Congenital adrenal hyperplasia (CAH) is a common autosomal recessive disorder caused mainly by defects in the steroid 21-hydroxylase gene (CYP21). The defective CYP21 genes in CAH fall into one of three categories: (a) small-scale conversions from CYP21P; (b) spontaneous mutations; and (c) chimeric RCCX modules that include the chimeric CYP21P/CYP21 gene (1) and the chimeric TNXATNXB gene (2–4). The RCCX module in chromosome 6p21.3 of the human MHC class III region is composed of a part of the RP gene (serine/threonine nucleotide protein kinase) (5), a full-complement C4 gene, a full CYP21(P) gene, and a portion of the TXN gene (6, 7). The C4 protein is coded by two genes, C4A and C4B. The occurrence of a long (20.4 kb) or short (14.1 kb) C4 gene is attributable to the presence of an endogenous retroviral sequence (6.7 kb), namely HERV-K (C4), in intron 9 (8). The TNX gene contains XA (TNXA) and TNXB. TNXB, in the downstream CYP21 gene, is partially duplicated in the downstream CYP21P gene, where a truncated gene is termed TNXA. Both TNXA and TNXB are transcribed on the opposite strand. There are two RP genes, RPI and RP2. The RP2 gene is truncated and corresponds to RPI adjacent to TNXA. These genes are arranged in the RPI-C4A-CYP21P-XA-RP2-C4B-CYP21P-TNXB gene sequence and are designated as the bimodule of RCCX (Fig. 1A).

The bimodule is composed of a long module including part of RPI, C4A (long), CYP21P, and TNXA, and a short module containing RP2, C4B (short), CYP21P, and part of the TNXB gene (2) (Fig. 1A). In Caucasians, the RCCX module has three possible forms: monomodule, bimodule, and trimodule; the bimodular form is the most frequently occurring form (9). To date, 75 different CYP21 mutations have been reported (10). Approximately 70% of cases of CYP21 mutations, including 15 mutations (10, 11), have been attributed to the intergenic recombination of DNA sequences from the highly homologous CYP21P pseudogene. The other 60 mutations (in ~10% of cases) are spontaneous. On the other hand, gross gene deletions of the ~30-kb genome, which leave behind the C4A and a single CYP21A-like gene, have been reported to occur in 20% of alleles in patients with CAH involving a 21-hydroxylase deficiency (12). Obviously, this kind of deletion (and/or conversion) is caused by loss of the -XA-RP2-C4B- gene array between these two RCCX modules. However, from recent studies, the consequence of such a gene deletion produces at least two different features of gene arrangement. One of them presents a -C4A-CYP21P/CYP21P-TNXB gene array, which produces four kinds of chimeric CYP21P/CYP21 genes (1, 13), whereas the other presents a -C4A-CYP21P-TNXA/TNXB array, which contains two kinds of chimeric TNXA/TNXB genes (2, 4).

Identification of such a 30-kb gross gene deletion (and/or conversion) in the RCCX region has traditionally required the Southern blot method with multiple isotopelabeled probes and separate restriction endonuclease digestion (14); TaqI generates 3.7- (functional) and 3.2-kb (pseudogene) fragments, whereas 2.5- (functional) and 2.0-kb (pseudogene) fragments are produced by double digestion with BglII/EcoRI. Although a nonisotopic Southern method has been used (15), the blocking procedure is still laborious. Furthermore, a heterozygous allele for the deleted CYP21 gene cannot be detected in the presence of a wild-type allele, and the results may be hard to interpret without a family study. It is possible that an allele with the trimodule CYP21P-XA-RP2-C4B-CYP21P-XA-RP2-C4B-CYP21P-TNXB may interfere with identification of a bimodule with the -XA-RP2-C4B- deletion. In such cases, densitometric screening of fragments is prone to errors and may produce a discordant diagnosis.

To circumvent these problems, we developed a long PCR product with allele-specific primers covering the 5′...
end of the CYP21P and/or the CYP21 and TNXB genes (Fig. 1A). The PCR product was subjected to TaqI and BglII/EcoRI digestions and analyzed by electrophoresis on an agarose gel. The samples used in development of the protocol were from two known CAH families. Both families provided informed consent.

For PCR amplification, genomic DNA, 2.0 U of Taq/ Pow DNA polymerase (Expand Long Template PCR System; Roche Diagnostics), primers (10 pmol each), deoxynucleotide triphosphates (250 μM each), and 10× PCR buffer (commercially supplied) were used for PCR amplification in an 80-μL reaction. The primers Tena30exF (5’-TGACAGATGCGACCCCTGACT-3’; nucleotides 77113–77133; GenBank accession no. AL049547) and 5-ENF (5’-CTCCATGACCCTACT-GTCTT-3’; nucleotides 88470–88450; GenBank accession no. AL049547; Fig. 1A) were used for amplification of the 11.357-bp PCR product (Fig. 1B, lane c) covering the 5’ end of nucleotide −1810 of the CYP21 gene and/or CYP21P and the TNXB gene of exon 31. According to GenBank (accession nos. AL049547 and AF019413) and past studies (16, 17), these two primers are specific for these two loci, and no polymorphic site exists.

From analysis of the 11.3-kb PCR product of TaqI digestion (Fig. 1B, lane c), we found a 3.7-kb fragment possessing the functional CYP21 gene and a 2.5-kb fragment containing a product of exon 45 to intron 36 of the TNXA and TNXB genes (1) in an apparently healthy individual (Fig. 1B, lane 1). A sample from carrier with a 30-kb deletion in one allele gave 3.7-, 2.5-, and 3.2-kb fragments (CYP21P; Fig. 1B, lane 2), whereas a sample from a CAH patient with two alleles containing a 30-kb deletion gave only 3.2- and 2.5-kb fragments (Fig. 1B, lane 3). After BglII/EcoRI double digestion, the sample from the healthy individual (Fig. 1B, lane 1) gave 8.1- (TNXB) and 2.6-kb fragments (the CYP21 gene, more commonly shown in the literature as being 2.5 kb long), whereas the sample from the carrier (Fig. 1B, lane 2) gave 8.1-, 2.6-, and 2.1-kb fragments (CYP21P, more commonly shown in the literature as being 2.0 kb long), and the sample from the patient (Fig. 1B, lane 3) gave the 8.1- and 2.1-kb fragments, but not the 2.6-kb fragment. Production of a 3.2-kb fragment by TaqI digestion (Fig. 1B, lane 2) and a 2.1-kb fragment by BglII/EcoRI double digestion (Fig. 1B, lane 5) resulted from the 5’-end sequence of CYP21P being replaced by a CYP21P-like sequence, which was caused by a 30-kb deletion of the -XA-RP2-C4B gene locus (Fig. 1A). In addition, the absence of the 3.7-kb fragment after TaqI digestion (Fig. 1B, lane 3) and the 2.6-kb fragment after BglII/EcoRI double digestion (Fig. 1B, lane 6) indicated that neither allele in the CAH patient carried the functional CYP21 gene, whereas they both carried the CYP21P-like gene. Obviously, most fragments produced by digestion with these restriction enzymes in the PCR product analysis (Fig. 1B) were also revealed by the conventional Southern blot analysis (Fig. 1A, bottom).

The allele with the heterozygous carrier defect in the single case (Fig. 1B, lane 2) could be directly verified by...
the restriction fragments. It is sufficient that the 3.2-kb fragment produced by TaqI analysis (Fig. 1B, lane 2) was used to verify deletion of the -XA-RP2-C4B gene locus in the RCCX region. It should be noted that the 2.5-kb fragment produced by TaqI may be replaced by a 2.4-kb fragment the case of the chimeric TNXA/TNXB (2), in which TNXB is replaced by TNXA, which extends to exon 36 beyond the 120-bp boundary. We therefore suggest that the use of TaqI for restriction enzyme digestion may produce a satisfactory result for diagnosing a 30-kb gross gene deletion (and/or gene conversion) in CAH. The use of EcoRI/BglIII double digestion does not seem to be absolutely necessary.

We also used PCR amplification products and amplification-created restriction site analysis (18) to analyze samples from 31 ethnic Chinese (i.e., Taiwanese; Table 1) CAH families with one chromosome carrying the 3.2-kb fragment produced by TaqI digestion. Members of 6 of these 31 (19%) CAH families carried the chimeric CYP21P/CYP21 gene (19), and members of 24 families (77%) carried the mutations IVS2−12A/C>G and 707−714delGAGACTAC (20), whereas only 1 CAH family (3%) carried a CYP21 deletion (4). Hence, we concluded that the 30-kb gene deletion producing the 3.2-kb fragment by TaqI digestion shows diversity and that mutations of IVS2−12 A/C and 707−714delGAGACTAC among the three CYP21 haplotypes are abundant and may be the most prevalent ones in ethnic Chinese CAH patients.

Our results indicate that PCR product analysis can reveal the continuity and entirety of fragments of analyzed gene loci and that these do not require densitometry for identification of the gene copy number or the use of family studies for interpreting the heterozygous defective allele. This procedure is saves time and is convenient and practical for the routine laboratory diagnosis of CAH patients.

The two CAH families that provided samples for demonstration of the PCR protocol were referred by Dr. Y.J. Lee from the Department of Pediatrics, Mackay Memorial Hospital, Taipei. This work was supported by the King Car Research Foundation from the King Car Food Industrial Co., Taiwan, Republic of China.

### Table 1. Frequency of the CYP21 gene with the 3.2-kb TaqI fragment in ethnic Chinese CAH patients.

<table>
<thead>
<tr>
<th>CYP21 with the 3.2-kb TaqI-produced fragment</th>
<th>Chromosomes</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS2−12A/C&gt;G combined with 707−714delGAGACTAC mutation</td>
<td>24</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Chimeric CYP21P/CYP21</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>CYP21 deletion</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>These six chromosomes include one with CH-1, two with CH-2, and three with CH-3. The designations CH-1, CH-2, and CH-3 are based on the studies of Lee et al. (1).

### References

 DOI: 10.1373/clinchem.2003.028597