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Localization of Cytochrome P-450 Gene Expression in Normal and Diseased Human Liver by *In Situ* Hybridization of Wax-embedded Archival Material

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The localization of the expression of several cytochrome P-450 genes in normal and diseased human liver was investigated by *in situ* hybridization of formalin-fixed, paraffin wax–embedded archival tissue samples with ^{35}S-labeled antisense RNA probes. The results demonstrated that genes coding for members of the cytochrome P-450 3A subfamily (CYP3A) were preferentially expressed in hepatocytes in acinar zone 3 (the centrilobular region), whereas genes coding for CYP1A2, CYP2A, 2B and 2C were expressed uniformly throughout the liver acinus. In cirrhotic livers, CYP2A and 2B genes (and to a lesser extent, CYP3A genes) were highly expressed in isolated hepatocytes located at the junction of parenchyma with fibrous septa. The cause and significance of the position-dependent expression of specific cytochrome P-450 genes in normal and diseased human liver are discussed. (HEPATOLOGY 1992;16:682-687.)

Cytochromes P-450 constitute a superfamily of hemoproteins that are of fundamental importance in normal liver function (1-3). They have been classified, on the basis of amino acid sequence homology into families (designated by Arabic numerals, e.g., CYP1, CYP2) and subfamilies (designated by capital letters, e.g., CYP2A, 2B) (4, 5). In a given species, members of different cytochrome P-450 families have less than 35% sequence similarity, whereas those of different subfamilies have between 35% and 65% identity. Individual components of a subfamily have more than 65% identity and are distinguished by Arabic numerals (e.g., CYP2A1, 2A2). To date, more than 25 distinct cytochromes P-450 have been identified in man (5).

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Cytochromes P-450 are located mainly in the membranes of the endoplasmic reticulum, where they are involved in the metabolism of endogenous compounds such as steroids, fatty acids and prostaglandins. They are also of primary importance in the detoxification or activation of many foreign hydrophobic compounds, including many therapeutic drugs, environmental pollutants and chemical carcinogens. The proteins are subject to developmental and tissue-specific regulation; most are expressed predominantly in adult liver, and many are inducible by the compounds they metabolize.

Analysis of human liver RNA samples by quantitative RNase protection assays reveals marked interindividual variations in the expression of cytochrome P-450 genes, ranging from tens of fold (for CYP2B6) to thousands of fold (for CYP2A6) (6, 7; Palmer CNA et al., Unpublished observations, 1990). Having quantified cytochrome P-450 messenger RNAs (mRNAs) in homogenized samples of human liver, we wished to investigate the localization in the various liver zones of the mRNAs and, hence, of the expression of the corresponding genes. This was done by *in situ* hybridization of formalin-fixed, paraffin wax–embedded archival biopsy samples with radiolabeled antisense RNA probes derived from complementary DNA (cDNA) clones coding for human cytochromes P-450. In this paper we describe the use of this approach to determine the regional localization of the expression of genes coding for CYP1A2 and for members of the CYP2A, 2B, 2C and 3A subfamilies in normal and diseased human liver.

MATERIALS AND METHODS

Tissues. Paraffin wax blocks of three wedge-biopsy and seven needle-biopsy specimens of liver tissue, all histologically normal in appearance, were retrieved from the files of the Department of Histopathology of St. Bartholomew's Hospital. Additionally, blocks from two needle-biopsy specimens, obtained from patients with abnormal liver function, showing histological signs of cirrhosis were studied. All of the tissues had been fixed in 10% formal saline solution and embedded in paraffin wax according to standard procedures. Sections 5 μm thick were cut, floated on warm water and collected onto

sialinized slides. Sections were stored at room temperature until they were used (up to 3 mo in some cases).

Preparation of Hybridization Probes. Templates for the synthesis of antisense RNA probes for *in situ* hybridization were constructed as follows. CYP1A2 – a 290-bp *Pvu*II/*Bg*1II fragment of pNF1 (8) that encodes a region free of the Alu family repetitive elements present elsewhere in the cDNA (9) – was inserted into a *Sma*I/*Bam*HI–digested pBluescript KS vector to form pBS1A2. CYP2A – a 288-bp *Bam*H1/*Pst*I fragment of pHP450(1) (10) – was inserted into *Bam*HI/*Pst*I–digested pBluescript KS to form pBS2A288. CYP2B – a 450-bp *Hind*III fragment of p7A22e (11) encoding a region shown to be devoid of the repetitive sequences present elsewhere in the cDNA (11) – was inserted into the *Hind*III site of pBluescript KS to form pBS2B450. CYP2C – a 206-bp *Eco*RI/*Bam*HI fragment of pB8 (12), was ligated to an *Eco*RI/BamHI–digested pBluescript KS to form pBS2C206. CYP3A – a 240-bp *Hind*III/*Sac*I fragment of pCYK – was inserted into *Hind*III/*Sac*I–digested pBluescript KS to form pBS3A240.

Radiolabeled antisense RNA probes were produced by *in vitro* transcription of the pBluescript constructs with T3 (for pBS2B450 and pBS2C206) or T7 (for pBS1A2, pBS2A288 and pBS3A240) polymerase. To produce sense RNA probes from these constructs, templates were transcribed with the alternative promoter. Plasmid DNA was linearized by digestion with a restriction endonuclease that cut in the polylinker at a site 5′ of the template strand of the cDNA insert. The sample was digested with proteinase K (50 μg/ml) at 37° C for 20 min, extracted once with phenol/chloroform (1:1, vol/vol) and ethanol precipitated. Linearized template (1 μg) was transcribed with an *in vitro* transcription kit (Stratagene Ltd., Cambridge, UK) according to the supplier's recommendations with 10 units of the appropriate RNA polymerase (T3 or T7) and 50 μCi [α-^{32}P]CTP (>600 Ci/mmol; ICN Flow, High Wycombe, Buckinghamshire, UK) for studies using autoradiography to x-ray film or [α-^{35}S]UTP (>400 Ci/mmol; Amersham Ltd., Buckinghamshire, UK) for microscopic localization with photographic emulsion.

After transcription, the DNA template was digested with RNase-free DNase (Stratagene Ltd.). The size of the transcript was checked by denaturing PAGE and autoradiography. After addition of 30 μg of transfer RNA, the probe was precipitated from ethanol and resuspended in 300 μl of 80% formamide, 40 mmol/L Pipes buffer (pH 6.4), 0.4 mol/L NaCl and 1 mmol/L EDTA. When diluted to a concentration of 1×10^4 cpm/μl, ^{32}P-labeled probes could be stored at −20° C in this buffer for several days without significant breakdown. For ^{35}S-labeled probes, dithiothreitol was added to a final concentration of 10 mmol/L; probes were stored at −70° C.

In Situ Hybridization. Our method is based on that of Angerer et al. (13), but the proteolytic enzyme digestion treatment has been modified for use on our formalin-fixed, paraffin wax–embedded tissues. Sections were dewaxed in xylene, washed in alcohol and air dried. Proteinase K (Boehringer Mannheim, Mannheim, Germany), at concentrations ranging from 0.5 to 500 μg/ml in 100 mmol/L Tris-HCl (pH 8.0) and 50 mmol/L EDTA, was applied for 30 min at 37° C. Sections were washed in 0.1 mol/L triethanolamine (pH 8.0). Sections were acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 10 min, rinsed in 2× standard saline citrate (1× standard saline citrate is 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.5), dehydrated through graded alcohols and air dried. The hybridization medium contained 50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 10% dextran sulfate, 1× Denhardt's solution (14) and yeast total RNA (500 μg/ml). When we used ^{35}S-labeled probes, 100 μmol/L dithiothreitol was included. Sense or antisense probes were included at a final concentration of 0.2 μg probe/ml/kb of probe complexity (approximately 1×10^4 cpm/μl for our probes). After addition of probe, the hybridization mixture was heated at 80° C for 3 min, and 15 to 30 μl was applied to each section. Sections were covered with a glass coverslip and incubated overnight at 55° C in a damp box. Coverslips were removed and sections were subjected to three 30-min washes in 2× standard saline citrate at room temperature. To remove unhybridized probe, slides were incubated for 30 min at 37° C in 0.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA and RNase A (20 μg/ml). Digested probe was washed off with 2× standard saline citrate at room temperature; sections were washed in 0.1× standard saline citrate for 30 min at 55° C and then for 30 min at room temperature. Sections were dehydrated through graded alcohols containing 0.3 mol/L ammonium acetate and air dried. As negative controls, tissue sections were hybridized with sense RNA probes or were digested with RNase A (20 μg/ml) for 30 min at 37° C before hybridization with antisense probes.

Signals from sections hybridized with ^{32}P-labeled probes were detected by autoradiography of the slides to x-ray film for 16 to 20 hr. For cellular localization of transcripts with ^{35}S-labeled probes, slides were dipped in liquid emulsion (Ilford K5 [Ilford Ltd., Knutsford, Cheshire, UK] diluted 1:1 in distilled water) and stored in a lightproof box for 2 or 3 wk at 4° C. Slides were developed with Kodak D19 (Eastman Kodak Co., Rochester, NY) for 3 min, fixed in Kodak fix and counterstained with hematoxylin or hematoxylin and eosin. Sections were viewed under bright-field illumination and with epipolarizing illumination, which increases the sensitivity of detection of autoradiographic signal and allows identification of vessels through the birefringence of connective tissue fibers. Photographs were taken under epipolarizing or bright-field illumination. To quantify hybridization signals, sections were viewed under oil immersion with an eyepiece graticule divided into 100 squares of equal size. The number of silver grains per square was recorded for cells in the capsule and zones 1 and 3.

RESULTS

Optimization of in Situ Hybridization. To optimize the conditions required for probe penetration and target retention in formalin-fixed, paraffin wax–embedded archival samples, sections of 10 histologically normal human livers were digested with a range of proteinase K concentrations before being hybridized with radiolabeled sense or antisense RNA probes for human CYP3A. The use of ^{32}P-labeled probes and autoradiography (as described in "Materials and Methods") enabled rapid macroscopical analysis of the samples. All 10 samples gave positive results when hybridized with the antisense probe. However, the strength of the hybridization signal varied with the concentration of proteinase K; negligible signals were obtained with the lowest and highest concentrations, and optimal signals were found with intermediate concentrations. It was observed that in general, wedge-biopsy specimens required more extensive digestion than did needle-biopsy specimens (~200 μg proteinase K/ml compared with ~50 μg/ml, respectively). The sense RNA probe gave negligible hybridization signals at all proteinase K concentrations (results not shown). The same conditions were found optimal for all probes used.

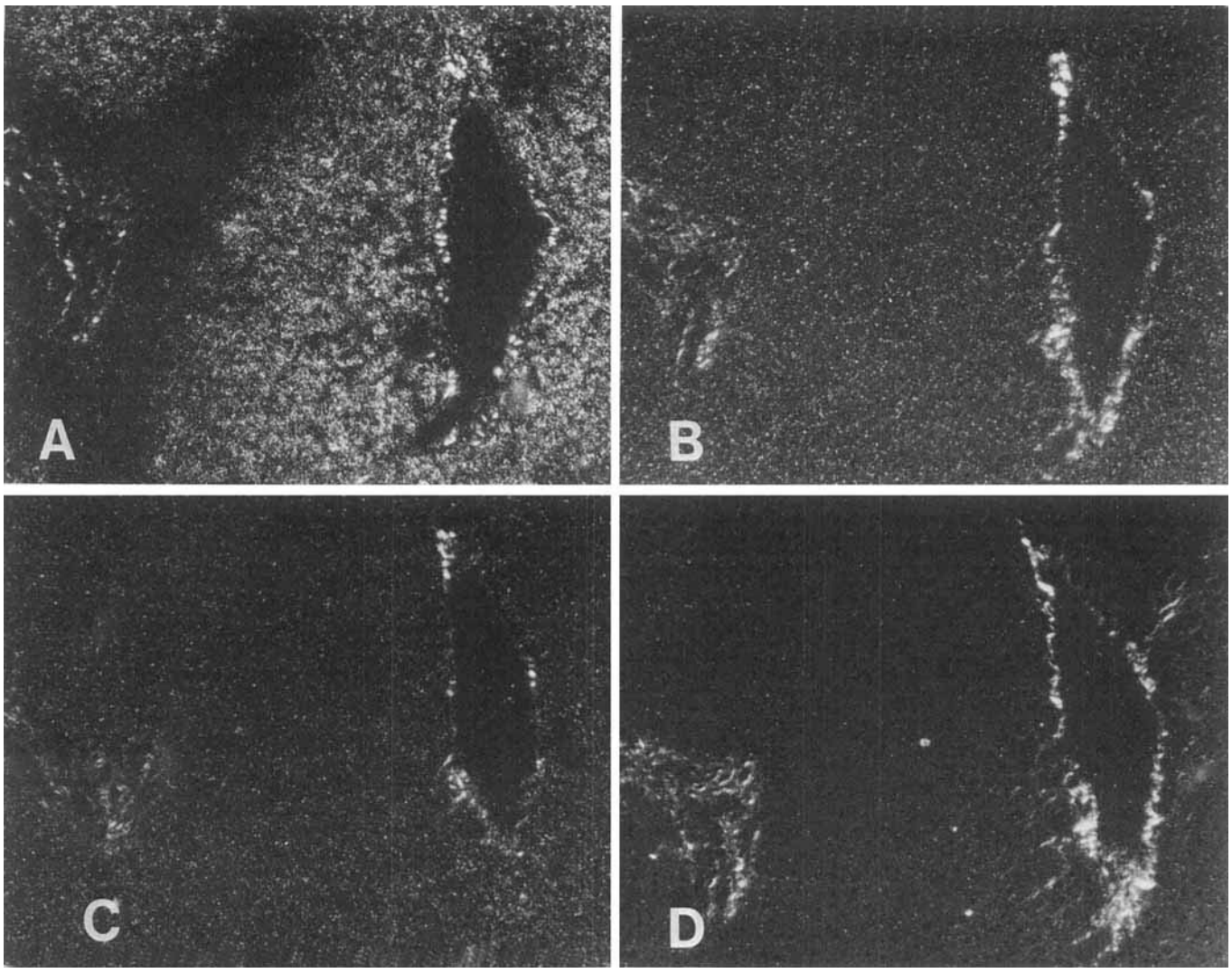

FIG. 1. Serial sections of histologically normal liver hybridized for cytochrome P-450 gene expression. Sections were viewed with epipolarizing illumination, which allows identification of vessels through the birefringence of connective tissue fibers. Each section shows a central vein *(right)* and a portal tract *(left)*. (A) CYP3A. (B) CYP2A. (C) CYP2C. (D) Control section hybridized with sense probe. Note the localization of CYP3A mRNA to zone 3. The patterns of expression are representative of all 10 normal livers analyzed, although signal intensities vary among individuals.

Normal Liver. The proteinase K digestion conditions determined to be optimal for each of the archival samples were used to investigate the regional localization of cytochrome P-450 mRNAs in normal human liver. For these experiments, ^{35}S-labeled sense and antisense probes were used.

The CYP3A antisense RNA probe gave clear hybridization signals for all 10 biopsy samples examined. However, the intensity of the signals varied between samples taken from different individuals. The distribution of silver grains indicated that CYP3A mRNAs are localized to hepatocytes and are not present in other, nonparenchymal cells. CYP3A mRNAs exhibited regional localization in liver acini; they were expressed predominantly in hepatocytes in zone 3 (surrounding terminal hepatic venules), with decreasing abundance toward portal tracts. They were virtually undetectable in zone 1 (Fig. 1 A, Table 1).

mRNAs encoding CYPIA2 and members of the CYP2A, 2B and 2C subfamilies were also found to be confined to hepatocytes (Figs. 1B and 1C; some data not shown). But these mRNA species were less abundant and more homogeneously distributed throughout liver zones than were CYP3A mRNAs (Table 1).

Hybridization with sense RNA probes gave low, background signals of equal intensity from hepatocytes, vessels and capsule (Fig. 1D; some data not shown). Background signals were also obtained from sections that had been treated with RNase before hybridization with specific antisense probes (data not shown).

Diseased Livers. *In situ* hybridization was also used to investigate the expression of cytochrome P-450 mRNAs in samples derived from needle-biopsy specimens of two histologically abnormal livers. The optimal conditions for proteinase K digestion for these specimens were found to be the same as those for needle-biopsy samples

from normal tissue (i.e., 50 μg/ml). Both samples showed evidence of cirrhosis, with extensive fibrosis surrounding nodules of parenchyma. Very similar results were obtained from both specimens. Strong hybridization signals were obtained with both CYP2A and 2B antisense probes throughout the parenchyma; they were particularly intense in isolated hepatocytes located at junctions between fibrous septa and nodules (Fig. 2A and B). This is shown more clearly at a higher magnification of cells at the junction of parenchymal and fibrous areas hybridized with the CYP2A antisense probe (Fig. 2D). A similar distribution was observed for CYP3A mRNAs (Fig. 2C), but in this case the signals were less intense than those obtained with the CYP2A and 2B probes. The antisense RNA probe for CYP2C mRNAs gave weak hybridization signals that were evenly distributed over all cells in the parenchyma (data not shown), similar to the results obtained with this probe in normal tissue. All probes gave weak signals over the fibrotic areas (Fig. 2). However, in these experiments these signals were not above background levels.

TABLE 1. Quantification of hybridization signals for cytochrome P-450 mRNAs in sections taken from a single subject

Probe	Capsule[a]	Zone 1	Zone 3
3A	6.2 ± 3.1 (50)[b]	8.8 ± 3.3 (50)	49.2 ± 13.8 (150)
2A	10.9 ± 3.4 (50)	17.4 ± 7.0 (100)	23.2 ± 5.0 (100)
2B	8.7 ± 4.9 (31)	31.2 ± 4.3 (100)	28.4 ± 3.6 (100)
2C	10.3 ± 4.3 (33)	20.0 ± 7.1 (100)	22.4 ± 7.6 (100)

[a]The numbers obtained for the capsule are not significantly different from background values.

[b]Data expressed as average number of silver grains per unit area ± S.D. (number of individual observations).

DISCUSSION

We have determined the regional localization of the expression of several cytochrome P-450 genes in human liver by means of *in situ* hybridization of formalin-fixed, paraffin wax–embedded archival biopsy samples. Although the ability to detect specific mRNAs in archival material by *in situ* hybridization was demonstrated some time ago (15), the use of such samples has been confined mostly to the study of sequences present in high or moderate abundance (15-17). Results obtained from quantitative RNase protection assays (6, 7) showed that all the cytochrome P-450 mRNAs investigated in this study were present in relatively small amounts in human liver. Nevertheless, *in situ* hybridization signals above background were obtained with each of the probes from all 12 of the liver samples analyzed, confirming the general applicability of *in situ* hybridization for the detection of low-abundance mRNAs in archival samples. Using the results of the RNase protection assays (6, 7) we estimate that our technique of *in situ* hybridization of wax-embedded archival tissue samples can detect as few as 10 to 20 mRNA molecules/cell. This compares favorably with the sensitivity obtained from cryostat sections (18, 19). However, to obtain this degree of sensitivity it was necessary to optimize the proteolytic digestion conditions for each sample. This was presumably due to variability of the duration of fixation in a series of archival samples.

The degree of sequence similarity between cytochromes P-450 (discussed earlier) indicates that, under the stringency conditions of the *in situ* hybridization, all the probes (with the exception of CYP1A2) will detect mRNAs coding for all known members of their respective cytochrome P-450 subfamilies but not those coding for members of other subfamilies. Indeed, we have shown this to be the case for Northern-blot hybridization (20). The lack of cross-hybridization between members of different subfamilies in *in situ* hybridization experiments is confirmed by the fact that in diseased livers the distribution of CYP2C mRNAs is completely different from that of CYP2A and 2B mRNAs.

Our results demonstrate that in normal human liver, the genes coding for CYP1A2 and for members of the CYP2A, 2B and 2C subfamilies are expressed uniformly throughout the various zones of the liver acinus. In contrast, CYP3A genes are preferentially expressed in zone 3 (the centrilobular region). Because the flow of blood in a liver acinus is from the portal tracts to the terminal hepatic venules, the heterogeneous distribution of CYP3A gene expression cannot be due merely to the exposure of hepatocytes to an inducing agent circulating in the blood. The heterogeneity could be due to a concentration gradient of a particular metabolite across the liver acinus (21, 22). However, the relatively sharp demarcation in the distribution of CYP3A mRNAs argues against this and suggests, instead, an effect related to the developmental stage of the cell in the hepatocyte lineage. Thus it may be that CYP3A mRNAs are expressed in the more fully differentiated hepatocytes, which are located around the terminal hepatic venules, and not in the less mature cells near the portal tracts. Thus they may represent useful phenotypical markers for the differentiated state of human hepatocytes. In contrast, the expression of the other cytochrome P-450 genes analyzed appears independent of cell-lineage position. The distribution of the expression of CYP3A genes in liver acini may well be controlled by position-specific signals, regulatory proteins or both. Position in the cell lineage is thought to be the major factor responsible for the nonuniform expression across the liver acinus of several other genes, including α-fetoprotein and insulinlike growth factor II (expressed by cells early in the lineage), albumin and transferrin (expressed in midlineage) and glutamine synthetase (expressed in terminally differentiated hepatocytes) (23-26).

The preferential expression of CYP3A genes in zone 3 (the centrilobular regions) of human liver is similar to that found in rat and mouse by immunohistochemical techniques (27). A similar heterogeneous pattern of expression is displayed in rats by the CYP2A, 2B and 2C genes (27-29). This differs from the more uniform pattern of expression of these genes that we have found in human liver.

It has been postulated that members of the CYP3A subfamily have a protective role against liver cancer

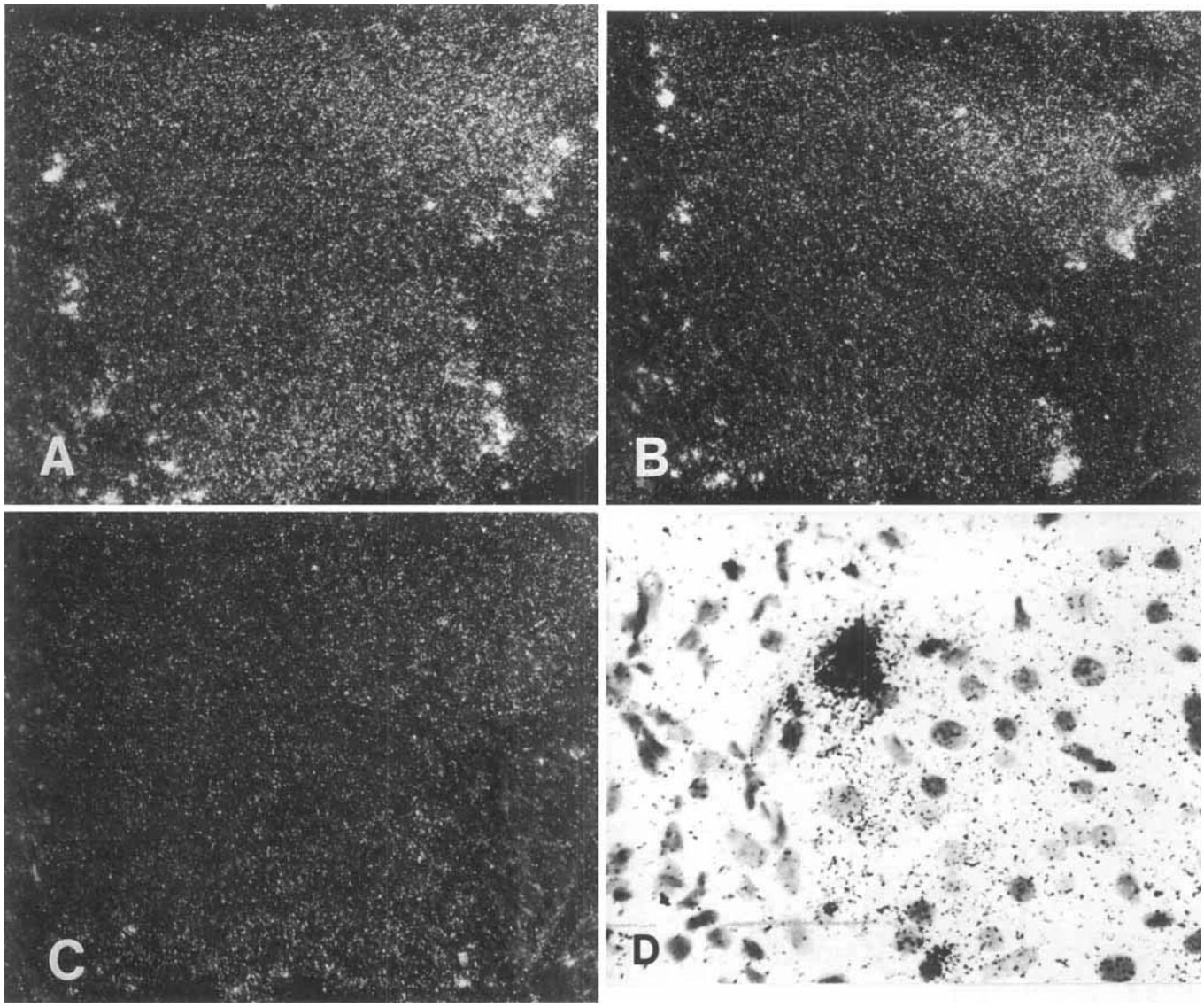

FIG. 2. Serial sections of cirrhotic liver showing an irregular nodule of liver bounded on either side by fibrous tissue (viewed with epipolarizing illumination). (A) CYP2A. (B) CYP2B. (C) CYP3A. Note the presence of intensely labeled cells at the junction of parenchymal and connective tissue. (D) Higher magnification of CYP2A under bright-field illumination. The photomicrograph shows liver parenchyma on the right and fibrous tissue on the left. A single, isolated, intensely hybridized cell is present at the junction of parenchymal and fibrous tissue.

(30). In this respect it is interesting to note that in human liver, CYP3A genes are expressed in functionally differentiated hepatocytes, which are thought not to be susceptible to carcinogenesis, but not in cells early in the lineage, which appear to be targets for oncogenesis (31). Other clinically important roles of CYP3As include the metabolism of steroid hormones (32), oral contraceptives (33), drugs such as nifedipine (34), antibiotics such as erythromycin and triacetyloleandomycin (35) and the immunosuppressant cyclosporine (36).

Patients with severe liver disease consistently have lower cytochrome P-450–specific contents and lower cytochrome P-450–catalyzed activities toward many substrates (37). However, our results indicate that in cirrhotic liver a marked increase occurs in the expression of specific cytochrome P-450 genes—namely, CYP2A and 2B and, to a lesser extent, CYP3A—in isolated hepatocytes. The fact that this increase in gene expression is confined to cells immediately adjacent to fibrotic tissue suggests that it is mediated either by direct contact with the fibrotic cells or by a diffusable agent released from these cells. However, because only some of the hepatocytes that are in contact with connective tissue appear to be affected, the increase in CYP2A and 2B gene expression may be a transitory phenomenon. It has been reported that microsomes isolated from normal tissue surrounding human and rat liver tumors contain higher cytochrome P-450 content and catalytic activities than do those isolated from normal tissue far removed from the tumor (38). Thus an increase in the expression of certain cytochrome P-450 genes in normal tissue immediately adjacent to abnormal tissue may occur in a wide range of liver diseases.

Our finding of a highly localized increase in the

expression of specific cytochrome P-450 genes in cirrhotic livers provides evidence at the molecular level for altered hepatocyte function in liver disease and merits further study. Although the significance of such position-dependent increases in cytochrome P-450 gene expression in diseased human liver is unclear, clearly there are implications for the detoxification and activation of therapeutic drugs and chemical carcinogens in patients with liver disease.

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