* genomic clone recently isolated by Zou *et al* (31). The *hGHR* gene regulatory regions were further characterized by precisely mapping the V7, V1, V4 and V8 sequences in series on the gene and identifying a transcriptional start site upstream of V1. Transcription initiation at this site leads to a gene product that includes the V1, V4 and V8 sequences. The present data also demonstrate that there are additional mRNA species extending upstream of V1 to include V7 and the 5' flanking region to V7. Differential splicing of these two transcripts leads to the V1, V4, V7 and V8 isoforms previously identified by 5'RACE (25) and RT-PCR (30), as well as to the novel mRNA transcript observed in this study using RT-PCR.

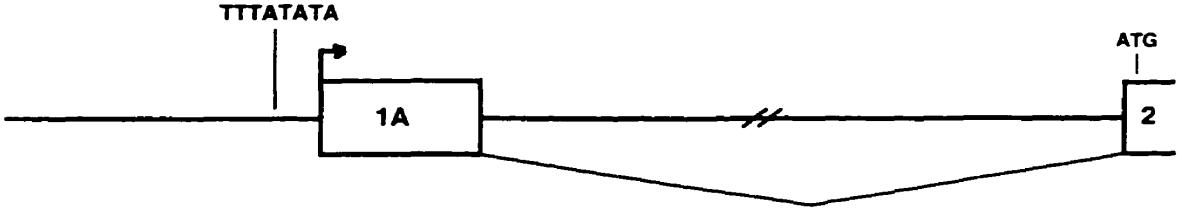
The expression profile of V1, together with the presence of a transcription initiation site in the region immediately 5' to V1 (Figure 4.7), suggests that RNA synthesis at this position is driven by a promoter that is downregulated in fetal tissues and hepatic tumours and activated in normal postnatal liver (20,34). The contributions of differential promoter usage (35), relative to alternative RNA splicing (36), in modulating the ontogeny and tissue distribution of V1 and V4 versus V3 will be more evident once the upstream transcription start site(s) and the V3 sequence have been mapped on the

Fig. 4.7. Comparison of human, ovine and murine *GHR* gene promoter regions. Exons are boxed and characterized splicing patterns are indicated (v-shaped lower lines) (25-27). A splice acceptor dinucleotide AG site is located in the genes of the three species at -12 bp from the start site of translation (ATG). The V7, V1, V4 and V8 sequences that have been previously cloned by 5'RACE (25) are mapped (broken lines) within the human 5'UTR. Defined (arrows) and potential (arrows with ?) transcriptional start sites as well as conserved TATA motifs are shown.

Human GHR



Ovine GHR



Murine GHR

hGHR gene. Tissue- and developmental-specific alterations in mRNA stability (37) may also play a role in modulating the cellular levels of specific hGHR mRNA isoforms. In addition, the 5'UTRs of many eukaryotic mRNAs are involved in modulating translation efficiency (29); it will be important to determine how the V1 and V4 isoforms, that are tightly regulated, influence translation in comparison to the widely expressed V3 mRNA.

Similar promoter regions in the human, ovine, and rodent *GHR* genes may be directing tissue and developmental regulation of mRNA expression (Figure 4.7). Studies in the ovine suggest that a hepatic-specific promoter drives synthesis of a 5'UTR variant (exon 1A) that is 76% identical to the human V1 (26): the sheep exon 1A mRNA isoform, like V1 (30) and V4 (present data), is detected only in postnatal liver (26). In the mouse and rat, expression of a GHR mRNA isoform (L1 and GHR1, respectively; both sharing 50% nucleotide sequence similarity with V7) is also modulated by a liver-specific promoter and detectable only in postnatal liver (27,28). Our present findings, together with these previous observations, reveal that the organizational architecture of the *hGHR* gene is a composite of the ovine and rodent counterparts. Therefore, multiple *hGHR* gene promoters appear to be sharing the responsibility of assuring postnatal liver-specific expression of hGHR mRNA, as opposed to the single promoter in sheep and rodents.

A TTTATTATA motif is appropriately located between -27 and -19 bp of the defined start site for V1 hGHR mRNA (Figures 4.6 and 4.7). Interestingly, this sequence is homologous to proximal TATA boxes in the ovine (26) and murine (27) liver-specific *GHR* gene promoters (Figures 4.5 and 4.7), suggesting that the conserved TATA box

motif may be an important cis element in regulating liver-enhanced *GHR* gene transcription. Likewise, putative binding sites for DBP (38) and HNF-4 (38) lie immediately upstream of the V1 transcription start site; this is analogous to the presence of responsive elements for liver-enriched transcription factors in the minimal promoters modulating synthesis of the sheep exon 1A (26) and mouse L1 (27) transcripts. It is noteworthy that the DBP transcription factor is absent in rat fetal liver and is expressed only postnatally (38). If expression of DBP is similar in the human, it may play a crucial role in hepatic-specific postnatal activation of V1 and V4 mRNA synthesis.

Comparison of the human TTTATTATA sequence with the ovine and mouse TTTATATA motifs reveals one potentially important difference. The human sequence contains an extra thymidine nucleotide which distinguishes it from the most frequently found eukaryotic consensus binding site (TATATA) for transcription factor IID (39). This discrepancy may render transcription initiation at this position weaker than the corresponding sites in the ovine and murine *GHR* genes, thereby creating a need for additional sites to drive expression of liver-specific *hGHR* gene transcripts. This hypothesis is supported by the presence of additional mRNA isoforms extending upstream of the transcriptional start site we identified, as well as the presence of a potential TTTATATA motif within the V1 sequence in the *hGHR* gene that may be regulating gene expression. Studies are currently underway to identify upstream promoter regions and to determine whether the TATA box within V1 is active. Interestingly, a similar genetic architecture is present in the mouse and may be responsible for the two mouse L1 isoforms that result from transcription initiation at different sites. The upstream

ATTATTAAT consensus (27), which is believed to mediate synthesis of the longer L1 transcript, does not appear to be a well conserved TATA box compared to the downstream TTTATATA motif.

The presence of V1, V4, V7 and V8 sequences in the same gene transcript suggests that V7 and V8, like V1 and V4, are under tissue-, developmental- and tumor-specific regulation. Additional transcription initiation sites downstream of V1 may be mediating synthesis of transcripts that include the V4 and/or V8 sequences. However, if regulated expression of V1 and V4 is the result of transcriptional modulation, all promoter regions mediating synthesis of the V1 and V4 mRNA isoforms should be under parallel control. Thus, activation of gene promoter regions driving synthesis of V1-, V4-, V7- and V8-containing transcripts in postnatal hepatocytes may account for the 6-fold increase in total hGHR mRNA isoforms we have observed in postnatal relative to fetal liver (20).

In summary, portions of the *hGHR* gene promoter regions have been cloned and characterized, and the V7, V1, V4 and V8 sequences have been precisely mapped in series on a 3.8 kb XbaI-BsaAI genomic fragment. A transcriptional start site upstream of V1 has been identified and evidence is provided for additional mRNA isoforms extending upstream of V1 to include V7 and the 5' flanking region to V7. These data suggest that the heterogeneity of the 5'UTR of hGHR mRNAs is regulated by differential promoter usage and splicing, and that the tissue-, developmental- and tumour-specific V1 and V4 mRNA expression profiles are due, at least in part, to transcriptional regulation.

4.6. Materials and Methods.

4.6.1. Tissues.

Human fetal tissues were obtained following therapeutic abortion (n=9; 10-16.5 wk FA). Fetal age was determined by foot length (40). Postnatal specimens were collected at the time of surgery (n=20; 6 months - 84 yr) or within 4-10 h following removal of the liver organs for transplant into pediatric patients (n=5; 11-62 years). Specimens were flash-frozen in dry-ice acetone and stored at -70°C until subsequent total RNA isolation using the guanidine thiocyanate/CsCl gradient method (41) and treatment with deoxyribonuclease I (DNAse I; Pharmacia Biotech, Baie D'Urfé, Québec, Canada). Normal human genomic leukocyte DNA samples (n=6) were prepared using the proteinase K-SDS method (42). Protocols for obtaining human tissues and blood samples were approved by local ethics committees and informed consent obtained.

4.6.2. Screening of a human genomic λ DASH library.

One million independent clones of a human lymphocyte genomic λ DASH library (Stratagene, La Jolla, CA) were screened with the 5A [^{32}P]-endlabelled V1-specific oligonucleotide probe (Table 4.1), using methods previously reported (43). Membranes received a final wash at 55°C with 1% (w/v) SDS, 40 mM sodium phosphate (pH 6.8) and 1 mM EDTA. After autoradiography, positive clones were subjected to two more rounds of plaque purification and phage DNA was isolated using the plate lysate method (44).

4.6.3. Restriction Enzyme Mapping Analysis.

Recombinant phage DNA was digested with combinations of SalI, XbaI, HindIII, EcoRI and BamHI. Southern blots were probed with the [^{32}P]-endlabelled 5A oligonucleotide (Table 4.1) under the same conditions used for genomic library screening. A 2.0 kb XbaI/BamHI V1-containing λ phage DNA fragment was subcloned into the SK$^-$ Bluescript vector (Stratagene) and sequenced using an ALF automated system (Pharmacia Biotech) located at the Sheldon Biotechnology Centre of McGill University. Genomic DNA was digested with the same restriction enzyme combinations used for the recombinant phage clones. The EcoRI/BamHI recombinant phage DNA fragment (Probe 1, Figure 2A) was random primer labelled with [^{32}P]-dCTP and used to probe the genomic blots.

4.6.4. PCR cloning of the *hGHR* gene from genomic DNA.

Five hundred ng of human genomic DNA were amplified using 0.3 μM of overlapping *hGHR* primer combinations (Table 4.1 and Figure 4.2B), 0.5 mM deoxyribonucleotides (dNTPs; Pharmacia Biotech), 22.5 mM $MgCl_2$, 2.5 U Taq/PWO polymerase mix (Boehringer Mannheim, Laval, Québec, Canada) and 1 x Expand Long Template PCR System Buffer #3 (Boehringer Mannheim). The reactions were heated at 92°C for 2 min, cycled 30 times for 10 sec at 92°C, 30 sec at 61°C and 2 min at 68°C, and terminated with a final elongation of 5 min at 68°C. The 4S-8A (2041 bp) amplified fragments were inserted into the PGEM-T TA cloning vector (Promega, Madison, WI) and sequenced.

4.6.5. RT-PCR and PCR analyses.

Five μg of total RNA were heated at 70°C for 5 min and then reverse transcribed for 1 h at 48°C with 2.5 U Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT; Life Technologies), 80 U RNAsin (Promega), 0.6 μM of the appropriate antisense *hGHR* primer (Table 4.1 and Figure 4.2B), 0.48 mM dNTPs, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 100 mM Tris-HCl (pH 8.3) and 50 mM KCl. The AMV-RT was denatured by heating the RT product for 5 min at 98°C. A parallel RT reaction was run in the absence of AMV-RT to control for genomic DNA contamination. 6 μl of RT product or 500 ng of DNA (human genomic or λ phage) were amplified for 25 cycles in 2.5 U Taq DNA polymerase (Life Technologies), 0.5 mM dNTPs, 0.25 μM *hGHR* sense and antisense primers (Table 4.1 and Figure 4.2B), 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 7% DMSO. The first cycle consisted of 3 min at 92°C, 1 min at 61°C and 3 min at 72°C. Subsequent cycles were 30 sec at 92°C, 1 min at 61°C and 1.5 min at 72°C. The reaction was terminated with a final elongation of 5 min at 72°C. The internal V4 standard was generated using the PCR method of Jin *et al* (45).

4.6.7. Southern blotting analysis of PCR products.

PCR products were resolved on 1-2% agarose gels and transferred to 0.45 μm positively charged nylon membranes (Schleicher & Schuell, Keene, NH). Blots were prehybridized for 3 h at 42°C in 6xSSPE, 1% (w/v) SDS, 10 x Denhardt's and 0.15 mg/ml denatured salmon sperm DNA (Sigma Chemicals, St. Louis, MO). Relative volumes of the prehybridization and hybridization solutions were 100 μl per cm^2 of

membrane. Hybridization was carried out overnight at a temperature (T_H) 5°C lower than the melting temperature of the hybrid formed between the probe and its complementary nucleic acid sequence. The hybridisation solution contained 6xSSPE, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA and 0.4 nCi/ml of endlabelled probe (Table 4.1) per cm^2 of nylon membrane. Blots were washed twice for 10 min at T_H with 6xSSPE/1%SDS and once for 10 min at T_H minus 5°C with 2xSSPE/1%(w/v) SDS. Bands were visualized by autoradiography following 3-24 h exposure to Kodak XAR-5 film using two intensifying screens.

4.6.8. Ribonuclease (RNase) protection analysis.

Riboprobes (Figures 4.3 and 4.4) were synthesized by *in vitro* transcription of 1 μg of the appropriate linearized plasmid for 1 h at 37°C with 30 U T3 polymerase (Life Technologies), 50 μCi α-^{32}P-UTP (ICN Pharmaceuticals Canada Ltd., Montreal, QC), 50 nM GTP (Promega), 1.5 mM ATP/CTP/GTP mix (Promega), 5 nM DTT, 20 U RNAsin, 40 mM Tris (pH 8), 10 mM $MgCl_2$, 1 mM spermidine-HCl and 25 mM NaCl. The DNA template was digested for 15 min at 37°C with 10 U of DNase I and the riboprobes purified by ethanol precipitation. For each RNase protection reaction, 0.7 μCi of purified riboprobe were ethanol precipitated with 200 μg of human postnatal liver total RNA or yeast tRNA (Life Technologies), and then resuspended in 50 μl of hybridization buffer (80% formamide, 8 mM PIPES (pH 6.4), 80 mM NaCl and 200 mM EDTA (pH 8)). The hybridization solution was heated for 10 min at 85°C and then incubated for 18 h at 45°C. Following hybridization, one-eighth of the reaction was ethanol precipitated.

The remaining solution was digested for 1 h at 37°C with 60 U/ml of T2 RNase (Life Technologies), 60 mM sodium acetate (pH 4.4), 120 mM NaCl and 12 mM EDTA (pH 8). Double-stranded RNA was precipitated at room temperature with isopropanol. Both T2 RNase-treated and -untreated samples were resuspended in water, heated for 5 min at 95°C and separated on a 5% acrylamide/7 M urea gel. The T2 RNase-untreated sample was run in parallel to check the integrity of the riboprobe. The dried gel was autoradiographed for 2 weeks at -70°C with two intensifying screens.

4.6.9. Primer extension analysis.

Ten picomoles of primer (Table 4.1 and Figure 4.5A) were endlabelled for 1 h at 37°C with [γ-^{32}]-ATP (ICN Pharmaceuticals Canada) using T_4 polynucleotide kinase (Life Technologies). Fifty μg of human postnatal liver total RNA or yeast tRNA were heated for 10 min at 70°C before allowing 1 pmol (1.5 μCi) of endlabelled primer to anneal at 55°C for 90 min in 150 mM KCl, 10 mM Tris (pH 8.3) and 1 mM EDTA. Subsequently, 30 μl of reaction buffer (30 mM Tris pH 8.3, 15 mM $MgCl_2$, 8 mM DTT, 225 μg/ml actinomycin D (Sigma Chemical Co.), 220 μM dNTPs and 2.5 U AMV-RT) were added to the 15 μl hybridization solution and the RNA was reverse transcribed for 1 h at 48°C. The AMV-RT was inactivated by incubating the reaction for 5 min at 98°C and the RNA was digested with 20 μg/ml RNase A (Life Technologies) for 15 min at 37°C. The primer extension products were purified by phenol:chloroform (24:1) extraction and ethanol precipitation, resuspended in water, heated for 5 min at 95°C and resolved on a 5% acrylamide/7 M urea gel. The dried gel was autoradiographed for 24

h at -70°C using two intensifying screens.

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4.8. References.

1. Rosenbloom AL, Rosenfeld RG and Guevara-Aguirre J 1997 Growth hormone insensitivity. Pediatr Clin North Amer 44: 423-442.

2. Gluckman PD and Harding JE 1997 The physiology and pathophysiology of intrauterine growth retardation. Horm Res 48 (suppl 1): 11-16.

3. Hay WW Jr, Catz CS, Grave GD and Yaffe SJ 1997 Fetal growth: its regulation and disorders. Pediatr 99: 585-591.

4. Breier BH, Ambler GR, Sauerwein H, Surus A and Gluckman PD 1994 The induction of hepatic somatotropic receptors after birth in sheep is dependent on parturition-associated mechanisms. J Endocrinol 141: 101-108.

5. Walker JL, Moats-Staats BM, Stiles AD and Underwood LE 1992 Tissue-specific developmental regulation of the mRNAs encoding the growth hormone (GH) receptor and the GH binding protein in rat fetal and postnatal tissues. Pediatr Res 31: 335- 339.

6. Ymer S and Herington AC 1992 Developmental expression of the growth hormone receptor gene in rabbit tissues. Mol Cell Endocrinol 83: 39-49.

7. Pantaleon M, Whiteside EJ, Harvey MB, Barnard RT, Waters MJ and Kaye PL 1997

Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis? Proc Natl Acad Sci USA 94: 5125-5130.

8. Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigamo K, Wagner TE, Baumann G and Kopchick JJ 1997 A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). Proc Natl Acad Sci USA 94: 13215-13220.

9. Zogopoulos G, Figueiredo RMO, Jenab A, Ali Z, Lefèbvre Y and Goodyer CG 1996 Expression of exon three retaining and deleted human growth hormone receptor mRNA isoforms during development. J Clin Endocrinol Metab 81: 775-782.

10. Simard M, Manthos H, Giaid A, Lefèbvre Y and Goodyer CG 1996 Ontogeny of growth hormone receptors in human tissues: an immunohistochemical study. J Clin Endocrinol Metab 81: 3097-3102.

11. Hill DJ, Riley SC, Bassett NS and Waters MJ 1992 Localisation of the growth hormone receptor, identified by immunocytochemistry, in second trimester human fetal tissues and in placenta throughout gestation. J Clin Endocrinol Metab 75: 646-650.

12. Werther GA, Haynes KM and Waters MJ 1993 Growth hormone (GH) receptors are expressed on human fetal mesenchymal tissues - identification of messenger ribonucleic acid and GH-binding protein. J Clin Endocrinol Metab 76: 1638-1646.

13. Hill DJ, Freemark M, Strain AJ, Handwerger S and Milner RDG 1988 Placental lactogen and growth hormone receptors in human fetal tissues: relationship to fetal plasma human placental lactogen concentrations and fetal growth. J Clin Endocrinol Metab 66: 1283-1290.

14. Figueiredo RMO and Goodyer CG Characterisation of the growth hormone receptor in human dermal fibroblasts and hepatocytes during development. Program of the 75th Annual Meeting of the Endocrine Society, Las Vegas, Nevada, 1993, p 167 (Abstract).

15. Strain AJ, Hill DJ, Swenne I and Milner RDG 1987 Regulation of DNA synthesis in human fetal hepatocytes by placental lactogen, growth hormone and insulin-like growth factor I/somatomedin C. J Cell Physiol 132: 33-40.

16. D'Souza Li L, Krackovitch S and Goodyer CG Actions of growth hormone (GH) and the GH receptor in human fetal hepatocyte cultures. Program of the 79th Annual Meeting of the Endocrine Society, Minneapolis, MN, 1997, p 339 (Abstract).

17. Otonkoski T, Knip M, Wong I and Simell O 1988 Effects of growth hormone and

insulin-like growth factor I on endocrine function of human fetal islet-like cell clusters during longterm tissue culture. Diabetes 37: 1678-1683.

18. Swenne I, Hill DJ, Strain AJ and Milner RDG 1987 Effects of human placental lactogen and growth hormone on the production of insulin and somatomedin C/insulin like growth factor I by human fetal pancreas in tissue culture. J Endocrinol 113: 297-303.

19. Formby B, Ullrich A, Coussens L, Walker L and Peterson CM 1988 Growth hormone stimulates insulin gene expression in cultured human fetal pancreatic islets. J Clin Endocrinol Metab 66: 1075-1079.

20. Zogopoulos G, Lerner S, Albrecht S, Pietsch T, Alpert L, von Schweinitz D, Giussani D, Nathanielsz P and Goodyer CG Regulation of growth hormone receptor (GHR) mRNA in primates. Program of the 79th Annual Meeting of the Endocrine Society, Minneapolis, MN, 1997, p 338 (Abstract).

21. Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ and Wood WI 1987 Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330: 537-543.

22. Barton DE, Foellmer BE, Wood WI and Francke U 1989 Chromosome mapping of the growth hormone receptor gene in man and mouse. Cytogenet Cell Genet 50: 137-141.

23. Urbanek M, Macleod JN, Cooke NE and Liebhaber SA 1992 Expression of a human growth hormone (hGH) receptor isoform predicted by tissue-specific alternative splicing of exon 3 of the hGH receptor gene transcript. Mol Endocrinol 6: 279-287.

24. Wickelgren RB, Landin KLL, Ohlsson C and Carlsson LMS 1995 Expression of exon 3-retaining and exon 3-excluding isoforms of the human growth hormone (GH) receptor is regulated in an individual- rather than a tissue-specific manner. J Clin Endocrinol Metab 80: 2154-2157.

25. Pekhletsky RI, Chernov BK and Rubtsov PM 1992 Variants of the 5'-untranslated sequence of human growth hormone receptor mRNA. Mol Cell Endocrinol 90: 103-109.

26. O'Mahoney JV, Brandon MR and Adams TE 1994 Identification of a liver-specific promoter for the ovine growth hormone receptor. Mol Cell Endocrinol 101: 129-139.

27. Menon RK, Stephan DA, Singh M, Morris SM and Zou L 1995 Cloning of the promoter-regulatory region of the murine growth hormone receptor. J Biol Chem 270: 8851-8859.

28. Baumbach WR and Bingham B 1995 One class of growth hormone (GH) receptor and binding protein mRNA in rat liver, GHR1, is sexually dimorphic and regulated by GH. Endocrinology 136: 749-760.

29. Jansen M, De Moor CH, Sussenbach JS and van den Brande JL 1995 Translational control of gene expression. Pediatr Res 37: 681-686.

30. Zogopoulos G, Albrecht S, Pietsch T, Alpert L, von Schweinitz D, Lefèbvre Y and Goodyer CG 1996 Fetal- and tumor-specific regulation of growth hormone receptor mRNA expression in human liver. Cancer Res 56: 2949-2953.

31. Zou L, Burmeister LA and Sperling M 1997 Isolation of a liver-specific promoter for the human growth hormone receptor gene. Endocrinology 138: 1771-1774.

32. Prestridge DS 1991 SIGNAL SCAN: A computer program that scans DNA sequences for eukaryotic transcriptional elements. CANBIOS 7: 203-206.

33. Wingender E, Kel AE, Kel OV, Karas H, Heinemeyer T, Dietze P, Knuppel R, Romaschenko AG and Kolchanov NA 1997 TRANSFAC, TRRD and COMPEL: towards a federated database system on transcriptional regulation. Nucleic Acids Res 25: 265-268.

34. Su TS, Liu WY, Han SH, Jansen M, Yang-Fen TL, Peng FK and Chou CK 1989 Transcripts of the insulin-like growth factors I and II in human hepatoma. Cancer Res 49: 1773-1777.

35. Ayoubi TAY and van de Ven WJM 1996 Regulation of gene expression by alternative promoters. FASEB J 10: 453-460.

36. Rio DC 1993 Splicing of pre-mRNA: mechanism, regulation and role in development. Curr Opin Gen Dev 3: 574-584.

37. Ross J 1995 mRNA stability in mammalian cells. Microbiol Rev 59: 423-450.

38. Zaret KS 1994 The Liver: Biology and Pathobiology, ed. 3, Raven Press Ltd, New York, NY, p 53-68.

39. Bucher P and Trifonov EN 1986 Compilation and analysis of eukaryotic PolII promoter sequences. Nucleic Acids Res 14: 10009-10026.

40. Munsick RA 1984 Human fetal extremity lengths in the inteval from 9 to 21 menstrual weeks of pregnancy. Am J Obstet Gynecol 149: 883-887.

41. Chirgwin JJ, Przbyla AE, MacDonald RJ and Rutter WJ 1979 Isolation of

biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299.

42. Gross-Bellard M, Oudet P and Chambon P 1973 Isolation of high molecular weight DNA from mammalian cells. Eur J Biochem 36: 32-38.

43. Mouland AJ, Bevan S, White JH and Hendy GN 1994 Human chromogranin A gene: molecular cloning, structural analysis and neuroendocrine cell-specific expression. J Biol Chem 269: 6918-6926.

44. Sambrook J, Fritsch EF and Maniatis T 1989 Molecular Cloning: A Laboratory Manual, ed. 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 2.118-2.120.

45. Jin CF, Mata M and Fink DJ 1994 Rapid construction of deleted DNA fragments for use as internal standards in competitive PCR. PCR Methods Appl 3: 252-255.

APPENDIX 2

APPENDIX 2 - ADDITIONAL RESULTS FROM THE *CLONING OF 5' REGULATORY REGIONS OF THE HUMAN GROWTH HORMONE RECEPTOR GENE*

APP2.1. Summary.

Previous results have demonstrated that the V1 and V4 hGHR mRNA isoforms follow a similar expression profile that is distinct from that of V3 transcripts. Based on these observations, we hypothesized that transcription of V1 and V4 mRNAs is regulated 3by a common promoter, while V3 mRNA synthesis is driven by a different gene regulatory region. In fact, Chapter 4 provides evidence to suggest that V1 and V4 transcripts are derived by alternative splicing of a common gene transcript. To further investigate our hypothesis, we mapped the V3 sequence to recombinant clones containing portions of human genomic DNA. One million independent clones of a human lymphocyte genomic λ DASH library (Stratagene, La Jolla, CA) were screened, according to the procedures listed in Chapter 4, using a V3-specific antisense oligonucleotide (5' ACC TTT CTC TGT CCC TTC CAA GGT CTC CT 3'). Three overlapping recombinant clones, with insert sizes ranging from 16 to 20 kb, were identified (data not shown). Southern blot analysis revealed that these recombinant clones did not contain overlapping sequences with the V1-encompassing 3.8 kb genomic fragment that we recently characterized (Chapter 4 and data not shown). Thus, using this approach we were not able to construct a continuous physical map of the 5' regions of the *hGHR* gene. However, a 4.3 kb HindIII-XbaI restriction fragment of the λ/V3.03

clone that hybridized with the V3-specific oligonucleotide (data not shown) was subcloned and analysed.

Nucleotide sequencing permitted the precise mapping of the V2 and V3 sequences to the 4.3 kb HindIII-XbaI genomic fragment (Figures A2.1 and A2.2). In addition to the V2 cDNA that was cloned by 5'RACE (252), a transcript that includes the V2 sequence but that extends further upstream has also been isolated from a placental cDNA library (247). The overlapping regions of the V2 and placental 5'UTR variants are indicated in figures A2.1 and A2.2.

Previously, Pekhletsky et al identified three V3 subvariants (V3a, V3b and V3c) in adult liver by 5'RACE (252), which are thought to arise from alternative splicing of a single gene transcript. Subvariants V3a and V3b differ in their 5'ends, whereas V3c contains an internal deletion. In the present study, although the common 5' end of the V3 subvariants was successfully mapped to the 4.3 kb HindIII-XbaI genomic fragment, the corresponding DNA regions for V3b-specific sequence were not present within the clone (data not shown). Southern blot analysis, using a V3b-specific sense oligonucleotide probe (5' CAG ACG CAG GAG AAT TGC TAA CTA GT 3'), revealed that none of the three overlapping recombinant clones - isolated using the common probe for all V3 variants - contained the unique V3b sequence (data not shown). In addition, we were unable to amplify V3b transcripts from liver RNA by RT-PCR using the V3b-specific sense primer and an antisense primer within exon 2 (5' GAA CCT CAT CTG TCC AGT GGC AT 3'). Together, these data suggest that the V3b subvariant reported by Pekhletsky et al may have been a cloning artifact. It should be noted that, since the initial

Fig. A2.1. Physical map of human DNA encoding the V3-containing portion of the *hGHR* gene. Restriction sites for HindIII (H) and XbaI (X) are shown. The broken horizontal line refers to the downstream *hGHR* genomic structure that has not yet been cloned and, therefore, the precise mapping of the downstream V3 exon is not yet possible. The sequence at 2271-2318 bp is common to both the V3a and V3c variants, while the V3a-specific sequence lies downstream. Boxes indicate the positions of the placental 5'UTR, V2 and V3 cDNA sequences within the genomic DNA. The portion of the placental 5'UTR extending upstream of V2 is shaded. Characterized splicing patterns (v-shaped lower lines) are indicated. The different 5'UTRs converge at -11 bp from the start site of translation in exon 2.

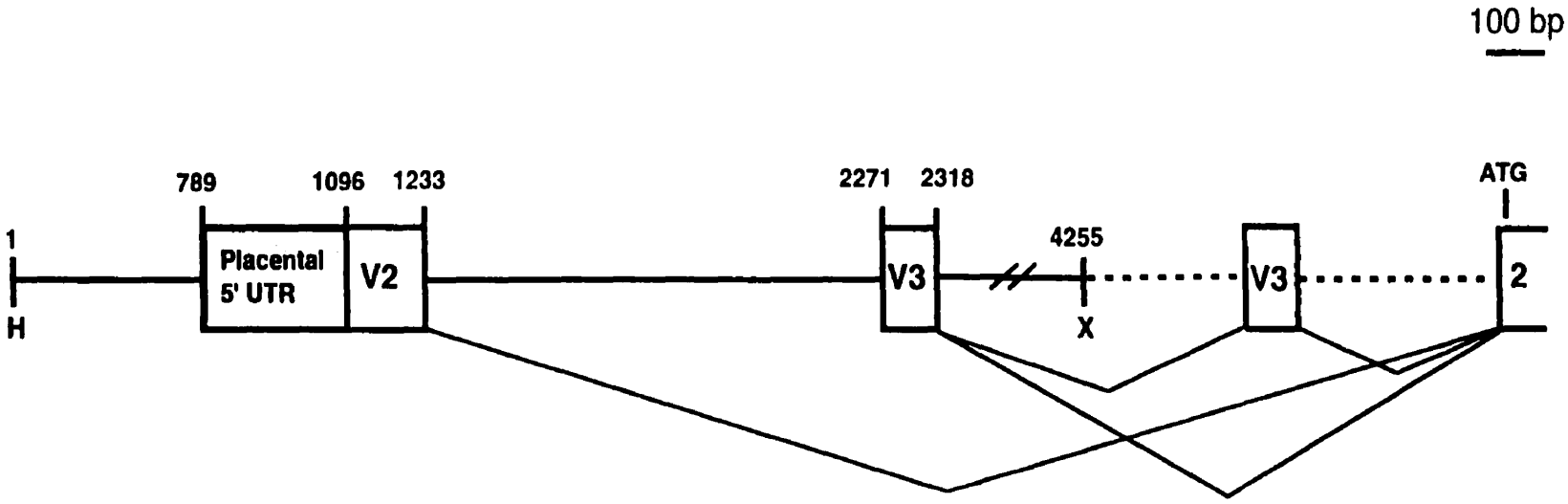
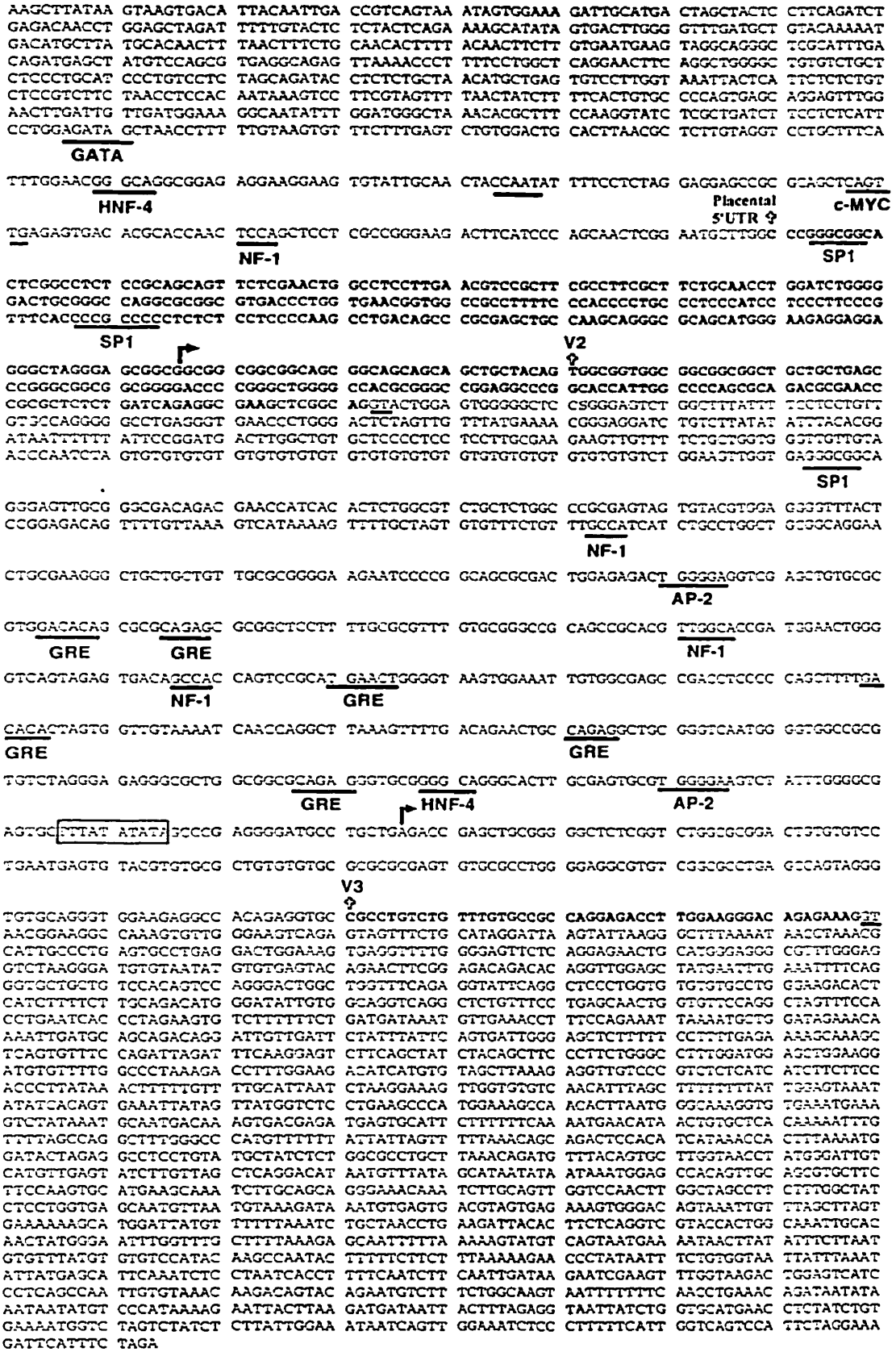

100 bp
789
1096
1233
2271
2318
ATG
1
Placental
5' UTR
V2
V3
4255
V3
2
H
X

Fig. A2.2. Nucleotide sequence of the 4255 bp genomic DNA fragment encompassing the placental 5'UTR, V2 and partial V3 sequences (in bold). Crosses mark the beginning of the three 5'UTR sequences previously identified by cDNA cloning. Splice donor dinucleotide GT sites at the 3' ends of the V2 and at the alternative splice site of V3 are indicated. The TSSG-predicted transcription start sites (arrows) and a TATA motif (boxed) proximal to the V3 sequence are indicated. Putative transactivator response elements (AP-2, CCAAT, C/EBP, c-MYC, GATA, GRE, HNF-4, NF-1 and SP1) in the region immediately upstream of the predicted transcription start sites are underlined.


GATA
HNF-4
Placental 5'UTR
c-MYC
NF-1
SP1
SP1
V2
SP1
NF-1
AP-2
GRE
GRE
NF-1
NF-1
GRE
GRE
GRE
HNF-4
AP-2
V3

cloning of the V3 subvariants, all V3 RT-PCR expression studies carried out in our laboratory as well as by others were designed in a manner that could not distinguish between V3a and V3b transcripts.

As shown in figure A2.2, a GT dinucleotide splice donor site is located at the 3' end of the mapped V3 sequence. This, together with the nucleotide sequences of the cloned V3a and V3c subvariants, suggests that splicing at this position can have two possible outcomes: 1) V3c transcripts can form by linking the sequence upstream of the GT donor site to -11 nucleotides from the AUG translational start site in exon 2 (Figure A2.1); 2) however, through alternative splicing, the region 5' to the GT splice donor site can also aquire additional intervening V3 sequences before connecting to the common site in exon 2 upstream from the AUG codon, resulting in the V3a subvariant (Figure A2.1).

Computer analysis of the 4.3 kb HindIII-XbaI DNA fragment, using the TSSG (307) and Signal Scan (308) software programs with the Transfac version 2.5 database, revealed several mammalian transactivator recognition sites (AP-2, CCAAT, C/EBP, c-MYC, GATA, GRE, HNF-4, NF-1 and SP1), and predicted two transcriptional start sites within the DNA sequence (Figure A2.2). The first start site is immediately upstream of the V2 sequence and is predicted to be driven by a GC-rich promoter (Figure A2.2). However, if this site is, in fact, biologically active, there must be multiple promoters regulating V2 expression since the placental 5'UTR extends further upstream of this transcriptional start site. The other TSSG-predicted start site is located just upstream of the V3 sequence (Figure A2.2) and has a proximal TATA box.

This preliminary characterization of genomic DNA encompassing V3 sequences supports the hypothesis that V3 and V1/V4 expression patterns are regulated by different gene promoters. It also suggests that there are at least two promoters driving synthesis of V2 transcripts. Future studies will have to verify the integrity of the 4.3 kb HindIII-XbaI genomic fragment by Southern blot analysis of human genomic DNA and test the TSSG-predicted start sites experimentally, as was done for the characterization of the V1-containing portion of the hGHR gene. In addition, a continous map of the hGHR gene regulatory regions will have to be constructed. To this end, we have screened by PCR, using V1- and V3-specific primers and protocols developed by Dr. Tom Hudson's laboratory (McGill University), BAC (309) and PAC (310) human genomic libraries. The PCR primers specific for V1- (sense, 5' AGA TTG AGA ATG ACT GAT TTG GGA G 3'; antisense, 5' GTT TCC TCC AGG CTT TAT ATC AAG AAC TTT 3') and V3- (sense, 5' GGA GAC CTT GGA AGG GAC AGA G 3'; antisense, 5' CAG AGC CTG ACC TGC CAC AAT A 3') encompassing genomic DNA regions were designed based on the V1- and V3-containing genomic sequences that were obtained by analysis of λ DASH recombinants. A BAC clone, BAC/hGHR.V1.V3, positive for both the V1 and V3 sequences as well as a PAC recombinant, PAC/hGHR.V3, encompassing V3 DNA, were isolated (data not shown). Southern blot analysis has confirmed that the BAC/hGHR.V1.V3 clone contains V1 as well as V3 sequences and has predicted an insert size of >100 kb (data not shown). The advantage of working with BAC (pBeloBAC11) and PAC (pCYPAC2) vectors is not only that they can hold inserts of 50-150 kb, but also that they are designed to minimize insert recombination. Therefore, it

is highly likely that characterization of the BAC/hGHR.V1.V3 and PAC/GHR.V3 clones will provide a complete and accurate physical map of the *hGHR* gene regulatory regions.

CHAPTER 5

CHAPTER 5 - *THE BABOON: A MODEL FOR THE STUDY OF PRIMATE GROWTH HORMONE RECEPTOR GENE EXPRESSION DURING DEVELOPMENT*

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(Submitted)

5.1. Preface.

Studies presented in the preceding chapters revealed that the regulatory regions of the *GHR* gene as well as the onset of GHR mRNA during development are substantially different in the human versus subprimates. These data suggest that a primate animal model might be a more appropriate test system in which to study regulation of human *GHR* gene expression during development. To investigate this, an RT-PCR approach, using human-specific primers, was employed to clone V1, V3 and V4 GHR cDNA homologues from postnatal baboon liver. A cocktail of DNA polymerases, containing a DNA "proof-reading" enzyme, was used to minimize the chance of PCR amplification errors. In addition, the integrity of the reported cDNA nucleotide sequences was confirmed by analyzing three independent recombinant clones. RT-PCR methodology, which permitted the precise sizing of the amplified fragments, was then used to characterize ontogenic- and tissue-specific expression of the three baboon 5'UTR isoforms. Southern blot analysis confirmed the identity of the PCR products.

5.2. Abstract.

Baboon growth hormone receptor (GHR) cDNAs were cloned from postnatal liver by RT-PCR, using hGHR-specific primers. The encoded baboon GHR precursor protein has an identical signal peptide sequence to that of human and rhesus monkey GHRs, and the mature baboon GHR is also 620 amino acids long with 95% and 98.5% amino acid identity to the human and rhesus monkey receptors, respectively. Previous studies in the human have identified eight 5' untranslated region (5'UTR) variants of the GHR mRNA (V1 to V8, numbered according to their relative abundance). We cloned the baboon V1, V3 and V4 homologues by RT-PCR; these variants have a high degree (>92%) of sequence identity with their human counterparts and also diverge at an identical point, 12 nucleotides upstream of the start of translation. The expression pattern of these three GHR mRNA isoforms in baboon liver during development was characterized. Strong expression of baboon V1 and V4 was evident by 49 days of postnatal life (n=5, 49 days & adult (18.6-19.6 kg)); very low levels of V1, but not V4, were observed in younger animals (n=2, 6 & 30 days). In contrast, V3 5'UTR variant mRNA was present in all fetal (n=4, 141-155 days gestation) and postnatal (n=7, 6-19.6 days & adult [18.6 kg]) hepatic specimens examined. Analysis of postnatal kidney and lung (n=2, 19 & 19.6 kg) revealed that V3 transcripts are present in these tissues, but not V1 and V4. Together, these data demonstrate that, as in the human, baboon V1 and V4 expression is developmentally regulated and tissue-specific, while the V3 isoform is more widely expressed. Therefore, it is likely that the regulatory regions of the baboon and human *GHR* genes are well conserved. Our findings suggest that the baboon is an appropriate

animal model in which to define the mechanisms regulating *GHR* gene transcription during primate development.

5.3. Introduction.

The growth hormone receptor (GHR) mediates multiple metabolic as well as growth-promoting effects in skeletal and soft tissues: expression of functional GHRs is crucial for normal postnatal mammalian growth and metabolic homeostasis (1). However, the physiological significance of the GHR during fetal development is poorly understood (2,3). Ontogenic studies in subprimates (rodent, sheep, cow, rabbit and pig) show that significant onset of GHR mRNA and protein synthesis occurs only after birth (4-7). In contrast, we have demonstrated that, in the human, GHR mRNA is widely expressed from the first trimester of fetal life (8). In addition, GHR immunostaining has been identified in human tissues as early as 8.5 weeks of fetal age and, by midgestation, the tissue distribution of the GHR is often identical to that found in the adult (9,10). Therefore, significant GHR mRNA and protein synthesis appears to begin earlier in human development than in lower species.

The diversity of the 5' untranslated region (5' UTR) of GHR mRNA in the human (11,12), ovine (13,14), bovine (15) and rodent (16,17) has suggested that, in all species, multiple promoter regions may regulate transcription of the GHR gene. In the human, eight different 5' UTR variants, numbered V1 to V8 according to their relative abundance, have been cloned from adult liver by 5' rapid amplification of cDNA ends (5' RACE) (11). All eight variants have distinct nucleotide sequences and only converge at -11 nucleotides prior to the start site of translation in exon 2. We have recently demonstrated that expression of two of these variants, V1 and V4, is tissue- and tumour-specific as well as developmentally regulated: V1 and V4 were only detected in postnatal

liver, and were of very low abundance in hepatoblastomas and hepatocellular carcinomas (12,18). In contrast, V3 GHR mRNA transcripts were expressed in all tissues examined, from as early as 10 weeks of fetal age (18). Our subsequent studies have mapped the V1 and V4 sequences to the same region in the human *GHR* gene and revealed that their transcription is regulated by a common promoter (12). The V3 sequence has been localized to a distinct genomic region and preliminary studies have suggested that it is transcribed from a unique start site (Zogopoulos, G., Goodyer, C.G. and Hendy, G.N., unpublished data).

Comparison of the 5' ends of the human (12) and subprimate (13-17) *GHR* genes has shown that the architecture of the human gene is a composite of the regulatory regions of lower species. These data suggest that subprimates may not be the best models for studying regulation of human *GHR* gene expression. We have hypothesized that another primate may be a more appropriate test system. In the present study, human GHR-specific primers were used to clone baboon GHR cDNAs by RT-PCR from postnatal baboon liver total RNA. Nucleotide sequencing of multiple baboon cDNA clones revealed that the baboon GHR has a high level of sequence identity with the human (95%) and rhesus monkey (98.5%) GHRs (present data, 19, 20). V1, V3 and V4 homologues were also cloned by RT-PCR from postnatal baboon liver. Subsequent semi-quantitative RT-PCR and Southern blotting analysis showed that, as in the human, baboon V1 and V4 GHR mRNA expression is developmentally regulated and tissue-specific, whereas V3 is widely expressed in fetal and postnatal tissues. Therefore, it is likely that the regulatory regions in the baboon and human *GHR* genes are well

conserved, resulting in similar GHR mRNA regulation in the two species. These data suggest that the baboon is an appropriate animal model in which to study GHR mRNA expression during primate development.

5.4. Materials and Methods.

5.4.1. Tissues.

Fetal and postnatal baboon tissues were obtained immediately after the animals were euthanized (n=8, 141 days gestation to adult). Those animals that were wild caught have been identified by weight at the time of study since their ages were unknown. The human postnatal liver specimen (transplant donor age = 43 years (yr)) was collected 5 h following death. Specimens were flash-frozen in dry-ice acetone and stored at -70 °C for RNA extraction. Total RNA was isolated using the guanidine thiocyanate/CsCl gradient method, and treated with deoxyribonuclease I (DNAse I; Pharmacia Biotech) to eliminate genomic DNA contamination. Animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee; animal facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

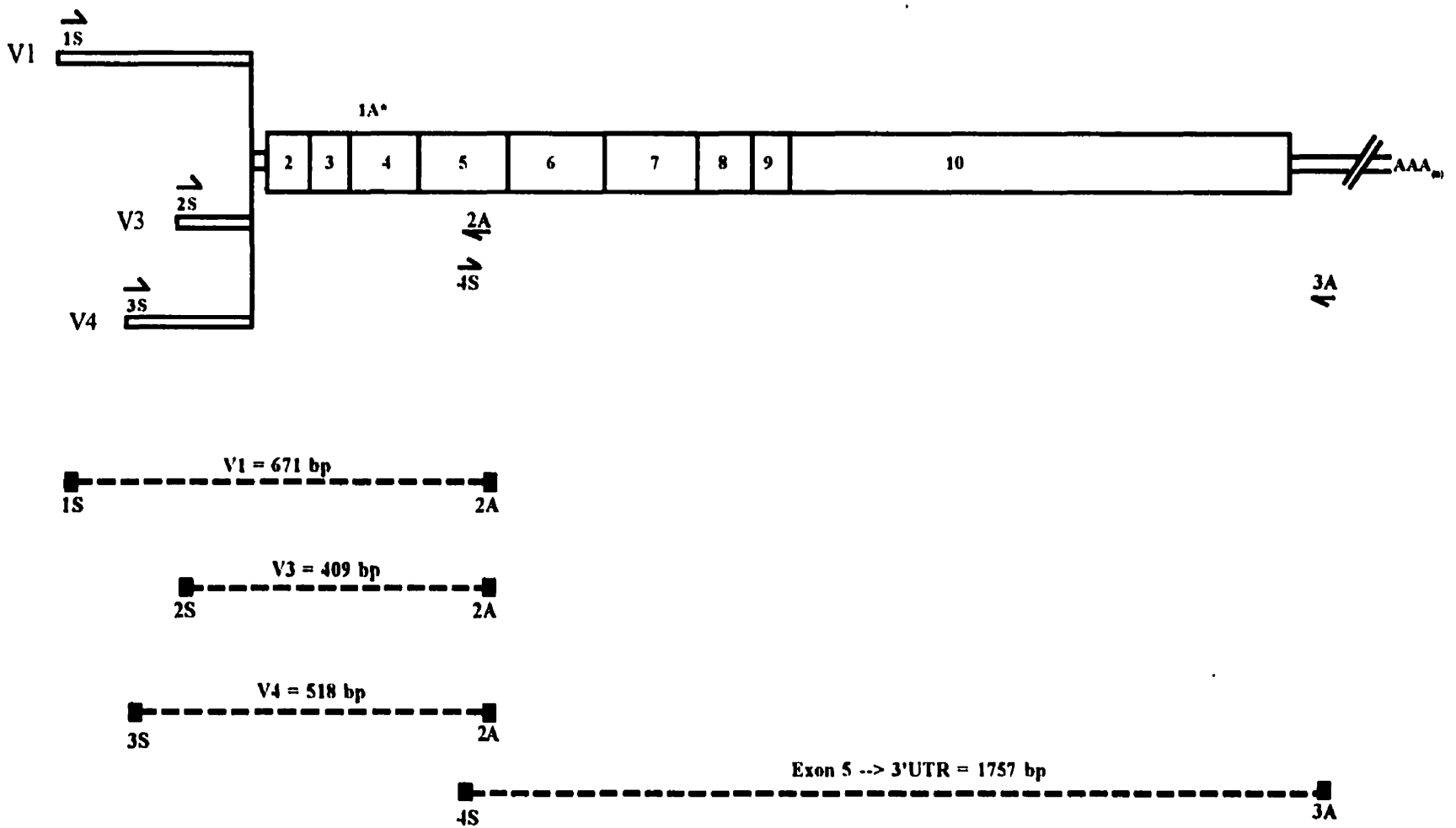
5.4.2. RT-PCR cloning of baboon GHR cDNAs.

Five μg of postnatal (49 days) baboon liver total RNA were heated at 70°C for 5 min and then reverse transcribed for 1 h at 48°C with 2.5 U Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT; Life Technologies), 80 U RNAsin (Promega), 0.6 μM of the appropriate antisense human GHR primer (Table 5.1 and Figure 5.1), 0.48 mM dNTPs, 10 mM $MgCl_2$, 10 mM dithiothreitol, 100 mM Tris-HCl (pH 8.3), and 50 mM KCl. The AMV-RT was denatured by heating the RT product for 5 min at 98°C. Parallel RT reactions were run in the absence of AMV-RT to ascertain that gene transcripts and not genomic DNA were being amplified. Six μl of RT product were

Table 5.1. Sequences of the oligonucleotide RT-PCR primers and the internal hybridization probe (*).

Oligonucleotide	Sequence
1S	5' AGA TTG AGA ATG ACT GAT TTG GGA G 3'
2S	5' GGA GAC CTT GGA AGG GAC AGA G 3'
3S	5' GAG TAG CAA AGA TGG ATT AAG TGA G 3'
4S	5' TCA AGA ATG GAA AGA ATG CCC TG 3'
1A*	5' GAA CCT CAT CTG TCC AGT GGC AT 3'
2A	5' AGG TAT CCA GAT GGA GGT AAA CG 3'
3A	5' TAG AAT CCA TAC CCC ATC CTG TC 3'

Fig. 5.1. Schematic representation of homologous baboon and human cDNA isoforms. Coding exons are indicated by numbered boxes and 5'UTRs are represented by empty boxes. The relative positions of the sense (S) and antisense (A) primers as well as the internal hybridization probe (1A*) are indicated (oligonucleotide sequences are provided in Table 5.1). Total RNA was reverse transcribed with primer 2A, and primer sets 1S/2A, 2S/2A and 3S/2A were used to specifically amplify the V1, V3 and V4 cDNAs, respectively. The remaining baboon cDNA coding region was cloned using primer 3A in the RT step, and the 2S/3A PCR primer combination. Broken lower lines indicate the resulting PCR products after amplification with these sets of primers. The V1 and V4 internal standards, used as positive controls in the semi-quantitative RT-PCR assays, both generate PCR products of 382 bp.


V1
1S
1A*
2
3
4
5
6
7
8
9
10
AAA(n)
V3
2S
2A
4S
V4
3S
3A
V1 = 671 bp
1S
2A
V3 = 409 bp
2S
2A
V4 = 518 bp
3S
2A
Exon 5 --> 3'UTR = 1757 bp
4S
3A

amplified for 30 cycles in 0.3 μM of appropriate sense and antisense human GHR primers (Table 1 and Figure 1), 0.5 mM deoxyribonucleotides (dNTPs; Pharmacia Biotech), 22.5 mM $MgCl_2$, 2.5 U Taq/PWO polymerase mix (Boehringer Mannheim), and 1 x Expand Long Template PCR System Buffer #3 (Boehringer Mannheim). The reaction was heated at 92°C for 2 min, cycled 30 times for 10 s at 92°C, 30 s at 61°C and 2 min at 68°C, and terminated with a final elongation of 5 min at 68°C. The amplified cDNAs were inserted into the PGEM-T TA cloning vector (Promega) and three independant clones were sequenced using a Li-Cor automated system (Li-Cor Biotech).

5.4.3. Semi-quantitative RT-PCR.

RT reactions were carried out as described above and 6 μl of RT product were amplified for 25 cycles in 2.5 U Taq DNA polymerase (Life Technologies), 0.5 mM dNTPs, 0.25 μM hGHR sense and antisense primers (Table 5.1 and Figure 5.1), 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 7% DMSO. The first cycle consisted of 3 min at 92°C, 1 min at 61°C and 3 min at 72°C. Subsequent cycles consisted of 30 s at 92°C, 1 min at 61°C and 1.5 min at 72°C. The reaction was terminated with a final elongation of 5 min at 72°C. The internal V1 and V4 standards were generated using the PCR method of Jin et al (21). Under the specified conditions, each amplification reaction was still in the exponential phase.

5.4.4. Southern blotting analysis of PCR products.

PCR products were resolved on 2% agarose gels and transferred to 0.45 μm

positively charged nylon membranes (Schleicher & Schuell). Blots were prehybridized for 3 h at 42°C in 6xSSPE, 1% (w/v) SDS, 10 x Denhardt's and 0.15 mg/ml denatured salmon sperm DNA (Sigma Chemicals). Relative volumes of prehybridization and hybridization solutions were 100 μl per cm^2 of membrane. Hybridization was carried out overnight at 63°C. The hybridization solution contained 6xSSPE, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 0.4 nCi/ml of end-labelled probe (Table 5.1 and Figure 5.1) per cm^2 of nylon membrane. Blots were washed twice for 10 min at 63°C with 6xSSPE/1%SDS and once for 10 min at 58°C with 2xSSPE/1%(w/v) SDS. Bands were visualized by autoradiography following 3-14 h exposure to Kodak XAR-5 film (Eastman Kodak) using two intensifying screens.

5.4.5. End-labelling reactions.

Approximately 150 nmol of the 1A* oligonucleotide (Table 5.1 and Figure 5.1) were end-labelled and purified through a Sephadex G-50 spin column (Pharmacia Biotech). The end-labelling reaction was at 37°C for 1 h in 2.5 μCi/μl of γ-^{32}P-ATP (ICN Pharmaceuticals Canada Ltd.), 20 U of T_4 polynucleotide kinase (Life Technologies), 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, and 1 mM 2-mercaptoethanol.

5.5. Results.

5.5.1. Cloning of GHR cDNAs from baboon liver.

Using human-specific primers and the RT-PCR strategy described in Figure 5.1, the V1, V3 and V4 baboon cDNA homologues were cloned from postnatal liver total RNA. Nucleotide sequencing revealed that, like their human counterparts, the three baboon 5'UTR variants diverge at -12 nucleotides from the start site of translation in exon 2 (Figure 5.2). The human (11) and baboon 5'UTR homologues are highly conserved: 96% for V1 and 92% for V4, while the shorter nucleotide stretch of the baboon V3 5'UTR matches its human counterpart precisely. It should be noted that an additional alternatively spliced V3 isoform of longer length and lower abundance has been identified in the human (11, 18). Evidence for the presence of this V3 subvariant in the baboon was obtained following longer exposure of the Southern blots to X-ray film (data not shown).

To obtain the complete baboon GHR mRNA coding sequence, a second RT-PCR, using human-specific primers within exon 5 and the 3'UTR, was carried out. The complete nucleotide sequence of the baboon GHR cDNA predicts a 620 amino acid mature protein, with 95% and 98.5% amino acid sequence similarity with the human (19) and rhesus monkey receptors (20), respectively (Figure 5.3). The characteristic features of the GHR are all well conserved in the three primates: the 18 amino acid signal peptide, the seven cysteine residues, five potential N-glycosylation sites and YGEFS motif of the extracellular domain, and the proline-rich Box 1 region of the cytoplasmic tail (Figure 5.3) (22).

Fig. 5.2. Alignment of the baboon V1, V3 and V4 cDNA variants with their human (11) counterparts. Stars indicate baboon nucleotides corresponding to the human-specific GHR primers used to clone the V1, V3 and V4 cDNAs from postnatal baboon liver. Italicized bold letters identify the 11 nucleotide stretch prior to the ATG start site of translation that is common to the three baboon and human 5'UTR variants.

V1

```
Baboon  ********** ********** *****gaatt ttgtgaaggt ctatatatca aagcagaaag
Human   agattgagaa tgactgattt gggag-g--- ---------- t--------- ----------

Baboon  tccaagaagt tagagattaa tacatgccaa gttgtgacca acaaacttct gtgggatccc
Human   a-------t- ---------- ---------- --g--a---- -g-------- ----------

Baboon  acctccaacc cctccctgtc tgccagattt taaccaaatc agtgtgatgt gatctgcttg
Human   ---------- -------c-- --t------- ---------- ---------- ----------

Baboon  aatatatgag tgaaagaaaa aggaaattta aaaagttctt gatataaagc ctggaggaaa
Human   c--------- ---------- ---------- ---------- ---------- ----------

Baboon  caatacgaaa atccagcctc tatttcagca atatctgccg gactattggt cctacaggtA
Human   ---------- ---------- ---------- ---------- ---------- ----------

Baboon  TG
Human   --
```

V3

```
Baboon  ********** ********** **aaaggtc ctacaggtAT G
Human   ggagaccttg gaagggacag ag-------- ---------- -
```

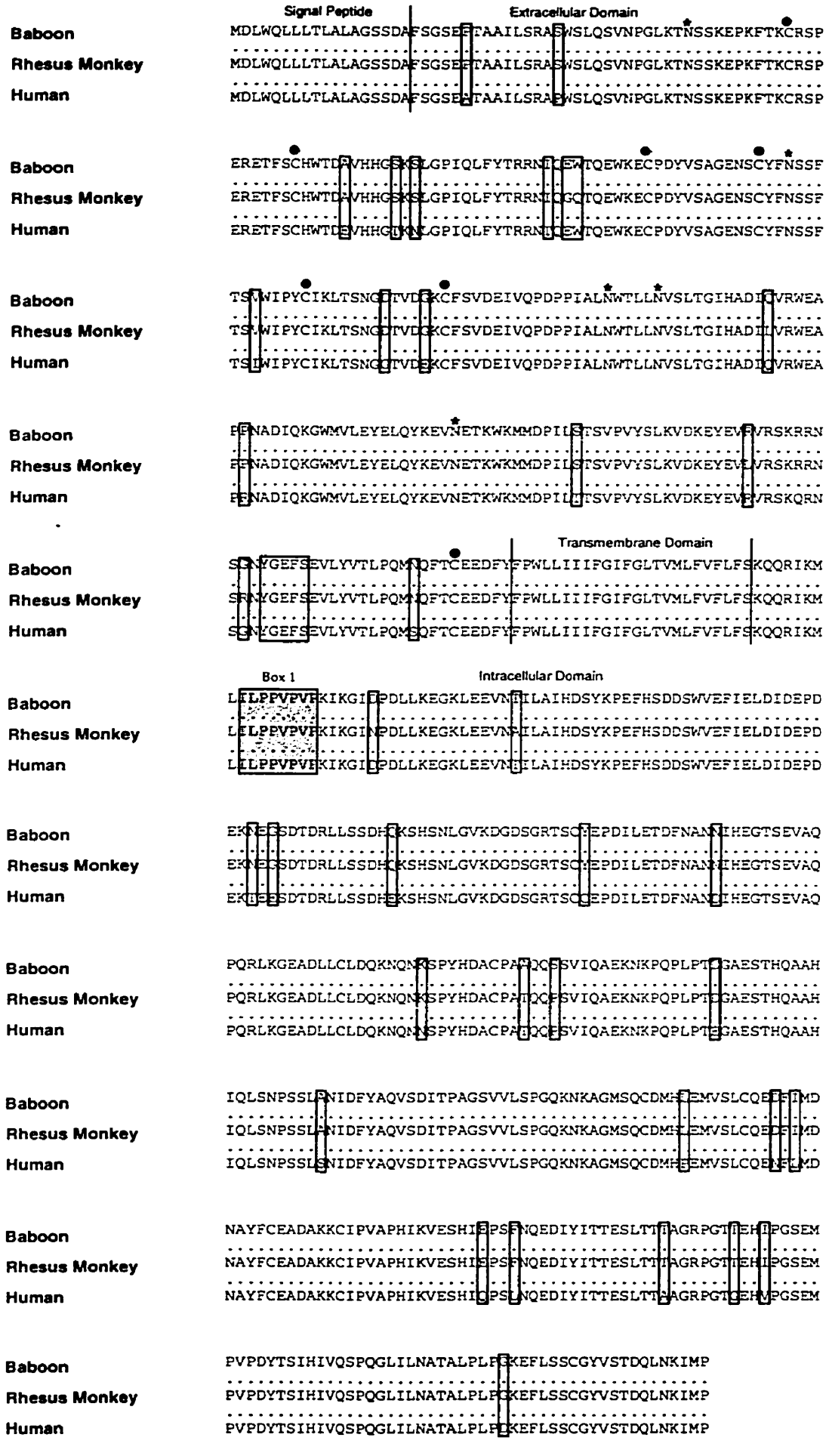
V4

```
Baboon  ********** ********** *****gaagt gagaagagga taggcggcag cggggccaga
Human   gagtagcaaa gatggattaa gtgag----- --------t- ----a----- -a--------

Baboon  ggtgtgaata gcctggaacc ttctgagaag atccctttgt agacagaaga aaggggagat
Human   ---------- --ca------ ---------- --t------- g---g----- --------g-

Baboon  ttgtggaaat gctggaagat aaggtcctaca ggtATG
Human   ---------- -g-------- ---------- ------
```

Fig. 5.3. Amino acid sequence comparison of the baboon, rhesus monkey (20) and human (19) GHRs. Solid vertical lines mark the boundaries of the signal peptide (18 amino acids) as well as the extracellular (246 amino acids), transmembrane (24 amino acids) and intracellular (350 amino acids) domains. Closed circles and stars identify conserved cysteine residues and N-glycosylation sites, respectively. Shaded boxes show the YGEFS and Box 1 motifs. Transparent boxes indicate differences in amino acids among the three species. The absence of an alignment dot between the sequences refers to a residue difference.


Signal Peptide
Extracellular Domain
Baboon
Rhesus Monkey
Human
Transmembrane Domain
Box 1
Intracellular Domain

5.5.2. Characterization of V1, V3 and V4 mRNA expression during development.

The RT-PCR/Southern blot strategy used to characterize V1, V3 and V4 mRNA expression is summarized in Figure 5.1. In each analysis, aliquots from the same RT reaction were tested for expression of each variant. V1 and V4 transcripts were of very low abundance in all fetal livers examined (n=4, 141-155 days gestation), and only readily detectable in hepatic samples at and following 49 days of life (n=4, 49 days & adult (18.6-19.6 kg)) (Figure 5.4). Low levels of V1 were also present in younger postnatal animals (n=2, 6 & 30 days); V4 5'UTR variant mRNAs were not detectable at this early postnatal stage in development. There was no evidence for expression of V1 and V4 transcripts in adult baboon kidney and lung specimens (n=2, 19 & 19.6 kg) (Figure 5.4). In contrast to V1 and V4, V3 was observed in all fetal and postnatal baboon livers examined, as well as the postnatal kidney and lung tissues (Figure 5.4). Therefore, similar to the human (12,18), baboon expression of V1 and V4 GHR mRNA transcripts is developmentally regulated and tissue-specific, while the V3 isoform is more widely expressed.

Fig. 5.4. Ontogenic and tissue-specific expression of the baboon V1, V4 and V3 5'UTR GHR mRNA variants. Southern blots showing the expression patterns of the GHR mRNA variants in baboon fetal liver (F-li; in days gestation) as well as postnatal liver (P-li; in days or kg), lung (P-lu; in kg) and kidney (P-ki; in kg) tissues. A human adult liver (HAL, 43 yr) specimen was run in parallel. Internal V1 and V4 standards (382 bp) served as a positive amplification control. H_2O served as a negative RT-PCR control. The sizes of the V1, V4 and V3 PCR products that were cloned and sequenced are indicated (in bp).

V1
V4
V3
671
382
518
382
409
HAL, 43 yr
F-li, 141 days
F-li, 142 days
F-li, 149 days
F-li, 155 days
P-li, 6 days
P-li, 30 days
P-li, 49 days
P-li, 19 kg
P-li, 18.6 kg
P-li, 19.6 kg
P-lu, 19 kg
P-lu, 196 kg
P-ki, 19 kg
P-ki, 196 kg
H2O

5.6. Discussion.

Using human-specific primers, we cloned by RT-PCR the coding exons as well as portions of three 5'UTRs (V1, V2 and V4) of the baboon GHR mRNA. Nucleotide sequencing analysis of the cDNA clones showed that the GHR is well conserved between the baboon, rhesus monkey (20) and human (19), with the few amino acid differences dispersed throughout the receptor sequence. The mature GHR protein sequences of the baboon and rhesus monkey are extremely well conserved (98.5%), and have a similar degree of variation from the human (baboon 95%, rhesus monkey 94%) (present data, 19,20). This is consistent with the idea that these primates are equi-distant evolutionary ancestors of the human (23).

The characteristic features of the GHR that are all well conserved in the baboon (present data), rhesus monkey (20) and human (19) include five potential N-glycosylation sites, seven cysteine residues and the YGEFS motif of the extracellular region, as well as Box 1 of the cytoplasmic domain. Given that both the human and rhesus receptors are glycosylated at several sites (19,20), it is likely that carbohydrate groups are added to the baboon GHR at the conserved asparagine residues. The formation of two extracellular domain disulfide bridges occurs in the GHRs of all species studied to date, and is also a feature of the class I division of the cytokine/GH/prolactin receptor superfamily (22). Another characteristic of class I receptors is the presence of a WSXWS (where X is any amino acid) motif in the transmembrane proximal region of the extracellular domain (22). In GHRs, this motif is replaced by the related YGEFS consensus (22). Mutation or deletion of the YGEFS domain impairs the receptor's intracellular signalling cascades

(24). It is thought that this motif directs conformational features in the GHR that are required for maximal biological activity of the receptor. The proline-rich Box 1 cytoplasmic GHR domain consists of the ILPPVPVP amino acid consensus sequences in the baboon as well as in all mammalian receptors cloned to date (22). Box 1 is crucial for GHR cellular responses since it is responsible for binding and activation of JAK 2 (25). Induction of JAK 2 tyrosine kinase activity is an early event in GHR intracellular signalling cascades and is thought to mediate the majority of GHR biologic responses (22).

Although the structural characteristics of the GHR are generally conserved among species, the amino acid sequence identities can be as low as 70% (22). The present cloning of the baboon GHR as well as the recent characterization of the rhesus monkey receptor (20) will permit comparative functional studies of primate versus subprimate GHRs. Martini et al have already demonstrated an intriguing difference between the monkey and rat GHRs (20). The rat GHR binds and activates the JAK 2 tyrosine kinase only after GH-induced receptor dimerization, while the rhesus monkey receptor is constitutively associated with the JAK 2 even though it only stimulates JAK 2 tyrosine kinase activity after ligand binding (20, 22).

The presence of 5'UTRs highly conserved between the baboon (present data) and human (11) GHR mRNAs suggests that the *GHR* gene regulatory regions will be similar in the two primates. The 5' end of the human *GHR* gene has been recently characterised: the V1 and V4 5'UTR variants are localized to the same region in the human gene and can be generated (along with V7 and V8) by transcription from a common promoter (12),

whereas V3 maps to a distant genomic region (with V2) and is thought to be regulated by a different promoter (Zogopoulos, G., Goodyer, C.G. and Hendy, G.N., unpublished data).

Investigations in subprimates have identified an ovine homologue (exon 1A) to V1, as well as mouse (L1) and rat (GHR1) transcripts with nucleotide sequences similar to the human V7 variant (11,13,16,17). Ontogenic studies have demonstrated that, like the human or baboon V1 and V4 isoforms, these subprimate transcripts are present only in postnatal liver (present data, 12,13,16-18). Thus, the 5' end of the GHR gene regulating postnatal-liver specific GHR mRNA expression in the human appears to be a composite of the homologous ovine and rodent DNA structures (12,13,16,17). It will be of interest to determine whether the comparable baboon *GHR* regulatory region is also a composite of the subprimate genomic DNA.

The 5'UTRs of many eukaryotic mRNAs are involved in modulating translation initiation (26). Tightly regulated genes, such as those of growth factors and proto-oncogenes, often have 5'UTRs with complex stem loop structures that hinder translation initiation. Given that V1 and V4 mRNA expression seems to be restricted to postnatal liver in primates (present data, 12,18), it will be important to determine how these two 5'UTR variants influence translation relative to the widely expressed V3 variant. It is also possible that differential control of fetal versus postnatal tissue expression of the receptor results in distinct GH biological responses in utero. Not too surprisingly, preliminary studies have indicated that regulation of the *GHR* gene in another primate, the rhesus monkey, may be similar to that in the human and baboon.

The RT-PCR and Southern blot approach employed in the present study of the baboon was used to test for expression of V1, V3 and V4 transcripts in rhesus monkey liver. As anticipated, V1 and V4 cDNAs were present in adult, but not fetal, liver, whereas V3 was present in hepatic tissue of adult as well as the fetal rhesus monkey (27).

Significant levels of GHR mRNA have been observed in a variety of human tissues from as early as the first trimester of gestation (8), whereas ontogenic studies in subprimates have demonstrated that *GHR* gene transcription begins during late fetal life with a marked onset after birth (4-7). An earlier developmental onset of GHR mRNA synthesis implies that regulation of the human *GHR* gene is different from that of the subprimates (rodent, ovine, bovine, rabbit and pig). Although the current study focused on characterizing the appearance of V1 and V4 mRNAs in the baboon liver, it also identified significant GHR mRNA expression in baboon hepatic tissues from the second half of gestation. To further validate the baboon as a primate model for *GHR* gene expression studies during development, the onset and distribution of GHR mRNA synthesis in fetal baboon tissues will have to be delineated in more detail.

In summary, baboon GHR cDNA isoforms have been cloned by RT-PCR from postnatal liver. The 620 amino acid mature baboon GHR protein is very similar to the human and rhesus monkey receptors. As in the human, expression of the baboon V1 and V4 5'UTR GHR mRNA homologues was found to be developmentally regulated and tissue-specific, whereas the V3 isoform was detected in all fetal and postnatal tissues examined. Together, these data suggest that the baboon is an appropriate animal model in which to study *GHR* gene expression during primate development.

5.7. Acknowledgements.

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5.8. References.

1. Rosenbloom, A.L., Rosenfeld, R.G., and Guevara-Aguirre, J. (1997). Growth hormone insensitivity. *Pediatric Clinics North America* 44: 423-442.

2. Gluckman, P.D. and Harding, J.E. (1997). The physiology and pathophysiology of intrauterine growth retardation. *Hormone Research* 48 (suppl 1): 11-16.

3. Hay, W.W. Jr, Catz, C.S., Grave, G.D., and Yaffe, S.J. (1997). Fetal growth: its regulation and disorders. *Pediatrics* 99: 585-591.

4. Breier, B.H., Ambler, G.R., Sauerwein, H., Surus, A., and Gluckman, P.D. (1994). The induction of hepatic somatotropic receptors after birth in sheep is dependent on parturition-associated mechanisms. *J. Endocrinology* 141: 101-108.

5. Walker, J.L., Moats-Staats, B.M., Stiles, A.D., and Underwood, L.E. (1992). Tissue-specific developmental regulation of the mRNAs encoding the growth hormone (GH) receptor and the GH binding protein in rat fetal and postnatal tissues. *Pediatric Research* 31: 335-339.

6. Ymer, S. and Herington, A.C. (1992). Developmental expression of the growth hormone receptor gene in rabbit tissues. *Mol. Cell. Endocrinol.* 83: 39-49.

7. Schnoebelen-Combes, S., Louveau, I., Postel-Vinay, M.C., and Bonneau, M. (1996). Ontogeny of GH receptor and GH-binding protein in the pig. *J. Endocrinol.* 148: 249 255.

8. Zogopoulos. G., Figueiredo, R.M.O., Jenab, A., Ali, Z., Lefèbvre, Y., and Goodyer, C.G. (1996). Expression of exon three retaining and deleted human growth hormone receptor mRNA isoforms during development. *J. Clin. Endocrinol. Metab.* 81: 775-782.

9. Simard, M., Manthos, H., Giaid, A., Lefèbvre, Y., and Goodyer, C.G. (1996). Ontogeny of growth hormone receptors in human tissues: an immunohistochemical study. *J. Clin. Endocrinol. Metab.* 81: 3097-3102.

10. Hill, D.J., Riley, S.C., Bassett, N.S., and Waters, M.J. (1992). Localisation of the growth hormone receptor, identified by immunocytochemistry, in second trimester human fetal tissues and in placenta throughout gestation. *J. Clin. Endocrinol. Metab.* 75: 646-650.

11. Pekhletsky, R.I., Chernov, B.K., and Rubtsov, P.M. (1992). Variants of the 5'-untranslated sequence of human growth hormone receptor mRNA. *Mol. Cell. Endocrinol.* 90: 103-109.

12. Zogopoulos, G., Goodyer, C.G., and Hendy, G.N. Cloning and characterization of promoter regions in the human growth hormone receptor gene. (submitted)

13. O'Mahoney, J.V., Brandon, M.R., and Adams, T.E. (1994). Identification of a liver-specific promoter for the ovine growth hormone receptor. *Mol. Cell. Endocrinol.* 101: 129-139.

14. Adams., T. (1995). Differential expression of growth hormone receptor messenger RNA from a second promoter. *Mol. Cell. Endocrinol.* 108: 23-33.

15. Heap, D., Lucy, M.C., Collier, R.J., Boyd, C.K., and Warren, W.C. (1995). Nucleotide sequence of the promoter and first exon of the somatotropin receptor gene in cattle. *J. Anim. Sci.* 73: 1529, 1995.

16. Menon, R.K., Stephan, D.A., Singh, M., Morris, S.M., and Zou, L. (1995). Cloning of the promoter-regulatory region of the murine growth hormone receptor. *J. Biol. Chem.* 270: 8851-8859.

17. Baumbach, W.R. and Bingham, B. (1995). One class of growth hormone (GH) receptor and binding protein mRNA in rat liver, GHR1, is sexually dimorphic and regulated by GH. *Endocrinology* 136: 749-760.

18. Zogopoulos, G., Albrecht, S., Pietsch, T., Alpert, L., von Schweinitz, D., Lefèbvre, Y., and Goodyer, C.G. (1996). Fetal- and tumor-specific regulation of growth hormone receptor mRNA expression in human liver. *Cancer Research* 56: 2949-2953.

19. Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J., and Wood, W.I. (1987). Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature* 330: 537-543.

20. Martini, J.F., Pezet, A., Guezennec, Y.C., Edery, M., Postel-Vinay, M.C., and Kelly, P.A. (1997). Monkey growth hormone (GH) receptor gene expression. *J. Biol. Chem.* 272: 18951-18958.

21. Jin, C.F., Mata, M., and Fink, D.J. (1994). Rapid construction of deleted DNA fragments for use as internal standards in competitive PCR. *PCR Methods Appl.* 43: 252-255.

22. Argetsinger, L.S. and Carter-Su, C. (1996). Mechanisms of signalling by the growth hormone receptor. *Physiol. Rev.* 76: 1089-1107.

23. Pausova, Z., Morgan, K., Fujiwara, T.M., and Hendy, G.N. (1995). Evolution of

a repeat sequence in the parathyroid hormone-related peptide gene in primates. *Mammalian Genome* 6: 408-414.

24. Baumgartner, J.M., Wells, C.A., Chen, C.M., and Waters, M.J. (1994). The role of the WSXWS equivalent motif in growth hormone receptor function. *J. Biol. Chem.* 269: 29094-29101.

25. Wang, Y.D. and Wood, W.I. (1995). Amino acids of the human growth hormone receptor that are required for proliferation and Jak-STAT signaling. *Mol. Endocrinol.* 9: 303-311.

26. Jansen, M., De Moor, C.H., Sussenbach, J.S., and van den Brande, J.L. (1995). Translational control of gene expression. *Pediatric Research* 37: 681-686.

27. Zogopoulos, G., Lerner, S., Albrecht, S., Pietsch, T., Alpert, L., von Schweinitz, D., Giussani, D., Nathanielsz, P., Hendy, G.N., and Goodyer, C.G. (1997). Regulation of growth hormone receptor mRNA in primates. *Program of the US Endocrine Society 79th Annual Meeting*, Abstract #P2-216.

PART III.

GENERAL DISCUSSION

AND

CLAIMS TO ORIGINAL RESEARCH

CHAPTER 6

CHAPTER 6 - *GENERAL DISCUSSION*

The presence of elevated fetal plasma levels of immunoreactive hGH and functional hGHRs in multiple fetal tissues suggested a role for hGH and its receptor during human fetal life (Chapter 1). To investigate this issue, the ontogenic pattern of hGHR mRNA expression was determined and the 5' regulatory regions of the *hGHR* gene were cloned and characterized. These data support the hypothesis that hGH and its receptor have a significant physiological role in early stages of human development.

hGHR mRNA was detected in all human tissues examined from as early as 4 weeks of gestation (Chapter 2). The ubiquitous distribution of hGHR mRNA suggests that the receptor is important for fetal as well as postnatal human development. Our analysis also demonstrated that expression of the exon 3 retaining and deficient hGHR mRNA transcripts is individual-, rather than tissue-, specific (Chapter 2). Similar studies by Wickelgren et al have shown that this individual-specific pattern is maintained in the adult (311).

There are a number of possible mechanisms regulating individual-specific expression of the two hGHR mRNA isoforms, the most obvious being deletion of exon 3 within the *hGHR* gene. However, we demonstrated the integrity of exon 3 in genomic DNA of fetuses expressing only the exon 3 deficient transcript. Thus, this transcript must be the result of a splicing event rather than genomic deletion. Genetic polymorphisms within the *hGHR* gene itself or within the different components of the RNA splicing machinery (reviewed in 312), the spliceosome, could also explain the individual-specific expression pattern. Indeed, a recent pedigree analysis of four Hutterite families has

suggested that the ability of an individual to express one or both of these two transcripts follows a simple type of Mendelian inheritance (313).

The eukaryotic splicing machinery precisely removes introns from precursor RNAs with high fidelity (reviewed in 312). This is due to the conservation of recognition sequences at intronic boundaries: the nucleotide sequences of all characterized eukaryotic introns begin with GT (splice donor site) and end with AG (splice acceptor site). Introns also have an internal consensus sequence, the lariat branch point, that is required for the splicing process and is located 20-50 nucleotides upstream of the 3' splice acceptor site. Since alterations in these conserved cis splicing elements have been shown to cause aberrant splicing of several gene transcripts (e.g., mutations in the human globin genes underlying thalassemia (314)), it is possible that polymorphisms in the intronic sequences between exons 2 and 3 of the *hGHR* gene regulate the individual-specific expression pattern of the exon 3 retaining and deleted mRNA isoforms. In a recent study of ten adults, Stallings-Mann et al found no correlation between hGHR mRNA expression pattern and alterations in the splice donor and acceptor sites in the intron immediately upstream of exon 3 (313). Thus, we will have to search for genetic variations in other regions of the *hGHR* gene, to see if there are specific "cis-acting" DNA elements regulating exclusion of exon 3 from the hGHR mRNA.

The spliceosome consists of five small nuclear RNAs (snRNAs) and more than 50 proteins (reviewed in 312). It is possible that certain polymorphisms within these components of the splicing apparatus cause the spliceosome to recognize exon 3 of the hGHR mRNA as part of the intronic sequence and splice it out to produce the exon 3

deficient isoform. If such "spliceosome" polymorphisms exist, then there are probably other, as yet unidentified, gene transcripts that undergo alternative splicing in an "individual-specific" manner. Expression of the exon 3 deficient GHR mRNA isoform has been detected only in primates (173), suggesting that the genetic factor(s) governing its synthesis must have arisen in the later stages of evolution.

Our ontogenic analysis revealed predominant expression of the exon 3 deficient transcript during the early stages of human development: >90% of the fetuses tested expressed this mRNA prior to 9 weeks of gestation, but only ~30% of individuals postnatally. However, due to the cross-sectional nature of the tissue samples tested, there is the possibility of ascertainment bias in the data. To clarify this issue, we are currently carrying out a longitudinal study in which expression of hGHR transcripts in early gestation fetal cells (12-14 week amniotic fluid cells from amniocenteses) and in term placental villi from the same pregnancy are being compared. We have already established that the full-length and exon 3 lacking mRNA isoforms are synthesized in an individual-specific pattern in amniotic fetal cells and in placental tissues, both of which are of fetal origin, permitting a valid comparison in these paired samples.

The developmental regulation of exon 3 deleted hGHR mRNA synthesis does not occur in all individuals: we and others have observed exon 3 deficient transcripts in ~30% of postnatal subjects. One explanation for this ontogenic variation in hGHR mRNA expression may be, again, polymorphisms within the *hGHR* gene. Developmental regulation of alternative splicing is thought to be the result of ontogenic changes in the composition of the spliceosome (e.g., FGF receptor-1 mRNA isoforms in the mouse

heart (315)). Thus, it is possible that only some allelic variations of the *hGHR* gene are compatible with particular spliceosome changes and permit developmental regulation of exon 3 splicing, while others are insensitive to developmental changes in the splicing apparatus. It is equally possible that certain exon 3 deficient-producing *hGHR* alleles are transcriptionally active only in early development and then silenced; potential mechanisms include methylation (316) as well as *hGHR* gene polymorphisms in DNA binding sites for developmentally regulated transcription factors. The mechanisms regulating individual-specific and developmental expression of the exon 3 deficient hGHR mRNA are likely to be completely separate processes.

As discussed in Chapter 2, transfection studies have demonstrated that both the full-length and exon 3 deficient hGHRs have equal ligand binding affinities and that they are both capable of being cleaved near the outer leaflet of the plasma membrane to yield the hGHBP (179,180,248). However, it is not yet known whether there are any functional differences in these two receptor isoforms. To address this question, co-transfection studies will have to be carried out to examine the signalling cascades and biological end-points activated by homo- and hetero-dimer complexes of the two receptor isoforms. In addition, the recent development of an antibody specific for the exon 3-containing hGHBP (317) in combination with the ligand-mediated immunofunctional assay (LIFA, which identifies both exon 3 retaining and deficient hGHBPs) (317a) will now permit the determination of the relative levels of the two hGHBP isoforms in individuals expressing both the exon 3 retaining and deficient hGHRs; if specific ratios can be related to a particular phenotype, these analyses may help us understand the

biological significance of the exon 3 deficient hGHR mRNAs. Finally, the availability of fetal (i.e., tissues obtained from theraputic abortions) and postnatal (i.e., liver donors for pediatric transplant) hepatic specimens and the development of a hepatocyte purification methodology (Chapter 2) will permit functional in vitro studies of the two hGHR mRNA isoforms in "physiologic" hGH target cells.

Two additional hGHR isoforms (hGHR-t1 and hGHR-t2) have recently been identified (see Chapter 1, section 1.3.7). Although these two receptors are truncated, they both insert in the plasma membrane and have an increased capacity to be cleaved to generate hGHBPs (249-251). The truncated hGHRs are of interest because transfection studies have shown that, when present in sufficient amounts, they can act as dominant negative inhibitors of full-length receptor signalling cascades, presumably through the formation of hGHR-t:hGHR heterodimer complexes (250). Our RT-PCR and Southern blot analysis of several fetal and postnatal tissues did not detect either of these two transcripts. One explanation is that our technique was not sensitive enough: in most postnatal tissues tested to date, hGHR-t1 and hGHR-t2 mRNAs constitute $\sim 1\%$ and $< 1\%$ of total hGHR mRNA levels, respectively (249,250). In addition, expression of the hGHR-t1 transcript has been found to account for a substantial fraction (approximately half) of the total hGHR mRNA levels only in mammary gland and adipose tissues (249), tissues that we did not investigate. Given this information, it will be important to determine whether the hGHR-t1 transcript is present in fetal adipose tissue and whether there are developmental changes in expression that may be of functional significance. Furthermore, it is presently unknown whether the hGHR-t1 and

hGHR-t2 mRNA isoforms exist as exon 3 deficient transcripts.

In addition to characterizing the tissue distribution and ontogeny of the exon 3 retaining and deleted hGHR mRNA isoforms, the studies presented in Chapter 2 also demonstrated significant expression of *hGHR* mRNA in multiple human tissues from as early as the third month of fetal life. A subsequent quantitative RT-PCR analysis identified tissue-specific developmental changes in total hGHR mRNA levels: there was a six-fold decrease in postnatal lung, no change in intestine or kidney, and a six-fold increase in liver that parallels a four-fold increase in [^{125}I]hGH binding to hepatic membranes (Appendix 1). Since these tissue data reflect hGHR mRNA concentrations of all cell-types in each tissue, in situ hybridization studies will have to be undertaken to identify the specific hGHR mRNA-producing cells that contribute to these developmental changes. It is possible that hGHR mRNA levels vary among the different cell-types of the organs examined. For example, using a cell purification approach, we have already demonstrated that fetal hepatocytes are actively synthesizing hGHR mRNA, while the progenitor blood cells of the fetal liver are not (Chapter 2). However, it is not known whether the other fetal hepatic cells express hGHR mRNA.

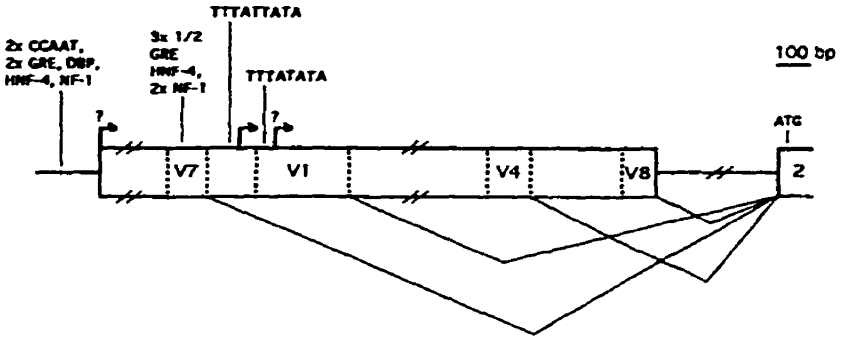
Control of gene transcription to ensure that different cell-types express the correct level of mRNA at the appropriate developmental stage can be accomplished by developmental- and tissue-specific gene promoter usage (e.g., the human *IGF-2* and *IGF-1* genes) (reviewed in 261,318,319). Our data suggest that this is the case for the *hGHR* gene as well: V1 and V4 5'UTR mRNAs were found to be developmentally regulated and tissue-specific, while the V3 variant was readily observed in all fetal and postnatal

tissues tested (Chapters 3 and 4). We have demonstrated that this heterogeneity in the 5'UTR of the hGHR mRNA arises from a combination of differential promoter usage and alternative splicing processes. The V1, V4 and V8 sequences map in series (5' V1-V4-V8 3') to nearby regions in the *hGHR* gene and their transcription is regulated by a common promoter to yield a transcript of at least 2 kb (Chapter 4) (Figure 6.1). In addition, the V7 sequence was determined to lie immediately upstream of V1: transcription from the, as yet unidentified, promoter(s) upstream of V7 results in a second set of long 5'UTRs (> 3.7 kb) that encompass the V7, V1, V4 and V8 sequences. Alternative splicing of these long transcripts can produce several mRNA isoforms, including the V1, V4, V7 and V8 cDNAs that were previously isolated by 5'RACE (Figure 6.1) (252). Finally, the V3 as well as the V2 sequences were localized in series (5' V2-V3 3') to a distinct genomic region and preliminary studies suggest that there are separate promoter regions upstream of both of the V2 and V3 genomic sequences (Appendix 2; Figure 6.2).

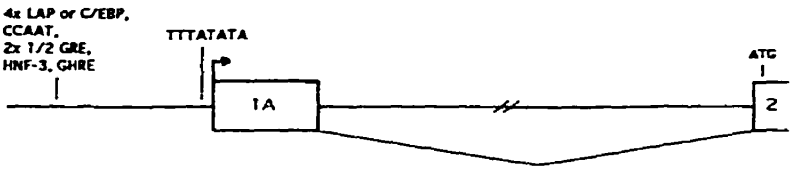
Further evidence that multiple promoters are regulating *hGHR* gene transcription comes from the ovine, bovine, rabbit, mouse and rat *GHR* gene structures (Figures 6.1 and 6.2) (12,227-231,234,252,255). Although only transcriptional start sites for the ovine (1A and 1B) and mouse (L1) 5'UTRs have been well characterized to date (227,228,230), there are substantial sequence identities in the different 5'UTR variants amongst species (Table 6.1). It is noteworthy that expression of at least one 5'UTR variant in each species is significantly expressed only in postnatal liver: human V1 and V4, ovine and bovine exon 1A, mouse L1 and rat GHR1 (Figure 6.1). In contrast,

Fig. 6.1. 5' regulatory regions of the *GHR* gene related to liver-specific expression of GHR. Comparison of analogous human, ovine, bovine, rabbit, murine and rat promoter regions and/or 5'UTRs. Exons are boxed and characterized splicing patterns are indicated (v-shaped lower lines). A splice acceptor dinucleotide AG site is located in the genes of the six species at -12 bp from the start site of translation (ATG). The V7, V1, V4 and V8 sequences that have been previously cloned by 5'RACE are mapped (broken vertical lines) within the human 5'UTR. Defined (arrows) and not yet characterized (arrows with ?) transcriptional start sites as well as conserved TATA motifs are shown. Regions with putative binding sites for transcription factors are indicated 5' to the transcriptional start sites.

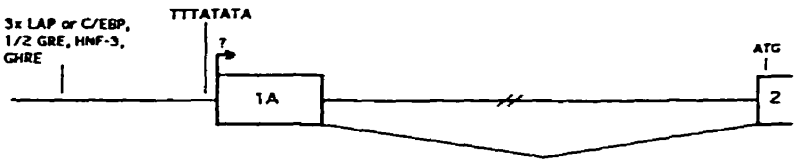
Human GHR



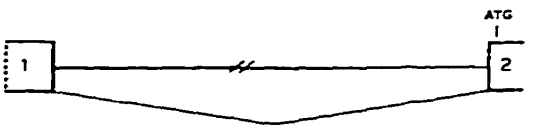
Ovine GHR



Bovine GHR



Rabbit GHR



Murine GHR

Rat GHR

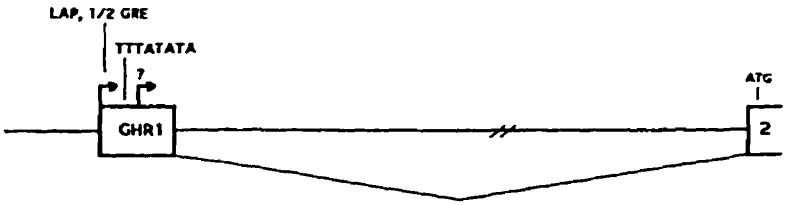
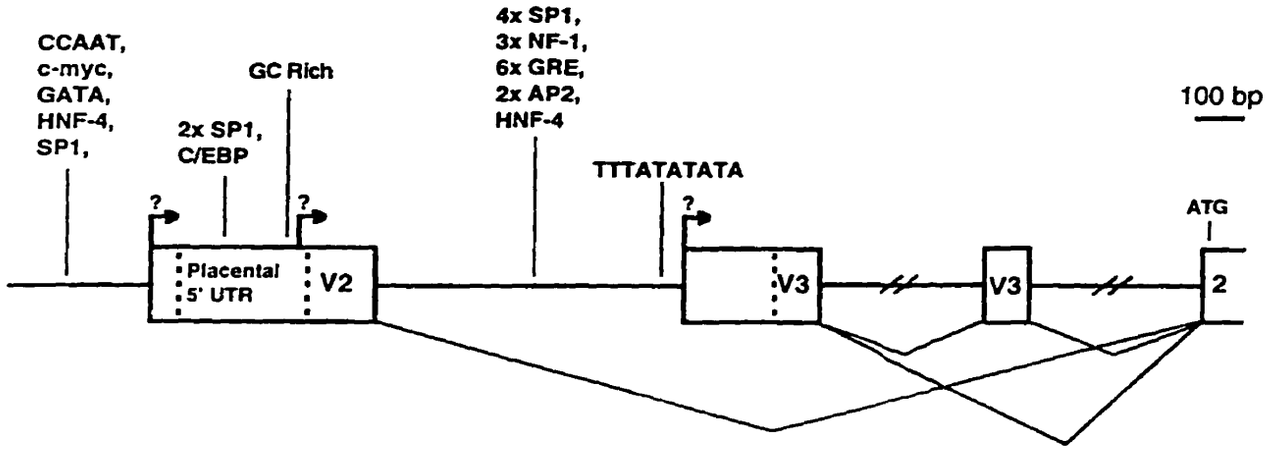


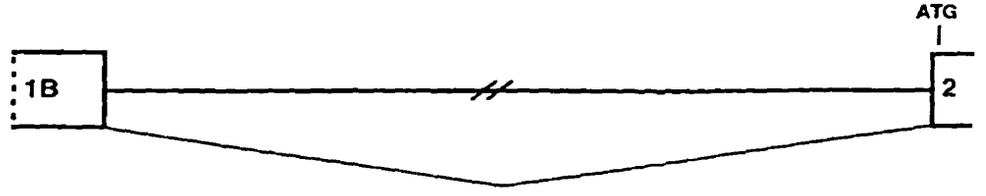
Fig. 6.2. 5' regulatory regions of the *GHR* gene related to ubiquitous expression of GHR mRNA. Comparison of analogous human and ovine promoter regions, as well as bovine, murine and rat 5'UTRs. Exons are boxed and characterized splicing patterns are indicated (v-shaped lower lines). A splice acceptor dinucleotide AG site is located in the genes of the six species at -12 bp from the start site of translation (ATG). The V2, placental 5'UTR (i.e., isolated from a human placental cDNA library) and V3 sequences that have been previously cloned are mapped (broken vertical lines) within the human 5'UTRs. Defined (arrows) and not yet characterized (arrows with ?) transcriptional start sites as well as conserved TATA motifs are shown. Regions with putative binding sites for transcription factors are indicated 5' to the transcriptional start sites.

Human GHR

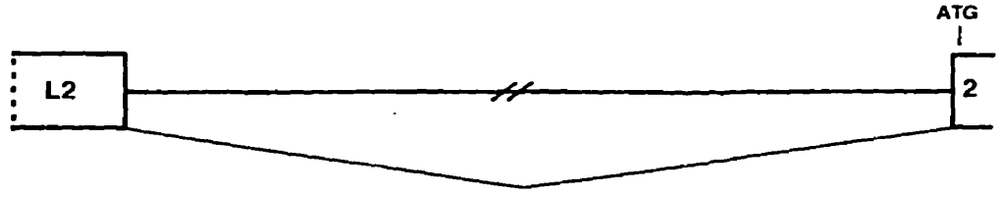


Ovine GHR

Bovine GHR



Murine GHR



Rat GHR

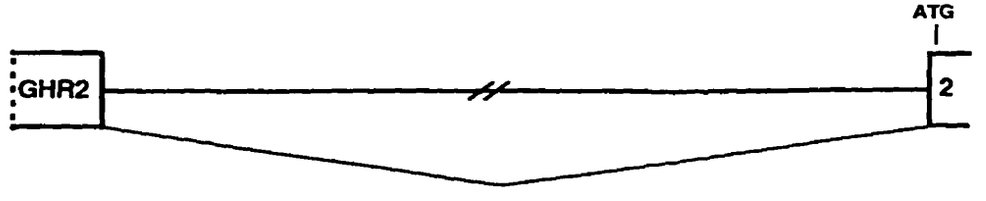


Table 6.1. Sequence homologies of subprimate 5'UTRs relative to the corresponding human variants.

									REF. #
Human	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	252
Ovine	76% (1A)	74% (1B)							227 228
Bovine	76% (1A)	74% (1B)							229 255
Rabbit	76%								12
Mouse		77% (L2)					50% (L1)		230 234
Rat		77% (GHR2)					50% (GHR1)		231

expression of human V3, ovine and bovine exon 1B, mouse L2 and rat GHR2 transcripts is not restricted to postnatal liver: several of these 5'UTR variants have been detected in other fetal and postnatal tissues (Figure 6.2). Given the expression pattern of its subprimate homologues (exon 1B, L2 and GHR2), it is likely that human V2 is also present in multiple fetal and postnatal tissues. The V2 variant has proven to be difficult to analyze with our present reverse-transcription (RT) and polymerase chain reaction (PCR) protocols due to its high GC content and secondary structure (G. Zogopoulos and C.G. Goodyer; personal communications with P. Rubtsov and T.E. Adams); therefore, its expression pattern has not yet been investigated.

The order of the human 5' V7-V1-V4-V8 3' and 5' V2-V3 3' genomic fragments relative to each other, as well as the genomic localization of the human V5 and V6 5'UTR sequences, remain unknown. Thus, future studies will have to focus on building a complete and continuous map of the 5' regulatory regions of the *hGHR* gene. The recently isolated bacterial artificial chromosome (BAC/hGHR.V1.V3, Appendix 2), containing a human DNA insert that encompasses both the V1 and V3 sequences, will likely provide this information. Analysis of the structural organization of these complicated 5' regulatory regions will permit investigators to determine whether there are additional transcriptional start sites (and/or 5'UTRs) as well as to characterize the various alternative splicing patterns that each primary *hGHR* gene transcript undergoes. Together these studies will provide a complete picture of the different 5'UTR isoforms of the hGHR mRNA.

The existence of a human counterpart for each subprimate 5'UTR variant suggests

that the architecture of the human gene is a composite of the regulatory regions of these lower species, and that there is more complex transcriptional control in the human (Figures 6.1 and 6.2). To determine whether there is a primate animal model in which to study this, we cloned the GHR as well as V1, V3 and V4 5'UTR homologues from postnatal baboon liver. Subsequent studies showed that, as in the human, baboon V1 and V4 expression are tissue-specific and developmentally regulated (i.e., detected only in postnatal liver), while V3 is ubiquitous in fetal and postnatal tissues (Chapter 5). To further validate the baboon as a primate model for *GHR* gene expression studies during development, the 5' end of the baboon gene will have to be characterized. In addition, since these investigations only examined hepatic specimens from the second half of gestation and no other fetal tissues, the onset and tissue-specificity of GHR mRNA expression in baboon fetal tissues will need to be analyzed in more detail. Recent investigations by Martini et al (173) have demonstrated that alternative splicing of the rhesus monkey GHR mRNA results in a GHBP-specific transcript similar to that reported for the mouse and rat. Using a parallel RT-PCR approach, we will now be able to determine whether GHBP-specific mRNA isoforms also exist in the baboon and human.

During the past decade, a large number of genes have been shown to contain multiple promoters (319). One of the most interesting cases, especially in relationship to hGHR, is the *hIGF-2* gene (reviewed in 261 and 318). Promoters P2, P3 and P4 of the *hIGF-2* gene regulate expression in all fetal tissues and, although their activity is substantially reduced after birth, they continue to ubiquitously transcribe hIGF-2 mRNA postnatally. In contrast, P1 of the *hIGF-2* gene regulatory region is active only in

postnatal liver. Therefore, *hIGF-2* gene transcripts synthesized from the P1 promoter have similar expression profiles as the V1 and V4 hGHR mRNA isoforms, while the P2, P3 and P4 derived IGF-2 mRNAs have comparable fetal and postnatal tissue distribution patterns with the V3 hGHR 5'UTRs. The similarities in their expression patterns suggest that the *hGHR* and *hIGF-2* genes may be under parallel transcriptional control.

Further evidence that the *hGHR* and *hIGF-2* genes are under similar regulation comes from the "fetal-like" expression patterns of these two genes in hepatic tumours. In a comparative study of three hepatoblastomas with paired normal liver specimens, Li et al found P1 promoter activity of the *hIGF-2* gene to be repressed, while the activity of the P2, P3 and P4 promoters was unaltered or upregulated (320). These data are similar to our findings demonstrating that expression of V1 and V4 mRNAs is suppressed in hepatoblastomas and hepatocellular carcinomas, while V3 transcripts were detected in all hepatic specimens (Chapters 3 and 4). It will be important to characterize whether the decrease in V1 and V4 mRNAs in the hepatic tumour state results in significantly lower total hGHR mRNA levels as compared to their paired normal livers, and whether there is a concomitant change in V3 mRNA expression.

Future studies will also have to delineate whether there are quantitative tissue-specific developmental changes in V3 as well as in any other hGHR 5'UTR variants that are expressed in fetal tissues (ie. V2?). It is also intriguing that the P2, P3 and P4 *hIGF-2* gene promoters are imprinted while P1 has biallelic expression (reviewed in 261,318). Because of similarities in the expression patterns of hIGF-2 and hGHR mRNAs, it will be important to test whether the ubiquitous *hGHR* promoters (eg. V3) are imprinted while

postnatal liver-specific expression of hGHR mRNA isoforms (eg. V1 and V4) is biallelic.

Specific liver-enriched transcription factors are involved in repressing P1 *hIGF-2* gene promoter activity in fetal liver (e.g., HNF-4), while other transactivators (e.g., C/EBPα, LAP) override this inhibition and stimulate P1 expression soon after birth (reviewed in 261,318). Liver-enriched transactivators are most likely also regulating activity of those *GHR* gene promoter regions transcribing postnatal liver-specific mRNAs: several DNA binding elements for these transcription factors have been identified upstream of the human V1, ovine exon 1A and mouse L1 transcriptional start sites, including HNF-1, HNF-3, HNF-4, C/EBPα, LAP and DBP (Figure 6.1) (Chapter 4) (227,230). It is noteworthy that these transactivators are themselves under developmental control: HNFs 1, 3, 4 and 6 are present only in rodent foregut derivatives and/or fetal liver, while C/EBPα, LAP and DBP are only detectable in postnatal rodent liver (318). Whether this is the case in the human has yet to be demonstrated.

Further support that differential promoter usage is responsible for the tissue- and developmental-specific expression patterns of GHR mRNAs comes from the presence of responsive elements for transactivators that are widely expressed (e.g., AP2, CCAAT, GATA, c-myc and NF-1) immediately upstream of the human V2 and V3 as well as the ovine exon 1B genomic sequences (Figure 6.2) (Appendix 2) (228). The presence of an HNF-4 binding site upstream of the human V2 and V3 gene sequences suggests that these potential gene promoters may be under tissue-specific regulation in fetal and/or postnatal liver.

Although it is likely, based on current data from human and lower species, that

tissue-specific and developmental regulation of the V1 and V4 versus V3 hGHR mRNAs is due to differential promoter usage, we have not excluded the possibility that alternative splicing is the major regulatory mechanism. To do this, we will have to demonstrate that the promoter region controlling V3 expression (Appendix 2) does not also give rise to either V1- or V4-containing transcripts.

Multiple gene promoters often have greater functional significance than merely regulating tissue- and developmental-specific levels of mRNA production. Studies have demonstrated that the use of differential gene promoter and/or alternative splicing can produce mRNA isoforms with variable translation efficiencies (e.g., the rabbit *ß-globin* gene as well the *c-myc* and *mdm2* proto-oncogenes) (reviewed in 321,322,323). This is because the 5'UTR of eukaryotic mRNAs can influence the rate of translation initiation. The purpose of the translation initiation step is to position the ribosome at the start of the coding region. Classically, this process begins with the binding of the ribosome to the 5'end (at the 7-methylguanylate cap site) of the mRNA. The ribosome then travels across the 5'UTR until it reaches a translational start site codon (AUG). Therefore, if open reading frames are present, as in all known 5'UTR variants of the hGHR mRNA except V2, upstream of the genuine AUG codon, translation of the mRNA can be inhibited, especially if these false initiation codons are in the favorable CC(Purine)CCAUGG consensus (253). In addition, long and/or GC-rich 5'UTRs with complicated secondary structures cannot be translated efficiently. Thus, it will be important to determine the relative efficacy with which the V1-containing transcripts of >2 kb (Figure 6.1) as well as the highly GC-rich V2 hGHR mRNA isoform are translated. Moreover, a comparison

of the translation efficiencies of the tissue-specific and developmentally regulated 5'UTRs (e.g., V1 and V4) versus those of the ubiquitously expressed isoforms (e.g., V3) will help delineate the biological significance for the tight regulation of the 5'UTR of the hGHR mRNA.

Translational efficiency can also be regulated by RNA binding proteins. The prototype for translational control by RNA binding proteins is the regulation of ferritin expression by iron (reviewed in 322). In the presence of low cellular iron levels, the 5'UTR of ferritin mRNA binds an inhibitory protein that causes a halt in its translation. However, as soon as the cellular iron levels increase, this inhibitory RNA binding protein is displaced by iron itself or by hemin so that translation of the ferritin mRNA can take place. More recently, translation of the P3-related hIGF-2 mRNA isoform has been shown to be stimulated in vitro by the direct binding of cytoplasmic proteins to the transcript (reviewed in 321). It is possible that RNA binding proteins are also involved in the regulation of hGHR mRNA translation, especially in fetal and postnatal tissues (e.g., lung) where hGHRs are undetectable although significant mRNA amounts are present (Chapter 2, plus references). One could hypothesize that a tissue-specific RNA binding protein, absent in lung at all developmental stages, is required for translation of the hGHR mRNA isoforms (e.g., V3) found in fetal and postnatal lung (Chapter 3).

Translation initiation of the vast majority of mRNA transcripts involves scanning of the entire 5'UTR (reviewed in 321,322). However, a number of eukaryotic mRNA transcripts, including the P1-driven hIGF-2 mRNA isoform, have been found to contain internal ribosomal entry sites (IRES). In these transcripts, the initiation complex enters

the 5'UTR at an IRES instead of at the 5'end of the mRNA. Thus, internal translation initiation provides an attractive mechanism for improving the translation efficiency of "difficult" 5'UTRs, especially those with multiple upstream open reading frames or complex secondary structures. The IRES motifs that have been characterized to date share little homology except for a polypyrimidine tract located approximately 20 nucleotides from the AUG translational start site so that identification of this motif within the 5' regions of the *hGHR* gene will require functional analyses. However, given the similarity in the expression pattern of the 5'UTRs of the hGHR and hIGF-2 mRNAs, it would be very interesting to characterize whether the postnatal liver-specific hGHR mRNA isoforms (e.g., V1 and V4), like the P1-driven *hIGF-2* gene transcripts, contain functional IRES motifs.

The expression patterns of the 5'UTR hGHR variants can also be influenced by their relative stability in different fetal and postnatal cell-types (reviewed in 324). An example of such regulation comes from the human HOX-5.1 gene which has two gene promoters that produce mRNA isoforms with altered half-lives in different cell-types (325). Thus, an important future study will be to test whether variations in the 5'UTR, the absence of exon 3 or alternative 3'ends (i.e., hGHR-t1 and-t2 mRNAs) alter the stability of the hGHR mRNA in different fetal and postnatal cell-types.

Finally, the role of the different 5'UTRs in regulating translation of the hGHR-t1 and -t2 transcripts as well as the exon 3 retaining versus deficient mRNA forms should be examined. In the case of the exon 3 retaining and deficient mRNAs, no specific correlation with V1, V2, V3 or V4 5'UTRs has been found; a similar analysis of hGHR-

t1 and -t2 transcripts has not been reported.

In summary, my doctoral research has demonstrated that there is significant hGHR mRNA expression throughout human development and that there are several tissue-specific ontogenic changes in the hGHR mRNA expression pattern. The significance of these changes in relation to hGHR biologic activity remains unknown. It is intriguing that the exon 3 retaining and deficient mRNA isoforms are expressed in an individual-specific manner and that, in some individuals, the exon 3 deficient transcript appears to be developmentally regulated. Are these variations in *hGHR* gene expression responsible for population differences in height and/or metabolic rates? Is one pattern of expression predisposing to growth-related pathophysiologies? These are at least two physiologically relevant questions that need to be addressed.

In addition, we have hypothesized that the use of multiple gene promoters is primarily responsible for the tissue-specific developmental changes in total hGHR mRNA and 5'UTR variants. The functional significance of these different 5'UTRs may be to regulate hGHR mRNA translation and, therefore, GHR synthesis in a tissue-specific fashion during development. It is fascinating that the *hGHR* and *hIGF-2* genes have similar ontogenic expression characteristics. In both cases, the changes in gene transcription most likely reflect the maturation of an endocrine system but may also confer fetal-specific control of hGHR and hIGF-II expression and biological activities. The potential ability to use the baboon as a primate model to study *GHR* gene expression is exciting since it would permit the characterization of *GHR* gene activity in tissues and at developmental stages that are not possible to examine in humans due to specimen

limitations and ethical considerations. Finally, once we have an understanding of the complete *hGHR* gene structure and the mechanisms regulating its expression, we can assess whether genetic alterations within the promoter or 5'UTR regions of the *hGHR* gene are involved in the pathophysiology of individuals who exhibit hGH insensitivity and abnormal fetal and/or postnatal growth.

Thus, the work in this thesis has significantly advanced our understanding of the hGHR during human development and has provided important "molecular tools" for future studies.

CHAPTER 7

CHAPTER 7 - *CLAIMS TO ORIGINAL RESEARCH*

1. Demonstration that transcription of the *hGHR* gene begins as early as the first trimester of fetal life in several human tissues.

2. Determination that fetal expression of the exon 3 retaining and deficient hGHR mRNA isoforms is individual-, and not tissue-, specific.

3. Demonstration that deletion of exon 3 from the *hGHR* gene is not the mechanism by which exon 3 deficient hGHR mRNA transcripts are generated.

4. Generation of cross-sectional data that show a statistically significant ($p<0.0002$) association of predominant expression of exon 3 deficient hGHR mRNA prior to 20 weeks of human fetal life.

5. Determination, using a quantitative RT-PCR assay, that there are tissue-specific developmental changes in hGHR mRNA expression: a six-fold increase in hGHR mRNA levels from fetal to postnatal hepatic tissues ($p<0.01$), no change in intestine or kidney, and a six-fold postnatal decrease in lung ($p<0.05$).

6. Demonstration that expression of the V1 and V4 5'UTR hGHR mRNA variants is developmentally regulated, tissue-specific and repressed in hepatic tumours, whereas V3 mRNA isoforms are more ubiquitously expressed throughout development.

7. Cloning of a 3.8 kb XbaI-BsaAI DNA fragment of the 5' end of the *hGHR* gene and precise placement of four of the eight known 5'UTR hGHR mRNA variants in series (5'-V7-V1-V4-V8-3') within this portion of the gene.

8. Identification of a transcriptional start site upstream of the V1 sequence in the 3.8 kb XbaI-BsaAI *hGHR* gene fragment, and evidence provided for a second transcriptional start site that is located further upstream.

9. Isolation of a bacteriophage recombinant clone with a 4.3 XbaI-HindIII human genomic DNA insert that contains sequences corresponding to the V2 and V3 5'UTR hGHR mRNA variants, with V2 being upstream of V3.

10. Isolation of a bacterial artificial chromosome recombinant clone that contains human genomic DNA corresponding to both the V1 and V3 cDNA sequences.

11. Cloning of the GHR cDNA from baboon liver and demonstration that the 5'UTR of the baboon GHR mRNA is heterogeneous, with at least three variants: transcripts sharing high sequence homology to the human V1, V3 and V4 5'UTR variants.

12. Demonstration that in the baboon, like in the human, expression of V1 and V4 5'UTR variants is developmentally regulated and tissue-specific, whereas V3 mRNA is more widely expressed.

PART IV.

REFERENCES

REFERENCES

1. Fiddle, O., Bates, R.W. and Dykshorn, S. The preparation, identification and assay of prolactin- a hormone of the anterior pituitary. Am. J. Physiol. 105: 191, 1933.

2. Li, C.H. and Evans, H.M. The isolation of pituitary growth hormone. Science 99: 183-184, 1944.

3. Frantz, A.G. and Kleinberg, D.L. Prolactin: evidence that it is separate from growth hormone in human blood. Science. 170: 745, 1970.

4. Fraiser, S.D. Session I: Landmarks in the history of the NCGS and recombinant growth hormone. The not so good old days: working with pituitary growth hormone in North America, 1956 to 1985. J. Pediatr. Suppl. 131: S1-S4, 1997.

5. Li, C.H. and Papkoff, H. Preparation and properties of growth hormone from human and monkey pituitary glands. Science 124: 1293-1294, 1956.

6. Knobil, E., Wolf, R.C., Greep, R.O. and Wilhelmi, A.E. Effect of primate pituitary growth hormone preparation on nitrogen metabolism in the hypophysectomized rhesus monkey. Endocrinol. 60: 166-168, 1957.

7. Beck, J.C., McGarry, E.E., Dyrenfurth, I. and Venning, E.H. Metabolic effects of human and monkey growth hormone in man. Science 125: 884-885, 1957.

8. Shepard, T.H. II, Nielsen, R.L., Johnson, M.L., Bernstein, N. and Ferrier, P. Human growth hormone I: metabolic balance studies carried out in a hypopituitary child. Am. J. Dis. Child. 99: 90-96, 1960.

9. Shepard, T.H. II, Nielsen, R.L., Johnson, M.L., Bernstein, N. and Ferrier, P. Human growth hormone II: further study of its effect on growth in dwarfism. J. Pediatr. 57: 363-369, 1960.

10. Hintz, R.L. A prismatic case: the prismatic case of Creutzfeld-Jakob disease associated with pituitary growth hormone treatment. J. Clin. Endocrinol. Metab. 80: 2298-2301, 1995.

11. Cronin, M.J. Pioneering recombinant growth hormone manufacturing: pounds produced per mile of height. J. Pediatr. 131: S5-S7, 1997.

12. Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G. Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330: 530-543, 1987.

13. Wells, J.A. Binding in the growth hormone receptor complex. Proc. Natl. Acad. Sci. USA 93: 1-6, 1996.

14. Fuh, G., Cunningham, B.C., Fukunaga, R., Nagata, S., Goeddel, D.V. and Wells, J. Rational design of potent antagonists to the human growth hormone receptor. Science 256: 1677-1680, 1992.

15. Postel-Vinay, M.C. Growth hormone- and prolactin-binding proteins: soluble forms of receptors. Horm. Res. 45: 178-181, 1996.

16. Cooke, N.E. and Liebhaber, S.A. Molecular biology of the growth hormone-prolactin gene system. In: Vitamins and Hormones. Ed. Litwark, G., Academic Press, San Diego. p. 385-457, 1995.

17. Argetsinger, L.S. and Carter-Su, C. Mechanism of signalling by the growth hormone receptor. Physiol. Rev. 76: 1089-1107, 1996.

18. Strobl, J. and Thomas, M.J. Human growth hormone. Pharm. Rev. 46: 1-34, 1994.

19. Gluckman, P.D. and Harding J.E. The physiology and pathophysiology of intrauterine growth retardation. Horm. Res. (Suppl. 1) 48: 11-16, 1997.

20. Hay, W.W.Jr., Catz, C.S., Grave, G.D. and Yaffe, S.J. Fetal growth: its regulation and disorders. Pediatr. 99: 585-591, 1997.

21. Walker, W.H., Fitzpatrick, S.L., Barrera-Saldana, H.A., Resendez-Perez, D. and Saunders, G.F. The human placental lactogen genes: structure, function, evolution and transcriptional regulation. Endo. Rev. 12: 316-328, 1991.

22. Owerbach, D., Rutter, W.J., Martial, J.A. and Baxter, J.D. Genes for growth hormonė, chorionic somatomammotropin and a growth hormone-like gene are located on chromosome 17 in humans. Science 209: 289-291, 1980.

23. Owerbach, D., Rutter, W.J., Cooke, N.E., Martial, J.A. and Shows, T.B. The prolactin gene is located on chromosome 6 in humans. Science 212: 815-816, 1981.

24. Goffin, V., Shiverick, K.T., Kelly, P. and Martial, J.A. Sequence-function relationships within the expanding family of prolactin, growth hormone, placental lactogen, and related proteins in mammals. Endo. Rev. 17: 385-410, 1996.

25. George, D.L., Phillips, J.A.III, Francke, U. and Seeburg, P.H. The genes for growth hormone and chorionic somatomammotropin are on the long arm of human chromosome 17 in region q21-qter. Hum. Genet. 57: 138-141, 1981.

26. Chen, E.Y., Liao, Y.C., Smith, D.H., Barrera-Saldana, H.A., Gelinas, R.E. and Seeburg, P. The human growth hormone locus: nucleotide sequence, biology and evolution. Genomics. 4: 479-497, 1989.

27. DeNoto, F.M., Moore, D.D. and Goodman, H.M. Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. Nucl. Acids Res. 9: 3719-3730, 1981.

28. Cooke, N.E., Ray, J., Emery, J.G. and Liebhaber, S.A. Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins. J. Biol. Chem. 263: 9001-9006, 1988.

29. Baumann, G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endo. Rev. 12: 424-449, 1991.

30. McWilliams, D. and Boime, I. Cytological localization of placental lactogen messenger ribonucleic acid in syncytiotrophoblast layers of human placenta. Endocrinol. 107: 761-765, 1980.

31. Seeburg, P.H. The human growth hormone gene family: nucleotide sequences show recent divergence and predict a new polypeptide hormone. DNA 1: 239-249, 1982.

32. Misra-Press, A., Cooke, N.E. and Liebhaber, S.A. Complex alternative splicing partially inactivates the human chorionic somatomammotropin-like (hCS-L) gene. J. Biol. Chem. 269: 23220-23229, 1994.

33. Golos, T.G., Durning, M., Fisher, J.M. and Fowler, P.D. Cloning of four GH/chorionic somatomammotropin-related cDNAs differentially expressed during pregnancy in the rhesus monkey placenta. Endocrinol. 133: 1744-1752, 1993.

34. Coóke, N.E., Szpirer, C. and Levan, G. The related genes encoding growth hormone and prolactin have been dispersed to chromosomes 10 and 17 in the rat. Endocrinol. 119: 2451-2454, 1986.

35. Robertson, M.C., Croze, F., Zchroedter, I.C. and Friesen, H.G. Molecular cloning and expression of rat placental lactogen-I complementary deoxyribonucleic acid. Endocrinol. 127: 702-710, 1990.

36. Deb, S., Faria, T.N., Roby, K.F., Larsen, D., Kwok, S.C.M., Talamantes, F. and Soares, M.J. Identification and characterization of a new member of the prolactin family, placental lactogen-I variant. J. Biol. Chem. 266: 1605-1610, 1991.

37. Duckworth, M.L., Kirk, K.L. and Friesen, H.G. Isolation and identification of a cDNA clone of rat placental lactogen II. J. Biol. Chem. 261: 10871-10878, 1986.

38. Campbell, W.J., Deb, S., Kwok, S.C.M., Joslin, J.A. and Soares M.J. Differential expression of placental lactogen-II and prolactin-like protein A in the rat chorioallantoic placenta. Endocrinol. 125: 1565-1574, 1989.

39. Duckworth, M.L., Peden, L.M. and Friesen, H.G. A third prolactin-like protein expressed by the developing rat placenta: complementary deoxyribonucleic acid sequence and partial structure of the gene. Mol. Endocrinol. 2: 912-920, 1988.

40. Deb, S., Roby, K.F., Faria, T.N., Larsen, D. and Soares, M.J. Identification and immunochemical characterization of a major placental secretory protein related to the prolactin-growth hormone family, prolactin-like protein-C. Endocrinol. 128: 3066-3072, 1991.

41. Roby, K.F., Deb, S., Gibori, G., Szpirer, C., Levan, G., Kwok, S.C.M. and Soares, M.J. Decidual prolactin-related protein: identification, molecular cloning, and characterization. J. Biol. Chem. 268: 3136-3142, 1993.

42. Dai, G., Liu, B., Szpirer, C., Levan, G., Kwok, S.C.M. and Soares, M.J. Prolactin-like protein-C variant: complementary deoxyribonucleic acid, unique six exon gene structure, and trophoblast cell-specific expression. Endocrinol. 137: 5009-5019, 1996.

43. Linzer, D.I.H. and Nathans, D. Nucleotide sequence of a growth-related member of the prolactin-growth hormone family. Proc. Natl. Acad. Sci. USA 81: 4255-4259, 1984.

44. Linzer, D.I.H. and Nathans, D. A new member of the prolactin-growth hormone family expressed in mouse placenta. EMBO J. 4: 1419-1423, 1985.

45. Jackson, L.L., Colosi, P., Talamantes, F. and Linzer, D.I.H. Molecular cloning of mouse placental lactogen cDNA. Proc. Natl. Acad. Sci. USA 83: 8496-8500, 1986.

46. Colosi, P., Talamantes, F. and Linzer, D.I.H. Molecular cloning and expression of mouse placental lactogen I complementary deoxyribonucleic acid. Mol. Endocrinol. 1: 767-776, 1987.

47. Lin, J., Poole, J. and Linzer, D.I.H. Three new members of the mouse prolactin/growth hormone family are homologous to proteins expressed in the rat. Endocrinol. 138: 5541-5549, 1997.

48. Lin, J., Poole, J. and Linzer, D.I.H. Two novel members of the prolactin/growth hormone family are expressed in the mouse placenta. Endocrinol. 138: 5535-5540, 1997.

49. Jackson-Grusby, L.L., Pravtcheva, D., Ruddle, F., and Linzler, D.I.H. Chromosomal mapping of the prolactin/growth hormone gene family in the mouse.

Endocrinol. 122: 2462-2466, 1988.

50. Jackson Laboratory. Mouse Genome Database, Mouse Genome Informatics. World Wide Web(URL:http://www.informatics.jax.org/), Release 32. Jackson Laboratory, Bar Harbor, 1997.

51. Wilder, E.L. and Linzer, D.I.H. Expression of multiple proliferin genes in mouse cells. Mol. Cell Biol. 6: 3283-3286, 1986.

52. Anthony, R.V., Liang, E.P.K. and Pratt, S.L. The growth hormone/prolactin gene family in ruminant placentae. J. Reprod. Fertil. Suppl. 49: 83-95, 1995.

53. Southard, J.N., Do, L., Smith, W.C. and Talamantes, F. Hamster placental lactogen-II contains a structural feature unique amoung the growth hormone-prolactin-placental lactogen family. Mol. Endocrinol. 3: 1710-1713, 1989.

54. Ebbitt, D.H., Urley, W.L., Kessler, M.A., McDonald, D.J. and Schuler, L.A. Characterization of the gene corresponding to bovine placental prolactin-related cDNA. I. Evolutionary implications. DNA 8: 161-169, 1989.

55. Kessler, M.A. and Schuler, L.A. Structure of the bovine placental lactogen gene and alternative splicing of transcripts. DNA Cell Biol. 10: 93-104, 1991.

56. Colosi, P., Thordarson, G., Hellmiss, R., Singh, K., Forsyth, A.A., Gluckman, P. and Wood, W.I. Cloning and expression of ovine placental lactogen. Mol. Endocrinol. 3: 1462-1469, 1989.

57. Dietz, A.B., Georges, M., Threadgill, D.W., Womack, J.E. and Schuler, L.A. Somatic cell mapping, polymorphism, and linkage analysis of bovine prolactin-related proteins and placental lactogen. Genomics 14: 137-143, 1992.

58. Kessler, M.A., Milosavljevic, M., Zieler, C.G. and Schuler, L.A. A subfamily of bovine prolactin-related transcripts distinct from placental lactogen in fetal placenta. Biochem. 28: 5154-5161, 1989.

59. Yamakawa, M., Tanaka, M., Koyama, M., Kagesato, Y., Watahiki, M., Yamamoto, M. and Nakashima, K. Expression of new members of the prolactin-growth hormone gene family in bovine placenta. J. Biol. Chem. 265: 8915-8920, 1990.

60. Tanaka, M., Yamakawa, M., Watahiki, M., Yamamoto, M. and Nakashima, K. Isolation of a novel prolactin-like cDNA clone from bovine placenta: occurrence of new family members. Biochem. Biophys. Acta. 1008: 193-197, 1989.

61. Goodyer, C.G. Ontogeny of pituitary hormone secretion. In: Pediatric Endocrinology, 2nd Ed., Ed. Collu, R., Ducharme, J.R., Guyda, H.J. Raven Press,

New York, pp. 125-170, 1989.

62. Theill, L.E. and Karin, M. Transcriptional control of GH expression and anterior pituitary development. Endocr. Rev. 14: 670-689, 1993.

63. Cooke, N.E., Ray, J., Watson, M.A., Estes, P.A. Kuo, B.A. and Liebhaber, S.A. Human growth hormone gene and the highly homologous growth hormone variant gene display different splicing patterns. J. Clin. Invest. 82: 270-275, 1988.

64. Lecomte, C.M., Renard, A. and Martial, J.A. A new natural hGH variant (17.5K) produced by alternative splicing. An additional consensus sequence must play a role in branch point selection. Nucl. Acids Res. 15: 6331-6348, 1987.

65. Lewis, U.J., Sinha, Y.N. and Haro, L. S. Variant forms and fragments of human growth hormone in serum. Acta Paediatr. Suppl. 299: 29-31, 1994.

66. Hill, D.J. What is the role of growth hormone and related peptides in implantation and the development of the embryo and fetus? Horm. Res. 38: 28-34, 1992.

67. Hartman, M.L., Veldhuis, J.D. and Thorner, M.O. Normal control of growth hormone secretion. Horm. Res. 40: 37-47, 1993.

68. Bertherat, J., Bluet-Pajot, M.T. and Epelbaum, J. Neuroendocrine regulation of growth hormone. Eur. J. Endocrinol. 132: 12-24, 1995.

69. Mayo, K.E., Godfrey, P.A., Suhr, S.T. Kulik, D.J. and Rahal, J.O. Growth hormone-releasing hormone: synthesis and signaling. Rec. Prog. in Horm. Res. 50: 35-73, 1995.

70. Deghenghi, R. The development of "impervious peptides" as growth hormone secretagogues. Acta Paediatr. Suppl. 423: 85-87, 1997.

71. Smith, R.G., van der Ploeg, L.H.T., Howard, A.D., Feighner, S.D., Cheng, K., Hickey, G.J., Wyvratt, J.J.Jr., Fisher, M.H., Nargund, R. and Patchett, A.A. Peptidomimetic regulation of growth hormone secretion. Endocr. Rev. 18: 621-645, 1997.

72. Howard, A.D., Feighner, S.D., Cully, D.F. et. al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273: 974-977, 1996.

73. Martha, P.M.Jr., Gorman, K.M., Blizzard, R.M., Rogol, A.D. and Veldhuis, J.D. Endogenous growth hormone secretion and clearance rates in normal boys, as determined by deconvolution analysis: relationship to age, pubertal status, and body mass. J. Clin. Endocrinol. Metab. 74: 336-344, 1992.

74. Tannenbaum, G.S. and Martin, J.B. Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinol. 98: 562-570, 1976.

75. Gevers, E.F., Wit, J.M. and Robinson, I.C.A.F. Growth, growth hormone (GH)-binding protein, and GH receptors are differentially regulated by peak and trough components of the GH secretory pattern in rat. Endocrinol. 137: 1013-1018, 1996.

76. Bluet-Pajot, M.T., Epelbaum, J., Gourdji, D., Hammond, C. and Kordon, C. Hypothalamic and hypophyseal regulation of growth hormone secretion. Cell. Mol. Neurobiol. 18: 101-123, 1998.

77. Goodyer, C., Branchaud, C.L. and Lefebvre, Y. In vitro modulation of growth hormone (GH) secretion from early to midgestation human fetal pituitaries by GH-releasing factor and somatostatin: role of G_s-adenylate cyclase-G_i complex and Ca^{2+} channels. J. Clin. Endocrinol. Metab. 76: 1265-1270, 1993.

78. Lin, C., Lin, S.C., Chang, C.P. and Rosenfeld, M.G. Pit-1 dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. Nature 360: 765-768, 1992.

79. McCormick A., Brady, H., Theill, L.E. and Karin, M. Regulation of the pituitary-specific homeobox gene GFH1 by cell-autonomous and environmental cues. Nature 345:

829-832, 1990.

80. Wu, D., Chen, C., Zhang, J., Bowers, C.Y. and Clarke, I.J. The effects of GH-releasing peptide-6 (GHRP-6) and GHRP-2 on intracellular adenosine 3',5'-monophosphate (cAMP) levels and GH secretion in ovine and rat somatotrophs. J. Endocrinol. 148: 197-205, 1996.

81. Dickson, S.L., Leng, G., Dyball, R.E.J. and Smith R.G. Central actions of peptide and nonpeptide growth hormone secretagogues in the rat. Neuroendocrinology 61: 36-43, 1995.

82. Patel, Y.C., Liu, J.L., Galanopoulou, A.S. and Papachristou, D.N. Production, action, and degradation of somatostatin. In: The Handbook of Physiology, The endocrine pancreas and regulation of metabolism. Ed. Jefferson L.S., Cherrington, A.D. Oxford University Press, New York, 1997.

83. Florio, T. and Schettini, G. Multiple intracellular effectors modulate physiological functions of the cloned somatostatin receptors. J. Mol. Endo. 17: 89-100, 1996.

84. Patel, Y.C. Molecular pharmacology of somatostatin receptor subtypes. J. Endocrinol. Invest. 20: 348-367, 1997.

85. Kumar, U., Laird, D., Srikant, C.B., Escher, E. and Patel, Y.C. Expression of the five somatostatin receptor (SSTR1-5) subtypes in rat pituitary somatotrophs: quantitative analysis by double-label immunofluorescence confocal microscopy. Endocrinol. 138: 4473-4476, 1997.

86. Carpentier, V., Vaudry, H., Mallet, E., Laquerriere, A., Tayot, J. and Leroux, P. Anatomical distribution of somatostatin receptors in the brainstem of the human fetus. Neuroscience 73: 865-879, 1996.

87. Thoss, V.S., Piwko, C., Probst, A. and Hoyer, D. Autoradiographic analysis of somatostatin $SRIF_1$ and $SRIF_2$ receptors in the human brain and pituitary. Naunyn-Schmiedeberg's Arch. Pharmacol. 355: 168-176, 1997.

88. Asa, S.L., Kovacs, K., Laszlo, F.A.,Domokos, I. and Ezrin, C. Human fetal adenohypophysis: histologic and immunocytochemical analysis. Neuroendocrinology 43: 308-316, 1986.

89. Begeot, M., Dubois, M.P. and Dubois, P.M. Growth hormone and ACTH in the pituitary of normal and anencephalic human fetuses: immunocytochemical evidence for hypothalamic influences during development. Neuroendocrinology 24: 208-220, 1977.

90. Li, J.Y., Dubois, M.P. and Dubois, P.M. Somatotrophs in the human fetal anterior

pituitary. Cell Tissue Res. 181: 545-552, 1977.

91. Kaplan, S.L., Grumbach, M.M. and Shepard, T.H. The ontogenesis of human fetal hormones: I. Growth hormone and insulin. J. Clin. Invest. 51: 3080-3093, 1972.

92. Matsuzaki, F., Irie, M. and Shizume, K. Growth hormone in the human fetal pituitary glands and cord blood. J. Clin. Endo. Metab. 33: 908-911, 1971.

93. Furuhashi, N., Takahashi, T., Fukaya, T., Kono, H., Shinkawa, O., Tachibana, Y. and Suzuki, M. Plasma somatostatin and growth hormone in the human fetus and its mother at delivery. Gynecol. Obstet. Invest. 16: 59-62, 1983.

94. Goodyer, C.G. Development of the anterior pituitary. In: Handbook of Human Growth and Developmental Biology. Ed. Timiras, P.S. and Meisami, E., CRC Press, Boca Raton, Fla., p. 21-48, 1989.

95. Goodyer, C.P., Branchaud, C.L. and Lefebvre, Y. Effects of growth hormone releasing factor and somatostatin on growth hormone secretion from early to mid-gestation human fetal pituitaries.J. Clin. Endocrinol. Metab. 76: 1259-1264, 1993.

96. Goodyer, C.G., Sellen, J.M., Fuks, M., Branchaud, C.L. and Lefèbvre, Y. Regulation of growth hormone secretion from human fetal pituitaries: interactions

between growth hormone releasing factor and somatostatin. Reprod. Nutr. Dev. 27: 461-470, 1987.

97. Shimon, I., Taylor, J.E., Dong, J.Z., Bitonte, R.A., Kim, S., Morgan, B., Coy, D.H., Culler, M. and Melmed, S. Somatostatin receptor subtype specificity in human fetal pituitary cultures: differential role of SSTR2 and SSTR5 for growth hormone, thyroid-stimulating hormone, and prolactin regulation. J. Clin. Invest. 99: 789-798, 1997.

98. Frohman, L. and Stachura, M. Evidence for possible precursors in the synthesis of growth hormone by rat and human fetal anterior pituitary in vitro. Mt. Sinai J. Med. 40: 414-421, 1973.

99. Belleville, F., Hartemann, P., Paysant, P. et al. Incorporation de la leucine marquée dans les protéines secrétées par les cellules hypophysaires en culture. C.R. Soc. Biol. 167: 305-309, 1973.

100. Eshet, R., Assa, S. and Laron, Z. Heterogeneity of pituitary and endogenous plasma human growth hormone from fetuses, premature and full-term infants. Biol. Neonate 29: 354-359, 1976.

101. Levina, S.E. Endocrine features in development of human hypothalamus,

hypophysis and placenta. Gen. Comp. Endocrinol. 11: 151-164, 1968.

102. Rice, B.F., Ponthier, R. and Sternberg, W. Luteinising hormone and growth hormone activity in the human fetal pituitary. J. Clin. Endocrinol. Metab. 28: 1071-1079, 1971.

102a. Chasalow, F., Blethen, S.L. and King, K.C. Growth hormone isoforms in growth, development and metabolic disorders. Endo. Metab. 1(suppl. B): 48, 1994.

103. Wollmann, H.A.and Ranke, M.B. Metabolic effects of growth hormone in children. Metabolism 44: 97-102, 1995.

104. Russell-Jones, D.L. and Umpleby, M. Protein anabolic action of insulin, growth hormone and insulin-like growth factor I. Eur. J. Endocrinol. 135: 631-642, 1996.

105. Spagnoli, A. and Rosenfeld, R.G. Growth and growth disorders. In: Endocrinology and Metabolism Clinics of North America, Ed. Rosenfeld, R.G., W.B. Saunders Co., pp 615-631, 1996.

106. Kelly, P., Djiane, J., Postel-Vinay, M.C. and Edery, M. The prolactin/growth hormone receptor family. Endocr. Rev. 12: 235-251, 1991.

107. Bichell, D.P., Kikuchi, K. and Rotwein, P. Growth hormone rapidly activates insulin-like growth factor I gene transcription in vivo. Mol. Endocrinol. 6: 1899-1908, 1992.

108. Ooi, G.T., Cohen, F.J., Tseng, L.Y.H., Rechler, M.M. and Boisclair, Y.R. Growth hormone stimulates transcription of the gene encoding the acid labile subunit (ALS) of the circulating insulin-like growth factor-binding protein complex and ALS promoter activity in rat liver. Mol. Endocrinol. 11: 997-1007, 1997.

109. Yoon, J.B., Towle, H.C. and Seelig, S. Growth hormone induces two mRNA species of the serine protease inhibitor family in rat liver. J. Biol. Chem. 262: 4284-4289, 1987.

110. Johnson, T.R., Rudin, s.D., Blossey, B.K. and Ilan, J. Newly synthesized RNA: simultaneous measurement in intact cells of transcription rates and RNA stability of insulin-like growth factor I, actin and albumin in growth hormone-stimulated hepatocytes. Proc. Natl. Acad. Sci. USA. 88: 5287-5291.

111. Potter, J.J., Yang, V.W. and Mezey, E. Regulation of the rat class I alcohol dehydrogenase gene by growth hormone. Biochem. Biophys. Res. Comm. 191:1040-1045, 1993.

112. Slootweg, M.C., deGroot, R.P., Hermann-Erlee, M.P.M., Koornneef, I., Kruijer,W. and Kramer, Y.M. Growth hormone induces expression of c-jun and jun B oncogenes and employs a protein kinase C signal transduction pathway for the induction of c-fos oncogene expression. J. Mol. Endocrinol. 6: 179-188, 1991.

113. Murphy, L.J., Bell, G.I. and Friesen, H.G. Growth hormone stimulates sequential induction of c-myc and insulin-like growth factor I expression in vivo. Endocrinol. 120: 1806-1812, 1987.

114. Lahuna, O., Fernandez, L., Karlsson, H., Maiter, D., Lemaigre, F.P., Rousseau, G.G., Gustafsson, J.A. and Mode, A. Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone. Proc. Natl. Acad. Sci. USA 94: 12309-12313, 1997.

115. Clarkson, R.W.E., Chen, C.M., Harrison, S., Wells, C., Muscat, G.E.O. and Waters, M.J. Early responses of trans-activating factors to growth hormone in preadipocytes: differential regulation of CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta. Mol. Endocrinol. 9: 108-120, 1995.

116. D'Ercole, A.J. Insulin-like growth factors and their receptors in growth, In: Endocrinology and Metabolism Clinics of North America, Ed. Rosenfeld, R.G., W.B. Saunders Co., pp 573-590, 1996.

117. LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, C.T.Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr. Rev. 16: 143-163, 1995.

118. Richelsen, B. Action of growth hormone in adipose tissue. Horm. Res. 48 (Suppl 5): 105-110, 1997.

119. Silverman, B.L. and Friedlander, J.R. Is growth hormone good for the heart? J. Pediatr. 131: S70-S74.

120. Feld, S. and Hirschberg, R. Growth hormone, the insulin-like growth factor system, and the kidney. Endocr. Rev. 17: 423-480, 1996.

121. Ohlsson, C., Isgaard, J., Tornell, J., Nilsson, A., Isaksson, O.G.P. and Lindahl, A. Endocrine regulation of longitudinal bone growth. Acta Paediatr. Suppl. 391: 33-40, 1993.

122. Ohlsson, C., Bengtsson, B.A., Isaksson, O.G.P., Andreassen, T.T. and Slootweg, M.C. Growth hormone and bone. Endocr. Rev. 19: 55-79, 1998.

123. Schoenle, E., Zapf, J., Humbel, R. E. et al. Insulin-like growth factor I stimulates growth in hypophysectomized rats. Nature 96: 252-253, 1982.

124. Hizuka, N., Takano, K., Shizume, K., Asakawa, K. Miyakawa, M., Tanaka, I. and Horikawa, R. Insulin-like growth factor I stimulates growth in normal growing rats. Eur. J. Pharmacol. 125: 143-146, 1986.

125. Van Buul-Offers, S., Ueda, I. and Van den Brande, J.L. Biosynthetic somatomedin C (SM-C/IGF-I) increases the length and weight of Snell dwarf mice. Pediatr. Res. 20: 825-827, 1986.

126. Skottner, A., Clark, R.G., Robinson, I.C.A.F. et al. Recombinant human insulin-like growth factor: testing the somatomedin hypothesis in hypophysectomized rats. J. Endocrinol. 112: 123-132, 1987.

127. Ranke, M.B., Savage, M.O., Chatelain, P.G. et al. Insulin-like growth factor I improves height in growth hormone insensitivity: two year's result. Horm. Res. 44: 253-264, 1995.

128. Savage, M.O., Blum, W.F., Ranke, M.B. et al. Clinical features and endocrine status in patients with growth hormone insensitivity (Laron syndrome). J. Clin. Endocrinol. Metab. 77: 1465-1471, 1993.

129. Isaksson, O.G.P., Jansson, J.O. and Gauses, I.A.M. Growth hormone stimulates longitudinal bone growth directly. Science 216: 1237-1239, 1982.

130. Bentham, J., Ohlsson, C., Lindahl, A. et al. A double staining technique for detection of growth hormone and insulin-like growth factor-I binding to rat tibial epiphyseal chondrocytes. J. Endocrinol. 137: 361-367, 1993.

131. Lindahl, A., Isgaard, J., Carlsson, L. et al. Differential effects of growth hormone and insulin-like growth factor I on colony formation of epiphyseal chondrocytes in suspension culture in rats of different ages. Endocrinol. 121: 1061-1069, 1987.

132. Lindahl, A. Nilsson, A., Isaksson, O.G.P. Effects of growth hormone and insulin-like growth factor-I on colony formation of rabbit epiphyseal chondrocytes at different stages of maturation. J. Endocrinol. 115: 263-271, 1987.

133. Collett-Solberg, P.F. and Cohen, P. The role of the insulin-like growth factor binding proteins and the IGFBP proteases in modulating IGF function. In: Endocrinology and Metabolism Clinics of North America, Ed. Rosenfeld, R.G., W.B. Saunders Co., pp 591-631, 1996.

134. Cianfarani, S., Boemi, S., Spagnoli, A. et al. Is IGF binding protein-3 assessment helpful for the diagnosis of GH deficiency? Clin. Endocrinol. 43: 43-47, 1995.

135. Hardouin, S., Gourmelen, M., Noguiz, P. et al. Molecular forms of serum insulin-like growth factor (IGF)-binding proteins in men: relationships with growth hormone and

IGFs and physiological significance. J. Clin. Endocrinol. Metab. 69: 1291-1301, 1989.

136. Glasscock, G.F., Hein, A.N., Miller, J.A. et al. Effects of continuous infusion of insulin-like growth factor I and II, alone and in combination with thyroxine or growth hormone, on the neonatal hypophysectomized rat. Endocrinol. 130: 203-210, 1992.

137. Gargosky, S.E., Wilson, K.F., Fielder, P.J. et al. The composition and distribution of insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) in the serum of growth hormone receptor-deficient patients: effects of IGF-1 therapy on IGFBP-3. J. Clin. Endocrinol. Metab. 77: 1683-1689, 1993.

138. Camacho-Hubner, C., Clemmons, D.R. and D'Ercole, A.J. Regulation of insulin-like growth factor (IGF) binding proteins in transgenic mice with altered expression of growth hormone and IGF-1. Endocrinol. 129: 1201-1206, 1991.

139. Cohen, P., Graves, H.C., Peehl, D.M. et al. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. 75: 1046-1053, 1992.

140. Chez, R.A., Hutchinson, D.L. Salazar, H. and Mintz, D.H. Some effects of fetal and maternal hypophysectomy in pregnancy. Am. J. Obstet. Gynecol. 208: 643-650, 1970.

141. Freemark, M. and Handwerger, S. Ovine placental lactogen stimulates glycogen synthesis in fetal rat hepatocytes. Am. J. Physiol. 246: E21-E24, 1984.

142. Scheven, B.A. and Hamilton, N.J. Longitudinal bone growth in vitro: effects of insulin-like growth factor I and growth hormone. Acta Endocrinol. 124: 602-607, 1991.

143. Pantaleon, M., Whiteside, E.J., Harvey, M.B., Barnard, R.T., Waters, M.J. and P.L. Kaye. Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis. Proc. Natl. Acad. Sci. USA 94: 5125-5130, 1997.

144. Fukaya, T., Yamanaka, T., Terada, Y., Murakami, T. and Yajima, A. Growth hormone improves mouse embryo develoment in vitro and the effect is neutralized by growth hormone receptor antibody. Tohoku J. of Exp. Med. 184: 113-122, 1998.

145. Zhang, C.Z., Li, H.K., Young, W.G., Bartold, P.M., Chen, C.M. and Waters, M.J. Evidence for a local action of growth hormone in embryonic tooth development in the rat. Growth Factors. 14: 131-141, 1997.

146. Strain, A.J., Hill, D.J., Swenne, I. and Milner, R.D.G. Regulation of DNA synthesis in human fetal hepatocytes by placental lactogen, growth hormone, and insulin-like growth factor I/somatomedin-C. J. Cell. Physiol. 132: 33-40, 1987.

147. D'Souza-Li, L., Krackovitch, S. and Goodyer, C.G. Actions of growth hormone (GH) and the GH receptor in human fetal hepatocyte cultures. Program of the 79th Annual Meeting of the Endocrine Society, Minneapolis, MN, p. 339 (Abstact), 1997.

148. Ontonkoski, R., Knip, M., Wong, I. and Simell, O. Effects of growth hormone and insulin-like growth factor I on endocrine function of human fetal islet-like cell clusters during longterm tissue culture. Diabetes. 37: 1678-1683, 1988.

149. Swenne, I., Hill, D.J., Strain, A.J. and Milner, R.D.G. Effects of human placental lactogen and growth hormone on the production of insulin and somatomedin C/insulin-like growth factor I by human fetal pancreas in tissue culture. J. Endocrinol. 113: 297-303, 1987.

150. Formby, B., Ulrich, A., Coussens, L., Walker, L. and Peterson, C.M. Growth hormone stimulates insulin gene expression in cultured human fetal pancreatic islets. J. Clin. Endocrinol. Metab. 66: 1075-1079, 1988.

151. Bang, P., Westgren, M., Schwander, J., Blum, W.F., Rosenfeld, R.G. and Stangeberg, M. Ontogeny of insulin-like growth factor-binding protein-1, -2, and -3: quantitative measurements by radioimmunoassay in human fetal serum. Pediatr. Res. 36: 528-536, 1994.

152. Ray, J., Jones, B.K., Liebhaber, S.A. and Cooke, N.E. Glycosylated human growth hormone variant. Endocrinol. 125: 566-568, 1989.

153. Cooke, N.E., Ray, J., Emery, J.G. and Liebhaber, S.A. Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins. J. Biol. Chem. 263: 9001-9006, 1988.

154. MacLeod, J.N., Lee, A.K., Liebhaber, S.A. and Cooke, N.E. Developmental control and alternative splicing of the placentally expressed transricipts from the human growth hormone gene cluster. J. Biol. Chem. 267: 14219-14226, 1992.

155. Lee, A.K., MacLeod, J.N., Ray, J., Cooke, N.E. and Liebhaber, S.A. The human growth hormone-variant gene encodes a novel membrane-associated protein product. Clin. Res. 296-302, 1990.

156. De Zegher, F., Vandershcueren-Lodeweychx, M., Spirz, B., Faijerson, Y., Blomberg, F., Beckers, A., Hennen, G. and Frankenne, F. Perinatal growth hormone (GH) physiology: effect of GH-releasing factor on maternal and fetal secretion of pituitary and placental GH. J. Clin. Endo. Metab. 77: 520-522, 1990.

157. Erikson, L., Frankenne, F., Eden, S., Hennen, G. and Von Schoultz, B. Growth hormone 24h serum profiles during pregnancy- lack of pulsatility for the secretion of

placental variant. Br. J. Obstet. Gynaecol. 96: 949-953, 1989.

158. Collu, R., Deal, C.L., Castagné, J., Legacé, G., Ong, H., Boulanger, L., Gaudreau, P., Goodyer, C.G., Howard, A.D., Smith, R.G. and Van Der Ploeg, L.H.T. Presence of GHRH and GHS receptors in human placenta. Program of the 79th Annual Meeting of the Endocrine Society, Minneapolis, MN, p. 158 (Abstract), 1997.

159. Frankenne, F., Closset, S., Gomez, F., Scippo, M.L., Smal, J. and Hennen, G. The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant. J. Clin. Endo. Metab. 66: 1069-1072, 1988.

160. MacLeod, J.N., Worsely, I., Ray, J., Friesen, H.G., Liebhaber, S.A. and Cooke, N.E. Human growth hormone-variant is a biologically active somatogen and lactogen. Endocrinol. 128: 1298-1302, 1992.

161. Ray, J., Okamura, H., Kelly, P.A., Cooke, N.E. and Liebhaber, S.A. Human growth hormone-variant demonstrates a receptor binding profile distinct from that of the normal pituitary growth hormone. J. Biol. Chem. 265: 7939-7944, 1990.

162. Selden, R.F., Wagner, T.E., Blethen, S., Yun, J.S., Rowe, M.E. and Goodman, H.M. Expression of the human growth hormone variant gene in cultured fibroblasts and transgenic mice. Proc. Natl. Acad. Sci. USA 85: 8241-8245, 1988.

163. Caufriez, A., Frankenne, F., Hennen, G. and Copinschi, G. Regulation of maternal IGF-1 by placental GH in normal and abnormal pregnancies. Am. J. Physiol. 265: E572-E577, 1993.

164. Mirlesse, V., Frankenne, F., Alsat, E., Poncelet, M., Hennen, G. and Evain-Brion, D. Placental growth hormone levels in normal pregnancy and in pregnancies with intrauterine growth retardation. Ped. Res. 34: 439-442, 1994.

165. Muller, J., Starup, J., Christiansen, J.S., Jorgensen, J.O.L., Juul, A. and Skakkebaek, N.E. GH treatment during pregnancy in a GH-deficient woman. Eur. J. Endocrinol. 132: 727-729, 1995.

166. Finidori, J. and Kelly, P.A. Cytokine signalling through two novel families of transducer molecules: Janus kinases and signal transducers and activators of transcription. J. Endocrinol. 147: 11-23, 1995.

167. Kitamura, T., Ogorochi, T. and Miyajima, A. Multimeric cytokine receptors. Trends Endocrinol. Metab. 5: 8-14, 1994.

168. Baumgartner, J.W., Wells, C.A., Chen, C.M. and Waters, M.J. The role of the WSXWS equivalent motif in growth hormone receptor function. J. Biol. Chem. 269: 29094-29101, 1994.

169. Goujon, L., Allevato, G., Simonin, G., Paquereau, L., Le Cam, A., Clark, J., Nielsen, J.H., Djiane, J., Postel-Vinay, M.C., Edery, M. and Kelly, P.A. Cytoplasmic domains of the growth hormone receptor necessary for signal transduction. Proc. Natl. Acad. Sci. USA 91: 957-961, 1993.

170. Dasilva, L., Howard, O.M.Z., Rui, H., Kirken, R.A. and Farrar, W.L. Growth signalling and JAK2 association mediated by membrane-proximal cytoplasmic regions of prolactin receptors. J. Biol. Chem. 269: 18267-18270, 1994.

171. Wang, Y.D. and Wood, W.I. Amino acids of the human growth hormone receptor that are required for proliferation and JAK-STAT sigalling. Mol. Endocrinol. 9: 303-311, 1995.

172. Ilkbahar, Y.N., Wu, K., Thordarson, G. and Talamantes, F. Expression and distribution of messenger ribonucleic acids for growth hormone (GH) receptor and GH-binding protein in mice during pregnancy. Endocrinol. 136: 386-392, 1995.

173. Martini, J.F., Pezet, A., Guezennec, C.Y., Edery, M., Postel-Vinay, M.C. and Kelly, P.A. Monkey growth hormone (GH) receptor gene expression: evidence for two mechanisms for the generation of the GH binding protein. J. Biol. Chem. 272: 18951-18958, 1997.

174. Hocquette, J.F., Postel-Vinay, M.C., Kayser, C., deHemptinne, B. and Amar-Costesec, A. The human liver growth hormone receptor. Endocrinol. 125: 2167-2174, 1989.

175. Cunningham, B.C., Ultsch, M., deVos, A.M., Mulkerrin, M.G., Clauser, K.R. and Wells, J.A. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science 254: 821-825, 1991.

176. deVos, A.M., Ultsch, M. and Kossiakoff, A.A. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255: 306-312, 1992.

177. Bass, S.H., Mulkerrin, M.G. and Wells, J.A. A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. Proc. Natl. Acad. Sci. USA 88: 4498-4502, 1991.

178. Clarckson, T. and Wells, J.A. A hot spot of binding energy in a hormone-receptor interface. Science 267: 383-386, 1995.

178a. Wada, M., Uchida, H., Ikeda, M., Tsunekawa, B., Natio, N., Banba, S., Tanaka, E., Hashimoto, Y. and Honjo, M. The 20 kilodalton (kDa) human growth hormone (hGH) differs from the 22-kDa hGH in the complex formation with cell surface hGH

receptor and hGH-binding protein circulating in human plasma. Mol. Endo. 12: 146-156, 1998.

179. Sobrier, M.L., Duquesnoy, P., Duriez, B., Amselem, S. and Goossens, M. Expression and binding properties of two isoforms of the human growth hormone receptor. FEBS 319: 16-24, 1993.

180. Urbanek, M., Russell, J.E., Cooke, N.E. and Liebhaber, S.A. Functional characterization of the alternatively spliced, placental human growth hormone receptor. J. Biol. Chem. 268: 19025-19032, 1993.

181. Goodman, H.M., Frick, G.P. and Souza, S. Species specificity of the primate growth hormone receptor. News Physiol. Sci. 11: 157-161, 1996.

182. Behncken, S.N., Rowlinson, S.W., Rowland, J.E., Conway-Campbell, B.L., Monks, T.A. and Waters, M.J. Aspartate 171 is the major primate-specific determinant of human growth hormone. J. Biol. Chem. 272: 27077-27083, 1997.

183. Cunningham, B.C., Bass, S., Fuh, G. and Wells, J.A. Zinc mediation of the binding of human growth hormone to the human prolactin receptor. Science 250: 1709-1712, 1990.

183a. Perterson, F.C. and Brooks, C.L. Identification of a motif associated with the lactogenic actions of human growth hormone. J. Biol. Chem. 272: 21444-21448, 1997.

184. Argetsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N. and Carter-Su, C. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74: 237-244, 1993.

185. Silva, C.M., Lu, H., Weber, M.J. and Thorner, M.O. Differential tyrosine phosphorylation of JAK1, JAK2, and STAT1 by growth hormone and interferon-gamma in IM-9 cells. J. Biol. Chem. 269: 27532-27539, 1994.

186. Smit, L.S., Meyer, D.J., Billestrup, N., Norstedt, G., Schwartz, J. and Carter-Su, C. The role of growth hormone receptor and JAK1 and JAK2 kinases in the activationof STATS 1, 3 and 5 by growth hormone. Mol. Endocrinol. 10: 519-533, 1996.

187. Johnston, J.A., Kawamura, M., Kirken, R.A., Chen, Y.Q., Blake, T.B., Shibuya, K., Ortaldo, J.R., McVicar, D.W. and O'Shea, J.J. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature Lond. 370: 151-153, 1994.

188. Sotiropoulos, A., Perrot-Applanat, M., Dinerstein, H., Pallier, A., Postel-Vinay, M.C., Finidori, J. and Kelly, P.A. Distinct cytoplasmic regions of the growth hormone receptor are required for activation of JAK2, mitogen activated protein kinase, and

transcription. Endocrinol. 135: 1292-1298, 1994.

189. Watanabe, S. and Arai, K. Roles of the JAK-STAT system in signal transduction via cytokine receptors. Curr. Opin. Gen. & Dev. 6: 587-595, 1996.

190. Treisman, R. Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. 8: 205-215, 1996.

191. Vanderkuur, J., Allevato, G., Billestrup, N., Norstedt, G. and Carter-Su, C. Growth hormone-promoted tyrosyl phosphorylation of Shc proteins and Shc association with Grb2. J. Biol. Chem. 270: 7587-7593, 1995.

192. Tollet, P., Haberg, M., Gustafsson, J.A. and Mode, A. Growth hormone signalling leading to CYP2C12 gene expression in rat hepatocytes involves phospholipase A_2. J. Biol. Chem. 270: 12569-12577, 1995.

193. Amselem, S., Sobrier, M.L., Duquesnoy, P., Rappaport, R, Postel-Vinay, M.C., Gourmelon, M. and Dallapiccola, B. Recurrent nonsense mutations in the growth hormone receptor from patients with Laron dwarfism. J. Clin. Invest. 87: 1098-11102, 1991.

194. Rivera, V.M., Miranti, C.K., Misra, R.P., Ginty, D.D., Chen, R.H., Blenis, J.

and Greenberg, M.E. A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. Mol. Cell. Biol. 13:6260-6273, 1993.

195. Hill, C.S. and Treisman, R. Transcriptional regulation by extra-cellular signals: mechanisms and specificity. Cell 80: 199-211, 1995.

196. Ridderstrale, M., Degerman, E. and Tornqvist, H. Growth hormone stimulates the tyrosine phosphorylation of the insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase in primary adipocytes. J. Biol. Chem. 270: 35471-3474, 1995.

197. Argetsinger, L.M., Hsu, G.W., Myers, M.G.Jr., Billestrup, N., Norstedt, G., White, M.F. and Carter-Su, C. Growth hormone, interferon-gamma and leukemia inhibitory factor promoted tyrosyl phosphorylation of insulin receptor substrate-1. J. Biol. Chem. 270: 14685-14692, 1995.

198. Ridderstrale, M. and Tornqvist, H. PI-3-kinase inhibitor wortmannin blocks the insulin-like effects of growth hormone in isolated rat adipocytes. Biochem. Biophys. Res. Commun. 203: 306-310, 1994.

199. Cheatham, B., Vlahos, C.J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C.R.

Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis and glucose transporter translocation. Mol. Cell. Biol. 14: 4902-4911, 1994.

200. Billestrup, N., Bouchelouche, P., Allevato, G., Ilondo, M. and Nielsen, J.H. Growth hormone receptor C-terminal domains required for growth hormone-induced intracellular free Ca^{2+} oscillations and gene transcription. Proc. Natl. Acad. Sci. USA 92: 2725-2729, 1995.

201. Bredt, D.S. and Snyder, S.H. Nitric oxide: a physiologic messenger molecule. Annu. Rev. Biochem. 63: 175-195, 1994.

202. Corbalangarcia, S. Degenhardt, K.R. and Barsagi, D. Insulin-induced dissociation of SOS from Grb2 does not contribute to the down regulation of Ras activation. Oncogene 12: 1063-1068, 1996.

203. Fernandez, L., Flores-Morales, A., Lahuna, O., Sliva, D., Norstedt, G., Haldosen, L.A., Mode, A. and Gustafsson, J.A. Desensitization of the growth hormone-induced Janus kinase 2 (Jak2)/signal transducer and activator of transcription 5 (Stat 5)-signaling pathway requires protein synthesis and phospholipase C. Endocrinol. 139: 1815-1824, 1998.

204. Roupas, P. and Herington, A.C. Intracellular processing of growth hormone receptors by adipocytes in primary culture. Mol. Cell. Endocrinol. 57: 93-99, 1988.

205. Eshet, R., Peleg, S. and Laron, Z. Direct visualization of binding, aggregation and internalization of human growth hormone in cultured human lymphocytes. Acta Endocrinol. 107: 9-15, 1984.

206. Allevato, G., Billestrup, N., Goujon, L., Galsgaard, E.D., Norstedt, G., Postel-Vinay, M.C., Kelly, P.A. and Neilsen, J.H. Identification of phenylalanine 346 in the rat growth hormone receptor as being critical for ligand-mediated internalization and down-regulation. J. Biol. Chem. 270: 17210-17214, 1995.

207. Goldsmith, J.F., Lee, S.J., Jiang, J. and Frank, S.J. Growth hormone induces detergent insolubility of GH receptors in IM-9 cells. Am. J. Physiol. Endocrinol. Metab. 273: E932-E941, 1997.

208. Strous, G.J., van Kerkhof, P., Govers, R., Rotwein, P. and Schwartz, A.L. Growth hormone-induced signal transduction depends on an intact ubiquitin system. J. Biol. Chem. 272: 40-43, 1997.

209. Lobie, P.E., Barnard, R. and Waters, M.J. The nuclear growth hormone receptor binding protein: antigenic and physiochemical characterization. J. Biol. Chem. 266:

22645-22652, 1991.

210. Waters, M.J., Rowlinson, S.W., Clarkson, R.W. et al. Signal transduction by the growth hormone receptor. PSEBM 206: 216-220, 1994.

210a. Waters, M.J. Growth hormone signalling to the nucleus. Program of the 10th International Congress of Endocrinology, San Francisco, CA. p. 35 (Abstact), 1996.

210b. Lobie, P.E., Woods, T.J.J., Chen., C.M., Waters, M.J. and Norstedt, G. Nuclear translocation and anchorage of the growth hormone receptor. J. Biol. Chem. 269: 31735-313746, 1994.

210c. Lobie, P.E., Mertani, H., Morel, G., Morales-Bustos, O., Norstedt, G. and Maters, M.J. Receptor mediated nuclear translocation of growth hormone. J. Biol. Chem. 269: 21330-21339, 1994.

211. Jans, D. Nuclear signalling pathways for polypeptide ligands and their membrane receptors? FASEB J. 8: 841-847, 1994.

212. Lobie, P.E., Ronsin, B., Silvennoinen, O., Haldosen, L.A., Norstedt, G. and Morel, G. Constitutive nuclear localization of Janus kinases 1 and 2. Endocrinol. 137: 4037-4045, 1996.

213. Love, D.W., Whatmore, A.J., Clayton, P.E. and Silva, C.M. Growth hormone stimulation of the mitogen-activated protein kinase pathway is cell type specific. Endocrinol. 139: 1965-1971, 1998.

214. Yamauchi, T., Uek, K., Tobe, K. et al. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature 390: 91-96, 1997.

215. Simard, M., Manthos, H., Giad, A., Lefèbvre, Y. and Goodyer, C.G. Ontogeny of growth hormone receptors in human tissues: an immunohistochemical study. J. Clin. Endocrinol. Metab. 81: 3097-3102, 1996.

216. Roupas, P. and Herrington, A.C. Cellular mechanisms in the processing of growth hormone and its receptor. Mol. Cell. Endocrinol. 61: 1-12, 1989.

217. Frick, G.P., Leonard, J.L. and Goodman, H.M. Effect of hypophysectomy on growth hormone receptor gene expression in rat tissues. Endocrinol. 126: 3076-3082, 1990.

218. Mulumba, N., Massa, G., Ketelslegers, J.M. and Maes, M. Ontogeny and nutritional regulation of the serum growth hormone-binding protien in the rat. Acta Endocrinol. 125: 409-415, 1991.

219. Saito, Y., Teshima, R., Yamazaki, T., Ikebuchi, H. and Sawada, J. Ligand-induced internalization and phosphorylation-dependent degradation of growth hormone in human IM-9 cells. Mol. Cell. Endocrinol. 106: 67-74, 1994.

220. Li, J., Owens, J.A., Owens, P.C., Saunders, J.C., Fowden, A.L. and Gilmour, R.S. The ontogeny of hepatic growth hormone receptor and insulin-like growth factor I gene expression in the sheep fetus during late gestation: developmental regulation by cortisol. Endocrinol. 137: 1650-1657, 1996.

221. Brameld, J.M., Weller, P.A., Saunders, J.C., Buttery, P.J. and Gilmour, R.S. Hormonal control of insulin-like growth factor-I and growth hormone receptor mRNA expression by porcine hepatocytes in culture. J. Endocrinol. 146: 239-245, 1995.

222. King, A.P.J., Tseng, M.J., Logsdon, C.D., Billestrup, N. and Carter-Su, C. Distinct cytoplasmic domains of the growth hormone receptor are required for glucocorticoid- and phorbal ester-induced decreases in growth hormone (GH) binding. J. Biol. Chem. 271: 18088-18094, 1996.

223. Swolin-Eide, D., Nilsson, A. and Ohlsson, C. Cortisol increases growth hormone-receptor expression in human osteoblast like cells. J. Endocrinol. 156: 99-105, 1998.

224. Baxter, R.C., Bryson, J.M. and Turtle, J.R. Somatogenic receptors of rat liver:

regulation by insulin. Endocrinol. 107: 1176-1181, 1980.

225. Yu, Y.M., Domene, H.M., Sztein, J., Counts, D.R. and Cassorla, F. Developmental changes and differential regulation by testosterone and estradiol of growth hormone receptor expression in the rabbit. Eur. J. Endocrinol. 135: 583-590, 1996.

226. Mullis, P.E., Wagner, J.K., Eble, A., Nuoffer, J.M and Postel-Vinay, M.C. Regulation of human growth hormone receptor gene transcription by human growth hormonė binding protien. Mol. Cell. Endocrinol. 131: 89-96, 1997.

227. O'Mahoney, J.V., Brandon M.R. and Adams, T.E. Identification of a liver-specific promoter for the ovine growth hormone receptor. Mol. Cell. Endocrinol. 101: 129-139, 1994.

228. Adams, T.E. Differential expression of growth hormone receptor messenger RNA from a second promoter. Mol. Cell. Endocrinol. 108: 23-33, 1995.

229. Heap, D., Lucy, M.C., Collier, R.J., Boyd, C.K. and Warren, W.C. Nucleotide sequence of the promoter and first exon of the somatotropin receptor gene in cattle. J. Anim. Sci. 73: 1529, 1995.

230. Menon, R.K., Stephan, D.A., Singh, M., Morris, S.M. and Zou, L. Cloning of

the promoter-regulatory region of the murine growth hormone receptor. J. Biol. Chem. 270: 8851-8859, 1995.

231. Baumbach, W.R. and Bingham, B. One class of growth hormone (GH) receptor and binding protein mRNA in rat liver, GHR1, is sexually dimorphic and regulated by GH. Endocrinol. 136: 749-760, 1995.

232. Tiong, T.S. and Herington, A.C. Ontogeny of messenger RNA for the rat growth hormone receptor and serum binding protein. Mol. Cell. Endocrinol. 83: 133-141, 1992.

233. Walker, J.L., Moats-Staats, B.M., Stiles, A.D. and Underwood, L.E. Tissue-specific developmental regulation of the messenger ribonucleic acids encoding the growth hormone receptor and the growth hormone binding protein in rat fetal and postnatal tissues. Pediatr. Res. 31: 335-339, 1992.

234. Southard, J.N, Barrett, B.A., Bikbulatova, L., Ilkbahar, Y., Wu, K. and Talamantes, F. Growth hormone (GH) receptor and GH-binding protein messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed in mouse liver and placenta. Endocrinol. 136: 2913-2921, 1995.

235. Garcia-Aragon, J., Lobie, P., Muscat, G.E.O., Gobius, K.S., Norstedt, G. and Waters, M. Prenatal expression of the growth hormone (GH) receptor/binding protein

in the rat: a role for GH in embryonic and fetal development? Development 114: 869-876, 1992.

236. Ohlsson, C., Lovstedt, K., Holmes, P.V., Nilsson, A., Carlsson, L. and Tornell, J. Embryonic stem cells express growth hormone receptors: regulation by retinoic acid. Endocrinol. 133: 2897-2903, 1993.

237. Ymer, S.I. and Herington, A.C. Developmental expression of the growth hormone receptor gene in rabbit tissues. Mol. Cell. Endocrinol. 83: 39-49, 1992.

238. Schnoebelen-Combes, S., Louveau, I., Postel-Vinay, M.C. and Bonneau, M. Ontogeny of GH receptor and GH-binding protein in the pig. J. Endocrinol. 148: 249-255, 1996.

239. Badinga, L., Collier, R.J., Thatcher, W.W., Wilcox, C.J., Head, H.H. and Bazer, F.W. Ontogeny of hepatic bovine growth hormone receptors in cattle. J. Anim. Sci. 69: 1925-1934, 1991.

240. Breier, B.H., Funk, B., Surus, A., Ambler, G.R., Wells, C.A., Waters, M.J. and Gluckman, P.D. Characterization of ovine growth hormone (oGH) and ovine placental lactogen (oPL) binding to fetal and adult hepatic tissue in sheep: evidence that oGH and oPL interact with a common receptor. Endocrinol. 135: 919-928, 1994.

241. Hill, D.J., Riley, S.C., Bassett, N.S. and Waters, M.J. Localization of the growth hormone receptor, identified by immunocytochemistry, in second trimester human fetal tissues and in placenta throughout gestation. J. Clin. Endocrinol. Metab. 75: 646-650, 1992.

241a. Goodyer, P.R., Torban, E., Zogopoulos, G., Ferretti, E., Guyda, H.J. and Goodyer, C.G. Role of growth hormone during development of human fetal kidney. Endo. Metab. 1(suppl. B): 54, 1994.

242. Barnard, R. and Waters, M.J. The serum growth hormone binding protein: pregnant with possibilities. J. Endocrinol. 153:1-14, 1997.

243. Werther, G.A., Haynes, K., Edmondson, S., Oakes, S., Buchanan, C.J., Herington A.C. and Waters, M.J. Identification of growth hormone receptors on human growth plate chondrocytes. Acta Pediatr. Suppl. 391: 50- 53, 1993.

244. Figueiredo, R.M.O. and Goodyer, C.G. Characterization of the growth hormone receptor in human dermal fibroblasts during development. 75th Annual Meeting of the Endocrine Society, Las Vegas, NV, (Abstract), 1993.

244a. Barnard, R., Quirk, P. and Waters, M.J. Characterization of the growth hormone-binding protein of human serum using a panel of monoclonal antibodies. J. Endocrinol.

123: 327-332, 1989.

245. Godowski, P.J., Leung, D.W., Meacham, L.R., Galgani, J.R., Hellmiss, R., Keret, R., Rotwein,P.S., Parks, J.S., Laron, Z. and Wood, W.I. Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. Proc. Natl. Acad. Sci. USA 86: 8083-8037, 1989.

246. Edens, A., Southard, J.N. and Talamantes, F. Mouse growth hormone-binding protein and growth hormone receptor transcripts are produced from a single gene by alternative splicing. Endocrinol. 135: 2802-2805, 1994.

247. Urbanek, M., Macleod, J.N., Cooke, N.E. and Liebhaber, S. Expression of a human growth hormone (hGH) receptor isoform predicted by tissue-specific alternative splicing of exon 3 of the hGH receptor gene transcript. Mol. Endocrinol. 6: 279-287, 1992.

248. Esposito, N., Paterlini, P., Kelly, P.A, Postel-Vinay, M.C. and Finidori, J. Expression of two isoforms of the human growth hormone receptor in normal liver and hepatocarcinoma. Mol. Cell. Endocrinol. 103: 13-20, 1994.

249. Dastot, F., Sobrier, M.L., Duquesnoy, P., Buriez, B., Goossens, M. and Amselem, S. Alternatively spliced forms in the cytoplasmic domain of the human growth

hormone (GH) receptor regulate its ability to generate a soluble GH-binding protein. Proc. Natl. Acad. Sci. USA 93: 10723-10728, 1996.

250. Ross, R.J.M., Esposito, N., Shen, X.Y., Von Laue, S., Chew, S.L., Dobson, P.R.M., Postel-Vinay, M.C. and Finidori, J. A short isoform of the human growth hormone receptor functions as a dominant negative inhibitor of the full-length receptor and generates large amounts of binding protein. Mol. Endocrinol. 11: 265-273, 1997.

251. Amit, T., Bergman, T., Dastot, F., Youdim, M.B.H., Amselem, S. and Hochberg, Z. A membrane-fixed, truncated isoform of the human growth hormone receptor. J. Clin. Endocrinol. Metab. 82: 3813-3817, 1997.

252. Pekhletsky, R.I, Chernov, B.K. and Rubtsov, P.M. Variants of the 5'-untranslated sequence of human growth hormone receptor mRNA. Mol. Cell. Endocrinol. 90: 103-109, 1992.

253. Kozak, M. Adherence to the first-AUG rule when a second AUG codon follows closely upon the first. Proc. Natl. Acad. Sci. USA 92: 2662-2666, 1995.

254. Jansen, M., DeMoor, C.H., Sussenbach, J.S. and Van den Brande, J.L. Translational control of gene expression. Pediatr. Res. 37: 681-686, 1995.

255. Hauser, S.D., McGrath, M.F., Collier, R.J. and Krivi, G.G. Cloning and in vivo expression of bovine growth hormone receptor mRNA. Mol. Cell. Endocrinol. 72:187-200, 1990.

256. Heap, D., Collier, R.J., Boyd, C.K. and Lucy, M.C. Expression of alternate growth hormone receptor mRNA in ovary and uterus of cattle. Domes Anim. Endocrinol. 13:421-430, 1996.

257. Domené, H.M., Cassorla, F., Werner, H., Roberts, C.T.Jr. and Leroith, D. Rat growth hormone receptor/growth hormone-binding protein mRNA's with divergent 5'-untranslated regions are expressed in a tissue-specific manner. DNA and Cell Biology 14: 195-204, 1995.

258. Underwood, L.E. and Van Wyk, J.J. Ch. 21, Normal and aberrant growth. In: Williams Textbook of Endocrinology 8th ed., Ed. Wilson, J.D. and Foster, D.W., W.B. Saunders Co., Montreal, p. 1079-1138, 1992.

259. Juul, A., Dalgaard, P., Blum, W.F., Bang, P., Hall, K., Michaelsen, K.F., Muller, J. and Skakkebaek, N.E. Serum levels of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) in healthy infants, children, and adolescents: the relation to IGF-I, IGF-II, IGFBP-1 and IGFBP-2, age, sex, body mass index, and pubertal maturation. J. Clin. Endocrinol. Metab. 80: 2534-2542, 1995.

260. Leger, J., Oury, J.F., Noel, M., Baron, S., Benali, K., Blot, P. and Czernichow, P. Growth factors and intrauterine growth retardation. I. Serum growth hormone, insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein 3 levels in normally grown and growth retarded human fetuses during the second half of gestation. Pediatr. Res. 40: 94-100, 1996.

261. Sussenbach, J.S., Rodenburg, R.J.T., Scheper, W. and Holthuizen, P.E. Transcriptional and post- transcriptional regulation of the human IGF-II gene expression. In: Current Directions in IGF Research, Ed. D Leroith and MK Raizada, Plenum Press, NY, p 63-71, 1994.

262. Gluckman, P.D., Cutfield, W., Harding, J.E., Milner, D., Jensen, E., Woodhall, S., Gallaher, B., Bauer, M. and Breier, B.H. Metabolic consequences of intrauterine growth retardation. Acta Paediatr. Suppl. 417: 3-6, 1996.

263. Liu, L., Harding, J.E., Evans, P.C. and Gluckman, P.D. Maternal IGF-1 infusion alters fetal-placental carbohydrate and protein metabolism in pregnant sheep. Endocrinol. 135: 895-900, 1994.

264. DiChiara, R., Efstratiadis, A. and Robertson, E. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth II gene disrupted by targeting. Nature 345: 78-80, 1990.

265. DiChiara, R., Robertson, E. and Efstratiadis, A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell 64: 849-859, 1991.

266. Forejt, J., Gregorova, S. Genetic analysis of genomic imprinting: an imprinter-I gene controls inactivation of the paternal copy of the mouse Tme locus. Cell 70: 443-450, 1992.

267. Filson, A.J., Louvi, A., Efstratiadis, A. and Robertson, E.J. Rescue of the T-associated maternal effect in mice carrying null mutations in Igf-2 and Igf-2r, two reciprocally imprinted genes. Development 118: 731-736, 1993.

268. Powell-Braxton, L., Hollingshead, P., Giltiman, D. et al.Inactivation of the IGF-I gene in mice results in perinatal lethality. Ann. New York Acad. Sci. 692: 300-304, 1993.

269. Baker, J., Liu, J.P., Robertson, E.J. et al. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75: 73-82, 1993.

270. Liu, J.P., Baker, J., Perkins, A.S. et al. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-I) and type I IGF receptor (Igf-Ir). Cell 75: 59-72, 1993.

271. Gluckman, P.D. The endocrine regulation of fetal growth in late gestation - the role of insulin-like growth factors. J. Clin. Endocrinol. Metab. 80: 1047-1050, 1995.

272. Woods, K.A., Camacho-Hubner, C., Barter, D., Clark, A.J.L. and Savage, M.O. Insulin-like growth factor I gene deletion causing intrauterine growth retardation and severe short stature. Acta Paediatr. Suppl. 423: 39-45, 1997.

273. Menon, R.K. and Sperling, M.A. Insulin as a growth factor. In: Endocrinology and Metabolism Clinics of North America, Ed. Rosenfield, R.L., W.B. Saunders Co., pp. 633-647, 1996.

274. Han, V.K.M. The ontogeny of growth hormone, insulin-like growth factors and sex steroids: molecular aspects. Horm. Res. 45: 61-66, 1996.

275. Rajaram, S., Baylink, D.J. and Mohan, S. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr. Rev. 18: 801-831, 1997.

276. Han, V.K.M., Matsell, D.G., Delhanty, P.J.D., Hill, D.J., Shimasaki, S. and Nygard, K. IGF-binding protein mRNAs in the human fetus: tissue and cellular distribution of developmental expression. Horm. Res. 45: 160-166, 1996.

277. Mayhew, T.M. Recent applications of the new stereology have thrown fresh light on how the human placenta grows and develops its form. J. Microscopy 186: 153-163, 1997.

278. Handwerger, S. Clinical counterpoint: the physiology of placental lactogen in human pregnancy. Endocr. Rev. 12: 329-336, 1991.

279. Stephanou, A. and Handwerger, S. Retinoic acid and thyroid hormone regulate placental lactogen expression in human trophoblast cells. Endocrinol. 136: 933-938, 1995.

280. Stephanou, A., Ross, R. and Handwerger, S. Regulation of human placental lactogen expression by 1,25-dihydroxyvitamin D3. Endocrinol. 135: 2651-2656, 1994.

281. Hill, D.J., Freemark, M., Strain, A.J., Handwerger, S. and Milner, R.D.G. Placental lactogen and growth hormone receptors in human fetal tissues: relationship to fetal plasma human placental lactogen concentrations and fetal growth. J. Clin. Endocrinol. Metab. 66: 1283-1290, 1988,

282. Hill, D.J., Crace, C.J. and Milner, R.D.G. Incorporation of [^{3}H] thymidine by isolated fetal myoblasts and fibroblasts in response to human placental lactogen (HPL): possible mediation of HPL action by release of immunoreactive SM-C. J. Cell. Physiol.

125: 337-344, 1985.

283. Rygaard, K., Revol, A., Esquivel-Escobedo, D., Beck, B.L. and Barrera-Saldana, H.A. Absence of human placental lactogen and placental growth hormone (HGH-V) during pregnancy: PCR analysis of the deletion. Hum. Genet. 102: 87-92, 1998.

284. Lee, S.J., Talamantes, F., Wilder, E., Linzer, D.I.H. and Nathans, D. Trophoblastic giant cells of the mouse placenta as the site of proliferin synthesis. Endocrinol. 122: 1761-1768, 1988.

285. Nieder, G.L. and Jennes, L. Production of mouse placental lactogen-I by trophoblast giant cells in utero and in vitro. Endocrinol. 126: 2809-2814, 1990.

286. Faria, T.N., Ogren, L., Talamantes, F., Linzer, D.I.H. and Soares, M.J. Localization of placental lactogen-I in trophoblast giant cells of the mouse placenta. Biol. Reprod. 44: 327-331, 1991.

287. Yamaguchi, M., Ogren, L., Endo, H., Thordarson, G., Bigsby, R.M. and Talamantes, F. Production of mouse placental lactogen-I and placental lactogen-II by the same giant cell. Endocrinol. 131: 1595-1602, 1992.

288. Carney, E.W., Prideaux, V., Lye, S.J. and Rossant, J. Progressive expression of

trophoblast-specific genes during formation of mouse trophoblast giant cells in vitro. Mol. Reprod. Dev. 34: 357-368, 1993.

289. Yamaguchi, M., Ogren, L., Endo, H., Soares, M.J. and Talamantes, F. Co-localization of placental lactogen-I , placental lactogen-II, and proliferin in the mouse placenta at mid-pregnancy. Biol. Reprod. 51: 1188-1192, 1994.

290. Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E. and Brinster, R.L. Metallothionein-human GH fusion genes stimulate growth of mice. Science 222: 809-814, 1983.

291. Zhou, Y., Xu, B.C., Maheshwari, H.G., He, L., Reed, M., Lozykowski, M., Okada, S., Cataldo, L., Coschigamo, K., Wagner, T.E., Baumann, G. and Kopchick, J.J. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). Proc. Natl. Acad. Sci. USA 94: 13215-13220, 1997.

292. Woods, K.A., Weber, A. and Clark, A.J.L. The molecular pathology of pituitary hormone deficiency and resistance. Bailliere's Clin. Endocrinol. and Metab. 9: 453-487, 1995.

292a. Johnston, L.B., Woods, K.A., Rose, S.J., Clark, A.J.L. and Savage, M.O. The

broad spectrum of inherited growth hormone insensitivity syndrome. Trends Endocrinol. Metab. 9: 228-232, 1998.

292b. Ayling, R.M, Ross, R., Towner, P. et al A dominant-negative muntation of the growth hormone receptor causes familial short stature. Nat. Genet. 16: 13-14, 1997.

292c. Iida, K., Takahashi, Y., Kaji, H. et al Growth hormone (GH) insensitivity syndrome with high serum GH-binding protein levels caused by a heterozygous splice site mutation of the GH receptor gene producing a lack of intracellular domain. J. Clin. Endocrinol. Metab. 83: 531-537, 1998.

292d. Thanakitcharu, K., Woods, K.A., Mullis, P.E, Savage, M.O. and Clark, A.J.L. Severe growth hormone (GH) insensitivity associated with homozygous missense mutations in intracellular coding regions of the GH receptor. Horm. Res. 48: 457, 1997.

293. Tatzumi, K., Miyai, K., Notomi, T. et al. Cretinism with combined hormone deficiency caused by a mutation in the pit-1 gene. Nature Genetics 1: 56-58, 1992.

294. Radovick, S., Nations, M., Du, Y. et al. A mutation in the POU-domain of pit-1 is responsible for combined pituitary deficiency. Science 257: 1115-1118, 1992.

295. Ohta, K., Nobukuni, Y., Mitsubushi, H. et al, Mutations in the PIT-1 gene in

children with combined pituitary hormone deficiency. Biochem. Biophys. Res. Comm. 189: 851-855, 1992.

296. Pfaffle, R.W., DiMattia, G.E., Parks, J.S. et al. Mutation of the POU-specific domain of pit-1 and hypopituitarism without pituitary hypoplasia. Science 257: 1118-1121, 1992.

297. Li, S., Crenshaw, E.B. III, Rawson, E.J. et al. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature 347: 528-533, 1990.

298. Phillips, J.A.III and Cogan, J. Genetic basis of endocrine disease, 6. Molecular basis of familial human growth hormone deficiency. J. Clin. Endocrinol. Metab. 78: 11-16, 1994.

299. Lin, S.C., Lin, C.R., Gukovsky, I., Lusis, A.J., Sawchenko, P.E. and Rosenfeld, M.G. Molecular basis of the little mouse phenotype and implications for cell type-specific growth. Nature 364: 208-213, 1993.

300. Wajnrajch, M.P., Gertner, J.M., Harbison, M.D., Chua, S.C.Jr. and Leibel, R. Nonsense mutation in the human growth hormone-releasing hormone receptor causes growth failure analogous to the little (lit) mouse. Nature Genetics 12: 88-90, 1996.

301. Baumann, G. and Maheshwari, H. The Dwarfs of Sindh: severe growth hormone (GH) deficiency caused by a mutation in th GH-releasing hormone receptor gene. Acta Paediatr. Suppl. 423: 33-38, 1997.

302. Gluckman, P.D., Gunn, A.J., Cutfield, W.S., Guilbaud, O., Ambler, G.R., Wilton, P. and Wikland, K.A. Congenital idiopathic growth hormone deficiency associated with prenatal and early postnatal growth failure. J. Pediatr. 121: 920-923, 1992.

303. AD Goddard, P Dowd, S Chernausek, M Geffner, J Gertner, R Hintz, N Hopwood, S Kaplan, L Plotnick, A Rogol, R Rosenfeld, P Saenger, N Mauras, R Hershkopf, M Angulo and K Attie. Partial growth hormone insensitivity: the role of growth hormone receptor mutations in idiopathic short stature. J Pediatr 131:S51-55, 1997.

304. KM Attie, LMS Carlsson, AC Rundle and BM Sherman Evidence for partial growth hormone insensitivity among patients with idiopathic short stature. J Pediatr 127:244-250, 1995.

305. Kratsch, J., Schreiber, G., Selisko, T., Keller, E., Pflaum, C.D. and Strasburger, C.J. Measurement of serum exon 3-retaining growth hormone binding protein in children and adolescents by radioimmunoassay. Horm. Res. 48:252-257, 1997.

306. Jin, C.F., Mata, M. and Fink, D.J. Rapid construction of deleted DNA fragments for use as internal standards in competitive PCR. PCR Methods Appl 3: 252-255, 1994.

307. Prestridge, D.S. SIGNAL SCAN: A computer program that scans DNA sequences for eukaryotic transcriptional elements. CANBIOS 7: 203-206, 1991.

308. Wingender, E., Kel, A.E., Kel, O.V., Karas, H., Heinemeyer, T., Dietze, P., Knuppel, R., Romaschenko, A.G. and Kolchanov, N.A. TRANSFAC, TRRD and COMPEL: towards a federated database system on transcriptional regulation. Nucleic Acids Res. 25: 265-268, 1997.

309. BAC protocols, WWW: http://tree.caltech.edu/protocols/BACs

310. PAC protocols, WWW: http://bacpac.med.buffalo.edu

311. Wickelgren R.B., Landin K.L.L., Ohlsson C. and Carlsson L.M.S. Expression of exon 3-retaining and exon 3-excluding isoforms of the human growth hormone (GH) receptor is regulated in an individual- rather than a tissue-specific manner. J. Clin. Endocrinol. Metab. 80: 2154-2157, 1995.

312. Rio, D.C. Splicing of pre-mRNA: mechanism, regulation and role in development. Curr. Opin. Gen. Dev. 3: 574-584, 1993.

313. Stallings-Maan, M.L., Ludwizak, R.L., Linger, K.W. and Rottman, F. Alternative splicing of exon 3 of the human growth hormone receptor is the result of an unusual genetic polymorphism. Proc. Natl. Acad. Sci. USA 93: 12394-12399, 1996.

314. Krawczak, M., Reiss, J. and Cooper, D.N. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes, causes and consequences. Hum. Genet. 90: 41-54, 1992.

315. Jin, Y., Pasumarthi, K.B.S., Bock, M.E., Lytras, A., Kardami, E. and Cattini, P.A.
Cloning and expression of fibroblast growth factor receptor-1 isoforms in the mouse heart: evidence for isoform switching during heart development. J. Mol. Cell. Cardiol. 26: 1449-1459, 1994.

316. Elson, D.A. and Bortolomei, M.S. A 5' differentially methylated sequence and the 3'-flanking region are necessary for H19 transgene imprinting. Mol. Cell. Biol. 17: 309-317, 1997.

317. Kratzsch, J., Shcreiber, G., Selisko, T., Keller, E., Pflaum, C.D. and Strasburger, C.J. Measurement of serum exon 3-retaining growth hormone-binding protein in children and adolescents by radioimmunoassay. Horm. Res. 48: 252-257, 1997.

317a. Carlson, L.M., Rowland, A.M., Clark, R.G., Gesundheit, N. and Wong, W.L. Ligand-mediated immunofunctional assay for quantitation of growth hormone-binding protein in human blood. J. Clin. Endocrinol. Metab. 73: 1216-1223, 1991.

318. Rodenburg, R.J.T. Transcriptional regulation of the liver-specific promoter of the human IGF-II gene. PhD Thesis, University of Utrecht, 1996.

319. Ayoubi, T.A.Y. and van de Ven, W.J.M. Regulation of gene expression by alternative promoters. FASEB J. 10: 453-460, 1996.

320. Li, X., Adam, G., Cui, H., Sandstedt, B., Ohlsson, R. and Ekstrom, T.J. Expression, promoter usage and parental imprinting of insulin-like growth factor II (IGF2) in human hepatoblastoma: uncoupling of IGF2 and H19 imprinting. Oncogene 11: 221-229, 1995.

321. Jansen, M., De Moor, C.H., Sussenbach, J.S. and van den Brande J.L. Translational control of gene expression. Pediatr. Res. 37: 681-686, 1995.

322. Kozak, M. An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Biol. 115: 887-903, 1991.

323. Kozak, M. Features in the 5' non-coding sequences of rabbit a and b-globin

mRNAs that affect translational efficiency. J. Mol. Biol. 235: 95-110, 1994.

324. Ross, J. mRNA stability in mammalian cells. Microbiol. Rev. 59: 423-450, 1995.

325. Cianetti, L., Di Cristofaro, A., Zappavigna, V., Bottero, L., Boccoli, G., Testa, U., Russo, G., Boncinelli, E. and Peschel, C. Molecular mechanisms underlying the expression of the human HOX-5.1 gene. Nucleic Acids Res. 18: 4361-4368, 1990.