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Evidence for the presence of Calbindin-D 28K (CaBP-28K) in the tibial growth cartilages of rats

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Summary. The distribution of the vitamin-D dependent calcium-binding protein (Calbindin-D 28K) (CaBP-28K) in the tibial growth plate cartilage of the rat has been studied immunohistochemically using an antibody raised against rat renal CaBP-28K. The protein was detected mainly in the nuclei of chondrocytes and occasionally in the juxtanuclear cytoplasm. The distribution was not uniform throughout the growth plate, but concentrated in the proliferatively active chondrocytes of the resting and proliferative zones. These findings raise the possibility that CaBP-28K may be involved in the mitotic activity of the chondrocytes, acting as a regulator of the proliferative process, perhaps via intranuclear calcium.

Key words: Calbindin-D 28K (CaBP-28K) – Immunohistochemistry – Growth cartilage – Vitamin D – Rat

The calcium-binding protein Calbindin-D 28K (CaBP-28K) was first reported by Wasserman and Taylor (1966) to be present in the duodenal mucosa of the chick and this same protein was subsequently found in other chick-derived tissues and in a number of mammalian tissues (Thomasset et al. 1982). This protein has certain structural features in common with other intracellular calcium-binding protein, but differs fundamentally from the better-known members of this family (e.g. parvalbumin, calmodulin) in its dependence on vitamin D. The distribution of CaBP-28K has been studied immunocytochemically in such soft tissues as the chick intestine (Thorens et al. 1982, Taylor 1983, Taylor et al. 1984) and kidney (Roth et al. 1981b) mammalian kidney (Roth et al. 1982; Schreiner et al. 1983) and nervous system (Roth et al. 1981a; Baimbridge and Miller 1982; Legrand et al. 1983; Rabie et al. 1983; Garcia-Segura et al. 1984) at both the light- and electron-microscope levels. More recently, CaBP-28K has been shown to be present in mineralized tissues. It was found in the ameloblasts during tooth formation (Taylor 1984; Celio et al. 1984) and in the calcified cartilage matrix of prenatal rats (Celio et al. 1984). However, there are insufficient data on the temporal and spatial distribution of CaBP-28K in cartilage to provide for a better understanding of the role that the protein may play in the metabolism of calcium in mineralized tissues.

The present study has examined the distribution of CaBP-28K in the growth cartilage of the normal rat. The protein was identified immunohistochemically using a specific antibody to rat CaBP-28K and its distribution correlated with the zonal differentiation of the growth plate cartilage.

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Materials and methods

Reagents

Hydrogen peroxide (30%) was purchased from Prolabo (France). Poly-L-lysine (m.w. 300000), bovine serum albumine (BSA), protein A-peroxidase conjugate and 3-3′ diaminobenzidine (DAB) were purchased from Sigma (USA); normal rabbit serum and buffered glycerine were from Institut Pasteur (Paris).

Tissue preparation

Female Sprague Dawley rats, 21-day-old, were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg). The proximal parts of the tibias were removed and cooled to 0° C. The growth plate cartilages were rapidly dissected out and immediately immersed in ice-cold (4° C) Carnoy's fixative or 4% paraformaldehyde with calcium chloride in 0.1 M sodium phosphate buffer, pH 7.4, then fixed for 16–24 hours at 4° C. The tissue samples were subsequently washed for 4 hours at 4° C with phosphate buffered saline, pH 7.4 containing 0.5% bovine serum albumin (PBS-BSA) before being frozen in a jet of carbon dioxide and cut with a cryostat (SLEE, London) at −20° C. Sections of 5 μm were mounted on glass slides which had been freshly coated with poly-L-lysine and stored at 4° C for 18 h.

Immunohistochemistry

CaBP-28K was detected using a specific antiserum to rat renal CaBP-28K (Brehier et al. 1983) and staining was performed by the indirect protein A-peroxidase method of Dubois-Dalcq and collaborators (Dubois-Dalcq et al. 1977; see also Brunangelo et al. 1983). PBS-BSA was used for antibody dilution and all washings between incubation steps to minimize nonspecific absorption. Tissue sections were maintained at room temperature in a moist atmosphere throughout the following procedure. Endogenous peroxidase activity was blocked by a preliminary incubation of the sections with 1% H_2O_2 in methanol for permeabiliza-

tion for 10 to 30 minutes. The sections were then washed with PBS-BSA to stop the reaction and incubated with anti-CaBP-28K antiserum (dilutions 1:10–1:80) for 3 h at room temperature or for 16 h at 4° C. They were washed in PBS-BSA (3 × 10 min) and incubated for 1 h with freshly-prepared protein A-peroxidase conjugate (diluted 1:100 with PBS-BSA). Excess reagent was removed by thorough washing with PBS-BSA and the bound peroxidase was visualized by incubating with a solution containing 0.05% DAB, 0.01% H_2O_2 in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.6 for 8 to 10 min. After a final thorough wash with Tris-NaCl (3 × 5 min), the slides were mounted in buffered glycerine and examined with a Zeiss Universal microscope.

Controls

The specificity of the immunostaining was checked by incubating sections as follows: (1) using non-immune rabbit serum instead of anti-CaBP antiserum; (2) using protein A-peroxidase complex alone; (3) using anti-CaBP serum, unconjugated protein A, and protein A-peroxidase; (4) using PBS-BSA in place of the primary anti-CaBP antiserum.

Results

The general distribution of the brown reaction product indicating the presence of CaBP-28K in growth cartilage is shown in Fig. 1. There was heavy staining of the chondrocyte nuclei (Figs. 1, 2A–C, 3). The chromatin areas showed intense granular staining (Fig. 3) while the nucleoli remained unstained (Fig. 3B). Immunoreactive granular material was also occasionally seen in the juxtanuclear cytoplasmic region (Figs. 2C, D, 3A). No staining was detected in the other regions of the chondrocyte cytoplasm or in the extracellular matrix of the longitudinal and transverse septa (Fig. 1 et seq.).

Immunoreactive material was not uniformly distributed throughout the entire growth plate cartilage. The larger hypertrophied chondrocytes lining the epiphysis and being involved in its mineralization remained unstained while in the resting zone the small chondrocytes which give rise to the subsequent proliferative chondrocytes, of the growth cartilage, showed both nuclear (Figs. 1, 2A) granular perinuclear staining (Fig. 3A). In the subsequent zones of the growth plate the chondrocytes of the proliferative (Figs. 2B, C) and some chondrocytes of the upper maturation (Fig. 2C) zones showed intense staining in their nuclei and in the perinuclear cytoplasmic region of some cells. The cytoplasm was unstained. Not all the cell nuclei were stained. The nuclei of lower mature chondrocytes were unstained with brown particles sometimes appearing near the nuclear membrane and no staining of the chromatin (Fig. 2D arrow). The chondrocytes of the hypertrophic zone never contained brown coloration neither inside the nucleus nor in the cytoplasm (Fig. 2E). All controls were negative.

Discussion

The presence of the calcium-binding protein or Calbindin-D 28K, has been demonstrated in the growth plate cartilage of the rat using a specific antiserum to rat renal CaBP-28K and protein A-peroxidase-coupled immunohistochemistry.

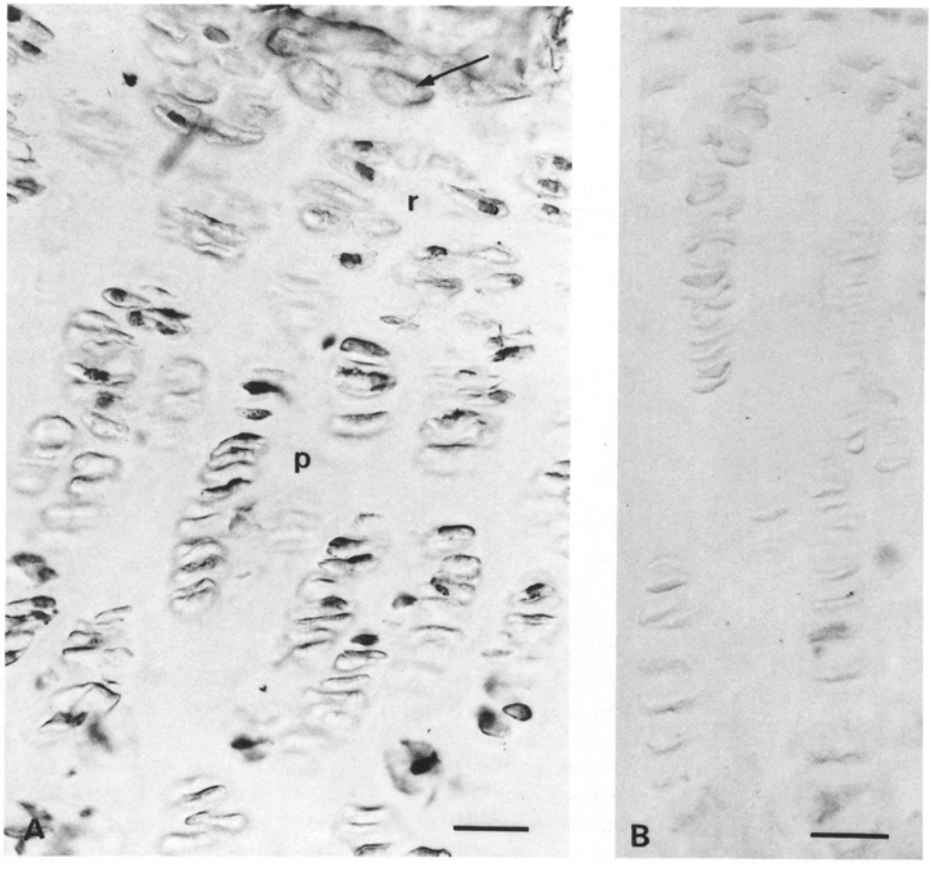

Fig. 1 A–B. Growth plate cartilage.
A Section reacted with anti-CaBP 28K and protein A-peroxidase.
B Control anti-CaBP replaced by non-immune rabbit serum. Dark deposits representing sites of CaBP-28K are seen in the nuclei of the zones of resting (*r*) and proliferative (*p*) chondrocytes. The cytoplasm is unstained. The hypertrophied chondrocytes lying towards the mineralizing epiphysis remain unstained. Bars: 24 μm. × 420

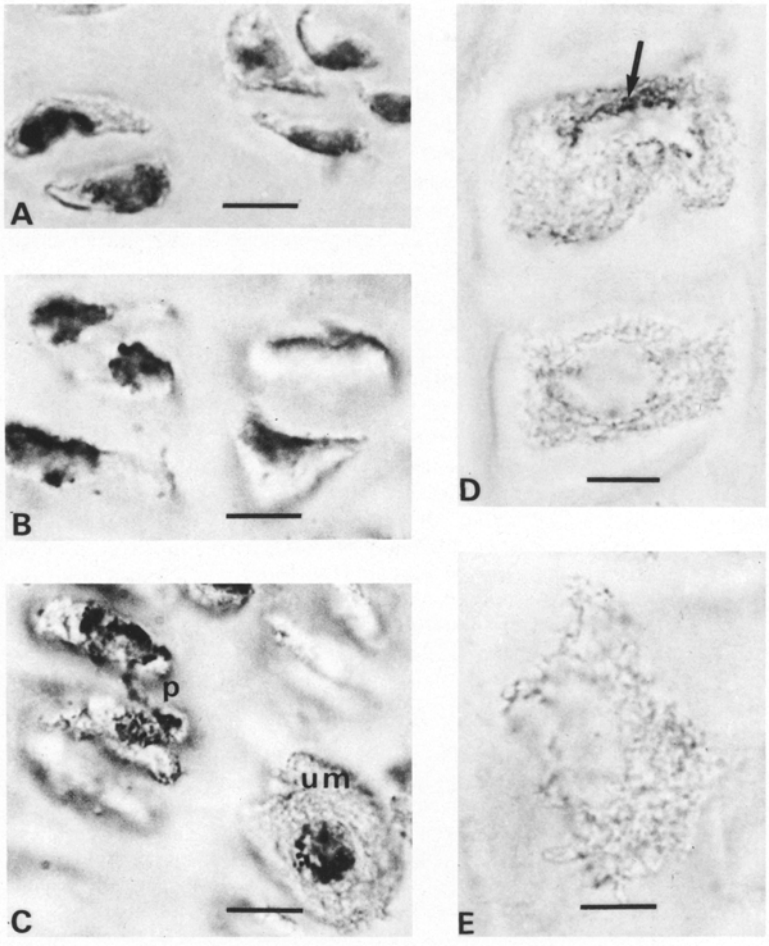

Fig. 2A–D. Chondrocytes of each zone of growth plate cartilage. Anti-CaBP-28K and protein A-peroxidase.
A Resting chondrocytes.
B Proliferative chondrocytes.
C Lower proliferative (*p*) and upper maturing (*um*) chondrocytes.
D, E Lower mature hypertrophic chondrocytes. Non-uniform immunocytochemical distribution: nuclear staining in the resting (**A**) proliferative (**B, C**) and upper mature chondrocytes (**C**); unstained nuclei in the hypertrophic chondrocytes (**D, E**). Some cells show juxtanuclear staining (**D**, *arrow*). Bars: 24 μm. ×1100

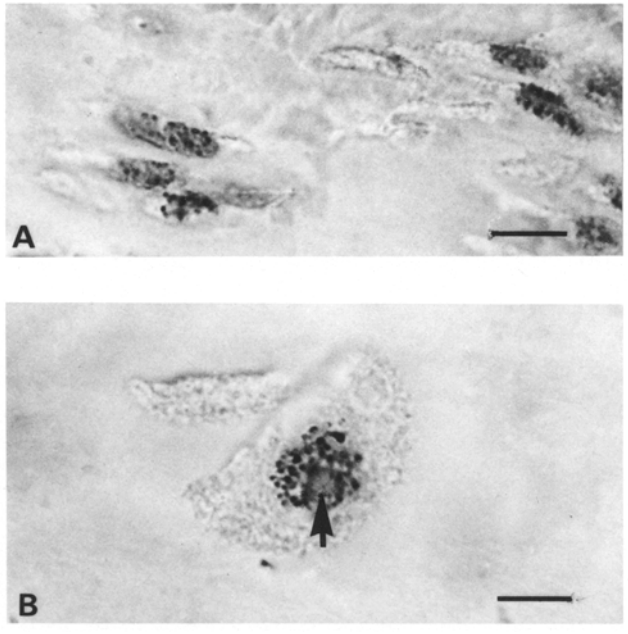

Fig. 3A–B. Resting (**A**) and upper mature (**B**) chondrocytes. Anti-CaBP 28K and protein A-peroxidase. Note the granular staining of the nuclei, the perinuclear staining (**A**); and the unstained nucleolus (**B**/*arrow*). The cytoplasm is unstained. Bars: 9 μm. ×1100

The immunoreactive material is found mainly in the chondrocytes of the cartilage zones undergoing proliferation, the resting and proliferative zones and not in non replicating hypertrophied chondrocytes. The protein is essentially intranuclear. The nucleoli are not colored and the cytoplasm shows no immunoreactivity, except for occasional staining in the juxtanuclear region. Above the resting zone and lining the epiphysis, the enlarged hypertrophied chondrocytes, which are involved in epiphysis calcification, contain no immunoreactive material. In the proliferative zone, not all the chondrocytes are stained. It would appear that the chondrocytes which have stopped CaBP-28K production are also those which are no longer multiplying. There is also no longer any immunoreactive material in the nuclear region of the chondrocytes when they have become mature, hypertrophied and in the hypertrophic calcifying zones.

It is unlikely that the antibody used in this study recognizes proteins other than CaBP-28K. While parvalbumin has been shown to be present in calcified tissues (Celio et al. 1984; Heizmann 1984), there is no evidence that it cross-reacts with the specific anti-CaBP-28K antiserum (Celio et al. 1984). Furthermore, parvalbumin is localized in the calcified trabeculae of vertebral bodies in the fetus and not in the cells (Celio et al. 1984; Heizmann 1984). Similarly, calmodulin, another calcium-binding protein, is biochemically and immunologically distinct from CaBP-28K (Christakos et al. 1984; Intrator et al. 1985).

One difference between the previously published reports on the localization of CaBP-28K and the present study concerns the intracellular localization of the protein. Other workers have described CaBP-28K in the cytoplasm in the chick intestine (Taylor 1980, 1983) and kidney (Taylor et al. 1982), in the mammalian kidney (Schreiner et al. 1983; Rhoten and Christakos 1981; Taylor et al. 1982), in mammalian central nervous system (Roth et al. 1981a; Garcia-Segura et al. 1984) and cochlear and vestibular hair cells (Rabie et al. 1983) and in mineralized tissues such as teeth (Taylor 1984; Celio et al. 1984). Other studies showed CaBP-28K to be present in the nucleus in addition to the cytoplasm but, when nuclear staining was reported, it was associated with heavy cytoplasmic staining (Taylor 1980; Jande et al. 1981; Roth et al. 1981b, 1982; Legrand et al. 1983).

Our results are different in that, in the growth plate cartilage, the major localization is nuclear, the cytoplasmic staining is minimal and in the perinuclear region. It is highly unlikely that this distribution could have occurred as the result of a diffusion artifact as the intranuclear staining is frequently seen as particulate granules. The techniques used for this study were chosen to avoid any such problems (Schreiner and Jande 1983). Moreover, previous studies in which nuclear staining has been reported have stated that the immunoreactivity was specifically found over the nuclear euchromatin (Roth et al. 1981b, 1982; Thorens et al. 1982). Nuclear staining of CaBP-28K in chondrocytes in no way precludes a cytoplasmic origin for the protein, but the absence of the protein from the greater part of the cytoplasm makes it highly unlikely that the nuclear CaBP-28K is the result of a diffusion artifact. The finding of nuclear staining in the nuclei of young chondrocytes in an early developmental stage may be compared with the results of Legrand et al. (1983) who showed in cerebellum that the nuclear staining is an early event.

Although vitamin D-dependent CaBP-28K has been known for almost 20 years, its exact function is still a matter of considerable debate. Many of the proposed functions are based upon its cellular localization. It has been suggested that it is involved in vitamin D-dependent intestinal calcium-transport processes (Wasserman and Taylor 1966), transmembrane translocation of calcium (Kowarski and Schachter 1980) or, based on electron-microscopical subcellular localization, that it plays a role in intracellular calcium translocation (Roth et al. 1981b, 1982; Norman et al. 1982). The finding of CaBP-28K in the incisor ameloblasts (Taylor 1984; Celio et al. 1984) and in the mineralizing fetal bones (Celio et al. 1984) has been used as the basis of a proposal that the protein is involved in mineralization.

The data reported here on the distribution of CaBP-28K in the growth plate cartilage of rats link the presence of the protein to the proliferative activity of the chondrocytes at an early developmental stage. This idea is supported by the association of CaBP-28K with the nuclear chromatin and by the discontinuous nature of its distribution. Although the protein is seen most frequently in the cells of the proliferative layer, not all the cell nuclei are stained suggesting that the presence of CaBP-28K depends on the phase of the chondrocyte cell cycle. The presence of a specific high affinity calcium-binding protein in the nuclei of the proliferative cells could be related with the localization of calcium in the nuclei of flattened chondrocytes in a zone corresponding to the proliferative zone, from studies with K-pyroantimonate in fetal bones (Burger and Matthews 1978; Carson et al. 1978).

Vitamin D has widespread effects on the metabolism of calcium, and one of its major target organs is the growing bone. However, the specific target cells and the way in which the steroid acts remains largely unknown. It has been suggested that vitamin D was not directly necessary for bone growth and mineralization (Underwood and DeLuca 1984), but $1,25(OH)_2D_3$ receptors have been found in chick cartilage and flattened chondrocytes (Suda et al. 1985). The demonstration of a calcium-binding protein whose synthesis is, in other tissues, dependent on the presence of vitamin D, in proliferative chondrocytes of the growth plate cartilage suggests that these cells are targets for the steroid, while the location of CaBP-28K within the nuclei of these cells and the importance of calcium in their proliferation, point to a specific role for CaBP-28K in the regulation of this process.

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References

Baimbridge KG, Miller JJ (1982) Immunohistochemical localization of calcium-binding protein in the cerebellum, hippocampal formation and olfactory bulb of the rat. Brain Res 245:223–229

Brehier A, Elion J, Thomasset M (1983) Vitamin D-dependent rat kidney CaBP: purification and development of a radioimmunoassay. In: Bernard B de (eds) Calcium binding protein in health and disease. Elsevier Science, pp 389–390

Brunangelo F, Taylor CR, Phil D (1983) New developments in immunoperoxidase techniques and their application. Arch Pathol Lab Med 107:105–117

Burger EH, Matthews JL (1978) Cellular calcium distribution in fetal bones studies with K-pyroantimonate. Calcif Tiss Res 26:181–190

Carson FL, Davis WL, Matthews JL, Martin JH (1978) Calcium localization in normal rachitic and D_3-treated chicken epiphyseal chondrocytes utilizing potassium pyroantimonate osmium tetroxide. Anat Rec 190:23–39

Celio MR, Norman AW, Heizmann CW (1984) Vitamin D-dependent calcium-binding protein and parvalbumin occur in bones and teeth. Calcif Tissue Int 36:129–130

Christakos S, Bruns EH, Mehra AS, Rhoten WB, Van Eldik LJ (1984) Calmodulin and rat vitamin D-dependent calcium-binding proteins: biochemical and immunochemical comparison. Arch Biochem Biophys 231:38–47

Dubois-Dalcq M, Mc Farland H, Mc Farlin D (1977) Protein A-peroxidase: a valuable tool for the localization of antigens. J Histochem Cytochem 25:1201–1206

Garcia-Segura LM, Baetens D, Roth J, Norman AW, Orci L (1984) Immunohistochemical mapping of calcium-binding protein immunoreactivity in the rat central nervous system. Brain Res 296:75–86

Heizmann CW (1984) Parvalbumin, an intracellular calcium-binding protein distribution, properties and possible roles in mammalian cells. Experientia 40:910–921

Intrator S, Elion J, Thomasset M, Brehier A (1985) Purification, immunological and biochemical characterization of rat 28K Da cholecalcin (cholecalciferol induced calcium-binding proteins). J Biochem 231:89–95

Jande SS, Tolnai S, Lawson DEM (1981) Immunohistochemical localization of vitamin D-dependent calcium-binding protein in duodenum, kidney, uterus and cerebellum of chickens. Histochemistry 71:99–116

Kowarski S, Schachter D (1980) Intestinal membrane calcium binding protein vitamin D-dependent membrane component of the intestinal calcium transport mechanism. J Biol Chem 255:10834–10840

Legrand Ch, Thomasset M, Parkes CO, Clavel MC, Rabie A (1983) Calcium-binding protein in the developing rat cerebellum. Cell Tissue Res 233:389–402

Norman AW, Roth J, Orci L (1982) The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium-binding proteins). Endocr Rev 3:331–366

Rabie A, Thomasset M, Legrand Ch (1983) Immunocytochemical detection of calcium-binding protein in the cochlear and vestibular hair cells of the rats. Cell Tissue Res 232:691–696

Rhoten WB, Christakos S (1981) Immunocytochemical localization of vitamin D-dependent calcium-binding protein in mammalian nephron. Endocrinology 109:981–983

Roth J, Baetens D, Norman AW, Garcia-Segura LM (1981a) Specific neurons in chick central nervous system stain with antibody against chick intestinal vitamin D-dependent calcium-binding protein. Brain Res 222:452–457

Roth J, Thorens B, Hunziker W, Norman AW, Orci L (1981b) Vitamin D dependent calcium-binding protein: immunocytochemical localization in chick kidney. Science 214:197–200

Roth J, Brown D, Norman AW, Orci L (1982) Localization of the vitamin D-dependent calcium-binding protein in mammalian kidney. Am J Physiol 243:F243–F252

Schreiner DS, Jande SS (1983) Immunohistochemical localization of low molecular weight water soluble protein-antigens. Immunol Commun 12:77

Schreiner DS, Jande SS, Parkes CO, Lawson DEM, Thomasset M (1983) Immunocytochemical demonstration of two vitamin D-dependent calcium-binding proteins in mammalian kidney. Acta Anat 117:1–14

Suda S, Takahashi N, Shinki T, Horiuchi N, Yamaguchi A, Yoshiki S, Enomoto S, Suda T (1985) 1α,25-dihydroxyvitamin D_3 receptors and their action in embryonic chick chondrocytes. Calcif Tissue Int 37:82–90

Taylor AN (1980) Immunocytochemical localization of the vitamin D-induced calcium binding protein: resolution of two divergent distribution patterns. In: Siegel FL (eds) Calcium-binding proteins: structure and function. Elsevier North Holland Inc., New York Oxford pp 393–400

Taylor AN (1983) Intestinal vitamin D-induced calcium-binding protein: time course of immunocytological localization following 1,25-dihydroxyvitamin D_3. J Histochem Cytochem 31:426–432

Taylor AN (1984) Tooth formation and the 28,000 Daltons vitamin D-dependent calcium-binding protein: an immunocytochemical study. J Histochem Cytochem 32:159–164

Taylor AN, McIntosh JE, Bourdeau JE (1982) Immunocytochemical localization of vitamin D-dependent calcium-binding protein in renal tubules of rabbit rat and chick. Kidney Int 21:765–773

Taylor AN, Gleason WA, Lankford GL (1984) Immunocytochemical localization of rat intestinal vitamin D-dependent calcium-binding protein. J Histochem Cytochem 32:153–158

Thomasset M, Parkes CO, Cuisinier-Gleizes P (1982) Rat calcium-binding proteins: distribution, ontogeny and vitamin D-dependence. Am J Physiol 243:E483–E488

Thorens B, Roth J, Norman AW, Perrelet A, Orci L (1982) Immunocytochemical localization of the vitamin D-dependent calcium binding protein in chick duodenum. J Cell Biol 94:115–122

Underwood JL, DeLuca HF (1984) Vitamin D is not directly necessary for bone growth and mineralization. Am J Physiol 246:E493–E498

Wasserman RH, Taylor AN (1966) Vitamin D_3-induced calcium-binding protein in chick intestinal mucosa. Science 152:791–793

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