

# Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism

Kyu-Ichiro Okuda,<sup>1,\*</sup> Emiko Usui,<sup>†</sup> and Yoshihiko Ohyama<sup>\*\*</sup>

Department of Surgery I,\* Miyazaki Medical College, Kiyotake, Miyazaki; Department of Biochemistry,<sup>†</sup> Hiroshima University School of Dentistry, Hiroshima 734; and Graduate Department of Gene Science,<sup>\*\*</sup> Faculty of Science, Hiroshima University, Higashi-Hiroshima, Japan

- I. Vitamin D<sub>3</sub> 25-Hydroxylase (25-Hydroxylase)
  - A. Subcellular localization of 25-hydroxylase
  - B. 25-Hydroxylase in liver microsomes
  - C. Purification of 25-hydroxylase from liver mitochondria
  - D. Identity of 25-hydroxylase with cholestanetriol 27-hydroxylase
  - E. Cloning of 25-hydroxylase cDNA in liver mitochondria
  - F. Expression of a cDNA encoding rat liver mitochondrial vitamin D<sub>3</sub> 25-hydroxylase
  - G. Abnormal vitamin D<sub>3</sub> metabolism in cerebrotendinous xanthomatosis
- II. 1 $\alpha$ -Hydroxylation of 25-Hydroxyvitamin D<sub>3</sub> (1 $\alpha$ -hydroxylase)
- III. 24R-Hydroxylation of 25-Hydroxyvitamin D<sub>3</sub> (24-Hydroxylase)
  - A. Identification of 24R, 25-dihydroxyvitamin D<sub>3</sub>
  - B. Purification of 24-hydroxylase
  - C. Cloning of 24-hydroxylase cDNA
  - D. Induction of 24-hydroxylase by vitamin D<sub>3</sub> metabolite
  - E. Subcellular location of 24-hydroxylase and the induction-responsive site
  - F. Further oxidation of the 24-hydroxylated vitamin D<sub>3</sub> derivatives
  - G. Gene structure of 24-hydroxylase and 1,25-dihydroxyvitamin D<sub>3</sub>-responsive elements
- IV. Conclusion

The early studies of Huldschinsky (1), Mellanby (2), McCollum et al. (3), Steenbock and Black (4), Askew et al. (5), and others indicated that an antirachitic agent capable of curing rickets was present in cod-liver oil and in irradiated foods. The culmination of these investiga-

tions was the isolation and identification of vitamin D<sub>3</sub> as the antirachitic factor followed by elucidation of its biosynthesis from cholesterol (6, 7).

In the ensuing three decades, relatively little additional work on vitamin D metabolism was done. Nonetheless, during this time there were great advances in methodology applicable to the study of vitamin D metabolism, which set the stage for a new era of vitamin D research. The advances included reverse-phase chromatography for vitamin D, development of a method for the biosynthesis of <sup>14</sup>C-labeled vitamin D, and competitive binding assays for quantification of vitamin D (7, 8).

In 1968, Blunt, DeLuca, and Schnoes (9) reported the isolation of 25-hydroxyvitamin D<sub>3</sub> (25-OH-D<sub>3</sub>). They also reported that this metabolite of vitamin D<sub>3</sub> had a slightly greater biological activity than its precursor, vitamin D<sub>3</sub>. Until this time, all known metabolites of vitamin D<sub>3</sub> isolated had been reported to be inactive (10). Lawson, Wilson, and Kodicek (11) studied the metabolism of 25-OH-D<sub>3</sub> using a doubly labeled compound with a tritium label at carbon-1 and <sup>14</sup>C at carbon-4. They observed that the tritium label, but not the carbon label, was lost in one of the metabolites of the compound found in intestinal nuclear fractions of chicks, a subcellular fraction known to retain the highest radioactivity

Abbreviations, trivial names, and systemic names (in brackets): vitamin D<sub>3</sub> [calcidiol]; 25-OH-D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub> [calcidiol]; 1 $\alpha$ -OH-D<sub>3</sub>, 1 $\alpha$ -hydroxyvitamin D<sub>3</sub>; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [calcitriol]; 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24R,25-dihydroxyvitamin D<sub>3</sub>; 1,24,25(OH)<sub>3</sub>D<sub>3</sub>, 1 $\alpha$ ,24R,25-trihydroxyvitamin D<sub>3</sub> [calcitriol]; 24-oxo-25-OH-D<sub>3</sub>, 24-oxo-25-hydroxyvitamin D<sub>3</sub>; 24-oxo-23,25(OH)<sub>2</sub>D<sub>3</sub>, 23S,25-dihydroxy-24-oxo-vitamin D<sub>3</sub>; 24-oxo-1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxy-24-oxo-vitamin D<sub>3</sub>; 24-oxo-1,23,25(OH)<sub>3</sub>D<sub>3</sub>, 1 $\alpha$ ,23S,25-trihydroxy-24-oxo-vitamin D<sub>3</sub>; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction.

<sup>†</sup>Formerly from the Department of Biochemistry, Hiroshima University School of Dentistry, Name was previously spelled as Kyuichiro Okuda.

of the labeled vitamin D<sub>3</sub> administered. Kodicek, Lawson, and Wilson (12) then examined the effect of this metabolite on <sup>45</sup>Ca transfer across the intestine. They showed that this compound was not only 2.9-times more active than vitamin D<sub>3</sub> but was also 2.5-times more active than 25-OH-D<sub>3</sub>. The polarity of the compound and the loss of hydrogen from carbon-1 suggested that an oxygen-containing function had been introduced at the 1-position. The eventual identification of the metabolite as 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] was independently accomplished by Lawson, Fraser, and Kodicek (13), Holick, Schnoes, and DeLuca (14), and Norman et al. (15). The α configuration of the 1-hydroxyl group was established by Semmler et al. (16), giving the definitive structure of 1α,25-dihydroxyvitamin D<sub>3</sub>. As no metabolite of vitamin D<sub>3</sub> that is more active than 1,25(OH)<sub>2</sub>D<sub>3</sub> has so far been isolated, it is considered to be an ultimate form of vitamin D<sub>3</sub>. Meanwhile, another metabolite of vitamin D<sub>3</sub>, 24,25(OH)<sub>2</sub>D<sub>3</sub>, was isolated by DeLuca's group (17, 18). Subsequent study showed that this vitamin was formed in animals in a calcium-replete status, while 1,25(OH)<sub>2</sub>D<sub>3</sub> was formed in animals in a calcium-depleted status (19). Therefore, both compounds were considered to be involved in maintenance of calcium homeostasis.

The metabolic pathway of vitamin D<sub>3</sub> is shown in Fig. 1. Enzymes responsible for hydroxylation in this pathway are members of the superfamily of P450 (20, 21). In this article, recent progress in enzymology and molecular biology of these hydroxylases is reviewed.

## I. VITAMIN D<sub>3</sub> 25-HYDROXYLASE (25-HYDROXYLASE)

### A. Subcellular localization of 25-hydroxylase

Horsting and DeLuca (22) showed that vitamin D<sub>3</sub> is hydroxylated at C-25 by rat liver homogenate. Later, DeLuca (23) reported that this 25-hydroxylation was catalyzed by liver mitochondria. Two years later, however, Bhattacharyya and DeLuca (24, 25) reported that vitamin D<sub>3</sub> 25-hydroxylase was present in microsomes and that its activity was dependent on the concentration of ionized calcium in serum. Björkhem and Holmberg (26, 27) reinvestigated the subcellular location of the enzyme using mass fragmentography for identification of metabolites. They found that NADPH-dependent 25-hydroxylation activity was present in both mitochondria and microsomes. In mitochondria NADPH could be replaced by isocitrate. Isocitrate is known to be an electron donor in mitochondria, as it can provide NADPH through oxidation by isocitrate dehydrogenase (21, 28). Madhok and DeLuca (29) reinvestigated the subcellular location of vitamin D<sub>3</sub> 25-hydroxylase. In accordance with their previous work (24, 25), they reported that the enzyme activity was present in both microsomes and mitochondria, when fortified with NADPH and cytosol. However, they attributed the mitochondrial activity to microsomal contamination, as they could not observe succinate-supported mitochondrial activity.

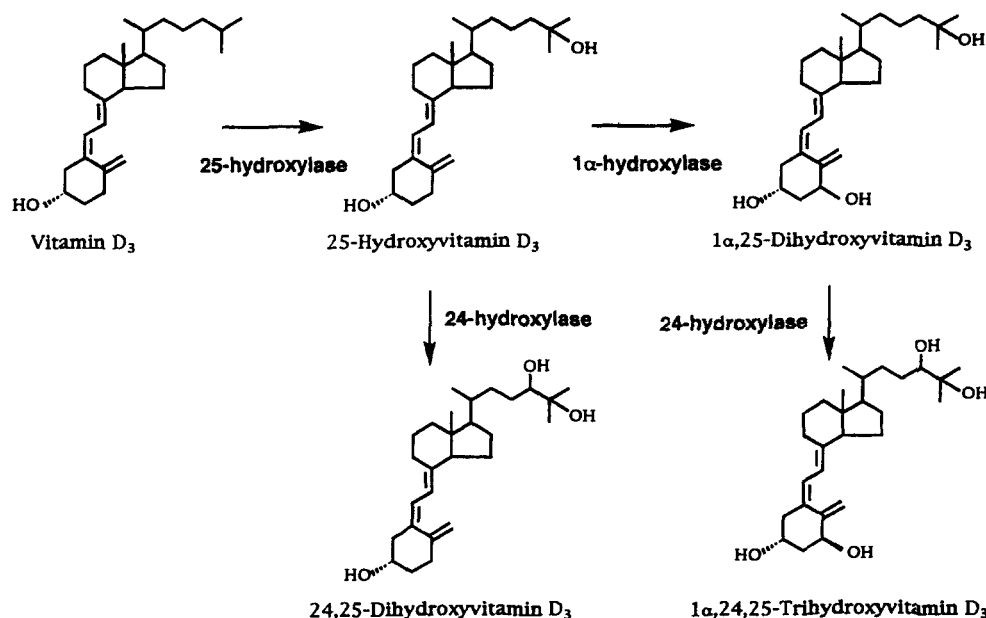


Fig. 1. Metabolic pathway of vitamin D.

## B. 25-Hydroxylase in liver microsomes

Confirmation that microsomal vitamin D<sub>3</sub> 25-hydroxylase was indeed a P450 enzyme was obtained by Andersson, Holmberg, and Wikvall (30). They isolated an electrophoretically homogeneous protein possessing vitamin D<sub>3</sub> 25-hydroxylation activity from rat liver microsomes and showed that the enzyme had spectral characteristics of a P450 enzyme. However, as they did not describe the amino acid sequence of the enzyme, it could not be ascertained whether the enzyme was new or was identical to other P450 enzymes already reported. Hayashi, Noshiro, and Okuda (31, 32) independently purified the microsomal P450 from rat liver that catalyzes vitamin D<sub>3</sub> 25-hydroxylation. They found that the enzyme had the same NH<sub>2</sub>-terminal amino acid sequence as CYP2C11 (33). This P450 is a constitutive P450 known to exist solely in male liver microsomes but not in the female. In fact, female liver microsomes showed a very low activity toward vitamin D<sub>3</sub> 25-hydroxylation catalyzed by a P450 different from CYP2C11 (about 1/5th that of male liver microsomes, 34).

## C. Purification of 25-hydroxylase from liver mitochondria

Previously, Taniguchi, Hoshita, and Okuda (28) and Okuda (21) had established that liver mitochondria contain a P450 catalyzing 27-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol (27-hydroxylase). This finding was in conflict with previous claims as Sottocassa et al. (35) and Brunner and Bygrave (36) had reported that P450 was not observable spectrophotometrically in liver mitochondria. Björkhem and Holmberg (27) thought it quite possible that vitamin D<sub>3</sub> 25-hydroxylase was a different entity than cholestanetriol 27-hydroxylase based on some criteria (21). In order to solve these problems unequivocally, Masumoto, Ohyama, and Okuda (37) began to purify the mitochondrial vitamin D<sub>3</sub> 25-hydroxylase based on enzyme activity. The enzyme was extremely labile compared to other P450s and these workers found it useful to use a rapid HPLC technique that enabled them to complete the anion exchange column chromatography within 1 h. They isolated a homogeneous preparation with a turnover number of 0.36 min<sup>-1</sup> toward vitamin D<sub>3</sub> (and 1.6 min<sup>-1</sup> toward 1 $\alpha$ -OH-D<sub>3</sub>) which were the highest values reported to date.

## D. Identity of 25-hydroxylase with cholestanetriol 27-hydroxylase

In parallel with the work of Masumoto et al. (37), Okuda, Masumoto, and Ohyama (38) purified a mitochondrial hydroxylase that catalyzed 27-hydroxylation of cholestanetriol. The turnover number of their preparation for vitamin D<sub>3</sub> 25-hydroxylation was found to be

the same as that of the enzyme purified by Masumoto et al. (37).

Ohyama et al. (39) performed additional experiments to test whether these two hydroxylation activities were due to a single enzyme. They concluded that both enzyme activities were due to a common active site of a single protein based on the following facts. 1) The two activities copurified using various column chromatographies. 2) 25-Hydroxylase was competitively inhibited by cholestanetriol. 3) Conversely, 27-hydroxylase was inhibited by 25-OH-D<sub>3</sub> in the same manner. 4) Both enzyme activities were inactivated by controlled heat-denaturation, or chemical modification of enzyme protein. Independently, Dahlbäck and Wikvall (40) also reported that vitamin D<sub>3</sub> 25-hydroxylation activity was present in their preparation of cholestanetriol 27-hydroxylase. However, they raised the possibility that their results were due to contamination. Indeed, Dahlbäck (41) prepared a monoclonal antibody to cholestanetriol 27-hydroxylase, which inhibited the 27-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol but not the 25-hydroxylation of vitamin D<sub>3</sub>, and she postulated that the two enzymes were, in fact, different entities.

## E. Cloning of 25-hydroxylase cDNA in liver mitochondria

Usui, Noshiro, and Okuda (42) prepared antibodies against rat liver mitochondrial vitamin D<sub>3</sub> 25-hydroxylase that had been isolated according to the method of Masumoto et al. (37). Using this antibody as a probe, they isolated a cDNA clone from a rat liver cDNA library. The isolated cDNA showed no similarity to any P450s known except to the cholestanetriol 27-hydroxylase isolated by Andersson et al. (43) from a rabbit liver cDNA library, to which the Usui cDNA had 73% homology. It contained a mitochondria-specific presequence consisting of 32 amino acid residues. The mature enzyme consisted of 501 amino acid residues corresponding to a predicted molecular weight of 57,182.

## F. Expression of a cDNA encoding rat liver mitochondrial vitamin D<sub>3</sub> 25-hydroxylase

To confirm whether a single enzyme is responsible for the two catalytic activities, vitamin D<sub>3</sub> 25-hydroxylation and cholestanetriol 27-hydroxylation, Usui et al. (44) prepared an expression plasmid encoding the rat liver mitochondrial vitamin D<sub>3</sub> 25-hydroxylase. This plasmid was transfected into COS cells and the mitochondrial fraction of the transfected cells was assayed for both hydroxylation activities. Usui et al. (44) observed both 25-hydroxylation of vitamin D<sub>3</sub> and 27-hydroxylation of cholestanetriol in this fraction from transfected cells but not in that from control cells. The ratio of both activities

was roughly equal to that observed with the purified enzyme from rat liver mitochondria.

Akiyoshi-Shibata et al. (45) expressed the cDNA encoding 25-hydroxylase under the control of the yeast alcohol dehydrogenase I promoter and terminator in *Saccharomyces cerevisiae* cells. Mitochondria prepared from the transfected cells exhibited both 25-hydroxylase and 27-hydroxylase activities in a reconstituted system containing bovine adrenodoxin and NADPH-adrenodoxin reductase. They thus ruled out the formal possibility that the mammalian cell line might contain an endogenous cholestanetriol 27-hydroxylation activity (although only very low levels of this activity are found in most cultured cells). Su et al. (46) independently isolated a cDNA encoding a rat ovary mitochondrial P450 catalyzing both cholesterol 27-hydroxylation and vitamin D<sub>3</sub> 25-hydroxylation.

Recently, Guo et al. (47) isolated a cDNA for the human liver mitochondrial vitamin D<sub>3</sub> 25-hydroxylase using PCR products of a fraction of rat liver 25-hydroxylase cDNA as a hybridization probe. The structure of the isolated human cDNA was virtually identical to that of cDNA of human cholestanetriol 27-hydroxylase cDNA (48). When the cDNA was transfected into COS cells with an adrenodoxin cDNA, the transfected cells revealed a 10- to 20-fold higher 25-hydroxylation activity than nontransfected cells toward vitamin D<sub>3</sub> and 1 $\alpha$ -OH-D<sub>3</sub>. The transfected cells also showed a much higher 27-hydroxylation activity than 25-hydroxylation activity as in the case of rat liver mitochondrial enzyme. Vitamin D<sub>2</sub>, which carries an ergosterol-like side chain, and dihydrotachysterol<sub>3</sub>, a stereo-isomer of vitamin D<sub>3</sub>, were hydroxylated at C-24 and 25, respectively, suggesting that the regioselectivity of the enzyme differs according to the structure of substrates (Fig. 2). Taken together with the finding of Cali and Russell (48), who observed that this cDNA expressed cholestanetriol 27-hydroxylation activity in COS cells, the evidence appears convincing that 25-hydroxylase and 27-hydroxylase are expressed by the same gene (*CYP27*) in humans as well. However, these experiments do not exclude the possibility that the two enzymes differ by a posttranscriptional modification as suggested by Wikvall (49). Very recently, Axén et al. (50) reported that CYP27 of pig and human catalyzes not only 27-hydroxylation of cholestanetriol and 25-hydroxylation of vitamin D<sub>3</sub>, but also 1 $\alpha$ -hydroxylation of 25-hydroxyvitamin D<sub>3</sub> (Fig. 2). As 1 $\alpha$ -hydroxylation activity toward 25-OH-D<sub>3</sub> is much lower compared to the other two activities and liver does not seem to be involved in 1 $\alpha$ -hydroxylation of 25-OH-D<sub>3</sub> at least in mammals, the physiological meaning of this activity remains to be established.<sup>2</sup>

<sup>2</sup>Kobayashi's group observed that the major activity of 1 $\alpha$ -hydroxylase exists in liver in fish (86) and in fetal rat (87). Whether 1 $\alpha$ -hydroxylation activity and that of 25-hydroxylation are due to a common enzyme in these animal organs remains unsolved.

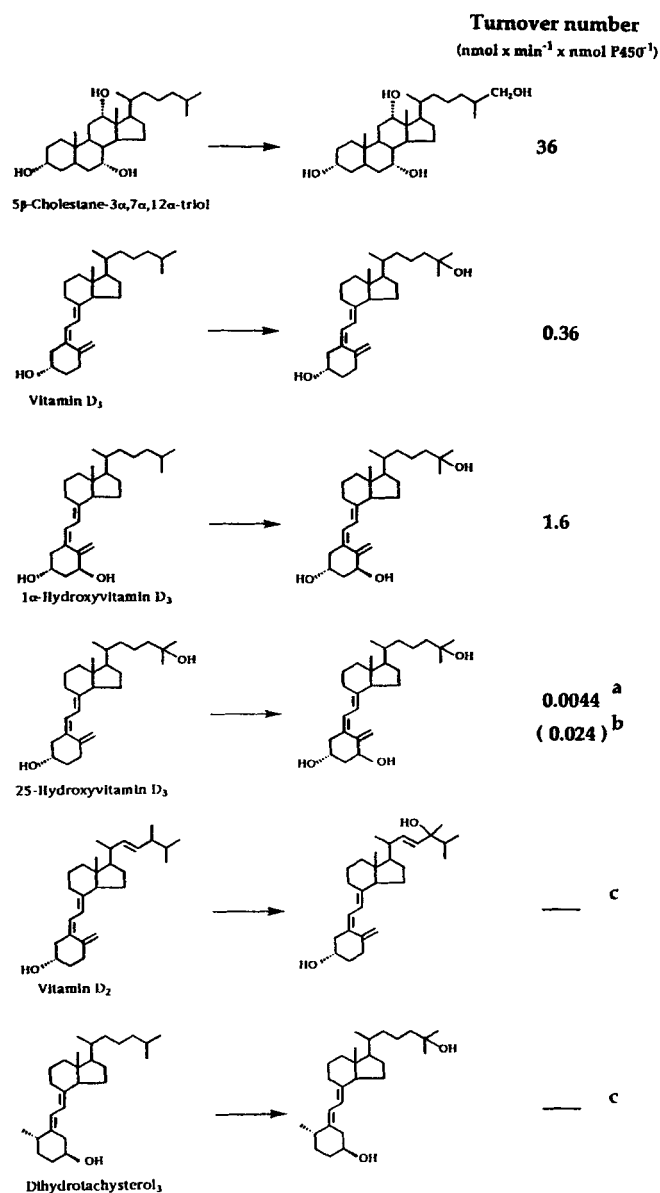


Fig. 2. Regioselectivity of CYP27 for various substrates. <sup>a</sup>CYP27 purified from pig liver mitochondria (50); <sup>b</sup>CYP27 purified from the COS cells transfected with human *CYP27* cDNA (50); <sup>c</sup>COS cells transfected with human *CYP27* cDNA (47).

### G. Abnormal vitamin D<sub>3</sub> metabolism in cerebrotendinous xanthomatosis (CTX)

Cerebrotendinous xanthomatosis is caused by an anomaly of the gene structure of 27-hydroxylase (21, 51, 52). Berginer et al. (53) found that extensive osteoporosis and increased risk of bone fractures occurred in some CTX patients. The serum level of some vitamin D<sub>3</sub> metabolites such as 25-OH-D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> was lower than normal. In particular, the serum level of 24,25(OH)<sub>2</sub>D<sub>3</sub> was more than 2-fold lower than the

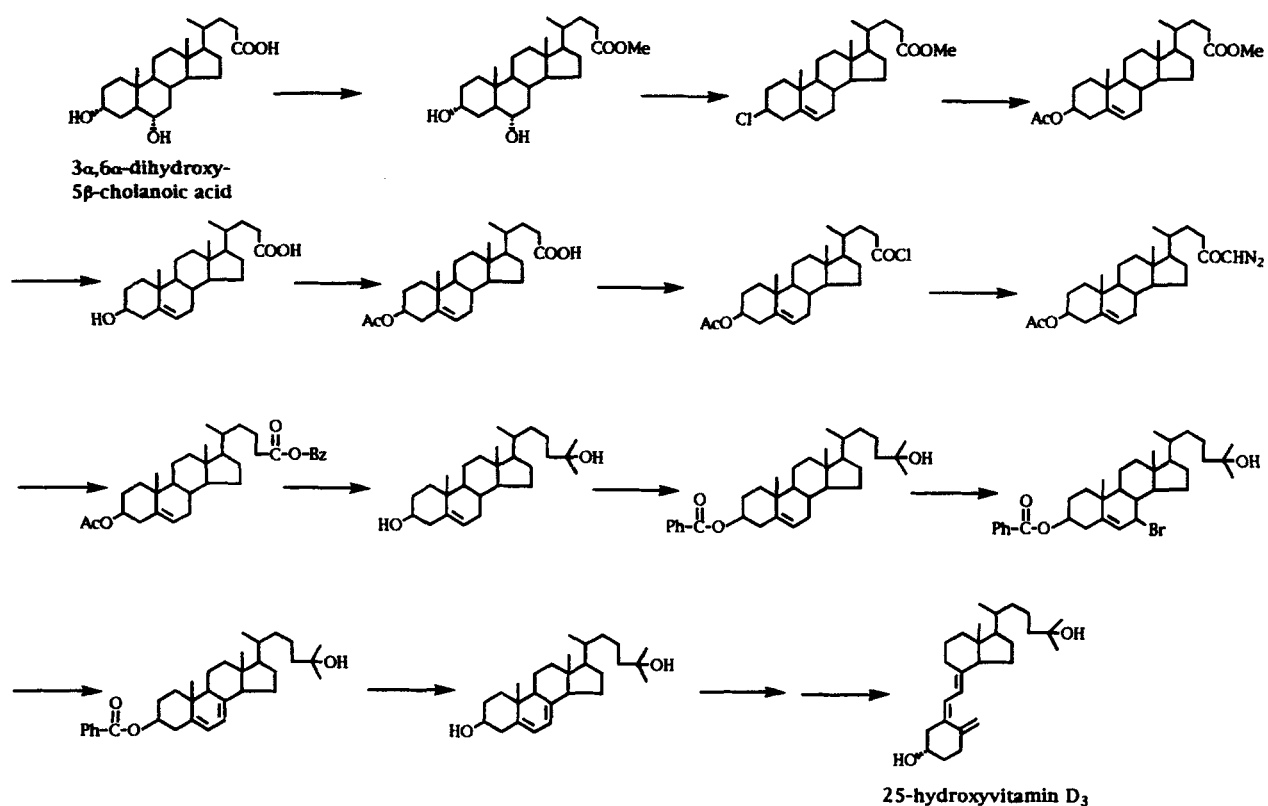


Fig. 3. Chemical synthesis of 25-hydroxyvitamin D<sub>3</sub> [Y. Ohyama and K.I. Okuda, unpublished].

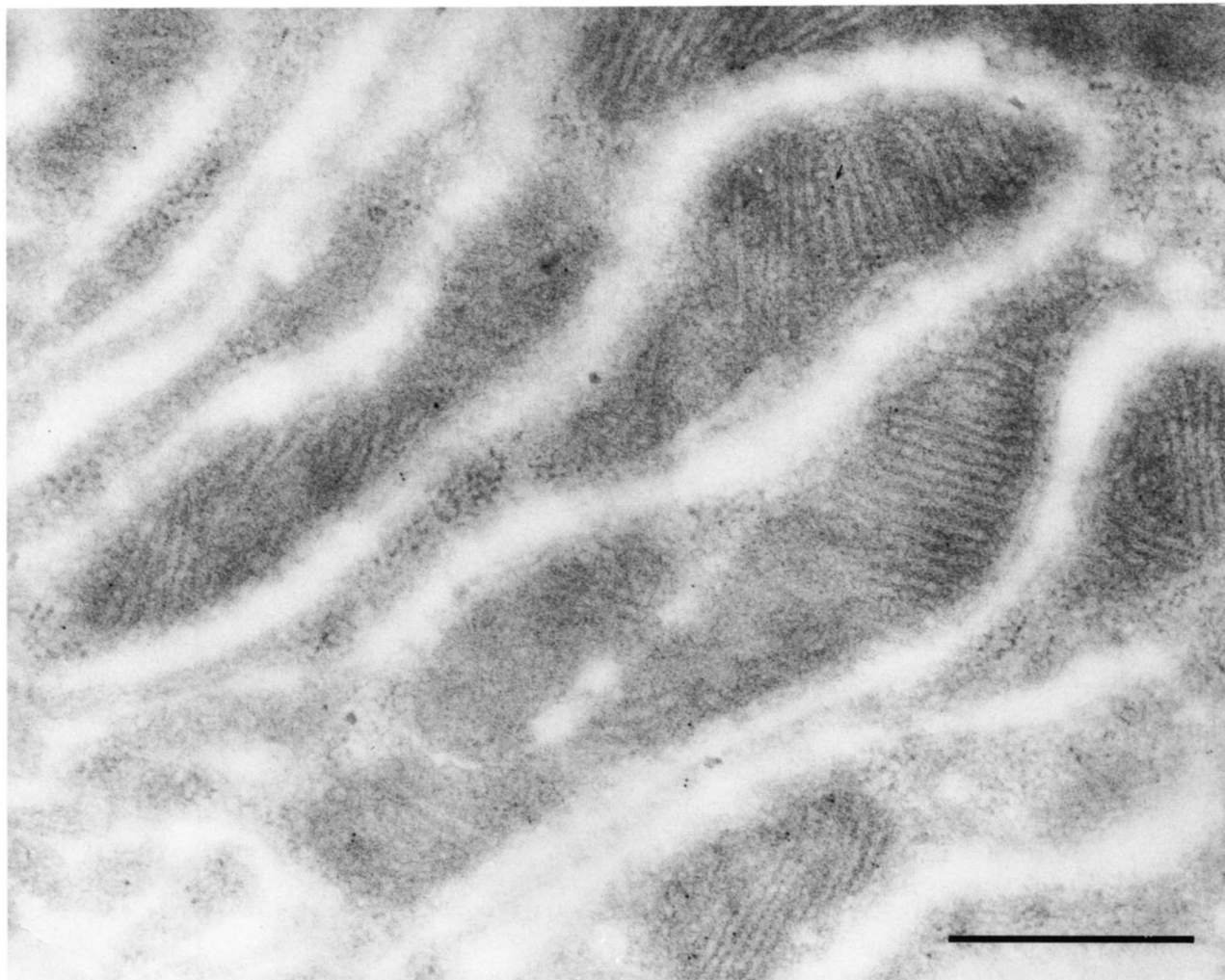
normal, while that of 1,25(OH)<sub>2</sub>D<sub>3</sub> was within normal limits. Affected subjects showed classic manifestations of CTX, including dementia, pyramidal and cerebellar insufficiency, peripheral neuropathy, cataracts, and tendon xanthoma, in association with elevated serum cholestanol concentrations. They therefore considered that extensive osteoporosis and increased risk of bone fractures were components of the inherited disease. It may be surmised that if 25-hydroxylase and cholestanetriol 27-hydroxylase are the same enzyme, the osteoporosis may be explained by low or null activity of liver mitochondrial vitamin D<sub>3</sub> 25-hydroxylation owing to the abnormal enzyme formed from the defective gene.

## II. 1 $\alpha$ -HYDROXYLATION OF 25-HYDROXYVITAMIN D<sub>3</sub> (1 $\alpha$ -HYDROXYLASE)

The major location of 1 $\alpha$ -hydroxylase is in the inner mitochondrial membrane of proximal convoluted cells of the kidney (54). Feeding a low calcium diet or a vitamin D-deficient diet to rats markedly stimulates 1 $\alpha$ -hydroxylase (55).

A monoclonal antibody against this enzyme was prepared by Moorthy, Mandel, and Ghazarian (56) and

Burgos-Trinidad et al. (57) from chick kidney. Moorthy et al. (56) immuno-purified both 1 $\alpha$ -hydroxylase and 24-hydroxylase, and determined the NH<sub>2</sub>-terminal amino acid sequence. The first 10 amino acid residues of the two enzymes were 100% homologous and amino acid compositions of the two enzymes were very similar. They thus suggested that 24-hydroxylase may be a proteolytic cleavage product of 1 $\alpha$ -hydroxylase. However, Burgos-Trinidad et al. (57) who also immuno-purified the two enzymes noticed that NH<sub>2</sub>-terminal amino acid sequences of 24-hydroxylase and 1 $\alpha$ -hydroxylase were homologous but definitely different in some residues. Furthermore, amino acid compositions of the two enzymes were significantly different in contrast to the finding reported by Moorthy et al. (56). Ettinger, Ismael, and DeLuca (58), however, recently isolated a cDNA clone using the monoclonal antibody of Burgos-Trinidad et al. (57) as a probe and determined nucleotide sequence from which the amino acid sequence of the protein was deciphered. As a result it was found that it did not have homology to P450 enzymes and did not have a heme consensus sequence and was considered to be a vitamin D<sub>3</sub> hydroxylase-associated protein, VDHAP. The cloning of a cDNA encoding a true renal 1 $\alpha$ -hydroxylase with the expected properties of a P450 enzyme has not yet been reported from any species.



**Fig. 4.** Immunoelectron microscopy of mitochondria in the S<sub>1</sub> and S<sub>2</sub> segments of the proximal tubules of rat kidney. Ultra-thin LR White sections prepared from normal (B) and vitamin D<sub>3</sub>-treated (A) rats were incubated with an anti-24-hydroxylase antibody as described by Iwata et al. (73). The typical gold particle is indicated by arrows. Original magnification,  $\times 50,000$ ; Bars = 0.2  $\mu\text{m}$ .

### III. 24R-HYDROXYLATION OF 25-HYDROXYVITAMIN D<sub>3</sub> (24-HYDROXYLASE)

#### A. Identification of 24R,25-dihydroxyvitamin D<sub>3</sub>

24R,25(OH)<sub>2</sub>D<sub>3</sub> was isolated by Suda et al. (17) from the plasma of pigs given large doses of vitamin D<sub>3</sub> and the structure was later established by Holick et al. (18) and Lam et al. (59). The compound is produced in kidney mitochondria when chicks are fed a high calcium diet, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> was produced when animals were fed a low calcium diet (18). 24,25(OH)<sub>2</sub>D<sub>3</sub> therefore seems to be involved in calcium homeostasis. It was also shown that the enzyme is located in intestine (60) and cartilage (61). However, the physiological function of this compound remains unresolved. Some authors consider that it is important for mineralization of bone

matrix (62), as massive doses of 24,25(OH)<sub>2</sub>D<sub>3</sub> stimulate bone formation without inducing hypercalcemia (63). Others consider that the real substrate of 24-hydroxylase is 1,25(OH)<sub>2</sub>D<sub>3</sub> (64) owing to the fact that the enzyme shows a much higher affinity for 1,25(OH)<sub>2</sub>D<sub>3</sub> than for 25-OH-D<sub>3</sub>. With this hypothesis, 1,24,25(OH)<sub>3</sub>D<sub>3</sub> is the end metabolic product formed whenever 1,25(OH)<sub>2</sub>D<sub>3</sub>, the active form of vitamin D<sub>3</sub>, is present in excess in the target tissue. Nonetheless, if the extensive osteoporosis and increased risk of bone fracture of CTX patients is due to the low level of 24,25(OH)<sub>2</sub>D<sub>3</sub>, the physiological importance of this vitamin should be reconsidered.

#### B. Purification of 24-hydroxylase

To elucidate the role of 24-hydroxylation in calcium homeostasis at a molecular level, we have purified two enzymes involved in this reaction. Ohyama et al. (65, 66)

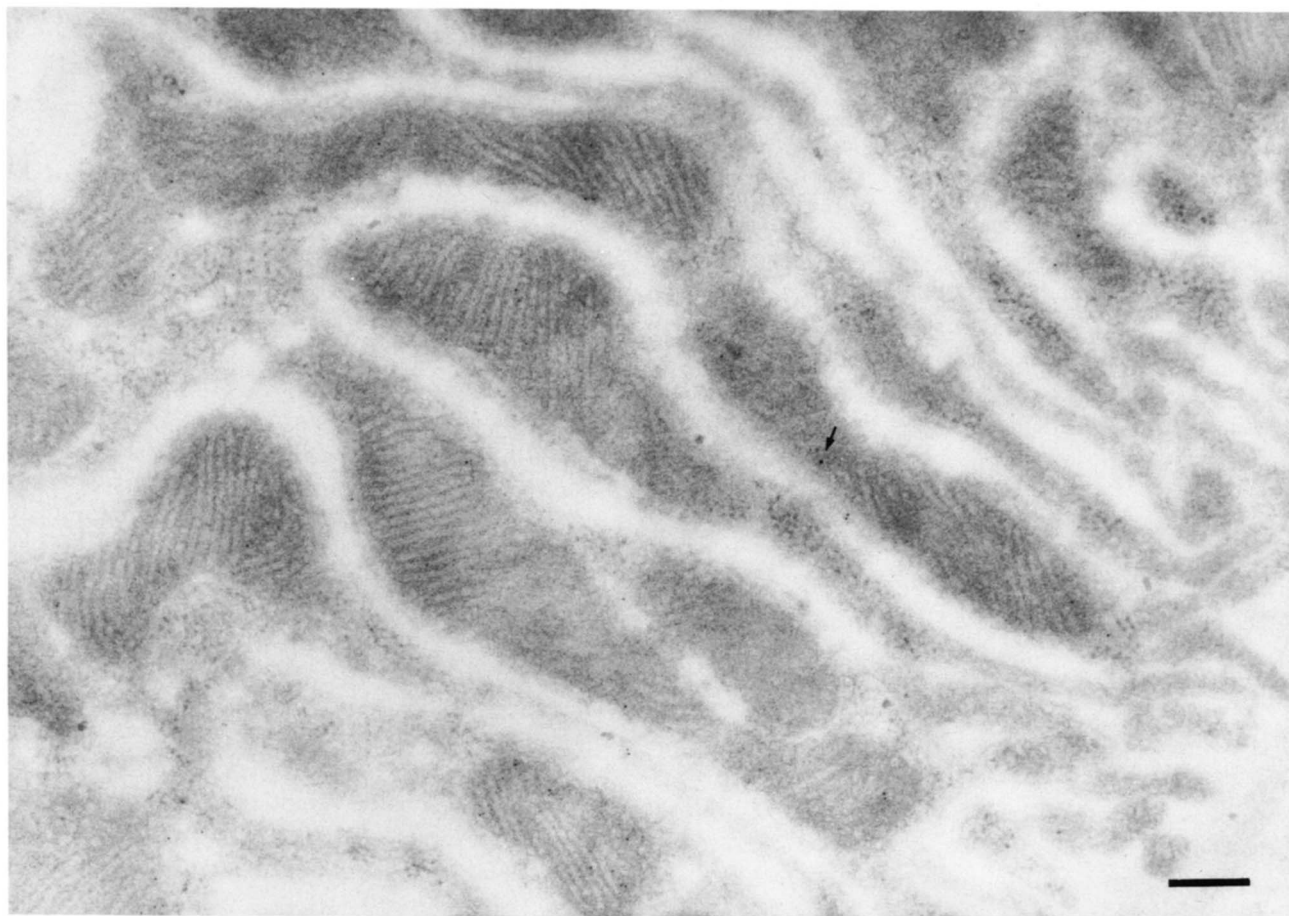


Fig. 4B.

attempted to purify the 24-hydroxylase enzyme based on its catalytic activity toward 25-OH-D<sub>3</sub>.<sup>3</sup> Although several methods for the synthesis of 25-OH-D<sub>3</sub> had been reported, Ohyama et al. developed an efficient approach to the chemical synthesis of this compound (Fig. 3). In order to facilitate isolation of the enzyme from the kidney, Ohyama et al. (65, 66) induced the enzyme 8-fold by injecting a large amount of vitamin D<sub>3</sub>.<sup>4</sup> Lubrol and/or Tween 20 were found to be the most suitable detergents and pentyl-Sepharose 4B was better than other hydrophobic columns tested for enriching 24-hy-

<sup>3</sup>It is important to purify a given P450 based on its catalytic activity, otherwise one may not obtain the genuine P450 responsible for the reaction concerned. In addition, some endogenous P450s are labile and lose their catalytic activity during purification. Finally, most if not all P450s show broad substrate specificity.

<sup>4</sup>According to the work of Armbrrecht et al. (69), this induction may have been caused by increased formation of 1,25(OH)<sub>2</sub>D<sub>3</sub>, which in turn undergoes 24-hydroxylation.

droxylase in the early stage of purification. After 7 years of effort, Ohyama et al. (65, 66) obtained a homogeneous preparation of 25-OH-D<sub>3</sub> 24-hydroxylase from rat kidney mitochondria. An HPLC technique that permitted rapid anion exchange chromatography of the enzyme was a key component of the isolation procedure as it greatly reduced loss of enzyme activity. The purified 25-OH-D<sub>3</sub> 24-hydroxylase had a molecular weight of 53,000 and showed an absorption spectrum characteristic of P450 with a peak at 453 nm in the reduced CO-difference spectrum. Upon reconstitution with the adrenal mitochondrial electron donors, adrenodoxin and NADPH-adrenodoxin reductase, the purified enzyme catalyzed 24-hydroxylation of 25-OH-D<sub>3</sub> with a turnover number of 22 min<sup>-1</sup>. Enzyme activity was inhibited by 7,8-benzoflavone, ketoconazole, and carbon monoxide, whereas it was only slightly inhibited by aminogluthetimide, metyrapone, and SKF-525A, which are well-known inhibitors of other species of P450. The enzyme not only 24-hydroxylated 25-OH-D<sub>3</sub> but also

24-hydroxylated 1,25(OH)<sub>2</sub>D<sub>3</sub> with a lower turnover number (6.0 min<sup>-1</sup>). However, the purified preparation did not catalyze 1 $\alpha$ -hydroxylation of 25-OH-D<sub>3</sub>. The enzyme followed Michaelis-Menten kinetics with a *K<sub>m</sub>* of 2.8  $\mu$ M toward 25-OH-D<sub>3</sub>.

The NH<sub>2</sub>-terminal amino acid sequence was Arg-Ala-Pro-Lys-Glu-Val-Pro-Leu-, which was different from any P450s reported to date, suggesting that it was a novel member of the P450 superfamily.

A specific monoclonal antibody and polyclonal antibodies were prepared against the purified enzyme. The latter inhibited the enzyme reaction about 70% and reacted with the protein in immunoblotting. The antibody did not react with any other P450 in rat liver microsomes or mitochondria, such as CYP2B1, CYP1A1, CYP7, CYP27.

### C. Cloning of 24-hydroxylase cDNA

Using the monoclonal antibody as a probe, Ohyama, Noshiro, and Okuda (67) isolated a cDNA clone encoding 25-OH-D<sub>3</sub> 24-hydroxylase from a rat kidney cDNA library. The isolated cDNA was 3.2 kb in length and contained an open reading frame encoding 514 amino acids. The deduced amino acid sequence contained a presequence in the NH<sub>2</sub>-terminal region typical of mitochondrial enzymes. The amino acid sequence showed less than 30% similarity to those of all other P450s reported to date, and thus is a member of a new P450 family. This enzyme was named CYP24 (trivial name, P450cc24; gene symbol *CYP24*) in the P450 superfamily (33).

Ohyama et al. (67) prepared an expression vector encoding the rat kidney mitochondrial 25-OH-D<sub>3</sub> 24-hydroxylase and introduced this DNA into COS cells. The transfected cells produced a protein that was reactive to the monoclonal antibody. Solubilized mitochondrial fractions of the transfected cells hydroxylated 25-OH-D<sub>3</sub> at C-24 in the presence of adrenodoxin and NADPH-adrenodoxin reductase, whereas mitochondria from mock-transfected cells had little activity (67).

Chen, Prahl, and DeLuca (68) recently isolated a cDNA of human 25-OH-D<sub>3</sub> 24-hydroxylase from a human HL-60 cell cDNA library using PCR products representing a fragment of rat 24-hydroxylase cDNA as a hybridization probe. The human protein whose sequence was predicted from the cDNA was 90% homologous (82% identical) to that of the rat.

### D. Induction of 24-hydroxylase by vitamin D<sub>3</sub> metabolite

Ohyama et al. (67) injected 50,000 IU of vitamin D<sub>3</sub> into rats and carried out Northern blot analysis of the kidney mRNA after 5 days using 24-hydroxylase cDNA

as a probe. Messenger RNA for 24-hydroxylase markedly increased in the vitamin D<sub>3</sub>-treated rats. Armbrecht and Boltz (69) have shown that the induction occurred after a single administration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. They also found that 24-hydroxylase mRNA increased in the intestine of rats injected with 1,25(OH)<sub>2</sub>D<sub>3</sub>.

The induction observed by Ohyama et al. (67) might be due to the 1,25(OH)<sub>2</sub>D<sub>3</sub> formed in vivo from the exogenous vitamin D<sub>3</sub>. Shinki et al. (64) demonstrated that induction of 24-hydroxylase mRNA by 1,25(OH)<sub>2</sub>D<sub>3</sub> was suppressed by the parathyroid hormone in the kidney but not in the intestine.

The induction of 24-hydroxylase by 1,25(OH)<sub>2</sub>D<sub>3</sub> was also observed in cultured human keratinocytes (70), the proliferation of which is known to be inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> and differentiation is promoted. Roy et al. (71) studied the effect of phosphate deprivation of *Hyp* mice that exhibit rachitic bone disease, hypophosphatemia, impaired renal phosphate reabsorption, and abnormal regulation of renal 1,25(OH)<sub>2</sub>D<sub>3</sub> metabolism. They found that phosphate deprivation of *Hyp* mice resulted in a 3-fold increase in the maximum velocity of 24-hydroxylase activity, in the amount of 24-hydroxylase-immunoreactive protein, and 24-hydroxylase mRNA in kidney. *Hyp* mice also exhibited an appropriate increase in 24-hydroxylase mRNA and catalytic activity in response to increasing doses of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Induction of 24-hydroxylase was also observed in bone. Nishimura et al. (72) have shown that 24-hydroxylase activity and mRNA increased when osteoblast cells were incubated with 1,25(OH)<sub>2</sub>D<sub>3</sub>.

### E. Subcellular location of 24-hydroxylase and the induction-responsive site

Recently, Iwata et al. (73) characterized the intracellular location of 24-hydroxylase using an immunogold technique. They found that the enzyme was distributed in mitochondria along the renal tubules of rat kidney in normal rat. In vitamin D<sub>3</sub>-treated rats, about a 12-fold increase in the amount of the enzyme was observed only in the S<sub>1</sub> and S<sub>2</sub> segments of kidney tubules, whereas much less increase was observed in other parts of the tubule (Fig. 4 and Fig. 5).

### F. Further oxidation of the 24-hydroxylated vitamin D<sub>3</sub> derivatives

Recently, Akiyoshi-Shibata et al. (74) expressed a rat 24-hydroxylase cDNA in *Escherichia coli* cells without modification of the cDNA and after insertion in the expression vector pKK223-3. They found that not only 24-hydroxylated compounds but also further oxidation products of these compounds were produced when the expressed enzyme was incubated with 25-OH-D<sub>3</sub> or



1,25(OH)<sub>2</sub>D<sub>3</sub>. They concluded that the following series of reactions are catalyzed by the 24-hydroxylase enzyme alone: *i*) 25-OH-D<sub>3</sub> → 24,25(OH)<sub>2</sub>D<sub>3</sub> → 24-oxo-25(OH)D<sub>3</sub> → 24-oxo-23,25(OH)<sub>2</sub>D<sub>3</sub>; *ii*) 1,25(OH)<sub>2</sub>D<sub>3</sub> → 1,24,25(OH)<sub>3</sub>D<sub>3</sub> → 24-oxo-1,25(OH)<sub>2</sub>D<sub>3</sub> → 24-oxo-1,23,25(OH)<sub>3</sub>D<sub>3</sub>. The enzyme thus catalyzes 24-hydroxylation, 24-OH-dehydrogenation, and subsequent 23-hydroxylation of the 24-oxo compounds. Similar multiple-oxidations by P450 were also observed in the formation of estrogen by CYP19 (75), lanosterol de-


methylation by CYP51 (76), and sterol-27 hydroxylation by CYP27 (77).

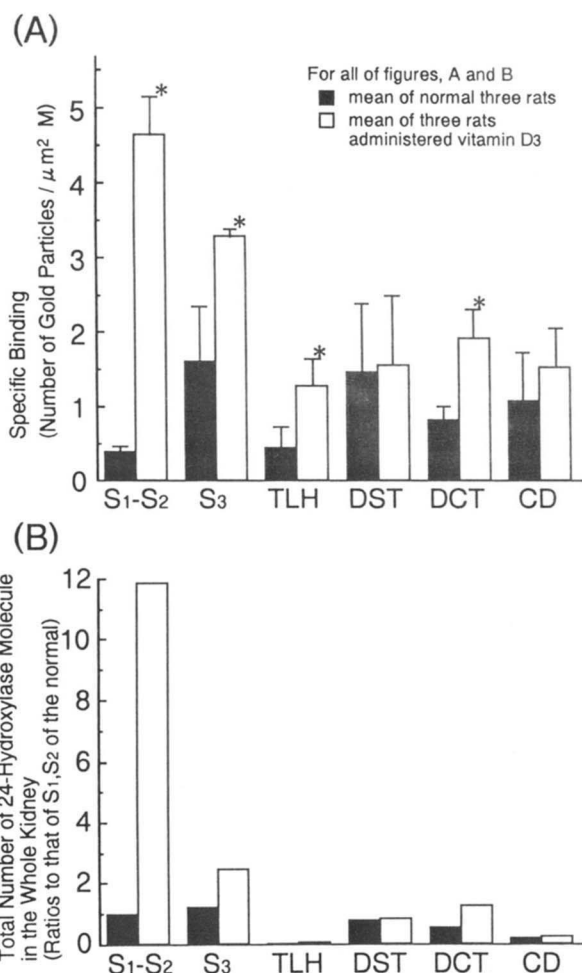
### G. Gene structure of 24-hydroxylase and 1,25(OH)<sub>2</sub>D<sub>3</sub>-responsive elements

Ohyama et al. (78) recently determined the structure of the gene encoding rat 24-hydroxylase. It spans about 15 kb, is composed of 12 exons, and exists in a single copy in the rat haploid genome.

Recently, Zierold, Darivish, and DeLuca (79) and Ohyama et al. (80) independently identified vitamin D-responsive elements in the 5'-flanking region of the 24-hydroxylase gene. Zierold et al. (79) used a gel retardation assay of PCR products for the promoter region of the 24-hydroxylase gene to identify a vitamin D-responsive element (VDRE) between nt -262 and nt -238. The DNA sequence of the VDRE identified was 5'-GGTTCAgcgGGTGCG-3', which resided on the antisense strand between nt -259 and nt -245, and was similar to that in the mouse osteopontin gene (81). On the other hand, Ohyama et al. (80) identified a vitamin D-responsive element between nt -167 and -102 by unidirectional deletion analyses of the 5'-flanking region as well as by a gel retardation study. By closer analysis they identified a direct repeat motif, 5'-AGGTGAggAGGGCG-3', between nt -151 and nt -137 on the antisense strand as a vitamin D-responsive element. Both VDREs of the 24-hydroxylase gene resemble those reported for vitamin D-inducible proteins such as osteocalcin (82), calbindin (83), and osteopontin (81). The promoter regions of these latter genes contain specific DNA sequence elements that bind the vitamin D receptor (VDR). VDR is a known member of the superfamily of steroid/thyroid/retinoid receptors that function as ligand-dependent transcription factors (84). Zierold, Darivish, and DeLuca (85) have recently shown that the two VDREs found in the promoter region of the 24-hydroxylase gene function in a cooperative manner to yield maximum activity.

### IV. CONCLUSION

As indicated in this review, the enzymes that metabolize vitamin D<sub>3</sub> are becoming increasingly well understood as a consequence of biochemical, molecular, and genetic studies. In turn, the cDNA and antibody probes arising from this body of work should allow still further insight into the physiological actions of this important vitamin. Several challenges remain, with the isolation of a bona fide vitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase as a Holy Grail. It seems likely that this prize will soon be realized by the current molecular crusade. 



**Fig. 5.** Ratio of the total number of the enzyme molecules<sup>a</sup> in the various segments in normal rats (solid bars) and vitamin D<sub>3</sub>-treated rats (open bars) S1-S2 (B): S1 and S2 segments of proximal tubules; S3; S3 segment; TLH: thin limb of Henle's loops; DST: distal straight tubules; DCT: distal convoluted tubules; CD: collecting ducts (only principal cells were analyzed). (Koji Iwata, personal communication). <sup>a</sup>Calculated according to the method of Stierhof et al. (88) using morphometric data of Pfaller (89) and immunological data of Iwata et al. (A) (73).

The authors wish to thank Dr. Alan F. Hofmann at the University of California and an anonymous reviewer for their editing of this manuscript. The author's appreciation is also extended to Dr. T. Setoguchi at Miyazaki Medical College for helpful discussion and Dr. Koji Iwata for providing electron microscopic figures.

Manuscript received 21 March 1995 and in revised form 22 May 1995.

## REFERENCES

1. Huldschinsky, K. 1919. Heilung von Rachitis durch künstliche Höhensonne. *Dtsch. Med. Wochenschr.* **45**: 712-713.
2. Mellanby, E. 1919. An experimental investigation on rickets. *Lancet.* **1**: 407-412.
3. McCollum, E. V., N. Simmonds, J. E. Becker, and P. G. Shipley. 1925. Studies on experimental rickets. A diet composed principally of purified food-stuffs for use with the "line test" for vitamin D studies. *J. Biol. Chem.* **65**: 97-100.
4. Steenbock, H., and A. Black. 1924. Fat-soluble vitamins. The induction of growth-promoting and calcifying properties in a ration by exposure to ultra-violet light. *J. Biol. Chem.* **61**: 405-422.
5. Askew, F. A., R. B. Bourdillon, H. M. Bruce, R. G. C. Jenkins, and T. A. Webster. 1931. The distillation of vitamin D. *Proc. R. Soc. Lond. Ser. B.* **107**: 76-90.
6. Windaus, A., F. Schenck, and F. von Werder. 1936. Über das antirachitisch wirksame Bestrahlungs-produkt aus 7-Dehydro-cholesterin. *Z. Physiol. Chem.* **241**: 100-103.
7. Omdahl, J. L., and H. F. DeLuca. 1973. Regulation of vitamin D metabolism and function. *Physiol. Rev.* **53**: 327-372 (review).
8. Kodicek, E. 1974. The story of vitamin D from vitamin to hormone. *Lancet.* 325-329 (review).
9. Blunt, J. W., H. F. DeLuca, and H. K. Schnoes. 1968. 25-Hydroxycholecalciferol, a biologically active metabolite of vitamin D<sub>3</sub>. *Biochemistry.* **7**: 3317-3322.
10. Kodicek, E. 1963. Turnover and distribution of vitamin D and its mode of action. In *The Transfer of Calcium and Strontium Across Biological Membranes*. R. H. Wasserman, editor. Academic Press, New York. 185-196 (review).
11. Lawson, D. E. M., P. W. Wilson, and E. Kodicek. 1969. New vitamin D metabolite localized in intestinal cell nuclei. *Nature.* **222**: 171-172.
12. Kodicek, E., L. A. W. Lawson, and P. W. Wilson. 1970. Biological activity of a polar metabolite of vitamin D<sub>3</sub>. *Nature.* **228**: 763-764.
13. Lawson, D. E. M., D. R. Fraser, and E. Kodicek. 1971. Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. *Nature.* **230**: 228-230.
14. Holick, M. F., H. K. Schnoes, and H. F. DeLuca. 1971. Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D<sub>3</sub> metabolically active in the intestine. *Proc. Natl. Acad. Sci. USA.* **68**: 803-804.
15. Norman, A. W., J. F. Myrtle, R. J. Midgett, H. G. Nowicki, V. Williams, and G. Popjak. 1971. 1,25-Dihydroxycholecalciferol: identification of the proposed active form of vitamin D<sub>3</sub> in the intestine. *Science.* **173**: 51-54.
16. Semmler, E. J., M. F. Holick, H. K. Schnoes, and H. F. DeLuca. 1972. The synthesis of 1 $\alpha$ ,25-dihydroxycholecalciferol—a metabolically active form of vitamin D<sub>3</sub>. *Tetrahedron Lett.* **40**: 4147-4150.
17. Suda, T., H. F. DeLuca, H. K. Schnoes, G. Ponchon, and Y. Tanaka. 1970. Dihydroxycholecalciferol, a metabolite of vitamin D<sub>3</sub> preferentially active on bone. *Biochemistry.* **9**: 2917-2922.
18. Holick, M. F., H. K. Schnoes, H. F. DeLuca, R. W. Gray, I. T. Boyle, and T. Suda. 1972. Isolation and identification of 24,25-dihydroxycholecalciferol, a metabolite of vitamin D<sub>3</sub> made in kidney. *Biochemistry.* **11**: 4251-4255.
19. Omdahl, J. L., R. W. Gray, I. T. Boyle, J. Knutson, and H. F. DeLuca. 1972. Regulation and metabolism of 25-hydroxycholecalciferol by kidney tissue in vitro by dietary calcium. *Nature New Biol.* **237**: 63-64.
20. Okuda, K-I. 1992. Vitamin D<sub>3</sub> hydroxylases—its occurrence, biological importance and biochemistry. In *Frontiers in Biotransformation*. K. Ruckpaul and H. Rein, editors. Vol. 6. Akademie Verlag. 114-146 (review).
21. Okuda, K-I. 1994. Liver mitochondrial P450 involved in cholesterol catabolism and vitamin D activation. *J. Lipid Res.* **35**: 361-372 (review).
22. Horsting, M., and H. F. DeLuca. 1969. In vitro production of 25-hydroxycholecalciferol. *Biochem. Biophys. Res. Commun.* **36**: 251-256.
23. DeLuca, H. F. 1971. Metabolism and mechanism of action of 25 hydroxycholecalciferol. In *Cellular Mechanism for Calcium and Homeostasis*. G. Nicoles, Jr. and R. H. Wasserman, editors. Academic Press, New York. 426-450 (review).
24. Bhattacharyya, M. H., and H. F. DeLuca. 1973. The regulation of rat liver calciferol-25-hydroxylase. *J. Biol. Chem.* **248**: 2969-2973.
25. Bhattacharyya, M. H., and H. F. DeLuca. 1973. Comparative studies on the 25-hydroxylation of vitamin D<sub>3</sub> and dihydrotachysterol<sub>3</sub>. *J. Biol. Chem.* **248**: 2974-2977.
26. Björkhem, I., and I. Holmberg. 1978. Assay and properties of a mitochondrial 25-hydroxylase active on vitamin D<sub>3</sub>. *J. Biol. Chem.* **253**: 842-849.
27. Björkhem, I., and I. Holmberg. 1979. On the 25-hydroxylation of vitamin D<sub>3</sub> in vitro studied with a mass fragmentographic technique. *J. Biol. Chem.* **254**: 9518-9524.
28. Taniguchi, S., N. Hoshita, and K-I. Okuda. 1973. Enzymatic characteristics of CO-sensitive 26-hydroxylase system for 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol in rat-liver mitochondria and its intramitochondrial localization. *Eur. J. Biochem.* **40**: 607-617.
29. Madhok, T. C., and H. F. DeLuca. 1979. Characteristics of the rat liver microsomal enzyme system converting cholecalciferol into 25-hydroxycholecalciferol. *Biochem. J.* **184**: 491-499.
30. Andersson, S., I. Holmberg, and K. Wikvall. 1983. 25-Hydroxylation of C<sub>27</sub>-steroids and vitamin D<sub>3</sub> by a constitutive cytochrome P-450 from rat liver microsomes. *J. Biol. Chem.* **258**: 6777-6781.
31. Hayashi, S., M. Noshiro, and K-I. Okuda. 1984. Purification of cytochrome P-450 catalyzing 25-hydroxylation of vitamin D<sub>3</sub> from rat liver microsomes. *Biochem. Biophys. Res. Commun.* **121**: 994-1000.
32. Hayashi, S., M. Noshiro, and K-I. Okuda. 1986. Isolation of cytochrome P-450 that catalyzes the 25-hydroxylation of vitamin D<sub>3</sub> from rat liver microsomes. *J. Biochem. (Tokyo).* **99**: 1753-1763.

33. Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Foreseen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K-I. Okuda, and D. W. Nebert. 1993. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12**: 1-51.
34. Hayashi, S., E. Usui, and K-I. Okuda. 1988. Sex-related difference in vitamin D<sub>3</sub> 25-hydroxylase of rat liver mitochondria. *J. Biochem. (Tokyo)*. **103**: 863-866.
35. Sottocassa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with outer membrane of liver mitochondria. *J. Cell Biol.* **32**: 415-438.
36. Brunner, G., and F. L. Bygrave. 1968. Microsomal marker enzymes and their limitations in distinguishing the outer membrane of rat liver mitochondria from the microsomes. *Eur. J. Biochem.* **8**: 530-534.
37. Masumoto, O., Y. Ohyama, and K-I. Okuda. 1988. Purification and characterization of vitamin D 25-hydroxylase from rat liver mitochondria. *J. Biol. Chem.* **263**: 14256-14260.
38. Okuda, K-I., O. Masumoto, and Y. Ohyama. 1988. Purification and characterization of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol 27-hydroxylase from female rat liver mitochondria. *J. Biol. Chem.* **263**: 18138-18142.
39. Ohyama, Y., O. Masumoto, E. Usui, and K-I. Okuda. 1991. Multi-functional property of rat liver mitochondrial cytochrome P450. *J. Biochem. (Tokyo)* **109**: 389-393.
40. Dahlbäck, H., and K. Wikvall. 1988. 25-Hydroxylation of vitamin D<sub>3</sub> by a cytochrome P-450 from rabbit liver mitochondria. *Biochem. J.* **252**: 207-213.
41. Dahlbäck, H. 1988. Characterization of the liver mitochondrial cytochrome P-450 catalyzing the 26-hydroxylation of 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol. *Biochem. Biophys. Res. Commun.* **157**: 30-36.
42. Usui, E., M. Noshiro, and K-I. Okuda. 1990. Molecular cloning of cDNA for vitamin D<sub>3</sub> 25-hydroxylase from rat liver mitochondria. *FEBS Lett.* **262**: 135-138.
43. Andersson, S., D. L. Davis, H. Dahlbäck, H. Jörnvall, and D. Russell. 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**: 8222-8229.
44. Usui, E., M. Noshiro, Y. Ohyama, and K-I. Okuda. 1990. Unique property of liver mitochondrial P450 to catalyze the two physiologically important reactions involved in both cholesterol catabolism and vitamin D activation. *FEBS Lett.* **274**: 175-177.
45. Akiyoshi-Shibata, M., E. Usui, T. Sakaki, Y. Yabusaki, M. Noshiro, K-I. Okuda, and H. Ohkawa. 1991. Expression of rat liver vitamin D<sub>3</sub> 25-hydroxylase cDNA in *Saccharomyces cerevisiae*. *FEBS Lett.* **280**: 367-370.
46. Su, P., H. Rennert, R. M. Shayiq, R. Yamamoto, Y-M. Xheng, S. Addya, J. F. Strauss III, and N. G. Avadhani. 1990. A cDNA encoding a rat mitochondrial cytochrome P450 catalyzing both the 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D<sub>3</sub>; gonadotropic regulation of the cognate mRNA in ovaries. *DNA Cell Biol.* **9**: 657-667.
47. Guo, Y-D., S. Strugnell, D. W. Back, and G. Jones. 1993. Transfected human liver cytochrome P450 hydroxylates vitamin D analogs at different side chain positions. *Proc. Natl. Acad. Sci. USA.* **90**: 8668-8672.
48. Cali, J. J., and D. W. Russell. 1991. Characterization of human sterol 27-hydroxylase. *J. Biol. Chem.* **266**: 7774-7778.
49. Wikvall, K. 1991. Cytochrome P450 in bile acid biosynthesis. In Proceedings of the 19th Nobel Conference on Biosynthesis, Regulation and Products of the Mevalonate Pathway at Södergarn, Sweden. 33-37.
50. Axén, E., H. Postlind, H. Sjöberg, and K. Wikvall. 1994. Liver mitochondrial cytochrome P450 CYP27 and recombinant-expressed human CYP27 catalyze 1 $\alpha$ -hydroxylase of 25-hydroxyvitamin D<sub>3</sub>. *Proc. Natl. Acad. Sci. USA.* **91**: 10014-10018.
51. Cali, J. J., C-L. Hsieh, V. Francke, and D. W. Russell. 1991. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266**: 7779-7783.
52. Leitersdorf, E., A. Reshef, V. Meiner, R. Levitzki, S. P. Schwartz, E. J. Dann, N. Berkman, J. J. Cali, L. Klapholz, and V. M. Berginer. 1993. Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews of Moroccan origin. *J. Clin. Invest.* **91**: 2488-2496.
53. Berginer, V. M., S. Shany, D. Alkalay, J. Berginer, S. Dekel, G. Salen, S. Tint, and D. Gazit. 1992. Osteoporosis and increased bone fractures in cerebrotendinous xanthomatosis. *Metab. Clin. Exp.* **42**: 69-74.
54. Paulson, S. K., and H. F. DeLuca. 1985. Subcellular location and properties of rat renal 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase. *J. Biol. Chem.* **260**: 11488-11492.
55. Armbrecht, H. J., T. V. Zenser, and B. B. Davis. 1982. Modulation of renal production of 24,25- and 1,25-dihydroxyvitamin D<sub>3</sub> in young and adult rats by dietary calcium, phosphorus, and 1,25-dihydroxyvitamin D<sub>3</sub>. *Endocrinology.* **110**: 1983-1988.
56. Moorthy, B., M. L. Mandel, and J. G. Ghazarian. 1991. Amino-terminal sequence homology of two chick kidney mitochondrial proteins immunisolated with monoclonal antibodies to the cytochrome P450 of 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase. *J. Bone Miner. Res.* **6**: 199-204.
57. Burgos-Trinidad, M., R. Ismael, R. A. Ettinger, J. M. Prah, and H. F. DeLuca. 1992. Immunopurified 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase and 1,25-dihydroxyvitamin D 24-hydroxylase are closely related but distinct enzymes. *J. Biol. Chem.* **267**: 3498-3505.
58. Ettinger, R. A., R. Ismael, and H. F. DeLuca. 1994. cDNA cloning and characterization of a vitamin D<sub>3</sub> hydroxylase-associated protein. *J. Biol. Chem.* **269**: 176-182.
59. Lam, H. Y., H. K. Schnoes, H. F. DeLuca, and T. C. Chen. 1973. 24,25-Dihydroxyvitamin D<sub>3</sub>: synthesis and biological activity. *Biochemistry.* **12**: 4851-4855.
60. Kumar, R., H. K. Schoer, and H. F. DeLuca. 1978. Rat intestinal 25-hydroxyvitamin D<sub>3</sub> and 1 $\alpha$ ,25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase. *J. Biol. Chem.* **253**: 3804-3809.
61. Garabedian, M., M. B. Dubois, M. T. Corvol, E. Pezant, and S. Balsan. 1978. Vitamin D and cartilage. In vitro metabolism of 25-hydroxycholecalciferol by cartilage. *Endocrinology.* **102**: 1262-1268.
62. Ornoy, A., D. Goodwin, D. Noff, and S. Edelstein. 1978. 24,25-Dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation. *Nature.* **276**: 517-519.
63. Nakamura, T., Y. Nagai, H. Yamato, K. Suzuki, and H. Orimo. 1992. Regulation of bone turnover and prevention of an atrophy in ovariectomized beagle dogs by the administration of 24R,25-(OH)<sub>2</sub>D<sub>3</sub>. *Calcif. Tissue Int.* **50**: 221-227.

64. Shinki, T., C. H. Jin, A. Nishimura, Y. Nagai, Y. Ohyama, M. Noshiro, K-I. Okuda, and T. Suda. 1992. Parathyroid hormone inhibits 25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase mRNA expression stimulated by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> in rat kidney but not in intestine. *J. Biol. Chem.* **267**: 13757-13762.
65. Ohyama, Y., S. Hayashi, and K-I. Okuda. 1989. Purification of 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase. *FEBS Lett.* **255**: 405-408.
66. Ohyama, Y., and K-I. Okuda. 1991. Isolation and characterization of cytochrome P450 from rat kidney mitochondria that catalyzes the 3 $\alpha$ -hydroxylation of 25-hydroxyvitamin D<sub>3</sub>. *J. Biol. Chem.* **266**: 8690-8695.
67. Ohyama, Y., M. Noshiro, and K-I. Okuda. 1991. Cloning and expression of cDNA encoding 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase. *FEBS Lett.* **278**: 195-198.
68. Chen, K-S., J. M. Prah, and H. F. DeLuca. 1993. Isolation and expression of human 1,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase cDNA. *Proc. Natl. Acad. Sci. USA.* **90**: 4543-4547.
69. Armbricht, H. J., and M. A. Boltz. 1991. Expression of 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 in kidney mitochondria and intestine. Effect of 1,25-dihydroxyvitamin D and age. *FEBS Lett.* **292**: 17-20.
70. Chen, M. L., G. Heinrich, Y. Ohyama, K-I. Okuda, J. L. Omdahl, T. C. Chen, and M. F. Holick. 1994. Expression of 25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase mRNA in cultured keratinocytes. *Proc. Soc. Exp. Biol. Med.* **207**: 57-61.
71. Roy, S., J. Martel, S. Ma, and H. S. Tenenhouse. 1994. Increased renal 25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase messenger ribonucleic acid and immunoreactive protein in phosphate-deprived *Hyp* mice. *Endocrinology.* **134**: 1761-1767.
72. Nishimura, A., A. Shinki, H. J. Cheng, Y. Ohyama, M. Noshiro, K-I. Okuda, and T. Suda. 1994. Regulation of messenger ribonucleic acid expression of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-24-hydroxylase in rat osteoblast. *Endocrinology.* **134**: 1794-1799.
73. Iwata, K., A. Yamamoto, S. Satoh, Y. Ohyama, Y. Tashiro, and T. Setoguchi. 1995. Quantitative immunoelectron microscopic analysis of the localization and induction of 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase rat kidney. *J. Histochem. Cytochem.* **43**: 255-262.
74. Akiyoshi-Shibata, M., T. Sakaki, Y. Ohyama, M. Noshiro, K-I. Okuda, and Y. Yabusaki. 1994. Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase itself: a study with the mature enzyme expressed in *Escherichia coli*. *Eur. J. Biochem.* **224**: 335-343.
75. Watanabe, Y., and Y. Ishimura. 1989. Aromatization of tetralone derivatives by Fe<sup>III</sup>PFP(Cl)PhIO and cytochrome P450-cam. *J. Am. Chem. Soc.* **111**: 410-411.
76. Aoyama, Y., Y. Yoshida, Y. Sonoda, and R. Sato. 1987. Metabolism of 32-hydroxy-24,25-dihydroxylanosterol by purified cytochrome P-450<sub>14DM</sub> from yeast. *J. Biol. Chem.* **262**: 1239-1243.
77. Holmberg-Betsholtz, I., E. Lund, I. Björkhem, and K. Wikvall. 1993. Sterol 27-hydroxylase in bile acid biosynthesis. *J. Biol. Chem.* **268**: 11079-11085.
78. Ohyama, Y., M. Noshiro, G. Eggertsen, O. Gotoh, Y. Kato, I. Björkhem, and K-I. Okuda. 1993. Structural characterization of the gene encoding rat 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase. *Biochemistry.* **32**: 76-82.
79. Zierold, C., H. M. Darwish, and H. F. DeLuca. 1994. Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin D<sub>3</sub>) 24-hydroxylase gene. *Proc. Natl. Acad. Sci. USA.* **91**: 900-902.
80. Ohyama, Y., K. Ozono, M. Uchida, T. Shinki, S. Kato, T. Suda, O. Yamamoto, M. Noshiro, and Y. Kato. 1994. Identification of a vitamin D-responsive element in the 5'-flanking region of the rat 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase gene. *J. Biol. Chem.* **269**: 10545-10550.
81. Noda, M., R. L. Vogel, A. M. Craig, J. Prah, H. F. DeLuca, and D. T. Denhardt. 1990. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D<sub>3</sub> receptor and 1,25-dihydroxyvitamin D<sub>3</sub> enhancement of mouse secreted phosphoprotein 1 (Supp-1 or osteopontin) gene expression. *Proc. Natl. Acad. Sci. USA.* **87**: 9995-9999.
82. MacDonald, P. N., C. A. Haussler, C. M. Terpening, M. A. Galligan, M. C. Reeder, G. K. Whitefield, and M. R. Haussler. 1991. Baculovirus-mediated expression of the human vitamin D receptor. *J. Biol. Chem.* **266**: 18808-18813.
83. Darwish, H. M., and H. F. DeLuca. 1992. Identification of a 1,25-dihydroxyvitamin D<sub>3</sub>-response element in the 5'-flanking region of the rat calbindin D-9k gene. *Proc. Natl. Acad. Sci. USA.* **89**: 603-607.
84. Baker, A. R., D. P. McDonnell, M. Hughes, T. M. Crisp, D. J. Mangelsdorf, M. R. Haussler, J. W. Pike, J. Shine, and B. W. O'Malley. 1988. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA.* **85**: 3294-3298.
85. Zierold, C., H. M. Darwish, and H. F. DeLuca. 1995. Two vitamin D responsive elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *J. Biol. Chem.* **270**: 1675-1678.
86. Takeuchi, A., T. Okano, and T. Kobayashi. 1991. The existence of 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase in the liver of carp and bastard halibut. *Life Sci.* **48**: 275-282.
87. Takeuchi, A., T. Okano, H. Sekimoto, and T. Kobayashi. 1994. The enzymatic formation of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> from 25-hydroxyvitamin D<sub>3</sub> in the liver. *Comp. Biochem. Physiol.* **109C**: 1-7.
88. Stierhof, Y-D., H. Schwarz, M. Dürrenberger, W. Villiger, and E. Kellenberger. 1991. Yield of immunolabel compared to resin sections and thawed cryosections. In *Colloidal Gold. Principles, Methods and Applications*. Vol. 3. M. A. Hayat, editor. Academic Press, New York. 87.
89. Pfaller, W. 1982. Structure function correlation on rat kidney. *Adv. Anat. Embryol. Cell Biol.* **70**: 1.