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**Study on the Action of Vitamin D in the
Egg Shell Gland of the Laying Hen**

A. Bar, M., R.H. Wasserman, C.S. Fullmer

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Title:

STUDY OF THE ACTION OF VITAMIN D IN EGG SHELL GLAND OF THE LAYING HEN

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ABSTRACT

The aims of the present study were to: (a) evaluate the relationship between eggshell gland (ESG) calbindin (calcium-binding protein; Mr 28,000) and eggshell deposition; and (b) elucidate the mechanism of regulation of calbindin mRNA and calbindin protein synthesis in the ESG.

A rapid, simple and quantitative solution-hybridization procedure for total calbindin mRNA employing synthetic oligodeoxynucleotide probes was developed. The assay is especially well-suited to multiple sample analyses. These probes, as well as chicken intestinal calbindin cDNA were used to visualize calbindin mRNA by Northern blot analysis.

The concentrations of calbindin mRNA and calbindin protein in the ESG were lower in hens forming thin shells, as compared with those forming thick shells. In old hens, which produced thinner shells than young ones, a significant correlation was found between ESG calbindin protein and shell thickness. Calbindin mRNA and calbindin protein appeared in the ESG during calcification of the first egg shell in both chickens and quail. Calbindin mRNA fluctuated markedly during the daily egg cycle, in close temporal association with egg shell calcification. In vitamin D-deficient quail, these changes in mRNA occurred without any change in calbindin protein. The formation and degradation rates of calbindin mRNA and calbindin protein were determined. Vitamin D₃ metabolites induced the synthesis of intestinal, but not ESG calbindin mRNA or protein, although 1,25(OH)₂D₃ increased the concentration of its occupied VDR in the ESG. Calbindin mRNA in the ESG was markedly reduced when egg shell calcification was interrupted. A Ca pump was co-localized with calbindin in the tubular gland cells of the ESG.

Administration of estradiol induced hypertrophy of the ESG and increased the concentration of VDR, but its effect on the synthesis of calbindin mRNA was very small. Progesterone decreased the synthesis of calbindin mRNA in laying and in non-laying birds. Parathyroid hormone, testosterone and corticosterone did not affect calbindin mRNA synthesis in the ESG. Dexamethasone decreased calbindin mRNA synthesis in the ESG, increased egg cycle length and increased shell Ca. Positive cotranscriptional regulation of intestinal calbindin gene expression by 1,25(OH)₂D₃ and glucocorticoids was observed in tissue culture.

The results suggest: (a) calbindin, in association with a Ca pump, is most likely involved with shell formation; (b) the major regulatory site of calbindin synthesis is at the transcriptional level; (c) the induction of calbindin mRNA in the ESG does not require vitamin D, although the translation of the mRNA may require 1,25(OH)₂D₃, (d) the appearance of calbindin mRNA in the ESG is associated with the actual calcium transport by the tissue, or with some intermediate such as the gonadal hormones, having the same temporal behavior; (e) the regulatory mechanism for the synthesis of calbindin mRNA in the ESG may involve a stimulator(s) and oscillating depressor(s), both of which appear to be closely linked to the reproductive cycle. Progesterone is most likely a depressor. Estrogens and Ca²⁺ transport may act as stimulators; (f) it is most likely that more than one hormone are involved in the regulation of calbindin

mRNA and, therefore, cotranscriptional regulation may occur in the ESG; (g) with the exception of $1,25\text{-(OH)}_2\text{D}_3$, the same factors act similarly on the intestine and ESG.

OBJECTIVES

In general, our objectives were to establish the relationships among vitamin D, eggshell gland (ESG) calbindin (28KDa), and shell deposition in the laying bird. Specific objectives were:

- (1) to relate shell quality to ESG calbindin;
- (2) to relate ESG calbindin to vitamin D metabolism;
- (3) to determine whether Ca transport by the ESG is facilitated by calbindin or whether calbindin synthesis is induced, or at least initiated, as a consequence of Ca transport (cause and effect);
- (4) to determine whether calbindin-mediated Ca transport by the ESG is facilitated by $1,25\text{(OH)}_2\text{D}_3$, $24,25\text{(OH)}_2\text{D}_3$ or PTH;
- (5) to examine whether the absence of calbindin in the ESG prior to first egg shell calcification is caused by inhibition at the receptor binding steps, the transcription or the translation step (the limiting step);
- (6) to search for possible factors capable of affecting this "limiting step"; and
- (7) to produce basic knowledge in order to enable further studies focusing on the ESG calbindin gene(s), as a possible means for the genetic improvement of egg shell quality.

DESCRIPTION OF COOPERATION

The cooperation experienced between our research groups has been very useful and mutually beneficial. At Cornell, Dr. Fullmer, Prof. Corradino and Prof. Wasserman concentrated mainly on the development of methods (calbindin mRNA and the Ca pump) or in their adaptation to our specific aims in the localization of the Ca pump and in basic studies using the intestine as a model. In Israel, Dr. Bar concentrated on regulatory mechanisms in the ESG and on the relationship between calbindin and shell deposition. During March 1988, Dr. Fullmer visited Dr. Bar's laboratory and consulted on the use of the solution hybridization technique for quantification of calbindin mRNA and the technology transfer was completed at this time, long before submission of the methods manuscript. Dr. Bar and Mrs. Striem visited the Cornell group in September 1989 and October 1991, respectively, and discussed methodological aspects of the study as well as the meaning of the findings and future research directions. All coinvestigators intensively employed the Bitnet system in order to transfer data, ideas and interpretations.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

Our 1st, 2nd, 4th and 5th objectives were entirely accomplished. The results suggest that calbindin is associated with shell formation and may be a major factor in the determination of shell quality (Obj. 1). At least transcription of the calbindin gene is not dependent on vitamin D (Obj. 2). Our data do not support the hypothesis that induction of ESG calbindin mRNA is vitamin D-dependent or influenced by PTH (Obj. 4). Our data suggest that, while regulation occurs also at the translational level, transcription is the limiting step in calbindin synthesis. (Obj. 5). Extensive sequence analysis of calbindin protein or calbindin cDNA was obviated by the successful application of synthetic oligonucleotide probes, based on intestinal calbindin. This approach provided the opportunity to initiate ESG calbindin mRNA measurements as early as March 1988.

Our 3rd, 6th and 7th objectives were not entirely accomplished. Calbindin in the ESG is well-correlated with shell thickness. However, it is not yet clear whether calbindin is the cause (regressor) or the result (dependent variable). Most tested factors affected similarly shell Ca deposition and ESG calbindin. Some factors (dexamethasone, Mifepristone) influenced shell

Ca and calbindin gene expression differently. These results suggest that calbindin gene expression is not a consequence of shell deposition, although Ca transport is a major factor in the regulation of calbindin gene expression in the ESG and intestine (Obj. 3).

Few factors were found to inhibit ESG calbindin synthesis. Only one, namely shell calcification, was found to induce its synthesis. Our findings suggest that positive cotranscriptional regulation (by several endocrine and other factors) is involved in the induction of calbindin gene expression (Obj.6).

Our research revealed basic physiological, kinetic, and endocrine knowledge. The regulatory mechanisms appear quite complicated and cannot be entirely resolved by *in vivo* techniques. However, it is clear from the whole animal studies that calbindin gene expression is differentially regulated in the intestine and the ESG. Intestinal calbindin synthesis and optimal Ca absorption (and, therefore, Ca supply) are absolutely dependent on $1,25(\text{OH})_2\text{D}_3$. Egg shell thickness is related to Ca supply and correlates well with ESG calbindin level. For future studies, it will be critical to adopt model systems which eliminate or minimize the indirect effect (intestinal) of the vitamin D endocrine system on ESG function.

PUBLICATIONS

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11. Wasserman, R.H., Smith, C.A., Smith, C.M., Brindak, M.E., Fullmer, C.S., Krook, L., Penniston, J.T., Kumar, R. (1991) Immunohistochemical localization of a calcium pump and calbindin D28K in the oviduct of the laying hen. *J. Histochem. Cytochem.* 96:413-418.