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Cell-specific immunohistochemical localization of a cellular retinol-binding protein (type two) in the small intestine of rat

(vitamin A/cellular retinol-binding protein/absorptive cells)

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ABSTRACT **One of us recently has reported the purification of a new retinol-binding protein that is distinctly different from the well-known cellular retinol-binding protein, CRBP. This protein, which we propose to name cellular retinol-binding protein type II [CRBP(II)], was found almost exclusively in the small intestine of the adult rat at levels 1000 times greater than that of CRBP. Here we have determined the cellular location of these two proteins in the small intestine of the rat. By using an immunohistochemical technique, the absorptive cells of the small intestine, from the duodenum to the ileum, were strongly stained when antiserum against CRBP(II) was used. More intense staining was observed in absorptive cells near the tips of the villi than in those located at the base of the villi. However, the proliferative cells in the crypts of Lieberkühn were stained only lightly if at all. In contrast to absorptive cells, goblet cells in the villi did not stain. When tissue sections containing the gastroduodenal junction were examined, no staining was observed in the gastric epithelium, while the epithelium of the most proximal portion of the duodenum showed very strong staining. In tissue sections containing the ileocecal junction, staining terminated abruptly at the end of the distal ileum. No staining was observed in the epithelium of the colon. In contrast, the cellular location of CRBP in the small intestine was quite different from the cellular location of CRBP(II). The epithelial cells of the small intestine showed no staining when affinity-purified anti-CRBP was used. Staining was observed for connective tissue cells in the lamina propria and in cells located within the gut-associated lymphoid tissue. The cell-specific localization pattern determined for these two proteins suggests that CRBP(II), rather than CRBP, is the protein that plays a role in the absorption of retinol.**

The transport and distribution of vitamin A is mediated by a related group of specific binding proteins. These proteins include a blood protein, retinol-binding protein, which transports retinol from the liver to target cells (1, 2); cellular retinol-binding protein (CRBP) (3) and cellular retinoic acid-binding protein (4), both of which are found within cells believed to utilize vitamin A (5, 6); and cellular retinaldehyde-binding protein and interstitial retinol-binding protein (7–10), both of which are unique to visual tissue. Recently, we have reported another protein that binds retinol. It is found at high levels in the small intestine of the adult rat (11). This protein is quite distinct from CRBP, which also is present in rat small intestine, albeit at 1/1000th the level. The organ-specific location of this new protein, which we propose to call cellular retinol-binding protein, type two [CRBP(II)], has suggested that it may have a role in the absorption of vitamin A. Such a role has been suggested previously for CRBP (12).

To address this question we have used the technique of immunohistochemical localization to determine the cellular location of CRBP(II) and CRBP in the small intestine of rat. Here we report that CRBP(II), and not CRBP, was observed in the absorptive cells of the epithelium of rat small intestine in a pattern quite consistent with it being involved in retinol absorption.

METHODS

Specificity of Antisera. The antisera used in these studies were raised against pure CRBP(II) (11) and against CRBP (6). Affinity-purified anti-CRBP was prepared as described (13). The immune reagents were checked for the possibility of cross-reaction with other proteins. Soluble extracts were prepared from jejunum mucosa, ileum mucosa, and colon mucosa of rat as described (6). Protein content was determined by a modification of the method of Lowry (14). Aliquots of the extracts (containing 70 μg of protein) and aliquots of pure CRBP(II) and of CRBP (each containing 200 ng of protein) were subjected to electrophoresis on an 11% polyacrylamide gel by the method of Laemmli (15). Proteins were then transferred from the gel to nitrocellulose paper (16).

Immunoreactive proteins were demonstrated by a modification of the method of Towbin *et al.* (16). Briefly, after blocking, the nitrocellulose paper was incubated sequentially with (*i*) a 1:1000 dilution of anti-CRBP(II) or 5 ml of affinity-purified anti-CRBP diluted to an A_{280} of 0.006, (*ii*) biotinylated second antibody, and (*iii*) avidin–biotin-peroxidase complex (ABC), with washing in 0.05% Tween/0.14 M NaCl/0.01 M sodium phosphate buffer, pH 7.5, between each incubation. A brown color, developed by incubating the paper with H_2O_2 and diaminobenzidene, revealed the presence of immunoreactive material. The biotinylated second antibody, the avidin, and the biotinylated peroxidase were components of a kit purchased from Vector Laboratories (Burlingame, CA).

Preparation of Tissue Sections. Rats were killed by decapitation. Tissue was rapidly removed and washed with 0.14 M NaCl/0.01 M sodium phosphate buffer, pH 7.5. Tissue samples were fixed in PerFix (Fisher), dehydrated in ethanol, and embedded in paraffin. Sections 5 μm in thickness were obtained for immunolocalization experiments.

Immunohistochemical Localization. CRBP(II) was localized by using the ABC method described by Hsu *et al.* (17). The reagents were purchased from Vector Laboratories and used as recommended by the company. Briefly, the tissue sections were deparaffinized in xylene, incubated for 30 min in 0.3% H_2O_2 in 100% methanol to inactivate endogenous peroxidases, and rehydrated. Sections were first incubated for 20 min in diluted normal goat serum and then incubated with anti-CRBP(II) (diluted 1:1000 in 1% normal goat

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Abbreviations: CRBP, cellular retinol-binding protein; CRBP(II), CRBP type two; ABC, avidin–biotin-peroxidase complex.

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serum/0.14 M NaCl/0.01 M sodium phosphate buffer, pH 7.5) for 48 hr at 4°C in a humid chamber. The sections were then incubated with affinity-purified biotinylated anti-rabbit IgG for 30 min at room temperature. Finally, the sections were incubated for 60 min with ABC. Sections were washed three times with 0.14 M NaCl/0.01 M sodium phosphate buffer, pH 7.5, between each incubation. Diaminobenzidine and H_2O_2 were used as substrates for the peroxidase to produce the brown stain that revealed the presence of immunoreactive substances in the sections. Some sections were counterstained with Gill's hematoxylin and/or were treated with a 1% OsO_4 solution. The sections were then dehydrated and covered with a coverslip.

CRBP also was localized by the ABC method. The sections were deparaffinized, the endogenous peroxidases were inactivated, and the sections were rehydrated as before. The sections then were incubated for 20 min at 37°C in 1.8% normal goat serum/2.5% bovine serum albumin/0.1% Triton X-100/0.9% NaCl/0.05 M Tris chloride, pH 7.4. This buffer was used for all subsequent dilutions. Affinity-purified anti-CRBP was diluted to an A_{280} of 0.04 and applied to the tissue sections for 1 hr at 37°C. The tissue sections were then treated as above.

In control sections, normal rabbit serum or antiserum treated with pure CRBP(II) (100-fold excess of the binding capacity of the antiserum) was used instead of anti-CRBP(II). A purified rabbit IgG solution with an A_{280} of 0.04 was substituted for anti-CRBP as a control.

RESULTS

Specificity of Antisera. After extracts of jejunum, ileum, and colon mucosa were resolved by gel electrophoresis, the proteins were transferred to nitrocellulose and examined for immunoreactivity with antiserum raised against pure CRBP(II) and affinity-purified anti-CRBP, respectively. The lanes containing tissue extracts were purposely overloaded to enhance detection of cross-reacting material. When antiserum raised against CRBP(II) was used, only one band of specific immunoreactive material was observed for the extracts of jejunum and ileum mucosa, tissues known to contain high levels of CRBP(II), and no bands were observed for the extract of colon mucosa, a tissue known to contain about 1/500th as much CRBP(II) (Fig. 1*A*). The position of the band corresponded to a M_r of 16,000 and was indistinguishable from the position of pure CRBP(II). As shown, this antiserum did not react with pure CRBP, confirming our previous report (11). The light bands observable at the top of the transfer are nonspecific staining, which appeared in all lanes, even those containing pure CRBP(II) or CRBP. Consequently, the antiserum was specific for CRBP(II) and recognized no other proteins in the tissues we planned to study.

With affinity-purified anti-CRBP, a light band was detected in the mucosal extracts of the intestine that was identical in position with the dark band of pure CRBP at M_r 15,000 (Fig. 1*B*). The intensity of the band was consistent with the low levels previously reported by us for these tissues (6). No reaction was observed with pure CRBP(II). Consequently, this affinity-purified preparation was shown to be specific for CRBP and, most importantly, would not recognize CRBP(II).

Immunolocalization of CRBP(II) and CRBP in the Jejunum. Tissue sections from the jejunum were analyzed for the location of CRBP(II). Reaction product, indicating the presence of CRBP(II), was observed only in epithelial cells lining the villi of the mucosa (Fig. 2*A*). No staining was observed in other cells of the mucosa, and no staining was observed in the smooth muscle of the jejunum. Control slides in which either preimmune serum or antiserum absorbed with pure CRBP(II) was substituted for immune serum showed no staining (Fig. 2*B*). Additionally, colon, which contains 1/1000th as much

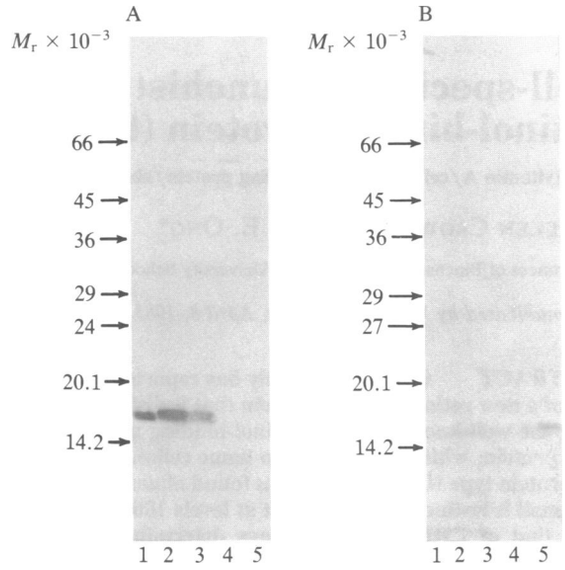

FIG. 1. Detection of immunoreactive proteins in mucosal extracts. Aliquots of mucosal extracts and aliquots of pure CRBP(II) and of pure CRBP were subjected to electrophoresis on an 11% polyacrylamide gel and transferred to nitrocellulose. Immunoreactive proteins were detected as described. Lanes: 1 and 5, 200 ng of pure CRBP(II) and pure CRBP, respectively; 2, 3, and 4, aliquots of extracts of jejunum mucosa, ileum mucosa, and colon mucosa, respectively, each containing 70 μg of total protein. Both anti-CRBP(II) (*A*) and affinity-purified anti-CRBP (*B*) were checked for specificity.

CRBP(II) as jejunum, was examined and no staining was observed. Thus, the staining observed was considered specific for the presence of CRBP(II).

Tissue sections of the jejunum were analyzed also for the location of CRBP. No staining was observed in the epithelial cells (Fig. 2*C*). Immunoreactive material was observed in the gut-associated lymphoid tissue and in connective tissue cells within the lamina propria (Fig. 2*D*). Control slides for which pure rabbit IgG diluted to the appropriate A_{280} was substituted for affinity-purified anti-CRBP showed no staining. Therefore, we concluded the staining was specific for CRBP.

Within the villi, only the absorptive epithelial cells were stained for CRBP(II) (Fig. 3). A gradation in the intensity of staining is apparent with cells at the tips of the villi stained more intensively than cells at the bases of the villi (Fig. 3*A*). The proliferative cells of the crypts of Lieberkühn were stained only lightly if at all. As shown in Fig. 3*B*, the transition from crypts to villi was clearly marked by the abrupt increase in immunostaining. There also appeared to be a light staining in the 10–12 crypt cells adjacent to the junction.

In contrast to the absorptive epithelial cells, goblet cells did not stain (Fig. 3*C*). Much of the cytoplasm of the goblet cell is compressed at its slender basal end, below the vacuole created when the mucigen droplets are lost from the theca during processing of the tissue sections. The cytoplasm could be visualized with Gill's hematoxylin in the sections we used here. Lack of staining is apparent below the vacuoles, suggesting the goblet cells contained little, if any, CRBP(II).

Investigation of the Gastroduodenal and Ileocecal Junctions. Tissue sections containing the gastroduodenal junction and the ileocecal junction were examined also for the location of CRBP(II) (Fig. 4). No staining was observed in the gastric epithelium, but the absorptive epithelial cells in the most proximal portion of the duodenum were stained darkly (Fig. 4*A*). The ileocecal junction showed a similar dramatic staining pattern (Fig. 4*B*). The absorptive epithelial cells in the

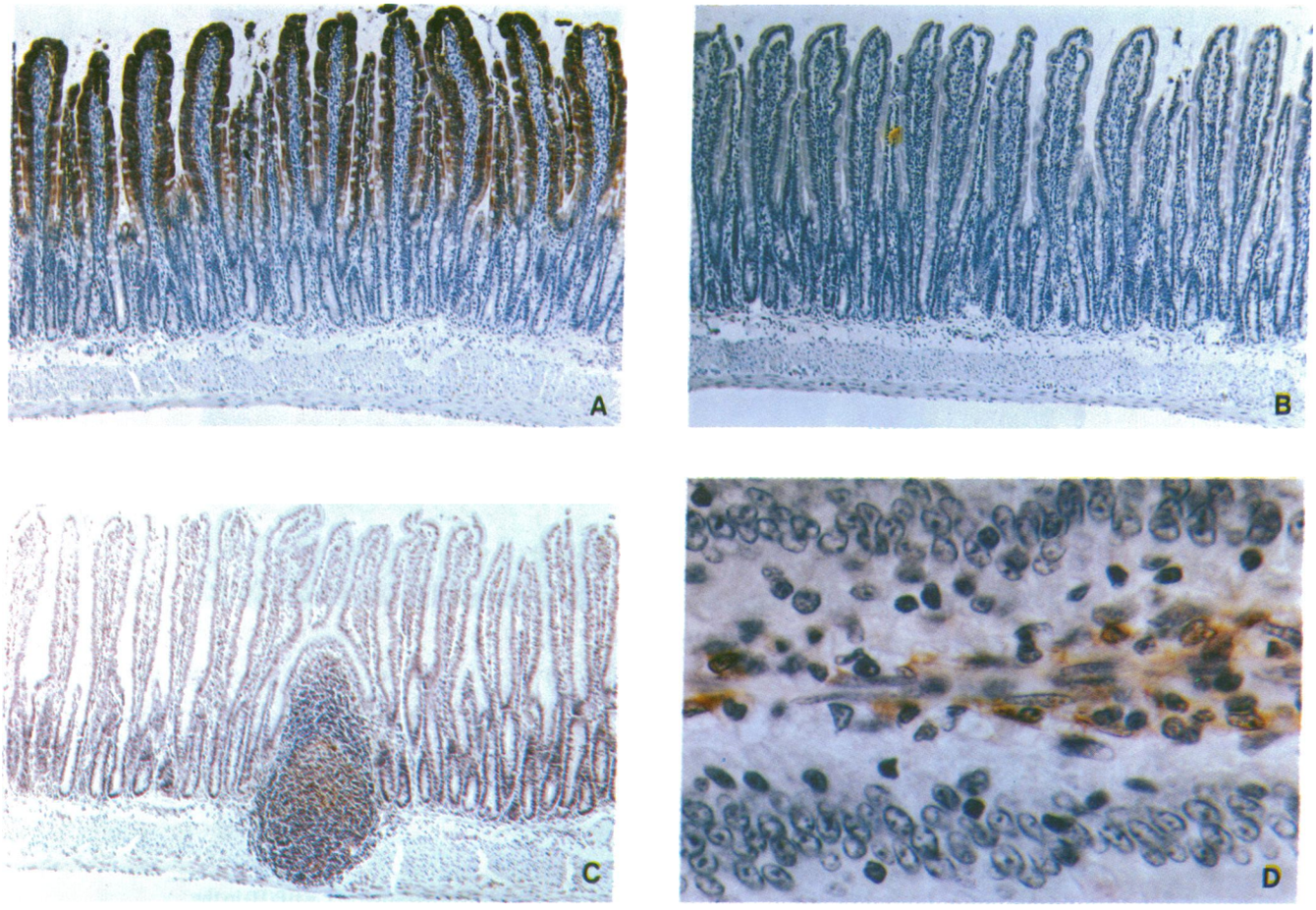

FIG. 2. Immunolocalization of CRBP(II) and CRBP. Sections (5 μm) of rat jejunum were stained by the ABC method with anti-CRBP(II) antiserum (*A*), anti-CRBP(II) antiserum pretreated with pure rat CRBP(II) (*B*), and affinity-purified anti-CRBP antiserum (*C* and *D*). (*A*) Staining is seen only in the epithelial cells of the villi. (×60.) (*B*) No staining is seen when the anti-CRBP(II) was pretreated with pure CRBP(II). (×60.) (*C*) Staining for CRBP is seen only in the gut-associated lymphoid tissue (center). (×60.) (*D*) Cell-associated staining for CRBP can be seen in the central portion of an individual villus—the lamina propria—here oriented horizontally. (×650.)

most distal portion of the ileum were stained, but the cecum was unstained. These results clearly show a very precise restriction of CRBP(II) to the small intestine. As can be seen in this figure, a gradient in staining intensity was obtained along the small intestine, with duodenum staining more intensely than ileum.

DISCUSSION

Our previous report on the discovery of CRBP(II) revealed that the small intestine of the rat contained much higher levels of the protein than in any other organ examined (11). The organ-specific location of CRBP(II) suggested a possible role in the absorption of retinol. The cell-specific location we report here strengthens that contention.

We previously had determined the level of CRBP(II) both in the mucosa, mechanically scraped from its underlying muscle layer, and in the scraped muscle and found a 3:1 ratio between the two fractions (11). Here, CRBP(II) was found only in the epithelial layer of the intestinal mucosa and not the muscularis, as would be expected for a protein with a role in absorption. The presence of CRBP(II) in the muscle fraction of the intestine in our previous study was perhaps due to either incomplete removal of the mucosa from the muscle or to the leaking of cytoplasm from epithelial cells damaged during the separation.

We also found that epithelial cells present on the villi were strongly stained for CRBP(II), whereas epithelial cells in the crypts of Lieberkühn were only lightly stained if at all. The epithelial cells on the villi in the small intestine arise from the rapidly dividing cells in the crypts (18). Cells migrate from the crypts up the villi as they mature into absorptive cells. The staining we observed increased abruptly at the crypt–villus junction suggesting a very rapid appearance of high levels of CRBP(II), which would correlate with the cell becoming a functioning absorptive cell. These absorptive cells continue to migrate up the villi and eventually are extruded from the tip (19). Thus, the most mature cells are located at the tips of the villi, and cells at the tips did appear to be more darkly stained. In contrast, goblet cells, which secrete mucin and may not function in the absorption of nutrients (20), did not stain for CRBP(II).

A similar crypt–villus distribution, correlating with the maturation of absorptive cells, has been determined for digestive enzymes such as alkaline phosphatase, maltase, invertase, and L-alanyl-L-proline dipeptidase (21) as well as fatty acid-binding protein (22). Those results were based in part on fractionation of the mucosa into crypt and villus fractions with subsequent assay. The results presented here demonstrate a dramatically similar distribution for CRBP(II).

Absorption of vitamin A can occur along the length of the small intestine (23). Consistent with that, we observed initiation of staining at the first demonstrable villus of the duodenum and an abrupt cessation of staining at the end of the ileum. Previously we had found by radioimmunoassay that CRBP(II) is present in higher levels in the jejunum than

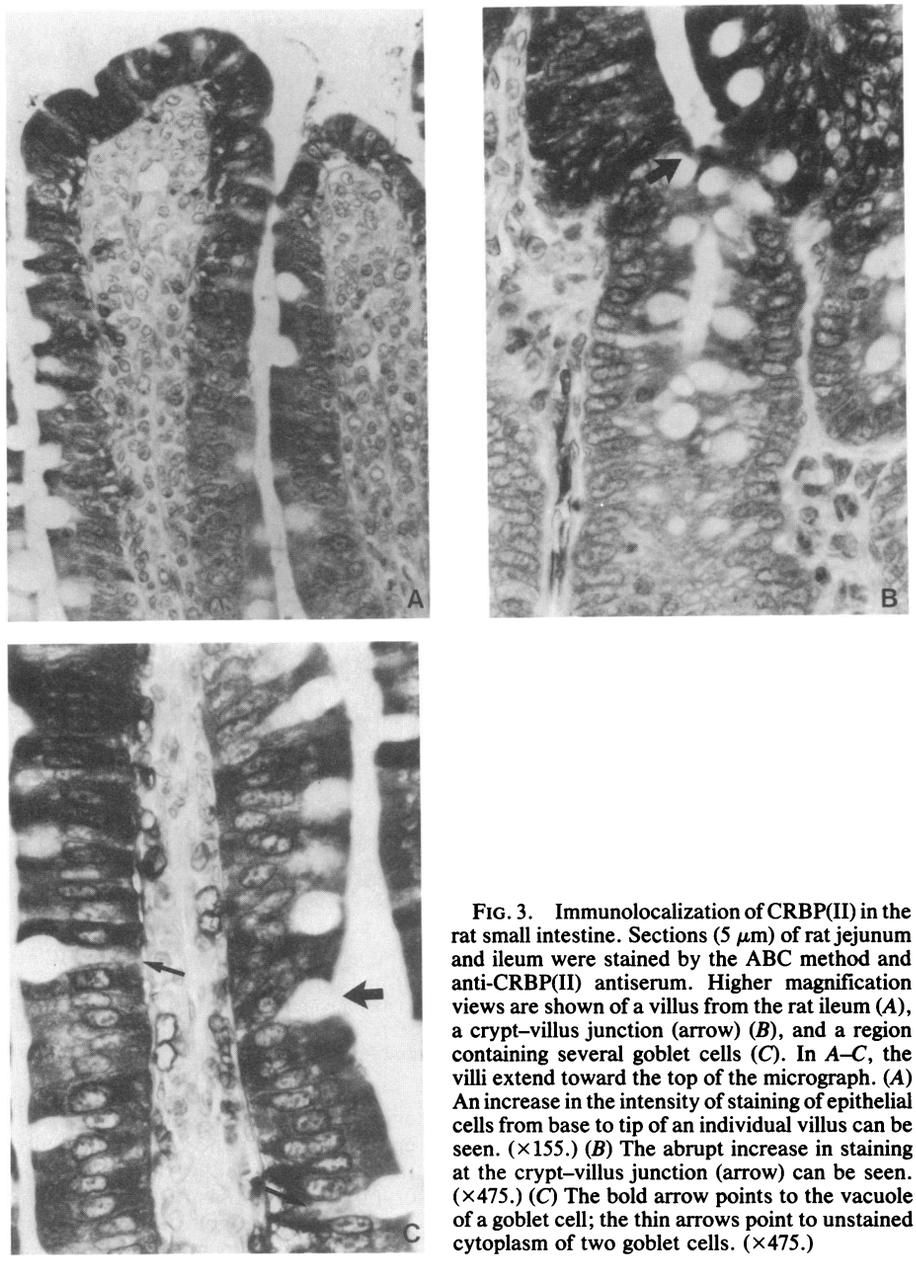

FIG. 3. Immunolocalization of CRBP(II) in the rat small intestine. Sections (5 μm) of rat jejunum and ileum were stained by the ABC method and anti-CRBP(II) antiserum. Higher magnification views are shown of a villus from the rat ileum (*A*), a crypt–villus junction (arrow) (*B*), and a region containing several goblet cells (*C*). In *A–C*, the villi extend toward the top of the micrograph. (*A*) An increase in the intensity of staining of epithelial cells from base to tip of an individual villus can be seen. (×155.) (*B*) The abrupt increase in staining at the crypt–villus junction (arrow) can be seen. (×475.) (*C*) The bold arrow points to the vacuole of a goblet cell; the thin arrows point to unstained cytoplasm of two goblet cells. (×475.)

in ileum. Here we observed that staining was more intense in the duodenum and jejunum sections than in ileum sections, consistent with that distribution.

We also determined the cell-specific location of CRBP in the small intestine. As suggested by the low level of CRBP found by radioimmunoassay (6), it was found in cells that were much less abundant than those containing CRBP(II). CRBP was found in connective tissue cells in the lamina propria as well as in cells in the gut-associated lymphoid tissue. However, CRBP was not detected in the epithelial cells of the small intestine. We have rigorously shown that both immune reagents used in these studies recognize specifically the pure protein against which they were raised and only that protein in the tissues under study here. Eriksson *et al.* (24) recently have reported the presence of immunoreactive material in the absorptive cells of the jejunum, using antibodies raised against CRBP. However, the possibility of cross-reaction of those antibodies with other proteins of the intestine was not examined as was done with extracts of liver and testis. Because of the large amounts of CRBP(II) compared with CRBP in the intestine and its similarity to CRBP, it seems likely that the antibodies used by Eriksson *et al.* were recognizing CRBP(II). Because liver and testis contain little or no CRBP(II), this would not have been evident from the analyses done. It should be pointed out that the existence of CRBP(II) had not been published at the time this earlier work was completed.

The results we report here indicate that CRBP(II) will be involved in the intestinal metabolism of retinol. Since retinol is esterified in the absorptive cell prior to its incorporation into chylomicrons, the role of CRBP(II) may be to present retinol to the appropriate esterifying enzyme. This hypothesis is not without precedence. A fatty acid-binding protein in the intestinal mucosa is believed to serve as a carrier transporting absorbed fatty acids to esterifying enzymes present in the endoplasmic reticulum (22). Thus, the protein serves to circumvent the hydrophobicity of the fatty acids. An analogous situation may well exist in the metabolism of intracellular retinol as suggested (25).

CRBP had been suggested (12) to serve as an intracellular transport protein for retinol in the intestinal mucosa. However, the discovery of CRBP(II), present in the intestinal

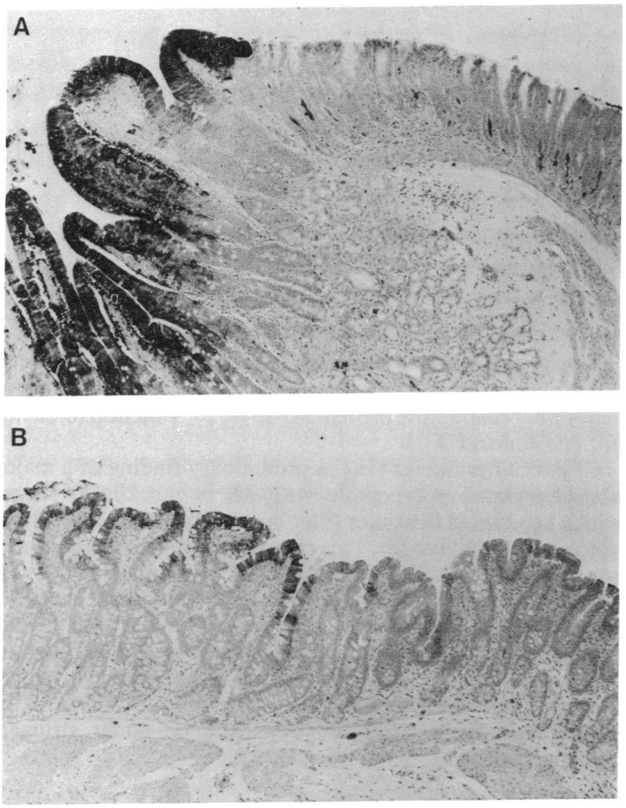

FIG. 4. Immunolocalization of CRBP(II) in tissue sections containing the gastroduodenal junction and the ileocecal junction. Sections (5 μm) containing the gastroduodenal junction (*A*) and ileocecal junction (*B*) were stained by the ABC method with anti-CRBP(II) antiserum. (*A*) The gastric pits and the muscle of the pyloric region are seen on the right side of the micrograph. In the center of the field, heavy staining can be seen at the first obvious villus of the duodenum. (*B*) Staining of the ileal villi can be seen extending from the left side of the micrograph. In the center of the field, staining terminates at the last recognizable villus and is not observed in the crypts of the cecum (right side). (×55.)

mucosa at levels 1000 times greater than CRBP, made it the more likely candidate. Certainly the cell-specific localization we report here is consistent with CRBP(II), rather than CRBP, serving as this postulated carrier protein.

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