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MOLECULAR CLONING AND EXPRESSION OF cDNAS ENCODING RAT ALDOSTERONE SYNTHASE: VARIANTS OF CYTOCHROME P-450$_{11\beta}$

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Summary: Two distinct forms of cDNA encoding rat aldosterone synthase were cloned from an adrenal capsular tissue cDNA library. The deduced amino acid sequences showed that one of the enzymes (P-450$_{11\beta}$,aldo-1) had a long extension peptide composed of 34 amino acid residues while the other (P-450$_{11\beta}$,aldo-2) had an extension peptide identical to that of rat P-450$_{11\beta}$. Glu at the 320th position of P-450$_{11\beta}$,aldo -1 was replaced with Lys in P-450$_{11\beta}$,aldo-2. The amino acid sequence of the aldosterone synthase was highly homologous (81%) to rat P-450$_{11\beta}$. Constructed expression vector containing the cDNA for extension peptide of P-450$_{11\beta}$ and the mature protein of P-450$_{11\beta}$,aldo-1 was transfected into COS-7 cells. The cells converted 11-deoxycorticosterone into corticosterone, 18-hydroxycorticosterone, and aldosterone.

The molecular nature of aldosterone synthase and its relation to cytochrome P-450$_{11\beta}$ are still under discussion. The bovine adrenocortical P-450$_{11\beta}$ purified to homogeneity has been shown to catalyze not only the 11β- and 18-hydroxylation of 11-deoxycorticosterone but also conversion of corticosterone to aldosterone (1-3). Recently it was shown that all these reactions indeed occurred in mitochondria prepared from COS-7 cells which had been transfected by a bovine P-450$_{11\beta}$ cDNA (4). Thus a single enzyme is responsible for the final step of aldosterone biosynthesis, *i.e.* the conversion of 11-deoxycorticosterone to corticosterone and to aldosterone. On the other hand two distinct forms of P-450$_{11\beta}$ seem to exist in rat adrenal cortex. A P-450$_{11\beta}$ with an apparent molecular weight of 51,000 is present in all zones of adrenal cortex and is probably responsible for the 11β -hydroxylation. Another P-450$_{11\beta}$ with an apparent molecular weight of 49,000 exists only in the zona glomerulosa mitochondria (5,6). Its level is elevated in the tissue of Na^+-depleted animals (7). This cytochrome is probably responsible for the aldosterone synthesis.

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These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number D00567 or D00568 for cytochrome P-450$_{11\beta}$,aldo-46 and pcP-450$_{11\beta}$,aldo-16, respectively.

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The two P-450$_{11\beta}$ species were isolated and their NH_2-terminal amino acid sequences were determined up to the 20th residue (8). They were identical except that the 11β-hydroxylase has Val at the 6th position, while the aldosterone synthase has a "Leu6" structure. We recently isolated and characterized a cDNA clone encoding the 11β-hydroxylase in a cDNA library constructed from the whole adrenal tissue obtained from ACTH-stimulated rats (9). In this communication, we describe an attempt to isolate a cDNA clone encoding the aldosterone synthase from a cDNA library constructed from the adrenal capsular tissue of Na^+-depleted and K^+-repleted rats. Two cDNAs having the "Leu6" structure were isolated, whose structures were highly homologous but not identical.

MATERIALS AND METHODS

Materials: An expression vector, pSVL, was purchased from Pharmacia. Cos-7 cells were generously given by Drs. K. Morohashi and T. Omura (Kyushu Univ.). Steroids were purchased from Makor Chemicals (aldosterone and 18-hydroxycorticosterone) or from Sigma (dexamethasone, 11-deoxycorticosterone and corticosterone).

Isolation of rat P-450$_{11\beta}$,aldo cDNA and determination of nucleotide sequence: Male Sprague-Dawley rats, weighing 200-250 g, were fed low Na^+- and high K^+- diet for 2 weeks. Adrenal capsular tissues were prepared from the animals as described before (5,6). Total RNA was extracted from the capsular tissues, and a cDNA library was constructed in bacteriophage λgt10 as described before (9). The library containing 7 x 10^5 phages was screened using a *Eco*RI-*Pst*I fragment of pcP-450$_{11\beta}$-62 (428-bp long) as a probe. Forty-three positive clones were isolated. The inserts were subcloned into the *Eco*RI site of pUC118 plasmid, and sequenced by the dideoxynucleotide chain termination method.

Determination of 5'-terminal region sequence by one-sided polymerase chain reaction (PCR): Three primers were designed according to the nucleotide sequence determined in this paper; the sequence of primer 1 was 5'-CAC-CATGGGGACAAAATTTTGAAC-3' (an oligonucleotide corresponding to 490-513), that of primer 2, 5'-TTTCAGTCGGTTGAAGCGCCATTCA-3' (an oligonucleotide, 435-459), and that of primer 3, 5'-GTGTAGGTTCTCTTGGCCCTGCTCC-3' (an oligonucleotide, 201-225). "Anchored primers" used were following two oligonucleotides; a short oligonucleotide, 5'-TAATACGACTCACTATAGGG-3', and a long oligonucleotide, 5'-TAAGATCTAATACGACTCACTATAGGGAAGCTTTTTTTTTTTTTTTT-3'. The cDNA was tailed with dA using terminal deoxynucleotidyl-transferase, and then amplified by PCR (10). In the first amplification, primer 1 and a mixture of the two "anchored primers" at a molar ratio of 5:1 (short *versus* long) were used. In the second amplification, primer 2 and the short "anchored primer" and in the third, primer 3 and the short "anchored primer" were used, respectively. The PCR conditions were as follows; 55°C, 30 s; 72°C, 1 min; 94°C, 30 s; 30 cycles. The amplified DNAs were subcloned into pUC118 plasmid and several independent clones were sequenced.

Construction of expression plasmids: The fragment containing the complete coding region of P-450$_{11\beta}$,aldo -1 was prepared by *Bam*HI digestion to eliminate polyadenylation signal and poly A tail, and then was inserted into the *Bam*HI site of expression vector, pSVL. The mutagenesis of fragments of pcP-450$_{11\beta}$,aldo -46 and pcP-450$_{11\beta}$,aldo -16 to those containing the complete mature protein regions and the extension peptide region having the same amino acid sequence as that of pcP-450$_{11\beta}$-62 was conducted by the PCR method using the mismatched primer, 5'-CTAGATGGCTCTCAGGGTGACAGCAGATG-3' and the matched primer, 5'-TTTTTGTGGGTCATGTCAAAATAAT-3' (an oligonucleotide corresponding to 1857-1881). The fragments digested with *Bam*HI were inserted into the *Sma*I and *Bam*HI digested fragment of expression vector, pSVL. Sequencing was

performed to confirm the 5' end structure. The *Eco*RI-*Kpn*I and *Eco*RI-*Xmn*I fragments of cloned bovine adrenodoxin reductase and adrenodoxin, respectively, were prepared and inserted into the *Sma*I site of pSVL.

Transfection of DNA into COS-7 cells: DNA transfection was performed by electroporation as described by Morohashi *et al.*(4) with some modification. COS-7 cells ($5x10^6$) were suspended in 0.5 ml saline G containing 50 μg, 10 μg, and 10 μ g of the expression plasmid DNAs of cytochrome P-450, adrenodoxin reductase, and adrenodoxin, respectively, and 250 μ g carrier DNA. After the cells were subjected to a single electric pulse (125 μ F at 450 V), they were divided into two 9 cm-dishes (200 μ l, each) and incubated for 24 h with 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Then the medium was changed and 200 nmol 11-deoxycorticosterone was added to the fresh medium. After 24 h, the medium was recovered, and 5 nmol of dexamethasone was added as an internal standard. Extraction and analysis of the steroids were performed as described previously (2).

RESULTS AND DISCUSSION

Among the forty-three clones isolated, forty of them contained the inserts whose deduced NH_2-terminal amino acid sequences had the "Leu6" structure, indicating that they were the clones containing the aldosterone synthase cDNA. Two clones having the longest cDNA insert were isolated. Restriction enzyme maps of the two inserts, named pcP-$450_{11\beta}$,aldo -46 and pcP-$450_{11\beta}$,aldo -16, are shown in Fig. 1. The two maps are highly similar except that pcP-$450_{11\beta}$,aldo -46 has a *Sac*I site while pcP-$450_{11\beta}$,aldo -16 has not. Complete nucleotide sequences of the two inserts are shown in Fig 2A. Alignment of the two inserts with that of rat P-$450_{11\beta}$ (pcP-$450_{11\beta}$-62) (9) revealed that pcP-$450_{11\beta}$,aldo -46 is a full-length cDNA having a 74-bp 5'-noncoding region, a 1,530-bp open reading frame and a 1,217-bp 3'-noncoding region. pcP-$450_{11\beta}$,aldo -16 begins with "G", lacks the initiation codon, "ATG", and has a 1,497-bp coding region and a 1,198-bp 3'-noncoding region. The two inserts have an identical nucleotide

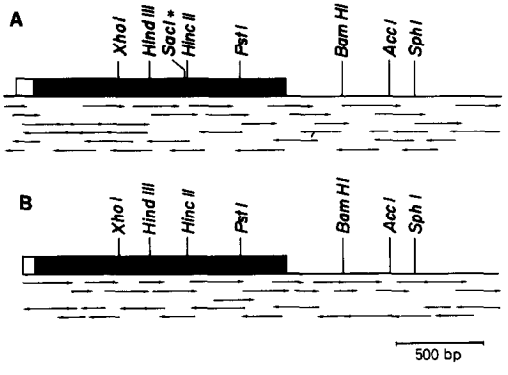
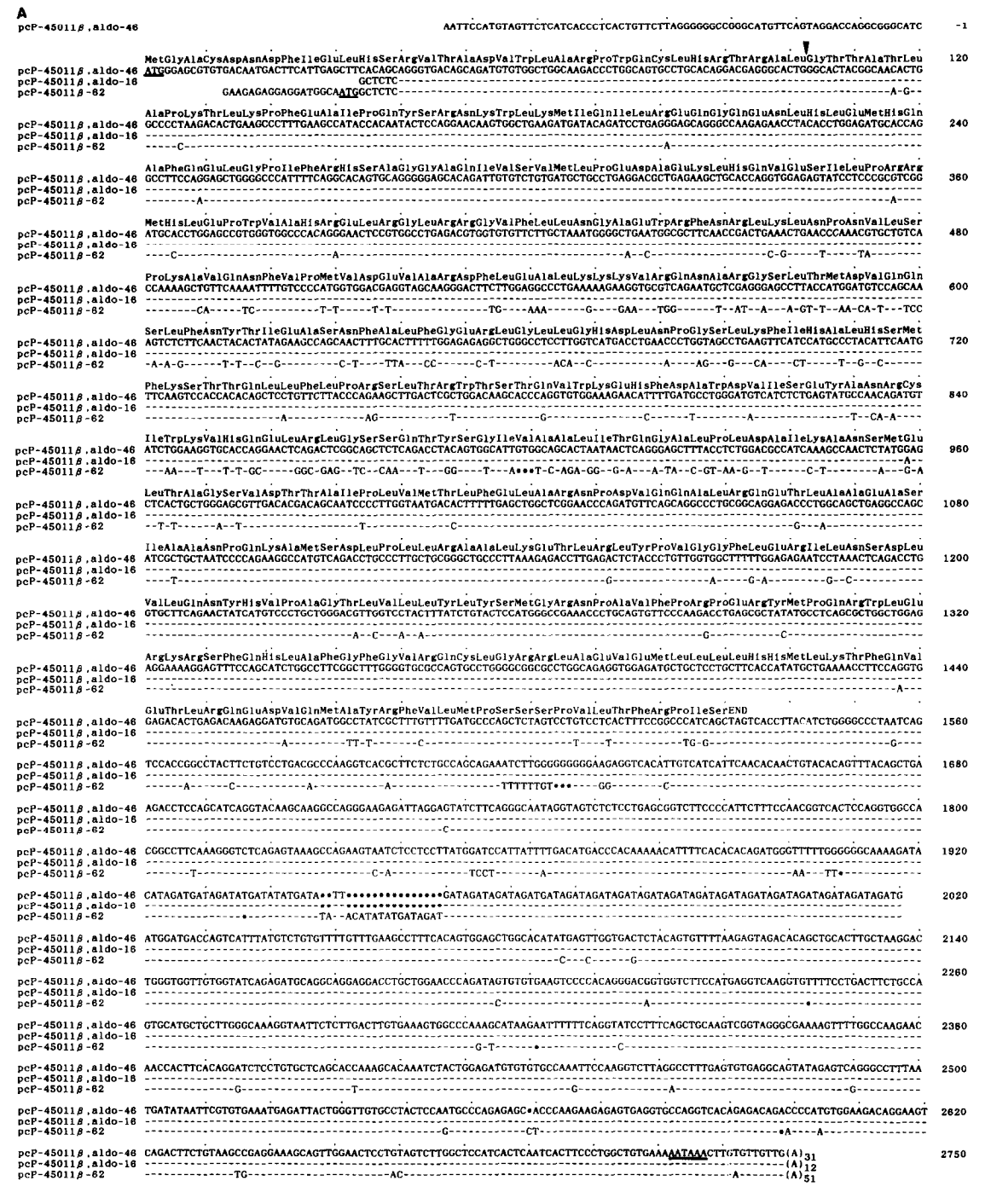
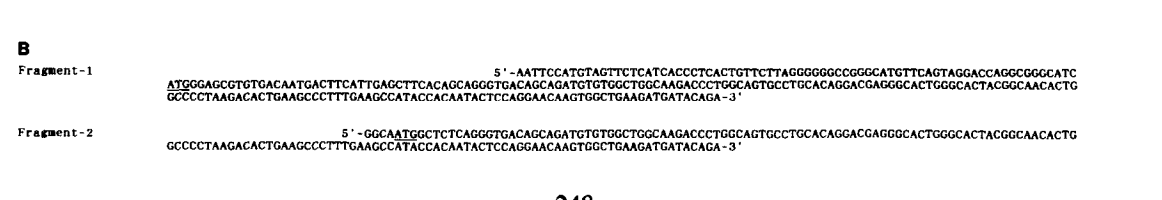


Fig. 1. **Restriction enzyme maps and sequence strategy of the P-$450_{11\beta}$,aldo cDNAs** . The panels A and B show the main restriction sites of pcP-$450_{11\beta}$,aldo-46 and pcP-$450_{11\beta}$,aldo-16, respectively. The rectangular boxes indicate the coding regions and the bars, the 5'- and 3'-untranslated regions. The open boxes represent the extension peptides, the closed boxes, the mature proteins. Arrows indicate the direction and extent of sequencings. The *Sac*I site was found only in pcP-$450_{11\beta}$,aldo-46.


A
pcP-45011β,aldo-46
pcP-45011β,aldo-16
pcP-45011β-62
MetGlyAlaCysAspAsnAspPheIleGluLeuHisSerArgValThrAlaAspValTrpLeuAlaArgProTrpGlnCysLeuHisArgThrArgAlaLeuGlyThrThrAlaThrLeu
AlaProLysThrLeuLysProPheGluAlaIleProGlnTyrSerArgAsnLysTrpLeuLysMetIleGlnIleLeuArgGluGlnGlyGlnGluAsnLeuHisLeuGluMetHisGln
AlaPheGlnGluLeuGlyProIlePheArgHisSerAlaGlyGlyAlaGlnIleValSerValMetLeuProGluAspAlaGluLysLeuHisGlnValGluSerIleLeuProArgArg
MetHisLeuGluProTrpValAlaHisArgGluLeuArgGlyLeuArgArgGlyValPheLeuLeuAsnGlyAlaGluTrpArgPheAsnArgLeuLysLeuAsnProAsnValLeuSer
ProLysAlaValGlnAsnPheValProMetValAspGluValAlaArgAspPheLeuGluAlaLeuLysLysLysValArgGlnAsnAlaArgGlySerLeuThrMetAspValGlnGln
SerLeuPheAsnTyrThrIleGluAlaSerAsnPheAlaLeuPheGlyGluArgLeuGlyLeuLeuGlyHisAspLeuAsnProGlySerLeuLysPheIleHisAlaLeuHisSerMet
PheLysSerThrThrGlnLeuLeuPheLeuProArgSerLeuThrArgTrpThrSerThrGlnValTrpLysGluHisPheAspAlaTrpAspValIleSerGluTyrAlaAsnArgCys
IleTrpLysValHisGlnGluLeuArgLeuGlySerSerGlnThrTyrSerGlyIleValAlaAlaLeuIleThrGlnGlyAlaLeuProLeuAspAlaIleLysAlaAsnSerMetGlu
LeuThrAlaGlySerValAspThrThrAlaIleProLeuValMetThrLeuPheGluLeuAlaArgAsnProAspValGlnGlnAlaLeuArgGlnGluThrLeuAlaAlaGluAlaSer
IleAlaAlaAsnProGlnLysAlaMetSerAspLeuProLeuLeuArgAlaAlaLeuLysGluThrLeuArgLeuTyrProValGlyGlyPheLeuGluArgIleLeuAsnSerAspLeu
ValLeuGlnAsnTyrHisValProAlaGlyThrLeuValLeuLeuTyrLeuTyrSerMetGlyArgAsnProAlaValPheProArgProGluArgTyrMetProGlnArgTrpLeuGlu
ArgLysArgSerPheGlnHisLeuAlaPheGlyPheGlyValArgGlnCysLeuGlyArgArgLeuAlaGluValGluMetLeuLeuLeuLeuHisHisMetLeuLysThrPheGlnVal
GluThrLeuArgGlnGluAspValGlnMetAlaTyrArgPheValLeuMetProSerSerSerProValLeuThrPheArgProIleSerEND
TCCACCGGCCTACTTCTGTCCTGACGCCCAAGGTCACGCTTCTCTGCCAGCAGAAATCTTGGGGGGGGAAGAGGTCACATTGTCATCATTCAACACAACTGTACACAGTTTACAGCTGA
ACGCCTTCAAAGGGTCTCAGAGTAAAGCCAGAAGTAATCTCCTCCTTATGGATCCATTATTTTGACATGACCCACAAAAACATTTTCACACACAGATGGGTTTTTGGGGGGCAAAAGATA
CATAGATGATAGATATGATATATGATA••TT••••••••••••••••GATAGATAGATAGATAGATGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATG
ATGGATGACCAGTCATTTATGTCTGTGTTTTGTTTGAAGCCTTTCACAGTGGAGCTGGCACATATGAGTTGGTGACTCTACAGTGTTTTAAGAGTAGACACAGCTGCACTTGCTAAGGAC
TGGGTGGTTGTGGTATCAGAGATGCAGGCAGGAGGACCTGCTGGAACCCAGATAGTGTGTGAAGTCCCCACAGGGACGGTGGTCTTCCATGAGGTCAAGGTGTTTTCCTGACTTCTGCCA
GTGCATGCTGCTTGGGCAAAGGTAATTCTCTTGACTTGTGAAAGTGGCCCAAAGCATAAGAATTTTTTCAGGTATCCTTTCAGCTGCAAGTCGGTAGGGCGAAAAGTTTTGGCCAAGAAC
AACCACTTCACAGGATCTCCTGTGCTCAGCACCAAAGCACAAATCTACTGGAGATGTGTGTGCCAAATTCCAAGGTCTTAGGCCTTTGAGTGTGAGGCAGTATAGAGTCAGGGCCTTTAA
TGATATAATTCGTGTGAAATGAGATTACTGGGTTGTGCCTACTCCAATGCCCAGAGAGC•ACCCAAGAAGAGAGTGAGGTGCCAGGTCACAGAGACAGACCCCATGTGGAAGACAGGAAGT
CAGACTTCTGTAAGCCGAGGAAAGCAGTTGGAACTCCTGTAGTCTTGGCTCCATCACTCAATCACTTCCCTGGCTGTGAAAATAAACTTGTGTTGTTG(A)31 (A)12 (A)51
2750


B
Fragment-1
5'-AATTCCATGTAGTTCTCATCACCCTCACTGTTCTTAGGGGGGCCGGGCATGTTCAGTAGGACCAGGCGGGCATC
ATGGGAGCGTGTGACAATGACTTCATTGAGCTTCACAGCAGGGTGACAGCAGATGTGTGGCTGGCAAGACCCTGGCAGTGCCTGCACAGGACGAGGGCACTGGGCACTACGGCAACACTG
GCCCCTAAGACACTGAAGCCCTTTGAAGCCATACCACAATACTCCAGGAACAAGTGGCTGAAGATGATACAGA-3'
Fragment-2
5'-GGCAATGGCTCTCAGGGTGACAGCAGATGTGTGGCTGGCAAGACCCTGGCAGTGCCTGCACAGGACGAGGGCACTGGGCACTACGGCAACACTG
GCCCCTAAGACACTGAAGCCCTTTGAAGCCATACCACAATACTCCAGGAACAAGTGGCTGAAGATGATACAGA-3'

sequence except for the differences present in their 5'-termini and at the 958th position where "G" in pcP-450$_{11\beta}$,aldo -46 is replaced with "A" in pcP-450$_{11\beta}$,aldo -16.

The obvious difference between the 5'-terminal region of pcP-450$_{11\beta}$,aldo -46 and that of pcP-450$_{11\beta}$-62 as well as the lack of the initiation codon in pcP-450$_{11\beta}$,aldo -16 prompted us to perform the primer extension experiment to confirm the 5'-terminal structures of these inserts. 5'-Termini of the cDNAs in the zona glomerulosa library were polydeoxyadenylated and the one-sided PCR was performed. The amplified DNA fragments were subcloned into pUC118 and sequenced. Among seventeen fragments analyzed, all were found to have the "Leu6" structure when the amino acid sequences were deduced from the nucleotide sequences. As shown in Fig. 2B, fifteen of the seventeen fragments had the structure identical to the 5'-terminus of pcP-450$_{11\beta}$,aldo -46 (Fig. 2B, fragment-1), whereas the 5'-terminal sequence of the remaining two fragments was 5'-GGCAATGGCTCTC-3'(Fig.2B, fragment-2). This sequence is consistent with that of pcP-450$_{11\beta}$,aldo -16 except that the latter is lacking in the first seven nucleotides. This sequence is also found in the 5'-terminus of pcP-450$_{11\beta}$ -62. These findings suggest that the coding region of pcP-450$_{11\beta}$,aldo -16 begins with the initiation codon "ATG" at the same position as that of pcP-450$_{11\beta}$ -62. Thus two distinct 5'-termini seem to be present in the cDNA library. It should be noted that the results of the PCR experiment do not deny existence of a cDNA having "G" at the 958th position and "5'-GGCAATGGCTCTC-3'" sequence at its 5'-terminus.

The deduced amino acid sequences of pcP-450$_{11\beta}$,aldo -46 (P-450$_{11\beta}$,aldo -1) and pcP-450$_{11\beta}$,aldo -16 (P-450$_{11\beta}$,aldo -2) are illustrated in Fig. 3. P-450$_{11\beta}$,aldo -1 is composed of 510 amino acid residues, and the estimated molecular weight is 58,237. The difference between P-450$_{11\beta}$,aldo-1 and P-450$_{11\beta}$,aldo-2 exists at their NH_2-termini. P-450$_{11\beta}$,aldo -1 has an extension peptide composed of 34 amino acid residues. The peptide contains four acidic amino acid residues, which is unusual as an extension peptide. The extension peptide of P-450$_{11\beta}$, aldo -2 is identical to that of P-450$_{11\beta}$ and is composed of 24 amino acid residues. Also different between the two P-450$_{11\beta}$,aldo s is the

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Fig. 2. **Nucleotide sequences of the P-450$_{11\beta}$,aldo cDNAs and the deduced amino acid sequence.** A; Two nucleotide sequences of pcP-450$_{11\beta}$,aldo and that of pcP-450$_{11\beta}$-62 are compared. The putative cleavage site by processing protease is indicated by an arrowhead. The initial ATG codons of pcP-450$_{11\beta}$,aldo -46 and pcP-450$_{11\beta}$-62, and a polyadenylation signal are underlined. The same nucleotides to those in pcP-450$_{11\beta}$,aldo-46 are indicated by hyphens, and the deleted nucleotides, by stars. The deduced amino acid sequence of pcP-450$_{11\beta}$,aldo-46 is depicted above its nucleotide sequence. B; The sequences found in the primer extension experiments are shown. The sequence of fragment-1 was identical to that of pcP-450$_{11\beta}$,aldo-46. The sequence of fragment-2 was identical to pcP-450$_{11\beta}$,aldo -16 except for the presence of 5'-GGCAATG-3' at the beginning. This heptanucleotide probably corresponds to the 5'-untranslated region and the initial ATG of pcP-450$_{11\beta}$,aldo-16.

320th amino acid residue. Glu320 in P-450$_{11\beta}$,aldo -1 is substituted for Lys310 in P-450$_{11\beta}$,aldo -2. Both P-450$_{11\beta}$,aldo s have the "Leu6" structure in the mature proteins. The extents of the amino acid sequence similarity of P-450$_{11\beta}$,aldo -1 to

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rat P-45011β,aldo-1   MGACDNDFIE LHSRVTADVW LARPWQCLHR TRALGTTATL APKTLKPFEA   50
rat P-45011β,aldo-2             MAL------- ---------- ---------- ----------   40
rat       P-45011β              MAL------- ---------- --------KV ----------   40
bovine  P-45011β-3              MALWAK-R-R M-G--LS--E A-L---RGAV ---AVL----   40
human     CYP11B1               MALRAK-E-C M-V--LS-Q- AQ----R-AR V-R-VL----   40

rat P-45011β,aldo-1   IPQYSRNKWL KMIQILREQG QENLHLEMHQ AFQELGPIFR HSAGGAQIVS  100
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------   90
rat       P-45011β    ---------- ---------- ---------- ---------- ----------   90
bovine  P-45011β-3    M-RCPG---M R-L--WK--- S--M--D--- T--------- YDV--RHM-F   90
human     CYP11B1     M-RRPG-R-- RLL--W---- Y-D----V-- T--------- YDL---GM-C   90

rat P-45011β,aldo-1   VMLPEDAEKL HQVESILPRR MHLEPWVAHR ELRGLRRGVF LLNGAEWRFN  150
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------  140
rat       P-45011β    ---------- --------H- -P-------- ---------- -----D----  140
bovine  P-45011β-3    ------V-R- Q-AD-RH-Q- -I----L-Y- QA--HKC--- ----PQ--LD  140
human     CYP11B1     ------V--- Q--D-LH-H- -S------Y- QH--HKC--- ----P-----  140

rat P-45011β,aldo-1   RLKLNPNVLS PKAVQNFVPM VDEVARDFLE ALKKKVRQNA RGSLTMDVQQ  200
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------  190
rat       P-45011β    --Q----M-- ---I-S---F --V-----V- N---RMLE-V H--MSINI-S  190
bovine  P-45011β-3    --R---D--- LP-L-KYT-L --G-----SQ T--AR-L--- -----L-IAP  190
human     CYP11B1     --R---E--- -N---R-L-- --A-----SQ ------L--- -----L---P  190

rat P-45011β,aldo-1   SLFNYTIEAS NFALFGERLG LLGHDLNPGS LKFIHALHSM FKSTTQLLFL  250
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------  240
rat       P-45011β    NM----M--- H-VIS----- -T----K-E- VT-T------- -------M--  240
bovine  P-45011β-3    -V-R------ TLV-Y----- --TQQPN-D- -N-----EA- L---V--M-V  240
human     CYP11B1     -I-H------ -L-------- -V--SPSSA- -N-L---EV- ----V--M-M  240

rat P-45011β,aldo-1   PRSLTRWTST QVWKEHFDAW DVISEYANRC IWKVHQELRL GSSQTYSGIV  300
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------  290
rat       P-45011β    -K-------- R-------S- -I----VTK- -KN-YR--AE -RQ-SW-V-*  289
bovine  P-45011β-3    --R-S--M-- NM-R---E-- -Y-FQ----A -QRIY---A- -HPWH-----  290
human     CYP11B1     ----S----P K------E-- -C-FQ-GDN- -Q-IY---AF SRP-Q-TS--  290

rat P-45011β,aldo-1   AALITQGALP LDAIKANSME LTAGSVDTTA IPLVMTLFEL ARNPDVQQAL  350
rat P-45011β,aldo-2   ---------- ---------K ---------- ---------- ----------  340
rat       P-45011β    SEMVA-ST-S M---H----- -I-------- -S-------- ----------  339
bovine  P-45011β-3    -E-LMRADMT --T----TID ---------- F--L------ ----E----V  340
human     CYP11B1     -E-LLNAE-S P--------- ---------V F--L------ ----N-----  340

rat P-45011β,aldo-1   RQETLAAEAS IAANPQKAMS DLPLLRAALK ETLRLYPVGG FLERILNSDL  400
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------  390
rat       P-45011β    ---S------ -V-------- ---------- ---------S -V---VH---  389
bovine  P-45011β-3    ---S-V---R -SE---R-IT E--------- ---------I TL--EVS---  390
human     CYP11B1     ---S---A-- -SEH----TT E--------- ---------L ----VAS---  390

rat P-45011β,aldo-1   VLQNYHVPAG TLVLLYLYSM GRNPAVFPRP ERYMPQRWLE RKRS***FQHLAF  450
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----***------  440
rat       P-45011β    ---------- -F-II----- ---------- ---------- ----***------  439
bovine  P-45011β-3    ------I--- ---KVL---L -------A-- -S-H-----D -QG-GSR-P----  443
human     CYP11B1     ------I--- ---RVF---L -----L---- ---N-----D I-G-GRN-Y-VP-  443

rat P-45011β,aldo-1   GFGVRQCLGR RLAEVEMLLL LHHMLKTFQV ETLRQEDVQM AYRFVLMPSS  500
rat P-45011β,aldo-2   ---------- ---------- ---------- ---------- ----------  490
rat       P-45011β    ---------- ---------- ---------- -------M-- VF--L-----  489
bovine  P-45011β-3    ---------- -V-------- ---V--N-L- ---E---IK- V---I----T  493
human     CYP11B1     ---M------ ---------- ---V--HL-- ---T---IK- V-S-I-R-SM  493

rat P-45011β,aldo-1   SPVLTFRPIS                                              510
rat P-45011β,aldo-2   ----------                                              500
rat       P-45011β    --F-----V-                                              499
bovine  P-45011β-3    L-LF---A-Q                                              503
human     CYP11B1     C-L----A-N                                              503
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Fig. 3. **The deduced amino acid sequences of rat P-450$_{11\beta}$,aldo s.** The amino acid sequences of rat(9), bovine(11), and human(12) P-450$_{11\beta}$ s are shown for comparison. The peptides indicated with single and double lines represent the putative heme/steroid binding site and the heme binding site (9), respectively. (-) indicates that the amino acid is identical to that of rat P-450$_{11\beta}$,aldo -1. The cleavage site by processing protease is depicted by arrowhead.

rat P-450$_{11\beta}$, bovine P-450$_{11\beta}$-3 and human CYP11B 1 were 81%, 63% and 67%, respectively. It should be noted that the amino acid sequences of a heme-binding site and a proposed steroid binding site (9) are well-conserved among the family of P-450$_{11\beta}$ s.

The coding region fragment of pcP-450$_{11\beta}$,aldo -46 was inserted into a eukaryotic expression vector, and the constructed plasmid was transfected into nonsteroidogenic cells (COS-7 cells). The cells were incubated with 11-deoxycorticosterone, and the conversion of the steroid was determined. A small amount of corticosterone was detected in the culture medium of the plasmid-transfected cells, indicating the presence of 11β -hydroxylase activity in the transformed COS-7 cells (data not shown). However, the rate of 11β-hydroxylation was low and also the production of aldosterone was undetectable under these conditions. We surmised that the unusual NH_2-terminal extension peptide structure of P-450$_{11\beta}$,aldo -1 hampered the expression of pcP-450$_{11\beta}$,aldo -46 insert in the heterologous cells such as COS-7 cells. Therefore, we decided to reconstruct the extension peptide of P-450$_{11\beta}$,aldo -1 so that the biosynthesized peptide could be more easily transported into the mitochondria of COS-7 cells. The results using the reconstructed plasmid are shown in Fig. 4. The mature

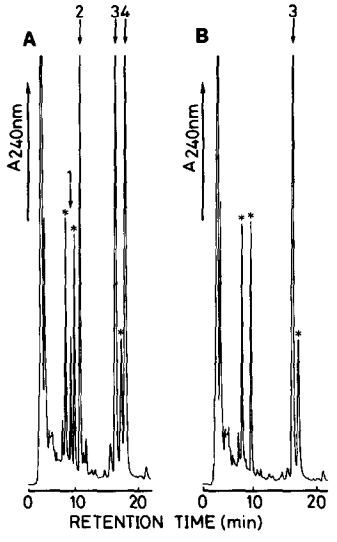

Fig. 4. **Chromatograms of the products in the medium obtained from the incubation of COS-7 cells with 11-deoxycorticosterone.** After introducing the signal peptide of pcP-450$_{11\beta}$-62 into the corresponding positions of pcP-450$_{11\beta}$,aldo-46 and pcP-450$_{11\beta}$ -16 by PCR method, each fragment was inserted into the expression vector, pSVL, and the vector was transfected to COS-7 cells. A; pcP-450$_{11\beta}$,aldo-46 was expressed. The chromatogram of the medium obtained from the incubation of the cells with 11-deoxycorticosterone showed peaks at the same retention times to authentic 1; aldosterone, 2; 18-hydroxycorticosterone and 4; corticosterone. The peak 3 is the internal standard, dexamathasone. B; The expression of pcP-450$_{11\beta}$,aldo -16 was also attempted. The chromatogram was essentially the same as that of mock transfection (data not shown). The peaks indicated by stars are unidentified substances derived from medium or degradation products derived from substrate.

protein expressed from the reconstructed plasmid containing pcP-450$_{11\beta}$,aldo -46 insert clearly mediated the aldosterone biosynthetic reaction from 11-deoxycorticosterone. The amounts of aldosterone, 18-hydroxycorticosterone, and corticosterone produced were 0.89, 3.39, and 6.13 nmol/24hr/dish, respectively. Interestingly the reconstructed plasmid containing pcP-450$_{11\beta}$,aldo -16 insert failed to show the steroid conversion activity, suggesting that the replacement of Glu with Lys at the 320th position might profoundly affect the steroid hydroxylation activity. Further study should be required, however, because undesirable misincorporation of nucleotides may occur during the construction of the expression vector using the PCR. In conclusion, we herein reported the isolation and the sequence determination of two cDNAs encoding rat aldosterone synthase.

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