Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice[S]

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Abstract **High-cholesterol diets elicit changes in gene expression via such transcription factors as sterol-regulatory element binding proteins (SREBPs) and liver X receptors (LXRs). We used Affymetrix microarrays to identify genes in mouse liver regulated by dietary cholesterol (0.0% vs. 0.5% cholesterol wt/wt). Three independent experiments were performed, and data were analyzed with Affymetrix Microarray Suite and ANOVA statistical software. There were 69 unique Unigene clusters consistently regulated by dietary cholesterol (37 downregulated and 32 upregulated). The array results were confirmed by quantitative RT-PCR (Q-PCR) for seven of nine downregulated genes and five of six upregulated genes. A time course of dietary cholesterol feeding over 1 week revealed different temporal patterns of gene regulation for these confirmed genes. Six downregulated genes were examined in transgenic mice overexpressing truncated nuclear forms of SREBP-1a and SREBP-2, and all were induced in these mice. A second microarray analysis of mice treated with the LXR agonist TO901317 confirmed that 13 of the 32 cholesterol upregulated genes were also LXR-activated. This array result was confirmed by Q-PCR for three of three genes. In summary, these studies identified and confirmed six novel dietary cholesterol-regulated genes, three putative SREBP target genes (calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, and proprotein convertase subtilisin/kexin 9), and three putative LXR target genes (a disintegrin and metalloprotease domain 11, apoptosis-inhibitory 6, and F-box-only protein 3).**—Maxwell, K. N., R. E. Soccio, E. M. Duncan, E. Sehayek, and J. L. Breslow. **Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J. Lipid Res.* 2003.** 44: **2109–2119.**

Supplementary key words dietary cholesterol • oligonucleotide arrays • gene expression profiling • sterol-regulatory element binding proteins • liver X receptor

Cholesterol is an important constituent of cellular membranes and serves as a precursor in the formation of bile acids and steroid hormones. Excessive cholesterol, however, is involved in atherosclerotic lesion and gallstone formation. Therefore, a balance must be maintained between cholesterol absorption and excretion and endogenous cholesterol synthesis. In this regard, the liver plays an important role. For example, increasing dietary cholesterol results in downregulation of lipoprotein receptors such as the LDL receptor and cholesterol biosynthetic enzymes like HMG-CoA reductase (HMGCR) and results in upregulation of the bile acid biosynthetic enzyme cholesterol 7-α-hydroxylase (CYP7A1) (1, 2).

Cholesterol can regulate expression at both transcriptional and posttranscriptional levels, and in the former, two key transcription factors have been implicated, sterol-regulatory element binding protein (SREBP) and the liver X receptor (LXR) (3, 4). The SREBP pathway utilizes three transcription factors, SREBP-1a, -1c, and -2, which are synthesized as transmembrane precursors in the endoplasmic reticulum with an N-terminal basic helix-loop-helix-Zip transcription factor domain facing the cytoplasm. When cellular sterols are low, the SREBP cleavage-activating protein (SCAP)-INSIG-1 complex brings the SREBP to the Golgi, where the site 1 protease cleaves intraluminally and the site 2 protease cleaves intramembraneously to release the N-terminal domain of the protein into the cytoplasm. This N-terminal domain translocates to the nucleus and activates gene transcription (5–7). Korn et al. (8) showed that the SREBPs are responsive to cellular sterol levels in vivo, as the amounts of nuclear SREBP-1 and SREBP-2 are

Abbreviations: Abcg5, ATP binding cassette, subfamily G, member 5; Acac, acetyl-CoA carboxylase; Acly, ATP citrate lyase; Adam11, a disintegrin and metalloprotease domain 11; Api6, apoptosis-inhibitory 6; Camk1D, calcium/calmodulin-dependent protein kinase 1D; CYP7A1, cholesterol 7-α-hydroxylase; FABP5, fatty acid binding protein 5; Fbxo3, F-box-only protein 3; HMGCR, HMG-CoA reductase; HMGCS, HMG-CoA synthase; Laptm5, lysosomal-associated protein transmembrane 5; LXR, liver X receptor; Pcsk9, proprotein convertase subtilisin/kexin 9; Rgs16, regulator of G-protein signaling 16; SAA3, serum amyloid A 3; Sqle, squalene epoxidase; SREBP, sterol-regulatory element binding protein; StarD4, StAR-related lipid transfer domain containing 4.

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[S] The online version of this article (available at http://www.jlr.org) contains one supplemental table.

Manuscript received 15 May 2003 and in revised form 25 July 2003.

Published, JLR Papers in Press, August 1, 2003.
DOI 10.1194/jlr.M300203-JLR200

decreased by a 1% cholesterol/0.5% cholic acid diet. Studies with mice overexpressing truncated nuclear forms of SREBP-1a, -1c, and -2 have revealed that SREBP-2 preferentially activates genes involved in cholesterol biosynthesis and metabolism, such as HMG-CoA synthase (HMGCS) and HMGCR, whereas SREBP-1c and -1a preferentially activate genes involved in fatty acid biosynthesis, such as acetyl-CoA carboxylase (Acac) and ATP citrate lyase (Acly) (9–12).

LXRs are nuclear hormone receptors that form obligate heterodimers with the retinoid X receptor. When bound to their oxysterol ligands, LXRs activate transcription of target genes (3). The importance of the LXR pathway was demonstrated in part by studies in knockout mice. When both wild-type and LXRα knockout mice were challenged with increased dietary cholesterol, LXRα knockout mice showed a greater increase in liver cholesterol, increased plasma cholesterol, blunted bile acid excretion, and the inability to upregulate CYP7A1, the rate-limiting step in bile acid production (13). It has also been shown that LXRs control genes involved in sterol movement across membranes, including ATP binding cassettes G5 and G8 (Abcg5 and Abcg8) and ABCA1, in lipoprotein metabolism, including cholesterol ester transfer protein, and in fatty acid synthesis, including SREBP-1c, among others (14).

While much information about cholesterol-responsive genes has been obtained from in vitro work and studies with genetically modified mice, it is important to understand the mechanisms an intact organism uses to respond to increased levels of dietary cholesterol. This study aims to identify genes in the mouse liver that are important in this regard by using microarrays. Affymetrix oligonucleotide microarrays were interrogated with liver RNA samples from mice fed 0.0% versus 0.5% cholesterol diets. The combined analysis of three experiments identified 69 genes regulated by dietary cholesterol, 37 downregulated and 32 upregulated. Regulation of a subset of the identified array genes was confirmed using quantitative RT-PCR (Q-PCR) analysis. Further studies are presented regarding the time course of regulation by dietary cholesterol and the role of the SREBP and LXR transcription factors. These studies have allowed the identification of three novel putative SREBP and three novel putative LXR target genes.

MATERIALS AND METHODS

Animals and diets

All animal protocols were approved by The Rockefeller University Animal Care and Use Committee. Wild-type C57BL/6 mice, SREBP-1a transgenic mice, and SREBP-2 transgenic mice were obtained from the Jackson Laboratory (stock numbers 00664, 002840, and 003311, respectively). Animals were housed in a humidity- and temperature-controlled room with a 12-h dark/12-h light cycle at the Laboratory Animal Research Center at The Rockefeller University. For dietary studies, 6-week-old C57BL/6 male and female mice were fed a semisynthetic modified AIN76a diet containing 10% kcal as fat and 0.0% cholesterol [Clinton/Cybulsky Rodent Diet; Research Diets D12102N (15)] for 1 week. The mice were then split into two groups and fed either the 0.0% cholesterol diet or the same diet supplemented with 0.5% (wt/wt) cholesterol (Research Diets D00083101; cholesterol is added as a powder prior to pelleting) for 1 week prior to sacrifice. For the time course studies, 20 male and 20 female 6-week-old mice were fed the 0.0% cholesterol diet for 1 week; four mice were sacrificed at the 1-week time point and the remaining mice were switched to a 0.5% cholesterol diet. Four mice were sacrificed at days 1, 2, 4, and 7 of feeding. For the LXR agonist study, 7-week-old male C57BL/6 mice were placed on the 0.0% cholesterol diet for 1 week and then gavaged with vehicle alone (5% ethanol, 95% sesame oil) or with vehicle plus 10 mg/kg TO901317 (Sigma T2320). This treatment was repeated after 24 h, and mice were sacrificed on the same day. Transgenic mice expressing truncated nuclear forms of human SREBP-1a or SREBP-2, backcross generations to C57BL/6 at N6/N7 and N2/N3, respectively, were used. Mice were genotyped by PCR from tail tip DNA (see supplementary data for primer sequences). Transgenic and littermate control mice of both sexes were fed standard rodent chow from birth to 8 weeks. They were then switched to a 65% protein, 10% carbohydrate diet (Purina TestDiet 8092) for 2 weeks to induce maximal transgene expression, because the SREBP transgenes were under the control of the phosphoenolpyruvate carboxykinase promoter. At the end of all mouse experiments, food was removed from the cage at 9 AM, and the mice were then fasted for 5 h, sedated with ketamine/xylazine, and sacrificed. Blood was collected by left ventricular puncture, and harvested liver slices were frozen in liquid nitrogen and stored at −80°C or stored in RNAlater (Ambion) according to the manufacturer's instructions.

Plasma and liver cholesterol measurements

Plasma was separated from whole blood by centrifugation. Plasma total cholesterol (mg/dl) was measured enzymatically using the Infinity Cholesterol Reagent (Sigma Diagnostics, 402-100P). Liver total, free, and esterified cholesterol (mg/g liver) were measured by gas chromatography with coprostanol as an internal standard as previously described (16).

Sample preparation for gene expression analysis

Liver tissue in RNA later was homogenized in TRIzol reagent (Invitrogen), and total RNA was isolated according to the manufacturer's instructions. Total RNA was then subjected to RNeasy Cleanup (Qiagen) for microarray and Q-PCR. For microarrays, 20 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and a poly-dT primer containing the T7 RNA polymerase binding site (Genset Corporation). Second-strand cDNA was then made using *Escherichia coli* DNA polymerase, *E. coli* DNA Ligase, and T4 DNA polymerase (Invitrogen). Double-stranded cDNA was purified using phenol-chloroform-isoamyl alcohol on Phase-Lock gel columns (Eppendorf), ethanol precipitated, and resuspended in water. cRNA was synthesized from the cDNA using biotin-labeled ribonucleotides and T7 RNA polymerase (Enzo Bioarray/Affymetrix) and purified on Qiagen RNeasy columns. cRNA was then fragmented for 30 min at 95°C in a solution of 40 mM *Tris*-acetate pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate. For Q-PCR analysis, total RNA was treated with Dnase I (Ambion), and 5 μg was reverse transcribed using Superscript II and a mixture of oligo-dT and random hexamer primers.

Affymetrix oligonucleotide microarrays

Three microarray experiments were performed using cholesterol-fed and control mice according to the protocol above. Six male mice were used for experiment 1 ($n = 3$ on each diet), and 10 male and 10 female mice ($n = 5$ on each diet) were used for

experiment 2. In these experiments, mice were housed together in cages. Equal amounts of liver RNA samples from mice within a sex and feeding group were pooled together and split into duplicate samples for cDNA and cRNA synthesis and application to the microarrays. In experiment 3, eight male and eight female mice ($n = 4$ on each diet) were housed in separate metabolic cages to prevent coprophagia and competition for food, and liver RNA from each mouse was processed for an individual microarray. For all three experiments, fragmented cRNA samples were combined with hybridization controls (Affymetrix), herring sperm DNA (Promega), and acetylated BSA (Invitrogen) and hybridized to microarrays for 16 h at 45°C. Each sample was hybridized to the Affymetrix Test3 microarray and the MGU74v2 microarray set (three chips: A, B, and C). This set contains >36,000 probe sets corresponding to >23,000 Unigene clusters (for information, see www.affymetrix.com). Washing, staining, and scanning of the microarrays was performed by the Gene Array Core Facility at Rockefeller University according to standard protocols (Affymetrix).

Data analysis of Affymetrix microarrays

The raw data from the microarrays was analyzed using the Microarray Suite Software version 5.0 (MAS5.0, Affymetrix). Each array was subjected to absolute analysis, and all chips showed proper behavior of spiked controls, signal ratios of 5′ to 3′ sequences from housekeeping genes approximately equal to 1, and low background and noise values. The signal values for all arrays were scaled using a scaling factor (SF) derived from the formula: SF × mean signal = 250. For experiments 1 and 2, there were duplicate arrays for each diet, so four pair-wise comparisons were performed between feeding groups within a sex. In both experiments, genes with a signal in at least one condition >250 and average fold for the pair-wise comparisons >|1.6| were included in the preliminary analysis. As experiment 3 had four mice per group, this allowed examination of biological variation in gene expression, and statistical analysis was performed to determine significant differences between the groups. From an initial power calculation, utilizing the average variance for all genes on the MGU74v2 chip set in the 0.0% cholesterol male group, it was determined that with four replicates, a fold >|1.6| could be reliably discerned if genes with a signal >250 were used. Therefore, the experiment 3 signals derived from the MAS5.0 absolute analysis were subjected to an ANOVA statistical analysis software package (17). Genes in experiment 3 were determined to be significantly regulated by dietary cholesterol if the signals in at least one group were >250, the fold >|1.6|, and the ANOVA P value < 0.001. Genes were also kept in the final analysis if in experiment 3 their ANOVA P value was between 0.01 and 0.001, and they also met the criteria specified above for the preliminary analysis in experiments 1 or 2. Information regarding each probe set identified in the combined statistical and MAS analysis was downloaded from www.affymetrix.com. This information included the gene names shown in **Tables 1** and **2**. Other information such as accession number and Unigene cluster can be obtained by searching with the probe set identifier. In cases where the probe set was not assigned to a gene by Affymetrix, the sequence was used to query the mouse genome (www.ensembl.org) and identify a putative gene. This information is in parentheses next to the Affymetrix name in Tables 1 and 2.

Real-time quantitative Q-PCR

Q-PCR was performed as described (18) with slight modifications. Briefly, cDNA was synthesized as above and 5 or 10 μl of diluted cDNA (1:100, v/v, or 1:250, v/v, in water) was used as template. Each sample was amplified in duplicate for the genes of interest and a housekeeping gene, cyclophilin A or hypoxanthine guanine phosphoribosyl transferase, on an Applied Biosystem 7900 or 7700 Sequence Detection System using the quencher dye TAMRA as a passive reference. Sequences of forward and reverse primers and TaqMan probes are available in the supplementary data. The threshold was set in the linear range of normalized fluorescence, and a threshold cycle (C_t) was measured in each well; data were analyzed as in Soccio et al. (18).

Cloning of calcium/calmodulin-dependent protein kinase 1D and proprotein convertase subtilisin/kexin 9

At the time these studies were performed, two genes of interest had not been cloned. The Affymetrix probe set sequence was used to query the mouse genome, and putative coding sequences were identified and confirmed by RT-PCR from mouse liver. PCR reagents were Advantage cDNA polymerase (Clontech) and Super Taq Plus polymerase (Ambion), primers were from GeneLink (sequences in the supplementary data), and thermal cycling was on a Perkin-Elmer 9700. The 5′ ends of both genes were identified by 5′ rapid amplification of cDNA ends (RACE) (Ambion 5′ RLM-RACE), and the 3′ end was deduced by identification of a poly-A signal (ATTAAA) in the genome sequence in agreement with transcript size on Northern blotting. PCR products were TA cloned with pCR-2.1-TOPO (Invitrogen) and the sequence verified. Sequences have been deposited in GenBank [Accession Numbers: proprotein convertase subtilisin/kexin 9 (Pcsk9)-AY273821, and calcium/calmodulin-dependent protein kinase 1D (Camk1D)-AY273822].

RESULTS

Dietary cholesterol feeding paradigm

In order to identify liver genes that respond to dietary cholesterol in an intact organism, C57BL/6 mice were fed a semisynthetic diet with either 0.0% cholesterol or supplemented with 0.5% cholesterol. As shown in **Fig. 1A**, this feeding paradigm did not significantly raise plasma total cholesterol levels in males or females. However, this feeding paradigm significantly raised the levels of total, free, and esterified cholesterol in the liver in both sexes (Fig. 1B, 1C, and 1D, respectively). Males increased liver total cholesterol, free cholesterol, and cholesterol ester by 4.4-fold, 1.2-fold, and 15.2-fold, respectively, and females increased liver total cholesterol, free cholesterol, and cholesterol ester by 6.0-fold, 1.8-fold, and 10.3-fold, respectively. Therefore, despite no effect on plasma cholesterol levels, this feeding paradigm raised liver cholesterol levels and allowed a study of the effect of dietary cholesterol on liver gene expression.

Oligonucleotide microarrays

Liver genes regulated at the mRNA level by cholesterol feeding were determined using the Affymetrix oligonucleotide microarray three-chip MGU74v2 set. Three experiments were performed. RNA was pooled from three male mice per diet in experiment 1 and separately from five male and five female mice per diet in experiment 2. For each of these pools, two aliquots of RNA were studied. In experiment 3, RNA from four individual male and four individual female mice per diet were studied separately. Microarray data were analyzed with MAS5.0 as described in Materials and Methods, which allows only pair-wise comparisons between chips. Genes were retained with signal

TABLE 1. Genes downregulated by a 1-week, high-cholesterol diet

NetAffx ID	Gene Name	Males MAS Fold Ex. 1	Males MAS Fold Ex. 2	Males MAS Fold Ex. 3	Males P	Females MAS Fold Ex. 2	Females MAS Fold Ex. 3	Females P	LXR Agonist Array
Cholesterol metabolism									
97518_at	FPP farnesyl transferase 1[a]	−1.7	−1.5	−2.0	4.30E-03	−1.6	−1.4	3.50E-04	NC
99098_at	FPP synthetase[a]	−5.1	−4.8	−8.7	1.20E-04	−3.9	−6.9	1.30E-04	NC
160424_f_at	FPP synthetase[a]	−3.8	−3.4	−6.3	9.50E-04	−2.9	−4.9	6.80E-04	NC
94325_at	Hypothetical protein MGC36662 (HMGCS)[a]	−2.7	−2.4	−4.4	8.20E-03	−2.5	−1.6	4.10E-02	NC
93868_at	NADP-dependent steroid dehydrogenase-like	−3.3	−2.9	−5.1	6.40E-04	−2.5	−3.0	3.00E-03	NC
98630_at	NADP-dependent steroid dehydrogenase-like	−2.3	−1.7	−2.6	9.30E-05	−1.6	−1.8	4.70E-03	NC
98631_g_at	NADP-dependent steroid dehydrogenase-like	−2.2	−2.5	−4.5	7.10E-05	−2.2	−2.8	1.30E-02	NC
105991_at	NADP-dependent steroid dehydrogenase-like	−5.1	−8.3	−5.7	1.80E-04	−2.2	−3.9	1.90E-03	abs
168360_f_at	NADP-dependent steroid dehydrogenase-like	−2.6	−3.5	−4.1	5.70E-04	−2.5	−3.9	9.40E-04	n.d.
111380_at	RIKEN cDNA 1110011E12 (PMVK-related)[a]	−2.1	−2.1	−4.1	2.30E-03	−2.2	−3.1	4.60E-03	NC
167266_f_at	RIKEN cDNA 1110011E12 (PMVK-related)[a]	−1.7	−2.1	−2.7	1.90E-04	−2.1	−2.4	7.60E-04	n.d.
96269_at	Similar to isopentenyl-diphosphate delta isomerase[a]	−4.1	−4.7	−6.6	1.10E-03	−4.8	−7.0	2.90E-03	NC
164172_at	Similar to isopentenyl-diphosphate delta-isomerase[a]	−2.5	−2.7	−3.5	4.90E-04	−3.1	−3.2	1.30E-03	NC
94322_at	Sqle[a]	−3.5	−2.3	−4.5	7.60E-03	−2.1	−3.5	1.10E-03	NC
160388_at	Sterol-C4-methyl oxidase-like	−3.0	−3.0	−3.8	2.60E-03	−3.1	−2.9	8.00E-03	NC
102768_i_at	Sterol-C5-desaturase	−1.9	−1.9	−2.2	6.50E-03	−1.7	−1.2	5.20E-02	NC
102769_f_at	Sterol-C5-desaturase	−1.9	−2.2	−2.4	3.60E-04	−1.7	−1.3	8.20E-02	NC
Fatty acid metabolism									
106070_at	Acetyl-CoA synthetase 1, AMP forming[a]	−2.4	−2.3	−4.9	3.90E-03	−2.5	−2.2	1.40E-02	2.7
97248_at	Diazepam binding inhibitor, acyl-CoA binding protein[a]	−1.0	−1.3	−1.4	1.50E-03	−1.2	−1.9	8.80E-05	1.6
160544_at	FABP5, epidermal	−2.8	−1.6	−2.8	7.10E-04	−1.9	−2.6	1.00E-01	2.9
101082_at	Malic enzyme[a]	−1.2	−1.5	−3.0	3.20E-04	−1.8	−1.4	2.10E-02	4.8
160391_at	RIKEN cDNA 0710001O03 (delta-5 desaturase)[a]	−1.5	−1.5	−2.3	1.80E-04	−1.5	−1.7	1.60E-03	NC
Other lipid metabolism (or hypothesized role)									
99592_f_at	Androgen-regulated short-chain dehydrogenase/reductase 1	−1.6	−1.5	−2.1	2.10E-04	−1.7	−1.5	2.70E-03	NC
100927_at	PLTP	−2.0	−1.0	−1.6	8.10E-03	−2.7	−2.2	2.50E-03	1.9
139034_at	RIKEN cDNA 1110033E03 (CTPCT related)[a]	−0.5	−1.2	−1.7	5.70E-03	−1.1	−1.6	1.10E-01	n.d.
169465_s_at	RIKEN cDNA 3110041O18 (TM7SF2, lamin-B receptor related)	−2.0	−2.4	−2.6	3.00E-04	−1.8	−3.2	1.10E-04	n.d.
92437_at	RIKEN cDNA 3110041O18 (TM7SF2, lamin-B receptor related)	−2.0	−1.6	−1.8	1.40E-03	−1.5	−2.2	5.70E-04	NC
113753_at	START domain containing 4	−1.4	−1.5	−2.1	1.90E-03	−2.3	−2.2	2.20E-03	NC
116072_at	START domain containing 4	−1.5	−1.7	−2.2	4.80E-05	−1.8	−2.6	8.40E-04	NC
Miscellaneous									
102200_at	Aquaporin 8	−1.5	−1.2	−1.8	8.00E-03	−1.4	−1.4	5.50E-02	NC
97334_at	Hairy and enhancer of split 6	−1.0	−1.2	−1.2	9.90E-02	−1.3	−1.6	3.90E-04	NC
138949_at	Heat shock 27 kDa protein 3	−1.1	−1.3	−1.0	8.30E-01	−1.5	−2.3	2.90E-03	n.d.
94378_at	Rgs16	−1.4	−1.9	−2.4	2.70E-03	−1.4	−1.6	1.40E-02	−2.8
161609_at	Rgs16	−1.5	−2.0	−2.2	2.60E-03	−1.4	−1.4	7.60E-02	−1.8
100758_at	Ribosomal protein S28	−1.1	−0.9	−1.1	2.20E-01	−0.9	−1.6	1.60E-05	NC
102098_at	RIKEN cDNA 2410038A03 (ribosomal protein L44-related)	−1.1	−1.0	−1.2	8.00E-02	−0.9	−1.6	1.90E-04	NC
99115_at	RIKEN cDNA 2610041P16 (ubiquinol cyt c reductase-related)	−1.1	−1.1	−1.2	1.80E-01	−0.9	−1.6	4.40E-04	NC
104072_at	Serum amyloid P-component	−1.9	−2.4	−1.8	6.10E-04	−1.1	−1.4	1.30E-01	NC
114299_at	T-complex testis expressed 1	−1.0	−1.4	−1.1	2.30E-01	−0.9	−1.7	1.80E-04	NC
111819_at	Ubiquitously-expressed transcript	−0.9	−0.9	−1.1	5.50E-01	−0.7	−1.6	3.40E-05	NC
Unclassified									
95749_at	Arginine-rich, mutated in early stage tumors	−1.5	−1.5	−1.1	5.90E-01	−0.8	−1.7	9.10E-04	NC
137560_at	ESTs	−0.9	−1.3	−1.6	3.70E-04	−1.2	−1.3	2.00E-01	n.d.
131854_at	ESTs, weakly similar to NED4_MOUSE	−1.7	−1.5	−1.5	6.90E-05	−1.0	−1.4	9.60E-04	n.d.
108752_at	Expressed sequence AI747682 (Pcsk9)	−1.3	−1.8	−2.1	1.20E-03	−1.9	−1.9	1.10E-03	1.7
109426_at	Liver-expressed antimicrobial peptide 2	−1.0	−1.5	−1.3	3.40E-01	−0.9	−1.9	1.10E-04	NC
168386_f_at	RIKEN cDNA 1810044O22 (cytochrome B5 related)	−1.0	−1.8	−1.6	1.90E-03	−1.0	−1.3	1.60E-01	n.d.
99854_at	Sulfotransferase-related protein SULT-X2	−0.8	−0.7	−1.5	4.60E-01	−2.0	−2.8	7.00E-03	abs
163290_at	UI-1-CF0-apo-c-08-0-UI.s1 Mus musculus cDNA (Camk1D)	−1.7	−1.7	−2.0	3.00E-03	−1.4	−1.3	8.70E-03	NC

abs, genes called absent; Camk1D, calcium/calmodulin-dependent protein kinase 1D; CTPCT, CTP-choline phosphate cytidyltransferase; EST, expressed sequence tag; Ex., experiment; FABP5, fatty acid binding protein 5; FPP, farnesyl diphosphate; HMGCS, HMG-CoA synthase; LXR, liver X receptor; NC, fold <|1.6|; n.d., genes on C chip of MGU74v2 set; Pcsk9, proprotein convertase subtilisin/kexin 9; PMVK, phosphomevalonate kinase; Rgs16, regulator of G-protein signaling 16; Sqle, squalene epoxidase; START, StAR-related lipid transfer.

[a] Published regulation by sterol-regulatory element binding protein (SREBP) transcription factors.

TABLE 2. Genes upregulated by a 1-week, high-cholesterol diet

NetAffx ID	Gene Name	Males MAS Fold Ex. 1	Males MAS Fold Ex. 2	Males MAS Fold Ex. 3	Males P	Females MAS Fold Ex. 2	Females MAS Fold Ex. 3	Females P	LXR Agonist Array[b]
Metabolism									
94354_at	ABCA1[a]	1.3	1.1	1.3	5.20E-02	1.3	1.6	1.70E-04	2.3
114831_at	Abcg5[a]	1.9	2.5	2.2	9.40E-03	1.8	1.6	1.40E-02	2.1
101638_s_at	Cytochrome P450, 3a16	1.5	3.4	3.1	3.00E-05	1.4	1.5	1.10E-04	2.3
93770_at	Cytochrome P450, steroid inducible 3a11	1.4	2.7	2.6	2.80E-05	1.3	1.2	1.40E-04	1.8
103581_at	Cytosolic acyl-CoA thioesterase 1	3.6	1.6	2.3	2.90E-03	1.0	1.1	4.90E-01	4.0
160074_at	Dopa decarboxylase	1.4	1.6	1.5	8.10E-02	1.7	1.6	3.70E-03	1.7
97317_at	Ectonucleotide pyrophosphatase/phosphodiesterase 2	1.2	1.2	1.3	1.10E-02	1.2	1.6	1.60E-04	NC
101587_at	Epoxide hydrolase 1, microsomal	1.4	2.0	2.0	3.50E-04	1.4	1.6	1.10E-02	1.9
112669_at	EST AI266984 (esterase related)	1.6	1.5	1.6	3.60E-04	1.6	1.4	8.90E-03	5.0
168829_r_at	EST AL022671 (START domain containing 7 homolog)	0.5	1.0	1.0	2.90E-01	3.2	4.9	7.90E-04	n.d.
100611_at	Lysozyme	0.9	1.9	2.0	1.60E-04	0.9	1.9	5.80E-03	abs
101753_s_at	P lysozyme structural	1.0	2.2	2.3	7.30E-05	0.9	1.7	2.20E-03	abs
99571_at	Protein MGC29978 (similar to peroxisomal 3-oxoacyl-CoA thiolase)	1.5	1.5	1.7	2.80E-04	1.1	1.1	1.50E-01	3.6
Immune/acute phase response									
92223_at	Complement component 1, q subcomponent, c polypeptide	1.0	1.4	1.6	1.40E-04	1.0	1.2	3.70E-02	NC
94285_at	Histocompatibility 2, class II antigen E β	1.0	1.7	1.7	3.60E-04	1.1	1.3	6.20E-03	NC
94734_at	Orosomucoid 2	1.3	0.9	2.2	1.00E-02	1.9	2.2	2.20E-03	abs
100333_at	SAA1	1.2	0.3	2.2	5.50E-03	1.6	1.7	1.20E-02	NC
103465_f_at	SAA2	1.4	0.2	3.6	1.20E-02	2.9	3.2	2.70E-03	NC
102712_at	SAA3	2.9	1.4	8.9	5.60E-03	2.1	3.7	2.00E-02	abs
114603_at	SAA4	1.1	0.7	1.6	7.40E-04	1.7	1.2	2.80E-01	−2.5
Transcription factors									
170720_r_at	Myeloid ecotropic viral integration site 1	2.1	1.3	1.4	1.10E-03	0.9	1.2	8.30E-01	n.d.
140898_at	Zinc finger protein, multitype 2	1.4	2.2	1.9	5.90E-04	0.4	1.0	6.00E-01	n.d.
Unclassified									
93445_at	Api6	1.0	1.7	1.7	6.50E-02	1.2	1.9	2.80E-04	1.7
116554_at	EST AW060611 (Adam11)	7.1	5.4	5.0	9.50E-03	4.4	7.8	6.10E-07	12.8
164261_at	ESTs	2.1	1.4	2.2	2.50E-03	1.0	1.1	6.20E-01	4.7
133804_at	ESTs	1.1	1.7	1.7	1.20E-03	1.4	1.7	1.10E-02	n.d.
103379_at	Fbxo3	1.1	1.2	1.3	1.20E-01	1.1	1.7	4.80E-04	1.6
129871_at	ESTs	0.7	1.7	1.9	5.00E-04	2.3	1.1	9.90E-01	n.d.
101872_at	Glutathione S-transferase, α 2, Yc2	1.6	2.9	1.8	5.40E-03	1.7	1.9	7.90E-02	1.6
100012_at	Laptm5	0.9	1.5	1.9	5.70E-04	1.6	1.2	1.70E-01	abs
99071_at	Macrophage expressed gene 1	1.0	1.5	1.6	6.70E-03	1.1	1.7	7.10E-04	1.6
96271_at	RIKEN cDNA 2310075C12 gene	1.1	1.1	1.2	7.40E-02	1.2	1.6	1.30E-04	NC

ABCA1, ATP binding cassette A1; Adam11, a disintegrin and metalloprotease domain 11; Api6, apoptosis inhibitory 6; Fbxo3, F-box-only protein 3; Laptm5, lysosomal-associated protein transmembrane 5; SAA3, serum amyloid A 3.

[a] Published regulation by LXR transcription factors.

values on at least one condition >250, fold >|1.6|, and denoted changed by MAS5.0 on at least 75% of pair-wise comparisons. This analysis yielded 39, 45, and 50 downregulated probe sets and 28, 66, and 38 upregulated probe sets in males in experiments 1, 2, and 3, respectively. This analysis yielded 32 and 39 downregulated probe sets and 16 and 25 upregulated probe sets in females in experiments 2 and 3, respectively.

The design of experiment 3 allowed a statistical analysis of the microarray results. Using an ANOVA statistical analysis software package (17), genes were determined to be significantly regulated by the 0.5% cholesterol diet if the signals in at least one group were >250, fold >|1.6|, and with an ANOVA P value < 0.001. Genes were also kept in the analysis if the ANOVA P value in experiment 3 was between 0.01 and 0.001, and if they also met the criteria for MAS5.0 comparison analysis in experiments 1 or 2. This more stringent analysis yielded 48 downregulated probe sets and 32 upregulated probe sets that showed regulation by dietary cholesterol in males and/or females.

Genes regulated by dietary cholesterol

Tables 1 and 2 show the 48 probe sets representing 37 Unigene clusters downregulated by dietary cholesterol and the 32 probe sets representing 32 Unigene clusters upregulated by dietary cholesterol in this study. The tables also show P values for experiment 3 as derived from the ANOVA analysis and the average folds for all pair-wise comparisons from each experiment as derived from the MAS5.0 software. The cholesterol-regulated genes were classified according to the categories specified by Affymetrix.

Among the 37 genes downregulated by dietary cholesterol (Table 1), 19 were classified in cholesterol metabolism, fatty acid metabolism, or the metabolism of other lipids. Eleven of these have previously been shown to be SREBP targets (marked by superscript *a*). Squalene epoxidase (Sqle), isopentenyl diphosphate delta isomerase, far-

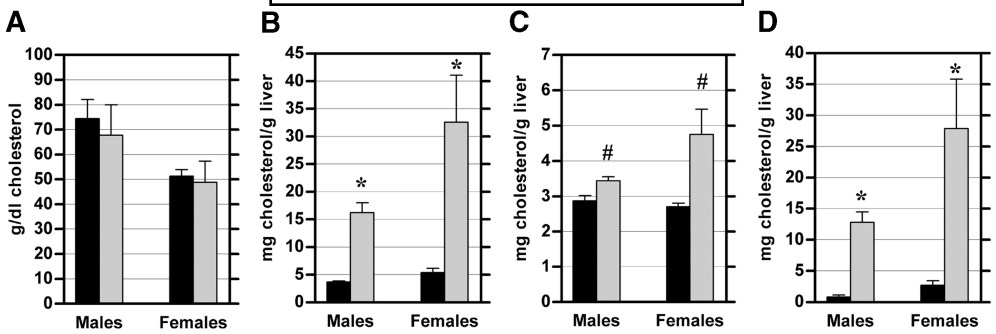

Fig. 1. The feeding paradigm does not raise plasma total cholesterol levels but does raise hepatic cholesterol levels. Male and female mice ($n = 5$ per group) were fed a semisynthetic diet with 0.0% cholesterol (black bars) or supplemented with 0.5% cholesterol (gray bars) for 7 days. Blood and liver tissue were collected as described, plasma cholesterol levels were measured by enzymatic assay, and liver cholesterol levels were measured by gas chromatography. The feeding paradigm did not change plasma total cholesterol levels in male or female mice (A). The feeding paradigm significantly raised hepatic total cholesterol levels (B), free cholesterol (C), and cholesterol esters (D) in both male and female mice. # $P < 0.01$; * $P < 0.001$.

nesyl diphosphate farnesyl transferase, phosphomevalonate kinase, farnesyl diphosphate synthetase, and HMGCS are genes in the cholesterol biosynthetic pathway (19–22). Malic enzyme and acetyl-CoA synthetase are involved in fatty acid synthesis (23, 24). Acyl-CoA binding protein is implicated in acyl-CoA transport and steroidogenesis (25). Delta-5 desaturase is necessary for the conversion of dietary linoleic acid (Ω3 series) and α-linolenic acid (Ω6 series) to the biologically active arachidonic acid, eicosopentenoic acid, and docosahexaenoic acid (26). CTP-choline phosphate cytidyltransferase is involved in phosphatidylcholine biosynthesis (27). The other eight genes in the metabolism categories, as well as the remaining 18 genes in the miscellaneous and unclassified categories, are not previously known SREBP targets.

Among the 32 genes upregulated by dietary cholesterol (Table 2), 13 genes were classified in metabolism. Two of these have previously been shown to be LXR targets (marked by superscript *a*). Abcg5 is involved in sterol transport in the intestine and liver (28). ABCA1 mediates cholesterol efflux, and its mRNA is highly regulated by LXR in the macrophage (29). Although in one previous study, liver ABCA1 mRNA levels were not increased by an LXR agonist (30), another study showed increased liver protein levels in mice fed a high-fat/high-cholesterol diet (31). In the current study, liver ABCA1 mRNA levels were increased by dietary cholesterol only in females. The other 11 genes in the metabolism category and the remaining 19 genes in the other categories (seven in immune/acute phase response, two as transcription factors, and ten unclassified) are not previously known LXR targets.

Confirmation of regulation by RT-PCR analysis for selected array genes

To confirm the results of the microarray study, a selection of genes that appeared to be highly regulated and/or possessed interesting domains suggestive of function were subjected to Q-PCR analysis, along with control SREBP and LXR targets (**Table 3**). For the downregulated genes, this analysis was performed for two known SREBP-1 target genes (Acac and Acly), three known SREBP-2 target genes (HMGCR, HMGCS, and Sqle), and six other genes. Acac was downregulated 2.3-fold in males only, and Acly was downregulated 2.9-fold in males only. HMGCR was downregulated 7.9-fold in males only, HMGCS was downregulated 4.5- and 2.3-fold in males and females, respectively, and Sqle was downregulated 38.6- and 27.8-fold in males and females, respectively. The six other genes were not known SREBP targets and four were previously cloned. Fatty acid binding protein 5 (FABP5) can bind long-chain fatty acids, but its function is unknown (32). FABP5 was downregulated 6.0- and 3.1-fold in males and females, respectively. Regulator of G protein signaling 16 (Rgs16) is a GTPase-activating protein that attenuates heterotrimeric

TABLE 3. Confirmation of selected array genes by Q-PCR

		Males		Females	
NetAffx ID	Gene Name	Array (Ex. 3)	TaqMan	Array (Ex. 3)	TaqMan
n/a	Acac[a]	ND	−2.3*	ND	NC
n/a	Acly[a]	ND	−2.9*	ND	NC
163290_at	Camk1D[b]	−2.0**	−2.2**	NC	NC
160544_at	FABP5[b]	−2.8**	−6.0**	−2.6*	−3.1**
104285_at	HMGCR	−2.0*	−7.9*	NC	NC
94325_at	HMGCS	−4.4*	−4.5*	−1.6*	−2.3**
108752_at	Pcsk9[b]	−2.1**	−6.5**	−1.9	−8.6
94322_at	Sqle[b]	−4.5*	−38.6*	−3.5**	−27.8**
113753_at	StarD4[b]	−2.1**	−2.3*	−2.2	ND
114831_at	Abcg5 [b]	2.2*	4.0*	1.6*	2.2**
116554_at	Adam11[b]	5.0*	11.7*	7.8**	10.5**
93445_at	Api6	1.7	1.7*	1.9**	1.8**
103379_at	Fbxo3	NC	NC	1.7**	2.0*
102712_at	SAA3	8.9*	11.4*	3.7*	6.0*

Acac, acetyl-CoA carboxylase; Acly, ATP citrate lyase; HMGCR, HMG-CoA reductase; $n = 5$ or six mice per group; ND, not determined; Q-PCR, quantitative RT-PCR; StarD4, StAR-related lipid transfer domain containing 4. * $P < 0.05$; ** $P < 0.005$.

[a] Acac and Acly were not array targets but are reported here as control lipogenic SREBP-1 targets.

[b] Confirmed by Northern blot.

G-protein signaling (33). Rgs16 was downregulated 1.8-fold in males and <1.6-fold in females, yet this did not reach significance in either case (data not shown). Ubiquitously expressed transcript, which may be a transcription factor, was not significantly regulated in males and females (data not shown). StAR-related lipid transfer domain containing 4 (StarD4), a recently cloned gene putatively involved in intracellular cholesterol transport, has previously been shown by Q-PCR analysis to be downregulated by dietary cholesterol in females (18). This was confirmed in the current study for males, in which there was 2.3-fold downregulation.

The other two downregulated genes were initially expressed sequence tags (ESTs). The probe set locations of these ESTs in the mouse genome allowed the deduction of the gene structures. In both cases, the probe sets were in the 3′ untranslated regions (UTRs) of the genes. The coding regions and 5′ UTRs were cloned by RT-PCR and 5′ RACE, respectively. One gene is the putative ortholog of a recently cloned cDNA from human leukocytes (34) and has been named Camk1D (GenBank Accession Number: AY273822). This gene is a member of the calcium calmodulin-dependent protein kinase family and is most closely related to CaMKI. Camk1D was downregulated 2.2-fold in males only (Table 3). The other gene is a member of the subtilisin family of serine proteases, many of which have been shown to be proprotein convertases (35). There are eight other mouse subtilases, including furin and the site 1 protease. This gene has been named protein convertase subtilisin kexin 9 (Pcsk9) (GenBank Accession Number: AY273821). Pcsk9 was downregulated 6.5- and 8.6-fold in males and females, respectively (Table 3).

To confirm the microarray results for the upregulated genes, this analysis was performed for one known LXR target gene (Abcg5) and five other genes. Abcg5 was upregulated 4.0- and 2.2-fold in males and females, respectively. The five other genes were not known LXR targets, and all were previously cloned. F-box-only protein 3 (Fbxo3) was upregulated 2.0-fold in females only (Table 3). Lysosomal-associated transmembrane protein 5 was upregulated 1.8-fold in males only, although this was not significant (data not shown). Serum amyloid A 3 (SAA3), an acute phase reactant, was upregulated 11.4- and 6.0-fold in males and females, respectively. Apoptosis-inhibitory 6 (Api6) is a member of the scavenger receptor family (36) and was upregulated 1.7- and 1.8-fold in males and females, respectively. Finally, a disintegrin and metalloprotease domain 11 (Adam11) family member (37) was the most highly upregulated gene in this study. It was upregulated 11.7- and 10.5-fold in males and females, respectively.

Temporal patterns of gene expression after dietary cholesterol feeding

The effect of dietary cholesterol on gene expression over time was determined by Q-PCR analysis for each of the confirmed genes above except StarD4. The downregulated genes showed two temporal patterns of regulation by dietary cholesterol, as shown in **Fig. 2A**. HMGCR, HMGCS, Pcsk9, and Sqle were downregulated 70–90% after 1 day of cholesterol feeding and remained down through day 7. In contrast, Acly, Camk1D, and FABP5 were downregulated only 20–40% after 1 day of cholesterol feeding, trended toward upregulation on day 2, and then were further downregulated between 50–75% through day 7.

The upregulated genes showed three temporal patterns of regulation, as shown in Fig. 2B. The known LXR target gene, Abcg5, was upregulated after 1 day of cholesterol feeding by 2.5-fold, and this increased by day 7 to 4.5-fold. Adam11 showed a similar pattern, as it was upregulated 5.9-fold after 1 day of cholesterol feeding and 12.1-fold by day 7. Api6 and Fbxo3 were significantly upregulated after 1 day of cholesterol feeding by 1.9- and 1.5-fold, respectively, but remained at approximately these levels through day 7. A third pattern was observed for SAA3, which was not significantly upregulated until day 4 of cholesterol feeding.

Expression of downregulated genes in SREBP transgenic mice

To confirm that the selected downregulated genes discussed above were SREBP targets, Q-PCR analysis was performed on liver RNA from transgenic mice overexpressing the transactivation domain of SREBP-1a and SREBP-2 (9, 11). In these mice, SREBP-1a or SREBP-2 activates transcription regardless of cellular cholesterol levels, and target genes should be upregulated relative to levels in littermate controls. In this experiment, the known SREBP-2 target genes, HMGCR, HMGCS, and Sqle, were significantly upregulated in both male and female SREBP-1a and SREBP-2 transgenic mice compared with littermate controls, as expected (**Table 4**). A similar pattern was observed for Pcsk9, which was significantly upregulated in male and female SREBP-1a transgenic mice by 14.7- and 6.1-fold and in SREBP-2 transgenic mice by 6.4- and 4.0-fold, respectively. The known SREBP-1 target genes, Acac and Acly, were highly upregulated in male and female SREBP-1a mice and marginally or not at all in SREBP-2 mice, as expected. A similar pattern was observed for FABP5, which was significantly upregulated in male and female SREBP-1a transgenic mice by 7.3- and 26.0-fold, respectively, and unchanged in SREBP-2 transgenic mice. Similarly, Camk1D was significantly upregulated in male and female SREBP-1a transgenic mice by 5.6- and 3.1-fold, respectively, and unchanged in SREBP-2 transgenic mice.

Expression of all dietary cholesterol-regulated genes in mice treated with the LXR agonist, TO901317

Because the LXR transcription factors are also major mediators of gene regulation by cholesterol, the regulated genes shown in Tables 1 and 2 were examined by microarray analysis using RNA from the liver of male mice treated with the LXR agonist, TO901317 (38). This experiment was performed like dietary cholesterol experiment 2 above, with two aliquots from a single RNA pool derived from five male mice per condition. Only the MGU74v2 A and B chips were used for this experiment. As shown in Table 1, all genes classified in cholesterol metabolism that were downregulated by dietary cholesterol showed no

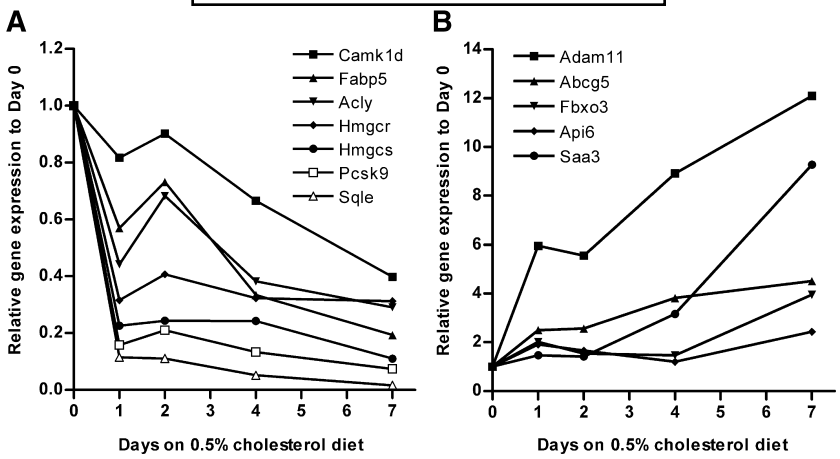

Fig. 2. Time course of regulation by dietary cholesterol for confirmed genes. Twenty male mice were fed a 0.0% cholesterol diet for 1 week. Four mice were sacrificed at the 1-week time point, and the remaining mice were switched to a 0.5% cholesterol diet. Four mice were sacrificed at days 1, 2, 4, and 7 of feeding. RNA was prepared from liver and subjected to quantitative RT-PCR. A: HMG-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS), proprotein convertase subtilisin/kexin 9 (Pcsk9), and squalene epoxidase (Sqle) mRNA levels were significantly ($P < 0.05$) downregulated beginning at day 1 and remained at similar downregulated levels through day 7. In contrast, ATP citrate lyase (Acly), calcium/calmodulin-dependent protein kinase 1D (Camk1D), and fatty acid binding protein 5 (FABP5) levels moderately declined at day 1, increased at day 2, and then slowly declined through day 7. B: The a disintegrin and metalloprotease domain 11 (Adam11) and ATP binding cassette G5 (Abcg5) levels increased immediately at day 1 to significant levels and continued to increase through day 7 of feeding. Apoptosis inhibitory 6 (Api6) and F-box-only protein 3 (Fbxo3) levels increased immediately to significant levels at day 1 and remained at similar levels through day 7. Serum amyloid A 3 (SAA3) levels did not significantly increase until day 4 of feeding and increased at day 7.

change in expression after treatment with the LXR agonist. In contrast, four of the five genes involved in fatty acid metabolism downregulated by dietary cholesterol were upregulated by the LXR agonist, consistent with the fact that LXRs activate SREBP-1c (39). Finally, only two other genes downregulated by dietary cholesterol were moderately upregulated by the LXR agonist, phospholipid transfer protein, recently shown to be regulated by LXR (40), and Pcsk9.

As shown in Table 2, nine of the 13 metabolism genes upregulated by dietary cholesterol were also upregulated by treatment with the LXR agonist. None of the genes classified as immune/acute phase response genes that were upregulated by dietary cholesterol were upregulated by the LXR agonist. Finally, 6 of the 10 unclassified genes were upregulated by the LXR agonist. Ten other cholesterol upregulated genes were not found as LXR target genes for several reasons: five were on the C chip and not evaluated, three were called absent in the LXR experiment, and two were unchanged. Because only two of the upregulated genes, Abcg5 and ABCA1, are previously demonstrated LXR targets, this experiment suggests 13 additional LXR-regulated genes. Three of these, Adam11, Api6, and Fbxo3, were tested by Q-PCR analysis and confirmed (data not shown).

TABLE 4. Expression of selected downregulated genes in SREBP transgenic mice

		SREBP-1a		SREBP-2	
NetAffx ID	Gene Name	Males	Females	Males	Females
104285_at	HMGCR	31.2*	8.2*	14.6**	7.3**
94325_at	HMGCS	7.4*	4.8**	8.8**	4.9**
108752_at	Pcsk9	14.7*	6.1*	6.4**	4.0**
94322_at	Sqle	9.5*	16.0*	17.1*	5.8**
NA	Acac [a]	13.1*	5.3*	2.8	NC
NA	Acly [a]	18.1*	9.8*	2.6*	NC
163290_at	Camk1D	5.6*	3.1*	NC	NC
160544_at	FABP5	7.3**	26.0**	NC	1.6

n = five or six mice per group; NA, not applicable. * $P < 0.05$; ** $P < 0.005$.

[a] Acac and Acly were not array targets but are reported here as control lipogenic SREBP-1 targets.

DISCUSSION

In the current study, we have established a model for examining the effects of dietary cholesterol on liver gene expression. Results from three microarray experiments were combined to identify 80 probe sets representing 69 unique genes that were regulated by dietary cholesterol. Of the 36,893 probe sets on the Affymetrix MGU74v2 set, ~8,900 hybridized to liver RNA (24%) (data not shown). Thus, <1% of the probe sets revealed consistent regulation by dietary cholesterol. The expression changes of 15 of the 69 unique genes regulated by dietary cholesterol were examined by Q-PCR analysis and confirmed for 12 genes. The expression of this subset of genes was further examined in a time course of cholesterol feeding and in SREBP transgenic mice by Q-PCR. In addition, the expression of all of the genes was further examined by Affymetrix microarray analysis in mice treated with the LXR ago-

nist. The combined analysis yielded three novel putative SREBP target genes, Camk1D, FABP5, and Pcsk9, and 13 novel putative LXR target genes in mouse liver, three of which were confirmed by Q-PCR, Adam11, Api6, and Fbxo3.

This study has two obvious strengths. The first strength was the model we used, which was based on a physiological paradigm, namely cholesterol feeding of an intact animal, whereas previous studies have used cell culture models or genetically manipulated mice, such as SREBP transgenic and LXR knockout mice. The second strength was performing three independent experiments, especially the third experiment, which examined responses of four mice per condition to assess the individual variation and to perform statistical analysis of significant differences between groups. Many microarray analyses rely on a single experiment using one microarray per condition. In the current study, compared with the 48 decreased and 32 increased probe sets listed for the combined analysis, had we taken only the first experiment and used one A chip per condition (0.5% vs. 0.0% cholesterol), the Microarray Suite software would have listed 82 decreased and 150 increased probe sets. Of the 82 listed as decreased, only 18 were on the final list, and of the 150 listed as increased, only 12 were on the final list. It is difficult to assess the sensitivity and specificity for regulated genes identified by microarray analysis for most studies in the literature. In the current study, despite the conservative nature in which genes were selected, three of 15 genes tested by Q-PCR were not confirmed.

This study also has potential disadvantages. First, many studies of regulated gene expression have used cell culture systems, and the current use of an in vivo model clearly is more complex and subject to greater variability. However, the use of an inbred mouse strain and strictly controlled environmental conditions minimizes this concern to the extent possible in a complex living organism. Second, C57BL/6J mice do not respond to cholesterol feeding with an increase in plasma cholesterol levels. However, we show in the current study that dietary cholesterol increases liver total, free, and esterified cholesterol, thus validating our approach for this tissue. Third, a number of well-characterized cholesterol-regulated genes were not identified by our statistical criteria as regulated by 0.5% dietary cholesterol for 1 week. This could be due to the stringency of the qualifying criteria and/or the experimental design. For example, HMGCR and 7-dehydrocholesterol reductase, two known SREBP target genes (20), did not make our list, even though they trended toward being decreased by cholesterol feeding, as they showed extensive array signal variability between mice and did not reach statistical significance. As another example, mevalonate kinase and diphosphomevalonate decarboxylase, also SREBP target genes (20), had very low signal values, presumably due to poor probe set design, and thus were not considered. Finally, the known SREBP target gene LDL receptor (5) and the known LXR target gene CYP7A1 (3) were not regulated by our feeding paradigm as analyzed by microarray or Q-PCR, although they have been shown to be regulated by higher concentrations of dietary cholesterol and by longer feeding times (13, 41). Perhaps if we had used a different concentration of cholesterol or length of feeding, these and other well-characterized cholesterol regulated genes would have been regulated on our microarrays.

In our experimental design, gene expression was analyzed after mice had been fed cholesterol for 1 week. To assess which genes were early and late responders, a subset of 12 responsive genes were examined at earlier time points. Genes involved in cholesterol biosynthesis and regulated by SREBP-2, such as HMGCR, HMGCS, and Sqle, were immediately downregulated at day 1 of feeding. As Pcsk9 also followed this pattern, we predict it may be involved in cholesterol biosynthesis. In contrast, a gene involved in fatty acid metabolism and regulated by SREBP-1, Acly, was downregulated later in the cholesterol feeding time course. Because Camk1D and FABP5 also followed this pattern, we predict they may be involved in fatty acid metabolism. There were several genes that were upregulated after 1 day of cholesterol feeding, such as Abcg5, Adam11, Api6, and Fbxo3. As Abcg5 is involved in reverse cholesterol transport, we speculate that the other three genes may also be important for this process. SAA3 was not induced until a later time point and probably represents a secondary response to the high-cholesterol diet, such as an inflammatory response.

Integration of information derived from the cholesterol feeding study, the LXR agonist study, and the SREBP-1a and SREBP-2 transgenic experiments also allows us to make predictions about the function of some of the novel genes we have identified that were downregulated by dietary cholesterol. For example, cholesterologenic SREBP-2 target genes should have the following profile: *1*) downregulated by dietary cholesterol due to the inhibitory effect of sterols on SREBP processing, *2*) upregulated in SREBP-2 transgenic mice and SREBP-1a transgenic mice, as SREBP-1a is a potent activator of all SREBP target genes (6), and *3*) unchanged by LXR agonist feeding, because LXR transcriptionally activates only SREBP-1c (39). In our study, this has been shown for HMGCR, HMGCS, and Sqle, three known cholesterol biosynthetic genes (see Tables 3 and 4). This is supported by the behavior of known SREBP-2 target genes in our dietary cholesterol and LXR agonist feeding studies (see cholesterol metabolism genes marked by superscript *a* in Table 1). Therefore, we propose that other genes downregulated by dietary cholesterol and unchanged by the LXR agonist are also cholesterologenic genes regulated by SREBP-2 (Table 1). Some examples include sterol C5 desaturase, sterol C4 methyl oxidase-like, NADP-dependent steroid dehydrogenase-like, StarD4, TM7SF2/lamin B receptor-related and androgen-regulated short-chain dehydrogenase/reductase 1.

In contrast, lipogenic SREBP-1c target genes should have the following profile: *1*) downregulated by dietary cholesterol, *2*) upregulated in SREBP-1a transgenic mice only, and *3*) upregulated by LXR agonist feeding. This has been shown in our study for Acac and Acly, known lipogenic genes (Tables 3 and 4). This is supported by the be-

havior of known SREBP-1 target genes in our dietary cholesterol and LXR agonist feeding studies (see fatty acid metabolism genes marked by superscript *a* in Table 1). Therefore, we propose that other genes downregulated by dietary cholesterol and upregulated by the LXR agonist are also lipogenic genes regulated by SREBP-1 (Table 1). An interesting example is FABP5, which we have shown to be regulated by SREBP-1a and not SREBP-2 in this study. FABP5 is a member of the fatty acid binding protein family and is expressed ubiquitously. It has been shown to bind long-chain fatty acids, but its physiological function is unknown (32, 42).

Two of the novel dietary cholesterol downregulated genes cloned here, the kinase Camk1D and the protease Pcsk9, do not fit neatly into this analysis. Camk1D expression was modestly downregulated by dietary cholesterol only in males, upregulated in SREBP-1a but not SREBP-2 transgenic male and female mice, but unchanged by LXR agonist feeding in males. Pcsk9 expression was highly downregulated by dietary cholesterol, highly upregulated in both SREBP-1a and SREBP-2 transgenic mice, but modestly upregulated by LXR agonist feeding. Thus, we cannot apply our algorithm exactly to these genes, but Camk1D expression resembles more a lipogenic gene and Pcsk9 a cholesterologenic gene. Mouse Camk1D has a human homolog, but nothing is known about its function. During review of this manuscript, Abifadel et al. (43) reported that mutations in Pcsk9 are present in all affected members of two families with autosomal dominant hypercholesterolemia (OMIM 603776), but absent in 200 control chromosomes. This strongly implicates mutations in Pcsk9 as causative for some forms of familial hypercholesterolemia. Their findings and our observation of strong regulation by dietary cholesterol imply an important role for Pcsk9 in cholesterol metabolism. This also provides exciting validation of our experimental approach to identifying novel genes important in cholesterol metabolism.

We can also predict the function of some novel genes upregulated by dietary cholesterol. For example, genes involved in reverse cholesterol transport should be upregulated by feeding either cholesterol or the LXR agonist (such genes should not be SREBP targets and were not examined in the SREBP-1a or SREBP-2 transgenic mice). We observe this pattern for ABCA1 and Abcg5, two genes with established roles in reverse cholesterol transport. Therefore, we propose that other genes upregulated by dietary cholesterol and upregulated by the LXR agonist may also be involved in reverse cholesterol transport (Table 2). Interesting examples include the cytochrome P450 3a mouse genes (Cyp3a11 and 3a16), which have been shown to be involved in the formation of lithocholic acid (44), and Api6, a member of the scavenger receptor family (36). The highest upregulated gene in the dietary cholesterol microarray, Adam11, was also very highly induced by the LXR agonist. This gene is a member of the disintegrin and metalloprotease family with an inactive metalloprotease domain (37). ADAMs are membrane bound and some interact with integrins (45). The physiological function of Adam11 remains to be determined.

This study combined the use of microarrays to identify liver genes regulated by dietary cholesterol in mice with investigation of the transcription factor pathways that may be responsible for the effects. In this way, we have identified three putative SREBP targets and three putative LXR targets. Further studies of gene promoters are necessary to confirm that these are indeed SREBP and LXR targets. Additional cell culture and in vivo studies will be necessary to reveal the physiological role these genes play in cholesterol metabolism.

The authors would like to thank the members of the Rogosin Institute at Rockefeller University, particularly Rimma Belenkya, for providing the ANOVA statistical software package, the members of the Gene Array Core Facility, particularly Greg Khitrov and Richard Pearson, for technical assistance with microarrays and Q-PCR, and Rachel Adams for technical assistance. This work was supported by National Institutes of Health Medical Scientist Training Program Grant GM-07739 (K.N.M. and R.E.S.) and by National Institutes of Health Grant HL-32435 (J.L.B.).

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