Structural Characterization of the Gene Encoding Rat 25-Hydroxyvitamin D_3 24-Hydroxylase[†,‡]

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ABSTRACT: The structural gene encoding 25-hydroxyvitamin D_3 24-hydroxylase (P-450cc24) was isolated from the rat genomic DNA. It spans ≈15 kb, is composed of 12 exons, and was demonstrated by Southern blot analysis to be present as a single copy. One major T residue was identified at the cap site, a putative TATA (ATAAATA) box was located at position −30, and a putative CCAAT box was at −58. Four possible vitamin D responsive elements that may be involved in regulation of 24-hydroxylase expression were found in the 5′-flanking region. Alignment with mitochondrial P-450 proteins showed that 7 out of 11 intron insertion sites of P-450cc24 gene occupied positions identical with those in the *CYP11* family ($P\text{-}450_{scc}$, $P\text{-}450_{11\beta}$). The structure of the gene is discussed in relation to present knowledge about the mechanism of regulation of the 25-hydroxyvitamin D_3 24-hydroxylase and calcium homeostasis.

Vitamin D_3 is metabolized by a network of P-450-containing hydroxylases. In rat liver, both microsomal and mitochondrial 25-hydroxylases are present: P-450cc25 (Björkhem & Holmberg, 1978; Björkhem et al., 1979; Hayashi et al., 1986; Andersson & Jörnvall, 1986) and P-450LMT25 (Björkhem et al., 1980; Masumoto et al., 1988; Usui et al., 1990), respectively. In kidney mitochondria, there are both a 1α-hydroxylase (Moorthy et al., 1991) and a 24-hydroxylase (Pedersen et al., 1983; Ohyama et al., 1989, 1991). Their activities are regulated by the level of serum Ca, phosphate, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),[1] and/or other physiological factors such as parathyroid hormone (PTH) (Brommage & DeLuca, 1985; DeLuca, 1988; Minghetti & Norman, 1988).

Brommage and DeLuca (1985) speculated that P-450cc24 may 24-hydroxylate not only $25(OH)D_3$ but also $1,25(OH)_2D_3$, which is the most biologically active form under physiological conditions. This hypothesis was experimentally verified by Ohyama and Okuda (1991) using purified 25-hydroxyvitamin D_3 24-hydroxylase. Indeed, the enzyme hydroxylated $1,25(OH)_2D_3$ at position 24 with a turnover number of 6 nmol min^{-1} (nmol of P450)$^{-1}$. Although the enzyme hydroxylates $25(OH)D_3$ more efficiently [turnover number 19.7 nmol min^{-1} (nmol of P450)$^{-1}$], this does not necessarily mean that $25(OH)D_3$ is the sole physiological substrate. In view of the fact that $1,24,25(OH)_3D_3$ has 60% of the activity of vitamin D_3 in curing ricket (Holick et al., 1973), the possibility must be considered that 24-hydroxylase is merely a detoxification enzyme working for clearance of $1,25(OH)_2D_3$. However, there are also data supporting a physiological function of $24,25(OH)_2D_3$ [e.g., see Henry and Norman (1978), Sömjen et al. (1987), and Matsumoto et al. (1988)]. At the present state of knowledge, there is no clear-cut answer to the question of which viewpoint should be preferred.

Recent successful purification of $25(OH)D_3$ 24-hydroxylase from rat kidney mitochondria (Ohyama et al., 1989) enabled us to prepare specific antibodies against 24-hydroxylase and to clone the cDNA encoding the enzyme (Ohyama et al., 1991). The cloning made it possible to study regulation at the gene expression level. Using the probes prepared on the basis of the cDNA sequence, it was demonstrated by Northern blot that the induction of 24-hydroxylase occurs at the pretranslational level in both kidney and intestine (Shinki et al., 1992; Ohyama et al., 1991; Armbrecht & Boltz, 1991). Such a mechanism had also been suggested by Tomon et al. (1990a,b) based on studies with inhibitors of transcription and translation.

In order to examine the induction mechanism of 24-hydroxylase in more detail at the molecular level, we have now isolated and characterized the gene encoding P-450cc24 (designated as the *CYP24*[2,3] according to the recent gene nomenclature). In addition, we have carried out alignment analyses to elucidate the structural similarity and phylogenetic relationship to the other mitochondrial P-450 genes.

EXPERIMENTAL PROCEDURES

All enzymatic manipulations were done according to standard procedures (Sambrook et al., 1989).

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[1] Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $1,24,25(OH)_3D_3$, 1α,24,25-trihydroxyvitamin D_3; PTH, parathyroid hormone; P-450, cytochrome P-450; P-450cc24, 25-hydroxyvitamin D_3 24-hydroxylase; $P\text{-}450_{scc}$, cholesterol side chain cleavage cytochrome P-450, gene product of *CYP11A*; $P\text{-}450_{11\beta}$, steroid 11β-hydroxylase cytochrome P-450, gene product of *CYP11B*; P-450LMT25, gene product of *CYP27*; kbp, kilobase pair(s); SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction.

[2] D. W. Nebert, personal communication.

[3] The P-450 gene and gene product are described according to the nomenclature recommended by Nebert et al. (1991): italics for the gene and cDNA; nonitalics for mRNA and protein.

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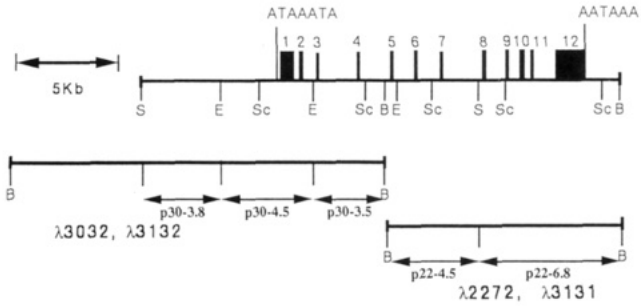

FIGURE 1: Restriction map and gene structure of rat 24-hydroxylase. Four clones were isolated from a λEMBL3 library constructed with a *Bam*HI digest of rat genomic DNA. The arrangement of two clones on the gene was determined by both genomic Southern blot analysis using the full-length cDNA as a probe and sequencing of the PCR product. The positions and relative sizes of the exons and introns are schematically presented in the upper portion of the figure. Exons are indicated by the numbered boxes. The positions of all cleavage sites for restriction enzymes used for Southern blot analysis are shown along the gene schematic: S, *Sal*I; B, *Bam*HI; E, *Eco*RI; Sc, *Sac*I. The fragments of 3.8 (p30-3.8), 4.5 (p30-4.5), 3.5 (p30-3.5), 4.5 (p22-4.5), and 6.8 kb (p22-6.8) were subcloned into pUC19 and Bluescript II for sequence analysis.

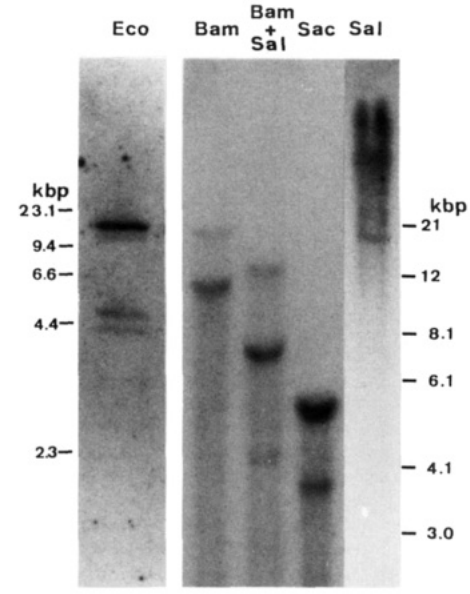

FIGURE 2: Southern blot analysis of rat genomic DNA. Ten micrograms of genomic DNA was digested with the indicated restriction enzymes and subjected to Southern blot analysis with the probe derived from the full insert of P-450cc24 cDNA as described under Experimental Procedures. The positions of standard size markers migrated in an adjacent well of the gel are shown on the left and right of the panels. Eco, *Eco*RI; Bam, *Bam*HI; Sal, *Sal*I; Sac, *Sac*I.

Materials. Restriction and modifying enzymes were purchased from Boehringer Mannheim GmbH (Germany), Toyobo Inc. (Osaka, Japan), and Takara Shuzo (Kyoto, Japan). Pharmacia LKB Biotechnology Inc. supplied Oligolabelling Kit, and λEMBL3 was obtained from Stratagene (San Diego, CA). [α-^{32}P]dCTP (111 TBq/mmol), [γ-^{32}P]-dATP (111 TBq/mmol), and [^{35}S]dCTP (50 TBq/mmol) were obtained from Du Pont—New England Nuclear.

Construction of the Genomic DNA Library. Genomic DNA prepared from Wistar rat spleen (Wong et al., 1987) was digested with *Bam*HI, and ligated into λEMBL3 phage vector. The recombinant DNA was packaged with Gigapack Gold II (Stratagene) and transfected into *Escherichia coli* P2392.

Cloning and DNA Sequencing. The library was plated and screened by plaque hybridization (Sambrook et al., 1989) using the full-length rat P-450cc24 cDNA as a probe (Ohyama et al., 1991), which was labeled with [α-^{32}P]dCTP by the random primer method. Hybridization was done at 65 °C for 16–20 h with 6 × SSC, 5 × Denhardt's, 10 mM EDTA, 1% SDS, and 100 μg/mL sonicated salmon sperm DNA. The filters were washed 3 times at room temperature for 15 min with 0.2 × SSC containing 0.2% SDS, and finally with 0.1 × SSC containing 0.2% SDS at 55 °C for 30 min. Positive clones were plaque-purified, and phage DNA was isolated. The inserts cut out with *Bam*HI were digested with selected restriction enzymes and subcloned into BluscriptII and pUC19. The deletion mutants of plasmids (Yanish-Peron et al., 1985) were prepared by a deletion kit (Takara Shuzo., Kyoto, Japan) for DNA sequencing analysis. Sequencing was carried out on alkaline-denatured plasmid DNA templates using a Sequenase kit (United States Biochemical Corp.) following the manufacturer's instructions. Sequence data were assembled with the DNASIS program (Hitachi Software Engineering Co., Japan).

PCR Study. PCR studies of rat genomic DNA were performed using peripheral blood leukocytes of Wistar rats (Mullis et al., 1986).

RNA Isolation. Total RNA was prepared from kidney of rat by guanidine hydrochloride extraction (Cox, 1968) and enriched for poly(A)$^+$ RNA by oligo(dT) affinity chromatography.

Southern Hybridization Analysis. Rat genomic DNA was digested with appropriate restriction enzymes, electrophoresed on a 0.7% agarose gel, and transferred to Nytran (Schleicher & Schuell). Hybridization and washing of filters were carried out as described above.

Primer Extension Analysis and S1 Nuclease Mapping. The transcription start site was determined by both primer extension and S1 mapping (Sambrook et al., 1989). Primer extension was carried out with 26-mer oligonucleotide corresponding to positions +83 to +108 (Figures 3 and 4). The primer was labeled by ^{32}P at the 5′ end (1 × 10^5 cpm) with polynucleotide kinase, annealed to 5 μg of rat poly(A)$^+$ RNA, and extended with 20 units of AMV reverse transcriptase (Life Sciences). For S1 nuclease protection, a 395 bp fragment encompassing the first exon and upstream region of the gene was isolated from genomic subclone p30-4.5 with the enzymes *Pst*I and *Alu*I which cleave at −287 and +108, respectively (Figure 4). The fragment was also labeled at the 5′ end by kinase reaction, denatured by heating at 100 °C, and loaded on a 5% polyacrylamide gel for strand separation. The single-stranded DNA (2 × 10^4 cpm) was hybridized with 5 μg of poly(A)$^+$ RNA and digested with S1 nuclease. S1 nuclease protected fragments and primer-extended materials were subjected to electrophoresis on an 8% polyacrylamide–urea sequencing gel adjacent to a sequencing ladder primed with the same 26-mer oligonucleotide.

Alignment of Amino Acid Sequences of Mitochondrial P-450 and the Phylogenetic Tree. Optimal alignment between a pair of P-450 protein sequences was obtained by the algorithm of Gotoh (1990). Differences in amino acid sequences were calculated by assuming a stretch of deletion/insertion as a single mismatch, and then converted to evolutionary distances in PAM units (Dayhoff et al., 1978). A phylogenetic tree of P-450 proteins was obtained from the distance values by the UPGMA (unweighted-pair-group method of analysis) method (Sneath & Sokal, 1973). The tree was used to align progressively protein sequences of various P-450s as described by Gotoh and Fujii-Kuriyama (1989).

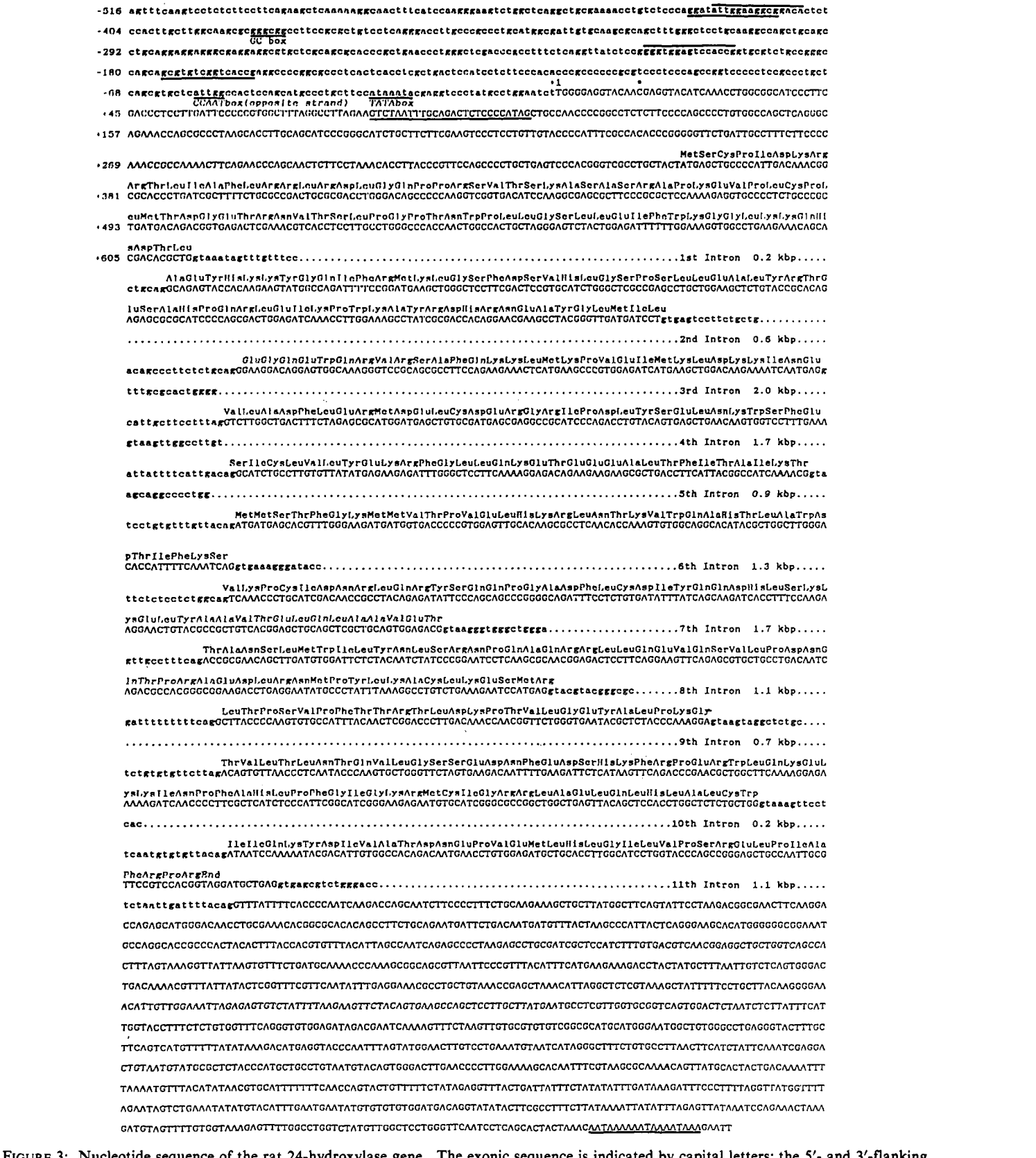

FIGURE 3: Nucleotide sequence of the rat 24-hydroxylase gene. The exonic sequence is indicated by capital letters; the 5′- and 3′-flanking sequences and those of introns are indicated by lower case letters. The exon–intron boundaries were determined in reference to the cDNA sequences and the G-T/A-G consensus sequence. The mRNA cap site, determined by S1 mapping and primer extension (see Figure 3), is designated +1. Most introns were not completely sequenced owing to their large size. Instead, the intron sizes were determined by agarose gel electrophoresis. The predicted amino acid sequence is indicated above the corresponding nucleotide sequence. The asterisk above the sequence indicates the 5′ end of 24-hydroxylase cDNA (Ohyama et al., 1991). Putative TATA box, CCAAT boxes, the poly(A)-additional signal (AATAAA), and the GC box (SpI binding site) are underlined. The sequences homologous to VDRE (vitamin D responsive element) of the rat osteocalcin gene reported by MacDonald et al. (1991) are also overlined in the 5′-flanking region. Two underlines in the 5′-flanking region indicate homologous sequences to VDRE (Darwish & DeLuca, 1992) of the rat calbindin D-9k gene. The 26-nucleotide primer used for primer extension analysis is also underlined in exon 1 (+83 to +108).

RESULTS

Isolation of Clones Containing the Rat 24-Hydroxylase Gene. Southern blot analysis of *Bam*HI-digested genomic DNA using a full-length P-450cc24 cDNA probe revealed two bands, one at 18 kb and the other at 11 kb (Figure 2). A rat genomic library was therefore constructed by digesting rat spleen DNA with *Bam*HI and inserting the resulting fragments into λEMBL3 phage vector. Four hybridization-positive clones were isolated after screening of approximately 3×10^5 recombinant clones using the same cDNA probe. The inserts in these genomic clones were subjected to restriction mapping. As a result, they were classified into two groups: one (λ3032, λ3132) containing an 18 kbp fragment and the other (λ2272, λ3131) carrying an 11 kbp fragment as shown in Figure 1. The sizes of these fragments agree with those of the *Bam*HI-digested fragments observed in the Southern blot analysis (Figure 2). The inserts of λ3032 and λ2272 were digested with appropriate restriction enzymes, subcloned into plasmid vectors, and used for sequence analysis.

Characterization of 24-Hydroxylase Gene Structure. λ3032 and λ2272 contained four exons (exons 1–4) and eight exons (exons 5–12), respectively (Figure 1). These 12 exons included the entire cDNA sequence of rat 24-hydroxylase. To confirm that these two clones were located side-by-side in the rat genome, Southern blot analysis was carried out using the full-length cDNA. As shown in Figure 2, a 16 kbp fragment from *Sal*I-digested genomic DNA, 4.2 kbp from *Eco*RI digestion, and 3.5 kbp from *Sac*I digestion were found to cover the junction of the two clones. That the clone λ3032 continues to the clone λ2272 was confirmed by cloning and sequencing the PCR product covering the junction (data not shown). The sizes and numbers of hybridizing fragments in different restriction enzyme digests agreed well with those predicted from the restriction map in Figure 1. These results indicate that the 24-hydroxylase gene is a single gene in rat and spans approximately 15 kbp consisting of 12 exons.

The sequences of the exons and parts of the introns of this gene are shown in Figure 3. The exons were found to be completely identical to those of the cDNA. An asterisk in Figure 3 indicates the 5′ end of the cDNA reported previously (Ohyama et al., 1991). Exons 1–12 consisted of 614, 191, 94, 97, 92, 112, 146, 167, 79, 198, 120, and 1312 base pairs, respectively. Exon 10 contains the heme binding region, and the last exon encodes the entire 3′-untranslated region of the mRNA. The DNA sequences of the intron–exon junctions were found to follow the canonical GT-AG rule of eukaryotic genes (Mount, 1982).

Characterization of the Transcription Start Site and the 5′-Flanking Sequence. As shown in Figure 4, the transcription start site was determined by both S1 nuclease mapping and primer extension. The *Pst*I–*Alu*I fragment (395 bp) derived from the clone p30-4.5 (Figure 1) was used in S1 nuclease analysis. Primer extension was carried out using a synthetic oligonucleotide (26-mer) located next to the *Alu*I site (underlined in Figure 3). Both S1 nuclease mapping and primer extension carried out with two separate preparations of mRNA (lanes 1 and 2 in Figure 4 for each method) indicated an identical nucleotide T as the transcription start site (shown by an arrow in Figure 4). This result, together with the fact that P-$450_{17\alpha}$ (Bhasker et al., 1989) and P-$450_{15\alpha}$ (Lindberg et al., 1989) have their transcription start site at a nucleotide T and the transcription of most eukaryotic genes including those of P-450s is known to start with a nucleotide A (Breathnach & Chambon, 1981), puts P-450cc24 into a small group of genes have a nucleotide T at the start site. This

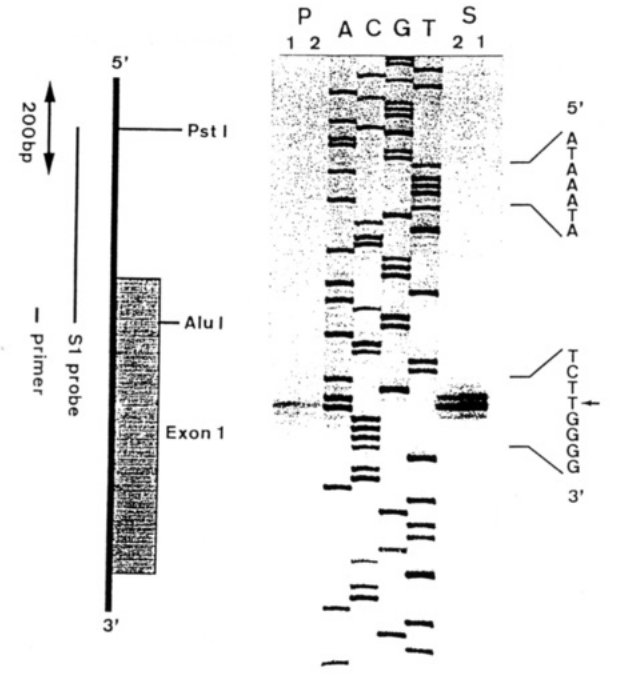

FIGURE 4: Transcription initiation site of the rat 24-hydroxylase gene. S1 nuclease mapping and primer extension were carried out on rat kidney poly(A)$^+$ RNA as described under Experimental Procedures. P, primer extensions; S, S1 nuclease mapping. Separate preparations of poly(A)$^+$ RNA were used in lanes 1 and 2, respectively. The 5′ end of the S1 mapping is coincident with the 5′ end of the extended primer. A DNA sequence ladder was prepared using the same primer for primer extension. The arrow indicates the initiation site of transcription. A putative TATA box is also indicated in the figure.

transcription start site was located at 357 base pairs upstream from the amino-terminal methionine. A putative TATA box (ATAAATA) was found at 30 bp upstream from the start site (Figure 3). A putative CCAAT box (ATTGG in the complementary strand) was also detected at 58 bp upstream from the start site (Figure 3). A computer-aided search for transcriptional regulation elements 5′ upstream of exon 1 was performed as shown in Figure 3 (see Discussion).

Comparison of Gene Structures of Mitochondrial P-450s. Gene structures of two mitochondrial P-450 subfamilies (P-450_{scc} and P-$450_{11\beta}$, both belonging to the *CYP11* gene family) have been reported previously. The two subfamilies have a similar exon–intron organization; i.e., nine exons are interrupted by eight introns. Figure 5 shows the alignment of protein sequences of these two P-450s together with P450cc24 with exhibition of exon/intron boundaries. Seven introns out of 11 in *CYP24* were found in *CYP11* at the corresponding codon positions with the same phases. Thus, all introns except intron 1 in the *CYP11* genes exist in *CYP24*, although the latter has four additional introns that are lacking in *CYP11*. Furthermore, some important amino acid residues conserved in most P-450s were also conserved in this enzyme (Figure 5). Figure 6 shows a phylogenetic tree obtained by analysis of the mitochondrial P-450 protein sequences. Among the known mitochondrial P-450s, P-450cc24 is most closely related to the *CYP27* family encoding a protein catalyzing both sterol 27-hydroxylation and vitamin D 25-hydroxylation (Usui et al., 1990). These two P-450s were estimated to have diverged 3.5×10^8 years ago.

DISCUSSION

We isolated and characterized a rat vitamin D 24-hydroxylase gene using a cDNA of the enzyme as a probe.

FIGURE 5: Alignment of mitochondrial P-450 amino acid sequences and comparison of the positions of introns. Locations of introns of the 24-hydroxylase gene (*CYP24*) on the aligned amino acid sequence were compared with those of human *CYP11A* (Morohashi et al., 1987) and bovine *CYP11B* (Hashimoto et al., 1989). Numbers on the left side indicate the position of amino acids. The locations of introns are indicated by triangles: (▼) intron lies between the codons for flanking amino acids; (▶) intron lies between the first and second nucleotide of the following amino acid; (◀) intron lies between the second and third nucleotide of the codon for the preceding amino acid. Some highly conserved peptides in the P-450 family (Gotoh & Fujii-Kuriyama, 1989) are underlined.

The gene spanned about 15 kbp and contained 12 exons. The number of exons in the 24-hydroxylase gene was relatively large compared to those of other mammalian P-450 species which contain 6–13 exons. Exons 2–11 of *CYP24* included 70–200 nucleotides as has been observed also in other P-450s. The first exon consisted of 614 bp containing 356 bp of 5′-noncoding region. To our knowledge, the sizes of exon 1 and the 5′-noncoding region are the largest of all the P-450 genes so far reported. The fact that the 5′-noncoding region is unusually long and rich in GC, as shown in Figure 3, may be related to the stability and transcriptional efficiency of mRNA, which are likely to be controlled through the secondary structure as suggested by Guan and Weiner (1989). Exon 12 contained only the 3′-noncoding region. This fact is similar to that observed with exon 13 of *CYP4A1*, which has the largest number of exons hitherto reported among p-450s (Kimura et al., 1989).

The positions of 11 introns of *CYP24* are compared to those of *CYP11A* and *CYP11B* on the basis of their aligned sequence (Figure 5). Apparently, 7 out of 11 total introns of *CYP24* are found in identical positions with those of *CYP11*. Since P-450cc24 is closely related to P-450LMT25 (Figure 6), it might be interesting to compare exon/intron boundaries of these genes. However, the gene structure of *CYP27* is so far unknown.

The divergence between microsomal and mitochondrial P-450s occurred more than 900 million years ago, and the gene duplications leading to the current P-450 families occurred earlier than 400 million years ago (Gotoh & Fujii-Kuriyama, 1989). The positions of introns in microsomal P-450 genes of individual families are grossly different from each other except those of *CYP17* and *CYP21* (Nishimoto et al., 1991). In contrast, as described above, at least two mitochondrial families (*CYP11*, *CYP24*) share many common

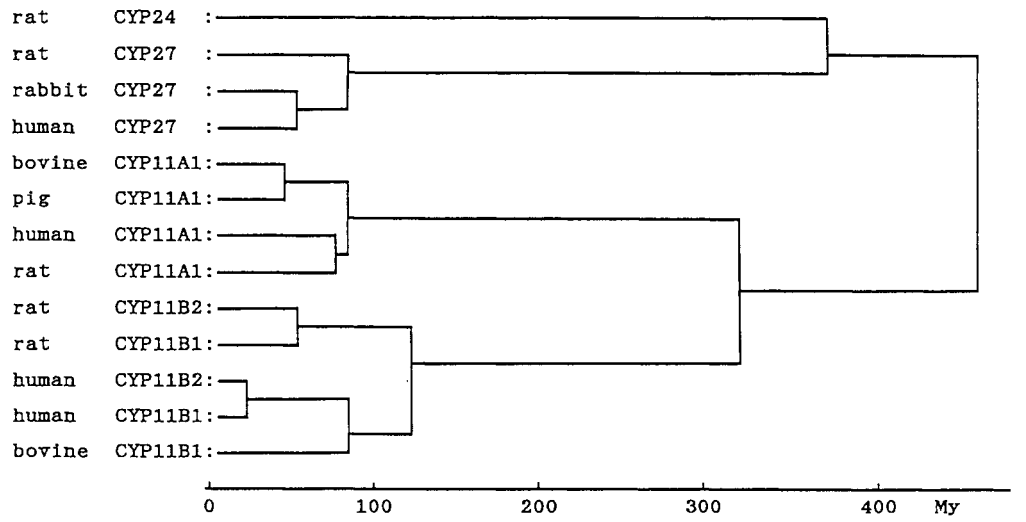

FIGURE 6: Phylogenetic tree of mitochondrial P-450 proteins. Mitochondrial P-450 proteins in various species were analyzed with the UPGMA method (Sneath & Sokal, 1973). This tree was calculated from the values for amino acid sequence divergence. My, million years. Sequences data are summarized and cited on the basis of the review of Nebert et al. (1991).

insertion sites for introns, although the sequence divergence between the two families (26%) is not so different from those observed within microsomal families (<32%). These observations suggest that the alteration rates of numbers and positions of introns in each P-450 family are different from the mutation rates of the amino acid sequences in the process of molecular evolution. On the basis of gene structure, *CYP24* seems to be close to *CYP11*, and the similarity is higher than those of any other P-450 families, although these two enzyme species belong to different P-450 families based on the amino acid sequence similarity.

There is a suggestion that the 24-hydroxylation reaction is catalyzed by two isozymes, one inducible and the other constitutive (Mandla et al., 1990). However, only one isozyme has been identified so far. The finding that the level of mRNA of P-450cc24 is increased after D_3 supplementation (Ohyama et al., 1991) and decreases in vitamin D deficiency (Shinki et al., 1992) indicates that this enzyme is of importance not only in vitamin D replete conditions but also in normal and vitamin D deficient conditions. Recently, Armbrecht and Boltz (1991) and Shinki et al. (1992) reported that the mRNA that hybridized with the cDNA of P-450cc24 was expressed in intestine as well as in kidney where the enzyme activity and mRNA are known to be regulated by both PTH and 1,25-$(OH)_2D_3$ in parallel. According to the present study, the vitamin D 24-hydroxylase is encoded by a single gene in the rat. It seems likely, therefore, that the same gene products are expressed in kidney and intestine.

One of the important roles of P-450 monooxygenation systems is to detoxicate internal and external toxic substrates, by which many P-450 species are often inducible. The inducibility of 24-hydroxylase by vitamin D reminds us of such characteristic induction of P-450, since vitamin D may be toxic when present in excessive amounts. It is therefore tempting to suggest that the 24-hydroxylase may be functional in regulation of the level of active hormone in target tissues in response to its concentration.

Induction of vitamin D 24-hydroxylase by 1,25$(OH)_2D_3$ is considered to be mediated by the vitamin D receptor. We therefore searched for sequences similar to those of the known rat vitamin D responsive elements, i.e., GGGTGTCG-GAAGCCC for the rat calbindin D-9k gene (Darwish & DeLuca, 1992) and GGGTGAATGAGGACA for the rat osteocalcin gene (MacDonald et al., 1991). As shown in Figure 3, four candidates for vitamin D responsive elements were found. Two were located in the region −427 to −409. Although these sequences were not identical with those described above, they were similar and located in the region −427 to −409, a similar position as that found in calbindin D-9 and osteocalcin. The other two candidates were located at −210 to −196 and −175 to −161, and contained the core sequence (GGGTGA), which is well conserved in vitamin D responsive elements. Interestingly, the sequence of −210 to −196 has a symemtrical feature similar to other steroid hormone responsive elements (glucocorticoid response element, estrogen response element). In a preliminary study, about 500 base pairs of DNA containing these regions were ligated to the chloramphenicol acetyltransferase (CAT) gene. When the fusion gene was introduced into COS-7 cells, expression of the CAT gene increased 20-fold in response of 5×10^{-8} M 1,25$(OH)_2D_3$ (Ohyama et al., unpublished observation). A more precise characterization of the vitamin D responsive elements is now performed in this laboratory.

It has been suggested that cAMP (Horiuchi et al., 1977) and protein kinase C (Mandla et al., 1990) are involved in the regulation of 24-hydroxylase. However, no canonical consensus regulatory elements of cAMP (TTCGTCA) or the AP1 binding site (TCAGTCA for protein kinase C) were found in the promoter region. A typical GC box (transcriptional factor Sp1 binding site, GGGCGG) was found at −385 to −380.

The most interesting finding here is perhaps that the putative vitamin D responsive elements, which exist in calcium binding proteins and are well characterized, were found in the 24-hydroxylase gene. This will enable us to connect studies on calcium binding proteins and calcium homeostasis to studies on the mechanism of regulation of 24-hydroxylase at the molecular level.

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