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CLONING AND EXPRESSION OF A cDNA FOR HUMAN CYTOCHROME P-450$_{aldo}$ AS RELATED TO PRIMARY ALDOSTERONISM[1]

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SUMMARY: A cDNA clone encoding human aldosterone synthase cytochrome P-450 (P-450$_{aldo}$) has been isolated from a cDNA library derived from human adrenal tumor of a patient suffering from primary aldosteronism. The insert of the clone contains an open reading frame encoding a protein of 503 amino acid residues together with a 3 bp 5'-untranslated region and a 1424 bp 3'-untranslated region to which a poly(A) tract is attached. The nucleotide sequence of P-450$_{aldo}$ cDNA is 93% identical to that of P-450$_{11\beta}$ cDNA. Catalytic functions of these two P-450s expressed in COS-7 cells are very similar in that both enzymes catalyze the formation of corticosterone and 18-hydroxy-11-deoxycorticosterone using 11-deoxycorticosterone as a substrate. However, they are distinctly different from each other in that P-450$_{aldo}$ preferentially catalyzes the conversion of 11-deoxycorticosterone to aldosterone via corticosterone and 18-hydroxycorticosterone while P-450$_{11\beta}$ substantially fails to catalyze the reaction to form aldosterone. These results suggest that P-450$_{aldo}$ is a variant of P-450$_{11\beta}$, but this enzyme is a different gene product possibly playing a major role in the synthesis of aldosterone at least in a patient suffering from primary aldosteronism.

Aldosterone, the most typical mineralocorticoid, is synthesized via enzymatic reactions involving several specific monooxygenases (1), termed P-450s (2), starting from cholesterol through a pathway including pregnenolone, progesterone, 11-deoxycorticosterone (DOC) and corticosterone as metabolic intermediates (3). Nature of the enzyme catalyzing the final step of aldosterone biosynthesis is not as yet fully understood. From clinical viewpoints, it is very important to characterize the nature of this enzyme, because several acquired and inborn errors in the synthesis or action of aldosterone such as primary aldosteronism and corticosterone methyl oxidase Type II (CMO II) deficiency have been reported (4,5).

[1]The sequence data in this paper will appear in GenBank/EMBL/DDBJ Nucleotide Sequence Databases under the accession number X54741.

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Abbreviations: P-450$_{11\beta}$, steroid 11β-hydroxylase; P-450$_{aldo}$, aldosterone synthase cytochrome P-450; DOC, 11-deoxycorticosterone; CMO II, corticosterone methyl oxidase Type II; 1xSSC, 150 mM NaCl and 15 mM sodium citrate.

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Recently, several investigators demonstrated that cytochrome P-450$_{11\beta}$ purified from bovine adrenocortical mitochondria catalyzes not only 11β-, 18- and 19-hydroxylations of DOC (6,7) but it also catalyzes the conversion of corticosterone to aldosterone via 18-hydroxycorticosterone (8-11). Morohashi et al. isolated two kinds of cDNA clone for bovine P-450$_{11\beta}$ (12,13) and demonstrated that both of these bovine P-450$_{11\beta}$s expressed in COS-7 cells catalyze the synthesis of aldosterone (14). In contrast, Ogishima et al. (15) isolated aldosterone synthase cytochrome P-450 (P-450$_{aldo}$) induced in sodium-depleted potassium-replete rat adrenal cortex (16-18) and demonstrated that the P-450$_{aldo}$ catalyzes three successive monooxygenation reactions of DOC to produce aldosterone as a final product whereas rat P-450$_{11\beta}$ does not substantially catalyze the reaction to form aldosterone. More recently, cDNAs for rat P-450$_{aldo}$ distinct from that for rat P-450$_{11\beta}$ (19) have been isolated by two groups (20,21).

In regard to human P-450$_{11\beta}$, Chua et al. (22) isolated a partial-length cDNA using a fetal adrenal cDNA library. Mornet et al. (23) have recently isolated human P-450$_{11\beta}$ gene (CYP11B1) and its related gene (CYP11B2). According to their report, only CYP11B1 is actively transcribed and CYP11B2 transcripts are not detectable in human adrenal mRNA or among cDNA clones. In these works, however, it remains to be elucidated whether human P-450$_{11\beta}$, a product of CYP11B1, has the ability to catalyze the formation of aldosterone.

As an initial step to investigate what kind of enzymes are responsible for aldosterone-dependent diseases in humans, we attempted to isolate cDNA clones for human P-450$_{11\beta}$ and its related enzymes, using a cDNA library derived from adrenal tumor of a patient suffering from primary aldosteronism. In our preceding paper (24), we reported the isolation of a full-length cDNA (pH11β1) coding for human P-450$_{11\beta}$ together with the regulatory properties of the corresponding gene. In this paper, we report the isolation of a cDNA clone (pH11β2) coding for human P-450$_{aldo}$ and present a line of evidence to show that the cDNA clone (pH11β2) corresponds to the transcript of the unidentified gene (CYP11B2) as reported by Mornet et al. (23) and that P-450$_{aldo}$ but not P-450$_{11\beta}$ as expressed in COS-7 cells is the enzyme responsible for catalyzing the synthesis of aldosterone using DOC as a substrate.

MATERIALS AND METHODS

Molecular cloning and nucleotide sequencing

cDNA was prepared as described previously (24) using poly(A)$^+$ RNA isolated from adrenal tumor of a patient suffering from primary aldosteronism. The cDNA was fractionated by gel filtration and cDNA species longer than 2 kb were collected and used to construct a λgt10 cDNA library as described by Huynh et al. (25). The *Eco*RI-*Sma*I fragment (-4 to 88 relative to the translational initiation site) of pH11β1 (24) and a synthetic 47-mer oligonucleotide (5'-TGCAAGACTAGTTAATCGCTCTGAAAGTGAGGAGGGGGGACGTGCCA-3') were used as screening probes. The synthetic oligonucleotide was designed on the basis of the nucleotide sequence of CYP11B2 (23). Two clones hybridized with both probes were isolated out of $1x10^6$ recombinants. One clone (pH11β2) containing the longer insert was subcloned into pUC plasmid for further analysis. Nucleotide sequence was determined by the dideoxy chain termination method (26,27).

Construction of expression plasmids and transfection of DNAs into COS-7 cells

The *Eco*RI-*Bam*HI fragment of pH11β1 (24) or pH11β2 was inserted into the *Sma*I-*Bam*HI site of the expression vector pSVL (Pharmacia) after filling-in the *Eco*RI sites of the cDNAs. The resulting plasmids designated as pSV11β1 and pSV11β2 were separately transfected into COS-7

cells as described below. For mock transfection as a control experiment, pSVL vector plasmid without insert was used.

DNA transfection was performed by electroporation as described by Neuman et al.(28) using Gene Pulser (Bio-Rad Laboratories). COS-7 cells ($5x10^6$) were suspended in 0.5 ml of saline G (29) containing 20 μg of the expression plasmid DNA and 250 μg of sonicated herring sperm DNA as a carrier. After cells were subjected to a single electric pulse (960 μF at 240 V), they were plated in a dish (φ90 mm) containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and incubated for 72 h. To overcome the variability inherent in transfection, β-galactosidase expression plasmid, pCH110, (30) was cotransfected and β-galactosidase activity was determined for compensating transfection efficiency.

Analysis of steroid hydroxylase activity of P-450s expressed in COS-7 cells

The cells transfected were collected from twelve dishes, suspended in 10 volumes of 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5) and homogenized with a Potter-Elvehjem homogenizer. Mitochondrial fraction was prepared by differential centrifugation at 400xg and 8000xg. The mitochondria were solubilized with sodium cholate and then assayed for steroid hydroxylase activity as described by Ohnishi et al. (11). Briefly, solubilized mitochondria were incubated at 37°C for 20 min with 1 μM [1,2-^{3}H] 11-deoxycorticosterone in 25 mM potassium phosphate buffer (pH 7.5) containing 4 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.5 U/ml glucose 6-phosphate dehydrogenase, 0.5 μM NADPH-adrenodoxin reductase, 15 μM adrenodoxin, 0.1% sodium cholate and 100 μM NADPH. The products formed were analyzed using HPLC system as described by Wada et al. (9).

Northern blot analysis

Poly(A)$^+$ RNA (0.3 μg) was electrophoresed on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane as described by Maniatis et al. (31). Hybridization was carried out using the same probes as those used for screening the cDNA library. The filter was washed at 65°C for 15 min with 2xSSC containing 0.1% SDS, then with 1xSSC containing 0.1% SDS and finally washed at 65°C for 30 min with 0.1xSSC containing 0.1% SDS. It was autoradiographed at -70°C with intensifying screens.

RESULTS

Fig.1 represents the restriction map of and the sequencing strategy for a cDNA clone (pH11β2) isolated. Fig.2 shows the nucleotide sequence of pH11β2 consisting of 2936 bp excluding a poly(A) tract. The open reading frame starting from the first ATG (Met) codon consists of 1509 nucleotides encoding a polypeptide composed of 503 amino acid residues, followed by the translational termination codon TAG. A poly(A) tract is attached to the 3'-terminus of 1424 bp 3'-untraslated region. For comparison, the nucleotides of pH11β1 and the deduced amino acid residues of P-$450_{11\beta}$ (24) distinct from that of pH11β2 and the corresponding deduced amino acid residues are also present in Fig.2. As observed in this figure, the nucleotide sequences of pH11β1 and pH11β2

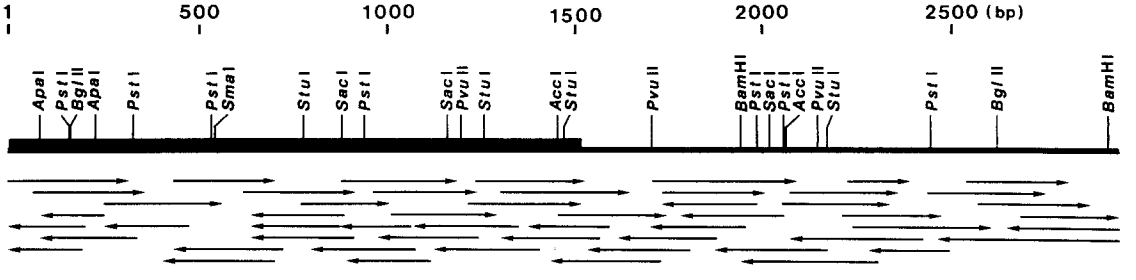
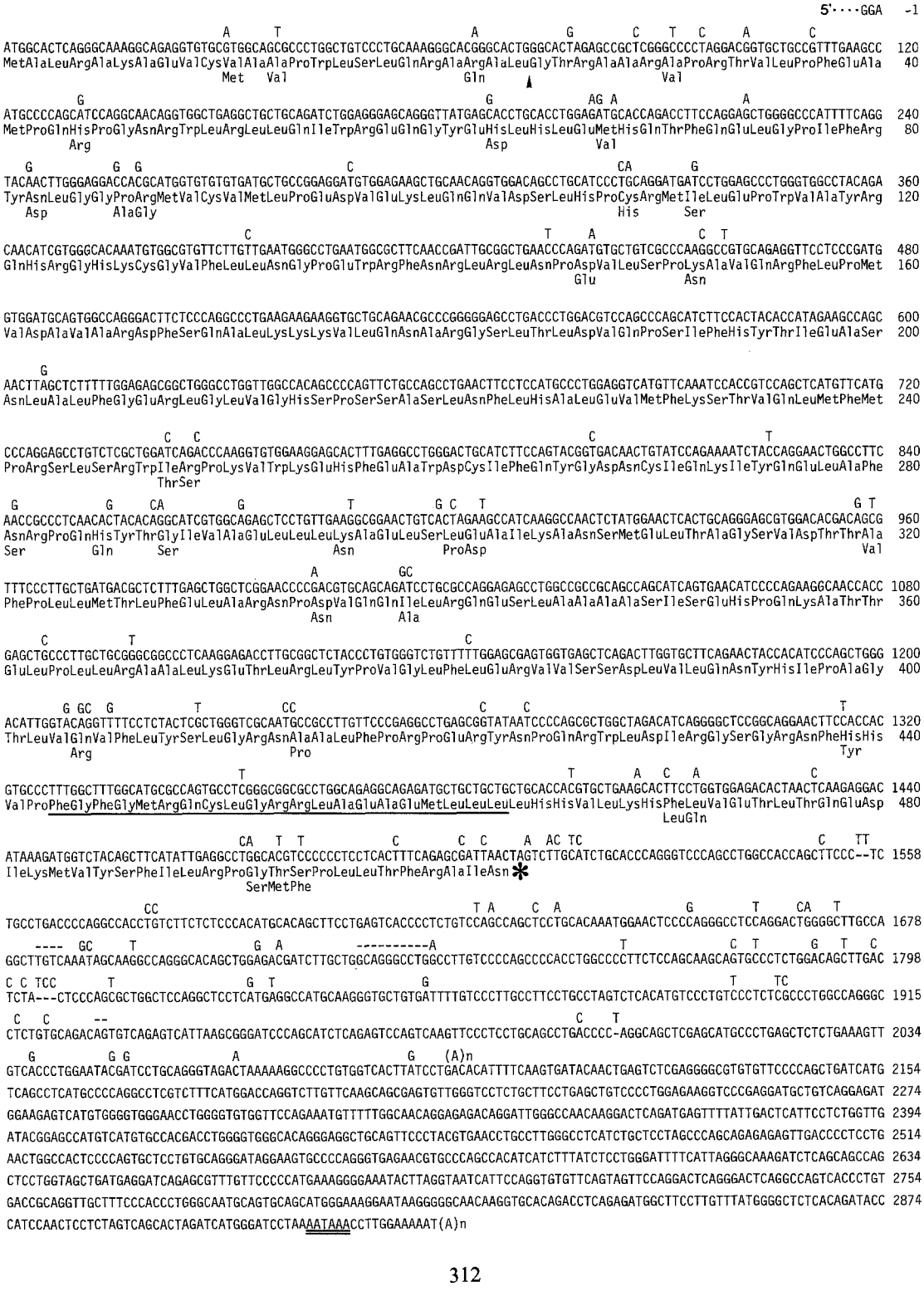


Fig.1. Restriction map of and sequencing strategy for the cloned cDNA (pH11β2) encoding human P-450_{aldo}. The restriction map displays only relevant restriction endonuclease sites. A closed box and a solid line represent the protein coding region and the untranslated regions, respectively. Horizontal arrows show the direction and the extent of sequence determination.

and deduced amino acid sequences are 93% identical to each other. The amino acid sequence deduced from the nucleotide sequence of pH11β2 is 73%, 69% and 64% identical to those of bovine P-450$_{11\beta s}$ (12,13,22), rat P-450$_{aldo}$ (20,21) and rat P-450$_{11\beta}$ (19), respectively.



In order to elucidate the functional characteristics of the products coded by these two P-450 cDNAs (pH11β1 and pH11β2), expression plasmids (pSV11β1 and pSV11β2 containing *Eco*RI-*Bam*HI fragments of pH11β1 and pH11β2, respectively) were transfected into COS-7 cells and steroid hydroxylase activity of these two P-450s expressed was determined. The results of a typical experiment are summarized in Table 1. The hydroxylation reaction of DOC to form corticosterone catalyzed by the P-450$_{11\beta}$ derived from pSV11β1 proceeds approximately two-fold faster than that of the P-450 derived from pSV11β2. The rate of conversion of DOC to 18-hydroxy-11-deoxycorticosterone by these two P-450s is almost equivalent. The hydroxylase activities of the P-450 derived from pSV11β2 at the 19 position of DOC and at the 18 position of corticosterone are approximately 4- and 10-fold higher than those derived from pSV11β1, respectively. Of particular interest is the fact that aldosterone synthase activity of the P-450 coded by pSV11β2 is remarkably high (more than 50-fold) as compared with that of P-450$_{11\beta}$ coded by pSV11β1. These results indicate that the clone pH11β2 is the cDNA encoding human aldosterone synthase cytochrome P-450 (P-450$_{aldo}$).

To determine the sizes and amounts of transcripts of human P-450$_{aldo}$ and P-450$_{11\beta}$ genes, we performed Northern blot analysis. As shown in Fig.3, a major band of 3.1 kb is detected using the oligonucleotide probe specific for pH11β2, while three other bands of 4.2 kb, 3.6 kb and 2.2 kb are detected when the restriction fragment of pH11β1 is used as a probe. These results suggest that the band of 3.1 kb represents the mRNA for P-450$_{aldo}$ and three other bands correspond to mRNAs for P-450$_{11\beta}$. This conclusion is consistent with the recent report by Mornet et al. (23), indicating that human P-450$_{11\beta}$ gene (CYP11B1) is expressed using multiple polyadenylation signals to form different sizes of mRNAs.

DISCUSSION

In the present study, we have isolated and sequenced a new cDNA clone, pH11β2, similar to pH11β1, the cDNA for P-450$_{11\beta}$ (24). Furthermore, we have demonstrated that the product coded by pH11β2 functions as aldosterone synthase cytochrome P-450 (P-450$_{aldo}$). The nucleotide sequence of pH11β2 agrees well with that of the presumed exons of the unidentified gene, CYP11B2, as reported by Mornet et al. (23). This fact indicates that pH11β2 corresponds to the transcript of CYP11B2.

Fig.2. The nucleotide sequence of human P-450$_{aldo}$ cDNA (pH11β2) and the deduced amino acid sequence. Nucleotides are numbered starting at 'A' residue of the first ATG codon. The deduced amino acid residues are shown blow the nucleotide sequence and numbered beginning with the first methionine. The nucleotides of pH11β1 and the deduced amino acid residues of P-450$_{11\beta}$ (24) different from the corresponding nucleotides of pH11β2 and the corresponding amino acid residues of P-450$_{aldo}$ are shown above and below each sequence, respectively. The dash represents that the corresponding nucleotides are deleted. A star symbol downstream of the protein coding region indicates the stop codon. The putative heme binding site is marked by an underline. The poly(A) addition signal is also marked by double-underlining. The cleavage site for human P-450$_{aldo}$ precursor polypeptide to form a mature protein is tentatively assigned to be between Leu24 and Gly25 and indicated by an arrowhead in the figure, because the same position is also postulated to be the cleavage site of rat P-450$_{aldo}$ precursor protein (20,21).

Table 1. Steroid hydroxylase activity of mitochondria in COS-7 cells transfected with pSV11β1 or pSV11β2

Product formed	Hydroxylase activity derived from	
	pSV11β1	pSV11β2
	(pmol)	
Corticosterone	519	234
18-hydroxy-11-deoxycorticosterone	20.2	25.9
19-hydroxy-11-deoxycorticosterone	3.7	16.0
18-hydroxycorticosterone	5.3	52.6
Aldosterone	< 0.03	2.3

The data in this table represent the amount of each product formed in 20 min incubation, using DOC as a substrate. Each value is corrected on the basis of the transfection efficiency of each plasmid by determining β-galactosidase activity taken as an internal standard for transfection as described under Materials and Methods. Expression plasmids, pSV11β1 and pSV11β2, contain cDNAs for P-450$_{11\beta}$ and P-450$_{aldo}$, respectively. Under the assay conditions employed, the amount of aldosterone, if any, formed by the product coded by pHSV11β1 is as low as that obtained by mock transfection using pSVL.

Using Northern blot analysis, we have observed that the P-450$_{aldo}$ gene, together with the P-450$_{11\beta}$ gene, is highly expressed in adrenal tumor of a patient suffering from primary aldosteronism. In contrast, Mornet et al. (23) reported that any kind of transcript of CYP11B2 is not detected in human adrenal mRNA. These results are consistent with the recent preliminary report by Ogishima et al. (32) that P-450$_{aldo}$ activity is detectable in adrenal tumor of a patient with the same disease, but the activity is hardly detectable in normal adrenal gland under the assay conditions employed. Therefore, all of these results, taken together, suggest that primary aldosteronism is caused by adrenal tumor in which the P-450$_{aldo}$ gene is highly expressed. In this connection, it is possible to postulate that CMO II deficiency is derived from a defect or mutation of this gene,

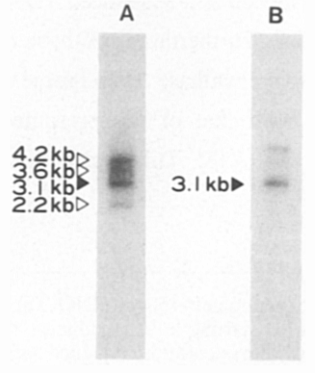


Fig.3. Northern blot analysis. Poly(A)$^+$ RNA of adrenal tumor of a patient suffering from primary aldosteronism was electrophoresed, transferred to a nylon membrane and hybridized with (A) the ^{32}P-labeled *Eco*RI-*Sma*I fragment of pH11β1 (24) or (B) the 47-mer oligonucleotide probe specific for the P-450$_{aldo}$ gene (23). Closed and open arrowheads indicate the positions of mRNAs for P-450$_{aldo}$ and P-450$_{11\beta}$, respectively. The size of each band is shown on the left side of each lane. Note that the band of 3.1 kb for P-450$_{aldo}$ mRNA is detectable in lane A, because it crosshybridizes with the *Eco*RI-*Sma*I fragment of pH11β1. The more slowly migrating band detected in lane B appears to be a nonspecific crosshybridized band.

because marked reduction in the concentration of aldosterone with concomitant accumulation of 18-hydroxysteroids in serum and urine (4,5,33) is observed in patients suffering from this disease.

More detailed molecular analyses of DNAs, mRNAs, cDNAs and protein products from patients suffering from primary aldosteronism, CMO II deficiency or 11β-hydroxylase deficiency will provide useful data for elucidating the exact roles of $P\text{-}450_{aldo}$ and $P\text{-}450_{11\beta}$ genes in humans.

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