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Molecular cloning and sequence analysis of cDNA encoding rat adrenal cytochrome P-450$_{11\beta}$

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A cDNA clone encoding cytochrome P-450$_{11\beta}$ of rat adrenal has been cloned and sequenced using a bovine P-450$_{11\beta}$ cDNA insert (pcP-450(11β)-2; (1987) J. Biochem. 102, 559–568) as a probe. The nucleotide sequence contains an open reading frame sufficient to encode the entire amino acid sequence of a P-450$_{11\beta}$ precursor protein consisting of 499 amino acids including an extension peptide of 24 amino acids at the NH_2-terminus. The cDNA contains 1247 nucleotides at the 3'-noncoding region including 51 nucleotides of poly A, but lacks the 5'-noncoding region. The deduced amino acid sequence shows 61% similarity to that of bovine P-450$_{11\beta}$. Putative binding sites for heme and steroid are highly conserved among steroidogenic P-450s of known structure.

cDNA cloning; Cytochrome P-450$_{11\beta}$; Steroidogenesis; Heme-binding site; Steroid-binding site; (Rat)

1. INTRODUCTION

Recent studies in our laboratory demonstrated that a highly purified preparation of bovine adrenocortical cytochrome P-450$_{11\beta}$ catalyzed not only the 11β-, 18- and 19-hydroxylations of 11-deoxycorticosterone but also the conversion of corticosterone to 18-hydroxycorticosterone and aldosterone [1–4]. More recently, by subjecting the regularly purified preparation of P-450$_{11\beta}$ to hydroxylapatite HPLC, Ogishima et al. [5] successfully resolved the preparation into two distinct P-450 peaks. Both enzymes were capable of catalyzing aldosterone synthesis as well as the 11β- and 18-hydroxylation of 11-deoxycorticosterone.

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The sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X15431, Cytochrome P-450 11 beta

The respective NH_2-terminal amino acid sequences of the two proteins were found to correspond with those deduced from the nucleotide sequences of two distinct cDNA clones previously reported by Kirita et al. [6]. It seems that at least two kinds of P-450$_{11\beta}$ mRNA are expressed in the adrenal gland of the individual animal, and each gene product has the oxygenation activity on multiple sites of a steroid nucleus.

In the case of rat adrenal cortex, the existence of 2 different forms of P-450$_{11\beta}$ was suggested by using another approach. Sodium restriction and potassium loading are known to stimulate markedly the production of aldosterone from corticosterone in rats [7]. In 1987 Lauber et al. [8] reported the induction of a protein of 49 kDa in zona glomerulosa mitochondria of rats on a low sodium and high potassium diet. This protein was immunocrossreactive with a monoclonal antibody raised against purified bovine P-450$_{11\beta}$. On the other hand, zonae fasciculata-reticularis mitochondria prepared from rats maintained on normal diet

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contained another immunocrossreactive protein of 51 kDa. According to these authors, the 49 and 51 kDa proteins were most likely aldosterone synthase and the 11β-hydroxylase of rat, respectively [9]. To investigate further the property of P-450$_{11\beta}$ of rat adrenal cortex, we have screened a cDNA library of rat adrenal gland using a bovine P-450$_{11\beta}$ cDNA fragment as a probe. In this communication we describe the isolation and the structural determination of a cDNA clone encoding one of rat P-450$_{11\beta}$s.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 200–250 g, were injected intraperitoneally with Cortrosyn (a synthetic ACTH); 0.67 μg/animal daily for 3 days. On day 4 animals were killed by decapitation and adrenal glands were dissected out immediately. Total RNA was extracted from the glands by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method [10]. The poly(A)$^+$ RNA was purified by oligo(dT) column chromatography. The mRNA was reverse-transcribed into cDNA and the cDNA was fractionated in agarose gel electrophoresis to obtain DNA species longer than 1 kbp. The cDNA was used to construct a library in the bacteriophage λ vector, λgt10. The library containing independent recombinants of approx. 1.3×10^6 phages was screened using the insert of pcP-450(11β)-2 as a probe. Five positively reacting clones containing long inserts (named 62, 65, 94, 110 and 127) were selected and recloned into the *Eco*RI site of pUC119 plasmid. DNA sequencing was performed by the use of single strand forward and reverse primers of M13 and Sequenase kit (United States Biochemical Corporation). Similarity search among amino acid sequences of various P-450s was performed as described by Miyata et al. [11].

3. RESULTS AND DISCUSSION

Among the five clones isolated by the plaque hybridization method, a clone having the longest cDNA insert (pcP-45011β-62) was selected. The restriction enzyme map of the insert and the sequencing strategy are illustrated in fig.1. Also shown in the figure are the four other independent clones, which are found to have the nucleotide sequences identical to that of pcP-45011β-62 up to about 200 bp. One of the clones (110) begins from the same position as pcP-45011β-62.

Fig.2 shows the complete nucleotide sequence of pcP-45011β-62. The overall length of the cDNA was 2747 bp including the poly(A) tail and is in good agreement with the mRNA size estimated by the Northern hybridization (2.7 kb, not shown). The open reading frame begins from the first

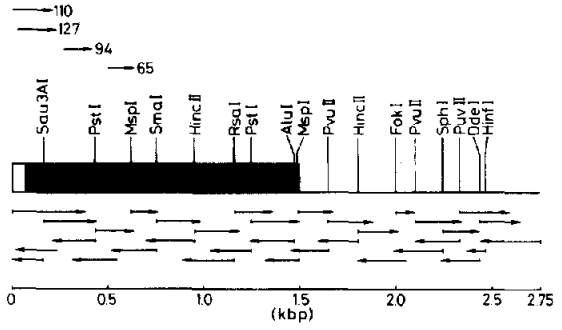

Fig.1. Restriction enzyme map and sequencing strategy for the P-450$_{11\beta}$ cDNA clone. Rectangular box shows the cDNA insert of pcP-45011β-62. The open box represents the extension-peptide, the closed box, the mature protein and the line, the 3′-noncoding region. Horizontal arrows in the lower space indicate the direction and extent of sequencing. Arrows with numbers in the upper space indicate the extent of sequencing of the other independent clones (65, 94, 110 and 127).

nucleotide 'A' at the 5′ terminus, and codes for a peptide consisting of 499 amino acids. Evidence that the peptide is that of rat cytochrome P-450$_{11\beta}$ is provided by striking sequence similarity between the amino acid sequence deduced and those of bovine, mouse, and human P-450$_{11\beta}$s. The cDNA of rat P-450$_{11\beta}$ has a long (1247 bp) noncoding region at the 3′-terminus.

As shown in fig.3, the predicted amino acid sequence at the NH_2-terminus is considerably similar to those of the bovine [6,12,13] and mouse [14] enzymes. It should be noted that the nucleotide sequences of coding regions of the rat and mouse cDNAs are identical in the first 78 nucleotides (residue 8 of the mouse enzyme, Val, should be Asp, when translated from the nucleotide sequence described in [14]). The Met codon at the 5′ terminus of the rat cDNA is thought to be the initiation codon, because the corresponding Met codons of the bovine and mouse enzymes have been identified as the true initiation codons by the use of S1 nuclease analysis [12,14]. Since the cleavage site of bovine P-450$_{11\beta}$ precursor peptide is Leu24-Gly25, that of the rat peptide is most likely at the same position. These considerations suggest that the extension-peptide of rat P-450$_{11\beta}$ precursor corresponds to a peptide of the NH_2-terminal 24 amino acid residues (2864 Da). It is interesting to note that the predicted extension-peptide is strongly basic in that it has 4 Arg and 1 His residues,

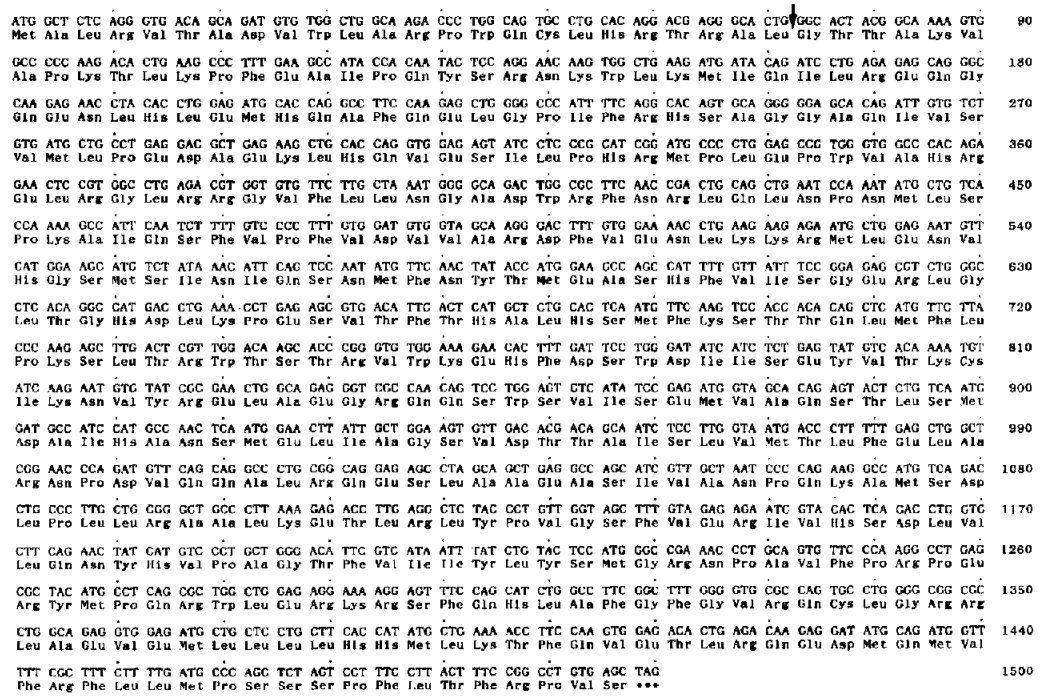

Fig.2. Nucleotide sequence of the P-450$_{11\beta}$ cDNA clone and the deduced amino acid sequence. The cleavage site by processing protease is indicated by an arrow. The underlined sequence within 3′-noncoding region indicates a polyadenylation signal.

though it has 1 Asp residue at the 8th position. These characteristics are compatible with the feature of the extension-peptides of mitochondrial precursor [15].

The entire amino acid sequence of rat P-450$_{11\beta}$ is similar to those of the bovine (61%), mouse and human enzymes. When the sequences of rat, bovine and human enzymes are aligned with one another, deletions are found at two portions, that is, around the 290th and the 433rd residues, and insertion is found around the 299th residue (the numbers correspond to those of the rat enzyme). These portions of the peptides may not play important roles in the enzymatic function or the maintenance of the three-dimensional structure of the enzyme.

A comparison of the sequences of rat and bovine P-450$_{11\beta}$s with those of the other steroidogenic P-450s, such as P-450$_{SCC}$ [15], P-450$_{C21}$ [16] and P-450$_{17\alpha}$ [17], shows that there are two highly identical regions. The first region is the heme binding site as depicted as a box marked with two asterisks in fig.3. Cys446 is thought to serve as the fifth ligand to heme. The sequence around it reveals a common feature of the heme-binding site of P-450, that is, F-X-X-G-X-R-X-C-X-G-X-X-X-A [18]. Also conserved among rat, bovine and human P-450$_{11\beta}$s is Glu453 (next to Ala in the above-

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rat      MALRVTADVW LARPWQCLHR TRALGTTAKV APKTLKPFEA IPQYSRNKWL      50
bovine   ---WAK-R-R M-G--LS--E A-L---RGA- ---AVL---- M-RCPG---M      50
mouse    -------V-- ---------- --------TL -----Q---- --             42

rat      KMIQILREQG QENLHLEMHQ AFQELGPIFR HSAGGAQIVS VMLPEDAEKL     100
bovine   R-L--WK--- S--M--D--- T--------- YDV--RHM-F ------V-R-     100

rat      HQVESILPHR MPLEPWVAHR ELRGLRRGVF LLNGADWRFN RLQLNPNMLS     150
bovine   Q-AD-RH-Q- -I----L-Y- QA--HKC--- ----PQ--LD --R---DV--     150

rat      PKAIQSFVPF VDVVARDFVE NLKKRMLENV HGSMSINIQS NMFNYTMEAS     200
bovine   LP-L-KYT-L --G-----SQ T--A-V-Q-A R--LTLD-AP SV-R--I---     200

rat      HFVISGERLG LTGHDLKPES VTFTHALHSM FKSTTQLMFL PKSLTRWTST     250
bovine   TL-LY----- -LTQQPN-D- LN-I---EA- L---V----V -RR-S--M--     250
human              W=AEA=SSA- ==-L--M=V- ----=----M --=======P

rat      RVWKEHFDSW DIISEYVTKC IKNVYRELAE GRQQSWSVIS *E**MVAQSTLSM 300
bovine   NM-R---EA- -Y-FQ-ANRA -QRI-Q---L -HPWHY-G-V A-LL-R-DM--** 301
human    K--------== -C-==-GDN- --K=-=---N S--Q=TS-= =-==LN-ELSP**

rat      DAIHANSMEL IAGSVDTTAI SLVMTLFELA RNPDVQQALR QESLAAEASI     350
bovine   -T-K--TID- T--------F P-L------- ---E----V- ----V---R-     351
human    ---=--C--- =-----R-V= =-=------- ---N------ ------A---

                *
rat      VANPQKAMSD LPLLRAALKE TLRLYPVGSF VERIVHSDLV LQNYHVPAGT     400
bovine   SE---R-ITE ---------- -------IT L--E-S---- -----I----     401
human    ==H----T== ---------- ------LI =--V-=---- -----=----

                                          **
rat      FVIIYLYSMG RNPAVFPRPE RYMPQRWLER KRS***FQHLAFG FGVRQCLGRR 450
bovine   L-KVL---L- ------A--- S-H-----D- QG-GSR-P---- ---------- 454
human    =-R=F---=- ----L----- --N------I -==-RN-Y-VP-- --M-------

rat      LAEVEMLLLL HHMLKTFQVE TLRQEDMQMV FRFLLMPSSS PFLTFRPVS     499
bovine   V--------- --V--N-L-- --E---IK-- Y--I----TL -LF---AIQ     503
human    ---AD----- Q-
```

Fig.3. Comparison of the amino acid sequence of rat P-450$_{11\beta}$ with those of bovine, mouse and human enzymes. The sequences of bovine, mouse and human P-450$_{11\beta}$ were taken from [6,14,13] respectively. The peptides enclosed with boxes with one and two asterisks indicate the putative heme/steroid binding site and the heme binding site, respectively. (–) and (=) indicate that the amino acid is identical to that of rat enzyme and to that of bovine enzyme, respectively. The cleavage site by processing protease is depicted by arrowhead.

mentioned peptide), which has been pointed out as a feature of mitochondrial type P-450s by Morohashi et al. [12].

Another region that is identical among the steroidogenic P-450s is found in a stretch spanning residues 361–376 (shown as a box marked with an asterisk in fig.3). Fig.4 illustrates the amino acid sequences of this region found in steroidogenic P-450s. Glu370 and Arg373 (indicated with asterisks) are conserved in all the P-450s whose structures have been so far determined [19,20]. Among the P-450s of known structure, a sequence, L(364)-X-A-X-X-X-E-X-L-R-X-X-P, is found only among steroidogenic P-450s, such as P-450$_{11\beta}$, P-450$_{SCC}$, P-450$_{C21}$ and P-450$_{17\alpha}$.

A comparison of the amino acid sequence of P-450$_{cam}$ [19] with that of rat P-450$_{11\beta}$ indicates the presence of the sequence, E-X-X-R, at the 288–291st of P-450$_{cam}$. This region resides in helix K (the peptide stretch of the 280–292nd amino acid residues) [21]. In the three-dimensional structure of P-450$_{cam}$, the helix constitutes a part of heme

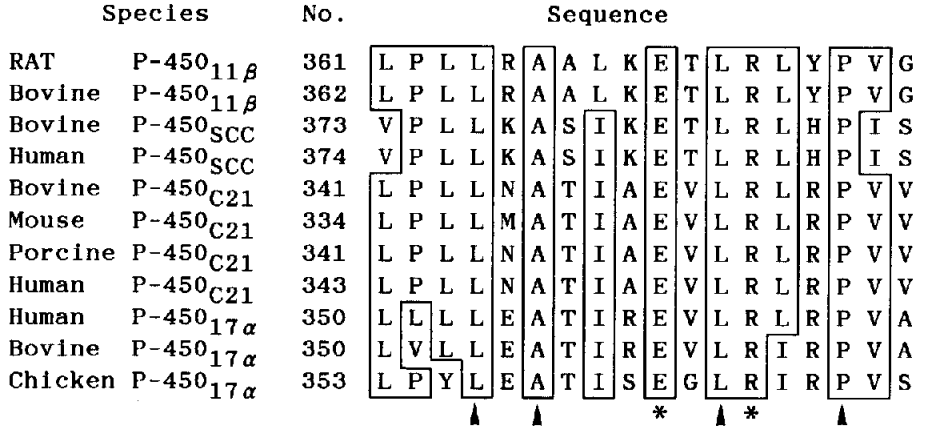

Fig.4. Amino acid sequences of a conserved region in steroidogenic P-450s. The amino acid sequences of a region highly conserved among steroidogenic P-450s are shown. The numbers are those of the amino acid residues depicted as the first in the peptides. Amino acid residues conserved in more than 9 P-450s are enclosed. Asterisks and arrowheads indicate the conserved amino acids among all the P-450s of known structure and those among steroidogenic P-450s, respectively. The amino acid sequences of bovine P-450$_{11\beta}$, bovine and human P-450$_{SCCs}$, porcine P-450$_{C21}$, and chicken P-450$_{17\alpha}$ were taken from [6, 15, 22, 23] respectively. The other sequences were found in [19].

pocket, and an amino acid residue located in the vicinity of this helix, Val295, directly interacts with the substrate (camphor) or the inhibitor (metyrapone). Val295 in P-450$_{cam}$ corresponds to Val377 in P-450$_{11\beta}$, which is the amino acid residue next to Pro376 in the above-mentioned consensus sequence. Taking these points into consideration, we propose that the peptide stretch from Leu364 to Pro376 and the following region constitute a part of the heme- and steroid-binding sites of P-450$_{11\beta}$.

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