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Molecular biology and vitamin D function

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The 1980s have seen the successful application of molecular biological techniques to furthering our understanding of calcium homeostasis and bone growth. The present article will review some of the findings which emerged from the use of these techniques to elucidate the role of vitamin D in controlling Ca homeostasis. The studies followed the recognition that 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$D) is the hormonal form of vitamin D responsible for all its activities. As with other steroid hormones, 1,25-$(OH)_2$D interacts with a specific receptor protein in its target tissues and the complex is taken up and retained within the nucleus. The concentrations of the mRNA of an increasing number of proteins have been recognized to be synthesized in a 1,25-$(OH)_2$D-dependent manner in part due to an increase in the transcription of their genes. However, it is only the expression of the calbindin gene in the intestine which is clearly 1,25-$(OH)_2$D-dependent (Emtage *et al.* 1973) even though there are serious doubts as to whether this is the only effect of 1,25-$(OH)_2$D (Spencer *et al.* 1976). The generally accepted view is that 1,25-$(OH)_2$D maintains Ca homeostasis in part by increasing the proportion of absorbed dietary Ca; this role is achieved by the hormone interacting with a specific-tissue receptor-binding protein and this steroid–protein complex interacts with the genome to permit at least calbindin-gene expression.

Using molecular biological techniques the amino acid sequence of both the 1,25-$(OH)_2$D receptor and calbindin were established and the obligatory role of the receptor in the molecular mechanism of 1,25-$(OH)_2$D action shown. Furthermore, evidence has been produced that the full biochemical effect of 1,25-$(OH)_2$D has still to be described as vitamin D is believed to have a physiological function in the egg-shell gland of hens and recent studies have shown that 1,25-$(OH)_2$D does not stimulate calbindin-gene transcription in this tissue.

1,25-$(OH)_2$D RECEPTOR

The classification of the various types of rickets according to aetiology is given in Table 1. Rickets is ultimately a consequence of a lack of 1,25-$(OH)_2$D but this in turn can arise from an inadequate supply of vitamin D and 25-hydroxyvitamin D. As implied by the name, privational rickets is caused by a lack of dietary vitamin D or inadequate exposure to ultraviolet light. However, inadequate amounts of the two precursors of 1,25-$(OH)_2$D can also arise from the increased metabolism of 25-hydroxycholecalciferol (25-OHD) and vitamin D occurring in diseases involving the liver and the gastrointestinal tract (Clements *et al.* 1987). Endocrinological causes of rickets are more directly related to

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Table 1. *Classification of rickets and osteomalacia*

Nutritional and metabolic	Endocrinological
Privational	Renal osteodystrophy
Elderly	
Immigrants to Europe	Other acquired renal disease
	Tumours
Gastrointestinal	Heavy metal poisoning
Small bowel disease	
Partial gastrectomy	Hereditary renal diseases
	Vitamin D resistance
Liver	Vitamin D dependence
Biliary cirrhosis	Acidosis
Alcoholism	Cystinosis
Chronic hepatitis	

1,25-$(OH)_2$D. In cases of acquired renal disease, the renal 25-OHD 1-hydroxylase (*EC* 1.14.13.13) is severely reduced leading to inadequate production of 1,25-$(OH)_2$D. In these conditions 1,25-$(OH)_2$D has been shown in double-blind trials to be useful prophylactively. Rickets can also arise in at least three types of hereditary renal disease. These are the last of the rickets cases to be explained in molecular terms and at present only a start has been made in providing the explanation using molecular biology.

It has long been known that rickets occurs in some individuals which either does not respond to physiological levels of vitamin D or requires continued treatment with very high doses. These cases were classed in a number of ways including vitamin D-resistant rickets or pseudo-vitamin D-deficiency rickets. The commonest form of this type is vitamin D-resistant or hypophosphataemic rickets, transmitted as a dominant X-linked characteristic. Vitamin D metabolism is not really changed in these patients, rather the defect appears to be in a phosphate-transporter gene mainly expressed in the kidney. Currently, a major research effort is under way to isolate this gene using chromosome-walking techniques. A second type of hereditary rickets is known as vitamin D-dependency rickets (type I) which is a rare, autosomal recessive disorder, very similar to privational rickets. Successful treatment can be effected with up to 4 mg vitamin D daily or 1–2 μg 1,25-$(OH)_2$D but the disease reappears if treatment lapses. The condition is, therefore, due to a defect in renal 1,25-$(OH)_2$D synthesis. The type II form of the disease has similar genetic characteristics, but the bone disease is severe, plasma 1,25-$(OH)_2$D levels are high, and 75% of patients have whole-body alopecia. 1,25-$(OH)_2$D has no effect so that the condition appears to result from a target-tissue defect.

During the course of investigating patients with vitamin D-dependency rickets (type II) the target-tissue defect was found to reside in the 1,25-$(OH)_2$D receptor. Studies on the nature of the defect were carried out in vitro on fibroblasts, keratinocytes or cultured bone cells from affected patients and shown to concern the 1,25-$(OH)_2$D receptor (Liberman, 1988). The interaction of the hormone with its receptor was assessed either using whole cells or extracts and the hormone–receptor complex was characterized by its sedimentation velocity through a sucrose gradient or its elution from a DNA–cellulose column. At least five different intracellular defects all related to the 1,25-$(OH)_2$D receptor are presently known.

Class I	Absence of 1,25-$(OH)_2D$ binding to cytosols
Class II	1,25-$(OH)_2D$ binding sites reduced by 90%
Class III	Reduced affinity of binding protein for 1,25-$(OH)_2D$
Class IV	Deficient nuclear uptake of 1,25-$(OH)_2D$
Class V	Abnormal binding of 1,25-$(OH)_2D$–receptor complex to DNA

The 1,25-$(OH)_2D$ receptor has a high affinity for its ligand with the K_d 10^{-10}M; the molecular weight of the avian and mammalian proteins of 60 kDa and 55 kDa (424 amino acids) respectively. 1,25-$(OH)_2D$ binds to its receptor in a hydrophobic pocket located in the C-terminal 30 kDa fragment. The centre of the molecule contains an exposed region that is antigenic and may act as a hinge between the hormone-binding region and the DNA-binding domain located towards the N-terminal end. This last domain is about 8 kDa and contains nine cysteine residues which coordinate two Zn atoms and form 'fingers' to anchor the receptor to the DNA (Haussler *et al.* 1988).

The chromosomal gene for the human vitamin D receptor covers about 44 kb DNA and contains nine exons, with the three protein domains described previously each requiring three exons to code for them (Pike *et al.* 1988). Comparison of the 1,25-$(OH)_2D$ receptor with the other nuclear hormone receptors shows that the DNA-binding regions are about 50% homologous whereas the hormone-binding region is only about 20% homologous. The vitamin D receptor is the smallest of all the proteins and is most similar to the thyroxine receptor (hc erbA).

There are three reports describing the cloning and sequencing of the 1,25-$(OH)_2D$-receptor gene from affected patients. In one report four children from three related families showed the classical syndrome of hereditary vitamin D-resistant rickets although all six parents were phenotypically normal (Ritchie *et al.* 1989). Fibroblasts from the affected children had no detectable binding of 1,25-$(OH)_2D$ and the receptor protein was undetectable by immunoblot analysis of fibroblast extracts (a class I defect, which is the commonest). DNA from these subjects was isolated and amplified by the polymerase chain reaction (PCR) technique.

PCR amplification showed the presence in these patients of the nine exons having the correct size for the 1,25-$(OH)_2D$ gene. DNA sequencing of these exons showed only a single point mutation in exon 7. The effect of this mutation was to convert a C at position 970 to an A in a codon (TAC) normally coding for tyrosine. The resulting TAA codon causes termination of the mRNA elongation process; the foreshortened mRNA produces a 291 amino acid protein lacking much of the 1,25-$(OH)_2D$-binding domain. All the parents tested (four) and four clinically unaffected children from these families were heterozygous for the vitamin D-receptor gene; i.e., they possessed both the normal TAC codon and the mutant TAA form.

A cDNA coding for the mutant 1,25-$(OH)_2D$ receptor was created and inserted into a eukaryotic expression vector. Cells transfected with this vector contained a 32 kDa protein but it was present in substantially smaller amounts than the wild-type receptor found in parallel experiments. The activity of the product of this recombinant vector was tested by cotransfecting cells with a vitamin D-receptor-responsive reporter gene. The latter was the chloramphenicol acetyltransferase (*EC* 2.3.1.28; CAT) gene under the control of the promoter for vitamin D-sensitive osteocalcin, i.e., in the presence of 1,25-$(OH)_2D$ and the 1,25-$(OH)_2D$-receptor the osteocalcin promoter can drive the expression of the CAT gene. The CAT and receptor expressing plasmids were cotransfected into eukaryotic cells (CV-1) which do not normally possess the 1,25-

$(OH)_2D$ receptor. The CAT gene was not expressed when cells were cotransfected with the mutant 1,25-$(OH)_2D$-receptor cDNA but it was expressed in a 1,25-$(OH)_2D$-dependent manner when cotransfected with normal cDNA showing that the mutation produced a functionally inactive protein.

Other families with hereditary resistant rickets in some children have been identified and the nature of the defect causing the rickets established. In these cases the vitamin D receptor was of normal size, abundance and affinity for 1,25-$(OH)_2D$ but it was unable to bind to the DNA (a class V defect). Similar techniques to those described previously were used to identify the site of the mutation in the receptor gene of these families and to show that the product of this mutation was the cause of the defect. Three sites for the missense point mutations producing single amino acid changes were identified in three families. All three mutations were in the DNA-binding domain (Hughes *et al.* 1988; Sone *et al.* 1990).

Conclusions. A number of conclusions and observations of general interest can be drawn from these studies which to the nutritionist may appear esoteric.

1. Cultured fibroblast cells may be a useful model with which to investigate 1,25-$(OH)_2D$ function as fully differentiated enterocytes still cannot be maintained in culture.
2. These patients provided evidence for a 1,25-$(OH)_2D$ action in the so-called non-classical target tissues. For example, in the affected individuals 1,25-$(OH)_2D$ did not stimulate the renal 25-hydroxyvitamin D-24-hydroxylase activity, osteocalcin production and cell proliferation. The extensive alopecia in the most severe cases suggest that 1,25-$(OH)_2D$ is necessary for the development of the hair follicle.
3. Two independent reports suggest that long-term infusions of Ca caused a healing of the rickets as judged by X-rays and bone histomorphometry suggesting that 1,25-$(OH)_2D$ does not affect bone mineralization directly (Balsan *et al.* 1986; Weisman *et al.* 1987).

THE CALBINDIN GENE AND ITS EXPRESSION

Molecular biology has also had a role to play in furthering our understanding of events in the target tissues controlled by 1,25-$(OH)_2D$. The major biochemical response of the intestine to vitamin D is an increase in calbindin biosynthesis. This protein occurs in two forms, a larger (molecular weight 30 500) more widely distributed form and a smaller (molecular weight 9000) form present in mammalian intestine, placenta and kidney. It seems that only mammalian kidney has both forms of this protein. Although the two proteins have a different amino acid sequence, obtained from analysis of their cDNA (Wilson *et al.* 1985) in almost all other respects they are very similar. Both calbindins are members of the calmodulin-gene superfamily and as with calmodulin they consist of a number of Ca-binding domains linked together by short peptide sequences. The structure of each domain consists of an α-helix–loop–α-helix with the loop having a characteristic sequence and being rich in amino acids with oxygen containing side-chains. The larger calbindin has six of these domains although it binds only four atoms of Ca. The two inactive domains are probably domain 2 in which glycine replaces aspartate and domain 6 the leading part of which may have been replaced with a foreign piece of DNA.

The gene for the larger calbindin is about 20 kb with the protein being coded for by eleven exons (Wilson *et al.* 1988). The structure of the calbindin gene provided further insight into the evolution of the members of the calmodulin superfamily. Proteins have

evolved with two, three, four or six Ca-binding sites but their only common ancestral protein is one with two Ca-binding domains. That is calmodulin with four domains (or sites) is not an ancestor of calbindin and other six-site proteins; instead separate gene duplication events of the two-site ancestor gave rise to calmodulin-type proteins and to the four-site ancestor of calbindin (Parmentier *et al.* 1989). A further gene-duplication event was required to convert the calbindin four-site ancestor to calbindin. The present-day position of the ten introns in the calbindin gene strongly suggests that the introns have been randomly inserted after the evolution of these genes (Wilson *et al.* 1988).

The function of calbindin has not been elucidated. The protein was first identified because of its vitamin D dependency and its importance was indicated by the finding that the highest concentration was in those tissues transporting large amounts of Ca, such as intestine, kidney and the egg-shell gland. The following observations correlated calbindin with intestinal Ca transport: (a) both are vitamin D dependent in the intestine, (b) both respond in a similar manner to low dietary Ca levels, (c) the order of affinity of divalent cations for calbindin is the same as the order of their rate of intestinal absorption. These ions are absorbed by the same route as Ca.

The mechanism of Ca transfer across the intestine and egg-shell gland of birds is generally assumed to be very similar. The evidence includes high 1,25-$(OH)_2$D production and raised plasma 1,25-$(OH)_2$D levels during egg production, the presence in both tissues of 1,25-$(OH)_2$D receptors and that Ca absorption and intestinal and egg-shell-gland calbindin also increase during egg formation. Although it is clear that intestinal calbindin is 1,25-$(OH)_2$D dependent, the position in the egg-shell gland is problematical as egg-laying birds on a vitamin D-deficient diet stop laying and any arrest of egg production causes a marked decline in egg-shell-gland calbindin concentration.

In a series of collaborative studies with Dr Y. Nys (France), and Dr A. Bar (Israel) the effect of 1,25-$(OH)_2$D and of egg laying on the concentration of calbindin and its mRNA was examined. 1,25-$(OH)_2$D increased the calbindin concentration in the intestine of vitamin D-deficient immature chicks but the steroid had no effect on egg-shell-gland calbindin in similar birds given oestradiol to stimulate the growth of this tissue (Bar *et al.* 1990; Nys *et al.* 1991). The egg-shell glands of these immature birds were shown to contain 1,25-$(OH)_2$D receptors (Bar *et al.* 1990). In normal laying birds, increased plasma 1,25-$(OH)_2$D concentrations are associated with an increase in intestinal calbindin concentration but egg-shell-gland calbindin is unaffected. Similar relationships were observed with the measurement of calbindin mRNA strongly suggesting that 1,25-$(OH)_2$D only has an effect on calbindin gene transcription in the intestine and not in the egg-shell gland (Bar *et al.* 1990; Nys *et al.* 1991).

It was also of interest that in normal laying birds the onset of egg production caused an increase in the concentration of calbindin and its mRNA in both the intestine and the egg-shell gland (Nys *et al.* 1988, 1991). This effect of the egg-production process was additional to any effect of 1,25-$(OH)_2$D and as the calbindin mRNA concentration was also increased it implies the effect is transcriptional in nature. The increases began during the onset of the formation of the first egg following a period in which no eggs were laid (Bar *et al.* 1990). Furthermore, the increases in the concentrations of calbindin and its mRNA were not observed if shell deposition was prevented. It is possible, therefore, that the process of Ca transfer across the egg-shell gland is associated in some way with an increase in the transcription of the calbindin gene in this tissue. But, whatever the

nature of the stimulus in the egg-shell gland, a similar stimulus is observed in the intestine. It is of course possible that there is an endocrinological stimulus to calbindin mRNA formation in both tissues linked to ovulation but an alternative explanation would involve the increased flux of Ca across the egg-shell gland requiring of necessity an increased intestinal flux and that calbindin gene expression is Ca sensitive.

The most recent studies of the calbindin gene have been concerned with the identification of the nucleotide sequence in the calbindin promoter sensitive to 1,25-$(OH)_2$D. Despite many attempts in several laboratories such a sequence has not been recognized. The approach has been to ligate sections of the calbindin promoter to the gene for CAT and to transfer this fragment into 1,25-$(OH)_2$D-sensitive cells. Although a number of cell types have been successfully transfected, in no case was the CAT gene regulated by 1,25-$(OH)_2$D. The problem may be the tissue specificity of the 1,25-$(OH)_2$D regulation of calbindin-gene expression. As mentioned earlier the vitamin D sensitivity of calbindin is clearly observed in the intestine and kidney only. Vitamin D-sensitive intestinal cells have not been cultured and the cultured kidney cells available may not contain calbindin. However, it is of interest that the vitamin D-responsive nucleotides in the osteocalcin gene recently identified (Kerner *et al.* 1989; Demay *et al.* 1990; Markose *et al.* 1990) appear not to be present in the calbindin promoter. Of course, this observation may be understood in that there may not be a high degree of homology between the vitamin D-responsive sequences in the promoters of the calbindin and osteocalcin genes. These sequences (or elements) are often only six bases in length, sometimes separated into two groups of three bases by one or more nucleotides which can make their recognition difficult. It seems that the vitamin D–calbindin system in the intestine, which for 20 years since its discovery has been such an apparently fruitful area of research to elucidate the molecular mechanism of vitamin D action, is no longer the one of choice for attempts to fully understand the function of this hormone.

Conclusions. 1. 1,25-$(OH)_2$D stimulation of calbindin formation occurring in intestine and kidney has still to be shown to be a direct effect of the hormone.

2. In tissues such as intestine and egg-shell gland which transport large amounts of Ca 1,25-$(OH)_2$D has other biochemical effects.

3. Calbindin-gene expression is affected by factors other than 1,25-$(OH)_2$D.

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