A retinol dehydrogenase, RoDH(I), which recognizes holo-cellular retinol-binding protein (CRBP) as substrate, has been cloned, expressed, and identified as a short-chain dehydrogenase/reductase (Chai, X., Boerman, M. H. E. M., Zhai, Y., and Napoli, J. L. (1995) J. Biol. Chem. 270, 3900–3904). This work reports the cloning and expression of a cDNA encoding a RoDH isozyme, RoDH(II). The predicted amino acid sequence verifies RoDH(II) as a short-chain dehydrogenase/reductase, 82% identical with RoDH(I). RoDH(II) recognized the physiological form of retinol as substrate, CRBP, with a $K_m$ of 2 nM. Similar to microsomal RoDH and RoDH(I), RoDH(II) had higher activity with NADP rather than NAD, was stimulated by ethanol and phosphatidyl cholines, was not inhibited by the medium-chain alcohol dehydrogenase inhibitor 4-methylpyrazole, but was inhibited by phenylarsine oxide and the short-chain dehydrogenase/reductase inhibitor carbenoxolone. Northern blot analysis detected RoDH(I) and RoDH(II) mRNA only in rat liver, but RNase protection assays revealed RoDH(I) and RoDH(II) mRNA in kidney, lung, testis, and brain. These data indicate that short-chain dehydrogenases/reductase isozymes expressed tissue-distinctively catalyze the first step of retinoic acid biogenesis from the physiologically most abundant substrate, CRBP.

Metabolic activation of retinol provides the hormone all-trans-retinoic acid (RA). RA produces a variety of biological responses by modulating the expression of genes that regulate the state of differentiation or entry into apoptosis of diverse cell types in numerous organs (1–5). A model of RA biosynthesis postulates that the enzyme(s) that catalyze RA synthesis physiologically recognize as substrate the predominant form of retinol bound within CRBP (6). The CRBP concentration exceeds that of retinol ($7 \text{ versus } 5 \text{ nM}$, respectively (7)), and CRBP has a high affinity interaction with retinol ($K_a \approx 0.1–1 \text{ nM}$), much higher than substrate-enzyme interactions (8, 9). Binding of retinol within CRBP shields the prohormone from the cellular environment and would confer specificity on RA biosynthesis by restricting access of retinol to enzymes capable of recognizing and interacting with the CRBP-retinol "cassette." This would prevent opportunistic oxidation by dehydrogenases/oxydases with inexact substrate tolerances, thereby contributing to precise spatial-temporal control over RA biogenesis. CRBP binding would also protect retinol from nonenzymatic oxidation and cells from the membrane-altering faculty of unbound retinol (10–12). A pathway of RA biosynthesis consistent with this hypothesis involves as the first and rate-limiting step production of retinol in microsomes with holo-CRBP as substrate, i.e. RoDH (13, 14). Retinal generated in microsomes from holo-CRBP by RoDH supports RA biosynthesis by cytosolic retinal dehydrogenases (15).

A microsomal RoDH has been partially purified, its active site has been identified with a 34 kDa polypeptide by chemical cross-linking with holo-CRBP, and its cDNA has been cloned and expressed (16–18). This RoDH, hereafter termed RoDH(I), belongs to the family of short-chain dehydrogenase/reductase (19). By Northern blot analysis, mRNA expression of RoDH(I) was detected only in rat liver, despite the well established occurrence of CRBP-recognizing RoDH activity in multiple tissues. These results suggested occurrence of multiple RoDHs.

This work reports the cDNA cloning and expression of a second RoDH, RoDH(II), shows that it is a previously unknown short-chain dehydrogenase/reductase that can catalyze the first step in RA synthesis with CRBP as substrate, and compares the expression of RoDH(II) mRNA in rat tissues with that of RoDH(I) by RNase protection assays.

### MATERIALS AND METHODS

**Library Screening**—A rat liver cgt11 cDNA library (Clontech) was screened through three rounds with probe A (nucleotides 653–975 of RoDH(I)), as described (18). DNA from one of the three plaques obtained was cloned into p-Direct to provide p-DirectRo3, which contained a 1.5-kilobase cDNA insert distinct from RoDH(I) but with no initiation codon. Probe B (nucleotides 587–909 of the final product) was prepared from p-DirectRo3 by Aval digestion and used to identify 36 plaques by screening the same library through three rounds. The inserts of these plaques were amplified by PCR and analyzed by Southern blot with a synthetic 32-base pair oligonucleotide (probe C, nucleotides 974-1005 of the final cDNA). Six PCR products hybridized at 42 °C to probe C and were washed at 68 °C in 0.1% SDS in 0.1 × SSC (pH = 10 NaCl and 15 mM sodium citrate). The longest was cloned into pBluescript to provide PBSK/RoDH(II). The insert in PBSK/RoDH(II) was sequenced by dideoxy chain termination with T7 DNA polymerase.

**Expression of RoDH**—The coding region of RoDH(II) of PBSK/RoDH(II) was amplified with the sense primer 5'-CGCGGATTCCCTCCAGGTGTCCTTAC-3' (nucleotides 206–223) and the antisense primer 5'-CGGAATTCCCTGCTTGTCTTCTAC-3' (nucleotides 1233 to 1216) containing BamHI and EcoRI cleavage sites, respectively (underlined). The PCR product was ligated into pUC3 DNA (Invitrogen) to yield pUC3/RoDH(II). pUC3/RoDH(II) was transfected by calcium phosphate/DNA precipitation into semi-confluent P19 cells as described (18). Mock transfections were done with pUC3DNA. 24 h after transfection, cells were harvested, the pellets were suspended in 10 mM Hepes,

### EXPRESSION OF ITS mRNA RELATIVE TO TYPE I*

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1 The abbreviations used are: RA, all-trans-retinoic acid; CRBP, cellular retinol-binding protein type I; PAO, phenylarsine oxide; PCR, polymerase chain reaction; 10Ksup, 10,000 × g supernatant of a cell homogenate.
Northern Blot Analysis—Northern blot analysis was described as described (18) with 5 mg of poly(A)+ mRNA hybridized at 65 °C for 16 h to a RoDH(II) probe of the 841 nucleotide FokI/SphiI product (nucleotides 1148–1988) from the 3’-untranslated region of PBSK/RoDH(II). The blot was probed with a glyceraldehyde dehydrogenase cDNA probe.

RNAse Protection Assays—The RoDH(II)-specific probe was obtained by amplifying a 341-base pair fragment from pDirectRoZ (18) by PCR with the sense primer 5’-CGCGGATCCTTTTCAAGACTCTCTCA-3’ (nucleotides 849–865) and the antisense primer 5’-CGGAATTCGGAAGGTAGCTCATG-3’ (nucleotides 1190–1173) containing BamHI and EcoRI cleavage sites, respectively (underlined). The RoDH(II)-specific probe was obtained by amplifyng a 341-base pair fragment from PBSK/RoDH(II) by PCR with the same sense primer used for RoDH(II) and the antisense primer 5’-CGGAATTCCGGAAGGTAGCTCATG-3’ (nucleotides 1140–1123) containing an EcoRI cleavage site (underlined). The fragments were subcloned into pCDNA3 in the anti-sense orientations, and the plasmids were linearized with BamHI.

2p-labeled antisense riboprobes were transcribed with SP6 RNA polymerase (Ambion) for 1 h at 37 °C in 10 mM dithiothreitol, 0.5 mM each ATP, CTP, and GTP, 50 mM UTP, and 50 mM α-32PUTP. DNA templates were removed with DNase I. The 450-base pair transcripts, which included 109 base pairs of plasmid, were purified with a 5% polyacrylamide/urea gel, and the fragments were resuspended in 20 ml of 80% deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate, and 1 mM EDTA, pH 6.4, and were incubated at 45 °C for 20 h. The same amounts of probes were hybridized with 10 mg of yeast RNA as control. After digestion with RNase A (2.5 units/ml) and RNase T1 (100 units/ml) for 30 min at 37 °C, the protected fragments were resuspended on 5% polyacrylamide 8% urea gels and visualized at 80 °C overnight with one intensifying screen. Quantification was done with a Bio-Rad Model GS-670 densitometer.

RoDH Assay—Assays for retinol metabolism were done in duplicate with retinol bound to excess CRBP for 30 min at 37 °C in 0.5 ml of 10 mM Hepes, 150 mM KCl, 2 mM EDTA, and 2 mM NADP, pH 8, with 2 mM MgCl2 and 0.8 mM phosphatidylcholine (added in 2 μl of ethanol). Retinal was quantified by high performance liquid chromatography (18, 20, 21). Hdo- and apo-CRBP were generated in Escherichia coli from pMCR-22 (12) and were purified (15).

RESULTS AND DISCUSSION

cDNA and Amino Acid Sequence—During screening of a cDNA library with a probe from RoDH(I), a 1.5-kilobase partial cDNA was identified with sequence distinct from RoDH(I). A probe from this cDNA was used to re-screen the library. The longest identified cDNA (2 kilobase) was subcloned to create PBSK/RoDH(II) and was sequenced in both directions to reveal a cDNA with a nucleotide sequence distinct from that of RoDH(I).

A single open reading frame in PBSK/RoDH(II) predicts an RoDH(II) of 317 amino acids with a calculated molecular mass of 35 kDa; the same as RoDH(I) (Fig. 1). Of the 23 amino acid residues conserved in >70% of the extended short-chain dehydrogenase/reductase family, 21 have been conserved in RoDH(II) (19). These include the residues typical of an short-chain dehydrogenase/reductase: the G(X)GXX cofactor binding site (Gly33), N-terminal to the active site; the sequence LXXNAG (Leu109), and the active site Y(X)K (Try176). C-terminal to the cofactor binding site. One of the two substitutions, D107W, represents a common substitution in short-chain dehydrogenase/reductase; the second, A191R, also occurs in the short-chain dehydrogenase/reductases 11β-hydroxysteroid and 18β-hydroxybutyrate dehydrogenases of rat (23, 24). RoDH(II) differs from RoDH(I) in 57 amino acid residues; most are non-conservative substitutions. Seven of these have been verified in RoDH(II) by microsequencing (18). Yet the predicted primary sequence of RoDH(II) diverges less from RoDH(I) compared with a bovine 11-cis-retinol dehydrogenase, a short-chain dehydrogenase/reductase expressed only in retinal pigment epithelium (25), or to other rat short-chain dehydrogenase/reductases (Table I). A second Y(X)K pattern 15 residues closer to the C terminus from the probable active site Y(X)K presents a curious feature of both RoDH(II) and RoDH(I). The first Y(X)K most likely serves as the active site because of its position in the primary sequence and because it resembles the Y(C/G/S)(A/I/S)(T/S)K sequence of other short-chain dehydrogenase/reductase active sites (26).
Fig. 1. Nucleotide and deduced amino acid sequences of RoDH(II).

The first line of amino acid sequence immediately below the nucleotide sequence shows the predicted amino acid sequence of RoDH(II). The underlined residues in this line identify the 21 amino acids in RoDH(II) conserved in >70% of the 57 members of the extended short-chain dehydrogenase/reductase superfamily (19). The second line of amino acid sequence represents RoDH(I). The blanks indicate residues identical to RoDH(II). The underlined residues in this second line are those that have been determined by microsequencing of RoDH(II) (18).

TABLE I

| Dehydrogenase | mRNA loci | Amino acid homology | Ref. |
|---------------|-----------|---------------------|-----|
|                |           | similarity         | identity | %  |
| Rat RoDH(II)   | Liver     | >90, kidney > brain > lung > testis | 100     | 100 |
| Rat RoDH(I)    | Liver     | >90, >> lung > testis > brain > kidney | 95.6   | 82  |
| Bovine 11-cis-retinol | Retinal pigment epithelium | 69.5 | 53.8 |
| Rat 11β-hydroxybutyrate | Liver | 58.9 | 37.8 |
| Rat 11β-hydroxysteroid | Kidney, liver, testis | 46.6 | 20.6 |

Retinol Dehydrogenase Type II

K_m compares well with the previously determined values for holo-CRBP of 1.6 mM for rat liver microsomal RoDH, 0.6 mM for partially purified RoDH(I), and 0.9 mM for recombinant RoDH(I) (13, 16, 18).

Tissue Distribution of RoDH mRNA—Northern blot analyses of RoDH(II) revealed a 1.8-kilobase mRNA in liver but did not detect mRNA in brain, kidney, lung, or testis (data not shown), just as had occurred with RoDH(I) (18). RNase protection as-
says of RoDH(I) and RoDH(II), however, showed wider tissue distribution of both mRNAs (Fig. 5; Table I). Liver was the major site of expression of both RoDHs. Expression of RoDH(I) in the extra-hepatic tissues screened was <2% of liver. RoDH(II), in contrast, had relatively abundant expression in kidney and more abundant expression in brain and lung than RoDH(I). Testis had equivalent expression of RoDH(II) and RoDH(I). RNase protection assays under high stringency conditions revealed many protected fragments that could not be rationalized from the nucleotide sequences of RoDH(I) and RoDH(II) and the sequences of the probes, consistent with occurrence of closely related mRNAs, possibly from additional isozymes of RoDH. This expression of RoDH mRNA among multiple tissues reflects the ability of multiple tissues to biosynthesize RA (12, 13, 36, 37) and the widespread tissue distribution of CRBP (38–40).

Concluding Summary—This work identifies a second RoDH as another isozyme that catalyzes the first step in RA biosynthesis. Occurrence of this heretofore unknown short-chain dehydrogenase/reductase indicates the importance of tissue-distinctive expression of RoDHs to RA biogenesis. The 5 mM concentration of holo-CRBP in normal rat liver exceeds the $K_m$ value for RoDH(I) and (II), consistent with physiological roles for them in RA biogenesis (7). Their ability to recognize CRBP provides a mechanism for accessing the major pool of retinol in vivo while allowing CRBP to control the availability and distribution of retinol. Two other classes of proteins important to
superfamily of steroid/lipid-specific proteins. Potentially important to retinoid action that belong to a larger tors (41–43) and sterol/lipid-binding proteins (44, 45), respectively. Thus, RoDHs represent a third class of proteins retinoid function are expressed in definite temporal-spatial patterns, the receptors RAR and RXR and the binding proteins CRBP and cellular retinoic acid-binding protein. These proteins also belong to superfamilies of sterol/lipid hormone receptors (41–43) and sterol/lipid-binding proteins (44, 45), respectively. Thus, RoDHs represent a third class of proteins potentially important to retinoid action that belong to a larger superfamily of sterol/lipid-specific proteins.

REFERENCES
1. Lotan, R. (1988) Prog. Clin. Biol. Res. 259, 261–271
2. Chytil, F., and ul-Haq, R. (1990) Crit. Rev. Eukaryotic Gene Expression 1, 61–73
3. Fesus, L., Davies, P. J., and Piacentini, M. (1991) Eur. J. Cell Biol. 56, 170–177
4. Lohnes, D., Dierich, A., Ghyselinck, N., Kastner, P., Lampron, C., LeMeur, M., Luftin, T., Mendelsohn, C., Nakshatri, H., and Chambon, P. (1992) J. Cell Sci. 16, 69–76
5. Semb, R. D. (1994) Clin. Infect. Dis. 19, 489–499
6. Napoli, J. L. (1993) J. Nutr. 123, 362–366
7. Harrison, E. H., Blaner, W. S., Goodman, N. C., Li, E., and Gordon, J. I. (1987) Biochemistry 26, 3622–3629
8. Li, E., Qian, S., Winter, N. S., d’Avignon, A., Levin, M. S., and Gordon, J. I. (1988) Biochemistry 27, 7027–7037
9. Cowan, S. W., Newcomer, M. E., and Jones, T. A. (1993) J. Mol. Biol. 230, 1225–1246
10. Napoli, J. L., Posch, K. C., Fiorella, P. D., and Boerman, M. H. E. M. (1991) Biomed. Pharmacother. 45, 131–143
11. Ong, D. E. (1994) Nutr. Rev. 52, suppl.) 24–31
12. Napoli, J. L. (1994) in Basic Science and Clinical Aspects of Vitamin A in Health and Disease (Blomhoff, R., ed) Chapter 6, Marcel Dekker, New York
13. Posch, K. C., Boerman, M. H. E. M., Burns, R. D., and Napoli, J. L. (1991) Biochemistry 30, 6224–6230
14. Napoli, J. L., Posch, K. C., and Burns, R. D. (1992) Biochim. Biophys. Acta 1120, 183–186
15. Posch, K. C., Burns, R. D., and Napoli, J. L. (1992) J. Biol. Chem. 267, 19676–19682
16. Boerman, M. H. E. M., and Napoli, J. L. (1995) Biochemistry 34, 7027–7037
17. Boerman, M. H. E. M., and Napoli, J. L. (1995) Arch. Biochem. Biophys. 321, 434–441
18. Chai, X., Boerman, M. H. E. M., Zhai, Y., and Napoli, J. L. (1995) J. Biol. Chem. 270, 3900–3904
19. Jörnvall, H., Persson, B., Krok, M., Attrian, S., González-Durante, R., J effery, J., and Ghosh, D. (1995) Biochemistry 34, 6003–6013
20. Napoli, J. L. (1986) Methods Enzyom. 123, 112–124
21. Napoli, J. L. (1990) Methods Enzymo. 189, 470–482
22. Levin, M. S., Locke, B., Yang, N. C., Li, E., and Gordon, J. I. (1988) J. Biol. Chem. 263, 17715–17723
23. Churchill, P., Hempel, J., Romovae, H., Zhang, W.-W., Brennan, M., and Churchill, S. (1992) Biochemistry 31, 3793–3799
24. Agarwal, A. K., Monder, C., Eckstein, B., and White, P. C. (1989) J. Biol. Chem. 264, 18929–18943
25. Simon, A., Hellman, U., Wernstedt, C., and Eriksson, U. (1995) J. Biol. Chem. 270, 1107–1112
26. PavloviS, J. E., and Penning T. M. (1994) J. Biol. Chem. 269, 13502–13510
27. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
28. Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) J. Mol. Biol. 120, 97–120
29. Marks, A. R., Mcintyre, J. O., Duncan, T. M., Erdjument-Bromage, H., Tempst, P., and Flierscher, S. (1992) J. Biol. Chem. 267, 15439–15463
30. Jauhiainen, M., Stevenson, K. J., and Dolphin, P. J. (1988) J. Biol. Chem. 263, 6525–6533
31. Berlieth, E. S., Kasperek, E. M., Grill, S. P., Braunscheidel, J. A., and C. R. (1989) J. Biol. Chem. 264, 17715–17723
32. Hoffman, R. D., and Lane, M. D. (1992) J. Biol. Chem. 267, 14005–14011
33. Monder, C., Stewart, P. M., Lakshmi, V., Valentino, R., Burt, D., and Edwards, C. R. (1989) Endocrinology 125, 1046–1053
34. Edwards, C. R., Walker, B. R., Benediktssson, R., and Seckel, Y. R. (1993) J. Steroid Biochem. Mol. Biol. 45, 1–5
35. Leatherbarrow, R. J. (1987) Endfitter: A Nonlinear Regression Data Analysis Program, Elsevier-Biosoft, Cambridge, UK
36. Napoli, J. L., and Race, K. R. (1987) Arch. Biochem. Biophys. 255, 95–101
37. Siegenthaler, G., Sauvat, J. H., and Ponec, M. (1990) Biochem. J. 268, 371–378
38. Ong, D. E., Crow, J. A., and Chytil, F. (1982) J. Biol. Chem. 257, 13385–13389
39. Gustafson, A.-L., Dencher, L., and Eriksson, U. (1993) Development 117, 451–460
40. Roberte, E., Friederich, V., Morris-Kay, G., and Chambon, P. (1992) Development 115, 973–987
41. Evans, R. (1988) Science 240, 889–895
42. Leid, M., Kastner, P., and Chambon, P. (1992) Trends Biochem. Sci. 17, 427–433
43. Petkovich, M. (1992) Annu. Rev. Nutr. 12, 443–471
44. Chytil, F., and Ong, D. E. (1987) Annu. Rev. Nutr. 7, 321–335
45. Clarke, S. D., and Armstrong, M. K. (1989) FASEB J. 3, 2480–2487

TABLE II
Relative mRNA levels of RoDH(II) and RoDH(I) in rat tissues.

| Tissue   | RoDH(I) | RoDH(II) |
|----------|---------|----------|
| Liver    | 100     | 41       |
| Kidney   | 0.2     | 10.2     |
| Brain    | 0.6     | 3.3      |
| Lung     | 1.3     | 1.8      |
| Testis   | 1.2     | 1.2      |

Signals were normalized to the signal for β-actin mRNA by densitometry.
