Identification of the DNA Binding Specificity and Potential Target Genes for the Farnesoid X-activated Receptor*

(Received for publication, July 9, 1999, and in revised form, January 11, 2000)

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The farnesoid X-activated receptor (FXR; NR1H4) is a member of the nuclear hormone receptor superfamily and functions as a heterodimer with the 9-cis-retinoic acid receptor (RXR). In order to determine the optimal DNA binding sequence for the FXR/RXR heterodimer, we have utilized the selected and amplified binding sequence imprinting technique. This technique identified a number of related sequences that interacted with FXR/RXR in vitro. The consensus sequence contained an inverted repeat of the sequence AGGTCA with a 1-base pair spacing (IR-1). This sequence was shown to be a high affinity binding site for FXR/RXR in vitro and to confer ligand-dependent transcriptional activation by FXR/RXR to a heterologous promoter. Electrophoretic mobility shift assays and transient transfection assays were used to investigate the importance of the core half-site sequences, spacing nucleotide, flanking sequences, and orientation and spacing of the core half-sites on DNA binding and ligand-dependent transcriptional activation by FXR/RXR. These studies demonstrated that the FXR/RXR heterodimer binds to the consensus IR-1 sequence with the highest affinity, although FXR/RXR can bind to and activate through a variety of elements including IR-1 elements with changes in the core half-site sequence, spacing nucleotide, and flanking nucleotides. In addition, FXR/RXR can bind to and transactivate through direct repeats. Three genes were identified that contain IR-1 sequences in their proximal promoters. These elements were shown to bind FXR/RXR in vitro and to confer FXR/RXR-dependent transcriptional activation to a heterologous promoter in response to a bile acid or synthetic retinoid. The endogenous mRNA levels of one of these genes, phospholipid transfer protein, were shown to be induced by FXR and FXR ligands. The identification of the IR-1 and related elements as high affinity binding sites and functional response elements for FXR/RXR and the identification of a target gene for FXR/RXR should assist in the identification of additional genes regulated by FXR/RXR.

Nuclear hormone receptors are ligand-activated transcription factors that belong to a superfamily consisting of over 150 different members (reviewed in Refs. 1–3). These receptors elicit their actions by binding to hormone response elements (HREs) in the promoters of target genes and regulating transcription in response to lipophilic ligands. HREs are typically composed of two 6-base pair half-sites that may be arranged as direct, inverted, or everted repeats (2–4). The HRE for a given receptor is determined by the nucleotide sequence of the half-sites, the orientation and spacing of those half-sites, and the nucleotide sequence flanking the half-sites (2–4). The nuclear hormone receptor superfamily can be divided into the following two major classes: type I or classic steroid receptors (which include estrogen, progesterone, glucocorticoid, and mineralocorticoid receptors) and type II or non-steroid receptors (which include the retinoic acid, retinoid X, thyroid hormone, and vitamin D receptors) (1–3). Type I receptors typically bind as homodimers to HREs that are inverted repeats separated by three nucleotides (IR-3) (3, 4). Type II receptors show a larger variety of modes of DNA binding. Most type II receptors bind DNA as a heterodimer with the 9-cis-retinoic acid receptor (RXR) (1–4). HREs for these heterodimers are generally direct or inverted repeats with between 0 and 5 base pair spacing (0 to 5 rule) (5–7). Some type II receptors bind DNA as monomers (e.g. NGFI-B) (8, 9) or homodimers (e.g. RXR) (1–4). The HRE recognized by a monomer consists of a single half-site with additional sequence requirements upstream of the half-site called the 5′-extended half-site (8, 9). HREs that are recognized by homodimers are typically inverted or direct repeats (10–12).

When a nuclear hormone receptor is bound to an HRE, it may activate or repress transcription depending on the presence of ligand, cell type, promoter, response element, or other signals. Recent studies demonstrate that HREs contain “signaling information” that the receptor must “interpret” to determine the activity of a receptor bound to that site. For example, the glucocorticoid receptor (GR) can activate transcription when bound to certain glucocorticoid response elements (GREs) or repress transcription when bound to other GREs (Ref. 13 and references therein). Yamamoto and co-workers (13) iden-

* This work was supported in part by National Institutes of Health Grant HL 30568 (to P. A. E.) and by the Laubisch fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by a United States Public Health Service National Research Service Award GM 07185.
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†§*** The abbreviations used are: HRE, hormone response element; FXR, farnesoid X-activated receptor; RIP14, RXR-interacting protein 14; SAAB, selected and amplified binding sequence imprinting; IR, inverted repeat; DR, direct repeat; EcR, edysone receptor; USP, ultraspirecle; RXR, 9-cis-retinoic acid receptor;RAR, retinoic acid receptor; LXR, liver X receptor; TR, thyroid hormone receptor; GR, glucocorticoid receptor; GREs, glucocorticoid response elements; EcE, edysone receptor element; PCR, polymerase chain reaction; hsp27, heat shock protein 27; CDCA, chenodeoxycholic acid; PLTP, phospholipid transfer protein; PNMT, phenylethanolamine N-methyltransferase, CPT II, carnitine palmitoyltransferase II; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; WT, wild type; DTT, dithiothreitol; FBS, fetal bovine serum; TTNBP, tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-1-propenyl benzoic acid; S.O.s, selected oligonucleotides.
tified a mutant GR (K461A) that activated transcription when bound to all GREs tested, implying that this residue was critical for interpreting the signal from the GRE. Another example of context-dependent activation is the liver-X receptor (LXR). LXR activates transcription when bound as a heterodimer with RXR to a direct repeat of the sequence AGTCTCA spaced by 4 base pairs (DR4T element or LXR element) (14, 15). However, LXR/RXR is unable to activate through a DR4 element with the sequence AGTCTA (DR4G element), even though LXR/RXR binds to DR4T and DR4G with similar affinities (14, 15). Thus, HREs represent not only a specific binding site for nuclear receptors but can also significantly affect the function of the receptor.

The FXR (NR1H4) (16) was originally isolated from a rat liver cDNA library by degenerate PCR, utilizing primers to the highly conserved DNA binding domain of nuclear hormone receptors (17). The FXR cDNA was found to be homologous (94% identity) to a previously identified mouse receptor called RXR-interacting protein 14 (RIP14) (17, 18). These receptors are expressed in liver, kidney, gut, and adrenal cortex of adult rats and mice and in several embryonic tissues (17, 18). Sequence comparison indicates that FXR is closely related to the edcysone receptor (EcR) from Drosophila, particularly in the DNA-binding domain where they share 81% identity (17). The EcR is known to function as a heterodimer with ultraspireacle (USP), the Drosophila homolog of RXR (19–21). The EcR/USP heterodimer binds to and transactivates through an EcR element (EcRE) that consists of an inverted repeat with 1-base pair spacing (IR-1) (19–21). The Drosophila heat shock protein 27 (hsp27) promoter contains an IR-1 that functions as an EcRE (22). Based on homology between FXR (or RIP14) and EcR, it was shown that FXR/RXR or RIP14/RXR bound to the hsp27-EcRE (17, 18). RIP14/RXR was also shown to bind to several other elements including the retinoic acid response element from the hRARβ2 promoter (an imperfect direct repeat with 5-base pair spacing; DR-5), synthetic DR-2, DR-4, and DR-5 elements (18); however, it is not known whether these various motifs also function as positive or negative transcriptional elements involved in the regulation of gene expression by FXR. A detailed investigation of the DNA sequences necessary for binding and activation of transcription by FXR/RXR has not been reported.

FXR/RXR was originally shown to activate reporter genes with multiple copies of the EcRE upstream of a heterologous promoter in response to the isoprenoid farnesol or the related retinoic acid or a synthetic retinoid (TTNPB) (23). In contrast, both FXR and RIP14 can be activated by all-trans-retinoic acid or a synthetic retinoid (TTNPB) (23). More recently, a number of bile acids, including chenodeoxycholic acid (CDCA), have been shown to bind to and potentially transactivate FXR and RIP14 (24–26). Neither farnesol, Juvenile Hormone III, nor all-trans-retinoic acid have been shown to bind directly to either FXR or RIP14, although TTNPB has been shown to displace CDCA from FXR (24). The FXR/RXR heterodimer can also be activated by RXR-specific ligands (including 9-cis-retinoic acid) through the RXR moiety (17, 18, 23).

Here we report the identification of high affinity binding sites for FXR using an unbiased in vitro selection technique. We identified an IR-1 element as a high affinity binding site and functional response element for FXR/RXR. In addition, a number of non-consensus IR-1 and DR sequences were shown to function as response elements for FXR/RXR when placed upstream of a minimal promoter. We identified naturally occurring IR-1 elements in the promoters of several genes that both bind FXR/RXR in vitro and confer transcriptional activation by FXR ligands in vivo. These results suggest that FXR may regulate expression of these genes. Consistent with this hypothesis, we demonstrate that FXR ligands increase the mRNA levels of at least one of these genes and that overexpression of FXR further increases these levels. These studies will assist in the identification of genes and pathways regulated by FXR.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Full-length cDNA for FXR (pKS-OR2) was kindly provided by Dr. Cary Weinberger (NIEHS, National Institutes of Health). FXR (amino acids 111–469) was amplified by PCR using pKS-OR2 as the template and primers incorporating a BamHI site upstream and an XhoI site downstream of the indicated coding region. This fragment was subcloned into BamHI/XhoI-digested pRSET B (Invitrogen, Carlsbad, CA) to generate pRSET-FXRα110. Mammalian expression vectors for FXR (pCMX-FXR) and hRXRα (pCMX-hRXRα) were kindly provided by Dr. Ron Evans (Salk Institute, La Jolla, CA). Full-length hRXRα was amplified by PCR using pCMX-hRXRα as the template and subcloned into pRSET B, as described above, to generate pRSET-hRXRα.

**Purification of Histidine-tagged FXR and hRXRα—**NH2-terminal histidine-tagged FXRα110 and histidine-tagged hRXRα were expressed using pRSET-FXRα110 and pRSET-hRXRα in Escherichia coli (strain BL21(DE3)pLysS) (Novagen). Cultures were grown to an A600 of 0.6 and then induced with 1 mM isopropylthio-β-D-galactoside for 2.5 h. Induced cells were collected and resuspended in MAC-CO (20 mM Tris, pH 7.9, 0.5 mM NaCl, 1 mM PMSF, 1.0 µg/ml leupeptin, 0.7 µg/ml pepstatin) and then sonicated three times. Lysates were centrifuged at 27,000 g, and the supernatant (crude extract) was loaded onto a nickel affinity column (Novagen). Columns were washed thoroughly with MAC-CO (20 mM Tris, pH 7.9, 40 mM imidazole, 0.5 mM NaCl, 1 mM PMSF, 1.0 µg/ml leupeptin, 0.7 µg/ml pepstatin), and proteins were eluted with MAC-CO (200 mM Tris, pH 7.9, 200 mM imidazole, 0.5 mM NaCl, 1 mM PMSF, 1.0 µg/ml leupeptin, 0.7 µg/ml pepstatin). Five 1-ml fractions were individually dialyzed against 25 mM HEPES, pH 7.6, 2 mM MgCl2, 20% glycerol, 1 mM EDTA, 100 mM KCl, 1 mM DTT, 1% Nonidet P-40, 200 µM PMSF, 0.7 µg/ml pepstatin, and 1.0 µg/ml leupeptin.

**Selection and Amplification of Binding Sequences (SAAB)—**The SAAB technique was performed essentially as described (27). Briefly, a randomized single-stranded oligonucleotide template (SAAB template, 5′-agtgggacgagctggagggggcagaaaagggggaagggctggaagcaggggaa-3′) (see Fig. 1A), a 5′ SAAB primer (5′-agtgggacgagctggagggggcagaaaagggggaagggctggaagcaggggaa-3′) and a 3′ SAAB primer (5′-gggggttacccctactgg-3′) were purchased (Life Technologies, Inc.). Double-stranded randomized templates were generated by annealing the 3′ SAAB primer to the SAAB template and extending with Klenow fragment of DNA polymerase (Life Technologies, Inc.) at 37 °C (28). The double-stranded SAAB templates were isolated from single-stranded oligonucleotides by electrophoresis on a 15% polyacrylamide gel, excised, and eluted in 0.5 ml of 0.5 mM ammonium acetate, 10 mM MgCl2, 1 mM EDTA, and 0.1% SDS at 37 °C for 12 h. Templates were then precipitated with ethanol, resuspended, and then precipitated using sodium acetate and ethanol. For first round selection, random templates were end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (NE Biolife Science Products). Labeled double-stranded templates were mixed with approximately 50 ng of FXRα110 and 25 ng of hRXRα proteins (described above) and incubated in binding buffer (10 mM HEPES, pH 7.6, 0.5 mM DTT, 40 mM NaCl, 2.5 mM MgCl2, 0.05% (v/v) Nonidet P-40, 10% (v/v) glycerol, 50 ng/µl poly(dI-dC) (Amersham Pharmacia Biotech), 25 mM non-fat milk) for 1 h at room temperature. The ratio of FXRα110 to hRXRα was 2:1 in order to favor formation of FXRα/hRXRα heterodimers rather than hRXRα homodimers. DNA-protein complexes were separated on a 6% non-denaturing polyacrylamide gel at 4 °C for 2 h. The gel was dried and visualized by autoradiography. An oligonucleotide probe containing a single EcRE, a known binding site for FXR/hRXRα, served as a marker for migration of the FXR/hRXRα-DNA complex. The FXRα/hRXRα-DNA complex was excised based on migration of the positive control. The SAAB-selected oligonucleotides (S.O.s) were eluted in 0.5 ml of 0.5 mM ammonium acetate, 10 mM MgCl2, 1 mM EDTA, and 0.1% SDS at 37 °C for 3 h. Glycogen (10 µg) was added, and the S.O.s were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The S.O.s were resuspended in H2O and then precipitated using sodium acetate and ethanol. Approximately one-fifth of the S.O. pool was amplified by PCR using standard conditions.
PCR products were purified on a 15% polyacrylamide gel as described above. These purified amplified templates were labeled for subsequent rounds of selection by PCR; approximately 5 ng of templates were amplified by one cycle of PCR with 100 ng each Saab 5’ primer and Saab 5’ primer, 30 µCi of [α-32P]dCTP, 0.25 mM each dATP, dGTP, and dTTP, 10 mM MgCl2, 10 mM dithiothreitol buffer. Unincorporated ATP removed by gel filtration on BioSpin 6 columns (Bio-Rad), and approximately 100 pg of labeled template was used for the next round of selection (EMSA). After three rounds of selection, selected oligonucleotides were cloned into the pcR2.1-TOPO cloning vector (Invitrogen). Twenty-six clones were picked and sequenced using the Sequenase sequencing version 2.0 kit (Amersham Pharmacia Biotech). Sequences corresponding to half-sites are capitalized.

**Oligonucleotides**—Single-stranded oligonucleotides were purchased (Life Technologies, Inc.), annealed, and analyzed by 15% non-denaturing polyacrylamide gel electrophoresis. For EMsas, double-stranded oligonucleotides were labeled with T4 polynucleotide kinase (New England Biolabs) and [α-32P]ATP (NEI Life Science Products) and unincorporated ATP removed by gel filtration on BioSpin 6 columns (Bio-Rad). Oligonucleotides used were as follows: IR-1-consensus, 5’-gatc-aagAGGTCAaTGACCTcggAGGTCAttttg-3’ and 5’-gatc-aagAGGTCAcTGACCTcggAGGTCAttttg-3’; hsp27-ECRE-WT, 5’-gatc-aagAGGTCAaTGACCTcggAGGTCAttttg-3’ and 5’-gatc-aagAGGTCAcTGACCTcggAGGTCAttttg-3’; PLTP, 5’-gatccaaaaTGACCTcggAGGTCAttttg-3’ and 5’-gatccaaaaTGACCTcggAGGTCAttttg-3’; CPTII, 5’-gatccaaaaTGACCTcggAGGTCAttttg-3’; PNMT, 5’-gatccaaaaTGACCTcggAGGTCAttttg-3’. Sequences corresponding to half-sites are capitalized. All IR-1 “mutant” oligonucleotides were identical to IR-1-CON, except where indicated in the figures. All EcRE mutant oligonucleotides were identical to sequences found in the “natural” promoters.

**Electrophoretic Mobility Shift Assays (EMsas)**—For EMsas, partially purified FXRα110 and hRXRα were incubated in binding buffer (10 mM HEPES, pH 7.6, 0.5 mM DTT, 2.5 mM MgCl2, 0.05% (v/v) Nonidet P-40, 10% (v/v) glycerol, 50 µg/ml poly(dI-dC) (Amersham Pharmacia Biotech), 25 mg/ml non-fat milk, and 50 mM NaCl on ice for 15 min. Labeled probe (20,000 cpm, 1.5 fmol) was added, and the binding reaction was incubated at room temperature for 30 min. Complexes were resolved by 4% non-denaturing polyacrylamide gel electrophoresis at 4 °C for 2 h. The gel was dried and analyzed by PhosphorImaging (ImageQuant™ software, Molecular Dynamics).

**RESULTS**

**Selection of High Affinity Binding Sites for FXR**—In order to determine the DNA sequences that represent high affinity binding sites for FXR, we utilized the SAAB technique (27). We synthesized a 60-base pair double-stranded oligonucleotide, which contained the canonical nuclear receptor half-site, AGGTCA, upstream of 11 randomized base pairs and is referred to as the “SAAB template” (Fig. 1A). This oligonucleotide should allow for selection of either direct or inverted repeats with 0–5-base pair spacing between half-sites. The SAAB template (Fig. 1A) was end-labeled and used in EMsas with recombinant, purified FXR and RXR proteins. An oligonucleotide containing the hsp27 EcRE sequence (EcRE-WT) was used as a positive control in the EMsas and allowed for identification of the FXR-RXR-DNA complex. Shifted DNA complexes were recovered from the gel, and the DNA was amplified by PCR. Following each round of selection, selected oligonucleotides were end-labeled with [32P]ATP and used in EMsas without or with FXR and/or RXR to test for enrichment of high affinity binding sites. The formation of specific complexes was largely dependent on the presence of both FXR and RXR, suggesting that FXR/RXR heterodimers were favored over FXR/FXR homodimers, RXR/RXR homodimers, or monomers (data not shown). However, a minor shifted complex, presumably containing RXR/FXR homodimers, was formed in the absence of FXR (data not shown). We utilized an amino-terminal truncated FXR protein (~41 kDa) along with full-length RXR (~54 kDa), which allowed us to distinguish between FXR/RXR heterodimers, RXR/RXR or FXR/FXR homodimers, or monomers. The migration of the major protein-SAAB template complex was consistent with the formation of FXR/RXR heterodimers since the complex migrated in the gel to a position approximately equal to that of the positive control (FXR-FXR-EcRE-WT complex, data not shown). Following three rounds of selection, selected oligonucleotides were subcloned, and 26 independent clones were chosen and sequenced. The cloned sequences were aligned, and the frequency which given nucleotides were selected in the randomized 11 nucleotide region is shown in Fig. 1B. Analysis of the data indicates that the selected sequences contain a consensus that consists of an inverted repeat with a downstream half-site sequence AGGTCA or AGTTCA (reading 5’ to 3’).
and migration of the FXR/RXR-IR-1-CON complex was identical to the migration of a FXR/RXR-EcRE-WT complex (Fig. 2D, lanes 1 and 5, data not shown). In direct binding experiments, a higher percentage of the IR-1-CON labeled probe was shifted by FXR/RXR compared with the EcRE-WT (Fig. 2D, lane 1 versus 5). To determine the relative affinity of FXR/RXR for the IR-1-CON versus the EcRE-WT, competitive EMSAs were performed. Radiolabeled IR-1-CON was incubated with FXR and RXR proteins in the absence (Fig. 2A, lane 1) or presence of increasing amounts of unlabeled IR-1-CON oligonucleotide (Fig. 2A, lanes 2–7) or EcRE-WT oligonucleotide (Fig. 2A, lanes 8–13). The IR-1-CON was an effective competitor, competing approximately 95% of the shifted probe at 250-fold molar excess, whereas the EcRE-WT was considerably less effective, competing only about 20% at the same concentration (Fig. 2A and B). When the EcRE-WT was used as the 32P-labeled probe, unlabeled IR-1-CON was a much better competitor than unlabeled EcRE-WT (data not shown). Scatchard plot analysis of the data indicates that the IR-1-CON had a 10-fold higher affinity for FXR/RXR than the EcRE-WT (data not shown). These results demonstrate that the FXR/RXR heterodimer can bind to the IR-1-CON with high affinity.

Since the EcRE-WT oligonucleotide differs from the IR-1-CON oligonucleotide in both half-sites (Fig. 2C), as well as in flanking nucleotides, we tested whether the difference in affinity of FXR/RXR for the IR-1-CON versus the EcRE-WT was due to differences in the half-site sequences or in flanking nucleotides. Oligonucleotides were generated where either the 5'- or 3'-half-site of the IR-1-CON oligonucleotide were mutated to the sequence of the EcRE-WT half-sites, respectively (Fig. 2C, IR-1-M1, -M2, and -M3). Conversely, half-sites of the EcRE-WT were mutated to consensus half-sites (Fig. 2C, EcRE-M1, -M2, and -M3). These oligonucleotides were end-labeled with 32P and used in EMSAs with purified FXR and RXR proteins. Mutation of either half-site of the IR-1-CON to half-site sequences from the EcRE-WT resulted in decreased formation of a shifted complex (Fig. 2D, lane 1 versus lanes 2 and 3). Mutation of both half-sites of the IR-1-CON to those of the EcRE (IR-1-M27) resulted in a significant decrease in complex formation (Fig. 2D, lane 4 versus lane 5), to a level that was approximately equal to that obtained with the EcRE-WT (Fig. 2D, lane 4 versus lane 5). Conversely, mutation of either binding site of the EcRE-WT to the consensus sequence of the IR-1-CON resulted in an increase in the formation of a shifted complex, whereas mutation of both half-sites increased complex formation to near that of IR-1-CON (Fig. 2D, lane 5 versus lanes 6–8). These results demonstrate that the core half-site nucleotides, not the flanking regions, represent the primary determinant for complex formation.

**The IR-1-CON Serves as a Functional Response Element for FXR/RXR—**To determine if the IR-1-CON can function as a response element for FXR/RXR, two copies of the IR-1-CON oligonucleotide were cloned upstream of the TK minimal promoter and luciferase reporter gene (pTK-luciferase vector). Two copies of the binding sites were chosen to allow for a relatively high level of expression of the reporter and to limit the synergistic activation that can occur when multiple binding sites are used. As a positive control, two copies of the EcRE-WT oligonucleotide were also cloned into the pTK-luciferase vector. The reporter plasmids were cotransfected into HepG2 cells along with expression vectors for FXR and RXR. As expected, the EcRE-WT reporter was activated by ligands for FXR (CDCA or TTNPB) or RXR (LG10153) (Fig. 2E). Addition of both FXR- and RXR-specific activators resulted in additive levels of expression of the EcRE-WT reporter (Fig. 2E) in agreement with a previous report (19). The IR-1-CON reporter con-
struct was also activated by ligands for either FXR or RXR (Fig. 2E). In the presence of both FXR- and RXR-specific ligands, additive levels of expression were observed (Fig. 2E). The IR-1-CON reporter construct was activated to a higher level than the EcRE-WT reporter (Fig. 2E), consistent with the higher affinity of FXR/RXR for the IR-1-CON versus the EcRE-WT (Fig. 2A). Coexpression of FXR, but not RXR, was required for activation of either the IR-1-CON or the EcRE-WT reporter.
genes in HepG2 (data not shown). However, coexpression of RXR, together with FXR, further increased the activity of both reporter genes (data not shown). These results demonstrate that the IR-1-CON can function as a high affinity binding site and a functional response element for FXR/RXR.

**Mutational Analysis of the IR-1-CON**—Several non-consensus IR-1 elements, which differ from the IR-1-CON by only a single nucleotide in the downstream half-site, were selected by the SAAB technique (Figs. 1C and 3A). To determine if the IR-1-CON represents the highest affinity binding site for FXR/RXR, we introduced single nucleotide mutations in the downstream half-site of the IR-1-CON. Some of these mutations were made to investigate non-consensus IR-1 sequences selected by SAAB (IR-1-M1, -M2, -M25, and -M26) (Figs. 1C and 3A). Other mutations were introduced to test whether specific sequences, not selected by SAAB, could also function as binding sites for FXR/RXR heterodimers (IR-1-M3, -M4, -M5, and -M6) (Fig. 3A). Each of the oligonucleotides shown in Fig. 3A were end-labeled and used in EMSAs with purified FXR and RXR proteins. Fig. 3B shows that FXR/RXR binds with high affinity to number of oligonucleotides containing non-consensus inverted repeats. Certain single point mutations resulted in a significant decrease in the formation of a shifted complex (Fig. 3B, lanes 6 and 9 versus lane 2). These results demonstrate that the SAAB technique resulted in isolation of sequences that are bound by FXR/RXR with a broad affinity range. Most of the sequences (22 of 26) identified by SAAB are high affinity FXR/RXR binding sites, although some were of lower affinity (Fig. 3B, IR-1-M25). In addition, some high affinity binding sites (IR-1-M3 and -M5, Fig. 3B) were not selected presumably because only 26 clones were analyzed (Fig. 1B). Nonetheless, these results demonstrate that the SAAB technique identified sequences that, in general, were of higher affinity than previously identified FXR/RXR-binding sites.

To test how mutations in the downstream half-site sequence of the IR-1-CON affect function as a response element for FXR/RXR, we generated luciferase constructs, each containing two copies of the indicated response element (IR-1-M1 through M6). The resulting reporter constructs were cotransfected into HepG2 cells along with expression vectors for FXR and RXR. The results demonstrate that the induced level of reporter gene activity, in the presence of the FXR ligand TTNPB, was M3 > M1 = M5 > M2 > CON > M4 > M6 (Fig. 3C). The rank order of activation was similar to the order of DNA binding affinity (Fig. 3B).

**Importance of the Spacer Nucleotide and Extended Half-site Sequence**—In addition to selecting a specific sequence for the downstream half-site, the SAAB technique also selected specific nucleotides in the spacer nucleotide (T or A) and “extended” half-site (TTTT) (Fig. 1B). To investigate the importance of these regions on DNA binding by FXR/RXR, we generated oligonucleotides with mutations in these regions (Fig. 4A). EMSAs showed a small decrease in binding affinity of FXR/RXR when the spacer nucleotide is changed from T to G (IR-1-M9) (Fig. 4B, lane 2 versus lane 1) or when the extended half-site was changed from TTTT to GGGG (IR-1-M14, -M19, or -M24) (Fig. 4B, lanes 1, 4 and 6 versus lanes 3, 5, and 7). These studies indicate that the spacer nucleotide and flanking nucleotides have a minor effect on the DNA binding affinity of FXR/RXR and together with previous results (Fig. 2D) demonstrate that the core half-sites are the critical determinants of FXR/RXR binding.

**FXR/RXR Binds to and Activates through Direct Repeats**—The SAAB technique selected two direct repeat elements (DR-1 and DR-3), in addition to the many inverted repeat elements (Fig. 1C). To test if other direct repeat elements could function as high affinity binding sites for FXR/RXR, EMSAs were performed using oligonucleotides with consensus DR-1 through DR-5 elements. All five DR elements were able to form a specific complex with FXR/RXR (Fig. 5A, lane 2–6). Competitive EMSAs indicated that the affinity of FXR/RXR for DR-4 and DR-5 was similar to that for EcRE (data not shown). Fig.
TABLE 1

| Binding site | Spacer | Downstream half-site | Extended half-site | Selected |
|--------------|--------|----------------------|-------------------|----------|
| IR-1-CON     | g      | T G A C C T           | ttttt             | S        |
| IR-1-M9      | g      | T G A C C T           | ttttt             | NS       |
| IR-1-M14     | g      | T G A C C T           | ttttt             | S        |
| IR-1-M1      | g      | T G A C C T           | ttttt             | NS       |
| IR-1-M19     | g      | T G A C C T           | ttttt             | S        |
| IR-1-M2      | g      | T G A C C T           | ttttt             | NS       |
| IR-1-M24     | g      | T G A C C T           | ttttt             | S        |

5B shows that reporter genes containing two copies of DR-4 or DR-5 were activated by ligands TTNPB or CDCA in the presence of coexpressed FXR and RXR. Activation required coexpression of FXR, but maximal activation was dependent on the coexpression of both FXR and RXR (Fig. 5C). The slight activation of the DR-5 element by TTNPB in the absence of coexpressed FXR (Fig. 5C, panels 5 and 7) likely results from the activation of endogenous RAR/RXR. The latter heterodimer (RAR/RXR) is known to bind to a DR-5 element and to be activated by TTNPB (28, 29). Taken together, these results demonstrate that FXR/RXR can bind to and activate expression through direct repeat elements.

Identification of Natural Response Elements for FXR/RXR—We performed a data base search using the IR-1-CON sequence and identified several candidate genes that contain sequences in their proximal promoters that correspond closely to the consensus IR-1 sequence. Three of these genes, phospholipid transfer protein (PLTP) (30), phenylethanolamine N-methyltransferase (PNMT) (31, 32), and carnitine palmitoyltransferase II (CPT II) (33), were chosen for further investigation. These genes are expressed in liver, kidney, adrenals, and/or gut (30–37), tissues where FXR expression has been observed (17, 18). The sequences from the promoters of these genes that correspond closely to the consensus IR-1 element are shown in Fig. 6A. To determine if FXR/RXR could bind to these elements, oligonucleotides were generated which correspond to the IR-1 elements from the PLTP, PNMT, and CPT II genes. Each of these elements was specifically bound by FXR/RXR with affinities that are similar to that of IR-1-CON (Fig. 6B, lanes 1–4). These elements also function as response elements for FXR/RXR when placed upstream of a minimal promoter and luciferase reporter gene (Fig. 6C). Interestingly, each of these reporters was activated by either TTNPB or CDCA to higher levels than the IR-1-CON reporter (Fig. 6C). The PLTP reporter was consistently activated to the highest level by TTNPB (35-fold) or CDCA (60-fold) (Fig. 6C). The PNMT reporter was also highly activated by TTNPB (15-fold) or CDCA (31-fold) (Fig. 6C). The IR-1 element from the PLTP promoter has a sequence of GGTTCA in both half-sites, whereas the PNMT element has this sequence in one half-site and the consensus AGGTCA in the other half-site (Fig. 6A). We noted earlier that the IR-1-M3 reporter, which has this same downstream half-site sequence (GGTTCA), was the most highly activated reporter (Fig. 3C). Taken together, these results may indicate a preference for a G nucleotide in the 1st position of the half-site for high level activation of IR-1 elements by FXR/RXR. These results demonstrate that FXR/RXR can bind to and activate expression through naturally occurring IR-1 elements contained within the promoters of the PLTP, PNMT, and CPT II genes.

Regulation of the Endogenous PLTP mRNA Levels by FXR/RXR—Based on its role in lipid transport and lipoprotein metabolism, PLTP appeared to be the most promising candidate gene and thus was chosen for a more detailed investigation. To determine if FXR/RXR could regulate expression of the endogenous PLTP gene, Northern blots were performed on RNA derived from two different cell types that express FXR. Northern blot analysis demonstrated that FAO cells, a hepatocyte-derived cell line, express endogenous FXR (data not shown). When FAO cells were treated with either of two different FXR ligands, the PLTP mRNA level was increased (1.7–2.5-fold) (Fig. 7A). To demonstrate that FXR mediates activation of PLTP, we generated cells overexpressing FXR by infecting HepG2 cells with a virus encoding FXR and the neomycin resistance gene (Fig. 7B, FXR) or the neomycin resistance gene only (Fig. 7B, neo). Neomycin-resistant cells were selected and then treated with CDCA or MeSO4 for 24 h. The PLTP mRNA levels showed a dramatic increase in cells infected with FXR when compared with cells infected with the empty vector (Fig. 7B). Furthermore, PLTP mRNA levels were induced by treatment with the FXR ligand CDCA (Fig. 7B). These results demonstrate that FXR/RXR regulates the level of expression of the endogenous PLTP mRNA.

DISCUSSION

No detailed study of the DNA binding specificity of FXR has been reported to date, even though such information is crucial for definitive analyses of FXR target genes. In this study, we utilized an unbiased, PCR-based selection technique (the SAAB technique) (27) to identify high affinity binding sites for the FXR/RXR heterodimer. The majority of the selected sequences contained elements that were identical to or highly related to the IR-1 consensus sequence. These findings are consistent with previous reports that demonstrate that FXR/RXR (or RIP14/RXR) can bind to an imperfect IR-1 (the EcRE-WT) or to an idealized IR-1 element (17, 18). The nucleotide sequences selected for high affinity binding by FXR/RXR (IR-1 consensus) in this study are similar to sequences selected (by...
EMSAs were performed using the indicated $^{32}$P-labeled oligonucleotides. A probe incubated with Me$_2$SO, TTNPB (5 μM), or CDCA (100 μM) was treated with FXR/RXR as described under “Experimental Procedures.” The percent of radioactive probe shifted was determined by the related EcR-USP complex from Drosophila (38) and sequences shown to bind Mosquito EcR/USP with high affinity (39, 40).

By using direct binding and competitive EMSAs, we demonstrate that the consensus IR-1 represents the highest affinity FXR/RXR-binding site known to date. Our studies show that FXR/RXR binds to the consensus IR-1 with greater than 10-fold higher affinity than to the previously identified EcRE-WT sequence (Fig. 2, A and B) or to DR elements (Fig. 5A). More significantly, the consensus IR-1 was shown to function as a response element for FXR/RXR and to be more effective than the EcRE-WT in conferring responsiveness to ligands for FXR (CDCA or TTNPB) or RXR (LG10153) (Fig. 2E). The level of transactivation by FXR/RXR through various IR-1 elements was shown to correlate with DNA binding affinity, determined from in vitro EMSAs (Fig. 3, B and C, data not shown).

Additional studies reveal that FXR/RXR can bind to and transactivate through various IR-1 elements. The highest level of transactivation by FXR/RXR was mediated by an IR-1 where one half-site had the sequence GGGTCA (IR-1-M3) (Fig. 3C). Interestingly, the optimal monomer binding sequence for RXX has been shown to be GGGTCA (41), and the optimal RXX homodimer binding site has been shown to be a DR-1 element with one half-site of the sequence GGGTCA (42). This may suggest that the FXR/RXR heterodimer favors an orientation on the IR-1-M3 element where the RXX moiety is bound to the GGGTCA half-site, and this orientation may result in a greater level of activation of the FXR/RXR heterodimer.

We also demonstrate that FXR/RXR binds to DR elements. These results are consistent with earlier reports that utilized RIP14, the mouse homolog of FXR, and RXX (18) or EcR/USP (38–40). However, it was not known if DR elements could function as response elements for RIP14/RXR or FXR/RXR. Importantly, we now demonstrate that FXR/RXR can activate expression of reporter genes through DR-4 and DR-5 elements (Fig. 5). DR-4 and DR-5 elements have been described as response elements for several nuclear receptor heterodimers, including LXR/RXR and TR/RXR (DR-4) and RAR/RXR (DR-5) (5, 6, 15, 16, 43, 44). Therefore, activation of genes through DR-4 or DR-5 elements by FXR/RXR will likely depend, in part, on the relative concentration of these different receptors in the cell and their relative binding affinities for these elements.

The identification of DNA sequences that both are recognized by FXR/RXR in vitro and serve as response elements in vivo has allowed us to identify three putative target genes that may be regulated by FXR. Each of these genes contained an IR-1 element in their proximal promoter region that was bound by FXR/RXR and when placed upstream of a heterologous promoter was able to confer ligand-dependent regulation by FXR/RXR. One of these potential targets was in the proximal promoter of the human phospholipid transfer protein (PLTP) gene (30). Reporter genes containing two copies of the PLTP IR-1 element (GGGTCAgTGACCC) are the most responsive to FXR ligands tested to date (Fig. 6). Importantly, we now demonstrate that FXR/RXR can activate reporter constructs (described above) were transiently cotransfected into HepG2 cells along with CMV-β-galactosidase, with or without 50-ng expression vectors for FXR and RXX, as indicated. Cells were treated with Me$_2$SO (black bars), TTNPB (5 μM) (gray bars), or CDCA (100 μM) (white bars) for 24 h before harvesting. The results are shown as normalized luciferase units and are representative of three independent experiments. The percent of radioactive probe shifted was EcRE, 23%; DR-1, 16%; DR-2, 2%; DR-3, 14%; DR-4, 26%; and DR-5, 25%. Oligonucleotides used in these experiments are as follows: IR-1, IR-1-CON; DR-1 through DR-5, direct repeats of the consensus sequence GGGTCA.
PLTP mRNA levels are increased in response to FXR ligands (Fig. 7). In addition, in HepG2 cells, PLTP mRNA levels are further increased in response to overexpression of FXR (Fig. 7B). Consistent with these observations, preliminary studies have shown that the natural PLTP promoter can be regulated by FXR/RXR or RIP14/RXR.2 Thus, PLTP represents only the second known target gene for activation by FXR (25). PLTP mediates transfer of phospholipids and cholesterol from triglyceride-rich lipoproteins to high density lipoproteins (reviewed in Refs. 45 and 46) (47). Taken together, these findings suggest that FXR may play a role in the regulation of lipoprotein metabolism by mediating expression of PLTP.

We identified other functional FXR/RXR-binding sites in the proximal promoters of the CPT II gene (33) and the PNMT gene (31). CPT II is important in the entry of acyl groups into the mitochondrion for \( \beta \)-oxidation of fatty acids (reviewed in Ref. 48). PNMT is primarily expressed in the adrenal medulla and encodes the final enzyme in the synthesis of epinephrine. Additional studies are required to demonstrate that these target genes are regulated by FXR \textit{in vivo}. Our studies, defining the DNA binding specificity for FXR/RXR, should both assist in the identification of additional genes and pathways regulated by FXR, bile acids, and retinoids.

**Acknowledgments**—We thank Drs. Johan Ericsson, Simon Jackson, Peter Tontonoz, and members of the Edwards’ laboratory for critical discussions; Dr. Cary Weinberger of the NIEH for the full-length FXR cDNA; Dr. Ron Evans for pCMX-hRXR \( ^a \) and pCMX-FXR plasmids; Dr. Roger Davis for FAO cells; and Dr. Richard Heyman of Ligand Pharm.

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2 A. M. Zavacki and D. D. Moore, unpublished observations.
maceuticals, Inc. (San Diego, CA), for the generous gift of LG10153. We also thank Song Wang and Fan Li for technical contributions.

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DNA Binding Specificity of FXR

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J. Biol. Chem. 2000, 275:10638-10647.
doi: 10.1074/jbc.275.14.10638

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