Cloning and expression of rat 25-hydroxyvitamin D₃-1 α -hydroxylase cDNA

(vitamin D metabolism/1 α -hydroxylase/24-hydroxylase/P450)

TOSHIMASA SHINKI*^{†‡}, HIROKO SHIMADA^{‡§}, SHU WAKINO[¶], HIDEHARU ANAZAWA[§], MATSUHIKO HAYASHI[¶], TAKAO SARUTA[¶], HECTOR F. DELUCA^{||}, AND TATSUO SUDA*

*Department of Biochemistry, School of Dentistry, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142, Japan; [§]Tokyo Research Laboratory, Kyowa Hakko Kogyo Co. Ltd., Asahimachi, Machida, Tokyo 194, Japan; [¶]Department of Internal Medicine, University of Keio, Shinanomachi, Shinjuku-ku, Tokyo 160, Japan; and ^{||}Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, 420 Henry Mall, Madison, WI 53706

Contributed by Hector F. DeLuca, September 16, 1997

ABSTRACT A full-length cDNA for the rat kidney mitochondrial cytochrome P450 mixed function oxidase, 25-hydroxyvitamin D₃-1 α -hydroxylase (P4501 α), was cloned from a vitamin D-deficient rat kidney cDNA library and subcloned into the mammalian expression vector pcDNA 3.1(+). When P4501 α cDNA was transfected into COS-7 transformed monkey kidney cells, they expressed 25-hydroxyvitamin D₃-1 α -hydroxylase activity. The sequence analysis showed that P4501 α was of 2,469 bp long and contained an ORF encoding 501 amino acids. The deduced amino acid sequence showed a 53% similarity and 44% identity to the vitamin D₃-25-hydroxylase (CYP27), whereas it has 42.6% similarity and 34% identity with the 25-hydroxyvitamin D₃-24-hydroxylase (CYP24). Thus, it composes a new subfamily of the CYP27 family. Further, it is more closely related to the CYP27 than to the CYP24. The expression of P4501 α mRNA was greatly increased in the kidney of vitamin D-deficient rats. In rats with the enhanced renal production of 1 α ,25-dihydroxyvitamin D₃ (rats fed a low Ca diet), P4501 α mRNA was greatly increased in the renal proximal convoluted tubules.

It has been established that vitamin D₃ is first metabolized in the liver to 25-hydroxyvitamin D₃ [25(OH)D₃] and subsequently in the kidney to 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] and 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃]. Of these two dihydroxyvitamin D metabolites, 1 α ,25(OH)₂D₃ has been demonstrated to be the active form of vitamin D₃ (1, 2). The production of 1 α ,25(OH)₂D₃ is regulated primarily at the final step in its synthetic pathway; 1 α -hydroxylation of 25(OH)D₃ by the renal mitochondrial P450 mixed-function oxidase (3, 4). The cytochrome P450 enzymes form a superfamily of heme-containing proteins that are bound to the membranes of the microsomes and mitochondria and serve as an oxidation-reduction component of the mixed-function oxidase system. In *in vivo* conditions, this system is involved in the oxidative metabolism of a number of steroids. The CYP family is large, with at least 74 families, and each mammalian species is estimated to have between 60 and 200 distinct superfamily members (5, 6). These P450 proteins have more than 40% amino acid homology within a single family and usually more than 55% identity within a subfamily. The cysteine-containing region associated with the heme-binding domain of the P450 protein is highly conserved among all eukaryotic species.

The amino acid sequence similarity between 25(OH)D₃-24-hydroxylase (CYP24) and vitamin D₃-25-hydroxylase (CYP27) enzymes within the heme-binding domain has been reported to be 60% (7, 8). The homology of the putative ferredoxin-binding domain between CYP27 and CYP24 was 70%. By

using a probe from the 3' region of the rat CYP24 cDNA, which encompasses the heme-binding domain of the molecule, St-Arnaud *et al.* (9) screened a cDNA library from the kidney of vitamin D-deficient rats under reduced stringency conditions to identify and clone the 25(OH)D₃-1 α -hydroxylase cDNA. They reported the partial amino acid sequence of P4501 α at the 18th Annual Meeting of the American Society for Bone and Mineral Research (September 9–11, 1996, Seattle, WA). The amino acid sequence similarity with CYP24 was calculated to be 78% within the heme-binding domain; the two proteins diverged significantly outside this region for the overall sequence similarity of 26%. However, neither the full-length sequence of 25(OH)D₃-1 α -hydroxylase nor its regulation in the kidney has been reported.

Recently, Nicoll *et al.* (10) cloned the third mammalian Na⁺-Ca²⁺ exchanger, NCX3, by using degenerate oligonucleotide primers derived from two highly conserved regions of NCX1 and NCX2. Shen *et al.* (11) also cloned a new form of P450 gene by reverse transcription-PCR (RT-PCR) by using degenerate primers containing inosine. To isolate a fragment of 25(OH)D₃-1 α -hydroxylase, a similar approach was used according to the method of mixed oligonucleotide-primed amplification of cDNA. We hypothesized that 1 α -hydroxylase should have a degree of homology with the products of known P450 genes. The P450 genes with homology considered to be likely candidates were CYP27 and CYP24. The primers were targeted at two conserved regions (heme-binding domain and ferredoxin-binding domain) of the products of CYP24 and CYP27. By using the method of mixed oligonucleotide-primed amplification of cDNA, we have cloned a new sequence of the P450 mixed function oxidase from the kidney of vitamin D-deficient rats. This clone designated as P4501 α has been sequenced and expressed. Here we report the cloning and sequencing of the P4501 α gene and the regulation of its mRNA expression in rat kidney.

MATERIALS AND METHODS

Animals. Male weanling rats (Sprague-Dawley strain) were maintained for 3 weeks on a synthetic vitamin D-deficient diet

Abbreviations: 25(OH)D₃, 25-hydroxyvitamin D₃; 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; 26,27-F₆-1 α ,25(OH)₂D₃, 26,27-hexafluoro-1 α ,25-dihydroxyvitamin D₃; 24,25(OH)₂D₃, 24,25-dihydroxyvitamin D₃; P4501 α , 25-hydroxyvitamin D₃-1 α -hydroxylase; CYP24, 25-hydroxyvitamin D₃-24-hydroxylase; CYP27, vitamin D₃-25-hydroxylase; PCT, proximal convoluted tubules; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; PTH, parathyroid hormone.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. 001992).

[†]To whom reprint requests should be addressed at: Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan. e-mail: shinki@dent.showa-u.ac.jp.

[‡]T. Shinki and H.S. contributed equally to this work.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1997 by The National Academy of Sciences 0027-8424/97/9412920-6\$2.00/0
PNAS is available online at <http://www.pnas.org>.

containing 0.03% Ca and 0.6% phosphorus (Teklad, Madison, WI). Rats fed the vitamin D-deficient diet were maintained in a room with incandescent lighting, and all potential sources of UV light and vitamin D were excluded. All chemicals were of a highly purified analytical grade. $1\alpha,25(\text{OH})_2\text{D}_3$ was purchased from Wako Pure Chemicals (Osaka). $24,25(\text{OH})_2\text{D}_3$ was a gift from Kureha Chemical Industries (Tokyo). $26,27\text{-Hexafluoro-}1\alpha,25(\text{OH})_2\text{D}_3$ [$26,27\text{-F}_6\text{-}1\alpha,25(\text{OH})_2\text{D}_3$] was kindly donated by Sumitomo Pharmaceuticals (Osaka). $10\text{-Oxo-}19\text{-nor-}25\text{-hydroxyvitamin D}_3$ was a gift from S. Yamada (Tokyo Medical and Dental University, Tokyo). [$26,27\text{-}^3\text{H}$]- $25(\text{OH})\text{D}_3$ was obtained from Amersham. [$\alpha\text{-}^{32}\text{P}$]- and [$\alpha\text{-}^{33}\text{P}$]dCTP were purchased from DuPont/New England Nuclear. *Taq* DNA polymerase, restriction enzymes, vectors, and other molecular biological reagents were obtained from Stratagene, Boehringer Mannheim, Takara Biomedicals (Shiga, Japan), and BRL. COS-7 cells were obtained from the American Type Culture Collection. Trypsin, penicillin G, streptomycin, cell culture media, and fetal bovine serum were obtained from GIBCO. Oligonucleotides described in the text were synthesized by Pharmacia.

Cloning of 1α -Hydroxylase cDNA Fragment. Total RNA was isolated from a vitamin D-deficient rat kidney by guanidine-hydrochloride method (12) and poly(A)⁺ RNA was purified through an oligo(dT) cellulose column. Poly(A)⁺ RNA was reverse-transcribed by a random hexamer primer. PCR with degenerate primers was used to identify a fragment of the 1α -hydroxylase. The primers were derived from the amino acid sequence of conserved regions of CYP24 and CYP27. The forward primer was a 23 mer with 36-fold degeneracy (5'-CTCTSAARGCHGTSATYAARGA-3') based on a portion of the proposed ferredoxin-binding site. The reverse primer was a 23 mer with 432-fold degeneracy (5'-CKCTTBC-CRAABCCRAARGGVA-3') based on a portion of the heme-binding region. cDNA from the rat kidney was used as a template for the PCR, which was carried out for 35 cycles (94°C for 30 sec; 42°C for 1 min; 72°C for 1 min). The PCR product was cloned into the pCRII (Invitrogen), and after transformation, four plasmids with appropriately sized inserts were sequenced. All inserts had the same sequence and had a previously undescribed sequence of P450.

PCR Cloning of Rat $25(\text{OH})\text{D}_3$ - 1α -Hydroxylase cDNA. Vitamin D-deficient rat kidney poly(A)⁺ RNA was used to direct synthesis of the first strand cDNA from the T17-tailed rapid amplification of cDNA ends (RACE) oligonucleotide primer. A region of rat mitochondrial $25(\text{OH})\text{D}_3$ - 1α -hydroxylase cDNA was amplified between the RACE primer and a specific oligonucleotide (5'-AAGGCTGTGATCAAAGAAGTGT-TGA-3'). The resulting PCR product, a 1.2-kbp rat kidney 1α -hydroxylase cDNA (confirmed by Northern blot analysis), was cloned into the pCRII (Invitrogen).

Cloning and Sequencing of Full-Length Rat $25(\text{OH})\text{D}_3$ - 1α -Hydroxylase. cDNA was prepared from the poly(A)⁺ RNA of the vitamin D-deficient rat kidney and ligated into Uni-ZAP XR vector arms by a ZAP cDNA Synthesis Kit (Stratagene). The recombinant DNA was packaged with a Gigapack II Gold Packaging Extract (Stratagene) and infected to *Escherichia coli*, XL1-Blue MRF'. The primary library size was 2.7×10^6 independent recombinants. The primary library was amplified once and suspended to a titer of 3.4×10^9 pfu/ml. The library was plated ($1\text{--}2 \times 10^4$ pfu/150 mm NZY [NZ amine (casein hydrolysate enzymatic) yeast extract] agar plate) and screened by plaque hybridization (13) by using the 1.2-kbp fragment as a probe which was labeled with digoxigenin (DIG) by a PCR DIG Probe Synthesis Kit (Boehringer Mannheim). Hybridization was done at 65°C for 16–20 h with $5\times$ standard saline citrate (SSC) containing 1% blocking reagent, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 50–100 ng/ml of probes. The filters were washed twice in $2\times$ SSC containing 0.1% SDS at 60°C for 5 min and once in $0.1\times$ SSC containing 0.1% SDS

at 60°C for 15 min. The detection was performed with a DIG Luminescent Detection Kit (Boehringer Mannheim). Positive plaques were isolated and verified by PCR with the specific primers for P4501 α . Each clone isolated was excised to a pBluescript by a Rapid Excision Kit (Stratagene) and sequenced by the dideoxy-chain termination method (14).

Expression of cDNA in COS-7 Cells. A *NheI*-*Bam*HI fragment of the isolated P4501 α was inserted into the same sites of the mammalian expression vector pcDNA 3.1 (+) (Invitrogen) to construct pcDNA-1 α . COS-7 cells were subcultured for 20 h before transfection and seeded in 100-mm plates at a density of 1×10^6 cells/dish. Cells were transfected with 10 μg of pcDNA-1 α according to the lipofection protocol by using Lipofectin (BRL) as described in the manufacturer's manual. Twenty-four hours after transfection, the medium was replaced and the cells were cultured for a further 24 h. Then the cells were incubated with [^3H] $25(\text{OH})\text{D}_3$ (2,000 Bq), and then methanol was added to terminate the reaction. An aliquot of the lipid soluble metabolites was applied to HPLC under the following conditions: column, Finepak SIL 4.6×250 mm; mobile phase, *n*-hexane/2-propanol/methanol (89:5.5:5.5); flow rate, 1.5 ml/min (15). The radioactivity of the eluate was counted. Generation of [^3H] $1\alpha,25(\text{OH})_2\text{D}_3$ was confirmed by comigration of the radioactivity and authentic $1\alpha,25(\text{OH})_2\text{D}_3$.

Northern Blot and RT-PCR Analyses. Total RNA was prepared by the method of Chomczynski and Sacchi (16), and poly(A)⁺ RNA was isolated by using the Oligotex dT 30 isolation system (Takara Biomedicals) (17). Poly(A)⁺ RNA was size-fractionated on a 1.4% agarose gel containing 6% formaldehyde. After electrophoresis, RNA was transferred to Hybond-N membrane (Amersham) by capillary diffusion in $20\times$ SSC and fixed by UV cross-linking. ^{32}P -labeled single-stranded DNA probes were hybridized to the membrane overnight at 42°C. The blot was washed for 15 min at 42°C with $2\times$ SSC in 0.1% SDS, followed by washing steps with increasing stringency up to $0.2\times$ SSC at 60°C for 15 min. RT-PCR of microdissected PCT segments were performed by the method as described in the previous papers (18, 19). Two mm of microdissected proximal convoluted tubule (PCT) segments were reverse-transcribed with a random hexamer primer. Synthesized cDNA was used for PCR. The sense (5'-AGTGAGCTGAACAAGTGGTC-3') and antisense (5'-GTCTGATTGTCAGGCAGCAC-3') primers used in PCR for CYP24 located in the coding region and corresponded to bases 956–975 and 1,418–1,437, respectively. The sense and antisense primers used in PCR for P4501 α were 5'-CTAAAGGCTGT-GATCAAAGAAGTGT-3', which corresponded to bases 1,110–1,134 and 5'-TTCGTTTGCCAAAGCCAAAAGGAAG-3', which corresponded to bases 1,338–1,362, respectively.

RESULTS

Amplification of a cDNA Fragment with Mixed Primers. PCR with degenerate primers was used to identify a fragment of 1α -hydroxylase. The primers were targeted at two conserved regions of the products of CYP24 and CYP27. After mRNA was isolated from rat kidney, it was reverse-transcribed into cDNA by using a cDNA synthesis kit (Stratagene), and then the cDNA was amplified with a set of degenerate primers derived from the heme- and ferredoxin-binding domains of the CYP24 and CYP27 genes. By using the method of mixed oligonucleotide-primed amplification of cDNA, we have cloned a novel sequence from the kidney of vitamin D-deficient rats. PCR amplification resulted in three bands on gel electrophoresis (Fig. 1). A PCR product with an approximate size of 255 bp was detected only in cDNA from the vitamin D-deficient rats. This PCR product was cut out, extracted, and cloned into the TA cloning vector pCR II for the sequence analysis. Rapid amplification of 3' end from P4501 α cDNA was carried out according to the manufacturer's manual (3'-RACE system for rapid amplification of cDNA ends, BRL). A single

FIG. 1. Electrophoretic bands (ethidium bromide fluorescence) produced by the method of mixed oligonucleotide-primed amplification of cDNA. Lanes 1 and 3, vitamin D-deficient rats D(-); lane 2, vitamin D-deficient rat treated for 48 h with 1 μ g of 26,27-F₆-1 α ,25(OH)₂D₃ (F6).

RACE product about 1.2 kbp was observed (data not shown). The PCR product was subcloned into a plasmid vector, and this insert was used for cloning of the full-length P4501 α cDNA.

DNA Sequences. The λ Zap cDNA library was screened by using the 1.2-kbp fragment as a probe. Eleven positive clones were detected from 8×10^4 plaques at the first screening. The longest insert of these clones was sequenced. A full-length cDNA clone obtained was 2,469 bp long (Fig. 2). The P4501 α clone contained an ORF of 1,503 nucleotides, starting at base 24 and ending at base 1,526, which encoded a protein of 501 amino acids. The predicted polypeptides were compared with the protein databases and found to possess the hallmark features of the CYP superfamily (Fig. 3). There was a significant homology to CYP members in the ferredoxin- and heme-binding domains. A heme-binding, highly conserved region could be recognized from residues 441 to 461, in which Cys-448 was considered as the heme-binding site. Amino acid characteristics of the ferredoxin-binding site in P450 (20) were observed at Lys-364, Lys-368, and Arg-378. A sequence search that used the University of Wisconsin Genetics Computer Group (GAP program) indicated that the deduced amino acid sequence of P4501 α has a 52.9% similarity and 43.6% identity with CYP27. Its similarity is 42.6% and its identity is 33.9% with CYP24. It is thus related more to the liver mitochondrial vitamin D 25-hydroxylase than to the kidney mitochondrial 24-hydroxylase. Based on these findings and the current nomenclature (5, 6), we propose P4501 α be known as CYP27B1.

Metabolism of 25(OH)D₃ in the P4501 α -Transfected COS-7 Cells. To confirm that P4501 α can catalyze the hydroxylation of 25(OH)D₃ at 1 α -position, we examined the metabolism of 25(OH)D₃ in COS-7 cells transfected with the plasmid expressing P4501 α . Expression of P4501 α mRNA was confirmed by RT-PCR. Control COS-7 cells did not express the P4501 α mRNA. A marked expression of P4501 α mRNA was observed in the cells transfected with cDNA P4501 α (data not shown). Following a 20-h incubation with 25(OH)D₃, COS-7 cells transfected with pcDNA-1 α generated a metabolite that comigrated with authentic 1 α ,25(OH)₂D₃ (Fig. 4). This experiment has been repeated three times with identical results. 1 α ,25(OH)₂D₃ was not generated in the cells transfected with a pcDNA empty vector under the same conditions. These results indicate that P4501 α cDNA catalyzes the 1 α -hydroxylation reaction of 25(OH)D₃.

Expression of CYP24 and P4501 α in the Kidney. Next, we examined the expression of CYP24 and P4501 α mRNAs in the kidney, the endocrine organ of vitamin D. The expression level of the CYP24 gene was increased by the administration of 26,27-F₆-1 α ,25(OH)₂D₃ (Fig. 5B). No CYP24 mRNA was detected in the kidney of vitamin D-deficient rats. In contrast, the expression level of renal P4501 α mRNA was greatly

FIG. 2. The nucleotide sequence of rat P4501 α cDNA and its deduced amino acid sequence. Arrows indicate primer positions for isolation of P4501 α fragment. The region of the heme-binding site is underlined. The conserved amino acids known to be important in ferredoxin-binding are circled. The start codon is shown in bold type.

increased in the kidney of vitamin D-deficient rats (Fig. 5A). No transcript was detected in the kidney of 26,27-F₆-1 α ,25(OH)₂D₃-treated rats. 1 α ,25(OH)₂D₃ is synthesized from 25(OH)D₃ by P4501 α exclusively in the mitochondria of renal PCT (21, 22). Fig. 6 shows the preferential expression of CYP24 and P4501 α mRNAs in microdissected PCT of each rat model. Interestingly, P4501 α mRNA in PCT was increased significantly in rats with enhanced renal 1 α -hydroxylase activity (rats fed a low calcium diet). In contrast, expression of CYP24 mRNA in PCT was down-regulated in rats fed a vitamin D-deficient or -repleted low Ca diet. The expression level of P4501 α mRNA in PCT was inversely correlated with the expression of CYP24 mRNA.

Effects of Low Calcium and Low Phosphorus Feeding on the Expression of P4501 α . Fig. 7 shows the expression of renal P4501 α mRNA after rats had been maintained on a vitamin D-replete diet containing 1.2% calcium and 0.6% phosphorus (Normal), 0.03% calcium and 0.6% phosphorus (Low Ca), or 1.2% calcium and 0.1% phosphorus (Low P) for 2 or 3 weeks. In the low calcium group, expression of P4501 α mRNA was greatly and time-dependently increased as compared with that

P4501 α	1	MT-QA-VKL--A---SRVF-H-----RVQ--LPSQL-----G-S	23
CYP24	1	--MSPIDKRRRTLIAFLRLRLD--GQPPRSVTSKASASRAPKEVPLCLPM	47
CYP27	1	MAVLSRRLRLWALLDTRVMGHLGCPQGA RAKAAI PAALRDHESTEGPGTG	50
P4501 α	24	-D-SVLRSLSDIPGPSTPSFL-AEL--FCKGGLSRHLQVHGAAARYGPI	86
CYP24	48	TD-GETRNVTSLPQPTNNPLGSLLEIFWKGGLKKQHDTLAEYHKYGGQI	96
CYP27	51	QDRPRLSLAELFPGPTLRFL-FQL--FLRGVYLHLQALNKAKYGP	97
P4501 α	89	WSGSFGLTRTVYVADPALVEQLLRQESHCPERCFSFSSWSEHRRRHQRACG	118
CYP24	97	FRMKLGSDSVHLGSPSLALYRTESAPQLRIKPKWAKYDRHRNEAYG	146
CYP27	98	WTTTFGTRTNVNLASAPLLEQVMRQEGKYPIRDSMEQWKEHDKHGLSVG	147
P4501 α	119	LLTADGEEWQRLRSLLAPLLLRPQAAAGYAGTLDVSVSDVLRRLRRQRGR	168
CYP24	147	LMILEGGQWQVRSAFQKLMKPEVIMKDKKINEVLADFLERMDELCEDE	196
CYP27	148	IFITQGGQWYHLRLSLNQRMLKPAEALYTDALNEVISDFIARLDQVRTE	197
P4501 α	169	GSGLPDLVLDVAGEFYKFGLEGIGAVLLGSRIGCLEAEVPPDTFTIEAV	218
CYP24	197	RGRIPDLYSELNKSF---ESICLVLYEKRFGLLKQKETEELTFITAI	242
CYP27	198	SAS-GDQVPDVAHLLYLALAEATCYILFEKRVGCLFSPIDETATFIRSV	246
P4501 α	219	GSFVSTLLTMAMP-SMLHRLIPGPWALRCRDWDQMFQAFQKHVEQREGE	267
CYP24	243	KTMSTFGKHMVTPEVLHRLNLTVMQAHTLAWDTIFK-SVKPCIDNRLQ	291
CYP27	247	GLMFKNVYVTFLP-KWSRPLLP-FWKRYNNWNIIFSFGKMKIHQVQ	294
P4501 α	268	AAVRNQKPEEDLPTGHHLTHFLF-REKVSQSVIGNVTELLLAGVDTVS	316
CYP24	292	-RYSQPGADFLCDIYQ--DH-LSKKELYA--AV--TEQLAAVETTA	332
CYP27	295	IEAQLQAAGPDGVQVSGYL-HFLLTKELLSPPQETVGTFFELLILAGVDTTS	343
P4501 α	317	NTLSWALVELSRHPEVQSAHLSBITGAUNPGSYAHL-QATALSQFLPLKA	365
CYP24	333	NSLMWILYNLSRNPQAQRRLQEVQSVL-PDN--QTPRAEDLRNMPYLKA	379
CYP27	344	NTLTWALYHLSKNPIQEAHKEVTGVV-P--FGKVPQNKDFAHMPLKA	390
P4501 α	336	VIKEVLRLLYPVVPNSR-VPRDIDICVGNVYIPQDTLVSLCHYATSRDPAQ	414
CYP24	380	CLKESMRLTPSVFTTR-TLDKPTVLGEYALPKGTVLTLNTQVLGSSDN	428
CYP27	391	VIKETRLYPVVTNSRIITEKETEINGFLFPKNTQFVLCHYVVSRRDPSV	440
P4501 α	415	FREPNSFNPARWL---GEGPA-PHPFASLPFGFGKRSCIGRRILAELEQ	459
CYP24	429	FEDSHKFRPERWLQK-E---KKI-NFPAHLFFGIGKRCIGRRILAEQLH	473
CYP27	441	FPEPESFQPHRWLRKRREDDNSGIFHPFGSVFPGYGVRSCLGRRILAELEQ	490
P4501 α	460	MALAQILTHFEVLPEPGALPVKPMTRTVLVPERSIHLQFVDR-	
CYP24	474	LALCMTIQKYDI-VATDNPEVMEHLGLVPSRELPIAFRPR-	
CYP27	491	LLLSRLIQKYEVVLSPGMGVSVSRIVLVPSSKKVSLRFLQKQ	

FIG. 3. Alignment of P4501 α with closely related other CYPs. The closest relatives to P4501 α were determined by using the BLASTP program provided by the National Center for Biotechnology Information, and the amino acid sequences were manually aligned to P4501 α . CYP27, the predicted protein from the rat mitochondrial vitamin D₃-25-hydroxylase (GenBank accession no. M38566); CYP24, the predicted protein from the rat mitochondrial 25(OH)D₃-24-hydroxylase (GenBank accession no. X59506). Identical residues are shown in bold type, and dashes represent gaps for alignment purposes.

in the normal group. In contrast, the expression level of P4501 α mRNA in the low P feeding group was moderately increased as compared with the low Ca group.

DISCUSSION

The present study describes the cloning and sequencing of 25(OH)D₃-1 α -hydroxylase (P4501 α). The P4501 α has been expressed in COS-7 cells and metabolism of 25(OH)D₃ into 1 α ,25(OH)₂D₃ could be observed, thus verifying that P4501 α encodes a 25(OH)D₃-1 α -hydroxylase. Vitamin D is metabolized by three major cytochrome P450-containing enzymes—the liver 25-hydroxylase (CYP27), the renal 1 α -hydroxylase, and the renal and intestinal 24-hydroxylase (CYP24) (3, 4). P4501 α and CYP24 are renal P450 enzymes synthesized from nuclear transcripts. P4501 α and CYP24 are both embedded in the inner membrane of the mitochondria facing the matrix. The major location of P4501 α and CYP24 is in the inner mitochondrial membrane of PCT cells of the kidney (21, 22). In agreement with the previous studies (18, 19, 21, 22), P4501 α and CYP24 mRNAs were expressed in the PCT of the renal nephron (Fig. 6). They receive electrons from a common electron donor, ferredoxin, and catalyze the vitamin D hydroxylation reactions. These common features must be related to the conserved region of the molecules. As described previously, the putative heme-binding portion is located in the highly conserved regions (5, 6). The peptide region that interacts with ferredoxin must also be conserved (20). The amino acid sequence of 1 α -hydroxylase (P4501 α) contained a conserved cysteine residue located at position 448 that was considered to be a ligand for the heme ion (Figs. 1 and 2).

FIG. 4. Metabolism of 25(OH)D₃ in COS-7 cells transfected with the expression plasmid for P4501 α . Metabolism of 25(OH)D₃ by the cells transfected with 10 μ g of pcDNA 1 α (A) and the same amount of pcDNA empty vector (B). The retention times of 25(OH)D₃, 24,25(OH)₂D₃, 10-oxo-19-nor-25(OH)D₃, and 1 α ,25(OH)₂D₃ are indicated. 25(OH)D₃ was metabolized into 1 α ,25(OH)₂D₃ only in the cells transfected with pcDNA 1 α . The fraction containing 1 α ,25(OH)₂D₃ was further applied to HPLC under the following two conditions: column, Finepak SIL 4.6 $\times$ 250 mm; mobile phase, 8% 2-propanol in hexane; flow rate, 1 ml/min; column, J'-sphere ODS-AM 4.6 $\times$ 150 mm (YMC Co., Kyoto, Japan); mobile phase, 10% water in methanol; flow rate, 1.0 ml/min (data not shown). In all cases the putative 1 α ,25(OH)₂D₃ comigrated with authentic 1 α ,25(OH)₂D₃. Similar results were obtained in three independent sets of experiments.

Subjecting the deduced amino acid sequence to a computer homology search, it was found that the enzyme is 43.6% identical and 53% similar to a cytochrome P450 isolated from rat liver mitochondria catalyzing CYP27. It is 42.6% similar and 34% identical to the 25-hydroxyvitamin D-24-hydroxylase. Based on current nomenclature (5, 6), we propose P4501 α be known as CYP27B1.

Recently, Guo *et al.* (23) isolated a cDNA for the human liver mitochondrial vitamin D₃-25-hydroxylase by 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 the human cholestetriol 27-hydroxylase cDNA. Axén *et al.* (24) also reported

FIG. 5. Expression of CYP24 and P4501 α mRNAs in rat kidney. Poly(A)⁺ RNAs (5 μ g) were prepared from rats that had been treated with either 26,27-F₆-1 α ,25(OH)₂D₃ or vehicle. 26,27-F₆-1 α ,25(OH)₂D₃ (1 μ g/rat) was intravenously administered to vitamin D-deficient low Ca rats. Rats were killed 48 h after administration of either 26,27-F₆-1 α ,25(OH)₂D₃ or vehicle. The blots were probed with [³²P]dCTP labeled P4501 α (A) and CYP24 (B). Similar results were obtained in three independent sets of experiments.

FIG. 6. Expression of CYP24 and P4501 α mRNAs in the kidney of each rat model. Radioautography of RT-PCR products of CYP24 and P4501 α mRNAs in microdissected PCT. Three-week-old rats were fed a vitamin D-replete or a vitamin D-deficient low Ca diet for 2 weeks. Microdissection and RT-PCR were performed as described (18, 19) with a slight modification. Final volume of the PCR reaction was 20 μ l, and composition of the solution was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, gelatin 0.01%, 200 nM sense and antisense primers, 0.1 mM dNTPs, 74 kBq of [³²P]dCTP, and 1 unit of *Taq* DNA polymerase. The program of PCR was as follows: 25 cycles of 94°C for 40 sec, 50°C for 1 min, and 72°C for 1 min. The expected size of RT-PCR products for P4501 α and CYP24 were 253 and 482 bp, respectively.

that CYP27 of pigs and humans catalyzes not only 27-hydroxylation of cholestanetriol and 25-hydroxylation of vitamin D₃, but also 1 α -hydroxylation of 25(OH)D₃. Because the 1 α -hydroxylase activity toward 25(OH)D₃ is much lower than that of the other two enzyme activities and also the liver does not seem to be involved in the 1 α -hydroxylation reaction of 25(OH)D₃ at least in mammals, the physiological meaning of this activity remains to be elucidated. Andersson *et al.* (25) reported that CYP27 is localized not only in the liver but also in the kidney, lung, and spleen. However, the degree to which the CYP27 is regulated hormonally is not clear.

P4501 α is the only enzyme physiologically responsible for the biosynthesis of 1 α ,25(OH)₂D₃ from 25(OH)D₃ (1, 2). The regulatory mechanism of 1 α -hydroxylase in the kidney remains unclear, because its cDNA has not yet been cloned. Renal 1 α -hydroxylase activity is regulated by a number of physiological factors including parathyroid hormone (PTH),

FIG. 7. Expression of CYP24 and P4501 α mRNAs in 3-week-old rats maintained for 2–3 weeks on a vitamin D-repleted diet containing 1.2% Ca and 0.6% P (Normal), 0.03% Ca and 0.6% P (Low Ca), or 1.2% Ca and 0.1% P (Low P). Poly(A)⁺ RNA (5 μ g) was electrophoresed and probed for P4501 α . Similar results were obtained in three independent sets of experiments.

1 α ,25(OH)₂D₃, calcitonin, insulin, calcium, phosphorus, growth hormone, and prolactin (1, 2). Among these factors, PTH is a major regulator of 1 α ,25(OH)₂D₃ synthesis in the kidney. The mechanism of action of PTH on renal 1 α -hydroxylase has not been fully elucidated. Previous studies have demonstrated that cAMP is a major intracellular mediator in the stimulation of 1 α -hydroxylase activity by PTH (26). On the other hand, 1 α ,25(OH)₂D₃ production is inhibited by high serum concentration of calcium and 1 α ,25(OH)₂D₃ *per se*. In agreement with the previous reports (17, 19), renal expression of P4501 α mRNA was increased in the state of the enhanced synthesis of 1 α ,25(OH)₂D₃ (Figs. 5 and 7).

In rats fed a low phosphorus diet, the direction of regulation of serum levels of PTH and calcium was opposite to that of intact rats fed a low calcium diet. A possible common event that can occur in both of the rats fed a low calcium diet and a low phosphorus diet may be a decrease of the intracellular phosphorus level in renal proximal tubules (19). Feeding of rats with a low calcium diet or a vitamin D-deficient diet markedly stimulates 1 α -hydroxylase activity (17, 19). In contrast, feeding a low phosphorus diet moderately stimulates 1 α -hydroxylase activity (19). To clarify whether this increase of the enzyme activity is caused by a pretranslational induction, changes of the mRNA level in the kidney were examined by Northern blot analysis. The results showed that expression level of 1 α -hydroxylase was regulated at the mRNA level (Fig. 7).

The cloning of the P4501 α gene will now make possible a full understanding how 1,25(OH)₂D₃ synthesis in the renal PCTs is regulated at the molecular level.

We thank Prof. Colin Jefcoate (University of Wisconsin, Madison) and Prof. Yoshihiko Ohya (Hiroshima University) for their help with P450 nomenclature as well as their helpful reviews.

- DeLuca, H. F. (1988) *FASEB J.* **2**, 224–236.
- Suda, T., Shinki, T., & Kurokawa, K. (1994) *Curr. Opin. Nephrol. Hypertens.* **3**, 59–64.
- Armbrecht, H. J., Okuda, K., Wongsurawat, N., Nemani, R. K., Chen, M. L., & Boltz, M. A. (1992) *J. Steroid Biochem. Mol. Biol.* **43**, 1073–1081.
- Okuda, K., Usui, E., & Ohya, Y. (1995) *J. Lipid Res.* **36**, 1641–1652.
- Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., & Nebert, D. W. (1996) *Pharmacogenetics* **6**, 1–42.
- Graham-Lorence, S. E., & Peterson, J. A. (1996) *Methods Enzymol.* **272**, 315–326.
- Usui, E., Noshiro, M., & Okuda, K. (1990) *FEBS Lett.* **262**, 135–138.
- Ohya, Y., Noshiro, M., & Okuda, K. (1991) *FEBS Lett.* **278**, 195–198.
- St-Arnaud, R., Moir, M., Messerlian, S., & Glorieux, F. H. (1996) *J. Bone Miner. Res.* **11** (Suppl. 1), S-124 (abstr.).
- Nicoll, D. A., Quednau, B. D., Qui, Z., Xia, Y.-R., Lusis, A. J., & Philipson, K. D. (1996) *J. Biol. Chem.* **271**, 24914–24921.
- Shen, Z., Wells, R. L., Liu, J., & Elkind, M. M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11483–11487.
- MacDonald, R. J., Swift, G. H., Przybyla, A. E., & Chirgwin, J. M. (1987) *Methods Enzymol.* **152**, 219–227.
- Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
- Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Miyamoto, Y., Shinki, T., Yamamoto, K., Ohya, Y., Iwasaki, H., Hosotani, R., Kasama, T., Takayama, H., Yamada, S., & Suda, T. (1997) *J. Biol. Chem.* **272**, 14115–14119.
- Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
- Shinki, T., Jin, C. H., Nishimura, A., Nagai, Y., Ohya, Y., Noshiro, M., Okuda, K., & Suda, T. (1992) *J. Biol. Chem.* **267**, 13757–13762.
- Iida, K., Taniguchi, S., & Kurokawa, K. (1993) *Biochem. Biophys. Res. Commun.* **194**, 659–664.

19. Iida, K., Shinki, T., Yamaguchi, A., DeLuca, H. F. Kurokawa, K. & Suda, T. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6112–6116.
20. Nebert, D. W. & Gonzalez, F. J. (1987) *Annu. Rev. Biochem.* **56**, 945–993.
21. Brunette, M. G., Chan, M., Ferriere, C. & Roberts, K. D. (1978) *Nature (London)* **276**, 287–289.
22. Kawashima, H., Torikai, S. & Kurokawa, K. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1199–1203.
23. Guo, Y.-D., Strugnell, S., Back, D. W. & Jones, G. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8668–8672.
24. Axén, E., Postlind, H., Sjöberg, H. & Wikvall, K. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 10014–10018.
25. Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H. & Russell, D. W. (1989) *J. Biol. Chem.* **264**, 8222–8229.
26. Horiuchi, N., Suda, T., Takahashi, H., Shimazawa, E. & Ogata, E. (1977) *Endocrinology* **101**, 969–974.