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Research Article

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Normal Genes for the Cholesterol Side Chain Cleavage Enzyme, P450scc, in Congenital Lipoid Adrenal Hyperplasia

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Abstract

Congenital lipoid adrenal hyperplasia is the most severe form of congenital adrenal hyperplasia. Affected individuals can synthesize no steroid hormones, and hence are all phenotypic females with a severe salt-losing syndrome that is fatal if not treated in early infancy. All previous studies have suggested that the disorder is in the cholesterol side chain cleavage enzyme (P450scc), which converts cholesterol to pregnenolone. A newborn patient was diagnosed by the lack of significant concentrations of adrenal or gonadal steroids either before or after stimulation with corticotropin (ACTH) or gonadotropin (hCG). The P450scc gene in this patient and in a previously described patient were grossly intact, as evidenced by Southern blotting patterns. Enzymatic (polymerase chain reaction) amplification and sequencing of the coding regions of their P450scc genes showed these were identical to the previously cloned human P450scc cDNA and gene sequences. Undetected compound heterozygosity was ruled out in the new patient by sequencing P450scc cDNA enzymatically amplified from gonadal RNA. Northern blots of gonadal RNA from this patient contained normal sized mRNAs for P450scc and also for adrenodoxin reductase, adrenodoxin, sterol carrier protein 2, endozepine, and GRP-78 (the precursor to steroidogenesis activator peptide). These studies show that lipoid CAH is not caused by lesions in the P450scc gene, and suggest that another unidentified factor is required for the conversion of cholesterol to pregnenolone, and is disordered in congenital lipoid adrenal hyperplasia. (*J. Clin. Invest.* 1991. 88:1955-1962.) Key words: pregnenolone • pseudohermaphroditism • cholesterol desmolase • polymerase chain reaction • sex steroids

Introduction

Congenital adrenal hyperplasia (CAH)¹ is a group of inborn errors of steroid hormone synthesis. Distinct autosomal recessive forms of CAH have been described for each step in the conversion of cholesterol to the principal secretory products of

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1. Abbreviations used in this paper: CAH, congenital adrenal hyperplasia; hCG, gonadotropin; PCR, polymerase chain reaction; PRA, plasma renin activity.

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the adrenals (for reviews see references 1, 2). Because the enzymes responsible for adrenal steroidogenesis also participate in gonadal steroidogenesis, many forms of CAH also affect gonadal sex steroid synthesis.

The most severe form of CAH is congenital lipoid adrenal hyperplasia (lipoid CAH). This disorder is characterized by an absence of significant circulating or urinary concentrations of all steroids, high basal concentrations of adrenocorticotropin (ACTH) and plasma renin activity (PRA), absent steroidal response to long-term treatment with high doses of ACTH or human chorionic gonadotropin (hCG), and grossly enlarged adrenals laden with cholesterol and cholesterol esters (3-6). These findings have suggested that the lesion is in the first step in steroidogenesis, the conversion of cholesterol to pregnenolone. Enzymologic studies (6-9) confirmed that adrenal and gonadal tissue from these patients failed to convert cholesterol to pregnenolone, consistent with the notion that the lesion is in the enzyme converting cholesterol to pregnenolone.

The conversion of cholesterol to pregnenolone is mediated by a single mitochondrial enzyme, cytochrome P450scc (EC 1.14.15.67) (for reviews see references 10-12). P450scc functions as the terminal oxidase in an electron transport chain. Electrons from NADPH are donated to a membrane-bound flavoprotein, termed adrenodoxin reductase, then passed to a soluble iron/sulfur protein, termed adrenodoxin, and then passed to P450scc. Congenital lipoid adrenal hyperplasia might conceivably be caused by a genetic lesion in any of the three components of the side-chain cleavage system. In one autopsied patient, P450scc protein was undetectable (9), thus implying that the lesion was in P450scc. A lesion in adrenodoxin reductase or adrenodoxin appears most unlikely. Adrenodoxin and adrenodoxin reductase have been isolated from various tissues with properties indistinguishable from the adrenal proteins (for references see 13). These proteins thus interact with many other mitochondrial cytochrome P450 enzymes, such as hepatic bile acid 26-hydroxylase/vitamin D 25-hydroxylase, (14, 15) or renal vitamin D 1-hydroxylase (16). There is only one human gene for adrenodoxin reductase, located on chromosome 17q24-q25 (17-19) that is expressed in all human tissues examined, (20) hence a disorder in this gene should have effects extending far beyond the steroidogenic tissues. Similarly, there are one (21) or two (22) nearly identical genes for adrenodoxin closely linked on chromosome 11q22 (19, 22, 23) that encode identical proteins (23); this mRNA is also found in all tissues examined (24). Thus, it has generally been agreed that congenital lipoid adrenal hyperplasia represents a genetic defect in the human P450scc gene (1, 25).

To date, only one study has directly examined the P450scc genes in patients with lipoid CAH. Matteson et al. (26) used oligonucleotide probes and a partial-length P450scc cDNA to probe Southern blots of DNA from three patients and various controls, finding no deletions, rearrangements, or restriction

Table I. Basal and Tropicly Stimulated Hormonal Values in Case 1

Steroid (ng/dl)	No treatment		0.25 mg ACTH gel 22 d	5000 U hCG M, W, F 24 d	Normal term infants at 7-30 d	Normal term infants post-hCG
	Age: 2 d	10 d				
Pregnenolone	50	114		<70	150-2000	
17OH pregnenolone		<20			100-3000	
17OH progesterone		30	1.9		60-150	
Dehydroepiandrosterone		<20	11	<10	50-760	
Androstenedione	16	29		6.0	20-290	
Corticosterone	<50	<50			70-850	
Cortisol (μg/dl)	<1.0	2.2	<1		2-11	
Aldosterone	8.5	3.8	1		5-175	
Testosterone				<3	60-400*	180-735
Dihydrotestosterone				<2.7	12-85*	13-183

* At 20-60 d age. Hormonal studies in patient No. 1.

fragment length variations in the P450scc gene. However, the more recent cloning and characterization of the single human P450scc gene (27) on chromosome 15q23-q24 (19) indicates that the oligonucleotides and partial-length cDNA used in that study could have missed deletions at the 5' end of the gene.

We now report a new case of lipoid CAH, and the sequencing of the cDNA and the exons for this patient's P450scc gene and of the gene from our previously reported patient (6). The normal P450scc gene sequences in these two patients and the grossly normal RNA blotting studies of all of the components of the cholesterol side chain cleavage system and known components of the cholesterol transport system indicate that new models of this disorder must be considered, possibly searching for other factors in the delivery of cholesterol to the mitochondria.

Case reports

Case 1. A 3,320-g newborn Korean infant without known consanguinity was referred to one of us (PS) because of a 6.5-yr-old female sibling had previously been diagnosed as having congenital adrenal hyperplasia due to 3β-hydroxysteroid dehydrogenase deficiency (see below). Physical examination at 2 d of age was unremarkable except for generalized increased pigmentation and normal female external genitalia. Initial laboratory data included serum Na, 139 mEq/liter; K, 6.1 mEq/liter; CO₂, 20 mEq/liter; glucose, 74 mg/dl; and various hormonal values (Table I). Urinary excretion of 17-ketosteroids was 0.05 mg/24 h. Pelvic sonography was technically unsatisfactory. The infant gained weight poorly and developed hypotonia and hyperthermia. At 10 d of age the respiratory rate was 36, the heart rate was 140. Laboratory data included serum Na, 113 mEq/liter; K, 9.0 mEq/liter; CO₂, 15 mEq/liter; Cl, 90 mEq/liter; glucose, 111 mg/dl; and further hormonal values (Table I). All steroid values in Tables I and II were determined by Endocrine Sciences, Tarzana, CA, by chromatographic extraction followed by specific immunoassays.

The infant was treated with intravenous saline, hydrocortisone, and fludrocortisone and did well. A karyotype obtained at 2 d of age was 46 XY. Further provocative testing of the adrenals and gonads was done by administering a single dose of 0.25 mg synthetic ACTH (1-39) gel (Acthar; Armour Pharmaceutical Co., Tarrytown, NY), and 5,000 U of hCG IM on Monday, Wednesday, and Friday before drawing blood sam-

ples on the following Monday, yielding the values shown in Table I. Other studies included normal thyroid function studies and a plasma ACTH of 585 pg/ml at 40 d of age (normal ≤ 100 pg/ml).

At 6.5 mo of age a gonadectomy was performed. The internal reproductive structures included undescended testes each measuring 9 × 5 × 5 mm, and no Müllerian structures (fallopian tubes, uterus, cervix) consistent with testicular synthesis of Müllerian inhibitory factor but not of steroids (28, 29). The histologic examination of the gonadal tissue showed normal testes bilaterally.

Recent hormonal reevaluation of the 7-yr-old 46 XX sibling showed low but detectable concentrations of adrenal steroids with no response to 0.25 mg of intravenous ACTH (1-24) (Cortrosyn; Organon Diagnostics, West Orange, NJ) at a time when steroidal replacement therapy was discontinued for 36 h (Table II). Thus, both siblings appear to have the same, presumably autosomal recessive defect in the synthesis of all steroid hormones.

Case 2. The clinical, laboratory, radiographic, and biochemical findings in case 2 have been reported in detail previously (6). Her DNA was also included in the study of Matteson et al. (26) as patient No. 1.

Table II. Hormonal Values in the Sibling of Case 1

Steroid (ng/dl)	Patient		NL prepubertal girls	
	Basal	60' ACTH	Basal	60' ACTH
Pregnenolone	15	27	31±16	77±23
Progesterone	<20	<20	26±12	144±15.5
17OH pregnenolone	53	<20	59±43	354±186
17OH progesterone	33	17	27±15	133±52
Dehydroepiandrosterone	25	14	98±38	180±105
Androstenedione	<5	<5	36±16	64±22
Cortisol (μg/dl)	1.3	2.4	8.3±3.4	27.4±3.4

Hormonal studies in the 46XX sister of patient No. 1, done at 7 yr, 10 mo of age, after glucocorticoid (but not mineralocorticoid) replacement therapy had been discontinued for 36 h. Basal ACTH concentration, before administration of ACTH, was 400 pg/ml (nl <100 pg/ml).

Methods

Preparation and blotting of DNA and RNA. Fibroblasts were cultured from skin obtained at surgery from patient 1, and peripheral leukocytes were obtained from patient 2. Genomic DNA was prepared from these cells by lysis in 0.32 M sucrose, 10 mM Tris pH 7.5, 5 mM MgCl₂, 1% Triton X-100 followed by centrifugation at 2,500 g for 15 min. The pelleted nuclei were resuspended in 10 mM Tris pH 7.5, 10 mM NaCl, 10 mM EDTA and lysed by adding NaDodSO₄ to 1% and proteinase K to 0.2 mg/ml. After gentle overnight agitation at 37° the solution was extracted with phenol, then extracted with chloroform and precipitated with ethanol. The precipitated DNA was harvested by spooling, washed in 70% ethanol, dried, and dissolved in 10 mM Tris, 1 mM EDTA. Restriction endonuclease digestions, gel electrophoresis, Southern blotting, and autoradiography were as described (30).

Gonadal tissue from patient 1 was frozen in liquid N₂ at the time of surgery. RNA was prepared, electrophoresed through agarose gel, transferred to nylon membranes, probed, and autoradiographed as described previously (31). Cloned cDNA probes for human P450scc (32), adrenodoxin (24), adrenodoxin reductase (17), sterol carrier protein 2 (33), endozepine (34), and hamster GRP-78 (35) were isolated from agarose gel and labeled with ³²P by random primer labeling.

Amplification and sequencing of genomic DNA. Samples of genomic DNA were used for enzymatic amplification of the exons of the P450scc gene by polymerase chain reaction (PCR) (36). Each 25-μl reaction contained 25 ng of genomic DNA, 10 mM Tris (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 15 μg/ml BSA, 200 μM each of dGTP, dATP, dTTP, and dCTP, 0.2 μM of each of the two primers used and 1 U *T. aquaticus* (*Taq*)—DNA polymerase. Amplifications were done in a commercial thermal cycler programmed as follows: (a) initial denaturation at 95° for 7 min without *Taq* polymerase; (b) addition of *Taq* polymerase followed by 40 cycles of denaturing at 94° for 1 min, annealing at 60° for 30 s and extension at 72° for 90 s; (c) final extension at 72° for 7 min. The sizes of the resulting PCR products were analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide and photographed under ultraviolet light.

PCR primers were designed to correspond to 18–20 base sequences of introns of the human P450scc gene (27). Where possible, these primers were 10–20 bases from intron/exon junctions to facilitate sequencing these junctions; however, for primers 3'scc1 and 5'scc6, the available sequence data for the introns did not permit this. The sequences of each pair of primers and their distances from the intron/exon junctions are shown in Table III.

PCR products were extracted with phenol/chloroform and purified by passing through a Centricon-100 filter (Amicon, Beverly, MA). This purified DNA was blunt-ended with Klenow polymerase, phos-

phorylated with ATP and T4 polynucleotide kinase and subcloned into pBluescript vectors. DNA sequences were determined from single- or double-stranded vectors by the dideoxynucleotide technique (37, 38) using ³⁵S [dATP] and Sequenase (United States Biochemical Corp., Cleveland, OH).

The sizes of the resulting PCR products are listed in Table III. Oligonucleotide pairs 5'scc1 and 3'scc1 through 5'scc7 and 3'scc7, respectively, amplified exons 1–7 as small fragments of 135–335 bp. These were cloned and completely sequenced on each strand from single reactions. Oligonucleotide pair 5'scc8 and 3'scc9 spanned exons 8 and 9, and intron 8, resulting in a fragment of ~ 850 bp. This larger fragment was cleaved into two smaller fragments, each containing one exon, with restriction endonuclease Acc I. These two smaller fragments were then subcloned and sequenced on each strand. For both patients, two completely separate enzymatic amplifications were made with all eight pairs of oligonucleotides and the products of all 16 PCR reactions were cloned and completely sequenced on both strands.

Amplification and sequencing of testicular cDNA. Testicular RNA from patient No. 1 was used to synthesize complementary DNA (cDNA). Each 20-μl reaction contained 1 μg of total cellular RNA in 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM each of dGTP, dATP, dCTP, and dTTP, and 2.5 μM of oligodeoxythymidine [(dT)₁₇]. After RNA denaturation at 65°C for 2 min the mixture was chilled rapidly and 1 U recombinant placental RNase inhibitor (Perkin Elmer Cetus, Norwalk, CT) and 2.5 U Moloney murine leukemia virus reverse transcriptase were added. Synthesis of cDNA was initiated by heating to 42°C for 1 h and terminated by heating to 95°C for 5 min.

To initiate PCR amplification of the synthesized cDNA, 5 μl of the above reaction was mixed with 20 μl of 70 mM Tris HCl (pH 8.0), 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 15 μg/ml BSA, 0.2 μM of each of the two primers (5'scc1 and 3'scc9; Table III) and 1 U *Taq* polymerase. Amplifications were done in a commercial thermal cycler programmed as for the amplification of genomic DNA (above), except that the extension program in step 2 was for 180 s rather than 90 s.

Results

Clinical studies. The diagnosis of a severe salt-wasting form of congenital adrenal hyperplasia in a sibling resulted in early referral and evaluation of patient No. 1. The patient's lack of clinically significant hyponatremia and hyperkalemia at 2 d of life followed by development of severe hyponatremia, hyperkalemia, acidosis, and shock by 7–10 d is typical of all forms of salt wasting congenital adrenal hyperplasia (1, 2). The minimal basal concentrations of the Δ⁵ steroids (pregnenolone, 17OH-

Table III. Oligonucleotide Primers Used for PCR Amplification

Name	Sense primers	Exons amplified	Antisense primer	Name	Size (bp)
5'SCC1	5'-GCTGAAGTGGAGCAGGTACA-3' (20)	EXON 1	(3) 5'-TCCCACCCCTGCGCAGGCT-3'	3'SCC1	334
5'SCC2	5'-TCCTTCCATCAGCCCCTCTC-3' (14)	EXON 2	(11) 5'-CCAGTCCCTGGGAGATGGC-3'	3'SCC2	221
5'SCC3	5'-GGTCTGTGACCATGAGGGCT-3' (21)	EXON 3	(10) 5'-CACTGCCAGCCAGGTGCAA-3'	3'SCC3	270
5'SCC4	5'-CGTCAGAAATGGCTCCTCAG-3' (19)	EXON 4	(40) 5'-AGGAGCCGGCTGAGGCT-3'	3'SCC4	301
5'SCC5	5'-CTGCAGGGAACCTCACTCTT-3' (23)	EXON 5	(7) 5'-GGTGCCGCCCTACAGCC-3'	3'SCC5	229
5'SCC6	5'-ATACCCTACTCCCCACCAG-3' (0)	EXON 6	(30) 5'-CCTGCCAGGGATTGGAGT-3'	3'SCC6	235
5'SCC7	5'-CCATCAGCTTCTGAGGTCCC-3' (11)	EXON 7	(6) 5'-CTGCAGCCTGCTGGCTGC-3'	3'SCC7	135
5'SCC8	5'-GCTTACTCAGGCTCTGATC-3' (12)	EXON 8 & EXON 9	(12) 5'-CTCCCATGTGGCTGCAGGC-3'	3'SCC9	~850

Numbers in parenthesis show the number of bases of intron DNA between the primer and the corresponding exon.

pregnenolone, and dehydroepiandrosterone) and their failure to rise in response to tropic hormonal stimulation rule out the diagnosis of 3β hydroxysteroid dehydrogenase deficiency that had been made (incorrectly) in the sibling. The elevated basal concentration of ACTH and the minimal concentrations of all adrenal and gonadal steroids and their unresponsiveness to stimulation with ACTH and hCG, respectively, establish the diagnosis of congenital lipid adrenal hyperplasia. The measurement of steroid values that are greater than zero does not necessarily mean a "partial enzyme deficiency." Some of the steroids measured at 2–10 d of age represent residual maternal steroids (6), as shown by the lower values, even after tropic stimulation, at 3–4 wk of age (Table I). Furthermore, extraadrenal and extragonadal steroidogenesis do occur, especially in the fetus (39, 40); however, this peripheral steroidogenesis is mediated by enzymes other than those found in the adrenals and gonad (41–43). Thus, the hormonal data unambiguously establish a defect in the synthesis of pregnenolone and all other steroid hormones. This has, to date, been interpreted as a lesion in P450scc (1, 6, 9, 12, 25). Similarly, the studies in the 7-yr, 10-mo-old sister suggest the presence of low concentrations of some adrenal steroids while other steroids, such as progesterone and androstenedione, were undetectable. However, these minimal steroid concentrations did not rise in response to ACTH (Table II). These values probably reflect immunocross-reactivity and/or peripheral metabolism of the replacement steroids administered.

DNA blotting studies. To determine if gross deletions, conversions, or rearrangements of the P450scc gene caused lipid CAH, genomic DNA from patients 1 and 2 and from a normal individual were digested with the restriction endonucleases Bam HI, Hind III, and Eco RI. Southern blots of these DNAs were probed with a full-length human P450scc cDNA. Fig. 1 shows that the pattern of P450scc gene fragments identified was identical among the three DNA samples with each of the endonucleases used. Bam HI yielded fragments of 1.3, 4.8, 5.2, and 12 kb; Hind III yielded fragments of 5.8 and 23 kb, and Eco RI yielded fragments of 6.0, 6.6, 8.5 kb. These patterns closely match the P450scc gene mapping and sequencing data of Morohashi et al. (27) (Fig. 1). Thus, the identity of the patterns from patients and a control, and their correspondence with the established map of this locus indicate that gross deletions, duplications, or rearrangements have not occurred in the P450scc genes of these patients with lipid CAH.

PCR amplification and sequencing of genomic DNA. To determine if point mutations or very small deletions, duplications, or rearrangements of the P450scc gene resulted in an abnormal P450scc mRNA in these patients, we amplified and sequenced the exons of their P450scc genes. Because the P450scc gene is ~ 15–20 kb long (27), the entire gene cannot be manipulated or sequenced easily. Therefore, we designed eight pairs of oligonucleotides that spanned the nine exons (Table III), permitting the enzymatic amplification and sequencing of all of the portions of the P450scc gene that encode the P450scc protein as eight discrete fragments of 135 to ~ 850 bases.

The sequencing was done a total of four times from independent amplifications, and any ambiguities were resequenced. No abnormalities in the P450scc gene in either patient. The sequences of the exons were consistent with the P450scc cDNA (32) and the sequences at the intron/exon boundaries were consistent with the human P450scc gene se-

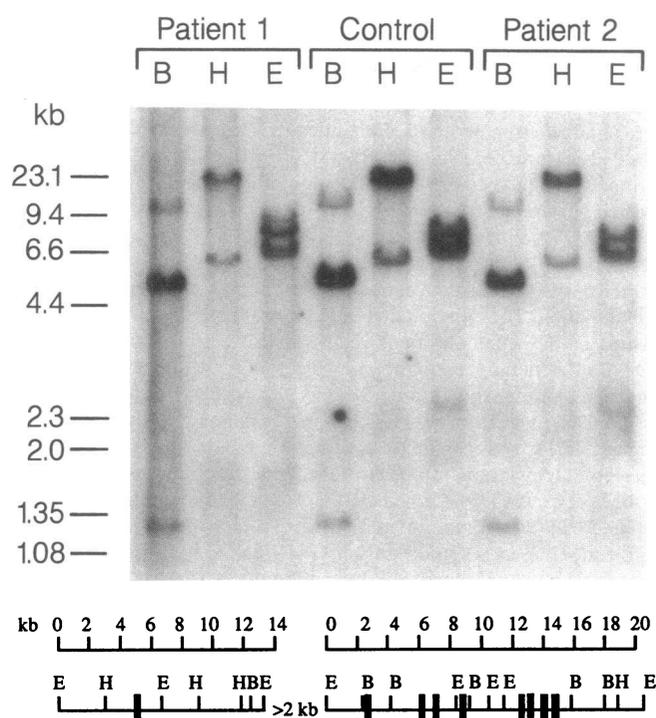


Figure 1. (Top) Southern blot. Genomic DNA was prepared from patients 1 and 2 and from a normal control. The DNA was digested with Bam HI (B), Hind III (H), or Eco RI (E) and displayed by electrophoresis through 1% agarose gel. The DNA fragments were transferred to a nylon membrane and probed with 32 P-labeled full-length human P450scc cDNA (32). Molecular size markers in kilobases (kb) shown at left were determined by the electrophoretic migration of non radioactive bacteriophage λ DNA cut with Hind III and bacteriophage ϕ x174 DNA cut with Hae III run in another lane and seen by staining with ethidium bromide. (Bottom) Map of the human P450scc gene as indicated by the sequencing studies of Morohashi et al. (27) and confirmed by the Southern blotting data shown in the upper panel. Mapped DNA is indicated by a solid bar with the exons indicated as superimposed rectangles. A region of at least 2 kb in the first intron has not been mapped, hence, the gene is shown as two noncontiguous segments. Note the scale in kb. Restriction endonuclease cleavage sites indicated are Bam HI (B), Hind III (H), and Eco RI (E).

quence (27). The only minor variations with those sequences were as follows. First, both of our patients and the human P450scc cDNA show the sequence GCATG, where ATG is the translational initiation signal in exon 1; by contrast Morohashi et al. (27) reported GCAATG. As our two patient genes and the cDNA agree, it is likely that the additional A residue is a sequencing error by Morohashi et al. (27). Second, both of our patients and the gene sequence of Morohashi et al. (27) show TGC (encoding cysteine) and ATC (encoding isoleucine) at codons No. 16 and No. 301, respectively. By contrast, the cDNA sequence of Chung et al. (32) shows TAC (tyrosine) and ATG (methionine) at codons 16 and 301, respectively, suggesting errors in the reverse transcriptase reaction or sequencing errors by Chung et al. (32). Third, at codon No. 274 in exon 4 both of our patients and the human P450scc cDNA show TTC (encoding phenylalanine) whereas Morohashi et al. (27) reported TTG (leucine). As the bovine sequence also has TTC (Phe) at this location and because Phe residues are generally

conserved, this suggests a sequencing error by Morohashi et al. (27). Finally, at codon No. 452 in exon 8, the previously published cDNA (32), the gene (27) and patient No. 1 have AAC (asparagine), whereas patient No. 2 had AAT (asparagine). As the amino acid is unchanged the nucleotide difference is inconsequential. Thus, both patients have P450scc genes capable of encoding P450scc mRNA and protein that are absolutely normal.

PCR amplification and sequencing of cDNA. To rule out undetected compound heterozygosity or RNA splicing errors, we confirmed the results from PCR of genomic DNA by PCR amplification and sequencing of P450scc cDNA. As PCR oligonucleotides 5' scc1 and 3' scc9 lie in the 5' and 3' untranslated regions of P450scc mRNA, we used them to amplify the protein-coding region of P450scc cDNA synthesized by oligo (*dT*)-primed reverse transcription of testicular RNA from patient No. 1. The resulting 1,637-bp PCR cDNA product (Fig. 2), including the 20-base primers on each end, was sequenced on both strands.

Because enzymatic errors in reverse transcription and, more commonly, in PCR amplification with *Taq* polymerase occur fairly commonly, we performed three separate cDNA syntheses, PCR amplifications, cloning, and complete cDNA sequencing. The first PCR/cDNA examined showed a change in codon No. 184 from AGG (arginine) to AAG (lysine). The second PCR/cDNA examined showed a change in codon No. 314 from GAG (glutamic acid) to GGG (glycine). The third PCR/cDNA examined was completely normal in these two regions, i.e., identical with the published cDNA (32) and gene (27) sequences, indicating that at least one and probably both of the changes are PCR artifacts. Thus, at least one allele in

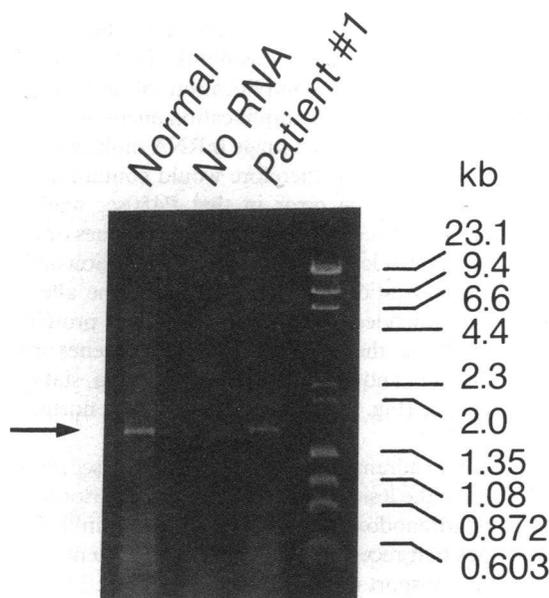


Figure 2. Agarose gel of PCR-amplified P450scc cDNA. The left lane shows the PCR-amplified 1,637 bp P450scc cDNA from a sample of RNA from a normal human fetal adrenal; the middle lane shows a control reaction with no RNA, and the right hand lane shows the products of the PCR amplification of P450scc cDNA from the testicular RNA of patient No. 1. The molecular size markers, shown on the right, are bacteriophage λ DNA cut with *Hind* III and bacteriophage ϕ x174 cut with *Hae* III. The gel was stained with ethidium bromide and photographed under ultraviolet light.

patient No. 1 was making P450scc mRNA encoding normal P450scc protein. Since reverse transcriptase and/or *Taq* polymerase errors occur about every 1–2,000 bases, finding PCR errors in these cDNA clones is to be expected. The finding of normal sequences for codons 184 and 314 in the four genomic PCR amplifications is strongly consistent with this, and suggests that both P450scc alleles are normal in these patients.

RNA blotting studies. It remained possible that the congenital lipid adrenal hyperplasia in these patients could be caused by a lesion in the promoter of the P450scc gene. In such a hypothetical case, the gene might be anatomically intact but transcriptionally inactive. In principle, this could be examined by sequencing the regulatory regions of the DNA. However, this would require a very precise knowledge of which bases are responsible for activity—a knowledge not yet available. Furthermore, we have recently established that sequences as far upstream as 2,300 bases from the transcriptional start site are involved in the transcriptional regulation of the human P450scc gene (31). Thus sequencing is not a useful approach for unknown promoter lesions.

To determine if the P450scc gene in patient No. 1 was able to make P450scc mRNA, we analyzed testicular RNA by Northern blotting analysis. As the patient's surgery was done at 6.5 mo of age, an age at which testicular steroidogenesis is normally diminishing, the patient was given 5,000 U of hCG 3 d before surgery. As seen in Fig. 3 A, a band of P450scc mRNA was readily detectable in the RNA from the patient's testis. This band is identified as P450scc mRNA by its comigration at the expected 2.0-kb size with P450scc mRNA from a control RNA sample (from the testis of a 20-wk gestation fetus) and by its intense hybridization under highly stringent hybridization and washing conditions. Thus the promoter of the P450scc gene in patient No. 1 was able to direct the synthesis of P450scc mRNA, and, as shown by the sequencing studies, that P450scc mRNA was completely normal.

As it has been deduced, but not experimentally proven, that a lesion in adrenodoxin reductase or adrenodoxin would have more devastating and widely ranging manifestations than those seen in lipoid CAH, we then examined these mRNAs in patient No. 1. We removed the P450scc probe from the northern blot in Fig. 3 A by boiling (and repeating the autoradiography to show that no residual ^{32}P remained on the membrane), then reprobbed the blot with ^{32}P -labeled cDNA for adrenodoxin (Fig. 3 B). This procedure was then repeated and the blot reprobbed for adrenodoxin reductase (Fig. 3 C). The mRNA from the patient's testis had mRNAs for adrenodoxin and adrenodoxin reductase, as expected. Each of these mRNAs comigrated with the corresponding mRNA in the control tissue, and each mRNA hybridized under highly stringent conditions, indicating the presence of normal-sized mRNA. Because the control was tissue from a 20-wk fetus, quantitative comparisons of the abundance of each mRNA in the patient's tissue and the control should not be made. While the hybridization pattern in Fig. 3 B might suggest a relative diminution in the patient's adrenodoxin mRNA abundance, this would have little effect on P450scc activity as it is binding of cholesterol to P450scc, rather than electron transport, that is rate-limiting in P450scc activity.

Because the sequencing and RNA blotting studies appear to rule out a lesion in the cholesterol side-chain cleavage system as the cause of lipoid CAH, we considered other possible explanations. Disorders of cholesterol synthesis, binding to serum

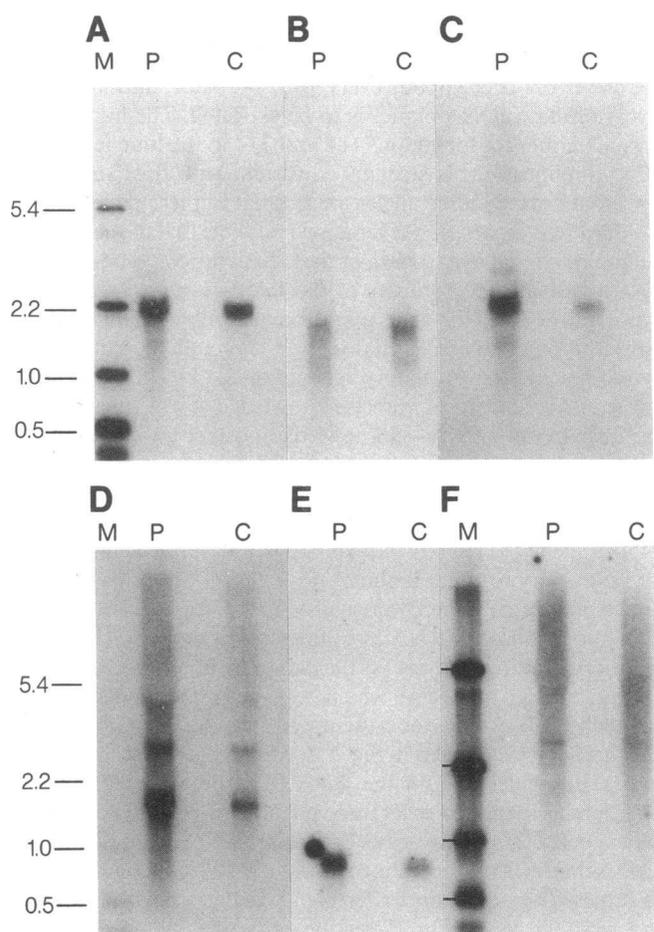


Figure 3. Northern blots. RNA was prepared from testicular tissue removed from patient No. 1 (P) and from control testicular tissue from a normal 20-wk-gestation human fetus (C), obtained from the International Institute for the Advancement of Medicine (Philadelphia, PA). Molecular size markers (M) are DNA from bacteriophage PM2 cleaved with Hind III and end-labeled by treatment with alkaline phosphatase followed by treatment with polynucleotide kinase and γ - 32 P ATP (sizes are in kb). (A–E) show the same blot wherein the patient's sample consisted of 25 μ g of total RNA and the control sample consisted of 10 μ g of total RNA. In each case the blot was autoradiographed then washed and reautoradiographed to ensure that no radioactive signal remained before the blot was reprobed. (F) Autoradiography of a separate blot containing 20 μ g of RNA from the patient and the control in each lane. The human cDNA probes used were (A) P450scc, (B) adrenodoxin, (C) adrenodoxin reductase, (D) sterol carrier protein 2, (E) endozepine, (F) hamster GRP-78.

LDL, and receptor-mediated uptake of LDL cholesterol are known causes of other diseases. However, recent data suggest that steroidogenic tissues employ specialized factors to store and mobilize cholesterol. Disorder of any such factor could conceivably cause the syndrome of lipoid CAH. At present, compelling data involve at least three proteins in the activation of the cholesterol side-chain cleavage process: sterol carrier protein 2 (44–46), endozepine (47, 48), and steroidogenesis activator peptide (49, 50), which is the carboxy-terminal 30 amino acids of glucose-regulated protein (51). Therefore, we reprobbed the Northern blot of testicular RNA from patient No. 1 with probes for each of these factors. As shown in Fig. 3, D–F,

the mRNAs for these proteins were also present in normal size in the patient's tissue. Thus, we have found neither a lesion in the P450scc system, nor in the known intracellular activators of steroidogenesis.

Discussion

The diagnosis of lipoid CAH is inherently indirect because, in contrast to other forms of CAH, there is no characteristic accumulation of a precursor steroid. Because no pregnenolone is made, this disorder was previously thought to involve P450scc, the enzyme converting cholesterol to pregnenolone. The failure of adrenal or gonadal mitochondria from such patients to convert cholesterol to pregnenolone appeared to confirm this view (6–9). We previously examined the P450scc genes of three patients with lipoid CAH by Southern blotting analysis, and found no gross lesions (26). However, because that study was not done with a full-length cDNA, gross lesions at the 5' end of the gene could have been missed. Furthermore, such gross lesions are much less common than are point mutations, which can only be detected by sequencing. PCR technology has now permitted a detailed search for possible mutations in the P450scc gene associated with lipoid CAH. No such lesion was found in the genomic DNA of two patients.

PCR amplification, cloning, and sequencing of a large gene in multiple segments can miss lesions in introns, causing RNA splicing errors, or might miss the lesions in compound heterozygotes. The chances of not detecting a lesion in a compound heterozygote would be $1/x (1/2)^n$, where x is the total number of lesions on both alleles and n is the number of independent clones examined from separate PCR amplifications. For a compound heterozygote having one lesion on each allele examined in four separate PCR amplifications as we did, the probability of missing all lesions is $1/32$ or 3.125%. To reduce this possibility to zero, and to exclude the possibility of RNA splicing errors, we performed PCR amplification of testicular cDNA from patient No. 1. As this amplification encompassed the entire protein-coding region of a single mRNA molecule, it arose from only one allele, and therefore would contain any missense mutation or splicing error in that P450scc allele. Complete sequencing of three independent cDNA clones only showed random artifactual lesions of reverse transcription and *Taq* polymerase amplification. Therefore, at least one allele (and probably both) encoded mRNA with a normal protein coding sequence. Thus, in these patients the P450scc genes are grossly intact (Fig. 1), and can be transcribed into stable mRNA of normal size (Fig. 3) that has a completely normal coding sequence.

If congenital lipoid adrenal hyperplasia is not P450scc deficiency, what, then, is the lesion? As discussed above, disorders in adrenodoxin or adrenodoxin reductase seem most unlikely. The other enzymes that receive their reducing equivalents via these two electron transport intermediates are apparently normal in lipoid CAH patients successfully treated for many years (5, 6). Furthermore, the mRNAs for both of these proteins were present in the testicular tissue from patient No. 1. A more promising area may be the steps proximal to P450scc. Cholesterol is stored as cholesterol esters in lipid droplets in steroidogenic tissues. A defect in cholesterol esterase causes a disorder of steroidogenesis similar to lipoid CAH. However, this disorder, Wollman's disease, affects all tissues, leads to hepatosplenomegaly and characteristic foam cells in the marrow, and

is inevitably fatal in the first year of life irrespective of adrenal replacement therapy (52). Thus, a candidate step for congenital lipid adrenal hyperplasia should occur after cholesterol esters are converted to free cholesterol (and before P450scc) and should be specific to steroidogenic tissues. As only the steroidogenic tissues need to transport large amounts of cholesterol into mitochondria, the factors involved in this transport might be attractive candidates. Sterol carrier protein 2, a cytoplasmic cholesterol-binding protein, is one of several factors involved in the flow of cholesterol to the inner mitochondrial membrane (44–46). Whereas its mRNA is present in patient No. 1, its sequence in patient No. 1 is unknown. However, because SCP 2 also is found in the liver, this does not appear to be a strong candidate to be the affected step in lipid CAH. Endozepine, an endogenous protein that binds to the benzodiazepine receptor, has recently been implicated in the transport of cholesterol across the outer mitochondrial membrane (47, 48). However, this protein is also expressed ubiquitously (34) and hence does not fulfill the criterion of specificity to steroidogenic tissues. Furthermore, its mRNA was also present in the testis of patient No. 1. Finally, Pederson and Brownie (49, 50) have reported the isolation and sequencing of a 30–amino acid steroidogenesis activator peptide (SAP) that appears to function in the flux of cholesterol across the inner mitochondrial membrane. Recent studies show that SAP is the carboxy-terminal 30 amino acids of a widely expressed heat shock protein, HSP-78 (also termed GRP-78) (51). This protein is also widely (if not ubiquitously) expressed. GRP-78 mRNA was also present in the testis of patient No. 1. Thus the molecular defect in congenital lipid adrenal hyperplasia remains unknown.

In the absence of a defined molecular lesion, congenital lipid adrenal hyperplasia remains a syndrome. It is possible that some patients will be found with P450scc gene lesions, and that such patients will have phenotypic and clinical manifestations of lipid CAH that are indistinguishable from the disease in these two patients. Although lipid CAH is rare, it appears in genetic clusters. Among the 32 cases compiled by Hauffa et al. (6) 18 (56%), including patient No. 2 in this study, were of Japanese heritage and another 5 (16%) were from Southern Germany or Switzerland. Patient No. 1, newly described in this report, is of Korean ancestry. As Japan and Korea are very close geographically and historically, patients 1 and 2 may represent the same gene pool. However, we have recently done one set of genomic PCR amplifications of the P450scc exons from a patient of Mexican Indian ancestry and again found normal sequences. Examination of the P450scc genes from European and other patients with lipid CAH will be of interest.

In addition to finding normal P450scc gene sequences and the normal mRNAs discussed above, any future molecular model of congenital lipid adrenal hyperplasia must also account for the following features of the disorder that are difficult to reconcile with a P450scc gene lesion. First, while the lesion in the adrenals and gonads is steroidogenically equivalent, only the adrenals accumulate cholesterol and cholesterol esters; even though fetal Leydig cells should be very active in steroidogenesis, they do not accumulate lipids like the adrenals. Second, the successful gestation of these patients as normal term infants suggests that placental synthesis of pregnenolone and progesterone should be intact, although the role of transplacental maternal progesterone has not been elucidated. Thus, a molecular model of lipid CAH should implicate a gene involved in adrenal and gonadal, but not in placental steroidogenesis.

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