Regulation of the angiopoietin-like protein 3 gene by LXR

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Abstract **Angiopoietins are members of the vascular endothelial growth factor family. One family member, angiopoietin-like protein 3 (Angptl3), was recently shown to be predominantly expressed in the liver and to play an important role in regulating lipid metabolism. In this study, we show that the Angptl3 gene is a direct target of the liver X receptor (LXR). Mice fed a high cholesterol diet exhibited a significant increase in Angptl3 expression in the liver. Oral administration to mice of T0901317, a synthetic LXR-selective agonist, increases levels of plasma lipids and Angptl3 mRNA in the liver. Treatment of HepG2 cells with LXR selective agonists led to a dose-dependent increase of Angptl3 mRNA. Analysis of the DNA sequence just 5′ of the Angptl3 transcriptional start site revealed the presence of several potential transcription factor binding sites, including that for LXR. When transfected into HepG2 cells, the promoter activity of Angptl3 was significantly induced by LXR- or retinoid X receptor-selective agonists. Mutation of the predicted LXR binding site (DR4 element) completely abolished the LXR agonist-mediated activation of the promoter. Together, these studies show that Angptl3 is transcriptionally regulated by LXR, and reveals a novel mechanism by which LXR may regulate lipid metabolism.**—Kaplan, R., T. Zhang, M. Hernandez, F. X. Gan, S. D. Wright, M. G. Waters, and T-Q. Cai. **Regulation of the angiopoietin-like protein 3gene by LXR. *J. Lipid Res.* 2003.** 44: **136–143.**

Supplementary key words lipid metabolism • angiopoietin • transcriptional regulation • nuclear receptor

The angiopoietins are a family of secreted growth factors. Together with their respective endothelium-specific surface receptors, such as the receptor tyrosine kinase Tie2, the angiopoietins play important roles in angiogenesis [as reviewed in ref. (1)]. One family member, Angiopoietin-like protein 3 (Angptl3), was previously found to be expressed in a liver specific manner (2), and more recently, it was found to play an important role in the regulation of lipid metabolism (3). Mutation of Angptl3 results in low levels of plasma lipids in KK/San mice (3). These hypolipidemic mice are derived from KK obese mice, which display a multigenic syndrome of moderate obesity and a diabetic phenotype including hyperinsulinemia, hyperglycemia, and hyperlipidemia (4–6). Administration or overexpression of Angptl3 in mice elicited a rapid increase in circulating levels of plasma cholesterol, triglycerides (TG), and non-esterified fatty acids (3). While these studies revealed a role of Angptl3 in lipid metabolism, they did not address the molecular mechanism by which a hyperlipidemic response was mediated by Angptl3. Understanding the regulation of Angptl3 expression is an important step toward a better comprehension of the physiological network that comprises lipid metabolism.

Many genes involved in lipid metabolism are regulated by nuclear receptors. Liver X receptors (LXRs), including LXRα and LXRβ, are members of the nuclear receptor superfamily [as reviewed in ref. (7)]. LXRs heterodimerize with retinoid X receptors (RXRs) and can be activated by both RXR and LXR ligands. Upon activation, LXRs regulate the expression of target genes by binding to specific promoter response elements (termed LXREs) that contain a hexameric nucleotide direct repeat separated by four bases (DR4). Recent studies have shown that these ligand-activated transcription factors play important roles in the regulation of genes that govern cholesterol homeostasis in the liver and peripheral tissues (7).

In addition to cholesterol metabolism, accumulating evidence suggests that LXRs also play important roles in regulating fatty acid metabolism. For example, the expressions of sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase, and lipoprotein lipase were all shown to be strongly activated by LXR agonists (8–10). It has been suggested that the regulation of SREBP-1c by LXR may contribute to increased levels of plasma TG when animals are treated with LXR agonists (11–12). However, SREBP-1c cannot be solely responsible for the elevated TG because mice overexpressing SREBP-1c had a reduced level of circulating TG (13). To determine if Angptl3 might be such a factor, we stud-

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Abbreviations: ABCA1, ATP-binding cassette transporter 1; Angptl3, angiopoietin-like protein 3; FCS, fetal calf serum; LXR, liver X receptor; OH Ch, hydroxycholesterol; 9-RA, 9-cis retinoic acid; SREBP, sterol regulatory element-binding protein; TG, triglycerides.

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TABLE 1. Sequences of primers and probes for real time quantitative PCR

Gene	Species	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Probe (5′ to 3′)
Angptl3	Human	ACCATTTATAAC AGAGGTGAACAT ACAAG	CCTGATATAACA TCACAGTAGACA TGAAAA	TGTATGCCATCAG ACCCAGCAACTCT CA
Angptl3	Mouse	GATTTGCTATGT TGGATGATGTCAA	CTTATGGACAAAA TCTTTAAGTCCA TGA	ATTTTAGCGAATG GCCTCCTGCAGCT
CYP7A	Mouse	CAAAACCTCCAA TCTGTCATGAGA	GCGTTAGATATC CGGCTTCAAA	AGGGATGTATGC CTTCTGCTACCGA GTGAT

ied regulation of Angptl3. Here we report that the discovery that Angptl3 is indeed regulated by LXR. Given the profound effect of Angptl3 on circulating levels of cholesterol and TG, our findings may offer a novel mechanism for a role of LXR in regulating lipid metabolism.

MATERIALS AND METHODS

Cell culture

HepG2 cells, a human hepatocellular carcinoma cell line obtained from the American Type Culture Collection (Rockville, MD), were maintained in MEM medium with 10% heat inactivated fetal calf serum (FCS), non-essential amino acids, and sodium pyruvate in an atmosphere containing 5% CO_2 and 95% air at 37°C. Before assay, cultured cells were trypsinized, washed once with PBS, and then resuspended in the assay medium (same as that of complete medium but with only 0.5% of FCS). All test compounds used in the experiments were diluted in the same assay medium.

RNA isolation

Total RNA was extracted from the cultured cells using TRIZOL reagent according to the protocol provided by the manufacturer (Life Technologies, Grand Island, NY). The RNA was treated with DNase (Ambion, Inc., Austin, TX) and was reverse transcribed using TaqMan reverse transcription reagents as described by the manufacturer (Applied Biosystems, Foster City, CA) before analysis by real-time quantitative RT-PCR.

Primers and fluorogenic probes

Oligonucleotide primers and TaqMan probes were designed using Primer Express software (Applied Biosystems) and were synthesized by Qiagen Operon (Alameda, CA). Sequences of probes and primers are listed in **Table 1**. Primers and probes for 18*S* rRNA were purchased from Applied Biosystems.

Real-time quantitative PCR

Real-time quantitative TaqMan PCR analysis was used to determine the relative levels of Angptl3 mRNA. TaqMan PCR reactions were performed according to the manufacturer's instructions (Applied Biosystems, TaqMan Universal PCR Master Mix). Target cDNA amplification was detected by the increased fluorescent signal of FAM (reporter dye) during the amplification cycles. Amplification of the 18*S* rRNA transcript was performed in the same reactions using a different reporter dye, VIC, as an internal control for variations in RNA amounts. Levels of the target mRNAs were subsequently normalized using 18*S* mRNA levels, and are presented as the ratio of treated cells to untreated cells.

Sequence analysis of the genomic region of Angptl3

BLAST searches were performed to identify genomic sequences encoding human and mouse Angptl3 (14). The genomic sequences of human and mouse Angptl3 were compared using mVISTA (15–16) and ClustalW (17). Putative binding sites for transcription factors were identified by searching against the TRANSFAC database (18) and the positional weight matrices for transcription factors constructed internally.

5′ Rapid amplification of cDNA ends PCR

5′ Rapid amplification of cDNA ends (RACE) PCR was performed with the GeneRacer kit (Invitrogen, Carlsbad, CA) using 3.5 μg of total RNA purified from C57Bl/6J mouse liver. The 5′ ends of the mRNA were dephosphorylated, decapped, and ligated with the GeneRacer RNA oligo. The ligated mRNA was then reverse transcribed using SuperScript II and a gene-specific primer 5′-CAGAATCAAATGATGAAAGGTCTGGAT -3′ (Qiagen Operon). The 5′ end of the cDNA was then amplified (hot start, 94°C 30 s, 68°C 30 s, 70°C 1 min, 5 cycles; then 94°C 30 s, 66°C 30 s, 70°C 1 min, 20 cycles; then 70°C 10 min) using PCR SuperMix, the GeneRacer 5′ primer, and the aforementioned gene-specific primer. A second PCR reaction was run (hot start, 94°C 30 s, 55°C 30 s, 68°C 1 min, 25 cycles; then 68°C 3 min followed by 15°C 10 min) using 1 ul of the original PCR reaction, PCR SuperMix, the GeneRacer 5′ nested primer and a nested gene-specific primer 5′-GTCTGGATCCACTCTGGATGCAATT-3′ (Qiagen Operon). The PCR product was run on a 1% agarose gel and the

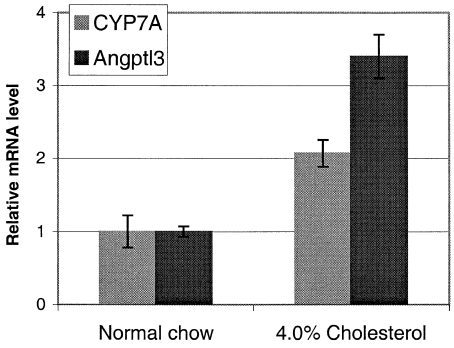

Fig. 1. Cholesterol feeding raises the level of Angptl3 mRNA in mouse liver. Mice were fed with a normal chow diet or with the same supplemented with 4% of cholesterol for 7 days. On the 8th day, mice were euthanized, RNAs were prepared from the liver samples, and the relative levels of Angptl3 and CYP7A mRNAs were measured as described in Materials and Methods. Results are normalized to the values obtained from normal chow diet fed mice, and data are shown as the means ± SE of each group (n = 5).

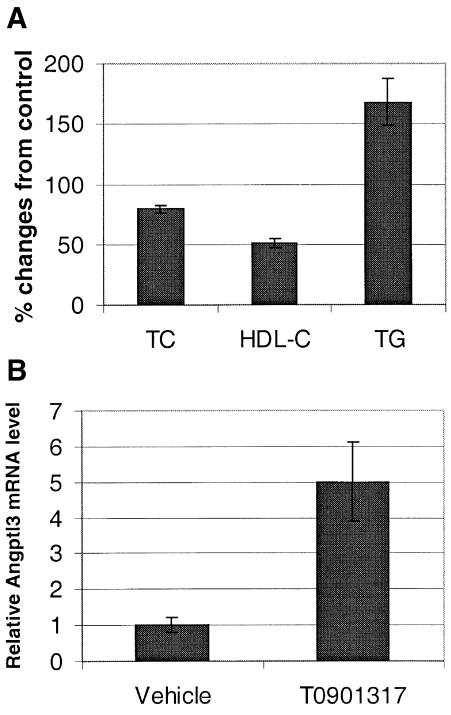

Fig. 2. LXR-selective agonist T0901317 increases levels of plasma lipids and Angptl3 mRNA in the liver. Mice were orally treated with either vehicle or 10 mpk (mg compound/kg of animal body weight/day) of T0901317 for 7 days. On the 8th day, mice were euthanized, and (A) plasma levels of total cholesterol (TC), HDL cholesterol (HDL-C), and triglycerides (TG), or (B) levels of Angptl3 mRNA in the liver were measured. Lipid data are reported as the percent of changes from control mice (A, n = 10); Relative Angptl3 mRNA levels in treated mice are calculated by normalizing to the values obtained from control mice (B, n = 5), and data are shown as the mean ± SE of each group.

band was excised, purified using a SNAP column, and TOPO TA cloned into pCR 4-TOPO vector. The plasmid was sequenced using the T3 and T7 priming sites by ACGT, Inc. (Northbrook, IL). All reagents listed were provided by Invitrogen, unless otherwise stated.

Construction and mutagenesis of the human Angptl3 promoter-luciferase construct

A firefly luciferase reporter construct was generated with 953 bp of the promoter of human Angptl3 and 49 bp of the 5′ UTR. Briefly, human genomic DNA (CLONTECH, Palo Alto, CA) was PCR-amplified with Angptl3 specific primers and a *Bgl*II restriction site 8 nucleotides from the 5′ end of the forward primer (Dexter-10F: 5′-GATCGATCAGATCTGGGAGGCCAAGGTAAAAGAA-3′) and a *Hind*III restriction site 8 nucleotides from the 5′ end of the reverse primer (Dexter-10R: 5′-GATCGATCAAGCTTTTTTATC TTGATTTTCAATTTCAAG-3′). After restriction with *Bgl*II and *Hind*III, this fragment was cloned into a similarly digested pGL3 Basic vector (Promega, Inc., Madison, WI) and confirmed by sequencing the entire insert and nearby vector (ACGT, Inc.) to create the human Angptl3 promoter-Luciferase (hpAngptl3-Luc) construct.

A similar construct with a mutation of the DR4 element of the human Angptl3 promoter (hpAngptl3/DR4Mut-Luc) was created by site directed mutagenesis of the 5′ half-site of the hpAngptl3 DR4 element. The mutagenesis was performed using the Gene-Editor system (Promega, Inc.) and mutagenic oligonucleotide according to the manufacturer's instructions. The mutagenic oligonucleotide, 5′-TCTAACTCAATGTGGAAGAGGGCCCCAT-TCGTGCAAGTTAACAC-3′, was designed to introduce an *Apa*I site into the 5′ half-site of the Angptl3 DR4 element. A restriction digestion with *Apa*I (Life Technologies, Inc., Bethesda, MD) was used to screen for mutants and the clones were sequenced (ACGT, Inc.) to confirm the lack of any additional mutations in the Angptl3 sequence.

DNA transfection and reporter gene assays

Transfections of HepG2 cells were performed in 96-well plates with FuGENE 6 reagent according to the manufacturer's instructions (Roche Diagnostics Corp., Indianapolis, IN). Immediately prior to transfection, cells were trypsinized and the complete media was replaced by assay medium to a concentration of 5×10^5 cells/ml. One hundred microliters of cells were added per well of the 96-well plates. The constructs, hpAngptl3-Luc or hpAngptl3/DR4mut-Luc, were co-transfected with phRL-null (*Renilla*, Promega, Inc.) at a total of 37.5 ng of reporter DNA and 12.5 ng of the *Renilla* internal control per well to maintain a FuGENE to DNA ratio of 3:1. Six hours after transfection, test compounds suspended in assay medium were added, and 42 h later the cells were harvested using Passive Lysis Buffer and assayed for firefly and *Renilla* luciferase activities with the Dual-Luciferase Reporter Assay System purchased from Promega. All transfections and subsequent steps were performed in triplicate.

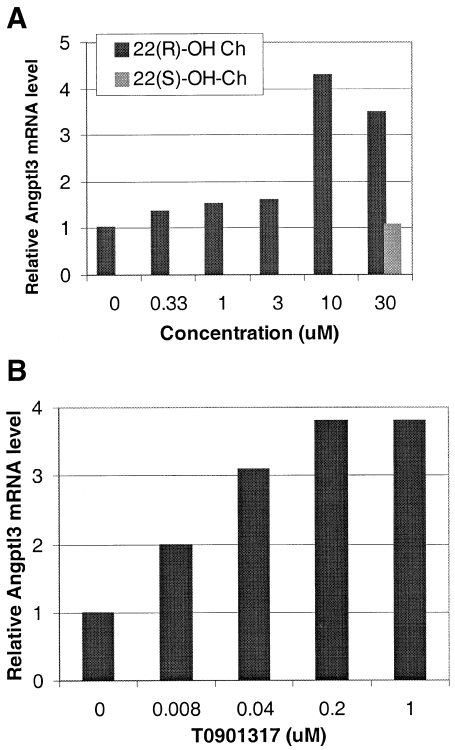

Fig. 3. Concentration-dependent induction of Angptl3 expression by LXR agonists. HepG2 cells (5×10^5 cells/well) were plated in 6-well plates and treated with (A) buffer, increasing concentrations of 22(*R*)-OH Ch or 30 μM 22(*S*)-OH Ch or (B) buffer, or increasing concentrations of T0901317. Following ~16 h of incubation at 37°C, RNA samples were prepared from the cultured cells, and expression levels of Angptl3 mRNA were measured as described in Materials and Methods. Results are normalized to the values obtained from control cells, and data are shown as the means of duplicate determinations.

Animal procedures

C57BL/6J mice were purchased from Taconic (Germantown, NY). Animals were maintained in normal light cycle, and were provided with water ad libitum. Mice were fed with normal chow diet either with or without 4% of cholesterol. Compound-treated mice were dosed once a day with either vehicle (0.5% of methylcellulose solution) or 10 mpk (mg compound/kg of animal body weight/day) of T0901317 by oral gavage. After 7 days of feeding or gavage dosing, mice were euthanized and blood samples were taken through cardiac puncture. Liver samples were collected for RNA analyses. Plasma total cholesterol, HDL cholesterol (HDL-C), and triglyceride concentrations were determined with assay kits from Sigma Chemical Co. (St. Louis, MO). All procedures were approved by the Institutional Animal Care and Research Advisory Committee at Merck.

RESULTS

Cholesterol feeding increases expression of Angptl3 mRNA in mouse liver

To determine if expression of Angptl3 mRNA may be subject to regulation in response to perturbations of cholesterol homeostasis, mice were fed for 7 days with a normal chow diet or with the same supplemented with 4% of cholesterol. Quantitative PCR analysis showed, as expected, that mice fed a high-cholesterol diet had an increased expression of CYP7A (**Fig. 1**), which encodes cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis (19). The high-cholesterol diet also resulted in a 3.4 ± 0.3-fold higher expression of Angptl3 in the liver compared with mice fed a normal chow diet ($P < 0.01$, n = 5) (Fig. 1). Thus, liver Angptl3 expression is responsive to alterations in dietary cholesterol intake.

Administration of LXR-selective agonist increases expression of Angptl3 mRNA in mouse liver

Recent studies reported that feeding mice with a high-cholesterol diet or loading cells with cholesterol may result in activation of the nuclear receptor LXR or increased expression of its target genes including CYP7A (8, 19–20). We thus asked if LXR could play a role in regulating the expression of Angptl3 by treating mice with T0901317, a synthetic LXR-selective agonist (11). Consistent with a previous report (11), oral treatment of mice with T0901317 resulted in significant elevations of plasma total cholesterol, HDL-C, and triglycerides (**Fig. 2A**). Analysis of liver mRNA levels revealed a significant increase of Angptl3 mRNA (Fig. 2B). Oral treatment of mice with 10 mpk of T0901317 for 7 days resulted in a 5.0 ± 1.1-fold increase of Angptl3 mRNA in the liver ($P < 0.01$, n = 5) (Fig. 2B). Thus, liver Angptl3 expression is responsive to the activation of LXR in vivo.

Activation of LXR increases expression of Angptl3 in HepG2 cells

To determine if the T0901317 treatment-induced changes of Angptl3 in mice could be mediated by direct effects on hepatocytes, we studied the impact of LXR agonists on Angptl3 expression in HepG2 cells. Cultured HepG2 cells were incubated for ~16 h with various oxy-

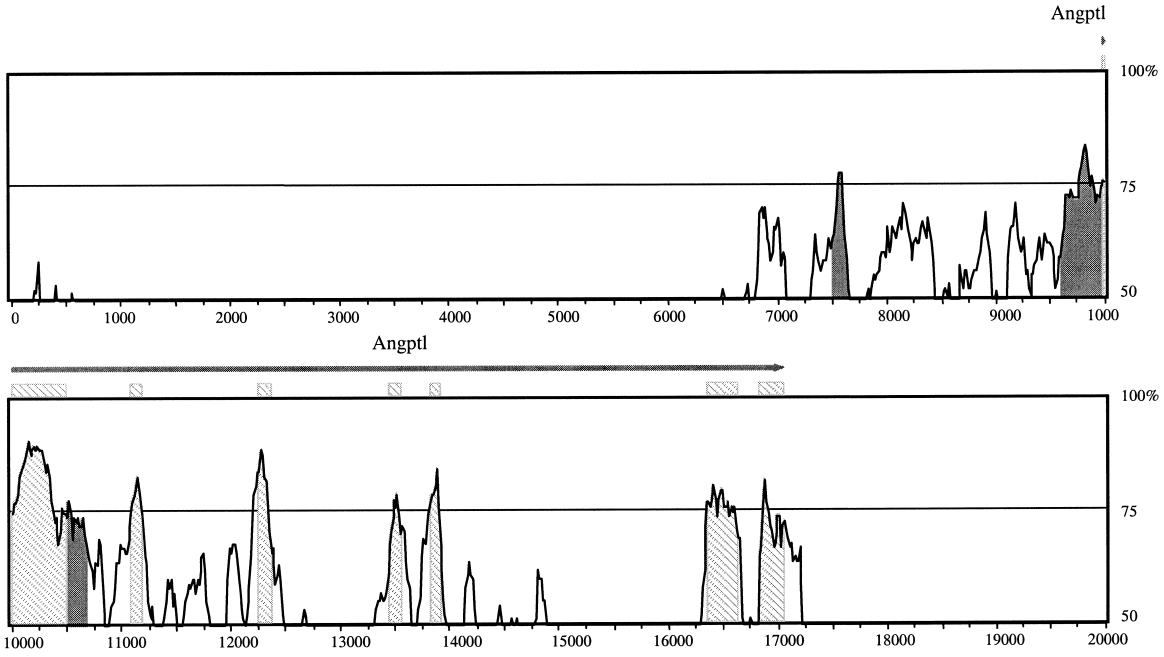

Fig. 4. Comparison of the genomic regions upstream of Angptl3 in human and mouse. Genomic regions spanning Angptl3 in human and mouse were compared using mVISTA (http://www.gsd.lbl.gov/vista). Conserved regions with an identity greater than or equal to 50% between human and mouse sequences are shown as peaks of similarity (vertical axes) relative to the positions along the mouse genomic sequence (horizontal axes). Peaks representing highly conserved regions (identity >75% over >100 bp) are shaded if they overlap with non-coding regions, or marked by slanted lines if they overlap with exons. There are two highly conserved non-coding regions upstream of human and mouse Angptl3: the 500 bp region immediately upstream and the 150 bp region 2.5 kb upstream of the gene.

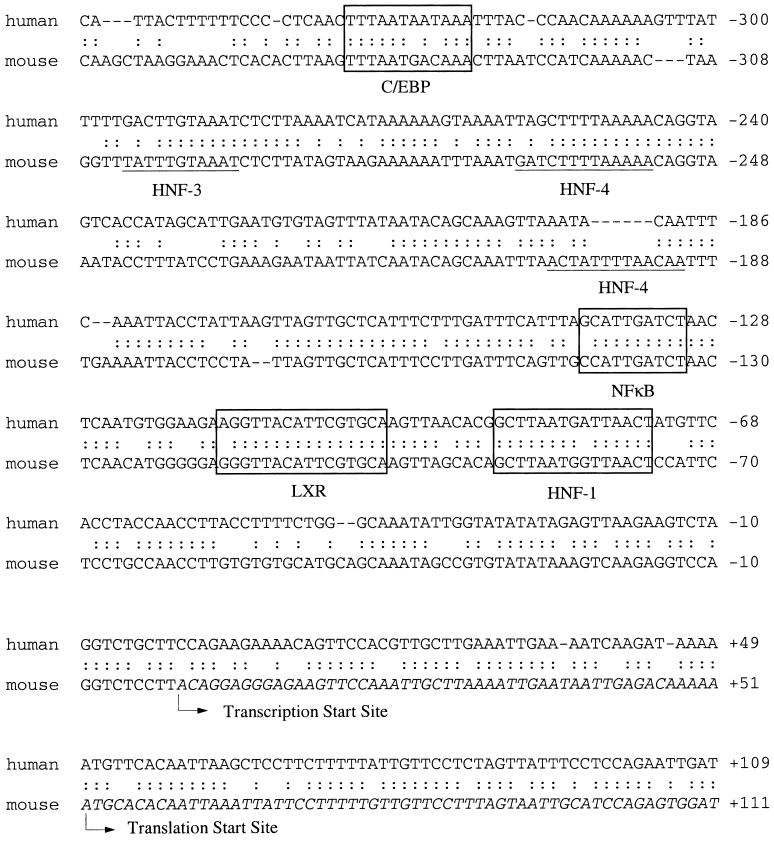

Fig. 5. Analysis of the promoter region of human and mouse Angptl3. The sequence segment overlapping the 5′ RACE product is shown in italic, and its 5′ end is marked by arrow as the putative transcription start sites (i.e., position +1 in the sequences). The translational start codon (i.e., ATG) is also marked by an arrow. A number of putative binding sites of transcription factors are predicted in this region. Those conserved between human and mouse are boxed, and those found in only one species are underlined.

sterols and T0901317. As shown in **Fig. 3A** and 3B, treatment of HepG2 cells with 22(*R*)-hydroxycholesterol (OH Ch) or T0901317, both of which activate LXR (7, 11), resulted in a dose-dependent increase of Angptl3 mRNA. Similar results were observed when cells were treated with 25-OH Ch, which also activates LXR (data not shown). In contrast, addition of 22(*S*)-OH Ch, which does not activate LXR (7), failed to significantly change the expression of Angptl3. Together, these results suggest that LXR can activate Angptl3 transcription in hepatocytes.

Identification of the Angptl3 promoter

To determine if the LXR-agonist induced effects in HepG2 cells could be due to direct action of LXR on the Angptl3 gene, we studied the Angptl3 promoter. We located the putative transcriptional start site by identifying the full 5′ UTR by 5′ RACE PCR using total RNA from C57Bl/6J mouse liver as the template. The reaction produced essentially a single PCR product, and sequence analysis identified the putative transcription start site as 51 bp upstream of the initiation methionine codon for mouse Angptl3. Comparison of the 5′ RACE product generated from the mouse with the human Angptl3 genomic sequence suggests that the putative human transcription start site is located 49 bp upstream of the initiation methionine codon.

Next we compared the genomic sequence 5′ of the mouse and human Angptl3 open reading frames. The comparison revealed that the 500 bp region adjacent to, and a 150 bp region ~2.5 kb upstream of, the initiation methionine codon are highly conserved with >75% sequence identity (**Fig. 4**). These regions may play a role in the regulation of the Angptl3 gene, thus we searched for putative binding sites of transcription factors in these conserved sequences. **Figure 5** shows the putative binding sites identified in the 400 bp region adjacent to the transcription start sites. Several putative binding sites for transcription factors, including HNF-1, LXR, NFκB, and C/EBP, are conserved between human and mouse, which merit further experimental verification. Similarly, putative binding sites for HNF-3, HNF-4, and C/EBP were also predicted in the conserved 150 bp region 2.5 kb upstream of the transcription start site (data not shown).

A truncated Angptl3 promoter is functional and responsive to LXR activation

To study the function of the Angptl3 promoter, we transfected HepG2 cells with a human Angptl3 promoter-luciferase construct (953 bp of the promoter and 49 bp of 5′ UTR fused to the luciferase coding sequence). To test if such a truncated promoter is functional and responsive to LXR activation, cells were treated for 42 h with various oxysterols, 9-*cis* retinoic acid (9-RA), or T0901317. As shown in **Fig. 6A**, treatment of cells with the LXR agonists, 22(*R*)-OH Ch, or 25-OH Ch, resulted in a 4- and 3-fold increase in promoter activity of Angptl3, respectively. In contrast, addition of 22(*S*)-OH Ch, an oxysterol that does not activate LXR, failed to significantly alter the Angptl3-promoter activity. In addition, a RXR agonist, 9-RA, in-

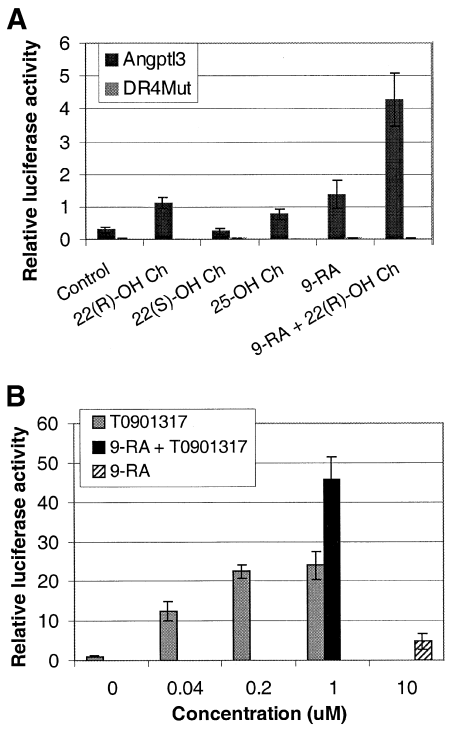

Fig. 6. Effect of LXR agonists on activity of a truncated Angptl3 promoter in HepG2 cells. A fragment of the human Angptl3 promoter (from −953 to +49 bp) with or without mutations at the DR4 element was linked to the firefly luciferase reporter gene. The resulting plasmid was cotransfected with a control reporter plasmid expressing *Renilla* luciferase into HepG2 cells. Cells were treated with (A) buffer, 10 μM of 22(*R*)-OH Ch, 25-OH Ch, 22(*S*)-OH Ch, 9-cis-retinoic acid (9-RA), or both 9-RA and 22(*R*)-OH Ch; or (B) buffer or increasing concentrations of T0901317 or combination of T0901317 (1 μM) and 9-RA (10 μM). After 42 h, the cells were lysed and relative luciferase activities were measured. Results are expressed as a ratio between the firefly and *Renilla* luciferase activities, and data are shown as the means ± SD of triplicates of a representative experiment. Three to four independent experiments were performed with similar results.

creased the promoter activity by about 5-fold. When 22(*R*)-OH Ch and 9-RA were combined for a single treatment, there was a synergistic ∼15-fold induction of the Angptl3 promoter activity. Similar but more profound inductions were observed when cells were treated with T0901317, a synthetic LXR-selective agonist (Fig. 6B). Treatment of cells with 1 μM of T0901317 increased the Angptl3 promoter activity by 24-fold. Cotreatment of 1 μM of T0901317 with 10 μM of 9-RA increased the Angptl3 promoter activity by 45-fold. These results suggest that transcriptional regulation of Angptl3 is transcriptionally regulated by LXR and RXR.

The DR4 sequence is a functional LXRE in the Angptl3 promoter

Earlier studies have shown that transcriptional regulation of several genes including ABCA1 (21–22), SREBP-1c (8), and LPL (10) by LXR requires the presence of the DR4 element. Analysis of the Angptl3 promoter identified a putative DR4 element (5′-AGGTTACATTCGTGCA-3′) 123 bp upstream of the transcription start site. To test if the DR4 element is indeed necessary for transcriptional activation of the Angptl3 gene by LXR, we introduced a mutation into the 5′ half site of the DR4 element within the Angptl3 promoter (5′-GGGCCCCATTCGTGCA-3′). As shown in Fig. 6A, this mutation completely abolished the inducible response of the human Angptl3 promoter to 22(*R*)-OH Ch, 25-OH Ch, 9-RA, or the combination of 22(*R*)-OH Ch and 9-RA. Therefore, these results strongly suggest that the identified DR4 element is necessary for LXR-responsiveness.

DISCUSSION

In light of the potential importance of Angptl3 in lipid metabolism (3), we studied the transcriptional regulation of Angptl3. We showed that expression of Angptl3 mRNA is upregulated in mice by cholesterol-feeding and by administration of the LXR agonist T0901317. Furthermore, treatment of HepG2 cells with natural or synthetic agents that selectively activate LXR increases Angptl3 expression. We cloned and analyzed the promoter of the human Angptl3 gene and identified an LXR response element, the DR4 element. We developed an assay that measures the activity of a truncated version of the human Angptl3 promoter. This truncated Angptl3 promoter is active in HepG2 cells and is induced by treatment of agents that activate LXR or RXR. Mutation of the DR4 element completely abolished the inducible response of the Angptl3 promoter to selective LXR or RXR agonists. Our data indicate that Angptl3 is a direct target gene of LXR. The DR4 element is conserved in the human and mouse Angptl3 promoters, suggesting that LXR responsiveness is also conserved.

In addition to the LXR response element, analysis of the Angptl3 promoter identified several potential binding sites for other transcription factors including HNF-1, HNF-4, NFκB, and C/EBP. Some of these potential binding sites were also conserved in both the mouse and human Angptl3 promoters, suggesting that those sites may be biologically relevant. HNF-1, HNF-4, and C/EBP are liver-restricted transcription factors, which are involved in live-specific gene expression (23–25). The identification of HNF-1 and c/EBP sites that are conserved in the mouse and human Angptl3 promoter may account for a restricted expression of Angptl3 in the liver.

Angiopoietins are members of the vascular endothelial growth factor family (26–27). Members of this family share the following characteristics: a signal peptide, an extended helical domain, a short linker peptide, and a globular fibrinogen-like domain. Several features distinguish Angptl3 from other angiopoietins, such as lack of a cysteine-based motif within the fibrinogen-like domain, lack of calcium binding motif, and almost exclusive expression in the liver (2).

Interestingly, it was recently found that mutation of Angptl3 is responsible for low plasma lipid levels in KK/

San mice, whereas administration of Angptl3 elicited a rapid increase in circulating levels of cholesterol, TG, and free fatty acid (3). Although these studies revealed a potential role for Angptl3 in lipid metabolism, they did not address the cellular and molecular mechanism(s) by which a hyperlipidemic response is mediated by Angptl3. Very recently, it was reported that Angptl3 may regulate levels of VLDL-TG via direct inhibition of lipoprotein lipase activity (28). Here we report for the first time a transcriptional regulation of Angptl3 by LXR, a critical lipid sensing transcription factor. Our findings are thus consistent with a role of Angptl3 in lipid metabolism, and will be useful for further elucidation of mechanism of the hyperlipidemic responses of Angptl3.

Administration of agents that selectively activate LXR results in an increase of total cholesterol (mainly HDL-C) and TG in plasma (11–12). ABCA1, a mediator of cholesterol efflux to apolipoprotein A-I, is a direct target gene of LXR (21–22). Thus, induction of ABCA1 by the treatment of LXR agonists may be responsible for an increase of HDL-C. However, a mechanism(s) for the LXR agonist-mediated increase of circulating TG remains to be elucidated. In addition to ABCA1, activation of LXR may also increase the expression of a number of genes involved in fatty acid metabolism, including SREBP-1c, fatty acid synthase, and lipoprotein lipase (8–10). Effects of LXR agonists on such lipogenesis genes are thus believed to be responsible for an increase of circulating TG. However, this interpretation is complicated by the fact that although overexpression of SREBP-1c in mice increases TG level in the liver, TG level in the plasma was reduced (13).

The exact role of Angptl3 in LXR-mediated hyperlipidemic response remains to be tested. Given the profound effect of Angptl3 on plasma lipid levels (3), it is possible that Angptl3 may play an important role in LXR-mediated increases of plasma lipids. It is interesting to note that administration of LXR agonists (11, 29) or Angptl3 (28) both result in increase of TG levels largely in the VLDL fraction. Administration of an LXR-agonist to the KK/San mouse, which has an Angptl3 mutation, could be used to test definitively an involvement of Angplt3 in LXR-mediated increases of plasma lipids.

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REFERENCES

1. Loughna, S., and T. N. Sato. 2001. Angiopoietin and Tie signaling pathways in vascular development. *Matrix Biol.* **20:** 319–325.
2. Conklin, D., D. Gilbertson, D. W. Taft, M. F. Maurer, T. E. Whitmore, D. L. Smith, K. M. Walker, L. H. Chen, S. Wattler, M. Nehls, and K. B. Lewis. 1999. Identification of a mammalian angiopoietin-related protein expressed specifically in liver. *Genomics.* **62:** 477–482.
3. Koishi, R., Y. Ando, M. Ono, M. Shimamura, H. Yasumo, T. Fujiwara, H. Horikoshi, and H. Furukawa. 2002. Angptl3 regulates lipid metabolism in mice. *Nat. Genet.* **30:** 151–157.
4. Kondo, K., K. Nozawa, T. Tomita, and K. Ezaki. 1957. Inbred strains resulted from Japanese mice. *Bull. Exp. Anim.* **6:** 107–112.
5. Nakamura, M. 1962. A diabetic strain of the mouse. *Proc. Jpn. Acad.* **38:** 348–352.
6. Nakamura, M., and K. Yamada. 1967. Studies on a diabetic (KK) strain of mice. *Diabetologia.* **3:** 212–221.
7. Repa, J. J., and D. J. Mangelsdorf. 2000. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu. Rev. Cell Dev. Biol.* **16:** 459–481.
8. Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J. M. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldstein, and D. J. Mangelsdorf. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* **14:** 2819–2830.
9. Joseph, S. B., B. A. Laffitte, P. H. Patel, M. A. Watson, K. E. Matsukuma, R. Walczak, J. L. Collins, T. F. Osborne, and P. Tontonoz. 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J. Biol. Chem.* **277:** 11019–11025.
10. Zhang, Y., J. J. Repa, K. Gauthier, and D. J. Mangelsdorf. 2001. Regulation of lipoprotein lipase by the oxysterol receptors, LXRalpha and LXRbeta. *J. Biol. Chem.* **276:** 43018–43024.
11. Schultz, J. R., H. Tu, A. Luk, J. J. Repa, J. C. Medina, L. Li, S. Schwendner, S. Wang, M. Thoolen, D. J. Mangelsdorf, K. D. Lustig, and B. Shan. 2000. Role of LXRs in control of lipogenesis. *Genes Dev.* **14:** 2831–2838.
12. Liang, G., J. Yang, J. D. Horton, R. E. Hammer, J. L. Goldstein, and M. S. Brown. 2002. Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *J. Biol. Chem.* **277:** 9520–9528.
13. Shimano, H., J. D. Horton, I. Shimomura, R. E. Hammer, M. S. Brown, and J. L. Goldstein. 1997. Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J. Clin. Invest.* **99:** 846–854.
14. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389–3402.
15. Dubchak, I., M. Brudno, G. G. Loots, L. Pachter, C. Mayor, E. M. Rubin, and K. A. Frazer. 2000. Active conservation of noncoding sequences revealed by three-way species comparisons. *Genome Res.* **10:** 1304–1306.
16. Mayor, C., M. Brudno, J. R. Schwartz, A. Poliakov, E. M. Rubin, K. A. Frazer, L. S. Pachter, and I. Dubchak. 2000. VISTA: visualizing global DNA sequence alignments of arbitrary length. *Bioinformatics.* **16:** 1046–1047.
17. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.
18. Wingender, E., X. Chen, R. Hehl, H. Karas, I. Liebich, V. Matys, T. Meinhardt, M. Pruss, I. Reuter, and F. Schacherer. 2000. TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acids Res.* **28:** 316–319.
19. Peet, D. J., S. D. Turley, W. Ma, B. A. Janowski, J. M. Lobaccaro, R. E. Hammer, and D. J. Mangelsdorf. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell.* **93:** 693–704.
20. Venkateswaran, A., J. J. Repa, J. M. Lobaccaro, A. Bronson, D. J. Mangelsdorf, and P. A. Edwards. 2000. Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J. Biol. Chem.* **275:** 14700–14707.
21. Schwartz, K., R. M. Lawn, and D. P. Wade. 2000. ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. *Biochem. Biophys. Res. Commun.* **274:** 794–802.
22. Costet, P., Y. Luo, N. Wang, and A. R. Tall. 2000. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J. Biol. Chem.* **275:** 28240–28245.
23. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science.* **243:** 1681–1688.

24. Ryffel, G. U. 2001. Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. *J. Mol. Endocrinol.* **27:** 11–29.
25. Sladek, F. M., W. M. Zhong, E. Lai, and J. E. Darnell, Jr. 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **4:** 2353–2365.
26. Davis, S., T. H. Aldrich, P. F. Jones, A. Acheson, D. L. Compton, V. Jain, T. E. Ryan, J. Bruno, C. Radziejewski, P. C. Maisonpierre, and G. D. Yancopoulos. 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell.* **87:** 1161–1169.
27. Maisonpierre, P. C., C. Suri, P. F. Jones, S. Bartunkova, S. J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T. H. Aldrich, N. Papadopoulos, T. J. Daly, S. Davis, T. N. Sato, and G. D. Yancopoulos. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science.* **277:** 55–60.
28. Shimizugawa, T., M. Ono, M. Shimamura, K. Yoshida, Y. Ando, R. Koishi, K. Ueda, T. Inaba, H. Minekura, T. Kohama, and H. Furukawa. 2002. ANGPTL3 decreases VLDL triglyceride clearance by inhibition of lipoprotein lipase. *J. Biol. Chem.* **277:** 33742–33748.
29. Grefhorst, A., B. M. Elzinga, P. J. Voshol, T. Plösch, T. Kok, V. W. Bloks, F. H. van der Sluijs, L. M. Havekes, J. A. Romijn, H. J. Verkade, and F. Kuipers. 2002. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J. Biol. Chem.* **277:** 34182–34190.