

Identification of Farnesoid X Receptor β as a Novel Mammalian Nuclear Receptor Sensing Lanosterol

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Nuclear receptors are ligand-modulated transcription factors. On the basis of the completed human genome sequence, this family was thought to contain 48 functional members. However, by mining human and mouse genomic sequences, we identified FXR β as a novel family member. It is a functional receptor in mice, rats, rabbits, and dogs but constitutes a pseudogene in humans and primates. Murine FXR β is widely coexpressed with FXR in embryonic and adult tissues. It heterodimerizes with RXR α and stimulates transcription through specific DNA response elements upon addition of 9-*cis*-retinoic acid. Finally, we identified lanosterol as a candidate endogenous ligand that induces coactivator recruitment and transcriptional activation by mFXR β . Lanosterol is an intermediate of cholesterol biosynthesis, which suggests a direct role in the control of cholesterol biosynthesis in nonprimates. The identification of FXR β as a novel functional receptor in nonprimate animals sheds new light on the species differences in cholesterol metabolism and has strong implications for the interpretation of genetic and pharmacological studies of FXR-directed physiologies and drug discovery programs.

Cholesterol metabolism is a tightly regulated enzymatic pathway. Deregulation of this pathway leads to accumulation of excess cholesterol and can result in diseases such as atherosclerosis and gallstone formation (10). The homeostatic balance between uptake and elimination of cholesterol is accomplished by regulation of three pathways: de novo cholesterol synthesis from acetate, uptake of cholesterol from the intestine, and elimination of cholesterol through the synthesis of bile acids.

Cholesterol catabolism into bile acids is controlled by transcriptional feedback and feedforward mechanisms that are mediated by members of the nuclear receptor family. Activation of cholesterol breakdown into bile acids is mediated by liver X receptor alpha (LXR α ; NR1H3), a nuclear receptor that binds oxysterols formed during the synthesis and metabolism of cholesterol (14, 18, 28). Together with another nuclear receptor, liver receptor homologue 1 (NR5A2), LXR α stimulates transcription of cholesterol 7 α -hydroxylase (CYP7A1), an enzyme catalyzing the rate-limiting step of this pathway. Suppression of cholesterol degradation into bile acids is triggered by the farnesoid X receptor (FXR; NR1H4), which binds to and is activated by bile acids (21, 27, 33). Ligand-bound FXR activates transcription of the short heterodimer partner (NR0B2) and subsequently downregulates transcription of CYP7A1 (11, 19). However, there are species differences in the regulation of

cholesterol metabolism and sensitivity to dietary cholesterol. While rodents respond to cholesterol feeding with induction of CYP7A1, humans and rabbits appear to lack this response and are left more sensitive to the cholesterolemic effects of dietary cholesterol (13, 35, 36). This difference was directly attributed to the ability of LXR to regulate CYP7A in different species (4, 24).

Nuclear hormone receptors form a family of evolutionarily conserved sequences, and a standardized nomenclature has been proposed (26). Six subfamilies are currently recognized; the nuclear receptors FXR and LXR α and - β form the subgroup NR1H together with the *Drosophila* ecdysone receptor (EcR), which recognizes the metamorphosis steroid ecdysone as its natural ligand (16). A set of 48 human nuclear hormone receptors was known well before the human genome was sequenced. In the *Drosophila* genome, only 21 nuclear hormone receptors were predicted (1), whereas for *Caenorhabditis elegans*, more than 270 sequences homologous to the nuclear hormone receptor family were identified (32). Publication of the sequence of the human genome allowed the identification of all genes homologous to the nuclear receptor family of proteins. We and others identified three novel nuclear receptor sequences apart from the 48 known nuclear receptors, all potentially representing pseudogenes in humans (6, 20, 29).

Given the known species-specific difference in cholesterol metabolism, our attention was particularly drawn to a pseudogene showing strong homology to FXR (NR1H4) that we and others designated FXR β (NR1H5). Here we demonstrate that FXR β is, in contrast to previous perception, a novel functional member of the NR1H subgroup of nuclear receptors. It con-

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stitutes a functional nuclear hormone receptor in all of the mammalian species investigated except primates and humans, where it encodes a nonfunctional protein. In mice, FXR β is able to activate transcription and its endogenous ligand was identified as the cholesterol precursor lanosterol. Since lanosterol is an intermediate in the cholesterol biosynthetic pathway, this nuclear receptor may represent an additional level at which cholesterol levels are controlled in nonprimate mammals.

MATERIALS AND METHODS

Bioinformatic analysis. A database of all nuclear receptor protein sequences was constructed and used to search the daily human genome updates (GenBank) by employing the BLASTX tool with default parameters (2). Two FXR β -containing bacterial artificial chromosomes (BACs) were identified: GenBanknew accession no. AC026039 and EMBLnew accession no. AL390235.

Molecular cloning of FXR β . The murine FXR β (mFXR β) genome locus was isolated by screening the BAC library Easy-to-Screen DNA Pools BAC Mouse ES (release II) from Incyte Genomics by using primers 5' TGGGGTCTTTG TTTTCCA3' and 5'GTGAAATGGACATGTACATGCG. FXR β -containing BACs were purchased, and the locus was sequenced by using PCR products generated with the above-described primers. Flanking regions were sequenced by primer walking. FXR β cDNAs from mice and other mammalian species except humans were isolated by using adult liver and testis RNAs from the respective species and the above-described primers. For amplification of primate FXR β sequences, genomic DNAs and RNAs from the respective species were derived from the collections at the S. Pääbo laboratory. Full-length cDNAs were isolated by rapid amplification of cDNA ends with a GeneRacer kit (Invitrogen). Human FXR β (hFXR β) cDNAs were isolated by reverse transcription (RT)-PCR of adult human testis RNA (Clontech) with primers 5' CCAGACCAACCTATTC TTCCTCGAGAAATAAGGGAC 3' and 5' TGGGGTCTTTGTTTCCAAG TGCTAAGTATTCTG 3' in 35 PCR cycles followed by 30 cycles of nested PCR.

Expression analysis. RT-PCR expression analysis of mFXR β was performed with primers 5' TCATCCAGCACCAGATCTGGGAAAG 3' and 5' GTCCTT TGTTTTCCACATGCCAAGG 3' and that of mFXR was performed with primers 5' GGGATGTTGGCTGAATGTTGTAACTG3' and 5' TCACTGCAC ATCCCAGATCTCACAG3' in 35 and 28 cycles PCR cycles, respectively, with a panel of adult (Clontech) and embryonic (Quantum Appligene) RNAs.

Plasmids. The gateway cloning system (Life Technologies) was used to insert the ligand binding domains (LBDs) of hFXR (amino acids 187 to 472) and mFXR β (amino acids 186 to 505) into pCMV-BD (Stratagene) and that of human retinoic X receptor alpha (hRXR α ; amino acids 197 to 461) into pCMV-AD (Stratagene) to generate mammalian two-hybrid vectors. pCMV-BD-hFXR and pCMV-BD-mFXR β express their respective FXR LBDs as fusion proteins with the Gal4 DNA binding domain (DBD). The hRXR α LBD is expressed fused with the activation domain (AD) of NF- κ B. As a Gal4-responsive luciferase reporter, pFR-LUC (Stratagene) was used. Full-length mFXR β (GenBank accession no. AY094586) was inserted into expression vector pCMV-AD containing an NF- κ B AD (Stratagene) and expression vector pcDNA4/TO (Invitrogen). Luciferase reporter plasmids were constructed by inserting synthetic response elements (REs) into the *Xho*I site of vector pGL2-Promoter (Promega). Oligonucleotides were designed containing two REs separated by 10 nucleotides. The REs consist of two consensus half sites with the sequence AGGTCA as inverted repeats (IRs), everted repeats (ERs), or direct repeats (DRs) with zero- to eight-nucleotide spacers between the half sites and flanking *Xho*I sites.

The I-BABP RE reporter construct contains two tandem copies of the oligonucleotide CCCCAGGGTGAATAACCTCGGGGCTCTGTCCCTCCAATCC CAGGGTGAATAACCTCGGGA from the human I-BABP promoter (12) ligated between the *Kpn*I and *Bgl*II sites of pGL2-Promoter (Promega). For the homogeneous time-resolved fluorescence (HTRF) assay, the mFXR β LBD (amino acids 186 to 505) and the hLXR α LBD (amino acids 155 to 447) were fused to glutathione *S*-transferase (GST), His tagged, and recombined into the pACGHLT polylinker (PharMingen) with a gateway cassette (Life Technologies). A GST fusion of the hFXR LBD (amino acids 187 to 476) and a His-tagged NR interaction domain of human Tif2 (hTif2; amino acids 548 to 878) were recombined into pDest15 and pDest17 (Life Technologies).

Transient transfections. Human embryonic kidney (HEK) 293 cells were maintained at 37°C in 5% CO₂ in Dulbecco modified Eagle medium (DMEM;

Invitrogen) containing 10% fetal calf serum. Cotransfection was performed in OptiMem (Invitrogen) with 50 ng of the appropriate expression vector, 50 ng of the reporter construct, and 10 ng of a thymidine kinase-*Renilla* luciferase control vector with Lipofectamine (Invitrogen) for 4 h. Cells were then treated with ligands and grown in DMEM for 18 h. Ligands and ketoconazole (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Lanosterol was freshly dissolved in ethanol at 35°C. When treated with lanosterol, cells were grown in the presence of 1% DMSO for 2.5 h to permeabilize cell membranes and facilitate uptake (38) and then kept in DMEM for 16 h. After ligand treatment, cells were harvested and luciferase assays were performed with the dual-luciferase reporter assay as described by the manufacturer's (Promega) manual. Transfection data were normalized to the *Renilla* control and expressed as mean numbers of relative light units from triplicate assays \pm the standard deviations (SDs).

Coactivator recruitment assay. The mFXR β and hLXR α LBDs were used in the Pharmingen Baculovirus Expression vector system. The hFXR LBD and hTif2 were expressed in *Escherichia coli*. The resulting proteins were GST or His affinity purified as recommended by Pharmacia and QIAGEN. His-hTif2 was biotinylated (Roche). A biotinylated mSCR1 peptide (amino acids 680 through 704) was purchased (SynPep Corp.). Proteins and compounds were mixed with 1 nM europium cryptate-anti-GST antibody and 100 ng of streptavidin-allophycocyanin in black 384-well microtiter plates (Nunc Fluotrac200). The reaction mixture volume was 25 μ l. Plates were shaken for 2 min at 800 rpm and incubated for 60 min at room temperature. Measurement of fluorescence from both fluorophores at 665 nm (acceptor signal) and 615 nm (donor signal) occurred in a time window of 50 to 400 μ s after excitation on a Victor V HTRF reader. Signals were plotted as ratios of light intensity at 665 nm versus light intensity at 615 nm. For analysis of dose-response curves, ratios were plotted against log concentrations and 50% effective concentrations (EC₅₀s) were calculated by Prism (GraphPad Software Inc.).

RESULTS

Identification of primate FXR β as a pseudogene. In an effort to identify novel nuclear hormone receptor proteins after the publication of the human genome sequence, we performed exhaustive database searches with the BLAST algorithm (2). As one result, we and others (6, 20, 29) detected a novel member of the NR1H subgroup, termed FXR β (NR1H5), on the basis of its homology to FXR. The novel gene has conserved intron positions compared to FXR, which, together with the sequence conservation, argues in favor for a functional nuclear receptor. In addition to previously published results (20), we identified several splice variants of FXR β in RNA from human testis (data not shown). However, by sequencing those cDNAs and three independent genome loci, we found that hFXR β contains two stop codons in exon 11 and three frame shifts at exon-intron junctions (Fig. 1A, arrows). The extremely low transcript levels determined by nested RT-PCR and the presence of two stop codons and three frame shifts in the cDNA strongly argue that FXR β is a pseudogene in the human lineage.

To assess whether FXR β constitutes a functional protein in other primate species, we performed PCR amplification of genome loci and analysis of the chimpanzee, gorilla, orangutan, and rhesus monkey FXR β sequences. In all cases, stop codons and frame shifts were identified at positions similar to those in the human mRNA and no transcripts could be detected by RT-PCR from liver and testis RNA sources (data not shown). Thus, FXR β seems to be a nonfunctional gene in these primate species as well.

FXR β codes for a functional receptor in other mammals. RT-PCR amplification of liver RNA and subsequent sequence analysis of nonprimate mammals, including mice, rats, rabbits, and dogs, revealed FXR β mRNAs showing continuous open

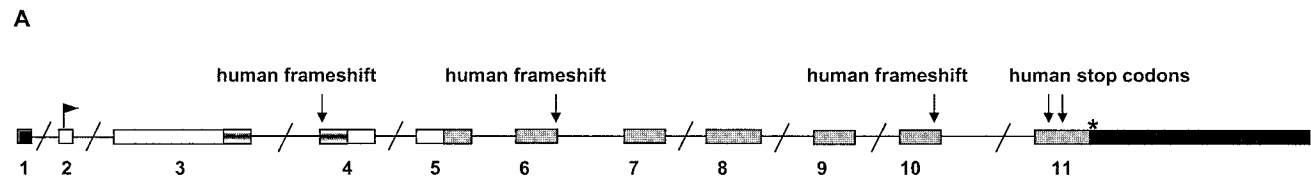


FIG. 1. FXR β genomic structures and molecular cloning. (A) Schematic representation of FXR β genomic structure. mFXR β and hFXR β loci show similar exon-intron structures. Here the murine locus is displayed, consisting of at least 11 exons spanning 26 kb of genomic DNA. Striped boxes represent the DBD, gray boxes represent the LBD, the asterisk represents the stop codon, and the flag represents the start codon. The 5' untranslated region is encoded by exon 1, while exon 11 encodes the 3' untranslated region (black boxes). Human stop codons and frame shifts are indicated by vertical arrows. Untranslated regions are not completely sequenced for the human locus. (B) Sequence of mFXR β . Shown are cDNA and protein sequences of mFXR β (GenBank accession no. AY094586). Start and stop codons are bold, and the poly(A) signal is underlined.

CTC ATT CCA TGA GGA GGA GGG CAG ACT GCA GGA ACA CTA CCC AAT TGT TTT GTT CTG AAA
 GTC TTT AGC ATT GCT GAA GGA AAC AAC TAG AAG CTG GAC CAG AGA ATG GCA AAT ACC TAT
 GTT GCP ACG TCT GAT GGG TAC TAC CTC GCT GAA CCC AGA CAG TAT TAT GAT ATT TTG CCA
 E Q P F H Y Q L C D T D F Q E P P Y C Q Y
 GAA CAA TTC CAT TAT CAG CTG TGT GAT ACA GAT TTC CAA GAA CCA CCC TAT TGT CAA TAT
 S T A Q P P P A L L Q S P S L Q S H F N T
 TCT ACC GCT CAG TTT CTT CCA GGG TTA CAG TCC CCA TCT TTA CAA AGP GAT TTC AAC ACA
 H C L D P C V S C G S W C G L D A R E S
 CAT GGT TTG GAT CCA CAG TAC AGT GCA GGC AGT TGG TGT GSA CTC GAG GCT CGA GAA TCT
 G Q S T Y V V V H D D E D P F P G A Q R
 GGT CAG TCC ACT TAT GTG GGT GTT CAC GAT GAT GAA GAT GAA TTC CCT GGG GCA CAA AGC
 C R A T C C S L R W K G Q D D M L C M V C
 TSS ASA GCA ACT TGT TCT TTA CCG TGG AAG GGT CAA GAT GAC ATG CTC TGC ATG GTC TGC
 G D R A S C Y H Y N A L T C F G C K G F
 GGT GAT AAG GCA TCA GSA TAT CAC TAC AAT GCA TTP ACT TGT GAG GGG TCC AAA GGC TTT
 F R R S I T K N A V Y S C K N G G H C E
 TTC GGG GGT AGC ATT ACC AAG AAT GCA ATG TAT TCT TGC AAG AAC GCP GCP CAA TGT GAA
 M D M Y M R R K K C Q E T C R L K K C K A V
 ATG GAC ATG TAC ATG CCG AGA AAA TSC CAA GAG TSC AGA CTG AAG AAG TGT AAG GCG GTG
 Q M L A E C L L T E Y Q C K S K F S G K
 GGT ATT TCG GAA TGT TTT CTT ACG AAG ATC CAG TTT AAG TCG AAG ASA CTT GCG AAG
 N P K H G P A L Y P A I Q V E D R G A D
 AAC TTC AAG CAC GGG CCT GCT CAG TCC GCT GGC ATC CAA GFG GAA GAT GAA GCA GCA
 T K H V S S S T R S G K G V Q D N M T L
 ACC AAA CAC CTG TCA TCC AGC ACC AGA TCT GGG AAA GGG GTT CAG GAC AAC ATG ACT CTA
 T Q F E H P L E H T V T A H Q K S M I
 ACT CAA GAG CAA CAT GGT CTT CTG AAT ACC ATA GTG ACT CCT CAC CAA AAA TCC ATG ATT
 P I G E T S K L L Q F E G S N P E L S F L
 CCC TTG GSA GAA ACA AGC AAA CTT CTG CAG GAG GGT TCC AAC CCC GAA CTA AGT TTT CTG
 R L S E V S V L H I Q G L M K F T K G L
 AGA CTT TCA GAG TCA GTC CTG CAG ATG CAA GGG CTA ATG AAG TTT ACC AAG GGA CTC
 P G F P E N L T T E D Q A A E Q A S K T
 CCA GGA TTT GAA AAT TTA ACC ACT GAG GAT CAG GCT GCA TTA CAG AAG GCG TCA AAA ACT
 H V M Z L H V A Q L Y G C K D S T S G S
 GAG GTG ATG TCT CTT CAT GTA GCC GAG CTT TAT GGT GSG AAA GAC TCA ACC TCT GGA AGT
 T M R P A K P S A G T L E V H N P S A D
 ACT ATA AGA CCA GCA AAG CCC TCA GCT GGG ACA CTA GAG GTG CAT AAT CCI AGC GCI GAT
 E S V H S P E N F L R E G Y P S P L T
 GAA AGT GTT CAT TCT CCG GAA AAG TTT CTC AAG GAA GGC TAC CCT TCG AGT GCT CTA ACT
 D I T K E P T A S L E V P Y R R M S E L
 GAT ATT ACT AAA GAA TTT ATT GCC TCA TCT TAC TTT CAC AGA AGA ATG AGT GAA CTT
 H V S D T E Y A I L T A T T V L F S D R
 CAT GTA TCG GAT ACT GAA TAT GCT CTT ACC GCG ACA ACA GTG CTT TTC TCA GAT GGT
 P C L K N K Q H V E N L Q E P V L L
 CCA TCC TTT AAA AAT AAG CAG CAT ATA GAA AAC CTA GAA CAG GTC CTS CAA CTT TGT
 F F F S H M Y H D E D P Q H E A H L E Q
 TTT AAG TTT TCA AAA ATG TAC CAT CCA GAA GAC CCA GAG CAT TTC GGC CAC CTC ATA GSG
 R L T E L R T L S H S H S E I L R M W K
 AGG CTT ACT GAA CTG AGA ACT CTG AGT CAC AGC CAC TCT GAA ATC CTT GCG ATG FGG AAA
 T K D P R L V M L D P S E K W D L H S F S
 ACA AAG GAC CCC AGG TTT GTG ATG TTA TTT TCT GAG AAA TGG GAT CTG CAG TCA TTT TCC
 +

indicating that these two genes are, in fact, FXR β orthologs. We identified *in silico* two different ORFs from the draft sequence of the pufferfish (*Fugu rubripes*) genome that show similarity to the FXR genes. *Fugu rupies* FXR1 is included with good confidence in the FXR lineage. FXR2 is building a separate deep branch at the root of the tree and appears to be ancestral to the duplication that led to the clusters of FXR and FXR β . Mutation rates are higher for FXR β than for FXR, as represented by respective branch lengths on the phylogenetic tree (Fig. 2).

Ubiquitous tissue expression of mFXR β . To investigate the potential function of the novel receptor, we analyzed mFXR β transcripts in embryonic and adult tissues. Five splice variants of mFXR β were isolated by RT-PCR from adult liver and testis RNA. Isoforms differentially splice out parts of exon 3, 8, or 10 and thereby differ in the regions encoding the DBD and the LBD (Fig. 3A). mFXR β is ubiquitously and strongly expressed during embryonic development, and its expression pattern largely overlaps that of FXR (Fig. 3B). This mRNA distribution suggests a function of mFXR β during embryonic development. In the adult, however, mFXR β expression is more tissue specific and restricted mainly to the liver, reproductive tissues, and heart. Coexpression with FXR is observed in the liver and intestine, main tissues of FXR expression and function, but not in the kidney (8).

The IR1 and ER2 REs are recognized by mFXR β . Nuclear receptors bind specifically to repeats of single DNA hexamers called REs (23). They bind either as monomers, homodimers, or heterodimers with RXR. As heterodimers, they bind to REs consisting of a repeated core motif that can be an IR, a DR, or an ER. To determine the DNA binding properties of mFXR β , we fused mFXR β to a constitutively active NF- κ B AD and screened against a panel of luciferase reporter constructs containing synthetic consensus IR, DR, and ER elements in transient cotransfection assays. Transactivation of the luciferase reporter gene was observed with the synthetic IR1 and ER2 elements, as well as the FXR RE from the I-BABP promoter, which is itself an IR1 element (12) (Fig. 4A). The specific interaction with typical FXR REs suggests that at least part of the FXR target genes may also be regulated by FXR β .

reading frames (ORFs). These mRNAs therefore potentially encode functional nuclear receptor proteins. RT-PCR and rapid amplification of cDNA ends of mFXR β verified 10 coding exons translating into a protein of 505 amino acids (Fig. 1A and B). The 5' untranslated region is encoded by exon 1, while exon 11 encodes the 3' untranslated region and is flanked by a poly(A) signal.

All isolated FXR β sequences form a robust phylogenetic group well separated from the FXR cluster (Fig. 2). The FXR orthologs recently isolated from *Xenopus laevis*, FOR1 and -2 (31), are included with high confidence in the FXR β branch,

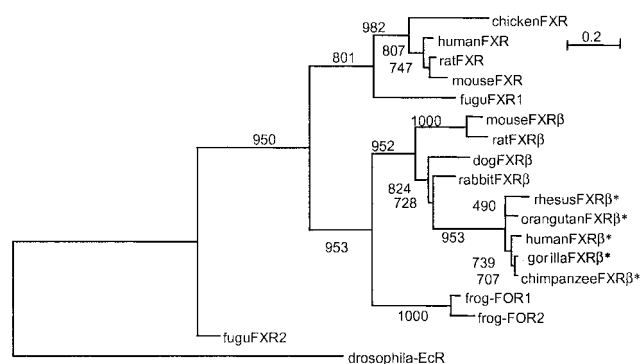
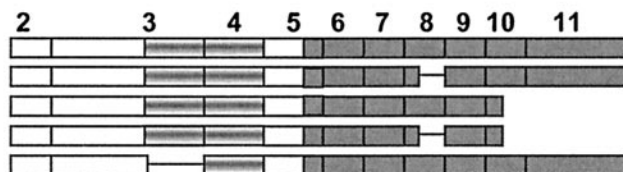


FIG. 2. Phylogenetic tree containing FXR and FXRβ genes from different species. The phylogenetic tree was calculated on the basis of protein alignments by using PHYLIP (phylogeny interference package, 1993; distributed by J. Felsenstein, University of Washington, Seattle, Wash.) on the basis of the following sequences (accession numbers): chicken FXR (AF492497), hFXR (U68233), rat FXR (U18374), mFXR (NM009108), *Fugu* FXR1 (S001361), mFXRβ (AY094586), frog FOR2 (AF456452), frog FOR1 (AF456451), *Fugu* FXR2 (S000248), and drosophila EcR (M74078). Numbers indicate the bootstrap support (out of 1,000) for the respective nodes. Asterisks indicate putatively nonfunctional proteins in primates.

mFXRβ heterodimerizes with RXRα. Since nuclear receptors are known to interact with their REs as either homodimers or RXR heterodimers (23), we examined the ability of mFXRβ to interact with RXR in response to its ligand, 9-*cis*-retinoic acid (9-*cis*-RA). For a mammalian two-hybrid assay, the LBDs of mFXRβ and hFXR were fused to the Gal4 DBD while hRXRα was fused to an NF-κB AD and cotransfected in HEK 293 cells with a luciferase reporter construct. Compared to the control, we observed the expected constitutive interaction between the hFXR and hRXRα LBDs (Fig. 4B). Addition of 9-*cis*-RA further enhanced this interaction. Compared to hFXR, mFXRβ exhibited a significantly stronger constitutive interaction with RXRα that was also potentiated by 9-*cis*-RA. These data indicate a constitutive mFXRβ-RXRα interaction and a responsiveness of the complex formed to the RXR ligand 9-*cis*-RA.

Lanosterol promotes coactivator recruitment by mFXRβ. FXR, LXRα, and LXRβ are activated by bile acids (21, 27, 33) and oxysterols (14, 18), respectively, all of which are intermediates of the cholesterol metabolic pathway. Since these receptors are evolutionarily related to FXRβ, we tested compounds

A



B

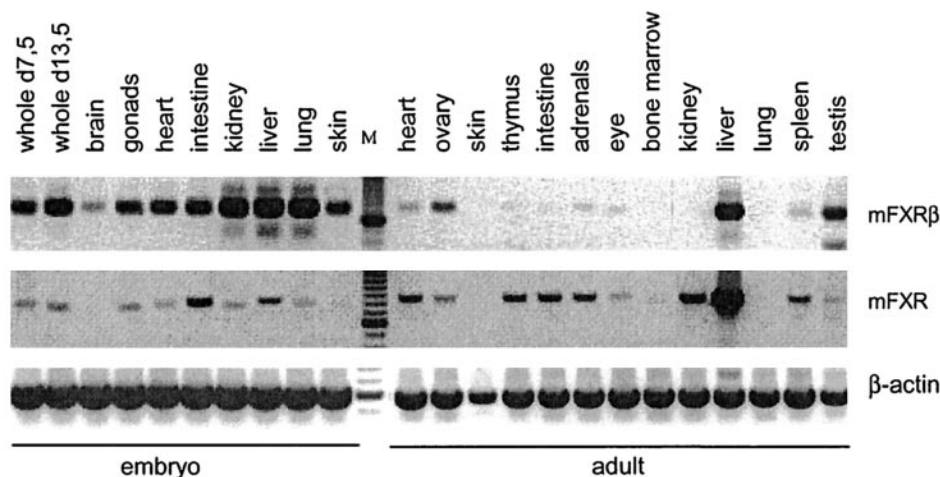
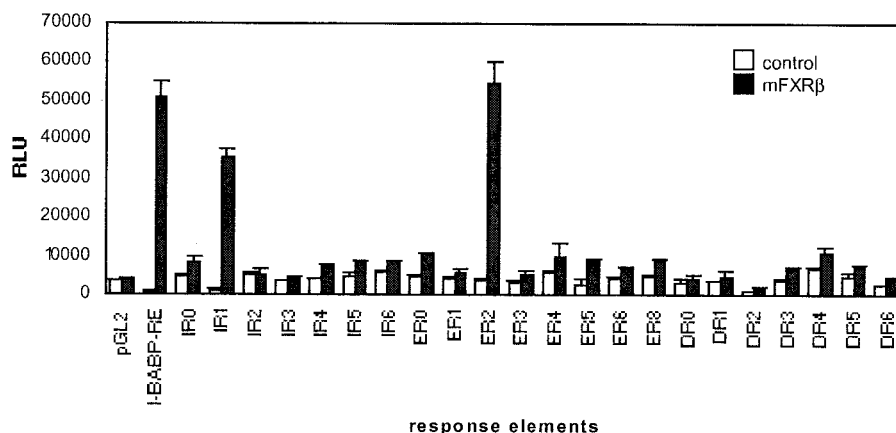


FIG. 3. Transcription of mFXRβ. (A) Splice variants of mFXRβ. Five splice variants were isolated and sequenced (GenBank accession no. AY094586 through AY094590). Striped boxes indicate exons encoding the DBD, and gray boxes indicate exons encoding the LBD. Numbers indicate exons. Shown are only coding regions. (B) Tissue distribution of mFXRs. mFXR and mFXRβ transcripts were determined by RT-PCR amplification of nonhomologous sequences derived from the LBDs of the respective receptors. β-Actin served as an internal control. RNAs from indicated embryonic and adult tissues were used as templates. Lane M, DNA size marker. d, day.

A



B

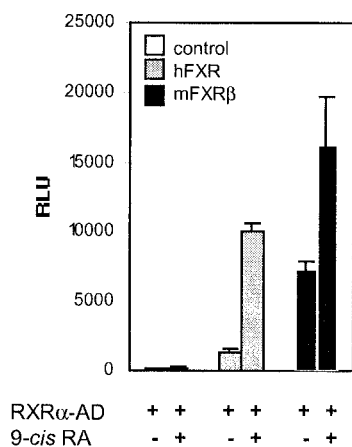


FIG. 4. mFXR β binds selectively to nuclear REs and forms heterodimers with RXR α . (A) DNA REs recognized by mFXR β . HEK 293 cells were cotransfected with expression plasmids containing mFXR β fused to an NF- κ B AD (black bars) or an empty vector (white bars) and the indicated plasmid from a panel of luciferase reporter constructs containing synthetic IR, ER, or DR REs with different spacers between the half sites (zero to eight nucleotides). Luciferase activities are means of triplicate experiments, and the SDs are indicated. (B) Interaction of mFXR β with RXR α is enhanced by 9-*cis*-RA. HEK 293 cells were cotransfected with the reporter vector pFR-Luc and RXR α fused to an NF- κ B AD (pCMV-AD-hRXR α) in combination with either pCMV-BD (white bars), FXR fused to the Gal4 DBD (pCMV-BD-hFXR; gray bars) or an mFXR β fusion with the Gal4 DBD (pCMV-BD-mFXR β ; black bars). Cells were treated with 1 μ M 9-*cis*-RA (+) or an equivalent volume of DMSO (-). Cells from triplicate experiments were assayed for luciferase activity, and means \pm SDs are shown. RLU, relative light units.

that are related to the respective ligands of these homologs for binding to mFXR β . This set of around 100 compounds includes cholesterol metabolites, steroid hormones, and other bile acid metabolites. We employed an HTRF ligand-sensing assay to measure the interaction between the mFXR β LBD and a short peptide derived from the coactivator mSRC-1 induced by a ligand. Interestingly, lanosterol, an intermediate of the de novo cholesterol biosynthetic pathway, showed the greatest affinity in the ligand-sensing assay. Lanosterol induces interaction between mSRC-1 and the mFXR β LBD with an EC₅₀ of \sim 1 μ M (Fig. 5A). Similar affinities are reported for the physiological ligands of other lipid receptors (14, 18, 34). Thus, lanosterol is a likely candidate for an endogenous ago-

nist ligand that induces coactivator recruitment by mFXR β . The FXR-specific compound GW4064 (22) has very poor affinity for mFXR β (Fig. 5B), suggesting different ligand specificities for both receptors. Furthermore, lanosterol displays a remarkable specificity for mFXR β as it binds neither to the closest relative of mFXR β , FXR (Fig. 5B), nor to the more distant relative LXR α (data not shown). In addition, mFXR β shows somewhat promiscuous ligand binding as it binds a quite diverse spectrum of chemical structures with lower affinity (Fig. 5C). Again, these compounds do not bind to FXR (data not shown). Finally it is important to note that various bile acids that, in part, can serve as endogenous ligands for FXR have no significant affinity for mFXR β (Fig. 5D). This again

indicates that there is no overlap between FXR and FXR β in terms of specificity for natural ligands

Lanosterol activates mFXR β as an agonist ligand. Finally, we sought to investigate the transcriptional activities of mFXR β on the I-BABP RE-containing reporter gene construct in a cellular context and the modulation of these activities by 9-*cis*-RA and lanosterol. Lanosterol is an intermediate of the cholesterol biosynthetic pathway (Fig. 6A). It is a precursor of the meiosis-activating sterols FF-MAS and T-MAS, which are synthesized from lanosterol by the cytochrome P-450 enzyme CYP51 (30). In transient transfection assays, we co-transfected a full-length mFXR β expression plasmid with the I-BABP reporter construct. mFXR β expression results in constitutive activation of reporter gene activity compared to the vector control (Fig. 6B). Addition of 9-*cis*-RA results in a dose-dependent increase in luciferase activity only in the presence of mFXR β . This supports the notion that mFXR β can exert its transcriptional activity as a heterodimer with RXR α . Furthermore, treatment of cells with 5 μ M lanosterol significantly stimulated reporter gene expression (Fig. 6C). This activation was potentiated by the simultaneous addition of 9-*cis*-RA. These data strongly suggest that mFXR β is, indeed, a functional hormone receptor, with lanosterol as its physiological agonist ligand.

However, as lanosterol is a highly water-insoluble compound and may only be able to enter cells to a small extent during the transfection assay, we sought to independently confirm the observed lanosterol effect. Inhibition of CYP51, the enzyme that demethylates lanosterol as the first step in the conversion to cholesterol, by ketoconazole (25, 37; Fig. 6A) should lead to accumulation of lanosterol within the cell. We therefore co-transfected HEK 293 cells with the full-length mFXR β expression vector and the I-BABP reporter construct and treated the cells with ketoconazole. We indeed observed transcriptional activation by mFXR β above the basal level (Fig. 6C). This effect could be potentiated by the addition of lanosterol and 9-*cis*-RA to the medium. Together, these results provide convincing evidence that lanosterol acts as an agonist ligand for mFXR β both in vitro and in vivo.

DISCUSSION

Nuclear hormone receptors are ligand-modulated transcription factors that mediate the transcriptional effects of endogenous ligands such as steroid, thyroid, and retinoid hormones (9). The importance of nuclear receptors as targets for the development of medicines raised strong interest in the identification of novel functional nuclear receptors in the human genome. Bioinformatic approaches did not identify novel functional receptors in the human genome in addition to the 48 previously known nuclear receptors. Only three additional pseudogenes were identified, including an FXR homologue designated FXR β (6, 20, 29).

We demonstrate in our report that in other mammalian species like mice, rats, rabbits, and dogs, the gene for FXR β has the capacity to code for a functional nuclear receptor protein. In a phylogenetic analysis, all isolated FXR β sequences form a phylogenetic group well separated from the FXR cluster. Since the FXR homologs from *X. laevis*, FOR1 and -2 (31), are, in fact, FXR β orthologs, our data indicate the

existence of an FXR β -type gene already in the common ancestors of amphibians and mammals. We identified in silico two different ORFs from the pufferfish (*F. rubripes*) genome, one of which is included in the FXR lineage while the other is building a separate deep branch at the root of the tree. Since no sequence with similarity to FXR β could be detected in the pufferfish genome, the phylogenetic data indicate an origin of the FXR β gene as part of the postulated second wave of gene duplications during tetrapod (land-living vertebrate) evolution that probably led to the diversification of receptors (7). FXR β later lost its function as a secondary event. However, it is unknown when during the evolution of primates the gene lost its function, since our analysis focused on great apes (gorillas, chimpanzees, and orangutans) and just one Old World monkey (rhesus monkey) was added. Further analyses of other branches of the primate lineage are necessary to solve this issue. To our knowledge, FXR β represents the first member of the nuclear hormone receptor family constituting a functional receptor protein in several mammalian species but a pseudogene in the primate lineage. It is therefore possible that other identified human pseudogenes in this family have functional counterparts in primate or nonprimate species as well.

FXR β was identified through its sequence similarity to FXR and was shown in our phylogenetic analysis to code for an independent nuclear receptor protein. However, when examining transcript levels in mouse tissues, we observed significant coexpression of FXR and FXR β . Both receptors are ubiquitously expressed in embryos, which argues for a role of both receptors during embryonic development. In addition, they are coexpressed in a subset of tissues in adult organisms. FXR has been shown to be involved in the regulation of cholesterol-bile acid metabolism and in the regulation of bile acid transporters in the intestine, which is accompanied by high expression levels in tissues such as those of the liver, kidneys, and intestine (8). Coexpression with mFXR β in the liver and intestine but not in the kidneys may indicate at least partly overlapping functions. The expression of mFXR β in tissues such as the testis and ovary suggests additional roles in reproductive functions.

Like most nonsteroidal receptors, such as LXR and FXR, mFXR β binds specific DNA elements as a heterodimer with RXR α , the 9-*cis*-RA receptor. Furthermore, we show that mFXR β can function through consensus IR1 and ER2 REs and is active as well on an FXR RE originating from the I-BABP promoter (12). Most published FXR target genes contain one or more IR1-type elements in the proximal promoter or distal enhancers that function as an FXR RE and are required for transcriptional activation (3, 11, 12, 17, 19, 21). Our finding that mFXR β recognizes consensus, as well as natural, IR1 elements strongly suggests the existence of common target genes for both FXR and FXR β . Moreover, FXR β activates transcription through ER2 but not ER8 elements, derivatives of which were recently described as targets for FXR (15). This finding, on the other hand, indicates possible differences in target genes between the two receptors.

Our results show that mFXR β is a nuclear hormone receptor that is activated by lanosterol, an intermediate in the cholesterol biosynthetic pathway. Importantly, mFXR β is not activated by bile acids, the endogenous ligands for its closest homologue, FXR. The ligand-bound nuclear receptors FXR and LXR are known to modulate cholesterol breakdown through

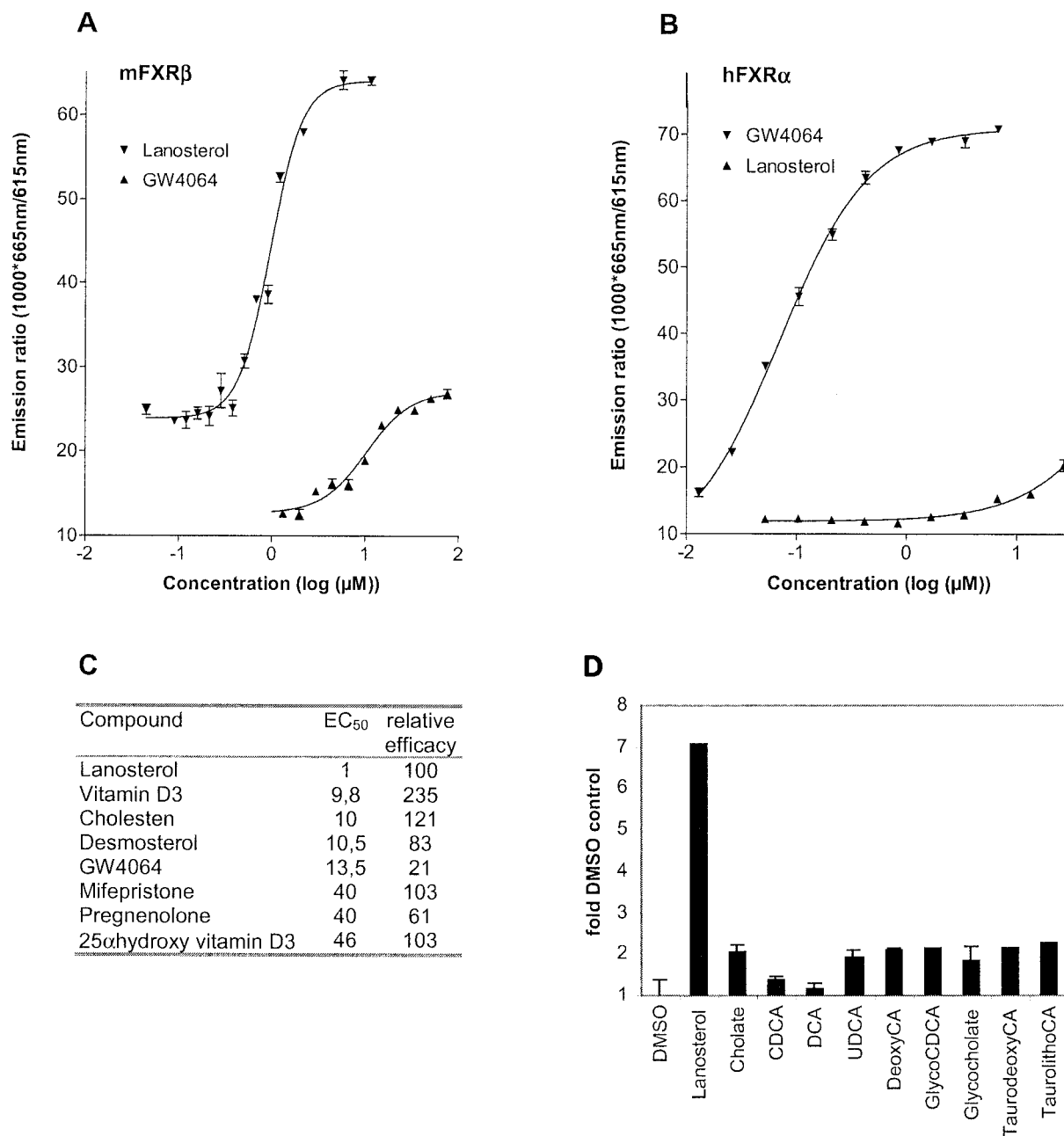


FIG. 5. Identification of ligands for mFXR β . (A) Lanosterol selectively induces SRC1 binding to mFXR β at physiological concentrations. GST-mFXR β LBD and biotinylated mSRC1 peptide were mixed, and the effect of lanosterol and the FXR agonist GW4064 on the interaction was determined by HTRF assay. Cofactor interaction is induced by lanosterol with an EC₅₀ of 1 μ M, whereas GW4064 only acts as a weak agonist. Signals are plotted as means of triplicates, and error bars indicate SDs. (B) GST-hFXR LBD was mixed with biotinylated, His-tagged hTif2, and the effects of lanosterol and GW4064 on the respective interaction were assayed. The FXR agonist GW4064 induces cofactor interaction with an EC₅₀ of 70 nM, whereas lanosterol does not show any effect. (C) A diverse set of compounds induces cofactor interaction with mFXR β but not FXR. Shown are EC₅₀s and the relative efficacy of SRC1 recruitment (the efficacy of lanosterol was set to 100%) calculated from dose-response experiments for a selected group of compounds found to be active for FXR β . (D) Cholates and cholic acids do not induce significant SRC1 binding to mFXR β , whereas lanosterol does induce SRC1 recruitment. GST-mFXR β LBD and biotinylated mSRC1 peptide were mixed. The effects of various compounds at a concentration of 40 μ M on the interaction was determined by HTRF assay. Ratios of signals versus DMSO controls are plotted as means of triplicates, and SDs are indicated. CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid.

regulation of CYP7A1, the rate-limiting enzyme of this pathway. However, there are observed differences between species in the regulation of cholesterol homeostasis. While rodents seem to be able to adapt to large fluctuations in sterol input

with little change in plasma cholesterol concentrations, humans do experience alterations in plasma cholesterol levels when sterol balances change (5). These observed differences have so far been explained by a rapid catabolism in rodents

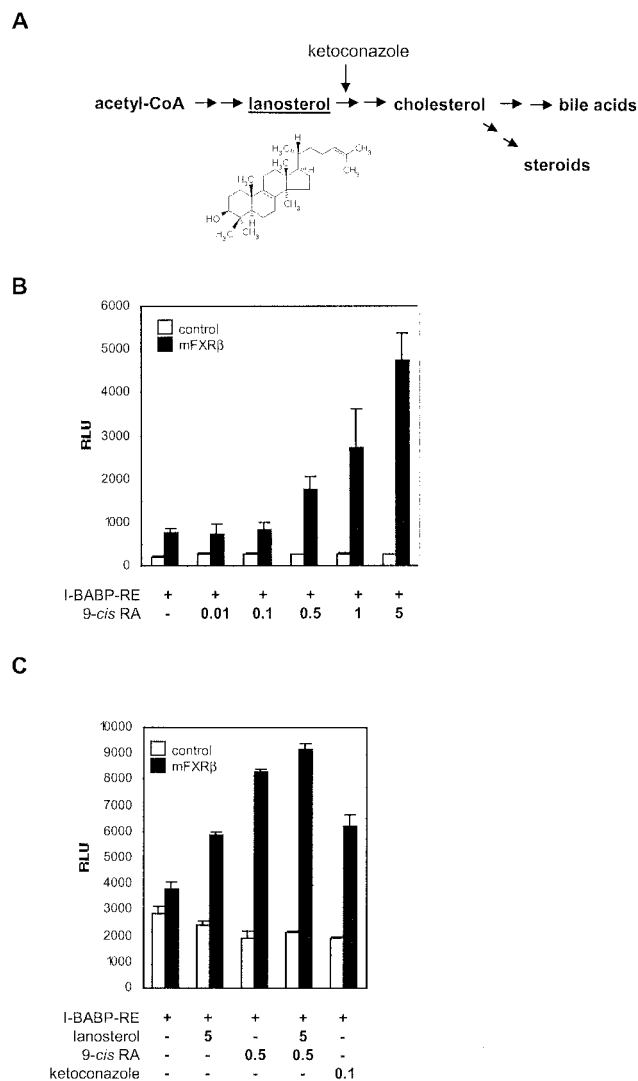


FIG. 6. Lanosterol activates mFXR β . (A) Lanosterol is an intermediate of the cholesterol metabolism pathway. Cholesterol is synthesized from acetyl coenzyme A (acetyl-CoA) via lanosterol and catabolized into bile acids and steroids. Ketoconazole inhibits CYP51, the enzyme catalyzing the demethylation of lanosterol. (B) mFXR β is activated by 9-cis-RA. HEK 293 cells were cotransfected with an mFXR β full-length expression vector (black bars) or an empty vector (white bars) and the I-BABP RE *luc* reporter. Cells were treated with the indicated micromolar concentrations of 9-cis-RA. Mean luciferase activities and SDs determined from triplicate experiments are shown. (C) mFXR β is activated by lanosterol. HEK 293 cells were cotransfected with an mFXR β full-length expression vector (black bars) or an empty vector (white bars) and the I-BABP RE *luc* reporter and treated with the indicated micromolar concentrations of lanosterol, 9-cis-RA, and ketoconazole. Mean luciferase activities and SDs determined from triplicate experiments are shown. RLU, relative light units.

caused by high CYP7A1 activity, which is balanced by a high rate of endogenous cholesterol synthesis. In humans, lower enzyme levels, together with a lack of inducibility of CYP7A1 by dietary input of cholesterol, seem to lead to a greater susceptibility to high levels of cholesterol intake. As FXR β is absent in humans but serves as a nuclear receptor in rodents, it may add an additional layer of regulation to cholesterol

biosynthesis in rodents that is absent in primates and in this way contribute to the observed species-specific differences.

Finally, our understanding of cholesterol metabolic pathways in humans, which in part is deduced from pharmacological studies and compound testing in nonprimate mammals, may have to be revisited because of our finding of a novel and additional nuclear receptor, FXR β , that is a pseudogene in primates.

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