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Cloning and expression of cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase

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A cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase (P450cc24) was isolated from a rat kidney cDNA library using specific antibodies to the enzyme. The isolated cDNA was 3.2 kbp long and contained a 1542-bp open reading frame encoding 514 amino acids. The deduced amino acid sequence contained a presequence typical of mitochondrial enzymes in the N-terminal region. The amino acid sequence shows less than 30% similarity to those of any other cytochrome P450s so far reported and, therefore, P450cc24 constitutes a novel family of P450. COS-7 cells transfected with the cDNA produced a protein that was reactive with the antibodies and catalyzed NADPH-dependent 24-hydroxylation of 25-hydroxyvitamin D_3 in the presence of adrenodoxin and NADPH-adrenodoxin reductase. Using the cDNA as a probe we demonstrated that the increase of 24-hydroxylation activity caused by administration of vitamin D_3 into rats was accompanied by an increase of the mRNA.

25-Hydroxyvitamin D_3 24-hydroxylase; P450cc24; cDNA cloning; cDNA expression

1. INTRODUCTION

Vitamin D_3 is indispensable in maintaining calcium homeostasis in animals. However, it must be modified before it exerts any physiological functions. It first undergoes 25-hydroxylation in liver mitochondria and/or microsomes. The product, 25-hydroxyvitamin D_3, is released into the blood stream and transported to kidney mitochondria where it is further converted to 1α,25-dihydroxyvitamin D_3 or 24,25-dihydroxyvitamin D_3 depending on the serum level of calcium [1]. According to recent reports [2–5], 24-hydroxylation activity is induced by 1α,25-dihydroxyvitamin D_3 itself in a dose-dependent manner.

To understand the molecular mechanism of calcium homeostasis, it is necessary to elucidate the structure of genes responsible for the modifications of vitamin D_3, and therefore isolation of cDNA for enzymes involved in these modifications is of pivotal importance. In this paper we report the cloning and expression of cDNA for 25-hydroxyvitamin D_3 24-hydroxylase of rat kidney mitochondria.

2. MATERIALS AND METHODS

2.1. *Preparation of anti-P450cc24 antibodies*

P450cc24 was prepared according to the previously described method [6]. Specific polyclonal antibodies against this P450 were prepared by immunizing BALB/c female mice with the purified protein mixed with Ribi adjuvant as described elsewhere (Ohyama and Okuda, submitted).

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2.2. *Cloning and sequencing of cDNA*

Total kidney poly(A)$^+$ RNA was prepared by the guanidine hydrochloride method [7] followed by oligo(dT) cellulose column chromatography from rats injected intraperitoneally with vitamin D_3 (50000 IU) for 5 days. λgt11 and λZAP cDNA libraries were prepared from the poly(A)$^+$ RNA as described [8]. Subcloning into pBluescript was performed by the in vivo excision method [9]. Deletion mutations of the clone were prepared by using an *Exo*III/Mung bean nuclease deletion system (Takara Shuzo Co., Tokyo) and nucleotide sequence was determined by the dideoxynucleotide chain termination method [10] using the deletion mutants as templates.

2.3. *Expression of cDNA in COS-7 cells*

A *Dde*I–*Pvu*II fragment (nucleotide 319 to 2134) of the isolated P450cc24 cDNA (containing the open reading frame) was inserted into the *Sma*I site of the mammalian expression vector pSVL to construct pSVL24. Control plasmid (pSVL24R) was prepared by inserting the cDNA fragment in the reverse direction. The constructed plasmids were then transfected into COS-7 cells by the DEAE dextran method, 5 μg of expression plasmid being used per 1×10^6 cells [11,12]. Cells were incubated for 72 h at 37°C under 5% CO_2 in 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

2.4. *Assay of the expressed 24-hydroxylase*

The mitochondrial fraction prepared from the incubated cells was solubilized with 0.8% cholate and the mixture was centrifuged at $8000\times g$ for 15 min. The enzyme activity of the supernatant was assayed in the presence of adrenodoxin and NADPH-adrenodoxin reductase as an electron transporting system. After incubation for 1 h, products were extracted and analyzed by normal phase HPLC. Identification of 24,25-dihydroxyvitamin D_3 was performed as described previously [6].

3. RESULTS AND DISCUSSION

Screening of 5×10^5 plaques of rat kidney λgt11 cDNA with anti-24-hydroxylase antibodies gave one positive clone having an insert of 1.6 kbp, which was

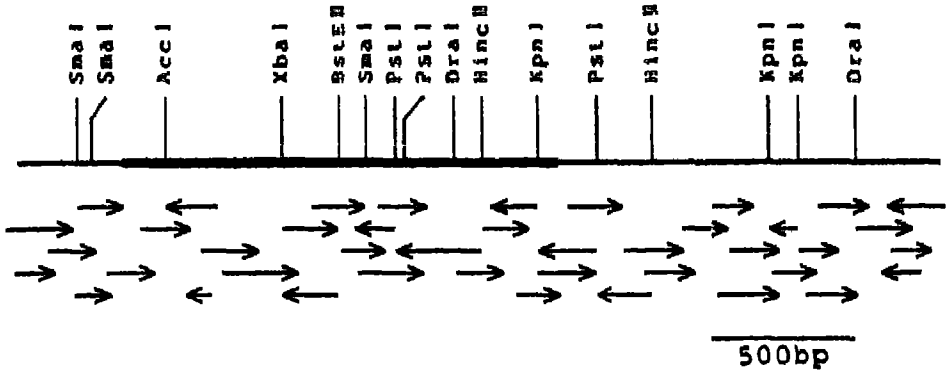

Fig. 1. Restriction map and sequencing strategy of pCC24-8. Arrows indicate the directions and the extents of sequence determination. A closed box indicates an open reading frame revealed by the sequencing.

shorter than the size of mRNA (3.4 kb, see Fig. 4) as estimated from Northern blot analysis. The λZAP library was therefore rescreened using the 1.6-kbp insert as a probe. Four positive clones were isolated, subcloned into pBluescript and subjected to restriction mapping. Since the four clones showed the same restriction map (Fig. 1), the plasmid named pCC24-8 containing the largest insert (3.2 kbp) was sequenced. Fig. 2 shows the entire nucleotide sequence of pCC24-8 insert and the predicted amino acid sequence. It contains a 1542-bp open reading frame encoding 514 amino acid residues (M_r 59445), a 343-bp 5'-untranslated region a 1324-bp 3'-untranslated region. The N-terminal amino acid sequence of purified P450cc24, determined by the automated Edman degradation method (unpublished data), agreed perfectly with the deduced sequence from residues 36 to 43, establishing that processing of the precursor occurs between residues 35 and 36. The 35-residue removed by processing is characteristic of a mitochondrial presequence. It contains 6 arginine and 2 lysine residues together with 16 hydrophobic amino acid residues distributed throughout the sequence, conferring characteristic amphiphilicity common to presequences that direct proteins into mitochondria. The mature enzyme comprises 479 amino acid residues corresponding to a molecular weight of 55 535 which is close to 53 000 estimated from SDS-polyacrylamide gel electrophoresis for the purified enzyme [6]. A heme-binding, highly conserved region can be recognized from residues 455 to 475, in which cysteine 462 is considered as the heme binding site. Amino acids characteristic of adrenodoxin binding site in P450scc [13,14] are observed at Lys-378, Lys-382, Arg-386. A computer homology search (NBRF data base) indicated that the deduced amino acid sequence of P450cc24 is not more than 30% identical with those of any of P450s so far reported. The highest homology observed was to P450LMT25 [15] (about 30%), a liver mitochondrial P450 that catalyzes vitamin D_3 25-hydroxylation. P450cc24 is thus concluded to constitute a novel P450 family.

That this cDNA is indeed encoding 25-hydroxyvitamin D_3 24-hydroxylase was confirmed by expression in COS cells. The plasmid pSVL24 and pSVL24R (control) were transfected to COS-7 cells. After 72 h culture, the mitochondrial fraction was prepared from the cells. The supernatant of cholate-solubilized mitochondria was assayed for its enzymatic activity. The cells transfected with pSVL24 showed 10× higher 24-hydroxylation activity toward 25-hydroxyvitamin D_3 than those transfected with pSVL24R (Table I). Western blot analysis of the expressed protein in the transfected cells (Fig. 3, lane 4) showed that a single protein band reactive with the antibodies was observed at a position corresponding to the purified P450cc24 (lane 1), whereas in control cells no such immunoreactive band was observable (lanes 2 and 3). Since no other band reacting with the antibodies was observed, the expressed protein seemed to have been processed to the mature form.

Many reports have been published to date demonstrating that the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3, induces 24-hydroxylation activity in vivo and in cultured cells [2-5]. Previously we also observed that the enzyme activity was elevated by injection of vitamin D_3 itself (unpublished observation). To clarify whether this increase of enzyme activity is due to pretranslational induction, the change of mRNA level in the kidney was examined by Northern blot analysis. It was thus found that in vitamin D_3-treated rats the amount of mRNA hybridizable with the 24-hydroxylase cDNA was actually increased (Fig. 4A), whereas no significant difference was observed for actin mRNA (Fig. 4B). It is therefore suggested that vitamin D_3 or its metabolite(s) works at the pretranslational level.

In conclusion we have isolated cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase, which is one of

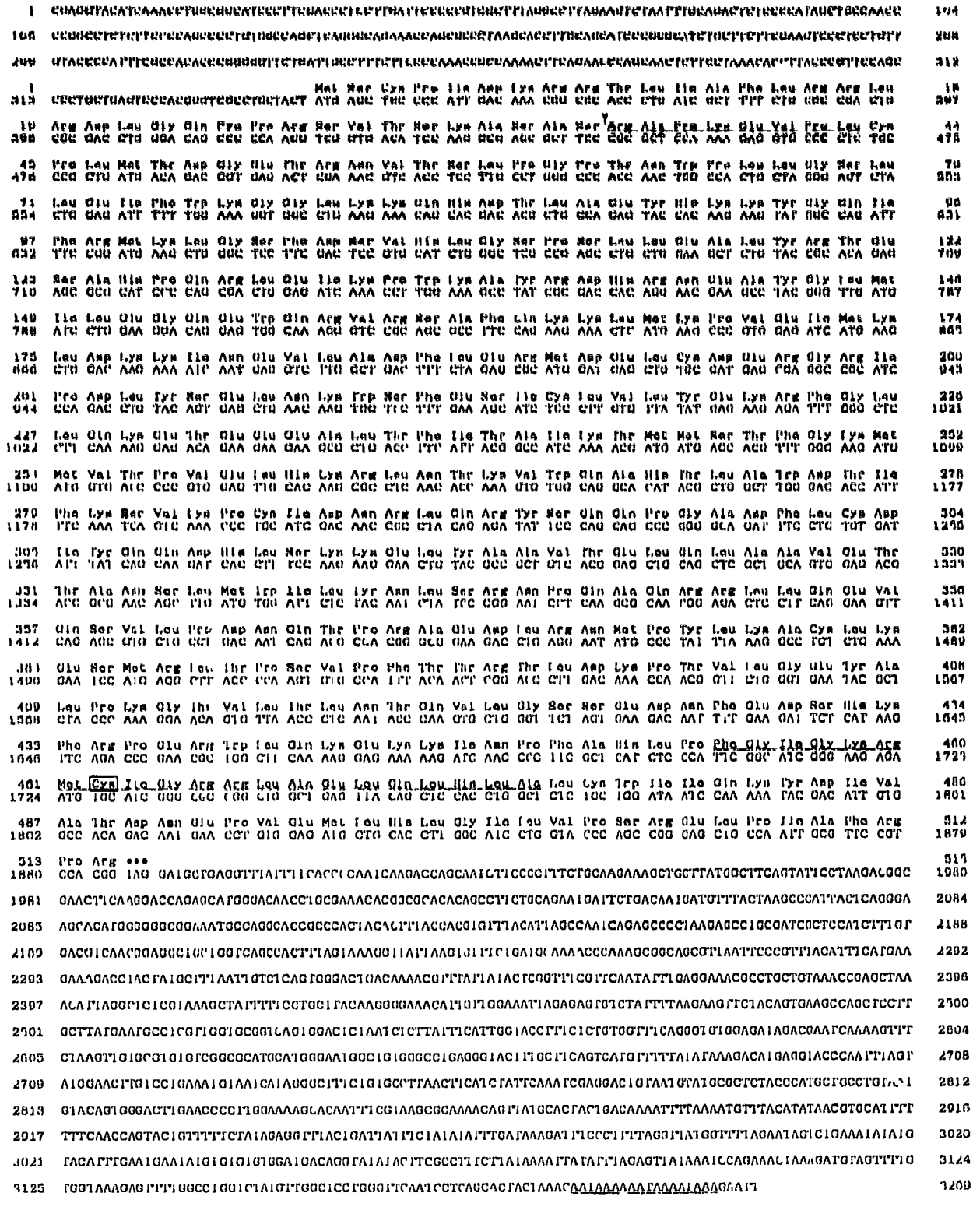

Fig 2 Nucleotide sequence of the pCC24-8 insert and predicted amino acid sequence A DNA fragment of cDNA (3 2 kbp) including the total coding region was sequenced The cleavage site of the presequence is shown by an arrowhead Corresponding amino acid sequence of the purified P450cc24 determined by Edman degradation is underlined (residue 36 to 43). The consensus sequence for the heme binding domain is marked by underline (residue 455 to 475) Poly(A) addition signals are observed from nucleotide 3187 to 3204

Table I

25-Hydroxyvitamin D_3 24-hydroxylation activity of the mitochondrial fraction of the transfected COS-7 cells

Experiment	pSVL24 (pmol/mg protein/h)	pSVL24R (pmol/mg protein/h)
1	850	20
2	580	110
3	750	< 20
4	840	70

The reaction mixture contained 50 μg protein, 2 nmol of adrenodoxin and 0.05 U of NADPH adrenodoxin reductase in a final volume of 0.5 ml.

the important enzymes in maintaining calcium homeostasis. The availability of the cDNA probe should pave the way to elucidate the mechanism of calcium homeostasis at the molecular level.

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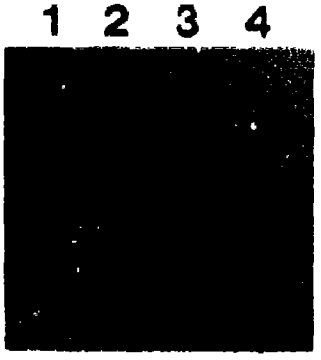

Fig 3 Western blot analysis of expressed protein in COS cells. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [16] and immunoblotting by the method of Towbin [17]. Lane 1, purified P450cc24 (0.06 μg), lane 2, untransfected cells, lane 3, pSVL24R (control)-transfected cells, lane 4, pSVL24-transfected cells. To each lane was applied 24 μg of the supernatant of solubilized mitochondria. The blot was stained by using horseradish peroxidase-conjugated goat anti-mouse IgG, 3,3'-diaminobenzidine and H_2O_2 [18].

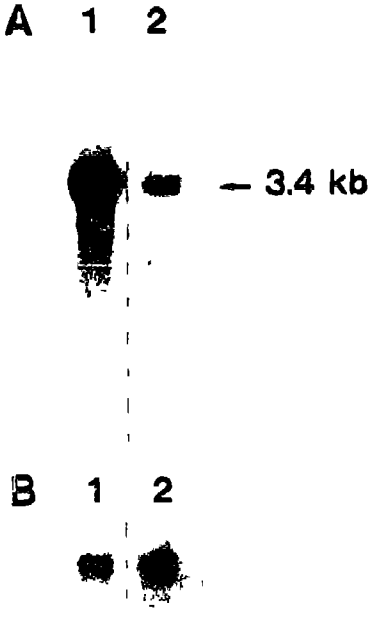

Fig 4 Northern hybridization analysis of kidney poly(A)$^+$ RNA Five microgram poly(A)$^+$ RNA was electrophoresed on 1% agarose gel containing formaldehyde and then blotted onto a Nytran (Schleicher and Schuell) Lane 1, vitamin D_3-treated rat (Treatment of rats with vitamin D_3 was as described for preparation of cDNA library), lane 2, normal rat Panel A, hybridized with ^{32}P-labeled insert of pCC24-8 (nucleotide 1 to 2134), panel B, hybridized with actin cDNA as control