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Deletion of the steroid 21-hydroxylase and complement C4 genes in congenital adrenal hyperplasia

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SUMMARY DNA was analysed from 20 patients with congenital adrenal hyperplasia due to cytochrome P-450 steroid 21-hydroxylase deficiency. Using probes recognising sequences in both the 21-hydroxylase gene and the adjacent fourth component of complement (C4), one patient was found to have a homozygous deletion of DNA which encompassed the C4B and 21-hydroxylase B genes. Evidence is presented for this deletion arising by recombination between homologous regions of 21-hydroxylase A and B. Seven patients appeared to be heterozygous for the same deletion, but no detectable alteration in the 21-hydroxylase gene could be demonstrated in others.

Congenital adrenal hyperplasia (CAH) is an inborn error of cortisol biosynthesis. The most common form (some 95% of cases) arises from an inability to convert 17-hydroxyprogesterone to 11-deoxycortisol, a step controlled by a steroid 21-hydroxylase enzyme (EC 1.14.99.10). It is of autosomal recessive inheritance with an incidence ranging from 1 in 5000 to 1 in 15 000 in Europe and the United States, that is, of the same order of magnitude as phenylketonuria. This has led to the development of neonatal screening programmes in some regions of the United Kingdom, a recent neonatal study in Scotland finding an incidence of 1 in 18 000.[1]

There are four recognised clinical forms of 21-hydroxylase deficiency: salt losing, simple virilising, late onset, and cryptic. Their presentation has been reviewed by New and Levine.[2] It has been known for some time that there is a close genetic linkage between the salt losing and simple virilising forms and the human leucocyte antigen (HLA) genes.[3] Kohn *et al*[4] and Levine *et al*[5] have shown that this is also true of the late onset and cryptic forms. It has been proposed[4] that these variants represent combinations of 'severe' and 'mild' alleles for 21-hydroxylase deficiency.

In recent months, the genes coding for 21-hydroxylase have been located on the short arm of chromosome 6 within the class III major histocompatability region coding for the complement components C2, factor B, and C4 (fig 1).[6 7] Two 21-hydroxylase genes (21A and B) exist, only one of which is thought to be active.[7] They are located at the 3′ ends of the genes for the fourth component of complement (C4).[6 7]

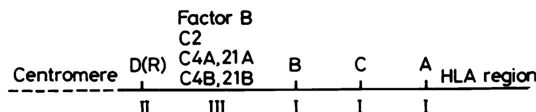

FIG 1 *Map of the HLA region on the short arm of chromosome 6 showing the approximate positions of the 21-hydroxylase genes in relation to the HLA class I genes (A,B,C), class II genes (DR), and class III genes, factor B, C2, C4A, and C4B.*

‡Dr R R Porter has died since this work was started.

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In this study we have analysed DNA from a number of patients presenting in the neonatal period with 21-hydroxylase deficiency, in an attempt to determine whether any particular restriction fragment length polymorphism or deletion can be found to be associated with the clinical phenotype.

Patients

All patients presented in the neonatal period with ambiguous genitalia with (18) or without (two) salt losing crises. CAH due to 21-hydroxylase deficiency was diagnosed by grossly raised 17-hydroxyprogesterone levels before treatment.

Methods

Genomic DNA was isolated from peripheral blood leucocytes by the method of Bell *et al.*[8] Samples of 10 μg were digested with restriction endonucleases according to manufacturer's instructions (Pharmacia Ltd). Digests were separated on 0·75% agarose gels, treated according to Wahl *et al*,[9] and blotted onto nitrocellulose.[10]

Probes were labelled with ^{32}P by nick translation.[11] Hybridisation was carried out at 65°C in a solution containing 5×SSC (1×SSC is 0·15 mol/l sodium chloride, 0·015 mol/l sodium citrate, pH 7·0), 10% w/v dextran sulphate, 0·02% w/v ficoll, 0·02%

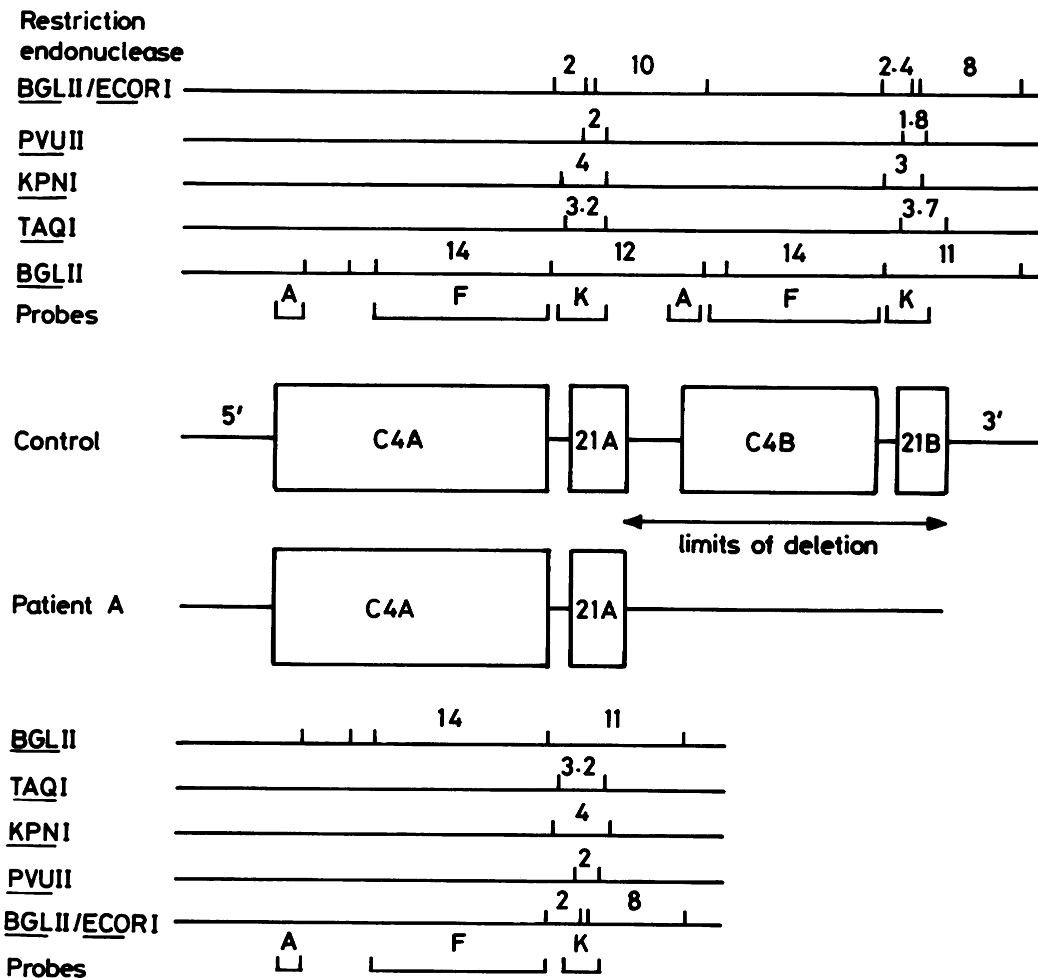

FIG 2 *Simplified restriction map of the C4 and 21-hydroxylase genes in a control subject and patient A showing the proposed limits of the deletion.*

w/v bovine serum albumin, 0·02% w/v polyvinylpyrollidine, 0·1% w/v sodium dodecyl sulphate (SDS), and 100 μg/ml denatured herring sperm DNA. Blots were washed for 4 × 5 minutes in 3 × SSC, 0·1% w/v SDS at room temperature, followed by 4 × 15 minutes in 1 × SSC, 0·1% SDS at 65°C. After drying they were exposed to Fuji 100-RX *x*-ray film at −70°C with an intensifying screen.

The probes used were as follows: probe K, unique sequences derived from a *Bgl*I digest of a 4 kilobase pair (kb) *Kpn*I genomic fragment isolated from cosmid Cos 1E3 and containing the 5′ end of the 21A gene[12]; probe F, cDNA extending from the 3′ noncoding region to within approximately 400 bp from the 5′ end of the coding region[13]; and probe A, 0·5 kb *Bam*HI/*Kpn*I fragment from the full length C4 cDNA insert of pAT-A[14] (fig 2).

Results

Owing to the proximity of the 21-hydroxylase and C4 genes, restriction fragments can be generated which contain parts of both genes (fig 2) and can therefore be detected by both C4 (A) and 21-hydroxylase (K) specific probes. By using a combination of three probes, a clear deletion of DNA in the 21-hydroxylase region was found in one of the 20 samples analysed. This patient (patient A) (HLA type A9,32 B44,13 Cw4 DR1,7) with the salt losing form of CAH was found to have a deletion of a 12 kb *Bgl*II fragment following hybridisation with probe K, the control showing two bands of 12 and 11 kb (data not shown). The 12 kb band is part of the 5′ C4B region and includes the 21-hydroxylase A gene[6] (fig 2). The presence of a deletion was supported by probe A which, in the control, hybridised to the other end of this fragment (fig 2). No other smaller fragments were detected with either probe K or A following *Bgl*II digestion, suggesting that the entire fragment was missing.

However, following hybridisation of a *Taq*I digest with probe K, a single band of 3·2 kb was seen, whereas the control contained two fragments of equal intensity at 3·2 and 3·7 kb (fig 3, lanes 1 and 2).

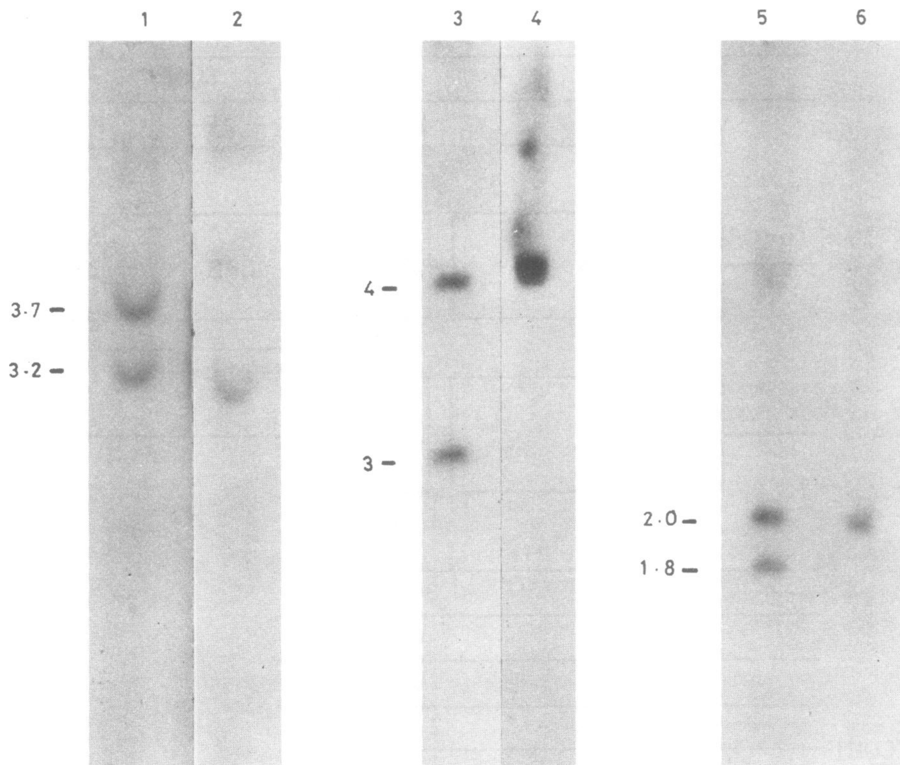

FIG 3 *Comparison of TaqI (lanes 1,2), KpnI (lanes 3,4), and PvuII (lanes 5,6) restriction enzyme digests of DNA from a control subject (lanes 1,3,5) and patient A (lanes 2,4,6) hybridised with probe K. Fragment sizes (kb) determined by inclusion of molecular size markers in the gel.*

The 3·2 and 3·7 kb bands are derived from the 21A and 21B genes respectively.[7] Thus, absence of the 3·7 kb fragment is consistent with a deletion of 21B and not of 21A. Further evidence for deletion of the 21B gene was found following hybridisation of probe K to DNA digested with two other enzymes, *Kpn*I and *Pvu*II. In the former case two bands of 4 and 3 kb were seen in the control. As probe K is derived from a 4 kb *Kpn*I genomic sequence in the 5′ region of the 21A gene, and is therefore a marker for 21A, the 3 kb fragment presumably arises from 21B. The latter is deleted in patient A (fig 3, lanes 3 and 4). Similarly, with the *Pvu*II enzyme two bands, 2·0 and 1·8 kb, were found in the control but only the 2·0 kb in this patient (fig, 3, lanes 5 and 6). These results also show that although the 21-hydroxylase genes share sufficient homology to hybridise to the same probe, they can be differentiated by restriction fragment length polymorphisms.

The extent of the deletion in patient A was found to include the C4B gene as all specific restriction markers for the C4B gene, for example, 1·7 kb *Bgl*II and 3·5 kb *Bam*HI fragments usually detected with probe F, were absent (data not shown). This is consistent with the finding of a homozygous null allele at the C4B locus on protein typing.

Fig 2 shows a simplified restriction map of the C4-21 hydroxylase gene region in a healthy subject and patient A. Our results are consistent with a deletion that extends from the 3′ end of the 21A gene to a homologous region at the 3′ end of the 21B gene including C4B, as indicated in fig 3. This could have arisen by recombination between two misaligned chromosomes, as depicted in fig 4.

Double digests with *Eco*RI and *Bgl*II were carried out on DNA from all patients. This digest has the advantage of yielding fragments which cover the entire 21A and 21B gene regions but, being smaller, can be more easily resolved than those produced by *Bgl*II alone. In the control sample, four bands of 10, 8, 2·4, and 2·0 kb were present (fig 5, lane 1), the 10 and 8 kb arising from the 3′ ends of 21A and 21B respectively. The 2·4 and 2·0 kb bands are from the 5′ ends of the gene, but as yet it is not clear which represents the 21A and B gene. However, as patient A showed a deletion of both the 10 and 2·4 kb bands (fig 5, lane 2), it would be reasonable to assume that the 2.4 kb fragment is part of the 21-hydroxylase B gene. Seven other patients (table, patients B to H) were heterozygous for a deletion of the 10 and 2·4 kb bands, as judged from the intensity of hybridisation and also by comparison with that of an obligate heterozygote (the father of patient A, fig 5, lane 3). They were also heterozygous for the *Pvu*II and *Kpn*I polymorphisms, from which it can be inferred that they share the same deletion as patient A on one chromosome. There appeared to be no correlation

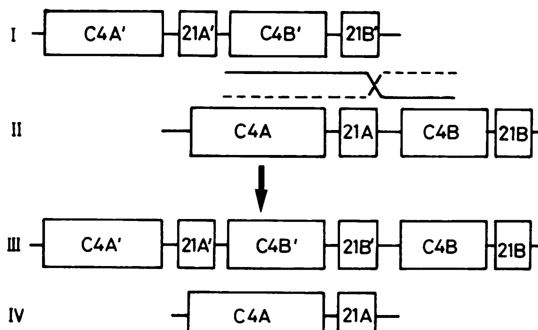

FIG 4 *Model describing unequal crossover between two homologous chromosomes (I and II) leading to duplication of C4B-21B on one chromosome (III) and deletion of C4B-21B on the other (IV).*

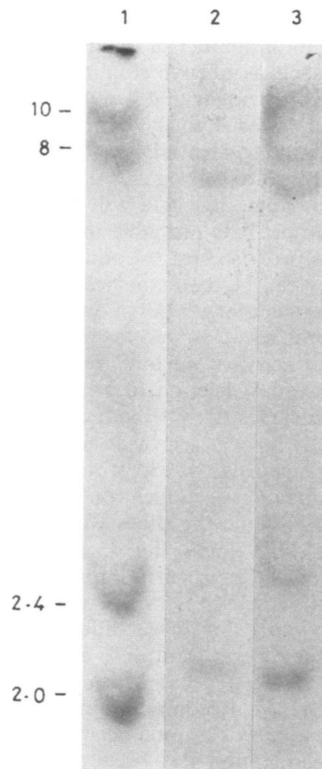

FIG 5 *BglII/EcoRI double digest of DNA from a normal control (lane 1), patient A (lane 2), and an obligate heterozygote (lane 3). Fragment sizes (kb) determined by inclusion of molecular size markers in the gel.*

TABLE *Summary of restriction enzyme data from eight patients with 21-hydroxylase deficiency of both salt losing (SL) and non-salt losing (NSL) phenotypes hybridised with probe K. Patients are identified by HLA-B types. Fragment sizes are given in kilobases (kb).*

Restriction enzyme	*Fragment size*	*Patient*							
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
BglII/EcoRI double digest	10	–	½	½	½	½	½	½	½
	8	+	+	+	+	+	+	+	+
	2·4	–	½	½	½	½	½	½	½
	2·0	+	+	+	+	+	+	+	+
Clinical phenotype		SL	NSL	SL	SL	SL	SL	SL	SL
HLA-B type		B44.13	B51	B35.7	B40.Bw3	NA	NA	B39.14	B7

+ = presence of a particular band, – = absence, ½ = heterozygote as judged by intensity of hybridisation, NA = data not available.

between the HLA-B type, the presence of this deletion, and the clinical phenotype as is shown in the table.

No further deletions or additional fragments were found in any other samples using probe K.

Discussion

In common with a number of other inborn errors of metabolism, such as phenylketonuria and β thalassaemia, it is now evident that a number of different mutations will give rise to the clinical phenotype of congenital adrenal hyperplasia.

The two 21-hydroxylase genes appear to share a high degree of homology and will hybridise to the same DNA probe. It is probable that they arose by duplication as proposed for the adjacent C4 genes.[15] However, they can be distinguished by a number of restriction fragment length polymorphisms as shown here and by others.[6 7 15]

Using these markers we have found one patient with a homozygous deletion of a large fragment of DNA which includes the C4B and 21-hydroxylase B genes. This may have arisen by an unequal crossover event during meiosis which would lead to one chromosome having a duplication of the C4B-21B locus (fig 4, III) while the other would be left with only the C4A-21A locus (fig 4, IV), making the recipient of the latter a carrier for 21-hydroxylase deficiency. Large deletions in this region have been reported previously for three different haplotypes, although in these cases leading to a loss of the C4B and 21-hydroxylase A genes which were not associated with CAH.[15] Recombination in this region appears to be a fairly common occurrence.

A strong association has been described for the different forms of CAH with various HLA-B haplotypes: the salt losing form with HLA-B47,[16] simple virilising with Bw51/5,[16] and late onset and cryptic forms with HLA-B14.[17] Linkage is to be expected in view of the proximity of 21-hydroxylase and HLA genes on chromosome 6.[6 7] The B47 haplotype also has a deletion of the 21B gene,[16] but the extent of the deletion has not been described. Although this haplotype is associated with a null allele at the C4B locus on protein typing,[7] Carroll *et al*[15] have shown that not all null alleles can be attributed to deletions of DNA. There were no HLA-B47 types among the 18 salt losing patients studied here, the most common HLA types found being B44, B14, and B7. Only one subject with HLA-B type B44, B13 was found to have a deletion of 21-hydroxylase B on both chromosomes. In those patients apparently heterozygous for this deletion, no gross abnormality could be demonstrated in the other 21-hydroxylase B gene. In such cases, sequencing of the remaining B gene may identify point mutations, that is, changes in a single nucleotide which could otherwise only be detected if they altered a restriction enzyme site.

In conclusion, this work indicates that the clinical phenotype of 21-hydroxylase deficiency is due to a heterogeneous collection of defects and only in occasional instances arises from a substantial deletion of DNA.

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