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Two genes encoding steroid 21-hydroxylase are located near the genes encoding the fourth component of complement in man

(congenital adrenal hyperplasia/*HLA* haplotype/autoimmunity/cytochrome P-450/recombinant DNA probe)

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ABSTRACT **Two genes encoding steroid 21-hydroxylase [21-OHase; steroid 21-monooxygenase; steroid,hydrogen-donor:oxygen oxidoreductase (21-hydroxylating); EC 1.14.99.10], a cytochrome P-450 enzyme, have been located within the *HLA* major histocompatibility complex. Congenital adrenal hyperplasia due to 21-OHase deficiency is a common inherited disorder of cortisol biosynthesis which is in genetic linkage disequilibrium with certain extended *HLA* haplotypes. These haplotypes include characteristic serum complement allotypes. A series of cosmid clones was isolated from a human genomic library by using a probe encoding part of the fourth component of complement, C4. These clones also hybridized with a probe encoding most of human 21-OHase. Restriction mapping and hybridization analysis showed that there are two *21-OHase* genes, each located near the 3′ end of one of the two *C4* genes. Hybridization with probes specific for the 5′ and 3′ ends of the *21-OHase* gene showed that the *21-OHase* and *C4* genes all have the same orientation. The *21-OHase* genes 3′ to *C4A* and *C4B* carry *Taq* I fragments of 3.2 and 3.7 kilobases (kb), respectively. Both of these fragments are found in genomic DNA of most individuals. In DNA from an individual with the severe, "salt-wasting" form of 21-OHase deficiency who was homozygous for *HLA-A3;Bw47;C4A*1;C4B*Q0(null); DR7*, the 3.7-kb *Taq* I fragment is absent, whereas hormonally normal individuals homozygous for *HLA-A1;B8;C4A*Q0; C4B*1;DR3* do not carry the 3.2-kb *Taq* I fragment. These data suggest that the *21-OHase "B"* gene (3.7-kb *Taq* I fragment) is functional, but the *21-OHase "A"* gene (3.2-kb *Taq* I fragment) is not.**

The class III region of the major histocompatibility complex contains genes encoding several components of the serum complement system (1–3). In the *HLA* complex in man, these include the genes for the second component of complement (*C2*) and for factor B (*Bf*) and genes encoding two forms of the fourth component of complement (*C4A* and *C4B*) (4). In the S region of the *H-2* complex of the mouse, there is a single *C4* gene, encoding hemolytically active C4, and a second gene *Slp*, which encodes a hemolytically inactive homolog of C4 that is expressed in males of certain strains of mice but not in most females (5, 6). Cosmid clones covering the class III regions of both mouse and man have been isolated and mapped (1–3), demonstrating that the *Bf* and the two *C4* (or *C4* and *Slp* genes) are similarly arranged in two species.

The gene for steroid 21-hydroxylase deficiency, a common inherited disorder of steroidogenesis in man, has, like the class III genes, been mapped between *HLA-B* and *HLA-DR* (7, 8). This disease is in genetic linkage disequilibrium with certain extended *HLA* haplotypes that include characteristic complement allotypes. The severe, "salt-wasting" form of the disease is often found in association with the haplotype *HLA-A3;Bw47;Bf*F,C2*C;DR7* (9, 10). This haplotype also carries a null allele at one of the *C4* loci, but as it is not possible to electrophoretically distinguish the patterns of *C4A*Q0(null);C4B*3* and *C4A*1;C4B*Q0*, it has not been clear whether the null allele is at the *C4A* or *C4B* locus.

It has been shown that this haplotype carries a deletion of all or part of a gene for the cytochrome P-450 specific for steroid 21-hydroxyation [hereafter referred to as 21-OHase; steroid 21-monooxygenase, steroid,hydrogen-donor:oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10] (11). Presumably, this *21-OHase* gene was adjacent to the *C4* locus carrying the null allele, which is probably also deleted in the *Bw47* haplotype.

There is no equivalent of 21-OHase deficiency in the mouse. Instead, the murine *21-OHase* genes have been mapped by examining a cluster of overlapping cosmid clones derived from the S region of the BALB/c mouse (1), using a bovine cDNA clone encoding part of 21-OHase (12). There are two mouse *21-OHase* genes, each located near the 3′ end of one of the two *C4*-like genes (13).

The human *21-OHase* genes have now been mapped equally precisely by studying a series of cosmid clones isolated from a cosmid library with a cDNA clone encoding part of C4 (14). There are also two *21-OHase* genes in man, each located near the 3′ end of one of the two *C4* genes. By examining DNA from individuals with null alleles at each of the *C4* genes, it is possible to draw tentative conclusions regarding the function of each of the *21-OHase* genes.

MATERIALS AND METHODS

DNA Probes. Plasmid pC4AL1 (kindly provided by S. Whitehead and H. Colton) contains a 950-base-pair (bp) cDNA insert in the *Pst* I site of pBR322, corresponding to the 3′ end of the mRNA encoding C4 (14).

Plasmid pC21/3c was isolated by screening a human fetal adrenal cDNA library (kindly provided by D. Russell) with the bovine cDNA clone pC21a (12). The library was produced by the Okayama–Berg protocol, which tends to yield nearly full-length copies (15); pC21/3c contains an insert of about 2000 bp and is shorter than a full-length copy by about 100–200 bp corresponding to the 5′ end of the mRNA (unpublished observations). The insert can be released from the vector by digestion with restriction endonuclease *Bam*HI.

Plasmids were digested with the appropriate restriction endonuclease (purchased from Boehringer-Mannheim and used according to the supplier's instructions) and the insert

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Abbreviations: 21-OHase, steroid 21-hydroxylase; C4, fourth component of serum complement; bp, base pair(s); kb, kilobase(s).

was separated from the vector by electrophoresis in 1% agarose (International Biotechnologies, New Haven, CT). The band containing each insert was sliced from the gel, and DNA was isolated by the glass powder method (16). Insert DNA was radioactively labeled with [α-^{32}P]dATP (New England Nuclear) by nick-translation (17).

Sources of Human DNA. Continuously proliferating lymphoblastoid cell lines were produced by *in vitro* transformation with Epstein–Barr virus (18). Cell line JY (19) is homozygous for *HLA-A2;B7*, and lines BRG and TER, for *HLA-A1;B8;DR3*. Line PLH was derived from a patient with 21-OHase deficiency who is homozygous for *HLA-A3;Bw47;DR7* (11). Individuals K.S. and J.C. are homozygous for *HLA-A1;B8;DR3*.

Construction of Cosmid Library. DNA was prepared from JY cells as described (20) and partially digested with *Mbo* I. Fragments 35–45 kilobases (kb) long were isolated by sucrose gradient sedimentation and ligated to the vector pJB8 essentially as described (21). Plasmids were packaged into bacteriophage λ particles *in vitro* and used to transduce *E. coli* strain HB101 cells (21).

Screening the Library. Transduced bacteria were plated on nitrocellulose filters (Millipore HATF) at a density of 1×10^5 colonies per 150-mm plate and replicated onto additional nitrocellulose filters (22). Replicas were hybridized with the insert of pC4AL1 as described (23). Apparently positive clones were purified by replating and rescreened.

Mapping of Restriction Sites. Cosmid DNA was prepared using the rapid-boil method (24) after amplification with chloroamphenicol. DNA was digested with various restriction endonucleases and with pairs of enzymes. In some cases, DNA was digested with a single enzyme, subjected to electrophoresis in low-melting-temperature agarose (Marine Colloids, Rockland, ME), and stained with ethidium bromide. Bands were sliced out and slices were melted at 65°C; after cooling to 37°C and adding the appropriate concentrated buffer, individual bands were digested with a second enzyme (25). Where necessary, fragments were end-labeled with the appropriate [α-^{32}P]dNTPs and the large fragment of DNA polymerase (Boehringer-Mannheim) (26) and visualized by autoradiography.

Hybridization Analysis. DNA samples (3–5 μg) prepared (21) from cultured cells or from peripheral blood leukocytes or cosmid DNA samples (0.2 μg) were digested with various restriction enzymes, subjected to electrophoresis in agarose, and transferred to nitrocellulose (Schleicher & Schuell) (27). Blots were hybridized overnight at 65°C with denatured radioactive probe in hybridization solution (100 μl/cm^2): 0.9 M NaCl/0.09 M Na citrate, pH 7/10% (wt/vol) dextran sulfate (omitted when cosmid DNA was analyzed)/0.5% NaDodSO$_4$/denatured herring sperm DNA (100 μg/ml)/0.1% Ficoll 400/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin. Genomic DNA was examined by using intact plasmid, labeled to a specific activity of 2×10^8 dpm/μg, at a concentration of 10^6 dpm/ml. When cosmid DNA was analyzed, the insert of each plasmid was labeled to a specific activity of 10^7 dpm/μg and used at a concentration of 5×10^4 dpm/ml. Blots were washed twice, for 10 min each at room temperature in 0.3 M NaCl/0.03 M Na citrate, pH 7/0.5% NaDodSO$_4$, and three times for 1 hr each at 65°C in 0.15 M NaCl/0.015 M Na citrate, pH 7/0.5% NaDodSO$_4$. Blots were autoradiographed on Kodak XAR film with an intensifying screen at −70°C.

RESULTS

Screening the Library. After screening, purification, and repeat screening, seven clones were isolated that hybridized with the insert of pC4AL1. Preliminary restriction mapping suggested that cosmids 4.5, 4.6, and 4.8 were very similar to each other, as were cosmids 4.1 and 4.7. Cosmids 4.2 and 4.4 appeared to be unique. Therefore, cosmids 4.1, 4.2, 4.4, and 4.5 were studied further.

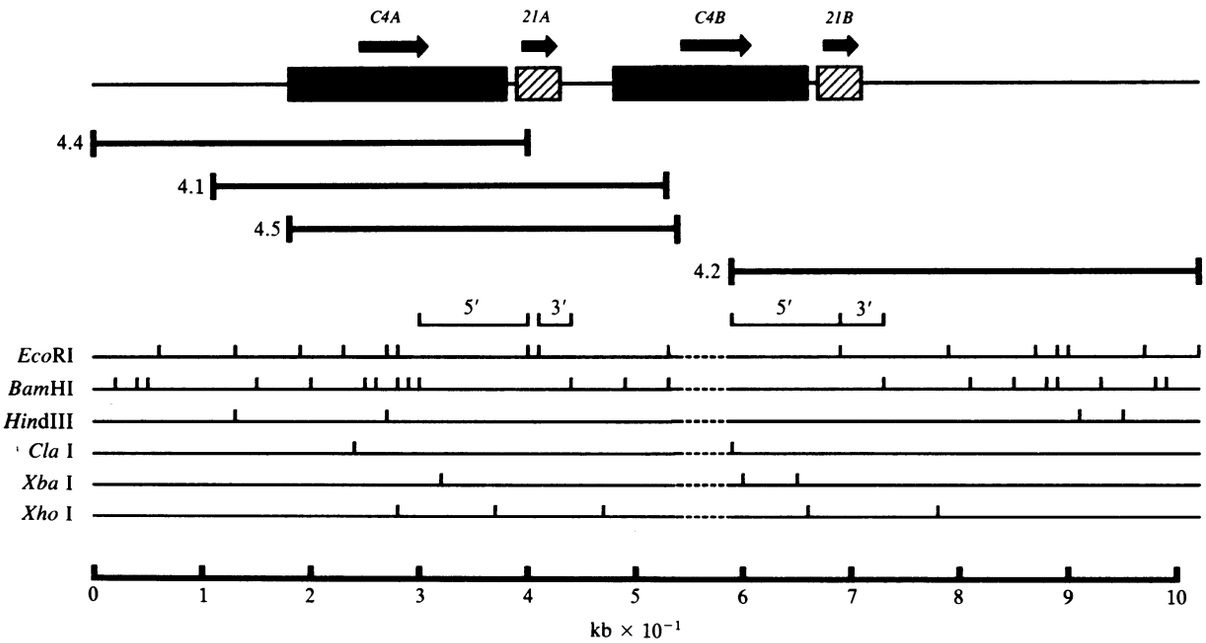

FIG. 1. Map of restriction endonuclease recognition sites in cosmid clones carrying the *C4* and *21-OHase* genes. The extents of individual cosmid clones are shown above maps of sites for six enzymes; the scale at the bottom is marked at 10-kb intervals. Cosmids 4.5 and 4.2 do not overlap, and the gap between them is indicated on each restriction map as a dotted line. The approximate boundaries of each gene are indicated by a box, and the direction of transcription of each gene is shown by an arrow. The *C4A* and *C4B* genes are marked; *21A* and *21B* are the corresponding *21-OHase* genes. The direction of transcription of each *21-OHase* gene was determined by hybridizing probes specific for the 5′ and 3′ ends of the gene with *Bam*HI/*Eco*RI double digests of cosmids 4.5 or 4.2. The hybridizing fragments in these cosmids are indicated above the *Eco*RI restriction map. The approximate borders and orientations of the *C4* genes are taken from published data (3); the genes for complement components C2 and factor B are to the left of the diagrammed region. The indicated size and position of each *21-OHase* gene are those of the *Taq* I fragment on which most of each gene is found.

Restriction Mapping and Hybridization Analysis. A map of these clones is shown in Fig. 1. Cosmids 4.4, 4.1, and 4.5 overlap each other; 4.2 does not overlap the other clones. By comparison with a published map of this region (2, 3), it is apparent that 4.4 is nearest the *Bf* and *C2* genes and that the 3′ end (relative to the direction of transcription of the *C4* genes) of 4.5 is about 5 kb from the 5′ end of 4.2.

The insert of pC21/3c hybridized weakly to a 13-kb *Eco*RI fragment, a 14-kb *Bam*HI fragment, and a 1.0-kb *Taq* I fragment from cosmid 4.4; this indicated that cosmid 4.4 contained only a small part of a *21-OHase* gene near one end. The probe hybridized more strongly to two 13-kb *Eco*RI fragments, a 14-kb *Bam*HI fragment, and a 3.2-kb *Taq* I fragment in cosmid 4.5 and to two 10-kb *Eco*RI fragments, a 16-kb *Bam*HI fragment, and a 3.7-kb *Taq* I fragment in cosmid 4.2. The hybridizations to *Taq* I digests are shown in Fig. 2. This located the two *21-OHase* genes within about 2 kb of the 3′ ends of the respective *C4* genes.

To orient the *21-OHase* genes, the insert of pC21/3c was digested with *Eco*RI, yielding a 500-bp fragment, corresponding to the 5′ end of the mRNA, and a 1600-bp fragment, corresponding to the 3′ end (unpublished observations). The fragments were isolated by electrophoresis in agarose, radioactively labeled, and hybridized with *Bam*HI/*Eco*RI double digests of cosmid DNA. The 500-bp 5′ probe hybridized to a 10-kb fragment in cosmid 4.5 and to a 9.5-kb fragment in 4.2. The 1600-bp 3′ probe hybridized to 3.5-kb fragments in both 4.5 and 4.2. These results demonstrated that the *21-OHase* genes are oriented in the same direction as the *C4* genes.

The hybridization patterns of these cosmids were compared with hybridization of pC21/3c to genomic DNA from several individuals (Fig. 2). Most individuals display two *Taq* I bands of 3.2 and 3.7 kb that hybridize with pC21/3c, as well as a much fainter band at 2.4 kb. The first two bands correspond to the two *21-OHase* genes carried on cosmids 4.5 and 4.2, respectively. The faint band does not correspond to any fragment found in the cosmids, and presumably represents cross-hybridization to a gene for a structurally related cytochrome P-450. It is not seen when the bovine cDNA clone encoding 21-OHase, pC21a, is used as a hybridization probe (11). With DNA from cell line PLH, which is homozygous for *HLA-A3;Bw47;DR7* and 21-OHase deficiency, the 3.7-kb *Taq* I fragment is not seen. With DNA from cell lines BRG and TER and from individuals K.S. and J.C., all homozygous for *HLA-A1;B8;DR3*, the 3.2-kb band is absent.

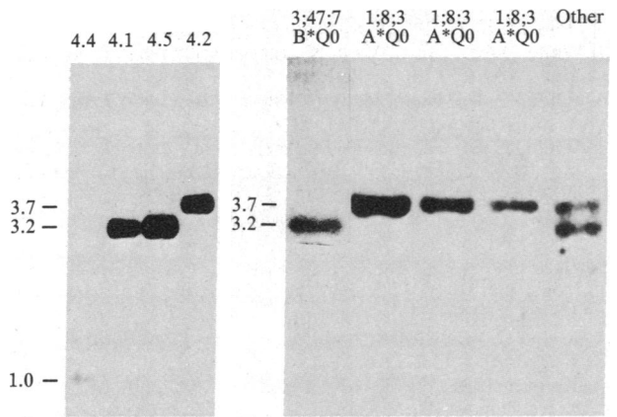

FIG. 2. Hybridization of pC21/3c to cosmids and to human genomic DNA. Sizes of hybridizing fragments (in kb) are indicated at the left; they were determined by comparing mobilities with a *Hin*dIII digest of bacteriophage λ DNA. (*Left*) Hybridization to *Taq* I digests of DNA from individual cosmid clones, as marked. (*Right*) Hybridization to *Taq* I digests of human DNA from unrelated *HLA*-homozygous cell lines or peripheral blood leukocytes. The *HLA-A;B;DR* haplotype of each individual is shown above each lane. Individuals carry homozygous null (*Q0*) alleles for *C4A* or *C4B* as indicated. The normal, "other," pattern is seen in DNA from most other individuals (ref. 11 and unpublished observations). The DNA samples are, from left, respectively, from cell lines PLH (derived from a patient with salt-wasting 21-OHase deficiency), BRG, and TER and from the peripheral blood leukocytes of individuals J.C. and T.A. (*HLA-A3,w31; Bw51,w44;DRw6,7*). DNA from individual K.S. (*HLA-A1;B8;DR3* homozygous), not shown, yields the same pattern as that from J.C.

DISCUSSION

In man, as in the mouse (13), there are two *21-OHase* genes, which alternate with two *C4* genes (Fig. 3). This is presumably due in both species to duplication of single adjacent *C4* and *21-OHase* genes. Certainly the *C4* and *21-OHase* genes became linked in the major histocompatibility complex before mouse and man diverged during evolution, but it will not be clear until other species are examined whether the duplication of these genes is a general phenomenon that also reflects an event occurring prior to mammalian speciation.

Whereas both *C4* genes in man encode functional proteins, it appears that this may not be true for the *21-OHase* genes. An individual homozygous for *HLA-A3;Bw47;DR7* has a homozygous deletion of the *21-OHase* gene carrying the 3.7-kb *Taq* I fragment. Although the other *21-OHase* gene carrying the 3.2-kb *Taq* I fragment is present, this individual suffers from the severe salt-wasting form of 21-OHase deficiency. Although we have examined only one patient homozygous

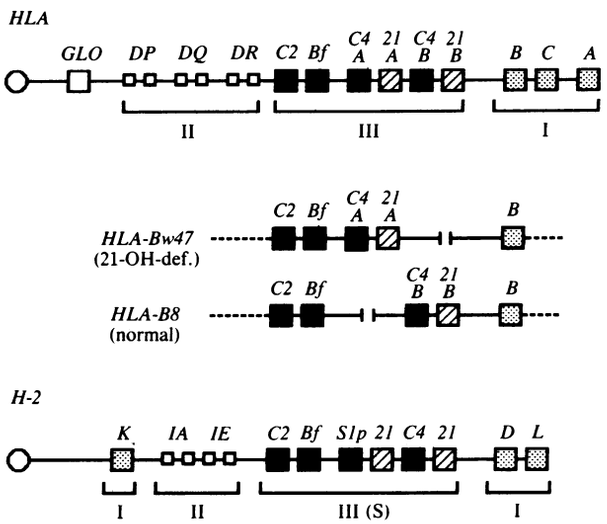

FIG. 3. Schematic of the major histocompatibility complex in man (*HLA*) and mouse (*H-2*). The centromere is indicated by a circle at the left of each map. *GLO* refers to the gene for the red cell enzyme glyoxalase I. The class I and class II transplantation antigens are described elsewhere (28); there are more class II genes than are diagrammed here. The class III region contains genes encoding the second component of complement (*C2*) and factor B (*Bf*). Normally, in man, the two genes encoding C4 (*C4A* and *C4B*) alternate with the two *21-OHase* genes (*21A* and *21B*). The *21-OHase B* gene and the adjacent *C4B* gene appear to be deleted on the chromosome carrying *HLA-Bw47* and the allele for salt-wasting 21-OHase deficiency (21-OH-def.). In contrast, the chromosome carrying the *HLA-A1;B8;DR3* haplotype [here labeled B8 (normal)] does not carry clinical 21-OHase deficiency, but is proposed to have a deletion of the *C4A* and *21-OHase A* genes. The mouse *H-2* complex is similarly arranged, but one class I gene (*H-2K*) is centromeric to the class II genes. The S region contains the class III genes. *Slp* encodes a hemolytically inactive homolog of C4, expressed only in males of certain strains of mice. The *21-OHase* genes again alternate with the *C4*-like genes (13).

for the *Bw47* haplotype, study of other patients with 21-OHase deficiency who are heterozygous for *Bw47* has shown that this haplotype always carries 21-OHase deficiency with a deletion of the 3.7-kb *Taq* I fragment (11). In contrast, four individuals who are homozygous for the *HLA-A1;B8;DR3* haplotype have an apparent deletion of the *21-OHase "A"* gene carrying the 3.2-kb *Taq* I fragment, whereas the *21-OHase "B"* gene carrying the 3.7-kb *Taq* I fragment remains intact. These individuals do not have 21-OHase deficiency, and, in fact, persons carrying this haplotype have a decreased likelihood of having 21-OHase deficiency (8).

These data suggest that, in man, the *21-OHase A* gene does not encode active 21-OHase. To demonstrate this conclusively, it will be necessary to determine the DNA sequence of both genomic genes as well as cDNAs. This should be technically straightforward as the *21-OHase* genes are relatively small; >90% of the coding region is carried on genomic fragments <4 kb long. Genes encoding other cytochromes P-450 are up to 14 kb long (29). It is not known whether the *21-OHase A* gene is a pseudogene, or encodes an inactive protein, or encodes an active cytochrome P-450 with a different substrate specificity, or is expressed in a different tissue. Although individuals homozygous for *HLA-A1;B8;DR3* synthesize cortisol normally, their ability to metabolize xenobiotics, for example, has not been examined. As the *21-OHase* genes are highly homologous, the term "*21-OHase A*" is used here, with the recognition that it may be a misnomer.

These deletions of *21-OHase* genes occur in association with null alleles at one or the other *C4* locus. Probably the corresponding *C4* gene is deleted (3) along with the *21-OHase* gene. Thus, the *HLA-A1;B8;DR3* haplotype carries a null allele at *C4A* (Rodgers antigen) (30), with presumed deletions of the *C4A* and *21-OHase A* genes. Although it is not clear from protein analysis whether the *A3;Bw47;DR7* haplotype carries a null allele at *C4A* or *C4B*, these data suggest that the full haplotype is *HLA-A3;Bw47;C4A*1;21-OHaseA* (intact); C4B*Q0; 21-OHaseB*Q0;Bf*F;C2*C;DR7*, with an apparent deletion of the adjacent *C4B* (Chido antigen) and *21-OHase B* genes.

Duplications of *C4* genes have also been noted. In particular, the *B14;DR1* haplotype carries a duplication of the *C4B* gene (3, 31). This haplotype is strongly associated with a mild, "cryptic" ("late-onset") form of 21-OHase deficiency (32). Preliminary hybridization analysis of DNA from patients with this form of 21-OHase deficiency suggests that this haplotype carries three *21-OHase* genes (unpublished observations). The role of this duplication in the development of late-onset disease is unknown.

The presence of duplicated *C4* and *21-OHase* genes may allow for frequent deletion or further duplication by unequal crossing-over during meiosis. Because the *A1;B8;DR3* haplotype only carries single *C4* and *21-OHase* genes, it may be less likely to lose the second, functional *21-OHase* gene by this mechanism. Possibly this is an explanation for the negative association of 21-OHase deficiency and *B8;DR3*.

Both the *A1;B8;DR3* and *A3;Bw47;DR7* haplotypes are remarkable for an apparent suppression of recombination over a genetic distance of at least 2 centimorgans. It has been proposed (33) that such haplotypes are the human equivalent of the murine T haplotypes, where recombination suppression seems to result from inversion of part of chromosome 17 (34). It is tempting to speculate that the deletions described in this study (or the duplication in *B14;DR1*) cause sufficient misalignment of chromosomes to reduce the likelihood of recombination in the *HLA* region, allowing maintenance of these extended haplotypes.

It is not known whether the deletion on the *HLA-A1;B8;DR3* haplotype has any other functional significance. It is, however, possible that the remarkable association between this *HLA* haplotype and autoimmune diseases, such as insulin-dependent diabetes mellitus and Graves disease (35), is directly related to the described genetic rearrangement within the *HLA* complex. This association might be a composite effect mediated by modifications in both serum complement activity and steroid hormone metabolism.

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