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Rat cellular retinol-binding protein II: Use of a cloned cDNA to define its primary structure, tissue-specific expression, and developmental regulation

(vitamin A/protein structure/gastrointestinal development)

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ABSTRACT **The primary structure of rat cellular retinol-binding protein (CRBP) II has been determined from a cloned cDNA. Alignment of this 134-amino acid, 15,580-Da polypeptide with rat CRBP revealed that 75 of 133 comparable residues are identical. Both proteins contain four tryptophan residues, which occupy identical relative positions in the two primary structures, providing a structural explanation for their similar fluorescence spectra when complexed to retinol. Two of the three cysteines in each single-chain protein are comparably positioned. Both polypeptides contain reactive thiol groups, but the rate of disruption of CRBP II–retinol complexes by *p*-chloromercuribenzoate is greater than that of CRBP–retinol. The small intestine contains the highest concentrations of CRBP II mRNA in adult rats. CRBP II mRNA is first detectable in intestinal RNA during the 19th day of gestation, a time that corresponds to the appearance of an absorptive columnar epithelium. Unlike in intestine, a dramatic fall in liver CRBP II mRNA concentration occurs immediately after birth. The CRBP II gene remains quiescent in the liver during subsequent postnatal development. These data suggest that ligand–protein interactions may be somewhat different for the two rat CRBPs. They also support the concept that CRBP II plays a role in the intestinal absorption or esterification of retinol and suggest that changes in hepatic metabolism of vitamin A occur during development.**

Vitamin A is necessary for growth, reproduction, differentiation of epithelial tissues, and vision (1). In the intestine, retinol, whether absorbed from the gut lumen or generated from β-carotene, is esterified and packaged into chylomicron particles prior to secretion. These retinyl esters are then taken up, hydrolyzed, reesterified, and stored by the liver (1). Mobilization of retinol from the liver and transport to peripheral tissues occurs by means of a well-characterized highly controlled process: hepatic retinyl esters are hydrolyzed and retinol is released to the blood stream complexed with a specific carrier protein, serum retinol-binding protein (2). Little is known about the trafficking of this extremely hydrophobic compound between various metabolic pathways *within* the cell. Specific intracellular retinol carrier proteins may play a role in the intracellular transport and metabolism of retinol.

To date, two low molecular weight cytosolic retinol-binding proteins have been identified in the rat, and they are known as cellular retinol-binding protein (CRBP) and cellular retinol-binding protein II (CRBP II) (3, 4). Both of these proteins bind retinol but not the retinol metabolite retinoic acid (3, 4). CRBP is present in a wide variety of adult rat tissues but is most abundant in liver and kidney (3–7). By contrast, the highest recorded concentrations of CRBP II in adult rats have been in the small intestine, where radioimmunoassays suggest it is 1000-fold more abundant than CRBP (3). Immunocytochemical localization studies have shown that CRBP II is present in villus-associated enterocytes (6). Ericksson *et al.* (5) reported that CRBP was also located in the villus cells of the proximal small intestine. However, using different antibodies, Crow and Ong were able to detect CRBP only in the lamina propria and in gut-associated lymphoid tissue (6). They also found that the concentration of CRBP II in neonatal (1-day-old) rat liver exceeded that of CRBP but that by adulthood CRBP II was barely detectable. In view of its high concentration in the enterocyte, these workers suggested that CRBP II plays a role in the absorption or esterification of retinol by the intestinal epithelium (6). The physiological role played by CRBP II in liver was less clear.

The amino acid sequence of CRBP has recently been determined by Sundelin *et al.* (8). To gain further insights about the function of CRBP II, we have used a cloned cDNA to define its structure, tissue-specific expression, and developmental regulation.

MATERIALS AND METHODS

Animals. Timed-pregnant, neonatal, and adult male Sprague–Dawley rats were obtained from Sasco (St. Louis, MO). Animals were fed a standard rat chow diet (Ralston Purina Rodent Laboratory Chow no. 5001) ad lib. This chow contains 15 international units of vitamin A per g and 4.5 ppm of carotene.

RNA Isolation. Total cellular RNA was isolated from fetal, neonatal, or adult tissues by using the guanidine thiocyanate extraction procedure of Chirgwin *et al.* (9).

Isolation of a cDNA Encoding CRBP II. CRBP II (2 nmol) (3) was subjected to automated sequential Edman degradation in an Applied Biosystems model 470A vapor-phase sequencer (10), and the NH_2-terminal 23 residues were identified: Thr-Lys-Asp-Gln-Asn-Gly-Thr-Trp-Glu-Met-Glu-Ser-Asn-Glu-Asn-Phe-Glu-Gly-Tyr-Met-Lys-Ala-Leu. Two oligonucleotides containing all possible codons for the amino acids spanning positions 1–6 and 8–14 of CRBP II were synthesized by using phosphoramidite chemistry (11) and an Applied Biosystems Model 380A DNA synthesizer. Oligonucleotides were labeled with T4 polynucleotide kinase and [γ-^{32}P]rATP. Screening (12) of an adult rat small intestinal cDNA library containing 3000 independent recombinants (13) yielded one clone, pCRPII-15, that reacted with both oligonucleotides. Restriction analysis revealed that its plasmid DNA contained a 300-base-pair (bp) insert (see Fig. 1). An *Ava* II–*Hin*fI restriction fragment from pCRPII-15 was

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Abbreviations: CRBP, cellular retinol-binding protein; bp, base pair(s).

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labeled with ^{32}P by the random primer method (14) and used to screen a 12-day suckling rat small intestinal cDNA library containing 8000 independent recombinants. This library was constructed in pUC13 (15) by using the method of Gubler and Hoffman (16). Six probe-positive clones were identified. One, pCRPII-6, had an insert in its plasmid DNA that was large enough (600 bp) to encode the entire CRBP II polypeptide.

DNA Sequencing. Restriction fragments from each plasmid were subcloned in M13 mp18 and mp19 phage (17) and sequenced by the dideoxy chain-termination method (18).

RNA Blot Hybridization Analyses. Dot blots were prepared by using a template manifold (19). Four different amounts (0.5, 1, 2, and 3 μg) of each total cellular RNA sample were applied to the filter after denaturation in formaldehyde. Yeast tRNA was added to the samples so that the total amount of RNA per dot was identical (3 μg). Stringencies used for hybridization and filter washing have been described elsewhere (20). The relative abundance of CRBP II mRNA in total cellular RNA preparations was calculated by quantitative scanning densitometry of filter autoradiographs (19). Signals in the linear range of film sensitivity (determined from reference standards) were analyzed (19). The reproducibility of the dot blot data was independently audited by probing the blots with a cloned rat cDNA for apolipoprotein A IV (13). The relative abundance of apolipoprotein A IV mRNA in fetal, suckling, weaning, and adult tissue RNAs was calculated from signals generated by these filters. They were within 15% of those determined previously with independently constructed blots (19). Blot hybridization after electrophoresis was according to Thomas (20).

Cell-Free Translation. Samples of total cellular RNA isolated from 14-day postnatal liver and adult small intestine were added to nuclease-treated rabbit reticulocyte lysates (Promega Biotec, Madison, WI) containing [^{35}S]methionine. The [^{35}S]methionine-labeled polypeptides were incubated with polyclonal rat CRBP II antiserum raised in rabbits (3, 6). Antigen–antibody complexes were adsorbed to staphylococcal protein A-Sepharose, washed, and denatured prior to electrophoresis through 15% polyacrylamide slab gels containing $NaDodSO_4$ (21). To calculate the abundance of CRBP II mRNA, radiolabeled CRBP II was recovered from gel slices by passive elution, its radioactivity was measured, and the data were expressed as a percentage of total *in vitro* protein synthesis (21).

RESULTS AND DISCUSSION

Nucleotide Sequence of CRBP II cDNA. cDNA clones encoding CRBP II were isolated from rat small intestinal libraries. The nucleotide sequencing strategies employed for the inserts contained in pCRPII-15 and pCRPII-6 are summarized in Fig. 1. There were no discrepancies between the defined nucleotide sequences of the cloned cDNAs. Translation of the compiled nucleotide sequence (Fig. 2) disclosed an initiation codon that begins at position 56, followed by an open reading frame that terminates with a stop codon at nucleotide 458. The 134-residue polypeptide encoded by this region has a calculated molecular weight of 15,580. The amino acids spanning positions 2–24 of the deduced primary translation product are identical to those identified by automated sequential Edman degradation of CRBP II (see *Materials and Methods*).

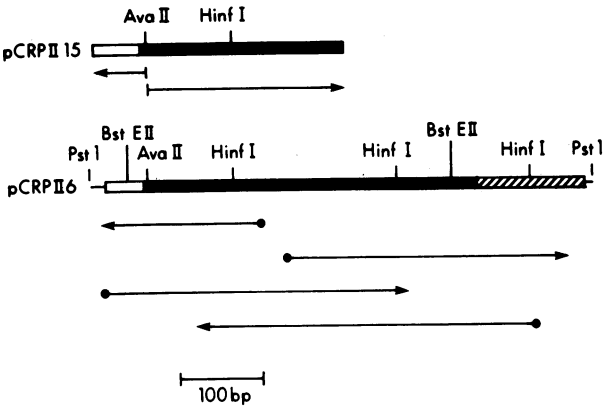

FIG. 1. Restriction endonuclease map and nucleotide sequencing strategy for cloned CRBP II cDNAs. Arrows beginning with closed circles represent sequencing reactions carried out with synthetic oligonucleotides as primers. Arrows beginning with vertical lines indicate that a "universal" 17-nucleotide M13 primer (Collaborative Research, Lexington, MA) was utilized. The open, closed, and hatched bars represent the 5′ nontranslated, coding, and 3′ nontranslated region of CRBP II mRNA, respectively.

Comparative Sequence Analysis of CRBP and CRBP II. We used the ALIGN algorithm (22) and the parameters specified in the legend to Fig. 3 to generate the optimal alignment shown in that figure. The degree of amino acid sequence identity is remarkable (75 out of 133 possible matches). The four tryptophan residues at positions 9, 89, 107, and 110 of CRBP II are entirely conserved in CRBP. Three of the four tryptophans are present in the context of a COOH-terminal oligopeptide domain, which exhibits the highest amount of amino acid sequence identity found between these proteins (16 of 22 residues spanning positions 89–110 are identical).

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      10        20        30        40        50        60        70        80        90       100
GCAGCTTGTTCCTTCACGGTCACCAAACGTCCGCATCAAACCAGAGGCCGCCATCATGACGAAGGACCAGAATGGAACCTGGGAAATGGAGAGTAATGAG
                                                          MetThrLysAspGlnAsnGlyThrTrpGluMetGluSerAsnGlu

     110       120       130       140       150       160       170       180       190       200
AACTTTGAAGGCTACATGAAGGCCCTAGATATTGATTTTGCCACCCGCAAGATTGCAGTGCGTCTGACTCAGACGAAGATCATCGTTCAAGACGGTGATA
AsnPheGluGlyTyrMetLysAlaLeuAspIleAspPheAlaThrArgLysIleAlaValArgLeuThrGlnThrLysIleIleValGlnAspGlyAspA

     210       220       230       240       250       260       270       280       290       300
ACTTCAAGACAAAAACCAACAGCACGTTCCGCAACTATGACCTAGATTTCACAGTGGGGGTGGAGTTTGACGAACACACAAAGGGTCTGGATGGCCGGAA
snPheLysThrLysThrAsnSerThrPheArgAsnTyrAspLeuAspPheThrValGlyValGluPheAspGluHisThrLysGlyLeuAspGlyArgAs

     310       320       330       340       350       360       370       380       390       400
CGTCAAGACCCTAGTCACCTGGGAAGGAAACACCCTGGTGTGTGTGCAGAAAGGGGAGAAGGAGAATCGTGGCTGGAAGCAGTGGGTCGAGGGAGACAAG
nValLysThrLeuValThrTrpGluGlyAsnThrLeuValCysValGlnLysGlyGluLysGluAsnArgGlyTrpLysGlnTrpValGluGlyAspLys

     410       420       430       440       450       460       470       480       490       500
CTGTACCTGGAGCTGACCTGCGGTGACCAGGTGTGTCGACAAGTGTTCAAAAAGAAGTGATGGGCCCAGGGGAAGCCTGGAACATGTGTAGAGTTCTCTG
LeuTyrLeuGluLeuThrCysGlyAspGlnValCysArgGlnValPheLysLysLys*op

     510       520       530       540       550       560       570
CCATTCTGAAAAGCAGCATTGGGACTCCCTGGTTCCTGACAGAGCCCCCCTTGCATCACTGCCTGGGTTTG
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FIG. 2. Nucleotide sequence of CRBP II DNA and the derived primary translation product. Residues that are underlined were verified by automated sequential Edman degradation of rat intestinal CRBP IIA. *op, Stop.

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                  10        20        30        40        50        60        70
CRBPII   MTKDQNGTWEMESNENFEGYMKALDIDFATRKIAVRLTQTKIIVQDGDNFKTKTNSTFRNYDLDFTVGVE
           : :: : : :::::: :  :::   : ::::  :   : ::::::     : ::::::  :: :: :
CRBP     -PVDFNGYWKMLSNENFEEYLRALDVNVALRKIANLLKPDKEIVQDGDHMIIRTLSTFRNYIMDFQVGKE
                  10        20        30        40        50        60

                  80        90       100       110       120       130
CRBPII   FDEHTKGLDGRNVKTLVTWEGNTLVCVQKGEKENRGWKQWVEGDKLYLELTCGDQVCRQVFKKK-
         : :   : : :   : : : :  : :::::::: ::: :: ::: : ::       : :::::
CRBP     FEEDLTGIDDRKCMTTVSWDGDKLQCVQKGEKEGRGWTQWIEGDELHLEMRAEGVTCKQVFKKVH
         70        80        90       100       110       120       130
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FIG. 3. Alignment of rat CRBP and CRBP II. The program ALIGN (22) was utilized. The standard one-letter code for amino acid residues is used, and identities are indicated by colons. The mutational data matrix was used with a bias of +2 (22). The gap penalty was set at 6, and 100 random runs were performed on a VAX 11/785 computer. The score for the alignment shown is 26.43 SD.

The absorption and fluorescence excitation–emission spectra of CRBP and CRBP II complexed with all-*trans*-retinol are very similar (3, 4). These spectra contain major peaks at 348 and 366 nm due to the bound ligand. This is distinct from the λ_{max} at 325–330 nm observed when retinol is dissolved in organic solvents. Both retinol–protein complexes also show a peak at 290 nm in their fluorescence excitation spectra. When emission is monitored at 480 nm (retinol fluorescence), this peak at 290 nm most likely represents energy transfer from tryptophan residues to the bound retinol (3, 4). Such transfer is a function of both distance and orientation between the all-*trans*-retinol and the tryptophan residue(s). The efficiency of this transfer appears quite similar for CRBP and CRBP II, since the ratios of fluorescence intensities at 290 nm and 348–350 nm are essentially identical for the two protein–retinol complexes (when monitored at 480 nm; see ref. 3). The identical positions of tryptophan residues in CRBP and CRBP II provide a structural explanation for the similarity in their spectral behavior. The spectral data suggest that one or more of these "reporter" residues in both CRBP and CRBP II may have similar orientations with respect to the bound all-*trans*-retinol and may be located close to it.

CRBP and CRBP II are single-chain polypeptides (3, 4). Both proteins contain three cysteine residues. In CRBP, the cysteine residues at positions 95 and 126 (see Fig. 3) are known to form a disulfide bond (8). The alignments reveal that these two cysteines are conserved in CRBP II. However, CRBP II and CRBP differ in the location of their third cysteine. In CRBP II, a cysteine is located at residue 122. In CRBP, the cysteine residue is located at position 82. If these residues contribute free thiol groups, then a reagent such as *p*-chloromercuribenzoate could perturb the fluorescence of retinol bound to CRBP and CRBP II. To test this hypothesis, an experiment was performed whose results are shown in Fig. 4. CRBP–retinol and CRBP II–retinol were incubated with *p*-chloromercuribenzoate. Fluorescence emission was measured at 480 nm after excitation at 348 nm. The decrease in emission at 480 nm indicated that binding of all-*trans*-retinol by CRBP and CRBP II was disrupted by *p*-chloromercuribenzoate [since bound retinol exhibits a 7- to 8-fold enhancement of fluorescence compared to free retinol (3, 4)]. The rate of disruption was greater with CRBP II. Two conclusions can be made. First, the data are consistent with the hypothesis that both cytosolic proteins contain reactive thiol groups. Second, the different rates of disruption suggest that these cysteine residues are not equally accessible to, or reactive with, *p*-chloromercuribenzoate. The experiment does not allow us to determine if the reactive thiol group is directly involved in ligand binding. These data also suggest that there are subtle differences between the interaction of CRBP and CRBP II with their ligands, and point to the need for further studies comparing the ligand specificities and affinities of the two CRBPs.

Tissue-Specific Expression of the CRBP II Gene in Adult Rats. The distribution of CRBP II mRNA in adult rat tissues was examined by using blot hybridizations. A unique 800-nucleotide RNA species was detected in a blot containing adult small intestinal RNA. No signal was noted in lanes containing adult liver RNA (data not shown). Samples of total cellular RNA, prepared from 10 different tissues harvested from 10 adult male Sprague–Dawley rats, were surveyed by using dot blots (Fig. 5). The hybridization and washing stringencies used for dot and electrophoretic blots were identical. CRBP II mRNA was most abundant in small intestine. It is ~2-fold more abundant in the proximal compared to distal half of the small intestine (data not shown). No signals were seen when colonic RNA was examined. Adrenal, testes, and brain RNA gave signals that were 2.3%, 0.8%, and 1.5% of the signal produced by small intestinal RNA. No CRBP II mRNA was detected in total cellular RNA prepared from adult liver, kidney, spleen, heart, or lung.

The 100-fold range of small intestinal RNA standards included in Fig. 5 illustrates the sensitivity of the dot-blot technique. Data from cell-free translation studies indicated that 0.2–0.3% of adult small intestinal mRNA encodes the 16-kDa CRBP II polypeptide. At this level of abundance, signals can be seen in dots containing as little as 30 ng of small intestinal total cellular RNA. Extrapolating this implies that the dot blots can detect CRBP II mRNA when its concentration in total cellular RNA is ≥0.003%. Despite this sensitivity, a question can be raised about the *specificity* of the signal—especially in light of the sequence similarities between CRBP and CRBP II. Comparable data on the distribution of CRBP mRNA in these adult rat tissues are not available. However, analysis of the distribution of CRBP by using a radioimmunoassay has shown that liver and kidney contain the highest concentrations of CRBP in adult rats (3–7). The lack of detectable signals in dot blots of kidney and liver RNA (Fig. 5*B*) provides one piece of evidence that the

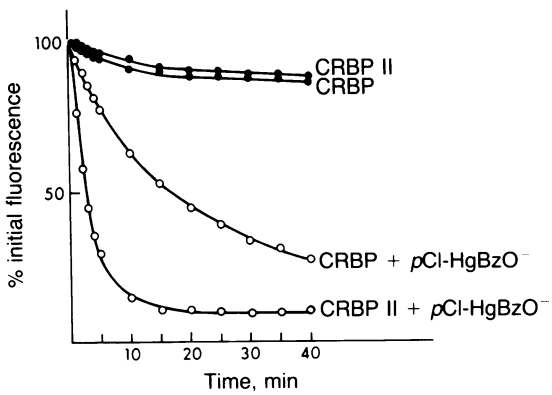

FIG. 4. Effects of *p*-chloromercuribenzoate on the fluorescence excitation and emission spectra of CRBP–retinol and CRBP IIA–retinol. Protein–ligand complexes (150 nM) were incubated in buffer alone (0.2 M potassium phosphate, pH 7.2) or buffer containing *p*-chloromercuribenzoate (*p*Cl-HgBzO⁻) (2 mM). Periodically during the incubation, the complex was excited at 348 nm and fluorescence was monitored at 480 nm (3).

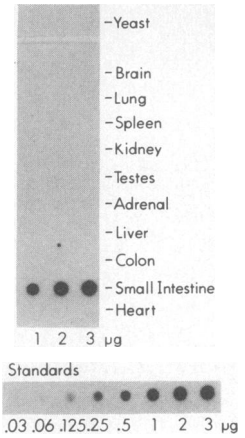

FIG. 5. Blot hybridization analysis of adult rat tissue RNAs. Tissues were pooled from 250-g male rats consuming a standard chow diet. Total cellular RNA was applied to a dot blot in the amounts indicated. The blot was probed with ^{32}P-labeled CRBP II cDNA. The filter was hybridized in a solution containing 50% (vol/vol) deionized formamide, 6× SSC, 50 mM sodium phosphate at pH 7, 1 mM EDTA, 1.5× Denhardt's solution, sheared single-stranded salmon sperm DNA at 50 μg/ml, and 10% dextran sulfate (19) (1× SSC = 0.15 M NaCl/0.015 M sodium citrate; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). After a 24-hr incubation at 42°C, the filter was washed in 1× SSC at room temperature followed by four 10-min exchanges in 0.1× SSC maintained at 55°C. An autoradiograph of the filter is shown. Control dots of the carrier yeast tRNA did not react with the probe.

hybridization and washing stringencies selected did not result in cross-reaction between CRBP II cDNA and CRBP mRNA.

Developmental Regulation of CRBP II Gene Expression. Cytosolic retinol-binding activity has been found in placenta (23). Dot-blot hybridization analysis of 8-day conceptus RNA as well as RNA prepared from placentas harvested during days 18–21 of pregnancy failed to detect CRBP II mRNA. We therefore concluded that CRBP II does not play a role in the placental transport or metabolism of vitamin A during the early and late stages of gestation.

A sagittal section of a 19-day fetal rat was stained with CRBP II antiserum (Fig. 6) to identify tissues where the gene is expressed. CRBP II was found only in the liver and in the villus epithelial cells of the small intestine. We further defined the developmental regulation of CRBP II gene expression in these two organs by using dot blots.

CRBP II mRNA was first noted in fetal intestine on the 19th day of gestation (see Fig. 7*A*). This coincides with the time that well-formed villi lined with well-differentiated columnar epithelium first appear (24). During the next 48 hr of fetal development, CRBP II mRNA levels rise sharply. By the 21st and final day of fetal life, intestinal concentrations are 11-fold higher than at day 19 and the highest recorded at any stage of intestinal development. CRBP II mRNA levels begin to fall immediately after parturition and continue to do so throughout the suckling period (postpartum days 1–12). During the weaning period (days 13–28) the concentration of this mRNA increases briefly, but by the time weaning has concluded (day 35), levels are only 15% of what they were immediately after birth. As the adult animal grows, concentrations increase once again. Changes in the concentration of intestinal CRBP II mRNA observed during late fetal life appear to correlate to a large extent with the appearance and proliferation of villi (24). Postpartum changes can be at least partly explained by the changes in the ratio of villus to crypt cells that occur during the suckling and weaning periods (25).

It is unclear whether absorption of vitamin A in amniotic fluid by fetal gut plays any role in the transport of retinoids

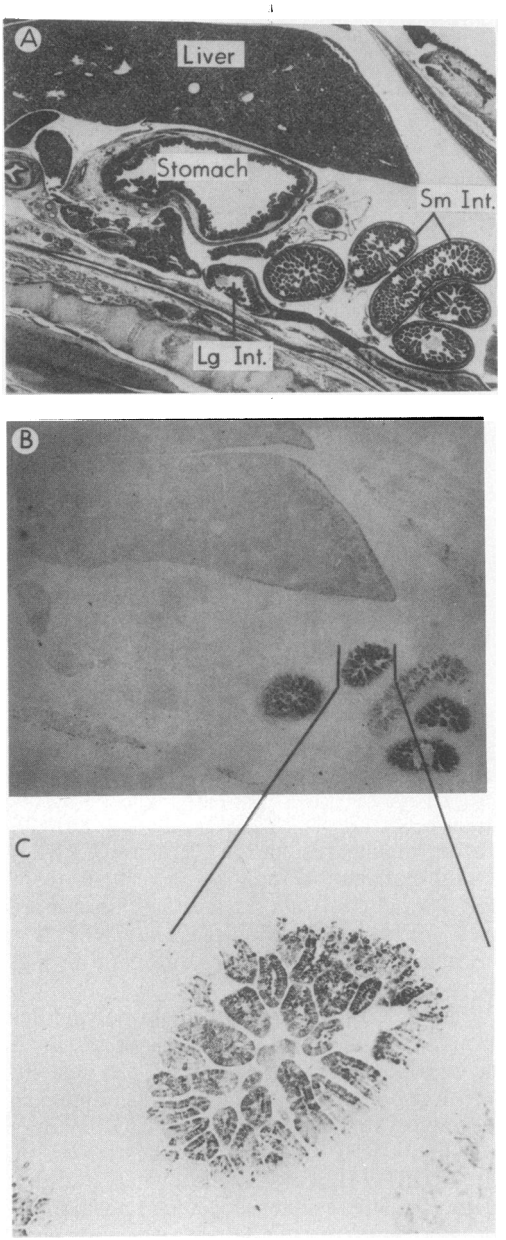

FIG. 6. Immunolocalization of CRBP II in a 19-day fetal Sprague–Dawley rat. Whole animal sagittal sections (5 μm) were stained by the ABC method with CRBP II antiserum (3, 6). (*A*) Hematoxylin and eosin stain of the sagittal section, ×9. (*B*) CRBP II visualized with the antibody is located in liver and small intestine. No staining is seen in other organs, including colon and stomach. (×9.) (*C*) An enlargement (×50) of the region of small intestine seen in *B* demonstrates immunolocalization of the binding protein in absorptive cells situated on villi, which have just formed at this developmental stage.

from mother to fetus. There is indirect evidence for such a pathway in some other species (26).

The developmental changes in liver CRBP II mRNA concentrations do not parallel those in the intestine (compare the upper and lower panels of Fig. 7). Liver CRBP II mRNA is detected earlier in fetal development (day 18 vs. day 19 in the intestine). The increase in CRBP II mRNA concentration seen during the late stage of fetal intestinal ontogeny is also seen in liver, but it is not as dramatic. The concentration of this mRNA falls in both liver and intestine after parturition, but in liver this fall is much more abrupt—no mRNA is found in material harvested from 1-day-old pups. The CRBP II gene becomes quiescent, and it remains so in the liver throughout adulthood. Postpartum changes in the levels of liver CRBP II

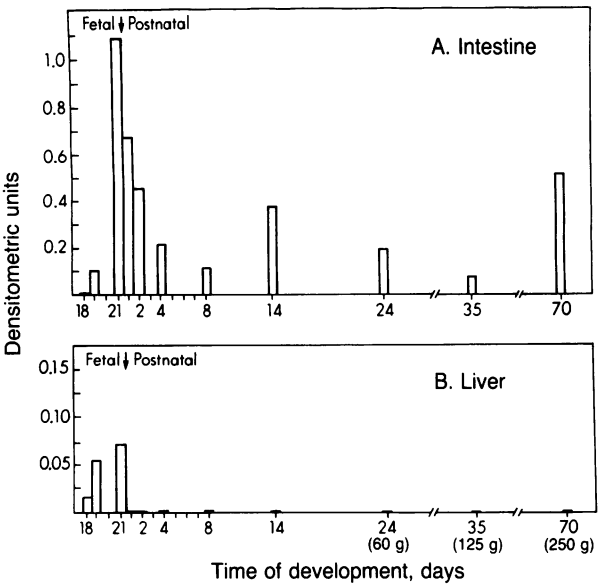

FIG. 7. Developmental changes in intestinal and liver CRBP II mRNA accumulation. Tissues were pooled from 10–30 animals for each developmental time point analyzed. Four concentrations of each total cellular intestinal (*A*) or liver (*B*) RNA sample were applied to nitrocellulose filters prior to probing with ^{32}P-labeled CRBP II cDNA. Autoradiographs of the dot blots were examined by an LKB UltroScan laser densitometer. Reference standards of adult intestinal RNA (0.03–3 μg) were included in each filter to define the linear range of film sensitivity and to permit comparison of values obtained from different blots probed with the same [^{32}P]cDNA preparation. Note the difference in scale between *A* and *B*. Densitometric absorbance units have been normalized to show the relative amounts of CRBP II mRNA in the two tissues.

mRNA cannot be simply attributed to known changes in the liver's constituent cell population.

The CRBP II mRNA detected in fetal rat liver was identical in size to that found in adult intestine (data not shown). This observation suggests that the signals observed in dot blots of fetal liver RNA were derived from CRBP II mRNA.

The abrupt decrease in CRBP II mRNA levels in the liver immediately after parturition may reflect changes in vitamin A delivery to, and metabolism within, the liver at this time. During gestation there is limited, albeit well-controlled, transfer of vitamin A from mother to fetus (27). On the basis of studies in the rat, Takahashi and co-workers suggested that transfer of vitamin A from dam to fetus at, and following, the 11th day of gestation occurs primarily by transplacental transport of retinol complexed with maternal serum retinol binding protein (27). By contrast, after birth dietary retinoids are delivered to the liver in the form of retinyl esters packaged in chylomicron particles (1).

There is relatively limited accumulation of vitamin A during late fetal life (27). Liver retinoid concentrations in fetal and suckling rats are much lower than in adult rats (28, 29). Rasmussen *et al.* (29) observed that, under steady-state conditions, retinoids in both fetal liver and adult liver are predominantly in the ester form (>95%). Little is known about the dynamics of retinol metabolism in the liver during development (i.e., rate of esterification vs. rate of mobilization to peripheral tissue vs. rate of conversion to other retinoid metabolites). The changes observed in liver CRBP II mRNA concentrations underscore the need for further studies on vitamin A delivery to, and metabolism within, the developing liver.

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