

## Steroid 21-hydroxylase deficiency: Three additional mutated alleles and establishment of phenotype–genotype relationships of common mutations

(congenital adrenal hyperplasia/DNA sequencing/PCR/genetic diagnosis/mutation screening)

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**ABSTRACT** Lesions in the gene encoding steroid 21-hydroxylase [steroid hydrogen-donor: oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10] result in defective adrenal steroid synthesis; the severe forms are known as congenital adrenal hyperplasia. To facilitate complete characterization of mutations in this region of tandemly repeated genes, we have developed selective PCR amplification and direct sequencing of full-length nonpseudogene steroid 21-hydroxylase genes. This technique identifies known mutations, characterizes or excludes unknown mutations, and determines the gene-copy number. Three additional defective alleles were found. A Gly-292 → Ser mutation and a frameshift mutation at Arg-484 (GG → C) were identified in patients with severe steroid 21-hydroxylase deficiency. An allele with three additional sequence variations—C → T at 4 bases upstream of translation initiation, Pro-106 → Leu, and Pro-454 → Ser—were identified in two siblings with late-onset deficiency. Pro-454 is conserved in four species, indicating its importance for normal enzyme function. Functional consequences of individual alleles have been determined *in vivo* by studying individuals with only one steroid 21-hydroxylase gene. Detailed analyses of clinical data revealed that genotyping could predict the clinical course of the disease. The locations of disease-causing mutations on different haplotypes of the steroid 21-hydroxylase gene region are described.

Steroid 21-hydroxylase [steroid hydrogen-donor: oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10; P450c21] is an essential enzyme in adrenal steroid synthesis. Inherited defects of this enzyme lead to various degrees of impaired cortisol and aldosterone synthesis accompanied with androgen excess (1, 2). Steroid 21-hydroxylase deficiency is inherited as an autosomal recessive trait. The disorder exists in a wide range of manifestations that traditionally have been divided into three groups: (i) simple virilizing, presenting with virilization of external genitalia at birth in girls and early pseudoprecocious puberty in boys; (ii) salt-wasting, which in addition shows severe aldosterone deficiency neonatally; and (iii) late-onset, which is associated with pseudoprecocious puberty, hirsutism, polycystic ovary syndrome, and decreased fertility (3–5). However, the three groups are not distinct. The severe forms of the disease occur in 1/12,000 Swedish newborns. Based on hormonal analyses, mild steroid 21-hydroxylase deficiency is estimated to occur in 1/500–1/1000 North Europeans (6).

There are two human steroid 21-hydroxylase genes (21OH)—one active gene, *CYP21*, and a highly homologous inactive pseudogene, *CYP21P*. These genes are located 3' of each of the two genes encoding the fourth component of complement, C4A and C4B, in the HLA class III gene region

on chromosome 6p21.3 (7, 8), forming tandem repeat units. Considerable variation in the number and gross structure of C4/21OH repeat units has been detected (9, 10). The *CYP21* gene consists of 10 exons spanning 3 kilobases (kb) of DNA (11–13). Studies of upstream sequences in mice have identified a number of potential regulatory elements within 300 base pairs (bp) 5' of the gene that influences gene expression *in vitro* (14). Considerable sequence variation has been described between human *CYP21* genes, some altering the encoded steroid 21-hydroxylase protein sequence. The protein variants include both apparently neutral amino acid substitutions (11–13, 15, 16), as well as substitutions causing impairment of steroid 21-hydroxylase activity (15, 17–22). All described sequence variants that impair enzyme function are also present in the pseudogene and are thought to be transferred by recombination from *CYP21P* to *CYP21*. The pseudogene-derived mutations represent a large part but do not represent all of the steroid 21-hydroxylase deficiency mutations in different populations (16, 21, 23).

Attempts to fully characterize the genetic lesions in the various manifestations of steroid 21-hydroxylase deficiency have been hampered by the complicated structure of the *CYP21* locus. We have developed rapid direct sequencing of selectively PCR-amplified full-length *CYP21* genes to obtain information of all nonpseudogenes present in individuals with various disease manifestations. This technique enabled identification of three additional mutated alleles and establishment of genotype–phenotype correlations for the common mutations. The functional contribution of individual *CYP21* alleles has been determined *in vivo* by studying subjects carrying only one copy of the gene (hemizygous).

### MATERIALS AND METHODS

**Patients.** The study comprises 2 healthy controls and 34 patients affected with steroid 21-hydroxylase deficiency of different severity. This group represents 61 unrelated alleles. The main symptoms and the time of their initial observation are given in Table 1. Urine from four patients—B32, B33, B103, and B108—carrying three additional mutated alleles, were collected over a 24-hr period, and the steroid pattern was analyzed by using gas/liquid chromatography, displaying typical increases of pregnanetriol. There were no metabolites suggesting any other defect in adrenal steroidogenesis. In addition, patients B32 and B33 showed pathological responses of serum cortisol and 17-hydroxyprogesterone concentrations during an adrenocorticotrophic hormone (ACTH) test (10). Two individuals hemizygous for the *CYP21* gene,

Abbreviations: *CYP21*, alleles of the active steroid 21-hydroxylase gene; *CYP21P*, alleles of the steroid 21-hydroxylase pseudogene; 21OH, any of the steroid 21-hydroxylase genes; C4, fourth component of serum complement; ACTH, adrenocorticotrophic hormone. ‡To whom reprint requests should be addressed.

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B21 and B31, exhibited normal hormone levels during the ACTH test. The C4/21OH haplotypes of the patients have previously been characterized and are given in Table 1 (10, 24). Haplotypes 1 and 2 contain one *CYP21P* and one *CYP21* gene, haplotype 5 contains only one *CYP21*, and haplotypes 6, 7, and 8 contain two *CYP21P* and one *CYP21* (Fig. 1). Linkage phases of haplotypes and mutations were established through segregation in families. The study was approved by the Ethics Committee at the Karolinska Hospital.

**PCR.** Genomic DNA was prepared from peripheral leukocytes. A specific restriction site for *Taq* I is present at base -210 in the *CYP21P* gene (Fig. 1), base numbering being identical to that reported by Rodrigues *et al.* (13). In cases where the *CYP21* alleles were completely sequenced from nucleotides -330 to 2735, DNA was cleaved with *Taq* I (New England Biolabs), and a first round of PCR was done by using primers P1 5'-TTCAGGCGATTTCAGGAAGGC (-418 to -399) and P4 5'-TCTGCACCCAGTATGACT (2892-2911) at concentrations of 0.5  $\mu$ M (Fig. 1). After an initial denaturation at 96°C for 3 min, 30 cycles at 96°C for 1 min, 56°C for 30 sec, and 72°C for 15 min were done in a buffer containing 1.0 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.4 at 70°C), and 0.1% Tween 20. Shorter extension time failed to reproducibly generate the desired fragment. One microliter of the 3.3-kb PCR product was used for subsequent amplifications to generate four overlapping fragments by incubating 30 cycles at 96°C for 1 min, 58°C for 30 sec, and 72°C for 3 min in 100  $\mu$ l, using one normal and one biotinylated primer at 0.1  $\mu$ M. *Taq* polymerase (Perkin-Elmer/Cetus) was used at 2 units per 100  $\mu$ l. For partially sequenced alleles, the first round of PCR was performed by using primers P1 together with P48 (5'-CAGAGCAGGGAGTAGTCTC) and P55 (5'-CCTGTCCTGGGAGACTACT) together with P4 (Fig. 1). Primers P48 and P55 are specific for the sequence in nonpseudogene DNA; P49 covers the corresponding part in the pseudogene containing an 8-bp deletion. To verify the specificity in distinguishing between *CYP21* and *CYP21P*, all DNAs were amplified with primers P48 and P49 together with P1, and the produced fragments were submitted to *Taq* I cleavage. In all cases only genes carrying the deletion were cleaved. Allele-specific PCR was performed from one patient (B108), where a previously unreported mutation was found in heterozygous form. Her *CYP21* alleles were followed by segregation from the parents, and a heterozygous position (base 1126) was used to design

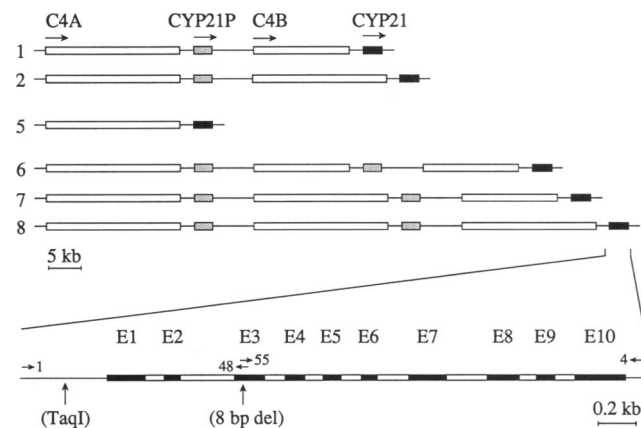


FIG. 1. Schematic illustration of the C4/21OH gene region showing haplotypes labeled 1, 2, 5, 6, 7, and 8, with differences in number and structure of C4/21OH repeat units (10). Thin arrows at top indicate direction of transcription. The enlargement shows the exon/intron organization of the *CYP21* gene. The *Taq* I site and the 8-bp deletion (del) within parentheses are present in corresponding parts of *CYP21P*. Horizontal arrows in the enlargement denote positions of PCR primers.

a primer with the most-3' nucleotide base-pairing only with the allele carrying the additional mutation.

**Direct Sequencing.** The biotinylated PCR fragments were immobilized by binding to streptavidin-covered magnetic beads (Dynabeads; Dynal, Oslo), and the nonbiotinylated strands were removed by incubating in 50  $\mu$ l of 0.15 M NaOH for 5 min at room temperature (25). The remaining DNA was rinsed three times, suspended in 13  $\mu$ l of distilled water, and sequenced by the dideoxynucleotide chain-termination method with the Auto-Read sequencing kit (Pharmacia). Sequencing was done with fluorescently labeled primers by using the Pharmacia automated laser fluorescent DNA sequencer. Thirteen out of 43 alleles were completely sequenced from nucleotides -330 to 2735. The remaining alleles were partly sequenced. Additional mutations were confirmed by sequence determination of both strands. All primer sequences for PCR amplification and DNA sequence determination are available upon request.

## RESULTS

**Hemizygous Individuals.** The *CYP21* alleles from two hemizygous individuals with normal steroid 21-hydroxylase function were completely sequenced from base -330 to base 2735. Patient B21 differed from patient B31 in four silent positions in exons and seven positions in introns (Table 1). In addition, this normal allele varied at base 1650, changing Ser-269 to threonine.

Twenty hemizygous patients with steroid 21-hydroxylase deficiency were analyzed (Table 1). Patient B103, affected with salt-wasting disease, carried an allele with a previously unreported G  $\rightarrow$  A transition at nucleotide 1718, changing Gly-292  $\rightarrow$  Ser in exon 7 (Fig. 2A). The only additional sequence variation between bases -330 and 2735, as compared with the two normal alleles, was a T  $\rightarrow$  C transition at position 398, 9 bases into the 5' end of intron 2. Two siblings affected with late-onset disease (B32, B33) carried three additional base substitutions in their only *CYP21* allele. These include a C  $\rightarrow$  T transition at 4 bases upstream of the initiation codon; a C  $\rightarrow$  T transition at nucleotide 696, changing Pro-106 to leucine in exon 3; and a C  $\rightarrow$  T transition at position 2584, changing Pro-454  $\rightarrow$  Ser in exon 10 (Fig. 2B). No other deviations from the normal alleles were detected.

Of the remaining hemizygous patients, seven (B14, B20, B60, B74, B100, B110, and B115) carried the splice mutation at base 659 located 13 bp upstream of exon 3 (16, 17), one *CYP21* allele (B68) carried the Arg-357  $\rightarrow$  Trp mutation at base 2113 (15), and one *CYP21* allele (B80) carried the thymine insertion at base 1768, creating a truncated protein. The Val-282  $\rightarrow$  Leu mutation at base 1688 (19) was identified in three patients (B57, B85, and B92). Five *CYP21* alleles from hemizygous patients carried the Ile-173  $\rightarrow$  Asn mutation (18) at base 1004 (B28, B37, B41, B73, and B101). Three Asn-173 alleles were completely sequenced from patients with different disease manifestations (B37, B73, and B101). Table 1 shows that only base 2252 differed among the alleles, resulting in a silent codon change at Ser-375.

**Heterozygous Individuals.** Fourteen steroid 21-hydroxylase deficiency patients carrying two *CYP21* genes on known C4/21OH haplotypes were sequenced. One heterozygous patient with severe steroid 21-hydroxylase deficiency (B108) carried the Arg-357  $\rightarrow$  Trp mutation on one chromosome, and a totally different mutation on the other. Bases 2675-2676, coding for GG in Arg-484 in exon 10, were replaced by cytosine, causing a frameshift and resulting in a predicted 45-amino acid-longer protein with 57 additional amino acids in the C-terminal end. This allele also carried the Asp-184  $\rightarrow$  Glu mutation at base 1126, which has been shown to encode an enzyme with normal function after transfection *in vitro* (16). The only other sequence variation was a T  $\rightarrow$  C

Table 1. Alleles of the steroid 21-hydroxylase gene with nucleotides at the variable positions displayed

No.	Sex	Degree* and age of symptoms	Haplo- type †	Nucleotide numbers, locations in the gene, and position for amino acid substitutions ‡																																					
				-4	28-30	118	138	398	422	456	562	563	605	659	687	696	697	1004	1109	1116	1126	1423	1426	1562	1591	1630	1688	1718	1768	2113	2252	2384	2675-6	2697	2705						
				5'	E1	E1	E1	I2	I2	I2	I2	I2	I2	I2	E3	E3	E3	E4	I4	I4	E5	I6	I6	I6	E7	E7	E7	E7	E8	E9	E10	E10	E10	E10							
				L10												R103	P106	I173				D184												S269	V282	G292	L308	R357	P454	R484	S494
<b>Hemizygous individuals</b>																																									
B31	F	Normal	2	C	CTG	T	A	T	A	C	G	G	C	C	G	C	C	T	A	C	C	A	C	T	G	G	G	G	G	C	C	C	GG	G	G						
B21	M	Normal	2	-	-	C	C	-	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
B14	F	SW/3mo	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
B20	F	V,US/b‡	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
B28	F	C,US/b	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
B32	M	PP/10yr	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B33	F	GA,C/8yr	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B37	F	V,US/b‡	8 or 2	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
B41	F	V/b	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B57	F	IF,H/19yr	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B60	M	SW/4wk	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B68	M	SW/2wk	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B73	M	P,GA/1yr	2	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
B74	M	SW/2wk	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B80	F	SW/b	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B85	M	SW/2wk‡	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B92	F	PP/7yr	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B100	M	SW/b	1 or 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B101	F	V/b,7yr‡	8 or 2	-	-	Δ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B103	M	SW/3wk	2	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B110	F	SW/b	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B115	F	V/b‡	8 or 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
<b>Heterozygous individuals</b>																																									
B4	M	-/2wk‡	1,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B44	F	SW/3wk	1,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B46	F	IF,H/23yr	2,6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B47	M	GA/7yr	6,7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B48	F	GA/4yr	2,7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B51	F	V/b,4yr‡	2,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B64	M	SW/3wk	1,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B65	F	V/b	1,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B82	F	SW/b	2,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B84	F	V/b,3mo‡	2,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B106	F	SW/3wk	1,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B112	M	ACTH	2,6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B121	F	SW/b	2,2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
B126	M	ACTH	5,6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						

Sequences are compared with the gene sequence of patient B31. Positions in the DNA sequence between -330 and 2735 that vary in the analyzed alleles are shown. A dash indicates that the base is identical with that of B31; a blank space indicates that the sequence was not analyzed in this region; Δ denotes deleted bases. The boldface boxes indicate the additional variants listed in Table 2. Thin boxes show the previously described mutations leading to steroid 21-hydroxylase deficiency.

\*Normal, normal steroid 21-hydroxylase activity in hemizygous ACTH-tested individuals (10); SW, salt-wasting is defined as sodium concentration <130 mmol/liter before treatment; in females SW is always accompanied with severe virilization of external genitalia at birth; V, virilization of female external genitalia, including clitoral enlargement and pseudoscrotal formation; US, common opening for urethra and vagina; b, at birth; C, clitoral enlargement; PP, pseudoprecocious puberty; GA, growth acceleration; IF, infertility; H, hirsutism; P, penile enlargement; ACTH, pathological ACTH test.

†C4/21OH haplotypes are shown in Fig. 1 (10). For heterozygous patients, mutations are assigned to corresponding haplotypes by their placement relative to the comma.

‡Nucleotide 1 is the adenosine in the start codon, 5' denotes the 5' untranslated region, E and I denote exons and introns, and the amino acids in one-letter code are given for B31 at the polymorphic positions.

§Patient B4 was identified by hormonal analysis before symptom onset; patients B20, B37, and B115 received hormonal substitution before developing clear salt-wasting; patients B51, B84, and B101 were virilized from birth but were not diagnosed until the indicated age; patient B85 showed salt-wasting during a gastrointestinal infection, but treatment was terminated at 2 yr (10).

transition at position 398, 9 bases into the 5' end of intron 2, the same as in allele B103 (Table 1). The GG → C frameshift mutation at Arg-484 is shown in heterozygous form (Fig. 2C), as well as after allele-specific PCR and sequencing (Fig. 2D).

Five patients (B44, B64, B65, B82, and B121) were homozygous for the splice mutation at base 659. Three patients (B4, B51, and B84) were heterozygous for the splice mutation and the Ile-173 → Asn mutation. Four patients (B46, B48, B112, and B126) were heterozygous for the splice mutation at base 659 and the Val-282 → Leu mutation. One patient (B47) was homozygous for the Val-282 → Leu mutation. An example of the DNA sequence covering the splice mutation at base 659 in heterozygous form is shown in Fig. 2E. This sequence illustrates that the number of CYP21 genes can be determined by selective PCR and direct sequencing.

**Allelic Sequence Variations.** The three additional mutant steroid 21-hydroxylase alleles are summarized in Table 2. As listed in Table 1, 32 nucleotide positions varied among the sequenced 3065 bases. Of the five additional nucleotide variations in our three additional mutated alleles, the four causing amino acid changes have not been seen in any

pseudogene. The -4 base variation has been found in two of the three reported CYP21P alleles and is also present in B68 together with the Arg-357 → Trp mutation at base 2113.

Previously studied mutations constitute another five of the 32 base variations, positions 659, 1004, 1688, 1768, and 2113 (15-19, 26, 27). All these variants have been seen in the sequence of at least two of the three reported CYP21 alleles. Other positions that varied among the CYP21 alleles include 11 intron substitutions, 6 silent codon substitutions, and 5 sequence variations leading to amino acid changes (deletion of Leu-10, Arg-103 → Lys, Asp-184 → Glu, Ser-269 → Thr, and Ser-494 → Asn). These amino acid changes have been seen in the four normal alleles reported by others and/or have resulted in normal enzyme activity after transfection (11-13, 15, 16, 28). Of the 11 polymorphic intron nucleotides, 6 vary between our two normal alleles B21 and B31. This leaves 5 variations in intron 2 (bases 398, 422, 456, 563, and 605). As shown in Table 1, the polymorphisms at bases 422 and 456 are in linkage disequilibrium with the Ile-173 → Asn mutation, and the 563 polymorphism is in linkage disequilibrium with the Val-282 → Leu mutation. These three intron polymor-

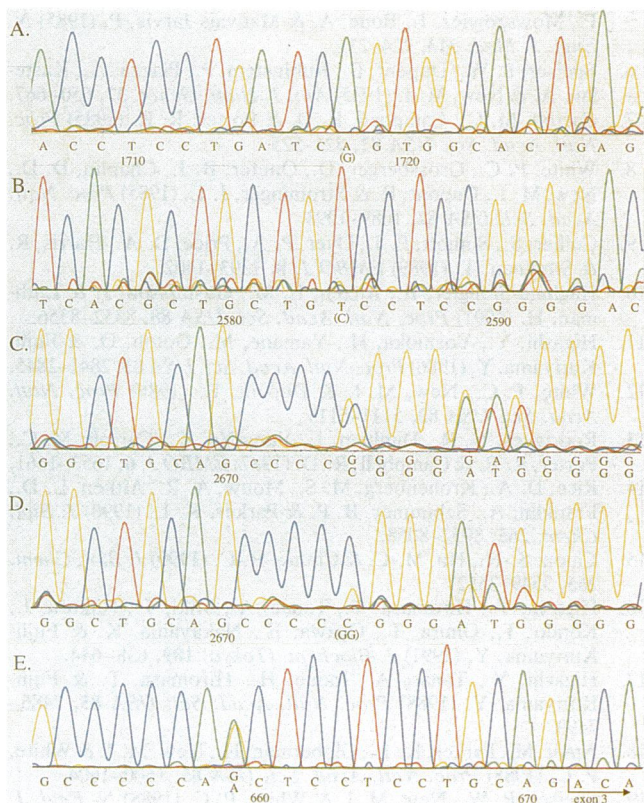


FIG. 2. Sequences of parts of the *CYP21* genes. (A) Sequence of Gly-292 → Ser mutation at base 1718 (patient B103). (B) Sequence of Pro-454 → Ser mutation at base 2584 (patient B33). (C) Sequence of GG → C frameshift mutation at bases 2675–2676 in heterozygous form (patient B108). (D) Same mutation after allele-specific amplification and sequencing. (E) Sequence in the intron 2/exon 3 region from an individual heterozygous for the splice mutation at base 659. Wild-type sequences are shown within parentheses below mutations. At heterozygous positions the sequence of both alleles are displayed.

phisms are also present in alleles reported normal by others (11, 12). The remaining 2 variations in intron 2, bases 398 and 605, have not been excluded as affecting gene expression. The variation at base 398 is present on our two additional severely affected alleles (B103, B108) and has also been seen together with the splice mutation (17) and the Gln-319 → stop mutation (20). The variation at position 605 is present in an allele also carrying the 659 splice mutation (B20).

**DISCUSSION**

We have developed a strategy for rapidly generating complete sequence information of all nonpseudogene steroid 21-hydroxylase genes present in individuals. In this way, we have identified three additional mutated alleles from steroid 21-hydroxylase deficiency patients. The single steroid 21-hydroxylase allele present in patient B103, affected with salt-wasting 21-hydroxylase deficiency, carried a single-amino acid substitution, Gly-292 → Ser. Gly-292 is conserved

Table 2. Three additional mutated *CYP21* alleles associated with steroid 21-hydroxylase deficiency

Allele	Base no.	Structural change	Patient
1	-4	C → T	B32, B33
	696	Pro-106 → Leu	
	2584	Pro-454 → Ser	
2	1718	Gly-292 → Ser	B103
3	2675–2676	Arg-484 → frameshift	B108

between human (11, 12), bovine (29, 30), murine (31), and porcine (32) steroid 21-hydroxylases, indicating its importance for normal enzyme function. The only additional base variation in this allele, which has not been demonstrated as neutral with regard to steroid 21-hydroxylase activity, is a T → C transition at base 398, 9 bases into the 5' end of intron 2. This nucleotide is unlikely to be important for the function of the allele, considering its location outside the consensus sequence in the 5' end of introns (33).

Three positions in the *CYP21* allele of the hemizygous siblings, B32 and B33, deviated from the normal sequence, nucleotide -4, Pro-106 → Leu, and Pro-454 → Ser. Pro-454 is conserved among human, bovine, murine, and porcine steroid 21-hydroxylases, which makes this amino acid substitution the most likely candidate for causing the mild, late-onset deficiency of these patients. Pro-106 does not show this species conservation. As long as a purine is located at position -3, deviations from the rest of the consensus sequence surrounding the AUG codon only slightly impair the initiation of translation (34), making the base variation at -4 an unlikely candidate.

A frameshift mutation, GG → C at Arg-484, was found in patient B108. This patient is a compound heterozygote for this mutation and the Arg-357 → Trp mutation and showed symptoms of salt-wasting at 3 weeks of age. The allele with the frameshift mutation at position 2675 also carried the variation at base 398, 9 bases into the 5' end of intron 2, which has not been excluded as affecting enzyme function. However, the predicted protein resulting from the frameshift mutation is 45 amino acids longer than the normal enzyme, making severe disruption of activity conceivable.

Eighteen of the 59 unrelated steroid 21-hydroxylase deficiency alleles carried a deletion of the entire *CYP21* gene. Of the abnormal nondeletion *CYP21* alleles, three base substitutions—the splice mutation at base 659, Ile-173 → Asn, and Val-282 → Leu—were identified in 35 out of 41 alleles (85%). One additional mutation, Arg-357 → Trp, occurred in more than one allele. These findings show no major deviation from results obtained in other populations (16, 21, 23).

Sequencing the complete *CYP21* allele of individuals hemizygous for this gene enabled evaluation of the contribution of individual polymorphisms with regard to steroid 21-hydroxylase function *in vivo*. The Ser-269 → Thr substitution at 1650 has been shown to result in normal enzyme activity after transfection *in vitro* (16) but has only been found *in vivo* in heterozygous form (35). Its presence in individual B21 confirmed that this amino acid substitution is neutral with regard to 21-hydroxylase deficiency. Six intron variations (positions 562, 1109, 1116, 1425, 1426, and 1562) were also assigned as neutral in this way. Similarly, correlations between genotype and phenotype could be made. Deletion of *CYP21* obviously leads to abolished enzyme function, which correlated with salt-wasting. The splice mutation at base 659 was associated with neonatal salt-wasting, except in one case. In two siblings homozygous for this mutation (B64 and B65), the elder was raised as a boy until 3 yr of age when he was diagnosed as a severely virilized girl, whereas the younger brother showed severe neonatal salt-wasting. In none of the other 11 patients who were hemi- or homozygous for this mutation could neonatal salt-wasting be excluded. This mutation has been shown to have low, but measurable, *in vitro* activity after transfection (16). The position 357 Arg → Trp mutation, the insertion at base 1768, and the two additional mutations, Gly-292 → Ser and the frameshift at Arg-484, were found in patients with salt-wasting at birth. All patients carrying the Val-282 → Leu mutation, either in hemi- or homozygous form or as compound heterozygotes with the splice mutation, showed mild symptoms with late-onset. However, the influence of environmental stress in modulating the phenotype is illustrated by the patient B85, carrying



the Val-282 → Leu mutation. He showed neonatal hyponatremia in conjunction with a gastrointestinal infection but later proved to exhibit a very mild enzyme deficiency (10). The Ile-173 → Asn mutation has been described as displaying the largest degree of clinical variation, the clinical spectrum ranging from late-onset to salt-wasting (16, 18, 21). This variation was also the result of routine classification of this patient material. When the medical records were scrutinized and the patients were thoroughly interviewed, however, it is clear that all female patients had symptoms of virilization before birth, as manifested by labial fusion or a common urogenital sinus, even when the diagnosis in some cases was delayed. The only case without symptoms or signs of steroid 21-hydroxylase deficiency at birth is a male, B73, with both clinical and hormonal signs of disease at 1 yr of age. As shown here, the apparent clinical heterogeneity is not due to co-existence of other mutations in the gene from nucleotide -330 to 2735. In conclusion, we find that the Ile-173 → Asn mutation correlates well to a phenotype with early virilization without salt loss. The additional allele identified in the mildly affected patients B32 and B33 encoded a phenotype intermediate to the Val-282 → Leu mutation and the mildest cases of the Ile-173 → Asn mutation.

Association has previously been observed between mild 21-hydroxylase deficiency and haplotypes with three C4/21OH repeat units (10, 24, 36, 37). All 7 Val-282 → Leu mutations occurred on the two nonidentical triplicated C4/21OH haplotypes 6 or 7 (Fig. 1). Among the other mutations, haplotype 2 clearly dominated. This haplotype is also the most common haplotype in the general population (B.H.-S., O. Olerup, A.W., E.M.R., and H.L., unpublished work).

The described approach using selective PCR and direct sequencing gives reliable information for carrier detection and genetic diagnosis because it identifies all mutations and indicates the number of *CYP21* genes of individuals. In cases of compound heterozygosity, the presence of the mutations on separate alleles can be verified after allele-specific amplification, obviating the need for segregating mutations in families. Categorizing patients according to clinical symptoms only is possible but has complications; environmental factors can affect the severity of disease temporarily, and boys are obviously easy to mis-diagnose. In addition, early treatment of these patients is desirable to prevent symptoms; female fetuses should be treated *in utero* to avoid virilizing malformations. Thus, *CYP21* genotyping is valuable for predicting the clinical course of individuals affected with steroid 21-hydroxylase deficiency.

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