

Molecular cloning of cDNA for vitamin D₃ 25-hydroxylase from rat liver mitochondria

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A cDNA clone encoding mitochondrial vitamin D₃ 25-hydroxylase was isolated from a rat liver cDNA library by the use of specific antibodies to the enzyme. The isolated cDNA clone was 1.9 kbp long and contained a 1599 bp open reading frame encoding 533 amino acid residues. The deduced primary structure contained a presequence typical for mitochondrial enzymes in the N-terminal region. The N-terminal sequence of the mature enzyme was determined to be Ala-Ile-Pro-Ala-Ala, which agrees perfectly with a portion of the deduced sequence, establishing the cleavage point of the precursor.

Vitamin D₃ 25-hydroxylase; Cytochrome P-450; cDNA cloning; 5 β -Cholestane-3 α , 7 α , 12 α -triol 27-hydroxylase

1. INTRODUCTION

In the conversion of vitamin D₃ into the active form (1 α ,25-dihydroxyvitamin D₃), the initial hydroxylation at position 25 of vitamin D₃ is essential for the subsequent hydroxylation at position 1 α [1]. The initial hydroxylation is catalyzed by vitamin D₃ 25-hydroxylases existing in rat liver microsomes and mitochondria [2–4]. In human liver, however, only the mitochondrial enzyme seems to play the major role in the initial hydroxylation of vitamin D₃ since vitamin D₃ 25-hydroxylation activity is observed only in mitochondria [5].

Recently, rat liver mitochondrial vitamin D₃ 25-hydroxylase was purified to homogeneity in this laboratory based on the catalytic activity [6]. In this paper we describe the isolation of a full-length cDNA encoding mitochondrial vitamin D₃ 25-hydroxylase from a rat liver cDNA library using specific antibodies.

2. MATERIALS AND METHODS

Cytochrome P-450 responsible for 25-hydroxylation of vitamin D₃ was purified from female rat liver mitochondria as described

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The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession no. Y07534

previously [6]. Specific polyclonal antibodies were prepared by immunizing BALB/c female mice with the purified protein mixed with Ribi adjuvant as described previously [7].

A lambda gt11 cDNA library was prepared from liver poly(A)⁺ RNA of male rat [8]. The liver lambda gt11 cDNA library was screened with specific antibodies to mitochondrial vitamin D₃ 25-hydroxylase as described by Young and Davis [9]. Positive plaques with immunoreactive signals were isolated, and their DNA inserts were excised by *Eco*RI digestion and subcloned in pUC19.

DNA sequence analysis was carried out by a modification of the dideoxy chain termination method [10], which utilizes 7-deaza-dGTP [11] and Sequenase [12]. Northern hybridization was performed as described previously [8]. Manual sequence analysis of protein was performed by the method described by Black and Coon [13].

3. RESULTS AND DISCUSSION

Specific polyclonal antibodies were produced against rat liver mitochondrial vitamin D₃ 25-hydroxylase. The antibodies recognized the hydroxylase specifically as examined by Western blotting and therefore were used to screen the liver cDNA library constructed in lambda gt11 to isolate cDNA clones encoding mitochondrial vitamin D₃ 25-hydroxylase. Out of 1.4×10^6 clones, 10 immunoreactive clones were isolated and analyzed. The 1.9 kbp insert was excised by *Eco*RI digestion from the longest clone and subcloned in a pUC19 plasmid (pLMT25). The size of vitamin D₃ 25-hydroxylase mRNA was checked by Northern hybridization using this cDNA clone as a probe. It was thus found that mRNA of liver mitochondrial vitamin D₃ 25-hydroxylase having a size of approximately 2.1 kb was present in both female and male rats (fig.1). The

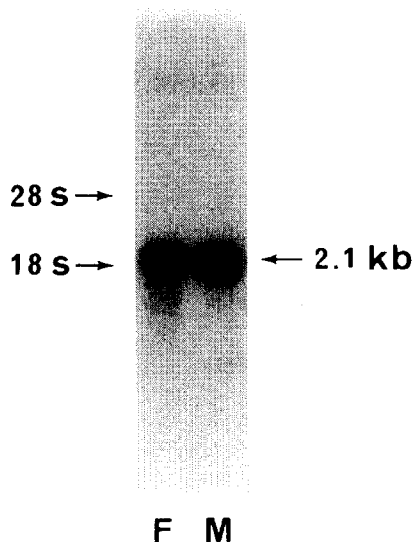


Fig.1. Northern hybridization of liver poly(A)⁺ RNA of normal rat. 5 µg of poly(A)⁺ RNA was electrophoresed on agarose gel containing formaldehyde [20]. A ³²P-labeled insert (1.9 kb) of the longest clone was used as a probe.

intensities of the hybridized bands of both sexes were not significantly different in contrast to mRNA of liver microsomal vitamin D₃ 25-hydroxylase, which is absent in female rat liver [14]. Since pLMT25 seemed to contain the full size of the coding region, it was subsequently used for DNA sequencing.

Fig.2 shows a restriction map of pLMT25 and the sequencing strategy. A 1.9 kbp DNA fragment was sequenced. Fig.3 shows the nucleotide sequence of the mitochondrial vitamin D₃ 25-hydroxylase cDNA as determined by analysis of pLMT25. The deduced amino acid sequence of the hydroxylase protein begins with a methionine residue and consists of 533 amino acid residues.

Most mitochondrial proteins are cytoplasmically synthesized as larger precursor forms and then translocated into mitochondria, during the course of which they are processed into the mature forms. To see if our purified mitochondrial vitamin D₃ 25-hydroxylase is the processed, mature form, its N-terminal amino acid sequence was determined. The sequence thus determined was Ala-Ile-Pro-Ala-Ala. This sequence agrees perfectly with the deduced sequence from residues 33 through 37 (fig.3). It was thus established that the peptide removed by processing comprises amino acid residues 1 through 32. This peptide has many of the hallmarks of a mitochondrial presequence. Namely it contains one lysine and 5 arginine together with 18 hydrophobic amino acid residues distributed throughout the 32 amino acid sequence, conferring a characteristic amphiphilicity common to presequences that direct proteins into the mitochondrion [15]. The mature enzyme consists of 501

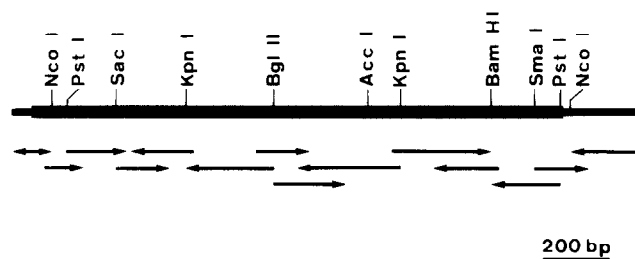


Fig.2. Restriction map of and sequencing strategy for pLMT25. Arrows indicate the directions and extents of sequencing.

amino acid residues corresponding to a molecular weight of 57182.

The protein sequence of mitochondrial vitamin D₃ 25-hydroxylase contains a conserved cysteine residue (located at position 447 in the mature enzyme) that is considered to be a ligand for the heme iron [16]. Subjecting the deduced amino acid sequence to a computer homology search (NBRF data base), it was found that the enzyme is 73% similar to a cytochrome P-450 isolated from rabbit liver mitochondria catalyzing 5β-cholestane-3α,7α,12α-triol 26(or 27)-hydroxylation [17]. However, we could not find out any other P-450s exhibiting more than 30% sequence similarity to mitochondrial vitamin D₃ 25-hydroxylase. The gene encoding this protein can therefore be referred to as CYP26 as suggested by Andersson et al. [17].

Since it was suggested that both 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol and 25-hydroxylation of vitamin D₃ in rat liver mitochondria are catalyzed by the same enzyme protein by Okuda et al. [18] and Ohyama et al. [19], the amino acid sequence described here may also represent the primary structure of rat 5β-cholestane-3α,7α,12α-triol 27-hydroxylase. Conclusive evidence, however, will be obtained by expression of the cDNA which is now under investigation in this laboratory and will be the subject of a future publication.

REFERENCES

- [1] DeLuca, H.F. and Schnoes, H.K. (1976) *Annu. Rev. Biochem.* 45, 631–666.
- [2] Hayashi, S., Noshiro, M. and Okuda, K. (1986) *J. Biochem.* 99, 1753–1763.
- [3] Andersson, S. and Jörnvall, H. (1986) *J. Biol. Chem.* 261, 16932–16936.
- [4] Björkhem, I. and Holmberg, I. (1979) *J. Biol. Chem.* 254, 9518–9524.
- [5] Oftebro, H., Saarem, K., Björkhem, I. and Pedersen, J.I. (1981) *J. Lipid Res.* 22, 1254–1264.
- [6] Masumoto, O., Ohyama, Y. and Okuda, K. (1988) *J. Biol. Chem.* 263, 14256–14260.
- [7] Noshiro, M. and Omura, T. (1978) *J. Biochem.* 83, 61–77.
- [8] Noshiro, M., Lakso, M., Kawajiri, K. and Negishi, M. (1988) *Biochemistry* 27, 6434–6443.
- [9] Young, R.A. and Davis, R.W. (1983) *Proc. Natl. Acad. Sci. USA* 80, 1194–1198.

1	1	TGCGCTGGATGGGGCGCGTAGTCTCTGGCTCTAAACTCTTGGCTTCTCAGACACGATCT																		Met	Ala	Val	Leu	Ser	
6	74	Arg	Met	Arg	Leu	Arg	Trp	Gly	Leu	Leu	Asp	Thr	Arg	Val	Met	Gly	His	Ala	Val	Leu	Ser	25			
		CGC	ATG	AGA	CTG	AGT	TGG	GCG	CTT	CTG	GAC	ACT	CGT	GTG	ATG	GGC	GCT	GCG	GTG	CTG	CGC	133			
26	134	Gln	Gly	Ala	Arg	Ala	Lys	Ala	Ala	Ile	Pro	Ala	Ala	Leu	Arg	Asp	His	Glu	Ser	Thr	Glu	45			
		CAA	GGG	GCC	AGA	GCC	AAG	GCC	GCG	ATC	CCT	GCA	GCC	CTC	CGG	GAT	CAC	GAG	AGC	ACG	GAG	193			
46	194	Gly	Thr	Gly	Thr	Gly	Gln	Asp	Arg	Pro	Arg	Leu	Arg	Ser	Leu	Ala	Glu	Leu	Pro	Gly	Pro	65			
		GGT	CCA	GGA	ACA	GGT	CAA	GAC	CGA	CCG	CGC	CTG	CGG	AGT	CTG	GCG	Glu	Leu	CCG	GGA	CCC	253			
66	254	Gly	Thr	Leu	Arg	Phe	Leu	Phe	Gln	Leu	Phe	Leu	Arg	Gly	Tyr	Val	Glu	His	Leu	His	Glu	85			
		GGA	ACG	CTA	CGC	TTT	TTA	TTC	CAG	CTA	TTT	CTA	CGA	GGC	TAT	GTG	CTG	CAC	TTG	CAC	GAG	313			
86	314	Leu	Gln	Ala	CTG	Asn	Lys	Ala	Lys	Tyr	Gly	Pro	Met	Trp	Thr	Ala	Thr	Phe	Gly	Thr	Arg	105			
		CTC	CAG	GCG	GCG	AAC	AAG	GCC	AAG	TAC	GGC	CCA	ATG	TGG	ACA	ACC	ACC	TTT	GGG	ACT	CGC	373			
106	374	Thr	Asn	Val	Asn	Leu	Ala	Ser	Ala	Pro	Leu	Leu	Glu	Gln	Val	Met	Arg	Gln	Gly	Gly	Lys	125			
		ACC	AAT	GTG	AAT	CTG	GCT	AGC	GCC	CCG	CTC	TTG	GAG	CAA	GTG	ATG	AGA	CAG	GAG	GGC	AAG	433			
126	434	Tyr	Pro	Ile	Arg	Asp	Ser	Met	Glu	Gln	Trp	Lys	Glu	His	Arg	Asp	His	Lys	Gly	Leu	Ser	145			
		TAC	CCC	ATA	AGA	GAC	AGC	ATG	Gln	CAG	TGG	AAG	GAG	CAC	CGA	GAC	CAC	AAA	GGC	CTC	TCC	493			
146	494	Tyr	Gly	Ile	Phe	Ile	Thr	Gln	Gly	Gln	Trp	Tyr	His	Leu	Arg	His	Ser	Arg	Leu	Asn	Gln	165			
		TAT	GGG	ATC	TTC	ATC	ACA	CAA	GGA	Gln	CAG	TGG	TAC	CAT	CTG	CGT	CAT	AGT	TTG	AAT	CAG	553			
166	554	Arg	Met	Leu	Lys	Pro	Ala	Glu	Ala	Leu	Tyr	Thr	Asp	Ala	Leu	Asn	Glu	Val	Ile	Ser		185			
		CGG	ATG	CTG	AAG	CCT	GCT	GAG	GCA	GCC	CTC	TAC	ACA	GAT	GCC	TTA	AAC	GAG	GTC	ATC	AGT	613			
186	614	Asp	Phe	Ile	Ala	Arg	Leu	Asp	Gln	Val	Arg	Thr	Glu	Ser	Ala	Ser	Gly	Asp	Gln	Val	Pro	205			
		GAC	TTT	ATT	GCC	CGG	CTG	AGC	Gln	GAG	CTG	ACA	Glu	AGT	GCA	TCG	GGG	CAT	GAG	GTG	CCA	673			
206	674	Asp	Val	Ala	His	Leu	Leu	Tyr	His	Leu	Ala	Leu	Glu	Ala	Ile	Cys	Tyr	Ile	Leu	Phe	Glu	225			
		GAT	GTG	GCA	CAT	CTT	CTC	TAC	CAC	CTT	GCC	TTG	GAA	GCC	ATC	TGC	TAT	ATC	CTG	TTT	GAG	733			
226	734	Lys	Arg	Glt	Gly	Cys	Leu	Glu	Pro	Ser	Ile	Pro	Glu	Asp	Thr	Ala	Ala	Phe	Arg	Ser		245			
		AAA	AGG	VAL	GGC	CTG	CTG	GAG	CCC	TCC	ATC	CCT	GAG	GAC	ACC	GCC	ACC	TTC	ATC	AGA	TCT	793			
246	794	Val	Gly	Leu	Met	Phe	Lys	Asn	Ser	Val	Tyr	Val	Thr	Phe	Leu	Pro	Lys	Trp	Ser	Arg	Pro	265			
		GTT	GGA	CTC	ATG	TTC	AAG	AAC	TCA	GTC	TAT	GTC	ACT	TTC	CTT	CCC	AAG	TGG	TCT	CGG	CCT	853			
266	854	Leu	Leu	Pro	Phe	Trp	Lys	Arg	Tyr	Met	Asn	Asn	Asp	Asn	Ile	Phe	Ser	Thr	Gly	Glu		285			
		CTG	CTG	CCC	TTT	TGG	AAG	CGA	TAC	ATG	AAT	AAC	TGG	GAT	AAC	ATT	TTC	TCC	TTC	GGG	GAG	913			
286	914	Lys	Met	Ile	His	Gln	Lys	Val	Gln	Glu	Ile	Glu	Ala	Gln	Leu	Gln	Ala	Ala	Gly	Pro	Asp	305			
		AAG	ATG	ATT	CAT	CAA	AAA	GTC	CAG	GAG	ATA	GAA	GCC	CAG	CTA	CAG	GCG	GCT	GGG	CCA	GAT	973			
306	974	Gly	Val	Gln	Val	Ser	Gly	Tyr	Leu	His	Phe	Leu	Leu	Thr	Ala	Glu	Leu	Leu	Ser	Pro	Gln	325			
		GGG	GTC	GAG	GTA	TCT	GGC	TAC	CTG	CAC	TTC	CTG	CTG	ACT	Lys	GAA	TTG	CTC	AGT	CCT	CAA	1033			
326	1034	Glu	Thr	Val	Gly	Thr	Phe	Pro	Glu	Leu	Ile	Leu	Ala	Gly	Val	Asp	Thr	Thr	Asn	Thr		345			
		GAG	ACT	GTC	GGC	ACC	TTT	CCT	GAG	CTG	ATC	TTG	GCT	GGG	GTA	GAC	ACG	ACA	TCC	AAT	ACA	1093			
346	1094	Leu	Thr	Trp	Ala	Leu	Tyr	His	Leu	Ser	Lys	Asn	Pro	Glu	Ile	Gln	Glu	Ala	Leu	His	Lys	365			
		CTG	ACC	TGG	GCC	CTG	TAT	CAC	CTT	TCA	AAG	AAC	CCA	GAG	ATC	CAG	GAA	GCC	TTG	CAC	AAG	1153			
366	1154	Glu	Val	Thr	Gly	Val	Val	Pro	Phe	Gly	Lys	Val	Pro	Gln	Asn	Lys	Asp	Phe	Ala	His	Met	385			
		GAA	GTG	ACT	GGT	GTG	GTA	CCC	TTC	GGG	AAG	GTG	CCC	CAG	AAC	AAG	GAC	TTT	GCC	ACT	ATG	1213			
386	1214	Pro	Leu	Leu	Lys	Ala	Val	Ile	Lys	Glu	Thr	Leu	Arg	Leu	Tyr	Pro	Val	Val	Pro	Thr	Asn	405			
		CCC	CTG	CTA	AAA	GCT	GTG	ATT	AAG	GAG	ACC	CTG	CGC	CTC	TAC	CCT	GTG	GTT	CCC	ACA	AAC	1273			
406	1274	Ser	Arg	Ile	Ile	Thr	Glu	Lys	Glu	Thr	Glu	Ile	Asn	Gly	Phe	Leu	Leu	Pro	Lys	Asn	Thr	425			
		TCC	CGG	ATC	ATC	ACA	GAA	Lys	GAA	ACT	GAA	ATT	AAT	Gly	TTT	CTC	Phe	CTC	AAG	AAT	ACA	1333			
426	1334	Gln	Phe	Val	Leu	Cys	His	Tyr	Val	Val	Ser	Arg	Asp	Pro	Ser	Val	Phe	Pro	Glu	Pro	Glu	445			
		CAG	TTT	GTG	TTA	TGC	CAC	TAC	GTG	GTG	TCC	CGA	GAT	CCC	AGT	GTG	TTT	CCT	GAG	CCC	GAG	1393			
446	1394	Ser	Phe	Gln	Pro	His	Arg	Trp	Leu	Arg	Lys	Arg	Glu	Asp	GAT	Asn	Ser	Gly	Ile	Gln	His	465			
		AGC	TTT	CAG	CCT	CAG	CGA	TGG	CTG	AGG	AGA	AGA	Glu	GAC	ASP	AAC	TCC	GGG	ATC	CAA	CAC	1453			
466	1454	Pro	Phe	Gly	Ser	Val	Pro	Phe	Gly	Tyr	Gly	Val	Arg	Ser	Cys	Leu	Gly	Arg	Arg	Ile	Ala	485			
		CCA	TTT	GGC	TCT	GTG	CCC	TTT	GGC	TAT	GGG	GTT	CGG	TCC	TGC	CTG	GCT	CGC	AGG	ATT	GCA	1513			
486	1514	Glu	Leu	Glu	Met	Gln	Leu	Leu	Leu	Ser	Arg	Leu	Ile	Gln	Lys	Tyr	Glu	Val	Val	Leu	Ser	505			
		GAA	CTG	GAG	ATG	CAA	CTC	CTG	CTG	TCA	AGG	CTG	ATA	CAA	AAG	TAT	GAG	GTG	GTC	CTG	TCT	1573			
506	1574	Pro	Gly	Met	Gly	Glu	Val	Lys	Ser	Val	Ser	Arg	Ile	Val	Leu	Val	Pro	Ser	Lys	Lys	Val	525			
		CCC	GGG	ATG	GGA	GAA	GTG	AAG	TCT	GTG	TCC	CGC	ATC	GTC	CTG	GTT	CCC	AGC	AAG	AAG	GTG	1633			
526	1634	Ser	Leu	Arg	Phe	Leu	Gln	Arg	Gln	***	TACCAAGCTGGGCTCCTGCTCCATGGGACTTGTGCCAGAAGCCC										533				
		AGC	CTA	CGC	TTT	CTG	CAG	AGA	CAG	TAG												1703			
1704		TGGCACAGAAAGTTCTTGGCCAGTCTCAGCTCACATGTCCAGATGCCAGATTCAACAGGGGACCTCTCTGCCCTTCCCAT																				1782			
1783		AGACACCAGACGTCTGGCACAATCTCTACTGAGCAGCACCCATTAAAGACATTAGAGCACCTCATATCACAGGACGGTG																				1861			
1862		CTTGGGTACAATTAAAAATAAAATTTAAAAATTCAAAAAA																				1900			

Fig.3. Nucleotide sequence of the pLMT25 insert and predicted amino acid sequence. A DNA fragment of cDNA (1.9 kbp) including the total coding region was sequenced. The cleavage site of the presequence is shown by an arrow. Amino acids determined by manual sequencing are underlined. The consensus sequence for the heme binding domain is marked by a broken line. A putative poly(A) addition signal (AATAAA) is overlined.

- [10] Sanger, F., Miklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [11] Mizusawa, S., Nishimura, S. and Seela, F. (1986) *Nucleic Acids Res.* 14, 1319–1324.
- [12] Tabor, S. and Richardson, C.C. (1987) *Proc. Natl. Acad. Sci. USA* 84, 4767–4771.
- [13] Black, S.D. and Coon, M.J. (1982) *J. Biol. Chem.* 257, 5927–5938.
- [14] Yoshioka, H., Morohashi, K., Miyata, T., Kawajiri, K., Hirose, T., Inayama, S., Fujii-Kuriyama, Y. and Omura, T. (1987) *J. Biol. Chem.* 262, 1706–1711.
- [15] Boise, D. and Schatz, G. (1988) *J. Biol. Chem.* 263, 4509–4511.
- [16] Nebert, D.W. (1987) *Annu. Rev. Biochem.* 56, 945–993.
- [17] Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H. and Russell, D.W. (1989) *J. Biol. Chem.* 264, 8222–8229.
- [18] Okuda, K., Masumoto, O. and Ohyama, Y. (1989) *J. Biol. Chem.* 263, 18138–18142.
- [19] Ohyama, Y., Masumoto, O. and Okuda, K. (1989) in: *Cytochrome P-450: Biochemistry and Biophysics*, vol. I (Shuster ed.), pp.105–108, Taylor and Fransis, London.
- [20] Thomas, P.S. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5201–5205.