Multiple mechanisms of ontogenic regulation of nuclear receptors during rat liver development

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Balasubramaniyan, N., Mohammad Shahid, Frederick J. Suchy, and M. Ananthanarayanan. Multiple mechanisms of ontogenic regulation of nuclear receptors during rat liver development. Am J Physiol Gastrointest Liver Physiol 288: G251–G260, 2005. Published September 23, 2004—Nuclear receptors (NRs) play pivotal roles in the regulation of genes contributing to hepatobiliary cholesterol and bile acid homeostasis. We have previously shown that transporters involved in bile formation are developmentally regulated and are poorly developed during the fetal stage, but their expression reached gradual maturity during the postnatal period. To define the molecular mechanisms underlying this regulation and the role that class II NRs and associated members [liver receptor homolog-1 (LRH-1) and short heterodimer partner (SHP)] play, we have analyzed the ontogeny of NR expression during rat liver development. Real-time PCR analysis of hepatic NR expression from fetal day 17 through adult revealed that steady-state mRNA levels for all NRs were very low during the embryonic period. However, mRNA levels peaked close to that of adult rats (≥6 wk-old rats) by 4 wk of age for farnesoid X receptor (FXR), pregnane X receptor (PXR), liver X receptor-α (LXRα), peroxisome proliferator-activated receptor-α (PPARα), retinoid acid receptor-α (RARα), LRH-1, and SHP, whereas RXRα mRNA levels lagged behind. FXR, PXR, LXRα, RARα, and PPARα functional activity in liver nuclear extracts assayed by gel EMSA demonstrated that the activity attained adult levels by 4 wk of age, exhibiting a strict correlation with mRNA levels. Surprisingly, PPARα activity was delayed as seen by EMSA assay. Protein levels for NRs also corresponded to the mRNA and functional activity except for RXRα. RXRα protein levels were higher than message levels, suggesting increased protein stability. We conclude that expression of NRs during rat liver development is primarily regulated by transcriptional mechanisms, which in turn, control the regulation of bile acid and cholesterol metabolic pathways.

Ontogeny; bile acids; cholesterol

Nuclear hormone receptors (NRs) are a group of transcription factors that control transcription of various genes after stimulation by hormones or other small lipophilic and xenobiotic compounds (24). Class II NRs comprise transcription factors that heterodimerize with a common partner retinoid X receptor (RXR; NR2B1) and bind to cis elements in the DNA that contain a dyad of six base pairs in direct, inverted or everted configuration separated by 0–8 bases (14). Recent investigations over the last 5 yr have revealed the important role that class II NRs and associated family members, such as liver receptor homolog-1 (LRH-1; also known as FTF and CPF) and short heterodimer partner (SHP), play in cholesterol and bile acid metabolism and transport (5). Among the class II NRs are “orphan receptors,” which, until recently, did not have physiological ligands but have been “adopted” after the identification of ligands that bind and activate them. Farnesoid X receptor (FXR; NR1H4) is a bile acid receptor that activates the transcription of ileal bile acid binding protein (IBABP), bile salt export pump (BSEP, ABCB11), and SHP, another member of the NR family (NR0B2), while inhibiting the transcription of cholesterol 7α-hydroxylase (CYP7A1) and sodium taurocholate cotransporting polypeptide (NTCP; also known as SLC10A1) (1, 4, 7, 10, 25). Further studies have shown that the repressive effect of FXR is predominantly mediated through its activation of SHP, although SHP-independent mechanisms may also play a role in certain situations (10). Pregnan X receptor/steroid xenobiotic receptor (humans) (PXR/SXR; NR1I2) and constitutively active retinoid receptor (CAR; NR1I3) are critical for the metabolism and transport of most xenobiotic compounds, including exogenously administered drugs, by influencing transcription of CYP3A4 (in humans) and MDRI genes (18). In addition, CAR has recently been shown (12) to mediate induction of genes involved in bilirubin and xenobiotic clearance such as UDP-glucuronosyl transferase (UGT1A1), multidrug resistance-associated protein 2 (MRP2), organic anion transporter Oatp2 (Slc21a6), GSTA1, and GSTA2. Peroxisome proliferator-activated receptor (PPAR)γ (NR1C1, NR1C3) influences a variety of enzymes involved in fatty acid catabolism and adipocyte differentiation, respectively, and agonists of PPARγ (thiazolidine diones) are currently being used in the treatment of type II diabetes mellitus (20). LRH-1/CPF/FTF (NR5A2) is an NR family member implicated in promoting liver X receptor-α (LXRα; NR1H3)-mediated activation of rodent Cyp7a1 (10), the rate-limiting step in bile acid biosynthesis. Retinoid acid receptor-α (RARα; NR1B1) binds to its cis element consisting of a DR-2 sequence and is shown to bind and activate Ntcp and Mrp2 promoters (7).

Bile acid secretion is a critical function of liver and is the combined result of bile acid synthesis from cholesterol and transport into bile mediated by bile acid transporter proteins (32). Bile acid pool size in liver is maintained under physiological levels by concerted regulation of bile acid uptake (Ntcp) and excretion [bile salt export pump (Bsep)] and a regulated synthesis from cholesterol. In addition, bile formation is an ontogenetically regulated process, which increases during postnatal life with the development and differentiation of hepatic tissue (2). Transport systems that contribute to bile formation are regulated by transcriptional as well as posttranscriptional mechanisms (11, 31). In view of the recent develop-
opments on the role of NRs in cholesterol and bile acid homeostasis and the lack of information of ontogenic regulation of NRs during liver development, we have, for the first time, carried out a detailed study of NRs in the rat at the fetal, postnatal, and adult levels. We show, on the basis of our data in this paper, that both transcriptional and posttranscriptional mechanisms are involved in the ontogenic regulation of NRs during liver development and may contribute significantly to the development of transporters involved in bile secretion.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats (200–250 g) and timed pregnant (embryonic days E17 to E21) dams or pups with dams were obtained from Taconic Farms (Germantown, NY) and reared in the animal facility of Mount Sinai School of Medicine. The animals were treated humanely according to criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by National Institutes of Health (NIH publication 86–23, revised 1985). Animal studies were conducted according to a protocol approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Animals were housed under constant temperature and humidity conditions with a 12:12-h light-dark cycle, had free access to water, and were fed rodent laboratory chow. Pups were raised by the mothers and suckled until weaning at postnatal day 21. For fetal stages (days E17, E18, E19, E20, and E21), postnatal ages (days P1, P7, P14, P21, and P28) and adult stages (defined as >6-wk-old males), livers were obtained after death, flash frozen in liquid nitrogen, and stored at −70°C until use. Data shown are the means ± SE of pools of liver tissue from three independent groups. To acquire fetal and postnatal tissues, pups from different dams were mixed to form separate pools without separation of males and females. All animals were killed after anesthetization with injection of a cocktail of ketamine and xylazine using standard dosage.

Chemicals, Isotopes, and Antibodies

Molecular biological reagents were obtained from Invitrogen (San Diego, CA) or New England Biolabs (Beverly, MA). [α-32P]dCTP (3,000 Ci/mmole) and [γ-32P]ATP (3,000 Ci/mmole) were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA). Random Primed Labeling Kit was from Boehringer-Mannheim (Indianapolis, IN). Bradford protein assay was purchased from Bio-Rad (Hermes Primed Labeling Kit was from Boehringer-Mannheim (Indianapolis, IN). Bradford protein assay was purchased from Bio-Rad (Herndon, VA). Nuclear Receptor Direction Sequence, 5'-ATGCTGAGGGAGATCCTCAGTGT-3' was reverse transcribed by using Superscript II (GIBCO-BRL) following manufacturer’s directions. The reaction mixture was diluted 50-fold, and 2 μl of the diluted product was used for PCR. The forward and reverse primers for the various NRs were chosen by using Primer Express software from PE Biosystems (Norwalk, CT) to yield an amplicon size of 150 bp and are listed in Table 1. The PCR cycles were carried on an ABI PRISM 7900HT Sequence Detection System from ABI Applied Biosystems (Foster City, CA) consisting of the following cycling parameters: 1) 1 cycle for 15 min at 95°C; 2) 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. After each reaction, the products were run on a 2% agarose gel to ensure that a single product of the correct size was obtained. Data were analyzed by using ABI SDS software version 2.1 to obtain critical (Ct) values. In all experiments, PCR reactions were also run for GAPDH, and the GAPDH Ct value for each sample was subtracted from target (NR) Ct values for normalization (Ct_Target − Ct_GAPDH). Change in expression during development was calculated as fold change over the day E17 (set to unity) level using the formula 2^[-ΔCt(sample) − ΔCt(target)].

Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Nuclear Receptor</th>
<th>Direction</th>
<th>Sequence, 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farnesoid x receptor, NR1H4</td>
<td>Forward</td>
<td>AGGCCATGTTCTGCGTCTCA</td>
</tr>
<tr>
<td>Liver X receptor, NR1H3</td>
<td>Reverse</td>
<td>TTCAGCTCCTGGGAAT</td>
</tr>
<tr>
<td>Liver receptor homolog-1 (LRH-1/FTF/CFP), NR5A2</td>
<td>Forward</td>
<td>GCTGCGCTGTGATCGATAC</td>
</tr>
<tr>
<td>Peroxisome-proliferator-activated receptor, NR1C1</td>
<td>Forward</td>
<td>GAGCCTTGATGTCGTCGACG</td>
</tr>
<tr>
<td>Pregnane X receptor, NR1H2</td>
<td>Forward</td>
<td>GGATGAGCAAGGGAGCTGTC</td>
</tr>
<tr>
<td>Retinoic acid receptor, NR1B1</td>
<td>Reverse</td>
<td>AGAGAGACGTGGTGGAGAG</td>
</tr>
<tr>
<td>Retinoid X receptor, NR2B1</td>
<td>Reverse</td>
<td>CTTTCCTCCAGGCCTCATCA</td>
</tr>
<tr>
<td>Short heterodimer partner, NR0B2</td>
<td>Forward</td>
<td>GTTGCGAGAGAAGGAAAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Reverse</td>
<td>GAGGTTGCGAGAGAAGGATA</td>
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Real-Time PCR Analysis

Real-time PCR was carried out to quantitate the expression of NRs messages during rat liver development using Brilliant SYBR Green QPCR kit from Qiagen (Valencia, CA). In brief, 2 μl of total RNA was reverse transcribed by using Supercript II (GIBCO-BRL) following manufacturer’s directions. The reaction mixture was diluted 50-fold, and 2 μl of the diluted product was used for PCR. The forward and reverse primers for the various NRs were chosen by using Primer Express software from PE Biosystems (Norwalk, CT) to yield an amplicon size of 150 bp and are listed in Table 1. The PCR cycles were carried on an ABI PRISM 7900HT Sequence Detection System from ABI Applied Biosystems (Foster City, CA) consisting of the following cycling parameters: 1) 1 cycle for 15 min at 95°C; 2) 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. After each reaction, the products were run on a 2% agarose gel to ensure that a single product of the correct size was obtained. Data were analyzed by using ABI SDS software version 2.1 to obtain critical (Ct) values. In all experiments, PCR reactions were also run for GAPDH, and the GAPDH Ct value for each sample was subtracted from target (NR) Ct values for normalization (Ct_Target − Ct_GAPDH). Change in expression during development was calculated as fold change over the day E17 (set to unity) level using the formula 2^[-ΔCt(sample) − ΔCt(target)].

Northern Blot Analysis

Total RNA was obtained by using TRizol reagent (GIBCO-BRL, Manassas, VA) according to manufacturer’s directions. PolyA+ RNA from total RNA was prepared by using PolyA Tract Kit IV (Promega, Madison, WI). PolyA+ RNA (5 μg) was fractionated on 1% formaldehyde-agarose gels and blotted overnight to nylon membranes (GeneScreen; Amersham Biosciences, Piscataway, NJ) in 10× SSC by capillary transfer followed by baking in a vacuum oven at 80°C for 2 h. Blots were prehydrized for 3 h followed by hybridization in a buffer of 50% formamide, 10% dextran sulfate, 1 M NaCl, and 1% SDS overnight at 42°C with 10⁶ counts·min⁻¹·ml⁻¹ (labeled by using a random priming kit and [α-32P]dCTP to a specific activity of 10⁹ counts·min⁻¹·μg⁻¹) of the cDNA probe for the respective NRs. Probes consisted of full-length cDNAs released by digestion with appropriate restriction enzymes to render the following size probes: rFXR, 1.4 kb; hRARα, 1.9 kb; mRXRα, 4.8 kb; hLXRx, 1.9 kb; mPIR, 1.3 kb; hPPARα and mSHP, 0.8 kb; and hLXRα, 1.1–1.7 kb. Blots were washed the next day as follows: 1) 2× SSC at room temperature for 5 min; twice; 2) 2× SSC, 0.5% SDS at 60°C for 30 min; and 3) 0.1× SSC at room temperature for 30 min. Blots were then exposed to X-ray films at a sensitivity of <1 for 1–2 days and developed in a Konica SRS-101A film developer.
Western Blot Analysis

To assess the functional capacity of the NRs, EMSA assays were conducted by using rat liver nuclear extracts prepared from selected developmental stages as previously described by us (1) using the consensus cis elements shown in Table 2. In brief, 20 μg of nuclear protein was incubated with the labeled probe (5 x 10⁶ counts/min) in a total volume of 20 μl binding buffer [in mM: 12 HEPES (pH 6.9), 60 KCl, 4 Tris-HCl, 1 EDTA, 1 dithiothreitol, 1 μg poly(dI-dC), plus 5% glycerol and 1 μg of salmon sperm DNA] on ice for 45 min. In competition assays, 50-fold excess of wild-type or mutant cold probes were added to the reaction mix 15 min before the addition of probe. DNA-protein complexes were fractionated by 4% native PAGE in TBE. The gel was dried and exposed to X-ray films for 10 days. The retardation bands were detected by using an enhanced chemiluminescence kit (Amersham) and quantitated in a Bio-Rad GS-800 densitometer and using Bio-Rad Quantity One software.

Statistical Analysis

All data are presented as means ± SE from three independent experiments. Student’s t-test was used to calculate statistical significance, and a P value of <0.05 was considered significant.

Table 2. Oligonucleotides used for EMSA

<table>
<thead>
<tr>
<th>Nuclear Receptor, cis element type</th>
<th>Type</th>
<th>Sequence, 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farnesoid x receptor, IR-1</td>
<td>Wild type</td>
<td>GAGATTGGGATTCATTTGTCAGTATGAGAA</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>GAGATTTCAACTCTGTCAGTATGAGAA</td>
</tr>
<tr>
<td>Liver X receptor, DR-4</td>
<td>Wild type</td>
<td>CAGGATTGGGATTCATTTGTCAGTATGAGAA</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>CAGGATTTCAACTCTGTCAGTATGAGAA</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor, DR-5</td>
<td>Wild type</td>
<td>GAGGATTTCAACTCTGTCAGTATGAGAA</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>GAGATTTCAACTCTGTCAGTATGAGAA</td>
</tr>
</tbody>
</table>

Only the sense strand sequence is shown for clarity. Mutated bases are indicated in lower case. Recognition sequence for the individual nuclear receptor is indicated by underlined bases.
Fig. 1. Real-time PCR quantitative analysis of nuclear receptors (NRs) during rat liver development. Liver tissues were collected from rats of embryonic days 17-21 (days E17-E21) and postnatal days 1, 7, 14, 21, and 28 (days P1, P7, P14, P21, P28) and adult males and females as described in MATERIALS AND METHODS. Total RNA was reverse transcribed and an aliquot of the product was used in real-time PCR reactions using Brilliant SYBR Green QPCR kit on an ABI Prism 7900HT Sequence Detection System machine. Primers used for the different NRs are listed in Table 1. Cycling parameters for the PCR are as described in MATERIALS AND METHODS. Fold changes in mRNA were calculated by the comparative method as described in MATERIALS AND METHODS using GAPDH levels for normalization. Data are shown as percentage of adult values for each NR obtained from 3 independent samples of RNA and are means ± SE. *P < 0.05 compared with expression level at age E17. A: FXR, farnesoid X receptor; B: RXRα, retinoid X receptor-α; C: LXRα, liver X receptor-α; D: PXR, pregnane X receptor; E: PPARα, peroxisome proliferator-activated receptor-α; F: RARα, retinoid acid receptor-α; G: SHP, short heterodimer partner; H: LRH-1, liver receptor homolog-1.
Northern blot analysis of SHP mRNA is low throughout the embryonic period (days E17–E20) remaining at 0.25 to 0.35% of the adult period. On day E21, mRNA was 8.3% but increased and was maintained at ~60% of adult values between days P1 and P14. The amount of mRNA reached adult levels by day P21 (Fig. 1G).

Northern Blot Analysis of NRs mRNA During Rat Liver Development

Although real-time PCR is an extremely sensitive technique, it does not give information regarding the mRNA size and information on the regulation of multiple transcripts (where it exists) for a single gene. Therefore, we prepared PolyA⁺RNA from day E20 through adult age (because of the need to collect a large number of liver tissue from day E17–E19 livers for preparation of mRNA, these ages were not studied), which were run on formaldehyde-agarose followed by hybridization with the individual NR cDNA probes. A representative Northern blot analysis is shown in Fig. 2. Quantitative analysis of the Northern blots using a Phosphorimager (model Storm 860) with ImageQuant software (Molecular Dynamics, Sunnyvale, CA) revealed that the relative expression levels of various NRs at the indicated ages highly correlated with those obtained by using real-time PCR, suggesting concordance between these techniques (data not shown). Two transcripts are observed for PXR and RARα consistent with previous reports in the literature. In confirmation of the data obtained by using real-time PCR analysis, mRNA levels for all NRs were low during the fetal stages followed by a rise to adult levels at around day P28. GAPDH mRNA, used as a control for mRNA loading, did not show any change during liver development. Thus our data on real-time PCR were verified by Northern blot analysis. There is little information on the functional role of the dual transcripts in the case of PXR and RARα and some controversy in the case of FXR. Therefore, no attempt was made to clarify the roles of these different-sized transcripts for these NRs during liver development.

**Functional Assessment of FXR, LXRα, PXR, RARα, and PPARα (Hetero-Dimerization Partners for RXR) in the Developing Rat Liver by EMSA Analysis**

NRs are synthesized in the cytoplasm and imported into the nucleus where they bind to the cis elements of target genes and activate their transcription. In the case of class II NRs (those that form heterodimers with RXR), it has been shown that they are constitutively bound to their cis elements. In the absence of ligands, the target gene is in a repressed state and ligand binding results in release of corepressors and recruitment of coactivators leading to gene activation. To determine the functional activity of these NRs during liver development, we prepared nuclear extracts from days E20, P7, and P28, and adult livers and subjected them to EMSA using the consensus recognition sequences listed in Table 2. To verify that authentic consensus element-NR complex was obtained in the assays with nuclear extracts, EMSA was also performed using pure proteins obtained by in vitro translation of the NR-encoding cDNAs (data not shown). Quantitative analysis of the retarded complex using a densitometer was carried out to estimate the relative functional activity of these receptors, and the results are shown in Fig. 3. EMSA data correlated well in most cases with mRNA measured by Northern blot analysis and real-time PCR analysis.

**FXR**. The complex formed by binding of FXR to its IR-1 element was 32% of the adult amount on day E20 and reached adult levels by day P28. Comparison of the degree of FXR binding in the EMSA to the amount of its mRNA demonstrates more activity than can be accounted for by the level of mRNA, implying additional levels of posttranscriptional control. However, in the postnatal period, the temporal pattern of the EMSA compares well with mRNA levels (full activity at day P28), indicating transcriptional regulation as the major mechanism of control (Fig. 3Aa).

**LXRα**. LXRα binding to its DNA response element (DR-4) as measured by EMSA was only at 3.5% of adult at day E20 rising to 39 and 83.7% on days P7 and P28, respectively. At day P28, the degree of amount of complex bound was similar to the adult (Fig. 3Ab).

**PXR**. The amount of PXR protein bound to its DR-3 element in the EMSA activity on day E20 was greater than that predicted from the abundance of mRNA at this stage. Thus, although the message levels were at 5.7% at day E20, there was
36.7% of adult consensus element-binding capacity at this age (Fig. 3A,c). Thus these data also suggest additional levels of control of PXR regulation including potential posttranscriptional mechanisms.

**RARα.** EMSA analysis reflecting RARα binding to a DR2 consensus element showed a low amount of binding consistent with the mRNA levels at this stage, exhibiting a 1:1 correlation (Fig. 3A,d). Thus there was 10.1% mRNA and 11% functional activity at day E20. EMSA and mRNA data correlated well at later time points.

**PPARα.** PPARα binding to its response element (DR-1) as shown by EMSA analysis lagged behind the mRNA levels.
Thus on day P28, mRNA was 103%, whereas the EMSA binding was only at 40% of the adult (Fig. 3A). These data may indicate poor translation of the available message and/or reduced import into the nucleus, resulting in reduced availability for binding to cognate cis elements on its target gene promoters.

Figure 3B shows a representative EMSA image for five NRs: FXR, LXRα, PXR, RARα, and PPARα that form heterodimers with RXR and bind to their recognition cis elements on the target genes. Specificity of receptor binding is also shown by its abrogation by incubation with a 50-fold excess wild-type sequence-containing DNA (Fig. 3B, WT lane), whereas a mutant sequence (Fig. 3B, Mut lane) fails to inhibit the formation of the complex.

Western Analysis of Protein Mass of NRs During Liver Development

Although the functional activity of NRs was ascertained as illustrated in Fig. 3, protein mass and the relative amount of NRs was examined by using commercially available antibodies by Western blot analysis of nuclear extracts obtained at different stages of liver development. Quantitative estimation of the protein levels by densitometric scanning is shown in Fig. 4 and a representative blot for each of the receptors analyzed is shown in Fig. 5. Due to the nonavailability of high affinity, good quality antibodies to all the receptors studied by RNA analysis, we limited our studies to those receptors for which we were able to obtain specific bands of the correct protein mass with the antibodies. As a loading control, we used an antibody to the constitutively expressed nuclear protein Histone H1, which is shown in Fig. 5.

FXR. The amount of FXR protein was at 16.8% of adult values on days E20 and P7. However, because FXR mRNA was at 100% of adult level on day P7, posttranscriptional regulatory mechanisms are likely involved in determining FXR expression in the postnatal period. At 4 wk of age, protein levels rose to 75.2% of the adult.

RARα. There was also significant disparity between mRNA and protein levels with regard to RARα in the developing liver. Whereas the mRNA levels remained at 32.8 to 69.9% at ages P7–P28 days, protein levels were at 113.6 and 96.5% at days P7 and P28, respectively. Because RARα heterodimerizes with all of the type II NRs, we speculate that its availability, possibly enhanced by a long half-life, is critical in not limiting the activity of these receptors in the postnatal period.

PXR. Nuclear protein levels for PXR on day E20 were at 42.3% of adult, whereas the PXR mRNA was at only 5.7% of adult value exhibiting a pattern similar to that of RXRα. However, during the postnatal period, there was a gradual increase in the protein amounts similar to the trend seen with PXR message levels.

PPARα. PPARα protein levels in developing rat liver nuclei were slightly higher than their corresponding message at day E20 (22.2% mRNA and 32.8% of adult protein). On day P28, the trend was reversed with higher mRNA (103.4%) and lower protein (60.9%). Once again, these data are reflective of transcriptional and posttranscriptional levels of control.

SHP. Despite the extremely low levels for SHP mRNA during fetal stages days E17–E20 (0.25–0.35% of adult), we observed 8.4% adult SHP protein at day E20. However, during the postnatal period, there was good correlation between the mRNA and protein levels for this potent repressor of cyp7a1 and Ntcp.

Because the available antibodies to LXRα did not examine LXRα protein levels.

DISCUSSION

Bile formation (both bile acid-dependent and bile acid-independent) is an ontogenically regulated process in that humans and rats exhibit decreased bile flow during the fetal and neonatal life, which slowly increases to the adult rates of flow and secretion of various solutes during liver development (30). In our previous work, we have systematically analyzed the regulation of basolateral and canalicular bile acid and organic anion transporters (Ntcp, Bsep, and Mrp2) during rat liver development (11, 31). On the basis of our data, we have hypothesized that expression of these transporter genes are regulated in proportion to the bile flow rate, thus providing a molecular mechanism for regulation of bile formation (2).

During the past 5 yr, the role of NRs in regulation of bile acid and cholesterol homeostasis has come to light on the basis of the work from a number of laboratories including our own (14). Class II NRs form a heterodimer complex with RXR and bind to cis elements of cognate genes that consist of AGGTCA (or slight variations thereof) arranged in tandem as a direct, inverted, or everted fashion with spacer nucleotides ranging from 0–8. Studies with FXR- and PXR-null mice have further supported their critical role in vivo (26, 29). We have speculated that expressions of class II NRs might also be developmentally regulated, providing a mechanism coordinating path-
ways for biosynthesis and transport. There is very little information on the development of hepatic NR. Recently, Huang et al. (12) examined expression of CAR mRNA during development in mouse and human livers. The authors showed that in mouse liver, expression of CAR message was significantly lower between 0.5 and 6.5 days after birth, reaching near adult values at 2 wk of age. CAR expression was also found to be lower in neonatal human livers compared with adult. To prove our hypothesis, in this study we have examined mRNA, functional protein, and total protein mass of several type II NRs (FXR, RXRα, LXRα, PXR, RARα, PPARα) and associated family members (SHP and LRH-1) in rat liver during development from age E17 days to the adult.

FXR, which acts as a bile acid receptor, undergoes significant ontogenic change with mRNA levels very low in the fetus but increasing to near adult levels by day P7 (Figs. 1 and 2). However, FXR protein content of nuclear extracts remains low, ~16% of the adult, even as EMSA shows DNA binding of FXR in excess of 60% of the adult. Thus transcriptional as well as posttranscriptional mechanisms appear to control FXR expression (Figs. 3 and 4). RXRα is the heterodimeric partner for FXR whose mRNA is significantly delayed during development (Figs. 1 and 2). However, RXRα protein levels are much higher than that of its mRNA, suggesting that, in this case, the stability of RXRα protein might control its activity and availability to heterodimerize with FXR and other class II NR (Fig. 5).

Complex changes in the ontogeny of FXR and RXRα are likely to be critically important to the expression of several target genes involved in bile acid homeostasis. Thus the near-adult expression of FXR at day P7 is consistent with 90% expression of Bsep mRNA, which is activated by FXR, as observed by us (31) and confirmed by Gao et al. (8) (123% of adult at day P5) using real-time PCR, as well as the decreased expression of cyp7a1 (23) at this stage of liver development. However, it cannot explain the adult-level expression of Ntcp mRNA at day P7 (11), suggesting the participation of other mechanisms in its regulation.

SHP is a transcriptional suppressor that has been shown to suppress Ntcp transcription by preventing RARα/RXRα binding to Ntcp promoter (6). SHP mRNA is <1% of the adult until
FXR. However, functional activity of LXR4th wk after birth, allowing potentially unimpeded transcription during the postnatal period, reaching a maximum during the postnatal period, it rises gradually to adult values by 4 wk. 

Protein mass for SHP (Fig. 5) seems to follow the mRNA postnatal period it rises gradually to adult values by 4 wk. 

Fig. 5. Western blot analysis of protein masses for NRs in rat liver nuclear extracts during development. Nuclear extracts prepared from livers of defined developmental stages were run on 10% SDS-PAGE and probed with commercial antibodies to NRs after blotting to nitrocellulose membranes. A representative blot for each NR is shown. Molecular masses of each NR in kilodaltons, which were consistent with that reported in the literature, are indicated on the left.

day E20 when it reaches of 8.3% of adult, and during the postnatal period it rises gradually to adult values by 4 wk. Protein mass for SHP (Fig. 5) seems to follow the mRNA expression. On the basis of these data, it is likely that the repressive effect of SHP on Ntcp and Mrp2 is significant only during the postnatal period, reaching a maximum during the 4th wk after birth, allowing potentially unimpeded transcription of Ntcp and Mrp2 during the postnatal period.

LXRα has been shown to activate the expression of rodent cyp7a1, which is further stimulated by LRH-1 (22). mRNA levels for LXRα during the fetal age are 1.5 to 15.6% of the adult (Figs. 1 and 2), which is significantly higher than that for FXR. However, functional activity of LXRα as measured by EMSA (Fig. 3) is at 39.4% of the adult on day P7. These data are consistent with undetectable cyp7a1 mRNA at day E18, a rise in the newborn followed by a decrease at day P4 and reaching adult levels at age P22 days in rat liver as reported by Massimi et al. (23). These values may also explain the need for bile acid supplementation of diet of cyp7a1-null mice during the immediate postnatal period due to immaturity of alternative bile acid synthetic pathways (13).

PXR is considered to be the major nuclear receptor involved in regulation of drug metabolizing enzymes, predominantly CYP3A4, in humans. Previous studies (3, 16, 17, 27) have shown that newborn rats are more sensitive to drug toxicity, especially during the first 4–5 wk after birth. Our data on PXR mRNA levels are consistent with these data because PXR message levels are only 40–60% adult during days P7–P28, suggesting that expression of PXR might influence the liver’s ability to detoxify ingested drugs or xenobiotics. EMSA data show that PXR activity is at 60% of adult by day P7 (Fig. 3).

Because PXR also controls Mrp2 expression (15) and because fetal hepatocytes are also highly sensitive to oxidative damage, decreased expression of Mrp2 during the perinatal period and inability to completely excrete glutathione conjugates of toxic compounds offer a molecular mechanism for this sensitivity.

RARα is involved in regulation of Ntcp and Mrp2 promoters by binding to a DR2 element in the promoters of both genes (6). RARα mRNA levels are higher than those for FXR or RXRα during the fetal period ranging from 10.1 to 24.7% of the adult (days E17–E21). The levels rise to 80% at day P7 but then decrease to ~60% on days P14 and P21. Adult values are attained by day P28. It is likely that this fluctuation in RARα levels as well as the slow increase in its functional activity (Fig. 3) might partly explain the gradual increase in Mrp2 mRNA levels reported by us earlier (31) and confirmed by recent studies of Gao et al. (8). Our previous data (11) showing rat Ntcp mRNA at adult levels by day P7 may be attributed to this postnatal increase in RARα expression.

PPARα plays a major role in fatty acid catabolism and is highly expressed in liver (9). mRNA levels for PPARα are fairly high during the fetal age (17–77% between days E16 and E21, Figs. 1 and 2) and exhibited a surge to 200–300% of adult values between days P7 and P21 before returning to 100% of adult values at day P28. The fall to adult values after weaning corresponds to a change from a high-fat, milk-based diet to laboratory chow. However, despite these high mRNA levels for PPARα, its functional activity was only at 30% of adult values even at day P28, suggestive of posttranscriptional level of control (Fig. 3).

Previous studies involved in cloning and characterization of the NRs showed that all of these receptors are expressed in fetal liver. However, their relative levels compared with adult liver (both mRNA and functional protein in nuclei) and how the levels are temporally regulated during liver maturation has not been examined rigorously in a systematic manner. In view of its relevance to cholesterol and bile acid biosynthetic and transport pathways, we have carried out an analysis of mRNA levels of NRs using the sensitive real-time PCR assay and functional activity using EMSA from day E17 through adult age. These studies revealed that for most receptors, both mRNA and functional activity remained low during the fetal period and rose gradually to adult values by the 4th wk after birth. Thus our data underscore the importance of ontogenic expression of NRs, which may in turn, control the expression of many enzymes and transporters contributing to bile formation in liver. The importance of these developmental changes in NRs to the expression of target genes remains unknown. It is also uncertain whether a critical amount of NR is required for activation. Intuitively, production of 5 to 10% transcription factors compared with the adult should be sufficient, but the dynamics of protein synthesis, import, and recruitment to the nucleosome are not well understood. There might even be competition among the many NRs that form heterodimers with RXRα. The pattern of NR expression, particularly those regulating the genes involved in bile acid homeostasis, mirror closely the expression of cyp7a1, Ntcp, and Bsep in fetal and neonatal liver. However, a focus on NRs alone is likely to be an over-simplification of a very complex process, because transcription requires the formation of multiple complexes of coactivator proteins necessary for chromatin remodeling, recruitment, and activation of RNA polymerase. Developmental changes may also occur in histone acetyltransferase and methyltransferase activities in complexes that are involved in...
ATP-dependent nucleosome remodeling and in recruitment/activation of the basal transcription machinery. For example, we have recently determined by chromatin immunoprecipitation that ligand-dependent activation of the human BSEP locus is associated with a simultaneous increase of FXR and CARM1 (coactivator-associated arginine methyl transferase 1) occupation of the promoter (1a). Very little is known about the ontogeny of these components and whether any could be rate limiting at a particular age. Finally, it is clear that bile acid pool size is lower and bile acid composition differs in the fetus and newborn compared with the adult. The bile acid pool of the developing animal contains unusual bile acids with multiple hydroxylations that are more hydrophilic and possibly hepatoprotective but may be poor ligands for the bile acid receptor FXR.

GRANTS

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