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Frequent Deletion and Duplication of the Steroid 21-Hydroxylase Genes

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SUMMARY

Congenital adrenal hyperplasia due to 21-hydroxylase (21-OHase) deficiency is an HLA-linked disorder resulting from a mutation in the *21-OHase B* gene encoding the adrenal cytochrome P450 specific for steroid 21-hydroxylation. To identify polymorphisms associated with 21-OHase deficiency, DNA samples from 22 unrelated patients with this disorder were examined with a human cDNA clone encoding the enzyme. Deletions of the active *21-OHase* gene were found in almost one-fourth of classical *21-OHase* deficiency alleles. In contrast, mild, "nonclassical" 21-OHase deficiency is associated with a duplicated *21-OHase* gene.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an inherited disorder of cortisol biosynthesis, caused in 95% of cases by a deficiency in steroid 21-hydroxylation, the penultimate step in the biosynthetic pathway [1, 2]. Because 17-hydroxyprogesterone (17-OHP) is not converted to 11-deoxycortisol, it accumulates and is shunted into the androgen biosynthesis pathway. Virilization is therefore a prominent feature of this disorder. In classical 21-hydroxylase (21-OHase) deficiency, which occurs in one of 5,000–10,000 births, symptoms begin prenatally, so that females are born with ambiguous genitalia. In more

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than half of classical cases, aldosterone biosynthesis is also impaired, and untreated individuals may die in the neonatal period from an inability to conserve urinary sodium ("salt-wasting"). In nonclassical 21-OHase deficiency, individuals remain asymptomatic or develop symptoms of androgen excess in childhood or at puberty; this extremely common, relatively mild syndrome is found in 0.3% of the general Caucasian population and in 1%–3% of European Jews [3].

This disorder is linked to the HLA major histocompatibility complex [4] on the short arm of chromosome 6 and is associated with particular HLA antigens or haplotypes [5]. Salt-wasting 21-OHase deficiency is associated with *HLA-A3;Bw47;DR7* and *HLA-Bw60* (40), while classical disease without salt-wasting, also called "simple virilizing" 21-OHase deficiency, is associated with *HLA-B51* (5). Nonclassical disease often occurs on the haplotype *HLA-B14;DR1*. The haplotype *HLA-A1;B8;DR3* is negatively associated with 21-OHase deficiency. These characteristic HLA haplotypes include particular allotypes for the HLA-linked complement proteins notably the fourth component of serum complement, C4 [6, 7]. The *Bw47;DR7* haplotype associated with salt-wasting 21-OHase deficiency includes the complotype *C4A*1; C4B*Q0(null);Bf*F;C2*C*, while the *B8;DR3* haplotype is *C4A*Q0;C4B*1; Bf*S;C2*C*. The *B14;DR1* haplotype includes a duplicated *C4B* gene [8, 9]: *C4A*2;C4B*1;C4B*2;Bf*S;C2*C*.

The HLA-linked gene affected in 21-OHase deficiency has been identified as a structural gene for the cytochrome P450 specific for steroid 21-hydroxylation [10]. Study of human cosmid clones has shown that there are two 21-OHase genes, each located immediately adjacent to the 3′ end of one of the two *C4* genes [11]. The *21-OHase B* gene is deleted on the *Bw47;DR7* haplotype associated with severe 21-OHase deficiency, while hormonally normal individuals who are homozygous for the *B8;DR3* haplotype have a homozygous deletion of the *21-OHase A* gene. This suggests that the *21-OHase A* gene product is not active in cortisol synthesis, and DNA sequence analysis has confirmed that the *21-OHase A* gene is a pseudogene [12].

While study of these characteristic haplotypes has yielded useful information about the function of the *21-OHase* genes, most cases of classical 21-OHase deficiency do not occur in association with such haplotypes. It therefore was of interest to examine additional patients with 21-OHase deficiency who did not carry the *Bw47;DR7* haplotype known to carry a deletion of the *21-OHase B* gene. We found that deletions of the *21-OHase B* gene are a common cause of classical 21-OHase deficiency. In contrast, nonclassical 21-OHase deficiency on the *B14;DR1* haplotype is associated with a duplicated *21-OHase* gene.

MATERIALS AND METHODS

Patient Evaluation

Patients with 21-OHase deficiency were followed by the Division of Pediatric Endocrinology at New York Hospital. Diagnostic evaluation included determination of serum 17-OHP levels before and 1 hr after intravenous administration of ACTH (Cortrosyn). Patients were diagnosed as having classical or nonclassical disease by comparing these

test results with published nomograms [13]. Fusion of labioscrotal folds in females was considered diagnostic of classical disease. Salt-wasting disease was diagnosed if electrolyte disturbances were documented in infancy or if inability to conserve sodium during a low-sodium diet and elevated plasma renin to aldosterone ratios were found during impatient evaluation while the patient was off mineralocorticoid replacement therapy. HLA typing was performed by standard microcytotoxicity methods [14].

Hybridization Studies

Plasmid pC21/3c was used as a hybridization probe. This plasmid was isolated from a human fetal adrenal cDNA library and contains a 2.0-kilobase (kb) insert corresponding to the complete 21-OHase mRNA, except for about 30 base pairs (bp) at the 5′ end [12]. The insert was separated from the vector by agarose gel electrophoresis after digestion with restriction endonuclease *Bam*HI [15]. It was labeled with [^{32}P]dATP to a specific activity of 2×10^8 dpm/mcg by nick-translation [16].

DNA samples were prepared from leukocytes as described [17] and digested overnight with 4 U/mg of various restriction endonucleases (purchased from Boehringer-Mannheim and used according to the manufacturer's directions). Digests were subjected to agarose gel electrophoresis; 0.7% gels were used except when analyzing *Taq*I digests, which were best resolved on 1.0% gels. Ten micrograms were loaded in each lane of a 20-cm-long gel and run at about 1 V/cm for 40 hrs. Gels were blotted to nitrocellulose as described [18]. Blots were prehybridized for 2 hrs at 65°C in 0.9 M NaCl/0.09 M Na citrate, pH 7.0/0.5% sodium dodecyl sulfate (SDS)/0.1%. Ficoll 400/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/100 mg denatured herring sperm DNA per ml. Hybridizations were performed overnight in the same solution with the addition of 10% dextran sulfate and 10^7 dpm/ml of boiled radioactive probe; 100 μl of hybridization solution were used per cm^2 of blot. Blots were washed twice for 10 min each at room temperature in 0.3 M NaCl/0.03 M Na citrate/0.5% SDS and three times for 1 hr each at 65°C in 0.03 M NaCl/0.003 M Na citrate/0.5% SDS. Blots were autoradiographed for about 2 days on Kodak XAR film using an intensifying screen at −70°C. Blots of *Taq*I digests were evaluated by scanning densitometry using a Beckman DU8 spectrophotometer.

RESULTS

Samples from 22 unrelated patients (table 1) were examined. These patients included 11 with the severe, salt-wasting (SW) form of 21-OHase deficiency and four with classical disease without salt-wasting (simple virilizing disease, SV). No patients with the *HLA-Bw47;DR7* haplotype were examined since the deletion of the *21-OHase B* gene on this haplotype has been established. Seven patients had mild, nonclassical (NC) disease, all of these carried at least one *B14;DR1* haplotype. Hormonal data obtained from two of these patients, both males, were also consistent with SV disease.

When DNA samples were digested with restriction endonuclease *Taq*I and hybridized with pC21/3c, two bands of 3.2 and 3.7 kb were detected (fig. 1). These are carried by the *21-OHase A* and *B* genes, respectively. These bands are of equal relative intensity in most normal individuals, and so decreased relative intensity of one band can, in general, be taken as evidence of a heterozygous deletion of one gene. Six of 11 SW patients and one of four SV patients had decreased intensity of the 3.7-kb band consistent with a heterozygous deletion of the *B* gene. No *HLA-B;DR* haplotype occurred more than once among patients with deletions of the *B* gene. One SW patient and one SV

TABLE 1

LIST OF PATIENTS WITH 21-OHase DEFICIENCY

PATIENT NO.	HLA TYPE				DIAGNOSIS	RATIO B/A	*Eco*RI POLY	RESULT
	B	*DR*	*B*	*DR*				
1	*18*	*1*	*18*	*5*	SW	0.43	–	B del
2	*27*	*2*	*27*	*4*	SW	0.40	–	B del
3	*7*	*7*	*15*	*4*	SW	0.41	–	B del
4	*35*	*7*	*w60*	*7*	SW	0.51	–	B del
5	*w60*	*1*	*w60*	*4*	SW	0.55	–	B del
6	*15*	*5*	*35*	*5*	SW	0.60	–	B del
7	*8*	*3*	*35*	*4*	SW	2.59	–	A del
8	*w22*	*4*	*39*	*4*	SW	0.90	+	– –
9	*8*	*4*	*27*	(4)	SW	1.10	–	– –
10	*w22*	*4*	*14*	(4)	SW	0.98	+	– –
11	*13*	*7*	*w60*	*w6*	SW	1.05	+	– –
12	*7*	*1*	*w60*	(1)	SV	0.95	–	– –
13	*7*	*6*	*44*	*1*	SV	0.28	–	B del
14	*39*	*1*	*39*	*4*	SV	1.62	–	A del
15	*15*	–	*18*	*5*	SV	0.95	–	– –
16	*14*	*1*	*5*	*2*	SV?	0.77	+	dup?
17	*14*	*1*	*14*	*1*	SV?	0.43	+	dup
18	*14*	*1*	*14*	*1*	NC	0.39	+	dup
19	*14*	*1*	*14*	*1*	NC	0.45	+	dup
20	*14*	*1*	*44*	*1*	NC	0.41	+	B del +dup?
21	*14*	*1*	*7*	*3*	NC	0.79	+	dup?
22	*14*	*1*	*w52*	*w6*	NC	0.76	+	dup?

NOTE: *HLA-B;DR* haplotypes are shown; DR antigens in parentheses indicate possible DR homozygosity that cannot be distinguished from a "blank" *DR* allele on the basis of available family data. Diagnosis of each patient is listed: SW, salt-wasting 21-OHase deficiency; SV, simple virilizing disease; NC, nonclassical disease. Patients 16 and 17 have SV disease by hormonal testing but carry the *HLA-B14;DR1* haplotype associated with NC disease; they are marked "SV?". In Southern blots of *Taq*I digests of human genomic DNA, the 21-hydroxylase probe hybridizes to fragments of 3.7 and 3.2 kb, corresponding to the *21-OHase B* and *A* genes, respectively; the ratio of intensity of hybridization in these bands, as determined by scanning densitometry, is shown. The presence (+) or absence (–) of 13- or 14-kb bands in *Eco*RI digests is also indicated. These results are interpreted: B del, heterozygous deletion of the *21-OHase B* gene; A del, heterozygous deletion of the *21-OHase A* gene; dup, homozygous duplication of a *21-OHase* gene; dup?, possible heterozygous duplication of a *21-OHase* gene; – –, normal pattern. The criteria for these scorings are described in the text.

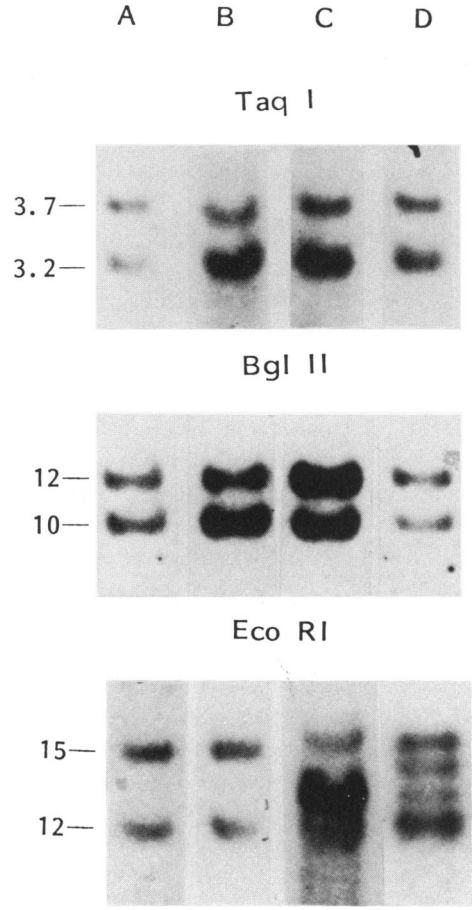

FIG. 1.—Examples of hybridization patterns using pC21/3c as a hybridization probe. The restriction endonuclease used for each digestion is indicated *above each row*, and sizes of hybridizing fragments (in kb) are shown *to the left of each row*. *Each column* represents a different individual: *A*, normal; *B*, salt-losing 21-OHase deficiency with deletion of *21-OHase B* gene; *C*, nonclassical disease, homozygous for *HLA-B14;DR1*; *D*, nonclassical disease, heterozygous for *B14;DR1*.

patient had decreased intensity of the 3.2-kb band consistent with deletion of the *A* gene; the SW patient carried the *A1;B8;DR3* haplotype, which is known to carry such a deletion. No patients with homozygous deletions were found in this study; one parent carried the same deletion as the patient in the five families where this was tested. Excluding the *Bw47;DR7* haplotypes that have previously been studied, seven of 30 or 23% of classical *21-OHase* deficiency alleles may carry deletions of the *21-OHase B* gene.

All three patients who were homozygous for *HLA-B14;DR1* had increased intensity of the 3.2-kb *Taq*I band in a pattern indistinguishable from patients with a heterozygous deletion of the *21-OHase B* gene. However, three of four patients who were heterozygous for *B14;DR1* had only a small difference in intensity between the two *Taq* I bands (the fourth patient had a readily apparent

difference in intensity). Because this haplotype is known to carry a third *C4* gene, it seemed possible that the pattern seen in *B14;DR1* homozygotes was due not to a heterozygous deletion of the 3.7-kb fragment but, rather, to a homozygous duplication of the 3.2-kb fragment, which might yield a similar 2:1 difference in intensity between the two bands. A heterozygous duplication would result in a 3:2 difference in intensity that would not be as readily detectable.

Samples were therefore digested with additional restriction endonucleases (fig. 1). After digestion with *Bgl*II, normal DNA contains two fragments of 10 and 12 kb that hybridize with the probe at equal intensity. The 12-kb band is missing in DNA from an individual homozygous for a deletion of the *21-OHase B* gene on the *Bw47;DR7* haplotype, and all eight individuals with presumed heterozygous deletions of the *21-OHase B* gene have diminished intensity of the 12-kb *Bgl*II fragment relative to the 10-kb fragment. In contrast, DNA samples from the three NC patients who are homozygous for the *B14;DR1* haplotype have *increased* relative intensity of the 12-kb fragment. A difference in relative intensity of these two bands cannot be reliably demonstrated in individuals who are heterozygous for *B14;DR1*.

The probe hybridizes to *Eco*RI fragments of 16 and 12 kb in DNA from most normal individuals and in all patients with a presumed heterozygous deletion of the *21-OHase B* gene. The cDNA probe has an *Eco*RI site; DNA from an individual with a homozygous deletion of the *21-OHase B* gene yields a relatively intense band at 16 kb, corresponding to the 3′ end of the *21-OHase A* gene, and a fainter band at 12 kb, corresponding to the 5′ end of the *21-OHase A* gene. A difference in intensity between these two bands is difficult to detect in DNA from individuals with a heterozygous deletion. In addition to these bands, samples from individuals carrying *HLA-B14;DR1* all displayed a band of 14 kb, which was sometimes resolved into two bands at 14 and 13 kb. These extra bands were also seen in DNA from three patients who did not carry *HLA-B14,* and they are also found in about one-third of normal individuals who do not carry *B14;DR1* (unpublished observations). It is likely that, in individuals who do not carry *B14;DR1,* this extra band represents a frequent polymorphism of the 3′ end of the *21-OHase B* gene, rather than a third *21-OHase* gene.

DISCUSSION

A single *C4* and *21-OHase* gene carried on about 30 kb of DNA were duplicated in the past, resulting in the present arrangement of two *21-OHase* genes alternating with two *C4* genes. Deletions or further duplications of one *C4* and/or one *21-OHase* gene could occur by unequal crossing over due to misalignment of the closely spaced homologous genes during meiosis. Such an unequal exchange between two sister chromatids would result in one set of *C4* and *21-OHase* genes on one chromosome and three sets on the other. The arrangements observed on the *HLA-B8;DR3* and *Bw47;DR7* haplotypes, which each carry only one *C4* gene and one *21-OHase* gene, and the *B14;DR1* haplotype, which contains three sets of *C4* and *21-OHase* genes, presumably resulted from such events. While all cases of 21-OHase deficiency on the *Bw47;DR7* hap-

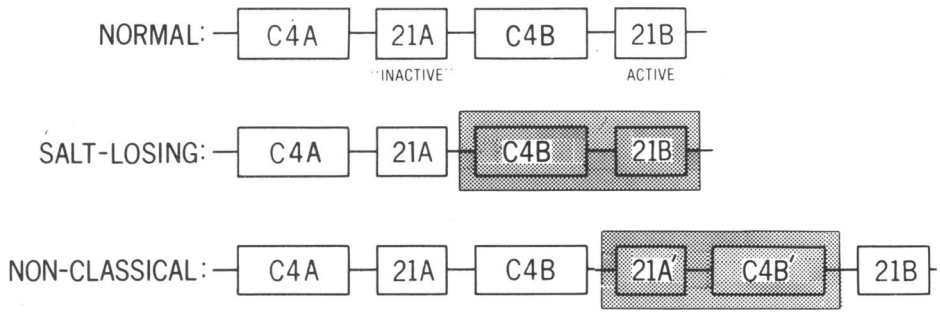

FIG. 2.—Schematic of the genes encoding the fourth component of serum complement (C4A and B) and 21-OHase (21A and B) in normal individuals and patients with 21-OHase deficiency. All genes are transcribed *from left to right*. The genes are located between *HLA-B* and *HLA-DR*, but their orientation on the chromosome has not been determined. In some but not all patients with severe, salt-losing 21-OHase deficiency, the 3.7-kb *Taq*I fragment carried by the *21-OHase B* gene is deleted. When this occurs on the *HLA-Bw47;DR7* haplotype, there is also a null allele at the *C4B* locus, presumably due to a deletion of all or part of the *C4B* gene. These deletions are diagrammed by shading of the *C4B* and *21-OHase B* genes, but the borders of the deletions are not known and probably vary in individuals with different salt-losing alleles; in some cases, the *C4B* gene may be completely unaffected. In nonclassical disease on the *HLA-B14;DR1* haplotype, the *C4B* gene and a *21-OHase* gene appear to be duplicated; the latter carries a 3.2-kb *Taq*I fragment like the *21-OHase A* gene. These duplicated genes are shaded; their actual arrangement is not yet known.

lotype may be descended from the same ancestral mutation, about one-fourth of classical alleles in this study carried deletions of the *21-OHase B* gene on a variety of HLA haplotypes. These are presumed to represent independent events, suggesting that unequal crossing over occurs relatively frequently as a cause of 21-OHase deficiency. Figure 2 is a schematic of this chromosomal region in the different forms of 21-OHase deficiency.

It is unclear whether the extra *21-OHase* gene on the *B14;DR1* haplotype contributes to the development of the mild, NC phenotype. It appears from both protein and molecular genetic analysis that a *C4B* gene has been duplicated, but the extra *21-OHase* gene carries a 3.2-kb *Taq*I fragment characteristic of the *21-OHase A* gene. If it is, in fact, a duplicated *A* gene, the gene product is expected to be enzymatically inactive, and the duplication is simply an associated polymorphism that does not contribute to 21-OHase activity. Determining the DNA sequences of the *21-OHase* genes on this haplotype would be expected to answer this question.

Based on the known associations of different forms of 21-OHase deficiency with particular HLA antigens, the inheritance of this disorder is consistent with the existence of multiple alleles (SW, SV, NC) at a single locus. On the molecular level, there is normally one active *21-OHase* gene on each chromosome. A complete deletion of the active *21-OHase B* gene is associated with a severe, SW allele on that chromosome; frameshifts, nonsense mutations, and presumably some point mutations would also result in complete abolition of the activity of the encoded enzyme. Other point mutations might affect the activity of the enzyme relatively mildly, resulting in SV or NC alleles. A single SV or NC gene probably results in enough 21-OHase enzyme activity to prevent SW

disease, even if the *21-OHase B* gene on the other chromosome is absent. Most nondeletional mutations cannot be detected by the methods used in this study. It is thus not possible to distinguish whether an individual with a heterozygous deletion of the *21-OHase B* gene has an SW, SV, NC, or unaffected phenotype, and it is not surprising that heterozygous deletions of the *21-OHase B* gene were found among the SV patients examined here.

While it appears that the *Bw47;DR7* haplotype always carries a deletion of the *21-OHase B* gene and the *B14;DR1* haplotype an extra *21-OHase* gene, in general, it cannot be assumed that every case of 21-OHase deficiency associated with a characteristic HLA antigen has the same mutation. For example, SW 21-OHase deficiency is strongly associated with HLA-Bw60 (40) and two SW patients in this study are heterozygous for this antigen, yet only one appears to have a heterozygous deletion of the *21-OHase B* gene. One SW patient is homozygous for HLA-Bw60, yet he has only a heterozygous deletion of the *B* gene, not a homozygous deletion. Thus, only two of four SW alleles associated with HLA-Bw60 carry a deletion of the *21-OHase B* gene.

Although the nomograms for 17-OHP levels appear to be reliable for diagnosis of 21-OHase deficiency, in some cases, hormonal testing, HLA typing, and hybridization analysis may yield different results. For example, *B14;DR1* is associated with NC 21-OHase deficiency and a third *21-OHase* gene, yet one patient homozygous for this haplotype and one heterozygote have been diagnosed as having SV disease on the basis of 17-OHP levels. While additional mutations may have occurred changing the usual NC alleles to more severely affected SV alleles, it is also possible that epigenetic factors have resulted in unusually high 17-OHP levels in patients who genotypically have NC 21-OHase deficiency. It has also been suggested that epigenetic factors can affect development of the SW phenotype [19]. Such factors might be best studied in patients who have been genotyped by hybridization methods. In addition to a cDNA probe as employed here, specific oligonucleotide probes [20] might be used to identify specific mutations after DNA sequence analysis is used to identify nondeletional mutations resulting in 21-OHase deficiency.

NOTE ADDED IN PROOF: Several other papers on this subject have been published recently [21–23].

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