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Stable Expression of Rat Cytochrome *P*450 11β-Hydroxylase (CYP11B1) and Aldosterone Synthase (CYP11B2) in MA-10 Cells

Mingyi Zhou, Di Xue, Mark F. Foecking and Celso E. Gomez-Sanchez*

Division of Endocrinology and Metabolism, Department of Internal Medicine, Harry S. Truman Memorial Veterans Hospital and University of Missouri, Columbia, MO 65201, U.S.A.

Glucocorticoids and mineralocorticoids are synthesized in the adrenal cortex through the action of two different cytochrome 11β-hydroxylases, CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) which are distributed in the zona fasciculata and glomerulosa, respectively. We have created stably transfected cell lines using the Leydig tumor cell line MA-10 with CYP11B1 and CYP11B2 cDNA-containing plasmids which have a selectable gene to confer resistance to geneticin. The expression of the transfected cDNA in the cells was characterized by Northern-blot and measurement of enzymatic activity. The cell lines express the enzymes stably for many generations. CYP11B1 transfected cells converted DOC into corticosterone, 18-OH-DOC and small amounts of 18-OH-corticosterone, in a time and concentration dependent manner. Incubation of the cells with corticosterone generated 18-OH-corticosterone especially at concentrations of 30 and 100 μM. The production of 18-OH-corticosterone from corticosterone at these doses was significantly higher than incubations with similar concentrations of DOC. CYP11B2 transfected cells converted DOC into corticosterone, 18-OH-corticosterone, aldosterone and small amounts of 18-OH-DOC in a time and concentration dependent manner. They converted corticosterone into 18-OH-corticosterone and aldosterone in a time and concentration dependent manner. The absolute and relative production of aldosterone from DOC was significantly higher than when cells were incubated with corticosterone, and the ratio of aldosterone to 18-OH-corticosterone was higher at all concentrations of DOC compared to corticosterone. CYP11B2 transfected cells (but not the CYP11B1 transfected cells) transform 18-OH-DOC into 18-OH-corticosterone, but can not convert 18-OH-DOC into aldosterone. In conclusion, stably transfected MA-10 cells with the cDNAs for the CYP11B1 and CYP11B2 enzymes were prepared and their enzymatic activity studied. These cells are useful in the study of inhibitors of the specific enzymes, as well as determining the roles that each enzyme plays in zone-specific steroidogenesis in the adrenal cortex.

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INTRODUCTION

Aldosterone, the most powerful mineralocorticoid, and corticosterone, the main glucocorticoid in rats, are synthesized from 11-deoxycorticosterone (DOC) by the actions of two different cytochrome *P*450 11β-hydroxylase enzymes. In the rat, cytochrome *P*450 11β-hydroxylase enzyme, or CYP11B1, has an apparent molecular mass of 51 kDa and is found in the zona fasciculata-reticularis (ZF) mitochondria [1, 2]. CYP11B1 transforms DOC into corticosterone, 18-hydroxydeoxycorticosterone (18-OH-DOC) and smaller quantities of 19-hydroxydeoxycorticosterone [3, 4]. The cytochrome *P*450 11β-hydroxylase/aldosterone synthase, or CYP11B2, enzyme has an apparent molecular mass of 49 kDa, is present in zona glomerulosa (ZG) mitochondria and is induced by subjecting the animals to a low sodium, high potassium diet [1, 2, 5, 6]. CYP11B2 catalyzes the formation of aldosterone, 18-hydroxycorticosterone (18-OH-B) and corticosterone from DOC [3, 4, 7, 8].

cDNAs encoding the two cytochrome *P*450 11β-hydroxylase enzymes have been isolated from rat adrenal cDNA libraries [9–12]. CYP11B1 mRNA has been localized by *in situ* hybridization in the adrenal ZF

*Correspondence to C. E. Gomez-Sanchez.
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[13, 14] and CYP11B2 mRNA in the ZG [15]. A low sodium diet with potassium supplementation stimulated CYP11B2 mRNA expression [6, 16–19]. ACTH produced a transient increase after 3 h of administration, but mRNA levels were unchanged after 24 h of ACTH [17], in agreement with the observations of the changes of aldosterone secretion after ACTH [20]. Expression of 11β-hydroxylase mRNA increased after ACTH [17]. Immunocytochemical studies using antibodies specific for 11β-hydroxylase stained the ZF well, but failed to demonstrate the enzyme in the ZG [8, 21].

Cos cells in which cDNA for CYP11B1 enzyme is transiently expressed convert DOC into corticosterone, 18-OH-DOC and 19-OH-DOC [3, 4, 22]. Small amounts of 18-OH-B were also formed when the cells were incubated with corticosterone, but 18-OH-DOC was not converted to 18-OH-B. Cells transiently transfected with CYP11B1 cDNA can also transform 19-OH-DOC into 19-oxoDOC [3, 4]. Cos cells transiently transfected with the CYP11B2 cDNA transform DOC into corticosterone, 18-OH-B and aldosterone and small amounts of 18-OH-DOC. These cells also transform 18-OH-DOC into 18-OH-B, but not into aldosterone [3]. The level of expression of transiently transfected cell lines varies with each experiment and the steroidogenic properties of the enzymes are difficult to establish with consistency. Stably transfected cell lines which express constant amounts of enzyme in all passages would be useful to explore mechanisms of steroid transformation by these enzymes. We report studies of steroid transformation by two cell lines derived from Leydig cell tumor MA-10 stably transfected with rat CYP11B1 and CYP11B2 cDNA.

MATERIALS AND METHODS

Materials

Waymouth MB 752/1 medium and steroids were from Sigma Chemical company (St Louis, MO). Lipofectin and Geneticin were from GIBCO (Grand Island, NY), [α-^{32}P]dCTP was from DuPont NEN (Boston, MA), and Zeta-Probe GT membrane was from Bio-Rad Laboratories (Richmond, CA). The rat MA-10 Leydig tumor cell line [23], the kind gift of Dr Mario Ascoli (University of Iowa), was chosen because it expresses adrenodoxin and adrenodoxin reductase, needed for the functioning of the mitochondrial *P*450 enzymes.

Stable transfection of rat cytochromes P450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) cDNAs into MA-10 cell lines

The plasmids pcDNAI/neo-11B1 and pcDNAI/neo-11B2, which contain the insert of the whole coding region cDNA of CYP11B1 and CYP11B2, respectively, were prepared as described previously [12]. Sequencing of the 11β-hydroxylase revealed an identical sequence to that described [9]. The pcDNA/neo-11B2 cloned by us [12] differs from the one described by others [10] in that it contains 6 nucleotide substitutions, but the sequence of the signal peptide is the same as described and used in transient expression studies [3, 4, 10].

MA10 cells were grown in Waymouth MB 752/1 media supplemented with 20 mM HEPES and 15% horse serum in a T-75 culture flask to about 40% confluency. The cells were washed with serum free medium twice and incubated with 10 ml of serum free medium pre-mixed with 3 μg of plasmid DNA and 80 μl of lipofectin for 4 h at 37°C. Additional medium (10 ml) containing 30% of horse serum was then added and the culture continued for 48 h. The cells were then transferred to 5 × 96-well culture plates (approx. 100,000 cells per well) cultured with medium containing 200 μg/ml of geneticin for 2–3 weeks with medium changes twice a week, until the surviving clones were large enough to be seen easily. Medium with 1 μM deoxycorticosterone was added to the wells containing clones and incubated overnight and media collected for RIA or ELISA assay of the conversion products to identify the clones expressing the cDNA. The positive clones were amplified and those with the highest enzymatic activity kept and used for the studies.

Cells expressing high enzymatic activity were cloned by limit dilution in 96-well plates by plating at a concentration of 0.3, 1 and 3 cells/well. Positive wells at the lowest dilution were expanded and aliquots frozen in liquid nitrogen.

Northern analysis of mRNA of stably transfected MA-10 cells

Total RNA was extracted from MA-10 cells with RNAzol B solution [24] and 10 μg RNA separated by electrophoresis on a formaldehyde-denatured 1% agarose gel. The RNA was blotted onto Zeta-probe GT membrane with a modified downward capillary transfer method [25]. DNA probes 345 and 354 bp specific for the 11β-hydroxylase and aldosterone synthase, respectively, were prepared by PCR amplification and labelled with a random primed DNA labeling kit (USB, Cleveland, OH). The standard hybridization protocol described by the manufacturer of the Zeta-probe membrane was followed.

Substrate metabolism by transfected MA-10 cells

Stably transfected MA-10 cells were cultured to confluence in 24-well plates. The cells were washed twice and 1 ml of medium containing 0.5% of BSA (studies done in medium containing horse serum gave similar results), 10 μM of deoxycorticosterone or other substrates, and 30 μM of cyanoketone, to inhibit conversion of endogenous pregnenolone to progesterone [26], was added to each well and the cells incubated for various times to determine the time course of the formation of conversion products. A dose–response

curve with different concentration of substrates (0–100 μM) was also done with cells incubated for 4 and 24 h. At the end of the incubations, media were collected and frozen until assay.

Determination of kinetic parameters in mitochondria from transfected MA-10 cell lines

Cells were grown in 225 cm^2 flasks until confluent, scraped and homogenized in a Polytron homogenizer, and the mitochondria prepared by differential centrifugation as previously described [27]. The mitochondria were then incubated with various concentrations of DOC (0.01–100 μM) for 20 min (the reaction is linear for 30–40 min) in an incubation reaction containing 10 mM isocitrate, fumarate and 1.5 mM NADP [28]. At the end of the incubation, the samples were placed in ice, extracted and assayed as detailed below.

Steroid measurements

Aldosterone, corticosterone, 18-OH-B and 18-OH-DOC, were determined by radioimmunoassay (RIA) or ELISA as described previously [29, 30]. All the experiments were done in triplicate.

RESULTS

Cell growth was detected in approx. 20% of wells at the end of 3 weeks. In turn, approx. 20% of these surviving wells converted DOC to either corticosterone (CYP11B1) or aldosterone (CYP11B2). Several clones which exhibited the highest conversion rates were selected for expansion and cloning. Cells were cloned by limit dilution until all clones exhibited similar conversion activity. Northern-blots from transfected cells indicated the presence of abundant message (Fig. 1); control MA-10 cells similarly treated did not express either enzyme mRNA.

Time-course and dose–response

CYP11B1 transfected cells converted DOC into corticosterone and 18-OH-DOC in a time dependent fashion (Fig. 2). Levels of 18-OH-DOC formed were approximately one half those of corticosterone and those of 18-OH-corticosterone were low. CYP11B1 transfected cells also converted corticosterone to 18-OH-corticosterone in small but clearly detectable amounts. As expected, the production of these steroids was dependent on the concentration of the

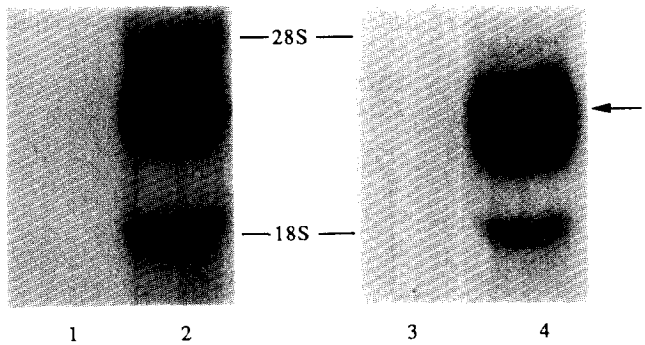

Fig. 1. Northern blot from total RNA extracted from MA-10 cells stably transfected with the CYP11B1 and CYP11B2 cDNAs. The relative mobility of 18S and 28S RNA is shown. Lanes 1 and 3 correspond to RNA from MA-10 cells. Lane 2 corresponds to RNA from CYP11B1-transfected cells and lane 4 corresponds to RNA from CYP11B2-transfected cells.

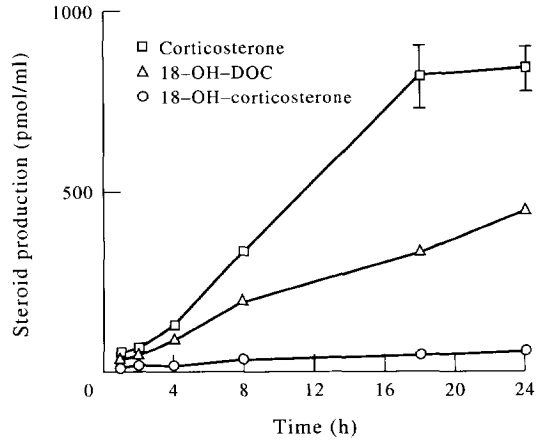

Fig. 2. Time-course of steroid production of MA-10 cells stably transfected with the CYP11B1 cDNA. Cells were grown in 24-well plates and incubated with 1 ml of media containing 0.5% bovine serum albumin and 10 μM DOC for the assigned times.

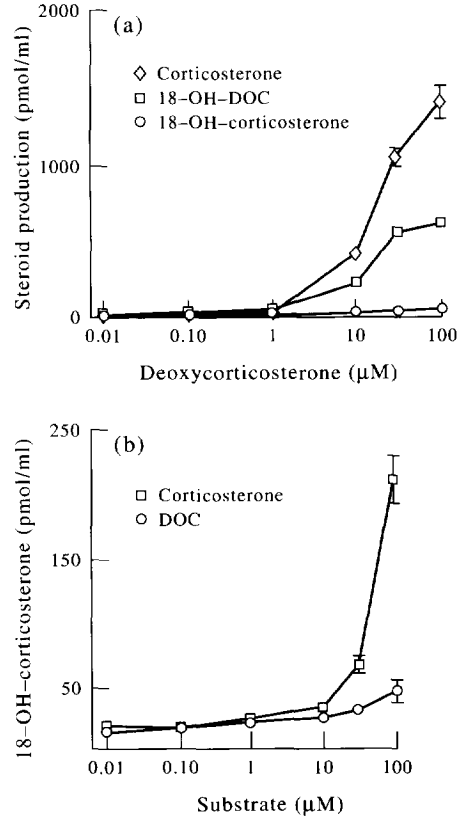

Fig. 3. (a) Steroid production by CYP11B1 transfected MA-10 cells incubated with various concentrations of DOC for 24 h. (b) Production of 18-OH-corticosterone by CYP11B1 transfected MA-10 cells incubated with various concentrations of DOC or corticosterone for 24 h.

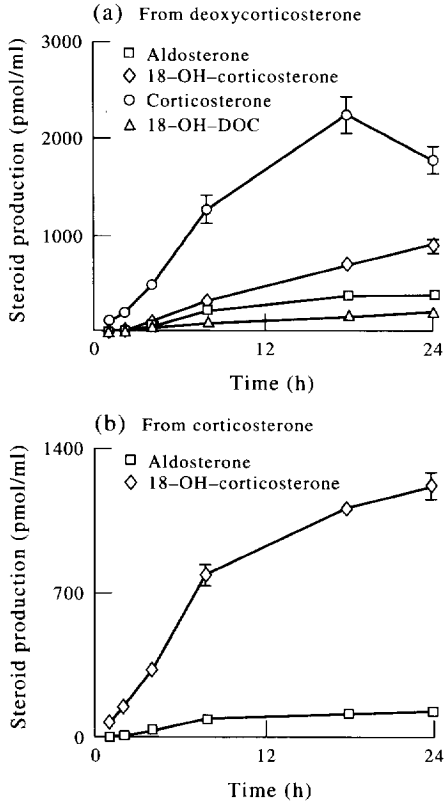

Fig. 4. (a) Time-course of steroid production by CYP11B2 transfected MA-10 cells incubated with 1 ml of medium containing 0.5% bovine serum albumin and 10 μM DOC. (b) Time-course of steroid production by CYP11B2 transfected MA-10 cells incubated with 1 ml of medium containing 0.5% bovine serum albumin and 10 μM corticosterone.

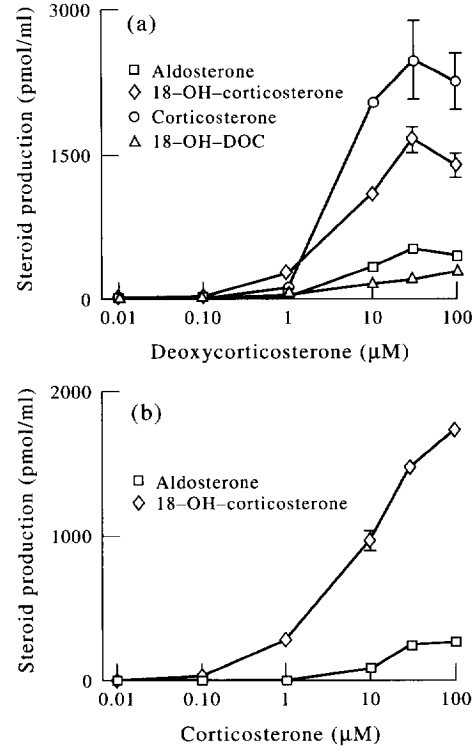

Fig. 5. (a) Steroid production by CYP11B2 transfected MA-10 cells incubated with various concentrations of DOC for 24 h. (b) Steroid production by CYP11B2 transfected MA-10 cells incubated with various concentrations of corticosterone for 24 h.

substrate [Fig. 3(a)]. The formation of 18-OH-corticosterone was higher when corticosterone instead of DOC was used as substrate, especially at the higher doses [Fig. 3(b)].

CYP11B2 transfected cells converted DOC into corticosterone,18-OH-corticosterone, aldosterone and 18-OH-DOC, and also corticosterone into 18-OH-corticosterone and aldosterone, in a time [Fig. 4(a, b)] and concentration dependent manner [Fig. 5(a, b)]. The production of 18-OH-DOC was significantly lower than that produced by CYP11B1 transfected cells. The formation of 18-OH-corticosterone and aldosterone from corticosterone was lower than when DOC was used as the substrate ($P < 0.05$, $n = 3$). The ratio of aldosterone to 18-OH-corticosterone was significantly lower ($P < 0.05$, $n = 3$) when corticosterone was used as substrate rather than DOC (Fig. 6).

Incubation of CYP11B1 transfected cells with 10 μM 18-OH-DOC for 16 h did not result in any detectable formation of 18-OH-corticosterone. Incubation of CYP11B2 transfected cells with 18-OH-DOC produced reasonable levels of 18-OH-corticosterone, but no aldosterone.

Mitochondria from cells transfected with CYP11B1 transformed DOC to corticosterone with an apparent K_m of 2 ± 0.2 μM ($n = 2$). Mitochondria from cells transfected with CYP11B2 transformed DOC into aldosterone with an apparent K_m of 0.7 ± 0.1 μM ($n = 3$).

DISCUSSION

We have prepared stably transfected cell lines expressing CYP11B1 and CYP11B2 using geneticin selection in MA-10 transfected cells. A high proportion of wells (20%) plated with cells transfected with the plasmids exhibited resistance to geneticin, but only approx. 20% of these wells had cells which expressed the cytochrome *P*450 enzymes. Levels of enzymatic activity were very different in the cells from various

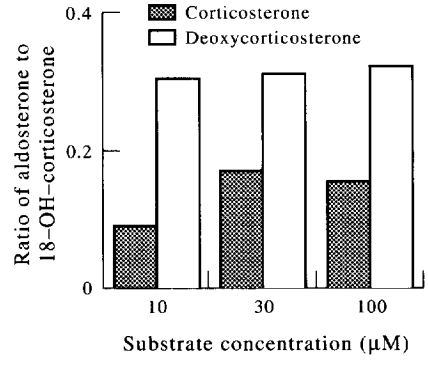

Fig. 6. Ratio of aldosterone to 18-OH-corticosterone of CYP11B2 transfected cells incubated with various concentrations of DOC or corticosterone.

wells, reflecting either a difference in the expression of the enzymes or an admixture of cells that did not express enzyme. Cells from wells with the maximal enzymatic activity were further cloned by limit dilution until all the wells exhibited similar enzymatic activity. A few of these clones were further expanded and preserved by freezing. The cloned cells have been stable and continue to express the enzymes after many generations.

The CYP11B1 enzyme is expressed only in the ZF of the rat [8, 21]. The major steroid products are corticosterone and 18-OH-DOC, with smaller amounts of 18-OH-corticosterone. The formation of 18-OH-B in the ZF is believed to be due to the action of the CYP11B1 on corticosterone, but the efficiency is very low [3, 43]. CYP11B1 transfected cells converted DOC into corticosterone and 18-OH-DOC. Corticosterone was the major product with 18-OH-DOC generation 53% that of corticosterone. Conversion of DOC to 18-OH-corticosterone was very low, but corticosterone conversion was also low at low substrate concentration and proportionally much higher at 30 and 100 μM corticosterone [Fig. 3(b)]. CYP11B1 cannot convert 18-OH-DOC into 18-OH-corticosterone. These results are similar to those reported with transiently transfected COS cells [3]. At the present time it is thus difficult to know the mechanism by which 18-OH-corticosterone is synthesized in the ZF, since both DOC and corticosterone are poor substrates for the formation of 18-OH-corticosterone by CYP11B1. Corticosterone is a fair substrate, at concentrations that are probably higher than those found in the adrenal.

CYP11B2 is the only 11β-hydroxylase expressed in the ZG of the adrenal [8, 13, 21] and expressed only in this zone. CYP11B2 catalyzes the conversion of DOC to corticosterone, 18-OH-corticosterone and aldosterone through a series of consecutive hydroxylations. It also can convert DOC into 18-OH-DOC, but the yields are significantly less than with CYP11B1. The production of aldosterone increases with incubation time and reaches a plateau at around 18 h. 18-OH-corticosterone continues to increase throughout the incubation period, and the concentration of corticosterone is lower at 24 h than at 18 h, suggesting that corticosterone becomes a substrate for the enzyme. The maximal steroid production in the transfected cells seem to occur at 30 μM concentration of DOC [Fig. 5(a)]. 18-OH-corticosterone and aldosterone continue to increase with higher concentration of corticosterone. The ratio of aldosterone to 18-OH-corticosterone production depended on the substrate, with the ratio significantly higher when cells were incubated with DOC than with corticosterone. This finding could be of importance in abnormal situations where the two enzymes, which are not normally present in the same cells, are expressed in the same cells, as in adrenal adenomas producing primary aldosteronism [31]. These patients secrete abnormally high amounts of 18-OH-corticosterone, which is useful in the differential diagnosis of aldosterone producing adenomas from other forms of hyperaldosteronism [32]. The presence of the CYP11B1 enzyme would generate corticosterone which would be an additional substrate for the CYP11B2 enzyme, and the combination of available substrates would generate the abnormal ratio of products.

DOC may be a better substrate for the CYP11B2 enzyme than corticosterone reflecting a higher affinity of the enzyme for DOC, or because the greater hydrophobicity of DOC makes it easier for DOC to penetrate the cell and/or mitochondrial membranes. We postulate that the CYP11B2 enzyme is a partially processive enzyme, in which DOC binds to the enzyme and is successively hydroxylated at the 11β- and 18-positions and where hydroxylation products can leave the enzyme at various stages of hydroxylation. The finding that 18-OH-DOC is transformed to 18-OH-corticosterone and not to aldosterone suggests that 18-OH-corticosterone (as the 18–20 hemiacetal form) is a terminal product of the CYP11B2 enzyme. At this point some of the 18-OH-corticosterone having assumed the stable hemiacetal form, leaves the enzyme, with the product bound to the enzyme as the open form hydroxylated sufficiently fast to the germinal diol which then spontaneously dehydrates to aldosterone. The products which leave the enzyme are either less efficient substrates (corticosterone) or are not substrates (18-OH-corticosterone). 18-OH-DOC and 18-OH-corticosterone can be converted to aldosterone by rat ZG cells [33], but the mechanism is unclear, since neither substrate is converted to aldosterone by cells transfected with the CYP11B2 enzyme [3].

The CYP11B2 cDNA transfected to produce the cell line described herein corresponds to a variant that we have cloned from a Sprague–Dawley rat cDNA library [12] and differs from those reported by others by 6 nucleotides [10, 11]. The coded nucleotides result in a difference of 3 amino acids from the originally described clones [10, 11]. It is of interest that these amino acids are homologous to those of the CYP11B1 enzyme. Additional differences between the various cDNAs identified by several laboratories include the length of the signal peptide. The present cDNA contains a 24 amino acid signal peptide which is identical to the one found in the CYP11B1 enzyme and found in some clones identified by us [12] and by Imai *et al* [10]. We also found a clone with a 30 amino acid signal peptide [12] and Matsukawa *et al* [11] found an additional clone containing a 34 amino acid signal peptide. Cells transfected with cDNA clones bearing the longer signal peptides expressed little enzymatic activity [11, 12]. It is at the present time unknown which of the cDNAs or which proportion of them are expressed normally in the adrenal gland. Final resolution of the dependence of enzymatic activity of expressed cDNAs upon signal

peptide constructs awaits the preparation of additional cell lines.

In conclusion, stably transfected cell lines have been prepared with cDNAs for the cytochrome *P*450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). These cells consistently retain enzymatic activity through many generations. The cells are useful tools for the study of the enzymes and in the development of specific inhibitors of a given pathway in steroidogenesis.

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