Biochemical properties, tissue expression, and gene structure of a short chain dehydrogenase/reductase able to catalyze *cis*-retinol oxidation

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Abstract We have identified a retinol dehydrogenase (cRDH) that catalyzes the oxidation of 9-*cis*- but not all-*trans*-retinol and proposed that this enzyme plays an important role in synthesis of the transcriptionally active retinoid, 9-*cis*-retinoic acid. There is little information regarding either the biochemical properties of cRDH or how its 9-*cis*-retinol substrate is formed. We now report studies of the properties and expression of human and mouse cRDH and of the characteristics and location of the murine cRDH gene. Additionally, we report mouse hepatic 9-*cis*-retinol concentrations and demonstrate that 9-*cis*-retinol is formed in a time- and protein-dependent manner upon incubation of all-*trans*-retinol with cell homogenate. Human and mouse cRDH display similar substrate specificities for *cis*-isomers of retinol and retinaldehyde. Moreover, human and mouse cRDH show marked sensitivity to inhibition by 13-*cis*-retinoic acid, with both being inhibited by approximately 50% by 0.15 μM 13-*cis*-retinoic acid (for substrate concentrations of 10 μM). Lesser inhibition is seen for 9-*cis*- or all-*trans*-retinoic acids. Immunoblot analysis using antiserum directed against human cRDH demonstrates cRDH expression in several tissues from first trimester human fetuses, indicating that cRDH is expressed early in embryogenesis. Adult mouse brain, liver, kidney, and to a lesser extent small intestine and placenta express cRDH. The murine cRDH gene consists of at least 5 exons and spans approximately 6 kb of genomic DNA. Backcross analysis mapped the mouse cRDH gene to the most distal region of chromosome 10. Taken together, these data extend our understanding of the properties of cRDH and provide additional support for our hypothesis that cRDH may play an important role in 9-*cis*-retinoic acid formation.—Gamble, M. V., E. Shang, R. P. Zott, J. R. Mertz, D. J. Wolgemuth, and W. S. Blaner. **Biochemical properties, tissue expression, and gene structure of a short chain dehydrogenase/reductase able to catalyze *cis*-retinol oxidation. *J. Lipid Res.* 1999.** 40: **2279–2292.**

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Retinoids are acquired from the diet and are needed for maintaining the general health of higher animals and humans (1). They are required for normal vision, for maintaining normal growth and differentiation, for an uncompromised immune response, for normal male and female reproduction, and for other essential biologic processes (1–5). Retinoids act primarily as ligands for transcription factors which modulate expression of a large number of genes including those encoding hormones, growth factors, transcription factors, membrane receptors, extracellular matrix proteins, structural proteins, and enzymes involved in diverse metabolic processes (4, 5). The transcriptional regulatory actions of retinoids are thought to be mediated primarily through the actions of all-*trans*- and 9-*cis*-retinoic acid which bind to members of the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families of ligand-dependent transcription factors (4). As members of the RXR family of receptors are able to serve as partners in forming heterodimers with the vitamin D receptor, the thyroid hormone receptors, the peroxisomal proliferator activator receptors, and several other ligand dependent transcription factors, 9-*cis*-retinoic acid likely plays an important role in regulating a broad spectrum of hormonally responsive genes (4).

The metabolism of retinoic acid within tissues is complex and includes both the activating metabolism of retinol to retinoic acid and the oxidative and conjugative metabolism of retinoic acid to more polar metabolites (6, 7). Retinoic acid is formed from retinol through two enzymatic oxidations, in a manner analogous to ethanol oxidation. The first of these oxidation reactions involves the ox-

Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; SCDR, short chain dehydrogenase/reductases; RDH, retinol dehydrogenase; 11cRDH, 11-*cis*-retinol dehydrogenase; cRDH, *cis*-retinol dehydrogenase; HPLC, high performance liquid chromatography; MAP, multiple antigen peptide; RALDH-2, retinaldehyde dehydrogenase, type 2; PBS, 10 mm sodium phosphate, pH 7.4, 150 mm NaCl.

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idation of retinol to retinaldehyde. Members of two families of alcohol dehydrogenases are actively being investigated as possible physiologically relevant candidates for in vivo catalysis of retinol oxidation (6, 7). The first family consists of the relatively abundant cytosolic class I and class IV alcohol dehydrogenases. Data that support in vivo involvement of these enzymes in retinoic acid formation include demonstrations of overlapping temporal and spatial patterns of retinoic acid presence and enzyme expression in tissues of the developing mouse embryo (8, 9). Several members of a second family of enzymes, the short-chain dehydrogenase/reductases (SCDR), are also being investigated as possibly essential for retinoic acid formation (10–19). Members of the SCDR family also are thought to be importantly involved in steroid and eicosanoid metabolism (20, 21). At present, there is not general agreement regarding the importance of members of each of these enzyme families in catalyzing retinol oxidation in vivo (7–19). However, based on the relatively large number of enzymes that can catalyze retinol oxidation, it would appear likely that there is redundancy in the enzymatic machinery needed for retinoic acid formation.

We have previously described an enzyme that we termed 9-*cis*-retinol dehydrogenase at the time of its report. However, as reported below, because of its broad substrate specificity for *cis*-retinols, we are now referring to it as *cis*-retinol dehydrogenase (cRDH). This enzyme is present in several human tissues including mammary tissue, kidney, liver, and testis (19). Based on the substrate specificity of this enzyme, we proposed that human cRDH plays an important role in the formation of 9-*cis*-retinoic acid by catalyzing the oxidation of 9-*cis*-retinol to 9-*cis*-retinaldehyde. Others recently also have reported the cloning of a stereospecific mouse retinol dehydrogenase (RDH) with 9-*cis*-retinol dehydrogenase activity and have characterized the expression pattern of this enzyme in some embryonic and adult mouse tissues (22, 23). We now extend our studies of cRDH by providing detailed biochemical characterizations of human and mouse cRDH, by describing cRDH distribution in fetal human and adult mouse tissues, and by characterizing the mouse gene for cRDH. In addition, we demonstrate that 9-*cis*-retinol can be formed from all-*trans*-retinol upon incubation in a cell homogenate in a time- and protein-dependent manner.

MATERIALS AND METHODS

Retinoids and chemicals

All-*trans*-retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, and 11-*cis*-retinaldehyde were obtained as gifts from Dr. Christian Eckhoff of Hoffmann-La Roche, Inc. (Nutley, NJ). All-*trans*-, 13-*cis*-, and 9-*cis*-retinaldehyde and 13-*cis*-retinol were purchased from Sigma Chemical Co. (St. Louis, MO). 9-*Cis*- and 11-*cis*-retinol were synthesized through $NaBH_4$ reduction of the corresponding retinaldehyde (24). The resulting 9-*cis*- or 11-*cis*-retinol was purified by normal phase HPLC essentially as we have described for 11-*cis*-retinol purification (24). Purity of each retinoid used as a substrate or an inhibitor was assessed by normal phase HPLC (see below) using a photodiode array detector providing the UV–Vis spectrum of each retinoid. Based on the retention time and the UV–Vis spectum and integrated peak areas (if impurities were present) we estimated the purity of each retinoid used in our studies. Normally, for use as a substrate or an inhibitor, at the start of an assay, the purity of each retinoid used in assays was at least 99% or greater.

Steroids used for our studies were purchased from Sigma. These include androsterone, testosterone, estrone, β-estradiol, 4-androstene-3,17dione, 5α-androstane-3,17dione, 5α-androstane-3α,17βdiol, 5α-androstane-3β,17βdiol, 5α-androstan-17βol-3one, cortisone, corticosterone, hydrocortisone, prednisone, and progesterone. NADH, NAD^+, NADPH, $NADP^+$, citral, disulfuran, phenylarsine oxide, carbenoxolone, 4-methyl pyrazole and 1,10-phenanthroline were also obtained from Sigma. Oleyl and petroselinyl alcohols and palmitic and oleic acids were obtained from Nu-Chek Chemicals (Elysian, MN).

HPLC and other organic solvents, all HPLC grade, were purchased from Fisher Chemical Company (Pittsburgh, PA). Other reagents used in these studies were purchased from standard commercial sources.

Enzymatic assays

Assays for human and mouse cRDH were carried out essentially as we described previously for human cRDH (19). For expression of mouse cRDH we used a cDNA clone obtained from a mouse liver cDNA library (see below). Recombinant human or mouse cRDH expressed in CHO cells was incubated in an assay mixture containing 10 μm 9-*cis*-retinol (added in 0.02 mL ethanol) and 2 mm NAD^+ in 10 mm Hepes, pH 8.0, containing 150 mm KCl, 1 mm EDTA, and 50 μg phosphatidylcholine for 15 min at 37°C (final assay volume of 0.6 ml) with agitation. This assay mixture was used routinely to measure oxidation of 9-*cis*-retinol to 9-*cis*-retinaldehyde and in studies assessing oxidation of 11-*cis*-, 13-*cis*-, and all-*trans*-retinol to their corresponding aldehydes. For some experiments, we assessed the ability of human or mouse cRDH to catalyze the reduction of 9-*cis*-, 11-*cis*-, 13-*cis*-, or all-*trans*-retinaldehyde. To assess these reduction reactions, incubations were carried out in 100 mm sodium acetate, pH 5.0, containing 50 μg phosphatidylcholine and 2 mm NADH (or 2 mm NADPH) in a final volume of 0.6 ml for 15 min at 37°C.

Immediately after incubation of the assay, the assay mixture was denatured with an equal volume of absolute ethanol (0.6 ml), and the retinoids were extracted into 2.5 ml of hexane. After one backwash of the hexane extract with 0.5 ml of deionized water, the hexane was evaporated to dryness under a gentle stream of N_2. The extracted retinoids were immediately redissolved in 120 μl of hexane and analyzed by normal phase HPLC as described below. The recovery of retinoids was assessed by summing the total amount of substrate and products present in the normal phase HPLC profiles (see below) and dividing by the amount of substrate originally added to the assay mixture.

To investigate whether human or mouse cRDH will catalyze oxidations or reductions of 3α-hydroxy-, 11β-hydroxy-, or 17β-hydroxysteroids, we incubated these steroids at 37°C for 1.5 h in the assay media described above for assessing oxidation/reduction of 9-*cis*-retinol/9-*cis*-retinaldehyde. The HEPES NAD^+-containing (or $NADP^+$-containing) assay buffer described above was used to assess hydroxysteroid oxidation, and to assess hydroxysteroid reduction the acetate buffer containing NADH (or NADPH) assay buffer (see above) was used. Immediately after incubation, the steroids were extracted as described above for retinoids. The final hexane extract was evaporated to dryness, redissolved in approximately 0.05 mL chloroform–methanol 2:1 (v/v) and spotted onto a silica high performance TLC plate (Whatman Inc., Clifton, NJ) and developed in hexane–diethyl ether–acetic acid 70:30:1 (v/v) or ethyl acetate–chloroform 25:75 (v/v). Steroids

were visualized by charring after treatment of the TLC plates with an aerosol of 10% (v/v) H_2SO_4 in methanol.

Inhibition studies of cRDH activity

For our inhibition experiments, each potential inhibitor tested was added to the recombinant cRDH in 5 μl of ethanol in the presence of 2 mm NAD^+, 10 mm HEPES, pH 8.0, containing 150 mm KCl, 1 mm EDTA, and 50 μg phosphatidylcholine (assay incubation mixture lacking the retinoid substrate). This mixture was allowed to sit on ice for 5 min prior to adding substrate. Upon addition of 10 μm 9-*cis*-retinol (final concentration), the mixture was incubated for 15 min at 37°C with agitation and extracted as described above for the standard enzyme assay.

The potential inhibitory properties of various hydroxysteroids towards blocking 9-*cis*-retinaldehyde reduction was also tested. For this purpose, incubations were carried out in 100 mm sodium acetate, pH 5.0, containing 50 μg phosphatidylcholine and either 2 mm NADH or 2 mm NADPH (both cofactors were used in independent determinations) in a final volume of 0.6 ml for 15 min at 37°C.

HPLC procedures

Stereoisomers of both retinol and retinaldehyde were separated on a 4.6 × 15 mm Supelco (Bellefonte, PA) LC-SI Supelcosil™ column using hexane–ethyl acetate–butanol 96.9:3:0.1 (v/v) as the mobile phase flowing at 0.8 ml/min. A silica guard column (Supelco) preceded the running column. Retinols and retinaldehydes were detected using a Waters 996 Photodiode array detector at absorbances of 325 and 365 nm, respectively. Retention times for all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinols and corresponding retinaldehydes were established using the purified compounds obtained as described above. Quantities of each retinol and retinaldehyde isomer present in extracts were determined by comparisons of the integrated areas under the HPLC peaks with a standard curve constructed relating integrated peak area with known masses of each retinoid isomer. The concentrations of each retinoid isomer were determined by UV–visible spectrophotometry using published extinction coefficients for each retinol, retinaldehyde, or retinoic acid isomer (25).

Tissue extractions

To measure hepatic concentrations of all-*trans*- and 9-*cis*-retinol, six 3-month-old chow-fed male mice were killed by CO_2 euthanasia and the livers were immediately dissected from the carcass. Livers were immediately minced and weighed, placed in 4 volumes of ice-cold 10 mm sodium phosphate, pH 7.4, containing 150 mm NaCl, and homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) to yield a 20% (w/v) homogenate. Immediately prior to homogenization of a tissue mince, approximately 1 μCi all-*trans*-[^{3}H]retinol was added to serve as an internal standard and to provide a measure of artifactual isomerization of all-*trans*-retinol to 9-*cis*-retinol. Total lipids present in each homogenate were extracted into 6 volumes of $CHCl_3$–CH_3OH 2:1 (v/v). After removal of the lower lipid-containing $CHCl_3$ phase, the $CHCl_3$ was evaporated under a gentle stream of N_2. The retinol-containing lipid film was redissolved in 1.0 ml of hexane. A portion of this material (0.1 ml) was taken for HPLC analysis and determination of retinol isomer concentrations using the HPLC procedures outlined above. During this HPLC analysis, fractions were collected from the materials eluting under the 9-*cis*- and all-*trans*-retinol peaks. These fractions were subjected to liquid scintillation counting to assess the precent of internal standard all-*trans*-[^{3}H]retinol that had undergone isomerization to the 9-*cis*-isomer. In addition, an aliquot of the remaining redissolved extract also was taken for liquid scintillation counting to assess the total recovery of retinol.

Antibody production

A peptide corresponding to amino acids 236–257 of the primary sequence of human cRDH (19) was synthesized by the Columbia University Howard Hughes Protein Core Facility. The peptide was linked to an eight-armed matrix (26) and this multiple antigen peptide (MAP) served as the immunogen. The MAP was sent to Pocono Rabbit Farm & Laboratory, Inc., Canadensis, PA, for antibody production. For this purpose, a New Zealand White rabbit was injected intradermally with 1 mg of MAP in Complete Freund's Adjuvant. One booster intradermal injection of 100 μg of MAP in Complete Freund's Adjuvant was given on day 14. On day 28, the rabbit was given a subcutaneous injection of 100 μg of the MAP in Incomplete Freund's Adjuvant. Test bleeds began 42 days after the initial injection, followed by injection of 50 μg of MAP in Incomplete Freund's Adjuvant on day 56 and every 4 weeks thereafter. Test bleeds were taken 2 weeks after each injection (days 42, 70, and 98). On day 126 after the initial immunization, the rabbit was exsanguinated.

Immunoblot analyses

For immunoblot analyses, tissues or cells were homogenized in 10 mm Tris-HCl, pH 7.4, using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Debris and unhomogenized materials were removed from the homogenate through centrifugation of the crude homogenate at 800 *g* at 4°C for 10 min. To an aliquot of homogenate containing 100 μg total protein, 6X-SDS treatment buffer (0.35 m Tris-HCl, pH 6.8, 10.28% SDS, 36% (v/v) glycerol, 0.6 m DTT, 0.012% (w/v) bromophenol blue) was added and, after brief vortexing, the mixture was boiled at 100°C for 10 min. The treated homogenate was loaded onto a 15% SDS-PAGE gel and electrophoresed at 150 mV until the tracking dye reached the bottom of the gel. Proteins were transferred from the 15% SDS-PAGE gel onto a nitrocellulose membrane at 30 volts and 4°C for 12 h. The nitrocellulose membrane was subsequently blocked with 5% non-fat dry milk in 10 mm sodium phosphate containing 150 mm NaCl at pH 7.4 (PBS) for 1 h, followed by incubation in the presence of the rabbit polyclonal anti-human cRDH antiserum at a 1:5000 dilution in PBS containing 0.2% Tween 20. The membrane was washed 4 times with PBS containing 0.2% Tween 20 for 15 min and then incubated for 1 h at room temperature in PBS containing 0.2% Tween 20 and a 1:1000 dilution of a second antibody consisting of donkey anti-rabbit IgG conjugated to alkaline phosphatase (Amersham, Chicago, IL). After incubation with the secondary antiserum, the nitrocellulose membrane was again washed 4 times for 15 min each with PBS containing 0.2% Tween 20. All washes and incubations were carried out with constant orbital agitation. Immunoblots were visualized on photographic films using the ECL kit purchased from Amersham (Amersham, Chicago, IL), following the supplier's instructions.

Human fetal tissues were obtained from several first trimester fetuses through the Laboratory for the Study of Human Embryos and Fetuses, University of Washington, Seattle, WA. These consisted of mixed adrenal and small intestine tissue from an 11-week fetus, kidney and brain from a 12-week fetus, and intestine, brain, and lung tissue from an 11-week-old fetus. These tissue samples had been frozen at −70°C prior to use for our studies. For immunoblot analysis, these fetal tissues were processed as described above.

Sources of mouse tissues and RNA

Normal mouse tissues were obtained from adult Swiss Webster mice (Charles River, Wilmington, DE). Embryos were obtained from timed pregnant Swiss Webster mice. Dissected tissues were frozen in liquid N_2 prior to RNA isolation.

Screening of cDNA libraries

Two mouse cDNA libraries were screened to obtain a cDNA clone for mouse cRDH: an adult mouse testis cDNA library and a day 14 mouse testis cDNA library. The libraries were generated using the Uni-Zap XR cDNA synthesis kit (Stratagene, La Jolla, CA) and were a generous gift from Dr. Kunsoo Rhee (Columbia University) (27). The probe used for screening was the cDNA corresponding to human cRDH (19). The cDNA probe was labeled with [α-^{32}P]dCTP using the random primer DNA labeling kit (Amersham, Arlington Heights, IL). All radioactive nucleotides used in this study was obtained from New England Nuclear (Wilmington, DE). The cDNA libraries were screened according to standard protocols (28). The hybridization conditions were: 500 mm NaCl, 50 mm Tris (pH 8.0), 5 mm EDTA, 1× Denhardt's solution, 100 μg/ml salmon sperm DNA, 1% SDS at 55°C overnight. The final washing conditions were: 0.2× SSC, 0.1% SDS at 55°C 30 min. For each library, 6×10^5 plaques were screened. Positive clones were plaque-purified twice and inserts from the tertiary screen were in vivo excised following the protocol provided by Stratagene. The phagemids contained the cDNA at the EcoRI and XhoI site of pBluescript SK$^-$. Clones isolated from these screens were sequenced using an Applied Biosystems Model 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Screening of a genomic library

A genomic library from mouse strain 129SV/J (Stratagene, constructed in λ Fix II vector) was screened using a 1.4 kb cDNA corresponding to cRDH isolated from an adult mouse testis cDNA library. (This cDNA is designated mt-cRDH1 in Fig. 2). ^{32}P-labeled probed was generated using the random primer method as described above. The conditions were identical to those used in the cDNA library screening except that the hybridization and final washing temperature was 65°C. λ DNA was prepared according to standard protocols (28). Briefly, after cell lysis, the culture was treated with DNase I and RNase A and centrifuged. The phage in the supernatant were precipitated by PEG 8000 and centrifuged. The phage particles were disrupted in SDS, EDTA at 65°C and then phenol-extracted (2 times) and phenol–chloroform-extracted. Single and double enzyme digestion was performed according to the manufacturer's recommended conditions.

Chromosome mapping

A genomic polymorphism in the murine cRDH gene was identified between samples of DNA from *M. musculus* strain C57Bl/6J and *M. spretus*. A small intron was selected as the marker region and a pair of primers was synthesized for use in PCR amplification of this intron. The Backcross DNA Panel and Service provided by The Jackson Laboratory (Bar Harbor, ME) was used for mapping the chromosomal site of cRDH. PCR analysis using the primer pairs was performed on the panel DNA and the products were electrophoresed on agarose gels and stained with ethidium bromide. The presence of specific PCR products was noted and the data submitted to The Jackson Laboratory for chromosomal assignment.

Northern blot hybridization analysis

Total RNA was isolated using standard procedures (29). Samples consisting of 10 μg of total RNA were electrophoresed on 0.8% agarose gels containing 2.2 m formaldehyde at 1 v/cm overnight. The probe used for Northern blot hybridization analysis was the 1.4 kb mouse testicular cDNA (mt-cRDH1 in Fig. 3), which was also used to screen the genomic library. Ethidium bromide staining of the 18S and 28S RNAs was used to demonstrate equal loading for each sample. After electrophoresis the gels were soaked in 20× SSC for 1 h and blotted overnight onto a nitrocellulose membrane using 10× SSC. The RNA was transferred to nitrocellulose membranes and baked at 80°C in a vacuum oven for 2 h. The cRNA probe was labeled using T7 RNA polymerase and [α-^{32}P]UTP. Hybridization was carried out at 65°C in 5× SSC, 20 mm sodium phosphate buffer (pH 7.0), 60% formamide, 1% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast RNA, and 7% dextran sulfate overnight.

RESULTS

Generation and characterization of antiserum to human cRDH

In order to characterize the distribution of human cRDH in tissues and the biochemical properties of this enzyme, we generated a rabbit polyclonal antibody that recognizes human cRDH. This antiserum was made against a multiple antigen peptide consisting of amino acids 236 to 256 of the human cRDH (21 out of 318 amino acids) (19). This peptide shows a large number of amino acid differences when compared with other closely related members of the SCDR family which share homology with human cRDH. For this region, the human cRDH shows 6 out of 21 amino acid differences with mouse cRDH (see below), 5 amino acid differences with bovine 11cRDH (15, 16), 15 differences with rat liver all-*trans*-retinol dehydrogenase type I (13), 14 differences with rat liver all-*trans*-retinol dehydrogenase, type II (14), 15 differences with rat liver all-*trans*-retinol dehydrogenase, type III (12), and 15 differences with the mouse liver *cis*-retinol/androgen dehydrogenase (18). A second reason for selecting this peptide as an antigen was that computer analysis using an amphipathic profiler indicated that this sequence scored high on an antigenicity scale.

Immunoblot analysis showed that the antiserum recognizes strongly and specifically recombinant human cRDH as a 32 kDa protein (the correct size predicted from the cDNA for human cRDH) (**Fig. 1**). Recognition of this protein band was effectively competed by preincubation of the antiserum with the peptide antigen (200 μg MAP in 20 ml blocking buffer). As can be seen from Fig. 1, neither sham-transfected CHO cell homogenates nor homogenates from CHO cells transfected with the mouse cRDH cDNA contained protein(s) recognized by this antiserum. Because the size of the protein recognized by the antiserum separated on the SDS-PAGE gel corresponds to a protein with a mass of 32 kDa, the mass of human cRDH predicted by its cDNA, it would appear that cRDH does not undergo substantial post-translational modification upon expression in CHO cells and further, that such modification is not essential for cRDH activity.

Upon incubation of recombinant human cRDH for 15 min at 37°C with up to 25 μl whole rabbit antiserum followed immediately by human cRDH enzyme assay, no differences in cRDH activities were observed for the mixtures containing or not containing the antiserum (data not shown). Thus, the rabbit anti-human cRDH antiserum does not inhibit human cRDH enzymatic activity. This could suggest that the peptide used for generating the antiserum resides at a site on the human cRDH molecule removed from the catalytic center of the enzyme.

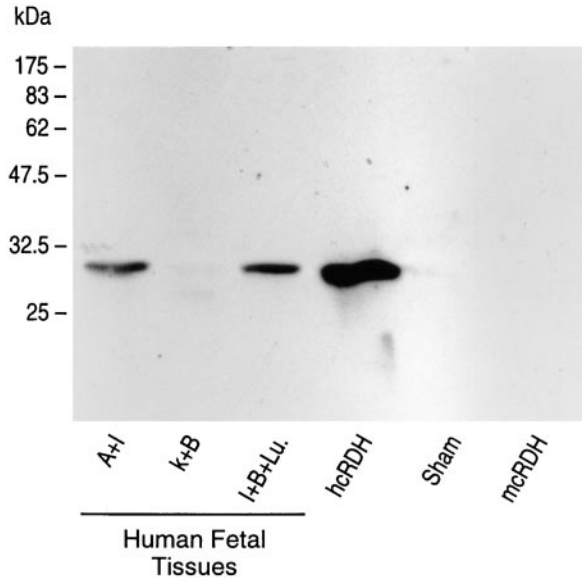


Fig. 1. Immunoblot analysis for cRDH expression in 11-week-old human fetal tissues. Equal protein loads from cell homogenates of CHO cells transfected with a cDNA for human cRDH, with a cDNA for mouse cRDH, or with the vector alone (Sham) were used as controls for this analysis. First trimester (week 11) human fetal tissue pools examined consist of adrenals and small intestine (A+I), kidney and brain (k+B), and small intestine, brain, and lung (I+B+Lu.). Tissues and immunoblots were processed exactly as described in Materials and Methods.

Immunoblot analysis of cRDH distribution in fetal human tissues

Using the anti-human cRDH antiserum described above, we carried out immunoblot analyses of the expression of cRDH in human fetal tissues. Immunoblot analysis of human fetal tissue homogenates indicates that cRDH is expressed in the first trimester of pregnancy. A representative immunoblot for tissues from an 11-week-old human fetus is provided in Fig. 1. Figure 1 demonstrates that cRDH is expressed in several fetal tissues including pooled samples of fetal adrenals + small intestine and small intestine + brain + lungs. As only pooled human fetal tissues were available for this experiment, we are not able to discriminate among which tissues expressed cRDH at 11 weeks gestation. Replicate immunoblot analyses of several tissues from a different 11-week-old human fetus indicated that cRDH is also expressed during the first trimester in spinal cord but not muscle (data not shown).

Isolation of cDNAs for mouse cRDH

As cRDH is expressed in the human adult testis, we chose to screen an adult mouse testis cDNA library and a day 14 mouse testis cDNA library for clones for mouse cRDH. A total of approximately 10^6 plaques was screened over a series of two experiments. Six positive cDNA clones for mouse cRDH were identified and characterized upon screening the libraries with a probe generated from the cDNA for human cRDH. Sequence analysis revealed that all six clones were indeed murine homologues of human cRDH but were either incomplete or exhibited abnormal splicing. As is shown diagrammatically in **Fig. 2**, panel C, one of the testis cDNA clones has 58 bp of intron 3 and 222 bp of intron 1 unspliced (see details regarding the mouse cRDH gene below). This clone did not extend to the first exon. A second testis clone has 340 bp of unspliced intron 3 did not extend to exon 3. A third testis clone did not contain any intron sequences but was missing the translation initiation codon. All unspliced intron sequences belonged to the 3′ region of the intron and were adjacent to the 5′ end of the next exon. We also obtained two cDNA clones from the mouse EST data bank, one derived from a liver cDNA library and one from a kidney library. Based on the human cRDH cDNA, the liver clone appeared to be nearly full-length (1281 bp). It has a 957 bp coding region corresponding to a protein of 318 amino acids, a 123 bp 3′ untranslated region, and a 199 bp 5′ untranslated region. The kidney clone was nearly identical to this liver clone except that the first 5 bp of intron 1 were unspliced. Sequences for the mouse liver cRDH cDNA (Accession No. AF033196) and mouse kidney cRDH cDNA (Accession No. AF033195) have been deposited with, and are available through, the NCBI GenBank.

Isolation and characterization of the murine cRDH gene

To elucidate the organization of the mouse and human cDNAs and their corresponding genes, we isolated mouse cRDH genomic sequences. Approximately 10^6 plaques of a genomic library from mouse strain 129SV/J were screened over a series of two experiments using a probe generated with the mouse testis cRDH cDNA. Four potential genomic clones were identified, subcloned, mapped for restriction sites, and characterized with regard to the position of exons. The clones span approximately 30 kb of DNA in total. Two of the clones contained exons corresponding to the 5′ region of the mouse cRDH cDNA; one contained 3′ exons, and the fourth contained the entire cRDH gene. All of the coding exons for the mouse cRDH cDNA were mapped to the genomic clone and sequenced. Exon–intron boundaries were confirmed at the sequence level. The murine cRDH gene has at least five exons and spans approximately 6 kb of genomic DNA. The largest intron, intron 3, is approximately 3.5 kb and the smallest, intron 2, is 114 bp. The other introns include intron 1 of 554 bp and intron 4 of 198 bp. The sizes of the five exons are: exon 1, 167 bp (this exon may be incomplete); exon 2, 342 bp; exon 3, 259 bp; exon 4, 164 bp; and exon 5, 349 bp. The translation initiation codon is contained within exon 2. Although it is possible that additional exons exist in the 5′ genomic DNA, they would not be very large as the cDNAs we have obtained for mouse cRDH are close in size to the transcripts observed in tissues examined to date (see below). The genomic organization data are summarized in Fig. 2 (panels A and B).

Northern blot analysis of cRDH expression in mouse tissues

Northern blot analysis of adult mouse tissues revealed that cRDH transcripts are most readily detected in RNA

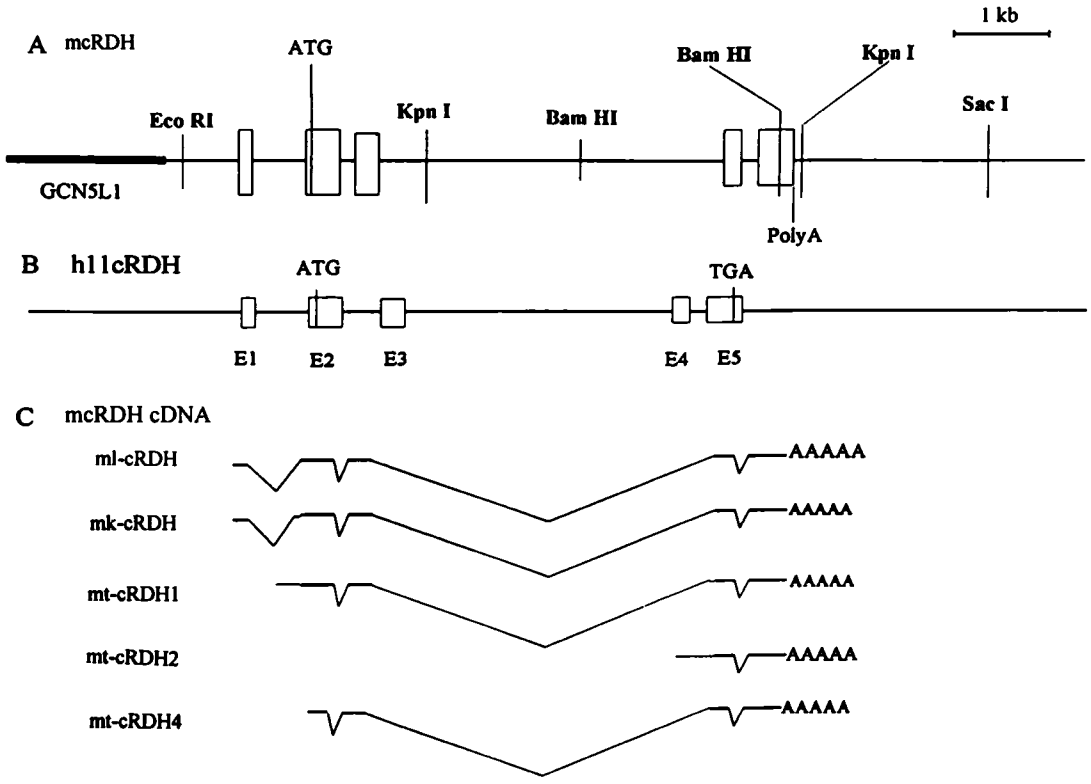


Fig. 2. Panel A: Exon–intron organization of mouse cRDH gene. The sizes of the five exons are: exon 1, 167 bp; exon 2, 342 bp; exon 3, 259 bp; exon 4, 164 bp; and exon 5, 349 bp. The sizes of the four introns are: intron 1, 554 bp; intron 2, 114 bp; intron 3, 3.5 kb; and intron 4, 198 bp. Panel B: Exon–intron organization of human 11cRDH gene as reported by Simon et al. (33). The sizes of exon 2, exon 3, and exon 4 are exactly the same for mouse cRDH and human 11cRDH. Panel C: Mouse cRDH cDNAs. The liver clone of 1281 bp aligned to the mouse cRDH gene. It appeared to be spliced normally according to the AG/GU rule. As compared to the liver clone, the kidney clone has 5 bp of intron 1 that is unspliced. Testis clone 1 has 58 bp of intron 3 and 222 bp of intron 1 that are unspliced; testis clone 2 has 340 bp of unspliced intron 3 and did not reach exon 3; testis clone 3 did not contain any intron sequences and was identical to the liver clone but was missing the translation initiation codon.

from kidney, liver, and brain and at somewhat lower levels in intestine, heart, and placenta (**Fig. 3**). The transcript identified by Northern blot analysis was approximately 1.5 kb, a size that agrees well with that predicted by the size of the longest of the murine cRDH cDNA clones. For some tissues, bands migrating under the 18S rRNA and for testis

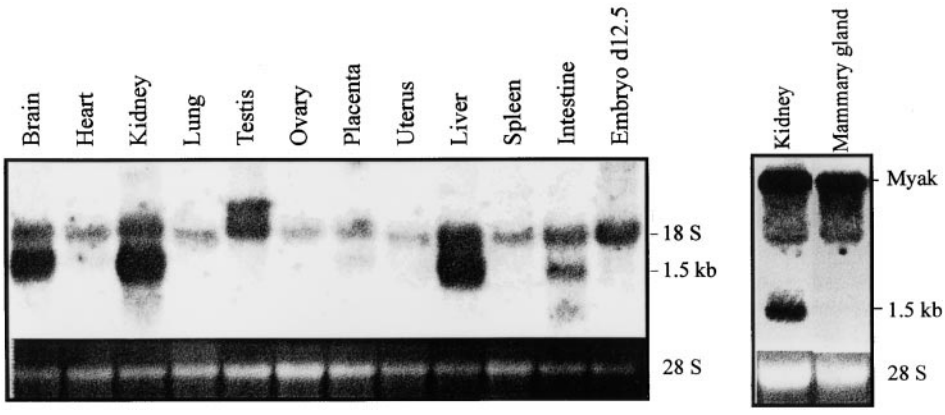


Fig. 3. Northern blot analysis of cRDH expression in mouse tissues. RNA was isolated from adult mouse brain, heart, kidney, lung, testis, ovary, placenta, uterus, liver, spleen, small intestine, a day-12.5 mouse embryo, and mammary gland. Each lane contains 10 μg of total RNA. For assessing cRDH expression in mouse mammary gland, kidney RNA was used as positive tissue control for cRDH expression and another gene, Myak, which is known to be expressed in mouse mammary tissue, was hybridized at the same time as a control for the quality of the mammary tissue RNA. A low level of cross-hybridization with the 18S ribosomal RNA band can also be seen in all samples.

a band migrating between the 18S and 28S rRNAs (at approximately 3.5 kb) were also detected. Although this may represent non-specific cross hybridization, given the heterogeneous cDNAs isolated from the liver, kidney, and testis libraries (see above), these bands may represent transcripts arising from differential splicing. Alternatively, we may be experiencing some cross hybridization with other known or unknown closely related dehydrogenases that are members of the SCDR family. We are not presently able to distinguish among these three possibilities. Nevertheless, the presence of a 1.5 kb transcript corresponding to the expected size of mouse cRDH mRNA is present in kidney, liver, brain, heart, intestine, and placenta.

Substrate specificity of mouse cRDH

The liver cRDH cDNA was expressed in CHO cells in order to characterize the biochemical properties of mouse cRDH. The specific activities of recombinant human and mouse cRDH expressed in CHO cells for 9-*cis*-, 13-*cis*-, 11-*cis*-, and all-*trans*-retinol oxidation along with those obtained for 9-*cis*-, 13-*cis*-, 11-*cis*-, and all-*trans*-retinaldehyde reduction are provided in **Table 1**. NAD^+ and NADH were much more effective than $NADP^+$ and NADPH in serving as cofactors for these reactions (data not shown). For no substrate could equimolar concentrations $NADP^+$/NADPH support reaction rates greater than 10% of those observed for NAD^+/NADH. As can be seen in Table 1, with regard to substrate and cofactor specificity, mouse cRDH is similar to human cRDH. For both human and mouse cRDH the three *cis*-isomers of both retinol and retinaldehyde were very good substrates for human and mouse cRDH. When all-*trans*-retinol was tested as a substrate for both human and mouse cRDH, we detected only low levels of all-*trans*-retinaldehyde being formed. However, we also observed significant amounts of 13-*cis*- and 9-*cis*-retinaldehyde formation upon addition of all-*trans*-retinol to the enzyme source. As can be seen from data presented below (see Fig. 5), some all-*trans*-retinol is isomerized by CHO cell homogenate to 13-*cis*- and 9-*cis*-retinol and presumably these *cis*-retinol isomers can be oxidized by cRDH to the corresponding aldehydes. We calculated the specific activity levels reported in Table 1 based on the amount of total products formed, and as all-*trans*-retinol can isomerize readily to the 13-*cis*- and 9-*cis*-retinol substrates, the specific activity levels of human and mouse cRDH given in Table 1 for all-*trans*-retinol should be seen as upper estimates.

TABLE 1. Substrate preference of human and mouse cRDH for isomers of retinol and retinaldehyde

Substrate	hcRDH	mcRDH
	picomoles/min/mg protein	
9-*cis*-retinol	204 ± 5	74 ± 2
11-*cis*-retinol	510 ± 14	60 ± 3
13-*cis*-retinol	301 ± 6	58 ± 2
all-*trans*-retinol	38 ± 2	24 ± 2
9-*cis*-retinaldehyde	958 ± 39	125 ± 39
11-*cis*-retinaldehyde	122 ± 4	57 ± 4
13-*cis*-retinaldehyde	1114 ± 298	298 ± 4
all-*trans*-retinaldehyde	5.9 ± 1.0	9.4 ± 1.0

cDNAs for human (hcRDH) and mouse cRDH (mcRDH) were expressed in CHO cells and homogenate protein from transfected CHO cells was used as the source of human cRDH and mouse cRDH activity. All assays were carried out under conditions (time = 15 min; protein = 150–180 μg/assay; and substrate = 10 μm) which gave linear rates of product formation, employing the standard cRDH assay conditions described in Materials and Methods. Data reported in this table represent the means ± 1 standard deviation obtained from 5 or more assays carried out using the same CHO cell homogenate protein. Replicate assays were carried out on two or three separate occasions using different sources of transfected CHO cell proteins. These replicate determinations gave similar specific activity values to those reported in this table.

Biochemical characterizations of human and mouse cRDH

Using recombinant human and mouse cRDH expressed in CHO cells as a source of enzymatic activity, we carried out a series of biochemical characterizations of the enzyme. We first asked whether reagents that inhibit other SCDRs or alcohol dehydrogenases also inhibit human or mouse cRDH activity. Study of the effects of phenylarsine oxide (15 μm final concentration), carbenoxolone (0.5 mm), 1,10-phenanthroline (1 mm), 4-methyl pyrazole (1 mm), citral (1 mm final concentration), disulfuran (1 mm final concentration), and zinc acetate (1 mm final concentration) indicated that none of these compounds influenced recombinant human or mouse cRDH activity. In this regard, human and mouse cRDH differ markedly from other members of the SCDR family that catalyze oxidation of all-*trans*-retinol (10, 11, 18).

In order to understand whether natural retinoids may serve as regulators of human and mouse cRDH activity, we asked whether the different retinoic acid isomers are able to act as modulators of human and/or mouse cRDH activity. As can be seen in **Fig. 4**, the all-*trans*-, 13-*cis*-, and 9-*cis*-isomers of retinoic acid proved to be very potent inhibitors of both human and mouse cRDH activity. By far the most potent inhibitor proved to be 13-*cis*-retinoic acid. Approximately 50% of cRDH activity (ID_{50}) was abolished when 13-*cis*-retinoic acid was included in the assay mixture at concentrations of 0.15 μm, a concentration that is less than 2% of that of the 9-*cis*-retinol substrate concentration (10 μm). Although both 9-*cis*-retinoic acid (ID_{50} = 2.6 μm) and all-*trans*-retinoic acid (ID_{50} = 4.6 μm) strongly inhibited cRDH activity; these inhibitory effects were observed at concentrations that are an order of magnitude greater than for 13-*cis*-retinoic acid.

Because of the large differences in the extent of inhibition observed for 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid, it seemed possible that the inhibition by the 9-*cis*- (18 times less potent than 13-*cis*-retinoic acid) and all-*trans*-isomers (30 times less potent than 13-*cis*-retinoic acid) could arise from isomerization to 13-*cis*-retinoic acid during incubation of the assay. To test this possibility, we measured the degree of isomerization of retinoic acid that occurs under our assay conditions. At a concentration of 10 μm retinoic acid (a concentration that results in 66 and 81% inhibition of cRDH activity for all-*trans*-, and 9-*cis*-retinoic acid, respectively), 1.0 ± 0.1% (n = 3) of all-*trans*-retinoic acid and 1.1 ± 0.2% (n = 3) of 9-*cis*-retinoic acid isomerize to

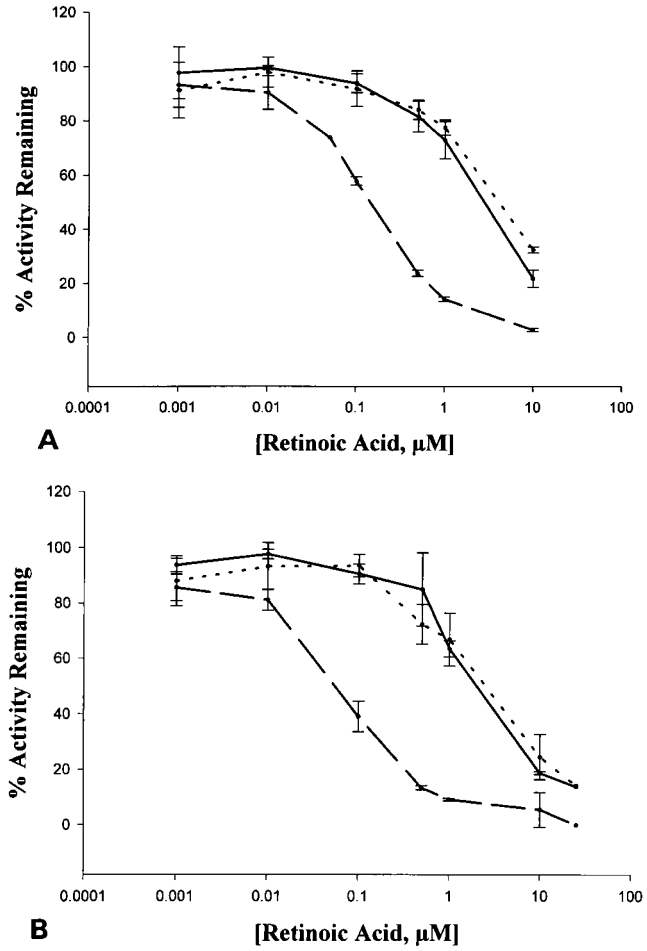


Fig. 4. Effects of all-*trans*-retinoic acid (short dashed line), 13-*cis*-retinoic acid (long dashed line), and 9-*cis*-retinoic acid (solid line) on human (panel A) and mouse (panel B) cRDH activity. Assays were carried out for 15 min at 37°C using 50 μg CHO cell homogenate protein per assay at a concentration of 9-*cis*-retinol substrate of 10 μm and NAD^+ of 2 mm. The error bars represent ± one standard deviation from the mean. When an error bar is not visible, the error bar is sufficiently small so as to be located within the symbol. The values presented in this figure are the means ± standard deviation for all data obtained from three fully independent experiments.

13-*cis*-retinoic acid. This would translate to a final concentration (at the end of the 15-min assay) of approximately 0.1 μm 13-*cis*-retinoic acid. This concentration of 13-*cis*-retinoic acid would be expected to inhibit approximately 40–50% of human cRDH activity if the 13-*cis*-retinoic acid were present in the assay for the full 15-min incubation. It is likely that the isomerization of all-*trans*- and 9-*cis*-retinoic acid occurs over the entire 15-min incubation period. Hence, the 0.1 μm 13-*cis*-retinoic acid concentration arising from isomerization of the all-*trans*- and 9-*cis*-isomers could account for some of the inhibitory actions observed for the all-*trans*- and 9-*cis*-retinoic acid isomers. Nevertheless, isomerization to 13-*cis*-retinoic acid would not seem to account fully for the inhibitory properties of all-*trans*- or 9-*cis*-retinoic acid.

To gain further insights into the chemical properties responsible for 13-*cis*-retinoic acid inhibition of cRDH activity, we tested the potential inhibitory properties of a variety of chemicals that share structural similarities with retinoic acid. Among several compounds tested, only oleic acid, which has one *cis* double bond and a carboxylic acid group, weakly inhibited cRDH enzyme activity. Palmitic acid, which lacks the *cis* double bond, was not an inhibitor nor were several fatty alcohols including petroselinyl alcohol and oleyl alcohol. Finally, all-*trans*-retinol also did not inhibit cRDH activity. Thus, the effects of these retinoic acid isomers on cRDH activity appear to be very specific as other structurally similar retinoids, fatty acids, and fatty alcohols did not markedly influence the activity of either human or mouse cRDH.

Biswas and Russell (30) have reported that rat liver all-*trans*-retinol dehydrogenase type I also uses several 3α-hydroxy- and 17β-hydroxysteroids as substrates. Chai, Zhai, and Napoli (18) also reported that hydroxysteroids are substrates for mouse liver *cis*-retinol dehydrogenases. As human cRDH is approximately 50% identical to the rat liver enzymes and shares approximately 63% homology to mouse liver *cis*-retinol dehydrogenase, we asked whether these steroids might also be substrates for cRDH. To test this possibility we determined whether human or mouse cRDH would catalyze oxidation of androsterone, testosterone, or dihydrotestosterone to corresponding ketone products (using either NAD^+ or $NADP^+$ as a cofactor). We also asked whether human and/or mouse cRDH could catalyze reduction of the corresponding ketones (using either NADH or NADPH as a cofactor). Neither human nor mouse cRDH were found to catalyze oxidations or reductions of potential hydroxysteroid substrates (data not shown). We estimate, based on our lower limits for hydroxysteroid detection by TLC, that we would be able to detect rates of hydroxysteroid oxidation as low as approximately 3 pmol/min per mg protein. This rate of hydroxysteroid oxidation is approximately 2 orders of magnitude lower than V_{max} values reported by Biswas and Russell (30) and Chai et al. (18) for oxidation of hydroxysteroids in their studies of enzymes that are able to oxidize both retinol and hydroxysteroids.

To gain additional insight into the possibility that human or mouse cRDH might possibly have some hydroxysteroid dehydrogenase activity, we also asked whether a relatively large number of 3α-hydroxy-, 11β-hydroxy-, and 17β-hydroxysteroids (a larger number of hydroxysteroids than we tested as potential substrates) are able to inhibit 9-*cis*-retinol oxidation by human or mouse cRDH activity. We reasoned that if a hydroxysteroid was found to be inhibitory then this would suggest that the steroid may be a substrate for cRDH and worthy of further investigation. Hence, we asked whether androsterone, testosterone, estrone, β-estradiol, 4-androstene-3,17dione, 5α-androstane-3,17dione, 5α-androstane-3α,17βdiol, 5α-androstane-3β,17βdiol, or 5α-androstan-17βol-3one, cortisone, corticosterone, hydrocortisone, prednisone, and progesterone could inhibit cRDH activity. For hydroxysteroid concentrations that were equimolar to the substrate concentration (10 μm), none of these hydroxysteroids significantly (i.e., >10%) inhibited either human or mouse cRDH activity when as-

sayed in either the forward (retinol oxidation) or reverse (retinaldehyde reduction) directions.

Endogenous 9-*cis*-retinol concentrations and 9-*cis*-retinol and 9-*cis*-retinoic acid formation

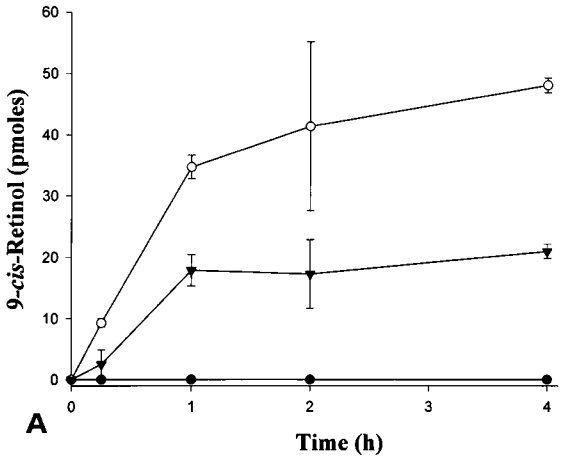
Although Labrecque and colleagues (31) have reported that approximately 10% of the total retinol present in kidneys from vitamin A-repleting rats is present as 9-*cis*-retinol, there is relatively little information regarding tissue concentrations of 9-*cis*-retinol in tissues of vitamin A-sufficient animals or of how this retinol isomer is formed within cells. As a first step towards better understanding the physiology of 9-*cis*-retinol, we measured levels of 9-*cis*- and all-*trans*-retinol in the livers of 3-month-old male mice that had been maintained on a vitamin A-sufficient chow diet. As it seemed possible that some 9-*cis*-isomer could be formed artifactually from all-*trans*-retinol arising from homogenization, extraction and/or analytical procedures, we added all-*trans*-[^{3}H]retinol to the liver minces immediately prior to homogenization. After normal phase HPLC analysis, we assessed the ^{3}H-cpm eluting under the 9-*cis*- and all-*trans*-retinol HPLC peaks. For six mouse livers, all-*trans*-retinol concentrations averaged 26.5 $\pm$ 3.5 μg/g liver (mean $\pm$ 1 standard deviation) whereas the mean 9-*cis*-retinol concentration was determined to be 0.42 $\pm$ 0.18 μg/g liver (mean $\pm$ 1 standard deviation). Thus, these measures indicate that 9-*cis*-retinol is present in mouse liver at a level that is approximately 1.6% of the level of all-*trans*-retinol. The mean level of ^{3}H-cpm detected for the fractions collected under the 9-*cis*-retinol HPLC peaks was approximately 0.53% of the level of ^{3}H-cpm detected for fractions collected from under the all-*trans*-retinol peaks. As artifactual isomerization of all-*trans*-retinol could not account fully for the 9-*cis*-retinol detected in mouse liver, we conclude that 9-*cis*-retinol, albeit at relatively low concentrations, is present endogenously in mouse liver. We did not assess 9-*cis*-retinyl ester concentrations in mouse liver.

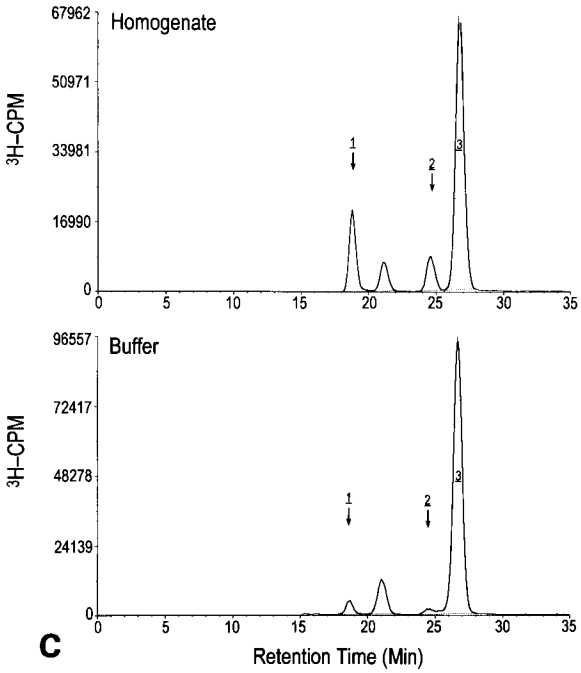
It is still not clear how the 9-*cis*-retinol substrate for cRDH is formed within cells. While carrying out studies of substrate specificity with all-*trans*-retinol, we noted that a small amount of 9-*cis*-retinaldehyde was formed when we tested all-*trans*-retinol as a substrate. We detected 9-*cis*-retinaldehyde formation even though neither 9-*cis*-retinol nor 9-*cis*-retinaldehyde could be detected in the stock solution of all-*trans*-retinol. This suggested that some 9-*cis*-retinol was being formed upon addition of all-*trans*-retinol to the recombinant cRDH/CHO cell homogenate. To investigate this possibility, we carried out a series of experiments to assess whether sham-transfected CHO cell homogenate (lacking cRDH) could catalyze formation of 9-*cis*-retinol from exogenously added all-*trans*-retinol. We found that 9-*cis*-retinol can be formed from all-*trans*-retinol by CHO cell homogenate in a manner that is both time- and homogenate-dependent. Results from this experiment can be seen in **Fig. 5**. We observed 9-*cis*-retinol formation when we added all-*trans*-retinol at a relatively high non-physiologic concentration (10 μm) (Fig. 5, panels A, B, D) to the incubation mixture and when it was added at a relatively low concentration (0.035 μm) (Fig. 5, panel C) more like those of free retinol (not bound to cellular retinol-binding protein, type I (CRBP I)) that might be encountered physiologically within cells that express CRBP I (11). Although the levels of 9-*cis*-retinol formed are not large, this amount of 9-*cis*-retinol formation could certainly account for what is needed to support 9-*cis*-retinoic acid biosynthesis. Thus, it would appear that the cellular environment possesses the capacity to bring about the formation of 9-*cis*-retinol from the more abundant all-*trans*-isomer.

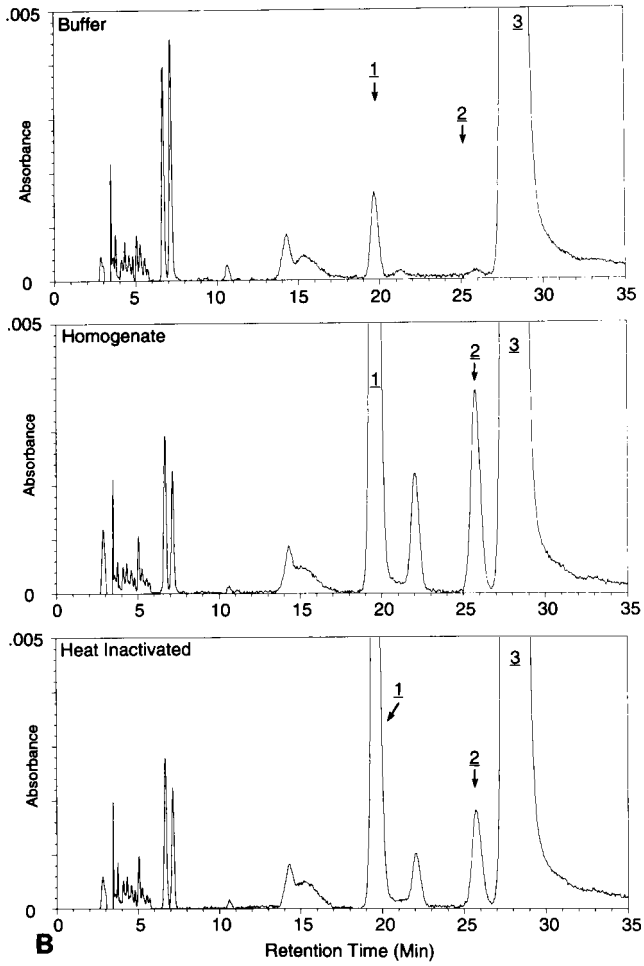
We have also observed in vitro that cRDH is able to support 9-*cis*-retinoic acid formation from 9-*cis*-retinol when coincubated with mouse retinaldehyde dehydrogenase, type 2 (RALDH-2), an enzyme that is reportedly able to cataylze 9-*cis*-retinaldehyde oxidation (31, 32). Because preliminary experiments indicated that CHO cells do not possess an activity able to oxidize 9-*cis*-retinaldehyde to 9-*cis*-retinoic acid in vitro, we transfected CHO cells with mouse cRDH and a cDNA encoding the full sequence for mouse RALDH-2. When 9-*cis*-retinol, at a concentration of 10 μm, was added to cell homogenate containing both enzymes or to sham-transfected CHO cell homogenates, we observed 9-*cis*-retinoic acid formation in the presence but not in the absence of these enzymes. 9-*Cis*-retinoic acid formation did show both time- and protein-dependence. Hence, it would appear that addition of 9-*cis*-retinol to cRDH in the presence of other needed enzyme activities like RALDH-2 does result in 9-*cis*-retinoic acid formation. While it is important to be cautious about extrapolating these data obtained from a very artificial in vitro system to conclusions about the physiologic role of cRDH in vivo, this experiment does provide circumstantial information that links cRDH expression, 9-*cis*-retinol availability, and 9-*cis*-retinoic acid formation.

Chromosome mapping of the murine cRDH

To characterize more definitively the relationship of the mouse and human cRDHs to other retinol dehydrogenases and SCDRs, we mapped the chromosomal location of cRDH in the mouse genome. The Jackson Laboratory interspecific backcross panels (C57BL/6J $\times$ *M. spretus*)F1 $\times$ C57BL/6J (BSB) and (C57BL/6JEi $\times$ SPRET/Ei)F1 $\times$ SPRET/Ei (BSS) were used to map the cRDH gene to its location on a mouse chromosome. To use these backcross panels, it is first necessary to find a polymorphism in cRDH between the two mouse species. For each backcross animal, some regions of its chromosomes are homozygous and some regions are heterozygous in a different pattern. If cRDH is located in the homozygous region, one band is produced; if it is located in a heterozygous region, two bands result. In order to use this approach, a PCR fragment length polymorphism search was first undertaken. As the exons are conserved, we selected PCR primers from the exons for this PCR screen. A polymorphism in the length of intron 4 between the mouse species C57BL/6J and *M. spretus* was observed. We examined 188 animals in order to identify with greater than 1 cM accuracy the chromosomal location of the cRDH gene. The data accumulated from the BSS and BSB panels indicated that the







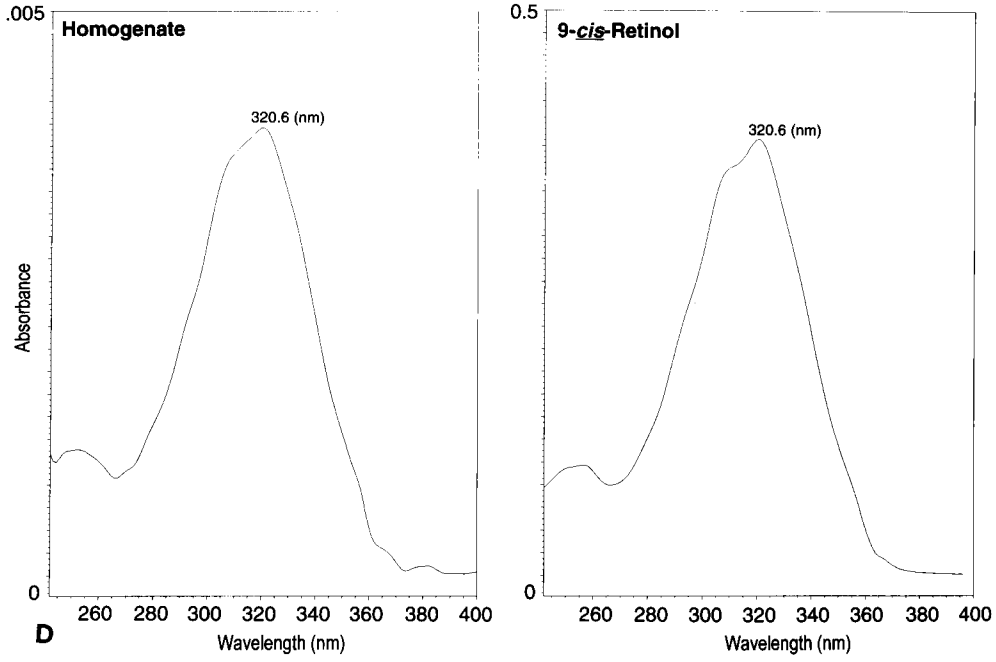


Fig. 5. Panel A: The time-dependence of 9-*cis*-retinol formation from all-*trans*-retinol (10 μm) in the presence of buffer alone (cRDH assay buffer) (closed circles), CHO cell homogenate (open circles), and heat-inactivated CHO cell homogenate (65 °C, 10 min) (closed triangles). Assays were carried out in triplicate and error bars give 1 SD from the mean. Panel B: Normal phase HPLC profiles for extracts made after 4 h incubation of all-*trans*-retinol in buffer alone (upper), in CHO cell homogenate (middle), or in heat-inactivated CHO cell homogenate (lower). The peak labeled "1" corresponds to authentic 13-*cis*-retinol, "2" to 9-*cis*-retinol, and "3" to all-*trans*-retinol. Panel C: Normal phase HPLC profiles for extracts made after 4 h incubation of all-*trans*-[^{3}H]retinol (1 μCi, 0.035 μm) in buffer alone (lower) or in CHO cell homogenate (upper). The numbered peaks in this panel are the same as those given in the legend to panel B above. Panel D: On the left side of panel D, the UV–Vis spectrum is shown for the compound eluting as peak "2" in the middle profile (for CHO cell homogenate) of panel B. On the right side of panel D is the UV–Vis spectrum for authentic 9-*cis*-retinol obtained using the same Waters 996 Photodiode array detector used to obtain the spectrum shown on the left half of this panel.

cRDH gene is located on the most distal region of mouse chromosome 10 (**Fig. 6**). This region is syntenic with human chromosome 12q, the region to which the human 11-*cis*-retinol dehydrogenase (11cRDH) has been mapped previously (33).

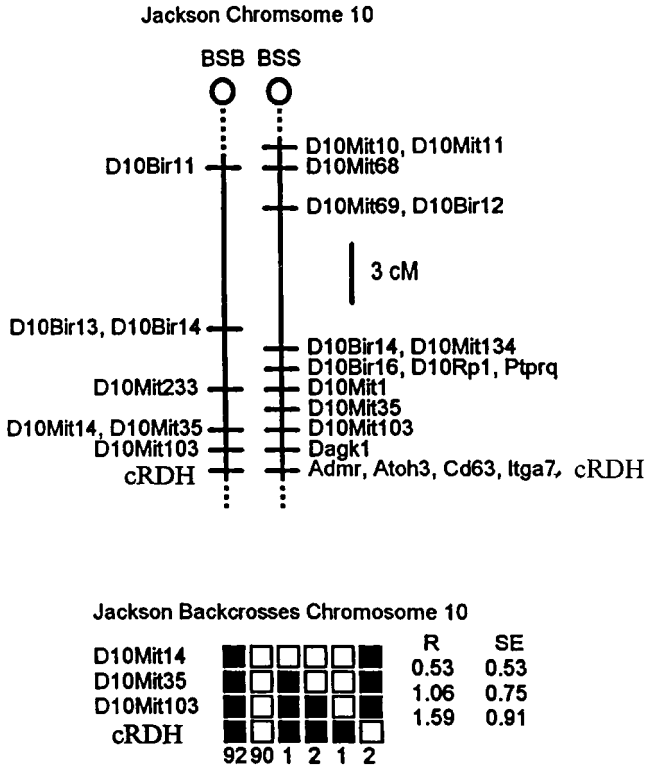


Fig. 6. Chromosome mapping of mouse cRDH gene. Panel A: Map figures from The Jackson Laboratory BSB and BSS backcrosses showing part of chromosome 10. The maps are depicted with the centromeres toward the top. A 3 cM scale bar is shown to the right of the figure. There are large portions of the chromosome not shown, as indicated by the dashed lines. Loci mapping to the same position are listed in alphabetical order. Missing typings were inferred from surrounding data where assignment was unambiguous. Panel B: Haplotype figure from the combined data of The Jackson Laboratory BSB and BSS backcross showing part of chromosome 10 with loci linked to cRDH. Loci are listed in order with the most proximal at the top. The black boxes represent the C57BL6/JEi allele and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percent recombination (R) between adjacent loci is given to the right of the figure, with the standard error (SE) for each R. Raw data from The Jackson Laboratory can be obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdatas.

DISCUSSION

It is generally accepted that 9-*cis*-retinoic acid is an important retinoid needed for regulating the actions of members of the RXR family of retinoid nuclear receptors (4, 5). There is only limited information available regarding how 9-*cis*-retinoic acid is formed and how it is oxidatively and/or conjugatively metabolized (6). Three possible pathways for 9-*cis*-retinoic acid formation have been proposed in the literature (6, 19, 31–36). It is clear that 9-*cis*-retinoic acid can be formed through isomerization of all-*trans*-retinoic acid (6, 34, 35). Cleavage of 9-*cis*-β-carotene, a carotenoid that is present in foods, has been proposed as a second pathway resulting in 9-*cis*-retinoic acid formation (36). This, however, cannot be an essential pathway for 9-*cis*-retinoic acid formation as animals maintained on a carotenoid-free diet are normal. Additionally, 9-*cis*-retinoic acid can be synthesized through oxidation of 9-*cis*-retinol to 9-*cis*-retinaldehyde followed by its oxidation to 9-*cis*-retinoic acid (19, 23, 31, 32). Several short and medium chain alcohol dehydrogenases able to catalyze 9-*cis*-retinol oxidation, and several aldehyde dehydrogenases able to catalyze 9-*cis*-retinaldehyde oxidation have been identified (6, 18, 19, 23). 9-*cis*-Retinol is reported to be present in liver and kidney of vitamin A-repleting rats where these enzymes are expressed (19, 23, 31, 32, 37). However, there is very little information available regarding endogenous 9-*cis*-retinol concentrations in vitamin A-sufficient animals or regarding how 9-*cis*-retinol is formed within tissues. Our data (see Fig. 5) show that 9-*cis*-retinol is readily formed, in a time- and protein-dependent manner, when free all-*trans*-retinol (not bound to cellular retinol-binding protein) is incubated in the presence of a homogenate prepared from CHO cells. Moreover, we have demonstrated that 9-*cis*-retinol, albeit at concentrations that are less than 1% of those of all-*trans*-retinol, is present endogenously in livers of mice maintained on a control diet. These findings that 9-*cis*-retinol is present endogenously in mouse liver, a tissue that expresses cRDH, and that 9-*cis*-retinol formation can be catalyzed upon addition of all-*trans*-retinol to a cell homogenate provide further support for the hypothesis that cRDH or other "*cis*-retinol dehydrogenases" (18, 22) are importantly involved in 9-*cis*-retinoic acid formation.

Our studies indicate that human and mouse cRDH have strikingly similar biochemical properties. The cDNA for mouse cRDH shares 85.5% homology with the human cRDH cDNA. At the amino acid level, mouse and human

cRDH are 87.1% identical. Both human and mouse cRDH will catalyze the NAD^+-dependent oxidations of 9-*cis*-, 11-*cis*-, and 13-*cis*-retinol and the NADH-dependent reductions of 9-*cis*-, 11-*cis*-, and 13-*cis*-retinaldehyde (see Table 1). $NADP^+$ and NADPH can substitute for the unphosphorylated cofactors but these will only support approximately 10% or less of the maximal reaction velocities observed for NAD^+ or NADH. Human and mouse cRDH are not inhibited by ethanol or zinc chelators (data not shown) and both human and mouse cRDH are potently inhibited by 13-*cis*-retinoic acid at concentrations as low as 0.05 μm, and by 9-*cis*- and all-*trans*-retinoic acid at concentrations in the 1 to 5 μm range, but not significantly by fatty acids or fatty alcohols. Similarly, human and mouse cRDH do not catalyze either hydroxysteroid oxidation or reduction and are not inhibited by a large number of hydroxysteroids. Based on these very similar biochemical characteristics, we believe the mouse cDNA described above encodes a protein that is truly the mouse homologue of the human cRDH. Moreover, the catalytic properties of cRDH seem to be well conserved across these two species, suggesting a highly defined physiologic role for the enzyme.

Both human and mouse cRDH share a great sensitivity to inhibition by 13-*cis*-retinoic acid (see Fig. 4). Both all-*trans*- and 9-*cis*-retinoic acid are at least one order of magnitude less potent inhibitors of cRDH activity. Approximately 50% of human cRDH activity is inhibited at 13-*cis*-retinoic acid concentrations around 0.1 μm. This is a concentration which is only 5- to 10-times greater than the normal physiologic concentrations of 13-*cis*-retinoic acid reported for rat and mouse tissues (38, 39). It is possible, considering the less than one order of magnitude difference in the concentrations of 13-*cis*-retinoic acid needed to inhibit cRDH and those that are present physiologically in blood and tissues, that fluxes in 13-*cis*-retinoic acid concentration could play a direct role in regulating cRDH activity under normal physiologic conditions. As the concentrations of 13-*cis*-retinoic acid reached in the blood and tissues of both animals and humans receiving 13-*cis*-retinoic acid pharmacologically can reach 10 μm or greater (38–40), it is probable that cRDH inhibition will be observed upon pharmacological use of 13-*cis*-retinoic acid.

In addition to its actions on cRDH activity, the literature indicates that 13-*cis*-retinoic acid can act as an inhibitor of several enzymes proposed to be important for retinol and hydroxysteroid oxidation. It is well established that both all-*trans*- and 13-*cis*-retinoic acid administration to frogs can act in vivo to block the visual cycle through a mechanism proposed to involve the 11-*cis*-retinol dehydrogenase (11cRDH) known to be present in the RPE (41). Biswas and Russell (30) have demonstrated that 13-*cis*-retinoic acid can inhibit the oxidation of 3α-adiol by rat liver and human prostate 17β-hydroxysteroid dehydrogenase and human and rat retinol dehydrogenase, type I in vitro. Interestingly, 9-*cis*-retinoic acid is an even more potent inhibitor of the human 17β-hydroxysteroid dehydrogenase than the 13-*cis*-isomer (K_i for 13-*cis*-retinoic acid of 4 μm compared to a K_i of 0.4 μm for 9-*cis*-retinoic acid) whereas 13-*cis*-retinoic acid is a more potent inhibitor than 9-*cis*-retinoic acid of the human and rat retinol dehydrogenase-catalyzed oxidation of 3α-adiol (30). Recently, Allali-Hassani and colleagues (42) have demonstrated in vitro inhibition by 13-*cis*-retinoic acid of purified human alcohol dehydrogenase type IV, an enzyme that has retinol dehydrogenase activity. However, for all of these studies, the concentration of 13-*cis*-retinoic acid needed to bring about significant inhibition of retinol oxidation was in the μm range. The inhibitory potency of 13-*cis*-retinoic acid for both human and mouse cRDH is at least an order of magnitude lower than that needed to bring about significant inhibition of these other retinoid-metabolizing enzymes. Taken together, these data are consistent with the hypothesis that 13-*cis*-retinoic acid may act as a regulator of enzymes that catalyze formation of physiologically active retinoids and steroids.

Although the biochemical properties of human and mouse cRDH are very similar, the tissue distribution of cRDH in the mouse is not identical to that of the human enzyme. One very striking difference between cRDH mRNA expression in the human and the mouse can be seen with mammary tissue (see Fig. 3). Mouse mammary tissue is essentially devoid of cRDH mRNA (Fig. 3). This is unlike the human situation where mammary tissue is the tissue site where cRDH is most highly expressed (19). Another striking difference in this pattern is seen for the testis. In the human, the testis shows a level of expression for cRDH mRNA that is exceeded by only mammary tissue and kidney (19). However, as assessed by Northern blot (Fig. 3), the mouse testis either does not express detectable cRDH mRNA or expresses an alternatively spliced species. It seems probable, considering our findings described above in the Results section regarding identification of alternatively processed cRDH cDNAs in testis cDNA libraries, that mouse testis does express cRDH mRNA, but as an alternatively spliced form. The human heart, lung, and placenta all express cRDH mRNA, albeit at relatively low levels, whereas no expression of mouse cRDH mRNA can be detected in these tissues by Northern analysis. Thus, it would seem that cRDH is more broadly and/or more highly expressed in the human than in the mouse. This would suggest that there may be differences across species in how tissues synthesize/obtain 9-*cis*-retinoic acid. Although we would expect these differences would have only a minor impact on whole body retinoid metabolism, these may be of significance for understanding known species differences in the levels of different retinoic acid isomers and their metabolites present in tissues and blood (43, 44).

Our data and the data of others raise an important question regarding cRDH and its identity (19, 23, 24, 33). We first identified cRDH activity through expression and enzymatic assay of a cDNA clone obtained from a human mammary tissue cDNA library (19). Two decades ago, Lion and colleagues (45) identified an enzyme, 11cRDH, in the bovine retinal pigment epithelium which catalyzed the formation of the visual pigment 11-*cis*-retinaldehyde from 11-*cis*-retinol. Later reports by other investigators

concerning this ocular enzyme indicated that the bovine enzyme will not catalyze 9-*cis*-retinol oxidation (46). In 1995, two groups reported the independent cloning of the cDNA for bovine 11cRDH. (15, 16). Both indicated that 11cRDH mRNA could be detected only in the retinal pigment epithelium (15, 16). With the publication of the description of the genomic clone for human 11cRDH (31), it seemed to us that human 11cRDH and human cRDH were very similar, if not identical. Alternatively, it was also possible that the gene described as human 11cRDH was actually that of cRDH as no measures of the enzymatic activity of the expressed cDNA clone for this gene were reported (33). The group reporting the genomic clone for human 11cRDH (and the earlier cloning of a bovine 11cRDH cDNA (15)) is the same group that recently described the cloning of mouse cRDH in Romert et al. (22). In the text of Romert et al. (22), these investigators express the belief that their cRDH is distinct from 11cRDH. The recent report of Driessen et al. (23), another group that reported cloning a cDNA for bovine 11cRDH (16), indicates that these investigators believe that cRDH and 11cRDH are the same enzyme, and that the earlier eye literature had been incomplete in its characterization of the tissue distribution and substrate specificities of 11cRDH (15, 16, 45–47). Although we have not worked with ocular tissue nor with recombinant preparations acknowledged to have 11cRDH activity, based on our cDNA cloning data, the biochemical properties of human and mouse cRDH and the genomic structure of the cRDH gene (see below), we are inclined to accept the view put forth by Driessen et al. (23) that cRDH and 11cRDH are indeed the same protein. Consequently, we would suggest that 11cRDH and cRDH, in the future, should simply be referred to as *cis*-retinol dehydrogenase (cRDH).

We have mapped the mouse gene for cRDH to the most distal region of mouse chromosome 10 (Fig. 6). This region of mouse chromosome 10 was also recently proposed to be the site of the mouse 11cRDH gene by Driessen et al. (47). These authors indicate that the gene is composed of 5 exons and they have provided some information about the approximate sizes of each exon. In addition, Driessen et al. (47) reported that the gene is probably a single copy gene. Based on this information from the literature and our data on the structure and location of the mouse cRDH gene, it would appear that we and Driessen et al. (47) have cloned the same gene. This too is consistent with the conclusion reached above that cRDH and 11cRDH are the same enzyme (i.e., cRDH) and moreover, this adds much weight to this conclusion.

In summary, we have characterized the tissue distribution of cRDH in adult and fetal mouse and fetal human tissues along with some of the biochemical properties of human and mouse cRDH. In addition, we report on the gene structure for mouse cRDH. Our demonstration of cRDH expression in first trimester human embryos coupled with our observation that 13-*cis*-retinoic acid is a specific and highly potent inhibitor of cRDH activity raises the possibility that one biochemical basis for the toxic actions of 13-*cis*-retinoic acid may arise from its effect on cRDH activity. It also is clear from our studies that 9-*cis*-retinol can be formed in a cellular milieu in a time- and protein-dependent manner from free all-*trans*-retinol. Overall, these findings, along with literature reports of the presence of 9-*cis*-retinol in some tissues that express cRDH (31, 35), are consistent with our earlier suggestion that cRDH plays a physiologic role in the formation of 9-*cis*-retinoic acid (19). Nevertheless, the physiologic importance of cRDH in a 9-*cis*-retinol-utilizing metabolic pathway for generating 9-*cis*-retinoic acid will require further investigation to establish this conclusively.

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