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Molecular cloning of a cDNA encoding aldosterone synthase cytochrome P-450 in rat adrenal cortex

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Using an oligonucleotide probe designed on the basis of the N-terminal amino acid sequence of purified rat aldosterone synthase cytochrome P-450 [(1989) J. Biol. Chem. 264, 10935] we have isolated from rat adrenal cDNA library a 2687 base pair cDNA that encodes a protein of 500 amino acid residues. The deduced amino acid sequence contained the regions well conserved among all cytochrome P-450s sequenced to date, and also a portion (residues 25–44) which was identical to the N-terminal peptide sequence of rat aldosterone synthase cytochrome P-450. These results indicate that the cDNA encodes a precursor form of rat aldosterone synthase cytochrome P-450.

Complementary DNA; Molecular cloning; Cytochrome P-450; Aldosterone; Steroid-11-hydroxylase; (Rat)

1. INTRODUCTION

Recently, we reported isolation and purification of a new cytochrome P-450 from zona glomerulosa mitochondria of rat adrenal cortex [1]. The cytochrome P-450 catalyzed 3 successive monooxygenation reactions of 11-deoxycorticosterone (DOC) giving aldosterone as a product, and hence was designated as cytochrome P-450_{aldo}. The enzyme was distinct from rat cytochrome P-$450_{11\beta}$ which formed corticosterone but not aldosterone from DOC. The cytochrome P-450_{aldo} was present only in the mitochondria of zona glomerulosa, while the cytochrome P-$450_{11\beta}$ was found in the mitochondria of all the 3 zones of rat adrenal cortex. They also differed from each other in their structures as exemplified by the difference in amino acid sequences at the N-terminus. Thus, two cytochrome P-450 species both of which metabolize DOC exist in the mitochondria of rat adrenal cortex. Subsequently, Nonaka et al. [2] have cloned and sequenced a cDNA for a cytochrome P-450 in rat adrenal gland. The amino acid sequence deduced from the cDNA had a putative extension peptide of 24 amino acid residues, and the subsequent 20 residues (25–44) were identical to the N-terminal peptide sequence of the purified cytochrome P-$450_{11\beta}$ [1]. The cDNA is therefore considered to code for the rat cytochrome P-$450_{11\beta}$.

We report here cloning and sequencing of a cDNA for another cytochrome P-450 in rat adrenocortical mitochondria, i.e. cytochrome P-450_{aldo}. The assignment was made based on the facts that residues 25–44 of the deduced amino acid sequence agreed with the N-terminal peptide sequence of the rat cytochrome P-450_{aldo} obtained by protein sequence analysis [1] and that the cDNA was highly homologous in both nucleotide and amino acid sequences to rat cytochrome P-$450_{11\beta}$ [2]. These results, taken together with the report by Nonaka et al. [2], indicate existence and expression in rat adrenocortical cells of two distinct genes for these closely related cytochrome P-450 species.

2. MATERIALS AND METHODS

Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). A rat adrenal cDNA library, constructed in pcD vector [3], was a generous gift from Dr H. Okayama (Osaka University). The library was screened by colony hybridization technique [4] using as a probe a 47-mer oligonucleotide, 5′TTCAAAGGGCTTCAGGGTCTTTGGGGCCAGTGTTGCCGTGGTGCCCA3′, that was synthesized, purified [5] and ^{32}P-labeled at the 5′ end [6]. The probe was designed on the basis of N-terminal peptide sequence of purified rat cytochrome P-450_{aldo} [1]. Cloned cDNAs were cleaved from pcD vector as *Bam*HI fragments and inserted into pUC18, and appropriate restriction fragments were subcloned into M13mp18 or M13mp19 for dideoxy chain termination sequencing [7]. The sequencing was carried out with a Sequenase kit (United States Biochemical Co., USA) according to the manufacturer's instructions. Some sequences were obtained using oligonucleotide primers specific to the cDNA. The sequence data were analyzed by using a sequence analysis program, Microgenie (Beckman, USA).

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Abbreviations: bp, base pairs; kb, kilobase pairs; cytochrome P-450_{aldo}, cytochrome P-450 with aldosterone synthase activity; cytochrome P-$450_{11\beta}$, steroid 11-β-hydroxylase; DOC, 11-deoxycorticosterone

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3. RESULTS AND DISCUSSION

Four rounds of the screening procedure selected 14 clones that exhibited strong signals when hybridized with the radiolabeled oligonucleotide probe. The clones carried cDNA inserts ranging from 2.6 to 3.1 kb in size. These positive clones were categorized into two groups upon restriction endonuclease mapping; two clones (pAld23 and pAld10) had identical restriction enzyme maps, while the other clones, identical to one another except near the 5′ end, showed restriction maps slightly different from the former two clones. Since the synthetic oligonucleotide probe should hybridize with cDNAs for both rat cytochrome P-450$_{aldo}$ and cytochrome P-450$_{11\beta}$ due to the sequence homology in between the N-terminal regions of these two enzymes, it was conceivable that each of the two clone groups corresponded to each of the two enzymes. We presumed the minority (2 out of 14 clones) to be for the cytochrome P-450$_{aldo}$ and the majority to be for the cytochrome P-450$_{11\beta}$, since rat adrenal cortex contained a smaller amount of the former than that of the latter [1]. The presumption seemed to be correct because the restriction enzyme maps so far obtained for clones of the major group were identical to that of rat cytochrome P-450$_{11\beta}$ [2]. Fig. 1 shows the restriction enzyme map of pAld23 cDNA and the sequencing strategy. We determined an entire nucleotide sequence of pAld23 and approximately 15 nucleotides from both 5′ and 3′ termini of pAld10. The partial nucleotide sequences determined for pAld10 were identical to the corresponding regions of pAld23 except that poly(A) tail of pAld10 started at 7 bp upstream to that of pAld23.

We show in Fig. 2 the complete nucleotide sequence of pAld23 cDNA and the deduced amino acid sequence. The cDNA is 2687 bp in length excluding poly(A) tail and lacks 5′ noncoding sequencing. It has an open reading frame spanning from nucleotide 1 to 1503 and encodes 500 amino acids. We consider the first ATG codon at the 5′ end as the initiation codon because the N-terminal peptide sequence of purified cytochrome P-450$_{aldo}$ agrees with the reading frame of the cDNA sequence. The N-terminus of the purified protein corresponds to the 25th residue in the deduced amino acid sequence of the cDNA, indicating the existence of an extension peptide of 24 residues. The cDNA has a long untranslated region at the 3′ end with the polyadenylation signal at about 20 bases upstream to a poly(A) tail.

When the nucleotide sequence of the cDNA was compared with that of rat cytochrome P-450$_{11\beta}$ cDNA [2], it showed 88% and 95% homology in the coding and 3′ noncoding regions, respectively. At the peptide level, the amino acid sequences deduced from these two cDNAs had an overall homology of 83%. Fig. 3 shows the comparison of the peptide sequences deduced from

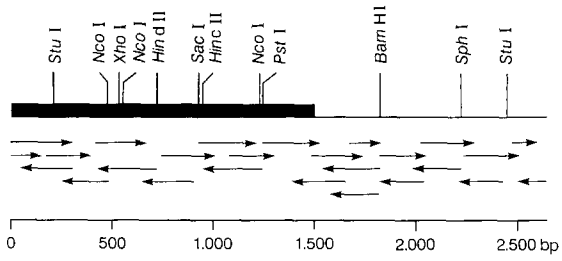

Fig. 1. Restriction enzyme map and sequencing strategy for pAld23 cDNA. The box and line represent the coding and 3′ noncoding region, respectively. Horizontal arrows show the direction and extent of sequencing.

cDNAs of pAld23 and rat cytochrome P-450$_{11\beta}$ [2]. Out of 500 amino acid residues, 415 which are indicated by *dashes* are common between the two amino acid sequences. There are two regions, however, where frequent amino acid replacements were found, and they resided in the mid-portion of the entire sequence as *boxed* in Fig. 3. One region is from residue 175 to 192 and the other from 277 to 300. Thus pAld23 codes for a protein very similar to but definitely different from that encoded by the rat cytochrome P-450$_{11\beta}$ cDNA. As already mentioned, residues 25–44 of the deduced amino acid sequence of pAld23 are identical to the N-terminal peptide sequence of purified rat cytochrome P-450$_{aldo}$. These findings indicate that the cDNA encodes the rat cytochrome P-450$_{aldo}$. Molecular mass of the mature form of cytochrome P-450$_{aldo}$ apoprotein calculated from the amino acid sequence was 54 282 Da.

Then we compared the peptide sequence of cytochrome P-450$_{aldo}$ and that of cytochrome P-450$_{11\beta}$ in more detail. The two sequences were found to be 100% homologous in the regions which were commonly conserved among the members of the cytochrome P-450 family including steroidogenic cytochrome P-450s [8]. These regions, enclosed in *boxes* with *asterisk(s)* in Fig. 3, are located in the carboxy-terminal half of the proteins. The heme-binding domain spans from residue 440 to 460 in the cytochrome P-450$_{aldo}$ sequence and contains a cysteine residue that can be thought of as the fifth ligand to the heme ion [9–11]. The second domain, residues 362–378 indicated by a single *asterisk*, can be allocated to Ozols' tridecapeptide [12] and might correspond to a portion of helix K of cytochrome P-450$_{cam}$ from *Pseudomonas putida* [11]. The third, the so-called aromatic region, spans residues 417–428 and lies between the two conserved domains mentioned above. Significance of such a strict conservation of the 3 regions between the two proteins is unknown at present, but may suggest some important roles of the domains in the metabolism of DOC.

At least two different types or modes of corticosteroidogenesis have been postulated to occur in

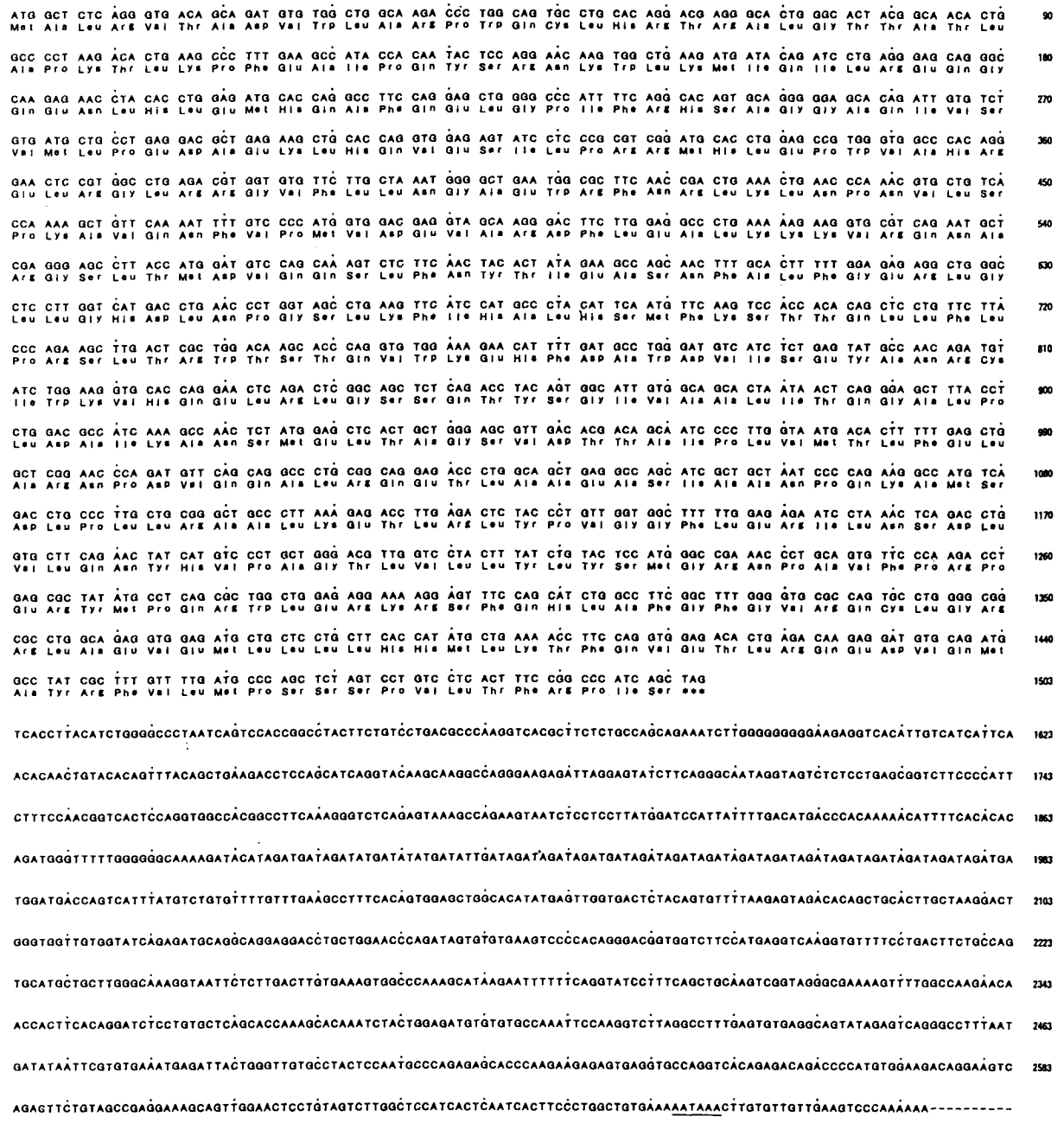

Fig. 2. Nucleotide sequence of pAld23 cDNA and deduced amino acid sequence. The nucleotides are numbered starting at the first 'A' in putative initiation codon. Termination codon is indicated by asterisks. Within the 3′ untranslated region the poly-adenylation site is underlined.

mammals [1]; one is such seen in cattle in which a single enzyme cytochrome P-450$_{11\beta}$ catalyzes the biosynthesis of both corticosterone and aldosterone from DOC [13] and another is observed in rat where two distinct enzymes exist for the biosyntheses of corticosterone (cytochrome P-450$_{11\beta}$) and aldosterone (cytochrome P-450$_{aldo}$) [1]. The present results, together with those of Nonaka et al. [2], provide further evidence for the above issue that rat adrenal cortex has two distinct enzymes, and also a means to gain an insight into the regulatory mechanisms of corticosteroidogenesis in rat.

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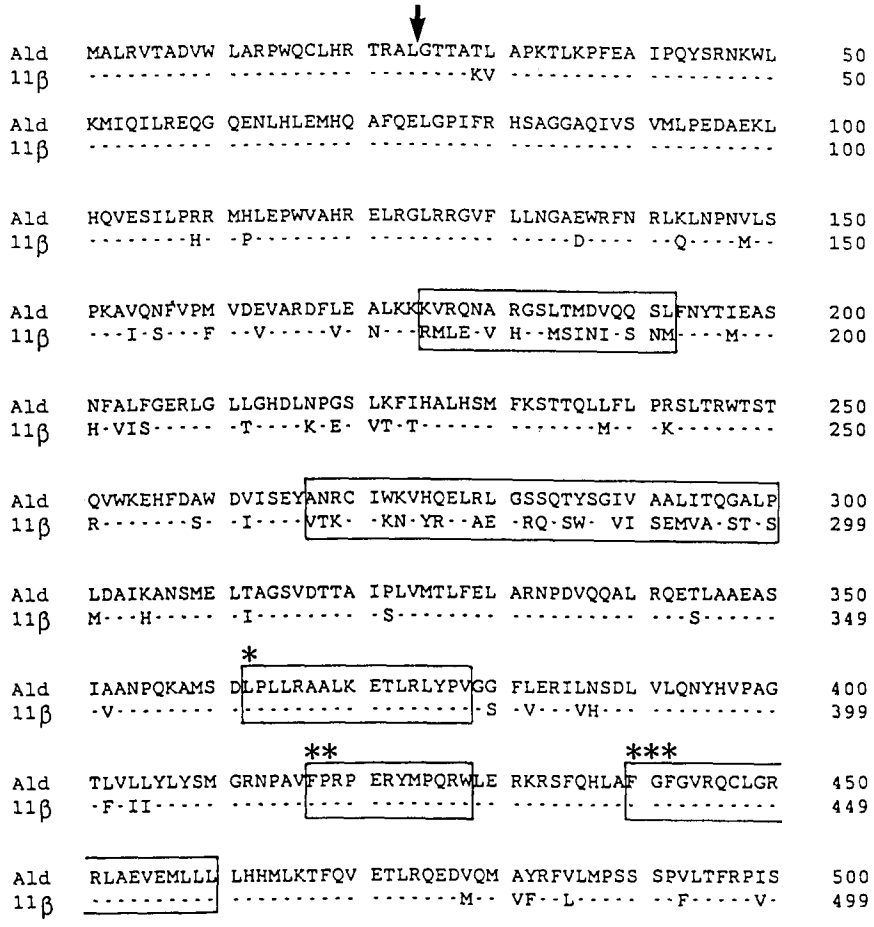

Fig. 3. Comparison of the entire peptide sequences predicted by rat cytochrome P-450$_{aldo}$ and rat cytochrome P-450$_{11\beta}$ cDNAs. Ald and 11β denote sequences derived from cDNAs for rat cytochrome P-450$_{aldo}$ and rat cytochrome P-450$_{11\beta}$, respectively. The amino acid sequence of the latter was taken from [2]. Dashes indicate the residues common in the two sequences. Boxed regions are: *helix-K region, **aromatic region, and ***heme binding domain conserved among all cytochrome P-450s sequenced to date. The arrow shows the putative cleavage site for the processing enzyme.

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