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ARTICLE

# *CYP21* and *CYP21P* variability in steroid 21-hydroxylase deficiency patients and in the general population in the Netherlands

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**Steroid 21-hydroxylase deficiency is caused by defectiveness of the *CYP21* gene. Such defects have presumably originated from interactions with the nearby *CYP21P* pseudogene during evolution. We studied these mechanisms by comparing the genetic variability of *CYP21*, *CYP21P*, and *CYP21P/CYP21* hybrids (resulting from large-scale rearrangements) at eight mutation sites in a group of Dutch steroid 21-hydroxylase deficiency patients, their family members, and controls. The most common *CYP21* defect in patients with salt-losing steroid 21-hydroxylase deficiency was a splice junction mutation in intron 2. The most common defect in the simple virilising form of the disease was ile172 → asn. *CYP21P* showed considerable sequence variation in its central and 3' sections; the 5' section was constant. A single nucleotide (T) insert in exon 7 was found in all *CYP21P* genes. During the course of evolution, this was probably the third defect introduced into *CYP21P* after the splice junction mutation in intron 2 and the 8 bp deletion in exon 3. Gene conversions introducing *CYP21*-like sequences contribute to *CYP21P* variability. Such an event has occurred *de novo* in one family. A comparison of *CYP21* and *CYP21P* mutations on the same chromosome shows that at least some of the small-scale gene conversions that supposedly transfer defects to *CYP21* involve interaction between homologous chromosomes. The majority of the putative *CYP21P–CYP21* transitions in hybrid genes appears to occur in a distinct zone that lies 5' of nucleotide 2108, which is further downstream than previously hypothesised. The other transitions lie upstream of nucleotide 999. Apparent 'large-scale' *CYP21–CYP21P* gene conversions lead to hybrid genes that are very similar to those found in *CYP21* deletions, so these haplotypes have probably resulted from a meiotic double unequal crossover.** *European Journal of Human Genetics* (2000) **8**, 827–836.

**Keywords:** *CYP21*; *CYP21P*; steroid 21-hydroxylase deficiency; congenital adrenal hyperplasia; Netherlands; hybrid genes; mutations; evolution

## Introduction

The human *CYP21* gene, located on chromosome 6 (6p21.3), encodes the adrenocortical enzyme steroid 21-hydroxylase.[1] *CYP21P* is a pseudogene sharing 98% sequence homology with *CYP21* but containing several alterations rendering it inactive.[2,3] *CYP21* and *CYP21P* are about 30 kb apart and are part of a cluster of duplicated genes that includes genes encoding the fourth component of complement (C4) and tenascin-X.[1,4,5]

Defectiveness of *CYP21* leads to steroid 21-hydroxylase deficiency, which is the cause of over 90% of all cases of congenital adrenal hyperplasia. Complete deficiency results in severe salt-loss crises in untreated paediatric patients due to lack of aldosterone ('salt-losing') 21-hydroxylase deficiency), while increased androgen levels cause virilisation

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even in patients with less severe defects ('simple virilising' 21-hydroxylase deficiency).[6–8]

Genetic analysis of steroid 21-hydroxylase deficiency is complicated by the existence of many different arrangements of *CYP21* and *CYP21P*, including gene deletions, duplications, and large-scale gene conversions. Analysis of *Taq*I and *Bgl*II restriction patterns is commonly used to determine the gross arrangement of the *CYP21/C4* region in steroid 21-hydroxylase deficiency patients and their family members.[1,9–15] This approach allows the definition of *CYP21/C4* haplotypes, some of which are associated with steroid 21-hydroxylase deficiency.[14–22].

Three categories of defects can be distinguished:

(a) Haplotypes with a single gene that features a *CYP21P*-like *Taq*I restriction pattern ('*CYP21* deletions'). These have resulted from a meiotic unequal crossover between *CYP21* and *CYP21P*, creating a gene consisting of the 5' section of *CYP21P* and the 3' section of *CYP21*.[1,9,23–25]

(b) Haplotypes with two genes, both with a *CYP21P*-like restriction pattern ('large-scale gene conversions'). Here, a section of *CYP21* that includes at least the *Taq*I site in the 5' flank has been replaced by a *CYP21P*-like sequence.[10,12,13]

(c) Haplotypes where a gene with a *CYP21*-like restriction pattern is present (by exclusion).

On chromosomes bearing steroid 21-hydroxylase deficiency, the *CYP21* gene carries one or more deleterious mutations.[8,17,26–28] With a few exceptions, these mutations are found in the consensus sequence of *CYP21P*[2,3] and have supposedly been transferred to *CYP21* during evolution ('small-scale gene conversions').

The nature and the frequencies of the mutations in *CYP21* have been well studied. They vary among different populations.[8,17,18,22,26–37] However, information on the variability of the *CYP21P* pseudogene, especially in association with the *Taq*I/*Bgl*II haplotypes that define the overall structure of the region, is rather limited.[18,38] Such information is highly relevant to the hypotheses describing the origin of the different categories of disease-causing mutations in *CYP21*: the location of crossover sites and the extent of conversion zones depend on the composition of the *CYP21P* gene involved. We compared *CYP21* and *CYP21P* in a single population, to assess the consequences of *CYP21P* variability for the hypothetical concepts of 'hybrid genes' and 'gene conversions'. The distribution of the 10 most common *CYP21* defects (gene deletion, large-scale conversion, and eight mutations) was determined in 38 steroid 21-hydroxylase deficiency patients from The Netherlands, a population which has not been studied before. The sites corresponding to these mutations were also studied in *CYP21P* in these families, and in 46 controls having only one *CYP21P* gene.

## Methods

### Patients, family members, and controls

Steroid 21-hydroxylase deficiency patients, their parents, and any available sibs from 38 families were included; in six families, only one of the parents was available. The number of independent chromosomes was 150 (due to consanguinity between two of the families), 75 of which carried a steroid 21-hydroxylase deficiency allele. Single patients and two families with apparent *de novo* gene rearrangements were not included. There were 29 families with salt-losing (SL) patients and nine families with simple virilising (SV) patients. From our group of 143 healthy controls from the general Dutch population, 46 had a heterozygous deletion of *CYP21P* and were selected for study of *CYP21P* separately. All subjects were informed about the purpose of the study and gave their consent.

### Haplotyping and mutation analysis

*Taq*I and *Bgl*II restriction analysis[1,9–15] was used as described earlier[19] to establish *CYP21/C4* haplotypes in all families and controls.

Polymerase chain reaction (PCR) amplification of *CYP21* and *CYP21P* followed by oligonucleotide hybridisation[17,18,27–29] was used to determine sequence variability. Three overlapping parts of either *CYP21* or *CYP21P* (see following sections 1, 2 and 3; Figure 1) were specifically amplified using the primers listed in Table 1. PCR conditions (annealing temperature, number of cycles, $MgCl_2$ concentration) were: *CYP21*, section 1: 54°C, 30 cycles, 2.5 mM; *CYP21P*, section 1: 55°C, 32 cycles, 2.5 mM; *CYP21*, section 2: 55°C, 33 cycles, 2.5 mM; *CYP21P*, section 2: 57°C, 32 cycles, 2.5 mM; *CYP21*, section 3: 56°C, 32 cycles, 2.4 mM; *CYP21P*, section 3: 56°C, 32 cycles, 2.4 mM. All reactions were done with 1 unit of GoldStar DNA polymerase (Eurogentec, Seraing, Belgium) in a final volume of 50 μl in the presence of 2% formamide. In each case, the reverse primer was non-specific (not distinguishing between *CYP21* and *CYP21P*) and downstream of the forward primer of the next section. The specificity of each reaction was checked by digesting 20% of the amplified product with *Pvu*II (section 1), *Nde*II (section 2), or *Pst*I (section 3), followed by electrophoresis in 2–2.5% agarose gels. These restriction sites are normally present in *CYP21* and absent from *CYP21P*. Section 1 was also digested with *Eco*RI; the *Eco*RI site is specific for *CYP21P* (see Figure 1). The *Pvu*II and *Eco*RI restriction patterns of the section 1 PCR were matched to the bands shown by *Taq*I/*Pvu*II and *Taq*I/*Eco*RI double digests of genomic DNA.[39,40] Control samples containing or not containing these sites were included in each reaction. The results for section 1 were confirmed by amplification of *CYP21* and *CYP21P* in a single reaction using the same (non-specific) reverse primer and a non-specific forward primer (see Figure 1; 53°C, 32 cycles, 3 mM $MgCl_2$) followed by *Eco*RI digestion to distinguish between *CYP21* and *CYP21P*, Southern blotting and hybridisation as described elsewhere.[40] The remainder of the product was slot-blotted

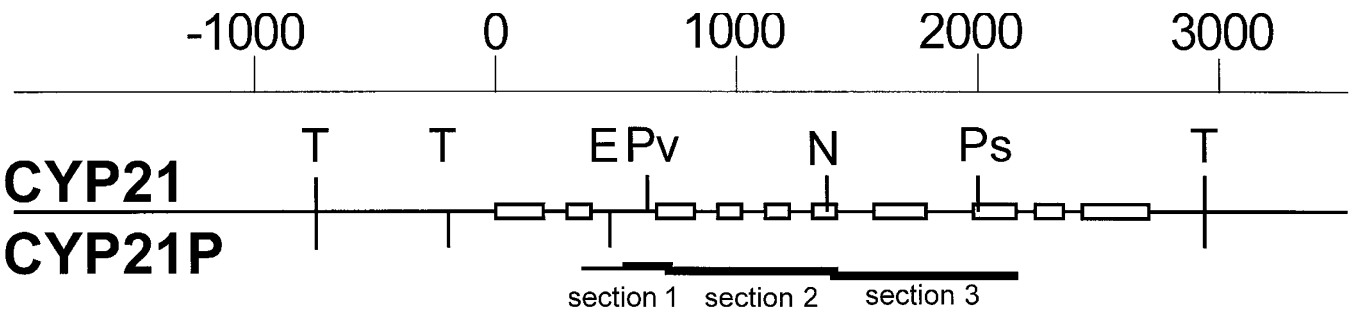


**Figure 1** Map of the consensus *CYP21* and *CYP21P* genes; open boxes are exons. Restriction sites used for identification (see text) are shown: E = *Eco*RI site, N = *Nde*II site, Ps = *Pst*I site, Pv = *Pvu*II site, T = *Taq*I site. The bold black lines below represent the three amplified sections. The amplification of *CYP21* and *CYP21P* in a single reaction (see text) matched section 1, extended further to the 5' side as indicated by the thinner black line to the left.

on to Hybond-N$^+$(Amersham Pharmacia, Little Chalfont, UK), and mutations were detected by hybridisation to allele-specific oligonucleotides, some of which were also used for amplification (see Table 1 and Figure 2).

## Results

### Arrangement of *CYP21/CYP21P* and complement *C4* genes

An overview of the *CYP21/CYP21P* arrangements in the 38 families and 143 controls investigated is presented in Table 2. Several *CYP21/C4* haplotypes not described in our earlier report[19] were detected in the larger population now studied. Three controls had two *CYP21* genes and no *CYP21P* genes on one chromosome, suggesting a 'large-scale' conversion of *CYP21P* to *CYP21*. Both *C4* genes were 'long' (22 kb) in this haplotype. One control carried one *CYP21* gene, three *CYP21P* genes, one long *C4* gene and three short (16 kb) *C4* genes on one chromosome. The mother of one patient had one *CYP21* gene, three *CYP21P* genes and four 'long' *C4*

**Table 1** Oligonucleotides used as primers and for mutation detection

| *Sequence* | *Description* | *Final wash*[a] |
|---|---|---|
| CAGACCTGAGCCACTTACCT | nt 367: forward primer *CYP21* and *CYP21P*, section 1 | |
| ATCCCCAATCCAGGTCCCT | nt 530: forward primer *CYP21*, section 1 | |
| TTTGTTAGAGATGGGGTCTTG | nt 430: forward primer *CYP21P*, section 1[b] | |
| CAGGAGGAGTTGGGGGCTG | A at nt 655: splice junction intron 2 (normal) | 52°C, 0.5×SSC |
| CAGGAGGAGGTGGGGGCTG | C at nt 655: splice junction intron 2 (normal) | 52°C, 0.5×SSC |
| CAGGAGGAGCTGGGGGCTG | G at nt 655: splice junction intron 2 (mutant) | 52°C, 0.5×SSC |
| TCCTTGGGAGACTACTCCCT | nt 700: absence of 8 bp deletion in exon 3 (normal); also forward primer *CYP21*, section 2 | 48°C, 0.4×SSC |
| TGTCGTTGGTCTCTGCTCTG | nt 698: presence of 8 bp deletion in exon 3 (mutant); also forward primer *CYP21P*, section 2 | 52°C, 0.4×SSC |
| TTCTTGTGGGCTTTCCAGAG | nt 721: reverse primer *CYP21* and *CYP21P*, section 1 | |
| GCGGCATCATCTGTTACCT | T at nt 999: ile172 (normal) | 48°C, 0.5×SSC |
| GCGGCATCAACTGTTACCT | A at nt 999: asn172 (mutant) | 48°C, 0.5×SSC |
| CACATCGTGGAGATGCAGCTG | TCGTGGAGAT at nt 1380–1389: ile val glu met236 (normal), also forward primer *CYP21*, section 3 | 52°C, 0.3×SSC |
| CACAACGAGGAGAAGCAGCTG | ACGAGGAGAA at nt 1380–1389: asn glu glu lys236 (mutant), also forward primer *CYP21P*, section 3[c] | 52°C, 0.3×SSC |
| CACAAGGAGGAGAAGCAGCTG | AGGAGGAGAA at nt 1380–1389: lys glu glu lys236 (mutant) | 52°C, 0.1×SSC |
| AGTCCCCACCTTGTGCTGCCT | nt 1397: reverse primer *CYP21* and *CYP21P*, section 2 | |
| CCATGTGCACGTGCCCTTC | G at nt 1683: val281 (normal) | 48°C, 0.5×SSC |
| CCATGTGCAAGTGCCCTTC | T at nt 1683: leu281 (mutant) | 48°C, 0.5×SSC |
| GTGAAGCAAAAAAACCACGG | nt 1760: absence of T insertion in exon 7 (normal) | 48°C, 0.4×SSC |
| GTGAAGCAAAAAAAACCACG | nt 1760: presence of T insertion in exon 7 (mutant) | 48°C, 0.4×SSC |
| GCTCCTCCTGCAGTCGCTG | C at nt 1994: gln318 (normal) | 48°C, 0.7×SSC |
| GCTCCTCCTACAGTCGCTG | T at nt 1994: stop318 (mutant) | 48°C, 0.7×SSC |
| CTGCGCCTGCGGCCCGTTG | C at nt 2108: arg356 (normal) | 52°C, 0.2×SSC |
| CTGCGCCTGTGGCCCGTTG | T at nt 2108:trp356 (mutant) | 52°C, 0.2×SSC |
| AACCCTCGGGAGTCACCTGCT | nt 2153: reverse primer *CYP21* and *CYP21P*, section 3 | |

nt = nucleotide number according to Higashi *et al*[2]; [a]the final wash in the oligonucleotide hybridisation was 20 min at these conditions; [b]primer site chosen about 100 bp upstream of the *CYP21* primer, to include the *Eco*RI site in exon 2; [c]section 3 of genes carrying lys glu glu lys236 was amplified normally using this primer, but it was easily washed off in the hybridisation experiments of the section 2 PCR product.

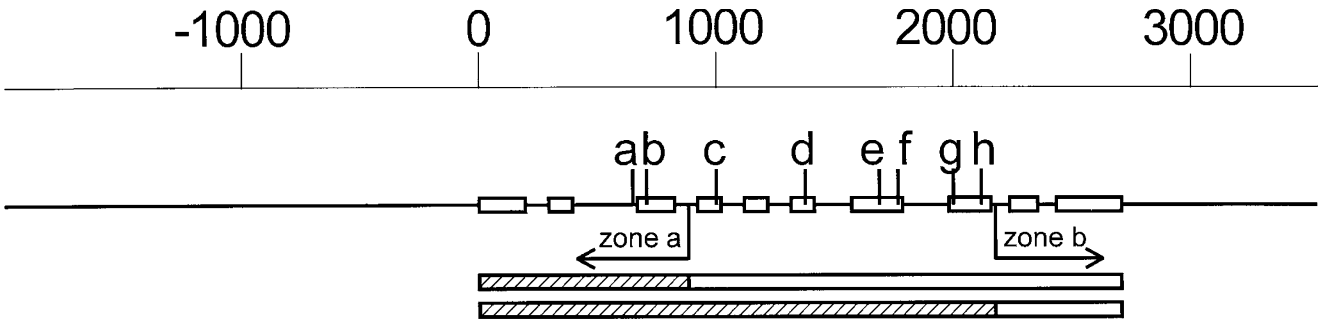


**Figure 2** *CYP21* intron–exon structure (open boxes are exons) and the mutations studied: a = intron 2 splice junction mutation (A/C/G) (nt 655); b = 8 bp deletion (present or absent) (nt 700); c = ile172/asn172 (nt 999); d = ile val glu met236/asn glu glu lys236/lys glu glu lys236 (nt 1380); e = val281/leu281 (nt 1683); f = 1 bp insertion between codons 306 and 307 (present or absent) (nt 1760); g = gln318/stop318 (nt 1994); h = arg356/trp356 (nt 2011). The characteristics of the consensus *CYP21* gene at each mutation site are, respectively: A or C at the splice junction mutation; no 8 bp deletion; ile172; ile val glu met236; val281; no 1 bp insertion; gln318; arg356. The characteristics of the consensus *CYP21P* gene are: G; 8 bp deletion; asn172; asn glu glu lys236; leu281; 1 bp insertion; stop318; trp356. The limits of the putative *CYP21P–CYP21* transition zones have been drawn at arbitrary places relative to ile172/asn172 (zone a, 5' limit) and arg356/trp356 (zone b, 3' limit), respectively, since no further distinction is possible because the sequences of *CYP21* and *CYP21P* are equal there. The arrows indicate that the actual transition site within these zones may vary for each hybrid. Bottom: hybrid genes with transition zones a and b, respectively; the hatched section is *CYP21P*-like.

**Table 2** *CYP21/CYP21P* haplotypes in the Dutch population

| | *Families, normal haplotypes*[a] | *Families, defective haplotypes*[b] | *Controls, all haplotypes* |
|---|---|---|---|
| Haplotype | | | |
| *CYP21-CYP21P* (normal) | 58 | 38 | 212 |
| *CYP21* (*CYP21P* deletion) | 11 | 9 | 55[c] |
| *CYP21-CYP21P-CYP21P* (*CYP21P* duplication) | 2 | 3 | 11 |
| *CYP21-CYP21-CYP21P* (*CYP21* duplication) | 0 | 1 | 3 |
| *CYP21-CYP21P-CYP21P-CYP21P* (*CYP21P* triplication) | 1 | 0 | 1 |
| *CYP21-CYP21* (*CYP21P* to *CYP21* conversion) | 0 | 0 | 3 |
| *CYP21P*[d] (*CYP21* deletion) | 0 | 15 | 1 |
| *CYP21P-CYP21P*[d] (*CYP21* to *CYP21P* conversion) | 0 | 9 | 0 |
| Total | 72 | 75 | 286 |

[a]haplotypes not carrying steroid 21-hydroxylase deficiency; in three families, these could not be deduced because one of the parents was missing; [b]haplotypes carrying steroid 21-hydroxylase deficiency; [c]the genotypes were: four persons with *CYP21/CYP21*, one person with *CYP21-CYP21/CYP21*, and 46 persons with *CYP21-CY21P/CYP21*, these 46 were used as controls in the *CYP21P* mutation analysis; [d]these haplotypes carry steroid 21-hydroxylase deficiency due to absence of the *CYP21* gene.

genes on one chromosome. These unusual haplotypes were confirmed by *Sac*II digestion and pulsed field gel electrophoresis (unpublished observations).

### Polymerase chain reaction and oligonucleotide hybridisation

The *Pvu*II and *Eco*RI restriction patterns for section 1 matched the genomic *Taq*I/*Pvu*II and *Taq*I/*Eco*RI results in each case. The specificity of the amplification of section 2 could then be deduced from the mutation analysis of section 1, which contains its forward primer sites. Similarly, section 2 contains the forward primer site of section 3. This strategy allowed assignment of each sequence variant to either *CYP21* or *CYP21P* in all cases.

Mutation detection by oligonucleotide hybridisation to slot blots or Southern blots usually produced straightforward results, with the exception of the (non-defective) 'C' variant at nucleotide 655 (the site of the splice junction mutation in intron 2; see Figure 2), which hybridised poorly and required careful washing steps. Similar weak signals with the 'C' variant were found by Rumsby *et al*.[41] The 'C' variant was readily detected in individuals homozygous for it, but the hybridisation signal was much weaker when the other chromosome carried either 'A' or 'G'. The (normal) 'A' and (defective) 'G' variants performed equally well in this analysis. In each parent carrying the 'G' allele on one chromosome, a non-defective ('A' or 'C') allele could also be detected, indicating that drop-out of one allele in the PCR[42] did not occur here. On the other hand, the amplification of both genes in a single reaction often produced a higher amount of *CYP21P* (containing 'G') than *CYP21*, especially when one or both of the *CYP21* genes carried a 'C' at nucleotide 655.

### *CYP21* mutations

In 89% of the patients' *CYP21* genes, the defect could be attributed to one of the deleterious mutations investigated here (see Table 3). In general, there was a good match between phenotype and genotype: a severe defect (deletion, large conversion, intron 2 splice mutation, 8 base pair deletion, asn glu glu lys236, 1 base pair (T) insertion, stop318, trp356 – mutation group A in the classification of Speiser *et al*[28]) was found on both chromosomes in 25 out of 29 families with the salt-losing variety of the disease. None of the SV patients had one of the more severe defects on both chromosomes. The *Eco*RI site in intron 2 was not found in any *CYP21* gene. The *Pvu*II site in intron 2 was missing from one *CYP21* gene that also carried the splice junction mutation, suggestive of a small-scale conversion transferring both alterations.

One patient had both the 1 bp insertion in exon 7 and stop318 in exon 8, which were probably transferred from *CYP21P* in a single event during evolution. Two patients had val281→leu, the 1 bp insertion (codon 306-307), gln318→stop, and arg356→trp in the *CYP21* gene, suggesting that a single conversion event has occurred in the past to introduce these defects into *CYP21*.

One patient had a defective allele with two *CYP21* genes. This haplotype carried the intron 2 splice junction mutation and gln318→stop, and is probably the same as a haplotype described in the Swedish population.[43]

### *CYP21P* mutations

*CYP21P* mutation analysis was complicated by the lack of clinical association and by the relatively frequent occurrence of chromosomes with more than one *CYP21P* gene. Only mutations that could be assigned to a specific haplotype with certainty were included. The distribution of *CYP21P* mutations in the most common haplotypes is shown in Table 4. On 36 chromosomes where the *CYP21* defect was found, at least one *CYP21P* gene was present. On 32 of these chromosomes, there was a match between the *CYP21* defect and the *CYP21P* sequence at the corresponding site – in these cases, *CYP21P* matched the consensus sequence.[2] Four *CYP21* genes had a defect not found in the accompanying *CYP21P* gene. These were: leu281 and stop318 and trp356 on *CYP21 vs* val281 on *CYP21P*; leu281 and stop318 and trp356 on *CYP21 vs* arg356 on *CYP21P*; trp356 on *CYP21 vs* lys glu glu lys236 and val281 and arg356 on *CYP21P*; and trp356 on *CYP21 vs* val281 and arg356 on *CYP21P*. Seven chromosomes carried a defective *CYP21* gene but no *CYP21P* gene. The *CYP21* defects were: all four asn glu glu lys236 mutations; two of the intron 2 splice junction mutations; and one stop318 mutation.

The variation in the central and 3' sections of 'normal' *CYP21P* genes (ie *CYP21P* genes on the *CYP21–CYP21P* haplotype) was considerable. The most frequent deviations from the consensus *CYP21P* sequences were lys glu glu lys236, val281, gln318, and arg356. On the other hand, the T insert between codons 306 and 307 in exon 7 was present in all pseudogenes on the *CYP21–CYP21P* haplotype.

Many *CYP21P* genes carry more than one mutation as compared with the consensus sequences.[2,3] The following combinations were found in the control group of individuals with a single *CYP21P* gene, where they could be unambiguously linked: asn glu glu lys236-leu281-stop318-trp356 (20×); asn glu glu lys236-leu281-stop318-arg356 (8×); lys glu glu lys236-val281-stop318-arg356 (8×); asn glu glu lys236-leu281-gln318-arg356 (4×) asn glu glu lys236-leu281-gln318-trp356 (1×); asn glu glu lys236-val281-stop318-arg356 (1×).

Additional combinations were found in the family studies: of the 16 possible combinations of asn glu glu lys/lys glu glu lys236, leu/val281, stop/gln318 and trp/arg356, only lys glu

**Table 3** *CYP21* defects in Dutch patients with classical steroid 21-hydroxylase deficiency

| *Defect* | *SL* | *SV* |
|---|---|---|
| *CYP21* deletion (5' *CYP21*P/3' *CYP21* hybrid) | 13 | 2 |
| *CYP21* large conversion (5' *CYP21P*/3' *CYP21* hybrid and *CYP21P* gene) | 8 | 1 |
| splice junction mutation in intron 2[a] | 21 | 1 |
| 8 bp deletion in exon 3 | 1 | – |
| ile172 → asn | 1 | 7 |
| ile val glu met236 → asn glu glu lys | 4 | – |
| ile val glu met236 → lys glu glu lys | – | – |
| val281 → leu | – | – |
| 1 bp insertion in exon 7 (between codons 306 and 307) | – | – |
| gln318 → stop | 1 | – |
| arg356 → trp | 2 | 1 |
| 1 bp insertion in exon 7 and gln318 → stop | 1 | – |
| val281 → leu and 1 bp insertion in exon 7 and gln318 → stop and arg356 → trp | 1 | 1 |
| splice junction mutation and gln318 → stop (2 *CYP21* genes on one allele) | 1 | – |
| no defect found | 3[b] | 5 |
| Total | 57[c] | 18 |

The numbers refer to the independent chromosomes carrying the steroid 21-hydroxylase deficiency allele (see text); [a]a G at position 655, of the remaining genes, 83 had an A and 18 a C (equally distributed between SL and SV patients); [b]in one of these genes, the milder defect pro30 → leu was detected in a separate experiment (results not shown); [c]one fewer than expected for 29 families due to known consanguinity (see text).

**Table 4** Distribution of *CYP21P* variability per haplotype

| *Mutation* | CYP21–CYP21P *(normal) families*[a] (n=*96)* | CYP21–CYP21P *(normal) controls*[b] (n=*46)* | CYP21P *alone (*CYP21 *del.) patients*[c] (n=*15)* | CYP21P–CYP21P *(large conv.) patients*[d] (n=*18)* | CYP21–CYP21P–CYP21P *(*CYP21P *dupl.) families*[e] (n=*10)* |
|---|---|---|---|---|---|
| presence of *Eco*RI site in intron 2 | 100 | 100 | 100 | 100 | 100 |
| absence of *Eco*RI site in intron 2 | 0 | 0 | 0 | 0 | 0 |
| absence of *Pvu*II site in intron 2 | 100 | 100 | 100 | 100 | 100 |
| presence of *Pvu*II site in intron 2 | 0 | 0 | 0 | 0 | 0 |
| splice junction in intron 2, 'G' | 100 | 100 | 100 | 100 | 100 |
| splice junction in intron 2, 'A' | 0 | 0 | 0 | 0 | 0 |
| splice junction in intron 2, 'C' | 0 | 0 | 0 | 0 | 0 |
| presence of 8 bp deletion in exon 3 | 100 | 100 | 100 | 100 | 100 |
| absence of 8 bp deletion in exon 3 | 0 | 0 | 0 | 0 | 0 |
| asn172 | 98 | 100 | 67 | 82 | 100 |
| ile172 | 2 | 0 | 33 | 18 | 0 |
| asn glu glu lys236 | 86 | 81 | 33 | 82 | 100 |
| lys glu glu lys236 | 13 | 19 | 33 | 18 | 0 |
| ile val glu met236 | 1 | 0 | 33 | 0 | 0 |
| leu281 | 72 | 79 | 25 | 64 | 87 |
| val281 | 28 | 21 | 75 | 36 | 13 |
| presence of 1 bp insertion in exon 7 | 100 | 100 | 73 | 82 | 100 |
| absence of 1 bp insertion in exon 7 | 0 | 0 | 27 | 18 | 0 |
| stop318 | 89 | 88 | 27 | 31 | 70 |
| gln318 | 11 | 12 | 73 | 69 | 30 |
| trp356 | 60 | 52 | 21 | 67 | 50 |
| arg356 | 40 | 48 | 79 | 33 | 50 |

The mutations are grouped per site; the variant matching the consensus sequence is mentioned first. The frequency of each mutation per haplotype is shown in percentages. The numbers in each column heading refer to the *CYP21P* genes, not to the chromosomes: for the haplotypes *CYP21-CYP21P-CYP21P* and *CYP21P-CYP21P*, the number of chromosomes is half the number of *CYP21P* genes. Mutations were only counted when they could be assigned to a haplotype with certainty. [a]steroid 21-hydroxylase deficiency families, numbers for chromosomes carrying and not carrying steroid 21-hydroxylase deficiency were added, 'standard' *CYP21-CYP21P* haplotypes, *n*=90 for val/leu281, *n*=93 for gln/stop318 and for arg/trp356; [b]controls with *CYP21–CYP21P* on one chromosome and a *CYP21P* deletion on the other, *n*=46 for mutations upstream of and including the 8 bp deletion, *n*=42 for the remaining mutations; [c]steroid 21-hydroxylase deficiency patients; hybrid genes due to a 30 kb deletion; *n*=12 for val/leu281, *n*=14 for arg/trp356; [d]patients, hybrid genes due to a large-scale gene conversion (two *CYP21P* genes were counted per chromosome), *n*=14 for val/leu281, *n*=16 for gln/stop318, *n*=12 for arg/trp356; [e]families, numbers for chromosomes carrying and not carrying steroid 21-hydroxylase deficiency were added, *CYP21P* duplication (two *CYP21P* genes were counted per chromosome), *n*=8 for val/leu281 and the arg/trp356.

glu lys-leu-gln-trp, lys glu glu lys-val-gln-trp, lys glu glu lys-leu-gln-arg, and lys glu glu lys-leu-stop-arg were not found. One *CYP21P* gene carried both ile172 and arg356; another carried both ile172 and ile val glu met236. There were no statistically significant differences in the frequencies of the *CYP21P* mutations between 'standard' (*CYP21–CYP21P*) chromosomes with functional or non-functional *CYP21* genes, or between 21-hydroxylase deficiency families and controls.

Table 5 shows all combined mutations on haplotypes carrying a single CYP21P-like gene (CYP21 'deletions') or two CYP21P-like genes ('large scale' CYP21 conversions). All these genes are CYP21P-like from their 5' flank to, at least, the 8 bp deletion in exon 3, and therefore defective.

### *De novo CYP21P* mutation in one family

In a family with two patients each having two *CYP21P* genes, one patient carried ile val glu met236 on one chromosome and asn glu glu lys236 on the other, whereas all other family members only had asn glu glu lys236 on their *CYP21P* genes. Paternity was confirmed by testing independent genetic markers (results not shown). All other investigated mutations and the *CYP21/C4* haplotypes were inherited in a normal Mendelian fashion in this family. A *de novo* conversion event locally changing *CYP21P* to a *CYP21*-like sequence is the most obvious explanation. All *CYP21* genes in this family carried ile val glu met236.

## Discussion

### Mutations in the *CYP21* gene

The *CYP21* mutations causing salt-losing 21-hydroxylase deficiency occurred at frequencies similar to those observed in other populations, the intron 2 splice mutation (a 'G' at nucleotide 655) being the most common. The frequency distribution is different for simple virilising 21-hydroxylase deficiency, where ile172 → asn is clearly more abundant. This finding is in agreement with some studies;[18,28,33,34] others found the intron 2 splice mutation more often in simple virilising patients as well.[17,22,26,36] The chance that a randomly selected Dutch individual who is a carrier of salt-

**Table 5** Combined mutations on chromosomes carrying *CYP21P* alone ('deletion') and *CYP21P–CYP21P* ('large-scale conversion')

| Family | Haplotype | 172 | 236[a] | 281 | 306–307[b] | 318 | 356 |
|---|---|---|---|---|---|---|---|
| Consensus *CYP21* | | ile | ile | val | no ins | gln | arg |
| Consensus *CYP21P* | | asn | asn | leu | ins | stop | trp |
| 1 | *CYP21P* | asn | asn | leu | ins | stop | arg |
| 2 | *CYP21P* | asn | asn | ... | ins | gln | ... |
| 5 | *CYP21P* | asn | lys | leu | ins | gln | arg |
| 6 | *CYP21P* | asn | lys | val | ins | gln | trp |
| 7 | *CYP21P* | asn | lys | val | ins | gln | trp |
| 8 | *CYP21P* | ile | ile | val | no ins | gln | arg |
| 9 | *CYP21P* | ile | ile | val | no ins | gln | arg |
| 13 | *CYP21P* | asn | lys | val | ins | gln | arg |
| 16 | *CYP21P* | asn | asn | ... | ins | gln | trp |
| 17 | *CYP21P* | ile | ile | val | no ins | gln | arg |
| 23 | *CYP21P* | ile | ile | leu | ins | stop | arg |
| 26 | *CYP21P* | asn | asn | val | ins | stop | arg |
| 30 | *CYP21P* | asn | asn | val | ins | stop | arg |
| 31 | *CYP21P* | asn | asn | ... | ins | stop | arg |
| 32 | *CYP21P* | asn | lys | val | ins | gln | arg |
| 3 | *CYP21P–CYP21P* | asn+ile | asn+ile | leu+val | ins+no ins | stop+gln | trp+arg |
| 14 | *CYP21P–CYP21P* | ile+... | ile+... | val+... | no ins+... | gln+... | arg+... |
| 14 | *CYP21P–CYP21P* | asn+asn | asn+asn | leu+leu | ins+ins | ...+... | trp+trp |
| 15 | *CYP21P–CYP21P* | asn+asn | asn+asn | leu+leu | ins+ins | stop+stop | trp+trp |
| 16 | *CYP21P–CYP21P* | asn+asn | asn+asn | ...+... | ins+ins | gln+gln | trp+trp |
| 18 | *CYP21P–CYP21P* | asn+asn | asn+asn | val+... | ins+ins | gln+... | ...+... |
| 19 | *CYP21P–CYP21P* | asn+asn | asn+asn | leu+val | ins+ins | gln+gln | trp+trp |
| 28 | *CYP21P–CYP21P* | asn+ile | asn+ile | leu+val | ins+no ins | stop+gln | trp+arg |
| 38 | *CYP21P–CYP21P* | asn+asn | asn+asn | leu+leu | ins+ins | gln+... | arg+arg |

Consensus sequences (top) are according to Higashi *et al*.[2] The sequence upstream of codon 172 matched the consensus *CYP21P* sequence in all cases (*Taq*I site in 5' flank at nucleotide –211; *Eco*RI site in intron 2; no *Pvu*II site in intron 2; 'G' at nucleotide 655 (splice junction mutation site), 8 bp deletion in exon 3); ...: both alternatives mentioned are possible: not counted in Table 4. Middle section: *CYP21* deletion, bottom: *CYP21* to *CYP21P* conversion. [a]mutation cluster in exon 6; asn: asn glu gly lys; ile: ile val glu met; lys: lys glu glu lys; ...: asn glu glu lys or ile val glu met; [b]306–307: location of the 1 bp insert (T) in exon 7.

losing steroid 21-hydroxylase deficiency can be detected by analysis of the mutations described here, is about 95% (Table 3). This degree of certainty is unlikely to become much higher by checking additional mutations because *de novo* events are relatively frequent (in our population, twice in 40 families). In all patients described here, a genetic defect was found on at least one of both *CYP21* alleles. Non-detection of both mutations in a single patient is unlikely, so it may indicate clinical misdiagnosis of steroid 21-hydroxylase deficiency.

### Variability and evolution of *CYP21P*

*CYP21P* is constant in its 5' section up to and including the 8 bp deletion in exon 3, but shows considerable variation in its central and 3' parts even on 'standard' chromosomes carrying one copy of *CYP21* and *CYP21P* each. The evolutionary origin of *CYP21P* variability is uncertain. The concept of 'back conversions' has been proposed to introduce *CYP21*-like sequences into *CYP21P*.[44] A *de novo* mutation introducing ile val glu met236 into *CYP21P* (not previously described in the literature) was observed in one 21-hydroxylase deficient patient, so 'back conversions' evidently play a role in maintaining CYP21P variability.

It is assumed that the duplicated arrangement of the *CYP21-C4-TNX* gene cluster has resulted from unequal crossover events that occurred independently in primates and other mammals during evolution.[4,5,45,46] Therefore, *CYP21*-like sequences in the pseudogene may also be a leftover predating such a duplication. Comparison of primate *CYP21P* genes[45] suggests that after the duplication, deleterious mutations gradually accumulated in the gene now known as *CYP21P*. The splice junction mutation in intron 2 (found in the gorilla) was first introduced into the pseudogene, the 8 bp deletion in exon 3 (found in the chimpanzee) came next, and other mutations (only found in humans) followed later. The T insertion in exon 7 seems to be the next in line, because it is found in all *CYP21P* genes on normal (*CYP21–CYP21P*) chromosomes. Two previous studies[18,38] found no variation at this site on the *CYP21–CYP21P* haplotype. The evolution of *CYP21P* may be further clarified by determining whether the T insert is ubiquitous in other human (especially, non-Western) populations as well.

CYP21P variability may influence the outcome of small-scale gene conversions, because the physical conversion zone may extend beyond the region of similarity between a mutated gene and the *CYP21P* consensus sequence. In addition, *CYP21P* variability may contribute to the creation

of 'mosaic' genes with multiple apparent *CYP21/CYP21P* transitions.

**Mechanisms of small-scale gene conversions**

By analogy to fungal genetics, the term gene conversion is used in higher eukaryotes to describe a non-reciprocal transfer of genetic information. The presence of *CYP21P*-like defects in *CYP21* is usually attributed to a gene conversion-like mechanism, although donor and recipient can only be studied directly in case of a *de novo* event. Collier *et al*[24] described a *de novo* ile172 → asn conversion from *CYP21P* to *CYP21*; all *CYP21P* genes involved carried asn172. The opposite, a *de novo* conversion from *CYP21* to *CYP21P* described in this report, was also non-reciprocal: asn glu-glu lys236 was changed to ile val glu met, while all *CYP21* genes retained ile val glu met. The necessary interaction between *CYP21P* and *CYP21* may occur either between sister chromatids or between homologous chromosomes, but the distinction could not be made in the two *de novo* conversions mentioned above, because all potential donor sequences were identical. Since small-scale gene conversions are primarily mitotic processes,[25] sister chromatid interaction might be expected to provide the donor sequence. Four of the chromosomes studied here carried a *CYP21* defect not found in the *CYP21P* gene on the same chromosome. In these cases, *CYP21P* matched the consensus non-mutated *CYP21* sequence at the sites involved, and cannot have provided the donor sequence for the *CYP21* conversion. This implies that at least some of the small-scale gene conversions involve interactions between homologous chromosomes rather than between identical sister chromatids.

***CYP21P–CYP21* transition zones in hybrid genes**

Absence of the *CYP21* gene (as judged by *Taq*I/*Bgl*II restriction analysis) is one of the major causes of steroid 21-hydroxylase deficiency. Allowing for rare exceptions,[16] there are two main categories of chromosomes without a *CYP21* gene: those with one, and those with two *CYP21P* genes. Chromosomes with one *CYP21P* gene (*CYP21* deletion; Table 5, middle) carry a single hybrid gene that has a *CYP21P*-like 5' section and a *CYP21*-like 3' section. Differences between the *CYP21* and *CYP21P* consensus sequences (Table 5, top) have frequently been used to define a putative *CYP21P–CYP21* transition zone, and thus to determine the breakpoint of the unequal crossover that created these hybrids during the course of evolution. However, several of such landmarks (val281, gln318, arg356, and to some degree even ile172 and ile val glu met236) are not necessarily specific for *CYP21* on normal chromosomes, but appear in *CYP21P* as well (Table 4). This causes ambiguity in locating the 5' limit of *CYP21P–CYP21* transition areas (unless a *de novo* mutation is available for analysis).

Two crossover regions have been proposed for hybrid genes: (a) between the 8 bp deletion in exon 3 and the ile172 → asn site in exon 4, and (b) between the end of exon 7 and the gln318 → stop site in exon 8.[23,35,47] In our patient group, the breakpoint lies in zone (a) in the families 8, 9, and 17 (see Table 5). Zone (a) extends further upstream, because it has been shown that *de novo* recombinations can also occur upstream of the 8 bp deletion in exon 3.[25] In the other 12 families with the 'deletion' haplotype, the breakpoint cannot lie immediately downstream of exon 7, because several of the hybrid genes carry either stop-318 or trp-356 instead of the expected *CYP21*-like sequence. We therefore hypothesise that the second putative conversion zone (b) lies downstream of the arg356 → trp site (see Figure 2), and that the differences between these haplotypes can be explained by variation between the *CYP21P* genes involved in the crossover. This model also explains the variability at the other sites listed in Table 5, and the differences at the val281 → leu and gln318 → stop sites previously found in the analysis of several HLA-B47 haplotypes.[35,47] However, the alternative of later introduction of these mutations in a pre-existing HLA-B47 associated hybrid gene cannot be excluded. A reliable marker for distinguishing between conversion zone (a) and conversion zone (b) in hybrid genes, is the absence (zone a) or presence (zone b) of the T insert in exon 7.

Chromosomes with two *CYP21P* genes ('large-scale conversions'; Table 5, bottom) carry one regular *CYP21P* gene and one *CYP21P–CYP21* hybrid. The transition zones in the hybrid gene are the same as for the 'single' hybrid genes described above: zone (a) in families 3, 14 (one of the two chromosomes), and 28, and zone (b) in the other cases.

Tusié-Luna and White[25] have found that *de novo* unequal crossovers leading to *CYP21* deletions occur during meiosis only. However, this also applies to apparent 'large-scale conversions', because the hybrid genes found in *CYP21* deletions and in large-scale conversions are so similar (see Table 5) that the PCR amplification used in that study does not distinguish between them.

Since *CYP21* 'deletions' and 'large scale' conversions are both meiotic and result in a very similar layout of the *CYP21P–CYP21* hybrid gene, they are probably created by similar processes. We therefore hypothesise that large-scale gene conversions of *CYP21* to a *CYP21–CYP21P* hybrid are actually meiotic double unequal crossovers. One putative crossover site lies within region (a) or (b) as shown in Figure 2, the other one lies upstream of the *Taq*I restriction site at nucleotide –211 in the 5' flank. Interestingly, the other product expected from such an event, a chromosome with two *CYP21*-like genes and no *CYP21P*-like genes, was found in three healthy controls. Contrary to the single unequal crossovers that lead to deletions and duplications of *CYP21* or *CYP21P*,[1,5,6,9,23] the proposed double unequal crossover does not alter the number of *C4-CYP21-TNX* units or the overall size of the region.

## Conclusions

Our mutation analysis detects 95% of all defects causing salt-losing steroid 21-hydroxylase deficiency in The Netherlands, and hence provides a guideline for the diagnosis of the most severe form of this disease in the Dutch population.

*CYP21P* shows a high variability, and non-consensus *CYP21P* genes are widespread in the general population. This necessitates a revised definition of *CYP21P–CYP21* transition zones in gene conversions and unequal crossovers generating *CYP21* defects.

At least some of the small-scale gene conversions between *CYP21* and *CYP21P* involve interaction between homologous chromosomes. Large-scale gene conversions appear to be the result of a completely different genetic mechanism: these rearrangements are probably generated by a meiotic double unequal crossover.

Future research on the genetic mechanisms underlying the variability in the *CYP21*/complement *C4* region should take account of the high variability of the *CYP21P* pseudogene. This notably applies to the study of *de novo* mutations, where information on donor and recipient DNA can be obtained more easily than in population studies.

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