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Studies on growth hormone regulation of the *CYP2C12* gene in rat liver

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In memory of my mother

Contents

Abstract

Growth hormone (GH) is secreted from the pituitary gland in a sexually dimorphic manner in the rat. This leads to a sexual dimorphism in the expression of a number of hepatic GH-target genes including members of the cytochrome P450 (*CYP*) family. Transcription of the female specific *CYP2C12* gene is dependent on continuous presence of GH in serum, which characterizes the female rat. To understand which component(s) of the GH secretion pattern that is recognized as male or female by the hepatocyte, it is essential to understand how GH transduces its signal into the cell. This thesis focuses on elucidating signaling events in response to continuous GH exposure and the expression of the female specific *CYP2C12* gene.

GH elicits its effects by dimerizing membrane bound receptors (GHR). When primary rat hepatocytes were exposed to increasing doses of rat GH (rGH), the expression of P450 2C12 mRNA reached a plateau. The induction of IGF-I, a classical GH response gene, appeared biphasic. This is in line with a sequential binding mechanism, as described for hGH. When hepatocytes were treated with a site 2 mutant, ^{G118R}rGH, an unexpected agonistic effect was observed. The lack of antagonism together with the difficulty in achieving bell-shaped dose-response curves with rGH, indicate that the binding sites of rGH have similar affinity. Moreover, the agonistic effect of ^{G118R}rGH was bell-shaped, indicating an interaction with two receptors. This could imply that ^{G118R}rGH, via its site 2, can interact with another receptor than GHR.

GH regulates expression of *CYP2C12* at the transcriptional level. In gel shift experiments, using an element in the proximal promoter of *CYP2C12*, binding of HNF-3 proteins was observed with liver nuclear extracts from rats devoid of GH, and a complex distinct from the HNF-3 complexes was detected with liver nuclear extracts from rats exposed to GH. The bound protein was identified as HNF-6, a liver enriched transcription factor. Transient co-transfection experiments showed that both HNF-3β and HNF-6 could transactivate *CYP2C12*-luciferase constructs, indicating a physiological function of these transcription factors in the regulation of the gene.

The *CYP2C12* gene harbors STAT5 binding elements both in the 5' flanking region and in the 3' untranslated region (UTR). Interestingly, several different STAT5 complexes were formed on the 3'UTR element. In addition to full-length STAT5 also carboxy-truncated STAT5 (STAT5β)-proteins, lacking their transactivating domain, bound to this element. Moreover, in transient transfection studies, we demonstrated that the presence of the 3' UTR element reduced GH activation of a *CYP2C12*-luciferase construct conveyed by the 5'-STAT5 elements. Thus, it is possible that binding of STAT5β to the 3' UTR element could be of relevance for the GH-dependent and sex-specific expression of *CYP2C12*.

The family of SOCS/CIS proteins represents negative regulators of cytokine signal transduction. We demonstrated that SOCS-2 and CIS expression in rat liver is dependent on the presence of GH. Furthermore, in cotransfection studies, overexpression of CIS, but not SOCS-2, inhibited GH-induced transactivation of a STAT5-responsive reporter gene construct. This suggests that GH induction of CIS could be one mechanism behind the desensitization of GH-dependent JAK/STAT5 signaling in liver cells continuously exposed to GH.

Main References

The thesis is based on the following papers, which will be referred to by their Roman numerals;

I **Helander H.**, Clark R.G., and Mode A. GH receptor dimerization mechanisms in primary rat hepatocytes. *manuscript*

II Lahuna O., Fernandez L., **Karlsson H.**, Maiter D., Lemaigre F.P., Rousseau G.G., Gustafsson J-Å., and Mode A. (1997) Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone. *Proc. Natl. Acad. Sci. USA*, 94, 12309-12313.

III **Helander H.**, Gustafsson J-Å., and Mode A. (2002) Possible involvement of truncated signal transducer and activator of transcription-5 in the GH pattern dependent regulation of *CYP2C12* gene expression in rat liver. *Mol Endocrinol*. 16, 1598-1611.

IV **Karlsson H.**, Gustafsson J-Å., and Mode A. (1999) Cis desensitizes GH induced Stat5 signaling in rat liver cells. *Mol. Cell. Endocrinol.* 154, 37-43.

Abbreviations

CBP	CREB-binding protein
C/EBP	CCAAT/enhancer binding protein
CIS	cytokine inducible SH2 protein
$cPLA_2$	cytosolic phospholipase A_2
CREB	cAMP response element binding protein
CYP	cytochrome P450
EPO	erythropoietin
JAK	janus kinase
GAS	IFN-γ activated sequence
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
GLE	GAS-like element
HNF	hepatocyte nuclear factor
IFN	interferon
IGF-I	insulin-like growth factor 1
IL	interleukin
IRE-ABP	insulin response element-A binding protein
MAPK	mitogen-activated protein kinase
p/CAF	p300/CBP-associated factor
PI 3-kinase	phosphatidylinositol 3-kinase
PIAS	protein inhibitor of activated STAT
PKC	protein kinase C
PRL	prolactin
PRLR	prolactin receptor
SH2	src-homology 2
SH3	src-homology 3
SHP-1/2	SH2 domain containing protein-tyrosine phosphatase 1/2
SIRP	signal regulatory protein
SOCS	suppressor of cytokine signaling
SS	somatostatin
STAT	signal transducers and activators of transcription
T_3	thyroid hormone (3,5,3'-triiodothyronine)
TTR	transthyretin

General Introduction

Growth hormone (GH) is secreted from the pituitary gland in a sexually dimorphic manner in most species (1, 2). In adult male rats, GH is secreted in regular peaks at intervals of 3 to 4 hours with low or undetectable levels in between. The episodic secretion in female rats occurs with lower peak amplitude and a higher basal level, resulting in a continuous presence of circulating GH (3). GH has a multitude of different effects, including the regulation of several genes encoded by the cytochrome P450 supergene family (*CYP*). Several members of the *CYP2C* subfamily expressed in rat liver are sex-differentiated because of the sexually dimorphic secretion of GH, *e.g.* the *CYP2C12* gene is expressed at high levels in female but not in male rat liver, whereas the opposite is true for the *CYP2C11* gene (4). This thesis is focused on GH regulation of the *CYP2C12* gene at different levels, from GH binding to its receptors at the cell membrane to the involvement of the transcription factors HNF6 and Stat5 in transcriptional activation of the gene and desensitization processes of GH induced JAK-STAT5 signaling via SOCS/CIS proteins.

CYP2C12

The *CYP2C12* gene encodes a hepatic cytochrome P450 enzyme that is responsible for the female specific 15β-hydroxylation of sulfo-conjugated steroids in the rat. A number of different steroid sulfates, including conjugates of deoxycorticosterone and 5α-androstane-3α,17β-diol, which have a sulfate group at positions 17β or 21, can act as substrates for this P450, as well as some xenobiotics. A "feminizing factor" from the pituitary was postulated to regulate the metabolism of drugs and steroid hormones in rat liver, including 15β-hydroxylase activity (5). This "factor" was later identified as the female-characteristic secretion of GH from the pituitary (6). GH regulation of 15β-hydroxylase activity was confirmed, as hypophysectomized male and female rats treated continuously, but not intermittently, with GH exhibited an increase in 15β-hydroxylase activity, and an increase in P4502C12 protein and mRNA levels (7, 8). The physiological role of the 15β-hydroxylase, encoded by the *CYP2C12* gene, is still unknown. However, one possibility is that it may be important for protecting target organs from unwanted steroids.

Growth hormone

GH structure

GH belongs to the helix bundle peptide hormone family, in which the members have four long α-helices arranged in an up-up-down-down configuration (9). This family of proteins also includes prolactin (PRL), placental lactogen (PL), interleukins (ILs), colony-stimulating factors and macrophage growth factors, where GH, PRL and PL are believed to stem from a common ancestral gene (10). The hormones in this family do not only share structural features, they have also overlapping actions, similar receptor design and common signaling mechanisms (reviewed in (11)).

In the rat, the GH gene encodes a single polypeptide chain of 190 amino acids that has the molecular weight of 22 kDa (10). In contrast, there are two genes encoding GH in human, GH-N, which is the "normal" gene that is expressed by somatotrophs in the anterior pituitary, and GH-V, a variant GH that is expressed in the placenta (12). GH-N gives rise to a 22 kDa, 191 amino acid long protein. The sequence homology between rat and human GH is around 75%, whereas rat and bovine GH share 95% identity.

Multiple forms of GH are formed by alternative mRNA-splicing, different posttranslational modifications including glycosylation, phosphorylation and proteolytic alterations (reviewed in (13)). Four different molecular weight forms of GH (17 kDa, 20 kDa, 22 kDa and 27kDa) have been demonstrated in human serum with Western blot analysis (13). The physiological significance of the different molecular forms of GH is unclear, but there are indications that they may have different receptor affinities and functions (14, 15).

GH secretion

The somatotrophs in the anterior pituitary are the major source of GH. Nearly 10% of the dry weight of the pituitary gland is thought to consist of GH (13). This is at least 800 times the amount of any other pituitary hormone. The secretion of GH is primarily regulated by two hypothalamic peptides, GH releasing hormone (GHRH) and somatostatin (SS). GHRH stimulates growth and differentiation of the somatotrophs, expression of the GH gene, and secretion of GH, whereas SS antagonizes GHRH induced production and secretion of GH (16, 17). Other hormones influencing GH expression are glucocorticoids, thyroid hormone (T_3) and insulin-like growth factor I (IGF-I); different species, however, respond differently to T_3 and glucocorticoids. For example, the rGH promoter is induced by T_3, whereas the hGH promoter is not. On the other hand, glucocorticoids have a stimulatory effect on the hGH promoter, but do not influence the expression of rGH (18). The secretion of growth hormone can also be stimulated by growth hormone secretagogues, a family of small peptides that act through a receptor distinct from that of GHRH. An endogenous example, ghrelin, a 28-amino acid peptide that is produced mainly in the stomach, potently induces GH secretion (reviewed in (19). GH secretion is controlled by feedback mechanisms, where GH regulates its own secretion by directly inhibiting the secretion of GHRH and stimulating the secretion of SS. An indirect feedback is exerted by GH induced IGF-I, that inhibits GH expression via stimulated secretion of SS. Other factors that affect GH secretion are high serum levels of glucose or free fatty acids, via increased SS levels, and amino acids and low glucose levels that inhibit the secretion of SS.

Like other pituitary hormones, GH is secreted episodically. The sexually dimorphic pattern of GH secretion was first described in the rat, but is also present in other species including humans (1, 2). Secretion of GH is developmentally regulated. It increases in late fetal life until postpartum when it decreases. A second increase takes place during puberty. This increase is followed by a life-long slow decline in secretion (20, 21). At puberty, GH secretion becomes sex-differentiated. In the male rat, GH is secreted intermittently with peaks every three to four hours and low to undetectable levels in between the peaks. A continuous presence of GH in the plasma is characteristic of the female rat, secreting GH in more frequent peaks of lower

amplitude than males and having a higher basal GH level (3). The regulation of the sex-differentiated GH secretion involves a gender difference in the mode of hypothalamic SS and GHRH signaling (16, 17). The secretion of GHRH has been shown to occur at a higher steady state level in female than in male rats and the secretion of SS to be continuous in females and cyclic in males (22). It has been suggested that testosterone and estradiol have opposite effects on the secretory pattern of SS. The continuous presence of testosterone appears to be necessary to maintain low basal levels of GH in adult male rats, whereas estradiol has been shown to elevate basal plasma GH levels and increase the pulsatility.

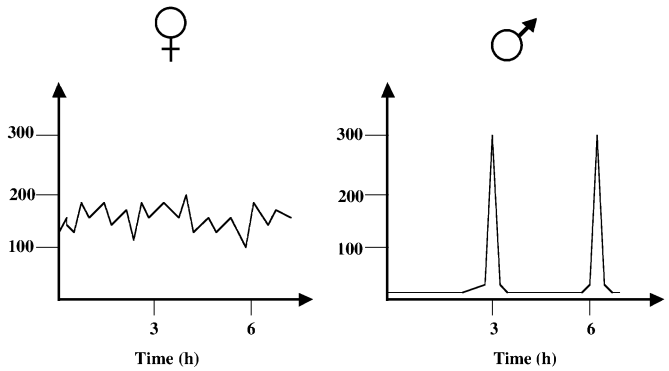

Figure 1. A schematic presentation of the GH secretory patterns in adult female and male rats. The level of GH (ng/ml) is indicated on the y-axis.

Biological effects of GH

As the name implies, one of the major functions of GH is promotion of linear growth (23). In young vertebrates, hyposecretion of GH during development leads to dwarfism and hypersecretion of GH during development leads to gigantism. In adults,

hypersecretion of GH results in the clinical condition known as acromegaly, characterized by enlarged bones of the face, hands and feet. As many as 25% of individuals with acromegaly develop type 2 diabetes, a condition that results from chronically elevated insulin levels in blood and subsequent insulin resistance (24). In healthy adults, GH exerts effects on protein, fat and carbohydrate metabolism. Moreover, GH may have effects on the central nervous system, including beneficial effects on certain functions like memory, mental alertness, motivation and working capacity (25).

Among the many metabolic effects of GH, two contradictory actions have been described: acute, early insulin-like effects and late insulin antagonistic, diabetogenic, effects. Acute insulin-like effects include hypoglycaemia, increased glucose and amino acid transport and metabolism and increased lipogenesis. The insulin-like effects are seen primarily *in vitro* or under circumstances *in vivo* where the individual has not been exposed to GH for a period of time, *e.g.* after hypophysectomy. The late insulin-antagonistic or diabetogenic effects of GH include hyperglycaemia, hyperinsulinaemia, increased lipolysis, decreased glucose transport, increased levels of free fatty acids, decreased glucose metablism and insulin resistance in humans and animals (26, 27). These insulin antagonistic effects require relatively long periods of GH treatment and are thought to reflect the major metabolic effects of GH.

The somatomedin hypothesis

The original somatomedin hypothesis postulated that GH acts primarily on the liver, which in response to GH produces somatomedin(s) that is released into the bloodstream (reviewed in (28)). Circulating somatomedin(s) would subsequently act on peripheral tissues to regulate somatic growth (29). In parallel experiments, two factors were identified and named insulin-like growth factors (IGF)-I and –II due to their functional and structural similarities with insulin. IGF-I was later shown to be the somatomedin substance that was regulated by circulating GH in rats (30). The first challenge to the original hypothesis came with the discovery that IGFs are expressed in most, if not all tissues (31). Moreover, direct effects of GH were observed. When GH was administered locally into the cartilage growth plate of one limb of hypophysectomized rats an increase in longitudinal bone growth was observed,

whereas the opposite limb, which received no GH did not show a significant increase in growth (32). Moreover, in β-cells the GH stimulated effect on proliferation was not mediated by IGF-I (33).

Recent studies in mice with targeted disruption of hepatic IGF-I gene expression, revealed no significant differences in growth and development compared with wild-type littermates, although the circulating levels of IGF-I were reduced to about 25% (34, 35). Thus, the current understanding is that somatic growth is due primarily to GH-stimulated, locally produced IGF-I, while circulating IGF-I serves mainly to provide negative feedback on GH secretion, as an increased GH level was observed in the serum of these mice.

GH regulation of cytochrome P450 genes

In addition to the *CYP2C12* gene other members of the *CYP2C* family, expressed in rat liver, are developmentally regulated at the transcriptional level by GH (4, 36). The *CYP2C11* and *CYP2C13* genes encode male-specific enzymes, whereas the *CYP2C7* gene, like the *CYP2C12* gene, is more expressed in female compared to male rat liver. Continuous GH treatment of normal male rats leads to feminization, *i.e.* induction of female characteristic genes and down-regulation of male characteristic genes. Hypophysectomy suppresses transcription of the *CYP2C7* and *CYP2C12* genes in both sexes and *CYP2C11* expression in males, and increases the expression of *CYP2C11* and *CYP2C13* genes in females (4, 37-40). The effects of hypophysectomy can be reversed by sex-characteristic administration of GH without any other hormone substitution showing that the GH secretory pattern is the major determinant in mediating the sexually differentiated expression of these *CYP2C* genes.

Evidently, GH has opposing effects on the liver depending on the temporal pattern by which the hormone is presented to the animal. Interestingly, dwarf rats maintain sex differences in GH secretion and liver steroid metabolism typical of normal rats despite a 95% reduction of pituitary GH levels (37, 41). Furthermore, restoration of the feminine-like plasma GH profile in hypophysectomized females to a concentration of 3% of the normal level restores the expression levels of *CYP2C12* (mRNA and protein) to 45% of normal (42). Normal female expression levels of

CYP2C12 require 12-25% of physiological hormone levels, whereas restoration of *CYP2C7* mRNA expression requires 50% of normal GH levels to reach female-like concentrations. Interestingly, as little as 3% of the circulating feminine GH profile could completely suppress *CYP2C11* expression in female rats. This illustrates that the suppression of *CYP2C11* is more sensitive to GH regulation than the *CYP2C12* induction.

Primary rat hepatocytes, continuously exposed to GH express both *CYP2C12* and *CYP2C7*, whereas the low basal expression of *CYP2C11* and *CYP2C13* is repressed (43-46). This suggests that the effects obtained *in vivo* by continuous GH treatment are due to a direct effect on the hepatocyte. However, although intermittent administration of GH alone in hypophysectomized rats induces a complete masculinization of *CYP2C11* expression, so far no study has demonstrated induction of *CYP2C11 in vitro* although attempts have been made. This could indicate that the *CYP2C11* induction *in vivo* does not result from the primary action of GH on the hepatocyte, that additional factors are needed together with GH, or that it has not been possible to fully mimic the male-specific GH pattern with GH pulses intervened by periods of no GH.

The GH receptor

Like other pituitary peptide hormones, GH exerts its biological effects through the interaction with specific membrane bound receptors on target cells. The GH receptor (GHR) was the first identified member of the cytokine/hematopoietin receptor superfamily, which includes receptors for PRL, ciliary neurotrophic factor (CNTF), erythropoietin (EPO), colony stimulating factors, interleukins, leptin and thrombopoietin (reviewed in (47, 48)). The receptors in this family are single membrane-spanning proteins with an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic domain with elements responsible for signal transduction, receptor internalization and downregulation. Receptor subunits that interact with more than one cytokine receptor, such as gp130, the β-subunit of leukemia inhibitory factor (LIF) receptor, the IL-3 receptor common β-chain and the γ-chain of the IL-2 receptor, are also members of this family. Although the overall sequence homology of the receptors in this family is low, the cytoplasmic domains of these receptors share some common motifs, like a membrane proximal proline rich motif, referred to as Box 1, and a cluster of hydrophobic amino acids ending with one or two positively charged residues referred to as Box 2. In GHR, Box 2 is located approximately 30 amino acids C-terminally of Box 1 (reviewed in (49)). Moreover, common motifs are also present in the extracellular domain, where two pairs of cysteins are usually found. However, the GHR has seven cysteins in this region. Furthermore, GHR has a divergent Trp-Ser-any-Trp-Ser motif in its extracellular domain that is conserved in all other members of the family.

The human (h) GHR and the hPRL receptor (PRLR) are 30% homologous, with the highest sequence identity in the extracellular, hormone binding domain (50, 51). Interestingly, hGH does not only bind to the hGHR but also to the hPRLR (52), whereas hPRL can only interact with the hPRLR. Also in the rat, hGH can interact with both GHR and PRLR, and mediate both somatogenic and lactogenic effects (53). This is in contrast to bovine and rat GH, which are considered to be exclusively somatogenic.

The primary transcript that generates the GHR mRNA can undergo alternative processing to produce several related mRNAs, including transcripts that encode for the circulating GH binding protein (GHBP), as has been demonstrated in rodents (see below), and a shorter membrane-anchored hGHR that lacks most of the intracellular domain (54-56). In addition to this, the use of alternative promoters and transcription start sites in the GHR regulatory region generates transcript forms that differ only in the 5'- untranslated region (UTR) (for review see (57)). Interestingly, continuous, but not intermittent, GH stimulates transcription of a specific GHR transcript, GHR_1, in rat liver (58, 59). The GHR_1, like other transcripts differing in the 5'UTR, is not thought to give rise to a different GHR protein. However, it has been suggested that there are different forms of GHR that may mediate insulin-like and lipolytic responses in rat adipose tissue (60), and there is immunological evidence for different GHR forms, that are probably generated through posttranslational modifications (61). It is known that GHR can undergo posttranslational modifications, such as glycosylation and ubiquitination (62).

GH binding protein

In addition to the membrane bound GHR, there is a circulating form of the GHR extracellular domain, called the GH binding protein (GHBP) (63). About 45% of GH in plasma is complexed with the high-affinity binding protein, GHBP, and another 5% of GH is bound to a low-affinity binding protein that is not related to the receptor (64). However, the exact role of GH binding to GHBP is unclear. Experimental evidence exists both for an inhibitory role of GHBP (by sequestering GH away from cellular GHR or by competing with surface GHRs for GH) (65) and for a positive role of GHBP (by delaying renal clearance of GH in the intact animal). However, *in vivo* the GHBP appears to have an agonistic effect on GH signaling (66). GHBP is formed by two distinct mechanisms in different species. In rodents, GHBP is mainly derived by alternative splicing of the primary GHR transcript, where the receptor's transmembrane and cytoplasmic domain-encoding regions are replaced by a short hydrophilic peptide (67). This results in a protein, largely composed of the GHR extracellular domain. On the other hand in humans and rabbits, GHBP is mainly formed by proteolytic cleavage of the membrane bound full-length receptor at or near the external face of the membrane such that the extracellular domain is released as

GHBP. A specific metalloprotease seems to be responsible for this cleavage (reviewed in (68)). GHBP shedding by this metalloprotease has also been demonstrated in a murine cell line, and it has therefore been suggested that proteolytic cleavage may be an additional route whereby GHBP is formed in rodents (69). Moreover in humans, later studies have revealed alternative splice forms of the human GHR that lacks the majority of the cytoplasmic domain (54). The expression of these human splice variants only accounts for a small proportion of the total GHR transcript, but it produces a short isoform that can modulate the function of the full-length receptor, inhibit signaling and generating large amounts of GHBP (55, 56). Thus, it seems like both alternative splicing and proteolytic cleavage can be involved in the formation of GHBP in both humans and rodents.

Receptor dimerization

GH transduces its effects by binding to its membrane bound receptors, via a sequential homodimerization mechanism (70). The crystal structure of hGH bound to the extracellular domains of two identical hGHR shows that GH has two distinct binding faces on either side of the molecule, known as site 1 and site 2, which interact with essentially identical receptor domains (71). It has been demonstrated that site 1 in hGH has a higher affinity than site 2 for hGHR. This leads to dose response curves with biphasic appearances due to reduced receptor dimerization when very high concentrations of hormone are used (72, 73).

GH dimerization involves three distinct molecular recognition events, where GH binding to first one and then to a second receptor, is followed by an inter-receptor interaction (for review see (74)). This leads to a conformational change that seems to be important for full biological response (75, 76). In addition, a large fraction of GHRs activated by GH undergo a GH dependent disulfide linkage (77), although the physiological role of this linkage is uncertain.

Receptor turnover

Down-regulation of signal transducing membrane receptors is part of a programmed cascade of events leading both to extinction of signaling pathway(s) and to rapid

degradation of primary messengers, the receptor and its ligand. The ligand-receptor complex is internalized via clathrin-coated vesicles and subsequently transported via endosomes to lysosomes (78, 79). Both the endocytosis and the transport to lysosomes require an active ubiquitin-conjugation system and can be inhibited by proteasome inhibitors (80-82). In particular, a 10 amino acid motif (ubiquitin-dependent endocytosis, UbE, motif) in the cytoplasmic part of the GHR seems to be essential for this process (62). Endocytosis and degradation of the GHR require the ubiquitin system, and occur in the absence of ligand binding. In addition to receptor degradation, there are data indicating that ligand bound, dimerized and internalized GHR can be transported to the nucleus where it may have some function (83, 84).

GH signal transduction

Janus kinases

The mechanisms by which GHR activation elicit the diverse responses to GH remained elusive for a long time. The cloning of the GHR, shed no light on the mechanisms, since the deduced amino acid sequence of the GHR bore no homology to proteins with known function (51). However in 1993, a GHR associated tyrosine kinase, Janus kinase 2 (JAK2), was identified (85). Upon GH induced dimerization of the extracellular domains of GHR the cytoplasmic domains, and thereby the associated JAK2 proteins are brought together. At close proximity, one JAK2 acts as a substrate for the other and they both become trans-phosphorylated and thus activated. Activated JAK2 can phosphorylate GHR at tyrosine residues, which then serve as docking sites for SH2 containing signaling molecules. In addition to the GHR, JAK2 tyrosine phosphorylates a number of signaling molecules triggering a signaling cascade (reviewed in (86)). In addition to the GHR, JAK proteins are associated with other receptors in the hematopoietin/cytokine family, and are important for the signal transduction initiated by ligand binding to these receptors.

The JAK family of tyrosine kinases includes JAK1, JAK2, JAK3 and TYK2 (reviewed in (87)). An interesting feature of these proteins is the absence of SH2 or SH3 domains, commonly found in other tyrosine kinases. Instead, the four JAK family members have seven highly conserved domains (JH1-JH7), where JH1 and JH2 are kinase related domains. However, only the JH1 kinase domain appears to be functional. The JH2 domain harbors considerable homology with tyrosine kinase domains but lacks certain critical amino acids required for a functional kinase. Both the tyrosine kinase domain (JH1) and the pseudo-kinase domain (JH2) are housed in the carboxy terminus of the protein. The precise functions of the JH3-JH7 domains and the pseudo-kinase domain are not fully understood. Considering the variety of interactions and functions performed by the JAK proteins it seems plausible that these domains facilitate key functions like protein-protein interactions, recruitment of substrates, etc.

STAT proteins

Important factors by which GH and other cytokines regulate gene transcription are the signal transducers and activators of transcription (STAT) proteins. STAT proteins, originally identified in interferon (IFN) signaling (88), are latent, SH2 domain containing cytoplasmic factors. Currently seven mammalian STAT proteins, encoded by individual genes, have been identified; STAT1, STAT2, STAT3, STAT4, STAT5a (originally identified as mammary gland factor, MGF), STAT5b and STAT6 (89). STAT proteins bind, via its SH2 domains, to tyrosine phosphorylated residues of receptors, where they can be subjected to tyrosine phosphorylation by JAK proteins. Phosphorylated STAT proteins dimerize by an interaction between the SH2 domain of the individual STAT proteins and a phosphorylated tyrosine of the other STAT protein, and translocate to the nucleus where they activate gene transcription by binding to specific target DNA elements (reviewed in (90)). GH has been shown to specifically activate STAT1, STAT3 and STAT5 in rat liver (91), where STAT5 seems to be most responsive to GH. The STAT5a and STAT5b proteins are highly homologous, being more than 90% identical at the amino acid level, and diverge primarily in the COOH-terminal transactivation part (92, 93). STAT5b has been shown to be more abundant in rat liver than STAT5a (94), and has been implicated to be the main isoform activated by the male characteristic intermittent GH pattern (91). Repeated application of GH pulses to cells of liver origin, stimulates repeated cycles of STAT5b activation, provided there is an interpulse interval of at least 2.5-3h (95). Furthermore, the intermittent stimulation of hepatocytes *in vivo* by successive plasma GH pulses found in adult male rats triggers a rapid and repeated tyrosine phosphorylation of STAT5b. By contrast, in adult female rats, the more continuous pattern of GH exposure results in lower JAK2 activation, and a low steady-state level of transcriptionally active, nuclear STAT5 than in males (96-98).

In addition to tyrosine phosphorylation, STAT molecules may undergo serine phosphorylation (reviewed in (99)), and GH has been shown to induce serine phosphorylation of STAT5 (91). STAT5b is serine phosphorylated at Ser730 and STAT5a at the corresponding Ser725 and also at Ser779, not present in STAT5b (100, 101). Phosphorylation of these serines can modulate the transcriptional activity of

STAT5a and STAT5b both negatively and positively in a promoter dependent manner (102).

DNA binding of STAT proteins

Most STAT proteins bind to GAS (IFN-γ activated sequence)-like elements (GLE), harboring the consensus $TTCN_nGAA$ with n = 4 for STAT6 and n = 3 for the optimal binding of other STAT proteins. However, fine differences in DNA binding specificity of the various STAT proteins depend also on the middle nucleotides in the GLE and on those immediately adjacent to it (reviewed in (103)). It has been demonstrated that homodimers of STAT5a and STAT5b bind to different GLEs with different affinities. This is thought to be a result of a glycine residue at position 433 in STAT5b, and a glutamic acid residue at the corresponding position in STAT5a (104). In general, the DNA sequence for optimal binding of STAT5a and STAT5b appears very similar (105), but a difference between STAT5a and STAT5b complex binding is the ability of STAT5a, but not STAT5b, to form tetrameric complexes on GLEs (see below) (105, 106).

Tetramers of STAT proteins

In addition to binding as dimers, STAT1, STAT4 and STAT5a have been shown to form tetrameric complexes on GLEs (106-111). Such cooperative binding can serve to selectively bind different STAT proteins on a promoter that contains multiple GLEs, and this kind of DNA setting seems common among several STAT5 responsive genes (106, 108, 112, 113). Interestingly, it has been suggested that STAT5 dimers are not competent to trans-activate all STAT5 responsive promoters and that further oligomerization (*e.g.* tetramerization) is necessary for the activation of certain genes (107). Moreover, tetramer formation enables STAT5 to extend its range of target sites by virtue of additionally utilizing non-consensus sites for binding, *e.g.* STAT5 tetramers can bind to tandem elements where only one is a perfect consensus binding element (106, 108). A spacing of 6 bp between the GLE sites has been determined as favorable for tetramer formation (105). Nevertheless, tetramers are formed on the promoters of CIS and IL-2 receptor α where the tandem elements are spaced by 8 and 10 bp respectively (106, 108). It has been suggested that

tetramers may form when the STAT5 molecules binding to the two sites face the same side of the DNA helix (108). Tetramer formation is mediated by interaction via the N-terminal regions of the STAT proteins (109-111); tryptophan 37 is a key residue at the interaction interface for STAT5 proteins (107). However, although both STAT5a and STAT5b have tryptophan 37, there are data indicating that only STAT5a can form tetramers (105, 106).

Truncated STAT5 proteins

The existence of naturally occurring carboxy-truncated forms of STAT proteins including STAT5a and STAT5b has been documented in myeloid cell lineages and in liver cells. Truncated STAT5 forms are produced either by alternative splicing (94, 114, 115) or by proteolytic cleavage (116-119), and it is possible that these mechanisms occur in different cell types or alternatively co-exist in the same cell. A physiological significance of truncated STAT5 is indicated by phenotypic changes of myeloid progenitor cells upon introduction of "non-cleavable" STAT5 (116). Furthermore, a specific nucleus associated STAT5 protease has been identified which is able to cleave both active and inactive STAT5 (116), and in a recent study, activity of a STAT3 and STAT5 specific protease was demonstrated both in cytosolic and nuclear extracts (119). The identified carboxy truncated STAT5 proteins lack their transactivation domain, and have consequently been shown to inhibit the expression of STAT5 regulated genes by either heterodimerization with full-length STAT5 proteins or by binding as homodimers (114). Noteworthy, since tetramer formation is mediated via the N-terminal part of STAT proteins (109-111), it is likely that carboxy-truncated STAT5, in addition to full-length STAT5 proteins, form tetrameric complexes.

Pysiological function of STAT5 proteins

To assess the role of the individual STAT5 proteins, mice have been derived in which STAT5a and STAT5b are individually or simultaneously disrupted. Despite the similarities of the two STAT5 proteins, different phenotypes are associated with the disruption of the individual genes (120-124). STAT5a deficient mice exhibit defective mammary gland development and as a consequence, female mice fail to lactate after

parturition. Moreover, disruption of either STAT5a or STAT5b led to the loss of a female-specific GH regulated hepatic *Cyp2b* enzyme in female mice (122). In male mice, disruption of STAT5b resulted in decreased body growth rate and reduced expression of male specific *Cyp* genes to levels observed in wild-type female mice. Another gene whose expression was negatively affected by STAT5b disruption was *igf-1* (123). In contrast, the expression in male mice of female-predominant *Cyp* genes, increased to a level intermediate to those observed in wild-type males and females when the STAT5b gene was disrupted. This indicates that STAT5b is essential for GH regulation of certain *Cyp* genes and *igf-1* and that STAT5b can have both positive and negative impact on expression of female specific *Cyp* genes in mice.

Other signaling pathways activated by GH

In addition to the JAK/STAT pathway, GH binding to its receptors can initiate the mitogen-activated protein kinase (MAPK) pathway and the pathway involving the insulin receptor substrate (IRS) and phosphatidylinositol 3-kinase (PI 3-kinase) (reviewed in (86, 125). Engagement of these pathways, like activation of STAT proteins, appears to require JAK2 activation. To date, the only reported JAK2-independent effect of GH is Ca^{2+} entry via L-type calcium channels (126). GH may induce phosphorylation of ERK1/2 via the indirect association of JAK2 to growth factor binding protein 2 (Grb2)-son of sevenless (SOS) complexes through SH2 containing, Shc, proteins, or via trans-phosphorylation of the epidermal growth factor receptor by JAK2 which then recruits the Grb2-SOS complex. This results in a signaling cascade starting with the activation of the small GTP-binding protein Ras, which in turn activates the serine/threonine kinase Raf. Raf activates the tyrosine/serine/threonine kinase MEK, which is followed by MEK activation of the extracellular signal regulated kinases ERK1 and 2. Activated ERK proteins can phosphorylate a number of proteins like cytoplasmic phospholipase A_2 ($cPLA_2$), phospholipase Cγ (PLCγ), and nuclear proteins including Elk-1, c-fos, and c-Jun (127). Of these potential substrates, GH has been shown to stimulate the phosphorylation of, $cPLA_2$ and Elk-1 (128, 129).

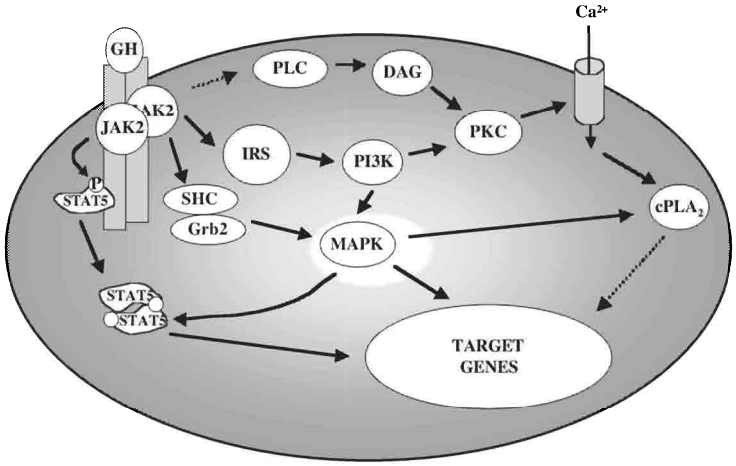

Figure 2. GH signaling pathways.

IRS is another molecule by which GH elicits some of its cellular effects. GH stimulates phosphorylation of IRS1 and IRS2, presumably via JAK2 activation. The phosphorylated IRS then recruits and activates several proteins, such as the p85 regulatory subunit of PI 3-kinase (130). Activated PI 3-kinase can activate ERK1/2, and regulate the activity of certain isoforms of protein kinase C (PKC). PKC activation in response to GH can also occur as a result of PLC activation leading to the formation of the PKC activator, 1,2-diacylglycerol (DAG).

Regulatory mechanisms of GH signal transduction

GH signaling pathways are modulated at several levels. This includes both negative and positive regulation, where the net effect of these regulatory processes determines the cellular response. Upon GH exposure internalization of the ligand bound receptor occurs, which is followed by recycling, where receptors reappear at the membrane within 2h (131). The internalization process leads to less GHR at the cell membrane after a GH pulse, and consequently a lowered GH responsiveness. A recent report

suggests that desensitization of the JAK/STAT pathway may be a result of down-regulation of GHR levels (132). Termination of GH activated JAK/STAT signaling is thought to involve activation and/or recruitment of protein tyrosine phosphatases to the GHR-JAK2 complex. Dephosphorylation of the GHR terminates the recruitment of signaling molecules to the complex and may also lead to internalization and degradation of the receptor. However, the responsible phosphatases are unknown. Two candidates are the SH2 domain-containing phosphatases SHP-1 and SHP-2, which have been implicated in dephosphorylation of tyrosines on JAK2 and GHR (133-135). Additionally, recent studies have indicated that the membrane spanning phosphatase, CD45, negatively regulates JAK/STAT signaling stimulated by IL-3, IL-4, EPO and IFN-γ (136). The activity of STAT proteins can also be inhibited by members of the protein inhibitors of activated STAT (PIAS) family that are constitutively expressed and suggested to titrate the amount of activated STAT (137, 138). Furthermore, STAT5 mediated transcription has been shown to be negatively affected by c-Cbl that is thought to direct STAT5 to proteosomal degradation (139). Another protein that is suggested to act as a negative regulator of GH-JAK2 signaling, is a member of the signal regulatory protein (SIRP) family of membrane bound proteins, SIRPα. Interestingly, GH induced tyrosyl phosphorylation of SIRPα leads to the recruitment of SHP-2 that in turn de-phophorylates SIRPα and most likely also JAK2 (140, 141). However, SHP-2 has also been suggested to have a positive role in GH signaling (142). Other positive regulatory mechanisms of GH JAK/STAT signaling have been described. Activated JAK2 recruits the SH2 domain containing protein SH2-B (143), where after SH2-B increases the kinase activity of JAK2. This leads to enhanced activation of downstream signaling proteins such as STAT5b (144).

Desensitization of JAK/STAT signaling via CIS/SOCS proteins

The family of the cytokine inducible SH2 protein (CIS)/ suppressors of cytokine signaling (SOCS) proteins have also been implicated in negative regulation of cytokine signal transduction via the JAK/STAT pathway. *Socs-1* was identified in 1997 by three different groups, on the basis of its interaction with JAKs (145), its antigenic cross-reactivity with part of the SH2 domain of STAT3 (146) and its ability to inhibit IL-6 signaling (147). Database searches using the predicted amino acid sequence of SOCS1 revealed that at least 20 proteins in mice and humans share

sequence homology within a 40-residue C-terminal motif referred to as the SOCS box (148). One of these genes, *cis*, had previously been cloned as an immediate early gene induced by several cytokines like IL-2, IL-3 and EPO (149). The SOCS box containing proteins were classified into five subfamilies based on their additional domains. Proteins with a central SH2 domain in addition to the SOCS box were termed CIS/SOCS proteins; CIS and SOCS1-7. Interestingly, a comparison of the primary amino acid sequence of CIS/SOCS subfamily members shows that pairs of CIS/SOCS proteins are more similar to each other than to other CIS/SOCS proteins. Thus, CIS and SOCS2, SOCS1 and SOCS3, SOCS4 and SOCS5, and SOCS6 and SOCS7 all form related pairs (148). Within the SOCS family, the actions of SOCS4-7 remain poorly understood.

Transcripts encoding SOCS1-3 and CIS are often present in cells at low or undetectable levels, but are rapidly induced by a broad spectrum of cytokines, both *in vitro* and *in vivo*. Clearly SOCS expression is tightly regulated at the transcriptional level, and it is apparent that the STAT family of transcription factors contributes significantly to the transcriptional upregulation of the *cis* and *socs1-3* genes (146, 150, 151). For example, the promoter of the *cis* gene contains four STAT5 binding sites, all of which are required for EPO dependent activation of the *cis* promoter in reporter assays (106, 150). Furthermore, the induction of CIS mRNA in response to IL-3 is inhibited in cell lines that express a dominant negative STAT5 (152) and CIS expression in mouse ovary is abolished in mice lacking both STAT5a and STAT5b (124).

Consistent with the discovery of SOCS1 as an inhibitor of IL-6 signaling, it is now clear that overexpression of CIS, SOCS1 and SOCS3 in cell lines results in the inhibition of signaling by a wide range of cytokines, hormones and growth factors (reviewed in (153)). Although SOCS2 also inhibits cytokine signaling, it is not as potent as CIS, SOCS1 and SOCS3. Furthermore, SOCS2 has been described to have a dual role; it inhibits GH induced STAT5 dependent gene transcription at low concentrations, and augments GH signaling at higher concentrations (154).

Molecular mechanisms of SOCS action

The members in the CIS/SOCS family can inhibit cytokine signaling by different mechanisms (Fig. 3). For example, SOCS1 has been shown to inhibit cytokine signaling via association with JAK proteins and inhibition of their catalytic activity (145, 146, 155). Also SOCS3 inhibits JAK activity; SOCS3 is associated with tyrosine phosphorylated receptors and thereby brought into vicinity of the JAK proteins (156). In contrast, neither SOCS2 or CIS inhibits JAK activity, instead CIS is thought to compete with STAT proteins for binding to tyrosine phosphorylated receptors and thereby inhibit STAT phosphorylation (157).

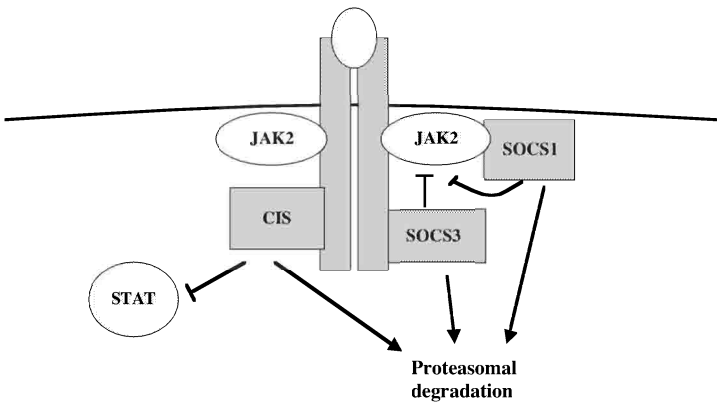

Figure 3. A schematic illustration of molecular mechanisms whereby SOCS/CIS proteins function as negative regulators of cytokine signaling. See text for details. Adapted from Krebs et al. (153).

An additional model for SOCS function has been described, where SOCS proteins activate signaling proteins for degradation by the proteasome (reviewed in (158)). All SOCS box containing proteins tested associate with a complex containing elongin B and C (elongin BC) via their SOCS box (159). In turn, the elongin BC complex binds

to a E3 ubiquitin ligase termed cullin-2. Given that SOCS proteins contain SH2 domains that interact with tyrosine phosphorylated proteins, it is possible that they can act as adapters that bring ubiquitin ligases into the vicinity of activated signaling proteins and cause their ubiquitination and degradation. Also the SOCS proteins could be ubiquitinated and degraded in this process.

The physiological function of SOCS proteins

To investigate the physiological function of SOCS proteins, knock-out and transgenic mouse models have been developed. Analysis of mice lacking SOCS1 suggests that it is critical for negative regulation of IFN-γ signaling and for differentiation of T cells (160-162). Studies on *socs3* suggest that it may play a role in the regulation of fetal liver erythropoiesis, probably through its ability to modulate EPO signaling (153, 163). In transgenic mice overexpressing CIS, the GH and prolactin induced tyrosine phosphorylation of STAT5 was significantly inhibited in liver and mammary gland, respectively (164). In addition, the phenotype of CIS transgenic mice resembled that of mice lacking STAT5a and/or STAT5b, indicating that CIS might contribute to the negative regulation of STAT5 (164). Interestingly, when the *socs2* gene was disrupted in mice, increased growth was observed; these mice grew 30-40% larger than their wild-type littermates (165). This suggests that SOCS2 acts to negatively regulate growth-promoting cytokines such as GH and IGF-I. Moreover, in favor for this hypothesis *socs2-/-* mice are phenotypically similar to both GH and IGF-I transgenic mice (165, 166). Surprisingly, SOCS2 transgenic mice are not growth deficient as one would expect from the phenotype of the *socs2-/-* mice, but grow larger than wild-type mice (167). This is in line with the *in vitro* data suggesting that SOCS2 may have dual effects on GH signaling (154).

Liver enriched transcription factors

At least six families of liver enriched transcription factors have been described. These are the families of hepatocyte nuclear factors (HNF) 1, HNF3 (also called HNF5), HNF4 (also called HNF2), D-binding protein (DBP), CCAAT/enhancer binding protein (C/EBP), and HNF6. These proteins were originally suggested to be liver specific transcription factors, but later studies have revealed their expression in other organs as well, and thus they are now termed liver enriched factors.

C/EBP proteins

There are six members, C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ, belonging to the family of C/EBP proteins (reviewed in (168)). Further diversity is added by the generation of differently sized polypeptides, *e.g.* C/EBPα mRNA can give rise to two polypeptides, 42 kDa and 30 kDa, with the latter having a lower activation potential, and C/EBPβ mRNA can produce at least three isoforms. The transcription factors in the C/EBP family have a highly conserved basic-leucine zipper (bZIP) domain at the C-terminus. The bZIP domain consists of a basic amino-acid-rich DNA binding region followed by a dimerization motif termed the "leucine zipper". Because of the high conservation in the bZIP domain, the different C/EBP proteins are able to form heterodimers, in addition to homodimers (169). In contrast to the bZIP domain, the N-termini of the C/EBP proteins are quite divergent except for three short subregions that are conserved in most members. These three regions are involved in interaction with components of the basal transcription apparatus.

HNF3 proteins

The HNF3 proteins were originally identified as factors that mediate liver-specific transcription of the transthyretin (*ttr*) gene via recognition of a consensus DNA binding site (reviewed in (170, 171)). These factors are mainly present in the liver, but all isoforms are also found in intestine and stomach, and one or two isoforms have been detected in lung, heart, adipose tissue and testis. The three members present in liver, HNF3α, β and γ, share over 90% homology in the DNA binding domain and

have nearly identical DNA recognition properties. HNF3 proteins are known to bind to their recognition elements as monomers. As the DNA binding domain of HNF3 proteins is homologous to a *Drosophila* protein called forkhead, this family is sometimes referred to as the fork head/HNF3 family of transcription factors. The binding motif, called the "winged helix", consists of a helix-turn-helix structure that together with protein loops resembles the shape of a butterfly with two "wings".

HNF4 proteins

HNF4 is primarily expressed in the liver, gut, kidney and pancreas and has been shown to play an essential role in embryonic development (172). In adult rat liver two main isoforms of HNF4 are generated via differential splicing, with the longest isoform, HNF4α2, being predominant (173, 174). As HNF4 is a member of the nuclear receptor family (175), it has two zinc finger motifs that serve as interfaces in both protein-DNA and protein-protein interactions. HNF4 has been considered as an orphan receptor, but fatty acyl-CoA thioesters have been suggested as potential ligands (176). Homodimers of HNF4 bind to specific elements resembling direct repeats with the spacing of 1-2 nucleotides (177). Interestingly, the DNA binding affinity of HNF4 to an element in the promoter of *hnf6* has been demonstrated to increase in response to GH exposure (178). Furthermore, CBP mediated acetylation of HNF4 appears crucial for proper nuclear retention of HNF4. Acetylation also increases HNF4 DNA binding activity and its affinity for CBP itself, and is required for target gene activation (179).

HNF6 proteins

The HNF6 protein was originally characterized as a transcriptional activator of the 6-phophofructo-2-kinase gene expressed in liver (180), but it is also present in the brain, spleen, pancreas and testis (180). Transfection studies have shown that HNF6 stimulates the expression of an array of different genes in the liver including the transcription factors HNF3β and HNF4 (180-183).

HNF6 is the founding member of the ONECUT/HNF6 class of transcription factors, characterized by a DNA-binding domain consisting of a single cut domain and an

unusual type of homeodomain. Three members of this class have been found in mammalian species, namely HNF6, One-cut-2 (OC-2) and OC-3 (184, 185). A cut homeodomain protein was originally described as the product of the *Drosophila cut* gene, which contains three cut domains upstream of a homeodomain (186, 187). In contrast, members of the HNF6 family have a single cut domain. Furthermore, the HNF6 homeodomain differs from homeodomains in other proteins, by the nature of residues 48 and 50 (180). Residue 48, part of the hydrophobic core, is a phenylalanine in HNF6, whereas it is usually a tryptophan in other homeodomain proteins, and residue 50, located in the DNA recognition helix and a key determinant of sequence-specific DNA binding, is a methionine in HNF6, an amino acid not found at this position in other homeodomain proteins. These characteristics of the homeodomain and the presence of a single cut domain have been evolutionarily conserved; a similar DNA binding sequence is encoded by the genome of *Caenorhabditis elegans* (180, 188).

HNF6 activation of different genes, such as the *hnf3β* and the *ttr* genes, involves different mechanisms (189). The cut domain of HNF6 is required for DNA binding to the specific response elements in both genes, whereas the HNF6 homeodomain is involved in DNA binding to the response element in *ttr* and in transcriptional avtivation of *hnf3β*. Transcriptional activation of a target gene to which HNF6 binds without requirement of the homeodomain, like the *hnf3β* gene, involves interaction between HNF6 and the coactivator CREB-binding protein (CBP) (189). This interaction depends both on the LXXLL motif of the cut domain and on the F48M50 dyad of the homeodomain. On the other hand, transcriptional activation of the *ttr* gene by HNF6, where the homeodomain is required for DNA binding but not for transcriptional stimulation, does not involve interaction with CBP but with the coactivator p300/CBP-associated factor (p/CAF) (189). Moreover, this interaction is independent of the LXXLL motif in the cut domain.

Two isoforms of HNF6, generated by alternative splicing, have been described in the rat, HNF6α (465 residues) and HNF6β (491 residues) (190). They differ only by an insert of 26 amino acids in the linker between the cut domain and the homeodomain

in HNF6β (180). HNF6α or HNF6β bind to target DNA elements as monomers with slightly different affinities for different DNA targets sites (181).

Network of transcription factors in liver

Liver enriched transcription factors make up a regulatory network with cross-regulatory and autoregulatory loops and it is evident that GH has a regulatory impact on this network (see Fig. 4). For example, up-regulation of the *hnf6* gene occurs via GH induced binding of STAT5 and HNF4 to the proximal promoter of *hnf6* (178). Furthermore, a single injection of GH to hypophysectomized rats causes a rapid and transient decrease in the amount of C/EBPα protein, which returns to normal levels 6h after the injection. This decline in C/EBPα levels correlates with a transient stimulatory effect of GH on the *hnf6* gene (191). However, in hepatocytes continuously treated with GH for longer time periods an increase in C/EBPα levels is evident (192). Moreover, the HNF6 protein stimulates expression of both the *hnf4* and *hnf3β* genes (182), and C/EBPα is known to stimulate the *hnf3β* gene (193), which in turn controls the expression of the *hnf4* gene (194). HNF4 expression has been demonstrated to precede the expression of HNF1α and HNF1β in all species where analyzed. Consistent with this a functional binding site for HNF4 has been demonstrated in the promoter of the *hnf1α* gene, however an HNF1 binding site has also been identified in the hnf4 promoter suggesting a reverse regulation as well (reviewed in (195)). Studies with *hnf6* knock-out mice suggest that HNF6 inhibits the expression of the *oc2* gene, a member of the ONECUT class of transcription factors to which also HNF6 belongs (196). Both OC2 and HNF6 act in trans on the *hnf3β* gene (185). In addition, auto-regulation of the *c/ebpα*, *hnf3β* and *hnf4* genes has been described (197-199). This network is likely to be even more complex and to involve additional factors. Moreover, the regulation of the factors in the network may be dependent on both temporal and spatial events.

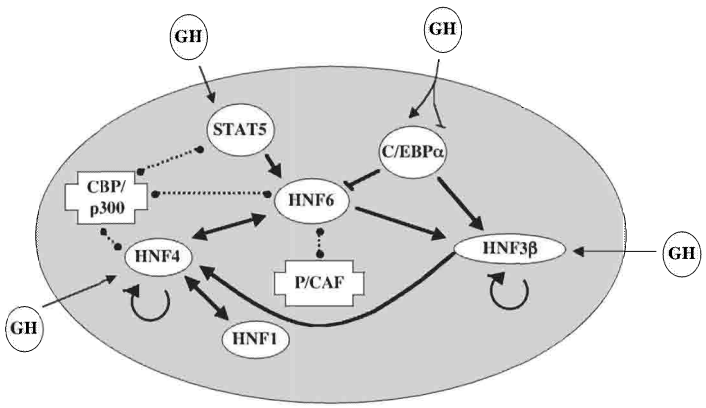

Figure 4. Scheme of the GH regulated network of transcription factors in rat liver. Suggested interactions with the coactivators CBP and p/CAF are indicated by dotted lines.

GH regulation of the CYP2C12 gene

The rat hepatic *CYP2C12* gene is transcriptionally activated by the female-characteristic continuous pattern of GH secretion. From studies using primary hepatocytes it is known that the induction requires on-going protein synthesis (44) and that PKC has a permissive role (200). Moreover, activation of $cPLA_2$ together with subsequent P450-catalyzed eicosanoid metabolism is thought to be involved (128).

Several different nuclear factors have been proposed to contribute to the transcriptional regulation of the gene. The distinct transcription factors C/EBPα and C/EBPβ bind to an element in the proximal promoter, and transient transfection of a C/EBPα expression vector into primary hepatocytes increases the expression of *CYP2C12* (201). In this study GH treatment of primary hepatocytes for up to 3h, did not alter C/EBP protein levels or its binding to a putative regulatory element in the proximal promotor of the gene. However, when primary hepatocytes are treated for longer time periods, an increase in C/EBPα levels is evident (192). Another liver-enriched transcription factor, HNF4, also binds to an element in the proximal promoter of *CYP2C12*, and upon co-transfection of an HNF4 expression vector and a *CYP2C12* reporter gene construct in COS7 cells, a 3-fold increase in reporter gene activity was observed (202). The importance of HNF4 in the regulation of the *CYP2C12* gene has been confirmed in *in vivo* transfection experiments (113). Moreover, the transcription factors HNF3α, HNF3β and HNF6 enhance transcription from a *CYP2C12* promoter construct, and a synergistic interaction between HNF3β and HNF6 has been suggested by Delesque-Touchard *et al.* (203). These authors propose that STAT5 down-regulates HNF3- and HNF6- induced expression of *CYP2C12*. Apparently the STAT5 mediated down-regulation does not involve DNA binding of STAT5 and the mechanism remains elusive. An inhibitory role of STAT5 in the regulation of *CYP2C12* gene expression would be in line with the finding that a subset of female dominant mouse *Cyp* genes is upregulated in livers of STAT5b deficient male mice (120, 122). However, STAT5 has been elegantly demonstrated to convey GH regulation of the *CYP2C12* gene by binding to elements in the upstream

5'flanking region, in concert with the transcription factors HNF4 and HNF6 binding to the proximal promoter of the gene (113). In addition, a GH-regulated nuclear factor (GHNF) with several binding sites in the promoter region has been proposed to be of importance for GH activation of the *CYP2C12* gene (204), but it has so far not been characterized.

Aims of the present investigation

Understanding of the molecular mechanisms of GH action includes understanding of how temporal and sex-different patterns of GH exposure direct differences in gene expression in target cells. The *CYP2C12* gene expressed in female rat liver constitutes the prototypical sex differentiated gene induced by the female specific pattern of GH secretion. Aspects on GH regulation of this gene are the main focus of this thesis work. The specific studies have concerned:

- The dimerization model for rat GH induction of *CYP2C12* and IGF-I in primary rat hepatocytes.

- The binding of liver nuclear proteins to potential regulatory elements in the *CYP2C12* gene, and the functional role of this in GH regulation of the gene.

- GH dependent desensitization mechanisms of the JAK/STAT pathway.

Comments on methodology

Primary hepatocytes

Primary rat hepatocytes maintain their adult liver-characteristic phenotype and respond to various inducers of P450 enzymes when cultured on a basement membrane matrix derived from the Engelberth-Holm Sarcoma (EHS) (43, 205). It was later shown that overlaying hepatocytes, cultured on plastic, with EHS-matrix was as effective in maintaining the degree of differentiation and GH responsiveness as when the cells were cultured on EHS-matrix. Overlaying of proliferating hepatocytes with EHS-matrix led to an increase in HNF4 protein and DNA binding activity. STAT5 DNA binding activity on the other hand was only slightly affected, whereas HNF3 and STAT3 DNA binding activities were reduced in the presence of EHS-matrix (206). To test whether overlaying of hepatocytes with EHS-matrix was applicable to our studies, we compared GH induction of *CYP2C12* gene expression in hepatocytes cultured on EHS-matrix with cells cultured on plastic and overlaid or not with EHS-matrix. In contrast to previous studies (43), we found no difference in GH induction of *CYP2C12* between the culture systems, and as yet we have not been able to explain this. There are great advantages being able to maintain differentiation and GH responsiveness of the cells in the absence of EHS. It reduces experimental cost and saves experimental animals (the EHS tumor is propagated in mice), but most important from an experimental point of view, it enables liposomal mediated transfection of the cells. In some way EHS interferes with this kind of transfection technique.

Solution hybridization

The mRNA levels of P4502C12 and IGF-I have been analyzed by specific solution hybridization assays. The method is based on specific hybridization in solution between an ^{35}S-labeled cRNA probe and the complementary mRNA of interest. An advantage with this method is that the hybridization signal is quantitative and directly proportional to the amount of specific mRNA in the sample (207). However, there is no separation or visualization of the transcripts hybridizing with the probe, and it is

therefore important to determine the specificity of the probes by analyzing them with electrophoretic techniques. The probes used in this study have previously been shown to give specific and accurate determinations of the mRNA levels (38, 208).

Antibodies in Western blot and gel shift experiments

Both in Western blot and gelshift experiments antibodies against STAT5 have been used. The STAT5a and STAT5b proteins are very similar (>90% identity) and diverge mainly in the most carboxy-terminal part (92, 93), therefore isoform specific antibodies are directed against this end. However, the unique carboxy-terminal part of STAT5b is very short and it is difficult to find specific antibodies for this isoform. The STAT5b antibody used in the Western blot experiments in paper III (06-554, Upstate Biotechnology, directed against amino acids 774-787 of human STAT5b) is by all means specific (93). Since this antibody is not suitable for use in gel shift experiments, another antibody (sc-835x, Santa Cruz Biotechnology, directed against amino acids 763-779 of mouse STAT5b), claimed to be STAT5b specific (95), was used for supershift analysis. However, the specificity of this antibody has been reevaluated and it is now considered to recognize STAT5a as well. This does not to any major extent affect the interpretations of our results, since a specific STAT5a antibody (see Fig. 5) (sc-1081x, Santa Cruz Biotechnology, directed against amino acids 774-793 of mouse STAT5a), has been used in parallel.

Figure 5. Alignment of peptide sequences of the carboxy teminal ends of the STAT5a (5a) and STAT5b (5b) proteins. The antibodies used in Western blot (WB) and/or gel shift (EMSA) experiments are directed towards the peptide sequences shown in grey.

Results and Discussion

Studies on GHR dimerization mechanisms in the rat (Paper I)

GH is believed to bind to its receptor via sequential homodimerization, a mechanism that has been suggested based on the crystal structure of the hGH$(GHR)_2$ complex (70). As was pointed out above, each hGH molecule contains two binding sites, a high affinity site that interacts with the first receptor and a low affinity site that interacts with the second receptor leading to a receptor homodimer. In agreement with a sequential dimerization mechanism and an affinity difference, where site 1 has a higher affinity than site 2, bell-shaped dose-response curves have been observed in experiments where the dose has been varied from low to very high (73).

It is well known that hGH can interact with receptors for both GH and PRL in the rat (52), and that GH from different species can have different affinities for a specific GHR. We have therefore used a homologous system of rat GH and primary rat hepatocytes expressing GHR to explore the mechanism of GHR activation in rat liver. The hepatocytes were treated with recombinant rGH or a site 2 mutant (^{G118R}rGH) and GH responses, P450 2C12 and IGF-I mRNA levels, were measured. In dose-response experiments, a tendency of a biphasic induction curve of IGF-I mRNA was observed, whereas the P450 2C12 mRNA response plateaued. These results do not contradict a sequential binding mechanism of rGH, similar to the mechanism described for hGH. When primary rat hepatocytes were treated with ^{G118R}rGH, no antagonistic but an agonistic effect on *CYP2C12* expression was observed. The difficulty in achieving bell-shaped dose-response curves together with the lack of an antagonistic effect of ^{G118R}rGH indicate that the binding sites of rGH have similar affinity for the receptor. Furthermore, the agonistic induction of ^{G118R}rGH on *CYP2C12* expression was bell-shaped, indicating that ^{G118R}rGH interacts with two receptors. This could imply that ^{G118R}rGH, via its site 2, can interact with other receptor molecules than GHR. Thus, it is tempting to speculate about a scenario where rGH activation of the *CYP2C12* gene in female hepatocytes could be transduced via heterodimers, in addition to GHR homodimers. One candidate is the PRLR since both hGH (53) and the analog site 2 mutant ^{G120R}hGH (209) have been shown to interact with the rPRLR. There are, however, conflicting data whether the GHR can heterodimerize with the PRLR.

Crystallographic and modeling studies suggest that hGHR and hPRLR are sterically hindered from forming a hybrid dimer with hGH (210). However, in this study the issue was whether a complex of hGH and hPRLR could interact with an hGHR to form a receptor dimer, and not vice versa, which is a more likely scenario if the site 2 mutant, ^{G118R}rGH, would form a GHR-PRLR heterodimer. Moreover, ovine placental lactogen is able to heterodimerize the extracellular domains of the ovine GHR (oGHR) and the bovine PRLR (bPRLR) (211). In this study, it was also demonstrated that GHR-PRLR heterodimers of chimeric receptors of the transmembrane and cytoplasmic domains of oPRLR and oGHR fused to the extracellular domains of hGM-CSF receptors, are capable of transmitting a hormonal signal with equal efficiency as the two corresponding homodimers (211). These results imply that in addition to GHR homodimers, formation of, and signaling via, a GHR-PRLR heterodimer could occur.

In contrast to the sequential homodimerization mechanism, recent data suggest that GHR is in fact dimerized in the absence of ligand (212). Binding of GH to this preformed dimer induces a conformational change, which enables activation of JAK2 molecules. In addition, other studies have indicated that site 2 mutants can bind to GHR dimers but that they are unable to initiate signaling transduction (213, 214). Thus, even if ^{G118R}rGH would interact with preformed GHR homodimers it is less likely that it would lead to activation of the receptor. The bell-shaped dose-response induction of P450 2C12 mRNA by ^{G118R}rGH argues for the involvement of two receptors and a sequential binding mechanism for this ligand. The induction of P450 2C12 mRNA by rGH reached a plateau, which may reflect similar affinities of the two binding sites for GHR, but can also be the result of activation via two different receptor complexes. Whether the rGHR heterodimerization partner would be the rPRLR or another receptor remains to be investigated. Another candidate is gp130, which has been shown to interact with other receptors in the hematopoietin/cytokine receptor family. It would be interesting to look for GHR interacting proteins by immunoprecipitation of the ligand bound receptor followed by Western blot experiments where the presence of potential receptor partners could be analyzed.

Binding of HNF3 or HNF6 proteins depends on the presence of GH (Paper II)

A GH dependent difference in the DNase I cleavage pattern in footprinting experiments with the *CYP2C12* promoter prompted further examination of a putative *cis*-element at –52 to –30. This element was found to bind the HNF-3α, β and γ isoforms in liver nuclear extracts from hypophysectomized rats. However, using liver nuclear extracts from normal rats or hypophysectomized rats treated "continuously" with GH, a complex distinct from the HNF-3 complexes was detected. The bound protein was identified as the transcription factor HNF-6, which is the founding member of the ONECUT family of transcription factors (180). Transient co-transfection experiments showed that both HNF-3β and HNF-6 could transactivate *CYP2C12*-luciferase constructs in the human hepatoma cell line HepG2, indicating a physiological function of these transcription factors in the regulation of the gene.

Although there was a clear GH dependent binding of HNF6, the difference in HNF6 binding between female and male nuclear extracts was only two-fold. This indicates that binding of HNF6 to this element may account only for a part of the female specific expression of the *CYP2C12* gene, and that other transcription factors are involved in the marked sexual dimorphism of *CYP2C12* expression. Interestingly, HNF6 is phosphorylated by PKA, which is suggested to be of importance for the transactivation potential of HNF6 (215). However, activation of PKA leads to reduced *CYP2C12* gene expression in primary hepatocytes (200). Another study has revealed that HNF6 makes contact with different co-factors depending on the target gene (189). The core element of an HNF6 binding site in the *CYP2C12* promoter, is identical to the corresponding element in the *hnf3β* promoter. When HNF6 is bound to the element in *hnf3β,* HNF6 interacts with the coactivator CBP (189), and it is therefore plausible that HNF6 activation of the *CYP2C12* promoter involves CBP. CBP has been demonstrated to interact with several different transcription factors, among them HNF4 (179), STAT5 (216), and CREB (217), which all have been shown to contribute to the GH activated transcription of the *CYP2C12* gene (113). Therefore it is tempting to suggest an involvement of CBP in this network of transcription factors regulating *CYP2C12*, and it would be interesting to test this hypothesis by immunoprecipitation and in cotransfection assays.

The involvement of STAT proteins in GH regulation of CYP2C12 (Paper III)

The *CYP2C12* gene harbors STAT5 binding elements both in the 5' flanking region and in the 3' untranslated region (3'UTR) (113, 218). Two neighboring identical consensus STAT5 binding elements are present in the 5' flanking region at about –4kb, whereas in the 3'UTR, one consensus STAT5 binding site is present in close proximity to a half site. In gel shift experiments, binding to the 5' flanking element resulted mainly in one complex, which was supershifted by STAT5 specific antibodies. Due to the slow migration of the complex we interpreted it as binding of either two dimers or a tetramer complex of STAT5. So far, only STAT5a has been shown to bind as tetramers (105, 106), and since the major STAT5 isoform in the complex was STAT5b it is conceivable that the complex represents binding of two dimers. It has been suggested that heterodimerization of STAT5a and STAT5b is an important determinant of sex-dependent GH effects, since both isoforms are required for full expression of a female specific *Cyp* gene in mice (122). Similarly, binding of STAT5 heterodimers to the 5' flanking region of the *CYP2C12* gene may be of significance for GH regulation of the gene.

In gel shift experiments, binding of liver nuclear extracts to the element in the 3'UTR revealed several different STAT5 complexes that bound to the element in an apparent GH pattern dependent manner. Moreover, in addition to the binding of full-length STAT5 also carboxy-truncated STAT5 (STAT5β) formed complexes on this composite element. We found that STAT5β proteins can be formed by proteolytic cleavage in rat liver nuclei, and that they co-localized with full-length STAT5 in the nuclei after GH exposure. Even though the protease was found to be constitutively active, STAT5 activation by the different GH patterns may indirectly result in different relative amounts of STAT5β in the nuclei. Upon continuous GH exposure, a low level of STAT5 is continuously translocated into the nucleus which is likely to result in an equilibrium of full-length and truncated STAT5. After a GH pulse, a rapid activation and nuclear translocation of STAT5 occurs. One can anticipate that immediately after the pulse, all STAT5 exist in the full-length form. With time, the STAT5 is cleaved by the protease, and since no STAT5 is translocated into the nucleus in the interpulse period, an augmented relative amount of STAT5β could

prevail in the interpulse period compared to after continuous GH exposure. STAT5β has a higher resistance to dephosphorylation than full-length STAT5 (114, 219), and this could further contribute to an unfavorable ratio of transcriptionally active and inactive STAT5.

STAT5β proteins lack their transactivation domain and have been shown to inhibit transcription of STAT5 regulated genes either by heterodimerization with full-length STAT5 proteins or by binding as homodimers (114). An inhibitory role of full-length STAT5 in the GH regulation of the *CYP2C12* gene has been postulated in a study where STAT5 was demonstrated to inhibit HNF3β and HNF6 induced expression of a *CYP2C12* promoter construct lacking classical STAT5 binding elements (203). However, the two adjacent STAT5 binding elements in the 5' flanking region of *CYP2C12* have been shown to be necessary for GH regulation of the gene in *in vivo* transfection experiments but there was no sex-differentiated expression of the construct (113). In transient transfection studies, we were able to demonstrate that the presence of the 3' UTR element reduced GH activation of a *CYP2C12*-luciferase construct harboring the 5'-STAT5 elements. It is therefore tempting to speculate that binding of STAT5β to the 3' UTR element could be of relevance for the GH-dependent and sex-specific expression of *CYP2C12*, where a postulated higher ratio of STAT5β in the interpulse period of the male specific GH pattern could be involved in the reduced expression of the gene in males rats. To test this hypothesis one could perform *in vivo* transfection experiments using a *CYP2C12* reporter gene construct harboring both the 5' flanking region and the 3'UTR STAT5 binding elements.

Desenstitzation of the JAK/STAT pathway (Paper IV)

Pronounced activation of the JAK2/STAT5 signaling pathway by GH is a rapid and transient process (220, 221). Conceivably, there may be several different mechanisms by which desensitization is exerted. The family of SOCS/CIS proteins has been identified as negative regulators of cytokine signal transduction (153). We have demonstrated that SOCS2 and CIS mRNA expression in rat liver is dependent on the presence of GH. Furthermore, cotransfection studies in the rat liver cell line BRL-4, stably transfected with rGHR, revealed that overexpression of CIS, but not SOCS2,

inhibited GH-induced transactivation of a STAT5-responsive reporter gene construct. This indicates a functional role for CIS in the desensitization of GH induced JAK/STAT5 signaling in rat liver cells. We have also shown that GH rapidly induces CIS mRNA expression in BRL-4 cells and in primary rat hepatocytes in culture. Upon prolonged treatment of primary rat hepatocytes with GH, up to 24 hours, an initial transient expression followed by a linear increase of CIS mRNA expression was observed. Our data suggest that GH induction of CIS could be one mechanism behind the desensitization of GH-dependent JAK/STAT5 signaling in liver cells continuously exposed to GH since 1) continuous exposure of hepatocytes to GH both *in vivo* and *in vitro* resulted in maintained expression of CIS mRNA and 2) that GH-induced JAK/STAT5 signaling was reduced by CIS expression.

It may seem contradictory that continuous GH treatment, characteristic of female rats, induces the expression of CIS mRNA, when GH regulation of the female specific gene *CYP2C12* is dependent on STAT5 activation. However, it has been demonstrated that a low level of activated STAT5 can be sustained even when the CIS gene is expressed (157). In female rat liver the level of activated STAT5 is much lower than the level found after a GH pulse (98), and this level is obviously sufficient for the expression of the *CYP2C12* gene.

Concluding remarks

The results in this study imply that rGH effects can be transduced both by homodimeric GHR complexes as well as by heterodimeric receptor complexes. The *CYP2C12* response elicited by GH binding to a putative heterodimer receptor complex is probably minute in comparison with the response elicited by homodimer receptor complexes. Thus it is less likely that heterodimer complex signaling has any significant physiological relevance for the GH- and sex-dependent expression of *CYP2C12* or is involved in the mechanism(s) by which the hepatocyte can translate continuous and intermittent GH exposure into different gene regulatory events.

One can anticipate that GH- and sex-dependent expression of *CYP2C12* is due to that continuous and intermittent GH secretion trigger different signaling mechanisms and/or that the same signaling events are triggered but to different extent. The latter applies to at least two of the identified transcription factors involved in the GH regulation of *CYP2C12*. The level of GH activated full-length STAT5 is lower in female than in male rat liver whereas a two-fold higher level of HNF-6 is present in female compared to male liver. That these differences *per se* should be responsible for the marked sex difference in *CYP2C12* expression appears less likely. The lower level of activated STAT5 in female compared to male liver may in part be explained by GH induced expression of CIS, which was found to inhibit STAT5 mediated expression of a reporter gene construct in liver derived cells. Other transcription factors involved in the GH dependent expression of *CYP2C12* include HNF4, CREB-like and HNF3-like factors, and possibly also C/EBPα. The identified *trans*-acting factors, binding in the proximal promoter and the 5'-flanking region of *CYP2C12*, mediate GH-stimulated transcription of a reporter gene construct in *in vivo* transfection assays independently of the pattern of GH exposure (113). However, it cannot be excluded that the sex-differentiated expression of the endogenous gene may be the result of differences in chromatin structure allowing access to the identified transcription factors only in the female rat. Techniques such as *in vivo* footprinting could help elucidate this issue. The identification of STAT5 binding elements in the 3'UTR of the gene and the demonstration of the formation of truncated, non-transcriptionally active STAT5β binding to these elements offer one possible mechanism that could contribute to the

sex-different expression of *CYP2C12*. A high ratio of STAT5β/STAT5 during periods when no further STAT5 activation occurs, *i.e.* between GH pulses in the male, could constitute a repressor mechanism. In order to test this, one could apply the *in vivo* transfection technique and compare the expression of *CYP2C12* promoter constructs harboring the 3'UTR in female and male rat liver.

Of further interest is the observation that HNF-4, HNF-6 and CREB can interact with the co-activator CBP (179, 216, 217). Whether the sex differentiated GH pattern has any impact on the interaction between CBP and these transcription factors should be investigated in future studies on the regulation of *CYP2C12*.

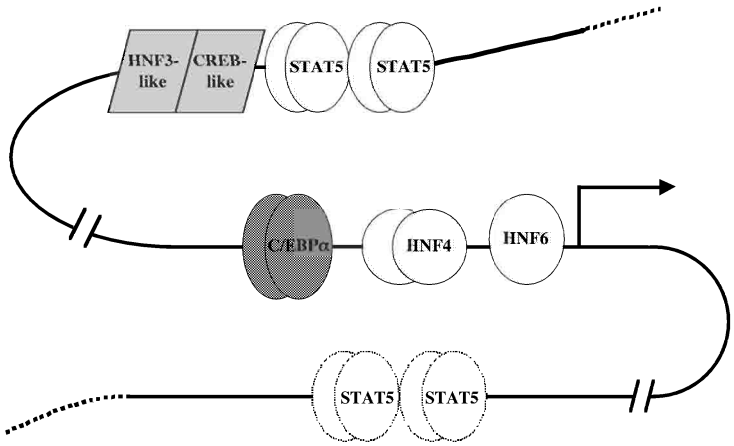

Figure 6. Current model of transcription factors involved in GH regulation of the *CYP2C12* gene.

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