Coexpression of the mRNAs Encoding Retinol Dehydrogenase Isozymes and Cellular Retinol-Binding Protein

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We used in situ hybridization of adult rat tissue to show that mRNAs encoding cellular retinol-binding protein (CRBP) and retinol dehydrogenase (RoDH) isozymes I/III and II were expressed in hepatocytes uniformly throughout the liver lobule, but were absent from Kupffer cells and endothelial cells of blood vessels and bile ducts. In kidney, CRBP, RoDH(I), and RoDH(II) were found in the proximal tubules of the cortex. Distal tubules, Henle's loops, collecting ducts, and glomeruli showed little, if any, expression. In testis, CRBP, RoDH(I), and RoDH(II) were found in Sertoli cells. Expression, albeit weaker, also occurred in spermatogonia and primary spermatocytes. Peritubular cells and other germ cells had even weaker expression. Only CRBP and RoDH(II) mRNA were detected in interstitial cells. In lung CRBP, RoDH(I) and RoDH(II) were expressed most intensely in the epithelium of the bronchi and bronchioli, but also occurred in the simple columnar epithelial cells of the alveolar duct and in alveolar type II cells. These data are consistent with the hypothesis that holo-CRBP serves as substrate for retinoic acid biosynthesis because they show that the substrate and the enzyme occur in the same cellular loci in vivo. These data also indicate that multiple cellular sites of retinoic acid biosynthesis occur throughout tissues. Also, the general concordance between mRNA localization and CRBP expression patterns, revealed by previous immunocytochemistry studies, supports and extends the conclusion that CRBP mRNA expression correlates with CRBP expression, based earlier on comparing RNA assays with radioimmunoassays. **J. Cell. Physiol. 173:36–43, 1997.** © 1997 Wiley-Liss, Inc.

Metabolism of vitamin A (retinol) produces the hormone retinoic acid (RA), which modulates numerous biological processes by regulating gene expression throughout the life span of vertebrates (Blomhoff, 1994). RA controls transcription by serving as a ligand for the nuclear RA receptors RARα, β, and γ (Evans, 1988; Giguére, 1994; Chambon, 1996). Liver, kidney, lung, and testis are among the tissues that express RARs and depend upon RA to direct stem-cell differentiation, as well as to regulate transcription in differentiated cells (Wolf, 1984). Lack of a source of RA, e.g., affects lung by allowing squamous metaplasia of epithelia (Chytil, 1996) and affects testis by causing aspermatogenesis, followed by testicular degeneration (Huang and Hembree, 1979; Van Pelt and de Rooiz, 1990). Biogeneration of RA occurs in situ in these retinoid-target tissues in a two-step process (Napoli, 1996). Retinol dehydrogenase (RoDH) isozymes catalyze the reversible, rate-limiting dehydrogenation of retinol into retinal; retinal dehydrogenase isozymes catalyze the irreversible conversion of retinal into RA.

Cellular retinol-binding protein type (CRBP) sequesters intracellular retinol in a protected pocket, secluded from the rest of the cellular environment (Ong et al., 1994). It is not clear that any tissues totally lack CRBP, but the tissues that express the highest levels include liver, kidney, lung and testis (Adachi et al., 1981; Ong et al., 1982; Eriksson et al., 1984). The CRBP concentration in rat liver (~7 μM), e.g., exceeds that of retinol (~5 μM) (Harrison et al., 1987). This pattern of excess CRBP relative to retinol pertains not only to whole liver, but also to the individual cell types in liver (Blomhoff et al., 1985; Hendriks et al., 1988). The CRBP in excess of retinol and the low K_d value relative to the concentration of CRBP would likely reduce unbound retinol to very low levels. Since the discovery in 1973 of CRBP, the purpose of this sequestering has been discussed actively (Bashor et al., 1973). Possibly, CRBP's effect of keeping retinol from accumulating in membranes confers long-term advantages to membrane integrity/function. Simultaneously, enclosure

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within CRBP would shield retinol from non-enzymatic oxidation and isomerization and impart enzyme specificity to retinol metabolism. Other functions of CRBP may include facilitating the cellular uptake of retinol, and perhaps influencing the ultimate intracellular retinol concentration (Lissoos et al., 1995). The concentration of apo-CRBP also affects the relative flow of retinol into storage as esters vs. its release from storage by inhibiting lecithin: retinol acyltransferase (LRAT) and stimulating cholate-independent retinyl ester hydrolase activities, respectively (Ong et al., 1994; Napoli, 1996).

The enzymes that catalyze the esterification of retinol, LRAT, and its dehydrogenation into retinal, RoDH, can access retinol in the CRBP-retinol complex, i.e., they do not require prior release of the lipophilic retinol from CRBP. This has been demonstrated by the kinetics of both enzymes with CRBP-retinol relative to their kinetics with unbound retinol, and also by the requirements of reactions catalyzed by subcellular fractions for the cosubstrates recognized by LRAT and RoDH with CRBP-retinol as substrate, relative to cosubstrate requirements with unbound retinol as substrate (Ong et al., 1988; Yost et al., 1988; Posch et al., 1992). This capability resolves the problem of accessing the tightly bound retinol, allows enzymatic access to the form of retinol in highest concentration in vivo, and imparts enzymatic discrimination to retinol metabolism.

Three microsomal and two cytosolic RoDH isozymes have been identified so far which recognize holo-CRBP as substrate (Posch et al., 1992; Ottonello et al., 1993; Boerman and Napoli, 1995, 1996). Little is known about the cytosolic enzymes, but cDNA encoding three distinct microsomal RoDH isozymes have been cloned (Chai et al., 1995a,b, 1996). Each microsomal isozyme belongs to the short-chain dehydrogenase/reductase gene family and the first 103 of the 317 amino acid residues of each are identical. Overall, RoDH(I) and RoDH(III) share 98% amino acid identity—they differ by just 7 amino acid residues; but RoDH(I) and RoDH(II) have only 82% amino acid identity. Liver alone expresses the mRNA of RoDH(III), whereas liver, kidney, lung, and testis are among the tissues that express the mRNAs of RoDH(I) and RoDH(II). Although individual functions have not been determined yet for these isozymes, each could occur in different cell types or could respond differently to regulatory signals.

The present work was done in several adult rat RA generating and target tissues to learn whether the microsomal RoDH isozymes localize to different cell types and to further test the hypothesis that holo-CRBP serves as a substrate for RoDH by determining whether CRBP and RoDH isozymes were coexpressed in the same cell types. The data indicate that RoDH isozyme mRNAs are both expressed in many RA target tissues and the cells surveyed which expressed RoDH isozymes also expressed CRBP.

MATERIALS AND METHODS

Sections

Tissues from adult male Sprague-Dawley rats (~150 g) were immersed overnight in freshly prepared, cold 4% paraformaldehyde/phosphate-buffered saline and were then xylene/ethanol-dehydrated and embedded in paraffin. In situ hybridization was done with coronal and/or sagittal serial sections of 5 µm collected onto 2% aminopropyltriethoxysilane-coated slides and stored with desiccant at −80°C.

Probes

Three digoxigenin-labeled antisense riboprobes were used under high-stringency conditions: CRBP specific; RoDH(I/III) specific; RoDH(II) specific. A polymerase chain reaction (PCR)-generated 862 bp fragment of RoDH(II) cDNA (nucleotides 1030–1891) was subcloned in both directions into the *Eco*RV site of pcDNA3 (Chai et al., 1995b). This fragment comprises the last 60 3′-coding nucleotides and the first 802 3′-untranslated nucleotides and has only 62% identity with the corresponding area of RoDH(I) and 72% identity with the same area of RoDH(III) and experimentally was specific for RoDH(II) (see below). After sequencing the junction between insert and vector, pcDNA3/RoDH-862 was linearized with *Kpn*I to produce an RoDH(II) antisense cRNA probe or with *Xho*I to produce a sense cRNA probe upon in vitro transcription with SP6 or T7 RNA polymerase and digoxigenin-11-UTP. Probe length was reduced to 0.15–0.2 kb by limited alkaline hydrolysis. Sense and antisense riboprobes were generated similarly from the 677 bp fragment of RoDH(I) cDNA, consisting of the last 60 3′-coding nucleotides and the first 617 3′-untranslated nucleotides (Chai et al., 1995a). This fragment shares >96% nucleotide identity with the corresponding area of RoDH(III). The 695 basepair probe for CRBP, representing the entire coding region, was generated by PCR with rat liver cDNA as template with the primers from the rat cDNA (the linkers are underlined): 5′-CGGGATCCTTCACCCGGAGCGCATTA and 3′-CGGAATTCTATTGGGGTAGCTTCATT (Sherman et al., 1987). The PCR product was subcloned into the *Eco*RI/*Bam*HI site of pcDNA3.

Protocol

A standard protocol was used with modification (Hillan, 1992). Briefly, paraffin was removed, the samples were rehydrated, and the protein was digested with proteinase K and post-fixed in 4% paraformaldehyde in phosphate-buffered saline. Charged sites were blocked by acetylation with acetic anhydride and triethanolamine. Hybridization was done at 42°C overnight with 2 µg/ml of riboprobe. Slides were incubated with RNase A/T1 mixture and washed at 65°C. Signals were detected with anti-digoxigenin conjugated with alkaline phosphatase, using nitroblue terazollium chloride/5-bromo-4-chloro-3-indolylphosphate as substrate and levamisole to inhibit endogenous alkaline phosphatase. Slides were photographed through a light microscope. Controls, done for each tissue and for each probe, consisted of sense riboprobes labeled with digoxigenin, antisense probes not labeled with digoxigenin, and antisense probes labeled with digoxigenin but without adding anti-digoxigenin Fab-alkaline phosphatase conjugate.

RESULTS

Probes

The cRNA probe used for RoDH(I) would also recognize RoDH(III), but because only liver expresses RoDH(III) mRNA, as determined by RNase protection assays (Chai et al., 1996), this would likely impact on the results with liver only. The RoDH(II) and CRBP

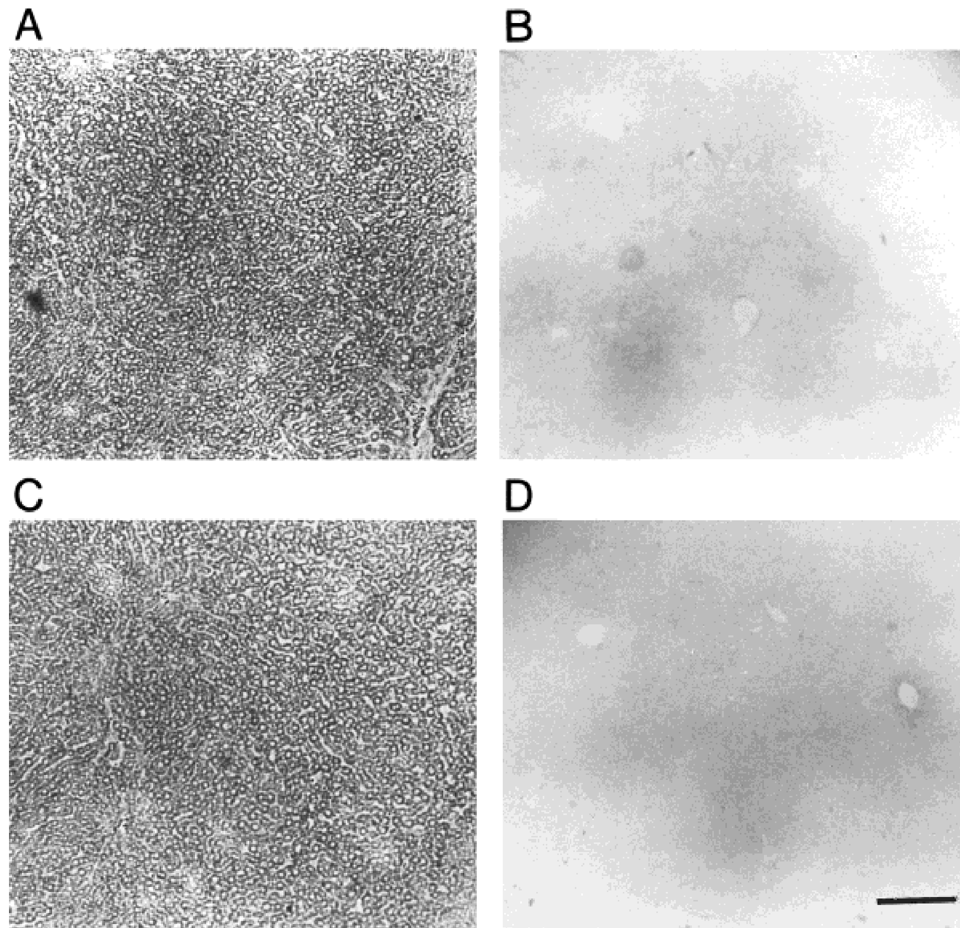

Fig. 1. Adult rat liver expression of RoDH(I/III) and RoDH(II). Antisense and sense probes, respectively, were used for RoDH(I/III) (**A,B**) and RoDH(II) (**C,D**). Bar = 200 µm.

cRNA probes specifically identify these two mRNA (Chai et al., 1995a,b; Busch et al., 1990).

Liver expression

CRBP (not shown), RoDH(I/III), and RoDH(II) mRNAs all showed signals distributed uniformly throughout the liver lobule (Fig. 1). All three signals were strongest in hepatocytes and revealed no obvious differences in the loci of their expression (Fig. 2A,C,E), although the signal with RoDH(II) was somewhat less intense than those for CRBP and RoDH(I/III). All signals were cytoplasmic and were excluded from the nuclei. No specific signals were detected in the Kupffer cells, the epithelial cells of the bile ducts, or the endothelial cells of blood vessels. Sense controls showed only non-specific background and relatively light staining (Fig. 2B,D,F). The other controls, omission of probes from hybridization, treating the section with RNase before hybridization, or using unlabeled antisense probes, yielded results similar to the sense probe control (data not shown). These results are consistent with previous studies which used immunocytochemistry to expose CRBP expression in hepatocytes, but not in Kuppfer cells or endothelial cells (Porter et al., 1983; Kato et al., 1984; Eriksson et al., 1984). CRBP also has been localized in stellate cells via immunocytochemistry. The strong signals produced by in situ hybridization, however, precluded identification of stellate cells in our studies, because they obscured visualization of the much smaller stellate cells.

Kidney expression

Both serial sagittal and transverse sections were obtained of kidney to aid in precise localization of the three mRNAs. All three probes produced intense signals in the cortex, but weak if any in medulla (Fig. 3). The signals within the cortex were localized to the proximal convoluted and straight tubules, and were especially intense in the basal portion of the cytoplasm. The relative intensities of all three signals were similar. Cells of the distal tubules, Henle's loops, collecting ducts, and glomeruli showed only low intensity signals, if any at all. These in situ results also agree well with immunocytochemical studies placing CRBP in the rat kidney cortex, and specifically predominantly in the proximal convoluted tubules, to a lesser extent in the proximal straight tubules, but not in the glomeruli (Kato et al., 1984; Eriksson et al., 1984).

Testis expression

All three probes produced strong signals within the seminiferous tubules of adult rat tissues. The signals

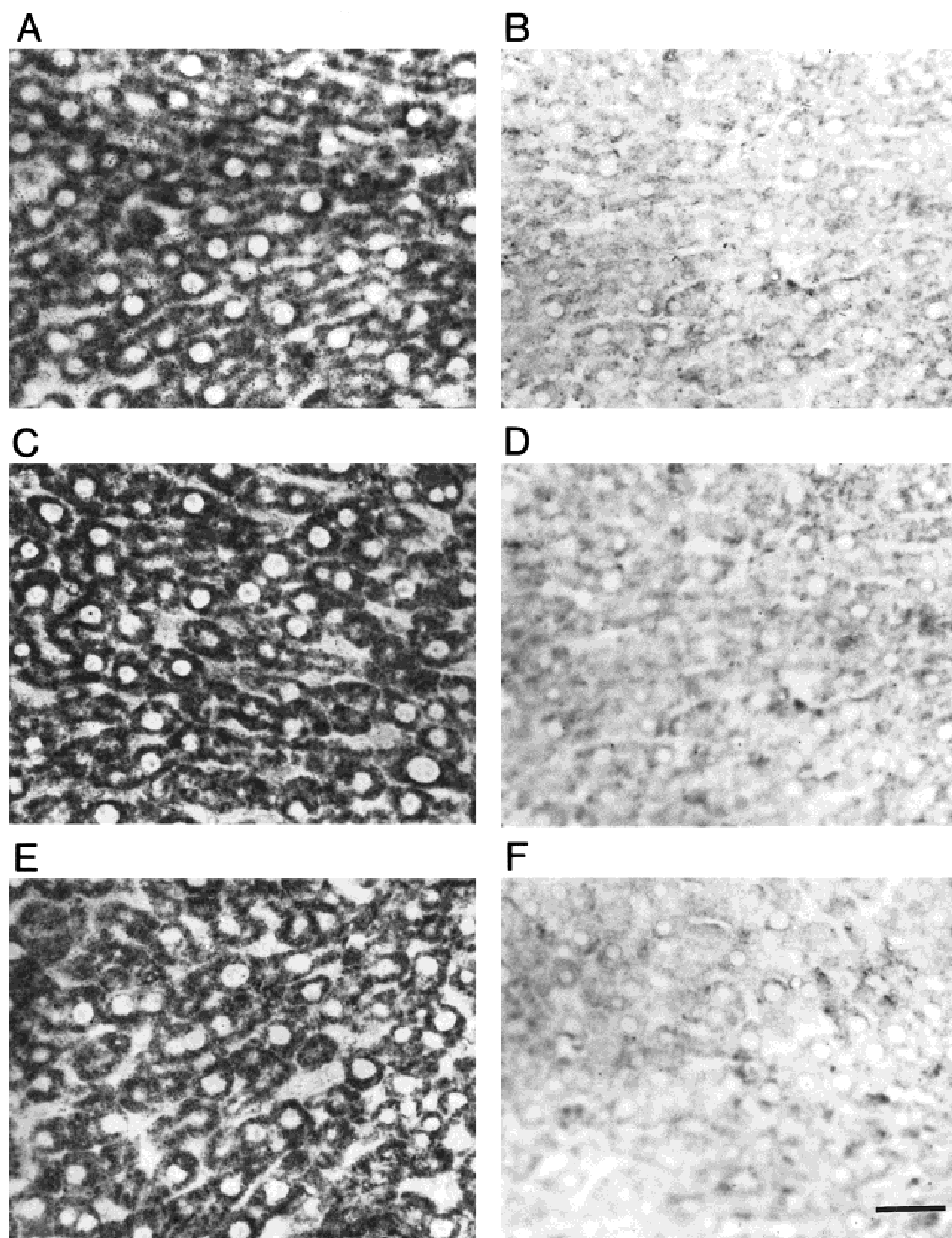

Fig. 2. Adult rat liver expression of CRBP and RoDH isozyme mRNA. Antisense and sense probes, respectively, were used for CRBP (**A,B**), RoDH(I/III) (**C,D**), and RoDH(II) (**E,F**). Bar = 50 µm.

were not uniform throughout the seminiferous tubules, most likely reflecting the different developmental stages of various sections (Schmitt and Ong, 1993). Within the germinal epithelium the three signals appeared as spokes on a wheel. All three signals appeared primarily in the Sertoli cells, which are slender, elongated, and irregularly shaped cells which extend from the basement membrane to the lumen of the seminiferous tubules (Fig. 4). Spermatogonia and primary spermatocytes were also positive, but the intensities of all three signals were much less than in the Sertoli cells. Peritubular cells and other germinal cells showed faint signals, including secondary spermatocytes, spermatids, and spermatozoa. Within the interstitial space signals for CRBP and RoDH(II) mRNA were detected in Leydig cells, but no signal was detected with the RoDH(I/III) mRNA probe. This represents the sole difference in cellular expression loci between RoDH(I/III) and RoDH(II) noted in the liver, kidney, lung, or testis.

Immunocytochemical studies have shown that adult rat testis expresses CRBP most intensely in Sertoli cells (Porter et al., 1983; Kato et al., 1985). Expression,

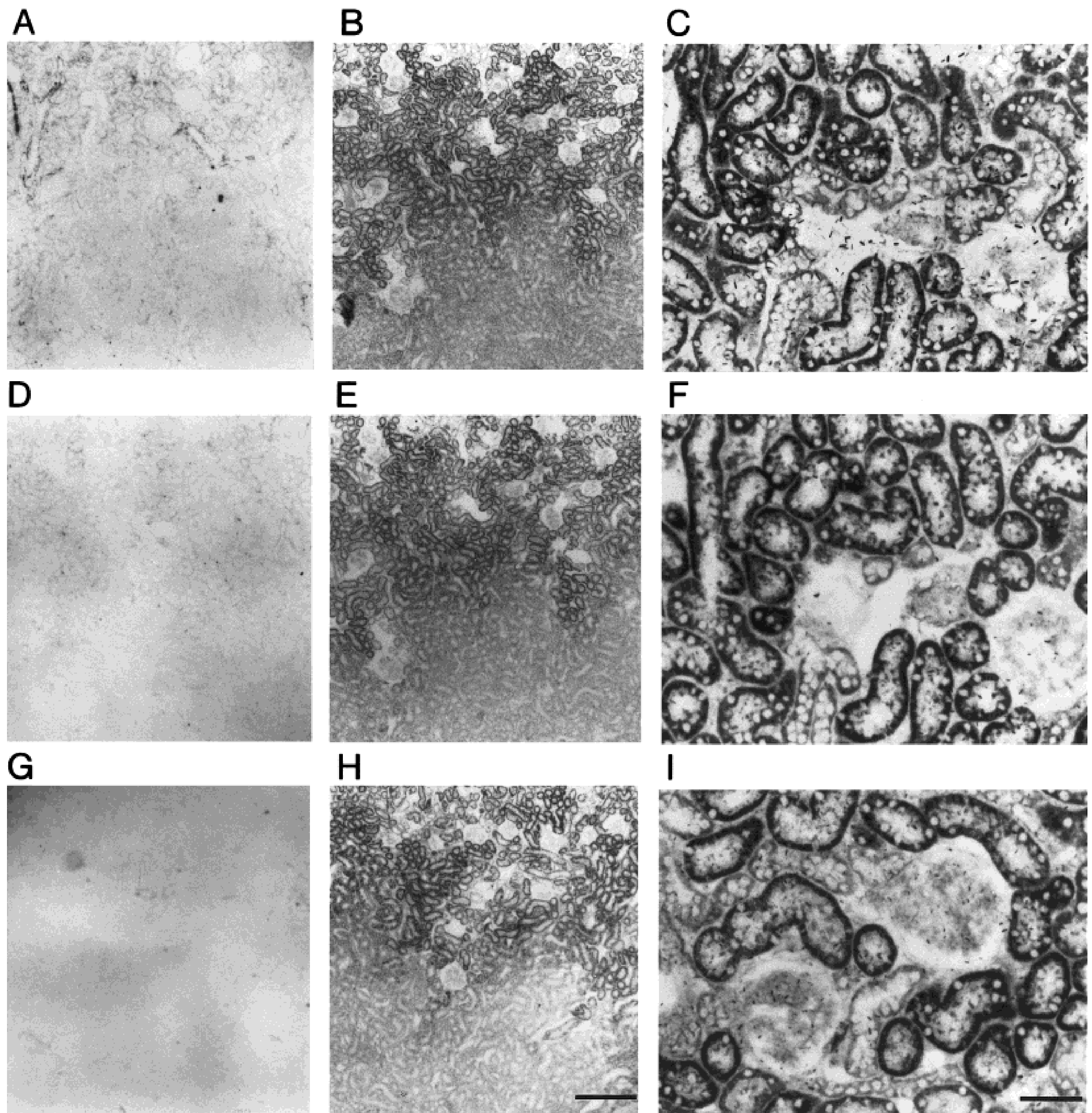

Fig. 3. Adult rat kidney expression of CRBP and RoDH isozyme mRNA. Sense probes were used for CRBP (**A**), RoDH(I/III) (**D**), and RoDH(II) (**G**). Antisense probes were used for CRBP (**B,C**), RoDH(I/III) (**E,F**), and RoDH(II) (**H,I**). A, B, D, E, G, and H show both the renal cortex (top sections of each) and the renal medulla (bottom sections of each). The higher magnification of C, F, and I reveals detail in the cortex. Bars = 500 µm (H); 100 µm (I).

albeit weaker, has also been detected in germ cells including spermatogonia, spermatids, and spermatocytes (Kato et al., 1985; Schmitt and Ong, 1993). Weak immunochemical staining has also been noted for CRBP in interstitial cells (Kato et al., 1985). A subsequent study also identified strong immunochemical staining for CRBP in rat Sertoli cells, but observed intense staining in spermatogonia and spermatoctyes, and somewhat less intense signals in Leydig cells (Busch et al., 1990). The reasons are not clear for these differences in degree of expression. Northern blot analysis of isolated Sertoli cells, peritubular cells, and Leydig cells revealed mRNA only in the first two cell types, and not in Leydig cells (Eskild et al., 1991). Northern blot analysis, however, can be insensitive relative to in situ hybridization. A recent study using in situ hybrid-

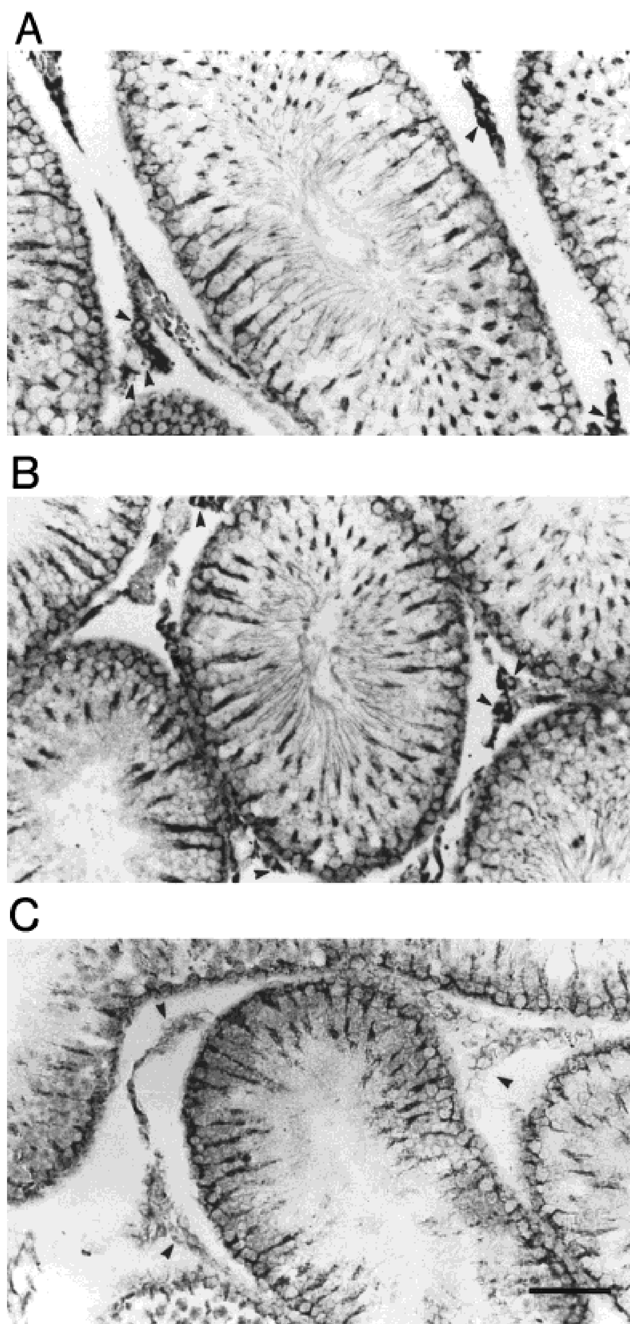

Fig. 4. Adult rat testis expression of CRBP and RoDH mRNA: (**A**) CRBP, (**B**) RoDH(II), and (**C**) RoDH(I/III). Note that the RoDH(II) photo has been placed under that of CRBP. Arrowheads point to interstitial cells. Bar = 100 μm.

ization with an [^{35}S]-labeled probe also confirmed intense CRBP mRNA expression in Sertoli cells, but because of technical considerations could not "comment with precision" about CRBP mRNA in germ cells (Rajan et al., 1990).

Lung expression

In the lung all three mRNAs were distributed throughout the entire epithelial region of the airways from the principle bronchi to the respiratory bronchioli, but were most intense in the epithelial layer of the mucosa (Fig. 5). Within the airway epithelium signals appeared to be localized to the cytoplasm of the pseudostratified columna or simple cuboidal epithelium and the goblet cells. Similarly, within the respiratory regions, signals were observed in the simple columnar cells of the epithelium of the alveolar duct and in the type II cells of alveoli. Only very weak signals were visible in the rest of the lung cell types. We are not aware of any other studies concerning the precise loci of CRBP expression in lung using either immunocytochemistry or in situ hybridization.

DISCUSSION

These data reveal the extensive expression of the genes for RoDH isozymes throughout multiple cell types in the retinoid-dependent tissues surveyed, consistent with multiple cellular sites of RA biosynthesis throughout tissues. Coexpression of RoDH(I/III) with RoDH(II) in liver and RoDH(I) with RoDH(II) in kidney and lung, however, does not help resolve possibly different roles for each of these isozymes. Localization of only RoDH(II) in testis interstitial cells, on the other hand, does suggest a unique function for RoDH(II) in testis, and was somewhat unexpected. This occurrence of an RoDH isozyme outside of the seminiferous tubules indicates a testicular function for RA not related directly to spermatogenesis. Dosing RA to vitamin A-deficient mammals sufficient to maintain growth and fulfill all other readily observable vitamin A functions does not support spermatogenesis and does not prevent testicular atrophy, although much larger doses do support testis function (Huang and Hembree, 1979; Van Pelt and de Rooiz, 1990). This indicates that RA normally cannot cross the blood-testis barrier of mammals and is generated physiologically within the seminiferous tubules from retinol. Low activity of RoDH(II) producing RA just sufficient for interstitial use and/or the resistance of the blood-testis barrier to low amounts of RA could prevent RA interstitially generated by RoDH(II) from fulfilling RA needs within the seminiferous tubules.

Perhaps the most important insight provided by these data was colocalization of RoDH isozymes in cell types that express CRBP, at least in liver, kidney, lung, and testis. This coexpression is consistent with the hypothesis that holo-CRBP serves as substrate for RA synthesis in vivo. Kinetic experiments have shown that the rate of retinal synthesis catalyzed by RoDH in vitro from the CRBP-retinol complex occurs too rapidly to reflect the concentration of only unbound retinol; and also that the rate of retinal synthesis in the presence of variable concentrations of holo-CRBP and a fixed concentration of unbound retinol or, vice versa with a fixed concentration of holo-CRBP and variable concentrations of unbound retinol, reflects the holo-CRBP concentration (Posch et al., 1992). Biochemically, with holo-CRBP as substrate and microsomes as the source of RoDH activity, NADP serves as preferred cofactor, but with unbound retinol either NAD or NADP will serve. RoDH expressed from the cDNA prefers NADP as cofactor (Chai et al., 1995a,b, 1996). Finally, holo-CRBP (and not apo-CRBP) specifically and covalently crosslinks with RoDH in microsomes only in the presence of cofactor, reflecting the ordered bisubstrate reac-

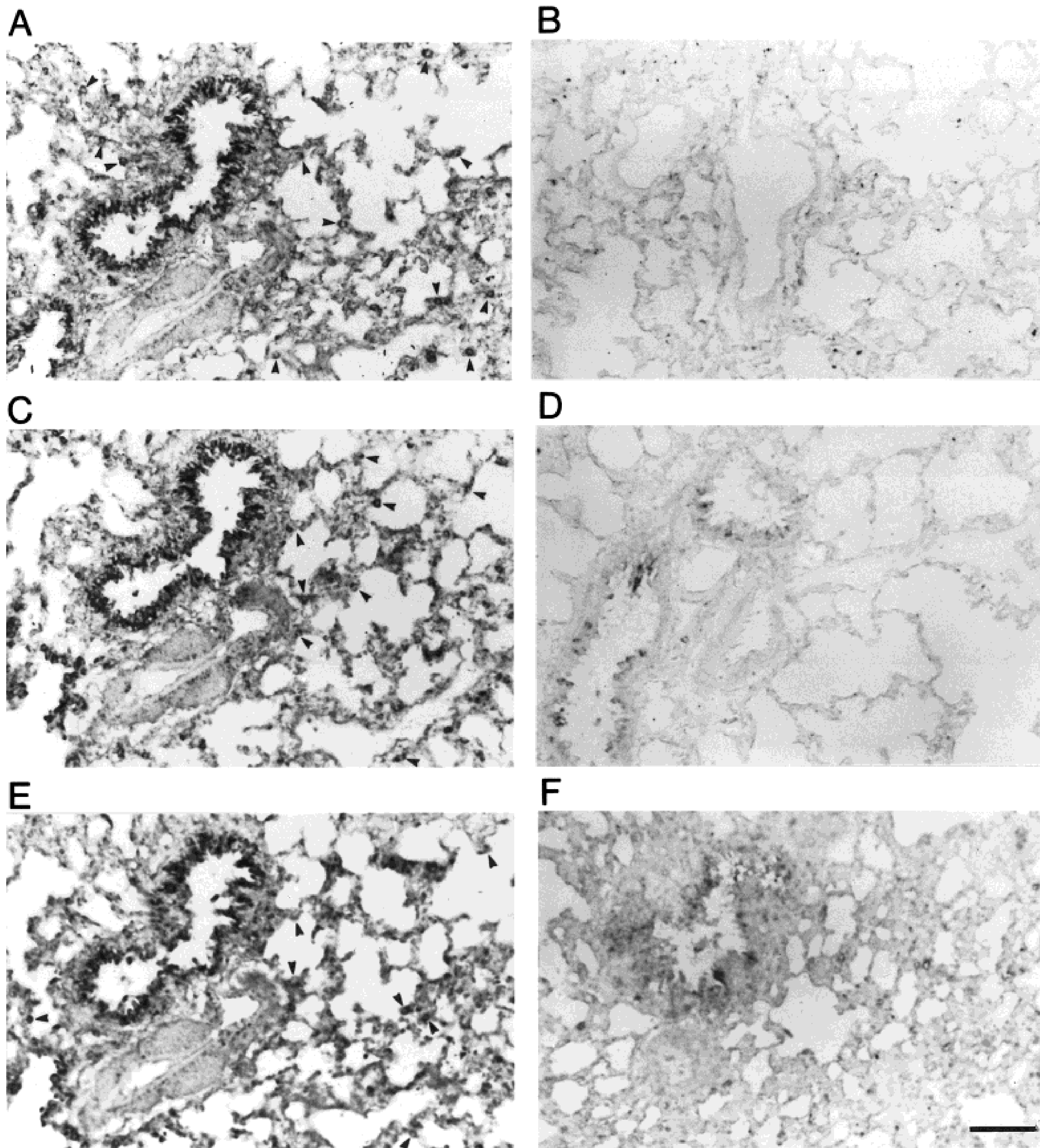

Fig. 5. Adult rat lung expression of CRBP and RoDH isozyme mRNA. Antisense and sense probes, respectively, were used for CRBP (**A,B**), RoDH(I/III) (**C,D**), and RoDH(II) (**E,F**). Arrowheads point to type II cells of alveoli. Bar = 100 μm.

tion mechanism of a dehydrogenase (Boerman and Napoli, 1995). The morphological data presented here sustain the model of holo-CRBP serving as substrate for RA biosynthesis, based on kinetic and biochemical evidence, by placing the holo-CRBP substrate and the RoDH enzyme in the same cellular loci in vivo.

Finally, these are the most extensive data reported on the cellular localization of CRBP in adult tissue using in situ hybridization. As such these results complement previous detailed studies delineating the loci of CRBP expression using immunocytochemistry and extend the limited work of CRBP mRNA expression by in situ hybridization in adult mammalian tissue. Previous work based on a comparison of data from either RNA blot analyses or RNase protection assays with radioimmunoassay results suggested that CRBP mRNA levels

tend to correlate with CRBP levels (Levin et al., 1987; Rajan et al., 1990). The general concordance between the in situ mRNA localization data reported here and the CRBP expression patterns revealed by the previous immunocytochemistry studies supports this conclusion and provides additional detail to CRBP expression patterns.

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