Regulation of the V1 mRNA variant of the human growth hormone receptor gene by Gfi-1, Gfi-1b, a GAGA element and the liver enriched transcription factor, HNF-4α

By

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"The master in the art of living makes little distinction between his work and his play, his labor and his leisure, his mind and his body, his information and his recreation, his love and his religion. He hardly knows which is which. He simply pursues his vision of excellence at whatever he does, leaving others to decide whether he is working or playing. To him he is always doing both." *James Michener*

For you created my inmost being; you knit me together in my mother's womb. I praise you because I am fearfully and wonderfully made; your works are wonderful, I know that full well. My frame was not hidden from you when I was made in the secret place. When I was woven together in the depths of the earth, your eyes saw my unformed body. All the days ordained for me were written in your book before one of them came to be.

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PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS

Zakaria Rhani, Cynthia Gates Goodyer

Expression of the hepatic specific mRNA variant (V1) of the human growth hormone receptor (hGHR) gene is regulated by P1 and P2 isoforms of HNF-4α. (manuscript in progress) [1, 2, 3, 4, 5, 6, 7, 9, 10].

Gurvinder Kenth, Yuhong Wei, Cynthia G Goodyer

Regulation of the human growth hormone receptor V1 variant by Gfi-1 and Gfi-1b, and a GAGA element. (manuscript in progress) [1, 2, 3, 4, 5, 7, 8, 9, 10].

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Regulation of the V1 transcript of the Human Growth Hormone Receptor (hGHR) Gene by two Liver-Enriched Transcription Factors: HNF-4α and DBP. The Endocrine's 88th Annual Meeting, Boston, MA, USA 2006 (Poster). [8,9,10]

Joy K Osafo, Yuhong Wei, Zakaria Rhani, Cynthia G Goodyer

Characterization of the Proximal Promoter of the V1 Variant of the Human Growth Hormone Receptor. The Endocrine's 87th Annual Meeting, San Diego, California, USA 2005 (Poster). [1, 2, 3, 4, 5, 6, 7, 9, 10].

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Regulation of the Human Growth Hormone Receptor Gene by HNF-4α. The Endocrine's 86th Annual Meeting, New Orleans, Louisiana, USA 2004 (Poster) [1, 2, 3, 4, 5, 9, 10].

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Contribution by members of the Dr. Cindy Goodyer laboratory

[1] Hong Zheng, a former research assistant created the V4P1 and V8P1 promoter constructs.

[2]Levon Igidbashian, a summer student created the V1P1, V1P2, V1P4 and V1P5 promoter constructs.

[3] Marylene Rousseau, a former research assistant created the V2P1, V1P3, V7V1P1 and V7P1 promoter constructs.

[4] David Ghattas, a summer student created the V_XP_A promoter construct.

[5]Jennifer Manalo, a former masters student and the candidate were responsible for the processing of human foetal hepatocytes from fresh human foetal liver.

[6]Zakaria Rhani, a former postdoctoral fellow labelled HNF-4 EMSA probes.

[7]Yuhong Wei performed the first three cotransfection experiments with GAF in HEK293 cells.

[8]Gurvinder Kenth ran the western blots for Gfi-1 and DBP analyses in human tissues.

[9]The candidate was responsible for the remaining aspects of the study but chose not to have her name on the manuscripts for reasons relating to her religious beliefs and the use of human foetal tissue.

[10]Human tissues were collected by Dr. Cindy Goodyer. She also supervised the writing of the manuscripts, abstracts and preparation of the posters.

ABBREVIATIONS

-/-:	homozygous null mutant
3+:	exon 3 retained GHR
3-:	exon 3 deleted GHR
5′ RACE:	5′ rapid amplification of cDNA ends
ACS:	acyl-CoA binding site
5′ UTR:	5′ untranslated region
ADA2:	alteration/deficiency in activation
ADAM:	a disintegrin and metalloproteinase
ALS:	acid labile subunit
AMBP:	α_1-microglobulin/bikunin precursor
ANOVA:	analysis of variance
Apo:	apolipoprotein
Apo B48:	truncated form of apoB
Apo B100:	full length apo B
Asn:	Asparagine
AT_1:	angiotensin II
ATCC:	American type culture collection
ATP:	Adenosine triphosphate
BAC:	bacterial artificial chromosome
BMD:	bone mineral density
$CaCl_2$:	Calcium chloride
CBP:	CREB binding protein
cDNA:	complementary DNA
CDP:	C/EBP displacement protein
C/EBP:	CCAAT enhancer binding protein
ChIP:	chromatin immunoprecipitation
CK2:	casein kinase 2

COUP-TF:	chicken ovalbumin upstream promoter transcription factor
CPHD:	combined pituitary hormone deficiency
CV1:	African green monkey kidney fibroblast cell line
CYP:	P450 cytochrome
DBD:	DNA binding domain
DBP:	D-binding protein
DMEM:	Dulbecco's modified eagle's medium
DNAse:	DNA nuclease
dNTP:	deoxyribonucleotides
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
EGF:	epidermal growth factor
EMSA:	electromobility shift assay
EMSSA:	electromobility supershift assay
ERK:	extracellular signal regulated kinase
FACT:	facilitates chromatin transcription
FAK:	focal adhesion kinase
FAP:	fatty acid binding pocket
FBS:	foetal bovine serum
FH:	foetal hepatocyte
FL:	foetal liver
FTF:	fetoprotein transcription factor
G-6-P:	glucose-6-phosphatase
GAS:	interferon-gamma activated sequence
GBP:	GAGA binding protein
Gfi:	growth factor independence
GH:	growth hormone
GHD:	growth hormone deficiency
GHBP:	growth hormone binding protein

GH_N:	growth hormone (pituitary-derived)
GHR:	growth hormone receptor
GHRE:	growth hormone response element
GHRH:	growth hormone releasing hormone
GHRHR:	growth hormone releasing hormone receptor
GHSR:	growth hormone secretagogue receptor
GH_V:	growth hormone variant (placenta-derived)
G:	G-protein
GLE:	GAS-like element
Gln:	glycine
Grb:	growth factor receptor bound
GPCR:	G-protein coupled receptor
hABBP:	human Apobec-1 binding protein
HAL:	human adult liver
HAT:	histone acetylase
HBS:	HEPES buffer solution
HCR:	hepatic control region
HDAC:	histone deacetylase
HDL:	high density lipoprotein
HEK293:	human embryonic kidney cell line
HEPES:	hydroxyethylpiperazine-N'-2-ethanosulfonic acid
HepG2:	human hepatoma cell line
hGH:	(human) growth hormone
hGHR:	human growth hormone receptor
HLF:	hepatic leukaemia factor
HNF:	hepatic nuclear factor
hsp:	heat shock protein
Huh7:	human hepatoma cell line
IDL:	intermediate density lipoprotein
IGF:	insulin-like growth factor

IGFBP-3:	IGF binding protein
IGHD:	isolated growth hormone deficiency
IL:	interleukin
IP:	immunoprecipitation
IP_3:	inositol triphosphate
IRS:	insulin receptor substrate
JAK:	janus kinase (just another kinase)
KCl:	potassium chloride
kDa:	kilodaltons
LCFA:	long chain fatty acid
LCR:	locus control region
LDL:	low density lipoprotein
LDLR:	low density lipoprotein receptor
LETF:	liver-enriched transcription factor
LRCC8:	human epithelial tubular kidney cells
M:	mean
MAPK:	mitogen-activated protein kinase
mCBF-A:	mouse CA-rich G box binding factor
MEK:	mitogen activated protein-ERK
$MgCl_2$:	magnesium chloride
mRNA:	messenger RNA
MSY-1:	mouse Y-box factor 1
NaCl:	sodium chloride
NaF:	sodium fluoride
Na_3VO_4:	sodium vanadate
NF-Y:	nuclear factor-Y
NMR:	nuclear magnetic resonance
NRR:	negative regulatory region
NURF:	nucleosome remodelling factor
OSM:	Oncostatin M

PBS:	phosphate buffered saline
PC4:	positive cofactor 4
PCR:	polymerase chain reaction
PDGF:	platelet derived growth factor
PI3K:	phosphatidylinositol-3-kinase
PIAS:	protein inhibitor of activated Stats
PIT-1:	pituitary specific factor
PKC:	protein kinase C
PL:	placental lactogen
PLC:	phospholipase C
PMSF:	phenylmethylsulfonyl fluoride
POZ/BTB:	pox viruses and zinc finger/broad-complex, tramtrack, bric-a-bric
PRL:	prolactin
PROP-1:	prophet of PIT-1
PRR:	positive regulatory region
PUFA:	polyunsaturated fatty acid
PVDF:	polyvinylidene difluoride
Raf:	serine threonine kinase
RNA:	ribonucleic acid
RT-PCR:	reverse transcription-PCR
SD:	standard deviation
SDS:	sodium dodecyl sulphate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
SEM:	standard error of mean
SH-2:	src-homology 2
SHC:	src-homology 2/α collagen-related
SHP:	src-homology-2-containing phosphatase
SHP:	small heterodimer protein
SIE:	sis inducible element

SIRP:	signal regulatory protein
SMRT:	silencing mediator of retinoid and thyroid receptors
SOCS:	suppressor of cytokine signalling
Spi:	serine protease inhibitor
SRC:	steroid coactivator
SST:	somatostatin
Stat:	signal transducer and activator of transcription
TAF:	TBP associated factor
Taq:	*Thermus acquaticus* DNA polymerase
TACE/ADAM:	TNF-α converting enzyme/
TBP:	TATA binding protein
TEF:	thyrotroph embryonic factor
TF:	transcription factor
TNF:	tumour necrosis factor
TSS:	transcription start site
UbE:	ubiquitin endocytosis motif
V;	variant 5'UTR hGHR exon
V1bR:	vasopressin 1b receptor
VLDL:	very low density lipoprotein
Wk FA:	weeks foetal age
Yr:	year old

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ABSTRACT

GH acts through its specific receptor, GHR. In the human liver, more than twelve hGHR mRNAs are transcribed from unique 5'UTR exons, seven of which are found in two small clusters: module A (V2,V9,V3) and module B (V7,V1,V4,V8). While module A mRNAs are ubiquitously expressed, module B transcripts are restricted to normal postnatal liver, suggesting developmental- and liver-specific regulation of the hGHR gene.

To begin characterising the elements regulating module B mRNA expression, I studied the promoter region of the V1 exon, the most abundantly expressing variant in liver. A 1.8 kb region upstream of the V1 transcriptional start site (TSS) was actively repressed in transient transfection assays (TTAs). However, 5' or 3' deletions relieved the suppression, suggesting the presence of multiple negative and positive regulatory elements. Two sites for growth factor independence-1 (Gfi-1) and Gfi-1b and a GAGA element were identified in the most 3' 300 bp regulatory region. Gfi-1 was detected by western blot in human foetal and postnatal liver. Gfi-1 and Gfi-1b strongly repressed while the *Drosophila* GAGA factor (GAF) stimulated V1 transcription through their specific sites, as determined by TTAs and site-directed mutagenesis (SDM). Six putative sites for hepatic nuclear factor-4 (HNF-4) were also identified in the 1.8 kb region. HNF-4α is developmentally regulated in human liver: HNF-4α2 and α8 proteins are expressed in foetal hepatocytes but only HNF-4α2 is

detected postnatally. TTAs and SDM demonstrated that HNF-4α2 and HNF-4α8 have a similar dual effect on V1 transcription: activation via site #1 in the proximal promoter and repression through site #6, ~1.7 kb upstream of the TSS. Results from EMSA/EMSSA/ChIP analyses suggest these sites are bound by HNF-4α.

Thus, V1 transcriptional activity is repressed by Gfi-1/Gfi-1b, stimulated through a GAGA element by GAF, and repressed or stimulated by HNF-4α (2+8) depending on the site. Similar sites are present in homologous regions of the bovine, ovine and mouse GHR genes suggesting that their regulatory roles are conserved. However, none of these factors individually appear to be responsible for the postnatal hepatic-specific expression of V1 mRNA. To define the specific regulatory mechanisms, future studies should examine their interactions with additional liver-enriched factors (e.g. C/EBPα).

ABRÉGÉ

L'hormone de croissance (GH) agit à travers son récepteur spécifique, le GHR. Dans le foie humain, on compte plus que douze ARNm qui sont transcrits à partir d'uniques exons 5'UTR, dont sept ont été trouvés répartis sur deux petits clusters: le module A (V2,V9.V3) et le module B (V7,V1,V4,V8). Si les ARNm du premier module sont exprimés d'une manière ubiquitaire, les transcrits du deuxième module sont, cependant, spécifiques au foie postnatal normal. Ceci suggère une régulation du gène hGHR spécifique au développement et à la régulation hépatique. Pour ainsi caractériser les éléments régulant l'expression des ARNm du module B, j'ai étudié la région du promoteur de l'exon V1, la variante la plus exprimée dans le foie. La région à 1,8 kb en amont du site de transcription (TSS) a été activement réprimée dans les essais de transfection transitoire (TTA).

Toutefois, des délétions 3' et 5 ' relâchent cette suppression, suggérant la présence de plusieurs éléments régulateurs positifs et négatifs. En effet, deux sites de facteurs de croissance independence-1 (Gfi-1), Gfi-1 et Gfi-1b, et un élément GAGA ont été identifiés dans la région régulatrice 3' 300 bp. Le Gfi-1 a été détecté par western blot dans le foie humain fœtal et postnatal. Comme il a été déterminé par mes expériences de TTAs et de mutagenèse site-dirigée (SDM), Gfi-1 et Gfi-1b répriment alors que le facteur GAGA de drosophile (GAF) stimule, via leurs spécifiques sites, la

transcription du V1. Six sites potentiels pour le nuclear factor-4 (HNF-4) ont été également identifiés dans la région 1,8 kb. HNF-4α est régulé, au cours du développement, dans le foie humain: les protéines HNF-4α2 et α8 sont exprimées dans les hépatocytes fœtaux, mais seulement HNF-4α2 qui est détectée à l'état postnatal.

TTAs et SDM ont démontré que HNF-4α2 et HNF-4α8 ont un effet dualiste similaire sur la transcription de V1 : d'une part, une activation via le site #1 dans le promoteur proximal et, d'autre part, une répression à travers le site #6, environ 1,7 kb en amont du TSS. Les résultas des analyses EMSA/EMSSA/ChIP suggèrent que HNF-4α s' s'attache à ces sites.

Aussi, l'activité «transcriptionnelle» du V1 est-elle réprimée par Gfi-1/Gfi-1b, stimulée par GAF à travers l'élément GAGA et désactivée ou activée par HNF-4α (2+8), dépendamment du site.

Des sites similaires sont présents dans des régions géniques GHR homologues chez les bovins, les ovins et chez la souris, indiquant que leurs rôles régulateurs ont été conservés. Toutefois aucun de ces facteurs ne semble être responsable, d'une manière individuelle, de l'expression postnatale spécifique du ARNm V1 pour définir de tels mécanismes régulateurs, les analyses ultérieures devront examiner de plus près les interactions de ces sites avec d'autres facteurs hépatiques (ex. C/EBPα).

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Growth Hormone (GH)

GH is well known as a major regulator of postnatal growth and metabolism (1). GH was first isolated from rat pituitary extracts, and subsequently from bovine (2;3). Studies with porcine and bovine GH showed that monkeys and humans did not respond to subprimate GH, suggesting species specificity [reviewed in (4)]. In the 1950's, GH was isolated from human and monkeys and tested in humans. It was discovered in the 1960's that GH could be used to treat GH-deficient children. Subsequently, GH was extracted from human cadavers and used in treating GH deficiency. However in 1985, Creutzfeldt-Jakob disease was diagnosed in several patients who had been treated with extracted pituitary hGH, thus the treatment was withdrawn from the North American market. At that time, a budding North American pharmaceutical company, Genentech, developed recombinant GH (rGH). The FDA (Food and Drug Administration) quickly approved Genentech's rGH (Protropin) for treatment in 1985 [reviewed in (4)]. Several other companies now produce rGH including Eli Lilly and Serono. rGH remains the chosen mode of treatment for GH deficiencies to this day.

1.1.1. Structure of GH

GH, a 191 amino acid polypeptide, belongs to the helix bundle peptide (HBP) hormone family that includes the closely related hormone, prolactin, and several haematopoietic cytokines (5). As the name suggests, HBP hormones are composed of four α-helices that run in an anti-parallel fashion (5;6).

The hGH gene consists of five exons, and four introns and is part of a highly conserved five gene cluster spanning ~66 kb on chromosome 17q22-24 (7;8). The five genes are in the order 5′ to 3′: pituitary GH (GH_N), Placental lactogen-L (PL-L), PL-A, GH variant (GH_V) and PL-B (9). GH_N, or GH1 as it is sometimes known, is expressed primarily in the somatotropic cells of the anterior pituitary; PL-A, PL-B and GH_V are produced in the syncytiotrophoblastic cells of the placenta. PL-L is considered a pseudogene even though mRNA transcripts have been detected (10). GH_V is produced by the placenta beginning ~6 weeks post conception and secreted only into maternal circulation, gradually replacing GH_N in the mother (10).

GH_N is the major circulatory form of GH. Secretion in the foetus has been detected from ~6 weeks, rising to 120-150 ng/ml at midgestation, followed by a slow decline although levels remain high into the first few weeks of postnatal life [reviewed in (11-13)]. It drops thereafter to ~10 ng/ml in children and adults. Alternative splicing of exon 3 of GH_N

transcripts produces two variants, 22 kDa (191 aa) and 20 kDa (176 aa), and an exon 3 deficient form, 17.5 kDa. The major forms (22 kDa and 20 kDa) account for 75-85% and 15% of circulating GH, respectively. Both isoforms have somatotrophic and lipolytic actions, although the 20K isoform has been shown to have weaker anti-insulin-like effects [reviewed in (11)].

1.1.2. Regulation of GH secretion

GH is secreted from the pituitary somatotrope in a pulsatile manner, with 7-10 episodic bursts per day, and a nocturnal surge (14). For several years, trough and peak patterns of GH release have been known to be primarily under the control of two hypothalamic hormones: the stimulatory GH releasing hormone (GHRH) and the inhibitory somatostatin (SST) (15;16). Both of these regulatory hormones are released into the median eminence and travel to the anterior pituitary via the hypothalamic-hypophyseal portal system. A third more minor regulatory factor, Ghrelin was identified a few years ago (Figure 1) (17).

GHRH, from the arcuate nucleus of the hypothalamus, interacts with its G-protein coupled receptor (GPCR), GHRHR, on the somatotropes of the anterior pituitary and causes the release of pre-packaged GH secretory granules by increasing intracellular cAMP concentrations and activating L-type calcium channels and, stimulates transcription of GH gene to increase

a readily releasable pool of GH. *In vitro* studies of foetal pituitary explants and clinical studies of premature babies have shown that the human foetus responds positively to GHRH [reviewed in (11)].

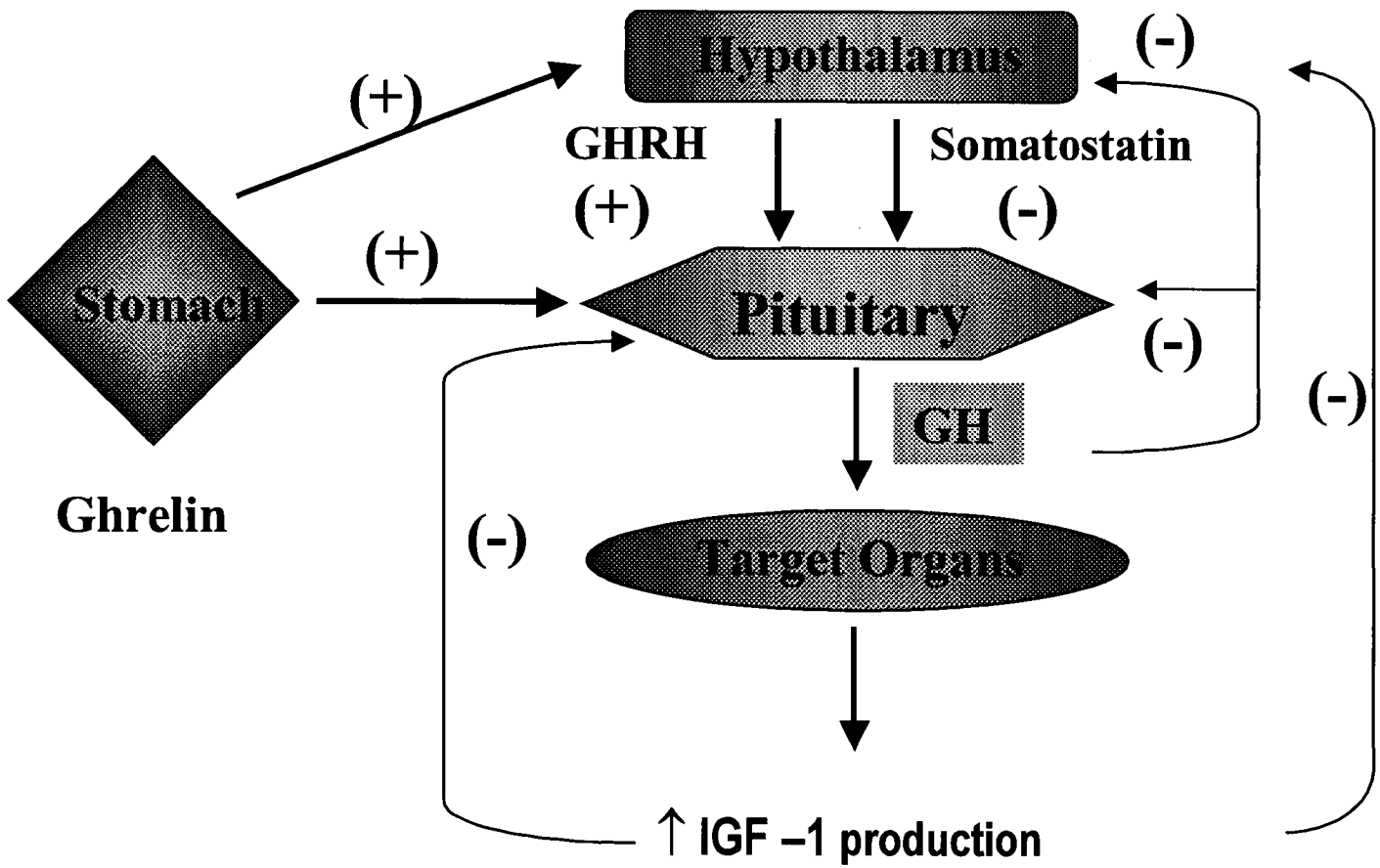
On the other hand, the SST inhibition of GH is not effective until after birth. SST is produced in the periventricular nucleus. There are five SST receptor genes (SSTR1-5), located on different chromosomes [reviewed in (18-20)]. The five receptors are GPCRs. SSTR5 is the predominant type in rodent and human somatotropes and mediates actions of SST on GH secretion [reviewed in (20)]. SST reduces intracellular cAMP by activating inhibitory G-proteins (G_i) and inhibits L-type calcium channels by G_O activation; it inhibits release of GH but has no effect on GH gene transcription [reviewed in (21)]. It has also been shown to reduce GH binding to hepatocytes, thereby inhibiting GH-activated pathways in hepatocytes (22).

Ghrelin was identified as an endogenous agonist of the GH secretagogue receptor (GHS-R), another member of the GPCR family (17). The major form of ghrelin, an n-octanoylated 28 amino acid peptide, was first purified from rat and human stomachs (17;23). A minor form, with a deletion of glycine 14, des-Gln14-ghrelin, was subsequently purified in both species (24;25).

Figure 1: Hypothalamic-GH-IGF-1-target organ axis.

Release of GH from the anterior pituitary is primarily regulated by the hypothalamic hormones GHRH and SST. Ghrelin, a third and more minor regulating hormone, is mainly produced in the stomach and stimulates GH release by acting on the hypothalamus and anterior pituitary.

GH: Growth Hormone, GHRH: Growth Hormone Releasing Hormone, SST: Somatostatin, IGF-1: Insulin-Like Growth Factor. Adapted from (17;26).


Hypothalamus
(+)
(-)
GHRH
Somatostatin
(+)
(-)
Stomach
(+)
Pituitary
(-)
GH
(-)
Ghrelin
(-)
Target Organs
↑ IGF –1 production

Ghrelin stimulates GH secretion by interacting with somatotrope GHS-R and increasing intracellular Ca^{2+} concentration through IP_3. This increases GH levels through GHRH stimulation of somatotropes (27;28). Maximal stimulatory effect of ghrelin is 2-3 fold higher than GHRH, however the synergistic effects of GHRH and ghrelin are greater than either factor alone [reviewed in (29)]. GHS-R, the receptor for ghrelin has been shown to be constitutively active. Pantel *et al.* described patients that presented with short stature (30). The patients had a missense mutation in the GHS-R that affected its constitutive activity. However the mutant GHS-R maintained its responsiveness to ghrelin. Thus the physiological role of ghrelin in GH secretion is still unknown. Other effects of ghrelin include appetite regulation through effects at the level of the hypothalamus [reviewed in (29)].

GH and IGF-1 negatively feedback on the hypothalamus and anterior pituitary to regulate GH secretion (Figure 1). High levels of IGF-1 inhibit GH secretion by direct inhibition of the somatotrope and, indirectly, by stimulating SST production from the hypothalamus. GH inhibits GHRH secretion from the hypothalamus and GH release from the somatotropes [(31) and reviewed in (26)].

Other factors that affect GH secretion include sleep, feeding, exercise, steroids (glucocorticoids, sex steroids, thyroid hormone), stress and obesity (1).

1.1.3. Gender differences

GH secretion is sexually dimorphic in rats, mice and, to a lesser extent, humans (32-34). In rodents, the sex differences in GH secretion are strikingly different. The amplitude of GH bursts is characterised by higher peaks and deeper troughs in males than in females. In humans, the patterns of GH secretion become gender dependent only after puberty and are not as markedly different as in rodents (35-37). In addition, women have 2-3 fold higher levels of GH than males due to the frequent and irregular pulses of GH release. However, IGF-1 levels are roughly the same in both sexes suggesting a relative insensitivity of the GHR-IGF-1 axis in females (38;39). Neonatal exposure to sex steroids sets the patterns of pituitary GH secretion that are later manifest in puberty (40;41). The sexual dimorphic pulsatile pattern of GH secretion is important in stimulating postnatal growth. This has been shown in GH-deficient animals where pulsatile rather than continuous GH infusion is more effective in restablishing growth (42).

1.2. Actions of GH

GH has major actions on growth and metabolism either directly or indirectly through IGF-1. GH stimulates longitudinal bone growth, glucose transport and metabolism in liver, lipolysis in adipose tissue and liver, and

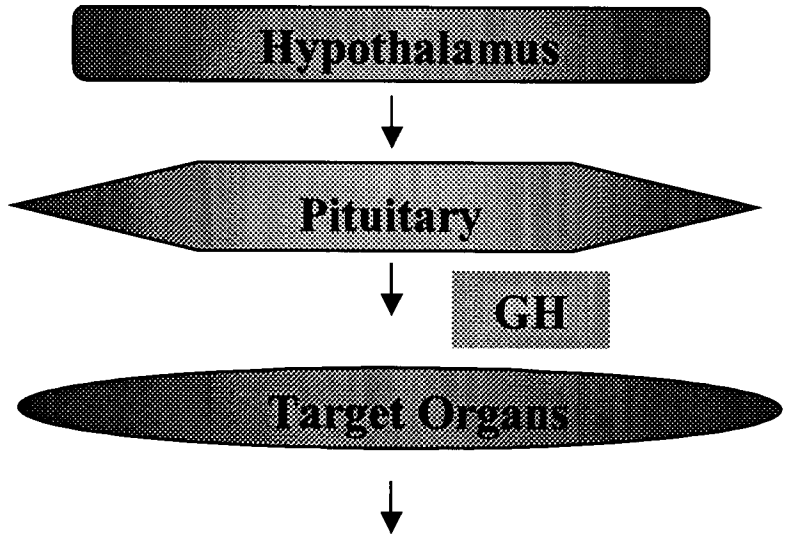
protein synthesis and amino acid metabolism and transport in muscle, adipose tissue and liver (Figure 2).

1.2.1. Growth

Effects of GH on bone growth and bone mineral density (BMD) are well known even though studies, especially in BMD area of research, are limited. Long bone growth occurs at the epiphyseal growth plate by a series of finely controlled steps. In the first few weeks of life, the skeletal system of the foetus forms as cartilage and connective tissue. Endochondral ossification, a process by which cartilage tissue is gradually replaced by bone tissue, begins around the eighth week of conception. The epiphyseal growth plate lies between the epiphyses and the metaphyses of long bones. Chondrocytes in the growth plate are split into four zones of progressive chondrocyte maturation: resting or germinal zone, proliferative (upper and lower), hypertrophic and calcifying. The most distal region is the transitional zone from cartilage to bone. Longitudinal bone growth comes to a halt during late puberty, when the growth plate becomes completely ossified. Sex steroids and GH are among the hormones that influence bone growth. Initially the somatomedin hypothesis suggested that GH stimulated longitudinal bone growth through liver production of IGF-1 that then circulated to the epiphyseal growth plate as an endocrine factor (43;44).

Figure 2: Effect of GH on target organs.

GH, secreted by the anterior pituitary exerts its effects on target cells both directly and indirectly (through the production of IGF-1) in the majority of cells.



- **Majority of cell types:** ↑ IGF –1 production
- **Liver:** ↑ glucose transport and metabolism, ↑ amino acid transport, ↑ lipid and lipoprotein metabolism
- **Adipose Tissue:** ↑ lipolysis, ↑ amino acid transport
- **Muscle:** ↑ protein metabolism, ↑ amino acid transport
- **Bone :** ↑ growth of long bones, ↑ bone remodelling

This theory was later challenged by the dual-effector hypothesis: GH was found to accelerate growth of the rat tibia epiphyseal plate when directly injected into the growth plate, suggesting that it can have direct effects on longitudinal bone growth (45). In agreement with this, GH has been observed to stimulate bone growth both directly and indirectly through local IGF-1 production (46). The direct role of GH on bone growth is demonstrated by the presence of functional GH receptors (GHRs) on chondrocytes of rat, rabbit and human growth plates and the fact that GH treatment in igf-1 null mice leads to the normal expansion of the germinal (resting) zone (47;48).

GH also affects bone mineral density: it stimulates both bone formation and resorption (49). Bone resorption is carried out by osteoclasts and formation of new bone by osteoblasts. Net decreases in bone mineral density occur when bone resorption exceeds bone formation as is the case in post-menopausal women due to a deficiency in oestrogen. GH treatment enhances bone turnover in post-menopausal women with osteoporosis, improving their bone mass (50;51). GH also stimulates bone turnover in young healthy males (52). GH deficient patients have a decrease in their bone mineral density and this condition is reversed by GH treatment [(53;54) and reviewed in (55)].

GH, GHR and IGF-1 deficiencies all result in severe growth retardation as well as changes in BMD. The effects of GH and GHR deficiencies are discussed in more detail in sections 1.2.7 and 1.4.1.

1.2.2. Metabolism

GH exerts a range of metabolic effects on target tissues (Figure 2). The metabolic effects depend on the length of exposure to GH. When administered acutely, GH has insulin-like effects and stimulates glucose uptake and lipogenesis. The effect of chronic exposure to GH is quite the opposite: anti-insulin-like effects are observed. This is especially evident in acromegalics that may also develop type II diabetes (26). In the upcoming sections of the literature review, the major focus of GH metabolic effects will be on the liver as the experimental work of this PhD thesis is focused on hepatic GHR expression.

1.2.2.1. Effect of GH on glucose metabolism in the liver

The effects of GH on glucose metabolism were evident even before its isolation in the 1940's (56). Anti-insulin-like effects of GH were first observed in experimental diabetic dogs in the 1930's by Houssay and Biasotti: hyperglycaemia was reduced in these animals following hypophysectomy (57). The factor responsible for this action was later isolated from the pituitary and identified as GH (2;3;56). Much of the known and documented effects of GH are in the postnatal individual.

However, there is evidence to suggest that GH plays a role in glucose metabolism in the foetal liver as well. In the developing foetus, the liver is a major site for haematopoiesis, until late gestation, when the site of haematopoiesis moves to the bone marrow and the liver begins to adopt the metabolic functions that are seen in the adult. Receptors for GH are present on hepatocytes from as early as 8 weeks of foetal life in the human (58-60). Furthermore, these receptors are similar in structure and size to those present on adult hepatocytes (59). The functionality of foetal GHRs is shown by stimulation of glucose uptake by primary cultures of hepatocytes from 15-20 week old foetuses in response to GH (59). In addition, newborns with GH deficiency are often born with hypoglycaemia, indicating an important role for GH in glucose metabolism of the late gestation foetus (61).

GH effects on glucose metabolism have been studied in detail in healthy individuals and type 1 diabetics [reviewed in (62)]. GH increases fasting glucose levels by stimulating gluconeogenesis and glycogenolysis in hepatocytes. It also decreases use of glucose in peripheral tissues by inhibiting glycogen synthesis and oxidation of glucose [reviewed in (62)]. PEPCK and GLUT-2, two proteins involved in glucose metabolism and transport, have been shown to be regulated by GH in the mouse (63).

1.2.2.2. Effect of GH on lipid metabolism in the liver

The liver is the main site of cholesterol (lipid) metabolism, which involves cholesterol synthesis, breakdown and transport. GH regulates lipid metabolism in both rodents and humans, however some species differences exist in the mode of action [reviewed in (64)]. Very low density lipoprotein (VLDL) is produced in the liver and transports esterified cholesterol from the liver to peripheral tissues. While in circulation, triglycerides and some apolipoprotein components are removed, converting VLDL into IDL (intermediate) and LDL (low). HDL (high) scavenges cholesterol from other tissues and transports it to the liver. VLDL, IDL, HDL and LDL are sequestered by the LDL receptor (LDLR) on the surface of cells and, through receptor-mediated endocytosis and degradation of the lipoproteins by lysosomal lipases, release amino acids, cholesterol and fatty acids into the cell (65).

In humans, LDLR is required for the lipid lowering effects of GH. GH has been shown to increase hepatic LDLR in cholecystectomy patients and cultured HepG2 cells, and to lower circulating plasma levels of LDL in healthy subjects (66-68). However, patients with familial hypercholesterolaemia (FH) do not express functional LDLRs and their plasma levels of LDL cholesterol do not decrease following GH treatment (69). GH is also required for the full restoration of stimulatory effects of oestrogen on LDLR expression in hypophysectomized animals. This is a direct effect of GH as IGF-1 does not produce the same effect (64). In

mice, however, GH action on lipid metabolism is LDLR independent, as GH is able to reduce plasma levels of LDL cholesterol in LDLR knock out mice by stimulating cholesterol 7-α hydroxylase (cyp7a1), the rate limiting enzyme in the breakdown of cholesterol into bile acids (70).

Apolipoproteins (apo) are the protein components of lipoproteins that transport lipids in various forms throughout the body. Of the nine known apolipoproteins, apo B100 (full length), apo B48 (truncated form of apo B100) and apo E are involved in cholesterol clearance (65). GH stimulates expression of apolipoprotein B (full length) and E in both the rat and humans (64;71). RNA editing of full length apoB mRNA by a multiprotein editosome complex produces apoB48 which has a higher affinity for the LDLR than apoB100 and results in faster clearance of lipoproteins (64;72-74). Unlike the rat where apoB editing occurs in both the liver and intestine, apoB editing occurs only in the intestine in humans (75;76).

Excess cholesterol is cleared from the body through the liver through conversion to bile acids by the neutral or acidic pathway, and faecal excretion of the salt conjugates (65). In rats and mice, GH stimulates the expression of cyp7a1, the rate-limiting enzyme of the neutral pathway, thereby stimulating bile acid synthesis and subsequent excretion (64;70;77). In contrast, GH does not stimulate bile acid synthesis in humans (69).

1.2.2.3. Effects of GH on amino acid metabolism and protein synthesis

GH is well known to increase uptake of amino acids in liver, skeletal muscle and adipose tissue in both rodents and humans [reviewed in (78)]. As an anabolic hormone, GH increases nitrogen retention by a combination of both increased protein synthesis and/or inhibition of protein degradation (79;80). In the liver, GH promotes positive nitrogen balance by decreasing degradation of amino acids through the urea cycle, resulting in increased protein synthesis (81). In light of the positive effects of GH on nitrogen balance, GH has been used to treat protein loss in a variety of patients: for example, GH prevents muscle wasting in AIDS patients, promotes protein synthesis in cholecystectomy patients, and stimulates protein synthesis and promotes wound healing in burn victims [(82-85) and reviewed in (86)]. Inhibition of lipolysis has been shown to block GH-induced protein retention, suggesting that lipid metabolites are required for GH promotion of nitrogen retention (87).

1.2.3. Detoxification: GH regulation of the P450 cytochrome family

In addition to its fuel metabolic functions, the liver also acts as a detoxifying organ, metabolizing endogenous steroids, fatty acids and foreign substances such as drugs and environmental carcinogens. This function is carried out by haem-containing, membrane-bound P450

enzymes (CYPs), in rodents and humans. CYP1, 2 and 3 are highly expressed in the liver and subject to transcriptional regulation by several factors including environmental insults (e.g. carcinogens) and hormones (e.g. GH).

Certain members of the CYP family are expressed in a sexually dimorphic manner, such as the male-specific testosterone 2α- and 16α-hydroxylases, cyp2c11 and cyp2d9, and the female-specific steroid sulphate 15β-hydroxylases, cyp2c12 and cyp2a4 [reviewed in (88)]. The sex differences in the expression of CYPs are attributed to the influence of sexually dimorphic GH secretory patterns and, in rodents, Stat5b has been identified as the intracellular mediator of these GH-mediated sexual dimorphic effects [reviewed in (88)]. In the liver of pubertal male rats, tyrosine phosphorylation and nuclear translocation of Stat5b is high during a GH pulse and knock out male mice models of STAT5b lose the distinct adult growth pattern and the expression of male-specific cyps seen in normal littermates (89-91). The reverse is observed in the liver of female rats: 90% of Stat5b activation is suppressed by the continuous exposure of female rats to GH (92). Although there is an increased expression of more female CYPs in the liver of the male Stat5b knock out mice, changes are not as pronounced in Stat5b female knock out mice (92).

Studies have also shown that Stat5b is not the sole determinant of the sexually dimorphic expression patterns of CYPs in the liver: several liver

enriched transcription factors (LETFs) have been implicated. Cyp2c12 is upregulated by HNF-6 and HNF-4α in the female rat liver (93). GH stimulates HNF-6 expression via STAT5b and HNF-4α (94). Hypophysectomy reduces HNF-6 mRNA and protein levels in rats, and is only restablished by continuous infusion of GH (95).

Male specific cyp4a12 and female specific cyp2b10, cyp2b13, cyp3a41 and cyp3a44 are also regulated by both GH and HNF-4α in mice (96).

Gender differences in the expression of CYPs also exist in the human. CYP3A4, a major CYP expressed in human liver and intestine is found at 2 fold higher levels in females than males (97). In addition, its activities such as the inactivation of cortisol and metabolism of certain drugs (e.g. erythromycin) are more rapid in women [reviewed in (88)]. GH regulation of CYP3A4 was shown by increased levels in response to the female pattern of GH secretion, and suppression by pulsatile GH treatment of primary human hepatocytes (98;99).

1.2.4. Pathologic conditions associated with GH

Clinical manifestations of GH undersecretion are known collectively as GH deficiency (GHD) and overexpression of GH as acromegaly or gigantism. GHD includes isolated GHD (IGHD) where only GH secretion is affected, and combined pituitary hormone deficiency (CPHD), where other anterior pituitary hormones as well as GH are affected. Four classes of IGHD exist

(Table 1) [reviewed in (12)]. The majority of IGHD are a result of molecular defects in the GH_N (GH) gene. CPHD is caused by mutations in different transcription factors and their binding sites that regulate the development of the anterior pituitary hormone secretory cells. These factors include PIT-1, PROP-1, HEX1, LHX-3 and –4 [reviewed (12)]. GH deficiency can also be acquired following head trauma or brain surgery. Patients with GH deficiencies are phenotypically short, they accumulate fat around the waist area, and have reduced muscle mass and energy. Biochemical analyses show that they have low to absent circulating levels of GH but respond to exogenous GH which leads to an increase in IGF-1. Excessive production of GH by GH-secreting pituitary adenomas leads to two clinical disorders of GH that are distinguished by the age of onset. Pre-pubertal onset of GH overexpression is rare and causes gigantism. Postpubertal onset of GH overproduction results in acromegaly, characterised by overgrowth of soft tissues. In both cases, biochemical characteristics include elevated levels of GH and IGF-1 as well as decreased insulin sensitivity [reviewed in (100)].

Table 1: Classification of Isolated Growth Hormone Deficiency (IGHD). There are four classes of isolated growth hormone deficiency (IGHD). IGHD-1A patients are completely GH1 deficient while IGHD-1B, IGHD-II and IGHD-III patients produce low levels of GH1. IGHD-III is associated with hypogammaglobulinaemia, an immunity disorder; however, no mutations have been identified in the GH1 gene. IGHD patients have severe growth defects, corresponding to GH function in the postnatal individual. Table from (12).

Type	Mode of inheritance	Features	Etiology
IGHD-IA	Autosomal recessive	• Total deficiency • Development of anti-GH antibodies	• GH1 deletions • Nonsense mutations
IGHD-IB	Autosomal recessive	Partial deficiency	Splicing mutations
IGHD-II	Autosomal dominant	Partial deficiency	Missense and splicing mutations
IGHD-III	X-linked	Partial deficiency	Unknown

Acromegalics are treated by surgery (tumour excision), radiation and pharmacological interventions, including pegvisomant, the GHR antagonist, dopamine agonists and long acting analogs of SST (octreotide LAR and lanreotide SR) [reviewed in (101)].

1.3. Growth Hormone Receptor (GHR)

1.3.1. Cytokine Receptor Family

Most members of the cytokine receptor superfamily are single transmembrane proteins that lack intrinsic kinase activity and share certain structural motifs, including multiple paired cysteine residues in the extracellular domain and box 1, an eight residue proline-rich sequence located 10-20 amino acids intracellular to the transmembrane domain (102). There are two classes of cytokine receptors. Class I receptors are distinguished by the presence of a conserved WSXWS motif extracellular to the transmembrane region which is involved in ligand binding and subsequent signalling (103). The GHR is a class I receptor and other family members include receptors for prolactin (PRL), leptin and erythropoietin (Figure 3). Interleukin-10 (IL-10) and IL-12 act through class II receptors.

The GHR contains a YGEFS motif in place of the WSXWS motif (Figure 4).

Figure 3: Cytokine Receptor Family

Diagram depicting members of the cytokine receptor family and their structural domains. The receptors are identified by their ligand. The intracellular domain is in blue; fibronectin type III module in pink; cytokine receptor module in yellow; immunoglobulin module is in light blue; and the fibronectin type III spacer is green. The conserved disulfide bonds are indicated in the cytokine receptor module and the position of the WSXWS box is also indicated in the fibronectin type III module. GH, growth hormone; PRL, prolactin; IL-4, interleukin 4; IL-7, interleukin-7; Epo, erythropoietin; G-CSF, granulocyte colony-stimulating factor; IL-2. interleukin 2; IL-3, interleukin 3; IL-5, interleukin 5; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-6, interleukin 6; LIF/OSM, leukemia inhibitory factor/oncostatin M; CNTF, ciliary neurotrophic factor; MPL, the cellular counterpart of the viral *mpl* oncogene product. ECD: extracellular domain; TMD: transmembrane domain; ICD: intracellular domain. Figure and text of legend from (104).

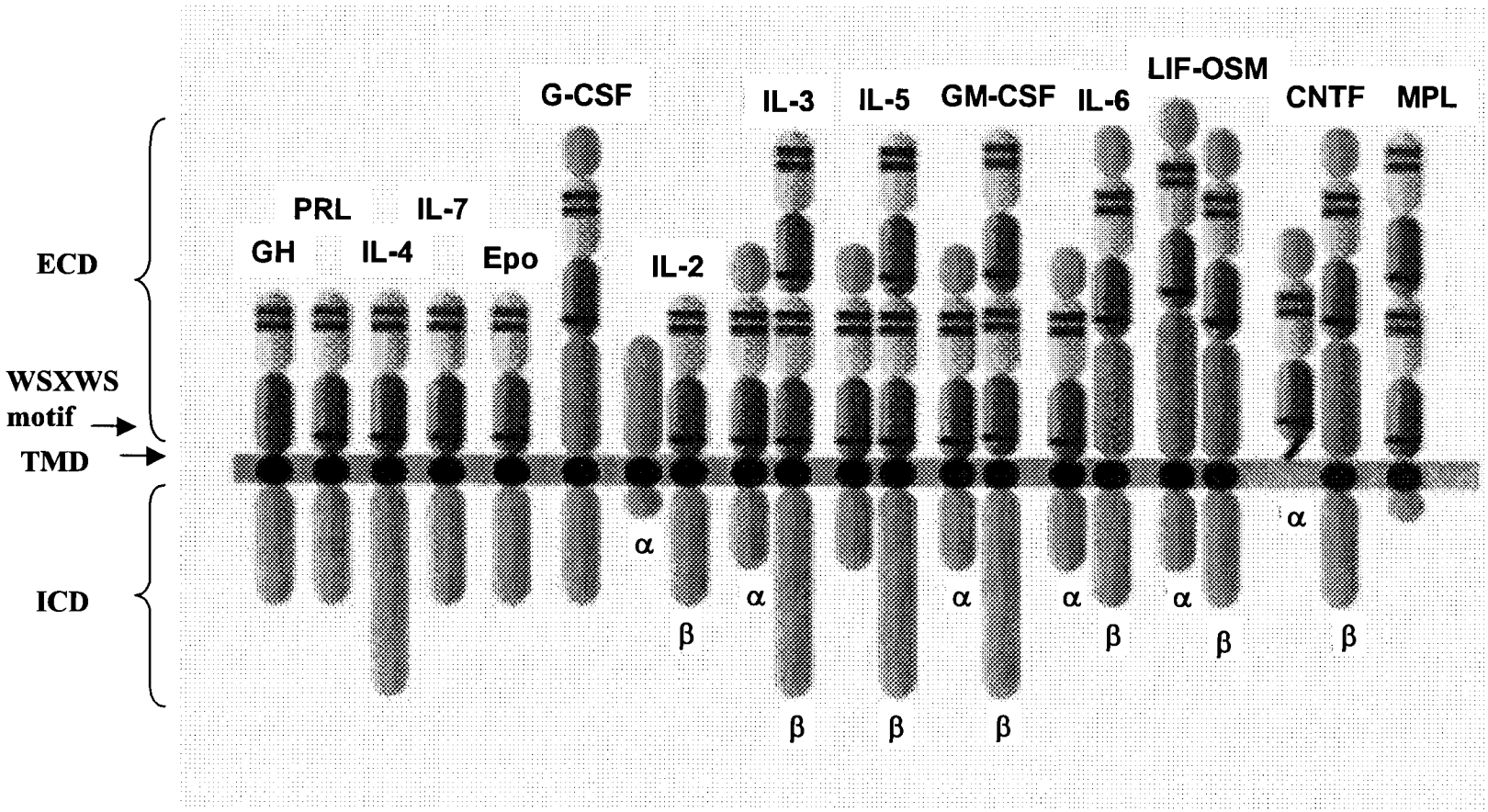
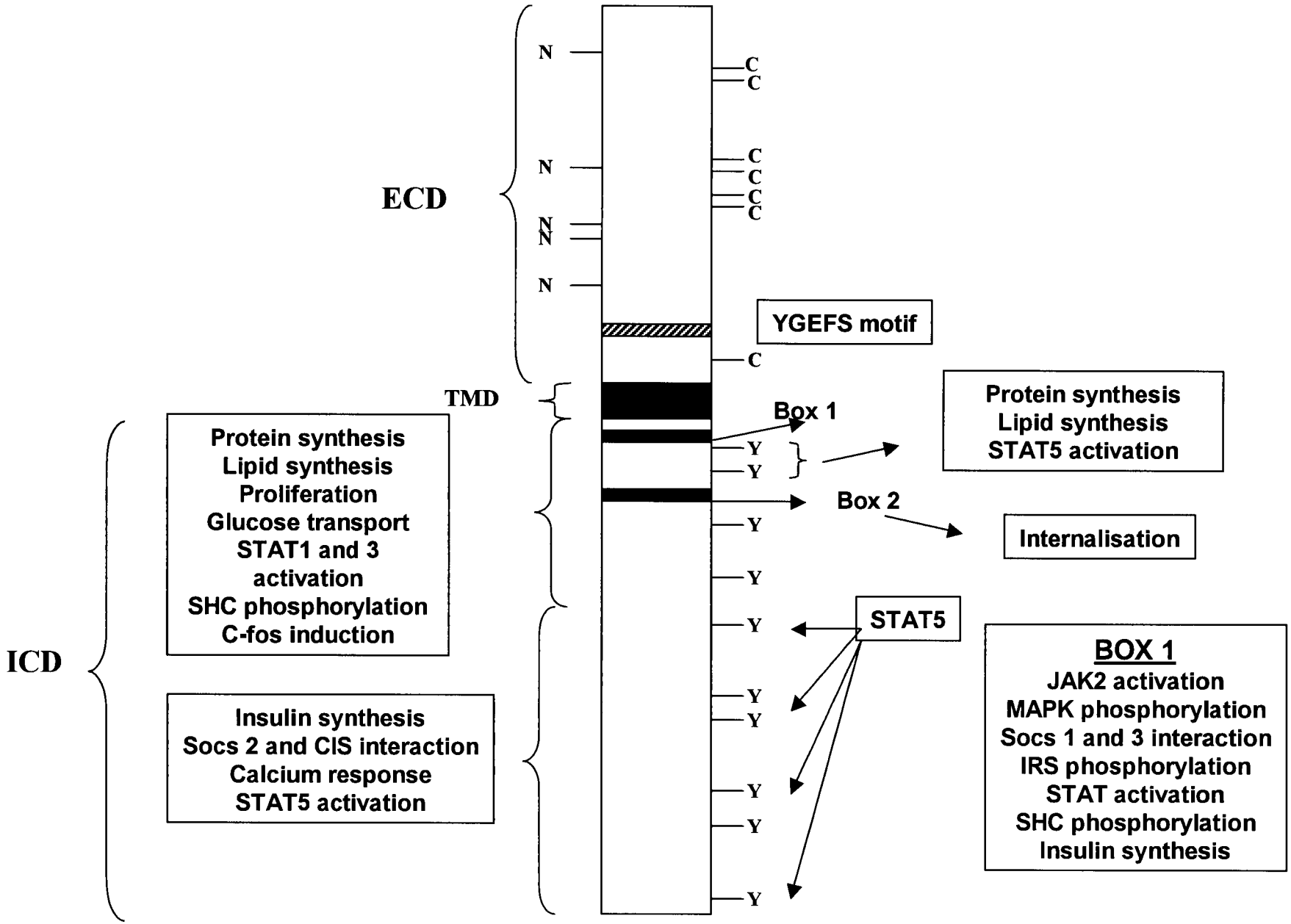

ECD
WSXWS
motif
TMD
ICD
GH
PRL
IL-4
IL-7
Epo
G-CSF
IL-2
IL-3
IL-5
GM-CSF
IL-6
LIF-OSM
CNTF
MPL
α
β

Figure 4: Structural and functional features of GHR.

The key structural features of GHR and their relation to GH function are shown in this diagram. The main protein domains; ECD, TMD and ICD are indicated. Five potential aspargine (Asn)-linked glycosylation sites (N), the YGEFS motif and seven cysteine residues (C) (six of them paired) are located in the ECD. Boxes 1 and 2, and tyrosine (Y) residues are indicated. ECD: extracellular domain; TMD: transmembrane domain; ICD: intracellular domain. Figure modified from (105).


ECD
N
N
N
N
C
C
C
C
C
C
YGEFS motif
TMD
Box 1
Box 2
Y
Y
Y
Y
Y
Y
Y
Y
Y
Y
Protein synthesis
Lipid synthesis
STAT5 activation
Internalisation
STAT5
ICD
Protein synthesis
Lipid synthesis
Proliferation
Glucose transport
STAT1 and 3
activation
SHC phosphorylation
C-fos induction
Insulin synthesis
Socs 2 and CIS interaction
Calcium response
STAT5 activation
BOX 1
JAK2 activation
MAPK phosphorylation
Socs 1 and 3 interaction
IRS phosphorylation
STAT activation
SHC phosphorylation
Insulin synthesis

Even though this motif does not directly interact with GH, alanine mutagenesis studies of the YGEFS motif have shown that it is important for ligand binding and signal transduction of GH (103). The ECD contains five potential N-linked glycosylation sites and seven cysteine residues. The ICD contains box 1 and box 2. Box 1 interacts with JAK2 and is required for the activation of GH signalling (106). The serine rich box 2 is 40 amino acids downstream of box 1 and is involved in internalisation of the GHR (26). There are several tyrosine residues in the ICD that are phosphorylated upon GHR activation by GH, and are involved in GH stimulation of lipogenesis and protein synthesis (107;108).

Upon ligand binding, cytokine receptor associated protein kinases are activated which, in turn, activate the receptors themselves as well as latent cytoplasmic proteins by phosphorylation. The phosphorylated proteins either phosphorylate other cytoplasmic proteins (e.g. the adaptor protein, IRS-1) and activate various signalling pathways or translocate into the nucleus (e.g. STATs) and affect gene transcription. Members of the cytokine receptor family activate similar signalling pathways due to the common structural motifs.

1.3.2. GHR Isoforms

Several isoforms of the GHR have been identified. A circulating form, the growth hormone binding protein (GHBP), exists in all animals, identical in

sequence to the extracellular region of the full-length receptor (109). The mouse and rat have an alternative exon, 8A, which introduces a premature stop codon; thus, two GHR mRNAs are produced by alternative splicing, one of which encodes the GHBP (110). In man, the cow and the rabbit, the GHBP is produced by proteolytic cleavage of the GHR at the cell surface (109). Both mechanisms of generating GHBP exist in the rhesus monkey (111). Cleavage of GHR by the metalloprotease TACE/ADAM 17 produces GHBP (ECD) and a remnant (TMD and ICD) which is cleaved further by γ-secretase (112-116). The major role of the GHBP seems to be to enhance the effects of GH by increasing its circulatory half-life.

Other variable forms of the hGHR have been detected exclusively in human tissues. Exon 3 of the GHR gene codes for 22 amino acids in the N-terminal region of the receptor. An exon-3 deleted GHR isoform is present in about 30% of individuals (117). Initially, studies from this laboratory and others reported that exon 3 deleted GHR isoforms are produced as a result of alternate GHR mRNA splicing in tissues and that expression is individual-specific (118;119). Subsequently, it was determined that individuals expressing the exon-3 deleted isoform do not have exon 3 of the hGHR in their genomic sequence as a result of a retroviral deletion mechanism (117;120).

Whether the exon 3 deleted isoform is functionally different from the exon 3 retaining isoform is still controversial. Both isoforms of the GHR (exon 3+ and 3-) have been shown to have similar binding properties and the presence of a single copy of either the exon 3 deleted or exon 3 retaining isoform has been shown to be sufficient for growth (121-123). A nonsense mutation in exon 3 affecting it's splicing has been identified in a patient with Laron syndrome. The other GHR allele of this patient has another mutation in exon 4, whilst the parents are heterozygous for each mutation and are of normal height (120). Thus, there is considerable evidence that having one normal GHR allele (3+ or 3-) is sufficient for normal function.

Three recent clinical studies of final adult height and growth responses to GH treatment in idiopathic short stature, SGA (small for gestational age) and GH deficient children, and Turner syndrome patients suggest that individuals homozygous for exon 3 retaining hGHR isoforms are less responsive to GH (124-126). In contrast, two groups have recently reported that the presence or absence of exon 3 of the GHR does not affect height in children with idiopathic short stature or CGD (127;128). Thus, the exon 3 controversy remains unresolved.

Two truncated (GHR277 and GHR279) forms resulting from alternate splicing of exon 9 are present as <1-<10% of all GHRs in GH target tissues (129;130). GHR279 is produced by the use of an alternative 3'

acceptor splice site 26 bp into exon 9 (Figure 5), while exon 9 is completely excluded from the GHR277 isoform. Both isoforms lack the necessary domains required for signalling but can heterodimerise with full length GHR and, therefore, act as dominant negative forms of the receptor (129). The truncated isoforms are highly resistant to internalisation and produce large amounts of GHBP (129-132). The actual mechanisms governing the physiological production of these alternate GHR isoforms are not yet known. However, relative amounts appear to be involved in GH insensitivity (GHIS). Three patients presenting with GHIS, characterized by high GHBP levels and partial responses to exogenous GH, were found to be heterozygous for a mutation in the donor splice site of intron 9 that resulted in the skipping of exon 9 (133). GHR 277, normally <1% of expressed GHR, was now at least 50% of expressed GHR in the lymphocytes of these patients (133). This unusually large proportion of the truncated GHR accounts for the high serum levels of GHBP, and the presence of one normal GHR allele allows for a partial response to GH administration (133;134). Aylin *et al.* have also reported mutations that result in exon 9 skipping and familial GHIS (131;132).

1.3.3. GH signalling pathways

Figure 5: Schematic representation of hGHR and the alternatively spliced truncated hGHRtr (GHR 279).

Frame-shifting by a 26-bp deletion results in a novel translation of 6 intracellular amino acids and an early termination, truncating 97.5% of the intracellular sequence. Box 1 is interrupted and Box 2 is deleted. The changed amino acids are indicated in bold. K: lysine; Q: glutamine; R: arginine; I: isoleucine; M: methionine; L: leucine; S: serine; D: aspartic acid. Figure and text of legend from (135).

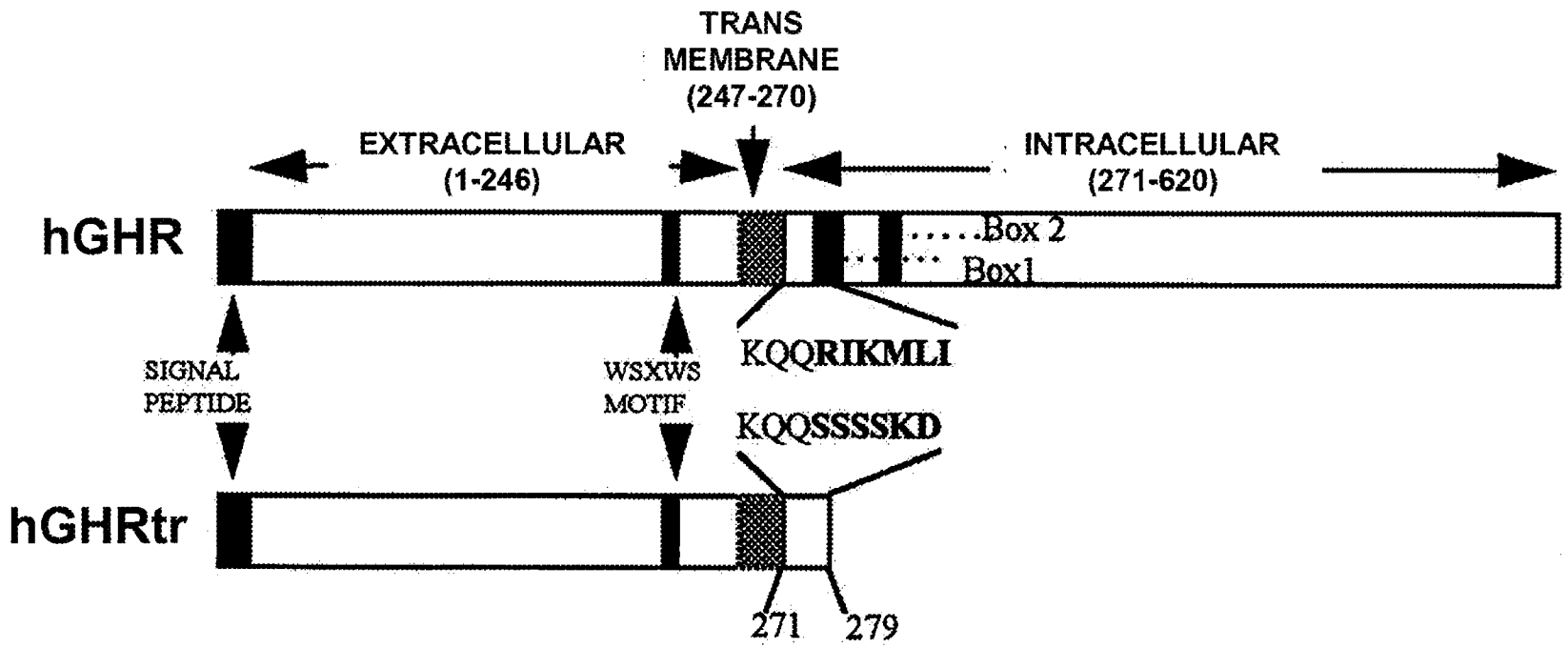
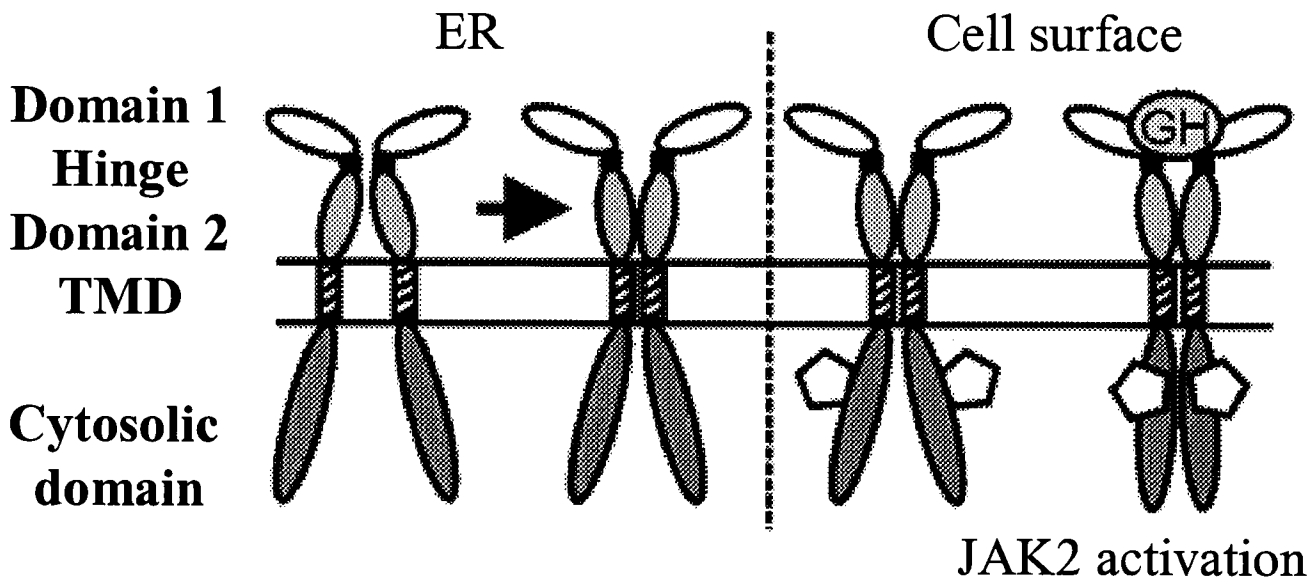

TRANS
MEMBRANE
(247-270)
EXTRACELLULAR
(1-246)
INTRACELLULAR
(271-620)
hGHR
Box 2
Box1
KQQRIKMLI
SIGNAL
PEPTIDE
WSXWS
MOTIF
KQQSSSSKD
hGHRtr
271
279

Figure 6: Hypothetical model of GHR dimerization.

Dimerization of GHRs occurs in the endoplasmic reticulum (ER) and the GHRs are transported to the cell surface as dimers. GH binding induces a conformational change in the GHR, activating JAK2. Figure from (136).


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

1.3.3.1. Receptor dimerization

For many years, it has been proposed that GH binding causes GHR dimerization. However, a recent report has challenged this, showing that, in the absence of ligand, GHR exist as preformed dimers both in the endoplasmic reticulum (ER) and at cell surfaces (Figure 6) (136).
One molecule of GH binds to a dimer of its specific membrane bound receptor. Site 1 of the GH molecule binds to one molecule of GHR while site 2 of GH interacts with a second receptor. The heterotrimer complex results in conformational change of the receptor and activation of a cascade of signalling pathways that mediate the effects of GH on a cell (Figure 7) (137).

JAK activation

Following receptor activation, Janus kinases (JAKs) are recruited to box 1 of GHRs. In turn, they transphosphorylate each other as well as the tyrosine residues within the intracellular domain of GHR (105). To date, experiments suggest that GH signalling mainly involves JAK1, JAK2, JAK3 and Tyk 2, a JAK related protein, but JAK2 is preferentially recruited (138;139). Apart from JAK 3, that is highly abundant in haematopoietic cells, all of the JAK family proteins are ubiquitously expressed [reviewed in (140;141)]. JAKs are required for cytokine signalling and are essential for several biological functions as determined by studies in knock out mice and natural mutations in humans [reviewed in (141)]. JAK 1 is an

important determinant in early development as disruption in mice is perinatally lethal (142). Targeted disruption of JAK2 in mice causes defective haematopoiesis and is also embryonically lethal (143;144). JAK3 -/- mice and humans with JAK3 mutations suffer from severe combined immunodeficiency (SCID), where lymphopoiesis is defective while myelopoiesis seems normal (145). As the mice age, defective myelopoiesis becomes apparent and mice have enlarged spleens and an increase of myeloid progenitors in peripheral blood (146). Thus JAK1 and JAK2 are important in early development, while JAK3 is involved in haematopoiesis. Information from studies of Tyk2 knock out mice suggest that it is most important in responses to IL-12 and LPS [reviewed in (141)].

JAK-STAT pathway

Many pathways have been described that are activated following phosphorylation by the JAKs. However, the most well characterised pathway involves the signal transducers and activators of transcription (STATs). Following activation of the GHR, depending on the cell type involved, STATs 1, 3 or 5 are recruited to the receptor and tyrosine phosphorylated by JAK2. STATs 1 and 3 interact with phosphotyrosine residues on JAK2 via their *src* homology (SH2) domains, while STAT5 associates with JAK2 as well as the phosphorylated GHR. Homo- and hetero-dimers of the phosphorylated STATs then translocate to the

nucleus where they bind to their specific response elements, namely gamma interferon activated sequences (GAS) and GAS-like (GLE) response elements found on GH responsive genes such as IGF-1, the early genes c-fos and c-jun, serine protease inhibitor 2.1 (spi 2.1) and β-casein [reviewed in (147)]. In liver, JAK2 preferentially phosphorylates tyrosine residues of STAT5 in response to GH.

Mitogen Activated Protein Kinase (MAPK) pathway

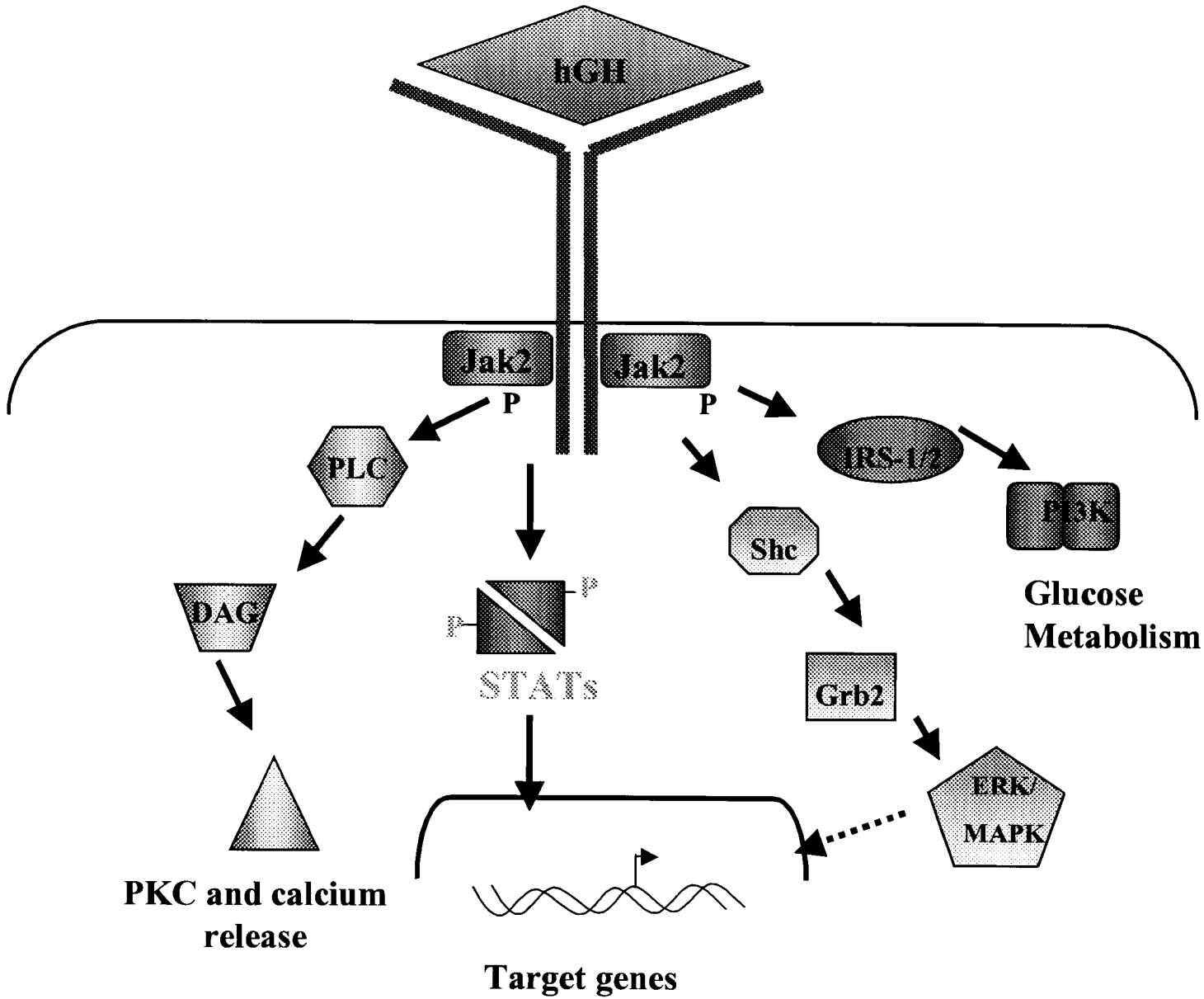
The MAP kinase family of proteins are serine-threonine protein kinases that mediate cellular responses to several factors, including GH. They include MAPKK kinase, MAPK kinase and MAPK. p38 MAPK is required for GH action on cytoskeletal organisation of the cell and cell proliferation (148). p44/42 MAPK and c-jun N-terminal kinase (JNK/SAPK) are also activated by GH (149;150). In 3T3-F442A cells, GH has been shown to activate the Ras-Raf-MEK pathway by the SHC-Grb2-SOS complex (151). The MAPK pathway involves several cytoplasmic adaptor and docking proteins that are common to other pathways, enabling cross-talk with several other pathways [reviewed in (150)].

Phosphatidylinositol-3-Kinase (PI3K) pathway

Insulin-like effects of GH include stimulation of lipogenesis, amino acid and glucose transport, protein synthesis, rearrangement of the cytoskeleton and mitogenesis (152).

Figure 7: GH activated signalling pathways.

Signal transduction pathways of GH. GH binds to its surface receptor, GHR, and, through the activation of JAK2 proteins, stimulates a series of signalling pathways. See text for details. Adapted from (147).


hGH
Jak2
P
Jak2
P
PLC
IRS-1/2
PI3K
Shc
DAG
P
P
STATs
Grb2
Glucose
Metabolism
ERK/
MAPK
PKC and calcium
release
Target genes

In common with signal transduction pathways activated by both factors are the insulin receptor substrates-1 and –2 (IRS-1 and –2). Tyrosine phosphorylation of IRS-1, –2 and –3 is stimulated by several cytokines, including GH, IL-2, IL-4 and oncostatin M (OSM) [reviewed in (153)]. GH stimulates tyrosine phosphorylation of IRS proteins via JAK2 activation of the docking protein, SH2-B (154). IRS proteins act as docking molecules for several proteins, including Grb-2, Nck, Csk and p85 (regulatory subunit of PI3K). Effects of GH mediated by PI3K include GH-induced organisation of actin cytoskeleton and anti-apoptotic actions through PI3K dependent phosphorylation of Akt (155;156). The Akt pathway also mediates GH actions on glucose transport by inducing GLUT-4 membrane translocation, and C/EBPβ-dependent regulation of *c-fos* by glucose synthase kinase 3 (GSK-3) (157;158).

Phospholipase C (PLC)/Protein kinase C (PKC)/Ca2+ pathway

All members of the cytokine receptor superfamily activate the PLC pathway. PLC hydrolyses phosphotidylinositol 4,5-bisphosphate into 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (65). IP3 increases intracellular Ca^{2+} concentrations, while DAG activates PKC. In addition, PLC-γ binds to and is tyrosine phosphorylated by GHR/JAK2 complexes (150). Effects of GH mediated through the PLC/PKC/Ca^{2+} pathway include lipogenesis, c-fos gene regulation, GHR internalisation and proteolytic cleavage (159-161).

GH activation of L-type calcium channels is JAK2-independent but requires the C-terminus (amino acids 454-506) of the GHR (162).

1.3.4. Regulation of GH signalling

Fine tuning of the GH/GHR signalling axis is dependent on the availability of GHR molecules on the cell surface as well as by both negative and positive regulation of signalling pathways (Figures 8, 9 and 10).

1.3.4.1. Hormonal regulation of GHR levels

Sex steroids, glucocorticoids, thyroid hormones and GH regulate the expression and availability of the GHR. GH-mediated postnatal growth is modulated by oestrogen in females and androgens in males [reviewed in (163)]. Hepatic GHR and GHBP protein levels are higher in female rats compared to their male counterparts (164-166). Studies in the rat have shown that ovariectomy or treatment with an oestrogen antagonist lead to a reduction in hepatic GHR and GHBP proteins, while they are increased by oestrogen treatment (167). However the effects of oestrogen are GH-dependent as the oestrogen receptor has been shown to be regulated by GH (168-170). In mice and humans, GHR protein levels are highest in the liver of the pregnant female (164;171). Oestrogen also regulates GHR at the transcriptional level, possibly through an ERE (oestrogen response element) on the mouse L1 promoter (172). In extra-hepatic tissues,

oestrogen regulation of GHR mRNA expression is cell-type specific: it increases GHR expression in osteoblasts but not in the uterus (173;174). Glucocorticoids generally stimulate GHR expression. A cortisol surge in the late gestational foetus increases hepatic GHR expression and cortisol treatment of human osteoblast-like cells increases GHR expression (175;176). However, dexamethasone has been shown to have dose-related effects on GHR expression in rat hepatocytes: stimulation at low levels and inhibition at high ($> 10^{-8}\mu m$) doses (177).

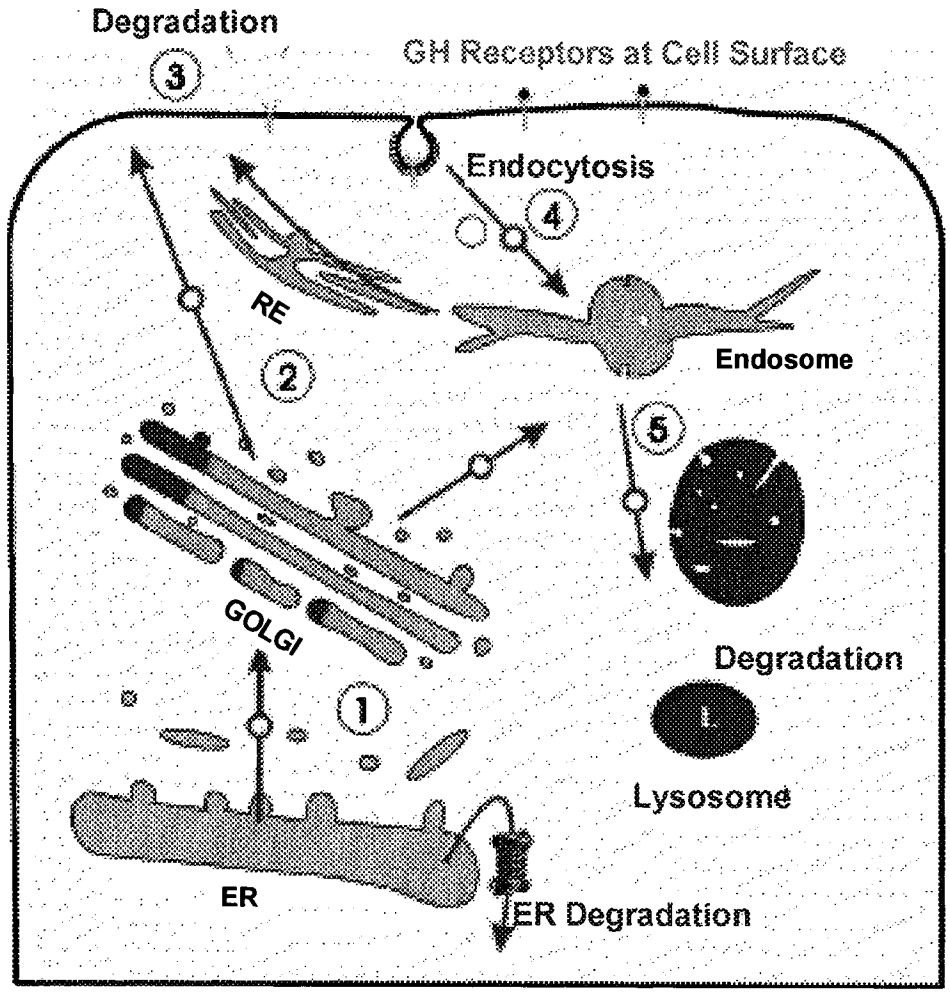
Thyroid hormones increase the transcription of GHR in ovine hepatic tissue, human hepatoma cells (Huh7) and porcine hepatocytes (178-180). GH upregulates its own receptor in hepatocytes, epiphyseal chondrocytes, mesangial cells and adipose tissue (181-188).

1.3.4.2. Bioavailability of GHR molecules

GH induces the internalisation and degradation of GHR via a proteasome-ubiquitination system.

Figure 8: Regulation of GHR bioavailability.

GHR are synthesized in the endoplasmic reticulum (ER) (1), sorted in the Golgi complex and transported to the cell surface (2). Receptors are shed into the extracellular space (3) or endocytosed into the cells (4) and transported to the lysosomes for degradation (5). Availability for GH at the cell surface is determined by: the rate of endocytosis by the ubiquitin–proteasome pathway (75%), the rate of shedding (10%), and other (unknown) mechanisms (15%). Figure and text of legend from (189).


Degradation
3
GH Receptors at Cell Surface
Endocytosis
4
RE
2
Endosome
5
GOLGI
Degradation
1
Lysosome
ER
ER Degradation

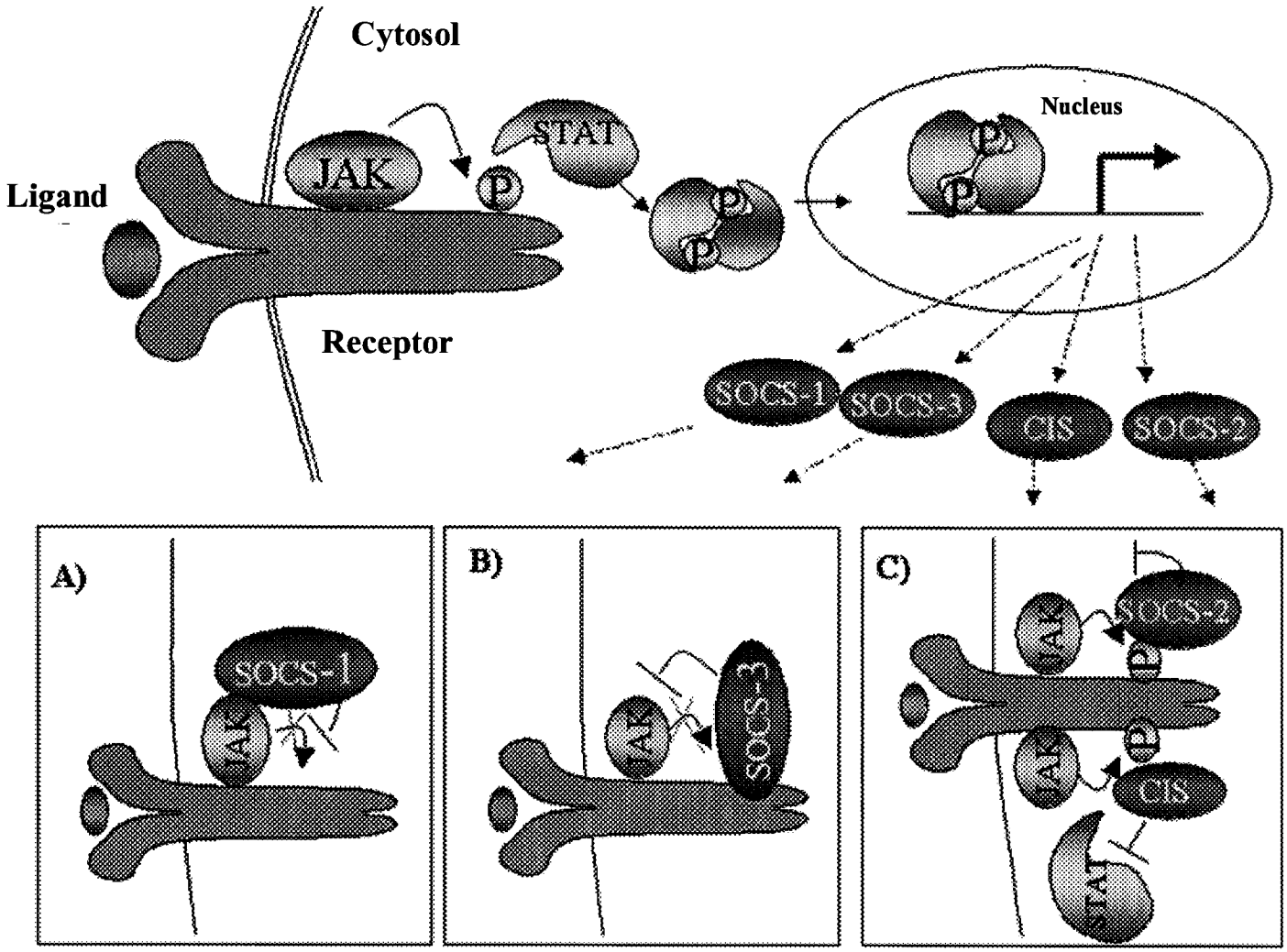
Ubiquitination is a three-step process: 1) E1, the ubiquitin-activating enzyme, activates ubiquitin, a 67 amino acid protein; 2) E2, the ubiquitin-conjugating enzyme, receives ubiquitin from the E1-ubiquitin complex; 3) E3, a ubiquitin ligase, catalyses the transfer of ubiquitin to the amino group of a lysine residue on the receptor [reviewed in (189-191)]. Polyubiquitinated substrates are then recognised and degraded by the 26S proteosome complex (190). A 10 amino acid UbE motif is located within box 2 of GHR (192). Mutation of the UbE motif increases the half-life of GHR (189).

However ubiquitination of GHR is dispensable in the internalisation of GHR by the proteosome: the internalisation of a truncated GHR lacking the UbE motif and having all 10 lysine residues in the ICD mutated is still dependent on the ubiquitin-proteosome pathway even though it is not ubiquitinated (192). Internalisation of GHR has been shown to occur in clathrin-coated pits and to involve association with caveolin (193-195).

In addition to preventing STAT5b binding to the GHR, cytokine-inducible SH2-containing protein (CIS) also targets GH-GHR-JAK2 complexes for the proteosome-ubiquitination pathway (See section 1.3.4.2) (196).

Figure 9: Negative regulation of GH signalling.

Cytokine stimulation activates the JAK-STAT pathway, leading to induction of CIS, SOCS1, SOCS2 and SOCS3. These SOCS proteins then inhibit the signalling pathway that initially led to their production. (A) SOCS1 binds to JAK and inhibits its catalytic activity, as does SOCS3 (B) after binding to the activated receptor. (C) CIS blocks STAT binding to the cytokine receptor thereby preventing STAT activation. The inhibitory mechanism of SOCS2 involves binding to two phosphorylated tyrosines on the ICD of the GHR. Figure and text of legend from (197).


Cytosol
Ligand
JAK
P
STAT
P
P
Nucleus
P
P
Receptor
SOCS-1
SOCS-3
CIS
SOCS-2
A)
SOCS-1
JAK
B)
SOCS-3
JAK
C)
SOCS-2
JAK
P
P
JAK
CIS
STAT

1.3.4.3. Additional Negative Regulation by Intracellular Molecules.

Additional proteins that negatively regulate GH signalling are either constitutive or induced. Tyrosine phosphatases and protein inhibitors of activated STATs (PIAS) are constitutive regulators, whereas suppressors of cytokine signalling (SOCS) are inducible (198).

SOCS

Four of the eight members of the SOCS superfamily are involved in the regulation of GH signalling, namely SOCS1, SOCS2, SOCS3 and CIS. Interestingly, all four proteins are induced in rat liver by GH, via STAT binding (199).

SOCS3 is rapidly and transiently induced, the production of SOCS2 is slow and sustained while the effect on CIS is rapid and prolonged [reviewed in (200)].

SOCS proteins suppress GHR signalling by interacting with JAK2 and inhibiting its activity, by binding to tyrosine residues of GHR usually phosphorylated by JAK2 or required for STAT interaction, or by targeting bound signalling molecules for degradation (201). SOCS1 and SOCS3 inhibit JAK activity by interacting with the activation loop of JAK2 and the receptor, respectively, whereas SOCS2 and CIS interact with the phosphorylated receptor and prevent access to STAT

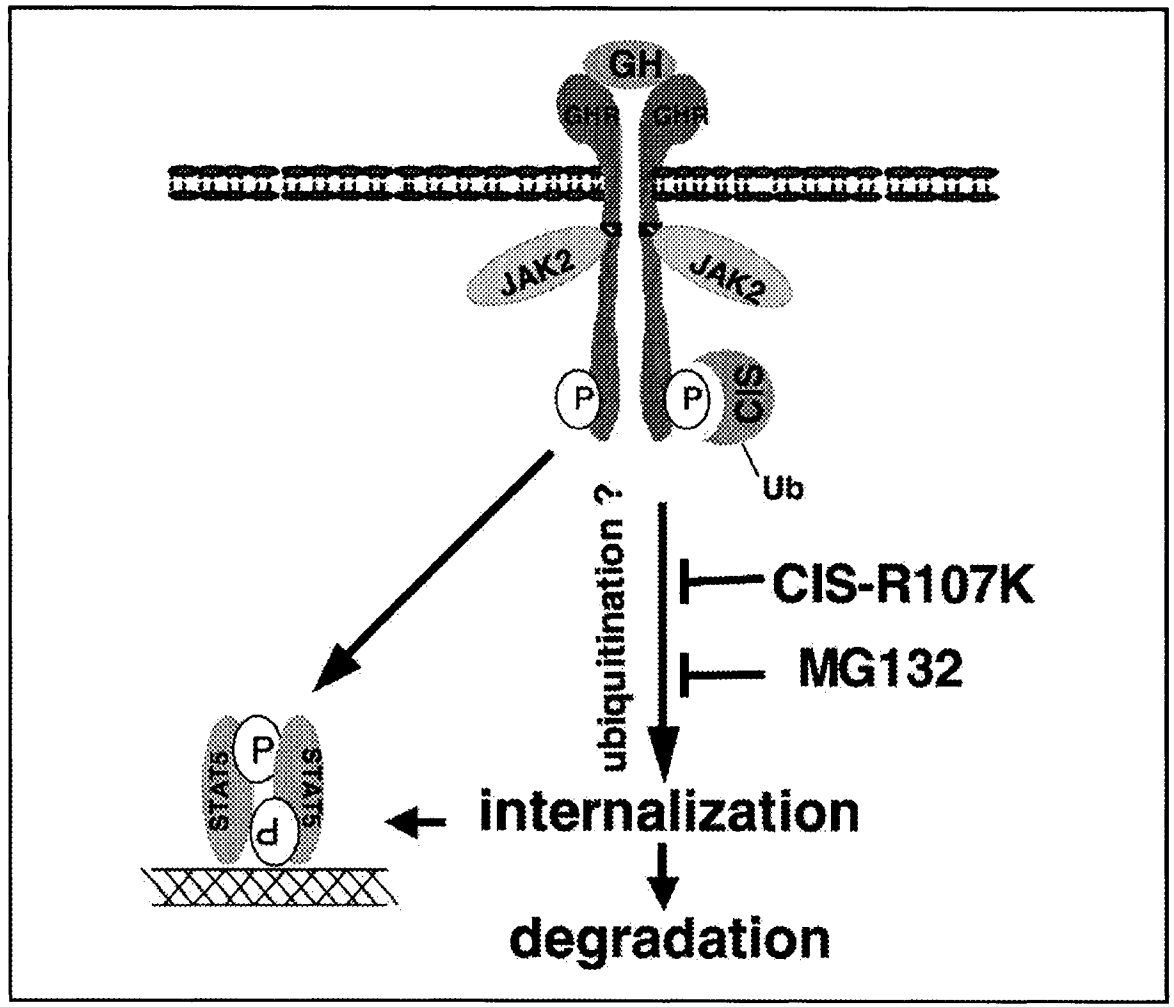
proteins (Figure 9) (201). CIS interacts with the GH-GHR-JAK2 complex and targets them for degradation by the proteosome (Figure 10) (196). The physiological role of SOCS proteins has also been studied by their targeted disruption in mice. SOCS1 -/- mice die around 3 weeks after birth of multiple organ failure (202;203). SOCS2 -/- mice develop gigantism whilst overexpression of SOCS2 leads to only mild gigantism (204). Titration studies of SOCS2 suggest a dual role in regulation of GH signalling: low protein concentrations of SOCS2 inhibit about 50% of GH induced STAT5 activation, whereas high levels enhance GH signalling possibly by inhibiting the activity of other members of the family (205). SOCS3 -/- is embryonically lethal: mice die from placental insufficiency and defective erythropoiesis (206;207). SOCS3 mRNA is more highly expressed in rat foetal liver than in adult tissues (206;208).

Tyrosine phosphatases

SH-2 domain containing protein-1 (SHP-1) and SHP-2 have been implicated in the regulation of GH signalling. SHP-1 is activated GH and binds to JAK2 (209). GH also causes SHP-1 to be translocated into the nucleus where it binds to STAT5b and attenuates signalling (209). SHP-2 has both negative and positive effects on GH signalling. It acts as a negative regulator of GH activated signalling pathways by attaching to the GHR (210).

Figure 10: Regulation of GHR internalization by CIS.

One of the pathways of GHR internalization involves CIS. CIS interacts with GH-GHR-JAK2 complexes and targets them for degradation by the proteosome. Transfection of CIS-R107K, the dominant-negative SH2 domain mutant form of CIS into cells inhibits GHR internalization. The proteosome inhibitor MG132 also inhibits GHR internalization. Once internalized, the GH-GHR-JAK2 complex continues to phosphorylate Stat5 proteins. Figure from (196).


GH
GHR
GHR
JAK2
JAK2
P
P
CIS
Ub
ubiquitination ?
CIS-R107K
MG132
STAT5
STAT5
P
P
internalization
degradation

Mutating the tyrosyl residues of GHR that are recognised by SHP-2 results in prolonged GH-induced tyrosine phosphorylation of GHR, JAK2 and STAT5b (210;211).

Protein inhibitors of activated STATs (PIAS)

The PIAS family of proteins was identified as interacting with STAT proteins using the yeast two hybrid assay (212;213). PIAS proteins have an N-terminal LXXLL motif also present in nuclear receptors and required for cofactor interaction, a putative zinc binding motif, an acid rich region and a serine/threonine rich region (214).

Recently a PINIT motif has been identified as being essential for the nuclear retention of PIAS (215). PIAS proteins have been shown to bind activated STAT1 and STAT3 dimers, to inhibit their DNA binding and to act as a corepressor of STAT4 (212;216;217). PIAS regulation of STAT activity has been shown in prolactin activated signalling pathways, although it remains to be determined whether it has the same effect on GH signalling (213).

Growth factor receptor bound protein-10 (Grb-10)

Grb-10 is an SH-2 and pleckstrin homology (PH) adaptor protein that regulates GH signalling by interacting with phosphorylated tyrosine residues 454-620 of GHR. In Huh7 cells, Grb10 inhibited GH-induced stimulation of c-fos and spi 2.1 promoter constructs via the SRE and

GHRE2 elements, respectively. Although it is known that Grb10 does not inhibit the phosphorylation of GHR, JAK2 or Stat5, the mechanism of action has not yet been determined (218).

1.3.4.4. Positive regulators

SHP-2

As mentioned earlier, SHP-2 has a dual role in the regulation of GH signalling. SHP-2 is tyrosine phosphorylated in response to GH and associates with Grb2 and SIRPα (211). The mechanism by which SHP-2 exerts a positive effect is unknown. It has been speculated that interaction with Grb2 will lead to activation of the Ras-Raf-MAPK pathway. Also, phosphorylated SHP-2 may dephosphorylate certain substrates such as the negative regulator, SIRPα (211).

SH2-B

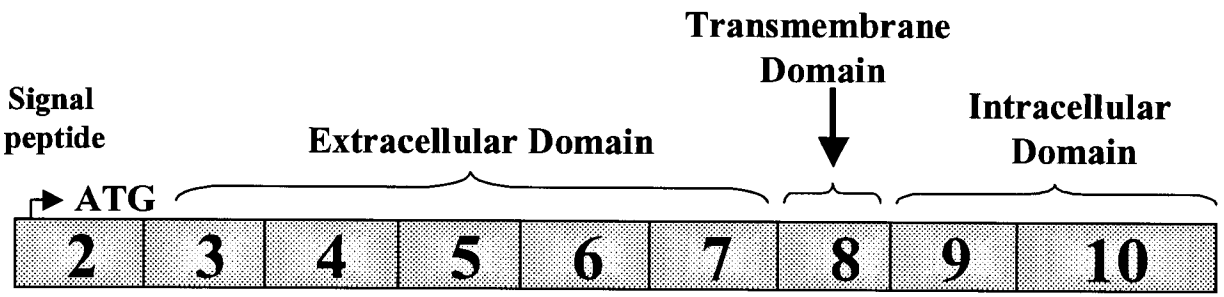
SH2-B is an adaptor protein that contains both an SH-2 and a PH domain. Of the three splice variants (α, β, γ), SH2-Bβ has been shown to interact with JAK2 via its SH-2 domain (150;219). It has been shown to specifically increase GH-stimulated tyrosine phosphorylation of JAK2 and the tyrosine phosphorylation of STAT5b by JAK2, thereby enhancing activation of the downstream signalling pathways (220).

Figure 11: The GHR gene, coding exons and protein domains.

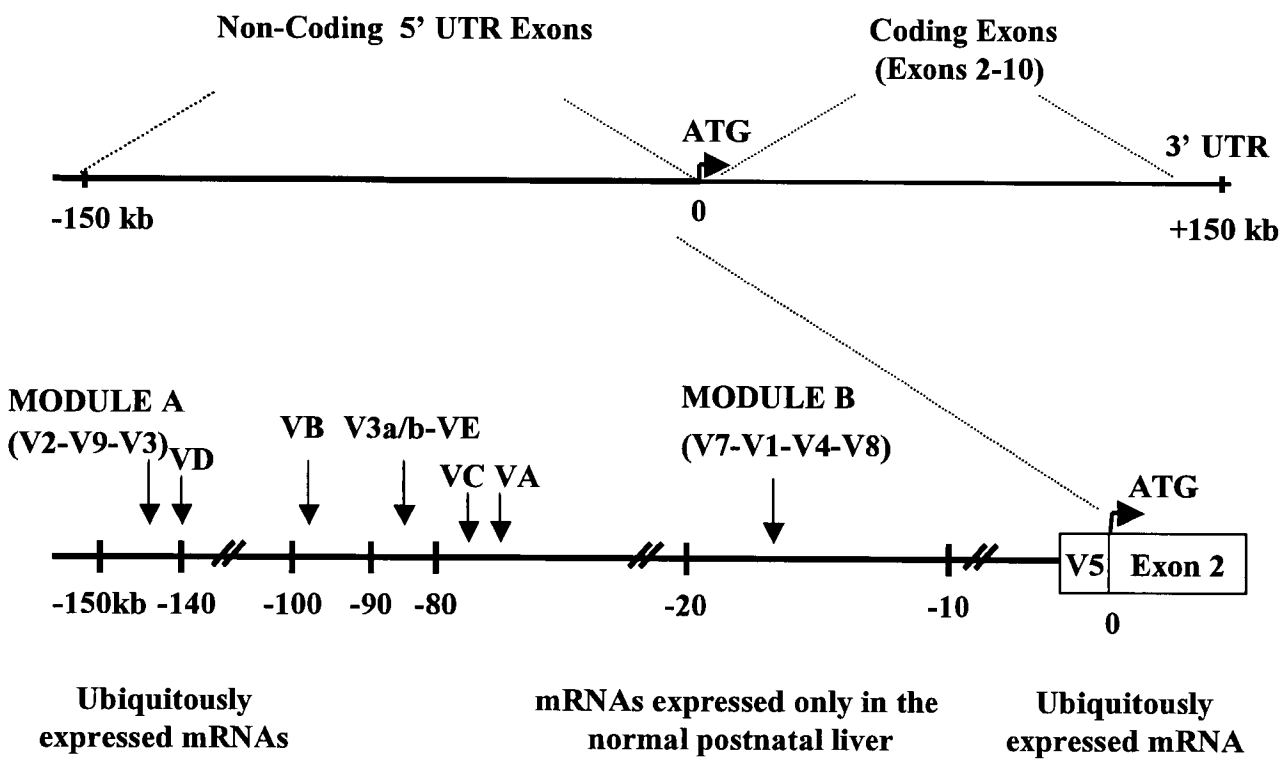
The hGHR is coded for by exons 2-10. (A) The ECD is coded for by exons 3-7, the TMD by exon 8, and ICD by exons 8 (part), 9 and 10.

(B) The hGHR gene is located on the short arm of chromosome 5 near the centrosome. Thirteen non-coding exons have been reported within the 300 kb upstream of exon 2 in the 5′ UTR. Seven of the non-coding exons are clustered in 2 small regions defined as Module A (~1.6 kb) and Module B (~2 kb); VA-VE and V3a/b are found between the two modules. V5 is located adjacent to the first coding exon, exon 2. hGHR: human Growth Hormone Receptor; ECD: extracellular domain; TMD: transmembrane domain; ICD: intracellular domain (102;221-224).

A


Transmembrane Domain
Signal peptide
Extracellular Domain
Intracellular Domain
ATG
2
3
4
5
6
7
8
9
10

B


Non-Coding 5' UTR Exons
Coding Exons (Exons 2-10)
ATG
3' UTR
-150 kb
0
+150 kb
MODULE A (V2-V9-V3)
VD
VB
V3a/b-VE
VC
VA
MODULE B (V7-V1-V4-V8)
ATG
V5
Exon 2
-150kb
-140
-100
-90
-80
-20
-10
0
Ubiquitously expressed mRNAs
mRNAs expressed only in the normal postnatal liver
Ubiquitously expressed mRNA

1.4. GHR gene

The hGHR gene has been mapped to chromosome 5p12-13, where a cluster of other receptors for growth factors such as PRLR, IL-5R, IL-3R, CSF-2R, GM-CSFR and PDGFR have also been localized (221). The gene locus spans approximately 300 kb (Figure 11B).

The coding region of the GHR gene consists of 9 exons (exons 2-10) that code for a 620 amino acid protein: exon 2 codes for 18 aa of the signal sequence and the first five amino acids of the extracellular domain (ECD), exons 3-7 code for the majority of the ECD, exon 8 for the last three amino acids of the ECD and the 24 amino acid transmembrane region (TMR) and exons 8 (part), 9 and 10 for the 350 amino acid intracellular domain (ICD) (Figure 11A) (102).

1.4.1. Growth hormone insensitivity: Defects of GHR (coding region)

Laron dwarfism, idiopathic short stature (ISS) and constitutional growth delay (CGD) are three classes of clinical defects associated with GH insensitivity (GHI), a term used to describe a condition characterised by low to non-detectable levels of circulating IGF-1 in parallel with (and in response to) normal or supraphysiological levels of GH (225).

1.4.1.1. Laron syndrome

In 1958-59 Laron *et al.* first described three Israeli siblings with marked short stature and hypoglycaemia (226). In the years following, the

purification of hGH and development of specific hGH radioimmunoassays (RIA) enabled them to define the biochemical reason for this disorder (227;228). Patients had elevated levels of GH, low circulating levels of IGF-1 and their hepatic membranes did not bind radiolabelled GH. Patients also failed to produce IGF-1 in response to GH stimulation (229). These observations suggested a primary defect in the GHR. Two years after cloning of the human and rabbit GHR, a partial gene deletion (resulting in the loss of part of the GHR ECD) in two patients with Laron-type dwarfism was characterised (102). Current nomenclature of this autosomal recessive disorder in the literature includes Laron dwarfism, Laron syndrome (LS), GH insensitivity (GHI) and GHR deficiency (GHRD). In addition to short stature and the biochemical abnormalities, patients have craniofacial abnormalities, sparse hair, blue sclerae (in Mediterranean and Middle-Eastern patients), high pitched voices in certain children and adult females, profuse sweating and hypoglycaemia in newborns and infants, delayed sexual maturation and hypogonadism (more pronounced in males), and obesity. To date several defects, including missense, nonsense and splice site mutations as well as whole gene deletions, have been found in the GHR coding sequence of patients with Laron syndrome [reviewed in (226;230)]. GHBP levels are low to undetectable in 80% of GHR-deficient patients (231). Patients with normal to high circulating levels of GHBP have GHR mutations that allow

for the production of high levels of binding protein (229;232). The only form of treatment available for these patients is exogenous recombinant IGF-1. Patients have been identified all over the world, and include patients from Ecuador, Brazil, South Africa, USA, various parts of Europe and the middle East (233).

The Laron syndrome mouse model is similar to the human in that it exhibits low IGF-1 levels, elevated levels of GH in serum, growth retardation and delayed sexual maturation (234). However, mice models do not present with hypoglycaemia, obesity or elevated levels of insulin as is seen in humans with Laron syndrome (235).

Only rare cases of mutations (<2%) in the GHR coding regions have been described in patients with ISS or CGD; thus the classification of these disorders remains "idiopathic".

1.4.1.2. Defects in GH signal transduction pathways

Defects in tyrosine phosphorylation of STAT5 have been observed in two patients with GHI, although no molecular mutation was identified (236). The patients were short and had elevated levels of GH and low IGF-1 and IGBP-3 levels (237). Kofoed *et al.* have recently reported the first patient with a homozygous autosomal molecular defect in her JAK-STAT pathway (238). The patient presented with severe growth retardation, immunodeficiency and low serum concentrations of IGF-1, IGFBP-3 and

ALS. A missense mutation at residue 630 in the SH-2 domain of STAT5b affected its tyrosine phosphorylation following GH binding to GHR (238;239). More recently, a complete absence of STAT5b in a GHI patient has been reported (240). The patient, who presented with short stature, also had normal to high GH levels whereas IGF-1, IGFBP-3 and ALS levels were barely detectable (240).

1.4.2. GHR 5'UTR mRNA isoforms

The 5'UTR of the hGHR gene locus is highly complex in that it contains several non-coding exons each of which gives rise to different mRNA transcripts [V1-V5, V7-V9, V3a/b, VA-E (223;224;241;242)] (Figure 11B). V6, initially described by Pekhletsky *et al.*, has now been reported to be an experimental artifact (241;243). Transcripts derived from these non-coding exons all splice into exon 2, eleven nucleotides upstream of the translational start site and, thus, encode the same protein. Seven of the 5'UTR exons are clustered in two small regions (1.6 kb and 2 kb), which we have named Module A and Module B, respectively (223).

V1, V4, V7 and V8 form the module B cluster which is 16-18 kb upstream of exon 2, as identified by 5' rapid amplification of cDNA ends (RACE), RNAse protection (RPA) and primer extension (PE) assays (223). V1, the major transcript in the human liver, has two TATA boxes and two transcriptional start sites (TSS) that are unique to the human, while the

second and downstream TATA/TSS complex is conserved across species (Figure 12) (223). The end of V7 is just 60 bp upstream of the first TSS start site for V1. Module A contains V2, V3 and V9 and is, according to the most recent Human Genome Map (NCBI), ~ 140 kb upstream of exon 2 (224). Module A exons are dominated by CpG islands that suggest possible regulation by methylation. V2, a highly GC rich exon produces the second major transcript in the liver and the most abundant transcript in non-hepatic tissues (223;224). Two V5 mRNA transcripts have been detected in human tissues: the longer transcript is continuous with exon 2 and more abundant than the shorter transcript (223;241). Wei *et al.* have shown that transcripts from exons VA-VE are ubiquitously expressed (224).

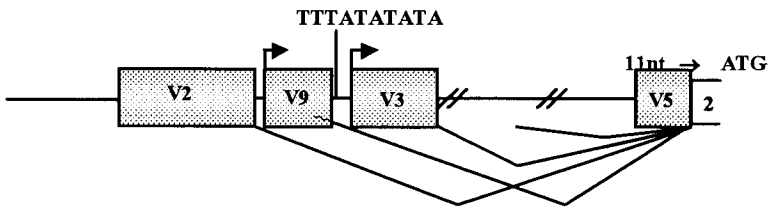
1.4.2.1. Modules A and B: Ubiquitous versus tissue- and developmental-specific expression of the GHR

The hGHR mRNA isoforms are expressed in both tissue- and developmental- specific patterns: mRNAs derived from the exons of Module A (V2, V3, V9), V5 and VA-E are ubiquitously expressed, whereas transcripts arising from Module B exons (V1, V4, V7, V8) are found only in normal human postnatal liver, where V1 is the most abundant transcript (Figure 12) (223;224;244).

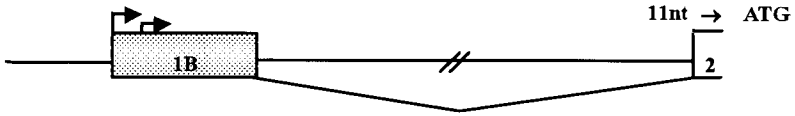
Figure 12: Comparing the 5'UTR of GHR in human and other species. Comparison of V2, V9, V3, and V5 (A) and of V7, V1, V4 and V8 (B) sequences of the human GHR gene (GenBank AJ002175 and AJ131868) with their equivalents in ovine, bovine, mouse and rat GHR genes. Figure from (59).

Module A/V5

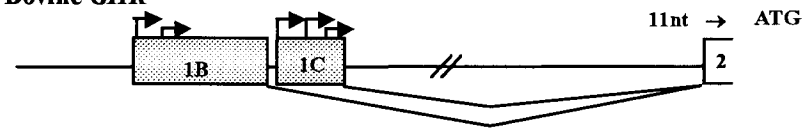
Human *GHR*



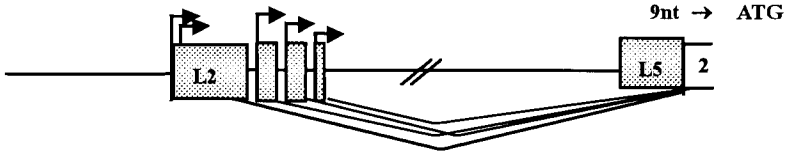
Ovine *GHR*



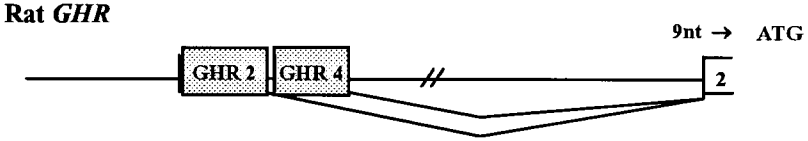
Bovine *GHR*



Mouse *GHR*

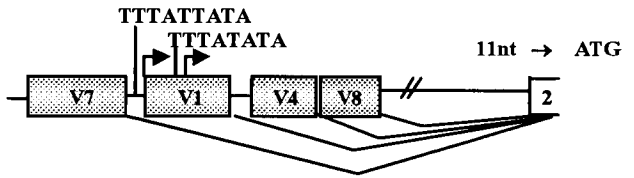


Rat *GHR*

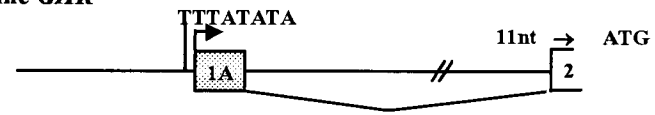


Module B

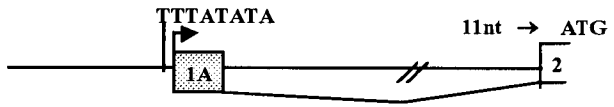
Human *GHR*



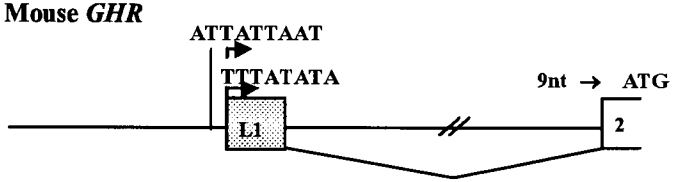
Ovine *GHR*



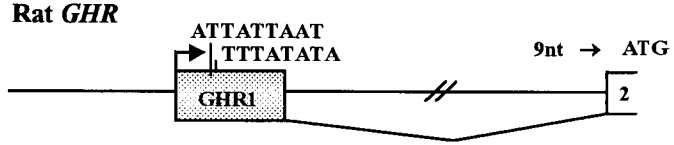
Bovine *GHR*



Mouse *GHR*



Rat *GHR*



1.4.2.2. Expression of GHR and its mRNA variants in other species

GHR genes have been identified in many species including the rabbit, mouse, rat, sheep, cow, chicken, pig, dog, monkey, fish, birds and turtles [NCBI and reviewed in (245)].

The complexity of the 5′ UTR of GHR is not restricted to the human gene. Five 5′ UTR exons have been identified in the rat and mouse, two in sheep and chicken, nine in the cow, and three in the rabbit and monkey (Figure 12 & data not shown) (222;246-252). Rat (GHR1), cow (b1A) and sheep (o1A) homologues of V1 are found exclusively in the liver (249;253;254). In rats, the expression of the liver-specific GHR1 is sexually dimorphic (255). GHR1 accounts for 30% and 2% of total hepatic GHR in female and male rats, respectively. Following gonadectomy, GHR1 levels are elevated to 7% in male mice and reduced to 16% in female mice (255). Furthermore, continuous infusion of GH to mimic the female GH secretory pattern results in a further increase in GHR levels in male rats to 17%, equivalent to females (255). In the mouse, the L1 variant is expressed only in the liver of the pregnant female (172;256). Therefore, although there is a certain degree of conservation in dynamics of GHR expression, there are also specific species differences.

The downstream TATA/TSS complex of the human V1 is conserved across species and has been described as the functional TATA for the ovine o1A, bovine b1A, and mouse L1 exons (256-258) (Figure 13). A V4 homologue is found in the baboon while V7 and V8 are, at present, unique to the human (252). Similar to V1, ovine o1A is ~17 kb upstream of the translational start site in exon 2 (246).

The human V2 mRNA variant is ubiquitously expressed, the most abundant transcript in non-hepatic tissues, and the second most abundant in normal postnatal liver. The V2 exon is located ~ 140 kb upstream of the translational start site (224). The region immediately upstream of the V2 exon is ~ 79% GC rich and does not contain a TATA box; instead it contains ETS1 and C/EBPα-CHOP sites and a CCAAT box.

The ovine equivalent, o1B, is at least 27 kb upstream of exon 2 and is ~ 78% GC rich (247). The putative promoter region is TATA-less, but it has a CCAAT box, an E-box, a GC box and a putative C/EBP site. The b1B promoter is ~ 75% GC rich and contains a single putative binding site for C/EBP, CTF/NF-1 and several Sp1 sites that are functional (259). Mouse L2 constitutes 50-80% of total hepatic transcripts in the liver of the non-pregnant mouse (260). The L2 exon is located at >26 kb upstream of the translational start site (245). Two GC boxes and a CCAAT box are found upstream of the 71% GC promoter region of the TATA-less L2 exon. L2 transcriptional activity has been shown to be regulated by Sp1 and Sp3,

and a repressosome complex containing NF-Y, BTEB1, HMG-Y/I and sin3b (260;261).

Interestingly, the level of expression of each mRNA transcript may not reflect the amount of GHR protein translated from it. The presence of multiple open reading frames (ORF) in the 5'UTR of several of the non-coding exons suggests there may be control of the GHR gene at the translational level (241;259). Translational efficiency studies have only been carried out in the bovine and have shown that, of the major isoforms in the liver (b1A, b1B and b1C), b1B is the least translated (249). This inhibition of translation could be caused by formation of a secondary structure due to the high GC content in the promoter region.

Indeed this might be the case in other species as well since tissues that express this isoform or its equivalent as the major transcript have low levels of the protein receptor, as determined by binding assays (249).

Chapter 2: OBJECTIVES

Previous findings in the laboratory of Dr. Cindy Goodyer have shown that the levels of hGHR mRNA and hGH binding in the liver are dramatically increased after birth (Figure 12) (223). These increases can be partially explained by the postnatal onset of expression of the module B liver-specific variant mRNAs (223;244).

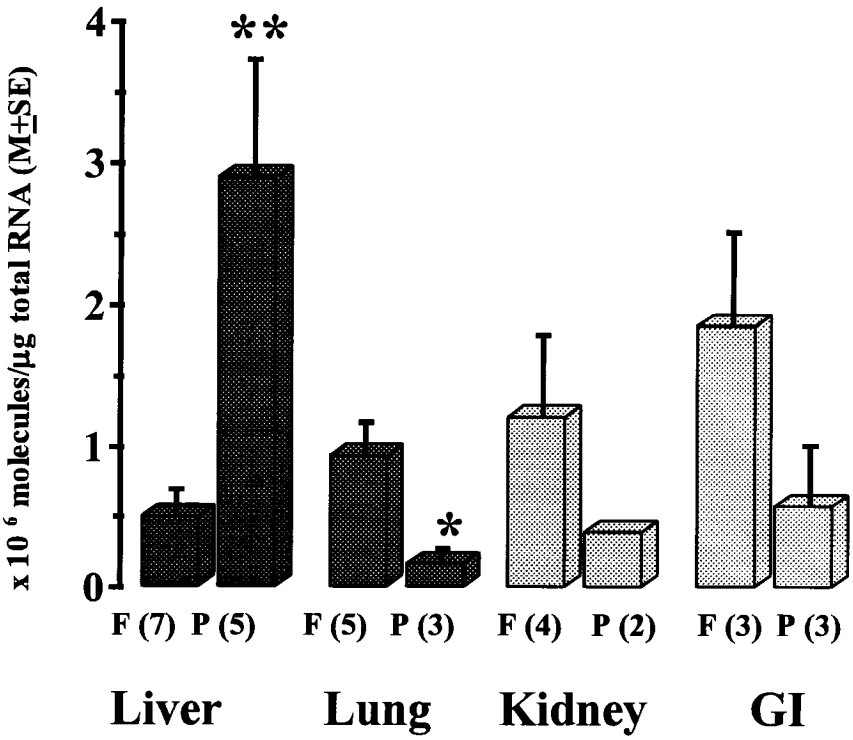
The overall goal of the Cindy Goodyer laboratory is to characterise the tissue- and developmental-specific mechanisms regulating the hGHR gene. The objectives of my PhD project have been

1) to do a preliminary characterisation of the hGHR gene promoter regions; and
2) to identify developmental- and liver-specific transcription factors that regulate expression of the V1 variant.

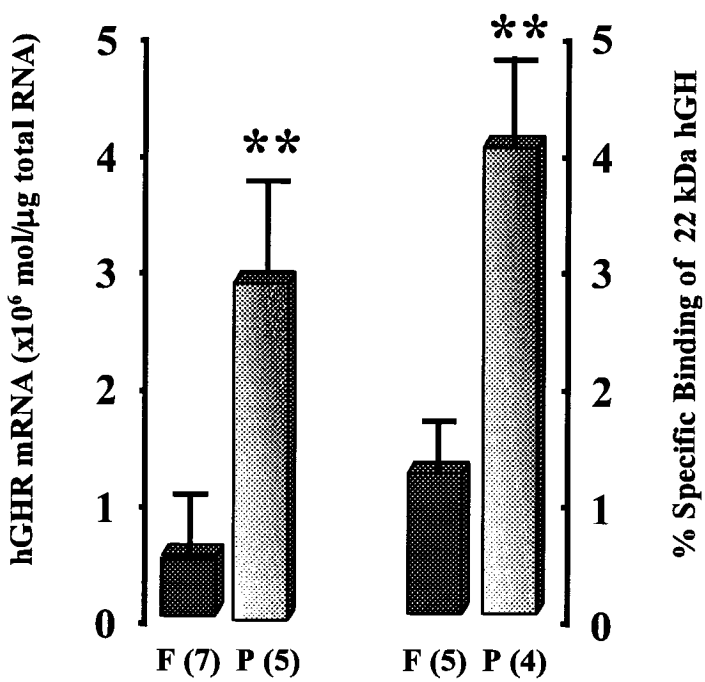
Figure 13: Tissue- and developmental-specific expression of the hGHR.

Several human foetal and postnatal tissues were analysed for (A) total GHR mRNA and (B) hGH binding. The sample number is indicated in parentheses. Both total hGHR mRNA expression and hGH binding were significantly increased in human postnatal liver. F: foetal; P: postnatal; Int: small intestine. Figure from (223).

A


x 10 6 molecules/µg total RNA (M±SE)
0
1
2
3
4
**
*
F (7) P (5)
F (5) P (3)
F (4) P (2)
F (3) P (3)
Liver
Lung
Kidney
GI

B


hGHR mRNA (x10⁶ mol/µg total RNA)
% Specific Binding of 22 kDa hGH
0
1
2
3
4
5
**
**
F (7) P (5)
F (5) P (4)

Chapter 3: MATERIALS AND METHODS

3.1. Materials

The following antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA): HNF-4α C-19 (sc-6556 and sc-6556X), HNF-4α H-171 (sc-8987X), Gfi-1 N-20 (sc-8558X), Gfi-1b (sc-22795X), anti-goat-HRP (sc-2020), goat IgG (sc-2028), rabbit IgG (sc-2027). Calnexin antibody (C45520) was obtained from Transduction Laboratories (Lexington, KY). Anti-mouse-HRP and anti-rabbit-HRP were purchased from NEN (NEN Life Sciences Products Inc., Boston, MA). An antibody to the *Drosophila* GAF protein was kindly provided by Dr. Carl Wu (National Cancer Institute, NIH, Bethesda, MD). CV1 (African Green Monkey Kidney fibroblasts), HEK293 (human foetal kidney epithelial cells) and HepG2 (human hepatoma cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA), while Huh7 (human hepatoma cells) were kindly provided by Dr. Ken K Ho (Garvan Institute of Medical Research, Sydney, New South Wales, Australia).

Expression vectors: HNF-4α1 was kindly provided by Dr. Elly Holthuizen (University of Utrecht, Netherlands), HNF-4α2 and α8 by Dr. Bernard Laine (U 459 INSERM, France), Gfi-1 and Gfi-1b by Dr. Lee Grimes (University of Louisville School of Medicine, KY), and GAF by Dr. Greti Aguilera (NICHD, Bethesda, MD). sp64 and pSV-β-galactosidase were

purchased from Promega Corporation (Madison, WI), and pA_3luc was kindly provided by Dr. Jacques Drouin (IRCM, Montreal, QC) (262).

3.2. Tissues

Human foetal livers were obtained at the time of therapeutic abortions (n=11, 13.5-19.5 wk), foetal age being determined by foot length (263). Normal human postnatal livers (n=5, 11-66 yr) were obtained 4-10 hr after the removal of a donor liver for paediatric transplantation and following perfusion to remove all blood cells. Tissues were collected following written consent and with the approval of the local ethics committees in compliance with CIHR guidelines. Tissues collected for RNA and protein studies were frozen at -80^{O}C until processed. Tissues for hepatocyte isolation were immediately processed, as detailed below.

3.3. Hepatocyte Isolation

Foetal hepatocytes were prepared as previously described (118). 2.5 mg of fresh human foetal liver were minced in 10 ml of warm filter-sterilised solution I (10 mM HEPES, 0.142 mM NaCl, 6.7 mM KCl, 1 mM EDTA.4Na). Minced tissues were washed 2X with warm sterile solution I, and transferred into a sterile scintillation vial with a mini-stir bar. 10 ml of filter sterilised warm solution II (100 mM HEPES pH 7.4, 67 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2.2H_2O$, 2 mg/ml collagenase type I [Invitrogen Corporation, Carlsbad, CA], 0.2 mg/ml DNAse I [Roche Diagnostics

Corporation, Indianapolis, IN]) was added to the minced tissue and stirred at 37°C, in 5% CO_2 for 20 min; the mixture was allowed to settle by unit gravity for 20 min and the supernatant discarded. This step was repeated. Cells were then resuspended in 5 ml of warm filter-sterilised solution III (0.96 g Minimum Essential Medium [Invitrogen]/100 ml of solution III (4.8 mM EDTA.4Na, 0.22 g $NaHCO_3$, 0.16 mg/ml DNAse I, pH 7.4) and centrifuged at 1000 RPM for 10 min. The cells were washed twice with warm sterile solution III and allowed to settle by unit gravity for 20 min. The final cell pellet was resuspended in William's Medium E (Invitrogen) supplemented with 10% FBS, 100 IU/ml penicillin G, 16 μg/ml gentamycin sulphate (Garamycin) and 39.2 μg/ml dexamethasone. Cells were then plated on 100 mm collagen-coated tissue culture dishes (BD Biosciences, Mississauga, ON) and cultured for 2 hr at 37°C in 5% CO_2. The cells were then washed 5-6X with warm sterile 1X phosphate buffered saline (PBS), until there was no trace of blood cells. Media were replenished and the cells were cultured overnight in the same conditions. This preparation gives a > 95% hepatocyte yield. Cells were washed twice with 1X PBS, scraped and centrifuged at 1000 RPM. The resulting pellets were immediately extracted for protein and RNA or frozen at –80°C until required.

3.4. RNA Extraction

1 ml of Trizol (Invitrogen) was added to cells (5-10 x 10^6) or ground tissue (200 mg) in an eppendorf tube and incubated at room temperature for 5 min to allow for complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added to the Trizol mixture and tubes vigorously shaken for 15 sec and subsequently incubated for a further 20 min at room temperature. The mixture was then centrifuged at 13,000 RPM for 15 min at 4^{O}C. The aqueous phase (clear upper layer) was transferred to a new eppendorf tube, 0.5 ml 100% isopropanol added, mixed, incubated at room temperature for 10 min and centrifuged for 10 min at 4^{O}C at maximum speed. The resultant pellet was washed with 75% ethanol (1 ml/1 ml Trizol), dried for 5 min and resuspended in 20 μl DEPC-treated water. The sample was heated at 55^{O}C for 5 min before being stored in aliquots at -80^{O}C.

3.5. Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) Assays

5μg of total RNA was transcribed in the presence of 200 U of Superscript II (Invitrogen), 200 ng of random primers (Invitrogen) or 5 μM specific antisense primers (Table 2) 0.1 M DTT, 10 mM dNTP and 1X first strand buffer (Invitrogen) in 20 μl of reaction mix. RNA and specific antisense primer solution were heated at 65^{O}C for 5 min before the reverse transcription reaction.

Table 2: Primers used in RT-PCR assays of hGHR variants.

Primer Name	Sequence
V2 sense	5'-CGGCTGCTGCTGAGCCCGGG-3'
V3 sense	5'-GGAGACCTTGGAGGGACAGAG-3'
V9 sense	ATGGAACTGGGGTCAGTAGAGTG-3'
V7 sense	5'-GTAATAAGGCCTCATGAGACTCCA-3'
V1 sense	5'-AGATTGAGAATGACTGATTTGGGAG-3'
V4 sense	5'-GAGTAGCAAAGATGGATTAGTGAG-3'
V8 sense	5'-TAGCTATGACAGCACGTATGAGC-3'
V5 sense	5'-TCGTTTGCTGTGAGGTGTTCTAT-3'
1S sense	5'-CTGCTGTTGACCTTGGCACTGGC-3'
Exon 4 sense	5'-ATTCACCAAGTGCCGTTCACCTGA-3'
Exon 5 antisense	5'-TAATCAGGGCATTCTTTCCATTC-3'
1A2 antisense	5'-AGGTATCCAGATGGAGGTAAACG-3'

Table 3: Primers used in RT-PCR assays of HNF-4α isoforms

Primer	Sequence
HNF-4α exon 1D sense	5'-CAGTGGAGAGTTCTTATGACACG-3'
HNF-4α exon 1A sense	5'-TAACCCCCACCCCTCCCCG-3'
HNF-4α exon 3/4 antisense	5'-CGGAAGCATTTCTTGAGCCTGC-3'
HNF-4α exon 3 antisense	5'-TCCCGCTCATTCTGGACGGCTT-3'
HNF-4α exon 7 sense	5'-GCCTACCTCAAAGCCATCATCTT-3'
HNF-4α C-terminal truncated antisense (intron 8)	5'-AGCGGCACAGTGGGGAAGCCA-3'
HNF-4α exon 9 sense #1	5'-CAGGAGATGCTGCTGGGAGG-3'
HNF-4α exon 9 sense #2	5'-GTTGCCAACACAATGCCCACTCACCTCAG-3'
HNF-4α exon 10 antisense #1	5'-CTGCTTGGTGATGGTCGGCTG-3'
HNF-4α exon 10 sense #2	5'-GGCTCCCGGCAGGAGCTTATAGG-3'

The RT was performed at 42°C for 50 min and the reaction inactivated at 70°C for 15 min. For reactions involving random primers, the primers were heated at 25°C for 10 min prior to the 42°C step. Products were stored at –20°C.

PCR assays were performed with Taq DNA polymerase (Invitrogen) and 3 μl of cDNA from the RT reactions. Primers (Tables 2 and 3) were purchased from Alpha DNA (Montreal, QC). PCR conditions were as follows: one cycle of 2 min at 94°C followed by amplification for 35 cycles at 94°C for 2 min, 60-70°C for 1 min, 72°C for 1.5 min, ending with 72°C for 5 min. Products were resolved on a 1.5% agarose gel, using ethidium bromide.

3.6. DNAse I Treatment of DNA-contaminated RNA

To ensure that PCR amplified RNA products were not contaminated with genomic DNA, total RNA was treated with DNase I. 9 U of DNase I (Invitrogen) was added to 10 μg of total RNA (100 ng/μl) and heated at 37°C for an hour, then at 70°C for 5 min to inactivate the DNase I. The DNA was ethanol precipitated overnight at –80°C. The DNA-ethanol mixture was spun at 13,000 RPM for 15 min at 4°C. The supernatant was discarded and the pellet was washed in cold 75% ethanol, and centrifuged for 15 min at 4°C at 13,000 RPM. The pellet was dried for 15 min at room temperature and resuspended in 15 μl DEPC water.

3.7. Cell Culture

CV1 and HepG2 cells were cultured in low glucose (1 g/L D-glucose) Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, cat # 11885-084) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin G, 1.6 mg/ml gentamycin sulphate (Garamycin) and 25 mM HEPES. HEK293 cells were grown in low glucose (1 g/L D-glucose) DMEM (Invitrogen), supplemented with 10% FBS:newborn calf serum (NCS) (1:1), 100 IU/ml penicillin G, 1.6 mg/ml gentamycin sulphate and 25 mM HEPES. Huh7 cells were cultured in Earle's salts MEM (Invitrogen), 10% FBS, 500 IU/ml penicillin G and 12.5 mM HEPES. All cells were maintained at $37^{O}C$ in 5% CO_2.

3.8. Plasmid Construction

2.7 kb (Module A) and 4 kb (Module B) of hGHR genomic DNA were subcloned from a Bac clone (hcit.102E14) into the Bluescript (pSK^{+}) vector (223). Various restriction enzymes were used to digest the DNA and specific fragments were subcloned upstream of pA_3luc (262), a luciferase reporter vector (Figure 14). Deletion constructs were created from V2V3P2 (Module A) and V1P1 (Module B) to study the putative promoter regions of the exons in the two modules (Figures 14 and 15).

Figure 14: Promoter constructs for modules A and B.

Human genomic DNA from a Bac clone (hcit.102E14) was digested using restriction enzymes and the fragments were cloned upstream of a luciferase vector, pA_3luc. Promoter constructs were created to study the promoter regions upstream of the exons in (A) module A (V2 and V3) [V9 has already been studied (223)]and (B) module B (V7, V1, V4 and V8).

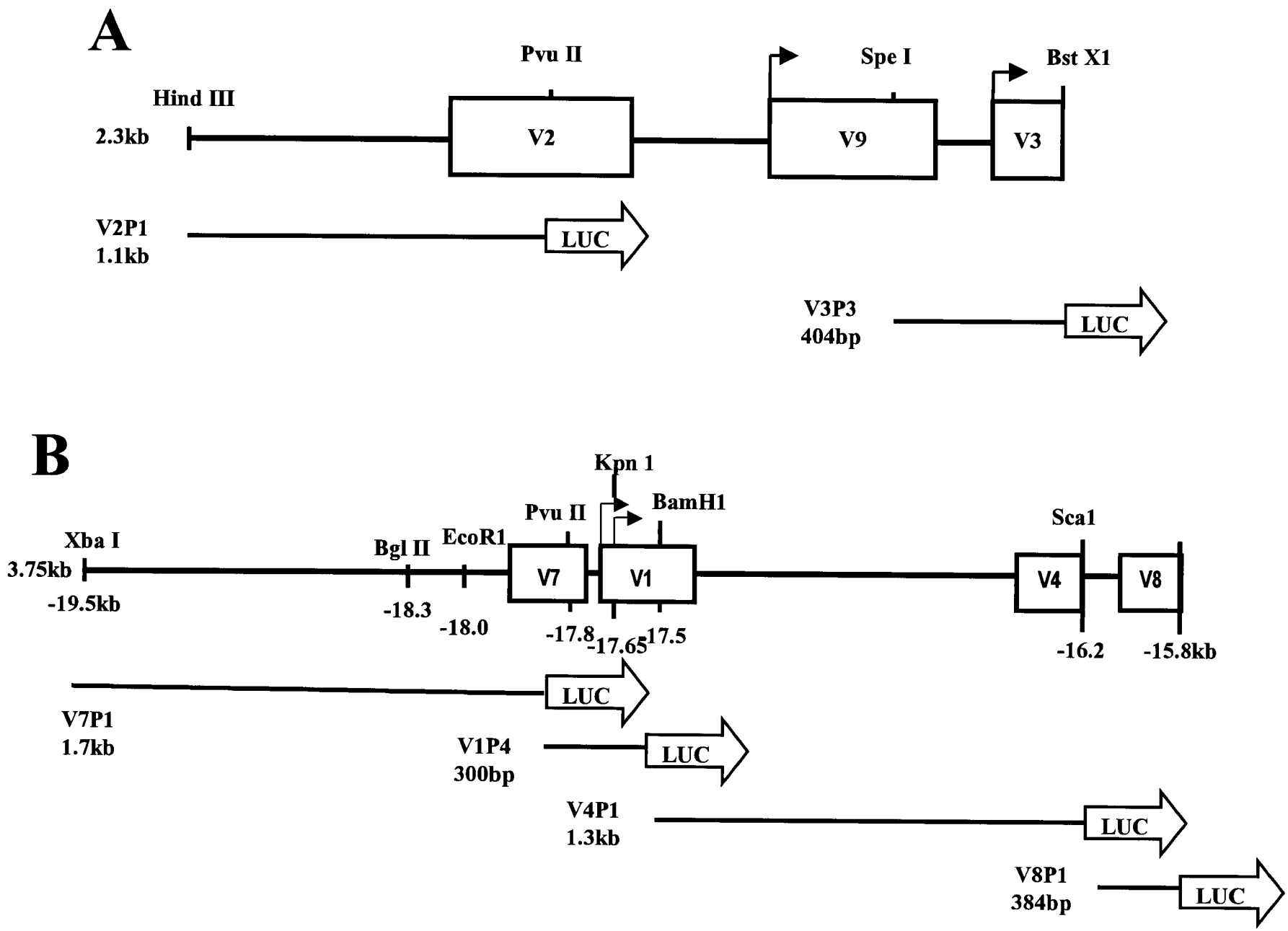
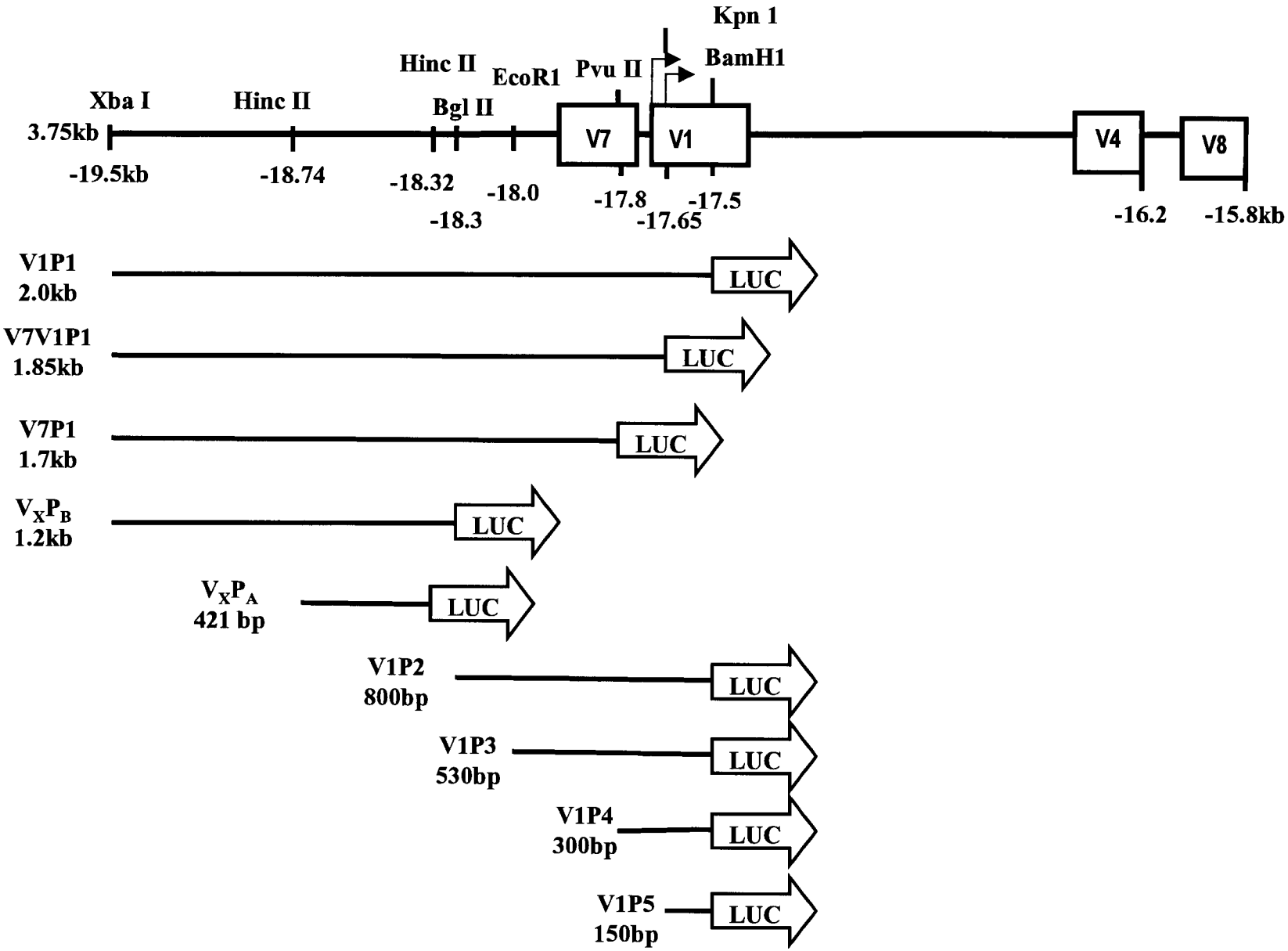

A
Pvu II
Spe I
Bst X1
Hind III
2.3kb
V2
V9
V3
V2P1
1.1kb
LUC
V3P3
404bp
LUC
B
Kpn 1
Pvu II
BamH1
Sca1
Xba I
Bgl II
EcoR1
V7
V1
V4
V8
3.75kb
-19.5kb
-18.3
-18.0
-17.8
-17.65
17.5
-16.2
-15.8kb
LUC
V7P1
1.7kb
V1P4
300bp
LUC
V4P1
1.3kb
LUC
V8P1
384bp
LUC

Figure 15: V1 deletion promoter constructs

In order to study the V1 promoter region in more detail, 5′ and 3′ deletion constructs were created from V1P1 (2 kb) using specific restriction enzymes sites as indicated. ⌐► = known transcriptional start sites. Kb numbers indicate distance from the translational start site in exon 2.


Kpn 1
BamH1
Hinc II
EcoR1
Pvu II
Xba I
Hinc II
Bgl II
3.75kb
V7
V1
V4
V8
-19.5kb
-18.74
-18.32
-18.0
-17.8
-17.5
-18.3
-17.65
-16.2
-15.8kb
V1P1
2.0kb
LUC
V7V1P1
1.85kb
LUC
V7P1
1.7kb
LUC
VXPB
1.2kb
LUC
VXPA
421 bp
LUC
V1P2
800bp
LUC
V1P3
530bp
LUC
V1P4
300bp
LUC
V1P5
150bp
LUC

3.9. Site directed mutagenesis

Mutations were introduced into plasmids using the Quikchange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA). In a PCR reaction, 20 ng of template DNA, 10 μM of sense and antisense primers carrying the desired mutations, 10 mM dNTPs, 1X Pfu turbo buffer and 2.5 U of Pfu turbo triplemaster Taq polymerase (Stratagene) were cycled under the following conditions: 1 cycle of 95°C/30 sec, 16 cycles of 95°C/30 sec, 55°C/60 sec and 68°C/ (2 min per kb of plasmid). The presence of the mutations was verified by sequencing. The primers used are listed in Tables 4-6.

3.10. Transient transfection assays

Two modes were used to deliver DNA into the cells for transient transfection assays: calcium phosphate and Polyfect (Qiagen, Mississauga, ON). 1×10^5 cells were seeded into 12-well plates (BD Biosciences) 24 hr prior to transfection.

Calcium phosphate method (for CV1, HEK293, HepG2 and Huh7 cells): DNA-mixes (2.5 μg of reporter luciferase plasmid, 0.5 μg RSV-β-galactosidase, and sufficient sp64 (empty) vector to bring the total DNA per well up to 5 μg) were prepared for triplicate wells in 5 ml polypropylene tubes. For HNF-4α1 studies in HEK293 and HepG2 cells, 1 μg of HNF-4α1 expression vector (per well) was added to the DNA mixes.

Table 4: Primers for site-directed mutagenesis of Gfi-1/1b sites.
Mutated nucleotides are in bold and small letters. Gfi-1/1b sites are underlined.

Primer name	Sequence
Gfi-1/1b mutant sense 1 (proximal site)	5′-GAGAGAGATTGAGAATGACTGA**g**TTG GGAGGGATTTTC-3′
Gfi-1/1b mutant antisense 1 (proximal site)	5′-CAAAATCCCTCCCAA**c**TCAGTCATTCT CAATCTCTCTC-3′
Gfi-1/1b mutant sense 2 (second site)	5′-GAGACAATGGTCTGATGTGA**g**TTAATT TACTCC-3′
Gfi-1/1b mutant antisense 2 (second site)	5′-GGAGTAAATTAA**c**TCACATCAGACC ATTGTCTC-3′

Table 5: Primers for site-directed mutagenesis of GAGA sites.
Mutated nucleotides are in bold and small letters. The GAGA element is underlined.

Primer name	Sequence
GAGA mutant sense 1	5′-TGTGTGTGTGCATG**tc**A**cc**GA**tt**G**t**TT GAGAATGACTGATTT-3′
GAGA mutant antisense 1	5′-AAATCAGTCATTCTCAA**a**C**aa**TC**gg**T **ga**CATGCACACACACA-3′

Table 6: Primers for site-directed mutagenesis of HNF-4 sites.
Mutated nucleotides are in bold and small letters; the HNF-4 sites are underlined.

Primer name	Sequence
HNF-4 site 1 (½) mutant sense	5′-AGAGCCCAGAGATGTACT**tcaag**AAGGT CAGGGATTTATTATATG-3′
HNF-4 site 1 (½) mutant antisense	5′-CATATAATAAATCCCCTGACCTT**cttga**AG TACATCTCTGGGCTCT -3′
HNF-4 site 1 mutant sense	5′-GCCCAGAGATGTACT**a**GGCAA**c**GGT**a**AG GGGATTTATTATATGAGA-3′
HNF-4 site 1 mutant antisense	5′-TCTCATATAATAAATCCCCT**t**ACC**g**TT GCC**t**AGTACATCTCTGGGC-3′
HNF-4 site 6 mutant sense	5′-AGGGGATGCTCA**gg**GCCCTGAAGGATG-3′
HNF-4 site 6 mutant antisense	5′-CATCCTTCAGGGC**cc**TGAGCATCCCCT-3′

Sterile water was added to the mix and 75 μl of 4X $CaCl_2$ (500 mM $CaCl_2$, 2 mM Tris-HCl, 0.2 mM EDTA) was added dropwise to each tube. 150 μl of 2X HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4) solution was introduced into the bottom of the tube and bubbles created into the mixture 5-6 times, using a pipette. The DNA-mixture was incubated at room temperature for 30 min to allow the precipitate to form. The cells were washed once with 1X PBS and fresh media was added. 100 μl of the DNA precipitate was added dropwise to each well. The cells were incubated at 37°C in 5% CO_2 for 48 hr.

Polyfect method: HEK293 cells were transfected with 0.5 μg of promoter reporter or empty reporter vector, 0.1μg of pSV-β-galactosidase (Promega), 0.001-0.02 μg of Gfi-1 or Gfi-1b expression vectors, 0.025-0.1 μg of GAF expression vector or 0.05-0.2 μg of HNF-4α1, α2 or α8 expression vectors; total DNA per well was made up to 1 μg with sp64. 150 μl of DMEM and 6 μl of polyfect reagent were added to triplicate well DNA mixes in 5 ml polypropylene tubes. The tubes were vortexed and complexes left to form at room temperature for 10 min. Media of cells was replaced with 1 ml of fresh complete media. 900 μl of complete media was added to the polyfect-DNA complex and 356 μl evenly dispersed in each well. The cells were then incubated at 37°C in 5% CO_2 for 48 hr.

3.11. β-Galactosidase and Luciferase Assays

48 hr after transfection, cells were washed twice with ice-cold 1X PBS and lysed for 15 min on a rotating platform at room temperature in 200 μl lysis buffer (0.5 mM DTT, 0.1 M Tris HCl, 0.005% NP-40).

β-galactosidase assay: 10 μl of lysate was added to the wells of a 96-well microtiter plate (Corning Inc., NY) and 100 μl of β- galactosidase solution composed of 0.5 μl of β- galactosidase and 99.5μl of reaction buffer diluent (100 mM Na_2PO_4 pH 7.5, 1 mM $MgCl_2$, and 5% sapphire-II enhancer [Tropix Galactonstar, Bedford, MA]) was added to the lysate. The plate was covered with a clear lid and the reaction incubated at room temperature for an hour.

Luciferase assay: 100 μl of lysate was added to the wells of a 96-well microtiter plate (Corning). 1X luciferin solution was prepared in 0.1 M Tris HCl (pH 7.9) from a 10X luciferin concentrate (2.5 mM ATP [Amersham Biosciences, Baie D'Urfe, QC], 0.1 mM Coenzyme A [Roche], 1 mM luciferin [BD Biosciences], 5 mM $MgCl_2$, 0.5 M Tris HCl pH 7.9) stored at –20°C.

Both β-galactosidase and luciferase activities were measured in relative light units (RLU) using an EG&G Berthold MicroLumat Plus bioluminometer. Luciferase data were normalised to the β-galactosidase activity and expressed as a fold change relative to the empty vector, pA_3luc.

3.12. Protein Extraction

Tissue: 500 mg of frozen liver tissue was minced in 1-2 ml of 1X radioimmunoassay immunoprecipitation (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) [modified from a protocol provided at www.upstate.com]. The solution was then homogenised in 1X RIPA buffer supplemented with phosphatase and protease inhibitors (50 mM NaF, 0.2 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 1 μg/ml each of aprotinin, leupeptin and pepstatin) in a Dounce homogeniser. The lysate was centrifuged at 12,000 RPM for 50 min at 4°C.

Cells: HEK293, Huh7 and HepG2 cells were trypsinised and centrifuged at 1000 RPM for 10 min at 4°C. Pellets were resuspended in 10X pellet volume of 1X lysis buffer (50 mM Tris HCl pH 7.5, 0.1% Triton-X 100, 2 mM EDTA) supplemented with phosphatase and protease inhibitors (50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml each of aprotinin and leupeptin) and incubated on ice for 15 min. The cell suspension was then spun at 13,000 RPM for 10 min at 4°C.

Supernatants from tissue and cell preparations were stored in aliquots at 20°C. Protein concentrations were determined by the Bradford assay (Biorad Laboratories Inc., Hercules, CA).

3.13. Nuclear Protein Extraction

Nuclear extracts were prepared from both cells and tissues using the NE-PER kit (Pierce Biotechnology Inc., Rockford, IL), following the

manufacturer's instructions. After resuspension in NER (nuclear extraction buffer), supplemented with protease inhibitors (Roche), the nuclear extract was dialysed (Slide-a-Lyser, Pierce) in ice cold PBS and stored in aliquots at -80^{O}C. Protein concentrations were determined by the Bradford assay (Biorad).

3.14. Western blot

1-75 μg of whole cell or nuclear protein extract were boiled in 1X SDS loading buffer (0.1% bromophenol blue, 0.1 M DTT, 10% glycerol, 2% SDS and 50 mM Tris pH 6.8) for 3 min, separated on a 12% SDS-PAGE gel and transferred onto Immobilin-P, a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Mississauga, ON) using a wet transfer mini trans-blot cell (Biorad Laboratories Inc.). The blots were blocked in 5% milk (PBS-T [PBS + 5% tween] or TBS-T [Tris-buffered saline + 5% tween]) and probed with various primary antibodies. The blots were then washed for cycles of 1X 15 min and 3X 10 min with PBS-T/TBS-T before being incubated for an hour at room temperature with 1:1000 fold dilution of corresponding secondary antibodies. The enhanced chemiluminescence (ECL) system (Perkin Elmer Life Sciences Inc., Boston, MA) was used to visualise the bands. In order to reprobe, the membrane was rinsed in double distilled water and rotated at 50^{O}C for 30 min in stripping buffer (2% SDS, 62.5 mM Tris pH 6.8, 100 mM β-mercaptoethanol). The membrane was rinsed in PBS-T and probed with

anti-calnexin (1:1000, BD Transduction Labs) and anti-mouse-HRP as a secondary antibody (1:1000, NEN), as a loading control. HNF-4α (1:1000, Santa Cruz) and GAF (1:3000) antibodies were probed for an hour at RT, and overnight for Gfi-1 (1:200) and Gfi-1b (1:100). The blot was treated with ECL kit reagents (Perkin Elmer) and visualised by autoradiography. Quantitation analysis of HNF-4α content were performed by densitometric analyses of the bands using the Biorad Gel Doc analysis system (Mississauga, ON), and data are expressed as a ratio of HNF-4α to calnexin.

3.15. EMSA and EMSSA (Electromobility Shift and Supershift Assays)

Double-stranded oligonucleotides were prepared by dissolving complementary oligonucleotides (Alpha DNA) (Tables 7-8) in annealing buffer (100 nM Tris HCl pH 7.5, 1 M NaCl, 10 mM EDTA) at a final concentration of 100 nmol/ml, incubated at 65^{O}C for 10 min, then at RT for 1-2 hr. The double-stranded oligonucleotides were end-labelled with [γ32P] ATP using T4 polynucleotide kinase (Invitrogen). Alternatively, the double-stranded oligonucleotides were labelled (fill-in reaction) with [α32P] CTP using Klenow fragment (Invitrogen). Labelled probes were purified by G-50 spin columns (Amersham).

6-10 μg of nuclear extract were preincubated on ice in 1X binding buffer and 1 μg of polydI.dC (Sigma Aldrich) for 20 min on ice. 0.5 ng of

labelled probe was added to the mixture and incubated for a further 20 min on ice. In supershift assays, 2 μg of antibody was added to the mixture after the 20 min incubation with the labelled probe and incubated for an hour at 4°C. In competition assays, 200X cold probe was added to the binding reaction.

The 20 μl reaction was then loaded on 5-10% pre-run polyacrylamide gels and electrophoresed at 100 V in cold 0.5X TBE. Gels were dried at 80°C for an hour and complexes detected by autoradiography.

3.15.1. EMSA and EMSSA Binding buffers

HNF-4 binding buffer: 20 mM HEPES, 5% glycerol, 100 mM KCl, 1 mM DTT, 1 mM EDTA, 113 mM $MgCl_2$.

GAGA binding buffer: 1.2 M KCl, 0.5 M Tris HCl (pH 7.9), 0.05 M EDTA, 23% glycerol, 0.005 M DTT.

3.16. Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (264). Briefly, cells (1 X 10^6) cultured in a 100 mm dish were crosslinked in 1% formaldehyde (ICN Biomedicals Inc., Aurora, OH) for 10 min at room temperature. Cells were scraped and pelleted, and the pellets were resuspended in 300 μl SDS lysis buffer on ice and sonicated twice for 15 sec at 50% input (VibraCell Sonicator, Sonics, Betatek Inc., Toronto, ON).

Table 7: Oligonucleotides for EMSA probes (GAGA).

Mutated nucleotides are in bold and small letters. GAGA element is underlined.

Oligonucleotide name	Sequence
GAGA sense 1	5′- TGTGCATGAGAGAGAGAGATTGAG AATGACTG-3′
GAGA antisense 1	5′-CAGTCATTCTCAATCTC-3′
GAGA mutant sense 1	5′-TGTGTGTGTGCATG**tc**A**cc**GA**tt**G**t**TT GAGAATGACTG-3′
GAGA mutant antisense 1	5′-CAGTCATTCTCAA**a**C**aa**-3′

Table 8: Oligonucleotides for EMSA probes (HNF-4).

Mutated nucleotides are in bold and small letters, HNF-4 sites are underlined. AS: antisense; ½: half-site mutant.

Oligonucleotide name	Sequence
HNF-4 site 1 sense	5′-GAGATGTACTGGGCAAAGGTCAGGG GA-3′
HNF-4 site 1 AS	5′-TCCCCTGACCTTTGC-3′
HNF-4 site 1 (½) mutant sense	5′-GAGATGTACT**tcaag**AAGGTCAGGGGA-3′
HNF-4 site 1 (½) mutant AS	5′-TCCCCTGACCTT**cttga**-3′
HNF-4 site 1 mutant sense	5′-CT**a**GGCAA**c**GGT**a**AGGGGATTTA-3′
HNF-4 site 1 mutant AS	5′-TCCCCT**t**ACC**g**TTGC-3′
HNF-4 site 5 sense	5′-GATGAAACAGGGGCAGAGGAGGAAG AAAAGAC-3′
HNF-4 site 5 AS	5′-GTCTTTTCTTCCTCCTC-3′
HNF-4 site 5 mutant sense	5′-GATGAAACA**ttc**GCA**c**AGGAGGAAGAAA AGAC-3′
HNF-4 site 5 mutant AS	5′-GTCTTTTCTTCCTCCT**g**-3′
HNF-4 site 6 sense	5′-AGGGGATGCTCATTGCCCTGAAGGA TG-3′
HNF-4 site 6 AS	5′-CATCCTTCAGGGCAATGAGCATCCCCT-3′
HNF-4 site 6 mutant sense	5′-AGGGGATGCTCA**gg**GCCCTGAAGGATG-3′
HNF-4 site 6 mutant AS	5′-CATCCTTCAGGGC**cc**TGAGCATCCCCT-3′

The suspension was centrifuged at 10,000 RPM for 10 min at 4°C. The lysate was diluted 10-fold in ChIP dilution buffer and 500 μl was kept aside as input. 2 ml of the remaining lysate was supplemented with 1X protease inhibitor cocktail (Roche) and precleared with 2 μg of BSA (NEB, Beverly, MA), 4 μg of sonicated herring sperm DNA (Sigma) and 45 μl of protein A/G plus agarose beads (Santa Cruz) for 30 min at 4°C on a rotating platform. The beads were pelleted and the supernatant immunoprecipitated for 3 hr at 4°C, rotating with 4 μg of either goat IgG (control) or anti-HNF-4α. Complexes were pulled down by incubating the above solution overnight with 2 μg of BSA, 4 μg of sonicated herring sperm DNA, and 45 μl of protein A/G plus agarose beads (Santa Cruz), rotating at 4°C. Beads were pelleted and washed sequentially with low salt buffer, high salt buffer, lithium chloride wash buffer and 1X Tris-EDTA (TE) for 5 min each. Bound protein was eluted twice from the beads by vortexing at the highest speed for 15 sec and gently rotating for 15 min in ChIP extraction buffer, at room temperature. Crosslinks were reversed for 4 hr at 65°C. DNA was purified with the Qiagen DNA purification kit (Qiagen). 2-5 μl of the 50 μl purified DNA in elution buffer was used per PCR reaction. PCR assays were performed with Taq DNA polymerase (Invitrogen) and 3 μl of cDNA from the RT reactions. Primers (Table 9) were purchased from Alpha DNA (Montreal, QC). PCR conditions were as

follows: one cycle of 2 min at 94^{O}C followed by amplification for 26-32 cycles at 94^{O}C for 30 sec, 58-62^{O}C for 1 min, 72^{O}C for 1 min, ending with 72^{O}C for 5 min. Products were resolved on a 2% agarose gel, using ethidium bromide.

3.16.1. ChIP buffers

ChIP lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1).

ChIP dilution buffer: 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1).

Low salt buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1).

High salt buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl (pH 8.1).

LiCl wash buffer: 0.25 M LiCl, 1% NP-40, 1% deoxycholate (Na salt), 1 mM EDTA, 10 mM Tris-HCl (pH 8.1).

1X TE: 10 mM Tris-HCl (pH 8.1), 1 mM EDTA.

Elution buffer: 1% SDS, 0.1 M $NaHCO_3$.

3.17. Statistical analyses

The significance of observed differences between groups was determined by ANOVA followed by Bonferroni's group comparison statistical test, using the Instat 3 program.

Table 9: Primers for ChIP analysis of HNF-4 sites #1, #5 and #6. Expected size of PCR products are indicated.

Primer Name	Sequence	Product size (bp)
ChIP site 1 HNF-4 sense	5′-GTAATAAGGCCTCATGAGACTCCA-3′	270
ChIP site 1 HNF-4 antisense	5′-AACCTTCACAAAATCCCTCCCA-3′	
ChIP site 5 HNF-4 sense	5′-CCAAGTGGGAAATGGTGGCTA-3′	200
ChIP site 5 HNF-4 antisense	5′-TTCTAAAACTAAACTCAGTGGTCCA-3′	
ChIP site 6 HNF-4 sense	5′-ATGTGTTCAGTGGTCCAGCCCA-3′	200
ChIP site 6 HNF-4 antisense	5′-TCCCATTTTCAGTTAGTAATAGAA-3′	
ChIP control sense	5′-AAAGAACATTTTGCTGACAAT-3′	210
ChIP control antisense	5′-TTGGGAGGTGGGGGGAAA-3′	

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Comparison of module A and B promoters

4.1.1. GHR variant expression in human foetal hepatocytes, adult liver and cell lines

Previous results from this laboratory have shown that module A mRNA variants are expressed in every tissue examined to date and at every developmental stage, including tumours (223;244). Module B mRNA variants, however, are expressed only in the normal postnatal liver; foetal liver, hepatocarcinomas and hepatomas do not express module B variants (223;244). These data suggested that hGHR expression is regulated by multiple promoters. To test this, the promoter activity of modules A and B exons were examined in two human hepatoma cell lines (HepG2 and Huh7) and two primate kidney cell lines (CV1 and HEK293), using transient transfections.

In order to determine the background expression of the hGHR variant expression in these cells as well as in human foetal and adult hepatocytes (controls), we first designed primers specific to the different hGHR variants for use in RT-PCR assays (Table 2). The results from the RT-PCR experiments show that both human foetal and adult hepatocytes and all the four cell lines express GHR mRNA, although HepG2 cells have extremely low to undetectable levels (Table 10). Adult hepatocytes express all known hGHR variants. Foetal hepatocytes express all but

module B variants, confirming what is already known (223;244). The GHR variant profile of the cell lines is similar to foetal hepatocytes: they do not express module B variants. However, they all express V5 and module A variants (V2, V3 and V9), except for HepG2 cells that have undetectable levels of V9, and CV1 cells that do not express V3 or V9. A recent publication has reported that V6 hGHR mRNA is an artifact and, therefore, it has not been studied further (243).

4.1.2. Promoter activity of Module A and Module B promoters

Promoter constructs were prepared to compare the individual promoters from modules A and B (Figure 14), and tested in all four cell lines except for V9 which has already been studied (223). We hypothesized that module A constructs will be more active than the liver-specific module B promoter constructs, as the cell lines do not express the liver-specific variants. Indeed, module A promoter constructs all showed very significant transcriptional activity, 30-200 fold over the promoterless vector in all cell lines (Figure 16), the activity being 4-5 times higher in Huh7 cells, the GH responsive hepatoma cell line (265), than in HepG2, CV1 and HEK293 cells.

Table 10: RT-PCR results of GHR mRNA variants in human adult liver and foetal hepatocytes, and four primate cell lines.

HepG2, Huh7: human hepatoma cell lines, HEK293: human foetal kidney cell line, CV1: African green monkey kidney cell line. ++: present (strong), +: present, +/-: barely detectable, -: not detected.

Sample/ Variant	Human Adult Liver (n=2-4)	Human Foetal Hepatocytes (n=4-9)	HepG2 (n=3)	Huh7 (n=3)	HEK293 (n=3)	CV1 (n=3)
Total GHR	++	+	+/-	+	+	+
(Module B) V7/V1/V4/V8	++	-	-	-	-	-
V2	++	+	+/-	+	+	+
V3	++	+	+/-	+	+	-
V5	++	+	+/-	+	+	+
V9	++	+	-	+	+	-

Module B promoter constructs were ~2-4 fold lower in activity than module A promoter constructs in all cell lines (Figure 16), as hypothesized. Surprisingly, V8, an hGHR mRNA variant that is not expressed in any of the cell lines, and is the least abundant mRNA variant in adult liver, was the most active, especially in Huh7 cells (mean±SE, n; 29.7±4.5, n=10) (Figure 16B).

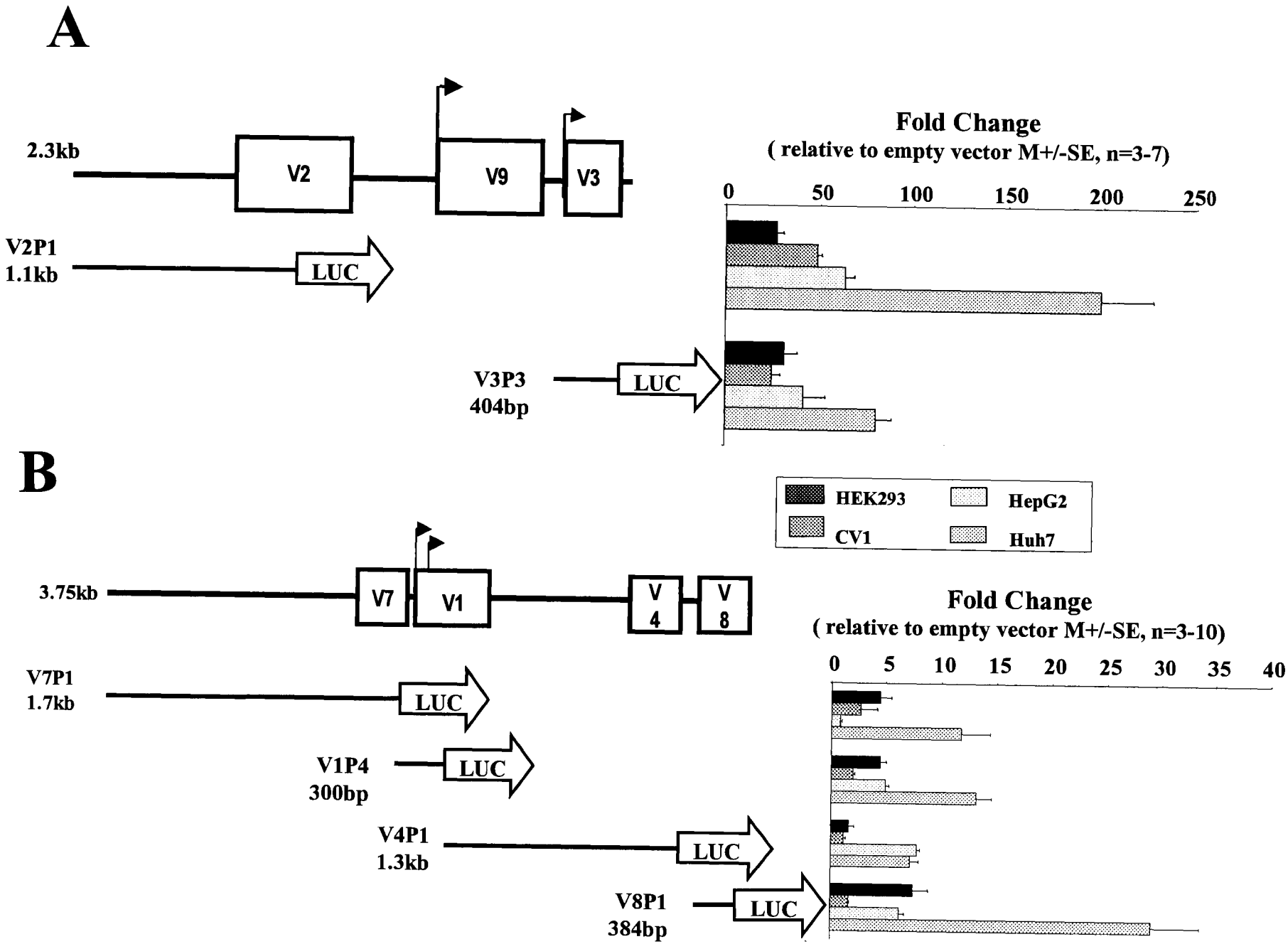
Thus, the information inferred from the cell lines, and in particular the hepatoma cell lines, may not always reflect what occurs in normal adult liver as the hGHR module B variant transcriptional profile is quite different. However from these data and the published V9 data, I can conclude that all seven of the non-coding exons in modules A and B have individual promoter activity.

4.2. Deletion analyses of the V7-V1 promoter

V1 is the most abundant variant in the human postnatal liver (59;223;241). Because this expression pattern suggests both developmental- and tissue-specific regulation, we decided to focus on characterising the elements controlling V1 transcriptional activity. The first objective was to characterise a 1.8 kb region upstream of the second transcriptional start site (TSS). In order to identify the regulatory domains, 5′ and 3′ deletional promoter fragments were cloned upstream of a luciferase vector and tested by transient transfection assays in the four cell lines (Figure 15).

Figure 16: Transcriptional activity of hGHR modules A and B promoter constructs.

Module A (A) and module B (B) constructs were transfected into HEK293, CV1, HepG2 and Huh7 cells by the $CaPO_4$ method. Cells were harvested 48 hr after transfection and assayed for luciferase and β-galactosidase (internal control) activity. The relative luciferase activity is presented as fold change over the promoterless vector. Data are presented as mean ± standard error, n=3-10 experiments.

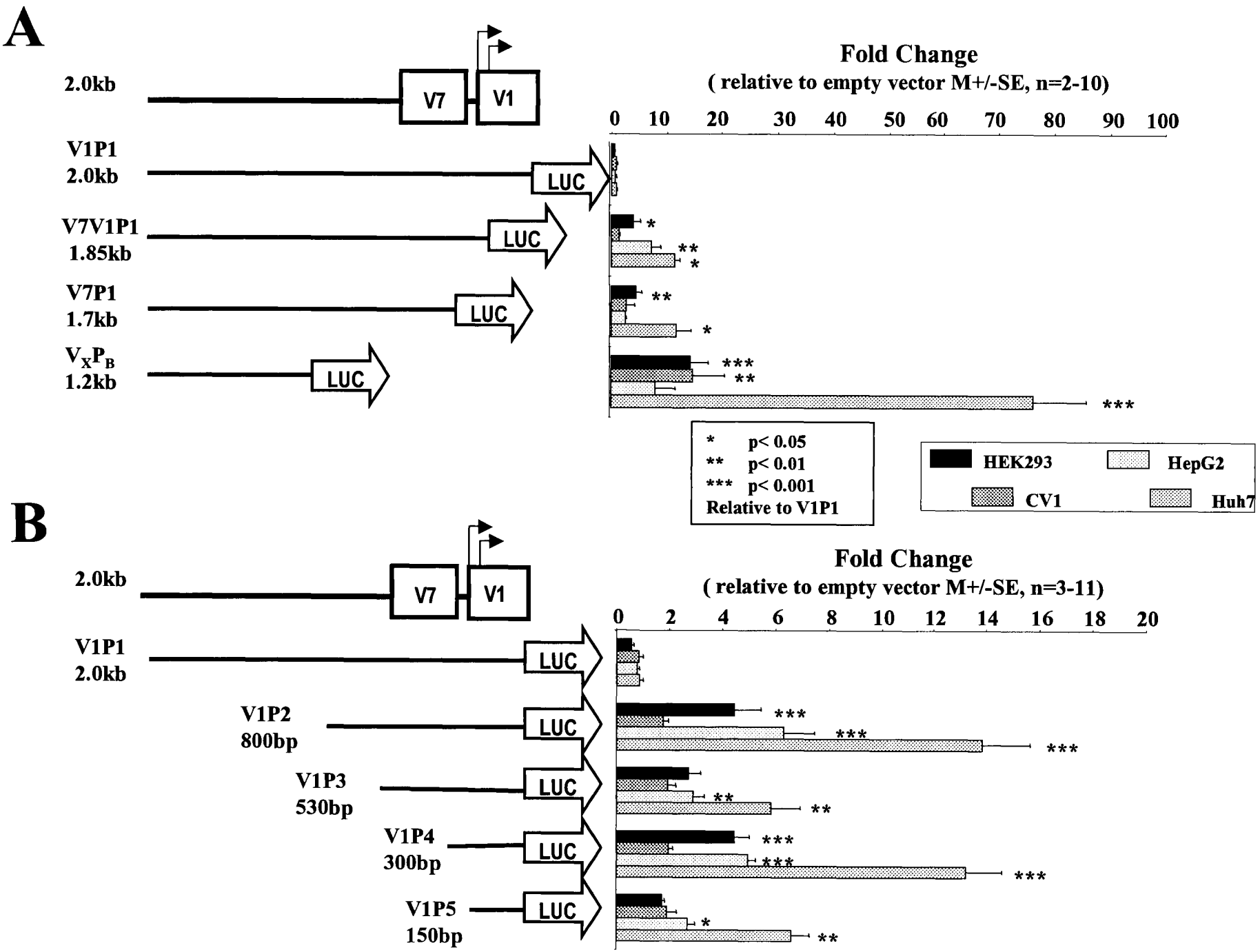

A
2.3kb
V2
V9
V3
V2P1
1.1kb
LUC
V3P3
404bp
LUC
Fold Change
(relative to empty vector M+/-SE, n=3-7)
0
50
100
150
200
250
B
HEK293
HepG2
CV1
Huh7
3.75kb
V7
V1
V 4
V 8
V7P1
1.7kb
LUC
V1P4
300bp
LUC
V4P1
1.3kb
LUC
V8P1
384bp
LUC
Fold Change
(relative to empty vector M+/-SE, n=3-10)
0
5
10
15
20
25
30
35
40

The longest construct, V1P1, containing the V7 exon and 46 bp of the V1 exon, was strongly repressed in all four cell lines (Figure 17).

When the 3′ 150 bp were deleted (V7V1P1 construct, 1.85 kb), there was a significant increase in luciferase activity in HEK293 (3.9±1.3, n=4, $p < 0.05$), HepG2 (7.2±1.8, n=5, $p < 0.01$) and Huh7 (11.5±0.9, n=7, $p < 0.05$) cells (Figure 17A). When another 3′ 150 bp were deleted (V7P1 construct: 1.7 kb), similar activities were observed. A further 3′ 0.5 kb deletion (V_XP_B construct: 1.2 kb) led to an even greater increase in reporter activity in all four cell lines: HEK293 (14.3± 3.2, n=4, $p < 0.001$), CV1 (14.8± 5.7, n=3, $p < 0.01$), HepG2 (7.88± 3.77, n=3, NS) and Huh7 (76.3± 9.5, n=5, $p < 0.001$) cells (Figure 17A). The significant changes in the activities of the 3′ deletion promoter constructs suggest that there are multiple inhibitory elements within the 3′ 0.8 kb region. When we analysed constructs with 5′ deletions, we found that a construct with deletion of the 5′ 1.2 kb region (V1P2) showed a significant increase in transcriptional activity in HEK293 (4.4±1.0, n=11, $p< 0.001$), HepG2 (6.3±1.1, n=4, $p< 0.001$), and Huh7 cells (13.9±1.8, n=8, $p< 0.001$) (Figure 17B).

Figure 17: Transcriptional activity of V1 hGHR deletional promoter constructs.

(A) 3′ deletion and (B) 5′ deletion V1 constructs were transfected into HEK293, CV1, HepG2 and Huh7 cells by the $CaPO_4$ method. Cells were harvested 48 hr after transfection and assayed for luciferase and β-galactosidase (transfection control) activities. The relative luciferase activity is presented as fold change over activity obtained using the promoterless vector. Data are presented as mean ± standard error from n=2-11 experiments, V_XP_B in CV1 is an n=2 experiments. The significance of the observed differences (compared to V1P1) was determined by Bonferroni's statistical test following an ANOVA analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A
2.0kb
V7
V1
V1P1
2.0kb
LUC
V7V1P1
1.85kb
LUC
V7P1
1.7kb
LUC
V_XP_B
1.2kb
LUC
Fold Change
(relative to empty vector M+/-SE, n=2-10)
0 10 20 30 40 50 60 70 80 90 100
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to V1P1
HEK293
HepG2
CV1
Huh7
B
2.0kb
V7
V1
V1P1
2.0kb
LUC
V1P2
800bp
LUC
V1P3
530bp
LUC
V1P4
300bp
LUC
V1P5
150bp
LUC
Fold Change
(relative to empty vector M+/-SE, n=3-11)
0 2 4 6 8 10 12 14 16 18 20

A further 5′ loss of 270 bp (V1P3) from V1P2 led to a general decrease in activity although levels were still higher than with V1P1 (HEK293: 2.7±0.4, n=7, NS; HepG2: 2.9±0.4, n=6, $p< 0.01$ and Huh7: 5.9±1.1, n=9, $p< 0.01$) (Figure 17B). Subsequent loss of another 230 bp (V1P4) resulted in a marked increase in activity in HEK293 (4.5±0.6, n=10, $p < 0.001$), HepG2 (5±0.3, n=5, $p < 0.001$) and Huh7 (13.2±1.4, n=5, $p < 0.001$) cells (Figure 17B), similar to V1P2. The V1P5 construct, containing the conserved downstream TATA box and TSS, ~100 bp of its promoter and 46 bp of the V1 exon, showed minimal promoter activity in HEK293 cells (1.7±0.9, n=7, NS) and CV1 cells (1.92±0.35, n=4, NS), and significant activity in HepG2 (2.7±0.3, n=6, $p < 0.05$) and Huh7 (6.6±0.7, n=8, $p < 0.01$) cells. The promoter constructs were generally active in CV1 cells but the changes were only significant with V_XP_B. Data from the 5′ deletional constructs suggests that there are additional inhibitory elements in the 5′ 1.2 kb region.

Based on these 3′ and 5′ deletional construct studies, I have defined three negative regulatory regions within the 1.8 kb V1 promoter (NRR 1-3) (Figure 18). The first (NRR1) is the most 3′ 300 bp end of the V1P1 construct containing both TATA/TSS complexes of V1. NRR2 is defined as the 230 bp region immediately upstream and finally NRR3 is the 5′ 1.2 kb region. Two positive regulatory regions (PRR 1-2) have also been identified in these studies: PRR1 is the 5′ 150 bp region of NRR1 and

contains the upstream TATA/TSS complex as well as ~ 130bp of its proximal promoter. PRR2 is the 270 bp region between NRR2 and NRR3 (Figure 18). Further investigations into possible repressor and activator response elements in these regions are discussed in sections 4.3, 4.4 and 4.5.

4.2.1. Discussion of the analyses of hGHR modules A and B promoter activity

Publications from the laboratory of Dr. Cindy Goodyer were the first to report the differential (ubiquitous versus developmental- and tissue-specific) expression of GHR mRNA variants in humans (223;244). V3 transcripts were detected in all foetal and postnatal tissues tested, and hepatic tumours. V1 transcripts, on the other hand, were only detected in normal postnatal liver tissue (223;244). Studies in other species (ovine, bovine, mouse and rat) have also shown that expression of V1 homologues are restricted to postnatal liver while the V2 homologues are ubiquitously expressed (246;248;251;266). To date, none of the promoter analyses on these other species have identified what TFs are responsible for the developmental switch in GHR expression. However, promoter studies of the V2 equivalent in ovine, bovine and mouse have shown that Sp1, a ubiquitously expressed TF, has a major role in regulating ubiquitous GHR expression (247;259;260).

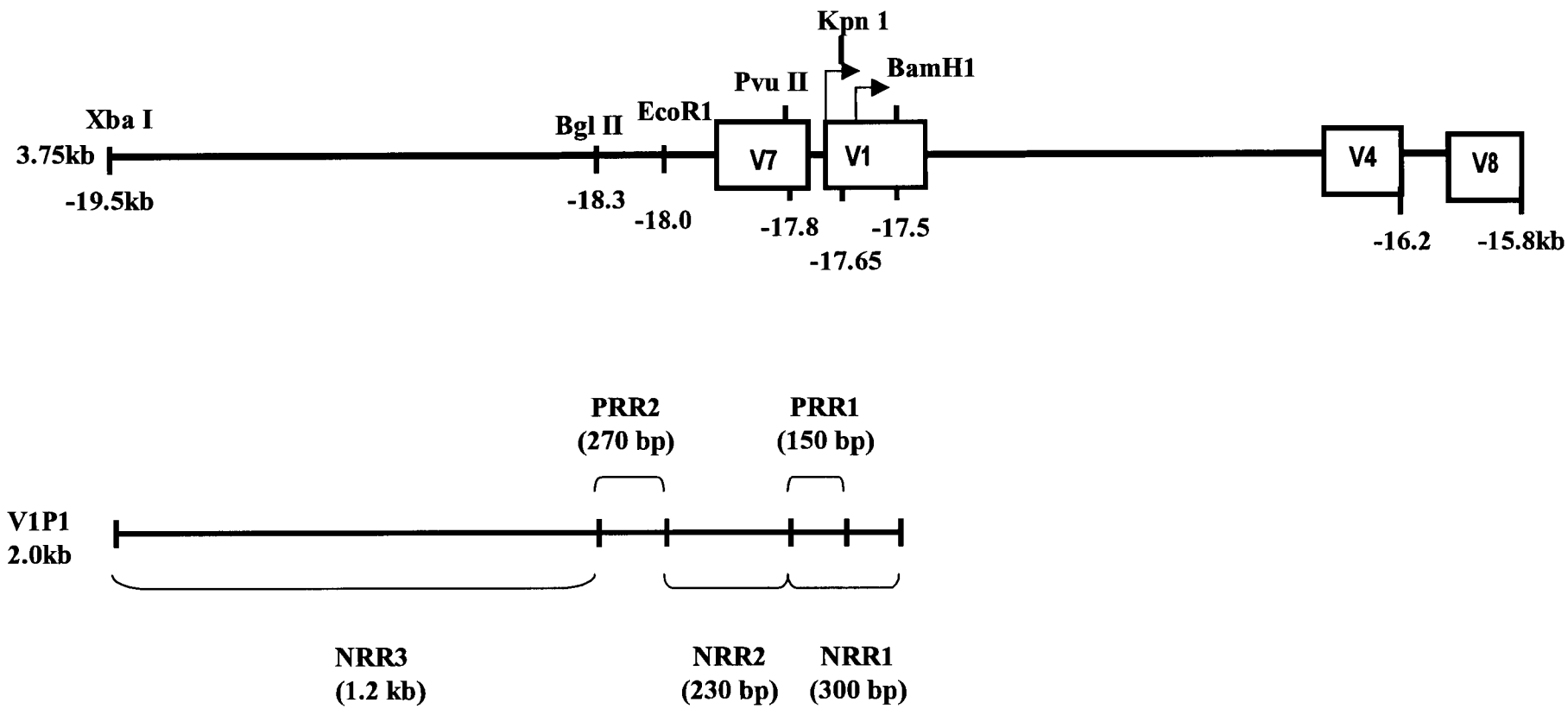
My comparative studies of putative promoters from modules A and B show that module A promoter constructs (V2, V3) are highly active in the cell lines. This was expected as all the cell lines express V2 and V3. Promoter studies of V9 have already been published, showing similar results (223). It is likely that the cell lines contain the necessary transcription factors to drive the ubiquitously expressing promoters and, thus, maintain a level of GHR expression. Module B variants on the other hand were understandably less active. It is assumed that the hepatoma cell lines do not express module B variants due to dysregulated hepatic function, which could include the presence of active repressors, the lack of transactivators and/or altered chromatin structure.

From the deletion promoter analysis of the 1.8 kb region upstream of the V1 start sites, three major negative regulatory regions and two positive regulatory regions have been identified (Figure 18).

NRR1: NRR1 is the 300 bp region at the 3′ end of V1P1 and includes the two TATA/TSS complexes. Very recently, Jiang *et al.* have reported stimulatory activity by a ubiquitous TF, ZBP-89, through its binding site on the b1A promoter, in a region similar to our NRR1 (267). This interaction was first identified by yeast one-hybrid assays as traditional computer assisted-TF searches did not reveal any binding sites for known TFs (267). Studies of a similar NRR in the V1 promoter by other laboratories or in the equivalent regions in other species have not been reported.

Figure 18: Putative negative (NRR) and positive (PRR) regulatory regions of the hGHR V1 promoter.

Regulatory regions of the V1 promoter as identified by deletion promoter studies. Kb numbers indicate distance from translational start site in exon 2. Specific restriction enzyme sites used in the cloning of the promoter constructs are indicated.


Kpn 1
BamH1
Pvu II
EcoR1
Bgl II
Xba I
3.75kb
-19.5kb
-18.3
-18.0
V7
V1
-17.8
-17.5
-17.65
V4
V8
-16.2
-15.8kb
PRR2
(270 bp)
PRR1
(150 bp)
V1P1
2.0kb
NRR3
(1.2 kb)
NRR2
(230 bp)
NRR1
(300 bp)

NRR2: By deletion promoter studies, Orlovskii *et al.* also describe a 115 bp repressor region in the human V1 promoter region that is equivalent to our NRR2 (243). Footprinting analyses of the human V1 promoter using HepG2 nuclear extracts have uncovered 17 bp (F1) and 28 bp (F2) footprints that overlap with our NRR2 (268). A 23 bp footprint that overlaps with our NRR2 has also been demonstrated with bovine liver nuclear extracts ($^{-}$446 - $^{-}$469) (258). In the ovine o1A, a ~ 191 bp region homologous to the NRR2 was found to be stimulatory (253).

NRR3: The most 5′ negatively regulated region, NRR3, occupies the 5′ 1.2 kb region of V1P1. Using deletion promoter studies, Rivers *et al.* describe a similar region (~ 900 bp) containing negative regulatory elements (268). The bovine b1A also has a 2.2 kb repressor region that overlaps with NRR3 (258). Ovine o1A deletion studies also show a 125 bp repressor region overlapping with the 5′ end of the NRR3 (253).

No further analyses of these NRRs in other species have been reported. Thus, data from other laboratories and in other species generally support our findings that NRR2 and NRR3 contain repressor elements that regulate the liver-specific expression of GHR. This study is the first to determine a third important NRR adjacent to the two V1 TSS.

PRRs: PRR1 is the 5′ 150 bp region of NRR1, and PRR2 is the 270 bp region between NRR2 and NRR3. Previous studies of the human V1

promoter have not uncovered any positive regulatory regions (243;268;269). However, footprinting analyses show that a 32 bp region within PRR2 is bound by endogenous HepG2 nuclear proteins (268). Jiang *et al.* have shown that HNF-4 α1, HNF-4γ and COUP-TFII stimulate b1A promoter activity through an HNF-4 response element in a region homologous to our PRR1 (258;270). This is discussed in more detail in section 4.7.13. Deletion promoter studies of the ovine equivalent of V1, o1A, have shown that a ~ 150 bp region similar to our PRR1 is also stimulatory (253). However our PRR2 overlaps with a ~ 196 bp region found to be inhibitory.

In summary, deletion promoter studies have led us to identify five major regulatory regions: two positive and three inhibitory. Regulation of the longest promoter construct (V1P1) suggests that the inhibitory regions interact in order to markedly repress V1P1 in all of the four cell lines. The most striking observation I made was the increase in the transcriptional activity of the V1 promoter construct in all four cell lines once the 3′ 150-300 bp region (NRR1) was removed. Therefore I next investigated the possible transcriptional repressors in this region (sections 4.3 to 4.4.7).

4.3. Putative binding sites of transcription factors in NRR1 of the V1 proximal promoter.

Noticeably, across all the cell lines, V1P1 was highly repressed. By removing 300 bp from the 3′ end, the repressive element was lost.

Figure 19: Putative TF response elements in the NRR1.

(A) Putative binding sites in the NRR1 for two related transcriptional repressors Gfi-1 (growth factor independence 1) and Gfi-1b, and a GAGA element were revealed by the MatInspector (www.genomatix.de) transcription factor binding site software. (B) Ovine and bovine equivalents of the human V1 have homologous sites for Gfi-1/1b and a GAGA element. L1, the mouse equivalent of V1, has binding sites for two alternative transcriptional repressors (RP58 and RBP JK) as well as a GAGA element in the same location.

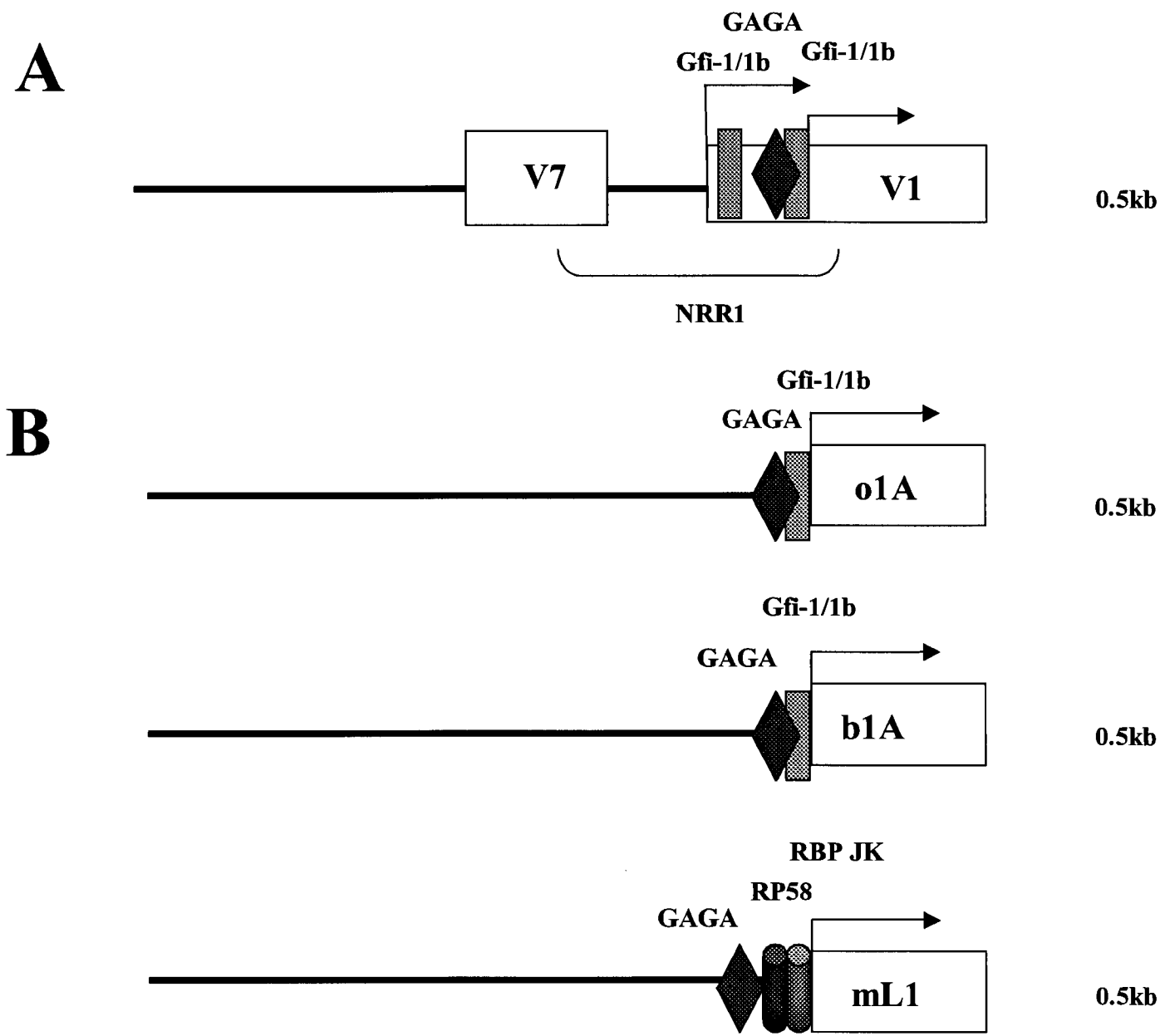
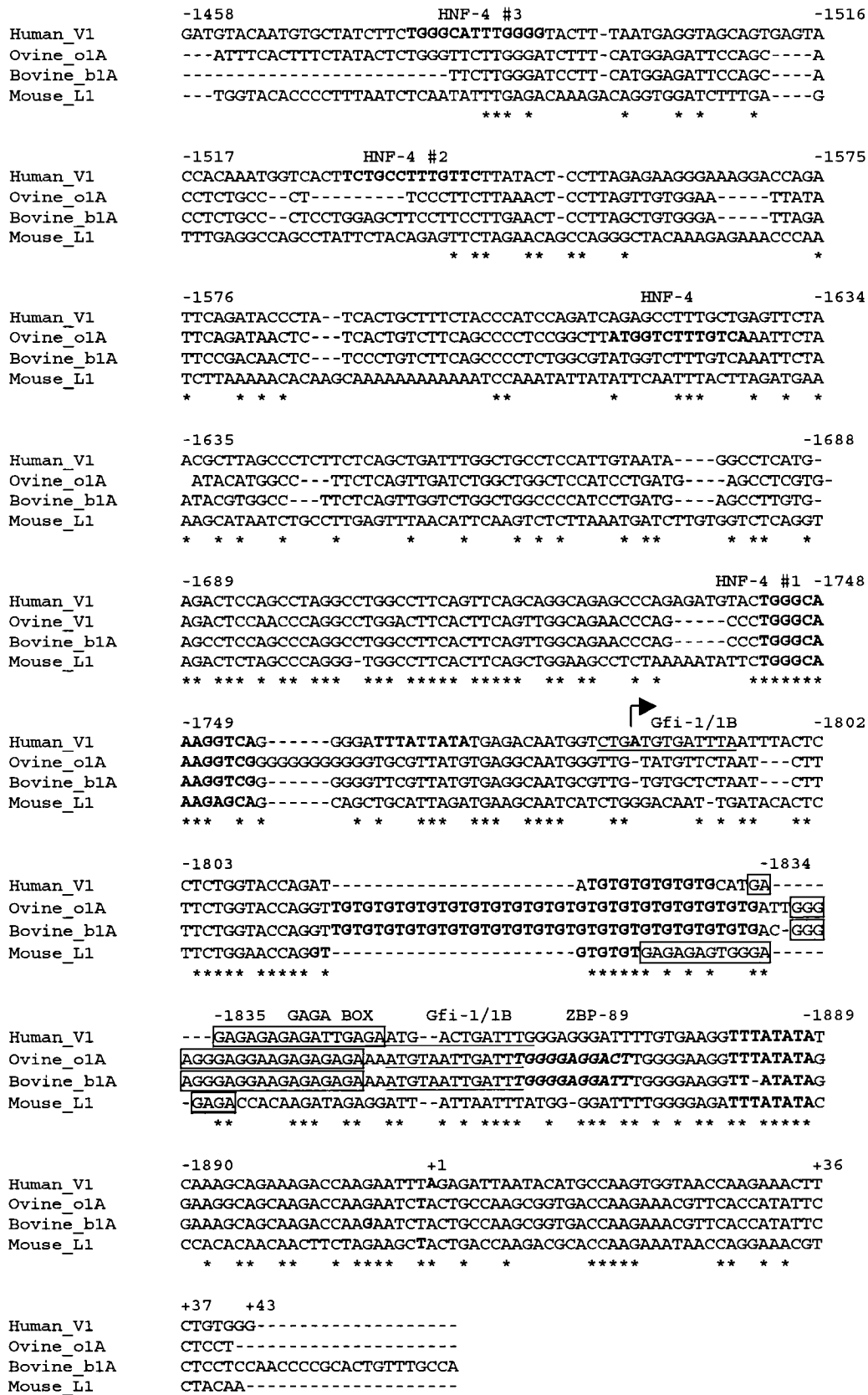

A
GAGA
Gfi-1/1b
Gfi-1/1b
V7
V1
0.5kb
NRR1
B
Gfi-1/1b
GAGA
o1A
0.5kb
Gfi-1/1b
GAGA
b1A
0.5kb
RBP JK
RP58
GAGA
mL1
0.5kb

Figure 20: Sequence comparison of human V1 to the ovine o1A, bovine b1A and mouse L1.

500 bp of the human V1 sequence (AF322015) aligned with the ovine, bovine (U15731) and mouse (NT_039747) equivalents (223;256;258;271). Transcription start sites (bold and italicised), TATA boxes (bold), Gfi-1/1b sites (underlined), GAGA element (boxed), GT repeat (bold), ZBP-89 site (bold and italicised) and putative HNF-4 sites (bold) are indicated. The human V1 has two TATA/TSS complexes, the downstream TATA/TSS is conserved in the ovine, bovine and mouse. * represents conserved nucleotides. ┌► : upstream transcriptional start site, +1: conserved transcriptional start site.


-1458 HNF-4 #3 -1516
Human_V1 GATGTACAATGTGCTATCTTCTGGGCATTTGGGGTACTT-TAATGAGGTAGCAGTGAGTA
Ovine_o1A ---ATTTCACTTTCTATACTCTGGGTTCTTGGGATCTTT-CATGGAGATTCCAGC----A
Bovine_b1A ----------TTCTTGGGATCCTT-CATGGAGATTCCAGC----A
Mouse_L1 ---TGGTACACCCCTTTAATCTCAATATTTGAGACAAAGACAGGTGGATCTTTGA----G
-1517 HNF-4 #2 -1575
Human_V1 CCACAAATGGTCACTTCTGCCTTTGTTCTTATACT-CCTTAGAGAAGGGAAAGGACCAGA
Ovine_o1A CCTCTGCC--CT---------TCCCTTCTTAAACT-CCTTAGTTGTGGAA-----TTATA
Bovine_b1A CCTCTGCC--CTCCTGGAGCTTCCTTCCTTGAACT-CCTTAGCTGTGGGA-----TTAGA
Mouse_L1 TTTGAGGCCAGCCTATTCTACAGAGTTCTAGAACAGCCAGGGCTACAAAGAGAAACCCAA
-1576 HNF-4 -1634
Human_V1 TTCAGATACCCTA--TCACTGCTTTCTACCCATCCAGATCAGAGCCTTTGCTGAGTTCTA
Ovine_o1A TTCAGATAACTC---TCACTGTCTTCAGCCCCTCCGGCTTATGGTCTTTGTCAAATTCTA
Bovine_b1A TTCCGACAACTC---TCCCTGTCTTCAGCCCCTCTGGCGTATGGTCTTTGTCAAATTCTA
Mouse_L1 TCTTAAAAACACAAGCAAAAAAAAAAAATCCAAATATTATATTCAATTTACTTAGATGAA
-1635 -1688
Human_V1 ACGCTTAGCCCTCTTCTCAGCTGATTTGGCTGCCTCCATTGTAATA----GGCCTCATG-
Ovine_o1A ATACATGGCC---TTCTCAGTTGATCTGGCTGGCTCCATCCTGATG----AGCCTCGTG-
Bovine_b1A ATACGTGGCC---TTCTCAGTTGGTCTGGCTGGCCCCATCCTGATG----AGCCTTGTG-
Mouse_L1 AAGCATAATCTGCCTTGAGTTTAACATTCAAGTCTCTTAAATGATCTTGTGGTCTCAGGT
-1689 HNF-4 #1 -1748
Human_V1 AGACTCCAGCCTAGGCCTGGCCTTCAGTTCAGCAGGCAGAGCCCAGAGATGTACTGGGCA
Ovine_V1 AGACTCCAACCCAGGCCTGGACTTCACTTCAGTTGGCAGAACCCAG-----CCCTGGGCA
Bovine_b1A AGCCTCCAGCCCAGGCCTGGCCTTCACTTCAGTTGGCAGAACCCAG-----CCCTGGGCA
Mouse_L1 AGACTCTAGCCCAGGG-TGGCCTTCACTTCAGCTGGAAGCCTCTAAAAATATTCTGGGCA
-1749 Gfi-1/1B -1802
Human_V1 AAGGTCAG------GGGATTTATTATATGAGACAATGGTCTGATGTGATTTAATTTACTC
Ovine_o1A AAGGTCGGGGGGGGGGGGTGCGTTATGTGAGGCAATGGGTTG-TATGTTCTAAT---CTT
Bovine_b1A AAGGTCGG------GGGGTTCGTTATGTGAGGCAATGCGTTG-TGTGCTCTAAT---CTT
Mouse_L1 AAGAGCAG------CAGCTGCATTAGATGAAGCAATCATCTGGGACAAT-TGATACACTC
-1803 -1834
Human_V1 CTCTGGTACCAGAT----------ATGTGTGTGTGTGCATGA-----
Ovine_o1A TTCTGGTACCAGGTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGATTGGG
Bovine_b1A TTCTGGTACCAGGTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGACGGG
Mouse_L1 TTCTGGAACCAGGT----------GTGTGTGAGAGAGTGGGA-----
-1835 GAGA BOX Gfi-1/1B ZBP-89 -1889
Human_V1 ---GAGAGAGAGATTGAGAATG--ACTGATTTGGGAGGGATTTTGTGAAGGTTTATATAT
Ovine_o1A AGGGAGGAAGAGAGAGAAAATGTAATTGATTTGGGGAGGACTTGGGGAAGGTTTATATAG
Bovine_b1A AGGGAGGAAGAGAGAGAAAATGTAATTGATTTGGGGAGGATTGGGGAAGGTT-ATATAG
Mouse_L1 -GAGACCACAAGATAGAGGATT--ATTAATTTATGG-GGATTTTGGGGAGATTTATATAC
-1890 +1 +36
Human_V1 CAAAGCAGAAAGACCAAGAATTTAGAGATTAATACATGCCAAGTGGTAACCAAGAAACTT
Ovine_o1A GAAGGCAGCAAGACCAAGAATCTACTGCCAAGCGGTGACCAAGAAACGTTCACCATATTC
Bovine_b1A GAAAGCAGCAAGACCAAGAATCTACTGCCAAGCGGTGACCAAGAAACGTTCACCATATTC
Mouse_L1 CCACACAACAACTTCTAGAAGCTACTGACCAAGACGCACCAAGAAATAACCAGGAAACGT
+37 +43
Human_V1 CTGTGGG----------
Ovine_o1A CTCCT----------
Bovine_b1A CTCCTCCAACCCCGCACTGTTTGCCA
Mouse_L1 CTACAA----------

The 300 bp NRR1 was analysed for putative response elements using the transcription factor scanning program MatInspector (Figure 19).
Two putative binding sites for the transcriptional repressors, Growth factor independence 1 and 1b (Gfi-1/Gfi-1b), were identified. The most proximal site is conserved in the ovine and bovine while the mouse has different transcriptional repressors in the same region (Figures 19 & 20). The upstream site is unique to the human and is located at the upstream V1 TSS (Figure 20).

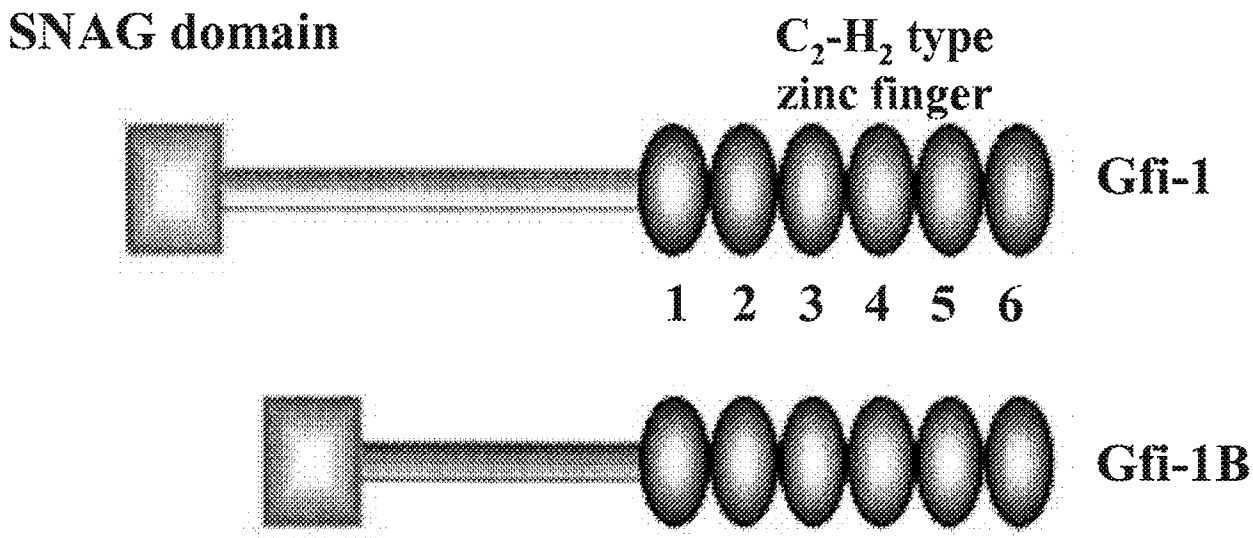
4.4. Gfi-1 and Gfi-1b

4.4.1. Protein domains

Gfi-1 and Gfi-1b are two related zinc finger transcription factor proteins that are predominantly expressed in the haematopoietic system. They contain an identical 20 amino acid SNAG domain in the N-terminus and six C2H2 type zinc fingers in the C-terminus, and only differ in the intervening sequence (21% similarity in amino acids) between the two termini (Figure 21) (272-274). Both factors bind to the same DNA sequence: TAAATCAC(A/T)GCA; however, only the core AATC sequence is required for binding (273).

Figure 21: Protein domains of Gfi-1 and Gfi-1b.

Diagrammatic representation of Gfi-1 and Gfi-1b proteins indicating the SNAG (green) and C_2H_2 zinc-finger (blue) domains. Gfi-1 and Gfi-1b proteins differ in the length of the intervening sequence between the SNAG and zinc finger domains. Figure from (274).


SNAG domain
C_2-H_2 type
zinc finger
Gfi-1
1 2 3 4 5 6
Gfi-1B

4.4.2. Expression patterns

Gfi-1 is predominantly expressed in the thymus, spleen, testis and bone marrow while Gfi-1b is expressed in bone marrow and spleen, specifically in haematopoietic stem cells, erythroblasts and megakaryocytes (275). Gfi-1b levels have been shown to be highest in human foetal liver and bone marrow (276). However, because the foetal liver is heavily populated with haematopoietic cells and this study did not differentiate between expression in hepatocytes versus blood cells, it was impossible to conclude whether foetal hepatocytes express Gfi-1b.

Gfi-1 is also expressed in human neuroendocrine tumour cells, human prostate cancer cells and the cochlear hairs of the inner ear (277-279). Gfi-1 is required for inner ear hair cell development (279). One of the transcription factors that regulates Gfi-1 in this process is *Pou4f3*, which, when mutated, causes deafness in humans and mice (280).

Mice homozygous for the Gfi-1 mutant allele have neutropaenia, lose their hearing and are sensitive to bacterial toxins (281). Interestingly, the body weight of these Gfi-1 null homozygous mice was 10-50% that of their normal littermates (281). Homozygous null Gfi-1b mice exhibit haemorrhaging, oedema and pallor and die by day E15 due to defective erythropoeisis (282). Humans with a defect in Gfi-1 are also neutropaenic but no hearing loss or susceptibility to endotoxins has been observed

(283). No growth or metabolic abnormalities resulting from Gfi-1 and/or Gfi-1b mutations have been reported in humans.

4.4.3. Repressor activity

Gfi-1 acts as a transcriptional repressor on the human cytomegalovirus major immediate-early (HCMV MIE), p21Cip/WAF1, Bax and 25 hydroxyvitamin D 1α hydroxylase genes (278;284). Gfi-1 represses transcription by interacting with the corepressors ETO, mSin3A and by direct interaction with histone deacetylases (HDAC-1, -2 and 3), thereby, modifying chromatin structure (Figure 22A) (284;285). Both the zinc finger and SNAG domains have been implicated in the repressive behaviour of Gfi-1. Grimes *et al.* showed that the SNAG domain was essential whereas McGhee *et al.* showed that the SNAG domain was dispensable, but the zinc finger domain was required for ETO-mediated repression (272;285). Gfi-1 suppresses p21Cip/WAF1 expression in an epigenetic fashion, by recruiting the histone methylase G9a and HDAC1, independent of the SNAG domain (284). In addition, it binds and suppresses its own expression in lymphoid cells (286).

Targets of Gfi-1b include SOCS1 and 3, the negative regulators of cytokine and GH signalling, Gfi-1 and GATA-2, a major regulator of primitive erythropoiesis (286;287). SOCS proteins inhibit cytokine induced cell proliferation and, thus, the role of Gfi-1b in suppressing their expression correlates with its role in enhancing erythroid expansion. In response to

Epo stimulation of the myeloid 32D cells, Gfi-1b is downregulated through STAT5 induced *de novo* synthesis of intermediate, and as yet unidentified, proteins (287). A similar role could be played by GH but this has not yet been investigated.

4.4.4. Activator activity

Gfi-1b has also been shown to act as a transcriptional activator on the α_1-antichymotrypsin (pACT) promoter by interacting with PIAS (Figure 22B) (288). PIAS associates with STAT 3 making it inactive; binding of Gfi-1 to PIAS releases STAT 3, enhancing its transactivating of the pACT promoter construct by ~ 5 fold (288). In addition, Gfi-1 has stimulatory roles in the renewal and proper function of haematopoietic stem cells (HSCs), pre-T cell survival, guanylyl cyclase expression (in concert with CCAAT binding factor) and erythroid expansion (275;289-292). The function of these transcription factors as repressors or activators appears to be both promoter and cell type specific (Figure 22) (274). For example, Gfi-1 is required for suppression of p21Cip/WAF1 in Jurkat cells and for its expression in HSCs (289;292).

Figure 22: Two possible mechanisms of Gfi-1 action.

(A) Gfi-1 as a repressor. Repression of Gfi-1 target genes occurs by recruiting HDACs to the promoter sequence. De-acetylated histones result in a more tightly packaged chromatin that silences transcription. (B) Gfi-1 as an activator. Gfi-1 can enhance STAT3 mediated transcriptional transactivation by binding and sequestering the STAT3 inhibitor PIAS3. Figure and text of legend from (274).

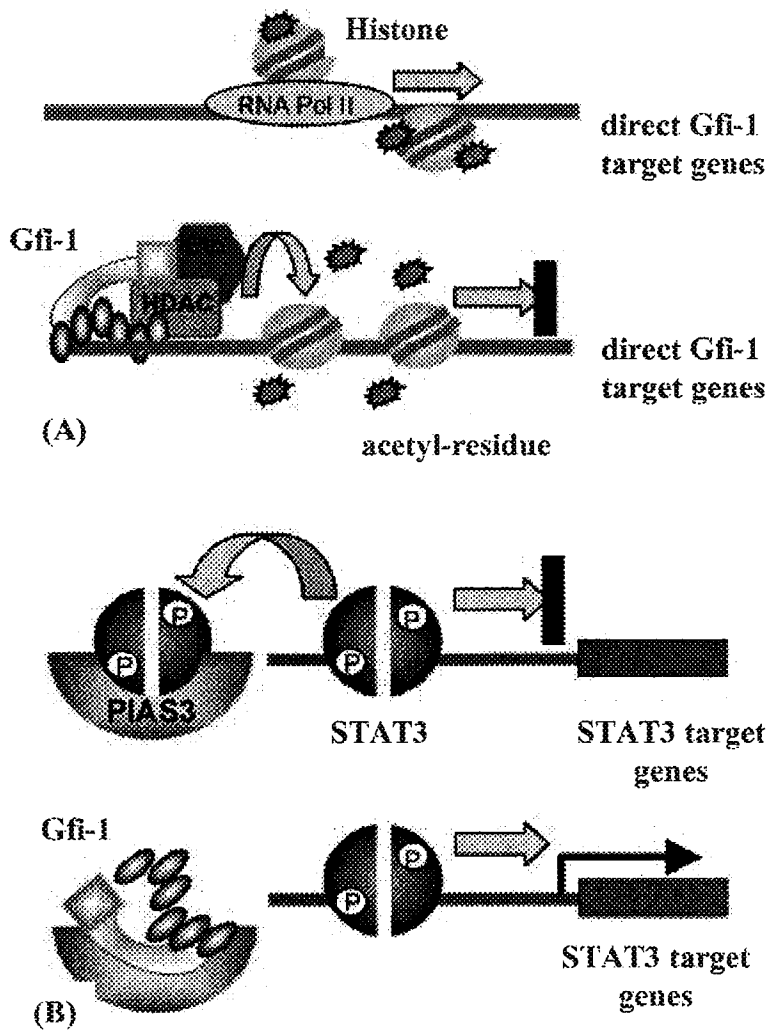
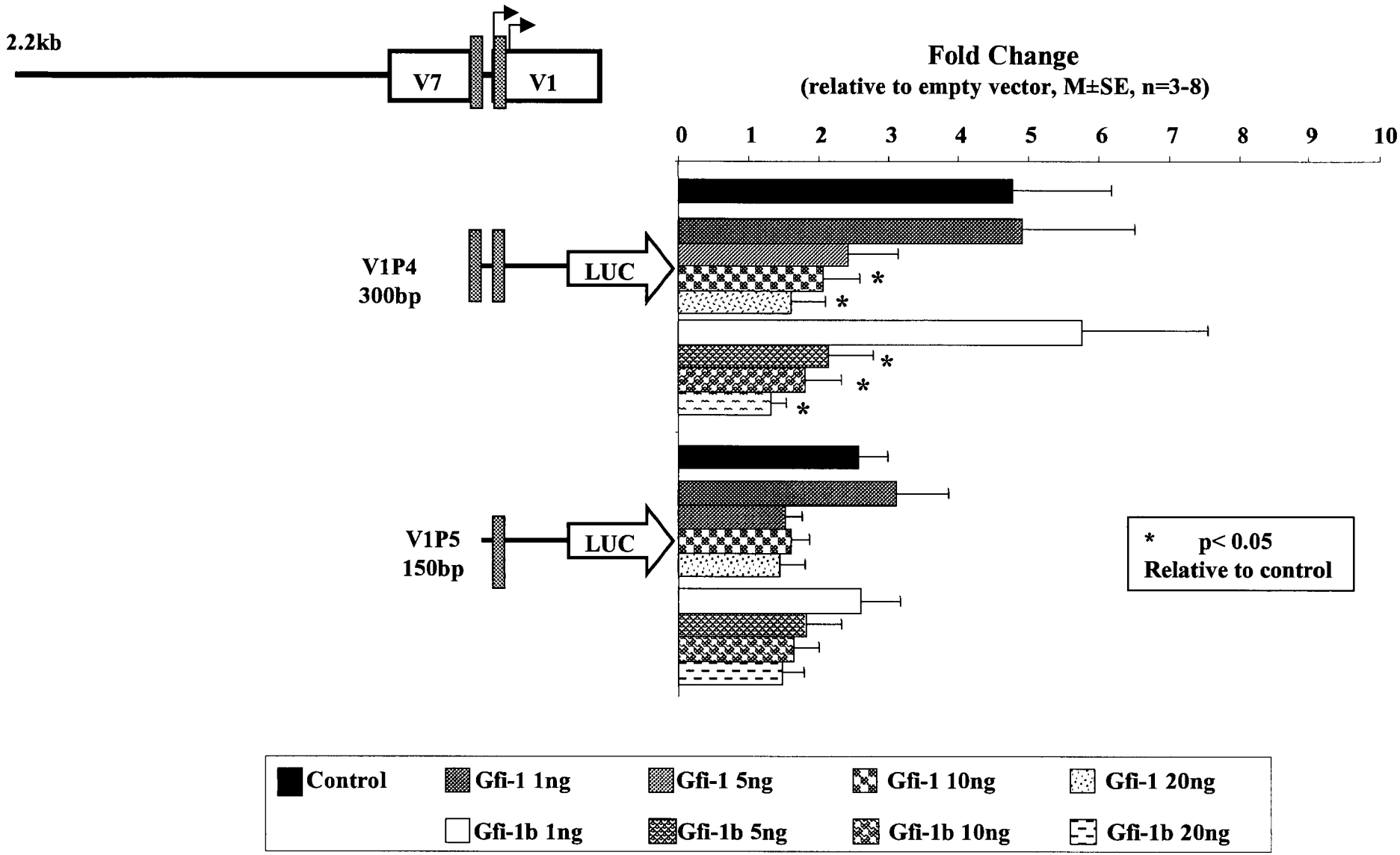

Histone
RNA Pol II
direct Gfi-1
target genes
Gfi-1
HDAC
direct Gfi-1
target genes
(A)
acetyl-residue
P
PIAS3
STAT3
STAT3 target
genes
Gfi-1
STAT3 target
genes
(B)

Figure 23: Effect of Gfi-1 and Gfi-1b on the transcriptional activity of V1.

Expression vectors for Gfi-1 and Gfi-1b were cotransfected with two V1 constructs, V1P4 containing two sites and V1P5 containing only one, into HEK293 cells with polyfect. Cells were harvested 48 hr later and assayed as before. Data are presented as mean ± standard error, n=3-8 experiments. The significance of the differences observed was determined by Bonferroni's statistical test. * $p < 0.05$.


2.2kb
V7
V1
Fold Change
(relative to empty vector, M±SE, n=3-8)
0
1
2
3
4
5
6
7
8
9
10
V1P4
300bp
LUC
V1P5
150bp
LUC
*
*
*
*
*
*
p< 0.05
Relative to control
Control
Gfi-1 1ng
Gfi-1 5ng
Gfi-1 10ng
Gfi-1 20ng
Gfi-1b 1ng
Gfi-1b 5ng
Gfi-1b 10ng
Gfi-1b 20ng

4.4.5. Transient transfection assay studies on the effects of Gfi-1 and Gfi-1b on the V1 proximal promoter

We hypothesized that Gfi-1 and Gfi-1b would repress the activity of V1. To test this, I carried out cotransfection experiments with a range of Gfi-1 and Gfi-1b expression vector doses and V1 constructs containing either the most proximal and conserved site (V1P5) or both sites (V1P4) in HEK293 cells. The results showed up to 75% repression by very low amounts of these factors (Figure 23). V1P4, containing both sites, was significantly repressed by 10 and 20 ng of Gfi-1, and 5,10 and 20 ng of Gfi-1b. V1P5 showed inhibition that was not significant when multiple doses were tested (Figure 23) but was significant when only a 20 ng dose was used (Figure 24).

4.4.6. Site-directed mutagenesis of two Gfi-1/1b sites

In order to further evaluate the functionality of these putative Gfi-1/1b response elements, I mutated the sites in V1P4 and V1P5 (Table 4) and tested their responses to 20 ng of Gfi-1 and Gfi-1b. Repression by both Gfi-1 and Gfi-1b was abolished in the V1P5 mutant (Figure 24). Mutating the conserved Gfi-1/1b site in V1P4 diminished the response to Gfi-1/1b, however the repressed pattern was still present, likely due to the presence of the upstream site (Figure 25). This pattern is lost when only the upstream site was mutated.

Figure 24: Mutational analyses of the Gfi-1/1b binding sites in the proximal promoter of V1 (V1P5).

V1P5 construct with the Gfi-1/1b binding site mutated (Table 4) was cotransfected with Gfi-1 and Gfi-1b; non-mutated V1P5 construct was transfected as control. Data are presented as mean ± standard error, n=4-11 experiments. The significance of the differences observed was determined by ANOVA followed by Bonferroni's statistical test. *$p < 0.05$.

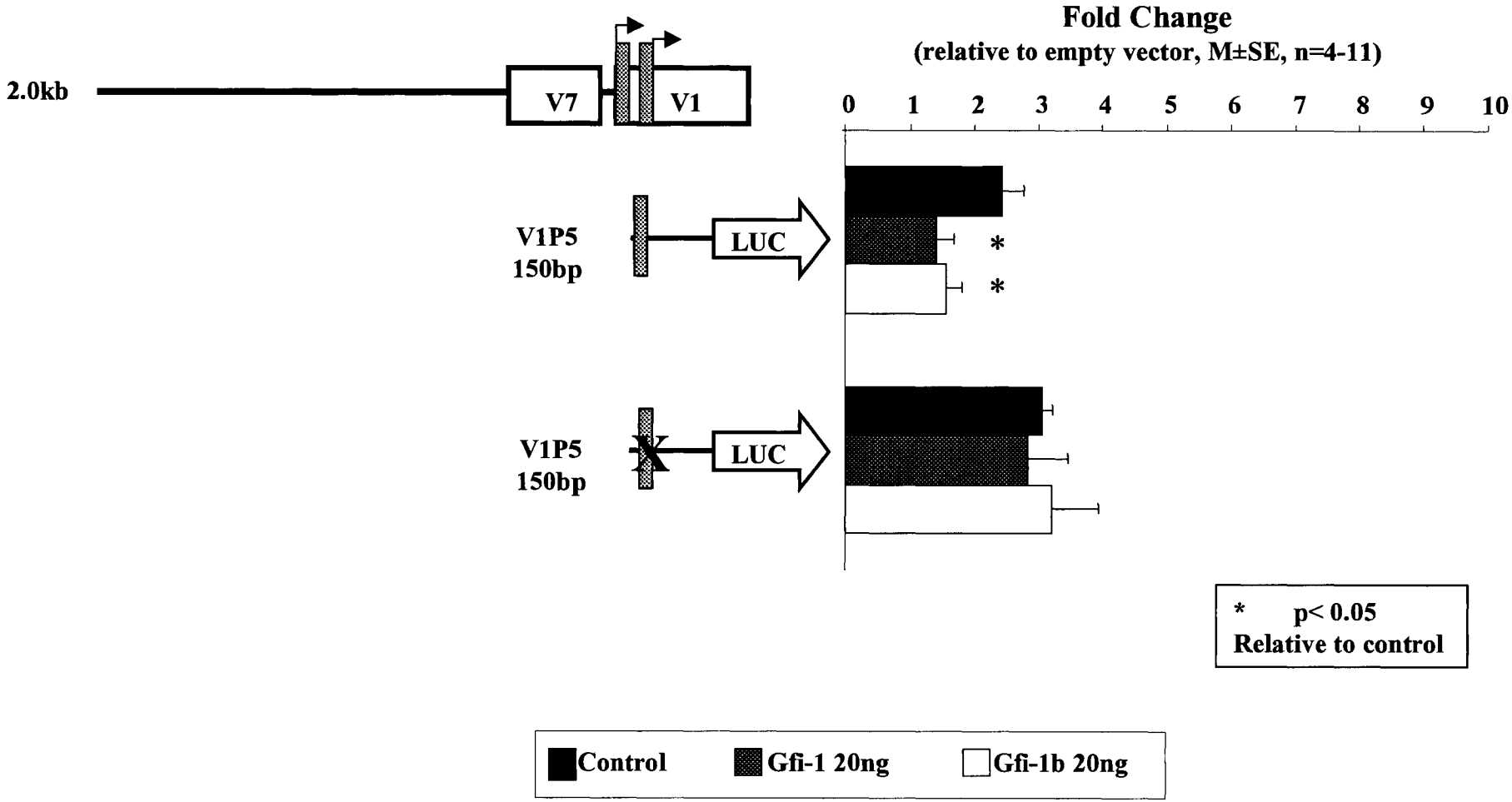

Fold Change
(relative to empty vector, M±SE, n=4-11)
0 1 2 3 4 5 6 7 8 9 10
2.0kb
V7
V1
V1P5
150bp
LUC
*
*
V1P5
150bp
LUC
* p< 0.05
Relative to control
Control
Gfi-1 20ng
Gfi-1b 20ng

Figure 25: Mutational analyses of the Gfi-1/1b binding sites in the proximal promoter of V1 (V1P4).

V1P4 constructs with either one or both of the Gfi-1/1b binding site(s) mutated (Table 4) were cotransfected with Gfi-1 and Gfi-1b; non-mutated V1P4 construct was transfected as control. Data are presented as mean ± standard error, n=3-17 experiments. The significance of the differences observed was determined by ANOVA followed by Bonferroni's statistical test. $^{**}p<0.01$, $^{***}p<0.001$.

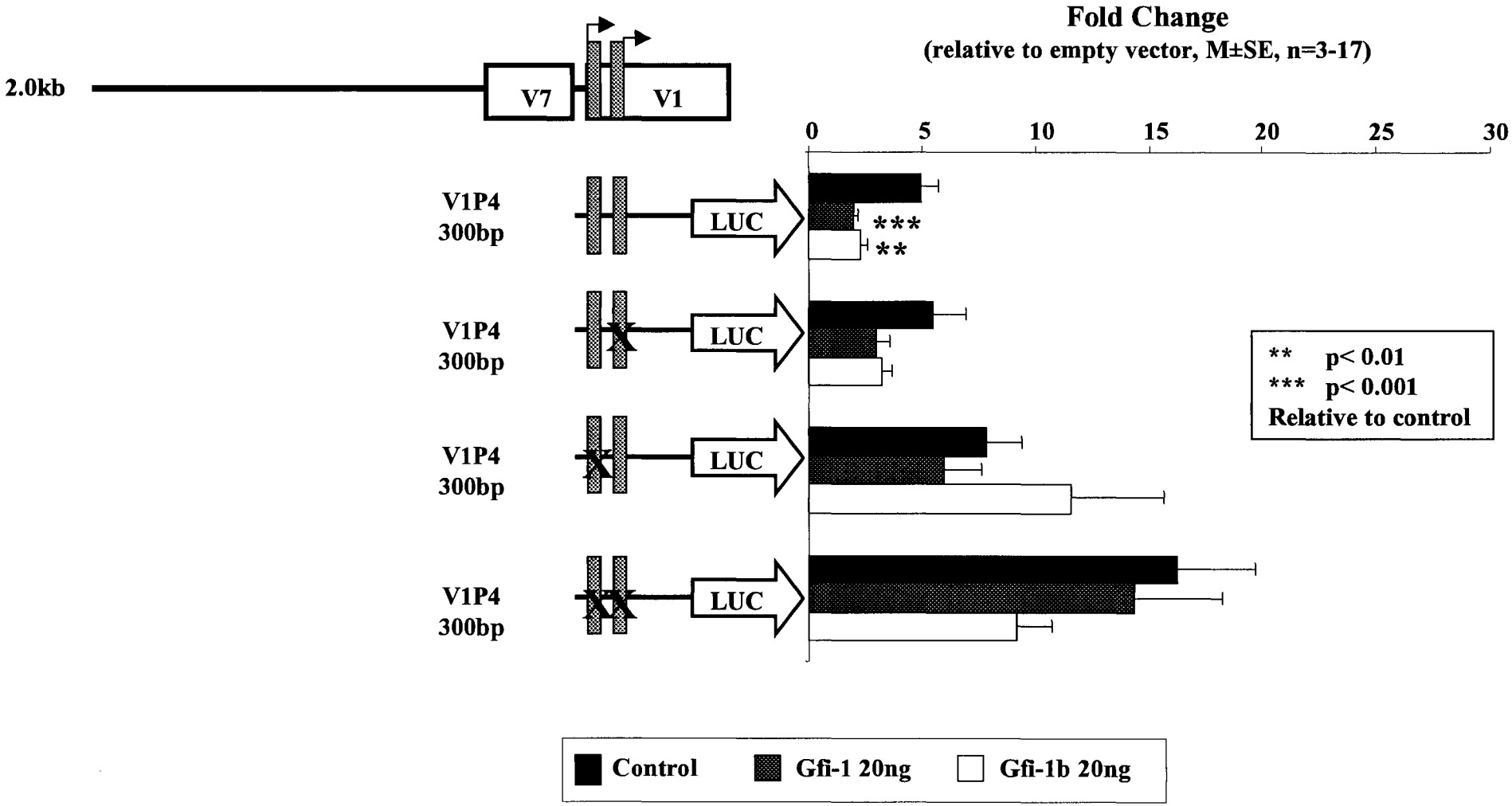
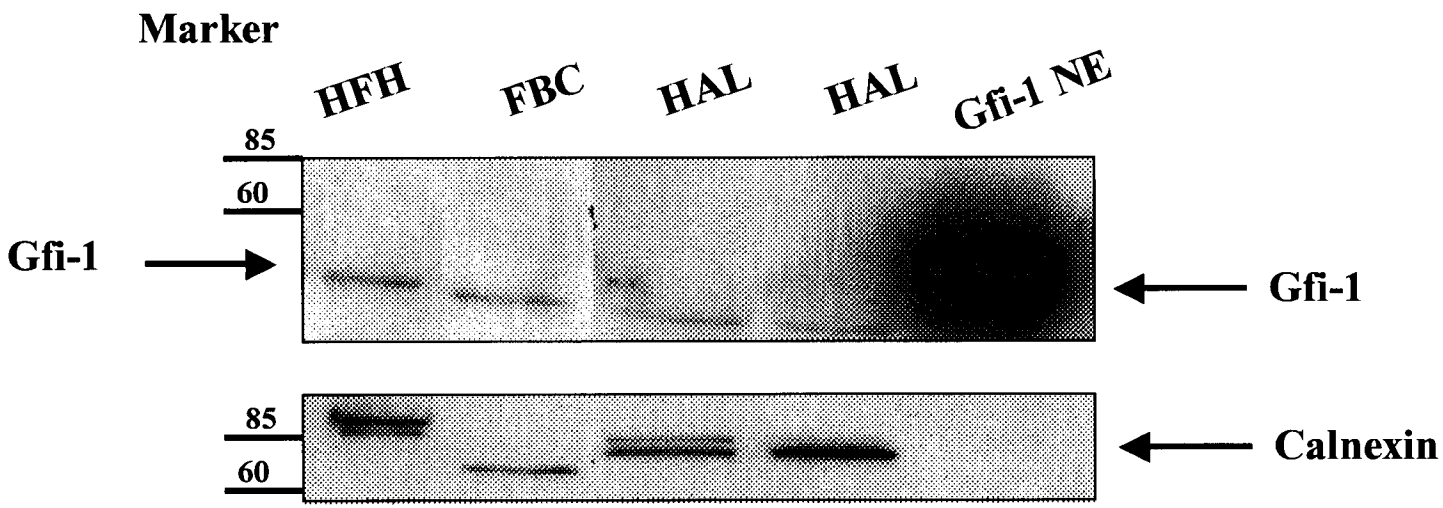

Fold Change
(relative to empty vector, M±SE, n=3-17)
2.0kb
V7
V1
0
5
10
15
20
25
30
V1P4
300bp
LUC
***
**
V1P4
300bp
LUC
V1P4
300bp
LUC
V1P4
300bp
LUC
** p< 0.01
*** p< 0.001
Relative to control
Control
Gfi-1 20ng
Gfi-1b 20ng

Figure 26: Detection of Gfi-1 immunoreactive protein in human liver by Western blot.

75 μg of whole cell (human foetal hepatocytes) and tissue (human postnatal liver) lysates were resolved on 12% SDS-PAGE and immunoblotted with anti-Gfi-1. 1 μg of nuclear lysate from HEK293 cells overexpressing Gfi-1 was run as a control. Calnexin was used as a loading control. Another lab member, Gurvinder Kenth, carried out this experiment.

HAL: human adult liver; HFH: human foetal hepatocytes; FBC: foetal blood cells.


Marker
HFH
FBC
HAL
HAL
Gfi-1 NE
85
60
Gfi-1
Gfi-1
85
60
Calnexin

In addition, loss of both sites resulted in a significant increase in basal activity (16.2±3.4, n=5) compared to the wild-type (4.9±0.8, n=17, $p<0.001$), upstream Gfi-1/1b (7.8±1.5, n=3, $p<0.01$) and proximal Gfi-1/1b (5.5±1.5, n=8, $p<0.01$) mutants (Figure 25).

Thus, by mutating the Gfi-1/1b sites, endogenous repressors, presumably Gfi-1 and/or Gfi-1b, can no longer inhibit the activity of the V1P4 promoter construct.

Protein expression of Gfi-1 was detected in human foetal hepatocytes, human foetal blood cells, human foetal liver and adult liver by immunoblotting (Figure 26). We did not detect this transcription factor in the cell lines (data not shown). However, Gfi-1 expression in these cells may be below the detection level of our immunoblots, as the transfection data show that very small amounts of both factors can cause significant repression and that endogenous Gfi-1-like proteins are present in HEK293 cells.

4.4.7. Discussion of Gfi-1 and Gfi-1b data

The restricted expression of V1 mRNA transcripts to normal postnatal liver has been intriguing and, thus, the mechanisms regulating it have been of great interest. From the deletion promoter studies of the V1 region, the transcriptional activity of V1P1 was markedly repressed across the four cell lines. The fact that this was not cell type specific suggests a common mechanism of repression. The 300 bp region (NRR1) contains two sites

for the transcriptional repressors, Gfi-1 and Gfi-1b: the proximal Gfi-1/1b site is conserved in the ovine and bovine GHRs but the upstream site is unique to the human. Using transient transfection assays and site-directed mutagenesis, I have shown that both Gfi-1 and Gfi-1b strongly repressed V1 promoter activity. The inhibitory effect was more pronounced in a construct containing the conserved site as well as the response element unique to the human V1 and promoter activity was significantly higher when both sites were mutated. This suggests that cooperation of the two sites is required for stronger repressive effects. Interestingly, NRR1 (the V1P4 construct), shows significant activity in all four cell lines (Figure 17B), and, therefore, the repressive effects of endogenous Gfi-1 and Gfi-1b (and other repressors) appear to be overridden by endogenous positive regulators acting within this region. Gfi-1 has been shown to repress transcription by interacting with the corepressor, Eto, and the recruitment of HDACs (285). It is also involved in epigenetic repression of p21Cip/WAF1 via interaction with the histone methylase, G9a (284). Regulation of GHR by DNA methylation seems unlikely as the V1 promoter has no CpG islands and few CpG dinucleotides. However, other epigenetic mechanisms may be involved. Initially the majority of the described targets of Gfi-1 and Gfi-1b were associated with the haematopoietic system [reviewed in (293;294)]. Since then, genes involved in deafness, prostate cancer and

neuroendocrine systems have also been described as targets [reviewed in (274)]. This is the first account of GHR as a target of both Gfi-1 and Gfi-1b. In addition, Gfi-1 proteins were shown to be present in human foetal liver, blood cells and hepatocytes, and adult liver for the first time. This suggests a role for Gfi-1 in regulating hepatic genes, including the hGHR. It has recently been reported that ZBP-89 stimulates b1A promoter activity through a site just downstream of and overlapping the conserved Gfi-1/1b response element (Figure 20) (267). DNAse I footprinting studies with a 190 bp fragment (-169 to +21) of the b1A promoter showed that this region is bound by endogenous proteins in bovine liver. Yeast one-hybrid and EMS/SA studies confirmed that the interacting protein was ZBP-89 (267). Interestingly, the conserved Gfi-1/1b site also located in the 190 bp fragment was not protected from DNAse I digestion suggesting that the site is not bound in bovine liver (267). In addition, the yeast one-hybrid screen did not pick up Gfi-1 or Gfi-1b. Since the human V1 promoter sequence shows a non-consensus ZBP-89 site, it is likely that there are major species-specific differences in regulation of the V1 equivalent proximal promoter.

In summary, my studies of two Gfi-1/1b sites in the proximal promoter of V1 have shown that V1 is a target of Gfi-1 and Gfi-1b repressive activity.

4.5. GAGA element

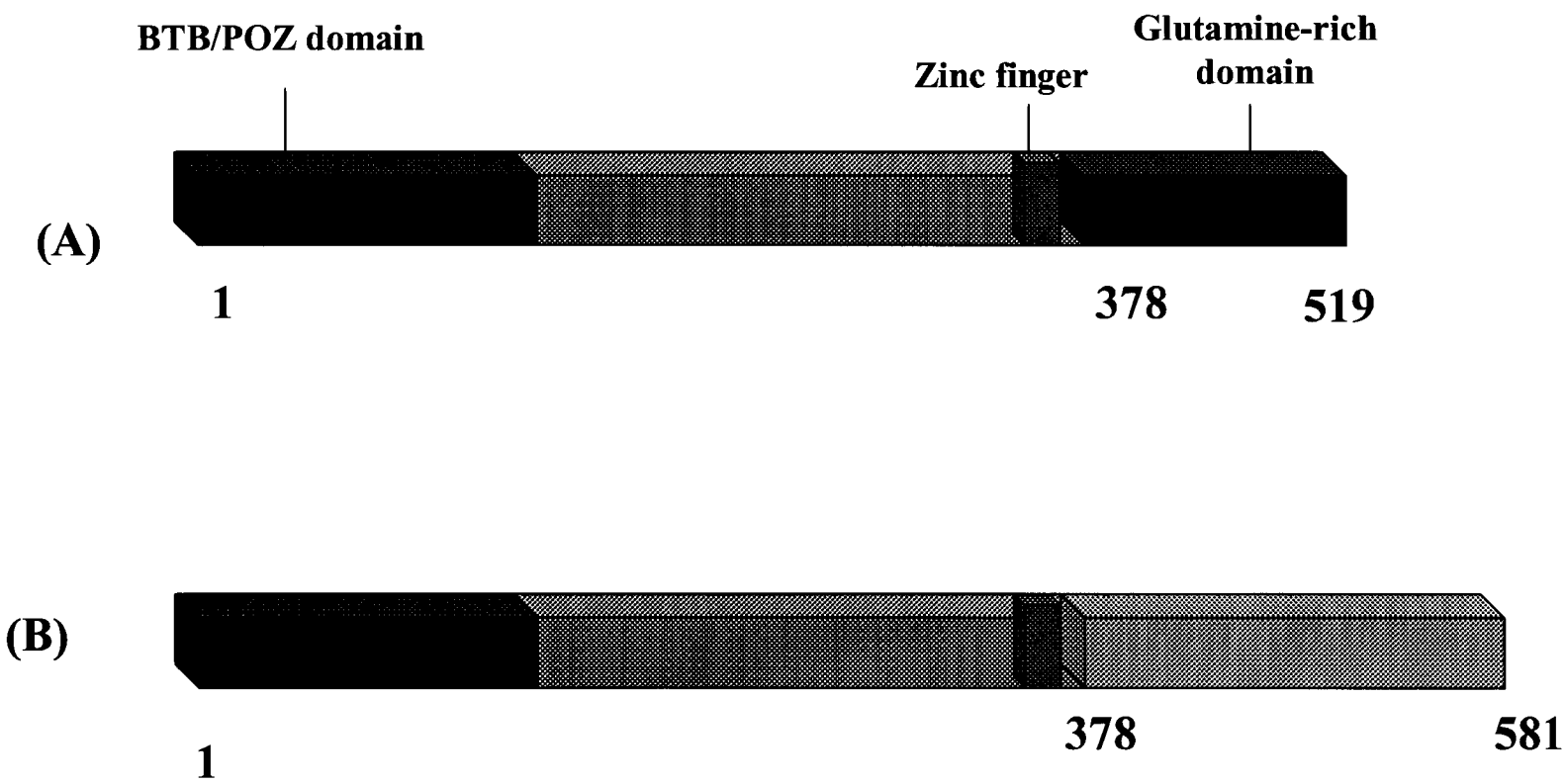
Also of interest within the NRR1 site, and overlapping with the conserved Gfi-1/1b site, was a 4X GAGA repeat (Figures 19 & 20). This GAGA element is conserved in the ovine, bovine and mouse (Figures 19 & 20), suggesting that it is an important element in regulation of the liver-specific variant of GHR. More exciting is the fact that GAGA elements have been described as GHREs in several vertebrate genes.

4.5.1. GAGA factor (GAF) in *Drosophila*

GAGA elements were first identified in *Drosophila* genes and subsequently, in genes of other species, including mammals [reviewed in (295)]. The GAGA factor (GAF) was first isolated in *Drosophila* as binding to GA_n repeats in the *Ultrabiothorax (Ubx)* promoter (296). GAF is encoded by the Trithorax-like gene, which is required for the regulation of several homeotic genes in *Drosophila* (297). Two alternatively spliced isoforms of GAF mRNA have been identified that translate into 519 and 581 amino acid proteins (~ 70-90 kDa) (Figure 27) (298;299). These GAF proteins share the first 377 amino acids that encode the following domains: an N-terminal pox viruses and zinc finger/broad-complex, tramtrack, bric-a-bric (POZ/BTB) domain and a single zinc finger DNA binding domain (DBD).

Figure 27: Protein domains of the GAGA factors.

GAF proteins: (A) the 519 amino-acid isoform and (B) the 581 amino-acid isoform. The two proteins share the first 377 amino acids at which point they diverge. The glutamine rich regions (light and dark blue), BTB/POZ domain (black) and single zinc finger (red) are indicated. Figure from (300).


BTB/POZ domain
Zinc finger
Glutamine-rich domain
(A)
1
378
519
(B)
1
378
581

They differ in the length of the glutamine (Q) rich C-terminus. NMR studies of GAF showed that it binds to a GAGAG pentamer (301). However, gel shift analyses and immunoprecipitation assays demonstrated that GAF recognises a minimal GAG trinucleotide (302). The POZ/BTB domain is involved in protein-protein interactions and the Q domain is involved in DNA distortion and melting, as well as multimerisation (303). Both isoforms form homo- and hetero-dimers, bind the same sequence, and have similar chromatin remodelling properties (298). DNA binding activity of the 519 GAF isoform is modified by the serine/threonine kinase, casein kinase 2 (CK2). CK2, phosphorylates S378 and S388 residues in the DBD of GAF and reduces its DNA binding activity (304). This region is absent in the 581 GAF isoform and, therefore, phosphorylation by CK2 is not common to the isoforms (304).

Effects of GAF on heat shock gene transcription have been extensively studied in *Drosophila*. GAF activates transcription by counteracting the repressive effects of histones (305). Addition of GAF before or after nucleosome assembly on the *hsp 70* promoter results in the disruption of chromatin structure and the appearance of hypersensitive sites (306). This process of chromatin remodelling requires ATP: the chromatin remodelling properties of GAF are facilitated by direct interaction with the ATP-dependent nucleosome remodelling factor (NURF) and FACT (facilitates chromatin transcription) protein (306-308). In addition to

chromatin disruption, GAF also maintains chromatin structure during the cell cycle as it is found associated with heterochromatin throughout the cell cycle (309). GAF and the GAGA element are also required for the enhancer blocking activity of the *Frontoabdominal-7 (Fab-7)*, a boundary element in the bithorax gene complex (310).

4.5.2. GAF studies in vertebrates

In vertebrates, the *Drosophila* GAF has been studied as a classical transactivator on genes. An unusually large inverted GAGA element, a 134 CT repeat, is found ~ 90 bp upstream of the transcriptional start site of the rat vasopressin 1b receptor gene (V1bR) (311). Basal activity of a promoter construct was drastically reduced when the GAGA element was deleted. In addition, *Drosophila* GAF-519 stimulated a V1bR promoter construct containing the GAGA element by 11-fold. Endogenous V1bR mRNA expression in the hypothalamic cell line, H32, and the breast cancer cell line, MCF-7, was induced 6-fold by GAF-519. Thus, the GAGA element is required for the basal activity of V1bR. No GAF homologue has been identified in mammalian systems. However, several GAGA binding proteins (GBPs) have been identified (see below). These proteins seem to be regulated by cytokines, GH being most well studied.

4.5.3. GAGA element in vertebrates as a GH response element (GHRE)

GAGA elements have been investigated in several vertebrate genes as GHREs. Promoter activities of the rat spi 2.1, carbamyl phosphate synthase 1 and the human type-1 angiotensin II (AT_1) receptor genes were significantly increased upon GH treatment, mediated by the GAGA element (312-314).

The rat spi 2.1 gene is one of the most well studied of the GH-regulated genes. GH upregulates spi 2.1 mRNA both *in vivo* and *in vitro* (312;315). Two GHREs were identified: the more distal element, GHRE II, is a position independent enhancer and contains GLEs that interact with STAT5 upon GH stimulation (312;316-318). The more proximal element, GHRE I, includes a GAGA element just upstream of the transcriptional start site and is responsible for both basal promoter activity and stimulatory response to GH (312;314). *In vitro* transfection studies suggest that JAK2 and the C-terminus of GHR are required for GH-induced activation of the rat spi 2.1 promoter through the GAGA element (314). An EMSA probe containing the GAGA element of the spi 2.1 gene bound liver nuclear extracts (314). DNAse I footprinting experiments show that binding to the lower strand of the GAGA element was abolished in liver nuclear extracts prepared from rats either hypophysectomised or in whom LPS was used to induce inflammation, suggesting cytokine regulation of the GAGA binding proteins (GBPs) (314).

Two heat stable proteins, p38 and p40, have been identified as interacting with the GAGA box of spi 2.1 (319). These two proteins are rat homologues of the mouse CA-rich G box binding factor (mCBF-A) and the human Apobec-1-binding protein (hABBP-1) (320;321). Both mCBF-A and hABBP-1 are type A/B heterogenous nuclear ribonucleoproteins (hnRNPs) that interact with both RNA and DNA (320;321). mCBF-A binds both single and double stranded DNA and RNA (SP6 kappa gene) (322). hABBP-1 interacts with apobec-1, the catalytic subunit of the apoB mRNA editing complex and also with apoB mRNA, and is thus involved in the editing of apoB (321). As discussed in section 1.2.4, GH stimulates editing of apoB in rats by as yet unidentified mechanisms. Though the effect of GH on the binding of the two proteins was not investigated in the study, the same group has shown that GH affects the binding of GBPs through an as yet unidentified JAK2 activated pathway (314).

It would be of great interest to know whether p38/40 are indeed regulated by GH, and whether GH regulation of apoB editing is through these proteins. Although the rat p38/40 proteins have no sequence homology to the *Drosophila* GAF, they may have similar functions in mammals. As type A/B hnRNPs with a potential ATP/GTP binding site, these GBPs may be involved in ATP-dependent chromatin remodelling. However they are unlikely to be involved in DNA melting as it was

demonstrated that they did not bind single strands (plus or minus) of the GAGA element (319).

The GAGA element from the promoter of the rat vasopressin V1b receptor interacts with endogenous proteins of a size similar to the 75 kDa GAF: dimers of a 70 kDa protein were identified by size-exclusion chromatography (311). This protein is likely different from the single zinc finger GAF, as it is not recognised by an antibody against the whole *Drosophila* GAF protein (311). The effect of GH on the binding activity of this protein has not been studied. Promoter activity of the AT_1 receptor gene is enhanced by GH treatment in LRCC8 (human epithelial tubular kidney) cells. Binding of an 18 kDa protein to a GAGA element in the AT_1 receptor gene was rapidly induced (2.5 min) by GH in these cells. This is probably due to post-translational modifications, such as phosphorylation of the GBP. Inhibition of protein synthesis by cyclohexamide did not abolish binding but rather increased it, suggesting that *de novo* synthesis was important in destabilizing the GBP (313). The 18 kDa and the 70 kDa proteins are yet to be sequenced and characterised. Thus, although several GBPs have been described, no known mammalian homologues of the *Drosophila* GAF have been identified.

4.5.4. Transient transfection assay studies on the effect of *Drosophila* GAF on the V1 proximal promoter

The ability of the GAGA element in the proximal promoter of V1 to respond to the *Drosophila* GAF factor was tested in cotransfection experiments using V1P5, the smallest GAGA containing construct, in HEK293 cells. GAF significantly stimulated V1P5 activity, although increasing doses > 25 ng to 100 ng of GAF made no significant difference on the activity of V1 [25 ng GAF: 12.5±2.8 fold (n=4, $p<0.05$); 50 ng GAF: 13.8±3.7 fold (n=7, $p<0.01$) and 100 ng GAF 14.8±3.6 fold (n=, $p<$ 0.001)] (Figure 28). The control construct, V_XP_A, showed no significant response to GAF. Overexpression of GAF in HEK293 cells was verified by western blot analyses (Figure 29).

4.5.5. Site directed mutagenesis of the GAGA element.

In order to confirm that the GAGA element is responsible for the response to GAF, the GAGA element was mutated and tested in cotransfection experiments with the *Drosophila* GAF (Table 5). The response to 25 ng GAF was completely ablated in the V1P5 GAGA element mutant (3.24±0.25, n=3) (Figure 30). However basal activity of the GAGA element mutant construct (2.75± 0.45, n=4) was comparable to the wild-type (2.65±0.35, n=7), suggesting that while GAF can act through the GAGA element in the V1 proximal promoter, HEK293 cells appear to be devoid of a protein with similar activating ability.

Figure 28: Effect of GAGA binding factor (GAF) on the transcriptional activity of V1.

Expression vector for GAF was cotransfected with V1P5 (containing the GAGA element) and V_XP_A was transfected as a control, in HEK293 cells with polyfect. Cells were harvested 48 hr later and assayed as before. Data are presented as mean ± standard error, n=4-7 experiments. The significance of the differences observed was determined by Bonferroni's statistical test. * $p< 0.05$, **$p<.0.01$, ***$p<.0.001$.

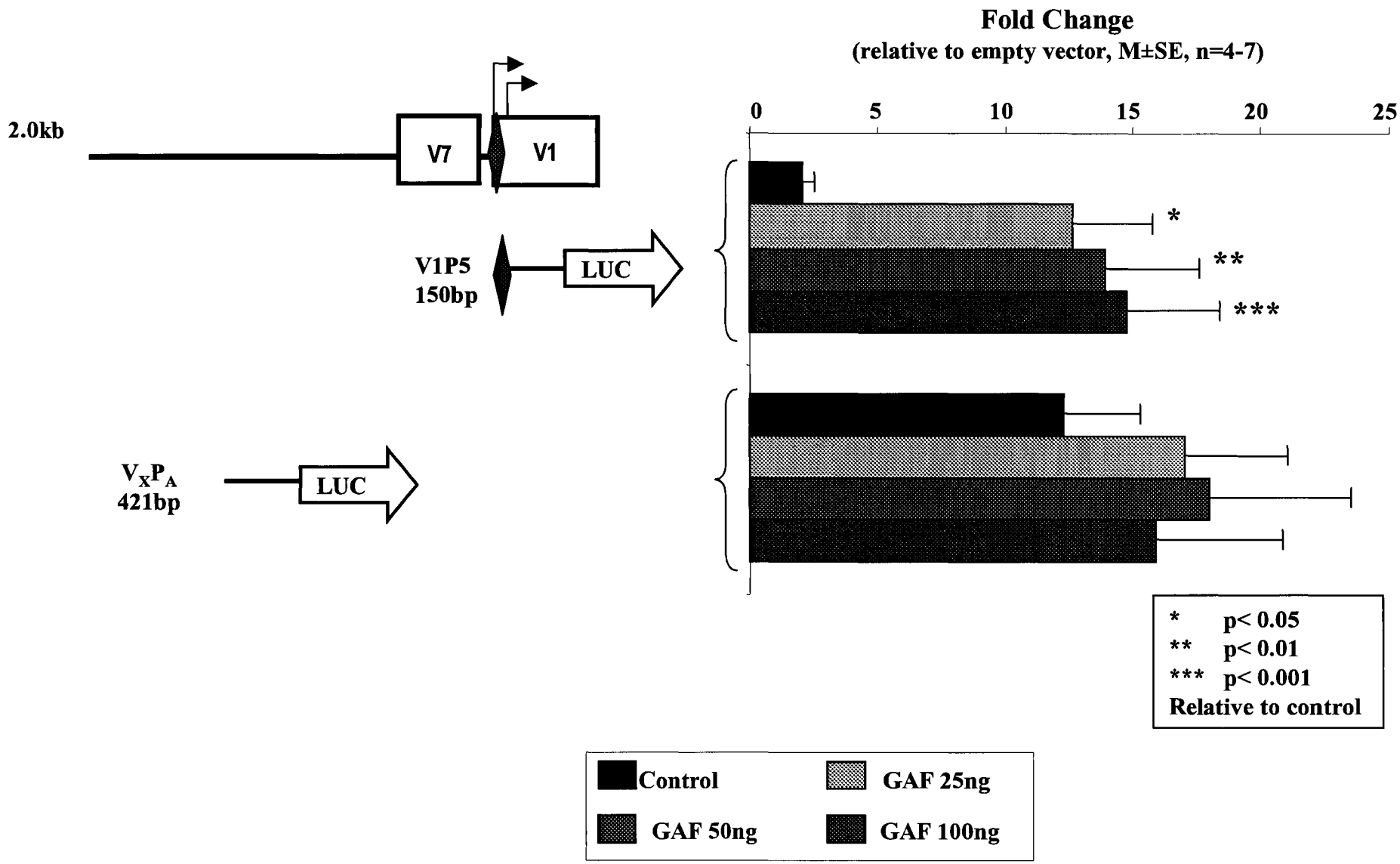

Fold Change
(relative to empty vector, M±SE, n=4-7)
0
5
10
15
20
25
2.0kb
V7
V1
V1P5
150bp
LUC
V_XP_A
421bp
LUC
*
**
***
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to control
Control
GAF 25ng
GAF 50ng
GAF 100ng

Figure 29: Western blot analyses of GAF overexpression in HEK293 cells. 8 μg of *Drosophila* GAF expression vector was transfected into HEK293 cells in a 100 mm tissue culture dish using the polyfect reagent. Cells were harvested 48 hr after transfection using the Pierce NE-PER nuclear extraction kit. 5 μg of nuclear extract from HEK293 cells and 20 μg of HEK293 whole cell extract were resolved on 12% SDS-PAGE and immunoblotted with an antibody directed against the whole *Drosophila* GAF protein. The immunoblot shows the overexpressed GAF protein at ~ 75 kDa. The faint band seen in HEK293 cells is non-specific as normal rabbit immune serum produces the same result.

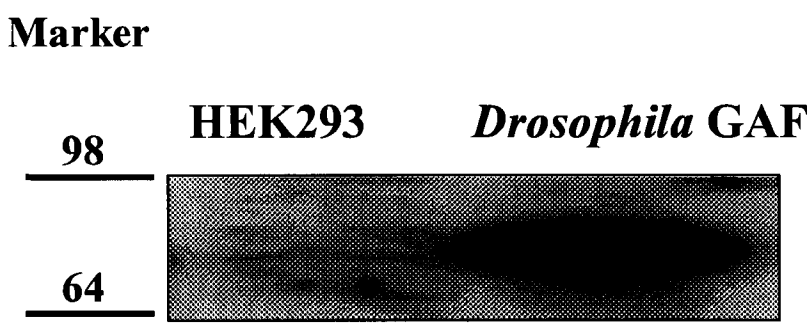
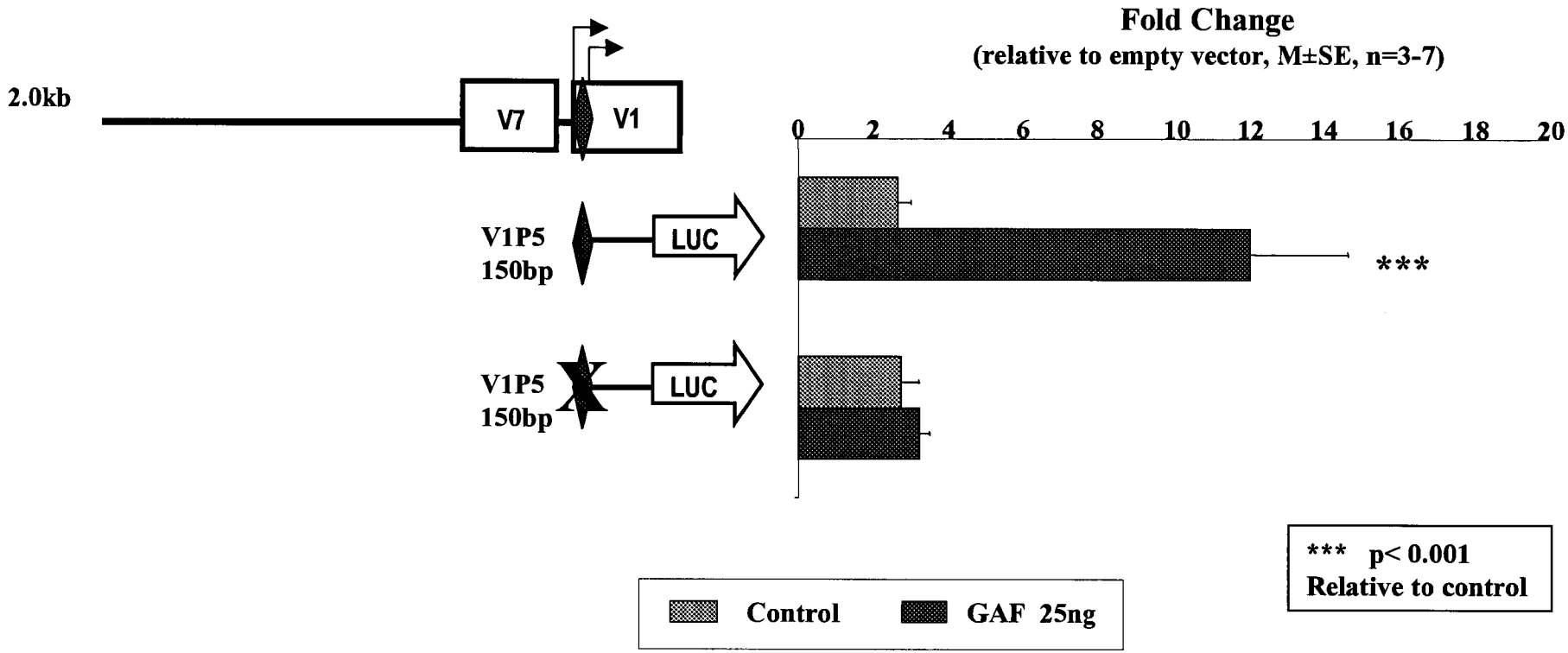

Marker
HEK293
Drosophila GAF
98
64

Figure 30: Mutational analyses of the GAGA element in the proximal promoter of V1.

Constructs with the GAGA element mutated (Table 5) were cotransfected with 25 ng of GAF; non-mutated constructs were transfected as controls. Data are presented as mean ± standard error, n=3-7 experiments. The significance of the differences observed was determined by ANOVA followed by Bonferroni's statistical test. *** $p < 0.001$.


Fold Change
(relative to empty vector, M±SE, n=3-7)
2.0kb
V7
V1
0
2
4
6
8
10
12
14
16
18
20
V1P5
150bp
LUC
***
V1P5
150bp
LUC
Control
GAF 25ng
*** p< 0.001
Relative to control

4.5.6. EMSA

A radioactively labelled probe encompassing the GAGA element bound endogenous nuclear proteins in HEK293 cells. A similar band was seen when the probe was incubated with nuclear extracts from HEK293 cells overexpressing *Drosophila* GAF 519, but the band was at least 50% less intense. The mutant probe did not show binding to nuclear proteins or GAF suggesting that the protein-DNA interaction is specific (Figure 31).

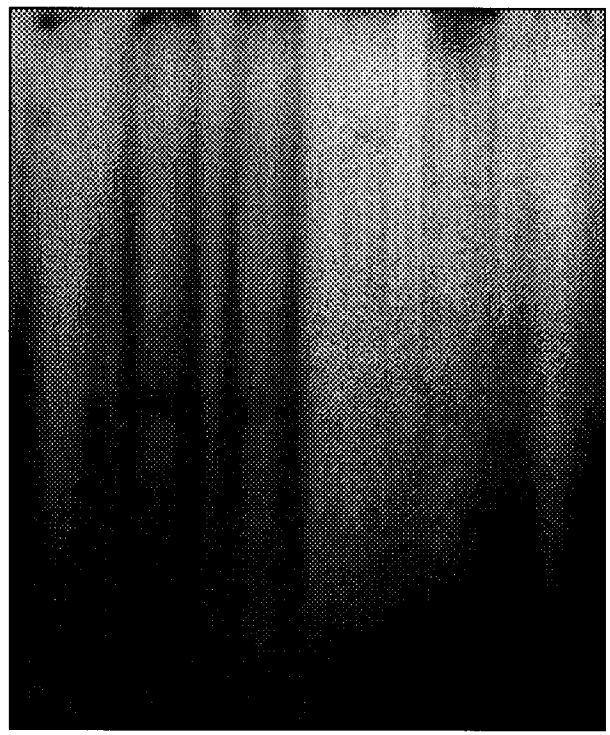
4.5.7. Interaction of Gfi-1/1b site and GAGA element in the 100 bp proximal promoter region of V1

Because of the overlap of the GAGA element with the Gfi-1/1b site in NRR1 (Figure 19), we studied the effect of increasing doses of the transcriptional repressors on GAF activation of V1P5, using Gfi-1 as both factors (Gfi-1 and Gfi-1b) have shown similar effects on the V1 promoter (Figure 23). Gfi-1 (10 ng) repressed GAF (25 ng) activation of V1P5 by ~75%. Increasing doses of Gfi-1 (25 and 50 ng) did not cause any further repression of the V1 construct although the level of significance increased (Figure 32). This suggests that, in a cell system where both factors are present, there will be competition of the two transcription factors for the same locus, and, thus, the levels of the two transcription factors will be critical in the regulation of V1.

Figure 31: Analyses of the binding of GAF and endogenous proteins to the GAGA element in the V1 promoter region by EMSA.

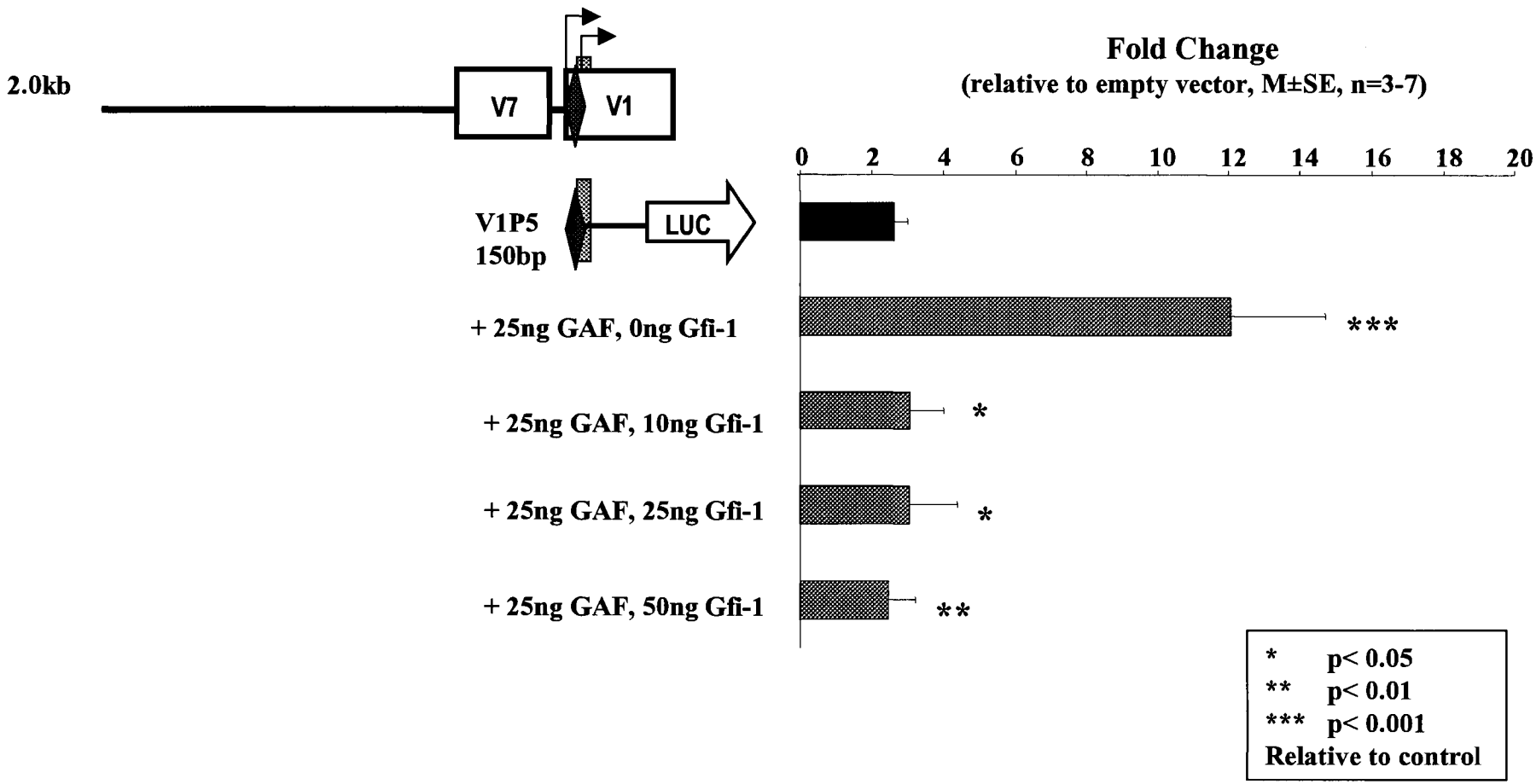
A double stranded oligonucleotide encompassing the GAGA site (Table 7) was radioactively labelled (α P32-CTP) and incubated with nuclear extracts from HEK293 cells and HEK293 cells overexpressing GAF. Mutant oligonucleotides (Table 7) were used to determine the specificity of binding. ⟹ S= shift.

S



GAGA element probe	+	+	+	-	-	-
GAGA element mutant probe	-	-	-	+	+	+
+ HEK293 cells (NE)	-	+	-	-	+	-
+ GAF (NE)	-	-	+	-	-	+

Figure 32: Interaction of GAF and Gfi-1 on the proximal promoter of V1. Constructs with the GAGA element were cotransfected with 25 ng GAF plus 0, 10, 25 and 50 ng of Gfi-1. Data are presented as mean ± standard error, n=3-7 experiments. The significance of the differences observed was determined by ANOVA followed by Bonferroni's statistical test. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.


2.0kb
V7
V1
V1P5
150bp
LUC
Fold Change
(relative to empty vector, M±SE, n=3-7)
0
2
4
6
8
10
12
14
16
18
20
+ 25ng GAF, 0ng Gfi-1
***
+ 25ng GAF, 10ng Gfi-1
*
+ 25ng GAF, 25ng Gfi-1
*
+ 25ng GAF, 50ng Gfi-1
**
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to control

4.5.8. Discussion of the GAGA element data

I have investigated the response of the GAGA element to GAF. The *Drosophila* GAF-519 protein strongly stimulates the activity of the proximal V1 promoter through the GAGA element and mutation of this element resulted in loss of GAF activation. Although the stimulatory mechanism is not known, the hGHR, rat V1bR, rat spi 2.1 and human AT_1 receptor genes all contain TATA boxes in very close proximity to the GAGA element and endogenous proteins binding to the GAGA element could possibly interact with transcriptional machinery and enhance transcription. However, at least in HEK293 cells, the proximal GAGA element is not required for basal transcriptional activity of V1 as is seen in the rat V1bR, rat spi 2.1 and human AT_1 receptor genes (311-313). These differences may be due to the fact that different test cell systems were used and the level of endogenous GBPs may differ significantly. It would be of interest to know whether the effect of GAF on V1 transcriptional activity is similar in other cell types (e.g. Huh7 cells) and whether there are coactivators and corepressors that can affect its activity depending on the cell type. In addition, the single GAGA element in the proximal promoter may not be sufficient for regulation of the basal activity of V1. There are two other GAGA elements within V1 that may be required as well: a 3X GA repeat ~ 1.5 kb upstream of the conserved TSS and/or another ~ 70 bp

downstream of the conserved TSS within the V1 exon. Finally, the proximal GAGA element could be important in growth factor-mediated stimulation of V1 by other than GH. This has been shown to be true for the human AT_1 receptor, rat spi 2.1 and rat V1b receptor genes (311-314). For example, EMSA studies of the human AT_1 receptor GAGA element showed increased binding to endogenous GBPs following treatment of the test cells with insulin, PDGF and EGF (313). Due to the time constraints of this PhD project, the effect of GH or any other growth factor on V1 transcriptional activity has not yet been investigated. Endogenous protein (s) in HEK293 cells formed a smiliar sized complex with the V1 GAGA element to that seen with nuclear extracts from HEK293 cells overexpressing the GAF protein, suggesting that there are endogenous GBPs interacting with the GAGA element. An antibody directed against the entire *Drosophila* GAF protein immunoreacted with endogenous proteins in HEK293, Huh7 and HepG2 cells, and shifted protein complexes formed between the GAGA element in the V1 promoter and HEK293 nuclear extracts. However, normal rabbit immune serum also caused a supershift in EMSA analyses. Thus, the question as to whether endogenous GAF-like proteins are expressed in the cell lines and whether the contribution of the GAGA element to the regulation of V1 is cell type specific remains unanswered.

In summary, I have shown that the *Drosophila* GAF-519 protein stimulates the activity of the V1 proximal promoter in HEK293 cells through the GAGA element. I have also demonstrated that Gfi-1 repressed the activity of GAF. The fact that the sites are adjacent and the transcription factors interact, suggests tight control of V1 transcriptional activity within the 50 bp proximal promoter of the conserved TATA/TSS complex.

4.6. Regulation of the longer 1.8 kb promoter of V1

4.6.1. Putative binding sites of liver-enriched transcription factors (LETFs) in the 1.8 kb V1 promoter region

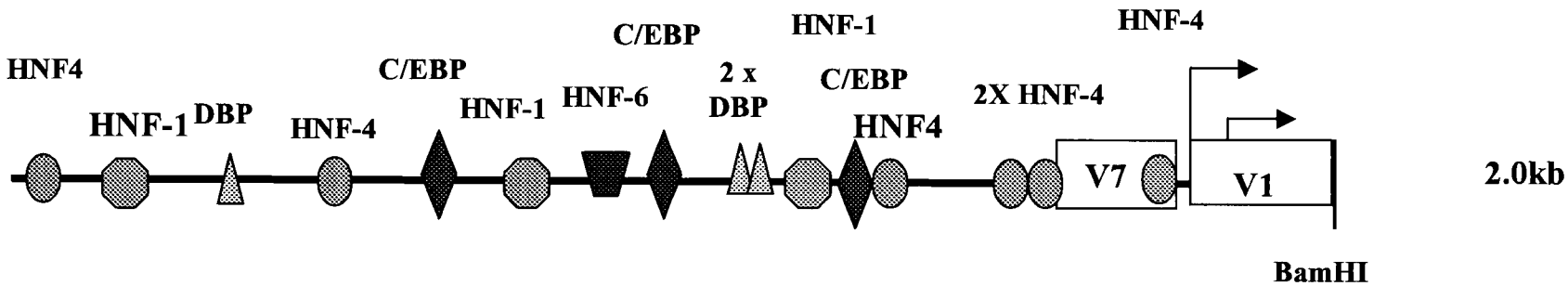
1.8 kb of the hGHR genomic sequence upstream of the first TSS for V1 was analysed by MatInspector and Signal Scan computer based transcription factor scanning programs. Putative binding sites for several LETFs were identified, including CCAAT enhancer binding protein (C/EBP), hepatocyte nuclear factor-1 (HNF-1), HNF-4, HNF-6 and D-binding protein (DBP) within the 1.8 kb promoter region of V1 (V1P1) (Figure 33).

4.6.2. Liver enriched transcription factor (LETFs)

Members of the LETF family that include C/EBP, HNF-1, HNF-3, HNF-4, HNF-6 and DBP cooperatively regulate expression of hepatic-specific genes.

Figure 33: Module B V1 Promoter: Putative binding sites for liver enriched transcription factor sites.

Several putative liver enriched transcription factor (LETF) binding sites were identified by MatInspector (www.genomatix.de) in the 1.8 kb promoter of V1. HNF: hepatocyte nuclear factor; DBP: D-binding protein; C/EBP: CCAAT enhancer binding protein.


HNF4
HNF-1
DBP
HNF-4
C/EBP
HNF-1
C/EBP
HNF-6
2 x
DBP
HNF-1
C/EBP
HNF4
HNF-4
2X HNF-4
V7
V1
2.0kb
BamHI

No single LETF family member is solely responsible for the transcription of a liver-specific gene as they form a complex network of transcriptional factors that define hepatic development as well as function (Figure 34) [reviewed in (323-325)].

In addition, it is well known that, in the rodent liver, GH regulates several members of the LETF family (Figure 35) and LETFs are involved in GH regulation of several liver genes [(326) and reviewed in (323;325;327)]. In addition, GH has been shown to regulate GHR mRNA levels in the liver but not in skeletal muscle of GH deficient mice (328). Thus, although my primary focus for the study of the hGHR V1 promoter by LETF family members has been HNF-4, it is important to understand the relationship between HNF-4, GH and other members of the LETF family.

C/EBP

C/EBP is part of a large family of basic leucine zipper (bZIP) transcription factors that include CCAAT response element binding protein (CREB) and AP-1 (c-fos and c-jun) [reviewed in (323)]. There are multiple isoforms of C/EBP (α, β, γ, δ, ε and ζ), that are involved in regulating metabolism, (acute phase) immune response, cellular differentiation, apoptosis, development, liver generation and liver tumour biology [reviewed in (323)]. C/EBPs contain an N-terminal activation domain, a bipartite dimerization domain and a leucine zipper DBD C-terminal domain (329).

Figure 34: Hierarchy of the expression of liver enriched transcription factors (LETFs) during rat liver development.

HNF-3α, HNF-3β, HNF-1α, HNF-1β, HNF-4 and HNF-6 are expressed in the developing liver whereas C/EBPα, C/EBPβ and DBP are expressed in the mature rat liver. Figure adapted from (325).

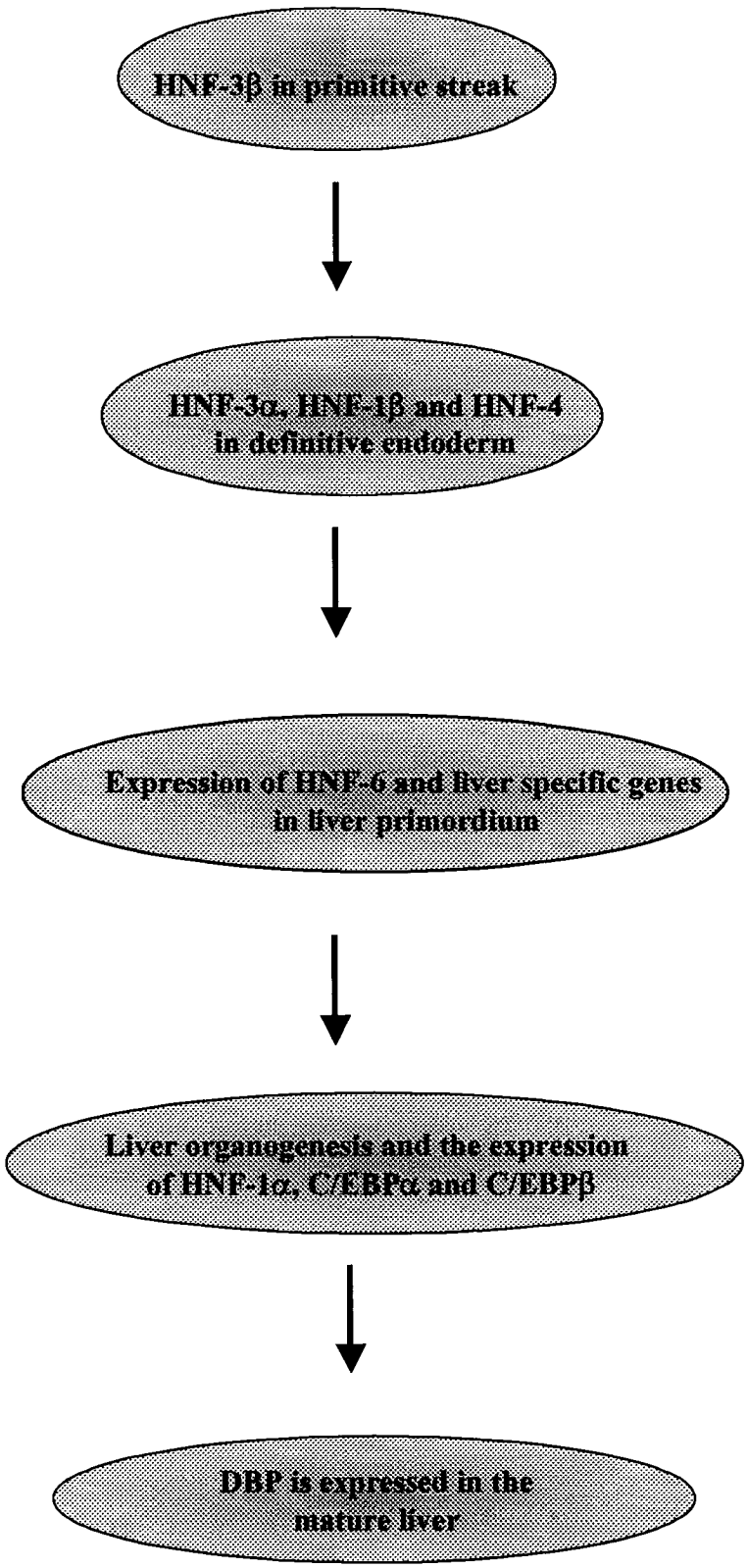
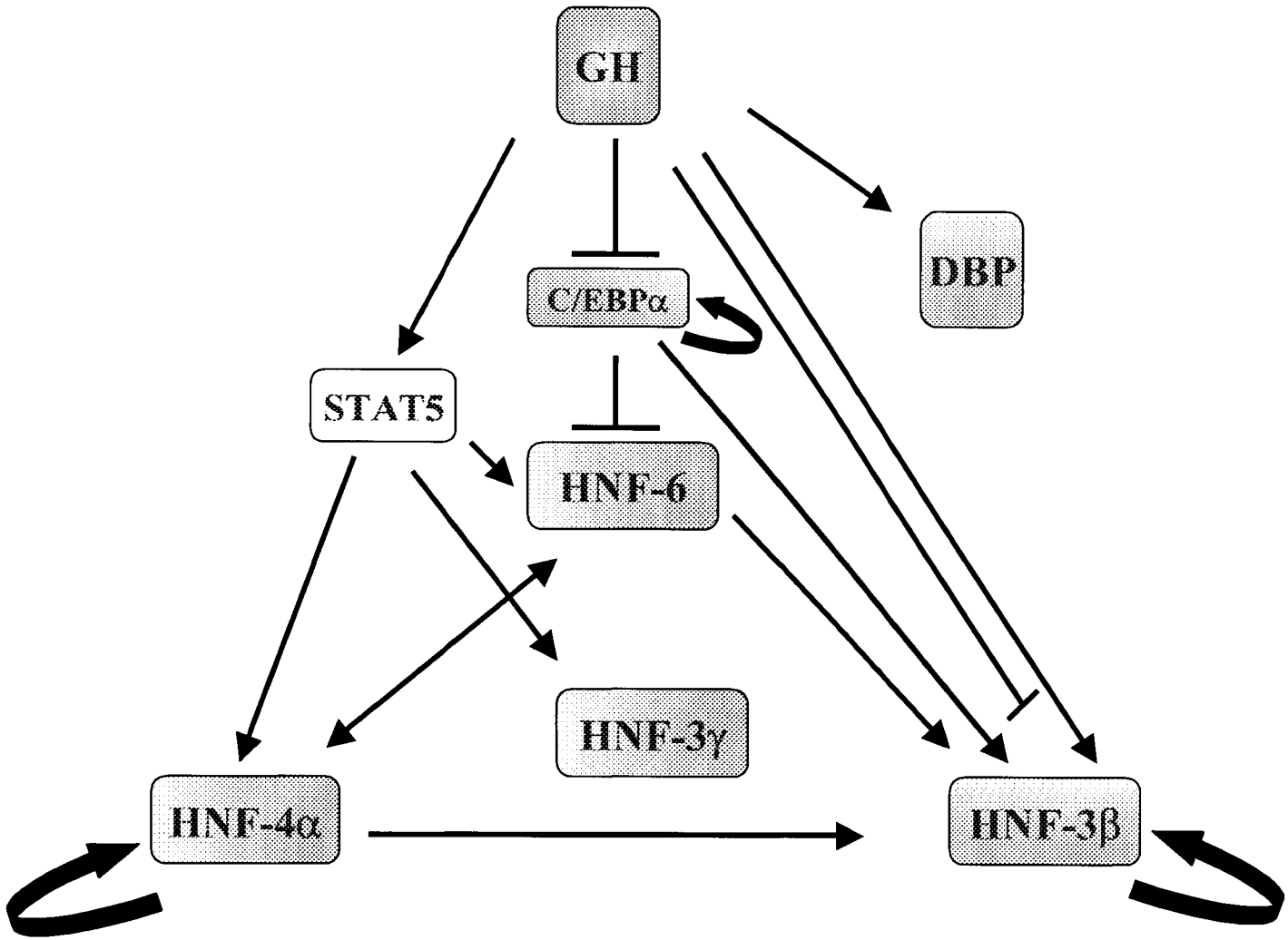

HNF-3β in primitive streak
HNF-3α, HNF-1β and HNF-4 in definitive endoderm
Expression of HNF-6 and liver specific genes in liver primordium
Liver organogenesis and the expression of HNF-1α, C/EBPα and C/EBPβ
DBP is expressed in the mature liver

Figure 35: GH regulation of LETFs.

GH stimulates the expression of DBP, HNF-6, HNF-3β, HNF-3γ and HNF-4, and suppresses C/EBPα expression in the rat and bovine liver. GH regulates HNF-6 and HNF-3γ by inducing STAT5 binding to their promoters. The mechanism by which the other LETFs are regulated by GH has not been reported (326). Figure adapted from (327).


GH
DBP
C/EBPα
STAT5
HNF-6
HNF-3γ
HNF-4α
HNF-3β

bZIP enhancing factor (BEF) interacts with C/EBPs to stabilise their binding to response elements and enhance their transactivating abilities (330). The highest levels of C/EBPα are in the placenta, and high levels are also found in the blood leukocytes, lung, liver, fat, adrenal gland, pancreas, small intestine, colon and skeletal muscle (331).

Hepatic expression of C/EBPα is restricted to terminally differentiated hepatocytes (332). Mice that are homozygous null mutants for C/EBPα die soon after birth due to failure to accumulate glycogen in hepatocytes and lipids in adipocytes, suggesting major defects in gluconeogenesis and adipogenesis (333). Adult mice that are liver specific null mutants for C/EBPα develop jaundice due to high levels of unconjugated bilirubin. In addition, the expression of several key enzymes involved in gluconeogenesis, such as PEPCK, were reduced (334). In a more recent study, mice with liver-specific deletion of C/EBPα have reduced serum levels of cholesterol, hyperammonaemia and impaired tolerance to glucose suggesting that C/EBPα regulates hepatic glucose, lipid and ammonia metabolism (335). Thus C/EBPα is required for the development of the liver, proper hepatic metabolic function and survival.

There are four forms of C/EBPβ due to the use of alternative translation initiation codons. Liver-enriched inhibitory protein (LIP), a 20 kDa isoform of C/EBPβ lacks the activation domain and acts to inhibit transcriptional activation of C/EBPs (336;337). Half of the C/EBPβ null homozygous mice

die before birth, and those that survive to birth die in the first few weeks of life (338). Thus, C/EBPβ is critical for development and survival of the foetus. C/EBPγ, like LIP, lacks the activation domain that is present in α and β isoforms (339). C/EBPδ is expressed during an acute phase response. C/EBPζ, also known as GADD (growth arrest and DNA damage), is ubiquitously expressed and is induced during growth arrest; it does not interact with DNA since it lacks the basic leucine zipper DBD (340).

Members of the C/EBP family heterodimerise with each other, with LIP, C/EBPγ and ζ acting as dominant negative factors. CCAAT displacement protein (CDP), an antagonist of C/EBP transactivation, is highly expressed in dedifferentiated cell types and down-regulated in terminally differentiated epithelial cells (341). It competes with C/EBPs for similar binding sites, and has been shown to negatively regulate cholesterol 7 α-hydroxylase (CYP7A1) gene in HepG2 cells by displacing C/EBPα and HNF-1α from their binding sites in the intron 1 of CYP7A1 (342). There are three putative C/EBP sites within the 1.8 kb V1 promoter region.

HNF-1

HNF-1 is a homeodomain TF and exists as two isoforms: HNF-1α and HNF-1β (also known as HNF-1v) (343;344). HNF-1 proteins contain an N-terminal dimerization domain that allows for homo- and heterodimer formation between the isoforms, a POU A domain, a homeodomain and a

C-terminal activation domain that is not conserved between the two isoforms (345). They can act as transactivators by interacting with coactivators that have intrinsic histone acetylase (HAT) activity (e.g. CBP, p300 and p/CAF), or directly as repressors of gene transcription by undefined mechanisms [reviewed in (323)]. Liver contains predominantly HNF-1α dimers; however, repression of hepatic specific genes in certain cell lines is correlated with liver expression of HNF-1β. Expression of HNF-1β is important in early development as it is expressed before HNF-1α (346;347). Mice knock outs for HNF-1β are embryonically lethal and thus studies have only been conducted on embryonic stem (ES) cell embryoid bodies (346). These ES embryoid bodies have decreased levels of HNF-4α1, HNF-1α and HNF-3γ suggesting that HNF-1β is required for their expression in the visceral endoderm (346). Mice lacking HNF-1α have multiple organ dysfunctions and die around time of weaning (348;349). Metabolic defects include non-insulin dependent diabetes (NIDDM), hyperlipidaemia, hepatomegaly and fatty livers (348). They also show growth retardation and, in certain strains of mice, Laron dwarfism (348). Humans with mutations in HNF-1α and β genes develop Maturity Onset Diabetes of the Young (MODY) –3 and –5, respectively, inherited diseases characterised by an early onset of Non-insulin dependent diabetes mellitus (NIDDM) due to defective pancreatic β-cell insulin production [reviewed in (350)].

HNF-4α has been shown to be essential for HNF-1α expression in embryonic liver and hepatic cell lines, and in pancreatic cells the hierarchy is reversed (351-353). Studies have shown that HNF-1α expression is not regulated by GH (326;327). Three putative HNF-1 binding sites have been identified within the 1.8 kb V1 promoter region.

HNF-6

HNF-6 belongs to the one-cut family of transcription factors; the other known member of this family is OC-2. Both contain a single cut domain and a homeodomain (354). The HNF-6 gene is made up of 3 exons, where exon 1 codes for the N-terminus and the one-cut domain, exon two for 26 amino acids found only in the β isoform and exon 3 for the homeodomain and the C-terminus (355). Alternative splicing of exon two produces two forms: HNF-6α and β (355). Both are expressed during development of the pancreas and liver, and in differentiating neuronal cells (356).

HNF-6 is regulated by GH through induced STAT5b binding to its response element (GLE) in the promoter region of the HNF-6 gene (94). GH also indirectly stimulates HNF-6 transcription by increasing HNF-4α transcription and binding, and by decreased transcription of its negative transcriptional regulator, C/EBPα, both of which have response elements in the promoter of the HNF-6 gene (94). Expression of HNF-6 is sexually dimorphic: normal female mice express higher levels of HNF-6 than their

male counterpart, whereas HNF-4α null male mice express HNF-6 at normal female liver levels suggesting that HNF-4α negatively regulates HNF-6 in male mice (96).

HNF-6 regulates genes encoding plasma proteins (e.g. protein C), enzymes involved in glucose metabolism (e.g. 6-phosphofructo-2-kinase) and CYPs (e.g. cyp2c12, as discussed in section 1.2.6) (95;354;357-359). There is one putative HNF-6 binding site in the 1.8 kb V1 promoter region.

DBP

DBP is a basic leucine zipper LETF and belongs to the proline and acidic-rich (PAR) family of proteins (360). DBP mRNA transcripts are detected in both foetal and postnatal rat liver and several non-hepatic tissues, but the protein is only detectable in the postnatal liver, thus showing developmental regulation at the translational level (360;361). DBP is involved in the regulation of several circadian-expressed genes in the brain and liver and is itself under circadian regulation by CLOCK, a major regulator of circadian oscillations (362-369). Although they are still rhythmic, dbp null mutant mice have disrupted circadian patterns in sleep and locomotor activity and expression of several liver genes (e.g. cyp2a4) is also disrupted (363;364;366). GH has been shown to upregulate DBP mRNA and diurnal expression of GHR mRNA has been shown in mice (326;328). Interestingly, this variation in GHR expression is only seen in

the liver of the mice and required GH (328). These data suggest that GH and DBP cooperatively regulate GHR expression in the murine liver. There are three putative DBP sites in the 1.8 kb V1 promoter region.

4.7. HNF-4

Hepatocyte nuclear factor 4 (HNF-4) family has three members: HNF-4α, HNF-4β, and HNF-4γ. Expression of HNF-4β has only been shown in the *Xenopus* (370). HNF-4α and HNF-4γ are 70% identical in their amino acid composition: the LBD and DBDs are identical while the A/B and the F domains differ slightly (371). They show similar distribution, being enriched in liver, and also expressed in kidney, pancreas, stomach and small intestine (270;372-374). However, HNF-4α mRNA is 10X more abundant in the liver than HNF-4γ (372).

We decided to study the effect of HNF-4α on the V1 promoter because of its relative abundance in the liver and it is the best studied member of the HNF-4 family.

4.7.1. Protein domains, structure and proposed ligands of HNF-4α

HNF-4α contains several domains: the A/B domain, a zinc finger DNA DBD (domain C), a hinge region (D), a large ligand binding (LBD) and dimerization domain (E), and a repressor domain (F) (Figure 36). The first 24 amino acids in the A/B domain code for activation function 1 (AF-

1) that has been shown to interact exclusively with coactivators (Table 11); some of these cofactors (e.g. CBP) have intrinsic HAT activity and have been implicated in chromatin remodelling. The DBD contains two zinc fingers, and this region is 100% conserved in vertebrates.

The LBD contains a large AF-2 domain, 11 α helices and 2 β sheets, common features in nuclear receptors (375;376) (Figure 37). X-ray crystallography experiments of the LBD show that two conformational states are present in each homodimer of HNF-4α: the open state where helix 12 is extended out and co-linear with helix 10, and the closed state in which helix 12 is folded against the LBD, forming a hydrophobic pocket with helices 3 and 4 and allowing the interaction of coactivators and corepressors via their LXXLL motifs (376-378). Although crystallography experiments were carried out in the absence of ligand, C14-C18 fatty acids were found to be constitutively bound to the LBD in both states and, thus, the bound fatty acids are viewed as a cofactor rather than a traditional ligand for HNF-4α (Figure 37) (377;379).

Nuclear receptors fall into two groups: members of the "classical" family are activated upon ligand binding, while members of the orphan receptor superfamily have no identified ligand (380). HNF-4α was considered an orphan nuclear receptor until recently when fatty acyl-CoA thioesters were identified as ligands for HNF-4α (381).

Figure 36: Protein domains of HNF-4α2.

HNF-4α2 is one of the nine protein isoforms of HNF-4α. HNF-4α is a member of the nuclear receptor superfamily. Members of this family have five distinct domains, A-F; A/B: activation domain, contains the AF-1 module; C: zinc finger DBD; D: hinge region; E: LBD, also contains AF-2 module; F: NRD, contains a repressor sequence. AF-1 and AF-2 modules have been shown to interact with coactivators. Numbers indicate amino acid residues.

AF: activation function, DBD: DNA binding domain, LBD: ligand binding domain, NRD: negative regulatory domain; rep: repressor sequence; aa: amino acid.

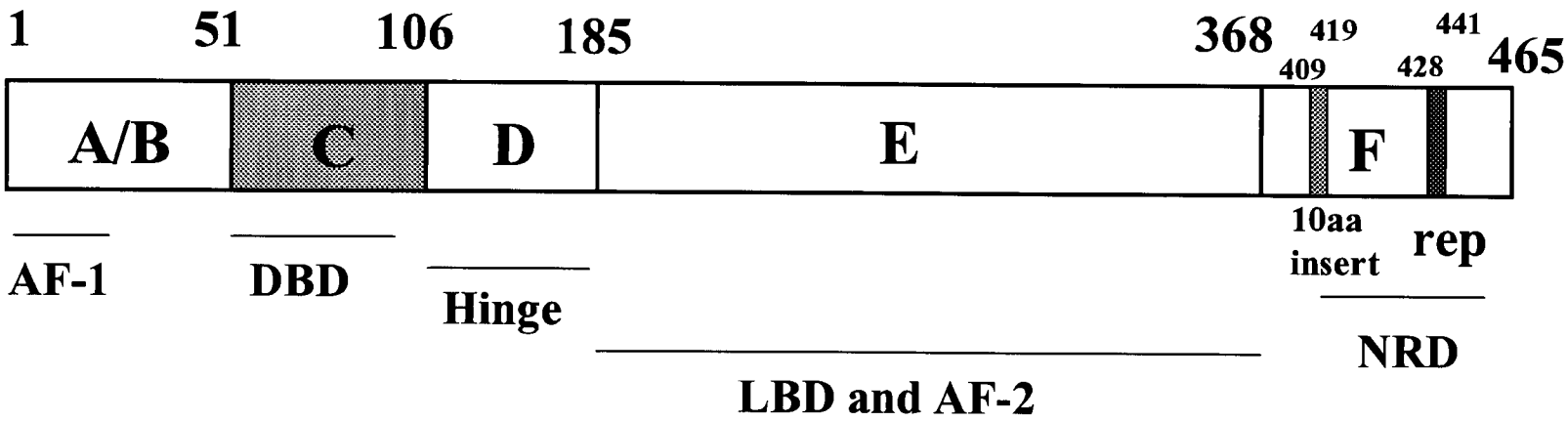

1
51
106
185
368
409
419
428
441
465
A/B
C
D
E
F
AF-1
DBD
Hinge
LBD and AF-2
10aa
insert
rep
NRD

Table 11: Coactivator and corepressor interactions of HNF-4α

Domain	Cofactor
AF-1: Coactivators	P160 (SRC-1, SRC-2, GRIP-1), CBP/p300, ADA2, PC4, TBP, TAFII31, TAFII80, TFIIB, TFIIH-p62
AF-2: Coactivators	SRC-1, SRC-2, SRC-3, CBP/p300,GRIP-1
AF-2: Corepressors	SMRT, SHP

Activation function (AF-1) domain has been shown to only interact with coactivators, whereas AF-2 interacts with both coactivators and corepressors. SRC-1: (steroid coactivator 1), GRIP-1 (glucocorticoid, receptor interacting protein-1), CBP (CREB-binding protein), ADA2 (alteration/deficiency in activation), PC4 (positive cofactor), TBP (TATA binding protein), TAF (TBP associated factor), TFIIB (transcription factor IIB), SMRT (silencing mediator of retinoid and thyroid receptors), SHP (small heterodimer protein) (323;371;383).

Figure 37: Structure of the HNF-4 ligand binding domain.

(A) A ribbon diagram of the homodimer reveals a "LBD" fold. Helices, shaded turquoise (1-9), yellow (10), or red (12), reveal two distinct conformations: open on the left and closed on the right. Strands are colored green, and the fatty acids (ffa) in the ligand binding pockets are colored magenta. (B) the ligand and elements of secondary structure are colored similarly in the schematic representations, with open and closed conformations in the same orientations for comparison. Figure and text of legend from (377).

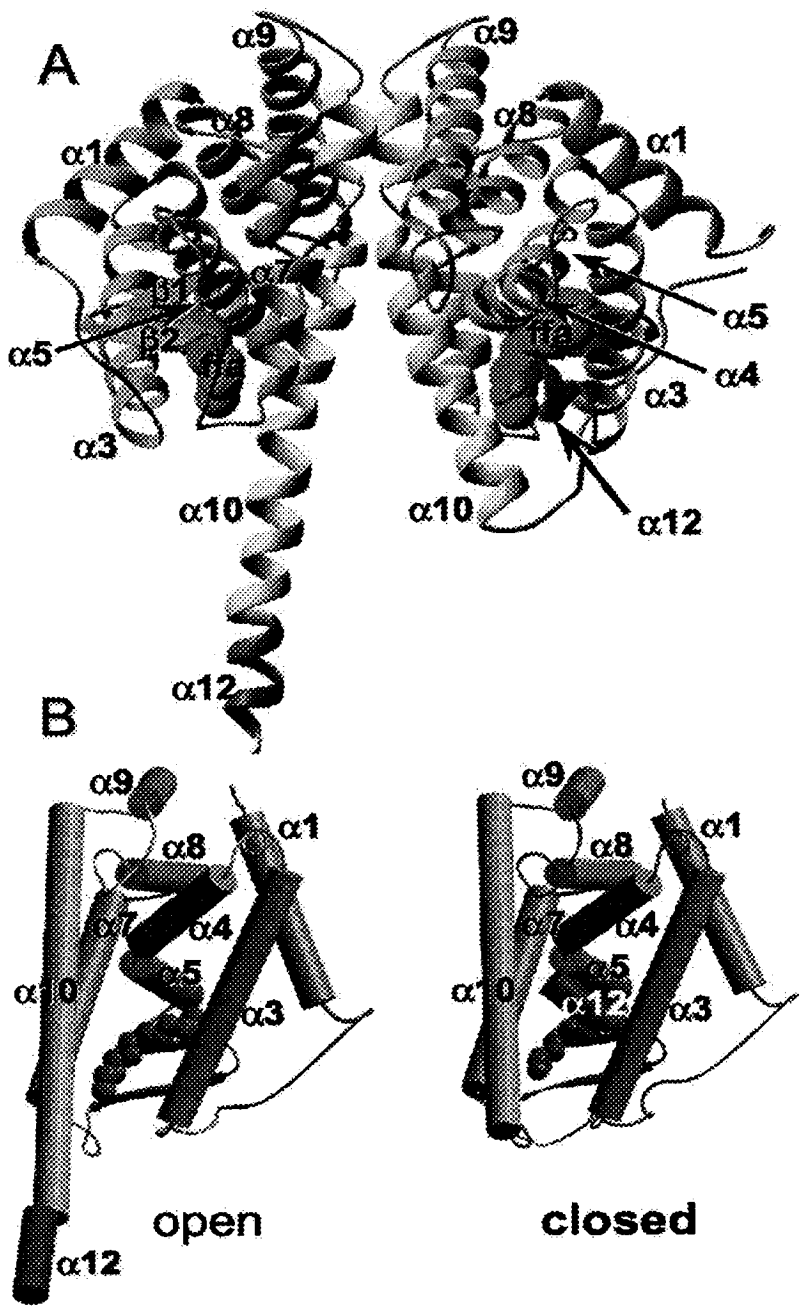
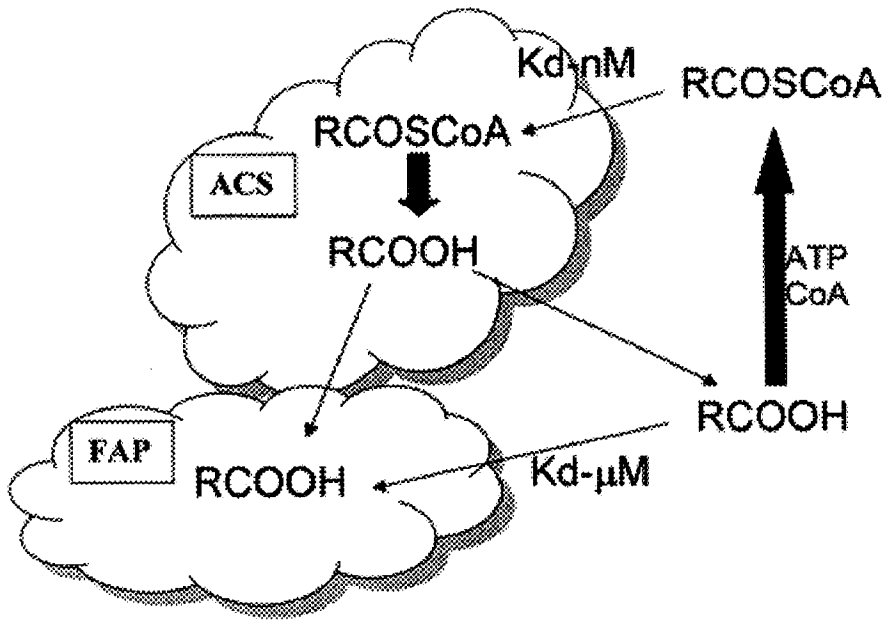

A
α9
α8
α1
α7
β1
β2
α5
α3
α10
α12
α4
B
open
closed

Figure 38: Ligand binding regions of HNF-4α

Two ligand binding regions have been identified within the LBD of HNF-4α1: the high affinity acyl-CoA binding pocket (ACS) stretches across the E and F domains, and the low affinity fatty acid binding pocket (FAP) is located in the E domain. It has been suggested that cross-talk between the ACS and FAP regions regulates HNF-4αactivity. Acyl-CoA esters bind to the ACS and are hydrolysed by the thioesterase. The hydrolysed product then interacts with the FAP. Alternatively, free fatty acids present in the medium could be exchanged with fatty acids already bound to the FAP. RCOOH: free fatty acid; RCOSCoA: Acyl CoA ester; CoA: coenzyme A. Figure from (382).


Kd-nM
RCOSCoA
RCOSCoA
ACS
RCOOH
ATP
CoA
RCOOH
FAP
RCOOH
Kd-μM

Two ligand binding pockets have been reported for HNF-4α: a low affinity fatty acid binding pocket (FAP) and high affinity acyl-CoA binding site (ACS) (Figure 38) (382). Crosstalk between the two binding pockets regulates the activity of HNF-4α.

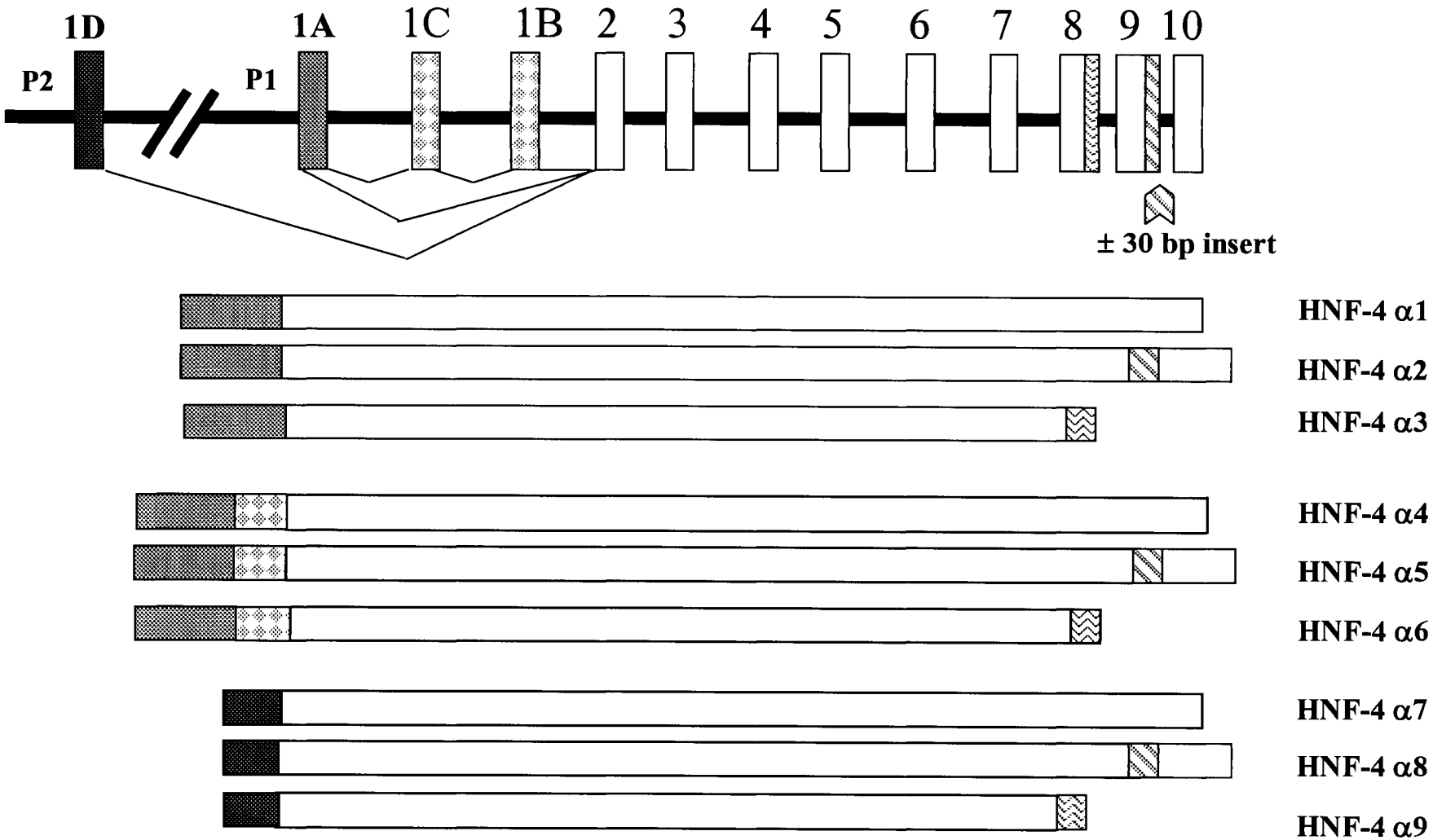
HNF-4α also functions as an acyl CoA thioesterase, which is linked to its ability to bind acyl CoA substrates at high affinity (382). Ligand binding generally acts as a switch for corepressor-coactivator exchange for nuclear receptors but this ligand-dependent exchange has not yet been described for HNF-4α (383).

4.7.2. Expression of HNF-4α and isoforms

HNF-4α is highly expressed in the liver and, to a lesser extent, in the kidney, GI tract, and pancreas (384). There are nine isoforms due to alternative promoter usage (P1 and P2), a possible 30 bp inclusion in the F domain, and a potential truncation of the carboxy terminus (Figure 39) (385). The P1 promoter regulates expression from exon 1A and produces isoforms α1-α6, whereas isoforms α7-α9 are transcribed from exon 1D under the control of the P2 promoter. Among the P1 isoforms, isoform α2 has a 30 bp insert in the C-terminus and is 10 amino acids longer than isoform α1. Isoform α3 is truncated at the C-terminal end. Isoforms α4-α6 are similar to isoforms α1-α3 except for the proposed two extra exons (1B and 1C) in the N-terminus (385).

Figure 39: HNF-4α gene and mRNA isoforms.

Expression of HNF-4α is under the control of two promoters (P1 and P2), giving rise to nine mRNA isoforms. See text for details. Figure adapted from (371).


1D
1A
1C
1B
2
3
4
5
6
7
8
9
10
P2
P1
± 30 bp insert
HNF-4 α1
HNF-4 α2
HNF-4 α3
HNF-4 α4
HNF-4 α5
HNF-4 α6
HNF-4 α7
HNF-4 α8
HNF-4 α9

P2 isoforms (α7-α9) are also similar to P1 isoforms α1-α3 except for the extreme N-terminus due to use of the P2 promoter and exon 1D.

P1 and P2 isoforms are differentially expressed: P1 isoforms are highly expressed in human, rat and mouse adult liver, and adult-like hepatoma cell lines (351;386-388) whereas P2 isoforms are predominant in murine foetal liver and foetal-like hepatoma cells, undifferentiated cell types and stem cell populations (386;389). In contrast to the above data, one group has reported the expression of P1 isoforms in the human pancreas and rodent pancreatic cell lines, and low levels of P2 isoforms have been reported in mouse adult liver by RT-PCR (386).

4.7.3. Actions of HNF-4α

HNF-4α is an important regulator of the expression of several liver-specific genes involved in regulating hepatic function, such as the metabolism of lipids, lipoproteins, carbohydrates, fatty acids, amino acids, blood coagulation, detoxification and haematopoiesis [reviewed in (371)]. It affects the transcription of target genes by binding as a homodimer to a DR-1 hexamer and recruits either coactivators to its AF-1 and AF-2 domains or corepressors to the AF-2 domain, thereby mediating activator or repressor functions (Table 11) (384) .

Tyrosine phosphorylation is required for its subnuclear localization and enhances DNA binding and subsequent activation potential (390). Serine/threonine phosphorylation also enhances DNA binding and

transactivation in bacterial and COS cells (391). High AMP:ATP ratios stimulate phosphorylation of serine 304 by the AMP-activated protein kinase, decreasing HNF-4α stability and dimer formation, and, thus reducing binding activity (392).

HNF-4α is constitutively active and forms dimers in the absence of added ligand; however, several physiological ligands have been shown to modulate its activity. HNF-4α binds acyl-CoA substrates with high affinity and converts them into fatty acids that directly interact with the FAP, thereby modifying its activity (Figure 38). Acyl CoA thioesters of long chain fatty acids (LCFA) increase oligomerization and decrease binding of HNF-4α to its cognate element, while those of C14-18 promote dimer formation and DNA binding, thus activating HNF-4α (381;382;393). Other antagonist ligands include lipid lowering polyunsaturated fats and hypolipidaemic drugs (393). Dietary status also affects the activity of HNF-4α, thereby modulating its effect on target genes. For example, polyunsaturated fatty acids (PUFA) inhibit the binding of HNF-4α to its response element on the promoter of the glucose-6-phosphatase (G-6-P) gene and, thus, repress its transcription (394). G-6-P is a gluconeogenic enzyme that acts to increase the amount of glucose available for release into the bloodstream. These studies suggest that hepatic metabolic homeostasis is, in part, maintained by modulating the activity of HNF-4α on target genes.

4.7.4. Role of HNF-4α during early development

In mice, HNF-4α is involved in formation of the primary layers of the embryo, organogenesis and the expression of early liver genes. HNF-4α is first detected in extra embryonic tissue and in the primitive endoderm of the E4.5 embryo (395). It is expressed in columnar visceral endoderm of the yolk sac at E5.5. Hepatic expression of HNF-4α first appears in the liver bud and hindgut at E8.5, when liver cells begin to proliferate and we first see the expression of liver-specific genes (e.g. AFP). The highest expression in liver is observed on day 18 and drops by day 20. There are low levels from birth throughout adult life due to the fact that P2 isoforms are no longer expressed and levels of P1 isoforms are decreased (383;386;395). HNF-4α is also expressed in the midgut (including the primordium of pancreas and gallbladder), the stomach, mesonephric tubules and the hindgut (intestine) ending at rectal-anus border (395). Homozygous mice knockouts are embryonically lethal and mice rescued with wild-type visceral endoderm develop a liver but fail to express several specific liver genes, including apolipoproteins (396;397). No homozygous mutants exist in the human population, presumably because the human knockout is also embryonically lethal.

4.7.5. Role of HNF-4 α in adult liver

Target hepatic genes of HNF-4α have been identified by the response elements on their promoters, their HNF-4α responses under *in vitro* test

conditions and their deregulation in HNF-4α defective models (Table 12) (371). Due to the lethality of the mice knockouts, conditional knockouts have been used to study the role of HNF-4α in adult mice. HNF-4α expression was barely detectable in the postnatal liver of these mice, they weighed less than their control littermates by 5 wk and had major defects in lipid metabolism and by 8 wk, 70% of the knockout mice were dead due to as yet undefined causes (398). The expression of other LETFs is also affected in HNF-4α null mice. HNF-3β which is normally expressed at higher levels in the liver of female mice is unaffected in the female knock outs of HNF-4α, but is expressed in the male knock out mice at normal female liver levels, suggesting that HNF-4α negatively regulates the expression of HNF-3β in male mice (96).

4.7.6. Transactivating properties

HNF-4α has been shown to act as a transactivator on the majority of its target genes (371). Isoforms with the 10 amino acid insert in the C-terminus (α2/α8) are stronger activators of transcription, reportedly because the insert exposes the AF2 domain to possible coactivator interactions (399). P2 isoforms are thought to be less potent transcriptional factors due to the fact that they lack the AF1 domain that interacts solely with coactivators (383;400;401).

4.7.7. Repressor properties

HNF-4α can also act as a transcriptional repressor on several hepatic genes including human IGF-II, HMG-CoA synthase, liver-specific arginase, acyl-oxidase and itself HNF-4α (402-407). However for the HNF-4α and liver-type arginase genes, the mode of repression by HNF-4α is not known as HNF-4α does not bind to these promoters (403-405).

HNF-4α has been shown to interact with SMRT and HDAC complexes that are involved in transcriptional repression (383;399).

4.7.8. HNF-4 binding sites in the 1.8 kb V1 promoter region

Studying the effect of HNF-4 on the hGHR V1 promoter was of particular interest since regulation of the liver-specific V1 is likely to involve a liver enriched transcription factor such as HNF-4α. In fact, six putative HNF-4 binding sites were identified by computer assisted TF scanning programs in the 1.8 kb upstream of V1 (Figure 40A) and GH as well as HNF-4α have been shown to be involved in the regulation of lipid, lipoprotein and bile acid metabolism [reviewed in (64;371)].

The HNF-4 site closest to the upstream V1 TSS is conserved across several species (ovine, bovine and murine) (Figures 20 and 40B,) (256;258;408-410).

Table 12: Hepatic target genes of HNF-4α.

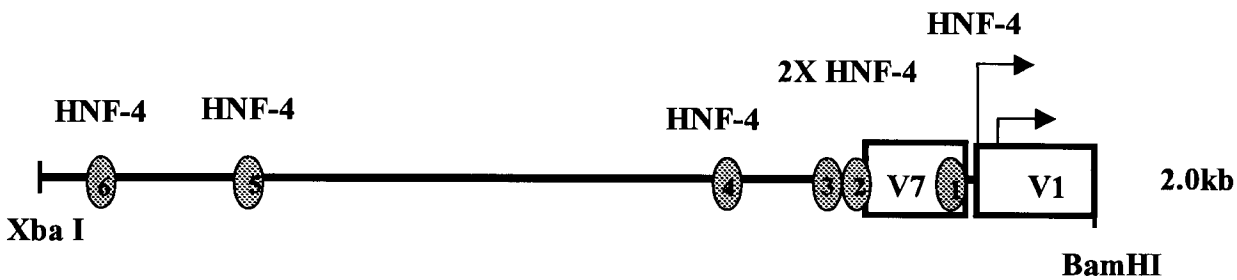
	Gene
Nutrient transport	
Lipid and retinol transport	Apolipoproteins (AI, AII, AIV, CIII) intestinal and hepatic fatty acid binding proteins
Other serum transport proteins	Transferrin, transthyretin
Nutrient metabolism	
Lipid and steroid metabolism	3-Hydroxy-3-methylglutaryl-CoA, P450 cytochromes (2A4, 2C1, 2D6, 7), cholesterol 7-α hydroxylase
Glucose metabolism	Aldolase B, phosphoenol pyruvate carboxy kinase, liver-type pyruvate kinase
Amino acid metabolism	Tyrosine aminotransferase, Ornithine carbamylase
Blood factors	
Coagulants	Factor VII, VIII, IX, S
Anticoagulants	Antithrombin III
Other	Erythropoietin
Transcription factors	HNF-1α, HNF-6, Fetoprotein transcription factor
Immune system	α1-Microglobulin and bikunin, Macrophage stimulating factor
Growth factors and receptors	Prolactin Receptor

Adapted from (323;371).

Figure 40: Putative HNF-4 binding sites identified by MatInspector and Signal Scan software programs.

Putative HNF-4 binding sites were identified by MatInspector and Signal Scan in (A) the 1.8 kb hGHR V1 promoter (AF322015) and in (B) ovine (o1A), bovine (b1A) (U15731) and mouse (mL1) (NT_039747) homologous regions (223;256;258;411).

A


HNF-4
2X HNF-4
HNF-4
HNF-4
HNF-4
6
5
4
3
2
V7
1
V1
2.0kb
Xba I
BamHI

B

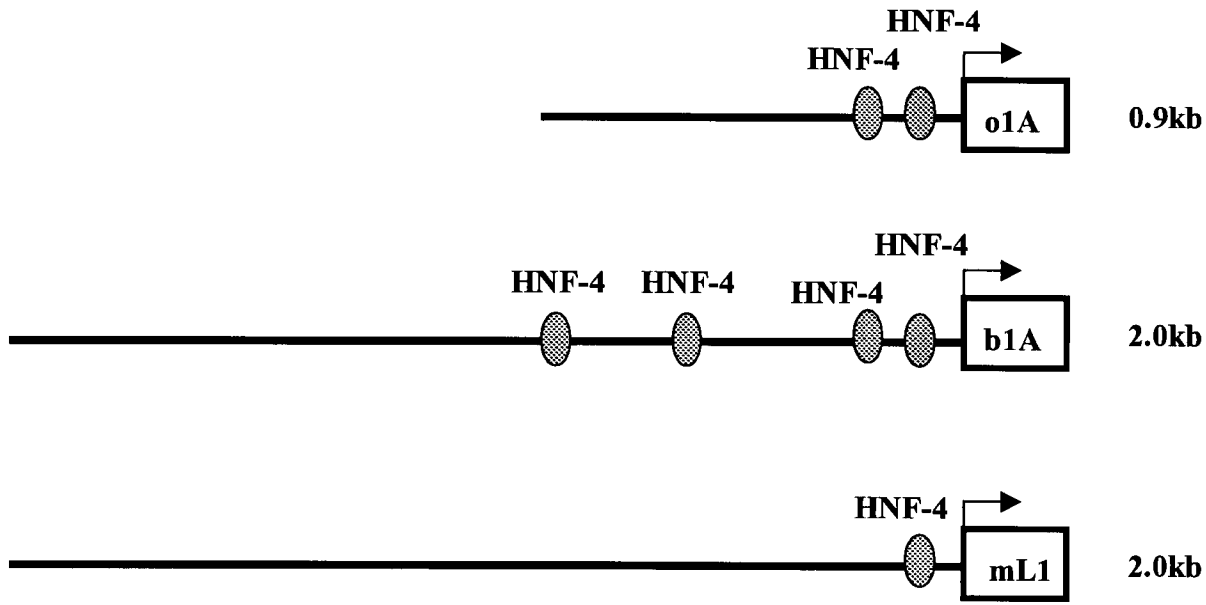

HNF-4
HNF-4
o1A
0.9kb
HNF-4
HNF-4
HNF-4
HNF-4
b1A
2.0kb
HNF-4
mL1
2.0kb

Table 13: Nucleotide sequences of the six putative HNF-4 sites in the 1.8 kb V1 promoter.

Comparison of the sequences of the putative HNF-4 sites in the V1 promoter with the published consensus sequence from Sladek *et al.* (371). The site 1 sequence is most like the consensus sequence (nucleotides not matching the consensus are in lower case type). (R): reverse strand.

Site	Sequence
Consensus (Sladek *et al*, 2001)	GGGTCA A AGGTCN A TCT G G TCT AG G
1	tGGGCA A AGGTCA
2 (R)	AGAaCA A AGGCaG
3	tGGGCA t ttGGgG
4 (R)	tGGGCA G AaGaaC
5	GGGGCA G AGGagG
6 (R)	AGGGCA A tGaGCA

Comparing the six putative V1 HNF-4 binding site sequences to the previously published sequences for HNF-4 revealed that the site closest to the TSS (#1) is most similar to what has been defined as the consensus element (Table 13) (371).

4.7.9. HNF-4α variant expression in human foetal and adult hepatocytes and cell lines (mRNA and protein)

In order to characterize the mRNA transcripts expressed in human foetal and adult hepatocytes, as well as in three human cell lines (HepG2, Huh7, and HEK293), primers were designed for specific regions of the HNF-4 gene and used in RT-PCR assays (Table 3). Transcripts arising from the P1 promoter were found in all cell types and in both foetal hepatocytes and adult liver (Table 14). Cells from the hepatic lineage also expressed transcripts for HNF-4α with or without the extra 30 bp in the C-terminus, the C-terminal truncated isoforms and isoforms transcribed from the P2 promoter. In contrast, HEK293 cells appear to be producing only the HNF-4α1 mRNA transcript.

An HNF-4α antibody that can identify isoforms 1,2,4,5,7 and 8 detected HNF-4α2 in human foetal hepatocytes, adult liver and the two hepatoma cell lines while HEK293 cells had undetectable levels of HNF-4α protein (Figures 41 A&B).

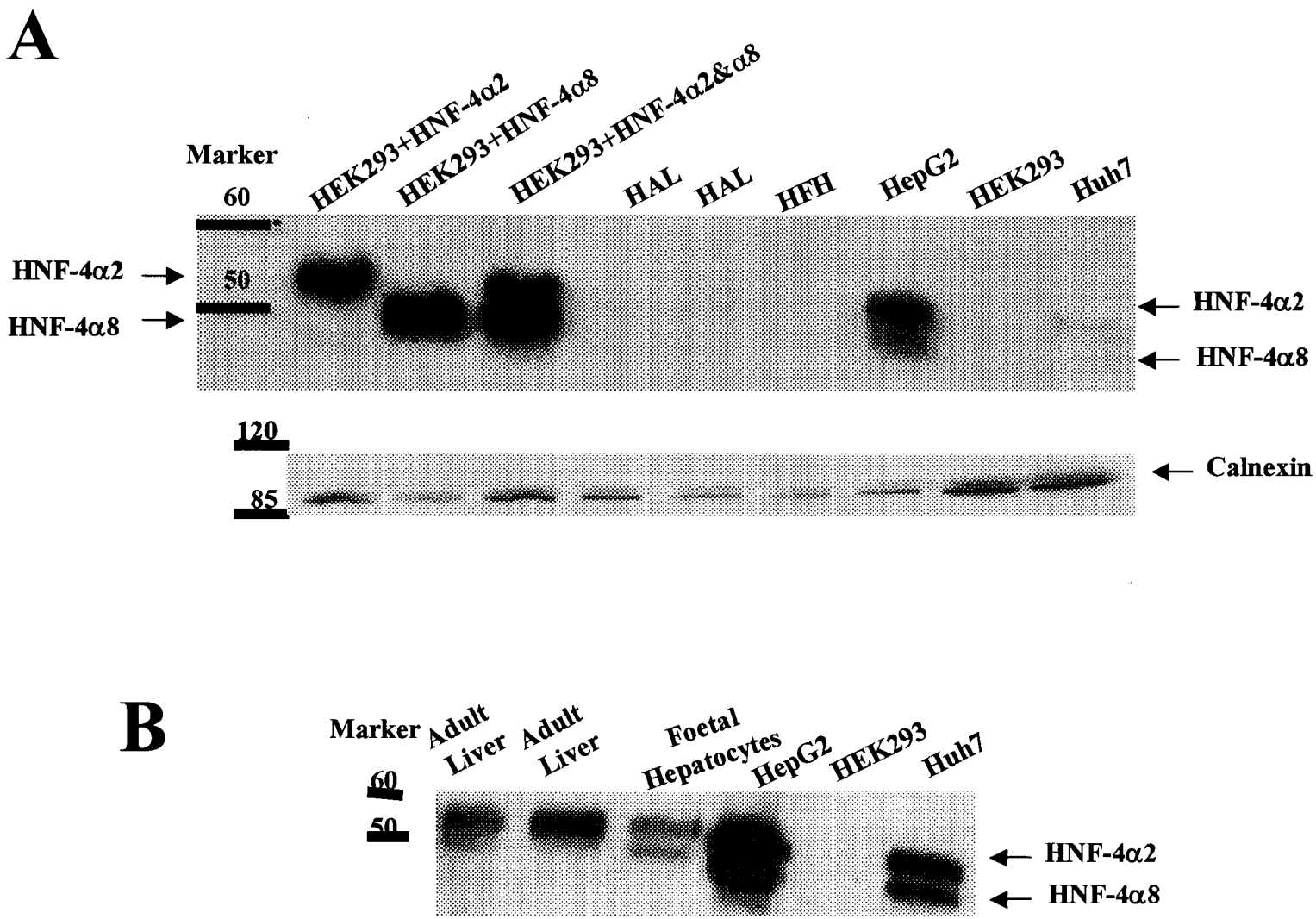
Table 14: Expression of HNF-4α mRNA isoforms in human liver and cell lines.

Primers were designed to specific sites in the hHNF-4α gene, in order to characterise the different mRNA variants present in three human liver tissues and cell lines (Table 3). The results from RT-PCR analyses are tabulated. * (HepG2, Huh7), ** (HEK293), # (Isoforms 4,5,6 were never detected), (+: present; - not seen).

Variant #	Foetal Hepatocytes (n=2-9)	Human Adult Liver (n=2-5)	Hepatoma Cell Lines* (n=2-4)	Kidney Cell Line ** (n=3-5)
P1 promoter: exon 1A Isoforms 1,2,3	+	+	+	+
P2 promoter: exon 1D Isoforms 7,8,9	+	+	+	–
W/out C-terminus 30 bp insert Isoforms 1,7	+	+	+	+
C-terminus +30 bp insert Isoforms 2,8	+	+	+	–
C terminus truncation Isoforms 3,9	+	+	+	–

Figure 41: Detection of HNF-4α protein in human liver and cell lines by Western blot.

(A) Western blot: 10 μg of nuclear lysates were resolved on 12% SDS-PAGE and immunoblotted with an HNF-4α antibody that recognises HNF-4α1,2,4,5,7,8. Calnexin was used as a loading control. (B) Extended exposure of Huh7, human adult liver (HAL) and human foetal hepatocytes (HFH) lanes to show HNF-4α2 as the major isoform expressed in HAL while HFH and Huh7 express both HNF-4α2 and α8 isoforms in equal amounts. HNF-4α was not detected (ND) in HEK293 cells. (C) Cumulative Western blot data expressed as mean or mean ± standard deviation, n=2-11. The significance of the observed differences (compared to adult liver) was determined by Bonferroni's statistical test following ANOVA analyses. * $p < 0.05$, ** $p < 0.01$.


A
Marker
HEK293+HNF-4α2
HEK293+HNF-4α8
HEK293+HNF-4α2&α8
HAL
HAL
HFH
HepG2
HEK293
Huh7
60
50
HNF-4α2
HNF-4α8
HNF-4α2
HNF-4α8
120
85
Calnexin
B
Marker
Adult Liver
Adult Liver
Foetal Hepatocytes
HepG2
HEK293
Huh7
60
50
HNF-4α2
HNF-4α8

C

HNF-4α (α2 or α2+α8) protein
levels relative to calnexin
2.0
1.6
1.2
0.8
0.4
0.0
Adult liver
(α2)
Foetal
Hepatocytes
(α2+ α8)
HepG2
(α2+ α8)
Huh7
(α2+ α8)
HEK293
(α2)
*
* *
* *
ND

Interestingly, foetal hepatocytes and hepatoma cell lines also expressed HNF-4α8 immunoreactive protein in approximately equivalent amounts to HNF-4α2. Quantitation of the relative amounts of total HNF-4 present in the samples reveals hepatoma cell types and foetal hepatocytes as having significantly higher amounts than adult liver and HEK293 cells (Figure 41 C; * $p< 0.05$, ** $p\leq 0.01$). Other groups have reported the expression of HNF-4α in hepatoma cell lines (387;388).

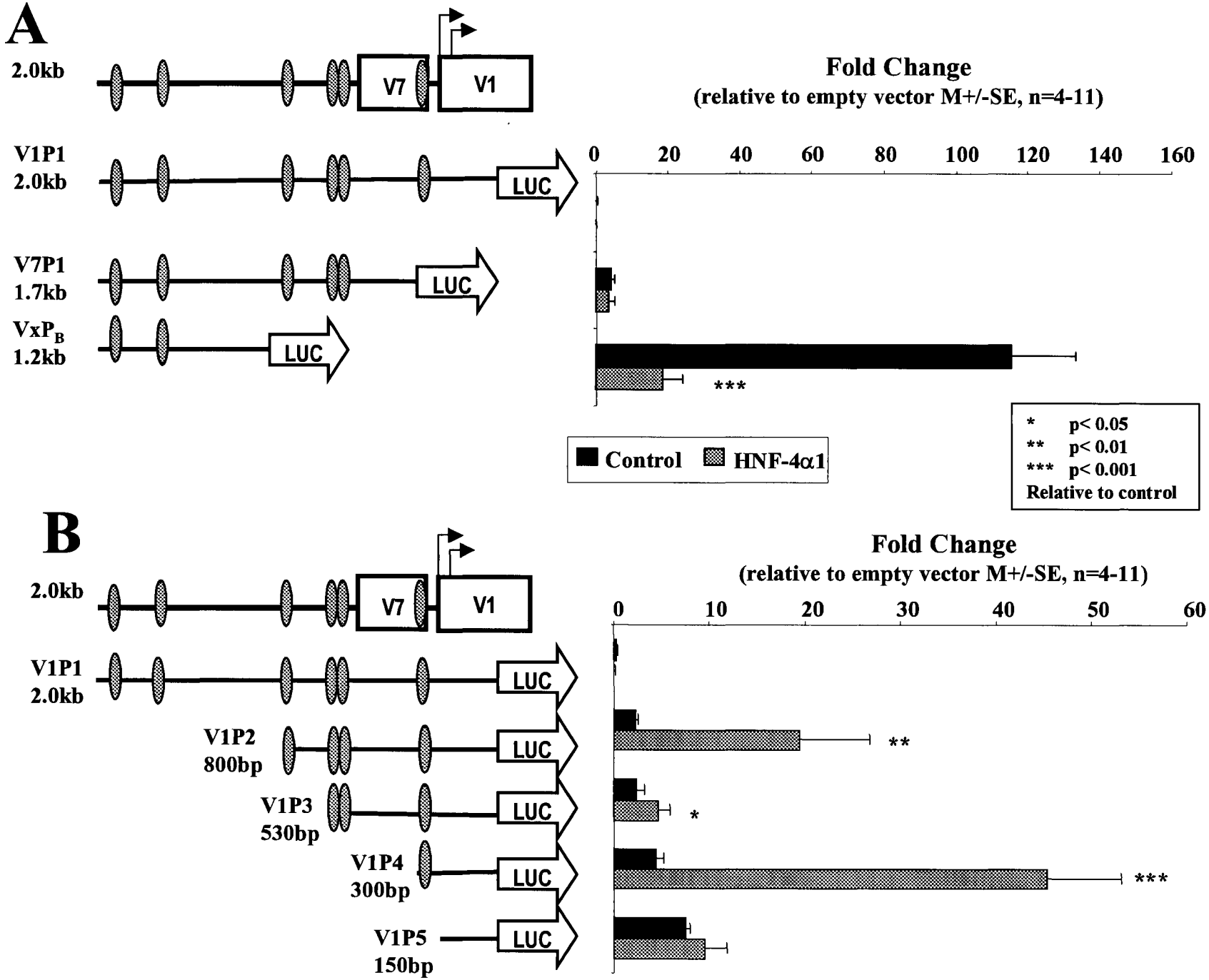
Even though immunoreactive HNF-4α protein is expressed in human and rat kidney tubules (387;388), and we have found HNF-4α mRNA in HEK293 cells, there was no detectable HNF-4α protein in HEK293 cells by western blot. Isoforms from the P1 promoter have been shown to be transcriptionally more active than those from the P2 promoter (400). Thus, the different developmentally regulated isoforms are good candidates for a role in regulating the expression of V1-derived GHR mRNAs.

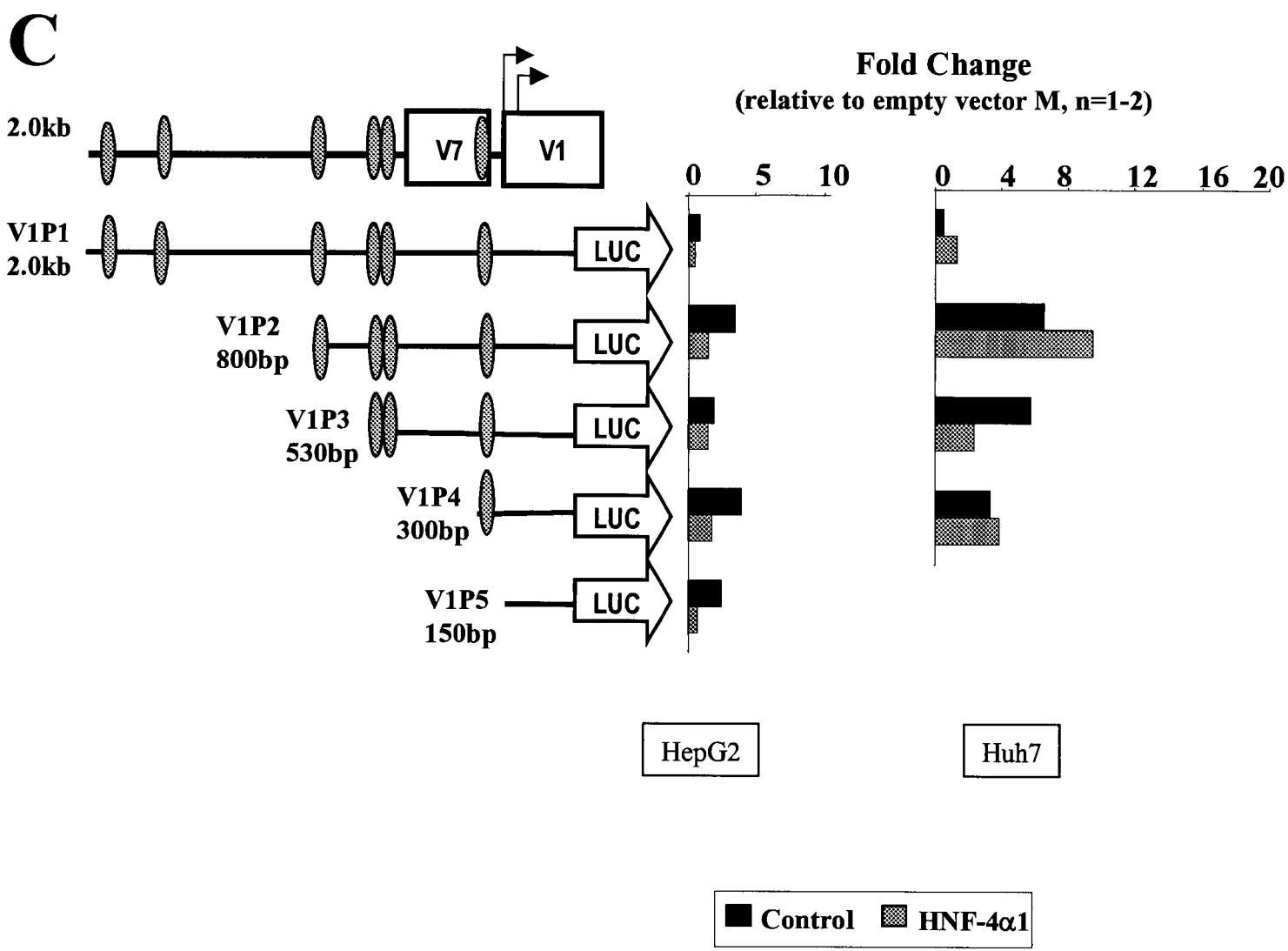
4.7.10. Transient transfection assay studies on the effect of HNF-4α1, α2 and α8 on the V1 promoter

Based on the review of the literature, we hypothesised that HNF-4α would have a stimulatory effect on the V1 promoter and that there might be a differential effect of the P1 versus P2 isoforms.

Figure 42: Effect of HNF-4α1 on the transcriptional activity of V1 promoter constructs.

HNF-4α1 (100 ng) expression vector was cotransfected with (A) 3′ and (B) 5′ deleted V1 constructs in HEK293 cells, (C) HepG2 and Huh7 cells using $CaPO_4$. Cells were harvested 48 hr later and assayed as before. Data are presented as mean ± standard error, n=4-11 experiments except for the HepG2 and Huh7 studies that were an n=1-2. The significance of the differences observed was determined by ANOVA, followed by Bonferroni's statistical test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. = HNF-4 response element.


A
2.0kb
V7
V1
V1P1
2.0kb
LUC
V7P1
1.7kb
LUC
VxP$_B$
1.2kb
LUC
Fold Change
(relative to empty vector M+/-SE, n=4-11)
0 20 40 60 80 100 120 140 160
***
Control
HNF-4α1
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to control
B
2.0kb
V7
V1
V1P1
2.0kb
LUC
V1P2
800bp
LUC
V1P3
530bp
LUC
V1P4
300bp
LUC
V1P5
150bp
LUC
Fold Change
(relative to empty vector M+/-SE, n=4-11)
0 10 20 30 40 50 60
**
*
***


C
Fold Change
(relative to empty vector M, n=1-2)
2.0kb
V7
V1
V1P1
2.0kb
V1P2
800bp
V1P3
530bp
V1P4
300bp
V1P5
150bp
LUC
0 5 10
0 4 8 12 16 20
HepG2
Huh7
Control
HNF-4α1

Initially, cotransfection studies were carried out with an HNF-4α1 expression vector, as this is the HNF-4 α isoform that has been used in the majority of studies on the transcriptional activity of HNF-4α (Figure 42). However, because we could not detect its protein expression in our human hepatic tissues, we subsequently changed to HNF-4α2 and α8, which we did find to be expressed at significant levels in the human hepatic tissues and cell lines (Figure 41). Transfections were carried out in HEK293 cells that did not express detectable endogenous HNF-4α protein.

V1P1, the longest and most repressed construct, with all six putative HNF-4 sites, did not respond to HNF-4α1, α2 or α8 (Figures 42A, 43A). The V7V1P1 construct, containing all six sites but having lost NRR1, also did not respond to HNF-4α2 or α8 (HNF-4α1 was not tested), suggesting that the NRR1 is not responsible for the lack of V1P1 response to HNF-4α (Figure 43A). There was also no response to the factors when the most 3' HNF-4 site was deleted (V7P1) (Figures 42A, 43A). Interestingly, the activity of the promoter region containing only the most 5' HNF-4 sites (#5 and #6) (V_XP_B) was markedly repressed by HNF-4α1 ($p< 0.001$), HNF-4α2 ($p< 0.001$) and, to a lesser extent, by HNF-4α8 ($p<0.01$) (Figures 42A, 43A). Cotransfection of equal amounts of HNF-4α2 and α8 were equally as effective in repressing V_XP_B ($p< 0.01$) (Figure 43A).

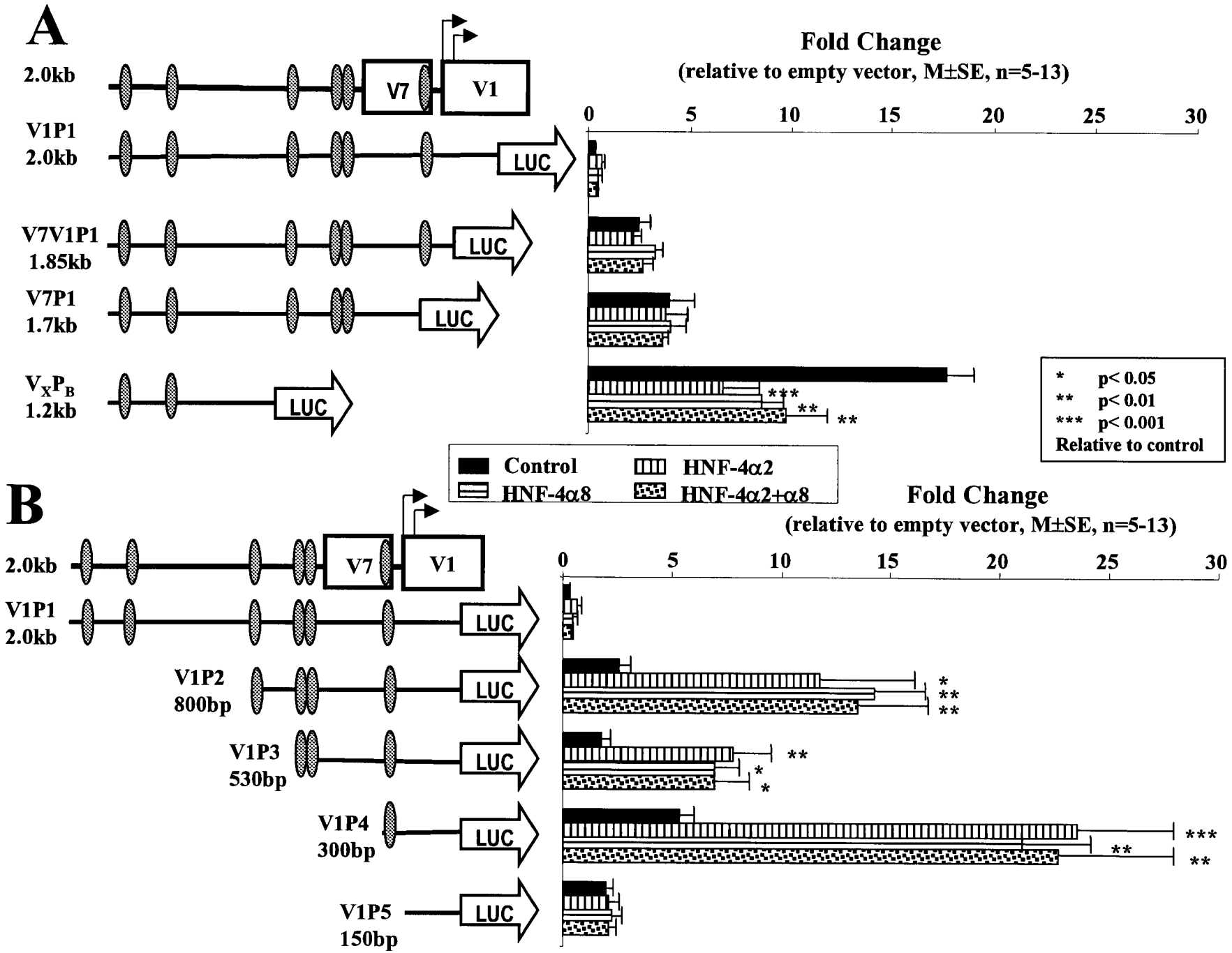
Loss of the 5′ 1.2 kb (sites #5 and #6 [V1P2]) led to a significant stimulation of transcriptional activity by all three isoforms (HNF-4α1, α2 [$p < 0.05$] and α8 [$p < 0.01$]) (Figures 42B, 43B). Deleting the #4 putative HNF-4 site (V1P3) caused a decrease in the stimulatory effect of the HNF-4 factors, while deleting sites #2 and #3 (V1P4), leaving only the most consensus HNF-4 binding site, led to a highly significant responsiveness to all three isoforms of HNF-4α1 and α2 ($p < 0.001$) and α8 ($p < 0.01$) (Figures 42B, 43B).

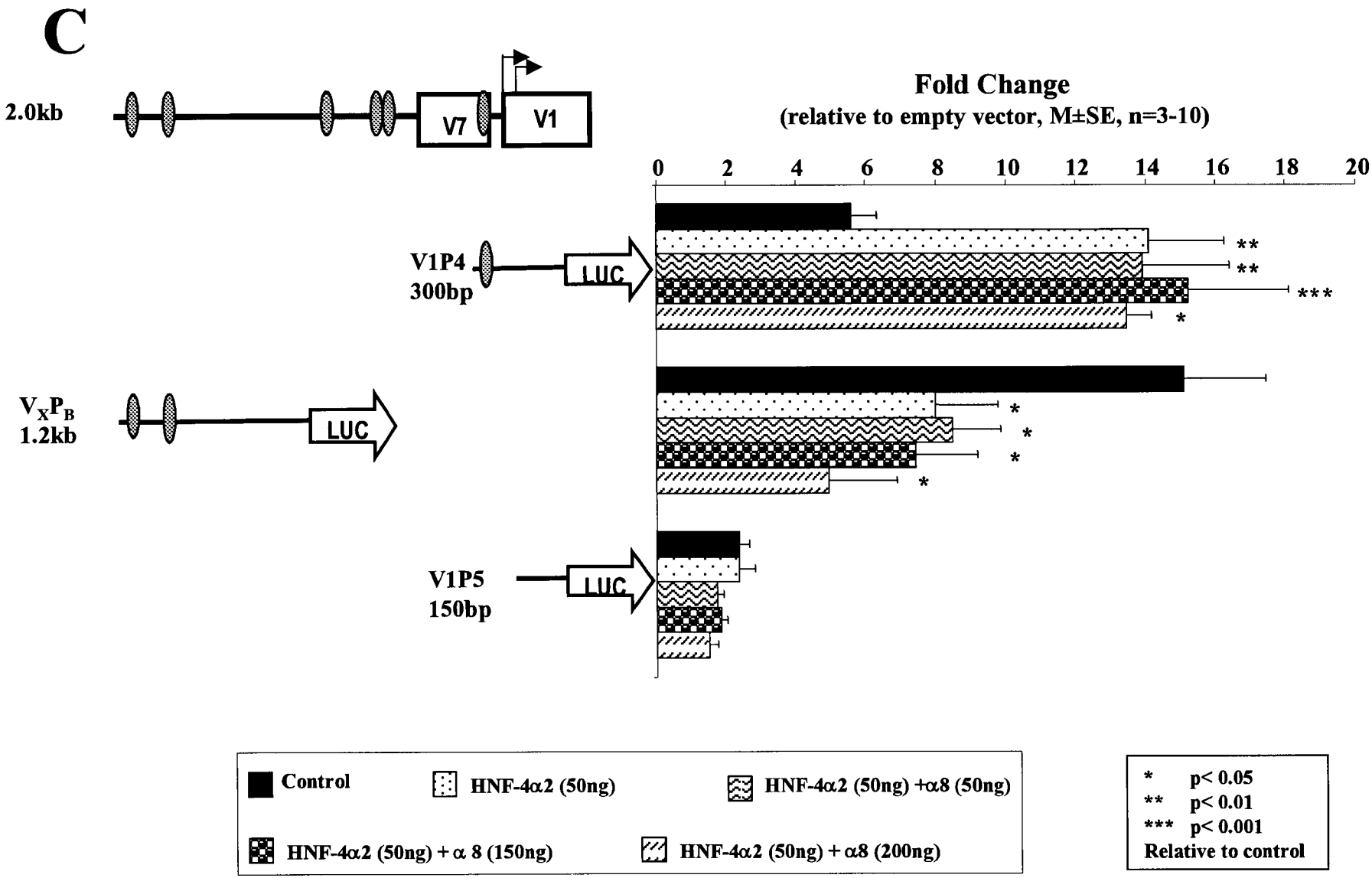
As expected, the V1P5 construct with no HNF-4 sites had no response to any of the HNF-4α isoforms. The effect of HNF-4α1 on the V1 promoter constructs was also tested in HepG2 and Huh7 cells but no response was observed likely due to the high levels of endogenous HNF-4α in these cells (Figure 42C).

P2 isoforms of HNF-4α contain one AF domain in the C-terminus, while the P1 isoforms have AF domains in both their N- and C-termini. On some gene promoters, the effects of P1 isoforms (e.g. α1) are greater than P2 isoforms (e.g. α7), probably due to the fact that the latter lack the N-terminal activation domain that has been shown to interact with coactivators (371;412). Since foetal hepatocytes and hepatoma cell lines express HNF-4α8 in addition to HNF-4α2 but not the V1 transcript, we hypothesised that the presence of HNF-4α8 would suppress the stimulatory effect of HNF-4α2 on V1 transcription.

Figure 43: Effect of HNF-4α2 and HNF-4α8 on the transcriptional activity of V1 promoter constructs.

HNF-4α2 (100 ng), HNF-4α8 (100 ng) and HNF-4α2+α8 (50 ng+50 ng) expression vectors were cotransfected with (A) 3′ and (B) 5′ deleted V1 constructs in HEK293 cells using polyfect. (C) 50 ng of HNF-4α2 and varying amounts of HNF-4α8 (50-200 ng) were cotransfected with V1P4, V_XP_B and V1P5 constructs into HEK293 cells. Cells were harvested 48 hr later and assayed as before. Data are presented as mean ± standard error, n=3-13 experiments. The significance of the differences observed was determined by ANOVA, followed by Bonferroni's statistical test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. = HNF-4 response element.


A
2.0kb
V7
V1
V1P1
2.0kb
LUC
V7V1P1
1.85kb
V7P1
1.7kb
V_XP_B
1.2kb
Fold Change
(relative to empty vector, M±SE, n=5-13)
0
5
10
15
20
25
30
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to control
Control
HNF-4α2
HNF-4α8
HNF-4α2+α8
B
2.0kb
V7
V1
V1P1
2.0kb
LUC
V1P2
800bp
V1P3
530bp
V1P4
300bp
V1P5
150bp
Fold Change
(relative to empty vector, M±SE, n=5-13)
0
5
10
15
20
25
30


C
2.0kb
V7
V1
Fold Change
(relative to empty vector, M±SE, n=3-10)
0
2
4
6
8
10
12
14
16
18
20
V1P4
300bp
LUC
**
**
***
*
VXPB
1.2kb
LUC
*
*
*
*
V1P5
150bp
LUC
Control
HNF-4α2 (50ng)
HNF-4α2 (50ng) +α8 (50ng)
HNF-4α2 (50ng) + α 8 (150ng)
HNF-4α2 (50ng) + α8 (200ng)
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to control

To test this, increasing amounts of HNF-4α8 were cotransfected with a constant amount of HNF-4α2, using our most consensus HNF-4 binding site (V1P4) and the most 5′ sites (V_XP_B) as promoter vectors (Figure 43C). Increasing doses of HNF-4α8 had no significant effect on the response to HNF-4α2 with either of the promoter vectors. Thus, HNF-4α8 does not affect the activating or inhibitory potential of HNF-4α2, under our test conditions, contrary to what was hypothesised.

4.7.11. Site directed mutagenesis of HNF-4 sites #1 and #6

To confirm that the proximal #1 and distal #6 putative HNF-4 sites are truly HNF-4α response elements, the sites were mutated (Table 6) and their responses to HNF-4α2 and α8 were tested in transient transfection assays. Mutagenesis of site #5 was not carried out because parallel EMSA and ChIP results indicated that this site was not functional (see below). Neither of the V1P4 HNF-4 mutant constructs (single and double) had a basal activity significantly different from the wild type V1P4 (4.7±0.8, n=10) (Figure 44). However, both mutant constructs showed no response to HNF-4α2 and α8. Significant repression of the 1.2 kb V_XP_B construct by HNF-4α2 and α8 was lost when the #6 HNF-4 site was mutated although, again, basal activities of both wild type and mutant constructs were similar (Figure 44).

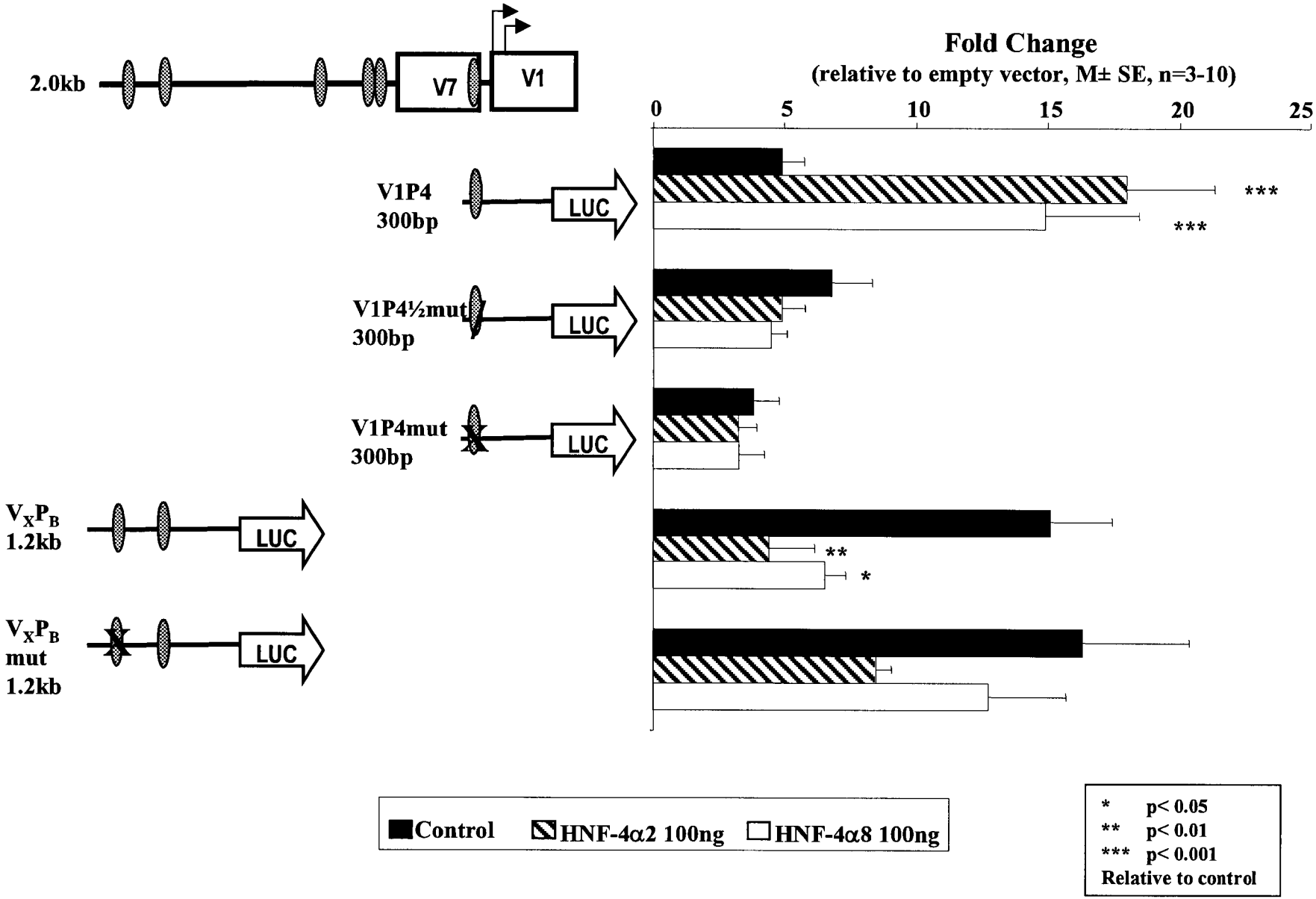

2.0kb
V7
V1
Fold Change
(relative to empty vector, M± SE, n=3-10)
0
5
10
15
20
25
V1P4
300bp
LUC
***
***
V1P4½mut
300bp
LUC
V1P4mut
300bp
LUC
VXPB
1.2kb
LUC
**
*
VXPB
mut
1.2kb
LUC
Control
HNF-4α2 100ng
HNF-4α8 100ng
* p< 0.05
** p< 0.01
*** p< 0.001
Relative to control

Figure 44: Mutational analyses of HNF-4 sites #6 (most 5′) and #1 (most 3′) in the proximal promoter of V1.

Constructs with either the (A) #6 or (B) #1 HNF-4 binding site mutated were cotransfected with HNF-α2 or α8; non-mutated constructs were transfected as controls. Data are presented as mean ± standard error, n=3-10 experiments. The significance of the differences observed was determined by ANOVA followed by Bonferroni's statistical test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

 = HNF-4 response element,  ½ mutated HNF-4 response element,  mutated HNF-4 response element (Table 6).

These data strongly suggest that the #1 site is responsible for the stimulatory effects of HNF-α2 and α8 while the #6 site is responsible for the inhibitory response to HNF-α2 and α8.

4.7.12. EMSA and EMSSA analyses of HNF-4 sites #1, #5 and #6

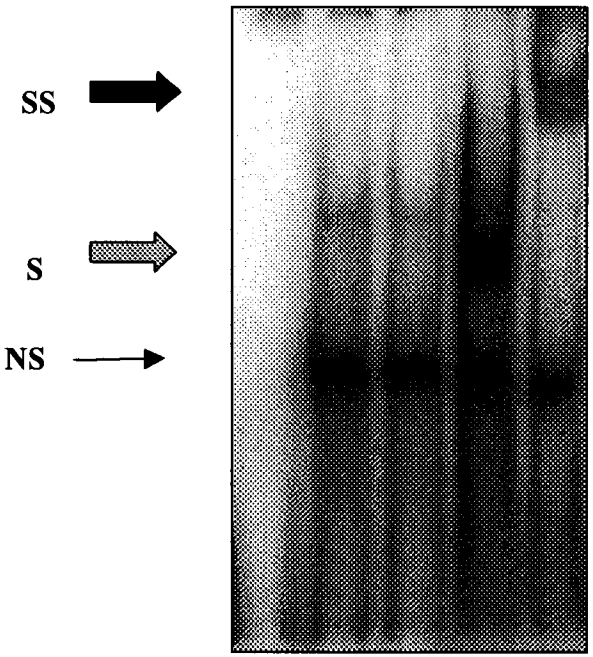
EMSA assays revealed that HNF-4α2 and α8 bind to the #1 site (Figure 45A). Whereas bands that were formed with HEK293 nuclear extract proteins did not change with an HNF-4α antibody that recognises isoforms α1, α2, α4, α5, α7 and α8, and, thus were non-specific, the bands formed with proteins from HEK293 cells overexpressing HNF-4α2 and α8 completely supershifted (Figure 45A). The partial and double mutants of this site showed only non-specific binding that was not supershifted by the HNF-4α antibody (Figure 45B).

The #5 HNF-4 site did not bind HNF-4α2 or α8 as detected by gel supershift analysis (Figure 45C), while the #6 site specifically bound HNF-4α2 and α8 and this binding was lost with the mutant probe (Figure 45D). Identical results were obtained when EMSA and EMSSA analyses were carried out with HEK293 nuclear extracts overexpressing either HNF-4α2 or α8 (data not shown).

Figure 45: EMSA and EMSSA analyses of HNF-4α proteins binding to putative HNF-4 sites #1, #5 and #6 in the V1 promoter.

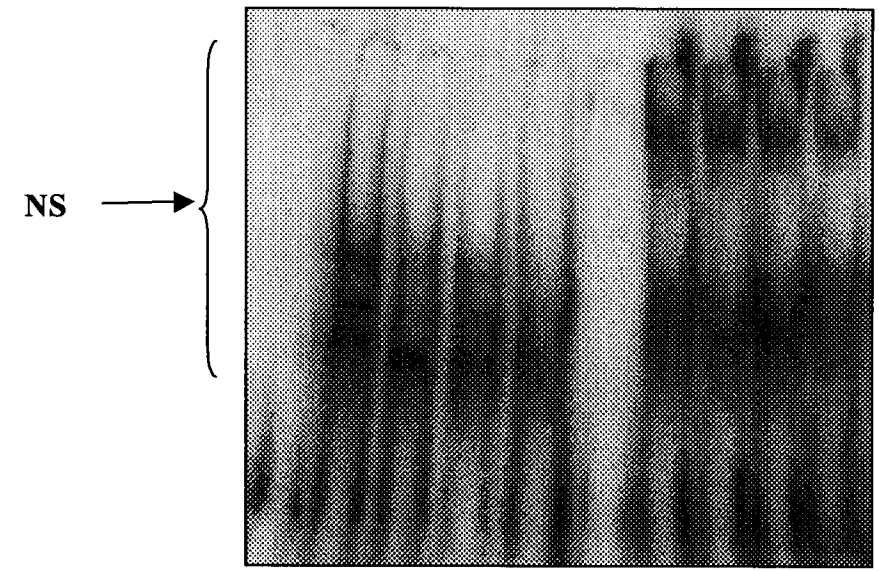
Double stranded oligonucleotides representing the (A, B) #1, (C) #5 and (D) #6 HNF-4 sites were radioactively labelled (αP32-ATP) and incubated with various nuclear extracts. Mutant oligonucleotides (Table 8) and a specific HNF-4α antibody that recognises HNF-4α2+8 were used to determine the specificity of binding. Nuclear extracts were from HEK293 cells overexpressing HNF-4α2 + α8. Arrows indicate → NS = non-specific, ⇒ S = shift, ➡ SS = supershift bands.

A



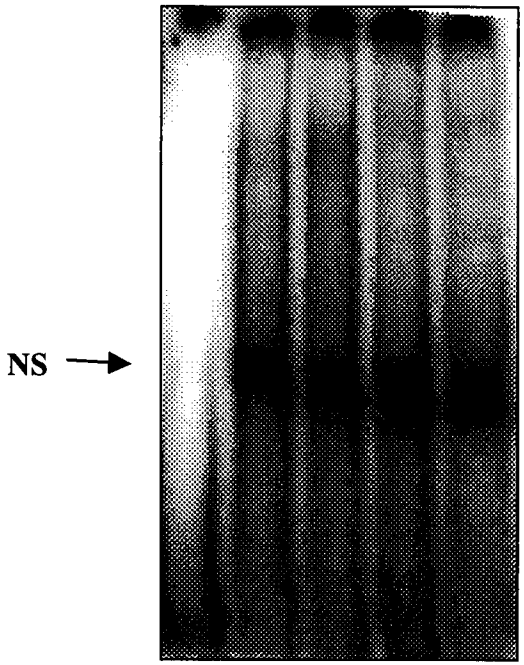
HNF-4 Site 1 probe	+	+	+	+	+
+ HEK293 cells (NE)	-	+	+	-	-
+ HNF-4α2+α8 (NE)	-	-	-	+	+
+ Anti HNF-4α	-	-	+	-	+

B



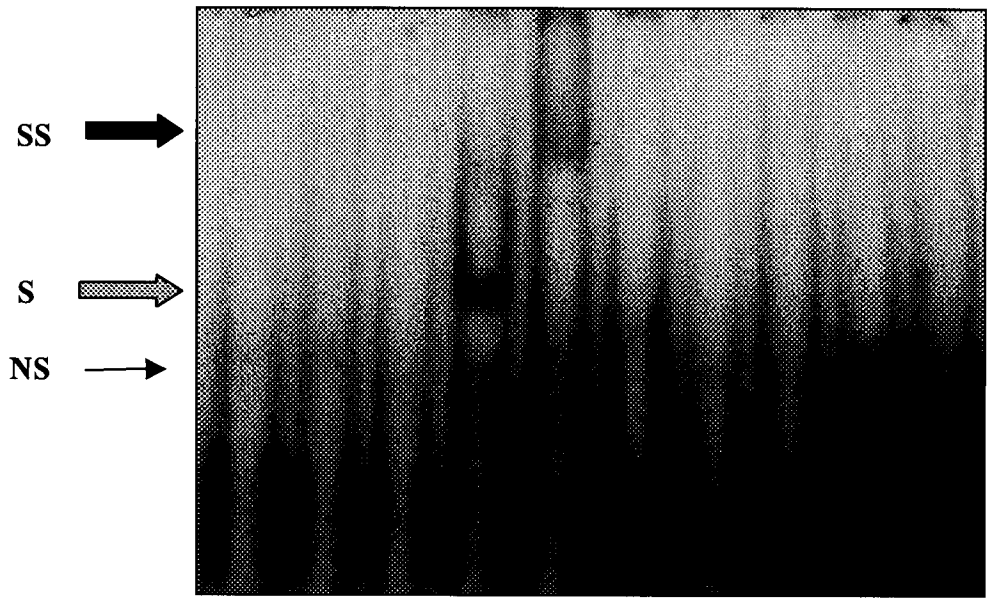
HNF-4 Site 1m (½ mutant) probe	+	+	+	+	+	-	-	-	-	-
HNF-4 Site 1m (double mutant) probe	-	-	-	-	-	+	+	+	+	+
+ HEK293 cells (NE)	-	+	+	-	-	-	+	+	-	-
+ HNF-4α2+α8 (NE)	-	-	-	+	+	-	-	-	+	+
+ Anti HNF-4α	-	-	+	-	+	-	-	+	-	+

C



HNF-4 Site 5 probe	+	+	+	+	+
+ HEK293 cells (NE)	-	+	+	-	-
+ HNF-4α2+α8 (NE)	-	-	-	+	+
+Anti HNF-4α	-	-	+	-	+

D



HNF-4 Site 6 probe	+	+	+	+	+	-	-	-	-	-
HNF-4 Site 6 mutant probe	-	-	-	-	-	+	+	+	+	+
+ HEK293 cells (NE)	-	+	+	-	-	-	+	+	-	-
+ HNF-4α2+α8 (NE)	-	-	-	+	+	-	-	-	+	+
+ Anti HNF-4α	-	-	+	-	+	-	-	+	-	+

4.7.13. ChIP analyses of HNF-4 sites #1, #5 and #6

In vivo analysis of the HNF-4 sites by ChIP in Huh7 cells revealed that the proximal (#1) and most distal (#6) sites are bound by endogenous HNF-4α; however, the distal site #5 is not (Figure 46).

From the site directed mutagenesis, EMS/SA and ChIP data, we conclude, therefore, that #1 and #6 are functional HNF-4 sites and #5 is not.

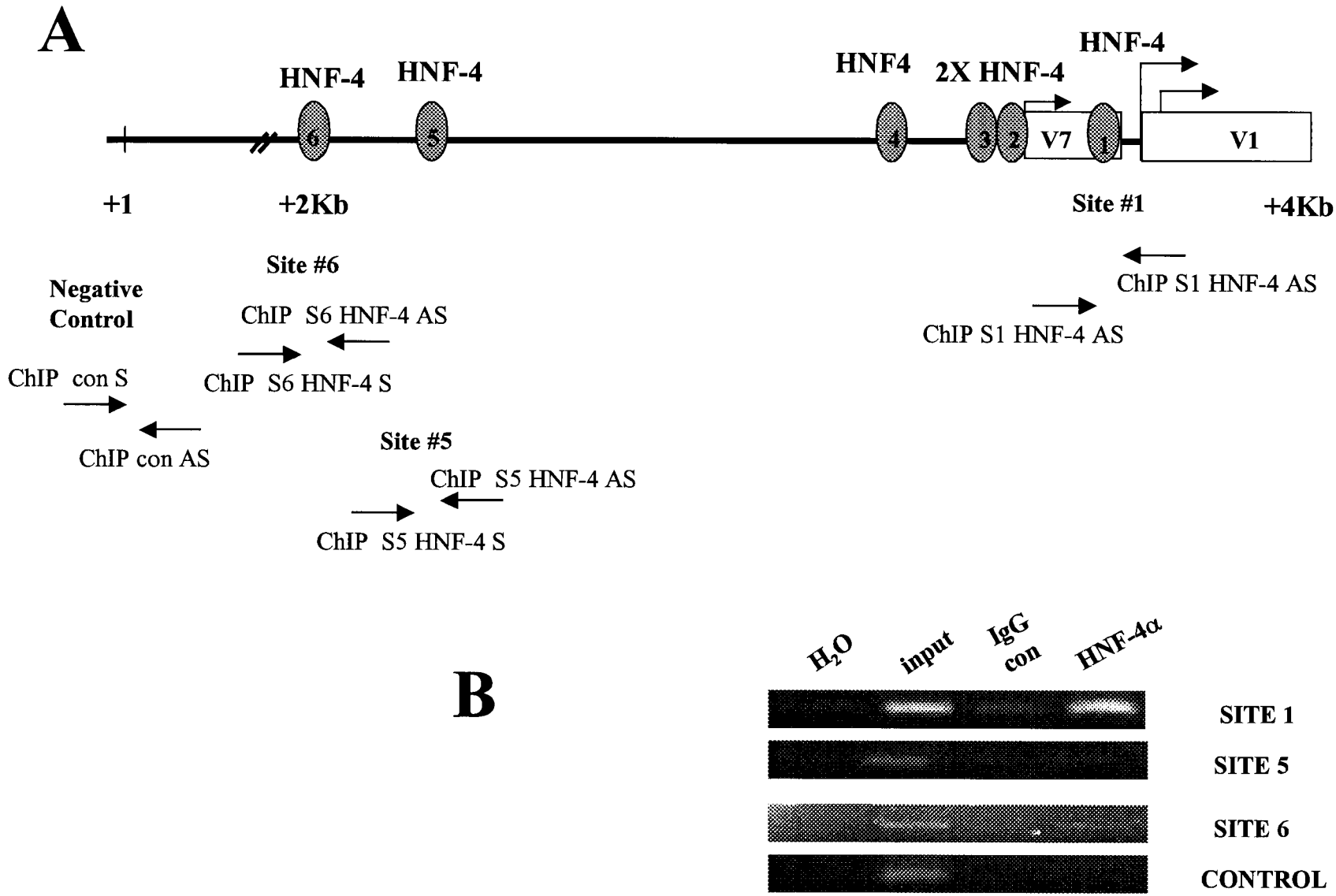
4.7.14. Discussion of the HNF-4α data

The negative regulatory regions (NRR 1-3) previously identified during the deletional studies of the V1 promoter coincide with the presence of five (HNF-4 sites #1, #2, #3, #5 and #6) of the six identified HNF-4 sites. Cotransfection studies with HNF-4 α2 and α8 expression vectors showed that constructs containing just the two most 5′ HNF-4 sites (in NRR3) were, in fact, repressed by both factors while the most proximal HNF-4 site (#1) is highly stimulated. Sites #2-#4 remain to be investigated in detail.

Studies by other labs have shown that regions homologous to our NRR2 interact with liver nuclear proteins. Using footprinting analyses, Jiang *et al.* showed that bovine nuclear extracts from liver, spleen and kidney bind to two regions (258;268). The first, ~ 23 bp footprint homologous to the 5′ end of NRR2 was weakly footprinted by nuclear proteins from liver, spleen and kidney.

Figure 46: *In vivo* ChIP analyses of proteins to three putative HNF-4 sites (#1, #5 and #6) in the V1 promoter region.

(A) Position of primer pairs used for ChIP analysis (Table 9). (B) Huh7 cells were crosslinked and the lysates immunoprecipitated with HNF-4α or goat IgG (IgG con). Following the reversal of the crosslinks, PCR assays were performed across HNF-4 binding sites #1, #5 and #6 and a region 2 kb upstream of HNF-4 site #6 as a control (control). Products were resolved on a 2% agarose gel. The intensity of the bands in the IgG con and HNF-4α of site #5 are equivalent and therefore there is no enrichment of HNF-4α at this site. There is no band in the IgG con lane of site #6 and a faint band in the HNF-4α lane of the same site; thus, HNF-4α is enriched at this site. However, HNF-4α cccupancy of site #6 is weaker compared to that of site #1.


A
HNF-4
HNF-4
HNF4
2X HNF-4
HNF-4
6
5
4
3
2
V7
1
V1
+1
+2Kb
Site #1
+4Kb
Site #6
Negative Control
ChIP S6 HNF-4 AS
ChIP S1 HNF-4 AS
ChIP S1 HNF-4 AS
ChIP con S
ChIP S6 HNF-4 S
Site #5
ChIP con AS
ChIP S5 HNF-4 AS
ChIP S5 HNF-4 S
B
H2O
input
IgG con
HNF-4α
SITE 1
SITE 5
SITE 6
CONTROL

MatInspector has identified no binding sites for transcription factors for this region. A second footprint (~ 20 bp), similar to a region in NRR2 that contains an HNF-4 site (#3) unique to the human, was specific to bovine liver nuclear extracts. Rivers *et al.* also showed that a region encompassing HNF-4 #3 (F2) in the V1 promoter was protected by HepG2 nuclear proteins in footprinting analyses, implying that HNF-4 site #3 is possibly bound by HNF-4 proteins (268).

Previous deletion promoter studies of the human V1 did not identify positive regulatory regions (268;269). However, footprinting analyses have identified two regions within the upstream positive region (PRR2) (Figure 19), between NRR2 and 3, that are bound by proteins in HepG2 nuclear extracts (F3 and F4) (268). Our HNF-4 response element #4 is located within the F4 footprint, suggesting that endogenous HepG2 nuclear proteins interacting at site #4 could be HNF-4, whereas F3 contains a putative TATA box and a C/EBP site (268). A similar regulatory region in the ovine does not contain any HNF-4 sites (Figure 19) and has been shown to be repressive in Huh7 cells (253).

PRR1, a 150 bp region containing the upstream V1 TATA/TSS complex as well as ~ 130 bp of its promoter, contains an HNF-4 site conserved in several species (Figure 19). The V1P4 construct containing this site (#1), was significantly stimulated by both HNF-4 α2 and α8. Ovine studies carried out in the laboratory of Dr. Timothy Adams identified a positive

regulatory region homologous to our PRR1 that contains an HNF-4 site although specific studies with HNF-4 were not carried out (253). In the bovine, extensive studies have been carried out to determine the functional significance of the regulatory domain equivalent to the V1 HNF-4 site #1 (258;270). Yeast one-hybrid experiments first identified HNF-4α, HNF-4γ and COUP-TFII as proteins interacting at this site, and this was then confirmed using bovine liver extracts in EMS/SAs and ChIP experiments (258;270). All three factors stimulated transcriptional activity of a 500 bp b1A promoter construct: the effect of COUP-TFII was about 15 fold higher than the HNF-4 factors. Deletion of the HNF-4 site ablated responses to HNF-4α and HNF-4γ but there was still some response to COUP-TFII. The -469 to –21 bp b1A construct contains a second putative HNF-4 site that is also present in the ovine but not in human or mouse but this site has not yet been investigated (Figures 20 & 40B). From Jiang *et al.* studies of b1A and the V1 studies carried out for my PhD research, I conclude that the HNF-4 site present in PRR1 and conserved across four species is functional and stimulatory in the bovine and human GHR genes, and that this is likely to be true for the ovine as well. It is difficult to speculate whether this is the case for the mouse L1 variant as this transcript is strongly upregulated only in the liver of pregnant mice and likely to be under additional regulatory mechanisms not common to other species expressing liver-specific variants of GHR (172;256;413). In fact,

studies of the mouse L1 promoter have identified a developmental enhancer element 3.5 kb upstream of the TSS that is only active in adult hepatocytes (256). This element binds NF-Y and MSY-1, a single stranded RNA binding protein. The amount of immunoreactive MSY-1 protein in the nuclei of non-pregnant mouse liver cells is much higher than in pregnant females and an MSY-1 expression vector repressed the activity of the 3.6 kb L1 promoter construct by 50-60%, suggesting that it normally suppresses mL1 expression in non-pregnant mice (413).

The importance of HNF-4α in the regulation of normal hepatic function has been demonstrated in liver-specific HNF-4α knock-out adult mice that show defects in hepatic lipid, lipoprotein and bile acid metabolism (398). These animals also weighed less compared to normal littermates (398). Studies in the rodent have clearly shown that expression of HNF-4α is under the control of two promoters: P1 and P2. Isoforms arising from the promoters are developmentally regulated, P2 isoforms being expressed only in the foetal hepatocytes and P1 isoforms in both foetal and adult hepatocytes (386;389). My immunoblotting data show, for the first time, a parallel situation in humans: the HNF-4α8 (P2) isoform was only expressed in the foetal hepatocytes while the HNF-4α2 (P1) isoform was found in both foetal and adult hepatocytes, using an HNF-4α antibody specific to the extreme C-terminus of HNF-4α. Other groups have reported the expression of the HNF-4α2 isoform in human adult

hepatocytes and hepatoma cells (HepG2, Huh7) using HNF-4α antibodies specific to the N-terminus that distinguish between P1 and P2 isoforms (387;388).

Since foetal hepatocytes and hepatoma cell lines express HNF-4α8 in addition to HNF-4α2, but not the V1 transcript, we initially hypothesised that the presence of HNF-4α8 would inhibit the stimulatory effect of HNF-4α2 on V1 transcription in foetal and tumour hepatic cells. Previous studies suggest that there are functional differences between the P1 and P2 isoforms: P2 HNF-4α isoforms that possess only the AF-2 domain are less potent transcription factors (400;401) but are stronger activators of early genes (412). In addition, insertion of ten amino acids in the F-domain of HNF-4α1 makes HNF-4α2 a more effective transactivator (414). Our observations of the effect of P1 and P2 HNF-4α isoforms on the V1 promoter did not reflect this: no significant differences in the potency of HNF-4 α2 (P1 isoform) and α8 (P2 isoform) on the hGHR V1 promoter were observed. Cotransfecting increasing amounts of HNF-4α8 with HNF-4α2 did not alter the effect of HNF-4α2 on the promoter fragments. Thus, I disproved our hypothesis that P2 isoforms repress the activity of P1 isoforms and, thereby, inhibit the expression of the liver specific V1 hGHR transcript in foetal hepatocytes and hepatoma cell lines. Instead, I showed that HNF-4α2 and α8 could have both stimulatory and repressive effects on the same gene (hGHR) through two different HNF-4

sites, #1 and #6. In agreement with the inhibitory effects, HNF-4α1 has been shown to act as a repressor on the HMG-CoA synthase gene, the liver-specific arginase gene, the acyl-oxidase gene, the liver-specific promoter of the human IGF-II gene, and the HNF-4α P1 and P2 promoters (403-407;415). Only one other example of the opposing effects of HNF-4α on the same gene via different sites has been reported: the human α_1-microglobulin/bikunin precursor (AMBP) gene that encodes two plasma glycoproteins (416). α_1-microglobulin is a retinal carrier protein and bikunin is a serine protease inhibitor protein (417;418). Although both proteins are unrelated in function, they are derived from the same precursor, AMBP, encoded by a single gene. ABMP expression is restricted to the liver and upregulated during the perinatal period, suggesting that this gene is under tissue and developmental regulation (418). Nine response elements (boxes) for the HNF family (HNF-1, HNF-3, HNF-4) have been described in an enhancer region, ~ 2.5 to 2.9 kb upstream of the TSS (416;419). Boxes 2, 7 and 8 are positive HNF-4 sites whereas HNF-4α1 exerts a negative effect through box 9 (416). The repressive effect is dependent on the absence of a functional box 8 and the presence of low HNF-4α: in conditions where HNF-4α is low, the occupancy of the low affinity box 8 will be low and, thus, the effect of box 9 will be more pronounced (416).

The V1 promoter is similar to the ABMP gene in terms of multiple HNF-4 sites in the promoter region and the expression is regulated in a developmental and tissue-specific pattern. At the same time they are different in the sense that the HNF-4 sites in ABMP are located in an enhancer region over 2 kb upstream of the TSS, whereas the HNF-4 sites in the V1 promoter are both proximal and distal.

HNF-4α2 and α8 both bind the same sites in the human V1 GHR promoter; this interaction is abolished by mutations in either one or both half sites of the direct repeat response element, showing that these are functional HNF-4 sites. The binding of P1 and P2 isoforms of HNF-4α to the V1 promoter suggests a regulatory role for HNF-4 in cells that express either one (adult hepatocytes) or both (foetal hepatocytes) of the isoforms.

Another level of regulation may lie in their association with SMRT-HDAC complexes. The laboratories of Drs. Frances Sladek and Mary Weiss have shown that HNF-4α1 (P1 isoform) and HNF-4α7 (P2 isoform) interact with SMRT-HDAC complexes *in vivo* and *in vitro*, and that HNF-4α1 is more strongly repressed by SMRT (383;412;420). Studies have shown that both class 1 (HDAC 1-2) and class II (HDAC 4-6) HDACs are required for active repression (421). The availability of different HDACs in a cell could affect the activity of nuclear receptors: the deacetylase domain of SMRT specifically activates HDAC 3, and, thus, although SMRT-HDAC-4

complexes may assemble, HDAC3 interaction is also required in order for repression to occur.

In addition, since no distinctive differences in HNF-4 binding sites have been identified that make it either inhibitory or stimulatory, activation or repression by HNF-4α is likely to be affected by other response elements in the region around it. In support of this, HNF-4α has been shown to repress PPAR activation of the mitochondrial HMG-CoA synthase gene, and HNF-4α repression of the human IGF-II gene is relieved by C/EBPα, suggesting competitive inhibition by other LETFs can occur at the same site (402;406). C/EBPα is expressed at very low levels in the rodent foetal liver and dedifferentiated epithelial cells and is upregulated in mature liver and in hepatomas (332). This could be the mechanism by which V1 expression is augmented in the liver postnatally.

The region containing the distal site #6 could be considered to be within the promoter of V7, which is upstream of the V1 exon, or to be a distant regulatory element of V1. As a promoter region of V7, repression of HNF-4 site #6 by both HNF-4α2 and α8, could be the mechanism regulating the suppression of V7 in the foetal liver and its low expression in adult liver. On the other hand, as a distal regulatory element for V1, it could account for the suppression of V1 expression in foetal liver and with increased expression of other LETFs such as C/EBPα around birth, these factors could alleviate the repression, and in cooperation with site #1,

upregulate V1 expression in the postnatal liver. All of these theories are yet to be investigated.

The HNF-4 sites chosen for this study (#1, #5, #6) had sequences closest to the published consensus and the constructs containing these sites showed striking responses to HNF-4α1, α2 and α8. Site #1 (V1P4), with only 1 bp mismatch was highly stimulated, and sites #5 and #6, with only 2 bp mismatches, were present in V_XP_B that was significantly inhibited in response to HNF-4α1, α2 and α8. Although site #2 was only a 2 bp mismatch and the promoter region (V1P3) was stimulated in response to HNF-4α1, α2 and α8, deletion of this site (including site #3) (V1P4) resulted in an increase in the magnitude of response, suggesting that site #2 (and possibly #3) is inhibitory.

Site #4 is 4 bp off from the consensus, yet deletion of the site led to a slight decrease in both basal activity and responsiveness to HNF-4α1, α2 and α8, suggesting that it may be stimulatory. Future experiments will investigate the functional significance of these three sites (#2, #3 and #4) and how all six sites interact to regulate transcription of the V1 mRNA transcript.

In summary, HNF-4α2 and α8 have comparable potency on the hGHR gene, stimulating and repressing V1 promoter activity via different sites. The stimulatory site is conserved in the bovine, ovine and mouse and studies by Jiang *et al.* have shown that it is also functional in the b1A

promoter. The distal site #6 interacts with, and is repressed by, HNF-4α2 and α8. Such duality in HNF-4α activity on the same gene may be important in the expression of V7 versus the V1 transcript in the adult liver and/or V1 repression in foetal liver and hepatomas.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS.

GH is an important regulator of postnatal growth and metabolism. It exerts its effects on target cells by interacting with its surface receptor, GHR. More than twelve mRNA transcripts of the hGHR have been identified, varying only in the 5'UTR sequence (224;241). Seven of the non-coding 5'UTR exons generating these mRNAs are grouped in two clusters which we have called modules A and B (59;223;224). Interestingly, mRNA variants arising from the exons in the two clusters are differentially regulated. While module A generated transcripts are ubiquitously expressed, module B variants are only switched on in the postnatal hepatocyte, likely accounting for the increase in GHR mRNA and GH binding observed beginning ~ 3 months after birth (223;244;269). It has been of great interest to determine what mechanisms regulate the expression of these latter variants. Several mechanisms could be in operation. First, the structure of the chromatin could be modified in a developmental- and tissue-specific manner. CpG islands have been shown to be involved in epigenetic regulation of the expression of several genes. However, the promoter regions of the module B exons did not contain any CpG islands or significant numbers of CpG dinucleotides. Second, developmental changes in the expression of transcriptional repressors in the foetal liver and non-hepatic cell types and/or transcriptional activators

in the postnatal liver could also be a mechanism by which the expression of module B mRNA variants are regulated. Whether these are acting to remodel the chromatin region relevant for module B exons' activation or are binding to already 'opened' chromatin regions remains to be determined.

I began my studies by comparing the promoter activities of the exons in modules A and B. The cell types used did not express module B variants but they did produce module A variants and, thus, the fact that module A promoters were much more active than those from module B was not surprising. More intriguing was the fact that a 2 kb V1 promoter construct, containing the two TSS/TATA complexes and about 1.8 kb of the promoter region, was completely repressed in all four cell lines. By removing 150-300 bp from the 3' end of this construct, promoter activity was significantly increased. Transcription factor scanning programs revealed two putative binding sites for the transcriptional repressors, Gfi-1 and Gfi-1b. Both factors were shown to bind to the same sequence on the V1 promoter and both factors repressed its activity, as shown by transient transfection assays and site-directed mutagenesis. Even though our protein detection systems did not identify Gfi-1 and Gfi-1b immunoreactive proteins in the cell lines, mutation of one or both sites in the 300 bp proximal promoter of V1 significantly increased the basal activity of the construct. This suggests that endogenous repressors were

actively repressing V1 transcriptional activity through the Gfi-1/1b sites. We still have not ruled out the possibility that these factors could be Gfi-1 and/or Gfi-1b, as the transient transfection assays have shown that small amounts of these factors (below the detection limits of our Western blots) strongly repress the activity of the V1 proximal promoter.

GH has been shown to regulate transcription of its own receptor in the human hepatocyte (181;182;185;186;188). Several response elements have been described as GHREs [reviewed in (147)]. Wang *et al* described a STAT5 site in the bovine b1A promoter through which GH regulates GHR in the hepatocyte (422). Although no consensus Stat response elements were detected in the 1.8 kb V1 promoter, a GAGA element is found ~ 20 bp upstream of the conserved TATA box of the V1 promoter. GAGA elements in vertebrates have been shown to interact with several unidentified endogenous GBPs (311;313-315). We discovered that the *Drosophila* GAF-519 protein strongly stimulates the activity of the 100 bp V1 proximal promoter through the GAGA element. The proximity of the proximal Gfi-1/1b site and the GAGA element led us to investigate the interaction of both factors. The effect of Gfi-1 was striking as it repressed ~ 75% GAF stimulation of the 100 bp V1 proximal promoter when expressed at very low levels. This leads us to hypothesise that fine control of the expression of Gfi-1/1b and mammalian homologues of the *Drosophila* GAF will be important in modulating V1 expression. As so little

is known about the mammalian homologues of the *Drosophila* GAF, it is difficult to know what its role in the regulation of V1 promoter activity may be. However, from our knowledge of the function of GAF in *Drosophila*, we can speculate that they might involve antirepressor activities and/or boundary functions (295). While the downstream Gfi-1/1b site and the GAGA element are conserved across the species, bovine studies of the proximal 190 bp promoter of b1A show that a ubiquitous transcription factor, ZBP-89, is bound to its site just downstream of the GAGA element and overlapping the Gfi-1/1b site (267). Comparison with the consensus ZBP-89 sequence suggests that this is present in the ovine but not in the human or mouse (Figure 20). Thus, regulation of the liver-specific variant of hGHR through ZBP-89 may be restricted to ruminants. Whether either Gfi-1/1b or GBPs regulate ruminant liver-specific GHR expression has not been examined.

In our search for a factor that could mediate the liver-specific expression of V1, we used computer-based TF scanning programs to analyse the 1.8 kb promoter region of V1 for binding sites for members of the LETF family. Several response elements were identified including for C/EBP (3 sites), DBP (3 sites), HNF-1 (3 sites), HNF-6 (1 site) and HNF-4 (6 sites). The HNF-4 site most proximal to the downstream TSS has a 1 bp mismatch with the published consensus sequence and has the potential to interact with HNF-4, COUP-TF and PPAR/RXR TFs. It is also conserved in

homologous regions in the bovine, ovine and mouse genes. This HNF-4 site has been shown in the bovine to interact with and be stimulated by HNF-4α1, HNF-4γ and COUP-TFII (258;270). HNF-4α and HNF-4γ are expressed in hepatocytes; however, HNF-4γ mRNA is 10X less abundant than HNF-4α mRNA (372). COUP-TFII has been shown to be expressed in human liver mesenchymal and ductal cells but not hepatocytes (423). However, a recent paper using a β-galactosidase marker driven by a COUP-TFII promoter did detect expression in murine hepatocytes, leaving the issue of whether COUP-TFII could be involved in liver-specific GHR expression controversial (424).

We chose to study the role of HNF-4α in regulating V1 transcription, in view of its abundance in the liver and its role in regulating several hepatic genes [reviewed in (371)]. Nine isoforms of HNF-4α have been described (385). Two leader exons, exon 1D and exon 1A regulate the transcription of all nine isoforms (386;389). The isoforms differ in the N-terminal and C-terminal regions. The leader exons also regulate the developmental- and, to a lesser extent, the tissue-specificity of HNF-4α isoforms. P1 isoforms, transcribed from exon 1A are the predominant isoforms in postnatal liver (386-389). However P2 isoforms are more abundant in foetal liver, dedifferentiated cell types, stem cells and non-hepatic HNF-4α expressing tissues (e.g. pancreas and small intestine) (351;386-389;401;425). Therefore, it appears that P2 HNF-4α isoforms are more

involved in the regulation of early genes and immature liver functions. We determined HNF-4α isoform expression in human liver and cell lines by RT-PCR and western blot analyses. The HNF-4α antibody used can detect HNF-4α isoforms 1,2,5,6,7 and 8. The results show that the P2 isoform (HNF-4α8) is expressed in foetal hepatocytes and in the dedifferentiated hepatic cell lines, HepG2 and Huh7. The P1 isoform (HNF-4α2) was detected in both foetal and postnatal liver and also in HepG2 and Huh7 cells.

Initially, we studied the effect of HNF-4α1 on V1 transcriptional activity. Subsequently, we switched to HNF-4α (2+8) as they were the isoforms we found to be expressed in the human tissues and cell lines. Transient transfection studies were conducted in HEK293 cells, as HNF-4α protein was not detectable. The V1 constructs tested contained a range of 1-6 putative HNF-4 sites. Constructs containing the two distal HNF-4 response elements were repressed by HNF-4α (1,2,8). The #1 site on its own was highly stimulated by HNF-4α. The contributions of the other sites were minor: sites #2 and 3 appeared to be slightly inhibitory whilst #4 was slightly stimulatory. Site-directed mutagenesis of sites #1 and #6 confirm that they are responsible for stimulatory and repressed responses to the HNF-4α isoforms (1,2,3). EMSA/EMSSA and ChIP analyses show that HNF-4α (2+8) proteins interact with sites #1 and #6, but not site #5 suggesting that the two major functional HNF-4 sites are #1 and #6.

Two paradoxical results were obtained from the HNF-4 studies. The first was that the highest levels of HNF-4α were found in the dedifferentiated hepatoma cell lines which didn't express V1. This was most surprising as HNF-4α is a strong regulator of hepatic genes including, as I have shown, hGHR, and yet V1 was not expressed in these cell lines. The second was that ChIP analyses carried out in one of these cell lines (Huh7) showed *in vivo* occupation of the HNF-4 stimulatory response element (#1). Thus, occupation of a response element does not confer transcriptional activity. However, HNF-4 expression in HepG2 cells has been reported to be uncoupled from its effects on target genes and, thus, the protein may not be functional.

Members of the nuclear receptor family to which HNF-4α belongs are activated upon ligand binding. Normally, HNF-4α has been shown to be constitutively active, and crystallography experiments have defined FFAs entrapped in its ligand binding pocket as essential cofactors (377). One of the features of hepatomas and hepatocarcinomas is defective lipid metabolism [reviewed in (426)]. Oleic acid, an LCFA that has lipid lowering capabilities, has been shown to inhibit HNF-4α activity and is also found at high levels in the hepatoma cell line, HTC 7288c (381;427;428). Assuming that the fatty acid profile is similar in Huh7 and HepG2 cells, it is highly likely that the HNF-4α ligand binding pockets are occupied by antagonists in the hepatoma cells. This would explain the inability of the

high levels of HNF-4α in the hepatic tumour cells to stimulate V1 mRNA expression.

HNF-4α activity has been shown to be modified by dietary and energy status (392;394). Non-esterified fatty acids (NEFA) are transported into the cell by fatty acid transporters and converted into fatty acyl coenzyme A (FACoA) thioesters by the acyl coenzyme A synthetase (429). The FACoA thioesters will then interact with the ACS of HNF-4α and modulate its activity and subsequent activation of its target genes (382). Low energy status (high AMP:ATP ratios) activates the AMP-activated protein kinase which in turn phosphorylates HNF-4α and inhibits HNF-4α dimer formation, DNA binding, protein stability and subsequent transcriptional activity (392). GHR expression is also affected by nutritional status: during fasting (and starvation), when energy needs to be conserved, GHR mRNA expression is downregulated and during feeding it is upregulated (430). Thus, dietary status may affect hepatic GHR mRNA expression probably by modulating the activity of HNF-4α and its subsequent transcriptional activation of V1.

The two aims of my PhD thesis were to carry out the screening of six putative hGHR promoter regions and then to characterise in more detail the promoter region of V1 and to identify developmental- and liver-specific factors that regulate its expression. The use of cell lines that do not express the V1 mRNA variant complicates the identification of regulatory

regions in the context of normal postnatal liver. Nevertheless, I was able to define three negative and two positive regulatory regions in the V1 promoter.

I began to characterise developmental- and liver-specific regulators of V1 expression: human foetal liver expresses both P1 and P2 isoforms of HNF-4α, whereas only the P1 isoform is present in adult liver. P1 isoforms have been shown to be more potent transcription factors than P2 isoforms, although I did not observe this in context of the HEK293 cell line used for the transient transfection studies. I had hypothesised that P2 isoforms (HNF-4α8) would suppress the activity of P1 isoforms (HNF-4α2) thus acting as a developmental switch. Unfortunately, I disproved this but speculate that other LETFs may act as foetal repressors or postnatal activators (e.g. C/EBPα).

I also identified two transcriptional repressors and began to define their role in suppressing V1 expression. A GAGA element was also found to mediate stimulation of the 100 bp proximal promoter activity by the *Drosophila* GAF. Obviously, since no mammalian homologues of GAF are known, we cannot assess if this is a developmental regulator. But if GH changes the levels or activities of endogenous GBPs,as has been shown for other genes with GAGA elements, there may be a developmental mechanism involving GAGA.

Finally we found what appear to be similar levels of Gfi-1 in foetal and postnatal tissues, making it unlikely that it is a developmental-specific regulator. However, we still need to assess if the ratios of GBPs and Gfi-1 in foetal versus postnatal hepatocyte are significantly different.

In the section following, I discuss possible experiments that could be carried out in the future to conclude the study of the mechanisms regulating the tissue- and developmental-specific regulation of V1 expression.

CHAPTER 6: FUTURE DIRECTIONS

The results of this project have opened up many interesting routes to explore. I will discuss future directions in module B-related experiments, especially those relating to the different factors that I have so far been investigated. Regulation of module A variants are being studied in the Goodyer lab at present by two PhD students: Yuhong Wei and Gurvinder Kenth.

Gfi-1/1b and the V1 promoter

Given that mutation of either one or both Gfi-1/1b sites in the 300 bp proximal promoter region of V1 increased basal activity of the promoter constructs, it would be interesting to see if the same mutations in the V1P1 construct would increase its activity. This would pinpoint the elements regulating the repression of the V1P1 promoter construct in the most 3′ 300 bp region.

In addition, two other Gfi-1/1b sites were identified by MatInspector, upstream of the 300 bp proximal promoter of V1 (Figure 47). Since it has been established that Gfi-1 and Gfi-1b are potent repressors of V1, it would be of interest to investigate the contribution of these other sites to the overall regulation of the V7-V1 promoter by transient transfection assays and ChIP analyses of all four sites in various types of cells and

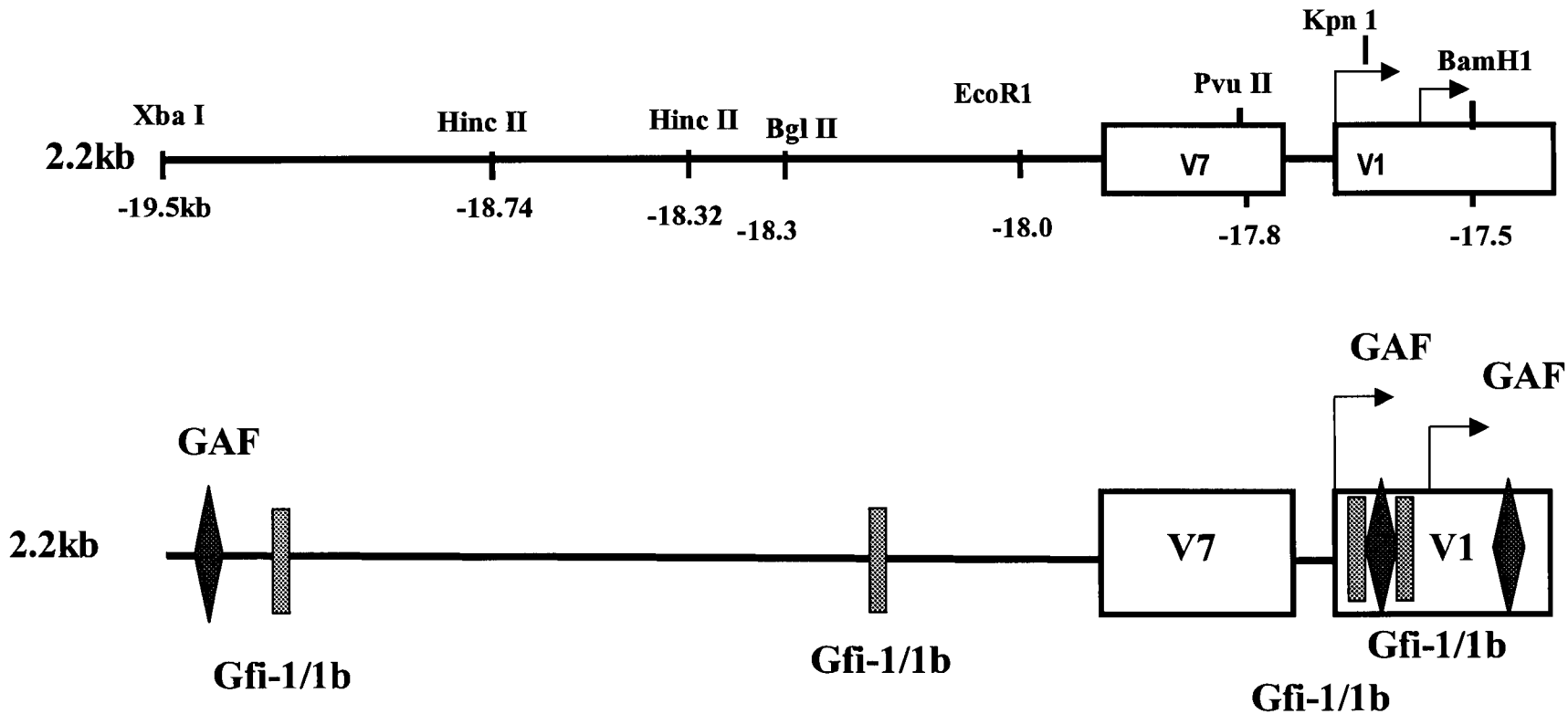
tissues. In module B, the V8 exon has a putative Gfi-1/1b site ~ 50 bp upstream of a TATA box. This also should be tested.

Dysregulation of Gfi-1 expression in certain cell types is tumorigenic (277;278). In T-cells, Gfi-1 has been shown to induce lymphomas together with myc and pim, and inhibit p21 WAF, a cell cycle regulator (431-433). GHR mRNA expression and GH binding are reduced in hepatic tumours and liver cirrhosis, and V1 expression is low or undetectable (244;434-437). So far, no link has been made between GHR and Gfi-1/1b expression in cancers. It would be interesting to determine whether the changes in GHR expression are associated with deregulated Gfi-1 and Gfi-1b activity in the cancerous cells.

GAGA element

I have shown that *Drosophila* GAF-519 stimulates the transcriptional activity of the V1 proximal promoter region through the GAGA element. EMSA results also show the interaction of endogenous GBPs with the GAGA element. To determine what GBPs are present in HEK293 as well as other cell types, we could use the GAGA element as bait in yeast one-hybrid assays; a cDNA library of choice (using data from EMSA experiments) could then be screened in order to identify any proteins that may be interacting.

Figure 47: Gfi-1/1b sites and GAGA elements within the V1 region. Two other Gfi-1b sites are located ~ 1.1 kb and 400 bp upstream of the TSS. A 3X GAGA element is found ~ 1.5 kb upstream of the TSS. A second 21 bp GAGA element is present ~ 70 bp downstream of the TSS, within the V1 exon.


Kpn 1
BamH1
Pvu II
EcoR1
Xba I
Hinc II
Hinc II
Bgl II
2.2kb
V7
V1
-19.5kb
-18.74
-18.32
-18.3
-18.0
-17.8
-17.5
GAF
GAF
GAF
2.2kb
V7
V1
Gfi-1/1b
Gfi-1/1b
Gfi-1/1b
Gfi-1/1b

Cloning of the cDNA encoding the protein would determine if it is a mammalian homologue to the *Drosophila* GAF. Once the sequence is known, antibodies can be raised and used to determine whether there is a tissue- or developmental-specific expression pattern. The cDNAs can be used in transient transfection assays to determine functional activity on the V1 promoter.

The function of the GAGA element as a GHRE in the proximal V1 promoter has also not been fully investigated. The effect of exposing different test cells to varying concentrations of hGH on the transcriptional activity of the V1 promoter should be studied to determine if there are GH effects on endogenous GBPs, as has been shown for the AT_1 receptor gene (313). A comparison of the different V1 promoter constructs would delineate any additional sequences that display responsiveness to hGH. In this way, although Stat5 response elements have not been detected by sequence analyses, alternative functional GHREs may be defined. Two other GAGA elements were identified by MatInspector, one in the 1.8 kb V1 promoter region, and the other within the V1 exon (Figure 47). These should be tested to determine if they have individual or "cooperative" activity.

HNF-4α

HNF-4α interacts with both coactivators and corepressors, and P1 and P2 isoforms of HNF-4α similarly repressed and activated V1 promoter activity.

Thus, determining cofactors interacting at each site by ChIP-on-ChIP analyses would shed light on the opposing effects of HNF-4α on the V1 promoter. In addition, only three of the six putative HNF-4 sites have been investigated to date. From the deletion promoter experiments, sites #2 and #3 have been hypothesised to be inhibitory, and site #4 stimulatory. Using transient transfection assays, site directed mutagenesis, EMSA, EMSSA and ChIP analyses, the function of these sites and their contribution to the regulation of V1 (and V7) should be investigated.

LETFS

Several putative sites for multiple LETFs were identified by computer based TF screening.

DBP is of major interest as it is expressed only in rat postnatal liver and it is involved in regulating the diurnal expression of several hepatic genes. However, preliminary data suggest that this is not the case in humans. We were able to detect DBP mRNA and immunoreactive protein in both human foetal hepatocytes and postnatal liver (438). The rat DBP protein is 87% homologous to the human DBP. Preliminary cotransfection experiments with either rat or human DBP did not yield any consistent results. However, several members of the PAR family of proteins (DBP, HLF and TEF) all bind to the same site, including E4BP4, a transcriptional

repressor (439;440). Studies have shown that E4BP4 is also under circadian regulation and that expression of E4BP4 and DBP is antiphasic (440). It would be interesting to test the hypothesis that DBP and E4BP4 are involved in regulating the postnatal diurnal expression of GHR mRNA in hepatocytes and that we have not observed DBP effects because of the presence of high levels of E4BP4 in our test cells. Since circadian rhythms are present in many cell lines (441), it would be of interest to determine, in cells that have been synchronized, the nuclear proteins interacting with the DBP sites, using EMSA, EMSSA and ChIP analyses, as the collection of human hepatocytes at specific times would be impossible.

Expression of human IGF-II is under the control of four promoters: P1-P4. P2, P3 and P4 are highly active during foetal life, whilst P1 is not. P1 is switched on in the postnatal liver, and P2-P4 are down regulated. Rodenburg *et al.* showed that the P1 promoter activity was stimulated by C/EBPα, C/EBPβ and HNF-3β in HEK293 and Hep3B cells whereas HNF-4α1 suppressed its activity only in Hep3B cells (442). C/EBPα and C/EBPβ also abolished HNF-4α1 mediated repression of the P1 promoter. There are three putative C/EBP sites in the 1.8 kb promoter region of V1. Using transient transfection assays, site-directed mutagenesis, EMSA/EMSSA and ChIP analyses, the function of the three C/EBP sites should be investigated. As has been done with studies of the liver-specific P1 promoter of the human IGF-II gene, analyses of the interactions of other

LETFs (e.g. HNF-1, HNF-6) on the V1 promoter should also be carried out (442).

A LOCUS CONTROL-like REGION (LCR-like)?

LCRs have been described for the hGH, globin and apolipoprotein cluster of genes [(443;444) and reviewed in (445;446)]. These LCRs are located several kb upstream of the gene clusters and are involved in remodelling the chromatin downstream to allow for regulated gene expression. The LCR region of the apolipoprotein gene cluster is referred to as a hepatic control region (HCR) and contains binding sites for HNF-3 and HNF-4 (446).

The expression of module B variants is switched on in the same tissue and at the same developmental stage, suggesting that there is some common activation mechanism. One possibility is an upstream region that mimics an LCR, with one or more transcription factors that act at a simple locus to "open" the chromatin of module B. A second possibility is that there is a common postnatal liver-specific transcription factor or set of transcription factors for all four exon promoters. All four contain TATA-like boxes although those of V7, V4 and V8 are not consensus TATA sequences. A comparison of the V7/V1, V4 and V8 promoter regions show some common putative elements (e.g. HNF-4) and unique sites (e.g. STAT6) that should be further investigated to test this hypothesis.

These suggested experiments are by no means exhaustive but they should provide greater insight into the mechanisms involved in regulating expression of this complex and intriguing gene that is essential to the postnatal growth of man.

CHAPTER 7: ORIGINAL CONTRIBUTIONS

1. I have demonstrated that V1, V2, V3, V4, V7 and V8 5′ UTR exons of the hGHR gene have individual promoter activities.
2. By promoter deletion studies of the 1.8 kb promoter region of the V1 exon, I have defined two positive regulatory regions, confirmed the presence of two negative regulatory regions and discovered a third negative regulatory element.
3. I have determined that the third negative regulatory region (NRR1), the 250 bp proximal promoter region of V1, contains functional binding sites for the transcriptional repressors, Gfi-1 and Gfi-1b, as well as a functional 4X GAGA element that could be stimulated by the *Drosophila* GAGA binding factor, GAF, as demonstrated by transient transfection assays and site-directed mutagenesis. Because the Gfi-1/1b site and the GAGA element in the 100 bp proximal promoter region overlap, I tested the effects of Gfi-1 and GAF in competition experiments and found that the two factors interact.
4. I have shown that isoforms of HNF-4α are developmentally regulated in the human hepatocyte as has been shown in the mouse: P1 (HNF-4α2) and P2 (HNF-4α8) protein isoforms were detected in human foetal hepatocytes at equal amounts while human postnatal liver contains only the P1 (HNF-4α2) protein isoform.

5. I have investigated in detail three of the six putative HNF-4 sites identified by computer-based transcription factor scanning programs in the 1.8 kb promoter region of the V1 exon. I have demonstrated that the HNF-4α (α2+α8) effects on the V1 promoter are complex: both isoforms stimulated V1 transcriptional activity through an HNF-4 response element (site #1) in the proximal promoter, and suppressed activity through a distal HNF-4 response element (#6). ChIP analyses suggest that in the Huh7 cell line, where HNF-4α (α2 and α8) proteins are abundant, both the proximal (#1) and distal HNF-4 sites are bound by HNF-4α (α2+α8). Thus, I have shown that the hGHR promoter is a transcriptional target of P1 (α2) as well as P2 (α8) HNF-4α isoforms and that their transcriptional effects are site specific.

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Ref Type: Thesis/Dissertation

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APPENDICES

1. Ethical approval for collection of human tissue
2. Waiver notices from publishers
3. Radioactivity certificates

Centre universitaire de santé McGill
McGill University Health Centre

Bureau d'éthique de la recherche
Office of Research Ethics

May 18, 2006

Dr. Cynthia G. Goodyer
Montreal Children's Hospital Research Institute
Endocrine Research Laboratory
Room 415/1
4060 Ste Catherine St. West
Westmount, QC
H3Z 2Z3

Re: "Use of Placental and Fetal Tissues for Research"

Dear Dr. Goodyer:

We have received an Application for Continuing Review of the Surgical Techniques/Medical Devices/Reproductiive Technologies (SDR) Committee for the research study referenced above and the report was found to be acceptable for ongoing conduct at the McGill University Health Centre. At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

The re-approval for the study was provided via expedited review of the Chair on May 16, 2006 and will be reported to the Research Ethics Board (REB) at its meeting of May 31, 2006. This decision will be entered accordingly into the minutes. The Research Ethics Board (REB) noted that no revision to the approved consent document (June 10, 2003) is required at this time.

All research involving human subjects requires review at a recurring interval and the current study approval is in effect until May 16, 2007. It is the responsibility of the principal investigator to submit an Application for Continuing Review to the REB prior to the expiration of approval to comply with the regulation for continuing review of "at least once per year".

However, should the research conclude for any reason prior to the next required review, you are required to submit a Termination Report to the Committee once the data analysis is complete to give an account of the study findings and publication status.

...2

Should any revision to the study, or other unanticipated development occur prior to the next required review, you must advise the REB without delay. Regulation does not permit initiation of a proposed study modification prior to REB approval for the amendment.

We trust this will prove satisfactory to you.

Sincerely,

Thomas Maniatis, MD, MSc, FRCPC
Chair
SDR Committee

Cc: SUR-03-031

Centre universitaire de santé McGill
McGill University Health Centre

November 30, 2005

Dr. C. Gates Goodyer
Anesthesia Department
Montreal Children's Hospital

Re: **PED-96-X04 The Human Growth Hormone Receptor: Regulation of Expression During Human Development**

Dear Dr. Goodyer,

We have received an Application for Continuing Review of the Montreal Children's Hospital Research Ethics Board for the research study referenced above and the report was found to be acceptable for ongoing conduct at the McGill University Health Centre. At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

The re-approval for the study and consent form (English and French version July 10, 2003) was provided via expedited review of the Chair on November 24, 2005, will be reported to the Research Ethics Board (REB) at its meeting of November 28, 2005, and will be entered accordingly into the minutes.

All research involving human subjects requires review at a recurring interval and the current study approval is in effect until November 23, 2006. It is the responsibility of the principal investigator to submit an Application for Continuing Review to the REB prior to the expiration of approval to comply with the regulation for continuing review of "at least once per year".

However, should the research conclude for any reason prior to the next required review, you are required to submit a Termination Report to the Committee once the data analysis is complete to give an account of the study findings and publication status.

Should any revision to the study, or other unanticipated development occur prior to the next required review, you must advise the REB without delay. Regulation does not permit initiation of a proposed study modification prior to REB approval for the amendment.

Sincerely,

Elizabeth Craven
Coordinator, Research Ethics Board
Montreal Children's Hospital

/ec

9. AUTHORIZING SIGNATURES

Signatures below certify that: ***(original ink signatures are required; no stamps or "as per" signatures)***

a) As Principal Investigator (Qualified Investigator), I will continue to comply with all relevant regulations and guidelines governing the conduct of research involving human subjects and with the requirements of the REB. I understand this research cannot be conducted without appropriate written REB approval.

- ***A copy of the appropriate REB-approved consent document has been signed by each person who has volunteered as a subject of the research unless otherwise authorized by the REB.***
- ***A copy of the signed document was offered to each subject, and each signed original is on file in a secure location.***
- ***If the study involves an MUHC patient receiving investigational treatment on a research protocol, a copy of the Executive Summary, and of signed consent document was sent to the patient's medical record and/or clinic file, and a "wallet card" was given to the subject.***

I have reviewed the content of this report including the above statements, and assure the REB of its accuracy.

Signature: _____ ***Date:*** 11/21/05

b) On behalf of the MCH **Research Ethics Board (REB), I confirm that:**

- ***the following decision was authorized by the REB:***
  - ☑ approval
  - ☐ approval with conditions
  - ☐ continuing review tabled
  - ☐ approval disallowed
  - ☐ other: _____
- ***decision was provided following:*** ☐ Full Board Review ☑ Expedited Review
- ***duration of the REB approval is provided for (no.) :*** 12 **months** ***or*** ☐ N/A

Name: Jane McDonald ***REB position:*** Chair.

Signature: _____ ***Review date:*** MAY 24, 2005

MUHC Study Number: PED-96-X04

(REO use only)

McGill University Health Centre – Royal Victoria Hospital

Consent Form

Title of the study: Use of placental and fetal tissues for research

Principal investigator: Dr. Cynthia Goodyer, Department of Pediatrics, McGill University

Introduction: You are about to have a surgical operation. This form asks your permission for the placental and fetal tissues removed during the operation to be used for biomedical and gene research projects. These projects will be carried out at the Royal Victoria Hospital, or at other research institutions in Quebec or outside Quebec.

Purpose of the study: The tissues will be used for research projects approved beforehand by the Research Ethics Committee of the Royal Victoria Hospital or the Research Ethics Committees at any other institution where research using the placental and fetal tissues is to be carried out. The projects (see list at the end of this form) have had a scientific review and are financed by Canadian and/or American government health research funding agencies, and/or pharmaceutical companies, and/or private sector foundations.

Risks: There is no physical risk associated with your giving permission for the placental and fetal tissues to be used for research purposes. The use of these tissues for research will not change either the usual surgical procedures or your care.

Advantages: There will not be any direct benefit to you as a result of agreeing to the use of the placental and fetal tissues for research. It is hoped, however, that the research carried out on these tissues will help other persons in the future.

Compensation: There is no financial compensation associated with your agreement that the tissues can be used for research.

Nature of the participation: You are free to give or to refuse your permission that the placental and fetal tissues removed during surgery may be used for research. Whatever your decision, it will not affect the quality of care that you receive or your future care. Since all tissues are obtained anonymously (without your name or any other form of identification), it is impossible to communicate the results from the research projects in which the tissues were studied.

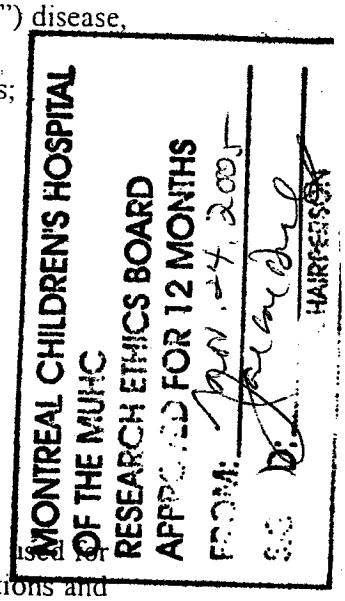
Confidentiality: Your medical file will remain confidential because we will collect the tissues anonymously; the researchers will not be able to link these tissues to you. In addition, the tissues and anything resulting from these tissues will be destroyed when the research project is completed. The results arising from the research will be the responsibility of the project's principal investigator and will be kept for as long as they are useful.

Persons to contact: If you have questions concerning the research projects, please call Dr Cynthia Goodyer, the principal investigator, at the Montreal Children's Hospital (Tel: 514-412-4400, extension 22481). If you have questions concerning your rights, you can call the Ombudsman at the Royal Victoria Hospital (Tel: 514-934-1934, extension 35655).

Summary of the Research Projects using Human Placental and Fetal Tissues

The placental and fetal tissues will be used in basic research studies of diseases that, as yet, have no cure. At present, the researchers are using these tissues to better understand:

- what genes are not functioning normally in children who grow too slowly;
- how the kidney loses its ability to control calcium levels in the body;
- what changes in genes may cause people to have a higher risk of developing diabetes;
- how to identify those cells in the pancreas that can produce insulin and prevent diabetes;
- how brain cells form and communicate with each other;
- what controls the death of brain cells in people with Alzheimer's, prion ("mad cow") disease, and following a stroke;
- how brain are injured and how they can be repaired in people with multiple sclerosis;
- how brain tumors form;
- how changes in the tissues that form bone joints may cause arthritis;
- the effect of toxic factors in the environment on the development of fetal tissues;
- how the lung develops.

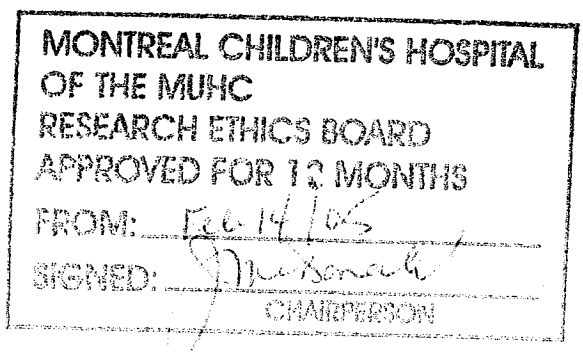


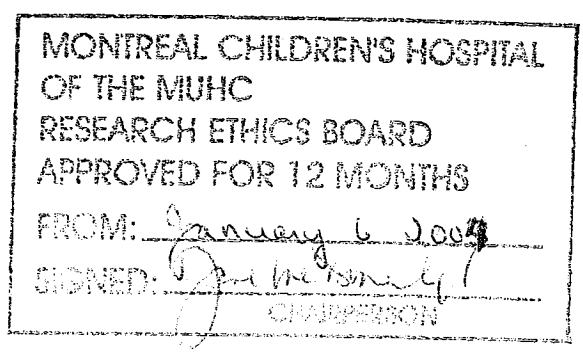
CONSENT

I agree that the placental and fetal tissues removed during the surgical procedure may be used for biomedical or gene research purposes. I have had the opportunity to ask all of my questions and these have been answered to my satisfaction. I have received a copy of this consent form.

Participant	Signature	Date

Study Nurse Coordinator	Signature	Date





Centre universitaire de santé McGill –Hôpital Royal Victoria

Formulaire de consentement

Titre de l'étude : Utilisation de tissus placentaire et fœtal à des fins de recherche

Chercheur principal : Dr Cynthia Goodyer, Département de Pédiatrie, Université McGill

Introduction : Vous allez recevoir une intervention chirurgicale. Le présent formulaire vise à obtenir votre consentement pour l'utilisation des tissus du placenta et du fœtus destinés à des projets de recherches biomédicales et de recherches sur les gènes réalisés à l'Hôpital Royal Victoria, ou à d'autres établissements de recherche au Québec ou à l'extérieur du Québec.

But de l'étude : Les tissus prélevés seront utilisés dans le cadre de projets de recherche préalablement approuvés par le comité d'éthique à la recherche de l'Hôpital Royal Victoria ou d'un autre établissement où seront menés des projets de recherche qui utilisent les tissus placentaires et foetaux. Les projets de recherche dont il est question (voir liste ci-jointe) ont été évalués pour leur mérite scientifique et ils sont financés par des organismes gouvernementaux de la recherche de la santé canadiens/américains, et/ou des sociétés pharmaceutiques et/ou des fonds du secteur privé.

Risques : Il n'existe pas de risque physique associé à votre consentement à donner vos tissus du placenta et du fœtus dans le but de la recherche. L'utilisation des tissus à des fins de recherche ne changera en rien la procédure usuelle chirurgicale ou vos soins.

Avantages : Il n'y aura aucun bienfait direct résultant de votre consentement à l'utilisation de tissus du placenta et du fœtus destinés à la recherche. On espère cependant que les recherches effectuées à partir des tissus aideront d'autres personnes à l'avenir.

Compensation : Il n'y a pas de compensation financière associée au consentement de l'utilisation des tissus dans le but de la recherche.

Nature de la participation : Vous êtes entièrement libre de consentir ou non à ce que les tissus placentaire et fœtal prélevés lors de la procédure chirurgicale soient utilisés à des fins de recherche. Peu importe votre décision, cela n'affectera pas la qualité des soins que vous recevez ou vos soins à l'avenir. Puisque tous les tissus sont obtenus anonymement (sans votre nom ou autre forme d'identification), il est impossible de vous communiquer les résultats des projets de recherche auxquels les tissus ont été examinés.

Confidentialité : Votre dossier médical demeurera confidentiel car nous recueillerons les tissus de façon anonyme. Les chercheurs ne pourront pas faire le lien entre ces tissus et vous. Par ailleurs, les tissus prélevés ainsi que tout ce qui ressort de ces tissus seront détruits lorsque le projet de recherche sera terminé. Les résultats découlant de la recherche seront sous la responsabilité de l'investigateur principal d'un projet et conservés aussi longtemps qu'ils seront utiles.

Personne ressources : Si vous avez des questions concernant les projets de recherche, vous pouvez communiquer avec le Dr Cynthia Goodyer, l'investigateur principal, à l'Hôpital de Montréal pour enfants ((514) 412-4400, poste 22481). Si vous avez des questions concernant vos droits, vous pouvez communiquer avec l'ombudsman de l'Hôpital Royal Victoria ((514) 842-1231, poste 35655).

Sommaire des projets de recherche qui utilisent les tissus humains du placenta et du fœtus

Les tissus placentaire et fœtal seront utilisés envers des projets de recherches fondamentales sur des maladies qui, jusqu'à présent, sont encore incurables. Présentement, les chercheurs utilisent ces tissus pour mieux comprendre :

- Quels gènes fonctionnent anormalement chez les enfants avec une croissance retardée.
- Comment les reins perdent leur capacité de régulation du calcium dans le corps.
- Quelles différences au niveau des gènes prédisposent certaines personnes au diabète.
- Comment identifier les cellules du pancréas qui produisent l'insuline et préviennent le diabète.
- Comment les cellules du cerveau se développent et communiquent entre elles.
- Comment les cellules du cerveau meurent chez les personnes atteintes de l'Alzheimer, les maladies du prion (vache folle), et accident vasculaire cérébral.
- Comment les cerveaux sont endommagés et pourraient être réparés chez les personnes atteintes de la sclérose en plaque.
- Comment les tumeurs cérébrales se développent.
- Comment les changements dans les tissus des articulations peuvent causer l'arthrite.
- Etudier les effets toxiques de l'environnement sur le développement des tissus fœtaux.
- Comment les poumons se développent.

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CONSENTEMENT

Je consens à ce que les tissus du placenta et du fœtus prélevés dans le cadre de la procédure chirurgicale soient utilisés à des fins de recherches biomédicales et de recherche sur les gènes. J'ai eu l'occasion de poser toutes mes questions et on y a répondu à ma satisfaction. J'ai reçu une copie du présent formulaire de consentement.

Participant	Signature	Date

Nom de l'infirmière coordonnatrice de l'étude	Signature	Date

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Journal: Endocrinology
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Title: Organisation and evolutions of the human growth hormone receptor gene 5' flanking region
Year: 2001
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Page Range: 1923-34
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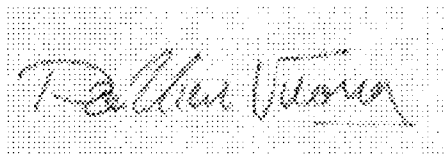

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