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An Essential Set of Basic DNA Response Elements Is Required for Receptor-Dependent Transcription of the Lecithin:Retinol Acyltransferase (*Lrat*) Gene

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Abstract

Lecithin:retinol acyltransferase (LRAT) is essential for vitamin A storage. Nuclear run-on assays demonstrated transcriptional regulation of the *Lrat* gene in vivo by *all-trans*-retinoic acid (RA) and other retinoids. Analysis of a 2.5 kb segment of rat genomic DNA revealed that the region ~300 bp upstream from the transcription start site (TSS) is necessary for high luciferase (Luc) reporter activity in HEK293T and HepG2 cells. Although this region lacks retinoid receptor binding elements, it responded to the nuclear receptors RAR α , RAR β or RAR γ , with RXR α , with and without ligand. Removal of -111 bp from the TSS, which is well conserved in human, rat and mouse genomes, completely eliminated activity. This region contains several basic elements (TATA box, SP3 site, AP-1 site, CAAT box), all of which were essential. Nuclear extracts from RA-treated cells exhibited enhanced binding. Therefore, this proximal region together with basal transcription factors may be sufficient to drive *Lrat* expression.

Keywords

liver; retinoid homeostasis; promoter analysis; cytochrome P450 *Cyp26*

Introduction

Lecithin:retinol acyltransferase (*Lrat*), a microsomal enzyme [1,2], catalyzes the synthesis of retinyl esters. A normal level of *Lrat* expression is required for the formation of RE in the intestinal during absorption, in the liver for storage, and in the retina to support the visual cycle. In the absence of *Lrat*, all of these functions are compromised, even when the diet is adequate in vitamin A [3,4]. In the liver and lungs, *Lrat* mRNA and enzymatic activity are coordinately down-regulated during vitamin A deficiency and up-regulated by treatment with *all-trans*-retinoic acid (RA), the principal active metabolite of vitamin A [5–10], while in other tissues *Lrat* mRNA is expressed in a constitutive manner [7]. *Lrat* expression is impaired or lost in certain tumor cell lines, despite being detectable in non-tumor cell lines originating from the same tissues [11–13]. *Lrat* appears to be a unique mammalian member of a large gene family of amidases and related activities present in archbacteria, plants, and viruses [14,15]. The gene organization of *Lrat* has been defined in both human and rat [16,17], and the transcription start site (TSS) has been identified [17].

It is now well described that RA functions as the main biological ligand for the RAR family (RAR α , β , and γ) of nuclear hormone receptors [18]. Together, pairs of RAR with the coreceptor RXR form RAR-RXR heterodimers that bind to DNA defined as retinoic acid response elements (RARE). These elements contain a core of two hexameric motifs of PuG (G/T)TCA(X)nPuG(G/T)TCA that generally are oriented as a direct repeat (DR) spaced by 2 or 5 nucleotides. The RARE are located upstream of the TSS in most RA-responsive genes [18–20]. Yet, only a minority of the many genes that have been shown to be physiologically responsive to retinoid treatment in vivo [19] have also been shown to be controlled directly by the transcriptional mechanism involving the direct binding of ligand-activated RAR-RXR to cognate RARE elements. Indeed, it appears that the majority of RA-responsive genes may be regulated indirectly [19]. Thus, elucidating alternative mechanisms is important for understanding the full potential for RA-regulated gene expression in vivo.

The mode of regulation of *Lrat* is unclear, as despite the significant regulation of *Lrat* mRNA by RA in tissues such as liver and lungs [7,9,21], no canonical RARE could be located in the regions more than 10 kbp upstream and downstream of the TSS, including in the intronic region of the *Lrat* gene [17]. Recently, Cai and Gudas [22] demonstrated that a 172-bp proximal region of human *Lrat* gene is essential for its transcription and responds to RA in cultured normal human prostate epithelial cells (PrEC), but not in human prostate cancer cell line PC-3. Because little is known regarding the regulation of *Lrat* expression in the liver, we have first analyzed rat liver for evidence of in vivo transcriptional regulation of the *Lrat* gene under physiological conditions, using nuclear run-on analysis, and then have analyzed the 5' putative promoter region of *Lrat* to identify regions that may confer regulation in response to retinoids. The results provide evidence that *Lrat* is transcriptionally regulated by RA in the intact liver, and that *Lrat* transcriptional activity is dependent on a group of generic and basal transcriptional elements in a manner dependent on retinoid receptors, but with little effect by their ligands. The results therefore suggest that basal transcriptional elements play a key role, and may even be sufficient, for modulating *Lrat* gene expression.

Materials and methods

Materials

Mouse RAR α , β , and γ , and RXR α , clones, each in pSG5 expression plasmid vector, were kindly provided by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/Collège de France, Illkirch-Cedex, Strasbourg, France). Rabbit polyclonal IgG antibodies against RAR α (C-20), RAR β (C-19), RXR α (D-20), SP1 (PEP-2, and SP3 (D-20) and mouse monoclonal antibodies against RAR γ (G-1) and actin (C-2) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with horseradish peroxidase were obtained from GE Healthcare (UK).

In vivo studies

Experimental protocols were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Liver tissues for LRAT mRNA analysis and nuclei preparation were obtained from rats fed after weaning a nutritionally adequate diet but deficient in vitamin A. When the rats were 55–60 day old they were divided into 3 groups, $N = 3/\text{group}$, treated orally with either vehicle (90% canola oil, 10% ethanol), vitamin A (0.7 mg retinol) in the form of retinyl palmitate, or the same amount of vitamin A combined with 0.1 mg of the RAR- α selective ligand Am580 [23]. Six h later, the rats were individually euthanized with carbon dioxide. The liver was rapidly dissected and weighed, and portions were snap frozen in liquid nitrogen for later extraction of RNA. Nuclei were prepared from fresh liver tissue of vitamin A-adequate and deficient rats by homogenization and isolation procedures described below.

RNA Extraction and Analysis

Total RNA was extracted from individual liver samples using Trizol (Invitrogen, Carlsbad, CA) and then treated further with guanidine hydrochloride and sodium acetate. Total RNA was quantified by spectrophotometry and analyzed for LRAT mRNA transcript levels by real-time PCR, with 18S rRNA as internal control [7].

Nuclei Preparation and Nuclear Run-On Assay

Nuclei were isolated from fresh liver tissues and then incubated in a reaction mixture containing ribonucleotides including [α - 32 P]-UTP (Amersham, Chicago, IL) to elongate the nascent RNA transcripts as previously described [24]. The labeled nuclear RNA was then extracted and hybridized to Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) that had been dot-spotted with cDNA probes for rat LRAT, CYP26A1, and beta-actin. The membrane was then washed extensively and exposed to X-ray film (Kodak BioMax, Eastman Kodak Co., Rochester, NY) for 1 h to 3 days at -80°C [24].

Cloning the 5' Upstream Region of the Rat *Lrat* Gene

A 3.2 kbp fragment spanning from -2541 bp upstream to 800 bp downstream of the rat *Lrat* TSS [7] was amplified by PCR from rat genomic DNA using 5' AGGTGCGACCCTGTCTCTAA3' as a forward primer and 5' CCTGCGGACTGATAGGAGAG3' as a reverse primer by high fidelity *pfx* DNA polymerase (Invitrogen) following the protocol recommended by the manufacturer. The cycling program was 94°C , 2 min for initial denaturation, and 40 cycles of 94°C , 15 s; 55°C , 30 s; 68°C , 3.5 min. The amplified fragment was run on agarose gel, the appropriately-sized band was cut from the gel and the DNA was extracted (Qiagen gel extraction kit (Qiagen, Valencia, CA)). The DNA was then ligated to the *Sma*I site of *pGL3-Luc* basic vector using T4 DNA ligase (Promega). After transformation into JM109 E. coli (Promega) for amplification, this clone was used to construct clones with different deleted parts from upstream and downstream of TSS using appropriate restriction enzymes. The constructed DNA clones encompassing from -2425 to $+257$ bp from TSS were first transformed, amplified and then purified as described above. Each clone was subjected to sequencing for confirmation at the Nucleic Acid Facility of the Pennsylvania State University (University Park, PA). Sequences were analyzed using the Transcription Element Search System (TESS) program from the Department of Biology of the University of Pennsylvania, <http://www.cbil.upenn.edu/cgi-bin/tess/tess>. For mutation of the sequence of basic transcriptional response elements including TATAA box, SP3 site, AP-1 site, CAAT box and SP1 site, a pair of oppositely directed primers was designed to contain EcoRV restriction site (GATATC) at the 5' end of each primer used to mutate the DNA binding site. Each primer was paired with a primer in the *pGL3*-basic vector harboring the rat *Lrat* putative promoter extending from -268 to $+257$ from the TSS as the template and used to amplify the mutated *Lrat* promoter. Each amplified DNA segment was digested with EcoRV and cloned. The cloned segments were ligated together and re-cloned into *pGL3*-basic vector, as above. The mutated clone was subjected to sequencing for confirmation as described above.

Transient Transfection and Luciferase Assays

Human embryonic kidney (HEK293T) cells and HepG2 hepatoma cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured as described [7]. One to two days before transfection the cells were seeded into 12- or 24-well plates with full growth media (DMEM medium with 10% fetal bovine serum [7]). When the cells were about 80% confluent, the plasmid-Luc DNA constructs were introduced into the same medium containing 5% fetal bovine serum with no antibiotics using Lipofectamine-2000 (Invitrogen). *pRLTK* containing the Renilla-Luc gene (Promega) was co-transfected to provide an internal standard for transfection efficiency. After 16 to 24 h of transfection, the cells were washed and incubated

with the full-growth medium with antibiotic in the absence and presence of 0.1 to 1 μ M RA in a final concentration of 0.1% ethanol. After incubation at 37°C for 24 h the cells, were harvested and assayed for firefly-Luc and Renilla-Luc activities using the DRL Luc assay system (Promega). Promoter activity was defined as the ratio of firefly-Luc to Renilla-Luc activity. Each experiment was performed at least 2 times, in duplicate, with consistent results within and between experiments.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed according to the methods previously described [25]. Briefly, single-stranded oligonucleotides spanning from -69 to -34 of the rat *Lrat* promoter containing AP-1 and SP3 sites were annealed in buffer containing 10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, pH 8.0, at 90°C for 10 min and cooled to room temperature overnight. The annealed double-stranded oligonucleotide was then 5'-end labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Promega). End labeled probes were purified using a G25 column (Roche Molecular Biochemicals) and counted in a Liquid Scintillation counter spectrometry. The end-labeled DNA probe (5×10^4 cpm) was incubated with the nuclear extract from HEK293T cells (5 μ g) for 30 min at room temperature. For competition assays, a 50-fold excess of an unlabeled oligonucleotide containing either, the wild type or mutated sequence was added to the reaction mixture and incubated for 10 min prior to the incubation with labeled probe. For EMSA antibody supershift assays, nuclear extracts were incubated with antibody to SP1, SP3, RAR α , RAR β , RAR γ , and RXR α , for 20 min prior to addition of labeled probe. Incubation mixtures were subjected to electrophoresis on 5% native polyacrylamide gels using 0.5 $\times$ TBE as electrophoresis buffer. Gels were then vacuum dried and then subjected to autoradiography as described above.

Western Blotting

Western blot analysis was performed according to a method previously described [25]. Briefly, nuclear protein extract samples (20 μ g protein) from HEK293T cells, along with Full Range Rainbow Recombinant Protein Molecular Weight Marker (GE Healthcare) were resolved in a 10% gel by SDS-PAGE and electroblotted onto a Pure Nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking in 5% non-fat dry milk in washing buffer (phosphate-buffered saline containing 0.05% Tween 20) for 3 h, the membrane was incubated with the primary antibody (1:200) in washing buffer containing 5% non-fat dry milk at 4°C overnight. The membrane was washed three times with washing buffer, each for 10 min, and then incubated with the appropriate secondary antibody (1:2000) conjugated with horseradish peroxidase for 1 h at room temperature in 1% non-fat dry milk in washing buffer and then washed as described above. Horse raddish peroxidase activity was analyzed through visualization by SuperSignal West Pico Chemiluminescent substrate solutions (Thermo Biotechnology, Rockford, IL) followed by exposure to Hyperfilm ECL (GE Healthcare). The density and area of the protein bands on the developed film were scanned and quantified in Photoshop (Adobe, San Jose, CA) using a continuous black and white scale. Total protein was measured by using the Bradford protein assay from Bio-Rad.

Statistical analysis

The data shown are the mean $\pm$ standard deviation (SD) or standard error of the mean. Analysis of variance was performed, followed by Fisher's Protected Least Significance Difference test, to determine differences with $P < 0.05$ among treatment groups.

Results

In vivo transcriptional regulation by nuclear run-on assay

To assess whether the in vivo response of vitamin A-deficient animals to retinoid treatment is due to an increase in the transcriptional activation of *Lrat*, we conducted nuclear run-on assays using nuclei prepared from fresh liver tissue from vitamin A-deficient rats and rats that had been treated for 6 h with vitamin A (retinol) or vitamin A+Am580. The *Lrat* signals obtained from nuclei from either vitamin A-deficient rats or retinol-treated rats were at or below the level of detection (Fig. 1A); however, the *Lrat* signal was readily detectable from rats treated with vitamin A plus Am580, a stable analog of RA [23], shown previously to increase LRAT enzyme activity in liver in vivo [23]. By quantification of the hybridized spots, *Lrat* gene run-on activity was increased ~3-fold, compared to vitamin A-deficient (control) rats. The run-on signals for β -actin, a prototypical reference gene, were equal for retinoid treated and control animals. Additionally, we measured on the same blot the nascent transcripts of *Cyp26a1*, used as a positive control because the *Cyp26a1* gene contains functional RAREs [26], and is highly responsive to RA in liver [27]. Whereas almost no *Cyp26a1* signal was detected in vitamin A-deficient and vitamin A-treated rats, it was increased 20-fold after treatment with vitamin A +Am580. The level of *Lrat* mRNA, quantified by PCR, was also assessed for *Lrat* and *Cyp26a1* (Fig. 1B). Similar to the run-on results, *Lrat* mRNA level was about 3 times higher ($P < 0.05$) in the vitamin A+Am580 group compared to the control group (Fig. 1B). *Cyp26a1* mRNA levels were elevated about 13- and 27-times in the vitamin A and vitamin A +Am580 groups, respectively, and thus these changes also agreed well with the transcription activity observed by nuclear run-on assay (Fig. 1B). Overall, nuclear run-on assays for *Lrat* in rat liver nuclei and *Lrat* mRNA in the liver agreed well, suggesting that transcriptional regulation of the *Lrat* gene, at a level less than for the highly inducible *Cyp26a1* gene, might account for the increase in mRNA after retinoid treatment in vivo.

Analysis of the *Lrat* T gene promoter

Previous results have shown that the region approximately 300 bp upstream from the TSS in both the human and rat *Lrat* genes is sufficient to drive LRAT expression in both primary cells and cell lines [22,24]. For further analysis the putative promoter of the rat LRAT gene, we cloned a fragment covering from -2425 to +257 bp from TSS [7,17] from rat genomic DNA (see Materials and Methods) into the promoterless pGL3-basic vector. This construct, and those with a series of deletions (see Figure 2), were transiently transfected into human embryonic kidney HEK293T cells, and human hepatoma HepG2 cells, and tested for Luc activity in comparison to Renilla-Luc as a control for transfection efficiency. Stepwise deletion of the 5' flanking region of the promoter region resulted in an increase in the activity of the Luc reporter gene in both HEK293T and HepG2 cells (Fig. 2). The highest activity was present in the region spanning from about -300 to +257 in both cells. Deletion of the 111 bp region immediately upstream of the TSS completely abolished Luc reporter activity in both cells. Treatment of the cells with RA (1 μ M) for 24 h following transfection had a small but consistent effect on the activity of the *Lrat* promoter construct containing the proximal region. Because expression of *Lrat* mRNA and enzyme activity have been shown to be up-regulated by ligands for RAR α in the liver of intact rats [23], we tested *Lrat* promoter activity by cotransfection of cells with the individual *Lrat* promoter constructs shown in Fig. 2 along with RAR α /RXR α , followed by treatment with RA and 9-cis-RA for 24 h as described in Methods. With the exception of the promoter construct covering from -111 to +257 of the TSS, cotransfection of RAR α together with RXR α increased the Luc activity of each construct in HEK293T cells (Fig. 3A) and in HepG2 cells (data not shown), both in the absence and presence of RA. Again however, treatment of the cells with RA had a small or no effect on the reporter activity. In a separate experiment, Am580 was compared with RA in HEK293T cells transfected with the promoter construct containing the proximal region (-268 to +257) without and with cotransfection of

RAR α plus RXR α expression vectors. Am580 was as effective as RA in causing a small induction in *Lrat* promoter activity in the absence and presence of RAR α and RXR α (Fig. 3B). Both RAR β and RAR γ had similar effect as RAR α when cotransfected with RXR α in HEK293T or HepG2 cells (data not shown).

Comparative nucleotide sequence analysis of the region proximal to the *Lrat* TSS

The cDNA nucleotide sequences for human and rat *Lrat* are about 80% similar [7]. Therefore, to determine whether the *Lrat* DNA sequences upstream of the TSS are also similar among species, we sequenced and aligned this region for the genes of both human and rat. The proximal region covering about 100 bp upstream of the TSS was found to be well conserved (Fig. 4), while no significant alignment was found between the human and rat *Lrat* genes beyond this region. Within the region just upstream of the TSS, several basic elements were found, which appear to be conserved in both species (Fig. 4). These include a TATA box, CAAT box, AP-1, and SP3 sites with GC-rich regions. There are several GATA sites present in human but not in the rat gene [22]. No RA response element was found in this conserved region of *Lrat* for either species (or for the mouse) using the TESS program.

Functional analysis of DNA response elements for basic transcription

To determine whether these basic nucleotide elements near the TSS of the *Lrat* gene are essential for transcriptional activity, we mutated the individual sites and conducted transfection assays in HEK293T cells. Again, the construct lacking -1 to -111 had no activity. Except for the distal SP site (-85 to -94), all other sites examined were required for the transcriptional activity of the *Lrat* gene (Fig. 5). For example, mutation of the proximal SP site alone (putative SP3 site) reduced the Luc activity by about 90%. Mutation in any of the other 3 sites resulted in a 65 to 70% reduction in Luc reporter activity.

Next, to further examine the complexes formed between nuclear proteins and these DNA binding sites we prepared a polynucleotide DNA probe containing both proximal SP site (SP3) and AP-1 site (-69 to -34) and used it with nuclear protein extracts from HEK293T cells in EMSA assay. Several protein-DNA complexes (designated C1–C6 in Fig. 6A) were detected. The unlabeled wild type sequence competed for binding with the labeled probe, as expected (Fig. 6A), shown by the reduction in complexes C1 to C3. When either the mutated AP-1 site or the mutated SP3 site was used as the competitor, complex C3 but not C1 or C2 was competed out, indicating either the latter two complexes may be bound to DNA more tightly than complex C3, or complex C3 is formed in the DNA region outside the mutated area. It appears that complex C1 is associated with the AP-1 site and complex C2 with the SP3 site. Results from the supershift analysis showed that complexes C4 and C6 formed with the concomitant disappearance of the complex C3 when SP3 antibody was added, while the migration of complex C5 was slowed when RAR γ antibody was added (Fig. 6A). No supershifted complex was observed when antibodies for SP1, RAR α , RAR β , or RXR α were included in the incubation (Fig. 6A). However, it appears that antibodies of all the RAR receptors, especially RAR β and RAR γ enhanced the formation of complexes C1 to C3 (darker signals indicating more complexes were formed). The treatment of nuclear extract directly with RA (e.g., added during the binding assay) had no effect on complex formation; however, nuclear extracts that were prepared from RA-treated HEK cells (RA in cells, Fig. 6A, right-most lane) exhibited enhanced formation of binding complexes C1 to C3. Thus, more of the nuclear proteins in these complexes were present in RA-treated cells.

To determine whether SP3 and RAR γ may interact in the formation of extra complexes in this region of the *Lrat* gene, separate EMSA supershift assays were performed using nuclear extract from RA-treated cells and antibodies for either SP3, RAR γ alone or in combination. The same complexes were detected whether either antibody was used alone or in combination (Fig. 6B).

However, the formation of complexes C1 to C3 was enhanced when RAR γ was used alone or in combination with SP3 antibody. These results indicated there may be an interaction between SP3 and RAR γ in their binding to these DNA sites.

To further examine whether SP3 has any role in driving *Lrat* promoter activity, the wild type *Lrat* promoter was cotransfected with siRNA against SP3, over a concentration range from 0 to 50 nM, overnight, and then assayed for Luc activity following 24 h incubation with full-growth medium. Luc activity was reduced by up to one-third by increasing the concentration of siRNA (data not shown).

Having shown that SP3 is involved in the regulation of LRAT gene expression and binding is increased in extracts of RA-treated cells, we next wanted to examine whether the expression of SP3 itself is regulated by RA. For this, HEK293T cells were treated with either vehicle or 1 μ M at-RA for 24 h and then nuclear protein extracts were prepared, and subjected to Western blot analysis. The polyclonal antibody against C-terminal region of the human SP3 detected at least 4 SP3 protein isoform bands, as shown previously [28], in our nuclear protein extract of HEK293T cells, with a molecular weight range from 60 to >100 kDa. Based on densitometric analysis the protein bands were increased 40 to 60% in the nuclear extracts of the RA-treated cells compared to vehicle-treated control cells (Fig. 7). Although SP1 is apparently not involved in binding, SP1 did increase with RA treatment. Based on densitometry and area, the intensity of the SP1 protein band was increased >2.5 times in RA-treated cells as compared to the control cells (Fig. 7).

Discussion

Most of the actions of vitamin A on gene expression are mediated through its major active metabolite, RA. It is becoming established that multiple mechanisms, both direct and indirect, account for the regulation of RA-responsive genes in vivo. The presence of RAREs of the DR2 and DR5 types in the promoter region of retinoid-responsive genes are often a signature for those genes that respond directly, and often very rapidly, to RA or to other retinoid receptor agonists [20,29]. However, RA-activated RAR receptors may also interact with other nuclear receptors such as AP1 [30–33], NF κ B [34,35], SP1 [36–40], and Nrf2 [41], and these may also regulate the expression of genes containing DNA sequences for these proteins. Balmer and Blomhoff [19] reported that whereas about 530 genes are known to be regulated by RA, only a few dozen of have definitively been shown to be directly regulated by RA. The results of the present study suggest that *Lrat* may be classified as an RA-responsive gene that is regulated, quite rapidly in vivo but within a fairly narrow range of expression, as compared to *Cyp26a1*, and may not be regulated through a classical DR2 or DR5-dependent mechanism, since these sequences were not detected.

In this study, we first determined by nuclear run-on assay if the level of nascent *Lrat* RNA transcripts is altered in the liver as a result of retinoid treatment. LRAT transcripts were barely detectable not only in the liver of vitamin A-deficient rats but also in the liver of the rats treated with vitamin A. This suggests that *Lrat* expression is low under normal dietary conditions. Because previous studies showed detection of *Lrat* mRNA by PCR in normal liver, and a reduction in the liver of vitamin A-deficient animals [7], it is likely that the *Lrat* gene is only slowly transcribed and that transcripts decay in the vitamin A-deficient state. However, nascent *Lrat* mRNA transcripts became measurable when the rats were treated with vitamin A+Am580, a stable analogue of RA [42], which is known to be a potent inducer of both *Lrat* and *Cyp26a1* gene expression in vivo. This result suggests that a prolonged retinoid signal may be necessary to induce the expression of the *Lrat* gene in vivo. The increase in nascent *Lrat* mRNA transcripts by vitamin A+Am580 was similar in magnitude to the increase in the steady state level of *Lrat* mRNA in the same livers, suggesting that mRNA induction by retinoids in vivo

may be due mostly, if not entirely, to the transcriptional activation of the *Lrat* gene. Similarly, the results of nuclear run-on assay and PCR assay were also well correlated for *Cyp26a1*, but both results were considerably stronger than for *Lrat*. This side-by-side comparison shows that both *Lrat* and *Cyp26a1* are concomitantly regulated transcriptionally by vitamin A in the presence of a stable retinoid signal, but also indicates that the induction of *Lrat* is considerably weaker than that of *Cyp26a1*.

Consistent with the relatively weaker transcriptional regulation of *Lrat*, we could not find any RARE-like response elements in the flanking regions upstream or downstream of the *Lrat* gene. By contrast, *Cyp26a1* contains at least two DR-5 RAREs, which interact to heighten activation [26]. Nonetheless, because transcriptional regulation of the *Lrat* gene was indicated by the *in vivo* studies, we analyzed the 5' flanking region of the *Lrat* gene covering 2.4 kbp upstream of the TSS with entire 5'UTR region as a putative and possibly basal *Lrat* promoter. Studies were conducted by transient transfection analysis in human kidney HEK293T cells and human liver HepG2 cells. Even though the expression of endogenous *Lrat* mRNA was low in HEK293T cells and below detection in HepG2 cells, the activity of *Lrat* promoter constructs was readily detected in both cells, which allowed for an examination of the effects of both retinoid ligands and nuclear receptors, RAR α and RXR α , the major nuclear retinoid receptors in liver [43,44], on *Lrat* gene activation.

Regarding features of the *Lrat* promoter sequence determined in this study, five features are notable: *i*) the DNA construct covering 2425 bp upstream from the TSS of the *LRAT* gene, including the entire 5'UTR, was active as a promoter in both the kidney and liver cells, but was not responsive to RA. *ii*) The gradual elimination of the 5' end of the *LRAT* promoter resulted in an increase in Luc activity in both kidney and liver cells, with the increase being significantly higher in HepG2 cells. This result may be due to the elimination of repressor(s) in the region that was deleted, which may put restraint on the basic transcription complex required for Luc expression. *iii*) Those DNA constructs that contained only the proximal region upstream of the *Lrat* TSS conferred a small increase in Luc activity when the cells were treated with RA following transfection. In addition, cotransfection of all of the constructs with RAR α /RXR α resulted in an increase in Luc activity both in the absence and presence of RA and 9-cis-RA, although there is no identifiable RARE (DR2 or DR5) present in the region used as the promoter. *iv*) The highest promoter activity resided in the proximal region upstream of the *Lrat* TSS, covering about -300 to -400 bp, and therefore this region may be considered as the optimal region for constitutive activity. *v*) The elimination of the -111 to +257 region (Fig. 2) completely knocked down the Luc activity. In this proximal region there are several *cis* elements including basic transcriptional response elements such as TATA box, SP3 site, AP-1 site and CAAT box. Since those elements are well conserved in rat, human, as well as in mouse, we mutated the individual sites in the promoter construct containing -268 to +257 and evaluated the activity of the constructs in human kidney cells. All of these elements and especially the SP3 site were absolutely required for *Lrat* promoter activity. Among the proteins that bind to this site include both SP3 and RAR γ with apparent interaction between these two proteins in binding to the site (Fig. 6). Whether these elements have any functional role in the expression of the gene remains to be determined. Specificity proteins including SP1 and SP3 which are expressed ubiquitously in many tissues including liver in both hepatocytes and stellate cells [45–48] have been shown to interact with RA nuclear receptors in expression of a few genes [36–40]. However, no specific pattern of interaction common to all these genes has been reported. The increase in promoter activity of *LRAT* by RA (Fig. 2 and 3), though at a relatively low level, might be due in part to an increase in SP3 (Fig. 7) in nuclear proteins which resulted in tighter binding to the DNA response element in the proximal region of *LRAT* promoter shown in Fig. 6.

Cai and Gudas [22] examined the human *Lrat* promoter including 2.0 kbp upstream of the transcription start site in cultured normal human prostate epithelial cells (PrEC) and human prostate cancer cell line PC-3. They found that the activity of the promoter in PC-3 cells was 40% of that in the normal PrEC cells. By deletion analysis, a 172-bp proximal promoter region was shown to be essential for *Lrat* transcription and to confer RA responsiveness in PrEC cells, but not in PC-3 cells. This region and the region identified by our studies agree well. They reported that this region in the human *Lrat* gene contains several GATA binding sites, and by cotransfection of GATA4 with either RAR β or RAR γ , but not RAR α , the promoter activity was doubled. However, we found no conserved GATA sites in either the rat or the mouse *Lrat* gene promoter, and thus the GATA sites may not have a general role across tissues or species.

In summary, the increase in the steady-state level of *Lrat* mRNA by retinoids in the liver of rats could be mostly, if not entirely, due to the transcriptional activation of *Lrat*, involving basal transcriptional factors. The proximal flanking region around the TSS is responsive to not only RAR/RXR, but exhibits a small effect of RA or Am580, a stable retinoid and RAR α -specific ligand. Given the relatively low level of transcriptional response of the *Lrat* gene to retinoid in rat liver in vivo, in comparison to *Cyp26a1* for which the response is very high and cooperating RAREs are known to be important control elements [26], a modest influence of receptors and ligand within this basic region of the *Lrat* gene might be sufficient to drive its expression to the levels normally observed. This region contains a group of DNA response elements, including a TATA box, SP3 site, AP-1 site, and CAAT box, each one of which is apparently essential for *Lrat* transcription, as the elimination by mutation of any one of these sites abrogated transcriptional activity. The highly conserved nature of these elements further suggests that they serve an essential function. Based on these results, we suggest that *Lrat* may belong to the category of RA-responsive genes that is regulated in vivo at the level of transcription, but at a relatively low level and in an apparently indirect manner [19].

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References

1. MacDonald PN, Ong DE. Biochem Biophys Res Commun 1988;156:157–163. [PubMed: 3178828]
2. Shi YQ, Furuyoshi S, Hubacek I, Rando RR. Biochemistry 1993;32:3077–3080. [PubMed: 8457568]
3. Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR, Bronson D, Possin D, Van Gelder RN, Baehr W, Palczewski K. J Biol Chem 2004;279:10422–10432. [PubMed: 14684738]
4. O'Byrne SM, Wongsiriroj N, Libien J, Vogel S, Goldberg IJ, Baehr W, Palczewski K, Blaner WS. J Biol Chem 2005;280:35647–35657. [PubMed: 16115871]
5. Randolph RK, Ross AC. J Biol Chem 1991;266:16453–16457. [PubMed: 1885578]
6. Matsuura T, Ross AC. Arch Biochem Biophys 1993;301:221–227. [PubMed: 8460936]
7. Zolfaghari R, Ross AC. J Lipid Res 2000;41:2024–2034. [PubMed: 11108736]
8. Zolfaghari R, Wang Y, Chen Q, Sancher A, Ross AC. Biochem J 2002;368:621–631. [PubMed: 12201819]
9. Zolfaghari R, Ross AC. J Nutr 2002;132:1160–1164. [PubMed: 12042426]
10. Ross AC. J Nutr 2003;133:291S–296S. [PubMed: 12514312]
11. Andreola F, Giandomenico V, Spero R, De Luca LM. Biochem Biophys Res Commun 2000;279:920–924. [PubMed: 11162450]
12. Zhan HC, Gudas LJ, Bok D, Rando R, Nanus DM, Tickoo SK. Clin Cancer Res 2003;9:4897–4905. [PubMed: 14581364]
13. Guo X, Ruiz A, Rando RR, Bok D, Gudas LJ. Carcinogenesis 2000;21:1925–1933. [PubMed: 11062150]

14. Anantharaman V, Aravind L. *Genome Biol* 2003;4:R11. [PubMed: 12620121]
15. Jahng WJ, Xue L, Rando RR. *Biochemistry* 2003;42:12805–12812. [PubMed: 14596594]
16. Ruiz A, Kuehn MH, Andorf JL, Stone E, Hageman GS, Bok D. *Invest Ophthalmol Vis Sci* 2001;42:31–37. [PubMed: 11133845]
17. Zolfaghari R, Ross AC. *Gene* 2004;341:181–188. [PubMed: 15474300]
18. Chambon P. *FASEB J* 1996;10:940–954. [PubMed: 8801176]
19. Balmer JE, Blomhoff R. *J Lipid Res* 2002;43:1773–1808. [PubMed: 12401878]
20. Altucci L, Leibowitz MD, Ogilvie KM, de Lera AR, Gronemeyer H. *Nat Rev Drug Discov* 2007;6:793–810. [PubMed: 17906642]
21. Ross AC, Ambalavanan N, Zolfaghari R, Li NQ. *J Lipid Res* 2006;47:1844–1851. [PubMed: 16685080]
22. Cai K, Gudas LJ. *Int J Biochem Cell Biol* 2009;41:546–553. [PubMed: 18652909]
23. Shimada T, Ross AC, Muccio DD, Brouillette WJ, Shealy YF. *Arch Biochem Biophys* 1997;344:220–227. [PubMed: 9244401]
24. Zolfaghari R, Cifelli CJ, Lieu SO, Chen Q, Li NQ, Ross AC. *Am J Physiol Gastrointest Liver Physiol* 2007;292:G1029–1036. [PubMed: 17185629]
25. Chen Q, Ma Y, Ross AC. *Immunology* 2002;107:199–208. [PubMed: 12383199]
26. Loudig O, Maclean GA, Dore NL, Luu L, Petkovich M. *Biochem J* 2005;392:241–248. [PubMed: 16053444]
27. Wang Y, Zolfaghari R, Ross AC. *Arch Biochem Biophys* 2002;401:235–243. [PubMed: 12054474]
28. Ross S, Best JL, Zon LI, Gill G. *Mol Cell* 2002;10:831–842. [PubMed: 12419227]
29. de Lera AR, Bourguet W, Altucci L, Gronemeyer H. *Nat Rev Drug Discov* 2007;6:811–820. [PubMed: 17906643]
30. Soprano DR, Scanlon E, Shukri M, Zhang ZP, Soprano KJ. *J Cell Biochem* 2000;77:604–614. [PubMed: 10771516]
31. Darwiche N, Bazzi H, El-Touni L, Abou-Lteif G, Doueiri R, Hatoum A, Maalouf S, Gali-Muhtasib H. *Radiat Res* 2005;163:296–306. [PubMed: 15733037]
32. Dedieu S, Lefebvre P. *Cell Signal* 2006;18:889–898. [PubMed: 16176868]
33. Zhang M, Thomas BC, Napoli JL. *Gene* 2003;305:121–131. [PubMed: 12594048]
34. Hoo RL, Chan KY, Leung FK, Lee LT, Leung PC, Chow BK. *Febs J* 2007;274:2695–2706. [PubMed: 17451432]
35. Saavalainen K, Tammi MI, Bowen T, Schmitz ML, Carlberg C. *J Biol Chem* 2007;282:11530–11539. [PubMed: 17307735]
36. Laliotis GP, Bizelis I, Argyrokastritis A, Rogdakis E. *Comp Biochem Physiol B Biochem Mol Biol* 2007;147:627–634. [PubMed: 17493856]
37. Wolf AT, Medcalf RL, Jern C. *Blood* 2005;105:1060–1067. [PubMed: 15466927]
38. Khan S, Wu F, Liu S, Wu Q, Safe S. *J Mol Endocrinol* 2007;39:289–304. [PubMed: 17909268]
39. Islam MR, Puri S, Rodova M, Magenheimer BS, Maser RL, Calvet JP. *Am J Physiol Renal Physiol* 2008;295:F1845–1854. [PubMed: 18922886]
40. Cheng YH, Yin P, Xue Q, Yilmaz B, Dawson MI, Bulun SE. *J Clin Endocrinol Metab* 2008;93:1915–1923. [PubMed: 18270252]
41. Wang XJ, Hayes JD, Henderson CJ, Wolf CR. *Proc Natl Acad Sci U S A* 2007;104:19589–19594. [PubMed: 18048326]
42. Hashimoto Y, Kagechika H, Shudo K. *Biochem Biophys Res Commun* 1990;166:1300–1307. [PubMed: 2154975]
43. Kato S, Mano H, Kumazawa T, Yoshizawa Y, Kojima R, Masushige S. *Biochem J* 1992;286(Pt 3):755–760. [PubMed: 1329717]
44. Zolfaghari R, Ross AC. *Arch Biochem Biophys* 1995;323:258–264. [PubMed: 7487086]
45. García-Ruiz I, de la Torre P, Díaz T, Esteban E, Fernández I, Muñoz-Yagüe T, Solís-Herruzo JA. *J Biol Chem* 2002;277:30551–30558. [PubMed: 12055194]
46. Hernández I, de la Torre P, Rey-Campos J, García I, Sánchez JA, Muñoz R, Rippe RA, Muñoz-Yagüe T, Solís-Herruzo JA. *DNA Cell Biol* 2000;19:341–352. [PubMed: 10882233]

47. Inagaki Y, Nemoto T, Nakao A, Dijke P, Kobayashi K, Takehara K, Greenwel P. J Biol Chem 2001;276:16573–16579. [PubMed: 11278686]
48. Ruiz IG, de la Torre P, Diaz T, Esteban E, Morillas JD, Muñoz-Yagüe T, Solís-Herruzo JA. DNA Cell Biol 2000;19:167–178. [PubMed: 10749169]

Fig. 1. Nuclear run-on analysis and steady-state expression of *Lrat* mRNA in liver of vitamin A-deficient rats treated with vitamin A, Am580, or both in combination

(A) Nuclear run-on assay. Nuclei were freshly isolated from the liver of vitamin A-depleted adult rats treated 6 h earlier with a single oral dose of retinyl palmitate as vitamin A or vitamin A combined with Am580 as an RAR α agonist (see Methods). Nuclei were incubated with ribonucleotides including [32 P]-UTP to elongate the endogenously initiated RNA transcripts. The labeled RNA was isolated and hybridized to membranes dot-blotted with cDNA probes for *Cyp26a1* (as a DR5 containing positive control gene), *Lrat*, and β -actin as a housekeeping control gene. The membranes were washed and exposed to X-ray film for 3 days for CYP26A1 and *Lrat* and for 8 h for β -actin. (B) Total RNA was isolated from individual liver tissue of rats ($N = 3$ /group), treated as above, and subjected to quantitative real time PCR analysis for *Cyp26a1* (left bars) and *Lrat* mRNA (right bars) as described in Methods. The data were analyzed by one-way ANOVA followed by Fisher's Least Squares test. Bars with different letters differed significantly (a,b,c, $P < 0.05$ for *Cyp26a1* and *Lrat*; a,c, $P < 0.0005$ for CYP26A1).

Fig. 2. The 5' upstream region of *Lrat* gene confers activity on the Luc reporter gene both in the absence and presence of RA

The firefly Luc reporter plasmids controlled by *Lrat* promoter constructs shown on the left were transiently transfected along with the control plasmid expressing Renilla-Luc under the thymidine kinase promoter into either HEK293T or HepG2 cells, followed by treatment of cells with either ethanol (vehicle) or *all-trans*-RA (RA) for 24 h. Values for firefly Luc activity were normalized to Renilla-Luc activity for each well and the average value for the construct covering the -2425 to +59 region of the *Lrat* gene was assigned a value of 1 for each cell type. The results shown are one representative experiment of 3 independent experiments (each with $N = 2/\text{treatment}$) in which the coefficient of variation within each experiment was less than 10%.

Fig. 3. The putative *Lrat* gene promoter is responsive to RARα/RXRα in the absence and presence of RA and Am580 in HEK293T cells

(A) The individual *Lrat* promoter constructs indicated were cotransfected with 0.2 μg of each of RARα/RXRα into HEK293T cells overnight. The cells were then treated with RA/9-cis-RA, each at 1 μM for 24 h and then harvested for Luc assay as described in the legend of Fig. 2. Except for the construct for the -111 to +257 region, the Luc activity of all promoter constructs was increased by cotransfection of RARα/RXRα both in the absence and presence of RA. RA increased the activity of the promoter constructs containing the proximal upstream region of the *Lrat* gene. (B) The promoter construct for the -268 to +257 region was cotransfected without and with RARα/RXRα into the cells as described above. The cells were

then treated with Am580/9-cisRA, each at $1 \text{ } \mu\text{M}$ for 24 h and then harvested for Luc assay. Am580 was as effective as RA both in the absence and presence of RAR α /RXR α pair. Data represent the mean $\pm$ SD of $N = 4$ wells/treatment.

Fig. 4. Alignment of nucleotide sequence and identification of consensus elements in the proximal region upstream from the *Lrat* TSS in human and rat

The clones containing the proximal 5' flanking region of *Lrat* from mouse and rat were sequenced and the nucleotide sequences were aligned using Clustal W program. The putative response elements were identified with the TESS program (See Methods).

Fig. 5. Effect of mutation of DNA response elements for individual basic transcription factors on the activity of *Lrat* promoter

The nucleotide response elements present in the proximal region of the rat *Lrat* promoter including TATA box, SP3 site, AP-1 site, CAAT box, and SP1 site were mutated individually into gatatc, atgatatcgg, ttctatagt, agatatc, and gatattcta, respectively, (see Materials and Methods for detail) in the construct spanning from -268 to +257 and cloned into pGL3-Luc vector. The individual constructs, each, were transfected into HEK293T cells and the expressed luciferase activity was measured and compared to vector with wild *Lrat* promoter. The results are from 4 different experiments, mean $\pm$ SD. The promoter construct lacking -1 to -111 from the *Lrat* TSS was used as the control.

Fig. 6. Binding of nuclear proteins to the DNA response element containing AP-1 and SP3
 (A) Nuclear proteins extract from HEK293T cells treated with either vehicle or RA was incubated with ^{32}P -end-labeled oligonucleotide spanning from -69 to -34 (see Fig. 5), which included both AP-1 and SP3 sites, and then subjected to electrophoretic mobility shift assay. For competition, the nuclear protein extract was incubated with cold oligonucleotide before incubation with probe. The two righthand lanes show results when RA was added to the nuclear extract (NPE+RA), and when cells were treated with RA before the preparation of the nuclear extract (RA in cells). (B) Similarly, for electrophoretic mobility supershift assay, the nuclear extract from cells treated with RA was first incubated with individual antibodies to SP3, RAR γ , or both, before incubation with the probe. The gels were dried under vacuum and exposed to X-ray film as described in detail in the Methods section. Labels C1 through C6 designate nuclear protein complexes with different migration characteristics (see Results text).

Fig. 7. Expression of SP proteins in the nuclear extracts of HEK293T cells

Nuclear protein extract samples (20 μ g protein) of HEK293T cells incubated with either vehicle (Veh) or all-trans RA (1 μ M) for 24 h in full-growth media were subjected to Western blot analysis using protocol as described in detail in the Methods section. Protein Molecular Weight Marker (MWM) ranged from 10 to 250 kD.