Cloning and molecular expression analysis of large and small lecithin:retinol acyltransferase mRNAs in the liver and other tissues of adult rats

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Retinyl ester, the most abundant form of vitamin A (retinol), is synthesized by the enzyme lecithin:retinol acyltransferase (LRAT). Previously, we cloned a 2.5 kb LRAT cDNA from rodent liver which codes for functional LRAT activity. However, Northern blots of tissues probed with the 2.5 kb cDNA revealed the presence of a larger transcript of ≈ 5 kb as well as several smaller transcripts. To elucidate the nature of the large LRAT transcript, a high-molecular-mass adrenal gland cDNA library was screened. Two similar clones of 3962 and 3187 nt were identified which appeared to be part of the 3'-untranslated region (UTR) of a 5358 nt LRAT mRNA. The 5.3 kb cDNA was then amplified from liver by reverse transcriptase PCR (RT-PCR) and demonstrated to encode functional LRAT activity. The 3'-UTR of the 5.3 kb cDNA contains several AAUAAA polyadenylation signals. Analysis of the 3' ends of LRAT mRNA transcripts from liver, intestine and testis showed the usage of both canonical and

non-canonical polyadenylation signals. To further analyse the LRAT mRNAs expressed *in vivo*, Northern blot analysis was performed using four probes spanning sections from the 5' end to the distal 3' end of the 5.3 kb LRAT cDNA. The results show that the major 5.3 kb transcript uses the canonical signal AAUAAA located at nt 5308, and the major short transcript of ≈ 1.5 kb uses the non-canonical signal AUUAAA located at nt 1330. The 5.3 kb LRAT transcript was predominant in the liver of retinoic acid-repleted vitamin A-deficient rats, coincident with increased quantitative expression of LRAT mRNA and enzyme activity. The differential usage of these polyadenylation signals can explain the presence of multiple LRAT mRNA transcripts which are expressed in different tissue-specific patterns.

Key words: PCR, polyadenylation, retinoic acid, retinol esterification, 3'-untranslated region.

INTRODUCTION

Lecithin:retinol acyltransferase (LRAT), a microsomal enzyme, is present in several retinol (vitamin A)-metabolizing tissues where it catalyses the esterification of retinol for storage or transport. The cDNA for LRAT has been cloned from human [1] and bovine [2] retinal pigment epithelium and rodent liver [3]. Neither sequence has significant similarity to any previously reported sequences and thus LRAT appears to belong to a unique gene family. The LRAT cDNAs from human retinal pigment epithelium and rodent liver are similar in length (2.7 and 2.5 kb respectively), each includes an open reading frame (ORF) coding for homologous proteins of 230 and 231 amino acids, respectively, and encodes functional retinol-esterifying activity in transfection assays [3]. In both of these cDNAs, the ORF is followed by a relatively long (≈ 1.5 kb) 3' untranslated region (UTR).

When the LRAT cDNAs of ≈ 2.5 kb, as reported above, were used as probes in Northern blot analysis of human and rat RNA from a number of tissues, several RNA bands were detected, including a species of ≈ 5 kb, as well as several lower-molecular-mass bands. These bands were expressed in different patterns in several of the tissues examined [1,3]. Other investigators reported the presence of an LRAT mRNA transcript of ≈ 5 kb together with other smaller-sized transcripts in normal human mammary epithelial cells [4,5] and renal epithelial cells [6], which also were capable of retinol esterification. The cause of the multiple forms of LRAT mRNA is not yet understood. These differences could

represent the presence of variant genes, or the various LRAT mRNA species could be the products of a single gene which is differentially processed as a result of differences in promoter usage, alternative splicing or the presence of different polyadenylation signals within the 3'-UTR of the LRAT mRNA. However, no canonical AAUAAA polyadenylation signal was found in the 2.5 kb cDNA clones of either mouse or rat LRAT mRNA [3], although two such sequences are present in the human LRAT mRNA [1]. Moreover, we ruled out the possibility of alternative splicing at least within the amino acid coding region because only a single amplicon was generated by PCR using primers that would specifically amplify this region [3].

Based on having observed an LRAT transcript of ≈ 5 kb on Northern blots, we initiated the present study to verify the presence of a larger LRAT mRNA species and determine its functionality in normal tissues. We describe herein the cloning of a 5358 nt LRAT cDNA which encodes the same functional ORF as found previously, and which has an extended 3'-UTR which contains multiple potential sites for polyadenylation. Transfection of cells with this cDNA resulted in expression not only of the 5 kb LRAT transcript, but of one significant band of lower mass and several minor bands. To characterize the LRAT mRNAs that are endogenous to normal tissues, we used anchored PCR to amplify and Northern blot analysis to identify the 3' end of LRAT mRNAs which are present endogenously in liver, intestine and testis, or which are expressed in LRAT-transfected cells. This analysis revealed the presence of multiple polyadenylation signals having either canonical or non-canonical sequences.

Abbreviations used: CRBP, cellular retinol-binding protein; LRAT, lecithin:retinol acyltransferase; RA, retinoic acid; ORF, open reading frame; UTR, untranslated region; RT-PCR, reverse transcriptase PCR.

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The 2.5 kb rat liver lecithin:retinol acyltransferase cDNA was deposited with the GenBank Nucleotide Sequence Database under accession no. AF255060 [3].

The amplification of LRAT mRNA 3' ends containing these sequences from normal tissues indicates that they are all used *in vivo*, although the efficiency of usage appears to differ. By Northern blot analysis, the major LRAT transcripts of ≈ 5 and ≈ 1.5 kb both share the same 5'-end. However, only the longer transcript contains the more proximal and distal segments of the 3'-UTR. While LRAT mRNA and enzyme activity were nil in liver of vitamin A-depleted rats, both increased rapidly and dose-dependently after treatment with all-*trans*-retinoic acid (RA): both high- and low-molecular-mass LRAT transcripts were expressed. These experiments have revealed a much greater complexity for LRAT mRNA than previously appreciated, and suggest that differential usage of polyadenylation signals accounts, at least in part, for the observed heterogeneity of LRAT mRNA amongst various tissues.

EXPERIMENTAL

Construction of a high-molecular-mass cDNA library

The cDNA library was constructed using the SMART cDNA construction kit from Clontech (Palo Alto, CA, U.S.A.) following the protocol furnished by the company with some modifications. Poly(A)⁺ RNA (1.5 μ g) from rat adrenal gland was used to make the first cDNA strand, followed by synthesis of the second strand using three cycles of PCR according to the primer-extension procedure of the Clontech protocol. The double-stranded cDNA library was cleaned of any protein and then digested with *Sfi*I enzyme. We modified the protocol by using 1% agarose gel for electrophoresis not only to remove the small *Sfi*I-digested oligonucleotide fragments but also to size-fractionate the cDNA library. The cDNA fractions of 3.5 kb and higher were cut from the gel and extracted using a Qiagen II Gel Extraction kit (Qiagen, Valencia, CA, U.S.A.). The fractionated cDNAs were ligated into the TrpI-Ex2 vector, which was then packaged using the Gigapack III Gold Packaging Extract from Stratagene (La Jolla, CA, U.S.A.). The library was infected into XL1-Blue strain *Escherichia coli* and then plated on agar for plaque formation. More than 10⁵ plaques were screened using the full-length (2.5 kb) rat liver LRAT cDNA labelled with [α -³²P]dCTP, using the Prime-a-Gene labelling kit (Promega, Madison, WI, U.S.A.), as the probe. After two rounds of screening, we identified two clones (clones 1 and 14; see the Results section) which were then converted from the phage into the plasmid vector via Cre-lox-mediated subcloning in *E. coli* BM25.8, exactly according to the protocol supplied by Clontech. The clones in plasmid pTrpI-Ex-2 were isolated, analysed for the LRAT cDNA insert and transformed into *E. coli* JM109 (Promega). DNA from a large-scale plasmid preparation was isolated and sequenced for the insert in both directions by gene walking, initially using the primers from the vector (Nucleic Acid Facility, Pennsylvania State University, University Park, PA, U.S.A.). The sequences were assembled and aligned with the rat liver LRAT cDNA sequence (accession no. AF255060) that we reported previously [3]. When we submitted the cDNA for the 3'-UTR sequence cloned from rat adrenal gland, GenBank determined that the same accession number should be used for the putative full-length 5358 nt LRAT cDNA, which we subsequently amplified intact from rat liver.

RNA isolation and analysis

Total RNA and poly(A)⁺ RNA were prepared as described previously [3,7]. For Northern blot analysis the isolated total RNA (≈ 20 μ g) or poly(A)⁺ RNA samples (4–5 μ g) were separated by electrophoresis in 1–1.2% agarose gel, transferred

to nylon membrane, and the membrane was hybridized to the LRAT cDNA probes labelled with [α -³²P]dATP (6000 Ci/mmol) using the Prime-a-Gene labelling kit. The membranes were washed and exposed to Kodak BioMax-MS film (Eastman Co., Rochester, NY, U.S.A.) for different times.

For experiments on the kinetics and dose-response of LRAT mRNA following treatment of rats with RA (see below), LRAT mRNA was measured by a quantitative real-time PCR procedure using a Perkin-Elmer ABI 7700 cyclor system with LRAT-specific primers and probe described previously [8]. The endogenous 18 S RNA transcript was measured as the internal control. Samples were analysed in triplicate, and the values for LRAT mRNA/18 S mRNA were then expressed relative to the mean value for the pair-fed vitamin A-sufficient group, defined as 1.00.

LRAT expression in HEK-293T cells

A pair of primers were designed from the extreme 5'- and 3'-ends of the large LRAT mRNA for reverse transcriptase PCR (RT-PCR) analysis of poly(A)⁺ RNA from rat liver and other tissues, including adrenal gland, eye, lung, small intestine and testes. The primers were 5'-*ATAGGATCCTGACCAACACTACATCCTCTC*-3' (nt 76–97) as the forward primer and 5'-*ATTCTC-GAGTCTAAGTTTATTGAAACCCAG*-3' (nt 5246–5270) as the reverse primer, for amplification of a fragment with 5194 nt (nucleotides in *italic* represent the *Bam*HI and *Xho*I restriction sites, respectively). The first strand was synthesized by Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, U.S.A.) and used for amplification of LRAT cDNA by Platinum Pfx DNA polymerase (Life Technologies) following the manufacturer's protocol. The amplified fragment was cloned unidirectionally into pcDNA3.1(+), subjected to sequencing for confirmation and permanently transfected into HEK-293T cells as described previously [3]. The cells were grown and then lysed for determination of LRAT activity. Cells containing the mouse LRAT ORF [3] and the empty vector only were used as positive and negative controls, respectively.

In vitro transcription/translation assay

The pcDNA3.1 vector containing the LRAT cDNA used for the transfection study above was also used as a template for an *in vitro* transcription/translation assay (TNT[®] Coupled Reticulocyte Lysate System from Promega) following the manufacturer's instructions exactly. Briefly, 1 μ g of DNA plasmid was used in a standard TNT reaction system of 50 μ l with T7 RNA polymerase in the presence of [³⁵S]methionine at 30 °C for 90 min. An aliquot of 5 μ l of the reaction product was subjected to electrophoresis in an SDS/polyacrylamide gel (12%) under reducing conditions to size fractionate the labelled protein products. The gel was dried under vacuum and then exposed to X-ray film as described above.

Analysis of the 3' ends of the rat LRAT mRNA transcripts

Anchored PCR with gene-specific primers was used to analyse and identify the 3' end of the rat LRAT mRNA. For this, 1 μ g of poly(A)⁺ RNA from small intestine, liver or testes was used to synthesize the 3' end of the first-strand cDNA using Superscript II reverse transcriptase and an anchored oligo(dT) primer supplied in the SMART RACE kit (Clontech). Following dilution, the first-strand reaction product was subjected to two rounds of PCR for amplification to increase specificity using *Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA,

U.S.A.) and *Taq* Extender (Stratagene). In the first round of the amplification, we used either the LRAT gene-specific primer 1 (5'-TTTGTGACCTACTGCAGATACGGC-3', nt 760–783) or primer 3 (5'-AATTGAATCACAGAATAGCCAAGCATG-3', nt 2543–2569) as the forward primers and the universal primer mix from the SMART RACE kit as the reverse primer with the following cycling program in a Perkin-Elmer model 2400 cycle sequencer: one cycle of 94 °C for 10 min, 5 cycles of 94 °C for 5 s and 72 °C for 4 min, 5 cycles of 94 °C for 5 s, 70 °C for 10 s and 72 °C for 4 min. Finally, 25 cycles of 94 °C for 5 s, 68 °C for 10 s and 72 °C for 4 min. The PCR reaction product was diluted and then subjected to the second round of PCR using either primer 2 (5'-TGCATCCCGTTCTGCTTGTGG-3', nt 931–951) or 4 (5'-CCAAGCATGTAATCCCAACC-3', nt 2561–2580) as the nested gene-specific primers (see Figure 4A, below) and the nested universal primer from the Clontech SMART RACE kit. The cycling program for PCR was as follows: one cycle of 94 °C for 10 min, 28–30 cycles of 94 °C for 5 s, 68 °C for 10 s and 72 °C for 4 min, and finally one cycle of 72 °C for 15 min. After each round of the amplification, the amplified PCR product was analysed by electrophoresis in 1% agarose gel. The PCR products after the second round of the amplification were then cloned into the pGEM-T Easy plasmid vector using a TA cloning ligase kit (Promega). The ligated DNA was transformed into *E. coli* strain JM109, which was then plated on to agar plates at 37 °C overnight. The clones (50–100 colonies) were picked individually and amplified in 3 ml of LB medium [9] at 37 °C overnight. The plasmid DNA from each clone was extracted using a DNA plasmid Miniprep kit (Qiagen) and then analysed by *NotI* restriction digestion. One clone of each size was subjected to sequencing in both directions using primers from the vector. The DNA sequence pieces were assembled, aligned and then compared with those sequences available in the NCBI database using the BLAST program.

RA treatment of vitamin A-deficient rats

Approval for these studies was obtained from the Institutional Animal Use and Care Committee. Lactating female rats of the Lewis strain were purchased with male pups (Harlan Sprague-Dawley, Indianapolis, IN, U.S.A.). The females and post-weaning male rats were fed a vitamin A-free purified diet (AIN93; Dyets, Bethlehem, PA, U.S.A.). When rats were 40 days old, they were randomly assigned to treatment groups ($n = 5$ for the vitamin A-deficient and vitamin A-sufficient groups, and $n = 3$ for each RA-repletion group), each with equal average body mass. For a 2 week experimental period, all of the rats except one group (vitamin A-sufficient) continued to receive the vitamin A-free diet. The vitamin A-sufficient group was fed diet of the same composition containing 4 mg of retinol, as retinyl palmitate, per kg of diet. To ascertain the kinetics of RA repletion, groups of vitamin A-deficient rats were treated with a single intraperitoneal injection of 100 µg of all-*trans*-RA in vehicle [10], and were killed 3–72 h later. For the dose-response study, groups of vitamin A-deficient rats received either an intraperitoneal injection of 5 or 50 µg or an oral dose of 500 or 5000 µg of all-*trans*-RA in soya bean oil and were killed either 8 or 18 h later. Oral dosing was used in this study because it was impractical to inject the larger doses of RA, and previous studies have indicated a similar LRAT response to retinoids administered intraperitoneally and orally [11]. At the indicated times the rats were killed individually by CO₂ asphyxiation, blood was drawn from the vena cava for preparation of plasma, and tissues were removed and immediately frozen in liquid nitrogen for storage at –80 °C until analysis [12].

LRAT enzyme activity assay

LRAT enzyme activity was measured in HEK-293T cell lysates and rat liver homogenates, as described previously [3,10], using [³H]retinol bound to cellular retinol-binding protein (CRBP) as the substrate under conditions in which product formation is proportional to incubation time and the amount of homogenate protein analysed.

Statistical analysis

Data are presented as means ± S.E.M. Analyses were conducted by unpaired Student's *t* test or by one-way ANOVA, as appropriate, and Fisher's protected least-significant difference test (SuperANOVA; Abacus Concepts, Sunnyvale, CA, U.S.A.).

RESULTS

Cloning of a 5.3 kb LRAT cDNA

To better understand the nature of multiple forms of LRAT mRNA observed in previous studies, a high-molecular-mass cDNA library was constructed from rat adrenal gland. This tissue was chosen because the larger-sized LRAT mRNA was the predominant form observed in adrenal gland by Northern blotting [3]. The cDNA library was screened using, as a probe, the 2.5 kb LRAT cDNA isolated previously from rat liver [3]. After two rounds of screening about 10⁵ plaques in total, we identified two clones with inserts of 3962 and 3187 nt (Figure 1A). The sequences of both clones matched perfectly with the 3'-UTR of the rat liver LRAT mRNA cloned previously [3], beginning at nt 1397 and 2172, respectively, and thus appeared to constitute the 3'-UTR of a larger LRAT transcript. Alignment of the sequence of either clone with that of the 2.5 kb liver LRAT cDNA resulted in an assembled clone with a size of 5358 bases. This sequence (Figure 1B) contains five polyadenylation signals within the 3'-UTR region, with the last signal located, as is typical, about 15 nt upstream of the polyadenylation region [13]. There are also 13 AUUUA sequence motifs scattered in the 3'-UTR. These motifs have been reported to play important roles in the stability of mRNA in other genes [14–16], although their function in LRAT mRNA remains to be determined.

In order to determine whether such an mRNA species is present in intact form in the liver and other tissues, a pair of primers was designed from the extreme regions of this clone (Figure 1B), and used to amplify this large fragment by RT-PCR. For this, the first strand was constructed from poly(A)⁺ RNA of different tissues and used to amplify a 5.2 kb fragment by PCR as described in detail in the Experimental section. The fragment is apparent in the liver (Figure 2A), and was also seen in other tissues, including adrenal gland, eye, lung, small intestine and testes (results not shown). The amplified fragment from the liver was cloned unidirectionally into a mammalian expression vector, pcDNA3.1, and then sequenced for comparison with the assembled sequence shown in Figure 1(B). Both sequences were identical. The plasmid containing the clone was then stably transfected into HEK-293T cells to determine whether it encodes functional LRAT activity, measured *in vitro* using [³H]retinol CRBP as substrate. Cells that had been transfected with either the previously tested LRAT ORF or an empty vector were used as positive and negative controls, respectively. As shown in Figure 2(B), the lysate of cells transfected with the large LRAT cDNA fragment had significant retinol esterification activity, comparable with the positive control cells transfected with only the LRAT ORF, while the lysate of the cells transfected with empty vector had no significant LRAT activity.

Figure 2 Amplification of a 5.2 kb LRAT cDNA from rat liver RNA by RT-PCR and analysis of its LRAT functional activity after expression in HEK-293T cells

(A) Ethidium bromide-stained agarose gel electrophoresis of the LRAT RT-PCR product amplified from rat liver poly(A)⁺ RNA. A pair of primers was designed from the 5' end (nt 76–97) and 3' end (nt 5246–5270) of the LRAT mRNA (see Figure 1B) and used for RT-PCR as described in the Experimental section. The LRAT amplicon from rat liver had the expected size of about 5.2 kb. MW, high-mass DNA marker (Life Technologies); C, negative control; rl-LRAT, rat liver LRAT. (B) The long LRAT cDNA in the pcDNA3.1 vector was stably transfected into HEK-293T cells, and the cells were then grown in T-25 flasks to confluence. One-twentieth of the cell lysate was incubated with [³H]retinol-CRBP (see the Experimental section). The vector plasmid alone and vector containing mouse liver LRAT cDNA for the ORF [3] were used as the negative and positive controls, respectively.

Northern blot analysis and translation analysis of expressed LRAT cDNAs

In order to compare the pattern of the LRAT mRNA expression in transfected HEK-293T cells with that of LRAT mRNA in rat liver, total RNA from wild-type and transfected HEK-293T cells, along with poly(A)⁺ RNA from rat liver, were subjected to Northern blot analysis using the rat LRAT 5'-end cDNA as a probe. No LRAT mRNA transcript was detected in the RNA samples from either wild-type cells or cells transfected with only the pcDNA3.1 vector (Figure 3A and its loading control, Figure 3B). The LRAT cDNA probe detected a single mRNA transcript, which was somewhat greater than 1 kb in length, in the RNA sample from HEK-293T cells that had been transfected with mouse LRAT cDNA encompassing only the ORF. However, similar to the Northern blot of rat liver RNA, a ≈ 5 kb LRAT mRNA transcript was the major transcript in cells transfected with the long (5.2 kb) rat LRAT cDNA. Another significant band of ≈ 1.5 kb was also present in cells transfected with the long LRAT cDNA.

The ORF of the previously described 2.5 kb mouse and rat LRAT cDNA predicts a protein of 25.8 kDa [3]. Based on our

Figure 3 Transcriptional expression of the long LRAT cDNA in HEK-293T cells and of LRAT protein in a cell-free system

(A) Northern blot analysis of RNA from wild-type and transfected HEK-293T cells as compared with that of rat liver. Total RNA (20 μg) from the cells and poly(A)⁺ RNA (5 μg) mixed with total RNA (12.5 μg) from rat liver were subjected to Northern blot analysis using ³²P-labelled probe A (see Figure 4). Lanes: 1, wild-type cells; 2, cells transfected with pcDNA3.1 vector plasmid; 3, cells transfected with mouse LRAT cDNA encompassing the ORF nucleotides; 4, cells transfected with long rat LRAT cDNA; 5, RNA from rat liver. (B) Ethidium bromide-stained agarose gel electrophoresis of the RNA samples before transfer to the membrane for Northern blot analysis. (C) SDS/PAGE of ³⁵S-labelled LRAT protein products expressed in a cell-free transcription/translation system. The LRAT cDNA containing pcDNA3.1 vector plasmid was used as the template to synthesize ³⁵S-labelled LRAT protein. The synthesized proteins were fractionated by electrophoresis under reducing conditions on an SDS/polyacrylamide gel, which was then dried and exposed to X-ray film.

nucleotide sequence analysis, the long rat LRAT cDNA contains exactly the same ORF and would therefore be expected to encode a protein of identical size. To evaluate this experimentally, we conducted an *in vitro* transcription/translation assay using the plasmid containing only the LRAT ORF insert or the pcDNA3.1 plasmid containing the long LRAT cDNA insert. The *in vitro*-translated [³⁵S]methionine-labelled proteins were

Figure 1 Relative map of the rat adrenal gland LRAT cDNA clones compared with that of the 2.5 kb rat liver LRAT cDNA, and nucleotide sequence of the 5.3 kb rat LRAT mRNA

(A) Two clones (nos 1 and 14) were isolated from a rat adrenal gland cDNA library (see the Experimental section). The nucleotide sequences of both clones matched perfectly with that of the 3'-UTR of the 2.5 kb rat liver LRAT cDNA [3] illustrated, and contained no ORF for LRAT protein. After alignment of the sequences of these two clones with that of the 2.5 kb liver LRAT cDNA, a putative LRAT transcript with a length of 5358 nt (see Figure 1B) was assembled. (B) The nucleotide sequence resulting from assembly of nucleotide sequences of clone 1 and of rat liver LRAT cDNA. It consists of 696 bases for the ORF (methionine bold and underlined) encoding the 231 amino acid residues of LRAT protein and a long 3'-UTR containing five AAUAAA (double-underlined) polyadenylation signals and 13 AUUUA (underlined) motifs.

Figure 4 Identification of LRAT mRNA transcripts in different tissues of adult rats

(A) Positions of the 3' end of major and minor LRAT mRNA transcripts relative to the large rat LRAT mRNA. As described in the Experimental section, two sets of anchored PCR, each followed by a nested PCR, were performed using gene-specific primers 1–4 to clone and identify the 3' end of the LRAT mRNA transcripts expressed in several tissues. (B, E) Agarose gel electrophoresis of the PCR products of the first (B) and second (E) set of anchored PCR from small intestine (I), liver (L) and testes (T). (C, F) The PCR products from liver in each set were cloned in pGEMT Easy vector and transformed in *E. coli* JM109 for amplification and isolation. The individual plasmid clones were digested with *NotI* and then separated in ethidium bromide-stained agarose gel; (C) clones a–e from the first set of anchored PCR; (F) clones f–m from the second set of anchored PCR. Lanes M1 and M2, DNA size markers (in kb). (D, G) Identification, size, 3'-end positions and polyadenylation sequences of the clones obtained from sets 1 and 2 of anchored PCR.

electrophoresed side by side on an SDS/PAGE gel. One major protein band of ≈ 25 kDa was detected from the translation of the long rat LRAT cDNA as well as the mouse LRAT ORF cDNA (Figure 3C). As expected, no protein was detected when empty pcDNA3.1 vector was used as the template.

Analysis of 3' end of the LRAT mRNA transcripts

To ascertain whether the multiple forms of LRAT mRNA transcripts observed by Northern blotting (see [1,3]) may be due to the presence of multiple polyadenylation signals in the 3'-UTR of the LRAT mRNA (see Figure 1B), we first identified the 3' ends of the LRAT mRNA transcripts. For this, we synthesized the first-strand cDNA from poly(A)⁺ RNA from small intestine, liver and testes of normal adult rats using an anchored oligo(dT) primer as described in detail in the Experimental section. As is illustrated in Figure 4(A), a gene-specific primer, primer 1, was designed for the anchored PCR and then, using the anchored PCR product as the template, another gene-specific primer, primer 2, was used as a nested primer for PCR to increase the specificity and efficiency. The nested PCR products were analysed by electrophoresis in ethidium bromide-stained agarose gel (Figure 4B). In total, five or six bands ranging in size from 400 to 1600 bp were observed (Figure 4B). In the testis the smallest band was most intense, while in the liver the largest band was most intense. In the intestine, however, both the largest and smallest bands were of nearly equal intensity. Additionally, three less-intense bands ranging in size from 800 to 1000 bp were clearly observed in the liver but were very faint in the intestine and testes. No band larger than 1600 bp was observed, although a band with a size of more than 4 kb was anticipated (see Figure 4A). When the products of nested PCR from liver were cloned into the pGEM-T Easy vector by TA cloning and screened, as described in detail in the Experimental section, we obtained five different clones (labelled a–e, see Figure 4C), which were then sequenced and compared with the sequence of the rat LRAT cDNA. As illustrated in Figures 4(C) and 4(D), these five clones ranged in size from 423 to 1591 bp. Four of these clones had a polyadenylation signal although none was as perfect as the canonical signal, AAUAAA. The smallest clone, which represents the major band in the PCR products of the intestine and testes, contained a non-canonical AUUAAA sequence beginning at nt 1330 in the LRAT cDNA (see Figures 1B and 4A). Such a sequence has been shown to be functional as the polyadenylation signal for some other genes [17–19]. The largest sized clone, however, contained no polyadenylation signal, although it appears as the major band in the nested PCR products from liver (Figure 4B). The 3' end of this clone terminates just after the region where there is a CA repeat and before a canonical polyadenylation signal, AAUAAA, immediately downstream of the clone within the 3'-UTR of the LRAT mRNA transcript (see Figure 1B). In fact, when we originally cloned the LRAT cDNA from liver of mouse and rat by a similar method we could not clone beyond this region [3].

Because we were unable to clone the entire 3'-end region of the 5.3 kb LRAT transcript using primers 1 and 2, we designed a new set of gene-specific primers, 3 and 4, as illustrated in Figure 4(A), and performed a second set of anchored PCR followed by nested PCR. As shown in Figure 4(E), the bands were similar in size in liver, intestine and testis, while there were differences in the intensity of most of the bands among these tissues. After cloning and sequencing, as described above, we obtained eight new clones (f–m), ranging in size from 77 to 2760 bp (Figures 4E and 4F). Two of these clones contained the canonical polyadenylation signal, AAUAAA, beginning at nt 3781 and 5308,

Figure 5 Northern blot analysis of RNA from rat tissues and HEK-293T cells

Poly(A)⁺ RNA (4 or 5 μ g) isolated from the pooled total RNA of rat small intestine (I), liver (L) and testes (T) and total RNA (20 μ g) from HEK-293T cells (HEK) transfected with the long rat LRAT cDNA (see Figure 2) were electrophoresed in 1% denatured-agarose gel. The fractionated RNA samples were transferred to membranes and then hybridized to one of the [³²P]dATP-labelled probes A, B, C or D (see Figure 4A) according to the procedure described in the Experimental section. The blots were exposed to X-ray film for different times and then scanned.

respectively (Figure 4G). The latter is just upstream of the last polyadenylation site of the large LRAT transcript which was the major band in the Northern blot. Three clones contained non-canonical polyadenylation signals of AUUAAA, AGUAAA and AAUAGA, respectively, and three other clones had no recognizable polyadenylation signal (Figure 4G).

Based on the results of the experiment above, we then designed four probes which span the putative 5.3 kb LRAT mRNA, as shown in Figure 4(A); these have been designated probe A (nt 77–1417), probe B (nt 1479–2443), probe C (nt 2561–4422) and probe D (nt 4500–5268). Northern blot analysis was then conducted on four replicate blots, each containing poly(A)⁺ RNA from rat small intestine, liver and testes, and total RNA from HEK-293T cells transfected with the entire region of rat LRAT cDNA (as shown in Figure 2). Probe A corresponding to the 5' end of LRAT cDNA hybridized to both large and smaller LRAT transcripts (Figure 5). The same result was found using probes spanning either 1–914 or 1–2522 nt (results not shown). In comparison, probes B, C and D from three regions in the 3' end also hybridized to the larger LRAT mRNA transcript, but they did not detect the lower-mass transcripts (Figure 5). This difference is particularly evident for the small intestine, testes and HEK-293T cells in which smaller-sized LRAT transcripts are most intense when hybridized with probe A but are not seen when hybridized with probes B, C or D (Figure 5).

Quantitative expression of LRAT mRNA and activity after RA repletion

LRAT mRNA expression and activity have been shown to be down-regulated by vitamin A deficiency as well as induced by

Figure 6 Time course of the LRAT mRNA and enzyme activity responses in liver of vitamin A-deficient rats to treatment *in vivo* with all-*trans*-RA

Data shown are the means $\pm$ S.E.M. from $n = 5$ vitamin A-sufficient (VAS) rats, $n = 5$ vitamin A-deficient (VAD) rats and $n = 3$ VAD rats repleted with $100 \mu\text{g}$ of RA at each time point. (A) LRAT mRNA, assayed by quantitative real-time PCR (QRT-PCR) is expressed relative to the mean of the VAS group which is defined as 1.0; the dashed line represents the mean. (B) LRAT enzyme activity was assayed with [^3H]retinol bound to cellular retinol-binding protein (see the Experimental section) and is also expressed relative to the mean of the VAS group, defined as 1.0 (mean value, $29.3 \text{ pmol of retinyl ester} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$). VAS and VAD groups were compared by Student's *t* test and the results are indicated; the time-course data were analysed by one-factor ANOVA. Groups not sharing a common letter are significantly different ($P < 0.05$).

RA in the liver of vitamin A-deficient rats [3,10], but detailed studies comparing the induction of both mRNA and enzyme activity simultaneously have not been conducted. For this purpose, we examined the kinetics of expression of LRAT mRNA level and activity induced by all-*trans*-RA in the liver of vitamin A-deficient rats, and compared these with steady-state levels in vitamin A-adequate control rats. Liver LRAT mRNA was assayed by a quantitative PCR procedure using fluorescently labelled nucleotide probes chosen from the coding region [8]; therefore both the longer and shorter LRAT-encoding transcripts were detected by this assay. Compared with vitamin A-sufficient rat liver (defined as 1.00 for comparison), the level of LRAT mRNA in vitamin A-deficient liver was reduced to about 1% of control at the outset of the study ($P < 0.0001$; Figure 6A). Plasma retinol levels were similarly reduced (results not shown). Vitamin A-deficient rats were then treated with $100 \mu\text{g}$ of all-*trans*-RA and LRAT mRNA and activity were assessed 3–72 h later. LRAT mRNA increased within 3 h, reached a maximum by ≈ 10 h, and then declined by 24 h. Similarly, LRAT enzyme activity increased within 3 h, reached a maximum a few hours after the peak of LRAT mRNA, and then declined (Figure 6B). Because pharmacokinetic studies have shown the half-life of RA *in vivo* to be on the order of an hour after either intravenous or oral administration [20], the decline in mRNA after its rapid induction suggests that RA must be present continuously to maintain LRAT gene expression in the 'on' position. At the time of maximum induction after RA treatment, LRAT mRNA was

Figure 7 Dose-response of LRAT mRNA and enzyme activity in liver of vitamin A-deficient rats treated with all-*trans*-RA

Data shown are the mean $\pm$ S.E.M. from $n = 5$ vitamin A-deficient (VAD) rats and $n = 3$ VAD rats repleted with ≈ 5 – $5000 \mu\text{g}$ of RA. (A) LRAT mRNA expressed relative to the mean of the control group (shown in Figure 6 by the dashed line). (B) LRAT activity. RE, retinyl ester. The dose-response data were analysed by one-way ANOVA and Fisher's least-significant difference test. Groups not sharing a common letter are significantly different ($P < 0.05$).

about twice that of vitamin A-sufficient controls, whereas LRAT activity did not rise above the control level.

Having determined the maximum times of RA-induced LRAT mRNA and enzyme activity, we examined the response to RA dosage over a range of 5– $5000 \mu\text{g}$ of RA. For this experiment, measurements of LRAT mRNA were made 8 h after treatment and measurements of LRAT activity 18 h after treatment with RA. A single $5 \mu\text{g}$ dose of RA significantly increased both LRAT mRNA (Figure 7A) and enzyme activity (Figure 7B), while higher doses resulted in higher expression. As observed in the kinetic study, the relative increase in LRAT mRNA exceeded that of LRAT activity.

To examine whether the multiple LRAT mRNA bands observed in the liver of control rats are induced by RA in vitamin A-deficient rats, we prepared pools of poly(A)⁺ RNA from the livers of animals in the kinetic and dose-response studies shown above and subjected them to Northern blot analysis using probe A (Figure 8). Several bands were revealed throughout the induction phase. At all times and doses, the larger LRAT mRNA was the predominant form.

Previously it was shown that inhibitors of RNA and protein synthesis nearly completely suppressed the induction of LRAT enzyme activity by RA in vitamin A-deficient rats [10]. To determine whether actinomycin D has a similar effect on LRAT mRNA expression, a group of vitamin A-deficient rats was treated with actinomycin D 2 h prior to administration of $100 \mu\text{g}$ of RA [10]. As compared with the response of vitamin A-

Figure 8 Northern blot analysis of liver LRAT mRNA after RA treatment in time-course and dose-response studies

Poly(A)⁺ RNA prepared from pooled total RNA from the groups of animals shown in Figures 6 and 7 was fractionated on agarose gel and probed using probe A (see Figure 4), which detects large and small LRAT mRNAs. Markers for 28 and 18 S RNA are shown. For equal loading of RNA on the gel, the residual 18 S ribosomal RNA signal is shown below in the ethidium bromide-stained agarose gel, before transfer of the fractionated RNA to the membrane for Northern blot analysis. C, vitamin A-sufficient control (control samples from two different experiments are shown); D, vitamin A-deficient.

deficient rats treated identically but without actinomycin D, the increase in LRAT mRNA expression was inhibited by 93% ($P < 0.001$) in actinomycin D-treated rats, and LRAT activity was similarly reduced ($P < 0.0001$; results not shown).

DISCUSSION

The presence of more than one LRAT transcript in the size range of 1.5–5 kb in several human and rodent tissues and cell lines [1,3–6] suggests that LRAT mRNA expression is likely to be a complex process. In the present work we focused first on defining the nature of the long species of LRAT mRNA of ≈ 5 kb. This band has been detected by Northern blot analysis in several normal tissues and it appears to be the predominant species of LRAT mRNA in the liver, adrenal gland, lung and several other tissues [3]. By screening an adrenal gland cDNA library, we identified two positive clones containing the same sequence yet of different lengths (Figure 1A). Although neither of these clones contains an LRAT ORF, both are completely homologous with a portion of the 3'-UTR of the rat liver LRAT mRNA we described previously [3]. The assembly of either of these 3'-end clones with that of the previously obtained rat liver LRAT cDNA resulted in a predicted clone of 5358 nt (Figure 1B). This predicted size is very close to the observed size of the large-molecular-mass LRAT mRNA species present in several tissues. Direct evidence that this assembled clone represents the large-molecular-mass LRAT mRNA species found *in vivo* was obtained in two ways. First, the full-length 5 kb LRAT cDNA was amplified by RT-PCR directly from poly(A)⁺ RNA samples of rat liver (Figure 2A), as well as several other tissues (adrenal gland, eye, lung, small intestine and testes; results not shown), using conditions optimized for long-distance PCR. Second, upon transfection of the long rat LRAT cDNA into HEK-293T cells, a 5 kb LRAT mRNA transcript was expressed (Figure 3A), and was shown to be functional for LRAT activity (Figure 2B).

Analysis of the nucleotide sequence of the 5358 nt rat LRAT cDNA (Figure 1B) showed that the 3'-UTR of the corresponding mRNA contains five canonical AAUAAA polyadenylation signals, which begin at positions 2527, 3781, 4490, 5257 and 5308. These results suggest that several LRAT RNAs may exist *in vivo*

which share the same or similar 5' ends but differ in the length of their 3'-UTR. It appears that this single cDNA can be transcribed and processed into more than one RNA band because transcripts of both ≈ 5 and 1.5 kb were clearly evident by Northern blot analysis of HEK-293T cells that had been transfected with the long form of LRAT cDNA (Figure 3A). These two bands are comparable in size with the bands detected in rat liver mRNA. The size of the *in vitro*-translated protein, 25 kDa, obtained from transcription/translation of the long LRAT cDNA is identical with that expressed by the cDNA encoding only the LRAT ORF (Figure 3C). These results demonstrated that the presence of the long 3'-UTR did not affect the translation of the LRAT ORF in this system.

Based on the multiple polyadenylation signals described above, we used anchored PCR to amplify LRAT cDNA transcripts that are present endogenously in liver and other tissues. Two rounds of anchored PCR yielded 13 clones in total (a–m, Figure 4), each ending in a different segment of the 3'-UTR of the LRAT mRNA. Sequence analysis showed that these clones contain a canonical polyadenylation signal, a non-canonical signal or no known signal. In two of the clones with the canonical polyadenylation signal, AAUAAA, one was located beginning at nt 3781 and the other at nt 5308, corresponding to two of the signals described in Figure 1(B) for the 5.3 kb LRAT cDNA. The polyadenylation signal beginning at 5308 appears to be used to process the major high-mass species of LRAT mRNA that is present in all of the tissues examined [1,3,8]. In contrast, the polyadenylation signal at position 3781 appears to be used to only a minor extent, as a tissue LRAT transcript of corresponding length was not evident by Northern blotting. It is likely that the greater sensitivity and efficacy of the PCR amplification procedure resulted in the detection of this minor LRAT mRNA species. The other three canonical polyadenylation signals, AAUAAA, located at nt 2527, 4490 and 5257, were apparently non-functional in the sense that no clone was obtained having this signal in the region immediately upstream of the polyadenylation site.

Besides the clones described above with a canonical polyadenylation signal, seven clones were found to contain a non-canonical polyadenylation signal, including AUUAAA, AAUAAU, AUUAAU, AGUAAA and AAUAGA. These signals apparently are used during the processing of LRAT mRNA as they are represented in clones amplified directly from rat tissue RNA samples. These non-canonical polyadenylation signals have been reported to be functional in other genes [17–19], although they are not used as efficiently as the canonical AAUAAA signal. However, it appears that the AUUAAA signal located at nt 1330 in the LRAT mRNA is used efficiently to process the major LRAT mRNA transcript of ≈ 1.5 kb that is present in the small intestine and testes, as well as in HEK-293T cells transfected with the long LRAT cDNA. Among the non-canonical polyadenylation signals, AUUAAA has been shown to be used relatively efficiently in other genes, although not as efficiently as the canonical signal AAUAAA. Moreover, the AUUAAA signal has been reported to be used with greater efficiency when it is located in the proximal 3' end of the UTR rather than the distal 3' end [17]. The usage of these alternative polyadenylation signals during the processing of LRAT mRNA seems to be regulated in a tissue-specific manner, because Northern blotting showed the major low-mass LRAT mRNA transcript of about 1.5 kb to be more intense in the small intestine and testes (known as a 'hotspot' for differential polyadenylation site usage [17]) than in liver, lung or several other tissues [3,8].

In addition to the majority of the LRAT 3'-UTR clones which contained either a canonical or non-canonical polyadenylation

signal near their 3' ends, we found four different LRAT-positive clones with no apparent polyadenylation signal. These clones seem to represent minor processing variants of LRAT mRNA because, whereas they were apparent after PCR amplification, a band of corresponding size was not visible on Northern blots. Overall, the strategy of using anchored PCR with LRAT-specific primers to amplify the 3'-UTR of the LRAT mRNA from liver, intestine and testis revealed a greater complexity of LRAT mRNAs than has previously been demonstrated.

We then returned to Northern blot analysis of poly(A)⁺ RNA from rat tissues as well as total RNA of LRAT-transfected HEK-293T cells (Figure 5) to test directly whether the larger- and smaller-molecular-mass bands seen previously each contain the 5' region of the LRAT mRNA encompassing the LRAT ORF, and whether the shorter transcript(s) lack the 3' terminal end, and perhaps even the more proximal region, of the 3'-UTR. For this analysis, four probes, as illustrated in Figure 4(A), were used to probe replicate blots all containing the same size-fractionated poly(A)⁺ RNA from small intestine, liver and testis, and total RNA from HEK-293T cells transfected with long LRAT cDNA. We hypothesized that probe A, which encompasses the 5' end of LRAT mRNA, would hybridize to LRAT mRNA transcripts of all of the sizes that were previously observed (5 to $\approx$ 1.5 kb). We also hypothesized that, in contrast, probes B, C and D would also hybridize to LRAT mRNA transcripts larger than $\approx$ 1.5 kb but would not bind to the smaller LRAT mRNAs. Hybridization of these labelled probes showed that only probe A bound to both the longer and shorter LRAT mRNA species in each tissue, whereas the three probes encompassing the 3' end of the LRAT 3'-UTR also bound to the longer LRAT mRNA but not to the shorter LRAT mRNA species, principally the 1.5 kb mRNA. This result further suggests that the different LRAT mRNA transcripts share a common 5' end, and are truncated at different positions within the 3'-UTR. The major large LRAT transcript of $\approx$ 5 kb uses the canonical AAUAAA signal located at nt 5308, and the major small transcript of $\approx$ 1.5 kb uses the non-canonical signal of AUUAAA located at nt 1330.

Another interesting feature of the 3'-UTR of the 5.3 kb LRAT mRNA (Figure 1B) is the presence of a number of AU-rich sequence motifs, including 13 copies of the AUUUA motif, most of which are located within the first half of the 3'-UTR. Such a conserved *cis*-acting AU-rich element has been found in the mRNA coding for a variety of cytokines and proto-oncogenes, and has been shown to control their half-life by enhancing the rates of deadenylation and subsequent mRNA degradation (reviewed in [14–16]). It will be interesting in the future to determine whether these AUUUA motifs play any role in the regulation of LRAT mRNA in normal tissues or cancer cells.

To better understand the qualitative and quantitative regulation of LRAT mRNA and enzyme activity in the liver, additional studies of RA repletion were conducted in vitamin A-deficient rats. LRAT mRNA was quantified by a real-time PCR assay and LRAT enzyme activity was assayed under conditions in which substrate concentration is not rate-limiting. Both LRAT mRNA and activity were shown to be up-regulated in a time- and dose-dependent manner by all-*trans*-RA, with the maximum expression of LRAT mRNA occurring prior to that of LRAT activity as expected for a precursor-product relationship. Both increases were blocked by actinomycin D. These results imply that LRAT mRNA expression is regulated, at least in part, at the level of gene transcription. However, it is not yet known whether this effect is directly on the LRAT gene or may be indirect through regulation of another gene. In theory this mechanism could be determined by using an approach such as a nuclear run-

on assay. Our attempts to use this assay to study LRAT have not yet been successful, however, despite success with other genes [21], perhaps because LRAT mRNA abundance is relatively low. While the level of LRAT mRNA and enzyme activity were significantly correlated ($r = 0.6$, $P < 0.0001$, $n = 41$ samples in the time and dose studies; results not shown), there was not an exact one-to-one relationship between them. Generally LRAT mRNA was increased by RA several fold above the level present in vitamin A-adequate animals, while LRAT enzyme activity increased to near the control level (Figures 6 and 7). This may indicate that not all of the LRAT mRNA is translated. We note, however, that the Northern blot patterns for liver LRAT mRNA after RA repletion shows expression of the two major bands as well as several minor bands (Figure 8), as would be predicted from the cloning and analysis of the several 3' ends for LRAT mRNAs as shown in Figure 4.

In conclusion, these experiments have revealed a newly characterized 5.3 kb LRAT cDNA which can be amplified from the mRNA of liver and several other tissues, and which when expressed is functional for LRAT activity. The presence of several potential signals for polyadenylation in the 3'-UTR of this longer transcript suggests that smaller transcripts arise through truncation at different points along the 3'-UTR. The complexity of LRAT gene expression is thus revealed to be greater than previously recognized. It seems very likely that the ultimate regulation of LRAT expression, in different tissues and under different physiological and pathological conditions, occurs through a combination of several mechanisms.

This work was supported by grants from the National Institutes of Health DK46869 and CA90214, and funds from the Howard Heinz Endowment.

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Received 13 June 2002/13 August 2002; accepted 29 August 2002

Published as BJ Immediate Publication 29 August 2002, DOI 10.1042/BJ20020918