Natural Structural Variants of the Nuclear Receptor FXR Affect Transcriptional Activation

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Running Title: FXR Isoforms Affect Gene Expression
Summary: The Farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily that has been shown to play an important role in bile acid and cholesterol homeostasis. Here we identify four murine FXR transcripts, derived from a single gene, that encode four isoforms: FXRα1, FXRα2, FXRβ1 and FXRβ2. FXRα and FXRβ differ at their amino terminus and FXRα1 and FXRβ1 have a four amino acid residue insertion in the hinge region immediately adjacent to the DNA binding domain. Real time PCR and 5’RACE followed by Southern blotting reveal that these four transcripts are expressed differentially in liver, intestine, kidney, adrenals, stomach, fat and heart. Electrophoretic mobility shift assays demonstrate that FXRα2 and FXRβ2 bind to FXR response elements with a higher affinity as compared to FXRα1 and FXRβ1, suggesting that the four amino acid insert may affect FXR function. Consistent with this idea, the results of transient transfection experiments demonstrate that the four FXR isoforms differentially transactivated a number of promoter-reporter genes; activation of an I-BABP promoter-reporter gene varied 20-fold depending on the FXR isoform; the rank order of activation was FXRβ2>FXRα2>>FXRα1=FXRβ1. In contrast, SHP-reporter or BSEP-reporter genes were activated to similar degrees by each of the FXR isoforms. Finally, NIH3T3 cells were stably infected with individual murine FXR isoforms and the cells were treated with FXR ligands. The endogenous I-BABP gene was activated by the four FXR isoforms with the same rank order as seen in transfections. This effect was gene specific, since induction of BSEP mRNA was independent of the FXR isoform. These observations suggest that there are four distinct murine FXR isoforms that differentially regulate gene expression in numerous tissues in vivo.
Introduction:

Nuclear hormone receptors are transcription factors that are involved in numerous processes, including reproduction, development and general metabolism (1). Most of these receptors are comprised of a ligand-independent transcriptional activation function (AF-1) at the amino terminus, a DNA-binding domain (DBD), a hinge region and a ligand-binding domain (LBD), a dimerization interface and a ligand-dependent activation function (AF-2) at the carboxy terminus (2,3). In many cases entry of a specific ligand into the pocket formed by the ligand binding domain results in a conformational change of the receptor, recruitment of co-activators and transcriptional activation (4-13).

Nuclear hormone receptors have been classified into sub-groups depending on whether they bind DNA as homodimers, heterodimers or monomers (14). A few family members have been identified that do not bind DNA directly but instead function by interacting with other transcription factors and altering their activity (15,16). Nonetheless, the major sub-group contains members that bind to DNA as heterodimers with the common partner, retinoid X receptor (RXR) (14). The farnesoid X receptor (FXR, NR1H4) falls into this category.

FXR was isolated by screening a rat cDNA library using PCR and degenerate primers corresponding to the highly conserved DNA-binding domain of nuclear receptors (17). Independently, two mouse homologues of rat FXR, termed RIP14-1 and RIP14-2, were isolated using the yeast two-hybrid assay and the human RXR ligand-binding domain as bait (18). Northern blot assays and in situ hybridization indicated that FXR expression was limited to the liver, small intestine, kidney and adrenal gland (17,19).

In the initial studies, supraphysiological levels of farnesol were shown to activate the rat (17), but not the murine FXR (20). In 1999, several groups independently identified bile acids as
endogenous ligands that activated FXR at physiological concentrations (21-23). The finding that bile acids not only bound to FXR but that this interaction resulted in recruitment of co-activators (21,22), provides compelling evidence that bile acids are physiologically important hormones that function to activate the FXR/RXR heterodimer.

The recent characterization of FXR null mice (24), the synthesis and utilization of a high affinity ligand for FXR (25) and the identification of a number of FXR target genes have provided important insights into the role of FXR in controlling lipid metabolism. FXR target genes include ileal bile acid binding protein (I-BABP) (21,26), phospholipid transfer protein (PLTP) (27,28), apolipoprotein C-II (29), multidrug resistance associated protein 2 (MRP2; ABCC2) (30), the bile salt export pump (BSEP) (31) and the small heterodimer partner receptor (SHP) (25,32) (reviewed in (33)). These genes are involved in various aspects of bile acid, lipoprotein and lipid metabolism (33). The demonstration that FXR null mice are unable to respond appropriately to diets enriched in fat or bile acids (24) further emphasized the critical role of FXR in controlling lipid homeostasis.

Two forms of murine FXR (RIP14-1 and RIP14-2) that differ at their amino terminus were originally isolated (18). RIP14-2, in contrast to RIP14-1, contained an additional 12 bp that results in the insertion of four amino acids in the hinge region, adjacent to the DBD. Analysis of the cDNA encoding rat FXR indicates that it does not contain the 12 bp insert, but otherwise corresponds to murine RIP14-1. It is not known whether these different isoforms have different functions.

Taken together, these results suggested that there might be at least four FXR isoforms that differ either at their amino terminus and/or at the site of the four amino acid insertion in the hinge region. Since the hinge region is thought to have a role in the DNA binding properties of
nuclear receptors (34-36), we hypothesized that these different isoforms might differentially bind to DNA and/or differentially activate target genes. The current report provides evidence to support these proposals.

**EXPERIMENTAL PROCEDURES**

*Animals*-C57BL/6J female mice were fed a standard rodent chow diet in a temperature-controlled room (23 ºC) on a 12 hr light/dark cycle. Eight to 12 week-old wild type mice were sacrificed and tissues were snap-frozen in liquid nitrogen and stored at –80 ºC until use.

*Plasmids and Reagents*-Four different mouse FXR cDNAs were isolated from the liver tissue using gene-specific primers (GSP1 and GSP2) and adapter primers (ADP1 and ADP2) in the Sure-RACE Panels according to the manufacture’s protocol (OriGene Technologies, Inc., Rockville, MD). The first-round of PCR utilized ADP1 and GSP1 (Fig. 1A). The generated cDNA was further amplified in a second round of PCR utilizing ADP2 and GSP2. The full-length coding regions of four murine FXR isoforms were amplified by PCR using gene-specific primers and cloned into BamHI/XhoI sites of CMX-PL1 vector to produce expression constructs CMX-FXR-\( \alpha_1 \), CMX-FXR\( \alpha_2 \), CMX-FXR\( \beta_1 \), CMX-FXR\( \beta_2 \). In order to make retroviral expression constructs, the full-length coding regions of four different isoforms were separately excised from the CMX expression constructs using BamHI/XhoI restriction enzymes, and subcloned into BglII/XhoI sites of the MSCV-IRES-neo vector to make constructs MSCV-FXR\( \alpha_1 \), -FXR\( \alpha_2 \), -FXR\( \beta_1 \), -FXR\( \beta_2 \). The mouse BSEP promoter (-1050 to +25) was amplified using gene-specific primers and cloned into Saci/XhoI digested-pGL3-LUC vector (Promega) to create pGL3-BSEP-Luc. All the plasmids have been confirmed by sequencing. Plasmids pIBABP\_1031-Luc and pIBABP\_142mut-Luc are kind gifts from Dr. David Mangelsdorf (University of Texas Southwestern Medical Center) (21). pGL3-hSHP-Luc was kindly provided by Dr.
Bryan Goodwin (GlaxoSmithKline, Research Triangle, NC) (25). The retroviral vector MSCV-IRES-neo plasmid was a gift from Dr. Owen Witte (University of California Los Angeles). The sources of other plasmids and synthetic ligands have been described elsewhere (30).

5′ RACE, Southern and Northern Blot Analysis-Sure-RACE Mouse Panels (OriGene Technologies, Inc., Rockville, MD) contain double-stranded cDNAs synthesized from 24 tissues. A 5′ adapter, containing sequences corresponding to ADP1 and ADP2, was ligated at the 5′ ends. The cDNAs were amplified using gene-specific primers (GSP1 and GSP2) and adapter specific primers (ADP1 and ADP2). The PCR products were then isolated on a 1.2% agarose gel, transferred to a nylon membrane, and the membranes were probed with a mouse FXR cDNA probe. The bands corresponding to FXRα or FXRβ were recovered from the gel and cloned into pCR2.1-TOPO vector (Invitrogen). After transformation, the white colonies were patched onto duplicate ampicillin-positive LB plates. The colonies from the plate were first screened to identify whether they represent FXRα or FXRβ using P3 and P4 (see Fig. 1), followed by using P1 (for screening the isoform with 12 bp insert) and then P2 (for screening the isoform without 12 bp insert) (Fig. 1). The sequences for P1 and P2 are: 5′-

TGGCTGAATGTATGTATACAGTTTGTTAA-3’ and 5′-

ATGTTGGCTGAATGTTTGTTAACTGA-3’, respectively. All positive colonies were further confirmed either by sequencing or PCR. For Northern blot analysis, total RNA was isolated using Trizol reagent (Invitrogen) and 10 µg of RNA was denatured, electrophoresed, transferred to a nylon membrane, and probed with the indicated cDNA probe.

Quantitative PCR—Real time PCR was performed essentially as described (37). Briefly, 1 µg of DNase I-treated total RNA was reverse transcribed with random hexamers using the Taqman Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s
protocol. Each amplification mixture (50 µl) contains 50 ng of cDNA, 900 nM forward primer, 900 nM reverse primer, 250 nM fluorogenic probe, and 25 ul of Universal PCR Master Mix (Applied Biosystems). PCR thermocycling parameters were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Real time PCR was carried out using Applied Biosystems 7700 sequence detector. Samples were analyzed simultaneously for cyclophilin expression. Quantitative expression values were extrapolated from separate standard curves. Each sample was assayed in duplicate and normalized to cyclophilin. The sequences for primers and probes are as follows: FXRα, 5’-TGGGCTCCAATCCTCCTTAXAGA (forward primer, F), 5’-TGGTCCTCAAATAAGATCCTTGG (reverse primer, R), 5’-CCTTGGACATCTCTGGCCCCCCAAGCA (Probe, P); FXRβ, 5’-GGGCTTAGAAAAATCCCAATTCAAGATTA-3’ (F), 5’-CGTCCCGCACAATAATCCTG-3’ (R), 5’-TCTTCACCACACGCCACCGGCTG-3’ (P); I-BABP, 5’-CAAGGCTACCGTGAAGATGGA-3’ (F), 5’-CAAGGCTACCGTGAAGATGGA-3’ (R), 5’-CCTTGGACATCTCTGGCCCCCCAAGCA (Probe, P); BSEP, 5’-ACAGAAGCAAAGGGTAGCCATC-3’ (F), GGTACGCGATGACGAGCCC-3’ (R), CCGCGGCTCATACGGAACC (P); cyclophilin, 5’-GGCCGATGACGAGCCC-3’ (F), 5’-TGGTCTTTGGAACTTTGTCTGCAA-3’ (R), 5’-TGGGCCGCGTCTCCTTCGA-3’ (P). All probes were dually labeled at the 5’end with 6-carboxyfluorescein (6-FAM) and at the 3’ end with 6-carboxytetramethylrhodamine (TAMRA).

**Electrophoretic Mobility Shift Assay (EMSA)**-EMSAs were performed essentially as described (27). Mouse FXR isoforms or hRXRα was synthesized in vitro using TNT T7 Coupled Reticulocyte System (Promega). To compare transcription/translation efficiency of the expression constructs expressing different mouse FXR isoforms, equal volumes of 35S-labeled lysates were loaded and separated on an 8% SDS-polyacrylamide gel. The gel was dried and
The bands were quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Binding reactions were carried out in a buffer containing 10 mM HEPES, pH 7.8, 100 mM KCl, 0.2 % Nonidet P-40, 6 % glycerol, 0.3 mg/ml BSA, 1 mM dithiothreitol, 2 µg of poly(dI-dC), 1-3 µl each of in vitro translated receptors and 32P end-labeled oligonucleotide. DNA-protein complexes were resolved on a 5 % polyacrylamide gel in 0.5 X TBE (45 mM Tris Borate, 1 mM EDTA) at 4 °C. Gels were dried and autoradiographed. The sequences for mI-BABP probe and hSHP probe are 5’-
GTTTTCCTTAAGGTGAATAACCTTGGGGCTC-3’ and 5’-
GTACAGCCTGGGTAAATGACCCTGTTTATGC-3’, respectively.

Cell Culture, Transfection, and Stable Cell lines-CV-1 and HepG2 cells were maintained in modified Eagle’s medium (MEM)/10% fetal bovine serum (FBS). Transient transfections were performed in triplicate in 48-well plates as described (27). Cells were treated with ligands or vehicle (DMSO) in super-stripped FBS (HyClone, Logan, UT) as indicated in the legends. Luciferase activity was measured and normalized to β-galactosidase activity. To produce stable cell lines, 293T cells were transfected with MSCV-FXRα1, -FXRα2, -FXRβ1, -FXRβ2 or MSCV-neo along with ΨE helper virus. The supernatants of the culture media were then used to infect NIH3T3 cells, followed by selection with 800 µg/ml G418 sulfate (Geneticin®, Invitrogen) for three to four weeks. The selected stable cells were subsequently treated with the indicated ligands, as described in the text.

RESULTS

Isolation of Four Murine FXR Isoforms—Based on the previous reports on rat (17) and murine (18) FXR, we hypothesized that there might be four murine FXR isoforms. For clarity,
the four isoforms that have been characterized in the present report have been termed FXRα1, FXRα2, FXRβ1 and FXRβ2 (Fig. 1A). FXRβ2 and FXRα1 correspond to RIP14-1 and RIP14-2, previously identified by Seol et al. (18).

In order to identify all possible FXR isoforms, we employed 5′RACE (5′ rapid amplification of cDNA ends) and cDNAs, generated from 24 murine tissues that had been ligated to a 5′ adaptor (Origene Technologies, Inc.). Gene specific primers (GSP1 or GSP2 in Fig. 1A) together with adapter-specific primers (ADP1 or ADP2) were used in a series of PCR reactions to amplify FXR specific cDNAs (see Experimental Procedures). Southern blot analysis identified the PCR products that corresponded to FXR cDNAs and these were subsequently cloned into pCR2.1-TOPO vector. Radiolabelled oligonucleotides P3 or P4 (Fig. 1A) were then used to differentiate colonies corresponding to FXRα from FXRβ (data not shown). Filters containing either FXRα or FXRβ positive colonies were probed with radiolabeled oligonucleotides P1 or P2 (Fig. 1A) to distinguish whether these colonies did, or did not contain, the 12 bp insert (Fig. 2A). This approach, coupled with DNA sequencing of selected inserts, identified four murine FXR isoforms (Fig. 1A). Analysis of the 5′ RACE data and various databases suggests that i) murine FXR consists of 11 exons and 10 introns, ii) FXRα transcription is initiated from exon 1, iii) FXRβ transcription is initiated from exon 3, iv) FXRα and FXRβ share exons 4 ~ 11, and v) the 12 bp insert is located at the 3′ terminus of exon 5 (Fig 1; Table I). Thus, alternative splicing between exon 5 (that contains the variable 12 bps) and exon 6 produces FXR isoforms that include (α1, β1) or exclude (α2, β2) the four amino acid (12 bp) insert (Fig. 1B). Table I provides details of the genomic organization and intron-exon junctions of the murine FXR gene: the gene encompasses 76,997 bps contains 11 exons that range in size from 100 to 572 bps and introns that vary from 328 to 16,388 bps. Almost all of
the exon/intron boundaries display the canonical GT/AG sequence (Table I). The data were obtained by comparison of the sequence of the FXR cDNA with the publicly available genomic sequence of murine Chromosome 10 [www.genome.ucsc.edu]. Fig. 1C illustrates the domain structures of the FXR isoforms and shows that the FXRβ isoforms contain an additional 37 amino acids at the amino terminus that are absent from FXRα. The four amino acid (MYTG) insert is located in the hinge region, adjacent to the DBD (Fig. 1C).

**FXR Isoforms Are Differentially Expressed in Tissues**—In order to determine the relative expression of FXRα1:FXRα2, and FXRβ1:FXRβ2 in different tissues, we transferred ≥33 bacterial colonies that contained DNA corresponding to either FXRα or FXRβ to filters. These filters were probed sequentially with probes P1 and P2 (Fig. 1A and Fig. 2A). As illustrated in Fig. 2A, this approach distinguished between FXRβ colonies that contained (Fig. 2A, left panel) or did not contain (Fig. 2A, right panel) the 12 bp insert. Thus, the ratio of FXRβ1:FXRβ2 in the liver was 1:1.9 (Fig. 2A; Table II). Table II summarizes the results obtained from similar assays utilizing cDNAs generated from six tissues. The data indicate that the ratio of FXRα1:FXRα2 and FXRβ1:FXRβ2 in different tissues varies significantly. For example, the ratio of FXRα1:FXRα2 (+12 bp/-12 bp) is 1:51 in the heart and 1:0.75 in the adrenal gland (Table II). Analysis of the PCR products also indicated that some tissues, including the heart, kidney, stomach, and adrenal gland, expressed predominantly one FXR isoform, either FXRα or FXRβ, whilst the liver expressed equivalent levels of FXRα or FXRβ (Table II). Because the DNA was analysed after a PCR amplification step, the ratios of the four FXR isoforms are considered to be semi-quantitative.

To determine the relative expression of FXRα versus FXRβ, total RNA from 13 tissues in C57BL/6J mice (n=5) fed a normal chow diet was isolated and real time quantitative PCR was
performed. As shown in Fig. 2B, FXRα and FXRβ were most abundantly expressed in the liver. The liver was the only organ that expressed similar levels of both isoforms. FXRβ was abundantly expressed in ileum, moderately in kidney, and at low levels in stomach, duodenum, and jejunum (Fig. 2B). FXRα was moderately expressed in ileum and adrenal gland. In addition, heart, lung, and fat contained low but measurable levels of both FXRα and FXRβ (Fig. 2B). In contrast, FXR was undetectable in brain, spleen, or muscle (Fig. 2B). Interestingly, the ratio of FXRα:FXRβ in different tissues varies significantly; the ratio was 7:1, 1:1, and 1:3 in the adrenals, liver and ileum, respectively (Fig. 2B). The differences in expression of the FXR isoforms in different tissues suggest that the physiological functions of the four isoforms may vary.

**FXR Target Gene Promoters Are Differentially Transactivated by FXR Isoforms**-In order to investigate if the four FXR isoforms have different binding affinities to DNA, the full-length coding regions of the four isoforms were cloned into an expression vector. *In vitro* transcription/translation experiments show that the expression constructs for FXRβ1 and FXRβ2 produce proteins of the expected molecular weight (Fig. 3A). Based on the incorporation of radioactive methionine into the proteins, more FXRα1 and FXRα2 isoforms were synthesized as compared to FXRβ1 and FXRβ2 (Fig. 3A). Consequently, different volumes of lysate, containing equivalent amounts of each individual FXR isoform, were used in EMSAs. The results of multiple EMSAs consistently show that FXRβ2 and FXRα2 bind to the mouse I-BABP (mI-BABP) FXR response element (FXRE) or the hSHP FXRE with a higher affinity, as compared to FXRβ1 or FXRα1 (Fig. 3B). The interaction of FXR/RXR with the radiolabeled DNA probe was attenuated in the presence of excess wild-type competitor DNA but not by the competitor DNA containing a mutated FXRE (data not shown). Similar results to those shown in
Fig. 3B were obtained when the radiolabeled DNA contained FXREs from mouse BSEP, mouse SHP, human PLTP, rat MRP2, or human apoC-II genes (data not shown).

Results from EMSAs indicate that inclusion of the four amino acid insert in the hinge region decreases the ability of FXR/RXR to bind to a number of FXREs (Fig. 3). To determine whether transactivation is dependent upon the FXR isoform, CV-1 cells, derived from the kidney of an African green monkey, were transiently transfected with various reporter genes, RXR and specific FXR isoforms. The cells were then treated with DMSO (vehicle), CDCA (physiological FXR ligand), GW4064 (synthetic FXR ligand) in the presence or absence of LG100153 (RXR ligand). The hSHP promoter (Fig. 4A) and mouse BSEP promoter (Fig. 4B) were transactivated to similar levels by each of the four FXR isoforms. In contrast, the ml-BABP promoter-reporter gene was differentially induced by the four ligand-activated FXR isoforms (Fig. 4C). The rank order of activation of the ml-BABP promoter-reporter gene was FXRβ2 > FXRα2 >> FXRα1= FXRβ1. When the FXRE in the ml-BABP promoter was mutated, the reporter gene was not longer activated by any of the FXR isoforms (Fig. 4D). These data suggest that activation of the ml-BABP promoter by the four FXR isoforms is mediated through the FXRE in the promoter.

In order to ensure that the results obtained with CV-1 cells were not cell- or species-specific, we also transiently transfected HepG2 cells, a human hepatoma cell line that has been used extensively in the study of FXR target genes, with the ml-BABP and mBSEP promoter-reporter genes. As shown in Fig. 4E, the ml-BABP reporter gene was potently activated in the presence of FXRβ2 and FXRα2 and ligands for FXR and RXR. The same reporter gene was refractory to activation by FXRα1 and FXRβ1, the isoforms containing the additional four amino acids in the hinge region (Fig. 4E). In contrast, the mBSEP-reporter gene was activated to similar levels by all four FXR isoforms, demonstrating that transactivation is target-gene
specific. Thus, the transfection experiments, that utilize either CV-1 or HepG2 cells and mI-
BABP and mBSEP promoter-reporter genes, give essentially identical results.

**Transcriptional Activation of Specific FXR Target Genes is Affected by the Ratio of FXR Isoforms**—The results illustrated above indicate that activation of certain murine genes, such as I-
BABP, might depend on the relative nuclear ratio of FXR isoforms that do, or do not contain the 
four amino acid insert in the hinge region. In order to test this hypothesis, we transiently 
transfected HepG2 cells with the mIBABP-reporter gene and different ratios of FXRβ1 (which 
contains the 4 amino acid insert) and FXRβ2 (which lacks the 4 amino acids). The data of Fig. 5 
show that relatively high levels of FXRβ1 (25 ng plasmid) induce the reporter gene less than 3-
fold in the presence of ligands for FXR and RXR. In contrast, the reporter gene was potently 
activated (28-fold) in the presence of low levels of FXRβ2 (5 ng plasmid) (Fig. 5). Most 
importantly, this high level of activation in response to ligand-activated FXRβ2 was greatly 
attenuated in cells following co-transfection of FXRβ1 (Fig. 5). The data demonstrate that the 
fold activation of the mI-BABP reporter gene decreased from 28-fold (in the presence of FXRβ2 
and no FXRβ1) to 15-, 11- and 8-fold as the ratio of FXRβ1:FXRβ2 was increased from 1:1 to 
2:1 to 5:1 (Fig. 5).

**Mouse I-BABP Gene Is Differentially Induced by FXR Isoforms**—Based on the transient 
transfection experiments, we hypothesized that the mI-BABP gene may be differentially 
regulated by the four FXR isoforms *in vivo*. To test this hypothesis, we utilized NIH3T3 cells, a 
murine fibroblast that lacks endogenous FXR (Fig. 6A). NIH3T3 cells were infected with a 
retrovirus that expressed one specific murine FXR isoform and stable cell lines were then 
selected by growth in media containing G418. Results from Northern blot assays indicate that
the stable NIH3T3 cell lines express murine FXRα1, FXRα2, FXRβ1 and FXRβ2 at similar high levels (Fig. 6A). No FXR was detected in NIH3T3 cells infected with the empty retroviral vector (Fig. 6A). These five different cell lines were each treated for 24 h with vehicle or ligands for FXR (GW4064) or FXR and RXR (LG100153), prior to quantitation of specific mRNAs. Fig. 6B shows that mouse BSEP mRNA levels were induced to similar levels by each of the four FXR isoforms. In contrast, the endogenous mI-BABP mRNA levels were induced from 2.6- to 141-fold in an FXR isoform-specific manner. The rank order of potency is the same as that observed in transient transfection experiments (FXRβ2 > FXRα2 >> FXRβ1 = FXRβ2) (Fig. 6C). Taken together, these results strongly suggest these four FXR isoforms function differentially in vivo.

**DISCUSSION**

Herein, we report the cloning and functional properties of four murine FXR isoforms, termed FXRα1, FXRα2, FXRβ1 and FXRβ2. Prior to this report, two murine FXR isoforms corresponding to FXRα1 and FXRβ2 had been identified but not characterized (18). The FXRβ isoforms are derived from the use of an internal promoter that generates transcripts which are 187 bp shorter than the FXRα transcripts, but encode proteins that contain an additional 37 amino acids at the amino terminus. However, our data suggest that it is the presence or absence of the four amino acid residues in the hinge region (Fig. 1) that plays a critical role in modulating the function of FXR. Similarly, a recent report, published after the completion of the current studies, identified four hamster as well as four human FXR isoforms (38). The fact that these isoforms appear to be conserved across a number of species, provides additional significance to these collective findings.
A limited number of studies have investigated the importance of the hinge region (D domain) of other nuclear receptors. For example, mutagenesis of the D domain reduces the transcriptional activation properties of the glucocorticoid receptor (34), but has no effect on the function of the estrogen receptor (35,36). The availability of FXR isoforms with natural variations in the amino acid sequence in the hinge region provided a unique opportunity that allowed us to investigate the importance of these changes on receptor function. EMSAs demonstrated that the isoforms containing the additional four amino acids (FXRα1, FXRβ1) bind to several FXREs with a lower affinity than FXRα2 and FXRβ2. Since the additional four amino acids are separated from the DBD by only four amino acids, it is possible that the extra amino acids result in minor alterations in the structure of the receptor that affect either DNA binding and/or the ability of FXR to dimerize with RXR on the FXRE and/or the interaction of FXR with co-repressors or co-activators.

Studies that involved FXR-dependent activation of various promoter-luciferase reporter genes and endogenous genes demonstrated that induction of mI-BABP by FXR ligands is particularly sensitive to the FXR isoforms, with the rank order being FXRβ2>FXRα2>>FXRα1 =FXRβ1 (Figs. 4, 6). I-BABP is thought to be primarily expressed in intestinal cells. The finding that murine duodenum, jejunum and ileal cells express at least 3-fold more FXRβ than FXRα mRNA (Fig. 2B) and more FXRβ2 than FXRβ1 (Table II), is consistent with the high expression of I-BABP in this organ. In contrast to I-BABP, two other genes (BSEP, SHP) are induced to similar levels by each FXR isoform. In preliminary studies we replaced the FXRE in the mI-BABP promoter-reporter with the FXRE from the mSHP gene; induction of this novel reporter gene by GW4064 was much less sensitive to the FXR isoforms than the original wild-type reporter gene (data not shown). These data suggest that the FXR isoforms differentially
regulate target genes in various tissues and that these differences are due, at least in part, to the specific sequences that comprise the FXREs.

Murine FXR expression has been reported to be limited to the liver, small intestine, kidney, and adrenal gland (17,19). In the current study we utilized real time PCR to demonstrate that FXR is also expressed in stomach, heart, lung, and fat, albeit at lower levels (Fig. 2B). The relative expression of the four FXR isoforms differs significantly in these eight tissues (Fig. 2B; Table 1). Since the FXR isoforms differentially activate specific genes, such as I-BABP, we hypothesize that a change in the relative ratio of the FXR isoforms will significantly affect gene expression. Studies are currently underway to determine whether the two promoters that control the expression of FXRα and FXRβ are differentially regulated. A number of FXR target genes have been identified in liver, intestine and kidney, tissues that are known to be involved in bile acid synthesis and metabolism. The current demonstration that there are multiple FXR isoforms in lung, adipose tissue, heart and stomach, tissues that are not currently known to be involved in bile acid metabolism, raises the possibility that additional FXR ligands remain to be identified that function in these tissues. Identification of these target genes and putative ligands may provide important clues as to the function of FXR in multiple murine tissues.

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Legends

**TABLE I.** Genomic organization of the murine FXR gene

**TABLE II.** Relative expression of FXRα1:FXRα2, and FXRβ1:FXRβ2 in mouse tissues.

Colonies (≥33 colonies) were screened for analysis of the relative expression of FXRα1 and FXRα2 or FXRβ1 and FXRβ2 using the method as described in Fig 2A. The ratio of FXRα1 to FXRα2, or FXRβ1 to FXRβ2 in different tissues is shown. ND, not detected.

**Legends for Figures**

**FIG. 1.** Isolation and characterization of four murine FXR isoforms. **A**, Schematic diagram of four mouse FXR mRNAs. Four mouse FXR isoforms, termed FXRα1, FXRα2, FXRβ1 and FXRβ2, were isolated as described in the text. The 5’ ends of FXRα1 and FXRα2 are different from those of FXRβ1 and FXRβ2. FXRα1 and FXRβ2 correspond to RIP14-2 and RIP14-1, respectively. An additional ten base pairs (GTTGCCGTGA) were identified at the 5’-end of FXRβ1 and FXRβ2 mRNAs compared to RIP14-1. The first ATG (asterisk) and stop codon (T) are denoted. The 12 bp insert is denoted by a vertical black bar. The probes (P1, P2, P3, P4) and gene specific primers (GSP1, GSP2) are indicated. **B**, Schematic representation of the organization of murine FXR gene. The mouse FXR gene consists of 11 exons and 10 introns. FXRα and FXRβ are transcribed from exon 1 and exon 3, respectively. The 12 bp insert is located at the 3’ end of exon 5. Alternative splicing between exon 5 and exon 6 produces two forms of FXR, that contain or do not contain the 12 bp insert. * initiation and/or inframe methionines are indicated. **C**, Schematic diagram of mouse FXR receptors. FXRα and FXRβ have the same amino acid sequence except FXRβ has an additional 37 amino acids at its amino
terminus. FXRα1 and FXRβ1 have a four amino acid insert (MYTG) which is located within the hinge region (D domain). DBD and LBD, DNA binding domain and ligand binding domain, respectively.

FIG. 2. **Relative expression of murine FXR isoforms in different tissues.** *A*, Southern blot analysis of the relative expression of FXRα1 to FXRα2 or FXRβ1 to FXRβ2. Mouse cDNAs were amplified using adapter primers and gene-specific primers and then separated on a 1.2% agarose gel. The DNA bands corresponding to FXRα or FXRβ were recovered from the gel and cloned into pCR2.1-TOPO vector. After transformation, the white colonies were transferred to a nylon membrane and the membranes were probed with 32P-labeled oligonucleotides P3 or P4, followed by P1 (containing the 12 bp insert) and then P2 (lacking the 12 bp insert). The results obtained with PCR products derived from the liver and corresponding to FXRβ1 (left panel) and FXRβ2 (right panel) are shown. *B*, Quantitative analysis of the relative expression of FXRα and FXRβ in different tissues. Total RNA was isolated from different tissues in mice (n = 5) fed a normal chow diet and real-time quantitative PCR assays were performed in duplicate as described in Experimental Procedures and normalized to cyclophilin. SI-D, SI-J, SI-I represent duodenum, jejunum, ileum portions of small intestine, respectively.

FIG. 3. **Mouse FXR isoforms bind to FXR response elements with different affinity.** *A*, CMX-FXRα1, -FXRα2, -FXRβ1, -FXRβ2 were synthesized *in vitro* in the presence of 35S-methionine. Three µl of *in vitro*-translated lysates were analysed on an 8% SDS-polyacrylamide gel. The gel was dried and subjected to autoradiography. *B*, Equivalent amounts of *in vitro* synthesized RXR and either FXRα1, FXRα2, FXRβ1 or FXRβ2 protein were incubated with 32P-
labeled DNA probes containing the mI-BABP or hSHP FXRE. The DNA-protein complex was resolved on a 5% polyarylamide gel and analyzed by autoradiography. Free probes are not shown. NS, non-specific.

FIG. 4. Four mouse FXR isoforms differentially transactivate reporter constructs. Promoter-reporter plasmids pGL3-hSHP-Luc (A), pGL3-mBSEP-Luc (B, F), pIBABP1031-Luc (C, E), or pIBABP142mut-Luc (D) were transiently co-transfected into CV-1 cells (A-D) or HepG2 cells (E, F) together with the indicated FXR isoform or no receptor (NR). Cells were then treated with DMSO (vehicle), CDCA (100 µM), GW4064 (1 µM) in the presence or absence of LG100153 (100 nM) for 42 h. Luciferase activities were assayed and normalized to β-galactosidase activity. The data (mean +/- SE) are derived from three experiments, each performed in triplicate. Numbers on the top of the bars refer to the fold increase of luciferase when the activity was compared to that obtained from DMSO-treated cells that were not transfected with nuclear receptors. RLU, relative light units.

FIG. 5. The Relative Ratio of FXRβ1:FXRβ2 Affects the Transcriptional Activation of the Murine I-BABP-Reporter Gene. HepG2 cells were transiently transfected in triplicate with the ml-BABP reporter gene and plasmids encoding RXR, FXRβ1, FXRβ2 and β-galactosidase. The amount (ng) of each plasmid used in the transfection is shown. Cells were treated with vehicle or the indicated ligand(s) for 24 h and the results (mean +/- SE; two experiments) were determined as described in the legend to Fig. 4.
FIG. 6. Mouse I-BABP mRNA levels are differentially induced in NIH3T3 cells stably expressing individual FXR isoforms. A, NIH3T3 cells were infected with retroviruses expressing neomycin alone or neomycin and either FXRα1, FXRα2, FXRβ1 or FXRβ2. After 3-4 weeks selection with G418 (800 ng/ml), total RNA was isolated from the cells and Northern blot analysis was performed. B, Stably infected NIH3T3 cells were treated with DMSO, GW4064 (1 µM) or GW4064 plus LG100153 (100 nM) for 24 hours. Total RNA was isolated and real-time quantitative PCR was performed as described in Experimental Procedures to test the relative expression of mBSEP (B) or mI-BABP (C) in response to ligands. Values were normalized to cyclophilin.

Abbreviations

The abbreviations used are: activation function, AF; DNA binding domain, DBD; ligand binding domain, LBD; retinoid X receptor, RXR; farnesoid X receptor, FXR; ileal bile acid binding protein, I-BABP; phospholipid transfer protein, PLTP; multidrug resistance associated protein 2, MRP2, bile salt export pump, BSEP; small heterodimer partner, SHP; gene-specific primer, GSP; adapter primer, AP; RACE, rapid amplification of cDNA ends; 6-carboxyfluorescein, 6-FAM; 6-carboxytetramethylrhodamine, TAMRA; LG100153, LG; fetal bovine serum, FBS; modified Eagle’s medium, MEM; electrophoretic mobility shift assay, EMSA, base pairs, bp
References


Galardi, C., Wilson, J. G., Lewis, M. C., Roth, M. E., Maloney, P. R., Willson, T. M.,


27. Laffitte, B. A., Kast, H. R., Nguyen, C. M., Zavacki, A. M., Moore, D. D., and Edwards,

**275**(50), 39313-7

29. Kast, H. R., Nguyen, C. M., Sinal, C. J., Jones, S. A., Laffitte, B. A., Reue, K., Gonzalez,
1728


31. Ananthanarayanan, M., Balasubramanian, N., Makishima, M., Mangelsdorf, D. J., and

32. Lu, T. T., Makishima, M., Repa, J. J., Schoonjans, K., Kerr, T. A., Auwerx, J., and


645-52.


# Table I

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### Table II

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<td>adrenal gland</td>
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</table>
Figure 1

A

FXR α1

FXR α2

FXR β1

FXR β2

B

FXRα

FXRβ

C

FXRα1

FXRβ1
Figure 2

A

FXRβ1 (Probe: P1)

FXRβ2 (Probe: P2)

B

Relative Expression

- FXRα
- FXRβ

brain
heart
lung
liver
stomach
SI-D
SI-J
SI-I
kidney
adrenal gland
spleen
muscle
fat
Figure 3

(A) Western blot analysis of FXR/RXR complexes. Lysates were resolved by SDS-PAGE and probed with antibodies specific for FXR/RXR heterodimers. The molecular weights of the bands were estimated to be 75 kDa and 50 kDa.

(B) Gel shift assay of FXR/RXR complexes. The gel was loaded with various concentrations of RXR and mI-BABP, and hSHP. The mobility of the FXR/RXR complexes was determined and compared to the non-specific (NS) control.
Figure 4

A hSHP-Luc

B mBSEP-Luc
Figure 4

C  
\[ \text{mI-BABP}_{1031}\text{-Luc} \]

- DMSO
- CDCA
- CDCA+LG
- GW4064
- GW4064+LG

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D  
\[ \text{mI-BABP}_{142\text{mut}}\text{-Luc} \]

- DMSO
- CDCA
- CDCA+LG
- GW4064
- GW4064+LG

<table>
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**Figure 4**

**E**

**mI-BABP\textsubscript{1031-Luc} (HepG2)**

- DMSO
- GW
- GW+LG

**F**

**mBSEP-Luc (HepG2)**

- DMSO
- GW
- GW+LG
Figure 5

The figure shows a bar graph representing the luminescence units (RLU) of mI-BABP1031-Luc under different conditions. The x-axis represents the concentrations of RXR (ng) and FXRβ1 (ng) with values 0, 5, 10, 25 ng for each respective category. The y-axis represents the RLU with a range from 0 to 60 RLU. The graph includes bars for DMSO, GW, and GW+LG treatments.

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x

- For RXR (ng) 5
  - DMSO: 1x
  - GW: 2.7x
  - GW+LG: 28.3x
Figure 6

A

Neo α1 α2 β1 β2
FXR
18S rRNA

B

mBSEP

DMSO
GW4064
GW4064+LG

Relative Expression

Neo FXRα1 FXRα2 FXRβ1 FXRβ2

C

mI-BABP

DMSO
GW4064
GW4064+LG

Relative Expression

Neo FXRα1 FXRα2 FXRβ1 FXRβ2

141x 5.2x 21x 2.6x
Natural structural variants of the nuclear receptor FXR affect transcriptional activation
Yanqiao Zhang, Heidi R. Kast-Woelbern and Peter A. Edwards

*J. Biol. Chem.* *published online October 19, 2002*

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