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Major-Histocompatibility-Complex Gene Markers and Restriction-Fragment Analysis of Steroid 21-Hydroxylase (CYP21) and Complement C4 Genes in Classical Congenital Adrenal Hyperplasia Patients in a Single Population

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Summary

The gene CYP21B, encoding the steroid 21-hydroxylase enzyme of adrenal steroid biosynthesis, has been mapped to the human major histocompatibility complex (MHC). Deficiency of this enzyme leads to congenital adrenal hyperplasia (CAH). We report the phenotypes of the HLA and complement C4 and Bf genes, which are closely linked to the CYP21B gene, together with a detailed analysis of the CYP21 and C4 RFLP, in 17 Finnish families with CAH. The RFLP analysis with six restriction enzymes suggested that, altogether, 35% of the affected chromosomes had a CYP21B+C4B gene deletion, 9% an obvious gene conversion of the CYP21B gene to a CYP21A-like gene, and 3% a CYP21A+C4B duplication. The remaining 53% gave the RFLP patterns also found in nonaffected chromosomes. We also found that a 14.0-kb *Eco*RI RFLP marker of the CYP21 genes was strongly associated with the presence of a short C4B gene, suggesting that some of the RFLP markers found with the CYP21 probe may actually derive from C4B gene polymorphism. Three particular MHC haplotypes, each with a characteristic RFLP pattern, were found in many unrelated families. These three haplotypes accounted for 59% of the affected chromosomes in our study group, the rest (41%) of the affected chromosomes being distributed among various subtypes. The results suggest that, within a single, well-defined population such as in Finland, only a few CYP21B gene defects may constitute a substantial part of the affected chromosomes. This finding will help in genetic studies of CAH in such populations.

Introduction

Congenital adrenal hyperplasia (CAH) is a defect of adrenal cortisol synthesis. The most common form results from a recessively inherited deficiency of the steroid 21-hydroxylase enzyme needed for mineralocorticoid and glucocorticoid synthesis (New and Speiser 1986; Miller and Levine 1987). Classical CAH has been subdivided into two clinical types. In the simple virilizing (SV) form, early virilization due to defective cortisol synthesis is the most prominent feature. Roughly two-thirds of patients have the more severe, salt-wasting (SW) form, where, in addition to virilization, they suffer from renal salt loss due to an inability to synthesize mineralocorticoids. The disease affects about 1/5,000–15,000 newborns in Caucasian populations, and as such it is one of the most common inborn errors of metabolism.

The 21-hydroxylase genes (CYP21) have been mapped to the human major histocompatibility complex (MHC) on chromosome 6 (Dupont et al. 1977). There are two homogeneous CYP21 genes, CYP21A and CYP21B, located adjacent to the genes C4A and C4B encoding the C4 component of the human complement system (fig. 1; Carroll et al. 1985*a*; White et al. 1985). DNA sequence data (Higashi et al. 1986; White et al. 1986) show that only the CYP21B gene is functional. Molec-

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ular maps derived from cloned DNA of nonaffected persons show that with certain restriction endonucleases (fig. 1) the two genes can be distinguished from each other in Southern analysis using a probe specific for CYP21 sequences. However, several recent more detailed analyses (Donohoue et al. 1986; Harada et al. 1987; Matteson et al. 1987; Higashi et al. 1988) have shown that a gene conversion mechanism can alter the size of certain RFLP markers. Thus the use of several restriction endonucleases is required. Parallel RFLP analysis of the C4 genes may further help to interpret the results, particularly, as the C4B genes are of two sizes—"short" and "long" (Prentice et al. 1986; Schneider et al. 1986)—and as the C4 gene deletions have been characterized (Carroll et al. 1985*b*; Schneider et al. 1986; Yu et al. 1986). The genes of the MHC are highly polymorphic (Dupont 1988), thus being exceptionally useful markers for genetic comparisons of CAH in different populations. Genetic (centimorgans [cM] and physical (kilobase pairs [kb] distances between different MHC gene markers are shown in figure 1.

In this study we describe the MHC gene markers, including the HLA antigens and complement phenotypes (complotypes), and detailed RFLP analysis of the CYP21 and C4 genes in Finnish families with classical CAH. As the patients were all from a single, ethnically homogeneous population of some 4.8 million people, it was possible to gain a picture of CYP21 gene mutations within a single population.

Subjects and Methods

Seventeen unrelated families with a total of 22 patients suffering from classical CAH due to 21-hydroxylase deficiency were analyzed. The number of children studied (see table 2) varied from one to 10 per family (mean 1.8). Two affected siblings were studied in three families, and three affected siblings were studied in one family. In four families only one of the parents could be analyzed. Four patients (families 6, 7, 10, and 11 of table 3) had the SV form of CAH, and the remainder had the SW type. CAH was classified as SV if a patient required only glucocorticoid therapy, whereas both glucocorticoids and mineralocorticoids were necessary for a patient to be classified as having the SW form. Except for the two homozygous cell lines of the 10th International HLA Workshop (Dupont, in press), control samples were obtained from the healthy blood donors described by Partanen (1987, 1988). All of the persons were of Finnish origin.

The HLA antigens were typed with the standard NIH cytotoxicity test. The HLA-DR alleles were additionally confirmed by hybridizing the *Taq*I-digested genomic DNA samples with the DR-beta-gene probe (Long et al. 1983). The definition of these HLA-DR "DNA alleles" was based on the results of Carlsson et al. (1987) and on our own unpublished experiments. The Bf and C4 alleles were typed essentially as described by Marcus and Alper (1986), except that in the C4 typing the samples were additionally treated with carboxypeptidase B (Sim and Cross 1986). The hemolytic overlay method (Marcus and Alper 1986) was applied to distinguish the C4A from the C4B allotype. The complement phenotypes have been abbreviated: e.g., S31 indicates the combination Bf*S C4A*3 C4B*1.

For the RFLP studies, samples of roughly 7 μg of genomic DNA were digested with 15–30 units each of the following restriction endonucleases: *Bgl*II, *Eco*RI, *Kpn*I, *Nco*I, *Taq*I, and *Xba*I (Promega, Ltd; Boehringer-Mannheim; and Bethesda Research Laboratories). After electrophoresis in 0.7% agarose, the DNA samples were transferred onto Biotrace filters (Gelman Sciences Inc.) with alkali blot as recommended in the protocols of the 10th International HLA Workshop (Dupont, in press). Prehybridization (16 h, 42°C) and hybridization (48 h, 42°C) were performed in 50% formamide, 1% SDS, 5% dextran sulfate, 5 × SSC, 0.1% Denhardt's, and 200 μg denatured DNA/ml (Dupont, in press). After hybridization the filters were washed twice in 2 × SSC, and for the stringent wash in 0.5 × SSC at 65°C for 30 min. The full-length cDNA probe specific for C4 genes, pAT-A, has been described by Belt et al. (1984), and the 21-OH probe p21-K4 has been described by Carroll et al. (1985*a*). The probes were provided by Dr. M. C. Carroll, Harvard University, Boston. The relative band intensities in the Southern blots were measured with a scanning densitometer (Helena Laboratories, Ltd).

Results

1. *HLA Antigens—and Complement Bf and C4 Phenotypes*

The frequencies of HLA-B and -DR antigens and of Bf and C4 phenotypes (complotypes) were compared between (1) 34 affected chromosomes of 17 index patients with classical CAH, (2) 30 nonaffected chromosomes of these families, and (3) the reference population (Lokki and Julin 1982; Partanen and Koskimies 1986). The most striking finding (table 1) was the exceptionally high frequency of the HLA B40 antigen in

Table 1

HLA B and Complement Phenotypes Showing Association with Classical CAH in the Finnish popularion: Frequencies in 34 Affected Chromosomes (A), in 30 Nonaffected Chromosomes (B), and in Reference Population (C)

	A		B		
	N (%)	X^2 (A vs. C)	*N* (%)	X^2 (B vs. C)	C: *N* (%)
HLA B:					
14	1 (3)		0		4 (.8)
w62	5 (15)		9 (30)	6.8[a]	71 (13)
40	16 (47)	48.2[b]	2 (7)		49 (9)
w47	2 (6)	10.7[a]	0		2 (.4)
Others	10 (29)		19 (63)		424 (77)
Total	34 (100)		30 (100)		550 (100)
Complotype:					
S01	8 (23)	8.6[a]	2 (7)		6 (9)
S30	9 (26)	7.3[a]	3 (10)		5 (7)
S31	7 (21)		10 (33)		28 (40)
S42	3 (9)		3 (10)		2 (3)
F91,0	4 (12)	. . .[c]	0		0
S2,1+2	1 (3)		0		0
S4′5′	1 (3)		0		0
S3′9′	1 (3)		0		0
Others	0		12 (40)		29 (41)
Total	34 (100)		30 (100)		70 (100)

[a] P (uncorrected) < .01.
[b] P (uncorrected) < .001.
[c] P = .01, owing to low N values; Fisher's exact test was used.

the affected chromosomes; this frequency was 47%, compared with 7% in the nonaffected chromosomes and 9% in the reference population ($P < .001$ in the χ^2 test). Also of interest was the distribution of the HLA Bw62 antigen. In the reference population its frequency was 13%, whereas in families with CAH it was remarkably higher (table 1). The HLA-DR frequencies showed no statistically significant differences (data not shown).

Only five different complotypes (table 1) were found more than once in the affected chromosomes, whereas in the controls up to eight and 10 different complotypes were found at least twice. Particular complotypes, such as F3+2,0 or S33, that were common in the normal population were absent from the affected chromosomes. The frequencies of S01 (23%), S30 (26%) and F91,0 (12%) were significantly increased in the affected chromosomes as compared with the controls (9%, 7%, and 0%, respectively). Altogether, 20% of the affected chromosomes carried complotypes F91,0, S4′5′, S2,1+2, or S3′9′ (the C4B alleles with ″ are rare variants typed without appropriate references), which were very rare in the controls.

2. *C4 and 21-Hydroxylase Genes*

The DNA samples from members of the 17 families and from the controls were digested with restriction endonucleases *Taq*I, *Kpn*I, *Xba*I, *Eco*RI, *Bgl*II, and *Nco*I and were subjected to Southern analysis with the C4 and CYP21 probes. The location of the restriction fragments relevant to this study are shown in figure 1. The usability of these RFLP markers was first tested by analyzing the control samples. These samples included two homozygous cell lines, "PLH" and "QBL," which at the 10th International HLA Workshop (Dupont 1988) were shown to carry a CYP21B+C4B and a CYP21A+C4B deletion, respectively. In addition, DNA samples from one blood donor homozygous for the HLA B8 S01 DR3 haplotype (this haplotype has been shown to carry a CYP21A+C4A deletion; see, e.g., Schneider et al. 1986; Partanen 1988) and from several controls without any known deletion were studied. The results from the controls (not shown) indicate that the enzymes *Taq*I and *Kpn*I were the most informative in the detection of deletions, confirming the results of many earlier studies (Schneider et al. 1986; White et al. 1988). With *Bgl*II and *Xba*I no distinction could be made be-

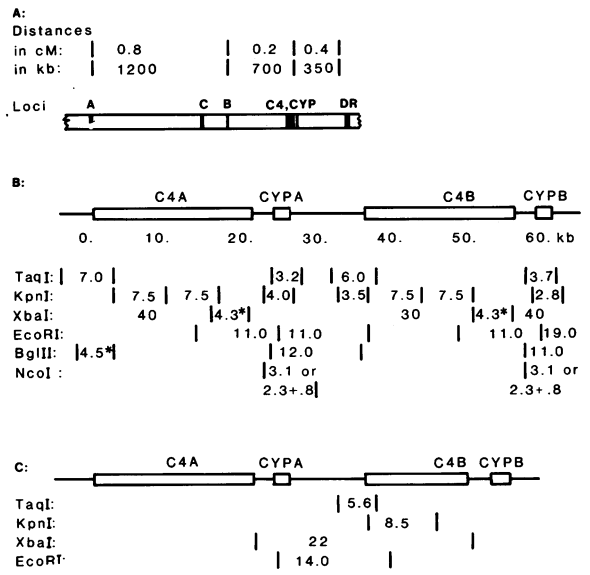

Figure 1 *A:*, Location of CYP21 and C4 genes in the HLA gene complex (not to scale). *B:* and *C:*, Simplified restriction-fragment maps of the C4 and CYP21 genes (*B:*, chromosomes with a long C48 gene; *C:*, chromosomes with a short C4B gene; only differences compared with the long gene are shown). Sizes are shown in kilobases, and polymorphic *Bgl*II and *Xba*I fragments are indicated by an asterisk (*). Maps are based on the following reports: Carroll et al. (1985*a*, 1985*b*), Prentice et al. (1986), and Schneider et al. (1986). The *Nco*I polymorphism has been described by Rodrigues et al. (1987), the 4.5-kb *Bgl*II by Schneider and Rittner (1988) and Partanen et al. (1988), and the 4.3-kb *Xba*I by, among others, Partanen (1988).

tween the CYP21A and CYP21B deletions; nor was the analysis with *Eco*RI and *Nco*I informative in picking up deletions. However, these four enzymes produced RFLP markers that could further subdivide the affected chromosomes.

Table 2 shows the relative band intensities expressed as the ratio of the CYP21B and CYP21A gene-specific RFLP markers in *Taq*I (3.7-kb/3.2-kb) and *Kpn*I (2.8-kb/4.0-kb) digestions and as the ratio of the C4B (5.6-kb, 6.0-kb, and 6.4-kb) and C4A (7.0-kb) gene-specific *Taq*I fragments. Samples without a known deletion gave a ratio of one. Thus, a change in the ratio obviously indicates a gene deletion or duplication.

A homozygous CYP21B+C4B gene deletion was indicated by the absence of the CYP21B and C4B gene-specific fragments, i.e., the ratios were zero in the patients of families 3, 8, and 14 of table 2. The *Taq*I, *Kpn*I, and *Bgl*II RFLP patterns of family 14 are shown in figure 2. The parents, as well as the obvious CAH carriers in these families, were heterozygous for this deletion.

A heterozygous CYP21B+C4B deletion was obvious in six families (i.e., in families 4–7, 10, and 13). In the patients of these families, the ratios of the CYP21 and C4 bands were about 0.5. The reduced ratio of the CYP21 bands was observed with both *Taq*I and *Kpn*I, indicating no discrepancy between the results obtained with these enzymes. The reduced ratios were also found in one of the parents of each family, except in family 13, where the analysis was not informative. Hence the MHC haplotype with the deletion could be determined. The importance of family studies was neatly demonstrated in family 5, where the mother gave equal CYP21A and CYP21B intensity but no C4B gene fragments at all. On the basis of analysis both of the other family members and of the C4B0,0 phenotype of the mother, it was most probable that there was a CYP21B+C4B deletion on one chromosome and a CYP21A+C4B deletion on the other chromosome, the former being the affected one.

In three chromosomes (families 1, 4, and 9), gene conversion of the CYP21B gene to a CYP21A-like gene was plausible (see Discussion). This is best demonstrated in the patient of family 4, who had no CYP21B gene-specific *Taq*I and *Kpn*I bands (table 2) but one C4B gene as deduced from the C4B/C4A ratio of 0.5. In the *Bgl*II and *Xba*I analysis with the CYP21 probe, the patterns (12.0 kb and 11.0 kb with *Bgl*II and 30 kb and 40 kb with *Xba*I; data not shown) indicated the presence of two CYP21 genes (compare with the map in fig. 1).

A possible C4B+CYP21A duplication was found in one affected chromosome (chromosome 17c). The chromosome expressed three C4 allotypes: C4A2, B1, and B2. Southern analysis suggested that there were one C4A gene, two C4B genes of short type, two CYP21A genes, and one CYP21B gene in this chromosome. As we could not analyze the entire family, the presence of the duplication could not be confirmed.

Altogether, the results of table 2 suggest that in the 34 affected chromosomes there were 12 (35%) CYP21B+C4B deletions, three (9%) obvious gene conversions, and one (3%) duplication. In 18 (53%) of the chromosomes, the type of the CYP21B mutation could not be determined, as all the RFLP markers found were also on the nonaffected chromosomes.

*Eco*RI digestions hybridized with the CYP21 probe classified the chromosomes into two subtypes. Sixteen (25%) of the 64 chromosomes in the CAH families had an additional 14-kb band, whereas the rest gave only the normal 19+12-kb pattern. The 14-kb *Eco*RI RFLP marker had an absolute association with the short C4B

Table 2

Relative Band Intensities between CYP21B and CYP21A Gene-specific RFLP Fragments and between C4B and C4A Specific Bands, Determined by Scanning Densitometer

Family No. and Chromosome Origin (clinical form)[a]	HLA B,compl,DR	*Taq*I, 3.7/3.2[b]	*Kpn*I. 2.8/4.0[b]	*Taq*I,C4B/C4A[b]	Interpretation[c]
Homozygous deletion:					
3:					
ab	62,F910,7/62,S33,4	.5	.7	.5	Bb – / +
cd	40,S30,1/62,S33,4	.7	.8	.5	Bb – / +
ac (SW)	62,F910,7/40,S30,1	0	0	0	Bb – /Bb –
8:					
ab	47,F910,7/18,S42,2	.5	.7	.5	Bb – / +
cd	47,F910,7/44,F31, –	.5	.7	.5	Bb – / +
ac (SW)	47,F910,7/47,F910,7	0	0	0	Bb – /Bb –
bc	18,S42,2/47,F910,7	.5	.5	.5	+ /Bb –
14:					
ab	40,S30,1/62,S42,1	.5	.7	.5	Bb – / +
cd	40,S30,1/13,S31,7	.5	.7	.5	Bb – / +
ac (SW)	40,S30,1/40,S30,1	0	0	0	Bb – /Bb –
ac (SW)	40,S30,1/40,S30,1	0	0	0	Bb – /Bb –
bc	62,S42,1/40,S30,1	.7	.5	.7	+ /Bb –
Heterozygous deletion:					
4:					
ab	ND				
cd	44,S45,4/50,S02,4	.5	.7	.7	Bb – / +
ac (SW)	18,F910,7/44,S45,4	0	0	.5	conv/Bb –
5:					
ab	40,S01,8/62,S33,4	.8	.8	1.0	+ / +
cd	40,S30,1/7,S30,1	1.0	1.0	0	Bb – /aB –
ac (SW)	40,S01,8/40,S30,1	.5	.5	.5	+ /Bb –
ac (SW)	40,S01,8/40,S30,1	.3	.3	.5	+ /Bb –
ac (SW)	40,S01,8/40,S30,1	.5	.3	.5	+ /Bb –
ad	40,S01,8/7,S30,1	.7	.6	.5	+ /aB –
bc	62,S33,4/40,S30,1	.3	.2	.5	+ /Bb –
bd	62,S33,4/7,S30,1	.7	.5	.5	+ /aB –
6:					
ab	7,S31,2/8,S01,3	3.2	(2.0)	2.0	+ /Aa –
cd	40,S30,1/7,S31,2	.6	.4	.7	Bb – / +
ac (SV)	7,S31,2/40,S30,1	.6	.4	.7	+ /Bb –
7:					
ab	40,S30,1/18,S31,5	.5	.5	.5	Bb – / +
cd	40,S01,8/62,S30,8	1.0	.9	.9	+ / +
ac (SV)	40,S30,1/40,S01,8	.6	(.5)	.5	Bb – / +
10:					
ab	ND				
cd	40,S01,8/35,F3 + 20,1	1.1	1.0	.8	+ / +
ac (SV)	40,S30,1/40,S01,8	.1	.5	.5	Bb – / +
13:					
ab	40,S01,8/27,S42,6	1.0	1.0	1.0	+ / +
cd	40,S30,2/8,S01,3	.5	.5	2.0	+ /Aa –
ac (SW)	40,S01,8/40,S30,2	.7	.5	.5	+ /Bb – ?
No cross-deletion:					
1:					
ab	62,S39,1/7,S61,7	.8	.8	1.0	+ / +
cd	18,S31,5/40,S31,5	.5	. 5	1.0	conv/ +
ac (SW)	62,S39,1/18,S31,5	.6	.4	.9	+ /conv
ac (SW)	62,S39,1/18,S31,5	(.5)	(.5)	(1.0)	+ /conv

(continued)

Table 2 (continued)

Family No. and Chromosome Origin (clinical form)[a]	HLA B,compl,DR	*Taq*I, 3.7/3.2[b]	*Kpn*I, 2.8/4.0[b]	*Taq*I, C4B/C4A[b]	Interpretation[c]
2:					
ab	40,S01,8/35,F3+20,1	1.0	1.0	1.0	+/+
cd	27,S42,1/40,S02,1	1.0	.9	2.0	+/A−?
ac (SW)	40,S01,8/27,S42,1	1.0	1.0	1.0	+/+
ac (SW)	40,S01,8/27,S42,1	1.1	1.1	1.0	+/+
9:					
ab	18,S31,5/62,S31,−	.1	.6	1.0	conv/+
cd	40,S01,5/62,S31,−	1.0	1.0	1.0	+/+
ac (SW)	18,S31,5/40,S01,5	.5	(.5)	1.1	conv/+
11:					
ab	62,S31,8/44,S31,5	2.0	(2.0)	2.0	+/Bbdupl?
cd	8,S31,−/7,S30,−	1.1	1.1	(1.0)	+/+
ac (SV)	62,S31,8/8,S31,−	1.0	1.2	(1.0)	+/+
12:					
ab	35,S30,5/62,F31,6	.1	(.5)	1.0	?
cd	62,S42,4/57,S61,7	.8	(1.0)	1.0	+/+
ac (SW)	35,S30,5/62,S42,4	.8	(1.0)	1.1	+/+
15:					
ab	27,S42,5/27,F3+20,1	.8	1.0	1.0	+/+
cd	ND				
ac (SW)	27,S42,5/40,S01,8	.8	1.0	1.0	+/+
16:					
ab	40,S01,8/35,S30,6	2.0	2.0	.5	+/aB−
cd	62,S31,8/44,S30,5	2.0	2.0	.5	+/aB−
cd (SW)	40,S01,8/62,S31,8	1.0	1.0	1.0	+/+
17:					
ab	7,S31,2/62,S42,4	1.0	1.0	1.0	+/+
cd	ND				
ac (SW)	7,S31,2/14,21+2,1	.6	.6	.7	+/aBdupl?

[a] Families are grouped into three categories on the basis of patients' RFLP results. The affected chromosomes are always a and c. ab = paternal origin; cd = maternal origin.

[b] Ratios in parentheses were estimated only visually, owing to a high background in the blots.

[c] − = Deletion, with the deleted C4 gene being denoted by A or B and the deleted CYP21 gene by "a" or "b"; + = normal chromosome; ND = not determined; conv = gene conversion of a CYP21B gene to a CYP21A-like gene; dupl = duplication. In addition to the scanning results, the interpretation is based on the *Bgl*II and *Xba*II RFLP markers and on the complement phenotypes.

gene (i.e., 5.6-kb *Taq*I band). This was observed in the affected and nonaffected chromosomes of the CAH families, as well as in the control blood donors (table 3).

3. *MHC Haplotypes Linked to 21-Hydroxylase Deficiency*

Three groups of particular MHC haplotypes were strongly associated with the CYP21B deficiency. Each had a characteristic HLA and complement phenotype and RFLP pattern (table 4). Group I (table 4) comprised eight haplotypes with the phenotype HLA (A24 Cw8) B40 S01 (DRw8). These chromosomes had no CYP21B or C4 deletions. The chromosomes were characterized by a long C4B gene (6.0-kb *Taq*I) and by a 4.3-kb *Xba*I band of C4 genes. In the controls (Partanen 1988), the S01 phenotype was almost exclusively associated with HLA B8 DR3 and carried a particular C4A+CYP21A deletion characterized by a unique 6.4-kb *Taq*I band and by the absence of the 4.3-kb *Xba*I band in the C4 hybridization. Thus, the S01 phenotype in the affected chromosomes was not identical to the S01 phenotype found frequently in the controls.

Group II (table 4) comprised eight chromosomes with the HLA (A2/A3) Cw3 B40 S30 DR1 phenotype. The common feature of group III was the F91,0 DR7 pheno-

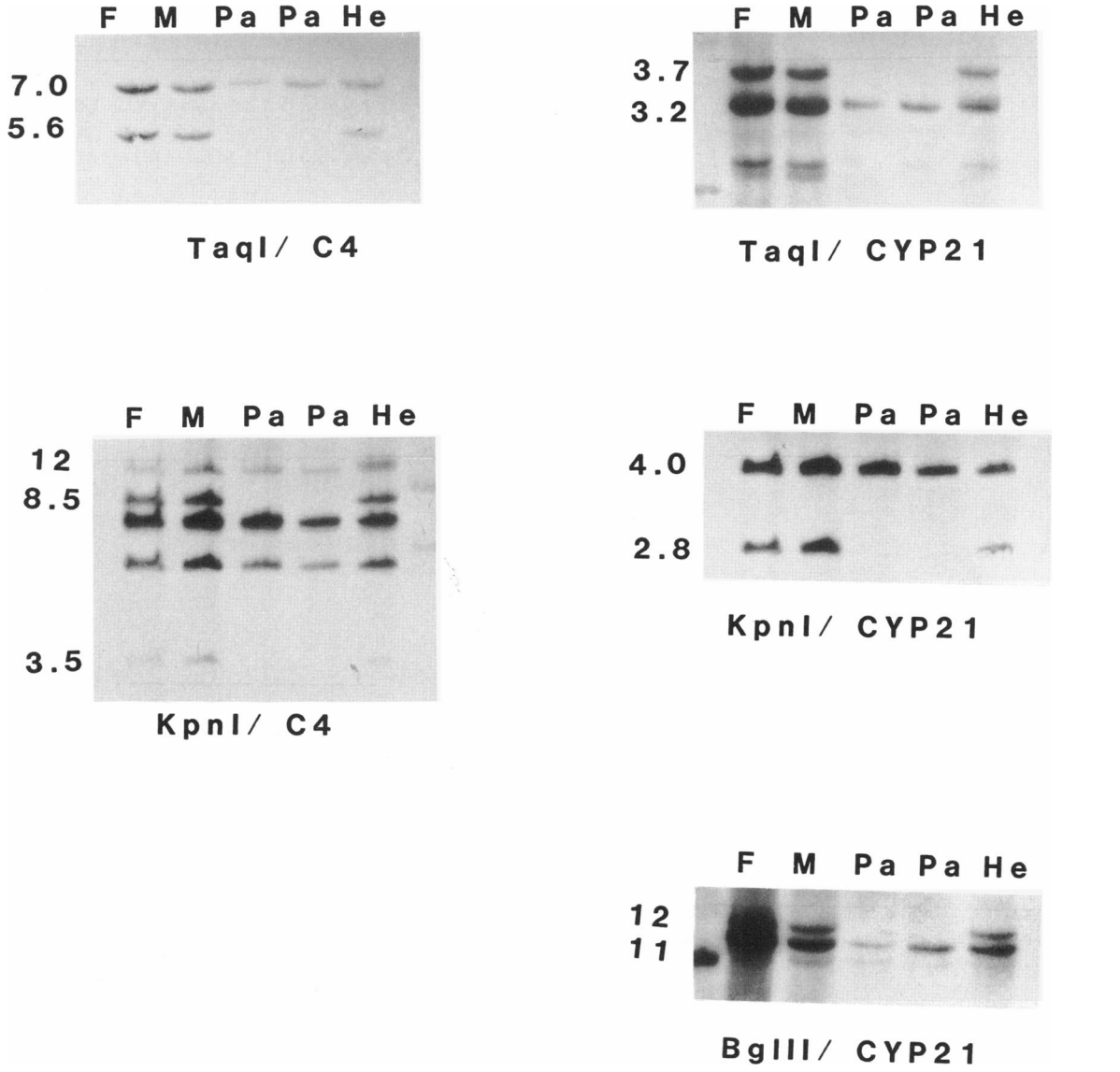

Figure 2 Genomic DNA, from family 14, digested with *Kpn*I, *Taq*I, and *Bgl*II and hybridized with the C4 and CYP21 gene probes. The two patients (Pa) are homozygous for the HLA Cw3 B40 S30 DR1 haplotype. They also have a homozygous CYP21B+C4B gene deletion, as the samples lack the 3.5-kb *Kpn*I and 5.6-kb (and 6.0-kb) *Taq*I fragments of the normal C4B gene and the 2.8-kb *Kpn*I and 3.7-kb *Taq*I fragments of the normal CYP21B gene. With *Bgl*II, only the 11-kb band is seen in these patients. The healthy sibling (He) is an obvious carrier of CAH, as deduced from the relative band intensities and HLA haplotypes (shown in table 2). The healthy chromosome has a short C4B gene giving the 8.5-kb *Kpn*I and 5.6-kb *Taq*I bands.

type, which only in two of the four chromosomes was associated with the expected (Fleischnick et al. 1983) HLA A3 Cw6 Bw47 haplotype. Both of the groups had a CYP21B+C4B deletion. Only two RFLP markers distinguished the two groups from each other. With *Bgl*II, group III, comprising four F91,0 haplotypes, gave an additional 4.5-kb band with the C4 probe, and with *Nco*I and the CYP21 probe it gave 2.3+0.8-kb bands. A 3.1-kb *Nco*I band was found in all the other affected chromosomes. The *Bgl*II RFLP has been mapped to the 5′ end of the C4A gene (Schneider and Rittner 1988) and has been observed in the Finns only with the F91,0 phenotype (Partanen et al. 1988). The *Nco*I RFLP maps to the CYP21A gene (Rodrigues et al. 1987). Altogether, groups I–III covered 20 (59%) of the 34 affected chromosomes.

Table 3

Association between the 5.6-kb *Taq*I RFLP Marker of the Short C4B Gene and the 14.0-kb *Eco*RI RFLP Marker of the CYP21 Gene

	5.6-kb *Taq*I/14.0-kb *Eco*RI			
	+ / +	+ / −	− / +	− / −
Families[a]	16	0	0	48
Blood donors[b]	9	0	0	17

[a] Sixty-four haplotypes of CAH families.
[b] Phenotypic association observed in 26 blood donors (family analysis not possible).

The rest (41%) of the affected chromosomes fell into various subtypes without a strong association with any particular HLA or RFLP pattern. For example, the RFLP patterns classified seven affected chromosomes having the S31 complement phenotype into three DNA variants (data not shown). Two of these chromosomes had an RFLP pattern identical to those of group I; three differed from these as being negative for the polymorphic 4.3-kb *Xba*I band of C4 genes; and two of the S31 phenotypes, both associated with HLA B18 DR5, had the supposed gene conversion described above.

4. *Association between Clinical Forms of Classical CAH and MHC Gene Markers*

A comparison was made between the SV and SW forms of CAH and the genetic markers described above. The clinical form of the disease of each patient is shown in table 2. The only salient finding was that the four index patients—and the HLA identical sibling in family 14—who obviously had no CYP21B genes at all had the more severe, SW form. No particular MHC haplotype or RFLP marker was found only in the SV form. Three of the patients with the SV form were deletion heterozygotes. If it is assumed that some CYP21 activity is required for the SV form, then the sister chromosome should express this activity in these cases. In two of these cases the sister chromosome had the HLA B40 S01 DRw8 phenotype, and in one case it had the HLA B7 S31 DR2 phenotype. The former phenotype, however, was also found in the SW form. Thus there appeared to be no strong association between the clinical subtypes and the genetic markers.

Discussion

The linkage between CAH and the polymorphic HLA gene complex provides a unique possibility to compare genetic markers of CAH in different populations. An increased frequency of up to 20% of a rare HLA Bw47 antigen has been described in patients of various Caucasian populations (Dupont et al. 1980; Coullin et al. 1982; Scholz et al. 1984). In addition, the HLA Bw47 antigen has been associated almost exclusively with a particular MHC haplotype, HLA A3 Cw6 F91,0 DR7 (Fleischnick et al. 1983). We found this MHC haplotype with a frequency of 6% in Finnish patients. In addition to the HLA Bw47 antigen, some population-specific HLA associations have been reported, e.g., by Scholz et al. (1984) and Dupont et al. (1980).

The special feature in Finnish patients was a very high frequency of the HLA B40 antigen: 47% of the chro-

Table 4

Three Most Common MHC Haplotypes Observed among Affected Chromosomes, and their Characteristic RFLP Markers

				Probe[a]:	4	C	C	4	C	C	C	C	C	C	4	4
				Enzyme[b]:	T	T	K	T	T	K	B	B	N	N	N	X
				Size[c]:							1	1				
				(per 100 bp)	7	3	4	6	3	2	2	1	3	2	0	4	4
Group No. (*N*)	MHC Haplotypes				0	2	5	0	7	5	0	0	1	3	8	5	3
I (8)	A24 (6/8)	B40 S01	DR8 (7/8)		+	+	+	+	+	+	+	+	0	0	0	0	+
	Others (2/8)		DR5 (1/8)														
II (8)	A2 (4/8)	B40 S30	DR1 (7/8)		+	+	+	0	0	0	0	+	+	0	0	0	0
	A3 (4/8)		DR2 (1/8)														
III (4)	A3 B47 (2/4)	F91,0 DR7			+	+	+	0	0	0	0	+	0	+	+	+	0
	Others (2/4)																

[a] 4 = C4; C = CYP21.
[b] T = *Taq*I; K = *Kpn*I; B = *Bgl*II; E = *Eco*RI; N = *Nco*I; X = *Xba*I.
[c] The size of each RFLP marker is shown vertically.

mosomes carried it. Family analysis, however, showed that the HLA B40 antigen was found in two totally different MHC haplotypes, either in HLA A24 Cw8 B40 S01 DRw8 or in HLA A2/3 Cw3 B40 S30 DR1. These actually were the two most common affected haplotypes. That these two MHC haplotypes covered almost one-half of the affected chromosomes but were very rare in the controls will help in, e.g., prenatal diagnosis of CAH. However, a possible heterogeneity in the CYP21B mutation in the HLA B40 S01 DRw8 haplotypes should first be tested using a mutation-specific DNA probe which can be constructed after sequencing of the CYP21B mutant gene. The most probable explanation for the high frequency of these two MHC haplotypes is a founder effect. It is interesting that Layrisse et al. (1987) also found a strong association between CAH and the B39 and Bw62 antigens in the Venezuelan population. Thus, patients of each ethnically well-defined population may carry characteristic MHC markers. The lack of strong association, except for that with HLA Bw47, in most studies of CAH may result from the mixed ethnic origin of the patients studied. Furthermore, frequent de novo mutations can be expected because CAH affects fertility.

As the CYP21 genes are located very close to the C4 genes (Carroll et al. 1985*a*; White et al. 1985), the determination of the complotypes was clearly relevant, especially as up to 20% of the affected chromosomes carried complotypes very rare in the controls. Moreover, the F91,0 complotype could be observed in four haplotypes but only twice with the expected HLA Bw47 antigen. Unfortunately, only limited data of complotypes in CAH are available (Pollack et al. 1981; Fleischnick et al. 1983). However, it should be noted that in some cases one complement phenotype, such as S31 and S01 in our study, can actually comprise several different RFLP variants.

We found that 35% of the affected chromosomes had an obvious CYP21B+C4B deletion. These deletions were exclusively associated with only two MHC haplotypes, (HLA Bw47) F91,0 DR7 and HLA B40 S30 DR1. Genomic cloning studies (Yu et al. 1986) have strongly suggested that the HLA Bw47 F91,0 DR7 haplotype carries a combined CYP21B+C4B gene deletion. The RFLP studies by other investigators and us are in accordance with this, as digestions with various endonucleases all pointed to the deletion. The two types of chromosomes with the CYP21+C4B deletion could be distinguished from each other by the BglII and *Nco*I digestions, which suggests their independent origin. With the other enzymes, the results of the HLA B40 S30 DR1 haplotypes were identical to those of the F91,0 phenotype. We therefore concluded that the B40 S30 DR1 haplotypes also had a CYP21B+C4B deletion. A deletion of about 30 kb in these two haplotypes also has been observed when pulsed-field gel electrophoresis has been used (J. Partanen et al., submitted), confirming the results derived from the standard Southern analysis. By comparison with many other genetic defects, CAH results relatively frequently from gene deletion. As frequent deletions of the CYP21A with the C4A or C4B gene have also been found in the normal population (e.g., Schneider et al. 1986; present report), this region may be exceptionally prone to deletions. Furthermore, frequent gene conversion types of events between the CYP21 genes (Donohoue et al. 1986; Harada et al. 1987; Matteson et al. 1987) indicate the intriguing genetic flexibility of this region. Harada et al. (1987) showed that the chromosomes giving only the 3.2-kb *Taq*I band but both the 11-kb and the 12-kb *Bgl*II bands with the CYP21 probe actually carried two CYP21A-like genes. One was located 3′ of the C4A gene, and the other was located 3′ of the C4B gene. We could assume the same kind of gene conversion in the three haplotypes of our report. If the CYP21B+C4B deletion indeed exists in 35% of the chromosomes and if gene conversion exists in the three chromosomes (9%), then at least 44% of the chromosomes lacked the functional CYP21B gene. In the other chromosomes the molecular basis of the CYP21 deficiency remained unclear. We tested six endonucleases, but these samples gave only the RFLP patterns found also in nonaffected chromosomes.

The enzymes *Bgl*II, *Eco*RI, and *Xba*I were not informative for the deletion mapping. White et al. (1988) have shown that most of the CYP21B deletions result in a hybrid gene with a 5′ end from the CYP21A gene and with a 3′ end from the CYP21B gene. Their findings explain the inability to distinguish between the CYP21A and CYP21B deletion in the *Bgl*II, *Xba*I, and *Eco*RI digestions. These enzymes, however, gave informative RFLP markers. An association between the polymorphic 14.0-kb *Eco*RI band of the CYP21 genes and CAH has been reported by many investigators (Mornet et al. 1986; Keller et al. 1987). We observed this RFLP marker in all the chromosomes having a short C4B gene. This suggests that the presence or absence of this *Eco*RI band per se does not tell us about the nature of CYP21 mutations. Determination of whether this CYP21 RFLP marker indeed derives from the 6.8-kb deletion in the short C4B gene (Prentice et al. 1986) requires more-

detailed mapping. Moreover, by *XbaI* analysis we could dissect, e.g., the S31 phenotypes into two groups, which clearly indicates their independent origin.

The more severe, SW form obviously results from an inability to synthesize both mineralocorticoids and glucocorticoids. Thus, the presence of the SW form in the four patients with a homozygous absence of the CYP21B gene was understandable, since by definition they cannot have a functional CYP21 enzyme. This, however, appeared to be the only clear association between the clinical subtypes and the MHC gene markers.

To summarize, we were able to show that in small and strictly defined populations, such as the Finns, the MHC phenotypes and RFLP patterns of the CYP21 genes were fairly homogeneous. The three most common MHC haplotypes, each with a characteristic RFLP pattern, accounted for 59% of the affected chromosomes. Determination of whether each of these haplotypes indeed carries only one unique CYP21B mutation awaits more-detailed mapping. If the variation in these mutations proves to be limited, both genetic screening of CAH and prenatal therapy of the affected fetus become more realistic. However, it should be noted that 41% of the mutations might represent more or less unique variants.

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