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FOR, a Novel Orphan Nuclear Receptor Related to Farnesoid X Receptor*

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We have identified and characterized a new amphibian orphan member of the nuclear receptor superfamily and termed it FOR1 (farnesoid X receptor (FXR)-like Orphan Receptor) because it shares the highest amino acid identity with the mammalian FXR. We also identified a variant of FOR1, called FOR2, which has 15 additional C-terminal amino acids. Both variants include an unusual insertion of 33 amino acids in the helix 7 region of the canonical ligand binding domain sequence, suggesting a unique structure for FOR. Northern blot analysis demonstrates that the *FOR* gene is highly expressed in adult and tadpole liver, kidney, and tail bud stage of the embryo. Detailed expression analysis using *in situ* hybridization indicates that FOR expression is first detectable at stage 30/31 in the presumptive liver region lasting until stage 41 with a peak level evident at stage 35/36. FOR forms heterodimeric complexes with retinoid X receptor (RXR) as demonstrated by biochemical and mammalian two-hybrid approaches. Gel mobility shift assays demonstrate that FORs form specific DNA-protein complexes on an FXR binding element consisting of an inverted repeat DNA element with 1 nucleotide spacing (IR1) from the *phospholipid transfer protein* gene promoter. Finally, although FORs do not exhibit constitutive transcriptional activity, frog gallbladder extract significantly augments the transcriptional activities of FORs.

The nuclear receptor superfamily comprises a large group of structurally related ligand-dependent transcription factors regulated by a variety of steroid and non-steroid hormones. It also includes a large number of related proteins that do not have known ligands, referred to as orphan nuclear receptors (reviewed in Refs. 1–3). The nuclear receptors modulate target gene transcription by direct binding to specific DNA sequences, called hormone response elements (HRE),[1] which are generally located in the promoter of the specific target genes. In general, both classic nuclear hormone receptors and orphan nuclear hormone receptors consist of four or five different modules or domains; A/B, C, D, E, and F (1). The non-conserved N-terminal region of nuclear receptors (A/B domain) is involved in transactivation in some cases but is of unknown function or is absent in others. The DNA-binding C-domain (DBD) shows the strongest sequence similarity among different nuclear receptors and is engaged in the binding of these receptors to cognate HREs. The C-domain consists of 65–68 amino acids, among which 8 cysteine residues are absolutely conserved and form two zinc-binding modules (1, 3, 4). The D-domain, called the hinge region, shows relatively low sequence similarity and contains sequences involved in HRE binding at its N terminus. The E-domain directly binds to ligands or hormones and is also involved in nuclear localization and receptor dimerization. The C terminus of the LBD contains a conserved motif that, together with other portions of the LBD, forms the binding site for transcriptional coactivators (1). The F-domain is an additional C-terminal extension found in only subset of receptors. The function of this non-conserved segment is unclear.

A large number of orphan nuclear receptor genes have been discovered by several different approaches. These include 1) screening cDNA libraries with conventional receptor cDNA probes at relaxed stringency (5) or with degenerate oligonucleotides based on the conserved regions (6), 2) performing PCR with degenerate oligonucleotide PCR primers from the DBD (7, 8), 3) screening cDNA libraries using nuclear receptor ligand binding domains (LBD) or receptor interaction domains of coactivators as bait in a yeast two-hybrid system (9, 10).

Although the biological functions of most orphan nuclear

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF456451, AF456452, and AF456453.

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[1] The abbreviations used are: HRE, hormone response element; FXR, farnesoid X receptor; FOR, FXR-like orphan receptor; DBD, DNA binding domain; LBD, ligand binding domain; PLTP, phospholipid transfer protein; RXR, retinoid X receptor; EcRE, ecdysone response element; hsp27, heat-shock protein 27; WISH, whole mount *in situ* hybridization; GST, glutathione *S*-transferase; MEM, modified Eagle's medium; cFXR, chicken FXR; rFXR, rat FXR; CMV, cytomegalovirus; TTNPB, 4-[(E)-2-(5,6,7,8,-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propeny)]benzoicacid; EcR, ecdysone receptor; SHP, small heterodimer partner; DAX-1, dosage-sensitive sex reversal, AHC critical region on the X chromosome, gene 1.

receptors remain to be elucidated, evidence indicates that orphan nuclear receptors can play key roles in cell growth, differentiation, and cell death. For example, NGF-induced clone B is involved in apoptosis of immune T cells (11) and SHP functions as a negative regulator of receptor-dependent signaling pathways (12). The regulation of steroidogenesis in gonad and adrenal gland (reviewed in Ref. 13) and homozygous loss of the *HNF-4* gene in mice causes early embryonic lethality, whereas loss of a single copy of this gene in human causes MODY (maturity onset diabetes of the young) (14).

The former orphan nuclear receptor originally called RIP14 was isolated from mouse liver using the yeast two-hybrid approach using the RXR ligand binding domain as a bait (15). Its rat homologue, FXR, was initially found to be activated by farnesol and its metabolites (16), and both proteins were later found to be activated by TTNPB and synthetic retinoids (17). More recently, bile acids, particularly chenodeoxycholic acid, have been shown to be endogenous ligands for this receptor, which is now referred to as FXR (18–20). This receptor shows 81% amino acid identity in the DBD to *Drosophila* nuclear receptor EcR, and both FXR and EcR bind to an ecdysone response element (EcRE) from the *Drosophila hsp27* gene promoter (15) as a dimer with either RXR or ultraspiracle (USP), the *Drosophila* homologue of RXR. More recently, natural and potential binding sites for FXR were discovered in the promoter of several genes, including intestinal bile acid binding protein (21), cholesterol 7α-hydroxylase (22), phospholipid transfer protein (PLTP) (23), SHP (24, 25), and ileal bile acid-binding protein (18, 21).

In the current study, we describe the isolation and characterization of a novel *Xenopus* orphan nuclear receptor, FOR, that associates with RXR and shares extensive sequence similarity to the orphan nuclear receptor FXR. Two isoforms of FOR were isolated from *Xenopus* liver cDNA library, termed FOR1 and FOR2. FOR1 and FOR2 share more than 90% amino acid sequence identity. FOR1 and FOR2 differ by a frameshift change at amino acid number 501 of FOR2 resulting in 15 extra amino acids in FOR2. Interestingly, when compared with other nuclear receptors, the FORs bear an unusual insertion in the helix 7 motif of the canonical LBD structure due to an addition of 33 extra amino acids. FOR mRNA is highly expressed in adult liver and kidney, and FOR expression is also detected in the liver and kidney of metamorphosing tadpoles. Electrophoretic mobility shift assays demonstrate that both FOR1 and FOR2 specifically bind an IR1 element from the *PLTP* gene promoter, previously described as an FXR target. Finally, both FOR1 and FOR2 show significant transcriptional activity upon treatment with frog gallbladder extract. These results suggest that FORs function as ligand-dependent transcription factors during frog development and in adult organ function.

MATERIALS AND METHODS

Isolation of FOR cDNAs—Degenerate primers derived from the most conserved regions of the nuclear receptor DBD were used to amplify PCR products from a *Xenopus laevis* liver cDNA library (Stratagene). The primers and PCR conditions were used precisely as previously described (8). The expected 130-bp PCR products were isolated by electrophoresis on a 1.5% agarose gel (high resolution, Sigma Chemical Co.) and cloned into the pGEM-T Easy system (Promega), and the clones were sequenced by dideoxy nucleotide sequencing (Sequenase, U.S. Biochemicals). A 130-bp DNA fragment showing high nucleotide sequence homology to FXR was labeled by random priming and used to screen a *X. laevis* liver cDNA library according to the manufacturer's protocols. Five positive clones were excised and subcloned into pBS SK(+) using *in vivo* excision by the Exassist system supplied with the library and sequenced. Two clones revealed an entire coding region corresponding to FOR1, and three clones represented FOR2.

Plasmids—FOR1 and FOR2 cDNAs from pBS SK(+) were subcloned into mammalian expression vector pCDNA3 (Invitrogen) at the *Not*I and *Apa*I sites. For mammalian two-hybrid assays, the LBD region of FOR corresponding to 319 amino acids for FOR1 and 346 amino acids for FOR2 was subcloned in-frame into pCMX-GAL4 in the *Xba*I and *Bgl*II sites downstream of the GAL4 DBD. VP16AD fusion constructs for FOR1 and -2 were generated by inserting fragments of FOR1 and -2 into pCMX-VP16. All the constructs were confirmed by sequencing.

In Vitro Translation—FOR1, FOR2, and RIP14/FXR cDNA in pBluescript (Stratagene) were transcribed and translated *in vitro* using a coupled rabbit reticulocyte system (TNT, Promega) in the presence of [^{35}S]methionine (Amersham Biosciences, Inc.) according to the manufacturer's instructions. The translated proteins were analyzed on 10% SDS-polyacrylamide gels and visualized by autoradiography.

Experimental Animals and Manipulation—Eggs were obtained from female *X. laevis* primed with 800 units of human chorionic gonadotropin (Sigma). After *in vitro* fertilization the embryos were dejellied in 2% cystein, pH 8.0, and cultured in 0.4× Marc's Modified Ringer (28) until stage 4 then transferred to 0.1× Marc's Modified Ringer. Embryos were staged according to Nieuwkoop and Faber (29).

Northern Blot Analysis—Approximately 30 μg of total RNA from *X. laevis* adult tissues was isolated, and Northern blot analysis was carried out as described previously (30). For embryonic stage blot, 10 μg of total RNA was isolated from whole specimens from stage 0 (ovary) and embryonic stages 33, 41, 45, 50, 54, 58, 62, and 66. Embryonic stage Northern blot analyses were carried out as described previously (31).

Whole Mount in Situ Hybridization—Whole mount *in situ* hybridization (WISH) was performed according to the standard protocols (32, 33) with minor modifications. Briefly, fixed whole embryos were hybridized with digoxigenin labeled sense or FOR1 riboprobes, followed by extensive washing and chromogenic detection with alkaline phosphatase conjugated to anti-digoxigenin antibody and BM Purple as an artificial substrate.

GST-Pull-down Assay—A GST pull-down assay was performed as described previously (10). Briefly, the GST fusion proteins and GST control protein were expressed in *Escherichia coli* BL21(DE3)pLys bacterial culture and purified using glutathione-Sepharose 4B beads (Amersham Biosciences, Inc.). GST fusion proteins bound to glutathione-Sepharose-4B beads were incubated for 2 h at 4 °C with various ^{35}S-labeled receptors expressed by *in vitro* translation. Bound proteins were eluted from beads with 15 mM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS-polyacrylamide gel electrophoresis and visualized by a phosphorimaging analyzer (BAS-1500, Fuji)

Gallbladder Extraction and Solvent Partition—The gallbladders (1 g) dissected from bullfrogs were freeze-dried and extracted twice with 2 ml of methanol and dichloromethane (1:1, v/v). The combined extract was concentrated under reduced pressure and partitioned between hexane and methanol. The methanol-soluble fraction was further partitioned three times between ethyl acetate and water. The final solvent partition was accomplished between 1-butanol and water. Each fraction was dried under vacuum and used in cotransfection assays. The butanol-soluble fractions were further fractionated by silica open column chromatography (230–400 mesh, 40–63 μm, EM science) and eluted with methylene chloride and methanol (3:1, v/v). These fractions were analyzed for luciferase activities as described below.

Cell Culture and Transient Transfection—For mammalian two-hybird assays, CV-1 cells were seeded in 24-well plates in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and transfected with the indicated plasmids using Superfect (Qiagen), according to the manufacturer's instructions. Cells were harvested at 48 h, and luciferase activities were assayed as described previously (15). Luciferase activities were normalized to the β-galactosidase activity expressed from the control plasmid CMX-β-GAL. For ligand testing, 293 human embryonic kidney cells were grown in minimal Eagle's medium (MEM) supplemented with 10% resin-charcoal-stripped fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate in humidified air containing 5% CO_2 at 37 °C. Transient transfections were carried out using SuperFect (Qiagen) according to the manufacturer's instructions. Cytomegalovirus-driven receptor expression vectors (0.19 μg/10^5 cells), and luciferase reporter construct containing the herpesvirus thymidine kinase promoter linked the corresponding response elements (1.04 μg/10^5 cells) and CMX-β-GAL (0.56 μg/10^5 cells) as an internal control were added as indicated. After 16 h, cells were treated with MEM-supplemented 5% resin-charcoal-stripped fetal bovine serum and antibiotics containing amphibian gallbladder extract dissolved in dimethyl sulfoxide for ~24 h. The cells were then harvested and assayed for luciferase and β-galactosidase activity.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were performed essentially as described previously (23). Briefly, 10,000 cpm of end-labeled IR1 or EcRE oligonucleotides (15, 23) was

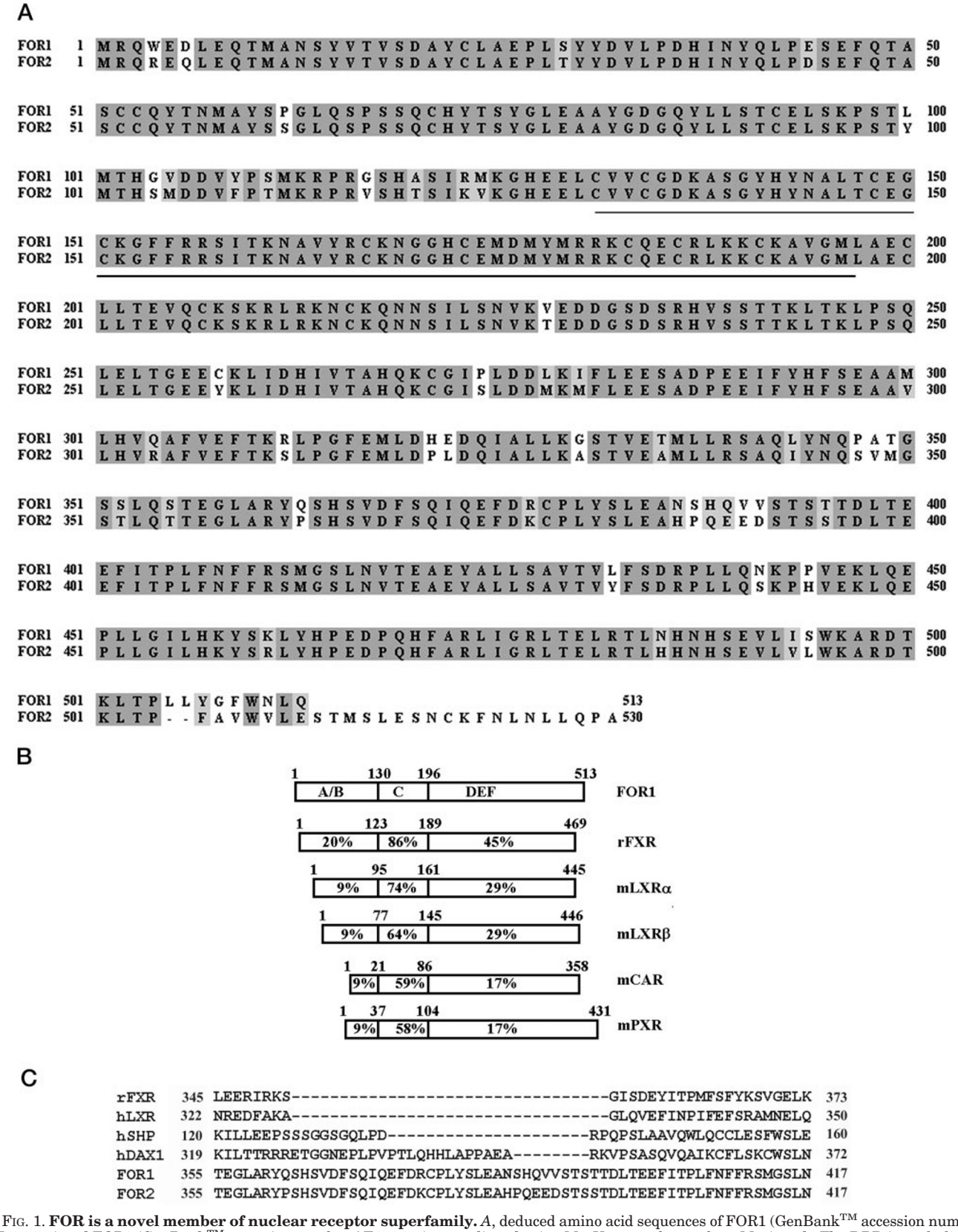

FIG. 1. **FOR is a novel member of nuclear receptor superfamily.** *A*, deduced amino acid sequences of FOR1 (GenBank™ accession number AF456451) and FOR2 (GenBank™ accession number AF456452) were aligned using MacVector software from Macintosh. The DBD is *underlined*, and the *numbers* represent corresponding amino acids. *B*, comparison of amino acid identities between FOR and related members of the nuclear receptor superfamily. The amino acid identities are indicated as percentages. The *numbers* represent position of amino acids corresponding to the A/B, C, or DEF domains. *C*, sequence alignment of the helix 7 motif in the LBD of FOR with related nuclear receptors. The unusual addition of amino acids in the helix 7 motif is characterized in SHP, DAX-1, and FOR. *Numbers* represent respective amino acid positions. *D*, genomic Southern blot analysis of the *FOR* gene. 20 μg of *Xenopus* genomic DNA was digested with indicated restriction enzymes and hybridized with ^{32}P-labeled FOR1 cDNA. *E*, deduced amino acid sequences of the A/B domain, DBD, or part of the LBDs of FOR, rFXR, and cFXR (GenBank™ accession number AF456453) were aligned as indicated. The hinge region is marked by a *gray bar*. Helices 1 and 2 are *underlined*. *Dark shading* represents identical amino acids, and *light shading* represents similar amino acids.

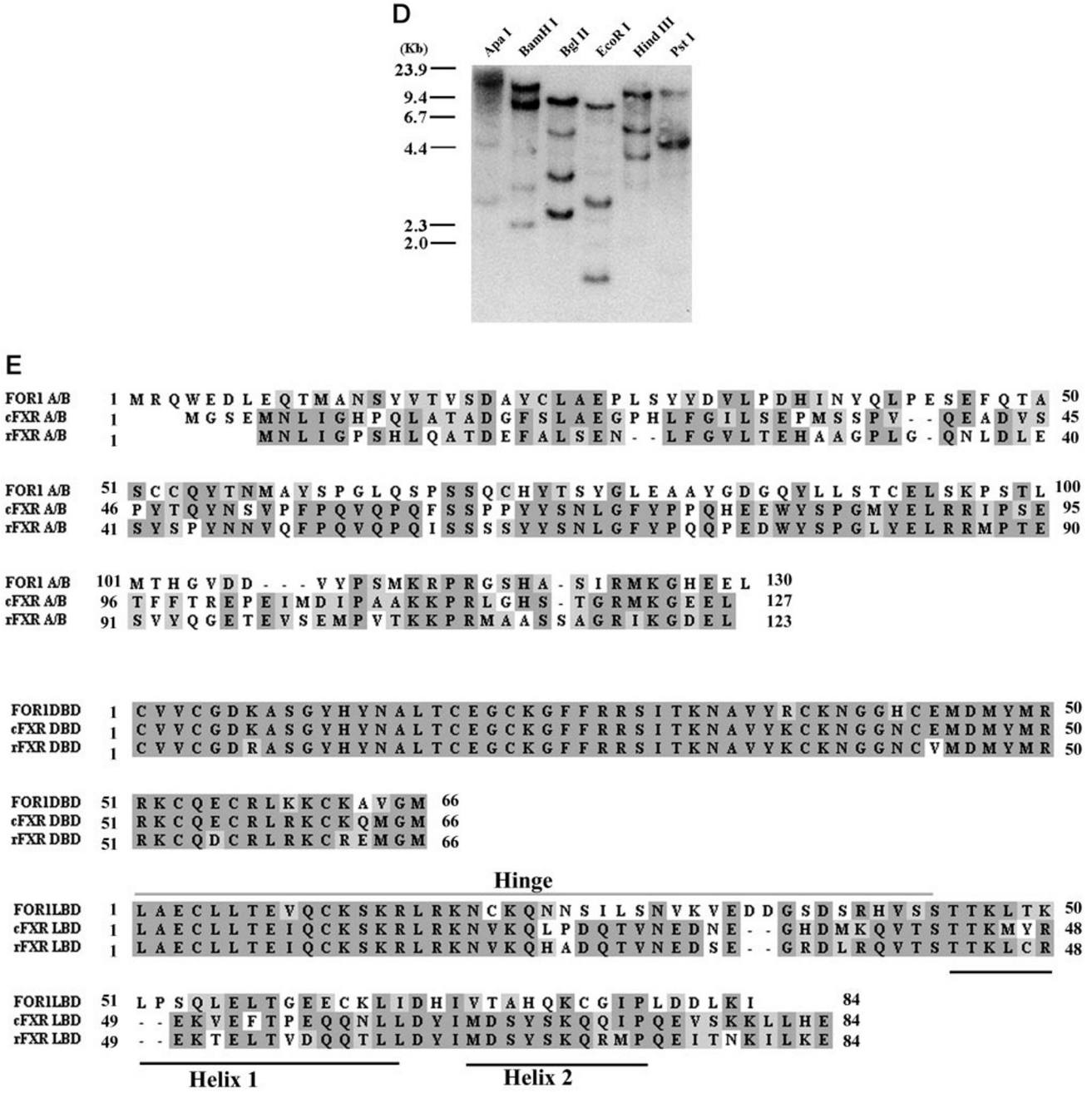

FIG. 1—*continued*

incubated with *in vitro* transcribed and translated FOR1, FOR2, and RXR in indicated combinations. The reaction mixtures were subjected to 5% non-denaturing gel electrophoresis followed by autoradiography.

RESULTS

Identification of FOR cDNA from X. laevis—To identify new members of the nuclear hormone receptor superfamily, degenerate oligonucleotide primers based on the most conserved region of the DBD (8) were used to amplify nuclear receptor-related cDNA fragments from a cDNA library of *X. laevis* liver. Amplified PCR fragments were subcloned and sequenced, revealing several known nuclear hormone receptors. Among these clones, one clone showed 86% amino acid identity with the DBD of rat FXR (rFXR). This fragment was further used as a probe to re-screen a *Xenopus* liver cDNA library to find the full-length receptor. As shown in Fig. 1*A*, two different isoforms of FOR, termed FOR1 and FOR2, were isolated. Based on the nucleotide sequence, these two isoforms are encoded by distinct, but highly related genes. The DBD of FOR1 and FOR2 shared identical amino acid sequences (with 98% nucleotide sequence identity), whereas the LBD showed 91% amino acid sequence identity. A single nucleotide insertion was found at the position of amino acid number 501 in the C-terminal region of FOR2, relative to FOR1, which caused an addition of 15 amino acids and eliminated the classical AF-2 consensus motif found in FOR1 (Fig. 1*A*). Both FOR1 and FOR2 were found to share 89% amino acid identity in the DBD with FXR, indicating a relatively close relationship. However, they share only 45% identity in the LBD (Fig. 1*B*). This is modestly higher than observed in pairwise comparisons with other members of the nuclear receptor subfamily 1, group H, which also includes the oxysterol receptors LXRα and β, the vitamin D receptor, and the xenobiotic receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR), and steroid and xenobiotic receptor (SXR) (Fig. 1*B*).

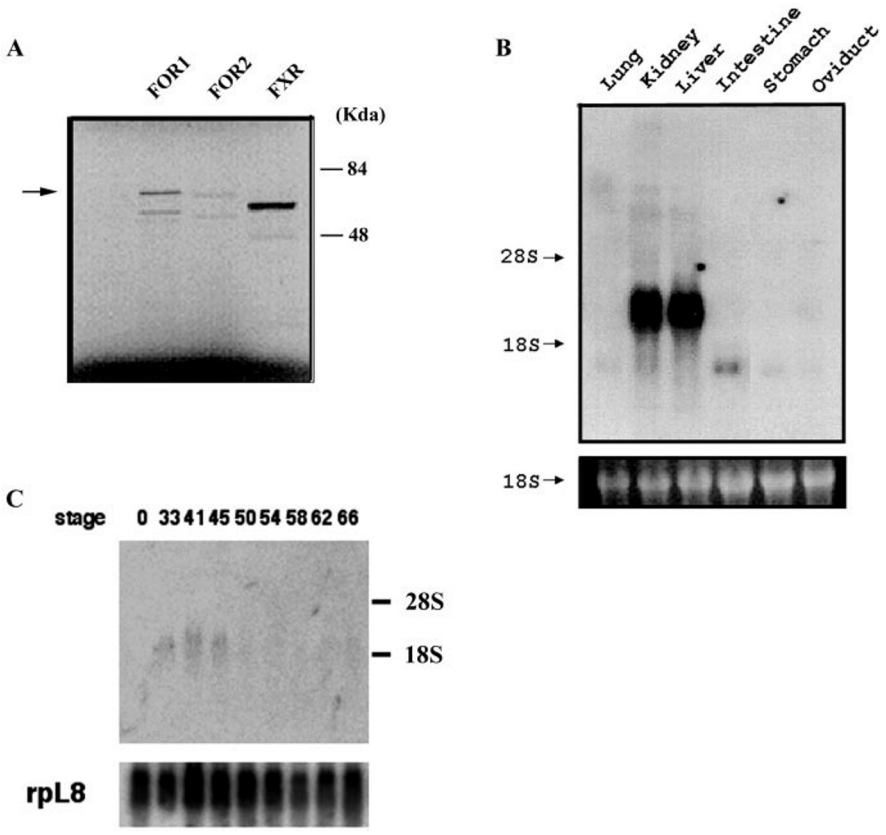

FIG. 2. **Expression of FOR.** *A*, SDS-PAGE analysis of *in vitro* transcribed and translated FOR1 and FOR2. FOR1 and FOR2 were transcribed and translated in the presence of [^{35}S]methionine *in vitro*, and resolved by 12% PAGE. Murine FXR was used as a positive control. *Numbers* on the *right* indicate molecular mass in kilodaltons. The positions of the protein bands are indicated by an *arrow*. *B*, expression of FOR in adult *Xenopus* tissues. A Northern blot containing ~30 μg of total RNA from the indicated *Xenopus* tissues was hybridized with FOR1 cDNA and following washing was autoradiographed. Equal loading of total RNA in each lane was demonstrated with 18 S ribosomal RNA. *C*, expression of FOR mRNA in developmental stages. 10 μg of total RNA was isolated from the indicated developmental stages, and Northern blot analysis was performed using the FOR1 cDNA as a probe. As an RNA loading control, the blot was reprobed with the cDNA for the ribosomal protein L8 (*rpL8*).

Although FOR1 and FOR2 include matches to conserved regions of the LBD, both showed an unusual 33-amino acid insertion in the putative helix 7 (Fig. 1*C*). A range of shorter amino acid additions are also present in this region of the mammalian orphan nuclear receptors SHP (9), DAX-1 (34), and zebrafish RXR delta and epsilon (35) (Fig. 1*C* and data not shown). However, the functional significance of this unusual structural feature remains unclear.

The high degree of homology between FOR1 and FOR2 and the fact that *X. laevis* is a pseudotetraploid animal prompted us to determine the copy number of the *FOR* gene. To this end, genomic Southern blot analysis was carried out. Several positive bands were obtained with the various restriction enzymes (Fig. 1*D*). Assuming that the *FOR1* and *FOR2* genes, like other nuclear receptor genes, contain a number of introns, these results are consistent with the possibility that there are only two *FOR* genes in the *Xenopus* genome. However, it is possible that there are a limited number of additional copies.

In search of homologues of FXR in other vertebrate species we also cloned a partial complementary DNA from chicken (cFXR) (Fig. 1*E*). Surprisingly, this clone demonstrated a very high amino acid identity with rat FXR (rFXR). FOR and rFXR shared an amino acid identity of 20% in the hyper variable A/B domain whereas cFXR and rFXR shared 53% amino acid identity in this region. The DBD of these two FXRs shared 92% identity, and the DE region comprising the hinge region, the complete helix 1, and helix 2 sequence showed amino acid identity of 76%, whereas the DBD and DE domain of FOR1 and rFXR shared amino acid identity of 86 and 46%, respectively. Fig. 1*E* demonstrates domain by domain alignment of FOR1 and rFXR with the partial cFXR cDNA. Taken together these results demonstrate that FORs are novel members of the FXR subfamily.

Expression of FOR—To confirm the predicted size of the FOR proteins, an *in vitro* translation assay was performed. As shown in Fig. 2*A*, SDS-PAGE analysis of *in vitro* translated FOR1 and FOR2 showed products close to 58 and 60 kDa, respectively. These results are consistent with the estimated protein sizes based on the open reading frames of the FOR1 and FOR2 cDNAs.

To characterize the expression of the *FOR* genes, Northern blot analysis was performed. An ~1.8-kb FOR mRNA transcript is dominantly expressed in liver and kidney in adult *Xenopus* (Fig. 2*B*). FOR mRNA was not detected at significant levels in early embryonic stages (morula, blastula, gastrula, and neurula, data not shown) but is transiently present in tadpoles between stages 33 and 45 (Fig. 2*C*). To further understand the spatio-temporal expression profile of FOR, whole mount *in situ* hybridization (WISH) was performed in a series of developing *Xenopus* embryos. The examined stages are following; one cell (stage 1), mid-blastula (stage 8), early gastrula (stage 10), yolk plug (stage 12), neural plate (stage 14), neural fold (stage 16), neural tube (stage 20), early tail bud (stage 25), mid-tail bud (stage 30), hatching larvae (stage 35), swimming larvae (stage 41), and feeding larvae (stage 45).

In agreement with the Northern blot analysis, FOR expression began to be detected around stage 30 in the presumptive liver region (Figs. 3, *A* and *B*), and the peak level of FOR expression was found at stages 35 to 36 (Fig. 3, *C* and *D*). Especially, at this peak stage, not only the intensity of hybridization signal was higher but also the expression domain was broader than those in the previous stages. Expression declined after stages 35 to 36, and only a low level of hybridization signal was detected in the stage 41 embryos (Fig. 3, *E* and *F*). Thereafter, FOR expression was no longer detected.

The expression profile of FOR was consistent among the *Xenopus* embryos examined by WISH, and very little variation was observed among sibling embryos in their FOR signal expression. Throughout the whole developmental stages, only a background level of hybridization signal was detected with sense probe (data not shown). Taken together, these results suggest that FOR expression primarily functions in liver and

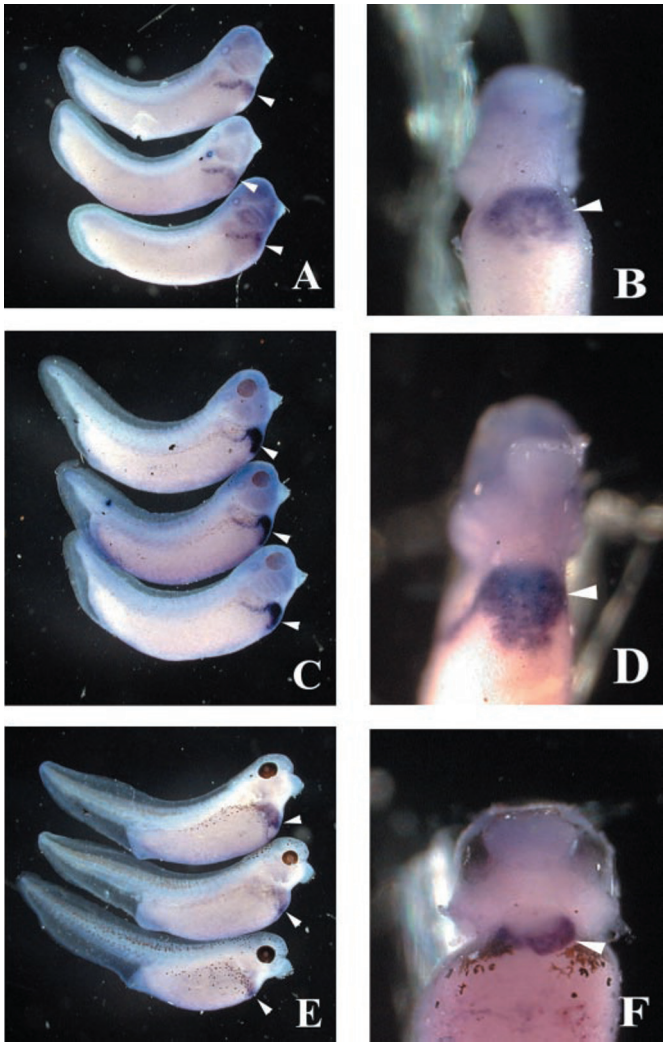

FIG. 3. **The expression profiles of FOR in the developing *Xenopus* embryos.** FOR expression was examined by whole mount *in situ* hybridization using digoxigenin-labeled antisense riboprobe. The expression of FOR is limited to the presumptive liver region indicated by the *white arrowhead*. *Panels A*, *C*, and *E* are lateral views of developing embryos and *B*, *D*, and *F* are ventral views, respectively. *A* and *B*, embryos at stage 30 (mid-tail bud stage). The *FOR* expression begins to be detected from this stage in the presumptive liver region (indicated by the *white arrowhead*). *C* and *D*, embryo at stage 35. The *FOR* expression in the embryo reaches a peak level at this stage. Note more intense staining in the broader expression domain compared with the embryos at stage 30. *E* and *F*, embryos at stage 41. The FOR expression declines remarkably at this stage.

kidney in adult *Xenopus* and may also play an important role in liver development during late embryonic stages.

Interaction of FOR with RXR—To determine whether FORs could form heterodimeric complexes with the universal heterodimeric partner RXR, GST pull-down assays were performed. As shown in Fig. 4 (*A* and *B*), FOR1 and FOR2 were able to form heterodimeric complexes with RXR *in vitro*. To further confirm the interaction between RXR and FOR1 or FOR2 *in vivo*, a mammalian two-hybrid assay was performed. The LBD of both FOR1 and FOR2 were fused to the GAL4 DBD in a CMV promoter-driven mammalian expression vector, and the RXR LBD was similarly fused to the VP16 activation domain. Transient transfection experiments were performed in CV-1 cells using a reporter in which luciferase expression is controlled by a promoter containing GAL4 DNA binding sites. As shown in Fig. 4*C*, the combination of the FOR and RXR hybrids resulted in strong activation of this reporter, demonstrating that FOR formed a heterodimeric complex with RXR. However, neither homodimerization nor heterodimerization between the two FOR isoforms were observed. These results demonstrate that FOR, as expected from its relationship with FXR, forms a heterodimeric complex with RXR.

DNA Binding Properties of FOR—Because the DBDs of FOR and FXR showed 86% sequence identity and an identical P box motif, which specifies the DNA hexamer recognized by receptor monomers (28), we examined whether FOR binds to previously reported FXR binding sites. Electrophoretic mobility shift assays were performed using an oligonucleotide containing the IR1 site from the *PLTP* gene promoter (23). As expected, both FOR1 and FOR2 formed specific DNA protein complexes with this element when combined with RXR, and this complex could be successfully competed with a 50-fold molar excess of cold IR1 but not with a 50-fold molar excess of unrelated oligonucleotide (Fig. 5*A*). Neither FOR1 nor FOR2 formed a monomeric complex with the IR1 site in the absence of RXR. Surprisingly, FORs did not bind other FXR binding sites, including the ecdysone response element (EcRE) from the *Drosophila* heat-shock protein 27 promoter (hsp27) (Fig. 5*B*) and various other direct and inverted repeat DNA elements that have been reported as potential FXR target sites (data not shown).

FORs Are Activated by Frog Gallbladder Extract—Solvent-partitioned organic extracts of tissues known to express FOR, gallbladder and kidney, were examined to identify natural FOR ligands. HEK 293 cells were cotransfected with fusion proteins consisting of the GAL4 DNA binding domain and the ligand binding domains of FOR1 or hRXRα, along with an appropriate luciferase reporter plasmid and a vector expressing the LBD of hRXRα as indicated. Following transfection, cells were treated with gallbladder tissue extracts. Interestingly, the 1-butanol extract of bullfrog gallbladder induced GAL-FOR1-mediated luciferase activity by 1.4-fold at 10 μg/ml, whereas GAL4-hRXRα and L-hRXRα (data not shown) demonstrated no response to the extract (Fig. 6*A*) indicating that the effect of this tissue extract was FOR-specific. Coexpression of the RXR ligand binding domain with GAL4-FOR1 resulted in a somewhat higher activation (1.8- fold) at the same concentration than observed with GAL4-FOR1 alone. These effects were dose-dependent (Fig. 6*B*), and similar results were obtained using GAL4-FOR2 (data not shown). These results indicate that the 1-butanol-soluble extract of bullfrog gallbladder contains activators of the two orphan nuclear receptors, FOR1 and FOR2. To further investigate this response, full-length FORs and a reporter construct driven by three copies of IR1 elements from PLTP promoter were cotransfected in HEK 293 cells followed by treatment with indicated doses of 1-butanol extract. Results similar to those with the chimeric FOR1 were obtained, and the 1-butanol extract was more effective in activating FOR2 than FOR1 (Fig. 6*C*). To further confirm the gallbladder extract-mediated FOR activity, the active 1-butanol-soluble fraction was further fractionated by a silica open column and eluted sequentially with methyl chloride and methanol (3:1, v/v) and 100% methanol containing 0.01% trifluoroacetic acid. Among 56 fractions collected, the sixth fraction (R_f = 0.75–0.85) significantly activated GAL4-FOR 2 (Fig. 6*D*, 3.8-fold, $p < 0.001$), and similar responses were obtained with GAL4-FOR1 (data not shown). These results showed that the amphibian gallbladder contains one or more distinct ligands for FORs. However, known FXR agonists, including chenodeoxycholic acid and synthetic retinoid TTNPB failed to activate the FORs indicating that the amphibian bile acids, which act as the agonist for FORs, may structurally differ from the mammalian bile acids.

Taken together, FORs are ligand-responsive and their transactivation functions were mediated through FOR1/RXR or

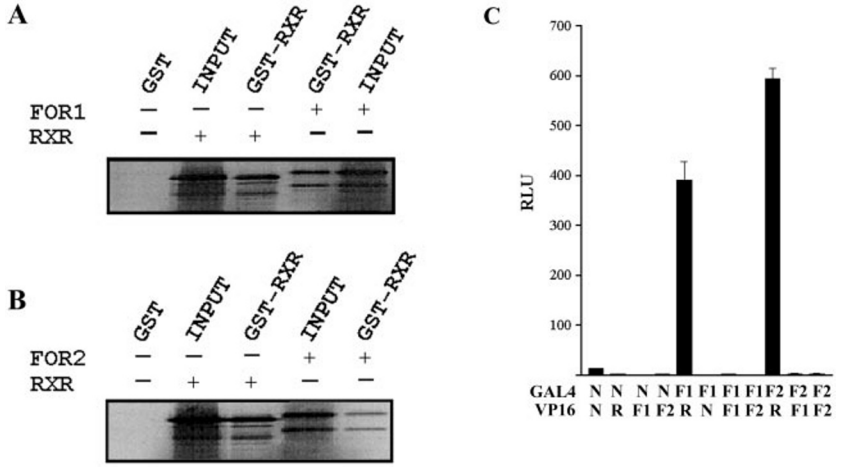

FIG. 4. **FOR interacts with RXR both *in vitro* and *in vivo*.** *A* and *B*, GST-pull-down assay of FOR1 and FOR2. Equal amounts of *in vitro* translated, [^{35}S]-methionine labeled FOR1 and FOR2 were incubated with either GST or GST RXR fusion protein. *In vitro* translated RXR was used as a positive control. The complex of GST-FOR1 and FOR2 was extensively washed, eluted with reduced glutathione, and resolved by SDS-PAGE. Specific bands corresponding to FOR1 and FOR2 were visualized by autoradiography. *C*, mammalian two-hybrid assay of FOR1 and FOR2. CV-1 cells were transiently cotransfected with a luciferase reporter construct driven by four copies of GAL4 upstream activating sequence and CMV promoter-driven expression vectors encoding the yeast GAL4-DBD alone, GAL4-FOR1 and FOR2-LBD, herpesvirus VP16 transactivation domain alone, or the VP16 activation domain linked to the amino-terminal end of RXR, *FOR1* LBD, and *FOR2* LBD, as indicated. 48 h post transfection, cells were lysed and luciferase activity was measured and normalized against β-galactosidase activity. The result shown is the mean of three independent experiments. *F1*, FOR1; *F2*, FOR2; *R*, RXR; *N*; empty vector.

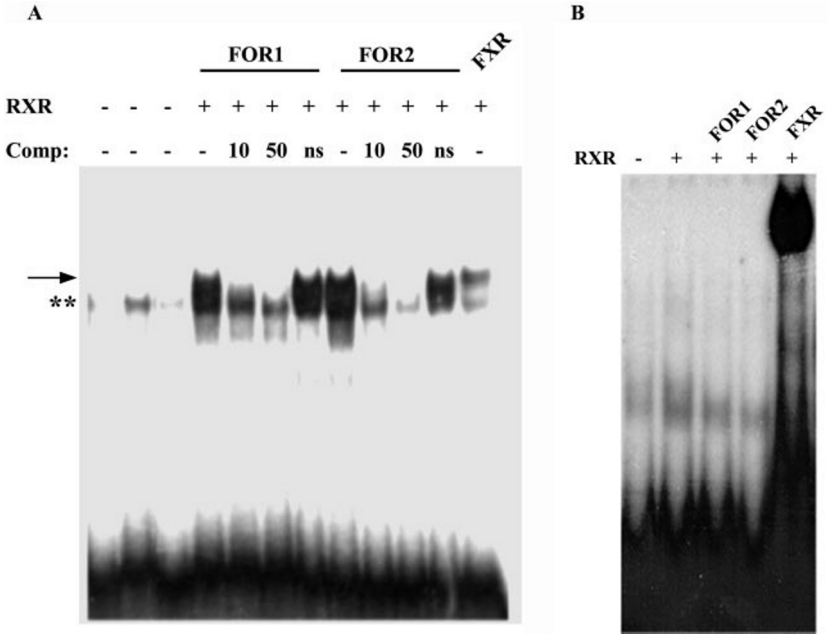

FIG. 5. **FORs specifically bind IR1 element from PLTP gene promoter.** End-labeled oligonucleotides corresponding to IR1 from *PLTP* (*A*) gene promoter or EcRE (*B*) of the hsp27 promoter were incubated with *in vitro* transcribed and translated FOR1, FOR2, and RXR in various combinations as indicated in the Fig. 10- or 50-fold molar excess of corresponding specific unlabeled oligonucleotides or unrelated oligonucleotides (*ns*) were included in the reactions as indicated. The DNA-protein complexes were resolved by 5% non-denaturing PAGE and analyzed by autoradiography.

FOR2/RXR heterodimers, and these results indicate that FORs are ligand-activated receptors and their specific ligands are present in the gallbladder of frogs. It is expected that these ligands would be bile acids, and currently the potential role of amphibian bile acids as ligands for FOR1 and FOR2 are under investigation.

DISCUSSION

Herein we describe the isolation and characterization of a novel orphan nuclear receptor that we have named FOR. The amino acid sequence of FOR is most closely related to the previously characterized orphan nuclear receptor FXR. Like FXR, FORs form heterodimeric complexes with RXR and are most abundantly expressed in adult liver and kidney (15, 16). Tissue-specific expression was also observed in metamorphosing tadpole liver. FOR is not expressed in early embryonic stages but shows a peak expression in early tadpole stages, raising the possibility that FOR plays a role in development of liver in amphibians.

Several lines of evidence demonstrate that FOR is not an orthologue of mammalian FXR. The simplest is that the LBD of FOR shows only 45% amino acid identity to that of FXR, whereas human and *Xenopus* RARα, for example, share 88% identity in the LBD. Moreover, comparison of FOR, rFXR, and a newly cloned cFXR amino acid sequences revealed that, although the cFXR and rFXR shared a very high amino acid identity, FOR exhibited significantly high homology with rFXR only in the DBD. To identify potentially closer relatives of FXR, several rounds of screening of appropriate *Xenopus* cDNA libraries with various low and high stringent conditions were performed using mouse FXR cDNA as a probe. However, no cDNA clones except FOR1 and FOR2 were isolated, indicating

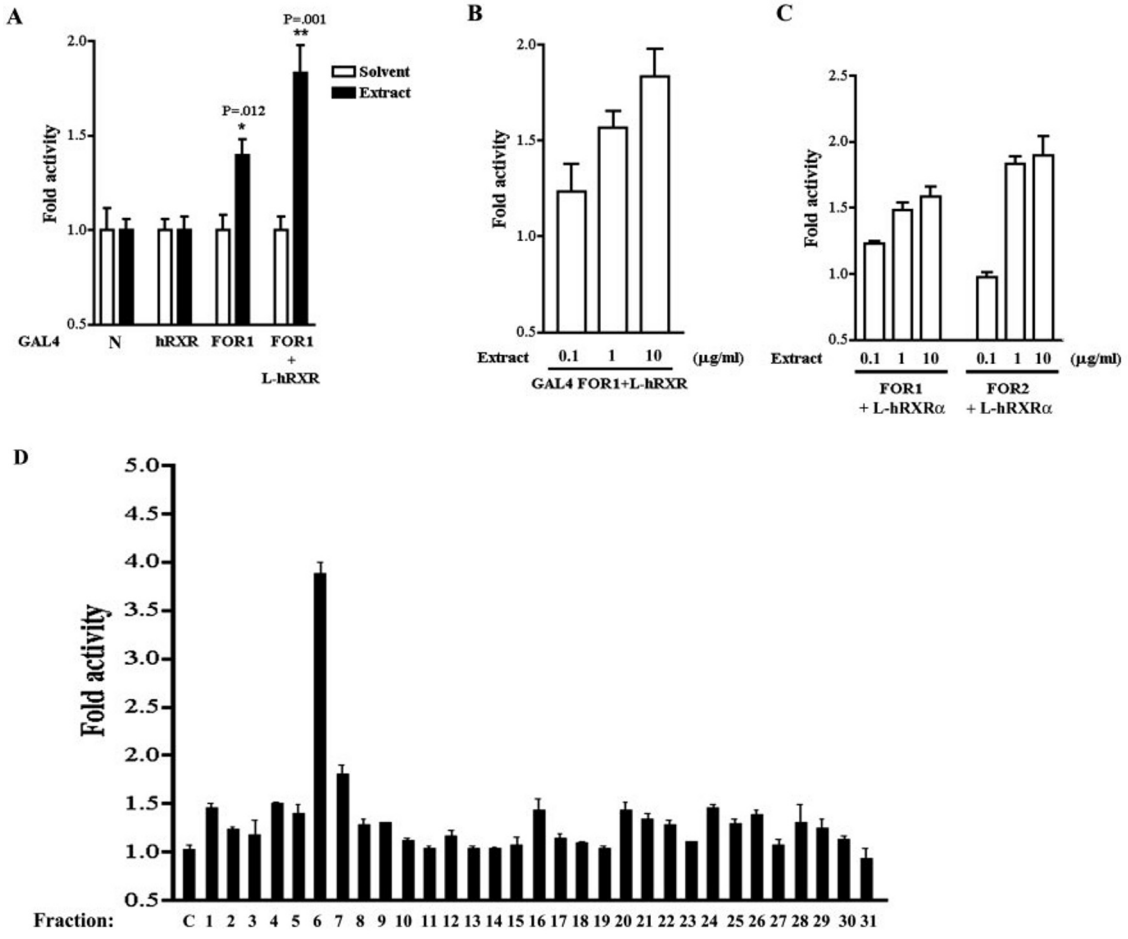

FIG. 6. **Transactivation of *FOR* genes induced by extract of bullfrog gallbladders.** *A*, GAL4 chimeric FOR1 was activated by extract of gallbladders on 293 cells. The 293 cells were transiently transfected with indicated receptors and reporter plasmids and treated with a 10 μg/ml 1-butanol-soluble extract of bullfrog gallbladders or vehicle alone in triplicate. Luciferase activity was normalized to the internal control (β-galactosidase) and plotted as -fold induction relative to untreated cells. Each experiment was repeated more than three times. *B* and *C*, dose-response profiles on the 293 cells transfected with indicated receptor and reporter that is regulated either by GAL4 (*B*) or by FOR response element (PLTP, *C*). *D*, following cotransfection of GAL4-FOR2 with GAS Luc, the cells were treated with indicated fractions (40 μg/ml) eluted from a silica open column with methyl chloride and methanol (3:1, v/v). The fractions dissolved in Me_2SO were tested in triplicate, and the experiments were performed at least three times. *Single* and *double asterisks* indicate the respective *p* values as determined by Student's one-tailed unpaired *t* test. *GAL4N*, empty vector containing GAL4 DBD only; *L-hRXR*α, human RXRα LBD; *C*, Me_2SO vehicle alone.

that FOR may be the sole representative of the FXR subfamily in *Xenopus*.

More direct evidence of the functional differences between the *Xenopus* and mammalian protein is provided by the finding that, although FOR binds the IR1 from *PLTP* gene promoter, it does not bind other preferential FXR recognition elements, including the EcRE from *Drosophila hsp27* gene promoter, despite the 86% amino acid identity between their DBDs. This result was somewhat surprising, because FXR and the ecdysone receptor (EcR), which share 81% identity, can both bind to this element. Furthermore, several potential FXR ligands, including TTNPB, chenodeoxycholic acid, and a large number of bile acid derivatives failed to cause any change in FOR activity, indicating that although FORs are structurally similar to the FXR their ligand selectivity is quite different.

The 33-amino acid insertion in the putative helix 7 of FORs may be responsible for the differences in DNA binding and transcriptional characteristics of FORs and FXR. A somewhat similar phenomenon has been observed in zebrafish, where zebrafish RXR delta and epsilon (35) contain an insertion in the similar region and these isoforms neither bind RXR recognition elements nor are activated by RXR ligands. However, the ability of the FOR LBD to interact with RXR demonstrates that this domain retains at least this function.

The ability of partially purified frog gallbladder extract to activate FORs indicates that FORs, like FXR, are ligand-activated receptors. Several reports have demonstrated the presence of bile acids and bile alcohols in amphibians that are markedly different from their mammalian counterparts (26, 27). Such amphibian bile acids or bile alcohols may represent potential FOR ligands. Alternatively, identification of the FOR ligand(s) may require purification and detailed characterization.

In summary, we have isolated a novel orphan nuclear receptor termed FOR, which is most closely related to the mammalian bile acid receptor FXR. FOR belongs to the nuclear hormone receptor superfamily 1 group H and, like several other members of this group, is expressed in liver. Both the limited amino acid sequence conservation of the LBD and the lack of functional similarities indicate that these relatives are not true orthologues. Thus, FOR may be involved in the regulation of metabolism of either endogenous or exogenous compounds in adult animals, and in liver development in tadpoles, but its physiological functions in amphibians remain to be identified.

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