

Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor

Xiulong Song, Yuan Chen, Leila Valanejad, Rajani Kaimal, Bingfang Yan, Matthew Stoner, and Ruitang Deng¹

Department of Biomedical and Pharmaceutical Sciences, Center for Pharmacogenomics and Molecular Therapy, University of Rhode Island, Kingston, RI 02881

Abstract Expression of bile salt export pump (BSEP) is regulated by the bile acid/farnesoid X receptor (FXR) signaling pathway. Two FXR isoforms, FXR α 1 and FXR α 2, are predominantly expressed in human liver. We previously showed that human BSEP was isoform-dependently regulated by FXR and diminished with altered expression of FXR α 1 and FXR α 2 in patients with hepatocellular carcinoma. In this study, we demonstrate that FXR α 1 and FXR α 2 regulate human BSEP through two distinct FXR responsive elements (FXRE): IR1a and IR1b. As the predominant regulator, FXR α 2 potently transactivated human BSEP through IR1a, while FXR α 1 weakly transactivated human BSEP through a newly identified IR1b. Relative expression of FXR α 1 and FXR α 2 affected human BSEP expression *in vitro* and *in vivo*. Electrophoretic mobility shift and chromatin immunoprecipitation assays confirmed the binding and recruitment of FXR α 1 and FXR α 2 to IR1b and IR1a. Sequence analysis concluded that IR1b was completely conserved among species, whereas IR1a exhibited apparent differences across species. Sequence variations in IR1a were responsible for the observed species difference in BSEP transactivation by FXR α 1 and FXR α 2. **In conclusion, FXR regulates BSEP in an isoform-dependent and species-specific manner through two distinct FXREs, and alteration of relative FXR isoform expression may be a potential mechanism for FXR to precisely regulate human BSEP in response to various physiological and pathological conditions.**—Song, X., Y. Chen, L. Valanejad, R. Kaimal, B. Yan, M. Stoner, and R. Deng. **Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor.** *J. Lipid Res.* 2013. 54: 3030–3044.

Supplementary key words bile acid transporter • canalicular secretion • gene transcription

This work was supported by the National Institutes of Health Grants R01 DK-087755, R01 GM-61988 (B.Y.), and R01 ES-07965 (B.Y.). The RI-INBRE Core Facility is supported by an Institutional Development Award (IDeA) from the National Institutes of General Medical Sciences of the National Institutes of Health under Grant 8 P20 GM-103430-12.

Manuscript received 29 March 2013 and in revised form 18 August 2013.

*Published, JLR Papers in Press, September 3, 2013
DOI 10.1194/jlr.M038323*

Bile acid homeostasis is achieved through an enterohepatic circulation. Canalicular secretion of bile acids through bile salt export pump (BSEP, ABCB11) is the rate-limiting step in the circulation (1, 2). Modulation of BSEP expression or function by inherited or acquired factors has profound impact on biliary and intrahepatic bile acid levels. Indeed, impairment of BSEP function or expression has been directly linked to such diseases as progressive familial intrahepatic cholestasis type 2 (3–6), benign recurrent intrahepatic cholestasis (7–9), intrahepatic cholestasis of pregnancy (10, 11), drug-induced cholestasis (12), and liver cancer (6, 13, 14).

BSEP expression is coordinately regulated by distinct but related transactivation pathways (15–19), notably the bile acids/farnesoid X receptor (FXR, NR1H4) signaling pathway (15, 16). Activation of FXR by bile acids strongly induces BSEP expression *in vitro* and *in vivo* (15, 16). Such feed-forward regulation of BSEP by bile acid/FXR is considered a major mechanism for preventing excessive accumulation of toxic bile acids in hepatocytes. Two FXR genes, FXR α and FXR β , have been identified (20–22). FXR α is functional in all the species tested, while FXR β is a pseudogene in humans (22). Four isoforms of FXR α (FXR α 1–4) have been identified as a result of alternative promoter and splicing (23, 24). Isoforms FXR α 1 and FXR α 2 are predominantly expressed in the liver and adrenal glands, whereas isoforms FXR α 3 and FXR α 4 are primarily expressed in the kidneys and intestines in humans (23).

Abbreviations: BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; ChIP, chromatin immunoprecipitation; DCA, deoxycholic acid; DIC, differential interference contrast; EMSA, electrophoretic mobility shift assay; ER2, everted repeat separated by two nucleotides; FXR, farnesoid X receptor; FXRE, FXR responsive element; FXREc, FXRE consensus; GDCA, glycodeoxycholic acid; GUG, guggulsterone; HCC, hepatocellular carcinoma; IR1, inverted repeat with one nucleotide space; LCA, lithocholic acid; NR1H4, nuclear receptor subfamily 1, group H, member 4; UDCA, ursodeoxycholic acid; wt, wild-type.

¹To whom correspondence should be addressed.
e-mail: dengr@mail.uri.edu

Copyright © 2013 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at <http://www.jlr.org>

We have recently showed that human BSEP expression was severely diminished in patients with hepatocellular carcinoma (HCC) and that such decreased expression of BSEP correlated with altered relative expression of FXR α 1 and FXR α 2 (25). Similar to human BSEP, FXR α 2 showed more potent activity than FXR α 1 in transactivating other hepatic FXR targets, including human syndecan-1 and fibrinogens (26, 27), whereas mouse bsep and small heterodimeric partner (SHP) were regulated by FXR α 1 and FXR α 2 to similar degrees (24). Currently, the underlying mechanisms for such isoform-dependent and species-specific regulation of BSEP by FXR are not understood.

In this study, we demonstrate that FXR α 1 and FXR α 2 transactivated human BSEP through two distinct FXR responsive elements (FXRE) that are inverted repeats spaced by one nucleotide (IR1): IR1b and IR1a, respectively. In addition to total FXR expression levels, relative expression of FXR α 1 and FXR α 2 affected human BSEP expression in vitro and in vivo. Further study revealed that sequence variations in IR1a were responsible for the observed species difference in transactivation of BSEP by FXR α 1 and FXR α 2.

EXPERIMENTAL PROCEDURES

Reagents and suppliers

Chemicals and reagents for cloning, site-directed mutagenesis, polymerase-chain reaction (PCR), gel-shift assays, cell cultures, transfection, and luciferase assays were described previously (28).

Plasmid constructs

Human, mouse, and rat bsep promoter reporters and mutants, including phBSEP (-2.6kb), pmBSEP(-2.6kb), prBSEP(-2kb), phBSEP(-125b), and phBSEP(-2.6kb)-IR1a-Mut, were prepared previously (18, 28, 29). Human pTK-3xhIR1a, mouse/rat pTK-3xm/rIR1a, and pTK-3xIR1b reporters were constructed by cloning three copies of the corresponding element into pTK-Luc vector. Element reporters pGL3/p-2xhIR1a and pGL3/p-2xhER2/IR1a were prepared by cloning two copies of IR1a and ER2/IR1a element in human BSEP promoter into the pGL3/p vector, respectively. Element reporters, including pElement 1–6, were generated by cloning the corresponding element into the pGL3/p vector. Expression plasmids for human nuclear receptors FXR α 2 and FXR α 1 were kindly provided by Dr. Matthew Stoner and Dr. David Mangelsdorf (University of Texas Southwestern Medical Center), respectively.

Liver samples

Snap-frozen healthy human liver samples were obtained through the Mid-Atlantic Division of the Cooperative Human Tissue Network (CHTN) (University of Virginia). The protocol for using human tissues was approved by the Institutional Review Board (IRB) at the University of Rhode Island.

Site-directed mutagenesis

Mutations were introduced into the templates using a QuickChange site-directed mutagenesis kit according to the manufacturer's manual (Stratagene) (29). Human BSEP promoter mutants phBSEP(-2.6kb)-IR1b-Mut, phBSEP(-2.6kb)-IR1ab-Mut, and phBSEP(-2.6kb)-ER2-Mut were prepared by mutating the IR1b, IR1a and IR1b, and half ER2 (everted repeat separated by two nucleotides) sites using phBSEP(-2.6kb) as a template. Six, four, and two nucleotides were mutated in IR1a (GACGCGTGAATTC),

IR1b (ATGCCCTTACT), and the half ER2 site (AACCT), respectively (mutated nucleotides are underlined). Rat bsep promoter mutants prBSEP(-2kb)-IR1a-Mut, prBSEP(-2kb)-IR1b-Mut, prBSEP(-2kb)-IR1ab-Mut, and prBSEP(-2kb)-ER2-Mut were prepared by mutating the IR1a, IR1b, IR1a and IR1b, and the half ER2 site using prBSEP(-2kb) as templates. Three, four, and three nucleotides were mutated in IR1a (GGGACATTGATCT), IR1b (ATGCCCTTACT), and the half ER2 site (AAACT), respectively. Replacement of human IR1a (hIR1a) with mouse/rat IR1a (m/rIR1a) was achieved through mutagenesis using phBSEP(-2.6kb) as the template, resulting in a chimeric reporter phBSEP(-2.6kb)-m/rIR1a. Chimeric reporter prBSEP(-2kb)-hIR1a was made through mutagenesis by replacing the mouse/rat IR1a (m/rIR1a) with the human IR1a (hIR1a) using prBSEP(-2kb) as the template. All the mutants were subjected to sequence confirmation.

Immunohistofluorescence

Normal human liver tissue sections embedded in paraffin (ab4348) were obtained from Abcam (Cambridge, MA). Deparaffinization, antigen retrieval, and immune staining of the sections were carried out basically according to the protocol recommended by the manufactures. Nonspecific binding was blocked by using 5% BSA in 1 \times TBS containing 0.1% TX-100 for 2 h on shaker at room temperature. Three types of primary antibodies (Abs) were applied to individual slides, including rabbit anti-FXR α 1-specific Abs (custom-made by the NeoBioLab, Woburn, MA), rabbit anti-FXR Abs (sc-13063), and rabbit IgG (sc-2027, Santa Cruz Biotechnology) as negative controls. AlexaFluor 594 goat anti-rabbit Abs (A11012, Invitrogen) was used as the secondary Abs. The sections were then mounted under a glass cover slip using Vectashield mounting medium containing 1.5 μ g/ml DAPI (H-1500, Vector Laboratories, Burlingame, CA) as nuclei counterstaining. Images were captured under a confocal microscope at a magnification of 40 \times (Zeiss AxioImager M2 Imaging System) with fluorescent and differential interference contrast (DIC) settings.

Reporter luciferase assay

The reporter luciferase activity assays were carried out using the human hepatoma Huh 7 cells and rat hepatocellular carcinoma

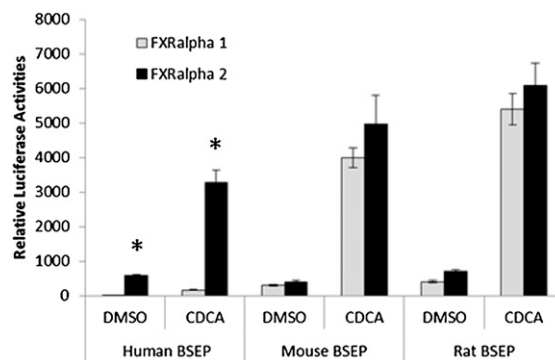


Fig. 1. Transactivation of BSEP by FXR α 1 and FXR α 2. Human, mouse, and rat bsep promoter reporters phBSEP(-2.6kb), pmBSEP(-2.6kb), and prBSEP(-2kb) were cotransfected into human hepatoma Huh 7 cells with either FXR α 1 or FXR α 2 expression plasmid, followed by treatment of the transfected cells with vehicle DMSO (0.1%) or CDCA (10 μ M) for 30 h. Reporter transactivation levels as luciferase activities were detected with a dual-luciferase reporter assay system. The firefly luminescence was normalized based on the *Renilla* luminescence signal. The data are presented as mean \pm SD of at least three separate experiments. * P < 0.05 between DMSO and CDCA-treated cells (Student *t*-test).

(HCC) L0 cells (kindly provided by Dr. Jonathan Reicher, Brown University) in a 24-well plate as described previously (28).

Electrophoretic mobility shift assays

Nuclear extracts of Huh 7 cells transfected with FXR α 1 or FXR α 2 were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). The electrophoretic mobility shift assays (EMSA) were performed using the LightShift Chemiluminescent EMSA kit (Pierce) as described (17). Supershift assays were carried out using polyclonal Abs against human FXR (sc-13063, Santa Cruz Biotechnologies).

Chromatin immunoprecipitation assays

Huh 7 cells seeded in 6-well plates were cotransfected with 400 ng of either FXR α 1 or FXR α 2 and either 400 ng phBSEP

(-2.6kb), phBSEP(-2.6kb)-IR1a-Mut, phBSEP(-2.6kb)-IR1b-Mut, or phBSEP(-2.6kb)-IR1ab-Mut. Sixteen hours posttransfection, cells were treated with 10 μ M CDCA for 24 h. Chromatin preparation and immunoprecipitation (ChIP) assays were performed essentially as described previously (28). A test set of primers, BSEP(-260b) and pGL4-R(122), flanking IR1a and IR1b were used to amplify a 462 bp fragment encompassing sequence from nucleotides -260 to +80 in the BSEP promoter and 122 nucleotides in the luciferase gene. A negative control set of primers was included in the assays (28).

Living imaging with in vivo imaging system

Sixteen female CD-1 mice between ages 5 and 8 weeks were obtained from Charles River Laboratory (Wilmington, MA) and randomly divided into four groups. Each group of mice was

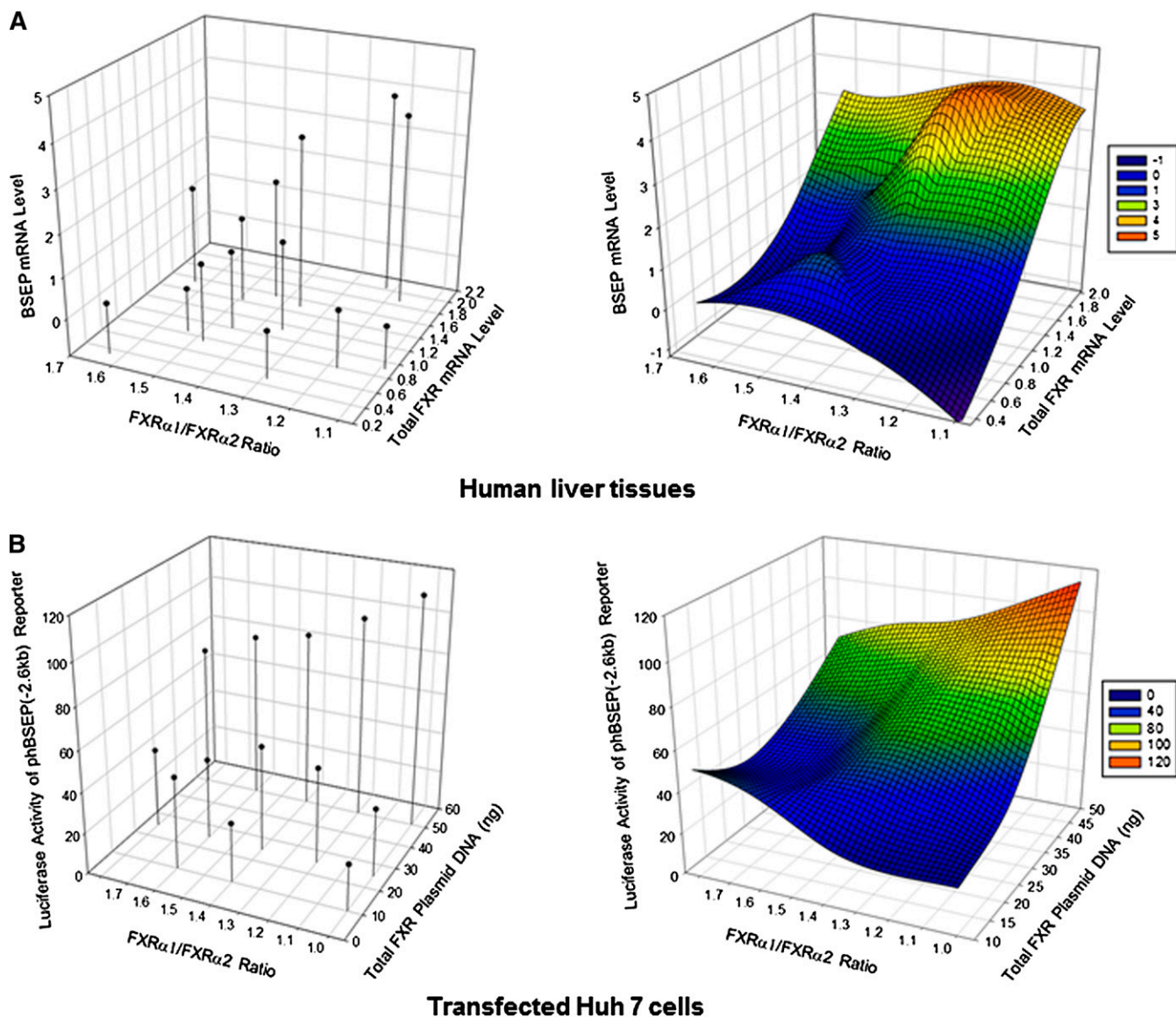


Fig. 2. The effects of relative expression levels of FXR α 1 and FXR α 2 on BSEP expression in human liver tissues and Huh 7 cells. (A) mRNA expression levels of BSEP and total FXR in healthy human liver tissues were determined by TaqMan real-time PCR and plotted against FXR α 1/FXR α 2 ratios using SigmaPlot version 11.0. The cycle time (Ct) values Ct24.92 for FXR and Ct27.12 for BSEP of one tissue sample were designated as a relative unit 1. (B) A series of mixtures of FXR α 1 and FXR α 2 plasmid DNA in various FXR α 1/FXR α 2 ratios (from 1.0, 1.2, 1.4, 1.6 to 1.8) with a constant amount of total FXR at 10, 25, or 50 ng were cotransfected with human BSEP promoter reporter in Huh 7 cells, followed by treatment of the transfected cells with CDCA for 30 h and subsequent detection of luciferase activity. FXR α 1/FXR α 2 ratios were plotted against total FXR and BSEP promoter transactivation as luciferase activities using SigmaPlot version 11.0. The left figure shows the level and position of each individual sample in the 3D diagram. The right figure is the smooth 3D model based on the data shown on the left.

hydrodynamically injected with phBSEP(-2.6kb), prBSEP(-2kb), phBSEP(-2.6kb)-m/rIR1a, or prBSEP(-2kb)-hIR1a promoter reporter as described previously (25). Luciferase activities were detected by living imaging with in vivo imaging system (IVIS) 48 h postinjection and captured by a CCD camera with an exposure time of 45 s (25). The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Rhode Island (URI) and followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Quantitative real-time PCR

Total RNA extraction from 14 healthy human and 8 CD-1 mice liver tissues and subsequent cDNA synthesis and TaqMan real-time PCR detection for total FXR and BSEP were carried out as reported (17, 25).

Semiquantification of FXR α 1 and FXR α 2

By using human liver cDNA samples as templates, the relative FXR α 1 and FXR α 2 mRNA levels were determined by the semiquantification methods described previously (25).

Statistical analysis

Student *t*-test was applied to pair-wise comparison to determine the statistical significance. Nonparametric Mann-Whitney test was used for pair-wise comparison for nonnormally distributed data. Values of 0.05 or less were considered significant.

RESULTS

Isoform-dependent and species-specific regulation of BSEP transactivation by FXR

Four FXR α isoforms (FXR α 1–4) have been identified, with predominant expression of FXR α 1 and FXR α 2 in the liver (23, 24). Our previous study showed that human BSEP was regulated by FXR in an isoform-dependent manner and that alteration of isoform expression was

associated with dysregulation of BSEP in patients with HCC (25). In this study, we extended such finding by testing FXR isoform-mediated transactivation of BSEP from three species, including human, mouse, and rat. Consistent with our previous findings, FXR α 2 exhibited much more potent activity in transactivating human BSEP than FXR α 1 in the presence or absence of endogenous FXR agonist chenodeoxycholic acid (CDCA) (Fig. 1). In contrast, both mouse and rat bsep were transactivated by FXR α 1 and FXR α 2 in comparable levels (Fig. 1). The results demonstrated that human BSEP was differentially regulated by FXR α 1 and FXR α 2 with FXR α 2 being the predominant regulator, whereas mouse and rat bsep were similarly transactivated by the two isoforms.

Relative FXR isoform and total FXR expression affected the expression levels of human BSEP

In our previous studies, it was shown that the relative expression levels of FXR α 1 and FXR α 2 correlated with BSEP expression (25). In this study, the comprehensive relationships among FXR isoforms, total FXR expression, and BSEP expression were further investigated. As shown in Fig. 2A, the expression levels of BSEP and total FXR in healthy human liver samples were plotted against FXR α 1/FXR α 2 ratios using SigmaPlot 3D program. Over 27- and 6-fold differences in BSEP and total FXR expression, respectively, were detected among the liver samples. Consistent with FXR's transactivating role in BSEP regulation, BSEP expression increased along with increase in total FXR. However, the relationship between FXR α 1/FXR α 2 ratios and BSEP expression did not exhibit a single trend (Fig. 2A). At higher levels of total FXR, as expected, BSEP expression decreased as the FXR α 1/FXR α 2 ratios increased. However, at lower levels of total FXR, BSEP expression increased as the FXR α 1/FXR α 2 ratios increased. The data thus indicated that BSEP expression in human

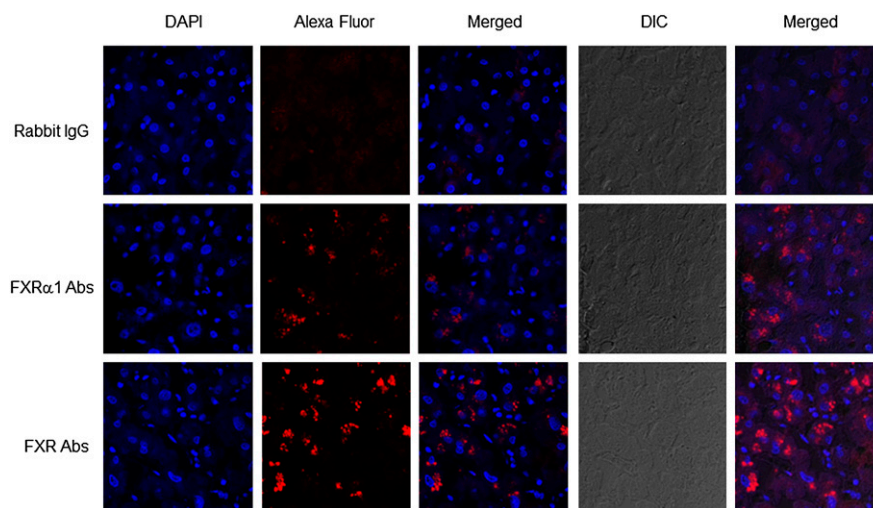


Fig. 3. Expression and localization of FXR α 1 and total FXR in human liver tissues. Liver sections were subjected to immunohistofluorescent assays. FXR α 1- and FXR-specific Abs and rabbit IgG as negative controls were used as primary Abs. Goat anti-rabbit Abs labeled with AlexaFluor 594 was used as the secondary Abs. Mounting medium containing DAPI (1.5 μ g/ml) was used as nuclei counterstaining. Images were captured under a confocal microscope at a magnification of 40 \times with both fluorescent and DIC settings.

livers was dictated by the FXR α 1/FXR α 2 ratios as well as by total FXR expression levels.

To directly evaluate the effect of FXR α 1/FXR α 2 ratios at various total FXR levels on BSEP transactivation in vitro, a series of mixtures of FXR α 1 and FXR α 2 plasmid DNA in various ratios (from 1:1 to 1.8:1) at various amounts of total FXR (10, 25, or 50 ng) were cotransfected with the human BSEP promoter reporter in Huh 7 cells, followed by treatment with CDCA. As shown in Fig. 2B, a relationship pattern comparable to human liver samples (Fig. 2A) was observed in transfected Huh 7 cells. BSEP transactivation gradually decreased as the FXR α 1/FXR α 2 ratios increased at a high level of total FXR plasmid DNA (50 ng) but increased at a low level of total FXR plasmid DNA (10 ng). The minor differences between the two data sets (Fig. 2) may reflect the fact that the sample size of human liver samples was relatively small. Taken together, the results demonstrated that the ratio of FXR α 1/FXR α 2 played an important role in

determining human BSEP transactivation and that its effects were influenced by the total FXR expression levels.

Expression and localization of FXR α 1 and total FXR protein in human liver tissue

Immunohistofluorescence was carried out to investigate the expression and localization of total FXR and FXR α 1. In our attempt to generate FXR isoform-specific Abs, antiserum raised against FXR α 1-specific peptide detected FXR α 1 protein specifically, whereas antiserum raised against FXR α 2-specific peptide recognized both FXR α 2 and FXR α 1 protein (data not shown) because there are only four extract amino acids inserted in FXR α 1 compared with FXR α 2. Therefore, FXR α 1-specific Abs and Abs recognizing both FXR α 1 and FXR α 2 were used in the immunohistofluorescent assays. As shown in Fig. 3, minimal red fluorescent signals were detected with rabbit IgG as negative controls while FXR α 1 and total FXR proteins were readily detected with FXR α 1- and FXR-specific Abs. Both FXR α 1 and total FXR proteins were predominantly present in the cytoplasm. As expected, relatively weaker signals were detected with FXR α 1-specific Abs than with FXR-specific Abs.

Isoform-dependent transactivation of human BSEP by FXR was not due to a difference in ligand-mediated activation

In the previous experiments, the potent endogenous FXR agonist CDCA was used in evaluating human BSEP transactivation by FXR isoforms. To determine whether FXR α 1- and FXR α 2-mediated differential transactivation of human BSEP is related to specific FXR ligands, a series of FXR endogenous and exogenous ligands were tested for their ability to induce FXR α 1- and FXR α 2-mediated transactivation of human BSEP. The ligands tested include six endogenous bile acids, including cholic acid (CA), CDCA, deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) and glycodeoxycholic acid (GDCA), a natural product guggulsterone, and two synthetic FXR agonists GW4064 and T0901317. As shown in Fig. 4A, all the FXR ligands exhibited similar FXR isoform-dependent transactivation of human BSEP regardless their agonistic potency. FXR α 2 consistently transactivated human BSEP at a much higher level than FXR α 1 with each of the ligands.

In contrast to human BSEP, both mouse and rat bsep promoters exhibited comparable transactivation activities by FXR α 1 and FXR α 2, with slightly higher levels with FXR α 2 in the presence of CDCA in Huh 7 cells (Fig. 1). Considering the possibility that rat or mouse bsep may exhibit a different transactivation pattern by FXR α 1 and FXR α 2 in rat or mouse cells, rat bsep promoter reporter prBSEP(-2kb) was cotransfected with FXR α 1 or FXR α 2 in rat HCC L0 cells, followed by treatment with various FXR ligands. As shown in Fig. 4B, consistent with the data from Huh 7 cells, FXR α 1 and FXR α 2 exhibited similar transactivation levels with most FXR ligands, except for CDCA and LCA. FXR α 2 transactivated rat bsep more potently than did FXR α 1 with CDCA and LCA. However, it should be emphasized that the differences between FXR α 2 and FXR α 1 in transactivating human BSEP in Huh 7 cells were

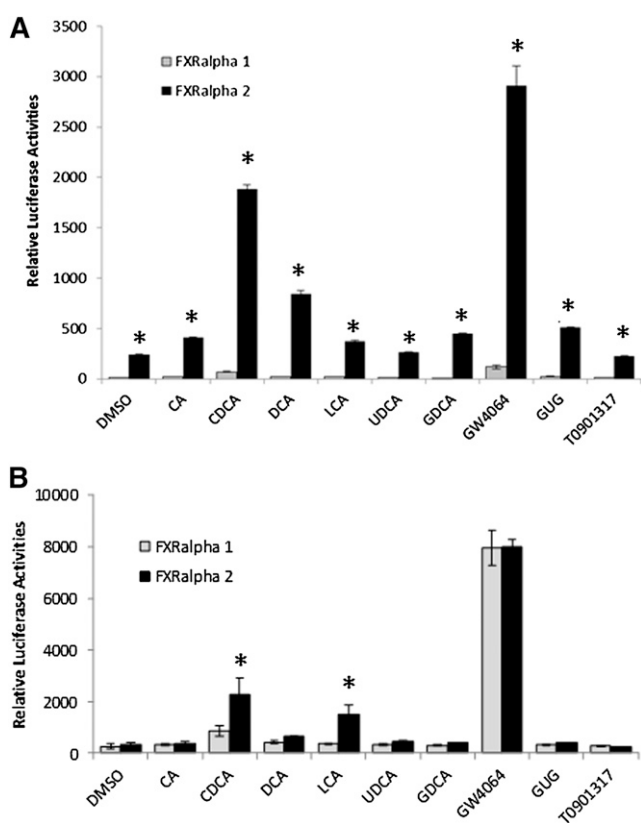


Fig. 4. Transactivation of BSEP promoters by FXR α 1 and FXR α 2 with various FXR ligands. (A) Human BSEP promoter reporter phBSEP(-2.6kb) and (B) rat bsep promoter reporter prBSEP(-2kb) were cotransfected into human hepatoma Huh 7 cells and rat HCC L0 cells, respectively, with FXR α 1 or FXR α 2, followed by treatment of transfected cells with various endogenous and exogenous FXR ligands, including CA (10 μ M), CDCA (10 μ M), DCA (10 μ M), LCA (10 μ M), UDCA (10 μ M), GDCA (10 μ M), GW4064 (1 μ M), GUG (10 μ M), and T0901317 (1 μ M). Thirty hours post treatment, reporter transactivation levels as luciferase activities were detected with a dual-luciferase reporter assay system. The firefly luminescence was normalized based on the *Renilla* luminescence signal. The data are presented as mean \pm SD of at least three separate experiments. * P < 0.05 between FXR α 1 and FXR α 2-transfected cells (Student *t*-test).

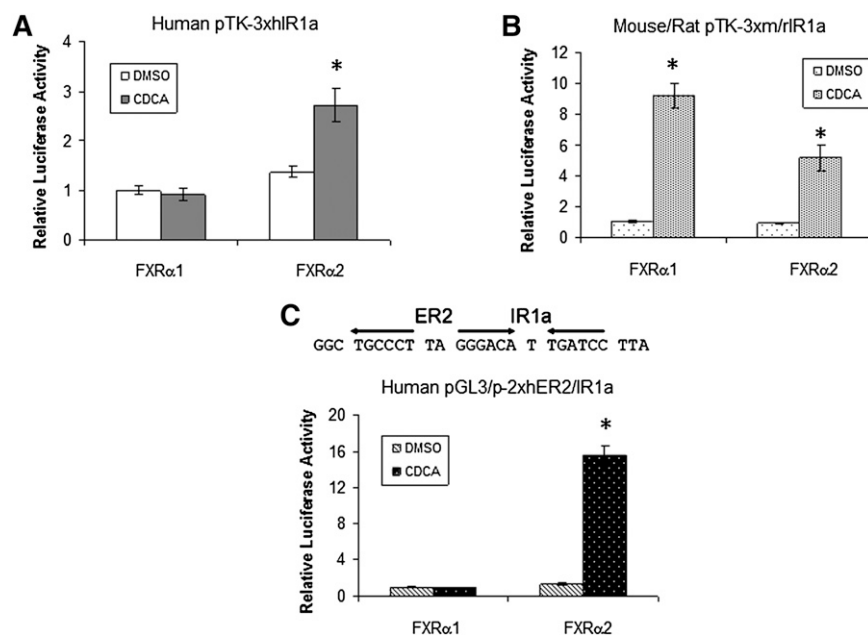


Fig. 5. Isoform-dependent and species-specific transactivation of IR1a reporters by FXR α 1 and FXR α 2. (A) Human BSEP IR1a reporter pTK-3xhIR1a, (B) mouse/rat bsep IR1a reporter pTK-3xm/rIR1a, and (C) human BSEP ER2/IR1a reporter pGL3/p-2xhER2/IR1a were cotransfected with FXR α 1 or FXR α 2, followed by treatment of the cells with DMSO (0.1%) or CDCA (10 μ M) for 30 h. Reporter luciferase activities were detected with the dual-luciferase reporter assay system. The data are presented as mean \pm SD of at least three repeated experiments. * P < 0.05 between DMSO and CDCA-treated cells (Student t -test).

much more striking than those with rat bsep in rat HCC L0 cells. FXR α 2 was 27.1- or 18.9-fold more potent than FXR α 1 in transacting human BSEP in Huh 7 cells in the presence of CDCA or LCA, whereas only 2.6- or 4.1-fold differences were observed between FXR α 2 and FXR α 1 in transactivating rat bsep in L0 cells with CDCA or LCA.

Taken together, the data indicated that the isoform-dependent transactivation of human BSEP by FXR was not due to a difference between FXR α 1 and FXR α 2 in ligand binding or ligand-mediated activation. On the other hand, FXR α 1 and FXR α 2 transactivated rat bsep comparably with most of the FXR ligands.

Isoform-dependent and species-specific transactivation of BSEP by FXR was due to a difference in FXRE in BSEP promoters

Early studies identified an FXRE (named IR1a) in the BSEP promoter (15, 16). To determine whether the isoform-dependent and species-specific transactivation of BSEP is due to a differential activation of the IR1a by FXR α 1 and FXR α 2, human and mouse/rat IR1a (hIR1a and m/rIR1a) reporters were constructed and tested for their ability to support FXR α 1 or FXR α 2 transactivation. It should be mentioned that the IR1a sequences are completely conserved in mouse and rat bsep promoters. As shown in **Fig. 5A**, FXR α 2 significantly transactivated hIR1a reporter, whereas no transactivation was detected in cells transfected with FXR α 1. Conversely, both FXR α 1 and FXR α 2 strongly transactivated m/rIR1a reporter (**Fig. 5B**). The results thus demonstrated that hIR1a only supported FXR α 2 transactivation, while m/rIR1a was capable of mediating both FXR α 1 and FXR α 2 transactivation.

Previous studies revealed that half nuclear receptor binding site and ER2 (everted repeat separated by two nucleotides) are commonly found adjacent to the IR1 (30). A half ER2 motif was identified immediately upstream the IR1a (**Fig. 5C**). Inclusion of the motif in hER2/IR1a reporter significantly enhanced FXR α 2-mediated transactivation (**Fig. 5C**), indicating that the half ER2 motif is required for maximal transactivation of human BSEP by FXR α 2. However, FXR α 1 still failed to transactivate the hER2/IR1a reporter, suggesting that FXR α 1 must regulate human BSEP through an additional FXRE.

FXR α 1 transactivated human BSEP through IR1b, a newly identified FXRE

To determine whether the potential additional FXRE is present in the minimal human BSEP promoter, the resultant phBSEP(-125b) reporter was cotransfected with FXR α 1, followed by treatment with DMSO or CDCA. As shown in **Fig. 6A**, similar to the full-length promoter reporter phBSEP(-2.6kb), FXR α 1 significantly transactivated the minimal promoter reporter, indicating that the potential additional FXRE is located in the minimal promoter region.

Since no FXREs were predicted by bioinformatic analysis in the region, a series of element reporters covering the entire region were constructed and evaluated for their ability to respond to FXR α 1 (**Fig. 6B, C**). Reporters pElement 1 and 4 exhibited significant induction of activity in response to CDCA in the presence of FXR α 1, whereas no induction was detected with other reporters. The data thus mapped a new functional FXRE (named IR1b) in the overlapped region between pElement 1 and 4 at positions from

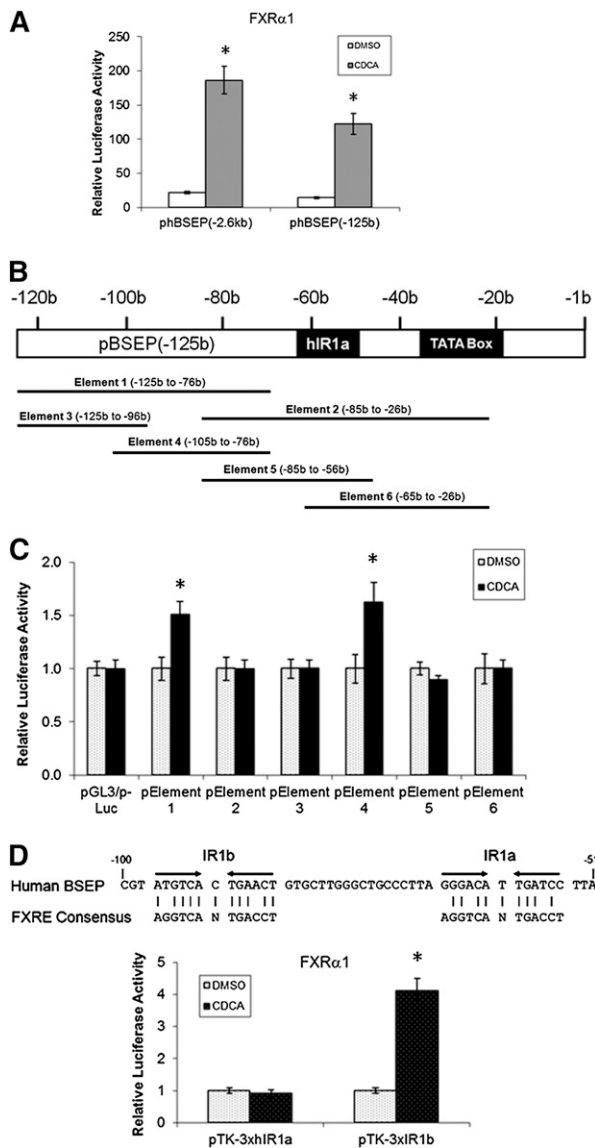


Fig. 6. FXR α 1 transactivated human BSEP through a newly identified FXRE, IR1b. (A) Full-length and minimal human BSEP promoter reporters phBSEP(-2.6kb) and phBSEP(-125b) were cotransfected with FXR α 1 in Huh 7 cells. Sixteen hours posttransfection, cells were treated with DMSO (0.1%) and CDCA (10 μ M) for 30 h, followed by detection luciferase activities with dual-luciferase reporter assay system. The data are presented as mean \pm SD of at least three repeated experiments. (B) A diagram showing the positions of the six elements in the minimal promoter region. (C) The six elements were cloned into the pGL3/p-Luc vector, resulting in six element reporters, pElement 1 to 6. Activation of the six element reporters by FXR α 1 in the presence of CDCA (10 μ M) was detected by the dual-luciferase reporter assay system. The data are presented as mean \pm SD of at least three repeated experiments. * P < 0.05 between DMSO and CDCA-treated cells (Student t -test). (D) The location and sequence of the newly identified IR1b are shown in the upper panel. Transactivation of hIR1a and IR1b reporters by FXR α 1 are presented in the lower panel. Reporters pTK-3xhIR1a and pTK-3xIR1b were cotransfected into Huh 7 cells with FXR α 1, followed by treating the cells with DMSO (0.1%) or CDCA (10 μ M) for 30 h prior to detection of luciferase activities as described in (A). * P < 0.05 between DMSO and CDCA-treated cells (Student t -test).

-97b to -85b (Fig. 6D). The IR1b was physically close to IR1a (18 bases from each other). To confirm that the newly identified IR1b is functional in supporting FXR α 1 transactivation, IR1b reporter pTK-3xIR1b was constructed and tested. As shown in Fig. 6D, FXR α 1 moderately transactivated IR1b reporter in the presence of CDCA, confirming that FXR α 1 regulates human BSEP through IR1b.

Mutational analysis of IR1a and IR1b in supporting FXR α 1- and FXR α 2-mediated transactivation of human and rat bsep

To determine the relative contribution of IR1a, IR1b, and the half ER2 sites to the transactivation of human and rat bsep by FXR α 1 and FXR α 2, mutational analyses were performed in those elements. Double mutation in IR1a and IR1b was also introduced.

For human BSEP, similar to the IR1a and IR1b double mutant, IR1b mutant completely lost its ability to respond to FXR α 1 (Fig. 7A), suggesting that FXR α 1 regulates human BSEP through IR1b. Although mutation in IR1a decreased FXR α 1-mediated transactivation, significant induction remained (Fig. 7A). More significantly, the fold induction by CDCA over DMSO was comparable between IR1a mutant and wild-type (wt) (13.1 versus 11.3), indicating that IR1a is required for basal activity but not FXR α 1-mediated transactivation. Conversely, mutation in IR1a almost entirely abolished FXR α 2-mediated transactivation as did the double mutant (Fig. 7B), consistent with regulation of human BSEP by FXR α 2 through IR1a. Similarly, the IR1b mutant retained the ability to respond to CDCA in the presence of FXR α 2 with almost identical fold inductions as the wt (7.9 versus 7.4) (Fig. 7B). Mutation in the half ER2 motif markedly reduced FXR α 2- but not FXR α 1-mediated transactivation (Fig. 7A, B). Taken together, the data demonstrated that FXR α 1 and FXR α 2 transactivated human BSEP through IR1b and IR1a, respectively, and that the ER2 motif is required for maximal FXR α 2 transactivation.

Similar mutational analyses were performed with rat bsep promoter to evaluate species differences. As shown in Fig. 7C, mutation in IR1a or IR1b resulted in a decrease in FXR α 1-mediated transactivation. However, significant activation by CDCA remained for both mutants. As expected, the double mutant almost completely lost its ability to respond to CDCA. The results indicated that different from human BSEP, FXR α 1 regulated rat bsep through both IR1a and IR1b. Conversely, mutation in IR1a almost totally abolished FXR α 2-mediated transactivation as did the double mutant, indicating that FXR α 2 regulates rat bsep through IR1a. Although mutation in IR1b decreased FXR α 2-mediated transactivation, significant induction by CDCA was detected with a comparable fold induction to the wt (7.7 versus 6.5) (Fig. 7D), indicating that similar to human BSEP, IR1b is required for basal activity but not for FXR α 2-mediated transactivation. Mutation in the half ER2 site had no detectable effect on FXR α 1- and FXR α 2-mediated transactivation (Fig. 7C, D), suggesting that different from human BSEP, the half ER2 in rat bsep does not play any roles in mediating FXR α 2 transactivation.

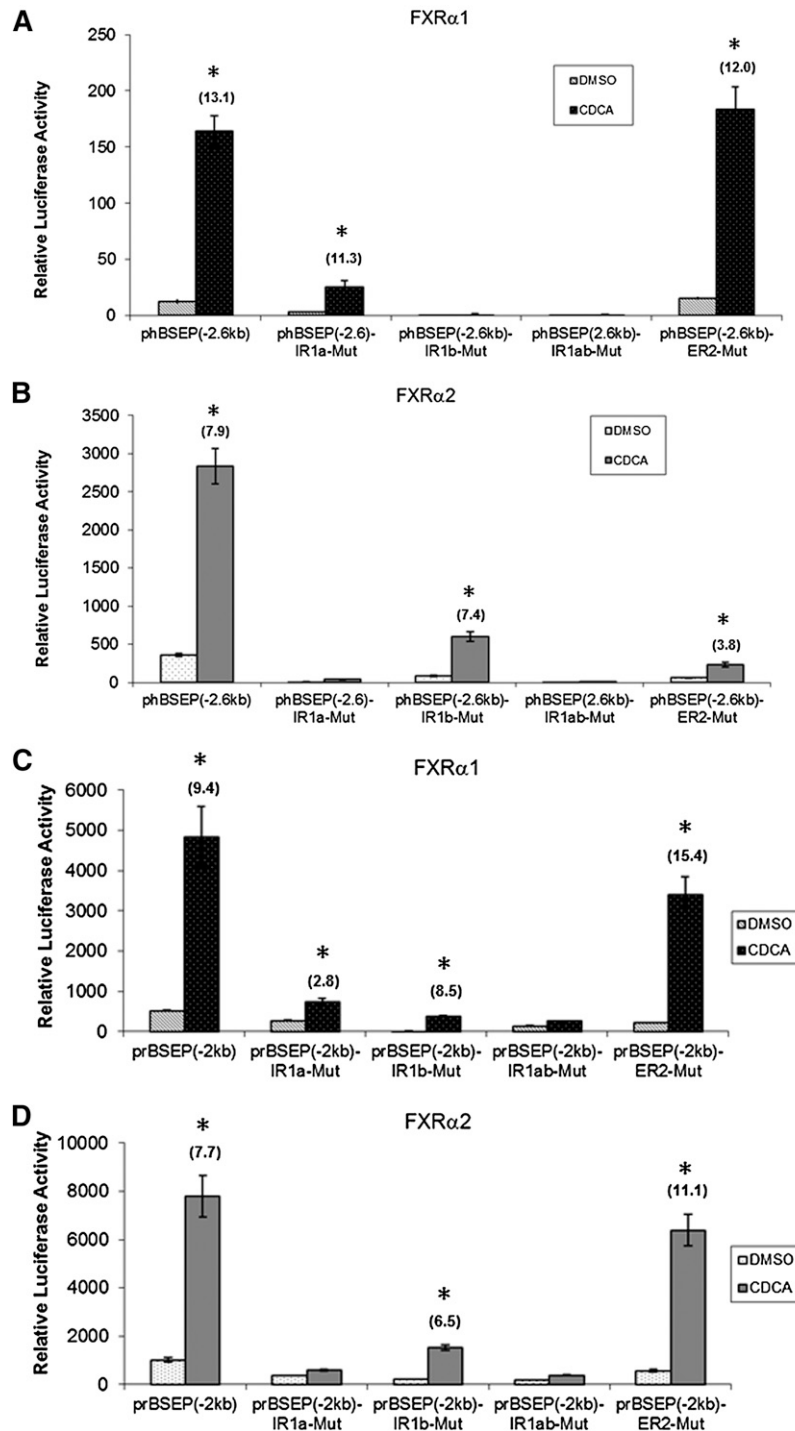


Fig. 7. Mutational analysis of IR1a and IR1b in BSEP promoters. Four human BSEP promoter mutants with mutation in IR1a, IR1b, the half ER2 site, or both IR1a and IR1b were made using human BSEP promoter reporter phBSEP(-2.6kb) as parental template, resulting in mutants phBSEP(-2.6kb)-IR1a-Mut, phBSEP(-2.6kb)-IR1b-Mut, phBSEP(-2.6kb)-IR1ab-Mut, and phBSEP(-2.6kb)-ER2-Mut. Four equivalent mutants were constructed using rat bsep promoter reporter prBSEP(-2kb) as parental template, resulting in prBSEP(-2kb)-IR1a-Mut, prBSEP(-2kb)-IR1b-Mut, prBSEP(-2kb)-IR1ab-Mut, and prBSEP(-2kb)-ER2-Mut. The four human BSEP promoter mutants and wt were cotransfected with (A) FXR α 1 or (B) FXR α 2 into Huh 7 cells. The four rat bsep promoter mutants and wt were cotransfected with (C) FXR α 1 or (D) FXR α 2. Sixteen hours posttransfection, cells were treated with DMSO (0.1%) or CDCA (10 μ M) for 30 h, followed by detection of luciferase activities by the dual-luciferase reporter assay system. The data are presented as mean \pm SD of at least three repeated experiments. * P < 0.05 between DMSO and CDCA-treated cells (Student t -test). The fold inductions by CDCA over DMSO are presented on the top of the bars.

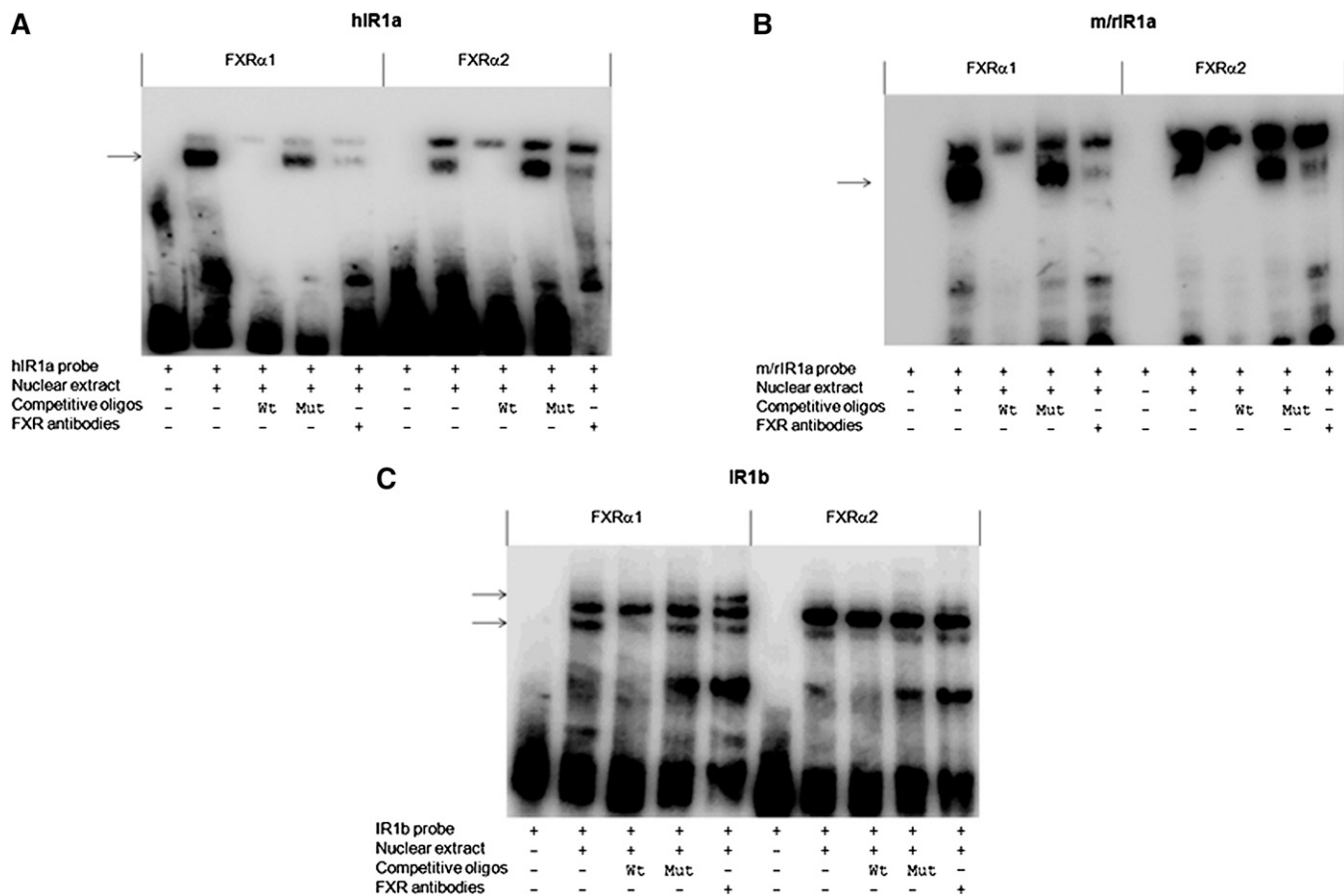


Fig. 8. Binding of FXR α 1 and FXR α 2 to IR1a and IR1b in vitro. EMSAs were performed using (A) human hIR1a, (B) mouse/rat m/rIR1a, or (C) IR1b as probe. Nuclear extracts were prepared from Huh 7 cells transfected with FXR α 1 or FXR α 2. The specificity of the binding was established with competition assays using competing oligonucleotides, including unlabeled or mutated probe with 10 \times concentration. Identification of FXR protein in the shifted complex was carried out by supershift assays using polyclonal Abs against human FXR. The shifted bands representing the specific binding of FXR to the probe or FXR Abs to FXR/DNA complex are indicated by arrows.

Binding of FXR α 1 and FXR α 2 to IR1a and IR1b in vitro

To determine whether FXR isoforms differentially bind to IR1a and IR1b in vitro, a series of EMSA assays were performed. With hIR1a and m/rIR1a probes, two distinct bands were shifted for both FXR α 1 and FXR α 2, indicating that two DNA/protein complexes were formed (Fig. 8A, B). To determine which band represents the specific binding of FXR α 1 or FXR α 2 to the probe, competition assays were carried out. The dominant bottom band was completely competed out by 10 \times unlabeled wt but not by mutated hIR1a or m/rIR1a oligonucleotide, indicating that the bottom band represented the probe-specific complex. To confirm that FXR α 1 or FXR α 2 is part of the complex, supershift assays with Abs against FXR were performed. Preincubation of FXR Abs with nuclear extracts markedly decreased the shifted band, suggesting that the binding of FXR Abs to FXR interferes with its binding to the hIR1a or m/rIR1a probe, decreasing the formation of the DNA/FXR complexes. The data thus demonstrated that both FXR α 1 and FXR α 2 bound to hIR1a or m/rIR1a abundantly in vitro. The abundant binding of FXR α 1 to hIR1a was not expected since FXR α 1 failed to transactivate hIR1a reporter.

With IR1b as a probe, two similar bands were shifted with FXR α 1 and FXR α 2. Competition EMSA assays concluded that the bottom band was the specific one. Incubation of FXR Abs with the complex resulted in a supershifted band (Fig. 8C), indicating that FXR is part of the complex. It should be emphasized that although similarly shifted patterns were observed for both FXR α 1 and FXR α 2, the bands shifted or supershifted with FXR α 1 were much more abundant than those with FXR α 2, suggesting that FXR α 1 has a higher binding affinity to IR1b than does FXR α 2.

FXR α 1 and FXR α 2 were specifically recruited to IR1b and IR1a, respectively, in human BSEP promoter in intact cells

Since IR1a and IR1b are located so close to each other, it was challenging to distinguish FXR recruitment to each individual site on the endogenous BSEP promoter. We used human BSEP promoter reporter wt and mutants to perform ChIP assays. Chromatins were prepared from Huh 7 cells cotransfected with FXR α 1 or FXR α 2 with human BSEP promoter wt, IR1a, IR1b, or IR1ab mutant. With FXR α 1, a PCR band was readily detected in cells transfected

with wt promoter when the chromatin was immunoprecipitated with anti-FXR Abs but not with IgG (Fig. 9A). More importantly, a PCR band with a relatively weaker signal was also detected in cells transfected with IR1a mutant. In contrast, no obvious bands were detected in cells transfected with either IR1b mutant or IR1ab double mutant (Fig. 9A). The data thus established that FXR α 1 was specifically recruited to IR1b but not IR1a in intact Huh 7 cells. On the other hand, with FXR α 2, a PCR band was readily detected in cells transfected with wt and IR1b mutant, whereas no signals were detected in cells transfected with IR1a and IR1ab double mutant (Fig. 9B). The data thus demonstrated that FXR α 2 was specifically recruited to IR1a but not IR1b.

FXR α 1 and FXR α 2 acted independently in transactivating human BSEP

To investigate whether FXR α 1 and FXR α 2 have additive or competitive effects on the transactivation of human BSEP IR1a element reporter and full-length promoter, increasing amounts of FXR α 1 were cotransfected with a constant amount of FXR α 2, followed by detection of the transactivation levels. As shown in Fig. 10A, FXR α 1 competitively decreased FXR α 2-mediated transactivation in cells transfected with IR1a element reporter pGL3/p-2xhIR1a. In contrast, FXR α 1 had minimal effects on FXR α 2-mediated transactivation of the full-length promoter reporter phBSEP (-2.6kb), even at the highest dose (Fig. 10B). The results

indicated that FXR α 1 competitively bound to IR1a with FXR α 2 in the IR1a element reporter, which is consistent with the data from EMSA (Fig. 8A). The results also indicated that no competitive binding of FXR α 1 to IR1a occurred in the full-length promoter, which is consistent with the data from the ChIP assays (Fig. 9A). Such discrepancy in FXR α 1's effects on FXR α 2-mediated transactivation of IR1a element and full-length promoter indicated that the context of IR1a played a determinant role in responding to FXR α 1. It was thus concluded that in the context of full-length promoter, FXR α 1 and FXR α 2 transactivated human BSEP independently through IR1b and IR1a, respectively and that BSEP expression levels were predominately determined by FXR α 2.

Combining the data from transactivation, mutational analysis, EMSA and ChIP assays, it was concluded that FXR α 1 and FXR α 2 regulated human BSEP independently through IR1b and IR1a. On the other hand, mouse and rat bsep are regulated by FXR α 1 through both IR1a and IR1b and by FXR α 2 through IR1a (Fig. 10C).

Sequence differences between hIR1a and m/rIR1a were primarily responsible for the species-specific transactivation of BSEP by FXR α 1 and FXR α 2

Sequence alignment of IR1a and IR1b in BSEP promoters from seven species including human, pongo pygmaeus, pan troglodytes, horse, dog, rat, and mouse revealed that IR1b was completely conserved (Fig. 11A), indicating the

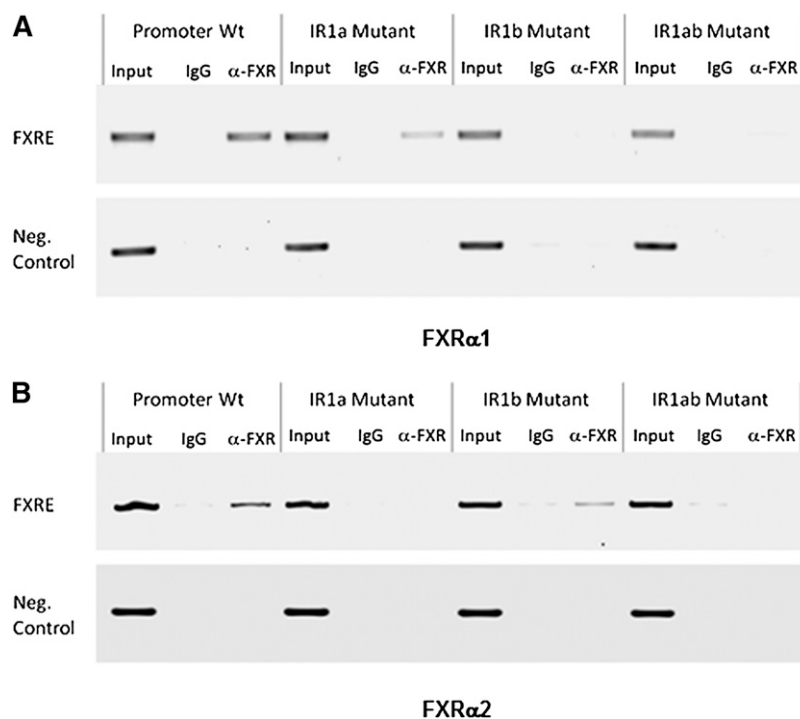


Fig. 9. Recruitment of FXR α 1 and FXR α 2 to IR1a and IR1b in intact cells. ChIP assays were carried out using chromatin prepared from Huh 7 cells cotransfected with (A) FXR α 1 or (B) FXR α 2 with human BSEP promoter reporter wt, IR1a, IR1b, or IR1ab double mutant. After immunoprecipitated with anti-FXR Abs or IgG as negative control, recruitment of FXR α 1 or FXR α 2 to IR1a or IR1b was detected by PCR using a set of primers, BSEP(-260b) and pGL-R(122), flanking the two IR1 sites in the reporter plasmid DNA. A negative control primer set, BSEP(-995b) and BSEP(-805b), was used to amplify a fragment approximately 700b upstream the IR1 sites. Input chromatin DNA was included as a positive PCR control.

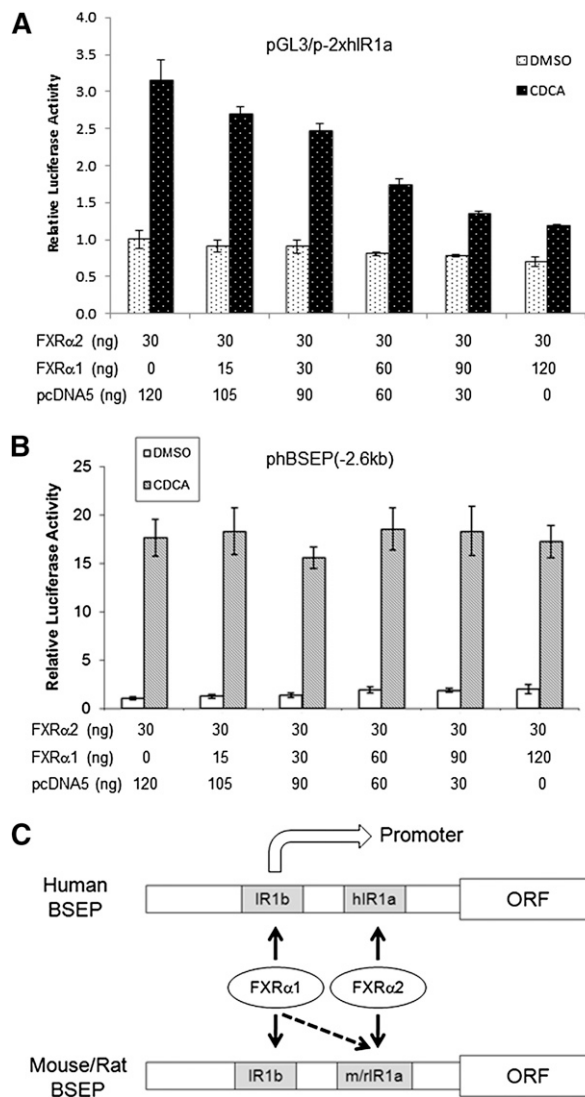


Fig. 10. Interrelationship between FXR α 1 and FXR α 2. The effects of FXR α 1 on FXR α 2-mediated transactivation of (A) human IR1a element reporter pGL3/p-2xhIR1a and (B) full-length human BSEP promoter reporter phBSEP(-2.6kb) were investigated. Increasing amounts of FXR α 1 were cotransfected with a constant amount of FXR α 2 and pGL3/p-2xhIR1a or phBSEP(-2.6kb). Vector pcDNA5 plasmid DNAs were also cotransfected to obtain a constant total plasmid DNA in each well. Sixteen hours posttransfection, cells were treated with DMSO (0.1%) or CDCA (10 μ M) for 30 h, followed by detection of luciferase activity. The data are presented as mean \pm SD of at least three repeated experiments. (C) A diagram summarizing FXR α 1- and FXR α 2-mediated transactivation of BSEP through IR1a and IR1b. In human, FXR α 1 and FXR α 2 regulate BSEP exclusively through IR1b and hIR1a, respectively. In mouse and rat, FXR α 1 is able to transactivate BSEP through both m/rIR1a and IR1b, whereas FXR α 2 transactivates exclusively through m/rIR1a.

importance of the element in regulating BSEP expression. On the other hand, IR1a was less conserved with 62% sequence identity among those species.

To determine whether the species-specific regulation of BSEP by FXR α 1 and FXR α 2 is due to the differences in IR1a, two chimeric promoter reporters in which the authentic IR1a

was replaced with the IR1a from other species were constructed and tested for their ability to respond to FXR α 1 and FXR α 2. As shown in Fig. 11B, replacement of the hIR1a in the human BSEP promoter reporter phBSEP(-2.6kb) with m/rIR1a resulted in a strong transactivation of the chimeric promoter reporter phBSEP(2.6kb)-m/rIR1a by both FXR α 1 and FXR α 2, a pattern comparable to mouse or rat promoter wt (Fig. 1). On the other hand, the reciprocal chimera prBSEP(-2kb)-hIR1a in which the m/rIR1a of rat bsep promoter reporter prBSEP(-2kb) was replaced with human hIR1a exhibited an isoform-dependent transactivation by FXR α 1 and FXR α 2 as human BSEP promoter wt (Fig. 11C).

To further confirm the results, the wt and chimeric promoter reporters were injected into mice, and their transactivation activities in vivo were evaluated by IVIS living imaging. As shown in Fig. 11D, strong luciferase signals were detected in mice injected with wt rat bsep promoter reporter prBSEP(-2.0kb), while no detectable signals were observed in mice received wt human BSEP promoter reporter phBSEP(-2.6kb), indicating that rat bsep promoter has much stronger activity than human BSEP promoter. Replacement of mouse/rat IR1a with human hIR1a in prBSEP(-2.0kb)-hIR1a markedly reduced the signal compared with wt rat bsep promoter reporter. On the other hand, substitution of the human IR1a with mouse/rat m/rIR1a in phBSEP(-2.6kb)-m/rIR1a resulted in noticeable elevation of luciferase signals compared with wt human BSEP promoter reporter. Taken together, the data firmly established that the sequence differences in IR1a were primarily responsible for the species-specific regulation of BSEP by FXR α 1 and FXR α 2.

To determine whether the observed differences in BSEP promoter activities between mouse and human is reflected by the endogenous BSEP transcription levels, the BSEP mRNA levels in human and mouse liver tissues were quantified by real-time PCR with GAPDH levels as internal controls. As shown in Fig. 11E, the mRNA level of mouse bsep was significantly higher than that of human BSEP. The data thus demonstrated that mouse bsep was transcribed at a much higher level than human BSEP in vivo.

DISCUSSION

As the limiting step in bile acid enterohepatic circulation, canalicular efflux of bile acids by BSEP is tightly regulated by the bile acids/FXR signaling pathway. In our previous study, it was found that human BSEP was isoform-dependently regulated by FXR, with FXR α 2 being the predominant regulator. The relative expression of FXR α 1 and FXR α 2 was altered in HCC patients and associated with dysregulation of BSEP (25). Similar FXR isoform-dependent transactivation has been reported for other FXR targets, including ileal bile acid-binding protein (24), fibrinogens (26), syndecan-1 (27), and α -crystallin (31). However, it is currently not understood how FXR α 1 and FXR α 2 exhibit different activity on those FXR targets. Based on EMSA data, it was postulated that FXR α 2 had a higher binding affinity than FXR α 1 to the FXRE of the targets (24). Such notion is consistent with the fact that

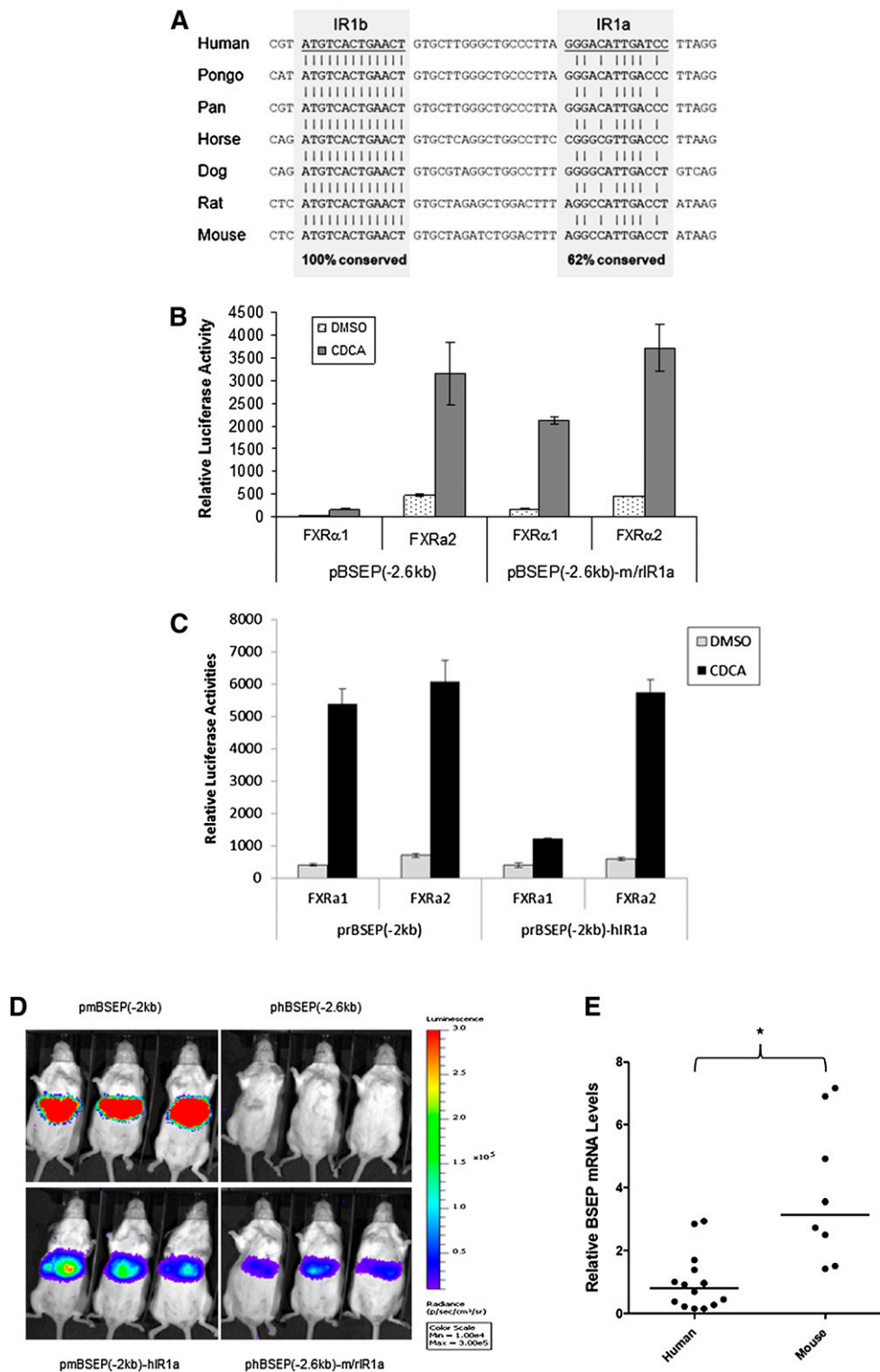


Fig. 11. IR1b is completely conserved among species, whereas IR1a is primarily responsible for the species difference in transactivation of BSEP by FXR α 1 and FXR α 2. (A) Sequences of the IR1a and IR1b from seven species including mouse, rat, dog, cat, horse, primate, and human were aligned. The overall conservation percentages of IR1a and IR1b are indicated. (B) The hIR1a in human BSEP promoter reporter phBSEP(-2.6kb) was replaced with mouse/rat m/rIR1a, generating a chimeric reporter pBSEP(-2.6kb)-m/rIR1a. (C) The rIR1a in rat bsep promoter reporter prBSEP(-2kb) was replaced with human hIR1a, resulting a chimeric reporter prBSEP(-2kb)-hIR1a. Human and rat bsep promoter wt and chimeric reporters were cotransfected with FXR α 1 or FXR α 2 into Huh 7 cells, followed by treatment of transfected cells with DMSO (0.1%) or CDCA (10 μ M) for 30 h. Reporter activities were detected by the dual-luciferase reporter assay system. The data are presented as mean \pm SD of at least three repeated experiments. (D) Female CD-1 mice were randomly divided into four groups. Each group of mice was hydrodynamically injected with either phBSEP(-2.6kb),

the four amino acid residues inserted in FXR α 1 are located immediately after the DNA binding domain (DBD). The insertion may alter the conformational structure in DBD, thus compromising its ability to bind to FXRE. However, in this study, we showed that FXR α 1 and FXR α 2 transactivated human BSEP through two FXREs, IR1b and IR1a, respectively. The question remains how FXR α 1-mediated transactivation through IR1b is much weaker than FXR α 2-mediated activation through IR1a in human BSEP. One possible explanation is that FXR α 1 might have a weaker intrinsic activity than FXR α 2. However, such notion is inconsistent with the results that FXR α 1 transactivated mouse and rat bsep as potently as did FXR α 2 (Fig. 1). Another more likely explanation is that the sequence and/or the content of the IR1b are not optimal for maximal activation by FXR α 1.

Considering that FXR α 2-mediated transactivation through IR1a is the predominant pathway regulating human BSEP expression, the question remains regarding the physiological significance of FXR α 1-mediated transactivation through IR1b in regulating human BSEP. One possible physiological function of FXR α 1/IR1b pathway is to support the maximal transactivation of BSEP by the FXR α 2/IR1a pathway. Consistent with such speculation are results from the mutational studies that mutation in IR1b markedly reduced FXR α 2-mediated basal as well as CDCA-induced transactivation of human BSEP (Fig. 7B). Searching the IR1b sequence in the database revealed that IR1b element is present in many promoter regions (data not shown). However, it remains to be determined whether IR1b element is involved in regulating any of those genes.

Our results showed that in addition to total FXR levels, relative FXR isoform expression levels (FXR α 1/FXR α 2 ratios) significantly affected human BSEP expression. In healthy human liver tissues, FXR α 1/FXR α 2 ratios ranged from 1.09 to 1.63, with an average of 1.37, while BSEP expression exhibited 24-fold differences. It was our expectation that as the FXR α 1/FXR α 2 ratios increased, BSEP expression would decrease. However, the actual data revealed that the relationship between FXR α 1/FXR α 2 ratio and BSEP expression was dependent on the total FXR expression levels (Fig. 2A, B). As expected, human BSEP expression decreased as the ratios increased at the higher levels of total FXR. However, in contrast to our expectation, BSEP expression increased as the FXR α 1/FXR α 2 ratio increased at the lower levels of total FXR. We were not able to provide a satisfactory explanation for the relationship exhibited at the lower levels of total FXR. Such phenomenon may be related to FXR α 1's specific activation through IR1b. Since IR1b is completely conserved among species, it is reasonable to speculate that FXR α 1-mediated

transactivation of human BSEP through IR1b is critical for maintaining BSEP expression in the events of low total FXR levels.


Although FXR α 1 abundantly bound to hIR1a and FXR α 2 weakly associated with IR1b *in vitro* in EMSAs (Fig. 8A, C), and FXR α 1 competitively decreased FXR α 2-mediated transactivation of IR1a reporter (Fig. 10A), it was concluded that FXR α 1 and FXR α 2 did not bind to hIR1a and IR1b in the full-length promoter content in intact cells, respectively, based on the following considerations: first, recruitments of FXR α 1 to hIR1a and FXR α 2 to IR1b were not detected in intact cells with the ChIP assays (Fig. 9A, B); second, FXR α 1 failed to transactivate hIR1a reporter (Fig. 5A); and third, overexpression of FXR α 1 had a negligible effect on FXR α 2-mediated transactivation of the full-length promoter (Fig. 10B), indicating no competition between the two for binding to IR1a. Taken together, the data strongly supported the conclusion that FXR α 1 and FXR α 2 exclusively bind to IR1b and IR1a, respectively, in intact cells. Such discrepancy of FXR binding to FXRE between *in vitro* and in intact cells indicates that FXR binding to FXRE within cells is governed not only by the sequence but also by the content. Such notion is supported by the finding that among the 1.7 million FXREs predicted in the mouse genome, only 1,656 (32) or 7,794 (30) FXREs were detected for FXR binding *in vivo* in the liver.

In the ChIP assays, it was noticed that compared with wt promoter, the recruitment of FXR α 1 to IR1b in the IR1a mutant or FXR α 2 to IR1a in the IR1b mutant was decreased (Fig. 9A, B). One possible explanation for such phenomenon is that mutation in one site has a negative impact on the binding of FXR α 1 or FXR α 2 to the other site due to the proximity of the two sites (18 bases apart). It should be noted that the ChIP assays were carried out on the transfected wt and mutant promoters. Definitive evidence for FXR α 1 binding to IR1b or FXR α 2 binding to IR1a in the endogenous BSEP promoter remains to be provided.

In contrast to human BSEP, mouse and rat bsep were comparably transactivated by FXR α 1 and FXR α 2 (Fig. 1). Mouse and rat m/rIR1a is able to support both FXR α 1- and FXR α 2-mediated transactivation as the FXRE consensus (FXREc: AGGTCA/TGACCT) does (data not shown). Comparing the sequences of hIR1a, IR1b, and m/rIR1a with FXREc, m/rIR1a is the closest to FXREc, followed by IR1b and hIR1a. Detailed analysis of the sequences revealed that a nucleotide G at position 2 is conserved among FXREc, m/rIR1a, and hIR1a but altered to T in IR1b. Therefore, the G at position 2 may play a determinant role in mediating FXR α 2 transactivation. On the other hand, nucleotides A and T in positions 1 and 13 are conserved

prBSEP(-2kb), phBSEP(-2.6kb)-m/rIR1a, or prBSEP(-2kb)-hIR1a promoter reporter. Luciferase activities as luminescent signals were detected by living imaging IVIS 48 h postinjection and captured by a CCD camera with an exposure of 45 s. (E) BSEP mRNA levels in human and mouse liver tissues were quantified by TaqMan real-time PCR. Using GAPDH mRNA levels as internal standards, the relative mRNA expression levels of BSEP in human and mouse liver tissues were compared. Median value of each group is indicated by a short line. Mann-Whitney nonparametric test was used for pair-wise comparison.

among FXREc, m/rIR1a, and IR1b, but they are substituted with G and C in hIR1a, suggesting that the A and T in positions 1 and 13 are important for mediating FXR α 1 transactivation. In this study, we also identified a half ER2 site immediately upstream of IR1a (Fig. 5C). Characterization of the ER2 half site revealed that it played an important role in achieving maximal transactivation of human BSEP by FXR α 2, but the site had no effects on FXR α 2-mediated transactivation of rat bsep. Such difference in requirement of ER2 for FXR α 2 to achieve maximal BSEP transactivation may relate to the fact that human hIR1a is more remote than mouse/rat m/rIR1a to the FXRE consensus. The imperfect hIR1a needs the half ER2 site to support maximal FXR α 2 transactivation, whereas the optimal m/rIR1a alone has the capability of fully supporting FXR α 2 transactivation.

In addition to the important role played by FXR in regulating bile acid homeostasis (33, 34), FXR regulates a myriad of other target genes critical for cholesterol, lipid, and glucose homeostasis (34–37), liver regeneration (38), and tumorigenesis (39, 40). Based on our new finding that FXR α 1 and FXR α 2 transactivated human BSEP through two distinct FXREs, it is reasonable to speculate that FXR α 1 and FXR α 2 may preferably regulate different sets of FXR target genes involved in various functional pathways. We are currently searching for those FXR isoform-specific target genes. 

The authors thank Drs. David Mangelsdorf and Jonathan Reicher for providing human FXR α 1 expression plasmid and rat HCC L0 cells, respectively. Technical and instrumental support from the RI-INBRE Core Facility are greatly appreciated.

REFERENCES

- Meier, P. J., and B. Stieger. 2002. Bile salt transporters. *Annu. Rev. Physiol.* **64**: 635–661.
- Kullak-Ublick, G. A., B. Stieger, and P. J. Meier. 2004. Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology.* **126**: 322–342.
- Jansen, P. L., S. S. Strautnieks, E. Jacquemin, M. Hadchouel, E. M. Sokal, G. J. Hooiveld, J. H. Koning, A. De Jager-Krikken, F. Kuipers, F. Stellaard, et al. 1999. Hepatocanalicular bile salt export pump deficiency in patients with progressive familial intrahepatic cholestasis. *Gastroenterology.* **117**: 1370–1379.
- Strautnieks, S. S., L. N. Bull, A. S. Knisely, S. A. Kocoshis, N. Dahl, H. Arnell, E. Sokal, K. Dahan, S. Childs, V. Ling, et al. 1998. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat. Genet.* **20**: 233–238.
- Wang, L., C. J. Soroka, and J. L. Boyer. 2002. The role of bile salt export pump mutations in progressive familial intrahepatic cholestasis type II. *J. Clin. Invest.* **110**: 965–972.
- Scheimann, A. O., S. S. Strautnieks, A. S. Knisely, J. A. Byrne, R. J. Thompson, and M. J. Finegold. 2007. Mutations in bile salt export pump (ABCB11) in two children with progressive familial intrahepatic cholestasis and cholangiocarcinoma. *J. Pediatr.* **150**: 556–559.
- van Mil, S. W., W. L. van der Woerd, G. van der Brugge, E. Sturm, P. L. Jansen, L. N. Bull, I. E. van den Berg, R. Berger, R. H. Houwen, and L. W. Klomp. 2004. Benign recurrent intrahepatic cholestasis type 2 is caused by mutations in ABCB11. *Gastroenterology.* **127**: 379–384.
- Kubit, R., V. Keitel, S. Scheuring, K. Kohrer, and D. Haussinger. 2006. Benign recurrent intrahepatic cholestasis associated with mutations of the bile salt export pump. *J. Clin. Gastroenterol.* **40**: 171–175.
- Lam, C. W., K. M. Cheung, M. S. Tsui, M. S. Yan, C. Y. Lee, and S. F. Tong. 2006. A patient with novel ABCB11 gene mutations with phenotypic transition between BRIC2 and PFIC2. *J. Hepatol.* **44**: 240–242.
- Eloranta, M. L., T. Hakli, M. Hiltunen, S. Helisalmi, K. Punnonen, and S. Heinonen. 2003. Association of single nucleotide polymorphisms of the bile salt export pump gene with intrahepatic cholestasis of pregnancy. *Scand. J. Gastroenterol.* **38**: 648–652.
- Keitel, V., C. Vogt, D. Haussinger, and R. Kubit. 2006. Combined mutations of canalicular transporter proteins cause severe intrahepatic cholestasis of pregnancy. *Gastroenterology.* **131**: 624–629.
- Lang, C., Y. Meier, B. Stieger, U. Beuers, T. Lang, R. Kerb, G. A. Kullak-Ublick, P. J. Meier, and C. Pauli-Magnus. 2007. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet. Genomics.* **17**: 47–60.
- Knisely, A. S., S. S. Strautnieks, Y. Meier, B. Stieger, J. A. Byrne, B. C. Portmann, L. N. Bull, L. Pawlikowska, B. Bilezikci, F. Ozcay, et al. 2006. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. *Hepatology.* **44**: 478–486.
- Strautnieks, S. S., J. A. Byrne, L. Pawlikowska, D. Cebecauerova, A. Rayner, L. Dutton, Y. Meier, A. Antoniou, B. Stieger, H. Arnell, et al. 2008. Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families. *Gastroenterology.* **134**: 1203–1214.
- Ananthanarayanan, M., N. Balasubramanian, M. Makishima, D. J. Mangelsdorf, and F. J. Suchy. 2001. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J. Biol. Chem.* **276**: 28857–28865.
- Plass, J. R., O. Mol, J. Heegsma, M. Geuken, K. N. Faber, P. L. Jansen, and M. Muller. 2002. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. *Hepatology.* **35**: 589–596.
- Deng, R., D. Yang, A. Radke, J. Yang, and B. Yan. 2007. The hypolipidemic agent guggulsterone regulates the expression of human bile salt export pump: dominance of transactivation over farnesoid X receptor-mediated antagonism. *J. Pharmacol. Exp. Ther.* **320**: 1153–1162.
- Song, X., R. Kaimal, B. Yan, and R. Deng. 2008. Liver receptor homolog 1 transcriptionally regulates human bile salt export pump expression. *J. Lipid Res.* **49**: 973–984.
- Weerachayaphorn, J., S. Y. Cai, C. J. Soroka, and J. L. Boyer. 2009. Nuclear factor erythroid 2-related factor 2 is a positive regulator of human bile salt export pump expression. *Hepatology.* **50**: 1588–1596.
- Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, et al. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* **284**: 1365–1368.
- Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science.* **284**: 1362–1365.
- Otte, K., H. Kranz, I. Kober, P. Thompson, M. Hoefler, B. Haubold, B. Rimmel, H. Voss, C. Kaiser, M. Albers, et al. 2003. Identification of farnesoid X receptor beta as a novel mammalian nuclear receptor sensing lanosterol. *Mol. Cell. Biol.* **23**: 864–872.
- Huber, R. M., K. Murphy, B. Miao, J. R. Link, M. R. Cunningham, M. J. Rupa, P. L. Gunyuzlu, T. F. Haws, A. Kassam, F. Powell, et al. 2002. Generation of multiple farnesoid-X-receptor isoforms through the use of alternative promoters. *Gene.* **290**: 35–43.
- Zhang, Y., H. R. Kast-Woelbern, and P. A. Edwards. 2003. Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. *J. Biol. Chem.* **278**: 104–110.
- Chen, Y., X. Song, L. Valanejad, A. Vasilenko, V. More, X. Qiu, W. Chen, Y. Lai, A. Slitt, M. Stoner, et al. 2013. Bile salt export pump is dysregulated with altered farnesoid X receptor isoform expression in patients with hepatocellular carcinoma. *Hepatology.* **57**: 1530–1541.
- Anisfeld, A. M., H. R. Kast-Woelbern, H. Lee, Y. Zhang, F. Y. Lee, and P. A. Edwards. 2005. Activation of the nuclear receptor FXR induces fibrinogen expression: a new role for bile acid signaling. *J. Lipid Res.* **46**: 458–468.
- Anisfeld, A. M., H. R. Kast-Woelbern, M. E. Meyer, S. A. Jones, Y. Zhang, K. J. Williams, T. Willson, and P. A. Edwards. 2003. Syndecan-1 expression is regulated in an isoform-specific manner by the farnesoid-X receptor. *J. Biol. Chem.* **278**: 20420–20428.

28. Deng, R., D. Yang, J. Yang, and B. Yan. 2006. Oxysterol 22(R)-hydroxycholesterol induces the expression of the bile salt export pump through nuclear receptor farnesoid X receptor but not liver X receptor. *J. Pharmacol. Exp. Ther.* **317**: 317–325.
29. Kaimal, R., X. Song, B. Yan, R. King, and R. Deng. 2009. Differential modulation of farnesoid X receptor signaling pathway by the thiazolidinediones. *J. Pharmacol. Exp. Ther.* **330**: 125–134.
30. Thomas, A. M., S. N. Hart, B. Kong, J. Fang, X. B. Zhong, and G. L. Guo. 2010. Genome-wide tissue-specific farnesoid X receptor binding in mouse liver and intestine. *Hepatology*. **51**: 1410–1419.
31. Lee, F. Y., H. R. Kast-Woelbern, J. Chang, G. Luo, S. A. Jones, M. C. Fishbein, and P. A. Edwards. 2005. Alpha-crystallin is a target gene of the farnesoid X-activated receptor in human livers. *J. Biol. Chem.* **280**: 31792–31800.
32. Chong, H. K., A. M. Infante, Y. K. Seo, T. I. Jeon, Y. Zhang, P. A. Edwards, X. Xie, and T. F. Osborne. 2010. Genome-wide interrogation of hepatic FXR reveals an asymmetric IR-1 motif and synergy with LRH-1. *Nucleic Acids Res.* **38**: 6007–6017.
33. Sinal, C. J., M. Tohkin, M. Miyata, J. M. Ward, G. Lambert, and F. J. Gonzalez. 2000. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell*. **102**: 731–744.
34. Eloranta, J. J., and G. A. Kullak-Ublick. 2008. The role of FXR in disorders of bile acid homeostasis. *Physiology (Bethesda)*. **23**: 286–295.
35. Zhang, Y., and P. A. Edwards. 2008. FXR signaling in metabolic disease. *FEBS Lett.* **582**: 10–18.
36. Kalaany, N. Y., and D. J. Mangelsdorf. 2006. LXRS and FXR: the yin and yang of cholesterol and fat metabolism. *Annu. Rev. Physiol.* **68**: 159–191.
37. Claudel, T., B. Staels, and F. Kuipers. 2005. The farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. *Arterioscler. Thromb. Vasc. Biol.* **25**: 2020–2030.
38. Huang, W., K. Ma, J. Zhang, M. Qatanani, J. Cu villier, J. Liu, B. Dong, X. Huang, and D. D. Moore. 2006. Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science*. **312**: 233–236.
39. Yang, F., X. Huang, T. Yi, Y. Yen, D. D. Moore, and W. Huang. 2007. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. *Cancer Res.* **67**: 863–867.
40. Kim, I., K. Morimura, Y. Shah, Q. Yang, J. M. Ward, and F. J. Gonzalez. 2007. Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice. *Carcinogenesis*. **28**: 940–946.