Phenotypic variability and origins of mutations in the gene encoding 3β-hydroxysteroid dehydrogenase type II

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ABSTRACT

Mutations in HSD3B2, the gene for 3β-hydroxysteroid dehydrogenase type II (3β-HSD II) have been detected and activities analysed through the *in vitro* expression of mutant cDNAs. Two full sibs with male pseudohermaphroditism were found to be double heterozygotes: N100S/266ΔA. This genotype leads to the most profound loss of 3β-HSD II enzyme activity (1·3% of normal) described to date in cases without severe salt-loss. One sib (N100S/266ΔA) is the first reported male case of type II deficiency affected with premature adrenarche. Three apparently independent kindreds had propositi affected with the HSD3B2 mutation A82T/A82T, which is associated with a non salt-losing phenotype with variable expressivity in females. These three families had the same extended HSD3B haplotype and are likely to have inherited the same ancestral mutation. The significance of this finding is discussed in the light of the presence of A82T mutation at a homologous position in pseudogene φ5 that is present in the HSD3B cluster.

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INTRODUCTION

3β-hydroxysteroid dehydrogenase (3β-HSD) is a NAD-dependent dehydrogenase that converts 3β-hydroxy-Δ^5-steroids to 3-keto-Δ^4-steroids and initiates the biosynthesis of mineralocorticoids, glucocorticoids and sex steroids (Mason 1993, Sutcliffe *et al*. 1996, Morel *et al*. 1997). Two forms of the enzyme have been described in humans (Lorence *et al*. 1990, Rheaume *et al*. 1991): type I enzyme is expressed principally in placenta and skin, and type II in adrenals and gonads. We have recently shown that the genes for these two enzymes are located in a cluster on chromosome 1p13·1 together with five closely related unprocessed pseudogenes, φ1–5 (McBride *et al*. 1999, Russell *et al*. 1994).

Null mutations of HSD3B2 lead to congenital adrenal hyperplasia (CAH), characterized by reduced levels of glucocorticoids and sex steroids, and severe salt wasting due to the loss of aldosterone synthesis in the zona glomerulosa of the adrenal cortex (Rheaume *et al*. 1992). Observations on a compound heterozygote affected with salt-losing 3β-HSD II deficiency led to the deduction that a reduction to 0–0·4% of normal type II enzyme activity led to a severe salt-losing phenotype (Sanchez *et al*. 1994). Mutations that cause a less severe loss of type II enzyme activity are associated

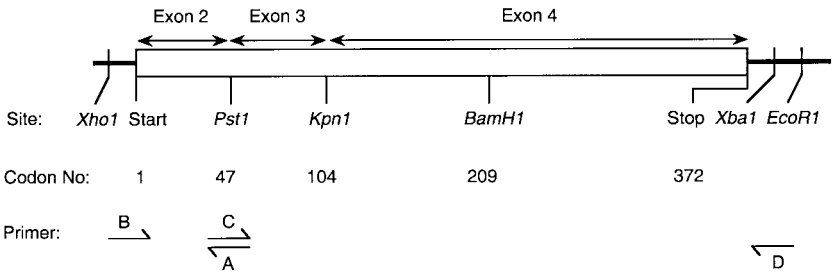

FIGURE 1. Diagram of the 3β-HSD type II expression cassette showing restriction sites used in constructing normal and mutant cDNAs. The positions of the primers A, B, C and D which were used to amplify cDNA fragments are shown. Engineered restriction sites are in italic.

with adequate production of aldosterone, and deficiencies in sex hormone synthesis. In males this causes insufficient synthesis of testosterone and its derivative, dihydrotestosterone, which leads to a failure in the development of male external genitalia and presents as hypospadias or pseudohermaphroditism (Rheaume *et al*. 1992, Simard *et al*. 1993, Russell *et al*. 1994). The effect of partial 3β-HSD II deficiencies on the female phenotype is more variable and extends from no phenotypic effect (Mendonca *et al*. 1994, Russell *et al*. 1994) through to premature pubarche, amenorrhoea, clitoromegaly and virilization (Mendonca *et al*. 1994, Paula *et al*. 1994, Rheaume *et al*. 1994, Sanchez *et al*. 1994). Here we report several cases of 3β-HSD type II deficiency. One, with genotype N100S/266ΔA, has the greatest reduction in type II enzyme activity yet reported in patients not affected with severe salt-wasting. We previously reported the occurrence of variable phenotypic effects in homozygotes for the HSD3B2 mutation, A82T (Mendonca *et al*. 1994). We now extend that observation with a further case report and to test whether these mutations are identical by descent. Finally, we use a haplotype analysis to investigate whether the presence of the corresponding A82T codon in pseudogene HSD3Bφ5 in the 3β-HSD gene cluster may provide evidence that the A82T mutation in HSD3B2 may have arisen through a gene conversion or recombination event involving another member of the gene cluster.

MATERIALS AND METHODS

Assays of plasma steroids were performed as previously described (Mendonca *et al*. 1994, Russell *et al*. 1994). Exons of the HSD3B2 were screened for mutations by denaturing gradient gel electrophoresis (DGGE), subcloned into pT7Blue T-vector (Novagen, Madison, WI, USA) and sequenced as previously described (Russell *et al*. 1994). The sequence of several independent PCR amplifications were determined to avoid mutations generated by PCR.

Expression cassette

For expression studies on 3-HSD II, segments of mutant alleles were subcloned into the type II cDNA and the resultant constructs were inserted into the eukaryotic expression vector pcDNAI/neo (Invitrogen, Groningen, The Netherlands). To facilitate the transfer of mutant exons, a PstI site was created by PCR-based mutagenesis (CTGCAG → CTGCAG) of codon 49, which is located at the extreme 5′ end of exon 3 (Fig. 1). This is a synonymous mutation (L49 L: CTGCAG) and does not alter the translational sense of HSD3B2. Mutagenesis was carried out by PCR amplification of type II cDNA with the following DNA primers (see Fig. 1). Primer A: 5′ GGTCCTGTTCT GCACGT 3′ (annealed to exon 3 sequence and contained codon 49 mutation, underlined); primer B: 5′ CTCGAGATGGGCTGGAGCTGCCTT GTGAC 3′ (annealed to the translational start site and included a 5′ XhoI site, underlined). The exons 3 and 4 of the cDNA were amplified together with primers C and D. Primer C: 5′ AGCTGCA GAACAGGACC 3′ (annealed to exon 3 sequence and contained codon 49 mutation, underlined); primer D: 5′ GAATTCTCTAGAGCACATCT CTGTCATCC 3′ (exon 4, 3 untranslated region with XbaI and EcoRI sites, underlined). DNA segments A-B and C-D were ligated, as respective XhoI-PstI and PstI and PstI-XbaI fragments, and inserted into pcDNAI/NEO. The resultant sequence was confirmed by DNA sequencing, and on expression yielded a K_m for wild type 3β-HSD II within the normal range. Mutations in exonic fragments of the type II gene were then subcloned into the full-length type II cDNA for expression.

Exon 3 mutations A82T and N100S were subcloned as PstI-KpnI fragments, and then transferred into eukaryotic expression plasmid pcDNAI/neo.

In vitro expression of 3β-HSD activity

In all expression experiments, equal quantities of newly prepared expression vector for the normal and mutant alleles were transfected into unit quantities of tissue culture cells. Thus COS1 cells in 100 mm dishes were transfected with 10 μg plasmid DNA by the DEAE-dextran method (Lorence *et al*. 1990). Cells at 24 h post-transfection were split into 12-well tissue culture dishes, and individual wells were assayed 24 h later for 3β-HSD activity by the addition of 0·5 ml media (Dulbecco's modified Eagle's medium (DMEM), 10% FCS, Life Technologies, Paisley, UK) containing 1 μM pregnenolone (Sigma, Poole, UK) and 0·025 μCi [^{3}H] pregnenolone (DuPont/NEN, Stevenage, UK) or dehydroepiandrosterone (DHEA) (Sigma) and 0·005 μCi [^{14}C]-DHEA (DuPont/NEN). Media were removed at recorded intervals and steroids were extracted into dichloromethane and separated by thin-layer chromatography (using 4:1 dichloromethane:ethyl ethanoate for pregnenolone incubations and 45:25:20:8:2 dichloromethane:cyclohexane:ethyl ethanoate:propanone:ethanol for DHEA incubations). Radiolabelled steroids were quantified using a phosphorimager (Fujifilm BAS-1500).

Cell homogenates were prepared by harvesting transfected cells into PBS/20% glycerol (v/v) using a rubber policeman, snap-frozen in a CO_2/ethanol bath and subjected to three cycles of freeze–thaw. Samples were sonicated and centrifuged at 800 r.p.m. for 1 min to pellet cellular debris. The protein content of the supernatant was determined by the method of Bradford (BioRad Protein Assay, Hemel Hempstead, UK) using bovine serum albumin as standard. The 3β-HSD activity was determined for pregnenolone and DHEA at 12 substrate concentrations ranging from 0·1 to 50 μM. Assays were performed at 37 °C in a total reaction volume of 0·5 ml consisting of 100 μg of total protein, 50 mM Tris pH 7·4, 1 mM NAD, of radiolabelled substrate (0·025 μCi [^{3}H]pregnenolone or 0·005 μCi [^{3}H]pregnenolone or 0·005 μCi [^{14}C] DHEA) and the desired concentration of the relevant unlabelled substrate. The reaction was stopped by the addition of dichloromethane and reaction steroids were separated and quantified as above.

Determination of genotypes

Mutation A82T were detected as previously described (Mendonca *et al*. 1994, Russell *et al*. 1994). Mutation N100S and 266ΔA were detected by denaturing gradient gel electrophoresis of HSD3B2 exons 3 and 4 respectively, followed by DNA sequencing of PCR products (following Russell *et al*. 1994). Microsatellite repeat polymorphisms DIS514 and DIS534 were amplified by PCR in the presence of [^{32}P]dCTP and resolved on 6% polyacrylamide 7 M urea sequencing gels; other polymorphisms were resolved as previously described (Russell *et al*. 1994).

RESULTS

Two brothers with HSD3B2 N100S/266ΔA

Two brothers (BS and GS; respectively 11 and 9 years) were reported by Fisher *et al*. (1988) with perineal hypospadias and normal testes. Both had elevated levels of 17-hydroxyprogesterone and dehydroepiandrosterone sulphate (DHEA-S). Urinary steroids indicated a defect in 3β-HSD, with elevations in pregnenolone, 16-hydroxypregnenolone and 16-hydroxy-DHEA. Both had basal levels within the normal range for plasma testosterone and aldosterone, and for urinary levels of cortisol metabolites. BS had perineal hypospadias, micropenis and no episodes of salt loss. He developed pubic hair at 7 years and his height was at the 97th percentile for his age. He was diagnosed as having premature adrenarche with advanced bone age and muscle development. GS was 2 years younger; he had no symptoms of virilization and his levels of testosterone were 15% of levels in BS. From age 2–9 years, GS experienced four episodes of pyrexia, vomiting and lethargy following infections. In three episodes serum sodium was <125 mmol/l. At 8 years he had a severe episode of hyponatraemia and he was noticed to have cutaneous hyperpigmentation suggestive of Addison's disease; this was excluded. GS had an elevated recumbent level of plasma renin activity, whereas BS had normal levels. On a low salt diet, BS and GS showed, respectively, 250% and 30% increases in the level of plasma renin activity and serum aldosterone concentrations were reduced by 46% (GS) and 34% (BS) of basal levels. In parallel, urinary aldosterone was unchanged for GS and elevated by only 28% for BS (compared with a mean elevation of 400% in controls). There was a poor response of plasma testosterone to human chorionic gonadotrophin (hCG), indicating that the deficiency was expressed in testis as well as in the adrenal cortex. Both brothers were treated with hydrocortisone (15 mg/day) and fludrocortisone (100 μg/day); this regimen continues. GS was not a typical severe salt-loser, as he did not present in the

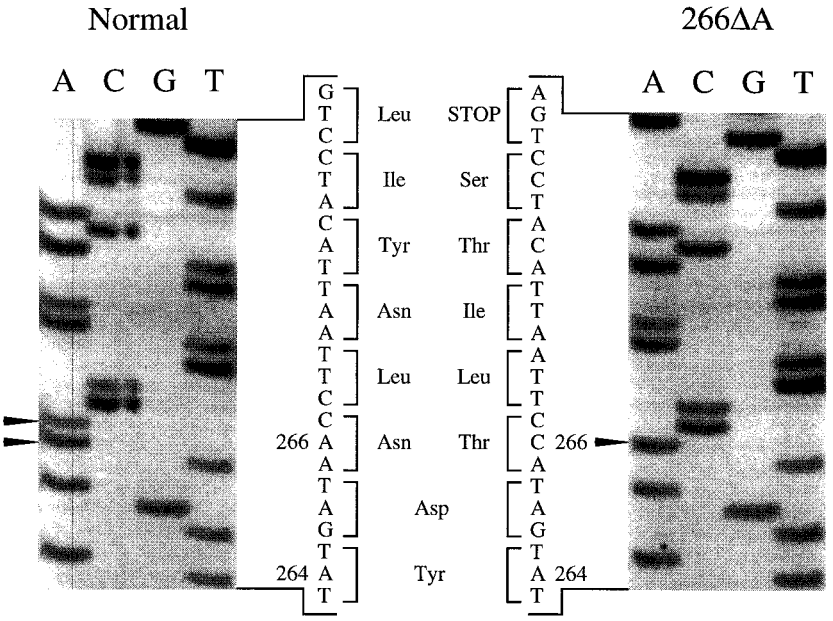

FIGURE 2. Sequence data from HSD3B2 exon 4 showing the normal sequence (left) and that of 266A, found in patient GS (right). The mutation creates a frameshift and a stop at codon 271. Deduced amino acid sequence is shown from codons 264 to 271.

first few weeks of life, and he is able to achieve a normal plasma aldosterone in a compensated state through elevated circulating concentrations of plasma renin activity.

The results of mutation screening showed that BS and GS are compound heterozygotes (N100S/266ΔA) for mutations in HSD3B2, having inherited N100S from their mother (N100S/+) and 266ΔA from their father (266ΔA/+). The deletion 266ΔA causes a stop at codon 271 and is a new mutation (Fig. 2). When expressed *in vitro*, the N100S allele was almost entirely devoid of activity with either pregnenolone or DHEA as substrates (Fig. 3A).

Three kindreds with the type II A82T mutation

The propositus in family Cam (Fig. 4) presented at 10 months as an XY pseudohermaphrodite without salt loss. He had perineal hypospadias with a blind vaginal pouch and inguinal testes. His parents were second cousins; he had eight siblings, two of whom had male pseudohermaphroditism and died of dehydration. He had normal serum levels of sodium, potassium, aldosterone, and raised circulating plasma renin activity (26·5 ng/ml per h; upper limit of normal (ULN) at 10 months is 13 ng/ml per h). He presented with normal circulating cortisol concentrations under basal conditions and after adrenocorticotrophin (ACTH) stimulation. When challenged with hCG, he had normal testosterone production (12·4 nmol/l; ULN on challenge 24 nmol/l) and an accumulation of DHEA (30·4 nmol/l; ULN 9·6 nmol/l). Serum 17-hydroxyprogesterone was also elevated both under basal conditions (20·3 nmol/l; ULN 3·2 nmol/l) and after ACTH stimulation (30·9 nmol/l; ULN 5 nmol/l). Of particular diagnostic importance was the elevated urinary Δ^5 steroid metabolite, pregnenetriol (5-PT) (9·1 mmol/24 h; ULN 1·9 mmol/24 h) compared with Δ^4 cortisol metabolites, the ratio of pregnenetriol/cortisol metabolites (5-PT/(tetrahydrocortisone + tetrahydrocortisol (THF) + 5α-THF)) being 0·67 compared with an expected value of 0·066. When expressed *in vitro*, the A82T allele was almost entirely devoid of activity with either pregnenolone or DHEA as substrates (Fig. 3B).

Two other kindreds with the A82T mutation have been previously reported (Vas and Lei). Because they were not known to be related, their A82T haplotypes were characterized using anonymous markers D1S514 and D1S534, two diallelic polymorphic markers in HSD3B1 and one simple sequence repeat in HSD3B2 (Table 1). D1S514 and D1S534 flank the HSD3B cluster and are genetically 1 cM apart, an estimate confirmed by fluorescence *in situ* hybridization experiments

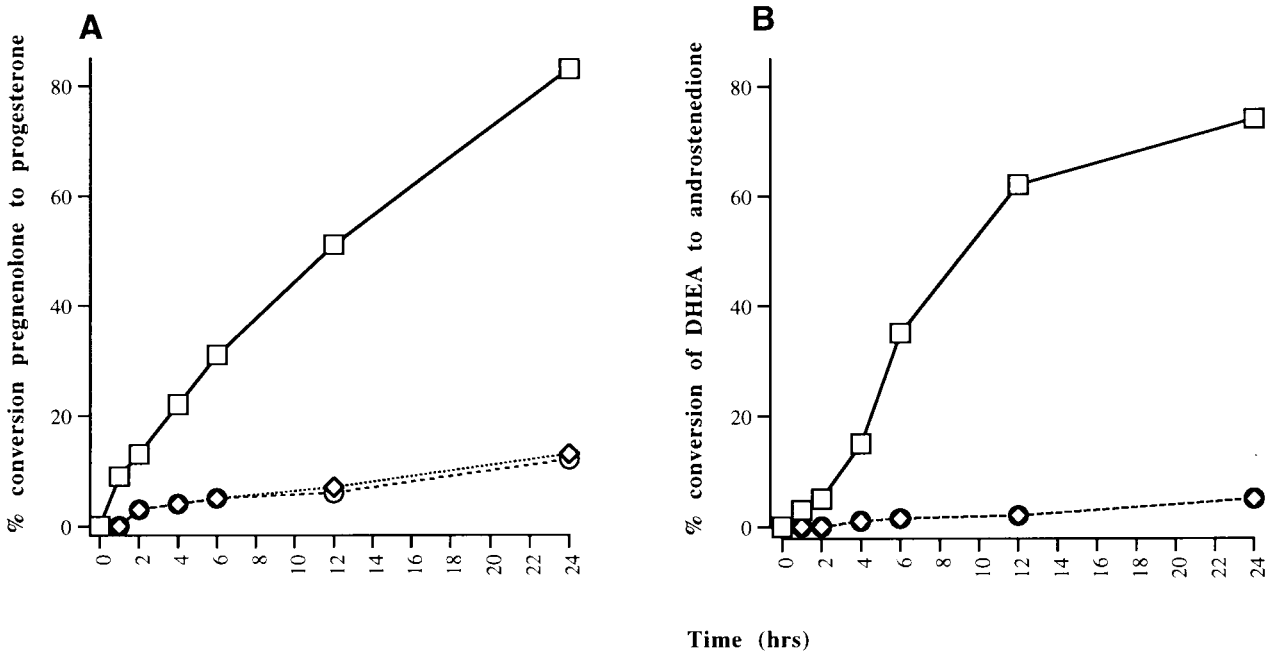

FIGURE 3. *In vitro* expression of 3β-hydroxysteroid dehydrogenase activity for normal (□), N100s (◇) and A82T (●) alleles. The conversion of pregnenolone to progesterone is shown in (A) and the conversion of DHEA to androstenedione in (B).

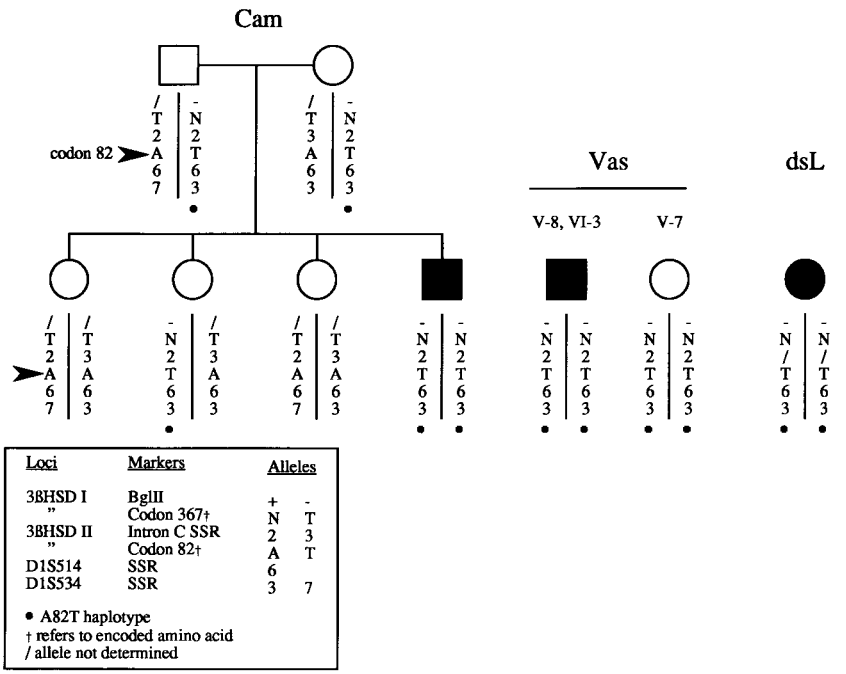

FIGURE 4. Haplotype analysis of family Cam and comparison with individuals homozygous for the A82T mutation in family Vas and patient dsL. Affected individuals are shaded. The A82T marker is indicated by an arrow.

TABLE 1. Expected equilibrium frequency of haplotype linked to A82T mutation. Expected equilibrium frequency of haplotype = 2.0×10^{-3} (calculated as product of allele frequencies for markers 1–3, 5, 6)

Marker no.	Locus	Marker	Genotype	Allele frequency
1	HSD3B1	Bgl II	-ve	0·5
2	HSD3B1	Codon 367	asn	0·65
3	HSD3B2	Intron CSSR	2	0·12
4	HSD3B2	Codon 82	thr	—
5	D1S514	SSR	6	0·21
6	D1S534	SSR	3	0·28

(Brintnell *et al*. 1997, and R G Sutcliffe, unpublished observations). Figure 4 shows that the haplotype associated with the propositus in family Cam also appears in individuals homozygous for A82T in families Vas and patient dsLei. Based on the population frequency of its polymorphic alleles (Table 1), the equilibrium frequency of haplotype (calculated as product of allele frequencies for the polymorphic markers 1–3, 5, 6 in Table 1) is calculated to be $2{\cdot}0 \times 10^{-3}$. It is therefore very likely that the A82T mutations observed in these three separate kindreds are related by descent from a single mutational event.

DISCUSSION

The effect of mutations of the activity of 3β-HSD type II may be assessed against emerging information on the active site of the enzyme. The 3β-HSD type I activity has been investigated using the steroid analogue, 2α-bromoacetoxyprogesterone (2α-BAP), which covalently alkylates the residues Cys183 and His262 in a reaction that is competed by pregnenolone (Thomas *et al*. 1993). The sequences that flank these residues are highly conserved in the 3β-HSDs of different mammals. Histidine residues are also implicated in the active sites of several steroid and other dehydrogenases (Sweet *et al*. 1972, Strickler *et al*. 1993), supporting the deduction that Cys183 and His262 are in close proximity in the type I substrate binding site. As the type II gene has one less codon than type I, the corresponding type II residues are Cys182 and His261. More recent studies have indicated that the isomerase activity of the type I enzyme locates to G251 to K274, hence G250 to K273 in the type II enzyme (Thomas *et al*. 1997). Of the 15 different missense mutations described in the type II enzyme to date, two are adjacent to Cys182 (L173R and P186L) and four locate adjacent to His261 (Y253N, Y254D, T259R and T259 M). A fifth, A245P is more distant and A245 is not conserved in the gene family.

Mutation N100S has previously been found to have a profound effect on phenotype (Mebarki *et al*. 1995), and the present patients BS and GS (N100S/266ΔA) were originally reported by Fisher *et al*. (1988). The clinical reports of Mebarki *et al*. (1995) and Fisher *et al*. (1987) are similar in that, in both cases, the affected children were male pseudohermaphrodites with the typical blood steroid pattern of 3β-HSD II deficiency. The N100S homozygote (Mebarki *et al*. 1995) had a craving for salt, salt-losing crises during childhood infections and, compared with GS, more elevated levels of plasma renin activity before and after a low salt diet. Patient GS (N100S/266ΔA) had very similar salt-losing crises and salt loss during a salt deprivation test. None of the three patients showed severe salt loss; none was treated with regular adrenocorticoid replacement therapy. They are all three capable of secreting marginally adequate levels of aldosterone with the compensating support of raised plasma renin activity. Although BS was unique in showing an accelerated growth rate and increased muscle mass, the N100S homozygote was too young at 2·8 years to show these signs.

When expressed *in vitro*, N100S has first-order rate constants 2·7% for pregnenolone and 11% for DHEA, relative to the activity of the normal type II allele (Mebarki *et al*. 1995). In contrast, patients BS and GS in the present report were compound heterozygotes (N100S/266ΔA) and would therefore be expected to have approximately half the amount type II activity compared with the homozygote, i.e. 1·3–5·5% for pregnenolone and DHEA respectively. In a case of male salt-losing type II 3β-HSD deficiency, Sanchez *et al*. (1994) detected a compound heterozygote, L108W/P186 L, in which the respective alleles had first-order rate constants of 0·3% and 0·2% of the normal allele respectively. This suggests that mutations that lead to type II enzyme activities of between 0·3% and 1·3% are associated with variable phenotypes, from severe salt loss to compensated non-salt loss.

We have now studied three kindreds with the A82T mutation. Although there is no evidence of a familial relationship between families Vas, Cam and Lei, they have in common the same HSD3B extended haplotype (Fig. 3). The population frequencies of the marker alleles in the haplotype (Table 1) lead to the expectation that, at equilibrium, the frequency of the haplotype would be $2{\cdot}0 \times 10^{-3}$. It is therefore most likely that the A82T mutation was inherited by these three kindreds from a single ancestral substitution of G→A at n5615.

Seven members of the human 3β-hydroxysteroid dehydrogenase (3β-HSD) gene family have now been cloned and physically mapped within 230 kb, on chromosome 1p131. These include HSD3B1 and 2, and five unprocessed pseudogenes HSD3Bφ1–5 which are 88–92% related to HSD3B1 and 2 but contain no corresponding open reading frames (Brintnell *et al*. 1997, McBride *et al*. 1999). The nucleotide substitution of N100S is not found in any other members of HSD3B gene family described to date, whereas the substituted nucleotide in A82T (HSD3B2 n5615A) appears in the homologous position in pseudogene HSD3Bφ5. However, the nucleotide sequence of HSD3Bφ5 diverges from exon 3 of HSD3B2 by 11·7%, and it is unlikely that such divergence would be compatible with non-homologous pairing of HSD3B genes during meiosis. Further, bases 12, 4 and 5 nucleotides away from n5615 have also diverged between the type II gene and HSD3Bφ5, but do not occur in the type II genes of the present patients. These observations strongly suggest that there is no evidence of intergenic recombination or conversion in the generation of mutations in HSD3B2. Intergenic recombination, conversion and microconversion have been reported between the 21-hydroxylase gene and its adjacent pseudogene, but these are 98% and 96% identical in exon and intron sequence respectively (Higashi *et al*. 1986, White *et al*. 1986).

All the present male cases of 3-βHSD type II deficiency showed pseudohermaphroditism, with clinical features in common with deficiencies in 17β-HSD type III. Mutations in either gene lead to reductions in the levels of testosterone and, as in the case of 5α-reductase II deficiency (Wilson *et al*. 1993) lead to inadequate levels of dihydrotestosterone (DHT). The severity of pseudohermaphroditism is generally not worse in salt-losing compared with non salt-losing cases, showing that substantial levels of 3-HSD type II activity are required to convert testosterone into adequate levels of DHT. Interestingly, a male pseudohermaphrodite homozygous for the missense mutation, L173R, is known to have vasa deferentia and seminal vesicles, showing that testosterone secretion was adequate for Wolffian duct development (Russell *et al*. 1994).

Patient CL and some other females affected with non salt-losing type II mutations show premature pubarche which may be interpreted as being due to the conversion of increased levels of Δ^5 steroid precursors to Δ^4 androgens through the activity of the type I enzyme (Mendonca *et al*. 1994, Rheaume *et al*. 1994). At adrenarche, the adrenals increase their output of the Δ^5 steroid, DHEA, leading to raised levels of testosterone in blood and the onset of pubarche. The 3β-HSD II deficiency may accentuate this process by increasing the levels of Δ^5 steroids, which can lead to premature pubarche. Child BS showed premature pubarche, high stature, increased muscle mass, and advanced bone age at 11 years, and is the first male case to be associated with these characteristics. Patient dsLei had twice the normal level of testosterone at 5 years when she entered premature pubarche. Notably, both BS and dsLei were affected with mutations that were non-salt-losing but which were sufficiently deleterious to cause elevations in plasma renin activity. Such mutations are expected to cause the greatest blockage in intra-adrenal conversion of Δ^5 substrates to Δ^4 products, and are expected to be associated with the greatest efflux of Δ^5 substrates into peripheral tissues, where they can be converted to androstenedione and testosterone. Nevertheless, in the present cases, the phenotypes of premature pubarche and accelerated gain of stature were not fully penetrant. They did not occur in child GS (N100S/266ΔA) or in an asymptomatic female homozygous for the A82T mutation (Mendonca *et al*. 1994).

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