

The Product of the CYP11B2 Gene Is Required for Aldosterone Biosynthesis in the Human Adrenal Cortex

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The steroid 11 β -hydroxylase (P450c11) enzyme is responsible for the conversion of 11-deoxycortisol to cortisol in the zona fasciculata of the adrenal cortex. Animal studies have suggested that this enzyme or a closely related isozyme is also responsible for the successive 11 β - and 18-hydroxylation and 18-oxidation of deoxycorticosterone required for aldosterone synthesis in the zona glomerulosa. There are two distinct 11 β -hydroxylase genes in man, CYP11B1 and CYP11B2, which are predicted to encode proteins with 93% amino acid identity. We used a sensitive assay based on the polymerase chain reaction to analyze the expression of the CYP11B1 and B2 genes. Transcripts of CYP11B1 were detected at high levels in surgical specimens of normal adrenals and also in an aldosterone-secreting adrenal tumor. Transcripts of CYP11B2 were found at low levels in normal adrenals, but at a much higher level in the aldosterone-secreting tumor. CYP11B2 mRNA levels were increased in cultured zona glomerulosa cells by physiological levels of angiotensin-II. The entire coding regions of both CYP11B1 and B2 cDNAs were cloned from the tumor mRNA. Expression of these cDNAs in cultured COS-1 cells demonstrated that the CYP11B1 product could only 11 β -hydroxylate 11-deoxycortisol or deoxycorticosterone, whereas the CYP11B2 product could also 18-hydroxylate cortisol or corticosterone. A small amount of aldosterone was synthesized from deoxycorticosterone only in cells expressing CYP11B2 cDNA. These data demonstrate that the product of CYP11B2 is required for the final steps in the synthesis of aldosterone. (*Molecular Endocrinology* 5: 1513–1522, 1991)

INTRODUCTION

The adrenal cortex produces glucocorticoids, mineralocorticoids, and androgens (1). Glucocorticoids (primarily cortisol in humans) are synthesized in the zona fasciculata under the control of corticotropin (ACTH) secreted by the anterior pituitary, whereas mineralocorticoids (mainly aldosterone) are synthesized in the zona glomerulosa. The hormonal control of mineralocorticoid synthesis is complex, but a primary stimulus is provided by angiotensin-II, which is, in turn, controlled by the secretion of renin by the renal juxtaglomerular apparatus (2).

Although cortisol and aldosterone have distinct physiological effects and are, therefore, regulated differently, the initial steps required to synthesize these hormones are identical; the side-chain of cholesterol is cleaved to yield pregnenolone, which is dehydrogenated at the 3 β position, producing progesterone. For cortisol (and androgen) biosynthesis, progesterone is 17 α -hydroxylated to 17 α -hydroxyprogesterone, but not all progesterone in the zona fasciculata is thus metabolized. Thus, both 17-deoxy and 17-hydroxy steroids are synthesized in the zona fasciculata.

In contrast, aldosterone and its precursors are not 17 α -hydroxylated, and the zona glomerulosa does not have significant levels of 17 α -hydroxylase activity. The syntheses of both cortisol and aldosterone then require 21-hydroxylation to 11-deoxycortisol and deoxycorticosterone, respectively, followed by 11 β -hydroxylation to cortisol and corticosterone. Aldosterone synthesis requires two additional steps: 18-hydroxylation to 18-hydroxycorticosterone and, finally, 18-oxidation. These latter steps do not normally occur to a significant degree in the zona fasciculata. This is of obvious importance for the appropriate regulation of mineralocorticoid biosynthesis, since relatively large amounts of corticosterone are normally synthesized in the zona fasciculata

and would otherwise be inappropriately converted to aldosterone.

It has been established that the cytochrome P450 enzymes catalyzing cholesterol side-chain cleavage (P450_{scc}) (3) and 21-hydroxylation (P450_{c21}) (4) are each encoded by a single active gene that is expressed in both the zona fasciculata and the zona glomerulosa (although the 21-hydroxylase gene, CYP21, is duplicated, the second copy, CYP21P, is a pseudogene). There are apparently multiple β -hydroxysteroid dehydrogenase genes, and their relative levels of expression in the zonae fasciculata and glomerulosa have not yet been determined (5, 6).

Humans carry two genes (7) on chromosome 8q21-q22 (8, 9) that potentially encode 11 β -hydroxylase (P450_{c11}) isozymes with predicted amino acid sequences that are 93% identical. One gene, CYP11B1, is expressed at high levels in normal adrenal glands, and a missense mutation in this gene has been found in patients with congenital adrenal hyperplasia due to 11 β -hydroxylase deficiency (10). Thus, CYP11B1 must encode the P450_{c11} enzyme required for cortisol biosynthesis. Transcripts of the other gene, CYP11B2, cannot be detected by hybridization to blots of normal adrenal RNA. Nonetheless, the gene contains no obviously deleterious mutations (e.g. nonsense mutations or frame shifts), although its 5' regulatory region has diverged considerably from that of CYP11B1. *A priori*, these findings are consistent with three possibilities: 1) CYP11B2 is a pseudogene for reasons that are not obvious from inspection of the sequence; 2) it duplicates the functions of CYP11B1 and is superfluous; or 3) it encodes a unique activity that is required in the adrenal gland (or possibly some other tissue) at low levels. The obvious candidate for such a unique activity is the 18-hydroxylation/oxidation required for aldosterone biosynthesis, because aldosterone is normally secreted at levels 100–1000 times lower than that of cortisol.

Studies of biosynthesis of aldosterone in animal adrenals have yielded contradictory results concerning the identity of the enzyme that catalyzes 18-hydroxylation and 18-oxidation. P450_{c11} purified from adrenal glands of pigs or cattle catalyzes these steps, even though the same enzyme in intact mitochondria from the zona fasciculata does not display 18-oxidation (11, 12). The mechanism of this inhibition is not known. In contrast, rats have a distinct P450_{c11} isozyme in the zona glomerulosa, termed P450_{aldo}, that catalyzes these reactions (13, 14). These biochemical findings have all been confirmed by cloning and expression of the corresponding cDNAs (15, 16). A recent report suggested that the product of the human CYP11B2 gene does, in fact, have 18-hydroxylase and 18-oxidase activities, but actual expression of this gene in the normal adrenal gland was not demonstrated (17).

To resolve these questions, we studied the expression of CYP11B2 in the normal adrenal gland and determined the enzymatic activity of the encoded protein. We found that this gene is indeed expressed at

low levels in normal adrenal glands, that its expression is appropriately increased by angiotensin-II, and that it alone encodes an enzyme that synthesizes aldosterone from deoxycorticosterone.

RESULTS

Relative Levels of Expression of CYP11B1 and CYP11B2

Although transcripts of CYP11B2 were not detected on Northern blots of total human adrenal RNA, a low level of expression could not be ruled out. Loading of large amounts of poly(A)⁺ adrenal mRNA might have permitted detection of CYP11B2 by blot hybridization, but only a limited amount of adrenal RNA was available. Accordingly, a more sensitive assay was used wherein RNA was reverse transcribed and then amplified using the polymerase chain reaction (RT-PCR) (18, 19).

Two controls were used in these PCRs. Primers corresponding to β_2 -microglobulin [a ubiquitous component of class I transplantation antigens (20)] were used as a control for RNA quantity and amplification efficiency. In some experiments these were added to all reactions. Primers amplifying transcripts of CYP17 (17 α -hydroxylase) (21) were used to assess the presence of zona fasciculata contamination in the zona glomerulosa preparations, because this gene is not active in the zona glomerulosa.

A 322-basepair amplified product corresponding to transcripts of CYP11B1 was detected after 20 PCR cycles in samples from normal adrenal glands. Although the CYP11B2 product could not be seen on ethidium bromide-stained gels after 20 cycles, this product could be readily detected by autoradiography of a blot hybridized with a CYP11B2-specific internal oligonucleotide. After 30 cycles, the CYP11B2 product was visible after staining with ethidium bromide (Fig. 1). Similar results were obtained using surgical specimens of adrenals from three different individuals, and no amplification of CYP11B1 or CYP11B2 was observed when RT-PCR was performed using RNA samples from other tissues (not shown). More exact quantitation of the comparative levels of CYP11B1 and B2 was not attempted because of possible differences in amplification efficiency using the various sets of primers.

Because the adrenal glands were obtained incidental to resections of renal carcinomas, it is likely that the patients had received sodium-containing iv fluids before surgery. This might have suppressed renin secretion and, consequently, aldosterone biosynthesis, thus possibly depressing levels of CYP11B2 mRNA. In support of this idea, rat P450_{aldo} could not be detected unless the animals had been maintained on a low sodium, high potassium diet (13), an intervention that was not possible in these surgical patients. As an alternative way of examining human adrenal tissue that was secreting increased amounts of aldosterone, a sample of an aldosterone-secreting adrenal tumor was obtained.

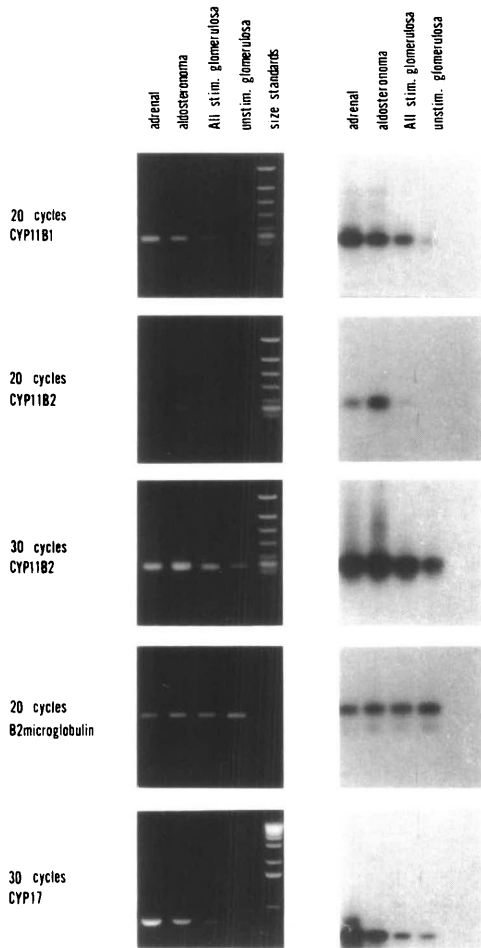


Fig. 1. Analysis of mRNA Levels Using RT-PCR
RNA samples were amplified for the indicated number of cycles with primers (no. 2–9 in Table 1) specific for the indicated gene transcripts. Samples were from a normal adrenal gland, an aldosterone-secreting tumor (aldosteronoma), and cultured zona glomerulosa cells stimulated with 10^{-7} M angiotensin-II or not stimulated. Panels on the *left* are ethidium bromide-stained agarose gels, whereas those on the *right* are autoradiograms of blots hybridized with internal oligonucleotides (no. 10–12 in Table 1) specific for the indicated gene. Size standards were a *Hae*III digest of ϕ X174 DNA, except that a 1-kilobase ladder (Gibco-BRL) was used in the *bottom panel*.

Whereas this tumor contained a concentration of CYP11B1 transcripts slightly lower than that of the normal adrenal, CYP11B2 transcripts were increased 5-fold over the normal gland by autoradiography, and the amplified CYP11B2 product was visible after 20 cycles by ethidium bromide staining (Fig. 1).
To determine if levels of CYP11B2 transcripts were appropriately regulated, the zona glomerulosa was dissected out of two human adrenal surgical specimens and cultured in the presence of angiotensin-II or corticotropin (ACTH) before preparing RNA.
In both experiments, levels of CYP11B1, CYP11B2, and CYP17 were markedly decreased in unstimulated cultured zona glomerulosa cells compared with those

in fresh intact adrenal glands. Culturing for 24 h in the presence of 10^{-7} M angiotensin-II (Fig. 1) resulted in increases in CYP11B1 and B2 transcripts of 7- and 3-fold, respectively, and a minimal increase in CYP17 transcripts. CYP17 product levels were still very low after 30 cycles of amplification, indicating minimal contamination of the culture with zona fasciculata cells. In a second experiment using a different adrenal specimen, 10^{-9} M angiotensin-II again increased transcript levels of CYP11B1 (14-fold) and B2 (which was not detectable in unstimulated cells), although absolute levels were lower than those in the first experiment. Incubation with 10^{-8} M ACTH increased CYP11B1 levels 25-fold, but had no effect on CYP11B2 levels (Fig. 2).

Enzymatic Expression of CYP11B1 and CYP11B2 cDNAs

The preceding experiments strongly suggested that CYP11B2 was regulated in a manner consistent with

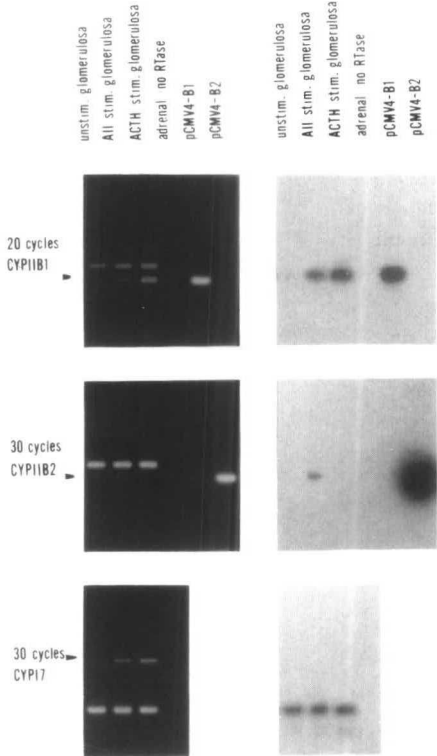
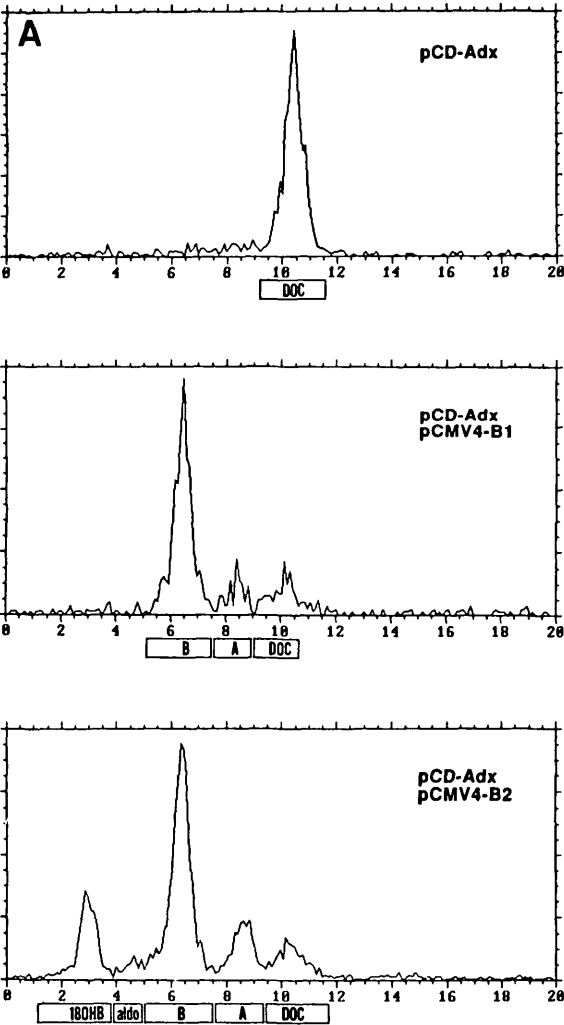
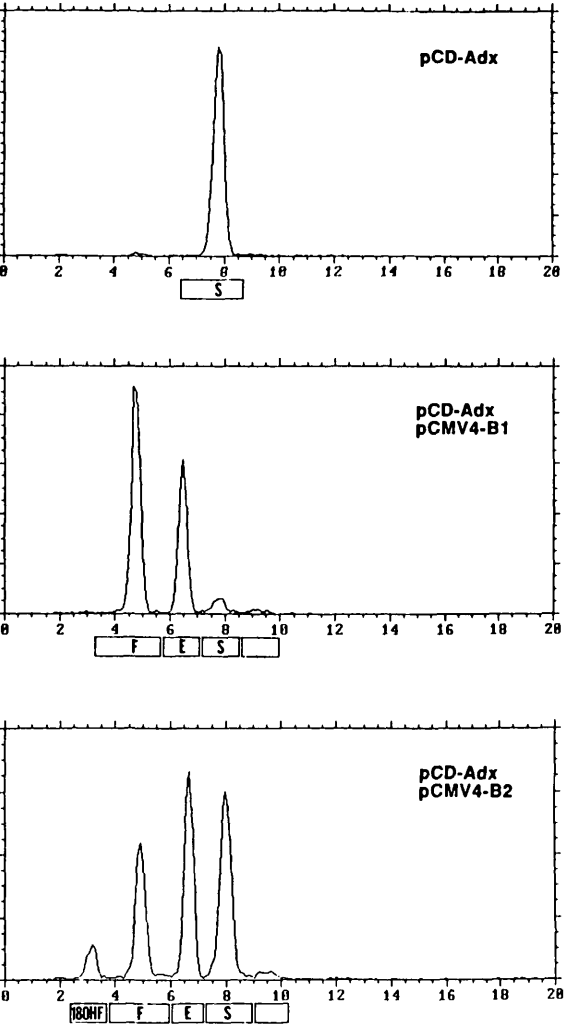


Fig. 2. Further Analysis of mRNA Levels by RT-PCR
RNA samples were taken from cultured zona glomerulosa cells that were unstimulated or stimulated with 10^{-9} M angiotensin-II or 10^{-8} M ACTH. Adrenal RNA that had not been incubated with reverse transcriptase was included as a negative control (adrenal, no RTase), and positive controls were cloned cDNAs for the CYP11B1 and B2 transcripts. Reactions were run with primers amplifying the indicated gene transcripts, and the positions of the specific products are indicated by *arrows*. All reactions in this experiment also contained primers amplifying β_2 -microglobulin; the corresponding product is the other band in the *first three lanes* of each panel. *Left panels* are ethidium bromide-stained gels, and *right panels* are autoradiograms. Note that the *bottom right panel* is hybridized with a β_2 -microglobulin probe.

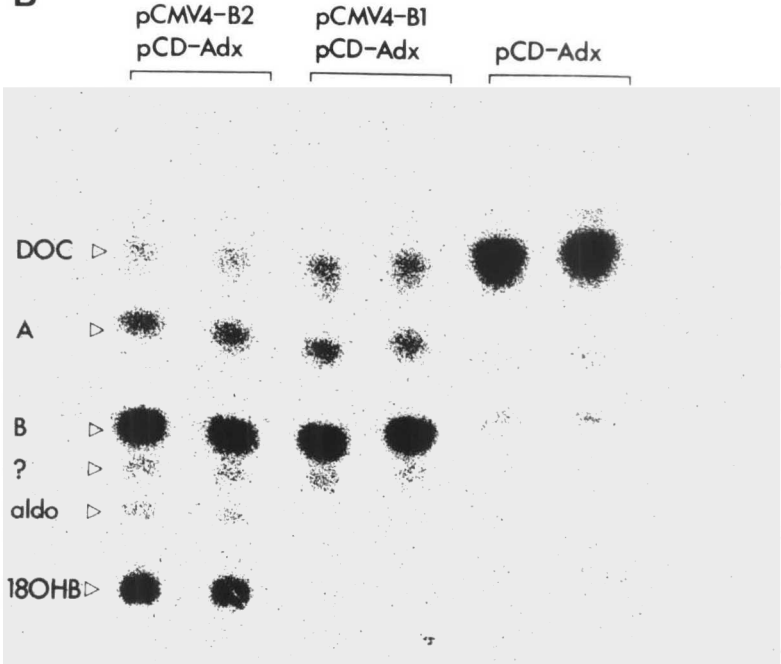
Transfected cells incubated with DOC



Transfected cells incubated with S



B



its involvement in aldosterone biosynthesis, but a contribution of CYP11B1 to aldosterone biosynthesis could not be excluded by these experiments. Therefore, the complete coding sequences of CYP11B1 and B2 mRNAs were amplified from the aldosterone-secreting tumor using RT-PCR, cloned into the eukaryotic expression vector pCMV4 (22), and sequenced. A number of deviations from the previously published genomic sequences were noted. These were confirmed by analysis of amplified exons from a number of normal individuals (our unpublished observations) and by comparison with subsequently published cDNA sequences (17, 23); they presumably represent sequencing errors and/or polymorphisms in the published genomic sequence (the Genbank entries for these sequences have now been corrected).

The constructs, respectively termed pCMV4-B1 and pCMV4-B2, were transfected into COS-1 cells along with pCD-Adx, another expression plasmid containing cDNA for bovine adrenodoxin (24). This last plasmid was included because previous experiments in which P450scc was transiently expressed in COS cells (25) indicated that these cells normally express inadequate amounts of adrenodoxin; P450scc and P450c11 are both mitochondrial enzymes that require adrenodoxin reductase and adrenodoxin for electron transfer from NADPH.

Transfected cells were then incubated for 24 h with [14 C]deoxycorticosterone (the precursor of aldosterone) or [3 H]11-deoxycortisol, and products and reactants were resolved by TLC (Fig. 3). Cells transfected with pCD-Adx alone did not metabolize either of these substrates. Cells transfected with pCMV-B1 converted most deoxycorticosterone to corticosterone and a small amount to another steroid that had a mobility identical to that of 11-dehydrocorticosterone. This was presumed to have been converted from newly synthesized corticosterone by an intrinsic 11 β -hydroxysteroid dehydrogenase activity of COS-1 cells. Cells transfected with pCMV4-B2 converted deoxycorticosterone to the same two products and, in addition, produced substantial amounts of 18-hydroxycorticosterone. A small peak with the mobility of aldosterone was also observed.

When parallel cultures were incubated with 11-deoxycortisol, cells transfected with pCMV4-B1 almost completely converted this substrate to cortisol and cortisone. Cells transfected with pCMV4-B2 produced an additional steroid with the mobility of 18-hydroxycortisol.

To confirm that the large amount of cortisone produced resulted from 11 β -hydroxysteroid dehydrogenase activity intrinsic to COS cells, cells transfected with pCD-Adx alone (*i.e.* negative controls) were incubated with [3 H]cortisol in the absence or presence of the 11 β -hydroxysteroid dehydrogenase inhibitor glycyrrhetic acid (26). In the absence of the inhibitor, almost 50% of the cortisol was converted to cortisone, and this reaction was almost completely inhibited by glycyrrhetic acid (Fig. 4).

Because only a small proportion of deoxycorticosterone was converted to aldosterone, an additional transfection was performed in which only nonradioactive deoxycorticosterone was added, and conversion to corticosterone and aldosterone was determined by RIA after Celite column chromatography (27, 28). Cells transfected with pCD-Adx alone produced only 2.4 ng/ml corticosterone; cells transfected with pCMV4-B1 produced 70.8 ng/ml corticosterone and no aldosterone, whereas cells transfected with pCMV4-B2 produced 30.5 ng/ml corticosterone and 2.35 ng/ml aldosterone (means of duplicate assays).

An attempt was made to identify the recombinant proteins encoded by pCMV4-B1 and -B2. However, the available rabbit antiporcine P450c11 serum reacted poorly with the human protein in these experiments. An overexposed autoradiogram of immunoprecipitates obtained after metabolic labelling (Fig. 5) suggests that the product of pCMV4-B1 migrates as a polypeptide of 52 kDa on an sodium dodecyl sulfate (SDS)-polyacrylamide gel, whereas the product of pCMV4-B2 migrates as a polypeptide of 50 kDa. These mobilities are very similar to those previously determined for the two rat P450c11 isozymes (13). Apparently, similar amounts of the two proteins were synthesized.

DISCUSSION

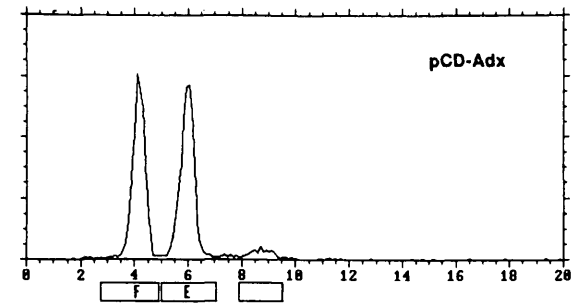
Physiological Functions of CYP11B1 and CYP11B2

The transcript of CYP11B1 is present at high levels in normal adrenal glands, is increased in cultured cells more by ACTH than by angiotensin-II, and can synthesize corticosterone, but not aldosterone, from deoxycorticosterone. The transcript of CYP11B2 is present at low levels in normal adrenal glands, is increased in an aldosterone-secreting tumor, is increased in cultured cells by angiotensin-II, but not significantly by ACTH,

Fig. 3. Analysis of Enzymatic Activities in Transfected Cells

A, Panels representing scans of TLC lanes. COS-1 cells were transfected with the expression plasmids indicated in each panel; pCD-Adx encodes bovine adrenodoxin, pCMV4-B1 contains the cDNA corresponding to CYP11B1, and pCMV4-B2 contains the cDNA corresponding to CYP11B2. In the *left panels*, cells were incubated with [14 C]11-deoxycorticosterone (DOC); in the *right panels*, cells were incubated with [3 H]11-deoxycortisol (S). The positions of unlabeled steroids run in the same lane are indicated. Other abbreviations for steroids are: A, 11-Dehydrocorticosterone; B, corticosterone; 18OHB, 18-hydroxycorticosterone; aldo, aldosterone; E, cortisone; F, cortisol; 18OHF, 18-hydroxycortisol. B, Two-dimensional scan of the incubations with [14 C] deoxycorticosterone. Abbreviations are the same as in A. The unknown product indicated by a question mark may be 18-hydroxy-11-deoxycorticosterone, but an unlabeled standard for this compound was not available.

Transfected cells incubated with F



Transfected cells incubated with F and glycyrrhetic acid

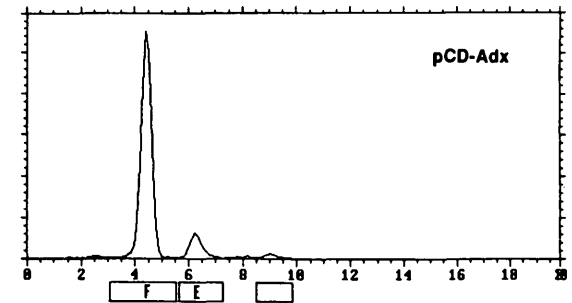


Fig. 4. Demonstration of 11 β -Hydroxysteroid Dehydrogenase Activity in COS-1 Cells

Cells were transfected with pCD-Adx, which does not encode this activity. They were then incubated with [3 H]cortisol (F) in the absence or presence of glycyrrhetic acid. The small peak is an unidentified contaminant of the cortisol preparation.

and is able to convert deoxycorticosterone to aldosterone. It may, thus, be concluded that the protein product of CYP11B2 (the systematic name of this product is P450XIB2) normally mediates the 18-hydroxylation and 18-oxidation steps required for aldosterone biosynthesis.

Because CYP11B1 transcripts are present at relatively high levels in an aldosterone-secreting tumor and are indeed increased in cultured glomerulosa cells by angiotensin-II, it remains possible that the product of this gene (P450XIB1) helps mediate 11 β -hydroxylation in the zona glomerulosa as well as the zona fasciculata. This might be important in providing adequate levels of corticosterone within the zona glomerulosa to permit the relatively inefficient 18-hydroxylation and 18-oxidation steps to proceed optimally. However, we have not eliminated the possibility that the observed expression of CYP11B1 in zona glomerulosa preparations might be the result of contamination with zona fasciculata cells. Definitive conclusions regarding the expression of the CYP11B1 and B2 genes in different adrenocortical zones will probably require *in situ* hybridization studies with gene-specific probes.

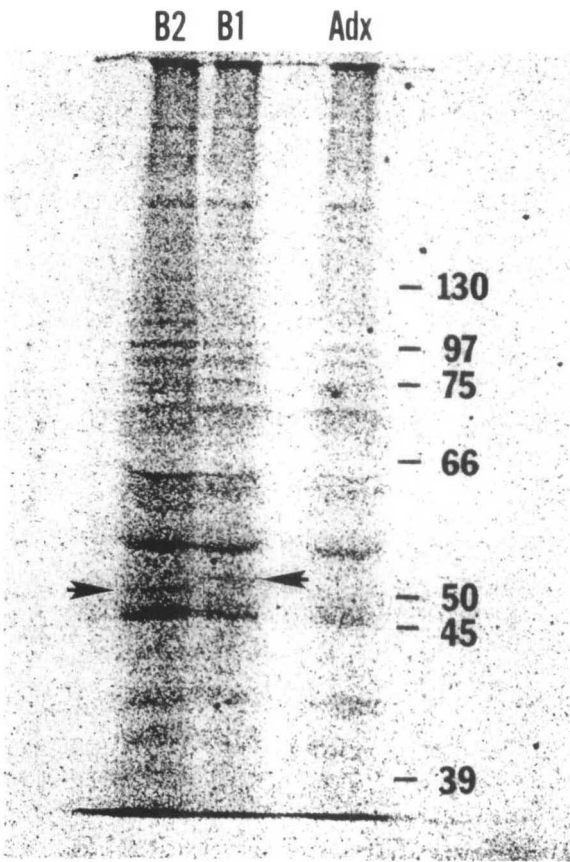


Fig. 5. Immunoprecipitates of 35 S-Labeled Proteins Expressed by Transfected Cells

COS-1 cells were transfected with pCD-Adx and pCMV4-B2, pCMV4-B1, or no additional plasmid; proteins were precipitated with a rabbit antiporcine P450c11 serum, subjected to electrophoresis, and visualized using a Phosphorimager. Positions of the presumed P450XIB2 and P450XIB1 are marked with arrows. Positions of size standards are also indicated.

Predicted Effects of Mutations Involving CYP11B1 and CYP11B2

The most common cause of congenital adrenal hyperplasia is 21-hydroxylase deficiency (1). All mutations causing this deficiency that have been described thus far are recombinations between the active CYP21 gene and the adjacent CYP21P pseudogene; about one fourth are net deletions of CYP21 due to unequal crossing-over during meiosis, whereas the remainder are transfers of deleterious sequences from the pseudogene to the active gene, a process termed gene conversion (29). Deficiency of 11 β -hydroxylase activity is the second most common cause of congenital adrenal hyperplasia. When it was first noted that the CYP11B genes were duplicated, it was tempting to hypothesize that recombinations similar to those observed in the CYP21 genes would account for many mutations causing 11 β -hydroxylase deficiency. The data presented here and previously (17) make it apparent that because CYP11B2 encodes an active enzyme,

such recombinations, if they occurred, would not yield a phenotype of 11β-hydroxylase deficiency. In fact, deletions have not been observed in 11β-hydroxylase-deficient patients, and the two mutations thus far identified are both *de novo* point mutations (Ref. 10 and our unpublished observations).

What phenotypes might be expected from recombinations between CYP11B1 and CYP11B2? A deletion would presumably yield a single hybrid gene depending on the exact position of the break point, but the function of such a gene is difficult to predict at this time because the relative positions of CYP11B1 and CYP11B2 on the chromosome are not yet known. If a sufficient amount of CYP11B1 coding sequences was transferred to CYP11B2 by gene conversion, the ability of P450XIB2 to synthesize aldosterone might be impaired. The inherited inability to synthesize aldosterone, a rare disorder termed corticosterone methyloxidase-II deficiency, is, in fact, genetically linked to the CYP11B genes, although causative mutations have as yet not been identified (30). Obviously, point mutations inactivating CYP11B2 would also be expected to cause corticosterone methyloxidase-II deficiency.

Conversely, transfers of sufficient coding sequences from CYP11B2 to B1 might confer the ability to synthesize aldosterone upon the encoded P450XIB1. If the mutant CYP11B1 gene remained normally regulated, this would result in the synthesis in the zona fasciculata of large amounts of aldosterone that would be inappropriately regulated by ACTH. A similar problem could result if CYP11B1 regulatory sequences were transferred to CYP11B2 in a gene conversion. In either case, a single copy of an abnormally regulated gene encoding P450XIB2 (or a P450XIB2-like enzyme) would be sufficient to cause abnormal aldosterone biosynthesis, and

the predicted disorder should, therefore, be inherited in an autosomal dominant manner. Such a disorder has, in fact, been observed and is termed glucocorticoid (or dexamethasone)-suppressible hyperaldosteronism. In this autosomal dominant form of hypertension (31), abnormally high levels of aldosterone secretion are stimulated by ACTH and suppressed by glucocorticoids, similar to the normal regulation of cortisol secretion. A prominent feature of this disorder is the abnormal secretion of 18-hydroxy- and 18-oxocortisol (32). As demonstrated here, P450XIB2 can synthesize 18-hydroxycortisol from 11-deoxycortisol, a finding consistent with the proposed genetic mechanism causing glucocorticoid-suppressible hyperaldosteronism.

Verification of this prediction will be aided by (and will, in turn, aid) identification of the structural features that account for the functional differences between P450XIB1 and B2.

MATERIALS AND METHODS

Tissues, Cells, and Plasmids

Specimens of normal human adrenal glands were obtained from nephrectomies for renal carcinoma or adrenalectomies during retroperitoneal lymph node dissections. Samples used for RNA preparation were obtained from the Tumor Procurement Service at the Memorial Sloan Kettering Center for Cancer Research, whereas those used as a source of zona glomerulosa cells were obtained from the City of Hope National Medical Center. Tissue from an aldosterone-secreting tumor was obtained from New York Hospital with the kind assistance of Dr. Jon Blumenfeld. COS-1 cells used in the transfection assays were purchased from the American Type Culture Collection (Rockville, MD).

The expression vector pCMV4 (22) was a gift from Dr.

Table 1. Sequences and Uses of Oligonucleotides

Oligonucleotide	Gene	Exon	Sense	Purpose
1. TTTGGATCCACC - ATGGCACTCAGGGCAAAGGCA	CYP11B1/2	1	+	PCR
2. ACATTGGTGCGCGTGTTCCTC	CYP11B1	8	+	PCR
3. ACATTGGTACAGGTTTCCTC	CYP11B2	8	+	PCR
4. GAGACGTGATTAGTTGATGGC	CYP11B1	9	-	PCR
5. CAGATGCAAGACTAGTTAATC	CYP11B2	9	-	PCR
6. TTCCTGAAGCTGACAGCATTC	B2M	1	+	PCR
7. CCTCCATGATGCTGCTTACAT	B2M	3	-	PCR
8. CATGCTGACACACTGATGC	CYP17	5	+	PCR
9. GGTGTATCTCTAAATCTGT	CYP17	8	-	PCR
10. GGGGGAACATGCTGGGCCTCA	CYP11B1	9	-	HYB
11. GGGGGACGTGCCAGGCCTCA	CYP11B2	9	-	HYB
12. CTGAGGCGAAGCAGCTCTCG	CYP17	6	-	HYB
13. TAGTGAACCGTCAGAATTG	pCMV4 vector		+	SEQ
14. AGGGAGCAGGGTTATGA	CYP11B1/2	1	+	SEQ
15. GGCACAAATGTGGCGTG	CYP11B1/2	2	+	SEQ
16. AGCCTGACCCTGGACGT	CYP11B1/2	3	+	SEQ
17. AGCCCAAGGTGTGGAA	CYP11B1/2	4	+	SEQ
18. GCAGGGAGCGTGGACAC	CYP11B1/2	5	+	SEQ
19. CCCTCAAGGAGACCTTG	CYP11B1/2	6	+	SEQ
20. GCTAGACATCAGGGGCT	CYP11B1/2	8	+	SEQ

David Russell, and pCD-Adx (24), an expression plasmid containing bovine adrenodoxin, was a gift from Dr. Michael Waterman.

Oligonucleotides

All oligonucleotides used were synthesized on an Applied Biosystems (Foster City, CA) model 391EP DNA synthesizer, except for those specific for 17 α -hydroxylase, which were a kind gift of Dr. Michael Waterman. The sequences and uses of all oligonucleotides are given in Table 1.

Primary Culture of Human Adrenal Glomerulosa Cells

Tissue culture reagents were purchased from Sigma (St. Louis, MO).

The outer adrenal cortical tissue was separated by a pathologist with microscopic assistance under sterile conditions. The outer glomerulosa tissue was minced in 1:1 Dulbecco's Modified Eagle's and F-12 media (DME/F12) containing 0.2% BSA, 100 U/ml penicillin G, and 100 μ g/ml streptomycin. Cell suspensions were prepared as previously described (33). Cells (2×10^5 /ml) in DME/F12 containing 10% fetal calf serum were plated on six-well (35 mm) culture plates, and the medium was changed every 48 h.

For each experiment, the medium was replaced with DME/F12 containing 0.5% fetal calf serum and 0.2% BSA, and after 24 h it was replaced again with the same medium. Synthetic human angiotensin-II (Peninsula Laboratories, Belmont, CA) or ACTH-(1-24) (Cortrosyn, Organon, West Orange, NJ) were added to some wells and added again after 12 h. After an additional 12 h, the cells were washed twice with ice-cold PBS, and RNA was extracted.

Amplification of Reverse Transcribed RNA Using the RT-PCR

RNA from both tissues and cultured cells was extracted using the acid guanidinium isothiocyanate method (RNAzol, BIOT-EXC Laboratories, Houston, TX) (34).

A 0.5- μ g aliquot of each RNA sample was reverse transcribed in a 10- μ l reaction using 100 U RNase-H⁻ reverse transcriptase (Superscript, purchased from Gibco-BRL, Gaithersburg, MD). The buffer provided by the manufacturer was used along with 30 U RNasin (U.S. Biochemical Corp., Cleveland, OH), 0.5 μ M random primers [pd(N)6, purchased from Pharmacia, Piscataway, NJ], and 1 mM of each dNTP (19). After incubation at 37 C for 1 h, the samples were placed in a boiling water bath for 5 min and chilled on ice. Forty microliters of PCR buffer [12.5 mM Tris-HCl (pH 8.3), 62.5 mM KCl, 1.9 mM MgCl₂, and 0.001% gelatin] containing the primers of interest and 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) were added to each reaction. The final concentration of each primer was 0.5 μ M, except that the β_2 -microglobulin oligonucleotides, when included in the reactions along with other primers, were used at a final concentration of 0.1 μ M. The conditions used in all PCRs were a denaturation step at 94 C for 1 min, annealing at 60 C for 1 min, and extension at 72 C for 1 min 35 sec, with the extension time increasing by 5 sec/cycle.

Gel Analysis and Blot Hybridization

The PCR products were analyzed on 1.5% agarose gels and, after staining with ethidium bromide and photography, transferred to nylon membranes (Micron Separation Industries, Westboro, MA) by capillary blotting. The oligonucleotides used as probes were labeled with ³²P using polynucleotide kinase (Gibco-BRL) and hybridized with the membrane overnight in 6 $\times$ SSC (1 $\times$ SSC contains 0.15 M NaCl and 0.015 M Na citrate), 0.25% nonfat dry milk powder, and 1% SDS. Blots were washed in 6 $\times$ SSC-0.1% SDS at 60 C (CYP11B1 and β_2 -

microglobulin probes), 65 C (CYP11B2 probe), or 55 C (17 α -hydroxylase probe).

Negatives of ethidium bromide-stained gels and autoradiograms were scanned using a Bio-Rad model 620 Video Densitometer (Richmond, CA), and the areas under the peaks of the profiles obtained were determined using the Bio-Rad 1-D Analyst II Data Analysis Software Package, version 3.1.

Cloning of cDNA for *in Vitro* Expression

Complementary DNA fragments containing the entire coding sequences of CYP11B1 and CYP11B2 were generated by RT-PCR using the exon 1 PCR primer containing the common coding sequences from the initiation codon (but with a modified 5' untranslated region to optimize translation efficiency) and either the CYP11B1 or CYP11B2 specific oligonucleotides from exon 9 (primers 1, 4, and 5 in Table 1). The PCR products were rendered blunt-ended by treatment with Klenow enzyme, digested with BamHI (U.S. Biochemicals), purified by gel electrophoresis, and subcloned into the BglII/SmaI sites of the eukaryotic expression vector pCMV4. The recombinants obtained were subjected to complete sequence analysis using a series of specific primers, modified T7 DNA polymerase (Sequenase II, U.S. Biochemicals) (35), and templates denatured in the presence of dimethylsulfoxide (36).

Large scale plasmid DNA preparations were purified by centrifugation in CsCl and ethidium bromide.

Transient Transfection Assay for Enzyme Activity

Plasmid DNA was transfected into COS-1 cells using Lipofectin reagent (Gibco-BRL) (37) according to the manufacturer's protocol. The procedure was scaled down for use in six (35-mm)-well plates. Approximately 5×10^5 cells at 80% confluence were exposed for 24 h to a mixture containing 15 μ g Lipofectin reagent, 5 μ g pCD-Adx, and 10 μ g pCMV4-B1, pCMV4-B2, or H₂O in 1 ml OptiMEM I Reduced Serum Medium (Gibco-BRL), 10 U penicillin G, 10 μ g streptomycin, 0.025 μ g amphotericin-B (antibiotic-antimycotic solution from Sigma). The cells were then incubated for 24 h in DME supplemented with 0.2 mM L-glutamine, 1-fold concentrated nonessential amino acids (100-fold solution from GIBCO-BRL), 1-fold antibiotic-antimycotic solution, and 20% newborn calf serum (Sigma) to facilitate cell recovery and allow expression of the introduced genes.

To assay enzyme activities of the transfected cells, the medium was replaced with 1 ml of the same medium containing 0.5 μ M [¹⁴C]deoxycorticosterone (0.001 μ Ci) or [³H]11-deoxycortisol (1 μ Ci; New England Nuclear, Boston, MA), and the cells were incubated for a further 24 h.

In one experiment, 5 μ M glycyrrhetic acid (an inhibitor of 11 β -hydroxysteroid dehydrogenase) was added when the Lipofectin reagent was replaced with fresh medium. Fresh glycyrrhetic acid was added at the same time as the steroid substrate (0.5 μ M [³H]cortisol). All transfections were performed in duplicate.

TLC

The medium from each well of transfected cells was transferred to borosilicate glass tubes and supplemented with 10 nmol of the unlabeled steroid that had been the substrate for each particular assay. The solution was then extracted with 2.5 vol methylene chloride, and the organic phase was dried down at 37 C under a stream of N₂. The steroids were dissolved in 15 μ l methanol and spotted onto glass-backed silica-coated TLC plates, which contained a 254-nm fluorescent indicator (Aldrich Chemical Co., Milwaukee, WI). An additional 10 nmol nonradioactive steroid corresponding to each of the expected radioactive steroid products were also spotted. Chromatography was performed in a solvent containing methylene chloride-methanol-H₂O in the ratio of 300:20:1. The

positions of the unlabeled steroids were identified under UV light, and the plates were analyzed using a Bioscan System 200 imaging scanner (Bioscan, Washington, DC). Plates containing ^{14}C -labeled steroids were also subjected to autoradiography using a Phosphorimager model 400E (Molecular Dynamics, Sunnyvale, CA).

RIAs

Transfected cells were treated identically to those used for the TLC assays, except that 2 μM unlabeled deoxycorticosterone was used. After extraction with methylene chloride, steroids were resolved by Celite column chromatography, and the concentrations of corticosterone and aldosterone were determined by RIA, as previously described (27, 28).

Biosynthetic Labeling and Immunoprecipitation

Transfected COS-1 cells were incubated for 48 h subsequent to removal of the Lipofectin reagent and washed in L-methionine/L-cysteine-free medium (made using the MEM Select-Amine Kit from Gibco-BRL), which had been supplemented with 0.2 mM L-glutamine, 1-fold concentrated nonessential amino acids, 1-fold concentrated antibiotic/antimycotic solution, and 20% newborn calf serum. They were then incubated for 2 h in 1 ml of the same medium supplemented with 250 μCi L-[^{35}S]methionine/L-[^{35}S]cysteine (Tran ^{35}S -label, ICN Biochemicals). After removing the medium, the cells were lysed in 1 ml 0.05 M Tris-HCl, 1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 100 U aprotinin, and 0.1 mM L-methionine and incubated at 4°C for 45 min. Nuclei and cell debris were removed by centrifugation for 2 min in a microcentrifuge. Each cell lysate was then precleared by incubation on ice for 30 min with 10 μl rabbit antiserum made to an unrelated protein, followed by incubation on a rocking platform at 4°C for a further 60 min with 25 μl of a 50% suspension of protein-A-Sepharose 4B (Pharmacia). The immune complexes formed were pelleted by centrifugation in a microcentrifuge for 10 sec, and the supernatant was transferred to a fresh tube. This preclearing procedure was repeated twice more.

A rabbit antiporcine P450c11 serum was a gift from Dr. Peter Hall. To diminish nonspecific bands immunoprecipitated by this antiserum, 1 μl of the antiserum was preincubated with 250 μl of an unlabeled lysate of COS-1 cells, containing a 10-fold excess of cells compared with the radiolabeled lysates. Two hundred and fifty microliters of each precleared labeled lysate were immunoprecipitated with the anti-P450c11 serum and 25 μl protein-A-Sepharose, as previously described. After centrifugation and removal of the supernatant, the beads were washed twice in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25% NP-40, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 10% newborn calf serum, and then once in each of the following solutions: 500 mM NaCl, 50 mM Tris-HCl (pH 7.8), and 0.5% NP-40; 150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 0.5% NP-40, and 0.1% SDS; and 150 mM NaCl, 50 mM Tris-HCl, and 0.5% sodium deoxycholate. Immunoprecipitated material was eluted from the beads by boiling in sample buffer and then analyzed by electrophoresis in a 8.5% SDS-polyacrylamide slab gel. Protein size standards were obtained from Gibco-BRL. The gel was soaked in Resolution (EM Corp., Chesnut Hill, MA) according to manufacturer's directions and dried. Radioactivity was detected using the Phosphorimager 400E.

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