9-*cis*-Retinoids: Biosynthesis of 9-*cis*-Retinoic Acid[†]

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ABSTRACT: Retinoids function through conformational alterations of ligand-dependent nuclear transcription factors, the retinoic acid receptors, and retinoid X receptors. 9-*cis*-Retinoic acid is a known biological ligand for retinoid X receptors, but its synthesis pathway in vivo is largely unknown. Recently, we identified a *cis*-retinol dehydrogenase (cRDH) that oxidizes 9-*cis*-retinol to 9-*cis*-retinal. Since both the expression of cRDH mRNA and its substrate are found in liver, we studied 9-*cis*-retinol metabolism and 9-*cis*-retinoic acid biosynthesis in two hepatic-derived cell types, Hep G2 hepatoma cells and HSC-T6 stellate cells. Both cell lines accumulate similar amounts of 9-*cis*-retinol provided in the medium. However, Hep G2 cells preferentially incorporate *all-trans*-retinol when equimolar concentrations of *all-trans*- and 9-*cis*-retinol were provided. In contrast, HSC-T6 cells did not exhibit a preference between *all-trans*- and 9-*cis*-retinol under the same conditions. Esterification of 9-*cis*-retinol occurred in both cell types, likely by acyl-CoA:retinol acyltransferase and lecithin:retinol acyltransferase. In vitro enzyme assays demonstrated that both cell types can hydrolyze 9-*cis*-retinyl esters via retinyl ester hydrolase(s). In Hep G2 cells, 9-*cis*-retinoic acid synthesis was strongly inhibited by high concentrations of 9-*cis*-retinol, which may explain the low levels of 9-*cis*-retinol in liver of mice. Cell homogenates of Hep G2 can convert *all-trans*-retinol to 9-*cis*-retinal, suggesting that the free form of *all-trans*-retinol may be used as a source for 9-*cis*-retinol and, thus, 9-*cis*-retinoic acid synthesis. Our studies provide the basis for identification of additional pathways for the generation of 9-*cis*-retinoic acid in specialized tissues.

Retinoids play an essential role in vertebrate growth and development, supporting cell differentiation (*1*−*3*); embryonic development (*4*, *5*), vision (*6*), the immune response (*7*), and reproduction (*8*). The actions of retinoids are mediated through binding and activation of the retinoic acid receptors (RARs)[1] or retinoid X receptors (RXRs), which function as ligand-dependent transcription factors. It is generally accepted that *all-trans*-retinoic acid serves as a physiologic ligand for the RARs and that 9-*cis*-retinoic acid acts as the preferred ligand for the RXRs (*9*, *10*).

Although the biochemical processes responsible for *all-trans*-retinoic acid formation from its precursor, *all-trans*-retinol, are beginning to be elucidated, there is still little information available regarding how 9-*cis*-retinoic acid is formed within cells and tissues (*11*, *12*). Three possible pathways for 9-*cis*-retinoic acid formation have been proposed. These consist of (1) the isomerization of *all-trans*-retinoic acid to 9-*cis*-retinoic acid, probably through nonenzymatic processes (*13*); (2) enzymatic oxidation of 9-*cis*-retinol to 9-*cis*-retinoic acid through a pathway similar to the oxidation of *all-trans*-retinol to *all-trans*-retinoic acid (*14*−*19*), and (3) the cleavage of 9-*cis*-β-carotene yielding directly 9-*cis*-retinoic acid or 9-*cis*-retinol/9-*cis*-retinal that is subsequently oxidized to 9-*cis*-retinoic acid (*20*, *21*). Although each of these pathways is possible, and the in vitro data supporting each of them are individually convincing, it is still unclear from the literature to what extent which of these pathways occur in vivo.

We have previously reported and characterized an enzyme that we originally termed 9-*cis*-retinol dehydrogenase (*16*). Because of the broad substrate specificity for *cis*-retinols and relative inability to catalyze *all-trans*-retinol oxidation (*22*), we now refer to this enzyme as *cis*-retinol dehydrogenase (cRDH). By Northern blot, the enzyme is expressed in liver, kidney, testis, and several other tissues in the human and mouse (*16*, *22*−*24*). On the basis of these and other data, we suggested that cRDH might be an important component of a biosynthetic pathway resulting in the formation of 9-*cis*-retinoic acid. In the present report, we provide further characterization of the biochemical properties of this enzyme relevant to 9-*cis*-retinoic acid formation and investigate the metabolism of 9-*cis*-retinoids by hepatic cells.

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[1] Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; cRDH, *cis*-retinol dehydrogenase; PGK, phosphoglycerate kinase; DTT, dithiothreitol; PVDF, poly(vinylidene difluoride); ECL, enhanced chemiluminescence; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; LRAT, lecithin:retinol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; REH, retinyl ester hydrolase; BSA, bovine serum albumin; 11cRDH, 11-*cis*-retinol dehydrogenase; ROL, retinol; RAL, retinal.

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EXPERIMENTAL PROCEDURES

Retinoids. *all-trans*-Retinal, 13-*cis*-retinal, 9-*cis*-retinal, *all-trans*-retinol, and 13-*cis*-retinol were purchased from Sigma. 11-*cis*-Retinal was a gift of Dr. Christian Eckhoff of Hoffmann-LaRoche, Inc. The internal standard, used to assess retinol and retinal recovery, was *all-trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (TMMP-ROH, Ro12-0586) (*25*), obtained from Dr. Louise H. Foley (Hoffmann-LaRoche, Inc). 9-*cis*-Retinol and 11-*cis*-retinol were prepared by reducing 9-*cis*-retinal and 11-*cis*-retinal, respectively, with $NaBH_4$ (Sigma) followed by purification by normal-phase high-performance liquid chromatography (HPLC) (*16*).

Cell Culture. L*X*SN/Hep G2 and L*RDH*SN/Hep G2 cells were cultured in α-modified Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). The HSC-T6 cells were cultured in Waymouth's medium supplemented with 10% FBS. All cells were cultured at 37 °C in 5% CO_2.

Endogenous Expression of cRDH by RT-PCR. RNA was extracted from parental Hep G2 cells and transduced Hep G2 cells (L*X*SN/Hep G2 and L*RDH*SN/Hep G2) by guanidine isothiocyanate/cesium chloride centrifugation following standard procedures (*26*). RNA from human liver was obtained from Dr. Ken Thummel (Department of Pharmaceutics, University of Washington). Reverse transcription (RT) was performed with 2 μg of total RNA with Superscript RT (Gibco) and random hexamers (Roche Molecular Biochemicals) at 42 °C for 1 h. The RT reaction (1 μL) was subsequently used for the PCR reaction. The cRDH primers were 587−605 (5′-GATCAACATCACCAGCGTC-3′) and 851−870 (5′-TGATGCGCTGTTGCATTTTC-3′) of the cDNA sequence (*16*), resulting in a 283 bp product. PCR conditions were in a total reaction volume of 25 μL with 4.8 pmol of each cRDH primer, 3 mM $MgCl_2$, 1.5 units of Taq polymerase (Roche Molecular Biochemicals), and 40 mM tetramethylammonium chloride (Sigma). Thermal cycling parameters were 94 °C for 5 min, (94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min) × 35 cycles, and 72 °C for 5 min. For a control, a housekeeping gene, phosphoglycerate kinase (PGK), was also amplified with primers reported by Sasaki et al. (*27*). The PCR conditions and thermal cycling conditions were the same as described above except for the annealing temperature of 55 °C. The resulting products for both the cRDH and PGK reactions (15 μL) were loaded onto a 1.5% agarose gel, separated by electrophoresis, and stained with ethidium bromide. The reverse image of UV-illuminated gel was obtained by the Stratagene Eagle-eye system, and densitometry was performed with NIH Image (version 1.60). Relative cRDH expression was determined by the ratio of cRDH (AU)/PGK (AU).

Transduction of Hep G2 Cells with cRDH. The full-length cRDH cDNA, including a polyadenylation signal, was directionally cloned from pcDNA3 (*16*) into the *Eco*RI and *Xho*I sites within the multiple cloning site of pL*X*SN (*28*) (Figure 1). Two packaging cell lines, PE501 and PA317, were used to generate viruses capable of infecting human cell lines following the method of Miller et al. (*29*). Viruses produced by PA317 cells with high titer and intact gene structure were used to infect Hep G2 cells, a human hepatocyte-derived cell line (*30*). The resulting cell lines are referred to as L*X*SN/Hep G2 (empty vector) and L*RDH*SN/

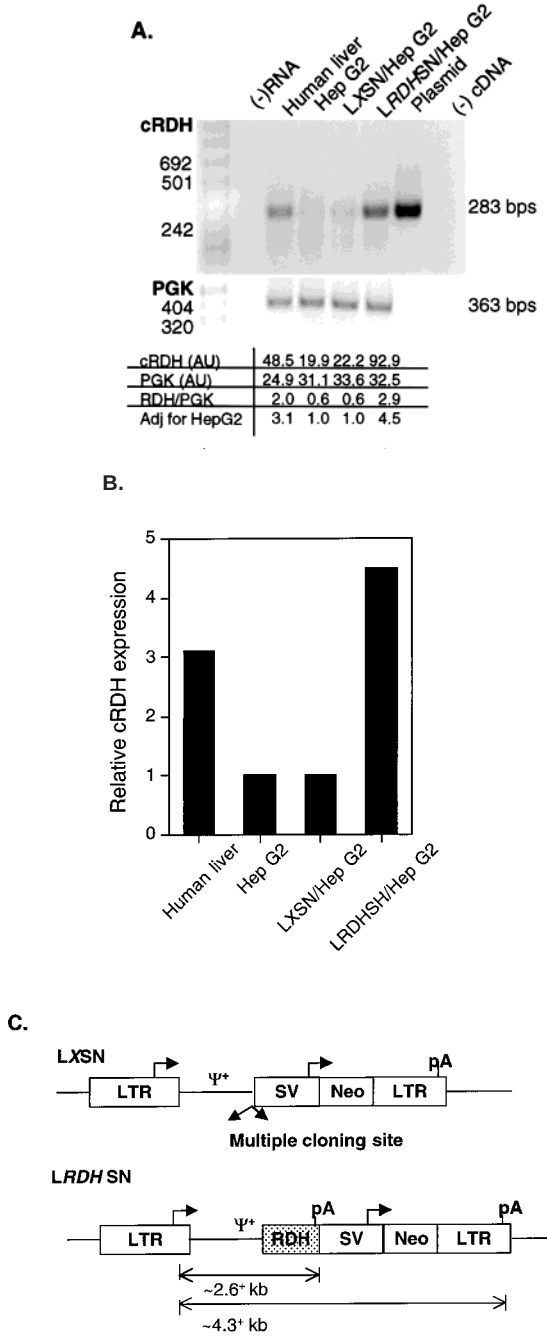

FIGURE 1: Expression of cRDH mRNA in human liver- and hepatocyte-derived cells and retroviral vector structures. (A) Total RNA (2 μg) of human liver, parental, and transduced Hep G2 cells was used for reverse transcription (RT) followed by PCR. cRDH primers were 587−605 (F) and 851−870 (R) of the cDNA sequence (upper gel). For a comparison, a housekeeping gene, PGK, was also amplified (lower gel) as described under Experimental Procedures. Negative controls were used for both RT (−mRNA) and PCR (−cDNA) reactions. A full-length cRDH plasmid was used as a positive control. Amplicons (15 μL each) were analyzed on 1.5% ethidium bromide-stained agarose gels. The reverse image of the UV-illuminated gel as detected by the Stratagene Eagle-eye system was analyzed by NIH image and expressed as arbitrary units (below the images). (B) Relative expression levels of cRDH were calculated by cRDH (AU)/PGK (AU), adjusted by the value of Hep G2 cells (arbitrarily = 1), and presented as a histogram. (C) Full-length cRDH cDNA, including its polyadenylation signal sequence, was cloned into L*X*SN. LTR, long terminal repeat; SV, simian virus 40 promoter; Neo, neomycin phosphotransferase; pA, polyadenylation signal. The expected sizes for mRNA transcripts of cRDH directed by the retrovirus are ~2.6 and 4.5 kb.

Hep G2. To ensure the success of the gene transduction, mRNA and protein expression were examined by Northern blot and Western blot analysis. The enzyme activity of the expressed protein was tested in vitro employing the assay procedure described below.

Northern Blot Analysis. RNA was extracted by guanidine isothiocyanate/cesium chloride centrifugation following standard procedures (*26*) from L*X*SN/Hep G2 and L*RDH*SN/Hep G2 cells, and 20 μg of total RNA was loaded onto 1.2% denaturing agarose gel and blotted onto a membrane (Zeta-probe membrane, Bio-Rad). A ^{32}P-labeled (α-dCTP) probe was prepared from the full-length cRDH cDNA by use of a Random prime labeling kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Hybridization was carried out overnight at 42 °C. The final wash was 2× SSC/0.1% SDS at 65 °C and the blot was exposed to X-ray film for autoradiography.

Western Blot Analysis. Microsomes of transduced Hep G2 cells were prepared by the method of Fleischer and Kervina (*31*) with some modification. Briefly, cells were grown to confluency on P 150 mm^2 tissue culture plates, harvested in a buffer containing 25 mM Tris, pH 7.4, 0.25 M sucrose, and 1 mM dithiothreitol (DTT), and homogenized with a Dounce homogenizer (B pestle) 50 times on ice followed by centrifugation at 12000*g* for 10 min at 4 °C. This process was repeated and supernatants were pooled and centrifuged at 100000*g* for 1 h at 4 °C. The resulting crude microsomal pellet was resuspended in 25 mM Tris, pH 7.4, and 1 mM DTT. Aliquots were snap-frozen in an ethanol−dry ice bath and stored at −70 °C. Protein concentration was determined by the Bradford assay (Bio-Rad) per the manufacturer's recommendation.

Crude microsomes (50 μg of protein) obtained from each cell line were loaded onto an SDS−15% polyacrylamide gel and, following electrophoresis, transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad) at 30 V overnight at 4 °C. Following incubation in 2% milk blocking solution for 1 h, the membrane was incubated with a rabbit-derived anti-polypeptide antibody (*22*) (1:5000 dilution) for 1 h at room temperature. After being washed with PBS containing 0.1% Tween 20, the membrane was incubated with a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2000 dilution, Santa Cruz) for 1 h at room temperature. The membrane was washed again as described above, and the signal was detected by enhanced chemiluminescent (ECL) reagent (Pierce) according to the manufacturer's instructions. Molecular weight standards were from Santa Cruz (sc-2035).

Enzyme Assays. Enzyme activity was measured by a modification of the method described by Mertz et al. (*16*). In a total reaction volume of 600 μL, total cell homogenates (100 μg of protein) of L*RDH*SN/Hep G2 or L*X*SN/Hep G2 were incubated at 37 °C for 1 h with 10 μM 9-*cis*-retinol (in ethanol) in the presence of 2 mM NAD^+ in a buffer containing 10 mM HEPES, pH 8.0, 150 mM KCl, and 2 mM EDTA (assay buffer). The total volume of ethanol used to dissolve 9-*cis*-retinol did not exceed 5% of the total reaction volume. Assays were carried out under the red light to minimize photoisomerization of retinoids. At the start of each assay, the reaction tubes were briefly flushed with N_2 before being sealed with Parafilm and covered with aluminum foil. These steps were taken to minimize contact with O_2 and light during incubation. At the end of the incubation, an equal volume of 100% ethanol (600 μL) was added to each reaction to denature the proteins and to stop the reaction. Retinol and retinal isomers were then extracted in 2.5 mL of hexane and analyzed by normal-phase HPLC as described below.

Retinoid Metabolism by Hepatocytes and Hepatic Stellate Cells. Both L*X*SN/Hep G2 human hepatoma cells and HSC-T6 rat hepatic stellate cells were employed to study the metabolism of 9-*cis*-retinoids by intact cells.

HSC-T6 stellate cells were isolated from male retired-breeder Sprague-Dawley rats as previously described (*32*) and maintained in primary culture on plastic culture dishes for 15 days in the presence of 10% fetal calf serum (FCS). On day 15 of culture, the stellate cells were transiently transfected with an expression plasmid containing the large T-antigen of SV40, and clones were isolated. One clone, designated HSC-T6, was expanded for further characterization. The use and characteristics of this cell line have been published (*33*). HSC-T6 stellate cells exhibit an activated phenotype as reflected in their fibroblastlike shape and rapid proliferation in culture. Also, the cells express cytoskeletal proteins including desmin, α smooth muscle actin, glial acidic fibrillary protein, and vimentin that are typical of activated stellate cells. Among the most striking features of hepatic stellate cells in vivo are the numerous vitamin A-rich lipid droplets that are present within the cytoplasm. When cultured in medium containing physiologic concentrations of retinol (2 mM), HSC-T6 stellate cells develop lipid droplets (*34*).

For studies of retinoid metabolism, medium was removed in the morning of the experiment and replaced with medium supplemented with different concentrations of either 9-*cis*-retinol, *all-trans*-retinol, or the combination. After predetermined times, ranging up to 5 h, the cells and medium were collected for extractions and HPLC analysis of retinyl ester, retinol, retinal, and/or retinoic acid isomer concentrations. For these measurements, cell pellets were homogenized in either 0.5 or 1.0 mL of PBS with a Polytron homogenizer (Brinkmann Instruments), and proteins were denatured through addition of an equal volume (0.5 or 1.0 mL) of 100% ethanol to the homogenate. Cell homogenates or medium was extracted with 2.5 mL of hexane, and the extracts were analyzed for retinol/retinal or retinyl ester concentrations by normal-phase HPLC. For retinoic acid measurements, cell homogenates and medium were extracted with 6 mL of chloroform/methanol (2:1 v/v) and the lower retinoid-containing chloroform phase was collected for normal-phase HPLC analysis carried out as described below.

Retinoid Analyses of Mouse Tissues. Concentrations of 9-*cis*-retinol and 9-*cis*-retinyl esters were measured in mouse serum and liver. For this purpose, 3-month-old male C57Bl/6J mice were exposed to CO_2, blood was collected by cardiac puncture, and the liver was dissected in a dark room under a red photographic light. The dissected livers were immediately frozen in liquid N_2 and stored for up to 1 week at −70 °C prior to HPLC analysis. Livers were homogenized using a Polytron homogenizer in 3 volumes of ice-cold PBS (25% w/v homogenate). To determine retinol concentrations, an aliquot of the homogenate was denatured with an equal volume of 100% ethanol and extracted twice into 5 mL of hexane. The two hexane extracts were combined and

evaporated to dryness under a gentle stream of N_2 followed by analysis by normal-phase HPLC for *all-trans*- and 9-*cis*-retinol as described below.

To assess *all-trans*- and 9-*cis*-retinyl esters, total *all-trans*- and 9-*cis*-retinol concentrations were determined after saponification of retinoids. The differences obtained from the measurement of total retinol and unesterified retinol were taken as retinyl esters. For total retinol measurements, an aliquot of the liver homogenate was extracted in 6 volumes of chloroform/methanol (2:1 v/v). The lower retinoid-containing chloroform phase was removed and evaporated to dryness under a gentle stream of N_2. The lipids were then redissolved in 2 mL of 50% (w/v) KOH in ethanol and allowed to incubate for 2 h at 65 °C. After the incubation, 2 mL of PBS was added and the saponified retinoids were extracted into 5 mL of hexane. The hexane extract was backwashed 5 times with 2 mL of PBS and evaporated to dryness under N_2. Concentrations of total *all-trans*- and 9-*cis*-retinol were determined by normal-phase HPLC.

HPLC Analysis. Retinol and retinal isomers were separated on a 4.6 × 150 mm Supelcosil LC-Si column (Supelco Inc.) preceded by a silica guard column (Supelco Inc.) with hexane/ethyl acetate/butanol (96.9:3:0.1 v/v/v) as the mobile phase and a flow rate of 0.8 mL/min. Isomers of retinol and retinal were detected by absorbance at 325 and 365 nm, respectively, on a Waters 996 Photodiode array detector (Waters 600E multisolvent delivery system, 717^+ autosampler, and Millennium^{32} software). Retinol and retinal peaks were identified by comparing retention times and spectra of experimental compounds to those of authentic standards. Each retinol and retinal isomer was quantitated by comparing its integrated area under the peak against those of known amounts of purified standards. The loss during extraction was accounted for by adjusting the recovery to that of the internal standard.

Retinyl ester concentrations were measured by normal-phase HPLC on the same silica columns described above but with hexane/diethyl ether (99.9:0.1 v/v) as the mobile phase at a flow rate of 0.8 mL/min (*35*). Retinoic acid isomer levels for cells and medium were also assessed by normal-phase HPLC employing two tandem silica columns (a 3.9 × 150 mm Resolve Silica column, Waters and a 4.6 × 150 mm Supelcosil LC-Si column, Supelco Inc.) and hexane/acetonitrile/acetic acid (99.5:0.4:0.1 v/v/v) as the running solvent (1.8 mL/min flow rate) (*36*).

Enzymatic Assays for Retinol Esterification and Retinyl Ester Hydrolase Activities. We employed standard assays to assess hepatic lecithin:retinol acyltransferase (LRAT), acyl-CoA:retinol acyltransferase (ARAT), and retinyl ester hydrolase (REH) activities (*37*−*39*). The enzymatic assays for ARAT and LRAT were carried out as described by Ross (*38*) and Randolph and Ross (*37*), respectively. Briefly, for the ARAT assay, cells were homogenized in 0.15 M potassium phosphate buffer, pH 7.4, with a Dounce homogenizer. The homogenate was centrifuged at 40 000 rpm in a Beckman TC-100 ultracentrifuge for 60 min at 4 °C to obtain a crude microsomal fraction. The crude microsomes were resuspended in 0.15 M potassium phosphate buffer, pH 7.4. To distinguish between retinyl esters formed during the in vitro assay and retinyl esters endogenous to the crude microsomal fraction, we used *n*-heptadecanyl-coenzyme A as the fatty acyl-CoA. Retinyl esters containing this fatty acyl group are not endogenously present in cells. For the assay (total volume 0.5 mL), 500 μg of microsomal protein was incubated for 1 h at 37 °C with 100 μM *n*-heptadecyl-CoA, 120 μM *all-trans*- or 9-*cis*-retinol in 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM DTT and 20 μM bovine serum albumin (BSA). For the LRAT assay, cells were homogenized in 150 mM potassium phosphate buffer, pH 7.25, and crude microsomes were obtained as described above. The microsomal protein (250 μg) was incubated with 5 μM of either *all-trans*- or 9-*cis*-retinol in a buffer containing 20 μM BSA and 5 mM DTT for 1 h at 37 °C. The enzymatic reaction was stopped with an equal volume of ice-cold ethanol and the retinoids were extracted in either 2 volumes of hexane (for ARAT) or 2.5 mL of hexane (for LRAT) and analyzed by normal-phase HPLC (see above). A control lacking either retinol isomer was included for both the ARAT and LRAT assays. In preliminary experiments prior to carrying out measures of LRAT and ARAT specific activities in microsomes prepared from HSC-T6, L*X*SN/Hep G2, and L*RDH*SN/Hep G2 cells, we established for both LRAT and ARAT, employing our assay conditions, that rates of retinyl ester formation were linear with respect to both time of incubation and microsomal protein concentration. Moreover, by varying the assay concentrations of retinol (both the *all-trans*- and 9-*cis*-isomers) for these trials, we established that the rate of product formation (retinyl ester) was independent of substrate concentration (i.e., was at substrate saturation).

Retinyl ester hydrolase (REH) assays were carried out on unfractionated homogenates prepared in 10 mM Tris-HCl, pH 8.0, with a Dounce homogenizer. Assays were carried out in 50 mM Tris−maleate buffer, pH 8.0, in the presence and absence of 1% (w/v) sodium taurocholate as described by Friedman et al. (*39*). The substrates for these assays consisted of either 10 μM *all-trans*-retinyl oleate or 10 μM 9-*cis*-retinyl oleate that had been synthesized through reaction of an individual retinol isomer with oleyl chloride in the presence of pyridine catalyst (*40*). Reaction mixtures (0.2 mL) were incubated for 1 h at 37 °C followed by addition of an equal volume of ice-cold ethanol to terminate the reaction. Extraction and analysis of retinoids were done as described above.

Determination of the Equilibrium Constant of Human cRDH. The equilibrium constant of cRDH at 37 °C and under our standard assay conditions was determined for 9-*cis*-retinal formation. For this purpose, we employed an approach previously used to calculate the equilibrium constant of bovine rod outer segment *all-trans*-retinol dehydrogenase (*41*). Briefly, the assay we routinely used to measure 9-*cis*-retinal formation was allowed to go to completion (2 h incubation). From the final concentrations of 9-*cis*-retinol and 9-*cis*-retinal measured by HPLC, the known initial concentrations of substrates, and the final pH, it is possible to calculate an equilibrium constant for the reaction. That a state of equilibrium had been established was verified through demonstration that addition of fresh cRDH had no effect on the final 9-*cis*-retinol and 9-*cis*-retinal concentrations. Addition of fresh substrate (9-*cis*-retinol and NAD^+) to the equilibrium mixture restarted the reaction, indicating the presence of active cRDH at equilibrium.

Production of 9-cis-Retinal. In some experiments, we explored the contribution of cRDH to the synthesis of 9-*cis*-

retinoic acid via oxidation of 9-*cis*-retinol to 9-*cis*-retinal. For these experiments, 1 μCi of *all-trans*-[^{3}H]retinol was incubated with either L*RDH*SN/Hep G2 or L*X*SN/Hep G2 cell homogenate (100 μg) or assay buffer alone in the presence of 2 mM NAD^+. The final reaction volume was 0.6 mL and the final concentration of *all-trans*-retinol was 0.03 μM. Immediately following incubation, 0.6 mL of ice-cold ethanol was added to the incubation mixture and retinoids were extracted into 2.5 mL of hexane. The presence of 9-*cis*-retinol and 9-*cis*-retinal in the reaction mixture was assessed by normal-phase HPLC as described above.

cRDH Enzyme Kinetics. Microsomes of L*RDH*SN/Hep G2 cells (50 μg) were incubated at 37 °C for 5 min with either 9-*cis*-retinol or 11-*cis*-retinol (in 100% ethanol) at various concentrations (0, 1, 2, 3, 4, 5, 7.5, 10, or 15 μM) in the assay buffer containing 2 mM NAD^+. Delipidated BSA (60 μM) (Sigma) was added to each reaction to ensure reproducible kinetics (*14*). Extraction and analysis of retinol and retinal were performed as described above.

RESULTS

Expression of cRDH in Hep G2 Cells. We wished to study the possible actions of cRDH in catalyzing the first oxidation step needed for the formation of 9-*cis*-retinoic acid from 9-*cis*-retinol. We previously employed cRDH expressed in CHO cells as a source of recombinant cRDH to study the biochemical properties of the enzyme (*16*). However, CHO cells lack endogenous expression (unpublished observation) and may not be a relevant physiologic context to study cRDH activity. We were interested in studying cRDH in a cellular environment where it would normally be present. Consequently, we expressed cRDH in Hep G2 cells. As seen in Figure 1A, RT-PCR analysis indicates that cRDH mRNA is expressed endogenously, albeit at low level, in Hep G2 cells. cRDH mRNA expression levels in parental Hep G2 cells as well as L*X*SN/Hep G2 cells were lower than that of a human liver tissue, approximately $^1/_3$ the expression level. The expression levels of cRDH in cRDH-transduced Hep G2 cells were similar to that of human liver. Potential amplification of genomic DNA was ruled out by using primers that span an intron of about 1.9 kb (*42*). No genomic DNA band (~2.2 kb) was detected.

Transduction of Hep G2 Cells with cRDH. Since the level of endogenous cRDH activity present in Hep G2 cells is very low (Figure 2C), and we wanted to characterize some of the biochemical properties of this enzyme in a cellular environment, we overexpressed cRDH in Hep G2 cells. This was accomplished by transducing Hep G2 cells with a retroviral vector containing the full-length cRDH cDNA, L*RDH*SN (Figure 1B). As a negative control, empty vector- (L*X*SN-) transduced Hep G2 cells were also created. To examine the success of the gene transduction, expression of mRNA and protein and enzyme activity of cRDH were analyzed by Northern blot analysis (Figure 2A), Western blot analysis (Figure 2B), and in vitro enzyme assay (Figure 2C), respectively.

Upon hybridization of Northern blot with ^{32}P-labeled full-length cRDH probe, the expected transcript sizes of 2.6 and 4.5 kb, resulting from two known polyadenylation sites (Figure 1B), were detected only in L*RDH*SN/Hep G2 cells (Figure 2A). No endogenous 1.4 kb cRDH transcript could be detected in the L*RDH*SN/Hep G2 cells or the L*X*SN/Hep G2 cells.

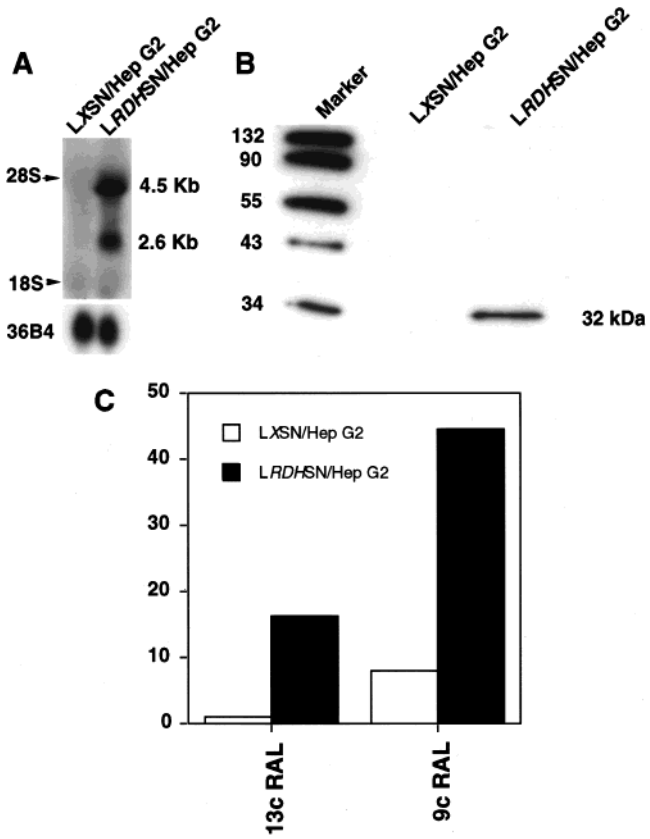

FIGURE 2: Expression and activity of cRDH in transduced Hep G2 cells. (A) Northern blot analysis of total RNA shows the expected size of mRNA transcripts (4.5 and 2.6 kb) from L*RDH*SN/Hep G2 (B) Immunoblot analysis of microsomes of transduced Hep G2 cells. An expected ~32 kDa protein was detected only from microsomes of LRDHSN/Hep G2 cells. (C) Whole-cell homogenates (100 μg) of L*X*SN/Hep G2 and L*RDH*SN/Hep G2 were analyzed for cRDH activity. L*RDH*SN/Hep G2 cells produced approximately 6 times more 9-*cis*-retinal compared to L*X*SN/Hep G2 cells. Detection of 13-*cis*-retinal was attributed to the isomerization from 9-*cis*-retinal.

To confirm expression of cRDH protein, an immunoblotting assay was performed with a polyclonal antipeptide antibody (*22*). As expected, we detected an approximate 32 kDa protein band in microsomal fractions obtained from L*RDH*SN/Hep G2 cells but not in microsomes from L*X*SN/Hep G2 cells (Figure 2B). cRDH protein was not detected from cytosolic fractions of either cell lines (data not shown). Multiple Western blot analyses of microsomal protein from L*X*SN/HepG2 cells did show a faint immunoreactive band that migrated at approximately 32 kDa, indicating that a low, endogenous level of cRDH protein is present in Hep G2 cells (data not shown). This is consistent with RT-PCR analysis showing expression of endogenous cRDH mRNA in non-transduced Hep G2 cells (Figure 1A).

To ascertain whether the cRDH protein expressed in Hep G2 cells was catalytically functional, an in vitro enzyme activity assay was performed. Whole-cell homogenates (100 μg of protein) of L*X*SN/Hep G2 or L*RDH*SN/Hep G2 were incubated with 10 μM 9-*cis*-retinol and 2 mM NAD^+ at 37 °C. Following a 60 min incubation period, retinoids were extracted and subjected to HPLC analysis. L*RDH*SN/Hep G2 cells possessed approximately 6-fold more cRDH-specific

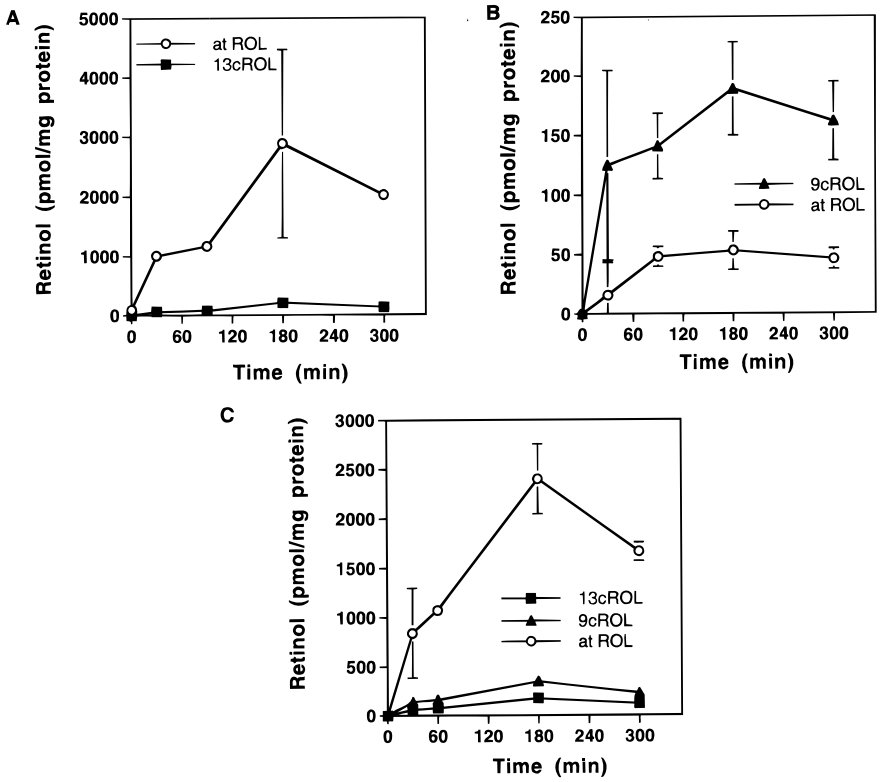

FIGURE 3: Retinol uptake by HCS-T6 cells. HSC-T6 cells were grown in the medium supplemented with either (A) *all-trans*-retinol (2.5 μM), (B) 9-*cis*-retinol (2.5 μM), or (C) the combination (2.5 μM each) over 5 h. Retinol levels associated with HSC-T6 cells were analyzed by HPLC at predetermined time points. Each data point represents a mean from triplicate plates. Note the scale differences in *y* axis among graphs.

activity than the L*X*SN/Hep G2 cells [1.55 vs 0.27 nmol h^{-1} (mg of protein)$^{-1}$] (Figure 2C).

Metabolism of 9-cis-Retinol by Human Hep G2 Hepatocytes and Rat Liver HSC-T6 Stellate Cells. Little is known about the presence and metabolism of 9-*cis*-retinoids within cells and tissues. It has been established that 9-*cis*-retinol is present, albeit at relatively low levels, in rat liver (*20*) and rat kidney (*15*), but as far as we are aware there has been no systematic study focusing on the metabolism of 9-*cis*-retinoids within these or other tissues. It is presently not known whether cells can take up 9-*cis*-retinol and maintain the stereochemical integrity of this retinol isomer. Moreover, there is limited information available regarding whether 9-*cis*-retinol can be esterified in cells to 9-*cis*-retinyl esters or whether 9-*cis*-retinyl esters can be hydrolyzed by cellular hydrolases to 9-*cis*-retinol. Our studies were designed to explore these possibilities. Since the substrate, 9-*cis*-retinol, and the enzymes that are capable of oxidizing 9-*cis*-retinol (*16, 18, 19*) are present in the liver, we chose to carry out our studies in liver cells, Hep G2 human hepatoma cell line, and HSC-T6, rat liver stellate cells (also called fat-storing cells, Ito cells, or lipocytes).

To study its intracellular metabolism, both Hep G2 and HSC-T6 cells were loaded with 9-*cis*-retinol. For cell loading, 9-*cis*-retinol was added to the tissue culture medium. Since we did not know whether the hepatic-derived cells have the ability to remove 9-*cis*-retinol from medium and maintain the *cis* configuration, a parallel study was carried out with *all-trans*-retinol. Intracellular levels of both *all-trans*- and 9-*cis*-retinol increased with time and retinol concentration in the two hepatic cell types. The time dependence of *all-trans*-retinol and 9-*cis*-retinol accumulation in HSC-T6 cells is shown in Figure 3, panels A and B, respectively. The cellular level of *all-trans*-retinol in HSC-T6 cells is approximately 20-fold greater than that of 9-*cis*-retinol. Relatively little of the *all-trans*-retinol associated with the HSC-T6 cells was isomerized to 9-*cis*-retinol during the experimental period. Approximately 25% of the 9-*cis*-retinol accumulated in HSC-T6 cells underwent isomerization to *all-trans*-retinol, but over the 5 h duration of this experiment, the relative amount of 9-*cis*-retinol associated with the HSC-T6 cells did not appreciably change with time (approximately 75% of the total retinol associated with the HSC-T6 cells remained in the 9-*cis* configuration throughout the experiment). This implies that the 9-*cis* configuration of retinol is stable within hepatic cells. Interestingly, as can be seen in Figure 3C, when equimolar concentrations of *all-trans*-retinol (2.5 μM) and 9-*cis*-retinol (2.5 μM) were provided together in the medium of HSC-T6 cells, the levels of *all-trans*- and 9-*cis*-retinol detected in the cells were not different from those observed when each retinol isomer was added individually to the culture medium.

L*X*SN/Hep G2 cells accumulate less *all-trans*-retinol from the culture medium compared to HSC-T6 cells (Figure 4A). After 5 h of incubation, *all-trans*-retinol concentrations in Hep G2 cells reached approximately 500 pmol/mg of protein (compared to 2000 pmol/mg of protein in HSC-T6 cells). The level of 9-*cis*-retinol detected in Hep G2 cells was very similar to that observed for HSC-T6 cells (Figure 4B) (160 vs 180 pmol/mg of protein, respectively). As was the case for HSC-T6 cells, approximately 25% of the total retinol associated with the Hep G2 cells exposed to 9-*cis*-retinol was present as *all-trans*-retinol (Figure 4B). However, unlike HSC-T6 cells, when equimolar concentrations of both *all-*

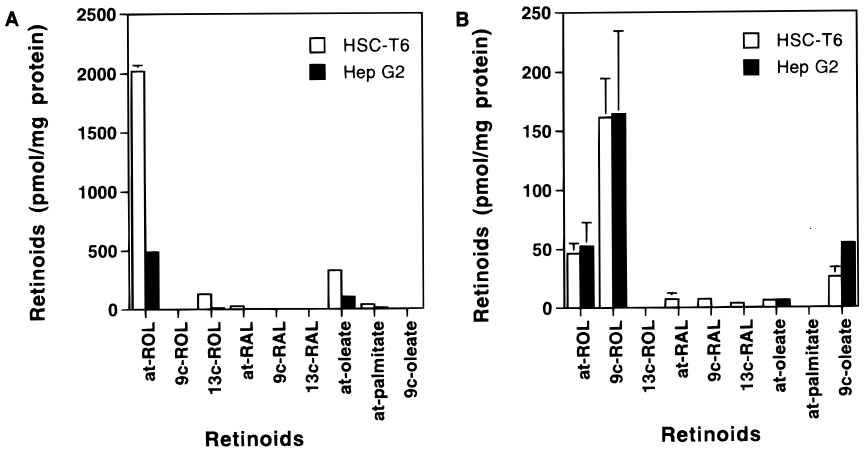

FIGURE 4: Retinoid metabolites by HSC-T6 and L*X*SN/Hep G2 cells. HSC-T6 and L*X*SN/Hep G2 cells were cultured in the medium containing (A) *all-trans*-retinol (2.5 μM) or (B) 9-*cis*-retinol (2.5 μM) for 5 h. Retinoids associated with cells were analyzed by HPLC. Results are from triplicate plates.

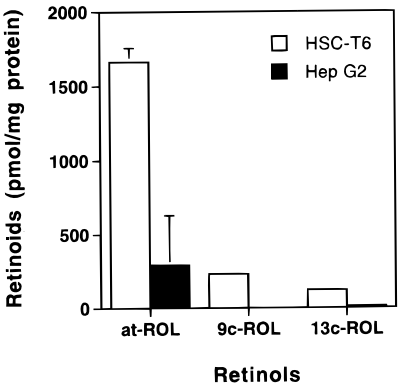

FIGURE 5: Retinol profiles in HSC-T6 and L*X*SN/Hep G2 treated with the combination of *all-trans*-retinol and 9-*cis*-retinol. HSC-T6 and L*X*SN/Hep G2 cells were cultured in medium containing both *all-trans*-retinol (2.5 μM) and 9-*cis*-retinol (2.5 μM) for 5 h. Concentrations of retinol isomers in cells were measured by HPLC analysis. Results represent the mean data from triplicate plates.

trans- and 9-*cis*-retinol were added to the L*X*SN/Hep G2 culture medium, only *all-trans*-retinol was found to be associated with the L*X*SN/Hep G2 cells (Figure 5).

Over the 5 h time period, both *all-trans*- and 9-*cis*-retinol were observed to undergo significant metabolism to retinal and retinyl esters within the HSC-T6 and L*X*SN/Hep G2 cells. The distributions of retinol, retinal, and retinyl ester metabolites associated with HSC-T6 cells and L*X*SN/Hep G2 cells following a 5 h incubation with medium supplemented with either *all-trans*-retinol (2.5 μM) or 9-*cis*-retinol (2.5 μM) are given respectively in Figure 4 panels A and B. Significant amounts of 9-*cis*-retinyl esters were formed upon addition of 9-*cis*-retinol to either L*X*SN/Hep G2 or HSC-T6 cells. Thus, not only can 9-*cis*-retinol accumulate within cells without loss of its geometric configuration but also it can be processed by the cells to 9-*cis*-retinyl ester, a putative storage form for this retinol isomer.

Since both hepatocyte- and stellate-derived cells were found to esterify 9-*cis*-retinol to 9-*cis*-retinyl esters, we measured in vitro the levels of retinol esterifying activities (for both *all-trans*- and 9-*cis*-isomers) associated with Hep G2 and HSC-T6 cells. Since two microsomal enzymes, ARAT and LRAT, have been proposed to participate in retinol esterification in liver cells (*37, 38, 43*), the specific activities of both LRAT and ARAT were measured in crude microsomes prepared from Hep G2 and HSC-T6 cells by standard assay procedures for the two enzymatic activities (*37, 38*). In preliminary studies carried out prior to these assays, we established that our assay conditions gave rates of product formation that were linear with respect to both time and microsomal protein concentration. In addition, we established in these preliminary experiments that the substrate (*all-trans*- and 9-*cis*-retinol) concentrations that we employed in the assays were saturating (giving rise to zero-order kinetics). Both LRAT and ARAT activities were observed to be present in the HSC-T6, L*X*SN/Hep G2, and L*RDH*SN/Hep G2 cells. *all-trans*-Retinol proved to be a better substrate for both the LRAT and ARAT activities present in each of these different cell lines. For the HSC-T6 cells, the LRAT activities directed against *all-trans*- and 9-*cis*-retinol were, respectively, 4.58 ± 0.30 and 0.42 ± 0.06 nmol of retinol consumed h^{-1} (mg of protein)$^{-1}$ (mean $\pm$ 1 standard deviation), whereas, for the HSC-T6 cells, the mean ARAT activities directed against *all-trans*- and 9-*cis*-retinol were, respectively, 1.69 ± 0.29 and 0.12 ± 0.03 nmol of retinol consumed h^{-1} (mg of protein)$^{-1}$. As we expected, we observed no significant differences for the specific activities that we determined for LRAT and ARAT between the L*X*SN/Hep G2 and L*RDH*SN/Hep G2 cell lines. Thus, a high level of expression of cRDH in the Hep G2 cells does not influence the levels of esterifying activities within the cells. For the L*X*SN/Hep G2 and L*RDH*SN/Hep G2 lines, mean LRAT specific activities toward the *all-trans*- and 9-*cis*-isomers of retinol averaged, respectively, 2.50 ± 0.34 and 1.72 ± 0.18 nmol of retinol consumed h^{-1} (mg of protein)$^{-1}$ (for L*X*SN/Hep G2 cells) and 2.61 ± 0.03 and 1.76 ± 0.05 nmol of retinol consumed h^{-1} (mg of protein)$^{-1}$ (for L*RDH*SN/Hep G2 cells). The microsomal hepatocyte ARAT activity showed a marked preference for the *all-trans*-retinol isomer over the 9-*cis*-isomer. For L*X*SN/Hep G2 and L*RDH*SN/Hep G2 cells, mean ARAT specific activities toward *all-trans*- and 9-*cis*-retinol were determined to be respectively, 1.83 ± 0.16 and 0.13 ± 0.01 nmol of retinol consumed h^{-1} (mg of protein)$^{-1}$ (for L*X*SN/Hep G2 cells) and 1.61 ± 0.02 and 0.13 ± 0.01 nmol of retinol consumed h^{-1} (mg of protein)$^{-1}$ (for L*RDH*SN/Hep G2 cells).

We similarly asked whether HSC-T6 and Hep G2 cells are capable of the enzymatic hydrolysis of *all-trans*- and

9-*cis*-retinyl esters. Since both bile salt-dependent and bile salt-independent retinyl ester hydrolases may play roles in the hydrolysis of hepatic retinyl esters (*43*), we carried out hydrolase assays in the presence and absence of 1% (w/v) sodium cholate. We were able to measure both bile salt-independent and bile salt-dependent retinyl ester hydrolase activities for microsomes prepared from HSC-T6, L*X*SN/Hep G2, and L*RDH*SN/Hep G2 cells for both *all-trans*- and 9-*cis*-retinyl oleate. Interestingly, the specific activities of the bile salt-dependent retinyl ester hydrolase directed against *all-trans*- and 9-*cis*-retinyl esters were not statistically different for the three cell lines. Thus, it would appear that the bile salt-dependent retinyl ester hydrolase activity present in each of these cell lines does not show a strong substrate preference for one isomeric configuration over another. The bile salt-independent retinyl ester hydrolase activities for the HSC-T6, L*X*SN/Hep G2, and L*RDH*SN/Hep G2 each showed a slight substrate preference for *all-trans*-retinyl oleate over the 9-*cis*-isomer. On the basis of these in vitro measures, we conclude that HSC-T6 stellate cells and Hep G2 hepatocytes possess the enzymatic machinery needed to hydrolyze both *all-trans*- and 9-*cis*-retinyl esters. This observation is consistent with our earlier observation that each of these cultured hepatic cell lines is able to take up and process both *all-trans*- and 9-*cis*-retinol.

Endogenous Level of Retinoids in Tissue. Data obtained from the study of cultured liver cells suggest that the liver has the capacity for accumulating and metabolizing 9-*cis*-retinol. However, there is limited information regarding 9-*cis*-retinol and/or 9-*cis*-retinyl ester levels in tissues of living organisms. To address this, we investigated the levels of 9-*cis*-retinol and 9-*cis*-retinyl ester present in serum and liver of 3-month-old fasting male mice. We could not detect 9-*cis*-retinol or 9-*cis*-retinyl esters in mouse serum. The lower limit of detection for these HPLC-based assays was less than 3 nM for 9-*cis*-retinol and 9-*cis*-retinyl esters. Thus, it would appear that neither 9-*cis*-retinol nor 9-*cis*-retinyl esters are present in the blood of fasted mice or are present at low levels. However, 9-*cis*-retinol and 9-*cis*-retinyl esters were detected in all livers obtained from these mice. As reported previously, the mean concentration of 9-*cis*-retinol measured from six livers was 0.42 ± 0.18 μg/g of liver and that of *all-trans*-retinol for the same livers was 28.5 ± 3.5 μg/g of liver (*22*). Here we report that the total 9-*cis*-retinol concentration (9-*cis*-retinol + 9-*cis*-retinyl esters) for these six livers was 2.31 ± 0.77 μg/g of liver and that of total *all-trans*-retinol (*all-trans*-retinol + *all-trans*-retinyl esters) was 898.5 ± 141.2 μg/g of liver. It is clear from these data that some endogenous 9-*cis*-retinol and 9-*cis*-retinyl ester is present in normal mouse liver, albeit at levels that are only approximately 0.25% of those of the all-trans isomers.

9-cis-Retinoic Acid Synthesis by LRDHSN/Hep G2 Cell Homogenates. In our studies of 9-*cis*-retinol metabolism, we observed some 9-*cis*-retinoic acid formation in Hep G2 cells. Since it is generally thought that the first step in retinoic acid biosynthesis (i.e., retinol oxidation) is rate-limiting, we wanted to assess whether changes in levels of cRDH activity in the Hep G2 cells would influence cellular 9-*cis*-retinoic acid levels. To test this, total cell homogenates from L*X*SN/Hep G2 and L*RDH*SN/Hep G2 were incubated with 10 μM 9-*cis*-retinol for 1 h and the amount of 9-*cis*-retinoic acid formed was measured by HPLC. Surprisingly, the amount of cRDH-specific activity present in the homogenates did not influence the amount of 9-*cis*-retinoic acid generated from 9-*cis*-retinol (data not shown). We then examined Hep G2 cells for the capacity to oxidize 9-*cis*-retinal when different concentrations of 9-*cis*-retinal were incubated with L*RDH*SN/Hep G2 cell homogenates. For this experiment the amount of 9-*cis*-retinoic acid formed was found to be dependent on time and 9-*cis*-retinal concentration (data not shown). Thus, Hep G2 cells are able to form 9-*cis*-retinoic acid from 9-*cis*-retinal. Previously, Bhat and colleagues (*15*) reported that 9-*cis*-retinol can act as a potent in vitro inhibitor of the rat kidney retinal dehydrogenase, with an apparent K_i of 5.4 μM. Thus, we asked whether the concentration of 9-*cis*-retinol would influence 9-*cis*-retinoic acid formation from 9-*cis*-retinal in Hep G2 cell homogenates. As can be seen in Figure 6, for two different 9-*cis*-retinal concentrations, the amount of 9-*cis*-retinol added to the assay markedly influenced the rate of 9-*cis*-retinoic acid formation. In good agreement with the K_i value for 9-*cis*-retinol reported by Bhat and colleagues (*15*), approximately 50% of the apparent 9-*cis*-retinal dehydrogenase activity was inhibited upon addition of 5 μM 9-*cis*-retinol. Thus, it would appear that 9-*cis*-retinol can act as a potent regulator of 9-*cis*-retinoic acid formation through action on the 9-*cis*-retinal dehydrogenase present in Hep G2 cells.

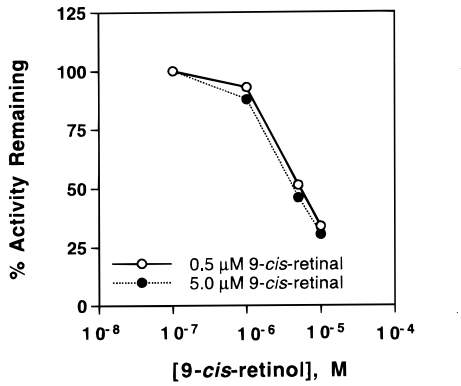

FIGURE 6: 9-*cis*-Retinol is a potent inhibitor of 9-*cis*-retinal oxidation. Whole-cell homogenates (100 μg) of L*X*SN/Hep G2 were incubated for 20 min with various concentrations of 9-*cis*-retinol in the presence of two fixed concentrations (0.5 μM or 5 μM) of 9-*cis*-retinal. 9-*cis*-Retinal oxidation was inhibited by 50% with 5 μM 9-*cis*-retinol. Each data point is a mean of duplicate reactions.

Equilibrium Constant of Human cRDH. In our preliminary experiments designed to establish assay conditions that give linear rates of 9-*cis*-retinal formation with respect to time, substrate, and cRDH (enzyme) concentrations, we were struck by the tendency of cRDH to catalyze 9-*cis*-retinol oxidation to near completion. Essentially, we observed that if reactions were incubated for a sufficiently long period, nearly all of the substrate, 9-*cis*-retinol, was converted to 9-*cis*-retinal. To better define this observation, we calculated the equilibrium constant for the oxidation reaction catalyzed by cRDH for our standard assay conditions at 37 °C. The equilibrium constant for the reaction catalyzed by cRDH was determined to be 4.9×10^{-9} M. Thus, for human cRDH under our assay conditions at pH 8.0 and 37 °C, the equilibrium concentration of 9-*cis*-retinal is nearly 100 times greater than that of 9-*cis*-retinol.

9-cis-Retinal Synthesis from all-trans-Retinol. Since in vivo liver 9-*cis*-retinol levels are very low compared to those

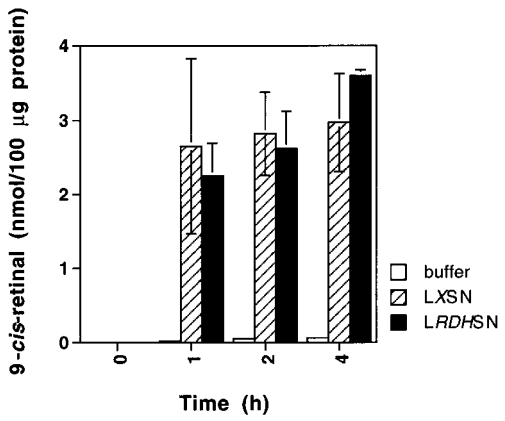

FIGURE 7: 9-*cis*-Retinal synthesis from *all-trans*-retinol. [^{3}H]-*all-trans*-Retinol (30 nM) was incubated with either L*X*SN or L*RDH*SN/Hep G2 cell homogenates for indicated time period. Formation of 9-*cis*-retinal was detected by normal-phase HPLC. Results are from triplicate reactions.

of *all-trans*-retinol (see above), we wondered whether cRDH participates in production of 9-*cis*-retinoic acid by efficiently oxidizing available 9-*cis*-retinol to 9-*cis*-retinal. We previously have demonstrated that 9-*cis*-retinol can be formed in a time- and protein-dependent manner when *all-trans*-retinol is incubated in the presence of CHO cell homogenate (*22*). Hence, we wanted to understand whether 9-*cis*-retinol, upon its formation from *all-trans*-retinol, will immediately be transformed to 9-*cis*-retinal in the presence of cRDH. If this were the case, then liver 9-*cis*-retinol levels would never need to be as great as those of *all-trans*-retinol in order for 9-*cis*-retinoic acid formation to occur. To test this hypothesis, we carried out assays in which *all-trans*-[^{3}H]retinol (1 μCi, 0.03 μM final concentration) was incubated in the presence of cell homogenates of L*RDH*SN/Hep G2 or L*X*SN/Hep G2 cells and 2 mM NAD^+. For this experiment, we chose a concentration of free *all-trans*-retinol (i.e., not bound to cellular retinol-binding protein, type I) that is thought to be present within the liver (*44*). As seen in Figure 7, 9-*cis*-retinal is formed from *all-trans*-retinol in the presence of either L*RDH*SN/Hep G2 or L*X*SN/Hep G2 cell protein but not in buffer alone. We observed nearly equal quantities of 9-*cis*-retinal formation for the two cell homogenates. This could arise if the conditions used in our experiments were sufficient to allow for an equilibrium or a state of near equilibrium to be reached. Since the concentration of enzyme does not influence equilibrium concentrations of reactants or products, this could account for why we observed equal concentrations of 9-*cis*-retinal for both L*RDH*SN/Hep G2 and L*X*SN/Hep G2 homogenates. Interestingly, for incubations involving both L*RDH*SN/Hep G2 and L*X*SN/Hep G2 homogenates but not buffer alone, a small amount of 9-*cis*-retinol was also detected in the incubation mixtures, indicating that 9-*cis*-retinol was being formed in a protein dependent manner from *all-trans*-retinol (data not shown).

Enzyme Substrate Preference and Kinetic Studies. The recent literature on cRDH suggests that the cRDH enzyme is identical to an enzyme, 11-*cis*-retinol dehydrogenase (11cRDH), that was originally reported to be expressed solely in the eye (*6, 16, 45, 46*). A recent study by Driessen et al. (*17*) indicates that recombinant mouse 11cRDH utilizes 9-*cis*-retinol as a substrate and that this enzyme, which was once thought to be restricted to the eye, is expressed in a variety of tissues outside of the eye. We asked the inverse of the question posed by Driessen et al. (*17*): will recombinant cRDH expressed in a hepatic cell line catalyze the oxidation of both 9-*cis*- and 11-*cis*-retinol with equal or near equal facility? To address this question, kinetic studies were performed with a crude microsome fraction obtained from L*RDH*SN/Hep G2 cells. Microsomal proteins (50 μg) were incubated with either 0, 1, 2, 3, 4, 5, 7.5, 10, or 15 μM 9-*cis*- or 11-*cis*-retinol for 5 min at 37 °C. Reactions were performed in duplicate for each substrate concentration. Each experiment was repeated at least three times. For different microsomal preparations, the calculated apparent K_m and V_{max} values for 9-*cis*- and 11-*cis*-retinol were reproducible. A representative result is shown in Figure 8. For both substrates, the enzymatic activity follows Michaelis−Menten kinetics, increasing linearly at the lower substrate concentrations but reaching a plateau (zero-order kinetics) for substrate concentrations exceeding approximately 5 μM. The calculated apparent K_m and V_{max} values for 9-*cis*-retinol were determined by Lineweaver−Burk plots to be approximately 0.44 μM and 0.126 nmol mg^{-1} min^{-1}, and for 11-*cis*-retinol, 1.1 μM and 0.06 nmol mg^{-1} min^{-1}, respectively. Thus, cRDH expressed in human Hep G2 hepatocytes will catalyze the oxidation of both 9-*cis*- and 11-*cis*-retinol with near equal affinity. This observation is consistent with the notion that cRDH and 11cRDH are the same enzyme.

DISCUSSION

Compared to the explosive development in understanding the actions of retinoid nuclear receptors, relatively little is firmly established regarding how their ligands, *all-trans*- and 9-*cis*-retinoic acid, are synthesized in vivo or how their levels are regulated in retinoid target tissues. It is generally accepted that *all-trans*-retinoic acid is formed by two consecutive oxidation events of *all-trans*-retinol (*11, 43, 47*−*49*), analogous to ethanol metabolism. Like ethanol oxidation, the rate-limiting step for this process is thought to be the first oxidation reaction, from which all-*trans*-retinal is generated. Several laboratories have recently cloned the cDNAs and genes of *cis*-retinol dehydrogenases that are capable of metabolizing *cis*-retinols to their aldehydes (*16*−*19, 24, 45, 46*). The identification of these enzymes argues for the existence of a pathway for 9-*cis*-retinoic acid synthesis in vivo, in which 9-*cis*-retinol is converted to 9-*cis*-retinoic acid by two-step oxidation, similar to *all-trans*-retinoic acid synthesis. For this scenario, the first oxidation step is proposed to be carried out by a *cis*-retinol-specific dehydrogenase(s), followed by a second step catalyzed by a retinal dehydrogenase that is capable of oxidizing either 9-*cis*-retinal or both *all-trans*- and 9-*cis*-retinals to the appropriate retinoic acid isomer (*15*).

Aside from the metabolism of 11-*cis*-retinoids in the eye, where 11-*cis*-retinal is the necessary visual pigment (*6*), very little information is available concerning how *cis*-retinoids are processed by cells and tissues. The existence of three distinct *cis*-retinol-specific dehydrogenases that are relatively widely distributed in different tissues (*16*−*19, 24*) and of retinal dehydrogenases able to catalyze 9-*cis*-retinal oxidation (*15*) underscores the importance of understanding how *cis*-retinoids are processed within nonocular tissues. Specifically, the existence of these enzymes raises a question as to if,

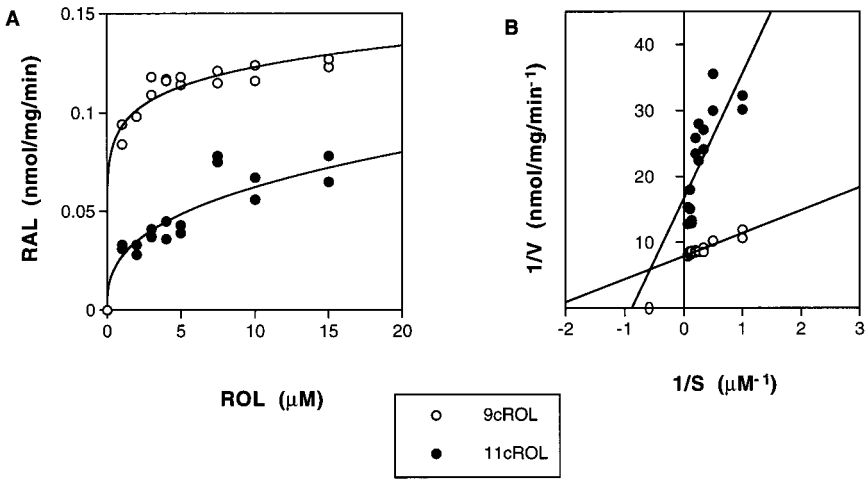

FIGURE 8: cRDH enzyme kinetics and substrate preferences. Microsomes isolated from L*RDH*SN/Hep G2 cells were incubated with either 9-*cis*-retinol (○) or 11-*cis*-retinol (●) at various concentrations for 5 min at 37 °C. (A) Production of 9-*cis*-retinal and 11-*cis*-retinal during the reaction period was plotted against substrate concentration. (B) Under our assay conditions, the apparent K_m and V_{max} were 0.44 μM and 0.126 nmol mg^{-1} min^{-1} for 9-*cis*-retinol and 1.1 μM and 0.06 nmol mg^{-1} min^{-1} for 11-*cis*-retinol, respectively. Shown here is a representative result from three independent experiments.

and how, 9-*cis*-retinoids are processed within tissues and cells. Our studies were carried out to provide insight into this question. First, we examined 9-*cis*-retinol metabolism in hepatocytes and stellate cells, the two main cell types responsible for retinoid storage and metabolism in the liver. We chose to investigate this question in hepatic cells, since both *cis*-retinol dehydrogenases and 9-*cis*-retinol and 9-*cis*-retinyl esters are found in the liver (*16*−*19, 24*). As seen in Figures 3 and 4, 9-*cis*-retinol can be loaded into both Hep G2 and HSC-T6 cells from the culture medium and can be used for synthesis of 9-*cis*-retinyl esters in these cells. Interestingly, the Hep G2 cells and the HSC-T6 cells show markedly different properties for 9-*cis*-retinol removal from the medium. For the Hep G2 cells, 9-*cis*-retinol cannot be effectively loaded into cells in the simultaneous presence of *all-trans*-retinol in the medium (Figure 5), while 9-*cis*-retinol loading into HSC-T6 cells is not influenced by the presence of *all-trans*-retinol in the medium (Figures 3C and 5). Moreover, both Hep G2 and HSC-T6 cells possess enzymatic activities for synthesis and hydrolysis of 9-*cis*-retinyl esters. Mata et al. (*50*) have previously reported that bovine liver possesses a neutral hydrolase activity that is able to hydrolyze *cis*-retinyl esters; however, as far as we are aware, the in vitro esterification of 9-*cis*-retinol by liver or liver cell homogenates has not been previously reported.

Since the two liver cell types can be loaded with 9-*cis*-retinol and maintain the 9-*cis*-configuration as well as metabolize 9-*cis*-retinol to esters, we wanted to explore how much 9-*cis*-retinol (or 9-*cis*-retinyl esters) is present within animal tissues. Although not routinely measured in tissues because of the need to use relatively tedious normal-phase HPLC procedures to resolve the geometric isomers of retinol and retinyl esters, 9-*cis*-retinol has been reported to be present in kidney and liver for various species (*15, 20, 51, 52*). Our data demonstrate that both 9-*cis*-retinol and its fatty acyl esters are present in liver obtained from 3-month-old male mice that had been maintained throughout their life on a control chow diet. These tissue levels of 9-*cis*-retinol and 9-*cis*-retinyl ester are low compared to tissue *all-trans*-retinol and/or *all-trans*-retinyl ester levels, approximately 0.25% that of total *all-trans*-retinol. Nevertheless, it is clear that 9-*cis*-retinol and its esters are present in liver. Since the three known *cis*-retinol-specific dehydrogenases are reported to be expressed in liver, where 9-*cis*-retinol and/or its esters are located, it would not seem unreasonable to speculate that either one or more of these *cis*-retinol dehydrogenases may act physiologically to catalyze 9-*cis*-retinol oxidation.

Since we could not detect 9-*cis*-retinoids in serum from fasted mice, we do not know the physiologic significance of differential accumulation of retinol isomers by the two liver cell types in vivo. However, we cannot rule out the presence of 9-*cis*-retinoid in serum, postprandially in our study. The source of 9-*cis*-retinol in the diet is largely unknown except for the observations of 9-*cis*-β-carotene being a possible precursor (*20, 21, 53*). Levin and Mokady (*9*) observed increased level of 9-*cis*-retinol in liver of chicks fed with high 9-*cis*-β-carotene diet. However, total liver retinol level was lower when chicks were fed high 9-*cis*-β-carotene diet compared to ones fed high *all-trans*-β-carotene diet (*9*). Thus, 9-*cis*-β-carotene appeared to be less efficient compared to *all-trans*-β-carotene as a precursor of retinol. In agreement with Levin and Mokady, You et al. (*54*) reported 9-*cis*-β-carotene to be a poor precursor of 9-*cis*-retinol in humans due to its high isomerization to *all-trans*-β-carotene upon absorption. In contrast, absorption of 9-cis-β-carotene appeared to be similar to all-trans configuration in ferrets whose intestine was perfused with either form of β-carotene (*21*). Moreover, total retinoic acid levels were similar regardless of configuration of β-carotene used except that 9-*cis*-retinoic acid was half the total retinoic acid formed when 9-*cis*-β-carotene was perfused (*21*). Whether the cell-specific accumulation of retinol isomers observed in our study plays a role in vivo needs to be considered in appropriate animal models.

Since Hep G2 cells endogenously express cRDH at low levels ($^1/_3$ the cRDH mRNA of human liver; Figure 1A), we generated L*RDH*SN/Hep G2, with expression levels of cRDH similar to that in human liver (Figure 1A), to study the pathway of 9-*cis*-retinoic acid synthesis. Using both empty vector-transduced Hep G2 cells (L*X*SN/Hep G2 cells) and L*RDH*SN/Hep G2 cells, we investigated the ability of these cells to generate 9-*cis*-retinoic acid from 9-*cis*-retinol

added to cell homogenates. Surprisingly, 9-*cis*-retinoic acid formation remained low regardless of cRDH overexpression, even though L*RDH*SN/Hep G2 cell homogenates showed 6 times more cRDH specific activity (for 9-*cis*-retinol oxidation to 9-*cis*-retinal) than the L*X*SN/Hep G2 in vitro (Figure 2C). When examined for the ability to oxidize 9-*cis*-retinal to 9-*cis*-retinoic acid, Hep G2 cells exhibited time- and substrate concentration-dependent production of 9-*cis*-retinoic acid (data not shown). Thus, the absence of retinal dehydrogenase could not be the primary reason for the low level of 9-*cis*-retinoic acid production as well as lack of differences in 9-*cis*-retinoic acid formation from two cell lines, L*RDH*SN/Hep G2 and L*X*SN/Hep G2.

Because Bhat and colleagues (*15*) have described a retinal dehydrogenase that uses 9-*cis*-retinal as a substrate, which is strongly inhibited by 9-*cis*-retinol (with a K_i of 5.4 μM), we asked whether 9-*cis*-retinal oxidation could be blocked by high concentrations of 9-*cis*-retinol in Hep G2 cell homogenates. Indeed, as seen in Figure 6, 5 μM 9-*cis*-retinol inhibited 9-*cis*-retinal oxidation by approximately 50% in the L*RDH*SN/Hep G2 cell homogenates. Thus, it would appear that Hep G2 cells possess a 9-*cis*-retinol-sensitive retinal dehydrogenase that is similar in this respect to the rat kidney enzyme described by Bhat and colleagues (*15*).

Low levels of liver 9-*cis*-retinol, compared to those of *all-trans*-retinol, in chow-fed male mice could suggest that 9-*cis*-retinol is not an important physiologic substrate for cRDH; however, we believe that this possibility is unlikely. One of the most striking features of cRDH activity that we have repeatedly observed is its capacity to catalyze 9-*cis*-retinol oxidation to near completion. Under our assay conditions, we have determined the equilibrium constant for the oxidation reaction catalyzed by cRDH to be 4.9×10^{-9} M. Consequently, at equilibrium, the concentration of 9-*cis*-retinal would be expected to be approximately 100 times greater than that of 9-*cis*-retinol. Thus, if some 9-*cis*-retinol were present together with cRDH and sufficient NAD^+, we would predict that the 9-*cis*-retinol would immediately be transformed to 9-*cis*-retinal. The total intracellular (cytoplasmic) NAD^+ concentration for rat liver is calculated to be approximately 1 mM (*55*, *56*) (a concentration similar to what we employed in our assay condition). Of the total NAD ($NAD^+ + NADH$) present in rat liver, approximately 99.9% will be present as NAD^+. Consequently, with our assay conditions, which reflect those of the liver, one would expect the reaction to go toward 9-*cis*-retinal. We tested this prediction in the experiment reported in Figure 7. When the Hep G2 cell homogenate was incubated with *all-trans*-[^{3}H]retinol, formation of 9-*cis*-retinal occurred. This finding indicates that the small amount of 9-*cis*-retinol produced through isomerization of *all-trans*-retinol is being oxidized to 9-*cis*-retinal, likely ensuring 9-*cis*-retinoic acid formation. This finding is also in keeping with our observation that high concentrations of 9-*cis*-retinol can strongly inhibit 9-*cis*-retinal oxidation and, hence, 9-*cis*-retinoic acid formation from 9-*cis*-retinol. Considering the inhibitory action of 9-*cis*-retinol on 9-*cis*-retinal oxidation (Figure 6), one would predict that 9-*cis*-retinol might never reach very high levels within cells or tissues, since this would have the effect of shutting down 9-*cis*-retinoic acid formation; a case of the precursor inhibiting product formation. On the basis of these considerations, we believe that cRDH is physiologically involved in catalyzing a first metabolic step needed for 9-*cis*-retinoic acid formation in the liver.

We and others have reported data that indicate that cRDH is identical to 11-*cis*-retinol dehydrogenase (11cRDH), an enzyme that was originally proposed to be specifically localized to the retinal pigment epithelium (RPE) and to be critically involved in 11-*cis*-retinal formation (*6*, *16*, *45*, *46*). Contrary to the earlier literature (*6*, *16*, *45*, *46*), it appears that recombinant human 11cRDH and bovine RPE 11cRDH may equally utilize both 11-*cis*-retinol and 9-*cis*-retinol as substrates (*17*, *23*). We asked whether cRDH, expressed in nonocular tissue, can also use both 9-*cis*- and 11-*cis*-retinol as substrates. As expected, both of these retinol isomers serve as substrates for cRDH derived from L*RDH*SN/Hep G2 cells. The apparent K_m values for 9-*cis*- and 11-*cis*-retinol were respectively 0.44 and 1.1 μM. Thus, cRDH expressed in nonocular cells can utilize with nearly equal affinity both retinol isomers as substrates. This observation is consistent with the conclusion that cRDH and 11cRDH are indeed the same enzyme.

Overall, our studies provide evidence that 9-*cis*-retinol is found within tissues of living animals and that hepatic cells are able to incorporate and metabolize 9-*cis*-retinol in a manner that resembles that of *all-trans*-retinol. These findings support the notion that several recently cloned *cis*-retinol-specific dehydrogenases likely catalyze 9-*cis*-retinoic acid formation. Moreover, our data provide new insights into how 9-*cis*-retinoic acid may be formed by tissues and cells and suggest that the metabolism of 9-*cis*-retinoids is highly regulated.

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