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Identification of a Nuclear Receptor That Is Activated by Farnesol Metabolites

Barry M. Forman,[1] Elizabeth Goode,[2] Jasmine Chen,[1] Anthony E. Oro,[1] David J. Bradley,[3]* Thomas Perlmann,[1] Daniel J. Noonan,[4] Leo T. Burka,[5] Trevor McMorris,[6] William W. Lamph,[7] Ronald M. Evans,[1,8] and Cary Weinberger[2]
[8] Howard Hughes Medical Institute
[1] Gene Expression Laboratory
The Salk Institute for Biological Studies
10010 North Torrey Pines Road
La Jolla, California 92037
[2] Receptor Biology Group
[5] Organic Chemistry Group
Laboratory of Reproductive and Developmental Toxicology
National Institute of Environmental Health Sciences
Research Triangle Park, North Carolina 27709
[3] Laboratory of Cell Biology
National Institutes of Health
Bethesda, Maryland 20892
[4] Department of Biochemistry
University of Kentucky
Lexington, Kentucky 40506
[6] Department of Chemistry
University of California, San Diego
La Jolla, California 92093
[7] Ligand Pharmaceuticals, Incorporated
9393 Towne Centre Drive
San Diego, California 92121

Summary

Nuclear hormone receptors comprise a superfamily of ligand-modulated transcription factors that mediate the transcriptional activities of steroids, retinoids, and thyroid hormones. A growing number of related proteins have been identified that possess the structural features of hormone receptors, but that lack known ligands. Known as orphan receptors, these proteins represent targets for novel signaling molecules. We have isolated a mammalian orphan receptor that forms a heterodimeric complex with the retinoid X receptor. A screen of candidate ligands identified farnesol and related metabolites as effective activators of this complex. Farnesol metabolites are generated intracellularly and are required for the synthesis of cholesterol, bile acids, steroids, retinoids, and farnesylated proteins. Intermediary metabolites have been recognized as transcriptional regulators in bacteria and yeast. Our results now suggest that metabolite-controlled intracellular signaling systems are utilized by higher organisms.

*Present Address: Department of Medicine, The Johns Hopkins Hospital, Baltimore, Maryland 21287.

Introduction

Molecular cloning studies have demonstrated that receptors for steroid, retinoid, vitamin D, and thyroid hormones comprise a superfamily of regulatory proteins that are structurally and functionally related (Evans, 1988; Leid et al., 1992a). Known as nuclear hormone receptors, these proteins bind to *cis*-acting elements in the promoters of their target genes and modulate gene expression in response to hormone. Nuclear receptors can be classified based on their DNA-binding properties (Evans, 1988; Glass, 1994). The glucocorticoid, estrogen, androgen, progestin, and mineralocorticoid receptors bind as homodimers to hormone response elements organized as inverted repeats (Glass, 1994). A second class of receptors, including the retinoic acid receptor (RAR), the thyroid hormone receptor (T_3R), the vitamin D_3 receptor, the fatty acid/peroxisome proliferator-activated receptor (PPAR), and the ecdysone receptor (EcR), bind as heterodimers with a common partner, retinoid X receptor (RXR) (Yu et al., 1991; Hallenbeck et al., 1992; Kliewer et al., 1992; Leid et al., 1992b; Marks et al., 1992; Zhang et al., 1992), the 9-cis retinoic acid receptor (Heyman et al., 1992; Levin et al., 1992).

An important advance in the characterization of this superfamily of regulatory proteins has been the delineation of a growing number of gene products (orphan receptors) that possess the structural features of hormone receptors, but that lack known ligands. The search for hormonal activators for these newly discovered orphan receptors has created exciting areas of research on previously unknown signaling pathways (Issemann and Green, 1990; Heyman et al., 1992; Levin et al., 1992). The ability to identify novel hormonal systems has important implications in physiology as well as human disease and its treatment.

As receptors have been identified for all known nuclear-acting hormones, a question arises as to the types of molecules that may activate orphan receptors. Products of intermediary metabolism act as transcriptional regulators in bacteria and yeast, suggesting that they may serve similar functions in higher organisms (Tomkins, 1975; O'Malley, 1989). A crucial biosynthetic pathway in higher eukaryotes is the mevalonate pathway, which leads to the synthesis of cholesterol, bile acids, porphyrin, dolichol, ubiquinone, carotenoids, retinoids, vitamin D, steroid hormones, and farnesylated proteins. The farnesol derivative, farnesyl pyrophosphate, is a key metabolic intermediate, since it is the last precursor common to all branches of the mevalonate pathway (Goldstein and Brown, 1990). We describe an orphan nuclear receptor named farnesoid X–activated receptor (FXR) that is activated by farnesol and related molecules. FXR provides an example of a vertebrate transcription factor that is regulated by an intracellular metabolite and suggests the existence of a potentially novel vertebrate-signaling pathway.

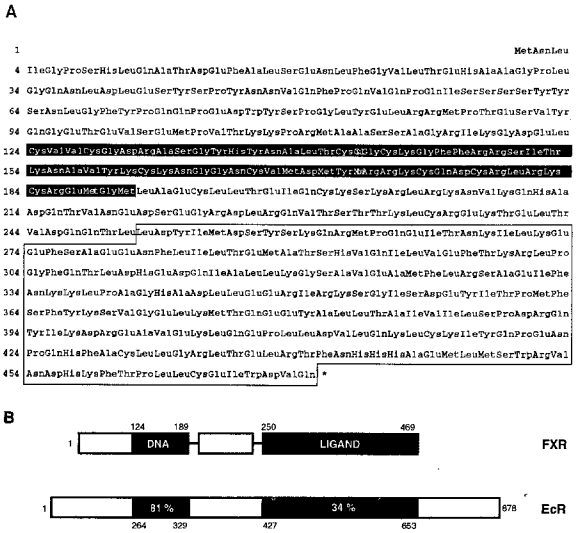

Figure 1. FXR Is a Member of the Nuclear Receptor Superfamily

(A) The nucleotide and amino acid sequence of rat FXR. The 2.1 kb cDNA encodes a 469 amino acid open reading frame (ORF). The ORF begins with an initiation codon that appears eight codons downstream of an in-frame stop codon and ends with a TGA stop codon (asterisk). In vitro translation of FXR-derived RNA results in a protein with a relative molecular mass (M_r) of 54,000, close to the predicted M_r of 53,934 (data not shown). Amino acids comprising the conserved DBD (Cys-124–Met-189) are in white letters on black background. The LBD (Leu-250–Gln-469) is boxed. The *FXRα1* cDNA contains a short interspersed repetitive DNA element (Sutcliffe et al., 1984) in the 3′ untranslated region followed by a polyadenylation signal.

(B) Amino acid sequence comparison between rat FXR and Drosophila EcR. Similarity between the DBDs and LBDs is schematically represented as percent amino acid identity. Amino acid regions comprising each domain are numbered accordingly.

Results and Discussion

Receptor Gene Isolation

A degenerate oligonucleotide probe corresponding to the highly conserved P box/DNA recognition helix (TCEGCK (G/V)FF) of the nuclear receptor superfamily DNA-binding domain (DBD) was used to isolate a cDNA for FXR from rat liver (Figure 1A). A similar cDNA has recently been cloned from mouse liver (Seol et al., 1995). Examination of the amino acid sequence of FXR reveals that it is a member of the nuclear receptor superfamily (Evans, 1988) and is most closely related to the insect EcR (Koelle et al., 1991). FXR and EcR share 81% amino acid identity within their DBDs (Figure 1B). The carboxy-terminal ligand-binding domain (LBD) is a complex region encoding subdomains for ligand binding, dimerization, and transcriptional activation (Forman and Samuels, 1990; Glass, 1994). FXR and EcR LBDs exhibit 34% sequence identity (59% similarity) throughout the LBD (Figure 1B). Closer inspection reveals that similarity is highest within the heptad repeats required for receptor dimerization (Forman and Samuels, 1990).

FXR–RXR Complexes Bind DNA

Previous studies have shown that the physiologically active form of EcR is an EcR–ultraspiracle (EcR–usp) heterodimer (usp is a Drosophila homolog of RXR) (Thomas et al., 1993; Yao et al., 1993). This prompted us to examine whether FXR also interacts with RXR. To do so, we made use of a two-hybrid system modified for use in mammalian cells (Nagpal et al., 1993). The LBD/dimerization domain

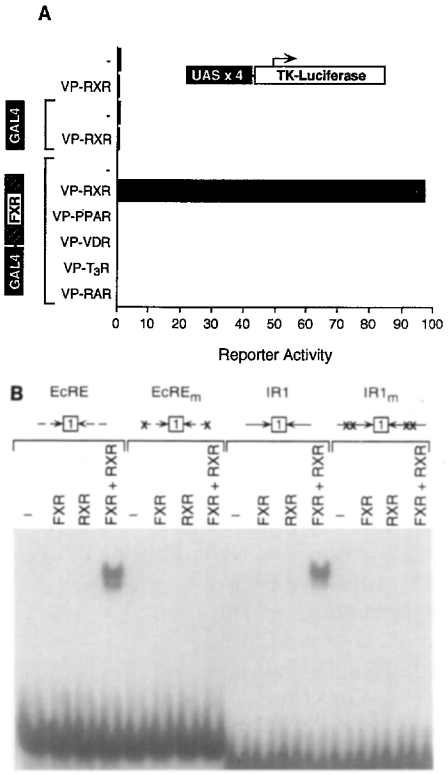

Figure 2. FXR and RXR Interact in Cells and In Vitro

(A) The LBDs of FXR and RXRα interact specifically in cells. CV-1 cells were transiently transfected, as indicated, with CMV promoter-driven expression vectors (100 ng per 10^5 cells) containing the yeast GAL4 DBD alone (GAL4[1–147]), GAL4 linked to the FXR LBD (GAL4–FXR[181–469]), the 78 amino acid herpesvirus VP16 transactivation domain alone (data not shown), or the VP16 domain linked to the amino-terminal end of the LBDs for human RXRα (VP–RXR[203–462]), mouse PPARα (VP–PPAR[155–468]), human vitamin D receptor (VDR) (VP–VDR[92–427]), human $T_3R\beta$ (VP–T_3R[173–456]), or human RARα (VP–RAR[156–462]). All cells were cotransfected with a thymidine kinase (TK)–luciferase reporter construct (300 ng per 10^5 cells) containing four copies of the yeast GAL4 upstream activating sequence and a CMV-driven β-galactosidase expression vector (500 ng/10^5 cells) as internal control. The only combination resulting in significant activation was GAL4–FXR plus VP–RXR. As expected from previous in vitro studies (Yu et al., 1991; Hallenbeck et al., 1992; Kliewer et al., 1992; Leid et al., 1992b; Marks et al., 1992; Zhang et al., 1992), VP–PPAR, VP–VDR, VP–T_3R, and VP–RAR interacted productively with GAL4–RXR (K. Umesono, personal communication; R. M. E, unpublished data), indicating that these VP16 chimeras are functionally expressed.

(B) FXR and RXR bind cooperatively to DNA. Mobility shift experiments were performed using the indicated ^{32}P-labeled DNA probes. Each probe is flanked by HindIII overhangs. Their sequences are as follows (Yao et al., 1992): hsp27–EcRE, GGTTCA A TGCACT; $EcRE_m$ (11N–hsp27–EcRE), CGTTCA A TGCACA; IR-1, AGGTCA A TGACCT; IR-1_m, AGAACA A TGTTCT. The position of mutations introduced into the hsp27–EcRE and the idealized IR-1 are underlined above and indicated in the figure (X). The gel was autoradiographed for 1.5 hr with an intensifying screen.

of FXR was fused to the yeast GAL4 DBD (GAL4–FXR). As seen in Figure 2A, the GAL4 DBD or the GAL4–FXR chimera is incapable of stimulating transcription from a reporter construct containing the GAL4 upstream activating sequence. Similarly, a fusion protein containing the herpesvirus VP16 transactivation domain linked to the RXRα LBD (VP–RXR) is inactive when expressed alone or with the GAL4 DBD (Figure 2A). However, when GAL4–FXR and VP–RXR are coexpressed, the reporter is activated 500-fold, indicating that FXR and RXRα interact efficiently in cells (Figure 2A). No interaction could be detected between FXR and other nuclear hormone receptors, indicating that FXR and RXRα associate in a highly specific fashion.

The amino acids comprising the DNA recognition helix (P box, Cys-141–Lys-145) of FXR and EcR are identical, suggesting that the FXR–RXRα complex could recognize an ecdysone response element (EcRE). Electrophoretic mobility shift analysis was performed using ^{32}P-labeled DNA and in vitro translated FXR and RXRα. As seen in Figure 2B, neither FXR nor RXRα was capable of high affinity binding to the hsp27–EcRE. However, when mixed, the two proteins bind cooperatively. Binding is specific, as indicated by the inability of the FXR–RXR complex to recognize the mutated EcRE ($EcRE_m$). The hsp27–EcRE consists of two imperfect core-binding sites arranged as inverted repeats separated by 1 nt (IR-1). Accordingly, the binding of FXR–RXR was further examined on an idealized IR-1 containing two consensus half-sites. As seen in Figure 2B, the FXR–RXR complex bound cooperatively to the idealized IR-1, but not to a mutant IR-1 containing substitutions within the half-sites ($IR\text{-}1_m$).

Activation by Farnesoids

We sought to determine whether FXR possessed transcriptional activity that could be hormonally controlled. Based on the identification of an EcRE as a DNA target, a reporter plasmid was constructed containing five copies of the hsp27 response element linked to a truncated mouse mammary tumor virus promoter (Yao et al., 1993). This reporter was cotransfected into CV-1 cells alone or with expression vectors for FXR, RXRα, or both. Cotransfected cells were treated with a variety of potential ligands and monitored for changes in luciferase activity. Candidate ligands or activators (or both) included a variety of low molecular weight molecules that have been reported to have specific biological activity. Unexpectedly, juvenile hormone III (JH III) (Moore, 1990) (50 μM) elicited a strong induction (10-fold) of luciferase activity in cells expressing both FXR and RXR (Figure 3A). Other potential ligands, including steroids, retroretinoids, eicosanoids, and bile acids, had no effect. JH III appears to be specific for the FXR–RXRα complex, since it failed to activate EcR–usp, RXR, T_3R, or RAR (Figure 3B).

Although JH III activates FXR–RXR, it fails to activate either FXR or RXR alone (Figures 3A and 3B). This is reminiscent of the Drosophila EcR, which requires RXR/usp for transcriptional activity (Yao et al., 1992, 1993; Thomas et al., 1993). EcR itself binds ecdysteroids with low affinity (Yao et al., 1993); high affinity binding and

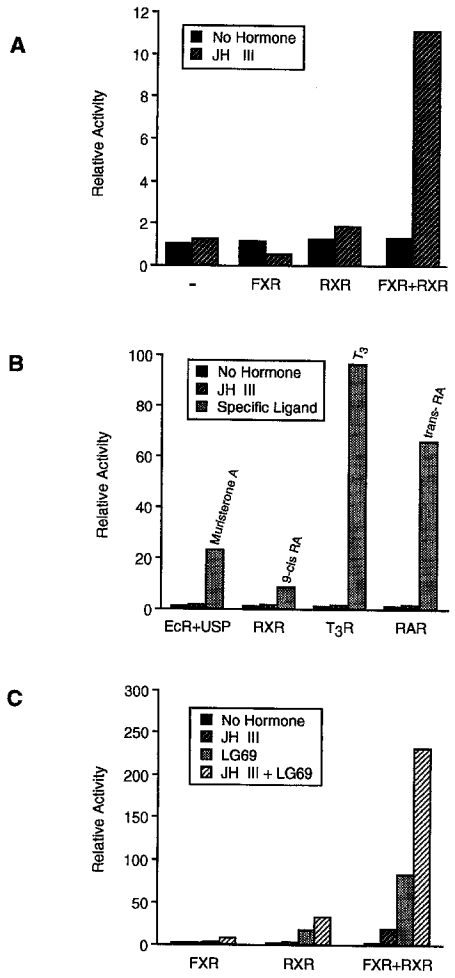

Figure 3. Hormonally Controlled Activity of FXR–RXR

(A) JH III activates FXR–RXR heterodimers. CV-1 cells were transiently transfected with hsp27–EcRE × 5 ΔMTV–luciferase (1000 ng per 10^5 cells) alone (minus sign) or with expression vectors for rat FXR, human RXRα, or both (50 ng per 10^5 cells). Reporter activity was assayed after treating cells with or without 50 μM JH III (*cis*- 10,11-epoxy-3,7,11-trimethyl-*trans-trans*-2,6-dodecadienoic acid methyl ester). JH III failed to activate FXR–RXR complexes using the parental ΔMTV reporter construct that lacked the EcREs (data not shown).

(B) JH III fails to activate other nuclear receptors. The activity of the following receptor/luciferase reporter pairs was assayed in the presence of 50 μM JH III or the indicated receptor-specific ligand: Drosophila G-EcR–usp/hsp27–EcRE × 5 ΔMTV (1000 ng per 10^5 cells), human RXRα/CRBPII–TK, human T_3Rβ/TREp × 2-TK, and human RARα/DR5 × 2-TK. TK–reporter constructs (300 ng per 10^5 cells) and CMV-driven receptor expression vectors (100 ng per 10^5 cells) were added as indicated.

(C) FXR–RXR is synergistically activated by JH III and LG69. CV-1 cells were transiently transfected as in (A), but treated without or with 50 μM JH III, 100 nM LG69, and JH III plus LG69. Datapoints were normalized for differences in transfection efficiency and plotted as relative activity where the untreated reporter is defined to have an activity of 1 U.

subsequent transcriptional activation require coexpression of EcR with RXR/usp. Thus, while the EcR–RXR/usp heterodimer is the physiologically active complex, the ability to respond to ecdysone is determined by the EcR component of the complex. Since the EcR–RXR hetero-

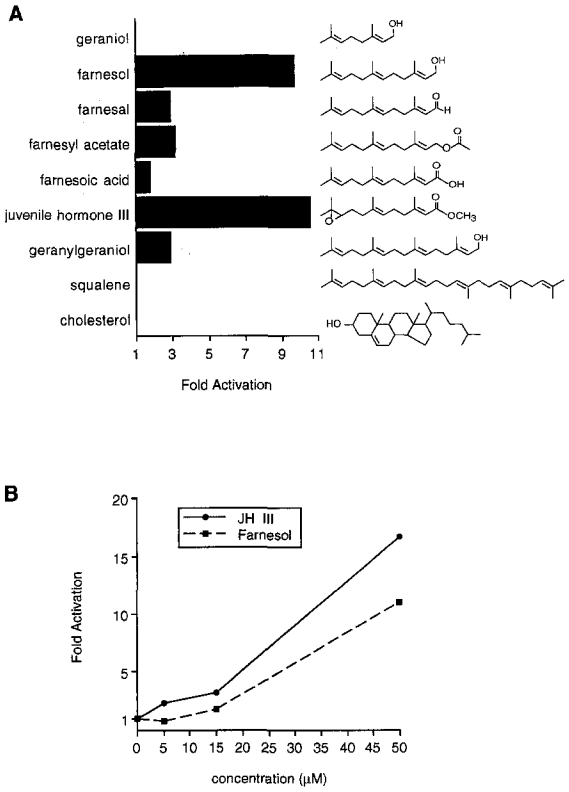

Figure 4. Profiles of FXR–RXR Activity Using Various Isoprenoids

(A) Activation of FXR–RXR by various farnesol metabolites. CV-1 cells were transiently transfected as in Figure 3A with expression vectors for rat FXR and human RXRα. Cells were treated with 50 μM concentrations of each compound. Similar results were obtained with all-*trans* and mixed isomers of farnesol and farnesoic acid. Data are plotted as fold activation relative to untreated cells. Transfections were performed as described in Figure 3. Farnesoic acid was synthesized as described above (Kulkarni et al., 1987).

(B) Dose response profile. The experiment was performed as in (A) with the indicated concentration of JH III and farnesol (mixed isomers).

dimer is composed of two functional receptors, the complex can be activated independently by ecdysteroids or 9-cis RA and can be activated synergistically by both ligands (Thomas et al., 1993). The structural and functional similarities between EcR and FXR prompted us to examine whether the FXR–RXR complex could also be synergistically activated by JH III and the RXR-specific ligand LG69 (Kurokawa et al., 1994). Using the hsp27–EcRE reporter (Figure 3C), we activated the FXR–RXRα complex 17-fold by 50 μM JH III, 76-fold by 100 nM LG69, and 212-fold by the combination of JH III and LG69. This synergistic activity required coexpression of FXR with RXRα (Figure 3C), RXRβ, or RXRγ (data not shown). The ability of JH III to synergize with saturating doses of LG69 or 9-cis RA (data not shown) suggests that these two compounds have distinct targets within the FXR–RXR complex. As LG69 is thought to be an RXR-specific ligand (Kurokawa et al., 1994), these results imply that JH III responsiveness is determined by the FXR component of the FXR–RXR complex.

JH III is a metabolic derivative of farnesyl pyrophosphate, a key precursor in the mevalonate biosynthetic pathway (Goldstein and Brown, 1990). Accordingly, we decided to test whether metabolites derived from the mevalonate pathway in mammalian cells could also serve as activators of the FXR–RXRα complex. Indeed, farnesol (*trans-trans* or mixed isomers, 50 μM) served as a strong activator of FXR–RXRα (Figure 4A), whereas other farnesoids such as farnesal, farnesyl acetate, and geranylgeraniol possessed weaker activity. In contrast, little or no activation was seen with 50 μM concentrations of geraniol, farnesoic acid, squalene (Figure 4A), methoprene, mevalonate, squalene epoxide, squalene dioxide, lanosterol, 24,25-epoxycholesterol, pregnenalone, dehydroepiandrosterone, bile acids, or 10 μM 25-hydroxycholesterol (data not shown). Mevalonate (200 μM) displayed weak activity, provided cells were cotransfected with a mevalonate transporter protein (Kim et al., 1992; data not shown). Since FXR–RXR is not activated by potent JH analogs (methoprene; data not shown) and since the JH receptor has not been identified, it remains unclear what relationship, if any, exists between FXR and the putative insect receptor for JH.

Activation of classical nuclear receptors occurs at physiological concentrations of circulating hormones. To be relevant, activation of FXR should occur at physiologic farnesoid concentrations. The levels of endogenous farnesoids have been difficult to determine, owing to their rapid metabolism and potential sequestration by intracellular and extracellular binding proteins. However, intracellular concentrations may be inferred from the Michaelis constant of enzymes that utilize isoprenoid substrates. These values have been reported to be in the range of 0.5–10 μM for protein farnesyltransferase and isopentenyl pyrophosphate isomerase (Rilling and Chayet, 1985; Reiss et al., 1990; Gomez et al., 1993). Furthermore, down-regulation of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase activity by a mevalonate-derived nonsterol occurs when mevalonate is added to cells at concentrations in excess of 100 μM (Brown and Goldstein, 1980; Nakanishi et al., 1988). Together, these reports suggest that physiologic concentrations of farnesoids are in the micromolar range. Accordingly, dose response studies were performed using increasing concentrations of farnesol and JH III, the two most effective FXR–RXR activators (Figure 4B). Activation of FXR–RXR required concentrations of 5–50 μM, suggesting that FXR–RXR activity is regulated at appropriate concentrations. Similarly, regulation of PPAR and sterol-regulatory element–binding protein 1 (SREBP-1) is known to require 5–100 μM doses of fatty acids (Green and Wahli, 1994) or cholesterol (Wang et al., 1994), levels that are consistent with the presumed intracellular concentrations of these compounds.

Expression of FXR mRNA in Isoprenoidogenic Tissues

One expectation of an intracellular metabolic activator is that it would be synthesized in the same tissues in which its receptor is expressed. Northern blot analysis revealed restricted expression of a 2.3 kb FXR-specific transcript in liver and kidney (Figure 5). In situ hybridization and

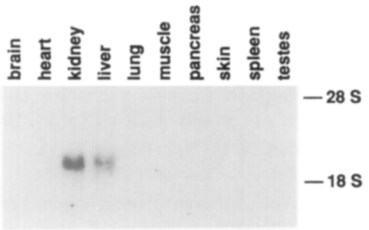

Figure 5. Expression of FXR mRNA

Northern blot analysis of rat tissues. The source of the poly(A)$^+$ RNA is shown above each lane. Muscle refers to skeletal muscle.

histochemistry (Figure 6) indicated that FXR expression is limited to the liver, kidney, and gut of rat embryonic day 19.5 (E19.5) embryo sections (Figure 6A). Background levels were seen in other tissues and in experiments using a control probe (Figure 6B). As expected (Mangelsdorf et al., 1992), mRNA for the heterodimerizing partner RXRβ is also found in the liver, kidney, and gut, as well as other embryonic tissues (Figure 6C). FXR expression in the gut is prominent in the intestinal villi (Figures 6D–6G). In the E19.5 kidney, highest levels of expression occur in the renal tubules (Figures 6D–6G). In the adult rat kidney, FXR is expressed in areas rich in renal tubules: the medullary rays and medullary stripe (Figure 6H). FXR expression is also detected in the adrenal cortex of the adult mouse (Figure 6I). Thus, FXR expression is restricted to the liver, gut, adrenal gland, and kidney: tissues known to have significant flux through the mevalonate pathway (Edmond et al., 1976; Righetti et al., 1976; Wiley et al., 1977).

Metabolic Intermediates as Transcriptional Activators

Our results identify an orphan nuclear receptor that is activated by farnesol derivatives. A question that remains unanswered is the mechanism by which farnesoids activate FXR. One possibility is that farnesoid treatment results in the prenylation of FXR. This seems unlikely, since FXR lacks a known prenylation sequence (Moores et al., 1991; Khosravi-Far et al., 1992) and is not prenylated by an in vitro prenyltransferase system (Khosravi-Far et al., 1992; data not shown). Alternatively, JH III or farnesol (or both) could potentially serve as ligands for FXR. To date, we have not been able to demonstrate specific binding of JH III or farnesol (or both) to the FXR–RXR complex. Thus, a more likely possibility is that a farnesoid, farnesoid metabolite, or farnesoid-induced metabolite may serve as an authentic ligand for FXR. This is conceptually similar to the activation of RXR by all-*trans* RA and ultimately leads to the identification of retinoid X as 9-cis RA (Mangelsdorf et al., 1990; Heyman et al., 1992; Levin et al., 1992).

The demonstration that FXR–RXR is regulated by farnesoid derivatives provides an example of gene regulation by intracellular metabolites. Other examples include the PPAR, a fatty acid-activated orphan receptor (Gottlicher et al., 1992) that regulates genes involved in fatty acid metabolism (Green and Wahli, 1994), and adipocyte differentiation (Tontonoz et al., 1994). Similarly, the low density

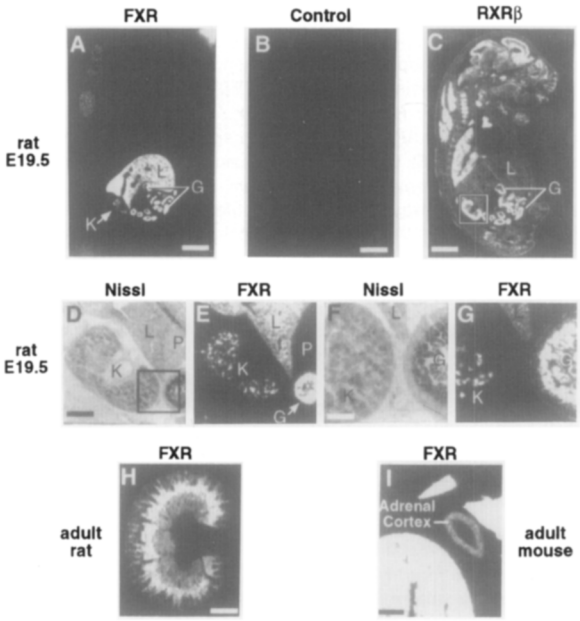

Figure 6. In Situ Hybridization and Histochemistry

Antisense cRNA probes from the truncated mouse FXR cDNA or full-length mouse RXRβ cDNA were used as indicated. Control represents a truncated rat glucocorticoid receptor sense cRNA probe. (A)–(C) represent serial sagittal sections from an E19.5 rat embryo. Areas of positive hybridization appear white. (D) and (E), respectively, are bright (Nissl) and dark-field photomicrographs of the boxed region in (C), hybridized with a FXR antisense probe. Similarly, (F) and (G) are derived from the boxed region in (D). (H) is a section from an adult rat (9-week old) kidney. The control probe revealed near-background hybridization (data not shown). (I) contains adult mouse (8-week old) adrenal gland and adjacent renal cortex and liver. Abbreviations: L, liver; G, gut; K, kidney; P, pancreas. Scale bars: (A–C) 4 mm; (D–E) 1 mm; (F–G) 0.3 mm; (H–I) 2 mm. Sections were apposed to Kodak X-OMAT film for 10 days (A–C and H–J) and then coated with nuclear emulsion (D–G) and exposed for 16 weeks.

lipoprotein receptor gene regulator SREBP-1 is maintained in an inactive form by cholesterol (Wang et al., 1994). As with PPAR and SREBP-1, the FXR ligand, as well as FXR target genes, remains to be identified. Nonetheless, these regulatory systems define a novel paradigm of metabolite-controlled intracellular signaling in vertebrates (O'Malley, 1989). Such signaling provides a means to regulate responses to intracellular metabolites in a cell-autonomous fashion. By transducing metabolic cues into genomic responses, FXR, PPAR, and SREBP-1 may provide examples for the metabolic code proposed by Tomkins (1975).

Experimental Procedures

cDNA Isolation

A degenerate 29-mer consensus oligonucleotide (5′-ACC TGT GAG GGC TGC AAR GKY TTC TTC AA 3′-) corresponding to the highly conserved P box/DNA recognition helix (TCEGCK(G/V)FF) of the nuclear receptor superfamily DBD was used to probe a λgt11 mouse hepatoma Hepa-1c1c7 cDNA library under low stringency conditions (Issemann and Green, 1990). An incomplete 850 bp mouse FXR cDNA clone was obtained. This clone was subsequently used to screen a regenerating rat liver cDNA library. The *FXRα1* clone was obtained from this screen and sequenced by the dideoxy sequencing method.

Cell Culture and Transfection

CV-1 cells were grown in DMEM supplemented with 10% AG1-X8 resin–charcoal–stripped calf bovine serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate (DMEM–CBS) at 37°C in 5% CO_2. Cells were plated to 50%–80% confluence 1 day prior to transfection using phenol red–free DMEM with 10% resin-charcoal–stripped fetal bovine serum (DMEM-FBS). Cells were transfected by lipofection using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-ammonium methyl sulfate (DOTAP) according to the instructions of the manufacturer (Boehringer Mannheim). Reporter constructs (300–1000 ng per 10^5 cells), cytomegalovirus (CMV)-driven receptor (50–100 ng per 10^5 cells), and β-galactosidase expression vectors (500 ng per 10^5 cells) were added as indicated. After 2 hr, the liposomes were removed, and cells were treated for 40 hr with phenol red–free DMEM-FBS containing the indicated compounds: 50 μM JH III, 100 nM muristerone A, 100 nM 9-cis RA, 100 nM T_3 (L-triiodothyronine), 1 μM *trans* RA (all-*trans* RA), or 100 nM LG69 (4-{1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-napthyl)-1-ethenyl}benzoic acid). Cells were harvested and assayed for luciferase and β-galactosidase activity. All points were performed in triplicate and varied by less than 10%. Experiments were repeated three or more times with similar results.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed using proteins translated in a rabbit reticulocyte lysate system (TNT; Promega). Proteins (1 μl) were incubated for 20 min at room temperature with 100,000 cpm of Klenow-labeled probes in 10 mM Tris (pH 8.0), 100 mM KCl, 6% glycerol, 0.05% NP-40, 1 mM DTT, 100 ng/μl poly(dI·dC) and then electrophoresed through a 5% polyacrylamide gel in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA).

RNA Expression Studies

For Northern blot analysis, poly(A)$^+$ RNA (10 μg) from the indicated rat tissues was electrophoresed through a 1% agarose gel under denaturing conditions and transferred to a filter. The filter was hybridized to the mouse FXR-truncated cDNA that was ^{32}P-labeled by the random primer method (Mangelsdorf et al., 1992) (5 × 10^8 cpm/μg). This probe corresponds to rat FXR sequences spanning amino acids 1–297, which encode the amino terminus, the DBD, and a portion of the LBD of FXR. Hybridization was performed overnight at 65°C in 500 mM sodium phosphate (dibasic:monobasic, 7:3), 1 mM ethylenediaminetetracetic acid, 1% bovine serum albumin, and 7% SDS. The filter was washed twice in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) at room temperature and twice in 1× SSC at 55°C and then autoradiographed with an intensifying screen at −70°C for 5 days. In situ hybridizations were performed as described elsewhere (Bradley et al., 1994).

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