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Vitamin D-Dependent Calcium-Binding Proteins (CaBPs) in Human Fetuses: Comparative Distribution of 9K CaBP mRNA and 28K CaBP during Development

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ABSTRACT. The vitamin D-dependent calcium-binding protein (CaBP) cholecalcin or calbindin, has been used as a molecular marker of 1,25-dihydroxyvitamin D_3 action. Mammals possess two CaBPs: a 9,000 mol wt (9K CaBP) and a 28,000 mol wt (28K CaBP). The distinct localization of each protein in the rat has been previously described with the aid of specific radioimmunoassays developed for each CaBP. Antibodies raised against the rat 28K CaBP can be used to detect this protein in a number of mammalian species including humans. In contrast, antibodies against rat 9K CaBP do not cross react with human 9K CaBP, but human 9K CaBP mRNA can be analyzed using a cDNA probe for rat 9K CaBP mRNA. Such a cross-hybridization between the rat cDNA probe and human CaBP mRNA was demonstrated by Northern analysis. We have documented the distribution and evolution of 28K CaBP and 9K CaBP mRNA in human tissues during fetal development from 14 to 32 wk of gestation. 28K CaBP was only present in kidney and cerebellum, and not detectable in duodenum. There was a 2-fold increase of 28K CaBP in the cerebellum between 14 and 24 wk of gestation. The 9K CaBP mRNA was unevenly distributed in human fetal tissues. 9K CaBP mRNA was present in classical vitamin D target tissues such as duodenum and placenta; high levels of 9K CaBP mRNA also were found in thymus and lung. These findings suggest a role for the hormone not only in the duodenum, placenta and kidney, but during the development of specific organs such as cerebellum, lung and thymus, and in agreement with many recent studies, point to a role of vitamin D in cellular differentiation and growth. (*Pediatr Res* 21: 362–367, 1987)

Abbreviations

$1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3
CaBP, calcium-binding protein
9K CaBP, 9,000 molecular weight calcium-binding protein
28K CaBP, 28,000 molecular weight calcium-binding protein
PGFα, prostaglandin
DEPC, diethylpyrocarbonate
SDS, sodium dodecyl sulfate

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The vitamin D-dependent CaBP, cholecalcin (1) or calbindin, is one of a group of intracellular proteins that bind calcium with an affinity constant Ka = 10^6 M^{-1} (2, 3). These proteins are the best known molecular markers of vitamin D action on target cells and have been characterized for a number of species (3). Birds have only one CaBP of 28,000 molecular weight, while two CaBPs have been described in mammals (3, 4). A small 9K CaBP having two calcium-binding sites was first located in the absorptive cells of the duodenum (5) but is also found in the placenta (6, 7). A larger 28K CaBP, similar to the chick 28K CaBP (3), possesses four calcium-binding sites and is concentrated in the distal tubules of the kidney (8) and the Purkinje cells of cerebellum (9). Although their exact molecular functions remain unclear, 9K CaBP in the duodenum and the placenta as well as 28K CaBP in the kidney are probably involved in calcium transfer. 28K CaBP has been proposed as a marker of Purkinje cell differentiation and development of the cerebellum (10). Vitamin D-dependence of rat intestinal 9K CaBP gene expression was documented using either a cell free translation system (11) or a specific cDNA probe synthesized from rat mRNA coding for 9K CaBP (12). This specific cDNA probe hybridizes to a homogeneous 500-600 nucleotide rat duodenal mRNA species which directs the synthesis of 9K CaBP, but not 28K CaBP, in a cell-free system (1).

Human CaBP purified from cerebellum and kidney has the same molecular weight as chick or rat 28K CaBP and there is a cross-reactivity between antibodies raised to these proteins and human 28K CaBP (13–15). 28K CaBP has been quantified in adult human postmortem tissues using a radioimmunoassay. The protein is found mainly in the kidney and cerebellum, but is also widely distributed throughout the nervous system (15).

As CaBPs are molecular markers of vitamin D action, we have studied these two markers during human fetal development. Antibodies raised to rat intestinal 9K CaBP do not cross-react with human 9K CaBP, and thus, cannot be used to quantify it. However, human 9K CaBP mRNA can be analyzed using the ^{32}P-labeled cDNA probe for rat 9K CaBP mRNA. In the present study we have used this ^{32}P-labeled cDNA probe to rat 9K CaBP and a radioimmunoassay for human 28K CaBP to examine the distribution of both 9K CaBP mRNA and 28K CaBP in human fetal tissues and the changes in their concentration during fetal development from 14 to 32 wk of gestation.

MATERIALS AND METHODS

Material. All radioisotopes were purchased from Amersham, Arlington Heights, IL. Nylon membranes were from New England Nuclear, and Oligo (dT) cellulose T7 from PL biochemicals. DNA polymerase, T_4 DNA ligase, baker's yeast tRNA, RNA

molecular mass marker were obtained from Boehringer Mannheim, Mannheim, West Germany.

Preparation of tissues. Tissues were removed from 15 fetuses obtained after therapeutic abortions at 14 to 32 wk of gestation: 21 trisomy ($n = 6$), Klinefeter's syndrome ($n = 1$), mucoviscidosis ($n = 2$), encephalocele ($n = 1$), maternal diseases ($n = 5$). All abortions were induced by PGFα injection and samples were obtained between 30 min and 2 h postmortem. Each tissue was dissected out on ice, washed in cold sterile 0.9% NaCl, immediately frozen in liquid nitrogen and stored at −80° C. Samples were obtained from placenta, duodenum (freed from pancreatic fragments), lung, sternum, thymus, liver, kidney, cerebellum, and diaphragm.

Radioimmunoassay of 28K CaBP. Prior to assay, each sample was homogenized with a Potter Elvehjem homogenizer in four volumes of 14 mM Tris-HCl pH 7.4, 120 mM NaCl, and 3 mM KCl. A clear solution was obtained by centrifugation at 100,000 × *g* for 1 h and stored at −20° C. Aliquots were taken for total protein estimation using the one-step method of Bradford (16) and CaBP quantification by radioimmunoassay (13). The 28K CaBP purified from rat kidney was used as the reference standard and to raise antibodies in rabbit. Serial dilutions of homogenates of human fetal samples produced curves parallel to the standard curve.

Preparation of cloned ^{32}P cDNA probe. The cloned cDNA (pC109) inserted at the PstI site of chimeric plasmid of pBR322 was obtained from bacterial cells by alkali extraction following chloramphenicol amplification as previously described (12). After isolation by electroelution and purification on a DEAE cellulose (DE52) column, the cDNA fragments were ligated by T_4 DNA ligase to increase the specific activity of the probe and then nick-translated in the presence of ^{32}P nucleotides (17). The labeled cDNA was isolated by chromatography on a Sephadex G50 microcolumn. The specific activity of the ^{32}P cDNA probe was about 2×10^8 cpm/μg.

Extraction of total RNA and poly A^+ RNA. Total RNA was extracted with phenol-chloroform according to Itoh *et al.* (18). Poly A^+ RNA was separated out by chromatography on oligo(dT)-cellulose T_7 as described by Aviv and Leder (19). All samples were stored at −80° C in tubes which had been pretreated with DEPC 0.1% at 37° C overnight and autoclaved.

RNA Northern hybridization. The electrophoresis apparatus was treated with 0.2% DEPC before use. Poly A^+ RNA was denatured with formaldehyde (20) and electrophoresed on 1.3% horizontal agarose gels for 1 h at 100 V in 10 mM NaH_2PO_4, pH 6.5, 3.3% (w/w) formaldehyde, 0.3% DEPC, and then tranferred from the agarose gel to a nylon membrane using 20 × SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) buffer (21). RNA transfer was complete in 12–16 h. The filters were dried and baked for 2 h at 80° C before hybridization.

RNA dot hybridization. Forty-five μg total RNA in 50 μl of sterile water were incubated with 20 μl of 20 × SSC and 30 μl 37% formaldehyde (w/w) at 60° C for 15 min. Eight hundred μl of 20 × SSC were added for analysis. Serially diluted samples (with 20 × SSC) were applied twice to a nylon filter using the Hybri Dot System (BRL, Bethesda, MD). The filters were dried under a lamp and baked for 2 h at 80° C before hybridization (22).

Hybridization assay. The filters were prehybridized in 50% (v/v) deionized formamide, 5 × SSC, 20 mM NaH_2PO_4, pH 7, containing bovine serum albumin, Ficoll 400 and polyvinyl pyrrolidone (all at 0.2% w/w), and 100 μg/ml of sonicated denatured salmon sperm DNA for at least 4 h at 42° C. The hybridization buffer consisted of the above buffer to which was added 10% (w/w) dextran sulfate and the nick-translated ^{32}P cDNA probe (10 ng cDNA/ml). After hybridization (16 h at 42° C), the filters were washed with four changes of 2 × SSC, 0.1% SDS for 10 min each at room temperature. The filters were given two further washes with 2 × SSC, 0.1% SDS at 50° C for 1 h. The blots were dried and autoradiographed at −80° C using Kodak X-O-Mat film. The autoradiograms of the dot blot assay were analyzed by spectrodensitometry (Shimadzu Scientific Instruments, Columbia, MD). Previous experiments have demonstrated that, under DNA excess conditions, a linear relationship exists between the degree of hybridization and the RNA concentration when dotted total RNA does not exceed 10 μg (23). Consequently, all measurements of human fetal 9K CaBP mRNA were carried out from total RNA in the range 1.25–10 μg/dot in the presence of DNA excess. Total RNA from rat duodenum was used as a standard. After hybridization with specific ^{32}P cDNA probe the amount of 9K CaBP mRNA in each RNA dot was quantified by spectrodensitometric analysis of the peak areas. Results were obtained from the spectrodensitometric analysis of the four total RNA concentrations dotted. Relative 9K CaBP mRNA levels were calculated from the slope of the hybridization curve and expressed as a ratio of standard rat 9K CaBP mRNA slope (arbitrary units).

Statistical analysis. All results were analyzed by variance analysis. When statistical significance was reached the groups were compared using Student Fisher's *t* test.

RESULTS

Extraction of total RNA from human fetal tissues. The yield of the different experimental steps of phenol-chloroform extraction was different for each tissue (Table 1). Nucleic acid and total RNA concentrations were the highest in liver and kidney, an intermediate group included duodenum, lung, and placenta, whereas the lowest levels were obtained in cerebellum, thymus, and sternum. In all tissues the value of A260/A280 and A260/A235 ratio, near 2, gives evidence of the quality of the extraction.

Northern analysis of 9K CaBP mRNA in human fetal tissues. Poly A^+ RNA preparations from placenta, lung, kidney, thymus, and liver of 20- to 23-wk-old fetuses electrophoresed under denaturing conditions on agarose gel, transferred to nylon membranes, and hybridized to the nick-translated ^{32}P cDNA showed that a single mRNA species was retained by the probe (Fig. 1). This mRNA contained approximately 500–600 bases and comigrated with the RNA from rat duodenum.

Distribution of 9K CaBP mRNA in 20- to 23-wk-old human fetuses. Dot hybridization was used to quantify the 9K CaBP mRNA in the total RNA extracted from human fetal tissues. Spectrodensitometric analysis of the autoradiograms indicated that the 9K CaBP mRNA was unevenly distributed in 20-wk-old human fetuses (Fig. 2). The major tissues in which 9K CaBP gene was expressed were thymus, lung, duodenum, kidney, placenta, and cerebellum, with a higher 9K CaBP mRNA concentration in the first two organs. There was a very low expression in liver, sternum, and diaphragm.

Statistical analysis of the results from six fetuses of approximately the same age (20–23 wk of gestation) showed three

Table 1. *Yield of extraction of total nucleic acid and total RNA from human fetal tissues**

Tissues	Total nucleic acid (mg/g tissue)	Total RNA (mg/g tissue)	A_{260}/A_{280}	A_{260}/A_{235}
Liver	9.8 ± 0.8	5.1 ± 0.5	1.9 ± 0.1	1.9 ± 0.2
Kidney	6.0 ± 0.5	3.0 ± 0.2	1.9 ± 0.1	2.0 ± 0.1
Duodenum	5.1 ± 0.4	1.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.1
Lung	5.0 ± 0.5	1.7 ± 0.1	1.9 ± 0.1	2.0 ± 0.1
Placenta	2.8 ± 0.3	1.6 ± 0.2	2.0 ± 0.1	2.1 ± 0.1
Cerebellum	2.3 ± 0.3	0.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1
Thymus	1.9 ± 0.1	0.8 ± 0.1	2.0 ± 0.1	2.4 ± 0.2
Sternum	1.8 ± 0.1	0.7 ± 0.1	1.9 ± 0.2	1.9 ± 0.1

* The values are the mean ± SEM of 15 different 14- to 32-wk-old fetuses.

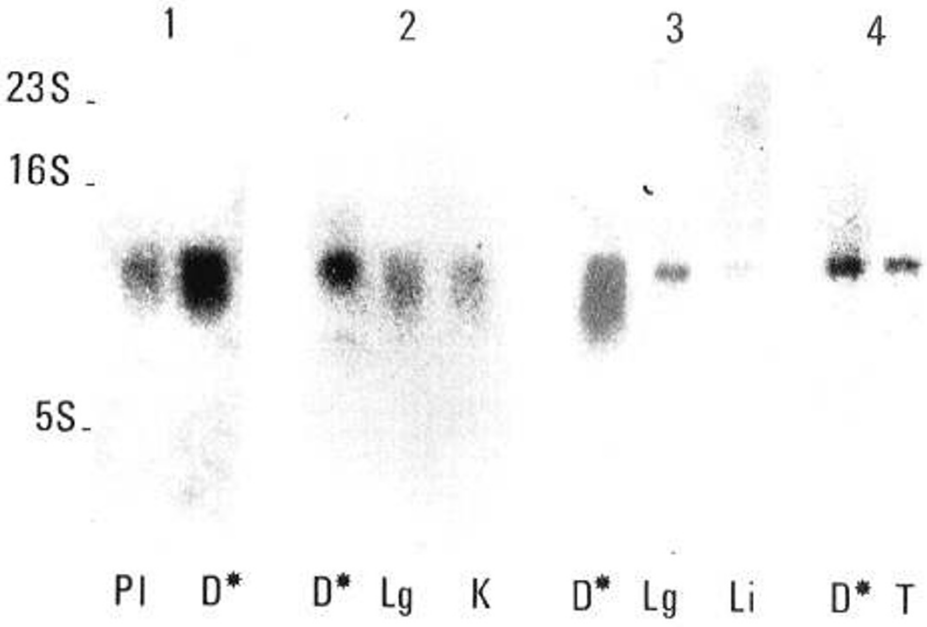

Fig. 1. Northern hybridization to ^{32}P cDNA probe of Poly A^+ RNA extracted from human fetal tissues at 20–23 wk of gestation. The autoradiograms resulting from four Northern hybridization assays (*1, 2, 3, 4*) of Poly A^+ RNA preparations (10 μg) from placenta (*Pl*), lung (*Lg*), kidney (*k*), liver (*Li*), and thymus (*T*) are shown. Total RNA extracted from rat duodenum (*D**) was used as control. The size scale was determined by coelectrophoresis of ribosomal *Escherichia coli* RNA (5S, 16S, 23S).

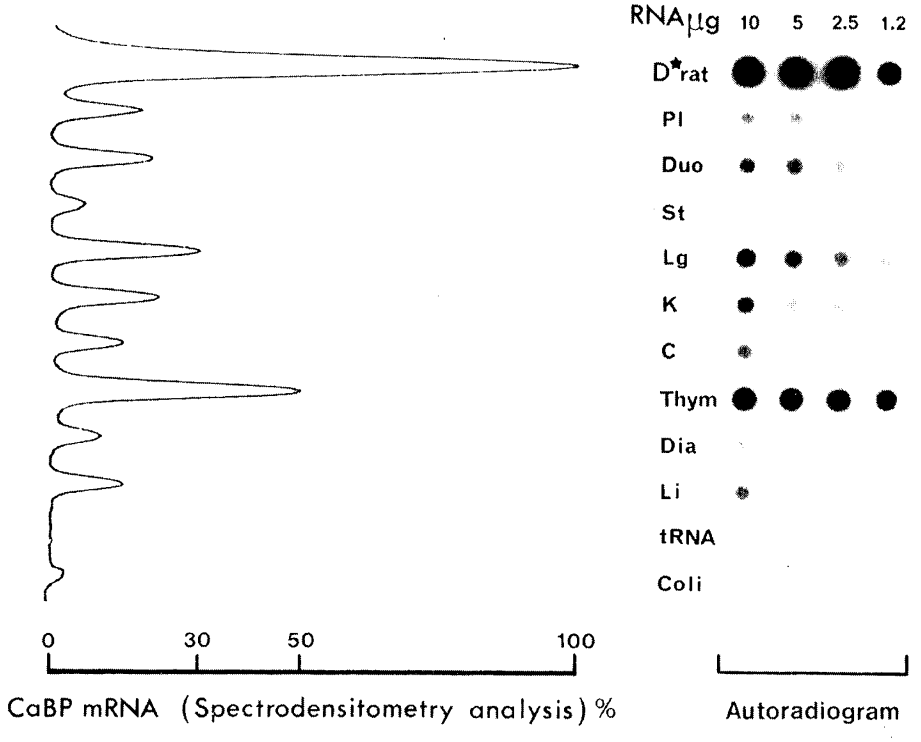

Fig. 2. Dot hybridization assay of 9K CaBP mRNA in a 20-wk human fetus. Spectrodensitometric analysis of an autoradiogram resulting from eight separate dot blot hybridization assays is shown. Total human fetal RNA (*10, 5, 2.5, 1.25*) from placenta (*Pl*), duodenum (*Duo*), sternum (*St*), lung (*Lg*), kidney (*K*), cerebellum (*C*), thymus (*Thym*), diaphragm (*Dia*), liver (*Li*), and rat duodenum (*D* rat*) were dotted; tRNA from yeast (*tRNA*) and RNA from *Escherichia coli* (*Coli*) were used as controls. The *curve* represents the scan of one of the four RNA dilutions analyzed.

significantly different groups of tissues. The highest levels of 9K CaBP mRNA appeared in thymus, an intermediate group included lung and kidney, and a third group was composed of duodenum, cerebellum, liver, and placenta (Fig. 3).

Distribution of 28K CaBP in 20- to 23-wk-old fetuses. The tissue distribution of 28K CaBP is shown in Table 2. The cerebellum and kidney contained 1750 and 500 ng/mg protein, respectively, whereas the level was low in sternum (30 ng/mg protein). 28K CaBP was undetectable in duodenum, liver, thymus, lung, and placenta.

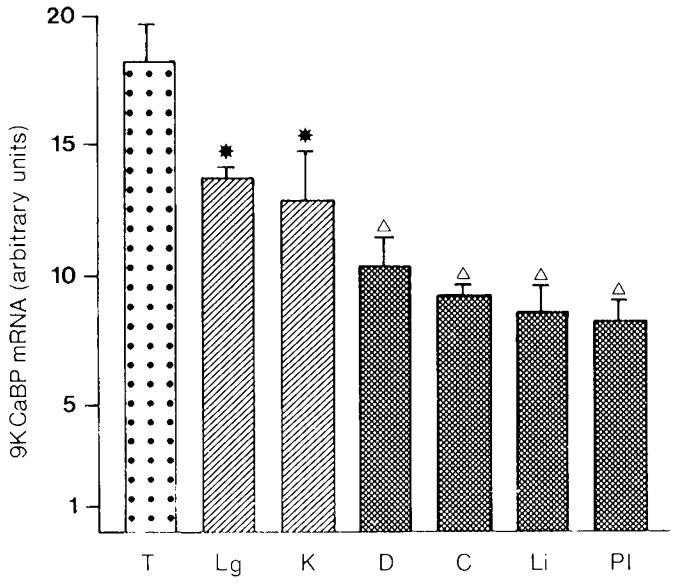

Fig. 3. Distribution of 9K CaBP mRNA in human fetal tissues. 9K CaBP mRNA concentration was analyzed by spectrodensitometry of dot hybridization assays of total RNA extracted from thymus (*T*), lung (*Lg*), kidney (*K*), duodenum (*D*), cerebellum (*C*), liver (*Li*), and placenta (*Pl*). Each *histogram* is the mean of six separate assays corresponding to the tissues of 6-, 20-, to 23-wk-old fetuses, except for cerebellum (3). * $p <$ 0.001 as compared to thymus. $\Delta\ p < 0.005$ as compared to lung and kidney.

Changes in 9K CaBP mRNA and 28K CaBP during human fetal development. 9K CaBP gene expression was detectable as early as 14 wk of gestation in thymus, lung, duodenum, placenta, liver, kidney, and cerebellum (Fig. 4). There was no significant change in 9K CaBP mRNA levels in these organs from 14 to 32 wk of gestation (Fig. 4).

The 28K CaBP concentration in the kidney remained unchanged between gestational wk 14 and 32 (Fig. 5). In contrast that of the cerebellum increased 2-fold between 14 and 20 wk of gestation (Fig. 5).

DISCUSSION

The number of human vitamin D-dependent CaBPs and their distribution reported in the adult is rather confused. Several studies have clearly demonstrated the presence of the large 28K

Table 2. *Distribution of 28K CaBP in human fetal tissues**

Tissues	28K CaBP (ng/mg soluble protein)
Cerebellum	1756 ± 132
Kidney	505 ± 40
Sternum	32 ± 3
Duodenum	0
Liver	0
Lung	0
Thymus	0
Placenta	0

* Values are means ± SEM of five fetuses at 20 wk of gestation.

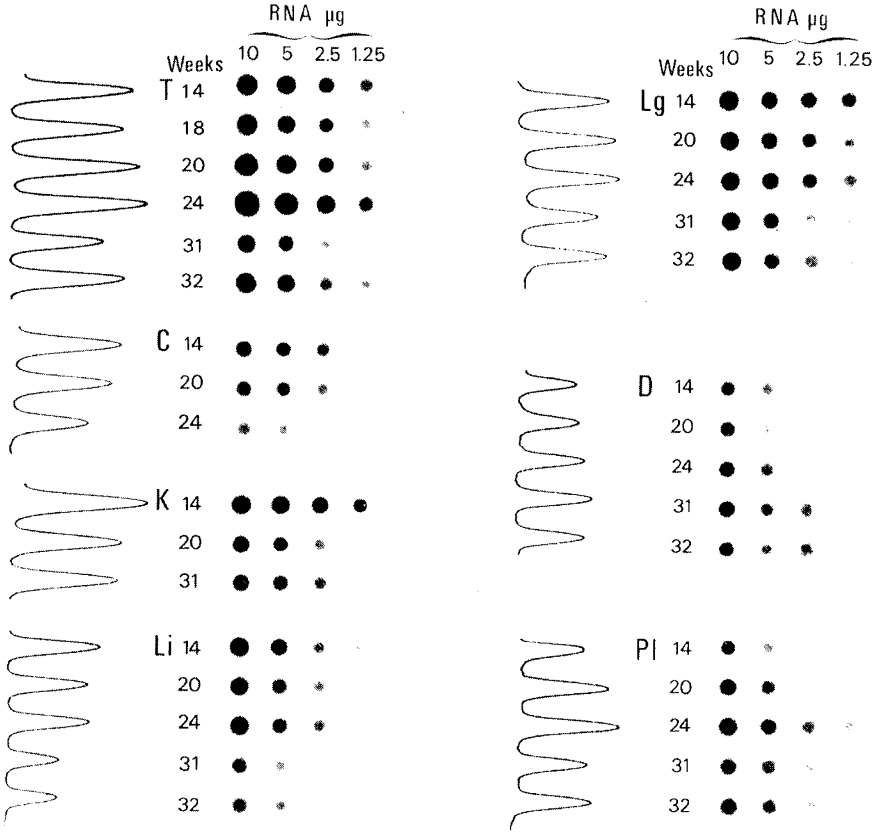

Fig. 4. Dot hybridization assay of 9K CaBP mRNA in fetal tissues between 14 to 32 wk of gestation. 9K CaBP mRNA concentration was analyzed by spectrodensitometry of dot hybridization assays of total RNA extracted from thymus (*T*), cerebellum (*C*), kidney (*K*), liver (*Li*), lung (*Lg*), duodenum (*D*), and placenta (*Pl*). Tissues from different fetuses at 14, 18, 20, 24, 31, and 32 wk of gestation were analyzed. One autoradiogram (*b*) for each tissue is shown and the curves (*a*) represent the scan of one of the four dilutions assayed.

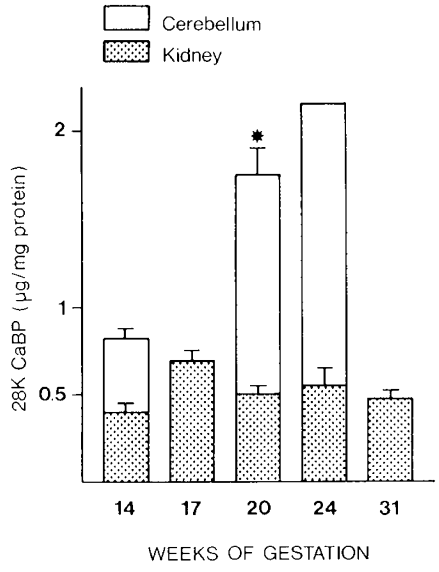

Fig. 5. Distribution of 28K CaBP in human fetal kidney and cerebellum between 14 and 31 wk of gestation. Values are means ± SEM of four fetuses for cerebellum except at 24 wk of gestation (1) and three fetuses for kidney. $*p < 0.05$ as compared to cerebellum at 14 wk of gestation.

CaBP in the kidney (14, 15) and cerebellum (15) from adult necropsy obtained 12–24 h postmortem. Our findings of 28K CaBP in kidney as early as 14 wk of gestation are consistent with the report based on immunocytolocalization (24) in which it was shown that the 28K CaBP is present at 11 wk of gestation in all medullary ducts and few distal tubules. We observed that the 28K CaBP level in the human fetal kidney did not increase from 14–31 wk of gestation. Immunochemical ontogenesis studies indicated that the distribution of human 28K CaBP changes as the kidney matures: the protein progressively disappears from the medullary ducts and its concentration increases in the distal tubules where it is found in adult (24). In the rat previous study has shown that the kidney content of 28K CaBP increases abruptly 1 day before birth (4). In the present study, kidneys from the very late period of human gestation were not available for testing. The 28K CaBP appears very early in the cerebellum, at 14 wk, and seems to increase between 14 and 24 wk of gestation. Although the vitamin D-dependence of cerebellum 28K CaBP has not been yet demonstrated (4), the appearance of 28K CaBP in the rat cerebellum is related to the Purkinje cell differentiation and the developmental increase of this protein reflects the overall maturation of Purkinje cells (9, 10). Such data suggest a possible role of the protein in the development of cerebellum. Further studies are clearly required on the immunocytolocalization of CaBP in human fetal cerebellum. The picture in the intestine is considerably less clear. Proteins of various sizes and immunological cross-reactivity have been reported (25). Our inability to detect 28K CaBP in human fetal duodenum is consistent with a previous study from our laboratory on adult human tissue (15).

Recent studies have shown that the rat cDNA probe recognizes human genomic DNA using Southern analysis. Hybridized rat and human genomic DNA have very similar restriction patterns when digested with the same enzymes (26). These data suggest that there is a single 9K CaBP gene in the rat and human. Northern analysis has shown that, in the rat, the cDNA probe hybridizes to a homogeneous 9K CaBP mRNA species 500–600 nucleotides long and that there is no cross-hybridization between 9K and 28K CaBP mRNAs (1, 27). The present analysis shows that the cDNA probe recognizes only one 500–600 nucleotides species of mRNA in human fetal tissues which comigrates with the hybridized 28K CaBP mRNA from rat duodenum, indicating that there is a specific cross-hybridization between the rat cDNA probe and human fetal 9K CaBP mRNA.

9K CaBP mRNA is widely and unevenly distributed in human fetal tissues. The presence of 9K CaBP mRNA in human fetal kidney and cerebellum correlates with that observed in the growing rats in the same organs (1). Such results in cerebellum and kidney should be confirmed by investigation in rat fetuses. The presence of 9K CaBP mRNA in human fetal duodenum and placenta also correlates with that observed in the rats (7). Only a single report has described the isolation of a small 10 000 Mr CaBP from human jejunum (28). The presence of a human mRNA which hybridizes to the specific cDNA encoding rat 9K CaBP mRNA is in agreement with the latter study and demonstrates that only 9K CaBP gene is expressed in fetal human duodenum. During rat fetal development, 9K CaBP appears in duodenum at the end of gestation (19–20 days) and the concentration increases dramatically after birth (4). 9K CaBP mRNA levels in the rat placenta represent 7 and 50% of rat duodenum CaBP mRNA at day 15 and 21 of gestation, respectively (29). The present study included no material from the late period of human gestation, precluding the detection of such an increase.

The presence of high concentrations of 9K CaBP mRNA in the thymus and lung of human fetuses revealed by the present study is somewhat unexpected. Such a level of 9K CaBP gene expression as that observed in these two tissues, which are not involved in calcium transfer, suggests that CaBP may be implicated in the modulation of differentiation and development. Many recent studies point to the role of vitamin D in cellular differentiation; *e.g.* the induction of bone marrow stem cells differentiation and maturation especially in monocyte-macrophage cell lines (30). There is also evidence that $1,25(OH)_2D_3$ acts as an immunoregulatory hormone by inhibiting lymphocyte interleukin 2 production and the proliferation of activated lymphocytes (31). All those actions occur via a receptor-mediated mechanism and involve physiological doses of the hormone. The $1,25(OH)_2D_3$ receptors in rat thymus gland are only found in the mitotically active medullary zone (32). In this organ, $1,25(OH)_2D_3$ inhibits the proliferation of active thymoblasts and induces their differentiation (33). Little is known of the vitamin D action in the lung. Rachitic rats show a distensibility of the lungs which results from disturbed development of alveolae and connective tissues (34). Maturation of the lung is closely linked to the appearance and proliferation of pulmonary macrophages. These cells, which are derived from the monocyte-cell line, have $1,25(OH)_2D_3$ receptors and are activated by the hormone (35). Immunohistological studies will be necessary before the role of 9K CaBP in the development of both thymus and lung can be described in more details.

The presence of the two vitamin D-dependent CaBPs in the human fetus indicates that this species follows the general pattern of calcium-binding protein distribution found in the rat, specially in classical vitamin D target tissues. Moreover, the demonstration of CaBPs gene expression in human fetal tissues which are not directly involved in calcium homeostasis, such as thymus, lung, and cerebellum, adds to the growing body of evidence for a role of vitamin D in the differentiation and growth at specific stage of development.

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