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Molecular Cloning and Functional Characterization of a Novel Nuclear Receptor Similar to an Embryonic Benzoate Receptor BXR

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Benzoate X receptor (BXR) is a member of the nuclear hormone receptor superfamily and is activated by alkyl esters of amino benzoic acids and expressed during early development stages. We report here a second BXR, which was cloned by screening *Xenopus laevis* embryonic cDNA libraries. This new BXR, termed BXRβ, exhibits 97% identity in the DNA-binding domain and 79% identity in the ligand-binding domain in amino acid sequence to previously reported *Xenopus* BXR. The BXRβ strongly binds as a heterodimer with retinoid X receptor (RXR) to direct repeats of an AGGTCA motif spaced 4 or 5 base pairs apart and activates transcription by addition of methyprednisolone and dexamethasone as well as amino- and hydroxybenzoates.

Key Words: **orphan nuclear receptor; benzoate X receptor; BXR; endocrine disrupter; dexamethosone**

The benzoate X receptor (BXR) is a member of the nuclear hormone receptor superfamily of ligand-modulated transcription factors (1, 2). These receptors exist in the nucleus and can bind to cognate ligands (3, 4). The conformational changes induced by ligand binding confers on them the ability to act as transcriptional activators through interaction with other factors and/or phosphorylation (5, 6). By these processes, ligand signals mediate cellular functions through expression of the target genes.

BXR was identified as a receptor for alkyl esters of amino and hydroxy benzoic acids (2). The amino acid sequence of *Xenopus* BXR is closely related to the mouse pregnane X receptor, PXR (7), and the human steroid and xenobiotic receptor, SXR (8). All of these receptors preferentially bind as heterodimers with the retinoid X receptor (RXR) (2, 7, 8) to direct repeats of the sequence AGTTCA spaced four nucleotides apart (DR4). The mRNA of BXR is only expressed during early developmental stages, suggesting that BXR plays some role in embryogenesis.

On the other hand, it has been hypothesized that the increasing occurrence of endocrine-related abnormalities in humans and wildlife may be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones (9). While the estrogen receptor (ER) has been thought to be a key target of such chemicals, other receptors may be influenced. We thought that BXR might be one such candidate because (i) BXR expressed in the early embryo stage is susceptible to endocrine disrupters, (ii) benzoates, the ligands of BXR, are abundantly synthesized as industrial chemicals, and (iii) the related receptors, PXR/SXR, are activated by a variety of xenobiotics.

In the course of our investigation of the possible involvement of BXR in endocrine-related abnormalities, we found a novel gene which is closely related to BXR. We report here the complete primary structure of this subtype of BXR and some properties of this new receptor.

MATERIALS AND METHODS

Chemicals. Dexamethasone (>98%), 17β-estradiol (>97%), progesterone (98%) refampicin (>90%), butyl *p*-aminobenzoate (98%), and butyl *p*-hydroxybenzoate (98%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 6α-Methylprednisolone (98%) and pregnenolone (98%) were purchased from Aldrich (Milwaukee, WI). 5α-Dihydrotestosterone (100%) was purchased from Fluka (Switzerland). Pregnenolone 16α-carbonitrile (>98%) was purchased from Sigma Chemical (St. Louis, MO). Diethylstilbesterol (99%) and *p*-nonylphenol (technical grade) were purchased from Nacalai Tesque (Kyoto, Japan).

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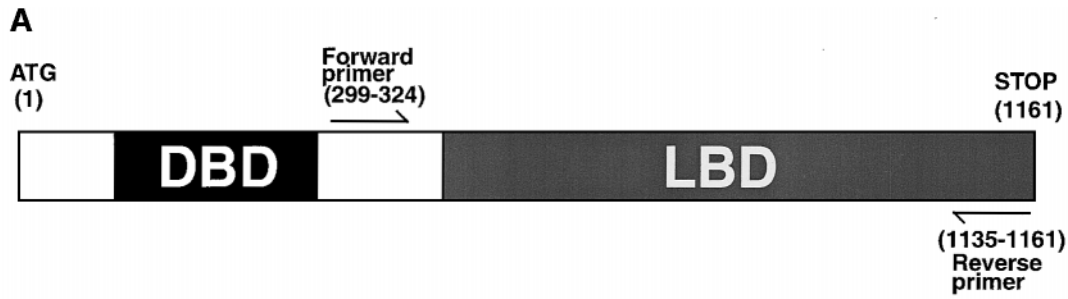
0006-291X/00 $35.00



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BXRalpha  299:TCGGCATGAGGAAAGAGTTGATCATGTCCGATGCAGCGGTGGAACAGAGACGAGCGCTAA 358
BXRbeta                                 TCAGATGAAGCGGTGGAACAGAGGAGAGCGCTAA
                                        ** **** ***************  *********
BXRalpha  359:TTAAGAGAAAACACAAATTAACGAAATTGCCCCCCACACCCCCAGGGGCCAGTCTGACTC 418
BXRbeta      :TTAAGAGAAAGCAGAACTTATCCCTGTCGCCCCCCACACCCCCAGGAGCCAGTCTGACTC
              ********** ** ** *** *    * ****************** *************
BXRalpha  419:CAGAGCAGCAGCACTTTCTCACTCAACTGGTTGGGGCCCACACCAAAACCTTTGACTTCA 478
BXRbeta      :CAGAGCAGCAGCACTTTATCACTGAACTGGTTGAGGCTCACACCAAAACCTTTGACTTCA
              ***************** ***** ********* *** **********************
BXRalpha  479:ACTTCACCTTCTCCAAGAACTTTCGGCCAATAAGAAGATCTTCAGACCCAACTCAGGAGC 538
BXRbeta      :ACTTCACCTTCTTCAAGAACTTTCGGCCCATAAGGAGATCTCCAGACCCAACTCAGGATC
              ************ *************** ***** ****** ***************** *
BXRalpha  539:CCCAAGCCACCTCTTCTGAAGCCTTTTTGATGCTACCTCATATATCCGACCTCGTTACCT 598
BXRbeta      :CCCAAGCTACCTCCTCTGAAGCCTTTTTGATGCTACCTCATATATCCGACCTTTTCACCT
              ******* ***** ************************************  * ****
BXRalpha  599:ACATGATCAAGGGCATCATCAGCTTTGCCAAAATGCTCCCCTACTTCAAGAGTCTGGACA 658
BXRbeta      :ACATGCTCAAGGGTGTCATCAGCTTTGCCAAAATGCTCCCCTACTTCAGGAGCCTGGCCA
              ***** *******  ********************************* **** **** **
BXRalpha  659:TTGAAGACCAAATTGCTCTCCTGAAAGGTTCTGTAGCGGAGGTTTCTGTGATCCGATTCA 718
BXRbeta      :TTGAAGACCAGATTGCTCTCCTGAAGGGTTCTGTCCTGGAGGTGTGTGTGATTCGATTCA
              ********** ************** ********   ****** * ****** *******
BXRalpha  719:ACACTGTGTTTAACTCTGACACCAATACGTGGGAGTGTGGCCCCTTCACCTATGACACTG 778
BXRbeta      :ACAGGATGTTCAACCCCAAGACCAATACGTGGGAGTGTGGCGCCTTCACCTATAATGCTG
              ***   **** *** *  * ********************** ********** *  ***
BXRalpha  779:AGGATATGTTCCTGGCCGGCTTCCGTCAGCTGTTCCTGGAGCCCCTGGTGAGGATTCATC 838
BXRbeta      :ACGATATGACCATGGCCGGCTTCAGTCAACAGTTCCTTGAGCCCCTGCTGAGAATACACT
              * ******  * ********** **** * ****** ********* **** ** **
BXRalpha  839:GCATGATGAGGAAACTGAATCTACAGAGTGAGGAATACGCCATGATGGCCGCTCTGTCCA 898
BXRbeta      :GCATGATGACAAAACTGAATCTAGAAAGTGAGGCATATGCCCTGATGGCTACCATGGCTC
              *********  *********** * ******* *** *** ******   *  ** *
BXRalpha  899:TTTTCGCTTCTGACCGACCGGGGGGTCTGCGACTGGGAGAAGATCCAGAAGCTGCAGGAAC 958
BXRbeta      :TTTTCTCTTCGGACCGACCGGGGGGTCTCTGACTGTGAGAAGATCCAGAACCTGCAGGAAC
              ***** **** ***************  ***** ************** **********
BXRalpha  959:ACATTGCCCTGACACTAAAAGATTTCATCGACAGCCAACGGCCCCCCTCCCCGCAGAACA 1018
BXRbeta      :ATATTGCCCTGATGCTGAAAGCATTCATCGAGAGCCATCGCCCACCCTCCCCACAGAACA
              * **********  ** ****  ******** ***** ** ** ******** *******
BXRalpha 1019:GGCTCCTGTACCCCAAGATCATGGAGTGTCTGACAGAGCTTCGGACAGTCAATGACATAC 1078
BXRbeta      :GGCTTCTATACCCCAAGATCATGGAGTGCCTGACAGAGCTTCGGACCATTAATGACATAC
              **** ** ******************** ****************  * **********
BXRalpha 1079:ACAGCAAGCAGCTCCTGGAGATCTGGGACATTCAGCCTGATGCCACCCCACTAATGCGAG 1138
BXRbeta      :ACAGCAAGCAGCTCATGGAGATATGGGACATACAGCCAGATGTTACCCCACTTATG
              ************** ******* ******** ***** ****  ******** ***
BXRalpha 1139:AAGTCTTTGGATCCCCTGAATGA                                    1161
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FIG. 1. Cloning of *Xenopus* BXRs. (A) Diagram of the previously described xBXR and the positions of primers used to amplify the LBD of BXR. (B) Nucleotide sequences of RT-PCR products. Identical residues are indicated by dark shading.

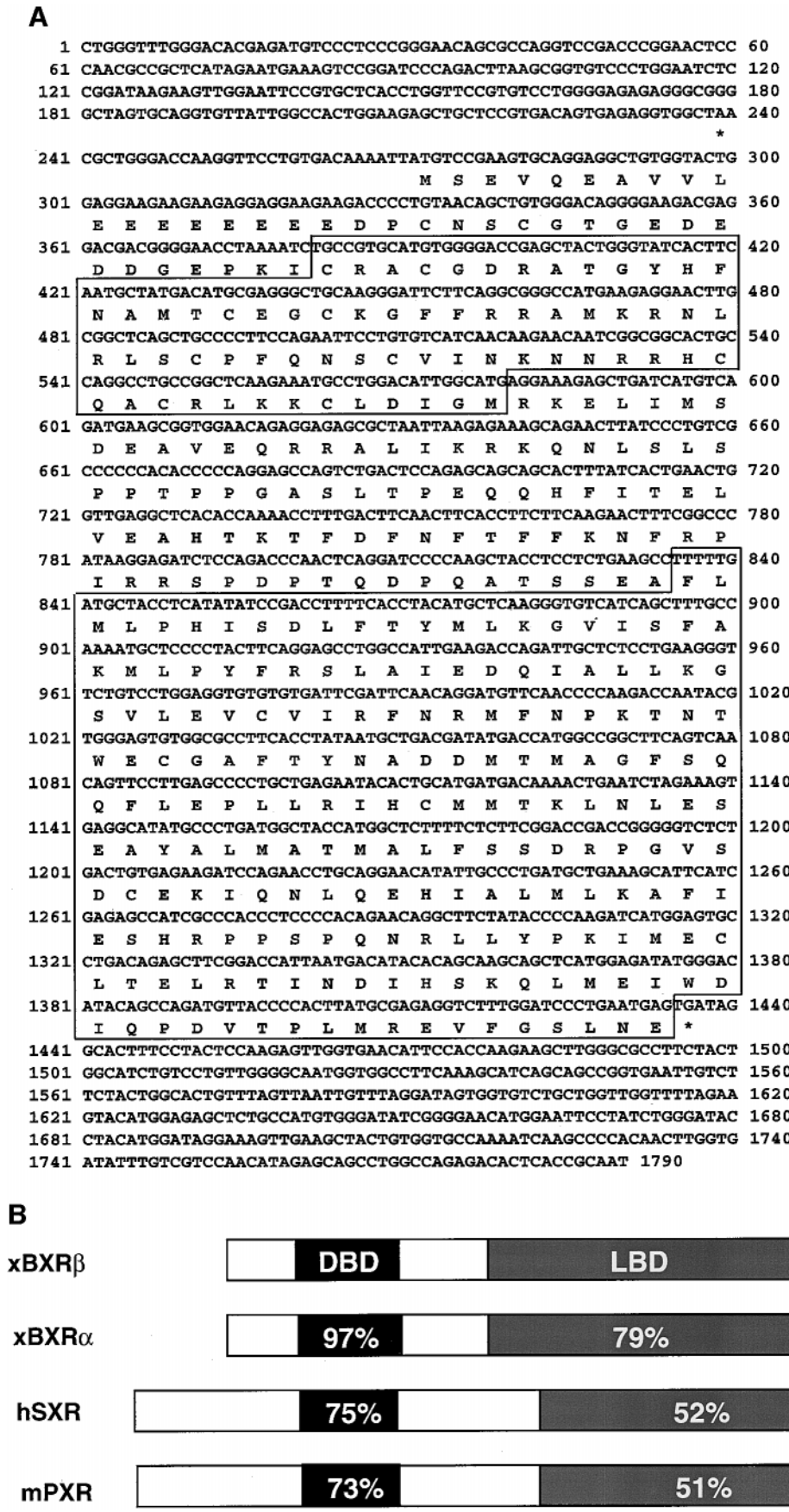

FIG. 2. BXRβ is a novel member of the nuclear receptor superfamily. (A) Sequence of the longest BXRβ cDNA clone. The DNA and ligand-binding domains are boxed. In-frame termination codons are indicated by asterisks. (B) A schematic representation of the BXR/PXR/SXR family is shown, along with the percentage of identical amino acids residues shared with BXRβ.

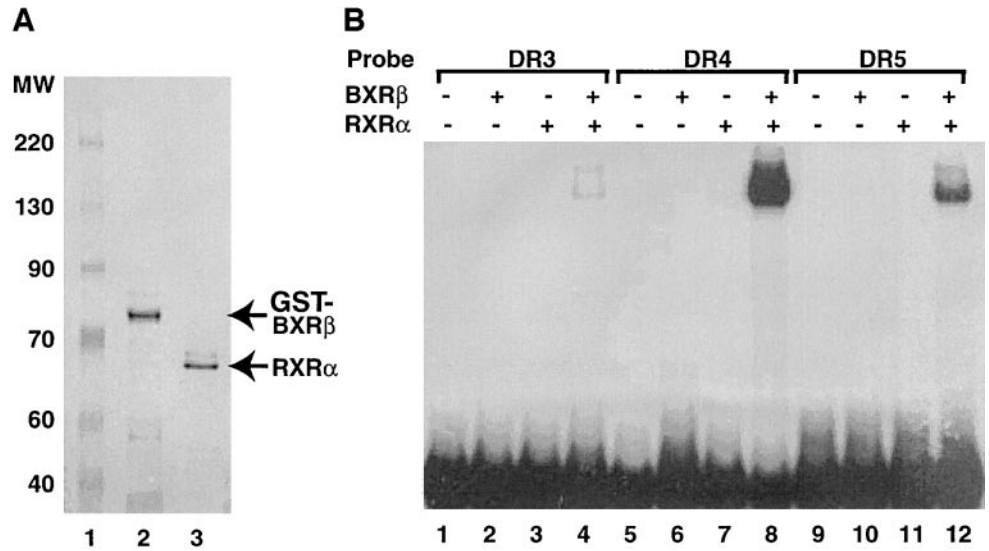

FIG. 3. DNA-binding properties of the BXRβ produced in *E. coli*. (A) SDS–polyacrylamide gel electrophoresis. Purified proteins of GST-BXRβ and RXRα were analyzed using 7.5% SDS–polyacrylamide gel electrophoresis and followed by Coomassie blue staining. The positions of each proteins are indicated by the arrow. Lane 1 shows molecular standard proteins (Gibco-BRL). (B) Gel shift assay of BXRβ. The ^{32}P-labeled oligonucleotides at the top were used as a probe for *in vitro* DNA binding analysis with the purified GST-BXRβ and RXRα. DR3 through DR5 are direct repeats of the sequence AGGTCA, separated by 3–5 nucleotides. The labeled probes were incubated with GST-BXRβ (10 ng) and/or RXRα (10 ng) and then electrophoresed on a 6% nondenaturing polyacrylamide gel.

Cloning of xBXRβ cDNA. To isolate a cDNA encoding the ligand binding domain of BXR, we designed oligonucleotide primers based on the published sequence of *Xenopus* BXR (2): +5′-TCGGCATGAGGAAAGAGTTGATCATG-3′ and –5′-TCATTCAGGGGATCCAAAGACTTCTG-3′. The primers were used in a reverse transcriptase-PCR protocol to amplify from total RNA prepared from *Xenopus laevis* eggs. The primers will produce a 863-bp fragment corresponding to nucleotides 299–1161 of the xBXR open reading frame (2). The PCR products were cloned into pBluescript KS$^-$ (Stratagene) for sequencing using an automated DNA sequencer DSQ 1000 (Shimadzu, Kyoto, Japan). The PCR products contained two genes: one is essentially identical to the xBXR previously reported (1, 2) and the other is a novel nuclear receptor.

A *X. laevis* oocyte 5′-stretch plus cDNA library (Clontech) was probed with the novel PCR product. Approximately 1×10^6 plaques were screened using a ^{32}P-labeled probe and 24 overlapping recombinant phage were isolated. pBluescript phagemids containing the inserts were excised and sequenced.

Preparation of recombinant proteins. The xBXRβ ORF was amplified by PCR using specific primers incorporating *Sal*I and *Not*I cloning sites, and cloned into pGEX4T-1 (Pharmacia Biotech). Glutathione *S*-transferase (GST) fusion protein was expressed in *E. coli* BL21 (DE3) (Novagen) and purified by glutathione-Sepharose 4B (Pharmacia Biotech). The mRXRα was expressed as a FLAG fusion protein in Sf9 cells via baculovirus vectors and affinity-purified with M2 agarose (Sigma).

DNA-binding analysis. Gel shifts were performed as described (10) with purified recombinant proteins. Proteins (10 ng each) were incubated for 30 min on ice with probes containing DR3, DR4 or DR5. Oligonucleotide sequences of DR3, DR4 and DR5 were as follows: DR3, 5′-ctagcgAGGTCAttgAGGTCAcg-3′; DR4, 5′-ctagcgAGGTCAattgAGGTCAcg-3′; DR5, 5′-ctagcgAGGTCAacaagGAGGTCAg-3′

Plasmid constructions. For construction of 2× DR3-luciferase, 2× DR4-luciferase, 2× DR5-luciferase, the synthesized oligonucleotides described above were multimerized as a 2-mer and cloned into the *Nhe*I site of PGV-P (Toyo Inc., Tokyo, Japan). The CMV-xBXRβ, xBXRβ expression vector, was constructed by subcloning the entire xBXRβ sequence into the *Sal*I–*Kpn*I site of pBK-CMV (Stratagene).

Cell culture and DNA transfection. HeLa cells were routinely maintained in phenol red-free Eagle's modified essential medium (EMEM) containing 10% charcoal-treated fetal calf serum in 10 cm plates. Six hours before transfection, 2×10^{-6} cells were seeded per 10 cm plate in phenol red-free EMEM containing 10% charcoal-treated fetal calf serum. Liposome-mediated transient transfections were performed using Lipofectamine reagent (Life Technologies Inc.), 3.5 μg of CMV-xBXRβ and 3.5 μg of reporter plasmids. Cells were incubated with liposome-DNA complexes at 37°C for 16 h, and then further incubated for 3 h after medium change. The cells were harvested, homogeneously mixed, and seeded into 96-well plates (2×10^{-4}/well) containing the medium with chemicals ($n = 6$). After 40 h incubation, cells were solubilized and luciferase activity was determined with a luminometer (Berthold MicroLumat LB96P).

RESULTS

Cloning of xBXRβ

To clone the BXR we initially performed RT-PCR using RNA prepared from *X. laevis* eggs and the primers shown in Fig. 1A. RT-PCR produced a single 860-bp DNA band of predicted size following electrophoresis on an agarose gel (data not shown). Cloning and sequencing of RT-PCR products revealed that the nucleotide sequences could be classified into two types (Fig. 1B). One type is completely identical to the previously described BXR (2). The second type shared 87% sequence identity, suggesting the presence of an additional BXR gene in *X. laevis*. Sequencing of 7 random PCR product clones showed that 4 of them encode the BXR and the remaining 3 represented the new gene.

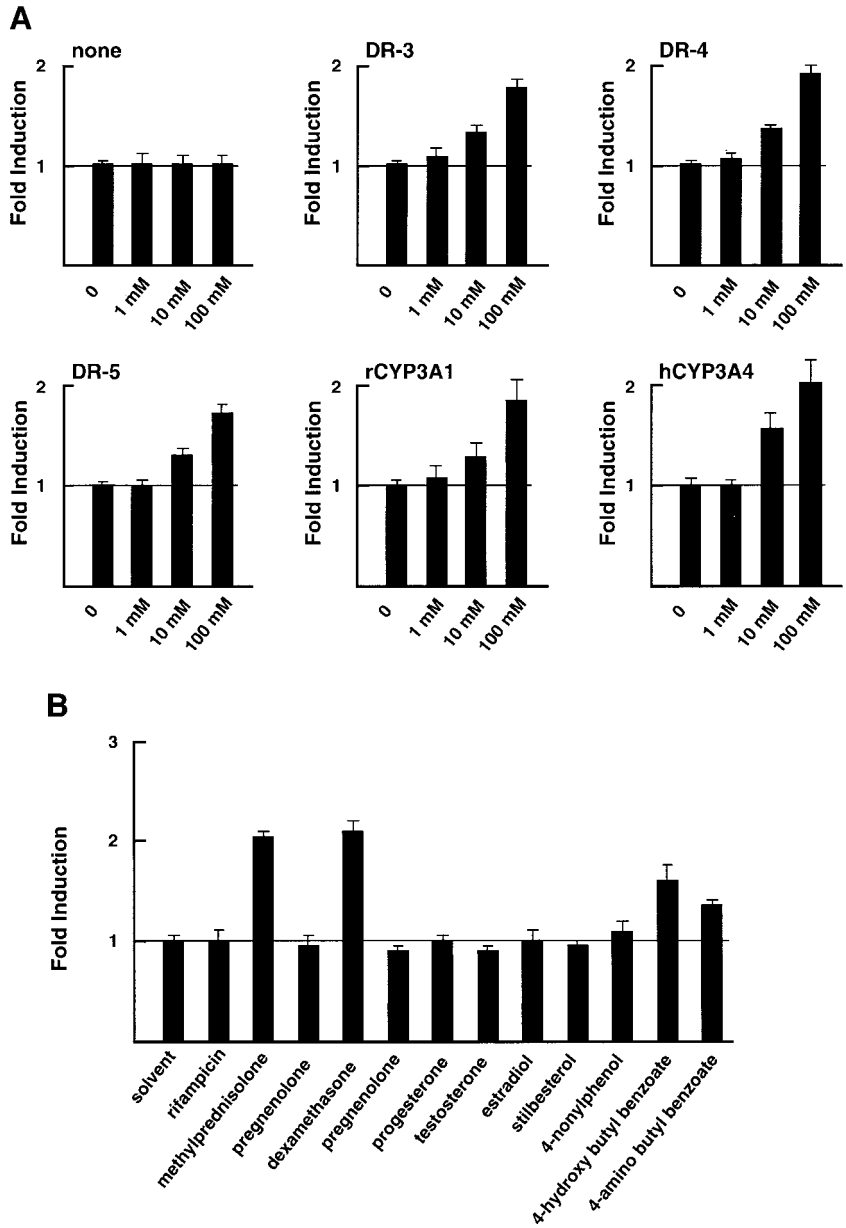

FIG. 4. Ligand-dependent transactivation of BXRβ. (A) BXRβ has a broad specificity for response elements. The oligonucleotide sequences used in this study were as follows: DR3, 5′-CTAGCG**AGGTCA**TTG**AGGTCA**CG-3′; DR4, 5′-CTAGCG**AGGTCA**ATTG**AGGTCA**CG-3′; DR5, 5′-CTAGCG**AGGTCA**ACAAG**AGGTCA**CG-3′; rCYP3A1, 5′-TAGAC**AGTTCA**TGA**AGTTCA**TCTAC-3′; hCYP3A4, 5′-TAGAATA**TGAACT**CAAAGG**AGGTCA**GTGAGTGG-3′. These oligonucleotides were connected to the SV40 promoter-luciferase gene, PGV-P. These reporter plasmids together with the BXRβ expression plasmid were transfected into HeLa cells by a liposome-mediated transfection technique. The indicated amounts of *p*-amino butyl benzoate were added. After 40 h incubation, cells were harvested and luciferase activities were determined with a luminometer. The fold stimulations against vehicle treatment are shown from six independent transfection analyses. The error bars indicate standard deviations. (B) BXRβ activation by pharmacological reagents. The ability of a panel of compounds to activate BXRβ was tested. Results are shown for 50 μM of compound by using the DR4 response element.

These results suggested that two closely related genes express equally in *X. laevis* eggs. To confirm the presence of a second BXR, a *X. laevis* egg cDNA library was screened at high stringency using the RT-PCR product as probe. Sequence analysis of 24 positive clones revealed the existence of a 1790-bp cDNA encoding a predicted protein of 389 amino acids which shows a very high homology with the previously described BXR (Fig. 2A). The highest homology with the BXR is in the DNA-binding domain where 97% of the amino acid residues are identical (Fig. 2B). The ligand-binding domain also shows considerable homology, sharing 79% amino acid identity (Fig. 2B), and implying that this novel receptor is also a receptor for benzoates.

Therefore, we termed this new receptor BXRβ. Sequence comparison with other members of the nuclear receptor family showed that the BXRβ is most closely related to hSXR (8) sharing 75% and 52% amino acid identity in the DNA-binding domain and ligand-binding domain, respectively (Fig. 2B). mPXR, the mouse homologue of hSXR (7), shares 73% and 51% amino acid identity in the DNA-binding domain and ligand-binding domain, respectively (Fig. 2B).

In Vitro DNA Binding

To further analyze the receptor, we attempted to express the ORF as a GST fusion protein in *E. coli*. A 72-kDa protein, which was almost consistent with the expected size, was efficiently expressed and purified by glutathione affinity chromatography (Fig. 3A, lane 2). Bearing in mind that BXR, PXR and SXR preferentially bind to DR-4 as heterodimers with RXR, we tested DNA binding of BXRβ alone and in combination with mRXRα on direct repeats of AGGTCA differing in their spacing by three to five nucleotides. As shown in Fig. 3B, strong binding was selective to a DR-4 motif with moderate binding to DR-5 and minimal binding to DR-3. Binding is strongly cooperative between BXRβ and RXR, as neither receptor alone showed binding at the protein concentrations used in the assay. These results demonstrate that BXRβ has similar DNA binding properties with subfamily proteins BXR, PXR and SXR.

Ligand-Dependent Transcriptional Activation

Whether benzoate derivatives function as bona fide ligands for BXRβ was addressed using transient cotransfection experiments. The suspected response elements were joined to a luciferase gene and transfected into HeLa cells together with the BXRβ expression plasmid. The levels of stimulation caused by addition of *p*-amino butyl benzoate are shown in Fig. 4A. Inconsistent with the DNA-binding data, DR-3, DR-4 and DR-5 worked equally well as a response element. The natural response elements derived from rCYP3A2 and hCYP3A4, which have been reported as PXR/SXR response elements (8), could also mediate ligand-dependent activation of BXRβ (Fig. 4A).

The homology of BXRβ with PXR/SXR led us to consider whether compounds known to activate PXR/SXR could be ligands for BXRβ. Transfected HeLa cells were systematically treated with a series of natural and synthetic compounds and reporter levels were measured. Interestingly, we found that the glucocorticoids methylprednislone (MP) and dexamethosone (DEX) stimulate BXRβ-mediated expression of the reporter gene on DR-4 (Fig. 4B). On the other hand, progesterone, pregnenolone 16α-carbonitrile (PCN), and rifampicin had no effect on BXRβ, although they have been reported to stimulate mPXR and/or hSXR (7, 8).

DISCUSSION

xBXRβ is a novel member of the nuclear receptor superfamily, and belongs to the subgroup of receptors which includes xBXR, hSXR and mPXR. Although the amino acid sequences of these receptors are extremely similar to each other in their DNA binding domains, the ligand binding domains are relatively divergent. Consistent with their homology, they share common properties in DNA binding despite the fact that their pharmacological properties are distinct from each other (7, 8, 11). Although many nuclear receptors activated by nonsteroidal ligands such as RAR, RXR, TR, LXR and PPAR have multiple subtypes (12–16), no such instance was reported in the BXR/PXR/SXR subfamily. The finding of a divergent form of BXR in a single vertebrate species may suggest the presence of several receptor subtypes of PXR/SXR in mammals. It is possible that this new branch of the nuclear receptor superfamily is considerably large and receptors of this subfamily could exert divergent physiological functions in differentiation or metabolism of xenobiotics.

Whereas the PXR/SXR is abundantly expressed in liver and intestine (7, 8), BXR exists in the unfertilized egg and remains until gastrulation whereupon it significantly decreases (2). This distinct expression pattern suggests functional differences between BXR and PXR/SXR. Although hSXR was isolated in a screen to identify a human homologue of xBXR, an embryo specific subtype of hSXR may possibly exist.

It is interesting that MP and DEX stimulate BXR-mediated transcription. It is reasonable that ligands of PXR/SXR bind to BXR, because the LBD of BXR considerably resembles PXR/SXR. This may raise concerns about the effects of glucocorticoids on embryogenesis. The spectra of ligand binding are partially overlapped among BXR, PXR and SXR, albeit not identical. On the contrary, natural substances examined in this study have no effect on BXRβ except for benzoates.

ACKNOWLEDGMENTS

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