



**HAL**  
open science

## The farnesoid X receptor induces fetuin-B gene expression in human hepatocytes

Takeshi Murakami, Robert Walczak, Sandrine Caron, Christian Duhem,  
Vincent Vidal, Raphaël Darteil, Bart Staels

► **To cite this version:**

Takeshi Murakami, Robert Walczak, Sandrine Caron, Christian Duhem, Vincent Vidal, et al.. The farnesoid X receptor induces fetuin-B gene expression in human hepatocytes. *Biochemical Journal*, 2007, 407 (3), pp.461-469. 10.1042/BJ20070658 . hal-00478806

**HAL Id: hal-00478806**

**<https://hal.science/hal-00478806>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**The farnesoid X receptor induces fetuin-B gene expression in human hepatocytes**

**Takeshi Murakami\***, **Robert Walczak†**, **Sandrine Caron‡§¶**, **Christian Duhem‡§¶**, **Vincent Vidal†**, **Raphaël Darteil†**, and **Bart Staels‡§¶1**

\*Tokyo New Research Laboratories I, Pharmaceutical Division, Kowa Company Ltd., 2-17-43  
Noguchicho, Higashimurayama, Tokyo, Japan

†GENFIT, Parc Eurasanté, Loos, France

‡Institut Pasteur de Lille, Lille, F-59019 France

§Inserm U545, Lille, F-59019 France

¶Université de Lille 2, Faculté de Pharmacie et Faculté de Médecine, Lille, F-59006 France

## ABSTRACT

Farnesoid X receptor (FXR), a nuclear receptor activated by bile acids, is a key factor in the regulation of bile acid, lipid and carbohydrate metabolism. The recent development of synthetic FXR agonists and knock-out mouse models has accelerated the discovery of FXR target genes. In this study we identify human Fetuin-B as a novel FXR target gene. Treatment with FXR agonists increased Fetuin-B expression in human primary hepatocytes and in the human hepatoma HepG2 cell line. In contrast, Fetuin-B expression was not responsive to FXR agonist treatment in murine primary hepatocytes. Fetuin-B induction by FXR agonist was abolished upon FXR knockdown by siRNA. In addition to the previously described P1 promoter, we show that the human Fetuin-B gene is also transcribed from an alternative promoter, termed P2. Transcription via the P2 promoter was induced by FXR agonist treatment, whereas P1 promoter activity was not sensitive to FXR agonist treatment. Two putative FXR response elements (IR-1) were identified in the region -1.6kb upstream of the predicted P2 transcriptional start site. Both motifs bound FXR/RXR complexes *in vitro* and were activated by FXR in transient transfection reporter assays. Mutations in the IR-1 sites abolished FXR/RXR binding and activation. Taken together, these results identify human Fetuin-B as a new FXR target gene in human hepatocytes.

Key word : farnesoid X receptor, fetuin-B, cystatin superfamily, alternative promoter, anti-cancer

Abbreviations : FXR, farnesoid X receptor ; RXR, retinoid X receptor ; BAs, bile acids ; CDCA, chenodeoxycholic acid ; IR, inverted repeat ; Cyp7A1, cholesterol-7 $\alpha$ -hydroxylase ; SHP, small heterodimer partner ; I-BABP, intestinal bile acid-binding protein ; BSEP, bile salt export pump ; NTCP, Na<sup>+</sup>-taurocholate cotransporting polypeptide ; apo, apolipoprotein ; PLTP, phospholipid transfer protein expression ; PPAR $\alpha$ , peroxisome proliferator-activated receptor  $\alpha$  ; ahsg, 2-Heremans and Schmid glycoprotein ; BCP, basic calcium phosphate ; FXRE, FXR binding element ; siRNA, small interfering RNA

1 To whom correspondence should be addressed : Tel: 33-320-87-73-88; Fax: 33-320-87-71-98; E-mail: bart.staels@pasteur-lille.fr.

## INTRODUCTION

The farnesoid X receptor (FXR, NH1H4) is a member of the nuclear receptor family that is expressed at highest levels in the liver, kidney, intestine and the adrenal gland [1,2]. FXR is activated by bile acids (BAs), such as chenodeoxycholic acid (CDCA) [3], or by synthetic FXR agonists, such as GW4064 [4]. In most cases, FXR binds to inverted repeat elements on DNA (IR-1) as a heterodimer with the retinoid X receptor (RXR). FXR regulates the expression of a variety of genes related to bile acid, cholesterol, triglyceride and carbohydrate metabolism. For example, FXR negatively regulates bile acid synthesis by decreasing the expression of cholesterol-7 $\alpha$ -hydroxylase (Cyp7A1), the rate limiting enzyme in the pathway of BA synthesis [5,6] through the induction of another nuclear receptor, SHP. Enterohepatic circulation of bile acids is also controlled by FXR, which modulates the expression of genes such as the intestinal bile acid-binding protein (I-BABP), bile salt export pump (BSEP) and Na<sup>+</sup>-taurocholate cotransporting polypeptide (NTCP)[7-9]. FXR is also an important factor in the control of cholesterol metabolism through the inhibition of apolipoprotein (apo) -AI expression and through the induction of phospholipid transfer protein expression (PLTP) [10,11]. Similarly, the expression of factors involved in the control of plasma triglyceride levels, such as apo-CII, apo-CIII and peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), is under the control of FXR [12-14]. As a consequence, FXR-deficient mice show increased liver and serum triglyceride levels [15]. It has also been postulated that the lipogenic response is negatively regulated by FXR [16,18]. Several recent reports have demonstrated that FXR activity is essential to maintain normal glucose homeostasis [19-21]. The role of FXR in the negative regulation of both hepatic glucose production and lipogenesis has been postulated [17,18]. Finally, *fxr* gene ablation in mice results in an impaired insulin sensitivity [19-21]. These reports suggest that FXR is a critical factor involved in lipid and carbohydrate homeostasis and a potential clinical target to treat the metabolic syndrome

Fetuin-A and fetuin-B are members of the fetuin family. Human fetuin-B (382 aa) shares 22% sequence similarity with fetuin-A. Also known as  $\alpha$ 2-Heremans and Schmid glycoprotein (Ahsg), fetuin-A is a major globular protein in foetal serum and is a member of the cystatin superfamily of cysteine protease inhibitors. Fetuin-A is the only known protein inhibitor of ectopic calcification that is systemic [22]. Due to a high affinity for hydroxyapatite, fetuin-A accumulates in the skeleton, where it inhibits the formation and precipitation of the apatite precursor mineral, basic calcium phosphate (BCP)[23]. In addition, Fetuin-A has been implicated in several processes related to osteogenesis, bone resorption and inhibition of vascular calcification [22, 24]. In addition, it has been reported that fetuin-A acts as a negative regulator of insulin receptor tyrosine kinase activity [25]. Fetuin-B is a paralogue of fetuin-A and functional analysis revealed that fetuin-B is able to act as calcium phosphate precipitation inhibitor albeit with lower affinity [26,27]. Since fetuin-B concentration in human serum tends to be low, a major quantitatively systemic contribution to calcium phosphate precipitate removal is rather unlikely. Other authors have reported that fetuin-B expression is upregulated in certain primary skin tumors and in a subset of human cancer cell lines [28]. Indeed, overexpression of fetuin-B in skin squamous carcinoma cells led to suppression of tumor growth in nude mice [28].

Based on a number of recent reports suggesting a role for FXR in certain cancers [29-31], we decided to search for novel FXR target genes in hepatocytes using a pharmacological approach and microarray technology. Our results identified the human fetuin-B gene as a FXR agonist regulated gene. Subsequently, we investigated the molecular mechanisms of fetuin-B regulation by FXR.

## MATERIALS AND METHODS

### **Culture and treatment of HepG2 cells and human primary hepatocytes.**

Human hepatoblastoma HepG2 cells (ATCC) were seeded in 6-well plates ( $5 \times 10^5$  cells/well) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, streptomycin/penicillin, sodium pyruvate, glutamine and non-essential amino acids (Invitrogen, Cergy-Pontoise, France) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Human primary hepatocytes were purchased from Biopredic International (Rennes, France) and grown in the culture medium that was furnished by the supplier at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

To induce FXR target gene expression, HepG2 cells and human primary hepatocytes were treated for 24 hours with chenodeoxycholic acid (CDCA; 75µM) (Sigma, Saint-Quentin, France) or with the synthetic FXR agonist GW4064 (5µM) in DMEM supplemented with 1% fetal calf serum. Small interfering RNA (siRNA) mediated FXR knockdown was performed as previously described by Sirvent et al.[32,33]. GW4064 was synthesized at GENFIT SA (Loos, France) according to Maloney et al. (4)

### **Culture and treatment of mouse primary hepatocytes.**

Mouse primary hepatocytes were isolated as previously described [18]. Before the addition of collagenase (0.0025%) (Sigma), livers were perfused with Hank's balanced salt solution (Sigma) at a rate of 5ml/min via the portal vein. Cell viability was assessed by the trypan blue exclusion test and it was always higher than 60%. Hepatocytes were cultured as monolayers in the serum free DMEM (Invitrogen) supplemented with 0.1% BSA, 50nM dexamethasone, 1% Glutamine, 1% Gentamycine, 25mM Glucose and 2% ULTROSER (Biosepra) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. FXR target gene expression was studied in mouse hepatocytes after 24 hours of treatment with either CDCA (50µM) or GW4064 (5µM).

### **RNA extraction, PCR analysis and DNA microarray analysis.**

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by the reverse transcription reaction from 1µg of total RNA, 200 ng of random hexamer primers and 200 units of M-MLV reverse transcriptase (Invitrogen). PCR analysis was performed using the following reaction buffer : 0.125 mM dNTP, 1.25 mM MgCl<sub>2</sub>, 1 unit of GoldStar *taq* polymerase (Eurogentec, Seraing, Belgium) and 100 nM specific primers. Standard PCR conditions were denaturation at 95°C for 5 minutes, followed by 40 cycles of 1 minute at 95 °C, 1 minute at 55° C and 1 minute at 72° C. DNA microarray analysis was performed as described previously [32,33].

### **Quantitative PCR analysis.**

Real-time quantitative PCR analysis was performed using Brilliant SYBR Green QPCR master mix (Stratagene) and specific primers according to the manufacturer's instructions. QPCR reactions were

run on MX4000 or MX3000 apparatus (Stratagene). Reaction conditions were denaturation at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. Fetuin-B mRNA levels were normalized to 28S mRNA. Statistical significance was assessed using student's *t*-test.

#### PCR primers

human Fetuin-A Fwd: 5'-ACAACGGCTCCAATTTTCAG-3'  
human Fetuin-A Rev: 5'-TACACTTGGCTGCCTCTGTG-3'  
human Fetuin-B P1 Fwd: 5'-AGTTGTAACAAAACCGCTCAA-3'  
human Fetuin-B P1 Rev: 5'-TCTGTGGAGAACAAGGCCAG-3'  
human Fetuin-B P2 Fwd: 5'-AACCTGAAGGTGTGACTGG-3'  
human Fetuin-B P2 Rev: 5'-GCACAGGACTAGGATGCAGA-3'  
human Fetuin-B ex7 Fwd: 5'-TGTGCATCTGGACCTAACCA-3'  
human Fetuin-B ex7 Rev: 5'-GCTTTTCTTTGGGGAAAGG-3'  
human Fetuin-B ex5 Rev: 5'-GTCGGAGGACTGAAGTGAACA-3'  
mouse Fetuin-B Fwd: 5'-CCCCATTGACTTGTCAAACC-3'  
mouse Fetuin-B Rev: 5'-ACACCCACTGGTTCATAGCC-3'  
28S Fwd: 5'-AAACTCTGGTGGAGGTCCGT-3'  
28S Rev: 5'-CTTACCAAAGTGGCCCACTA-3'  
cyclophilin Fwd: 5'-GCATACGGGTCCTGGCATCTTGTCC-3'  
cyclophilin Rev: 5'-ATGGTGATCTTCTTGCTGGTCTTGC-3'

#### **Plasmid cloning and site-directed mutagenesis.**

A human Fetuin-B promoter sequence (fragment between -1978 and +263 with respect to the transcriptional start site) was amplified from human genomic DNA by PCR with the following primers: (5'-TGTGGTACCGTTTAAATAACTTGTATTGAG-3') and (5'-TGTACGCGTCACAGTTGTGCTGGTTTATGA-3'). The resulting PCR product was cloned into the pGL3 basic reporter vector (Promega Corp., Madison, WI) using KpnI and MluI to yield the reporter plasmid phFetuin-B-pGL3. The sequence of the FXR response elements was mutated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The following oligonucleotides (5'-TAAATCAGTTCCCAGAATCATTGTTTTCTGATGTAAGCCCACCAG-3') and (5'-GTAAGCCCACCAGAAATCAGGGATTTTTGCGTTAGCAGAAGCTGG-3') were used to introduce mutations into the FXRE1(-1592) and FXRE2(-1559) sites, respectively. Reporter plasmids, which contain three copies of each FXR response element were produced by the ligation of a synthetic dsDNA cassette into the TK-pGL3 vector cut with SmaI.

The following oligonucleotides were used to assemble 3xFXRRE cassettes.

FXRE1(1592)wt oligo sense (5'-TTCCCAGGGTCATTGTCCTCTGATGTTCCC

AGGGTCATTGTCCTCTGATGTTCCCAGGGTCATTGTCCTCTGATG-3'), and oligo antisense (5'-CATCAGAGGACAATGACCCTGGGAACATCAGAGGACAATGACCCTGGGAACATCAGAGGACAATGACCCTGGGAA-3')

FXRE1(-1592)mt oligo sense (5'-TTCCCAGAATCATTGTTTTCTGATGTTCCAGAATCATTGTTTTCTGATG-3') and oligo antisense (5'-CATCAGAAAACAATGATTCTGGGAACATCAGAAAACAAATGATTCTGGGAACATCAGAAAACAATGATTCTGGGAA-3')

FXRE2(-1559)wt oligo sense (5'-CACCAGAGGTCAGGGACCTTTGCGTCACCAGAGGGACCTTTGCGT-3') and oligo antisense (5'-ACGCAAAGGTCCCTGACCTCTGGTGACGCAAAGGTCCCTGACCTCTGGTG-3')

FXRE2(-1559)mt oligo sense (5'-CACCAGAAATCAGGGATTTTTGCGTCACCAGAAATCAGGGATTTTTGCGT-3') and oligo antisense (5'-ACGCAAAAATCCCTGATTTCTGGTGACGCAAAAATCCCTGATTTCTGGTG-3')

All constructs were verified by restriction enzyme digestion and sequence analysis

#### **Cell culture and transfection assays.**

Reporter assays were performed in HepG2 cells. The standard transfection procedure with the cationic lipid RPR 120535B was described in detail elsewhere [34]. Shortly, reporter plasmids (100 ng) were cotransfected with both pcDNA3 human FXR (30 ng) and pSG5 human RXR $\alpha$  (30 ng). pSG5 (Stratagene, La Jolla, California, USA) and pcDNA3 (Invitrogen, Leek, The Netherlands) empty vectors were used as negative controls. All samples were complemented with pBSK<sup>+</sup> plasmid (Stratagene) to bring the total amount of DNA to 500 ng. Following three hours of incubation, transfection medium was replaced with a medium containing 1% FCS with or without CDCA. Luciferase activity was measured 36 hours later using a TR717 luminometer (Applied Biosystems). Transfection efficiency was monitored by a cotransfection with 10 ng of a CMV-driven  $\beta$ -galactosidase expression plasmid. Transfection experiments were done at least three times in triplicate.

#### **Electrophoretic mobility shift assays (EMSA).**

Human FXR and human RXR were produced in vitro using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, Wisconsin, USA). Double stranded probes were produced by annealing sense and anti-sense oligonucleotides (5  $\mu$ g each) in the following buffer (50mM Tris-HCl(pH8.0), 50mM KCl, 5mM MgCl<sub>2</sub>, 1mM dithiothreitol). Oligonucleotides were denatured at 95°C for 5 min, then the temperature was reduced to 65°C for 10 min and finally the mix

was incubated at room temperature for 16hr. The probe was subsequently labeled with  $^{32}\text{P}$   $\gamma$ -ATP using T4-polynucleotide kinase. FXR and/or RXR proteins preps (2  $\mu\text{l}$ ) were incubated for 15 min at room temperature in a total volume of 20  $\mu\text{l}$  with 1  $\mu\text{g}$  poly(dI-dC) and 1  $\mu\text{g}$  herring sperm DNA in binding buffer (10 mM HEPES, pH 7.6, 2.5 mM  $\text{MgCl}_2$ , 10% glycerol, 2.5 mg/ml BSA, 50 mM NaCl, and 0.5mM DTT) before the radiolabeled probe (1 ng) was added. Binding reactions were further incubated for 15 min and resolved by 6% nondenaturing polyacrylamide gel electrophoresis in 0.25x Tris-Borate-EDTA (TBE) buffer at room temperature. Excess of the unlabeled probe was included in the binding reaction, just before adding the labeled probe, to perform competition assays.

Oligonucleotides used in the study :

FXRE1wt sense : (5'-GTTCCCAGGGTCATTGTCCTCTGAT-3'),

FXRE1wt antisense : (5'-ATCAGAGGACAATGACCCTGGGAAC-3'),

FXRE1mt sense : 5'-GTTCCCAGAATCATTGTTTTCTGAT-3',

FXRE1mt antisense : (5'-ATCAGAAAACAATGATTCTGGGAAC-3');

FXRE2wt sense : (5'-ACCAGAGGTCAGGGACCTTTGCGTT-3'),

FXRE2wt antisense : (5'-AACGCAAAGGTCCCTGACCTCTGGT-3'),

FXRE2mt sense : (5'-ACCAGAAATCAGGGATTTTTGCGTT-3'),

FXRE2mt antisense: (5'-AACGCAAAAATCCCTGATTTCTGGT).

IBABP IR-1 sense : (5'-GATCTCAAGAGGTCATTGACCTT TTTG-3'),

IBABP IR-1 antisense: (5'- CAAAAGGTCAATGACCTCTTGAGATC -3').

A FXR-specific antibody SC-1204 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the binding reaction (2  $\mu\text{l}$ /tube) before addition of the probe, to perform supershift assays.

## RESULTS

### **FXR agonists induce Fetuin-B expression in human but not in murine primary hepatocytes.**

To identify new FXR target genes, we have performed DNA microarray analysis using human primary hepatocytes treated with 5 $\mu$ M GW4064 for 24hrs. The results revealed fetuin A (1.6 fold) and fetuin B (4.5 fold) were induced by FXR agonist (data not shown). To confirm these results, Fetuin-A and fetuin-B regulation by FXR was studied in a human hepatoma cell line HepG2 (Fig.1A) and in primary human hepatocytes (Fig.1B). Gene expression analysis by real-time quantitative PCR (Q-PCR) revealed that Fetuin-B but not Fetuin-A mRNA level was significantly increased in cells treated with either the natural (CDCA) or synthetic (GW4064) FXR agonist. Surprisingly, fetuin-B expression in mouse primary hepatocytes was completely insensitive to FXR agonist treatment (Fig.1C). These results suggest that the effect of FXR agonists on fetuin-B expression is species-specific.

### **Induction of Fetuin-B expression by CDCA and GW4064 requires FXR expression.**

Gene silencing by siRNA was used to determine whether the regulation of Fetuin-B expression by FXR agonists observed in human liver cells was dependent on FXR expression. HepG2 cells were therefore transfected with siRNA complexes (anti-FXR and/or negative control) followed by FXR ligand treatment. Basal Fetuin-B expression level was reduced by approximately 49% in FXR siRNA treated cells as compared to the negative control (Fig.2, white columns). Moreover, CDCA and GW4064-dependent increase in Fetuin-B expression was strongly attenuated in FXR siRNA treated cells (Fig.2, mesh and black columns). As control, FXR agonist induction of SHP expression was abolished by FXR siRNA and GAPDH expression was not affected by either FXR siRNA or the negative control siRNA in the same experiment [32, 33].

### **Induction of Fetuin-B through FXR occurs via the P2 but not the P1 promoter.**

A previous report (28) has identified a fetuin-B promoter (Fig.3A, P1) and three transcripts, which arise by differential splicing of 5'-terminal exons. We were unable to identify any putative FXR binding sites in the vicinity of this fetuin-B promoter. In addition, a 1.1kb reporter construct (sequence -1000 through +102 bp) derived from the published promoter sequence was not regulated by FXR over-expression and agonist treatment (not shown). We therefore suspected that the fetuin-B gene has other, alternative promoters. A data base search identified one expressed sequence tag (DA449793), which mapped both to the 5'-terminal fetuin-B exons and to a genomic sequence that is located 6 kb upstream of the published exon 1. Subsequent RT-PCR analysis confirmed the existence of transcripts which originate at an alternative promoter (Fig. 3 AB). Detailed analysis with both P1 or P2 specific primers revealed that each promoter produces three different transcripts that differ in size (Fig.3B). Sequencing of P1 promoter transcripts identified the Fetuin-B P1 $\alpha$ , P1 $\beta$  and P1 $\gamma$  isoforms, which have been previously described by Hsu et al [28]. Three novel Fetuin-B forms, called P2 $\alpha$ ,  $\beta$  and  $\gamma$  were identified by sequencing the PCR fragments obtained with the P2 specific primer (Fig.3A). The fetuin-B P2 $\alpha$  transcript contains the untranslated exon that follows the P2 promoter, the coding part of the

exon 1 and exons 2 through 7. Fetuin-B P2 $\beta$  is missing exon 3. Finally, exons 4 through 7 are directly spliced to the P2 specific 5'-untranslated exon in the fetuin-B P2 $\gamma$  transcript.

Real-time quantitative PCR assays, specific for either P1-derived or P2-derived transcripts were developed to determine which of the two Fetuin-B promoters is responsive to FXR. As shown in Fig.3C, the level of P2 specific transcripts was increased over three times by both CDCA and GW4064 in HepG2 cells, whereas P1 specific transcripts did not respond. Similarly, FXR agonist treatment increased the abundance of P2 specific transcripts 20-fold in human primary hepatocytes (Fig.3D). The level of P1 specific transcripts was not affected by FXR agonist treatment in human primary hepatocytes.

### **Functional FXR response elements are present in the human Fetuin-B P2 promoter.**

The P2 promoter flanking sequence, obtained from NCBI human genome resources (build 36.2 at <http://www.ncbi.nlm.nih.gov/genome/guide/human/>), was analyzed by VISTA tools (<http://genome.lbl.gov/vista/index.shtml>) for putative FXR binding sites. Two IR-1 motifs GGGTCATTGTCCT (-1592bp to -1577bp) and AGGTCAGGGACCT (-1559bp to -1546bp) were identified as putative FXR binding sites. Genomic sequence alignment with fetuin-B orthologs from different species (Fig.4A) showed that only one half site was conserved in each FXRE motif in mice. G to A conversion at the third position of an IR-1 half-site is usually detrimental for receptor binding.

The human Fetuin-B P2 promoter (positions : -1978bp / +263bp) was tested in transient transfection reporter assay in HepG2 cells. As shown in Fig.4B, CDCA treatment increased the transcriptional activity of the reporter plasmid (8-fold over control) in the presence of cotransfected FXR and RXR expression plasmids. The control plasmid (pGL3-basic) was not affected. Mutations introduced in each IR-1 motif compromised this induction. These results demonstrate that both FXRE1 and FXRE2 motifs are important for the activation of the Fetuin-B P2 promoter by FXR.

Next, both IR-1 motifs were tested in the context of a heterogenous promoter to check if those motifs are sufficient to direct FXR-mediated regulation. HepG2 cells were transfected with reporter constructs, which carried three copies of either FXRE1 or FXRE2 motif in the TKpGL3 vector. As shown in Fig.4C, CDCA-activated FXR/RXR strongly induced the FXRE1 motif (12-fold increase over the control), whereas the FXRE2 motif was only activated 2.5-fold in the same conditions. This difference in the response strength to FXR might probably be attributed to the presence of a guanine at the last position in the second half site in FXRE2. This position is usually occupied by an adenine in nuclear receptor binding motifs. Mutations introduced in either FXRE1 and FXRE2 motifs completely abolished FXR/RXR mediated regulation (Fig.4C), which confirm that these motifs are functional FXREs mediating FXR specific regulation of the human fetuin-B promoter.

### **FXRE1 and FXRE2 motifs identified in the human Fetuin-B P2 promoter bind FXR/RXR complexes in an EMSA assay.**

Electrophoretic Mobility Shift Assays (EMSA) were performed to test direct binding of the FXR/RXR complexes to the FXRE1 and FXRE2 motifs. A complex formed by the in vitro translated hFXR/hRXR but not by FXR alone, was able to bind both FXRE 1 (Fig.5A, lanes 8 through 17) and FXRE 2 (Fig.5B, lanes 8 through 17) motifs. FXR/RXR binding was easily displaced with a wt (Fig.5AB, lanes 9 through 12) but not with a mutant (Fig.5AB, lanes 13 through 16) unlabeled probe. The identity of the FXR/RXR complex was confirmed by supershift analysis (Fig.5AB, lane 17) using a specific anti-FXR antibody. These results demonstrate that the FXR/RXR heterodimer is able to bind specifically to both FXRE motifs identified in the human fetuin-B promoter P2.

## DISCUSSION

Fetuin-A is abundant foetal serum  $\alpha$ -globulin, which belongs to the cystatin family of cysteine protease inhibitors. The vast majority of physiological data available to date comes from studies on fetuin-A (mouse) or on its human orthologue  $\alpha$ 2-Heremans and Schmid glycoprotein (AHSG), which acts as a systemic inhibitor of ectopic calcification [22, 23]. In clinic, AHSG concentrations in serum were significantly lower in patients on haemodialysis than in healthy controls [35]. Low concentrations of the glycoprotein were associated with raised amounts of CRP and with enhanced cardiovascular and all-cause mortality [35]. In addition, sera from patients on long-term dialysis with low AHSG concentrations showed impaired ex-vivo capacity to inhibit calcium phosphate precipitation. Reconstitution of sera with purified AHSG returned this impairment to normal. Similarly, low serum concentrations of AHSG in peritoneal dialysis patients are associated with valvular calcification, higher cardiovascular mortality and development of malnutrition, inflammation, atherosclerosis/calcification (MIAC) syndrome [36].

Fetuin-A is also implicated in a variety of other metabolic regulations, through the inhibition of the TGF- $\beta$  pathway in relation to bone growth and remodelling [24] and acting as a negative regulator of insulin receptor tyrosine kinase activity [24,25]. High levels of fetuin-A in subjects with coronary artery disease were associated with the metabolic syndrome and with a pro-atherogenic lipid profile in the Heart and Soul Study [37].

Fetuin-A and fetuin-B share overlapping but not identical tissue distribution patterns and functions. At the RNA level, the highest expression of both fetuin-A and fetuin-B was reported in the liver, followed by kidney and tongue [27]. Fetuin-B but not fetuin-A mRNA has been detected in mouse ovaries. Interestingly, unlike fetuin-A, fetuin-B concentration in serum is higher in females [27]. On a structural basis, fetuin-B is clearly homologous to fetuin-A, as illustrated by the shared arrangement in three domains, including two cystatin-like domains D1 and D2 [26]. Mutational analysis showed that in the case of fetuin-A, the basic calcium precipitation (BCP) activity resides in the NH<sub>2</sub>-terminal cystatin-like domain D1 [38]. Functional experiments have shown that fetuin-B is indeed capable to inhibit BCP, although with lower efficiency [27]. In addition, both fetuin-B expression in human hepatocytes and its protein level in the serum are low as compared to fetuin-A. Therefore, fetuin-B can potentially function as a BCP inhibitor in vivo, but it is unlikely to make a major systemic contribution.

Here, we show that in human primary hepatocytes the expression of the gene coding for fetuin-B is directly up-regulated by agonist activated FXR through binding sites, which are located in the alternative P2 promoter. Three novel fetuin-B transcripts, which originate at the P2 promoter and arise by differential splicing of their 5'-terminal exons were identified in this study. Interestingly, the untranslated exon P2 is directly connected to the exon 4 in the fetuin-B P2 $\gamma$  transcript. This situation implies that the translation of the P2 $\gamma$  transcript is initiated at a different site. Indeed, a putative alternative start codon exists in the exon 4 at the position 21bp downstream from splicing site in exon

4. This putative, P2 $\gamma$  derived protein should only contain the cystatin-like domain D2. The abundance and the functional importance of this new isoform remains to be explored.

Interestingly, fetuin-B expression in primary mouse hepatocytes was not affected by FXR agonist treatment. Genomic sequence alignment of fetuin-B promoters from different species revealed that neither the first, nor the second FXRE is conserved between mouse and human. In addition, those putative FXRE elements in the mouse promoter are positioned relatively far (3.9kb) from the nearest putative transcriptional start site. Finally, basal fetuin-B concentration in serum is 31 times higher in mouse than in humans [27]. Therefore, we postulate that the mouse fetuin-B gene is regulated by different mechanisms than its human orthologue.

Recently reports show FXR regulates apoptosis and metastasis in breast cancer cells [29,30]. FXR agonists induce apoptosis in human breast carcinoma cells and this effect is diminished by knockdown of FXR [29]. On the other hand, the transfection of fetuin-B expression plasmids suppresses the growth of carcinoma cell line, B9 cells [28]. These results suggest that the FXR-fetuin B pathway might regulate proliferation and apoptosis in cancer cells.

In summary, we have demonstrated that FXR agonist treatment results in the activation of fetuin-B gene expression in human hepatocytes. This effect is selectively mediated through the P2 promoter and is species-specific. Since the functional importance of fetuin-B protein has not been firmly established, it is difficult to speculate on the metabolic consequences of FXR mediated fetuin-B regulation.

### **ACKNOWLEDGEMENTS**

This work was supported by grants from the EU Grant Hepadip (n° 018734) and the Agence Nationale de la Recherche (n° A05056GS).

## REFERENCE

1. Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., Perlmann, T., Noonan, D.J., Burka, L.T., McMorris, T., Lamph, W.W., Evans, R.M., Weinberger, C. (1995) Identification of a nuclear receptor that is activated by farnesol metabolites *Cell* **81**, 687-693
2. Seol, W., Choi, H.S., Moore, D.D. (1995) Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. *Mol. Endocrinol.* **9**, 72-85
3. Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J., Shan, B. (1999) Identification of a nuclear receptor for bile acids. *Science* **284**, 1362-1365
4. Maloney, P.R., Parks, D.J., Haffner, C.D., Fivush, A.M., Chandra, G., Plunket, K.D., Creech, K.L., Moore, L.B., Wilson, J.G., Lewis, M.C., Jones, S.A., Willson, T.M. (2000) Identification of a chemical tool for the orphan nuclear receptor FXR. *J. Med. Chem.* **43**, 2971-2974
5. Lu, T.T., Makishima, M., Repa, J.J., Schoonjans, K., Kerr, T.A., Auwerx, J., Mangelsdorf, D.J. (2000) Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol. Cell* **6**, 507-515
6. Goodwin, B., Jones, S.A., Price, R.R., Watson, M.A., McKee, D.D., Moore, L.B., Galardi, C., Wilson, J.G., Lewis, M.C., Roth, M.E., Maloney, P.R., Willson, T.M., Kliewer, S.A. (2000) A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol. Cell* **6**, 517-526
7. Grober, J., Zaghini, I., Fujii, H., Jones, S.A., Kliewer, S.A., Willson, T.M., Ono, T., Besnard, P. (1999) Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. *J. Biol. Chem.* **274**, 29749-29754
8. Ananthanarayanan, M., Balasubramanian, N., Makishima, M., Mangelsdorf, D.J., Suchy, F.J. (2000) Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J. Biol. Chem.* **276**, 8857-8865
9. Denson, L.A., Sturm, E., Echevarria, W., Zimmerman, T.L., Makishima, M., Mangelsdorf, D.J., Karpen, S.J. (2001) The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. *Gastroenterology* **121**, 140-147

10. Claudel, T., Sturm, E., Duez, H., Torra, I.P., Sirvent, A., Kosykh, V., Fruchart, J.C., Dallongeville, J., Hum, D.W., Kuipers, F., Staels, B. (2002) Bile acid-activated nuclear receptor FXR suppresses apolipoprotein A-I transcription via a negative FXR response element. *J. Clin. Invest.* **109**, 961-971
11. Urizar, N.L., Dowhan, D.H., Moore, D.D. (2000) The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. *J. Biol. Chem.* **275**, 39313-39317
12. Kast, H.R., Nguyen, C.M., Sinal, C.J., Jones, S.A., Laffitte, B.A., Reue, K., Gonzalez, F.J., Willson, T.M., Edwards, P.A. (2001) Farnesoid X-activated receptor induces apolipoprotein C-II transcription: a molecular mechanism linking plasma triglyceride levels to bile acids. *Mol. Endocrinol.* **15**, 1720-1728
13. Claudel, T., Inoue, Y., Barbier, O., Duran-Sandoval, D., Kosykh, V., Fruchart, J., Fruchart, J.C., Gonzalez, F.J., Staels, B. (2003) Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. *Gastroenterology* **125**, 544-555
14. Pineda, Torra, I., Claudel, T., Duval, C., Kosykh, V., Fruchart, J.C., Staels, B. (2003) Bile acids induce the expression of the human peroxisome proliferator-activated receptor alpha gene via activation of the farnesoid X receptor. *Mol. Endocrinol.* **17**, 259-272
15. Sinal, C.J., Tohkin, M., Miyata, M., Ward, J.M., Lambert, G., Gonzalez, F.J. (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**, 731-744
16. Watanabe, M., Houten, S.M., Wang, L., Moschetta, A., Mangelsdorf, D.J., Heyman, R.A., Moore, D.D., Auwerx, J. (2004) Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *J. Clin. Invest.* **113**, 1408-1418
17. Yamagata, K., Daitoku, H., Shimamoto, Y., Matsuzaki, H., Hirota, K., Ishida, J., Fukamizu, A. (2004) Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *J. Biol. Chem.* **279**, 23158-23165
18. Duran-Sandoval, D., Cariou, B., Percevault, F., Hennuyer, N., Grefhorst, A., van Dijk, T.H., Gonzalez, F.J., Fruchart, J.C., Kuipers, F., Staels, B. (2005) The farnesoid X receptor modulates hepatic carbohydrate metabolism during the fasting-refeeding transition. *J. Biol. Chem.* **280**, 29971-29979

19. Ma, K., Saha, P.K., Chan, L., Moore, D.D. (2006) Farnesoid X receptor is essential for normal glucose homeostasis. *J. Clin. Invest.* **116**, 1102-1109
20. Zhang, Y., Lee, F.Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F.J., Willson, T.M., Edwards, P.A. (2006) Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. *Proc. Natl. Acad. Sci. USA* **103**, 1006-1011
21. Cariou, B., van Harmelen, K., Duran-Sandoval, D., van Dijk, T.H., Grefhorst, A., Abdelkarim, M., Caron, S., Torpier, G., Fruchart, J.C., Gonzalez, F.J., Kuipers, F., Staels, B. (2006) The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. *J. Biol. Chem.* **281**, 11039-11049
22. Schafer, C., Heiss, A., Schwarz, A., Westenfeld, R., Ketteler, M., Floege, J., Muller-Esterl, W., Schinke, T., Jahn-Dechent, W. (2003) The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J. Clin. Invest.* **112**, 357-366
23. Schinke, T., Amendt, C., Trindl, A., Poschke, O., Muller-Esterl, W., Jahn-Dechent, W. (1996) The serum protein alpha2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. *J. Biol. Chem.* **271**, 20789-20796
24. Szweras, M., Liu, D., Partridge, E.A., Pawling, J., Sukhu, B., Clokie, C., Jahn-Dechent, W., Tenenbaum, H.C., Swallow, C.J., Grynepas, M.D., Dennis, J.W. (2002) alpha 2-HS glycoprotein/fetuin, a transforming growth factor-beta/bone morphogenetic protein antagonist, regulates postnatal bone growth and remodeling. *J. Biol. Chem.* **277**, 19991-19997
25. Mathews, S.T., Singh, G.P., Ranalletta, M., Cintron, V.J., Qiang, X., Goustin, A.S., Jen, K.L., Charron, M.J., Jahn-Dechent, W., Grunberger, G. (2002) Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene. *Diabetes* **51**, 2450-2458
26. Olivier, E., Soury, E., Ruminy, P., Husson, A., Parmentier, F., Daveau, M., Salier, J.P. (2000) Fetuin-B, a second member of the fetuin family in mammals. *Biochem. J.* **350**, 589-597
27. Denecke, B., Graber, S., Schafer, C., Heiss, A., Woltje, M., Jahn-Dechent, W. (2003) Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. *Biochem. J.* **376**, 135-145

28. Hsu, S.J., Nagase, H., Balmain, A. (2004) Identification of Fetuin-B as a member of a cystatin-like gene family on mouse chromosome 16 with tumor suppressor activity. *Genome* **47**, 931-946
29. Swales, K.E., Korbonits, M., Carpenter, R., Walsh, D.T., Warner, T.D., Bishop-Bailey, D. (2006) The farnesoid X receptor is expressed in breast cancer and regulates apoptosis and aromatase expression. *Cancer Res.* **66**, 10120-10126
30. Silva, J., Dasgupta, S., Wang, G., Krishnamurthy, K., Ritter, E., Bieberich, E. (2006) Lipids isolated from bone induce the migration of human breast cancer cells. *J. Lipid Res.* **47**, 724-733
31. Kim, I., Morimura, K., Shah, Y., Yang, Q., Ward, J.M., Gonzalez, F.J. (2007) Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice. *Carcinogenesis* **228**, 940-946
32. Sirvent, A., Claudel, T., Martin, G., Brozek, J., Kosykh, V., Darteil, R., Hum, D.W., Fruchart, J.C., Staels, B. (2004) The farnesoid X receptor induces very low density lipoprotein receptor gene expression. *FEBS Lett.* **566**, 173-177
33. Sirvent, A., Verhoeven, A.J., Jansen, H., Kosykh, V., Darteil, R.J., Hum, D.W., Fruchart, J.C., Staels, B. (2004) Farnesoid X receptor represses hepatic lipase gene expression. *J. Lipid Res.* **45**, 2110-2115
34. Raspé, E., Madsen, L., Lefebvre, A., Leitersdorf, I., Gelman, L., Peinado-Onsurbe, J., Dallongeville, J., Fruchart, J., Berge, R., Staels, B. (1999) Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARalpha activation. *J. Lipid Res.* **40**, 2099-2110
35. Ketteler, M., Bongartz, P., Westenfeld, R., Wildberger, J.E., Mahnken, A.H., Bohm, R., Metzger, T., Wanner, C., Jahnke-Dechent, W., Floege, J. (2003) Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. *Lancet* **36**, 827-833
36. Wang, A.Y., Woo, J., Lam, C.W., Wang, M., Chan, I.H., Gao, P., Lui, S.F., Li, P.K., Sanderson, J.E. (2005) Associations of serum fetuin-A with malnutrition, inflammation, atherosclerosis and valvular calcification syndrome and outcome in peritoneal dialysis patients. *Nephrol. Dial. Transplant.* **20**, 1676-1685
37. Ix, J.H., Shlipak, M.G., Brandenburg, V.M., Ali, S., Ketteler, M., Whooley, M.A. (2006) Association between human fetuin-A and the metabolic syndrome: data from the Heart and Soul Study. *Circulation* **113**, 1760-1767

38. Heiss, A., DuChesne, A., Denecke, B., Grotzinger, J., Yamamoto, K., Renne, T., Jahnen-Dechent, W. (2003) Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. *J. Biol. Chem.* **278**, 13333-13341

## Figure legends

### **Figure 1 Fetuin-B expression is induced by FXR agonists in human but not in mouse hepatocytes.**

HepG2 cells (A), human primary hepatocytes (B) or mouse primary hepatocytes (C) were treated with either 50 $\mu$ M CDCA or with 5 $\mu$ M GW4064 for 24 hours. Total RNA was extracted and human Fetuin-A, human Fetuin-B and mouse Fetuin-B mRNA levels were measured by real-time quantitative PCR and normalized to 28S mRNA levels. Data are expressed as fold change over the DMSO; mean  $\pm$  S.E.(n=3). Statistical significance was assessed using student's *t*-test. \**P*<0.05 compare with control.

### **Figure 2 The induction of fetuin-B expression by CDCA and/or GW4064 is FXR dependent**

HepG2 cells were transfected with either small interfering RNA (siRNA) targeting FXR or with negative control siRNA. Twelve hours post siRNA transfection cells were treated with either 75 $\mu$ M CDCA or with 1 $\mu$ M GW4064 for the next 24 hours. Human fetuin-B mRNA levels were then measured by the real-time quantitative PCR and normalized to cyclophilin mRNA levels. Data are expressed as a relative expression versus DMSO in the control group; mean  $\pm$  S.E.(n=3)

### **Figure 3 FXR dependent increase in fetuin-B expression is mediated through the P2 promoter.**

(A) Three human fetuin-B transcripts, which originate from the P1 promoter, have previously been described by Hsu et al. (25). Fetuin-B P1 $\alpha$  contains exons 1 through 7. Fetuin-B P1 $\beta$  is missing exon 2. Fetuin-B P1 $\gamma$  is missing exons 2 and 3. Fetuin-B P2  $\alpha,\beta,\gamma$  isoforms arise from the alternative P2 promoter. Fetuin-B P2 $\alpha$  contains the untranslated exon P2, the translated part (black box) of exon 1 and exons 2 through 7. Fetuin-B P2 $\beta$  is missing exon 3. Fetuin-B P2 $\gamma$  is missing exons 1, 2 and 3. Solid boxes and empty boxes indicate translated and untranslated regions, respectively. Arrows indicate P1 and P2 promoters. Arrow heads indicate PCR primers.

(B) The differential splicing pattern of fetuin-B gene was validated by PCR analysis with P1 or P2 specific primers in human primary hepatocytes. PCR products were analyzed by gel electrophoresis on 1.5 % agarose with TBE and verified by sequencing.

Selective, promoter dependent fetuin-B regulation by FXR in both HepG2 cells (C) and in human primary hepatocytes (D). Relative expression level of human fetuin-B transcripts, which originate either at the P1 or at the P2 promoter, was measured by real-time quantitative PCR and normalized to 28S mRNA. Data are expressed as fold change vs DMSO in each cell type, mean  $\pm$  S.E.(n=3)

### **Figure 4 Functional FXR response elements are present within the Fetuin-B P2 promoter.**

(A) Human Fetuin-B P2 promoter sequence was aligned with genomic sequences from different species. Two putative FXR binding sites (FXRE1 and FXRE2) were identified in the human sequence within 1.6 kbp upstream of the predicted transcriptional start site. Bold characters and arrows indicate

putative IR-1 sites. (Mouse chromosome 16 :position 19829761 to 19829857bp; Bovine chromosome 1: position 48748 to 48848bp. Canis chromosome 34: position 6763848 to 6763947bp).

HepG2 cells were transfected (B and C) with the reporter construct in the presence of either empty vector (control) or FXR and RXR expression vectors. Cells were treated with 50 $\mu$ M CDCA for 36hrs. RLU signal was normalized to the  $\beta$ -galactosidase activity. FXR agonist mediated induction is expressed as a fold change over DMSO.

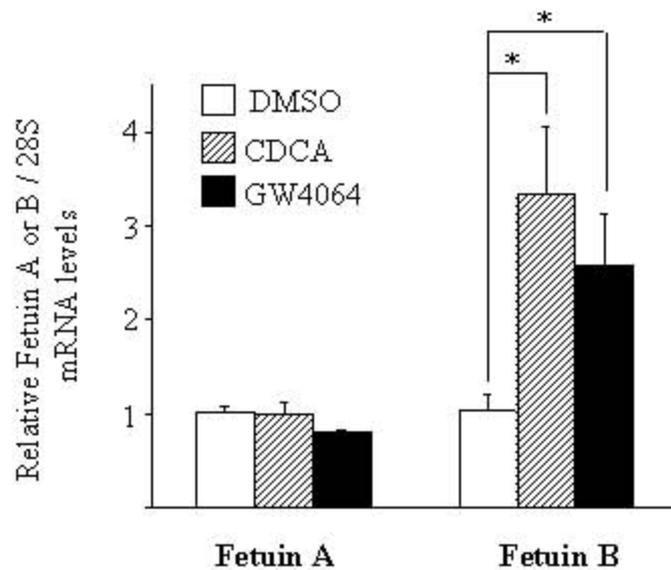
(B) Human Fetuin-B P2 promoter (-1978bp / +263bp) was cloned in the pGL3-basic reporter vector. Mutations were introduced in the FXRE 1 or FXRE 2 elements (E1mt, E2mt) as described in Methods. values, mean +/- S.E. (n=3).

(C) HepG2 cells were transfected with reporter constructs containing three copies of either FXRE1 or FXRE2 element. TK-pGL3 plasmid was used as a control. Mutations were introduced in the FXRE 1 or FXRE 2 elements (E1mt, E2mt) as described in the text.

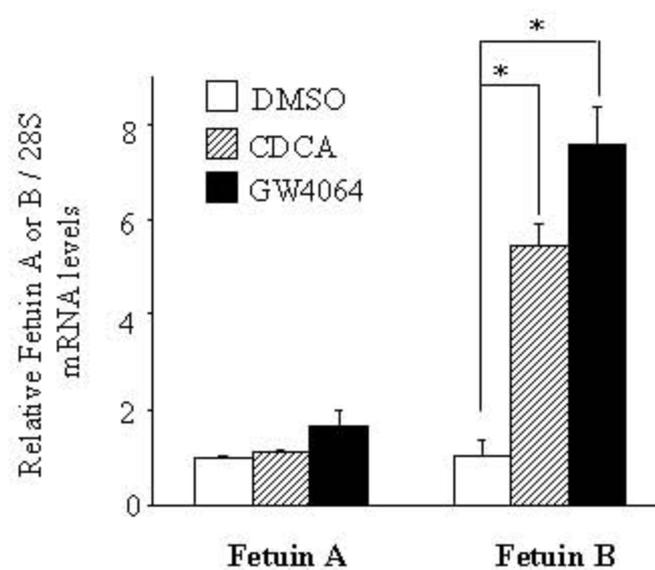
**Figure 5 The FXR/RXR complex binds in vitro to the human fetuin-B FXRE 1 and FXRE 2 motifs.**

Electrophoretic mobility shift assays were performed using end-labeled fetuin-B FXREs (wild type probe, lanes 5-17; mutant probe, lanes 18-19). An IR-1 element, previously identified in the IBABP promoter (lanes 1-4), was used as a positive control. Unprogrammed reticulocyte lysate (lanes 1,5 and 18), hRXR (lanes 2 and 6), hFXR (lanes 3 and 7), hRXR and hFXR (lanes 4, 8-17 and 19). Competition was performed by adding 10 (lanes 9 and 13), 50 (lanes 10 and 14), 100 (lanes 11 and 15), or 200 (lanes 12 and 16) fold excess of cold, wild type (lines 9-12) or mutated probe (lines 13-16). Anti-FXR antibody (2 $\mu$ l per tube) was added to the binding reaction before addition of the probe (lane 17) to obtain the supershift.

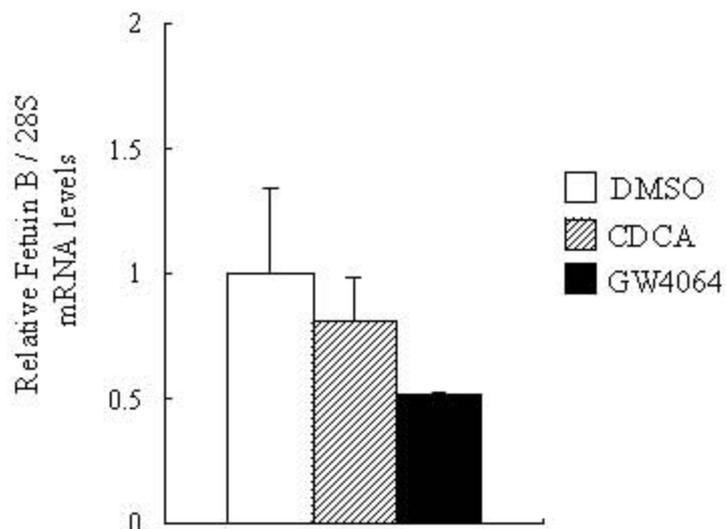
(A) HepG2 cells

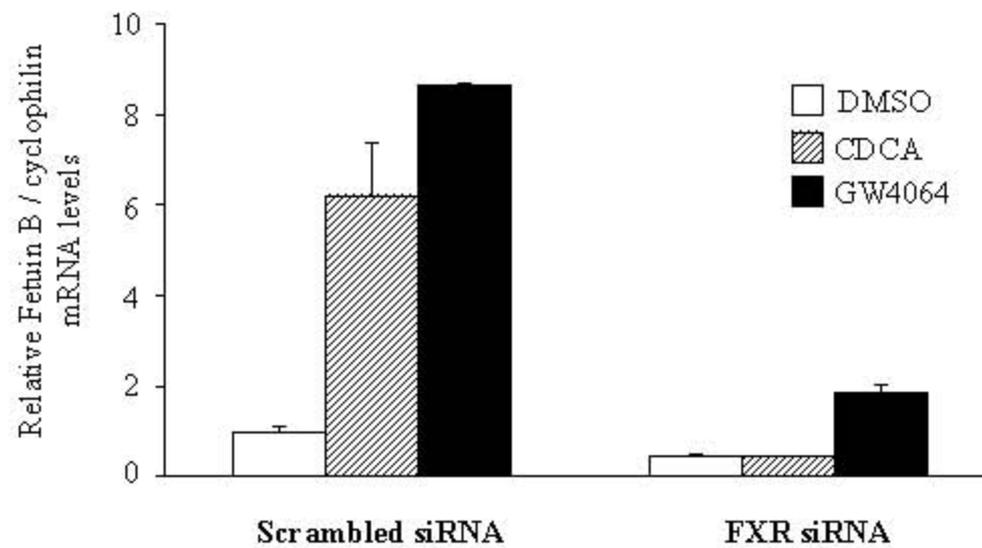


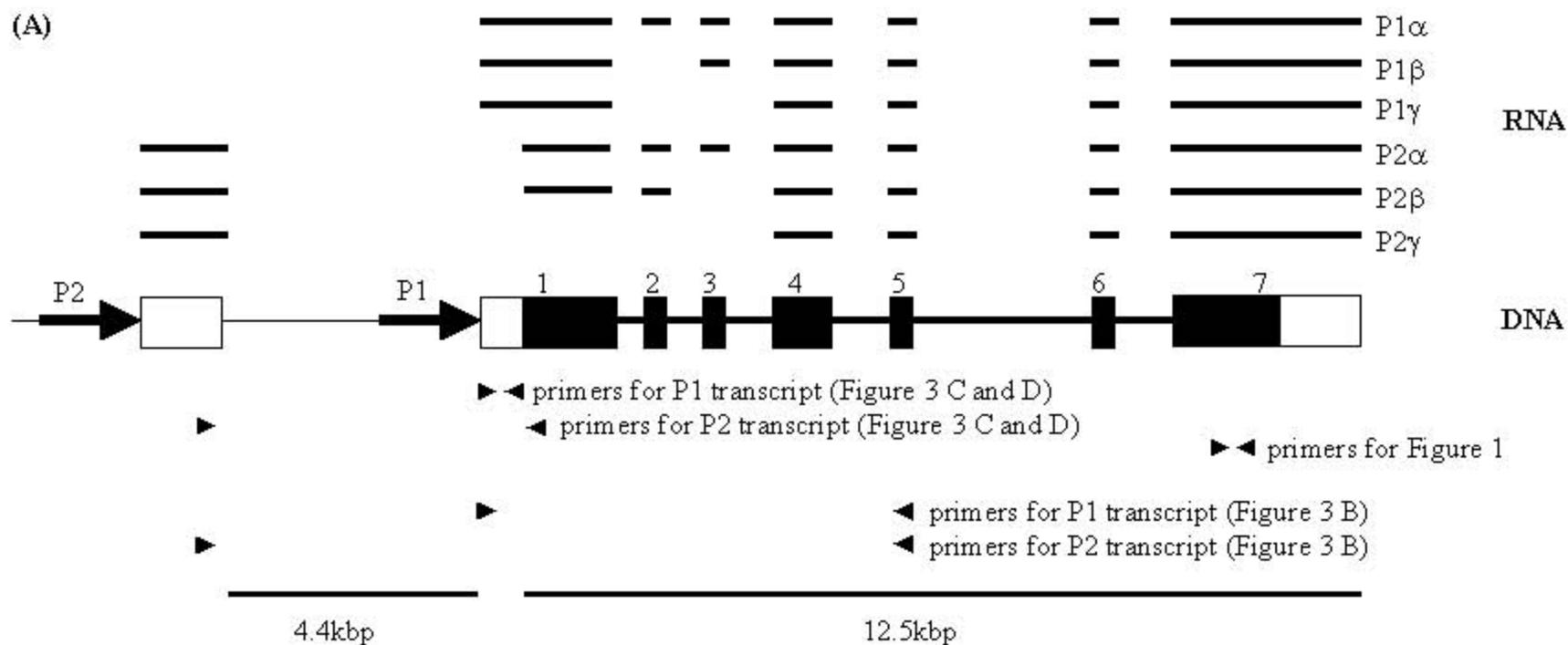
(B) Human primary hepatocytes

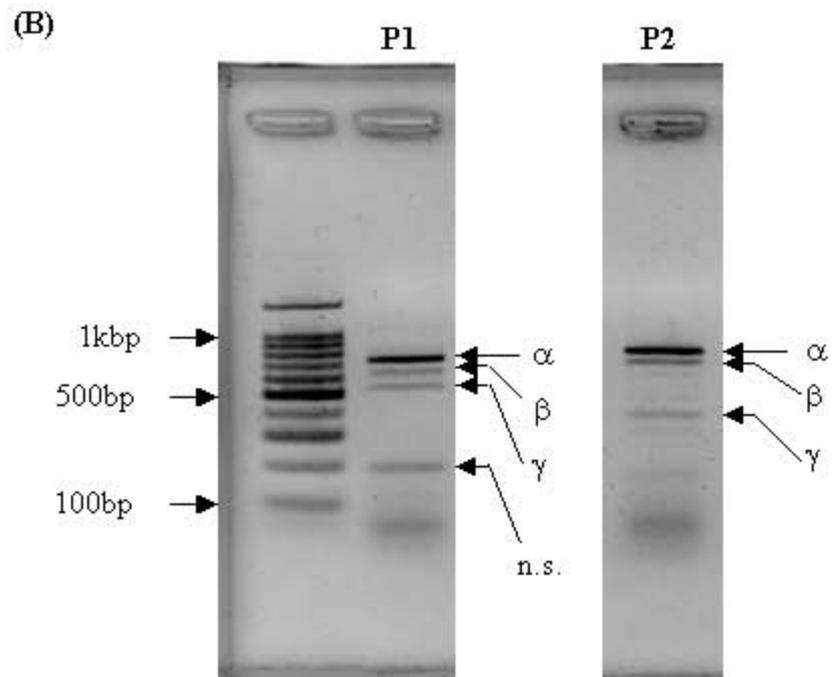


(C) Mouse primary hepatocytes

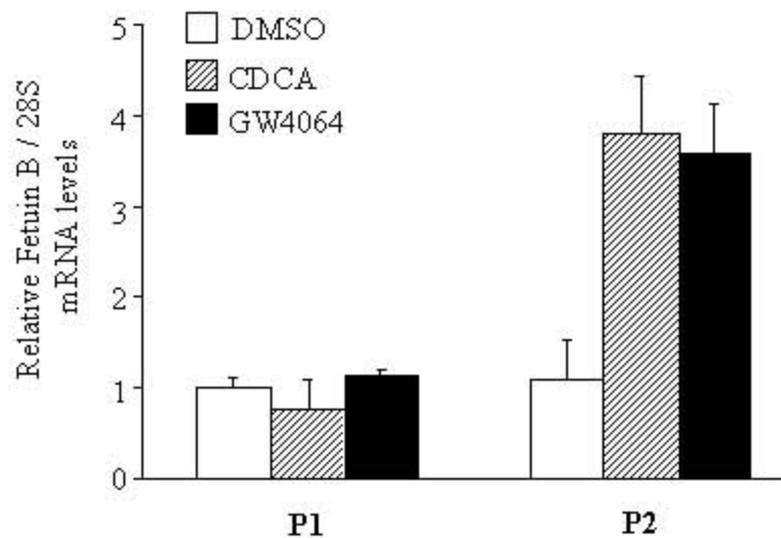




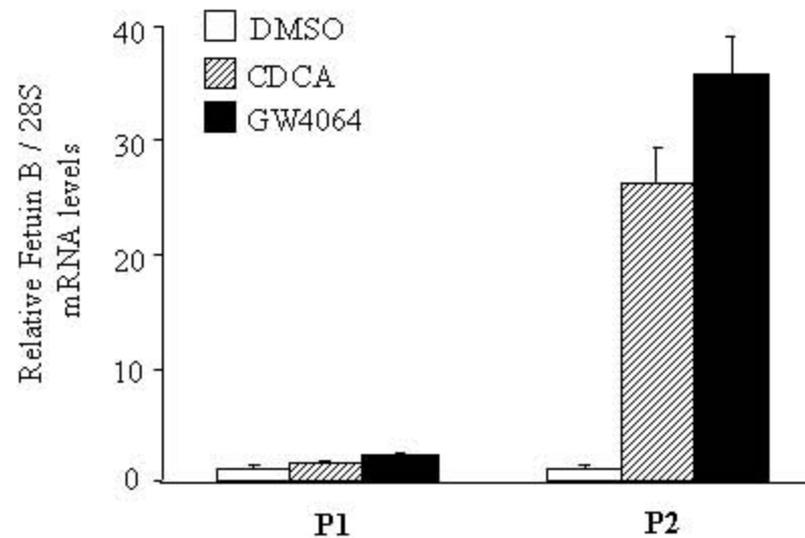




(C) HepG2 cells



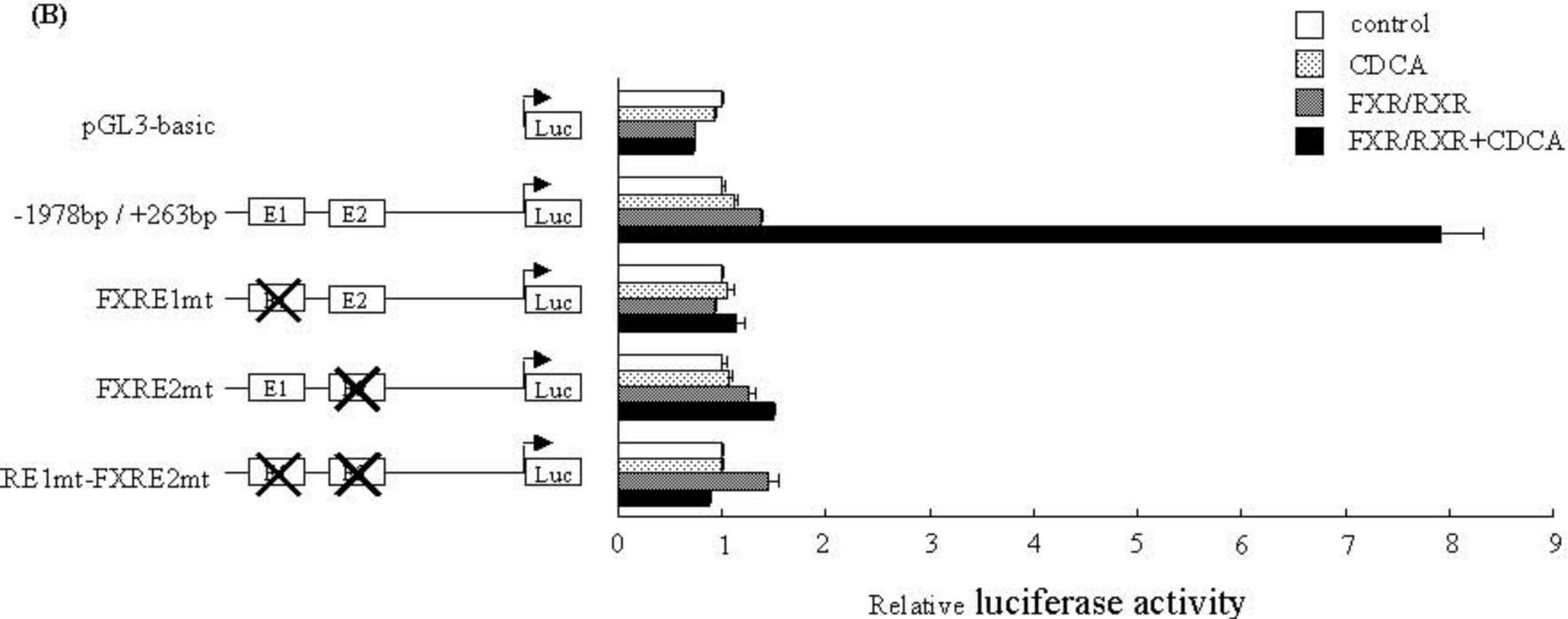
(D) Human primary hepatocytes



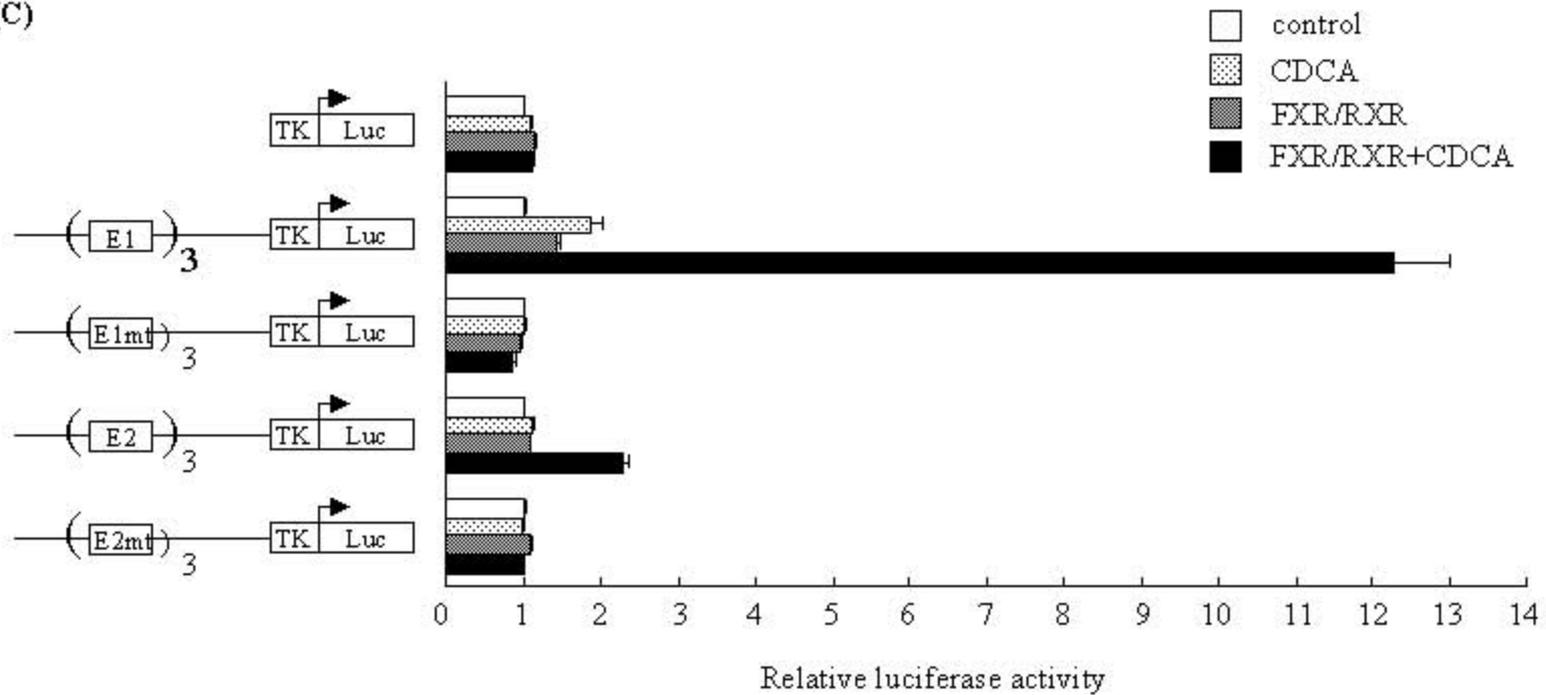
(A)

	<b>FXRE 1</b> →
Human Fetuin B P2 promoter	GTCAGCACTGAGAAGGGTGAAAATAAATCAGTTCCCA <b>GGGCATTGTCCT</b>
Mouse genome sequence	GGCCAGCATGATAGGGATGAAAAACAAGCCAGTCCCTGAGATCATTGTCCT
Bovine genome sequence	GTCGGCACTGGGAAAGGTGAAAACAAATTCATTCCCAAGATCGATGTCCT
Canis genome sequence	GCCCGCGCTGAGAAGGGTAGAAACAAATTAGCTCCTGAGGTCATTGTCCT
	* *        * * * * * * * *        * *    * * * * * * * *
	<b>FXRE 2</b> →
Human Fetuin B P2 promoter	CTGATGTAAGCCCACCAG <b>AGGTCAGGGACCT</b> TTTGC GTTAGCAGAAGCTGG
Mouse genome sequence	CTGATGTACCC----TGGAGGCCAATGATCCTTGCATCCAGGAAACCAGA
Bovine genome sequence	CTAATGTAAGCCCACCAGAGGTCAGGGACCCCTTGCATTAGCAGAAGCTGT
Canis genome sequence	CTGATGTAAGCCTAC-AGAGGCCAGGGACCCCTTGCAGTAGTAGAAATTGG
	* * * * * * * *    *        * * * * * * * *        * *        * *

(B)



(C)





(B)

