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Molecular cloning of cDNA for vitamin D_3 25-hydroxylase from rat liver mitochondria

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A cDNA clone encoding mitochondrial vitamin D_3 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 D_3 25-hydroxylase; Cytochrome P-450; cDNA cloning; 5β-Cholestane-3α, 7α, 12α-triol 27-hydroxylase

1. INTRODUCTION

In the conversion of vitamin D_3 into the active form (1α,25-dihydroxyvitamin D_3), the initial hydroxylation at position 25 of vitamin D_3 is essential for the subsequent hydroxylation at position 1α [1]. The initial hydroxylation is catalyzed by vitamin D_3 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 D_3 since vitamin D_3 25-hydroxylation activity is observed only in mitochondria [5].

Recently, rat liver mitochondrial vitamin D_3 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 D_3 25-hydroxylase from a rat liver cDNA library using specific antibodies.

2. MATERIALS AND METHODS

Cytochrome P-450 responsible for 25-hydroxylation of vitamin D_3 was purified from female rat liver mitochondria as described 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 D_3 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].

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

3. RESULTS AND DISCUSSION

Specific polyclonal antibodies were produced against rat liver mitochondrial vitamin D_3 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 D_3 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 D_3 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 D_3 25-hydroxylase having a size of approximately 2.1 kb was present in both female and male rats (fig.1). The

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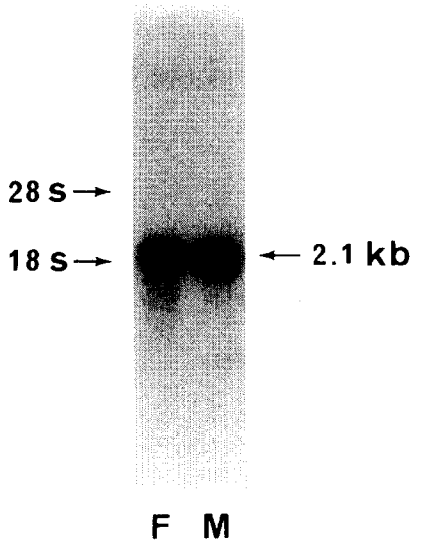

Fig.1. Northern hybridization of liver poly(A)$^+$ RNA of normal rat. 5 μg of poly(A)$^+$ RNA was electrophoresed on agarose gel containing formaldehyde [20]. A ^{32}P-labeled insert (1.9 kb) of the longest clone was used as a probe.

intensities of the hybridized bands of both sexes were not significantly different in contrast to mRNA of liver microsomal vitamin D_3 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 D_3 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 D_3 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

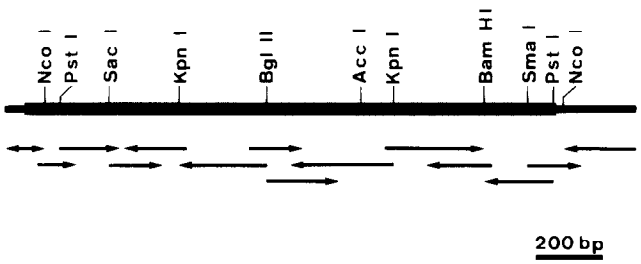

Fig.2. Restriction map of and sequencing strategy for pLMT25. Arrows indicate the directions and extents of sequencing.

amino acid residues corresponding to a molecular weight of 57182.

The protein sequence of mitochondrial vitamin D_3 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 D_3 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 D_3 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.

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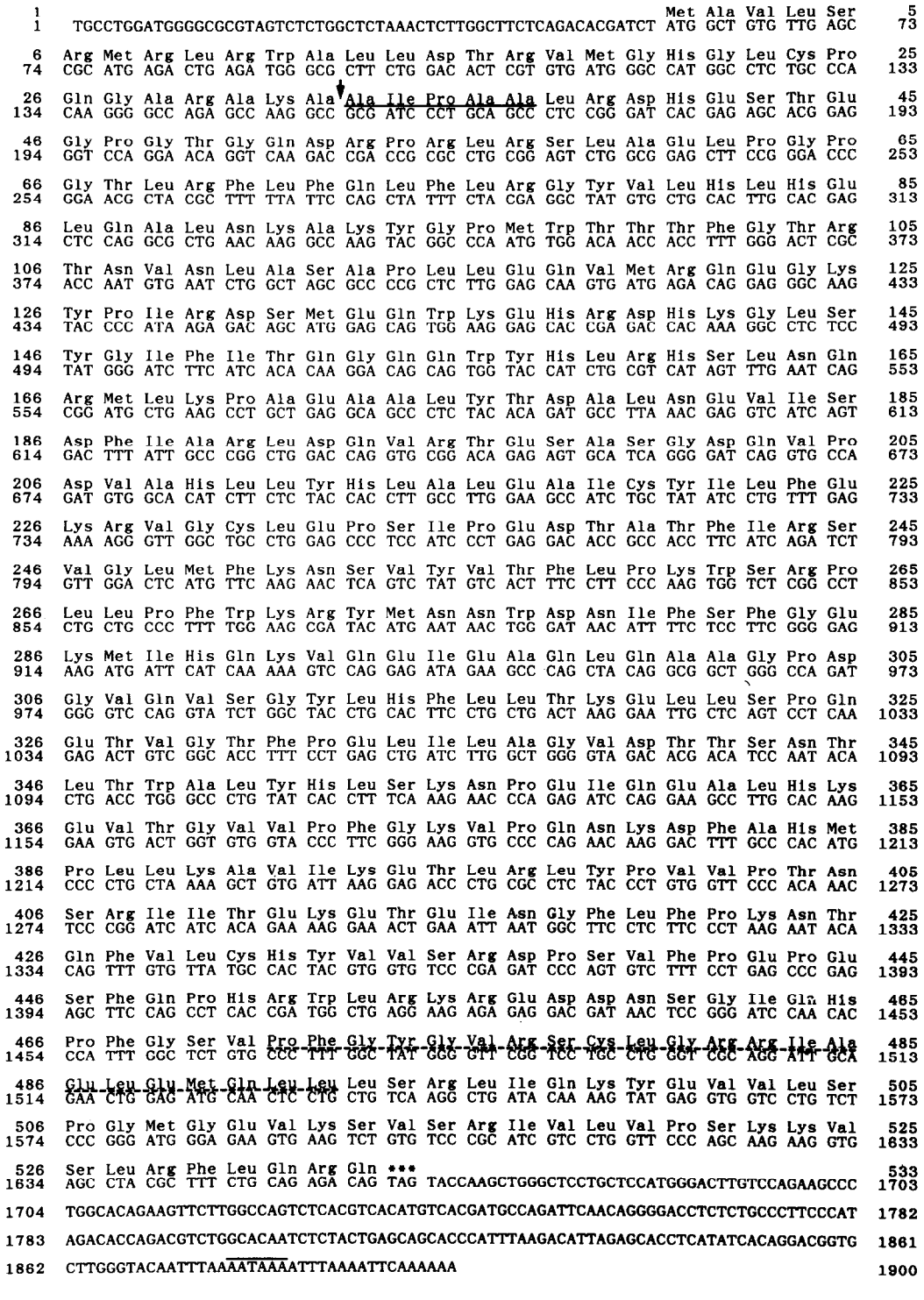

Fig.3. Nucleotide sequence of the pLMT25 insert and predicted amino acid sequence. A DNA fragment of cDNA (1.9 kbp) including the total coding region was sequenced. The cleavage site of the presequence is shown by an arrow. Amino acids determined by manual sequencing are underlined. The consensus sequence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

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