Identification and characterization of two alternatively spliced transcript variants of human liver X receptor alpha

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Abstract The liver X receptor α (LXRα) is a member of the nuclear hormone receptor superfamily that plays an important role in lipid homeostasis. Here we characterize two alternative human LXRα transcripts, designated LXRα2 and LXRα3. All three LXRα isoforms are derived from the same gene via alternative splicing and differential promoter usage. The LXRα2 isoform lacks the first 45 amino acids of LXRα1, and is generated through the use of a novel promoter and first exon. LXRα3 lacks 50 amino acids within the ligand binding domain and is generated through alternative recognition of the 3'-splice site in exon 6. LXRα2 and LXRα3 are expressed at lower levels compared with LXRα1 in most tissues, except that LXRα2 expression is dominant in testis. Both LXRα2 and LXRα3 heterodimerize with the retinoid X receptor and bind to LXR response elements. LXRα2 shows reduced transcriptional activity relative to LXRα1, indicating that the N-terminal domain of LXRα is essential for its full transcriptional activity. LXRα3 is unable to bind ligand and is transcriptionally inactive. These observations outline a previously unrecognized role for the N terminus in LXR function and suggest that the expression of alternative LXRα transcripts in certain biological contexts may impact LXR signaling and lipid metabolism.—Chen, M., S. Beaven, and P. Tontonoz. Identification and characterization of two alternatively spliced transcript variants of human liver X receptor alpha. J. Lipid Res. 2005. 46: 2570–2579.

Supplementary key words nuclear receptor • cholesterol metabolism • transcriptional regulation • RXR

Nuclear hormone receptors are transcription factors that are involved in numerous biological processes, including reproduction, development, and metabolism (1). Most of these receptors are comprised of a ligand-independent transcriptional activation function (AF1) domain at the N terminus, a DNA binding domain (DBD), a hinge region, and a ligand binding domain (LBD). The LBD possesses a dimerization interface, and a ligand-dependent activation function (AF2) region at the carboxyl terminus (2). The transcriptional activity of most nuclear hormone receptors is stimulated by specific small-molecule ligands. Binding of ligand to the LBD results in a conformational change of the receptor, release of corepressors, recruitment of coactivators, and transcriptional activation (3, 4).

The liver X receptors (LXRs) are nuclear hormone receptors that play a key role in the regulation of lipoprotein metabolism (3, 5). LXRs are activated by oxidized derivatives of cholesterol that serve as ligands (7–9). Two different LXRs have been described, LXRα (NR1H3) and LXRβ (NR1H2). LXRα is expressed at high levels in liver, adipose tissue, macrophages, intestine, kidney, and spleen, whereas LXRβ is expressed ubiquitously (9). Both LXRs heterodimerize with the retinoid X receptor (RXR) and stimulate transcription through binding to DR-4 response elements in target gene promoters (10).

To date, more than a dozen LXR target genes have been identified. They are involved in hepatic bile acid and fatty acid synthesis, glucose metabolism, and sterol efflux (11–16). In the liver, LXRs regulate gene expression of CYP7A (17) and sterol-regulatory binding element protein 1c (18), which are involved in cholesterol and fatty acid metabolism. In macrophages and other peripheral cell types, LXRs control the transcription of several genes involved in cellular cholesterol efflux, including ATP binding cassette transporter A1 (ABCA1) (19, 20), ABCG1 (21), and apolipoprotein E (14). LXRs also influence lipoprotein metabolism through the control of modifying enzymes such as lipoprotein lipase (22), cholesteryl ester transfer protein (11), and phospholipid transfer protein (13). Ligands for LXR have been shown to inhibit intestinal cholesterol absorption, promote hepatic sterol excretion, and reduce atherosclerosis in murine models (18, 23–25).

Abbreviations: AF, activation function; DBD, DNA binding domain; LBD, ligand binding domain; LXR, liver X receptor; LXRE, LXR response element; RXR, retinoid X receptor.
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Multiple isoforms have been identified for many members of the nuclear hormone receptor family. In several cases, different receptor isoforms have been found to have distinct activities and to play distinct biological roles (2, 26). Here we describe the identification and characterization of two isoforms of human LXRα that have distinct expression patterns and altered transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Reagents and plasmids**

GW3965 and T0901317 were provided by T. Willson and J. Collins at GlaxoSmithKline. Ligands were dissolved in DMSO prior to use in cell culture. The full-length coding regions of human LXR isoforms were amplified by PCR using specific primers and subcloned into BamHI/XhoI sites of the mammalian expression vector pCMX-PL1, to create pCMX-LXRα1, pCMX-LXRα2, and pCMX-LXRα3, respectively. Three isoforms of human LXRα were also subcloned into pEGFP-C1 vector using Xhol/BamHI sites to allow expression of N-terminal GFP-hLXRα fusion proteins. For retroviral expression constructs, inserts were excised from the pEGFP vectors using BglII/XhoI restriction enzymes and subcloned into BamHI/Sall sites of the pBabe vector to generate pBabe-GFP and pBabe-GFP-LXRα1, -LXRα2, and -LXRα3. The isoforms were also cloned into pShuttle-1 vector, which includes three repeats of FLAG tag in the N terminus. The dominant negative (ΔAF2) of human LXRα was generated by cloning of amino acids 1–435 of hLXRα1 to pCMV-Tag3C (Stratagene) vector via BamHI/XhoI sites. All plasmids were confirmed by DNA sequencing.

**Cell culture, transfection, and reporter gene assays**

HepG2 and HEK293 cells were cultured in modified Eagle’s medium containing 10% fetal bovine serum or lipopolysaccharide-deficient fetal bovine serum (LPDS). Transient transfections were performed in triplicate in 48-well plates. Cells were transfected with reporter plasmid (100 ng/well), receptor plasmids (5–50 ng/well), pCMV-β-galactosidase (50 ng/well), and pTKCIII (to a total of 205 ng/well) using Lipofectamine 2000 reagent (Invitrogen). Following transfection, cells were incubated in modified Eagle’s medium containing 10% LPDS and the indicated ligands or vehicle control for 24 h, and the results (mean ± SE; three experiments) were determined. Luciferase activities were assayed and normalized to β-galactosidase activity.

**Quantitative PCR**

Real-time quantitative PCR assays were performed using an Applied Biosystems 7700 sequence detector. Total RNA was reverse transcribed with random hexamers by using TagMan reverse-transcription reagents (Applied Biosystems) according to the manufacturer’s protocol. Real-time PCR Sybergreen assays for LXRα transcript levels were performed essentially as described (15). Samples were analyzed simultaneously for 36B4 expression. Quantitative expression values were extrapolated from separate standard curves. Each sample was assayed in duplicate and normalized to 36B4. The sequences for primers are as follows: hLXRα1, 5‘–3‘ (forward primer, CTGTGAGCTAGATCTCTCTG), 5‘–3‘ (reverse primer, CTGGCTGTTGCTCTCCTG); hLXRα2, 5‘–3‘ (forward primer, TGCCGAGAGGATAGAAGAAG), 5‘–3‘ (reverse primer, CTGGCTGTTGCTCTCCTG); hLXRα3, 5‘–3‘ (forward primer, GACCCGTCGGATGCTACGGTGA), 5‘–3‘ (reverse primer, CACTTCCAGGGTGTACCTCC).

**Gel shift assays**

Human LXRα isoforms and human RXRα were synthesized in vitro using the TNT T7-coupled reticulocyte system (Promega). To compare transcription/translation efficiency of the expression constructs expressing different human LXR isoforms, equal volumes of 35S-labeled lysates were loaded and separated on an 8% SDS-polyacrylamide gel. Gel shift assays were performed as described (15) using in vitro-translated proteins. Binding reactions were carried out in a buffer containing 10 mM HEPES, pH 7.8, 100 mM KCl, 0.2% Nonidet P-40, 6% glycerol, 0.3 mg/ml BSA, 1 mM diithiothreitol, 2 μg of poly(dI-dC), 1–μl each of in vitro-translated receptors and 35S-end-labeled oligonucleotide. DNA-protein complexes were resolved on a 5% polyacrylamide gel. The sequence of the rat FAS LXRE oligonucleotide was (only one strand shown): 5‘-gatacagtgaacggtagtaacccggc-3‘.

**Fluorescence microscopy**

Cells were transfected with retroviral vectors pBABE-GFP, pBABE-GFP-LXRα1, pBABE-GFP-LXRα2, and pBABE-GFP-LXRα3, and selected with puromycin to generate stable cell lines. The cells were seeded in 4-well chamber slides and fixed in 4% paraformaldehyde for 10 min at room temperature. Slides were mounted in Vectashield medium for fluorescence with 4′,6-diamidino-2-phenylindole (Vector) and analyzed under a Zeiss fluorescence microscope.

**Western blot analysis**

Cells transiently or stably transfected with FLAG-LXRα constructs were lysed in radioimmunoprecipitation assay buffer. Supernatants were collected, and protein content was assayed using the Bio-Rad protein reagent. Samples containing equal amounts of protein were boiled in 250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 2% mercaptoethanol, and then size-separated in 8% SDS-PAGE. Proteins were transferred to nitrocellulose membrane. Protein expression was detected with HRP-anti-FLAG antibody (M2) from Sigma, and visualized by the ECL technique.

**RESULTS**

By searching the expressed sequence tag (EST) database, we identified two cDNA clones similar to human LXRα (BC041172 and BC008819). Primers based on the EST sequences were used to amplify these LXRα transcripts from cdna, and the products were subcloned and sequenced. Comparison of these sequences to the publicly available genomic sequence of human chromosome 11 (www.genome.ucsc.edu) revealed that these two new LXRα transcripts were generated by alternative RNA splicing. For clarity, we refer to the original isoform as LXRα1 and the two new isoforms as LXRα2 and LXRα3, respectively. Details of the genomic organization of the human LXRα gene and the origin of various LXRα transcripts are shown in Fig. 1A. Our data indicate that the gene encompasses more than 20 kbp and contains 12 potential exons. Three distinct LXRα transcripts are produced through alternative splicing and promoter usage. The original isoform, LXRα1, and the newly identified LXRα3 are transcribed from a promoter upstream of exons 1a and 1b (15). The LXRα2 mRNA is transcribed from an alternative promoter and exon 1c, located approximately 10 kb upstream of exon 1a. Figure 1B shows an alignment of the
The predicted amino acid sequences of the three LXRα isoforms. The LXRα1 protein has 447 amino acids with a predicted size of 50.4 kDa. Because exons 1a and 1b are noncoding, the choice of these exons does not impact protein sequence. By contrast, translation of the LXRα2 mRNA starts in exon 3, leading to a truncated protein lacking the N-terminal 45 amino acids of LXRα1. The LXRα3 mRNA is generated by the removal of exon 6 through alternative splicing, leading to an in-frame deletion of 50 amino acids from the LBD.

To determine the absolute level of expression of LXRα isoforms in different tissues, total RNA from 20 human tissues in various stages of the human androgen response was analyzed by quantitative RT-PCR.

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**Table:** Predicted Amino Acid Sequences of LXRα Isoforms

<table>
<thead>
<tr>
<th>LXRα isoform</th>
<th>Predicted Amino Acid Sequence</th>
<th>Predicted Size (kDa)</th>
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<td>LXRα2</td>
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<tr>
<td>LXRα3</td>
<td>GGTAGVGLEAEAPATLLEPSEPTERQPKAPKMLGNECSV</td>
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**DNA Binding Domain**

<table>
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<tr>
<th>LXRα isoform</th>
<th>Predicted Amino Acid Sequence</th>
<th>Predicted Size (kDa)</th>
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<tbody>
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<tr>
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**Fig. 1.** Identification of new hLXRα isoforms. A: Schematic representation of human LXRα genomic structure and the corresponding isoform protein structure. Distinct modulator domains can be generated by alternative promoter usage and splicing (linked exons). Alternative splicing involving exons 1 and 2 generates the isoform LXRα1. Translation begins in exon 3 resulting in the truncation of the N-terminal 45 amino acids. LXRα3 is generated by the alternative splicing of exon 6, leading to an in-frame deletion of 50 amino acids in the ligand binding domain (LBD). DNA binding domain is shown (DBD). B: Alignment of the predicted amino acid sequence among human LXRα isoforms.
sues was reverse transcribed and real-time quantitative PCR was performed. As shown in Fig. 2A, the various LXRα isoforms differ in their patterns of expression. In normal tissues, the highest hLXRα1 expression was detected in liver, heart, brain, spleen, and kidney. LXRα2 was highly expressed in testis, where it was the predominant isoform.

LXRα3 was expressed at relatively lower levels in lung, thyroid gland, and spleen. In addition to normal tissues, transformed cell lines representing lymphoma, melanoma, osteosarcoma, medulloblastoma, and glioma were analyzed (Fig. 2B). Interestingly, the alternative isoforms α2 and α3 were somewhat more highly expressed in tumor cells com-

![Fig. 2. Differential expression of human LXRα isoforms. A: Real-time quantitative PCR analysis of LXRα isoform expression in various human tissues. B: Real-time quantitative PCR analysis of human LXRα isoforms in various human tumor cell lines.](image)

![Fig. 3. Functional characterization of human LXRα isoforms. A: Analysis of in vitro-translated proteins. pCMX-LXRα1, LXRα2, and -LXRα3 were synthesized in vitro in the presence of [35S]methionine. Three microliters of in vitro-translated lysates were analyzed on an 8% SDS-polyacrylamide gel. B: LXRα isoforms bind DNA by electrophoretic mobility shift assay. Equivalent amounts of in vitro-synthesized retinoid X receptor (RXR) in combination with LXRα1, LXRα2, or LXRα3. Protein was incubated with 32P-labeled FAS LXRE DNA probe, and the DNA-protein complex was resolved on a 5% polyacrylamide gel. C: hLXRα2 and LXRα3 proteins exhibit altered transcriptional activity. pCMX-LXRα1, -LXRα2, and -LXRα3 expression vectors were transfected into HEK-293 cells along with a pTk3×1XRE-Luc reporter construct. Each point is the average of triplicate experiments. Cells were treated with DMSO or T1317 (synthetic LXR agonist, 1 μM) for 24 h. The dominant-negative ligand-dependent activation function region (ΔAF2) construct is a mutant of LXRα lacking the AF2 domain (20). * P < 0.05 versus LXRα1 by Student’s t-test (2-tailed). Data are presented as mean ± SEM.](image)
pared with normal tissues. Furthermore, the cell type-specific nature of alternative transcript expression was also evident from these samples.

To investigate whether the alternative LXRα2 and α3 proteins were competent to bind DNA, they were produced in vitro using reticulocyte lysates. In vitro transcription/translation experiments confirmed the production of LXRα1, LXRα2, and LXRα3 proteins with expected molecular weights (Fig. 3A). Electrophoretic mobility shift assays revealed that both LXRα2 and LXRα3 retain the ability to heterodimerize with RXR and to bind the LXR response element (LXRE) from the fatty acid synthase (FAS) gene (12) (Fig. 3B). To address the transcriptional activity of the LXRα2 and LXRα3 isoforms, we performed transient transfections into HEK-293 cells. As expected, transfection of an LXRα1 cDNA expression vector stimulated activity of an LXRE-driven luciferase reporter in a ligand (T1317, 1 μM)-dependent manner (Fig. 3C). A low level of basal activity was observed with the LXRE reporter in the absence of transfected LXR due to the expression of endogenous LXRβ in HEK-293 cells. Transfection of an expression vector encoding the LXRα2 cDNA also promoted LXRE reporter expression, but it was clearly less active than LXRα1. By contrast, expression of LXRα3 cDNA did not stimulate reporter expression above basal levels. As a result of the deletion of the 50 amino acids encoded by exon 6, the LXRα3 protein lacks helixes 3 and 4 and part of helix 5, which comprise the ligand binding pocket of LXRα1 (27). On the basis of this structure, and consistent with our results in transient transfection assays, LXRα3 is predicted to be unable to bind ligand (T. Willson, personal communication). The results shown in Fig. 3 demonstrate that although both LXRα2 and LXRα3 bind DNA, they show altered transcriptional activity compared with LXRα1. Similar differences in activity between isoforms were observed when the natural LXR agonist 22(R)-hydroxycholesterol or the synthetic ligand GW3965 was used in place of T1317 (data not shown).

Presently, reliable antibodies recognizing the different human LXRα isoforms are not available. We therefore utilized GFP fusion proteins to study expression of the LXRα protein isoforms. We utilized retroviral transduction to generate HEK-293 cell lines expressing GFP-LXRα1, GFP-LXRα2, and GFP-LXRα3 fusion proteins. The cellular localization of the LXRα1, LXRα2, and LXRα3 proteins was visualized by fluorescence microscopy. Expression of the GFP-LXRα1 fusion protein in HEK-293 cells led to an exclusively nuclear distribution of fluorescence (Fig. 4A).
Identical cellular localization was observed with GFP-LXRα2 and GFP-LXRα3 fusion proteins. Furthermore, the alternative LXRα cDNAs were expressed and translated at rates comparable to those of LXRα1. An equivalent amount of fusion protein was produced by the three expression vectors, as judged by fluorescence microscopy (Fig. 4A) and Western blotting, using an anti-GFP antibody (data not shown). When the activity of the three GFP-LXRα fusion proteins was compared in transient transfection assays, the results were similar to those obtained with native LXR isoform expression vectors. GFP-LXRα2 showed reduced activity compared with GFP-LXRα1, and GFP-LXRα3 was inactive (Fig. 4B).

To determine whether the various LXRα isoforms also showed differential activity on endogenous target genes, we analyzed ABCA1 expression in HEK-293 cells transfected with retroviral GFP-LXRα fusion vectors. As shown in Fig. 4C, expression of LXRα1 strongly stimulated expression of ABCA1 mRNA. Consistent with their behavior in transient transfection assays, LXRα2 showed reduced activity, whereas LXRα3 actually reduced ABCA1 expression. Thus, LXRα2 and LXRα3 display altered transcriptional activity on the endogenous ABCA1 promoter as well as in transient transfection reporter assays.

The reduced activity of LXRα2 compared with LXRα1 suggests an unexpected function for the LXRα N terminus in transcriptional regulation. Studies on other nuclear receptors have shown that the N terminus can function to augment (e.g., RXRα, Ref. 28 and estrogen receptor, Ref. 29) or inhibit (e.g., PPARγ) transcriptional activity (30). However, the ability of the N terminus of LXRα to contribute to overall receptor activity has not been explored previously. To address the role of the LXR N terminus in more detail, we constructed serial deletions. As shown in Fig. 5, transfection of the deletion constructs into HEK-293 cells revealed that the N-terminal 20 amino acids are required for full receptor activity. Furthermore, activity declined further with the deletion of the N-terminal 45 amino acids. This observation suggests that sequences between amino acids 5 and 45 are important for receptor function. Similar results were obtained when GW3965 or T1317 was used as the LXR ligand (data not shown).

To ensure that the GFP fusion itself was not influencing the activity of the various LXR isoforms, we also explored the function of LXRα2 and LXRα3 using FLAG-tagged LXR fusion proteins. Transfection of these expression vectors into HEK-293 cells produced equivalent levels of LXRα protein, as determined by Western blotting using an anti-FLAG antibody (Fig. 6A). Consistent with the results presented above, FLAG-LXRα2 showed reduced activity compared with FLAG-LXRα1, whereas FLAG-LXRα3 was inactive (Fig. 6B). Furthermore, a FLAG-tagged receptor containing two repeats of the N-terminal 74 amino acids showed increased activity compared with the wild-type LXRα1 (Fig. 6B). Finally, the isolated AF1 domain enhanced the transcription of a GAL4-luciferase reporter when it was fused with GAL4 DBD (Fig. 6C). Thus, the N terminus of LXRα contains a bona fide transcriptional activation function.

The observation that LXRα3 binds DNA but is unable to activate transcription suggests that it might act to antagonize the function of wild-type LXRα1 when expressed in the same cell. Because mutations in the LBDs of other nuclear receptors have been shown to give rise to dominant-negative receptors, we considered the possibility that LXRα3 might function as a dominant negative. However, the effect of expression of LXRα3 in transfection assays was distinct from that of an AF2 deletion mutant (ΔAF2) that we have previously shown to function as a dominant negative (20). Expression of the ΔAF2 mutant effectively blocked basal LXR in the transfected cells, whereas that of LXRα3 did not (Fig. 7A). Moreover, when expressed together with LXRα1 in transfection assays, LXRα3 was able to function as a competitive inhibitor, but not a dominant-negative inhibitor, of LXRα1 (Fig. 7B). Expression of a large molar excess of LXRα3 was required to inhibit the function of LXRα1. Similar results were obtained when GW3965 or T1317 was used as the LXR ligand (data not shown). This observation is consistent with LXRα3 forming inactive heterodimers with RXR and simply competing for LXRα1 on the target promoter.
DISCUSSION

The LXRs are oxysterol-activated nuclear hormone receptors that regulate the expression of genes involved in cholesterol and fatty acid metabolism (5, 6). In previous studies, the human LXRα gene was mapped to chromosome 11 at 11p11.2. It was shown to cover 10.82 kb of genome sequence and to contain 11 exons (10). The present study expands our knowledge of the LXRα genomic locus. We show that the locus spans more than 20 kb of sequence and contains 12 possible exons. By searching the EST database, we have identified two new isoforms of human LXRα, termed hLXRα2 and hLXRα3. These two new LXRα transcripts are generated by alternative splicing and alternate promoter usage and exhibit altered transcriptional activity relative to LXRα1. These results add additional complexity to the known mechanisms of transcriptional regulation of lipid metabolism by LXRs.

In this study, a new upstream exon of the human LXRα gene was identified that is incorporated into the LXRα2 isoform. LXRα2 varies from LXRα1 in its 5’ untranslated region as a result of the use of this alternative first exon.
The N-terminal (AF1 domain or A/B region) of nuclear receptors is the least-conserved domain across the family, varying considerably both in length and in sequence (2, 26, 31). Furthermore, heterogeneity in the
N-terminal region is a common feature of many nuclear receptors. Several receptors have functionally distinct isoforms arising from differential promoter usage and/or alternative splicing. For example, tissue-specific alternative-promoter usage generates multiple transcripts of PPARγ (32), PPARα (33), CAR (34), RAR (35), RXR (36), estrogen receptor (ER) (37), glucocorticoid receptor (GR) (38), and others. In addition, the activity of the AF1 domain has been shown to vary in both a tissue- and promoter-specific manner for several nuclear receptors (2). We have shown here that LXRα, like several other members of the nuclear receptor superfamily, contains a ligand-independent activation domain in its N-terminal AF1 domain. The AF1 activities of other nuclear receptors serve significant functions in transcriptional regulation, not only by providing ligand-independent activation, but also by synergizing with AF2 (28, 29, 31, 39, 40). Interaction of AF1 regions with transcriptional coactivators has also been reported for several receptors (4, 29, 39–41). Other studies have suggested that the N-terminal domain can influence ligand binding (30). It is possible that truncation of the AF1 domain in human LXRA2 alters coactivator recruitment. It is also possible that LXRA undergoes posttranslational modification in the AF1 domain. For example, its partner receptor, RXR, can be phosphorylated at several serine and threonine residues in its N-terminal domain (42). Sequence analysis reveals several predicted phosphorylation and sumoylation sites that are conserved with LXRA proteins in other species (unpublished observations). Loss of these posttranscriptional modifications in LXRA2 may alter receptor function. The identification of interaction partners of the AF1 domain, the analysis of posttranslational modifications in the AF1 domain, and the examination of possible intramolecular communication involving the AF1 domain of LXRA are important subjects for future studies.

The observation that LXRA2 is the predominant isoform expressed in tests provides an interesting avenue for future investigation. Alternative splicing in the tests is important for sex determination, meiotic gene regulation, and spermatogenesis. A network of tests-specific splicing factor interactions has been uncovered (43). During male meiosis, there is a switch from active to inactive or from inactive to active transcription factors, directed by alternative splicing. Therefore, the differential expression of human LXRA isoforms in tests may play a role in the differential transcriptional regulation of LXRA target genes. The implications of alternative LXR isoform expression for LXR-dependent gene expression and lipid homeostasis in various cell types in vivo remain to be explored.

The authors are grateful to Dr. Mathew Kennedy for sharing the human tissue RNAs. In addition, the authors thank Brenda Mueller for administrative support. This work was supported by a postdoctoral fellowship from the American Heart Association (M.C.). P.T. is an Investigator of the Howard Hughes Medical Institute at the University of California, Los Angeles. This work was supported by National Institutes of Health Grants HL-66088 and HL-30568.
ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. J. Biol. Chem. 275: 14700–14707.