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MOLECULAR AND ENDOCRINE CHARACTERIZATION OF A MUTATION INVOLVING A RECOMBINATION BETWEEN THE STEROID 21-HYDROXYLASE FUNCTIONAL GENE AND PSEUDOGENE

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Summary—The gene encoding steroid 21-hydroxylase activity, *P*450c21B, is located in the major histocompatibility complex (MHC) class III region, in close proximity to a highly homologous pseudogene, *P*450c21A. Recombinations between *P*450c21B and *P*450c21A have been shown to result in deficiency of 21-hydroxylase activity, the usual cause of congenital adrenal hyperplasia (CAH). A mutant *P*450c21 gene from a patient with simple virilizing CAH was identified and shown to be consistent with a recombination between *P*450c21A and *P*450c21B. Sequence analysis of the mutant gene showed the recombination site to be located between the first exon and the second intron. The mutant gene encodes a leucine instead of the normal proline at codon 31. This mutation resides on a chromosome bearing the HLA-B44 serotype. A comparison of mutations associated with HLA-B44 and that normally found with the HLA-Bw47 serotype suggests that the HLA-B44 mutations are of more ancient origin. The patient's homologous chromosome has a deletion of *P*450c21B. Endocrinological testing therefore allows for testing of the mutant gene in genetic isolation. Such testing demonstrated that the patient was capable of producing aldosterone and retaining sodium in response to a low-sodium diet, indicating that the mutant gene encodes an enzyme with partial 21-hydroxylase activity.

INTRODUCTION

The genetic locus of steroid 21-hydroxylase (EC 1.14.99.10) has been localized to the short arm of chromosome 6 in humans, within the major histocompatibility complex (MHC) class III region [1–3]. This locus normally contains a functional gene encoding 21-hydroxylase activity, designated *P*450c21B [4], and a highly homologous pseudogene, designated *P*450c21A, which are arranged in tandem with the genes encoding complement proteins *C4B* and *C4A* (Fig. 1). Mutations in *P*450c21B give rise to a deficiency of adrenal 21-hydroxylase activity with characteristic clinical and biochemical abnormalities, inherited as an autosomal recessive trait and responsible for at least 90% of cases of congenital adrenal hyperplasia (CAH) [5].

Comparison of the DNA sequences of *P*450c21A and *P*450c21B has revealed several mutations in the coding sequence of *P*450c21A, which render it non-functional: an 8 base-pair (bp) deletion in exon 3 which introduces a frame-shift mutation, a single-base insertion in the 7th exon and a nonsense mutation in exon 8 [6–8]. In addition, a *P*450c21A-specific probe failed to hybridize to total adrenal RNA suggesting that *P*450c21A is not transcribed or that its mRNA has reduced stability [6]. Mutations in the functional gene, *P*450c21B, give rise to CAH. These mutations can be classified as gene conversion-like recombinations, gene deletions and point mutations. Of these, gene conversion-like events involving the incorporation of deleterious sequences from *P*450c21A into *P*450c21B appear to account for the majority of mutations.

*P*450c21B and *P*450c21A may be distinguished on Southern analysis by their characteristic fragment lengths following digestion of genomic DNA with various restriction enzymes. In particular, *P*450c21B is associated with *Taq*I 3.7 and *Kpn*I 2.9 kb fragments, while *P*450c21A is associated with *Taq*I 3.2 and *Kpn*I 4.0 kb fragments. Both of these restriction fragment length polymorphisms are due to sequence

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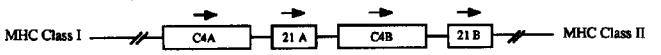

Fig. 1. Arrangement of *P*450c21B (21B) and *P*450c21A (21A) in tandem with the complement C4 genes, *C4A* and *C4B*. Arrows indicate the orientation of transcription.

differences in the 5′ upstream regions of these genes (Fig. 2) [6].

During an analysis of chromosomes containing 21-hydroxylase deficiency alleles, we observed a chromosome which has absence of the *Taq*I 3.7 and *Kpn*I 2.9 kb DNA fragments which are characteristic of *P*450c21B. However, hybridization using an oligonucleotide probe specific for the 8 bp in the 3rd exon of *P*450c21B, which are absent in *P*450c21A, indicated the presence of these 8 bp. This probe hybridized to the *Taq*I 3.2 kb fragment normally associated with *P*450c21A. Thus this chromosome, which is disease-associated, contains a *P*450c21 gene characterized by sequences from *P*450c21A within its 5′ region (the location of the *Taq*I and *Kpn*I restriction sites) and sequences from *P*450c21B at the 3rd exon (Fig. 2).

To characterize this mutant allele further, the region between the *Taq*I site, which is located 211 bp upstream from the 1st codon, and the 8 bp element in the 3rd exon was amplified and sequenced. The patients' other chromosome 6 contains a true deletion of *P*450c21B thus affording the opportunity to evaluate the endocrine consequence of the mutation in genetic isolation.

EXPERIMENTAL

Subjects

Subjects are designated as follows: H32 is an adult female with simple virilizing CAH due to 21-hydroxylase deficiency, which has been documented by standard clinical and biochemical testing since infancy. H36 and H29 are the father and mother, respectively, of H32. H9 is an unrelated female with salt-wasting 21-hydroxylase deficiency CAH. H11 and H10 are the father and mother, respectively, of H9. H68 is another unrelated female with salt-wasting steroid 21-hydroxylase deficiency CAH. NC1 is a normal individual. Approval for this study was obtained from the University of Minnesota's Human Subjects Committee.

Southern analysis

Preparation of genomic DNA and Southern analysis were performed by standard techniques as described in Ref. [9]. DNA probes used were as follows: p21OHB is a full-length genomic clone of the *P*450c21B gene. Owing to the very high sequence homology between *P*450c21B and *P*450c21A, it hybridizes to both genes under standard hybridization conditions. pAT-A (a kind gift of Dr Michael Carroll, Harvard University, Boston, MA) is a cDNA clone of a complement 4 gene [10]. A 600 bp *Bam*HI–*Bgl*II fragment corresponding to the 5′ end of the C4 gene was isolated and used as the C4 probe. This probe hybridizes to both the *C4A* and *C4B* genes.

Oligonucleotides used as *P*450c21B-specific probes were ON1: a 30-mer recognizing an 8 bp sequence in the 3rd exon of *P*450c21B which are absent in *P*450c21A (sequence: 5′-TGTGGGCTTTCCAGAGCAGAGAGTA-

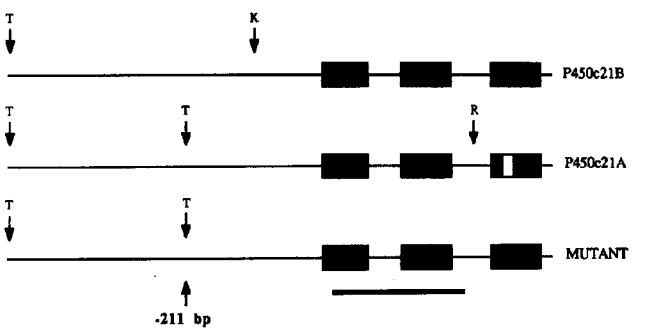

Fig. 2. Partial restriction map of the *P*450c21 genes T, K and R are *Taq*I, *Kpn*I and *Eco*RI. The shaded boxes indicate the first 3 exons. Exon 3 of *P*450c21A has an 8 bp deletion indicated by a white box. The recombinational event in the mutant gene occurred in the region shown by the heavy line.

GTCTC) and ON2: a 23-mer recognizing a sequence in the 8th exon of *P*450c21B which differs by a single base substitution from *P*450c21A (sequence: 5′-ATTCAGCAGCGA-CTGCAGGAGGA). Oligonucleotide probes were end-labeled by T4 polynucleotidyl kinase as described [11]. Final stringency conditions for washing filters probed with ON1 were 65°C and 6 × SSC for 10 min; for ON2 65°C and 5 × SSC for 10 min.

Restriction enzymes and the Klenow fragment of DNA polymerase I were from BRL (Bethesda, MD). T4 polynucleotidyl kinase was from Pharmacia LKB (Piscataway, NJ).

Polymerase chain reaction

Polymerase chain reaction (PCR) amplification primers were PR1: a 27-mer designed to prime in an upstream direction from the 3rd exon of *P*450c21B (sequence: 5′-GGGCTTTC-CAGAGCAGGGAGTAGTCTC) and PR2: a 27-mer designed to prime in a downstream direction from the region around the *Taq*I site at −211 bp upstream from the first codon (sequence: 5′-CCTTGCTTCTCGATGGGTGAT-TAATTT). PR1 should not anneal to the 3rd exon of *P*450c21A because the sequence of that gene is missing the 8 3′-terminal base-pairs of the complementary sequence required for PR1 binding. PCR was performed in a Perkin–Elmer/Cetus DNA thermal cycler in a total volume of 100 μl with reaction conditions as follows: 10 mM Tris–Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.25 mM each dNTP, 1 μM each PR1 and PR2, 1 μg genomic DNA and 2.5 U Ampli*Taq*™ DNA polymerase (United States Biochemical, Cleveland, OH). Following a 5 min denaturation step at 94°C, 30 cycles of PCR were performed with 2 min annealing at 60°C, extension at 72°C for 90 s (with 5-s increments/cycle) and a final extension of 12 min duration.

DNA sequencing

PCR-amplified DNA from subject H32 was blunt-ended with the Klenow fragment of DNA polymerase I and ligated into *Sma*I-cut pUC119. The resulting plasmids were used to transform *E. coli* JM101 cells by the $CaCl_2$ procedure [11]. Plasmids were recovered from cultures of transformed bacteria by alkaline lysis followed by ultracentrifugation in CsCl [11]. DNA inserts were isolated after double digestion with *Xba*I and *Sst*I. A series of overlapping subclones of the DNA inserts were made in pUC118 and pUC119. Bacterial cells containing these subclones were infected with the bacteriophage M13K07 to obtain single-stranded DNA. Sequencing was performed by the dideoxynucleotide method using a modified T7 DNA polymerase (Sequenase™ version 2.0; United States Biochemical Cleveland, OH) according to the manufacturer's recommendations.

Analysis of complement and HLA markers and endocrine assays

Phenotypes of C4 and Factor B (Bf) were determined as described previously [12] with a modification for C4 typing [13]. HLA serotypes were determined in the tissue typing laboratory of the University of Minnesota. Steroid assays were performed with the following RIA kits: aldosterone ('Coat-A-Count' Diagnostic Products, Los Angeles, CA); 17-hydroxyprogesterone ('SB-OHP', International-CIS, Polymedco, Yorktown Heights, NJ). Plasma renin activity was measured by the angiotensin I RIA kit (Du Pont, Billerica, MA). Electrolytes were measured on an Ektachem (Kodak, Rochester, NY).

Endocrine evaluation of steroid 21-hydroxylase activity

Steroid 21-hydroxylase activity was determined by evaluating salt retention and aldosterone synthesis in response to a low-sodium diet. Subjects were admitted to the University of Minnesota Clinical Research Center and placed on a sodium-restricted diet (10 mmol/day) for 4 days. To prevent possible endogenous synthesis of cortisol (which has a salt-retaining effect), subjects were also administered dexamethasone orally (2 mg/day) to suppress the hypothalamic–pituitary–adrenal axis [14]. Blood and urine samples were obtained for electrolyte and hormone assays by standard collection procedures.

RESULTS

Southern analysis

Probing of *Taq*I-restricted DNA with the 21-hydroxylase probe, p21OH, revealed the presence of the *P*450c21B 3.7 kb fragment in both H36 and H29, but not in their affected daughter, H32 (Fig. 3A), In addition, DNA from H32 revealed an absence of the *P*450c21B *Kpn*I 2.9 kb band (not shown). All of these subjects show the *P*450c21A-associated *Taq*I 3.2 kb band (Fig. 3A) and *Kpn*I 4.0 kb band (not shown).

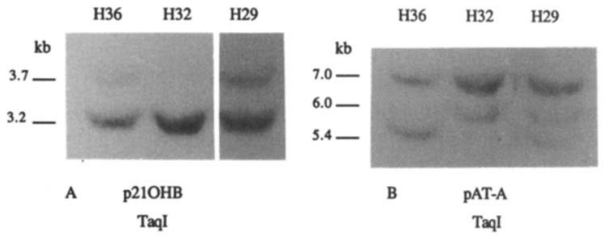

Fig. 3. Southern blots of *Taq*I-cut DNA probed with the steroid 21-hydroxylase probe, p21OHB (A) and the complement component C4 probe, pAT-A (B). The identity of the subjects is shown above the lanes.

When *Taq*I-restricted DNA from this family, and from another, unrelated family with 21-hydroxylase deficiency was probed with ON1 probe, which is specific for *P*450c21B, hybridization to the *P*450c21B *Taq*I 3.7 kb band occurred in all individuals except the patients H9 and H32 (Fig. 4). However, ON1 unexpectedly hybridized to the 3.2 kb band in H29 and in her daughter, H32. This indicates that H29 has a *P*450c21 gene which contains the restriction site profile of *P*450c21A at its 5′ region, but sequences from *P*450c21B at the 3rd exon. This gene has been transmitted to H32.

Further probing of *Taq*I-restricted DNA from H32 with the C4 probe pAT-A revealed the presence of hybridizing bands of 7.0 and 6.0 kb (the latter has been inherited from H29) (Fig. 3B). The 7.0 kb band is normally associated with the *C4A* genes, while the 6.0 kb band is normally associated with a *C4B* gene [15]. (The 5.4 kb bands seen in H36 and H29 also represent *C4B* genes but have not been transmitted to H32.)

These data indicate that the chromosome transmitted to H32 from H36 contains a deletion of a *P*450c21B gene and the neighboring *C4B* gene. On the other hand, the chromosome transmitted to H32 from H29 appears not to contain a simple deletion of a *P*450c21B gene but instead to have arisen by a recombination event leading to a hybrid gene which contains the restriction site profile of *P*450c21A at its 5′ end and sequences identifiable by ON1 as being derived from the 3rd exon of *P*450c21B. This recombination has not removed the *C4B* gene sequences which hybridize to pAT-A. In further support of this conclusion, scanning densitometry of the autoradiogram in Fig. 3A revealed that the ratio of the density of the *Taq*I 3.7 kb band to the 3.2 kb band is 0.47 in H36, consistent with a deletion of a *P*450c21B gene. In H29 the density ratio is 0.36, consistent with there being fragments of 3 *P*450c21 genes in the *Taq*I 3.2 kb band and 1 in the 3.7 kb band. Patient H68 has a homozygous deletion of the *P*450c21B and *C4B* genes (data not shown).

HLA serotyping and complement protein phenotyping indicated the presence of the following chromosome-6 haplotypes in H32: HLA-A3, HLA-Bw47, C4A91, C4BQ0, BfF (paternal) and HLA-A2, HLA-B44, C4A3, C4BQ0, BfF (maternal). The former haplotype

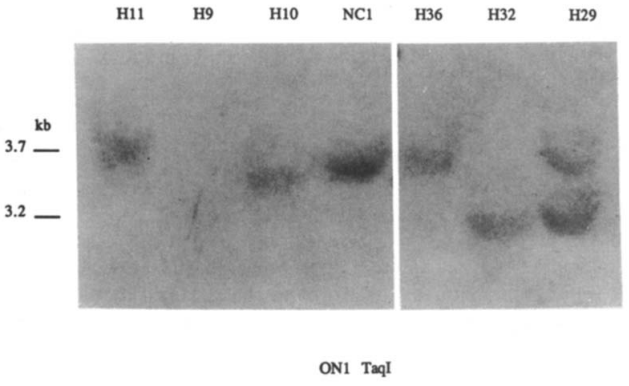

Fig. 4. Southern blot of *Taq*I-cut DNA probed with the *P*450c21B exon 3 specific oligonucleotide, ON1. The identity of the subjects is shown above the lanes.

has been demonstrated to be in strong positive linkage disequilibrium with 21-hydroxylase deficiency and to contain a deletion extending from the 3′ end of *P*450c21A to the 3′ end of *P*450c21B, eliminating the *C4B* gene, and most of *P*450c21B. The transition site has been localized between the 6th and 8th exons of the remaining *P*450c21 [16].

In agreement with this, ON2 hybridized to the *Taq*I 3.2 kb band in DNA from H36. As expected, DNA from H32, who has inherited this deletion from her father, H36, also showed this hybridization pattern. However, hybridization of ON2 to the *Taq*I 3.2 kb fragment in the mother, H29, is consistent with the more 3′ sequences of the mutant gene being derived from *P*450c21B (data not shown).

PCR amplification and DNA sequence analysis

In order to analyze the region involved in the gene conversion. PCR was performed using primers designed to hybridize specifically to the 3rd exon of *P*450c21B and to the region around the *Taq*I site at −211 bp from the initiation codon. This should result in specific amplification of the region of the gene conversion in H32, and of the normal *P*450c21B in other subjects. *P*450c21A should not be amplified because the downstream primer, PR1, cannot hybridize to that gene because it is missing the 8 bases complementary to the 3′ end of the primer.

PCR amplification of genomic DNA yielded a fragment of approx. 980 bp (the distance between the *Taq*I site and the 8 bp element in exon 3) in all subjects expected to have the 8 bp element characteristic of *P*450c21B, including H32 (Fig. 5, lanes B–E), but not in subject H9 whose DNA has a true deletion of *P*450c21B (Fig. 5, lane F). In addition, the PCR products from NC1 as well as H36, H32, and H29 did not show cleavage with *Eco*RI as would be expected if *P*450c21A had been amplified (data not shown). These data imply that under the PCR conditions used, the normal *P*450c21A is not amplified geometrically.

Therefore the sequences which are amplified geometrically are, as expected, the normal *P*450c21B gene and the mutant gene containing the *P*450c21B 8 bp element in exon 3. These two PCR products can be distinguished by digestion with *Kpn*I (Fig. 2). The normal *P*450c21B has a *Kpn*I site at −107 while the mutant gene, having the sequence from *P*450c21A at this site, is not cut by *Kpn*I. Digestion with *Kpn*I therefore shortens the *P*450c21B PCR product by 104 bp (Fig. 5, lanes H and K) but does not cut the mutant gene (Fig. 5, lane I). Subject H29, who has both a normal *P*450c21B and a mutant gene, shows both a cut and an uncut PCR product after digestion with *Kpn*I (Fig. 5, lane J).

Sequence comparison of the region between the *Taq*I site at nucleotide (nt) − 211 and the 8 bp element in the 3rd exon in H32 with published sequences of the normal *P*450c21B and *P*450c21A genes [6] is shown in Fig. 6. The comparison reveals that the 5′ portion of the mutant gene is derived from *P*450c21A as far as the substituted base at nt 92 which causes a proline-to-leucine substitution in the 1st exon. The 5′ flanking region contains the

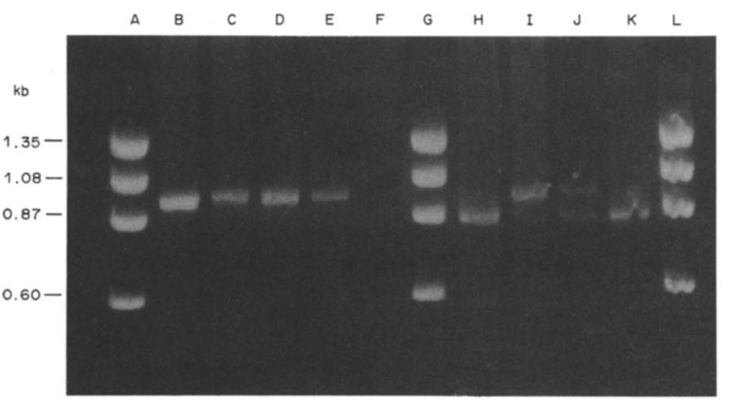

Fig. 5. Amplified DNA products following PCR. Lanes A, G and L are size markers. Lanes B, C, D and E show amplified DNA from subjects H36, H32, H29 and NC1, respectively. DNA from H9 did not amplify (lane F). Lanes H, I, J and K show the effect of *Kpn*I digestion of DNA from H36, H32, H29 and NC1, respectively.

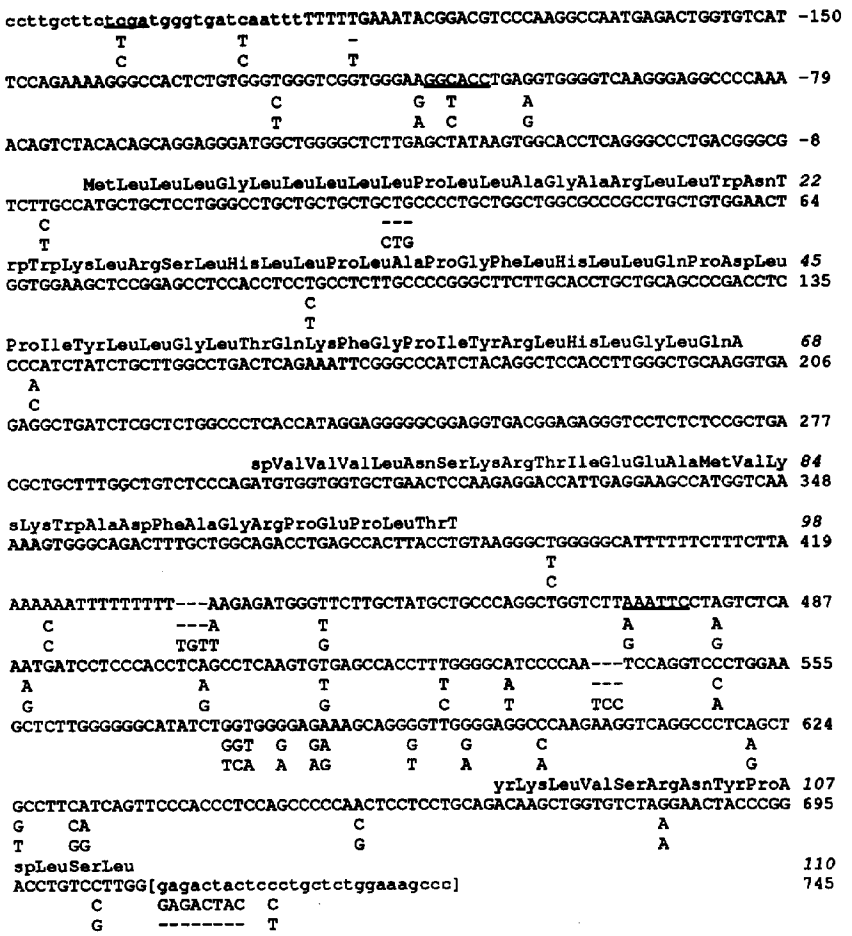

Fig. 6. Sequence of the region between the *Taq*I site at −211 bp from the initiation codon to the 8 bp element in exon 3. The full sequence is that of the mutant gene. The 2nd and 3rd lines show sequence differences from the *P*450c21B and *P*450c21A reported by Higashi [6]. The sequence of the PCR primers is shown in lower-case letters. For clarity, the sequence of the complementary bases to the downstream primer are presented [in square brackets]. A "-" indicates a base deletion. The *Taq*I, *Kpn*I and *Eco*RI sites shown in Fig. 2 are underlined.

characteristic base substitution which removes the *Kpn*I site at nt − 107. In addition, there are a further 5 bases characteristic of *P*450c21A as well as the presence of the *Taq*I site at nt − 211 (seen by restriction analysis). The presence of the extra leucine at codon 10 is not specific for *P*450c21A because it has been seen in one published *P*450c21B sequence [8] as well as the two sequenced pseudogenes [6, 7]. Beginning in the 2nd intron, at nt 398, the sequence diverges from *P*450c21A and matches that of *P*450c21B. The point of transition therefore lies between the 1st exon and the 2nd intron within a region of 300 bp. A more precise resolution of the transition site is not possible because of the sequence identity of *P*450c21A and *P*450c21B between these two points.

Endocrine studies

The residual 21-hydroxylase activity in H32 was analyzed by performing a salt-balance study. In response to sodium deprivation, H32 showed a 9-fold rise in her urinary aldosterone excretion with concomitant retention of urinary sodium (Table 1). For comparison, another adult female, H68, who has the salt-wasting phenotype was evaluated. As previously mentioned, H68 has a homozygous deletion of *P*450c21B. As is shown in Table 2, H68 showed a salt-wasting response to sodium deprivation in

Table 1. Results of salt-balance study, H32

24-h Urine	Day 1	2	3	4
17-Hydroxyprogesterone (pmol/24 h)		970	320	300
(nmol/mmol creatinine)		90	28	29
Na (mmol/l)		88	40	20
K (mmol/l)		16	27	43
Aldosterone (nmol/24 h)		8.3	33	75
(nmol/mmol creatinine)		0.77	2.9	7.3
Plasma				
Aldosterone (nmol/l)	<0.04			1.8
Renin activity (ng/l/s)	0.28			3.9

that she was unable to conserve urinary sodium, and showed only a minimal rise in aldosterone production.

DISCUSSION

Mutation analysis

The mutant gene characterized here is the result of a recombination between *P*450c21A and *P*450c21B without evidence of a deletion of either a *C4* or a *P*450c21 gene. A single unequal crossover event at the *P*450c21 locus (I) would be expected to produce two products (Fig. 7). The first (II) is a hybrid gene consisting of sequences from the 5′ region of *P*450c21A and from the 3′ region of *P*450c21B. This product should be associated with a deletion of *C4B* and a *P*450c21 gene. This pattern is found in association with HLA-Bw47. The second (III) expected product of a single unequal crossover contains an additional *P*450c21 gene characterized by sequences from the 5′ end of *P*450c21B and from the 3′ end of *P*450c21A. This product should also show duplication of *C4B*. The organization of the mutant gene described here is not consistent with either of these products of a single unequal crossover, but is consistent with a gene conversion which has directly transferred sequences from the 5′ region of *P*450c21B to the homologous region of *P*450c21A.

Gene conversion between *P*450c21A and *P*450c21B has been previously suggested as a mechanism involved in the generation of mutations at the *P*450c21B locus [8, 17–23] although in human genetic studies it is impossible to prove that a recombination has arisen by a classical conversion mechanism rather than by a double crossover event. Slightom *et al.* [24] have suggested that intergenic conversion may be a mechanism for regulating the co-evolution of related duplicated genes. If this mechanism is operating at the duplicated *P*450c21 loci, then it may contribute to the generation of new mutant *P*450c21 genes by bringing deleterious sequences from *P*450c21A into *P*450c21B.

The principal change in the sequence of the mutant gene is the proline-to-leucine substitution at codon 31. This change would be expected to alter the tertiary structure of the enzyme because a proline residue introduces a bend in protein conformation [25]. Such a mutation might alter the structure of the enzyme either at some critical site, or introduce some folding disruption which affects the overall tertiary structure. The known critical sites for the *P*-450 enzymes include the heme-binding region, the iron atom of which is believed to co-ordinate with the cysteine residue at amino acid 428 in the human 21-hydroxylase enzyme [6]. It has been suggested that the steroid binding region of this enzyme is between residues 338–361 based on similarities of sequence at this region between a range of steroid binding proteins (both receptors and enzymes) [26]. However, a specific function for the region around amino acid residue 31 has yet

Table 2. Results of salt-balance study, H68

24-h Urine	Day 1	2	3	4
17-Hydroxyprogesterone (pmol/24 h)		650	350	420
(nmol/mmol creatinine		69	35	46
Na (mmol/l)		84	59	72
K (mmol/l)		26	19	31
Aldosterone (nmol/24 h)		25	28	31
(nmol/mmol creatinine)		2.7	2.8	3.4
Plasma				
Aldosterone (nmol/l)	0.12			0.36
Renin activity (ng/l/s)	1.5			8.9

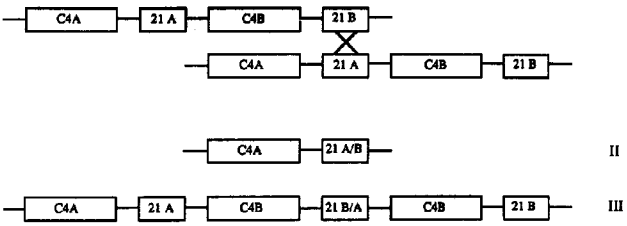

Fig. 7. A single unequal crossover event at the *P*450c21 locus (I) and its expected products (II and III).

to be described. In addition to the human gene, the murine [27], bovine [28] and swine [29] steroid 21-hydroxylases all have a proline residue at amino acid 31. Interestingly, the murine pseudogene has the same codon change which results in a proline-to-leucine substitution at residue 31 as does the human pseudogene [27]. Unlike the human pseudogene however, the murine pseudogene contains a large deletion which spans 215 bp, suggesting that the mutations in the pseudogenes of the two species are unrelated.

Implications of partial 21-hydroxylase activity

The demonstration of 21-hydroxylase activity sufficient to prevent salt-wasting in subject H32 with this mutant gene is of interest for several reasons. Firstly, patients with true homozygous deletions of *P*450c21B have the salt-wasting form of the disease, at least in infancy [5], while the patient described here, H32, can synthesize aldosterone, conserve sodium in response to a low-sodium dietary challenge, and does not required supplementation with mineralocorticoids. It is possible that the residual 21-hydroxylase activity is encoded by another gene. We have demonstrated excretion of tetrahydrocorticosterone (a 21-hydroxylated steroid) in two subjects with true homozygous deletions of *P*450c21B by gas-chromatography/mass spectrometry (unpublished observations). Both of these subjects have CAH with salt-wasting. Other workers have provided strong experimental support for the existence of multiple 21-hydroxylases [30, 31].

However, the observation that patients with true homozygous deletions of *P*450c21B have salt-wasting, at least during infancy, indicates that these alternative 21-hydroxylases cannot compensate for loss of the HLA-linked 21-hydroxylase activity. The observation that subject H32 described here does not have salt-wasting strongly suggests that the mutant gene encodes an enzyme with partial activity sufficient to prevent salt-wasting.

Secondly, Higashi *et al.* [6] were unable to demonstrate hybridization of *P*450c21A-specific probes to adrenal RNA which suggests that *P*450c21A is not transcribed or that the mRNA has reduced stability. Our data suggest that the 5′ flanking region of *P*450c21A, which contains a number of minor sequence differences from *P*450c21B, can support transcription of a *P*450c21 gene.

The diversity of HLA-B44-associated 21-hydroxylase mutations

A number of other investigators have reported mutations in *P*450c21B genes which are linked to HLA-B44. Rodrigues *et al.* [8] cloned and sequenced a mutant *P*450c21B allele on a haplotype containing the same markers as those associated with the mutation described here (HLA-A2, HLA-B44, C4A3, C4BQ0). That gene, however, has sequence differences from the one reported here and did not contain the proline-to-leucine substitution seen at codon 31. White *et al.* [16] reported one case of a possible gene conversion involving an HLA-B44-associated 21-hydroxylase allele which was associated with a *C4B* 6.0 kb fragment, and where an oligonucleotide probe specific for the 8 bp element of *P*450c21B hybridized to a *Taq*I 3.2 kb fragment. Further analysis is needed to determine if that allele is the same as the one described here. Higashi *et al.* [32], using a series of oligonucleotide probes specific for *P*450c21A and *P*450c21B described an HLA-B44-associated, mutant 21-hydroxylase allele which they suggested was the product of a gene conversion. In that case, the point of conversion occurred 3′ of the exon-3 marker. Other mutant *P*450c21 alleles linked to HLA-B44 have a different pattern of RFLPs from that described here or are linked to other genetic markers [15].

Thus, there are a variety of different mutations in the 21-hydroxylase locus which are found in association with HLA-B44. In marked contrast to this, haplotypes bearing the

HLA-Bw47 marker and a mutation at the *P*450c21B locus almost invariably appear to have a deletion of most of the *P*450c21B gene and the neighboring *C4B* gene. In addition, HLA-Bw47 is generally in strong linkage disequilibrium with CAH while HLA-B44 is not [33]. One possible explanation for these differences is that the mutations associated with HLA-B44 are of more ancient origin than that associated with HLA-Bw47 and consequently there has been time for linkage equilibrium to evolve between HLA-B44 and a number of different mutations at the *P*450c21B locus. Supporting evidence for this comes from sequence analysis which suggests that the *HLA-Bw47* gene may have arisen from the *HLA-B44* gene [34], and by the observation that HLA-Bw47 is found in only 1% of the population frequency of HLA-B44, suggesting that *HLA-Bw47* is itself of more recent origin. The observation that the mutation in linkage disequilibrium with HLA-Bw47 is a deletion of about 30 kb [16] suggests that it may have arisen from a single unequal crossover event, while the mutation described here appears to be the result of a gene conversion or a double crossover.

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