Stimulation of Intestinal Calcium-Binding-Protein mRNA Synthesis in the Nucleus of Vitamin D-Deficient Chicks by 1,25-Dihydroxycholecalciferol

By R. SPENCER, M. CHARMAN and D. E. M. LAWSON
*Dunn Nutritional Laboratory, University of Cambridge and
Medical Research Council, Cambridge CB4 1XJ, U.K.*

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Stimulation of intestinal calcium transport by the hormone 1,25-dihydroxycholecalciferol appears to involve RNA transcriptions and the synthesis of new proteins. Although one of these proteins has been identified as calcium-binding protein, no RNA molecules specifically induced by the hormone in the nucleus have been identified. Nuclear RNA from intestine of vitamin D-deficient chicks before and at various time intervals after treatment with the hormone or cholecalciferol was tested for its ability to code for calcium-binding protein in a cell-free system. Calcium-binding-protein mRNA could only just be detected in the intestinal nuclei 2 h after dosing with these steroids which is the same time that it was first observed in the polyribosomes. Thus 1,25-dihydroxycholecalciferol induces the production of new calcium-binding protein by stimulating the formation and rapid release from the nucleus of new mRNA molecules for this protein. Polyribosomal translation of the mRNA continued only as long as it was being synthesized, and the maximum rate of synthesis following a pulse dose of 125 ng of the hormone was the same as that observed after prolonged stimulation with cholecalciferol. The possibility that other 1,25-dihydroxycholecalciferol-dependent events may be occurring in the nucleus in the lag period between accumulation of the hormone in the intestine and the appearance of active calcium-binding-protein mRNA, and that these may ultimately control the synthesis of that mRNA, is discussed.

The intestinal nucleus is a major site of action of the hormonal metabolite of vitamin D₃, 1,25-dihydroxycholecalciferol, as was shown by dosing rachitic animals with the hormone and observing subsequent changes occurring in the intestine. It was found that after the rapid accumulation of the hormone in chick intestinal nuclei (Lawson & Emtage, 1974) nuclear RNA synthesis is stimulated (Tsai & Norman, 1973), as is RNA polymerase activity (Zerwekh *et al.*, 1974) and DNA template capacity (Zerwekh *et al.*, 1976), and the physiological response is inhibited *in vitro* by α -amanitin and actinomycin D (Corradino, 1973). Thus RNA transcription and protein synthesis were implicated in the mechanism of action of the hormone.

One of the earliest responses of vitamin D-deficient chicks to the action of 1,25-dihydroxycholecalciferol is the synthesis in intestinal cells of calcium-binding protein as a consequence of the appearance of active mRNA for the protein on polyribosomes (Spencer *et al.*, 1976a). In such chicks calcium-binding protein is first detectable as a nascent polypeptide chain on intestinal polyribosomes 2 h after dosing with 1,25-dihydroxycholecalciferol. Since this is in agreement with the time by which the most hormone has accumulated in the nucleus (Lawson & Emtage, 1974)

and with the reported increase in RNA polymerase II activity in the nucleus (Zerwekh *et al.*, 1974), it has been assumed by many workers that the transcription and translational events referred to above were those required for the synthesis of calcium-binding protein. Thus it is thought that by 2 h *in vivo* calcium-binding-protein mRNA has been transcribed, release from the nucleus, and its translation into protein begun. However, there is so far no direct evidence for the nuclear origin of calcium-binding-protein mRNA, nor that its transcription from the calcium-binding-protein gene in dosed animals is due to direct stimulation of the gene by the hormone.

In this paper we report the first evidence that in rachitic chicks dosed with 1,25-dihydroxycholecalciferol calcium-binding-protein mRNA is synthesized in the nuclei of intestinal cells, and some properties of this nuclear RNA are described. Calcium-binding-protein mRNA activity in the nucleus at various time intervals after dosing with the hormone is correlated with the accumulation of the mRNA in polyribosomes, and the significance of these events with respect to the mode of action of 1,25-dihydroxycholecalciferol in the intestine is discussed.

Materials and Methods

Chemicals

L-[4,5-³H]Leucine (57 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). 1,25-Dihydroxycholecalciferol was obtained from Dr. N. Pollitt, Roche Products, Welwyn Garden City, Herts., U.K., and cholecalciferol from Sigma (London) Chemical Co., Kingston upon Thames, U.K. The source of other biochemicals was given by Emtage *et al.* (1974).

Animals

Chicks (1 day old) (Rhode Island Red × Light Sussex) obtained from the National Institute for Research in Dairying (Shinfield, Reading, U.K.) were fed on a cholecalciferol-deficient diet (Lawson *et al.*, 1969). Vitamin D-dosed chicks received 12.5 µg of cholecalciferol (500 units) or 125 ng of 1,25-dihydroxycholecalciferol intracardially in 0.1 ml of ethanol/propylene glycol (1:9, v/v), before being killed at the times stated. All chicks were starved for 18 h before death.

Adult piebald rats were also starved for 18 h before death.

Preparation of chick intestinal nuclear RNA

Nuclei were prepared from chick ileal mucosa by the method described by Cox *et al.* (1974), in order to minimize contamination with ribosomes on the outer nuclear membrane (Knowler *et al.*, 1973), and RNA was isolated from the pellet by a hot phenol/sodium dodecyl sulphate extraction (Ruiz-Carrillo *et al.*, 1973). The aqueous phase was adjusted to 0.3 M-NaCl and RNA precipitated by the addition of 2 vol. of ethanol at -20°C for a minimum of 3 h. The precipitate was collected by centrifugation (38 000 g_{av.}; 15 min), washed with ethanol and then redissolved in 10 mM-disodium EDTA/10 mM-Tris/HCl (pH 7.4)/0.2% sodium dodecyl sulphate. The RNA was reprecipitated by the addition of 2 vol. of ethanol at -20°C, washed with ethanol and finally dissolved in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.6, before storage at -20°C. This method yielded 0.4–0.6 mg of nuclear RNA/g of mucosa initially homogenized.

It was important for this study to demonstrate that the calcium-binding-protein mRNA designated 'nuclear' did originate from the nucleus and not from contaminating cytoplasmic RNA. In an attempt to obtain a maximum estimate of the extent to which nuclear RNA could be contaminated by cytoplasmic RNA, mucosa from vitamin D-deficient birds (with no calcium-binding-protein mRNA) was homogenized in the post-nuclear supernatant obtained from vitamin D-dosed birds (containing calcium-binding-protein mRNA), and the contamination of nuclei from the deficient birds with trapped cytoplasmic calcium-binding-protein mRNA from the

dosed birds was determined by extracting and translating the nuclear RNA in the wheat-germ cell-free system. It was found that a maximum of 10% of the nuclear calcium-binding protein in RNA depicted in Fig. 3 could have arisen from cytoplasmic contamination.

Preparation of chick intestinal polyribosomes and polyribosomal-free cytoplasmic RNA

Chick ileal mucosa was homogenized in rat liver cell sap and polyribosomes were prepared as described before (Spencer *et al.*, 1976a).

In the experiments where polyribosomal-free cytoplasmic RNA was required, the supernatant from the discontinuous sucrose gradient was decanted (leaving behind the polyribosome pellet) and then adjusted to 10 mM-EDTA/50 mM-sodium acetate/0.5% sodium dodecyl sulphate (pH 5.2). RNA was extracted by the hot phenol/sodium dodecyl sulphate method (Ruiz-Carrillo *et al.*, 1973) and precipitated exactly as described above. The final RNA pellet was dissolved in 20 mM-Hepes, pH 7.6, and stored at -20°C. As a precaution against ribonuclease, all glassware and aqueous solutions used in both the RNA preparations were treated with 0.1% (v/v) diethyl pyrocarbonate (Palmiter, 1974).

Polypeptide synthesis *in vitro*, mRNA assay in the wheat-germ system, immunoprecipitation and analysis of the products obtained by the use of anti-serum against calcium-binding protein were carried out as described previously (Spencer *et al.*, 1976a).

Results

Behaviour of nuclear RNA in the wheat-germ cell-free protein-synthesizing system

As shown in Fig. 1, with typical preparations of nuclear RNA from chick intestine, maximum incorporation of [4,5-³H]leucine by the wheat-germ system into acid-precipitable products (6–12 times endogenous rate) was obtained at RNA concentrations of 4–6 µg/50 µl assay; 5 µg/50 µl assay was therefore routinely used in subsequent experiments, and this amount gave the same stimulation of protein synthesis in various wheat-germ extracts as 20 µg of polyribosomal RNA/5 µl assay.

The nuclear RNA coded for a range of polypeptides of molecular weights up to larger than 50 000, as shown in Fig. 2(a), where the wheat-germ system was primed with intestinal nuclear RNA obtained from vitamin D-dosed chicks and the ³H-labelled products were analysed by polyacrylamide-gel electrophoresis. After centrifuging of the incubation mixture to pellet the ribosomes, the supernatant was treated with antiserum to calcium-binding protein, and a single component, of identical mobility with that of marker calcium-binding protein, was obtained (Fig. 2b). This component was absent from the

Fig. 1. Stimulation of [^3H]leucine incorporation into acid-precipitable products by chick intestinal nuclear RNA in the wheat-germ system

Samples were incubated for 90 min at 25°C. Duplicate 20 μl samples were assayed. ●, ○, RNA extracted from two different nuclear preparations.

products of the wheat-germ system primed with nuclear RNA obtained from deficient chicks (Fig. 2c).

Time course of the appearance and activity of mRNA coding for calcium-binding protein in intestinal nuclei of rachitic chicks dosed with 1,25-dihydroxycholecalciferol

Nuclear RNA was extracted from the ileal mucosa of rachitic chicks at various time intervals after dosing intracardially with 125 ng of 1,25-dihydroxycholecalciferol. The nuclear RNA extracts were translated in the wheat-germ system and the ^3H -labelled products were treated with antiserum to calcium-binding protein. The dissociated immunoprecipitates were analysed by sodium dodecylsulphate/polyacrylamide-gel electrophoresis. Meanwhile polyribosomes were obtained from the duodena of the same birds and their ability to synthesize calcium-binding protein in a cell-free system *in vitro* was tested.

The changes in calcium-binding-protein mRNA activity in the nuclei with time were very closely related to the changes in polyribosomal synthesis of this protein (Fig. 3). Calcium-binding-protein mRNA was not detectable in either nuclei or polyribosomes until 2 h after dosing and at this stage the activity was very low at both sites: calcium-binding protein translated from nuclear RNA was 0.1% of total

Fig. 2. Gel electrophoresis of [^3H]polypeptides synthesized by the wheat-germ system primed with nuclear RNA

(a) Range of ^3H -labelled polypeptides translated from nuclear RNA obtained from chicks dosed with 12.5 μg of cholecalciferol 48 h before being killed (b) Proteins translated by the cell-free system from RNA as in (a) after precipitation with antiserum to calcium-binding protein. (c) Protein translated by the cell-free system from nuclear RNA obtained from vitamin D-deficient chicks, after precipitation with antiserum to calcium-binding protein. C, calcium-binding protein; H and L, heavy and light immunoglobulin chains respectively.

protein synthesis in the wheat-germ system, and 0.015% of total protein synthesis by polyribosomes. Calcium-binding-protein mRNA activity was not apparent in the nucleus at any time when it was not also detectable on polyribosomes, and the activity in both systems reached a maximum 12 h after dosing, declining thereafter. By 24 h virtually no calcium-binding-protein mRNA activity was detectable in nuclei, whereas a low activity remained in the polyribosomes.

Thus it appeared that, after being formed in the nucleus, the calcium-binding-protein mRNA was

released rapidly and immediately incorporated into polyribosomes for translation. During periods of active calcium-binding-protein production, the proportion of cytoplasmic calcium-binding protein mRNA not present in polyribosomes was found to be only 5% of the total extranuclear mRNA for this protein (Table 1).

Fig. 3. Time course of the appearance of calcium-binding-protein mRNA activity in nuclei and polyribosomes from chicks dosed with 125 ng of 1,25-dihydroxycholecalciferol

Groups of four chicks were dosed intracardially with the hormone and killed at the indicated times. Polyribosomes prepared from the pooled duodenal mucosae and RNA extracted from nuclei prepared from ileal mucosae of the same birds were translated *in vitro* and in the wheat-germ system respectively. ^3H radioactivity in immunoprecipitable calcium-binding protein at each time interval is expressed as a percentage of total acid-precipitable radioactivity. Calcium-binding-protein mRNA activity: $\circ$, polyribosomal; $\bullet$, nuclear.

Production of nuclear mRNA for calcium-binding protein after treatment with cholecalciferol

Rachitic birds were dosed intracardially with 12.5 μg of cholecalciferol and were killed either 48 h or 72 h later. Nuclei were prepared from the ileal mucosa and the RNA was extracted. Polyribosomes were prepared from the duodena of the same birds. Nuclear RNA was translated in the wheat-germ system and polyribosomes *in vitro*, and calcium-binding protein production was determined as a percentage of total acid-precipitable protein synthesis. The nuclear calcium-binding-protein mRNA activity at both time intervals was similar to that observed 12 h after 1,25-dihydroxycholecalciferol, whereas the polyribosome activity was far higher (Table 2). This result provided further evidence that the calcium-binding-protein mRNA activity observed in the nuclei obtained from dosed birds was not due to contamination of the nuclei by polyribosomes. Furthermore the observation that the mRNA activity in the nucleus is the same after both the hormone and the vitamin must mean that there is a maximum pool size of this RNA and that this concentration of the hormone in chicks (125 ng/200 g chick) is sufficient to achieve a maximum response.

Discussion

Our previous investigations into the events underlying the production of intestinal calcium-binding protein in response to 1,25-dihydroxycholecalciferol (Spencer *et al.*, 1976a,b, 1978) suggested the possibility that the hormone-induced synthesis of the protein could occur solely by cytoplasmic events resulting in the activation of pre-existing mRNA. The transcriptional event referred to in the introduc-

Table 1. Cellular distribution of cytoplasmic mRNA for calcium-binding protein

The pooled ileal mucosae from four rachitic chicks dosed with 125 ng of 1,25-dihydroxycholecalciferol intracardially 24 h before being killed were used to prepare polyribosomes in the usual way. RNA was extracted from the final polyribosome-free supernatant and then translated in the wheat-germ system at an optimum concentration of 10 $\mu\text{g}/100 \mu\text{l}$ assay. The radioactivity in the immunoprecipitated calcium-binding protein obtained from ten pooled assays and from polyribosomal protein synthesis *in vitro* was determined after electrophoresis of the dissociated precipitates, and the distribution of calcium-binding-protein mRNA between polyribosomes and polyribosome-free cytoplasm calculated.

Source of RNA	Yield of RNA (mg/g of mucosa)	RNA translated <i>in vitro</i> (μg)	^3H radioactivity of total acid-precipitable proteins (d.p.m.)	^3H radioactivity of calcium-binding protein (d.p.m.)	Calcium-binding-protein synthesis (% of total protein synthesis)	Total yield of calcium-binding protein (d.p.m./g of mucosa)	Percentage of total calcium-binding protein production
Polyribosomes	0.84	500	899 000	5210	0.58	8760	94.9
Polyribosome-free supernatant	0.89	100	275 000	50	0.018	445	5.1

Table 2. Comparison of nuclear calcium-binding-protein mRNA activity in birds dosed with cholecalciferol or with 1,25-dihydroxycholecalciferol

Nuclei were prepared from the ileal mucosae of birds dosed intracardially with either 125 ng of 1,25-dihydroxycholecalciferol or 12.5 µg of cholecalciferol at the indicated times before being killed, and the RNA was extracted. Polyribosomes were prepared from the duodenal mucosae of the same birds. Nuclear RNA was translated in the wheat-germ system and polyribosomes *in vitro*. Calcium-binding protein production was determined as a percentage of total acid-precipitable protein synthesis in both systems.

Dose and time of killing	Calcium-binding protein synthesis (% of total protein synthesis)		Nuclear activity (% of polyribosomal)
	Nuclear	Polyribosomal	
1,25-Dihydroxycholecalciferol (12h)	0.49	1.75	28.0
Cholecalciferol (48h)	0.36	4.5	8.0
Cholecalciferol (72h)	0.42	8.0	5.2

tion might therefore not reflect the synthesis of calcium-binding-protein mRNA.

We investigated the presence of functional calcium-binding-protein mRNA in intestinal nuclei of chicken before and at various time intervals after dosing with 1,25-dihydroxycholecalciferol or the vitamin. Our approach involved the translation of nuclear RNA into protein with the wheat-germ cell-free system. Although it is now clear that mRNA originates in the eukaryotic nucleus (Molloy & Puckett, 1976) there are very few reports in the literature of the successful translation of nuclear RNA into protein *in vitro* (Ruiz-Carrillo *et al.*, 1973; Knochel & Teidemann, 1975), and some workers have found that nuclear RNA inhibits protein synthesis *in vitro* (Cox *et al.*, 1974). We found that nuclear RNA stimulated protein synthesis in the wheat-germ system at concentrations up to 5 µg/50 µl assay, and ascertained that the observed messenger activity was not due to contamination of the nuclear RNA with cytoplasmic RNA.

From our findings several conclusions may be drawn concerning the nuclear and cytoplasmic events leading to the production of calcium-binding protein in response to stimulation by 1,25-dihydroxycholecalciferol. Firstly, calcium-binding-protein mRNA activity was only detected in the nuclei of hormone-stimulated chicks. Vitamin D-deficient animals therefore do not contain functional calcium-binding-protein mRNA in the intestinal nucleus or cytoplasm, and after dosing with the hormone the functional mRNA is produced *de novo* in the nucleus. From this result it is clear that the role of the hormone is not merely to regulate the release of mRNA from the nucleus; also the possibility that synthesis of the protein is stimulated by hormone-induced cytoplasmic events only is eliminated. 1,25-Dihydroxycholecalciferol, either directly or indirectly, is implicated in the regulation of transcriptional or maturation events within the nucleus, resulting in the formation of active calcium-binding-protein mRNA.

Secondly, there does not appear to be any lag period between the appearance of functional calcium-binding-protein mRNA in the nucleus and its translation on polyribosomes. Minute amounts of the mRNA were first detectable in chick intestinal nuclei 2 h after dosing with the hormone. This was the same time that nascent calcium-binding protein was first detectable on polyribosomes, so an initial accumulation of the mRNA in the nucleus does not appear to be a prerequisite for its release into the cytoplasm.

Subsequent changes with time in nuclear calcium-binding-protein mRNA activity were closely related to the changes in polyribosomal synthesis of the protein (Fig. 3), but between 2 and 10 h after dosing nuclear mRNA accumulated more rapidly than it could be released and taken up by polyribosomes. Between 12 and 18 h after the pulse dose of hormone both nuclear and polyribosomal activities decreased rapidly to low values.

From the results, some points concerning the regulation of calcium-binding-protein mRNA transcription and translation emerge. During periods when calcium-binding protein synthesis was occurring at a significant rate, only 5% of the extranuclear calcium-binding-protein mRNA molecules were not being translated by polyribosomes, so the decrease in calcium-binding-protein mRNA synthesis and translation observed at these later time intervals suggests that both phenomena are dependent on the continued presence of the hormone in the mucosa. The activity of the mRNA in the cytoplasm must be regulated either by a very short half-life of the RNA (3-4 h) in the absence of inducing hormone, or by fine translational control of the RNA exerted by another 1,25-dihydroxycholecalciferol-dependent factor.

In birds dosed with cholecalciferol for 48 or 72 h the nuclear mRNA activity was the same as that observed after a pulse dose of the hormone (Table 2). Thus the pool size of mature calcium-binding-protein mRNA in the nucleus observed 10-12 h after 125 ng

of the hormone is the largest attainable and at this time transcription of the gene is occurring at its maximum rate. Despite this, after doses of cholecalciferol intestinal polyribosomes synthesizing calcium-binding protein continue to accumulate for at least 72 h, so that in the continuing presence of the hormone the active half-life of this mRNA is very much longer.

In the chick intestine, calcium-binding-protein mRNA is only just detectable in the nucleus at 2 h after hormone administration, and requires a further 8 h to reach a maximum (Fig. 3). Yet the amount of hormone accumulated in the nucleus (Lawson & Emtage, 1974), together with the increased RNA polymerase II activity (Zerwekh *et al.*, 1974), are maximal within 2 h. In other systems, newly transcribed mRNA is processed very rapidly, with a half-life of minutes, into mature mRNA (Curtis & Weissmann, 1976; Bastos & Aviv, 1977). The delay observed in the chick intestine between maximum accumulation of the hormone and maximum calcium-binding-protein mRNA synthesis implies that additional events occurring in the nucleus affect the formation of the mRNA. If the transcription of the calcium-binding-protein gene is directly controlled by 1,25-dihydroxycholecalciferol, then maturation of the newly transcribed RNA into functional mRNA must be regulated by a second hormone-controlled factor. Alternatively the hormone could have no effect on transcription of calcium-binding-protein mRNA and instead regulate the expression of other genes, the products of which, directly or indirectly, control the formation of functional calcium-binding-protein mRNA from its initial transcript. Resolution of these alternatives, however, requires the preparation of complementary DNA from pure calcium-binding-protein mRNA, which with present technology is difficult because of its low tissue concentration.

In conclusion it is clear that in the chick intestine calcium-binding-protein mRNA originates in the nucleus, and is rapidly released and translated by

polyribosomes as long as mRNA synthesis continues. Release of the mature mRNA from the nucleus does not appear to be regulated by 1,25-dihydroxycholecalciferol, but, in the lag period between dosing chicks and the beginning of synthesis of active calcium-binding-protein mRNA, other hormone-dependent events may be occurring in the nucleus and it may be these which ultimately control the synthesis of calcium-binding-protein mRNA.

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