Regulation of Cellular Retinol Binding Protein Type II by 1,25-Dihydroxyvitamin D_3†

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ABSTRACT: Previously we purified and sequenced an 18-kDa chick duodenal protein that was modulated by 1,25-dihydroxyvitamin D_3. The N-terminus of this protein has striking sequence homology to cellular retinol binding protein type II (CRBP II). Furthermore, this purified chick protein binds retinol. Antibodies have now been generated to the chick protein and used for immunoblot analysis to demonstrate that the chick protein has molecular weight, tissue distribution, and subcellular localization similar to rat CRBP II. These antibodies also cross-reacted with rat CRBP II. Antibodies to rat CRBP II cross-react with the chick protein. Northern analysis using a cDNA probe for rat CRBP II showed a single 860 base pair mRNA in both chick and rat intestinal RNA preparations. These results demonstrate that the 1,25-dihydroxyvitamin D_3 modulated protein in chick embryonic organ culture is chick CRBP II. Pulse-chase experiments in chick embryonic duodenal organ culture strongly suggest that 1,25-dihydroxyvitamin D_3 markedly decreases the synthesis of CRBP II, while not changing the degradation rate. The concentration of 1,25-dihydroxyvitamin D_3 required for the decrease in CRBP II synthesis is approximately that required to stimulate calcium uptake into embryonic chick duodenal organ cultures.

The most active form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$),[1] regulates the concentration of serum calcium and phosphorus in mammals and birds by acting on three major target organs: intestine, bone, and kidney. In the intestine, 1,25-$(OH)_2D_3$ stimulates calcium and phosphorus transport from the lumen to the plasma. In conjunction with parathyroid hormone, 1,25-$(OH)_2D_3$ causes mobilization of calcium and phosphorus from the skeleton and reabsorption of calcium in the distal renal tubule (DeLuca, 1988). A major current effort is to elucidate the molecular mechanism by which 1,25-$(OH)_2D_3$ carries out these actions.

It is generally believed that 1,25-$(OH)_2D_3$ acts through the synthesis of proteins in carrying out these activities. This belief is based on (1) the finding that both RNA and protein synthesis are required for 1,25-$(OH)_2D_3$ stimulated calcium uptake in the chick embryonic duodenal organ culture (EDOC) system (Corradino, 1973a; Franceschi & DeLuca, 1981a) and (2) the existence of a high-affinity receptor protein for 1,25-$(OH)_2D_3$ (Brumbaugh & Haussler, 1974; Kream et al., 1976), whose structure is very similar to that of other steroid hormone receptors (Burmester et al., 1988; Baker et al., 1988). Therefore, the mechanism of 1,25-$(OH)_2D_3$ action is expected to be similar to that of steroid hormones. Steroid hormone receptors bound to the hormone act by binding to specific regulatory sites on chromatin and modulate the transcription of specific hormone-dependent genes (Yamamoto, 1985). It is thought that the cellular response to the hormone is mediated by the subsequent change in concentration of these hormone-dependent proteins.

Since 1,25-$(OH)_2D_3$ performs its actions on the intestine independently of parathyroid hormone, this is perhaps the simplest target tissue in which to study its molecular mechanism. One of the approaches taken is to study the proteins modulated by 1,25-$(OH)_2D_3$ in the intestine. The chick embryonic duodenal organ culture (EDOC) is a well-characterized system that responds to 1,25-$(OH)_2D_3$ by increased calcium uptake and synthesis of the 27-kDa (kilodalton) vitamin D dependent calcium-binding protein (CaBP) (Corradino, 1973b) in a manner similar to rachitic chicks in vivo (Franceschi & DeLuca, 1981b). This system can be used to radiolabel intestinal proteins so that their metabolism can be specifically and sensitively monitored by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and fluorography (Bishop et al., 1983).

Work done by Bishop et al. (1985) in our laboratory led to the discovery of an 18-kDa, 5.1 isoelectric point (pI) protein whose incorporation of radioactive leucine is changed dramatically in the chick EDOC system upon exposure to 1,25-$(OH)_2D_3$. To identify this 1,25-$(OH)_2D_3$ modulated protein, we purified and obtained its N-terminal sequence (Finlay & DeLuca, 1988). The first 28 amino acids of the protein have striking homology to rat cellular retinol binding protein, type II (CRBP II), and the purified chick protein–retinol complex has a very similar fluorescence spectrum to that of the rat CRBP II–retinol complex (Finlay & DeLuca, 1988).

CRBP II is a protein, first isolated from a rat (Ong, 1984), located exclusively in the small intestine (Crow & Ong, 1985) that has *all-trans*-retinol as its endogenous ligand. Further work indicates that the protein exists in human intestine (Page & Ong, 1987) and the gene is highly conserved in rats, mice,

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; EDOC, embryonic duodenal organ culture; kDa, kilodalton; CaBP, 27-kDa vitamin D dependent calcium binding protein; pI, isoelectric point; CRBP II, cellular retinol binding protein type II; LRAT, lecithin–retinol acyltransferase; MAPS, monoclonal antibody purification system; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; 2D, two dimensional; 1D, one dimensional; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; RIA, radioimmunoassay; bp, base pair; SEM, standard error of the mean.

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and humans (Demmer et al., 1987). One function of CRBP II is to direct newly absorbed retinol to a lecithin–retinol acyltransferase (LRAT) for esterification (MacDonald & Ong, 1988). Retinol must be esterified in order to be incorporated into chylomicrons and secreted into the lymph (Goodman & Blaner, 1984). Retinol travels to and is stored in the liver where it is released as needed (Goodman, 1984).

In this paper the 18-kDa protein, we have previously purified (Finlay & DeLuca, 1988), has clearly been shown to be CRBP II. The experiments described herein also provide strong evidence that 1,25-$(OH)_2D_3$ regulates CRBP II primarily by decreasing the synthesis of the protein without changing its degradation rate.

Materials and Methods

Chemicals. 1,25-$(OH)_2D_3$ was a gift from the Hoffmann-La Roche Co. (Nutley, NJ). The 0.24–9.5-kb RNA ladder, the low-range prestained protein molecular weight standards, and *p*-nitrophenyl β-D-galactopyranoside were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Sepharose CL-4B and Protein A Sepharose CL-4B were obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Freund's complete adjuvant and incomplete adjuvant were purchased from GIBCO (Grand Island, NY). Protein A–β-galactosidase and rabbit IgG were obtained from Sigma (St. Louis, MO). Anti-rabbit IgG–alkaline phosphatase conjugate was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). [^{35}S]Methionine or translabel [a less purified and hence less expensive preparation of ^{35}S-labeled methionine (>800 Ci/mmol)] was obtained from ICN Radiochemicals (Irvine, CA). Monoclonal antibody purification system (MAPS) buffers were obtained from Bio-Rad (Richmond, CA). ^{14}C-labeled protein molecular weight markers were purchased from NEN Research Products (Boston, MA). Hybond-N nylon membrane and [α-^{32}P]dCTP were obtained from Amersham (Arlington Heights, IL). Plasmid pGEM2 containing the cDNA for rat CRBP II was generously provided by Jeffery I. Gordon, Washington University School of Medicine (St. Louis, MO).

Animals. One-day-old white Leghorn chickens were obtained from Northern Hatcheries (Beaver Dam, WI) and maintained for 4 weeks on a previously described 1.2% calcium, 0.7% phosphorus, vitamin D deficient diet (Finlay & DeLuca, 1988). Alternately, 4-week-old white Leghorn chickens, maintained on a standard (vitamin D sufficient) diet, were obtained directly from the University of Wisconsin—Madison Poultry Science Department. Live chick embryos were obtained at 18–19 days of incubation from either Sunnyside Hatcheries (Oregon, WI) or the Poultry Science Department. They were maintained until use, at day 19, at 38 °C in a humidified incubator. Vitamin A deficient and replete rats were obtained as previously described (Silva et al., 1987). Porcine intestine and bovine kidney were obtained from the University of Wisconsin—Madison Meat and Animal Science Department. Fourteen-week-old, male, New Zealand White rabbits (5–7 lb) were obtained from Hazelton Research Animals (Denver, PA).

Buffers. The composition of the buffers was as follows: T, 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5 (25 °C); T-10x, 50 mM Tris-HCl, pH 7.5; TN, 50 mM Tris-HCl and 150 mM NaCl, pH 7.5; PBS, phosphate-buffered saline, pH 8.0; 2.68 mM KCl, 1.15 mM KH_2PO_4, 137 mM NaCl, and 8.10 mM Na_2HPO_4; 5% Blotto, 5% (wt/vol) Carnation nonfat dry milk and 0.05% NaN_3 in PBS; 0.5% Blotto, 0.5% (wt/vol) Carnation nonfat dry milk and 0.05% NaN_3 in PBS; 0.5% Blotto and Tween, 0.5% (wt/vol) Carnation nonfat dry milk, 0.05% Tween 20 and 0.05% NaN_3 in PBS; prehybridization buffer, 250 mM $NaPO_4$, 5% (wt/vol) SDS, 1 mM EDTA, 10% (wt/vol) poly(ethylene glycol) and 0.5% (wt/vol) Carnation nonfat dry milk, pH 7.4; 2X SSC, 300 mM NaCl, 30 mM $Na_3C_6H_5O_7$, 0.1% (wt/vol) SDS, pH 7.0; 0.5X SSC, 75 mM NaCl, 7.5 mM $Na_3C_6H_5O_7$, 0.1% (wt/vol) SDS, pH 7.0. The alkaline phosphatase substrate solution was prepared by adding 0.0066 volume of 50 mg of nitro blue tetrazolium/mL of 70% dimethylformamide (DMF) and 0.0033 volume of 50 mg of 5-bromo-4-chloro-3-indolyl phosphate/mL of DMF to one volume of 100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5, and used immediately.

Media. Waymouth's 752/1 medium containing 335 μM methionine was obtained from GIBCO. Nonradiolabeled and ^{35}S-labeled low methionine medium, containing 75 μM methionine, was prepared according to the published formulation for Waymouth's 751/2 medium (Waymouth, 1959) by reducing the methionine concentration. Medium containing 1,25-$(OH)_2D_3$ was prepared by adding the hormone in a 0.001 volume of ethanol. The concentration of 1,25-$(OH)_2D_3$ used was 150 nM unless otherwise indicated. Control medium contained a 0.001 volume of ethanol alone. A 0.005 volume of penicillin–streptomycin solution (10000 units of potassium penicillin G/mL and 10000 μg of streptomycin sulfate/mL) was added to all media before use.

Embryonic Duodenal Organ Culture and Metabolic Labeling of Proteins with [^{35}S]Methionine. Duodena were cultured and labeled with [^{35}S]methionine by a method similar to that of Bishop et al. (1983, 1984, 1985) for labeling with radioactive leucine. A 24-h culture period was used in all experiments. Duodena from three embryos were cultured in 40 mL of medium on one small stainless steel grid (6.5 × 2.5 cm and 0.2 cm high), which was placed on top of a larger grid described by Corradino (1973b). (If duodena were not incubated with low methionine or radioactive low methionine media, they were cultured only on the large grids in 35 mL of Waymouth's medium.) When low methionine or radioactive low methionine media were used, the small grid alone was placed in 5 mL of the medium for the labeling period. If cultures were incubated with nonradiolabeled medium after labeling with [^{35}S]methionine, then the small grid supporting the duodena was removed from the radioactive solution, rinsed with normal Waymouth's medium, blotted, and placed back on top of a large grid in 40 mL of normal Waymouth's medium. At the end of the culture period, each duodenum was rinsed in 4 °C buffer TN and placed in 0.5 mL of buffer T. The samples were either frozen on dry ice and stored at −70 °C or used immediately. All other manipulations of the extracts were carried out at 4 °C unless otherwise noted.

Preparation of Cytosolic Extracts. Preparation of embryonic chick, cytosolic extracts was similar to the method of Bishop et al. (1984). Each duodenum in 0.5 mL of buffer T was transferred to a glass–Teflon homogenizer containing 1 mL of buffer T and homogenized with six strokes. Homogenates were centrifuged at 170000*g* for 30 min and the supernatant cytosolic extract was removed. For the radioactive embryonic chick duodena samples, the specific activity of total cytosolic protein was measured after trichloroacetic acid (TCA) precipitation. The amount of protein in the precipitates was measured with the Bio-Rad protein microassay using bovine serum albumin as the standard and the radioactivity was measured by liquid scintillation counting.

All other cytosolic extracts were obtained in a similar fashion except they were homogenized in T-10x and their protein

concentration was measured directly.

Polyacrylamide Gel Electrophoresis. One-dimensional (1D) discontinuous sodium dodecyl sulfate (SDS) polyacrylamide slab gels with a 15% separating gel and a 4.75% stacking gel were electrophoresed by using the buffer system described by O'Farrell (1975). The gels used for the separation of the immunoprecipitates contained two times the stated concentration of Tris-HCl in both the separating gel and the running buffer (Fling & Gregerson, 1986) and ^{14}C-labeled protein molecular weight markers were electrophoresed next to the samples to estimate the molecular weights. Prestained molecular weight markers were electrophoresed next to the immunoblot samples and transferred to nitrocellulose to estimate molecular weights.

Two-dimensional (2D) polyacrylamide gel electrophoresis (2D-PAGE) was performed according to the method of O'Farrell (1975) by Kendrick Laboratory (Madison, WI) as previously described (Finlay & DeLuca, 1988).

Preparation of Antisera. For preparation of antisera to the chick 18-kDa protein the following protocol was used. Before immunization, rabbits were bled 2 times for preimmune serum. Injection solutions were made up by mixing 1 mg in 250 μL of chick CRBP II, purified by the method of Finlay & DeLuca (1988), with 750 μL of complete adjuvant. Two rabbits were injected at multiple sites subcutaneously in the back with 1 mg each of the 18-kDa protein. Animals were bled once or twice a week and the serum was checked for antibody titer by an enzyme-linked immunoabsorbent assay (ELISA). Briefly, microtiter wells were coated with partially purified 18-kDa chick protein, rabbit serum to be assayed, and protein A galactosidase conjugate. Then the wells were incubated with *p*-nitrophenyl β-D-galactopyranoside to indicate the presence of antibodies to the chick 18-kDa protein. After 24 days the animals were boosted with a single injection in the hip of 0.5 mg of 18-kDa chick protein in 188 μL mixed with 600 μL of incomplete adjuvant. Ten days after the boost, antibodies to the 18-kDa chick protein were detected in both rabbits.

The antiserum to rat CRBP II was prepared as described by Ong (1984).

Immunoblots. Immunoblots were performed by a procedure similar to that used by Brown et al. (1988). All procedures were done at room temperature. After electrophoresis the proteins were electrophoretically transferred to nitrocellulose and blocked in 5% Blotto and incubated for 2 h with anti-18-kDa chick protein or anti-rat CRBP II serum diluted 1:250 with 0.5% Blotto. Next the filter was washed three times for 20 min each with 0.5% Blotto and Tween, incubated with 0.5 ng/mL anti-rabbit IgG–alkaline phosphatase conjugate in 0.5% Blotto, and washed again three times for 20 min with 0.5% Blotto and Tween. The blots were visualized by incubating at room temperature for 20 min in phosphatase substrate buffer. The reaction was stopped in 20 mM Tris-HCl and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0.

Preparation of 18-kDa Chick Protein Sepharose. Sepharose CL-4B was activated with CNBr by the method of Kohn & Wilcheck (1982) using 16 mg of CNBr/g of wet Sepharose. Approximately 0.9 mg of 18-kDa chick protein in 0.2 M $NaHCO_3$ was coupled to 1.7 mL of activated Sepharose overnight at 4 °C. The 18-kDa chick protein–Sepharose was then blocked with 1 M Tris-HCl pH 9.0 and stored at 4 °C in 50 mM Tris-HCl, 1.5 mM EDTA, and 0.05% NaN_3, pH 7.4, until use.

Preparation of Anti-18-kDa Chick Protein Immunoaffinity Resin. All manipulations were carried out at 4 °C. First IgG was purified from the anti-18-kDa chick protein with MAPS buffers and Protein A Sepharose CL-4B according to manufacturer's instructions. The acid eluent containing IgG was collected in tubes containing 1 M Tris-HCl pH 9.0 in order to immediately neutralize the solution. The fractions containing IgG were concentrated by ultrafiltration with YM 30 membranes and an apparatus from Amicon (Danvers, MA). The IgG solution, after dialysis against PBS, was applied to a 1.7-mL 18-kDa chick protein–Sepharose column equilibrated in PBS and the column was then washed with PBS until the absorbance at 280 nm was 0. The antibodies specific for the 18-kDa chick protein were eluted with 0.1 M glycine–HCl pH 2.5. The eluent was collected in tubes containing 1 M Tris-HCl pH 9.0 and the fractions containing IgG were concentrated as above and dialyzed against 40 mM borate pH 9.0. The antibodies specific to 18-kDa chick protein were coupled to activated Sepharose as indicated above for the 18-kDa chick protein except that about 1 mg of protein was coupled to about 1 mL of resin. After coupling, the resin was blocked overnight with 1 M ethanolamine pH 9.0. Then the resin was washed alternately in 0.1 M acetate and 0.5 M NaCl pH 4.0 and then 0.2 M $NaHCO_3$ and 0.5 M NaCl pH 9.0 several times to remove noncovalently bound protein. Once prepared the affinity resin was stored in 50 mM Tris-HCl and 0.05% NaN_3, pH 7.5. Nonimmune resin was prepared in the same way using nonspecific rabbit IgG.

Immunoprecipitation of the 18-kDa Chick Protein. The ^{35}S-labeled cytosols were precleared by adding approximately 20 μL of nonimmune resin/600 μg of protein and incubating with gentle mixing for 2 h at 4 °C. The resin was removed by centrifugation at 11500*g* for 5 min and 20 μL of anti-18-kDa chick protein resin (or 20 μL of nonspecific rabbit IgG resin for nonimmune control) was added to equal amounts of cytosolic protein from each animal. The samples were then incubated with gentle mixing at 4 °C for 16–24 h. Next the resin was collected by centrifugation at 11500*g* for 5 min; washed twice in 50 mM Tris-HCl, 0.5% Triton X-100, and 0.5 M NaCl pH 7.5 and once in 50 mM Tris-HCl and 0.5% Triton X-100, pH 7.5; and layered on top of 50 mM Tris-HCl, 0.5% Triton X-100, 0.05% NaN_3, and 1 M sucrose, pH 7.5. Resin was centrifuged at 3000*g* for 10 min through the sucrose solution, the supernatant removed, and the resin resuspended in 60 μL of 125 mM Tris-HCl, 4% SDS, 20% glycerol, and 10 mM dithiothreitol pH 6.8. The samples were boiled for 90 s and then subjected to SDS–PAGE as described for the immunoprecipitations in the Materials and Methods section. After electrophoresis the gel was fixed in 50% methanol and 10% acetic acid and treated with En^{3}Hance according to manufacturer's instructions. Treated gels were dried and exposed at −70 °C to Kodak XAR film (Rochester, NY). Films were developed in an automatic film processor and subjected to densitometry on a Model SL-504 XL scanning densitometer (Biomed Instruments, Fullerton, CA). Radioactivity and film density were directly proportional for all film densities reported.

Northern Analysis of Chick and Rat Total RNA. Total RNA was prepared, electrophoresed, and transferred to a nylon membrane by procedures described in *Current Protocols in Molecular Biology* (Ausubel et al., 1988). The RNA ladder was electrophoresed next to the samples to estimate size. The ^{32}P-labeled probe was synthesized from linearized plasmid containing CRBP II cDNA with a Random Primed DNA labeling kit obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) according to manufacturer's instructions. The RNA was then cross-linked to the nylon membrane with UV light, incubated with prehybridization buffer at 42 °C for

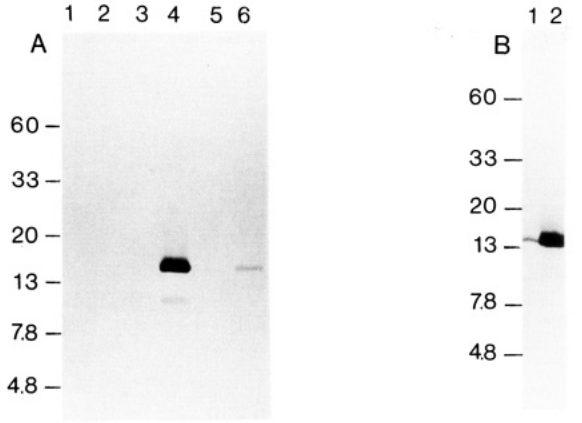

FIGURE 1: Demonstration of the 18-kDa protein in chick intestine. SDS–PAGE and immunoblots were performed as described in the Materials and Methods section using the antiserum to the 18-kDa chick protein. Three hundred micrograms of cytosolic protein was loaded in each lane. Panel A: lane 1, bovine kidney; lane 2, chick kidney; lane 3, chick liver; lane 4, chick duodena; lane 5, porcine intestine; lane 6, rat duodena. Panel B: lane 1, embryonic chick duodena; lane 2, duodena of 4-week-old chick. The molecular mass markers are indicated in kilodaltons.

at least 2 h, and then incubated with ^{32}P-labeled probe overnight. The membrane was rinsed three times in 2X SSC, washed with 0.5X SSC for 30 min at 42 °C, and exposed to film overnight.

Results

Tissue, Species, and Developmental Distribution of the 18-kDa Chick Protein. Immunoblot analysis of cytosolic protein showed immunoreactive proteins only in rat and chick duodena and not in porcine intestine, chick liver, or chick and bovine kidney (Figure 1A). The chick duodena had the greatest reactivity of all tissues examined showing an intense band at 14 kDa (lane 4). A minor band can be seen at 11 kDa. The rat duodena also contained a band at 14 kDa that was less intense than the chick (lane 6). Both the 4-week-old chick and the 19-day embryonic chick showed the band at 14 kDa, but the embryonic band was much less intense (Figure 1B). Immunoblots of the embryonic chick postcytosolic pellet fraction solubilized with 2% SDS revealed no immunoreacting proteins (data not shown). Incubation of similar immunoblots with preimmune serum in the place of anti-18-kDa chick protein serum showed no reactivity (data not shown).

Two-Dimensional Immunoblots of 4-Week-Old and Embryonic Chick Duodenal Proteins. When cytosol from the duodena of 4-week-old chicks was subjected to 2D-PAGE and stained with Coomassie blue, there were two major proteins observed at 15 kDa, with pIs of 6.6 (vertical arrow) and 7.1 (Figure 2A). When the cytosol was separated in the same way and then subjected to immunoblot analysis, the most prominent staining spots matched well with the 15-kDa/6.6 pI protein (Figure 2B). Additional less intense staining can be seen for three areas of the 15-kDa region. Two areas were seen at a higher pI and one at a lower pI. Cytosol from the embryonic chick intestine had less total staining but did also have reactivity at the 15-kDa/6.6 and 7.1 pI areas (Figure 2C). Some reactivity of the antibodies can be seen above the 60-kDa position in both the 4-week and embryonic chick blots. No reactivity was found in immunoblots of proteins solubilized with 1% SDS from the embryonic chick, postcytosolic pellet fraction. Incubation of similar immunoblots of cytosol from 4-week-old chicks, embryonic chick cytosol, or pellet with preimmune serum in the place of anti-18-kDa chick protein serum showed no reactivity (data not shown).

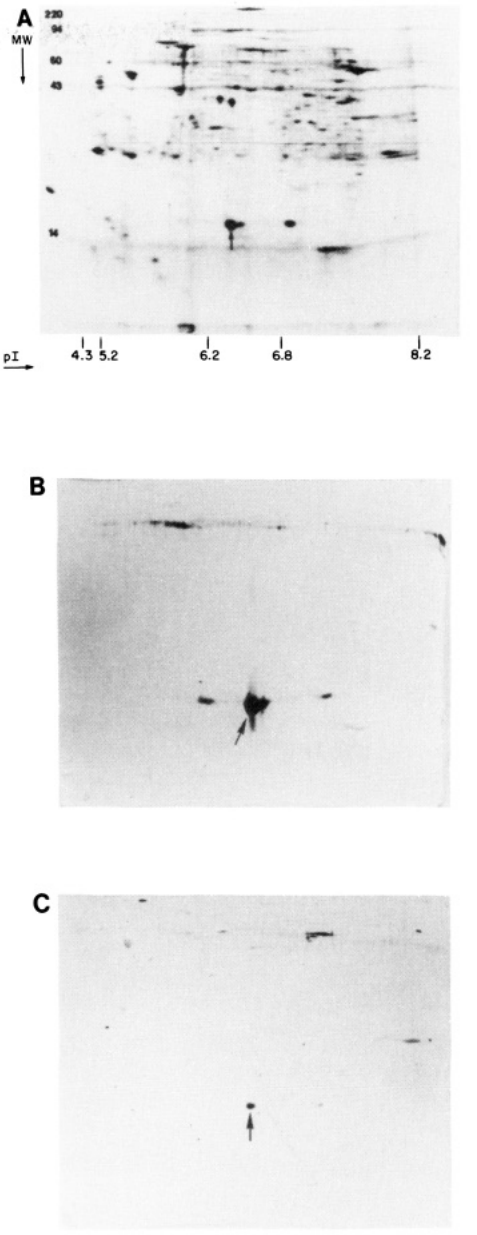

FIGURE 2: Molecular weight and pI of putative CRBP II in the embryonic chick and 4-week-old chick are the same. Panel A: 320 μg of chick duodenal cytosol was subjected to 2D-PAGE as described in Materials and Methods and the gel was stained with Coomassie blue. Positions of molecular mass (kDa) and pI markers are indicated. The chick protein is indicated by the vertical arrow. Then 2D gel electrophoresis and immunoblots were performed as described under Materials and Methods using the antiserum to the 18-kDa chick protein on 300 μg of cytosolic protein from 4-week-old chick duodena (panel B) and 300 μg of cytosolic protein from embryonic chick duodena (panel C).

Immunoblots of Rat and Chick Cytosol with Anti-Rat CRBP II Serum. When the rat and chick cytosolic proteins were analyzed by immunoblots with anti-18-kDa chick protein serum, results were obtained similar to Figure 1A (Figure 3A). However, when a similar immunoblot was probed with anti-rat CRBP II serum, equal intensity bands were seen at 14 kDa, and the 11-kDa band was not seen in the chick cytosol (Figure 3B). A blot similar to Figure 3A probed with preimmune serum showed no reactivity (data not shown).

The Effect of 1,25-$(OH)_2D_3$ and Chase Time on the Amount of Radioactive 18-kDa Chick Protein Immunoprecipitated from Embryonic Duodena. Considerable radioactive 14-kDa protein was immunoprecipitated from the embryonic duodena not exposed to 1,25-$(OH)_2D_3$, while treatment with

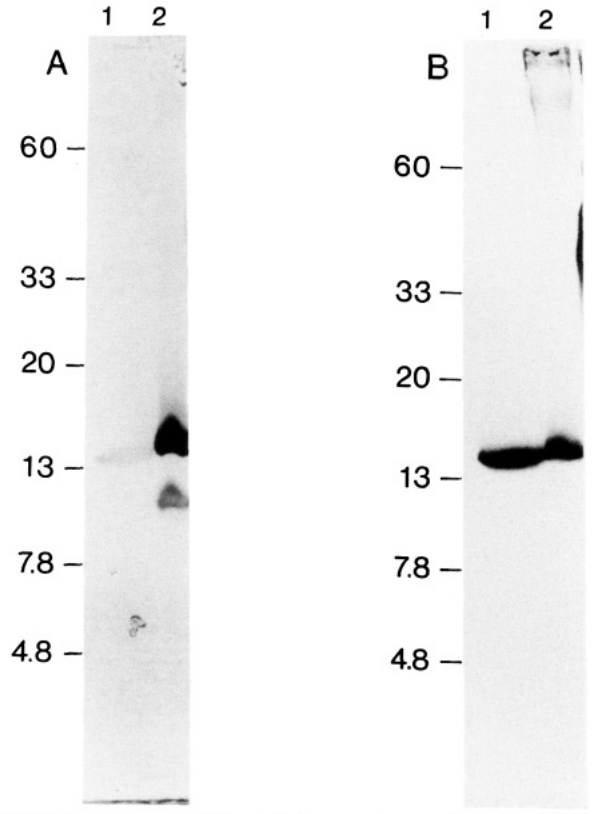

FIGURE 3: Rat and chick proteins are immunologically similar. SDS–PAGE and immunoblot analyses were performed as described under Materials and Methods. Lane 1, 300 μg of cytosolic protein from rat duodena; lane 2, 300 μg of cytosolic protein from 4-week-old chick duodena (panels A and B). Panel A: a nitrocellulose filter was incubated with the antiserum to the 18-kDa chick protein. Panel B: a nitrocellulose filter was incubated with the antiserum to the rat CRBP II.

1,25-$(OH)_2D_3$ markedly reduced immunoprecipitable radioactivity (Figure 4A, lanes 2–9). This 14-kDa protein was not precipitated with nonspecific rabbit IgG resin (Figure 4A, lane 1). The densities of the 14-kDa bands were determined and a significant difference in the means for three animals could be seen between the control and 1,25-$(OH)_2D_3$ treated duodena at each time examined (Figure 4B). There was a 92% decrease in the amount of radioactive 14-kDa protein precipitated after exposure of the cultures to 1,25-$(OH)_2D_3$ versus control in the absence of a chase period. This amount of decrease in the radioactive protein precipitated due to 1,25-$(OH)_2D_3$ treatment was similar at each subsequent chase time examined.

In an experiment similar to that described in Figure 4 where the pulse time was 10, 20, 40, or 60 min and the chase time was 4 h, the amount of [^{35}S]methionine incorporation into the protein increased at each time point. There was much less incorporation of radioactivity into the protein in the 1,25-$(OH)_2D_3$-treated cultures at every time point measured (data not shown).

Separate control cultures were examined for the ability to respond to 1,25-$(OH)_2D_3$ under conditions identical with those used for the [^{35}S]methionine labeling. Cultures responded to 1,25-$(OH)_2D_3$ by (1) increased calcium uptake as assayed by the method of Franceschi and DeLuca (1981b) and (2) synthesis of CaBP as assayed by the immunoblot procedure similar to that described in the Materials and Methods section (data not shown).

The specific activity of the total cytosolic protein extract increased during the 2-h chase period and declined afterward

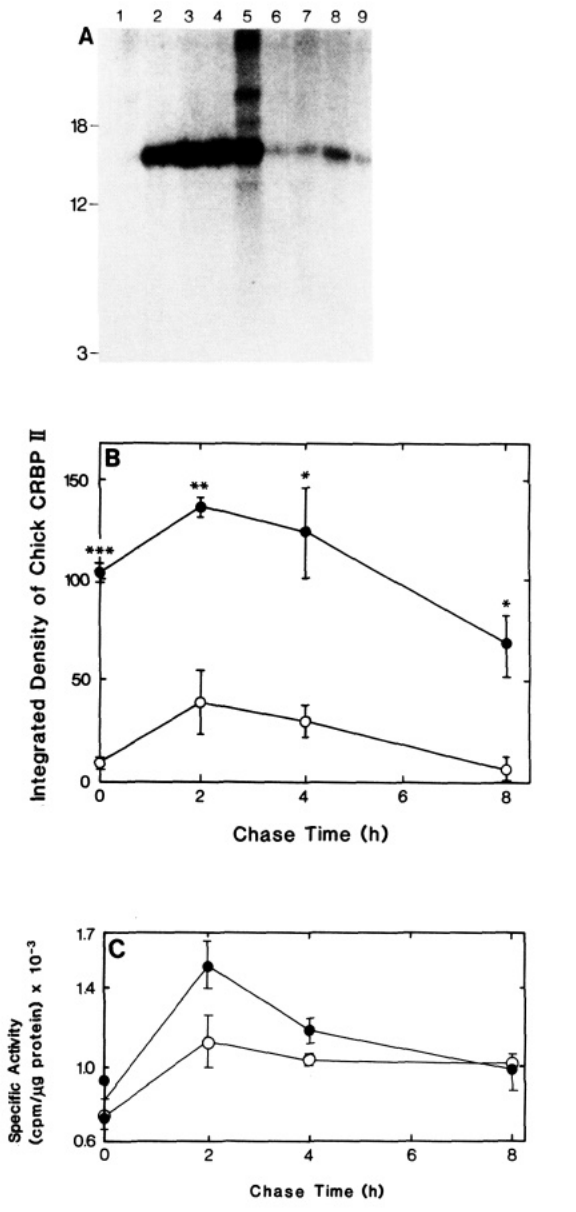

FIGURE 4: Effect of 1,25-$(OH)_2D_3$ and chase time on the amount of radioactive putative chick CRBP II immunoprecipitated from embryonic duodena. Panel A: Embryonic chick duodenal organ cultures treated with vehicle (lanes 1–5) or 1,25-$(OH)_2D_3$ (lanes 6–9) for 15.3–23.3 h were labeled with [^{35}S]methionine (130 μCi/mL) for 40 min and then incubated with excess nonradiolabeled methionine for 0 (lanes 1, 2 and 6), 2 (lanes 3 and 7), 4 (lanes 4 and 8), or 8 h (lanes 5 and 9). Cytosols were prepared from the labeled tissue and 600 μg of protein was subjected to immunoprecipitation, SDS–PAGE, and fluorography as described under Materials and Methods. Lane 1 was a sample immunoprecipitated with nonspecific rabbit IgG resin. Lanes 2–9 were samples precipitated with anti-chick 18-kDa resin. Three duodena were used for each vehicle and 1,25-$(OH)_2D_3$ time point. Panel B: All three fluorographs were subjected to densitometry and the intensity of the putative chick CRBP II band was measured and averaged for each time point. The closed circles represent duodena treated with vehicle and the open circles represent duodena treated with 1,25-$(OH)_2D_3$ ± SEM. The vehicle and 1,25-$(OH)_2D_3$ treated samples were significantly different, when compared by the Student's *t* test, at all time points as indicated: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.025$. Panel C: The specific activity of the total cytosolic protein in the samples indicated in panels A and B was measured as described under Material and Methods and plotted the same way as in panel B except there were only two samples for the control 0-h chase time group so both points are graphed instead of their mean. There was no sigificant difference between the vehicle and the 1,25-$(OH)_2D_3$ treated samples when the data were subjected to the Student's *t* test.

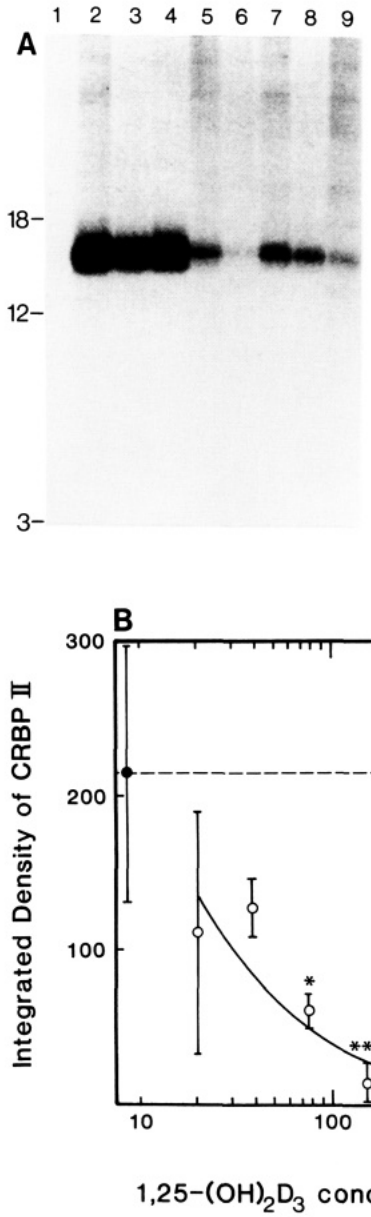

FIGURE 5: 1,25-$(OH)_2D_3$ causes a decrease in newly synthesized "chick CRBP II" in a dose-dependent manner. Embryonic chick duodena were incubated with different concentrations of 1,25-$(OH)_2D_3$ for 19.3 h, labeled for 40 min with [^{35}S]methionine, and incubated in medium containing excess methionine for 4 h. The immunoprecipitations were performed as described under Materials and Methods. Panel A: lane 1, sample immunoprecipitated with nonspecific rabbit IgG resin. Lanes 2–9 were cytosols precipitated with anti-chick 18-kDa resin. The nanomolar concentration of 1,25-$(OH)_2D_3$ used was as follows: lanes 1 and 2, 0; lane 3, 18.8; lane 4, 37.5; lane 5, 75; lane 6, 150; lane 7, 300; lane 8, 600; lane 9, 1200. Three duodena were used for the control and each 1,25-$(OH)_2D_3$ concentration. Panel B: All three fluorographs were subjected to densitometry, the chick 18-kDa band was measured and averaged for each concentration, and the integrated density is plotted. The closed circle represents duodena treated with vehicle and the open circles represent duodena treated with 1,25-$(OH)_2D_3$ ± SEM. The concentration of 1,25-$(OH)_2D_3$ at which the amount of radioactive CRBP II between the vehicle and the 1,25-$(OH)_2D_3$ treated samples is significantly different when compared to the Student's t test is as indicated: ***, $p < 0.01$; **, $p < 0.02$; *, $p < 0.05$.

for both the control and 1,25-$(OH)_2D_3$ treated samples (Figure 4C). However, there was no significant difference (by the Student's t test) between the specific activity of the control and 1,25-$(OH)_2D_3$ treated samples at any of the time points examined.

The Effect of 1,25-$(OH)_2D_3$ Concentration on Immunoprecipitation of Radioactive 18-kDa Chick Protein from Duodenal Cytosols. Considerable radioactive 14-kDa protein was immunoprecipitated from embryonic chick duodena (Figure 5A, lane 2); however, after exposure to increasing concentrations of 1,25-$(OH)_2D_3$, decreasing amounts of radioactive 14-kDa protein were recovered (Figure 5A, lanes 3–9). Again the 14-kDa band was not precipitated with nonspecific rabbit IgG resin (Figure 5A, lane 1). The densities of the 14-kDa bands were determined and a significant decrease in the means from three animals of recovered radioactive protein was observed at all 1,25-$(OH)_2D_3$ concentrations greater than 37.5 nM.

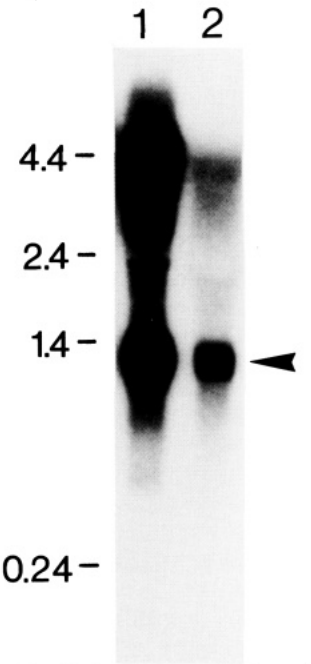

FIGURE 6: mRNA for chick and rat proteins have the same molecular weight. Total RNA from rats and chicks were subjected to Northern analysis using a rat CRBP II cDNA probe as described under Materials and Methods. Twenty-five micrograms of total RNA from rat (lane 1) or chick (lane 2) duodena was electrophoresed in a 1.5% agarose, 1.2% formaldehyde gel and transferred to a nylon membrane as described under Materials and Methods. The positions of the RNA ladder markers are indicated in kilobases, and the position of the 860 bp CRBP II RNA is indicated by the arrow.

Northern Analysis of Rat and Chick Duodenal RNA. Northern analysis of total RNA from both rat and chick revealed two bands of approximately 4400 and 860 base pairs (bp) in the rat and the chick (Figure 6). The 860 bp band from the chick was not as intense as that from the rat. If more stringent wash conditions were used, the 860 bp band in the chicken lane and the 4400 bp bands in the rat and chicken lanes were removed but the 860 bp band in the rat lane remained approximately the same intensity (data not shown). No hybridizing species could be detected in either total or poly$(A)_4^+$ RNA isolated from the embryonic chick duodena (data not shown).

Discussion

This paper demonstrates that the previously described protein (Finlay & DeLuca, 1988) from chick duodenal organ culture modulated by 1,25-$(OH)_2D_3$ (Bishop et al., 1985) is chick CRBP II, which is similar to but not identical with rat CRBP II. This is based on the (1) sequence homology between rat CRBP II and the 1,25-$(OH)_2D_3$ regulated 18-kDa chick protein, (2) similarity of the fluorescence spectra for retinol–CRBP II and retinol–1,25-$(OH)_2D_3$ regulated 18-kDa chick protein complexes (Finlay & DeLuca, 1988), (3) cross

reactivity of the antibodies to rat CRBP II with the 1,25-$(OH)_2D_3$ regulated 18-kDa chick protein, and (4) cross reactivity of the antibodies to the 1,25-$(OH)_2D_3$ regulated 18-kDa chick protein with rat CRBP II. We found large amounts of this protein present in intestine but none in liver or kidney (Figure 1A, lanes 1–4). The localization in the intestine and similar molecular weight for both rat CRBP II and the 1,25-$(OH)_2D_3$ regulated protein (Figure 3B) provided further evidence that the protein is chick CRBP II since rat CRBP II is localized in the intestine and is 16 kDa (Ong, 1984). Porcine intestinal cytosol showed no proteins that reacted with anti-chick CRBP II serum, strong reactivity was found in the chicken, and weak reactivity was found in the rat (Figure 1A, lanes 4–6). This could be due to differences in cross reactivity of our antibodies, made against the chicken protein, between species, or it could reflect differences in the abundance of the protein. Figure 1B clearly demonstrates that the antibodies prepared against the purified chick duodenal protein react with the same, albeit less abundant, protein in the embryonic chick duodena. Similar results were seen when 4-week-old chick and embryonic chick cytosols were subjected to 2D immunoblot analysis (Figure 2).

The 11-kDa protein detected in Figure 3A (lane 2) was probably a proteolytic fragment of the native 14-kDa protein since it is not always detected in immunoblots of cytosol (Figure 1A, lane 4; Figure 1B, lane 2).

2D immunoblots provided us with information about the different isoelectric forms of chick CRBP II. The most intense reaction in both cytosols was to the 15-kDa/6.6 pI abundant chick protein we used to make the antibodies (Figure 2B,C). However, we also saw reactivity with the pI 7.1 form that appears to be present in both the embryonic chick and 4-week-old chick. Recently two forms of CRBP II, CRBP IIA and CRBP IIB, have been characterized (Schaefer et al., 1989) and CRBP IIB is the N-terminally acetylated form of CRBP IIA. It is possible that the 15-kDa/6.6 pI form we found (Figure 2B,C) was CRBP II(A) and the 15-kDa/7.1 pI form is acetylated CRBP II (CRBP IIB). However, we would expect the unacetylated form to have a higher pI than the acetylated form. Our earlier data (Finlay & DeLuca, 1988) suggest that the 15-kDa/6.6 pI form is unacetylated since it is the most abundant form of the protein present in the preparation that we sequenced. Nevertheless, it is possible that we sequenced the less abundant pI 7.1 form or that the form with a pI less than 6.6 (Figure 2B) was the acetylated form. CRBP IIA and IIB were found to exist in equal abundance in the rat in vivo (Schaefer et al., 1989). In the chick cytosol (Figure 2A) two 15-kDa/pI 6.6 and 7.1 proteins appear to exist in approximately equal abundance but this was clearly not the case when immunoblot analysis was used to detect the forms of CRBP II (Figure 2B,C). The discrepancy between the abundance of the forms detected with Coomassie blue stain (Figure 2A) when compared with the immunoblots (Figure 2B) could be a result of preferential binding of antibodies to either the acetylated or unacetylated form of chick CRBP II. It is known that antibodies generated against acetylated CRBP II recognize both the acetylated and unacetylated form of rat CRBP II to differing degrees (Schaefer et al., 1989). The identification of the different pI forms in the chicken and their significance in both rat and chicken await further clarification. However, both Figures 1B and 2 confirmed our earlier results, which show this abundant protein in 4-week-old chick duodenum is the same protein as the less abundant 1,25-$(OH)_2D_3$ regulated protein in embryonic chick (Finlay & DeLuca, 1988).

Immunoblots or 2D immunoblots of embryonic chick solubilized postcytosolic pellet fraction revealed that chick CRBP II is cytosolic. This is consistent with the cytosolic localization for CRBP II found by Ong (1984).

The experiment in Figure 4 provided strong evidence that 1,25-$(OH)_2D_3$ caused a decrease in radioactive chick CRBP II by decreasing the synthesis rate of the protein. An excess of CRBP II affinity resin was used for immunoprecipitations so that complete recovery of all the CRBP II present in the samples could be expected. Figure 4A indicates that there was much more newly synthesized CRBP II present in the control duodena (lanes 2–5) than in the 1,25-$(OH)_2D_3$ treated duodena (lanes 6–9). We found that in the absence of a chase period there was a 92% decrease in newly synthesized chick CRBP II after exposure to 1,25-$(OH)_2D_3$ (Figure 4B). This was consistent with the 85% decrease in the protein reported by Bishop et al. (1985). The disappearance of CRBP II can be estimated from the slope of the lines of the 2–8-h times points (Figure 4B). The rate of disappearance of CRBP II was similar for the control and 1,25-$(OH)_2D_3$ treated cultures. Therefore, we concluded that 1,25-$(OH)_2D_3$ does not cause the decrease in CRBP II by increasing the degradation or disappearance rate especially since the disappearance of the protein appears to be slightly higher in the control cultures (Figure 4B). Since we were able to detect the approximately 90% 1,25-$(OH)_2D_3$ dependent decrease in newly synthesized CRBP II in the absence of the chase period and we could detect very little difference in the disappearance of CRBP II due to incubation with 1,25-$(OH)_2D_3$, we concluded that 1,25-$(OH)_2D_3$ acts by decreasing the synthesis rate of the protein.

The effect of 1,25-$(OH)_2D_3$ is specific for CRBP II since the specific activity of the total cytosolic protein extracted from the cultured duodena was not significantly changed by exposure to 1,25-$(OH)_2D_3$ (Figure 4C). We were surprised to see an increase in the specific activity of both total cytosolic protein and newly synthesized CRBP II during the chase period. The specific activity would be expected to decline immediately after the start of the chase period, but perhaps the general increase in specific activity seen even after the 2-h chase period (Figure 4C) was due to a long turnover time for the amino acid pools in this system.

The decrease in CRBP II synthesis was dependent on the concentration of 1,25-$(OH)_2D_3$ with maximum effect achieved by 150 nM (Figure 5B). This is similar to the saturating value of 75 nM for 1,25-$(OH)_2D_3$ stimulated calcium uptake found by Franceschi and DeLuca (1981b) in the chick EDOC.

The slight differences in molecular weight found for the 18-kDa chick protein [18 kDa for SDS–PAGE, 15 kDa for 2D-PAGE (Finlay & DeLuca, 1988) and 14 kDa for immunoblots and immunoprecipitations] were probably only due to the different techniques used to visualize the protein, since data from immunoblot analyses (Figure 3) and activity assays (Finlay & DeLuca, 1988) provided strong evidence that the 18-kDa chick protein previously characterized (Finlay & DeLuca, 1988) was CRBP II.

It is clear from Figure 6 that a single mRNA of approximately 860 bp exists for CRBP II in both the rat and the chick duodena. [The large bands at 4400 bp were due to nonspecific binding of the probe to the extremely abundant ribosomal RNA present in the total RNA samples (Figure 6, lane 1).] Previously, Li et al. (1986) have detected an 800 bp RNA in rat intestine for CRBP II but Southern analysis of cellular DNA from chickens using a rat CRBP II cDNA probe failed to detect the gene (Demmer et al., 1987). The Southern

analysis may not have been able to detect chick CRBP II because higher stringency washes were used. The differences between species in intensity of the CRBP II mRNA band (Figure 6) may indicate that the C-terminal homology between rat and chick CRBP II was less than in the first 28 amino acids. This is consistent with our comparison of the chick and rat proteins by immunoblots. The inability to detect mRNA to chick CRBP II in embryonic duodena may have been because it was much less abundant there than in the 4-week-old chick and the chick CRBP II mRNA clearly had less homology to the rat cDNA probe than rat mRNA.

We do not know the physiologic significance of 1,25-$(OH)_2D_3$ causing a reduction in the protein that plays a role in vitamin A absorption. However, there are reports that the toxic effects due to high intakes of vitamin A can be alleviated by intake of additional vitamin D in birds (Veltmann et al., 1987; Metz et al., 1985) or rats (Vedder & Rosenberg, 1938). Our observation of 1,25-$(OH)_2D_3$ regulation of the intestinal vitamin A binding protein may be important to understanding this phenomenon. Retinol or vitamin A appears to be absorbed in the intestine by means of a specific carrier protein (Said et al., 1988; Hollander & Muralidhara, 1977). Normally all of the retinol in the intestinal villus cell is probably bound to CRBP II (MacDonald & Ong, 1988). The function of CRBP II may be to accept retinol from its specific carrier protein in the villus membrane and direct it to the endoplasmic reticulum where LRAT enzyme resides to esterify the bound retinol. Therefore, regulation of CRBP II concentration could control the amount of retinol that either enters the cell or is able to leave the cell after esterification. It is possible that the 1,25-$(OH)_2D_3$ mediated decrease in CRBP II may cause a decrease in intestinal retinol absorption or esterification. A decrease in retinol absorption or esterification may then diminish vitamin A toxicity since less of the vitamin would enter the circulation. If this is the case, vitamin D would not be expected to decrease toxicity resulting from administration of retinoic acid. However, only continued investigation can provide insight into these possibilities.

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