

# Growth hormone regulates the expression of hepatocyte nuclear factor-3 gamma and other liver-enriched transcription factors in the bovine liver

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## Abstract

Growth hormone (GH) regulates the expression of many genes in the liver, and for some genes this regulation may be mediated through liver-enriched transcription factors (LETFs). As part of the long-term goal to investigate the role of LETFs in GH regulation of gene expression in the liver, in this study we determined the effect of GH administration on the expression of 10 LETFs, including hepatocyte nuclear factor (HNF)-1 $\alpha$ , HNF-1 $\beta$ , HNF-3 $\alpha$ , HNF-3 $\beta$ , HNF-3 $\gamma$ , HNF-4 $\alpha$ , HNF-6, CCAAT/enhancer-binding protein (C/EBP) $\alpha$ , C/EBP $\beta$ , and albumin D-element binding protein (DBP) in the bovine liver. Eighteen non-lactating and non-pregnant Angus cows were assigned randomly to three groups ( $n=6$  per group) and each cow received a single intramuscular injection of 500 mg slow-release recombinant bovine GH. Liver biopsy samples were taken from group 1 cows 6 h after GH administration, from group 2 cows 24 h after GH administration, and from group 3 cows 1 week after GH administration. Liver biopsies were also collected from group 3 cows 1 day before GH administration, serving as pre-GH controls. The LETF mRNAs in these liver samples were quantified using ribonuclease protection assays with probes generated from bovine LETF cDNAs cloned by standard reverse transcription-polymerase chain reaction. The levels of HNF-3 $\gamma$  and HNF-6 mRNAs were higher ( $P<0.05$ ) in the cows 24 h and 1 week after GH administration than in the untreated cows or the cows 6 h after GH administration. The levels of HNF-4 $\alpha$

mRNA were higher ( $P<0.05$ ) in the cows 1 week after GH administration than in the other three groups of cows. The levels of C/EBP $\alpha$  mRNA were higher ( $P<0.05$ ) in the cows 24 h after GH administration than in the untreated cows or the cows 6 h after GH administration. The levels of HNF-3 $\beta$  mRNA were higher ( $P<0.05$ ) in the cows 6 h after GH administration but were lower ( $P<0.05$ ) in the cows 24 h or 1 week after GH administration compared with those in the untreated cows. The levels of DBP mRNA were higher ( $P<0.05$ ) in the cows 6 h after GH administration but were lower ( $P<0.05$ ) in the cows 24 h after GH administration compared with those in the untreated cows. The levels of HNF-1 $\alpha$ , HNF-3 $\alpha$ , and C/EBP $\beta$  mRNAs were not different ( $P>0.05$ ) between groups. The expression of HNF-1 $\beta$  mRNA was not detectable. Thus, the expression of six LETFs including HNF-3 $\gamma$ , HNF-3 $\beta$ , HNF-4 $\alpha$ , HNF-6, C/EBP $\alpha$ , and DBP mRNAs in the bovine liver is regulated by GH, and these six LETFs may play a role in mediating GH regulation of gene expression in the liver. Among the 10 LETFs, the response of HNF-3 $\gamma$  to GH is most significant. Cloning and sequencing the promoter region of this gene revealed multiple putative binding elements for signal transducers and activators of transcription 5 (STAT5), suggesting that GH regulation of HNF-3 $\gamma$  expression in the liver may be mediated through direct binding of STAT5 to the HNF-3 $\gamma$  promoter.

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## Introduction

Growth hormone (GH) is a pituitary polypeptide hormone that plays a central role in animal growth and metabolism (Harvey *et al.* 1995). A major target organ of GH is the liver, where GH regulates the expression of many genes. Earlier gene expression studies using Northern blots showed that GH regulated the expression of insulin-like growth factor-I (IGF-I) (Mathews *et al.* 1986), IGF

binding protein (IGFBP)-1 (Seneviratne *et al.* 1990), IGFBP-3 (Lemmey *et al.* 1997), the acid labile subunit (ALS) of the IGF binding complex (Ooi *et al.* 1997), serine protease inhibitor (Spi) 2.1 (Yoon *et al.* 1990), suppressors of cytokine signaling (SOCS) genes (Tollet-Egnell *et al.* 1999), phosphoenol pyruvate kinase C and glucose transporter GLUT-2 (Valera *et al.* 1993), as well as the transcription factors c-fos and c-jun (Gronowski & Rotwein 1995). More recent gene expression studies

employing the microarray technology have identified many more GH-regulated genes in the liver. A gene expression analysis with a cDNA array containing 588 rat cDNAs identified eight novel transcripts from the rat liver that were regulated by GH (Thompson *et al.* 2000). Using a cDNA microarray representing 3000 rat sequences, Flores-Morales *et al.* (2001) found that 58 of 720 detectable mRNAs were regulated by GH in the rat liver, many of which were not previously known to be GH responsive. Using a microarray containing 11 000 rat expression sequence tags (ESTs), Olsson *et al.* (2003) identified 58 metabolic genes (i.e. genes involved in metabolism) whose expression in the liver was altered by overexpressed bovine GH in transgenic mice. Using a microarray consisting of 5889 unique rat genes, Ahluwalia *et al.* (2004) identified 27 female- and 44 male-predominant genes that were regulated by GH in the rat liver. These GH-regulated genes are involved in metabolism, detoxification, growth control and other functions of the liver (Thompson *et al.* 2000, Flores-Morales *et al.* 2001, Olsson *et al.* 2003, Ahluwalia *et al.* 2004). Thus, regulation of gene expression appears to be a major mechanism by which GH affects the function of the liver.

The signaling pathways by which GH regulates gene expression in the liver are only beginning to be understood (Herrington & Carter-Su 2001, Schwartz *et al.* 2002), including the identification of the signal transducers and activators of transcription (STAT), especially STAT5B and STAT5A, as key transcription factors mediating GH regulation of the expression of Spi 2:1 (Bergad *et al.* 1995), IGF-I (Woelfle *et al.* 2003) and several other members of the GH/IGF axis in the liver (Davey *et al.* 1999, Woelfle & Rotwein 2004). However, for most of the GH-regulated genes, the signaling pathways and the transcription factors involved remain to be elucidated. The expression of thousands of genes in the liver is directly or indirectly controlled by liver-enriched transcription factors (LETFs), including hepatocyte nuclear factor (HNF)-1 $\alpha$ , HNF-1 $\beta$ , HNF-3 $\alpha$ , HNF-3 $\beta$ , HNF-3 $\gamma$ , HNF-4 $\alpha$ , HNF-6, albumin D-element binding protein (DBP), and CCAAT/enhancer-binding proteins (C/EBP) $\alpha$  and C/EBP $\beta$  (Schrem *et al.* 2002, Odom *et al.* 2004). Thus, GH regulation of gene expression of at least some genes in the liver may be mediated through LETFs. Supporting the involvement of LETFs in GH regulation of gene expression in the liver are recent demonstrations that C/EBP $\beta$  contributes to GH regulation of c-fos expression (Liao *et al.* 1999), HNF-3 $\beta$  contributes to GH regulation of cytochrome P450 (CYP) 2A2, CYP4A2 and CYP2C11 expression (Park & Waxman 2001), and that HNF-3, HNF-4 and HNF-6 contribute to GH regulation of CYP2C12 expression (Sasaki *et al.* 1999, Delesque-Touchard *et al.* 2000) in the liver.

To identify LETFs that might be involved in GH regulation of gene expression in the liver of cattle, in this study we have determined the effect of

administration of GH on the expression of 10 LETFs in the bovine liver.

## Materials and Methods

### Animal experiments

A total of 18 non-lactating and non-pregnant Angus beef cows were used in this study. Each cow had free access to grass and water. The cows were randomly assigned to three groups (groups 1, 2, and 3) with each group containing six cows. Each cow received a single intramuscular injection of 500 mg recombinant bovine GH in a slow-release formula (Monsanto Company, St Louis, MO, USA). Liver biopsy samples were taken from group 1 cows 6 h after GH administration, from group 2 cows 24 h after GH injection, and from group 3 cows 1 week after GH administration. Liver biopsy samples were also taken from group 3 cows 1 day before GH administration, serving as pre-GH controls.

The liver biopsy was performed as described before (Oxender *et al.* 1971). Briefly, the skin area between the 11th and 12th ribs was washed, sterilized with 70% ethanol, shaved, and 10 ml lidocaine were administered subcutaneously. A small incision was made between the 11th and 12th ribs and the biopsy needle (Sontec Instruments, Inc., Englewood, CO, USA) was introduced to the liver through the skin incision. A liver sample of 100–300 mg was collected from each cow. Once taken, liver samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The animal-related procedures were approved by the Virginia Tech Animal Care Committee.

### RNA and DNA extraction

Total RNA from liver tissue samples was isolated by using TRI reagent (MRC, Cincinnati, OH, USA) and poly(A) RNA was isolated from total RNA using the Oligotex mRNA kit (Qiagen, Chatsworth, CA, USA), according to the manufacturers' instructions. Genomic DNA from bovine liver tissue was isolated by standard proteinase K digestion followed by phenol–chloroform extraction. The concentration and quality of extracted RNA and DNA were determined by spectrometry and gel-electrophoresis respectively.

### Reverse transcription–polymerase chain reaction (RT-PCR) and PCR

The RT-PCR was used to clone the cDNA fragments for nine bovine LETFs, including HNF-1 $\alpha$ , HNF-1 $\beta$ , HNF-3 $\alpha$ , HNF-3 $\beta$ , HNF-3 $\gamma$ , HNF-6, C/EBP $\alpha$ , C/EBP $\beta$ , and DBP. The reverse transcription (total volume 20  $\mu\text{l}$ ), containing 2  $\mu\text{g}$  bovine liver mRNA, 1  $\mu\text{g}$  oligo (dT) primer or 0.5  $\mu\text{g}$  random hexamer primers

**Table 1** Oligonucleotide primers used for PCR amplification of bovine LETF cDNAs and DNA

	Primers*	Corresponding region
<b>LETF</b>		
HNF-1 $\alpha$	Forward primer: GCCTATGAGAGGCCAGAAGAA Reverse primer: CGTGCCATGGCCAGCTTG	Coding
HNF-1 $\beta$	Forward primer: GAGCCCACCAACAAGAAGAT Reverse primer: CATGGCCAGCTTCTGCCGG	Coding
HNF-3 $\alpha$	Forward primer: CCGTTCTCCATCAACAACCT Reverse primer: GTGTTTAGGACGGGTCTGGA	Coding
HNF-3 $\beta$	Forward primer: TGGGAGCGGTGAAGATGGA Reverse primer: GCCCGCGCCGGGGGACAT	Coding
HNF-3 $\gamma$	Forward primer: CTTCAAGCTGGAGGAG Reverse primer: GCTGGTGTCTGTTCTGACAT	Promoter
HNF-3 $\gamma$	Forward primer: GCCGCCGGGAAATGGAGTCC Reverse primer: CCATCTTCACTGAGCCCAGCAT	Coding
HNF-6	Forward primer: CTGCAGGAGCCGGAGTTCCA Reverse primer: CCACTTGTCAGACTCCTCC	Coding
DBP	Forward primer: CCTCGAAGACATCGCTTCTC Reverse primer: GCACCGATATCTGGTTCTCC	Coding
C/EBP $\alpha$	Forward primer: TGGCCGACCTGTTCCAACA Reverse primer: CCCCAGCCCGCTCGTACA	Coding
C/EBP $\beta$	Forward primer: ATCGACTTCAGCCCCTACCT Reverse primer: CGTAGTCGTCGGAGAAGAG	Coding

\*All sequences are written 5' to 3'.

(Promega, Madison, WI, USA), 0.01 M dithiothreitol, 0.5 mM deoxyribonucleotide triphosphates (dNTP) (Invitrogen, Carlsbad, CA, USA), 200 U SuperScript II reverse transcriptase (Invitrogen) and 4  $\mu$ l 5 $\times$  reverse transcription buffer (Promega), was performed at 42 °C for 2 h. For the PCR amplification of each bovine LETF cDNA, 2  $\mu$ l of the reverse transcription products were mixed with 12.5  $\mu$ l 2 $\times$  PCR master mix (Promega) and 10 pmol LETF-specific forward and reverse primers (Table 1), which were designed based on the mRNA sequences of the human, ovine, or rodent LETFs, in a total volume of 25  $\mu$ l. The PCR amplification was initiated by heating at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 60 °C (for C/EBP $\beta$  and DBP) or 55 °C (for other LETFs), and 1 min at 72 °C. The PCR products were resolved on 1.5% agarose gels containing ethidium bromide. The DNA bands with the expected sizes were extracted from the gel using gel extraction kits (Qiagen), according to the manufacturer's instructions.

A standard PCR was used to amplify the promoter region of the bovine HNF-3 $\gamma$  gene, with forward and reverse primers (Table 1) that were designed based on the homologous DNA sequences between the 5'-flanking regions of the human and mouse HNF-3 $\gamma$  genes. The conditions of this PCR were 35 cycles of 30 s at 94 °C, 1 min at 60 °C, and 2 min at 72 °C. The PCR products were analyzed on agarose gels as described above.

#### Cloning and subcloning

The gel-extracted LETF cDNAs were ligated into pGEM-T Easy vector (Promega), essentially according to

the manufacturer's instructions. The gel-extracted bovine HNF-3 $\gamma$  promoter DNA was inserted into pGL2B vector (Promega) at the NheI and HindIII sites. The ligation was transformed into competent DH10B *Escherichia coli* (Invitrogen) by electroporation and the positive clones were selected on the LB/Ampicillin/5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal)/isopropyl thiogalactoside (IPTG) plates. The plasmid DNA from the selected *E. coli* cells was extracted using the Qiagen miniprep kit (Qiagen) according to the manufacturer's instructions and analyzed for inclusion of inserts by digestion with restriction enzyme EcoRI. In addition, the HNF-1 $\alpha$  and HNF-3 $\gamma$  cDNA inserts in their respective pGEM-T Easy plasmids were released with restriction enzymes SpeI and EcoRI and were subcloned into pGEM-4Z vector (Promega) between cloning sites EcoRI and XbaI.

#### Sequencing and sequence analysis

The sequencing reaction was set up with 400 ng of the plasmid and 5 pmol vector specific primers in a volume of 10  $\mu$ l using the ABI Prism Big Dye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA), run at 96 °C for 1 min, followed by 49 cycles of 96 °C for 10 s, 50 °C for 10 s, and 60 °C for 4 min. The sequencing reactions were analyzed on ABI 377 Automated DNA Sequencers (Applied Biosystems). The nucleotide sequences of the cloned bovine LETF cDNAs and HNF-3 $\gamma$  promoter DNA were compared with the sequences in GenBank using the BLAST program at <http://www.ncbi.nlm.nih.gov>. Multiple

**Table 2** Cloning information of the bovine LETF cDNA plasmids

Plasmid name*	Insert size (bp)	GenBank accession number	Cloning sites	Restriction enzymes to linearize plasmid for antisense RNA synthesis	RNA polymerase to synthesize antisense riboprobe
pGEM-4Z-bHNF-1 $\alpha$	207	AY621540	EcoRI/XbaI	EcoRI	T7
pGEM-TE-bHNF-1 $\beta$	273	AY621541	EcoRI/EcoRI	SpeI	T7
pGEM-TE-bHNF-3 $\alpha$	218	AY621542	EcoRI/EcoRI	SpeI	T7
pGEM-TE-bHNF-3 $\beta$	251	AY621543	EcoRI/EcoRI	SpeI	T7
pGEM-4Z-bHNF-3 $\gamma$	312	AY621544	EcoRI/XbaI	EcoRI	T7
pGEM-TE-bHNF-6	273	AY621545	EcoRI/EcoRI	SpeI	T7
pGEM-TE-bDBP	184	AY621548	EcoRI/EcoRI	SpeI	T7
pGEM-TE-bC/EBP $\alpha$	216	AY521546	EcoRI/EcoRI	NcoI	SP6
pGEM-TE-bC/EBP $\beta$	163	AY621547	EcoRI/EcoRI	NcoI	SP6

\*pGEM-TE, pGEM-T Easy vector (Promega); b, bovine

sequence alignment was performed with the ClustalW program at <http://www.ebi.ac.uk>.

#### In vitro transcription

The bovine LETF cDNA-containing plasmids cloned in this study as well as the bovine HNF-4 $\alpha$  cDNA (311 bp) plasmid cloned in a previous study (Jiang & Lucy 2001) were linearized with appropriate restriction enzymes (Table 2). About 0.5  $\mu$ g of each linearized plasmid was transcribed *in vitro* with either T7 or SP6 RNA polymerase (Table 2) in the presence of [ $\alpha$ -<sup>32</sup>P]CTP to generate antisense riboprobe for each LETF mRNA. The *in vitro* transcription was carried out using the Riboprobe Combination System kit (Promega), essentially according to the manufacturer's instructions. After transcription, free [ $\alpha$ -<sup>32</sup>P]CTP was removed from the probe by phenol-chloroform extraction and filtration through quick spin Sephadex G-50 columns (Roche Molecular Biochemicals, Indianapolis, IN, USA). The specific activity of the purified probe was estimated by liquid scintillation counting.

#### Ribonuclease protection assay (RPA)

The RPA was carried out using the RPA II kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Briefly, 20  $\mu$ g total liver RNA,  $1 \times 10^5$  c.p.m. of one or two LETF riboprobes (C/EBP $\alpha$  and C/EBP $\beta$  mRNAs) were analyzed in the same RPA, as were DBP and HNF-3 $\beta$  mRNAs), and  $1 \times 10^5$  c.p.m. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, which was synthesized as described before (Wang *et al.* 2003), were mixed in 20  $\mu$ l hybridization buffer. The mixture was incubated at 42 °C for about 16 h and then digested with 200  $\mu$ l 1:100 diluted ribonucleases A and T<sub>1</sub> at 37 °C for 45 min. The undigested RNA fragments were precipitated and resolved on 6% polyacrylamide gels containing 7 M urea. The gels were dried, exposed to phosphor-

screens, and scanned on a Molecular Imager FX System (BioRad, Hercules, CA, USA). The intensity of each protected band was measured using the Alpha Imager program (Alpha Innotech Corporation, San Leandro, CA, USA) and was used to represent the abundance of the corresponding mRNA. The intensity of the LETF mRNA band was adjusted to that of the GAPDH mRNA in the same sample to normalize variations in the starting amounts of RNA and variations in performing RPA.

Besides LETF mRNAs, IGF-I mRNA was also quantified in the liver samples, serving as a positive control for GH regulation of gene expression in the liver of cows. This RPA was performed essentially as described before (Wang *et al.* 2003).

#### Statistical analysis

The LETF mRNA levels of different groups of cows were compared using General Linear Model ANOVA and Tukey's procedures of SAS (SAS Institute, Cary, NC, USA). Differences were considered significant if  $P < 0.05$ . All data were expressed as means  $\pm$  S.E.M. (standard error of the mean).

## Results

#### Sequences of bovine LETF cDNAs

cDNA fragments between 150 base pair (bp) and 350 bp for bovine HNF-1 $\alpha$ , HNF-1 $\beta$ , HNF-3 $\alpha$ , HNF-3 $\beta$ , HNF-3 $\gamma$ , HNF-6, DBP, C/EBP $\alpha$ , and C/EBP $\beta$  were cloned and sequenced (Table 2). The sequences have been deposited in GenBank (Table 2). The sequences of the cloned bovine LETF cDNA fragments, except HNF-3 $\gamma$ , share more than 90% and 84% identities with the corresponding human and mouse LETF sequences respectively. The bovine HNF-3 $\gamma$  sequence is 85% and 78% identical to the human and mouse HNF-3 $\gamma$  sequences respectively.

*All 10 LETFs except HNF-1 $\beta$  mRNA were detectable by RPA in the cow liver*

The RPAs of 20  $\mu$ g total RNA detected significant expression of all ten LETFs except HNF-1 $\beta$  in the liver of both GH-treated and untreated cows (Figs 1A and 2A). (The RPA image for HNF-1 $\beta$  is not shown.) However, HNF-1 $\beta$  mRNA was detectable in fetal bovine liver (data not shown). The HNF-1 $\beta$  mRNA is probably expressed at very low levels in the adult cow liver and GH probably does not strongly stimulate HNF-1 $\beta$  expression in the cow liver, if it has any stimulatory effect.

*GH strongly stimulated HNF-3 $\gamma$  expression*

As revealed by RPAs (Fig. 1A), the liver expression of HNF-3 $\gamma$  mRNA in the cows 24 h and 1 week after GH administration was higher ( $P < 0.05$ ) than in the untreated cows or in the cows 6 h after GH administration (Fig. 1B). The liver levels of HNF-3 $\gamma$  mRNA were, however, not different between the untreated cows and the cows 6 h after GH administration, or between the cows 24 h after GH administration and the cows 1 week after GH administration (Fig. 1A,B). GH administration increased the HNF-3 $\gamma$  mRNA expression by more than fourfold (Fig. 1B). This magnitude of increase was the greatest among the GH-induced increases in all LETF mRNAs (Fig. 1B) and was comparable to that of the GH-induced increase in the expression of IGF-I mRNA (Fig. 1A,B), a widely known target of GH action in the liver.

*GH stimulated HNF-6, HNF-4 $\alpha$ , and C/EB- $\alpha$  expression weakly with different kinetics*

The levels of HNF-4 $\alpha$  mRNA in the liver of the cows 1 week after GH administration were higher ( $P < 0.05$ ) than in the untreated cows (Fig. 1A,B). The levels of HNF-4 $\alpha$  mRNA were not different between the cows 6 h or 24 h after GH administration and the untreated cows (Fig. 1A,B). Thus, GH administration increased the expression of HNF-4 $\alpha$  mRNA in the liver of cows, but this effect appeared to take a longer time than did the effect of GH on HNF-3 $\gamma$  or IGF-I expression (Fig. 1A).

The RPA with a probe transcribed from the cloned HNF-6 cDNA generated two ribonuclease-protected mRNA fragments across the liver RNA samples (Fig. 1A). The longer band was apparently at the size (273 bp) of the cloned HNF-6 cDNA and the shorter protected fragment was approximately 225 bp. These two protected bands corresponded to the HNF-6 mRNA splice variants, HNF-6 $\alpha$  mRNA and HNF-6 $\beta$  mRNA, which contains an insertion 43 bp downstream from the 5' end of the cloned HNF-6 $\alpha$  cDNA, based on the sequences of the mouse HNF-6 $\alpha$  and HNF-6 $\beta$  mRNAs (Lemaigre *et al.* 1996). As in the mouse liver (Lemaigre *et al.* 1996), the HNF-6 $\alpha$  mRNA was expressed at a higher level than the

HNF-6 $\beta$  mRNA in the bovine liver (Fig. 1A). The levels of both HNF-6 $\alpha$  and HNF-6 $\beta$  mRNAs in the liver of the cows 24 h and 1 week after GH administration were higher ( $P < 0.05$ ) than in the untreated cows or the cows 6 h after GH administration (Fig. 1B). However, the levels of HNF-6 $\alpha$  and HNF-6 $\beta$  mRNAs were not different between the cows 6 h after GH administration and the untreated cows, or between the cows 24 h after GH administration and the cows 1 week after GH administration (Fig. 1B).

GH administration also increased C/EBP $\alpha$  mRNA expression in the liver, as the levels of C/EBP $\alpha$  mRNA were higher ( $P < 0.05$ ) in the cows 24 h after GH administration than in the untreated cows (Fig. 1A,B). In contrast to the stimulation of GH on HNF-3 $\gamma$ , HNF-6, and IGF-I mRNA expression, GH stimulation of C/EBP $\alpha$  expression appeared to be temporary, as the levels of C/EBP $\alpha$  mRNA were not different between the cows 1 week after GH administration and the untreated cows (Fig. 1A,B).

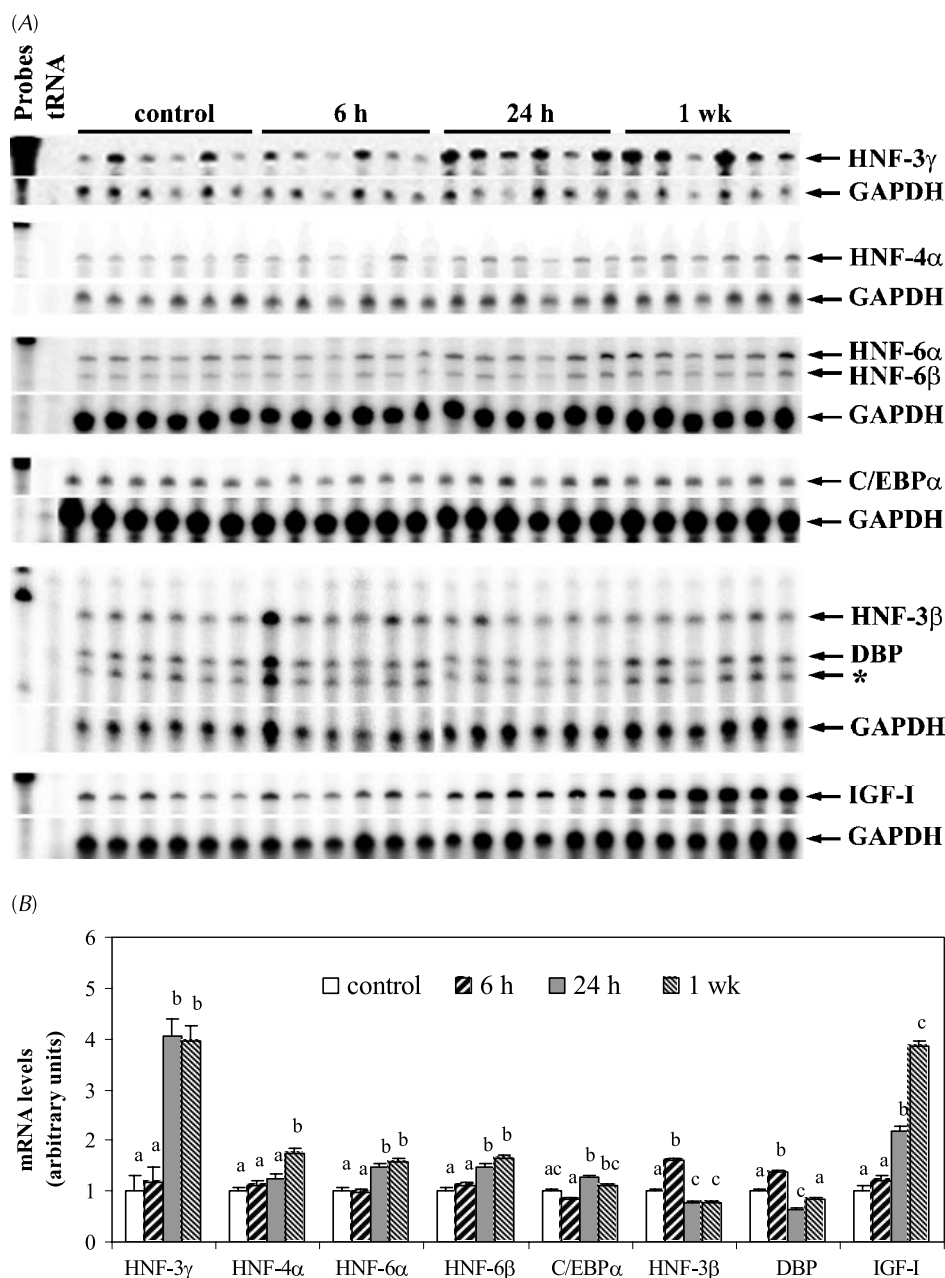
*GH had initially a stimulatory effect and subsequently an inhibitory effect on liver expression of HNF-3 $\beta$  and DBP mRNAs*

Growth hormone appeared to have a transient stimulatory but a sustained inhibitory effect on the expression of HNF-3 $\beta$  mRNA in the bovine liver, as the levels of HNF-3 $\beta$  mRNA were higher ( $P < 0.05$ ) in the cows 6 h after GH administration but lower ( $P = 0.05$ ) in the cows 24 h and 1 week after GH administration compared with those in the untreated cows (Fig. 1A,B).

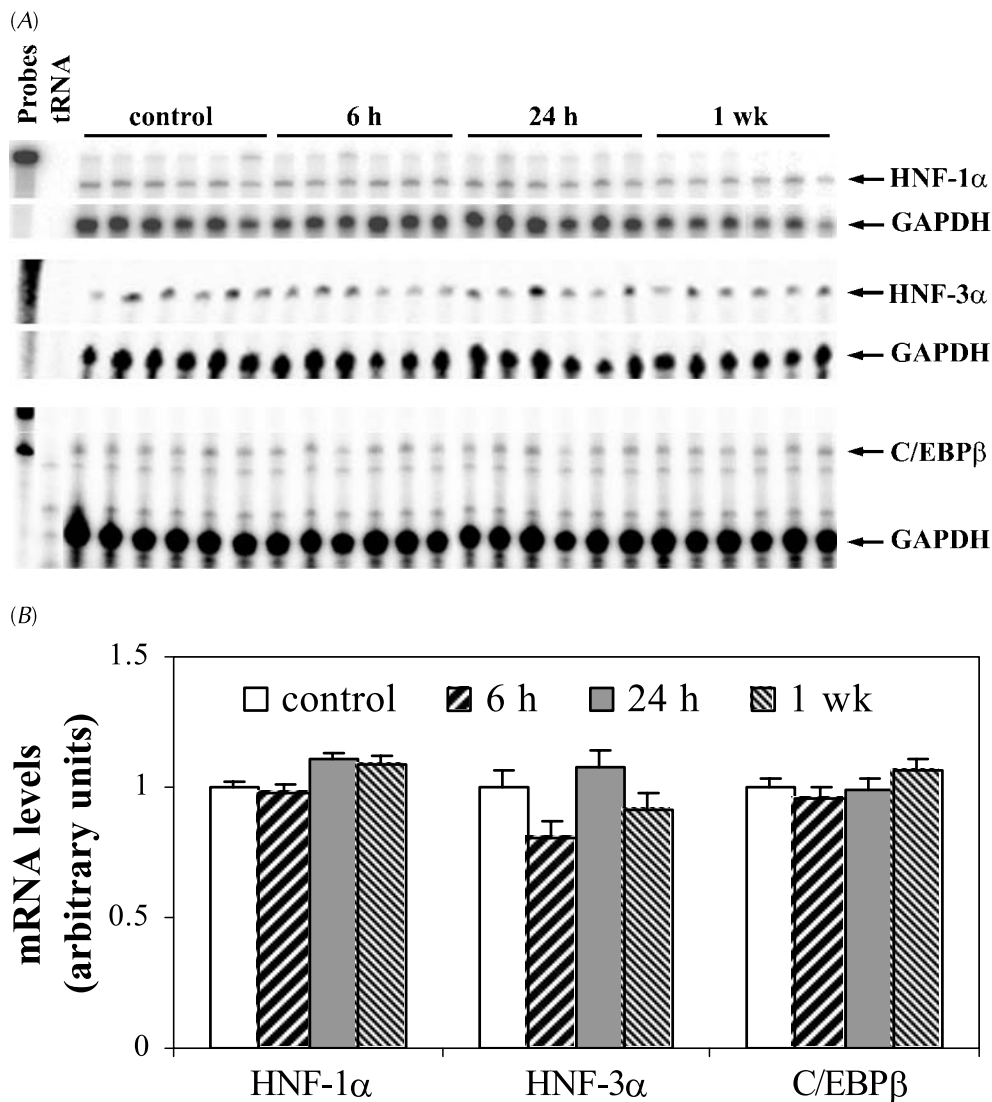
The RPA with a probe transcribed from the cloned DBP cDNA generated two ribonuclease-protected mRNA fragments across the liver RNA samples (Fig. 1A). The longer band, in the size (184 bp) of the cloned DBP cDNA insert, corresponded to the cloned bovine DBP mRNA sequence. The shorter band, in the same expression pattern as the longer band across the samples (Fig. 1A), probably represented an unidentified splice variant of DBP mRNA. Compared with the untreated cows, the cows 6 h after GH administration had increased expression ( $P < 0.05$ ) of DBP mRNA (both the long and the short bands), the cows 24 h after GH administration had decreased expression ( $P < 0.05$ ), and the cows 1 week after GH administration had similar levels of DBP mRNA in the liver (Fig. 1A,B). Thus, like its effect on HNF-3 $\beta$ , 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-1 $\alpha$ , C/EBP $\beta$  and HNF-3 $\alpha$  mRNA expression*

The liver mRNA levels of other LETFs, including HNF-1 $\alpha$ , HNF-3 $\alpha$ , and C/EBP $\beta$ , were not different between the cows 6 h, 24 h, and 1 week after GH administration



**Figure 1** RPAs of HNF-3 $\gamma$ , HNF-4 $\alpha$ , HNF-6, C/EBP $\alpha$ , HNF-3 $\beta$ , and DBP mRNAs in the livers of cows 6 h, 24 h, and 1 week after GH administration as well as untreated cows (control). (A) Phosphor images of the RPAs. In the RPA of each LETF mRNA, a probe specific for GAPDH mRNA was also included as a control for variations in starting amounts of RNA and the variations in performing RPA. Yeast tRNA (tRNA) was included in the RPA as a negative control. The ribonuclease-protected fragments corresponding to specific LETF and GAPDH mRNAs are indicated with arrows. The RPA of HNF-6 mRNA generated two protected bands that corresponded to splice variants HNF-6 $\alpha$  and HNF-6 $\beta$  (see text for details). The asterisk indicates a potentially unidentified DBP mRNA splice variant. The RPA for IGF-I mRNA served as a positive control for GH regulation of gene expression in the liver. (B) Relative abundance of HNF-3 $\gamma$ , HNF-4 $\alpha$ , HNF-6 $\alpha$ , HNF-6 $\beta$ , C/EBP $\alpha$ , HNF-3 $\beta$ , and DBP mRNAs as well as IGF-I mRNA. The relative abundance of each LETF mRNA or IGF-I mRNA was obtained by densitometric analysis of the RPA images in A. The density of each protected LETF or IGF-I mRNA band in each sample was normalized against that of protected GAPDH mRNA measured in the same RPA. All values are expressed as means  $\pm$  S.E.M. (pooled). For each mRNA species, the means with different letters are significantly different ( $P < 0.05$ ).



**Figure 2** RPAs of HNF-1 $\alpha$ , HNF-3 $\alpha$ , and C/EBP $\beta$  mRNAs in the livers of cows 6 h, 24 h, and 1 week after GH administration as well as untreated cows (control). (A) Phosphor images of the RPAs. (B) Relative abundance of HNF-1 $\alpha$ , HNF-3 $\alpha$ , and C/EBP $\beta$  mRNAs of different groups of cows. All values are expressed as means  $\pm$  S.E.M. (pooled). There are no significant differences between the means ( $P>0.05$ ).

and the untreated cows (Fig. 2A,B), indicating that GH had no significant effect on the expression of these three LETF mRNAs in the cow liver.

*The promoter of HNF-3 $\gamma$  contains multiple potential STAT5-binding sites*

Among the LETFs, the response of HNF-3 $\gamma$  to GH appeared to be the strongest and was similar in magnitude and time-course to the IGF-I response to GH (Fig. 1). The GH regulation of the expression of IGF-I (Woelfle *et al.* 2003) and several other genes (Bergad *et al.* 1995, Davey *et al.* 1999, Woelfle & Rotwein 2004) in the liver

has been shown to be mediated by STAT5. To determine if GH stimulation of HNF-3 $\gamma$  expression is also mediated by STAT5, a 967 bp promoter region of the bovine HNF-3 $\gamma$  gene was cloned and sequenced (Fig. 3A). A search of this promoter region for transcription factor binding sites revealed three potential STAT5 binding sites (Fig. 3A) that are nearly identical to the consensus STAT5 binding sequence TTCN<sub>3</sub>GAA (where N is any nucleotide) (Ehret *et al.* 2001). Aligning the corresponding DNA regions of the bovine, human, and mouse HNF-3 $\gamma$  genes further revealed that two of the putative STAT5 binding sites are highly conserved among these three species (Fig. 3B). The two STAT5 binding sites are also

## A

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gcgcgcggga aatggagtcc actctgtacc agcgcctccg cccctgccgc gcgagcggcc 60
ttccgccttc tccttctgcg aagcccgggc agcccttcat ttccgtcttt cgggcgccat 120
gctcttggga aatgtagtcc tgtgacaggg gggcgggacg gaaggggggc tggaggcttc 180
tgggaaatgg agttcacgaa gactctggct ccctggacgg ctgatgttag attgaacaga 240
gtcggatagg cgaagctcct aggtcttgca ggttatctcc agtctcagcg ggatatgagc 300
agaggccccg atccccctcca ccttttgtcc tggccattta cacaatctgg agcacttcca 360
gttatcagac gctcagttct ccagtccgtc caaagggaaa tgtggtttcg agtgtctctc 420
accggtcctt atagtaatag taacacaacg ggacctctgg agaccactcg gctctgagcc 480
ccctttccac tccaacaagt cttgctggct gttcgcctgc tccagttgag atgatgaaa 540
ccctaagtag aagggaccta gatttctgcc ctcgaggcct ttagaatgag caattgtttc 600
ccagttatag ctccctcact cttcagccgt ccccagctct ctctccccg ggctgcggat 660
cccggcctcct gaggtctctc tcctcgcaat cccgcagggg gcgccccaat ccgggcgcac 720
cgccccgggg cgggagcggg cgggatccga ggggagggcg gaggggctcc cgctgtccc 780
ggctataaag cgtggccgcc tcccgcggcg cccgggacga ctgggaccct gggcgatcgg 840
acggacgggc gcgggtcggg actcggcagt gccggccgag agatccgaaa ctctcgggtt 900
ctccccgggc cggagagggg gtgggtgggg gcgcaggccc ggggagtgct gggctcagtg 960
aagatgg

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## B

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hHNF-3γ -GTCCGGCTAGGCCTTCTGGGAAATGTAGTCC--CTTCGAGGCACCGGGCGGAA 52
mHNF-3γ CGAGCG-CCATGCCTTCTGGGAAATGTAGTCCGCGCTGCGCAGGCGCCGGACGGAA 55
bHNF-3γ CGGGCG-CCATG-CTTGGGAAATGTAGTCC-TGTGACAGGGGGGGCGGGACGGAA 53

hHNF-3γ AGGGAGCTAGAGCATTCTGGGAAATGGAGTTCTCAGTGGTGCTGAGTC 100
mHNF-3γ ATGGGAACGAAGGCTTCTGGGAAATGGAGTTCCCAGCGGTCCGGAGT- 102
bHNF-3γ GGGGGGCTGGAGGCTTCTGGGAAATGGAGTTCACGAAGACTCTGGCT- 100

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**Figure 3** The promoter of HNF-3 $\gamma$  contains putative STAT5-binding sites. (A) Sequence of a 967 bp promoter region of the bovine HNF-3 $\gamma$  gene. Underlined are three putative STAT5-binding sites; in bold is the translation start codon (ATG) for HNF-3 $\gamma$  protein. (B) Alignment of corresponding regions of the human (h), mouse (m) and bovine (b) HNF-3 $\gamma$  promoters. Shaded are two conserved STAT5 binding sites. The bovine sequence corresponds to the region between 111 and 210 in panel A of this figure. The human and mouse sequences correspond to the region between 92919 and 93018 in GenBank accession number AC008623.4 and the region between 185588 and 185689 in GenBank accession number AC145199.3 respectively.

conserved in the corresponding rat genomic region (data not shown).

## Discussion

The expression of many genes in the liver has been shown to be regulated by GH (Thompson *et al.* 2000, Flores-Morales *et al.* 2001, Olsson *et al.* 2003, Ahluwalia *et al.* 2004). As part of the effort to understand the mechanism by which GH regulates gene expression in the liver, we have analyzed the effect of administration of slow-release GH (equivalent to continuous GH administration) on the expression of 10 LETF genes in the liver of cows. Our data indicate that continuous GH administration has a sustained stimulatory effect on the expression of HNF-6, HNF-4 $\alpha$ , and C/EBP $\alpha$ , and initially has a stimulatory and subsequently an inhibitory effect on the expression of HNF-3 $\beta$  in the bovine liver. These findings in general agree with the observations from studying the effect of GH on the

expression of HNF-6 (Lahuna *et al.* 1997), HNF-4 $\alpha$  and HNF-3 $\beta$  (Lahuna *et al.* 2000) and C/EBP $\alpha$  (Rastegar *et al.* 2000, Strand *et al.* 2000) in the liver of hypophysectomized rats or cultured primary hepatocytes. The only major difference is that the GH-induced changes in these LETF mRNAs in cows were much smaller (less than onefold) than those observed in hypophysectomized rats (several folds). The reason for this difference is probably because hypophysectomized rats are much more sensitive to GH action than pituitary-intact cows.

The data from the present study also indicate that during the one-week course of GH treatment, the expression of HNF-1 $\alpha$  mRNA in the bovine liver was not changed. In earlier studies by others (Le Stunff *et al.* 1996, Rastegar *et al.* 2000), the liver levels of HNF-1 $\alpha$  protein in rats were not altered by hypophysectomy or GH replacement. These observations together suggest that GH does not regulate the expression of HNF-1 $\alpha$  in the liver. The effect of GH on other LETFs, including C/EBP $\beta$ , DBP,



and HNF-3 $\gamma$ , has not been reported or suggested by the literature. Our data from this study show that GH administration for one week had no effect on the expression of C/EBP $\beta$  mRNA but had a strong, sustained stimulatory effect on the expression of HNF-3 $\gamma$  mRNA and a biphasic effect, like that on HNF-3 $\beta$ , on the expression of DBP mRNA in the bovine liver. Thus, HNF-3 $\gamma$  and DBP are two new LETFs the expression of which is regulated by GH in the liver.

The mechanism by which GH regulates the expression of HNF-3 $\gamma$ , HNF-3 $\beta$ , HNF-6, HNF-4 $\alpha$ , C/EBP $\alpha$ , and DBP mRNAs in the bovine liver was not a focus of this study. However, cloning and sequencing the promoter region of the HNF-3 $\gamma$  gene, the LETF that was most sensitive to GH regulation, revealed two putative binding sites for STAT5, the same transcription factor that has been shown to be directly involved in GH regulation of the expression of Spi 2.1 (Bergad *et al.* 1995), IGF-I (Woelfle *et al.* 2003), ALS (Ooi *et al.* 1997), and HNF-6 (Lahuna *et al.* 2000). A sequence alignment further indicated that the sequences of the putative STAT5 binding sites are also conserved in the promoter regions of the human, mouse, and rat HNF-3 $\gamma$  genes. Functionally important *cis*-regulatory regions are often conserved during evolution (Cooper & Sidow 2003, Thomas *et al.* 2003). Containing multiple and evolutionally conserved sequences of STAT5 binding sites suggests that GH may regulate HNF-3 $\gamma$  mRNA expression in the liver through direct binding of STAT5 to the HNF-3 $\gamma$  promoter. This potential mechanism, as well as the mechanisms underlying GH regulation of other LETFs, warrants further studies.

Given the role of LETFs as major transcription factors controlling the expression of many genes in the liver (Cereghini 1996, Hayashi *et al.* 1999, Schrem *et al.* 2002), being regulated by GH suggests that they may be part of the transcription factor network mediating GH regulation of other genes in the liver. A cross examination of the list of genes that have been shown to be regulated by GH (Thompson *et al.* 2000, Flores-Morales *et al.* 2001, Olsson *et al.* 2003, Ahluwalia *et al.* 2004) and the list of genes that have been identified to contain binding sites for HNF-3 $\gamma$ , HNF-3 $\beta$ , HNF-6, HNF-4 $\alpha$ , C/EBP $\alpha$ , or DBP (Schrem *et al.* 2002, Odom *et al.* 2004) could identify a number of candidate genes whose expression in the liver may be regulated by GH through these LETFs. For example, the expression of CYP3A4 (Liddle *et al.* 1998, Jaffe *et al.* 2002), tyrosine-aminotransferase (TAT) (Ahluwalia *et al.* 2004), and transferrin (Tf) (Flores-Morales *et al.* 2001) in the liver were shown to be stimulated by continuous GH administration. The expression of CYP3A4 (Rodriguez-Antona *et al.* 2003), and TAT and Tf (Kaestner *et al.* 1998) in the liver has also been shown to depend on HNF-3 $\gamma$ . Now that HNF-3 $\gamma$  expression is found to be stimulated by GH in the liver, it is reasonable to suspect that GH may stimulate CYP3A4, TAT, and Tf expression in the liver through increased HNF-3 $\gamma$  expression.

Being regulated by GH also suggests that the genes containing binding sites for HNF-3 $\gamma$ , HNF-3 $\beta$ , HNF-6, HNF-4 $\alpha$ , C/EBP $\alpha$ , and DBP might also be target genes of GH regulation in the liver. The promoters of many genes have been found to contain binding sites for LETFs (Schrem *et al.* 2002). A recent study employing promoter microarrays identified that 12%, 1.7%, and 1.6% of 13 000 gene promoters were bound by HNF-4 $\alpha$ , HNF-1 $\alpha$ , and HNF-6 in hepatocytes respectively (Odom *et al.* 2004). Thus, the number of genes whose expression in the liver is regulated by GH might be much larger than has been indicated by gene expression studies using cDNA or oligonucleotide microarrays (Thompson *et al.* 2000, Flores-Morales *et al.* 2001, Olsson *et al.* 2003, Ahluwalia *et al.* 2004).

Compared with HNF-3 $\gamma$ , HNF-3 $\beta$ , HNF-4 $\alpha$ , HNF-6, C/EBP $\alpha$ , and DBP, the mRNA expression of HNF-1 $\alpha$ , HNF-3 $\alpha$ , and C/EBP $\beta$  in the bovine liver was not affected by GH. This, however, does not necessarily suggest that HNF-1 $\alpha$ , HNF-3 $\alpha$ , and C/EBP $\beta$  cannot contribute to GH regulation of gene expression. The ability of a transcription factor to affect gene transcription can be controlled at both concentration and activity, each at multiple levels (Calkhoven & Ab 1996). It is possible that GH regulates these LETFs (as well as other LETFs) at the translational level, thereby changing their protein concentrations; it is also possible that GH regulates these LETFs (as well as other LETFs) at the posttranslational level and hence the DNA binding activity or the ability of them to interact with other regulatory proteins. These possibilities remain to be tested in future studies.

In summary, the results of this study indicate that GH can regulate the mRNA expression of HNF-3 $\gamma$  and five other LETFs including HNF-3 $\beta$ , HNF-6, HNF-4 $\alpha$ , C/EBP $\alpha$ , and DBP in the bovine liver. These LETFs, therefore, have great potential to mediate GH regulation of other genes in the liver. The study also suggests that GH regulation of HNF-3 $\gamma$  expression in the bovine liver may be mediated by direct binding of STAT5 to the HNF-3 $\gamma$  promoter.

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