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Cloning and Expression of Cytochrome *P*450(11β) of Porcine Adrenal Cortex

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Four cDNA clones were isolated from a porcine adrenal gland library by using a bovine cytochrome *P*450(11β) cDNA fragment as a probe. Nucleotide sequences† of the four clones overlapped with each other. The deduced amino acid sequences indicated that these clones were derived from a porcine *P*450(11β) cDNA. Consecutive alignment of these clones covered almost 70% of a coding region of the cDNA, but its 5′-terminus was missing. The adrenal mRNA was reverse-transcribed, and polymerase chain reaction was used to obtain a cDNA fragment including the 5′-terminus. A cDNA constructed from this fragment and the isolated four fragments covered the entire apparent open reading frame of the enzyme, which was thus concluded to comprise 503 amino acids including a putative extension peptide of 24 amino acids at the NH_2-terminus. The amino acid sequence was 82% identical to that of bovine *P*450(11β)-3. The cDNA was transfected into COS-7 cells, and steroidogenic activity of the cells was measured. The cells not only converted 11-deoxycorticosterone to corticosterone and 18-hydroxycorticosterone, but also produced aldosterone. Thus we conclude that the primary sequence of porcine *P*450(11β) which plays a role in the biosynthesis of glucocorticoids as well as mineralocorticoids was determined.

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INTRODUCTION

Bovine adrenocortical cytochrome *P*450(11β) purified to homogeneity has been shown to catalyze not only the 11β- and 18-hydroxylation of 11-deoxycorticosterone (DOC), but also the conversion of corticosterone (B) to aldosterone (ALDO) [1, 2]. Experiments using mitochondria isolated from COS-7 cells transfected with *P*450(11β) cDNA confirmed that a single enzyme is responsible for the biosynthesis of glucocorticoids and mineralocorticoids in the bovine adrenal cortex [3]. Results of an investigation of porcine adrenal cortex also indicated that a single *P*450 enzyme produces B as well as ALDO [4]. The primary structure of the porcine enzyme, however, has not been determined yet.

Two distinct genes encoding two *P*450(11β) isozymes were identified in rat [5–8], mouse [9] and human [10, 11] adrenal cortex. One of these two isozymes was shown to be ALDO synthase, whereas the other was shown to be 11β-hydroxylase. The former enzyme is thought to exist only in the zona glomerulosa of adrenal cortex of these animals, while the latter is thought to function in the zona fasciculata-reticularis.

An interesting question immediately raised by reviewing these previous reports is: why is the step of conversion of DOC to ALDO catalyzed by one enzyme in some mammalian species, but by two enzymes in the other species? Before getting a reasonable answer to this question, we need more information on the molecular nature of this enzyme(s) of the other animal species. This paper describes an attempt to elucidate the primary structure of porcine *P*450(11β) that catalyzes not only 11β- and 18-hydroxylation of DOC but also conversion of DOC to ALDO.

MATERIALS AND METHODS

Isolation of poly(A)⁺ RNA, construction of a cDNA library and determination of the nucleotide sequence

Porcine adrenal glands were obtained from a local slaughterhouse. Total RNA was extracted from the adrenal glands by the acid guanidinium thiocyanate/

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†The nucleotide sequence data reported in this paper will appear in the GSBD, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number, D38590, Porcine mRNA for cytochrome *P*450 11 beta, aldo.

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phenol/chloroform method [12]. The poly(A)$^+$ RNA was purified with Oligo-dT30 (First Chemical Reagent Co., Ltd) as recommended by the manufacturer.

The mRNA was reverse-transcribed, and the resulting cDNA was fractionated by Sepharose CL-4b column chromatography to obtain cDNA species longer than 1000 bp. The cDNA was used to construct a library containing independent recombinants of approx. 2×10^6 phages. Using bovine *P*450(11β) cDNA Bgl II-Sph I fragment as a probe [13], four positively reacting clones containing long inserts were isolated. These inserts were recloned into the EcoRI site of pUC 118 plasmid and sequenced by the dideoxynucleotide chain-termination method.

Rapid amplification of cDNA ends

Since the clones obtained lacked the remainder of the 5′-region of the cDNA, this was obtained using the rapid amplification of cDNA ends (RACE) procedure [14]. This technique uses the DNA polymerase chain reaction (PCR) technique to amplify copies of the region between a single location in the known transcript and the unknown 5′-end. A single stranded cDNA was synthesized using poly(A)$^+$ RNA, a *P*450(11β)-specific primer (3′-TCGGACTGGGAC-CTGTAGTT-5′, from 547th to 567th in Fig. 2), and murine leukemia virus-reverse transcriptase. A poly(C) tail was added to the 3′-end of the single strand cDNA with terminal deoxynucleotidyl transferase, so that in the subsequent PCR step, the cDNA could be amplified using another specific *P*450(11β) primer (3′-ACGACAGCGACGTCCGGTACGTC-5′, from 443rd to 465th in Fig. 2), a poly (G) primer, and AmpliTaq DNA polymerase. The amplified cDNAs were subcloned into pCR II vector (Invitrogen). Three cDNA clones of approx. 500 bp were isolated and sequenced to obtain the remainder of the coding region.

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

A cDNA fragment containing the complete coding region of porcine *P*450(11β) was constructed from the RACE-produced cDNA end, Clones J and G employing restriction enzyme sites of Sma I and Nar I (Fig. 1). It was recloned into the Sma I site of the polylinker region of the expression vector, pSVL. COS-7 cells were suspended in 0.5 ml isotonic saline G containing 50 μg *P*450(11β)-expression plasmid and 50 μg bovine adrenodoxin-expression plasmid (pSVADX). Transfection was performed by electroporation with some modification (125 μF at 450 V). Aliquots were transferred to two 10 cm dishes (200 μl each) containing 10 ml Dulbecco's modified Eagle's medium and 10% fetal calf serum, and then the dishes were incubated at 37°C under an atmosphere of 5% CO_2/95% air for 24 h. After changing the medium to 10 ml fresh

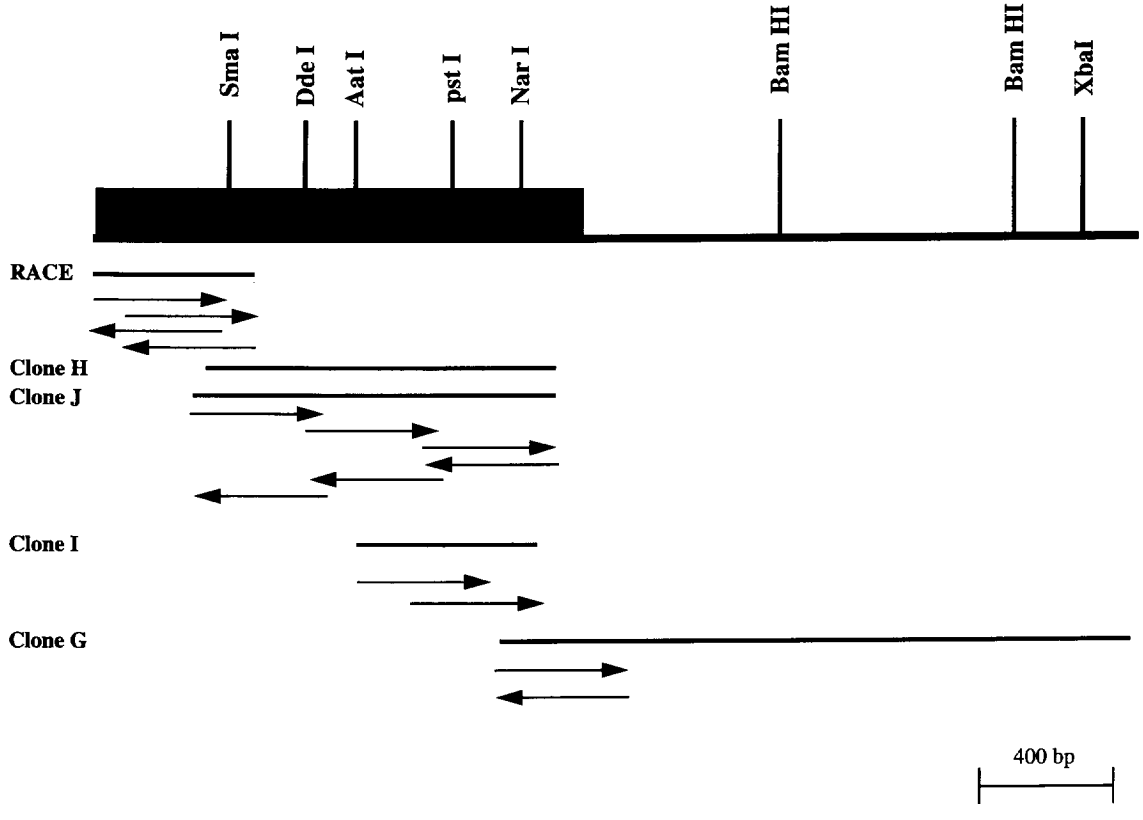

Fig. 1. Restriction enzyme map and sequencing strategy for the porcine *P*450(11β) cDNA clones. The rectangular box and the bar represent the coding region and 5′- and 3′ untranslated regions, respectively. Arrows indicate the direction and the extent of sequencing.

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                                                                ACACCACCAGG      -1

ATGGCCATCTGGGCAAAGGCAGAAGCGTGGCTGGCAGGGCCTTGGCTGGCCCTGAACAGGGCACGAACGTTGGGCACC      78
 M  A  I  W  A  K  A  E  A  W  L  A  G  P  W  L  A  L  N  R  A  R  T  L  G  T       26

AGAGCCGTTTTGGCCCCTAAGGGGGTGCTGCCCTTCGAAGCCATACCCCAGTTTCCCGGAAAAAAGTGGATGCGGGTG     156
 R  A  V  L  A  P  K  G  V  L  P  F  E  A  I  P  Q  F  P  G  K  K  W  M  R  V       52

CTGCAGCTTTGGCGGGAGCAGGGTTTCGAGAACAATCACCTGGAAATGCATCAGACCTTCCAGGAGCTGGGGCCCATT     234
 L  Q  L  W  R  E  Q  G  F  E  N  N  H  L  E  M  H  Q  T  F  Q  E  L  G  P  I       78

TTCAGATTTGACGTGGGAGGCAGGAACATGGTTCTTGTTATGCTTCCTGAGGACGTTGAGCGGTGTCAGAAGGTGGAA     312
 F  R  F  D  V  G  G  R  N  M  V  L  V  M  L  P  E  D  V  E  R  C  Q  K  V  E      104

GGCCTTCACCCCCAGCGGGATGTTCCTGGACCCTGGCTGGCCTACCGACACCTCCGCGGGCACAAGTGTGGCGTGTTC     390
 G  L  H  P  Q  R  D  V  P  G  P  W  L  A  Y  R  H  L  R  G  H  K  C  G  V  F      130

TTGCTAAACGGGCCCACCTGGCGTCTGGACCGACTGCAGCTGAACCCGGGCGTGCTGTCGCTGCAGGCCATGCAGAAG     468
 L  L  N  G  P  T  W  R  L  D  R  L  Q  L  N  P  G  V  L  S  L  Q  A  M  Q  K      156

TTCACGCCCCTGGTGGACGGGGTGGCCAGGGATTTCTCCCAGGCCCTGCGGGCGAGGGTCATGCAGAATGCCAGGGGG     546
 F  T  P  L  V  D  G  V  A  R  D  F  S  Q  A  L  R  A  R  V  M  Q  N  A  R  G      182

AGCCTGACCCTGGACATCAAGCCCAGCATCTTCCGCTACACCATCGAAGCCAGCAACTTAGTCCTTTTTGGAGAGCGG     624
 S  L  T  L  D  I  K  P  S  I  F  R  Y  T  I  E  A  S  N  L  V  L  F  G  E  R      208

CTGGGCCTCCTGGCCCATCAGCCGAATCCCGAAAGCCTGGACTTCATCCACGCGCTGGAGGTCATGTTCAAGTCCACT     702
 L  G  L  L  A  H  Q  P  N  P  E  S  L  D  F  I  H  A  L  E  V  M  F  K  S  T      234

GTGCAGCTCATGTTCATGCCCAGGAGCCTGTCGCGCTGGACGAGCACCGGCACGTGGAAGGAGCACTTTGAGGCCTGG     780
 V  Q  L  M  F  M  P  R  S  L  S  R  W  T  S  T  G  T  W  K  E  H  F  E  A  W      260

GACTGCATCTTCCAGTATGCCAACAAAGCCATCCAGAGGCTCTATCAGGAGCTGACCCTGGGCCATCCGTGGCACTAC     858
 D  C  I  F  Q  Y  A  N  K  A  I  Q  R  L  Y  Q  E  L  T  L  G  H  P  W  H  Y      286

AGCGGCGTCGTGGCAGAGCTGCTGACACACGCAAACATGACCGTGGATGCCATCAAGGCCAACTCAATCGACCTCACG     936
 S  G  V  V  A  E  L  L  T  H  A  N  M  T  V  D  A  I  K  A  N  S  I  D  L  T      312

GCCGGAAGCGTGGACACGACAGCCTACCCCCTGCTGATGACTCTGTTCGAGCTGGCCCGGAACCCAGAAGTGCAGCAG    1014
 A  G  S  V  D  T  T  A  Y  P  L  L  M  T  L  F  E  L  A  R  N  P  E  V  Q  Q      338

GCCCTCCGCCAGGAGAGCCTGGCAGCCGCGGCCAGGATCTCGGAAAACCCCCAGAAGGCAATCACCGAGCTGCCCCTG    1092
 A  L  R  Q  E  S  L  A  A  A  A  R  I  S  E  N  P  Q  K  A  I  T  E  L  P  L      364

CTGCGGGCGGCCCTCAAGGAGACCCTGAGGCTGTACCCCGTGGGTATCTTCTTGGACCGATGCGTGACTTCAGACTTG    1170
 L  R  A  A  L  K  E  T  L  R  L  Y  P  V  G  I  F  L  D  R  C  V  T  S  D  L      390

GTCCTGCAGAACTACCACATCCCGGCCGGGACCCTGGTGAAGGTGCTACTCTACTCCCTGGGTCGAAACCCCGCCGTG    1248
 V  L  Q  N  Y  H  I  P  A  G  T  L  V  K  V  L  L  Y  S  L  G  R  N  P  A  V      416

TTCGCCAGACCGGAGCGCTATCACCCCCAGCGCTGGCTGGACAACCAGGGCTCGGGCACCAGGTTCCCACACCTGGCC    1326
 F  A  R  P  E  R  Y  H  P  Q  R  W  L  D  N  Q  G  S  G  T  R  F  P  H  L  A      442

TTCGGCTTTGGCATGCGCCAGTGCCTGGGGCGGCGCCTGGCACAGGTGGAGATGCTACTGCTGCTGCACCACGTACTG    1404
 F  G  F  G  M  R  Q  C  L  G  R  R  L  A  Q  V  E  M  L  L  L  L  H  H  V  L      468

AAAAACTTCCTGGTGGAGACGCTGGTGCAAGAGGACATAAAGATGATCTACCGCTTCATAATGACGCCCTCCACCCTC    1482
 K  N  F  L  V  E  T  L  V  Q  E  D  I  K  M  I  Y  R  F  I  M  T  P  S  T  L      494

CCCCTCCTCACCTTCCGGGGCCATCAGCTAGTCCCGCCGCCGCGCTGTCCGCCCAGCCGCACCACAGTTCCCCTCTTGC    1560
 P  L  L  T  F  R  A  I  S  •                                                       503

CTTGATTCCAGGGCGACCGTGTTTTTCTTCCAATGGGCCTTGTTGTTTCACGGCAAAAATTTTGCCCCGGCAAAGAAT    1638
TTGGGGGAAAAGGGGAAAAAGC                                                           1660
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Fig. 2. Nucleotide sequence of the porcine *P*450(11β) and the deduced amino acid sequence. The cleavage site by processing protease is indicated by arrowhead.

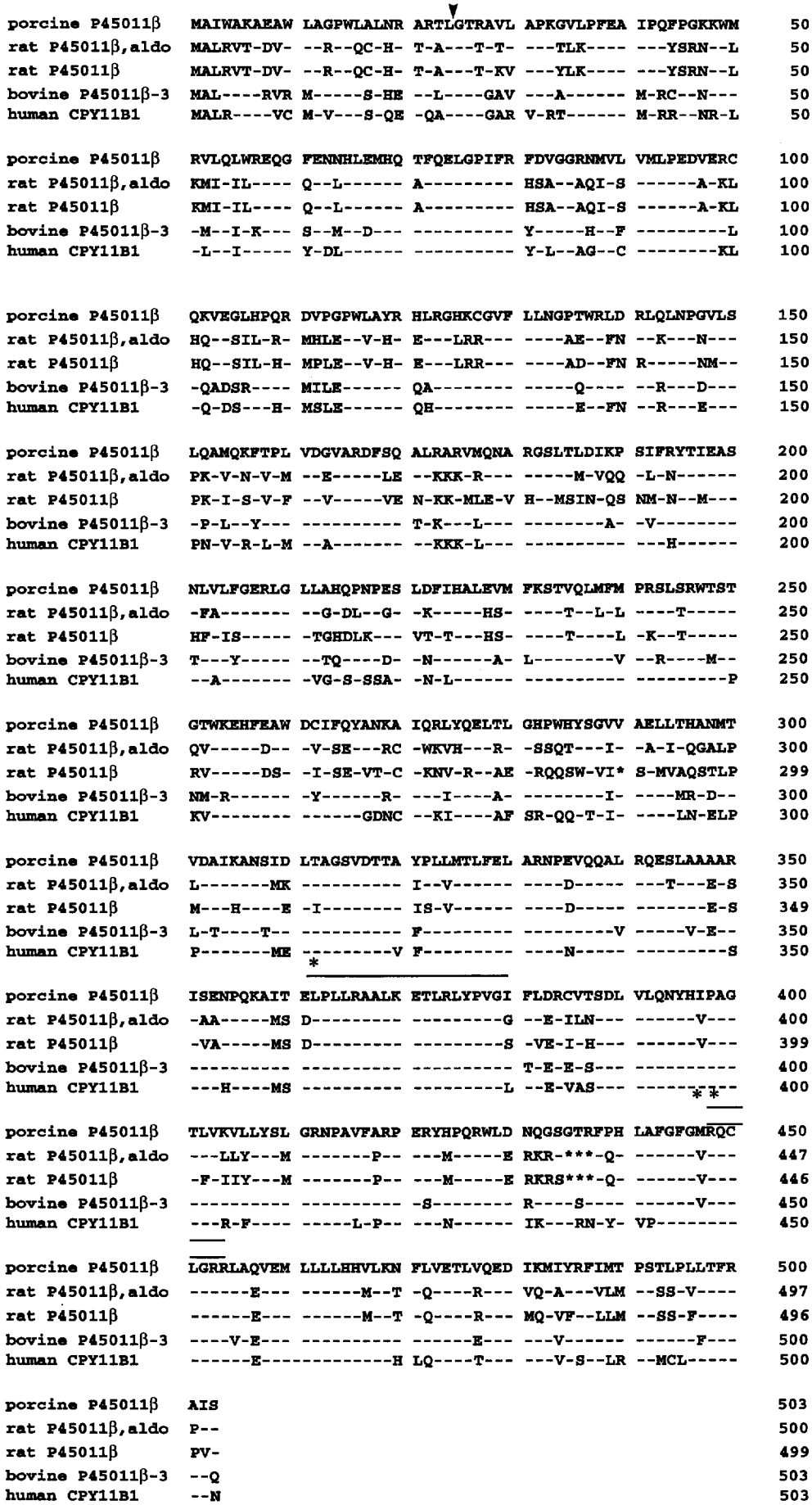

Fig. 3—*caption opposite.*

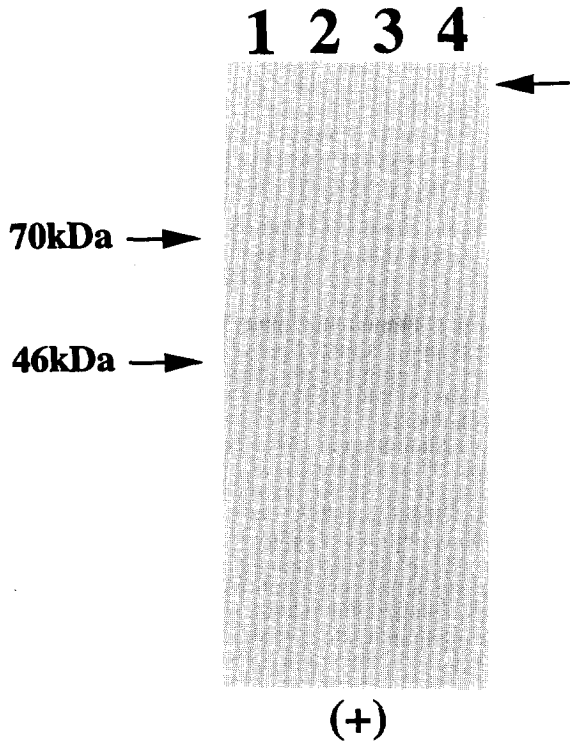

Fig. 4. Immunoblot analysis of porcine P450(11β) expressed in COS-7 cells. Cells were collected for preparing mitochondria after transfection. Solubilized proteins from cells or mitochondria were subjected to the electrophoresis. Lane 1, mitochondria prepared from porcine adrenal cortex; lane 2, cells transfected with porcine P450(11β); lane 3, mitochondria prepared from cells transfected with porcine P450(11β); lane 4, mitochondria prepared from cells transfected with pSVL vector only.

medium, 200 nmol of DOC in ethanol solution was added to the medium and incubated for a further 24 h. The medium was recovered and steroid products were analyzed by high performance liquid chromatography (HPLC) as previously described [8].

Analytical methods

Protein concentrations were determined with the BCA kit (Pearce). Immunoblot analysis of P450(11β) was performed as previously reported [15]. Anti-bovine P450(11β) antibody, which was generously given by Drs Fumiko Mitani and Yuzuru Ishimura of Keio University, Japan, was used for immunostaining.

RESULTS AND DISCUSSION

Northern blot analysis of mRNA prepared from a porcine adrenal gland using a bovine P450(11β) cDNA as a probe revealed the presence of a message of about 3.8 kb in size, which hybridized well with the probe (data not shown). Thus the bovine probe was used to screen a porcine adrenal gland cDNA library.

Four clones (clones G, H, I and J) were isolated. Their nucleotide sequences suggested that they overlapped with each other, but none of these were of full-length as shown in Fig. 1. Compared with the sequence of bovine cDNA, these clones, if consecutively aligned, seemed to cover almost 70% of the coding region of porcine P450(11β) from its COOH-terminus.

To obtain a DNA fragment including the 5′-terminal region of the porcine cDNA, the mRNA was first reverse-transcribed. Then PCR was used to amplify the fragment containing the 5′-terminus, as described under Materials and Methods and shown in Fig. 1. The amplified fragment was subcloned. A cDNA clone encoding the entire coding region of the protein, named pP450(11β), was constructed by employing restriction enzyme sites of Sma I and Nar I to ligate RACE-produced 5′-terminus, clones J and G together (Fig. 1).

Figure 2 illustrates the nucleotide sequence and the deduced amino acid sequence of the porcine P450(11β) cDNA. The precursor protein has an open reading frame comprising 503 amino acids. Comparison of its amino acid sequence with those of other animals suggested that cleavage of the precursor protein occurs at a peptide bond between Leu24 and Gly25 during transport of the mature protein into mitochondria (indicated by an arrowhead in Figs 2 and 3).

The amino acid sequence of the porcine enzyme and those of P450(11β) isozymes of the other animal species are illustrated in Fig. 3. The porcine enzyme displayed greatest similarity to bovine P450(11β)-3 (82% identical in amino acids and 71% identical in nucleotides). The amino acid sequence similarity of the porcine enzyme to rat P450(11β), rat P450(11β, aldo) and human P450(11β) was 61, 67 and 71%, respectively.

There are several highly conserved regions among these P450s. A region around Cys450 has been reported to be the heme binding site, the Cys serving as the fifth coordinating ligand to the heme iron. Within this region, it should be noted that Gln457 of the porcine enzyme replaced Glu457 of the other enzymes. Glu457 has been considered a residue particularly conserved among mitochondrial P450s [13].

Another region that is highly conserved among these P450(11β)s was found between residues 362 and 379. A sequence, Leu365-X-Ala-X-X-X-Glu-X-Leu-Arg-X-X-Pro, has been found only among the steroidogenic P450s, such as P450(11β), P450scc, P450(17α) and P450c21 [5].

Fig. 3 (opposite). Comparison of the amino acid sequence of porcine P450(11β) with those of the rat P450(11β, aldo), rat P450(11β), bovine P450(11β) and human P450(11β). The peptides indicated with single and double lines represent the putative heme/steroid binding site and the heme binding site, respectively. The cleavage site by processing protease is depicted by arrowhead.

Table 1. Hydroxylation activities of COS-7 cells co-transfected with pSVpP450(11β) and pSVADX. The activities were assayed by using DOC as substrate as described under Material and Methods

Product	Activity (nmol/24 h/10 ml medium)	Product/B × 100
B	5.7	100
18OH-DOC	1.28	22.5
18OH-B	2.27	39.8
ALDO	0.6	10.5

B, corticosterone; 18OH-DOC, 18-hydroxy-11-deoxycorticosterone; 18OH-B, 18-hydroxycorticosterone; ALDO, aldosterone.

To confirm that the cDNA described in this paper indeed encodes an active *P*450(11β) enzyme, we constructed an expression vector containing the insert, and transfected it into COS-7 cells. The cell homogenates and the mitochondria prepared from the cells were subjected to immunoblot analysis as shown in Fig. 4. They, and not the mock-transfected cells, contained single proteins that were immunostained with anti-bovine *P*450(11β) antibody, their mobility being the same as that of a protein present in porcine adrenocortical mitochondria (lane 1). The estimated molecular weight of the porcine *P*450(11β) was 51,000.

COS-7 cells were transfected with the porcine *P*450(11β) expression vector as well as a vector containing bovine adrenodoxin cDNA. The cells were assayed for steroidogenic activity by using DOC as the substrate (Table 1). The cells indeed converted DOC into B (11β-hydroxylation), 18-hydroxy-11-deoxycorticosterone (18-hydroxylation), 18-hydroxycorticosterone (11β- and 18-hydroxylation) and ALDO. It should be noted that the ratio of these products was very similar to those obtained employing the bovine *P*450(11β)-3-expressing cells reported previously [3].

In conclusion, we herein report the isolation and sequence determination of a porcine adrenal cDNA which encodes a *P*450(11β) protein having the 11β- and 18-hydroxylation activity as well as ALDO production activity.

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