Genesis by meiotic unequal crossover of a *de novo* deletion that contributes to steroid 21-hydroxylase deficiency

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Communicated by Mary F. Lyon, December 4, 1989

ABSTRACT The HLA-linked human steroid 21-hydroxylase gene *CYP21B* and its closely homologous pseudogene *CYP21A* are each normally located centromeric to a fourth component of complement (C4) gene, *C4B* and *C4A*, respectively, in an organization suggesting tandem duplication of a *ca.* 30-kilobase DNA unit containing a *CYP21* gene and a C4 gene. Such an organization has been considered to facilitate gene deletion and addition events by unequal crossover between the tandem repeats. We have identified a steroid 21-hydroxylase [steroid, hydrogen-donor:oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10] deficiency patient who has a maternally inherited disease haplotype that carries a *de novo* deletion of a *ca.* 30-kilobase repeat unit including the *CYP21B* gene and associated *C4B* gene. This disease haplotype appears to have been generated as a result of meiotic unequal crossover between maternal homologous chromosomes. One of the maternal haplotypes is the frequently occurring HLA-DR3, B8, A1 haplotype that normally carries a deletion of a *ca.* 30-kilobase unit including the *CYP21A* gene and *C4A* gene. Haplotypes of this type may possibly act as premutations, increasing the susceptibility of developing a 21-hydroxylase deficiency mutation by facilitating unequal chromosome pairing.

Steroid 21-hydroxylase [21-OH; steroid, hydrogen-donor:oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10] deficiency is a recessively inherited disorder of cortisol metabolism that accounts for >90% of the cases of congenital adrenal hyperplasia (1). Although clinically heterogeneous, the disorder is always characterized by accumulation of 17-hydroxyprogesterone and by excessive androgen production, which, in the classical form of the disorder, results in masculinization of the external genitalia in affected female newborns. In addition, ≈70% of classical 21-OH deficiency patients show an inability to conserve dietary sodium ("salt-wasting") as a consequence of parallel deficiency in aldosterone production. The disorder maps within the class III region of the HLA complex on the short arm of chromosome 6, and molecular genetic investigations have revealed that most normal haplotypes contain, in addition to a functional 21-OH gene, *CYP21B*, a pseudogene, *CYP21A*, which shows 97% sequence homology to the functional gene. The *CYP21A* and *CYP21B* loci are positioned ≈2 kilobases (kb) centromeric to the duplicated fourth component of complement (C4) genes, *C4A* and *C4B*, respectively (see Fig. 1), and the extant organization of these genes is presumed to reflect tandem duplication of an ancestral compound DNA unit ≈30 kb long and containing a single 21-OH gene and a single C4 gene (2, 3). Because of C4 gene length heterogeneity (4), the repeat units may be short (*ca.* 26 kb in length) or long (*ca.* 33 kb).

The tandemly repeated organization of the 21-OH and C4 genes has been considered to facilitate gene deletion and

addition events by unequal crossover mechanisms. Although the existence of haplotypes with variable numbers of C4 and 21-OH genes has been inferred by indirect short-range restriction mapping and complement C4 allotyping analyses, confirmation of the copy number variation has only recently been obtained by direct long-range restriction mapping approaches (5–7). Such studies have shown that gene expansion and contractions are frequent in the 21-OH/C4 gene cluster. Also, the size variation of haplotype-specific long-range restriction fragments that span the array of 21-OH and C4 genes is always consistent with integral numbers of the *ca.* 30-kb repeat units. Consequently, a considerable amount of circumstantial evidence has been accumulated in favor of the involvement of unequal crossover in the 21-OH/C4 gene cluster. In the present report, we present direct evidence for the occurrence of unequal crossover in this chromosomal region: a 21-OH deficiency patient has inherited a maternal haplotype that, as a result of meiotic recombination, has a *ca.* 30-kb *de novo* deletion that eliminates the functional *CYP21B* gene and a companion *C4B* gene.

MATERIALS AND METHODS

17-Hydroxyprogesterone Assays. Serum 17-hydroxyprogesterone levels were assayed as described (8).

HLA Antigen and Complement Typing. HLA-A, HLA-B, HLA-DR, factor B, and C4 typing was performed as described (8).

DNA Probes and Southern Blot-Hybridization. The origin of the C4B550 probe has been described (8). The 21-OH-specific probe, 21A-2.8, is a 2.8-kb *EcoRI*–*Bam*HI fragment isolated from a *CYP21A* subclone and defines a region spanning from the equivalent of exon 4 to a position 0.6 kb 3' to the *CYP21A* gene. Radiolabeling procedures, Southern blot-hybridization conditions, and laser scanning densitometry were as described (8).

Pulsed-Field Gel Electrophoresis Analysis. High molecular weight genomic DNA samples were digested with *Bss*HII or *Sac* II prior to size fractionation by pulsed-field gel electrophoresis as described (5). Restriction nuclease digestion conditions were as follows: *Bss*HII, 12 units per sample at 50°C overnight in the buffer recommended by the manufacturer (New England Biolabs); *Sac* II, 12 units per sample overnight at 37°C. Molecular weight markers were provided by concatemers of λ c1857S7.

RESULTS

HLA typing of members of the Irish 21-OH deficiency family whose pedigree is shown in Fig. 2 reveals that the affected

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Abbreviations: 21-OH, 21-hydroxylase; C4, fourth component of complement; RFLV, restriction fragment length variant; RFLP, restriction fragment length polymorphism; VNTR, variable number of tandem repeats.

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FIG. 1. Diagnostic restriction fragments used in short- and long-range restriction mapping of the CYP21 and C4 genes. The common two-repeat unit haplotype is depicted with genes shown in the conventional order for genes in the HLA complex (centromere to the left). Arrows mark the direction of transcription from 5' to 3' of the expressed genes. Numbers refer to the size in kb of indicated restriction fragments. Bars indicate the span of genomic DNA restriction fragments recognized by the indicated DNA probes. In the case of contiguous *Taq* I restriction fragments recognized by 21-OH-specific probes, numbers on the right (3.7, 3.2) denote CYP21-specific fragments that can distinguish the *CYP21A* and *CYP21B* genes, whereas numbers on the left (2.5, 2.4) represent fragments that can distinguish between sequences 3' to the *CYP21A* and *CYP21B* genes. Variable restriction fragment sizes indicated as numbers separated by a slash correspond to length heterogeneity of *C4B* genes, with the lower figure referring to short *C4B* genes and the higher figure referring to long *C4B* genes. *, In the case of haplotypes that exhibit a deletion of *CYP21A* and *C4A* the residual *C4B* gene is marked by a diagnostic 6.4-kb *C4-5'*-specific *Taq* I fragment. **, In the case of single-repeat unit haplotypes the corresponding *Bss*HII restriction fragment length polymorphism (RFLP) sizes are *ca.* 80 kb (long) and *ca.* 75 kb (short), and the equivalent *Sac* II RFLP sizes are *ca.* 40 kb (long) and *ca.* 35 kb (short).

boy has inherited the same paternal haplotype as his two unaffected older brothers, but he differs from them in having inherited a maternally transmitted haplotype that appears to be a recombinant of the maternal HLA haplotypes: HLA-DR7, B13, A30; and HLA-DR3, B8, A1.

To investigate the recombinational event we have hybridized complement C4-specific and 21-OH-specific DNA probes to Southern blots of *Taq* I-digested genomic DNA from individual family members. Such analyses are informative because, although the tandem *ca.* 30-kb repeats show very high levels of sequence homology, certain restriction nucleases reveal restriction fragment length variants (RFLVs)—i.e., nonallelic restriction fragments that show size differences that distinguish between closely homologous but distinct loci (5). For example, as summarized in Fig. 1, hybridization of a *C4-5'*-specific probe, C4B550, to *Taq* I-digested genomic DNA can differentiate *C4A* genes (usually associated with a 7.0-kb *Taq* I RFLV) from *C4B* genes (normally associated with either a 6.0-kb or a 5.4-kb *Taq* I

RFLV in the case of long or short *C4B* genes, respectively). Similarly, a 21-OH gene probe, 21A-2.8, distinguishes *CYP21A* genes (3.2-kb and 2.4-kb *Taq* I hybridization bands) from *CYP21B* genes (3.7-kb and 2.5-kb *Taq* I hybridization bands). Of these, the 3.2-kb and 3.7-kb bands represent *Taq* I RFLVs corresponding to CYP21 gene sequence, whereas the 2.4-kb and 2.5-kb bands represent *Taq* I RFLVs corresponding to DNA sequence immediately 3' of the CYP21 genes (Fig. 1). In the case of the paternal DNA sample the C4B550 probe detects 7.0-kb, 6.0-kb, and 5.4-kb *Taq* I hybridization bands (see Fig. 2 Center) whose intensities show a ratio of 2:1:1, respectively (densitometric data not shown). In the same sample the 21A-2.8 probe reveals equality in intensity for *Taq* I RFLVs representing the *CYP21A* gene (3.2 kb) and the *CYP21B* gene (3.7 kb) and similarly equality in intensity between *Taq* I RFLVs representing sequence 3' of the *CYP21A* and *CYP21B* genes (2.4 kb and 2.5 kb, respectively). The maternal sample shows *C4-5'* *Taq* I bands of 7.0 kb and 5.4 kb and also a 6.4-kb *Taq* I RFLV

FIG. 2. Short-range restriction mapping of the 21-OH/C4 gene cluster and complement C4 allotyping analyses in members of a 21-OH deficiency family. (Left) Pedigree of an Irish 21-OH deficiency family. HLA haplotypes are as follows: a, DR4, B44, A2; b, DR6, B7, A30; c, DR7, B13, A30; d, DR3, B8, A1; c/d, DR3, B13, A30. F, father; M, mother. Resting serum 17-hydroxyprogesterone levels of the affected child, his father, and his mother were 1322 nmol/liter, 5.7 nmol/liter, and 1.0 nmol/liter, respectively. One hour after intravenous administration of 0.25 mg of corticotropin [ACTH (Synacthen)] to the parents, the 17-hydroxyprogesterone levels of the father and mother were 7.8 nmol/liter and 3.0 nmol/liter, respectively. (Center) Composite autoradiograph of a Southern blot of *Taq* I-digested genomic DNA samples from indicated family members probed with 21-OH-specific and C4-specific DNA probes. *Taq* I-digested genomic DNA samples from indicated family members were size fractionated on a 0.8% agarose gel and transferred to a nylon membrane prior to hybridization with a *C4-5'*-specific DNA probe, C4B550, and subsequently with a CYP21-specific DNA probe, 21A-2.8. Numbers represent the size in kb of indicated bands on the corresponding autoradiograph. (Right) Complement C4 allotyping analysis of indicated family members by immunofixation assay.

that is associated with the HLA-DR3, B8, A1 haplotype and is well recognized to be a marker of a deletion haplotype in which there is absence of a *ca.* 30-kb DNA segment containing *CYP21A* and *C4A*-specific DNA sequence (9). In accordance with this interpretation, the ratio of the intensities of the *CYP21A*-associated 3.2-kb and *CYP21B*-associated 3.7-kb *Taq* I RFLVs is $\approx 1:2$ and similarly there is a 1:2 ratio between the 2.4-kb and 2.5-kb *CYP21*-3' RFLVs. The two unaffected boys show the same DNA profiles as their father.

From the above observations, paternal haplotypes *a* and *b* are inferred to have a conventional two-repeat unit organization with the *C4B* gene on haplotype *a* being of the long variety (6.0-kb *Taq* I marker at the 5' end) and that on haplotype *b* being of the short variety (5.4-kb *Taq* I marker; see Table 1). The gene organization on maternal haplotype *c* resembles that on paternal haplotype *b*, whereas maternal haplotype *d* appears to exhibit a deletion of *C4A* and *CYP21A*, which is characteristic of the HLA-DR3, B8, A1 haplotype and is marked by a diagnostic 6.4-kb *C4*-5' *Taq* I fragment (see Table 1). However, when the analyses are extended to include DNA from the affected boy, it is apparent that the recombinant haplotype is associated with an unexpected gene organization in the 21-OH/C4 gene cluster. This individual reveals a 2:1 ratio between the following sets of markers: the *C4*-5'-specific 7.0-kb and 6.0-kb *Taq* I RFLVs; the *CYP21*-specific 3.2-kb and 3.7-kb *Taq* I RFLVs; and the *CYP21*-3'-specific 2.4-kb and 2.5-kb *Taq* I RFLVs (Fig. 2 Center; densitometric data not shown). These results suggest that the affected boy has inherited a deletion haplotype that lacks a *CYP21*-*C4* unit. However, he does not appear to have inherited the maternal deletion haplotype as he lacks the maternal 6.4-kb *C4*-5' band and shows a comparative deficiency of *Taq* I RFLVs associated with the *CYP21B* locus (3.7 kb, 2.5 kb) instead of those associated with the *CYP21A* locus.

Supportive evidence for the occurrence of a *de novo* deletion on the maternally inherited disease haplotype has also been obtained from complement C4 allotyping studies (Fig. 2 Right). Both parental samples show only *C4A3* and *C4B1* bands but, whereas the phenotype of the father is the same as that of his two unaffected sons in showing a comparative deficiency of *C4B*, the maternal sample shows a comparative deficiency of *C4A*, as expected from the maternal deletion haplotype *d* that was inferred from the *Taq* I mapping analyses above to lack *C4A* and *CYP21A* markers. The complete absence of a *C4B* allotype in the affected child is therefore indicative of a *de novo* deletion on the maternally inherited disease haplotype that has resulted in the elimination of *C4B* gene sequence.

The above *Taq* I and C4 allotyping analyses are necessarily indirect in that they measure the combined contributions of genes or gene products on two haplotypes in each individual, and the interpretation of gene organization in the 21-OH/C4 gene cluster by such indirect methods has been the subject of much recent controversy (10–12). To assess the gene organization at the 21-OH/C4 gene cluster in individual haplotypes we have used a direct long-range variable number of

FIG. 3. Long-range VNTR mapping analyses of the 21-OH/C4 gene organization in indicated members of a 21-OH deficiency family. High molecular weight genomic DNA samples from indicated family members were digested with *Bss*HII (Left) or with *Sac* II (Right) prior to size fractionation by pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis gels were dried down prior to direct probing with the 21-OH-specific probe, 21A-2.8. Numbers to the right of the individual panels refer to the size in kb of indicated hybridization bands.

tandem repeats (VNTR) mapping approach. High molecular weight genomic DNA samples are digested with rare cutter restriction nucleases that cleave at well-conserved recognition sites flanking the 21-OH/C4 gene array, and the resulting haplotype-specific large restriction fragments are size fractionated by pulsed-field gel electrophoresis prior to detection with a 21-OH-specific DNA probe (5). Size variation in such large restriction fragments has been found to reflect integral numbers of the tandem *ca.* 30-kb repeat units that contain a single *CYP21* gene and a single *C4* gene, the individual repeat units showing length heterogeneity as a result of the presence or absence of a *ca.* 6.5-kb insert in a 5' intron of the *C4* genes. The *Bss*HII mapping analyses shown in Fig. 3 Left reveal two RFLPs in the paternal sample, whose sizes (≈ 110 kb and 105 kb) correspond with those of haplotypes that possess two *CYP21*-*C4* containing repeats (5).

In the case of the 110-kb RFLP, the corresponding haplotype contains two repeats of the long variety—i.e., they contain long *C4* genes (a *C4A* gene and a *C4B* gene associated with a 5' 6.0-kb *Taq* I RFLV), whereas the 105-kb *Bss*HII RFLP is a marker of a haplotype where one of the repeats contains a long *C4* gene (*C4A*) and the other repeat contains a short *C4B* gene (associated with the 5.4-kb *Taq* I RFLV). The maternal sample also exhibits a 105-kb *Bss*HII RFLP and also a 75-kb RFLP that represents the HLA-DR3, B8, A1-associated deletion haplotype that contains a single short *C4B* gene and a *CYP21B* gene. Of the parental RFLPs, the paternal 110-kb RFLP is transmitted to all three sons, whereas the two unaffected boys have inherited the maternal 105-kb RFLP. However, the maternally inherited disease haplotype in the affected boy is associated with a new *Bss*HII RFLP at 80 kb, as expected in the case of a deletion haplotype that has a single long repeat unit. The analogous *Sac* II mapping analyses show the same pattern (Fig. 3 Right). A novel RFLP is apparent in the affected boy (*ca.* 40 kb) that is associated with a deletion haplotype where the single *C4* gene is of the long variety and is distinct from the maternal deletion haplotype, which has a single *C4* gene of the short variety (*ca.* 35-kb RFLP) (6). The combined results from HLA and complement allotyping and

Table 1. Serologically defined markers and DNA markers associated with inferred haplotypes

Haplotype	Serologically defined HLA/C4 marker					DNA marker, kb					
						Short-range				Long-range	
	DR	C4B	C4A	B	A	21B (<i>Taq</i> I)	C4B (<i>Taq</i> I)	21A (<i>Taq</i> I)	C4A (<i>Taq</i> I)	21/C4 (<i>Bss</i> HII)	21/C4 (<i>Sac</i> II)
<i>a</i>	4	Qo	3	44	2	2.5, 3.7	6.0	2.4, 3.2	7.0	110	70
<i>b</i>	6	1	3	7	30	2.5, 3.7	5.4	2.4, 3.2	7.0	105	65
<i>c</i>	7	1	3	13	30	2.5, 3.7	5.4	2.4, 3.2	7.0	105	65
<i>d</i>	3	1	Qo	8	1	2.5, 3.7	6.4	—	—	75	35
<i>c/d</i>	3	Qo	3	13	30	—	—	2.4, 3.2	7.0	80	40

FIG. 4. Genesis by meiotic unequal crossover of a *de novo* deletion that contributes to 21-OH deficiency. *CYP21A* and *CYP21B* genes are indicated by 21A and 21B, respectively. The positions of *Taq* I sites that are relevant to the short-range mapping analyses at the *CYP21* and *C4* loci are represented by T. Flanking *Bss*HII sites are denoted by B. Numbers refer to the size in kb of the DNA segment between the indicated *Taq* I sites or between the flanking *Bss*HII sites. Note that both maternal *C4B* genes are of the short variety, whereas the *C4A* gene is long. Consequently, maternal haplotype *c* contains one long and one short repeat unit, maternal haplotype *d* contains a single short repeat unit, and recombinant haplotype *c/d* contains a single long repeat unit.

from short-range and long-range DNA mapping analyses (see Table 1) indicate a *de novo* deletion of a *CYP21B*-*C4B* unit on the maternally inherited disease haplotype as a result of meiotic unequal crossover between maternal haplotypes as illustrated in Fig. 4.

DISCUSSION

Unequal crossover has been widely assumed to be an important contributor to polymorphism at loci containing clustered repeat elements. However, although experimental verification of the involvement of unequal crossover has been obtained in certain cases in lower organisms, such as the rRNA-encoding DNA (rDNA) clusters in yeast (13), direct evidence for a similar role in higher organisms has not been so forthcoming. In mammals, a considerable amount of circumstantial evidence for unequal crossover mechanisms has been amassed from intraspecific and interspecific comparisons between the haplotypic organizations of DNA sequences at various loci, and unequal crossover events have been suggested in the molecular pathology of various inherited disorders in humans, including thalassaemia (14), growth hormone deficiency (15), 21-OH deficiency (5), color blindness (16), hypercholesterolaemia (17), X chromosome-linked ichthyosis (18), Tay-Sachs disease (19), adenosine deaminase deficiency (20), Duchenne muscular dystrophy (21), Fabry disease (22), and diseases associated with *C4* deficiency (23). Two basic classes of homologous unequal crossover have been inferred depending on whether the location of the unequal crossover is defined by analogous positions within homologous repeat unit members of a tandemly repeated array or by homologous interspersed repeat elements (24). In the first case, unequal crossover is envisaged to generate haplotypes with a variable number of tandem repeats, and a diagnostic criterion of VNTR polymorphism will be a size variation between haplotypes that is defined by integral multiples of the repeat unit length (25). The human 21-OH/*C4* gene cluster represents an example of this kind where the observed variation in haplotype length conforms to integral multiples of the *ca.* 30-kb unit repeat length (5). In the

second case, unequal recombination events are thought to be mediated by interspersed repeated elements occurring within or in the vicinity of a single-copy gene or clustered multigene family, and examples of this type have been implicated in the molecular pathology of several disorders (17, 19, 20, 22, 26).

In addition to the above haplotypic variation in gene organization, hybrid genes that individually show characteristics of two nonallelic loci—e.g., hemoglobins Lepore and Kenya (14)—also provide strong circumstantial evidence in support of a previous unequal crossover event. However, direct evidence for unequal crossover minimally requires the demonstration of difference in gene organization between known parental haplotypes and an identifiable recombinant haplotype, and evidence for this is rare in higher organisms. In the case of postulated unequal sister chromatid exchanges the inability to demonstrate exchange of flanking markers admits the possibility of alternative mechanisms. For example, in humans, although family studies of tandemly repeated hypervariable loci have permitted identification of a few spontaneous mutations to new length alleles (27, 28), no exchange of flanking DNA was detected in the one case that has been subjected to extensive molecular characterization (28). Although unequal crossover between sister chromatids, but not between non-sister chromatids, is a possible mechanism in this case, alternative possible mechanisms can be invoked, such as polymerase slippage and loopout deletion.

The case for unequal crossover is stronger when recombination is envisaged between homologous regions of non-sister chromatids, and DNA sequence organization can be shown to be altered on a recombinant haplotype when compared to known parental haplotypes. Densitometry-based analyses have suggested sequence duplication/deletion variation on mouse recombinant *t* locus haplotypes (29) and in the mouse pseudoautosomal region (30). However, the analyses have been indirect and have not included molecular characterization of sequences flanking the proposed crossover point. In the present report, family-based HLA-typing studies have permitted the identification of an intra-HLA recombinant haplotype in a 21-OH deficiency patient that has been generated by meiotic recombination between maternal homologous chromosomes. Indirect *C4* allotyping studies and short-range restriction mapping studies using 21-OH and *C4* probes have suggested that the recombinant haplotype lacks the normally functional 21-OH gene (*CYP21B*) and also a companion *C4B* gene, suggesting a *de novo* deletion of a *ca.* 30-kb repeat unit containing the two genes. Additionally, direct *Bss*HII and *Sac* II long-range mapping analyses confirm the absence of a *ca.* 30-kb repeat unit on the recombinant haplotype, and the observed size variation of the haplotypes in this family again conforms with that expected from a VNTR-type polymorphism (5). Taken together, these data provide the most direct evidence yet for the natural occurrence of an unequal crossover event in mammalian genomes. In relation to these observations, an analogous meiotic recombination event has been reported in the mouse H-2 class III region, wherein indirect short-range restriction mapping and *C4* allotyping analyses have suggested that the recombinant H-2^{aw18} haplotype differs from the parental H-2^a and H-2^{bm7} haplotypes in lacking a *C4* gene and a functional 21-OH gene (31). Although the structures of mouse *C4* and 21-OH genes show close resemblances to their human homologues, the chromosomal organizations are different in that the mouse 21-OH/*C4* pairs are separated by about 80 kb of DNA and it is not known whether the regular expansion/contraction events that underpin the VNTR model in humans are also applicable in mouse.

The observed HLA typing, complement *C4* allotyping, and DNA probing results (see Table 1) indicate that the unequal crossover has taken place in the *ca.* 5.5-kb *C4B*-*CYP21A* gene interval, resulting in a *de novo* deletion that eliminates

a functional *CYP21B* gene and a neighboring *C4B* gene. The junction between the tandem repeat units is also known to occur within the *C4B-CYP21A* interval as inferred from the observation of strong sequence homology between the DNA regions found to flank the 5' ends of the *C4A* and *C4B* genes (M. Anderson and R. D. Campbell, personal communication) and also between the regions found to flank the 3' ends of the *CYP21A* and *CYP21B* genes (2.4-kb and 2.5-kb *Taq* I RFLVs; see *Results*). Consequently, the crossover maps to a region ≈ 2 kb long, defined centromerically by the junction between the two tandem repeat units and telomerically by the location of the variable *Taq* I site that defines the 2.4-kb/2.5-kb *Taq* I RFLV system at a position ≈ 2.4 kb 3' to the *CYP21* genes.

In the present study one of the maternal haplotypes involved in crossover is the common HLA-DR3, B8, A1 haplotype, which is known to occur at high frequencies in Western populations, and in the Irish population accounts for about 24% of normal haplotypes (Tony Barnes, personal communication). This haplotype is normally associated with a *ca.* 30-kb deletion and has a single repeat unit containing the functional *CYP21B* gene and a *C4B* gene. Consequently, meiotic pairing between chromosomes with this organization and homologous chromosomes with the conventional two-repeat unit organization would be expected to lead to mispairing such that a *ca.* 30-kb region of the latter would be expected to loop out. Unequal pairing between the *CYP21B* and *C4B* genes on the HLA-DR3, B8, A1 haplotype and the *CYP21A* and *C4A* genes of conventional haplotypes would be expected to occur in approaching 50% of meioses. Sequence interaction between the *CYP21A* and *CYP21B* genes following unequal chromosome pairing has been implicated in the origin of all known pathological mutations in the 21-OH gene (2, 3). Consequently, by promoting the probability of unequal chromosome pairing, such single-repeat unit haplotypes may be envisaged as being analogous to premutations in increasing the susceptibility of developing subsequent pathological mutation (32).

Finally, the above data suggest that on rare occasions, one of the biological parents of a 21-OH deficiency patient may not be a carrier of the disorder. The biochemical data are consistent with the father being a carrier of 21-OH deficiency, but not the mother (see legend to Fig. 2). In this family, therefore, the risk of having another affected child could be expected to be $<1\%$ instead of the usual 25%.

We are grateful to Duncan Campbell for providing a parent *CYP21A* subclone from which 21A-2.8 was derived. This work was supported by a grant from the Wellcome Trust.

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