

# A Mutation in CYP11B1 (Arg-448 → His) Associated with Steroid 11 $\beta$ -Hydroxylase Deficiency in Jews of Moroccan Origin

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## Abstract

Steroid 11 $\beta$ -hydroxylase (P450c11) deficiency (failure to convert 11-deoxycortisol to cortisol) causes less than 10% of cases of congenital adrenal hyperplasia in most populations, but it is relatively frequent in Jews of Moroccan origin. P450c11 is encoded by the CYP11B1 gene which is located on chromosome 8q22 along with a homologous gene of unknown function, CYP11B2. To identify mutations in CYP11B1 associated with 11 $\beta$ -hydroxylase deficiency in Moroccan Jews, oligonucleotides were used that selectively amplified portions of CYP11B1 in polymerase chain reactions without amplifying CYP11B2. Sequence analysis of amplified fragments from one patient revealed a single base substitution in exon 8, codon 448 from CGC (arginine) to CAC (histidine). This residue is within the "heme binding" peptide that contains a cysteine that is a ligand to the heme group. The equivalent of Arg-448 is found in every known eukaryotic P450, and therefore it seems likely that a mutation of this residue would adversely affect enzymatic activity. 11 of 12 affected alleles from six Moroccan Jewish families carried the mutation in codon 448. This mutation is not normally present in CYP11B2 and thus appears to have arisen in CYP11B1 as a true point mutation rather than a gene conversion. (*J. Clin. Invest.* 1991. 87:1664–1667.) Key words: cytochrome P450 • congenital adrenal hyperplasia • hypertension • polymerase chain reaction • autosomal recessive disorder

## Introduction

Cortisol is synthesized from cholesterol in the zona fasciculata of the adrenal cortex in five enzymatic steps: cleavage of the cholesterol side-chain to yield pregnenolone, 3 $\beta$ -dehydrogenation to progesterone, and successive hydroxylations at the 17 $\alpha$ , 21, and 11 $\beta$  positions by three distinct cytochrome P450 enzymes (P450c17, P450c21, and P450c11). Congenital adrenal hyperplasia, an inherited disorder of cortisol biosynthesis, can result from a defect in any of these steps, but in > 90% of cases steroid 21-hydroxylase activity is deficient (1). The molecular genetic basis of 21-hydroxylase deficiency has been extensively studied (reviewed in 2–4).

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Classic 11 $\beta$ -hydroxylase (P450c11) deficiency comprises 5–8% of cases of congenital adrenal hyperplasia, and occurs in about 1/100,000 births in the general Caucasian population (5). A large number of cases have been reported in Israel among Jewish immigrants from Morocco, a relatively inbred population (6, 7). The incidence in this group is currently estimated to be 1/5,000–1/7,000 births, with a gene frequency of 1.2–1.4% (8).

Patients with this disorder are unable to convert 11-deoxycortisol to cortisol. Elevated levels of ACTH cause steroid precursors to accumulate proximal to the blocked step. Many of these precursors are shunted into the pathway for androgen biosynthesis as occurs in 21-hydroxylase deficiency. Thus, female patients with this disorder are born with masculinized external genitalia and affected individuals of both sexes undergo rapid somatic growth with premature epiphyseal closure, resulting in short adult stature.

A parallel defect usually exists in the synthesis of 17-deoxy steroids, so that deoxycorticosterone is not converted to corticosterone. This pathway is required for aldosterone biosynthesis in the zona glomerulosa, but most of the excessive deoxycorticosterone produced in this disorder originates in the much larger zona fasciculata. Because deoxycorticosterone and some of its metabolites have mineralocorticoid activity, elevated levels may cause hypertension and hypokalemia. About two thirds of untreated patients become hypertensive, sometimes early in life (9). This clinical feature distinguishes 11 $\beta$ -hydroxylase deficiency from 21-hydroxylase deficiency, in which poor aldosterone synthesis causes renal salt wasting in the majority of patients.

The 11 $\beta$ -hydroxylase gene, CYP11B1, is located along with the highly homologous CYP11B2 gene on chromosome 8q22 (10, 11). Although there are no obviously deleterious mutations (e.g., nonsense mutations or frameshifts) in CYP11B2, transcripts are not detectable in Northern blots of normal human adrenal RNA. It is not yet known if CYP11B2 is a pseudogene, which would be reminiscent of the close linkage of the 21-hydroxylase gene, CYP21, with the CYP21P pseudogene. Alternatively, the product of CYP11B2 may be required for aldosterone synthesis (corticosterone methyloxidase II activity) as is the case with the second 11 $\beta$ -hydroxylase gene in the rat (12).

Because all 21-hydroxylase deficiency alleles characterized thus far are the result of recombinations (unequal crossovers or gene conversions) between CYP21 and CYP21P, it was of interest to see if similar mechanisms were responsible for 11 $\beta$ -hydroxylase deficiency alleles. An Israeli population was examined because of the relatively high frequency of this disorder therein. We found that 11/12 mutant alleles contained the same mutation, which was a new point mutation rather than a gene conversion.

Table I. Clinical Data from Patients with 11 $\beta$ -Hydroxylase Deficiency

Patient	Sex	Genital virilization*	BP	THS <sup>‡</sup>	Plasma steroids <sup>‡</sup>					PRA <sup>‡</sup>
					S	DOC	$\Delta$ 4	T	Aldo	
			mmHg	mg/24 h	ng/dl					ng/ml per h
A-2	M	Yes	160/125	13.0	—	395	610	90	0.9	0.1
A-5	M	Yes	175/120	5.8	—	443	515	100	1.0	0.8
B-2	M	Yes	130/90	—	1600	—	—	—	—	—
B-3	F	Yes	130/90	—	1800	—	—	90	—	—
C-2	F	Yes	100/70	10.5	—	247	1010	150	1.0	5.8
D-1	M	Yes	150/100	3.9	2300	183	712	230	1.5	0.4
D-2	M	Yes	95/60	2.5	—	1150	794	100	0.7	1.8
E-1	M	No	75/45	1.3	4270	—	545	—	—	—
E-2	F	Yes	130/90	—	1470	—	—	—	5.0 <sup>§</sup>	11.0 <sup>§</sup>
F-2	M	No	145/80	0.7 <sup>  </sup>	2200 <sup>  </sup>	642 <sup>  </sup>	4910 <sup>  </sup>	—	8.8 <sup>  </sup>	8.3 <sup>  </sup>
F-4	F	Yes	85/50	5.0	3340	267	—	110	1.5	0.5

\* Genital virilization in males consisted of a large penis, small testes, and precocious appearance of pubic hair. Genital virilization in females consisted of ambiguous genitalia requiring genitoplasty. <sup>‡</sup> Hormone abbreviations and normal laboratory values for prepubertal children (in parentheses) are: THS, urinary tetrahydrodeoxycortisol (< 0.05 mg/24 h); S, deoxycortisol (20–150 ng/dl); DOC, deoxycorticosterone (3–20 ng/dl);  $\Delta$ 4, androstenedione (50–165); T, testosterone (10–60 ng/dl); aldo, aldosterone (2–20 ng/dl); PRA, plasma renin activity (2–11 ng/ml/h). Except as noted, blood samples were obtained before patients were treated with hydrocortisone. <sup>§</sup> Blood sample obtained during therapy with hydrocortisone. <sup>||</sup> Blood sample obtained at 6 d of age while therapy was interrupted for 1 d.

## Methods

**Patient population.** Six families carrying an allele for 11 $\beta$ -hydroxylase deficiency were studied (Table I); all were Jews who originated from Morocco. None of the families were related, but there was known consanguinity within one family (family C).

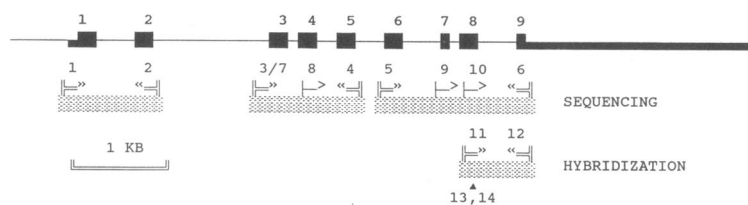
Deficiency of 11 $\beta$ -hydroxylase activity was suspected on clinical grounds and confirmed on the basis of elevated urinary excretion of tetrahydro-11-deoxycortisol (the main urinary metabolite of 11-deoxycortisol, the immediate precursor for the 11 $\beta$ -hydroxylase reaction) and/or plasma 11-deoxycortisol levels. Additional biochemical parameters supporting this diagnosis consisted of elevated concentrations of testosterone, androstenedione, and 11-deoxycorticosterone, and suppressed levels of aldosterone and plasma renin activity (6, 7).

**DNA amplification, cloning, and sequence analysis.** DNA was prepared from peripheral blood leukocytes from patient A-2, digested with HindIII, phenol-chloroform extracted, and precipitated with ethanol. 1  $\mu$ g of each sample was run in the polymerase chain reaction (13) using reagents supplied by Perkin-Elmer Cetus, Emeryville, CA, and each of three sets of primers (200 ng each) in parallel reactions (Table II, Fig. 1). These specifically amplified exons 1–2, 3–5, and 6–9 of CYP11B1 without amplifying CYP11B2. Specificity of each reaction was tested using cloned CYP11B1 and CYP11B2 genes as control templates. Expected fragment sizes for these three segments were 1.3, 1.7, and 1.8 kb, respectively. Reactions were carried out in a Perkin-Elmer Cetus thermocycler using 35 cycles of 94°C denaturing for 1 min, 65°C annealing for 1 min, and 72°C extension for 3.5 min, followed by a single 10-min incubation at 72°C.

Table II. Oligonucleotides Used in This Study

Oligonucleotide	Location	Sense	Purpose*
(TTTGAATTC)TCGAAGGCAAGGCACCAG	5' flanking	+	A, exons 1–2
(GGGGGATCC)TGCTCCAGCTCTCAGCT	Intron 2	–	A, exons 1–2
(CCCGAATTC)AGAAAAATCCCTCCCCCTA	Intron 2	+	A, exons 3–5
(CCCGGATCC)GACACGTGGGCGCCGTGTGA	Intron 5	–	A, exons 3–5
(CCCGGATCC)TGACCTGCAGCTGTGTCT	Intron 5	+	A, exons 6–9
GAGACGTGATTAGTTGATGGC	Exon 9, 3'UT <sup>‡</sup>	–	A, exons 6–9
ACAAGGAGGATGGGATA	Intron 2	+	S, exon 3
GGACTGAAGGGAGTGTG	Intron 3	+	S, exon 4
GGTGACAGAGACACAGG	Intron 6	+	S, exon 7
TCGAGCTGAGAACCCTC	Intron 7	+	S, exon 8
TACTCTCTGGGTCGCAACCCC	Exon 8	+	A, exons 8–9
GGGGGCACATGCTGGGCCTCA	Exon 9, 3'UT <sup>‡</sup>	–	A, exons 8–9
TTGGCATGCACCAGTGCCCT	Exon 8	+	H, mutant
AGGCACTGGCGCATGCCAA	Exon 8	–	H, normal

Positions at which the sequences of CYP11B1 and B2 differ are underlined. Restriction sites and arbitrary bases added for cloning are in parentheses. \* A, amplification; S, sequencing; H, hybridization. <sup>‡</sup> UT, untranslated.



**Figure 1.** Amplification and sequencing of CYP11B1. The exons are numbered in bold type. Positions of primers for polymerase chain reactions are indicated by heavy arrows. The amplified fragments indicated by bars immediately below the diagram of the gene were subcloned and sequenced with universal primers at the ends of each insert and with specific primers indicated by the light arrows. The segment containing exons 8 and 9 that was amplified for allele-specific oligonucleotide hybridization is also shown; the position of

the Arg-448 → His mutation is marked by an arrowhead. Numbers above each arrow and below the arrowhead refer to the sequence of each primer listed in Table II.

Amplified segments were cloned in pBluescriptKS+ (Stratagene Inc., La Jolla, CA) and supercoiled DNA was sequenced by the chain termination method (14) using modified T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH) (15) and primers corresponding to polylinker sequences and specific internal primers (Fig. 1). At least four clones of each amplification reaction were analyzed.

**Allele-specific oligonucleotide hybridization** (16). A 0.6-kb fragment containing exons 8 and 9 was amplified from each HindIII-digested sample using primers (Fig. 1) that were demonstrated to be specific for CYP11B1 under the reaction conditions used (the same as above, but with a 3-min extension time). Success of each reaction was confirmed by agarose gel electrophoresis. 300 ng of each amplified sample were dotted on to a nylon membrane (Micron Separation Industries) using a filtration manifold. Membranes were irradiated with 1,200 J of ultraviolet light and hybridized with oligonucleotide probes specific for the Arg-448 → His mutation (see below) and the corresponding normal sequence that were end-labelled with [<sup>32</sup>P]. Membranes were washed in 3 M tetramethylammonium chloride for 10 min at 65° and exposed to Kodak XAR film.

## Results and Discussion

**Arg-448 → His associated with 11β-hydroxylase deficiency.** A single mutation in exon 8 of CYP11B1 was identified by sequence analysis of clones from patient A-2. Codon 448, CGC, encoding arginine, was changed to CAC, histidine. This mutation was identified on all four clones of the exons 6–9 region.

Hybridization to dot blots (Fig. 2) demonstrated that 11/12 mutant alleles carried the same mutation.

The sulfhydryl of Cys-450 in P450c11 is presumed to constitute the fifth ligand to the iron atom of the heme prosthetic group. This residue is completely conserved in all cytochrome P450 enzymes and the surrounding heme binding peptide is

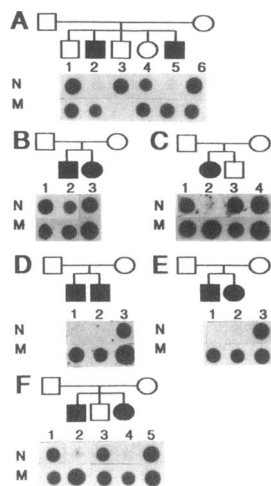
also highly conserved (Fig. 3). In particular, Arg-448 is conserved in all eukaryotic P450 enzymes examined thus far (see references in [17]) suggesting that substitutions at this position are poorly tolerated. Indeed, mutation of the analogous arginine residue in another P450 enzyme (P450IA2 from rat liver) results in an unstable enzyme (18). Thus, it is reasonable to speculate that the Arg-448 → His mutation interferes with binding or functioning of the heme functional group. Testing of this hypothesis will require expression of normal and mutant P450c11 in cultured cells, an endeavor that awaits isolation of the full-length cDNA coding sequence.

**Molecular mechanism generating the mutation.** The Arg-448 → His mutation is a change from CGC to CAC, which is a CpG → TpG mutation on the noncoding strand. This is presumably due to deamination of <sup>Me</sup>CpG (19), which is the most frequent type of point mutation in humans (20). It is notable that this mutation is not normally present in CYP11B2, and so it cannot have been transferred to CYP11B1 in a gene conversion event. This is in contrast to congenital adrenal hyperplasia due to 21-hydroxylase deficiency, for which all mutations described thus far are deletions or gene conversions. Of course, unless it is determined that the product of CYP11B2 is inactive, it is possible that gene conversions that transfer sequences from CYP11B2 to CYP11B1 do not produce 11β-hydroxylase deficiency alleles.

Deletions of CYP11B1 have not yet been detected in patients with 11β-hydroxylase deficiency ([21], and unpublished observations). Taken together, these findings suggest that intergenic recombination is not as important a mechanism for generating 11β-hydroxylase deficiency alleles as it is for 21-hydroxylase deficiency, a hypothesis consistent with the 10-fold lower incidence of 11β-hydroxylase deficiency seen in most populations.

**Deficiency of 11β-hydroxylase in the Israeli population.** Because all of the families studied here originate from the Moroccan Jewish community, it is likely that the results presented here reflect a founder effect. There was relatively little intermarriage in many relatively small Sephardic (non-European) Jewish communities before emigration to Israel, and so genetic heterogeneity at certain loci may be limited. In a similar example, Israeli patients of Iranian Jewish origin with a defect of aldosterone biosynthesis due to corticosterone methyl oxidase II deficiency all had a unique restriction fragment length polymorphism in CYP11B1; however, the causative mutation was not identified (20).

Although patients in 5/6 families in this study were presumably genotypically identical, there were significant differences in signs and symptoms of androgen and mineralocorticoid excess, even within families. For example, all affected females were born virilized, but only 5/7 males had an abnormally



**Figure 2.** Dot-blot hybridizations using oligonucleotide probes specific for the normal (N) and Arg-448 → His mutant (M) alleles. Families are designated as in Table I. Only individuals for whom samples were available are numbered. Individuals affected with 11β-hydroxylase deficiency are indicated by filled squares (males) and circles (females). Heterozygous carriers and genetically unaffected individuals are not distinguished.

	448										450									
<b>XIB</b>	Phe	Gly	Phe	Gly	Met	Arg	Gln	CYS	Leu	Gly	Arg	Arg	Leu	Ala	Glu	Val	Glu			
<b>XIB*</b>	Phe	Gly	Phe	Gly	Met	Arg	Gln	CYS	Leu	Gly	Arg	Arg	Leu	Ala	Glu	Val	Glu			
<b>XIA</b>	Phe	Gly	Trp	Gly	Val	Arg	Gln	CYS	Val	Gly	Arg	Arg	Ile	Ala	Glu	Leu	Glu			
<b>XXI</b>	Phe	Gly	Cys	Gly	Ala	Arg	Val	CYS	Leu	Gly	Glu	Pro	Leu	Ala	Arg	Leu	Glu			
<b>XVII</b>	Phe	Gly	Ala	Gly	Pro	Arg	Ser	CYS	Val	Gly	Glu	Met	Leu	Ala	Arg	Gln	Glu			
<b>II</b>	Phe	Ser	Thr	Gly	Lys	Arg	Ile	CYS	Leu	Gly	Glu	Gly	Ile	Ala	Arg	Asn	Glu			
<b>I</b>	Phe	Gly	Met	Gly	Lys	Arg	Lys	CYS	Ile	Gly	Glu	Thr	Val	Ala	Arg	Trp	Glu			
<b>III</b>	Phe	Gly	Asn	Gly	Pro	Arg	Asn	CYS	Ile	Gly	Met	Arg	Phe	Ala	Leu	Met	Asn			

**Figure 3.** Comparisons of heme binding regions from various families of cytochromes P450 (listed by roman numerals [17]). Highly conserved residues are boxed, and the cysteine that is the fifth ligand to the heme iron is boxed with a double line. *XIB*, steroid 11 $\beta$ -hydroxylase; *XIB\**, mutant 11 $\beta$ -hydroxylase; *XIA*, cholesterol desmolase; *XXI*, steroid 21-hydroxylase; *XVII*, steroid 17 $\alpha$ -hydroxylase; *II*, P450 induced by phenobarbital; *I*, P450 induced by dioxin; *III*, P450 induced by pregnenolone 16 $\alpha$ -carbonitrile.

large penis in infancy. Only 8/11 patients were hypertensive when untreated and, as has been previously noted (6), development of hypertension was poorly correlated with blood levels of 11-deoxycorticosterone. Thus, as is the case with 21-hydroxylase deficiency, other epigenetic or nongenetic factors probably influence the clinical phenotype of the disorder.

Because a large proportion of 11 $\beta$ -hydroxylase deficiency alleles in Israel carry the Arg-448  $\rightarrow$  His mutation, prenatal diagnosis of 11 $\beta$ -hydroxylase deficiency in this population should be informative in most cases if this single mutant allele is screened for. This would provide a useful supplement to the current hormonal methods of prenatal diagnosis (9). In using this approach for prenatal diagnosis, it will be important to avoid unintentional amplification of CYP11B2, which could lead to a spurious signal with the probe for normal allele, making it difficult to distinguish affected and heterozygous individuals.

It is also possible to do carrier screening among Jews of Moroccan ancestry, who comprise  $\sim$  13.5% of the current population of Israel (22). This strategy is probably most cost-effective when at least one parent comes from a family known to carry the disease.

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