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Genetics

HLA-linked congenital adrenal hyperplasia results from a defective gene encoding a cytochrome P-450 specific for steroid 21-hydroxylation

(21-hydroxylase deficiency/recombinant DNA probe/DNA hybridization/inborn error of metabolism/prenatal diagnosis)

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ABSTRACT **We have determined the molecular genetic basis of congenital adrenal hyperplasia due to 21-hydroxylase (21-OHase) deficiency. This common disorder of cortisol biosynthesis is *HLA*-linked. The haplotype *HLA-(A3);Bw47;DR7* is strongly associated with 21-OHase deficiency and always carries a null allele at the locus encoding the C4A (Rodgers) form of the fourth component (C4) of complement. It seemed likely that this haplotype carries a deletion encompassing the genes encoding both C4A and 21-OHase. We hypothesized that the *HLA*-linked defect involved a structural gene for the adrenal microsomal cytochrome P-450 specific for steroid 21-hydroxylation. Using a plasmid with a 520-base-pair bovine adrenal cDNA insert encoding the middle third of the cytochrome P-450 polypeptide, we compared hybridization patterns in DNA from normal and 21-OHase-deficient individuals. Normal human DNA yielded two fragments that hybridized with the probe after digestion with either restriction endonuclease *Eco*RI [12- and 14-kilobase (kb) fragments] or *Taq* I (3.7 and 3.2 kb). One of these bands (the first mentioned in each digest) was absent in DNA from a cell line derived from a patient homozygous for *HLA-Bw47*. DNA from six unrelated patients homozygous for 21-OHase deficiency who were heterozygous for *HLA-Bw47* yielded diminished relative intensity of the 3.7-kb *Taq* I band in five patients, consistent with a heterozygous deletion, and complete disappearance of the 3.7-kb band in one. This deletion segregated with *HLA-Bw47* in a large pedigree carrying 21-OHase deficiency and *HLA-Bw47*. Thus, 21-OHase deficiency sometimes results from the deletion of a specific cytochrome P-450 gene and sometimes, presumably, from smaller mutations. This gene is probably located very near the *C4A* gene.**

Congenital adrenal hyperplasia affects approximately 1 in 5000 births and, thus, is one of the most common inborn errors of metabolism (1). It results from a deficiency in one of the enzymes of cortisol biosynthesis. In 95% of the cases, 21-hydroxylation is impaired in the zona fasciculata of the adrenal cortex (2), so that 17-hydroxyprogesterone (17-OHP) is not converted to 11-deoxycortisol. Due to deficient cortisol synthesis, corticotropin (ACTH) levels increase, resulting in overproduction and accumulation of cortisol precursors, particularly 17-OHP, proximal to the block. This causes excessive production of androgens, resulting in virilization of varying degree. In female newborns, the external genitalia are masculinized (gonads and internal genitalia are normal). Postnatally, untreated males as well as females may manifest rapid somatic growth, penile or clitoral enlargement, precocious adrenarche, and ultimately early epiphyseal closure and short stature. In about half of the cases, there is an additional defect in aldosterone synthesis (conversion of progesterone to 11-deoxycorticosterone) in the zona glomerulosa (2). If untreated, this can result in shock or death in the neonatal period from inability to conserve urinary sodium ("salt wasting").

Congenital adrenal hyperplasia due to 21-hydroxylase (21-OHase) deficiency is inherited as a monogenic autosomal recessive trait closely linked to the human *HLA* major histocompatibility complex (3). Different forms of 21-OHase deficiency are associated with genes for characteristic HLA antigens (4). An isolated defect in cortisol synthesis, referred to as "simple virilizing" disease, is typically associated with *HLA-Bw51/5*. *HLA-(A3);Bw47;DR7* and *HLA-Bw60/40* are associated with the severe, "salt-wasting," form of the disease. There are two related "variant" forms associated with *HLA-B14;DR1* (5) in which patients have a relatively mild defect in cortisol synthesis, as measured by elevated baseline levels of 17-OHP, and either remain asymptomatic ("cryptic" form) or develop symptoms of virilization in late childhood or at puberty ("late-onset" form).

In addition to these associations with HLA-B;DR antigens, genetic linkage disequilibrium has been documented between complement allotypes and 21-OHase deficiency (6, 7). Of greatest interest, patients carrying the HLA-Bw47 antigen invariably have the unique haplotype *HLA-Bw47;C4A*Q0 (null);C4B*3;Bf*F;C2*C*, so that they have simultaneous deficiencies of 21-OHase activity and the C4A (Rodgers) form of the fourth component of complement (C4). The HLA-Bw47(w4) antigen is serologically very similar to the more common antigen HLA-B13(w4), and these two antigens form identical banding patterns on isoelectric focusing (8). Therefore, we proposed that the haplotype *HLA-Cw6;Bw47;21-OHase*NULL;C4A*Q0;C4B*3;DR7* arose by a large genetic deletion or rearrangement of the original *HLA-Cw6;B13;21-OHase*NORMAL;C4A*3;C4B*1;DR7* haplotype (7). This implied that genomic DNA containing the loci for 21-OHase and C4A might be identified by comparing DNA from an individual with 21-OHase deficiency who was homozygous for *HLA-Bw47* and DNA from a normal individual homozygous for *HLA-B13*.

The underlying biochemical defect in 21-OHase deficiency has never been directly determined. However, *in vitro* steroid 21-hydroxylation can be produced with two microsomal proteins from the adrenal cortex, an NADPH-dependent cytochrome reductase and a cytochrome P-450 specific for steroid 21-hydroxylation termed P-450$_{C21}$ (9). P-450$_{C21}$ is a heme-containing monooxygenase with a molecular weight of 52,000 and is one of four cytochromes P-450 required to con-

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Abbreviations: P-450$_{C21}$, cytochrome P-450 specific for steroid 21-hydroxylation; 21-OHase, steroid 21-hydroxylase; C4, fourth component of complement; ACTH, corticotropin; bp, base pair(s); kb, kilobase(s); 17-OHP, 17-hydroxyprogesterone.

vert cholesterol to cortisol (10). As P-450$_{C21}$, but not cytochrome reductase, is substrate specific, we hypothesized that the *HLA*-linked defect involved a structural gene for this protein and isolated a bovine adrenal cDNA clone encoding part of P-450$_{C21}$ (11) in order to test this hypothesis. We now report that a P-450$_{C21}$ structural gene is deleted in patients carrying 21-OHase deficiency on the *HLA-Bw47* haplotype.

MATERIALS AND METHODS

DNA Probe. Plasmid pC21a (11) contains a 520-base-pair (bp) insert in the *Pst* I site of pBR322 that encodes approximately the middle third of the P-450$_{C21}$ polypeptide. Plasmid DNA was prepared (12) and digested with restriction endonuclease *Pst* I, yielding, in addition to the vector, 430- and 90-bp fragments. The 430-bp fragment was purified by sucrose gradient sedimentation and labeled by nick-translation (13) with [^{32}P]dATP at 3000 Ci/mmol (1 Ci = 37 GBq) to a specific activity of 10^9 dpm/μg.

Patient Evaluation. Plasma levels of 17-OHP were measured in patients with 21-OHase deficiency, parents, and sibs before and after an intravenous infusion of ACTH. Individuals were identified as homozygous or heterozygous carriers of 21-OHase deficiency by means of published nomograms (14). *HLA* typing was performed as described (15, 16).

Cell Lines. Continuously proliferating lymphoblastoid cell lines were created by *in vitro* transformation with Epstein–Barr virus (17). Cell line PLH was derived from a patient with 21-OHase deficiency who was homozygous for *HLA-Bw47;DR7*, and line GMA, from a normal individual homozygous for *HLA-B13*.

DNA Analysis. DNA was extracted from cultured cells or peripheral blood leukocytes as described (18). Restriction endonucleases were purchased from Boehringer Mannheim and used according to the supplier's instructions. Each DNA sample (10 μg) was digested with 4 units of restriction endonuclease per μg for 16 hr. Digests were subjected to agarose gel electrophoresis and blotted to nitrocellulose (Millipore) (19). Blots were hybridized for 36 hr at 65°C with 10^6 dpm/cm^2 of the denatured radioactive probe in 100 μl/cm^2 of hybridization solution: 0.9 M NaCl/0.09 M Na citrate, pH 7/10% dextran sulfate/0.5% NaDodSO$_4$/0.1% Ficoll 400/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/100 μg of denatured herring sperm DNA per ml. Blots were washed twice for 10 min each at room temperature in 0.3 M NaCl/0.03 M Na citrate/0.5% NaDodSO$_4$ and three times for 1 hr each at 65°C in 0.15 M NaCl/0.015 M Na citrate/0.5% NaDodSO$_4$. Blots were autoradiographed for 2 days on Kodak XAR film using an intensifying screen at −70°C.

RESULTS

Initial studies were performed with the GMA and PLH cell lines (Fig. 1). The normal DNA sample from GMA yielded two fragments that hybridized with the probe at equal intensity after digestion with either *Eco*RI [12 and 14 kilobases (kb)] or *Taq* I (3.7 and 3.2 kb). One of these bands (the first mentioned in each digest) was absent in digests of DNA from the 21-OHase-deficient PLH cell line.

DNA was extracted from peripheral blood leukocytes of 10 unrelated normal individuals and six unrelated patients with homozygous 21-OHase deficiency who were heterozygous for *HLA-Bw47;DR7* (Fig. 2). After digestion with *Taq* I, all samples from normal individuals yielded two hybridizing bands of equal intensity. Samples from the six patients yielded, in five, diminished intensity of the 3.7-kb band relative to the 3.2-kb band, consistent with a heterozygous deletion; in one patient, the 3.7-kb band was completely absent.

A family study was performed to demonstrate Mendelian segregation of this deletion (Fig. 3). DNA samples were obtained from members of the family of one of the 21-OHase-deficient patients and again analyzed by digestion with *Taq* I and Southern hybridization with the pC21a probe. In individuals who carried *HLA-Bw47*, the heterozygous deletion of the 3.7-kb band could be detected by comparison with the intensity of the 3.2-kb fragment. This cosegregation of *HLA-Bw47* and the 3.7-kb deletion extended over three generations.

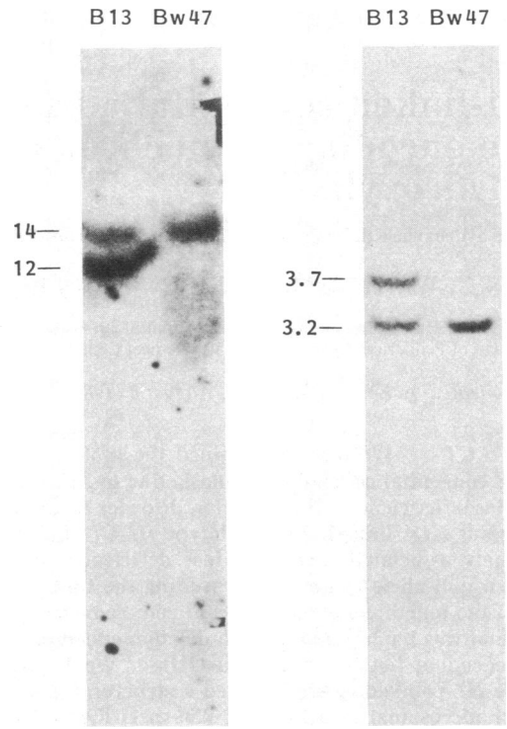

FIG. 1. Hybridization of pC21a to DNA from normal and 21-OHase-deficient cell lines. DNA was digested with restriction endonucleases *Eco*RI (*Left*) or *Taq* I (*Right*) as indicated. Digests were subjected to electrophoresis in, respectively, 0.6% or 1.0% agarose, blotted to nitrocellulose, and hybridized with the radioactively labeled insert of pC21a. Lanes labeled "B13" contained DNA from line GMA (normal, *HLA-B13* homozygous) and lanes labeled "Bw47" contained DNA from line PLH (21-OHase deficient, *HLA-Bw47* homozygous). Sizes of hybridizing fragments were determined by comparing electrophoretic mobilities with a *Hin*dIII digest of bacteriophage λ DNA. Sizes are shown in kb.

DISCUSSION

A bovine cDNA clone, pC21a, encoding part of the cytochrome P-450 specific for steroid 21-hydroxylation, called P-450$_{C21}$, hybridizes to human genomic DNA under conditions of moderate stringency, suggesting that the structure of this enzyme is highly conserved. Two hybridizing fragments are seen in normal DNA after digestion with either *Eco*RI or *Taq* I, but DNA from a patient with 21-OHase deficiency who is homozygous for *HLA-Bw47* yields a single hybridizing fragment after digestion with either of these two restriction endonucleases. This implies that there are normally two P-450$_{C21}$ genes, and that one of these genes is deleted on the *HLA-Bw47* haplotype. It is unlikely that the presence of two bands in normal individuals results from heterozygosity for a frequent DNA polymorphism of a single gene because, in that case, some normal individuals should be homozygous and display a single hybridizing band. If there were normally a single P-450$_{C21}$ gene with internal recognition sites for both *Eco*RI and *Taq* I, deletion of part of this gene should result in a change in the size of a hybridizing fragment in either the *Eco*RI or *Taq* I digest (or both), rather than complete disap-

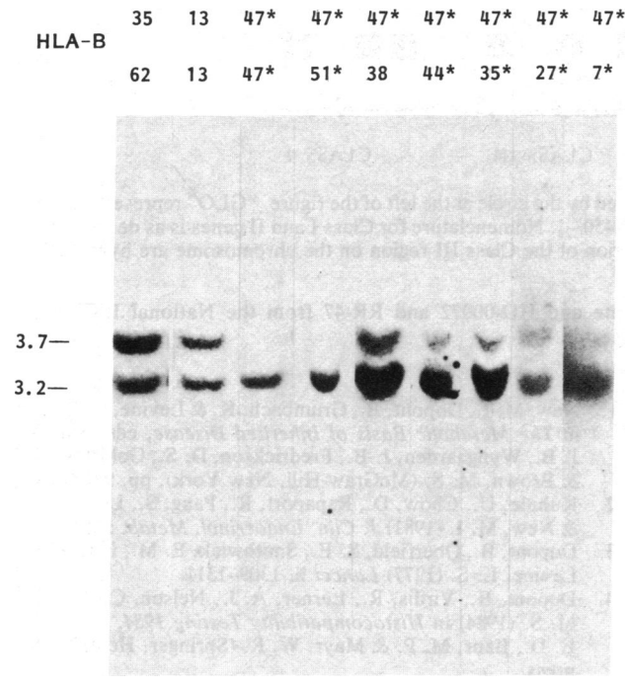

FIG. 2. Hybridization of pC21a to DNA from additional patients with 21-OHase deficiency. DNA from peripheral blood leukocytes was digested with *Taq* I and analyzed as described in Fig. 1. Lanes are labeled with the HLA-B antigens carried by each individual; asterisks indicate alleles carrying the 21-OHase deficiency. The second and third lanes from the left are copies of the *Taq* I digests displayed in Fig. 1. DNA from nine additional normal individuals yielded a pattern identical to that in the first and second lanes. Sizes are shown in kb.

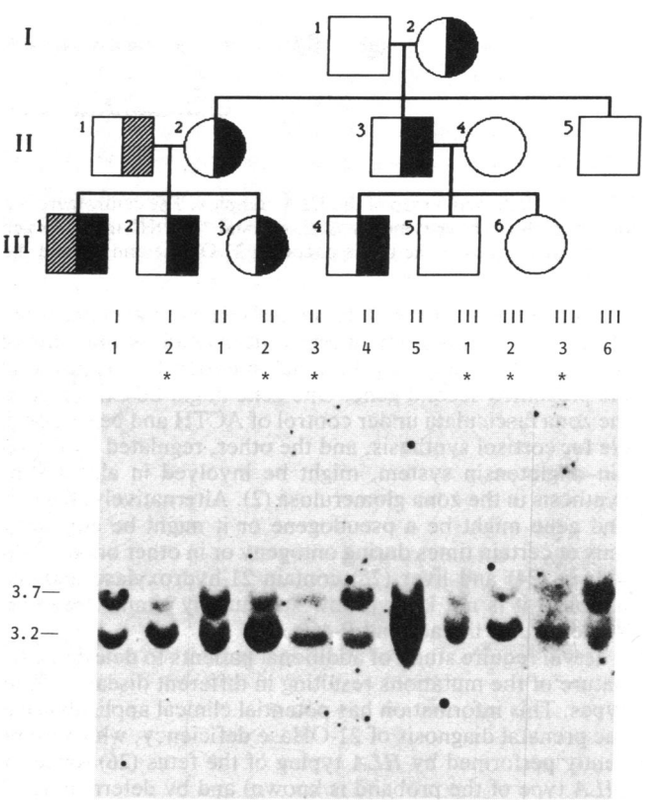

FIG. 3. Segregation of *Taq* I polymorphism in a family carrying the 21-OHase deficiency. (*Upper*) Pedigree of the family. The proband is III-1; III-2, III-3, II-1, and II-2 have been identified as heterozygous carriers of 21-OHase deficiency by hormonal testing. Black alleles indicate 21-OHase deficiency with *HLA-Bw47*, and cross-hatched alleles indicate 21-OH deficiency with *HLA-B35*. (*Lower*) Analysis of *Taq* I digests of DNA from family members, as described in Fig. 1. Lanes are labeled with pedigree number as defined in *A*. Asterisks indicate carriers of the *HLA-Bw47* haplotype. Sizes are shown in kb.

pearance of one fragment from each digest. A DNA rearrangement also would be likely to produce a restriction fragment length polymorphism rather than an apparent deletion.

The only available individual who was homozygous for *HLA-Bw47* was the donor of the PLH line. To determine if all cases of 21-OHase deficiency on the *HLA-Bw47* haplotype result from a deletion of a P-450$_{C21}$ gene, it was necessary to examine patients who were homozygous for 21-OHase deficiency but only heterozygous for *HLA-Bw47*. Detection of a heterozygous deletion is often difficult because it may not be possible to reliably quantitate the amount of DNA and the resulting strength of hybridization in different samples. In this case, digestion of DNA with *Taq* I normally yields two readily resolvable hybridizing bands of equal intensity and similar size (3.7 and 3.2 kb). As only one band (3.7 kb) is deleted in DNA from the *HLA-Bw47* homozygote, the unaffected band (3.2 kb) provides an internal standard, and so diminished relative intensity of the 3.7-kb band can be taken as evidence of a heterozygous deletion. DNA samples from five patients yield this pattern, whereas the sixth, in fact, carries a homozygous deletion. Thus, when 21-OHase deficiency is associated with *HLA-Bw47*, there is a deletion of a P-450$_{C21}$ structural gene ($\chi^2 = 15.9$; $P < 0.0001$). When 21-OHase deficiency is associated with other HLA-B antigens, there is occasionally (one of six in this experiment) a deletion of at least part of a gene, but often there is no polymorphism detectable with *Taq* I. In other words, it is not usually possible to distinguish those *HLA-Bw47* heterozygotes who are homozygous for 21-OHase deficiency from those who are heterozygous carriers.

These data demonstrate that 21-OHase deficiency results from a mutation involving the structural gene for P-450$_{C21}$, a finding that has not as yet been demonstrated by direct examination of affected adrenal glands. This gene is located within the *HLA* complex and represents a class of protein distinct from the other gene products of this complex (20). It is likely that this gene is located near the *C4A* locus because the *C4A* locus carries a null allele (7), and is presumably deleted, on the same *HLA-Bw47;DR7* haplotype that carries a deletion of a P-450$_{C21}$ gene.

As we have not yet found a DNA polymorphism of the presumed second P-450$_{C21}$ gene (3.2-kb *Taq* I and 14-kb *Eco*RI fragments), it is not possible to map it within the *HLA* complex by this approach. However, several human cosmid clones (kindly provided by D. Grossberger and J. Strominger) isolated with a cDNA probe for C4 (21) also hybridize with pC21a, and, in different clones, this hybridization is confined to 3.7- or 3.2-kb *Taq* I fragments (unpublished observations). When these cosmid clones are fully characterized, the arrangement of the genes in this region will be better defined.

The precise arrangement of the genes for C4 and P-450$_{C21}$ has already been determined in the mouse, where the major histocompatibility complex (*H-2*) has been studied extensively. A set of overlapping cosmid clones covering the "S" region containing the *C4* genes (22) has been probed with pC21a in collaboration with D. Chaplin and J. G. Seidman. We have found that there are two genes for P-450$_{C21}$ in the *H-2* complex, one located less than 6 kb from the 3′ end of each of the two *C4* genes (32). A hypothetical schematic map of the *HLA* complex, with alternating genes for C4 and P-450$_{C21}$ as found in the mouse *H-2* complex, is displayed in Fig. 4.

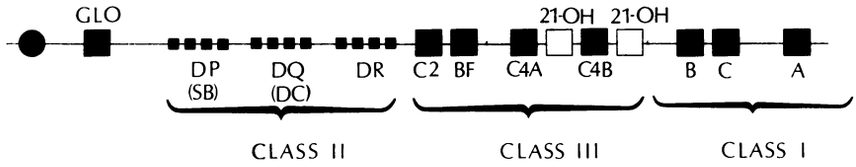

FIG. 4. Schematic map of the *HLA* complex. The centromere is represented by the circle at the left of the figure. "GLO" represents the gene for the erythrocyte enzyme glyoxalase I, and "21-OH" indicates genes for P-450$_{C21}$. Nomenclature for Class I and II genes is as described (23). The relative order of the genes encoding 21-OHase and C4 and the orientation of the Class III region on the chromosome are hypothetical.

It is notable that 21-OHase deficiency associated with *HLA-Bw47* results from an apparent deletion of only one of two genes; possibly an additional mutation has occurred in the presumed second gene. One gene might be expressed in the zona fasciculata under control of ACTH and be responsible for cortisol synthesis, and the other, regulated by the renin–angiotensin system, might be involved in aldosterone synthesis in the zona glomerulosa (2). Alternatively, the second gene might be a pseudogene or it might be expressed only at certain times during ontogeny or in other organs. The kidney (24) and liver (25) contain 21-hydroxylase activity, although it is not known how structurally similar these enzymes are to the adrenal P-450.

It will require study of additional patients to determine the nature of the mutations resulting in different disease phenotypes. This information has potential clinical applicability to the prenatal diagnosis of 21-OHase deficiency, which is currently performed by *HLA* typing of the fetus (26) (once the *HLA* type of the proband is known) and by determining 17-OHP levels in the amniotic fluid (27). Both of these tests require an amniocentesis at about 16 wk of gestation. When a sufficient number of informative DNA polymorphisms have been found, it should be possible to obtain sufficient material by chorionic villus biopsy (28) to offer prenatal diagnosis to many families before the end of the first trimester. If a female fetus is diagnosed as being affected with 21-OHase deficiency, treatment of the mother with dexamethasone would be expected to suppress the fetal adrenal gland and, thus, prevent virilization of the external genitalia (29).

The major histocompatibility complex contains several classes of genes, which are all involved in immune regulation (30). A teleologic explanation for the presence of a gene encoding a steroidogenic enzyme in the *HLA* complex (and for its frequent deficiency) is not obvious. Variations in glucocorticoid or androgen metabolism could certainly affect lymphocyte function. It is interesting that the *HLA-B8;DR3* haplotype, which is negatively associated with 21-OHase deficiency, has strong positive associations with many diseases with an autoimmune etiology, such as insulin-dependent diabetes mellitus and Graves disease (31). Determining the molecular genetic basis of one *HLA*-linked disease may thus make it possible to study the inherited component of other *HLA*-associated endocrinopathies.

Note Added in Proof. On reexamining the data on the C4 allotypes associated with the *HLA-Bw47* haplotype (6, 7), it is not possible to electrophoretically distinguish *C4A*QO;C4B*3* from *C4A*1; C4B*QO*. Therefore, our data are also consistent with a P-450$_{C21}$ gene located near the *C4B* (Chido) gene, with both these genes being deleted on the *HLA-Bw47* haplotype.

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