MECHANISMS OF GROWTH HORMONE REGULATION OF INSULIN-LIKE GROWTH FACTOR-I GENE EXPRESSION IN LIVER

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Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
In
Animal and Poultry Sciences

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February 02, 2009

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Key Words: Growth hormone, Hepatocyte nuclear factor-3γ, Insulin like growth factor-I, Liver, Signal transducer and activator of transcription 5

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By

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Abstract

The overall objective of this research was to understand the mechanims by which growth hormone (GH) regulates insulin-like growth factor-I (IGF-I) gene expression in liver. Previous studies have suggested that GH regulation of IGF-I gene expression in liver is mediated by binding of the transcription factor signal transducer and activator of transcription (STAT) 5 to four binding sites located distantly from the IGF-I promoter. The first specific objective of this research was to determine whether additional STAT5 binding sites were involved in GH stimulation of IGF-I gene expression in liver. Sequence analysis of 170 kb of mouse genomic DNA revealed nineteen consensus STAT5 binding sequences corresponding to fourteen ~200 bp chromosomal regions that were conserved in the corresponding human DNA sequence. Eight of these chromosomal regions were able to mediate STAT5 activation of reporter gene expression in cotransfection experiments. Two of these chromosomal regions corresponded to those previously identified. Gel-shift assays indicated that the eight new STAT5 binding sites and three of the four previously identified STAT5 binding sites could bind GH-activated STAT5 from mouse liver. Together, these results suggest that GH stimulation of IGF-I

gene transcription in the mouse liver may be mediated by at least eleven STAT5 binding sites located distantly from the IGF-I promoter. In a previous study, I found that liver expression of liver-enriched transcription factor hepatocyte nuclear factor 3γ (HNF-3γ) was increased by GH in cattle. Therefore, the second specific objective of this research was to determine how GH stimulates HNF-3γ gene expression and whether the increased HNF-3γ mediates GH stimulation of IGF-I gene expression in bovine liver. Sequence analysis of the bovine HNF-3γ promoter revealed the presence of two putative binding sites for STAT5. The proximal putative STAT5 binding site appears to be conserved in other mammals. Chromatin immunoprecipitation (ChIP) assays demonstrated that GH increased the binding of STAT5 to the HNF-3γ promoter in bovine liver and that this binding was associated with increased HNF-3γ expression. Gel-shift assays demonstrated that the proximal STAT5 binding site in the HNF-3γ promoter could bind GH-activated STAT5 from bovine liver. Cotransfection analyses showed that the proximal STAT5 binding site was necessary for the HNF-3γ promoter to be activated by GH. The promoter of the bovine IGF-I gene contains three putative HNF-3 binding sites that seem to be evolutionarily conserved. ChIP assays indicated that GH stimulated the binding of HNF-3γ to the IGF-I promoter in bovine liver. Gel-shift assays showed that one of the putative HNF-3 binding sites could bind HNF-3γ protein from bovine liver. Co-transfection analyses demonstrated that this HNF-3 binding site was necessary for HNF-3γ activation of reporter gene expression from the IGF-I promoter. In summary, the results of this dissertation research suggest that GH-activated STAT5 directly stimulates IGF-I gene transcription in liver by binding to at least eleven distantly located STAT5 binding sites in the IGF-I locus and indirectly stimulates IGF-I gene transcription by enhancing HNF-3γ gene expression in the liver.

Key Words: Growth hormone, Hepatocyte nuclear factor-3γ, Insulin-like growth factor-I, Liver, Signal transducer and activator of transcription 5

Acknowledgements

I am very grateful for the opportunity provided by Dr. Honglin Jiang to pursue a doctoral degree. I am also thankful for his experienced guidance, encouragement, inspiration and assistance throughout this graduate program and in the preparation of this dissertation. I wish to express my sincere appreciation to my committee members, Drs. Edward Smith, Eric Wong, Robert Grange, and Zhijian Tu for their valuable comments and suggestions in conducting my research project and support throughout my graduate study.

Special thanks also go to Bettina Heid and Lee Johnson for helping me in collecting samples for my study and for their daily uplift and encouragement. I would like to also extend my sincere thanks to my previous and current lab members Ying Wang, Gregory Bernard, Yinli Zhou, Miaozong Wu, Zhiliang Gu, Aihua Wang, and Xiaomei Ge for their friendship, technical discussions, and support during my graduate study. I will forever cherish the memories of the good times I had with them.

I also thank the faculty, staff, and graduate students of the Department of Animal and Poultry Sciences for their friendship and support during my graduate study. I would also like to thank Virginia Tech for providing me an opportunity to pursue my graduate study.

I give my deepest appreciation to my parents, E.D. Rama Rao and E.V. Hymavathi for their support and encouragement during my graduate study. I would also like to thank my friends, Vedavyas Duggirala, Naresh Budhavaram, Jeevan Patlola Murali Mallela, Sumanth Pudupakkam, Sachi Nekkanti and Hima Bindu Vanimisetti for their encouragement and support during my graduate study.

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Introduction

Growth hormone (GH) is a polypeptide hormone produced and released from the anterior pituitary gland. GH regulates a broad range of physiological processes involved in development, somatic growth, and metabolism (LeRoith, et al. 2001). A major target organ of GH is liver because it has highest GH receptor expression compared to other tissues. In this tissue, GH controls the expression of many genes (Lichansaka and Waters 2008a). The signal transducer and activator of transcription (STAT) 5 is one of the major transcription factors mediating GH regulation of gene expression in liver (Herrington and Carter-Su 2001). GH also regulates gene expression in liver by regulating the expression or activity of liver-enriched transcription factors (LETFs) (Wiwi and Waxman 2004). GH strongly stimulates the expression of insulin-like growth factor-I (IGF-I), one of the major target genes of GH in liver, through STAT5 (Chia, et al. 2006; Wang and Jiang 2005; Woelfle, et al. 2003b). However, the mechanism by which STAT5 mediates GH regulation of IGF-I gene expression in liver was not completely understood. GH also strongly stimulates the expression of hepatocyte nuclear factor (HNF)-3γ, a liver-enriched transcription factor (Eleswarapu and Jiang 2005). The mechanism by which GH stimulates the expression of HNF-3γ and whether HNF-3γ is involved in GH regulation of IGF-I were not known. Therefore, the overall goal of the studies reported in this dissertation was to understand these mechanisms. This research contained two specific objectives: 1) To identify the STAT5 binding sites mediating GH regulation of IGF-I gene expression in mouse liver; 2) To determine the mechanism by which GH regulates HNF-3γ gene expression and whether HNF-3γ is involved in GH regulation of IGF-I gene expression in bovine liver.

Chapter I Review of Literature

Introduction

Growth hormone (GH) is an anabolic hormone released from the anterior pituitary gland. It is the main regulator of postnatal growth and metabolism (Lichanska and Waters 2008a). Liver is the major target organ of GH. Many physiological processes in liver are controlled by GH, including glucose metabolism, lipid metabolism, steroid metabolism, and xenobiotic detoxification (Lichanska and Waters 2008a; Wiwi and Waxman 2004). These processes are often controlled by GH through regulation of gene expression. GH regulates gene expression in liver through direct and indirect mechanisms. As a direct mechanism, GH regulates gene expression through transcription factors directly activated by any of its signaling pathways (Herrington and Carter-Su 2001). As a indirect mechanism, GH regulates gene expression through transcription factors, such as some of the liver-enriched transcription factors (LETFs), whose expression is controlled by the transcription factors directly activated by the GH signaling pathways (Cesena, et al. 2007; Waxman and O'Connor 2006). IGF-I is one of the major target genes of GH in liver and is regulated at the level of transcription. Liver is the major source of circulating IGF-I. IGF-I plays an important role in growth, development, metabolism, aging, and tumorigenesis. This review summarizes the current understanding of actions of GH, mechanisms of GH action, and regulation of gene expression by GH. Since IGF-I is one of the important genes regulated by GH and is the focus of this dissertation research, I will also discuss the structure and function of the IGF-I gene and regulation of its expression.

Growth hormone (GH)

Growth hormone, also called somatotropin, is a peptide hormone produced and secreted mainly by the anterior pituitary gland. It is also synthesized in small quantities in other tissues, including reproductive tissues, lymphoid tissues and the gastrointestinal tract (Hartman, et al. 1991). GH secretion from the anterior pituitary occurs in a pulsatile pattern and is regulated by hypothalamic hormones (Figure 1.1). The secretion of pituitary GH is induced by growth hormone releasing hormone (GHRH) and inhibited by somatostatin (Sherlock and Toogood 2007; Velloso 2008). In addition, GH secretion is also stimulated by ghrelin, which is synthesized in the hypothalamus, pituitary, and stomach (Kojima, et al. 1999). GH release is also regulated by IGF-I. The GH-stimulated IGF-I in the circulation provides a negative feedback effect, suppressing the further release of GH from the anterior pituitary. In addition to these factors, various stimuli affect the frequency and magnitude of the GH pulses, including gender, age, adiposity, sleep, diet and exercise. Consequently, serum GH levels vary greatly throughout the day (Sherlock and Toogood 2007; Velloso 2008). The balance between these factors determines the serum GH levels. The gene coding for bovine GH is approximately 1800 base pairs in length and is composed of five exons and four introns. The molecular weight of bovine GH is 22 kilo Dalton (KDa). The coding regions of the GH gene are highly conserved among species (Gordon, et al. 1983; Hampson and Rottman 1987).

Recombinant GH has many applications in human health and animal agriculture. GH has been used to treat disorders related to GH deficiency and other conditions such as cystic fibrosis and Crohn’s disease (Smith and Thorner, 2000). In addition, GH has also been used for treatment of burn wounds (Smith and Thorner, 2000). The recombinant GH has been extensively used in animal agriculture, especially in the dairy industry to increase milk yield in the dairy cows

(Sirotkin 2005). Due to its ability to decrease back-fat and increase lean muscle, GH has also been used to improve carcass quality in the swine industry (Etherton 2000).

One of the major functions of GH in the body is to promote postnatal growth. In addition, GH also plays important roles in lipid and carbohydrate metabolism (Osafo, et al. 2005), xenobiotic and steroid metabolism (Wiwi and Waxman 2004), immunity (Meazza, et al. 2004), reproduction, and lactation (Sirotkin 2005) (Figure 1.2).

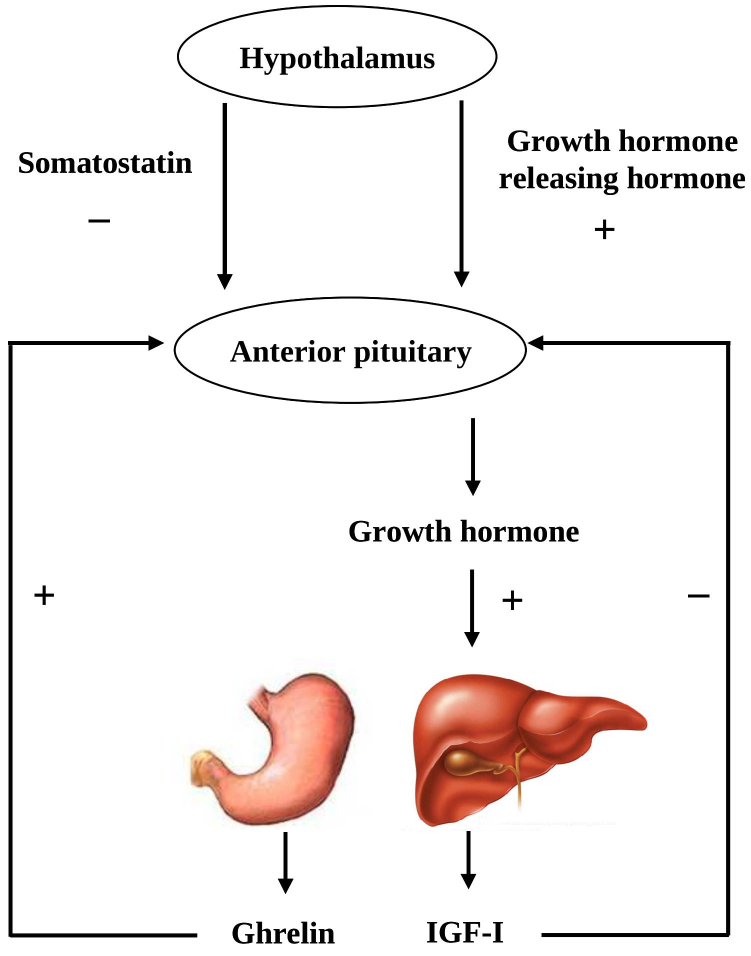


Figure 1.1. Regulation of GH secretion. Pituitary GH production and secretion is regulated by hypothalamic hormones (somatostatin and GH releasing hormone) and stomach hormone ghrelin. GH releasing hormone stimulates, while somatostain inhibits GH production. GH acts on liver and leads to production of IGF-I. IGF-I, in turn, regulates GH secretion by negative feedback. Ghrelin is mainly produced from the stomach and positively regulates GH secretion.

Actions on growth

In humans and animals, lack of GH or GH receptors results in growth retardation; GH replacement therapy improves growth (Walenkamp and Wit 2007). Mice lacking STAT5b, the major transcription factor of GH signaling, also show severe growth retardation (Udy, et al. 1997). Growth retardation was also reported in human subjects with mutations in GH receptor or STAT5b (Kofoed, et al. 2003; Laron 2004; Rosenbloom 1999; Savage, et al. 1993; Vidarsdottir, et al. 2006). These findings demonstrate that GH is important for postnatal growth. Moreover, all the above mentioned cases have very low serum IGF-I levels, suggesting that IGF-I mediates the actions of GH on postnatal growth. The role of IGF-I in GH-dependent postnatal growth is best evidenced by profound growth retardation of individuals with GH receptor mutations. These individuals produce GH, yet cannot generate IGF-I (Laron 2004). The body size of IGF-I knockout mice decreases progressively from 60% of normal at birth to 30% of normal at 8 weeks. Double knockout mice, lacking GHR and IGF-I, have a postnatal growth pattern of 17% of normal (Lupu, et al. 2001). Individuals lacking the IGF-I gene or containing inactivating mutations in IGF-I gene have low serum IGF-I levels and show growth retardation (Walenkamp, et al. 2005; Woods and Savage 1996). These findings demonstrate that GH-dependent IGF-I action is the main determinant of postnatal growth. Circulating IGF-I is primarily derived from liver (Yakar, et al. 1999); however, knockout studies in mice support the concept that the locally generated IGF-I at the target sites might be more important to control of growth than the systemic IGF-I (Sjogren, et al. 2002). IGF-I knock out mice show severe growth retardation. On the other hand, mice lacking the hepatic IGF-I gene show a 70% decrease in circulating IGF-I concentration, yet normal postnatal growth, indicating that IGF-I generated in the tissues other than the liver is critical for promotion of linear growth (Butler and Le Roith 2001). The importance of hepatic IGF-I over local IGF-I in post natal growth has not been resolved yet. Although IGF-I has been used to treat growth retardation in humans with GH receptor mutations and other forms of GH insensitivity, GH treatment results in a better growth response than IGF-I treatment in growth-retarded individuals (Savage, et al. 2006). This suggests that a direct GH effect independent of IGF-I is also necessary for optimal postnatal growth.

Actions on metabolism

GH is involved in carbohydrate, lipid, protein, and steroid metabolism. In healthy individuals, GH increases fasting hepatic glucose output, by stimulating hepatic gluconeogenesis and glycogenolysis, and decreases peripheral glucose oxidation (Holt, et al. 2003; Ridderstrale 2005). Excess GH is associated with insulin resistance, hyperinsulinemia, hyperglycemia and decreased glucose uptake. These effects of excess GH are associated with reduced expression of proteins involved in carbohydrate metabolism, including glycogen synthase, glycogen phosphorylase, glucokinase and glucose transporter (GLUT)-2 mRNA concentrations. On the other hand, GH-deficiency increases insulin sensitivity, leading to decreased fasting glucose, decreased insulin secretion, and decreased hepatic glucose production (Holt et al. 2003; Ridderstrale 2005).

GH also controls plasma cholesterol levels by regulating hepatic expression of low density lipoprotein (LDL) receptors, LDL production, and triglyceride production (Lind, et al. 2004). GH treatment in both normal and GH-deficient humans increases hepatic LDL receptors, resulting in reduced LDL cholesterol and increased lipoprotein A. GH-deficient adults show increased fat mass and that is significantly reduced with GH therapy. In addition, GH receptor-defective Laron syndrome patients progress towards obesity following birth. GH receptor knockout mice also have an increased percentage of body fat. These data demonstrate that GH has a role in fat metabolism (Mauras and Haymond 2005; Osafo et al. 2005). GH induces lipid breakdown, reduces triglyceride accumulation as well as decreases glucose uptake in mature adipocytes (Nam and Lobie 2000; Osafo et al. 2005). GH prevents lipid accumulation by inhibiting the activity of lipoprotein lipase enzyme (LPL), which hydrolyzes triglycerides in the circulation to release free fatty acids. Thus, it reduces free fatty acids that can be taken up by adipocytes. On the other hand, GH promotes lipolysis by prolonging the hormone-sensitive lipoprotein lipase activity (HSL), which is the rate-limiting enzyme that breaks down triglycerides in adipocytes (Nam and Lobie 2000; Osafo et al. 2005).

GH is an important protein-anabolic hormone. GH administration to healthy individuals results in increased whole body protein synthesis rates, with no effect on proteolysis (Horber and Haymond 1990), whereas when given to severely GH-deficient subjects, both protein synthesis and protein degradation increase with a net anabolic effect (Mauras, et al. 2000). GH administration can prevent the decline in ribosomal and polyribosomal concentrations in human skeletal muscle after surgery, suggesting that muscle protein synthesis is preserved (Welle 1998). In surgically stressed rats, growth hormone significantly stimulates muscle protein synthesis (Lo and Ney 1996).

Actions on lactation

One of the important actions of GH in cattle is on mammary gland development and lactation. GH plays an important role in ductal elongation and differentiation of ductal epithelia into terminal buds during mammary gland development (Coleman, et al. 1988). During lactation, GH administration stimulates milk yield in cows (Knight 1992). GH increases milk yield by increasing the milk volume and also by proportionately increasing the synthesis of milk protein, fat and lactose in the mammary gland. The mechanism by which GH increases milk yield is not completely understood. Whether GH directly acts on the mammary gland has been controversial. Earlier, it was thought that GH had no direct effect on mammary gland, as GH receptor could not be detected by a ligand-binding assay in mammary epithelial cells (Akers 1985). However, recent evidence suggests that GH may regulate milk production by direct action on mammary gland. This evidence includes detection of GH receptor mRNA and protein expression in both stromal and epithelial tissues of bovine mammary gland (Plath-Gabler, et al. 2001; Sinowatz, et al. 2000). Furthermore, GH receptor protein expression is much lower in dry cows compared to lactating cows (Sinowatz et al. 2000). Many studies have shown that the quantity of milk produced from the mammary gland depends on the activity of mammary secreting cells, the balance between cell proliferation and cell death, and the regulation of milk protein genes (Boutinaud, et al. 2004). Therefore, GH may increase milk yield by regulating any of the above mentioned processes in mammary gland. GH stimulates proliferation of mammary epithelial cells without a significant effect on apoptosis in midlactating cows (Capuco, et al. 2001). Thus, GH may increase persistence of lactation. However, whether the increased proliferation of mammary epithelial cells was due to direct action of GH or mediated by IGF-I was not known. GH stimulated the expression of β-casein mRNA in both mammary gland of lactating cows and mammary explant culture, suggesting that this effect may be due to direct action of GH on the mammary gland (Yang, et al. 2005). Furthermore, GH stimulates the expression of several milk protein mRNAs in mammary epithelial cell lines (Sakamoto, et al. 2005; Zhou, et al. 2008). These observations suggest that GH may stimulate the expression of milk protein genes in the mammary gland by directly acting on the epithelial cells.

Other actions of GH

GH modulates gut function by enhancing the uptake of macro- and micro nutrients (Waters, et al. 1999). GH is also involved in functioning and regulation of the immune system. In the immune system, GH stimulates the proliferation of T and B cells, synthesis of immunoglobulins and the maturation of myeloid progenitor cells. In addition, GH also modulates cytokine response (Meazza et al. 2004). GH is believed to play a role in the ageing process. Mice lacking GH or GH receptor live longer than their littermate controls. In addition, the oldest living mouse was a GH receptor-deleted animal (An and Xiao 2007; Bartke and Brown-Borg 2004; Chen, et al. 2004). GH is believed to have a protective effect on liver, especially during cardiopulmonary bypass surgery (CBP). The CBP may provoke a systemic inflammatory response syndrome and this response can cause injury to various organs and may lead to mortality. The liver is one of the vulnerable organs during systemic inflammatory response syndrome. GH administration has been shown to prevent acute liver injury associated with CBP (An and Xiao 2007; Chen et al. 2004).

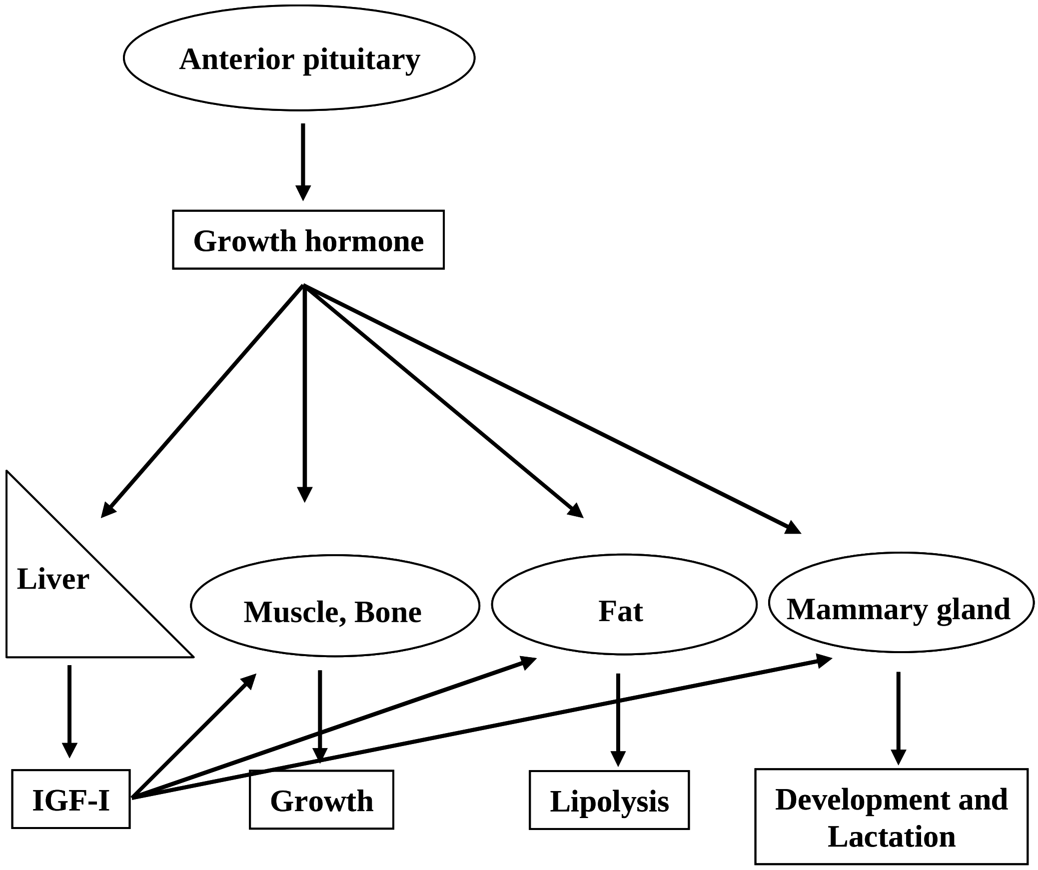


Figure 1.2. Actions of GH. GH has direct effects on muscle, bone, fat, and mammary gland for growth, lipolysis, mammary gland development and lactation. GH also has indirect effects on these organs by stimulating liver to secrete IGF-I.

Mechanisms of GH action

GH receptor

GH receptor is a single-pass transmembrane protein without tyrosine kinase activity, which belongs to a cytokine receptor superfamily. GH receptor has a ligand binding extracellular domain (ECD), transmembrane domain (TCD), and intracellular cytoplasmic domain (ICD) (Brooks, et al. 2008; Ridderstrale 2005). The ICD contains a proline rich sequence proximal to the plasma membrane referred to as Box 1 and a more C-terminal Box 2. Box 1 binds to tyrosine kinase JAK2 and Box 2 contains several tyrosine residues that are substrates for phosphorylation by JAK2 and so become binding sites for Src homology (SH) 2 domain proteins (Brooks et al. 2008; Ridderstrale 2005). The GH receptor is activated upon binding GH. One molecule of GH binds to two molecules of GH receptor. The GH molecule has two binding sites for the GH receptor. Site 1 binds with strong affinity with one of the GH receptors and site 2 forms a relatively weaker bond with the second receptor. Earlier, it was thought that GH binding to GH receptor causes receptor dimerization and consequent activation (Lichanska and Waters 2008b). However, recent studies have shown that GH receptor exists as a dimer on the surface of the cell even in the absence of GH (Brown, et al. 2005). Therefore, according to the new model, GH binds to a constitutively homodimerized GH receptor, which causes rotation of ICDs of GH receptor molecules, resulting in the activation of the associated tyrosine kinases and signal transduction (Brown et al. 2005). GH receptor is expressed in many tissues, including liver, fat, muscle, kidney, heart, prostrate, lymphocytes, and skin fibroblasts, with greatest expression in liver (Ballesteros, et al. 2000). Liver GH receptor expression is regulated by GH (Kopchick and Andry 2000; Schwartzbauer and Menon 1998). Liver GH receptor expression is reduced in undernutrition, diabetes, and cirrhosis (Donaghy, et al. 2002; Kopchick and Andry 2000; Schwartzbauer and Menon 1998).

Signaling via activation of JAK2

As mentioned earlier, JAK2 is associated with a Box 1 motif in the ICD of the GH receptor. Binding of GH to a GH receptor dimer causes relative rotation of the two GH receptors and this is believed to bring the JAK2 molecules on the GH receptors into close proximity (Brown et al. 2005). As a result, the JAK2 molecules activate each other by phosphorylating the tyrosine residue in their kinase domains and subsequently phosphorylate multiple tyrosine residues in the GH receptor. The phosphorylated tyrosines within JAK2 and the associated GH receptor form high-affinity binding sites for a variety of signaling proteins containing SH2 and other phosphotyrosine-binding domains. Recruitment of these signaling molecules to the GH receptor-JAK2 complexes initiates a variety of signaling pathways (Brooks et al. 2008; Ridderstrale 2005) (Figure 1.3). Analysis of GH receptor mutant mice has shown that activation of JAK2 is necessary for the majority of GH actions (Rowland, et al. 2005).

Signaling via activation of Src

In addition to JAK2, Src tyrosine kinase has also been shown to be activated by GH (Manabe, et al. 2006; Zhu, et al. 2002). This observation suggests that not all GH signaling pathways are activated by JAK2. GH causes activation of Src tyrosine kinase independent of JAK2 (Zhu et al. 2002). Manabe et al. (2006) showed that GH binding to GH receptor modestly stimulates Src activity in addition to JAK2. They further demonstrated that Src can bind to and phosphorylate the GH receptor. Therefore, it is possible that Src along with JAK2 may be involved in the activation of downstream signaling pathways. In support of this dual activation, recent studies have shown that GH activation of Ras-like small GTPases Ra1A, Ra1B, Rap1 and Rap2 require both Src and JAK2 (Lanning and Carter-Su 2006). Alternatively, Src and JAK2 may act independently to activate the downstream signaling molecules of GH signaling pathway. Zhu et al. (2002) have shown that GH activation of Src is linked to activation of extracellular signal-regulated kinases (ERKs) 1 and 2. In contrary, a recent study has shown that GH activates only JAK2, but not Src tyrosine kinase, in GH-responsive cell lines (Jin, et al. 2008). Obviously, further studies are needed to elucidate the role of Src in GH signaling.

GH signaling pathways

Major signaling pathways activated by GH include the signal transducers and activators of the transcription (STAT) pathway, mitogen-activated protein kinase (MAPK) pathway, and phosphatidyl inositol 3' (PI-3) kinase pathway (Cesena et al. 2007; Herrington and Carter-Su 2001; Lanning and Carter-Su 2006; Piwien-Pilipuk, et al. 2002) (Figure 1.3).

The STAT pathway: The STAT proteins are a family of latent transcription factors that are abundantly produced in many cell types. The STATs comprise a family of seven structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. It is believed that GH activates only STAT 1, 3, 5a, and 5b (Levy and Darnell 2002; Paukku and Silvennoinen 2004). Upon activation of GH signaling, the STAT molecules, which are present in the cytoplasm, are recruited to the GH receptor through their SH2 domain. Subsequently, the activated JAK2 phosphorylates STAT proteins on a single tyrosine residue at the carboxyl end of the molecule. The phosphorylated STAT molecules form homodimers (STAT1, STAT3, STAT5a and STAT5b) as well as heterodimers (STAT1 and STAT3) and are subsequently released from the receptor. The STAT-STAT dimerization occurs through reciprocal interactions between the SH2 domain of one monomer and the phospho-tyrosine of the other. Dimeric STATs enter the nucleus where they bind to specific DNA elements and activate transcription (Hennighausen and Robinson 2008; Herrington, et al. 2000).

Although GH activates STAT1, STAT3, and STAT5, many studies have shown that STAT5 is most important for a variety of GH functions. There are two highly related genes encoding STAT5a and STAT5b proteins, which are 96% similar at the amino acid level. They differ in their tissue-specific expression pattern. While STAT5a is the predominant STAT5 in the mammary gland, STAT5b is more abundant in liver and muscle (Hennighausen and Robinson

2008). In liver, these two proteins probably regulate the expression of common as well as distinct genes (Hennighausen and Robinson 2008; Herrington et al. 2000). The STAT5a deficient mice show defects in lactation with impaired lobuloalveolar proliferation of the mammary epithelium during pregnancy, indicating defective prolactin signaling (Liu, et al. 1997). STAT5a-deletion had no effect on body size, suggesting that it is not critical for GH-dependent postnatal growth. In contrast, STAT5b-deficient mice have a similar phenotype to that observed in GH receptor-deficient mice (Rowland et al. 2005; Udy et al. 1997). The STAT5b-deficient male mice were 25% smaller than the wild-type littermates. However, there were no significant decreases in body size of the STAT5b-deficient female mice. Mice deficient in both STAT5a and STAT5b showed further decreases in their body size in both sexes (Teglund, et al. 1998). Absence of STAT5b signaling due to mutations in STAT5b resulted in severe growth retardation in humans (Kofoed et al. 2003). Mutations in ICD of the GH receptor, which prevent STAT5 activation, decrease postnatal growth of both sexes by up to 50%. This decrease was comparable to the 55% decrease in GH receptor-deficient mice (Rowland et al. 2005). These observations suggest that STAT5 is critical for GH-dependent postnatal growth (Liu et al. 1997; Rowland et al. 2005; Teglund et al. 1998; Udy et al. 1997).

STAT5 is also believed to mediate some of the GH actions on body composition. Male mice lacking STAT5b developed obesity during later part of their life (Teglund et al. 1998; Udy et al. 1997). In addition, STAT5b deletion in a mature human was associated with striking obesity (Vidarsdottir et al. 2006). GH receptor mutant mice, which lack STAT5 activation, also exhibit striking obesity. These mice have adipocyte hypertrophy and increased subcutaneous adipose depot. These mice also exhibit hepatosteatosis (Lichanska and Waters 2008a). Moreover, fat pads from STAT5b deficient mice showed no lipolytic response to GH (Clodfelter, et al.

2006). These observations suggest that STAT5 may mediate GH actions on fat metabolism. STAT5 also mediates GH actions on muscle. Mice deficient in muscle STAT5 showed less postnatal growth to a decreased skeleton size. In addition, muscle STAT5-deficient mice showed reduced body mass due to decreased lean mass. These mice also exhibited glucose intolerance (Klover and Hennighausen 2007). Taken together, these observations suggest that STAT5 mediates GH dependent muscle and skeletal growth.

The MAPK pathway: The MAPK pathway is also activated by GH (Cesena et al. 2007; Frank 2001; Herrington and Carter-Su 2001; Lanning and Carter-Su 2006; Piwien-Pilipuk et al. 2002). Activation of the MAPK pathway appears to require only the proximal part of the cytoplasmic GH receptor, which includes the Box1 region. GH stimulates the binding of the adapter protein Src homologous and collagen-like (Shc) to GH receptor-JAK2 complexes. This binding leads to tyrosyl phosphorylation of Shc protein. Subsequently the phosphorylated Shc binds to growth factor receptor bound protein (Grb) 2 and the guanine nucleotide exchange factor (SOS) complexes. This leads to sequential recruitment and activation of a the small GTP-binding protein called Ras, serine/theronine kinase Raf, mitogen-activated protein kinase (MAPK) and finally extracellular-signal regulated protein kinase (ERKs) 1 and 2. The GH-sensitive MAPK substrates include the ternary complex factor or E-twenty-six-like kinase (ELK)-1, early growth response (Egr) 1, JunB and CAAT-enhancer binding protein (CEBP) β.

The PI-3’ kinase pathway: The PI-3’ kinase pathway or insulin receptor substrate (IRS) pathway represents another pathway by which GH may elicit some of its cellular effects. GH stimulates the tyrosine phosphorylation of IRS-1, IRS-2, and IRS-3 (Lanning and Carter-Su 2006; Piwien-Pilipuk et al. 2002; Zhu, et al. 2001). However, GH receptor does not appear to contain the NPXY consensus sequence required for the association of IRS proteins with receptors such as those for insulin and IGF-I (Zhu et al. 2001). Box 1 of GH receptor was shown to be required for GH-stimulated phosphorylation of IRS-1, 2, and 3. Since Box 1 is associated with JAK2 and NPXY consensus is absent in JAK2, it is possible that IRS proteins may associate with JAK2 via an adaptor molecule (Zhu et al. 2001). After phosphorylation, the IRS proteins associate with many downstream signaling molecules, including p85 subunit of PI-3' kinase, which is a pivotal effector molecule downstream of IRS proteins. It is believed that GH promotes association of the p85 subunit of PI-3'kinase with IRS proteins (Lanning and Carter-Su 2006; Zhu et al. 2001). In contrast, GH activated or tyrosine phosphorylated PI-3'kinase. GH might activate the PI-3' kinase pathway through direct binding of p85α and p85β subunits of PI-3' kinase to the carboxy terminus of GH receptor (Lanning and Carter-Su 2006; Zhu et al. 2001). In any case, activated PI-3'kinase activates serine/theronine protein kinase B (PKB/AKT). The GH-dependent activation of AKT was dependent on the JAK2 binding region of the GH receptor. AKT mediated PI-3' kinase-dependent events, including regulation of kinases, transcription factors and other regulatory molecules (Lanning and Carter-Su 2006; Zhu et al. 2001).

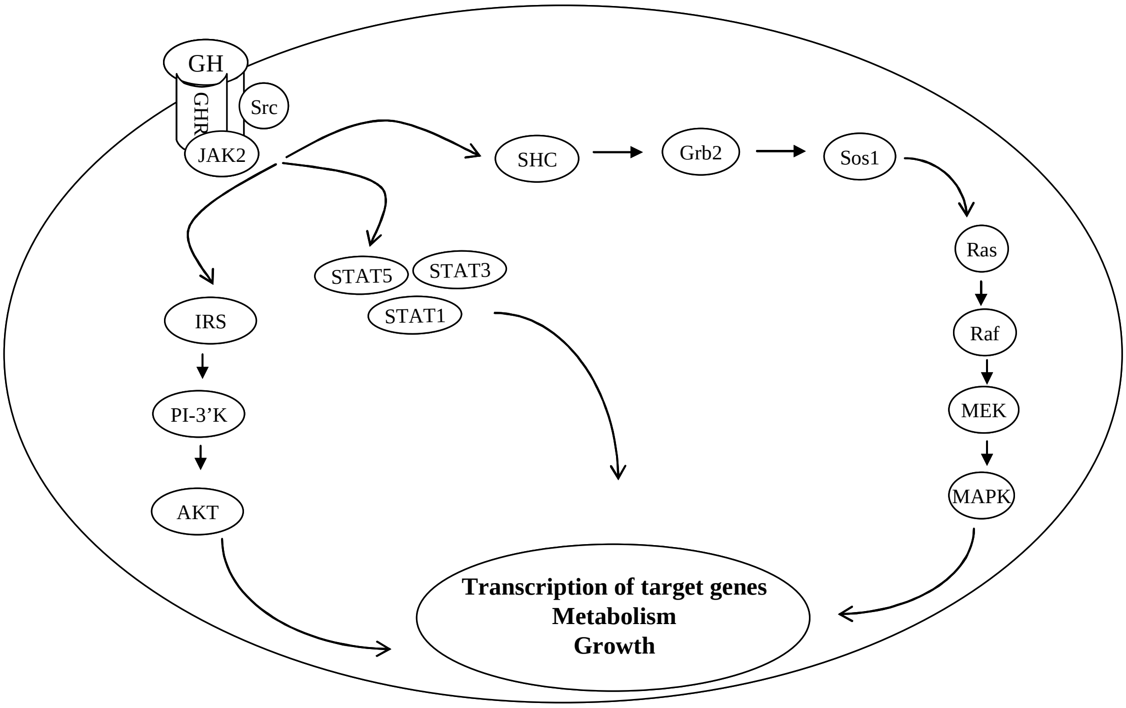


Figure 1.3. Intracellular signaling cascades from GH receptor. GH binds to the constitutive GH receptor dimer and causes rotation of GH receptor. This relative rotation leads to phosphorylation and activation of GH receptor, janus kinase (JAK)-2, and Src. Activated JAK2 and Src catalyze the following three primary tyrosine phosphorylation events: (1) Signal transducer and activator of transcription (STAT) pathway leads to direct stimulation of target gene transcription; (2) Activation of insulin receptor substrate (IRS) 1to 3 leads to activation of PI-3'K and subsequently AKT, leading to changes in gene expression; (3) Activation of the SHC-Grb2-Ras-Raf-MEK-MAPK pathway results in the activation of mitogen-activated protein kinase (MAPK) pathway leading to changes in gene expression. Adapted from Herrington and Carter-Su (2001).

Negative regulation of GH signaling

Three major classes of proteins regulate the GH signaling pathway and they are: suppressors of cytokine signaling (SOCS), protein tyrosine phosphatases (PTP), and protein inhibitors of activated STATs (PIAS) (Flores-Morales, et al. 2006; Lanning and Carter-Su 2006). **SOCS**: The SOCS proteins are a family of at least eight members: cytokine-inducible SH2 domain (CIS) and SOCS1-SOCS7. The members of this family contain an N-terminal domain of varying length, a central SH2 domain, and a conserved C-terminal motif termed SOCS-box (Flores-Morales et al. 2006). The expression of SOCS-1, 2, 3 and CIS are induced by GH. The activated STATs stimulate transcription of the SOCS genes and the resulting proteins regulate the GH signaling by a negative feedback mechanism (Flores-Morales et al. 2006; Lanning and Carter-Su 2006). The SOCS can effect their negative regulation by three means. First, by binding phosphotyrosines on the receptors, SOCS physically block the recruitment of STATs to the receptor. Second, SOCS proteins can bind directly to JAKs or to the receptors to specifically inhibit kinase activity. Third, SOCS interact with the elongin BC complex and cullin 2, facilitating the ubiquitination of JAKs and, presumably, receptors. Ubiquitination of these targets decreases their stability by targeting them for proteasomal degradation (Levy and Darnell 2002; Rawlings, et al. 2004; Shuai and Liu 2003). SOCS-1 regulates GH signaling pathway by inhibiting the activity of JAK2 by binding to its tyrosine kinase domain. SOCS-3 binds to GH receptor and thereby inhibits the activity of JAK2. SOCS-2 and CIS inhibit GH signaling by inhibiting the binding of STAT5 to GH receptor. SOCS-1, SOCS-2, and SOCS-3 also regulate GH signaling by promoting ubiquitination of the GH receptor-JAK2 complex. CIS appears to stimulate GH receptor internalization and proteasomal degradation (Flores-Morales et al. 2006; Lanning and Carter-Su 2006). The physiological importance of the SOCS-1, SOCS-3, and CIS is not clear, as mice lacking SOCS-1 or CIS or liver-specific SOCS-3 did not show increased growth. SOCS-2-deficient mice showed increased growth, suggesting that only SOCS-2 may be important for negative regulation of GH signaling (Flores-Morales et al. 2006).

PTPs: The second class of negative regulators is the phosphatases, which include SH2 domain-containing PTP (SHP1), SHP2, PTP-H1, PTP1, T-cell PTP (TC-PTP), and PTP1b (Flores-Morales et al. 2006; Lanning and Carter-Su 2006; Zhu et al. 2001). These phosphatases negatively regulate GH signaling by dephosphorylating the proteins involved in the GH signaling pathway. Upon activation by GH, SHP1 translocates to the nucleus and dephosphorylates

STAT5. SHP1 also inhibits GH signaling by controlling the duration of GH-dependent JAK2 phosphorylation in liver. Moreover, liver nuclear extracts from SHP1-deficient mice show prolonged phosphorylation of JAK2 and DNA binding of STAT5 in response to GH, suggesting that SHP1 is involved in negative regulation of GH signaling. It is believed that SHP2 negatively regulates GH signaling by dephosphorylating GH receptor, JAK2, and STAT5. In addition, SHP2 may also regulate GH signaling by binding to JAK2 associated membrane protein signal regulatory protein-α (Flores-Morales et al. 2006; Lanning and Carter-Su 2006). The PTP-H1, PTP1, TC-PTP, and PTP1b may act by dephosphorylating the GH receptor. Mice deficient in PTP1b display increased JAK2, STAT5, and STAT3 phosphorylation in response to GH compared to wild type mice (Flores-Morales et al. 2006; Lanning and Carter-Su 2006; Zhu et al. 2001).

PIAS: The third class of negative regulator is the PIAS proteins: PIAS1, PIAS2 (PIASx), PIAS3, and PIAS4 (PIASy). PIAS proteins were named for their ability to interact with and inhibit STAT factors (Palvimo 2007; Shuai and Liu 2005). These proteins have four structural motifs: an N-terminal scaffold attachment factor-A/B/acinus/PIAS (SAP) motif, a "PINIT" motif, a putative RING-type zinc-binding structure, and a small ubiquitin-related modifier-interacting (SIM) motif (Palvimo 2007; Shuai and Liu 2005). The PIAS proteins bind to activated STAT dimers and prevent them from binding DNA. PIAS2 and PIAS3 can interact with STAT4 and STAT3 respectively, while PIAS1 and PIAS4 can interact with STAT1 (Shuai and Liu 2005). In addition, PIAS3 interacts with STAT5 to repress its transcriptional activity (Rycyzyn and Clevenger 2002). Some studies have suggested that PIAS1 and PIAS3 proteins act by blocking the DNA binding activity of STATs, whereas PIAS2 and PIAS4 repress STAT activity by recruiting histone deacetylases (Valentino and Pierre 2006). However, the mechanism by which PIAS proteins act is not fully understood.

GH regulation of gene expression in liver

Many genes are regulated by GH in liver

Liver is the major target of GH, as GH receptor is expressed more abundantly in liver than any other organ. In liver, GH controls the expression of many genes (Lichanska and Waters 2008a). Different animal models have been employed to study GH regulation of gene expression in liver. These models include hypophysectomized rats (Ahluwalia, et al. 2004; Flores-Morales, et al. 2001; Gardmo, et al. 2002; Thompson, et al. 2000) or mice (Gronowski and Rotwein 1995), mice overexpressing bovine GH (Olsson, et al. 2003), and GHR knockout mice (Rowland et al. 2005).

Using cDNA microarrays, Flores-Morales et al. (2001) found that 58 of 720 detectable mRNAs were regulated by GH in liver of hypophysectomized rats. Many of these genes were not previously known to be GH responsive. Flores-Morales et al (2001) also found that GH upregulated hepatic expression of genes involved in carbohydrate and energy metabolism (transketolase, cytochrome b5, and ubiquinol-cytochrome c-reductase hinge protein), fatty acid or cholesterol metabolism (stearyl-CoA desaturase, acyl-CoA synthetase 5, and fatty acid transporter), signal transduction or trophic factors (hepatic fibrinogen, fibrinogen γ-chain, and 14-3-3 protein), and detoxification or xenobiotic metabolism (CYP2C12, CYP2C7, and gluthatione-S-transferase).

Olsson et al. (2003) compared liver gene expression in transgenic mice over-expressing the bovine GH with that in non-transgenic mice using DNA microarrays. They found that the transgenic mice had decreased hepatic expression of genes involved in fatty acid synthesis (fatty acid synthase, ATP-citrate lyase, carbonic anhydrase, long-chain fatty acyl CoA synthetase), β-oxidation (liver carnitine palmitoyl transferase 1, sterol-carrier protein X, peroxisomal dienoyl-CoA isomerase), and production of ketone bodies (HMG-coA synthetase, acetoacyl-CoA thiolase). The decreased expression of these hepatic metabolic genes resulted in reduced ability of these transgenic mice to form ketone bodies in both fed and fasted conditions (Olsson et al. 2003). In the same study, the expression of peroxisome proliferator activated receptor α (PPARα) and sterol-regulatory element binding protein (SREBP-I) genes were also reduced in the liver of the transgenic mice.

Using suppression subtractive hybridization and microarrays, Gardmo et al. (2002) identified a number of rat liver genes that are differentially regulated by GH. Examples of these genes include steroid and drug metabolism enzymes, CYP2A1, CYP2C7, 5α-reductase, and 17β-hydroxy steroid dehydrogenase type 6, a secreted protein, β-2 glycoprotein I and an intermediary metabolism enzyme S-adenosylmethionine synthetase. All of these genes are expressed at higher levels in the female liver than the male liver (Gardmo, et al. 2002).

Another study using cDNA microarrays (Thompson et al. 2000) also showed that GH up-regulates a diverse set of genes, which included signal transducer and activator of transcription (STAT)-3, mitogen activated protein kinase (p38MAPK), apurinic endonuclease (APEN), growth arrest and DNA damage 45 (GADD45) and monocorboxylate (lactate) transporter gene (MCT-1).

In another study, Ahluwalia et al. (2004) identified 27 female and 44 male-predominant genes that were regulated by GH in rat liver. These GH-regulated genes are involved in metabolism, detoxification, growth control and other functions in liver.

In hypophysectomized mice, GH administration down-regulated the expression of insulin-like growth factor binding protein-1 and albumin (Gronowski and Rotwein 1995). In contrast, GH up-regulated expression of c-fos, c-jun, hepatocyte nuclear factor (HNF)-6 and HNF-3β in liver (Gronowski, et al. 1996; Rastegar, et al. 2000a).

In a recent study, using GH receptor deficient mice, Rowland et al. (2005) identified nearly 330 genes regulated by GH. Some of the genes that were down- regulated in GH receptor mutant mice include IGF-I, HNF-3β, insulin-like growth factor binding protein (IGFBP)-3, acid labile subunit (ALS), SOCS-2, major urinary protein (MUP)-1, 3, 4, and 5, epidermal growth factor receptor (EGFR), amyloid P component, serum (APCS), catechol-O-methyl transferase (COMT), and cysteine sulfinic acid decarboxylase (CSAD). The genes that were up-regulated in GH receptor deficient mice, include angiogenin, corticosteroid-binding globulin (SERPINA6), P450 cytochrome (CYP) 17A1, 2B9, sulfotransferase family 2A, dehyroepiandrosterone (DHEA)-preferring member 2(Sth2), cyclin-dependent kinase inhibitor (CDKN) 1C, heat shock protein (HSPA) 4, and hydroxyacid oxidase (HAO) 3.

GH regulation of LETFs

The LETFs include HNF-1α, 1β, 3α, 3β, 3γ, 4α, 4β, 4γ, 6α, 6β, albumin D-element binding protein (DBP), CCAAT/enhancer-binding proteins (C/EBP)- α and β. These LETFs are abundantly expressed in the liver compared to other tissues and hence called LETFs (Hayashi, et al. 1999; Schrem, et al. 2002). Of all the LETFs, HNF-3γ is most strongly stimulated by GH (Eleswarapu and Jiang 2005). GH also appears to regulate the expression of HNF-3β in rodent liver. The HNF-3β mRNA expression was increased within 3 hours of GH injection in hypophysectomized rats (Lahuna, et al. 2000). The expression of HNF-3β mRNA decreased to values below the normal levels 6 hours after GH administration. GH was also able to stimulate the expression of HNF-4α mRNA in cattle (Eleswarapu and Jiang 2005). However, the increase in HNF-4α mRNA was weak and delayed. The levels of HNF-4α mRNA were increased by nearly two fold 1 week after GH administration compared to the untreated cows. The expression of HNF-4α mRNA, however, did not change in cows 6 hours or 24 hours after GH treatment. GH appears to regulate HNF-4 expression in rats as well (Lahuna et al. 2000). In hypophysectomized rats, mRNA levels of HNF-4 increased by nearly two fold 6 hours after GH administration.

The expression of HNF-6 was stimulated by GH in bovine liver (Eleswarapu and Jiang 2005). The expression of both HNF-6α and β mRNAs increased about two fold 24 hours and 1 week after GH administration in cows. GH had similar effects on HNF-6 in rat liver (Lahuna, et al. 1997), where the expression of both HNF-6α and β mRNAs increased 3 hours after GH administration. It was also shown that GH stimulated HNF-6 transcription by stimulating the binding of STAT5 to the HNF-6 promoter and also by increasing the binding affinity of HNF-4 to the HNF-6 promoter (Lahuna et al. 2000).

GH administration also increased C/EBPα mRNA expression in bovine liver (Eleswarapu and Jiang 2005).The C/EBPα mRNA levels were increased by about two fold 24 hours after GH administration. This increase was temporary, as the levels of C/EBPα mRNA decreased to the normal levels in cows one week after GH administration. In rat liver, GH caused increases in the mRNA levels of C/EBPα but decreased its protein levels (Rastegar, et al. 2000b), suggesting that GH may act on C/EBPα at the post-translational level. Similarly, GH regulates the activity of C/EBPβ at the post- translational level (Cesena et al. 2007). GH rapidly and transiently phosphorylates C/EBPβ through the MAPK-dependent pathway. The expression of DBP mRNA was also stimulated by GH in bovine liver (Eleswarapu and Jiang 2005). GH increased DBP mRNA levels by almost two fold 6 hours after GH injection. However, DBP mRNA levels decreased 24 hours and 1 week after GH administration. Thus, GH initially had a stimulatory effect and then an inhibitory effect on DBP mRNA expression in the liver of cows.

GH had no effect on HNF-1α mRNA expression in bovine liver. Similarly, GH did not affect HNF-1α expression in rats (Le Stunff, et al. 1996; Rastegar et al. 2000a). Hypophysectomy or GH replacement did not alter the HNF-1α protein levels in rat liver. These observations together suggest that GH does not regulate the expression of HNF-1α in the liver.

LETFs in GH regulation of gene expression in liver

The promoters of many liver-specific genes contain multiple consensus binding sites for LETFs (Schrem, et al. 2002). In hepatocytes, 12%, 1.7%, and 1.6% of 13,000 gene promoters were bound by HNF-4α, HNF-1α and HNF-6, respectively (Odom, et al. 2004). LETFs have been shown to directly control the expression of many genes in liver (Hayashi et al. 1999; Schrem et al. 2002). LETFs may play a role in GH regulation of gene expression in liver. For example, HNF-3, HNF-4 and HNF-6 contribute to GH regulation of cytochrome P450 (CYP) 2C12 expression (Delesque-Touchard, et al. 2000; Sasaki, et al. 1999) in liver. The expression of cyclin D1 and CYP7A1 was shown to be regulated by GH (Wang, et al. 2008) and expression of these genes has also been shown to depend on HNF-6 (Tan, et al. 2006; Wang, et al. 2004). Since HNF-6 is also regulated by GH (Lahuna et al. 2000), it is possible that HNF-6 may be involved in GH regulation of cyclin D1 and CYP7A1 genes in liver. HNF-3β is involved in GH activation of CYP2A2, CYP4A2 and CYP2C11 genes in liver (Park and Waxman 2001). C/EBPβ has been involved in GH regulation of c-fos expression in liver (Liao, et al. 1999). In addition, the expression of tyrosine-aminotransferase (TAT) (Ahluwalia, et al. 2004), transferrin (Tf) (Flores-Morales, et al. 2001) and CYP3A3 (Jaffe, et al. 2002) in liver was shown to be regulated by GH and expression of these genes has also been shown to depend on HNF-3γ (Kaestner, et al. 1998; Rodriguez-Antona, et al. 2003). Since HNF-3γ is also regulated by GH (Eleswarapu and Jiang 2005), it is possible that HNF-3γ may be involved in GH regulation of TAT, Tf and CYP3A3 genes in liver.

IGF-I

A variety of genes are regulated by GH in liver, including IGF-I. IGF-I is of significance because of its role in postnatal growth, ageing, metabolism, and cancer. IGF-I is a polypeptide hormone with a similar structure to insulin that is produced primarily in liver as an endocrine hormone. IGF-I is also produced locally from peripheral tissues and functions in a paracrine/autocrine fashion. In circulation, more than 85% of IGF-I is bound in a ternary complex with IGFBP-3 and ALS. On target tissues, IGF-I binds to its specific receptor, the IGF-I receptor, and activates intracellular signaling pathways (Jones and Clemmons 1995).

Actions on cell proliferation and apoptosis

IGF-I has a mitogenic effect on a variety of cells, including fibroblasts, chondrocytes, osetoblasts, keratinocytes, smooth muscle cells, skeletal muscle cells, neuronal cells, mammary epithelial cells, and several cell lines (Jones and Clemmons 1995). IGF-I stimulates proliferation of primary chick chondrocytes under serum-free conditions (Bohme, et al. 1992). Proliferation of human erythroid progenitor cells and T lymphocytes is stimulated by IGF-I (Kooijman, et al. 1992; Merchav, et al. 1992). On the other hand, blocking of IGF-I receptor function using antibodies or antisense vectors inhibited cell growth in fibroblasts (Pietrzkowski, et al. 1992; Porcu, et al. 1992) and in other malignant cell lines (Reiss, et al. 1992; Sell, et al. 1993).

IGF-I is a major cellular survival factor. IGF-I can protect cells from apoptosis induced by a wide variety of agents, including growth factor withdrawal, etoposide, and oncogene overexpression (Grothey, et al. 1999). For example, c-Myc induced apoptosis in fibroblasts was inhibited by IGF-I (Harrington, et al. 1994). IGF-I was also able to inhibit apoptosis in interleukin-3-dependent hemopoietic cells (Rodriguez-Tarduchy, et al. 1992). In addition, apoptosis of human erythroid progenitor cells due to serum starvation was inhibited by IGF-I (Muta and Krantz 1993). Moreover, apoptosis of rat fibroblasts was inhibited by IGF-I and this anti-apoptotic action of IGF-I was mediated by IGF-I receptor through activation of the PI-3'-kinase and AKT pathway (Kulik, et al. 1997).

Actions on body growth

IGF-I is believed to mediate most of the GH actions on body growth. Individuals with GH receptor or STAT5b mutation, who have decreased IGF-I levels, show severe growth retardation (Kofoed et al. 2003; Rosenbloom 1999). Treatment of these individuals with recombinant human IGF-I stimulated body growth (Savage et al. 2006). Mice over-expressing human IGF-I exhibited a 30% increase in size compared to normal mice (Mathews, et al. 1988). Overexpression of IGF-I stimulated normal growth in GH-deficient transgenic mice (Behringer, et al. 1990). More convincing evidence of the role of IGF-I in growth came from humans or animals with IGF-I deficiency. Complete IGF-I deficiency due to deletion of the IGF-I gene results in severe growth retardation in humans (Woods and Savage 1996). Similarly, a patient with an inactivating mutation of the IGF-I gene showed decreased postnatal growth (Walenkamp et al. 2005). The size of adult mice lacking IGF-I was 30% of normal mice (Yakar, et al. 2002). Moreover, injection of recombinant GH to IGF-I deficient mice failed to stimulate their growth (Liu and LeRoith 1999). In addition to postnatal growth, IGF-I is also believed to play a role in intrauterine growth. Mice lacking IGF-I or IGF-I receptors have birth weights of 60 and 45% of normal body weight, respectively (Baker, et al. 1993; Liu, et al. 1993; Powell-Braxton, et al. 1993). Similarly, severe intrauterine growth retardation was observed in an IGF-I-deficient patient (Walenkamp et al. 2005; Woods and Savage 1996).

IGF-I is also important for skeletal muscle growth. Mice lacking IGF-I or IGF-I receptor had severely diminished muscle mass (Liu et al. 1993; Powell-Braxton et al. 1993). Similarly, transgenic mice over-expressing dominant negative human IGF-I receptor in muscle exhibited decreased muscle mass (Fernandez, et al. 2002). In contrast, overexpression of IGF-I in muscle of transgenic mice caused myofiber hypertrophy (Coleman, et al. 1995) and could compensate for the decline in muscle mass and strength seen in muscular dystrophy (Barton, et al. 2002).

Actions on metabolism

Some of the metabolic actions of IGF-I are opposite to GH and similar to insulin, especially on glucose and lipid metabolism (Kaplan and Cohen 2007). IGF-I treatment of rats causes hypoglycemia by stimulating peripheral glucose uptake, glycolysis and glycogen synthesis, but has only a minimal effect on hepatic glucose production (Holt et al. 2003). Similarly, IGF-I infusion caused hypoglycemia in humans (Zenobi, et al. 1992). In a recent study in humans, high circulating IGF-I levels were associated with reduced risk of development of impaired glucose tolerance and type-2 diabetes (Sandhu, et al. 2002). IGF-I treatment of diabetic patients reduced hepatic glucose production and increased peripheral glucose uptake (Simpson, et al. 2004).

IGF-I also plays a role in lipid metabolism. In humans, effect of IGF-I on fat mass and lipid metabolism depends on duration of IGF-I treatment. Several studies have shown that short-term IGF-I treatment decreased body fat mass in patients with GH deficiency and in hypophysectomized rats (Guler, et al. 1988). It has been also shown that IGF-I treatment was accompanied by elevated energy expenditure and lipid oxidation and reduced protein oxidation in humans (Hussain, et al. 1993). Therefore, short-term IGF-I treatment may decrease body fat mass by increasing lipid oxidation and elevated energy expenditure. This action of IGF-I is through an indirect mechanism by inhibiting insulin secretion and thereby decreasing lipogenesis in adipose tissue (Frick, et al. 2000). In contrast, prolonged IGF-I treatment of patients with IGF-I gene deletion or GH insensitivity resulted in substantial gain in fat mass (Guevara-Aguirre, et al. 1997; Woods, et al. 2000). More recently, it was reported that long-term treatment of IGF-I in patients with GH insensitivity resulted in increased body adipose tissue (Laron, et al. 2006). Therefore, long-term IGF-I treatment may result in increased fat mass.

IGF-I stimulates protein synthesis in GH-deficient mice and increases total body weight in hypophysectomized rats (Jones and Clemmons 1995). Whole body protein synthesis was increased in individuals receiving IGF-I treatment, without any effect on proteolysis (Mauras et al. 2000). In addition, IGF-I stimulated amino acid uptake as well as protein synthesis in calorically restricted volunteers (Clemmons and Underwood 1992).

Other actions of IGF-I

Progesterone production in granulosa cells (Seto-Young, et al. 2003) and thymic factor secretion from epithelial cells of thymus (Timsit, et al. 1992) are stimulated by IGF-I. Thymic factor is believed to be involved in T-cell differentiation and enhancement of T and natural killer (NK) cell actions (Bach 1977). IGF-I is believed to play an important role in modulating the peripheral metabolism of glucocorticoids mainly through its effects on the isoenzyme 11β-hydroxysteriod dehydrogenase (HSD)-1 metabolism (Agha and Monson 2007). Isoenzyme 11β-HSD-1 functions as a reductase catalyzing the conversion of cortisone to cortisol. It is believed that IGF-I mediates GH inhibition of 11β-HSD-1 expression and activity in liver and adipose tissue. *In vitro* studies have shown that 11β-HSD1 is inhibited by IGF-I but not directly by GH (Moore, et al. 1999).

IGF-I also plays a role in immunity. IGF-I can potentiate B lymphopoiesis by promoting maturation of CD45R pre-B cells from CD45R precursors (Landreth, et al. 1992). Treatment of normal mice with IGF-I results in an increase in the total number of B lineage cells in the bone marrow (Jardieu, et al. 1994). Additionally, IGF-I can synergize with interleukin-7 to stimulate proliferation of CD45R cells (Gibson, et al. 1993). IGF-I can also stimulate immune cell function. Treatment of nine-month-old mice with recombinant human IGF-I significantly enhanced the initial response to a T-dependent antigen and subsequent rechallenge with the antigen (Clark, et al. 1993).

IGF-I also appears to have neuroprotective effects. It promotes neurogenesis, development, differentiation, synapse formation and glucose utilization throughout the brain. It also inhibits glial-inflammatory reactions by antagonizing tumor necrosis factor-α (Gasparini and Xu 2003). Nitric oxide production in endothelial cells is induced by IGF-I and in this regard it may function as an endogenous vasodilator (Tsukahara, et al. 1994).

IGF-I has also been implicated in longevity and ageing. Studies on GH-resistant, GH-deficient, and IGF-I receptor knockout mice provide evidence for the role of IGF-I in longevity and ageing. All these mutant animal models have very low serum IGF-I, reduced insulin and glucose levels, improved stress resistance, and reduced incidence of cancer (Bartke and Brown-Borg 2004; Berryman, et al. 2008). In animals with altered IGF-I signaling, genes important for carbohydrate metabolism, DNA repair, anti-oxidant resistance, apoptosis, and cell cycle arrest are upregulated (Greer and Brunet 2005) Since ageing is associated with DNA damage (Finkel and Holbrook 2000; Sohal and Weindruch 1996), it is believed that activation of DNA repair and anti-oxidant genes under reduced IGF-I conditions provide a potential mechanism for longevity enhancement in the IGF-I deficient models (Hinkal and Donehower 2008).

Mechanism of IGF-I action

IGF-I signaling

The IGF-I system contains two ligands, IGF-I and IGF-II; three cell membrane receptors, IGF-I receptor, insulin receptor (IR), and IGF-II receptor; and six high-affinity IGF binding proteins, IGFBP-1 to 6 (Jones and Clemmons 1995; Samani, et al. 2007). More than 85% of circulating IGF-I is bound in a ternary complex with IGFBP-3 and ALS. IGF-I binds to its receptor on target tissue and mediates its biological actions. IGF-I binds IGF-I receptor with highest affinity. In addition, it can also bind IGF-II receptor and IR with lower affinity. IGF-I receptor is composed of two extracellular α subunits and two intracellular β subunits. The α subunits bind IGF-I receptor ligands, while the β subunits transmit ligand-induced signals (Jones and Clemmons 1995; Samani et al. 2007).

The binding of IGF-I receptor by IGF-I activates two major signaling pathways, which include MAPK and PI-3’K pathways (Jones and Clemmons 1995; Samani et al. 2007). IGF-I receptor is a tyrosine kinase receptor and IGF-I binding induces its tyrosine kinase activity. This results in autophosphorylation of tyrosines on the intracellular portion of the β-subunit. The phosphorylated tyrosines serve as docking sites for several receptor substrates, including IRS 1 to 4 and Shc. Phosphorylated IRS activates PI-3’K, which in turn activates p70S6 kinase and AKT. Phosphorylated AKT, in turn, activates mammalian target of rapamycin (mTOR) and inactivates Bcl-2-associated death promoter (BAD). In parallel to PI-3’K-driven signaling, recruitment of Grb2/SOS by phosphorylated IRS or Shc leads to the activation of the MAPK pathway (Jones and Clemmons 1995; Samani et al. 2007).

IGF-I production

IGF-I gene

The IGF-I gene has been sequenced in many species. In cattle, it has been mapped to chromosome 5 and appears to be more than 70 kb (Chr5: 71125137-71198012) in size (Bovine October 2007 Assembly at the UCSC Genome Browser http://genome.ucsc.edu). The IGF-I gene in cattle consists of 5 exons and 4 introns (Figure 1.4), whereas human, rat and mouse IGF-I genes contain 6 exons and 5 introns (Woelfle, et al. 2005; Yakar et al. 2002). Despite these differences in gene structures, IGF-I protein is conserved in most species and is coded from exons 3 and 4 (Nagamatsu, et al. 1991), indicating that IGF-I may have similar functions in all the species.

IGF-I mRNA structure and tissue distribution

IGF-I gene contains distinct promoters, named P1 and P2. P1 initiates transcription from exon 1 and P2 from exon 2 in several species, including cattle (Wang, et al. 2003; Woelfle et al. 2005; Yakar et al. 2002). Exon 1-containing IGF-I mRNA variants are termed as class 1 IGF-I mRNA and exon 2-containing IGF-I mRNA variants as class 2 IGF-I mRNA (Figure 1.4). In cattle, class 1 IGF-I mRNA variants are transcribed from at least three different start sites in exon 1, whereas class 2 IGF-I mRNA variants from a single transcription start site in exon 2 (Wang et al. 2003). In class 1 IGF-I mRNAs, exon 1 is spliced onto exon 3 and in class 2 IGF-I mRNAs, exon 2 is spliced onto exon 3 (Wang et al. 2003; Woelfle et al. 2005; Yakar et al. 2002). Therefore, in most species, IGF-I mRNAs containing different 5’ ends are generated. In addition, IGF-I mRNA variants differing in the 3' end sequence have also been detected in humans, rats, and mice (Yakar et al. 2002). For example, in mouse two IGF-I mRNA transcripts, IGF-I-Eb and IGF-I-Ea, differing in the 3' end sequence, are generated (Bell, et al. 1986; Roberts, et al. 1987). IGF-I-Eb mRNA contains a 52 bp insert (exon5) between exon 4 and 6, whereas IGF-I-Ea mRNA transcript does not contain the 52 bp insert. Similarly, in humans three different IGF-I mRNA variants, IGF-I-Ea, IGF-I-Eb, and IGF-IEc, differing in the 3' end sequence, are generated (Chew, et al. 1995; Jansen, et al. 1991; Rotwein, et al. 1986).

In cattle, both class 1 and class 2 IGF-I mRNAs are expressed in many tissues, including adrenal gland, brain, fat, hypothalamus, kidney, liver, lung, mammary gland, skeletal muscle, pituitary, rumen, small intestine, spleen, and testis (Wang et al. 2003). Both classes of IGF-I mRNAs are expressed at high levels in liver. In cattle, class 1 IGF-I mRNA variants are more abundant than class 2 IGF-I mRNAs in all tissues (Ohlsen, et al. 1993; Wang et al. 2003). The tissue distribution patterns of class 1 and class 2 IGF-I mRNAs in cattle are very similar to that in sheep (Ohlsen et al. 1993) but are somewhat different from that in humans (Jansen et al. 1991) and rats (Lowe, et al. 1987), in which class 1 IGF-I mRNAs are expressed at a relatively high level in all tissues, but class 2 IGF-I mRNAs are only expressed in a few tissues at a very low level. Class 1 and class 2 IGF-I mRNAs encode multiple IGF-I protein precursors, from which, the same mature IGF-I polypeptide is generated, however (Woelfle et al. 2005; Yakar et al. 2002).

Mature IGF-I protein

The mature IGF-I peptide is composed of 70 amino acid residues and arranged in four highly conserved domains A-D in many species including cattle (Figure 1.4). The B and A domains of IGF-I have a 50% sequence similarity to the B and A chains of insulin (Denley, et al.

2005) and hence called insulin-like growth factor. The IGF-I has three alpha helices, helix 1, helix, 2 and helix 3. Helix 1 is in the B domain, whereas helix 2 and helix 3 are both located in the A domain. Three disulfide bonds hold together the three-dimensional fold of the IGF-I (Denley et al. 2005). The mature IGF-I protein contains six cysteine residues, which form intraprotein disulfide bonds. All the six cysteine residues in mature IGF-I protein are evolutionarily conserved (Rotwein et al. 1986; Wong, et al. 1989; Yakar et al. 2002).

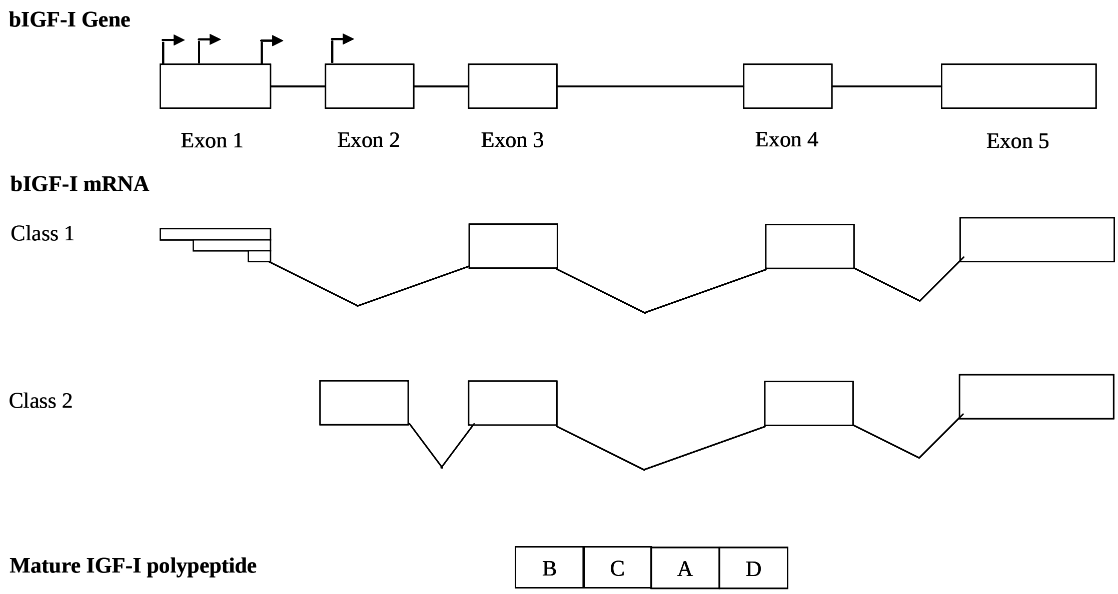


Figure 1.4. Schematic representation of bovine IGF-I gene, mRNA and protein structures. Exons are shown as boxes, and introns are represented by straight lines (GenBank accession number DQ851589 (exons 1 through 4)). Exon 5 shown here corresponds to exon 6 in mouse IGF-I mRNA. Exon 1 is spliced onto exon 3 to generate class I IGF-I mRNA (GenBank accession number AY277405). Exon2 is spliced onto exon 3 to generate class 2 IGF-I mRNA (GenBank accession number AY277406). The arrows above exons 1 and 2 indicate transcription start sites. Different domains (B, C, A, D) of mature IGF-I protein are shown in boxes. Sizes of exons, introns, and protein domains are not drawn to scale. Actual numbers of transcription start sites may be more than indicated (Wang et al. 2003).

Regulation of IGF-I gene expression

Hormonal regulation

Growth hormone: IGF-I is mainly produced from liver and the hepatic IGF-I constitutes 75% of total circulating IGF-I (Lichanska and Waters 2008a; Yakar et al. 2002). Studies on GH-deficient animal models have shown that there is a significant decrease in serum IGF-I and hepatic IGF-I mRNA levels in the absence of GH (Lichanska and Waters 2008a). GH treatment of these GH-deficient animals restored these levels to normal, indicating that IGF-I gene expression is mainly regulated by GH (Lichanska and Waters 2008a). In addition to liver, skeletal muscle, adipose tissue, and kidney also respond to GH administration by increasing IGF-I transcription (Bichell, et al. 1992; Le Roith, et al. 2001). GH rapidly and potently induces IGF-I gene transcription (Bichell et al. 1992) and this leads to sustained production of IGF-I mRNA and protein (Bichell et al. 1992; D'Ercole, et al. 1984). The rapid induction of IGF-I gene expression by GH is independent of new protein synthesis (Gronowski, et al. 1996; Gronowski and Rotwein 1995).

Conventional promoter transfection experiments and DNA-protein binding studies did not yield much information about how GH regulates IGF-I expression (An and Lowe 1995; Mittanck, et al. 1997; Wang, et al. 2000; Wang, et al. 1998; Wang and Jiang 2005). The first clue to how GH regulates IGF-I expression was the identification of a GH-regulated DNase-I hypersensitive site (HS7) (Bichell et al. 1992; Thomas, et al. 1995). This DNase-I hypersensitive site appeared just prior to induction of IGF-I gene transcription by GH, and disappeared with the fall in IGF-I transcription 6 h after GH treatment. Studies on STAT5 knock-out animal models implicated STAT5 in GH regulation of IGF-I gene. The STAT5b-null mice had 50% less liver IGF-I mRNA and 30% lower serum IGF-I concentrations than wild-type mice (Davey, et al. 2001; Udy et al. 1997). GH treatment of these STAT5b-null mice did not increase liver IGF-I mRNA abundance or serum IGF-I concentrations (Davey et al. 2001). Furthermore, overexpression of a dominant-negative STAT5b mutant completely prevented GH-induced IGF-I gene expression in liver, whereas that of a constitutively active STAT5b mutant led to robust, GH-independent IGF-I gene expression in the hypophysectomized rats (Woelfle, et al. 2003a). Mutation in STAT5b gene or GHR gene caused decreases in serum IGF-I concentrations in human patients (Hwa, et al. 2005; Kofoed et al. 2003; Milward, et al. 2004). Moreover, GH treatment was unable to increase IGF-I mRNA abundance in cultured fibroblasts isolated from the patient or serum IGF-I concentrations (Hwa et al. 2005; Kofoed et al. 2003; Milward et al. 2004). All these studies indicated that STAT5b may be the major transcription factor involved in GH regulation of IGF-I gene transcription. In addition to STAT5b, STAT5a has also been implicated in GH regulation of IGF-I gene expression. Supporting this role of STAT5a is the observation that STAT5a and STAT5b double knock-out mice had lower serum IGF-I levels and showed greater growth retardation than STAT5b knock-out mice (Teglund et al. 1998).

Based on these observations, Woelfle et al. (2003b) identified two adjacent putative STAT5 binding sites corresponding to HS7 in intron 2 of the rat IGF-I gene. These two STAT5 binding sites were conserved among different mammalian species. Using chromatin immunoprecipitation assays, they showed that GH rapidly induced the interaction of STAT5 with this DNA segment. They also showed that this interaction occurred just prior to initiation of IGF-I gene transcription from both promoters in the liver (Woelfle, et al. 2003b). In addition, the HS7 DNA region could confer GH-stimulated and STAT5-mediated transcriptional activation to IGF-I promoter, but when the two STAT5 binding sites within HS7 were mutated, GH activation of transcription was eliminated (Woelfle et al. 2003b). These observations showed that those two STAT5 binding sites mediated GH-induced STAT5 activation of IGF-I gene expression. More recently, using a shotgun cloning approach, Wang and Jiang (2005) identified two functional STAT5 binding sites 70 kb upstream from the human IGF-I gene. They showed that these two STAT5 binding sites were able to bind to STAT5 proteins and that binding of STAT5 to this region was associated with increased IGF-I mRNA expression in human hepatocellular carcinoma (HepG2) cells. They also demonstrated that these STAT5 binding sites can mediate GH activation of gene expression from IGF-I promoter and that mutation of these two STAT5 binding sites abolished GH activation of gene expression (Wang and Jiang 2005). These two

STAT5 binding sites were later found to also mediate GH activation of IGF-I gene expression in the rat liver (Chia, et al. 2006).

Insulin: In addition to GH, IGF-I gene expression is also regulated by insulin. Diabetic animals display lower levels of IGF-I mRNA and protein than normal animals, but IGF-I levels are normalized by insulin treatment (Goldstein, et al. 1988; Pao, et al. 1992). Nuclear run-on studies in diabetic rats and cultured hepatocytes indicated that IGF-I expression is regulated by insulin at the transcriptional level (Pao et al. 1992; Pao, et al. 1993). A DNase I hypersensitive site 360 bp from the transcription start site in exon 1, designated as region V, appears to be critical for IGF-I regulation by insulin and diabetic status (Pao, et al. 1995). This region was shown to contain a GC-rich element and an AT-rich element adjacent to a GC-rich element (Zhu, et al. 2000). Both Sp1 and Sp3 were able to act through the GC-rich region of the IGF-I gene (Kaytor, et al. 2001). The AT-rich element can be bound by the insulin response binding protein (IRBP) 1 (Chahal, et al. 2008; Kaytor et al. 2001). Furthermore, it has also been demonstrated that insulin causes enrichment of the IGF-I promoter region bound by IRBP1 (Chahal et al. 2008). It has been suggested that IRBP1 may interact with Sp1 to stimulate binding to region V and expression of the IGF-I gene (Kaytor et al. 2001). There is enough evidence suggesting that insulin may regulate the binding of IRBP1 to the IGF-I gene through the PI'3K signaling pathway (Villafuerte, et al. 2004).

Other hormones: In rat hepatocytes, glucagon increases IGF-I gene expression (Kachra, et al. 1991). In contrast, glucocorticoids decreased IGF-I production in osteoblasts (Delany and Canalis 1995). Chronic estrogen administration decreased GH-induced hepatic IGF-I expression and serum concentrations, but acted in a synergistic fashion when administered with GH and resulted in significantly greater accumulation of IGF-I mRNA in rats (Murphy and Friesen

1988). Thus, the growth retardation effect of estrogen in chronic treatments involves inhibition of GH-dependent hepatic IGF-I expression. Thyroid hormone has both direct and indirect effects on IGF-I expression in rats (Dorshkind and Horseman 2000). Thyroxine (T4) alone could directly stimulate IGF-I expression and increase its serum levels (Chernausek, et al. 1982). On the other hand, Triiodothyronine (T3) stimulates IGF-I expression indirectly by stimulating GH-releasing-hormone receptor mRNA expression in rat pituitary culture cells and thereby stimulating GH transcription (Mayo 1992).

Nutritional regulation of IGF-I gene expression

Several studies have shown that energy, protein, and certain specific nutrients can regulate IGF-I expression (Estivariz and Ziegler 1997; Ketelslegers, et al. 1995; Thissen, et al. 1994). For example, IGF-I levels in plasma continued to decrease steadily over a 9 day period of fasting, but were increased to only 50% of baseline with refeeding for 3 days. In obese individuals or patients with hyperphagia, IGF-I levels are either normal or only modestly increased (Thissen et al. 1994). In rodents, serum concentrations of IGF-I are significantly reduced during fasting, but rise over time in response to refeeding (Ketelslegers et al. 1995). Similarly, protein and/or calorie restriction reduced serum IGF-I concentrations (Lemozy, et al. 1994; Radcliff, et al. 2004). The level of protein intake markedly affects IGF-I mRNA and protein production in rat liver, as well as IGF-I levels in human plasma (Thissen et al. 1994). Moreover, protein refeeding with 80% of the dietary protein as essential amino acids significantly increased plasma IGF-I levels compared with diets providing 80% of protein as nonessential amino acids (Clemmons, et al. 1985).

Several studies have shown that decreases in serum IGF-I concentrations during undernutrition are associated with decreased expression of IGF-I mRNA in liver (Adamo, et al.

1991; Lemozy et al. 1994). Since liver produces 75% of serum IGF-I (Yakar et al. 1999), it is possible that a fasting-induced decrease in serum IGF-I in part may be caused by reduced liver IGF-I mRNA. Some studies have shown that during fasting, IGF-I expression is regulated at the transcriptional level (Hayden, et al. 1994; Straus and Takemoto 1990), whereas others have shown that it is regulated at the post-transcriptional level (Hayden and Straus 1995; Zhang, et al. 1998). Therefore, fasting may decrease IGF-I transcription or the stability of IGF-I mRNA.

During undernutrition or fasting, GH secretion and thereby serum GH levels are increased in all species, except rodents (Buonomo and Baile 1991; Pierce, et al. 2005). Furthermore, GH administration fails to increase serum IGF-I levels in these animals (Maes, et al. 1988; Miller, et al. 1981). These observations indicate that nutritional deprivation induces GH resistance. Since fasting decreases GH receptor abundance (Maes, et al. 1983; Postel-Vinay, et al. 1982), it was believed that GH resistance during fasting was due to decreased binding of GH to its receptor. However, in a recent study (Beauloye, et al. 2002), reduced phosphorylation of GHR, JAK2, and STAT5 was observed in fasted rats, suggesting that GH resistance during fasting was due to impaired JAK-STAT signaling.

More than 85% of circulating IGF-I is stabilized by forming a ternary complex with IGFBP-3 and ALS. The levels of IGFBP-3 and ALS were decreased in serum of fasted animals. Furthermore, IGF-I was more quickly degraded in the sera of fasted animals *in vitro* (Wu, et al. 2008). Therefore, in addition to decreased IGF-I production from liver, increased degradation of circulating IGF-I may also be responsible for reduced IGF-I levels during fasting.

Conclusions and perspectives

In summary, the liver is the major source of circulating IGF-I. Production of IGF-I in the liver is regulated by GH at the transcriptional level. STAT5 is the major transcription factor involved in GH regulation of IGF-I gene expression. Four STAT5 binding sites, two upstream and two downstream of the IGF-I promoter, are involved in the GH-activated STAT5 regulation of IGF-I gene expression in liver. Whether additional STAT5 binding sites are involved in GH regulation of IGF-I expression is not known. In addition, STAT5 has been shown to interact with co-activators, such as cAMP response element binding protein (CBP) and p300 (Pfitzner, et al. 1998; Ye, et al. 2001). Whether these co-activators are also involved in GH-stimulated STAT5 activation of IGF-I gene expression remains to be determined. The previously identified four STAT5 binding sites involved in GH regulation of IGF-I expression are distantly located from the IGF-I promoter. However, the mechanism by which these STAT5 binding sites mediate STAT5 action on the IGF-I promoter from distant locations remains to be determined. In GH receptor mutant mice, decreases in the IGF-I levels were much greater than that reported in STAT5a/b-null mice (Rowland et al., 2004), suggesting that other GH-regulated transcription factors are involved in GH regulation of IGF-I gene expression in liver. Liver-enriched transcription factors, HNF-3β and HNF-3γ, have been shown to be regulated by GH in liver (Eleswarapu et al. 2005; Lahuna et al. 2000). Moreover, GH receptor mutant mice had decreased expression of HNF-3β in liver (Rowland et al. 2004). Whether these HNF-3 proteins are involved in GH regulation of IGF-I gene expression in liver remains to be determined. Since IGF-I is involved in growth, metabolism, tumorigenesis and aging, understanding the mechanisms involved in regulation of IGF-I gene expression may help develop new approaches to improve animal productivity and to design therapeutic agents.

Recently, four STAT5 binding sites located distantly from IGF-I promoter were found to mediate GH regulation of IGF-I gene expression in liver (Chia, et al. 2006; Wang and Jiang 2005; Woelfle, et al. 2003b). Considering the size of the IGF-I gene (> 100 kb), we hypothesized that there may be additional STAT5 binding sites in the IGF-I gene that may be involved in the GH regulation of the IGF-I gene expression in liver. Therefore, the first objective of this research was to determine whether additional STAT5 binding sites were involved in the GH regulation of the IGF-I gene expression in liver. The expression of some of the LETFs is also regulated by GH (Eleswarapu and Jiang 2005). Of the all the LETFs, GH potently stimulates the expression of HNF-3γ in liver. Since STAT5 is the major transcription factor involved in the GH regulation of gene expression in the liver, we hypothesized that STAT5 may be involved in GH regulation of HNF-3γ expression in liver. Since HNF-3γ is involved in regulation of many genes in liver and also regulated by GH, we also hypothesized that HNF-3γ may be involved in the GH regulation of the IGF-I gene expression in the liver. Therefore, the second objective of this research was to determine the mechanism by which GH regulates HNF-3γ gene expression and whether HNF-3γ is involved in GH regulation of IGF-I gene expression in liver.

Chapter II Growth Hormone Regulation of IGF-I Gene Expression May Be Mediated by Multiple Distal STAT5 Binding Sites*

Abstract

Insulin-like growth factor-I (IGF-I) is a polypeptide hormone mainly produced from liver. Liver expression of IGF-I is mainly regulated by growth hormone (GH) through the transcription factor signal transducer and activator of transcription 5 (STAT5). Previous studies have suggested that STAT5 might exert this effect by binding to two STAT5 binding sites in the intron 2 region and two STAT5 binding sites in the distal 5' -flanking region of the IGF-I gene. The objective of this study was to determine whether additional STAT5 binding sites are involved in GH stimulation of IGF-I gene expression. Analysis of IGF-I pre-mRNA and mRNA expression indicated that IGF-I transcription in mouse liver increased from 30 min to 6 hours after GH administration. Sequence analysis of 170 kb mouse genomic DNA revealed nineteen consensus STAT5 sequences that were conserved in the corresponding human DNA sequence. These putative STAT5 binding sites were located in fourteen chromosomal regions with each region containing one or two STAT5 binding sites. Eight of these chromosomal regions were able to mediate STAT5 activation of reporter gene expression in cotransfection experiments. Two of these chromosomal regions corresponded to those previously identified. Gel-shift assays indicated that the eight new STAT5 binding sites and three of the four previously identified STAT5 binding sites could bind to GH-activated STAT5 from mouse liver. Together, these results suggest that GH stimulation of IGF-I gene transcription in the mouse liver may be

* These data have been recently published in Eleswarapu et al. Endocrinology 149: 2230-2240, 2008.

mediated by at least eleven STAT5 binding sites located in distal intronic and 5’ -flanking regions of the IGF-I gene.

Key words: Growth hormone, IGF-I, STAT5, liver, promoter, transcription

Introduction

Insulin-like growth factor-I (IGF-I), a polypeptide hormone, is essential for normal development and growth in mammals (Stewart and Rotwein 1996). IGF-I gene- or IGF-I receptor gene-deleted mice die at or shortly after birth, and those that survive are 70% smaller than wild-type mice, have defects in brain, bone, muscle and lung, and are infertile (Baker, et al. 1993; Liu, et al. 1993). IGF-I may also play a role in tumorigenesis and ageing, as high concentrations of circulating IGF-I are associated with increased risk of developing breast and prostate cancers (Pollak, et al. 2004; Samani et al. 2007) and low IGF-I levels are linked to extended longevity (Bartke 2005; Kenyon 2005). Although the IGF-I gene is widely expressed in the body (Daughaday and Rotwein 1989), most (~75%) of the circulating IGF-I comes from its gene expression in the liver (Yakar et al. 1999). Liver expression of the IGF-I gene is mainly controlled at the transcriptional level by growth hormone (GH) from the pituitary (Daughaday and Rotwein 1989). Therefore, understanding the mechanism by which GH regulates IGF-I gene expression in the liver is important to understanding how circulating IGF-I concentration is regulated.

The mechanism by which GH regulates IGF-I gene expression in the liver is only beginning to be understood. Studies on signal transducer and activator of transcription 5 (STAT5) knockout mice (Davey et al. 2001; Udy et al. 1997; Woelfle et al. 2003a) and on rats overexpressing a dominant-negative STAT5b mutant in the liver (Woelfle et al. 2003a) demonstrate that GH-increased IGF-I gene expression in the rodent liver is primarily mediated by STAT5. The same transcription factor also appears to mediate GH regulation of IGF-I gene expression in the human liver because a missense mutation in the STAT5b gene was associated with reduced serum IGF-I concentration in a man (Kofoed et al. 2003). Through mapping GH-induced DNase I hypersensitive sites, two STAT5 binding sites in intron 2 of the rat IGF-I gene were indicated to mediate STAT5 activation of IGF-I gene expression in the liver (Woelfle et al. 2003b). Through mapping STAT5 binding enhancers, two functional STAT5 binding sites were identified ~70 kb upstream from the human IGF-I gene (Wang and Jiang 2005) and these two STAT5 binding sites were later found to also mediate GH activation of IGF-I gene expression in the rat liver (Chia et al. 2006). The identification of four STAT5 binding sites upstream and downstream from the IGF-I promoter prompted us to conduct this study to determine whether there are additional STAT5 binding sites mediating GH regulation of IGF-I gene expression in the liver. Our results suggest eleven distantly located STAT5 binding sites may be involved in GH-induced STAT5 activation of IGF-I gene expression in the mouse liver.

Materials and Methods

Animal experiments

Breeding pairs of C57BL/6J-*Ghrhr*^{lit} mice, which contained a mutation in the growth hormone releasing hormone receptor (Ghrhr) gene (Godfrey, et al. 1993; Lin, et al. 1993a), were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed on automatically timed 12-h dark/light cycles and had free access to standard rodent diet (Harlan Tekland, Madison, WI, USA) and 5% sucrose water. These mice were bred to generate GH-deficient homozygous *lit/lit* mice. The *lit/lit* mice were identified based on smaller size compared to their littermates and confirmed by genotyping. For genotyping, genomic DNA was extracted from tail clips using a DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA, USA) and the *Ghrhr* DNA region containing the suspected mutation was amplified by PCR using gene-specific primers (Table 2.1). The amplified DNA was gel-purified and sequenced. *Lit/lit* male mice 4 to 5 months old were administered subcutaneously with 2 μg/g body weight of recombinant bovine

GH (The National Hormone and Peptide Program, Torrance, CA, USA) or equal amount of phosphate buffered saline (PBS). At this or similar supraphysiological dosages, bovine GH has been shown to be effective in restoring normal gene expression or normal growth in GH-deficient mice (Davey, et al. 1999; Davey et al. 2001; Noshiro and Negishi 1986; Pell and Bates 1992). The mice were killed for liver collection at 5 min, 15 min, 30 min, 2 h, 6 h, and 24 h after the GH administration. *Lit/lit* male mice administered with PBS served as controls. The animal-related procedures were approved by the Virginia Tech Animal Care Committee.

DNA and RNA extraction

Mouse (C57BL/6) genomic DNA was isolated by standard proteinase K digestion followed by phenol-chloroform extraction. Total RNA from mouse liver tissue was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA), essentially according to the manufacturer's instructions. Nuclear RNA or pre-mRNA were extracted from mouse liver nuclei. About 200 mg of fresh liver sample was immediately homogenized at low speed in 6 ml of ice-cold PBS. The homogenate was centrifuged at 3,000 rpm for 10 min at 4 ºC, and the pellet was washed once in 10 ml of ice-cold hypotonic buffer composed of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT. The pellet was resuspended in 10 ml of the same hypotonic buffer and homogenized in a glass Dounce homogenizer. The nuclei were collected by centrifugation at 3,000 rpm for 10 min at 4 ºC. Nuclear RNA was extracted from the liver nuclei as described for total RNA. The concentration and quality of extracted DNA and RNA were determined by spectrophotometry and gel-electrophoresis, respectively.

Plasmid construction

Fourteen mouse IGF-I gene and 5'-flanking regions containing putative STAT5 binding sites were amplified using standard PCR conditions. The sequences of the forward and reverse primers for these PCRs are shown in Table 2.1. These DNA regions were designated as S1 to S14 and were 200-400 bp long. The products of these PCR reactions were gel-purified after gel electrophoresis and digested with Sma I and Kpn I restriction enzymes. Subsequently, the digested DNA was purified and cloned into pGL2TK, a minimal thymidine kinase (TK) promoter-luciferase reporter vector constructed previously (Wang and Jiang, 2005). The resulting constructs were named pGL2TK-S1, -S2, -S3, -S4, -S5, -S6, -S7, -S8, -S9, -S10, -S11, -S12, -S13, and -S14, respectively. Similarly, mouse cytokine inducible SH2-containing protein gene (Cis) promoter region containing four STAT5 binding sites (Matsumoto, et al. 1997; Verdier, et al. 1998) was cloned to generate the construct pGL2TK-Cis. The inserts in all the plasmids were verified by sequencing at the Virginia Bioinformatics Institute Core Laboratory Facility (Blacksburg, VA, USA).

Cell culture, transient transfection and luciferase assay

The CHO cells, a Chinese hamster ovary cell line (Puck, et al. 1958), were grown in minimum essential medium (MEM) supplemented with 1 mM of sodium pyruvate, 1 mM of glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (FBS). The cells were cultured at 37 ºC in a humidified 5% CO_2 atmosphere. All reagents used in cell culture were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). In all transfection analyses, the CHO cells were plated in 24-well plates at a density of 5×10^4 / well 24 h before transfection. At 50% confluence, the cells were transfected with 0.2 μg of pGL2TK-promoter-reporter gene plasmid, 0.2 μg of bovine GHR expression plasmid, 0.2 μg of mouse STAT5b expression plasmid, and 1 ng of pRL-CMV (transfection efficiency control) using FuGENE 6 as the transfection reagent (Roche Applied Science, Indianapolis, IN, USA). The transfection efficiency was 40%. The medium was replaced with serum-free MEM 24 h after the transfection, and the cells were further cultured for 16 h. Subsequently, the cells were treated with 500 ng/mL of recombinant bovine GH (National Hormone and Peptide Program) or PBS (the vehicle for GH) for 8 h. The cells were lysed for dual-luciferase assay. In the cell lysates, the firefly luciferase activity and renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luciferase activity expressed from a promoter construct was divided by that from pRL-CMV in the same well to normalize the variation in transfection efficiency.

Nuclear protein extraction

Nuclear proteins were isolated from livers of *lit/lit mice* 30 min after GH or PBS injection. About 200 mg of fresh liver sample was immediately homogenized at low speed in 6 ml of PBS supplemented with a protease inhibitor cocktail tablet (Roche Applied Science), 0.5 mM sodium orthovanadate, 10 mM sodium beta-glycerophosphate, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate. The homogenate was centrifuged at 3,000 rpm for 10 min at 4 ºC, and the pellet was washed once in 10 ml of ice-cold PBS. The nuclei were resuspended in low-salt buffer (20 mM Hepes, pH 7.9, 25 % glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA) supplemented with protease and phosphatase inhibitors as described above. The nuclei were lysed by addition of an equal volume of high-salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA) and incubation on ice for 30 min. The lysed nuclei were pelleted by centrifugation at 14,500 rpm for 30 min. The supernatant was collected and dialyzed in a dialysis buffer (20 mM Hepes, pH 7.9, 25 % glycerol, 100 mM KCl, 0.2 mM EDTA) for 2 h at 4 ºC. The dialyzed nuclear protein was centrifuged at 12,000 rpm for 20 min at

4 ºC and the supernatant was collected. Protein concentration was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoretic mobility shift assay (EMSA)

Complimentary oligonucleotides (Table 2.2) corresponding to nineteen putative STAT5 binding sites were annealed by heating to 90 ºC for 10 min in DNA polymerase buffer and slowly cooling to 25 ºC over 1 h. Approximately 500 ng of double-stranded oligonucleotides were end-labeled with ^{32}P using 1 μL of T_4 polynucleotide kinase (promega) and 2 μL [γ-^{32}P] ATP (30 Ci/mmol, 2 mCi/mL) (PerkinElmer Life and Analytical Sciences, Inc.) for 1 h at 37 ºC. The ^{32}P-labeled probes were purified with phenol-chloroform extraction followed by filtration through Quick Spin Sephadex G-25 columns (Roche Applied Science). The activity of the probes was estimated by liquid scintillation counting.

Ten μg of nuclear proteins were incubated with 1 x 10^5 dpm of ^{32}P-labeled oligonucleotide probe in reaction buffer containing 20% glycerol, 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 2 μg poly(dI-dC) for 90 min at 4 ºC. For the super-shift assays, 10 μg of nuclear protein was incubated with 2 μg of anti-STAT5 antibody (sc-835, Santa Cruz biotechnology, Inc., CA, USA) or 2 μg of rabbit preimmune serum in the reaction buffer for 1 h at 4 ºC before being incubated with the labeled oligonucleotide. The labeled oligonucleotide was added and the reaction was further incubated for 1 h at 4 ºC. For competitive gel-shift assays, the ^{32}P-labeled oligonucleotide was incubated with nuclear protein in the presence of 1×, 10×, and 100× molar excess of unlabeled oligonucleotide. Following the incubation, the DNA-protein mixtures were resolved on native 6% polyacrylamide gels. After electrophoresis, the gels were dried, exposed to phosphor screens, and scanned on a Molecular Imager FX System (Bio-Rad).

Real-time reverse transcription PCR

Reverse transcription was performed using TaqMan Reverse transcribing reagents (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Briefly, two μg of total RNA or nuclear RNA were reverse transcribed in a total volume of 20 μl, containing 0.5 mM dNTPs, 2.5μM random hexamers, 0.4 U/μl RNase inhibitor, 1.25 U/μl reverse transcriptase, 5.5 mM $MgCl_2$ and 1× RT buffer. The conditions for reverse transcription were 10 min at 25 °C, followed by 30 min at 48 °C, and 5 min at 95 °C. The real-time PCRs of IGF-I, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and albumin cDNAs were performed using Power SyberGreen PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7300 Real-Time PCR System. PCR was performed in a total volume of 25 μl, containing 12.5 μl of SyberGreen Master Mix, 2 μl (0.2 μg) of the cDNA, 0.2 μM of forward and reverse primers. The primers for these PCRs are shown in table 2.1. The conditions of the PCR were 95 °C for 10 min, then 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 min. GAPDH and albumin were used as internal controls for real-time PCR reactions of IGF-I mRNA and IGF-I pre-mRNA, respectively. For reverse transcription-PCR of nuclear RNA, a reverse transcription that did not contain the reverse transcriptase was included for each sample to control for genomic DNA contamination. The reverse transcription-PCR of each sample was performed in duplicate. The real-time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method, as recommended by Applied Biosystems.

Statistical analysis

Comparisons between two means were done using *t* test. Comparisons between multiple means were done using ANOVA followed by the Tukey test. These statistical analyses were performed using the General Linear Model of SAS (SAS Institute Inc., Cary, NC). All data are expressed as mean ± standard error of the mean.

Results

GH stimulated IGF-I pre-mRNA and mRNA expression in mouse liver

We first conducted a time-course experiment to determine GH-stimulated changes in IGF-I pre-mRNA and mRNA expression in mouse liver. As shown in Figure 2.1, liver expression of IGF-I pre-mRNA was significantly higher at 30 min after than before GH administration ($P < 0.05$) and continued to rise by 2 h after administration; it started to decline at 6 h after the administration and was near its basal level by 24 h after the administration. As also shown in Figure 2.1, GH administration caused a similar but delayed time-dependent increase in liver IGF-I mRNA: liver IGF-I mRNA expression was significantly higher at 6 h after than before GH administration ($P < 0.05$), and it returned to its basal level by 24 h after the administration (Figure 2.1). The delayed response of IGF-I mRNA to GH obviously reflected the fact that IGF-I mRNA is processed from IGF-I pre-mRNA. As expected, GH had no effect on liver expression of albumin pre-mRNA or GAPDH mRNA (Figure 2.1).

Nineteen of ninety STAT5 binding consensus sequences in ~170 kb of mouse IGF-I gene and flanking regions were conserved in the corresponding human sequence

Analysis of 170 kb mouse genomic sequence (GenBank accession numbers AC125082 and AC139754) consisting of ~78 kb IGF-I gene (exon 1 to exon 6), ~80 kb 5' –flanking region and ~10 kb 3' –flanking region revealed ninety putative STAT5 binding sites (TTCNNNGAA, where N is any nucleotide) (Darnell 1997; Ehret, et al. 2001). A sequence alignment of the corresponding DNA regions of mouse and human genomes (GenBank accession number

AC010202), using VISTA program (Frazer, et al. 2004), revealed that nineteen of the ninety 9-bp STAT5 consensus sequences were conserved in highly similar (> 70% identical) DNA regions (> 100 bp) between the two species (Figure 2.2). These nineteen conserved STAT5 binding sites corresponded to fourteen chromosomal regions and were designated as S1 to S14. The binding sites S1a, S1b, S2, S3, and S4 were located in the distal 5' -flanking region, S5a, S5b in intron 2, S6, S7, S8, S9, S10a, S10b, S11a, and S11b in intron 3, S12 in intron 5, and S13, S14a, and S14b in exon 6 of the IGF-I gene (Figure 2.2). Among the nineteen conserved STAT5 binding sites, ten (S1a and S1b, S5a and S5b, S10a and S10b, S11a and S11b, and S14a and S14b) were less than 250 bp apart in the genome (Figure 2.2). S1a, S1b, S5a, and S5b corresponded to the previously identified STAT5 binding sites in the rat and human IGF-I loci (Chia et al. 2006; Wang and Jiang 2005; Woelfle et al. 2003b).

S1, S2, S5, S7, S8, S9, S10, and S11 were able to mediate GH-induced STAT5 activation of reporter gene expression

We next determined whether the conserved STAT5 binding site-containing IGF-I DNA regions can mediate GH-induced STAT5 activation of gene expression, using cotransfection analysis. In these cotransfection experiments, a luciferase reporter gene construct bearing a minimal TK promoter and one of the fourteen STAT5 binding site-containing IGF-I DNA regions (i.e., S1, S2...S14) was cotransfected with a GHR expression plasmid and a STAT5b expression plasmid into CHO cells, and reporter gene response to GH was measured. As shown in Figure 2.3, GH significantly increased reporter gene expression from the constructs containing S1, S2, S5, S7, S8, S9, S10, and S11 as compared to PBS ($P < 0.05$), and GH had no effect on reporter gene expression from promoter-only plasmid. However, GH did not stimulate reporter gene expression from the constructs containing S3, S4, S6, S12, S13, and S14 (Figure 2.3).

These data indicate that S1, S2, S5, S7, S8, S9, S10, and S11 can mediate GH-induced STAT5 activation of gene expression.

The putative STAT5 binding sites in S1, S2, S5, S7, S8, S9, S10, and S11 were able to bind to STAT5 in vitro

We next performed EMSA to determine whether the nineteen putative STAT5 binding sites can bind directly to STAT5 *in vitro*. As shown in Figure 2.4, fifteen of the nineteen oligonucleotides, including S1a, S1b, S2, S3, S5a, S7, S8, S9, S10a, S10b, S11a, S11b, S12, S13, and S14a, formed DNA-protein complexes, which appeared to have the same mobility, with liver nuclear proteins from the GH-injected mice. The same complexes were not formed between the oligonucleotides and liver nuclear proteins from PBS-treated *lit/lit* mice. To confirm the presence of STAT5 in and the specificity of these DNA-protein complexes, the oligonucleotide S1a was further analyzed in supershift and competitive gel-shift assays. As shown in Figure 2.4, the DNA-protein complexes formed between the oligonucleotides S1a and the GH-treated liver nuclear proteins were partially supershifted by a STAT5 antibody. The same complexes were completely competed away by a molar excess (10- or 100-fold) of unlabeled oligonucleotide S1a but were barely affected by the same molar excess of an oligonucleotide that did not contain a STAT5 consensus binding site (Figure 2.4).

Based on these gel-shift assays, all of the putative STAT5 binding sites in S1, S2, S5, S7, S8, S9, S10, and S11 except the 3' end of STAT5 binding site in S5 were able to bind to GH-activated liver STAT5 (Figure 2.4).These gel-shift data were consistent with transfection data that the putative STAT5 binding sites in S1, S2, S5, S7, S8, S9, S10, and S11 were able to stimulate reporter gene expression in response to GH (Figure 2.3). Despite putative STAT5 binding sites in S3, S12, S13, and S14 binding to STAT5 *in vitro;* constructs containing these binding sites were unable to stimulate reporter gene expression in response to GH (Figure 2.3), indicating that STAT5 binding does not always lead to transcriptional activation.

Discussion

GH rapidly and potently stimulates IGF-I gene transcription in liver (Gronowski and Rotwein 1995). Our time course experiment in this study has shown that IGF-I gene transcription begins within 30 min of GH administration and lasts for at least 6 h, with peak at 2 h. These time dependent GH responses of IGF-I mRNA and pre-mRNA in mice were similar to those in rats (Bichell et al. 1992; Carlsson, et al. 1998; Lin et al. 1993a; Lin, et al. 1993b). It should be noted that IGF-I mRNA was not completely absent in the liver of the *lit/lit* mouse (Figure 2.1). This observation is consistent with the studies that indicate the presence of some GH and IGF-I in the serum of the *lit/lit* mouse (Cheng, et al. 1983; Donahue and Beamer 1993). STAT5 activation and nuclear translocation can occur within 5-15 min of GH administration (Ram, et al. 1996). Moreover, it has been demonstrated that GH stimulates binding of STAT5 to rat IGF-I intron 2 (corresponding to S5 region) prior to activation of IGF-I gene transcription (i.e., within 15 min) and this binding persisted for at least 60 min (Woelfle et al. 2003b). Therefore, it is possible that GH stimulates STAT5 binding to these sites to increase IGF-I gene transcription to mouse liver.

Considering the strong expression of GH-stimulated IGF-I in liver, we wondered whether additional STAT5 binding sites were involved. In accordance with our hypothesis, analysis of 170 kb mouse genomic DNA revealed nineteen consensus STAT5 sequences that were conserved in the corresponding human sequence. These nineteen putative STAT5 sequences corresponded to fourteen regions. Two (S1 and S5) of these chromosomal regions corresponded to those previously identified (Chia et al. 2006; Wang and Jiang 2005; Woelfle et al. 2003b). Our cotransfection analyses have shown that eight of the fourteen STAT5 binding site-containing

IGF-I DNA regions (S1, S2, S5, S7, S8, S9, S10, and S11) were able to mediate GH-induced STAT5 activation of gene expression. However, the magnitude of GH-induced STAT5 activation of gene expression was relatively small. Similar modest GH response was observed for STAT5 binding site containing regions of human and rat IGF-I genes (Chia et al. 2006; Wang and Jiang 2005; Woelfle et al. 2003b). One possible reason for the modest GH response of STAT5 binding sites in this study is that the CHO cell signaling system may lack additional components that are required for maximal GH-response of these STAT5 binding sites. Another possible reason could be that each of these STAT5 binding sites is transactivationally weak, and combined activation is needed to have a significant effect on IGF-I gene transcription. Considering the strong effect of GH on IGF-I expression, involvement of multiple STAT5 binding sites in GH regulation of IGF-I seems to be a plausible mechanism. Moreover, the STAT5 binding sites in these IGF-I DNA regions (S1, S2, S3, S5, S7, S8, S9, S10, and S11) could bind to GH-activated STAT5, suggesting that these STAT5 binding sites may be involved in GH-stimulated activation of gene expression. Therefore, it is possible that all the eleven STAT5 binding sites in these nine IGF-I DNA regions may be involved in GH regulation of IGF-I gene expression in liver. However, it remains to be seen which of these and/or how many of these eleven STAT5 binding sites are activated during GH-stimulated changes in IGF-I gene transcription in liver.

One interesting observation of this study was that GH increased reporter gene expression from the constructs containing S1, S2, S5, S9, S10, and S11 by 50% to 220%, whereas constructs containing S7 and S8 showed less than 50% increase in reporter gene expression in response to GH. As mentioned earlier, S1, S5, S10, and S11 each contain a pair of STAT5 binding sites. Although S9 contains only one conserved STAT5 binding site, there is a non-conserved STAT5 consensus site 240 bp upstream from the conserved STAT5 binding site. Based on our gel-shift and cotransfection analysis data STAT5 binding site in S2 appears to be a high affinity and/or strong transactivation site. Two adjacent STAT5 binding sites located on the same side of the DNA helix can stabilize each other's binding to STAT5 through formation of STAT5 tetramers (Meyer, et al. 1997). It is therefore tempting to speculate that the paired STAT5 binding sites in S1, S5, S9, S10, and S11 might allow for the formation of STAT5 tetramers or some type of STAT5-STAT5 interaction, thereby strengthening their binding to DNA to enhance activation of gene expression. Though several STAT5 binding site-containing IGF-I DNA regions (S3, S12, S13, and S14) can bind to GH-activated STAT5 *in vitro*, these DNA regions did not mediate GH-induced STAT5 activation of gene expression. One possible reason is that, in addition to STAT5, these IGF-I DNA regions may contain binding sites for other transcriptions factors, which are required for GH-induced STAT5 activation of gene expression and these transcription factors may be not be expressed in CHO cells.

The GH-responsive STAT5 binding sites identified in this study are located distantly from the transcription start site in the IGF-I gene. For example, S1 and S2 are located more than 60 kb 5' from the transcription start site. Similarly, S9, S10, and S11 are located at least 26 kb downstream from the transcription start site. The distal locations of these GH-responsive STAT5 binding sites are in line with the increasing identification of long-range enhancers (Dean 2006; Kleinjan and van Heyningen 2005; Lomvardas, et al. 2006). But how do these STAT5 binding sites mediate STAT5 action to the IGF-I promoter over seemingly very long distances? Two major models have been proposed for distant enhancer-promoter interaction (Dean 2006; Kleinjan and van Heyningen 2005). One is the looping model, in which the long intervening DNA loops out to allow direct contact between the transcription factor bound to a distant enhancer and the general transcription machinery or sequence-specific transcription factors bound at the promoter. Another is the tracking model, in which, the transcription factor and perhaps other associated proteins bound at the distant enhancer track along the intervening DNA to the promoter. Increasing evidence favors the looping model (Dean 2006). Which of these mechanisms is used by eleven distal STAT5 binding sites in mediating GH induced IGF-I gene transcription remains to be tested.

In summary, the results of this study suggest that GH-induced STAT5 activation of IGF-I gene expression in the mouse liver may be mediated by at least eleven STAT5 binding sites that are all located distantly from the IGF-I promoter. The identification of multiple distal STAT5 binding sites underscores the complexity of the mechanism that mediates GH regulation of IGF-I gene expression.

Table 2.1. Primers used in study 1

Name	Sequence[1]	Gene and location	Amplicon size (bp)	Application
S1F	GAAAGTGGGTTTGGCTTGG			
S1R	TGTGCAAACCAACCAGTCAT	IGF-I 5' flanking	301	Cloning
S2F	TCTGTTCTGGGCAAGGTCAT			
S2R	GGTTTGGAACCAAGGACAGA	IGF-I 5' flanking	252	Cloning
S3F	GGCAATTTTCCAAGAGTCCA			
S3R	GCTCTTCTAGACTCCCAAGTGC	IGF-I 5' flanking	239	Cloning
S4F	CCTAGCCCCAGCAAAGGTAT			
S4R	TGGGGGAAAGCAATGAATAG	IGF-I 5' flanking	230	Cloning
S5F	GGGTGGCTCACCTCATACTC			
S5R	GCCGATGGTTAGTAGCCAAA	IGF-I intron 2	245	Cloning
S6F	GAGCAAAGGTGAAAAGGGAAT			
S6R	ACCATCACCTTCTGCCAAAC	IGF-I intron 3	227	Cloning
S7F	GTAGAAGGCGAGGCAGTAGC	IGF-I intron 3		
S7R	ACAGCACTGTTGCTGGGTTA		249	Cloning
S8F	TCCACCATCCCTTGAGTAGG	IGF-I intron 3		
S8R	TCTGTTTGAGTGTAGACATTCTGCT		251	Cloning
S9F	AAGGTGGAGGTGGCCTTTAG	IGF-I intron 3		
S9R	GCCTGAGAATGACCTTTGGA		232	Cloning
S10F	ATTCCTCCCAGCTGTGTGTC	IGF-I intron 3		
S10R	GGACTTGGTCTGAGGCAATG		199	Cloning
S11F	AAAGGAAGGCTGGGTGGTAG	IGF-I intron 3		
S11R	GTCCTGCATGTCTGTGGAAG		278	Cloning
S12F	GCCTCGCTCCTAAAGAGTCA			
S12R	ATTGACAGGTGGCACAGACA	IGF-I intron 5	299	Cloning
S13F	CAACACAGTAAAAGGAGAAAGCAA			
S13R	AAAGAAACCAGGACTCCCAAA	IGF-I exon 6	251	Cloning
S14F	CCACCCCACACACACCTATT			
S14R	AGCTGGCCAAACAGTAAAGG	IGF-I exon 6	260	Cloning
CisPF	GTCCAGCGATACGATTGGTC			
CisPR	GAACAGCTTGGAAGGACGAG	Cis promoter	264	Cloning
IGFIE2F	TGTAAACGACCCGGACCTAC			
IGFIE3R	CACGAACTGAAGAGCATCCA	IGF-I mRNA	171	mRNA qPCR
IGFII3F	CATGGGAAGGAGACAGAGGA			
IGFII3R	GGGGTTCACTGAGGTGATTT	IGF-I intron 3	252	pre-mRNA qPCR
AlbI3F	TGGGAGCTTGACAGTGACAG			
AlbI3R	GGGATGACCATTGGTATTGG	Albumin intron 3	166	pre-mRNA qPCR
IGFIE3F	CTTGCTCACCTTCACCAGCT			
IGFIE4R	TACATCTCCAGTCTCCTCAG	IGF-I mRNA	210	Cloning
GAPDHF	ACCCAGAAGACTGTGGATGG			
GAPDHR	GGATGCAGGGATGATGTTCT	GAPDH mRNA	81	Cloning
GHRHRE2F	TGAGCTTGCATGTCTTCAGG	Ghrhr exon 2 and		
GHRHRI3R	GGTGAAGTGGACGATGAGGT	intron 3	482	Genotyping

[1]All sequences are written from 5' to 3'. The top sequence of a pair of primers is the forward primer and the bottom sequence the reverse primer.

Table 2.2. Oligonucleotides used in the gel-shift assays of study 1

Name	**Sequence**[1]	**Chromosomal Location**[2]
S1a*	AAA**TTCTAAGAA**ACT	chr10:87250569-87250583
S1b*	TTT**TTCTTAGAA**GTA	chr10:87250802-87250816
S2	TCC**TTCCTTGAA**ACT	chr10:87258619-87258633
S3	GAG**TTCTGGGAA**TGT	chr10:87261780-87261794
S4	TTA**TTCATAGAA**TGA	chr10:87279703-87279717
S5a*	GCC**TTCCTGGAA**GAA	chr10:87325725-87325739
S5b*	TGC**TTCTTAGAA**TGA	chr10:87325801-87325815
S6	GTC**TTCCATGAA**GAA	chr10:87339430-87339444
S7	ATT**TTCTGTGAA**CTA	chr10:87340981-87340995
S8	TGT**TTCAGGGAA**AAA	chr10:87346761-87346775
S9	TCT**TTCAGGGAA**ATC	chr10: 87348654-87348668
S10a	CAG**TTCTCAGAA**AGG	chr10:87364876-87364890
S10b	AAA**TTCGCAGAA**GTG	chr10:87364891-87364905
S11a	TGA**TTCCTAGAA**GAG	chr10:87369686-87369700
S11b	TAG**TTCACAGAA**AAA	chr10:87369821-87369835
S12	GAA**TTCCTTGAA**GTC	chr10:87388381-87388395
S13	GCC**TTCCAAGAA**GAA	chr10:87394585-87394599
S14a	CAT**TTCTTTGAA**AGT	chr10:87396823-87396837
S14b	TCC**TTCTTTGAA**TGT	chr10:87396881-87396895

[*]These correspond to the previously identified STAT5 binding sites in the rat and human IGF-I loci (Woelfle et al., 2003; Wang and Jiang, 2005; Chia et al., 2006). [1]Sequences of sense strand written from 5' to 3'. The core sequences of the STAT5 binding sites are indicated in bold. [2] These correspond to the locations in the Bovine October 2007 Assembly at the UCSC Genome Browser (http://genome.ucsc.edu).

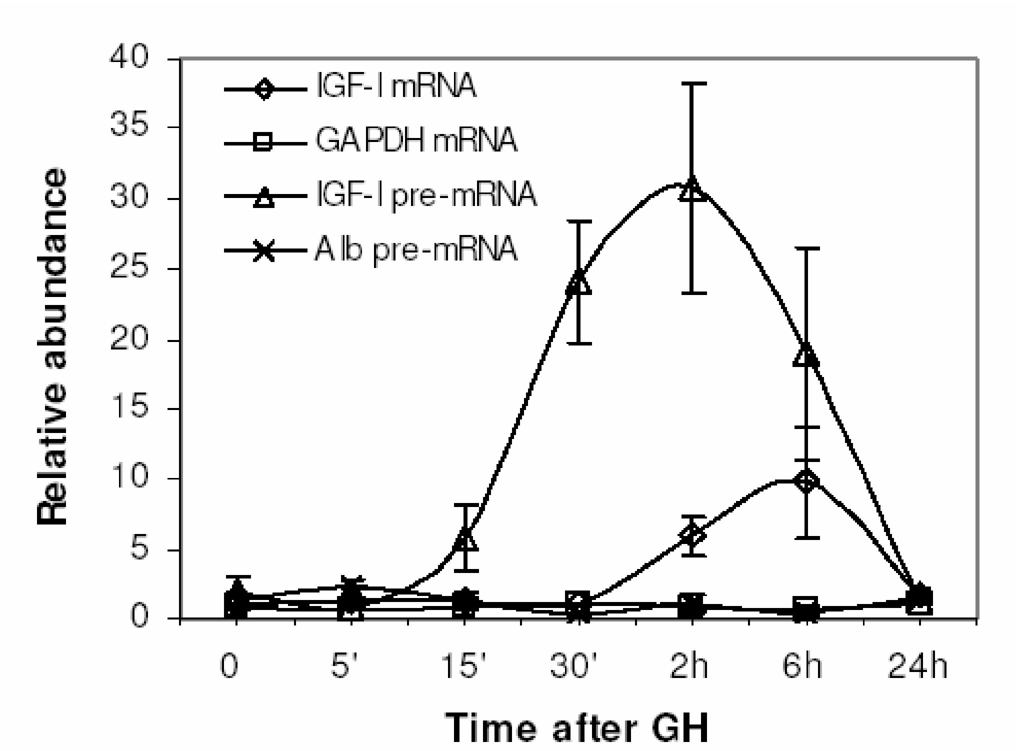


Figure 2.1. Time courses of GH-stimulated expression of IGF-I mRNA and pre-mRNA. Total RNA and nuclear RNA from GH-deficient *lit/lit* mice (n=3) at different times after GH administration were analyzed for the abundance of IGF-I mRNA, IGF-I pre-mRNA, GAPDH mRNA (as a total RNA loading control), and albumin pre-mRNA (as a nuclear RNA loading control) by quantitative real-time RT-PCR. Relative mRNA or pre-mRNA abundance at different times after GH administration is presented as changes over that in *lit/lit* mice not administered with GH (Indicated as time 0 in this figure). Note that the X-axis is not drawn to scale. * $P < 0.05$, compared to the zero time value.

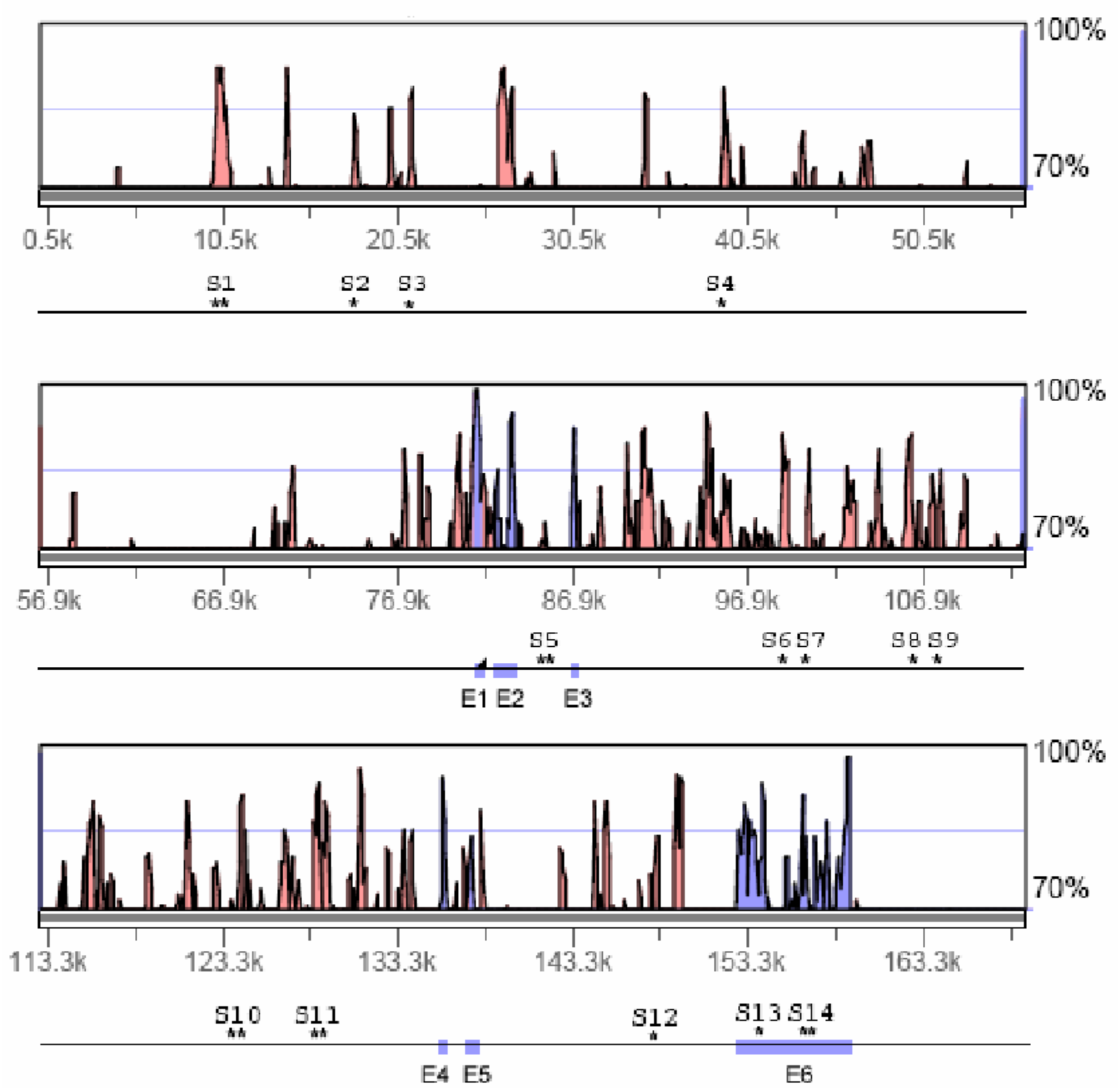


Figure 2.2. Nineteen STAT5 binding consensus sequences are conserved across the mouse and human IGF-I gene and flanking regions. The conserved STAT5 binding consensus sequences (TTCNNNGAAA) were identified by comparative sequence analysis using VISTA. The highly similar regions (>70% identical over >100 bp) between the mouse and human IGF-I gene (~80 kb 5'-flanking and ~10 kb 3'-flanking) sequences are indicated by peaks. The IGF-I exons are indicated by rectangles labeled with E1 (exon 1), E2, E3, E4, E5, and E6. The locations of the 19 conserved STAT5 binding consensus sequences are marked by asterisks. The 200-300 bp DNA regions containing these conserved STAT5 consensus sequences are designated S1 to S14.

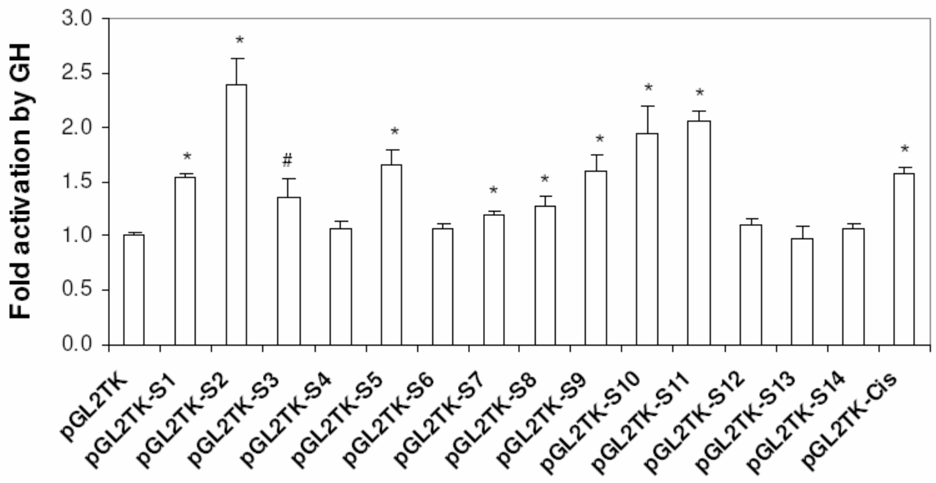


Figure 2.3. The IGF-I DNA regions S1, S2, S5, S7, S8, S9, S10, and S11 mediated GH-induced STAT5 activation of gene expression. The CHO cells were transfected with a GHR expression plasmid, a STAT5b expression plasmid, a renilla luciferase plasmid (as a transfection efficiency control), and a pGL2TK-Based firefly luciferase reporter plasmid that contained S1 to S14, or Cis. The CHO cells were treated with PBS or GH for 8 hours before dual luciferase assay. This experiment was repeated four times. Fold activation on the Y axis represents the ratio of reporter gene expression in the presence of GH to that in the presence of PBS. * and # indicate $P < 0.05$ and $P < 0.1$, respectively, compared to pGL2TK.

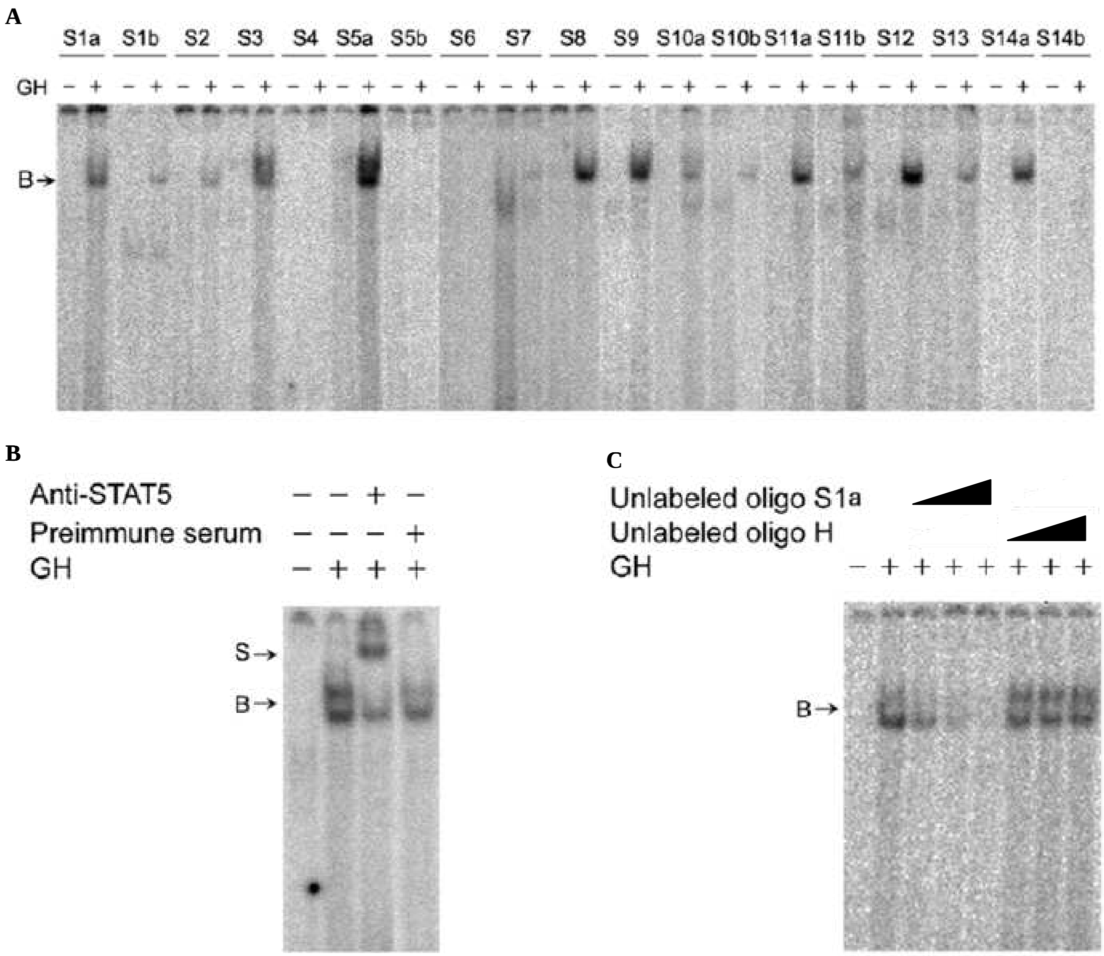


Figure 2.4. Eleven of the twelve putative STAT5 binding sites in S1, S2, S5, S7, S8, S9, S10, and S11 sequences bound to STAT5 *in vitro*. (A). Electrophoretic mobility shift assays of the nineteen putative STAT5 binding sites. A ^{32}P-labeled double-stranded oligonucleotides (15 bp) corresponding to a putative STAT5 binding site (i.e., S1a to S14) was incubated with liver nuclear proteins from the *lit/lit* mice injected with GH (+) or PBS (-), followed by polyacrylamide gel electrophoresis. "B" indicates DNA-protein complexes formed specifically between some of the oligonucleotides and the GH-treated liver nuclear proteins. (B). Supershift assay of the oligonucleotide S1a. In this assay, the ^{32}P-labeled oligonucleotide S1a was incubated with GH-treated liver nuclear proteins in the presence of anti-STAT5 antibody (anti-STAT5) or rabbit preimmune serum. "S" indicates a partial supershift of the DNA-protein complexes "B". Due to extended electrophoresis, the DNA-protein complex in panel A was separated into two bands, which probably reflected phosphorylation of STAT5 at two distinct sites (Ram et al., 1996). (C). Competitive gel-shift assay of the oligonucleotide S1a. In this assay, the ^{32}P-labeled oligonucleotide S1a was incubated with GH-treated liver nuclear proteins in the presence of 1×, 10×, or 100× molar excess of unlabeled S1a or unlabeled oligonucleotide H that did not contain a STAT5 binding site.

Chapter III Hepatocyte Nuclear Factor (HNF)-3γ Mediates Growth Hormone Stimulation of Insulin-Like Growth Factor (IGF)-I in Bovine Liver

Abstract

Insulin-like growth factor I (IGF-I) is abundantly expressed in liver under stimulation of growth hormone (GH). In a previous study we showed that GH stimulates the expression of hepatocyte nuclear factor (HNF)-3γ in bovine liver. The overall objective of this study was to determine whether and, if so, how liver-enriched transcription factor HNF-3γ is involved in GH stimulation of IGF-I expression in bovine liver. The first specific objective of this study was to determine the mechanism by which GH regulates HNF-3γ expression in bovine liver. Sequence analysis of the bovine HNF-3γ promoter revealed the presence of two putative binding sites for signal transducer and activator of transcription 5 (STAT5), whose role in GH regulation of gene expression has been well documented. The proximal STAT5 consensus sequence in the HNF-3γ promoter appears to be also conserved in other mammals. Chromatin immunoprecipitation (ChIP) assays demonstrated that GH increased the binding of STAT5 to the HNF-3γ promoter in bovine liver and that this binding was associated with increased HNF-3γ mRNA levels. Gel-shift assays demonstrated that the proximal STAT5 binding site was able to bind to GH-activated STAT5 from bovine liver. Cotransfection analyses showed that the proximal STAT5 binding site was necessary for the HNF-3γ promoter to be activated by GH. The second specific objective of this study was to determine whether HNF-3γ contributes to GH regulation of IGF-I gene expression in bovine liver. The promoter of the bovine IGF-I gene contains three putative HNF-3 binding sites that seem to be evolutionarily conserved. ChIP assays indicated that GH stimulated

the binding of HNF-3γ to the IGF-I promoter in liver. Gel-shift assays showed that one of the three putative HNF-3 binding sites, HNF-3 binding site 1, was able to bind to HNF-3γ protein from bovine liver. Co-transfection analyses demonstrated that this HNF-3 binding site was necessary for HNF-3γ activation of reporter gene expression from the IGF-I promoter. These results together suggest that in addition to the well-established mechanism of direct action, GH-activated STAT5 may also indirectly stimulate IGF-I gene transcription through enhancing HNF-3γ gene expression in the liver.

Key words: Bovine, GH, HNF-3γ, IGF-I, promoter, STAT5

Introduction

Hepatocyte nuclear factor-3 (HNF-3) proteins, including HNF-3α, -3β, and -3γ, are liver-enriched transcription factors (Schrem et al. 2002). The HNF-3α, -3β, and -3γ belong to the forkhead box family of transcription factors and are therefore also known as forkhead box (Fox) A1, FoxA2, and FoxA3, respectively. In adult liver, HNF-3γ is expressed at higher levels than HNF-3α and HNF-3β (Eleswarapu and Jiang 2005; Friedman and Kaestner 2006; Kaestner, et al. 1998). Gene-targeting studies have shown that HNF-3γ activates the expression of several genes in the liver, including phosphoenolpyruvate carboxykinase, tyrosine-aminotransferase, transferrin (Kaestner et al. 1998) and glucose transporter 2 (Shen, et al. 2001). In addition to these genes, the expression of cytochrome P450 3A4 (CYP3A4) and several members of the human CYP2C gene subfamily, a group of genes that play key roles in detoxification in the liver are also controlled by HNF-3γ (Bort, et al. 2004; Rodriguez-Antona, et al. 2003). The expression of HNF-3γ mRNA was increased during malnutrition and HNF-3γ mRNA expression was decreased by insulin (Imae, et al. 2000). The mechanism by which HNF-3γ is regulated by malnutrition or insulin is not understood.

Insulin-like growth factor-1 (IGF-I) is one of the most abundantly expressed genes in the liver (D'Ercole et al. 1984; Daughaday and Rotwein 1989). Liver IGF-I is the major source of circulating IGF-I (Yakar et al. 1999). At the cellular level, IGF-I stimulates cell proliferation, inhibits apoptosis and induces cell differentiation (Jones and Clemmons 1995). In addition, IGF-I also plays a role in postnatal growth, development of cancer, and ageing process (Hinkal and Donehower 2008; Samani et al. 2007; Stewart and Rotwein 1996). Liver expression of IGF-I is primarily regulated by GH at the transcriptional level (Bichell et al. 1992). Signal transducer and activator of transcription 5 (STAT5) has been shown to be the major transcription factor for GH regulation of IGF-I gene expression in the liver (Davey et al. 2001; Udy et al. 1997). More recent studies have suggested that STAT5 mediates GH regulation of IGF-I expression in liver by binding to multiple STAT5 binding sites that are located distantly from the IGF-I promoter (Chia et al. 2006; Eleswarapu, et al. 2008; Wang and Jiang 2005; Woelfle et al. 2003b).

In a previous study (Eleswarapu and Jiang 2005), we found that HNF-3γ expression in bovine liver was potently stimulated by GH and that other liver-enriched transcription factors, including HNF-1, HNF-3α, HNF-3β, HNF-4α, and HNF-6, were only moderately affected or not affected by GH. In this study, we determined whether GH stimulates HNF-3γ expression in bovine liver through STAT5. Since the human IGF-I promoter was previously shown to contain binding sites for HNF-3 proteins (Nolten, et al. 1996), we also tested the hypothesis that GH-stimulated HNF-3γ contributes to GH regulation of IGF-I expression in bovine liver. Our results demonstrate that GH stimulation of HNF-3γ gene expression is mediated by binding of STAT5 to a conserved STAT5 binding site in the HNF-3γ promoter and that GH-activated HNF-3γ mediates IGF-I gene expression by binding to a conserved HNF-3 binding site in the IGF-I promoter.

Materials and Methods

Animal experiments

Six non-lactating and non-pregnant cows were used in this study. Liver biopsy samples were taken from each cow 1 week before and 24 hours (h) after injection of 500 mg of recombinant bovine GH in a slow-release formula (Monsanto Company, St Louis, MO, USA). The liver biopsy samples taken before GH injection served as controls. The liver biopsy was performed as described previously (Eleswarapu and Jiang 2005). Upon collection, the liver biopsy samples were immediately frozen in liquid nitrogen and stored at -80 °C for future use or were processed immediately for liver nuclei extraction. All other tissue samples used in this study were taken from adult cows or bulls at slaughter. The animal-related procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

RNA and DNA extraction

Total RNA from bovine tissues was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA), essentially according to the manufacturers' instructions. Genomic DNA was isolated by standard proteinase K digestion followed by phenol-chloroform extraction. The concentration and quality of extracted RNA and DNA were determined by spectrophotometry and gel-electrophoresis, respectively.

Plasmids construction

pGEM-TbHNF-3γ239: To generate a probe for ribonuclease protection assay (RPA) to determine bovine HNF-3γ mRNA transcription start site, a 239 bp bovine HNF-3γ genomic DNA region that was predicted to cover the putative transcription start site based on the 5' end sequences of the human, mouse and rat HNF-3γ mRNAs in GenBank (GenBank accession numbers NM_004497, NM_008260, and NM_017077, respectively), was amplified by a standard PCR. The sequences of the forward and reverse primers for this PCR are shown in Table 1. This PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and was named pGEM-TEbHNF-3γ239.

pGL2B-bHNF-3γP: A standard PCR was used to amplify a 961 bp promoter region of the bovine HNF-3γ gene with primers bHNF3gPF1 and bHNF3gPR1 (Table 1). The 961 bp HNF-3γ promoter DNA was cloned into the pGL2B vector (Promega) between the Nhe I and Hind III sites to generate the plasmid pGL2B-bHNF-3γP.

pGL2B-bIGF-IP: A 2185 bp bovine IGF-I promoter region, spanning from 231 bp upstream of exon 1 to 42 bp downstream of exon 2, was amplified by PCR with primers bIGF-IP1921F1 and bIGF-IPR2 (Table 1). The 2185 bp IGF-I promoter DNA was cloned between the Kpn I and Xho I sites in pGL2B (Promega) to generate the plasmid pGL2B-bIGF-IP.

pGL2B-bHNF-3γPm: The putative STAT5 binding site in the bovine HNF-3γ promoter was mutated into a Not I restriction site by PCR-based site-directed mutagenesis. Briefly, two PCR amplifications were performed using plasmid pGL2B-HNF-3γP as template, and primers bHNF3gPF1 and bHNF3gPm2R in one PCR, and primers bHNF3gPm2F and bHNF3gPR1 in the other PCR (Table 1). The products of these two PCR amplifications were purified following gel electrophoresis and were combined to serve as template in a third PCR using primers bHNF3gPF1 and bHNF3gPR1 (Table 1). The product of this PCR was digested with the restriction enzymes Nhe I and Hind III and cloned into the pGL2B vector to generate the STAT5 binding site-mutated construct pGL2B-bHNF-3γPm.

pGL2B-bIGF-IPm1, pGL2B-bIGF-IPm2, and pGL2B-bIGF-IPm1m2: The HNF-3 binding sites in the bovine IGF-I promoter were similarly mutated. The mutation construct pGL2B-bIGF-IPm1, in which the first HNF-3 binding site was mutated, was generated through a PCR reaction using pGL2B-bIGF-IP as template and bIGF-IP1921m1F1 and bIGF-IPR2 as primers (Table 1). The mutation construct pGL2B-bIGF-IPm2, in which the second HNF-3 binding site was mutated, was generated by performing two PCR reactions using pGL2B-bIGF-IP as template. Primers bIGF-IP1921F1 and bIGF-IP1921m2R1 (containing a Not I restriction enzyme site) were used in one PCR, and primers bIGF-IP1921m2F1 (containing a Not I restriction enzyme site) and bIGF-IPR2 were used in the other PCR (Table 1). The products of these two PCR reactions were purified after gel electrophoresis and digested with the Not I restriction enzyme.

Subsequently, the digested products were ligated using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) and the ligated product was purified after gel electrophoresis. A single PCR reaction was performed using the purified ligated product as template with primers bIGF-IP1921F1 and bIGF-IPR2 (Table 1) to generate the plasmid pGL2B-bIGF-IPm2. The mutation construct pGL2B-bIGF-IPm1m2, in which two putative HNF-3 binding sites were mutated, was generated by a similar strategy with sequence-specific primers (Table 1). All the IGF-I promoter mutation plasmids were cloned between the Kpn I and Xho I restriction sites in pGL2B. The inserts in all the plasmids and all the mutations were verified by sequencing at the Virginia Bioinformatics Institute Core Laboratory Facility (Blacksburg, VA, USA).

Other plasmids: The plasmid encoding the wild-type STAT5b was provided by Dr. Kouichi Ariyoshi (The University of Tokyo, Tokyo, Japan) (Ariyoshi, et al. 2000). The expression plasmid encoding the bovine growth hormone receptor (bGHR) was previously generated in our laboratory (Wang and Jiang 2005). The expression plasmid for bovine HNF-3γ protein (pcDNA3.1-bHNF-3γ) was cloned previously by another laboratory member. The pGEM4Z-bHNF3g311 plasmid containing a 311 bp HNF-3γ cDNA was used to generate an antisense probe to measure HNF-3γ mRNA expression (Eleswarapu and Jiang 2005). The cDNA plasmid used to synthesize the antisense probe for ribonuclease protection assay (RPA) of total IGF-I mRNA was a pGEM-T-Easy based plasmid containing a 200 bp cDNA insert that corresponded to 137 bp of exon 3 and 63 bp of exon 4 of the bovine IGF-I gene (provided by Dr Matthew C. Lucy from University of Missouri, Columbia, MO, USA) (Kobayashi, et al. 1999). The bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA plasmid was described before (Kobayashi et al. 1999).

Ribonuclease protection assay (RPA)

To synthesize antisense RNA probes, 150 ng of linearized plasmids were mixed with 10 nmol of ATP, UTP, GTP, and 100 pmol of CTP (Promega), 50 μCi of ^{32}P-CTP (3,000 Ci/mmol, 10 mCi/mL) (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA), 250 nmol of DTT, 20 U of RNase inhibitor (Promega) and 15 U of T7 or SP6 RNA polymerase (Promega) in 1 × transcription buffer (Promega). The mixture was incubated at 37 °C for 1 h and then treated with 2 U of DNase I (Promega) at 37 °C for 20 minutes (min). The ^{32}P-labeled probes were purified with phenol-chloroform extraction followed by filtration (1,100 × g at 4 °C for 4 min) through Quick Spin Sephadex G-50 columns (Roche Applied Science, Indianapolis, IN, USA).

The RPA was carried out using the RPA II kit (Ambion, Austin, TX, USA). Briefly, 20 μg of total RNA was mixed with 1×10^5 dpm of HNF-3γ or IGF-I antisense probe in a total volume of 20 μL hybridization buffer. In the RPA of IGF-I and HNF-3γ mRNAs, 1×10^4 dpm of GAPDH antisense probe synthesized at 10-fold lower specific activity was included as a loading control. The mixture was incubated at 42 ºC for 16 h and then digested with 200 μL of 1:100 diluted ribonucleases A and T_1 at 37 °C for 45 min. The ribonuclease-protected RNA fragments were then precipitated and resolved on 6% polyacrylamide gels containing 7 M urea. After gel electrophoresis, the gels were dried and exposed to phosphor screens. Exposed phosphor screens were scanned on a Molecular Imager FX System (Bio-Rad Laboratories, Hercules, CA, USA). The densities of the protected bands were measured using the *ImageJ* software (http://rsb.info.nih.gov/ij), and were used to represent the abundance of the corresponding mRNA.

A sequencing ladder of the antisense strand of the 239 bp HNF-3γ DNA fragment was generated by using the *fmol* DNA sequencing system (Promega) and primer 5’CATCTTCAC

TGAGCCCAGCAT3'. The primer was labeled with ^{32}P-γ-ATP and T4 polynucleotide kinase as described previously (Xu, et al. 2004). This sequencing ladder served as a reference in the RPA to determine the HNF-3γ mRNA transcription start site.

Cell culture, transient transfection and luciferase assay

The CHO cells, a Chinese hamster ovary cell line (Puck et al. 1958) (ATCC, Manassas, VA) were grown in minimum essential medium (MEM) supplemented with 1 mM of sodium pyruvate, 2 mM of L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (FBS). The cells were cultured at 37 ºC in a humidified 5% CO_2 atmosphere. All reagents used in cell culture were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). In all transfection analyses, the CHO cells were plated in 24-well plates at a density of 5×10^4 / well 24 h before transfection.

In the transfection analyses to determine GH response of the HNF-3γ promoter, 0.5 μg of promoter construct pGL2B-bHNF-3γP or pGL2B-bHNF-3γPm, 0.5 μg of bovine GHR expression plasmid, 0.5 μg of STAT5b expression plasmid, and 1 ng of pRL-CMV (transfection efficiency control) were transfected using FuGENE 6 as the transfection reagent (Roche Applied Science). The medium was replaced with serum-free MEM 24 h after the transfection, and the cells were further cultured for 16 h. Subsequently, the cells were treated with 500 ng/mL of recombinant bovine GH (National Hormone and Peptide Program, Torrance, CA, USA) or phosphate buffered saline (PBS) (the vehicle for GH) for 8 h. The cells were lysed for dual-luciferase assay. In the transfection analyses to determine the ability of HNF-3γ to activate the IGF-I promoter, the CHO cells in each well were transfected with 0.5 μg promoter construct pGL2B-bIGF-IP, pGL2B-bIGF-IPm1, pGL2B-bIGF-IPm2, pGL2B-bIGF-IPm1m2, 0.5 μg of pcDNA3.1-bHNF-3γ, or empty vector, and 1 ng of pRL-CMV. The cells were lysed 48 h after the transfection. The transfection efficiency was 40%.

In the cell lysates, the firefly luciferase activity and renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activity expressed from a promoter construct was divided by that from pRL-CMV in the same well to normalize the variation in transfection efficiency.

Nuclear protein extraction

Nuclear proteins were isolated from bovine liver. About 200 mg of fresh liver sample was immediately homogenized at low speed in 6 ml of PBS supplemented with a protease inhibitor cocktail tablet (Roche Applied Science), 0.5 mM sodium orthovanadate, 10 mM sodium beta-glycerophosphate, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate. The homogenate was centrifuged at 3,000 rpm for 10 min at 4 ºC, and the pellet was washed once in 10 ml of ice-cold PBS. The nuclei were resuspended in low-salt buffer (20 mM Hepes, pH 7.9, 25 % glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA) supplemented with protease and phosphatase inhibitors as described above. The nuclei were lysed by addition of an equal volume of high-salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA) and incubation on ice for 30 min. The lysed nuclei were pelleted by centrifugation at 14,500 rpm for 30 min. The supernatant was collected and dialyzed in a dialysis buffer (20 mM Hepes, pH 7.9, 25 % glycerol, 100 mM KCl, 0.2 mM EDTA) for 2 h at 4 ºC. The dialyzed nuclear protein was centrifuged at 12,000 rpm for 20 min at 4 ºC and the supernatant was collected. Protein concentration was measured using a Bio-Rad Protein Assay Kit (Bio-Rad).

Electrophoretic mobility shift assay (EMSA)

Complimentary oligonucleotides corresponding to putative STAT5 or HNF-3 binding sites (Table 2) were annealed by heating to 90 ºC for 10 min in DNA polymerase buffer and slowly cooling to 25 ºC over 1 h. Approximately 500 ng of double-stranded oligonucleotides were end-labeled with ^{32}P using 1 μL of T_4 polynucleotide kinase (promega) and 2 μL [γ-^{32}P] ATP (30 Ci/mmol, 2 mCi/mL) (PerkinElmer Life and Analytical Sciences, Inc.) for 1 h at 37 ºC. The ^{32}P-labeled probes were purified with phenol-chloroform extraction followed by filtration through Quick Spin Sephadex G-25 columns (Roche Applied Science). The activity of the probes was estimated by liquid scintillation counting.

Ten μg of nuclear proteins were incubated with 1 x 10^5 dpm of ^{32}P-labeled oligonucleotide probe in reaction buffer containing 20% glycerol, 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 2 μg poly(dI-dC) for 90 min at 4 ºC. For the STAT5 oligonucleotide super-shift assays, 10 μg of nuclear protein were incubated with 2 μg of anti-STAT5 antibody (sc-835, Santa Cruz biotechnology, Inc., CA, USA) or 2 μg of rabbit preimmune serum in the reaction buffer for 1 h at 4 ºC before being incubated with the labeled oligonucleotide. Similar reactions were set up for HNF-3 oligonucleotide super-shift assays, where 2 μg of anti-HNF-3α (sc-6553), anti-HNF-3β (sc-6554), anti-HNF-3γ (sc-5361) (Santa Cruz Biotechnology Inc.), or goat preimmune serum were used. The labeled oligonucleotide was added and the reaction was further incubated for 1 h at 4 ºC for the STAT5 oligonculeotide and overnight at 4 ºC for the HNF-3 oligonucleotide. For competitive gel-shift assays, the ^{32}P-labeled oligonucleotide was incubated with nuclear protein in the presence of 1×, 10×, and 100× molar excess of unlabeled oligonucleotide. Following the incubation, the DNA-protein mixtures were resolved on native 6% polyacrylamide gels. After electrophoresis, the gels were dried, exposed to phosphor screens, and scanned on a Molecular Imager FX System (Bio-Rad).

Chromatin immunoprecipitation assay (ChIP)

The principle of this assay is presented in Figure 3.1. Nuclei were isolated from 200-300 mg of fresh liver samples as described for nuclear protein extraction. The pelleted nuclei were resuspended in 350 μl of shearing buffer supplemented with 2 μl of phenyl methyl sulphonyl fluoride (PMSF) and 2 μl of protease inhibitor cocktail (PIC) from the ChIP-IT kit (Active Motif, Carlsbad, CA, USA). The nuclei were subsequently sheared on ice with 10 pulses of 20 s sonication using a sonic dismembrator Model 100 at setting 3 (Fisher Scientific). Under these conditions, the chromatin was sheared to fragments 200 to 500 bp long. The sheared chromatin was either stored at -80 ºC or immediately immunoprecipitated. The immunoprecipitation of the sheared chromatin was performed using a ChIP-IT kit, following the manufacturer's directions (Active Motif) with minor modifications. Briefly, 4 μg of sheared chromatin was mixed with 25 μl of protein G-Dynal magnetic beads (Invitrogen), 3 μg of STAT5 antibody (sc-835; Santa Cruz Biotechnology, Inc.) or 3 μg of HNF-3γ antibody (sc-5361; Santa Cruz Biotechnology, Inc.) in a final volume of 200 μl of ChIP buffer 1 and was incubated overnight at 4 ºC. The immunocomplexes binding to the protein G-Dynal magnetic beads were collected using a magnetic stand. The pelleted magnetic beads were washed stringently by resuspending them in 800 μl of ChIP buffer 1. The beads were collected using a magnetic stand and the supernatant was discarded. This washing step was repeated twice. Subsequently, the magnetic beads were washed six times with 800 μl ChIP buffer 2. After washing, the immunocomplexes were resuspended in 200 μl of elution buffer and cross-linking was reversed by overnight incubation at 65 ºC. Simultaneously, 1 μg of sonicated chromatin without being immuno-precipitated, i. e., "input" chromatin, was also reverse cross-linked. Subsequently, the magnetic beads from reverse cross-linked input and immunoprecipitated chromatin samples were pelleted and the supernatant was collected. The eluted chromatin was treated with 2 μl of proteinase K at 37 ºC for 1 h. Subsequently, 200 μl of phenol-chloroform mixture was added to the sample and centrifuged for 10 min at 13,000 rpm. The supernatant was collected in a fresh tube and three times the volume of 100% ethanol and 1/10 th of total volume of 3 M sodium acetate (pH 5.2) were added and the DNA was precipitated by placing it in -80 ºC for more than 2 h. The DNA was pelleted by centrifuging the sample at 13,000 rpm for 10 min at 4 ºC. The supernatant was discarded and the DNA pellet was washed with 500 μl of 70% ethanol. After washing, the DNA pellet was air-dried and resuspended in sterile water, and the DNA concentrations were measured using a spectrophotometer at 260 nm wavelength.

The enrichment of DNA fragments from their corresponding antibody-precipitated chromatin samples was determined by semi-quantitative PCR using 2× PCR Master Mix (Promega) and sequence-specific primers (Table 1). In each PCR, a GAPDH promoter region was also amplified. The GAPDH promoter was not expected to be bound by STAT5 or HNF-3 because it does not contain a putative STAT5 or HNF-3 binding site, and is not regulated by GH (Eleswarapu and Jiang, 2005). Similar PCR was performed on "input" DNA. The PCR conditions were 30 cycles of 94 °C for 30 seconds, 60 °C for 1 min, and 72 °C for 2 min. The PCR products were resolved through standard agarose gels. Relative enrichment of DNA was obtained by densitometric analysis of the PCR products. The density of the promoter DNA in a ChIP sample was normalized against that of the same DNA in the input sample the normalized intensities were compared between the GH-treated and control samples.

Statistical analysis

The statistical significance of the difference between two means in a given experiment was determined using student's *t* test. Means of more than two groups were examined for statistical significance using ANOVA followed by the Tukey's test. All the statistical analyses were performed using the General Linear Model of SAS (SAS Institute, Inc., Cary, NC). All data are expressed as mean ± standard error of the mean.

Results

Tissue distribution of HNF-3γ mRNA in cattle

Based on RPA, HNF-3γ mRNA was expressed at high levels in the liver. HNF-3γ mRNA was also detectable in the small intestine (Figure 3.2). Expression of HNF-3γ mRNA was not observed in other tissues examined, including the skeletal muscle, lung, spleen, heart, kidney, testis, mammary gland, rumen, pituitary, cerebral cortex, and hypothalamus (Figure 3.2). In both liver and small intestine, three ribonuclease-protected HNF-3γ mRNA fragments were detected (Figure 3.2). These three fragments corresponded to HNF-3γ mRNA transcripts transcribed from three different start sites (see below).

Transcription start sites for HNF-3γ mRNA in liver

The RPA using the antisense probe generated from a 239 bp HNF-3γ genomic DNA region, which was expected to cover the transcription start site, generated three protected HNF-3γ mRNA fragments from liver (Figure 3.3). Each fragment reflected a different transcription start site. Based on the sequencing ladder of the antisense strand of the same 239 bp DNA fragment, the most abundant fragment corresponded to HNF-3γ mRNA transcribed from the nucleotide 29 bp downstream from a putative TATA box (Figure 3.3). The two less abundant bands placed two additional transcription start sites at 23 bp and 33 bp downstream from the TATA box (Figure 3.3).

Effects of GH on hepatic HNF-3γ and IGF-I gene expression in cattle

Liver expression of HNF-3γ mRNA in the cows 24 h after GH administration was higher ($P < 0.05$) than before GH injection (Figure 3.4A). The difference in HNF-3γ mRNA expression was more than two fold (Figure 3.4B). The RPA of IGF-I mRNA generated two ribonuclease-protected fragments across the liver samples (Figure 3.4A). The higher molecular weight band corresponded to major IGF-I mRNA sequences. The lower molecular weight band, which appeared in the same expression pattern as the higher band across the samples (Figure 3.4A), probably represented a splice variant of IGF-I mRNA. The GH administration increased liver IGF-I mRNA expression ($P < 0.05$) by three fold (Figure 3.4B). The GH administration had no effect on liver expression of GAPDH mRNA (Figure 3.3A).

Bovine HNF-3γ promoter contains a conserved STAT5 binding consensus site

Since STAT5 is the major transcription factor involved in GH regulation of gene expression in the liver (Herrington and Carter-Su 2001), we thought that STAT5 might also mediate GH regulation of HNF-3γ expression and therefore searched for the presence of putative STAT5 binding sites in the HNF-3γ promoter. The search revealed two consensus STAT5 binding sequence (Figure 3.5A), TTCNNNGAA, where N is any nucleotide (Darnell 1997). A sequence alignment of the corresponding DNA regions of the bovine, mouse, rat, horse, dog, human, and chimpanzee genomes revealed that the proximal putative STAT5 binding site was conserved across these species (Figure 3.5B). Since regulatory elements are often evolutionarily conserved (Boffelli, et al. 2004; Prakash and Tompa 2005), the conserved STAT5 binding site in HNF-3γ promoter might play a role in mediating GH regulation of HNF-3γ gene expression.

GH stimulated STAT5 binding to the HNF-3γ promoter in the bovine liver

Since the HNF-3γ promoter contains two putative STAT5 binding sites, we next determined whether the HNF-3γ promoter is bound by STAT5 in bovine liver, using ChIP assays. As shown in Figure 3.6, anti-STAT5 antibody precipitated more of the HNF-3γ promoter DNA containing the two putative STAT5 binding sites from GH-treated liver than from control liver. On the other hand, anti-STAT5 antibody did not precipitate detectable GAPDH promoter DNA (Figure 3.6), which does not contain a putative STAT5 binding site, from either GH-treated or control liver. In input (i. e., no antibody) liver chromatin, the abundance of HNF-3γ promoter DNA was not different between GH-treated and control livers (Figure 3.6). These ChIP assays indicate that GH increased the binding of STAT5 to the HNF-3γ promoter in bovine liver. This increased binding was associated with GH-stimulated HNF-3γ gene transcription in liver (Figure 3.4), suggesting that GH may increase HNF-3γ expression in liver by stimulating the binding of STAT5 to the HNF-3γ promoter.

The conserved STAT5 binding site in the HNF-3γ promoter was able to bind to STAT5 in vitro

We next performed EMSA to determine whether the two putative STAT5 binding sites in the HNF-3γ promoter can bind directly to HNF-3γ protein. As shown in Figure 3.7A, an oligonucleotide corresponding to the proximal STAT5 binding site, or STAT5 site 2, formed two DNA-protein complexes (denoted B1 and B2 in the figure 3.7A) with liver nuclear proteins from the GH-injected cows. STAT5 can bind to DNA sequence in the form of dimer or tetramer (John, et al. 1999). As complex B2 moved more slowly than B1, we speculate that B1 complex might contain a dimeric form of STAT5 and the B2 complex a tetrameric form of STAT5. The oligonucleotide corresponding to the distal STAT5 site, or STAT5 site 1, did not form a DNA-protein complex with bovine liver nuclear proteins (Figure 3.7A), indicating that this putative STAT5 binding site is not a real STAT5 binding site. Competitive gel-shift and supershift assays were performed to confirm the specificity and presence of STAT5 in the DNA-protein complex formed with the putative STAT5 binding site 2. In the competitive gel-shift assay, the DNA-protein complex was competed away by a molar excess (10- or 100-fold) of unlabeled oligonucleotide corresponding to STAT5 binding site 2, but was not affected by the same molar excess of an oligonucleotide unrelated to STAT5 binding site (Figure 3.7B). The same DNA-protein complex was supershifted by addition of the STAT5 antibody, whereas it was not affected by addition of the preimmune serum (Figure 3.7C). The results of these competitive gel-shift and supershift assays demonstrated the presence of the STAT5 protein in the DNA-protein complex formed between the proximal STAT5 binding site in the bovine HNF-3γ promoter and GH-activated STAT5 protein from bovine liver.

The HNF-3γ promoter was able to mediate GH-induced STAT5 activation of reporter gene expression in a STAT5 binding site-dependent manner

We determined whether the HNF-3γ promoter can mediate GH-induced STAT5 activation of reporter gene expression, using a cotransfection analysis. In this cotransfection experiment, a bovine HNF-3γ promoter-reporter construct was cotransfected with a bGHR expression plasmid and a STAT5b expression plasmid into CHO cells. GH treatment of the transfected CHO cells caused a 2-fold increase in luciferase activity expressed from the HNF-3γ promoter plasmid ($P < 0.05$, Figure 3.8). We next determined whether this response of the HNF-3γ promoter to GH is dependent on the proximal STAT5 binding site in the promoter. As shown in Figure 3.8, mutation of the proximal STAT5 binding site completely abolished the GH response of the promoter ($P < 0.05$). This indicates that the proximal STAT5 binding site in the HNF-3γ promoter is essential for the GH response of the promoter. The results of the above gel-shift, ChIP assays, and co-transfection analyses support the hypothesis that GH increases HNF-

3γ expression in bovine liver by stimulating the binding of STAT5 to the proximal STAT5 binding site in the HNF-3γ promoter.

The bovine IGF-I promoter contains three putative HNF-3 binding sites

Sequence analysis of a 2-kb bovine IGF-I promoter revealed three putative HNF-3 binding sites (Figure 3.8A). All of them were nearly identical to consensus HNF-3 binding sequence, 5' WRRRYMAAYA 3', where W is A or T; R is A or G; Y is C or T; M is A or C (Roux, et al. 1995). The putative HNF-3 binding site 1 corresponded to a HNF-3 binding site previously identified in human IGF-I promoter (Nolten et al. 1996). A sequence alignment of the corresponding DNA regions of the bovine, mouse, rat, horse, dog, human, and chimpanzee genomes revealed that all three putative HNF-3 binding sites were conserved in these species (Figure 3.9B).

GH stimulated HNF-3γ binding to the IGF-I promoter in bovine liver

Whether GH increased HNF-3γ binds to the IGF-I promoter in bovine liver was determined using ChIP assays. The assays indicated that a HNF-3γ antibody precipitated more of the IGF-I promoter region containing the putative HNF-3 binding sites from GH-treated liver than from control liver (Figure 3.10). In one of the ChIP assays, the HNF-3γ antibody did not precipitate detectable GAPDH promoter from either the GH-treated liver or the control liver (Figure 3.10). In two ChIP assays, the HNF-3γ antibody appeared to precipitate some GAPDH promoter DNA, but the amount of DNA precipitated was not different between GH-treated liver and control liver. This precipitation of GAPDH promoter by HNF-3γ antibody probably does not indicate HNF-3γ binding to the GAPDH promoter, as the GAPDH promoter lacks any putative HNF-3γ binding sites. Rather, it probably reflected either incomplete removal of the unbound chromatin during the ChIP procedure or high level of non-specific binding of the HNF-3γ antibody to the GAPDH promoter chromatin. There was no difference in the IGF-I promoter abundance amplified from input chromatin from GH-treated and control livers (Figure 3.10). These results together indicated that GH increased the binding of HNF-3γ to the IGF-I promoter in bovine liver.

Two putative HNF-3 binding sites in the IGF-I promoter were able to bind to HNF-3γ in vitro

Whether the putative HNF-3 binding sites in the bovine IGF-I promoter can bind directly to the HNF-3 protein from bovine liver was determined by gel-shift assays. The gel-shift assays showed that oligonucleotides corresponding to both the HNF-3 binding site 1 and site 2 formed a DNA-protein complex with nuclear proteins from control and GH-injected cows (Figure 3.11A), whereas the putative HNF-3 binding site 3 did not (Figure 3.11A). The HNF-3 site 1 complex was much stronger than the site 2 complex (Figure 3.11A). Competitive gel-shift and supershift assays were done to determine the specificity of the complex and the presence of HNF-3 proteins in the complex, respectively. The DNA-protein complex formed by the HNF-3 binding site 1 oligonucleotide was completely competed away by 100-fold molar excess of the same unlabeled oligonucleotide (Figure 3.11B), indicating that the interaction between this site and liver nuclear protein was specific. The DNA-protein complex formed by putative HNF-3 binding site 2 was not competed by as much as 100-fold molar excess of the unlabeled oligonucleotide (Figure 3.11B), suggesting that this complex might be non-specific. The DNA-protein complex formed by the putative HNF-3 binding site 1 was supershifted by addition of the HNF-3γ antibody, but not by the HNF-3α or preimmune serum (Figure 3.11C). These results indicated the presence of HNF-3γ in the DNA-protein complex formed with putative HNF-3 binding site 1. The complex formed between the oligonucleotide and GH-treated liver nuclear protein was denser than the complex with control liver nuclear protein. This difference reflected the increased expression of HNF-3γ protein in the GH-treated liver.

The IGF-I promoter was able to mediate HNF-3γ activation of reporter gene expression

Cotransfection analyses were performed to determine whether HNF-3γ can activate reporter gene expression from IGF-I promoter. We cotransfected a bovine IGF-I promoter reporter construct with either HNF-3γ expression plasmid or pcDNA3.1 (empty vector). As shown in Figure 3.12A, cotransfection of HNF-3γ increased luciferase activity from the IGF-I promoter construct more than 8-fold as compared to cotransfection of pcDNA3.1 ($P < 0.05$). We next determined whether the putative HNF-3 binding site 1 and site 2, which were indicated to bind to liver HNF-3γ by the above gel-shift experiments, are necessary for the response of IGF-I promoter to HNF-3γ. As shown in Figure 3.12B, mutation of HNF-3 binding site 1 (pGL2B-bIGF-IPm1) decreased ($P < 0.05$) the response of IGF-I promoter to HNF-3γ by 64%, suggesting that HNF-3 site 1 is a strong transactivation site. On the other hand, mutation of HNF-3 site 2 (pGL2B-bIGF-IPm2) did not cause a significant decrease ($P > 0.1$) in the response of IGF-I promoter to HNF-3γ (Figure 3.12B), or did not further reduce the response of the site 1 mutated IGF-I promoter to HNF-3γ (Figure 3.12B). These results indicated that most of the response of the IGF-I promoter to HNF-3γ is mediated by HNF-3 site 1. However, mutation of HNF-3 binding site 1 or both HNF-3 binding sites 1 and 2 did not completely block HNF-3γ activation of the IGF-I promoter (Figure 3.12B), suggesting that HNF-3γ may bind to other regions or induce other factors to activate IGF-I promoter.

Discussion

In this study, we have shown that HNF-3γ is expressed in liver and small intestine of cattle. In mice HNF-3γ mRNA is expressed not only in liver but also in testis, heart, and several other tissues (Kaestner, et al. 1994). Expression of HNF-3γ mRNA was not observed in bovine testis and heart, indicating a potential species-specific difference in HNF-3γ mRNA expression. It is also possible that the animals used in this study were not in the right physiological stage for expressing HNF-3γ mRNA in those tissues. In this study, we also determined the transcription start site of bovine HNF-3γ. Bovine HNF-3γ mRNA is transcribed from three start sites, located 23, 29 and 33 bp downstream from a putative TATA box in the HNF-3γ gene. Similar locations of transcription start sites have been reported for the mouse and human HNF-3γ mRNAs (Kaestner et al. 1994; Navas, et al. 2000).

We previously showed that GH caused strong and sustained increases in HNF-3γ mRNA expression in bovine liver (Eleswarapu and Jiang 2005). This effect of GH on HNF-3γ expression was confirmed in this study. STAT5 is the major transcription factor involved in GH regulation of gene expression in liver (Herrington and Carter-Su 2001). The bovine HNF-3γ promoter contains two consensus STAT5 binding sites. Here we have shown that GH increased binding of STAT5 to the HNF-3γ promoter in liver and that this increased binding was associated with increased HNF-3γ mRNA expression in liver. These results suggest that STAT5 mediates GH regulation of HNF-3γ expression in liver. The two STAT5 binding sites in the HNF-3γ promoter are located in the proximal promoter region and both adhere to the STAT5 consensus sequence TTCNNNGAA (Darnell 1997). However, based on gel-shift assays, only the conserved proximal STAT5 binding site in the HNF-3γ promoter bound to GH-activated STAT5 from bovine liver, suggesting that it is this STAT5 binding site that mediates the binding of

STAT5 to HNF-3γ promoter in bovine liver. Moreover, in cotransfection analyses, the proximal STAT5 binding site in the HNF-3γ promoter was found to be necessary for GH-activation, further suggesting that the proximal STAT5 binding site plays an important role in mediating GH regulation of HNF-3γ gene expression in liver. Although the distal STAT5 binding site is identical to the consensus STAT5 binding sequence, it did not bind to the GH-activated STAT5 from bovine liver. Unlike the proximal STAT5 binding site, the distal STAT5 binding site in the HNF-3γ promoter is not conserved. Furthermore, the sequence of this STAT5 site is not identical to any of the experimentally demonstrated STAT5 binding sites found in IGF-I (Eleswarapu et al. 2008; Wang and Jiang 2005; Woelfle et al. 2003b), GH receptor (Jiang, et al. 2007)(Jiang, et al. 2007), SOCS2 (Vidal, et al. 2007), HNF-6 (Lahuna, et al. 2000), CYP2A2, CYP4A2, and CYP2C11 genes (Park and Waxman 2001). In a previous study, we also found that some STAT5 consensus sequences did not bind to STAT5 (Eleswarapu et al. 2008). Thus, a consensus STAT5 binding site is not necessarily a true STAT5 binding site. In other words, the nucleotides in NNN of TTCNNNGAA might determine the affinity of the sequence for STAT5 too.

The promoter of bovine IGF-I gene contains three putative HNF-3 binding sites. This study showed that GH increased the binding of HNF-3γ to the IGF-I promoter in bovine liver and that this increased binding was associated with increased IGF-I mRNA expression in liver. In cotransfection analyses, HNF-3γ was able to increase reporter gene expression from the IGF-I promoter. These results suggest that HNF-3γ might mediate GH regulation of IGF-I gene expression in liver. Of the three putative HNF-3 binding sites, only putative HNF-3 binding site 1 was able to bind to GH-stimulated HNF-3γ from bovine liver. In cotransfection assays, mutation of this HNF-3 binding site caused significant reduction in HNF-3γ activation of IGF-I gene expression. This HNF-3 binding site is also identical to one of the two HNF-3binding sites in the human IGF-I promoter (Nolten et al. 1996). These results suggest that it is this HNF-3 binding site that mediates the binding of HNF-3γ to the IGF-I promoter in bovine liver. However, the mutation of putative HNF-3 binding site 1 did not totally abolish the response of IGF-I promoter to HNF-3γ. This suggests that HNF-3γ may activate IGF-I promoter by binding to additional HNF-3 binding sites in the IGF-I promoter. Alternatively, HNF-3γ may activate the IGF-I promoter through an indirect mechanism. One such additional HNF-3 binding site might be located 16 bp downstream from the HNF-3 binding site identified in this study, because the corresponding region in the human IGF-I promoter was demonstrated to bind to HNF-3γ (Nolten et al. 1996). This potential HNF-3 site was not studied in this work because it was not identified as a putative HNF-3 binding site by the TF search program (Heinemeyer, et al. 1999). Taken together, these results suggest that GH-increased HNF-3γ may bind to IGF-I promoter in liver and thereby increase IGF-I expression.

GH increases mRNA expression levels of HNF-3γ but not HNF-3α in bovine liver. GH also increases HNF-3β expression in bovine liver, but this increase is transient (Eleswarapu and Jiang 2005). In this study, we have shown that GH increases the HNF-3γ gene transcription through STAT5 and that the GH-increased HNF-3γ binds to the IGF-I promoter in bovine liver. Therefore, GH-increased HNF-3γ may contribute to GH-increased IGF-I mRNA expression in bovine liver. Hypophysectomy decreases HNF-3γ expression in rat liver, and GH increases HNF-3γ expression in the hypophysectomized rat liver (Lahuna, et al. 1997). The identified STAT5 binding site in the bovine HNF-3γ promoter is also conserved in other mammals, including rodents. Therefore, GH may regulate HNF-3γ expression through STAT5 in rodent liver as well. Some earlier studies, however, implicate a role of HNF-3β in GH regulation of liver IGF-I gene expression in rodents. The mRNA expression of HNF-3β was increased by GH in rat liver (Lahuna et al. 2000). Mice with truncated GH receptor had reduced HNF-3β protein in liver (Rowland et al. 2005). Furthermore, the rat HNF-3β was able to bind to human IGF-I promoter and was also able to transactivate IGF-I promoter in cotransfection experiments (Nolten et al. 1996). These studies seem to suggest that GH may regulate the expression of IGF-I in rodent liver by increasing the expression of HNF-3β. However, transgenic mice over-expressing HNF-3β showed decreases in IGF-I mRNA expression in liver (Rausa, et al. 2000). Adenoviral overexpression of HNF-3β also decreased IGF-I mRNA expression in mouse liver (Tan, et al. 2002). These results suggest that even though GH might increase HNF-3β expression, this increased HNF-3β may not necessarily lead to increased IGF-I gene expression in the liver. The transcriptional activity of HNF-3β in hepatocytes is dependent on its phosphorylation state (Wolfrum, et al. 2003). The HNF-3β contains a phosphorylation site for protein kinase B (PKB/AKT), whereas HNF-3γ does not contain this phosphorylation site (Wolfrum et al. 2003). Upon activation of the phosphatidylinositol-3-kinase (PI-3K) and AKT pathway in liver, HNF-3β gets phosphorylated and this phosphorylation decreases its transcriptional activity (Wolfrum et al. 2003). Since GH can activate the PI-3K-AKT pathway (Ji, et al. 2002), it would be reasonable to speculate that the transcriptional activity of HNF-3β is inhibited by GH despite its increased expression is by GH in liver.

The mechanism by which HNF-3γ regulates IGF-I gene expression in bovine liver was not further investigated in this study. However, based on the literature, HNF-3γ may regulate IGF-I gene expression by two possible mechanisms. The first mechanism is that HNF-3γ binding to the IGF-I promoter alters the IGF-I promoter chromatin structure and thereby enhances the binding of RNA polymerase II and other transcription factors. This reasoning is supported by the observation that HNF-3 proteins can open highly compacted chromatin in a manner not requiring the SWI/SNF chromatin remodeling complex (Cirillo, et al. 2002; Holmqvist, et al. 2005; Zhao, et al. 2007) and enhance the binding of RNA polymerase II to the gene promoters (Zhao et al. 2007) and/or promote binding of other transcription factors (Cirillo et al. 2002). The chromatin remodeling activity of HNF-3 proteins is due to the ability of their C-terminal domains to interact with core histones H3 and H4 (Holmqvist et al. 2005). The second possible mechanism is that HNF-3γ directly recruits RNA polymerase II to the IGF-I promoter independent of chromatin structure because HNF-3γ can increase reporter gene expression from "naked" IGF-I promoter in cotransfection analyses.

HNF-3γ binding induces DNA bending and this bending may facilitate interaction between proteins bound to distant sites by looping out the intervening DNA (Pierrou, et al. 1994). STAT5 is the major transcription factor involved in the IGF-I gene expression. Moreover, the STAT5 binding sites in IGF-I gene are distantly located from the IGF-I promoter. Therefore, it is reasonable to speculate that binding of HNF-3γ to the IGF-I promoter may induce bending of the IGF-I DNA and this bending may facilitate interaction between HNF-3γ and distantly bound STAT5 to regulate IGF-I gene expression. However, we did not observe any synergistic effect of HNF-3γ and STAT5 on GH-activation of IGF-I promoter plasmid containing STAT5 enhancer regions in CHO cells (unpublished data). It remains to be studied whether there is any interaction between HNF-3γ and STAT5 in GH regulation of IGF-I gene expression in the liver.

In summary, the results of this study suggest that GH increases the HNF-3γ gene transcription through STAT5 and that the GH-increased HNF-3γ binds to the IGF-I promoter and thereby increases IGF-I gene transcription in bovine liver. GH-activated STAT5 is known to directly stimulate IGF-I transcription in liver (Eleswarapu et al. 2008; Wang and Jiang 2005; Woelfle et al. 2003). Therefore, in addition to the well-established mechanism of direct action, GH-activated STAT5 may also indirectly stimulate IGF-I gene transcription through enhancing HNF-3γ gene expression in the liver.

Table 3.1. Primers used in study 2

Name	Sequence[1]	Chromosomal Location[2]	Application	Amplicon size (bp)
bHNF3gRPAF1 bHNF3gRPAPR1	GAGCGGGCGGATCCGAGG CATCTTCACTGAGCCCAGCAT	chr18 53170446-53170685	Cloning	239
bHNF3gPF1 bHNF3gPR1	ATGCTAGCCGCCGGGAAATGGAGTC GCAAGCTTCATCTTCACTGAGCCCAGCAT	chr18 53169726-53170686	Cloning	961
bHNF3gPChIPF1 bHNF3gPChIPR1	AGCCCTTCATTTCCGTCTTT AGGGAGCAGAGTCTTCGTGA	chr18 53169816-53169935	ChIP	120
bIGF-IP1921F1 bIGF-IPR2	TTCGGTACCACAGTGTCTGTGTTTTGTA AAACTCGAGCAGCAAAATTTGAGGGCAAT	chr5 71195851-71198044	Cloning	2185
bIGF-IPChIPF1 bIGF-IP1ChIPR1	TTTGCCAGAAGAGGGAGAGA GCAGGCTCTATCTGCTCTGAA	chr5 711987941-71198101	ChIP	161
bGAPDHPF1 bGAPDHPR1	ACTACTCTCCCGCAGTGCTC AGTAGTCGGCCTACCGCTTT	chr5 110663999-110664184	ChIP	185
bHNF3gPm2F	TGGAGGCTGCGGCCGCATGGAGTTCA	chr18 53169726-53169921	STAT 5 site mutagenesis	
bHNF3gPm2R	TGAACTCCATGCGGCCGCAGCCTCCA	chr18 53169726-53169921	STAT 5 site mutagenesis	
bIGF-IP1921m1F1	TTCGGTACCACAGTGTCTGTGTTTTGTGCG GCCGCGTGAGGATTTTCTCTAAAT	chr5 71198000-71198044	HNF-3 site 1 mutagenesis	
bIGF-IP1921m2F1	GTGATTTCTTGAGCGGCCGCGCGATTTCTT ACTC	chr5 71197734 -71197764	HNF-3 site 2 Mutagenesis	
bIGF-IP1921m2R1	GAGTAAGAAATCGCGGCCGCGCTCAAGAA ATCAC	chr5 71197734 -71197764	HNF-3 site 2 mutagenesis	

[1]All sequences are written from 5' to 3'. The top sequence of a pair of primers is the forward primer and the bottom sequence the reverse primer. Underlined are restriction enzyme recognition sites added for cloning or mutagenesis. [2] These correspond to the locations in the Bovine October 2007 Assembly at the UCSC Genome Browser (http://genome.ucsc.edu).

Table 3.2. Oligonucleotides used in the gel-shift assays of study 2

Name	Sequence[1]	Chromosomal Location[2]
bHNF3gP STAT5 site1 F1	CCTCTCC**TTCTGCGAA**GCCC	chr18: 53169792-53169811
bHNF3gP STAT5 site1 R1	GGGC**TTCGCAGAA**GGAGAGG	
bHNF3gP STAT5 site2 F1	GAGGC**TTCTGGGAA**ATGGA	chr18: 53169897-53169916
bHNF3gP STAT5 site2 R1	TCCAT**TTCCCAGAA**GCCTC	
bIGF-IP HNF-3 site1 F1	TTG**TAGATAAATG**TGA	chr5: 71198041-71198057
bIGF-IP HNF-3 site1 R1	TCA**CATTTATCTA**CAA	
bIGF-IP HNF-3 site2 F1	TTG**AAGGTAAATA**TTT	chr5: 71197733-71197756
bIGF-IP HNF-3 site2 R1	AAA**TATTTACCTT**CAA	
bIGF-IP HNF-3 site3 F1	G**TGTACTGTTTGCTTC**T	chr5: 71197673-71197702
bIGF-IP HNF-3 site3 R1	A**GAAGCAAACAGTACA**C	

[1]All sequences are written from 5’ to 3’. The top sequence of a pair of oligonucleotides is the sense oligo and the bottom sequence the antisense oligo. The core sequences of the STAT5 and HNF-3 binding sites are indicated in bold. [2] These correspond to the locations in the Bovine October 2007 Assembly at the UCSC Genome Browser (http://genome.ucsc.edu)

Table 3.3. Plasmids constructed and used in study 2

Construct name	Vector	Cloning sites	Corresponding locations in GenBank[1]	Notes
pGEM-TbHNF-3γ239	pGEM-T easy	EcoR I	chr18 53170446-53170685	Probe for RPA to determine transcription start site
pcDNA3.1-bHNF-3γ	pCDNA3.1	EcoR I, Not I	Acession # DQ157763*	Bovine HNF-3γ expression plasmid
pGL2B-bHNF-3γP	pGL2B	Nhe I, Hind III	chr18 53169726-53170686	Bovine HNF-3γ promoter
pGL2B-bHNF-3γPm	pGL2B	Nhe I, Hind III	chr18 53169726-53170686	STAT5 binding site-mutated bovine HNF-3γ promoter
pGL2B-bIGF-IP	pGL2B	Kpn I, Xho I	chr5 71195851-71198044	Bovine IGF-I promoter
pGL2B-bIGF-IPm1	pGL2B	Kpn I, Xho I	chr5 71195851-71198044	HNF-3 binding site 1-mutated bovine IGF-I promoter
pGL2B-bIGF-IPm2	pGL2B	Kpn I, Xho I	chr5 71195851-71198044	HNF-3 binding site 2-mutated bovine IGF-I promoter
pGL2B-bIGF-IPm1m2	pGL2B	Kpn I, Xho I	chr5 71195851-71198044	HNF-3 binding site 1 & 2-mutated bovine IGF-I promoter

[1]These correspond to the locations in the Bovine October 2007 Assembly at the UCSC Genome Browser (http://genome.ucsc.edu). * corresponds to GenBank at http://www.nlm.nih.gov.

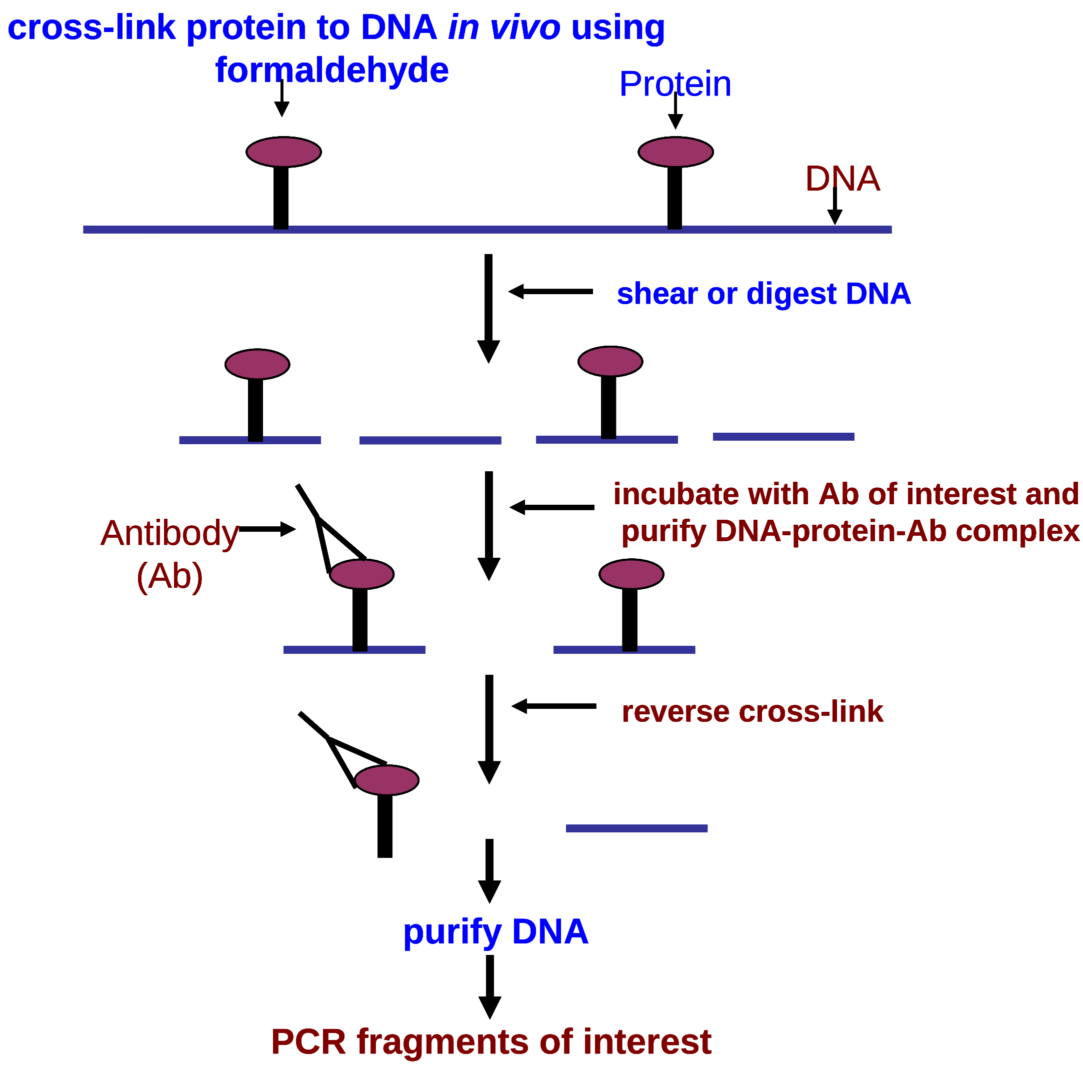


Figure 3.1. Schematic representation of chromatin immunoprecipitation assay (ChIP). The DNA-protein interactions are cross-linked by formaldehyde followed by shearing of chromatin by sonication. The DNA-protein complexes are immunoprecipitated with antibodies of interest. The cross-linking is reversed and the DNA is extracted and the target DNA is anlyazed by PCR.

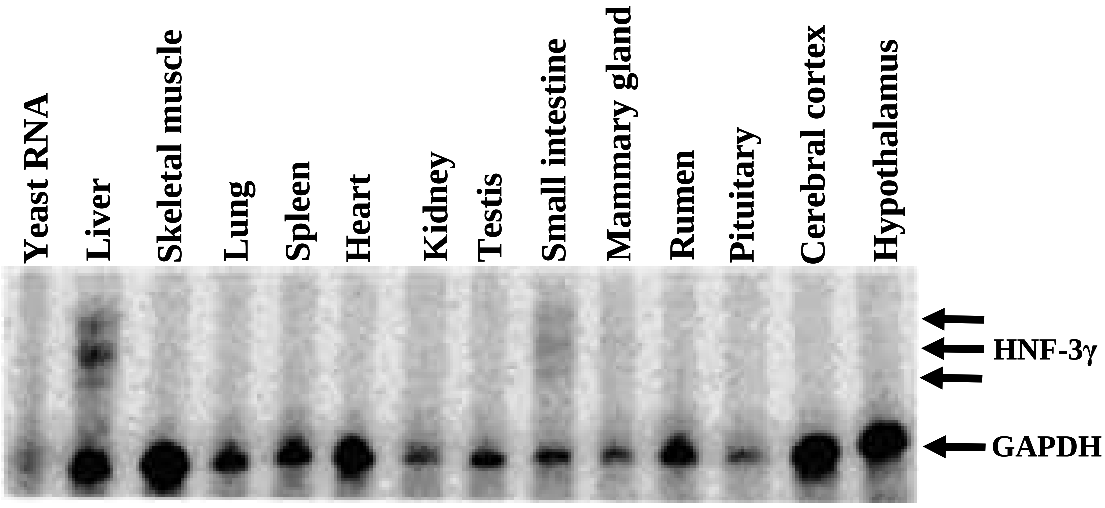


Figure 3.2. Expression of HNF-3γ mRNA in bovine tissues. All tissues were from two adult cows or bulls. HNF-3γ mRNA expression was analyzed by ribonuclease protection assay using a probe specific for the 5' portion of HNF-3γ mRNA. In the same assay, GAPDH mRNA was measured as a loading control and yeast RNA was included as a negative control. Three ribonuclease-protected HNF-3γ mRNA bands and one GAPDH mRNA band are indicated. The three protected HNF-3γ mRNA fragments correspond to three transcripts differing in the transcription start site.

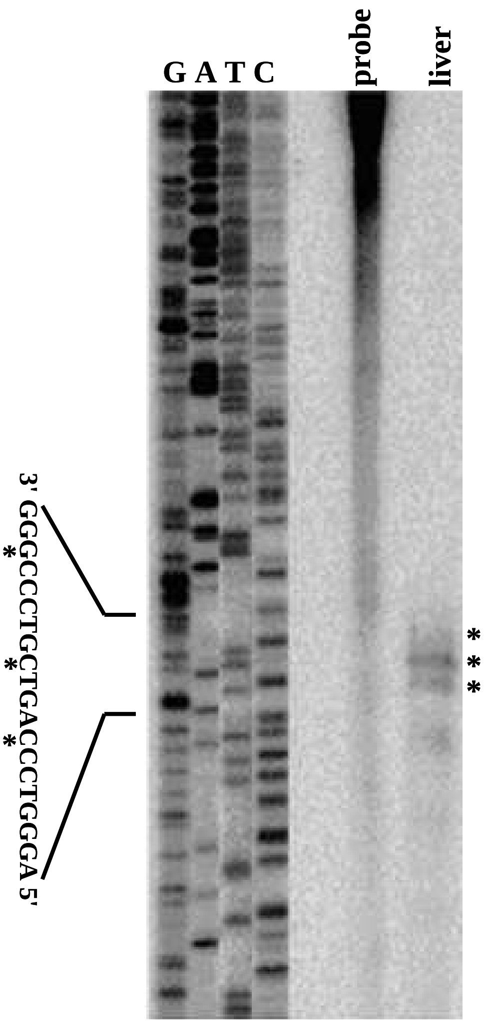
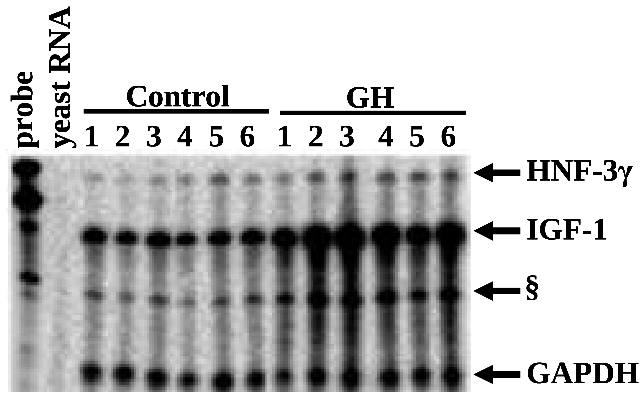


Figure 3.3. Mapping of the transcription start site for HNF-3γ mRNA. Bovine liver total RNA was analyzed by RPA using a riboprobe generated from a 239 bp HNF-3γ DNA region spanning the putative transcription start site. The ribonuclease-protected HNF-3γ mRNA fragments were resolved in parallel with the sequencing ladder (G, A, T, C) of the antisense strand of the 239 bp HNF-3γ DNA fragment. Three protected RNA bands (indicated with asterisks) place three transcription start sites at 23, 29 and 33 nucleotides downstream from a putative TATA box.

A.



B.

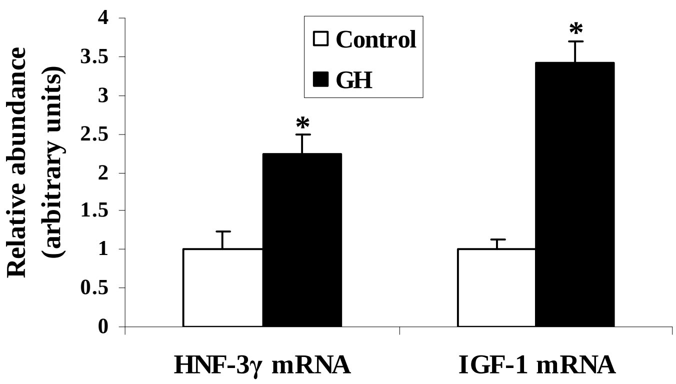


Figure 3.4. Effect of GH on HNF-3γ and IGF-I mRNA expression in cow liver. Liver RNA from six cows 24 h after GH administration and 1 wk before GH administration (Control) were analyzed for HNF-3γ and IGF-I mRNA expression. (A) Ribonuclease protection assay (RPA) of HNF-3γ, IGF-I, and GAPDH (loading control) mRNAs. Yeast RNA served as a negative control. The ribonuclease-protected fragments of HNF-3γ, IGF-I, and GAPDH mRNAs are indicated with arrows. § indicates a potential IGF-I mRNA splice variant. (B) Relative abundance of HNF-3γ and IGF-I mRNAs. The relative abundance of HNF-3γ and IGF-I mRNAs was obtained by densitometric analysis of the RPA images in panel A. The density of the protected HNF-3γ and IGF-I mRNA band in each sample was normalized against that of the protected GAPDH mRNA. All values are expressed as means ± S.E.M (n=6). '*' indicates $P < 0.05$, compared to control.

A.

gtcgctggga aatggagtcc actctgtacc agcgcctccg cccctgccgc gcgagcggcc -752
putative STAT5 site 1
ttccgccctc tcc**ttctgcg** **aa**gcccgggc agcccttcat ttccgtcttt cgggcgccat -692
putative STAT5 site 2
gctcttggga aatgtagtcc tgtgacaggg gggcgggacg gaaggggggc tggaggc**ttc** -632
tgggaaatgg agttcacgaa gactctggtt ccctggacgg ctgatgttag attgaacaga -572
gtcggatagg cgaagctcct aggtcttgca ggttatctcc agtctcagcg ggatatgagc -512
agaggccccg atcccctcca ccttttgtcc tggccattta cacaatctgg agcacttcca -452
gttatcagac gctcagtctt ccagtccgtc caaagggaaa tgtggtttcg agggtctcct -392
accggtcctt atagtaatag taacacaacg ggacctctgg agaccactcg gctctgagcc -332
ccctttccac tccaacaagt cttgctggct gttcgcctgc tccagttgag atgatgaaaa -272
ccctaagtag aagggaccta gagttctgcc ctcgaggctt ttagaatgag caattgtttc -212
ccagttatag ctccctcact cttcagccgt ccccagctct ctcgtccccg ggctgcggat -152
cccggcgcct gaggtctctc tcctcgcaat cccgcagggg gcgccccaat ccgggcgcac -92
cgccctcggg gaggcgggag gggagcctag ggcgggcgag ggcgggggtg tcccggc***tat*** -32
+1
aaagcgtggc cgcctcccgc ggcgccc***c***ggg ac***g***act***g***gga ccctgggcga tcggacggac +28
gggcgcgggt cggaactcgg gcagtgccgg ccgagagatc cgaaactctc ggttctcccc +88
gggccggaga gggggtgggt gggggcgcag gcccggggg**a tg**ctgggctc ggtgaagatg +148
g +149

B.

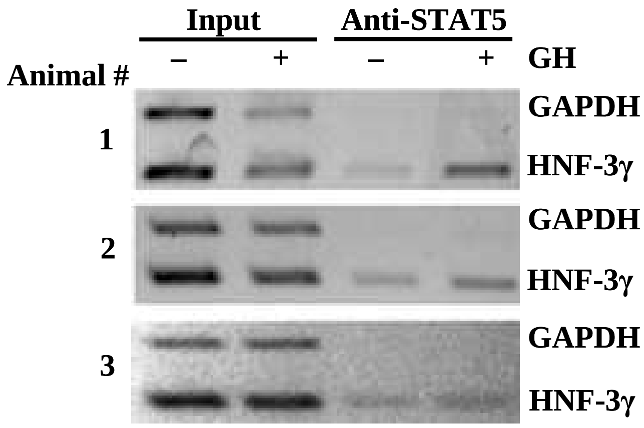
STAT5 site 1
Cow ccgccctctcc**ttctgcgaa**gc-----ccgggcagcccttcatttccg 43
Mouse ccgcccgccctccccgggcgtccac--cggagcggc-cgccatttccg 45
Rat ccgcccgccctccccgggcgtccac--cggggcggc-cgccatttccg 45
Horse ccgccc-tcccccgcaaatgacgactccgggccagcacttcatttccg 47
Dog cccccgcccctacccgacgagg----cccgggagcattttatttccg 44
Human ctgcccgcctccatactacgg------cgagcagacccttcatttccg 42
Chimpanzee ctgcccgcctccatactacgg------cgaccagccccttcatttccg 42

STAT5 site 2
Cow aggc**ttctgggaa**atggagttcacgaagactctggttccctggacggctg 50
Mouse aggc**ttctgggaa**atggagttcccagcggtccggagtccctctccc-tag 49
Rat aggc**ttctgggaa**atggagttcccagcggtccggagtccatctccc-tag 49
Horse aggc**ttctgggaa**atggagttcacagaggttctgagtccctggacggctg 50
Dog aggc**ttctgggaa**atggagtacacggtggttctgagtccctggacggctg 50
Human agca**ttctgggaa**atggagttctcagtggtgctgagtccctggacggctg 50
Chimpanzee agca**ttctgggaa**atggagttctcagtggcgctgagtccctggacggctg 50

Figure 3.5. The bovine HNF-3γ promoter contains two putative STAT5 binding sites. (A) Sequence of a 961 bp promoter region of the bovine HNF-3γ gene. The sequence corresponds to a region between 53169726 and 53170686 of chromosome 18 in the Bovine October 2007 Assembly at the UCSC Genome Browser (http://genome.ucsc.edu). In bold underlined are the two putative STAT5 binding sites. In bold italicized is the putative TATA box. Three transcription start sites are indicated in bold italicized underlined and the major transcription start site is denoted as +1. The translation start codon ATG for HNF-3γ protein is highlighted in a shaded box. (B) Alignment of corresponding regions of cow, mouse, rat, horse, dog, human, and chimpanzee HNF-3γ promoters. In bold are the putative STAT5 binding sites. The cow sequence corresponds to region between -749 and -589 in panel A of this figure. The mouse, rat, horse, dog, human, and chimpanzee sequences correspond to regions 19609673-19609510 of

chromosome 7 (July 2007 Assembly), 78377632-78377795 of chromosome 1 (November 2004 Assembly), 16415901-16416066 of chromosome 10 (January 2007 Assembly), 112649271-112649433 of chromosome 1 (May 2005 Assembly), 51058348-51058508 of chromosome 19 (March 2006 Assembly), 51493282-51493440 of chromosome 19 (March 2006 Assembly) respectively, at the UCSC Genome Browser.

A.



B.

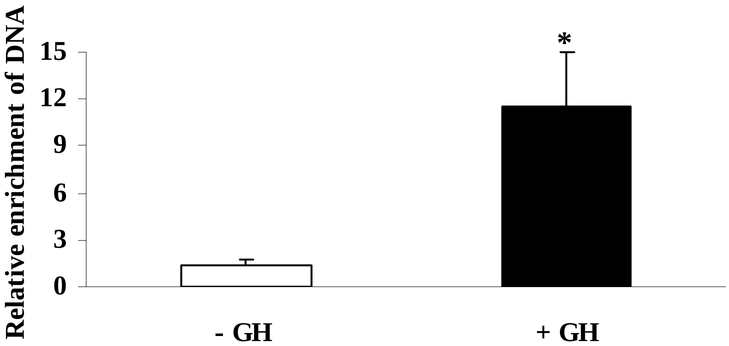


Figure 3.6. Chromatin immunoprecipitation (ChIP) assays of STAT5 binding to the HNF-3γ promoter containing the putative STAT5 binding sites in bovine liver. Cross-linked liver chromatin from GH administered (+) and control (-) cows was precipitated with anti-STAT5 antibody or was not immunoprecipitated (input), and the abundance of STAT5 binding sites-containing HNF-3γ promoter region and the GAPDH promoter region, which does not contain a STAT5 binding site (negative control), was quantified by PCR. Shown are the agarose gel images of PCR analyses of liver chromatin from 3 animals. (B) Relative enrichment of HNF-3γ promoter DNA. The relative enrichment of HNF-3γ was obtained by densitometric analysis of the ChIP images in panel A. The density of the HNF-3γ promoter DNA in Anti-STAT5 sample was normalized against that of the HNF-3γ promoter DNA in input sample. All values are expressed as means ± S.E.M (n=3). '*' indicates $P < 0.05$, compared to control.

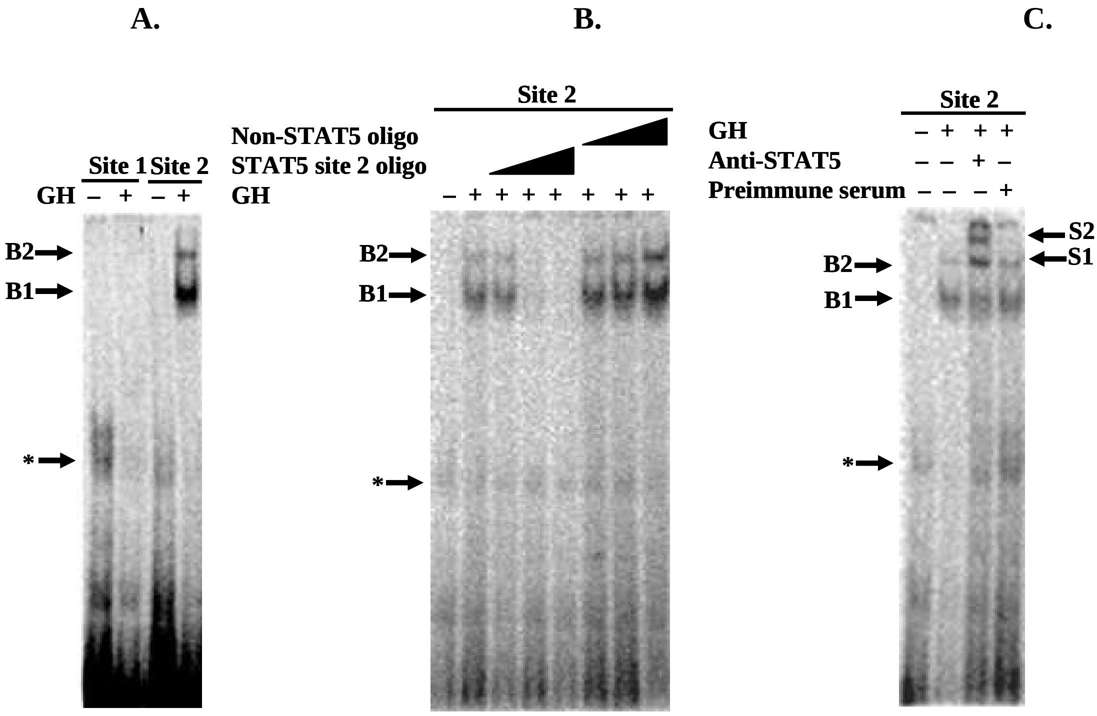


Figure 3.7. Electrophoretic mobility shift assay of the putative STAT5 binding sites in the HNF-3γ promoter. (A) A ^{32}P-labeled double-stranded oligonucleotide corresponding to a putative STAT5 binding sites (Site 1, and Site 2) were incubated with liver nuclear protein from GH-treated (+) and control (-) cattle followed by polyacrylamide gel electrophoresis. STAT5 can bind DNA sequence as a dimer or tetramer (John et al., 1999). We speculate that "B1" and "B2" indicate dimer and tetramer DNA-protein complexes, respectively. (B) Competitive gel-shift assay of the putative STAT5 binding site 2. In this assay, the ^{32}P-labeled STAT5 site 2 oligonucleotide was incubated with GH-treated liver nuclear proteins in the presence of 1 x, 10 x, 100 x molar excess of unlabeled STAT5 site 2 or unlabeled oligonucleotide that did not contain a STAT5 binding site. (C) Supershift assay of the STAT5 site 2 oligonucleotide. In this assay, the ^{32}P-labeled STAT5 site 2 oligonucleotide was incubated with GH-treated liver nuclear proteins in the presence of anti-STAT5 antibody (anti-STAT5) or rabbit preimmune serum. "S" indicate supershifts of the DNA-protein complexes "B1" and "B2", respectively to "S1" and "S2". * represents non-specific bands.

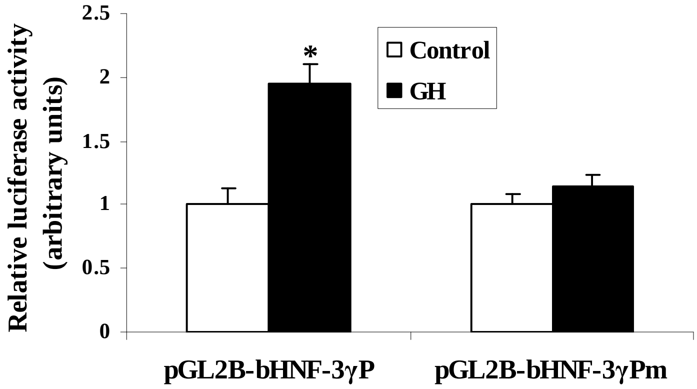


Figure 3.8. Co-transfection analysis of bovine HNF-3γ promoter in CHO cells. pGL2B-bHNF3γP is a plasmid containing bovine HNF-3γ promoter with an intact STAT5 binding site. pGL2B-bHNF3γPm contained a mutation in STAT5 binding site compared to pGL2B-bHNF3γP. The plasmid was co-transfected with GHR and STAT5 expression plasmids. Twenty-four h after transfection, the cells were serum-starved for 16 h, followed by 500 ng/mL of GH or PBS treatment for 8 h before dual-luciferase assay. Variation in transfection efficiency was controlled by co-transfecting pRL-CMV plasmid that encodes the Renilla luciferase. '*' indicates $P < 0.05$ (n=4), compared to control.

A.

putative HNF-3 site 1 **C1**
acagtgtctg tgttttg**tag ataaatg**tga ggattttctc taaat**c**cctc ttctgtttgc -1979
taaatctcac tgtcactgct aaattcagag cagatagagc ctgcgcaatg gaataaagtc -1919
ctcaaaattg aaatgtgaca ttgctctcaa catctcccat ctccctggat ttctttttgc -1859
ctcattattc ctgctaacca attcatttcc agactttgca cttcagaagc a**atg**ggaaaa -1799
putative HNF-3 site 2
atcagcagtc ttccaaccca attatttaag tgctgctttt gtgatttctt g**aaggtaaat -**1739
putative HNF-3 site 3
atttcttact ctttgaagtc attggggaat tttctttaaa ttgtgtac**tg tttgcttc**tg -1679
cttagaaatg ttcttcactt tagaattttc attgtttcgg cactgggagt tatttataaa -1619
ttgctgaata tgcaattctg tgggatctga aaaaatagct ccgggagata aatgcctttg -1559
cacagatatc tgtatgagta aaaactattg caaggtactt atgctaaatc ctccacttct -1499
tcagggcttg agtggtgtca ttatagaaga ttcctttaaa tcctgtctat ggttaagggc -1459
tatagggcat ggatatgaac ttttggattt tttttgcagg tgcagatgtt ttatttttaa -1399
gaccatgttc atgtgtatgt aggactgtgt gtggttgtgt gtgtgtgtgt gtgtgtgtgt -1339
aacttgccag gacttttgat tacaaggcat gccacataca aagagttacg ttttaaatga -1279
tattaaagct tttaaatatg atctttggag ctaaggtccc ggaactctct gcacttatgc -1219
ccagagagtc aaagttagag tgaagtttca tttgctcttc tgaaaaagaa ctccttaaga -1159
actcctgctg accttgcata ttcggataat ttaaacaaat gcacactgta tatggaaagc -1099
ggaaactttc tagcaactgc tattccaagt tttttctttt gaagaggact ctcagggggcg -1039
aagtttggac ttggggtttt gtgttgtaaa acgcggattt tgtatttggg attgtaaagt -979
ctctttaggt aaatttggct agcgttgtca atgcactgac ttcgcctttc caataactgg -919
ccctagttca agtttccatt ctcagcaaaa ttatatcttt caagacttgt atttttccaa -859
tttgcaagca tttttaagct gctgtcactg gctccccgat gcaattgcct gtgggctcaa -799
ttcatgatac gtccctaccg cttagtccag cactcgtttg ccgctttcaa gtactccacc -739
actacgactt ttcttagtca agtcagtggc ttaggagtta agaatacatc cttgtcgggc -679
acctgatcct gccgcccttg agatgccaag atgcacactg gctaccttgc ttttaaaaga -619
aatgaagtca gtatacacat atgctatatg gtctgacagc tggggatttt gtctccctac -559
ttagactatc ctaaaagggg ctgtgtggtg ttatctctgc attaattaca agtttggaaa -499
actccaaatg aactttccat gctgtgtatg ctgaactttt cagaagtaga gctagctagc -439
cataagttgt tgctttttcc tgtacttgga gcaggaagtg gtttcaggga gctacgtggt -369
tctttcaaat gtaattcaat gagtaaaggt gtctgccagg cagagctcac aagctgattg -309
tactgtgagt ctcaagatat ttccaagtgt ttgagtcaga gggaagaggg cacaggggag -249
gactggagtt tcggcccctg tccaggacgg ctacaatagg cacacgatgg aaatcggtgg -189
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tggacaaaag gcagtttacc caggctcata gcatacctgc ctgggtgtcc aaatgtaact -69
agatgctttc acaaacccca cccacaaagc agcacatgtt tttaagtcct cagttttcta -9
C2 +1
ttcacatc***a***g tctcataata cccaccctga cctgctgtaa aagatctgga acaaacaaaa +52
atggttacac ctacagtgag tattttcttg actattgccc tcaaattttg ctgggcattt +112
ttattataac ccagacatct ggaaccaatt gata +146

B.

HNF-3 Site 1
Cow tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50
Mouse tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50
Rat tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50
Horse tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50
Dog tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50
Human tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50
Chimpanzee tgtttcctgtctacagtgtctgtgttttg**tagataaatg**tgaggattttc 50

HNF-3 Site 2

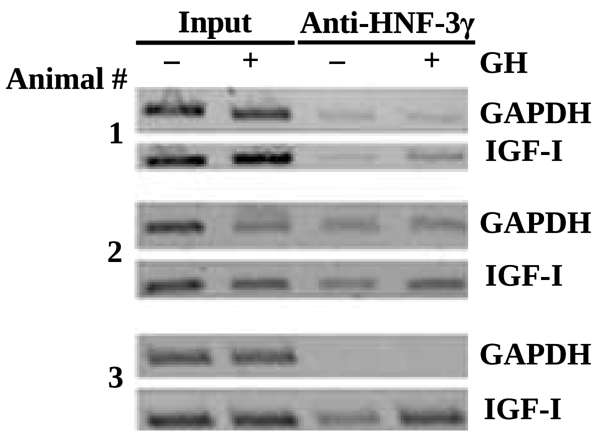
Cow tttaagtgctgcttttgtgatttcttg**aaggtaaata**tttcttactcttt 50
Mouse tttaagatctgcctctgtgacttcttg**aaggtaaata**tctcttacttttt 50
Rat tttaagatctgcctctgtgacttcttg**aaggtaaata**tctcttacttttt 50
Horse tttaagtgctgcttttgtgatttcttg**aaggtaaata**tttcttactcttt 50
Dog tttaagtgctgcttttgtgatttcttg**aaggtaaata**tttcttactcttt 50
Human tttaagtgctgcttttgtgatttcttg**aaggtaaata**tttcttactcttt 50
Chimpanzee tttaagtgctgcttttgtgatttcttg**aaggtaaata**tttcttactcttt 50

HNF-3 Site 3

Cow gaagtcattgggg-aattttctttaaattgtgtac**tgtttgcttc**tgctta 50
Mouse taactcgttggag-aattgtatttaaactgtgtac**tgttttcttc**tgccta 50
Rat gcagtcgttggat-aattgtatttaaactgtgtac**tgtcttcttc**tgcctg 50
Horse ggagtcattgggg-aattttctttaaattgtgtac**tgtttgcttc**tgccta 50
Dog gaagtcattgggggaattttctttaaattgtgtac**tgtttgcttc**ttct-a 50
Human gaagtcattgggg-aattctatttaaattgtgtac**tgtttgcttc**tgccta 50
Chimpanzee gaagtcattgggg-aattttatttaaattgtgtac**tgtttgcttc**tgccta 50

Figure 3.9. The bovine IGF-I promoter contains putative HNF-3 binding sites. (A) Sequence of a 2185 bp promoter region of the bovine IGF-I gene. The sequence corresponds to region between 71195851 and 71198044 of chromosome 5 in the Bovine October 2007 Assembly at the UCSC Genome Browser (http://genome.ucsc.edu). In bold underlined are the three putative HNF-3 binding sites. The transcription start site for class 2 IGF-I mRNA (C2) is indicated in bold italicized underlined and denoted as +1. The major transcription start site for class 1 IGF-I mRNA (C1) is indicated in bold italicized underlined. The translation start codons ATG for class 1 and class 2 IGF-I mRNAs are highlighted in shaded box. (B) Alignment of corresponding regions of cow, mouse, rat, horse, dog, human, and chimpanzee IGF-I promoters. In bold are the conserved putative HNF-3 binding sites. The cow sequence corresponds to region between -2040 and -1677 in panel A of this figure. The mouse, rat, horse, dog, human, and chimpanzee sequences correspond to regions between 87321852-87322234 of chromosome 10 (July 2007 Assembly), 24531706-24532095 of chromosome 7 (November 2004 Assembly), 26118807-26119182 of chromosome 28 (January 2007 Assembly), 44285630-44286004 of chromosome 15 (May 2005 Assembly), 101398157-101398532 of chromosome 12 (March 2006 Assembly), 103599713-103600088 of chromosome 12 (March 2006 Assembly) at the UCSC Genome Browser.

A.



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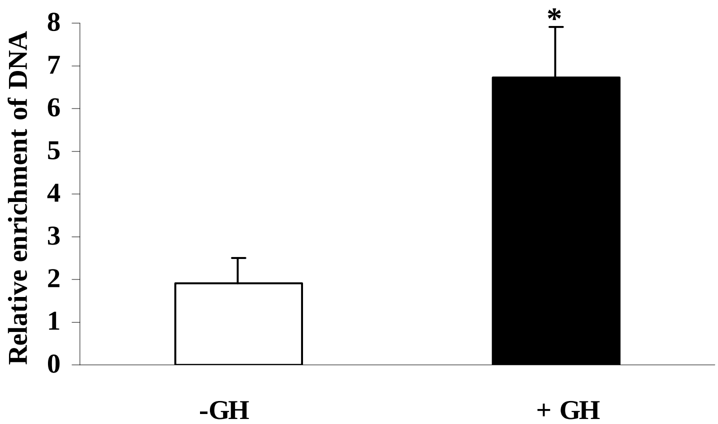


Figure 3.10. Chromatin immunoprecipitation (ChIP) assays of HNF-3γ binding to IGF-I promoter in bovine liver. (A) Cross-linked liver chromatin from GH administered (+) and control (-) cows was precipitated with anti-HNF-3γ antibody or without antibody (input), and the abundance of the putative HNF-3 binding sites-containing IGF-I promoter region and a GAPDH promoter region that does not contain a putative HNF-3 binding site (negative control) was quantified by PCR. Shown are the ChIP results of 3 animals. (B) Relative enrichment of IGF-I promoter DNA. The relative abundance of IGF-I promoter DNA was obtained by densitometric analysis of the ChIP images in panel A. The density of the IGF-I promoter DNA in anti-HNF-3γ sample was normalized against that of the IGF-I promoter DNA in input sample. All values are expressed as means ± S.E.M (n=3). '*' indicates $P < 0.05$, compared to control.

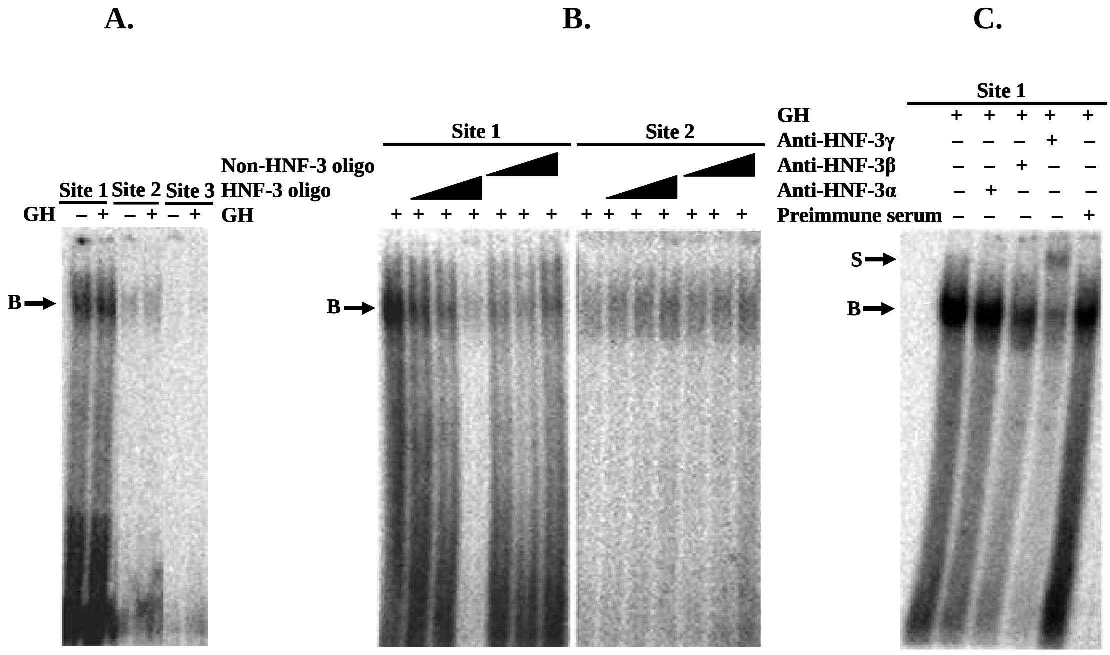
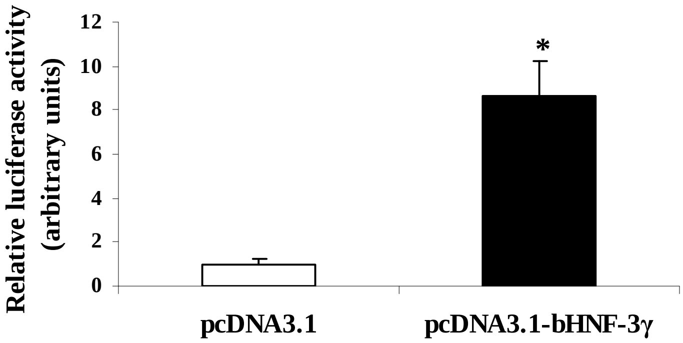


Figure 3.11. Electrophorectic mobility shift assay of the putative HNF-3 binding sites in IGF-I promoter. (A) A ^{32}P-labeled double-stranded oligonucleotide corresponding to the putative HNF-3 binding site 1, 2, or 3 was incubated with liver nuclear protein from cattle injected with GH (+) or control cattle followed by polyacrylamide gel electrophoresis. "B" indicates a DNA-protein complex. (B) Competitive gel-shift assay of the putative HNF-3 site 1 and site 2. In this assay, the ^{32}P-labeled HNF-3 oligonucleotide was incubated with GH-treated liver nuclear proteins in the presence of 1 x, 10 x, 100 x molar excess of unlabeled HNF-3 or an un-related oligonucleotide. (C) Supershift assay of HNF-3 site 1. In this assay, the ^{32}P-labeled HNF-3 site 2 oligonucleotide was incubated with GH-treated liver nuclear protein in the presence of anti-HNF-3α, anti-HNF-3β, or anti-HNF-3γ antibody, or goat preimmune serum. "S" indicates a partial supershift of the DNA-protein complex.

A.



B.

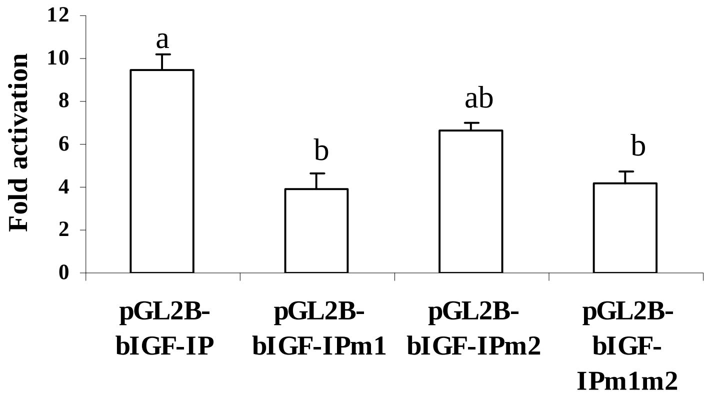


Figure. 3.12. Co-transfection analysis of bovine IGF-I promoter (bIGF-IP) in CHO cells. (A) HNF-3γ activated reporter gene expression from the IGF-I promoter. The pGL2B-bIGF-IP construct was co-transfected with bovine HNF-3γ expression plasmid (pcDNA3.1-bHNF-3 γ) or empty vector (pcDNA3.1). Variation in transfection efficiency was controlled by co-transfecting the pRL-CMV plasmid. '*' indicates $P < 0.05$ (n=4), compared to pcDNA3.1. (B) One HNF-3 binding site was required for the bovine IGF-I promoter to mediate HNF-3γ activation of gene expression. In pGL2B-bIGF-IP construct, the two putative HNF-3 binding sites were intact. In pGL2B-bIGF-IPm1, the first HNF-3 binding site was mutated. In pGL2B-bIGF-IPm2, the second HNF-3 binding site was mutated. In pGL2B-bIGF-IPm1m2, both HNF-3 binding sites were mutated. The values are represented as fold activation, which corresponds to the ratios of the reporter gene activity in the presence of pcDNA3.1-HNF-3γ to that in the presence of pcDNA3.1. Means with different letters are significantly different, $P < 0.05$ (n=4).

Epilogue

IGF-I is a polypeptide hormone that plays an important role in growth, development, aging, and tumorigenesis. In the body, IGF-I is mainly produced in the liver under the control of GH. Based on this dissertation research, GH regulates IGF-I gene expression in the liver with both direct and indirect mechanisms (Figure 4.0). With the direct mechanism, GH regulates IGF-I gene expression in liver by stimulating binding of STAT5 to at least eleven STAT5 binding sites located distantly from the IGF-I promoter. With the indirect mechanism, GH regulates IGF-I gene expression in liver by enhancing the expression of another transcription factor, HNF-3γ. Considering the significance of IGF-I in the body, I speculate that the presence of two different mechanisms of regulation of IGF-I gene expression may help to more tightly control the levels of IGF-I in the body.

Since IGF-I plays important roles in growth and milk production in cattle, the findings of this dissertation research have implications for practices in cattle production. I recommend that the breeder stock of cattle be screened for the presence of right sequences for not only the protein-coding region but also the regulatory regions identified in this research of the IGF-I gene. Since increased IGF-I has been associated with cancer in humans and various other metabolic disorders, the transcription factors involved in the regulation of IGF-I gene expression could serve as targets for therapeutic interventions to treat cancer.

This dissertation research also raises many questions to be addressed in future studies. Given the location of the eleven STAT5 binding sites from the IGF-I promoter, it makes one wonder how these distantly located STAT5 binding sites ineteract with the promoter to affect IGF-I gene transcription in liver. Therefore, it would be interesting to study the mechanisms by which these STAT5 binding sites mediate STAT5 action on the IGF-I promoter from distant locations. Since both STAT5 and HNF-3γ contribute to GH regulation of IGF-I gene transcription in liver, there is a possibility that STAT5 and HNF-3γ interact in this regulation. This possibility needs to be investigated. It is known that STAT5 interacts with co-activators, such as CBP and p300 (Pfitzner et al. 1998; Ye et al. 2001), it remains to be determined whether these co-activators are also involved in GH-induced STAT5 activation of IGF-I gene expression. More recently, glucocorticoid receptor (GR) has been implicated in regulation of IGF-I gene expression in liver (Engblom, et al. 2007). The involvement of GR in GH regulation of IGF-I gene expression in liver could be another question to study in the future.

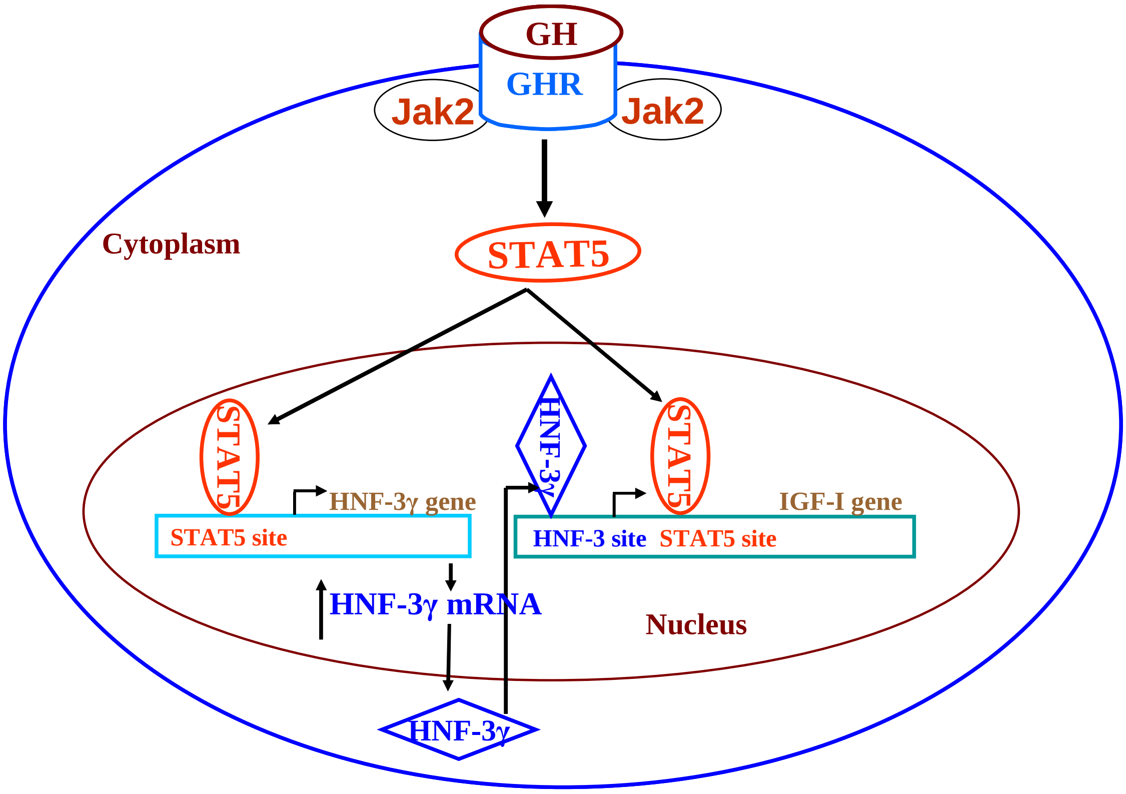


Figure 4.0. Mechanism of GH stimulation of gene expression in liver. GH may stimulate IGF-I gene transcription by both direct and indirect mechanisms. In the direct mechanism, GH stimulates IGF-I gene expression by increasing the binding of STAT5 protein to the STAT5 sites in the IGF-I gene (Eleswarapu et al. 2008; Wang and Jiang 2005; Woelfle et al. 2003b). In the indirect mechanism, GH stimulates HNF-3γ expression by increasing STAT5 binding to the HNF-3γ promoter. The increased HNF-3γ inturn binds to the IGF-I promoter and stimulates IGF-I gene expression.

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