Multiplex Minisequencing of the 21-Hydroxylase Gene as a Rapid Strategy to Confirm Congenital Adrenal Hyperplasia

Nils Krone,1 Andreas Braun,2 Stefanie Weinert,1 Michael Peter,1 Adelbert A. Roscher,3 Carl-Joachim Partsch,1 and Wolfgang G. Sippell1*

Background: Congenital adrenal hyperplasia (CAH) is a frequent autosomal recessive disease, with a wide range of clinical manifestations, most commonly attributable to mutations in the 21-hydroxylase gene (CYP21). Large gene deletions, large gene conversions, a small 8-basepair deletion, and eight point mutations in CYP21 account for ~95% of all enzyme deficiencies. We developed a new strategy for a rapid CYP21 analysis.

Methods: DNA samples from 40 CAH patients previously genotyped by direct DNA sequencing were reanalyzed by allele-specific amplification of the functional CYP21 gene followed by a multiplex minisequencing reaction using 13 primers. In addition, a second PCR that amplified a part of exon 3 was used to demonstrate the presence or absence of at least one functional gene.

Results: The assay detected the P453S mutation and nine of the most common mutations (P30L, intron 2 splice, Δ8bp, I172N, exon 6 cluster, V281L, F306t, Q318X, and R356W) caused by microconversions from the CYP21P pseudogene. The concordance was 100% for detecting these mutations, including gene deletions and large gene conversions. The 40 patient DNA samples were analyzed in 1.5 working days by one technician (actual hands-on time, 3.5 h). The material cost for analyzing one sample was approximately €10.00 (US $9.00).

Conclusions: This novel mutation screening strategy rapidly detects 90–95% of all mutations associated with CAH and appears applicable as a tool for confirmation of increased 17-hydroxyprogesterone found in neonatal CAH screening.

© 2002 American Association for Clinical Chemistry

Congenital adrenal hyperplasia (CAH)4 is one of the most common inborn errors of metabolism. This autosomal recessive disease is caused by the loss of or severely decreased activity of one of the five steroidogenic enzymes necessary for cortisol biosynthesis. Steroid 21-hydroxylase deficiency accounts for 90–95% of CAH cases. In addition to decreased cortisol production, aldosterone biosynthesis may be impaired. The syndrome can be classified into classic and nonclassic forms. Classic CAH occurs in a frequency of 1 in 7000 to 15 000 live births in most populations and presents phenotypically as the simple virilizing or the salt-wasting form. Classic CAH occurs in a frequency of 1 in 7000 to 15 000 live births in most populations and presents phenotypically as the simple virilizing or the salt-wasting form. The simple virilizing form is characterized by virilization of the external genitalia in female newborns and by hypoglycemia and precocious pseudopuberty in both sexes, caused by adrenal androgen overproduction. In the salt-wasting form, severe renal salt loss occurs as a consequence of aldosterone deficiency. The less severe nonclassic form is estimated to occur in a frequency of 1 in 1000 in various Caucasian populations and manifests predominantly in female patients with precocious pseudopuberty and/or hirsutism and decreased fertility (1–3).

The 21-hydroxylase gene (CYP21) and a nonfunctional pseudogene (CYP21P) are located on the short arm of chromosome 6 (6p21.3). CYP21 and CYP21P each consist of 10 exons, and they share a high homology with a

1 Laboratory of Molecular Endocrinology, Division of Pediatric Endocrinology, Department of Pediatrics, Universitätsklinikum Kiel, Schwanenweg 20, D-24105 Kiel, Germany.
2 Sequenom Inc., 3595 John Hopkins Court, San Diego, CA 92121-1331.
3 Department of Clinical Chemistry, Metabolites and Molecular Genetics, University Children's Hospital, Ludwig-Maximilians-Universität, München, Lindwurmstrasse 4, D-80337 Munich, Germany.

*Address correspondence to this author at: Division of Pediatric Endocrinology, Department of Pediatrics, Universitätsklinikum Kiel, Schwanenweg 20, D-24105 Kiel, Germany. Fax 49-431-597-1675; e-mail sippell@pediatrics.uni-kiel.de.

Received January 25, 2002; accepted March 22, 2002.

4 Nonstandard abbreviations: CAH, congenital adrenal hyperplasia; bp, basepair; and 17OHP, 17-hydroxyprogesterone.
nucleotide identity of 98% in their exons and 96% in their intron sequences (4, 5).

In most cases, inactivating CYP21 mutations are generated by unequal crossing-over or gene conversion events. The most frequent mutations originating from this mechanism are complete gene deletions, large gene conversions, eight point mutations, and an 8-basepair deletion (Δ8bp) (2). The inactivating point mutations are transferred by microconversions from the inactive CYP21P to the active CYP21 gene. Novel mutations and de novo mutations, which are not present in the pseudogene, are rare and occur in ~1-3% of the alleles in large studies (6).

Studies establishing mutation groups of different severity demonstrated a good correlation between CYP21 genotype and clinically expressed CAH phenotype (7, 8). Calculation of positive predictive values for each mutation group was shown to improve phenotype prediction (9).

Because CAH fulfills international criteria for newborn screening, determination of 17-hydroxyprogesterone (17OHP) in filter-paper blood spots was introduced to neonatal screening programs in many countries around the world (10). Previous shortcomings regarding the precision of this screening method were greatly decreased by relating 17OHP reference ranges to variables such as birth weight and gestational age (11, 12). Nevertheless, the rate of false-positive screening results is still a major problem. The resulting need for hormonal retesting and refined steroid assays (with extraction) leads to extra costs and causes unnecessary uncertainty for families and primary care physicians.

Genotyping has been described as a diagnostic component in comprehensive neonatal screening for CAH (13, 14). Several methods for mutation analysis of the CYP21 gene have been published, including allele-specific oligonucleotide hybridization (15–17), amplification-created restriction sites (18, 19), single-stranded conformation polymorphism analysis (20, 21), allele-specific PCR (22), the ligation detection reaction (23), and direct DNA sequencing (24–26). Some of these can be used for mutation screening in 21-hydroxylase deficiency.

In this study, we describe a rapid screening procedure that uses PCR, followed by a 13-plex minisequencing reaction to test for the 10 most common mutations that lead to 21-hydroxylase deficiency. This strategy complements 17OHP screening and hormonal retesting and could be performed as an independent second-step analysis of positive screening results for 21-hydroxylase deficiency.

**Materials and Methods**

**PATIENTS AND DNA PREPARATION**

Peripheral venous blood samples were collected from 40 CAH patients diagnosed at the Division of Paediatric Endocrinology, Department of Paediatrics, Christian-Albrechts-Universität, Kiel, Germany. Hormone measure-

<table>
<thead>
<tr>
<th>Table 1. Distribution of genotypes in the 40 CAH patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation group*</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>C</td>
</tr>
</tbody>
</table>

* Mutation groups according to Speiser et al. (7), Wedell et al. (8), and Krone et al. (9).

* Patients 1146, 647, 1065, and 1011 were typed only with the 8-bp deletion PCR.

* SW, salt-wasting form; SV, simple virilizing form; NC, nonclassic form.

* del, gene deletion; conv, large gene conversion. Results were obtained by Southern blotting (26). The allele frequencies shown are not comparable to population-based frequencies.
hormonal diagnosis of 21-hydroxylase deficiency. The genomic DNA preparation was carried out using the QIAamp DNA Blood Mini Kit (Qiagen). Table 1 shows the distribution of genotypes in the 40 CAH patients.

8-bp deletion test

The PCR was performed in a total volume of 50 µL using 45 µL of PCR Supermix (Life Technologies), 10 pmol each of primers 8-bp-F (5'-6-carboxyfluorescein-CCTGCAGACAGCTGGTGTC-3'), 318 F (5'-CACAGAACTCCTGGGTCAGC-3'), and 200 ng of genomic DNA. PCR was performed as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 53 °C for 45 s, and 72 °C for 1 min, with a final extension at 94 °C for 40 s, 53 °C for 45 s, and 72 °C for 5 min. The samples were separated on an ABI PRISM 310 Sequencer and analyzed using ABI GeneScan 3.1 software (Applied Biosystems). Size determinations were performed using the GeneScan™-350 size calibrator and the local Southern method, which is integrated in the GeneScan 3.1 software.

PCR amplification

The Expand High Fidelity PCR System (Roche) was used for allele-specific hot-start PCR amplification of the active CYP21 gene. Reactions were carried out in a volume of 100 µL containing 10 µL of 10× PCR reaction buffer 3, 2.5 mM/µL MgCl₂, 2.5 U of DNA polymerase mixture, 400 µM each deoxynucleotide triphosphate, 10 pmol each of primers 8-bp-F (5'-CGGTTCCGGAGGGAATGTA-3') and CYP21-R (5'-GCGATTCGAGCAGGACTGTTGAGT-3'), and 200 ng of genomic DNA. Cycling conditions for PCR were as follows: initial denaturation at 94 °C for 4 min; 14 cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 6 min, followed by 18 cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 6 min plus cycle elongation of 2 s for each cycle; and a final extension at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 15 min. The CYP21P amplicons were synthesized under the same conditions using the allele-specific primers CYP21P-F (5'-GTGGTCCGGGAGGGAATGTA-3') and CYP21P-R (5'-CGATTCGAGCAGGACTGTTGAGT-3').

MULTIPLE MINISEQUENCING

The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). The multiplex minisequencing reaction was carried out in a total volume of 6 µL containing 2.5 µL of SNaPshot Multiplex Ready Reaction reagent (Applied Biosystems), 2.5 µL of primer mixture (5 pmol of each primer; see Table 2), and 1 µL of purified PCR product. The SNaPshot Multiplex Ready Reaction reagent set contains AmpliTaq DNA polymerase, fluorescently labeled dideoxynucleotide triphosphates, and reaction buffer. The multiplex minisequencing was performed as follows: 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. After extension, the samples were treated with shrimp alkaline phosphatase according to the manufacturer's protocol. The samples were electrophoresed on an automated ABI PRISM 310 Sequencer and analyzed.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Sequence in 5'→3' direction</th>
<th>5' Site</th>
<th>Sense/Antisense</th>
<th>Color, size, and nucleotide of primer peaks</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>P30L</td>
<td>P30L-F</td>
<td>TCC GGA GCC TCC ACC TCC</td>
<td>71</td>
<td>+</td>
<td>black 24.7 C</td>
<td>red 26.9 T</td>
</tr>
<tr>
<td>I2 G</td>
<td>I2 G-R</td>
<td>TCC GCT TGT CAG GAG GAG</td>
<td>675</td>
<td>-</td>
<td>red blue 32.9 31.4 A C</td>
<td>black 30.8 G</td>
</tr>
<tr>
<td>I172N</td>
<td>I172N-R-T11</td>
<td>TGGCT AGG TGA GGT AAC CCA</td>
<td>1019</td>
<td>-</td>
<td>green 36.4 T</td>
<td>red 36.1 A</td>
</tr>
<tr>
<td>I235N</td>
<td>I235N-F-T40</td>
<td>TGGCT AGA AGA AGG AGA ACA A</td>
<td>1359</td>
<td>+</td>
<td>red 66.6 T</td>
<td>green 63.0 A</td>
</tr>
<tr>
<td>V236E</td>
<td>V236E-R-T40_W</td>
<td>TGGCT ACC TCA GCT AGC W TCT EC</td>
<td>1403</td>
<td>-</td>
<td>green 67.8 T</td>
<td>red 88.8 A</td>
</tr>
<tr>
<td>M238K</td>
<td>M238K-R-T48</td>
<td>TGGCT TGT GCC TCA GCT GCT</td>
<td>1409</td>
<td>-</td>
<td>green 69.6 T</td>
<td>red 70.9 A</td>
</tr>
<tr>
<td>V281L</td>
<td>V281L-F-T17</td>
<td>TGGCT AGG CAG CTC CGG GAG CAC</td>
<td>1682</td>
<td>+</td>
<td>blue 40.4 G</td>
<td>red 41.8 T</td>
</tr>
<tr>
<td>Q318X</td>
<td>Q318X-F-T25</td>
<td>TGGCT AGC ATT CAG CAG CAG CTG</td>
<td>1973</td>
<td>+</td>
<td>blue 45.7 G</td>
<td>red 50.2 T</td>
</tr>
<tr>
<td>R356W</td>
<td>R356W-F-T32</td>
<td>TGGCT ATC GAG GAG GTG GCT GCT GCT</td>
<td>2087</td>
<td>+</td>
<td>blue 54.8 G</td>
<td>red 57.3 T</td>
</tr>
<tr>
<td>F306+1</td>
<td>F306+1-F-T48</td>
<td>TGGCT ATC CAG ACT CTC CTC GGC GCT GGT TTT TTT</td>
<td>1736</td>
<td>+</td>
<td>blue 78.7 G</td>
<td>red 79.7 T</td>
</tr>
<tr>
<td>Δ8bp</td>
<td>Δ8bp-F-T82_S</td>
<td>TGGCT ATC CAG ATT CAG CAG CAG CTG</td>
<td>687</td>
<td>+</td>
<td>blue 84.3 G</td>
<td>red 86.8 T</td>
</tr>
<tr>
<td>Δ8bp-R-T86_R</td>
<td>TGGCT ATC CAG ATT CAG CAG CAG CTG</td>
<td>733</td>
<td>-</td>
<td>blue 88.7 G</td>
<td>black 88.6 G</td>
<td></td>
</tr>
<tr>
<td>P453S</td>
<td>P453S-F-T71</td>
<td>TGGCT AGC ATT CAG CAG CAG CTG</td>
<td>2557</td>
<td>+</td>
<td>blue 94.6 C</td>
<td>red 96.0 T</td>
</tr>
</tbody>
</table>

* Oligonucleotide primers were synthesized by Metabion (Martinsried, Germany).
* T(n) indicates the number of thymidine nucleotides of the poly(T) track elongating the specific primer sequence. Bases highlighted in yellow represent wobble bases (Y = C or T; W = A or T; S = G or C; R = A or G).
* Specific 5' primer binding site is given according to Higashi et al. (5).
* The primer peak sizes represent the mean value of the detected fragment length at this position.
with the ABI GeneScan 3.1 software. Size determinations were performed using the GeneScan-120 LIZ size calibrator and the local Southern method, which is integrated in the GeneScan 3.1 software.

**Results**

**8-bp deletion test PCR**

The 8-bp deletion test PCR discloses all mutant alleles leading to a null genotype, such as gene deletions, large conversions, and the 8-bp deletion in exon 3 in homozygous or compound heterozygous states. A fragment containing a part of exon 3 spanning the 8-bp deletion was amplified from genomic DNA. In the presence of at least one active CYP21 gene, two fragments (150 and 158 bp) were amplified (Fig. 1A). A 150-bp fragment was amplified in those cases where only genes bearing an 8-bp deletion were present, e.g., CYP21 gene with an 8-bp deletion and CYP21P pseudogene (Fig. 1B). All samples previously analyzed by Southern blotting could be unequivocally typed by this method (data not shown).

**PCR amplification of the CYP21 gene and multiplex minisequencing**

To detect point mutations in the CYP21 gene, an allelespecific PCR was performed, followed by a highly multiplexed minisequencing reaction. In this step, the 10 most common mutations were detected by single nucleotide extension of primers annealing directly adjacent to the nucleotide of interest. The sizes of gene-specific primer sequences ranged from 18 to 27 nucleotides. These primers were then synthetically elongated at their 5’ end with a poly(T) track, which varied in size to facilitate electrophoretic separation of diagnostic products.

Table 2 summarizes the various primer features and the potential reaction results after extension of primers with a fluorescently labeled dideoxynucleotide triphosphate mixture depending on the corresponding base of the template. All 13 primers were used in one multiplex reaction. A wild-type analysis can produce 13 or 14 peaks because a benign A/C polymorphism exists in addition to the disease-causing intron 2 splice site mutation (I2 G).

Fig. 2B shows the analysis of a CYP21 wild-type sample that is heterozygous for this A/C polymorphism. To demonstrate that all mutations can be detected in a heterozygous state, we analyzed a mixture of two PCR products. One fragment was obtained using CYP21-specific oligonucleotides, and the other one was derived using primers specific for the CYP21P pseudogene (Fig. 2C). Fig. 2D shows the pattern of peaks for all mutant positions derived after exclusive amplification of the pseudogene. The mutation P453S is not present in the pseudogene but occurs in ~1–2% of disease-causing alleles.

In the case of the P30L mutation, a wild-type allele (nucleotide, C) produced a black peak (see Fig. 2B, first primer peak position, and Table 2). In contrast, in the heterozygous state (nucleotides, C and T), a black and a red peak were detected (Fig. 2C and Table 2), whereas a homozygous P30L mutation showed a red peak (nucleotide, T; Fig. 2D and Table 2). In the case of the I2 G minisequencing reaction, the wild-type allele can produce a red (nucleotide, A) and/or a blue (nucleotide, C) primer peak (Fig. 2B and Table 2) according to the polymorphic status of this position. A heterozygous point mutation showed a red and a black peak (nucleotides, A and G) or a blue and a black peak (nucleotides, C and G; Table 2). In the homozygous state of an intron 2 splice site mutation (nucleotide, G), a black peak was detected (Fig. 2D and Table 2). The pattern in Fig. 2C demonstrates the detection of an “artificial” heterozygous state when a mixture of all possible alleles was analyzed using amplification products derived from the functional gene as well as the pseudogene. The remaining eight mutations can be clearly detected and interpreted.

Analysis of the 8-bp deletion in exon 3 was also
Fig. 2. GeneScan analysis of the multiplex minisequencing reaction.

(A), the *CYP21* gene shown schematically with the nine most common mutations, transferred by apparent gene conversions from the *CYP21P* pseudogene. The P453S mutation is not present in the pseudogene, but occurs in ~1−2% of mutant alleles. (B), *CYP21* wild-type gene with heterozygosity for the A/C polymorphism at the intron 2 splice site (I2 G) position. (C), mixture of *CYP21* and *CYP21P* gene fragments demonstrating the detection of heterozygous mutations at every peak position. (D), *CYP21P* pseudogene amplicon with all common *CYP21*-inactivating mutations, demonstrating the detection of all mutations in a homozygous state.
performed in the sense and the antisense direction. When a deletion was present, the detected base was located 9 bp upstream or downstream, respectively, compared with the wild-type sequence. The wild type was indicated by two blue peaks (Fig. 2B and Table 2), whereas a red and a black peak were detected when a patient carried the 8-bp deletion (Fig. 2D and Table 2). In the presence of a heterozygous state, two blue peaks, a red peak, and a black peak were detected, as shown in Fig. 2C.

DNA samples from 40 CAH patients were analyzed by this approach. Direct DNA sequencing of the complete intron and exon regions had been performed previously. According to the pattern demonstrated in Fig. 2, all genotypes could be confirmed, demonstrating the reliability of this diagnostic procedure. The reproducibility of the minisequencing analysis was confirmed by repetitive analysis with subsequent identical results.

**PRACTICABILITY AND COSTS**

The time required for the entire laboratory procedure was ≤1.5 working days. Forty samples could be analyzed simultaneously by one technician. Five samples could be analyzed within 8 h with an actual working time of 3.5 h. The material costs per patient sample were approximately €10.00 (US $9.00).

**Discussion**

In this study we describe a rapid and comprehensive procedure for mutation screening in 21-hydroxylase deficiency. Analysis of the CYP21 gene still presents a challenge because of the presence of a highly homologous CYP21P gene; for this reason, selective CYP21 gene amplification and mutation detection procedures have been developed within the last few years (23, 26, 28, 29). Major methodologic advances offer improved overall diagnostic sensitivity, speed, and reduced laboratory and material costs.

In the present study, a rapid 8-bp deletion PCR-based test was designed to exclude large deletions, large gene conversions, and the 8-bp deletion in homozygous or compound heterozygous states. However, with this strategy, analysis of peak heights and/or peak areas did not enable the detection of complex rearrangements or heterozygous gene deletions. For this, time-consuming Southern blot analyses of the CYP21 locus are still necessary (26, 30). For example, back-conversion of CYP21 to CYP21P may lead to difficulties in interpreting the results of the 8-bp deletion PCR if the 8-bp sequence is transferred to the pseudogene. This would produce an 8-bp deletion PCR fragment pattern, suggesting the existence of at least one CYP21 gene (Fig. 1A). In this rare case, the allele-specific CYP21 gene amplification would fail and multiplex minisequencing of the CYP21P pseudogene would be needed. This would reveal the presence of the 8-bp sequence in the homozygous or heterozygous state in the CYP21P pseudogene. Overall, back-mutation of the 8-bp sequence is not a frequent occurrence, in contrast to the back-conversions of exons 7 and 8, which have been described as common events (23).

The 13-plex minisequencing assay developed here requires only one reaction per DNA or patient sample. The subsequent interpretation of peak patterns is simple and can be automated. The use of a size calibrator assures reproducibility between various analytical runs and, in addition, facilitates automated allele calling. However, the electrophoretic mobility of such small diagnostic extension products is strongly influenced not only by their total length, but also by their base composition and by the fluorescent label used. Therefore, allele calling by measuring the length of the product only cannot be achieved.

The multiplex minisequencing technique enormously increases both the practicability and handling in routine laboratory work. In this respect the minisequencing strategy is comparable to approaches using ligase-mediated mutation detection schemes (23). However, only one mutation detection primer is needed, rather than two.

All nine common mutations (P20L, intron 2 splice, Δ8bp, I172N, exon 6 cluster, V281L, F306+t, Q318X, and R356W) and the P453S point mutation yielded clearly distinct peaks that allowed easy detection of wild-type and mutant alleles in homozygous as well as in heterozygous states. All patients previously genotyped by direct DNA sequencing showed concordant results in the minisequencing strategy. The expected diagnostic sensitivity for CYP21 mutations in a Caucasian cohort is 92–97%, with a rate of uncommon mutations of 1–3% and of unrevealed mutations of 2–5%, as described in several large studies (7–9).

In the case of compound heterozygosity with gene deletions/large gene conversions and point mutations, the “exact” genotype cannot be obtained with this minisequencing procedure, as with all strategies that do not include Southern blot analysis (26, 30). Therefore, in a few cases our approach, although of potentially high diagnostic sensitivity, may need additional steps for definitive molecular confirmation of CAH attributable to 21-hydroxylase deficiency.

A major issue in mutation analysis of the CYP21 gene is the phenomenon of allele drop-out (23, 31). This phenomenon should be considered whenever the results of clinical, biochemical, and genetic data are discordant. Investigating the genetic status of both parents is particularly important to clarify this artifact (32). Furthermore, the segregation of linked microsatellite markers and characterization of frequent single-nucleotide polymorphisms located in the CYP21 gene can provide further information (14, 31, 33). The analysis of chimeric CYP21P/CYP21 genes as described by Lee et al. (34) can be helpful in the identification and further elucidation of this phenomenon.

The described analytical strategy not only allows classification into mutation groups (7, 8), but also phenotype prediction by calculating positive predictive values (9).
This diagnostic improvement makes minisequencing for mutation detection a valuable new tool to supplement hormone testing in prenatal diagnosis of 21-hydroxylase deficiency and CAH newborn screening when rapid molecular genetic analysis of the CYP21 gene is desired. Confirmation and segregation analysis based on the parents' genotypes can also be performed rapidly with this method. This should be conducted whenever possible, particularly in view of the existence of alleles carrying multiple common mutations (2, 6, 9). The presence of multiple mutations in cis, which are the result of a large gene conversion, or in trans can not be clearly differentiated when only the patient's DNA is analyzed. Therefore, the genotypes of both parents must be completely determined to correctly define the genetic status of suspected patients (32).

Although the benefit of CAH screening by 17OHP detection methods is well documented, the rate of false-positive screening results caused by neonatal stress, immature adrenal function, or increased concentrations of cross-reacting fetocortical steroid metabolites in premature babies still represents a major problem. To overcome these shortcomings, gestational age or birth-weight-adjusted cutoff values have been introduced (11, 12, 35).

The general value of genotyping as a complementary method for confirming or discarding the diagnosis of CAH subsequent to neonatal screening has already been demonstrated (13). The challenge to screening laboratories is to use the high specificity of CAH genotyping to improve 17OHP screening so that most of the otherwise necessary recalls can be avoided. As a model system in neonatal screening, the high specificity of molecular testing as a second-tier test is already successfully used to compensate for the low specificity of cystic fibrosis screening by immunoreactive trypsin determinations (36). In this context, the features of our minisequencing technique for molecular testing for CAH attributable to 21-hydroxylase deficiency, such as speed, reliability, and low cost, appear to make this test applicable as a second-tier approach for blood-spot samples with increased 17OHP results. Pending evaluation of such an approach in larger newborn cohorts, most cases of increased 17OHP in CAH screening may be clarified when only 17OHP measurements are used.

We appreciate the expert technical assistance of Gisela Hohmann and thank Joanna Voerste for linguistic help with the manuscript.

References
19. Oriola J, Plensa I, Machuca I, Pavia C, Rivera-Fillat F. Rapid


