Biochemical Evidence for the Presence of Two Vitamin D-dependent Calcium-binding Proteins in Mouse Kidney*

Anne-Cécile Delorme‡, Jean-Louis Danan, and Henri Mathieu
From the Unité de Recherches sur le Métabolisme Hydro-Minéral, Institut National de la Santé et de la Recherche Médicale, U.120, 44 Chemin de Ronde, 78110 Le Vesinet, France

Mouse kidney, a vitamin D target organ, was investigated for the presence of vitamin D-dependent calcium-binding proteins (CaBP). Mouse kidney cytosol was fractionated by several biochemical methods including gel filtration chromatography, gel permeation high performance liquid chromatography, and chromatofocusing. Mouse kidney was found to possess two CaBPs which completely differed biochemically and exhibited no cross-immunoreactivity. One had a molecular weight of 25,000, and a pl of 5.9. The other, with a molecular weight of 10,000 and a pl of 4.9, was biochemically identical with mouse duodenal 10,000 CaBP. In addition, mouse renal and duodenal 10,000 CaBPs were immunologically identical. Moreover, the 10,000 CaBP was the predominant CaBP in mouse kidney since the latter contained about twice as much 10,000 CaBP as 25,000 CaBP (in mol/mg of renal cytosolic protein). In vitro incorporation of [3H]leucine into renal 10,000 CaBP demonstrated that it is synthesized in situ by mouse kidney. Renal 10,000 CaBP was already present during fetal life, and reached its adult level during the first week after birth. The vitamin D dependency of both mouse renal 10,000 and 25,000 CaBPs was assessed by their decrease in vitamin D-deficient mice and subsequent rise after 1,25-dihydroxyvitamin D$_3$ injection. The concomitant presence of substantial amounts of two vitamin D-dependent CaBPs in mouse kidney is peculiar to this organ, which might consequently provide a unique model for studying the hormonal expressions of 1,25-dihydroxyvitamin D$_3$.

A vitamin D-dependent CaBP$^1$ was first discovered in the chick (1) and was subsequently reported in a wide variety of species (2). CaBP is considered as the molecular expression of 1,25(OH)$_2$D$_3$, the hormonal form of vitamin D$_3$ (2). However, its physiological function remains unclear. In the chick, CaBP appears to have the same characteristics in all the organs examined to date and has a molecular weight of 25-28,000 (3-6). In mammals, on the other hand, there are CaBPs which are biochemically and immunologically different (2, 7, 8). In addition, they possess their own specific organ distribution. Thus, mammalian kidney contains a CaBP with a molecular weight of 25-28,000 (9, 10). By contrast, the mammalian duodenum contains a CaBP with a molecular weight of 9-10,000 (10-16).

Purification of duodenal 10,000 CaBP from rat and mouse allowed us to develop two specific RIAs for each of these two species (14, 16). When studying the tissue distribution of 10,000 CaBP in these species, we found a CaBP identical with the duodenal one in the uterus, choioallantoic placenta, and yolk sac of both rat and mouse (16, 17). When we tested mouse kidney cytosol by RIA, we detected substantial amounts of immunoreactive material. This was surprising in view of the fact that rat and other mammalian kidneys contain little or no immunoreactive 10,000 CaBP (1). This prompted us to define biochemically the immunoreactive material from mouse kidney and to investigate its dependency on vitamin D.

**MATERIALS AND METHODS**

**Animals and Diets**

For biochemical and immunological studies, normal Swiss mice, 6-8 weeks old, were obtained from CERJ, France and fed on a normal diet (UAR, France).

For vitamin D dependency studies, 89 normal weaning Swiss mice (CERJ) were randomized into 3 groups. All were raised in the dark on a vitamin D-free diet (containing 0.56% Ca and 0.36% P) for 5 weeks and then on a low-calcium vitamin D-free diet (0.03% Ca and 0.36% P) for 1 week. In one group, 2000 IU of vitamin D$_3$/kg dry diet were added to the daily feed (vitamin D-repleted group). In another group, each mouse received a subcutaneous injection of 1 mg of 1,25(OH)$_2$D$_3$/g of body weight in 10 µl of 95% ethanol and 24 h before killing (vitamin D-deficient + 1,25(OH)$_2$D$_3$ group). A third group received one subcutaneous injection of 10 µl of solvent alone, 48 and 24 hours before killing (vitamin D-deficient group). Animals were fed ad libitum and were not fasted before killing. In each group of 23 mice, tissue samples from 16 were pooled, and samples from the remaining 7 were treated individually.

For developmental studies, normal pregnant Swiss mice were obtained at 15-16 days of gestation. In this strain, parturition occurred after 19 days of gestation. Litter size was reduced to 8-10 pups within 48 h of birth. Mothers were removed from cages 20 days after birth. On given days, pups were taken at random from the litters and killed.

Chick duodenal mucosa samples were obtained from normal 5-week-old chickens (Ruel, France).

**Preparation of Tissues**

Mice were bled by decapitation, and the kidneys were removed, decapsulated, and weighed. They were minced and suspended (1:4, w/v) in ice-cold Tris-buffer (15.7 mM Tris-HCl, pH 7.4, containing 120 mM NaCl and 3 mM KCl), to which 4 mM phenylmethylsulfonyl fluoride and 1% aprotinin (Sigma) were added. They were homogenized in a Potter Elvehjem homogenizer and centrifuged at 100,000 g for 1 h at 4°C. The supernatant without the fluffy lipid layer was taken as the cytosol fraction and kept frozen at −30°C. Other mouse or chick tissues studied were similarly processed.

For the vitamin D dependency studies, the proximal 5 cm of the small intestine were excised in addition to the kidneys, rinsed in ice-cold Tris-buffer, and everted. The duodenal mucosa was scraped and treated as described above.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.

1 The abbreviations used are: CaBP, calcium-binding protein; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; GP-HPLC, gel permeation high performance liquid chromatography; RIA, radioimmunoassay.
For the developmental studies, in addition to the kidneys, the entire intestine, from the pylorus to the ileocaecal junction, was dissected from the pancreas and peritoneum, opened along its whole length, and thoroughly rinsed. Kidneys were excised, and all the samples were frozen.

Biochemical and Immunological Methods

**Gel Chromatography**—Gel chromatography was performed with Sephadex G-75 (Pharmacia, France) in 0.1 M ammonium acetate buffer, pH 7.2, with 1 mM mercaptoethanol. The column (2.6 × 100 cm) was calibrated with 4 proteins of known molecular weight: bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and cytochrome (12,500) (all from Boehringer) detected by their absorbance at 280 nm. The void volume of the column was determined by blue dextran exclusion. The partition coefficient between the liquid and the gel phases (Kd) was plotted against the log (molecular weight) of the protein and yielded a straight line.

**Chromatofocusing** was then carried out using Polybuffer Exchanger 74 (Pharmacia), packed in a column (0.9 × 30 cm). The two pH gradients (7-4 and 8-5) were respectively produced with Polybuffer 74 at pH 4.0 and with a mixture of Polybuffer 96 (30%) and Polybuffer 74 (70%) at pH 5. Collected fractions were monitored for pH with a Tacussel pH meter.

**Ouchterlony Double Immunodiffusion**—Plates that contained 1.5% (w/v) agar in a solution of 0.05 M sodium veronal, 0.01 M veronal, and 0.06% Tris, pH 9.0 (Sebia, France) were incubated overnight at 15 °C. Immunoprecipitates were stained with 0.5% Amido black in methanol/water/acetic acid (54:1, v/v).

**45Ca-Chelx Method**—The calcium-binding activities eluted from the different columns were detected by the 45Ca-Chelx method (12), using Chelx 100 (200-400 mesh, Bio-Rad). When chromatofocusing was performed in the presence of 1 mM CaCl2, the eluted fractions had to be freed of calcium prior to the Chelx assay. Therefore 0.1 ml of the Chelx 100/Tris HCl buffer, pH 7.4 (4:1) suspension was added to 0.3 ml of aliquots of each fraction. Samples were shaken for 30 min and centrifuged for 5 min at 5000 g. Calcium-binding activities were then assayed on 0.25 ml of aliquots of the supernatant incubated with 45Ca and Chelx 100 suspension according to the Chelx method. Radioactivity was counted in 7 ml of Picofluor 15 (Packard Instrument Co.) in an SL 40 Intertechnique scintillation counter (Kontron, France).

**Radioimmunoassay of CaBP—CaBP concentrations in cytosol samples were directly measured by an RIA using immunoserum directed against the duodenal 10,000 CaBP from rats, with pure mouse 10,000 CaBP as standard and 125I-labeled pure mouse 10,000 CaBP as tracer, as previously described (16). Results were expressed as mg of CaBP/mg of cytosolic proteins. Immunoreactivity was also tested on fractions eluted from the different columns, by incubating aliquots with the immunoserum and 125I-labeled pure mouse 10,000 CaBP. The results are expressed as 125I-labeled CaBP bound to the immunosorbent in the presence of aliquots (B) vs. 125I-labeled CaBP bound to the immunosorbent in the absence of CaBP (B). Radioactivity was counted in a CG 2000 Intertechnique γ counter (Kontron, France).

**In Vitro Synthesis and Immunoprecipitation of 10,000 CaBP from Mouse Kidney—** In a 25 ml-Erlenmeyer flask, mouse kidneys (200 mg) were minced in 3 ml of BME Earle’s salt solution (Glace) containing 250 μCi of [3H]leucine (160 Ci/mmol, Amersham Corp.). The flask was gassed with 95% O2, 5% CO2 and incubated for 8 h in a shaking water bath at 37 °C. The cytosol was prepared, and the total protein synthesis was estimated as described by Bruns et al. (15). Since, in earlier preliminary experiments, we observed nonspecific absorption by Protein-A (Pansorbin, Calbiochem) of high molecular weight [3H]labeled proteins, [3H]leucine-labeled cytosol was precipitated with 2000 mg of solid (NH4)2SO4/ml for 30 min at 4 °C before immunoprecipitation. After centrifugation, the supernatant was then dehydrated through a short column of Sephadex G AO eluted with 0.1 M ammonium acetate, pH 7.2. After lyophilization, the proteins were dissolved in 0.4 ml of a buffer containing 0.01 M Tris-HCl, pH 7.4, with 0.15 M NaCl and 1.5% Triton X500. Following exactly the procedure described by Bruns et al. (15), 0.2 ml of aliquots were then immunoprecipitated with 20 μl of the anti-duodenal rat 10,000 CaBP immunoserum. 20 μg of pure unlabeled rat 10,000 CaBP were added to the remaining 0.2 ml before immunoprecipititation, to assess the latter’s specificity. After three washings, the immunoprecipitate was dissociated by heating in buffer containing 0.1 M Tris-HCl, 2% sodium deoxycholate, 10% glycerol, and 15% dithiothreitol. [3H]-labeled 10,000 CaBP synthetized with mouse kidney was characterized by both sodium deoxycholate 15% (w/v)-polyacrylamide gel electrophoresis and by GP-HPLC.

**RESULTS**

Biochemical Evidence for Two CaBPs in the Mouse Kidney

To investigate the calcium-binding activity, mouse-kidney cytosol was fractionated by several biochemical techniques including gel filtration chromatography, GP-HPLC, and chromatofocusing. The eluted fractions were analyzed for calcium-binding activity and immunoreactivity using the anti-duodenal rat 10,000 CaBP immunoserum and 125I-labeled CaBP purified from mouse duodenum as described under "Materials and Methods."

**Sephadex G-75 Chromatography—** Chromatography of mouse kidney cytosol showed that [3H]leucine-labeled activity was distributed into two major [3H]leucine peaks (Fig. 1). The two peaks were well separated and of roughly similar size. Immunoreactive material was present as a single peak, exactly superimposable on the low molecular weight [3H]Ca-binding activity. The estimated molecular weight of the protein which exhibited immunoreactivity simultaneously with calcium-binding activity was approximately 10,000. Duodenal mouse 10,000 CaBP exhibited the same property (15, 16). The other [3H]Ca-binding peak corresponded to a CaBP with molecular weight of 25-27,000.

**GP-HPLC—** The recent progress achieved in the field of protein GP-HPLC (21) led us to use this powerful tool to further compare these two calcium-binding proteins. Thus, each of the two calcium-binding activities, 25,000 and 10,000 obtained by gel filtration of the mouse kidney cytosol, was submitted to GP-HPLC (Fig. 2). GP-HPLC performed at a flow rate of 0.4 ml/min was capable of discriminating between these two calcium-binding activities in 30 min. The 25,000 calcium-binding activity eluted at a volume of 8.0 ml, essen-
45Ca-binding rate was successively applied to a Waters gel permeation column in each calcium-binding activity eluted from the Sephadex column. The respective effects of calcium and EDTA on the elution of renal calcium-binding activities from the GP-HPLC column were investigated. The 10,000 CaBP was chosen for this study because we could easily detect it by its immunoreactivity. As shown in Fig. 3, the mobility of the 10,000 CaBP depended on whether calcium or EDTA was present in the buffers. In the presence of 1 mM CaCl₂, the elution volume was 8.8 ml, but in the presence of 1 mM EDTA, this was always decreased to 8.4 ml, and the apparent molecular weight therefore increased. Similar changes in the apparent molecular weight were reported for a few other calcium-binding proteins such as calmodulin using electrophoresis under denaturing conditions (22, 23). GP-HPLC thus provides a satisfactory new approach to detection of the particular changes induced by calcium binding and might prove useful in identifying and purifying CaBPs.

Chromatofocusing—Further characterization of both the 25,000 and 10,000 CaBPs from mouse kidney required determination of their isoelectric point. For this purpose, we chose chromatofocusing, a very convenient method for estimating pI of partially purified proteins (Fig. 4). The two calcium-binding activities (25,000 and 10,000) were applied together to tially the same as the elution volume obtained for the chick CaBP we prepared from duodenal mucosa. The 10,000 calcium-binding activity eluted at a volume of 9.2 ml and was also associated with immunoreactivity. The latter elution volume was exactly the one we found for the 10,000 CaBP from mouse duodenal mucosa. These results for GP-HPLC are in complete agreement with those for Sephadex G-75 filtration, but GP-HPLC has the marked advantage of being faster and more reproducible.

The two peaks of calcium-binding activities from mouse kidney were pooled (fractions 54 to 78 from the Sephadex G-75 column) and lyophilized. A, half the pool was redissolved in 0.025 M imidazole buffer, pH 7.4, and applied on a column of Polybuffer Exchanger 94; the elution buffer was Polybuffer 74, pH 4.0. B, half the pool was redissolved in 0.025 M Tris-acetate buffer, pH 8.3, containing 1 mM CaCl₂ and applied on a column of Polybuffer Exchanger 94; the elution buffer was a mixture of 30% Polybuffer 96 and 70% Polybuffer 74, pH 5, and contained 1 mM CaCl₂. For both columns, the flow rate was 19 cm/h. Fractions (1.1 ml) were collected and tested for 45Ca-binding (●) and immunoreactivity (○). The immunserum was anti-duodenal rat 10,000 CaBP. Arrows indicate the elution volume of bovine serum albumin (BSA) and CaBPs from chick and mouse duodenal mucosa.

**Fig. 2.** GP-HPLC of CaBPs from mouse kidney. Aliquots of each calcium-binding activity eluted from the Sephadex G-75 column were successively applied to a Waters 125 gel permeation column in 50 μl of 0.1 M Tris-acetate, pH 7.0, containing 0.15 M NaCl. The flow rate was 0.4 ml/min. Fractions (0.4 ml) were collected and tested for 45Ca-binding (●) and immunoreactivity (○). The immunserum was anti-duodenal rat 10,000 CaBP. A, 400 μg of the 25,000 calcium-binding activity eluted from the Sephadex G-75 column; B, 140 μg of the 10,000 calcium-binding activity eluted from the Sephadex G-75 column. Arrows indicate the elution volume of bovine serum albumin (BSA) and CaBPs from chick and mouse duodenal mucosa.

**Fig. 3.** Effect of Ca²⁺ and EDTA on CaBP mobility in GP-HPLC. 140 μg of the 10,000 calcium-binding activity, eluted from the Sephadex G-75 column, were applied on a Waters 125 gel permeation column. Elution was in 0.1 M Tris-acetate, pH 7.0, containing 0.15 M NaCl and 1 mM CaCl₂ (●) or 1 mM EDTA (○). The flow rate was 0.4 ml/min. Fractions (0.4 ml) were collected and tested for immunoreactivity. The immunserum was anti-duodenal rat 10,000 CaBP.

**Fig. 4.** Chromatofocusing of mouse kidney CaBPs. The two peaks of calcium-binding activities from mouse kidney were pooled (fractions 54 to 78 from the Sephadex G-75 column) and lyophilized. A, half the pool was redissolved in 0.025 M imidazole buffer, pH 7.4, and applied on a column of Polybuffer Exchanger 94; the elution buffer was Polybuffer 74, pH 4.0. B, half the pool was redissolved in 0.025 M Tris-acetate buffer, pH 8.3, containing 1 mM CaCl₂ and applied on a column of Polybuffer Exchanger 94; the elution buffer was a mixture of 30% Polybuffer 96 and 70% Polybuffer 74, pH 5, and contained 1 mM CaCl₂. For both columns, the flow rate was 19 cm/h. Fractions (1.1 ml) were collected and tested for 45Ca-binding activity (●) and immunoreactivity (○). The immunserum was anti-duodenal rat 10,000 CaBP. Arrows indicate the elution volume of CaBPs from chick duodenal mucosa, mouse duodenal mucosa, and mouse cerebellum.
Two Vitamin D-dependent Ca-binding Proteins in Mouse Kidney

a column of Polybuffer Exchanger 94 with a pH gradient descending from 7 to 4 formed by Polybuffer 74. Chromatofocusing of mouse kidney cytosol displayed two well separated peaks of specific calcium-binding activity. One calcium-binding activity eluted at a pH of 4.9 and was associated with immunoreactivity. Furthermore, this acid pH was identical with that of duodenal mouse 10,000 CaBP as determined by chromatofocusing and isoelectric focusing (16). The other calcium-binding component peaked at a pH of 5.9 and corresponded to the 25,000 calcium-binding activity. In addition, we found that mouse cerebellum cytosol contained a calcium-binding activity which eluted at 25,000 on a Sephadex G-75 column (not shown) and which also peaked at a pH of 5.9 on the same chromatofocusing column. Lastly, after fractionation on a Sephadex G-75 column, cytosol from chick duodenal mucosa exhibited a calcium-binding activity with a pH of 4.1. The latter value is in complete agreement with the one previously obtained by other methods (24), thus underlining the validity of the pH values we determined for mouse CaBPs. In addition, this result for chick CaBP indicates that despite the immunological cross-reactivity reported between chick and mammalian renal CaBPs (2,25) there are biochemical differences between the 25,000 CaBPs from one species to another.

To study an influence of calcium on the apparent pH values of mouse renal calcium-binding activities, we performed chromatofocusing in the presence of 1 mM CaCl₂ in all the buffers, with a pH gradient descending from 8 to 5. Two calcium-binding activity peaks were again obvious. In the presence of calcium, the apparent pH of the calcium-binding activity associated with immunoreactivity was 6.8. This pH of 6.8 is identical with what we found for the calcium-bound form of duodenal mouse 10,000 CaBP (16). The 25,000 calcium-binding activity had an apparent pH of 7.0 in the presence of calcium. Therefore, the apparent pH of the two calcium-binding activities from mouse kidney changes in the presence or absence of calcium. However, the variations in pH for each renal CaBP were not the same (2 pH units for the 10,000 CaBP and 1 pH unit for the 25,000 CaBP), but the reason for this is not known.

Note that the ⁴⁵Ca baseline in chromatofocusing experiments rose as pH decreased since the action of Chelex resin is known to be very dependent on pH (12).

**Ouchterlony Double Immunodiffusion**—The two CaBPs present in mouse kidney were compared from an immunochromatographic point of view using Ouchterlony double immunodiffusion (Fig 5). With anti-duodenal rat 10,000 CaBP immunserum (A) a single precipitating immunocomplex was formed with mouse duodenal cytosol, in agreement with our previous report (16). A single precipitation line was also observed when this immunoserum was incubated with a similar amount of mouse kidney cytosolic proteins. In addition, both precipitation lines from mouse kidney and mouse duodenum fused without spur, indicating the presence of apparently identical antigenic determinants in both mouse organs. Furthermore, in RIA, the immunodilution curve for mouse kidney cytosol was parallel to that of mouse duodenum cytosol (results not shown), thus confirming the complete immunological identity of renal and duodenal 10,000 CaBP. This immunoreactive material corresponded to the 10,000 calcium-binding activity eluted from the Sephadex G-75 column. Unlike mouse kidney, rat kidney cytosol gave no reaction with this anti-duodenal rat 10,000 CaBP immunserum.

When anti-human cerebellar 25,000 CaBP immunserum² was used (Fig 5B), a precipitating immunocomplex was formed with mouse kidney cytosol. This immunoreactive material was only associated with the 25,000 calcium-binding activity and not with the 10,000 one. In addition, a precipitating immunocomplex was formed with rat kidney cytosol. The precipitating line from this cytosol completely fused with that from mouse kidney cytosol. Lastly, no cross-reacting material was detected in mouse duodenum cytosol by this anti-25,000 CaBP immunserum.

All these results demonstrate that two CaBPs, which have completely different biochemical and immunological properties, are present in mouse kidney cytosol. One is identical with the cerebellar mouse CaBP, has a molecular weight of 25–27,000 and a pH of 5.9, and will be referred to as mouse renal 25,000 CaBP. The other is identical with the duodenal mouse CaBP, has a molecular weight of 9–10,000 and a pH of 4.9, and will be termed the mouse renal 10,000 CaBP.

**In Vitro Synthesis of Mouse Kidney 10,000 ³H-CaBP**

To show that the 10,000 CaBP we found in mouse kidney was an intrinsic protein, we tested mouse kidney homogenates for their ability to incorporate [³H]leucine into CaBP. Radioactive 10,000 CaBP was detected by immunoprecipitation using the anti-duodenal rat 10,000 CaBP immunserum. Fig. 6 shows the GP-HPLC pattern of the immunoprecipitate from newly synthesized cytosolic protein in mouse kidney. It exhibited a single radioactive peak which eluted at exactly the same place as mouse duodenal and renal 10,000 CaBP. This radioactive peak was no longer detected when an excess of unlabeled pure 10,000 CaBP was added to the cytosol prior to incubation with the anti-duodenal rat 10,000 CaBP immunserum. This radioactive peak was also absent when the immunoserum was omitted or replaced by non immunserum. The same results were obtained when the immunoprecipitates were analyzed by sodium dodecyl sulfate-15% (w/v)-polyacrylamide electrophoresis (not shown). The above experiments suggested that the 10,000 CaBP present in mouse kidney is in fact synthesized in situ.

**Distribution of 10,000 CaBP within Mouse Kidney**

8 mouse kidneys were separated into cortical and medullary regions, and their 10,000 CaBP concentrations were measured by RIA. These concentrations were not significantly different in both regions; they were 4.15 ± 0.31 μg of CaBP/mg of

² The anti-human cerebellar 25,000 CaBP immunserum was a gift from Dr. O. Parkes, Vancouver, Canada.
Changes in Renal 10,000 CaBP during Normal Development in Mice

Measurements of 10,000 CaBP in baby mouse kidney were made simultaneously with those of 16,000 CaBP in the entire small intestine, between 1 day before birth and day 23 after birth (Fig. 8). Before birth, substantial amounts of 10,000 CaBP were not only present in mouse fetal small intestine, but also in mouse fetal kidney. After birth, the developmental pattern of 10,000 CaBP in mouse kidney completely differed from that of intestinal 10,000 CaBP. In the kidney, the 10,000 CaBP concentration increased in the first week of life, since it was $1.96 \pm 0.07 \mu g/mg$ of cytosolic proteins on day 2 and rose to $4.16 \pm 0.11 \mu g/mg$ of cytosolic proteins on day 8. Thereafter, the 10,000 CaBP concentration in mouse kidney remained unchanged until the postweaning period. In mouse small intestine, the 10,000 CaBP concentration remained unchanged from birth until day 18, when it increased to $3.94 \pm 0.42 \mu g/mg$ of cytosolic proteins. The developmental pattern of 10,000 in mouse intestine was similar to that described in rat intestine (17, 26).

Vitamin D Dependency of CaBPs in Mouse Kidney

We investigated the influence of vitamin D status on both CaBPs from mouse kidney. For this purpose, native kidney cytosols from the 3 groups of mice were prepared, were assessed to contain the same amount of proteins, and were successively applied on the same Sephadex G-75 column (Fig. 7). In the cytosol from the vitamin D-repleted group (Fig. 7A), both calcium-binding activities were of similar importance. In the vitamin D-deficient group, both calcium-binding activities diminished but there was no change in their relative importance (Fig. 7B). In the vitamin D-deficient + 1,25(OH)$_2$D$_3$ group (Fig. 7B), both activities rose. However, the increase in 10,000 calcium-binding activity was seen to be larger than that observed for 25,000 activity.

The variations noted for the renal 10,000 calcium-binding activity led us to measure the mouse renal 10,000 CaBP directly by RIA, to obtain individual and more precise data (Table I). In kidneys from vitamin D-deficient mice, 10,000 CaBP concentrations ($0.7 \pm 0.07 \mu g/mg$ of cytosolic proteins) were significantly lower than those found in the vitamin D-repleted group ($2.9 \pm 0.17 \mu g/mg$ of cytosolic proteins). Furthermore, renal 10,000 CaBP followed almost the same pattern as duodenal CaBP. Injection of 1,25(OH)$_2$D$_3$ in vitamin D-deficient mice completely restored the 10,000 CaBP contents in the duodenum and kidney. However, renal 10,000 CaBP concentrations rose to $4.5 \pm 0.34 \mu g/mg$ of cytosolic proteins, a value higher than that for the vitamin D-repleted group, thus confirming the results obtained by the Chelex method.

Table I

| Mice | Body weight | Plasma calcium | Duodenal 10,000 CaBP | Renal 10,000 CaBP |
|------|-------------|----------------|----------------------|------------------|
| Vitamin D-replete (7) | 28 ± 0.7 | 9.5 ± 0.16 | 9.1 ± 0.81 | 2.9 ± 0.17 |
| Vitamin D-deficient (7) | 27 ± 1 | 7.4 ± 0.22 | 3.0 ± 0.25 | 0.7 ± 0.07 |
| Vitamin D-deficient + 1,25(OH)$_2$D$_3$ (7) | 26 ± 0.6 | 10.6 ± 0.86 | 10.0 ± 0.55 | 4.5 ± 0.34 |

* Value significantly different from the vitamin D-repleted group ($p < 0.05$).
when a pool of under "Materials and Methods." Results are given as mean ± S.E. Each point represents the average of small intestines and pairs of kidneys except on day 1 of postnatal life, when 6 pools of 3 newborn mice were used. Arrows indicate time of birth (B) and weaning (W).

**DISCUSSION**

Our data clearly demonstrate that two different vitamin D-dependent CaBPs are simultaneously present in the mouse kidney. The existence of the 25,000 CaBP that we partially characterized was to be expected since the kidneys of several species possess a 25–28,000 CaBP. By contrast, the synthesis of substantial amounts of a 10,000 CaBP by mouse kidney was a surprising result. Biochemical characterization of this renal 10,000 CaBP revealed its complete identity with the 10,000 CaBP of mouse intestine during normal fetal and postnatal development (from pylorus to ileocaecal junction). This lends support to the idea that the cytosolic and nuclear 1,25(OH)2D3 receptors in mouse kidney are functional (34, 35). Therefore, mouse kidney appears as a peculiar organ in which the hormonal action of 1,25(OH)2D3 results in two different molecular expressions. Consequently, it could provide a unique model for studying the genomic action of 1,25(OH)2D3.

The vitamin D dependency of both mouse renal 25,000 and 10,000 CaBPs was illustrated by the fact that they decreased in vitamin D-deficient mice and rose after 1,25(OH)2D3 injection. This lends support to the idea that the cytosolic and nuclear 1,25(OH)2D3 receptors in mouse kidney are functional (34, 35). Therefore, mouse kidney appears as a peculiar organ in which the hormonal action of 1,25(OH)2D3 results in two different molecular expressions. Consequently, it could provide a unique model for studying the genomic action of 1,25(OH)2D3.

Acknowledgements—We are indebted to Dr. J. M. Garel for 25,000 CaBP and to Dr. O. Parkes for his gift of anti-human cerebellar 25,000 CaBP immunserum. We also appreciated the skillful assistance of A. Porteu de la Morandiere and C. M. Fattaccini. Finally, we would like to thank M. Courat, M. Dreyfus, and P. Schuman for their help in the preparation of the manuscript.

**REFERENCES**

1. Wasserman, R. H., and Taylor, A. N. (1966) Science (Wash. D. C.) 152, 791–793
2. Wasserman, R. H., and Fullmer, C. S. (1982) in Calcium and Cell Function (Cheung, W. Y., ed) pp. 175–216, Academic Press, New York
3. Taylor, A. N., and Wasserman, R. H. (1967) Arch. Biochem. Biophys. 119, 536–540
4. Corradino, R. A., Wasserman, R. H., Pubols, M. H., and Chang, S. I. (1966) Arch. Biochem. Biophys. 125, 278–380
5. Taylor, A. N., and Brindak, M. E. (1974) Arch. Biochem. Biophys. 191, 100–108
6. Christakos, S., Friedlander, E. J., Frandsen, B. R., and Norman, A. W. (1979) Endocrinology 104, 1495–1503
7. Laouari, D., Pavlovič, H., Devecseri, G., and Blaisson, S. (1980) FEBS Lett. 111, 285–289
8. Price, P. A., and Baukol, S. A. (1980) J. Biol. Chem. 255, 11686–11683
9. Hermsdorff, C. L., and Bronner, F. (1975) Biochim. Biophys. Acta 379, 553–561
10. Arnold, B. M., Kuttner, M., Willis, D. M., Hitchman, A. J. W., Harrison, J. E., and Murray, T. M. (1975) Can. J. Physiol. Pharmacol. 53, 1135–1140
11. Fullmer, C. S., and Wasserman, R. H. (1975) Biochim. Biophys. Acta 393, 134–142
12. Freund, T., and Bronner, F. (1975) Am. J. Physiol. 228, 861–869
13. Bruns, M. E. H., Flesher, E. B., and Avioli, L. V. (1977) J. Biol. Chem. 252, 4345–4350
14. Marche, P., Pradelles, P., Gros, C., and Thomasset, M. (1977) Biochem. Biophys. Res. Commun. 76, 1020–1025
15. Bruns, M. E. H., Vollmer, S., Wallhein, V., and Bruns, D. E. (1981) J. Biol. Chem. 256, 4649–4653
16. Delorne, A. C., Duman, J. L., Ripoche, M. A., and Mathieu, H. (1982) Biochim. Biophys. Acta 705, 49–57
17. Delorne, A. C., Marche, P., and Garel, J. M. (1979) J. Dev. Physiol. (Oxf.) 1, 181–194
18. Fullmer, C. S., and Wasserman, R. H. (1973) Biochim. Biophys. Acta 317, 172–180
19. Bruns, M. E. H., Feustal, A., and Avioli, L. V. (1978) J. Biol. Chem. 253, 3186–3190
20. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70,
Two Vitamin D-dependent Ca-binding Proteins in Mouse Kidney

21. Pfeifer, R., Skea, W. M., Warraska, J., Cohen, C., and Burnwourth, L. (1982) in Biological and Biochemical Applications of Liquid Chromatography (Hawk, J. ed) pp. 43-55, Marcel Dekker, Inc., New York.

22. Klee, C. B., Crouch, T. H., and Krinks, M. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6270-6273.

23. Burgess, W. H., Jemiolo, D. K., and Kretsinger, R. H. (1980) Biochim. Biophys. Acta 623, 257-270.

24. Ingersoll, R. J., and Wasserman, R. H. (1971) J. Biol. Chem. 246, 2808-2814.

25. Rhoten, W. B., and Christakos, S. (1981) Endocrinology 109, 981-983.

26. Bruns, E. M., Bruns, D. E., and Avioli, L. V. (1979) Endocrinology 5, 934-938.

27. Fullmer, C. S., and Wasserman, R. H. (1977) in Calcium-binding Proteins and Calcium Function (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., Mac Lennan, D. H., and Siegel, F. L., eds) pp. 303-312, Elsevier North-Holland, Amsterdam.

28. Bredderman, P. J., and Wasserman, R. H. (1974) Biochemistry 13, 1687-1694.

29. Roth, J., Thorens, B., Hunziker, W., and Norman, A. W. (1981) Science 214, 197-200.

30. Jande, S. S., Tolnai, S., and Lawson, D. E. M. (1981) Histochemistry 71, 99-116.

31. Bonvalet, J. P., Champion, M., Courtalon, A., Farman, N., Vandewalle, A., and Wanstock, F. (1977) J. Physiol. (Lond.) 269, 627-641.

32. Speller, A. M., and Moffat, D. B. (1977) J. Anat. 123, 487-500.

33. Henning, S. J., and Kretschmer, N. (1973) Enzyme (Basel) 15, 3-23.

34. Colston, K. N., and Feldman, D. (1979) J. Clin. Endocrinol. Metab. 49, 798-800.

35. Colston, K., and Feldman, D. (1980) J. Biol. Chem. 255, 7510-7513.
Biochemical evidence for the presence of two vitamin D-dependent calcium-binding proteins in mouse kidney.
A C Delorme, J L Danan and H Mathieu

*J. Biol. Chem.* 1983, 258:1878-1884.

Access the most updated version of this article at [http://www.jbc.org/content/258/3/1878](http://www.jbc.org/content/258/3/1878)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/258/3/1878.full.html#ref-list-1](http://www.jbc.org/content/258/3/1878.full.html#ref-list-1)