Molecular genetic investigations of patients with defects in steroid hormone production

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Nomenclature of cytochrome P450:

CYP11A1	cytochrom P450 cholesterol-desmolase or P450scc
CYP11B1	11β-hydroxylase cytochrome P450 or P450-11β
CYP11B2	aldosterone synthase cytochrome P450 or P450-Aldo
CYP17	17α-hydroxylase/17,20-lyase cytochrome P450 or P450c17
CYP19	aromatase cytochrome P450 or P450arom
CYP21	21-hydroxylase cytochrome P450 or P450c21

Abbreviations:

3βHSD	3β-hydroxysteroid dehydrogenase
18-OH-B	18-hydroxy-corticosterone
ACTH	adrenocorticotrophin hormone
AdR	adrenodoxin reductase
Adx	adrenodoxin
Aldo	aldosterone
ANGII	angiotensin II
ASD	aldosterone synthase deficiency or CMO deficiency
ATP	adenosine triphosphate
B	corticosterone
BSA	bovine serum albumin
CAH	congenital adrenal hyperplasia
cAMP	cyclic adenosine monophosphate
CMO	corticosterone methyloxidase
CO	carbon monoxide
CYP	cytochrom P450
d	deoxy-
dATP	deoxy-adenosine-triphosphate
dCTP	deoxy-cytidine-triphosphate
ddH_2O	double distilled water
dGTP	deoxy-guanosine-triphosphate
DMEM	Dulbecco's modified Eagle's medium

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxy-NTPs
DOC	deoxycorticosterone
DTT	dithiothreitol
dTTP	deoxy-thymidine-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenedinitrilotetraacetic acid
F	cortisol
FAD	flavine adenine dinucleotide
FBS	fetal bovine serum
FMN	flavine mononucleotide
GC-MS	gas chromatography - mass spectrometry
GRA	glucocorticoid remediable aldosteronism or GSH
GSH	glucocorticoid-suppressible hyperaldosteronism
HPTLC	high performance thin layer chromatography
K	potassium
IPTG	isopropyl-b-D-thiogalactoside
MALDI-MS	matrix assisted laser desorption mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PKA	protein kinase K
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
S	11-deoxycortisol
SDS	sodium dodecylsulfate
StAR	steroidogenic acute regulatory protein
Taq	*Thermus aquaticus*
TEMED	*N,N,N',N'*-tetramethylethylenediamine
UV/vis	ultraviolet and visible wavelength range

WT	wild type

Standard abbreviations for amino acids:

A	Ala	Alanine	L	Leu	Leucine
R	Arg	Arginine	K	Lys	Lysine
N	Asn	Asparagine	M	Met	Methionine
D	Asp	Aspartic acid	F	Phe	Phenylalanine
C	Cys	Cysteine	P	Pro	Proline
Q	Gln	Glutamine	S	Ser	Serine
E	Glu	Glutamic acid	T	Thr	Threonine
G	Gly	Glycine	V	Val	Valine
H	His	Histidine	W	Trp	Tryptophan
I	Ile	Isoleucine	Y	Tyr	Tyrosine

Units:

Length	Meter	m
	Centimeter	cm
Mass	Gram	g
Molecular weight	Dalton	Da
Current strength	Ampere	A
Tension	Volt	V
Electricity	Watt	W
Temperature	Celsius	C
Volume	Liter	L
	Mililiter	mL
	Microliter	μL
Wave length	Nanometer	nm
Time	Second(s)	sec
	Minute(s)	min
	Hour(s)	hr

Summary

A) Zusammenfassung *(German version)*

Die Corticosteroide Cortisol und Aldosteron werden in der Nebennierenrinde in einer umfangreichen Reaktionskaskade, an der mehrere Cytochrome P450 (CYP) beteiligt sind, synthetisiert. CYPs gehören zu den Monooxygenasen, die molekularen Sauerstoff in ihre Substrate einbauen. Im Menschen werden die Endschritte der Cortisol und Aldosteron Synthese durch CYP11B1 beziehungsweise CYP11B2 katalysiert. CYP11B1, welches die Steroid 11β-Hydroxylierung katalysiert, wird im großen Maße in der Zona fasciculata/reticularis der Nebennierenrinde synthetisiert und durch ACTH reguliert. CYP11B1 ist verantwortlich für die Umwandlung von 11-Deoxycortisol (RSS) zu Cortisol. Die Defizienz der 11β-Hydroxylase ist, neben der 21-Hydroxylasedefizienz, die zweithäufigste Ursache für die angeborene Nebennierenhyperplasie (congenital adrenal hyperplasia, CAH), eine Erbkrankheit, die die Synthese von Cortisol aus 11-Deoxycortisol unterbindet. Im Gegensatz dazu wird die Aldosteronsynthase CYP11B2 in der Zona glomerulosa gebildet und hauptsächlich durch die Mengen an Angiotensin II und Kalium im Serum reguliert. CYP11B2 katalysiert die dreistufige Umwandlung von Deoxycorticosteron (DOC) zu Aldosteron: der erste Schritt ist eine Hydroxylierung an Position 11β und es entsteht Corticosteron (B). Eine weitere Hydroxylierung an Position 18 führt zur Bildung von 18-Hydroxycorticosteron (18OHB) und eine abschließende Oxidation an gleicher Stelle zu Aldosteron (Aldo). Die Defizienz der Aldosteronsynthase zeigt sich im Säuglingsalter als ein, bedingt durch Mutationen im *CYP11B2* Gen, lebensbedrohliches Elektrolytungleichgewicht. Allerdings wurden CAH und Aldosteronsynthasedefizienz, bedingt durch Mutationen sowohl im *CYP11B1* als auch im *CYP11B2* Gen, auf molekularer Ebene bis jetzt nur oberflächlich untersucht. Folglich würde eine gründliche Untersuchung der Mutationen und ihrer enzymatischen Aktivitäten Informationen für die Diagnose und Behandlung von CAH und Hypoaldosteronismus, verursacht durch CYP11B1 und CYP11B2 Defizienzen, liefern.

Die Ziel der vorliegende Arbeit waren:

1) Die Entdeckung von Mutationen in den *CYP11B2* und *CYP11B1* Genen von Patienten, die eine Fehlstörung der Nebennierensteroide (Mineralocorticoide und Glucocorticoide) aufweisen.

2) Die Untersuchung der Effekte dieser Mutationen auf die Steroidbiosynthese durch Expression der mutierten cDNAs in der Zellkultur. Damit einher geht der Vergleich zwischen COS-1 (Nierenzellen der Grünen Meerkatze) und HCT116 (humanes Coloncarzinom) Zellen als geeignetes, heterologes Expressionssystem für CYP11B1 und CYP11B2.

Um die Mutationen von Patienten, die an Aldosteronsynthasedefizienz litten bzw. Patienten mit CAH auf Cortisoldefizienz zu untersuchen, wurden *CYP11B1* und *CYP11B2* Gene dieser Patienten und ihrer Eltern mittels PCR aus der genomischen DNA, amplifiziert. Alle neun Exons und die Grenzen zwischen Exon und Intron wurden mittels automatischer Sequenzierung analysiert. Sechs neue Mutationen (L451F, S315R, R374W, R490Δ1, R181Q, und eine stille Mutation im letzten Nucleotid des Exons Nr. 5 wurden insgesamt bei den vier Patienten mit Aldosteronsynthasedefizienz entdeckt. Eine Mutation (L299P) war von dem Patient mit CAH bereits bekannt. Um die Auswirkungen der CYP11B1-Mutante auf die Cortisolsynthese und der CYP11B2-Mutanten auf die Aldosteronsynthese zu untersuchen, wurden diese Mutanten mit Hilfe des Expressionplasmidconstrukts pSVLhCYP11B1/11B2 mittels Ortsgerichteter Mutagenese hergestellt. Die enzymatische Aktivität der Mutanten wurde sowohl in COS-1 Zellen als auch in HCT116 $p53^{-/-}$ Zellen durch Co-Expression mit bovinem Adrenodoxin (Plasmid pbAdx) bestimmt. Die Expression der mutierten Proteine der Zelllinien wurde mittels Western Blots analysiert.

Zur Optimierung des Analyseverfahrens zur Messung der Aktivität der Genprodukte von CYP11B1 und CYP11B2 wurden HCT116 $p53^{-/-}$ Zelllinien auf ihre Aktivitäten von CYP11B1 und CYP11B2 mit und ohne einer Cotransfektion von bovinem oder humanem Adrenodoxin, einem Elektronendonor der P450, verglichen Obwohl sowohl humanes als auch bovines Adrenodoxin die Produktbildung von CYP11B1 und CYP11B2 steigerten, war bovines Adrenodoxin effektiver als das humane. Im Vergleich der COS-1 zu den HCT116 $p53^{-/-}$ Zellen, wandelten beide mit CYP11B und Adrenodoxin transfezierten Zelllinien die Substrates mit einer ähnlichen Effizienz um. Dies legt nahe, dass die Effizienz 1) der Transfektion, 2) des Transports des Precursorproteins vom Cytoplasma in die Mitochondrien, 3) des Elektronentransports vom NADPH zu den mitochondrialen P450 und 4) der Substratdurchlässigkeit, sowohl in den Nierenzellen der Grünen Meerkatze als auch in den humanen Coloncarzinomzellen, gleich ist. Das Produktprofil ist nicht von der Zelllinie abhängig. Daher schlussfolgern wir, dass beide Zelllinien gleich nützlich für Aktivitätsassays von CYP11B1 und CYP11B2 sind.

Während der Analyse der Patienten konnte gezeigt werden, dass Patient Nr.1, ein Homozygot, eine neuartige Missens-Mutation (L451F) im *CYP11B2* Gen besaß. Heterologe Expression zeigte, dass die L451F Mutante eine gleichen Expressionlevel wie der Wildtyp besaß, das gebildete Protein komplett inaktiv war. Darüber hinaus zeigt die Analyse unseres 3-D Computermodels einen sterischen Effekt in der unmittelbaren Nachbarschaft des Häms auf, die den beobachteten Verlust an Aktivität erklärt. Diese Ergebnisse weisen darauf hin, dass die

L451F Mutation die Enzymaktivität des CYP11B2 komplett unterdrückt. Dies stimmt mit dem in der GC-Ms gewonnenen Profil der Harnsteroide überein.

Patient Nr.2 war heterozygot. Ein Allel besaß eine Nucleotiddeletion bei R490 (R490Δ1nt), die einen Frame-Shift verursachte und 170 Aminosäuren zu dem Protein hinzufügte. Auf dem anderen Allel besaß das *CYP11B2* Gen im letzten Nucleotid des Exon 5 eine stille Mutation. Die Expressionstudien zeigten, dass R490Δ1nt die Enzymaktivität des CYP11B2 komplett unterdrückte. Die Substitution des Nucleotids G durch A als letztes Nucleotide des Exon 5 beeinflusste das Pre-mRNA Spleißen. Obwohl es sich um einen stille Mutation handelte , kam es zu einer Retention im Intron 5 (812 bp). Die genetischen und *in vitro* enzymatischen Analysen der beiden Mutanten legt eine CMO I Defizienz nahe. Allerdings ist das Profil der Blutsteroide nicht eindeutig und präsentiert eher eine Zwischenform aus CMO I und CMO II, denn das klassische CMO I.

Patient Nr. 3 war heterozygot und besaß zwei neuartige Mutationen, S315R und R374W, in dem *CYP11B2* Gen. Die S315R und R374W Mutanten waren enzymatisch inaktiv, d.h. sie waren nicht in der Lage, DOC in Aldo umzuwandeln. Zusätzlich zeigte die Analyse des Computermodells Wasserstoffbrückenbindungen sowohl von R315 als auch von W374 und somit die Bildung eines neuen Wasserstoffbrückennetzwerks der CYP11B2-Mutanten im Vergleich zum CYP11B2-WT. Somit ist die Kombination aus *in vitro* Enzymeassay und Computermodellierung des Wasserstoffbrückennetzwerks ein ausgezeichnetes Hilsmittel zum besseren Verständnis der klinischen Daten der Aldosteronsynthasedefizienz.

Patient Nr. 4 war heterozygot. Es gab eine Missens-Mutation, R181Q, auf einem Allel des *CYP11B2* Gens. Die Proteinexpression der CYP11B2-R181Q in COS-1 Zellen zeigt, dass diese Mutation die Bildung von Corticosteron steigerte, die von 18-Hydroxycorticosteron reduzierte und die von Aldosteron unterdrückte. Dieses Ergebnis stimmt mit dem mittels GC-MS gewonnen Profil der Harnsteroide überein. Allerdings konnte bis jetzt keine Mutation auf dem zweiten Allel gefunden werden, so dass im Moment der Phenotyp-Genotyp Beziehung noch nicht eindeutig ist.
Patient Nr. 5 war einer von zwei Geschwistern mit einem 46,XX Karyotyp und vollständiger Virilizierung. Dieser Patient mit einer Defizienz der 11β-Hydroxylase besaß eine homozygote L299P Mutation im *CYP11B1* Gen. Die Expression der L299P-Mutante in HCT116 $p53^{-/-}$ Zellen zeigte, dass L299P die 11-Hydroxylaseaktivität im Vergleich zum Wildtyp auf einen Wert zwischen 0,8 und 1,6 % reduzierte.

B) Version in English

The corticosteroids cortisol and aldosterone are synthesized in the adrenal cortex by a complex reaction cascade, catalyzed by several cytochromes P450 (CYP). CYPs belong to the monooxygenases that introduce molecular oxygen into their substrates. In humans the final steps of the cortisol and aldosterone synthesis are catalyzed by CYP11B1 and CYP11B2, respectively. CYP11B1 catalyzing the steroid 11β-hydroxylation is expressed at a high level in zona fasciculata/reticularis of the adrenal cortex and is regulated by ACTH. CYP11B1 is responsible for the conversion of 11-deoxycortisol (RSS) to cortisol. The deficiency of 11β-hydroxylase, besides 21-hydroxylase deficiency, is the second most common cause of congenital adrenal hyperplasia (CAH), an inherited disease with the inability to synthesize cortisol from 11-deoxycortisol. In contrast, CYP11B2, aldosterone synthase, is expressed in the zona glomerulosa and is mainly regulated by serum levels of angiotensin II as well as potassium. CYP11B2 catalyzes the conversion of deoxycorticosterone (DOC) to aldosterone, a process that requires three steps: a hydroxylation at position 11β to form corticosterone (B), and another one at position 18 to form 18-hydroxycorticosterone (18OHB), and finally an oxidation at position 18 to form aldosterone (Aldo). Aldosterone synthase deficiency usually finds its expression in infancy as a life-threatening electrolyte imbalance, caused by mutations in the *CYP11B2* gene. However, CAH and aldosterone synthase deficiency caused by the mutations of *CYP11B1* gene and *CYP11B2,* respectively, have been poorly investigated at molecular level. Therefore, in depth study of mutations and their enzymatic activities will provide information for the diagnosis and management of CAH and hypoaldosteronism caused by CYP11B1 and CYP11B2 deficiencies.

The aim of the present study was:

1) To detect mutations in the *CYP11B2* and *CYP11B1* genes of patients exhibiting disordered adrenal steroids (mineralocorticoids and glucocorticoids).

2) To investigate the effects of these mutations on the steroid biosynthesis by expressing the mutant cDNAs in cell cultures, which includes the comparison of monkey kidney COS-1 and human colonic carcinoma HCT116 cells as heterologous expression systems suitable for the study of CYP11B1 and CYP11B2.

In order to investigate mutations in patients suffering aldosterone synthase deficiency and patients with CAH of cortisol deficiency, *CYP11B1* and *CYP11B2* genes of the patients and their parents were amplified by PCR of genomic DNA. All 9 exons and the boundaries of exon/intron were analyzed by automated sequencing. Six new mutations (L451F, S315R, R374W, R490Δ1, R181Q, and a silent mutation in the last nucleotide of exon 5) were detected from the four

patients with aldosterone synthase deficiency. One mutation (L299P) was found before in the patient with CAH of cortisol deficiency. To analyze the consequences of the mutant of CYP11B1 in cortisol synthesis and the mutants of CYP11B2 in aldosterone synthesis, mutations were generated using the expression plasmid constructs pSVLhCYP11B1/11B2 by site-directed mutagenesis. The enzymatic activity of mutants was determined in COS-1 cells or HCT116 $p53^{-/-}$ cells by co-expression with bovine adrenodoxin using the plasmid pbAdx. The expression of the mutant proteins in the cell lines was analyzed by Western blots.

To optimize the analytical method for measuring the activities of gene products of CYP11B1 and CYP11B2, HCT116 $p53^{-/-}$ cell lines were compared for their activities of CYP11B1 and CYP11B2 with and without cotransfection of bovine or human adrenodoxin, an electron donor to P450. Although both human and bovine adrenodoxin increased the products from CYP11B1 and CYP11B2, bovine adrenodoxin was more effective than the human one. In comparison of COS-1 and HCT116 $p53^{-/-}$ cells, both cell lines cotransfected with CYP11B and adrenodoxin convert the substrates with a similar efficiency, suggesting that the efficiencies of 1) transfection, 2) transportation of precursor proteins synthesized in cytoplasm to mitochondria, 3) electron transportation from NADPH to P450 in mitochondria, and 4) substrate permeability, are similar in both monkey kidney and human colonic cell lines. The product pattern is not dependent on the cell line. Therefore, we concluded that both cell lines are similarly useful for the activity assay of CYP11B1 and CYP11B2.

When analyzing the patients, it was demonstrated that patient 1 was homozygous having a novel missense mutant (L451F) in the *CYP11B2* gene. Upon the heterologous expression experiments, the L451F mutant showed an expression level comparable with the wild type but the protein was completely inactive. Furthermore, the analysis of our 3-D computer model indicated a steric effect in the immediate vicinity of the heme which explains the observed loss in activity. These results demonstrated that in the L451F mutant the enzyme activity of CYP11B2 is completely abolished, being in perfect agreement with the urinary steroid profile by GC-MS.

The patient 2 was heterozygous. One allele contained a nucleotide deletion at R490 (R490Δ1nt) that causes a frame-shift adding 170 amino acids to the protein. In the other allele, the *CYP11B2* gene had a silent mutation in the last nucleotide of exon 5. The expression studies indicated that R490Δ1nt mutant completely abolished the enzyme activity of CYP11B2. The substitution of nucleotide from G to A at the last nucleotide of exon 5 affected pre-mRNA splicing although it was a silent mutation which resulted in the intron 5 retention (812 bp). The genetic and *in vitro* enzymatic analyses of the two mutants clearly suggest CMO I deficiency.

However, the steroid spectrum in the blood of the patient present an intermediate form between CMO I and CMO II rather than the classical CMO I.

The patient 3 was heterozygous having two novel mutations, S315R and R374W, in the *CYP11B2* gene. The S315R and R374W mutants were enzymatically inactive, i.e. not capable of converting DOC to Aldo. In addition, the analysis using computer modelling of hydrogen bonds of R315 and W374 demonstrated the formation of a new hydrogen bond network of CYP11B2 in the mutants compared to CYP11B2-WT. Thus, the combination of *in vitro* enzyme assay and computer modeling of the hydrogen network provides a valuable tool for better understanding of the clinical data of aldosterone synthase deficiency.

The patient 4 was heterozygous. There was one missense mutation R181Q in the *CYP11B2* gene of one allele. The protein expression of CYP11B2-R181Q in COS-1 cells indicated that this mutant increased corticosterone, reduced 18-hydroxycorticosterone and abolished aldosterone formation. This result is in agreement with the urinary steroid profile obtained by GC-MS. However, so far no mutation has been found in the second allele so that the phenotype-genotype correlation is not clear at the moment.

The patient 5 was one of two siblings having the 46,XX karyotype with complete virilization. This patient with 11β-hydroxylase deficiency had a homozygous L299P mutation in the *CYP11B1* gene. The expression of the mutant L299P in HCT116 $p53^{-/-}$ cells showed that L299P mutation reduces 11-hydroxylase activity to 1.6 - 0.8% for the conversion of 11-deoxycortisol to cortisol.

Abstract

The overall goal of this work consisted in investigations of the genotype of patients suffering from aldosterone synthase deficiency and patients with cortisol synthase disorders on the biosynthesis of steroid hormones. The aim of the first part of this work was to detect mutations in genomic DNA of patients with aldosterone synthase deficiency, and cortisol synthase disorders. Sequence analysis of the CYP11B1/CYP11B2 gene revealed that six new mutations were detected from the four patients with aldosterone synthase deficiency; one mutation was found before in the patient with CAH of cortisol deficiency.

The second part of this work consisted in analyzing effects of detected mutations of CYP11Bs by expressing the mutant proteins in the COS-1 and HCT116 cells. The combination of the functional analysis of the enzyme in the cell culture and 3-D computer model study may explain phenotypical characteristics of the patient.

Kurze Zusammenfassung

Das Gesamtziel dieser Arbeit bestand in der Erforschung des Genotyps sowohl von Patienten, die an Aldosteronsynthasedefizienz erkrankt sind, als auch von Patienten, die unter Störungen der Cortisolsynthase während der Biosynthese der Steroidhormone leiden. Im ersten Teil dieser Arbeit wurden die Mutationen in der genomische DNA von beiden Patientengruppen bestimmt. Die Sequenzanalyse des CYP11B1/CYP11B2 Gens zeigten, dass insgesamt sechs neue Mutationen bei den vier Patienten mit Aldosteronsynthase Defizienz auftraten; eine Mutation wurde bereits bei dem Patienten mit CAH der Cortisoldefizienz gefunden.

Der zweite Teil dieser Arbeit umfasst die Analyse der gefundenen Mutationen. Dafür wurden die CYP11B-Mutanten in COS-1 und HCT116 Zellen expremiert und auf veränderte funktionelle Eigenschaften hin untersucht. Die Kombination aus Zellkulturexperimenten und dem 3-D Computermodell könnte zur Klärung der phenotypische Charakteristika der Patienten führen.

1. Introduction

1. 1 Biosynthesis of the steroid hormones

Steroid hormone research began with crystallization of sex steroid hormones in about 1929-1935, the glucocorticoids in 1935-1938, and finally of aldosterone in 1953. The adrenal cortex produces a complex array of steroid hormones including glucocorticoids, mineralocorticoids, androgens, and estrogens. This gland surrounds the adrenal medulla and consists of different regions that produce different steroids. Just below the capsule of the adrenal lies the region of the adrenal cortex named the glomerulosa in which the major mineralocorticoid, aldosterone is produced. Between the glomerulosa and the adrenal medulla lie two regions of the cortex called the fasiculata and the reticularis that are required for the synthasis of glucocorticoids and the adrenal androgens (see Figure 1.1). Steroid hormones are produced in multi-step pathways that involve the participation of up to six P450s (see Figure 1.2): CYP11A1 (cholesterol side chain cleavage cytochrome P450 or P450scc), CYP17 (17α-hydroxylase/17,20-lyase or P450c17), CYP21 (21-hydroxylase cytochrome P450 or P450c21), CYP11B1 (11β-hydroxylase or P45011β), CYP11B2 (aldosterone synthase or P450aldo) and CYP19 (aromatase or P450arom) (Bernhardt 1996; Bureik et al. 2002; Hakki et al. 2006; Lisurek et al. 2004).

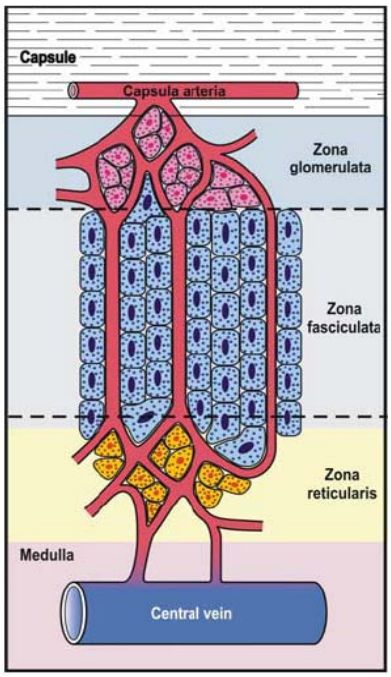


Figure 1.1 Zones in adrenal gland. The outmost hard connective tissue (white) is covering the zona glomerulosa (light blue), the products are mineralocorticoids specially aldosterone. The zona fasciculata (gray) is the most prominent area, and produces glucocorticoids, importantly cortisol. The inner zone of adrenal cortex is the zona reticularis (light yellow) that produces sex steroids, specially testosterone and estradiol. Just beyond the zona reticularis, the medulla starts (Asif 2004).

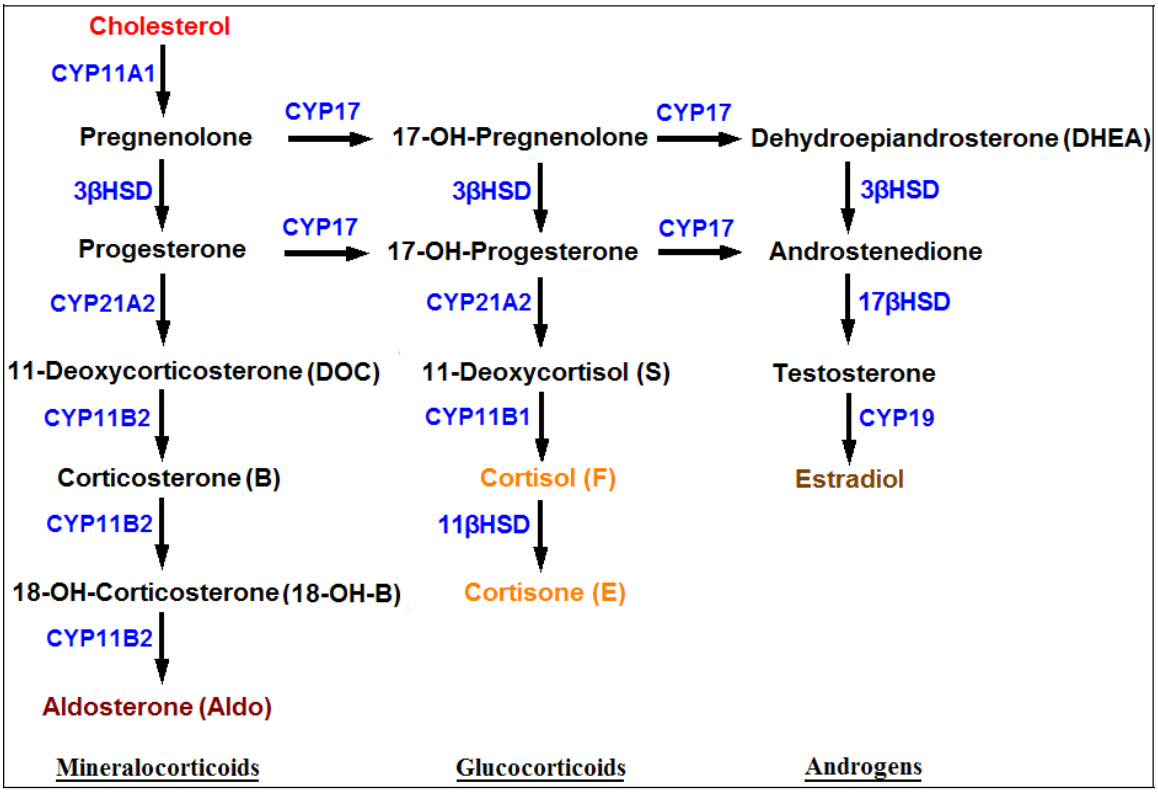


Figure 1.2 Principal pathways of human adrenal steroidogenesis (Ghulam et al. 2003).

The precursor of steroid hormones is cholesterol, which is a 27-carbon steroid. The cells of the steroidogenic tissues can *de novo* synthesize cholesterol from acetate, mobilize the intracellular cholesterol ester pools, or import lipoprotein cholesterol from the plasma. Cholesterol is stored as cholesterol acetate in neutral lipid droplets, which serves as a pool of readily available cholesterol for corticosteroid biosynthesis (Vinson et al. 1992). About 80% of cholesterol is usually provided by circulating plasma lipoproteins as low-density lipoproteins (Gwynne et al. 1982). Cholesterol is converted to steroid hormone intermediates and mature hormones by cytochrome P450 enzymes in the mitochondria and smooth endoplasmic reticulum. Synthesis begins in the mitochondria, continues in the endoplasmic reticulum, and is completed in the mitochondria. Therefore, shuttling of steroid hormone precursors between the mitochondria and cytoplasmic compartments is important in the multiple steps of hormone synthesis.

The rate-limiting step in the steroidogenesis is the cholesterol transport across the outer to the inner mitochondrial membranes and the CYP11A1 (20, 22 R-hydroxylase cholesterol

side-chain cleavage) active site. For acute steroid biosynthesis, cholesterol has to be mobilized and delivered from the lipid droplets to the CYP11A1 active site, which is associated with the inner mitochondrial membrane. The protein factor responsible for this transport, and as such regulating the acute production of steroids, has been identified and named steroidogenic acute regulatory protein (StAR) (Zenkert et al. 2000). Pregnenolone then passes from mitochondria to the endoplasmic reticulum for further metabolism. On the one hand, it can be directly converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD). Alternatively it can be hydroxylated at the 17α-position by 17α-hydroxylase (CYP17) to produce 17α-hydroxypregnenolone. 17α-hydroxypregnenolone can be converted to 17α-hydroxyprogestrone by 3βHSD or to a C19 steroid, dehydroepiandrosterone, by the 17,20 lyase activity of CYP17. Dehydroepiandrosterone (DHEA) can also be converted by 3βHSD to androstenedione, which serves as precursor of sex hormones. Progesterone or 17α-OH-pregnenolone can be hydroxylated at the 21-position by 21-hydroxylase (CYP21A2), producing 11-deoxycorticosterone and 11-deoxycortisol, respectively. The products of CYP21A2 must re-enter the mitochondria, where the final steps of steroidogenesis in the adrenal cortex occur. The two isoforms of CYP11B; 11β-hydroxylase/aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1), catalyze the conversion of deoxycorticosterone and 11-deoxycortisol to the glucocorticoids; corticosterone and cortisol respectively. On the other hand, the outer zone of the adrenal cortex, the zona glomerulosa, produces the potent mineralcorticoid aldosterone from deoxycorticosterone by the function of CYP11B2. The enzymatic difference between the zona glomerulosa and the zona fasciculata is the absence of CYP17 in zona glomerulosa. The glomerulosa cells predominantly express CYP11B2. In zona reticularis, androstenedione is converted to testosterone and estrone/estradiol by 17-ketosteroid reductase (17βHSD) and 19-hydroxylase (CYP19), respectively. Cortisol is produced in greater amounts compared to corticosterone in humans and represents approximately 80% of the glucocorticoids. The androgens, DHEA and androstenedione, produced by the zona reticularis can be metabolized to testosterone or estrogens by the cortical cells themselves or by metabolic pathways in other organs, such as the gonads. Species that produce predominantly corticosterone (such as rats and mice) have little sex

hormone production by the adrenal glands (Harvey 1996; Kroboth et al. 1999; Rainey et al. 2002; Wilson et al. 1992).

1.2 Cytochrome P450s

1.2.1 General aspects

Cytochrome P450 enzymes represent a superfamily of b-type hemoproteins i.e. containing a noncovalentely bound heme group which are found in almost all life forms. From an evolutionary point of view it seems like all cytochromes P450 have evolved from a common ancestor molecule (Nebert et al. 1989). The name of these enzymes is derived from their unusual spectral properties displaying a typical absorption maximum of the reduced CO-bound complex at 450 nm: cytochrome stands for a hemoprotein, P for pigment and 450 reflects the absorption peak of the CO complex at 450 nm. The ability of reduced P450 to produce an absorption peak at 450 nm upon CO binding is still used for the estimation of the P450 content (Omura et al. 1964). The red shift of about 30 nm as observed in cytochromes P450 indicates that the distribution of electron density at the heme is significantly perturbed as compared to other cytochromes. It has been documented that the cause of red shift is the thiolate sulphur that directly binds to the heme iron.

Cytochromes P450 are ubiquitously distributed enzymes that are able to metabolize a variety of different substrates. The field of activity of these enzymes includes many different reactions such as hydroxylation, N-, O- and S-dealkylation, sulfoxidation, epoxidation, deamination and N-oxide reduction (Bernhardt 1996; Ruckpaul 1993). For instance, cytochrome P450 plays an important role in the metabolism of many distinct drugs, carcinogens, alkaloids, pesticides and other important xenobiotics (Bernhardt 1996; Bernhardt 2006). Additionally, these proteins are involved in a variety of physiological processes such as the steroid hormone, vitamin D and bile acid biosyntheses. Taking this into account, it is not surprising that these enzymes have attracted the attention of different research fields such as biochemistry, pharmacology, physiology, organic chemistry and biotechnology (Bernhardt 1996). In 1991, efforts to establish a uniform classification of these enzymes were undertaken by Nebert (Nebert et

al. 1991). This systematic arrangement divides the P450 superfamily according to their sequence similarity into families, subfamilies and finally into the individual species. More than 6000 different P450 genes have been cloned up to date from animals, plants, fungi, other eukaryotes, and bacteria (for details see: http://drnelson.utmem.edu/CytochromeP450.html). Species belonging to the same family usually possess a sequence similarity of > 40 % whereas members of the same subfamilies are > 55 % identical (Nebert & Nelson 1991). P450s from different families usually display a sequence identity below 30%, which in part reflects the high number of different substrates of these enzymes. This classification resulted in a nomenclature for all cytochromes in which CYP stands for cytochrome P450 followed by the number for the respective family, a letter referring to the subfamily and finally a number that identifies the individual member.

All cytochromes P450 are monooxygenases that catalyze the incorporation of a single atom of molecular oxygen into the substrate. The reduction equivalents needed for this reaction are provided by an external substrate that is the reason why these enzymes are called external monooxygenases (Hayaishi et al. 1969). In general, cytochromes P450 catalyze the following reaction as shown below in the simplified reaction scheme:

$$\mathbf{RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+}$$

Cytochromes P450 are generally divided into two major classes: the microsomal type and the bacterial/mitochondrial type cytochromes (see Figure 1.3) (Bernhardt 2006; Harikrishna et al. 1993). In the case of microsomal type P450s the reduction equivalents are transferred from a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytochrome P450 reductase to the cytochrome. The P450 reductase contains a flavine adenine dinucleotide (FAD) as well as a flavine mononucleotide (FMN) group. Both enzymes are membrane bound (Bernhardt 1996; Bernhardt 2006). This class of cytochromes is mainly responsible for the metabolism of drugs and xenobiotics (Ruckpaul 1990). The second class of cytochromes P450, namely the mitochondrial and most of the bacterial cytochromes P450, require an additional electron carrier protein. In these systems reduction equivalents are provided to the cytochrome via an electron

transfer chain that consists of a FAD-containing reductase (AdR) and a soluble iron-sulfur protein named adrenodoxin (Adx). AdR is associated with the inner mitochondrial membrane (Bernhardt 1996; Bernhardt 2006). In addition to the two typical electron transfer pathways, Hannemann et al. (Hannemann et al. 2007) have also described other electron transfer pathways, for example, pathways mediated by fusion proteins of the electron transfer components.

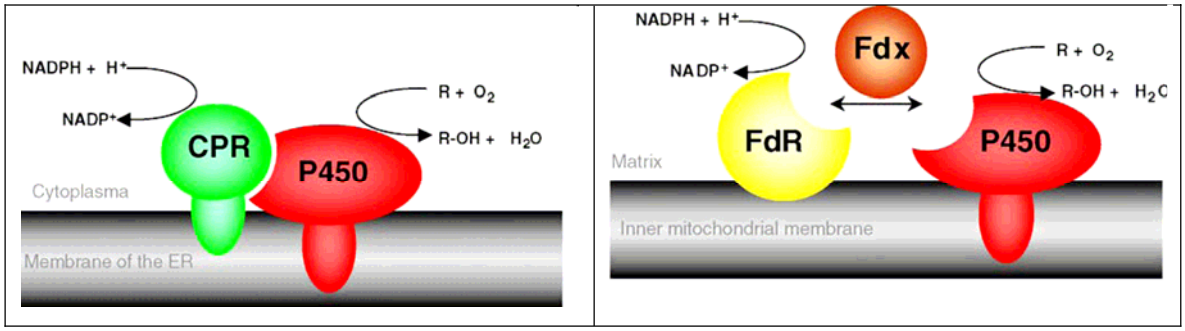


Figure 1.3: Schematic organisation of different cytochrome P450 systems (Bernhardt 2006)**.**

Poulos and coworker (Poulos et al. 1987) described a crystal structure of $P450_{cam}$ (CYP101) from *Pseudomonas putida*. In the early 1990s, the structures of P450s have been determined, namely, $P450_{BM3}$ (CYP102) (Ravichandran et al. 1993), $P450_{terp}$ (CYP108) (Hasemann et al. 1994), $P450_{eryF}$ (CYP107) (Cupp-Vickery et al. 1995) and $P450_{nor}$ (CYP55) (Nakahara et al. 1994). Until now crystallographers have been able to solve the structure of many bacterial P450s and recently of solubilized microsomal cytochromes P450, CYP2C5 (Wester et al. 2003), CYP2C8 (Schoch et al. 2004), CYP2C9 (Williams et al. 2003), CYP2B4, CYP2A6, CYP2D6, CYP1A2, and CYP3A4 (Williams et al. 2004a) (for more information about the resolved 3-dimensional structures of CYP450 visit: http://www.expasy.org/). Analysis of these structures has revealed that even if the sequence identities between the different cytochromes P450 tends to be very low, all cytochromes display a characteristic overall fold and topology. In general, P450s exhibit an interesting folding pattern with respect to how the N-terminal and C-terminal sequences are arranged around the heme. The conserved P450 structural core consists of

six-helices: D, E, I, L, J and K. There are two sets of structurally conserved β sheets: β sheet 1 containing five strands and β sheet 2 containing two strands. Heme is located between helix I and L (Peterson et al. 1998). Based on the alignment of CYP2C family members, Gotoh (Gotoh 1992) proposed 6 regions that are involved in substrate recognition, designated SRS (substrate recognition sites). There is spatial hypervariability in SRS-1 (B' helix), SRS-2 (carboxyterminal region of the F helix), and SRS-3 (amino-terminal region of the G helix). In contrast, SRS-4 (central I helix), SRS-5 (β6-1/β6-4), and SRS-6 (β4-hairpin) show only limited spatial variability. In addition, consensus sequence motifs include the well-recognized EXXR motif at the C-terminal end of helix K, the CXGXXLA motif in the Cys-pocket and the AGXXT motif in helix I, comprising some of the conserved residues among most CYPs, and the DXXXF motif in helix K' (Mestres 2005).

1.2. 2 CYP11B1 and CYP11B2

1.2.2.1 Structure and function

CYP11B1 and CYP11B2 (P450c11B1 and P450c11B2) are located in the inner mitochondrial membrane. In humans, the CYP11B family contains two members, CYP11B1 and CYP11B2, producing cortisol and aldosterone, respectively. Cortisol is the main glucocorticoid in humans. It regulates energy mobilization and thus the stress response. Furthermore, cortisol is formed by 11β-hydroxylation of 11-deoxycortisol (S) (see Figure 1.4) and is normally secreted 100 to 1000-fold in excess over aldosterone. Aldosterone is the most important human mineralocorticoid. It is involved in the regulation of the salt and water household of the body and thus in the regulation of blood pressure. The terminal 3 steps in aldosterone biogenesis in humans are the 11β-hydroxylation of 11-deoxycorticosterone (DOC) that leads to corticosterone (B), which is then 18-hydroxylated to yield 18-hydroxycorticosterone (18-OH-B) and finally oxidized to aldosterone (see Figure 1.4). In the course of cloning and analyzing the *CYP11B1* gene, White and coworkers isolated a cross-hybridizing gene, *CYP11B2* (Mornet et al. 1989), whose sequence was about 95% identical to the well-known *CYP11B1* gene in

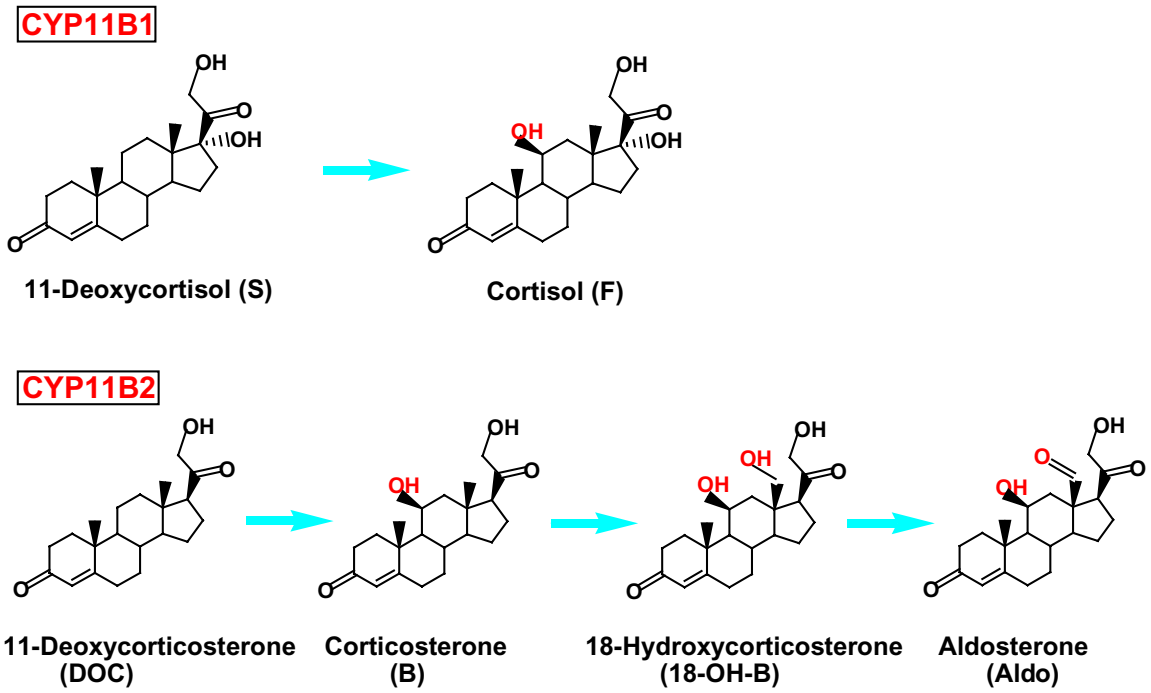


Figure 1.4: Reactions catalyzed by human CYP11B1 and CYP11B2. CYP11B1 catalyzes the 11β-hydroxylation reaction that produces cortisol from 11-deoxycortisol (S). CYP11B2 converts 11-deoxycorticosterone (DOC) via corticosterone (B) and 18-OH-corticosterone (18-OH-B) to aldosterone (Aldo).

coding regions and 90% identical in introns. The proteins are synthesized in cytoplasm and transported into the inner mitochondrial membrane and thus are synthesized including a leader sequence of 24 amino acids that is cleaved in the mitochondria to yield a mature protein of 479 amino acids in humans and 476 amino acids in mice. Although CYP11B1 and CYP11B2 consist of the same number of amino acids, the apparent molecular mass of the human enzymes was reported as 51 and 49 kDa on SDS-PAGE, respectively (White et al. 1994a), and the rat enzymes as 51.5 and 49.5 kDa, respectively (Ogishima et al. 1991). The 5′ upstream region of the CYP11B2 gene had considerably diverged from that of CYP11B1, suggesting that this second gene, if expressed, may be regulated differently. Mornet et al (Mornet et al. 1989) determined that the *CYP11B1* and *CYP11B2* genes both contain nine exons. Both genes are located on chromosome 8q21. CYP11B enzymes of other species have also been studied. In bovine (Wada et al. 1985),

porcine (Yanagibashi et al. 1986), and frog (Nonaka et al. 1995) adrenal cortexes, synthesis of gluco- and mineralocorticoids is catalyzed by single enzyme, while humans, baboon (Hampf et al. 1996; Swart et al. 2000) rats (Matsukawa et al. 1990), mice (Domalik et al. 1991), and guinea pigs (Bulow et al. 2002; Bulow et al. 1996) contain two distinct isoforms specialized in the formation of either mineralo- or glucocorticoids. The reason for these interspecies differences is unknown. Enzymes with 11β-hydroxylase activity have also been found in several fungi (Megges et al. 1990).

Understanding the structure-function relationships of CYP11B enzymes requires information about their three-dimensional structure. Protein structure determination by X-ray diffraction is often problematic in case of membrane-bound proteins such as CYP11B1 and CYP11B2, and nuclear magnetic resonance (NMR) structure determination is restricted to smaller proteins (see Figure 1.5 A). Models have been evaluated and used to explain the significance of a number of residues that were identified either by mutagenesis studies or mutations found in patients (Belkina et al. 2001). These models suggest that the main difference between the two proteins is the position of the heme (see Figure 1.5 B). An angle of approximately 20° between the hemes of the two models has been proposed, apparently dependent on the interaction of side-chains forming the heme environment and the orientation of its binding loop. In case of CYP11B1, one heme propionate group forms a hydrogen bond with Arg448 while the second one interacts with Arg384, whereas in CYP11B2 both heme propionate groups are involved in hydrogen bond interaction with Arg448. Both Arg448 and Arg384 mutations have been found in CYP11B1 of patients suffering from congenital adrenal hyperplasia (CAH) (Curnow et al. 1993; Nakagawa et al. 1995; White et al. 1991); all known mutations in positions 384 and 448 led to a complete loss of enzyme activity, most probably due to destabilization of the holoprotein. As a consequence of the different hydrogen bonding network around Arg384, Arg448, and the heme propionates, the active site of CYP11B2 is predicted to be smaller than that of CYP11B1. Besides, the models of Ulmshneider et al. (Ulmschneider et al. 2005) focus on describing protein-inhibitor interactions and structure activity relations of their developed inhibitors. Furthermore, the models of the CYP11B family of Roumen et al. (Roumen et al. 2007) provide insights

into the regioselectivity of the natural ligands within the enzymes and to protein-ligand interactions.

A **B**

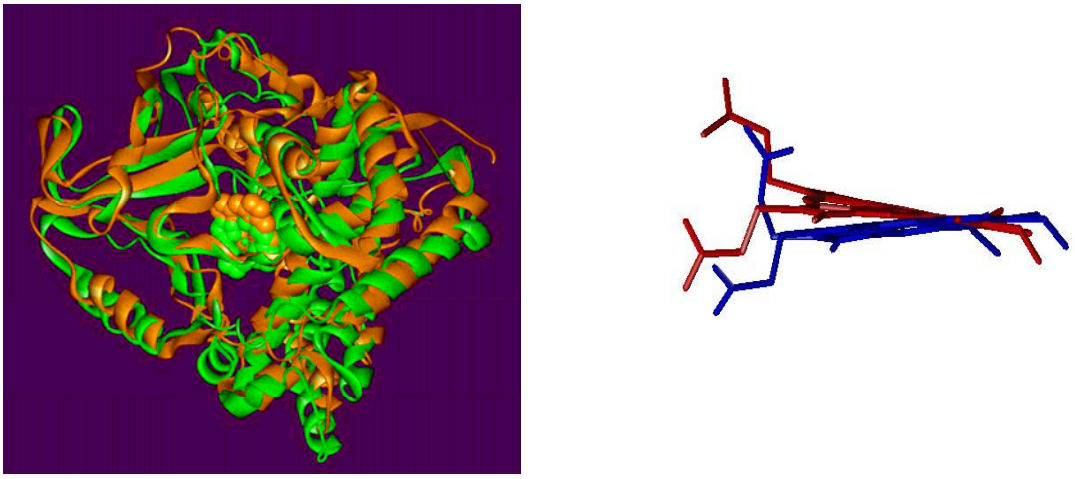


Figure 1.5: A, Superposition of the ribbon structures of the homology models of human CYP11B1 (green) and CYP11B2 (orange) (Belkina et al. 2001). **B**, the main difference between the two proteins is the position of the heme.

1.2.2.2 Regulation of steroid hydroxylase

The regulation of adrenocorticosteroid synthesis involves the hypothalamus and anterior pituitary (see Figure 1.6). The hypothalamus releases corticosteroid releasing hormone (CRH) into the portal blood, which goes to the anterior pituitary gland. CRH stimulates the anterior pituitary to release adrenocorticotropin (ACTH) into the systemic circulation. ACTH stimulates the glands of the adrenal cortex to convert cholesterol to pregnenolone. Pregnenolone then forms glucocorticoids and sex hormones in the zona reticularis and zona fasciculata and aldosterone in the zona glomerulosa. In addition, angiotensin II and high K^+ stimulate the aldosterone synthase (CYP11B2) in the zona glomerulosa to form aldosterone. With negative feedback, glucocorticoids and androgens inhibit both the release and action of CRH in the hypothalamus, and the formation and release of ACTH in the anterior pituitary. Aldosterone does not inhibit the release of ACTH or CRH with the negative feedback.

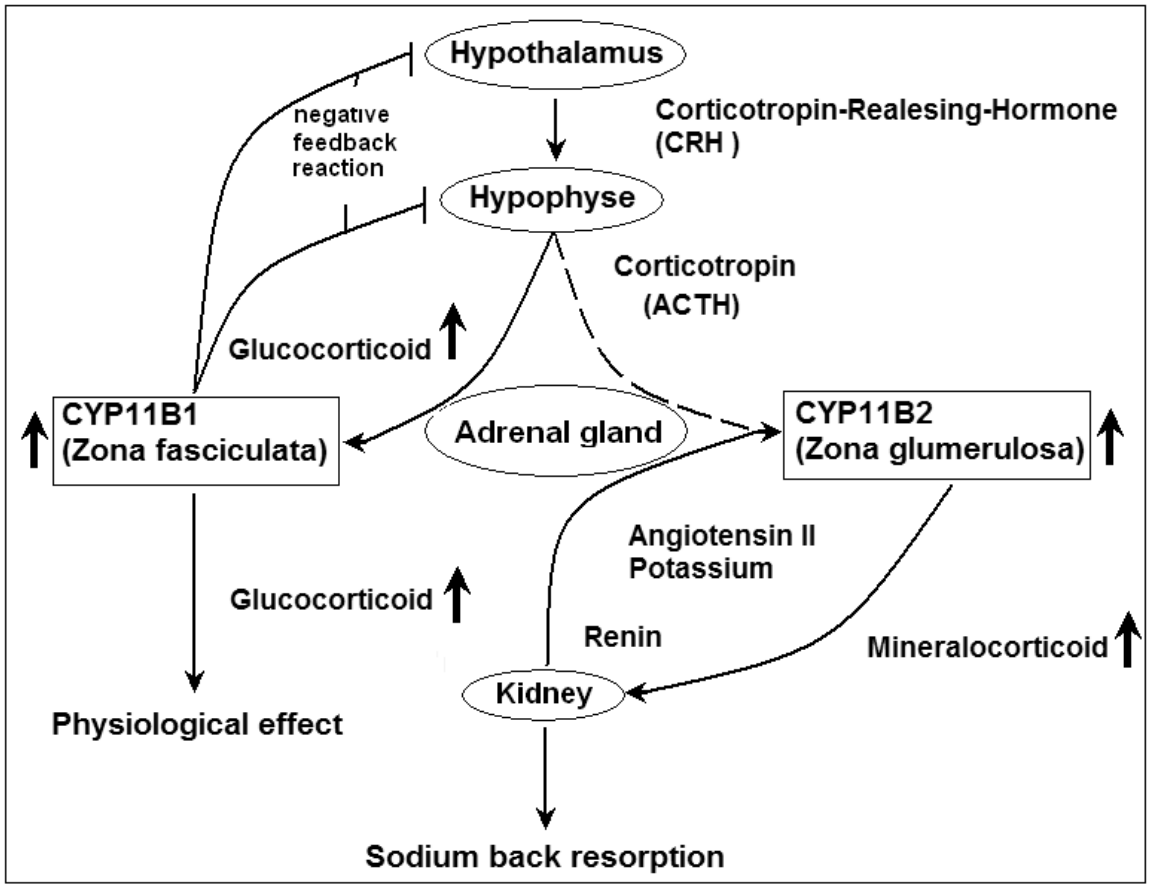


Figure 1.6: Regulation of 11-hydroxylase and aldosterone synthase (Hampf 2001a).

Cortisol synthesis is primarily controlled by ACTH (corticotropin) (Waterman et al. 1989). ACTH acts through a specific G protein-coupled receptor on the surface of cells of the adrenal cortex (Mountjoy et al. 1992), to increase levels of cAMP (adenosine 3', 5' monophosphate). Cyclic AMP has short-term (minutes to hours) effects on transport of cholesterol into mitochondria through increasing the synthesis of a short lived protein, steroidogenic acute regulatory (StAR) protein (Stocco et al. 1996). The increased level of intracellular cAMP also has longer term (hours to days) effects on transcription of genes encoding the enzymes required for cortisol biosynthesis including CYP11B1 (Waterman et al. 1997), and preferentially increases CYP11B1 mRNA expression over that of CYP11B2 (Curnow et al. 1991; Denner et al. 1996).

StAR is found to play a key or even essential role in mediating transport of cholesterol from outside the outer mitochondrial membrane into the inner mitochondrial membrane where cholesterol can enter the active site of CYP11A1 and be converted to

pregnenolone (Baker et al. 2005; Miller et al. 1999). A cyclic AMP response-element binding protein (CREB) is responsible for the protein kinase A (PKA) mediated response between ACTH and elevated StAR levels (Manna et al. 2002). The biophysical basis by which StAR stimulates cholesterol transport remains unclear, however, two important findings provide insight into this process. Mutagenesis studies of StAR suggest that StAR activity requires a pH-dependent protein globule transition on the outer mitochondrial membrane (Baker et al. 2005). Further, the peripheral-type benzodiazepine receptor (PBR) interacts with StAR on the outer mitochondrial membrane to facilitate cholesterol transfer across this membrane to the inner mitochondrial membrane, and then to CYP11A1 (Hauet et al. 2005).

Aldosterone synthase (CYP11B2) expressed in the zona glomerulosa is regulated by angiotensin II and potassium, with ACTH having mostly a short-term effect on expression (White et al. 2005). Because the necessary precursors for aldosterone biosynthesis (in particular, deoxycorticosterone) are synthesized in the much larger zona fasciculata, it is apparent that there must be uniquely regulated steps in aldosterone biosynthesis in the zona glomerulosa or this process simply would be regulated by ACTH.

Angiotensin is an oligopeptide in the blood that causes vasoconstriction and sodium retention (Lavoie et al. 2003), increased blood pressure, and release of aldosterone from the adrenal cortex. It is derived from the precursor molecule angiotensinogen, which is a member of the serine protease inhibitor gene superfamily. The effects of angiotensin II can be inhibited by antagonists against type 1 angiotensin II (AT1) receptor (Kakiki et al. 1997). Angiotensin II receptors are predominantly expressed in the zona glomerulosa, suggesting a role of angiotensin II in the glomerulosa-specific expression of CYP11B2 (Breault et al. 1996).

Renin is a proteolytic enzyme secreted by the juxtaglomerular apparatus of the nephron in response to decreased volume as sensed by stretch receptors in the afferent arteriole. Renin digests angiotensinogen to angiotensin I, a decapeptide which is converted by the angiotensin converting enzyme to an octapeptide, angiotensin II.

Angiotensin II occupies a G protein-coupled receptor (Curnow et al. 1992; Murphy et al. 1991; Sasaki et al. 1991), activating phospholipase C.

Potassium is secreted into the tubule in exchange for the sodium, which is reabsorbed. Potassium signaling in glomerulosa cells involes membrane depolarization leading to an influx of calcium through T and L-type channels. Consistent with this, elevating intracellular calcium with the calcium channels agonist BAYK8644 increases expression of CYP11B2 mRNA in H295R adrenal cells. Moreover, calcium channel blockers such as nifedipine block K^+-dependent induction of CYP11B2 (Clyne et al. 1997; Denner et al. 1996; Pezzi et al. 1997).

1.3 Defect in CYP11B isozymes

1.3.1 Steroid 11β-hydroxylase deficiency

Congenital adrenal hyperplasia (CAH), the inherited inability to synthesize cortisol, usually presents with signs of androgen excess such as virilization of female external genitalia. More than 90% of cases are caused by 21-hydroxylase deficiency (Pang et al. 1988). This usually affects both aldosterone and cortisol biosynthesis, leading to signs of aldosterone deficiency including hyponatremia, hyperkalemia, and hypovolemia that may, if untreated, progress to shock and death within weeks after birth (see Figure 1.2). Most of CAH associated with hypertension are due to 11β-hydroxylase deficiency (White et al. 1994a). It has been estimated in most populations that about 5 - 8% of CAH cases are due to 11β-hydroxylase deficiency (Zachmann et al. 1983), which occurs in approximately 1 in 200,000 births (White et al. 1994a). Large numbers of cases of 11β-hydroxylase deficiency have been reported in Israel among Jewish immigrants from Morocco, a relatively inbred population. The incidence in this group is currently estimated to be l/5000-l/7000 births (Rosler et al. 1992).

Clinical and biochemical presentation of 11β-hydroxylase deficiency

As mentioned above, 11β-hydroxylase deficiency, besides 21-hydroxylase deficiency, is the second most common cause of CAH. It is an inherited disease with the inability to

synthesize cortisol from 11-deoxycortisol. Characteristically this disease leads to androgen excess and hypertension.

Hypertension is a common disorder that affects a large heterogeneous patient population. Subgroups can be identified on the basis of their responses to hormonal and biologic stimuli. These subgroups include low-renin hypertensives and nonmodulators. It has been estimated that approximately two thirds of patients with classic 11β-hydroxylase deficiency present with elevated blood pressure (Rosler et al. 1992), often beginning in the first few years of life (Mimouni et al. 1985; Zachmann et al. 1983). Mutations in CYP11B1 result in impaired activity of 11β-hydroxylase, leading to accumulation of the steroid precursors 11-deoxycortisol. In the result of the feed back from the lack of cortisol, this deficiency leads to mineralocorticoid hypertension. Although the hypertension is usually of mild to moderate severity, left ventricular hypertrophy and/or retinopathy have been observed in up to one-third of patients, and deaths from cerebrovascular accidents have been reported (Hague et al. 1983; Rosler et al. 1992). Other signs of mineralocorticoid excess such as hypokalemia and muscle weakness or cramping occur in a minority of patients and are not well correlated with blood pressure. Plasma renin activity is usually suppressed in older children and levels of aldosterone are consequently low even though the ability to synthesize aldosterone is actually unimpaired (White et al. 1994b).

For the androgen excess, females affected with classic 11β-hydroxylase deficiency are born with masculinization of their external genitalia. This is caused by secretion of adrenal androgens during embryonic and fetal development. In contrast to the external genitalia, the gonads and the internal genital structures are normal. Rapid somatic growth in childhood, accelerated skeletal maturation leading to premature closure of the epiphyses, and short adult stature are signs of postnatal androgen excess in both sexes. Additionally, affected children may have premature development of sexual and body hair and acne (Peter et al. 1999). Patients with nonclassic 11β-hydroxylase deficiency are born with normal genitalia and present with signs and symptoms of androgen excess as children. Adult women may present with hirsutism and

oligomenorrhoea. However, only a small percentage of women with hirsutism and hyperandrogenic oligomenorrhoea has nonclassic 11β-hydroxylase deficiency (Azziz et al. 1991; Carmina et al. 1988; Joehrer et al. 1997).

For biochemical presentation, in 11β-hydroxylase deficiency, 11-deoxycortisol and 11-deoxycorticosterone are not efficiently converted to cortisol and corticosterone respectively. Decreased cortisol production leads via poor feedback control to increased ACTH secretion. This stimulates the zona fasciculata to overproduction of steroid precursors prior to the blocked 11β-hydroxylase step. These precursor steroids are excreted in the urine as tetrahydro-metabolites, but the greater part of the massively elevated 11-deoxycortisol and its precursor 17-OH progesterone is shunted into the androgen pathway, resulting in marked androgen excess and virilization. Because 11-deoxycorticosterone and certain metabolites, e.g. 19-Nor-DOC, are mineralocorticoid agonists, plasma renin activity is suppressed and levels of aldosterone are low even though the ability to synthesize aldosterone is not impaired (Levine et al. 1980).

Genetic analysis of 11β-hydroxylase

Deficiency of 11β-hydroxylase is caused by mutations in CYP11B1 (see Figure 1.7). The first mutation described in Moroccan Jews patients with the classical form was a single base exchange in codon 448 leading to an amino acid substitution Arg448His. Arg448 is adjacent to Cys450, which is the fifth ligand of the heme iron atom (White et al. 1991). This probably represents a founder effect, but this mutation has also occurred independently in other ethnic groups, and another mutation of the same residue (R448C) has also been reported (Geley et al. 1996). Subsequently, more than 35 different mutations in the CYP11B1 have been identified (see Figure 1.7).

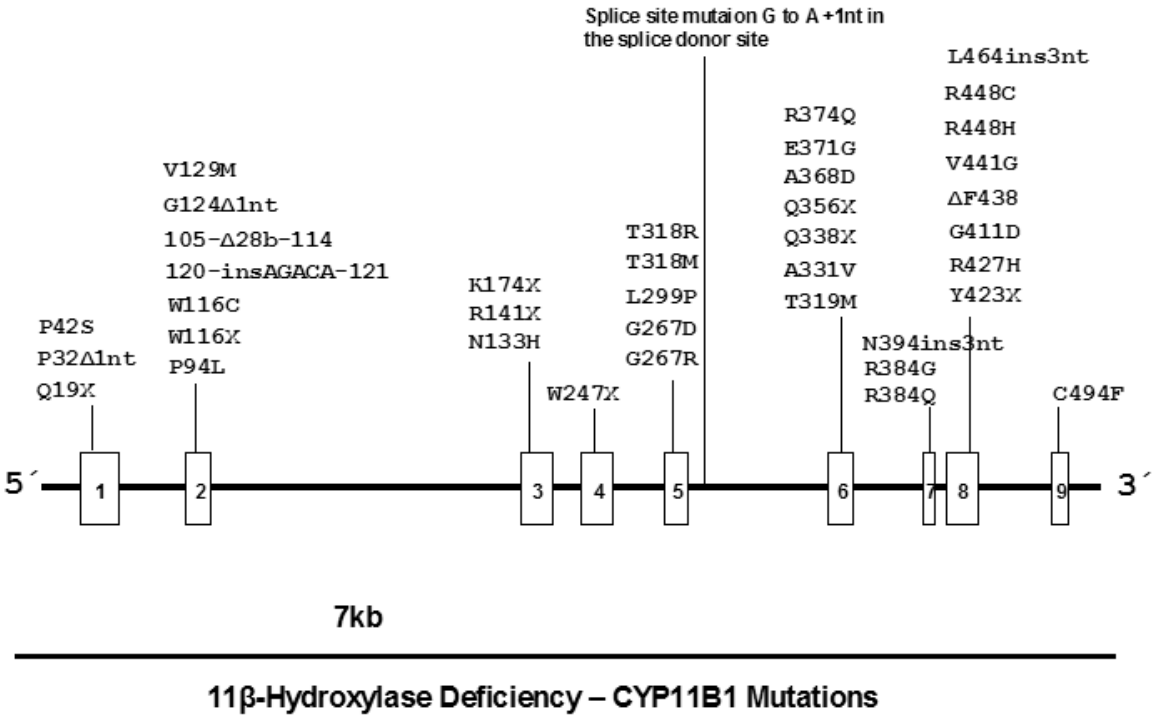


Figure 1.7: Schematic representation of the genomic structure of the human *CYP11B1* gene and positions of mutations reported to date. Exons are represented by boxes; and open boxes represent the non-coding regions.

Table 1.1. List of published missense mutations in the human *CYP11B1* gene leading to abolished 11β-hydroxylase activity in cell culture

Mutation	Ethnic group	Exon	Putative 3D	Effect in vitro	Reference
P42S	Not described	1	A-helix	15% reduced 11β-hydroxylase activity	Joehrer et al. 1997
P94L	North German	2	B-helix	0.05% reduced 11β-hydroxylase activity	Krone et al. 2006
W116C	Turkish	2	B-C loop	2.9 % reduced 11β-hydroxylase activity	Krone et al. 2005
V129M	Caucasian	2	C-helix	Complete loss of 11-hydroxylase activity	Geley et al. 1996
N133H	Not described	3	C-helix	17% reduced 11β-hydroxylase activity	Joehrer et al. 1997
L299P	Iraq	5	I-helix	1.2% reduced 11β-hydroxylase activity	Krone et al. 2005
T318M	Yemenite	5	I-helix, active site	Complete loss of 11β-hydroxylase activity	Curnow et al. 1993
T319M	Not described	6	I-helix, active site	37% reduced 11β-hydroxylase activity	Joehrer et al. 1997
A331V	Caucasian	6	I-helix	Complete loss of 11β-hydroxylase activity	Geley et al. 1996
A368D	North German	6	K-helix	1.17% reduced 11β-hydroxylase activity	Krone et al. 2006
E371G	Caucasian	6	K-helix	Complete loss of 11β-hydroxylase activity	Geley et al. 1996
R374Q	Lebanese Arab	6	K-helix	Complete loss of 11β-hydroxylase activity	Curnow et al. 1993
R384G	Japanese	7	Heme propionate neutralisation	Complete loss of 11β-hydroxylase activity	Yang L et al. 1995
V441G	White	8	Meander	Complete loss of 11β-hydroxylase activity	Curnow et al. 1993
R448C	Iranian	8	Heme propionate neutralisation	Complete loss of 11β-hydroxylase activity	Geley et al. 1996
R448H	Moroccan Jew	8	Heme propionate neutralisation	Complete loss of 11β-hydroxylase activity	White et al. 1991

Many missense mutations, which have been found in patients suffering from this disease, lead to functionally disturbed enzymes after expression in cell culture (see Table 1.1). Curnow (Curnow et al. 1993) indicated that mutations T318M, R374Q, R384Q and V441G in exons 5, 6, 7 and 8 lead to complete dysfunctional CYP11B1 activity. Later, mutations V129M, A331V, E371G and R448C were described by Geley and colleague (Geley et al. 1996) to be defective for 11β-hydroxylase activity. Mutations P42S, N133H and T319M lead to only partially disturbed 11β-hydroxylase (Joehrer et al. 1997). Furthermore, Krone and colleagues (Krone et al. 2006; Krone et al. 2005) described

W116C, L299P, DeltaF438, P94L and A368D in patients suffering from classical CYP11B1 deficiency. Analysing of these mutants in the cell culture indicated reduced enzymatic activities. Moreover, the combination of enzyme function analyzed by site-directed mutagenesis and molecular modeling provides valuable insights in cytochrome P450 structure function relationships. As can be seen in Table 1.1, mutations leading to 11β-hydroxylase deficiency are distributed over the entire coding region, but with a slightly enhanced frequency in exon 6 and 8. This might reflect the presence of functionally important amino acid residues in these regions or, alternatively, mutations in this region are more likely to have deleterious effects on the enzyme activity.

Other mutations detected in patients with the classic form of the disease are nonsense or frameshift mutations that also abolish enzyme activity (Cerame et al. 1999; Helmberg et al. 1992; Joehrer et al. 1997; Merke et al. 1998; Naiki et al. 1993). For example, a nonsense W247X has been described in several unrelated kindreds in Austria and also probably represents a founder effect (Geley et al. 1996). An African-American patient was found to be a compound heterozygous for a codon 318+1G→A substitution at the 5'-splice donor site of intron 5 and a previously reported nonsense mutation (Q356X) in exon 6 (Merke et al. 1998).

1.3.2 Aldosterone synthase deficiency

By far the most frequent defect of aldosterone biosynthesis is congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency. Two thirds of patients with classic 21-hydroxylase deficiency are unable to synthesize adequate amounts of aldosterone and are said to have the "saltwasting" form of the disorder. Rare patients have aldosterone deficiency without hyperandrogenism. In some cases, this is caused by CYP11A1 or 3β-hydroxysteroid dehydrogenase deficiencies that are unusual causes of congenital adrenal hyperplasia (White et al. 1987). Other patients have aldosterone deficiency in the context of entirely normal cortisol and sex steroid synthesis. In the early 1960s, the diagnosis of a selective mineralocorticoid deficiency was established by urinary steroid metabolite determinations utilizing improved laboratory methods such as gas chromatography. In

1964, Visser and Cost (Visser et al. 1964) were the first to suggest a biosynthetic defect with autosomal recessive inheritance, causing selective hypoaldosteronism due to deficient 18-hydroxylation of corticosterone. Subsequently, 2 patients with deficient 18-oxidase were described by Ulick et al. (Ulick et al. 1964) and Rappaport et al. (Rappaport et al. 1968). In the early 1970s, Ulick (Ulick 1976) suggested that the two biochemically different forms of selective aldosterone deficiency maybe termed corticosterone methyl oxidase (CMO) deficiency type I and type II. In 1996, the nomenclature was changed to aldosterone synthase deficiency type I and type II, since it was clear that one single P450 enzyme, termed aldosterone synthase, catalyses all three steps of the terminal aldosterone biosynthesis (Ulick 1996). In both aldosterone synthase deficiency types, aldosterone biosynthesis is impaired, while corticosterone of zona glomerulosa origin, under the primary control of the renin-angiotensin system, is produced in excess. The two defects biochemically differ in that 18-hydroxycorticosterone is deficient in aldosterone synthase deficiency type I, but overproduced in aldosterone synthase deficiency type II.

Clinical and biochemical presentation of aldosterone synthase deficieny

Mineralocorticoid deficiency leads to excessive sodium excretion and potassium retention in the renal distal tubule and cortical collecting duct, causing hyponatremia and hyperkalemia. In untreated infants with aldosterone synthase deficiency, serum sodium is usually in the range of 136-145 mM/L (normal value), whereas serum potassium ranges from 3.5-4.5 mM/L (normal value). Children older than 3-4 yr of age usually have normal serum electrolytes even if untreated. Plasma renin activity is markedly elevated (up to 100 times normal) in affected infants and young children, but it may be normal in adults (White et al. 1994a).

Two types of aldosterone synthase deficiency can be differentiated. Levels of deoxycorticosterone are increased and urinary excretion of corticosterone metabolites is elevated in both type I and type II deficiencies relative to excretion of cortisol metabolites. Whereas excretion of 18-hydroxycorticosterone is mildly decreased in type I deficiency, urinary and serum levels of this steroid are dramatically increased in patients

with type II deficiency. Aldosterone and its metabolites may be undetectable in patients with type I deficiency, whereas urinary excretion is mildly decreased in type II deficiency and serum levels of aldosterone are usually within normal limits. CMO II deficiency may thus be readily diagnosed by marked (often 100-fold) elevation of the ratio of 18-hydroxycorticosterone to aldosterone in either urine or serum; the ratio does not vary with age in affected individuals and may be the sole biochemical abnormality in adults. Peter et al. (Peter et al. 1995) reported 16 CMO-deficient infants diagnosed by using specific steroid determinations from plasma sample. The plasma level of 18-hydroxycorticosterone distinguishes between aldosterone synthase deficiency type I (where it is decreased or low-normal) and aldosterone synthase deficiency type II (where it is markedly elevated). The clearest distinguishing parameter between the two aldosterone synthase deficiency types reflecting impaired 18-hydroxylation is the ratio of plasma corticosterone/18-hydroxycorticosterone, which is elevated (>40) in aldosterone synthase deficiency type I and decreased (<10) in aldosterone synthase deficiency type II. The ratio of plasma 18-hydroxycorticosterone/aldsterone can also discriminate between the two aldosterone synthase deficiency variants (type I <10; type II >100). In some case, the ratio of 18-hydroxycorticosterone to aldosterone is not useful for diagnosis of CMO I deficiency because the usually undetectable levels of aldosterone render the ratio meaningless (Ulick et al. 1992). An ACTH test is not necessary for the diagnosis.

Recently, Wudy and coworker (Wudy et al. 2004) have measured steroid excretion rates in a 24-hr urine sample (quantitative urinary steroid profile), which represent the integrated output of adrenocortical and gonadal steroid production. Gas chromatography-mass spectrometry (GC-MS) urinary steroid profiling from spot urine samples allows to diagnose inborn errors of steroid biosynthesis by identifying characteristic steroid metabolites and by calculating ratios between precursor metabolites and product metabolites. In aldosterone synthase deficiency type I, the urinary steroid profile in 18-hydroxylase deficiency is charaterized by increased excretion of corticosterone and metabolites of corticosterone while 18-hydroxylated corticosterone metabolites are absent or very low. The exretion of cortisol metabolites is normal. In aldosterone synthase deficiency type II (18-hydroxysteroid dehydrogenase deficiency), the urinary steroid

profile shows in addition to high amounts of corticosterone metabolites also 18-hydroxylated corticosterone metabolites (18-OH-THA, 18-OH-THB).

The clinical presentation of aldosterone synthase deficiency varies with age (Rosler 1984; Ulick et al. 1992). Infants may develop signs and symptoms of mineralocorticoid deficiency at a few days to weeks of age. These include vomiting and dehydration leading to hypovolemia that may cause cyanosis, tachycardia, hypotension, acidosis, and prerenal azotemia. As discussed, hyponatremia and hyperkalemia are also characteristic of aldosterone deficiency. These problems may end in circulatory collapse. Although fatalities have occasionally occurred, the morbidity of aldosterone synthase deficiency is usually not as severe as that engendered by the salt-wasting form of congenital adrenal hyperplasia. This presumably reflects normal synthesis of deoxycorticosterone, corticosterone, and cortisol in aldosterone synthase deficiency, which ameliorate the development of shock.

Some children are diagnosed in early childhood with failure to thrive, anorexia, mild dehydration, and electrolyte abnormalities. Although electrolytes usually normalize by 4 yr of age (even with a low sodium diet), growth retardation may persist throughout childhood. Adults are usually asymptomatic but occasionally tolerate severe salt loss (for example, from gastroenteritis) less well than unaffected individuals. Asymptomatic adults with aldoterone synthase deficiency are occasionally ascertained through family studies by the persistently elevated ratio of 18-hydroxycorticosterone to aldosterone (Kayes-Wandover et al. 2001b; Peter et al. 1997; Rosler 1984).

It is difficult to distinguish variations in clinical severity between individuals from the marked improvement that occurs with age in all patients. All affected individuals from the Iranian Jewish community have identical mutations (see below), so that any existing individual variations in severity cannot reflect allelic variation. They must instead represent effects of other genetic loci or nongenetic factors.

Genetic analysis

Both types of aldosterone synthase deficiency are caused by mutations in the *CYP11B2* gene. So far, 18 mutations were detected in the *CYP11B2* gene in patients with

aldosterone synthase deficiency (see Figure 1.8). One mutation (V386A) was found in CMO I and CMO II. In CMO I deficiency, the completely inactive mutation has been found to cause aldosterone synthase deficiency type I (see Table 1.2), although the homozygous genotype with double mutations R181W/V386A until now is the only variant shown by *in vitro* activity assay to result in type II deficiency (see Table 1.3). For example, patients with CMO I deficiency carried a frameshift mutation (Mitsuuchi et al. 1993) and one carried a missense mutation, R384P, that eliminates the enzyme activity when expressed in cultured cells (Geley et al. 1996). In CMO II deficiency, mutants (T185I, T318M, V386A and T498A) reduced the 18-hydroxylase activity in the conversion of deoxycorticosterone to aldosterone (see Table 1.3). Iranian Jewish patients with CMO II deficiency are homozygous for two mutations, R181W and V386A. These mutants were expressed in cultured cells. V386A alone had a minimal effect on activity, whereas R181W and the double mutant (R186W/V386A) had intact 11β-hydroxylase activity, markedly decreased 18-hydroxylase activity and undetectable 18-oxidase activity (Pascoe et al. 1992a).

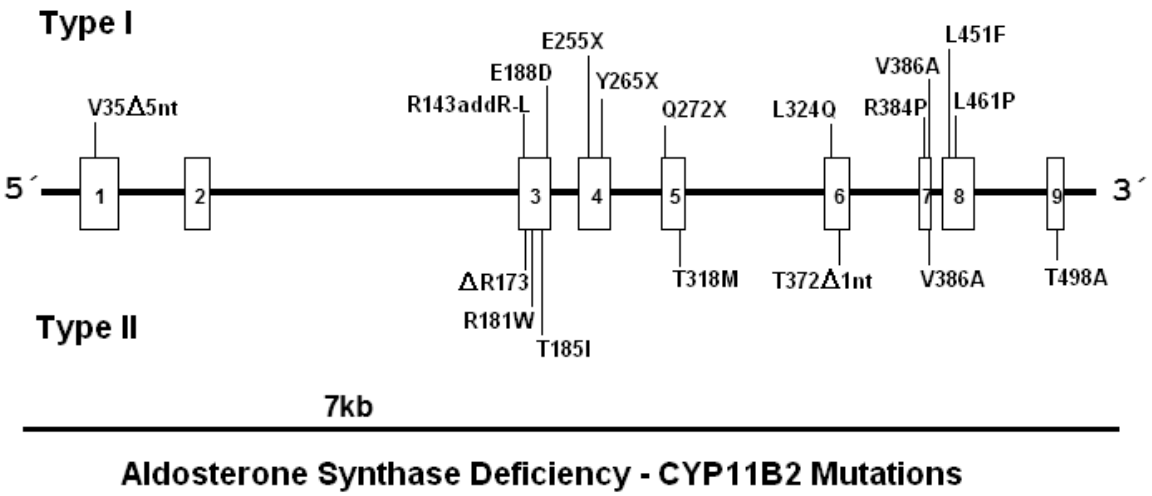


Figure 1.8: Schematic representation of the genomic structure of the human *CYP11B2* gene and positions of mutations reported to date. Exons are represented by boxes; and open boxes represent the non-coding regions.

Table 1.2. Aldosterone synthase deficiency type I

Mutation	Ethnic group	Exon	Putative 3D	*in vitro* activity	Reference
V35ΔTGCTC (homozygous)	North America	1	A-helix	No test, frameshift to form a stop codon in the same exon	Mitsuuchi et al. 1993
R143addArg-Leu (homozygous)	Caucasian	3	C-helix	Completely inactivity	Kayes-Wandover et al. 2001
E255X (homozygous)	Turk	4	G-helix	No test, premature stop codon	Peter et al.1997
E255X/Q272X (heterozygous)	Caucasian	4/5	G-helix	No test, premature stop codon	Williams et al. 2004b
L324Q/Y265X (heterozygous)		6/4	I-helix/G-helix	No test	Lopez-Siguero et al. 1999
R384P (homozygous)	Caucasian	7	β1-4	Completely inactivity	Geley et al. 1995
V386A/R188D (homozygous)	Not described	7/3	β1-4/between D- and E-helix	No test	Lopez-Siguero et al. 1999
V386A/E198D (homozygous)	French	7/3	1-4/E-helix	decreased 11β- and 18-hydroxylase activity, no detectable 18-oxidase activity (residual activity consistent with type II phenotype)	Portrat-Doyen et al. 1998
L451F (homozygous)	Turk	8	L-helix	Completely inactivity	Nguyen et al. 2008
L461P (homozygous)	Turk	8	L-helix	Completely inactivity	Nomoto et al. 1997

Table 1.3. Aldosterone synthase deficiency type II

Mutation	Ethnic group	Exon	Putative 3D	*in vitro* activity	Reference
R173del (homozygous)	Not described	3	D-helix	No test	Peter et al. 1998b
T185I (homozyogus)	Not described	3	E-helix	Reduced 18-hydroxylase activity, no detectable 18-oxidase activity	Peter et al.1998a
R181W/T372Δ1nt T318M/V386A (heterozygous) (2 mutation/allele) V386A (heterozygous)	Iranian Jew	3/6 5/7	K-helix/I-helix/β1-4	T318M mutant has less activity, T372Δ1nt has no activity	Zhang et al. 1995
V386A/R181W (homozygous)	Iranian Jew	7/3	β1-4/between D- and E-helix	0.2% activity of wild-type CYP11B2	Pascoe et al. 1992a
T498A/T185I (heterozygous)	Macedonian	9/3	β-sheet 3, strands 1 and 2	Reduced C_{18} activity of aldosterone synthase	Dunlop et al 2003

1.3.3 Glucocorticoid-Suppressible Hyperaldosteronism

Glucocorticoid-suppressible hyperaldosteronism (GSH), also known as dexamethasone-suppressible hyperaldosteronism, glucocorticoid-remediable aldosteronism (GRA) or familial hyperaldosteronism type I (FH-I) is a form of hypertension inherited in an autosomal-dominant manner with high penetrance (New et al. 1980). In 1966, Sutherland (Sutherland et al. 1966) described the first familial cases of hypertension due to a dexamethasone suppressible form of hyperaldosteronism. GRA is characterized by bilateral adrenal hyperplasia, or rarely, adrenal adenoma (Pascoe et al. 1995). However, the absence of reliable biochemical or genetic markers has made this disease difficult to ascertain. GRA accounts for approximately 1% of cases of primary hyperaldosteronism (Torpy et al. 2000).

Biochemically, hypokalemia is not consistently present and, if present, is usually mild. Absolute levels of aldosterone secretion are usually moderately elevated in the untreated state but may be within normal limits. Plasma rennin activity is strongly suppressed, so that the ratio of aldosterone secretion to renin activity is always abnormally high. Moreover, 18-hydroxycortisol and 18-oxocortisol are elevated to 20-30 times of normal levels (Connell et al. 1986; Gomez-Sanchez et al. 1988; Stockigt et al. 1987; Stowasser et al. 1995; Ulick et al. 1990). The ratio of urinary excretion of tetrahydro-metabolites of 18-oxocortisol to those of aldosterone exceeds 2.0 whereas this ratio averages 0.2 in normal individuals. Elevation of 18-oxocortisol is the most consistent and reliable biochemical marker of the disease, although it may also be elevated in cases of primary aldosteronism (Hall et al. 1986; Hamlet et al. 1988).

18-Hydroxycortisol and 18-oxocortisol are 17α-hydroxylated analogs of 18-hydroxycorticosterone and aldosterone, respectively. Because 17α-hydroxylase is not expressed in the zona glomerulosa, the presence of large amounts of a 17α-hydroxy, 18-oxo-steroid suggests that an enzyme with 18-oxidase activity (i.e. aldosterone synthase, CYP11B2) is being abnormally expressed in the zona fasciculata (White et al. 1991).

It is important to distinguish glucocorticoid-suppressible hyperaldosteronism from aldosterone-producing adenomas, considering that the latter condition is best treated by surgical removal of the affected adrenal gland (Melby 1991). Secretion of 18-hydroxy-

and 18-oxocortisol may be increased in patients with adenomas, but the ratio of urinary excretion of tetrahydro metabolites of 18-oxocortisol and aldosterone is rarely greater than 1.0 (Hamlet et al. 1988; Ulick et al. 1990). Suppression of aldosterone secretion with glucocorticoids (Hamlet et al. 1988; Kato et al. 1988) and familial aggregation (Gordon et al. 1992), although both reported, are unusual findings in adenomas. In addition, presentation of an adenoma during childhood is exceedingly rare.

Genetically, all patients with glucocorticoid-suppressible hyperaldosteronism have the same type of mutation, a chromosome that carries three *CYP11B* genes instead of the normal two (Lifton et al. 1992b; Lifton et al. 1992a). The middle gene on this chromosome is a chimera with 5' and 3' ends corresponding to *CYP11B1* and *CYP11B2*, respectively. The chimeric gene is flanked by presumably normal *CYP11B2* and *CYP11B1* genes (see Figure 1.8). Published cross-over breakpoints in the GRA patients are located between intron 2 and exon 4 (Lifton et al. 1992a; MacConnachie et al. 1998; Pascoe et al. 1992b). The presence of *CYP11B1* promoter and regulatory elements ensures that the gene is expressed in the zona fasciculata/reticularis under the control of ACTH and the 3' *CYP11B2* coding sequences lead to encoded the enzyme having the three activities required for aldosterone synthesis. Consequently, aldosterone is inappropriately synthesized and secreted in excess by the zona fasciculata/reticularis under the control of ACTH. The opposite case of a chimeric gene containing *CYP11B2* promoter and the *CYP11B1* structural gene was detected in the patients with steroid 11β-hydroxlase deficiency and congenital adrenal hyperplasia (Hampf et al. 2001b; Portrat et al. 2001).

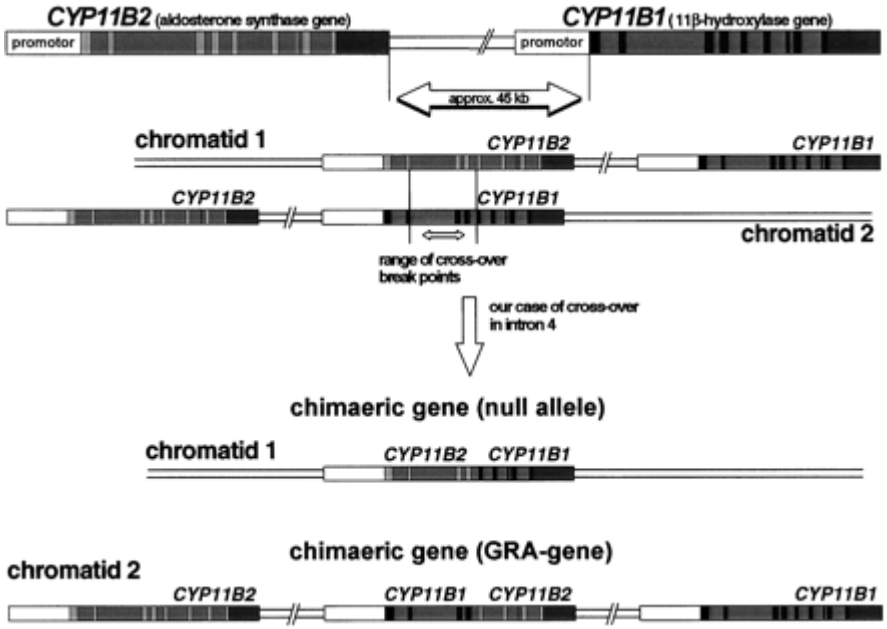


Figure 1.8: Unequal crossing-over of aldosterone synthases (CYP11B2) and 11β-hydroxylase (CYP11B1) genes (Hampf et al. 2001b). The genes are depicted as bars. The exons are colored light gray for *CYP11B2* and black for *CYP11B1*.

The figure outlines the genetic recombination of the examined patients. The high similarity of *CYP11B1* and *CYP11B2* enabled two chromatids to misalign for the meiotic cross-over, from which one chromatid emerged with only one (chimeric) *CYP11B* gene and the other with three (the reciprocal chimera between normal *CYP11B2* and *CYP11B1*). In the investigated patient, the CYP11B2/CYP11B1 chimera of the former product, a chromosome 8 carrying only this chimeric *CYP11B* gene, was detected by PCR and subsequent sequencing. Furthermore, the chimeric genes causing glucocorticoid-suppressible hyperaldosteronism may be readily detected by hybridization to Southern blots of genomic DNA, or they may be specifically amplified using the polymerase chain reaction. As these techniques are widely used in molecular genetics laboratories, direct molecular genetic diagnosis may be more practical in many cases than assays of 18-oxocortisol levels, which are not routinely available. Pascoe and coworker (Pascoe et al. 1995) demonstrated abnormal expression of chimeric gene in the zona fasciculata by in situ hybridization studies of an adrenal gland from a patient this disorder.

1.4 Aim of the work

The overall goal of this work consisted of investigations of the genotype of four patients suffering from aldosterone synthase deficiency and two patients with cortisol synthase disorders of the biosynthesis of steroid hormones. The combination of the functional analysis of the enzyme in the cell culture and the molecular modeling study may explain phenotypical characteristics of the patients. The main objectives of the present thesis were:

1) to detect mutations in genomic DNA of patients with aldosterone synthase deficiency and cortisol synthase disorders.

2) to analyze effects of detected mutations of CYP11Bs by expressing the mutant proteins in cell culture and analyze missense mutations using the three-dimensional model of CYP11B2.

3) to compare the usefulness of human colonic carcinoma, HCT116 $p53^{-/-}$ cells with COS-1 cells for the transfection analysis of CYP11B2.

2. Materials and methods

2.1 Materials

2.1.1 Bacteria strains

DH5α^{TM}-T1^{R} (invitrogen): This strain was used for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage with the following genotype:

F^{-} φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1 *endA*1 *hsdR*17(rk^{-}, mk^{+}) *phoA supE*44 *thi*-1 *gyrA*96 *ton*A1.

One Shot™ TOP10F' (invitrogen): This strain was used for general cloning and blue/white with IPTG. This strain overexpresses the Lac repressor (lacI^{q} gene) with the following genotype:

F' mcrA {*lac*I ^{q} Tn10(Tet^{R})} *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G

2.1.2 Cell lines

To investigate steroidogenic activity of CYP11B1 and CYP11B2, COS-1 cells and HCT116 p53$^{-/-}$ cells were used for transfection with plasmids containing the required cDNA of wild-type or mutated components of the CYP11B2 and CYP11B1.

COS-1 cells: This is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. This line contains T antigen, retains complete permissiveness for lytic growth of SV40, and supports the replication of pure populations of SV40 mutants with deletions in the early region. The line was derived from the CV-1 cell line by transformation with an origin defective mutant of SV40 which codes for wild type T antigen. The cells contain a single integrated copy of the complete early region of the SV40 genome (Gluzman, 1981).

HCT116 p53$^{-/-}$: These cells were derived from human colonic carcinoma with wild type *p53* gene knock-out (p53$^{-/-}$). The cells are positive for keratin by immunoperoxidase staining. HCT116 cells are positive for transforming growth factor beta 1 (TGF beta 1) and beta 2 (TGF beta 2) expression. This line has a mutation in codon 13 of the

protooncogene, and can be used as a positive control for PCR assays of mutation in this codon.

2.1.3 Vectors

pCR®2.1-TOPO	Cloning vector, Invitrogen.
pCR4Blunt-TOPO	Cloning vector, Invitrogen.
pRc/CMV	Expression vector, Invitrogen.
pSVL	Expression vector, Amersham Pharmacia, designed for high-level transient expression in eukaryotic cells.
pSVL/CYP11B2	This construct contains the cDNA encoding human aldosterone synthase which was published by Kawamoto et al. (Kawamoto et al., 1992) with one variation at position 249, where we found Ser instead of Arg, as described by Mornet *et al*. (Mornet *et al.*, 1989).
pSVL/CYP11B1	This construct contains the cDNA encoding human 11β-hydroxylase which was published by Mornet *et al*. (Mornet *et al.*, 1989).
pGlow-TOPO	Expression vector, Invitrogen. pGlow-TOPO vector was designed with promoter sequences upstream of GFP for *in vitro* or *in vivo* analysis of promoter function.
pBAdx4	Bovine adrenodoxin plasmid. The plasmid includes the cDNA of bovine adrenodoxin under the control of CMV (Okamura et al., 1987). The plasmid was kindly made available from Dr. M. R. Waterman (Nashville, the USA).
phAdx	Human adrenodoxin plasmid. The plasmid includes the cDNA of human adrenodoxin under the control of the CMV.

2.1.4 Primers

All oligonucleotides were synthesized by BioTez (Berlin, Germany). The oligonucleotides were cleaned by gel filtration or HPLC. 5' fluorescence labeled oligonucleotides (fluorophore IR800) used for DNA sequencing with a Licor^{TM}-DNA sequencer 4000 were purchased from MWG Biotech. "F" and "R" are forward and

reverse primers, respectively. The red letters are mutation sites. The under line letters are restriction sites. The sequences used in this work are given below:

Table 2.1: **Primers**

Primers for amplification of *CYP11B2* gene					
	Description	**F**	**R**	**Sequence (5´=>3´)**	**Position of 5´-base in *CYP11B2* sequences**
1	E16F	X		ACCAGACTTCTCCTTCATCTACCTT	440
2	E39F	X		TCAGCACCTGTGGGCAGAAGCTACAG	2961
3	E35F	X		TCAGCACCTGTGGGCAGAAGCTACAG	3264
4	E35F		X	CGCCCTCAACACTACACAGGCATCG	4072
5	E16R		X	GAGCGTCATCAGCAACGGAAACGCT	5021
6	E39R		X	CCCGGATCCAGGCCCTGCCAGCAAGAT	6691
Primers for amplification of *CYP11B2* gene for exon 9 and exon 4					
	Description	**F**	**R**	**Sequence (5´=>3´)**	
7	E9F	X		AACCCAGCCTCTGTCCTAGG	
8	E9R		X	ACCCTGGGTGCAGATGCAAG	
9	E4F	X		GAGGCAGCCAGGAGGCCTGGGGCTG	
10	E4R		X	GGAGAAATTGGGCCCCCATGGTGTC	
Primers for mutations of *CYP11B2*-cDNA					
	Description	**F**		**Sequence (5´=>3´)**	
11	E9M	R490X		AGCTTCATATTGA-GCCTGGCACGTC	
12	E3/4M	F168F/R173K		AGGGACTTTTCCCAGGCCCTGAGGAAGAAGG	
13	E3M	R181Q		GCTGCAGAACGCCCAGGGGAGCCTG	
14	E8M	L451F		CATGCGCCAGTGCTTCGGGCGGCGCCTG	
15	E5M	S308P		CCATCAAGGCCAACCCTATGGAACTC	
16	E5M1	S315R		CACTGCAGGGAGGGTGGACACGACAGC	
17	E6M	R374W		CAAGGAGACCTTGTGGCTCTACCC	
18	E5SF	splicing		GAGCGTGGACACAGTCAGGCCAGCA	
Primer for mutation of *CYP11B1*-cDNA					

	Description	Mutation	Sequence (5´=>3´)
19	B1-229F	L229P	CTGTTGAATGCGGAACCGTCGCCAGATGCC
20	B1-229R	L229P	GGCATCTGGCGACGGTTCCGCATTCAACAG

Primers for amplification of *CYP11B2* from exon 1/exon 4 to exon 6

	Description	F	R	Sequence (5´=>3´)
21	E1E6sF	*X*		AAGGGAGCGGCCGCATGGCACTCAGGGCAAAGGCAG AGGTGTGCG *Not* I
22	E1E6sR		X	AGCCAGCATCTAGATCTAGGTCTCCTTGAGGGCCGCC *Xba* I
23	E4E6sF	X		CATCCTCCCTCGAGCCATGCAACTTAGCTCT *Xho* I
24	E4E6sR1		X	GCACCCACCTCTAGATCTCATTGAGGGCC *Xba* I
25	E5E6sF	X		GTGACAACTCTAGACAGAAAATCTACCAGG *Xba* I
26	E19sR		X	GGGACCCTGGGTCTAGATGCAAGACTAGTTA *Xba* I

Primers for sequence of *CYP11B2* gene

	Description	F	R	Sequence (5´=>3´)
27	E1 115-18	X		CAGTTCTCCCATGACGTG
28	E1 3932-20		X	GGGAATGGCAGTGCTGAGTG
29	E2 663-18	X		AGGGTGGACAGGAGACAC
30	E2 3935-20		X	CCTGCTCCCAGCTCTCAGCT
31	E3 3933-20	X		TGCAGGCCGATTCCCCTTGG
32	E3 3934-20		X	CTCCTGGCTGCCTCCCCACA
33	E4 3936-20	X		GTGGGGAGGCAGCCAGGAGG
34	E4 180		X	CTTCCCCATAGCACTGC
35	E5 3938-22	X		AGGAGGAGGACACTGAAGGATG
36	E5 3939-21		X	TGGCATCACCCTCTCTGGGTG
37	E6 3940-20	X		GGTGTCCCGGGGGCTGAGTC

38	E6 664-19		X	ATAGCCCAGATTCTGTCTG
39	E7/E8 117-18	X		TAGGAAGGGTGCAGAGAG
40	E8 3156-23	X		CTCAGACTTGGTGCTTCAGAACT
41	E9 4396-20	X		TGTTCCCCCTTCAGCATAAT
42	E9 85(515-18)		X	TGACTCAGGAAGCTGTGC
43	E5F	X		GAACTCACTGCAGGGAGCGTGGACA
44	E4R		X	TCCTGGGCATGAACATGAGCTGGAC
45	E7F	X		CTCAGACTTGGTGCTTCAGAACTACC
Primers for vector				
	Description	**F**	**R**	**Sequence (5´=>3´)**
46	T7	X		TAATACGACTCACTATAGGG
47	M13R		X	CAGGAAACAGCTATGAC
48	M13F	X		GTAAAACGACGGCCAG

2.2 Methods

All standard DNA techniques were performed according to procedures published by Sambrook et al. (Sambrook et al., 1989; 2001) unless indicated.

2.2.1 Purification of genomic DNA from blood

To analyze the genomic DNA, we purified genomic DNA from blood of patients and and healthy donors. Genomic DNA purification from blood was performed using a commercially available kit from QIAGEN (QIAamp DNA Blood Midi/Maxi preparation kits) according to the manufacturers instructions. Genomic DNA of patients and healthy donors was purified from 2 ml of whole blood. After lysis, the lysate is loaded onto the QIAamp spin column. DNA binds to the QIAamp membrane while impurities are effectively washed away by centrifugation. Finally, genomic DNA can be eluted in 200 µl distilled water. After purification, the genomic DNA concentration was determined spectroscopically by measuring the absorption at 260 nm.

2.2.2 Polymerase chain reaction (PCR) protocol

PCR, now a common technique, is used in molecular cloning and analysis of DNA: PCR is performed to amplify a large number of copies of a specific region of DNA using DNA plymerase. A DNA polymerase is an enzyme that assists in DNA replication. These enzymes catalyze the polymerization of deoxyribonucleotides alongside a DNA strand. PCR, as currently practiced, requires several basic components: DNA template, two oligonucleotide primers, DNA polymerase, dNTP and buffer solution. The PCR usually consists of a series of 20 to 40 cycles involving the denaturalisation of template, the annealing of primer, and the extension of the annealed primers by DNA polymerase.

The *CYP11B2* gene was selectively amplified in two segments (exons 1-6, 4.5 kb, using primers E16F and E16R shown in 2.1.4; exons 3-9, 3.7 kb, using primers E39F and E39R shown in 2.1.4) or in one segment (exons 1-9, 5.9 kb, using primers E19F and E19R in 2.1.4). Amplification of the *CYP11B2* gene was performed using *BIO-X-ACT* DNA polymerase (Bioline) for long segments of DNA. Mutations in the human *CYP11B2* cDNA and human *CYP11B1* cDNA were generated in the vector pSVL by site-directed mutagenesis using *Pfu* DNA Polymerase (Stratagene) according to

manufacturer's instruction of the Quik Change kit. The primers used for these purposes are listed in section 2.1.4. Furthermore, E.*coli* colony PCR was performed using *Tag* DNA polymerase (Q BIOgene) which is cheaper than other DNA polymerase.

Standard PCR reaction mix for genomic DNA amplification

Components		Volume	
Genomic DNA (100-400 ng)		x	µl
10X OptiBuffer		5	µl
$MgCl_2$ Solution (2-2.5 mM)		2	µl
dNTPs (100 mM)		2	µl
Primer 1 (0.2 µM)		1	µl
Primer 2 (0.2 µM)		1	µl
BIO-X-ACT DNA polymerase (4u/µl)		0.5	µl
Water (ddH_2O)	Up to	50	µl

PCR program for the amplification of *CYP11B2* gene:

95°C for 5 min
60°C for 1 min
68°C for 5 min
95°C for 30secs — x 30-35 cycles
60°C for 30secs
68°C for 10 min

Program used to perform E. *coli* colony PCR:

95°C for 3 min
95°C for 1 min
55°C for 1 min — x 30 cycles
72°C for 2 min
72°C for 10 min

PCR program for the introduction of mutations into the human *CYP11B2* cDNA:

95°C for 3 min	
95°C for 1 min	x 20 cycles
60°C for 1 min	
72°C for 15 min	
72°C for 15 min	

2.2.3 DNA sequencing

The PCR product of the *CYP11B2* gene was purified after excision the agarose gel by using NucleoSpin columns (Extract kit Macherey-Nagel). All exons and exon/intron boundaries were sequenced directly from PCR products using the primers E1-E9 shown in section 2.1.5. Plasmids were purified from selected clones using the NucleoBond kit (Macherey-Nagel) according to the supplied instructions. Mutated plasmids and subcloned plasmids were sequenced using a slightly modified protocol of the didesoxynucleotide method developed by Sanger et al. (Sanger et al., 1977). Primers used for DNA sequencing were 5' fluorescence labeled (MWG Biotech) enabling a laser-scan detection on a Licor™ 4000 DNA sequencer (MWG Biotech, Ebersberg, Germany). PCRs were performed with the Thermo-Sequenase™ Cycle Sequencing Kit from Amersham according to the manufactures instructions. All DNA sequencing reactions being part of this work were thankfully carried out by Mrs. Katharina Bompais.

2.2.4 Site-directed mutagenesis

Site-directed mutagenesis was used to make point mutations, and delete or insert single or multiple amino acids. In this method, P*fu* DNA polymerase replicated both plasmid strands with high fidelity. The procedure was performed using a recombinant vector with an inserted gene of interest and two oligonucleotide primers containing the desired mutation. The primers were extended during temperature cycling by P*fu* DNA polymerase. This incorporation of primers generated a mutant plasmid containing staggered nicks. The treatment of the product with D*pn* I resulted in the digestion of the parental DNA template.

Mutants R181Q, S315R, R374W, L451F and R490Δ1nt in the human *CYP11B2* cDNA as well as mutant L299P in the human *CYP11B1* cDNA were generated in the vector pSVL by site-directed mutagenesis using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene Ltd, Cambridge, UK) according to manufacturer's instruction and using mutagenic primers listed under point 2.1.4.

PCR reaction mix for plasmid DNA

Components		Volume	
plasmid DNA (50 ng)			
10xPfu reaction buffer		5	µl
dNTPs (100 mM)		2	µl
Primer 1 (0.2 µM)		1	µl
Primer 2 (0.2 µM)		1	µl
P*fu* DNA polymerase (2.5 u/µl)		0.5	µl
Water (ddH_2O)	Up to	50	µl

Reactions were carried out in the thermal cycler PT-100, MJ Research Inc. with PCR program (see in 2.2.2). The PCR product was digested with *Dpn*I for 4 hours at 37°C, and then 1µl was used for transformation of competent TOP10F´ *Escherichia coli*. After screening of directed colonies on an aga plate with ampicillin, plasmids were purified following the procedure described below under point in 2.2.7. All changes were confirmed by automatic sequencing (see 2.2.3).

2.2.5 Preparation of competent cells

A single colony of *E. coli* TOP10F' strain was inoculated in 5 ml nutrient broth I (NB) medium and shaked at 180 rpm, 37°C for 12-16 hours (Sambrook et al., 1989). The culture was diluted 100 folds in 100 ml fresh NB medium and continuously shaked at 180 rpm, 37°C until OD600 to be 0.4 - 0.6 (about 3 hours). Subsequently, the cell culture was harvested by centrifugation at 4000 rpm for 15 min at 4°C. The pellet was suspended in 30 ml RF1 buffer and then kept on ice for 1 hour. After a further centrifugation, the cells were resuspended in 5 ml RF2 buffer on ice. Aliquots of 100 µl volume of the competent cells were transferred into a pre-chilled sterile eppendorf tube. The competent cells can

be stored at -70°C for 3 months. The quality of competent cells was confirmed by transformation with control plasmid DNA (pUC18).

RF 1

75 mM KCl
50 mM KCH_3COOH
50 mM $MnCl_2$
10 mM $CaCl_2$
15 % Glycerol

RF 2

10 mM MOPS
10 mM KCl
75 mM $CaCl_2$
15 % Glycerol

2.2.6 Heat shock transformation

Heat shock transformation was applied to introduce the plasmid DNA into cells (Sambrook et al., 1989; 2001). Approximately 20-100 ng plasmid DNA or the ligation mix was added to 100 µl freshly thawed competent cells in a reaction tube and incubated for 30 min on ice. The heat shock was performed at 42°C for 90 sec followed by incubation on ice for 5 min. Then 500 µl SOC medium was added to the cells and incubated at 37°C for 1 h, 180 rpm shaking. 200 µl of the culture were plated on NB agar plates containing appropriate antibiotic. The plates were incubated at 37°C overnight.

SOC medium

2 % Trypton
0.5 % Yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
pH = 7.0
autoclave
Add 20mM glucose and sterilize by filtration through a 0.22-micron filter

2.2.7 Plasmid purification and determination of the nucleic acid concentration

Plasmid purification was performed using a commercially available kit from Macherey-Nagel (Nucleobond® maxi or midi plasmid preparation kits) according to the

manufacturers instructions. After purification, the plasmid concentration was determined spectroscopically by measuring the absorption at 260 nm. The principle of this method is based on the absorption ability of UV light by the ring structure of purines and pyrimidines in the DNA or RNA. According to Hagemann (Hagemann, 1990) 1 AU260 corresponds to a dsDNA concentration of 50 µg/ml.

The concentration of nucleic acids was calculated by following formula:

C mg/ml = OD260*xε*f

C = concentration in mg/ml

f = dilution factor

xε = 50 (double strand DNA) or 40 (single strand DNA and RNA)

2.2.8 Minigene structure

Minigene is one segment of the gene including at least one exon and intron. Minigene structures are used in studying splicing. Minigene constructs consisting of exon 1 to exon 6 were subsequently cloned into the vector pRc/CMV (Invitrogen). They were generated from the genomic DNA of a healthy person and a patient. pRc/CMV containing both SP6 and T7 promoters for *in vitro* transcription is designed for high-level stable and transient expression in eukaryotic hosts. PCR products from exon 1 to exon 6 of the *CYP11B2* gene using the primers listed in section 2.1.5, excised with *Not*I and *Xba*I (restriction sites in primers), were cloned into the *Not*I and *Xba*I sites of pRc/CMV (Invitrogen). The splice sites in the subclone of pRc/CMV were sequenced to verify the integrity of the inserts (see section 2.1.4 for primer information).

2.2.9 Cell culture

COS-1 cells were grown in petri-dishes at 37°C and 6% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 1 mM pyruvate and 4 mM L-glutamine (DMEM+). Likely, HCT116 $p53^{-/-}$ cells were grown in petri-dishes at 37°C and 6% CO_2 in McCoy's medium supplemented with 5% fetal bovine serum, 0.1 mg/ml streptomycin, 100 U/ml penicillin (McCoy's+). Cells were grown to maximal 90 % confluence and

subculture. The culture medium was changed every 2-3 days. COS-1 and HCT116 $p53^{-/-}$ cells were splited and frozen the same way below.

2.2.9.1 Splitting cell lines

Prior to splitting the cells were washed twice briefly with 3 ml PBS. Then the cells were detached from the petri-dishes (10 cm) by adding pre-warmed 1x trypsin-EDTA (3 ml) (30 mg/ml streptomycin, 10000 U/ml penicillin) at 37°C for 2-3 min. After addition of FBS (3 ml) (GibcoBRL or Sigma), cells were splitted by transferring the cells to new plates (10 cm) containing fresh medium (10 ml) (split cells no more than 1:10).

2.2.9.2 Freezing cell lines for long term storage

After loosed with pre-warmed 1x trypsin-EDTA and addition of FBS, cells were transferred to a falcon. Then the cells were collected by centrifugation at 3500 rpm for 2-3 min. In order to prevent the formation of ice crystals within the cells, the pellet was suspended in media containing 20% DMSO and was slowly cooled down. This can be accomplished either by freezing the cells at –20°C for an hour, and then transferring them to –70°C overnight or by placing the cell-culture inside a cooler (Nalgene) for an hour, afterwards incubating the cells at –70°C overnight. In both cases, on the next -day, the cells were stored either in liquid nitrogen cell incubator (Nalgene) or at –80°C.

2.2.9.3 Transient transfections and enzymatic assays

Transfection of COS-1 cells and HCT116 cells was achieved using the non-liposomal lipid "Effectene Transfection Reagent®" from Qiagen according to the provided manual. This reagent is used to obtain higher transfection rates compared to other methods e.g. calcium-phosphate method. The Effectene method takes advantage of the fact that transient transfection in cells is most efficient when supercoiled plasmid DNA is used. Therefore, by using the provided Effectene reagents the plasmid DNA is first condensed followed by the formation of uniform lipid-based micelle structures (Qiagen). Binding of the positively charged Effectene-DNA complexes to the cell surface is then mediated through negatively charged groups such as sialylated glycoproteins. The

number of cells plated and grown overnight prior to transfection as well as the plasmid DNA concentrations were set as recommended by the manufacturer, depending only on the culture format to be used.

HCT116 cells or COS-1 cells were plated with a density of 2-6 x 10^5 cells per 6 cm dish and were grown overnight. The dishes should be 40-80% confluent on the day of transfection. Cell lines were co-transfected with 1.5 µg bovine adrenodoxin plasmids (pBAdx) or 1.5 µg human adrenodoxin plasmids (phAdx) and 1.5 µg pSVL vector containing *CYP11B2* cDNA (CYP11B2-WT) or *CYP11B1* cDNA (CYP11B1-WT). Transfection then was performed according to the instruction of the kit (Qiagen). Subsequently, the cells with the transfection complexes were incubated under their normal growth conditions (37°C and 6% CO_2).

2.2.10 Extraction of total RNA

The cells were incubated for 24 hr after transfection and harvested and disrupted in Qiagen (RLT) buffer containing guanidine isothiocyanate of the Rneasy Mini kit (Qiagen) and homogenized. After removing the medium, 350 µl of RLT buffer was added in the cell-culture dish (6 cm) to disrupt the cells. The cells then were collected with cell scraper (Sarstedt, USA) and pipetted into an eppendorf tube. In the cell lysate 350 µl of 70% ethanol was added and mixed well by pipetting. Then, extraction of total RNA was performed according to the instruction of the kit. After purification, the concentration of total RNA was determined spectroscopically by measuring the absorption at 260 nm. According to Hagemann (Hagemann, 1990) 1 AU260 corresponds to a RNA concentration of 40 µg/ml.

2.2.11 RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

The First-Strand cDNA synthesis was performed according to the instructions of the M-MLV Reverse Transcriptase kit (Invitrogen). The reverse transcription was used to synthesize cDNA from RNA templates. The mixture of total RNA, oligo (dT) 12-18 or random primer and dNTP was incubated at 65°C for 5 min and then quickly chilled on ice. After this, the First-Strand Buffer, DTT, Rnase OUT and M-MLV RT were added in the mixture. The reactions were incubated at 37°C for 50 min and heat inactivation was

done at 70°C for 15 min. The cDNA can be used as a template for amplification in PCR. The PCR was performed with the forward primer and reverse primer shown in Table 2.1.

PCR reaction mix for RT-PCR

Components		Volume	
cDNA (50 ng)		2-5	µl
10x Q BIOgene buffer		5	µl
dNTPs (100 mM)		2	µl
Primer 1 (0.2 µM)		1	µl
Primer 2 (0.2 µM)		1	µl
Tag- DNA polymerase (2.5 u/µl)		0.5	µl
Water (ddH_2O)	Up to	50	µl

Program used to perform RT-PCR:

95°C for 2 min	
95°C for 1 min	x30 cycles
55°C for 55 sec	
72°C for 1 min 30 sec	
72°C for 8 min	

The PCR products were purified after excision form the agarose gel by using NucleoSpin columns (Extract kit Macherey-Nagel) and sequenced by using T7 promoter primer (see Table 2.1).

2.2.12 Extraction of total protein

To analyze the expression of the wild type and mutated forms of aldosterone synthase in the transfected cells, after 6 hrs transfection, the cells were incubated with a medium containing DOC (2 µM). After incubation for 48 hrs, the COS-1 cells were washed three times with ice-cold PBS and lysed in 300 µl lysis buffer. Cell debris was removed by centrifugation at 12,000 x *g* for 20 min at 4°C, and the supernatant was retained for protein determination (Bio-Rad Laboratories, Inc., Regent Park, Australia). Equivalent amounts of protein were separated onto 10% or 12% SDS-PAGE gel.

Lysis buffer

10 mM Tris-HCl (pH 7.4)

50 mM NaCl

2 mM EDTA

1% Triton X-100

1 mM PMSF

2.2.13 Determination of the protein concentration

Protein concentration was determined using BC (BicinChoninic acid) Assay protein quantitation kit (Uptima Interchim, Montluçon, France). Proteins have traditionally been quantified by the spectrometric measurement of a colour produced by a reaction between proteins and a reagent. The principle of the BC Assay is a colorimetric assay which involves the reduction of Cu^{2+} to Cu^{+} by peptidic bonds of proteins. Bicinchoninic acid chelates Cu^{+} ions with very high specificity to form a water soluble purple coloured complex (see Figure 2.1).

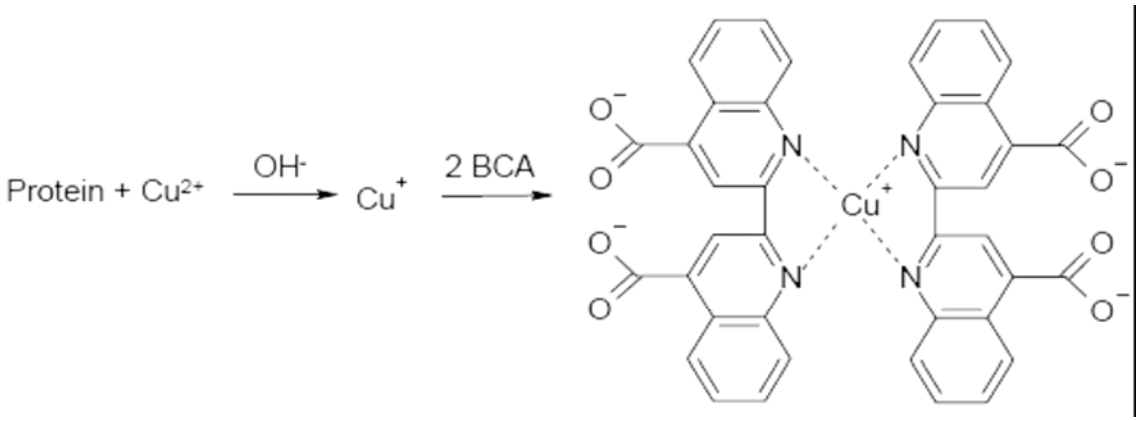


Figure 2.1 BC Assay reaction

The reaction was read at defined time and temperature conditions, otherwise it continues over time or is increased by high temperature. The reaction is measured by the high optical absorbance of the final Cu^{+} complex at 562 nm. Absorbance is directly proportional to the protein concentration with a broad linear range between 20 – 2000 µg/ml. Protein concentration is calculated with a reference curve obtained for a standard protein. Bovine serum albumin (BSA) was used as standard protein. Fresh standard

proteins were arranged from 2 mg/ml to 20 μg/ml in Table 2.2 and were diluted from the stock solution in the same buffer according to the kit.

Table 2.2 Standard protein (20 μg/ml-2 mg/ml)

	x μl BSA standard (2 mg/ml)	x μl Water/Buffer	Protein content (mg/ml)	Σ (μl)
A	100	0	2	100
B	50	50	1	100
C	37.5	62.5	0.75	100
D	25	75	0.5	100
E	20	140	0.25	160
F	40 of (E)	60	0.1	100
G	20 of (E)	80	0.2	100
H	0	100	0	100

BSA: bovine serum albumin

The protein concentration must fall in the range of the standard curve. Therefore it may be useful to prepare several dilutions to meet this requirement. The standard proteins and samples were incubated at 37^0C for 30 min or 2 hours at room temparature and then were merasured absorbance at 562 nm.

2.2.14 SDS (sodium dodecylsulfate) polyacrylamid gelelectrophoresis

Separation of proteins according to their molecular mass was conducted using the Laemmli discontinuous gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). For preparing the gel, the separating gel solution (see Table 2.3) was carefully poured between glass plates, overlaid with ddH_2O and allowed to polymerize at room temperature for 30 min. After removing the overlaying H_2O, the stacking gel solution (see Table 2.4) was poured in and a comb was inserted. The gel was allowed to polymerize for further 30 min. The gels were stored in soaked papers with water at 4°C until use.

A portion of the protein sample was mixed with 2x SDS loading buffer (1:1 v/v) and heated at 100°C for 5 min. The samples were applied to the slots of the stacking gel for 15 min at 120 V and then separated on a separating gel at 150 V until the bromophenol blue front reached the bottom of the gel.

Table 2.3 Recipes for polyacrylamide separating gel

Stock solutions	Final acrylamide concentration		
	10%	12%	15%
4X LT	3.75 ml	3.75 ml	3.75 ml
10% APS	75 µl	75 µl	75 µl
dest.H_2O	ad 15 ml	ad 15 ml	ad 15 ml
30% AA/Bis	5.0 ml	6.0 ml	7.5 ml
TEMED	7.5 µl	7.5 µl	10 µl

Table 2.4 Recipes for polyacrylamide stacking gel

Stock solutions	5% acrylamide concentration
4X UT	2.5 ml
10% APS	50 µl
Dest.H_2O	ad 10 ml
30% AA/Bis	1.6 ml
TEMED	5 µl

AA/Bis: Acrylamide /Biss

4x Buffer for separating gel (4X LT)

1,5M Tris/Cl, pH=8,8

0,4% SDS

4x Buffer for stacking gel (4X UT)

0,5M Tris/Cl, pH=6,8

0,4% SDS

2x SDS loading buffer

125mM Tris/Cl, pH=6.8

20% Glycerol

4% SDS

10% β-Mercaptoethanol

0.004% Bromphenolblue

2.2.15 Staining of proteins in polyacrylamide gels with Coomassie brilliant blue (G-250)

The gel was stained with coomassie staining solution for 1 h at RT and then incubated in destaining solution until the bands of the proteins were clearly visible. The gels were then slowly dried on a gel dryer (Model 583 gel dryer, BioRad) for storage.

Staining solution	Destaining solution
0.1% Coomassie Brilliant Blue G-250	25% Methanol
40% Methanol	10% Acetic acid
10% Acetic acid	

2.2.16 Western blot

Western blot analysis was performed to demonstrate the expression of the wild type and mutant of aldosterone synthase in the transfected cells. The antihuman-CYP11B rabbit antiserum was kindly provided by Dr. H. Takemori (Department of Molecular Physiological Chemistry, Osaka University Medical School, Osaka, Japan). After the separation on SDS-PAGE, 200 µg proteins were transferred onto a nitrocellulose membrane (pore size 0.2 µm) using a tank-blotting apparatus (Invitrogen). The transfer was carried out overnight with 10 mA of current. Blotting buffer consisted of 25 mM Tris-HCl, 0.2 M glycine, 20% methanol. The membrane was blocked in 20 ml blocking buffer (3 - 5% not-fat dried milk in TBST) for 1 hour at RT. After removed blocking buffer, the membrane was incubated in 20 ml TBST buffer containing primary antibody (1:2000) for 1 hour at RT on an orbital shaker. Subsequently, the membrane was washed 3 times for 5 min in 25 ml TBST and was incubated with 20 ml TBST buffer containing the secondary antibody (Polyclonal Goat anti-Rabit Imunoglobulins/HRP) for 1 hour.

Once the membrane was washed 3 times with 25 ml TBST, the specific protein was detected with 4-Chlor-1-naphtol or the ECL kit (Amersham Pharmacia).

TBST

10 mM Tris/Cl, pH = 7.6

150 mM NaCl

0.1% Tween 20

2.2.17 Extraction of steroids

To examine for CYP11B2 or CYP11B1 activity, cells were incubated for 6 hrs after transfection and media was replaced with 3 ml complete medium (DMEM+ for COS-1 cells or McCoy's+ for HCT116 cells) containing 2 µM DOC and 5 nCi of ^{14}C-labelled DOC or 5 µM 11-deoxycortisol and 0.6 µCi ^{3}H-labelled 11-deoxycortisol. After incubation for 72 hrs, steroids were extracted twice from the 800 µl media with 800 µl chloroform/each time and the organic phase was dried in a vacuum centrifuge (SpeedVac) for 3 hours. The residuum was dissolved in 10 µL chloroform and spotted onto glass-baked silica-coated high performance thin layer chromatography (HPTLC) plates (Merck; Darmstadt, Germany) which were incubated at 50°C for 1 hour. The HPTLC plates were developed twice in chloroform/methanol/water (300: 20: 1, v/v/v). The reaction products were identified by comigration of unlabeled steroid references and quantified after a 3-day exposure on a bioimaging analyser (BAS-2500, Fuji Photo Film Co., Ltd) and analyzed with the program TINA 20. All data presented were calculated from three independent experiments.

2.2.18 Bioinformatic methods

2.2.18.1 Splice site scores

In order to analyze splice junctions in the *CYP11B2* gene, splice site sequence motifs were scored using the splice site models introduced by Yeo and Burge (Yeo and Burge, 2004) and the available software at: http:// genes.mit.edu/burgelab/maxent/5ss. Briefly, splice site models that take into account adjacent and nonadjacent dependencies are built under the MaxENT using large datasets of human splice sites (Yeo and Burge, 2004). These splice site models assign a log-odd ratio (MaxENT score) to a 9bp (3 bp of

exon and 6 bp of intron) (5' splice site). The higher the score, the higher the probability that the sequence is a true splice site. Also, it can be argued that given two sequences of differing scores, the higher scoring sequence has a higher likelihood of being used.

2.2.18.2 Sequence analyses

The alignment of sequences was performed using the program CLUSTALW 1.8. (http://www.ebi.ac.uk/clustalw/). Translations of amino acids were carried out by using the Translate tool (Gasteiger et al., 2003) and the software available at http://au.expasy.org/.

2.2.18.3 Molecular modeling

We used the three-dimensional model of CYP11B2 from our laboratory (Belkina et al., 2001). The changes of residues in CYP11B2 were modelled by using the spdbv program (http:www.expasy.org/spdbv/) (Guex and Peitsch, 1997). The obtained model structures were energy minimized using the steepest descent algorithm implemented in the spdbv program. The structural representations were generated by using the ViewerLite program.

3. Results

The overall goals of the work in my thesis were to detect mutations in *CYP11B2* and *CYP11B1* genes of patients exhibiting disordered adrenal steroids (mineralocorticoids and glucocorticoids). To investigate the effects of these mutations on the steroid biosynthesis, the mutants were expressed in COS-1 and HCT116 cell lines and the conversion of steroids was studied.

In order to address each case separately, the results presented in this section as well as the subsequent discussion were divided into independent sections with describing each patient separately.

First, a new expression system for the CYP11B genes, the human cell line HCT116-p53$^{-/-}$ cells, has been investigated and compared with the well-established COS-1 cell line.

3.1 Evaluation of CYP11Bs expression in HCT116 p53$^{-/-}$ cells

To compare the expression of CYP11Bs in HCT116 p53$^{-/-}$ cells with COS-1 cells, first we analyzed optimal conditions for expression of CYP11Bs in HCT116 p53$^{-/-}$ cells. The pSVL vector containing cDNA of CYP11B1 (CYP11B1-WT) or CYP11B2 (CYP11B2-WT) was transfected into HCT116 p53$^{-/-}$ cells. The HCT116 p53$^{-/-}$ cells transfected with the plasmids were incubated with different concentrations of DOC (ranging from 1 to 20 µM) and different incubation times were used to optimize the incubation conditions to analyze the metabolites. The unlabeled steroid standards (DOC, B, 18-OH-B, Aldo) were visualized by UV light. Positions of steroid products on HPTLC plates are compared with those of the unlabeled steroid standards. The other compounds were unidentified. The optimal conditions were found to be a substrate concentration of 2 µM DOC and an incubation time of 72 h (see Figure 3.1).

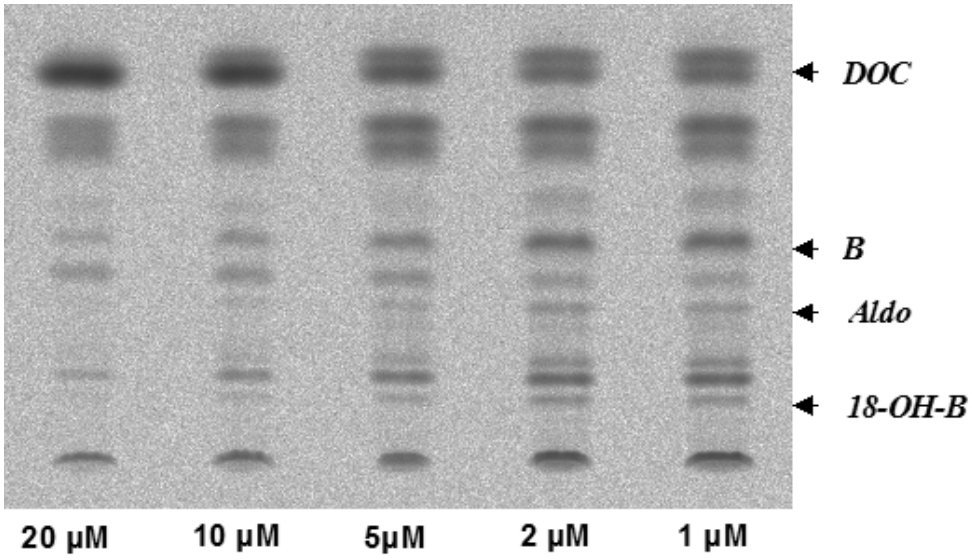


Figure 3.1: Autoradiography of thin layer chromatography of steroids produced by HCT116 p53$^{-/-}$ cells transfected with cDNA of CYP11B2 constructs and incubated with DOC. The transfected cells were incubated with substrate DOC (20 µM, 10 µM, 5 µM, 2 µM and 1 µM) and 5 nCi of 11-[^{14}C] deoxycorticosterone. Positions of steroids are marked on the autoradiogram as follows: DOC, 11-deoxycorticosterone; B, corticosterone; 18-OH-B, 18-hydroxycorticosterone; Aldo, aldosterone.

Furthermore, HCT116 p53$^{-/-}$cells were incubated with different concentrations of 11β-deoxycortisol (RSS) (ranging from 5 μM to 60 μM) and various incubation times were examined to optimize the incubation conditions. Non-radioactive steroid markers (RSS and F) that were visualized by UV light correspond to each of the expected radioactive steroid product. The optimal conditions in these concentrations were found to be a substrate concentration of 5 μM RSS and an incubation time of 72 h (see Figure 3.2).

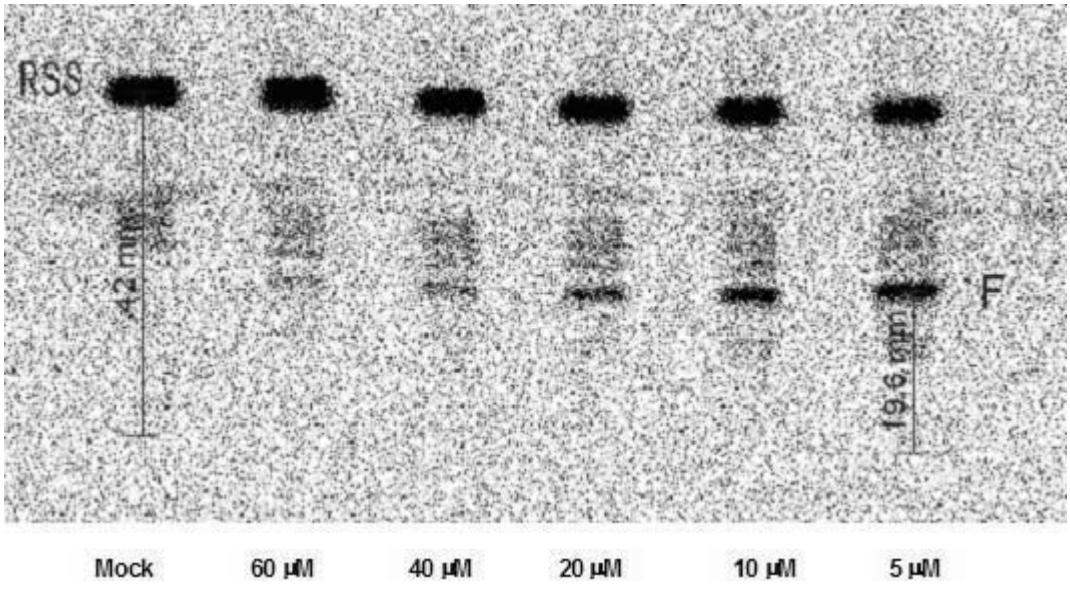


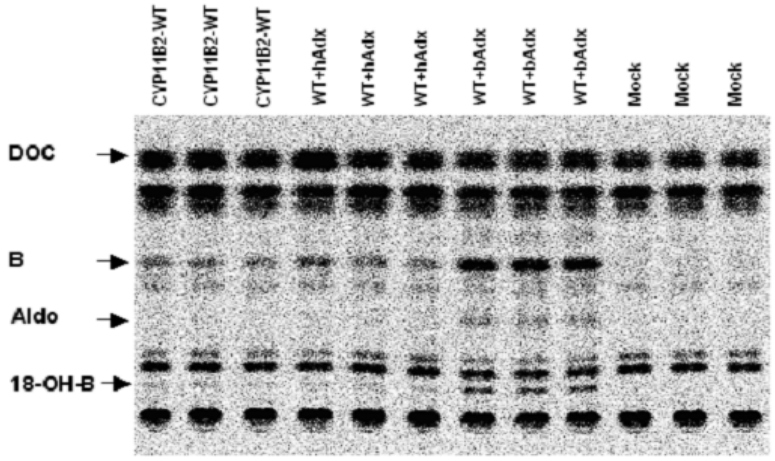
Figure 3.2: Autoradiography of thin layer chromatography of steroids produced by HCT116 p53$^{-/-}$ cells transfected with cDNA of CYP11B1 constructs and incubated with 11β-deoxycortisol with concentrations: 60 μM, 40 μM, 20 μM, 10 μM and 5 μM and 0.6 μCi of ^{3}H-RSS to follow the coversion of 11β-deoxycortisol to cortisol. Positions of steroids are marked on the autoradiogram as follows: RSS, 11β-deoxycortisol; F, cortisol.

To optimize the level of the electron transport mediator for the steroidogenic activity of the cell line, HCT116 p53$^{-/-}$ cells were co-transfected with bovine adrenodoxin plasmids (pBAdx) or human adrenodoxin plasmids (phAdx) and the pSVL vector containing *CYP11B2* cDNA (WT). The percentage of steroid products from HCT116 p53$^{-/-}$ cells co-transfected with WT and phAdx, as compared with WT alone, showed a 1.2 fold increase of corticosterone (B), a 1.3 fold increase of 18-hydroxycorticosterone (18-OH-B) but no statistically significant increase of aldosterone (Aldo). In case of co-transfection with WT

and pBAdx, the percentages of steroid products were increased by a factor of 2.9 for B, 2.8 for 18-OH-B and 3.1 for Aldo, compared with the percentage of steroid products from WT alone (see Figure 3.3). Thus, the co-expression of bovine adrenodoxin was demonstrated to be a useful approach to increase the activity of human CYP11B2 in the HCT116 $p53^{-/-}$ cell system, which is in accordance with data on the co-expression of bovine adrenodoxin in COS-1 cells (Bottner et al., 1998; Bottner et al., 1996; Cao and Bernhardt, 1999a).

Comparison of the enzyme activities in HCT116 $p53^{-/-}$ and COS-1 cell lines upon co-expression with bovine adrenodoxin showed that the percentages of steroid products in the HCT116 $p53^{-/-}$ cell line were the same as those in the COS-1 cell line (see Figure 3.3). This means that the product pattern is not dependent on the cell line used so that both cell lines are applicable for studying the effect of mutations in human steroid hydroxylase genes.

A



B

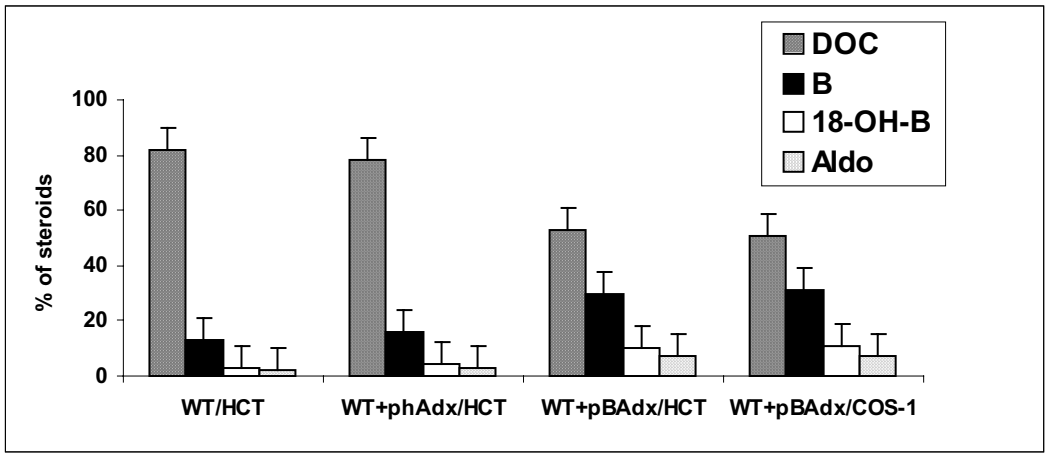


Figure 3.3: A, Autoradiography of thin layer chromatography of steroids produced by HCT116 $p53^{-/-}$ cells transfected with cDNA of CYP11B2 constructs and incubated with DOC. HCT116 $p53^{-/-}$ cells were transfected with 1.5 µg of hAdx (coding for human adrenodoxin) or pBAdx (coding for bovine adrenodoxin) and 1.5 µg of pSVL containing the cDNA of CYP11B2-WT or the empty vector pSVL as a negative control (Mock). The transfected cells were incubated with substrate DOC (2 µM DOC and 5 nCi of 11-[^{14}C] deoxycorticosterone). Positions of steroids were marked on the autoradiogram as follows: DOC, 11-deoxycorticosterone; B, corticosterone; 18-OH-B, 18-hydroxycorticosterone; Aldo, aldosterone. **B,** Enzyme activities of aldosterone synthase, co-expression of bovine adrenodoxin in HCT116 $p53^{-/-}$ cells and COS-1 cells. Steroid patterns of DOC conversion are given as mean ± SEM of four similar independent experiments performed in duplicate. The amounts of the substrate, the intermediates B and 18-OH-B and the final product Aldo are presented as percentages of total enzymatic activity.

3.2 Patient 1: *Aldosterone synthase deficiency caused by a homozygous L451F mutation in the CYP11B2 gene*

3.2.1 Case report *

The diagnosis of aldosterone synthase deficiency type I (corticosterone methyl oxidase type I - CMO I) was made on the basis of a GC-MS spot urinary steroid profile. An apparently normal male infant of Turkish parents started to vomit repeatedly from the age of 2 wk onwards. The serum sodium was 122 mM/L (normal range, 136-145 mM/L) and the serum potassium 7.1 mM/L (normal range, 3.5-4.5 mM/L). Spot urinary samples can be taken instead of 24h urine samples because the steroid profile of young children is not subject of the circadian rhythm. The pattern of urinary steroid metabolites showed on the one hand normal neonatal cortisol metabolites thus excluding all forms of congenital adrenal hyperplasia (cortisol biosynthesis defects) with salt wasting. In particular, spot urine concentrations [µg/L] of all major cortisol metabolites were normal, such as THE (tetrahydrocortisone, 416 µg/L, healthy controls [n = 47] mean ± SD: 875 ± 525 µg/L), 6α-OH-THE (6α-hydroxy-tetrahydrocortisone, 714 µg/L, controls: 542 ± 447 µg/L), β-CL (β-cortolone, 247 µg/L, controls: 235 ± 217 µg/L), 6α-OH-α-CL (6α-hydroxy-α-cortolone, 356 µg/L, controls: 785 ± 800 µg/L), and 6α-OH-β-CL (6α-hydroxy-β-cortolone, 486 µg/L, controls: 1001 ± 1086µg/L). On the other hand, metabolites of aldosterone precursors lacking 18-hydroxylation such as THA (tetrahydro-11-dehydrocorticosterone, 321 µg/L, controls: 68 ± 51 µg/L), and THB (tetrahydrocorticosterone, 36 µg/L, controls: 4 ± 10 µg/L) were elevated. Furthermore, compounds such as 6α-OH-THA (6α-OH-tetrahydro-11-dehydrocorticosterone), and hexahydro-11-dehydrocorticosterone (HHA) which are not present in healthy controls were clearly detectable. The urinary steroid profile obtained in the group of Prof. Wudy did not contain any 18-oxygenated metabolites (see Figure 3.4).

* Clinical data were obtained in the group of Prof. Stefan A. Wudy, Steroid Research Unit, Division of Pediatric Endocrinology and Diabetology, Center of Child and Adolescent Medicine, Justus-Liebig-University, Giessen, Germany.

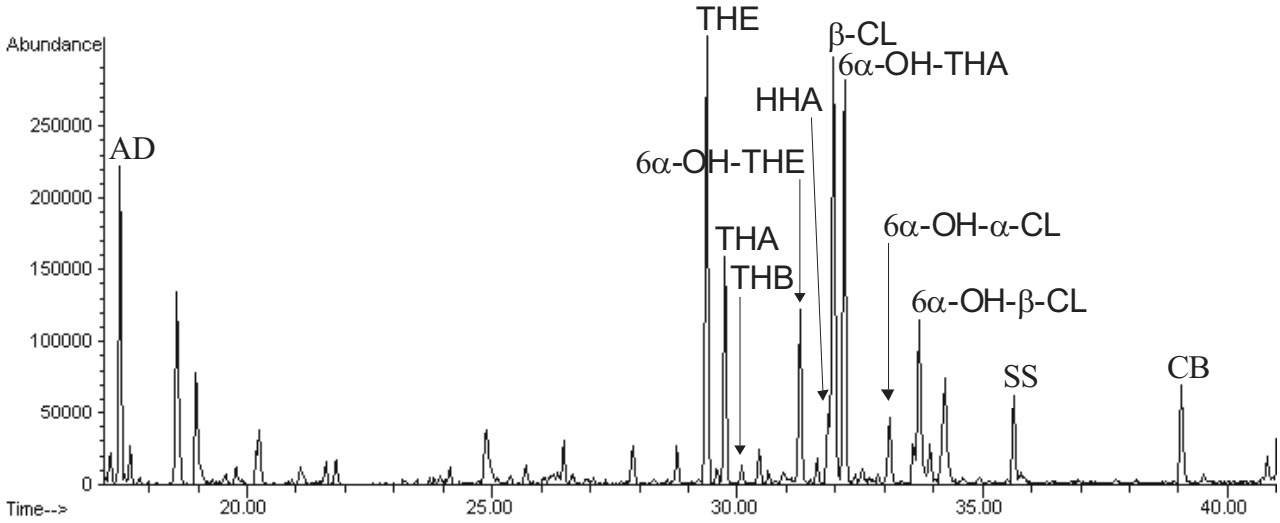


Figure 3.4: GC-MS urinary steroid profile from a sample of a newborn with aldosterone synthase deficiency type I. The profile was dominated by the huge peaks reflecting highly elevated corticosterone metabolites: THA (tetrahydro-11-dehydrocorticosterone), 6α-OH-THA (6α-hydroxy-tetrahydro-11-dehydrocorticosterone), HHA (hexahydro-11-dehydrocorticosterone), THB (tetrahydrocorticosterone). 18-oxygenated corticosterone metabolites were not detectable. Excretion of cortisol metabolites (THE, tetrahydrocortisone; 6α-OH-THE, 6α-hydroxy-tetrahydrocortisone; β-CL, β-cortolone; 6α-OH-α-CL, 6α-hydroxy-α-cortolone; 6α-OH-β-CL, 6α-hydroxy-β-cortolone) was normal. AD (5α-androstane-3α, 17α-diol), SS (stigmasterol) and CB (5-cholestene-3β-ol-butyrate) indicate internal standards.

Table 3.1: Urinary tetrahydrosteroid metabolite levels measured by gas chromatography-mass spectrometry.

	B THmetab (µg/24h)	18OHB THmetab (µg/24h)	Aldo THmetab (µg/24h)	B/18OHB metabolite ratio	18OHB/Aldo metabolite ratio
CMO I patients	**2870 ± 735**	**42.9 ± 25.1**	**0**	**84 ± 36**	∞
CMO II patients	**2930 ± 1250**	**1590 ± 1090**	**7.67 ± 4.87**	**1.76 ± 0.86**	**207 ± 106**
Normal subjects	**13.8 - 117**	**3.0 - 20.0**	**0.2 - 20**	**7.8 ± 3.1**	**3.34 ± 1.28**
SH-patient	**357**	**0**	**0**	∞	**0**

THmetab, tetrahydrometabolites; **B**, corticosterone; **18-OH-B**, 18-hydroxy corticosterone; **Aldo**, aldosterone.

In addition, the comparison of urinary metabolite ratios of B/18OHB and 18OHB/aldosterone of this patient with those of normal subjects, CMO I patients and CMO II patients (see Table 3.1) indicated that the patient had aldosterone synthase deficiency type I.

3.2.2 Screening for mutations

To confirm the diagnosis of an aldosterone synthase defect in the CMO I patient, the *CYP11B2* genes of the patient and his parents were amplified specifically from the genomic DNA, and all nine exons and the exon/intron boundaries were sequenced. The *CYP11B2* gene was selectively amplified in two segments (exons 1-6, 4.5 kb, using primers E16F and E16R shown in Table 2.1; exons 3-9, 3.7 kb, using primers E39F and E39R shown in Table 2.1) because the *CYP11B2* gene is too long (7 kb) to obtain by a single PCR in figure 3.5. Two fragments contained overlapping regions for all *CYP11B2* gene. PCR products were loaded on 1% agarose (see Figure 3.6).

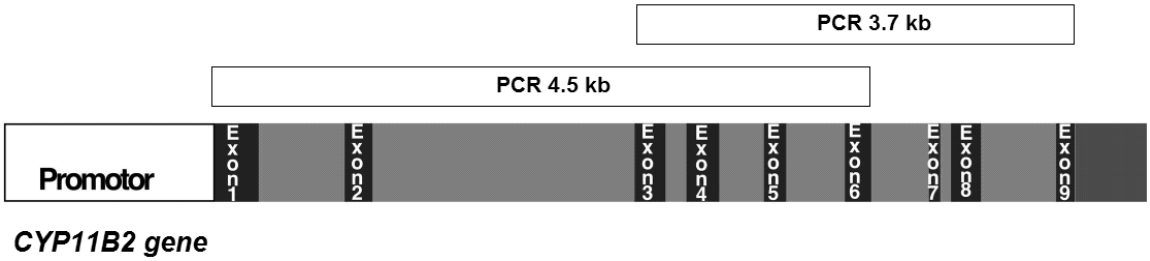


Figure 3.5: Amplification of *CYP11B2* gene. The two fragments contained overlapping regions.

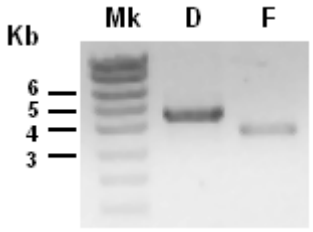


Figure 3.6: PCR of *CYP11B2* gene. Mk is marker lader, D fragment is 4.5 kb and F fragment is 3.7 kb. PCR products using *BIO-X-ACT* DNA polymerase (Bioline) were loaded onto 1% agarose gel.

After the *CYP11B2* gene of the patient and his parents was amplified specifically from the genomic DNA, all nine exons and the exon/intron boundaries were sequenced. One missense mutation was identified in exon 8 (see Figure 3.7A). The patient was found to be homozygous for the as-yet-unknown T to C point mutation at position bp 5869 (corresponding to c.1351T>C) in exon 8 of the *CYP11B2* gene. This mutation results in a substitution of leucine to phenylalanine at amino acid position 451 (L451F) of the CYP11B2 protein. Sequencing of the patient's *CYP11B2* gene revealed that the mutation in exon 8 was inherited from mother and father. Both parents bore the sequence change on one of the alleles and were heterozygous for the mutant allele (see Figure 3.7B and C). Furthermore, this patient was analyzed to be homozygous for a previously described R173K polymorphism in exon 3 (Portrat-Doyen et al., 1998). The *in vitro* expression of the R173K polymorphism in HCT116 $p53^{-/-}$ cells demonstrated that the polymorphism did not affect the CYP11B2 activity (data not shown), which is in agreement with a previous study (Portrat-Doyen et al., 1998).

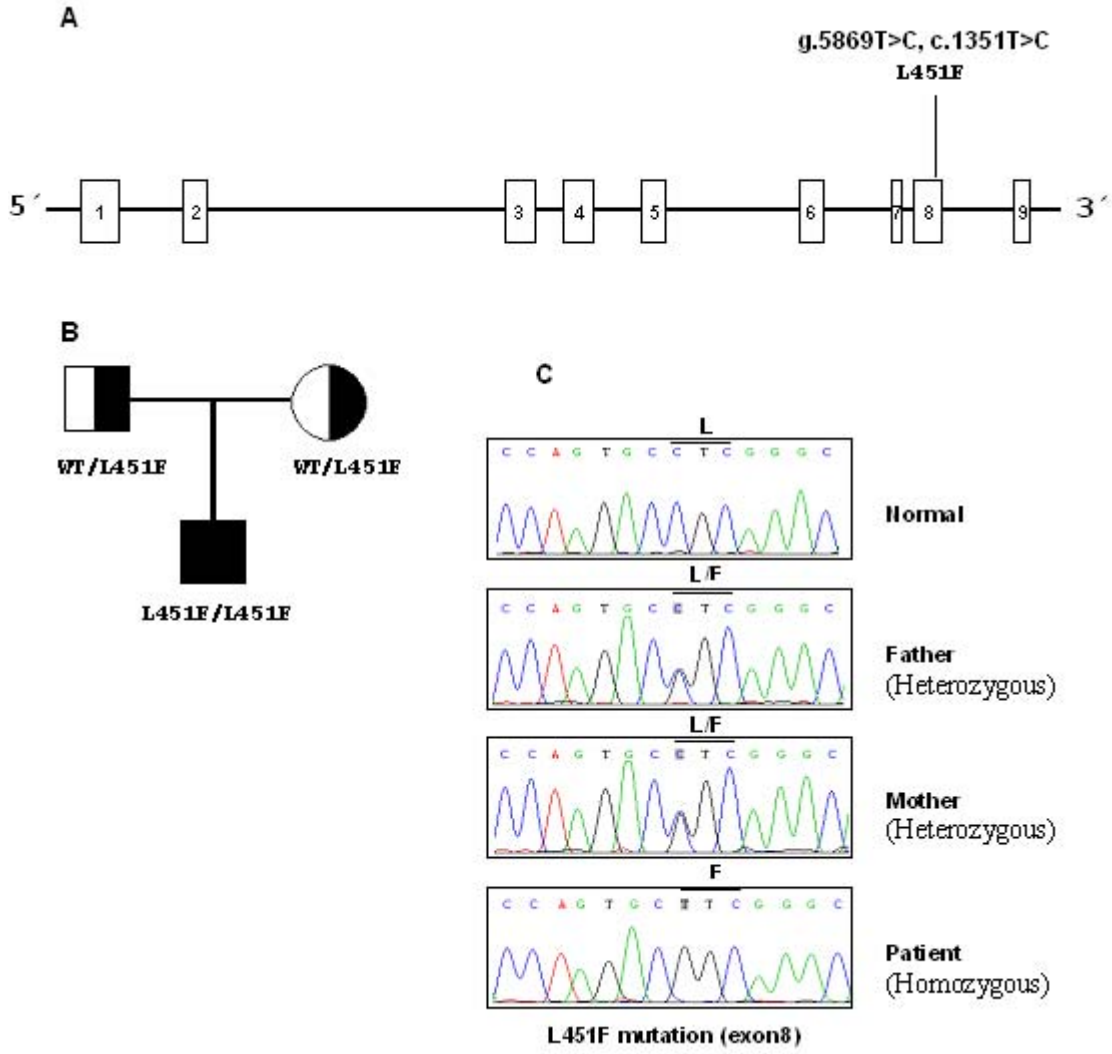


Figure 3.7: Mutant analysis by direct DNA sequencing. A, Location of novel mutation in the *CYP11B2* gene. **B**, Family pedigree showing inheritance of homozygous chromosomal segments. The father and the patient are represented by squares, and the mother is presented by circle. Both parents are heterozygous. The patient is homozygous and has two mutant alleles. **C**, The base change from T to C at position bp 5869 (corresponding to c.1351T>C) of *CYP11B2* cDNA leads to the substitution of leucine by phenylalanine at amino acid position 451.

3.2.3 *In vitro* expression of mutant L451F and assays of enzyme activity

To investigate whether the replacement L451F affects enzyme activity, a single mutation, L451F, was created by site-directed mutagenesis in the CYP11B2-WT. The successful insertion of the intended mutation was confirmed by sequence analysis.

The expression level of CYP11B2-WT and L451F mutant in COS-1 cells was confirmed by Western blot analysis (see Figure 3.8). All sample lanes on the SDS PAGE contained the same amount of proteins (200 µg), which could be confirmed by a single nonspecific band at approximately 32.5 kDa present in each lane on Western blot. Upon the immunoblot analysis, the expression of both the CYP11B2-WT aldosterone synthase as well as the L451F mutant was detected as a band of the correct size of approximately 48.5 kDa. However, the expression level of the L451F mutant appears to be significantly lower as compared with that of the CYP11B2-WT.

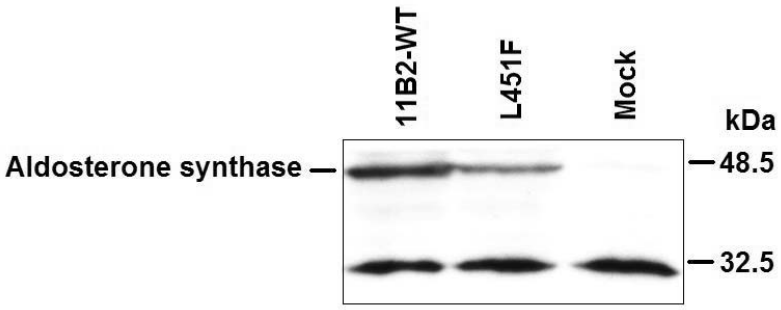


Figure 3.8: Western blot analysis of CYP11B2 expression in COS-1 cell line. Transfected cells were lysed after 48-h incubation with medium containing substrate (DOC). The proteins of wild-type (11B2-WT), mutant (L451F) and Mock (pSVL without 11B2cDNA) were separated by SDS/PAGE. After transfer to nitrocellulose membrane, the specific proteins were detected with an antihuman-CYP11B rabbit antiserum and visualized by ECL Western blot kit.

After the expression of the proteins was confirmed by Western blot analysis, the transfected HCT116 $p53^{-/-}$ cells as well as COS-1 cells were incubated either with DOC or B as substrate to check their ability of the expressed enzymes to convert the steroids. Steroids were extracted from medium after 72 h of incubation and separated by thin layer chromatography (see Figure 3.9). The profiles of steroid metabolites from cells expressing the CYP11B2-WT enzyme contained all expected reaction products (corticosterone, 18-hydroxycorticosterone and aldosterone). In contrast to this, the profiles of cells expressing the L451F mutant were similar to the profiles of mock transfected cells (vector pSVL as a negative control), which indicated that none of the steroid products was produced. Thus, the L451F mutant was enzymatically inactive concerning the 11-hydroxylation with DOC as well as the 18-hydroxylation with B as

substrate (see Figure 3.9). These results demonstrated that in the L451F mutant the enzyme activity of CYP11B2 is completely abolished, being in perfect agreement with the urinary steroid profile of a CMO I deficiency.

Taken together, a novel missense mutation (L451F) was detected within the CYP11B2 of a patient suffering from CMO I deficiency. The expression of the mutant in HCT116 $p53^{-/-}$ and COS-1 cells demonstrated that the L451F mutant abolished the CYP11B2 activity. This explained the relationship between genotype and phenotype of patient.

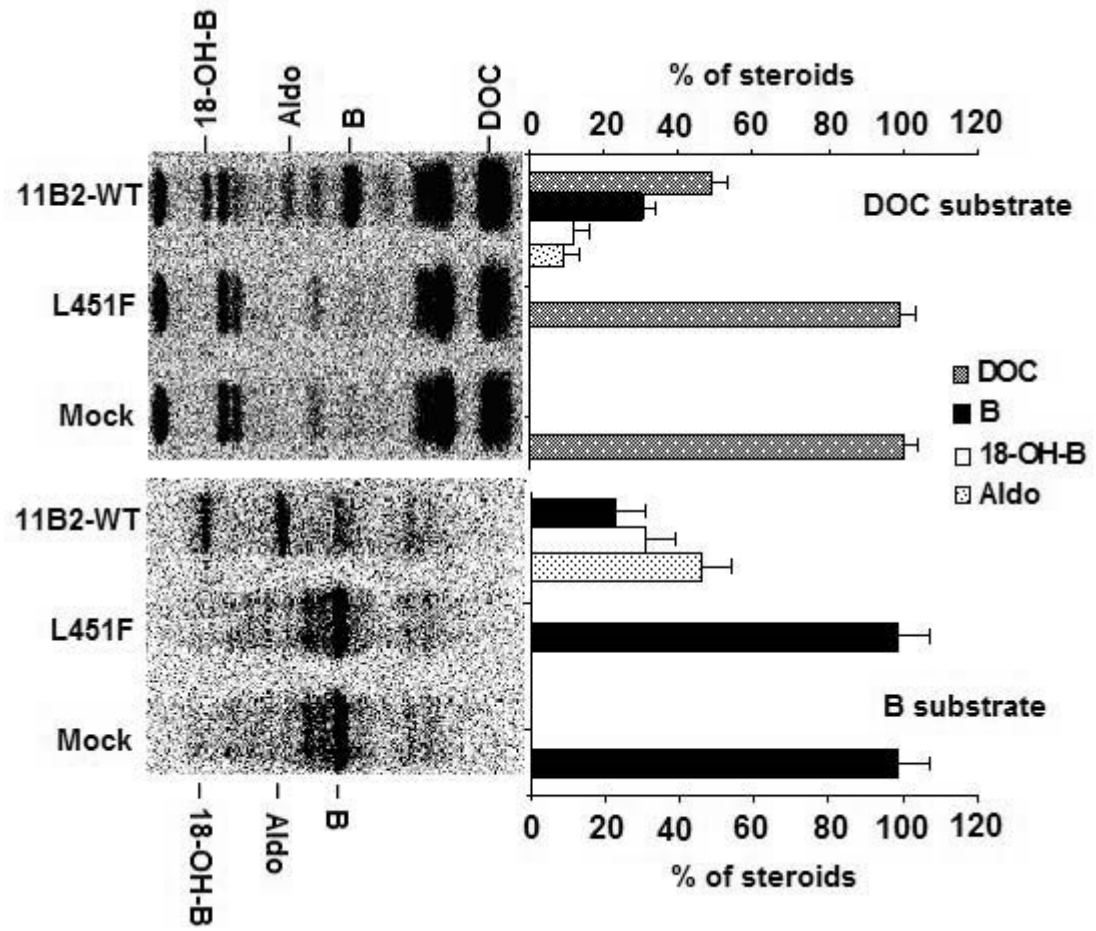


Figure 3.9: Autoradiography of thin layer chromatography of steroids and enzyme activities of CYP11B2 and L451F mutant expressed in HCT116 $p53^{-/-}$ cells and COS-1 cells. HCT116 $p53^{-/-}$ cells (upper TLC) and COS-1 cells (lower TLC) were transfected with 1.5 µg of pBAdx (coding for bovine adrenodoxin) and 1.5 µg of pSVL containing the cDNA of CYP11B2-WT or the mutant constructs encoding the substitution L451F or the empty vector pSVL as a negative control (Mock). The transfected cells were incubated with substrate DOC (2 µM DOC and 5 nCi of 11-[^{14}C] deoxycorticosterone) as well as substrate B (0.5 µM B and 0.6 µCi of 11-[^{3}H] corticosterone) and resulting metabolites were identified by TLC and autoradiography. Positions of steroids are marked on the autoradiogram as follows: DOC, 11-deoxycorticosterone; B, corticosterone; 18-OH-B, 18-hydroxycorticosterone; Aldo, aldosterone. Steroid patterns of DOC and B conversion are given as mean ± SEM of four similar independent experiments performed in duplicate. The amounts of the substrate, the intermediates B and 18-OH-B and the final product Aldo are presented as percentages of total activity.

3.3 Patient 2: *The effect of amino acid substitutions R490Δ1nt and silent mutant in the last nuculeotide of exon 5 in CYP11B2 on aldosterone synthase*

3.3.1 Case report ♣

HK patient is a few weeks old girl, which the age of a few life weeks was suffering from salt loss. The diagnosis showed a lack of 18-hydroxylase activity. At that time, she was treated with 0.1 mg Astonin (9-alpha-fluorohydrocortisone) daily. The patient's weight normalized and her further development has been uneventful since then.

Table 3.2. Plasma steroid levels (After 16 yrs treatment)

	B (ng/dl)	18OHB (ng/dl)	Aldo (ng/dl)	B/18OHB	18OHB/Aldo
HK-Patient	1420	105	25	13.5	4.2
CMO I pattients	700-5300	2.3-16	<3	>40	N.A.
CMO II patients	700-5300	438-2090	< 3-normal	<10	>100
Normal subjects	100-1000	12-55	5-60		

Aldo: aldosterone; B: corticosterone; 18OHB: 18-hydroxy-corticosterone; N.A.: Not applicable.

After 16 yrs treatment with Astonin, thebiochemical parameters presented sodium, 139 mM/liter (normal range, 136-145mM/liter); potassium, 4.0 mM/liter (normal range, 3.5-5.0 mM/liter); chloride, 103 mM/liter; ACTH, 10.9 pg/ml; aldosterone, 7.4 ng/dl (standard 5-60 ng/dl). During 4 wks without 0.1 mg Astonin dose per day, she felt in good physical condition. After 4 wks, the biochemical parameters showed sodium, 135 mM/liter, chloride, 105 mM/liter; potassium, 4 mM/liter. The multi-steroid analyses of the patient presented aldosterone, 25 ng/dl; corticosterone, 1420 ng/dl (clearly increase);

♣ Clinical data were obtained by Prof. Michael B. Ranke in Pediatric Endocrinology Section, University-Children's Hospital, Tuebingen, Germany.

11-deoxycorticosterone, 22 ng/dl (normal); cortisone, 700 ng/dl (normal); 18-OH-B, 105 ng/dl (clearly increase); 18-OH-DOC, 34 ng/dl (easily increase); a ratio of B/18-OH-B, 13.5, a ratio of 18-OH-B/Aldo, 4.2 (normal). The ratios of B/18-OH-B and 18-OH-B/Aldo were not typical of either CMO I or CMO II (see Table 3.2). Nevertheless, due to the original diagnosis of CMO I deficiency, the patient have been investigated in more detail.

3.3.2 Screening for mutations

To confirm the diagnosis of an aldosterone synthase defect in the HK patient, the *CYP11B2* gene of the patient and her family was amplified specifically from the genomic DNA, and all nine exons and the exon/intron boundaries were sequenced. The patient was found to be heterozygous for a deleted G point mutation at position bp 6424 (corresponding to c.1470GΔ1nt) in exon 9 of the *CYP11B2* gene. To confirm the c.1470GΔ1nt in one allele in exon 9, the PCR product of exon 9 was subcloned into a TOPO vector. The plasmid was sequenced to verify the deletion. This novel mutation (R490Δ1nt) shifted the translational reading frame of CYP11B2 by adding 170 amino acids, which resulted in a change of CYP11B2 protein. Furthermore, one silent mutation at position bp 4182 (corresponding to c.954G>A) was detected at the last nucleotide of exon 5 (T318T) in the other allele (see Figure 3.10A and C). Sequencing of the *CYP11B2* gene of her parents revealed that the c.1470GΔ1nt (R490Δ1nt) mutation was inherited from the mother and silent mutation c.954G>A at the last nucleotide of exon 5 was inherited from the father. Both parents bore the sequence change on only one allele. The family pedigree indicated inheritance of the heterozygous chromosomal segments (see Figure 3.10B). Thereafter, the R490Δ1nt mutant and the silent mutation at the last nucleotide of exon 5 were analyzed *in vitro* as shown below.

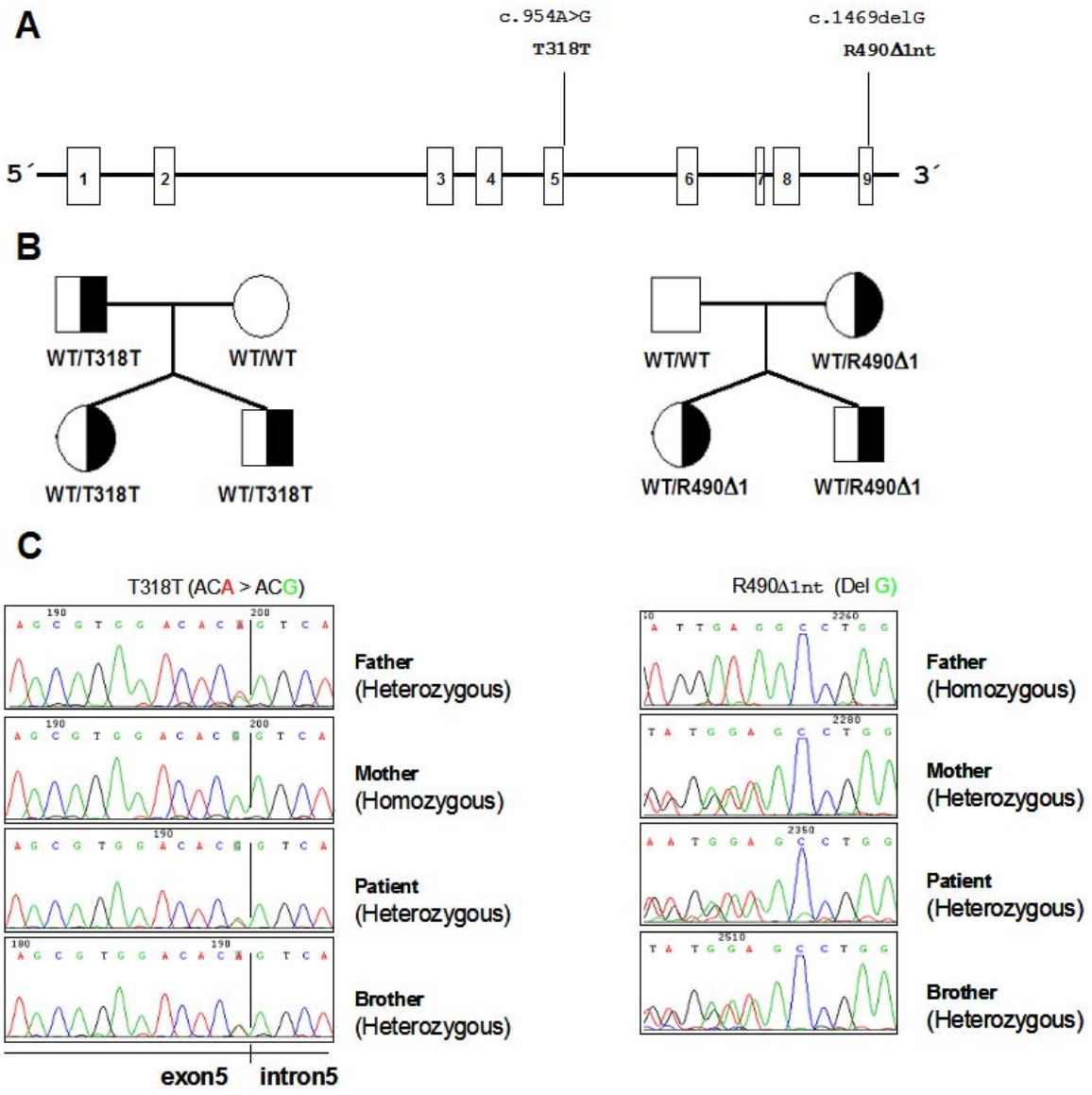


Figure 3.10: Mutation analysis by direct DNA sequencing. **A**, Location of novel mutations in the *CYP11B2* gene. **B**, Family pedigree showing inheritance of heterozygous chromosomal segments. The mother and the patient are represented by circles; the father and the brother are represented by squares. Both parents are heterozygous and have one mutant allele. The patient and brother are heterozygous and have two mutant alleles. **C**, a G deletion at position bp 1470 of *CYP11B2* cDNA leading to frameshift for the next residues of CYP11B2 protein of patient, brother and mother, was detected in one allele. The A replacement of G at the last nucleotide of exon 5 was detected in paternal, brotherly, and patient allele. The parents were found to be heterozygous for each mutation.

3.3.3 *In vitro* expression and assays of enzyme activity for R490Δ1nt mutant

In order to study the effect of the mutant R490Δ1nt on the aldosterone synthesis of this patient, one single R490Δ1nt mutant was created by site-directed mutagenesis in pSVL vector containing *CYP11B2*cDNA (CYP11B2-WT). The successful insertion of the intended mutations was confirmed by sequence analysis.

Western blot analysis of the CYP11B2-WT and mutant protein expressed in COS-1 cells demonstrated that R490Δ1nt mutant did not affect the translation efficiency (see Figure 3.11).

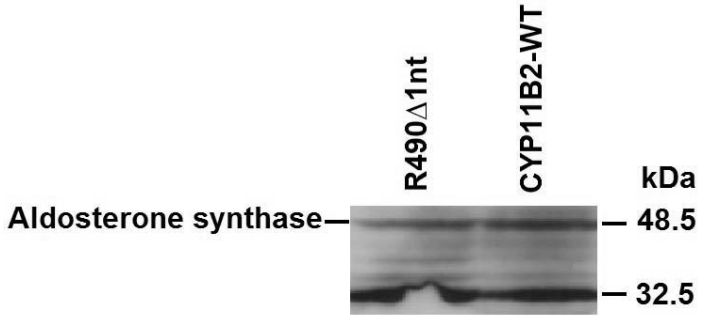


Figure 3.11: Western blot analysis of CYP11B2 expression in COS-1 cell line. Transfected cells were lysed after 48-h incubation with medium containing substrate (DOC). The proteins of CYP11B2-WT and mutant R490Δ1nt were separated by SDS/PAGE. After transfer to nitrocellulose membrane, the specific proteins were detected with an antihuman-CYP11B rabbit antiserum and visualized by ECL Western blot kit.

To check the enzymatic activity of mutation (R490Δ1nt), the transfected COS-1 cells were incubated with DOC as substrate. Medium of transfected cells was extracted after 72 h incubation, and steroids were separated by thin layer chromatography. The profiles of steroid metabolites from mutant R490Δ1nt were identical to the pattern of the Mock sample (vector pSVL as a negative control). There was no production of corticosterone, 18-hydroxycorticosterone and aldosterone, as compared to the WT production. The

expression studies showed that R490Δ1nt mutant displayed no activity with DOC as substrate (see Figure 3.12). These results demonstrated that the R490Δ1nt mutant completely abolished the enzyme activity of CYP11B2.

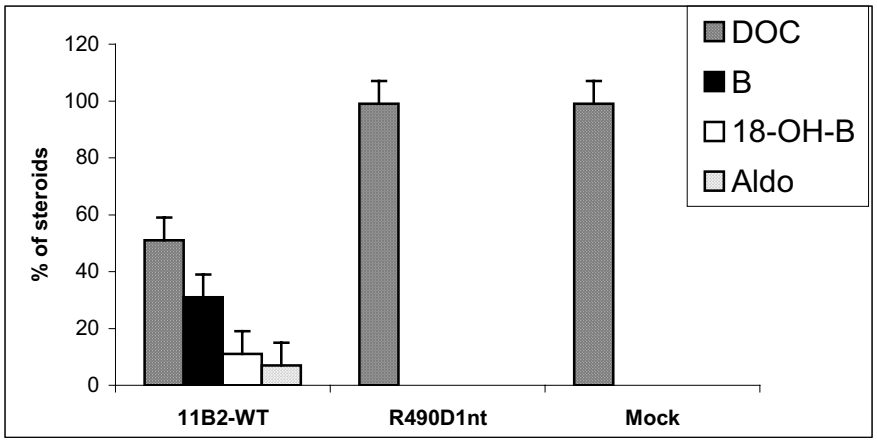


Figure 3.12: Enzyme activities of aldosterone synthase in COS-1 cells. COS-1 cells were transfected with 1.5 μg of pBAdx (bovine adrenodoxin) and 1.5 μg of pSVL containing the cDNA of CYP11B2-WT or the mutant constructs encoding the substitution R490Δ1nt or the empty vector pSVL as a negative control (Mock). 30 μM DOC and 5 nCi of [^{14}C]-DOC were used. Steroid patterns of DOC conversion are given as mean ± SEM of four similar independent experiments performed in duplicate. The amounts of the substrate, the intermediates corticosterone (B) and 18-hydroxycorticosterone (18-OH-B) and the final product aldosterone (Aldo) are presented as percentages of total activity.

3.3.4 Analysis of the splice site scores

In order to predict that the silent mutation at the last nucleotide of exon 5 in the other allele affects splicing, the replacement of adenine (A) to guanine (G) was analyzed with the splice site scores. The splice site scores were generated with (http://genes.mit.edu/burgelab/maxent/Xmaxent.html) a web-based resource. MaxEntScan is based on the approach for modeling the sequences of short sequence motifs such as those involved in RNA splicing, which simultaneously accounts for non-adjacent as well as adjacent dependencies between positions. A larger score generally indicates a larger 'strength' of the corresponding splice site. In this case, splice site score of the gene without mutation by using the maximum entropy model (MaxEnt) is 4.63 and the splice site score of silent mutation (g.4182G>A or c.954G>A) by using MaxEnt is -

5.75. Furthermore, splice site score of the gene without mutation by using the position weight matrix (PWM) is 4.91 and the splice site score of the mutation (g.4182G>A or c.954G>A) is 1.74. These results predicted that the substitution of A to G at the postion c.954 of the *CYP11B2* gene alternates the splice site.

3.3.5 Minigene construction

Mutants occurring in the boundary of exon and intron could affect the splicing of the gene. To analyze effects of the A to G mutation on pre-mRNA splicing, two minigene constructs designated pWT (normal) and pNH (mutation), consisting of exon 1 to exon 6 cloned into pRc/CMV vector, were generated from genomic DNA of a healthy subject and the patient. The fragment from exon 1 to exon 6 was amplified by using the primers shown in Table 2.1. PCR products (4.5 kb) of the healthy subject and the patient were excised with *Not*I/*Xba*I (restriction sites in primers) and cloned into *Not*I and *Xba*I sites of pRc/CMV vector (Invitrogen). The splice sites in the pWT and pNH were sequenced to verify the integrity of the inserts. The two minigenes possess the same sequence, except for the A to G mutation, as comfirmed by sequence analysis (see Figure 3.13).

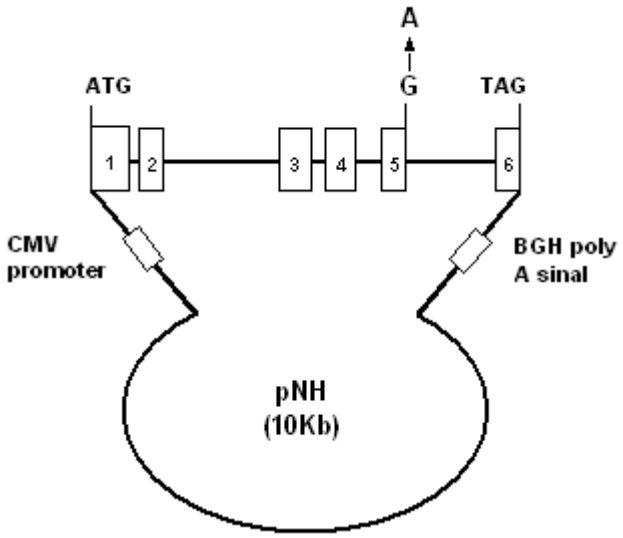


Figure 3.13: Minigene construction. The minigene (pNH) and (pWT) contained of exon 1 to exon 6 of *CYP11B2* gene cloned into pRc/CMV vector. The minigenes contained a cytomegalovirus (CMV) enhancer-promoter and a bovine growth hormone gene (BGH) polyadenylation signal for complete synthesis of mRNA. The pNH vector possessed A and pWT vector possessed G at the last nucleotide of exon 5.

3.3.6 Transcription analysis of the silent mutation in COS-1 cells

To confirm the effects of the silent mutation (T318T - c.954G>A) on the transcription of *CYP11B2* gene, COS-1 cells were transiently transfected with the minigene constructs consisting of *CYP11B2* genomic DNA from exon 1 to exon 6 with the mutation (pNH) or without the mutation (pWT). After incubation for 24hrs, the cells were harvested and then extraction of total RNA was performed according to the instruction of RNAesy kit (Qiagen). Total RNA was loaded into 1% agarose gel (see Figure 3.14). The concentration of total RNA was 1 μg/μl.

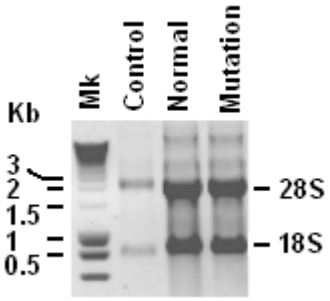


Figure 3.14: Total RNAs were loaded into 1% agrose gel. Total RNAs (28S and 18S of rRNA) were extracted from COS-1 cells that were transfected with plasmid containg exon 1 to exon 6 mutation (pNH) and normal (pWT).

The cDNAs were synthesized from total RNA of mutant and normal fragment. Two micrograms of total RNA and oligo (dT) primer were used for reverse transcription, which was performed according to the instructions of the kit (Invitrogen). To detect the fragment of the *CYP11B2* gene in the cDNA products, the PCR was performed with E4E6sF, E4E6sR1 primers shown in Table 2.1 using cDNA products as template. PCR products were separated using 0.8% agarose (see Figure 3.15). The PCR product from normal cDNA is 0.5 kb, but the PCR product from mutant cDNA is 1.3 kb. This result indicates that there is an alteration in the transcription of pNH.

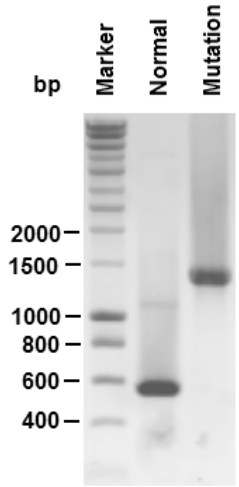


Figure 3.15: RT-PCR products of minigene transcriptions. RNA was extracted from the transfected COS-1 cells. Then, RT-PCR was performed to detect the alternative splicing by using the forward primer in exon 4 and reverse primer in exon 6. The RT-PCR product (0.5 kb) of the gene fragment without mutations consists of exon 4, exon 5 and exon 6, whereas the RT-PCR product (1.3 kb) of the mutant fragment keeps intron 5 (0.8 kb), leading to intronic retention in transcription of the *CYP11B2* gene.

To investigate the two fragments in more detail, we performed the direct sequencing of the PCR products (1.3 kb and 0.8 kb). Sequencing the RT-PCR product from the normal fragment showed correct splicing 526 bp (from exon 4 to exon 6) while the sequencing of the RT-PCR product from the mutant presented intron 5 retention (812 bp) in the RT-PCR product (1.3 kb) (see Figure 3.16). Thus, the *in vitro* data confirmed that the pWT could be spliced correctly, while the A replacement of G at the last nucleotide of exon 5 was responsible for the abnormal pre-mRNA splicing (intronic retention).

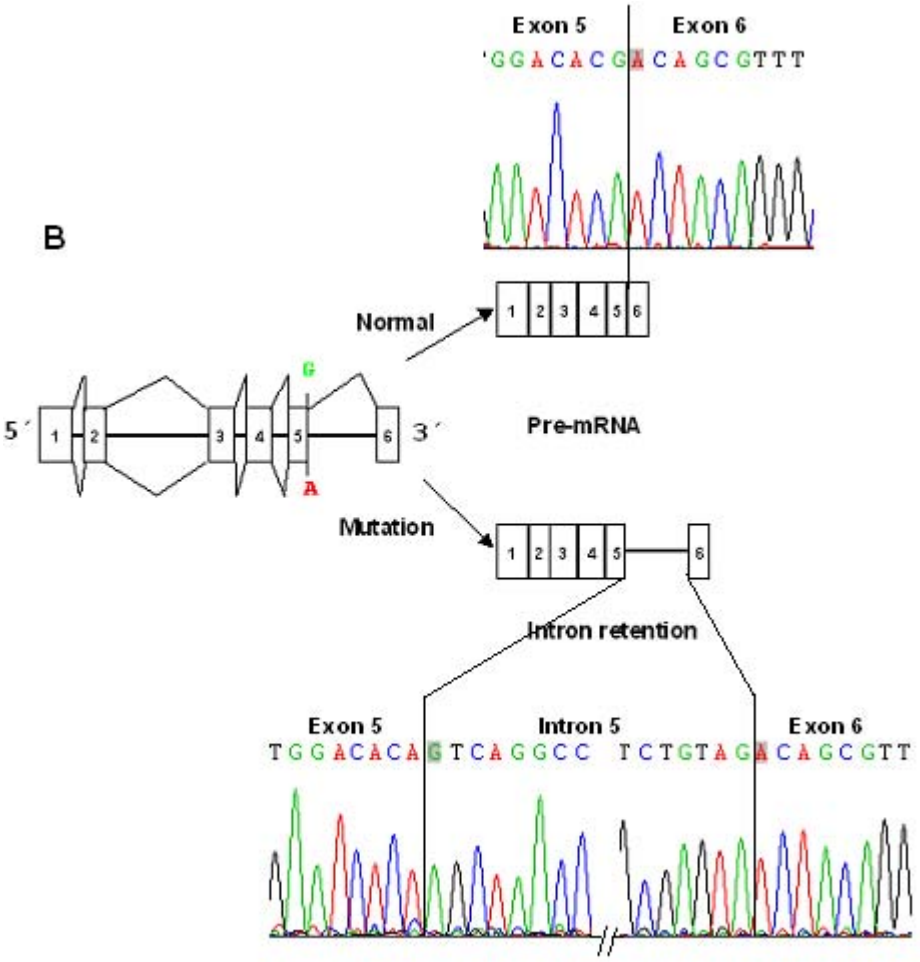


Figure 3.16: Sequencing of RT-PCR products from minigene transcriptions. The sequence (526 bp) of the normal fragment consists of exon 4, exon 5 and exon 6, whereas the sequence (1338 bp) of the mutant fragment keeps intron 5 (812 bp) that leads to intronic retention in transcription of the *CYP11B2* gene.

Taken together, a A replacement of G at the last nucleotide of exon 5 and deletion mutant R490Δ1nt were detected in *CYP11B2* gene from the patient with aldosterone synthase deficiency. The *in vitro* expression studies indicated that R490Δ1nt mutant completely abolished the enzyme activity of CYP11B2. Furthermore, the A replacement of G at the last nucleotide of exon 5 affected pre-mRNA splicing by intronic retention. These results suggest that the two mutants completely abolished the enzyme activity of CYP11B2.

3.4 Patient 3: *Aldosterone synthase deficiency type I caused by a compound heterozygous S315R and R374W mutations in the CYP11B2 gene*

3.4.1 Case report *

Urinary tetrahydrosteroids of the WA patient were measured by GC/MS. Metabolites of aldosterone precursors lacking 18-hydroxylation such as THA (tetrahydro-11-dehydrocorticosterone, 203 µg/L, controls: 68 ± 51 µg/L), and THB (tetrahydrocorticosterone, 49 µg/L, controls: 4 ± 10 µg/L) were elevated. Furthermore, compounds such as 6α-OH-THA (6α-OH-tetrahydro-11-dehydrocorticosterone), and hexahydro-11-dehydrocorticosterone (HHA) which are not present in healthy controls were clearly detectable. The urinary steroid profile did not contain any 18-oxygenated metabolites but low tetrahydroaldosterone (THAldo), 3 µg/L. In addition, we compared urinary metabolite ratios of B/18OHB and 18OHB/Aldo of this patient with those of normal subjects, CMO I patients and CMO II patients (see Table 3.3). Urinary metabolite ratios of B/18OHB and 18OHB/Aldo indicate that the patient has aldosterone synthase deficiency type I.

Table 3.3: Urinary tetrahydrosteroid metabolite levels measured by gas chromatography-mass spectrometry

	B THmetab (µg/24h)	18OHB THmetab (µg/24h)	Aldo THmetab (µg/24h)	B/18OHB metabolite ratio	18OHB/Aldo metabolite ratio
CMO I patients	**2870 ± 735**	**42.9 ± 25.1**	**0**	**84 ± 36**	∞
CMO II patients	**2930 ± 1250**	**1590 ± 1090**	**7.67 ± 4.87**	**1.76 ± 0.86**	**207 ± 106**
Normal subjects	**13.8 - 117**	**3.0 - 20.0**	**0.2 - 20**	**7.8 ± 3.1**	**3.34 ± 1.28**
WA-patient	**252**	**0**	**3**	∞	**0**

THmetab, tetrahydrometabolites; **B**, corticosterone; **18-OH-B**, 18-hydroxy corticosterone; **Aldo**, aldosterone.

* Clinical data were obtained in the group of Prof. Stefan A. Wudy, Steroid Research Unit, Division of Pediatric Endocrinology and Diabetology, Center of Child and Adolescent Medicine, Justus-Liebig-University, Giessen, Germany.

3.4.2 Screening for mutations

In order to confirm the diagnosis of aldosterone synthase deficiency I of this patient, we amplified *CYP11B2* gene as shown for patient 1. All nine exons and boundaries of exon/intron were sequenced. Two missense mutants were identified in exon 5 and exon 6 (see Figure 3.17).

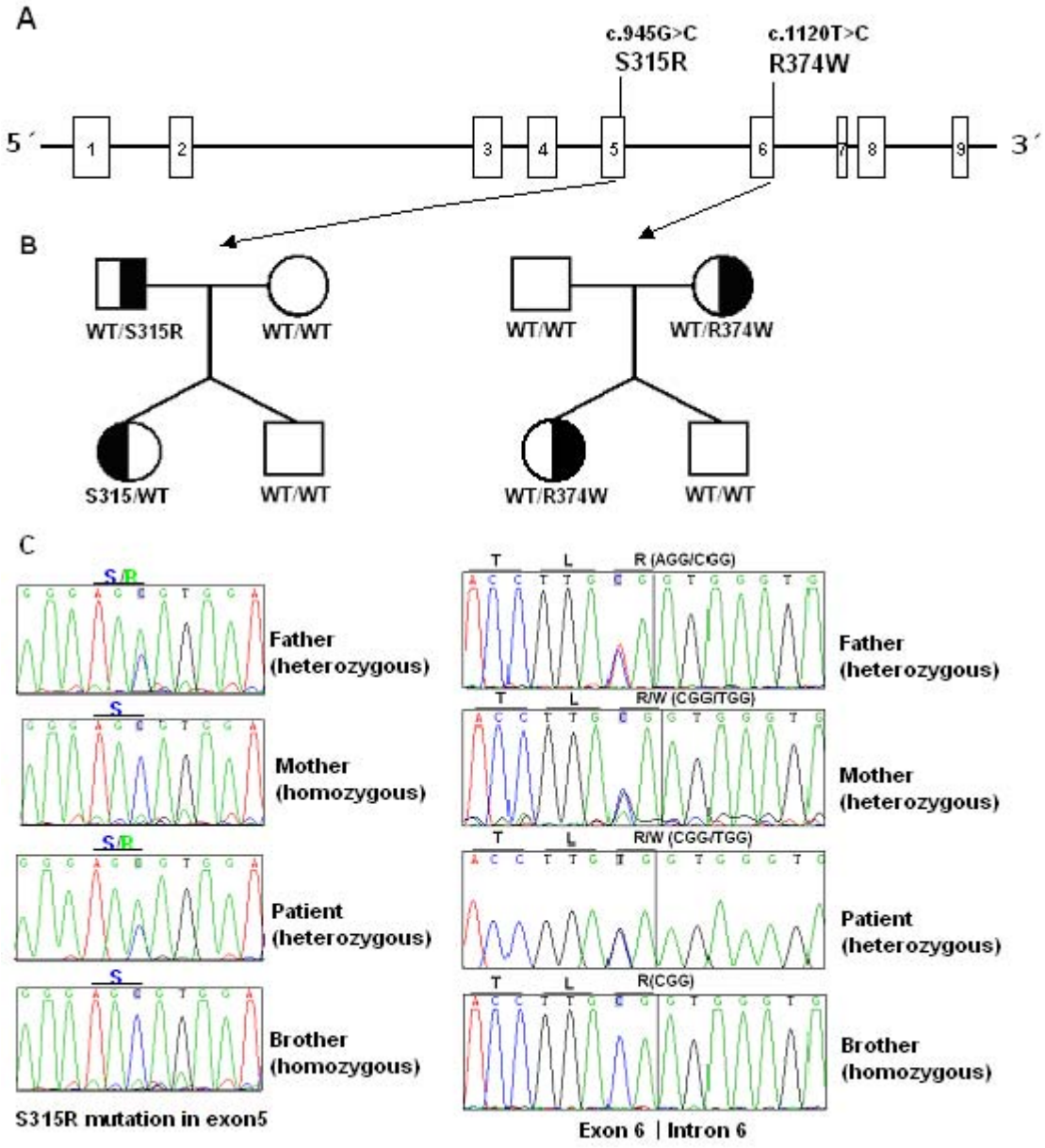


Figure 3.17. Mutant analysis by direct DNA sequencing. A, Location of point mutations in the *CYP11B2* gene. **B**, the family pedigree showing inheritance of heterozygous chromosomal segments. The father and brother are represented by squares, and the mother and the patient are

presented by circle. Both parents are heterozygous and have one mutant allele. The patient is heterozygous and has two mutant alleles. Mutations occur in one allele. The brother is homozygous and has two normal alleles. **C**, The base changes from G to C at position bp 4173 (corresponding to c.945G>C) and T to C at position bp 5160 (corresponding to c.1120T>C) lead to the substitution of serine by arginine at amino acid position 315 and arginine by tryptophan at amino acid position 374, respectively.

The patient was found to be heterozygous for the as-yet-unknown G to C point mutation at position bp 4173 (corresponding to c.945G>C) in exon 5 and the as-yet-unknown T to C point mutation at position bp 5160 (corresponding to c.1120T>C) in exon 6 in *CYP11B2* gene. The base change from G to C at position bp 4173 (corresponding to c.945G>C) of *CYP11B2* cDNA leads to the substitution of serine by arginine at amino acid position 315. The base change from T to C at position bp 5160 (corresponding to c.1120T>C) of *CYP11B2* cDNA leads to the substitution of arginine by tryptophan at amino acid position 374. Sequencing of the parent's *CYP11B2* gene revealed that the mutation in exon 5 was inherited from father and the mutation in exon 6 inherited from mother. The brother did not possess mutations in two alleles (see Figure 3.17B and C).

3.4.3 Functional analysis of enzyme activity

To analyze the two amino acid replacements (S315R, R374W) on the enzyme activity, two mutants were created by site-directed mutagenesis in the CYP11B2-WT using the oligonucleotides listed in Table 2.1. The successful insertion of the intended mutations was confirmed by sequence analysis.

The expression level of CYP11B2-WT and the two single mutants in COS-1 cells was examined by Western blot analysis (see Figure 3.18). All sample lanes of the SDS PAGE contained a similar amount of proteins (200 µg), which might be checked by a single nonspecific band at approximately 32.5 kDa present in each lane. The expression of aldosterone synthase of the CYP11B2-WT and the two single mutants was detected as a band of the correct size of approximately 48.5 kDa. The expression level of the two single mutants was similar to that of the CYP11B2-WT. Thus, Western blot analysis of the CYP11B2-WT and the two mutants (S315R, R374W) proteins expressed in the cells

demonstrated that none of the mutations apparently affected the translation efficiency (see Figure 3.18).

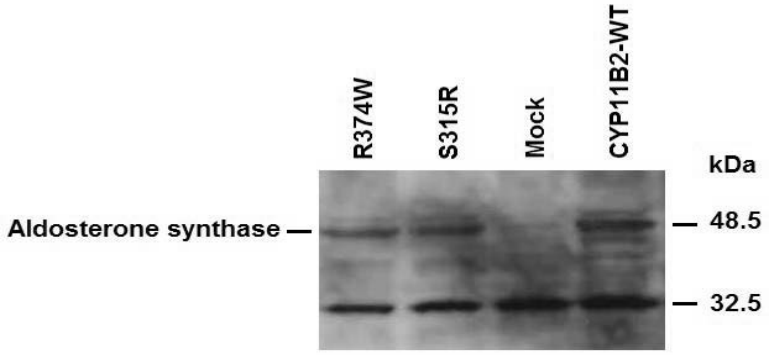


Figure 3.18: Western blot analysis of CYP11B2 WT and mutants (S315R and R374W) expression in COS-1 cell line. Transfected cells were lysed after 48-h incubation with medium containing substrate (DOC). The proteins of CYP11B2-WT, S315R, R374W and Mock (pSVL without 11B2cDNA) were separated by SDS/PAGE. After transfer to nitrocellulose membrane, the specific proteins were detected with an antihuman-CYP11B rabbit antiserum and visualized by ECL Western blot kit.

After the expression of the proteins was confirmed by Western blot analysis, the transfected COS-1 cells were incubated with DOC as substrate to check the ability of the expressed enzymes to convert the steroids. Steroids were extracted from medium after 72 h of incubation and separated by thin layer chromatography. The profiles of steroid metabolites from cells expressing the CYP11B2-WT enzyme contained all expected reaction products (corticosterone, 18-hydroxycorticosterone and aldosterone). In contrast to this, the profiles of cells expressing the single CYP11B2 mutant R374W were similar to the profiles of Mock transfected cells (vector pSVL as a negative control), which indicated that none of the steroid products was produced. In the steroid product from cells expressing the single mutant S315R, 9% of B was converted from DOC but no further products were produced, suggesting that mutant S315R dramatically decreased 11β-hydroxylation and abolished 18-hydroxylation and 18-oxidation. Thus, the expression studies showed that the two single mutants were enzymatically inactive i.e. not capable of converting DOC to Aldo (see Figure 3.19).

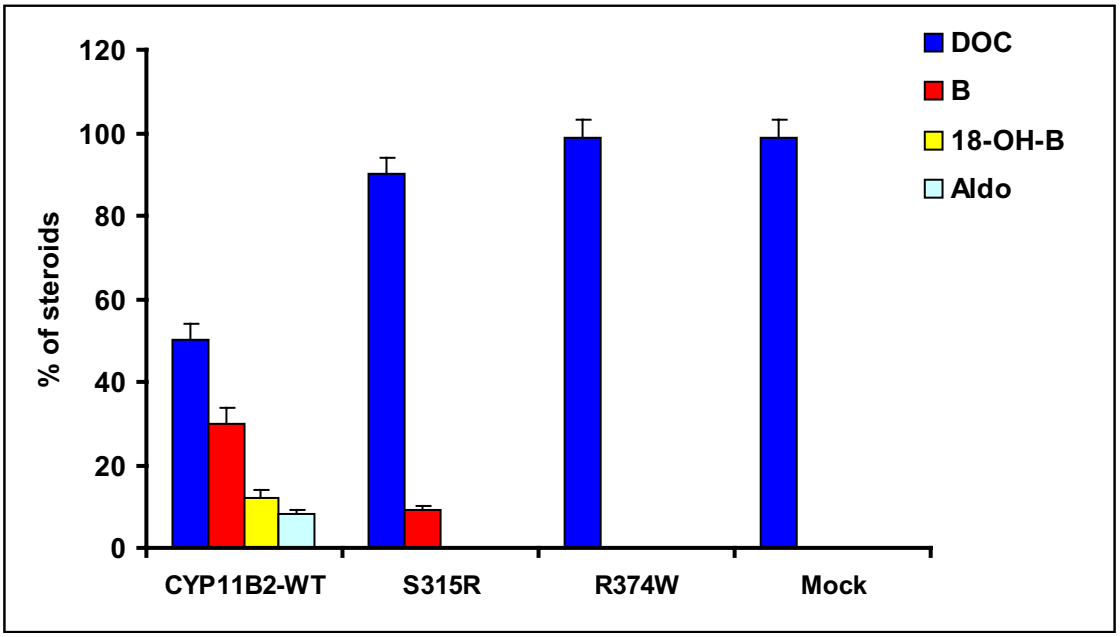


Figure 3.19: Enzyme activities of aldosterone synthase in COS-1 cells. COS-1 cells were transfected with 1.5 µg of pBAdx (bovine adrenodoxin) and 1.5 µg of pSVL containing the cDNA of CYP11B2-WT or the mutant constructs encoding the substitution S315R and R374W or the empty vector pSVL as a negative control (Mock). The transfected cells were incubated with substrate DOC (30 µM DOC and 5 nCi of 11-[^{14}C] deoxycorticosterone). Steroid patterns of DOC conversion are given as mean ± SEM of four similar independent experiments performed in duplicate. The amounts of the substrate, the intermediates corticosterone (B) and 18-hydroxycorticosterone (18-OH-B) and the final product aldosterone (Aldo) are presented as percentages of total activity.

In summary, two new missense mutants (S315R, R374W) were detected in the *CYP11B2* gene of this patient suffering from CMO I deficiency. The expression of the two single mutants in the COS-1 cells indicated that S315R and R374W mutants abolished enzyme activity.

3.5 Patient 4*: Mutant R181Q in CYP11B2 gene was detected in the patient with CMO II*

3.5.1 Case report *

Urinary tetrahydrosteroids were measured by GC/MS (Table 3.4). It is observed that the ratios of B/18OHB and 18OHB/Aldo of the patient match the symptoms of aldosterone synthase deficiency type II as compared with data from CMO II patients.

Table 3.4. Urinary tetrahydrosteroid metabolite levels measured by gas chromatography-mass spectrometry

	B THmetab (µg/24h)	18OHB THmetab (µg/24h)	Aldo THmetab (µg/24h)	B/18OHB metabolite ratio	18OHB/Aldo metabolite ratio
CMO I patients	**2870 ± 735**	**42.9 ± 25.1**	**0**	**84 ± 36**	∞
CMO II patients	**2930 ± 1250**	**1590 ± 1090**	**7.67 ± 4.87**	**1.76 ± 0.86**	**207 ± 106**
Normal subjects	**13.8 - 117**	**3.0 - 20.0**	**0.2 - 20**	**7.8 ± 3.1**	**3.34 ± 1.28**
WA-patient	**705**	**592**	**3**	**1.19**	**197.3**

THmetab, tetrahydrometabolites; **B**, corticosterone; **18-OH-B**, 18-hydroxy-corticosterone; **Aldo**, aldosterone.

3.5.2 Screening for mutations

To identify mutations in the *CYP11B2* gene that cause aldosterone synthase deficiency II of the patient, we amplified the *CYP11B2* gene as described for patient 1. All nine exons and boundaries of exon/intron were sequenced. One missense mutation was identified in exon 3 (see Figure 3.22A and C). A heterozygous G to A point mutation at bp 3348 in exon 3, corresponding to c.543G>A, that leads to a change from arginine to glutamine at amino acid position 181 of CYP11B2 was found in the patient and in the father. Mother and brother are normal. The pedigree of the family with the mutation R181Q in the

* Clinical data were obtained in the group of Prof. Stefan A. Wudy, Steroid Research Unit, Division of Pediatric Endocrinology and Diabetology, Center of Child and Adolescent Medicine, Justus-Liebig-University, Giessen, Germany.

CYP11B2 gene is shown in Figure 3.22B. Furthermore, this patient was found to be the same as a previously described R173K polymorphism in exon 3 (Portrat-Doyen et al., 1998), as described the patient 1.

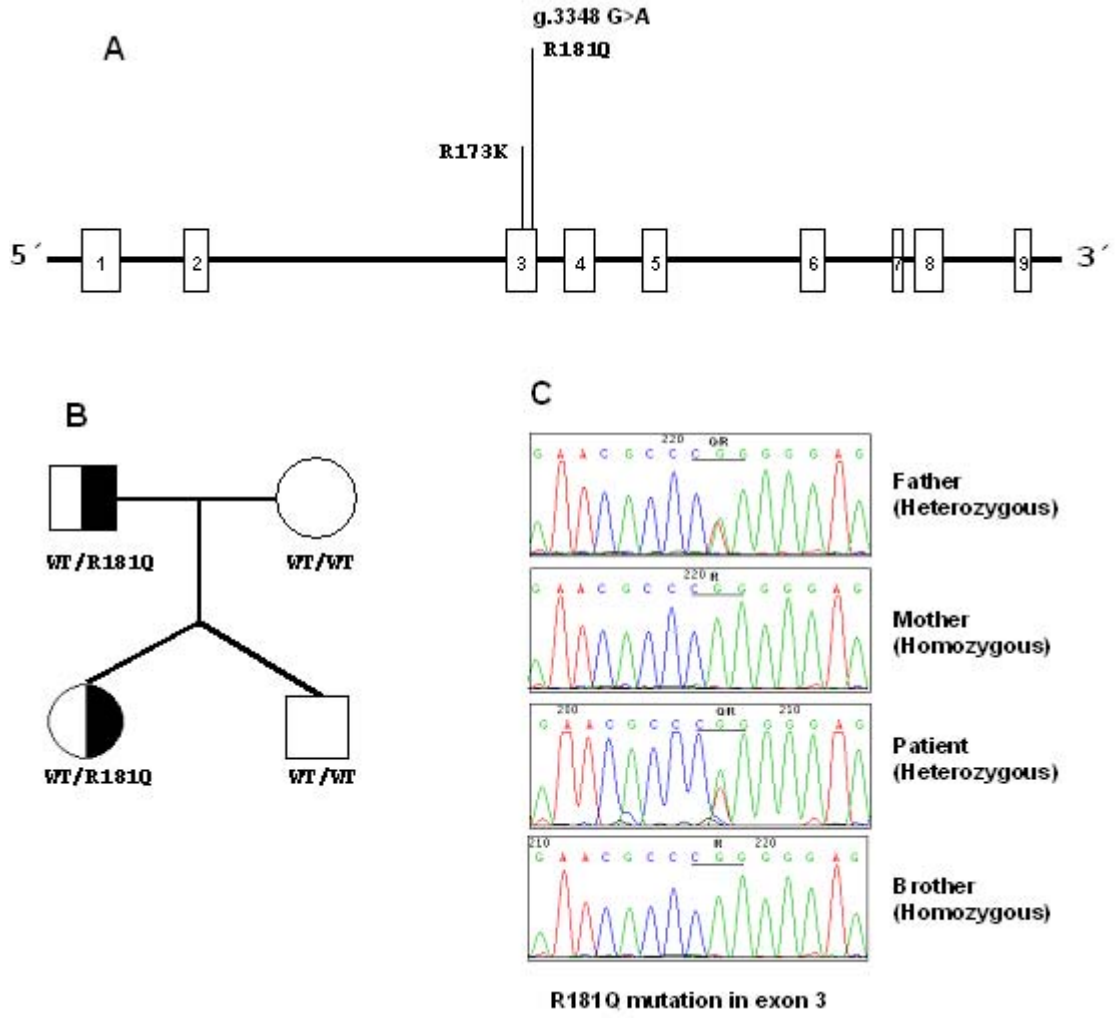


Figure 3.22: Mutant analysis by direct DNA sequencing. **A**, Location of point mutations and a polymorphism in the *CYP11B2* gene. **B**, the family pedigree showing inheritance of heterozygous chromosomal segments. The father and brother are represented by a square, the patient and the mother are represented by a circle. The father and the patient are heterozygous having one mutant allele. **C**, the base change from from G to A at position bp 3348 in exon 3 leads to the substitution from arginine to glutamine at codon 181. One mutant at amino acid 173, R173K, is considered to be a polymorphism in exon 3, which is in agreement with a previous study (Portrat-Doyen et al., 1998).

3.5.3 Expression of mutant R181Q and assays of enzyme activity

To analyze whether mutant R181Q affects enzyme activity, a single mutation, R181Q, was created by site-directed mutagenesis in the CYP11B2-WT using the primers shown in Table 2.1. The successful insertion of the intended mutation was confirmed by sequence analysis.

The expression level of CYP11B2-WT and the R181Q mutant in COS-1 cells was confirmed by Western blot analysis (see Figure 3.23). All sample lanes of the SDS PAGE contained the same amount of proteins (200 µg), which was confirmed by a single nonspecific band at approximately 32.5 kDa present in each lane. The expression of both the CYP11B2-WT aldosterone synthase as well as the R181Q mutant was detected as a band of the correct size of approximately 48.5 kDa. However, the expression level of the R181Q mutant appears to be significantly lower as compared with the CYP11B2-WT.

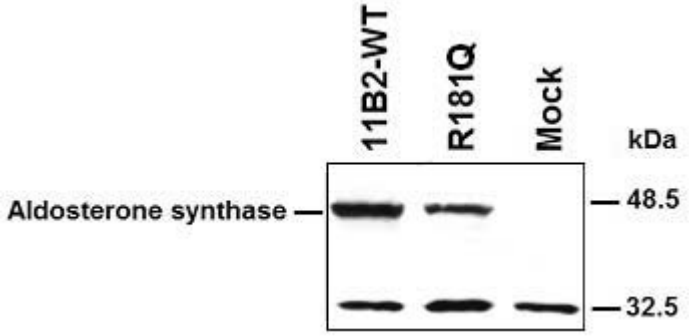


Figure 3.23: Western blot analysis of CYP11B2 expression in COS-1 cell line. Transfected cells were lysed after 48-h incubation with medium containing substrate (DOC). The proteins of CYP11B2-WT, mutant R181Q and Mock (pSVL without 11B2cDNA) were separated by SDS/PAGE. After transfer to nitrocellulose membrane, the specific proteins were detected with an antihuman-CYP11B rabbit antiserum and visualized using ECL kit from Amersham Pharmacia Biotech.

After the expression of the proteins was confirmed by Western blot analysis, the transfected COS-1 cells were incubated with DOC as substrate to check the ability of the expressed enzymes to convert the steroids. The profiles of steroid metabolites from cells expressing the CYP11B2-WT enzyme contained all expected reaction products (corticosterone, 18-hydroxycorticosterone and aldosterone). In the steroid profile from

the mutant R181Q, corticosterone (B) was increased, 18-hydroxycorticosterone (18-OH-B) was reduced, and no aldosterone (Aldo) was produced, as compared with the CYP11B2-WT and Mock sample (see Figure 3.24). Thus, these results indicated that the mutant R181Q abolished 18-oxidation.

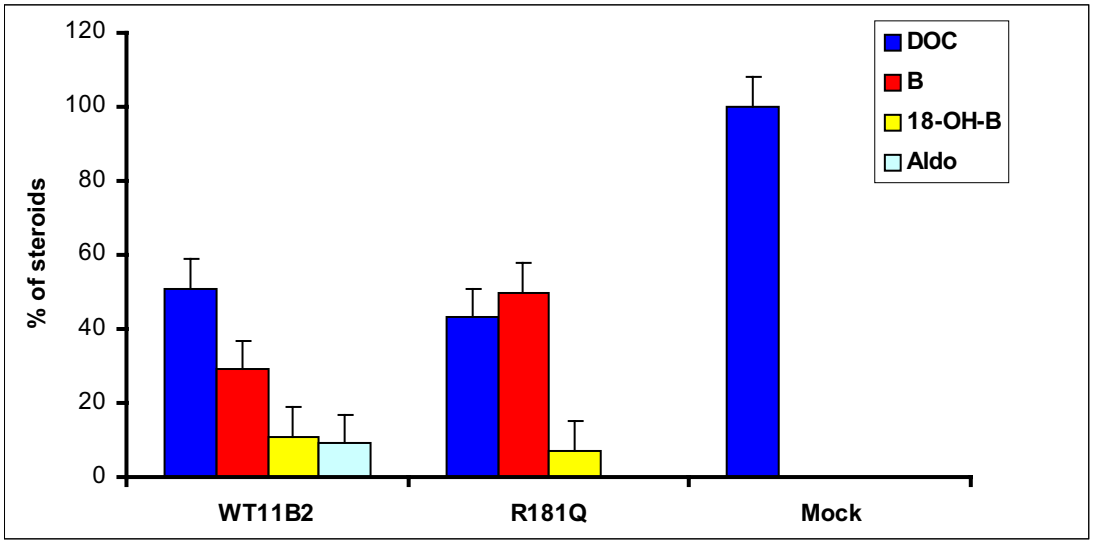


Figure 3.24: Enzyme activities of aldosterone synthase in COS-1 cells. COS-1 cells were transfected with 1.5 µg of pBAdx (bovine adrenodoxin) and 1.5 µg of pSVL vector containing the cDNA of CYP11B2-WT or the mutant constructs encoding the substitution R181Q or the empty vector pSVL as a negative control (Mock). 30 µM DOC and 5 nCi of [^{14}C]-DOC were used. Steroid patterns of DOC conversion are given as mean ± SEM of four similar independent experiments performed in duplicate. The amounts of the substrate, the intermediates corticosterone (B) and 18-hydroxycorticosterone (18-OH-B) and the final product aldosterone (Aldo) are presented as percentages of total activity.

Taken together, one missense mutant (R181Q) was found in *CYP11B2* gene of this patient with aldosterone synthase deficiency type II. The expression of mutant R181Q in COS-1 cells using the DOC substrate showed that mutant R181Q affected the 11β-hydroxylation, 18-hydroxylation and abolished 18-oxidation. No mutation has been detected in the second allele of 9 exons and boundaries of exon/intron.

3.6 Patient 5: *Complete virilisation in two 46,XX siblings with 11-β-hydroxylase deficiency due to homozygous L299P mutation in the CYP11B1 gene*

3.6.1 Case report ♠

There were two siblings from consanguineous Turkish parents with complete external virilisation. The karyotype of the two siblings was 46,XX. The MY patient had been raised as a boy and was diagnosed incidentally at the age of 19 months during a hospital admission for severe combined bacterial (urosepsis) and viral (CMV and EBV) infection. 11-deoxycortisol levels were increased and urine ketosteroid analysis showed increased excretion of tetrahydrometabolites of 11-deoxycortisol and DOC. Complete virilisation with slight glandular hypospadia and a hypoplastic empty scrotum was found. The JY patient (the younger sibling) was diagnosed at the age of 5 months. This child was born soon after diagnosis in the elder sibling. Biochemical test results (see Table 3.5) of adrenal androgens and urinary metabolites investigated by Dr. Riedl and coworker were in line with the diagnosis of congenital adrenal hyperplasia (CAH) due to 11β-hydroxylase deficiency (11β-OH-D).

Table 3.5: Biochemical parameters

	MY patient (at 19 months)	JY patient (at 5 months)	Normal subject
ACTH (pmol/L)	257	72	2-11
Cortisol (nmol/L)	472	599	138-690
11-Deoxycortisol (nmol/L)	1208	-	0.3-23
Androstendione (nmol/L)	106	-	0.35-1.75
DHEAS (µmol/L)	23.8	1.9	0.26-1.28
17-OHP (nmolL)	123.6	23.1	0.3-4.5
Testosterone (nmol/L)	8.4	2.0	0.07-0.35
Aldosterone (nmol/L)	0.55	2.25	0.11-0.86
PRA (ng/mL/h)	suppressed	2.6	2-10

ACTH, adrenocorticotrophin hormone**; DHEAS,** dehydroepiandrosterone**; 17-OH-P,** 17α-hydroxyprogesterone; **PRA,** plasma renin activity.

♠ Clinical data were obtained by Dr. Stefan Riedl in Paediatric Department, Medical University of Vienna, Austria.

3.6.2 Mutation analysis ♠

To confirm the diagnosis of the cortisol synthase defect in the patient, the *CYP11B1* genes of the patient and his family were sequence by Riedl and co-workers from the genomic DNA. All nine exons and the exon/intron boundaries were sequenced. A homozygous T to C point mutation at bp 3522 in exon 5, corresponding to c.T896T>C and leading to a change from leucine to proline at amino acid position 299 of CYP11B1 was found in both patients. The parents as well as 2 of 3 older daughters were heterozygous carriers of the mutation. The pedigree of the family with the mutation L299P in the CYP11B1 studied by Riedl and co-workers is shown in Figure 3.25.

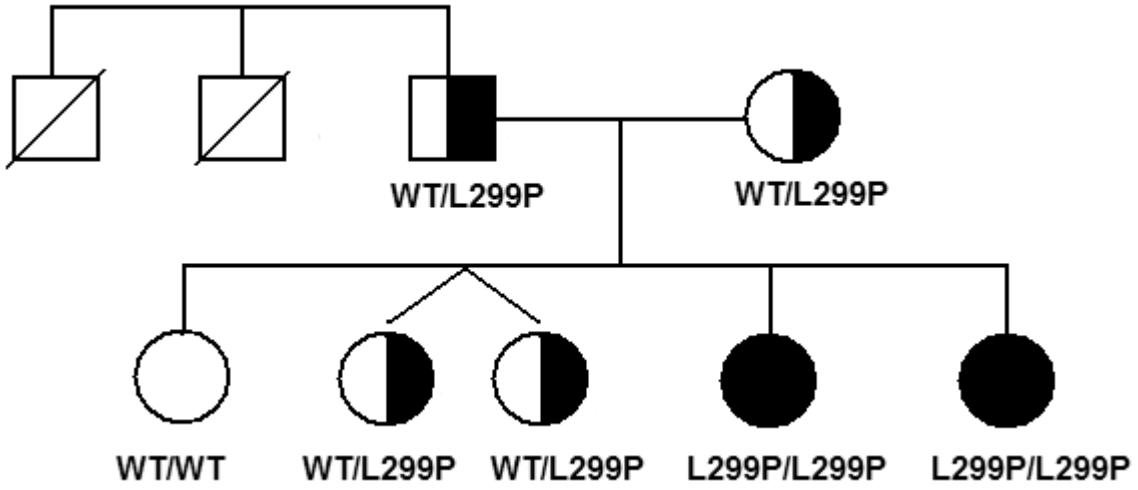


Figure 3.25: Pedigree of the family with the mutation L299P in the CYP11B1. Squares are presented as male; the cycles are presented as female. The MY patient and the JY patient (younger sibling) are homozygous carriers of the mutation. The parents as well as 2 of 3 three older daughters are heterozygous. Two brothers of then father, died at age 14 and 16 years, repectively, from heart disease.

3.6.3 Functional analysis of enzyme activity

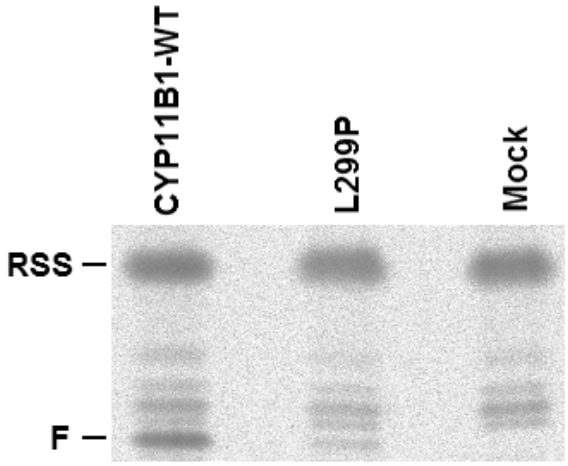
To analyze mutant L299P affect enzyme activity, a single mutation, L299P, was created by site-directed mutagenesis in the CYP11B1-WT using the B1-229F and B1-299R

♠ Mutaion analyses were obtained by Dr. Stefan Riedl in Paediatric Department, Medical University of Vienna, Austria.

primers shown in the Table 2.1. The successful insertion of the intended mutation was confirmed by sequence analysis. Mutant L299P and CYP11B1-WT were expressed with bAdx in the HCT116 $p53^{-/-}$ cells. The transfected HCT116 $p53^{-/-}$ cells were incubated with 11-deoxycortisol as substrate to check the ability of the expressed enzymes to convert 11-deoxycortisol to cortisol. The unlabeled steroid standards (RSS and F) were visualized by UV light. Positions of steroid products on HPTLC plates are compared with those of the unlabeled steroid standards. Transfection experiments demonstrated a reduction of 11β-OH activity to 1.6 ± 0.8% for the conversion of 11-deoxycortisol to cortisol (see Figure 3.26).

In conclusion, one homozygous mutant (L299P) was detected in two homozygous 46, XX siblings with 11β-hydroxylase deficiency. The expression of cDNA constructs containing mutation L299P in HCT116 $p53^{-/-}$ cells showed that the L299P replacement reduced 11-hydroxylase activity to1.6 ± 0.8% for the conversion of 11-deoxycortisol to cortisol.

A



B

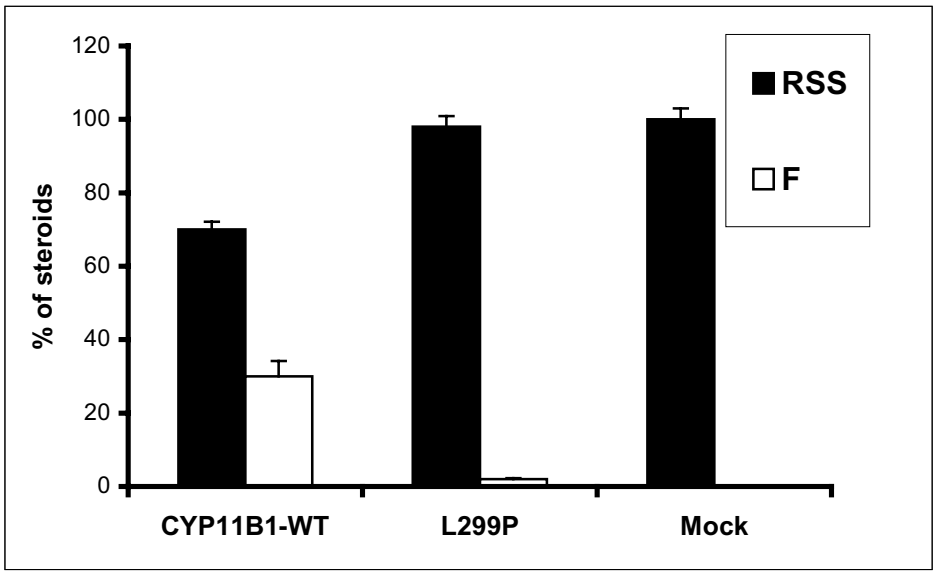


Figure 3.26: A, autoradiography of thin layer chromatography of steroids produced by HCT116 $p53^{-/-}$ cells transfected with cDNA of CYP11B1 constructs, L299P mutant and pbAdx and incubated with 11β-deoxycortisol (RSS) with 5 µM concentration and 0.6 µCi of ^{3}H-RSS to conversion of RSS to cortisol (F). Positions of steroids are marked on the autoradiogram as follows: RSS, 11β-deoxycortisol; F, cortisol. **B**, measurement of 11β-hydroxylase activity of CYP11B1 mutant L299P in transiently transfected HCT116 $p53^{-/-}$ cells. Steroid patterns of RSS conversion are given as mean ± SEM of three similar independent experiments performed in duplicate. The amount of cortisol is presented as percentages of total activity.

4. Discussion

4.1 Evaluation of CYP11Bs expression in HCT116-p53$^{-/-}$ cells

In previous studies, enzymatic characterization of CYP11B2 has been frequently investigated using the heterologous expression of this protein in COS-1 cells or COS-7 cells (Dunlop et al. 2003; Nomoto et al. 1997; Portrat-Doyen et al. 1998). Although COS cells are very popular as a mammalian cell system for the heterologous expression of proteins, COS cells may not be optimal for the enzymatic characterization of CYP11B proteins. Since CYP11B proteins are a mitochondrial P450s, human CYP11B1 and CYP11B2 may be more efficiently transported and/or folded in mitochondria of human cells compared to COS cell lines derived from monkey kidney. Because HCT116 p53$^{-/-}$ cells (human colon carcinoma cell line) are well established in our group, fast-growing, and easy to handle we decided to analyze human CYP11B2 in those alls and compare the usefulness of this cell line with COS-1 cells. Therefore, HCT116 p53$^{-/-}$ and COS-1 cells were transfected with an empty vector as control, with the human CYP11B2 expression vector and the bovine Adx (adrenodoxin, an electron transporter to mitochondrial P450s) vector, as well as with the human CYP11B2 expression vector and the human Adx expression vector.

To optimize the level of the electron transport mediator for the steroidogenic activity of the HCT116 p53$^{-/-}$ cells, we compared the CYP11B2 activities upon coexpression with human and bovine Adx. CYP11B2 with bovine Adx converted the substrates more efficiently to the products although both, human and bovine Adx, enhance the CYP11B2 activities in HCT116 p53$^{-/-}$ cells (see Figure 3.3A). The difference in the conversion efficiency of substrate can be explained by a different expression level of the ferredoxins, by a less efficient electron transfer from the human Adx to CYP11B2 than from bovine Adx, or by differences in the efficiency of electron transport from endogenous Adx-reductase to the two Adx forms. Thus, in the HCT116 p53$^{-/-}$ cell system, the activity of human CYP11B2 is most efficiently analyzed with the co-expression of bAdx but not with human Adx. Our conclusion is consistent with the results of co-expressing bAdx in COS-1 cells (Bottner et al. 1998; Bottner et al. 1996; Cao et al. 1999b).

As shown in Figure 3.3B, CYP11B2 activities in HCT116 p53$^{-/-}$ were indistinguishable from those in COS-1 cells upon co-expression of bAdx, suggesting that both cell lines have similar transfection efficiency under our experimental conditions, and that human CYP11B2 is transported and folded in the HCT116 p53$^{-/-}$ cells as efficiently as in the COS-1 cells. Further, the results also suggest that the availability of electrons for the heterologously expressed CYP11B2 is at a similar level in both cell lines.

Taken together, the human cell line HCT116 p53$^{-/-}$ is applicable for studying the effect of mutations in steroid hydroxylase genes from patients being diagnosed with a steroid biosynthesis defect. We concluded that HCT116 p53$^{-/-}$ cell line does not respond an advantage compared with the COS-1 cell line.

4.2 Aldosterone synthase deficiency (CMO I and CMO II)

The human aldosterone synthase CYP11B2 is expressed in the *zona glomerulosa* of the adrenal glands. The enzyme catalyzes three consecutive mono-oxygenation reactions to convert 11-deoxycorticosterone to aldosterone, the most important mineralocorticoid in humans (see Figure 1.2). Aldosterone biosynthesis disorder is an autosomal recessively inherited disorder caused by mutations in the *CYP11B2* gene. Due to the reduced adrenal aldosterone synthase activity, 11-deoxycorticosterone is not efficiently converted to aldosterone and insufficient aldosterone secretion leads to decreased sodium resorption and potassium secretion into the urine of patients (Peter et al. 1999) causing the symptoms of hypoaldosteronism.

The urinary tetrahydroderivatives of mineralocorticoids may be used as an indirect index of plasma mineralocorticoids (see Figure 4.1). The major urinary metabolites of mineralocorticoids are tetrahydroaldosterone (Thaldo) for aldosterone, tetrahydrocorticosterone (THB) and tetrahydro-11-dehydrocorticosterone (THA) for corticosterone. Likewise, tetrahydrodeoxycorticosterone (THDOC) and 18-hydroxytetrahydrocompound (18-hydroxy- tetrahydro-11-dehydrocorticosterone (18-OH-THA) and 18-hydroxy- tetrahydro-corticosterone (18-OH-THB)) correspond to 11-deoxycorticosterone (DOC) and 18-hydroxycorticosterone (18-OH-B), respectively (Ghulam et al., 2003). To delineat disorders of steroid metabolism, the application of GC-

MS urinary steroid profiling (Wudy et al. 2004) has proved to be a decisive step in determining further rational strategies regarding treatment and molecular genetic studies. Normally, measurements of plasma steroids and urinary steroid metabolites are used in patients to hormonally distinguish between aldosterone synthase deficiency type I (corticosterone methyl oxidase type I – CMO I) and type II (corticosterone methyl oxidase type I – CMO II) (Dunlop et al. 2003; Kayes-Wandover et al. 2001b; Ulick et al. 1992).

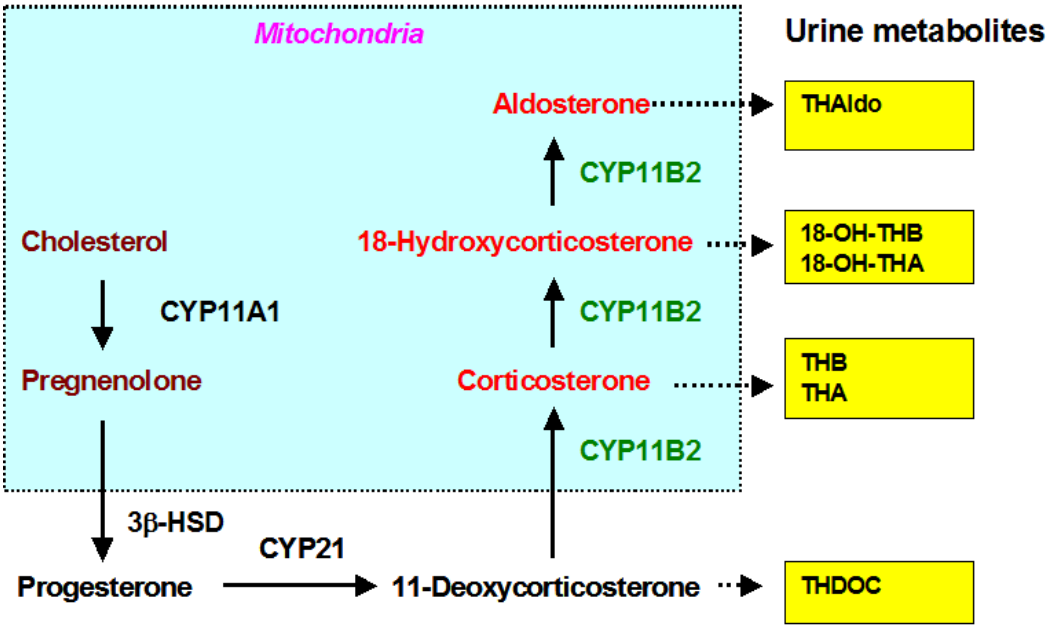


Figure 4.1: Unrinary metabolites of mineralocorticoid synthesis. **THDOC**: tetrahydrodeoxycorticosterone; **THA**: tetrahydro-11-dehydrocorticosterone, **THB**: tetrahydrocorticosterone; **18-OH-THA**: 18-hydroxy- tetrahydro-11-dehydrocorticosterone; **18-OH-THB**: 18-hydroxy- tetrahydro-corticosterone; **THAldo**: tetrahydroaldosterone.

The majority of patients suffering from aldosterone synthase deficiency type I generally reflect mutations in the *CYP11B2* gene causing a complete loss of aldosterone synthase activity because of a blockade of 18-hydroxylation of corticosterone and, at least in some cases, also 11β-hydroxylation of 11-deoxycorticosterone. In our patients 1 and 3, the results of GC-MS urinary steroid profiling did not show any 18-oxygenated metabolites. This indicates that the patients had symptoms of CMO I deficiency. As was predicted, 3 mutations (L451F, S315R and R374W) were detected in CYP11B2 of patient 1 and 3.

The results from transfection analysis in cell lines showed that L451F, S315R and R374W mutants were completely inactive. These mutations can be compared with previous studies. For example, a single point mutation (R384P) has been found in a male Caucasian patient suffering from CMO-I deficiency (Geley et al. 1995). The expression of this mutant in COS-1 cells showed complete loss of 11β- and 18-hydroxylase activities of CYP11B2. The other single point mutation CTG to CCG at codon 461 in exon 8 of CYP11B2 was reported by Nomoto et al (Nomoto et al. 1997) leading to an amino acid replacement L461P. This residue was shown to be involved in the putative heme binding site of CYP11B2 resulting in a complete loss of aldosterone synthase activity. Furthermore, mutants leading to deletion or insertion of nucleotides were detected in *CYP11B2* gene from patients suffering from aldosterone synthase deficiency type I. A patient was analyzed showing a truncated enzyme which was derived from five nucleotide deletions in exon 1 resulting in a frameshift to form a stop codon in the same exon (Mitsuuchi et al. 1993). The five nucleotide deletion occurred in two alleles. Another patient was also identified homozygous for a premature stop codon in exon 4 (E255X) (Peter et al. 1997). These mutations resulted in a completely inactive CYP11B2. Another patient had a homozygous duplication of six nucleotides at codon 143 in exon 3 of CYP11B2, leading to the insertion of two amino acid residues (Arg-Leu) (Kayes-Wandover et al. 2001b). The *in vitro* assay of this mutant in human embryonic kidney 293 cells showed a complete inactivity of CYP11B2. One nucleotide deletion (R490del1nt) also was found in one allele from our patient 2. The transfection assay of R490del1nt in COS-1 cells showed a complete inactivation of CYP11B2. Further, one silent mutation (c.954G>A at the last nucleotide of exon 5) was detected in the other allele of patient 2. This mutation obviously affects splicing of CYP11B2 pre-mRNA. Silent mutations in the boundaries of exon/intron, which alter the splicing, were not previously detected in patients with aldosterone synthase deficiency. Therefore, CMO I is biochemically defined as deficiency with no available CYP11B2 activity in the patients caused by mutations in the *CYP11B2* gene. When a patient with CMO I is homozygous, the mutant CYP11B2 protein expressed in cell cultures will be inactive in a transfection experiment. When a patient with a CMO I phenotype is heterozygous and each allele has a mutation in the *CYP11B2* gene, both of the mutant CYP11B2 proteins should be

inactive in the transfection assay. In those cases, the other member of the CYP11B subfamily, CYP11B1, is normal and present in zona faciculata/reticularis. Although the major role of CYP11B1 is to convert 11-deoxycortisol to cortisol, CYP11B1 is also able to catalyze the 11β-hydroxylation of 11-deoxycorticosterone for the production of corticosterone and 18-hydroxycorticosterone (very small amounts) when the substrate is available, but no conversion of 18-hydroxycorticosterone to further derivatives can be observed. Therefore, in the plasma and urine of patients with CMO I, 11β-hydroxylated steroids, corticosterone and cortisol, are observed, but 18-hydroxylated derivatives, 18-hydroxycorticosterone and aldosterone, are not present since CYP11B2 is the only enzyme catalyzing 18-hydroxylation reactions.

On the contrary to CMO I deficiency, CMO II deficiency is related with high levels of 18-OH-corticosterone and low levels of plasma aldosterone or subnormal to occasionally normal levels of urinary tetrahydroaldosterone (Ulick et al. 1992). For example, a patient with CMO II was found to have two mutations T185I, T498A in the *CYP11B2* gene. The transfection analysis in COS cells for the determination of enzyme activities of the mutants showed significantly reduced 18-oxidation of aldosterone synthase (Dunlop et al. 2003). In Iranian-Jewish patients sufferring from CMO II, two missense mutations (R181W and V386A) were identified in the *CYP11B2* gene (Pascoe et al. 1992a). Furthermore, the compound heterozygous (R181W/del C372 and T318M/V386A) reported by Zhang et al. (Zhang et al. 1995) showed a clinical phenotype of CMO II deficiency, with both detectable serum aldosterone and elevated 18-hydroxycorticosterone, but the *in vitro* activity is completely abolished. In our case, patient 4 suffering from CMO II deficiency had a heterozyogous mutation (R181Q). The activity assay of the R181Q mutant in COS-1 cells showed that corticosterone (B) was increased, 18-hydroxycorticosterone (18-OH-B) was reduced, and no aldosterone (Aldo) was found. In biochemical aspects, therefore, the CMO II deficiency is defined as a partial loss of CYP11B2 activities by mutations that may slightly or severely reduce the second 18-hydroxylation activity of CYP11B2.

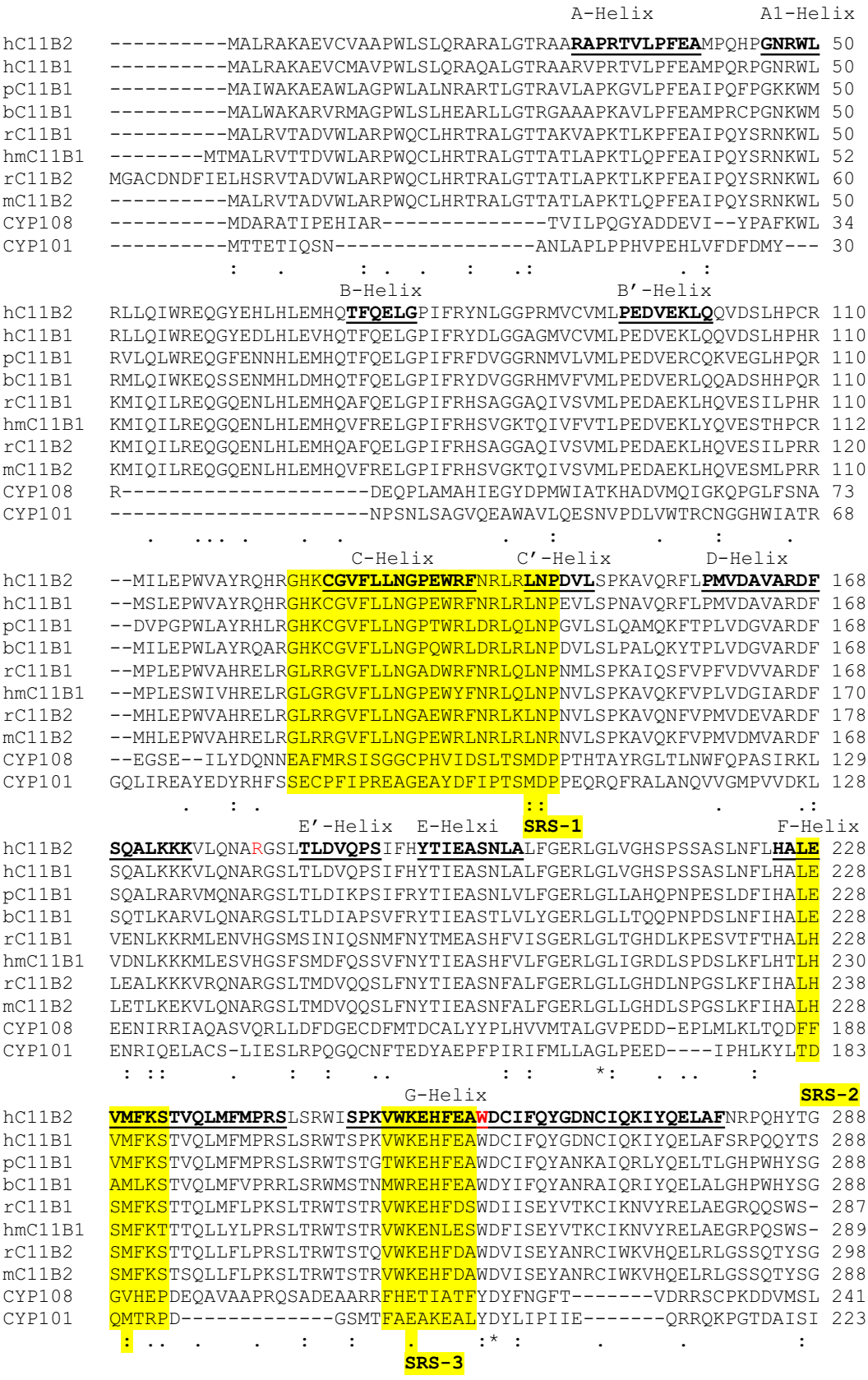
4.2.1 Patient 1: Homozygous mutant L451F

At the age of 3 wk a steroid profile from a random spot urinary sample of the patient was recorded. The patient in this case showed a steroid profile typical for a CMO I deficiency because the GC-MS result exhibits no 18-oxy-derivatives and, in addition, elevated levels of the corticosterone metabolites THA (tetrahydro-11-dehydrocorticosterone) and THB as compared with control (see clinical data of patient 1 in the result). This is a typical picture because functionally impaired CYP11B2 does not convert corticosterone (B) efficiently to the 18-oxygenated metabolites and, as a consequence, the B-derived metabolites THA and THB increase in the urine. B can, however, still be produced from 11-deoxycorticosterone by CYP11B1, a 93% identical mitochondrial cytochrome P450 isozyme (Mornet et al. 1989). However, CYP11B1 can only catalyze the 18-hydroxylation of B to form 18-OH-B to very small extent (Pascoe et al. 1992a; Portrat-Doyen et al. 1998) but can not support the following 18-oxidation to form aldosterone. Thus, it leads to an accumulation of derivatives of corticosterone.

In our case, we detected a novel missense mutation (L451F) in the CYP11B2 of the patient. The *in vitro* protein expression of CYP11B2-L451F analyzed by the Western blot (see Figure 3.8) showed that the mutant can be expressed in COS-1 cells but the expression level of the mutant was lower than that of the wild type. The replacement is next to the invariant heme coordinating ligand C450. This means that the mutation might influence the assembly of the heme into the apo-CYP11B2 and lead to an unstable mutant protein. When testing the CYP11B2 activity using the substrates DOC and B in COS-1 and HCT116 cells, the mutant L451F showed neither B nor 18-oxygenated metabolites, suggesting the complete loss of CYP11B2 activity. This fits with the clinical data, which indicated also no 18-oxygenated metabolites, whereas the detected B metabolites in urine samples were most probably formed due to the activity of the isoenzyme CYP11B1.

When considering the alignment of amino acid sequences (see Figure 4.2) it can be seen that the L451 residue is highly conserved in CYP11B1 and CYP11B2 enzymes of humans, mice, cow and pig, as well as in the microbial enzymes CYP108 and CYP101. This indicates a functionally or structurally important role of L451 in cytochromes P450. Another replacement (L461P) in this region has been described previously to completely block the enzymatic activity (Nomoto et al. 1997). It can be assumed that L451 plays an

important role in the interaction of the apo-P450 with its prosthetic group and/or in stabilizing the protein structure. To get deeper insight into this role, a computer model of the mutated CYP11B2 using the three-dimensional model of CYP11B2 (see Figure 4.2A) derived previously in our group as a template had been constructed (Belkina et al. 2001). It can be seen that L451 is located at the beginning of the L-helix, which is part of the P450 core region and of the heme-binding region. The heme of P450s is coordinated to the invariant cysteine found in a β-bulge region called the Cys-pocket. The sequence conservation extends from the Cys-pocket through most of the L helix (Hasemann et al. 1995). The L-helix belongs to a conserved structural core, which is associated with substrate recognition, substrate binding, and redox partner binding. This conserved P450 structural core consists of six-helices: D, E, I, L, J and K. In addition, there are two structurally conserved β-sheets (β sheet 1 containing five strands and β sheet 2 containing two strands), which are part of the hydrophobic substrate access channel. There is a structurally conserved consensus sequence on the proximal face of the heme containing the absolutely conserved cysteine residue (see Figure 4.2), which comprises the 5^{th} iron ligand, as well as L451 (Peterson et al. 1998). Residue L451 is invariant in a 10-residue signature motif which includes the thiolate (cysteine) ligand, and this region (which precedes the L helix) is highly conserved across the entire P450 superfamily of over 1200 genes (Nelson 1995). The heme is located between helices I and L where certain hydrophobic and π-π stacking interactions bind the iron protoporphyrin IX complex (Cupp-Vickery et al. 1995; Modi et al. 1995). When considering our model it becomes obvious that the side chain of F451 compared with that of L451 besides probably influencing the dynamics around the heme-coordinating C450 also may cause steric hindrance of nearby residues (Q449, G452, R453, R454, L455, D147, R143, L106) (see Figure 4.2 B-E). The larger phenylalanine side chain gets closer to residue D147 on the CYP11B2 surface (see Figure 4.2 D-E), which is involved in the regulation of CYP11B isoform specific substrate conversion (Bechtel et al. 2002). Thus, the computer model gives a conclusive explanation of the molecular effects which have been observed when analyzing the mutant protein in the cell cultures. And this, on the other hand, correlates excellently with the results of the Western blot experiments and the clinical picture.


A-Helix
A1-Helix
B-Helix
B'-Helix
C-Helix
C'-Helix
D-Helix
E'-Helix
E-Helxi
SRS-1
F-Helix
G-Helix
SRS-2
SRS-3

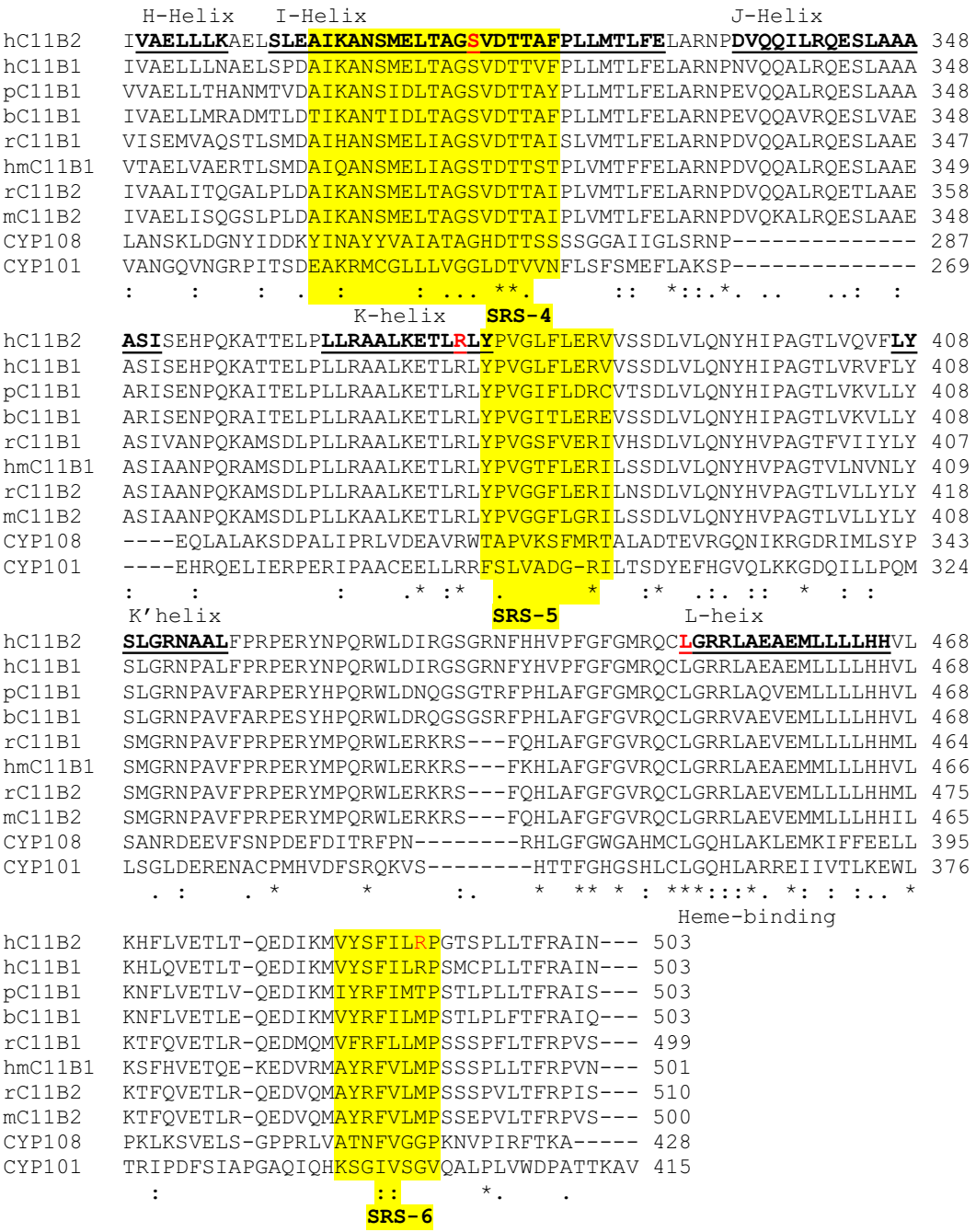


Figure 4.2: Multiple sequence alignment of CYP11B1 and CYP11B2 of human, bovine, mouse, rat and pig with CYP108 and CYP101. The alignment was done using the program CLUSTALW 1.8 (Berman et al. 2000). The underlined part in the CYP11B2 sequence corresponds to the helix. The red residues indicate mutations which were detected in patiens. Yellow areas indicate SRS (substrate recognition site) in CYP2 family members indentified by Gotoh (Gotoh 1992).

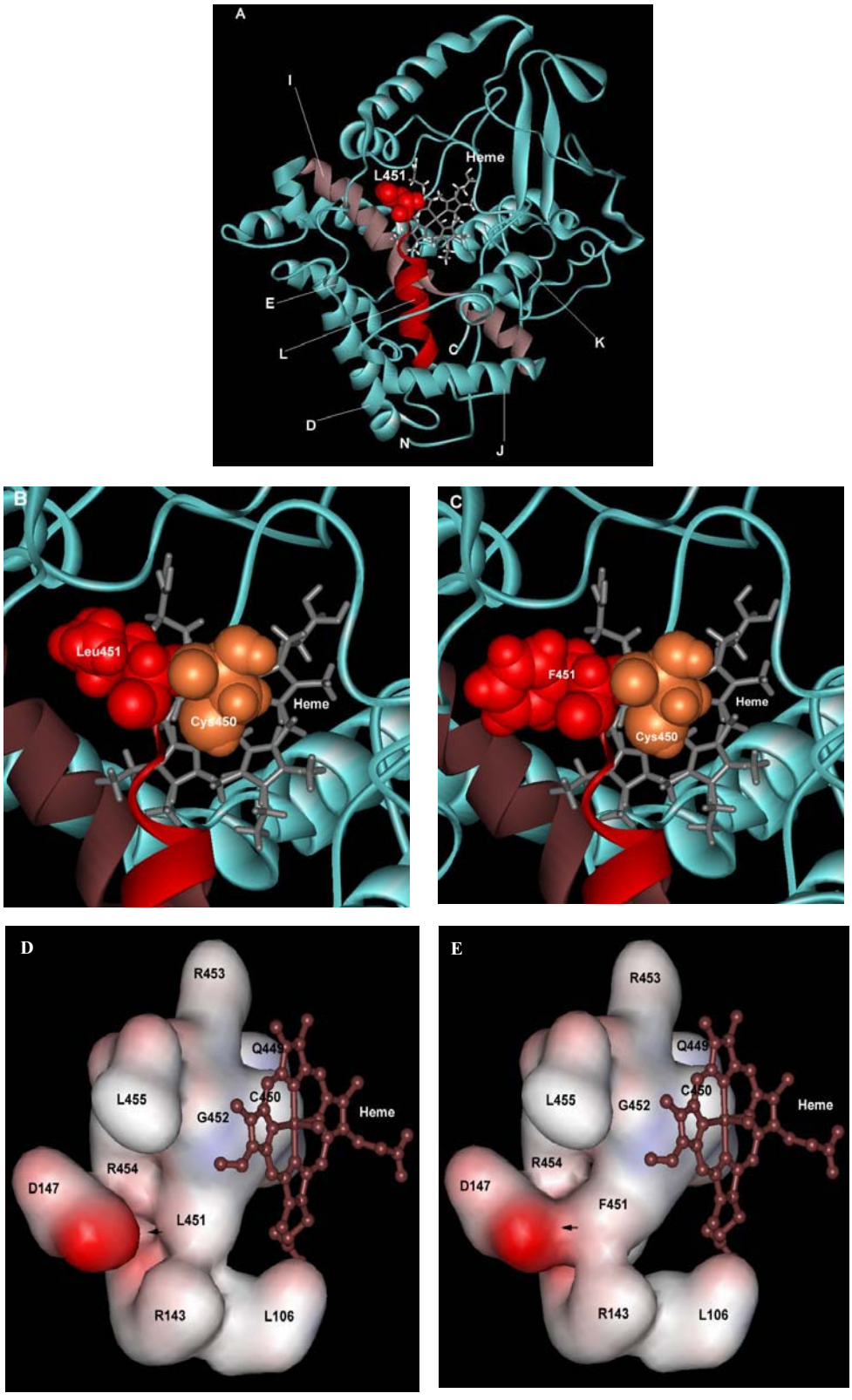

A
I
L451
Heme
E
L
C
K
D
N
J
B
Leu451
Cys450
Heme
C
F451
Cys450
Heme
D
R453
Q449
L455
C450
G452
Heme
R454
D147
L451
R143
L106
E
R453
Q449
L455
G452
C450
Heme
R454
D147
F451
R143
L106

Figure 4.3: Three-dimensional molecular model of CYP11B2. A, Amino terminus and carboxyl terminus are marked by N and C, respectively. The helix I is colored in brown, the helix L is colored in red. **B and C**, model of wild type CYP11B2 and mutant L451F in the immediate heme environment. The larger size of F451 compared with L451 can be clearly seen. **D and E**, model of wild type CYP11B2 and mutant L451F in the heme environment showing the surfaces of neighboring residues (Q449, C450, G452, R453, R454, L455, D147, R143, L106). The larger size of the phenylalanine side chain after the L451F replacement causes steric effects with the heme and residue D147 (black arrows) which is involved in the regulation of CYP11B isoform specific substrate conversion (Nguyen et al. 2008).

In conclusion, we detected a novel missense mutation (L451F) within the *CYP11B2* gene of a patient suffering from CMO I deficiency. The *in vitro* expression of cDNA constructs containing the sequence change in HCT116 $p53^{-/-}$ and COS-1 cells demonstrated that the L451F mutant abolished the CYP11B2 activity. Furthermore, our molecular model indicated a steric effect in the immediate vicinity of the heme which could provide new insights for the understanding of cytochrome P450 structure-function relationships.

4.2.2 Patient 2: Compound heterozygous mutant (R490Δ1nt and silent mutation in the last nuculeotide of exon 5)

The genotype/phenotype correlation in aldosterone synthase deficiency is not always straightforward. Kayes-Wandover et al 2001 (Kayes-Wandover et al. 2001b) reported a 47-year-old man who first presented with CMO type I deficiency after developing hyperkalemia in preparation for a barium enema but past medical history was notable for failure to thrive in infancy. This patient was homozygous for a duplicaton of six nucleotides at codon 143 in exon 3. Later, a *CYP11B2* gene of a girl has discovered being a compound heterozygous for nonsense mutations that encode a truncated protein, but displays biochemical features intermediate between those of CMO I and CMO II (Williams et al. 2004b). Besides, five patients in four unrelated kindreds with hyperreninemic hypoaldosteronism presenting in early infancy have been described in

whom no mutations in the *CYP11B2* could be detected (Kayes-Wandover et al. 2001a). In addition, one girl has been studied displaying aldosterone synthase deficiency type I, but only one heterozygous mutation was detected in the *CYP11B2* gene (Wasniewska et al. 2001).

In our case, the patient had symptoms of aldosterone synthase deficiency with low aldosterone and a small increase of costicosterone and 18-hydroxycosticosterone. The ratios of B/18-OH-B and 18-OH-B/Aldo (after 16 yrs treatment) were not typical of either CMO I or CMO II. The genotype described clearly a new compound heterozygous showing a silent mutation at the last nucleotide of exon 5 and R490Δ1nt which both abolished *in vitro* enzyme activity. The phenotype of her brother was not reported although the genotype is the same as the patient.

Silent mutations at the last nucleotide of exons (substitution of G to A; or C to A; or G to C) have been also identified in some other genetic diseases as seen in Table 4.1. These silent mutations influenced transcription by skipping or retaining an intron. The splice site consensus sequences are located at exon-intron junctions and are conserved phylogenetically (Cartegni et al. 2002; Shapiro et al. 1987). Each nucleotide in the splice site motif has a differing rate of variance, with the 5' GT and the 3' AG motifs being the most highly conserved. Recognition of such motif is important for understanding splicing mechanisms and helps to predict the consequences of changes identified in genomic DNA. In our study, the results of maximum entropy (MaxEnt) showed that the splice site score of the normal gene is 4.63 and the splice site score of silent mutation (c.954G>A) is -5.75. This predicted that mutation c.954G>A alters the splice site.

The present c.954G>A mutation at the last nucleotide of exon 5 resulted in aberrant splicing. *In vitro* splicing analysis clearly showed that the RT-PCR product from c.954G>A mutant (T318T) resulted in an intron 5 retention (812 bp) (see Figure 3.17). This indicated that c.954G>A mutant was responsible for a missing splicing at this position of *CYP11B2* mRNA. However, a c.954G>C mutation at the last nucleotide of exon 5 of the *CYP11B1* gene in a patient with severe steroid 11β-hydroxylase deficiency

did not affect the correct splicing of *CYP11B1* mRNA (Chabre et al. 2000). We suggested that most of the G>A mutations at the last nucleotide of exon were responsible for the abnormal splicing of mRNA in comparison to G>C mutation. Furthermore, we identified a novel deletion mutant (R490Δ1nt) which leads to a shift in the translational reading frame of CYP11B2 (no stop codon at position 504 and addition of 170 amino acids in the CYP11B2). The expression of *CYP11B2* cDNA containing this mutation in COS-1 cells clearly showed that the R490Δ1nt mutation led to an impaired activity of CYP11B2. Taken together, the *in vitro* analysis of c.954G>A mutation at the last nucleotide of exon 5 and transfection analysis of deletion mutant (R490Δ1nt), leading to frame-shift and addition amino acids, indicate that the patient got CMO I phenotype rather than CMO II phenotype.

The elevated corticosterone (B) and 18-OH-11-deoxycorticosterone (18-OH-DOC) plasma levels in this patient might be explained by the fact that CYP11B1 catalyzes 11β - and 18- hydroxylation to form B and 18-OH-DOC, respectively in the zona glomerulosa. However, CYP11B1 can not catalyze the 18-hydroxylation at position 18 of B to form 18-OHB as well as oxidation of the 18-hydroxy group to form aldosterone. Rosler (Rosler 1984) presented that in each affected individual with aldosterone synthase deficiency the clinical severity of the disease decreases with age. Continued mineralocorticoid replacement therapy after childhood is not always necessary, as on clinical observation compensatory extra-adrenal salt-conserving mechanisms mature with age. Plasma renin activity is markedly elevated (up to 100 times normal) in affected infants and young children, but it may be normal in adults (White et al. 1994a).

In conclusion, we have described here the first example that c.954G>A mutation at the last nucleotide in exon 5 of the *CYP11B2* gene leads to an abnormal splice site by a retention of intron 5 upon transcription of the mRNA of this gene. In addition, *in vitro* analysis of the R490Δ1nt mutation in COS-1 cells indicated that this mutation effected activity of CYP11B2. The *in vitro* analysis of the two mutants coincides with a clinical picture of a patient exhibiting CMO I deficiency. However, the biochemical features of the patient present an intermediate form between CMO I and CMO II rather than the

classical CMO I. Further studies are required to identify all of the factors involved in aldosterone synthase deficiency and the regulatory sequences that influence the complex genotype-phenotype of this disease. In addition, the biochemical features of the patient's brother should be reported and compared with the data of this patient.

Table 4.1 Silent mutations in the last nucleotide of the exon

Gene	Nucleotide	Mutation	Exon	Consequence	Ref.
ATM	G⇒A	S709S	16	Skipping	(Teraoka et al. 1999)
ATM	G⇒A	S1135S	26	Skipping	(Teraoka et al. 1999)
HEXA	G⇒A	L187L	5	Skipping	(Akli et al. 1990)
LIPA	G⇒A	Q277Q	8	exon 8 deletion	(Tadiboyina et al. 2005)
PTS	G⇒A	E81E	4	Skipping	(Imamura et al. 1999)
LMNA	C⇒A	K171K	2	Skipping	(Todorova et al. 2003)
ALDOB	G⇒A	K320K	3	12 nucleotide deletion	(Sanchez-Gutierrez et al. 2002)
KCNQ1	G⇒C	A344A	6	Skipping	(Murray et al. 1999)
PK	G⇒A	A423A	9	Skipping	(Kanno et al. 1997)
HS	G⇒C	L100L	3	intron 3 retention	(Garbarz et al. 1998)
Aldo	G⇒A	T318T	5	Intron 5 retention	This study

ATM, ataxia telangiectasia mutated; *HEXA*, hexosaminidase; *LIPA*, lipase A; *PTS*, 6-pyruvoyltetrahydropterin synthase; *LMNA,* laminopathies; *ALDOB,* aldolase B*; KCNQ1* previously named *KVLQT1,* potassium channel of long-QT syndrome; *PK*, pyruvate kinase; *HS,* hereditary spherocytosis; *Aldo*, aldosterone.

4.2.3 Patient 3: Compound heterozygous mutant (S315R and R374W)

Clinically, the patient exhibited a growth failure as a child. Biologically, no 18-hydroxy-tetrahydro-corticosterone and high tetrahydrocorticosterone in urinary samples were observed by GC/MS measurement, a pattern typical of CMO I deficiency. This is the typical picture for CMO I deficiency because functionally impaired CYP11B2 does not

convert corticosterone (B) efficiently to the 18-oxygenated metabolites and, as a consequence, the B-derived metabolites THA and THB increase in the urine. B can, however, still be produced from 11-deoxycorticosterone by CYP11B1, a 93% identical mitochondrial cytochrome P450 isozyme (Mornet et al. 1989). However, CYP11B1 only catalyzes the 18-hydroxylation of B to form 18-OH-B with very small extent (Pascoe et al. 1992a; Portrat-Doyen et al. 1998), but poorly supports the following 18-oxidation to form aldosterone. Thus, it leads to an accumulation of derivatives of corticosterone. Sequencing of the *CYP11B2* gene confirmed the presence of mutations in this gene. Two novel missense mutations (S315R and R374W) were detected in the CYP11B2 of the patient. The protein expression of S315R and R374W mutants analyzed by Western blot (see Figure 3.19) showed that the two mutants could be expressed in COS-1 cells as the wild type. When testing the CYP11B2 activity using the substrates DOC in COS-1 cells it was shown that the replacement R374W led to impaired activity of CYP11B2; and the replacement S315R reduced the production of B and abolished the formation of 18-OH-B and Aldo. This means that the pattern of activity of the mutant enzyme coincides with the aldosterone synthase deficiency type I. Therefore, molecular genetic results may explain clinical data, which also indicated no 18-oxygenated metabolites, whereas the detected B metabolites in urine samples were formed more probably due to the activity of the isoenzyme CYP11B1.

Besides, we analyzed the functional and structural consequences of the two point mutations (S315R, R374W) in CYP11B2 on the basic of a computer model of this protein. When considering the alignment of amino acid sequences (see Figure 4.2) it can be seen that the arginine 374 residue is highly conserved in CYP11B1 and CYP11B2 enzymes of humans, mice, bovine and pig, as well as in the microbial enzymes CYP108 and CYP101. This indicates a functionally or structurally important role of R374 in cytochromes P450. In addition, residue R374 is invariant in a Glu-X-X-Arg motif which may be involved in stabilizing the core structure. It is on the proximal side of the heme (the putative redox partner binding site) (Peterson & Graham 1998) and also involved in the substrate recognition site 5 (SRS 5) (see Figure 4.2). Hasemann (Hasemann et al. 1995) indicated that a variable-length β6-1 segment is anchored at one end by the K-helix

Glu-X-X-Arg motif and at the other end by the conserved arginine/ histidine propionate ligand in β1-4. Natural mutants occurring in the Glu-X-X-Arg motif have been shown to have abolished CYP11B1 activity (Mestres 2005).

Residue S315 in CYP11B2 has a non-polar character and plays a role in stabilizing the interaction of helix I with adjacent helices which are in close relation to the heme cofactor (see Figure 4.2) (Belkina et al. 2001; Gotoh 1992; Mestres 2005). The helix I of CYP11B1 and CYP11B2 also contains many hydrophobic amino acids and is the putative active site of CYP11B1 and CYP11B2 (Belkina et al. 2001). In addition, it has been demonstrated by changing CYP11B2 to CYP11B1 corresponding residues and vice versa (Bottner et al. 1998; Bottner et al. 1996) that the helix I of CYP11B1 and CYP11B2 is responsible for substrate specificity. The residue S315 is located within the central I-helix which is involved in heme binding and substrate recognition site (SRS4) (Belkina et al. 2001; Gotoh 1992; Mestres 2005). The residue S315 is highly conserved in human, mouse, bovine and rat CYP11B1 and CYP11B2. Furthermore, residue S315 in CYP11B2 is equivalent to residue G268 of CYP108 and residue G250 of CYP101 which are located in the AGXXT motif in the I-helix (Mestres 2005).

Hydrogen bonds are important landmarks in protein conformation. They contribute to the stability of secondary structures and of interactions between specific side chain and main chain polar atoms. Thus, the change of the hydrogen bond network of CYP11B2 can influence substrate recognition and stability. To investigate whether the two mutants (S315R, R374W) affect the structure of CYP11B2, the changes of those residues in CYP11B2 were modelled by using the spdbv program (http:www.expasy.org/spdbv/) (Guex et al. 1997). The hydrogen bonds of K370, E371, T372, L373, R374, L375, F406, L407, Y408, S409, L410, R412, A414, L416, F417, P420, E421, and R422 residues were depicted in Figure 4.3A. The side chain of Y372 interacts with the side chain of L375 by one hydrogen bond. Seven hydrogen bonds are generated by interactions of R374 side chains with K370, E371, P420 and R422 residues. One backbone of F417 interacts with the side chain of R422 by two hydrogen bonds. Two hydrogen bonds are generated by interaction the backbone of A414 residue with backbone of F417 residue and R412 residue. The backbone of L407 interacts with backbone of LS409 and L410 residue by

two hydrogen bones. One backbone of Y408 residue interacts with side chain of R412 by two hydrogen bonds. When the residue arginine at position 374 was substituted by tryptophan, six hydrogen bonds were removed between E371, P420 and R422 residues and W374 residue (see Figure 4.3B). One hydrogen bond was removed between A414 residue and F417 residue but one hydrogen bond was generated between A414 residue and backbone of R412 residue. Furthermore, two hydrogen bonds between residues 307 and S309, and between Y409 and R412 residue were lost but one hydrogen bond was created between the backbone of S409 residue and side chain of R412 residue. Thus, the replacement R374W (see Figure 4.3B) will result in a new hydrogen bond network of CYP11B2.

When considering the hydrogen bond network around amino acid S315 of CYP11B2, there were two hydrogen bonds between the backbone of S308 with either the backbone of T312 or the side chain of L311. Two hydrogen bonds were generated in the interaction of M309 with A313, and between E310 and G314. In addition, the backbone of S315 interacts with the backbone of L311 (see Figure 4.4A). In this situation, the replacement of serine by arginine at amino acid position 315 again changes the hydrogen bond network of the protein close to the active site. One hydrogen bond was added between the backbone of L311 and the side chain of G314, but one hydrogen bond between the backbone of S308 and the side chain of S311 was lost, as compared with hydrogen bond around S315 (see Figure 4.4B).

Thus, a point mutation (S315R) within the I-helix and the other point mutation (R374W) of the Glu-X-X-Arg motif, might generate deleterious conformational changes of the enzyme and of the the substrate recognition site due to the formation of an alternative hydrogen bond network.

In conclusion, we detected two novel missense mutants (S315R; R374W) within the CYP11B2 of a patient suffering from CMO I deficiency. The expression of the two single mutants in the COS-1 cells indicated that both, S315R and R374W, mutants abolished enzyme activity. Moreover, our computer model indicated a formation of a new hydrogen bond network that could provide valuable insights into cytochrome P450 structural-functional relationships.

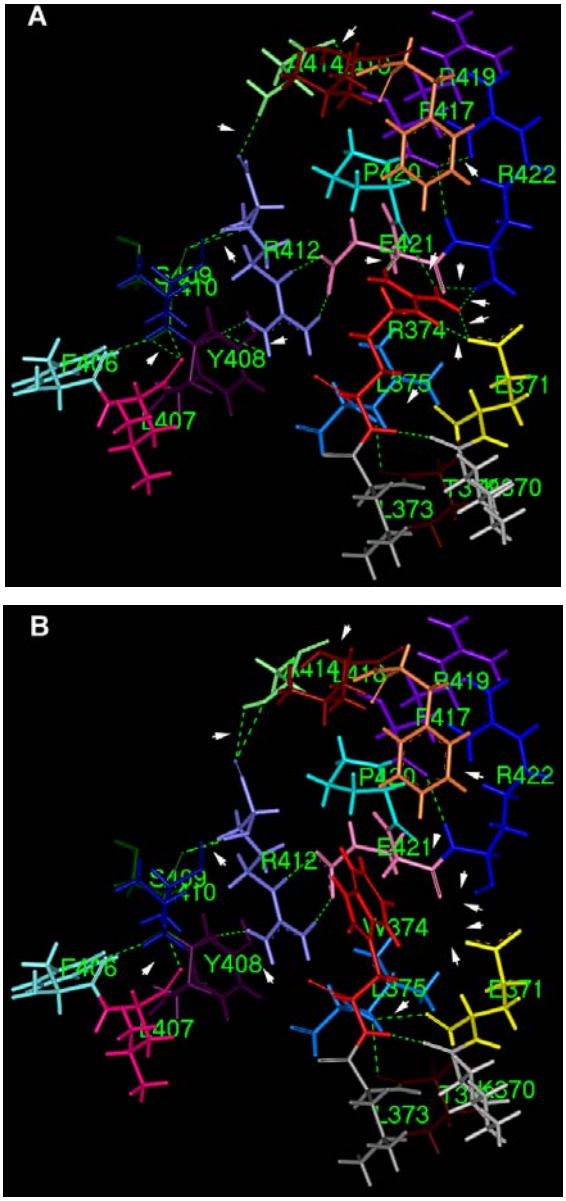


Figure 4.3: **Hydrogen bond network around residue 374 of different CYP11B2 species.** The criteria for the hydrogen bonds were a minimum distance between 1.2 Å and a maximum distance of 2.8 Å between acceptor and donor as well as a minimum angle of 120° between acceptor and donor. Models were generated as described under materials and methods. Blue dashed lines: hydrogen bonds of CYP11B2. Twelve white arrows show that hydrogen bonds were changed when arginine 374 was substituted by trytophan. A, Hydrogen bonds of WT (hydrogen bond around R372). B, Hydrogen bonds of mutant (hydrogen bond around W374).

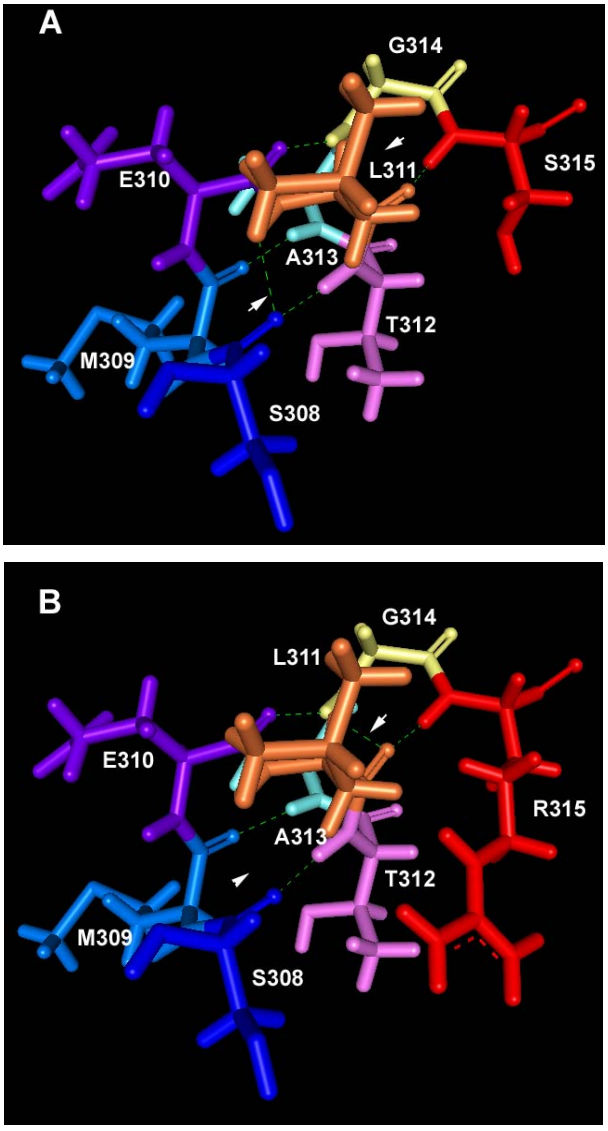


Figure 4.4: Hydrogen bond network around residue 315 of different CYP11B2 species. The criteria for the hydrogen bonds were a minimum distance between 1.2 Å and a maximum distance of 2.8 Å between acceptor and donor as well as a minimum angle of 120°C between acceptor and donor. Models were generated as described under materials and methods. Blue dashed lines: hydrogen bonds of CYP11B2. The white arrows show that hydrogen bonds were changed when serine 315 was substituted by arginine. A, Hydrogen bonds of WT (hydrogen bond around S315). B, Hydrogen bonds of mutant (hydrogen bond around R315).

4.2.4 Patient 4: Heterozygous mutant R181Q leading to CMO II deficiency

CMO II deficiency is characterized by high serum concentrations of corticosterone and 18-hydroxycorticosterone and low concentrations of aldosterone, i.e. is a specific disorder only in the 18-methyloxidase activity of CYP11B2. As mentioned above, CMO II deficiency has been studied in seven Iranian Jewish kindreds in which all 12 patients were homozygous for 2 different point mutations, R181W and V386V (Pascoe et al. 1992a). These mutants only cause disease when the individual is homozygous for both mutants or compound heterozygous of R181W and T318M (Zhang et al. 1995). When these mutations were individually introduced into the *CYP11B2* cDNA and expressed in cultured cells, R181W reduced 18-hydroxylase and abolished 18-oxidase activities but left 11β-hydroxylase activity intact, whereas V386A caused a small but consistent reduction in the production of 18-hydroxycorticosterone.

In our case, analysis of the results of urinary steroid profile indicated that the patient has symptoms of CMO II deficiency: high levels of the B-TH-metab (tetrahydro-11-corticosterone metabolites) derived from B, 18-OH-B-metab (tetrahydro-18-hydroxy-corticosterone metabolites) derived from 18-OH-B, and a low level of Aldo. In addition to this, the ratios of the steroid profile are indicative for CMO-II deficiency.

Genetically, we detected a heterozygous missense mutation (R181Q) in the CYP11B2 of this patient. The protein expression of CYP11B2-R181Q analyzed by the Western blot (see Figure 3.22) showed that the mutant could be expressed in COS-1 cells. When testing the CYP11B2 activity using the substrates DOC in COS-1 cells it was shown that the mutant R181Q led to an increased corticosterone (B), reduced 18-hydroxylcorticosterone and abolished aldosterone (Aldo) level as compared with the CYP11B2-WT and the mock sample. This result can explain part of the urinary steroid profile which exhibited high levels of the B-TH-metab. As discussed above, B and 18-OH-B can be produced from 11-deoxycorticosterone by CYP11B1, but CYP11B1 can not convert 18-OH-B to form Aldo. In addition, it is possible that the enzyme carrying

R181Q has more 18-oxidase activity in zona glomerulosa cells than is displayed in COS-1 cells. So far, we did not find other mutations in the second allele (9 exons and boundaries of exon/intron). Therefore, it is not possible at the moment to fully explain the genotype-phenotype correlation of this patient. The disease could be due to other undetected mutations in the *CYP11B2* gene, such as a mutation in the promoter or in an intron which disrupts splicing.

Summarizing, a heterozygous missense mutant R181Q was detected in the patient suffering from CMO II deficiency. The protein expression of CYP11B2-R181Q in COS-1 cells indicated that this mutant increased corticosterone, reduced 18-hydroxycorticosterone and abolished aldosterone.

4.3 11β-hydroxylase deficiency - Patient 5: replacement L299P in the *CYP11B1* gene

Congenital adrenal hyperplasia(CAH) ranks among the most frequent inborn errors of metabolism following an autosomal recessive trait. It is caused by the loss or severe decrease in activity in one of the five steroidogenic enzymes involved in cortisol biosynthesis. Approximately 90–95% of all cases are due to steroid 21-hydroxylase deficiency (White et al. 2000), and about 5–8% are caused by 11β-hydroxylase deficiency of CYP11B1 (Speiser et al. 2003). *CYP11B1* gene mutations were found within the entire encoding region (see Figure 1.7).

In our case, the index patient was presented with severe illness consisting of urosepsis, CMV, and EBV infection. Besides from haemodynamical stabilization, glucocorticoids seem to exert anti-inflammatory rather than immunosuppressive effects in severe septic infections and their administration have proven beneficial in septic shock patients without adrenal insufficiency (Keh et al. 2003). Additionally, decreased mortality has been observed in acutely ill patients who demonstrated functional adrenal insufficiency with lower random cortisol levels or attenuated cortisol response to corticotropin-releasing hormone when glucocorticoids were supplemented (Cooper et al. 2003). Therefore, the patient's clinically severe state might have been provoked by hypocortisolism. Both our

siblings showed a consistent genital phenotype and borderline hypertension (95th Pc). However, pronounced differences of serum androgen levels (17-OHP, testosterone, DHEAS) could be detected. The diverse biochemical phenotype might be explained by the different age at the time of diagnosis and cross-reactivity of antibodies or interference of matrix using immunoassays (Makela et al. 1988; Wudy et al. 1995).

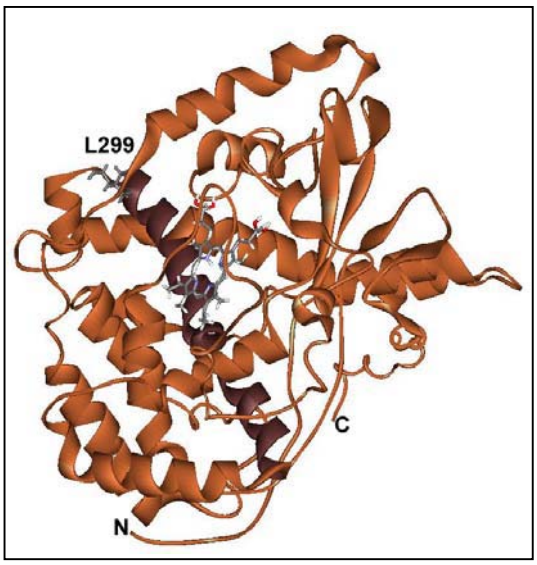


Figure 4.5: Three-dimensional molecular model of CYP11B1. Amino terminus and carboxyl terminus are marked by N and C, respectively. The helix I is colored in brown. The L299 residue is located N terminal from the I helix of the CYP11B1 protein.

Furthermore, genotype of our cases demonstrate for the first time the genital phenotype in 46,XX patients with 11β-hydroxylase deficiency due to a homozygous L299P mutation in the *CYP11B1* gene. Complete virilisation occurred in both siblings leading to male gender assignment after birth. The identical mutation has been found only recently in a compound heterozygous male patient from Iraq (L299P/c.1180delA, resulting in a premature termination at codon 429) who was first seen for symptoms of genital hyperplasia at the age of 2.5 years (Krone et al. 2005). Our expression studies using the HCT116 cells showed that the L299P mutant reduced the 11β-hydroxylase activity to 1.6 ± 0.8% for the conversion of 11-deoxycortisol to cortisol. This result corresponds very well to the result of (Krone et al. 2005), describing a residual activity of this mutant of 1.2 ± 0.9% after expression in COS-7 cells. Functional analysis of L299P mutation

revealed markedly reduced enzymatic activity, assumedly caused by a change of the I helix position (see Figure 4.5) which results in a steric disarrangement of the polar and apolar parts of the heme group relative to the enzyme and hence altered three-dimensional molecular structure (Krone et al. 2005).

In conclusion, a homozygous mutation (L299P) was detected in two 46,XX siblings with 11β-hydroxylase deficiency, leading to a near total loss of enzymatic activity and complete virilisation. The expression of cDNA constructs containing mutation L299P in HCT116 cells demonstrated that the L299P mutant reduces 11-hydroxylase activity to 1.6 ± 0.8% for the conversion of 11-deoxycortisol to cortisol.

4.4 Outlook

Mutations in CYP11B1 and CYP11B2 have been identified in patients sufferring from congenital adrenal hyperplasia and aldosterone synthase defficiency. The results from the present study will help to understand the relationship between phenotype and genotype. Moreover, the combination of heterologous expression analysis in cell culture and molecular modeling provides insight into structural-functional relationship of cytochrome P450s.

In the patients having no mutations or only one mutation in one allele, the *CYP11B2* gene may have mutaions in the promoter regions or in introns, a mutation in which disrupts splicing. The effects on splice site by silent mutations should also be investigated. Identification of mutations in all the patients will provide information for diagnosis and for timely treatment of the patients.

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Appendix

A. Publications resulting from this work

1) Aldosterone Synthase Deficiency Caused by a Homozygous L451F Mutation in the CYP11B2 Gene
   Huy-Hoang Nguyen[1], Frank Hannemann[1], Michaela F. Hartmann[2], Stefan A. Wudy[2], Rita Bernhardt[1]. Mol Genet Metab. Vol 93/4 pp 458-467.

2) Complete virilisation in two 46,XX siblings with 11-beta-hydroxylase deficiency due to homozygous L299P mutation in the *CYP11B1* gene
   Stefan Riedl, Huy-Hoang Nguyen, Franz Waldhauser, Susanne Clausmeyer *, Egbert Schulze *, Rita Bernhardt. Hormone research (in preparation).

B. Chemicals and enzymes

All commercially obtained materials used in this work were of the finest quality available purchased from the companies listed below:

Chemicals:

Amersham Pharmacia Biotech
Bio-Rad Laboratories
BioTez GmbH
Biozym GmbH
Clontech Laboratories GmbH
Difco Laboratories
Dulbeco
Fluka AG
Gibco/BRL GmbH
Invitrogen BV
Kodak
Merck
MWG Biotech GmbH

New England Biolabs

Promega

Qiagen

Roche Molecular Biochemicals

Serva GmbH & Co KG

Sigma-Aldrich

Stratagene

Enzymes:

Bioline

Stratagene Ltd, Cambridge, UK

New England Biolabs

Boehringer

CLONTECH Laboratories

MBI Fermentas

Promega Corporation

Radioactive-labeled chemicals:

Amersham Pharmacia Biotech

NEN DuPont

Consumables:

Amersham Pharmacia Biotech

Fisher Scientific

Falcon

Machery-Nagel GmbH & Co KG

Millipore

Carl Roth GmbH

Qiagen GmbH

C. Scientific apparatus

Autoclave	Zirbus IMM 20
CD-spectrometer	Jasco 715 spectropolarimeter
DNA-Sequencer	Licor-DNA sequencer 4000
Electrophoresis and blotting apparatus	Sigma-Aldrich system and
	EPR Bruker ESP300E spectrometer
Iamging plates	Fuji
Incubator	NewBrunswick Scientific
Phosphoimager	Fuji Bas-2500
SpeedVac	UniEquip Univapo
Thermomixer	Eppendorf thermomixer
Thermocycler	MJ Research
UV/Vis spectrophotometer	Shimadzu UV-2101 PC
	Gene-QuantII-Photometer
Centrifuges	Sigma 3K30 (rotors 19776, 12153)
	Sigma 2K15 (rotor 12148)
	Hitachi Himac CP75β(rotors P45, P30)

D. Genomic DNA of CYP11B2 and CYP11B1

CYP11B2 gene was received from Kawamoto *et al*. (1992) publishes under the Accession number D13752. The exons are red. The start codon is bold. Primers are shown in under lines.

```
GGATCCTGCAAGGAGGGATACAAATTACATACATTTGTCAAAACCCACAGCATGTTGACCACCAG    65

GAGGAGACCCCATGTGACTCCAGGACCCTGGTTGATAACAACGTATCGAGATTCCTCACATGGAA    130

CCAGTGCGCTCCTGTGGTGGAGGGTGTACCTGTGTCAGGGCAGGGGGTACGTGGACATTTTCTGC    195

AGTTTTTGATCAATTTTGCAATGAACTAAATCTGTGGTATAAAAATAAAGTCTATTAAAAGAATC    260

CAAGGCTCCCTCTCATCTCACGATAAGATAAAGTCCCCATCCATTTTACTCCTCTCAGCCCTGGA    325

GAAAGGAGAGGCCAGGTCCCACCACCTTCCACCAGCATGGACCCCCAGTCCAGACCCCACGCCTT    390
                                                        E16F⇒
TTCTCAGCATCCTCAGACCAGCAGGACTTGCAGCAATGGGGAATTAGGCACCTGACTTCTCCTTC    455

ATCTACCTTTGGCTGGGGGCCTCCAGCCTTGACCTTCGCTCTGAGAGTCTCAGGCAGGTCCAGAG    520

CCAGTTCTCCCATGACGTGATATGTTTCCAGAGCAGGTTCCTGGGTGAGATAAAAGGATTTGGGC    585
```

EXON 1 E1E6sF⇒

TGAACAGGGTGGAGGGAGCATTGGA**ATG**GCACTCAGGGCAAAGGCAGAGGTGTGCGTGGCAGCGC 650

CCTGGCTGTCCCTGCAAAGGGCACGGGCACTGGGCACTAGAGCCGCTCGGGCCCCTAGGACGGTG 715

CTGCCGTTTGAAGCCATGCCCCAGCATCCAGGCAACAGGTGGCTGAGGCTGCTGCAGATCTGGAG 780

GGAGCAGGGTTATGAGCACCTGCACCTGGAGATGCACCAGACCTTCCAGGAGCTGGGGCCCATTT 845

TCAGGTAAAGCCCTCCCTGGCCCTCGCTGGGAACACCCAGATCCCTGCCCCTGCTGCCCAGGACC 910

⇐E1R

CTGCCAGGCACTCAGCACTGCCATTCCCAGCAGGTCCCGGCACTCTGCATCCTTTGGAGGATGGG 975

GAAGGAGTGCAGCACATGCTGGTCTGTGGTGCTGCCAGGGCAGGGGATAGTGCAGAGAAAACCCC 1040

AGCTCACTGCAGAGAGGGCAGGACTCAGAAGCACTAAAGTTGAAAGGTTCCAGGGAGCCAGCAGG 1105

AGGGCTTTAGCTGTGAAGCCGCTAATCCAGGAGCAGGGAGGGTGGACAGGAGACACTTTGGATTG 1170

GGACTGCAGGGTGGGGCCACGAGGGACATGACCCCGTCCAGCAGGGCCTCCTGCTTGGCCCCACA 1235

EXON 2

GGTACAACTTGGGAGGACCACGCATGGTGTGTGTGATGCTGCCGGAGGATGTGGAGAAGCTGCAA 1300

CAGGTGGACAGCCTGCATCCCTGCAGGATGATCCTGGAGCCCTGGGTGGCCTACAGACAACATCG 1365

⇐E2R

TGGGCACAAATGTGGCGTGTTCTTGTTGTAAGCGGCGAGTTGGGAGCTGAGAGCTGGGAGCAGGG 1430

TGGGCAGCCTGGGTGTAGGGGGGAGGCGAGAGAGGTAGGACCCAAAAGCACATCTGCCCTGGGCC 1495

CCTGTGGTGGGCAGTGAGGGTGAGCACCCGGCCCAGAGGACGGCCATCCTGTGGGGTCGCGTCTG 1560

CACTGTGGGTTGGGGAAGCAGGGCGGTGGTGGAGAAATGGGCACGGGCACCTCTGCAGAGAAGAC 1625

GCAGAGCAATGAGCCCTTCTGTGTAGTGAGAACCCGCTCTGCACCAACCTCGGCGGCTGCTTTCT 1690

CTTGCGGTCTGGGGACTGTCCTTCCCATAGGTCAGAAAACTGAGGCCCTGAGAAGGGGACTTCCA 1755

CTGGCCCAGGTCACAGGCTGAGTGCTGAGCCTGGTGTTCGCCGGGGCCGCAGCCTCCCTCAGGGC 1820

GCTCAGGGTCCCTGCAGTCCTGGCAAACCTTCCTGATGGGGACAGTCCGGGGCAGGAGGCAGGTG 1885

GGGACGCAGGTGGCTGGTGGTTCCGTTGTTCTCAGAAGCAAGGCACAAGGTGGGGCGGTTGATGG 1950

CACTGGGGAGGATGTTTCCTGGCCCGTGGAGAGGGTGGCGCCTGGTCAGGTGGGCAGGGAGAGGC 2015

TGATGCTTGGAGTCGGTCACCTGCAGGGATGTTGTCATTAGGACGGGGGAAGGACTGGATGAGGA 2080

TGTCACAGTGGTGACAGCCCCCACTCCATGGTAGGAAGGGAACGCTATTGGGAATAGTGGGGTTT 2145

AGGTAAAAGGGCACCCGTGGGTCGGGGCCTTCACTGAGGCTGGCCTATAGATGACATCTGGGAGA 2210

GAGTCAGGACCCAGGAAGGCAGGTCCAGGAGGCTGGGTGCGCATAATGGAAGGAAGGGGAGCGCT 2275

CCTGTCTGTGTGTGTGTCTTGCATCTGTGCACATGCTGTGTGTTTCTCTGTACCTGCATTGCACA 2340

TGTGTAGTGTGTGCACGTGTCGTGTGTGAATGTATGTGTGGTGTGTGTGCACAAGTGTCTGTGTG 2405

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TGTGCATGTGCAGGTGCCGGCATGGGTGTAGTGTTTGTGCACACATGCACATGCGTCTCTTCACA    2470

CATGGTGTTGAGGTCTTGCATGGGCGCACGTGTGCATGTGCATCTTCTGCCTGTCATCACTGTCA    2535

ACAGCTCACAGCAGCCAGCTGGACATAAATAAAGGAGTTTTGCAGGAATGTGGCTGACAGGGGAA    2600

ATTCCTCCCCACCATTCCCTGGGGGCATCCATGGAGCCCCCACGCACTCTGGCTGTGGGTAGGAT    2665

GGCATGAAGCACAAAGCTTGGTTTCTGTCCTGCAGAAGATATAGATGCTTCACAGAGACAGCAGA    2730

GCAGATGCCCCAGAGGCACTGTGCCCAGGGCGGGGAAGGGTGGGGAGGAGAGGGCAGCCAGGGGC    2795

TCTCCCCTCAGGACACTGTGTGGGTGAGGTGGGCAAAGCTTGACAACAGGGGTCACCTCCTTTCT    2860

TGGAGAAAAGCCCTACCCTGTTACTACAGGGAGGGCCCGCATGGGTGAGGTGGTGCCAGACTTGG    2925
                                                  E46F⇒
GTCGCCAGGTCCCGGGAATGACCTCAGTTACCCTGTCAGCACCTGTGGGCAGAAGCTACCATCTC    2990

ATCCCTGCTTAGACCTGAGTGGCCTTTGCCCAGCACCTGGAGGCCGCTCTGAGAAAAGGCTGCAG    3055
                                                        E3F⇒
CTCGAACACAAACAGGCAGCTTCTACCAGGGCCCCCAGTCAGCTCCCTGCAGGCCGATTCCCCTT    3120

GGGGACAAGGAGGATGGGATACGGGTCAGGGCCTGTGTCTTGCTGGGGCGGCCTCACAAGCTCTG    3185
                EXON 3                                          ⇐E45R
CCCTGGCCTCTGTAGGAATGGGCCTGAATGGCGCTTCAACCGATTGCGGCTGAACCCAGATGTGC    3250

TGTCGCCCAAGGCCGTGCAGAGGTTCCTCCCGATGGTGGATGCAGTGGCCAGGGACTTCTCCCAG    3315

GCCCTGAAGAAGAAGGTGCTGCAGAACGCCCGGGGGAGCCTGACCCTGGACGTCCAGCCCAGCAT    3380

CTTCCACTACACCATAGAAGGTGTGGGCCATGCGGGAAGGTCCAGCCCCAGAGACCCTGGAGTGG    3445
                                                  E4F⇒
CCAGGGATGGGGATGGAGGACTGAAGGGAGTGTGGGGAGGCAGCCAGGAGGCCTGGGGCTGCCTT    3510
                E4E6sF⇒     EXON 4
GTGCTCAGCAGTGCATCCTCCCCGCAGCCAGCAACTTAGCTCTTTTTGGAGAGCGGCTGGGCCTG    3575

GTTGGCCACAGCCCCAGTTCTGCCAGCCTGAACTTCCTCCATGCCCTGGAGGTCATGTTCAAATC    3640
                ⇐E4R
CACCGTCCAGCTCATGTTCATGCCCAGGAGCCTGTCTCGCTGGATCAGCCCCAAGGTGTGGAAGG    3705

AGCACTTTGAGGCCTGGGACTGCATCTTCCAGTACGGTGAGGCCAGGGACCCGGGCAGTGCTATG    3770

GGGAAGGGACACCATGGGGGCCCAATTTCTCCTTCTCCACCACCCAGTGGGGAATGGAGGCCACA    3835

GGGAGGGGTCGGGGATTCCTCACCTTCCTGCCGGGGAGATTGGTGCGAGGCTGGGGCTGGGCTGG    3900

GCTGATCCGGAGAATTTGGGATGAGAGCAGGGAGATTTGGGTGTCGGGGCAGTCTGGGCAGGAGG    3965
E5F⇒
AGGACACTGAAGGATGCTTCCCAGCACCAAGATCTGAGGGCTGTCCCCTGCTCCCTGGACAGGTG    4030
EXON 5          E5E6sF⇒          E48F⇒
ACAACTGTATCCAGAAAATCTACCAGGAACTGGCCTTCAACCGCCCTCAACACTACACAGGCATC    4095

GTGGCGGAGCTCCTGTTGAAGGCGGAACTGTCACTAGAAGCCATCAAGGCCAACTCTATGGAACT    4160
        E5F⇒
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CACTGCAGGGAGCGTGGACACGGTCAGGCCAGCAACCAGCCCCACCCAGAGAGGGTGATGCCAAG    4225

CCTGCCTCCCAGGCACTGCCTGCCAATGCCACACGGCACCCACGTTCCCCATCCCCAGGCTACAG    4290

GCCCCACATTTCTGTTGCCCTCAGCCTTCCCCCTCCTTTGTTAAGGGATGAGATTTGCAGGGGAG    4355

GGGAAATGTGAGCTCCCCCTCACATGAGACTGAGTTTGCAGTTACCTGTGTGGGGATCCATGCTC    4420

CAGGCTGGAAGAAAGTTGGATGAGGCCCTGGACACACAGCAGCTCTGTCCCCACTGGAAAGCTCT    4485

GGGTGTACAAGGAGAAGGAGGGTTGAGAGGCAGCTGGAGGACTCCACTGGGCACCCTTCCCAGTG    4550

TGCCCGGTCACCTTGGGCCAGAAATGTAGATGCATGGGAGGGCAGGGTTGTGGGGAAGACAGCAG    4615

CACAGGCTCCAGCCAGTGCAGAGGGGCCTGTGGGTGCACAGTGGGGAGAACTCAATGGAAGCAGA    4680

GGGAGCTGGGGCTCCAGAACTCCCTGGATGATGCTGAGGTGTGGCCCCCTGCCCTAATGGTGGCT    4745

GTGAGAACCCGCCCTGAAGAGGCTGCAGGGGACCTGGGCCTTGGTGGAGATGGGGGTCACCTTTC    4810

CCTGAAGAAGTCAGGGAATCTGGCCCAAGTGGTCATCAAGGTTTCAGATCCGGGGTCCCAGGGCT    4875

CTGTTTTTGCTCAGGGCATGGATGTCTCCACCCCTCAGAGGGAGGTTGTCCTGGGAGGGGTGTCC    4940
E6F⇒                                                    EXON 6
CGGGGGCTGAGTCCTCCTGTGCAAGGTCTGACCCTGCAGACATGGCTTCTGTAGACAGCGTTTCC    5005
⇐E47R
CTTGCTGATGACGCTCTTTGAGCTGGCTCGGAACCCCGACGTGCAGCAGATCCTGCGCCAGGAGA    5070

GCCTGGCCGCCGCAGCCAGCATCAGTGAACATCCCCAGAAGGCAACCACCGAGCTGCCCTTGCTG    5135
        ⇐E1E6sR
CGGGCGGCCCTCAAGGAGACCTTGAGGTGGGTGCTGGCTGAGGCCTCCCTGTGGCCCTGGCCCCC    5200

TGCTGGAGAGCAGCCCCCACTGGGTGGTGGCAGACAGAATCTGGGGCTGATAAACAGCGTCACCC    5265

AGCAGCCCATTCCCCTGCACCTGCTCTTCCTCCCCCTCAAGGACAGGGAGCTCTTCTTCCTCTGG    5330

AATCCCTCTTCAACGCCCTGGGGATTAACGTGGGGCATGTCCTTCTGCGCTCGGGGCTGCTTAAG    5395

TTAGGGGAGGTTTGGCTGGGCTCAGCAGGTGCAAGGAAGCACTTCCTACGACCTGGGCTTCCCAT    5460
                                    E7/8F⇒
GGATCTGGGACCTCTGCGGGGTCTTCGGTAGGAAGGGTGCAGAGAGCACAGGGAGCCCCATCCAG    5525
                                          EXON 7
CTGAGGACCCTTTCTGTGGATGCCCCCACCTCCAGGCTCTACCCTGTGGGTCTGTTTTTGGAGCG    5590
                E7F⇒
AGTGGTGAGCTCAGACTTGGTGCTTCAGAACTACCACATCCCAGCTGGGGTGAGTGAGCCCCACA    5655

CCCCTCGAGCTGAGAACCTCCCTCCCCAGTCATTCCCTGATCCCTGCTCTGCACCGTCCGCAGAC    5720
EXON 8
ATTGGTACAGGTTTTCCTCTACTCGCTGGGTCGCAATGCCGCCTTGTTCCCGAGGCCTGAGCGGT    5785

ATAATCCCCAGCGCTGGCTAGACATCAGGGGCTCCGGCAGGAACTTCCACCACGTGCCCTTTGGC    5850

TTTGGCATGCGCCAGTGCCTCGGGCGGCGCCTGGCAGAGGCAGAGATGCTGCTGCTGCTGCACCA    5915

CGTAAGCAGGCCTGGGGGCGGGGGCGGGACCTGGGCAGCAGAGGCGGGACCTGCACACTGGGGGC    5980
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GGGGCTTGCATGGTGTGATTGACACCTGGGAACAGTGGATGGGGCCTTGGTTGGTTGAGGTCGGC    6045

GTGACCAGGGAGGATCTGTGCTGAGCAAGACAGGGTAGGATCTGGGTGAGGTTGCTTCTAAACAT    6110

TGAAATGGGGACTAGGGGAGTGGGGTGGAGCCTGTACAGAATAATGGGGCTTGGGCAAGACCTGG    6175

GCAGGATTCAGTCTGGGCCTGGTCCGCAAGGTGGGGCTGGTCAGAAATGGGATAGGTTGGGGCCC    6240

AGGCTGCTGCTCCCCCTTCAGCATAATTGTTGCACCTGGGACGATGGGAGGAAGCTGCCCCAGGT    6305
                                                      EXON 9
CCATGGGCTACTGACCAGGCCAGATGGAAACCCAGCCTCTGTCCTAGGTGCTGAAGCACTTCCTG    6370

GTGGAGACACTAACTCAAGAGGACATAAAGATGGTCTACAGCTTCATATTGAGGCCTGGCACGTC    6435
                                              ⇐E19sR
CCCCCTCCTCACTTTCAGAGCGATTAACTAGTCTTGCATCTGCACCCAGGGTCCCAGCCTGGCCA    6500
                                                                ⇐E9R
CCAGCTTCCCTCTGCCTGACCCCAGGCCACCTGTCTTCTCTCCCACGTGCACAGCTTCCTGAGTC    6565

ACCCCTCTGTCCAGCCAGCTCCTGCACAAATGGAACTCCCCAGGGCCTCCAGGACTGGGGCTTGC    6630
        ⇐E49R
CAGGCTTGTCAAATAGCAAGGCCAGGGCACAGCTGGAGACGATCTTGCTGGCAGGGCCTGGCCTT    6695

GTCCCCAGCCCCACCTGGCCCCTTCTCCAGCAAGCAGTGCCCTCTGGACAGCTTGACTCTACTCC    6760

TCCCAGCGCTGGCTCCAGGCTCCTCATGAGGCCATGCAAGGGTGCTGTGATTTTGTCCCTTGCCT    6825

TCCTGCCTAGTCTCACATGTCCCTGTCCCTCTCGCCCTGGCCAGGGCCTCTGTGCAGACAGTGTC    6890

AGAGTCATTAAGCGGGATCC                                                  6910
```

Sequence of human *CYP11B2* cDNA

Nucleotide sequence translated an amino acid sequence. The sequence begins with the start codon (M-methionine) and ends with the stop codon (-). Numbers refer on the right to the nucleotides and the amino acids.

```
atggcactcagggcaaaggcagaggtgtgcgtggcagcgccctggctgtccctgcaaagg   60
 M  A  L  R  A  K  A  E  V  C  V  A  A  P  W  L  S  L  Q  R    20
gcacgggcactgggcactagagccgctcgggcccctaggacggtgctgccgtttgaagcc   120
 A  R  A  L  G  T  R  A  A  R  A  P  R  T  V  L  P  F  E  A    40
atgccccagcatccaggcaacaggtggctgaggctgctgcagatctggagggagcagggt   180
 M  P  Q  H  P  G  N  R  W  L  R  L  L  Q  I  W  R  E  Q  G    60
tatgagcacctgcacctggagatgcaccagaccttccaggagctggggcccattttcagg   240
 Y  E  H  L  H  L  E  M  H  Q  T  F  Q  E  L  G  P  I  F  R    80
tacaacttgggaggaccacgcatggtgtgtgtgatgctgccggaggatgtggagaagctg   300
 Y  N  L  G  G  P  R  M  V  C  V  M  L  P  E  D  V  E  K  L   100
caacaggtggacagcctgcatccctgcaggatgatcctggagccctgggtggcctacaga   360
 Q  Q  V  D  S  L  H  P  C  R  M  I  L  E  P  W  V  A  Y  R   120
caacatcgtgggcacaaatgtggcgtgttcttgttgaatgggcctgaatggcgcttcaac   420
 Q  H  R  G  H  K  C  G  V  F  L  L  N  G  P  E  W  R  F  N   140
cgattgcggctgaacccagatgtgctgtcgcccaaggccgtgcagaggttcctcccgatg   480
```

```
 R  L  R  L  N  P  D  V  L  S  P  K  A  V  Q  R  F  L  P  M   160
gtggatgcagtggccagggacttctcccaggccctgaagaagaaggtgctgcagaacgcc  540
 V  D  A  V  A  R  D  F  S  Q  A  L  K  K  K  V  L  Q  N  A   180
cgggggagcctgaccctggacgtccagcccagcatcttccactacaccatagaagccagc  600
 R  G  S  L  T  L  D  V  Q  P  S  I  F  H  Y  T  I  E  A  S   200
aacttagctcttttggagagcggctgggcctggttggccacagccccagttctgccagc  660
 N  L  A  L  F  G  E  R  L  G  L  V  G  H  S  P  S  S  A  S   220
ctgaacttcctccatgccctggaggtcatgttcaaatccaccgtccagctcatgttcatg  720
 L  N  F  L  H  A  L  E  V  M  F  K  S  T  V  Q  L  M  F  M   240
cccaggagcctgtctcgctggatcagccccaaggtgtggaaggagcactttgaggcctgg  780
 P  R  S  L  S  R  W  I  S  P  K  V  W  K  E  H  F  E  A  W   260
gactgcatcttccagtacggtgacaactgtatccagaaaatctaccaggaactggccttc  840
 D  C  I  F  Q  Y  G  D  N  C  I  Q  K  I  Y  Q  E  L  A  F   280
aaccgccctcaacactacacaggcatcgtggcggagctcctgttgaaggcggaactgtca  900
 N  R  P  Q  H  Y  T  G  I  V  A  E  L  L  L  K  A  E  L  S   300
ctagaagccatcaaggccaactctatggaactcactgcagggagcgtggacacgacagcg  960
 L  E  A  I  K  A  N  S  M  E  L  T  A  G  S  V  D  T  T  A   320
tttcccttgctgatgacgctctttgagctggctcggaaccccgacgtgcagcagatcctg 1020
 F  P  L  L  M  T  L  F  E  L  A  R  N  P  D  V  Q  Q  I  L   340
cgccaggagagcctggccgccgcagccagcatcagtgaacatccccagaaggcaaccacc 1080
 R  Q  E  S  L  A  A  A  A  S  I  S  E  H  P  Q  K  A  T  T   360
gagctgcccttgctgcgggcggccctcaaggagaccttgcggctctaccctgtgggtctg 1140
 E  L  P  L  L  R  A  A  L  K  E  T  L  R  L  Y  P  V  G  L   380
tttttggagcgagtggtgagctcagacttggtgcttcagaactaccacatcccagctggg 1200
 F  L  E  R  V  V  S  S  D  L  V  L  Q  N  Y  H  I  P  A  G   400
acattggtacaggttttcctctactcgctgggtcgcaatgccgccttgttcccgaggcct 1260
 T  L  V  Q  V  F  L  Y  S  L  G  R  N  A  A  L  F  P  R  P   420
gagcggtataatccccagcgctggctagacatcaggggctccggcaggaacttccaccac 1320
 E  R  Y  N  P  Q  R  W  L  D  I  R  G  S  G  R  N  F  H  H   440
gtgccctttggctttggcatgcgccagtgcctcgggcggcgcctggcagaggcagagatg 1380
 V  P  F  G  F  G  M  R  Q  C  L  G  R  R  L  A  E  A  E  M   460
ctgctgctgctgcaccacgtgctgaagcacttcctggtggagacactaactcaagaggac 1440
 L  L  L  L  H  H  V  L  K  H  F  L  V  E  T  L  T  Q  E  D   480
ataaagatggtctacagcttcatattgaggcctggcacgtccccctcctcactttcaga  1500
 I  K  M  V  Y  S  F  I  L  R  P  G  T  S  P  L  L  T  F  R   500
gcgattaactag                                                  1512
 A  I  N  -                                                    503
```

genomic DNA of CYP11B1

```
AGTTTTGGATCTTTCCTGCTTTCTCTTGTGGGCATTTAGTGCTATAAATTTCCCTCTACACACTG    65

CTTTGAATGTGTTCCAGAGATTCTGGTATGCTGTGTCTTTGTTCTCGTTGGTTTCAAGAACATCT   130

TTATTTCTGCCTTCATTTTGTTACGTACCCAGTAGTCATTCAGGAGCAGGTTGCTCAGTTTCCAT   195

GTAATTGAGCGGTTTTGAGTGAGTTTCTTAATCCTGAGTTCTAGTTTGATTGCACTAAAATTTTT   260

AAAAAGTAAAAAAAATACATGTGGTTTAATACAATTCATGCCAACTCATTCCCTCGTTTTTTGCT   325

ATAAACCTTGCAAGGAGATGAATAATCCAAGGCTCTTGGATAAGATAAGGGCCCCATCCATCTTG   390
```

H1F⇒

CTCCTCTCAGCCCTGGAGGAGGAGGGAGAGTCCTTTTCCCCTGTCTACGCTCATGCACCCCCAAT 455

GAGTCCCTGCCTCCAGCCCTGACCTCTGCCCTCGGTCTCTCAGGCAGATCCAGGGCCAGTTCTCC 520

H2F⇒

CATGACGTGATCCCTCTCGAAGGCAAGGCACCAGGCAAGATAAAAGGATTGCAGCTGAACAGGGT 585

EXON 1

GGAGGGAGCATTGGA**ATG**GCACTCAGGGCAAAGGCAGAGGTGTGCATGGCAGTGCCCTGGCTGTC 650

CCTGCAAAGGGCACAGGCACTGGGCACGAGAGCCGCCCGGGTCCCCAGGACAGTGCTGCCCTTTG 715

AAGCCATGCCCCGGCGTCCAGGCAACAGGTGGCTGAGGCTGCTGCAGATCTGGAGGGAGCAGGGT 780

TATGAGGACCTGCACCTGGAAGTACACCAGACCTTCCAGGAACTAGGGCCCATTTTCAGGTAAAG 845

CCCTCCCTGGCCCTCGCTGGGAACACCCAGAGCCCTGCCCTTGCTGCCCAGGACCCTGCCGGGCA 910

CTCAGCACTGCCATTCCCAGCAGGTCCCGGCACTCTGCATCCTTTGGAAGATGGGGAAGGAGTGC 975

AGCACGTGCTGGTCTGTGGCGCTGCCAGGGCAGGGGATGGTGCAGAGCAAATCCCAGCTCGCTGC 1040

AGAGAGGGCAGGACTCAGAGGCACTGAAGTTAAGAGGTTCCGGGCAGTCAGCAAGAGGGCTTTAG 1105

CTGTGAAGCCGCTAATCCAGGAGAGGGGAGGGTGGACAGGAGACACTTTGGATTGGGACTGCAGG 1170

EXON 2

GTGGGGCTAGCGGGGACATGGTCCCATCCAGCACGGCCTCGTGCTTGGCCCCACAGGTACGACTT 1235

GGGAGGAGCAGGCATGGTGTGTGTGATGCTGCCGGAGGACGTGGAGAAGCTGCAACAGGTGGACA 1300

GCCTGCATCCCCACAGGATGAGCCTGGAGCCCTGGGTGGCCTACAGACAACATCGTGGGCACAAA 1365

TGTGGCGTGTTCTTGCTGTAAGCGGCGAGCTGAGAGCTGGGAGCAGGGTGGGCAGCCTGGGTGTA 1430

GGGGGGAGGCGAGAGAGGCAGGACCCAAAAGCACATCTGCCCTGGGCCCCTGTGGTGGGCAGTGA 1495

GGGTGAGCACCCGACCCAGAGGACGGCCATTCCGTGGGGTCGTGTCTGCCCTGTGGGTTGGGGAA 1560

GCAGGGCGGTGGTGGAGAAATGGGCACGGGCACCTCTGCAGAGAAGATGCAGAGCAATGAGCCCT 1625

TCTGTGTAGTGAGAACCCGCTCTGCACCAACCTTGGCCGATGCTTTCTCTTGCGGTCTGGGGACT 1690

GTCCTTCCCATAGGTCAGAAAACTGAGGCCCTGAGAAGGGTACTCCCACTGGCCCAGGTCACAGG 1755

CTGAGTACTGAGCCTGGTGTTCGCCGGGGCCGCAGCCTCCCTCAGGGCGCTCAGGGTCCCTGCAG 1820

TCCTGGCAAACCTTCCTGATGGGGACAGTCCGGGGCAGGAGGCAGGTGGGGATGCAGGTGGCTGG 1885

TGGCTCCATTGTTCTCAGAAGCAAGGCACGAGGTGGGGCGGTTGATGGCACTGGGGAGGATGTTT 1950

CCTGGCCCGTGCAGAGGGTGGCGCCTGGTCAGGTGGGCAGGGAGAGGCTGATGCTTGGAGTCAGT 2015

CACCTGCAGGAATGTTGTCATTAGGACGGGGGAAGGACTGGACGAGGATGTCACAGTGGCGACAG 2080

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CAGGACACTGTGTGGGTGAGGTGGGCAAAGCTTGACAACAGGGGTCAGTTCCTTTCTTGCAGAAA 2860

ATCCCTCCCCCCTACTACAGGGAGGGCCTGCATGGGTGAGGTGGTGCCAGACTTGGGGTGCCAGG 2925

H4F ⇒

TCCCGGGAATGACCTCAGTTACCCTGTCAGCACCTGTGGGCAGAAGCTACCATCTCATCCCTGCT 2990

TAGACCTGAGTGGCCTTTGTCCAGCACCTGGAGGCCGCTCTGAGAAAAGGCTGCAGCTCGAACAC 3055

AAACAGGCAGCTTCTACCAGGGCCCCCAGTCAGCTCCCTGCAGGCCGATTCCCCTTGGGGACAAG 3120

⇐ H3R

GAGGATGGGATACGGGTCAGGGCCTGTGTCTTGCTGGGGCGGCCTCACAAGCTCTGCCCTGGCCT 3185

EXON 3

CTGTAGGAATGGGCCTGAATGGCGCTTCAACCGATTGCGGCTGAATCCAGAAGTGCTGTCGCCCA 3250

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EXON 4

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CACCATGGGGGCCCAATTTCTCCCTCTCCACCACCCAGTGGGGAATGGAGGCCACAGGGAGGGGT 3835

CGGGGATTCCTCACCTTCCTGCCAGGGAGATTGGTGCGAGGCTGGGGCTGGGCTGGGCTGATCCG 3900

GAGAATTTGGGATGAGAGCAGGGAGACTTGGGTGTCGGGGCAGTCTGGGCAGGAGGAGGACACTG 3965

EXON 5

AAGGATGTCTCCCAGCACCAAAGTCTGAGGGCTGCCTCCCGCTCCCCGGATAGGCGACAACTGTA 4030

H6F ⇒

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TCCAGAAAATCTATCAGGAACTGGCCTTCAGCCGCCCTCAACAGTACACCAGCATCGTGGCGGAG    4095

CTCCTGTTGAATGCGGAACTGTCGCCAGATGCCATCAAGGCCAACTCTATGGAACTCACTGCAGG    4160

GAGCGTGGACACGGTCAGGCCGGCAACCAGCCCCACCCAGAGAGGGTGATGCCAAGCCTGCCTCC    4225

CAGGCACTGCCTGCCAATGTCACACGGCGCCCACGTGTCCCATCCCCAGGCTATGGGCCCCACAT    4290

TTCTTACTTGGGATTGTGATGTGATAAACACGTTTGCAGGTTGCCATGGTTGGAATGGGGGGTTC    4355

CTTTCTGTGGAGGACTCAGGGAAAGGGGTTTGGATGGGCATTAGGATTTGAAGTCTTGGGCTCTG    4420

TCGTTCTCAGGGTATGCATGTCTGCACCCCTCACAGGGAGGTTGTCCTGGGAGGGGTGTCCCGGG    4485
                                                    ⇐ H5R
GGCTGAGTCCTCCTGTGCAAGGTCTGACCCTGCAGCTGTGTCTCCTGCAGACGGTGTTTCCCTTG    4550
                  EXON 6
CTGATGACGCTCTTTGAGCTGGCTCGGAACCCCAACGTGCAGCAGGCCCTGCGCCAGGAGAGCCT    4615

GGCCGCCGCAGCCAGCATCAGTGAACATCCCCAGAAGGCAACCACCGAGCTGCCCTTGCTGCGTG    4680

CGGCCCTCAAGGAGACCTTGCGGTGGGTGCTGGCTGAGGCCTCCCTGTGGCCCTGGCCCCCTGCT    4745

GGAGAGCAGCCCCCACTGGGTGGTGGCAGACAGAATCTGGGGCTGATAAACAGCGTCACCCAGCA    4810

GCCCATTCCCCTGCACCTGCTCTTCCTCCCCCTCAAGGACAGGGAGCTCTTCTTTCTCTGGAATC    4875

CCTCTTCAACGCCCTGGGGATTAACGTGGAGCATGTCCTTCTGCGCTCGGGGCTGCTTAAGTTAG    4940

GGGAGGTTTGGCCGGGCTCAGCAGGTGCAAGGAAGCACTTCCTACGACCTGGGCTTCCCATGGAT    5005

CTGGGACCTCTGCGGGTTCTTCGGTAGGAAGGGTGCAGAGAGCACAGGAAGCCCCATCCAGCTGA    5070
                                    EXON 7
GGACCCTTTCTATGGATGCCCCCACCTCCAGGCTCTACCCTGTGGGTCTGTTTCTGGAGCGAGTG    5135

GCGAGCTCAGACTTGGTGCTTCAGAACTACCACATCCCAGCTGGGGTGAGTGAGCCCCACACCCC    5200
                                                                EXON 8
TCGAGCTGAGAACCTCCCTCCCCAGTCATTCCCTGATCCCCGCTCTGCACCGTCCGCAGACATTG    5265

GTGCGCGTGTTCCTCTACTCTCTGGGTCGCAACCCCGCCTTGTTCCCGAGGCCTGAGCGCTATAA    5330

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GCATGCGCCAGTGCCTTGGGCGGCGCCTGGCAGAGGCAGAGATGCTGCTGCTGCTGCACCATGTG    5460

AGCAGGCCCGGGCTGGGGAGGGGCCTGGGCGGGGTCTGGGCAGCATGGGCGGGGCTTGAGCAATG    5525

TGGGACTGGCCTCGGCAGAGTGGGAGTGGCCTGCATGTTTCCTGGACTGGGCAGAGCCGGTACTG    5590

GGAGAACCTGGGCCAGGTTGAGGCTGTGTAGGTCCTGGGCAGGAGTTGGTATGGTGAGGAGCGTA    5655

CCATCTGGGTGAGGTTGCTGCTAAACCGGGTCAGGTGGGAACTGGGGAAGTCGGGTGGAGCCTGT    5720

ACAGGATAGTGGGGCTTGGGCAATACCTGGGCTGGATGAATTCTGGGCCTGGGCTGTAAGGTGGG    5785

GCTGGTCAGGAATGAAACAGGTTGGAGGCCAGGCTGCTGTTCCCCCTTCAGCATAATCTCTGCAA    5850

CTTTGAGGGTCTGAGAAGGCTGCACCACGTGCATGGGCTGCGGACCAAGCCAGATGGAAACCCGG    5915
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                EXON 9
CTTCTGTCCTAGGTGCTGAAACACCTCCAGGTGGAGACACTAACCCAAGAGGACATAAAGATGGT    5980

CTACAGCTTCATATTGAGGCCCAGCATGTTCCCCCTCCTCACCTTCAGAGCCATCAACTAATCAC    6045

GTCTCTGCACCCAGGGTCCCAGCCTGGCCACCAGCCTCCCTTTCTGCCTGACCCCAGGCCACCCC    6110

TCTTCTCTCCCACATGCACAGCTTCCTGAGTCACCCCTCTGTCTAACCAGCCCCAGCACAAATGG    6175

AACTCCCGAGGGCCTCTAGGACCAGGGTTTGCCAGGCTAAGCAGCAATGCCAGGGCACAGCTGGG    6240

GAAGATCTTGCTGACCTTGTCCCCAGCCCCACCTGGCCCTTTCTCCAGCAAGCACTGTCCTCTGG    6305

                                                  ⇐H7R
GCAGTTTGCCCCCATCCCTCCCAGTGCTGGCTCCAGGCTCCTCGTGTGGCCATACAAGGGTGCTG    6370

TGGTTTTGTCCCTTGCCTTCCTGCCTAGTCTCACATGTCCCTGTTCCTCTTCCCCTGGCCAGGGC    6435

CCCTGCGCAGACTGTCAGAGTCATTAAGCGGGATCCCAGCATCTCAGAGTCCAGTCAAGTTCCCT    6500

CCTGCAGCCTGACCCCTAGGCAGCTCGAGCATGCCCTGAGCTCTCTGAAAGTTGTCACCCTGGAA    6565

TAGGGTCCTGCAGGGTAGAATAAAAAGGCCCCTGTGGTCACTTGTCCTGACATCCCCATTTTCAA    6630

GTGATACAACTGAGTCTCGAGGGACGTGTGTTCCCCAGCTGATCGTGTCAGCCTCATGCCCCAGG    6695

CCTCATCTTTCATGGACCAGGCCTTGTTCCAGGAGTGGGTGTTGGGTCCTCTGCTTCCTGTGCTG    6760

TCCCCTGGGGAAGGTCCCGAGGATGCTGTCAGGAGATGGAAGAGTCATGTGGGGTGGGAACCTGG    6825

GGTGTGGTTCCAGAAATGTTTTTGGCAACAGGAGAGACAGGATTGGGCCAACAAGGACTCAGACG    6890
```

Sequence of human *CYP11B1* cDNA

Nucleotide sequence translated an amino acid sequence. The sequence begins with the start codon (M-methionine) and ends with the stop codon (-). Numbers refer on the right to the nucleotides and the amino acids.

```
Atggcactcagggcaaaggcagaggtgtgcatggcagtgccctggctgtccctgcaaagg   60
 M  A  L  R  A  K  A  E  V  C  M  A  V  P  W  L  S  L  Q  R    20
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Atgccccggcgtccaggcaacaggtggctgaggctgctgcagatctggagggagcagggt   180
 M  P  R  R  P  G  N  R  W  L  R  L  L  Q  I  W  R  E  Q  G    60
Tatgaggacctgcacctggaagtacaccagaccttccaggaactagggcccattttcagg   240
 Y  E  D  L  H  L  E  V  H  Q  T  F  Q  E  L  G  P  I  F  R    80
Tacgacttgggaggagcaggcatggtgtgtgtgatgctgccggaggacgtggagaagctg   300
 Y  D  L  G  G  A  G  M  V  C  V  M  L  P  E  D  V  E  K  L   100
Caacaggtggacagcctgcatccccacaggatgagcctggagccctgggtggcctacaga   360
 Q  Q  V  D  S  L  H  P  H  R  M  S  L  E  P  W  V  A  Y  R   120
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Caacatcgtgggcacaaatgtggcgtgttcttgctgaatgggcctgaatggcgcttcaac  420
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 V  D  A  V  A  R  D  F  S  Q  A  L  K  K  K  V  L  Q  N  A   180
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 R  G  S  L  T  L  D  V  Q  P  S  I  F  H  Y  T  I  E  A  S   200
Aacttggctctttttggagagcggctgggcctggttggccacagccccagttctgccagc  660
 N  L  A  L  F  G  E  R  L  G  L  V  G  H  S  P  S  S  A  S   220
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 L  N  F  L  H  A  L  E  V  M  F  K  S  T  V  Q  L  M  F  M   240
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Gactgcatcttccagtacggcgacaactgtatccagaaaatctatcaggaactggccttc  840
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 V  P  F  G  F  G  M  R  Q  C  L  G  R  R  L  A  E  A  E  M   460
Ctgctgctgctgcaccatgtgctgaaacacctccaggtggagacactaacccaagaggac  1440
 L  L  L  L  H  H  V  L  K  H  L  Q  V  E  T  L  T  Q  E  D   480
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 I  K  M  V  Y  S  F  I  L  R  P  S  M  F  P  L  L  T  F  R   500
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 A  I  N  -   503
```
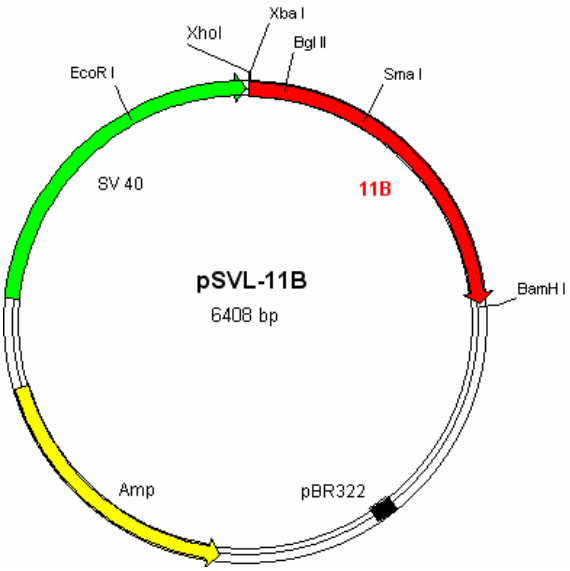
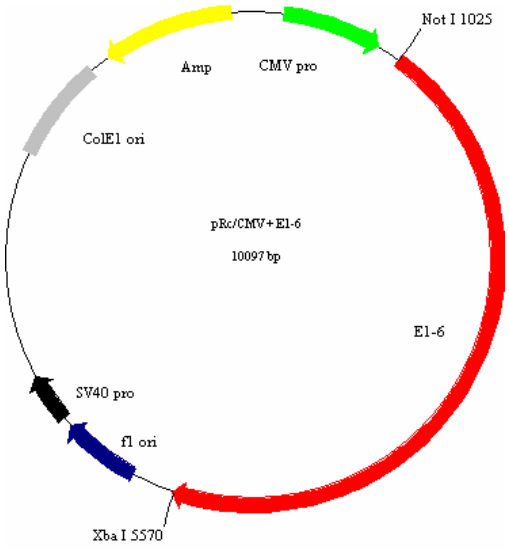

E. Plasmid map of 11B-pSVL and E16-pRc/CMV





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