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Synergistic Effect of Partially Inactivating Mutations in Steroid 21-Hydroxylase Deficiency*

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ABSTRACT

Lesions in the gene encoding steroid 21-hydroxylase result in congenital adrenal hyperplasia, with impaired secretion of cortisol and aldosterone from the adrenal cortex and overproduction of androgens. Mild forms of the disease cause late-onset symptoms of hyperandrogenism and are thought to be largely underdiagnosed. A limited number of mutations account for the majority of mutated alleles, but additional rare mutations are responsible for the symptoms in some patients. We previously reported a rare allele in two siblings with late-onset disease. This allele contained three sequence alterations, a C to T transition 4 bases upstream of translation initiation, a CCG to CTG change at codon 105 (P105L), and a CCC to TCC transition at codon 453 (P453S). The latter mutation has been found in other ethnic groups, whereas P105L seems to be unique to this family. We have now analyzed the functional consequences of the −4, P105L, and P453S sequence alterations by *in vitro* translation and after expression of mutant enzyme in cultured cells. As expected, the base substitution at position −4 had no measurable effect on gene expression. The P105L mutation reduced enzyme activity to 62% for 17-hydroxyprogesterone and 64% for progesterone, and the P453S mutation reduced activity to 68% and 46%, respectively. When present in combination, the two mutations caused a reduction of enzyme activity to 10% for 17-hydroxyprogesterone and 7% for progesterone. These results indicate that P105L and P453S can be expected to result in a very subtle disease manifestation when not found in combination, motivating their inclusion when genotyping to ascertain undiagnosed patients with the mildest forms of 21-hydroxylase deficiency. (*J Clin Endocrinol Metab* **82:** 194–199, 1997)

IN THE ADRENAL cortex, steroid 21-hydroxylase (P450c21) normally converts 17-hydroxyprogesterone (17-OHP) into 11-deoxycortisol and progesterone into 11-deoxycorticosterone. These steroids are subsequently converted into cortisol and aldosterone, respectively. Steroid 21-hydroxylase deficiency is the most frequent cause of congenital adrenal hyperplasia (CAH), causing impaired adrenal cortisol and aldosterone production together with increased androgen secretion (1, 2). This recessively inherited disorder exists in a wide range of manifestations, including complete cortisol deficiency and severe prenatal androgen stimulation with ambiguous genitalia in females with or without neonatal salt-wasting, moderate androgen excess with growth acceleration in childhood and precocious puberty, and mild forms with hirsutism and anovulatory amenorrhea in adulthood as the only symptoms. The severe forms occur in 1 of 10,000 Swedish newborns. Based on hormonal analyses, the mildest forms are estimated to occur in 1 of 500 to 1 of 1,000 North Europeans (3), but they often escape diagnosis due to inconspicuous symptoms.

The 21-hydroxylase locus has a complicated structure, with an active form of the gene (CYP21) and an inactive pseudogene (CYP21P). The genes are located 3′ of each of the genes encoding the fourth component of complement, C4A and C4B. One 21-hydroxylase gene and one C4 gene form a unit, these units are tandemly repeated in the HLA class III gene region on chromosome 6p21.3 (4, 5). There is substantial interindividual variation in the numbers and gross structure of these C4/21-hydroxylase units (6, 7). The majority of the disease-causing mutations in CYP21 have arisen through interaction with CYP21P, and are either deletions of CYP21 resulting from misalignment followed by unequal crossing-over or aberrations that represent deleterious sequences that have been transferred from CYP21P into CYP21 (8–10). By typing for nine pseudogene-derived mutations by allele-specific PCR, approximately 95% of all mutated CYP21 alleles are detected (11). The remaining rare mutations need to be characterized by sequencing of the CYP21 gene.

We have found a good association between genotype and degree of disease manifestation (12). One mutation (V281L) is responsible for the vast majority of mild CAH cases (13). Genotyping for mild CAH mutations is a valuable diagnostic complement for this form of the disease, which is believed to be largely underdiagnosed. We previously described a rare allele carrying three base substitutions occurring in two siblings with late-onset disease (14). These were a C to T transition 4 bases upstream of translation initiation, a CCG to CTG change at codon 105 (P105L), and a CCC to TCC transition at codon 453 (P453S). A thymine is usually present at position −4 in CYP21P, whereas the two missense mutations are not normally found in the pseudogene. A similar allele, containing the −4 base change and P453S mutation together with yet another missense mutation (R339H) has previously been described in a patient of Turkish origin (15). The P453S mutation is also relatively frequent among mild CAH patients in Texas (16). The functional consequences of R339H

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and P453S have been analyzed after expression of mutant enzymes carrying each of these two mutations separately (15), whereas the −4 base change and P105L mutation have not been studied functionally. We have now analyzed the degree of functional impairment resulting from the C to T transition at base −4 and the P105L and P453S mutations by *in vitro* translation and after expression of mutant enzymes in cultured cells. The effects of the two missense mutations were studied both individually and in combination. The results have implications for the identification of subjects with the very mildest forms of CAH.

Experimental Subjects

Patients B32 and B33 were a brother and a sister born in 1974 and 1976, respectively, before the Swedish neonatal screening program for CAH was initiated. They had inherited the CYP21 allele with the three sequence alterations from their father, whereas their maternal CYP21 allele was deleted (14, 17). The girl presented with growth acceleration and premature adrenarche, with progressive virilization from 7 yr of age. The diagnosis of late-onset CAH was established at 8.5 yr of age, at which time she had clitoromegaly, pubic and axillary hair, and a muscular habitus. The diagnosis of the girl led to the diagnosis of her brother, who at the age of 10.5 yr could report penile growth, pubic and axillary hair, and acne for at least 1 yr. At the time of diagnosis, his gonadal puberty had commenced. The girl's height was −1.5 SD until 4–5 yr of age, after which it reached +1.5 SD, and her bone age was +3.3 SD at diagnosis. The boy showed a similar degree of growth acceleration and bone age advancement. Hormonal data at diagnosis for the girl include a 17-OHP value of 166 nmol/L, increasing to 480 nmol/L after ACTH loading (reference values <5 nmol/L with minimal increment after loading). Dehydroepiandrosterone (DHEA) was 41.2 nmol/L (reference values <7 nmol/L), DHEA sulfate was 9.40 μmol/L (reference values <1.75 μmol/L), and androstenedione was 16.8 nmol/L (reference values <5 nmol/L). The boy's 17-OHP was 91 nmol/L, increasing to 450 nmol/L during ACTH loading, DHEA was 28.4 nmol/L, DHEA sulfate was 7.21 μmol/L, and androstenedione was 16.7 nmol/L.

Materials and Methods

Construction of plasmids

The human full-length P450c21 complementary DNA (cDNA) clone phc21, cloned into M13 mp18 (18), was kindly provided by Dr. Bon-chu Chung. A *Bam*HI fragment containing the complete cDNA from the initiation codon together with about 300 bp of 3′-untranslated sequences was inserted into plasmid pGEM3Z. This plasmid was modified to include 10 bp of 5′-untranslated sequences from CYP21, creating pGEM-CYP21. From pGEM-CYP21, a *Bgl*II/*Bam*HI fragment containing the CYP21 cDNA together with 10 bp of 5′- and approximately 300 bp of 3′-untranslated sequences was isolated and cloned into the *Bgl*II site of the expression vector pCMV4, generating pCMV4-CYP21. A shorter construct, containing only 28 bp of 3′-untranslated sequences, was generated by *Kpn*I cleavage of pCMV4-CYP21.

Introduction of mutations

The 5′-end of pGEM-CYP21 was PCR amplified with an upstream primer containing the −4 sequence alteration. The resulting fragment was inserted into pGEM-CYP21, generating pGEM-CYP21(−4). The P105L and P453S mutations were introduced separately into pGEM-CYP21 by site-directed mutagenesis, using two phosphorylated primers (19). One primer overlapped restriction sites for *Sal*I and *Bgl*II, but contained a sequence that destroyed the *Sal*I site, and the other primer covered the sequence of each of the two missense mutations. The two primers were annealed to the denatured, double stranded plasmid and directed the synthesis of a new second strand containing the destroyed *Sal*I site and each of the missense mutations separately. The resulting DNA was electroporated into a mismatch repair-defective *Escherichia coli* strain (BMH 71–18 mutS). Plasmid DNA was purified, *Sal*I treated, and used for electrotransformation of competent *E. coli* XL-1 blue. DNA from recovered colonies was purified, and *Sal*I-resistant plasmids were screened for the P105L and P453S mutations by PCR, using one primer specific for each mutant sequence. Plasmids pGEM-CYP21(P105L) and pGEM-CYP21(P453S) were cleaved with *Eco*RI, which cleaves between the two mutations, and *Kpn*I, which cleaves in the 3′-untranslated part of CYP21. The *Eco*RI/*Kpn*I fragment in pGEM-CYP21(P105L) was replaced by the corresponding fragment from pGEM-CYP21(P453S), generating pGEM-CYP21(P105L+P453S). Finally, *Bgl*II/*Bam*HI fragments from the four pGEM-CYP21 constructs were inserted into the *Bgl*II site of pCMV4. The 3′-*Kpn*I fragment was removed from all mutant pCMV4-CYP21 constructs, generating pCMV4-CYP21(−4), pCMV4-CYP21(P105L), pCMV4-CYP21(P453S), and pCMV4-CYP21-(P105L+P453S).

In vitro translation

Estimation of translation efficiency was performed using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. One microgram of plasmids pGEM-CYP21, pGEM-CYP21(−4), pGEM-CYP21(P105L), pGEM-CYP21(P453S), and pGEM-CYP21(P105L+P453S) was used; incubation times were 15, 30, 45, and 60 min. [^{35}S]Methionine incorporation was measured by scintillation counting after trichloroacetic acid precipitation of translation products. The translation products after 60-min incubation were also subjected to SDS-PAGE to verify the integrity of the 55-kDa 21-hydroxylase protein.

In vitro expression and assay of enzyme activity

Approximately 7×10^6 COS-1 cells were transiently transfected by electroporation (Bio-Rad Gene Pulser, Richmond, CA; 1200 V, 25 μF) with 10 μg of each of the pCMV4-CYP21 constructs together with 2 μg of the β-galactosidase vector pCH110 (Pharmacia, Sweden), seeded in 6-cm petri dishes, and incubated in DMEM supplemented with 5% calf serum for 24–36 h.

To determine 21-hydroxylase activity in intact cells, 0.2 μCi ^{3}H-labeled substrate (progesterone or 17-OHP) was added to the medium together with 0.5 μmol/L unlabeled steroid. After incubations for 10 min, 240 μL medium were collected in duplicate samples, and steroids were extracted with 300 μL methylene chloride (Sigma Chemical Co., St. Louis, MO), evaporated to dryness, and dissolved in ethanol. The steroids were separated by thin layer chromatography in chloroform-ethylacetate (80:20), and radioactivity was measured by liquid scintillation spectrophotometry. Subsequently, the cells were trypsinized, washed three times with PBS, and sonicated. Debris was centrifuged, and protein concentration and β-galactosidase activity were measured.

To determine 21-hydroxylase activity in cell homogenates, the cells were washed twice with phosphate-buffered saline (Life Technologies, Gaithersburg, MD), and approximately 2×10^6 cells/plate were recovered for homogenization by sonication for 20 s in 500 μL hypotonic buffer [10 mmol/L HEPES (pH 6.2), 10 mmol/L NaCl, and 1.5 mmol/L $MgCl_2$]. The homogenate was centrifuged at 350 × *g* for 10 min to remove nuclei and whole cells, and the supernatant was collected. To 50 μL supernatant, 4 mmol/L NADPH and 200 mmol/L phosphate buffer, pH 7.2, were added to a final volume of 300 μL, both with and without 20% glycerol. Subsequently, 0.2 μCi ^{3}H-labeled substrate (progesterone or 17-OHP) was added together with 0.5, 2.0, 4.0, or 6.0 μmol/L unlabeled steroid. After incubation at 37 C for 1–2 h, steroids were extracted and analyzed as described above.

Enzyme activities were determined as a percentage of substrate conversion compared to that in cells transfected in parallel by pCMV4-CYP21 encoding wild-type 21-hydroxylase, after correction for total

protein content. Assays were performed under initial rate conditions, determined by preliminary time-course experiments. The ratio of β-galactosidase activity/total protein content was measured in each experiment to verify reproducibility of transformation efficiency. Apparent kinetic constants were calculated after linear regression of the data derived from determinations of enzymatic activity in cell homogenates at each of the four different substrate concentrations, using SigmaPlot and SigmaStat (Jandel Scientific, Erkrath, Germany).

To ascertain the amount of translated P450c21 in transfected cells, proteins from supernatants of homogenized cells were size-separated on a polyacrylamide gel and transferred to a nitrocellulose filter. Immunoblot analyses were performed according to standard procedures, using sera from patients with Addison's disease containing autoantibodies against P450c21 (20).

Results

Expression of recombinant P450c21

Recombinant P450c21 was transiently expressed in COS-1 cells. The effect of cell culture time after transfection was investigated. Enzyme activity increased with extended cell culture time up to 18 h, after which point it did not increase further. This pattern was the same for the allele carrying the −4 mutation and the wild-type CYP21 gene, indicating that this base substitution does not affect the rate of translation in transfected cells. As any difference in expression levels would affect the apparent maximum velocity of the enzyme, we performed immunoblot analyses of homogenates from cells transfected with the different constructs. Similar amounts of the protein were obtained in each case (data not shown).

Deletion of around 270 bp of the 3′-untranslated sequence of CYP21 in the expression vector pCMV4 resulted in approximately 2-fold higher expression, as determined by enzyme activity, when cells were harvested 8 h after transfection (data not shown). This difference was hardly detectable when the cells were harvested and assayed for enzymatic activity 24 h after transfection. Due to the higher expression early after transfection, constructs with shortened 3′-untranslated sequences were used in all subsequent experiments. Many determinants of messenger ribonucleic acid stability are known to be located in the 3′-untranslated region of genes. Multiple degradative pathways recognize AT-rich motifs (21), and several AT-rich stretches are present in the sequences that were removed.

In vitro translation

To further investigate the effect on translation efficiency of the different sequence alterations, we incubated the four mutant alleles and wild-type CYP21 in a coupled transcription-translation system. None of the mutations, including the base substitution at position −4, affected the rate of translation in reticulocyte extracts (data not shown).

Enzyme activity

To assess the influence of the different base substitutions on 21-hydroxylase activity, we compared the activity of the normal protein with those of the various mutant forms. All mutant forms of CYP21 (−4, P105L, P453S, and P105L+P453S) were transiently expressed in COS-1 cells, and enzyme activities toward the two natural substrates for 21-hydroxylase, 17-OHP and progesterone, were assayed in intact cells. As illustrated in Fig. 1, the P105L mutation reduced enzyme activity to 62% for 17-OHP and 64% for progesterone, and the P453S mutation reduced activity to 68% and 46%, respectively, with the wild-type activity being arbitrarily defined as 100%. As the mutations were found in the same allele in the two patients, the combined effect of the two missense mutations was analyzed. The mutations were synergistic, resulting in a reduction of enzyme activity to 10% of normal for 17-OHP and 7% for progesterone. As expected, the construct containing the −4 base substitution gave rise to normal enzyme activity (not shown).

We also analyzed enzyme activities in cell homogenates from transfected cells and investigated the kinetic properties of the normal enzyme compared to those of the enzyme containing the P105L and P453S mutations. Figure 2 shows the kinetic results from a representative experiment, and the corresponding apparent kinetic constants are given in Table 1. The experiments were performed both with and without the addition of 20% glycerol, with essentially the same results. As shown, K_m was in the same range among the various P450c21s, whereas the maximum velocity was decreased for the mutant enzymes.

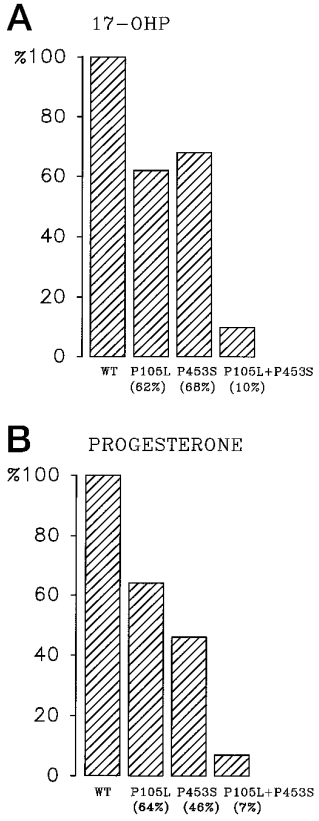

FIG. 1. Enzymatic activities of P450c21 mutants (P105L, P453S, and P105L+P453S) in intact COS-1 cells. Activities are expressed as a percentage of the wild-type activity, which is arbitrarily defined as 100%. Percent conversion values are shown for 17-OHP to 11-deoxycortisol (A) and progesterone to 11-deoxycorticosterone (B), using a substrate concentration of 0.5 μmol/L in both cases. All values are from three separate transfections; actual values (mean ± SEM) are: A, 62 ± 9, 68 ± 18, and 10 ± 2; B, 64 ± 12, 46 ± 8, and 7 ± 3.

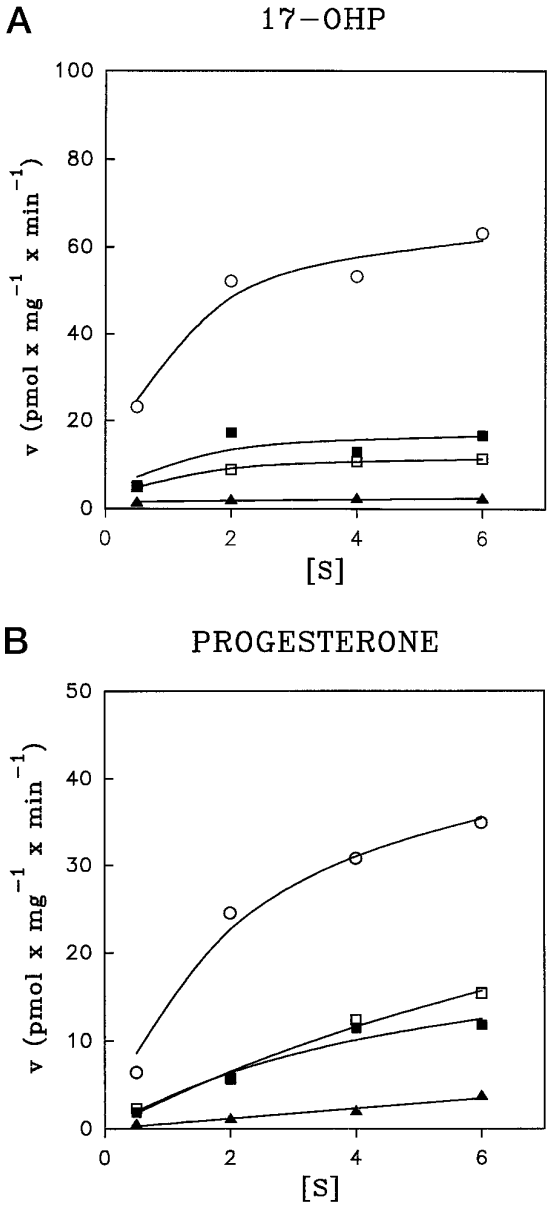

FIG. 2. Kinetic analyses of normal and mutant human P450c21 performed in preparations of cell homogenates. Plots of velocity (V; picomoles per mg total protein/min) against substrate concentration ([S]; micromoles per L) are shown. A, Conversion of 17-OHP to 11-deoxycortisol. B, Conversion of progesterone to 11-deoxycorticosterone. Values are from a representative experiment, except in the case of the normal protein, where the means from five (17-OHP) and two (progesterone) experiments are given. ○, Normal P450c21; ■, P105L; □, P453S; ▲, P105L+P453S.

Genotyping of the pseudogene

The presence of the P105L and P453S mutations in the pseudogene was investigated in 34 healthy parents of CAH patients from families in which C4/21-hydroxylase haplotypes have previously been determined (17, 22). These subjects represent 74 unrelated CYP21P alleles. Genotyping was performed by allele-specific PCR, essentially as previously described (11). Neither of the 2 mutations was present in any of the CYP21P alleles analyzed.

TABLE 1. Kinetic analysis of normal and mutant P450c21

	17-OHP[a]		Progesterone[a]	
	K_m	V_{max}	K_m	V_{max}
WT	1.3	88.5	6.5	85.8
P105L	1.5	25.6	5.5	22.2
P453S	0.8	12.6	4.6	22.8

[a] Measurements were made in nuclear free cellular lysates using 17-OHP and progesterone as substrates for wild-type (WT) and mutant P450c21 (P105L and P453S). K_m values are in micromoles per L, and V_{max} values are in picomoles per mg total protein/min. Values are from one representative experiment, except in the case of the wild-type protein, where the means from five (17-OHP) and two (progesterone) experiments are given. The P105L + P453S mutant did not approach substrate saturation, and apparent kinetic constants were not calculated.

Discussion

We have studied the effects of three base substitutions in CYP21, which were found together in one CYP21 allele identified in two siblings with late-onset CAH (14, 17). The two missense mutations, P105L and P453S, each caused a partial reduction of enzyme activity, displaying a synergistic effect when present in combination. The cytosine to thymine transition at 4 bp upstream of translation initiation did not impair translation efficiency or enzyme activity and represents a normal polymorphism.

The known missense mutations in CYP21 that have been shown to result in partial enzyme inactivation are listed in Table 2. I172N, V281L, and P30L all derive from the pseudogene and are relatively common among CAH patients, accounting for roughly 20%, 6%, and 2% of all affected CYP21 alleles in Scandinavia (12) (Wedell, A., unpublished observations). The I172N mutation is associated with prenatal virilization of females, whereas the V281L mutation is associated with mild CAH without virilizing malformations such as clitoromegaly. It is practical to relate to these two mutations because they are common, whereas for the rare alleles, large numbers of patients are not available for phenotypic evaluation. Based on the synergistic effect of the two mutations *in vitro*, it is reasonable to expect that P105L and P453S both would result in the mildest form of CAH if they were present separately in a CYP21 allele, *i.e.* the form of CAH that is believed to be largely underdiagnosed. It is interesting that the only other allele containing P453S that was completely sequenced also carried an additional mutation, R339H (15). This allele was found in a markedly hirsute woman with clitoromegaly. In this case, the combined effect of the two mutations was not studied in cultured cells.

Kinetic analyses have been performed for P30L, I172N, and V281L (23–25). In the present study, K_m was not impaired in the mutant enzymes, whereas V_{max} was decreased. The above three mutations affected both K_m and V_{max}, at least for one of the two substrates. Thus, P105L and P453S seem to interfere less with steroid binding, impairing enzyme function by different mechanisms. Proline mutants generally cause conformational changes by disrupting helixes, but we cannot explain the exact mechanisms behind the partial loss of function of these mutants. Limited information exists regarding structure-function relationships for the P450c21 pro-

TABLE 2. Summary of different studies characterizing partially inactivating mutations in CYP21 after expression of mutant enzyme in cultured cells

	17-OHP[a]		Progesterone[a]		Ref. no.[b]
	Whole cells	Lysates	Whole cells	Lysates	
I172N	2	<1	2		23
	<10		<10		18
	<10		<10		37
V281L	75	50	30	20	23
	25–50		25–50		37
P30L	60	8	25	5	24
	25–50		25–50		37
R339H	67		46		15
P453S	66		54		15
	68	20	46	30	Present study
P105L	62	20	64	30	Present study
P105L + P453S	10	10	7	10	Present study

[a] The percent conversion of 17-OHP and progesterone is shown for each mutation compared to the normal P450c21 enzyme.
[b] Different expression systems were used, as described in the indicated references.

tein. Four bacterial P450 enzymes have been crystallized (26–29), and this information can be used for cautious predictions of structurally vital residues in P450c21 (30). In addition, the location of the heme- and steroid-binding regions of the enzyme can be predicted on the basis of sequence homologies to other proteins with similar motifs (31). The P453S mutation affects an amino acid that is conserved between 21-hydroxylases from bovine (32, 33), murine (34), and porcine (35) species. Furthermore, it is located in proximity of the heme-binding region of the enzyme, and it is possible that it interferes with this vital property of the protein. The P105L mutation, on the other hand, affects a residue that is not conserved between species, and it is not located close to any region predicted to be of importance to the structure or function of the protein. When analyzing partially active mutants in cell lysates, the addition of glycerol usually has a positive effect on enzyme activity. We found no stabilizing effect of glycerol, possibly indicating that membrane binding or overall structure was not seriously altered by either of the mutations.

The C to T transition at position −4 did not affect CYP21 gene function, as expected. It is known that as long as a purine is present in position −3 before the initiation codon, translation is usually not impaired (36). As a thymine is usually present at position −4 in the pseudogene, it can be assumed that this base substitution has arisen by the same mechanism as the most common disease-causing CYP21 mutations, *i.e.* by transfer of a CYP21P sequence into CYP21. We could not detect the P105L or P453S mutations among Scandinavian CYP21P alleles. However, P453S has been reported to occur in the pseudogene in the Texan population, where this mutation is quite frequent (16). It is likely that there exists a rare, distantly related CYP21P allele carrying the P453S mutation in the Scandinavian population, and that P453S can be expected to occur in additional CAH patients also in this ethnic group.

We have analyzed approximately 350 Scandinavian CAH alleles and only found the P105L and P453S mutations in a single family of Scandinavian ancestry. The siblings of this family had a slightly more severe phenotype than the mildest forms of CAH; the girl had clitoromegaly, which is generally not seen in association with the mild V281L mutation. The functional studies of mutant enzymes show that the mutations would produce a more subtle phenotype when found alone than when combined, as was the case in this family. This together with the fact that the global frequency of P453S is not negligible motivates us to include these mutations when using genotyping to ascertain mild, undiagnosed cases of CAH.

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