Developmental and Growth Hormone Regulation of the Expression of Liver-Enriched Transcription Factors in the Bovine Liver

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ABSTRACT

Liver gene expression changes during development and is affected by growth hormone (GH). These changes in gene expression may be due to the differential expression of the liver-enriched transcription factors (LETFs). To study the potential involvement of LETFs in the regulation of gene expression in the bovine liver, we cloned the cDNA fragments of nine bovine LETFs, including hepatocyte nuclear factor (HNF)-1α, 1β, 3α, 3β, 3γ, 6, albumin D-element binding protein (DBP), and CCAAT/enhancer-binding proteins (C/EBP) -α and β, and compared the expression levels of them between adult and fetal bovine liver and between GH-treated and untreated adult bovine liver. The mRNA abundance of the LETFs was determined by ribonuclease protection assay (RPA). The cloned bovine LETF cDNA sequences showed high degrees of similarity (79 % to 99 %) to the LETF sequences of other species. The mRNA levels of HNF-1β, HNF-3γ, and HNF-6 were significantly higher ($P < 0.05$) in the fetal liver (n=3) than in the adult liver (n=7). There were significant increases ($P < 0.05$) in the mRNA expression of HNF-3γ and HNF-6 in the liver of cows 24 h (n=6) and 1 w (n=6) after GH administration. The results of this study suggest that HNF-1β, HNF-3γ, and HNF-6 may play a role in differential regulation of gene expression between the fetal and adult bovine liver and that HNF-3γ and HNF-6 may be also involved in GH regulation of gene expression in the bovine liver.

Keywords: Liver; Bovine; Growth Hormone; Liver-Enriched Transcription Factors

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Table of Contents

List of Tables

Table

List of Figures

Figures

CHAPTER I

REVIEW OF LITERATURE

Introduction

The liver is a vital organ in the body and performs many metabolic functions. In addition, the liver is also one of the major detoxification organs in the body. The liver also regulates growth through the expression of certain growth promoting proteins, including insulin-like growth factor-I (IGF-I), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGR). In addition, the liver expresses growth hormone receptors (GHR) and is a major target for GH, which is a major hormone stimulating growth.

Functions of the liver change during development and are also affected by hormonal and nutritional levels. Compared to the adult liver, the fetal liver has few of the metabolic functions. The fetal liver functions as the major hematopoietic organ in the mid-to-late fetal stage. With embryonic development, the liver gradually loses hematopoietic activity. Nutrient supply is an important factor for proper functioning of the liver. Food deprivation results in increased fatty acid oxidation in the liver, thereby causing increased levels of serum fatty acids and ketone bodies (Yamada et al., 2003). On the other hand, increases in the levels of certain nutrients may improve certain liver functions. For example, inclusion of catalytic quantities of fructose in a carbohydrate meal improves glucose tolerance and increases liver glucose uptake (McGuinness and Cherrington, 2003). In contrast, depletion of certain nutrients may adversely affect liver

function. It was shown that hepatic amino acid transport was decreased significantly in suckling and weanling rats fed on a low-protein diet (Gomez et al., 1995). Similarly, hormonal imbalance also affects liver functions. Glucose deposition in the liver is significantly reduced in glucocorticoid-deficient rats, which is ultimately reflected in higher blood glucose levels (Bishayi and Ghosh, 2003). In mice over- expressing GH, there is a decreased response to insulin in the liver, resulting in poor liver glucose metabolism (Bartke et al., 2002). Increased GH alters liver lipid metabolism and results in decreased hepatic triglyceride secretion (Olsson et al., 2003). These changes in function are often characterized by changed gene expression patterns in the liver. For example, during the late fetal and neonatal stages, the liver initiates the expression of several metabolic genes such as glucose-6-phosphatase, tyrosine aminotransferase and various types of cytochrome P450s, thereby preparing for increased metabolic and detoxificating duties after birth. These changes in gene expression in the liver may be regulated by the liver-enriched transcription factors (LETFs). This review summarizes the current understanding of regulation of liver gene expression at different developmental stages, the regulation of liver gene expression by GH and the potential involvement of LETFs in these regulations.

Differential expression of genes between the fetal and adult liver

Using differential display reverse transcription-polymerase chain reaction (RT-PCR), Malhotra et al. (1999) identified several known and unknown genes that are differentially expressed between human fetal and adult liver. They found that α-fetoprotein, stem cell factor, erthyriod α-spectrum, 2,3-bisphosphoglycerate mutase, porphobilinogen deaminase and insulin-like growth factor-II (IGF-II) mRNAs are highly

expressed in the fetal liver but only weakly expressed in the adult liver. In the same study, they also demonstrated that nicotinamide deaminase, human fibrinogen-related protein, and α-acid glycoprotein mRNAs are expressed in the adult liver but not in the fetal liver (Malhotra et al., 1999). The differential expression of these genes between fetal and adult livers is apparently consistent with their differential roles between the fetal and adult liver.

Using a high-density oligonucleotide DNA array, Nagata et al. (2003) identified 33 genes that are differentially expressed between the fetal and postnatal human liver. These 33 genes include proliferating cell nuclear antigen (PCNA), cell division cyclin-7 like-1 (CDC7L1), and cyclin D3 (CCND3), which regulate cell cycle; replication factor-4 (RFC4), recq-like type 2 (RECQ2), and nucleosome protein 1-like-1 (NAP1L1), which control DNA replication and repair; insulin-like growth factor-II (IGF-II) and insulin-like growth factor binding protein-2 (IGFBP-2), which control cell growth; steriod-5-alpha reductase (SRD5A1), nuclear receptor subfamily 1, group 1, and member 3 (NR1I3), which are components of hormonal signaling; and cytochrome P450 enzymes (CYP2C9, CYP2E1, CYP2A7, CYP2A13, CYP4F2, CYP3A4), which regulate cellular metabolism.

Tellgren et al. (2003) studied the expression of differentially expressed genes between the neonatal and adult liver using cDNA microarrays. They found that L-type pyruvate kinase, alanine aminotransferase GPT, apolipoprotein A-IV, carbonic anhydrase III, and glutathione peroxidase are down-regulated in the neonatal liver compared to the adult liver. In the same study, they found that tubulin-beta (class I), tubulin-alpha, and protein phosphatase are up-regulated in the neonatal liver compared to the adult liver. All of these genes play an important role in liver development and metabolism.

Growth hormone regulation of gene expression in the liver

Growth hormone (GH) is a polypeptide hormone released from the anterior pituitary gland. The GH regulates a broad range of physiological processes involved in development, somatic growth, and metabolism (Le Roith, 2001). A major target of GH is the liver. In this tissue, GH controls the expression of many genes.

Olsson et al. (2003) compared liver gene expression in transgenic mice over-expressing the bovine GH with that in the non-transgenic mice using DNA microarrays. They found 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, it was shown that the expression of peroxisome proliferator activated receptor α (PPARα) and sterol-regulatory element binding protein (SREBP-I) genes was 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 sex-specific temporal patterns of GH exposure. 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 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 liver expression of several regulators of transcription, including c-fos, c-jun, hepatocyte nuclear factor-6 and hepatocyte nuclear factor (HNF)-3β (Gronowski et al., 1996; Rastegar et al., 2000).

Liver-enriched transcription factors

The unique phenotype of hepatocytes arises from the expression of genes in a liver-specific fashion, which is controlled primarily at the level of transcription to make relevant mRNAs (Derman et al., 1981). Many nuclear transcription factors have been isolated from liver nuclear extracts, characterized and their corresponding genes have been cloned. These liver nuclear transcription factors include HNF-1α, 1β, 3α, 3β, 3γ, 4α, 4β and 4γ, albumin D-element binding protein (DBP), CCAAT/enhancer-binding proteins (C/EBP)-α and β, which are highly expressed in the liver and hence called liver-enriched transcription factors (LETFs) (Schrem et al., 2002; Hayashi et al., 1999). The promoter regions of many genes, including those encoding albumin, α-fetoprotein, α1-antitrypsin, transthyretin, fibrinogen, and certain members of the cytochrome P450 2C

family that are highly expressed in the liver, have been shown to contain binding sites for different LETFs (Cereghini et al., 1988; Hardon et al., 1988; Courtois et al., 1987; and Costa et al., 1988). The LETFs appear to act in coordination to regulate liver-specific gene expression during development. For example, C/EBP-α regulates phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) transcription early in the prenatal period, while C/EBP-β and DBP appear to play a more prominent role in regulating these genes later in development (Wang et al., 1995). The LETFs also appear to play a role in each other's expression. For instance, there is a putative HNF-4α binding site in the promoter region of the HNF-1α gene (McNair et al., 2000; Deryckere et al., 1996). Conversely, there is a putative HNF-1α binding site in the HNF-4α gene promoter (Bailly et al., 2001). Together, these imply a reciprocal regulatory loop. Determination of the tissue distribution of these factors and analysis of their hierarchical relations led to the hypothesis that the combinatorial action of LETFs together with ubiquitous transcriptional machinery of the cell is necessary, and maybe even sufficient for the maintenance of liver-specific gene expression (De Simone and Cortese, 1991).

Hepatocyte nuclear factor-1 (HNF-1) family

The HNF-1 family includes HNF-1α and HNF-1β. Two different genes encode HNF-1α and HNF-1β. The HNF-1α gene is located on murine chromosome 5 and human chromosome 12 (Schrem et al., 2002); whereas HNF-1β maps to murine chromosome 11 and human chromosome 17 (Kuo et al., 1990; Bach et al., 1991). These proteins are characterized by a homeobox- containing DNA binding domain with different transactivational properties (Kuo et al., 1991; Song et al., 1998). HNF-1β is expressed during early embryonic development, in the endoderm of the foregut, whereas HNF-1α is

activated later, upon condensation of hepatic parenchyma (De Simone et al., 1991; Mendel et al., 1991; Cereghini et al., 1992). In addition to liver, both HNF-1α and HNF-1β are expressed in kidney, stomach, and intestine (Baumhueter et al., 1990; Blumenfeld at al., 1991). Both HNF-1α and HNF-1β appear to bind to the same DNA consensus sequence GTTAATNATTAAC (where, N = G, A, T, or C) (Kuo et al., 1991). The promoters of many liver-specific genes, such as albumin, α-fetoprotein, α-fibrinogen, α1-antitrypsin, aldolase B, PEPCK, G6Pase, and P450, have HNF-1 binding sites and these promoters can be activated by HNF-1α (Akiyama et al., 2002; Schrem et al., 2002, and Hayashi et al., 1999). In addition to the above-mentioned genes, HNF-1α transactivates the expression of HNF-3γ in the liver (Hiemisch et al., 1997). It was also shown that over-expression of HNF-1α in a hepatoma cell line, HepG2, induces a regulatory mechanism that leads to down-regulation of HNF-4 dependent liver-specific genes, including the HNF-1α gene itself (Kitstaki and Talianidis, 2003). HNF-1β regulates the expression of liver-specific genes including albumin, α-fetoprotein, α1-antitrypsin and sucrose-isomaltase (Schrem et al., 2002; Boudreau et al., 2001).

Hepatocyte nuclear factor-3 (HNF-3) family

The HNF-3 family comprises three proteins, HNF-3α, HNF-3β, and HNF-3γ. All three members of the HNF-3 family share a strong similarity in the winged-helix/fork head DNA binding domain (Quian and Costa, 1995) and recognize the same consensus DNA sequence TATTGAC/TTTA/TG (Samadani and Costa, 1996; Pani et al., 1992; Costa et al., 1989). They also share similarity in two short similar regions in their C-terminal and N-terminal domains (Schrem et al., 2002). The HNF-3α, β, γ genes have been mapped to three different chromosomes, suggesting that the HNF-3 family genes

are widely dispersed and may require independent activation of each member (Avraham et al., 1992). HNF-3α, HNF-3β, and HNF-3γ are activated sequentially during development and are required for node and notochord axis formation and endodermal cell differentiation in the mouse embryo (Weinstein et al., 1994; Ang et al., 1993). The HNF-3β mRNA is expressed first in the primitive streak and the node before the expression of any liver genes. Slightly later than HNF-3β, HNF-3α along with HNF-3β is expressed in the definitive endoderm that lines the developing gut and subsequently forms the components of the liver, lung, pancreas, and alimentary canal. The HNF-3γ is expressed in the early liver and more posterior endoderm after the gut has formed (Monaghan et al., 1993). HNF-3γ is also expressed in the ovary, testis and heart. In combination with other LETFs, HNF-3α and HNF-3β bind and transactivate numerous liver-specific genes, such as albumin, transthyretin, α1-antitrypsin, PEPCK, transferrin, and α-fetoprotein (Schrem et al., 2002). In addition to the liver, HNF-3α and HNF-3β may regulate gene expression in the intestinal, pancreatic and bronchiolar epithelia (Rausa et al., 1997; Clevidence et al., 1998). The concentration of HNF-3α expression increases when hepatocyte derived cell lines are cultured on an extracellular matrix gel substratum. Extracellular matrix gel substrata coordinately induce differentiated cell morphology and liver gene transcription in primary hepatocyte cultures (DiPersio et al., 1991; Caron, 1990). Thus, HNF-3α may be important in transducing extracellular signals in the maintainence of hepatocyte differentiation. In another study (Vallet et al., 1995), overproduction of truncated HNF-3α protein lacking any transactivating activity induced a dramatic decrease in the expression of liver-specific genes, including those of albumin, transthyretin, transferrin, PEPCK, and aldolase B, whereas the expression of the L-type pyruvate kinase gene

containing no HNF-3 binding sites was unaltered, indicating that HNF-3α protein is needed for the proper expression of a set of liver-specific genes. HNF-3γ has been found to regulate the expression of cytochrome P4503A4, GLUT-2, albumin, catalase, and transcarbamylase (Rodriguez-antona et al., 2003; Shen et al., 2001; Nakamura et al., 1998).

Hepatocyte nuclear factor-4 (HNF-4)

The HNF-4 family includes HNF-4α, HNF-4β, and HNF-4γ. The HNF-4α and HNF-4γ genes have been mapped to different chromosomes (Schrem et al., 2002). The HNF-4 family proteins have different transcriptional activation potential, with HNF-4β and HNF-4γ having less transactivational activity than HNF-4α (Drewes et al., 1996; Holewa et al., 1997). The consensus DNA sequence for HNF-4 protein is NG/AGGNCAAAGG/ TTCA/GN (Fraser et al., 1998). In addition to the liver, HNF-4α is also highly expressed in the kidney and intestine in the adult (Sladek et al., 1990). HNF-4β is expressed in the lung, stomach, ovary and testis (Holewa et al., 1997). In contrast, HNF-4γ is not expressed in the liver but expressed in other tissues, such as pancreas, kidney, and small intestine (Drewes et al., 1996). During mouse development, HNF-4α is expressed in the primary endoderm at 4.5 days and then restricted to the visceral endoderm from 5.5 days (Hayashi et al., 1999). The HNF-4α shows capability to transactivate HNF-1α, HNF-6 and other liver-specific genes, such as transthyretin, α1-antitrypsin, apolipoprotein CIII, transferrin, ornithine transcarbamylase (Schrem et al., 2000), and a growth hormone receptor mRNA variant, GHR1A (Jiang and Lucy, 2001). The H5 cell line is a dedifferentiated hepatoma cell line; the H5 cells express neither HNF-1α nor HNF-4α. However, stable transfection of exogenous HNF-4α resulted in

expression of previously silent HNF-1α and activation of some hepatic proteins, but not of the endogenous HNF-4α in H5 cells (Spath and Weiss, 1997), implying the presence of transcriptional hierarchy and importance of HNF-4α for liver-specific gene expression.

Hepatocyte nuclear factor-6 (HNF-6)

The HNF-6 is the prototype of the novel ONECUT class of cut-homeoproteins (Lannoy et al., 1998, Jacquemin et al., 1999). The members of this class are characterized by a divergent homeodomain and by the presence of a single cut domain. Two HNF-6 isoforms α (456 residues) and β (491 residues) have been cloned in the rat. They differ only by the length (27 or 53 amino acids) of the linker between the cut domain and homeodomain. They originate from the same gene by differential splicing (Lannoy et al., 1998; Samadani and Costa 1996). HNF-6α and HNF-6β bind to the same DNA target sequence DHA/TATTGAT/CTA/TA/TD (where D is A, G, or T and H is A, C, or T) but they differ in binding affinity. The HNF-6α and β are expressed in tissues that originate from endoderm cells lining the foregut, liver, pancreas, brain, and spinal cord (Landry et al., 1997; Rausa et al., 1997). The onset of HNF-6 gene transcription is detected in the liver at embryonic day 9, correlating with the onset of liver differentiation and its expression disappears transiently from the liver between embryonic days 12.5 and 15, but is present again in the liver after embryonic day 15 (Landry et al., 1997). This pattern of HNF-6 expression is paralled by HNF-3β. HNF-6 binding sites have been identified in the promoter regions of HNF-3β and HNF-4α, as well as some of the liver-specific genes, such as α1-antitrypsin, α-fetoprotein, P450, PEPCK, and transthyretin (Schrem et al., 2002). Thus, HNF-6 is important for liver-specific gene expression and it may act through HNF-3β and HNF-4α.

CCAAT/enhancer-binding proteins (C/EBPs)

C/EBPs comprise a family of transcription factors that have a DNA-binding basic region and a leucine zipper (b/ZIP) dimerization domain, and they all bind to similar DNA sequences (Landschulz et al., 1988). The consensus DNA sequence for C/EBP is ACCACTTTCACAATCTGCTA (Crossely and Brownkee, 1990). Identified members of this family include C/EBP-α, C/EBP-β, C/EBP-γ, C/EBP-δ, and C/EBP-ε. The C/EBP-α, C/EBP-β, and C/EBP-δ homodimerize or heterodimerize and have different activation effects (Hayashi et al., 1999). The C/EBP proteins have strong similarity in their C-terminal sequences and divergence in their N-terminal transactivation domains (Landschulz et al., 1988). The C/EBP proteins except C/EBP-ε are expressed at a later stage in development and are abundant in liver and fat tissues, particularly in fully differentiated cells (Cao et al., 1991; Landschulz et al., 1988). C/EBP-ε is a myeloid tissue specific transcription factor and plays an important role in granulocyte differentiation (Gery et al., 2003). In pre-adipocytes, transfection of C/EBP-α cDNA results in suppression of clonal cell growth and promotion of adipogenic differentiation by inducing the expression of adipocyte-specific genes (Umek et al., 1991; Lin et al., 1992; Freytag et al., 1994). Thus, C/EBP-α plays an important role in differentiation of adipocytes. The transcription of C/EBP-α closely correlates with the passage of hepatocytes through the cell cycle of the regenerating liver (Mischoulon et al., 1992). Proliferating hepatocytes in the partially hepatectomized liver demonstrate an abrupt reduction of C/EBP-α mRNA within the first three hours of surgery, corresponding to the transition from the G0 to the G1 phase of the cell cycle. The C/EBP-α returns to a normal level by 72 hours, through a gradual increase after the S-phase (Mischoulon et al., 1992).

All of these observations indicate that C/EBP-α regulates two aspects of terminal differentiation: 1) induction of differentiation specific genes, and 2) cessation of mitotic growth. Apart from maintaining the hepatocyte differentiation, C/EBP-α binds and regulates the transcription of liver-specific genes, such as albumin, transthyretin, α1-antitrypsin, P450, and HNF-6 (Friedman et al., 1989; Herbst et al., 1989; Costa et al., 1989; Rastegar et al., 2000). Activated C/EBP-β can transactivate multiple cytokine genes and promote differentiation of macrophages and granulocytes (Akira et al., 1990; Screpanti et al., 1995). C/EBP-β also regulates the expression of several liver-specific genes, which include alcohol dehydrogenase, cycloxygeanse, and apolipoprotein (He et al., 2002; Caivano et al., 2001; Schrem et al., 2002). It has been shown that C/EBP-δ regulates the expression of the IGF-1 gene in osteoblasts and also the cycloxygenase gene in the liver (Caivano et al., Ji et al., 2003). C/EBP-γ increases the alcohol dehydrogenase transcription in conjunction with C/EBP-β in the liver (He et al., 2002). Thus, all C/EBP proteins appear to play a role in liver-specific gene expression.

Albumin D-element binding protein (DBP)

The DBP is a member of proline and acid-rich (PAR) domain subfamily of b/ZIP proteins and is involved in transcriptional regulation of genes in liver. The DBP was initially isolated because of its ability to interact with the D-element of the albumin gene promoter (Mueller et al., 1990). It was shown that the PAR domain is important and required for DBP activation, which occurs through a regulated, p-300 dependent process (Lamprecht and Mueller, 1999). In addition to the liver, DBP is also expressed in the thymus, kidney, spleen, brain and lung and can only be detected after birth. The expression of DBP displays a strong circadian rhythm in rat liver; maximal levels occur

in the evening and are 50-to150-fold higher than levels observed in the morning (Wuarin and Schibler, 1990; Mueller et al., 1990). In the liver, DBP binds with high affinity to a DNA sequence G/CTTAC/TGTAAC/T and may regulate many liver-specific genes (Li and Hunger, 2001), which include cholesterol 7α-hydroxylase, albumin, factor VIII, factor IX, CYP2C6, angiotensinogen, PEPCK and P450 (Lavery and Schibler, 1993; Yano et al., 1992; Begbie et al., 1998; Narayanan et al., 1998; Akiyama and Gonzalez, 2002; Roesler et al., 1992). The circadian amplitude of steroid 15α-hydroxylase and coumarin 7-hydroxylase mRNAs and protein expression in the liver was significantly impaired in DBP null mice (Lavery et al., 1999). Similarly, DBP may be involved in the regulation of the circadian rhythm of cholesterol 7α-hydroxylase gene in liver (Lee et al., 1994).

CHAPTER II

cDNA Cloning of Liver-Enriched Transcription Factors and Comparison of Their Expression Between Adult and Fetal Bovine Liver

ABSTRACT

The liver performs essential functions in the body that include growth, metabolism and detoxification. The functions of the liver change as it progresses from the fetal to the adult stage. Accompanying these functional changes are the changes in the gene expression profile between the fetal and adult liver. The promoter and enhancer regions of many liver-specific genes contain binding sites for liver-enriched transcription factors (LETFs). It is, thus, possible that differential expression of genes between the fetal and adult liver is due to differential expression of these LETFs. As part of the long-term goal to test this hypothesis, we cloned partial cDNAs of nine bovine LETFs, including hepatocyte nuclear factor (HNF)-1α, 1β, 3α, 3β, 3γ, HNF-6, albumin D-element binding protein (DBP), CCAAT/enhancer-binding proteins (C/EBP)-α and β, and compared their expression levels between adult and fetal bovine liver. The nine bovine LETF cDNAs were generated by standard reverse transcription-polymerase chain reaction (RT-PCR) and the mRNA expression levels were determined by ribonuclease protection assays with riboprobes generated from these cloned LETF cDNAs. Sequence analyses revealed 84 % to 100 % similarity of the bovine LETF cDNA sequences to the corresponding human, mouse, ovine or rat LETF cDNA sequences. The mRNA levels of HNF-1β, HNF-3γ, and HNF-6 were significantly higher ($P < 0.05$) in the fetal bovine liver than the adult bovine liver. However, the mRNA levels of the remaining LETFs, including HNF-1α, HNF-3α, HNF-3β, DBP, C/EBP-α and C/EBP-β, were not different between fetal and adult liver. These results suggest a role for

HNF-1β, HNF-3γ, and HNF-6 in differential expression of genes between fetal and adult bovine liver.

Keywords: Liver; Bovine; mRNA; Liver-Enriched Transcription Factors

Introduction

The adult liver performs unique functions in the body by expressing liver-specific genes encoding plasma proteins and enzymes involved in gluconeogenesis and glycogen storage, glucose metabolism, cholesterol homeostasis, synthesis of bile salts and somatic growth (Cereghini,1996). These functions of the adult liver are not prominent or are absent in the fetal liver, which functions as a major hematopoietic and lymphopoietic organ.

These changes in the function of the liver from the fetal stage to the adult stage can be attributed to differences in gene expression profiles between fetal and adult liver. Many liver-specific genes are differentially expressed between fetal and adult liver (Malhotra et al., 1999; Nagata et al., 2003; Lucy et al., 1998). For instance, α-fetoprotein, a main component of the mammalian fetal serum, is highly expressed in fetal liver; its expression decreases significantly in adult liver (Lazarevich, 2000). Similarly, the expression of growth hormone receptor mRNA variant 1A (GHR1A), which is believed to play an important role in mediating post-natal growth, is dramatically increased in adult liver (Lucy et al., 1998). The molecular mechanisms responsible for differential gene expression between fetal and adult liver are poorly understood.

Functional analyses of numerous liver-specific gene promoter and enhancer regions reveal that they are composed of multiple cis-acting DNA sequences that bind to different families of liver-enriched transcription factors (LETFs) (Cereghini,1996). The LETFs include hepatocyte nuclear factors (HNF) -1α, 1β, 3α, 3β, 3γ, 4α, 4β, 4γ and 6, as well as albumin D-element binding protein (DBP) and CCAAT/enhancer-binding proteins α and β (C/EBP-α and C/EBP-β). Although none of these LETFs are entirely liver-specific, combined action of them plays an important role in achieving abundant transcriptional activity and maintaining liver-

specific gene expression (Cereghini, 1996). Hence, it is possible that differences in gene expression patterns between fetal and adult liver may be regulated by changes in the expression of LETFs. As a step toward the long-term goal of understanding the molecular mechanism by which gene expression is differentially controlled between adult bovine liver and fetal bovine liver, we have cloned the cDNA fragments of nine bovine LETFs and compared their expression levels between fetal and adult bovine liver.

Materials and Methods

Tissue and RNA

The liver samples from bovine fetus (sex and age unknown), cows (non-lactating and non-pregnant), and bulls were collected from a slaughterhouse at the time of slaughter. Once collected, the liver tissue samples were immediately frozen in liquid nitrogen and stored at -80°C**.** Total RNA from liver tissue samples was isolated by using TRI regent (MRC, Cincinnati, OH), according to the manufacturer's instructions. The mRNA was isolated by using the Oligotex mRNA kit (Qiagen Inc., Valencia, CA). The RNA concentrations were determined by measuring absorbance at 260 nm and RNA quality was verified by electrophoresis on formaldehyde-agarose gels.

Reverse transcription-polymerase chain reaction (RT-PCR)

Two μg of bovine liver mRNA were incubated with 1 μg of oligo (dT) primer or 500 ng of random hexamer primers (Promega, Madison, WI) in a volume of 11 μl at 70°C for 10 min and then chilled on ice for 2 min. The tube containing random hexamer primers was additionally incubated for 5 min at room temperature. After addition of 4 μl of 5X reverse transcription buffer (Promega), 2 μl of 0.1 M dithiothreitol (DTT) and 10 mM deoxyribonucleotides triphosphates

(dNTP) (Invitrogen, Carlsbad, CA), each tube was incubated at 42°C for 2 min. Reverse transcription was initiated by the addition of 200 U of SuperScript^{TM}II reverse transcriptase (Invitrogen). The mixture was then incubated at 42°C for 2 h, followed by cooling the tubes on ice to terminate the reaction. The synthesized cDNA was stored at -20°C.

For the PCR amplification of each target cDNA, 2 μl of the reverse transcription mixture were mixed with 10 pmol forward and reverse primers (Table 1), and 12.5 μl 2X PCR master mix (Promega) in a total volume of 25 μl. The PCR amplification was initiated by heating at 94°C for 3 min, followed by 35 cycles of the following conditions: 30 s at 94°C, 1 min at 60°C (for C/EBPβ and DBP) or 55°C (for other LETFs), and 1 min at 72°C. The products of PCR were run on a 1.5% agarose gel containing ethidium bromide. The DNA bands at expected sizes were cut out from the gel and extracted using a Qiagen gel extraction kit (Qiagen Inc.), according to the manufacturer's instructions.

Cloning and subcloning of LETF cDNAs

The gel-extracted cDNAs were ligated into pGEM-T Easy vector (Promega), according to the manufacturer's instructions. The ligation was transformed into competent DH10B bacterial cells (Invitrogen) by electroporation and positive clones were selected on the LB/Ampicillin/5-bromo-4-choloro-3-indolyl-β-D-galactoside (X-Gal)/Isopropyl thiogalactoside (IPTG) plates. The plasmid DNA from the selected bacterial cells was extracted using the Qiagen mini prep kit (Qiagen Inc.) according to the manufacturer's instructions and analyzed for inclusion of inserts by digestion with appropriate restriction enzymes.

The HNF-1α and HNF-3γ cDNAs in their respective pGEM-T Easy plasmids were excised with restriction enzymes Spe I and EcoR I and were subcloned into pGEM-4Z vector (Promega) between cloning sites EcoR I and Xba I.

Sequencing of the LETF cDNAs and sequence analysis

The sequencing reaction was set up with 400 ng of the plasmid and 5 pmol of T7 or SP6 promoter primer in a volume of 10 μl using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, CA), and was 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. Later, the reaction was sent to Virginia Bioinformatics Institute (Blacksburg, VA) to obtain the nucleotide sequence. The nucleotide sequence of the cloned LETF cDNA inserts was compared to the LETF sequences of other species using the BLAST program (http://www.ncbi.nlm.nih.gov).

In vitro transcription to synthesize riboprobes

The direction of the cDNA insert in the plasmid was determined and then digested with an appropriate restriction enzyme (Table 2) to linearize the plasmid. The linearized plasmids were used in *in vitro* transcription in the presence of [α-^{32}P] CTP to generate antisense riboprobes for quantification of LETF mRNA levels. The in vitro transcription was carried out using the Riboprobe Combination System Kit (Promega), essentially according to the manufacturer's instructions. The RNA polymerases used in the in vitro transcription are listed in Table 2. After transcription, free [α-^{32}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). 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). Each hybridization tube contained 5 x 10^4 cpm of each probe and 15 μg of total liver RNA in a total volume of 20 μl of hybridization buffer. The tubes were incubated at 90-95°C for 4 min to denature the probe and

RNA and then the hybridization reaction was carried out at 42°C for 16 h. Following the hybridization, the samples were digested with 1 µl of RNase A/T1 mix (250 units/ml of RNase A and 10,000 units/ml RNase T1) at 37°C for 40 min in 100 µl of RNase digestion buffer. Later, the RNase was inactivated and samples precipitated by adding 150 µl of RNase inactivation/precipitation buffer from the kit and incubating at -80°C for at least 1 h. The samples were centrifuged at 13,000 rpm at 4°C for 30 min and the supernatant was discarded. The RNA pellet was washed with 500 µl of 75% ethanol and air dried for 10 min. After air-drying, the pellet was resuspended in 6 µl of RNA loading dye and denatured at 90-95°C for 4 min. The ribonuclease protected RNA fragments were resolved by electrophoresis on 6% acrylamide gels containing 7 M urea. After gel electrophoresis, gels were dried and exposed to phosphor screens. Exposed phosphor screens were scanned on a Molecular Imager system (Bio-Rad Laboratories, Hercules, CA). The densities of the protected bands were quantified by using the Quantity One software (Bio-Rad) and were used to represent the abundance of the corresponding mRNA.

Statistical analysis

The densities of the protected LETF mRNAs were adjusted to the densities of GAPDH mRNA in the same sample. The adjusted densities were analyzed using the General Linear Model procedure of SAS (SAS Institute, Cary, NC). The PDIFF option was used to estimate the pair wise difference between the means. Differences were considered significant if $P < 0.05$. The adjusted densities of the protected LETF mRNAs for cow and bull liver samples were grouped as adults, if there were no significant differences between them.

Results

HNF-1α

A 207 base pair (bp) of bovine HNF-1α cDNA was initially cloned into pGEM-T easy vector and then sub-cloned into pGEM-4Z plasmid. The bovine HNF-1α sequence was 97% identical to the ovine HNF-1α mRNA and 91% identical to the human HNF-1α mRNA (Figure 1). An RPA with a bovine HNF-1α riboprobe generated a single protected fragment across liver RNA samples from cows, bulls and fetal cattle (Figure 2A). There were no significant differences ($P > 0.05$) in the levels of HNF-1α mRNA between the cow, bull and fetal liver samples (Figure 2B,). Later cow and bull samples were grouped as adults and the mRNA expression levels comapred with fetal liver samples. There was no significant difference in the HNF-1α mRNA expression levels between adult and fetal liver samples (Figure 2C).

HNF-1β

The cloned bovine HNF-1β cDNA was 273 bp long and 98% identical in sequence to the ovine HNF-1β mRNA and 95% identical to the human HNF-1β mRNA (Figure 3). An RPA with a bovine HNF-1β specific riboprobe revealed that HNF-1β mRNA was expressed at higher levels ($P < 0.05$) in fetal livers than in cow or bull livers (Figure 4A). There was no significant difference ($P > 0.1$) in the mRNA levels of HNF-1β between cow and bull livers (Figure 4B). When the cow and bull samples were grouped as adults, the HNF-1β mRNA expression levels were significantly lower in the adult livers than in the fetal livers (Figure 4C).

HNF-3α

The length of the cloned bovine HNF-3α cDNA insert was 218 bp. The nucleotide sequence of the bovine HNF-3α cDNA was 90% identical to the human HNF-3α mRNA) and rat HNF-3α mRNA (Figure 5). The RPA detected HNF-3α mRNA in the liver samples from

cows, bulls and fetal cattle (Figure 6A). There were no significant differences ($P > 0.1$) in the levels of HNF-3α mRNA between the three groups (Figures 6B, 6C).

HNF-3β

The cloned bovine HNF-3β cDNA was 251 bp long and 96% identical in sequence to the human HNF-3β mRNA and 90% identical to the mouse HNF-3β mRNA (Figure 7). The RPA showed the presence of HNF-3β mRNA in the livers of both cows and bulls as well as fetal cattle (Figure 8A). There were significant differences ($P < 0.05$) in the levels of the HNF-3β mRNA between the cow and bull samples (Figure 8B). However, there was no significant difference between cow and fetal, and bull and fetal liver samples (Figure 8B). There was also no significant difference between adult cattle and fetal cattle liver samples (Figure 8C).

HNF-3γ

A 310 bp bovine HNF-3γ cDNA was cloned and it was 85% identical to the human HNF-3γ mRNA and 78% identical to the mouse HNF-3γ mRNA (Figure 9). An RPA with a HNF-3γ specific riboprobe generated a single protected fragment across the liver RNA samples from cows, bulls and fetal cattle (Figure 10A). There were significant differences ($P < 0.05$) in the HNF-3γ mRNA expression levels between fetal, cow and bull liver samples, with fetal cattle livers containing higher expression of HNF-3γ mRNA than cow and bull liver samples (Figure 10B). However, there were no significant differences ($P > 0.1$) in the HNF-3γ mRNA expression levels between cow and bull samples. There was also a significant difference ($P < 0.05$) in the HNF-3γ mRNA levels between fetal liver samples and adult liver samples combined from cow and bull samples (Figure 10C).

HNF-6

The length of the cloned bovine HNF-6 cDNA fragment was 270 bp. Its nucleotide sequence was 96% and 94% identical to the corresponding regions of human HNF-6 mRNA and

mouse HNF-6 mRNA respectively (Figure 11). The RPA revealed two protected HNF-6 mRNA fragments across the liver RNA samples from cows, bulls and fetal cattle (Figure 12A). The longer band, which is approximately 270 bp, represents HNF-6α mRNA, a splice variant of HNF-6 mRNA. The shorter protected fragment, which is approximately 230 bp, may correspond to another splice variant of HNF-6 mRNA, HNF-6β mRNA which contains an 78 bp insertion compared to HNF-6α mRNA in rats (Lemaigre et al., 1996). Both HNF-6α and HNF-6β mRNA levels were greater in the fetal livers than in the cow and bull livers ($P < 0.05$) (Figures 12B, 12D). However, there was no significant difference ($P > 0.05$) in the HNF-6α or 6β mRNA expression levels between cows and bulls. When cow and bull samples were grouped as adults, the HNF-6α and-6β mRNAs in these samples were also significantly lower ($P < 0.05$) than in the fetal cattle liver samples (Figures 12C, 12E).

DBP

The cloned bovine DBP cDNA was 183 bp long and 93% identical in sequence to the human DBP mRNA and 84% identical to the mouse DBP mRNA BC018323.1) (Figure 13). The RPA generated two protected fragments across the lanes (Figure 14A). The upper protected fragment indicated in Figure 14A is the band for the DBP mRNA as the size of the band was approximately 183 bp. The lower protected fragment could correspond to a splice variant of DBP mRNA. There were no significant differences ($P > 0.05$) in the densities of the protected DBP mRNA fragments between the groups (Figures 14B, 14C).

C/EBP-α

The length of the cloned bovine C/EBP-α cDNA was 215 bp. Its nucleotide sequence was 100% identical to the bovine C/EBP-α cDNA already present in the GenBank (Figure 15). It was also 97% and 99% identical to human and rat C/EBP-α cDNA already present in the GenBank (Figure 15). An RPA with a C/EBP-α specific riboprobe generated a single weak band

across the liver RNA samples of cows, bulls and fetal cattle (Figure 16A). Although the C/EBP-α mRNA expression levels in the cow liver samples appeared to be higher than that in bull or fetal cattle samples, the differences were not significant ($P > 0.1$) (Figures 16B, 16C).

C/EBP-β

A 161 bp bovine C/EBP-β cDNA was cloned and was 98% identical to the bovine C/EBP-β cDNA sequence already present in the GenBank (Figure 17). The RPA for C/EBP-β mRNA generated two protected fragments across the lanes (Figure 18A). The upper protected band corresponded to the C/EBP-β mRNA. The lower fragment, which showed a similar expression pattern across lanes to the upper band, might correspond to a C/EBP-β mRNA splice variant. There were no significant differences ($P > 0.1$) in the densities of the protected fragments between the groups (Figures 18B, 18C).

Discussion

Gene expression differs substantially between the fetal and adult liver. This differential gene expression is potentially controlled by the combinatorial actions of LETFs and ubiquitous transcription factors. In this study, we cloned nine bovine LETF cDNA fragments and compared the expression patterns of these LETF mRNAs in fetal and adult bovine livers, as a step toward understanding the role of LETFs in regulating differential gene expression between fetal and adult livers. We used GAPDH as an internal control for RPA reactions since GAPDH is a house-keeping gene and its expression does not change between adult and fetal liver (Cantz et al., 2003).

Comparison of the cloned bovine LETF cDNA sequences with the human, ovine, mouse, or rat LETF sequences in GenBank showed high degrees of similarity, ranging from 84 % to 100

%. The high degrees of sequence similarity between the LETFs of different species suggest that LETFs are highly conserved genes among species, and that they have important functions.

We found that HNF-1β mRNA was expressed at higher levels in the bovine fetal liver than the bovine adult liver. This result is in agreement with earlier studies, which showed that HNF-1β mRNA expression was decreased in the adult mouse liver as compared to the fetal mouse liver (Rey-Compos et al., 1991; Desimone et al., 1991; Mendel et al., 1991). A higher-level expression of HNF-1β mRNA in the fetal liver is also in line with the role of HNF-1β in activating the expression of several fetal serum proteins, such as α-fetoprotein (Schrem et al., 2002, Hayashi et al., 1999).

In contrast, there was no significant difference in the expression of HNF-1α mRNA between the fetal and adult bovine liver. A differential expression of HNF-1α mRNA through development has not been reported. The unchanged expression of HNF-1α mRNA between the fetal and adult bovine liver suggests that HNF-1α may not be involved in differential expression of genes in the liver during development.

As for HNF-3, no previous studies have compared the expression pattern of HNF-3α, 3β, or 3γ mRNAs between the fetal and adult liver. However, there have been reports that the expression of the three HNF-3 family members varies during different developmental stages of embryogenesis (Kaestner et al., 1994; Monaghan et al., 1993). Our study found that HNF-3γ was expressed at a higher level in the fetal bovine liver than the adult bovine liver but that the levels of HNF-3α and HNF-3β mRNAs were not different between the adult and fetal bovine liver, suggesting that HNF-3γ may be more important than HNF-3α and HNF-3β for fetal liver-specific gene expression.

In our study, the expression of HNF-6 mRNA was higher in the fetal liver than in the adult bovine liver, suggesting the importance of HNF-6 in fetal regulation of liver-specific gene expression. A specific role of HNF-6 in fetal liver development is in B cell lymphopoiesis, as HNF-6 inactivation led to the B cell lymphopenia (Bouzin et al., 2003). HNF-6 controls B cell lymphopoiesis in the fetal liver likely through regulation of the genes required for the B cell lymphopoiesis. A greater expression of HNF-6 mRNA in the fetal liver is also consistent with its role in stimulating the transcription of the α-fetoprotein gene in the fetal liver. (Nacer-Cherif et al., 2003).

We found that the mRNA levels of DBP between the adult and fetal bovine liver were not different. This result does not agree with the finding in rats that DBP is only strongly transcribed in the adult liver (Mueller et al., 1990). Similarly, we did not find any significant differences in the expression levels of C/EBP-α and C/EBP-β mRNA between the adult and fetal bovine liver, whereas both of them were reported to increase from the fetal stage to adulthood in rats (Jochhein et al., 2003; Cereghini et al., 1996; Birkenmeier et al., 1989). These discrepancies are perhaps due to species difference or differences in the stages of fetal liver between our study and those studies.

In summary, the results of this study indicate that HNF-1β, HNF-3γ, and HNF-6 are expressed at higher levels in the fetal bovine liver than in the adult bovine liver, whereas the expression of the remaining LETFs, including HNF-1α, HNF-3α, HNF-3β, DBP, C/EBP-α, and C/EBP-β does not differ between the adult and fetal liver. The differential expression of HNF-1β, HNF-3γ, and HNF-6 mRNAs between the fetal and adult liver suggests that these three LETFs may play a role in the differential expression of other genes between the adult and fetal liver.

Table 1

Oligonucleotide primers used for PCR-amplification of bovine LETF cDNAs

LETF	Primers
HNF-1α	Forward primer: 5' GCCTATGAGAGGCAGAAGAA 3'
	Reverse primer: 5' CGTGTCCATGGCCAGCTTG 3'
HNF-1β	Forward primer: 5' GAGCCCACCAACAAGAAGAT 3'
	Reverse primer: 5' CATGGCCAGCTTCTGCCGG 3'
HNF-3α	Forward primer: 5' CCGTTCTCCATCAACAACCT 3'
	Reverse primer: 5' GTGTTTAGGACGGGTCTGGA 3'
HNF-3β	Forward primer: 5' TGGGAGCGGTGAAGATGGA 3'
	Reverse primer: 5' GCCCGCGCCGGGGGACAT 3'
HNF-3γ	Forward primer: 5' CTTCAAGCTGGAGGAG 3'
	Reverse primer: 5' GCTGGTGTCTGTTCTGACAT 3'
HNF-6	Forward primer: 5' CTGCAGGAGCCGGAGTTCCA 3'
	Reverse primer: 5' CCACTTGTCCAGACTCCTCC 3'
DBP	Forward primer: 5' CCTCGAAGACATCGCTTCTC 3'
	Reverse primer: 5' GCACCGATATCTGGTTCTCC 3'
C/EBP-α	Forward primer: 5' TGGCCGACCTGTTCCAACA 3'
	Reverse primer: 5' CCCCGACCCGCTCGTACA 3'
C/EBP-β	Forward primer: 5' ATCGACTTCAGCCCCTACCT 3'
	Reverse primer: 5' CGTAGTCGTCGGAGAAGAG 3'

Table 2

Cloning information of bovine LETF cDNA plasmids

Plasmid name	Insert size (bp)	Cloning sites	Restriction enzymes to linearize plasmid for antisense RNA synthesis	RNA polymerase to synthesize antisense riboprobe
HNF-1α/pGEM-4Z	207	EcoR I/Xba I	EcoR I	T7
HNF-1β/ pGEM-T Easy	273	EcoR I/EcoR I	Spe I	T7
HNF-3α /pGEM-T Easy	218	EcoR I/EcoR I	Spe I	T7
HNF-3β/ pGEM-T Easy	251	EcoR I/EcoR I	Spe I	T7
HNF-3γ/ pGEM-4Z	312	EcoR I/Xba I	EcoR I	T7
HNF-6/ pGEM-T Easy	273	EcoR I/EcoR I	Spe I	T7
DBP/ pGEM-T Easy	184	EcoR I/EcoR I	Spe I	T7
C/EBP-α/ pGEM-T Easy	216	EcoR I/EcoR I	Nco I	SP6
C/EBP-β /pGEM-T Easy	163	EcoR I/EcoR I	Nco I	SP6

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bHNF-1α GCCTATGAGAGGCAGAAGAACCCCAGCAAGGAGGAGCGGGAGGCGCTGGTGGAGGAGTGC 60
hHNF-1α GCCTATGAGAGGCAGAAGAACCCTAGCAAGGAGGAGCGAGAGACGCTAGTGGAGGAGTGC 60
oHNF-1α --CTATGAGAGGCAGAAGAACCCCAGCAAGGAGGAGCGAGAGGCGCTCGTGGAGGAGTGC 60

bHNF-1α AACAGGGCGGAGTGCATCCAGAGGGGGGTGTCACCGTCGCAGGCCCAGGGACTGGGCTCC 120
hHNF-1α AATAGGGCGGAATGCATCCAGAGAGGGGTGTCCCCATCACAGGCACAGGGGCTGGGCTCC 120
oHNF-1α AACAGGGCAGAGTGCATCCAGAGGGGCGTGTCACCGTCGCAGGCCCAGGGACTGGGCTCC 120

bHNF-1α AACCTCGTCACAGAGGTGCGTGTCTACAACTGGTTTGCCAATCGCCGCAAGGAAGAAGCC 180
hHNF-1α AACCTCGTCACGGAGGTGCGTGTCTACAACTGGTTTGCCAACCGGCGCAAAGAAGAAGCC 180
oHNF-1α AACCTCGTCACAGAGGTGCGTGTCTACAACTGGTTTGCCAATCGCCGCAAGGAAGAAGCC 180

bHNF-1α TTTCGGCACAAGCTGGCCATGGACACG  207
hHNF-1α TTCCGGCACAAGCTGGCCATGGACACG  207
oHNF-1α TTTCGGCACAAGCTGGCCATGGACACG  205
```

FIGURE 1. Nucleotide sequence of the cloned bovine (b) HNF-1α cDNA and its alignment with the human (h) and ovine (o) HNF-1α cDNA sequences. The nucleotides different among the species are indicated in bold. The gaps in the sequence are indicated by dashes (-). The human HNF-1α sequence in the alignment corresponds to the region from 672 to 878 in GenBank accession No. NM000545.3; the ovine HNF-1α sequence in the alignment corresponds to the region from 1 to 205 in GenBank accession No. OAR409149.

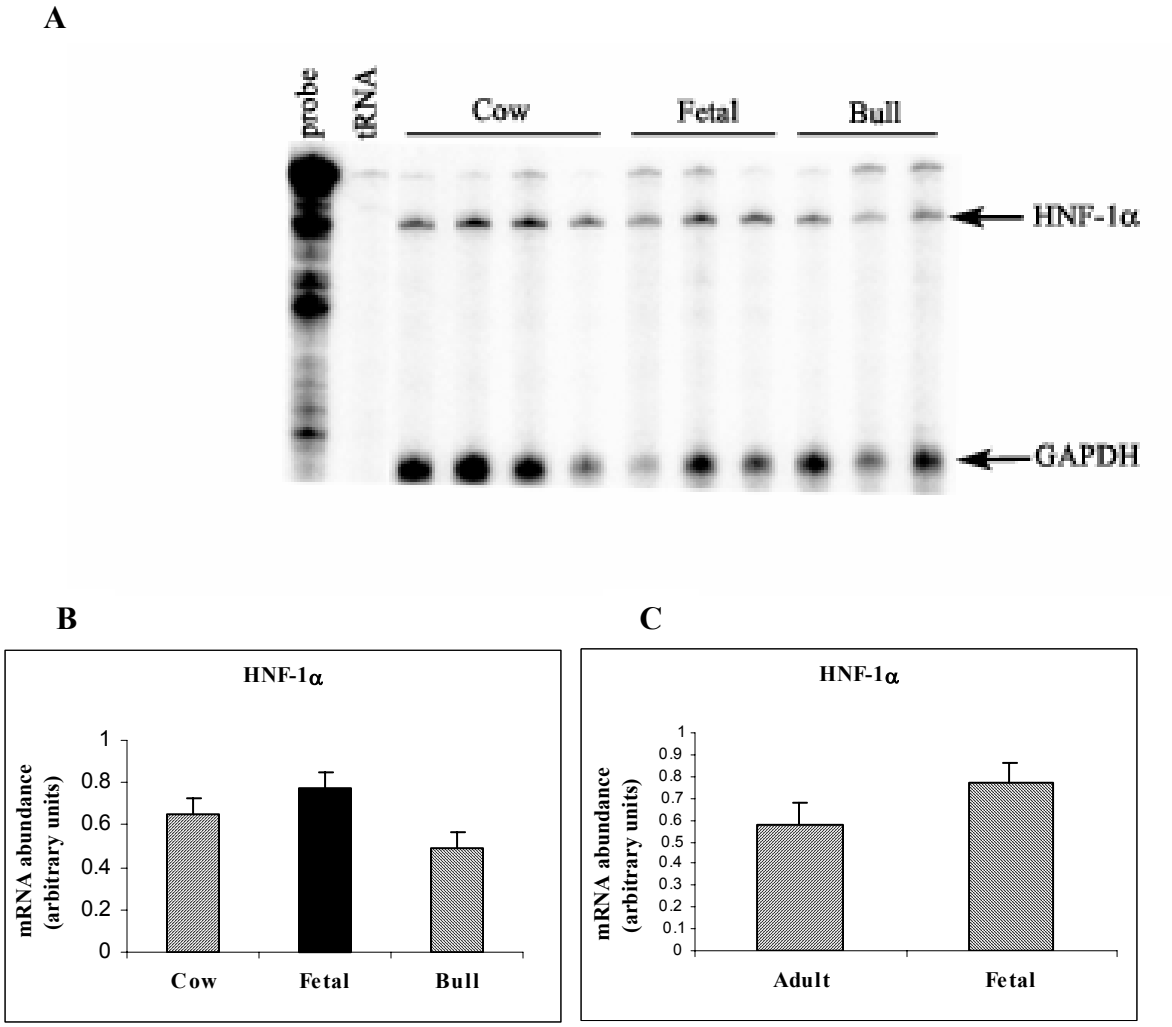

FIGURE 2. RPA of HNF-1α mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. In the RPA, a probe specific for the bovine GAPDH mRNA was also included as a loading control. Yeast tRNA (tRNA) was included in the RPA as a negative control. (B) Relative abundance of HNF-1α mRNA in cow, bull and fetal cattle liver samples. (C) Relative abundance of HNF-1α mRNA in adult cattle and fetal cattle livers. Values are expressed as mean ± SEM (standard error of the mean). There are no significant differences between the means ($P > 0.1$).

```
bHNF-1β CATGGCCAGCTTCTGCCGGAACGCCTCCTCCTTCCTGCGGTTTGCGAACCAGTTGTAGAC 60
hHNF-1β CATGGCCAGCTTTTGCCGGAATGCCTCCTCCTTCCTGCGGTTTGCAAACCAGTTGTAGAC 60
oHNF-1β CATGGCCAGCTTCTGCCGGAACGCCTCCTCCTTCCTGCGGTTTGCGAACCAGTTGTAGAC 60

bHNF-1β GCGGACCTCAGTGACCAGGTTGGAGCCCAGGCCGTGGGCTTTGGAGGGGGAGACGCCTCG 120
hHNF-1β ACGGACCTCAGTGACCAAGTTGGAGCCCAGGCCGTGGGCTTTGGAGGGGGACACCCTCG 120
oHNF-1β GCGGACCTCAGTGACCAGGTTGGAGCCCAGGCCGTGGGCTTTGGAGGGGGAGACGCCTCG 120

bHNF-1β CTGCAAACATTCTGCCCTGTTACACTCCTCCACTAAGGCCTCCCTCTCTTCCTTGCTGGG 180
hHNF-1β CTGCAAACATTCTGCCCTGTTGCATTCCTCCACTAAGGCCTCTCTCTCTTCCTTGCTGGG 180
oHNF-1β CTGCAAACATTCTGCCCTGTTGCACTCCTCCACTAAGGCCTCCCTCTCTTCCTTGCTGGG 180

bHNF-1β GTTCTTTTGCCGGTCGTAGGCCTGGTACAAGATTTGCTGGGACGCGGGCCCCCATTTGAA 240
hHNF-1β GTTCTTTTGCCGATCGTAGGCCTGGTACAAGATTTGCTGGGACGCGGGCCCCCATTTGAA 240
oHNF-1β GTTCTTTTGCCGGTCGTAGGCCTGGTACAAGATTTGCTGGGACGCGGGCCCCCACTTGAA 240

bHNF-1β CCGGTTGCGACGCATCTTCTTGTTGGTGGGCTC 273
hHNF-1β CCGGTTGCGGCGCATCTTCTTGTTGGTGGGCTC 273
oHNF-1β CCGGTTGCGACGCATCTTCTTGTTGGTGGG--- 273
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FIGURE 3. Nucleotide sequence of the cloned bovine HNF-1β cDNA and its alignment with the human and ovine HNF-1β cDNA sequences. The human HNF-1β sequence in the alignment corresponds to the region from 863 to 1133 in GenBank accession No. BC017714.1; the ovine HNF-1β sequence in the alignment corresponds to the region from 1 to 270 in GenBank accession No. OAR311788.

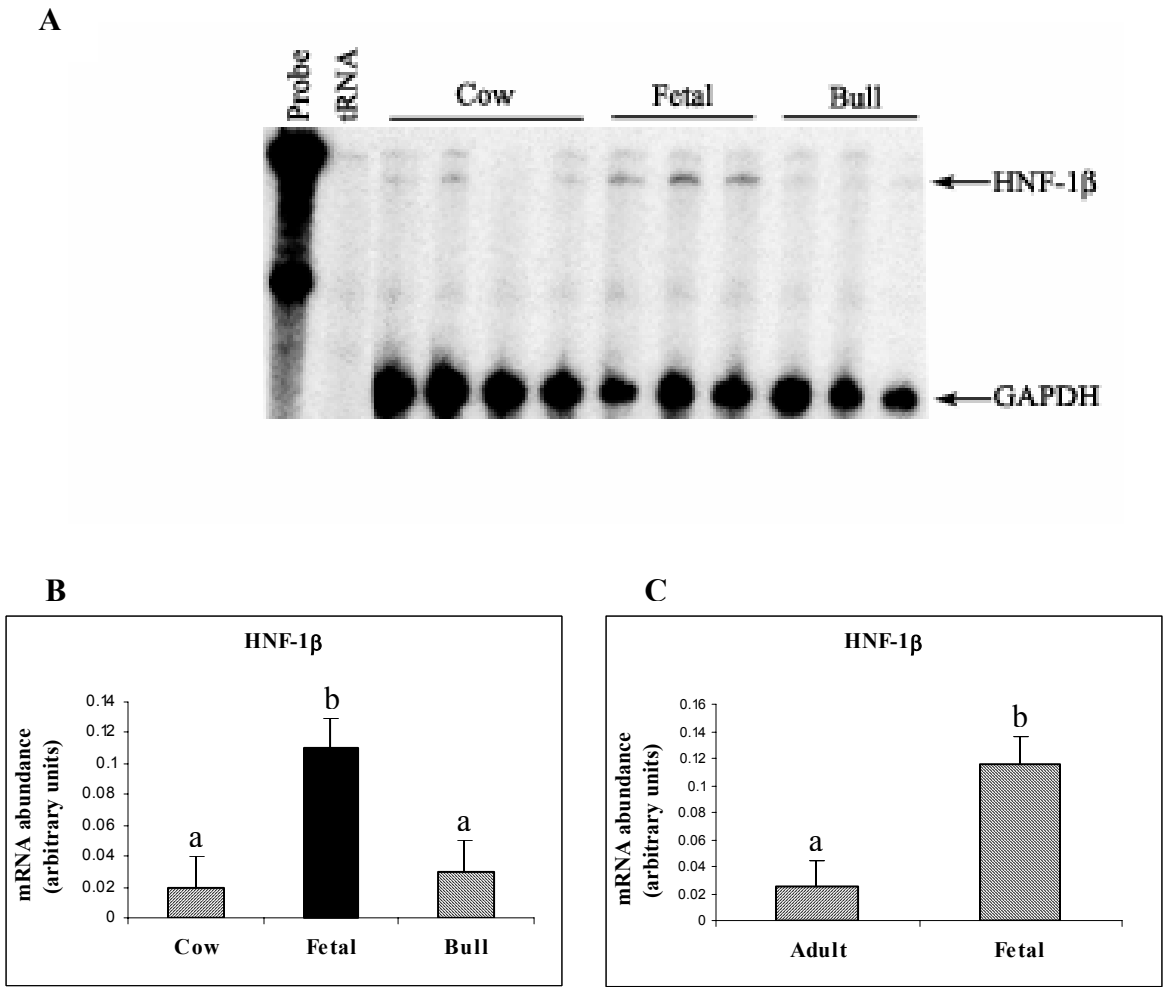

FIGURE 4. RPA of HNF-1β mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. (B) Relative abundance of HNF-1β mRNA in cow, bull and fetal cattle liver samples. (C) Relative abundance of HNF-1β mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$).

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bHNF-3α GTGTTTAGGACGGGTCTGGAATACACACCTTGGTAGTAGGCTGGCTCCAGGGCTGAGGGC 60
hHNF-3α GTGTTTAGGACGGGTCTGGAATACACACCTTGGTAGTACGCCGGCTCCAGGGCTGAGGGC 60
rHNF-3α ---TTTAGCACGGGTCTGGAATACACACCTTGGTAGTAGGCCGGCTCCAGGGCTGAGGGC 60

bHNF-3α TCAATGGGGCTCCTCGTGGCCACCGAGGCGCCGCCGAGGGGCAAGCTGGCGGGCAATGCG 120
hHNF-3α TCGATGGGGCTCCTGGTGGTCACCGAGGCGCTGCCTAGAGGCAGGCTGGCGGGCAACGTA 120
rHNF-3α TCGATGGGGCTCCTCGTGGCCACTGAGGCGCCGCCAAGGGGCAGACTGGCGGGCAAGGTG 120

bHNF-3α CTGCCGTAGGGCGAGTACTGTAGTGCCTGCTCATATGCCTTGAAGTCCAACTTGTGCTGC 180
hHNF-3α GAGCCGTAAGGCGAGTATTGCAGTGCCTGTTCGTATGCCTTGAAGTCCAGCTTATGCTGC 180
rHNF-3α GCGCCGTAAGGAGAGTACTGCAGTGCCTGCTCATATGCCTTGAAGTCCAGCTTGTGCTGC 180

bHNF-3α TGCTCCGAGGAGGACATGAGGTTGTTGATGGAGAACGG 218
hHNF-3α TGCTCCGAGGAGGACATGAGGTTGTTGATGGAGAACGG 218
rHNF-3α TGCTCCGAGGAGGACATGAGGTTGTTGATGGAGAA--- 218
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FIGURE 5. Nucleotide sequence of the cloned bovine HNF-3α cDNA and its alignment with the human and rat HNF-3α cDNA sequences. The human HNF-3α sequence in the alignment corresponds to the region from 1507 to 1724 in GenBank accession No. NM004496.2; the rat HNF-3α sequence in the alignment corresponds to the region from 1237 to 1448 in GenBank accession No. NM012742.

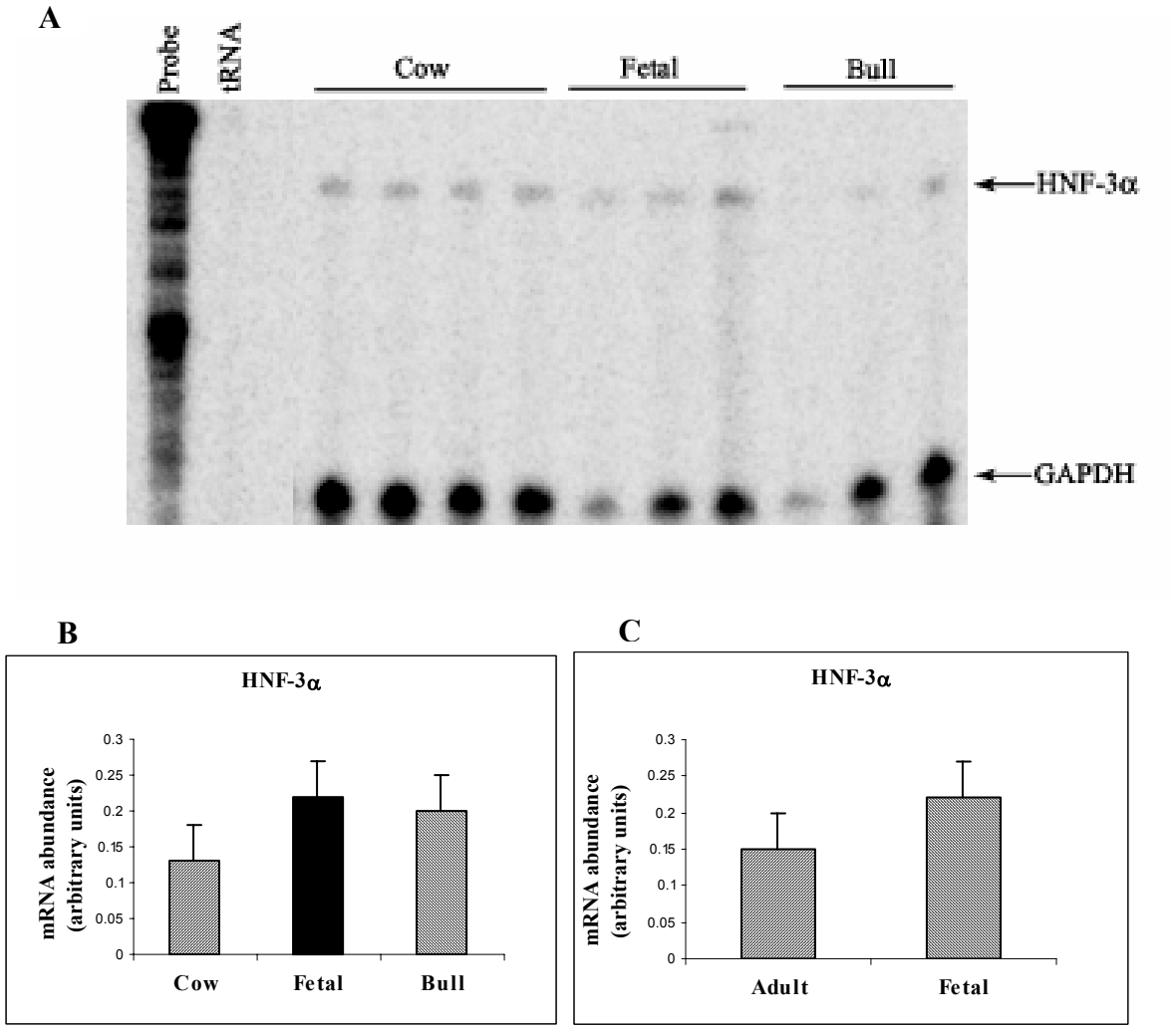

FIGURE 6. RPA of HNF-3α mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. (B) Relative abundance of HNF-3α mRNA in cow, bull and fetal cattle liver samples. (C) Relative abundance of HNF-3α mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. There are no significant differences between the means ($P > 0.1$).

```
bHNF-3β TGGGAGCGGTGAAGATGGAAGGGCACGAGCCGTCCGACTGGAGCAGCTACTACACCGAGC   60
hHNF-3β TGGGAGCGGTGAAGATGGAAGGGCACGAGCCGTCCGACTGGAGCAGCTACTATGCAGAGC   60
mHNF-3β TGGGAGCCGTGAAGATGGAAGGGCACGAGCCATCCGACTGGAGCAGCTACTACGCGGAGC   60

bHNF-3β CCGAGGGCTACTCCTCGGTGAGCAACATGAACGCCGGCCTGGGGATGAACGGCATGAACA  120
hHNF-3β CCGAGGGCTACTCCTCCGTGAGCAACATGAACGCCGGCCTGGGGATGAACGGCATGAACA  120
mHNF-3β CCGAGGGCTACTCTTCCGTGAGCAACATGAACGCCGGCCTGGGGATGAATGGCATGAACA  120

bHNF-3β CGTACATGAGCATGTCGGCGGCCGCCATGGGCAGCGGCTCGGGCAACATGAGCGCCGGCT  180
hHNF-3β CGTACATGAGCATGTCGGCGGCCGCCATGGGCAGCGGCTCGGGCAACATGAGCGCGGGCT  180
mHNF-3β CATACATGAGCATGTCCGCGGCTGCCATGGGCGGCGGTTCCGGCAACATGAGCGCGGGCT  180

bHNF-3β CCATGAACATGTCGTCGTACGTGGGCGCGGGCATGAGCCCGTCCCTGGCCGGCATGTCCC  240
hHNF-3β CCATGAACATGTCGTCGTACGTGGGCGCTGGCATGAGCCCGTCCCTGGCGGGGATGTCCC  240
mHNF-3β CCATGAACATGTCATCCTATGTGGGCGCTGGAATGAGCCCGTCGCTAGCTGGCATGTCCC  240

bHNF-3β CCGGCGCGGGC  251
hHNF-3β CCGGCGCGGGC  251
mHNF-3β CGGGCGCCGGC  251
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FIGURE 7. Nucleotide sequence of the cloned bovine HNF-3β cDNA and its alignment with the human and mouse HNF-3β cDNA sequences. The human HNF-3β sequence in the alignment corresponds to the region from 1 to 251 in GenBank accession No. BC019288; the mouse HNF-3β sequence in the alignment corresponds to the region from 17 to 267 in GenBank accession No. L1049.1.

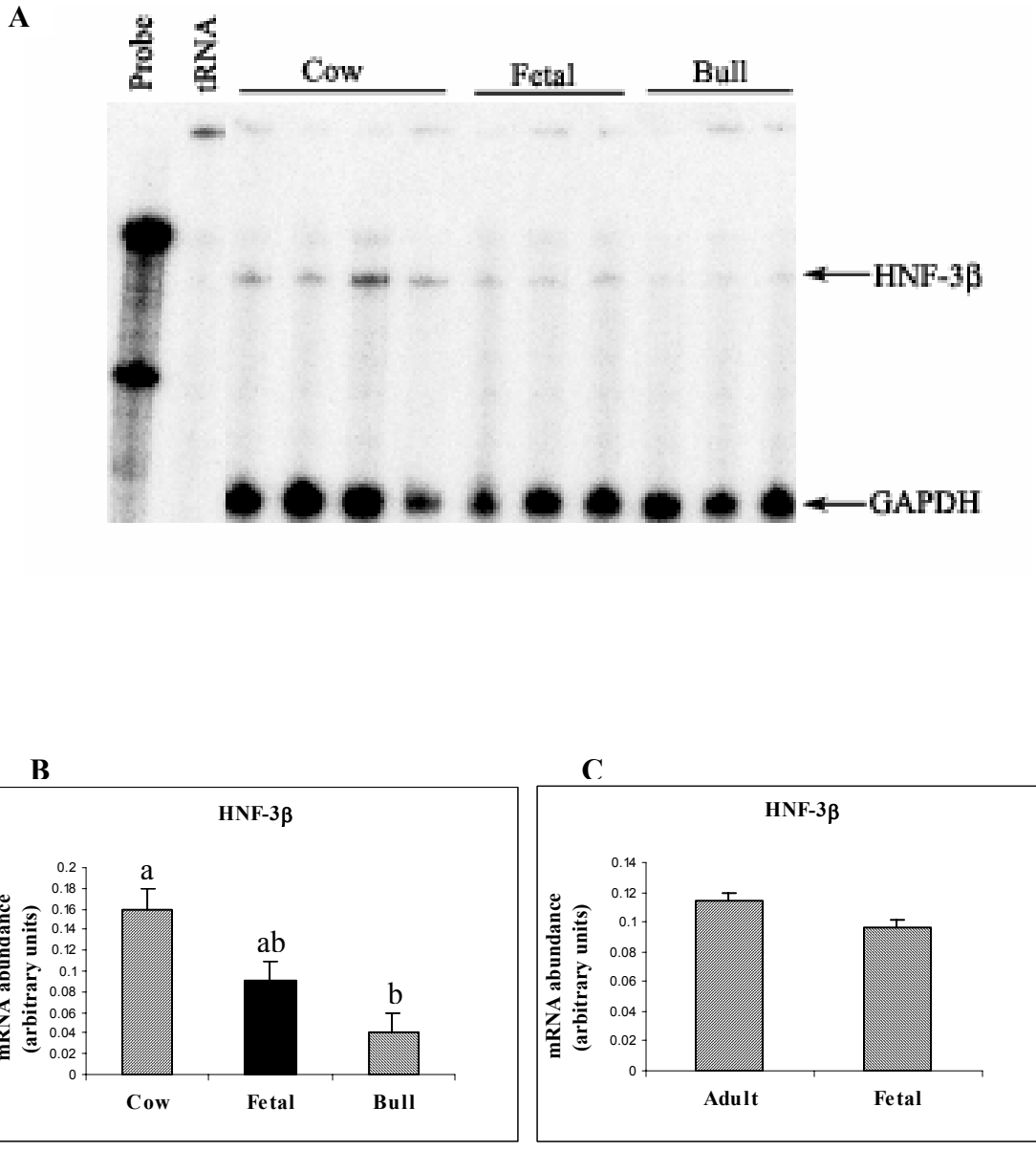


FIGURE 8. RPA of HNF-3β mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. (B) Relative abundance of HNF-3β mRNA in cow, bull and fetal liver samples. Values are expressed as mean ± SE. Means with different letters differ significantly ($P < 0.05$). (C) Relative abundance of HNF-3β mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. There are no significant differences between the means ($P > 0.1$).

```
bHNF-3γ CTTCAGCTGGAGGAGAAGGTGAAGAAAGGGGCGGCGGG---AGCTCGGCCTCCAGGAAC 60
hHNF-3γ ----AGCTGGAGGAGAAGGTGAAAAAAGGGGCAGCGGGCTGCCACCACCACCAGGAAC 60
mHNF-3γ ----AGCTGGAGGAGAAGGCAAAGAAAGGAAACAGCGCC---ATATCGGCCAGCAGGAAT 60

bHNF-3γ AG---TGCGGGGTCGGCCTCCACGGCCACCGCCCCTGCCGCCACT---GTGGCCTCCACG 120
hHNF-3γ GG---GACAGGGTCTGCTGCCTCGACCACCACCCCCGCGGCCACA---GTCACCTCCCCG 120
mHNF-3γ GGTACTGCGGGGTCAGCCACCTCTGCCACCACTACAGCTGCCACTGCAGTCACCTCCCCG 120

bHNF-3γ CCGCAGCCGCAGCCCCCGCCCCTGAGCCGGAGGCCCAGGGTGGGGACGAGGTGGGGGCT 180
hHNF-3γ CCCCAGCCCCCGCCTCCAGCCCCTGAGCCTGAGGCCCAGGGCGGGGGAAGATGTGGGGGCT 180
mHNF-3γ GCTCAGCCCCAGCCTACGCCATCTGAGCCCGAGGCCCAGAGTGGGGATGATGTGGGGGGT 180

bHNF-3γ CTGGACTGTGGCTCACCCGCTGCTCCCTCCACACCCTACTTCACTGGCCTGGAGCTCCCA 240
hHNF-3γ CTGGACTGTGGCTCACCCG---CTTCCTCCACACCCTATTTCACTGGCCTGGAGCTCCCA 240
mHNF-3γ CTGGACTGCGCCTCACCTC---CTTCGTCCACACCTTATTTCAGCGGCCTGGAGCTCCCG 240

bHNF-3γ GGGGAGCTAAAGCTGGATGCGCCCTACAACTTCAATCACCCTTTCTCCATCAACAACCTG 300
hHNF-3γ GGGGAGCTGAAGCTGGACGCGCCCTACAACTTCAACCACCCTTTCTCCATCAACAACCTA 300
mHNF-3γ GGGGAACTAAAGTTGGATGCGCCCTATAACTTCAACCACCCTTTCTCTATCAACAACCTG 300

bHNF-3γ ATGTCAGAACAGACACCAG 310
hHNF-3γ ATGTCAGAACAGACACCAG 310
mHNF-3γ ATGTCAGAACAGACA---- 310
```

FIGURE 9. Nucleotide sequence of the cloned bovine HNF-3γ cDNA and its alignment with the human and mouse HNF-3γ cDNA sequences. The human HNF-3γ sequence in the alignment corresponds to the region from 838 to 1143 in GenBank accession No. BC016024; the mouse HNF-3γ sequence in the alignment corresponds to the region from 772 to 1076 in GenBank accession No. BC037083.1.

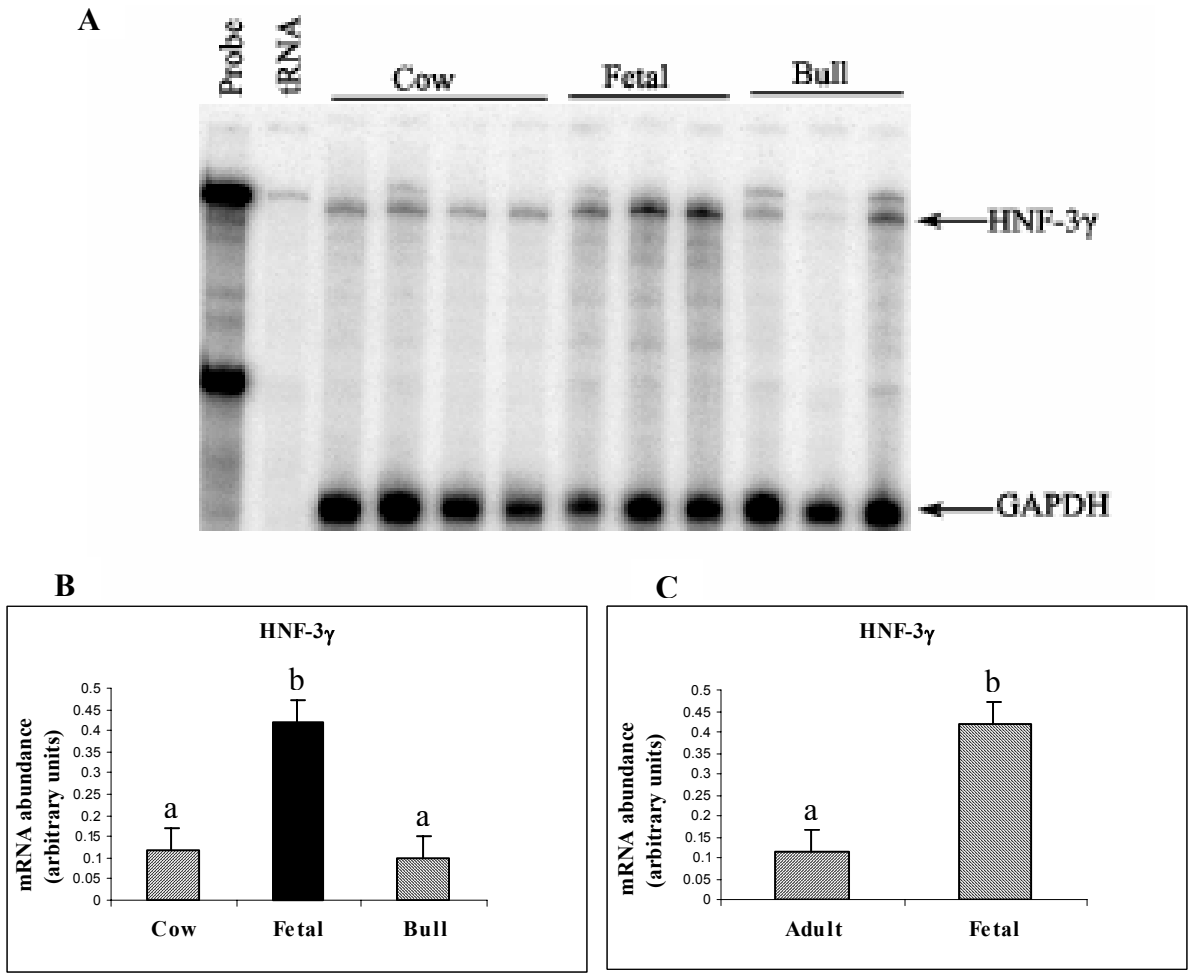


FIGURE 10. RPA of HNF-3γ mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. (B) Relative abundance of HNF-3γ mRNA in cow, bull and fetal liver samples (C) Relative abundance of HNF-3γ mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$).

```
bHNF-6 ACTTGTCCAGACTCCTCCTCCTCGCATTCATGAAGAAGTTGCTGACGGTGCTCAGCTCCA 60
hHNF-6 -CTTGTCCAGACTCCTCCTTCTTGCGTTCATGAAGAAGTTGCTGACAGTGCTCAGCTCCA 60
mHNF-6 ACTTGTCCAGACTCCTCCTCCTGGCATTCATGAAGAAGTTGCTGACAGTGCTCAGCTCCA 60

bHNF-6 ACCCCAGCTGCTGGGAAATGGTGATTTGCAATTCTTTGGATGGACGCTTATTTTCCTTGA 120
hHNF-6 ACCCCAGCTGCTGGGAAATGGTGATTTGCAATTCTTTGGATGGACGCTTATTTTCCTTGA 120
mHNF-6 ACCCCAGCTGCTGGGAGATGGTGATTTGTAATTCTTTGGACGGACGCTTATTTTCCTTGA 120

bHNF-6 ATATTGCATGTAGAGTTCGACGCTGGACATCTGTGAAGACCAACCTGGGCTTTTTGGGTG 180
hHNF-6 ATATTGCATGTAGAGTTCGACGCTGGACATCTGTGAAGACCAACCTGGGCTTTTGGGGTG 180
mHNF-6 ATATTGCATGTAGAGTTCGACGTTGGACGTCTGTGAAGACCAGCCTGGGCTTTTTGGGGG 180

bHNF-6 TGCTGCCTCTATCCTTCCCGTGTTCTTGCTCTTTCCTTTTGCATGCTGCTAAGCGGAGCG 240
hHNF-6 TGTTGCCTCTATCCTTCCCATGTTCTTGTTCTTTCCTTTTGCATGCTGCTAAGCGGAGCG 240
mHNF-6 TGTTGCCTCTGTCCTTCCCATGTTCTTGCTCTTTCCGTTTGCAGGCTGCTAAGCGGAGCG 240

bHNF-6 CAGACATACGCTGGAACTCCGGCTCCTGCA- 270
hHNF-6 CGGACATGCGCTGGAACTCCGGCTCCTGCAG 270
mHNF-6 CCGACATGCGCTGGAACTCCGGCTCCTGCA- 270
```

FIGURE 11. Nucleotide sequence of the cloned bovine HNF-6 cDNA and its alignment with the human and mouse HNF-6 cDNA sequences. The human HNF-6 sequence in the alignment corresponds to the region from 541 to 810 in GenBank accession No. HS077975.1; the mouse HNF-6 sequence in the alignment corresponds to the region 1437 to 1706 in GenBank accession No. BC024053.1.

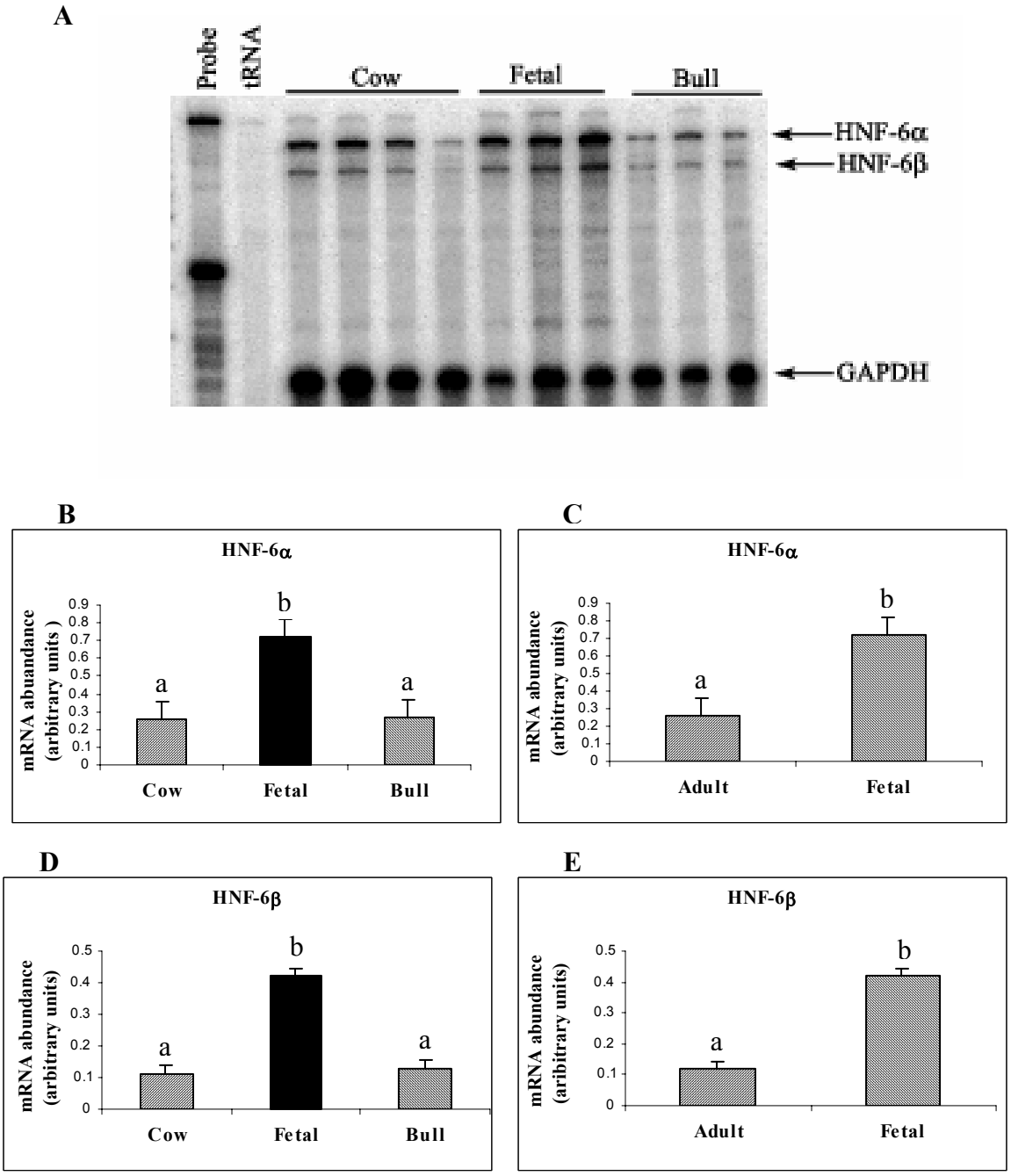


FIGURE 12. RPA of HNF-6α and HNF-6β mRNAs in cow, bull and fetal cattle livers. (A) Phosphor image of RPA. (B)& (D) Relative abundance of HNF-6α and HNF-6β mRNAs in cow, bull and fetal liver samples. (C) & (E) Relative abundance of HNF-6α and HNF-6β mRNAs in adult and fetal cattle livers. Values are expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$).

```
bDBP GCACCGATATCTGGTTCTCCTTGAGCCTCCGGGCGTCGCGGGACCGCTTGGCTGCCTCGT 60
hDBP GCACCGATATCTGGTTCTCCTTGAGCCGCCGGGCGTCACGGGACCGCTTGGCTGCCTCGT 60
mDBP GCACAGATATCTGGTTCTCCTTGAGTCTTCTTGCATCTCTCGACCTCTTGGCTGCTTCAT 60

bDBP TATTCTTGTACCGTCGGCTCCAGTATTTCTCGTCCTTCTGCTCCTCTGGCACCTGGATCT 120
hDBP TGTTCTTGTACCGCCGGCTCCAGTATTTCTCATCCTTCTGCTCCTCCGGCACCTGGATTT 120
mDBP TGTTCTTGTACCTCCGGCTCCAGTACTTCTCATCCTTCTGTTCCTCAGGCACCTGGACTT 120

bDBP TCCTCGCCTTCTTCATGATTGGCTGGGGCTTGAGTTCCTCTTCGGAGAAGCGATGTCTTC 180
hDBP TTCTTGCCTTCTTCATGATTGGCTGGGGCTTAAGTTCCTCTTCTGAGAAGCGATGTCTTC 180
mDBP TCCTTGCCTTCTTCATGATTGGTTGAGGCTTCAGTTCCTCCTCTGAGAAGCGGTGTCTCC 180

bDBP GAG 183
hDBP GAG 183
mDBP GAG 183
```

FIGURE 13. Nucleotide sequence of the cloned bovine DBP cDNA and its alignment with the human and mouse DBP cDNA sequences. The human DBP sequence in the alignment corresponds to the region from 1011 to 1193 in GenBank accession No. BC011965.1; the mouse DBP sequence in the alignment corresponds to the region from 1014 to 1196 in GenBank accession No. BC018323.1.

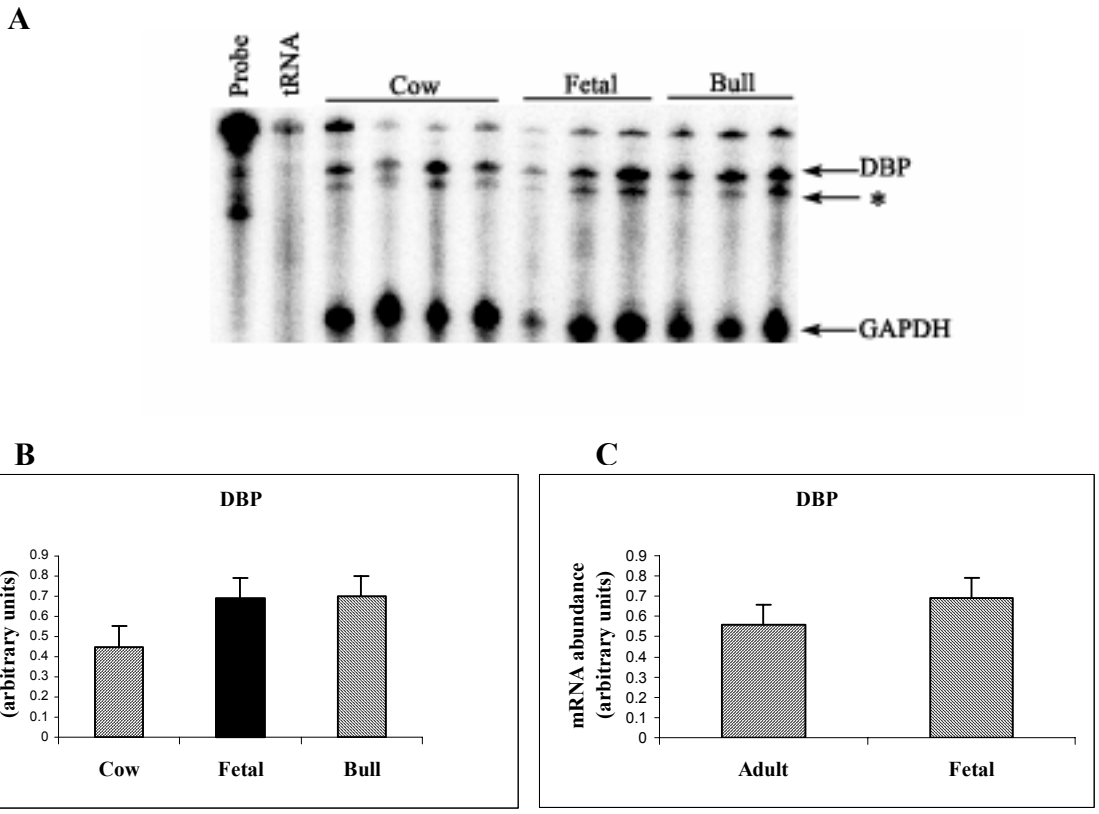


FIGURE 14. RPA of DBP mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. * is shorter than the DBP and could be an spliced variant of DBP (B) Relative abundance of DBP mRNA in cow, bull and fetal liver samples. (C) Relative abundance of DBP mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. There are no significant differences between the means ($P > 0.1$).

```
bC/EBP-α TGGCCGACCTGTTCCAACACAGCCGGCAGCAGGAGAAGGCCAAGGCGGCCGCGGCCCCCG 60
hC/EBP-α TGGCCGACCTGTTCCAACACAGCCGGCAGCAGGAGAAGGCCAAGGCGGCCGCGGCCCCCG 60
rC/EBP-α TGGCCGACCTGTTCCAACACAGCCGGCAGCAGGAGAAGGCCAAGGCGGCCGCGGCCCCCG 60

bC/EBP-α CAGGAGGCGGCAACGACTTTGACTACCCGGGCGCCCCCGTGGGCCCCGGCGGCGCCGTCA 120
hC/EBP-α CAGGAGGCGGCAACG------ACTTTGACTACCCGGGCGCCCCCGTGGGCCCCGGCGGCG 120
rC/EBP-α CAGGAGGCGGCAACGACTTTGACTACCCGGGCGCCCCCGTGGGCCCCGGCGGCGCCGTCA 120

bC/EBP-α TGCCCGGGGGGACGCACGGTCCCCCTCCTGGCTACGGCTGCGCGGCAGCCGGCTACCTGG 180
hC/EBP-α CCGTCATGCCCGGGGGGACGCACGGTCCCCCTCCTGGCTACGGCTGCGCGGCAGCCGGCT 180
rC/EBP-α TGCCCGGGGGGACGCACGGTCCCCCTCCTGGCTACGGCTGCGCGGCAGCCGGCTACCTGG 180

bC/EBP-α ACAGCAGGCTGGAGCCTCTGTACGAGCGGGTCGGGG----- 220
hC/EBP-α ACCTGGACAGCAGGCTGGAGCCTCTGTACGAGCGGGTCGGG 220
rC/EBP-α ACAGCAGGCTGGAGCCTCTGTACGAGCGGGTCGGG------ 220
```

FIGURE 15. Nucleotide sequence of the cloned bovine C/EBP-α cDNA and its alignment with the human and rat cDNA sequences. The bovine C/EBP-α sequence in this figure is 100 % identical of the bovine C/EBP-α sequence region from 401 to 616 in GenBank accession No D82984.1. The human C/EBP-α sequence in the alignment corresponds to the region from 824 to 1024 in the GenBank accession No.HSU34070.1; the rat C/EBP-α sequence in the alignment corresponds to the region from 233 to 447 in GenBank accession No.X12752.1.

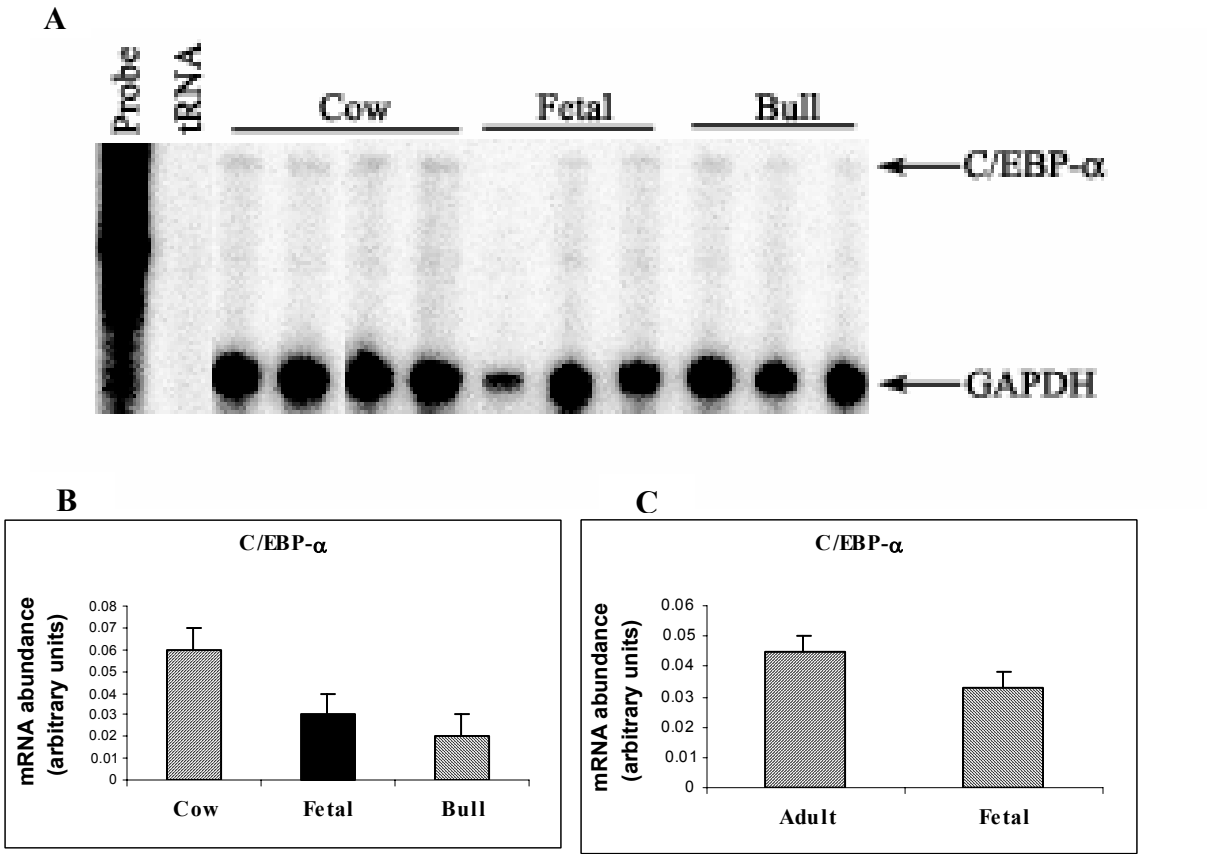


FIGURE 16. RPA of C/EBP-α mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. (B) Relative abundance of C/EBP-α mRNA in cow, bull and fetal liver samples. (C) Relative abundance of C/EBP-α mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. There are no significant differences between the means ($P > 0.1$).

```
bC/EBP-β ATCGACTTCAGCCCCTACCTGGAGCCGCTGGGCGCGCCGCAGGCCCCGGCACCCACCACG 60
hC/EBP-β ATCGACTTCAGCCCCTACCTGGAGCCGCTGGGCGCGCCGCAGGCCCCGGCACCCACCACG 60

bC/EBP-β GCCTCGGACACCTTCGAGGCGGCTCCGCCCGCGCCCGCCCCCCGCGCCCGCCTCCTCCGGG 120
hC/EBP-β GCCTCGGACACCTTCGAGGCGGCTCCGCCCGCGCCCGCCCCCGCGCCCGCCTCCTCCGGG 120

bC/EBP-β CAGCACCACGACTTCCTCTCCGACCTCTTCTCCGACGACTA 161
hC/EBP-β CAGCACCACGACTTCCTCTCCGACCTCTTCTCCGACGACTA 161
```

FIGURE 17. Nucleotide sequence of the cloned bovine (b) C/EBP-β cDNA and its alignment with the human (h) cDNA sequence. The bovine C/EBP-β sequence in this figure is 98 % identical to the bovine C/EBP-β cDNA sequence in GenBank (accession No D82985.1). The human C/EBP-β sequence in the alignment corresponds to the region from 412 to 572 in GenBank accession No.BC021931.1.

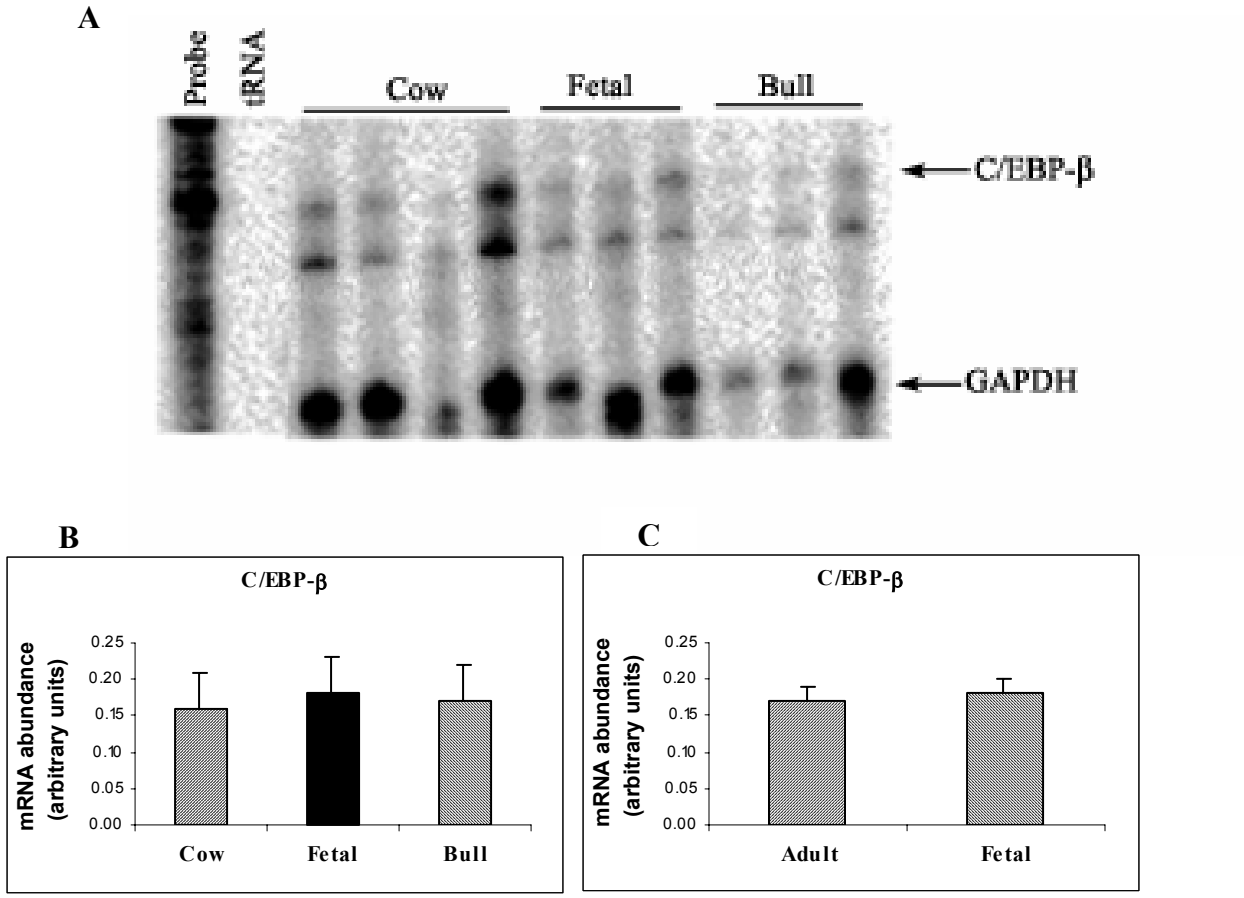


FIGURE 18. RPA of C/EBP-β mRNA in cow, bull and fetal cattle livers.
(A) Phosphor image of RPA. (B) Relative abundance of C/EBP-β mRNA in cow, bull and fetal liver samples. (C) Relative abundance of C/EBP-β mRNA in adult and fetal cattle livers. Values are expressed as mean ± SEM. There were no significant differences between the means ($P > 0.1$).

CHAPTER III

Effect of Growth Hormone on the Expression of Liver-Enriched Transcription Factors in the Bovine Liver

ABSTRACT

Growth hormone (GH) regulates a wide variety of physiological processes. The major target of GH is the liver, where GH controls the expression of many genes involved in metabolism, detoxification and other functions of the liver. A potential mechanism by which GH controls gene expression in the liver is that GH regulates the expression of liver-enriched transcription factors (LETFs), which in turn regulate the expression of other genes. The objective of this study was to identify the LETFs whose expression is regulated by GH in the bovine liver. Nonpregnant and nonlactating cows received a single intramuscular injection of 500 mg slow-release recombinant bovine GH and liver biopsies were collected 6 h (n = 6), 24 h (n = 6) and 1 w (n = 6) after GH injection. Liver biopsy samples were also taken from six untreated cows. The mRNA abundance of hepatocyte nuclear factor (HNF) -1α, HNF-1β, HNF-3α, HNF-3β, HNF-3γ, HNF-6, albumin D-element binding protein (DBP), CCAAT/ enhancer-binding proteins (C/EBP)-α and β in the liver samples were determined by ribonuclease protection assay (RPA). The levels of HNF-3γ and HNF-6 mRNAs in the liver of cows were significantly increased ($P < 0.05$) 24 h and 1w after GH administration. The mRNA expression of HNF-1α, HNF-1β, HNF-3α, HNF-3β, DBP, C/EBP-α, and C/EBP-β was not affected by GH ($P > 0.1$). These results suggest that HNF-3γ and HNF-6 may be involved in GH regulation of gene expression in the bovine liver.

Keywords: Growth Hormone; Liver; Bovine; Liver-Enriched Transcription Factors

Introduction

Growth hormone (GH) is a polypeptide hormone released from the anterior pituitary gland. The GH regulates a broad range of physiological processes, including development, somatic growth, and metabolism (Le Roith, 2001). A major target of GH is the liver. In this tissue, GH controls the expression of many genes, such as insulin-like growth factor (IGF)-1, phosphoenol pyruvate kinase C, and glucose transporter (Glut2) (Lemaigre et al., 1996; Pierreux et al., 1999; Lannoy et al., 1998; Gronowski et al., 1996) involved in metabolism, growth or detoxification. GH controls the expression of many of these genes primarily at the transcriptional level. A potential mechanism by which GH regulates the expression of these genes in the liver is that by regulating the expression of the liver-enriched transcription factors (LETFs), including hepatocyte nuclear factor (HNF) -1α, HNF-1β, HNF-3α, HNF-3β, HNF-3γ, HNF-6, albumin D-element binding protein (DBP), CCAAT/ enhancer-binding proteins (C/EBP)-α and β (Tollet et al., 1990; Bichell et al., 1992; Thomas et al., 1995; Rastegar et al., 2000), which in turn regulate the expression of other genes in the liver. Such a mechanism is indeed supported by the observations on hypophysectomized rats that GH administration causes changes in the expression of HNF-6, HNF-3β, C/EBP-α, and HNF-4 (Lahuna et al., 2000; Rastegar et al., 2000). To identify the LETFs that may be involved in GH regulation of gene expression in the bovine liver, we have performed a systematic analysis of the effect of GH administration on the mRNA levels of nine LETFs in the liver of cows.

Materials and Methods

Animals and experimental design

A total of 18 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 (Monsanto Company, St. Louis, MO). Liver biopsy samples were taken from group 1 cows 6 h after GH injection, from group 2 cows 24 h after GH injection, and from group 3 cows 1 w after GH injection. Liver biopsy samples were also taken from group 3 cows 1 d before GH injection (these samples served as pre-GH controls).

The liver biopsies were taken following a standard procedure (Oxender et al., 1971). After restraining the cows, the skin area between the 11^{th} and 12^{th} ribs was washed and sterilized with 70% ethanol. Thereafter, the skin in that area was shaved and a subcutaneous injection of lidocaine (10 ml) was given, and a small incision was made between the 11^{th} and 12^{th} ribs. The biopsy needle was introduced through the body wall and peritoneum and a liver sample (100-300 mg) was collected. Following the biopsy, the cutaneous incision was sutured and antiseptic agent was applied to prevent infection. Once taken, liver samples were immediately frozen in liquid nitrogen and stored at -80°C**.**

RNA extraction

Total RNA from liver tissue samples were isolated by using TRI regent (MRC, Cincinnati, OH) according to the manufacturer's instructions. The RNA concentrations were determined by measuring absorbance at 260 nm and RNA quality was verified by electrophoresis on formaldehyde-agarose gels.

In vitro transcription and ribonuclease protection assay (RPA)

To synthesize riboprobe for ribonuclease protection assay (RPA), the plasmids with LETF cDNA inserts were digested with an appropriate restriction enzyme (Table 2) to linearize the plasmid. The in vitro transcription in the presence of [α-^{32}P] CTP was carried out using the Riboprobe Combination System kit (Promega), essentially according to the manufacturer's instructions. The specific activity of the purified probe was estimated by liquid scintillation counting. The RPA was carried out on 10 μg of total liver RNA, using the RPA II kit (Ambion, Austin, TX) according to the manufacturer's instructions. The ribonuclease protected RNA fragments were resolved by electrophoresis on 6% acrylamide gels containing 7 M urea. After gel electrophoresis, gels were dried and exposed to phosphor screens. Exposed phosphor screens were scanned on a Molecular Imager system (Bio-Rad Laboratories, Hercules, CA). The densities of the protected bands were quantified by using the Quantity One software (Bio-Rad) and were used to represent the abundance of the corresponding mRNA.

Statistical analysis

The densities of the protected LETF mRNAs were adjusted to the densities of GAPDH mRNA in the same sample. The adjusted densities were analyzed using the General Linear contrast Model procedure of SAS (SAS Institute, Cary, NC), in which the liver mRNA levels between different groups were compared. Differences were considered significant if $P < 0.05$.

Results

The levels of HNF-1α, HNF-1β, HNF-3α, HNF-3β, mRNAs in the liver of cows 6 h, 24 h or 1 w after GH administration (Figures 19B, 20B, 20C, 21B, 22B). Whereas, the levels of HNF-6 and HNF-3γ mRNAs were higher ($P < 0.05$) in the liver of cows 24 h and 1w after GH

administration than in the untreated cows (Figures 23B, 24B). The levels of these two mRNAs also tended (P =0.01) to increase 6 h after GH administration (Figures 23B, 24B). The levels of DBP, C/EBP-α, and C/EBP-β mRNAs (25B, 26B, and 26C) were not different (P > 0.1) from that in the liver of untreated cows. Thus, the expression of these LETFs in the bovine liver was not affected by GH.

Discussion

As a step toward the long-term goal of understanding how GH regulates gene expression in the liver, we analyzed the effect of exogenous GH on the expression of nine LETFs in cattle liver.The expression of HNF-1α and HNF-1β mRNAs was not affected by GH administration. This result is consistent with the findings in hypophysectomized rats that the HNF-1α protein in liver extracts did not increase after GH administration (Rastegar et al., 2000), suggesting that HNF-1α expression in the liver is not regulated by GH. However, Meton et al. (1999) showed that the IGF-1 promoter contains a functional HNF-1α binding site. Thus, whether and how HNF-1α is involved in GH stimulation of IGF-1 gene expression in the liver remains an intriguing question.

Our results indicated that HNF-3γ expression in the liver increased in response to GH injection. To the best of our knowledge, this finding is new. There seems to be no effect of pre GH treatment biopsy on the expression levels of HNF-3γ mRNA in the 1w group because of the absence of generalized effect of biopsy on the expression levels of other LETFs. HNF-3γ has been shown to be involved in the regulation of catalase, transcarbamylase and cytochrome P4503A4 gene expression (Schrem et al., 2002). Thus, the expression of these genes may be regulated indirectly by GH, through HNF-3γ. In this study, we found that GH had no effect on

HNF-3β mRNA expression in the bovine liver. This result does not agree with that of Lahuna et al. (2000) in which liver HNF-3β mRNA expression increased significantly within the first 6 h after GH administration (100 μg/100g BW) in hypophysectomized rats. It is possible that compared to its effect on HNF-3γ, GH has a very short-term effect on HNF-3β expression.

GH injection also increased the HNF-6 mRNA expression in the liver of cows. This result is consistent with a similar finding in hypophysectomized rats (Lahuna et al., 2000). There seems to be no effect of pre GH treatment biopsy on the expression levels of HNF-6 mRNA in the 1 w group because of the absence of generalized effect of biopsy on the expression levels of other LETFs. Induction of HNF-6 mRNA has also been observed in isolated hepatocytes (Lahuna et al., 2000). These results together indicate that GH can directly stimulate HNF-6 expression in the liver. The transcription of many liver-specific genes, such as several cytochrome P450 genes and the genes that code for many enzymes of glucose metabolism, is regulated by HNF-6 (Lemaigre et al., 1996; Pierreux et al., 1999; Lannoy et al., 1998). The expression of some of the genes of glucose metabolism, such as aldose reductase and glucose-6-phosphate dehydrogenase (G6PDH) (Olsson et al., 2003), has been shown to be stimulated by GH too. It is, thus, possible that GH may regulate the expression of these genes indirectly by modulating the expression of HNF-6.

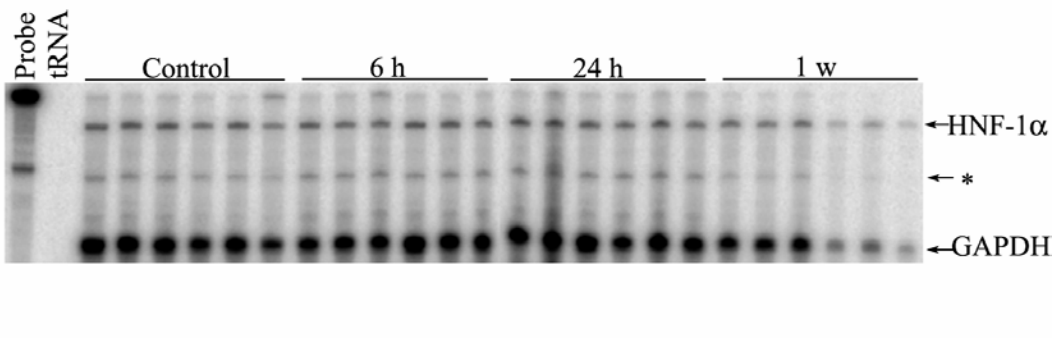
In this study, C/EBP-α mRNA expression did not increase after 6, 24 h, or 1 w after GH injection. However, Rastegar et al. (2000) found that in hypophysectomized rats liver C/EBP-α mRNA expression increased significantly within the first 3 h of GH injection. Thus, it is possible that GH has only a short-term effect on C/EBP-α mRNA expression in the liver. Similarly, we found that C/EBP-β mRNA expression did not increase 6 h, 24 h and 1 w after GH injection.

There have been no previous reports on the effect of GH on C/EBP-β. Hence, it is possible that GH does not affect the expression of C/EBP-β in the liver.

There have been no previous reports on the effect of GH on HNF-3α and DBP. This study indicated that GH did not increase the mRNA expression of HNF-3α and DBP after 6 h, 24 h, and 1 w of administration in the liver of cows. Thus, GH may not control HNF-3α and DBP expression in the liver.

In summary, the results of this study indicate that among the nine LETFs, HNF-6 and HNF-3γ expression in the bovine liver is increased by GH, whereas the expression of the remaining LETFs is not affected by GH. The increase in the expression of HNF-6 and HNF-3γ after GH administration suggests that these two LETFs may be part of GH regulated network of liver transcription factors mediating GH regulation of gene expression in the liver.

A



B

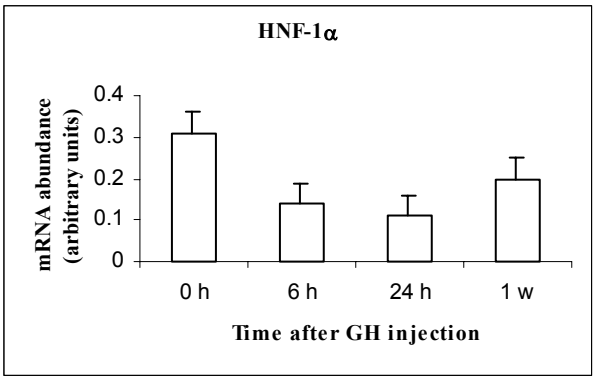
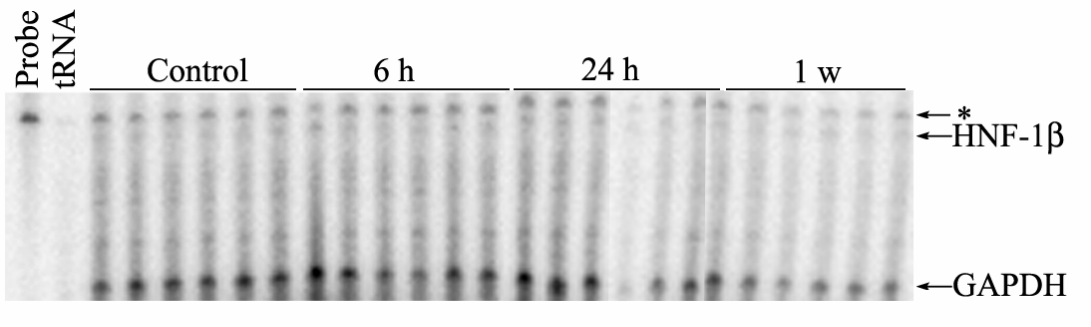


FIGURE 19. RPA of liver HNF-1α mRNA in cows 0, 6, 24 h and 1w after GH injection. (A) Phosphor image of the RPA. In the RPA, a probe specific for the bovine GAPDH mRNA was also included as a loading control. Yeast tRNA (tRNA) was included in the RPA as a negative control. The ribonuclease-protected fragments corresponding to HNF-1α and GAPDH mRNAs are indicated with arrows. * could be an alternative splice variant of HNF-1α. (B) Relative abundance of HNF-1α mRNA. Values are expressed as mean ± SEM (standard error of the mean). There are no significant differences between the means ($P > 0.1$).

A



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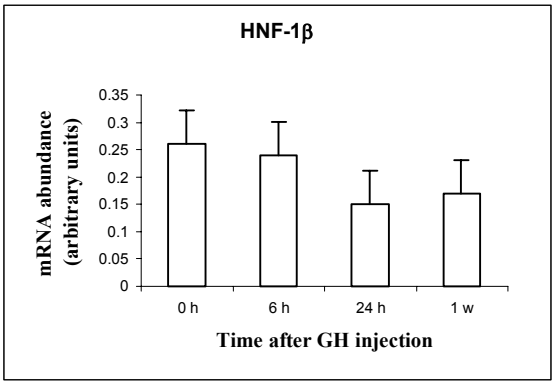
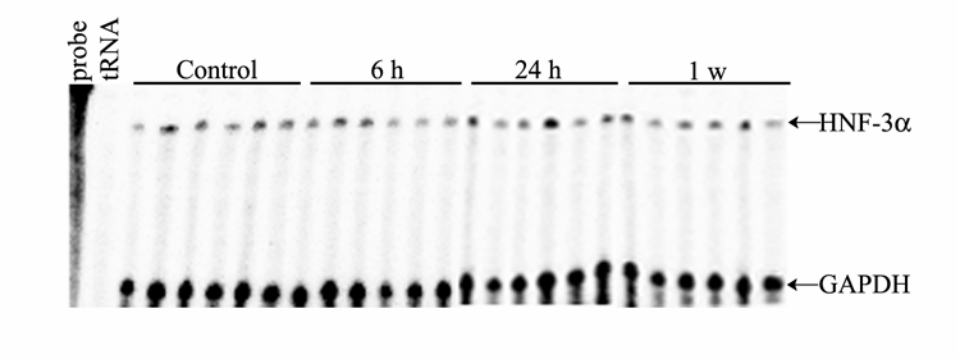


FIGURE 20. RPA of HNF-1β mRNA in cattle liver 0, 6, 24 h, and 1w after GH injection. (A) Phosphor image of the RPA. The ribonuclease-protected fragments corresponding to HNF-1β and GAPDH mRNA are indicated with arrows. * corresponds to undigested HNF-1β probe. (B) Relative abundance of HNF-1β mRNA. Values are expressed as mean ± SEM (standard error of the mean). There are no significant differences between the means ($P > 0.1$).

A



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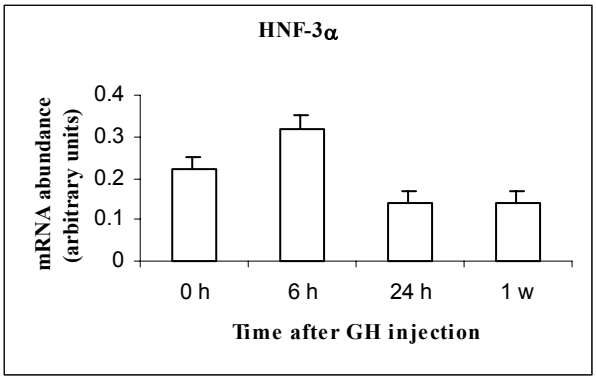
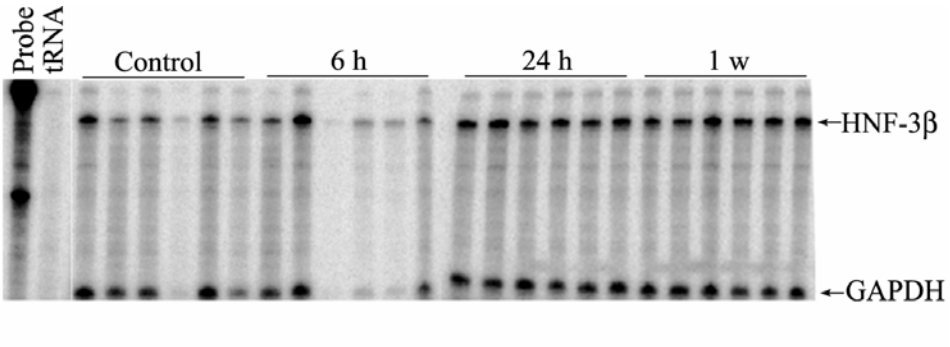


FIGURE 21. RPA of HNF-3α mRNA in cattle liver 0, 6, 24 h and 1w after GH injection (A) Phosphor images of the RPA. The ribonuclease-protected fragments corresponding to HNF-3α and GAPDH mRNA are indicated with arrows. (B) Relative abundance of HNF-3α mRNA. Values are expressed as mean ± SEM (standard error of the mean). There are no significant differences between the means ($P > 0.05$).

A



B

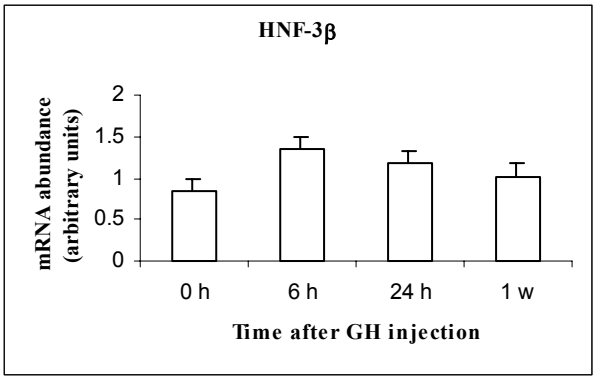
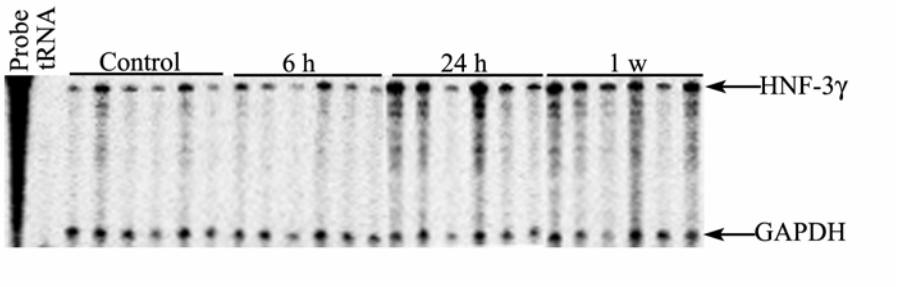


FIGURE 22. RPA of HNF-3β mRNA in cattle liver 0, 6, 24 h and 1w after GH injection. (A) Phosphor images of the RPA. The ribonuclease-protected fragments corresponding to HNF-3β and GAPDH mRNA are indicated with arrows. (B) Relative abundance of HNF-3β mRNA. Values are expressed as mean ± SEM (standard error of the mean). There are no significant differences between the means ($P > 0.05$).

A



B

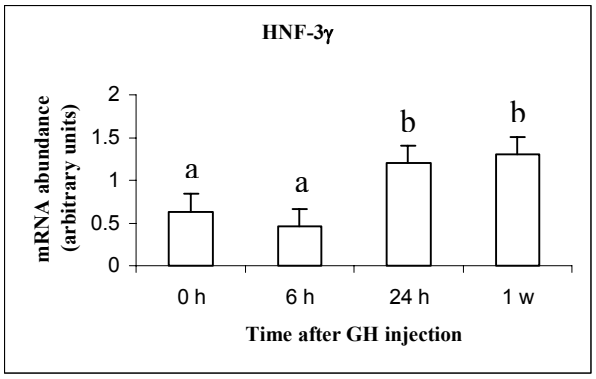
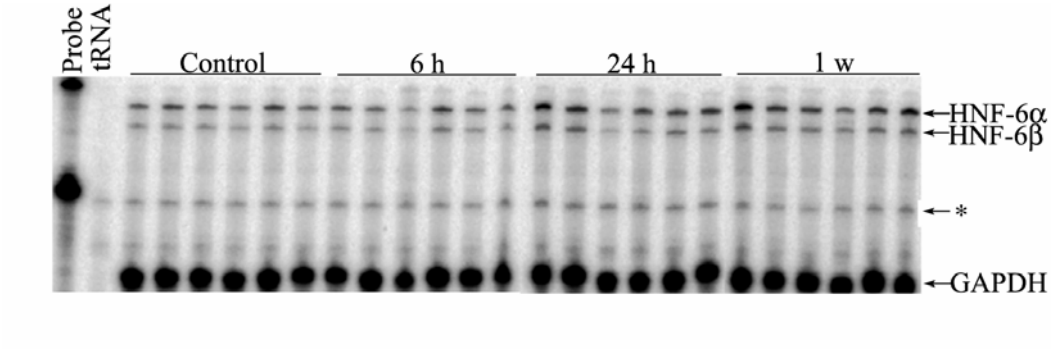


FIGURE 23. RPA of HNF-3γ mRNA in cattle liver 0, 6, 24 and 1w after GH injection. (A) Phosphor images of the RPA. The ribonuclease-protected fragments corresponding to HNF-3γ and GAPDH mRNA are indicated with arrows. (B) Relative abundance of HNF-3γ mRNA. Values are expressed as mean ± SEM (standard error of the mean). Means with different letters differ significantly ($P < 0.05$).

A



B

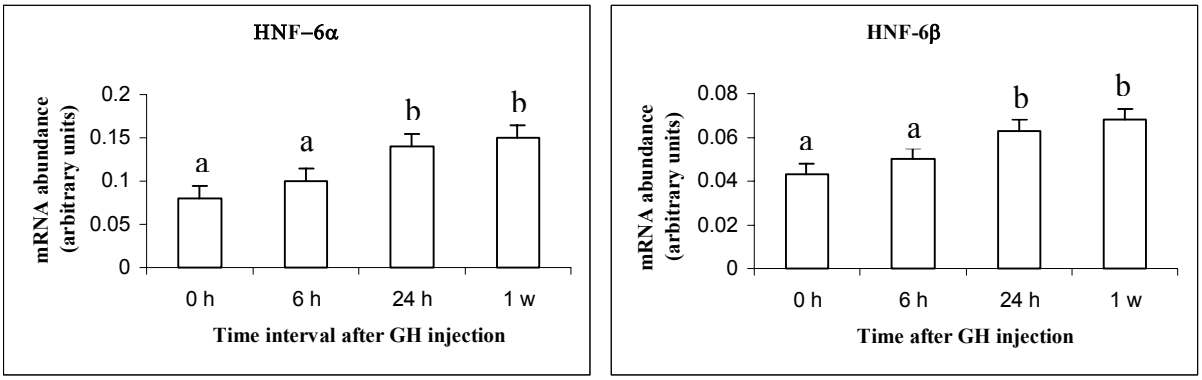
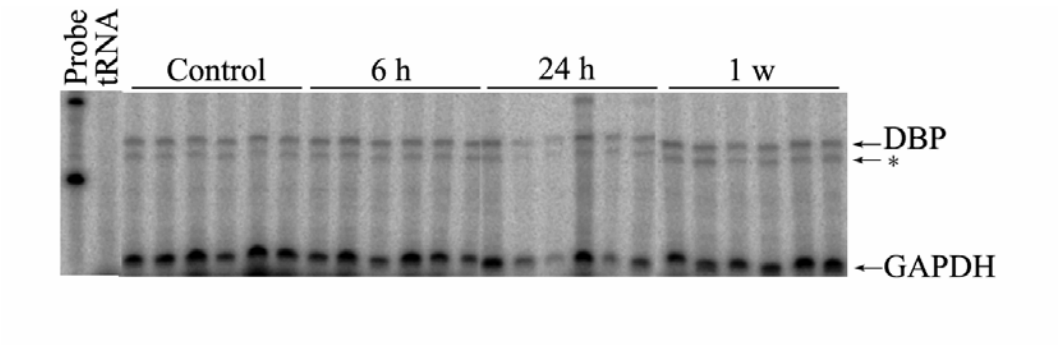


FIGURE 24. RPA of HNF-6 mRNA in cattle liver at 0, 6, 24 h, and 1w after GH injection. (A) Phosphor images of the RPA. The ribonuclease-protected fragments corresponding to HNF-6 and GAPDH mRNA are indicated with arrows. * corresponds to undigested GAPDH probe. (B) Relative abundance of HNF-6 mRNA. Values are expressed as mean ± SEM (standard error of the mean). Means with different letters differ significantly ($P < 0.05$).

A



B

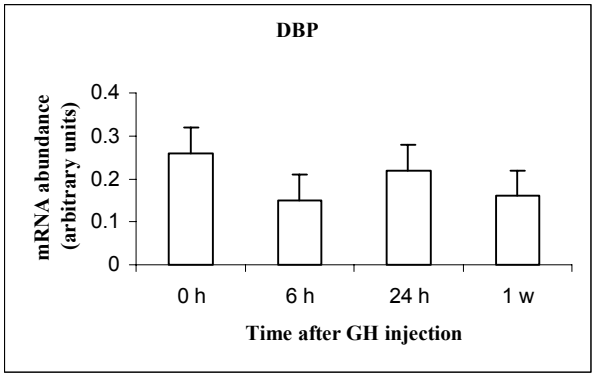
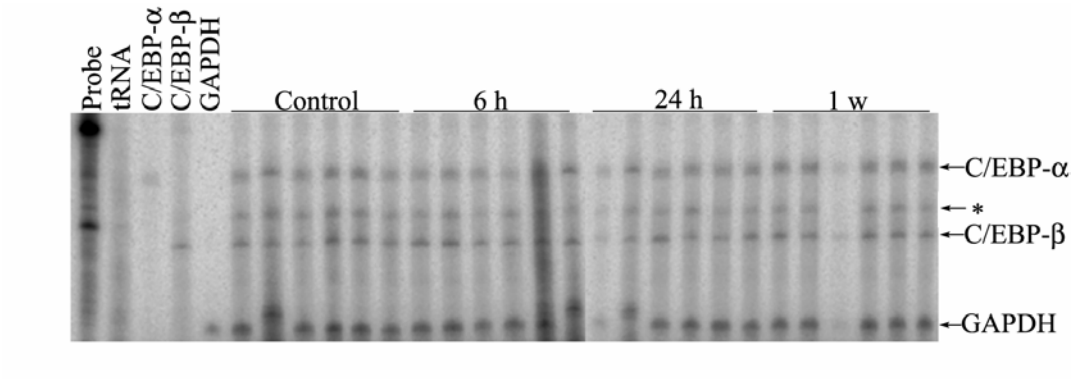
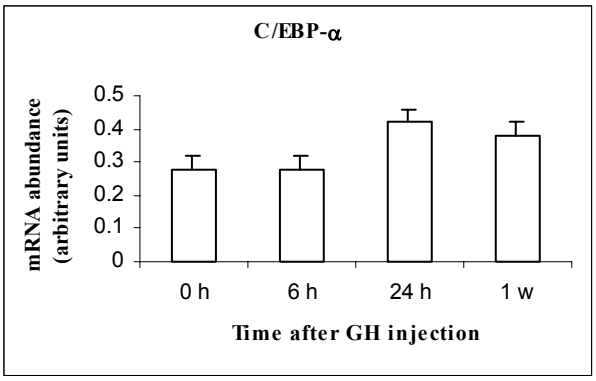


FIGURE 25. RPA of DBP mRNA in cattle liver 0, 6, 24 h, and 1w after GH injection. (A) Phosphor image of the RPA. The ribonuclease-protected fragments corresponding to DBP and GAPDH mRNA are indicated with arrows. * could be an alternatively spliced DBP. (B) Relative abundance of DBP mRNA. Values are expressed as mean ± SEM (standard error of the mean). There are no significant differences between the means ($P > 0.1$).

A



B



C

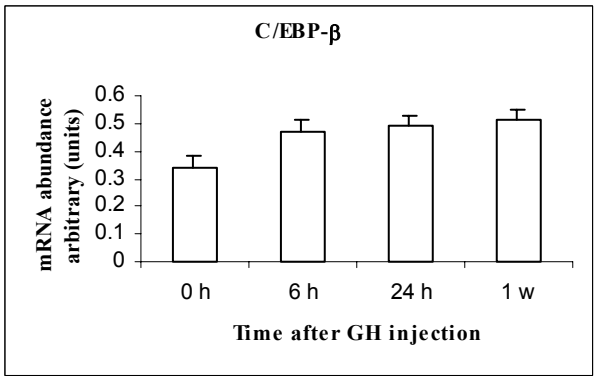


FIGURE 26. RPA of C/EBP-α and C/EBP-β mRNA in cattle liver at 0, 6, 24 h, and 1w after GH injection.
(A) Phosphor images of the RPA. The ribonuclease-protected fragments corresponding to C/EBP-α, C/EBP-β and GAPDH mRNA are indicated with arrows. * could be an alternative splice variant of C/EBP-α. (B) Relative abundance of C/EBP-α mRNA. There are no significant differences between the means ($P > 0.1$). (C) Relative abundance of C/EBP-β mRNA. There are no significant differences between the means ($P > 0.1$).

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