Messenger Ribonucleic Acids from Pig Intestinal Mucosa direct Synthesis of Calcium-Binding Protein in a Cell-Free Translation System

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mRNA from pig duodenal mucosa directs synthesis, in a wheat-germ cell-free system, of two products precipitated by antiserum to pure calcium-binding protein. One of these products has a higher molecular weight than authentic calcium-binding protein, but, like the authentic protein, is heat-stable. The other protein co-migrates with pure calcium-binding protein on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, is stable on heating to 70°C for 15 min and alters its elution position on ion-exchange chromatography depending on whether Ca^{2+} is present in or absent from the elution buffer. The synthesized protein has these properties in common with authentic calcium-binding protein.

The precise mechanism of calcium absorption from the gastrointestinal tract has not yet been established, but there is now considerable evidence that calcium-binding protein is involved in calcium transport in the intestine. Proteins that bind calcium have been found in the intestinal mucosa of several species, including chick (Wasserman & Taylor, 1966), rat (Kallfelz *et al.*, 1967), man and pig (Hitchman & Harrison, 1972). Some of these proteins appear to be dependent for their synthesis on the hormonal metabolite of vitamin D, 1,25-dihydroxycholecalciferol (Spencer *et al.*, 1978; Harrison *et al.*, 1975*a,b*). It would be of some interest to investigate the role of vitamin D sterols in the synthesis of intestinal calcium-binding protein in a similar way to studies of the influence of reproductive steroids on the expression of the chick ovalbumin gene (O'Malley & Means, 1974).

We have chosen as a model the synthesis of calcium-binding protein from pig intestinal mucosa. This protein has been isolated and purified and its amino acid sequence determined (Hitchman *et al.*, 1973; Hofmann *et al.*, 1977). It has been shown by radioimmunoassay that there are relatively large amounts of the calcium-binding protein in duodenal mucosa (Murray *et al.*, 1974), and there is evidence that it is at least partly dependent on 1,25-dihydroxycholecalciferol for its biosynthesis (Harrison *et al.*, 1975*b*).

In order to begin to study the possible role of vitamin D sterols in the expression of the gene for

Abbreviation used: SDS, sodium dodecyl sulphate.

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calcium-binding protein, it was first important to establish that mRNA extracted from pig intestinal mucosa could direct synthesis of the protein in a cell-free translation system.

Materials

Duodenum from freshly slaughtered pigs was obtained from the abattoir. DEAE-Sephadex A25 was from Pharmacia (G.B.), Ealing, London W5 5SS, U.K.; Ultrogel AcA 54 was from L.K.B. Instruments, South Croydon, Surrey, U.K.; Bio-Gel P10, acrylamide and bisacrylamide were from Bio-Rad Laboratories, St. Albans, Herts., U.K.; oligo(dT)-cellulose T2 was from Collaborative Research, Waltham, MA, U.S.A.; proteinase K, GTP and ATP were from Boehringer (London), Lewes, E. Sussex, U.K.; freshly milled (untoasted) wheat germ was from General Mills, Minneapolis, MN, U.S.A.; L-[4,5- ^3H]leucine and L-[^{35}S]methionine were from The Radiochemical Centre, Amersham, Bucks., U.K.; antiserum to purified Ca-binding protein was a gift from Dr. T. M. Murray (Toronto, Canada); antiserum raised in goats against rabbit immunoglobulin G, and spermidine, dithiothreitol, creatine kinase, phosphocreatine, L-amino acids, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], cytochrome *c* and Coomassie Brilliant Blue R were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K.; X-ray film (X-omat RP film), developer (Dx-80) and fixer (FX-40) were from Kodak, Manchester, U.K.; 2,5-diphenyloxazole was from Koch-Light Laboratories,

Colnbrook, Bucks., U.K. All other solutions and chemicals were from BDH Chemicals, Poole, Dorset, U.K. All reagents and chemicals were A.R. grade.

Preparation of solutions

To prevent digestion of RNA by ribonuclease, gloves were worn at all times during experiments involving RNA, all buffers used for RNA extraction were autoclaved in the presence of 0.025% diethyl pyrocarbonate at 104 kPa (15 lb/in²) for 10 min, plastic pipette tips and centrifuge tubes were autoclaved at 69 kPa (10 lb/in²) for 10 min, and all glassware was sterilized at 180°C for 1 h. Phenol was re-distilled before use. Scintillation fluid (modified Bray's) contained 0.4% 2,5-diphenyloxazole, 0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene, 6% (w/v) naphthalene, 2% (v/v) ethylene glycol and 10% (v/v) methanol in 1,4-dioxan.

Methods

Isolation and purification of pig intestinal calcium-binding protein

Ca-binding protein was isolated and purified by the method of Hitchman *et al.* (1973), except that preliminary gel filtration was done with Ultrogel AcA 54 and Bio-Gel P10. After the two ion-exchange-chromatography steps on DEAE-Sephadex, first with EDTA in the elution buffer, then with CaCl₂ in the elution buffer, the Ca-binding protein was found to migrate as a single band on SDS/polyacrylamide-gel electrophoresis. The purified Ca-binding protein obtained in this way was used as a molecular-weight marker and in immunoprecipitation experiments.

Extraction of total cellular nucleic acids

Duodenal mucosa from freshly slaughtered pigs was obtained by scraping the intestine with a glass slide after gently washing the mucosa with 0.9% NaCl. Total nucleic acids were extracted as described by Hall *et al.* (1979), with some modifications: the extraction was done with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and, instead of proceeding with CsCl-step-gradient centrifugation, the aqueous phase was re-extracted twice more with phenol/chloroform/3-methylbutan-1-ol, followed by three more extractions, each with 1 vol. of chloroform/3-methylbutan-1-ol (24:1, v/v). The concentration of NaCl in the aqueous phase was adjusted to 0.2 M; 2 vol. of cold ethanol was added and the nucleic acids were precipitated overnight at -20°C. The precipitate was collected by centrifugation at 10000 *g*_{av.} at -15°C for 10 min. It was then washed twice with cold 70% (v/v) ethanol containing 40 mM NaCl, and finally once more with ethanol, dried *in vacuo* and dissolved in sterile water to a final

concentration of 80–100 *A*₂₅₈ units/ml (1.0 *A*₂₅₈ unit = 50 µg of nucleic acid).

Isolation of mRNA

This was done by the method of Craig *et al.* (1976), except that the total nucleic acids solution was diluted with an equal volume of 20 mM-Tris/HCl (pH 7.5) containing 1 M-NaCl, 1% SDS and 2 mM-EDTA, and the bound RNA-oligo(dT)-cellulose was washed three times with 10 mM-Tris/HCl (pH 7.5) containing 0.5 M-NaCl, 0.5% SDS and 1 mM-EDTA before being poured into a column. The mRNA was eluted at 20°C by using 10 mM-Tris/HCl buffer (pH 7.5) containing 0.05% SDS and 1 mM-EDTA. After the second wash of the pellet with 70% ethanol containing 40 mM-NaCl, it was finally washed with ethanol and dried *in vacuo*. Total polyadenylated mRNA was isolated from lactating guinea-pig mammary glands as described by Hall *et al.* (1979).

Translation of polyadenylated mRNA from pig duodenal mucosa in a wheat-germ cell-free translation system

Wheat-germ lysate was prepared by the method of Roberts & Paterson (1973). Protein synthesis in the wheat-germ system was done in a final volume of 25 µl at room temperature for 3 h. The incubation mixture contained 50 mM added potassium acetate, 0.5 mM added magnesium acetate, 25 µM of 19 unlabelled L-amino acids, 12 µCi of L-[4,5-³H]leucine (sp. radioactivity 105 Ci/mmol) or 28 µCi of L-[³⁵S]methionine (sp. radioactivity 1080 Ci/mmol), 0.25 mM-spermidine, 8 mM-phosphocreatine, 2 mM-dithiothreitol, 50 µM-GTP, 1.0 mM-ATP, 24 mM-Hepes, pH 7.0, 8 µl of wheat-germ S-30 fraction and 0.05 *A*₂₅₈ unit (2.5 µg) of mRNA. To determine the mRNA activity, the incorporation of the labelled amino acid into trichloroacetic acid-precipitable protein was measured at the end of the incubation period; 2.5 µl samples were removed for spotting on filter papers (Whatman 3M; 1 cm × 1 cm), which were immediately dropped into cold 10% trichloroacetic acid, and then boiled in 5% trichloroacetic acid containing casein hydrolysate for 10 min. The filter papers were washed twice in ethanol and once in diethyl ether, dried and counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter.

Immunoprecipitation studies

Product analysis by immunoprecipitation was achieved by using a double-antibody technique. Translations were scaled up 10–20-fold for immunoprecipitation. To a 250–500 µl translation reaction mixture Triton X-100 was added (final concn. 1%), followed by rabbit antiserum raised against pure Ca-binding protein (final dilution 1:400) and incubated at 4°C overnight. Sufficient antiserum (50 times

volume of first antiserum) against rabbit immunoglobulins raised in goats was then added to produce optimum precipitation of the antiserum against Ca-binding protein. This was also incubated overnight at 4°C. The immunoprecipitate was pelleted by centrifugation at 10000 g_{av} for 10 min, the supernatant retained, and the pellet washed three times with antibody-wash buffer [20 mM-Tris/HCl (pH 7.4), 150 mM-NaCl, 2% Triton X-100] and dried *in vacuo*. This was then dissolved in sample buffer [160 mM-Tris/HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol] and heated at 100°C for 5 min. Portions (2.5 μ l) of the supernatant and the immunoprecipitate solution were used for trichloroacetic acid precipitation as described above, and their radioactivity was measured.

Analysis of the immunoprecipitates was by gel filtration on Bio-Gel P10 (200–400 mesh) and the column (29 cm $\times$ 1 cm) was eluted under gravity with 150 mM-ammonium acetate (pH 7.4); 200 μ l portions of each of the 1 ml fractions were added to 5 ml of scintillation fluid and the radioactivity was measured in a Packard liquid-scintillation counter.

Analysis of the immunoprecipitates by SDS/polyacrylamide-gel electrophoresis was done on 20% or 10–20% gradient polyacrylamide slab gels (Laemmli, 1970) by using unlabelled authentic Ca-binding protein and cytochrome *c*, also dissolved in the sample buffer, as molecular-weight markers. After fixing and staining in 0.2% Coomassie Blue in 7% (v/v) acetic acid/50% (v/v) methanol for 1 h, and destaining in 7% acetic acid/50% methanol overnight, the gels were fluorographed (Laskey & Mills, 1975).

Heating experiments

A 250–500 μ l portion of translation reaction mixture was centrifuged at 49000 g_{av} for 60 min, the pellet discarded and the supernatant divided into two, half of which was kept as control, and the other half was heated at 70°C for 15 min and then centrifuged at 49000 g_{av} for 30 min. The pellet was discarded. To this and the unheated supernatant, 0.25 vol. of 0.32 M-Tris/HCl (pH 6.8), containing 50% (v/v) glycerol, 10% (w/v) SDS and 25% (v/v) 2-mercaptoethanol, was added. The mixture was then boiled for 3 min and electrophoresed on 20% polyacrylamide gel containing SDS.

Ion-exchange chromatography

Heat-treated translation reaction mixtures were chromatographed on Bio-Gel P10 (55 cm $\times$ 1 cm), and eluted under gravity with 150 mM-ammonium acetate buffer (pH 7.4). The radioactivity of the fractions was measured and the fractions eluted with authentic Ca-binding protein were pooled, freeze-dried, and freeze-dried three times more from water, and dissolved in 2 ml of 20 mM-imidazole/HCl buffer, pH 7.0, containing 20 mM-NaCl and 1 mM-

EDTA, and chromatographed on a column of DEAE-Sephadex (30 cm $\times$ 1.5 cm) previously equilibrated with the same buffer. The column was run under gravity with a linear 0–0.7 M-NaCl gradient (total volume 200 ml) and the radioactivity of the fractions was measured. The fractions containing the major peaks of radioactivity eluted at high salt concentration were pooled, freeze-dried, desalted on a column of Bio-Gel P10 and then chromatographed on another column of DEAE-Sephadex (30 cm $\times$ 1.5 cm) equilibrated with 20 mM-imidazole/HCl (pH 7.0)/20 mM-NaCl/1 mM-CaCl₂. The column was run under gravity with the same buffer with a NaCl gradient (0–0.7 M) and the radioactivity of the fractions was measured.

Results

The yield of mRNA after two cycles of affinity chromatography on oligo(dT)–cellulose was about 0.5% of the total extracted nucleic acids. In the wheat-germ system this fractionated mRNA stimulated incorporation of the labelled amino acid into trichloroacetic acid-precipitable protein by 8–10-fold over the endogenous wheat-germ protein synthesis without added mRNA. Addition of wheat-germ tRNA in a concentration from 100 to 600 μ g/ml in the translation reaction decreased intestinal-mucosa mRNA activity by up to 33%. A similar phenomenon has been observed when wheat-germ tRNA at concentrations greater than 200 μ g/ml was used for translation of bovine parathyroid mRNA in a linked transcription/translation system (Kronenberg *et al.*, 1977). The proportion of Ca-binding protein translated in the system, based on the immunoprecipitation of the L-[4,5-³H]leucine-labelled translated products, was 4–10% of the total protein synthesized, but only about 2–5% for L-[³⁵S]methionine-labelled proteins. Analysis on Bio-Gel P10 of immunoprecipitates of the translation products with L-[4,5-³H]leucine in the assay revealed that only the translation mixture to which immune serum (against pure Ca-binding protein) was added showed a peak of radioactivity corresponding to the elution position of authentic Ca-binding protein (Fig. 1). No peak in this position was observed when non-immune serum was added to the translation mixture, nor when immune serum was used in the presence of excess purified Ca-binding protein. Similarly, no peak of radioactivity was observed in this position when antiserum to Ca-binding protein was added to endogenous wheat-germ translation products or to the products of translation of guinea-pig mammary-gland mRNA. When translations were performed by using L-[³⁵S]methionine and similar immunoprecipitation experiments carried out, analysis on Bio-Gel P10 showed a single peak of radioactivity that was eluted at a position corres-

ponding to cytochrome *c* (mol.wt. 12 500) (Fig. 2) when antiserum against pure Ca-binding protein was used, but again no peaks were observed when

Fig. 1. Gel filtration of L-[4,5-³H]leucine-labelled immunoprecipitated translation products

Translation reaction mixtures were immunoprecipitated as described in the Methods section and chromatographed on a column (29 cm × 2 cm) of Bio-Gel P10 (200–400 mesh) equilibrated with 150 mM-ammonium acetate, pH 7.4; 1 ml fractions were collected. Effluent fractions were analysed by liquid-scintillation counting. ●, Antiserum raised in rabbits against pure Ca-binding protein (CaBP); ■, non-immune rabbit serum; ○, antiserum to Ca-binding protein and excess unlabelled authentic Ca-binding protein. Abbreviations: V_0 , void volume; V_s , elution position of NaCl.

Fig. 2. Gel filtration of L-[4,5-³H]leucine- and L-[³⁵S]-methionine-containing translation products immunoprecipitated with antiserum to pure Ca-binding protein

Translations were performed with either L-[4,5-³H]leucine or L-[³⁵S]methionine. The reaction mixtures were immunoprecipitated as described in the Methods section, then chromatographed on a column (29 cm × 1 cm) of Bio-Gel P10 (200–400 mesh) equilibrated with 150 mM-ammonium acetate, pH 7.4; 1 ml fractions were collected. Effluent fractions were analysed by liquid-scintillation counting. ○, [³⁵S]Methionine-containing products; ●, [³H]leucine-containing products. Abbreviations are as in Fig. 1.

control immunoprecipitation experiments were performed.

Analysis of immunoprecipitates by electrophoresis on SDS/20% polyacrylamide gels showed that when L-[³⁵S]methionine had been incorporated into the specifically immunoprecipitated protein, only one band of radioactivity was visible on fluorography. This band corresponded to a mol.wt. of 12 500. However, when L-[4,5-³H]leucine had been incorporated into the specifically immunoprecipitated protein, the single peak of radioactivity observed on Bio-Gel P10 was now resolved into two bands on fluorography; one co-migrated with the single band observed when L-[³⁵S]methionine had been used in the translation reaction, but the other

Fig. 3. Densitometric scan after SDS/polyacrylamide-gel electrophoresis of immunoprecipitates

Immunoprecipitated translation products were analysed by SDS/10–20%-gradient-polyacrylamide-gel electrophoresis. The gel was fluorographed (Laskey & Mills, 1975) and exposed to Kodak X-omat RP film. The radioautograph was developed and the immunoprecipitated protein bands were scanned with a Vitatron TLD 100 densitometer. (a) L-[³⁵S]Methionine-containing immunoprecipitated translation product; (b) L-[4,5-³H]leucine-containing immunoprecipitated translation product. Absorption is given in arbitrary units. The migration position of authentic Ca-binding protein was determined by co-electrophoresis of 15 μg of pure Ca-binding protein on the same gel followed by staining with Coomassie Blue before fluorography.

co-migrated with authentic Ca-binding protein. These bands were absent when non-immune rabbit serum was added to the translation reaction containing either L-[35 S]methionine- or L-[4,5- 3 H]leucine-labelled proteins. Addition of unlabelled authentic Ca-binding protein to the immunoprecipitation assay also abolished both bands of radioactivity (Fig. 3).

Electrophoresis of the heat-treated L-[35 S]methionine-labelled translated products on SDS/20% polyacrylamide gels and subsequent fluorography revealed several bands (Fig. 4), one of which co-migrated with cytochrome *c*, but none corresponded to the authentic Ca-binding protein. The heat-stable L-[4,5- 3 H]leucine-containing proteins on SDS-polyacrylamide-gel electrophoresis showed a clear band corresponding to authentic Ca-binding protein which is heat-stable (Hitchman & Harrison, 1972). These bands co-migrated with

Fig. 4. SDS/polyacrylamide-gel electrophoresis of supernatant obtained after heat treatment of translation products

Heat treatment of translation reaction mixtures was performed as described in the Methods section. Control (unheated) reaction mixtures and supernatants obtained after final centrifugation of heated reactions were analysed by SDS/20%-polyacrylamide-gel electrophoresis. The gel was fluorographed (Laskey & Mills, 1975), then exposed to Kodak X-omat RP film. The radioautograph was then developed. Lanes: (1) L-[35 S]methionine-containing control (unheated) translation products; (2) L-[35 S]methionine-containing heated translation products; (3) L-[4,5- 3 H]leucine-containing control (unheated) translation products; (4) L-[4,5- 3 H]leucine-containing heated translation products. (a) High-molecular-weight product stable on heating that co-migrated with cytochrome *c*. (b) Low-molecular-weight product stable on heating that co-migrated with authentic Ca-binding protein. Cytochrome *c* (15 μ g) and authentic Ca-binding protein (15 μ g) were electrophoresed on the same gel and detected by staining with Coomassie Blue.

those observed when heat-stable L-[4,5- 3 H]leucine-containing products were specifically immunoprecipitated as described above. When lactating guinea-pig mammary-gland translation products were heated and electrophoresed, none of the milk-protein bands was present.

Ion-exchange chromatography on DEAE-Sephadex of heat-treated L-[4,5- 3 H]leucine-containing proteins (Fig. 5) showed that when 1 mM-EDTA was present in the elution buffer the major peak of radioactivity was eluted only at high salt concentration (approx. 0.3 M-NaCl). After desalting on Bio-Gel P10, material from this peak of radioactivity was then eluted from a column of DEAE-Sephadex with 1 mM-CaCl₂ in the elution buffer. On this occasion the major peak of radioactivity was eluted at low salt concentration (0.02 M-NaCl) in the void volume of the column (Fig. 6). Approx. 40% of the total translated proteins were found to be heat-stable. About half of this was recovered from the major peak from the DEAE-Sephadex column at high salt concentration when the elution buffer contained EDTA. When material from this peak was re-chromatographed on DEAE-Sephadex with CaCl₂ in the buffer, 40% of the major peak from the first ion-exchange-chromatography step with EDTA in the elution buffer shifted its elution position to the void volume of the column. It can be calculated therefore that at least 8% of the total translated

Fig. 5. Ion-exchange chromatography of heat-stable L-[4,5- 3 H]leucine-containing translation products in the presence of 1 mM-EDTA

Translation reaction mixtures were heated to 70°C for 15 min. After centrifugation at 49 000 *g* for 30 min the supernatant was chromatographed on a column of Bio-Gel P10 as described in the Methods section. The peak of radioactivity eluted at the position of Ca-binding protein was pooled, freeze-dried and dissolved in 2 ml of 20 mM-imidazole/HCl (pH 7.0)/20 mM-NaCl/1 mM-EDTA. This was then applied to a column (30 cm $\times$ 2.5 cm) of DEAE-Sephadex A25 previously equilibrated with the same buffer. Fractions (2 ml) were eluted with a gradient of NaCl (----) and analysed by liquid-scintillation counting.

Fig. 6. Ion-exchange chromatography of heat-stable L-[4,5-³H]leucine-containing translation products in the presence of 1 mM-CaCl₂.

The fractions containing the major peak of radioactivity from the ion-exchange chromatography described in the legend to Fig. 5 were freeze-dried and chromatographed on Bio-Gel P10 as described in the Methods section. The peak of radioactivity eluted at the position of authentic Ca-binding protein on Bio-Gel P10 was pooled, freeze-dried then dissolved in 2 ml of 20 mM-imidazole/HCl (pH 7.0)/20 mM-NaCl/1 mM-CaCl₂. This was then applied to a column (30 cm × 1.5 cm) of DEAE-Sephadex A25 previously equilibrated with the same buffer. Fractions (2 ml) were eluted with a gradient of NaCl (----) and analysed by liquid-scintillation counting.

protein was Ca-binding protein; this does not take into account the losses sustained at each chromatography step.

Discussion

We have shown that active mRNA can be extracted from normal pig duodenal mucosa. This mRNA stimulates protein synthesis in a wheat-germ cell-free system by 8–10-fold over the endogenous wheat-germ protein synthesis without added mRNA. Approx. 4–10% of the proteins synthesized in the wheat-germ system by this mRNA appear to be Ca-binding protein as determined by immunoprecipitation, heat-stability and ion-exchange chromatography.

Since Ca-binding protein contains no methionine residues and the initiator methionine can be cleaved off after translation of the mRNA in some cell-free systems (Housman *et al.*, 1970), it was expected that there might be no specific immunoprecipitate obtained by addition of antiserum against pure Ca-binding protein to the L-[³⁵S]methionine-labelled translation product, derived from the wheat-germ system. No authentic Ca-binding protein was detectable on gel chromatography or SDS/polyacrylamide-gel electrophoresis when methionine was used in the translation, but chromatography on Bio-Gel P10 of this L-[³⁵S]methionine-labelled immunopre-

cipitate revealed a peak of radioactivity that was eluted earlier than authentic Ca-binding protein. This protein also migrated more slowly than authentic Ca-binding protein on SDS/polyacrylamide-gel electrophoresis, and in addition co-migrated with a protein which was larger than its companion Ca-binding protein when L-[4,5-³H]leucine-labelled translation products were immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis. Moreover, this higher-molecular-weight methionine-containing protein was also heat-stable, and preliminary studies (results not shown) of these heat-stable methionine-containing proteins show that they too like authentic calcium-binding protein, have a component that shifts its elution position depending on whether Ca²⁺ is present in or absent from the elution buffer on ion-exchange chromatography.

It is possible that the antiserum used in these studies was raised against impure Ca-binding protein, or two related proteins with the same antigenic domain were synthesized in the cell-free system. It is also possible that the larger protein is a precursor of Ca-binding protein that is partially processed in the wheat-germ translation system.

It seems unlikely that the antiserum was raised against impure Ca-binding protein, since the material against which the antiserum was raised was purified by the method of Hitchman *et al.* (1973), which yields a protein that is a single band on SDS/polyacrylamide-gel electrophoresis. It cannot be excluded that these two proteins are related and possess similar antigenic domains. Examples of such related peptides are gastrointestinal hormones, which are probably the products of duplication and evolution of a common ancestral gene (Dockray, 1978).

Ca-binding protein from pig intestinal mucosa might be synthesized as a high-molecular-weight precursor; however, it now seems clear from the work of Spencer *et al.* (1978) that chick Ca-binding protein is the primary translation product of the mRNA coding for that protein. It has also been suggested that the rat intestinal Ca-binding protein may be synthesized as a precursor, which is cleaved to give the active Ca-binding protein, and that this processing activity is probably influenced by vitamin D metabolites (Drescher & DeLuca, 1971).

It is generally thought that the wheat-germ system is devoid of proteolytic enzymes, and therefore processing of precursor proteins does not occur in this system, but it has been reported that cleavage of prelactogen to lactogen in the wheat-germ system is prevented by the presence of 0.04% Triton X-100 (Peeters *et al.*, 1979). We ourselves did not find any change in the pattern of immunoprecipitated bands on SDS/polyacrylamide-gel electrophoresis when the intestinal-mucosal mRNA was translated in the presence of 0.04% Triton X-100. However, it is

possible that mRNA for proteolytic enzymes is extracted from the intestinal cells together with other cellular mRNA, and the activity of these enzymes is not inhibited by this concentration of Triton X-100. Whatever the nature of the higher-molecular-weight material, we believe that the mRNA from pig duodenal mucosa directs the synthesis of authentic Ca-binding protein, because a protein synthesized in the system is specifically immunoprecipitated by antiserum raised against pure pig Ca-binding protein and the immunoprecipitated protein is co-eluted with pure Ca-binding protein on gel chromatography. Furthermore, the immunoprecipitated protein co-migrates with authentic Ca-binding protein on SDS/polyacrylamide-gel electrophoresis. In addition, a protein that is heat-stable (like authentic Ca-binding protein) co-migrates with the immunoprecipitated protein on gel electrophoresis, and this heat-stable protein is precipitated by the addition of antiserum to pure Ca-binding protein. Moreover, a significant proportion of the heat-stable proteins synthesized in the translation system, like authentic calcium-binding protein, is negatively charged in the absence of Ca^{2+} and neutral in its presence. It therefore shares several properties with authentic Ca-binding protein.

mRNA from pig intestinal mucosa may be a suitable starting material for synthesis of complementary DNA. Isotopically labelled complementary DNA coding for Ca-binding protein could be used as a probe to monitor alterations in the amounts of mRNA for Ca-binding protein caused by dietary restriction or administration of various vitamin D sterols. In addition, the complementary DNA could also be used for cloning and nucleotide-sequence analysis.

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