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Research Article

Congenital adrenal hyperplasia (CAH) is caused by disorders of the P450c21B gene, which, with the P450c21A pseudogene, lies in the HLA locus on chromosome 6. The near identity of nucleotide sequences and endonuclease cleavage sites in these A and B loci makes genetic analysis of this disease difficult. We used a genomic DNA probe that detects the P450c21 genes (A pseudogene, 3.2 kb; B gene, 3.7 kb in Taq I digests) and the 3' flanking DNA not detected with cDNA probes (A pseudogene, 2.4 kb; B gene, 2.5 kb) to examine Southern blots of genomic DNA from 68 patients and 165 unaffected family members in 57 families with CAH. Of 116 CAH-bearing chromosomes, 114 could be sorted into five easily distinguished haplotypes based on blots of DNA digested with Taq I and Bgl II. Haplotype I (76 of 116, 65.6%) was indistinguishable from normal and therefore bore very small lesions, presumably point mutations. Haplotype II (4 of 116, 3.4%) and haplotype III (8 of 116, 6.9%) had deletions and duplications of the P450c21A pseudogene but had structurally intact P450c21B genes presumably bearing point mutations; point mutation thus was the genetic defect in 88 of 116 chromosomes (75.9%). Haplotypes IV and V lack the 3.7-kb Taq I band normally associated with the P450c21B gene. Haplotype IV (13 of 116, 11.2%) retains [...]

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Rearrangements and Point Mutations of P450c21 Genes Are Distinguished by Five Restriction Endonuclease Haplotypes Identified by a New Probing Strategy in 57 Families with Congenital Adrenal Hyperplasia

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Abstract

Congenital adrenal hyperplasia (CAH) is caused by disorders of the P450c21B gene, which, with the P450c21A pseudogene, lies in the HLA locus on chromosome 6. The near identity of nucleotide sequences and endonuclease cleavage sites in these A and B loci makes genetic analysis of this disease difficult. We used a genomic DNA probe that detects the P450c21 genes (A pseudogene, 3.2 kb; B gene, 3.7 kb in Taq I digests) and the 3' flanking DNA not detected with cDNA probes (A pseudogene, 2.4 kb; B gene, 2.5 kb) to examine Southern blots of genomic DNA from 68 patients and 165 unaffected family members in 57 families with CAH. Of 116 CAH-bearing chromosomes, 114 could be sorted into five easily distinguished haplotypes based on blots of DNA digested with Taq I and Bgl II. Haplotype I (76 of 116, 65.6%) was indistinguishable from normal and therefore bore very small lesions, presumably point mutations. Haplotype II (4 of 116, 3.4%) and haplotype III (8 of 116, 6.9%) had deletions and duplications of the P450c21A pseudogene but had structurally intact P450c21B genes presumably bearing point mutations; point mutation thus was the genetic defect in 88 of 116 chromosomes (75.9%). Haplotypes IV and V lack the 3.7-kb Taq I band normally associated with the P450c21B gene. Haplotype IV (13 of 116, 11.2%) retains all other bands, indicating that the P450c21B gene has undergone a gene conversion event, so that it is now also associated with a 3.2-kb band. Haplotype V (13 of 116, 11.2%) lacks the 2.4-kb Taq I fragment and the 12-kb Bgl II fragments normally associated with the P450c21A pseudogene, as well as lacking the 3.7-kb Taq I fragment, indicating deletion of ~ 30 kb of DNA, resulting in a single hybrid P450c21A/B gene. Most (114 of 116, 98%) CAH al-

les thus can easily be classified with this new probing strategy, eliminating many ambiguities resulting from probing with cDNA.

Introduction

Congenital adrenal hyperplasia (CAH)¹ due to deficient 21-hydroxylase activity is an autosomal recessive disorder linked to the MHC locus on the short arm of chromosome 6 (1, 2). 21-hydroxylase activity is mediated by P450c21, a microsomal cytochrome P450 enzyme (for review, see reference 3). Two highly homologous genes encoding P450c21, formally termed P450XXIA1 and P450XXIA2 (4) and commonly termed P450c21A and P450c21B, are linked in tandem with C4A and C4B, the genes encoding the fourth component of complement (5, 6). Only the P450c21B gene is active; P450c21A is an inactive pseudogene due to an 8-base deletion causing a frame shift plus a point mutation creating a stop codon (7-9). The order and transcriptional orientations of these genes have been established (2, 10). The P450c21 genes are ~ 3.4 kb long and contain 10 exons (7, 8, 11). A region of ~ 35 kb encompassing a C4 gene and a P450c21 gene has been duplicated in evolution, resulting in this tandem array of C4 and P450c21 genes in mice (12), probably cattle (13), and human beings (5, 6). The two human P450c21 genes have 98% nucleotide identity (7-9), even though this duplication occurred before mammalian speciation; thus, the two loci are evolving in tandem (concerted evolution) (14, 15). This extremely high degree of nucleotide identity thus indicates extremely frequent crossover events, and makes genetic distinction of the loci difficult (16).

Some restriction enzymes can distinguish the two P450c21 genes in normal persons. The P450c21A pseudogene is characterized by 3.2-kb Taq I and 12-kb Bgl II fragments, whereas the functional P450c21B gene is characterized by 3.7-kb Taq I and 11-kb Bgl II fragments. A deletion of the P450c21B gene, evidenced by an absent 3.7-kb Taq I fragment, was reported in a patient homozygous for HLA-A3, -B47, -DR7, C4A91 C4BQ0 (17). In hormonally normal individuals, including homozygotes for HLA-A1, -B8, -DR3, C4A90 C4B1, absence of the 3.2-kb Taq I fragment suggested deletion of the P450c21A pseudogene (17, 18). Deletions simultaneously affecting the P450c21 gene have been reported in selected populations hav-

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1. *Abbreviations used in this paper:* CAH, chronic adrenal hyperplasia; NC, nonclassical form; 17OHP, 17-hydroxyprogesterone; SV, simple virilizing form; SW, salt-wasting form.

ing certain HLA and complement types (18–20). Schneider et al. used Taq I digests alone to report deletion of the P450c21B gene in 10 of the 16 chromosomes from eight CAH patients who also carried a C4B null allele (20). However, two of these retained the C4A and C4B genes but lacked the 3.7-kb Taq I fragment and hence probably had gene conversions. Werkmeister et al., excluding CAH patients with HLA-A3, -B47, -DR7, C4A91 C4BQ0, reported seven deletions among 30 CAH alleles (21). Rumsby et al. (22) found the same result for 9 of 40 CAH haplotypes.

However, patients lacking the 3.7-kb Taq I fragment who nonetheless retain the P450c21B gene have been reported (23). This phenomenon is generally termed “gene conversion,” as the structure of the P450c21B gene is apparently converted to that of the P450c21A pseudogene (15, 22–27). The relative frequency of gene conversion versus gene deletion in CAH has been highly controversial. Jospe et al. reported nine deletions and three gene conversions among 28 salt-losing CAH chromosomes (24). Matteson et al. reported that the apparent gene deletions seen with Taq I (8 out of 20 salt-losing CAH chromosomes) were inconsistent with results in four other restriction enzymes; they proposed that those apparent deletions represented gene conversions, unequal crossovers or polymorphisms, but not physical loss of the P450c21B gene (25). Higashi et al. found that 9 of 22 CAH chromosomes lacked the 3.7-kb Taq I fragment, but that all retained at least part of the P450c21B gene (26). In other Japanese patients, Harada et al. found 6 of 21 unrelated affected chromosomes lacking the 3.7-kb Taq I fragment, of which four were due to gene conversion; this was confirmed for one chromosome by DNA sequencing (27). By contrast, White et al. recently showed that in their patient population 13 of 15 CAH chromosomes lacking the 3.7-kb Taq I fragment had bona fide gene deletions (28). The frequency of gene deletion versus gene conversion thus varies greatly among published studies of small series of patients.

To determine the frequency of various types of genetic defects causing CAH, we studied 57 French families with 68 affected and 165 unaffected family members. For this study we used a new DNA probing tactic using a P450c21 genomic probe, rather than a cDNA probe. This genomic probe can distinguish the P450c21A and B genes by the 3.7- and 3.2-kb Taq I fragments, as seen with a cDNA probe, and also by the 2.4- and 2.5-kb fragments lying immediately downstream (3') from these; this greatly simplifies the analysis of this complex locus, and permits the assignment of 114 of 116 affected chromosomes bearing CAH to one of five DNA haplotypes. We find that ~76% of chromosomes have grossly intact P450c21B genes and, hence, probably carry point mutations. Chromosomes bearing gene deletions and gene conversions were equally abundant, each ~11% of chromosomes carrying CAH.

Methods

Patients and families. 68 CAH patients in 57 families were studied including 47 families with 1 patient, 6 families with 2 patients, and 3 families with 3 patients. As two parents themselves had CAH, these 57 families bore a total of 116 affected chromosomes [(2 × 57) + 2 = 116]. These families were unrelated and had no known consanguinity. 17 family studies were incomplete because some members were deceased, unavailable for study, or refused study. All available family members underwent HLA-B typing.

All patients were diagnosed by elevated plasma 17-hydroxyprogesterone (17OHP) and were seen at least twice per year to monitor their hormonal replacement therapy. The patients were classified as having one of the three clinical forms of CAH defined according to standard criteria (2). CAH with salt wasting (SW) was characterized by onset of hyperkalemia, hyponatremia, dehydration, shock, hyperreninemia, and extremely elevated serum concentrations of 17OHP in the first month of life, which required treatment with both mineralocorticoids and glucocorticoids. Females had ambiguous genitalia and males had enlarged hyperpigmented genitalia. The simple virilizing form (SV) was usually diagnosed in late infancy or early childhood; females had ambiguous genitalia recognized at various ages, and males were referred for sexual precocity. Heights and bone ages were markedly advanced, and plasma renin activities were elevated in all SV patients. The nonclassical form (NC) was characterized in girls by normal external genitalia, precocious adrenarche, and elevated 17OHP responses to ACTH but no clinical or chemical signs of salt loss. Among our 57 CAH families, 42 had SW, 11 had SV, 4 had NC, and 2 parents also had NC.

HLA and complement typing. Leukocyte HLA typing was done by the stage microlymphotoxicity test (29, 30). Complement C2, Bf, and C4 types were determined as described (31), and C4 variants were identified by high-voltage agarose gel electrophoresis with subsequent immunofixation and hemolytic overlay or immunoblotting using anti-C4A and anti-C4B MAb (gifts from Dr. G. O'Neill, American Red Cross, Miami, FL) (32).

DNA blotting studies. Human leukocyte DNA was digested with restriction endonucleases, electrophoresed in 0.7 or 1.0% agarose gels, and analyzed by Southern blotting to nylon membranes by standard procedures. Blots were probed with a 2.7-kb Eco RI fragment of the murine P450c21A gene (12) under low-stringency conditions and with human P450c21 cDNA (25) under high-stringency conditions. High-stringency conditions were 2× SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate), 50% deionized formamide, 50 mM Tris-HCl (pH 7.5), 0.02% wt/vol each of polyvinylpyrrolidone, Ficoll, and BSA, 1% NaDodSO₄, and 100 μg/ml denatured salmon sperm DNA (Sigma Chemical Co., St. Louis, MO). Low stringency conditions substituted 6× SSC and 40% deionized formamide in the above recipe. Overnight prehybridization and 24-h hybridization were at 42°C. After hybridization, nylon membranes were washed twice with 2× SSC at 22°C, then three times with 0.1× SSC, 1% NaDodSO₄ (high stringency) or 2× SSC 1% NaDodSO₄ (low stringency) for 30 min at 65°C. After a final wash at 22°C, the wet membranes were exposed to Kodak XAR-5 films for 5–10 d at –70°C with intensifying screens. The validity of the data obtained with the mouse genomic DNA probe was confirmed by re-probing all Taq I digests with the human P450c21 cDNA (see examples in Fig. 3, below). Some Taq I blots were also hybridized with a 476-bp Bam HI-Kpn I restriction fragment from the full-length C4 cDNA clone, pAT-A (33) corresponding at the 5' C4 region, using high-stringency conditions.

Results

Study population. All affected individuals, obligate heterozygotes, and other family members underwent HLA-B typing. The distribution of HLA-B alleles among 78 unaffected chromosomes from phenotypically normal family members was the same as among a published normal French population (Table I, columns *a* and *b*). By contrast, the distribution of HLA-B alleles among affected chromosomes differed from our unaffected chromosomes. HLA-B8 was found less frequently and HLA-B14 and -B47 were found more commonly on chromosomes bearing CAH (Table I). The incidence of HLA-B47 in our patient population is lower than in some other studies (34, 35).

DNA haplotyping procedure. A DNA haplotype of Taq I and Bgl II fragments was determined for 116 chromosomes

Table I. Allele Frequencies (Percentages) at the HLA-B Locus among the Chromosomes Studied

HLA-B alleles	General population* (n = 124) (a)	Unaffected haplotypes (n = 78) (b)	Affected haplotypes				Chi square test
			Total (n = 110) (c)	SW (n = 77) (d)	SV (n = 22) (e)	NC n = 11 (f)	
5 (w51-w52)	8.0	7.7	10.0	5.1	0.0	—	
7	5.8	10.2	6.4	6.5	9.1	—	‡ b vs. c, § b vs. d
8	8.0	9.0	0.9	1.3	0.0	—	
12 (w44-w45)	16.9	17.9	9.1	10.4	9.1	—	
14	3.4	2.6	9.1	3.9	13.6	36.3	§ b vs. e, ‡ b vs. f
15 (w62-w63)	6.8	5.1	9.1	11.7	0.0	—	
18	5.8	6.4	3.6	3.9	4.1	—	
27	4.1	5.1	5.5	5.2	4.5	—	
35	8.4	14.1	9.1	10.4	4.5	—	
40 (w60-w61)	7.5	2.6	5.5	5.1	0.0	—	
47	1.2	0.0	7.3	9.1	4.5	—	b vs. c, b vs. d
Other HLA-B alleles	24.1	19.3	24.4	27.4	50.6	—	

n, number of haplotypes studied; * data from Betuel et al. (29). Genotype frequencies (g) are calculated from the phenotype frequencies (p) according to the formula $g = 1 - \sqrt{1 - p}$. Significance was tested with the Chi square test. P values are: ‡ <0.01, § <0.05, and || <0.025.

bearing CAH and 84 normal chromosomes from family members (including the 78 chromosomes in Table I). Taq I digests of DNA were probed with a murine 2.7-kb Eco RI genomic DNA fragment extending over 800 bases downstream from the 3' Taq I site of the P450c21 genes (12). This probe detects the 3.2- and 3.7-kb fragments characteristic of the P450c21A and B genes, respectively, and also detects the adjacent downstream Taq I fragments that cannot be detected by the cDNA probe (Fig. 1). For the P450c21A pseudogene, the next Taq I fragment downstream from the 3.2-kb fragment is 2.4-kb. For the P450c21B gene, the next Taq I fragment

downstream from the 3.7-kb fragment is 2.5 kb. The 3' flanking DNA as well as the genes themselves thus can be distinguished. Bgl II digested DNA yields fragments of 12 kb bearing the entire P450c21A pseudogene, and 11 kb, bearing the P450c21B gene. There is no difference in the Bgl II pattern seen on blots probed with the mouse genomic DNA probe or the human cDNA probe, as the 3' Bgl II restriction site lies far downstream from both probes (Fig. 1).

Five different restriction pattern haplotypes were identified among the 116 affected CAH chromosomes (Fig. 2). In haplotypes II to V, lane a shows a typical patient homozygous for the pattern and therefore represents the restriction pattern haplotype. By contrast, lane b shows a compound heterozygous patient carrying one chromosome of the indicated haplotype and one chromosome with haplotype I.

CAH haplotype I. Haplotype I has 3.7- and 3.2-kb Taq I bands of equal intensity, 2.5- and 2.4-kb Taq I bands of equal intensity, and 12- and 11-kb Bgl II bands of equal intensity (Fig. 2). This haplotype represents 65.6% (76 of 116) of affected chromosomes (Table II). It does not differ from the normal pattern, and hence indicates very small changes in the affected genes, presumably point mutations.

CAH haplotype II. Haplotype II lacks the 3.2- and 2.4-kb Taq I bands and the 12 kb Bgl II band indicating deletion of the P450c21A pseudogene. This haplotype represents only 3.4% (4 of 116) of the affected chromosomes. A patient heterozygous for this haplotype has decreased autoradiographic intensity of the 3.2- and 2.5-kb Taq I bands compared with the 3.7- and 2.5-kb Taq I bands, respectively, and similarly has a decreased 12-kb Bgl II band compared with the 11-kb Bgl II band.

Deletion of the P450c21A pseudogene in haplotype II is confirmed further by probing Taq I-digested DNA with the 476-bp Bam HI-Kpn I 5' fragment of C4 cDNA. Such Southern blots show absence of the 7.0-kb Taq I fragment but retention of the 6.4-kb fragment, consistent with deletion of the C4A gene. Haplotype II was found just as frequently or more so among clinically normal chromosomes (14.3% of our 84 normal chromosomes, see below), as it was among chromo-

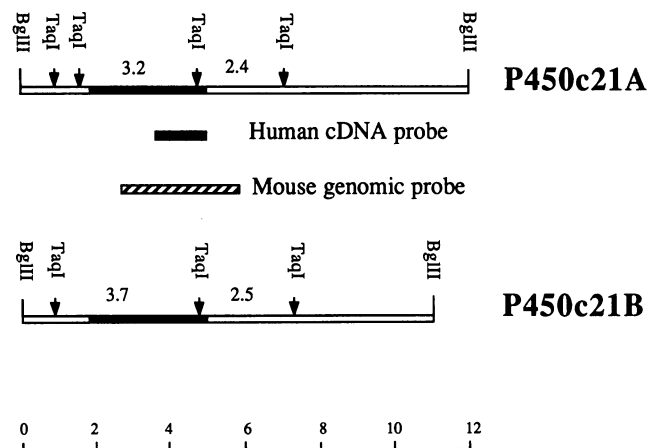


Figure 1. Diagram of the P450c21 genes (derived from references 4, 6, and 24). The 12-kb Bgl II DNA fragment bearing the P450c21A pseudogene is shown above and the 11-kb Bgl II fragment bearing the functional P450c21B gene is shown below. The P450c21 genes, spanning ~ 3.5 kb, are shown in black. The locations of the Taq I recognition sites are indicated by solid arrowheads pointing downward. The 3.2- and 3.7-kb Taq I fragments bearing the P450c21 A and B genes, respectively, are indicated, along with the downstream 2.4- and 2.5-kb fragments. The regions of the DNA hybridizing to the probes used in this study are indicated between the two gene diagrams. The scale below indicates distance in kilobases.

RESTRICTION PATTERNS OF P450c21 GENES IN CAH PATIENTS

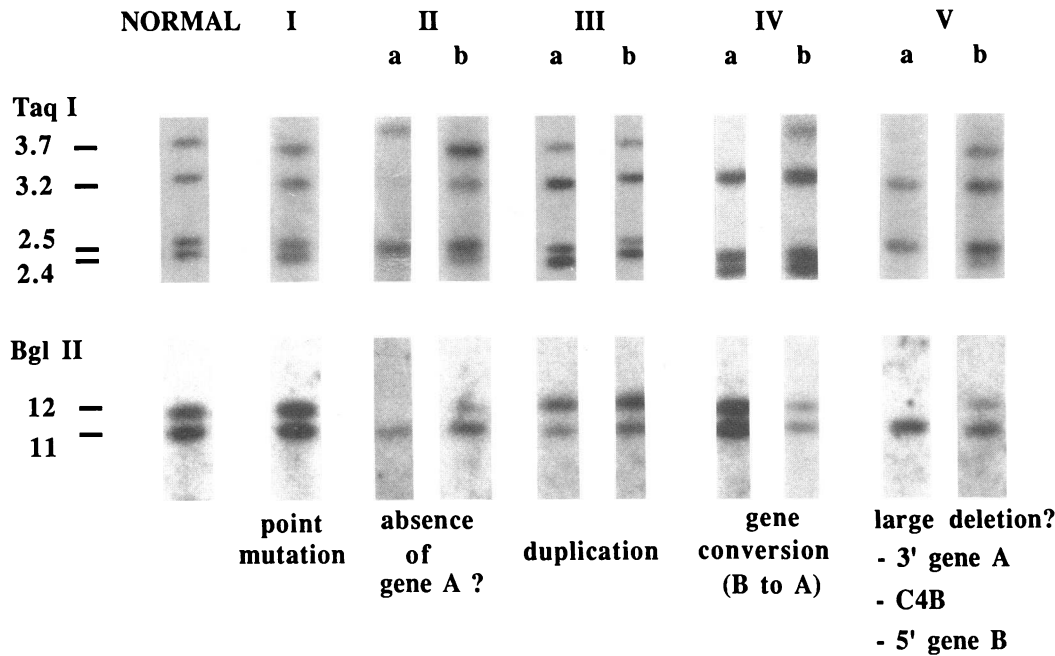


Figure 2. Southern transfer blots typical of the five restriction endonuclease haplotypes. Blots of DNA digested with Taq I are shown above; those digested with Bgl II are shown below. The sizes of the fragments (in kilobases) are shown at the left. Normal, DNA from a normal person; the four Taq I bands are of equal intensity and the two Bgl II bands are of equal intensity. Haplotype I is identical to the normal pattern but comes from a patient with severe salt-wasting CAH. For haplotypes II–V, lane *a* shows a patient homozygous for that haplotype and lane *b* shows a patient heterozygous with a chromosome bearing haplotype I. Hap-

lotype II, deletion of the P450c21A pseudogene. In the IIa homozygote, the 3.2- and 2.4-kb Taq I fragments, and the 12-kb Bgl II fragment, all characteristic of the P450c21A pseudogene, are absent. In the I/II heterozygote shown in lane IIb, these bands appear in diminished intensity because only the type I chromosome contributes them to the pattern. Haplotype III, duplication of the P450c21A pseudogene. The 3.2- and 2.4-kb Taq I bands and the 12-kb Bgl II bands are darker because there is an extra P450c21A pseudogene on each chromosome. In the IIIb heterozygote this difference in intensity is less, about the 3:2 ratio expected; however, Southern blots are inherently non-quantitative and thus assignment of a patient as type IIIb must be done in conjunction with HLA and complement data and family studies. Haplotype IV, gene conversion. In the IVa homozygote, the 3.7-kb Taq I band is absent but the other five bands remain. In the IVb heterozygote, the 3.7-kb band is fainter than the 3.2-kb band because only the heterozygous chromosome bearing haplotype I contributes this band. Haplotype V, gene deletion. The 3.7- and 2.4-kb Taq I bands and 12-kb Bgl II band are absent, indicating a large deletion of ~ 30 kb resulting in a single hybrid P450c21A/B gene. In the Vb heterozygote the intensities of these bands are diminished.

somes associated with CAH. Deletion of the P450c21A pseudogene thus does not cause CAH but is simply associated with point mutations in the P450c21B gene causing some cases of CAH.

CAH haplotype III. Haplotype III homozygotes have increased autoradiographic intensities of the 3.2- and 2.4-kb Taq I fragments, but the 3.7- and 2.5-kb Taq I fragments are still present. They also have both the 11- and 12-kb Bgl II bands, but the autoradiographic intensity of the 12-kb bands is increased. This haplotype, which represents 6.9% (8 of 116) of the chromosomes in our study, probably arises from a duplication of the P450c21A pseudogene. A patient heterozygous for this haplotype and haplotype I will have a 2:3 ratio of the 3.7- and 3.2-kb Taq I fragments. Because of the inherently nonquantitative nature of Southern blotting, these may be mistaken for a 2:2 ratio (normal or haplotype I) or a 1:2 ratio (haplotypes IV and V). As with haplotype II, haplotype III may be found in normal persons, indicating that the duplicated P450c21A pseudogene is merely associated with CAH but does not cause it. Therefore, patients having CAH and haplotype III must have point mutations in their grossly intact P450c21B genes. The incidence of point mutation causing CAH in our study thus is the sum of the incidences of haplotypes I, II, and III (88 of 116, 75.9%) (Table II).

CAH haplotypes IV and V. Haplotypes IV and V are the ones of greatest interest, representing gene conversion and

gene deletion, respectively. Among our study of 116 chromosomes, haplotypes IV and V each represent 11.2% of the total (13 of 116). In the homozygous state, both haplotypes completely lack the 3.7-kb Taq I band normally associated with the functional P450c21B gene, a pattern generally interpreted as diagnostic of gene deletion. However, haplotype IV retains both the 2.4- and 2.5-kb Taq I bands seen only with the genomic probe, whereas haplotype V lacks the 2.4-kb band. Homozygotes for haplotype IV retain both the 11- and 12-kb Bgl II fragments yielding equal autoradiographic intensities, whereas homozygotes for haplotype V lack the 12-kb Bgl II fragment normally associated with the P450c21A pseudogene. In haplotype IV both P450c21 genes are present, but there is a change in the structure of the 5' end of the P450c21B gene, converting it to the structure of a P450c21A gene. Thus, a chromosome bearing haplotype IV has two 3.2-kb Taq I fragments and no 3.7-kb Taq I fragments, but most other restriction endonuclease recognition sites are the same as in normal DNA, and both the C4A and C4B genes are retained and function normally (Table V).

Haplotype V represents a physical loss of ~ 30 kb of DNA, probably from an unequal crossover event. Disappearance of the 2.4-kb Taq I band normally associated with the P450c21A pseudogene, rather than of the 2.5-kb Taq I band associated with the deleted P450c21B gene, and of the 12-kb Bgl II fragment (A pseudogene) rather than the 11-kb Bgl II fragment (B

Table II. Frequency of Five Restriction Pattern Haplotypes in 57 CAH Families

Haplotype	Clinical form	Homozygous	Heterozygous	Haplotypes (n = 116)	Frequency	Unaffected haplotypes (n = 84)
						%
I						
Point mutation	SW	18	17	53	65.6	84.5
	SV	6	3	15		
	NC	3	3	8		
II						
Absence of A pseudogene	SW	1	2	4	3.4	14.3
III						
Duplication of A pseudogene	SW	—	2	2	6.9	1.2
	SV	1	1	3		
	NC	—	3	3		
IV						
Gene conversion	SW	2	7	11	11.2	0.0
	SV	—	2	2		
V						
Large deletion	SW	4	5	12	11.2	0.0
	SV	—	1	1		
Others	SW	—	1	1	1.7	0.0
	SV	—	1	1		

The numbers under "homozygous" or "heterozygous" refer to numbers of patients. The numbers under "haplotype" refer to the number of affected chromosomes (total, 116). The unaffected haplotypes are from 84 normal chromosomes borne by obligate heterozygotes.

Table III. Gene Frequencies (Percentages) of the C4A and C4B Alleles

Alleles	Unaffected chromosomes			CAH chromosomes		Chi square test
	I (n = 38) (a)	II (n = 10) (b)	Total (n = 48) (c)	I (n = 50) (d)	Total (n = 91) (e)	
C4A locus						
AQ0	10.5	60.0	20.8	14.0	9.9	a vs. b*
A1	2.6	—	2.1	—	—	
A2	—	—	—	6.0	7.7	c vs. e [‡]
A3	65.8	30.0	58.3	64.0	49.4	
A4	13.1	10.0	12.5	10.0	5.4	
A6	5.3	—	4.2	—	—	
A91	—	—	—	—	6.7	
A duplication	2.6	—	2.1	2.0	3.3	
C4B locus						
BQ0	18.4	40.0	22.9	6.0	18.7	
B1	55.3	40.0	52.1	68.0	47.3	
B2	10.5	10.0	10.4	10.0	6.6	
B3	7.9	10.0	8.3	10.0	5.5	
B6	5.3	—	4.2	2.0	—	
B1B2	2.6	—	2.1	—	7.7	

I and II correspond to restriction pattern haplotypes shown on Fig. 2: Column c labeled "total" is the sum of columns a and b; n, number of chromosomes studied. Significance was tested with a Chi square test. P values are: * <0.001, † 0.05. For C4AQ0, c vs. e is: >0.05 and <0.1.

gene), is not inconsistent and helps to define the limits of the DNA deletion. The deletion in haplotype V probably extends from exon 3 of the P450c21A pseudogene to the homologous site in the P450c21B gene, thus deleting the 2.4-kb Taq I fragment but retaining the 2.5-kb Taq I fragment. The 5' deletion site in the P450c21A gene and the 3' deletion site in the P450c21B gene must correspond very closely, because the electrophoretic mobility of the resulting Taq I-digested hybrid product is indistinguishable from a normal 3.2-kb Taq I band associated with the P450c21A gene. Similarly, the 11-kb Bgl II fragment seen in haplotype V is a hybrid consisting of the upstream (5') portion of the 12-kb P450c21A pseudogene and the downstream (3') portion corresponding to the P450c21B gene (Fig. 1). Deletion of the P450c21B gene in haplotype V is confirmed by probing Taq I-digested genomic DNA with the C4 cDNA, showing absence of either the 6.0 kb or 5.4 kb C4 gene fragments (Fig. 3).

2 of the 116 chromosomes examined could not be classified as bearing one of the five haplotypes discussed above. These two chromosomes bore Taq I patterns compatible with haplotype IV but had Bgl II patterns compatible with haplotypes III and V, respectively. These two chromosomes show that the five haplotypes discussed here account for most but not all chromosomes bearing CAH. Chromosomes bearing uninterpretable and seemingly contradictory restriction endonuclease patterns have been described in a series of families with salt-losing CAH (25).

DNA haplotypes of unaffected chromosomes. We assigned different Taq I restriction patterns to 84 unaffected chromosomes from obligate CAH heterozygotes. Among these, 71 (84.5%) had normal Taq I restriction patterns, but 12 (14.3%) had haplotype II and 1 had haplotype III (Table II). Deletion of

Table IV. Unaffected Haplotypes with a Detectable Alteration of P450c21 Genes

Types	HLA			Complotype				C4A	C4B
Pseudogene deletion (Haplotype II)	A1	B8	DR 3	C2C	BfS	C4 AQ0	C4 B1	—	6.4
	A3	B8	DR 3	C2C	BfS	C4 AQ0	C4 B1	—	6.4
	A3	B8	DR 3	C2C	BfF	C4 AQ0	C4 B3		
	A24	B8		C2C	BfS	C4 AQ0	C4 B1		
	A1	B8	DR 13	C2C	BfS/F	C4 A3	C4 BQ0		
	A2	B18	DR 3	C2C	BfF1	C4 A3	C4 BQ0	7.0	6.0
	A3	B18	DR 1	C2C	BfF1	C4 A3	C4 BQ0		
	A2	B18		C2C	BfS	C4 AQ0	C4 B1		
	A3	B27	DR 13	C2C	BfS	C4 A4	C4 BQ0	7.0	—
	A2	B41	DR 1	C2C	BfF	C4 AQ0	C4 B2.1		
	A2	B44	DR 13						
	Pseudogene duplication (Haplotype III)	A30	B14	DR 8					

Column C4A represents the sizes (in kilobases) of fragments that hybridized to the 5' C4 probe: 7.0 kb (C4A), 6.0 kb (long C4B), 6.4 kb (C4B with a deletion of C4A).

the P450c21A pseudogene has been reported in some normal subjects (17, 18); finding haplotype II on normal chromosomes confirms that the 2.4-kb Taq I fragment segregates with the P450c21A pseudogene.

Associated deletions of the C4A and C4B genes were indicated by HLA typing for 11 and complement typing for 10 of these 12 normal chromosomes bearing haplotype II (Table IV). Five of these carried HLA-B8, which is associated with deletion of the P450c21A pseudogene (18–20), and complement typing showed six C4AQ0 and four C4A3,BQ0 alleles.

To confirm apparent deletions of the C4A or C4B genes, four normal chromosomes were probed with C4 cDNA (Table IV and Fig. 3). Absence of a 7.0-kb Taq I band and presence of a 6.4-kb Taq I band are characteristic of deletion of the C4A gene (20). This pattern segregates with the complotype C4AQ0 and confirms deletion of the C4A gene and the P450c21A pseudogene (Fig. 3, family C, haplotype *b*). By contrast, presence of the 7.0-kb band and absence of the C4B Taq I fragments (6.0 or 5.4 kb) is characteristic of deletion of the C4B gene. This pattern segregates with the complotype C4A4,BQ0 and confirms a deletion encompassing the C4B gene and the P450c21A pseudogene (Fig. 3, family D, haplotype *c*).

These studies of unaffected chromosomes indicate that three Taq I patterns are compatible with normal 21-hydroxylase activity: pattern I (normal); pattern II (deletion of the P450c21A pseudogene); and pattern III (duplication of the P450c21A pseudogene). As discussed above, CAH-bearing chromosomes having haplotypes II or III are thus presumed to have point mutations in their P450c21B genes.

Correlation between restriction endonuclease haplotypes and clinical form of CAH. Table II shows the distribution of SW, SV, and NC CAH among the five haplotypes. Homozygotes for Type I (point mutation) may have any form of CAH. This indicates there are many different point mutations causing this disease, some of which eliminate essentially all enzymatic activity of P450c21, while others affect the enzyme only slightly. Because DNA haplotyping by restriction endonuclease digestion cannot distinguish among various different point

mutations, persons who are homozygous for Type I may be compound heterozygotes of two different point mutations. The one homozygous patient available with haplotype II had SW CAH and the one homozygote with haplotype III had SV CAH. These patients further indicate that a disorder in the P450c21B gene is causing the CAH; it is doubtful the presence or absence of the P450c21A pseudogene could have a phenotypic manifestation. All homozygotes for haplotypes IV and V had SW CAH, as would be expected if the functional P450c21B gene were deleted or converted to a nonfunctional gene.

Correlation of restriction endonuclease haplotypes with HLA and complement haplotypes. Linkage disequilibrium between certain HLA types and CAH (34–36) is also found in our study, indicating that our patient population is typical and representative (Table I). Complement typing should be even more informative because the C4 loci are so closely linked to the P450c21 genes; however, the great technical difficulty in performing C4 typing prevented its unambiguous determination in eight families. C4AQ0 was closely associated with haplotype II (deletion of the P450c21A pseudogene), but its overall incidence was lower on chromosomes bearing CAH alleles (9.9%) than on normal chromosomes (20.8%) (Table III). By contrast, C4BQ0 was found with equal frequency among normal and CAH-bearing chromosomes, but all chromosomes bearing a P450c21B gene deletion also bore C4BQ0 (12 of 12), consistent with a 30-kb deletion encompassing the C4B gene. One chromosome bearing haplotype IV also bore C4BQ0 and C4A2A2, suggesting that the C4B gene was converted to a C4A in conjunction with conversion of the P450c21B gene to a P450c21A pseudogene. Similar events have been described on other chromosomes encoding CAH (37, 38), but all our other chromosomes bearing haplotype IV had markers for both C4 genes.

Some extended supratypes are clearly linked to CAH (Table V). Among the 12 chromosomes bearing haplotype V, 6 carry HLA-A3,-B47,-DR7,C2C,BfF,C4A91,C4BQ0. Although HLA-B47 is strongly associated with P450c21B gene

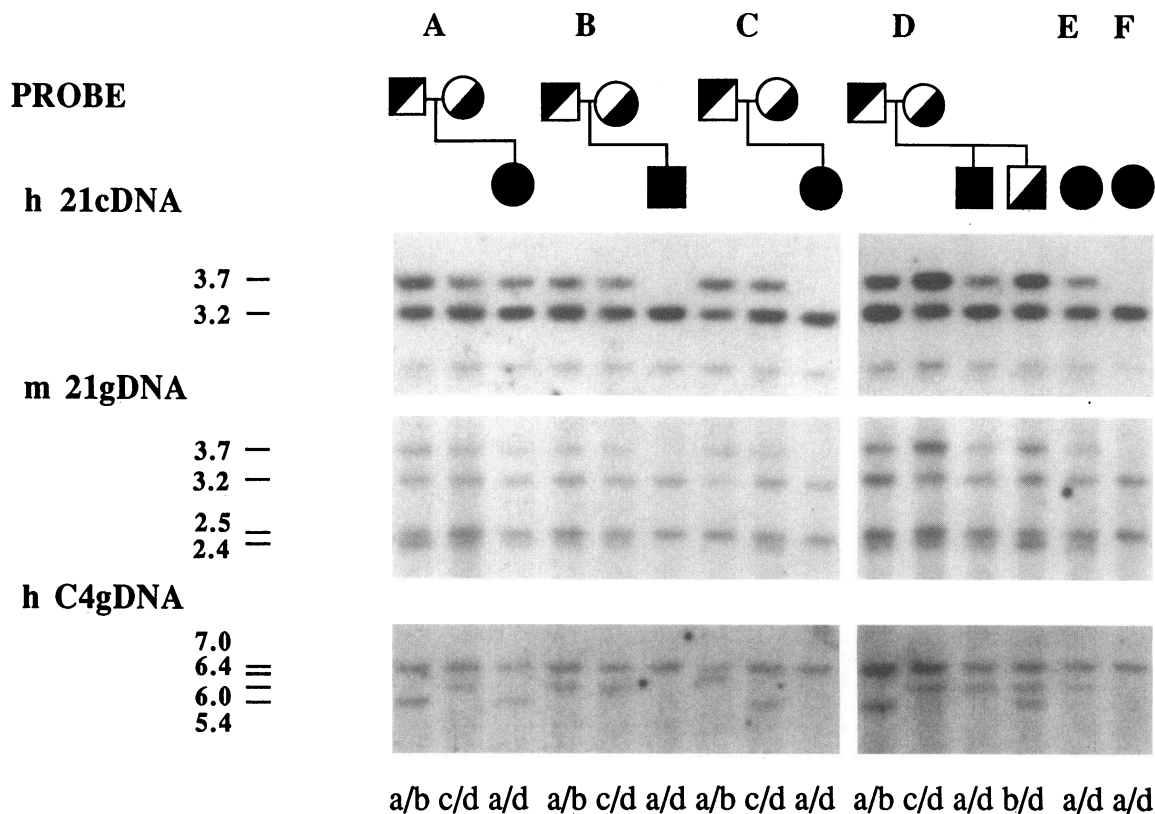


Figure 3. Studies of pedigrees bearing CAH haplotype V. ■ and ●, males and females, respectively; solid symbols and half-open/half-solid symbols represent homozygous affected individuals (21-hydroxylase deficiency) and heterozygotes, respectively. The haplotype for each individual is indicated at the bottom of the figure: *a* and *d* designate chromosomes bearing CAH; *b* and *c* represent unaffected chromosomes. The affected individuals in families B, C and F are homozygous for haplotype V. The extended haplotype, the sizes (in kilobases) of the fragments hybridizing to the 5' C4 cDNA probe (hC4DNA) and the restriction pattern haplotype defined in Fig. 2 are shown below. The P450c21 probes used are human P450c21 cDNA (h21cDNA), and mouse P450c21 genomic DNA (m21gDNA). The validity of the mouse genomic probing strategy is shown by finding the same pattern of 3.7- and 3.2-kb Taq I fragments with this probe and with the human cDNA probe. The complement and HLA types of the chromosomes, the sizes of their C4A and C4B genes digested with Taq I and their Southern blotting haplotypes are as follows:

Family	HLA haplotype	Complotype	C4A	C4B	Haplotype	
A	a	A24 B58 DR2/7	C2C BfF C4 A91 BQ0	7.0	—	V
	b	A3 B35 DR2/7	C2C BfS C4 A3 B1	7.0	6.0	I
	c	A29 B55 DR2/7	C2C BfS C4 A3 B1	7.0	6.0	I
	d	A3 B47 DR2/7	C2C BfF C4 A91 BQ0	7.0	—	V
B	a	A26 B5 DR1	C2C BfS C4 A3 B1	7.0	6.0	I
	b	A24 B35 DR11	C2C BfS C4 A3 B1	7.0	6.0	I
	c	A29 B44 DR11	C2C BfS C4 A3 B1	7.0	6.0	I
	d	A3 B47 DR7	C2C BfF C4 A91 BQ0	7.0	—	V
C	a	A3 B47 DR7	C2C BfF C4 A91 BQ0	7.0	—	V
	b	A1 B8 DR3	C2C BfS C4 A91 BQ0	—	6.4	II
	c	A3 B35 DR7	C2C BfS C4 A91 BQ0	7.0	5.4	I
	d	A3 B35 DR1	C2C BfF C4 A3 B1	7.0	—	V
D	a	A3 B47 DR7	C2C BfF C4 A91 BQ0	7.0	—	V
	b	A2 B18 DR7	C2C BfS C4 A3 B1	7.0	5.4	I
	c	A3 B27 DR13	C2C BfS C4 A4 B1	7.0	—	II
	d	A30 B49 DR13	C2C BfS C4 A3 B1	7.0	6.0	I
E	a	A3 B47 DR7	C2C BfF C4 A91 BQ0	7.0	—	V
	d	A32 B12 DR5	C2C BfS C4 A3 B1	7.0	6.0	I
F	a	A1 B41 DR2	C2C BfS C4 A3 B1	7.0	—	V
	d	A11 B7 DR1	C2C BfS C4 A3 B1	7.0	—	V

Table V. CAH Haplotypes with a Detectable Alteration of P450c21 Genes

Types		HLA			Complotype				C4A	C4B
Pseudogene deletion (Haplotype II)	a	A3	B35	DR 3	C2C	BfS	C4 AQ0	C4 B1	—	6.4
	b	A1	B8	DR 3	C2C	BfS	C4 AQ0	C4 B1	—	6.4
	c	A11	B35	DR 1	C2C	BfF	C4 A3	C4 BQ0		
Pseudogene duplication (Haplotype III)	a(4)	A28	B14		C2C	BfS	C4 A2	C4 B1B2		
	b(2)	A33	B14	DR 1	C2C	BfS	C4 A2	C4 B1B2		
	c*	A1	B17	DR 1	C2C	BfS	C4 A2	C4 B1B2		
	d	A29	B44	DR 8	C2C	BfF	C4 A3A1	C4 B1		
Gene conversion (Haplotype IV)	a(2)	A31	B14	DR 7	C2C	BfS	C4 A3	C4 B1		
	b(2)	A1	B27	DR 5	C2C					
	c	A26	B27	DR 1	C2C	BfS	C4 A3	C4 B1		
	d	A24	B62	DR 4	C2C	BfF	C4 A2A2	C4 BQ0		
	e	A2	B37	DR 13	C2C	BfF	C4 A3	C4 B1		
	f	A32	B44	DR 11	C2C	BfS	C4 A3	C4 B1		
	g	A32	B44		C2C	BfS	C4 A2	C4 B2		
	h	A1	B35	DR 7	C2C					
	i	A2	B51	DR 2	C2C	BfS	C4 A3	C4 B1	7.0	6.0
	j	A1	B57	DR 1	C2C					
	k	A3	B7	DR 8	C2C					
Deletion (Haplotype V)	a(6)	A3	B47	DR 7	C2C	BfF	C4 A91	C4 BQ0	7.0	—
	b	A26	B5	DR 1	C2C	BfS	C4 A3	C4 BQ0	7.0	—
	c	A3	B35	DR 1	C2C	BfS	C4 A3	C4 BQ0	7.0	—
	d	A1	B41	DR 2	C2C	BfS	C4 A3	C4 BQ0	7.0	—
	e	A11	B7	DR 1	C2C	BfS	C4 A3	C4 BQ0	7.0	—
	f	A26	B62	DR 8			C4 A3	C4 BQ0		
	g	A2	B40						7.0	—
	h	A28/32	B18/35	DR 13/7			C4 A3/A3	C4 B1/BQ0		
Undeterminable	a	A30	B27	DR 1	C2C	BfS	C4 A3	C4 BQ0	7.0	6.0
	b	A29	B27	DR 1	C2C	BfS	C4 A13	C4 BQ0		

c*: Recombination with HLA B14; a(4), number of same haplotype; and —, absence of bands.

deletions, we found two chromosomes bearing HLA-B47 and type I CAH haplotypes (P450c21B point mutations). These two chromosomes bore very different supratypes from the haplotype V chromosomes bearing HLA-B47: one was HLA-A30,-B47,-DR2,C2C,BfS,C4A3,B1, and the other was HLA-A3,-B47,-DR13. Nondeletion alleles bearing B47 have been reported in SW CAH (25, 26), and in an isolated normal population (39). A similar conservation of supratypes is seen in six of the eight chromosomes having haplotype III, which have either HLA-A28 or -A33 associated with -B14, BfS,C4A2, C4B1B2 (Table V). The latter supratype has a duplication of both the P450c21A pseudogene and the C4B locus, and is frequently found in patients with NC CAH. We have also found six other recombinations in addition to the gene rearrangements described above. These include three recombinations between HLA-A and -B and three between HLA-B and -DR (data not shown).

Discussion

We used Taq I and Bgl II digests to analyze DNA from 57 French families having one to three children with CAH, and two affected parents, for a total of 68 patients. The distribution

of clinical forms of CAH in our study of affected children is similar to that in other studies (17–22), but clearly different from a study of adults emphasizing NC CAH (35). The distribution of HLA-B types in our French Caucasian population does not differ from other studies (34–36), so our patient population is wholly typical. Our study differs from previous reports in the size of the population examined and the use of the genomic probe, which detects the DNA 3' to the P450c21 genes. This unique probing strategy permitted us to assign 114 of 116 CAH-bearing chromosomes to five haplotypes. The remaining two chromosomes presented a confusing array of restriction endonuclease patterns. This is not wholly surprising, as Matteson et al. (25) have shown that the restriction endonuclease patterns found in some CAH patients can be inconsistent and inexplicable by the simple genetic models accounting for haplotypes I–V. Such restriction endonuclease patterns might arise from compound heterozygosity among haplotypes II–V and/or other, uncharacterized gene rearrangements.

Among the 116 CAH chromosomes characterized, 76 (65.9%) had haplotype I, indicating point mutations or other changes sufficiently small to be undetectable on Southern blots. Four chromosomes (3.4%) bore haplotype II, which in-

icates deletion of the P450c21A pseudogene and may, or may not, be associated with a deletion of either of the C4 genes encoding the fourth component of serum complement. Haplotype II is not specific for CAH, because 14.2% of chromosomes not bearing CAH alleles carry haplotype II. Thus, the genetic lesion in CAH chromosomes bearing haplotype II must be an associated point mutation in the P450c21B gene.

Eight chromosomes (6.9%) bore a duplication of P450c21A pseudogene (haplotype III) and duplication of either C4A or C4B, in association with HLA-B14 (21, 35). The supratype HLA-A28/A33-B14 C4A2, B1B2 was frequently associated with P450c21A duplication (six of eight chromosomes). Although this supratype has been associated with NC CAH (21, 35) and is found in 3 of our 11 NC patients, our one patient homozygous for this supratype had SV CAH. Haplotype III thus shares a crucial characteristic with haplotype II: it can be found on unaffected chromosomes. Therefore, the lesion causing CAH on these chromosomes must be an associated point mutation in the P450c21B gene, and the CAH is not directly caused by duplication of the P450c21A pseudogene. This hypothesis is strongly supported by recent sequencing data showing a point mutation in the P450c21B gene of a patient homozygous for HLA-B14-DR1 and duplication of the P450c21A pseudogene (40).

When applied to gene alterations associated with CAH, the term gene conversion refers to a change in part or all of the P450c21B gene, converting part or all of its structure to a P450c21A pseudogene (16, 23–28, 37). Gene conversion can be most easily conceptualized as resulting from two unequal crossover events, but many other mechanisms may also be involved (see reference 15 for review and references). Gene conversions in CAH have been documented by Southern blotting studies (23–27) and by DNA sequencing (9, 27, 41). Simple Taq I digests alone probed with P450c21 cDNA cannot distinguish gene conversion (haplotype IV) from gene deletion (haplotype V); hence, the relative incidence of these two classes of mutations has been unclear and somewhat controversial. The use of both Taq I and Bgl II digests in conjunction with a genomic probe detecting the Taq I fragments 3' to the P450c21 genes now greatly simplifies this task. We found equal numbers of chromosomes bearing deletion or conversion, 13 of 116 or 11.2% for each lesion. Other studies report greatly varying relative incidences of these two lesions (24–28), presumably due to the small number of patients studied or to population differences, or both.

The available data now localize the region where the presumed gene conversion event(s) must occur. No reported examples of gene conversion modify the sizes of the 11- and 12-kb Bgl II fragments or, in our study, of the 2.4- and 2.5-kb Taq I fragments. These observations and the published sequencing data (27, 42) delineate the 3' end of the conversion event as lying downstream from the 8-bp deletion in exon 3 and upstream from exon 4 of the P450c21A pseudogene. Because C4B protein is detected in seven of eight gene conversion haplotypes, the 5' limit probably lies downstream (3') from the C4B gene. Similarly, the limits of the gene deletion in chromosomes bearing haplotype V should include the 3' end of the P450c21A pseudogene to the 5' end of the P450c21B gene, in a 30-kb deletion producing a hybrid, nonfunctional P450c21A/B pseudogene giving 3.2- and 2.5-kb Taq I fragments.

Our studies thus show that most chromosomes encoding CAH carry point mutations of the P450c21B gene. These may

be found on three haplotypes, here termed haplotypes I, II, and III, which together encompass 75.9% of the chromosomes in our study. Determination of the genetic lesions in these chromosomes requires cloning and sequencing the affected DNA. Specific amplification of affected sequences by polymerase chain reaction technology (43) is not effective, because the 98% sequence identity of the P450c21A and B genes prohibits suitably specific priming (Y. Morel and W. L. Miller, unpublished observations). Of the remaining CAH chromosomes, equal numbers (11.2%) bear gene deletions (haplotype V) and gene conversions (haplotype IV), while a small number (1.7%) still escape characterization by our procedures. Our procedures, using a genomic probe extending 3' to the P450c21A and B genes, now permit simple and accurate characterization of > 98% of chromosomes encoding CAH. We propose that Taq I digests alone are sufficient for accurate genetic diagnosis in ~ 98% of cases if family studies are done and if the blots are examined with a genomic probe extending 3' to the P450c21 genes (e.g., the 3.1-kb Eco RI/Bam HI fragment of the human genes); examination of these blots with a 5' C4 cDNA probe can provide useful confirmation.

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