

An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro

Silke Vogel,* Roseann Piantedosi,* Jorge Frank,[†] Avraham Lalazar,** Don C. Rockey,^{††} Scott L. Friedman,** and William S. Blaner^{1,*,\$}

Department of Medicine,* Department of Dermatology,[†] and Institute of Human Nutrition,[§] College of Physicians and Surgeons of Columbia University, New York, NY, 10032; Division of Liver Diseases,** The Mount Sinai School of Medicine, New York, NY 10029; and Duke Liver Center,^{††} Duke University Medical Center, Durham, NC 27710

Abstract Hepatocytes and hepatic stellate cells play important roles in retinoid storage and metabolism. Hepatocytes process postprandial retinyl esters and are responsible for secretion of retinol bound to retinol-binding protein (RBP) to maintain plasma retinol levels. Stellate cells are the body's major cellular storage sites for retinoid. We have characterized and utilized an immortalized rat stellate cell line, HSC-T6 cells, to facilitate study of the cellular aspects of hepatic retinoid processing. For comparison, we also carried out parallel studies in Hepa-1 hepatocytes. Like activated primary stellate cells, HSC-T6 express myogenic and neural crest cytoskeletal filaments. HSC-T6 cells take up and esterify retinol in a time- and concentration-dependent manner. Supplementation of HSC-T6 culture medium with free fatty acids (up to 300 μM) does not affect retinol uptake but does enhance retinol esterification up to 10-fold. RT-PCR analysis indicates that HSC-T6 cells express all 6 retinoid nuclear receptors ($\text{RAR}\alpha$, $-\beta$, $-\gamma$, and $\text{RXR}\alpha$, $-\beta$, $-\gamma$) and like primary stellate cells, HSC-T6 stellate cells express cellular retinol-binding protein, type I (CRBP) but fail to express either retinol-binding protein (RBP) or transthyretin (TTR). Addition of retinol (10^{-8} – 10^{-5} M) or all-*trans*-retinoic acid (10^{-10} – 10^{-6} M) rapidly up-regulates CRBP expression. Using RAR -specific agonists and antagonists and an RXR -specific agonist, we show that members of the RAR -receptor family modulate HSC-T6 CRBP expression. **Thus, HSC-T6 cells display the same retinoid-related phenotype as primary stellate cells in culture and will be a useful tool for study of hepatic retinoid storage and metabolism.**—Vogel, S., R. Piantedosi, J. Frank, A. Lalazar, D. C. Rockey, S. L. Friedman, and W. S. Blaner. **An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro.** *J. Lipid Res.* 2000. 41: 882–893.

Supplementary key words hepatic cells • Ito cells • fat-storing cells • vitamin A • oleic acid • cellular retinol-binding protein, type I (CRBP-I) • retinol-binding protein (RBP) • transthyretin (TTR) • Hepa-1 hepatocytes • retinoid nuclear receptor

Retinoids (vitamin A and its analogs) must be obtained from the diet by all higher organisms to maintain normal cell growth and differentiation, the immune response,

normal male and female reproduction, embryogenesis, and vision (1–5). The liver plays a central role in retinoid storage by ensuring their availability for the maintenance of gene expression (6). Two hepatic cell types, hepatocytes and stellate cells, are important for hepatic retinoid processing. Hepatocytes are responsible for the uptake of postprandial vitamin A as chylomicron remnant retinyl esters. After endocytosis, chylomicron remnant retinyl esters undergo hydrolysis to retinol (7). Neutral and acid bile salt-independent retinyl ester hydrolases localized in early and late endosomes have been proposed to be involved in hydrolysis (7–10). The hepatocyte is the sole cellular site of retinol-binding protein (RBP) synthesis and secretion from the liver (11). Consequently, the hepatocyte is essentially involved in maintaining circulating levels of retinol.

Hepatic stellate cells (also known as fat-storing cells, Ito cells, or lipocytes) are non-parenchymal cells, located perisinusoidally in the Space of Disse. These cells constitute approximately 1% of hepatic protein and they are the major cellular storage site for vitamin A in the liver (12). In well-fed animals about 70–90% of the total hepatic retinoid is located in these cells (13–16). Lipid droplets laden with retinyl esters are the distinguishing morphologic feature of stellate cells in animals on a vitamin A-sufficient diet (12, 13, 16–18).

Both primary rat liver hepatocytes and stellate cells are enriched in cellular retinol-binding protein, type I (CRBP), which is needed for the intracellular transport of retinol and to facilitate retinol metabolism, and enzymes including neutral and acid retinyl ester hydrolases, lecithin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyl-

Abbreviations: RBP, retinol-binding protein; CRBP, cellular retinol-binding-protein; TTR, transthyretin; RT-PCR, reverse transcription polymerase chain reaction; LRAT, lecithin:retinol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; RARE, retinoic acid response element.

¹ To whom correspondence should be addressed.

transferase (ARAT), which are essential for retinyl ester hydrolysis and retinyl ester formation, respectively (7, 19–25). As hepatocytes are responsible for uptake of dietary retinyl ester and for mobilization of retinol-RBP and the stellate cells store the bulk of hepatic retinoid as retinyl ester, there must be substantial communication between hepatocytes and stellate cells. At present, very little is known regarding how these two hepatic cell types interact to coordinate retinoid uptake, storage, and mobilization.

While primary stellate cell cultures are a useful tool for studying hepatic retinoid storage and metabolism, their isolation is extremely time-consuming, yields are modest, and there is considerable preparation-to-preparation variability. To overcome these limitations, we have developed and characterized an immortalized hepatic rat stellate cell line (HSC-T6 cells) (26) for use in the study of retinoid processing by stellate cells. Here, as a first step towards validating the use of HSC-T6 cells for further studies, we focused on retinoid uptake and processing and on the regulation of CRBP gene expression.

EXPERIMENTAL PROCEDURES

Establishment of the rat HSC-T6 stellate cell line

Stellate cells were isolated from male retired-breeder Sprague-Dawley rats as previously described (27) and maintained in primary culture on plastic culture dishes for 15 days in the presence of 10% fetal bovine calf serum. On day 15 of culture, approximately 2×10^6 rat hepatic stellate cells were transiently transfected for 24 h with lipofectamine (Gibco BRL), containing a cDNA in which the expression of the large T-antigen of SV40 is driven by the Rous sarcoma virus promoter. After 5–7 days, emerging clones were harvested and plated to limiting dilution, and single cell clones were isolated and amplified in the absence of any antibiotic selection. One of over 20 clones, designated HSC-T6, was expanded for further characterization based on preliminary cytoskeletal analysis that revealed a phenotype most closely resembling primary rat stellate cells. A stable phenotype has been documented for over 40 passages, including sustained expression of T-antigen by immunocytochemistry and Western blot analysis.

Cell culture procedures

HSC-T6 stellate cells were maintained in Waymouth MB 752/1 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 IU/ml and 100 mg/ml media, respectively) (Gibco, Grand Island, NY). Murine Hepa-1 hepatocytes were obtained from Dr. Max Gottesman, Institute of Cancer Research, Columbia University. The Hepa-1 cell line was originally derived from a transplantable tumor carried in C57 leaden/J mice (28, 29). Different sublines from the original Hepa cells line have been established and the present study uses the Hepa-1 cell line. Hepatocytes were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% FBS and penicillin–streptomycin (100 IU/mL and 100 mg/mL media, respectively) (Gibco, Grand Island, NY) according to Darlington (29). Both cell lines were maintained at 37°C in a humidified environment of 5% CO₂.

Immunocytochemistry

Cell cultures were washed with PBS and fixed with fresh paraformaldehyde (4%) in PBS for 5 min, then 0.3% Triton

X-100 for 2 min. After washing, cells were incubated overnight at 4°C in PBS containing rabbit antisera against either smooth muscle α -actin antibody (Clone 1A4, Sigma, St. Louis, MO), vimentin antibody (Amersham, Arlington Heights, IL), glial fibrillary acidic protein (Dako Z0334), or desmin (Sigma D-8281) each diluted 1:200, and Oregon Green conjugated phalloidin (Molecular Probes, Eugene, OR). Subsequently, the cells were washed and incubated with biotinylated mouse anti-rabbit IgG (Amersham, Arlington Heights, IL) for 2 h. After washing with PBS, samples were incubated with streptavidin-linked Texas Red (Amersham, Arlington Heights, IL) for 30 min, washed again, and mounted. Cells were visualized by epifluorescence and photomicrographs were taken using a Nikon TE 300 Photomicroscope (Nikon Co., Tokyo, Japan), Nikon N6006 automatic camera (Nikon Co.), and Ilford Plus film (Cheshire, England). Control specimens consisted of HSC-T6 cells exposed to irrelevant isotype matched antibody and processed as described above.

Oil red O staining of HSC-T6 stellate cells

HSC-T6 cells were stained with oil red O essentially as described by Ramirez-Zacarias, Castro-Munozledo, and Kuri-Harcuch (30). The HSC-T6 cells were incubated with media containing either 10% FBS or in media supplemented with 10% FBS and 5 μ m retinol for 24 h. After incubation, media were aspirated and the cells were fixed in 10% formalin for 90 min. After washing thoroughly with distilled water, cells were incubated with a working solution of oil red O for 3 h (30). Hematoxylin was used as the counterstain. The staining of lipid droplets in HSC-T6 cells was evaluated under a phase contrast microscope.

Studies of retinol uptake and processing by HSC-T6 and Hepa-1 cells

For several experiments designed to study retinol uptake and esterification by HSC-T6 stellate cells and Hepa-1 hepatocytes, the cells were treated with different concentrations of retinol for various time intervals. Cells were plated onto 10-cm plastic culture dishes and incubated overnight in the appropriate medium. The next morning, culture media were removed and replaced with fresh media containing different concentrations of retinol. For this purpose, a concentrated stock solution of retinol was diluted in a small volume of ethanol (<1% of the aqueous volume) and then added with vigorous mixing to a 5% (w/v) solution of fatty acid-free bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS). (All-*trans*-retinol was obtained as a gift from Dr. Christian Eckhoff of Hoffman La Roche Inc., Nutley, NJ.) The retinol-containing BSA solution was then added to the HSC-T6 or Hepa-1 media to achieve final media retinol concentrations of either 0.5 μ m, 1 μ m, or 5 μ m. Treated hepatic cells were incubated at 37°C in the dark for 2, 4, 8, 20, 32, or 48 h in this media. At each time, for triplicate cultures, media were removed from the cells and stored at –20°C for analysis. Cells were washed twice with ice-cold PBS, scraped into 2 mL ice-cold PBS, and the cell suspension was stored at –20°C until analysis. Control cells were cultured in normal medium containing 10% FBS.

Effect of fatty acids on retinol uptake and esterification

To investigate the effects of exogenous fatty acids on the uptake of retinol and on intracellular esterification of retinol, cultures of HSC-T6 stellate cells were incubated with oleic acid, palmitic acid, or linoleic acid (NuChek Prep Inc., Elysian, MN). Each fatty acid was dissolved in ethanol and added to a 3% (w/v) solution of fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO) in PBS. Cells were incubated for 12 h with the fatty acids (100 μ m), after which cells were treated with 5 μ m retinol for an additional 12 h. The effects of different concentrations of oleic

acid on retinol esterification by the stellate cells were also evaluated. Oleic acid was added bound to fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO) as a 3% BSA solution to achieve media oleic acid concentrations of 0, 10, 100, and 300 μM . Cells were incubated overnight and retinol was added the next morning and the cells were allowed to incubate for 12 additional h. The effect of retinol alone, fatty acid alone, or the combined supplementation of retinol and oleic acid on retinol uptake and retinol esterification in Hepa-1 hepatocytes was also studied. Cell cultures were treated with 3% fatty acid-free BSA alone or oleic acid bound to fatty acid-free BSA (final concentration 100 μM) overnight, after which retinol was added to half of the cultures. Cells were harvested at different times after retinol treatment and analyzed for cellular retinol and retinyl ester concentrations.

In vitro ARAT and LRAT assays

Enzymatic assays for ARAT and LRAT were carried out as described by Ross (21) and Randolph and Ross (24), respectively. For the ARAT assay, HSC-T6 cells were homogenized in 0.15 M potassium phosphate buffer, pH 7.4, using a Dounce homogenizer. The homogenate was centrifuged at 40,000 rpm in a Beckman TC-100 ultracentrifuge for 60 min to obtain a crude microsomal fraction. The crude microsomes were resuspended in 0.15 M potassium buffer, pH 7.4. In order to distinguish between retinyl esters formed during the in vitro assay and retinyl esters endogenous to the crude microsomal fraction we used n-heptadecanoyl-coenzyme A as the fatty acyl-CoA. Retinyl esters containing this fatty acyl group are not endogenously present in HSC-T6 cells. For the assay (total volume 0.5 mL), 500 μg microsomal protein was incubated for 1 h at 37°C with 100 μM n-heptadecyl-CoA, 120 μM retinol in 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM dithiothreitol and 20 μM BSA. The enzymatic reaction was stopped with an equal volume of ice-cold ethanol and the retinoid was extracted into 2 volumes of hexane and analyzed by reverse phase HPLC (see below). For the LRAT assay, HSC-T6 cells were homogenized in 150 mM potassium phosphate buffer, pH 7.25, and crude microsomes were obtained as described above. After resuspension of the microsomal fraction in this buffer, 250 μg of the microsomal protein was incubated with 5 μM retinol in a buffer containing 20 μM BSA and 5 mM DTT for 1 h at 37°C. The enzymatic reaction was stopped by addition of an equal volume of ice-cold ethanol and the retinoid was extracted into 2 volumes of hexane. A control lacking retinol was included for both the ARAT and LRAT assays.

HPLC analyses of retinol and retinyl esters

Retinol and retinyl esters were analyzed by reversed phase HPLC as described by Yamada et al. (16). Briefly, 1 mL of cell pellet, homogenized in PBS, or 1 mL of media was extracted with hexane after precipitation of the proteins with 1 mL absolute ethanol. Retinyl acetate was added to the absolute ethanol as internal standard to each sample to assess recovery of the retinoids during the extraction procedure. The hexane extracts (2 mL) were evaporated to dryness under a gentle stream of N_2 and the dry retinoid-containing film was immediately redissolved in benzene and taken for injection onto the HPLC column. The HPLC mobile phase consisted of acetonitrile–methylene chloride–methanol 70:15:15 (v/v). The instruments used for the HPLC analysis consisted of a Waters 510 HPLC pump (Waters Associates, Milford, MA) operating at a flow of 1.8 mL/min, an Ultrasphere C_{18} column (5 μm ; 4.6 mm \times 25 cm) (Beckman Instruments), and a Waters 996 Photodiodearray detector. Retinol and retinyl esters were monitored at 325 nm and quantified using standard curves relating known mass to mass ratio of retinol or retinyl esters to that of internal standard retinyl acetate. This HPLC method allows for detection and quantitation of different

retinyl esters, including retinyl palmitate, oleate, stearate, linoleate, and myristate.

Radioimmunoassay for RBP

RBP concentrations in cells and medium of Hepa-1 cells for the different treatment conditions were measured using a sensitive and accurate radioimmunoassay (RIA) exactly as described previously (31). This assay is specific for rat and mouse RBP and the rabbit anti-rat RBP antiserum does not recognize bovine RBP present in the fetal bovine serum-containing media (31). Prior to RIA, proteins present in media samples were concentrated using a Centriprep-10 centrifugal concentrators (Amicon Corp., Beverly, MA).

RNA preparation

Total RNA was isolated from HSC-T6 stellate cells and Hepa-1 hepatocytes using a modification of the procedure described by Chomczynski and Sacchi (32). Briefly, media were removed from over the cells and a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate-HCl, 0.1 M β -mercaptoethanol, and 0.5% sacrosyl directly added to lyse the cells. The resulting cell lysates were collected, transferred to Corex tubes, and 0.8 mL of 2 M sodium acetate, pH 4.0, 5 mL Tris saturated phenol, and 3.0 mL of chloroform–isoamyl alcohol 49:1 were added to the tubes. The lysate was mixed well with these reagents, cooled on ice for 15 min, and subsequently centrifuged at 10,000 g for 30 min at 4°C. All subsequent steps of the extraction procedure were carried out exactly as described by Chomczynski and Sacchi (32).

Northern blot analyses

Levels of RBP, CRBP, or TTR mRNA were determined by Northern blot analyses of total RNA samples prepared from HSC-T6 and Hepa-1 cells. For this purpose, total RNA was resolved by gel electrophoresis (1% agarose containing 0.98 M formaldehyde). For all samples, the ratio of intensities of the 28S and 18S ribosomal RNA bands after staining with ethidium bromide was approximately 2. Immediately after electrophoresis the RNA was transferred to a positively charged Nylon membrane (Amersham Life Sciences, Arlington Heights, IL) and subsequently hybridized at 65°C with ^{32}P -labeled probes for rat RBP, rat CRBP, or rat TTR (33). Random priming using a kit and accompanying procedures obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN) generated the ^{32}P -labeled cDNA probes. Hybridized membranes were washed at a final stringency of $1 \times \text{SSC}$, 1.0% SDS at 65°C and exposed to Kodak AR-2 film at -80°C .

RBP, TTR, and CRBP expression by HSC-T6 and Hepa-1 cells.

For estimation of RBP, CRBP, and TTR mRNA levels and to assess the influence of retinoid on the expression of these transcripts in HSC-T6 stellate cells and Hepa-1 hepatocytes, cells were plated and incubated overnight as described above. After incubation, this media was removed and cells were incubated for 12 h at 37°C in the dark with media containing different concentrations of either retinol or all-*trans*-retinoic acid added to the complete culture media (DMEM and Waymouth for Hepa-1 hepatocytes and HSC-T6 stellate cells, respectively). Cells were incubated in these media for various times. After incubation, the media was removed by aspiration and total cellular RNA was prepared as described above.

To investigate the dose-dependent effects of retinol and all-*trans* retinoic acid on the level of CRBP mRNA expression in HSC-T6 stellate cells, cell cultures were incubated for 12 h with unsupplemented complete media or with complete media con-

taining 0.01 μM , 0.05 μM , 0.1 μM , or 1 μM retinol or 10^{-4} μM , 10^{-3} μM , 0.01 μM , 0.1 μM , or 1 μM all-*trans*-retinoic acid. To establish the time course of induction of CRBP mRNA expression, HSC-T6 cell cultures were treated with 1 μM all-*trans*-retinoic acid and total RNA was extracted at different times after the start of the treatment (0.5, 1, 2, 4, 8, 12, and 24 h). Each of these analyses was carried out in duplicate on three separate occasions.

The effect of pan-RAR and pan-RXR agonists and a pan-RAR antagonist on CRBP expression in HSC-T6 cells was also investigated. The pan-agonists and the pan-antagonist were a kind gift from Galderma Inc. (San Diego, CA) and included the pan-RAR agonist CD367, pan-RXR agonist CD3127, and pan-RAR antagonist CD3106. HSC-T6 cells were incubated with media containing 10% FBS alone, media supplemented with 10^{-8} M all-*trans*-retinoic acid or media supplemented with 10^{-8} M of CD367, CD3127, or CD3106 in the presence of 10^{-8} M all-*trans*-retinoic acid. Cells were incubated for 12 h and RNA was extracted as described above.

Expression of the retinoid nuclear receptors

RT-PCR was used to identify which retinoid nuclear receptors are expressed by HSC-T6 cells. For that purpose cDNA was generated from total RNA prepared from HSC-T6 cells cultured under basal conditions using a Superscript Preamplification kit (Gibco, Grand Island, NY). The specific oligonucleotide primers used for the PCR reaction for amplification of the RAR α and RAR γ forms and for β -actin were originally described by Wan, Wang, and Wu (34). The remaining oligonucleotides were generated based on the published full-length murine mRNA sequences for the specific receptor. The specific oligonucleotides for each receptor and β -actin used are shown in **Table 1**. PCR amplification was carried out on 1 μg total RNA obtained from HSC-T6 cells according to the following program: initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 45 s, annealing at primer specific temperatures for 45 s (Table 1), and extension at 72°C for 1 min; followed by a final extension of 72°C for 7 min, in a Stratagene Robo Cycler Gradient 96 thermal cycler (Stratagene, La Jolla, CA). Each amplification reaction contained 50 ng/ μL of the forward and the reverse primer, respectively, 0.05 mM of each dNTP (Boehringer Mannheim, Indiana, IN), 1.5 μL DMSO (Fisher Scientific Co., Pittsburgh, PA), 5 μL of $10\times$ PCR buffer with 1.5 mM MgCl₂ (Boehringer Mannheim, Indianapolis, IN), and 2 U of Platinum Taq DNA Polymerase (Gibco-BRL, Gaithersburg, MD), in a total volume of 50 μL . The PCR products were electrophoresed on a 2% agarose gel.

Protein assay

Protein concentrations were determined by the modified Lowry method using a protein assay kit and instructions supplied by Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis

To assess the possible presence of RBP protein, HSC-T6 and Hepa-1 cell homogenates were analyzed by Western blot. For this purpose, 100 μg of total cellular protein was separated on a 15% SDS-PAGE gel. After completion of electrophoresis, the proteins were transferred to a PVDF membrane and probed with rabbit anti-rat RBP as antibody.

Statistical analysis

Data were analyzed statistically using the statistical package SAS (SAS Institute, Cary, NC). Data are expressed as mean \pm one standard deviation. Analysis of variance was used to compare intracellular retinyl ester concentrations in HSC-T6 cells after treatment with retinol to intracellular concentrations after incubation with 10% FBS alone.

RESULTS

Characteristics of rat liver HSC-T6 stellate cells

HSC-T6 stellate cells exhibit an activated phenotype as reflected in their fibroblast-like shape and rapid proliferation in culture. Also, the cells express cytoskeletal proteins including desmin, alpha smooth muscle actin, glial acidic fibrillary protein, and vimentin that are typical of activated stellate cells (**Fig. 1**).

Retinol uptake, esterification, and storage by HSC-T6 stellate cells and Hepa-1 hepatocytes

Among the most striking features of hepatic stellate cells in vivo are the numerous lipid droplets that are present within the cytoplasm of the cells. These lipid droplets are the major storage sites for retinyl ester in the liver and in vivo both the size and number of lipid droplets depend on dietary vitamin A intake (12). Consequently, we asked whether HSC-T6 stellate cells also form lipid droplets in response to retinol availability in the culture media. HSC-T6 stellate cells were cultured in media containing different levels of retinol and then stained with oil red O, a stain commonly used to visualize lipid-rich particles (12). When HSC-T6 cells were cultured in media containing 10% FBS, providing a final media retinol concentration of approximately 0.1 μM , lipid droplets were not detected within the cells. However, when the cells were exposed to media containing higher concentrations of retinol (1 and 5 μM), levels similar to those which

TABLE 1. Oligonucleotides, expected sizes of PCR products, and annealing temperatures for semi-quantitative RT-PCR analysis of nuclear retinoid and β -actin expression in HSC-T6

Receptor	Oligonucleotides		Expected Size of PCR Product	Annealing Temperature
	5'-3'	3'-5'		
RAR α	CTGTAAGGGCTTCTTCCG	AGTCTTAATGATGCACTT	397	55
RAR β	CTGGCTTGCTGTGTCATAATTC	GGTACTCTGTGTCTCGATGGAT	336	55
RAR γ	GTGGAGACCGAATGGACC	GACAGGGATGAACACAGG	512	55
β -Actin	GAGCTATGAGCTGCCTGACG	AGCACTTGCGGTGCACGATG	410	55
RXR α	TTCCTGCCGCTCGACTTCTCT	ACCGTTCCGCTGTCTCTTGTGTC	538	56
RXR β	TCCCCTTCCCAGTCATCAGTT	AGGAAGGTGTCATGGGGTGT	1153	56
RXR γ	CACCCAGCTACACAGACCC	CTGCCCGAGTAGAATGACCTG	914	48

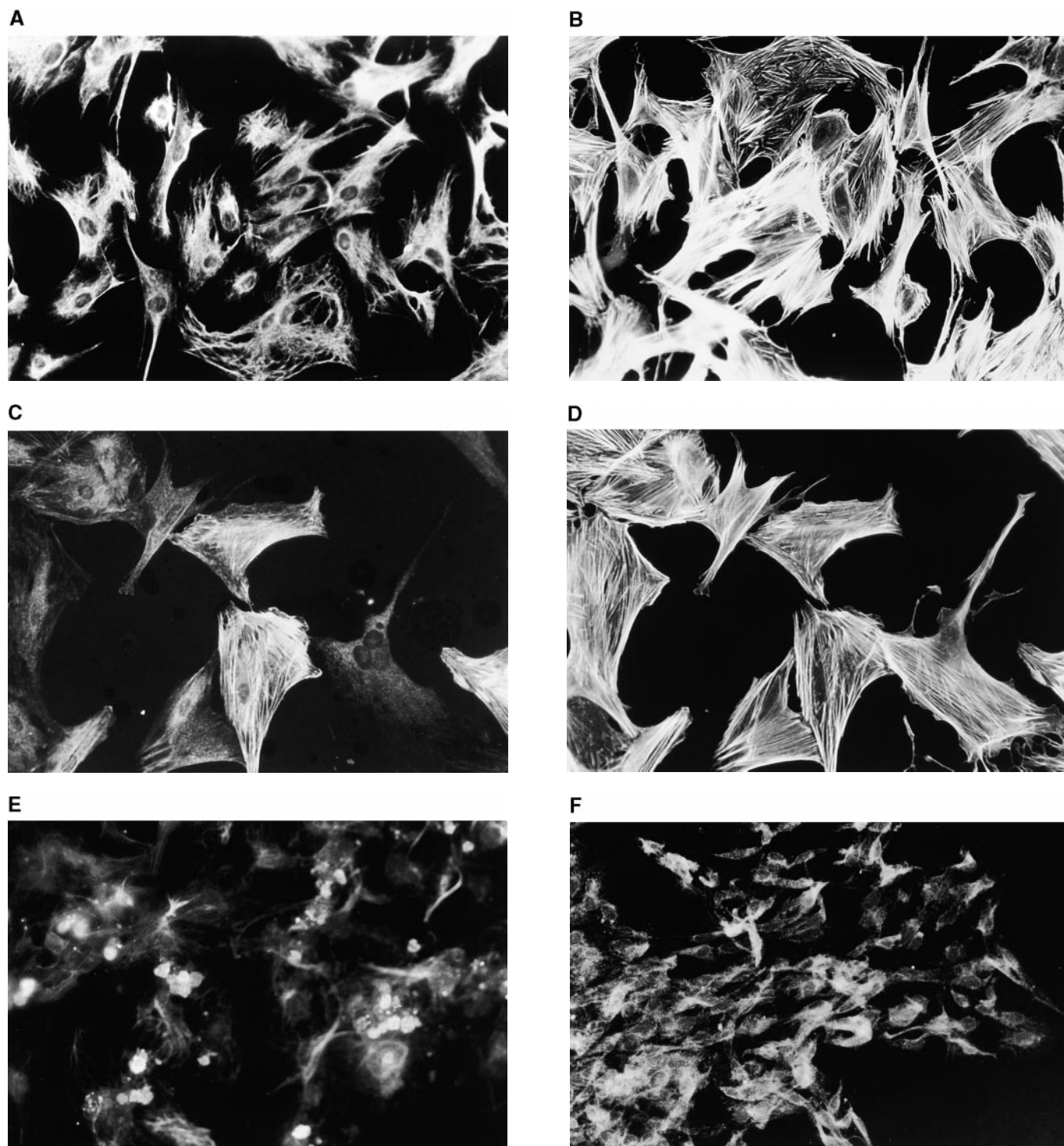
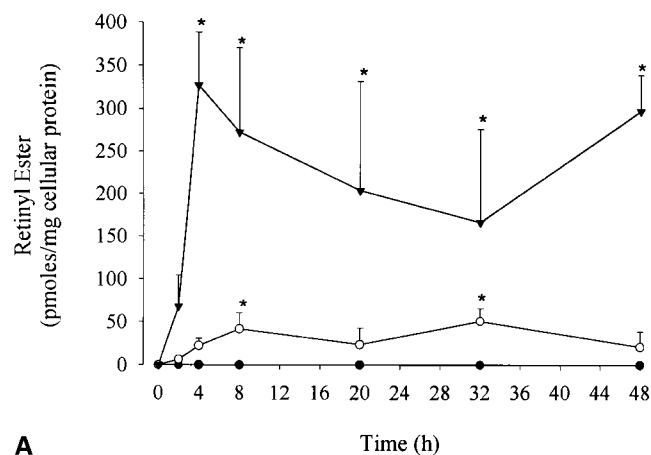


Fig. 1. Immunocytochemical detection of smooth muscle α -actin, vimentin, glial fibrillary acidic protein, and desmin for cultures of HSC-T6 stellate cells. Cells were plated, washed, and fixed and processed for immunocytochemistry as described in Experimental Procedures. In panel A and panel B, HSC-T6 cells were dual-labeled with anti-vimentin antibody and F-actin. HSC-T6 cells were labeled with anti-smooth muscle α -actin antibody in panel C and F-actin antibody in panel D. Panel E: HSC-T6 stellate cells were labeled with anti-GFAP and in panel F with anti-desmin. Magnifications were $300\times$ at the full 5×7 inch size photograph.

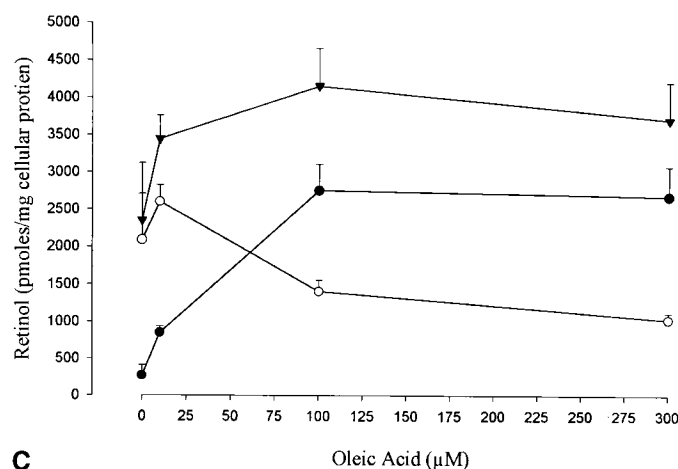
might be encountered physiologically in blood (11), cytosolic lipid droplets were detected by oil red O staining (data not shown). A greater number of oil red O stained droplets was observed for the cultures maintained in media containing $5 \mu\text{m}$ retinol than for $1 \mu\text{m}$. Based on these

morphologic studies, it appears that HSC-T6 stellate cells are able to form lipid droplets in a manner that reflects retinol content of the culture media.

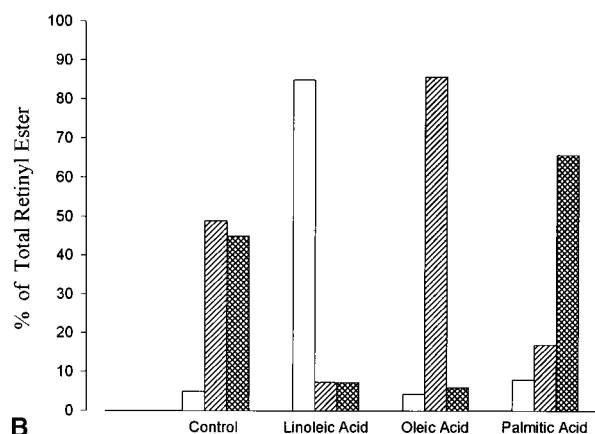
In keeping with the results obtained from the oil red O staining studies, when no supplemental retinol (other



A



C



B

Fig. 2. Retinol accumulation by cultured HSC-T6 stellate cells. Panel A: Effect of media retinol concentrations on cellular retinyl ester concentrations. HSC-T6 cells were incubated in media supplemented with retinol at concentrations of 0.5 μM (●), 1 μM (○), or 5 μM (▼) and retinyl ester concentrations were determined at different times after the start of retinol treatment. The data represent means \pm one standard deviation for triplicate cultures at each time. * $P < 0.05$, significance as compared to $t = 0$ hours. Panel B: Effect of media free fatty acid content on retinyl ester formation by HSC-T6 cells. HSC-T6 cells were incubated with either 100 μM palmitic acid, oleic acid, or linoleic acid and 5 μM retinol. Cellular retinyl ester concentrations were analyzed 12 h after the start of treatment with retinol and 24 h after start of treatment with the free fatty acids, exactly as described in Experimental Procedures. Panel C: Effect of oleic acid media supplementation on HSC-T6 levels of retinol (○), retinol oleate (●), and total retinol (retinol + retinol oleate) (▼). The HSC-T6 cells were cultured in media supplemented with 5 μM retinol and different oleic acid concentrations ranging from 0 to 300 μM . The data represent means \pm one standard deviation for triplicate HSC-T6 cultures at each oleic acid concentration.

than that present in the 10% FBS) was added in the culture media, no retinyl ester (our low limit of detection is 21 nm) could be detected in the HSC-T6 cells. However, as depicted in Fig. 2, panel A, retinyl esters accumulated in the HSC-T6 stellate cells upon treatment with culture media supplemented with either 1 or 5 μM retinol. Maximum intracellular retinyl ester concentrations were observed within 4 h after start of treatment with 5 μM retinol. At that time, approximately 6% of the total cellular retinol content was present as retinyl esters. Retinyl esters were present only intracellularly and none could be detected in the medium. Although primary rat liver stellate cells contain predominantly retinyl palmitate and lower levels of retinyl oleate, retinyl stearate, and retinyl linoleate (13), the retinyl ester composition of cultured HSC-T6 cells consisted of primarily retinyl oleate along with some retinyl palmitate and retinyl linoleate.

It is well established that the fatty acyl composition of cells in culture is dependent on the fatty acid profile of the culture media (35). Enrichment of the media with exogenous fatty acids leads to acyl substitutions in the phospholipids that are reflective of the exogenous added

FFA and to an increase in the total mass of triglyceride present in cells treated with supplemental fatty acids (35). To investigate whether changes in the cellular fatty acyl composition also modify the fatty acyl composition of retinyl esters, HSC-T6 stellate cells were co-incubated with both different free fatty acid bound to BSA and retinol. As is shown in Fig. 2, panel B, the predominant intracellular retinyl ester formed in the HSC-T6 cells is reflective of the fatty acid content of the culture media. Under culture condition with only 10% FBS (i.e., no exogenously added fatty acids), retinyl oleate and retinyl palmitate were the predominant retinyl esters observed. However, when HSC-T6 stellate cells were co-incubated with different fatty acids and retinol, the acyl group of the predominant intracellular retinyl ester always reflected the fatty acid added to the media (Fig. 2, panel B).

Enrichment of the media with exogenous fatty acids also increased the total concentrations of intracellular retinyl esters. For concentrations of supplemental oleic acid (10–300 μM), a significant increase of intracellular retinyl oleate was observed up to a concentration of 100 μM where retinyl ester formation reached a plateau (Fig. 2, panel

C). At this concentration, intracellular retinyl oleate concentrations were approximately 10-fold higher than those measured in cells cultured in unsupplemented medium. As seen in Fig. 2, panel C, the increase in intracellular retinyl oleate concentrations after supplementation with oleic acid was not due to increased uptake of retinol by the cells. Rather, this increase resulted from enhanced retinol esterification, while intracellular free retinol concentrations decreased as intracellular retinyl oleate concentrations increased in the HSC-T6 stellate cells (Fig. 2, panel C). After supplementation with 100 μM oleic acid and 5 μM retinol, an average of 60% of the total retinol present in the cells was as retinyl ester compared to only 15% when no exogenous free fatty acid was added to the culture medium.

For Hepa-1 hepatocytes, treatment with retinol led to a rapid increase in intracellular retinol concentrations as early as 2 h after the start of treatment (Fig. 3). As seen in Fig. 3, addition of exogenous oleic acid to the culture medium did not alter the intracellular total retinol concentrations in the Hepa-1 hepatocytes. This is unlike human HepG2 hepatocytes where the addition of dispersed retinol together with exogenous fatty acids leads to an approximate 15–20% increase in retinol uptake by those cells (36). Also, in contrast to the HSC-T6 stellate cells, only low levels of retinyl esters were ever observed in Hepa-1 hepatocyte cultures. Although the Hepa-1 cells acquired substantial amounts of retinol from the culture medium, retinyl esters were not detected at 2, 4, 8, or 20 h after start of the treatment (our low detection limit is 21 nm). Only at later time intervals, 32 and 48 h after start of treatment with 5 μM retinol, were retinyl esters detected (21.1 \pm 6.2 pmol retinyl ester/mg cellular protein and 64.7 \pm 28.7 pmol retinyl ester/mg cellular protein at 32 and 48 h, respectively) in cultured Hepa-1 cells. Thus, unlike HSC-T6 cells, Hepa-1 hepatocytes do not readily esterify retinol to retinyl ester. However, like HSC-T6 stellate cells, retinol esterification by Hepa-1 cells can be increased when the culture medium is supplemented with exogenous oleic acid. Retinyl ester concentrations for oleic acid-

treated Hepa-1 cells were approximately 10-times greater than those of untreated cells. Nevertheless, when expressed on the basis of cellular protein, regardless of the treatment, retinyl ester levels were always greater, by about 10- to 20-fold, for HSC-T6 stellate cells compared to those of Hepa-1 cells. The average intracellular concentrations in the Hepa-1 cells after co-incubation of retinol and oleic acid reached 160.9 \pm 44.2 pmol/mg cellular protein in contrast to concentrations of 2757 \pm 358 pmol/mg cellular protein in the HSC-T6 cells.

Both LRAT and ARAT activities are reportedly present in rat liver homogenates (19, 21–24). LRAT specific activity is high in primary rat stellate cells (6, 19). Both LRAT and ARAT activities are also expressed in HSC-T6 cells. For five independent in vitro assays of ARAT activity, a mean ARAT specific activity was determined to be 211.4 \pm 29.8 pmol of retinyl heptadecanoate formed/h per mg microsomal protein. For five independent in vitro assays of LRAT, on average LRAT specific activity of 90.8 \pm 45.2 pmol retinyl ester formed/h per mg microsomal protein was observed.

Northern and Western blot analyses

To characterize the HSC-T6 and Hepa-1 cell lines, the expression of CRBP, RBP, and TTR mRNAs by each line was evaluated. Total RNA samples were prepared from cells treated with no exogenous retinoids (containing only 10% FBS), 5 μM all-*trans*-retinoic acid, or 5 μM retinol for 12 h. Analyses of these RNA samples by Northern blot and hybridization with ^{32}P -labeled cDNA probes are shown in Fig. 4. As expected, in Hepa-1 hepatocytes, RBP and CRBP transcripts were present at nearly identical levels for all treatment conditions. While no significant increase in RBP mRNA level was observed after treatment with the retinoids, CRBP mRNA levels increased approximately 2-fold in the Hepa-1 hepatocytes upon treatment with either all-*trans*-retinoic acid or retinol. Unexpectedly, TTR mRNA was not detected in the Hepa-1 hepatocytes. Thus, unlike primary hepatocytes or HepG2 hepatocytes (6, 11, 37), Hepa-1 hepatocytes do not express TTR.

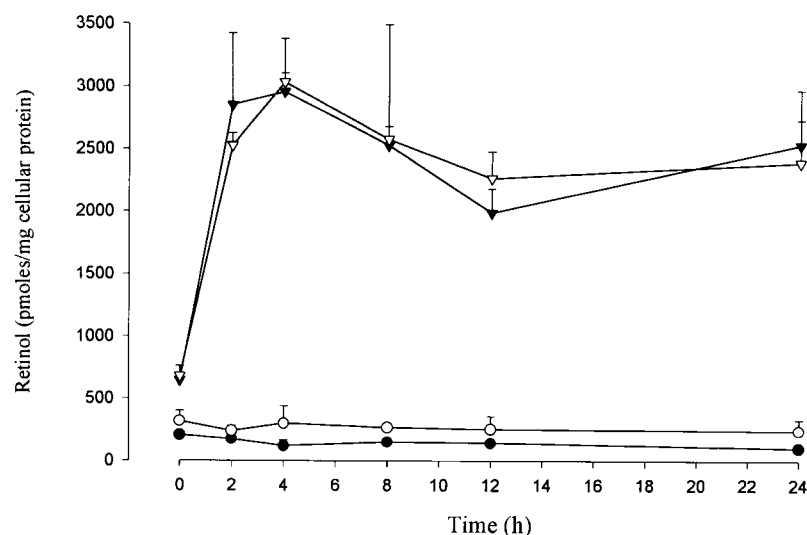


Fig. 3. Retinol uptake and processing by cultures of Hepa-1 hepatocytes. Effect of media retinol and oleic acid concentrations on cellular total retinol levels for Hepa-1 hepatocytes. Hepa-1 hepatocytes were cultured in media containing 10% FBS only (●), or media supplemented with 5 μM retinol (▼), 100 μM oleic acid (○), or 5 μM retinol and 100 μM oleic acid (△). The data give the mean \pm one standard deviation for triplicate cultures at each time after the start of treatment.

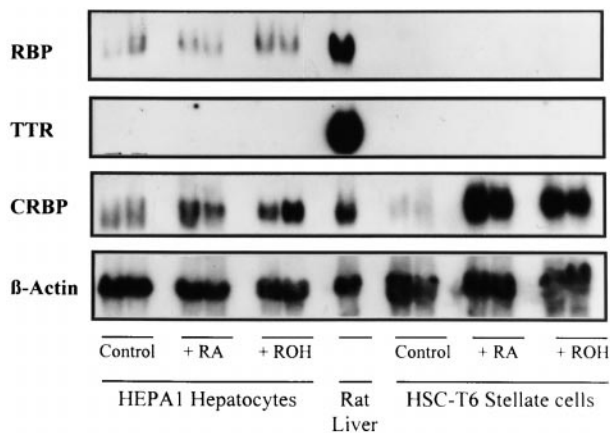


Fig. 4. Northern blot analyses for retinol-binding protein (RBP), transthyretin (TTR), and cellular retinol-binding protein (CRBP) expression in HSC-T6 stellate cells and Hepa-1 hepatocytes. Cells were cultured in media containing 10% FBS (control) or media supplemented with either 5 μ M all-*trans*-retinoic acid (+RA) or 5 μ M retinol. For HSC-T6 stellate cells and Hepa-1 hepatocytes, each lane was loaded with 20 μ g total cellular RNA and, to serve as a positive control for each of these RNA species, a lane was loaded with 5 μ g total RNA isolated from rat liver.

Extending the results from the Northern blot analyses, RBP protein was detected in the Hepa-1 hepatocytes by Western blot (**Fig. 5, panel A**) and by RIA analyses (mean cellular concentration 1050 ± 378 ng RBP/mg cellular protein). RBP was secreted by Hepa-1 cells into the media at a rate that was linear throughout the entire 24 h duration of this experiment (**Fig. 5, Panel B**).

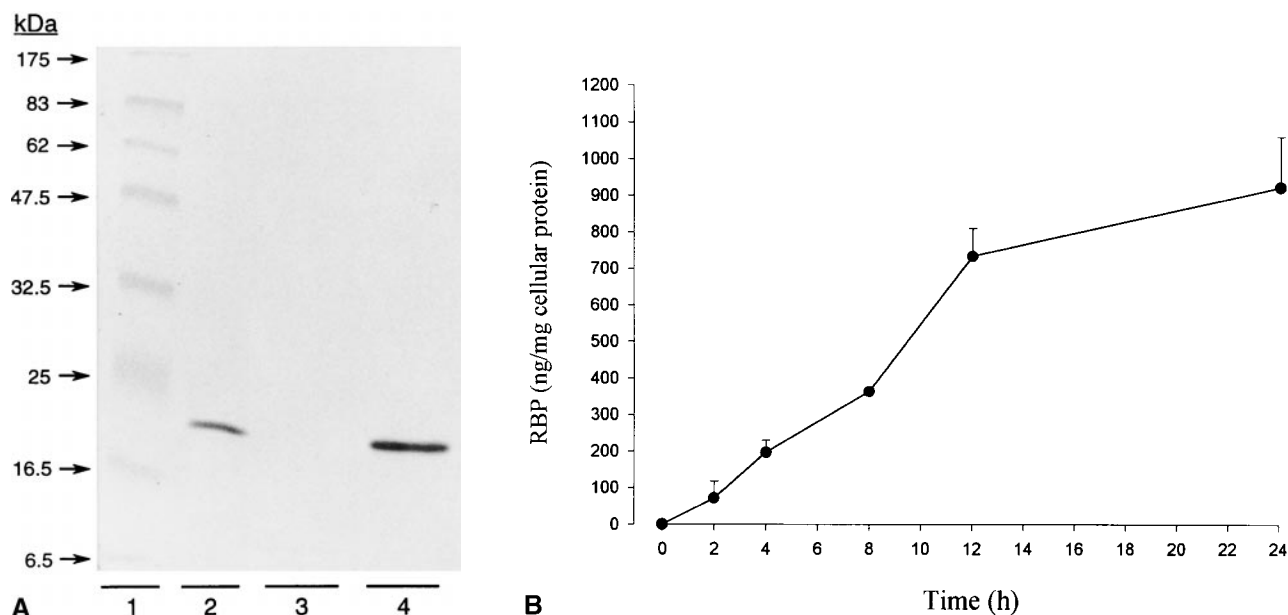


Fig. 5. RBP synthesis and secretion by Hepa-1 hepatocytes. Panel A: Western blot analysis for mouse RBP. This analysis was carried out as described in Experimental Procedures. Lane 1, molecular weight markers; lane 2, 100 μ g of total homogenate protein from Hepa-1 hepatocytes cultured in 10% FBS; lane 3, 100 μ g of total homogenate protein from HSC-T6 stellate cells cultured in 10% fetal calf serum; and lane 4, recombinant mouse RBP. Panel B: Time-dependent accumulation of RBP in the media of Hepa-1 hepatocytes cultured over 24 h. Hepa-1 cells were cultured in media containing 10% FBS and aliquots of the media were taken and analyzed for RBP content by RIA at different times after start of incubation.

As expected from studies of primary stellate cells (16, 38), HSC-T6 stellate cells did not express detectable levels of RBP or TTR mRNA nor was expression induced with retinoid treatment. Under basal culture conditions, only low levels of CRBP mRNA could be detected in HSC-T6 cells. However, a 10-fold increase in CRBP mRNA level was observed after treatment of the stellate cells with either retinol or all-*trans*-retinoic acid (**Fig. 4**).

To understand the effect of all-*trans*-retinoic acid on CRBP mRNA expression in HSC-T6 cells and to establish whether regulation of CRBP expression is similar to that reported for other cell types, a dose response and a time course of CRBP expression were established. HSC-T6 cells were treated with different concentrations of retinol (ranging from 10^{-8} M to 10^{-6} M) and all-*trans*-retinoic acid (ranging from 10^{-10} M to 10^{-7} M) for 12 h. As shown in **Fig. 6, panel A**, CRBP expression was induced by all-*trans*-retinoic acid even at treatment levels as low as 10^{-10} M.

To study the time course of CRBP mRNA induction by all-*trans*-retinoic acid, HSC-T6 cells were treated with 1 μ M all-*trans*-retinoic acid and total RNA was extracted at different time intervals (**Fig. 6, panel A**). By 4 h after the start of treatment, we observed induction of CRBP mRNA expression. This relatively early induction of CRBP expression suggests that new protein synthesis may not be required for this effect.

The induction of CRBP expression by all-*trans*-retinoic acid has been reported to be mediated through the nuclear receptors RARs and RXRs (39). Therefore, we asked which RAR and RXR subtypes are expressed in the HSC-T6 stellate cells and which of these may play a role in the up-

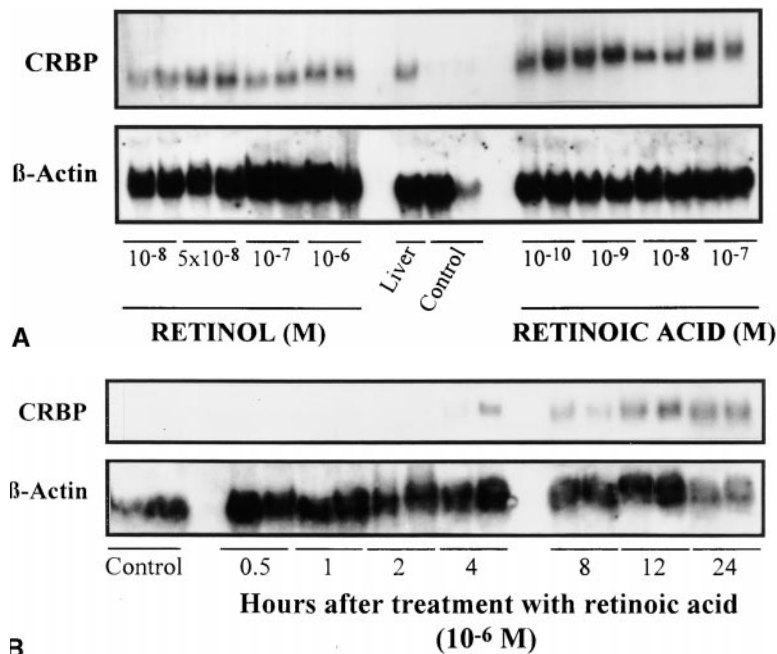


Fig. 6. Induction of cellular retinol-binding protein (CRBP) expression in HSC-T6 stellate cells by retinoids. Panel A: The concentration-dependence of retinoid induction of CRBP expression. HSC-T6 stellate cells were treated with different concentrations of retinol (10^{-8} to 10^{-6} m) and all-*trans*-retinoic acid (10^{-10} to 10^{-7} m) for 12 h; total RNA was extracted from the cells and analyzed for CRBP expression by Northern blot. Control RNA was extracted from cells incubated in unsupplemented media containing 10% FBS. Expression of β -actin was used as a control for sample loading. Panel B: The time-dependence of retinoid induction of CRBP expression. HSC-T6 stellate cells were incubated with 10^{-6} m all-*trans*-retinoic acid for different times and CRBP expression level was assessed by Northern blot. Control HSC-T6 stellate cells were incubated for 24 h in unsupplemented media containing 10% FBS.

regulation of CRBP expression. To identify the RAR and RXR subtypes present in the HSC-T6 cells, semi-quantitative RT-PCR was used (Fig. 7). The expression of RARs and RXRs in the HSC-T6 cells was compared to the expression of β -actin. As depicted in Fig. 7, all RAR and RXR types are expressed in the HSC-T6 stellate cells under basal culture conditions. Among the RARs, RAR α was present at the highest levels compared to RAR β and RAR γ . For the RXRs, lowest expression was observed for RXR γ for which only a very faint band can be observed.

Knowing that all six of the retinoid nuclear receptors are expressed in the HSC-T6 cells, we were interested in identifying which nuclear receptor confers the retinol/retinoic acid-dependent induction of CRBP expression in HSC-T6 cells. In a preliminary experiment we used RAR and RXR selective pan-agonists and a RAR selective pan-antagonist to

understand CRBP expression in these cells. As shown in Fig. 8, the RAR pan-agonist induces the expression of CRBP and, in agreement with this, the RAR pan-antagonist inhibits all-*trans*-retinoic acid-induced expression of CRBP in the HSC-T6 cells. In addition, treatment of the HSC-T6 with a RXR pan-agonist leads to enhanced CRBP expression.

DISCUSSION

It is well established that both hepatocytes and stellate cells play important roles in hepatic retinoid uptake, stor-

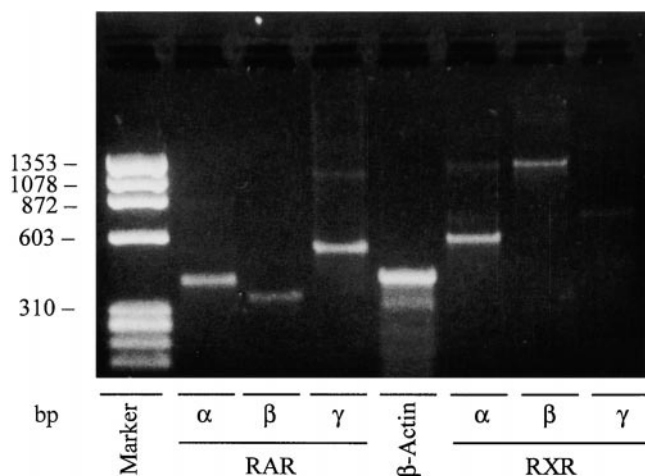


Fig. 7. Semi-quantitative RT-PCR analysis for RAR α , β , and γ and RXR α , β , and γ expression by HSC-T6 stellate cells.

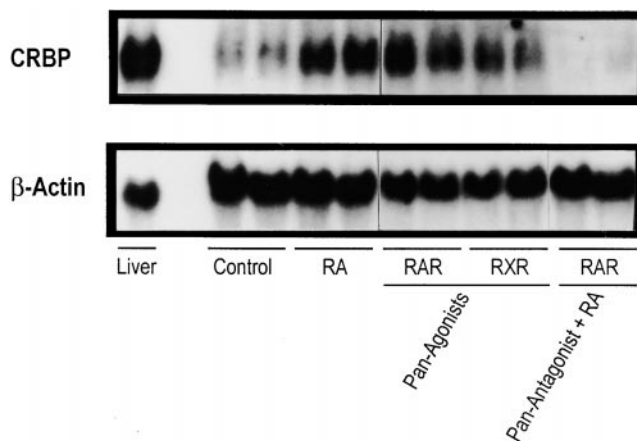


Fig. 8. The effects of pan-RAR (CD367) and pan-RXR (CD3127) agonists and a pan-RAR (CD3106) antagonist on expression of cellular retinol-binding protein (CRBP) in HSC-T6 stellate cells. HSC-T6 stellate cells were incubated in 10% FBS with no exogenously added retinoid (control), 10^{-8} m all-*trans*-retinoic acid (RA), 10^{-8} m pan-RAR agonist, 10^{-8} m pan-RXR agonist, or 10^{-8} m pan-RAR antagonist plus 10^{-8} m all-*trans*-retinoic acid (pan-antagonist + RA) for 24 h. After total RNA isolation from the cells, CRBP expression was evaluated by Northern blot analysis.

age, mobilization, and metabolism (6). Yet, mechanistic understanding of how hepatocytes and stellate cells interact to coordinate hepatic retinoid uptake, storage, and mobilization is still very limited. As hepatic stellate cells represent only 5–7% of the total cells present in the liver (12), it is technically difficult to isolate sufficient quantities of hepatic stellate cells for use in such studies. Equally limiting is the dual phenotype exhibited by hepatic stellate cells (40). In the context of the healthy liver, stellate cells have a quiescent phenotype characterized by a low proliferative rate and numerous lipid droplets containing retinyl esters. In contrast, stellate cells from fibrotic livers or isolated stellate cells maintained in culture exhibit an activated phenotype, which is characterized by high proliferative rates, expression of fibroblastic cell markers, and rapid loss of retinoid from the cells (27, 38, 40, 41). For cultured primary rabbit stellate cells undergoing a transition from the quiescent to the activated phenotype, Troen, Norum, and Blumhoff (41) report that the total retinol content of the stellate cells decreased by 20-fold from 144 nmol to 7.8 nmol/mg protein within the first 4 days of culture.

To overcome these limitations for studying primary cultures of hepatic stellate cells, several stellate cell lines have been reported in the literature. Two groups have reported establishing human stellate cell lines that can be maintained in culture (42, 43). However, these two stellate cell lines were derived from pathologic livers. The GRX cell line was derived from connective liver tissue (42, 44) and the L190 cell line was derived from human hepatic mesenchymal tumor cells (43). Nevertheless, both GRX and L190 cells, like hepatic stellate cells, are reported to be able to take up retinol from the culture medium and to form intracellular retinyl esters (42–44). Vincente et al. (44) have also demonstrated that CRBP is expressed by GRX cells. However, the usefulness of these two human lines for the study of hepatic retinoid physiology is not clear as cell lines with activated phenotypes isolated from fibrotic livers often have very reduced or no expression of some of the nuclear retinoid receptors (45–47). Moreover, these two lines have not been fully characterized with respect to their capacity for retinoid uptake, storage, and metabolism. Finally, in addition to these two human stellate cell lines, a stellate cell line derived from healthy mouse liver stellate cells and immortalized through infection with a temperature-sensitive mutant SV40 has been reported but the utility of this mouse line for studies of retinoid metabolism has not been established in the literature (48).

In this study we have generated an immortalized rat hepatic stellate cell line (HSC-T6 cells) and have explored the potential usefulness of this stellate cell line for study of hepatic retinoid metabolism. Our studies demonstrate that HSC-T6 cells express retinoid-related parameters that are characteristic of freshly isolated quiescent rat hepatic stellate cells. This suggests that the HSC-T6 will be a useful model cell system to study retinoid metabolism and intercellular communication between hepatocytes and stellate cells. Most importantly and in contrast to the other established stellate cell lines, all six nuclear retinoid receptors,

including the three subtypes of RARs and RXRs, are expressed in HSC-T6 cells. A few reports have described the expression patterns of RAR receptors and RXR receptors in quiescent as well as activated stellate cells (45–47, 49). All three RAR subtypes are reported to be expressed in quiescent stellate cells (44, 48). The expression pattern of the RXR subtypes in quiescent stellate cells is not definitively established. Ohata et al. (47) report the expression of all three RXR subtypes in freshly isolated rat stellate cells, whereas Ulven et al. (49) report being unable to detect RXR γ in primary rat stellate cell isolates. The activated phenotype of stellate cells is associated with the loss of intracellular retinoids and with reduced or nearly absent retinoid signaling by the activated cells. This change in phenotype is also associated with diminished expression of the nuclear retinoid receptors. The expression of RAR β is reported to be reduced in stellate cells isolated from livers with induced fibrosis through CCL4 or bile duct ligation (45, 48). RXR α expression is also reported to be depressed in the L190 cells and stellate cells from fibrotic liver as compared to cells from healthy livers (46, 47). Thus, with regard to the expression of the nuclear receptors, the rat HSC-T6 stellate cells are more like quiescent stellate cells as found in vivo or in primary culture rather than an activated stellate cell.

When HSC-T6 culture media are supplemented with retinol to physiologic levels (blood retinol concentration range from 2–3 μM (11)), intracellular retinyl ester accumulated in lipid droplets in HSC-T6 cells. These lipid droplets can be seen upon oil red O staining and cellular retinyl ester can be readily detected by HPLC analysis. These results are in keeping with evidence that retinol addition leads to accumulation of retinyl esters in cultured primary stellate cells and in human and mouse stellate cell lines (41–43, 48). Initially, we observed that incubation of HSC-T6 stellate cells with media supplemented with physiologic levels of retinol brought about significant accumulation of intracellular retinyl ester. However, these intracellular retinyl ester concentrations were still much lower than those reported for primary rat stellate cell isolates (13–16, 41, 50). It has been previously demonstrated that exogenous fatty acids added to the medium can lead to increased retinol uptake and retinol esterification by cells in culture (36, 41, 51). For HepG2 hepatocytes and MCF-7 mammary carcinoma cells, exogenous free fatty acids (at levels of 210 μM) increased the uptake of free retinol into cells by about 20% and retinol esterification by 50–60% (36). Troen et al. (41) observed an almost 3-fold increase in intracellular retinyl ester concentrations when primary rabbit stellate cells were co-incubated with 300 μM oleic acid and retinol (41). For the HSC-T6 stellate cells, addition of both oleic acid and retinol to the culture medium brought about a 10-fold increase in intracellular retinyl ester concentrations.

Several specific retinoid-transport proteins, including RBP, TTR, and CRBP, are involved in hepatic retinoid metabolism (6). Based on studies of primary liver cells in culture and immunohistochemical studies of the intact liver, distinct differences exist with regard to the presence of

these proteins in stellate cells and hepatocytes. While RBP, TTR, and CRBP are present in hepatocytes (6), only CRBP is found in primary rat stellate cells (16, 38, 52). Although RBP, TTR, and CRBP were all expressed in Hepa-1 hepatocytes, we detected only CRBP expression and no expression of either RBP or TTR in HSC-T6 stellate cells.

Retinoid-induced expression of CRBP in the HSC-T6 stellate cells must be mediated by the actions of retinoid nuclear receptors that are expressed constitutively in these cells. A retinoic acid response element (RARE) is present in the rat CRBP gene (53). Husmann et al. (39) have previously demonstrated that co-transfection of RAR α and RXR α resulted in a retinoic acid-dependent activation of a reporter gene driven by the RARE present in the gene for CRBP. Subsequent binding studies have shown that retinoic acid nuclear receptors bind as RAR/RXR heterodimers to the DNA domain. In HSC-T6 stellate cells, the RAR pan-agonist CD367 induced CRBP expression while the RAR pan-antagonist CD3106 suppressed the expression of CRBP mediated by retinoic acid, indicating a role for RAR in regulating CRBP expression. As RXRs are also constitutively expressed in HSC-T6 stellate cells, it seemed possible that transactivation of the CRBP gene might occur via heterodimerization with an RXR subtype. This possibility is supported by our observation that the RXR pan-agonist CD3127 also induces CRBP in HSC-T6 stellate cells. Based on these preliminary experiments involving receptor agonists and antagonists, it is not clear how the RXR pan-agonists induce expression of CRBP but it seems unlikely that a homodimer of RXR-RXR mediates the effect as the RARE of CRBP contains a DR2 motif and it has been shown that an RXR-RXR homodimer will not bind to such an element (54). It is, however, clear that CRBP expression in HSC-T6 cells is remarkably responsive. This is evidenced by Fig. 6, which indicates that CRBP mRNA is maximally induced at retinoic acid concentrations at or below the normal physiologic range and by Fig. 8, which shows that a RAR pan-antagonist completely blocks all-*trans*-retinoic acid induction of CRBP expression in HSC-T6 cells.

Although the HSC-T6 stellate cells express retinoid-related proteins and process retinoids in a manner that resembles quiescent rat stellate cells, the immortalized HSC-T6 cells do exhibit an activated phenotype in that they do proliferate and display a fibroblast-like morphology. Because these cells express each of the RAR and RXR subtypes, CRBP, LRAT, and ARAT activities and not RBP or TTR, and because of their strong ability to take up, accumulate, and esterify retinol, the ability of HSC-T6 cells to process retinoids like quiescent stellate cells appears to be uncoupled from the morphologic and proliferative characteristics observed for activated stellate cells. Based on the characteristics of the HSC-T6 stellate cells that we report here, we believe that these immortalized cells will prove useful to explore cellular aspects of hepatic retinoid storage and processing and possibly for use in co-cultures with hepatocyte lines like the Hepa-1 cells. ■

This work was supported by grants DK52444 and DK37340 from the National Institutes of Health and a grant from the

United States Department of Agriculture. The excellent technical assistance of Gudrun Theiss is gratefully acknowledged.

Manuscript received 9 August 1999 and in revised form 22 February 2000.

REFERENCES

1. Goodman, D. S. 1984. Vitamin A and retinoids in health and disease. *N. Engl. J. Med.* **310**: 1023–1031.
2. Saari, J. C. 1994. Retinoids in photosensitive systems. *In* The Retinoids: Biology, Chemistry, and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, Ltd., New York. 351–386.
3. Hofmann, C., and G. Eichele. 1994. Retinoids in development. *In* The Retinoids, Biology, Chemistry, and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, Ltd., New York. 387–442.
4. Mangelsdorf, D. M., K. Umesono, and R. M. Evans. 1994. The retinoid receptors. *In* The Retinoids: Biology, Chemistry, and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, Ltd., New York. 319–350.
5. Gudas, L. J., M. B. Sporn, and A. B. Roberts. 1994. Cellular biology and biochemistry of the retinoids. *In* The Retinoids: Biology, Chemistry, and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, Ltd., New York. 443–520.
6. Blaner, W. S., and J. A. Olson. 1994. Retinol and retinoic acid metabolism. *In* The Retinoids: Biology, Chemistry, and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, Ltd., New York. 229–255.
7. Harrison, E. H. 1993. Enzymes catalyzing the hydrolyses of retinyl esters. *Biochim. Biophys. Acta.* **1170**: 99–108.
8. Gad, M. Z., and E. H. Harrison. 1991. Neutral and acid retinyl ester hydrolases associated with rat liver microsomes: relationships to microsomal cholesteryl ester hydrolases. *J. Lipid Res.* **32**: 685–693.
9. Hagen, E., A. M. Myhre, T. E. Tjelle, T. Berg, and K. R. Norum. 1999. Retinyl esters are hydrolyzed in early endosomes of J14 macrophages. *J. Lipid Res.* **40**: 309–317.
10. Harrison, E. H., M. Z. Gad, and A. C. Ross. 1995. Hepatic uptake and metabolism of chylomicron retinyl esters: probable role of plasma membrane/endosomal retinyl ester hydrolases. *J. Lipid Res.* **36**: 1498–1506.
11. Soprano, D. R., and W. S. Blaner. 1994. Plasma retinol-binding protein. *In* The Retinoids: Biology, Chemistry, and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, Ltd., New York. 257–281.
12. Wake, K. 1980. Perisinusoidal stellate cells (fat storing cells, interstitial cell, lipocytes), their related structure in and around liver sinusoids, and vitamin A storing cells in extrahepatic organs. *Int. Rev. Cytol.* **66**: 303–353.
13. Hendriks, H. F. J., W. A. M. M. Verhoofstad, A. Brouwer, A. M. de Leeuw, and D. L. Knook. 1985. Perisinusoidal fat-storing cells are the main vitamin A storage sites in rat liver. *Exp. Cell. Res.* **160**: 138–149.
14. Lakshman, M. R., P. R. Sundaresan, L. L. Chambers, and P. K. Shoff. 1988. Cellular retinyl esters and retinol among parenchymal and stellate cells in normal rat liver. *Lipids.* **23**: 144–147.
15. Blomhoff, R., T. Berg, and K. R. Norum. 1988. Distribution of retinol in rat liver cells: effects of age, sex and nutritional status. *Br. J. Nutr.* **60**: 233–239.
16. Yamada, M., W. S. Blaner, D. R. Soprano, J. L. Dixon, H. M. Kjeldbye, and D. S. Goodman. 1987. Biochemical characteristics of isolated rat liver stellate cells. *Hepatology.* **7**: 1224–1229.
17. Batres, R., and J. A. Olson. 1988. Relative amount and ester composition of vitamin A in rat hepatocytes as a function of the method of cell proliferation and of total liver stores. *J. Nutr.* **117**: 77–82.
18. Moriwaki, H., W. S. Blaner, R. Piantadosi, and D. S. Goodman. 1988. Effects of dietary retinoid and triglyceride on the liver composition of rat liver stellate cells and stellate cell lipid droplets. *J. Lipid Res.* **29**: 1523–1534.
19. Blaner, W. S., A. M. van Bennekum, A. Brouwer, and H. F. J. Hendriks. 1990. Distribution of lecithin-retinol acyltransferase activity in different types of rat liver cells and subcellular fractions. *FEBS Lett.* **274**: 89–92.

20. Azais-Braesco, V., I. Dodeman, S. Delpal, M. C. Alexandre-Gouabau, A. Partier, P. Borel, and P. Grolier. 1995. Vitamin A contained in the lipid droplets of rat liver stellate cell is substrate for acid retinyl ester hydrolase. *Biochim. Biophys. Acta.* **1259**: 271–276.
21. Ross, C. A. 1982. Retinol esterification by rat liver microsomes. Evidence for a fatty acyl coenzyme A:retinol transferase. *J. Biol. Chem.* **257**: 2453–2459.
22. Ong, D. E., P. N. MacDonald, and A. M. Gubitosi. 1988. Esterification of retinol in rat liver. Possible participation by cellular retinol-binding protein and cellular retinol-binding protein (II). *J. Biol. Chem.* **263**: 5789–5796.
23. Matsuura, T., M. Z. Gad, E. H. Harrison, and A. C. Ross. 1997. Lecithin:retinol acyltransferase and retinyl ester hydrolase activities are differentially regulated by retinoids and have distinct distributions between hepatocyte and nonparenchymal cell fractions of rat liver. *J. Nutr.* **127**: 218–224.
24. Randolph, K. R., and A. C. Ross. 1991. Vitamin A status regulates hepatic lecithin:retinol acyltransferase activity in rats. *J. Biol. Chem.* **266**: 16453–16457.
25. Mercier, M., A. Forget, P. Grolier, and V. Azais-Braesco. 1994. Hydrolysis of retinyl esters in rat liver. Description of lysosomal activity. *Biochim. Biophys. Acta.* **1212**: 176–182.
26. Kim, Y., V. Ratziu, S-G. Choi, A. Lalazar, G. Theiss, Q. Dang, S. J. Kim, and S. L. Friedman. 1998. Transcriptional activation of TGF- β 1 and its receptors by the Kruppel-like factor Zf9/CPBP and Sp1; potential mechanisms for autocrine fibrogenesis in response to injury. *J. Biol. Chem.* **273**: 33750–33758.
27. Friedman, S. L. 1993. Isolation and culture of hepatic nonparenchymal cells. *Methods Toxicol.* **1**: 292–310.
28. Darlington, G. J., H. P. Bernhard, R. A. Miller, and F. H. Ruddle. 1980. Expression of liver phenotypes in cultured mouse hepatoma cells. *J. Natl. Cancer Inst.* **64**: 809–815.
29. Darlington, G. J. 1987. Liver cell lines. *Methods Enzymol.* **151**: 19–38.
30. Ramirez-Zacarias, J. L., F. Castro-Munozledo, and W. Kuri-Harcuch. 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. *Histochemistry.* **97**: 493–497.
31. Blaner, W. S. 1990. Radioimmunoassay for RBP, cellular RBP and cellular retinoic acid-binding protein. *Methods Enzymol.* **189**: 270–281.
32. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
33. Soprano, D. R., K. J. Soprano, M. L. Wyatt, and D. S. Goodman. 1988. Induction of the expression of retinol-binding protein and transthyretin in F9 embryonal carcinoma cells differentiated to embryoid bodies. *J. Biol. Chem.* **263**: 17897–17900.
34. Wan, Y. J., L. Wang, and T. C. J. Wu. 1992. Detection of retinoic acid receptors mRNA in rat tissues by reverse transcriptase-polymerase chain reaction. *J. Mol. Endocrinol.* **9**: 291–294.
35. Spector, A. A., and M. A. Yorek. 1985. Membrane lipid composition and cellular function. *J. Lipid Res.* **26**: 1015–1035.
36. Randolph, R. K., and A. C. Ross. 1991. Regulation of retinol uptake and esterification in MCF-7 and HepG2 cells by exogenous fatty acids. *J. Lipid Res.* **32**: 809–820.
37. Blaner, W. S., M. J. Bonifacia, H. D. Feldman, R. Piantedosi, and M. J. M. Saraiva. 1991. Studies on the synthesis of transthyretin by the human hepatoma cell line HepG2. *FEBS Lett.* **287**: 193–196.
38. Friedman, S. L., S. Wei, and W. S. Blaner. 1993. Retinol release by activated rat hepatic lipocytes: regulation by Kupffer cell-conditioned medium and PDGF. *Am. J. Physiol.* **264**: G947–G952.
39. Husmann, M., B. Hoffmann, D. G. Stump, F. Chytil, and M. Pfahl. 1992. A retinoic acid response element from the rat CRBP I promoter is activated by an RAR/RXR heterodimer. *Biochem. Biophys. Res. Commun.* **187**: 1558–1564.
40. Friedman, S. L. 1993. The cellular basis of hepatic fibrosis. *N. Engl. J. Med.* **25**: 1828–1835.
41. Troen, G., K. R. Norum, and R. Blomhoff. 1994. Characterization of liver stellate cell retinyl ester storage. *Biochem. J.* **300**: 793–798.
42. Borojevic, R., R. M. Guaragna, R. Margis, and H. S. Duta. 1990. In vitro induction of the fat-storing phenotype in a liver connective tissue cell line GRX. *In vitro Cell. Dev. Biol.* **25**: 361–368.
43. Murakami, K., T. Abe, M. Miyazawa, M. Yamaguchi, T. Masuda, T. Matsuura, S. Nagamori, K. Takeuchi, K. Abe, and M. Kyogoku. 1995. Establishment of a new human cell line, LI90, exhibiting characteristics of hepatic Ito (fat-storing) cells. *Lab. Invest.* **72**: 731–739.
44. Vincente, C. P., V. A. Fortuna, R. Margis, L. Trugo, and R. Borojevic. 1998. Retinol uptake and metabolism, and cellular retinol binding protein expression in an in vitro model of hepatic stellate cells. *Mol. Cell. Biochem.* **187**: 11–21.
45. Weiner, F. R., W. S. Blaner, M. J. Czaja, A. Shah, and A. Geerts. 1992. Ito cell expression of a nuclear retinoic acid receptor. *Hepatology.* **15**: 336–342.
46. Imai, S., M. Okuno, H. Moriwaki, Y. Muto, K. Murakami, K. Shudo, Y. Suzuki, and S. Kojima. 1997. 9,13-di-*cis*-Retinoic acid induces the production of tPA and activation of latent TGF- β via RAR in a human liver stellate cell line, LI90. *FEBS Lett.* **411**: 102–106.
47. Ohata, M., M. Lin, M. Satre, and H. Tsukamoto. 1997. Diminished retinoic acid signaling in hepatic stellate cells in cholestatic liver fibrosis. *Am. J. Physiol.* **272**: G589–G596.
48. Kitamura, Y., T. Tanigawa, T. Katsumoto, K. Tomita, H. R. Wang, K. Hirai, K. Ichihara, and T. Terada. 1997. Cell growth and differentiation of a novel mouse Ito (fat-storing) cell line transformed by a temperature-sensitive mutant of simian virus 40. *Hepatology.* **26**: 323–329.
49. Ulven, S. M., V. Natarajan, K. B. Holven, T. Lovdal, T. Berg, and R. Blomhoff. 1998. Expression of retinoic acid receptor and retinoid X receptor subtypes in rat liver cells: Implication for retinoid signaling in parenchymal, endothelial, Kupffer and stellate cells. *Eur. J. Cell. Biol.* **77**: 111–116.
50. Blaner, W. S., H. F. Hendriks, A. Brouwer, A. M. de Leeuw, D. L. Knook, and D. S. Goodman. 1985. Retinoids, retinoid-binding proteins, and retinyl palmitate hydrolase distributions in different types of rat liver cells. *J. Lipid Res.* **26**: 1241–1251.
51. McCloskey, H. M., J. M. Glick, C. A. Ross, and G. H. Rothblatt. 1988. Effect of fatty acid supplementation and cholesterol and retinol esterification in J774 macrophages. *Biochim. Biophys. Acta.* **963**: 456–467.
52. Kato, M., K. Kato, and D. S. Goodman. 1984. Immunocytochemical studies on the localization of plasma and of cellular retinol-binding proteins and transthyretin (prealbumin) in rat liver and kidney. *J. Cell. Biol.* **98**: 1696–1704.
53. Smith, W. C., H. Nakashatri, P. Leroy, J. Rees, and P. Chambon. 1991. A retinoic acid response element is present in the mouse cellular retinol-binding protein I promoter. *EMBO J.* **10**: 2223–2230.
54. Giguere, V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocr. Rev.* **15**: 61–79.