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STRUCTURE OF GENES ENCODING STEROIDOGENIC ENZYMES

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Summary—Synthesis of adrenal steroid hormones from cholesterol entails the actions of only five enzymes, four of which are specific forms of cytochrome P450. These cytochrome P450 enzymes have all been isolated and their activities reconstituted *in vitro*, showing that each enzyme catalyses multiple steroidal conversions. Genes or complementary DNAs have been cloned for human P450scc (the cholesterol side-chain cleavage enzyme), P450c17 (17α-hydroxylase/17,20 lyase) and P450c21 (21-hydroxylase). The sequences for microsomal P450c17 and P450c21 are much more closely related to one another than either is to the sequence for mitochondrial P450scc. Each of these P450 enzymes is encoded by a single human gene; the gene for P450scc lies on chromosome 15, that for P450c17 lies on chromosome 10, and that for P450c21 lies on chromosome 6. The human, mouse and bovine genomes each have two P450c21 genes. While only one of these is active in mouse and man, both genes may be active in cattle. A wide variety of lesions in the human P450c21(B) gene causes congenital adrenal hyperplasia, a common genetic disorder.

P450scc

Conversion of cholesterol to pregnenolone is the first, rate-limiting [1] and hormonally regulated step in the synthesis of all steroid hormones [2–5]. This involves three distinct chemical reactions, 20α-hydroxylation, 22-hydroxylation, and scission of the cholesterol side-chain at the bond between carbon atoms 20 and 22 to yield pregnenolone and isocaproic acid. Steroidal studies have shown that 20-hydroxycholesterol, 22-hydroxycholesterol, and 20,22-hydroxycholesterol can all be isolated from bovine adrenals in significant quantities. These findings suggested three separate and distinct enzymes were involved: 20-hydroxylase, 22-hydroxylase and a 20,22 lyase, and, in fact 3 mol of NADPH are consumed per mole of cholesterol converted to pregnenolone. However, protein purification studies and *in vitro* reconstitution of enzymatic activity show that a single enzyme, termed P450scc (where scc refers to the side-chain cleavage of cholesterol) is responsible for all the steps between cholesterol and pregnenolone [6–12] on a single active site [13].

More recently, differential enzymologic conversions of native cholesterol and a variety of cholesterol esters have been interpreted to imply the presence of several isozymes of P450scc, each relatively specific for a group of cholesterol esters or salts [14]. Protein purification data suggest the existence of a single immunologically identifiable species of P450scc. This P450 is found bound to the inner mitochondrial membrane as a multimer of 16 subunits totalling over 850,000 D [9, 10]. P450scc receives electrons from an iron–sulfur protein termed adrenodoxin which is found in solution in the mitochondrial matrix [15–18]. Adrenodoxin, in turn, receives electrons from a flavoprotein that is loosely bound to the inner mitochondrial membrane [6, 15, 16, 19]. This flavoprotein, termed adrenodoxin reductase, receives electrons from NADPH.

Based on the sequence of tryptic fragments of bovine P450scc [20], Morohasi *et al.* [21] synthesized degenerate-sequence oligonucleotides and used these to screen a bovine adrenal cDNA library. They ultimately identified a full-length cDNA encoding bovine adrenal P450scc. These cDNA sequence data subsequently permitted our investigation of a variety of physiologic and genetic questions concerning P450scc in human physiology and disease.

Since the bovine cDNA cloned by Morohashi *et al.* was not available to us, we used a non-commercial oligonucleotide synthesizer of unique design [22] to synthesize one 62-base and three 72-base oligonucleotides having sequences identical to various regions of the bovine P450scc cDNA [23]. Long oligonucleotides of this length (and longer) have been produced previously and used to probe heterologous species, but these earlier experiments employed ligation of numerous small oligos into a double-stranded form, followed by insertion into a vector, cloning and amplification [24, 25]. Our approach entailed a single, unique synthesis and gel purification of the longest species. This approach was validated by using the bovine-sequence oligonucleotides to clone human P450scc cDNA. Subsequent Southern blotting studies with both the human cDNA and with the bovine-sequence oligonucleotides gave identical results [23]. Our data with the human cDNA, and similar data in cattle [26], clearly indicate that both of these mam-

mals possess single, unique P450scc genes, negating the enzymologic hypothesis that multiple P450scc isozymes catalyze the side-chain cleavage of various cholesterol esters.

The partial-length human P450scc cDNA isolated as described above [23] was then used to isolate full-length human adrenal and testis P450scc cDNA clones [27]. The cloned cDNA is 1821 bases long, encoding 44 bases of the 5′ untranslated region plus the entire coding and 3′ untranslated regions. Primer extension studies show only a single cap site for P450scc mRNA, indicating that differential hormonal regulation of this gene does not function through different promoters. Human and bovine P450scc share 82% amino acid homology and 72% nucleotide homology, typical of bovine and human sequences. However, these nucleotide differences are not evenly distributed in the sequence, unlike other bovine and human sequences such as growth hormone [28], prolactin [29] and proopiomelanocortin [30].

As predicted from the Southern blotting data showing the presence of a single human P450scc gene, the human adrenal and testicular P450 cDNA clones have identical sequences. Examination of Southern blots of DNA from a panel of mouse/human somatic cell hybridoma cell lines shows the unique human P450scc gene lies on chromosome 15 [27].

Genetic lesions in P450scc activity are rare, as absent P450scc activity results in an inability to synthesize any steroid hormones, with consequent neonatal cardiovascular collapse and death. However, very early diagnosis can lead to treatment of the affected infant with glucocorticoid and mineralocorticoid steroid hormone replacement. Only 32 such infants lacking P450scc activity have been reported in the medical literature, and only 11 have survived [31]. Examination of Southern blots of DNA from three of these 11 survivors showed no gross deletions in their P450scc genes, suggesting small deletions or point mutations are responsible for the lethal autosomal recessive condition. While lesions in adrenodoxin or adrenodoxin reductase cannot be ruled out, the absence of detectable P450scc protein in the adrenal of one autopsied patient [32] suggests this rare but interesting disorder represents a lesion in P450scc.

3β-HYDROXYSTEROID DEHYDROGENASE/ ISOMERASE

The enzyme(s) catalyzing 3β-hydroxysteroid dehydrogenation and isomerization of Δ^5 steroids to Δ^4 steroids are poorly characterized. Although it has been widely assumed that there is a single 3β-hydroxysteroid dehydrogenase (3βHSD), early enzymatic studies suggested the presence of two or three different isozymes of the isomerase specific for the three different steroidogenic pathways [mineralocorticoid, glucocorticoid, and sex steroid] [33–35]. Subsequent indirect enzymatic data have supported this concept that there is a single 3βHSD enzyme but two or three distinct isozymes of isomerase [36–38]. The clinical identification of patients with "3βHSD deficiency" confined to a single steroidogenic pathway [39, 40] also suggests the presence of multiple isozymes. However, similar enzymologic and clinical genetic inferences have suggested multiple forms of P450scc, P450c17 and P450c21, and in all three cases molecular biology has proven there is only one functional gene for each enzyme. Recent protein purification data indicate a single protein carries all these activities [44].

P450c17

Progesterone and pregnenolone may undergo 17α-hydroxylation to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively. These latter 17-hydroxylated steroids may then undergo scission of the C17,20 carbon bond to yield dehydroisoandrosterone and androstenedione, respectively. It is now well demonstrated that all four of these reactions are mediated by a single enzyme, P450c17. This P450 is bound to smooth endoplasmic reticulum, where it accepts electrons from a flavoprotein immunologically distinct from the adrenodoxin reductase employed in mitochondria, and without benefit of an iron/sulfur protein [41–43].

17α-Hydroxylase and 17,20 lyase have traditionally been regarded as separate enzymes. The adrenals of prepubertal children synthesize ample cortisol but virtually no sex steroids (i.e. have 17α-hydroxylase activity but not 17,20 lyase activity) until a peripubertal event, termed adrenarche, initiates production of adrenal androgens (i.e. turns on 17,20 lyase activity) [45, 46]. Furthermore, patients have been described lacking 17,20 lyase activity but retaining normal 17α-hydroxylase activity [47, 48]. However, purification of pig testicular microsomal P450c17 to homogeneity and *in vitro* reconstitution of enzymatic activity clearly proved that both 17α-hydroxylase and 17,20 lyase activities reside in a single protein, P450c17 [49–51]. However, adrenal P450c17 from the same species prepared by the same investigators was found to differ slightly but significantly in its amino acid composition and N-terminal sequence [52, 53]. Thus, it appeared that the pig has two isozymes of P450c17, one expressed in the adrenal and the other in the testis.

We sought to study this complex situation in man. Based on the sequence of a portion of the porcine adrenal enzyme, we produced a degenerate-sequence oligonucleotide with which we probed a porcine adrenal cDNA library. A small 380 bp cDNA fragment was identified and sequenced, showing it encoded the carboxy terminus of porcine adrenal P450c17. This cDNA was used to screen our human adrenal cDNA library identifying

a series of clones. The longest of these is 1754 bp long encoding 41 bp of the 5′ untranslated region and the entire coding and 3′ untranslated regions. This human adrenal cDNA was used to screen our human testis cDNA library, identifying a 1303 bp clone lacking the 5′ untranslated region, the first 99 codons, and part of the 3′ untranslated region. However, all 1303 bp were identical to the corresponding region of the adrenal cDNA. To examine the uncloned regions, we used 5′ and 3′ restriction fragments of the adrenal cDNA to protect human adrenal and testicular mRNA from digestion with S_1 nuclease. The results were identical, indicating there was no detectable sequence disparity between the adrenal cDNA and testis RNA. Thus, the same P450c17 gene is expressed in both tissues [54].

It remains possible that other isozymes of human P450c17 may exist, as there are multiple P450c17 genes. To study gene copy number, we used a small 650 bp cDNA clone to minimize the detection of multiple bands on Southern blots due to restriction endonuclease sites lying in introns. A Southern blot of human DNA cut with eight different restriction endonucleases showed a single band in each lane when the blot was probed with human P450c17 cDNA under highly stringent conditions (42°C, 50% formamide). However, when the blot was reprobed under conditions of low stringency (37°C, 20% formamide), a complex pattern was seen containing at least three bands in each lane. Examination of Southern blots of DNA from a panel of mouse/human somatic cell hybrid lines, probed under the same highly stringent conditions, indicates the "authentic" P450c17 gene lies on chromosome 10. Re-examination of this blot under low-stringency conditions yielded uninterpretable smears of background hybridization due to the excess mouse DNA [55]. Thus, the human genome appears to have a single gene on chromosome 10 strongly homologous to, and presumably encoding, both adrenal and testis P450c17. However, the human genome also appears to have two other gene sequences that are more distantly related to P450c17 but still hybridize under conditions detecting about 70% nucleotide homology. The nature and location of these sequences are not yet known.

P450c21

The nature of the 21-hydroxylating step has been of great clinical interest, since about one in every 5000 persons has a genetic lesion in this enzyme inherited in an autosomal recessive fashion. The clinical symptoms associated with this common genetic disease are complex and devastating. Decreased cortisol and aldosterone synthesis often lead to sodium loss, hypotension, cardiovascular collapse and death within the first 2 weeks of life. Decreased synthesis of cortisol *in utero* leads to overproduction of ACTH and consequent stimulation of adrenal steroid synthesis; as the 21-hydroxylase step is impaired, 17-hydroxyprogesterone is converted to androstenedione and testosterone, resulting in severe prenatal virilization of female fetuses [56]. The increased ACTH also stimulates intra-adrenal synthesis of insulin-like growth factor II [56a] causing adrenal hyperplasia, as well as stimulating accumulation of MRNAs for P450scc and P450c17 [56b].

This disorder, generally termed congenital adrenal hyperplasia, has been extensively studied clinically. Variations in the manifestations of the disease, and especially the presence of many patients with little or no impairment in DOC and aldosterone production, led to the widely held hypothesis that there were two separate 21-hydroxylating enzymes under separate genetic control that were differentially expressed in the zones of the adrenal, specifically synthesizing aldosterone or cortisol [57].

21-Hydroxylation is mediated by P450c21 found in smooth endoplasmic reticulum. 21-Hydroxylation was the first biological activity ascribed to any cytochrome P450 [58]. Like P450c17, P450c21 employs a flavoprotein intermediate but no iron–sulfur protein in the electron transport chain from NADPH. Isolation of P450c21 from bovine adrenals showed a single species of protein [42] but it was not until the cDNAs and genes were cloned that it was proven that there is only a single functional 21-hydroxylase gene in man.

Using an immunologic screening tactic, White *et al.*[59] cloned a small 486 bp fragment of bovine P450c21 cDNA. This led to rapid development of our understanding of this gene in three mammalian species. We synthesized oligonucleotides based on White's sequence and used these to clone a bovine P450c21 gene, and demonstrate that there are two P450c21 genes in cattle. We suggested both genes might be active, since Northern blots of bovine adrenal RNA showed two bands, whereas human RNA showed only one [60]. Sequencing of the entire gene showed it is 3447 bp long and is divided into 10 exons and 9 introns. This gene is much smaller and has a distinctly different intron/exon pattern from other known P450 genes [61]. The gene contains several notable features. First, the site of initiation of mRNA transcription, located by both primer extension studies and S_1 nuclease protection experiments, is only 11 bases from the ATG translation initiation codon. Second, the ninth intron is only 48 bases long, the smallest mammalian intron reported to date, and apparently near the lower limit of the size that can yield lariat RNA splicing intermediates. Third, the seventh intron contains a region of repetitive DNA that can apparently form Z-DNA structures [61].

Two other groups cloned bovine P450c21 cDNA at the same time. One of these, while not full length, is identical in sequence to the corresponding regions of the gene we sequenced [62], confirming that this

gene is expressed, as first shown by our S_1 protection experiments [61]. The other cDNA, a full length clone, has numerous striking differences, including a 121 base 5′ untranslated region and several nucleotide differences resulting in two amino acid differences [63]. The differences between these two cDNAs appear too great to be due to variation among individual strains of cattle. Thus, it is most likely that both bovine P450c21 genes are expressed and thus encode these two different cDNAs.

However, in mice and men, only one gene is expressed. Using their bovine P450c21 cDNA fragment, White *et al.*[64] showed that the murine genome contains two P450c21 genes located in the S region of the murine H-2 histocompatibility locus. From a totally different approach, Amor *et al.*[65] found two P450c21 genes duplicated in tandem with the murine genes for the fourth component of complement (C′4). Carroll *et al.*[66] and White *et al.*[67] found the same tandem duplication of P450c21 and C′4 genes in the human genome. In both species, all four of these genes are oriented in the same transcription orientation: 5′ C′4(A)–P450c21(A)–C′4(B)–P450c21(B) 3′. Southern blotting of DNA from a patient having severe deficiency of 21-hydroxylase activity and also having deficient complement activity showed a large deletion encomposing both the C′4(B) and P450c21(B) loci, leading to the hypothesis that only the human P450c21(B) gene is active, and that the P450c21(A) gene is nonfunctional [67]. The two human P450c21 genes have now been cloned and sequenced by two groups, showing that the human P450c21(A) gene contains an 8 bp deletion in exon 3, thus rendering the A gene inactive [68, 69].

In mice, however, the B gene is inactive. Parker *et al.*[70] isolated cosmids of both murine genes and transfected these into cultured mouse Y-1 adrenal tumor cells. Only cells transfected with the A gene exhibited 21-hydroxylase activity. These investigators also synthesized oligonucleotides specific for the murine P450c21(A) and (B) genes and showed that all murine adrenal P450c21 mRNA was derived from the P450c21(A) gene. Sequencing of the mouse genes shows that the P450c21(B) gene has a 215 base deletion encompasing the entire second exon, plus other base changes resulting in premature stop signals [70a]. Thus, in mice, the P450c21(B) gene is a pseudogene. Since different genes are inactive in these two mammals, it suggests that these mutations occurred after mammalian speciation about 85 million years ago. The occurrence of such mutations after mammalian speciation is consistent with finding that both P450c21 genes are active in cattle and keeps open the question of different forms of 21-hydroxylase in other mammalian species.

21-Hydroxylase activity has been described in a broad range of human adult and fetal extra-adrenal tissues [71, 72], but the nature of the enzyme(s) mediating such activity is unknown. Certainly other P450 enzymes may have "gratuitous" 21-hydroxylase activity. In the rabbit, hepatic cytochrome P450-1 readily 21-hydroxylates progesterone, but its cDNA is structurally unrelated to P450c21 [73]). However, Amor *et al.*[65] detected P450c21 mRNA in Northern blots of mouse liver. We found considerable P450c21 mRNA in bovine testis but could not detect it in bovine brain, liver, kidney or ovary RNAs examined by S_1 nuclease protection [61]. We have also examined human fetal adrenal testis, ovary, placenta, kidney, muscle, liver, intestine and spleen RNAs by Northern blotting, and found detectable amounts of P450c21 mRNA only in adrenal [74]. Thus, it is likely that extra-adrenal 21-hydroxylase activity is principally mediated both by other enzymes having 21-hydroxylase activity.

Congenital adrenal hyperplasia due to deficient 21-hydroxylase activity is caused by a large number of different lesions in the human P450c21(B) gene. Several groups have suggested that about 25% of these patients have complete deletions of this gene, as Southern blots of their *Taq*I-digested DNA lack the 3.7 kb fragment corresponnding to the P450c21(B) gene. However, when multiple restriction enzymes are used, such "deletions" cannot be confirmed with any consistency. In a study of 10 affected families, apparent deletions of the 3.7b kb *Taq*I fragment were found in 7 of the 20 P450c21(B) alleles, but in all cases the intact P450c21(B) gene could still be detected in DNA digested with other enzymes [70b]. Thus, a wide variety of gene conversions, point mutations, crossovers and polymorphisms account for most cases of 21-hydroxylase deficiency.

P450c11

The final enzyme in the synthesis of adrenal mineralocorticoids and glucocorticoids is P450c11. This single enzyme mediates the 11-hydroxylation of 11-deoxycortisol to cortisol and also mediates all three final steps in the synthesis of aldosterone from desoxycorticosterone [75]. Thus, this single P450 has 11-hydroxylase, 18-hydroxylase, and 18-oxidase (aldehyde synthetase) activities. Like P450scc, P450c11 is found in the inner membrane of mitochondria [76, 77] and uses adrenodoxin and adrenodoxin reductase to receive electrons from NADPH [78–90]. Like P450scc, P450c11 also has a subunit molecular weight near 50,000 but exists as a large multimer *in vivo* [81, 82]. Both immunochemical studies [83] and reconstitution of purified P450c11 with adrenodoxin and adrenodoxin reductase *in vitro* [84, 85] show that a single species of P450c11 mediates both 11β-hydroxylase and 18-hydroxylase activities. Yanagibashi *et al.*[75] recently refined the purification protocol for P450c11 and purified the enzyme from mitochondria from bovine adrenal zona glomerulosa and zona fasciculata cells. Intact mitochondria from the fas-

ciculata lacked aldehyde synthetase activity, but when the P450c11 from these mitochondria was prepared, it had this activity to an extent indistinguishable from the P450c11 of the zona glomerulosa. Furthermore, the P450c11 from both zones was indistinguishable enzymologically, immunologically, by amino acid composition, and by N-terminal amino acid sequence. Spectral studies suggested the three enzymatic activities (i.e. 11β-hydroxylation, 18-hydroxylation and aldehyde synthetase) all occur on the same active site. Finally, both 11β-hydroxylase and 18-hydroxylase activities respond in parallel to hormonal stimuli in man [86]. Thus, P450c11, like all other adrenal steroidogenic P450 enzymes, is a multifunctional enzyme mediating several different steps in the steroidogenic pathways. The differential activity of P450c11 in the adrenal glomerulosa and fasciculata is probably due to zone-specific inhibitors.

Little is known about the molecular biology of P450c11. The cloning of bovine [87] and human [88] P450c11 cDNA have been reported without sequence data. These reports indicate that Southern blotting studies show there is a single P450c11 gene in both cattle and human beings. The mRNA for P450c11 is unusual in that Northern blotting studies indicate it is about 4 kb long [87, 88]. This is twice as long as the mRNA for any other P450 studied to date and suggests that P450c11 mRNA will have a 3′ untranslated region over 2 kb long.

EVOLUTION OF STEROIDOGENIC P450 GENES

Since only bovine and human genes for P450c21 have been sequenced, it is premature to speculate extensively about the evolution of these genes. Comparison at the level of cDNA and protein sequences are possible for P450scc, P450c21 and P450c17, but not P450c11. The human sequences for P450c21 and P450c17 show nearly 30% amino acid homology, while neither is more than 12% homologous with P450scc. In fact, P450c17 and P450c21 share greater homology with rat liver P450s induced by phenobarbital or 3-methylcholanthrene than with P450scc [54]. This presumably reflects a very ancient divergence between nuclear genes encoding the mitochondrial P450 enzymes (P450scc, P450c11) from genes encoding the P450s of the smooth endoplasmic reticulum (P450c17, P450c21, rat hepatic P450s induced by phenobarbital and 3-methylcholanthrene). We speculate that the P450c11 sequence and gene structure will more closely resemble P450scc than P450c17 or P450c21 and, similarly, that P450c17 will share some domains in common with P450c21 [54]. The determination of the structures of these genes will add greatly to our understanding of the evolution of steroidogenic and other P450s.

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