

Lecithin:retinol acyltransferase from mouse and rat liver: cDNA cloning and liver-specific regulation by dietary vitamin A and retinoic acid¹

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Abstract Lecithin:retinol acyltransferase (LRAT), present in microsomes, catalyzes the transfer of the *sn*-1 fatty acid of phosphatidylcholine to retinol bound to a cellular retinol-binding protein. In the present study we have cloned mouse and rat liver LRAT cDNA and tested the hypothesis that LRAT mRNA, like LRAT activity, is regulated physiologically in a liver-specific manner. The nucleotide sequences of mouse and rat liver LRAT cDNA each encode a 231-amino acid protein with 94% similarity between these species, and approximately 80% similarity to a cDNA for LRAT from human retinal pigment epithelium. Expression of rat LRAT cDNA in HEK293T cells resulted in functional retinol esterification and storage. RNA from several rat tissues hybridized with liver LRAT cDNA. However, LRAT mRNA was virtually absent from the liver of vitamin A-deficient animals, while being unaffected in intestine and testis. LRAT mRNA was rapidly induced by retinoic acid (RA) in liver of vitamin A-deficient mice and rats ($P < 0.01$). LRAT mRNA and enzymatic activity were well correlated in the same livers of rats treated with exogenous RA ($r = 0.895$, $P < 0.0001$), and in a dietary study that encompassed a broad range of vitamin A exposure ($r = 0.799$, $P < 0.0001$). Liver total retinol of <100 nmol/g was associated with low LRAT expression ($<33\%$ of control). We propose that RA, derived exogenously or from metabolism, serves as an important signal of vitamin A status. The constitutive expression of liver LRAT during retinoid sufficiency would serve to divert retinol into storage pools, while the curtailment of LRAT expression in retinoid deficiency would maintain retinol for secretion and delivery to peripheral tissues.—Zolfaghari, R., and A. C. Ross. Lecithin:retinol acyltransferase from mouse and rat liver: cDNA cloning and liver-specific regulation by dietary vitamin A and retinoic acid. *J. Lipid Res.* 2000. 41: 2024–2034.

Supplementary key words retinyl ester • hepatic retinoid metabolism • liver retinol • nutritional status

Retinol (vitamin A) and its metabolites are essential for embryonic development (1, 2) and maintenance of the normal differentiation state of numerous tissues and cell types (3). Retinol is absorbed from the intestine as chylomicron retinyl esters and rapidly taken up by hepatic pa-

renchymal cells (4). In the vitamin A adequate state, the liver contains nearly 75% of total body vitamin A; of this, nearly 95% is present as esterified retinol located mainly within lipid droplets in stellate cells (5). Esterified retinol is also the predominant form of tissue retinoid in most peripheral tissues, although typically at lower concentrations than in liver. The liver contains several retinyl ester hydrolases capable of hydrolyzing diet-derived retinyl esters or mobilizing retinol from hepatic retinyl ester stores (6). Thereafter, retinol may be secreted into plasma bound to retinol-binding protein (RBP) (7) or may become a substrate for various dehydrogenases and oxidases that generate retinal and retinoic acid (RA) (8). RA exerts the broad pleiotropic activities attributed to vitamin A by binding to two families of retinoid-activated nuclear receptors, RAR and RXR, which are expressed widely in tissue-specific patterns [see (9) for review]. These receptors bind to cognate response elements in the regulatory region of numerous genes that code for structurally and functionally diverse proteins. Retinoid-regulated gene expression is concentration dependent both for retinoid receptors and their ligands. Thus, understanding the metabolic processes that regulate retinol metabolism is fundamental to understanding the physiological control of retinoid-related functions.

In the present research, we have investigated the molecular regulation of retinol esterification by lecithin:retinol acyltransferase (LRAT). Two retinol esterification reactions have been described, acyl-CoA:retinol acyltransferase (ARAT) (10–12) and LRAT (11, 13–15), which are located in the microsomal fraction of the small intestine,

Abbreviations: ARAT, acyl-CoA:retinol acyltransferase; CRBP, cellular retinol-binding protein; HEK293T cells, human embryonic kidney 293 cells with large T antigen; HPLC, high performance liquid chromatography; LRAT, lecithin:retinol acyltransferase; RA, retinoic acid; RBP, retinol-binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction.

¹The nucleotide sequences reported in this article have been released to the GenBank Data Bank with accession numbers AF255061 (mouse) and AF255060 (rat).

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liver, retinal pigment epithelium, testis, and other tissues. Whereas ARAT is relatively nonspecific in the acyl species of retinyl esters formed (10, 16), LRAT is unique in esterifying retinol bound to the cellular retinol-binding proteins, CRBP and CRBP-II, and in utilizing the *sn*-1 fatty acid from phosphatidylcholine to form mainly retinyl palmitate, stearate, and oleate [reviewed in (17)], the species of esterified retinol that are predominant in most tissues. Moreover, only LRAT has been shown to be subject to regulation by vitamin A (18). Previously we reported that LRAT activity was undetectable in the liver of vitamin A-depleted rats; however in marked contrast to liver, intestinal LRAT was unaffected (18). Liver LRAT activity increased within several hours after rats were repleted orally with retinol, and was induced even more rapidly by RA (in the absence of retinol) (19). However, the LRAT activity induced by RA decayed back to a low level within 48–72 h after RA administration, a finding consistent with the rapid metabolism and short half-life of RA (20). The induction of liver LRAT activity, assayed in isolated microsomes with CRBP-retinol as substrate, correlated well with the restoration of retinol esterification in the liver of intact animals (21, 22). LRAT activity was also induced in vivo by several RAR-selective retinoids (23) and by 4-hydroxyphenylretinamide (21, 23), a retinoid analog with chemopreventive potential (24). However, LRAT had not been purified and molecular probes were not available to further test this hypothesis.

Ruiz et al. (25) reported the affinity labeling and micro-purification of LRAT from human retinal pigment epithelium, which enables the molecular cloning of LRAT cDNA. Their study revealed a novel 230-amino acid protein that was not homologous with any previously reported sequences and that, when expressed, resulted in retinol esterification activity. In the present experiments, we have conducted studies with three objectives: first, to clone LRAT cDNA from liver and to compare these sequences with those of the human LRAT cDNA from retinal pigment epithelium; second, to ascertain whether liver LRAT mRNA, like LRAT activity, is regulated by exogenous RA; and third, to evaluate the regulation of LRAT mRNA in a chronic model that included rats exposed to a broad range of vitamin A exposure. On the basis of these new data and previous results, we postulate that RA serves as an important signal of vitamin A adequacy that is interpreted, in liver, through the regulation of LRAT gene expression and enzymatic activity, and that serves the important function of diverting retinol into storage during vitamin A adequacy, and away from hepatic storage during vitamin A depletion, preserving retinol for secretion and other functions.

MATERIALS AND METHODS

Materials

Animals were obtained from Charles River Breeding Laboratories (Wilmington, DE). Retinoic acid (*all-trans*) was from Sigma (St. Louis, MO). All chemicals and solvents were of molecular biological grade or high performance liquid chromatography

(HPLC) grade, as appropriate; where specific reagents were used, they are noted below. [³²P]dCTP (6,000 Ci/mmol), was purchased from Amersham Pharmacia (Piscataway, NJ). The HEK293T cell line expressing large T-antigen was obtained from the American Type Culture Collection (Rockville, MD). DNA nucleotide sequencing was performed by the dideoxy method in the Nucleic Acid Facility of Pennsylvania State University (University Park, PA), using a Perkin-Elmer (Norwalk, CT) model 770 sequencer and reagents.

Animal experiments

Animal experiments were approved by the University of Pennsylvania Institutional Animal Use and Care Committee. Tissues were obtained from rats fed a nutritionally adequate purified diet, which was either vitamin A sufficient, containing 4 mg of retinol per kg of diet, or vitamin A free, as described previously (26). The study of vitamin A deficiency and RA repletion comprised four groups (*n* = 4 per group): fed a vitamin A-free diet throughout the study (vitamin A-deficient group); pair-fed the vitamin A-sufficient diet (control group); fed the vitamin A-deficient diet and then treated with 100 μg of *all-trans*-RA intraperitoneally 20 h before the end of the experiment (1 × RA group); or similarly treated with 100 μg of RA two times, at 40 and 20 h, before the end of the experiment (2 × RA group) (26). When rats were 58 days old, individual tissues were collected, immediately frozen in liquid nitrogen, and stored at –70°C until used for RNA extraction (27) and LRAT enzyme assay (19). Livers from rats in a chronic dietary study (28) were also used; in this study, male Lewis rats were raised from the time of weaning until they reached the ages of 2–3 months (designated young), 8–10 months (middle-aged), or 18–20 months (old) on the same semisynthetic diet (29), which contained either 0.35 mg retinol equivalents per kg diet (vitamin A marginal), 4 mg/kg (control), or 50 mg/kg (vitamin A supplemented) (28). Plasma (28) and liver retinol (30), LRAT enzymatic activity (30), and various biochemical indices of health status have been reported previously for these animals (28). BALB/c mice, as reported elsewhere (31), were fed the same vitamin A-free or vitamin A-containing purified diets for 12 weeks. Some mice received 100 μg of *all-trans*-RA orally 1 and 2 days before liver collection.

Cloning strategies

The published nucleotide sequence of human retinal pigment epithelium LRAT cDNA (25) was used to search the BLAST database and a mouse expressed sequence tag (EST) database [BLAST 2.0; National Center for Biotechnology Information (NCBI), Bethesda, MD]. Two EST sequences with GenBank accession numbers AA177870 and AI604132 were found to have greater than 80% homology with the human retinal pigment epithelium LRAT cDNA sequence (25) corresponding to nucleotide sequence numbers bp 501–bp 907. Two pairs of primers were designed from the mouse EST cDNA AI604132. The two forward primers included 1) 5'-TACAGCCTACTGTGGAACAACTGC-3' (corresponding to nucleotides 63–87 of the mouse EST) and 2) 5'-AGGTGGCAGCAGAGCTGAGC-3' (corresponding to nucleotides 22–42), and the two reverse primers were 3) 5'-ATGGGATACAGATTGCAGGAAGGG-3' (corresponding to nucleotides 250–273) and 4) 5'-ATGCTAATCCCAAGACAGCCGAAG-3' (corresponding to nucleotides 193–216). Primers 1 and 4 were used as the gene-specific primers to clone mouse LRAT cDNA by rapid amplification of cDNA ends (RACE), using the Smart-RACE kit (Clontech, Palo Alto, CA). Total RNA from mouse liver was used to synthesize both the 5'-end and 3'-end first-strand cDNA, using Superscript-II reverse transcriptase (Life Technologies, Gaithersburg, MD). After dilution of the first-strand products, primers 1 and 4 were used to amplify the 3'

and 5' ends of the mouse LRAT cDNA, respectively, using an Advantage-2 PCR amplification kit (Clontech) with the following cycling program: 5 cycles of 94°C for 10 s and 72°C for 4 min; 5 cycles of 94°C for 10 s, 70°C for 10 s, and 72°C for 4 min; and finally, 30 cycles of 94°C for 10 s, 68°C for 10 s, and 72°C for 4 min in a Perkin-Elmer model 2400 cycler. The polymerase chain reaction (PCR) products were separated by electrophoresis in a 1% agarose gel and the largest bands were cut and then extracted with a Qiagen (Valencia, CA) II gel extraction kit. The extracted DNA for each band was first tested by reamplification by PCR for the presence of the sequence encompassed by primers 1 and 4 and then cloned in the pGEM-T vector, using a TA cloning ligase kit (Promega, Madison, WI). After screening several clones for the presence of the LRAT insert, one clone for each RACE was selected for sequencing both strands of DNA. The DNA sequence pieces were assembled and aligned using both DNaid and DNAsis sequence analysis programs (Hitachi Software, San Bruno, CA). The 2,541-nucleotide sequence cloned from mouse liver was deposited in GenBank, accession number AF255061.

To clone LRAT cDNA from rat liver, primers based on the mouse EST sequence described above were used to amplify cDNA fragments by reverse transcriptase (RT)-PCR, using poly(A)⁺ RNA. After the first-strand cDNA was synthesized as described above, one-twentieth of the reaction was used for PCR in a reaction containing 1× *Taq* Gold DNA polymerase buffer, 1.5 mM MgCl₂, 0.3 mM dNTPs, 0.5 mM primers, and 2.5 U of *Taq* Gold polymerase (Perkin-Elmer). The cycling program was 94°C for 10 min for initial activation of the enzyme and 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR fragments were isolated by agarose gel electrophoresis, and then cloned and sequenced as described above. Two primers were designed from these sequenced clones and then used to clone and sequence rat LRAT cDNA by RACE as described above for mouse clones. The primers were as follows: 5'-TTTGTGACCTACTGCAGATACGGC-3' as the forward primer and 5'-CATATATGATGCCAGGCCTGTGTAG-3' as the reverse primer. The 2,522-nucleotide sequence cloned from rat liver was deposited in GenBank, accession number AF255060.

LRAT expression in HEK293T cells

A 736-bp fragment of mouse liver LRAT cDNA encompassing the entire protein-coding region was amplified from mouse liver total RNA by RT-PCR with two primers, 5'-tcctcgcAGCCAGTG GTTGGTTGCCTTAGG-3' as forward primer and 5'-tgttctaGAC CTGTGGACTGGAAGCTAGCC-3' as reverse primer, where nucleotides in lowercase represent the *Xho*I and *Xba*I restriction sites, respectively. The amplified fragment was cloned unidirectionally into the *Xho*I/*Xba*I site of the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) containing a cytomegalovirus promoter-enhancer element. The cloned plasmid vector was then transfected into HEK293T cells expressing large T-antigen. The empty plasmid vector was also used as a transfection control. After 4 days, the transfected cells were grown in medium containing G418 at 400 µg/ml. When the cells were ~80% confluent, the medium was removed, cells were washed with phosphate-buffered saline, and then collected and lysed in 0.15 M phosphate buffer, pH 7.4, for LRAT enzyme assay and protein determination.

RNA extraction and analysis

Total RNA was extracted from tissue samples of individual animals, using either guanidine salts (27) or Trizol reagent (Life Technologies). For poly(A)⁺ RNA isolation, total RNA samples from individual livers for each treatment group were pooled and then passed through oligo(dT)-cellulose (27). The isolated poly(A)⁺ RNA sample (4 µg per lane) was fractionated by

electrophoresis and transferred to Nytran membrane (Schleicher & Schuell, Keene, NH) by capillary action for Northern blot analysis (27). The rat LRAT cDNA, comprising the whole 5' untranslated region (UTR) and most of the amino acid-coding region (914 bp), was labeled with [³²P]dCTP and hybridized to the membranes as described previously (26). After washing, the membranes were exposed to Biomax X-ray film (Eastman Kodak, Rochester, NY) at -80°C for 10 days. The membranes were then stripped of the LRAT cDNA probe and rehybridized with radiolabeled rat β-actin cDNA, which was used as the control probe, and re-exposed to X-ray film for up to 2 h.

A semiquantitative RT-PCR method was used to measure the relative level of the LRAT mRNA in the liver tissue of individual animals; details of this method have been described elsewhere (32). The PCR products were separated by electrophoresis on an ethidium bromide-stained agarose gel, scanned, and analyzed using NIH Image 1.56 as previously reported (26, 32). For samples from the dietary study, each sample represented equal amounts of RNA from two rats, with three pools per treatment group (30). LRAT mRNA was determined by the method of quantitative real-time PCR on a Perkin-Elmer ABI 7700 system, using LRAT-specific primers and a fluorescently labeled probe optimized for this procedure, and 18S RNA as an internal control for each sample. Samples were analyzed in triplicate, averaged, and the values for LRAT mRNA for each sample were then expressed relative to the average value for the young control diet group, which was defined as 1.00.

LRAT activity assay

For determination of LRAT enzyme activity, 0.5 g of liver tissue was homogenized in 4 ml of 0.02 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM dithiothreitol and then incubated for 4 min (23) with [³H]retinol-CRBP (18) as substrate. Lysates of cultured cells were homogenized by passing the cells through 20-gauge needles several times before incubation with [³H]retinol-CRBP for 1 h. LRAT activity is defined as picomoles of esterified retinol formed per minute and LRAT-specific activity is defined as picomoles of esterified retinol formed per minute per milligram of protein.

HPLC analysis

Retinol and retinyl esters in HEK cells were determined by HPLC as described previously (33). Liver retinol values for rats in the chronic dietary study are those reported by Dawson et al. (30).

Statistics

Statistical analyses were performed by one-way analysis of variance and linear regression analysis (SuperAnova; Abacus Concepts, Sunnyvale, CA); *P* < 0.05 was considered significant.

RESULTS

Cloning of mouse and rat liver LRAT cDNA

Using a pair of primers designed from mouse EST sequences (see Materials and Methods), the 5' and 3' regions of LRAT cDNA were cloned by RACE from both mouse and rat liver. After alignment and assembly of the 5'- and 3'-end fragments, cDNA clones with 2,541 and 2,522 bases were obtained for mouse and rat liver LRAT, respectively (data not shown). The nucleotide sequences of both mouse and rat liver LRAT cDNA predicted a single open reading frame encoding a protein with a molecular

A

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.....10.....20.....30.....40.....50
Rat  MKNSMLEAASLLLEKLLLSNFKIFSVCAPGGGTGKKHPYEINSSFLRGDV: 50
Mouse MKNPMLEAASLLLEKLLLSNFKLFSVSVPGGGTGKNRPYEISSFVRGDV: 50
Human MKNPMLEVSLLEKLLLSNFTLFSSCAAGKDKGRNSFYETSSFHRGDV: 50

.....60.....70.....80.....90.....100
Rat  LEVSRTHFTHYGIYLGDNRVAHLMPDILLALTSDKERTQKVVSNKRLLG:100
Mouse LEVSRTHFTHYGIYLGNRVAHLMPDILLALTNDKERTQKVVSNKRLLLG:100
Human LEVPRTHFTHYGIYLGDNRVAHMMPDILLALTDDMGRTQKVVSNKRLLLG:100

.....110.....120.....130.....140.....150
Rat  VICKVASIRVDTVEDFAYGADILVNHLDETLKKKSLLNEEVARRAEQQLG:150
Mouse VICKVASIRVDTVEDFAYGADILVNHLDSTLKKSLLNEEVARRAEQQLG:150
Human VIVKVASIRVDTVEDFAYGANILVNHLDESLOKKALLNEEVARRAEKLLG:150

.....160.....170.....180.....190.....200
Rat  LTPYSLLWNNCEHFVTYCRYGSPISPQAEKFHETVKIIRDQRSCLASAV:200
Mouse LTPYSLLWNNCEHFVTYCRYGSRISPQAEKFYDTVKIIRDQRSSLASAV:200
Human FTPYSLLWNNCEHFVTYCRYGTFISPQSDKFCETVKIIRDQRSVLASAV:200

.....210.....220.....230.
Rat  LGLVSIIYTGLASYMTLPAVCIPFCLWMMSG:231
Mouse LGLASIVTGLASYMTLPAICIPFCLWMMSG:231
Human LGLASIVCTGLVSYNTLPAIFIPFFLWMAG:230

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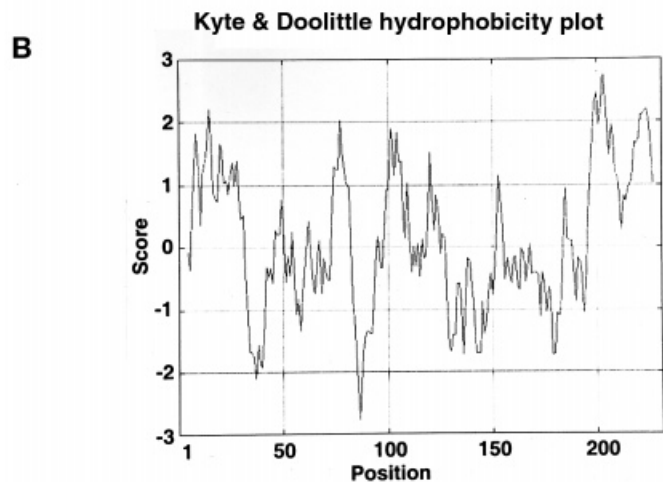


Fig. 1. Deduced amino acid sequences of mouse and rat liver LRAT. (A) Amino acid sequences of the LRAT proteins of mouse and rat liver, compared with the sequence reported for human retinal pigment epithelium LRAT (25). (B) Hydrophobicity plot [Kyte and Doolittle (40)] of the amino acid sequence for rat liver LRAT. Positive scores indicate regions of hydrophobicity and negative scores indicate regions of hydrophilicity. Gray boxes highlight conservative amino acid differences and black boxes nonconservative amino acid differences.

mass of 25.8 kDa and a pI of about 8.86 for mouse and 8.13 for rat (Fig. 1A). The first ATG initiation codon and the termination codon were perfectly matched in the LRAT cDNA sequence of both mouse and rat, giving an open reading frame encoding 231 amino acid residues. There is 94% amino acid similarity between the LRAT sequences for mouse and rat liver (Fig. 1A), but less than 80% homology between either the mouse or rat protein as compared with retinal pigment epithelium LRAT, which comprises 230 amino acid residues (25). A hydrophobicity plot (Fig. 1B) indicated the presence of at least two major membrane-spanning regions, one located in the amino-terminal region and the other near the carboxyl-terminal end of the protein. There are also two major hydrophilic regions corresponding to amino acid residues 67–103 and 155–167, respectively. A predicted glycosyl-

ation site, N-X-T, located at positions 21–23 in the human retinal pigment epithelium LRAT sequence (25), is not apparent in the deduced mouse or rat liver LRAT proteins. The rodent liver LRAT proteins also differed from human retinal pigment epithelium LRAT at their carboxyl-terminal end, where two cysteine residues at positions 221 and 225 in the rodent sequences are phenylalanine residues in the human sequence.

Expression of retinol-esterifying activity in HEK293T cells

To confirm the functional activity of the cloned LRAT cDNA, human embryonic kidney HEK293T cells were transfected with a 736-bp nucleotide fragment of liver LRAT cDNA that includes the entire amino acid-coding region. Both LRAT cDNA-transfected cells and appropri-

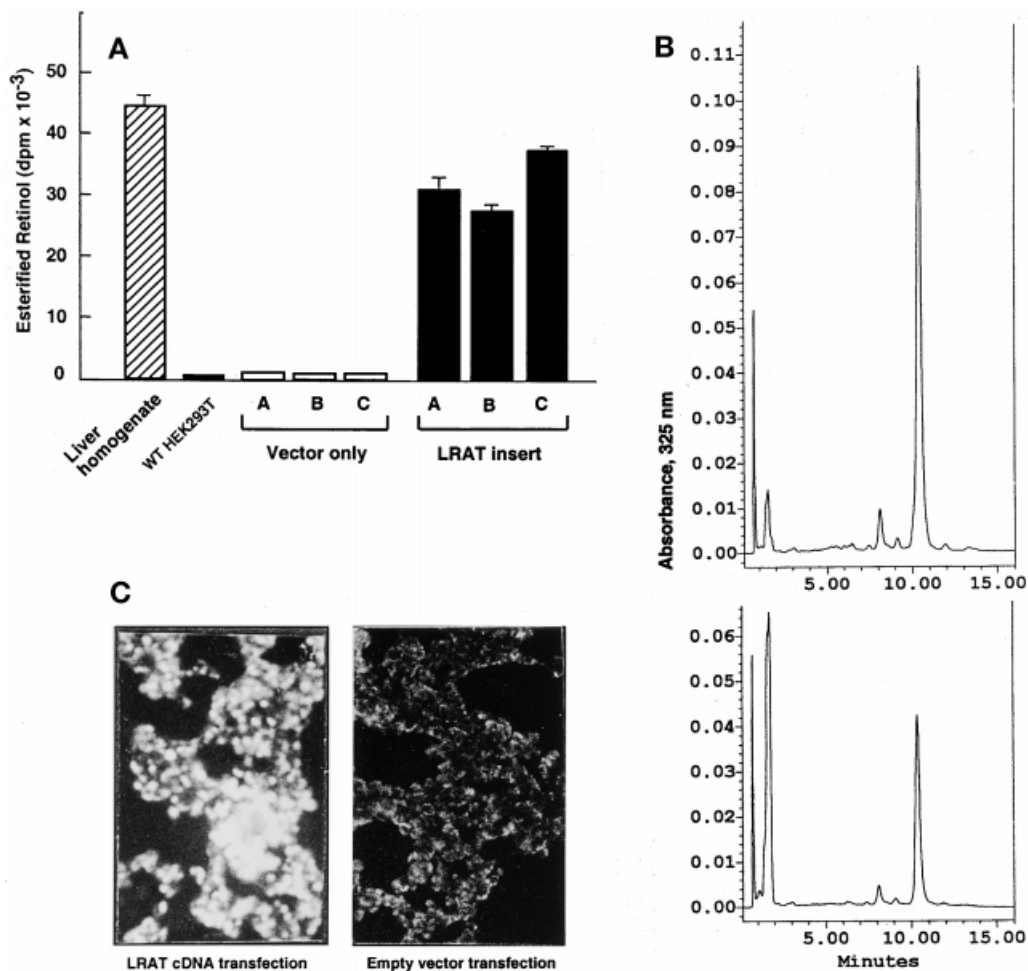


Fig. 2. Esterification of retinol by human HEK293T cells transfected with liver LRAT cDNA. Mouse liver LRAT cDNA ligated in pcDNA3.1 vector plasmid, or vector DNA plasmid alone, was transfected in three separate dishes each and cells were grown in Dulbecco's modified Eagle's medium in the presence of 10% fetal bovine serum and G418 (400 $\mu\text{g}/\text{ml}$) to obtain stably transfected cells. (A) An equal amount of each cell lysate, or rat liver homogenate protein as a positive control, was incubated with [^3H]retinol-CRBP as substrate at 37°C for 1 h, after which [^3H]retinyl ester was isolated and quantified (see Materials and Methods). (B) Cells were incubated with 2 μM retinol and oleic acid (50 $\mu\text{g}/\text{ml}$) for 3 h, and then analyzed for the presence of retinyl ester by HPLC. (C) LRAT cDNA-transfected (left) or vector-transfected (right) HEK293T cells were cultured in the presence of 2 μM retinol at 37°C for 24 h and then observed by fluorescence microscopy. The fluorescence shown in LRAT cDNA-transfected cells represents the dim, rapidly fading autofluorescence that is characteristic of retinol. Fluorescence was not observed in the absence of retinol (not shown) or in empty vector-transfected control cells.

ate controls were selected and grown in the presence of G418 prior to subsequent experiments. To assay the capacity of cell membranes to esterify CRBP-bound retinol, lysates prepared from confluent cell monolayers were incubated with [^3H]retinol-CRBP under standard LRAT assay conditions. Both wild-type HEK293T cell lysate and rat liver homogenates were used as controls. As shown in **Fig. 2A**, the lysates from three independent transfections with LRAT cDNA had significant retinol-esterifying activity, whereas no esterification activity was detected in either wild-type HEK293T cells or HEK293T cells transfected with vector alone.

To demonstrate esterification activity in intact cells, both wild-type and transfected cells were incubated with 2 μM retinol and oleic acid (50 $\mu\text{g}/\text{ml}$) for 1 to 3 h, and then subjected to extraction and analysis by HPLC. Ret-

inol was esterified in a time-dependent manner by LRAT cDNA-transfected cells incubated with retinol and oleic acid (**Fig. 2B**). Retinol esterification was also observed morphologically in LRAT-cDNA-transfected cells cultured with 2 μM retinol plus oleic acid for 24 h, and then viewed by UV fluorescence microscopy. The punctate pattern of rapidly fading autofluorescence shown in **Fig. 2C** (left) is similar to that observed for retinyl ester-containing lipid droplets in stellate cells (34).

Tissue distribution of LRAT mRNA

To study the tissue distribution of LRAT mRNA, we used the 5'-end fragment of the rat LRAT cDNA spanning the 5' UTR and most of the amino acid-coding region as a probe for the Northern blot analysis of the poly(A)⁺ RNA

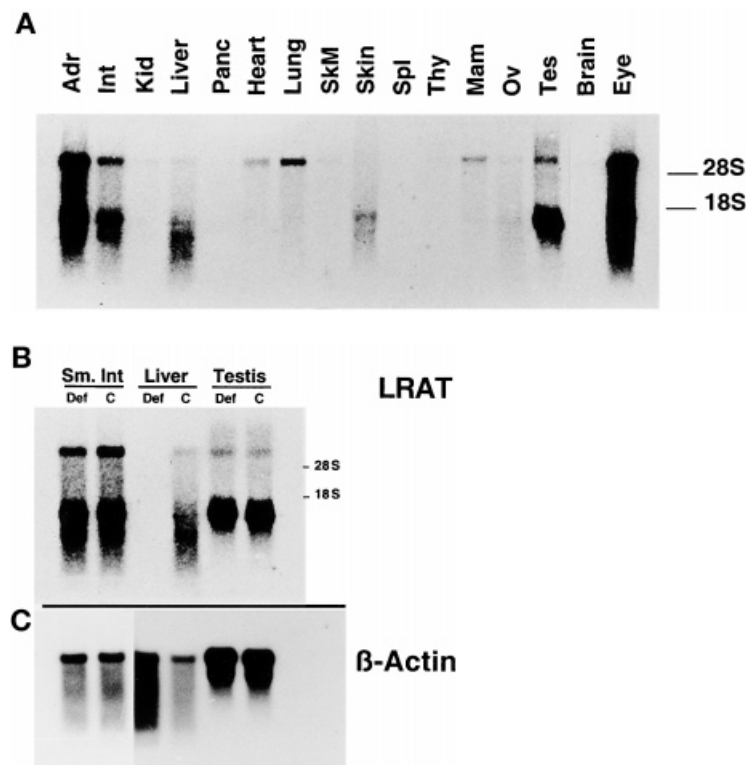


Fig. 3. Expression of LRAT mRNA in various tissues of adult rats. Poly(A)⁺ RNA (4 μ g) for each tissue was isolated from pooled total RNA prepared from tissues of four vitamin A-deficient and four pair-fed vitamin A-sufficient rats, separated by electrophoresis in a formaldehyde-denatured agarose gel, and transferred to Nytran membrane as described previously (27). The membranes were hybridized to ³²P-labeled rat liver LRAT cDNA. (A) Northern blot of the poly(A)⁺ RNA from vitamin A-sufficient rat tissues. (B) Northern blot of the poly(A)⁺ RNA from three tissues of vitamin A-deficient and vitamin A-sufficient rats. (C) The same blot was stripped and reprobed with cDNA to β -actin (26), followed by a 10-min film exposure for intestine and a 2-h exposure for liver and testis.

from various tissues of normal rat. Similar to the Northern blot results reported for human tissues (25), the rat liver cDNA probe hybridized to RNA species of at least two different sizes. One of higher molecular weight migrated just above the 28S ribosomal RNA, and the others of lower molecular weight with broad appearance migrated near the 18S ribosomal RNA band (Fig. 3A). Hybridization was observed for most tissues, with the adrenal gland, small intestine, testis, and eye tissue having a relatively high level of LRAT transcripts and other tissues such as liver, heart, lung, skeletal muscle, skin, and mammary tissue with lower expression. LRAT mRNA is apparently absent from or expressed at low levels in rat pancreas, ovary, brain, thymus, and spleen.

Liver-specific regulation of LRAT expression

Because LRAT enzymatic activity has been shown to be regulated by vitamin A status in the liver but not in the small intestine, experiments were conducted to determine whether LRAT mRNA expression is similarly regulated by vitamin A. RNA was prepared from the liver of individual rats that had been fed vitamin A-deficient diet until they had a mean plasma retinol concentration <0.15 μ M, compared with >1.2 μ M for controls, and no detectable liver retinol (26). Poly(A)⁺ RNA was prepared from pooled total RNA from either vitamin A-deficient or control rats for three different tissues and subjected to Northern blot analysis using ³²P-labeled liver LRAT cDNA as a probe, as described above. Whereas vitamin A has no effect on the LRAT mRNA signal from either small intestine and testis, there was no hybridization signal with liver poly(A)⁺ RNA from vitamin A-deficient rats (Fig. 3B).

When the membrane was reprobed using β -actin cDNA, β -actin was expressed in both vitamin A-deficient and control liver (Fig. 3C), with slightly higher expression in vitamin A-deficient liver, as shown previously (26).

Because LRAT mRNA appears to be a relatively rare mRNA in all rat tissues examined, including liver, we used an RT-PCR assay to examine relative LRAT mRNA expression in the liver of individual rats. The results were quantified by densitometry and expressed relative to the mean value of the vitamin A-sufficient group, which is defined as 1.00. As shown in Fig. 4A and B, LRAT mRNA was barely detected in vitamin A-deficient rat liver. However, after vitamin A-depleted rats were treated with 100 μ g of all-*trans*-RA, either once or twice, the level of LRAT mRNA expression increased significantly toward that of the vitamin A-sufficient group, even though there was no endogenous retinol available for esterification in the liver of the vitamin A-deficient animals. When the LRAT mRNA level of the individual rats in all four treatment groups was plotted versus the LRAT enzymatic activity in homogenates from the same livers, there was a strong correlation ($r = 0.895$, $P < 0.0001$, Fig. 4C). To confirm that liver LRAT is regulated similarly by vitamin A and RA in mice, we evaluated LRAT mRNA expression by RT-PCR in liver samples from vitamin A-deficient, RA-treated, and vitamin A-sufficient mice (Fig. 4D). Similar to results for the rat, LRAT mRNA was present in liver of vitamin A-sufficient control mice (Fig. 4D, lane 1), but was not detected for vitamin A-deficient mice (Fig. 4D, lane 2). However, after administration of 100 μ g of all-*trans*-RA orally, LRAT RT-PCR products were comparable for RA-repleted mice (Fig. 4D, lanes 4 and 5) and con-

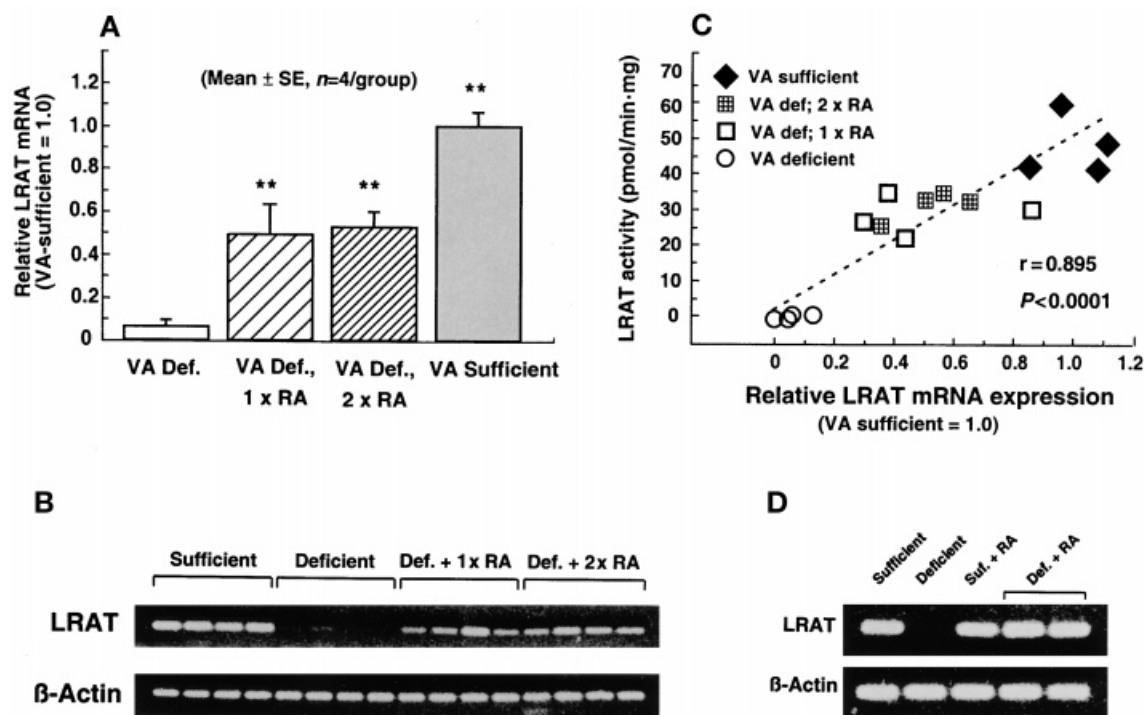


Fig. 4. Regulation of LRAT mRNA expression and LRAT enzymatic activity in rat and mouse liver. (A) Relative LRAT mRNA levels, as determined by RT-PCR analysis of total RNA, in the liver of vitamin A-deficient, retinoic acid (RA)-treated and vitamin A-sufficient rats. Asterisks indicate statistically significant differences, $P < 0.005$, between vitamin A-deficient and $1\times$ and $2\times$ RA-treated groups, and $P < 0.0001$ between vitamin A-deficient and control groups (Fisher's least significant difference test). (B) Ethidium bromide-stained agarose gel of the LRAT RT-PCR products amplified from rat liver total RNA, as quantified in (A). (C) Correlation of the LRAT mRNA levels and LRAT enzymatic specific activities in the liver of the individual rats described in (A). (D) Ethidium bromide-stained agarose gel of the LRAT RT-PCR products amplified from mouse liver total RNA of vitamin A-sufficient mice (lane 1), vitamin A-deficient mice (lane 2), vitamin A-sufficient, RA-treated mice (lane 3), and vitamin A-deficient, RA-treated mice (lanes 4 and 5).

control mice. LRAT mRNA was expressed at a similar level in the liver of vitamin A-sufficient mice with and without RA administration.

Relationship of LRAT mRNA to liver retinol concentration

Having shown that exogenous RA induces LRAT mRNA and enzymatic activity, we wanted to study LRAT regulation under dietary conditions. For this purpose, we analyzed LRAT mRNA expression by quantitative, real-time PCR in the liver of rats that had been fed diets having a marginal, control, or elevated level of vitamin A for three lengths of time (2–3, 8–10, and 18–20 months); these treatments resulted in a wide range of vitamin A exposure, and liver retinol concentrations that spanned a >4 -log range (30). LRAT activity had been measured previously, before reagents for mRNA analysis were available. The expression of LRAT mRNA correlated well with the assay of enzymatic activity ($r = 0.799$, $P < 0.0001$) (Fig. 5A). Values for LRAT mRNA, normalized so that the average value of young control rats equaled 1.0, was then plotted versus liver total retinol concentration (Fig. 5B). LRAT mRNA was low, <0.3 as compared with control, for all vitamin A-marginal rats for which liver retinol concentration was <100 nmol/g. For all control and vitamin A-supplemented rats, LRAT

mRNA was in the range of 0.6–1.6 times compared with the average young control group.

DISCUSSION

The nucleotide sequence of LRAT cDNA cloned from the liver of both mouse and rat comprises an open reading frame corresponding to 231 amino acids and a calculated protein molecular mass of 25.8 kDa. The mouse and rat proteins show about 91% identity, and 94% similarity, with one another, but less than 80% similarity to the human retinal pigment epithelium LRAT protein (25). These differences may be intrinsic to the species or due to tissue differences, or both. In contrast to the exact alignment of nucleotides within the open reading frame region of mouse and rat liver LRAT cDNA, nucleotide sequence homology is much lower in both the 5' and 3' UTR (data not shown), where there are several regions of local alignment but also a number of gaps between the mouse and rat cDNA sequences. The predicted molecular mass of LRAT protein agrees with that reported for human retinal pigment epithelium LRAT protein as determined by Western blot analysis (25). The predicted LRAT protein (Fig. 1B) is relatively hydrophobic, with two major membrane-spanning regions located near the amino- and carboxyl-terminal ends of the protein, a

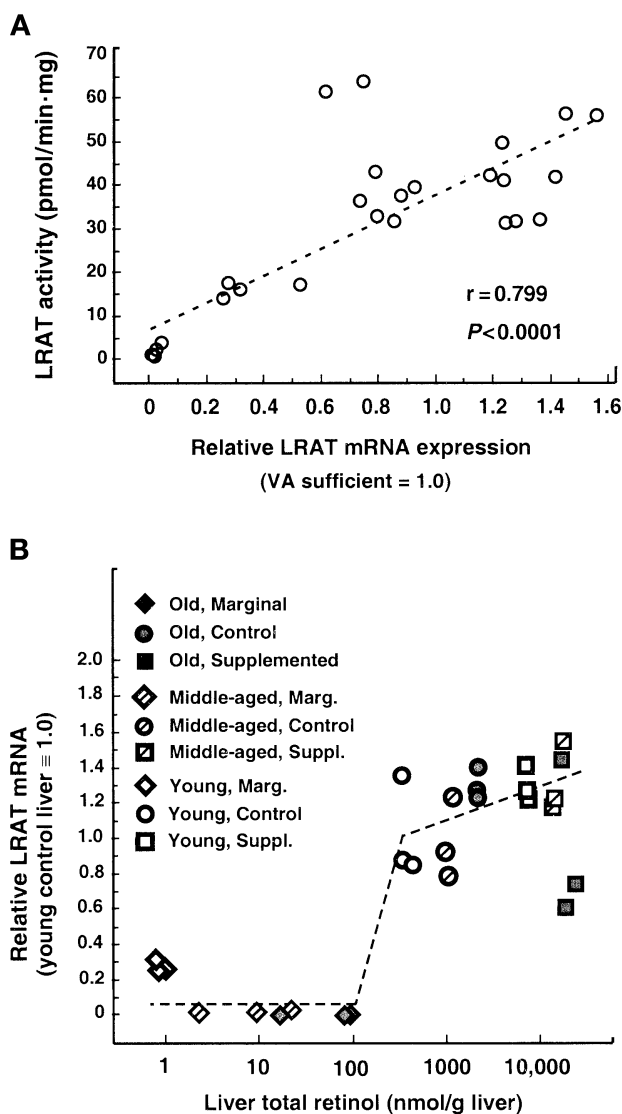


Fig. 5. Correlation of LRAT activity and mRNA, and of mRNA and hepatic retinol, in liver of rats exposed to three levels of dietary vitamin A during aging. (A) Correlation of LRAT activity and LRAT mRNA, analyzed by quantitative real-time PCR, under steady state dietary conditions produced by feeding rats one of three levels of dietary vitamin A (see Materials and Methods). Young, middle-aged, and old rats were 2–3, 8–10, and 18–20 months of age, respectively. (B) Relationship of LRAT mRNA expression to liver total retinol. For both (A) and (B), each point is a pool of two rat livers (three pools per treatment group); values for LRAT activity and total retinol (30) are the average value for the two livers in each pool, which were in close agreement.

number of potential phosphorylation sites scattered throughout the protein, and three closely spaced sites of potential myristoylation located near the amino-terminal end but outside the membrane-spanning region.

The predicted size of the LRAT protein, 25.8 kDa, is somewhat surprising because, on the basis of previous experiments in which radiation inactivation was used to estimate the functional molecular mass of LRAT activity in native rat liver microsomes, the size was estimated to be 52 ± 3 (mean \pm standard error) kDa (35). Radiation in-

activation analysis (36) assesses the target size of the functional unit that must be inactivated to incur loss of catalytic activity. Thus, the discrepancy between the predicted size based on sequence and the functional size based on radiation inactivation suggests that the LRAT protein might function as a dimer, or in concert with another protein(s) yet to be identified. It should be noted, however, that the results of transfection studies with HEK293T cells [(25) and Fig. 2], which do not express LRAT mRNA endogenously (25), demonstrated a gain of retinol esterification function by the transfection of single LRAT cDNAs cloned from human retinal pigment epithelium or rodent liver. Thus, if it is the case that another protein, besides the LRAT we have cloned, is required for retinol esterification, it must have been present within the HEK293T cells used as an LRAT expression system. LRAT activity was observed in HEK293T cell lysates, using either retinol bound in a physiological complex to CRBP (37) (Fig. 2A), or unbound retinol as substrate (25) and retinol esterification was also demonstrated in intact, LRAT cDNA-transfected cells by HPLC analysis and by increased autofluorescence of retinol and its esters (Fig. 2C). Thus the cellular expression of the LRAT cDNAs that have been cloned was both necessary and sufficient for the gain of retinol-esterifying function in these cells.

The cDNAs for human retinal pigment epithelium LRAT and rodent liver LRAT do not have significant homology with any previously cloned cDNA or gene sequences, and thus seem to represent a novel gene family. On the basis of the results of Northern blot studies in which poly(A)⁺ RNA from various tissues was hybridized with LRAT cDNAs from human retinal pigment epithelium (25) and rat liver (Fig. 3A), there are at least two mRNA species with substantially different sizes, differentially expressed in different tissues. It is not yet known whether these mRNA species represent variant genes or may be the product of a single gene but differ as a result of differences in promoter utilization, alternative splicing, stability of mRNA, or the presence of different poly(A) signals within the 3' UTR of the cDNA. We found no classic poly(A) signal, for example, AAUAAA, in the 3' UTR of the liver LRAT mRNA of either mouse or rat. Similarly, Ruiz et al. (25) observed more than one mRNA species that hybridized with the cDNA probe from human retinal pigment epithelium LRAT and suggested these might originate from the same gene, but have different poly(A) signals. We may rule out the possibility of alternative RNA splicing within the region coding for LRAT protein because amplification by PCR of the entire amino acid-coding region, as used for HEK293T cell transfections (see Materials and Methods) resulted in the production of not more than a single amplicon (data not shown). Whatever the origin of these differently sized mRNAs, all the transcripts that hybridized with liver LRAT cDNA were missing from the liver of vitamin A-deficient rats (Fig. 3B).

Although liver LRAT mRNA was not detected in a few of the tissues examined by Northern blot analysis, each tissue examined, except spleen, showed an appropriately sized LRAT amplicon after RT-PCR amplification of poly(A)⁺

RNA (data not shown). Although there are reports on the presence of LRAT activity in the eye, intestine, testis, and liver, LRAT activity in several of the other tissues examined for mRNA, specifically adrenal gland, lung, and skeletal muscle, has not been reported. On the basis of the presence of LRAT mRNA in these organs, their capacity to esterify CRBP-bound retinol should be examined.

Liver-specific, dietary regulation of LRAT expression

Previous research had demonstrated that LRAT enzymatic activity is regulated by vitamin A in the liver, but not in the small intestine (18). The present studies have demonstrated that LRAT mRNA expression is highly regulated by vitamin A in a liver-specific manner (Fig. 3B), while being expressed constitutively in other organs (small intestine or testis). We infer from the strong correlation (Fig. 4C) between liver LRAT mRNA and enzymatic activity that LRAT expression is curtailed in the absence of vitamin A, while vitamin A (as RA) is capable of transcriptionally inducing and/or stabilizing the expression of new LRAT mRNA in liver. Through future analysis of the LRAT gene promoter it should be possible to clarify the mechanism by which LRAT mRNA expression is regulated in liver, versus being expressed constitutively, even in vitamin A deficiency, in other tissues.

Although the observation that LRAT is regulated acutely by RA is likely to be relevant to its regulation by vitamin A, we wished to know whether there is sensitive regulation of LRAT mRNA expression under steady state dietary conditions. For this, we took advantage of samples from a long-term nutritional study conducted in aging rats, which resulted in differences in hepatic retinol concentration that spanned more than a 4-log range, for which LRAT activity was known (30). The same strong linear relationship between LRAT mRNA and LRAT activity (Fig. 5A) was observed in the steady state as was observed after acute treatment with RA (Fig. 4C). A comparison of LRAT expression and liver total retinol revealed that while low LRAT expression was associated with liver retinol <100 nmol/g (equal to <28.6 μg/g), vitamin A supplementation resulted in only a modest elevation of LRAT

mRNA expression above normal levels. Thus liver LRAT expression is mainly regulated as vitamin A reserves are depleted, consistent with the downregulation of LRAT expression being a mechanism to maintain the liver's pool of nonesterified retinol, to the extent possible, as would benefit retinol secretion and retinol delivery to target organs.

A model of peripheral tissue-liver interaction in the regulation of liver LRAT

The ability of RA to regulate hepatic LRAT mRNA expression and activity suggests a closely autoregulated network of retinoid homeostasis in which retinol's own active metabolite, RA, serves as the principal signal of the body's vitamin A adequacy. In liver, this system would subserve several biologically important functions. First, it would conserve vitamin A as retinyl esters for future utilization. Given that relatively few foods are rich sources of vitamin A, a mechanism of conserving this essential nutrient may have conferred a strong evolutionary advantage. Second, esterification may be a key means by which to control the concentration of unesterified retinol, thus regulating the availability of retinol for complexation with apo-RBP, secretion from liver into blood, and uptake by peripheral tissues. Third, the local availability of retinol as substrate for hepatic retinol dehydrogenases may, in a similar manner, be limited by the diversion of unesterified retinol into retinyl esters.

With respect to RA production, it is interesting that, although the liver contains several enzymes shown to be capable of oxidizing retinol and retinal and producing RA (8), apparently the preponderance of RA is produced in peripheral tissues. On the basis of the results of isotope kinetic studies conducted in intact rats, Kurlandsky et al. (38) concluded that the liver receives the greatest portion (~75%) of its RA by uptake from plasma, presumably derived from oxidation of retinol in peripheral tissues. On the basis of this information and our results of liver-specific regulation of LRAT expression by RA, we propose a model of peripheral tissue-liver interaction (Fig. 6) in which RA produced peripherally provides a critical "signal of vitamin A adequacy" that, in liver, serves to regulate vitamin A

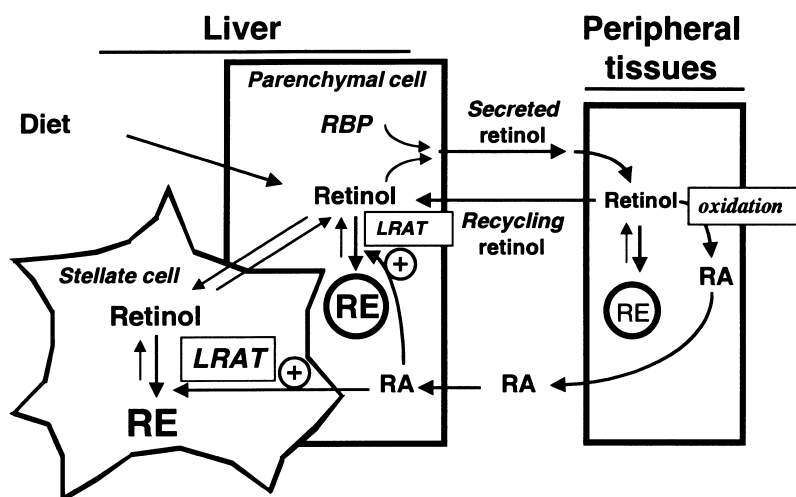



Fig. 6. Model of the proposed interaction between peripheral tissue RA production and hepatic regulation of LRAT expression and retinyl ester (RE) storage. Retinol derived either from diet or by recycling from peripheral tissues is directed into RE storage pools in parenchymal cell and stellate cells when the production of RA in the periphery is adequate; thus liver LRAT is maintained in its induced state (+). When vitamin A is limiting, delivery of retinol to peripheral tissues falls. When RA production in peripheral tissues falls, the RA signal returning to liver would no longer be adequate, and liver LRAT mRNA and activity would decline. The absence of LRAT activity could serve to divert any available retinol into the secretory pathway (via RBP), preserving target tissue functions as long as possible.

storage. In this model, liver LRAT is a primary target of RA's action. Liver LRAT remains in a constitutively induced state as long as the RA signal is in adequate supply. When vitamin A intake becomes inadequate and plasma retinol falls (39) then peripheral RA production would be expected to fall, and, in turn, the concentration of RA recirculating from peripheral tissues to liver would no longer be adequate to maintain expression of hepatic LRAT (Figs. 4 and 5). The transitory increase and decline in LRAT activity that is observed after giving a single dose of RA to vitamin A-deficient animals (19) is consistent with the short half-life of RA (20) and implies that continuous production of RA is necessary to maintain hepatic LRAT expression. During vitamin A adequacy, the liver LRAT is proposed to play a decisive and pivotal role in diverting excess retinol, whether obtained from diet or recirculation from extrahepatic tissues, into storage as retinyl esters. The model thus implies the existence of a well-controlled homeostatic feedback system. In this model, the liver not only regulates the provision of retinol, a prohormone, to peripheral tissues but also responds as an end organ to peripherally and/or locally produced RA. This signal, in turn, regulates the expression of LRAT and thereby the retention of retinol as retinyl esters within liver cells. 

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