Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and hepatocyte nuclear factor 4 γ (HNF-4 γ) and HNF-4 α regulate the bovine growth hormone receptor 1A promoter through a common DNA element

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Abstract

The growth hormone receptor (GHR) 1A promoter is responsible for transcription of the liver-specific GHR mRNA variant 1A in several mammalian species. We previously found that the region between nucleotide –218 and nucleotide –151 (relative to the major transcription start site) of the bovine GHR 1A promoter contained a binding site between –196 and –178 for the liver-enriched transcription factor hepatocyte nuclear factor 4 α (HNF-4 α) and that the same region might also interact with additional transcription factors in the liver. Using the –218/–151 region as bait to screen a bovine liver cDNA library in a yeast one-hybrid analysis, we identified chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and HNF-4 γ , as well as HNF-4 α , as binding proteins to the –218/–151 region. These binding proteins were also identified in a second yeast one-hybrid analysis using the –196/–178 region as bait, suggesting that COUP-TFII, HNF-4 γ and HNF-4 α all bind to the –196/–178 region. Electrophoretic mobility shift assays confirmed the ability of these three transcription factors in bovine liver nuclear extracts to interact with the –196/–178 region. These interactions also appeared to exist *in vivo*, as the GHR 1A promoter containing the –196/–178 region could be recovered by immunoprecipitation of the bovine liver chromatin with antibody against COUP-TFII, HNF-4 γ or HNF-4 α . In co-transfection analyses, each of these three transcription factors could activate GHR 1A promoter and the activation was dependent on the –196/–178 region. These results together identify COUP-TFII, HNF-4 γ and HNF-4 α as transcription factors regulating GHR 1A promoter activity through binding to a common DNA element.

Journal of Molecular Endocrinology (2004) **32**, 947–961

Introduction

Growth hormone (GH) is essential for postnatal growth and metabolism in animals (Harvey *et al.* 1995). The GH actions are mediated by a specific cell surface receptor, the growth hormone receptor (GHR) (Kelly *et al.* 1991). Binding of GH to GHR triggers a signal transduction cascade involving Janus kinase 2 (JAK2) and signal transducer and activator of transcription proteins (STAT), leading to changes in gene expression, enzymatic activity and metabolite transport (Carter-Su *et al.* 1996). GHR is absolutely required for the actions of GH.

GHR knockout mice have much smaller body size than wild-type mice and are also impaired in several other physiological processes (Zhou *et al.* 1997, List *et al.* 2001). Defects in GHR sequence or deficiencies in GHR levels are associated with growth retardation in animals and humans (Rosenfeld *et al.* 1994, Ross 1999).

GHR is expressed throughout the body with the highest level in the liver (Kelly *et al.* 1991, Harvey *et al.* 1995). A major effect of binding of GH to GHR in the liver is increased expression of insulin-like growth factor-I (IGF-I), a critical growth factor for cell proliferation and differentiation (Jones &

Clemmons 1995, Stewart and Rotwein 1996, Liu & LeRoith 1999). In several species, GHR mRNA in the liver is composed of variants that differ in the 5'-untranslated region (5'-UTR), due to transcription from different leader exons by different promoters (Edens & Talamantes 1998). One variant named GHR 1A is expressed exclusively and is the predominant GHR mRNA variant in the liver of postnatal cattle (Lucy *et al.* 1998, Jiang & Lucy 2001b), sheep (Adams *et al.* 1990) and humans (Zou *et al.* 1997). Changes in GHR 1A expression are accompanied by similar changes in liver IGF-I expression and blood IGF-I concentrations as well as growth rate in cattle (Liu *et al.* 1999, Smith *et al.* 2002). Thus, understanding how GHR 1A expression is regulated should help understand how animal growth is controlled.

In a previous study (Jiang & Lucy 2001a), using DNase I footprinting analysis, we observed that bovine liver nuclear extracts were able to generate an extensive footprint in the region between nucleotide -218 and nucleotide -151 (relative to the major transcription start site designated +1) of the GHR 1A promoter (previously named GHR promoter 1 or GHR P1). The same region (-218/-151) also appeared to form multiple DNA-protein complexes with bovine liver nuclear protein extracts in electrophoretic mobility shift assays (EMSA). In the same study (Jiang & Lucy 2001a), using a candidate factor approach, we demonstrated that liver-enriched transcription factor hepatocyte nuclear factor 4 α (HNF-4 α) could bind to the -196/-178 region within the -218/-151 region. We hypothesized that the -218/-151 region interacts with additional transcription factors in the bovine liver. The objective of this study was, therefore, to identify these additional transcription factors.

Materials and methods

Tissue collection and RNA extraction

All bovine tissues used in this study were collected at slaughter. Total RNA was extracted using TRI reagent (MRC, Cincinnati, OH, USA). Poly(A) RNA was isolated using the Oligotex mRNA kit (Qiagen). RNA concentrations, integrity and purity were verified by spectrometry and gel-electrophoresis.

cDNA library construction

A bovine liver cDNA library was constructed in the pGAD424 vector (BD Biosciences, Palo Alto, CA, USA) using the SuperScript plasmid system for cDNA synthesis and plasmid cloning kit (Invitrogen). Briefly, 2 μ g liver poly(A) RNA pooled from six cattle were converted into cDNA using oligo(dT) as primers. The cDNA fragments larger than 1 kb were ligated into the SalI and NotI sites of pGAD424 that was engineered to contain additional cloning sites including NotI. The ligated vector-cDNA was electroporated into the *Escherichia coli* (*E. coli*) strain DH10B (Invitrogen). The library contained approximately 2×10^6 independent colony forming units, of which more than 90% contained inserts averaging 2.2 kb. The library was expanded in a semi-solid medium according to a protocol provided by the manufacturer (Invitrogen). Plasmid DNA from the library was prepared using a plasmid maxiprep kit (Qiagen).

Plasmid constructs preparation

DNA fragments containing three repeats of the -218/-151 or the -196/-178 region of the bovine GHR 1A promoter (Jiang & Lucy 2001a) were cloned into the EcoRI and XbaI sites of the reporter vector pHISi (BD Biosciences) to generate constructs pHISi-(GHR -218/-151)₃ or pHISi-(GHR -196/-178)₃ respectively. These two constructs were used as bait constructs in the yeast one-hybrid analysis.

A 4.5 kb bovine HNF-4 α (bHNF-4 α) cDNA insert and a 3.7 kb bHNF-4 γ cDNA insert, which both contained the complete coding regions, were excised from their respective pGAD424-cDNA clones at EcoRI and NotI sites and inserted into the pcDNA3.1(+) vector (Invitrogen) at the same sites to generate bHNF-4 α and bHNF-4 γ expression vectors pcDNA3-bHNF-4 α and pcDNA3-bHNF-4 γ respectively. A 1.5 kb bCOUP-TFII cDNA containing the entire coding region was obtained by a reverse transcription-PCR (RT-PCR) and inserted into the BamHI and NotI sites of pcDNA3.1(+) to generate bCOUP-TFII expression vector pcDNA3-bCOUP-TFII. A rat HNF-4 α (rHNF-4 α) expression vector pMT7-rHNF4 α 1 (kindly provided by Dr Frank Sladek, University of California at Riverside) and a mouse COUP-TFII (mCOUP-TFII) expression vector pCR3-mCOUP-TFII (kindly provided by Dr

Ming-Jer Tsai, Baylor College of Medicine) were also used in this study.

A 200 bp bHNF-4 γ cDNA and a 370 bp bCOUP-TFII cDNA were amplified from their respective pGAD424-cDNA plasmids using PCR with bHNF-4 γ -specific primers 5'-GGAGGATCCG GAATGAAAAAGAAGCTGT-3' and 5'-GAAG AATTCACAGACATCACCAAT-3', and bCOUP-TFII-specific primers 5'-GGAGGATCCGCAGC GTGCGGAGGAA-3' and 5'-GAAGAATTCAC TCGACGGCGCT-3' respectively. These two cDNA fragments were cloned into the pGEM-4Z vector (Promega) at the EcoRI and BamHI sites to generate constructs pGEM4Z-bHNF-4 γ 200 and pGEM4Z-bCOUP-TFII370 respectively. The plasmid pGEMTe-bHNF-4 α 311, which contains a 311 bp of bHNF-4 α cDNA in the pGEM-T Easy vector (Promega), was prepared in a previous study (Jiang & Lucy 2001a). The plasmid pGEMTe-28S130 containing a 130 bp bovine 28S ribosomal cDNA insert and the plasmid pGEMTe-bGAPDH90 containing a 90 bp bovine GAPDH cDNA insert were prepared by standard RT-PCR. These cDNA plasmids were used in the *in vitro* transcription reactions to synthesize riboprobes for the ribonuclease protection assay.

The construct pGL2-GHR(-469/+21) containing a 490 bp GHR 1A promoter upstream of a firefly luciferase reporter gene and the construct pGL2-GHR(-469/+21) Δ (-196/-178) containing a deletion in the -196/-178 region were prepared in a previous study (Jiang & Lucy 2001a). These promoter-reporter constructs were used in the transient transfection analysis.

The inserts in all plasmid constructs prepared in this study were verified by DNA sequencing. The protein expression constructs were also verified for their ability to make the expected proteins by *in vitro* transcription and *in vitro* translation in the presence of S³⁵-labeled methionine.

Yeast one-hybrid analysis

The yeast one-hybrid analysis was performed using the MATCHMAKER one-hybrid system (BD Biosciences). Briefly, the pHISi-(GHR -218/-151)₃ or pHISi-(GHR -196/-178)₃ construct was linearized with restriction enzyme XhoI and used to transform the yeast strain YM4721. The YM4721 transformants were selected for those that were able to grow on medium lacking histidine

(His⁻ medium) but were unable to grow on His⁻ medium supplemented with 30 mM 3-amino-1,2,4-triazole (3-AT). Two of such YM4721 transformants, designated YM4721-pHISi-(GHR -218/-151)₃ and YM4721-pHISi-(GHR -196/-178)₃, were selected to be transformed with 30 μ g pGAD424 plasmid containing bovine liver cDNA using the lithium acetate method, according to the manufacturer's instructions. The transformation efficiency was approximately 2×10^4 colony forming units/ μ g cDNA plasmid. The yeast transformants were plated onto synthetic dropout (SD) medium lacking histidine and leucine but containing 45 mM 3-AT (SD/-His/-Leu/+3-AT). After 5-7 days of growth at 30 °C, large yeast colonies were picked for plasmid DNA extraction according to the manufacturer's instructions. Plasmid DNA extracted from each yeast colony was amplified in DH10B bacteria and sequenced.

DNA sequencing and sequence analysis

Sequencing reactions were carried out using the ABI Prism BigDye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA) and vector- or insert-specific primers. The sequencing reactions were analyzed on an ABI 377 automated DNA sequencer (Applied Biosystems). Sequence analyses were performed using the Blast and ORF Finder programs at <http://www.ncbi.nlm.nih.gov/> and the ClustalW program at <http://www.ebi.ac.uk/>.

Electrophoretic mobility shift assay

Nuclear proteins from the bovine liver were extracted essentially as described previously (Widen & Wilson 1991). Approximately 1×10^5 c.p.m. of ³²P-labeled oligonucleotide probe was mixed with 10 μ g bovine liver nuclear extracts in a total volume of 20 μ l containing 10% glycerol, 10 mM Tris pH 7.5, 50 mM KCl, 0.5 mM dithiothreitol (DTT), 10 mM EDTA, 2 μ g poly d(A-T) and 2 μ g poly d(I-C). The mixture was incubated at 4 °C for 45 min and then added with 1 μ l of antiserum specific for HNF-4 α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HNF-4 γ (Santa Cruz Biotechnology), or COUP-TFII (kindly provided by Dr Ming-Jer Tsai) or a combination of them. The binding reactions were further incubated at 4 °C overnight. DNA-protein complexes were

resolved by electrophoresis on a native 5% polyacrylamide gel in a buffer containing 100 mM Tris pH 8.3, 100 mM boric acid, and 2 mM EDTA (i.e. 1 × TBE). Following electrophoresis, the gel was dried and analyzed by phosphorimaging or autoradiography.

Chromatin immunoprecipitation (ChIP) assay

Nuclei from bovine liver were isolated as described previously (Gorski *et al.* 1986) with modifications that the nuclei homogenization buffer was supplemented with additional proteinase inhibitors, including 1 mM benzamidine, 0.5 mM leupeptin, 1 µg/ml pepstatin and 5 µg/ml aprotinin (all from Sigma). Approximately 2×10^7 nuclei were crosslinked with 1% formaldehyde and lysed essentially as described previously (Shang *et al.* 2000). The nuclei were sonicated with ten pulses of 10 s each at power setting 37% with the sonic dismembrator Model 300 (Fisher Scientific, Pittsburg, PA, USA) to fragments 500 bp to 1500 bp. The fragmented chromatin was collected and diluted with four volumes of ChIP dilution buffer containing 0.01% SDS, 1.0% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl, 0.4 mM phenylmethylsulfonylfluoride (PMSF), 0.01 mg/ml aprotinin and 0.02 mg/ml leupeptin. The diluted chromatin fragments were pre-cleared by incubation with 80 µl protein A/G PLUS-agarose slurry (Santa Cruz Biotechnology) for 30 min at 4 °C with agitation and then divided into five aliquots. One aliquot was saved as 'input' in the subsequent DNA extraction and PCR analysis. The other four aliquots were each added with 5 µg antiserum against HNF-4α, HNF4γ or COUP-TFII, or 5 µg of goat or rabbit preimmune serum and incubated overnight at 4 °C with gentle rotation. Sixty microliters of protein A/G PLUS-agarose were added and the incubation continued for 2 h. Washing of the immune complexes to remove nonspecific antibody binding, elution of the immune complexes from the agarose beads, degradation of RNA and reversal of the crosslinking were all performed essentially as described previously (Shang *et al.* 2000).

DNA from each treatment was isolated as described in Shang *et al.* (2000). Purified DNA was resuspended in 50 µl water. Two microliters of each DNA were amplified by PCR for a 255 bp GHR 1A promoter region between nucleotide

–299 and nucleotide –45 (relative to the transcription start site +1), using primers 5'-ATTCTAATACGGGCCTTCTC-3' and 5'-CAAATCAATTACATTTTCTCTCTCT-3', under 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min. Two microliters of each DNA were also amplified by PCR for a 297 bp growth hormone releasing hormone (GHRH) promoter region with primers 5'-GACGCTGACAACGCTTAGG-3' and 5'-ATATTCCCGAGCCCTAACA-3', under 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min. The PCR products were resolved on agarose gels containing ethidium bromide.

Co-transfection analysis

MAC-T cells, a bovine epithelial cell line (Huynh *et al.* 1991) were cultured in 24-well plates and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂ for 24 h. Cells in each well were then transfected with 0.5 µg pGL2-GHR(–469/+21) or pGL2-GHR(–469/+21)Δ(–196/–178), 0.05 ng pRLCMV (Promega) and indicated amounts of pcDNA3-bHNF-4α, pcDNA3-bHNF-4γ and pcDNA3-bCOUP-TFII expression constructs using Eugene-6 (Roche). The total amount of DNA transfected into each well was made equivalent with appropriate amounts of pcDNA3.1 plasmid. Cells were harvested 48 h after transfection. The reporter gene activity was measured using the Dual luciferase reporter assay system (Promega), essentially according to the manufacturer's instructions.

Ribonuclease protection assay (RPA)

RPA was used to determine the expression of HNF-4γ, COUP-TFII and HNF-4α mRNA in bovine tissues. The ³²P-labeled antisense probes for the RPA were synthesized by *in vitro* transcription of appropriately linearized pGEM4Z-bHNF-4γ200, pGEM4Z-bCOUP-TFII370 and pGEMTe-bHNF-4α311 plasmids, as described previously (Jiang *et al.* 1999). The RPA were carried out on 20 µg total RNA, using the RPAs II kit (Ambion, Inc., Austin, TX, USA), according to the manufacturer's instructions with modifications described previously (Jiang *et al.* 1999). The levels of GAPDH mRNA or 28S rRNA in the same RNA sample were also

quantified to correct for technical variations in RPA. The ribonuclease-protected RNA fragments were resolved by electrophoresis on 6% polyacrylamide gels containing 7 M urea and analyzed by phosphorimaging. The densities of protected bands were quantified by using the Alpha Imager program (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

Each transfection analysis was repeated three times, each time in duplicate. The luciferase activity data from co-transfection of increasing amounts of expression plasmids were analyzed by two-factor ANOVA followed by the Tukey test for multiple comparisons of the mean luciferase activity for each construct at each amount of the expression plasmid. The luciferase activity data from co-transfection of fixed amounts of expression plasmids were analyzed by one-factor ANOVA followed by the Tukey test for multiple comparisons. All statistical analyses were performed using the respective programs of SAS (SAS Institute, Cary, NC, USA). All data are expressed as means $\pm$ S.E.M. Differences at $P < 0.05$ were considered significant.

Results

Binding of HNF-4 γ , COUP-TFII and HNF-4 α to the GHR -218/-151 region in yeast

To identify the liver proteins that can bind to the GHR -218/-151 region, a reporter construct pHISi-(GHR -218/-151)₃ containing three concatenated copies of the GHR -218/-151 region inserted upstream of the HIS3 reporter gene was used to screen a bovine liver cDNA library constructed in the pGAD424 vector. Forty-two positive colonies from approximately 6×10^5 yeast transformants were picked for plasmid isolation and amplification in *E. coli*. DNA sequencing of these plasmids revealed that four of them contained cDNA for HNF-4 α , one for HNF-4 γ and one for COUP-TFII, which are all known as transcription factors. None of the remaining colonies appeared to contain cDNA encoding transcription factors and the majority of them contained cDNA for abundant liver proteins, such as albumin,

fibrinogen and transferrin, which do not appear to have specific DNA-binding ability.

Binding of HNF-4 γ , COUP-TFII and HNF-4 α to the GHR -196/-178 region in yeast

HNF-4 α , HNF-4 γ and COUP-TFII all belong to the nuclear receptor superfamily of transcription factors. They and some other nuclear receptors including the retinoic acid receptor (RAR) and retinoid X receptor (RXR) have been shown to bind to DNA regions containing a direct repeat of AGGTCA separated by one nucleotide (DR1) (Tsai & Tsai 1997, Sladek & Seidel 2001). We therefore hypothesized that the identification of HNF-4 α , HNF-4 γ and COUP-TFII in the above yeast one-hybrid analysis was indeed due to the presence of a DR1-like site in the -196/-178 region (CCCTGGGCAAAGGTCGGGG, the DR1-like sequence is underlined). To test this hypothesis and also to identify potential additional nuclear receptors (i.e. RAR or RXR) binding to the GHR -196/-178 region, a second yeast one-hybrid analysis was done using pHISi-(GHR -196/-178)₃ as a bait construct to screen the bovine liver cDNA library. A total of 46 positive colonies from this screening were picked for further analyses. Four, one and four of these colonies were found to contain cDNA for bovine HNF-4 α , HNF-4 γ and COUP-TFII respectively. None of the rest appeared to encode RAR, RXR or other nuclear receptors, or other transcription factors.

The specificity of the interaction of HNF-4 α , HNF-4 γ and COUP-TFII with the GHR -196/-178 region was further verified by re-transforming yeast YM4721-pHISi-(GHR -196/-178)₃ with pGAD424 plasmid containing cDNA encoding bovine HNF-4 α , HNF-4 γ or COUP-TFII. As shown in Fig. 1, yeast YM4721-pHISi-GHR(-196/-178)₃ was able to grow on SD/-Leu/-His/+3-AT-selective medium when transformed with a pGAD424 plasmid expressing bHNF-4 α , bHNF-4 γ or bCOUP-TFII protein, whereas the same yeast could not grow on the same selective medium when transformed with a pGAD424 plasmid encoding an unrelated protein named p53. Conversely, yeast YM4721-pHISi-p53, which did not contain the GHR -196/-178 DNA, was unable to grow on the selective medium when transformed with the pGAD424 plasmid

Figure 1 Specific activation of HIS3 reporter gene transcription by HNF-4α, HNF-4γ and COUP-TFII through binding to the GHR 1A promoter region –196/–178 in yeast YM4271. Yeast colonies carrying either *pHISi-(p53)₃* or *pHISi-(GHR –196/–178)₃* reporter construct were transformed with pGAD424-cDNA plasmids encoding GAL4-p53, GAL4-bHNF-4α, GAL4-bHNF-4γ, or GAL4-bCOUP-TFII fusion proteins and were streaked onto SD/–Leu medium or SD/–His/–Leu/+3-AT medium. Only yeast transformants containing the right combination of DNA-binding site and DNA-binding protein were able to grow on the SD/–His/–Leu/+3-AT medium.

encoding HNF-4α, HNF-4γ or COUP-TFII (Fig. 1).

cDNA sequence and deduced amino acid sequence of the bovine HNF-4α, HNF-4γ and COUP-TFII

The longest bHNF-4α, bHNF-4γ and bCOUP-TFII cDNA obtained from the yeast one-hybrid analyses were completely sequenced. The sequenced bHNF-4α cDNA was 4489 bp (not including the poly(A) tail), contained the complete coding sequence and appeared to equal the size of the full-length bHNF-4α mRNA on Northern blot analysis (data not shown). It shared 93 and 90% identity in the coding sequence with the human HNF-4α cDNA (GenBank accession: X87870, 2288 bp; X76930, 1441 bp; NM_000457,

2289 bp) and the rat HNF-4α cDNA (NM_022180, 1446 bp; X57133, 1758 bp) respectively (these human and rat HNF-4α cDNA sequences appear to lack most of the 3'-UTR). The deduced bovine HNF-4α amino acid sequence was 455 amino acids (a.a.) in length (Fig. 2) and shared 97 and 96% identity with the human (GenBank accession: CAA61133, 455 a.a.) and the rat (GenBank accession: A36471, 455 a.a.) HNF-4α amino acid sequences respectively.

The sequenced bHNF-4γ cDNA was 3737 bp (not including the poly(A) tail), contained the complete coding sequence and appeared to be full-length based on Northern blot analysis (data not shown). The bHNF-4γ cDNA sequence was 90 and 85% identical in the coding sequence to the human (GenBank accession: NM_004133, 1731 bp) and the mouse (GenBank accession:

Figure 2 Amino acid sequence alignment of bHNF-4 α , bHNF-4 γ and bCOUP-TFII proteins. The bHNF-4 α and bHNF-4 γ (indicated as bHNF-4 alpha and bHNF-4 gamma in the Figure) amino acid sequences are deduced from their respective cDNA clones obtained from the yeast one-hybrid analysis. The bCOUP-TFII amino acid sequence is adopted from GenBank accession number CAB55624. Identical amino acids between the three sequences are shaded. The locations of functional domains of the transcription factors are indicated with <> above the sequences. The cysteine residues involved in DNA-binding are indicated with an asterisk below the residues. The nucleotide sequences of bHNF-4 α , bHNF-4 γ and COUP-TFII cDNA have been deposited in GenBank under access numbers AY318752, AY318753 and AY318754 respectively.

NM_013920, 1706 bp) HNF-4 γ cDNA sequences respectively. The deduced bovine HNF-4 γ amino acid sequence was 425 amino acids in length (Fig. 2) and shared 93% and 92 identity with the human (GenBank accession: NP_004124, 408 a.a.)

and the mouse (GenBank accession: NP_038949, 418 a.a.; Q9 QUU6, 408 a.a.) HNF-4 γ amino acid sequences respectively.

The longest bCOUP-TFII cDNA obtained in the yeast one-hybrid analyses was 2246 bp.

Aligning this sequence with the known bovine (GenBank accession: AJ249441, 1289 bp), the human (GenBank accession: BC042897, 3049 bp) and the mouse (GenBank accession: NM_009697, 2495 bp) COUP-TFII cDNA sequences indicated that the bovine COUP-TFII sequence cloned in this study was truncated at the 5' end, perhaps due to incomplete reverse transcription during the cDNA synthesis. The COUP-TFII cDNA sequence determined in this study differed from the bovine COUP-TFII cDNA in GenBank (GenBank accession: AJ249441, 1289 bp) by two nucleotides and was 95 and 93% identical to the human and mouse COUP-TFII cDNA sequences respectively. The bovine COUP-TFII cDNA cloned in this study contained 271 amino acids of the 414-amino-acid full-length bCOUP-TFII (GenBank accession: CAB55624) and shared 99% identity with the corresponding portions of the human and mouse COUP-TFII amino acid sequences (GenBank accession: AAH42897, 414 a.a. and NP_033827, 414 a.a. respectively).

Aligning the amino acid sequences of bHNF-4 α , bHNF-4 γ and bCOUP-TFII revealed that the bHNF-4 α amino acid sequence shared an overall 72% identity with bHNF-4 γ amino acid sequence, with a greater similarity (81%) being in the DNA-binding domain (C domain), the hinge region (D domain) and the ligand-binding domain (E domain) (Fig. 2). The bHNF-4 α and bHNF-4 γ amino acid sequences shared 37 and 35% identical residues respectively, with the bCOUP-TFII amino acid sequence (Fig. 2). The amino acid sequences of the three transcription factors were less similar in the A/B domain and F domain, which are believed to exert activation function (Tsai & Tsai 1997, Sladek & Seidel 2001), than in other domains (Fig. 2). In addition, bHNF-4 γ had a truncated A/B domain and bCOUP-TFII had an extended A/B domain but a truncated F domain compared with bHNF-4 α (Fig. 2).

Distribution patterns of HNF-4 α , HNF-4 γ and COUP-TFII mRNA in bovine tissues

The expression of HNF-4 α , HNF-4 γ and COUP-TFII mRNA in a variety of adult bovine tissues, including the adrenal gland, brain (cerebral cortex), fat, heart, kidney, liver, lung, skeletal muscle, pituitary gland, rumen, small intestine and spleen, was quantified by RPA (Fig. 3). HNF-4 γ mRNA

Figure 3 Ribonuclease protection assays (RPAs) of HNF-4 α , HNF-4 γ and COUP-TFII mRNA in bovine tissues. Bands corresponding to HNF-4 α , HNF-4 γ , COUP-TFII and 28S rRNA (loading control) are indicated with arrows. tRNA was included in the assays as a negative control.

was found in the kidney, liver and small intestine, with higher levels in the liver than in the other two tissues (Fig. 3). COUP-TFII mRNA was present in all tissues examined, with higher levels in the adrenal gland, kidney, liver and lung than in other tissues (Fig. 3). HNF-4 α mRNA was expressed at high levels in the liver and kidney, at moderate levels in the small intestine, and at low levels in the skeletal muscle, spleen, fat and lung (Fig. 3). A densitometric analysis revealed that in the liver, the levels of HNF-4 α mRNA were about five times higher than that of HNF-4 γ mRNA and ten times higher than that of COUP-TFII mRNA (Fig. 3).

Binding of HNF-4 α , HNF-4 γ and COUP-TFII in bovine liver nuclear protein extracts to the GHR -196/-178 region

The yeast one-hybrid analyses indicated that HNF-4 α , HNF-4 γ and COUP-TFII were able to interact with the GHR -196/-178 region in yeast; we next determined whether HNF-4 α , HNF-4 γ and COUP-TFII proteins expressed in the bovine liver were also able to bind to the GHR -196/-178 region using an EMSA (Fig. 4). An oligonucleotide probe corresponding to the GHR -196/-178 region formed four major DNA-protein complexes with bovine liver nuclear protein extracts (Fig. 4). Three DNA-protein complexes (C1, C2 and C3 in Fig. 4) were super-shifted by

Figure 4 Electrophoretic mobility shift assay (EMSA) of binding of HNF-4 α , HNF-4 γ and COUP-TFII from bovine liver nuclear protein extracts to the GHR -196/-178 oligonucleotide. Incubation of bovine liver nuclear extracts with an oligonucleotide probe corresponding to the GHR -196/-178 region generated four major DNA-protein complexes, C1, C2, C3 and C4 that are indicated with arrows. Addition of antibody against HNF-4 α , HNF-4 γ or COUP-TFII or addition of preimmune serum (+) had differential effects or no effects on the mobility of different DNA-protein complexes. S, super-shifted DNA-protein complexes; F, free probe.

HNF-4 γ antibody, COUP-TFII antibody, HNF-4 α antibody or a combination of them, and were not affected by addition of goat preimmune serum (Fig. 4), rabbit preimmune serum, or antibody against a transcription factor named Sp3 or the antibody against the firefly luciferase (data not shown), demonstrating the presence of HNF-4 γ , COUP-TFII and HNF-4 α in these three DNA-protein complexes. The mobility of the fourth DNA-protein complex (C4 in Fig. 4) was not affected by any of these antibodies, suggesting that this complex was formed by proteins not immunoreactive with HNF-4 α , HNF-4 γ or COUP-TFII antibody.

Figure 5 Immunoprecipitation of the GHR 1A promoter-containing chromatin from bovine liver nuclei with HNF-4 α , HNF-4 γ and COUP-TFII antibodies. Nuclei from bovine liver were crosslinked with formaldehyde and immunoprecipitated with the antibody against HNF-4 α , HNF-4 γ or COUP-TFII, or with preimmune serum. Genomic DNA from the nuclei treated with antibody or preimmune serum and the nuclei not treated with antibody (input) were isolated and amplified by PCR for a 255 bp GHR 1A promoter region and a 297 bp GHRH promoter region using GHR- and GHRH-specific primers. PCR products were resolved on agarose gels containing ethidium bromide. 'Water' indicates negative controls for PCR.

Binding of HNF-4 α , HNF-4 γ and COUP-TFII to the GHR 1A promoter in the context of chromatin

HNF-4 α , HNF-4 γ and COUP-TFII can interact with the GHR -196/-178 region in both the yeast one-hybrid analysis and the EMSA using bovine liver nuclear protein extracts. We next determined whether the GHR 1A promoter in the context of chromatin is bound by these three proteins using a ChIP assay. Immunoprecipitation of the bovine liver chromatin with HNF-4 α , HNF-4 γ or COUP-TFII antibody each resulted in the recovery of a GHR 1A promoter region containing the -196/-178 region, whereas preimmune serum did not precipitate the same promoter region (Fig. 5). Meanwhile, immunoprecipitation of bovine liver chromatin with HNF-4 α , HNF-4 γ or COUP-TFII antibody did not precipitate the promoter of GHRH gene (Fig. 5), which normally is not active in the liver and does not appear to contain a consensus binding site for HNF-4 α , HNF-4 γ or COUP-TFII. These results together suggest that the GHR 1A promoter containing the -196/-178 region was bound by HNF-4 α , HNF-4 γ and COUP-TFII in the liver.

Transactivation of the GHR 1A promoter by HNF-4 α , HNF-4 γ and COUP-TFII via the -196/-178 region in MAC-T cells

To determine the effect of binding of HNF-4 α , HNF-4 γ or COUP-TFII to the GHR -196/-178 region on the activity of the GHR 1A promoter, we co-transfected the bovine HNF-4 α , HNF-4 γ or COUP-TFII expression plasmid with the GHR 1A promoter-luciferase construct into MAC-T cells. Co-transfection of the bHNF-4 α , bHNF-4 γ or bCOUP-TFII expression plasmid significantly increased the activity of the GHR 1A promoter ($P < 0.05$) (Fig. 6A, B and C). Co-transfection of the same expression plasmids had no effect on the promoterless plasmid pGL2B (Fig. 6A, B and C), demonstrating the specificity of the effect of HNF-4 α , HNF-4 γ and COUP-TFII on the GHR 1A promoter. The transactivation of the GHR 1A promoter by HNF-4 α or HNF-4 γ was completely blocked (Fig. 6A and B) and the transactivation of the GHR 1A promoter by COUP-TFII was nearly completely blocked (Fig. 6C) by deletion of the -196/-178 region from the GHR 1A promoter, demonstrating that the effect of HNF-4 α , HNF-4 γ and COUP-TFII on the GHR 1A promoter was dependent on the -196/-178 region. Interestingly, the -196/-178 region-deleted GHR 1A promoter retained some response to COUP-TFII (Fig. 6C), suggesting that COUP-TFII could also enhance GHR 1A promoter activity independent of the -196/-178 region. Similar activation of the GHR 1A promoter was also observed in co-transfection analyses using a rat HNF-4 α expression vector (pMT7-rHNF4 α 1) or a mouse COUP-TFII expression vector (pCR3-mCOUP-TFII), or using human hepatoma-derived HepG2 cells and Chinese hamster ovary-derived CHO cells, or using a promoter-reporter construct containing a longer (2.7 kb) GHR 1A promoter (data not shown).

Co-transfection of the COUP-TFII expression plasmid appeared to increase GHR 1A promoter activity by as much as 50-fold (Fig. 6C), whereas co-transfection of the HNF-4 α and HNF-4 γ expression plasmids increased GHR 1A promoter activity by a maximum of 4-fold (Fig. 6A and B). (Transfection of 160 or 320 ng of the HNF-4 γ expression plasmid did not increase GHR 1A promoter activity further.) These differences indicated that either COUP-TFII had a greater

activation potential than HNF-4 α or HNF-4 γ on the GHR 1A promoter, or COUP-TFII mRNA was translated more efficiently than HNF-4 α and HNF-4 γ mRNAs in MAC-T cells.

The untransfected MAC-T cells do not express HNF-4 α , HNF-4 γ or COUP-TFII mRNA (data not shown). Transfection of HNF-4 α , HNF-4 γ or COUP-TFII expression plasmid alone was able to increase the activity of the co-transfected GHR 1A promoter, suggesting that HNF-4 α , HNF-4 γ or COUP-TFII do not depend on each other to activate the GHR 1A promoter in MAC-T cells. Given the fact that these three transcription factors are co-expressed in the liver (Fig. 3), we tested the possible cooperation between these three transcription factors in regulating GHR 1A promoter activity in MAC-T cells. As can be seen in Fig. 6D, the increase in GHR 1A promoter activity caused by transfection of the HNF-4 α and HNF-4 γ expression plasmids together was more than the sum of the increases caused by transfection of them alone, suggesting that HNF-4 α and HNF-4 γ may activate the GHR 1A promoter in a cooperative manner. The increase in GHR 1A promoter activity caused by transfection of the HNF-4 α and COUP-TFII plasmids together, or the HNF-4 γ and COUP-TFII expression plasmids together, was not greater than the sum of the increases in GHR 1A promoter activity resulting from transfection of them alone (Fig. 6D), suggesting that HNF-4 α and COUP-TFII, or HNF-4 γ and COUP-TFII may not cooperate in enhancing GHR 1A promoter activity. Alternatively, the relative amounts of expression plasmids or other conditions used in this experiment were not appropriate for cooperation between HNF-4 α and COUP-TFII or cooperation between HNF-4 γ and COUP-TFII.

Discussion

In a previous study of ours (Jiang & Lucy 2001a), the -218/-151 region of the liver-specific GHR 1A promoter appeared to interact with proteins in addition to HNF-4 α in the bovine liver nuclear extracts. This prompted us to conduct the present study to identify these additional binding proteins. Using a yeast one-hybrid analysis, we identified HNF-4 γ and COUP-TFII as well as HNF-4 α as potential binding proteins to the -218/-151 region. These three transcription factors all belong

Figure 6 Enhancement of GHR 1A promoter activity by overexpressed HNF-4 α , HNF-4 γ and COUP-TFII in MAC-T cells. (A)–(C) Co-transfection of increasing amounts of bHNF-4 α , bHNF-4 γ or bCOUP-TFII expression vector into MAC-T cells with the –196/–178 region-containing GHR 1A promoter–reporter construct pGL2-GHR(–469/+21), the –196/–178 region-deleted GHR 1A promoter–reporter construct pGL2-GHR(–469/+21)Δ(–196/–178), or the promoter-less construct pGL2B. (D) Co-transfection of 20 ng bHNF-4 α , 4 ng bHNF-4 γ or 2 ng bCOUP-TFII expression vector or a combination of them with pGL2-GHR(–469/+21) into MAC-T cells. The luciferase activity under each treatment is expressed as fold increase over the luciferase activity (normalized to 1) of pGL2-GHR(–469/+21) in the absence of expression vector. Values are expressed as means $\pm$ S.E.M. from three independent transfection analyses. The different letters in (A), (B) and (C) indicate significant statistical differences ($P < 0.05$) at the same amount of co-transfected expression plasmid. The different letters in (D) indicate significant statistical differences ($P < 0.05$) between groups.

to the nuclear receptor superfamily of transcription factors that have been shown to recognize DNA regions containing a direct repeat of AGGTCA separated by one adenosine named DR1 (Tsai & Tsai 1997, Burris 2001, Sladek & Seidel 2001). The –218/–151 region contains a DR1-like region

between nucleotide –196 and nucleotide –178 (the –196/–178 region), which was previously shown to interact with HNF-4 α (Jiang & Lucy 2001a). These results further prompted us to ask whether COUP-TFII and HNF-4 γ bind to the same –196/–178 region as HNF-4 α . We found that

the GHR – 196/ – 178 region was able to interact with these three transcription factors in yeast and was also able to interact with these three transcription factors in bovine liver nuclear extracts.

In addition to being recognized by COUP-TFII, HNF-4 γ and HNF-4 α , the DR1 is also the core of the response elements for some other members of the nuclear receptor superfamily of transcription factors, including the RAR, the RXR and the peroxisome proliferator-activated receptor (PPAR) (Burris 2001). Several DR1-containing sequences have been reported to be recognized by multiple nuclear receptors. A DR1-containing region in the human apolipoprotein CIII promoter binds COUP-TFI, COUP-TFII and HNF-4 α (Mietus-Snyder *et al.* 1992); a DR1-containing region in the rat ornithine transcarbamylase promoter binds COUP-TFI and HNF-4 α (Kimura *et al.* 1993); a DR1-containing region in the rat cholesterol 7 α -hydroxylase promoter binds COUP-TFII and RXR (Stroup *et al.* 1997); a DR1-containing region in the rat retinol-binding protein II promoter binds RXR, COUP-TFII and HNF-4 α (Nakshatri & Chambon 1994); and a DR1-containing region in the human dihydrodiol dehydrogenase 4/aldo-keto reductase 1C4 gene binds HNF-4 α and HNF-4 γ (Ozeki *et al.* 2001). The results from this study add the GHR – 196/ – 178 region to this list as the first example of a DR1-containing DNA region being recognized by HNF-4 α , HNF-4 γ and COUP-TFII. Interestingly, none of these DR1-containing regions, including the GHR – 196/ – 178 region, have been reported to bind to the same set of nuclear receptor transcription factors. Perhaps the specificity of nuclear receptor transcription factors binding to these DR1-containing regions is determined by the varying nucleotides surrounding the DR1 core.

Nuclear receptor transcription factors usually bind to their response elements as dimers (Burris 2001). It has been believed that HNF-4 α binds to its response elements exclusively as homodimers (Jiang *et al.* 1995, Jiang & Sladek 1997) and that COUP-TFII binds to its response elements preferentially as homodimers (Butler & Parker 1995). In this study, we did not intend to determine whether HNF-4 α , HNF-4 γ and COUP-TFII bind to the GHR – 196/ – 178 as homodimer or heterodimers, but the result that one of the DNA–protein complexes (C1 in Fig. 4) in the

gel-shift assay could be super-shifted nearly completely by either anti-HNF-4 γ or anti-COUP-TFII antibody suggests that HNF-4 γ and COUP-TFII may bind to the GHR – 196/ – 178 region as heterodimers between them. Although the potential heterodimeric formation between HNF-4 γ and COUP-TFII in binding to the GHR – 196/ – 178 region needs to be verified with techniques that are more appropriate for analyzing protein–protein interactions, such as immunoprecipitation, the presence of HNF-4 γ and COUP-TFII heterodimers should not be totally surprising, for COUP-TFII has been reported to form heterodimers with other nuclear receptors including RXR, RAR and thyroid hormone receptor (TR) (Berrodin *et al.* 1992, Cooney *et al.* 1992, 1993) and HNF-4 γ has been shown to form heterodimers with HNF-4 α in binding to a DR1 region of the human dihydrodiol dehydrogenase 4/aldo-keto reductase 1C4 gene (Ozeki *et al.* 2001).

The ChIP experiment in this study indicated that the GHR 1A promoter in bovine liver was bound by COUP-TFII and HNF-4 γ as well as HNF-4 α , suggesting that all of these three transcription factors may play a role in GHR 1A transcription *in vivo*. When co-transfected with a GHR 1A promoter–reporter construct into MAC-T cells in culture, each of the transcription factors was able to significantly enhance the activity of the GHR 1A promoter in a dosage- and sequence-dependent manner, suggesting a positive role for COUP-TFII, HNF-4 γ and HNF-4 α in controlling the GHR 1A promoter *in vivo*. The positive effect of HNF-4 α on the GHR 1A promoter is highly expected, as HNF-4 α has always been found to transactivate its target genes (Sladek & Seidel 2001). Compared with numerous studies on the function of HNF-4 α , there has been only one reported study on the function of HNF-4 γ , in which HNF-4 γ was found to activate gene transcription when binding to a DNA element originally identified for HNF-4 α (Drewes *et al.* 1996). Drewes *et al.* (1996) also proposed that HNF-4 γ plays a differential role in controlling gene transcription from HNF-4 α , based on their finding that HNF-4 γ mRNA was not expressed in the liver where HNF-4 α was highly expressed. In this study, we found that HNF-4 γ mRNA shared the same tissue-distribution pattern as HNF-4 α mRNA, being expressed at high levels in the liver, kidney and small intestine and expressed at low levels or absent in other tissues.

This observation, as well as the fact that HNF-4 α and HNF-4 γ share a remarkable sequence similarity in the DNA-binding and ligand-binding domains, suggest to us that both HNF-4 γ and HNF-4 α may activate the expression of the same set of genes in the liver, kidney or small intestine. A functional difference between HNF-4 γ and HNF-4 α might be in their transactivation potential, as they differ significantly in the sequence of the A/B and F domains, which are believed to be responsible for activation function (Sladek & Seidel 2001).

Unlike HNF-4 α , which is a transcriptional activator, COUP-TFII can be either a transcriptional activator or inhibitor (Tsai & Tsai 1997). COUP-TFII has been found to repress the transcription of many genes through several different mechanisms, including competition with other transactivating nuclear receptors such as HNF-4 α for common binding sites or for heterodimeric partners such as RXR (Tsai & Tsai 1997). COUP-TFII has also been reported to activate the transcription of several genes, including the human apolipoprotein AI gene (Malik & Karathanasis 1995) and the rat cholesterol 7 α -hydroxylase gene (Stroup *et al.* 1997) through direct binding to DR1 elements, the mouse vHNF-1 (HNF-1 β) gene (Power & Cereghini 1996), the rat HNF-1 (HNF-1 α) promoter (Ktistaki & Talianidis 1997) and the mouse mammary tumor virus promoter (Kadowaki *et al.* 1995) through DR1 element-independent mechanisms. In this study, we found that COUP-TFII activated the GHR 1A promoter through the DR1-like site in transfected cells, thus adding the GHR gene to the short list of genes whose expression can be activated by COUP-TFII through binding to the DR1 element. Malik & Karathanasis (1995) reported that COUP-TFII possesses intrinsic transcription activation potential through binding to the basal transcription factor TFIIB, but this activation potential depends on physiological stimuli and cell and promoter contents. In this study, we found that the strong, DR1-dependent transactivation of the GHR 1A promoter by COUP-TFII could occur in different cell types (the transfection data in CHO and HepG2 cells were not shown), in the presence or absence of other nuclear receptors (i.e. HNF-4 α and HNF-4 γ), and with both bovine COUP-TFII and rat COUP-TFII (the transfection data using rat COUP-TFII were not shown). These observations

suggest that the DR1 site in the GHR 1A promoter and/or the GHR 1A promoter context may be unique in bringing out the activation potential of COUP-TFII.

In summary, the data from this study clearly indicate that not only HNF-4 α but also HNF-4 γ and COUP-TFII can bind to the $-196/-178$ region of the bovine GHR 1A promoter. The data also indicate that although each of these three transcription factors can activate the expression from the GHR 1A promoter, the degree of activation varies among them and depends on their expression levels as well, suggesting that changes in the relative amounts or activity of these three transcription factors could be a mechanism for fine-tuning the expression from the GHR 1A promoter in the liver. The expression of GHR 1A mRNA in cattle has been shown to be up-regulated in the postnatal period (Lucy *et al.* 1998), down-regulated during the peri-parturient period (Kobayashi *et al.* 1999a), and regulated by the levels of nutrition (Smith *et al.* 2002, Wang *et al.* 2003) as well as the levels of GH (Kobayashi *et al.* 1999b). It will be interesting to see if the expression levels and/or activity of HNF-4 α , HNF-4 γ and COUP-TFII correlate with the levels of GHR 1A mRNA under these conditions. The $-196/-178$ element of the bovine GHR 1A promoter is conserved in the human and ovine GHR 1A promoter (Jiang & Lucy 2001a); it will be also interesting to see if this element in the human and ovine GHR 1A promoters can also interact with HNF-4 α , HNF-4 γ and COUP-TFII.

Acknowledgements

We thank Dr Ming-Jer Tsai for providing COUP-TFII antibody and expression vector, Dr Frank Sladek for providing HNF-4 α antibody and expression vector, Dr Eric Wong for critical reading of the manuscript, and Lynn Springer and Julia Pugh for excellent technical assistance.

Funding

The work was supported by National Research Initiative Competitive Grant USDA CSREES 2001–35205–11732 (to H.J).

There is no conflict of interest that would prejudice the impartiality of this research.

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Received in final form 11 March 2004

Accepted 19 March 2004

Made available online as an

Accepted Preprint 29 March 2004