Natural Structural Variants of the Nuclear Receptor Farnesoid X Receptor Affect Transcriptional Activation

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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′-rapid amplification of cDNA ends followed by Southern blotting reveal that these four transcripts are expressed differentially in liver, intestine, kidney, adrenals, stomach, and heart. Electrophoretic mobility shift assays demonstrate that FXRα2 and FXRβ2 bind to FXR response elements with a higher affinity as compared with 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 ileal bile acid-binding protein 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 ileal bile acid-binding protein 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 bile salt export pump 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.

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, a hinge region and a ligand binding domain, a dimerization interface, and a ligand-dependent activation function (AF-2) at the carboxyl 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 subgroups 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 subgroup contains members that bind to DNA as heterodimers with the common partner, retinoid X receptor (RXR)³ (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 FXR ligand binding domain as bait (18). Northern blot assays and in situ hybridization indicate 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 provide 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 (27, 28), apolipoprotein C-II (29), multidrug resistance-associated protein 2 (ABCC2) (30), the bile salt export pump (BSEP) (31), and the small heterodimer partner receptor (SHP) (25, 32) (for review, see Ref. 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 DNA binding domain. 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. Because 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 °C) on a 12-h 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 °C 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 panel according to the manufacturer’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 different 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-FXRα1, CMX-FXRα2, CMX-FXRβ1, and CMX-FXRβ2. 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 MCV-IRE5-neo vector to make constructs MSCV-FXRα1, MSCV-FXRβ1, and MSCV-FXRβ2. The mouse BSEP promoter (−1050 to +25) was amplified using gene-specific primers and cloned into SacII/XhoI-digested-PSL3-LUC vector (Promega) to create pGL3-BSEP-Luc. All the plasmids have been confirmed by sequencing. Plasmids pBABPα1-Luc and pBABPα2-Luc were kindly provided by Dr. Bryan Goodwin (GlaxoSmithKline) (25). The retroviral vector MSCV-IRE5-neo plasmid was a gift from Dr. Owen Witte (University of Texas Southwestern Medical Center) (21). pGL3-bshP-Luc was kindly provided by Dr. Bryan Goodwin (GlaxoSmithKline) (25). The retroviral vector MSCV-IRE5-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′ Rapid Amplification of cDNA Ends (5′ RACE) and Southern and Northern Blot Analysis—**Sure-RACE mouse panels (OriGene Technologies) 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 and 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 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).

**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).

To identify all possible FXR isoforms we employed 5′-RACE and cDNAs generated from 24 murine tissues that had been ligated to a 5′ adaptor (OriGene Technologies). 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
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FXR Isoforms Are Differentially Expressed in Tissues—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:

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 “Experimental Procedures.” The 5′ ends of FXRα1 and FXRα2 are different from those of FXRβ1 and FXRβ2. FXRα1 and FXRβ2 correspond to RIPC4-2 and RIPC4-1, respectively. An additional 3′-terminal 12 base pairs (GTGCGCGTGA) were identified at the 5′ end of FXRβ1 and FXRβ2 mRNAs compared with RIPC4-1. The first ATG (asterisk) and stop codon (∗) 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 in-frame 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) that is located within the hinge region (D domain). The DNA binding domain (DBD) and ligand binding domain (LBD) are indicated.

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 a pCR2.1 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 under “Experimental Procedures” and normalized to cyclophilin. SI-D, SI-I, SI-J represent duodenum, jejunum, and ileum portions of small intestine, respectively.

76,997 bp and contains 11 exons that range in size from 100 to 572 bp and introns that vary from 328 to 16,388 bp. 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 DNA binding domain (Fig. 1C).

Procedures”), Southern blot analysis identified the PCR products that corresponded to FXR cDNAs, and these were subsequently cloned into pCR2.1 vector. Radiolabeled 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 bp) 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...
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TABLE I
Genomic organization of the murine FXR gene

| Exon | Exon size (bp) | Intronic size (bp) | Splice acceptor site (intron-exon) | Donor acceptor site (exon-intron) |
|------|---------------|-------------------|-----------------------------------|-------------------------------|
| 1    | 184           | 16,388            | GTCACCCAGG                        | GCAATCAGT-taagttc            |
| 2    | 134           | 9,602             | tgcctctcaq-GACTTCAATT             | AGCTATTTTG-taagttgct        |
| 3    | 121           | 8,086             | tccctctcaq-CTCGCGTGA              | CCAGCTAAG-taaggtcct         |
| 4    | 372           | 14,615            | tttactctcaq-GATGTCACT             | GGGTCGCAAAG-tgagagttgct     |
| 5    | 142           | 2,749             | ttcctccacag-GTCTTCCGG            | TGGTCTGAAAT-tgatgtacatc     |
| 6    | 138           | 1,613             | cccagcagag-AGTTGTAAAC            | GGTTGCAGAG-taagtgctgc      |
| 7    | 100           | 328               | gccagcctag-GAGAAAACGGG           | AAAATAACAT-tgatgtcctgc     |
| 8    | 101           | 4,450             | tasaaggtag-TAAAGAAAGG           | AAGCTCCGAG-tatattttt       |
| 9    | 148           | 15,454            | atccccatag-GCCCTCCGG            | CGAAGAGTGC-taagtgacac      |
| 10   | 115           | 1,685             | ccccacagag-ACACAGCAGTA          | CTCTCCTCGAG-taattgccg      |

FXRα2 and FXRβ1-FXRβ2 in different tissues vary significantly. For example, the ratio of FXRα2: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 (Table II). Because the DNA was analyzed 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:FXRβ1 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—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 with 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 mI-BABP FXR response element (FXRE) or the hSHP FXRE with a higher affinity as compared with 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 phospholipid transfer protein, rat multidrug resistance-associated protein 2, 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 Me2SO (vehicle), CDCA (physiological FXR ligand) or 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 mI-BABP promoter-reporter gene was differentially induced by the four ligand-activated FXR isoforms (Fig. 4C). The rank order of activation of the mI-BABP promoter-reporter gene was
When the FXRE in the mI-BABP promoter was mutated, the reporter gene was no longer activated by any of the FXR isoforms (Fig. 4D). These data suggest that activation of the mI-BABP promoter by the four FXR isoforms is mediated through the FXRE in the promoter.

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 extensively in the study of FXR target genes, with the mI-BABP and mBSEP promoter-reporter genes. As shown in Fig. 4E, the mI-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 FXRβ1 and FXRβ2, 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, which utilize either CV-1 or HepG2 cells and mI-BABP and mBSEP promoter-reporter genes, give essentially identical results.

**Fig. 4. Four murine FXR isoforms differentially transactivate reporter constructs.** Promoter-reporter plasmids pGL3-hSHP-Luc (A), pGL3-mBSEP-Luc (B and F), pIBABP<sup>1031</sup>-Luc (C and E), or pIBABP<sup>142mut</sup>-Luc (D) were transiently co-transfected into CV-1 cells (A–D) or HepG2 cells (E and F) together with the indicated FXR isoform or no receptor (NR). Cells were then treated with Me<sub>2</sub>SO (DMSO, vehicle), CDCA (100 μM) or GW4064 (1 μM) in the presence or absence of LG100153 (LG; 100 nM) for 42 h. Luciferase activities were assayed and normalized to β-galactosidase activity. The data (mean ± S.E.) 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 with that obtained from Me<sub>2</sub>SO-treated cells that were not transfected with nuclear receptors. RLU, relative light units.

FXRβ2 > FXRα2 ≫ FXRα1 = FXRβ1. When the FXRE in the mI-BABP promoter was mutated, the reporter gene was no longer activated by any of the FXR isoforms (Fig. 4D). These data suggest that activation of the mI-BABP promoter by the four FXR isoforms is mediated through the FXRE in the promoter.

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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. To test this hypothesis, we transiently transfected HepG2 cells with the mI-BABP reporter gene and different ratios of FXRβ1 (which contains the four-amino acid insert) and FXRβ2 (which lacks the four amino acids). The data of Fig. 5 show that relatively high levels of FXRβ1 (25 ng of 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 of plasmid) (Fig. 5). Most importantly, this high level of activation in response to ligand-activated FXRβ2 was greatly attenuated in cells after 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 5 different cell lines were then treated with 24 h with vehicle or ligands for FXR (GW4064) or FXR and RXR (LG100153) before 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 was the same as that observed in transient transfection experiments (FXRβ2 > FXRα2 > FXRβ1 = FXRα1) (Fig. 6C). Taken together, these results strongly suggest these four FXR isoforms function differentially in vivo.

Herein, we report the cloning and functional properties of four murine FXR isoforms, termed FXRα1, FXRα2, FXRβ1, and FXRβ2. Before 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. Because the additional four amino acids are separated from the DNA binding domain 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 and 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 I). Because 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 under way to determine whether the two promoters that control the expression of FXRα and FXRβ are differentially regulated. A
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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 there are multiple FXR isoforms in lung, adipose tissue, intestine, and kidney, tissues that are known to be involved in bile acid synthesis and metabolism. The current demonstration of these tissues. Identification of these target genes and putative 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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