

Transcript encoded on the opposite strand of the human steroid 21-hydroxylase/complement component C4 gene locus

(21-hydroxylase deficiency/congenital adrenal hyperplasia/overlapping genes/HLA class III genes/*CYP21* locus)

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ABSTRACT The gene encoding human adrenal steroid 21-hydroxylase (P450c21) and its highly similar pseudogene are duplicated in tandem with the two genes encoding the fourth component of human serum hemolytic complement (C4). This 60-kilobase gene complex, which lies within the major histocompatibility complex on the short arm of human chromosome 6, has been studied in considerable detail because genetic disorders in steroid 21-hydroxylation and in C4 are common. We have cloned a cDNA encoded by a previously unidentified gene in this region. This gene lies on the strand of DNA opposite from the strand containing the P450c21 and C4 genes, and it overlaps the last exon of P450c21. The newly identified gene encodes mRNAs of 3.5 and 1.8 kilobases that are expressed in the adrenal and in a Leydig cell tumor but are not expressed in nonsteroidogenic tissues. The sequence of the longest cDNA (2.7 kilobases) shows no similarity to known sequences available in two computerized data bases. The 5' end of this sequence is characterized by three repeats, each encoding about 100 amino acids flanked by potential sites for proteolytic cleavage. Although numerous studies have shown that gene deletions causing congenital adrenal hyperplasia occur in this region, none of these gene deletions extends into this newly identified gene, suggesting that it encodes an essential function.

The class III region of the human leukocyte antigen (HLA) locus on chromosome 6p21 contains tandemly duplicated genes for the fourth component of serum complement (C4) and for the P450 enzyme (P450c21) steroid 21-hydroxylase [steroid,hydrogen-donor:oxygen oxidoreductase (21-hydroxylating), EC 1.4.99.10] (reviewed in refs. 1 and 2). This locus has attracted considerable attention, in part because 1 in 7000 persons has deficient 21-hydroxylase activity causing congenital adrenal hyperplasia (CAH), a life-threatening disease (reviewed in ref. 1). The C4 genes, *C4A* and *C4B*, are transcribed in the same orientation as the P450c21B gene [also termed P450XXIA2 (3) or *CYP21B* (4)]. The P450c21A locus (*CYP21A*) also has the same transcriptional orientation but has several mutations rendering it a nonfunctional pseudogene (5, 6). A map of this region is shown in Fig. 1A. Genetic rearrangements in this locus are common (12). Deletions extending from the middle of the P450c21A gene to the middle of the P450c21B gene occur in 11% of patients with CAH (7); however, no deletion has been described extending to the 3' end of the P450c21B gene. We present evidence for the existence of a gene lying on the DNA strand opposite from that encoding the C4 and P450c21 genes. This gene overlaps exon 10 of the P450c21 gene(s) and extends 3' to the P450c21 gene(s). This gene(s) encodes 3.5- and 1.8-kb mRNAs expressed in the adrenal and in a Leydig cell tumor but not in nonsteroidogenic tissues. The sequence of the available cDNA shows no similarity to known sequences.†

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Because no deletion of this gene has been found in numerous studies of this region of DNA, we propose that it encodes an essential function.

MATERIALS AND METHODS

RNA was prepared from tissues either by guanidinium isothiocyanate extraction followed by CsCl ultracentrifugation (8) or by LiCl precipitation (13); RNA was prepared from cultured cells as described (14). Electrophoresis of total RNA in 1% agarose gel, its transfer to nylon membranes, and the hybridization and washing of such membranes were done as described (14).

RNA was reverse-transcribed into cDNA, cloned into λ gt11, and screened with a human P450c21 cDNA (10), all as described previously (15). Positively hybridizing plaques were purified and the sizes of the inserts were determined by agarose gel electrophoresis followed by blotting and hybridization with the P450c21 cDNA. The longest insert obtained (2.7 kb) was subcloned in M13 and pUC18 and sequenced by the dideoxy chain-termination technique using either single-stranded M13 templates or double-stranded sequencing (16). The cDNA sequence and the encoded protein sequence were compared to the GenBank and National Biomedical Research Foundation data bases on April 28, 1989.

Southern blots of normal human genomic DNA were prepared and probed with cDNA fragments isolated from their cloning vectors and labeled with 32 P with random primers, as described (7).

RESULTS AND DISCUSSION

Cloning of cDNA Encoded by this Gene. To determine the genetic lesion causing one form of 21-hydroxylase deficiency, we obtained adrenal tissue from a homozygously affected human fetus aborted at week 20. The diagnosis had been established hormonally and by HLA identity to an affected sibling (1). The adrenal cDNA library of 9×10^5 clones prepared from this tissue was screened with human P450c21 cDNA (10), identifying 36 cDNA clones of 1.0–2.7 kb. As human P450c21 mRNA is about 2.0 kb long (17), we considered that the cDNAs longer than 2.0 kb might be copies of incorrectly spliced mRNA precursors and that such splicing error(s) might account for the 21-hydroxylase deficiency. Therefore, we studied the 2.7-kb cDNA insert further.

Location of the Gene Encoding the 2.7-kb cDNA. Restriction endonuclease mapping of the 2.7-kb cDNA suggested that only a portion of it corresponded to the P450c21 gene. When

Abbreviations: CAH, congenital adrenal hyperplasia; C4, fourth component of complement; P450c21, cytochrome P450 specific for steroid 21-hydroxylation.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25813).

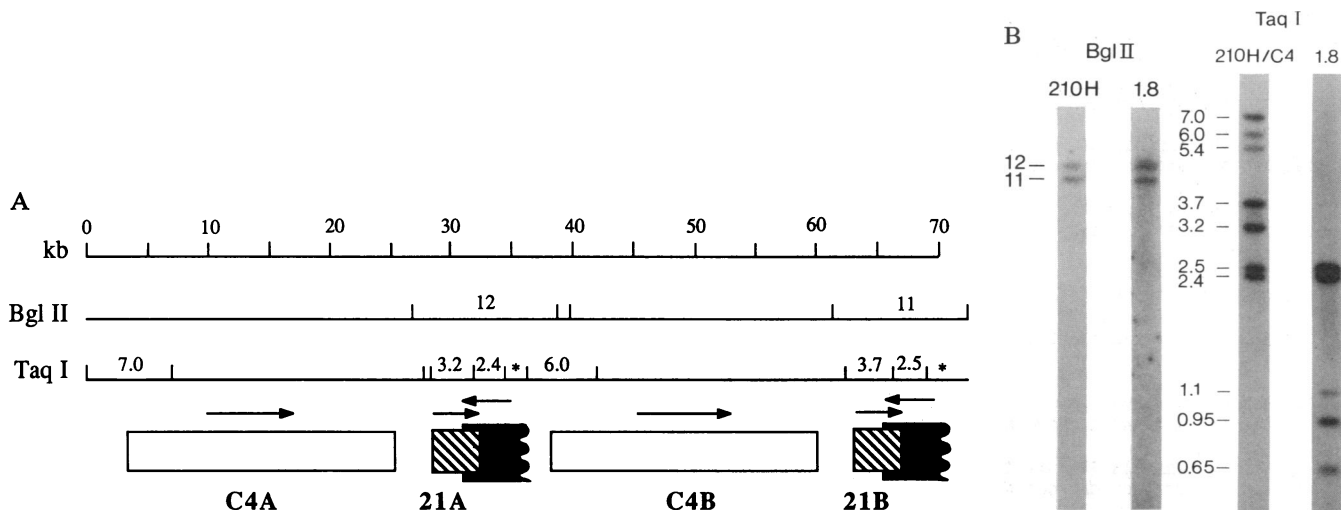


FIG. 1. (A) Map of the P450c21/C4 gene locus in the class III region of the HLA locus on human chromosome 6p21. The top line shows the scale in kilobases (kb). Boxes show the extent of *C4A* and *C4B* (open boxes), the P450c21A and -B genes [designated 21A and 21B (hatched boxes)], and the approximate extent of the detected portions of the opposite-strand gene(s) described in this report (solid boxes). Some of the known loci cleaved by restriction endonucleases *Bgl* II and *Taq* I, derived from published studies (5-7, 9, 12), are shown. Numbers indicate sizes (in kilobases) of DNA fragments hybridizing to the 1.8-kb *Bam*HI-*Eco*RI fragment of the 2.7-kb cDNA. The asterisks 3' to the 2.5- and 2.4-kb *Taq* I fragments indicate that several small fragments lie in those regions. (B) Southern blots of genomic DNA from a normal person. DNA was cleaved with *Bgl* II or *Taq* I and probed with the indicated ³²P-labeled cDNAs: 21OH, a human P450c21 cDNA fragment comprising the 1229 bases at the 3' end of P450c21 mRNA (10); 21OH/C4, a mixture of the 476-base-pair *Bam*HI-*Kpn* I fragment from the 5' end of the full-length human C4 cDNA clone pAT-A (11) and the 21OH probe; and 1.8, the 1.8-kb *Bam*HI-*Eco*RI fragment of the 2.7-kb cDNA excluding all P450c21 sequences. The sizes of the hybridizing bands seen in these blots were determined by comparison to migration of *Hind*III-cleaved DNA from bacteriophage PM2. The map locations of the hybridizing bands are shown in A. The 12- and 11-kb *Bgl* II bands, respectively, correspond to the P450c21A and -B genes (and their 3' flanking DNA). The 7.0-kb *Taq* I band corresponds to the 5' end of *C4A*, whereas the 6.0- and 5.4-kb *Taq* I bands correspond to the 5' ends of the long and short alleles of the *C4B*, respectively (11). The 3.2- and 2.4-kb *Taq* I fragments correspond to the P450c21A pseudogene and its 3' flanking DNA, respectively, whereas the 3.7- and 2.5-kb *Taq* I fragments correspond to the functional P450c21B gene and its 3' flanking DNA, respectively (7). The 1.1-, 0.95-, and 0.65-kb *Taq* I fragments correspond to the map region(s) designated by the asterisk(s).

Southern blots of human genomic DNA cleaved with *Bgl* II and *Taq* I were probed with the 2.7-kb insert, DNA fragments corresponding to the P450c21 genes and their 3' flanking DNA hybridized (data not shown). When these blots were probed with a 1.8-kb *Bam*HI-*Eco*RI fragment of the 2.7-kb cDNA that lacks P450c21 sequences, only the 3' flanking DNA hybridized (Fig. 1B). This suggested that the cDNA did not correspond to P450c21 or C4 mRNA, but to another transcript encoded by a gene lying downstream (3') from the P450c21 gene. Such a transcript could have two possible origins. First, it might have the same transcriptional orientation as the P450c21 gene and be initiated within that gene and extend downstream from it. Second, it might be initiated downstream from the P450c21 gene and be transcribed in the opposite orientation (i.e., from the opposite strand) and extend back to the P450c21 gene. Inspection of the sequence of the P450c21A and -B genes (5, 6) showed no sequences similar to transcriptional initiation complexes within either P450c21 gene; however, an AATAAA sequence, generally implicated as a polyadenylation signal (18), was found on the antisense strand 40 bases 3' to the TGA translational stop codon of P450c21. These observations were consistent with the second possible origin of the 2.7-kb cDNA. To confirm this hypothesis, we determined the nucleotide sequence of the 2.7-kb cDNA. The 3' end of the 2.7-kb cDNA was identified by a 13-base poly(A) tail; the adjacent DNA had a nucleotide sequence identical to that of the antisense strand of the 3' untranslated region of the P450c21 gene and contained the AATAAA sequence in the predicted location. Therefore, the 2.7-kb cDNA was encoded by a gene on the opposite DNA strand from the P450c21 and C4 genes that had a transcriptional initiation site downstream from the P450c21 locus and whose 3' end overlapped P450c21 exon 10 (Fig. 2).

To determine whether the 2.7-kb cDNA arose from the opposite strand of the P450c21A or P450c21B gene, we

compared its 3' sequence to the known P450c21A and -B gene sequences. The P450c21A pseudogene and P450c21B gene differ in only 87 of the 3400 bases comprising each of these two genes (5). Within the P450c21 3' untranslated region

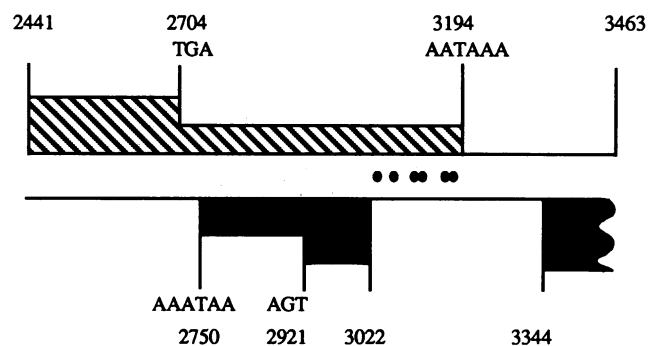


FIG. 2. Detailed map of the overlapping portions of the P450c21 gene(s) and the gene(s) encoding the 2.7-kb cDNA. Upper line: exon 10 of the P450c21B gene; the broader region of the bar indicates sequences encoding P450c21 protein and the narrower region indicates the 3' untranslated region of P450c21 mRNA; the thin line indicates 3' flanking DNA. Lower line: presumed structure of the 3' end of the gene encoding the 2.7-kb cDNA, inferred from partial cDNA sequencing. The narrow region of the bar shows the presumed 3' untranslated portion of the corresponding mRNA. The broad bar corresponds to protein-coding sequences, and the thin lines correspond to 3' flanking DNA and the last intron of this gene. Nucleotide numbers correspond to the P450c21B gene sequenced by Higashi *et al.* (5). The six dots between the two diagrams indicate the positions of the only nucleotides differing between the P450c21A and -B genes in exon 10. The diagram only extends to the last available base of the genomic DNA sequence of the 3' flanking region of P450c21B (5). Only those regions of the cDNA whose genomic locations could be confirmed from the genomic DNA sequence (5) are shown.

1 Gly Leu Ser Gly Arg Lys Arg Leu Gly Pro Ile Ser Ala Asp Ser Thr Thr Ala Pro Leu Glu Lys Glu Leu Pro Pro His Leu Gly Glu
1 GGT CTG TCA GGC AGG AAA CGA CTG GGC CCC ATC TCT GCT GAC AGC ACC ACA GCT CCC CTG GAG AAG GAG CTA CCT CCC CAC CTG GGG GAA

31 Leu Thr Val Ala Glu Glu Thr Ser Ser Ser Leu Arg Leu Ser Trp Thr Val Ala Gln Gly Pro Phe Asp Ser Phe Val Val Gln Tyr Arg
91 CTG ACC GTG GCT GAG GAG ACC TCC ACC TCT CTG CGC CTG TCC TGG ACG GTA GCC CAG GGC CCC TTT GAC TCC TTC GTG GTC CAG TAC AGG

61 Asp Thr Asp Gly Gln Pro Arg Ala Val Pro Val Ala Ala Asp Gln Arg Thr Val Thr Val Glu Asp Leu Glu Pro Gly Lys Lys Tyr Lys
181 GAC ACG GAC GGG CAG CCC AGG GCA GTG CCT GTG GCC GCA GAC CAG CGC ACA GTC ACC GTA GAG GAC CTG GAG CCT GGC AAG AAA TAC AAG

91 Phe Leu Leu Tyr Gly Leu Leu Gly Gly Lys Arg Leu Gly Pro Val Ser Ala Leu Gly Met Thr Ala Pro Glu Glu Asp Thr Pro Ala Pro
271 TTT CTG CTC TAC GGG CTC CTT GGG GGA AAG CGC CTG GGC CCG GTC TCT GCC CTG GGA ATG ACA GCC CCA GAA GAG GAC ACA CCA GCC CCA

121 Glu Leu Ala Pro Glu Ala Pro Glu Pro Pro Glu Glu Pro Arg Leu Gly Val Leu Thr Val Thr Asp Thr Thr Pro Asp Ser Met Arg Leu
361 GAG TTA GCC CCA GAG GCC CCT GAG CCT Pro GAA Glu GCC CGC CTA GGA GTG CTG ACC GTG ACC GAC ACA ACC CCA GAC TCC ATG CGC CTC

151 Ser Trp Ser Val Ala Gln Gly Pro Phe Asp Ser Phe Val Val Gln Tyr Glu Asp Thr Asn Gly Gln Pro Gln Ala Leu Leu Val Asp Gly
451 TCG TGG AGC GTG GCC CAG GGC CCC TTT GAT TCC TTC GTG GTC CAG TAT GAG GAC ACG AAC GGG CAG CCC CAG GCC TTG CTC GTG GAC GGC

181 Asp Gln Ser Lys Ile Leu Ile Ser Gly Leu Glu Pro Ser Thr Pro Tyr Arg Phe Leu Leu Tyr Gly Leu His Glu Gly Lys Arg Leu Gly
541 GAC CAG AGC AAG ATC CTC ATC TCA GGC CTG GAG CCC AGC ACC CCC TAC AGG TTC CTC CTC TAT GGC CTC CAT GAA GGG AAG CGC CTG GGG

211 Pro Leu Ser Ala Glu Gly Thr Thr Gly Leu Ala Pro Ala Gly Gln Thr Ser Glu Thr Ser Arg Pro Arg Leu Ser Gln Leu Ser Val Thr
631 CCC CTC TCA GCT GAG GGC ACC ACA GGC CTG GCT CCT GGT CAG ACC TCA GAG GAG TCA AGG CCC CGC CTG TCC GAC CTG TCT GTG ACT

241 Asp Val Thr Thr Ser Ser Leu Arg Leu Asn Trp Glu Ala Pro Pro Gly Ala Phe Asp Ser Phe Leu Leu Arg Phe Gly Val Pro Ser Pro
721 GAC GTG ACC ACC AGT TCA CTG AGG CTC AAC TGG GAG GCC CCA CCG GGG GCC TTC GAC TCC TTC CTG CTC CGC TTT GGG GTT CCA TCA CCA

271 Ser Thr Leu Glu Pro His Pro Arg Pro Leu Leu Gln Arg Glu Leu Met Val Pro Gly Thr Arg His Ser Ala Val Leu Arg Asp Leu Arg
811 AGC ACT CTG GAG CCG CAT CCG CGT CCA CTG CTG CAG CGC GAG CTG ATG GTG CCG GGG ACG CGG CAC TCG GCC GTG CTC CGG GAC CTG CGT

301 Ser Gly Thr Leu Tyr Ser Leu Thr Leu Tyr Gly Leu Arg Gly Pro His Lys Ala Asp Ser Ile Gln Gly Thr Ala Arg Thr Leu Ser Pro
901 TCC GGG ACT CTG TAC AGC CTG ACA CTG TAT GGG CTG CGA GGA CCC CAC AAG GCC GAC AGC ATC CAG GGA ACC GCC CGC ACC CTC AGC CCA

331 Val Leu Glu Ser Pro Arg Asp Leu Gln Phe Ser Glu Ile Arg Glu Thr Ser Ala Lys Val Asn Trp Met Pro Pro Pro Ser Arg Ala Asp
991 GTT CTG GAG AGC CCC CGT GAC CTC CAA TTC AGT GAA ATC AGG GAG ACC TCA GCC AAG GTC AAC TGG ATG ACC CCA CCA TCC CGG GCG GAG

361 Ser Phe Lys Val Ser Tyr Gln Leu Ala Asp Gly Gly Glu Pro Gln Ser Val Gln Val Asp Gly Gln Ala Arg Thr Gln Lys Leu Gln Gly
1081 AGC TTC AAA GTC TCC TAC CAG CTG GCG AAC GGA GGG GAG CCT CAG AGT GTG CAG GTG GAT GGC CAG GCC CGG ACC CAG AAA CTC CAG GGG

391 Leu Ile Pro Gly Ala Arg Tyr Glu Val Thr Val Val Ser Val Arg Gly Phe Glu Glu Ser Glu Pro Leu Thr Gly Phe Leu Thr Thr Val
1171 CTG ATC CCA GGC GCT CGC TAT GAG GTG ACC GTG GTC TCG GTC CGA GGC TTT GAG GAG AGT GAG CCT CTC ACA GGC TTC CTC ACC ACG GTT

421 Pro Asp Gly Pro Thr Gln Leu Arg Ala Leu Asn Leu Thr Glu Gly Phe Ala Val Leu His Trp Lys Pro Pro Gln Asn Pro Val Asp Thr
1261 CCT GAC GGT CCC ACA CAG TTG CGT GCA CTG AAC TTG ACC GAG GGA TTC GCC GTG CTG CAC TGG AAG CCC CCC CAG AAT CCT GTG GAC ACC

451 Tyr Asp Val Gln Val Thr Ala Pro Gly Ala Pro Pro Leu Gln Ala Glu Thr Pro Gly Ser Ala Val Asp Tyr Pro Leu His Asp Leu Val
1351 TAT GAC GTC CAG GTC ACA GCC CCT GGG GCC CCG CCT CTG CAG GCG GAG ACC CCA GGC AGC GCG GTG GAC TAC CCC CTG CAT GAC CTT GTC

481 Leu His Thr Asn Tyr Thr Ala Thr Val Arg Gly Leu Arg Gly Pro Asn Leu Thr Ser Pro Ala Ser Ile Thr Phe Thr Thr Gly Leu Glu
1441 CTC CAC ACC AAC TAC ACC GCC ACA GTG CGT GGC CTG CGG GGC CCC AAC CTC ACT TCC CCA GCC AGC ATC ACC TTC ACC ACA GGG CTA GAG

511 Ala Pro Arg Asp Leu Glu Ala Lys Glu Val Thr Pro Arg Thr Ala Leu Leu Thr Trp Thr Glu Pro Pro Val Arg Pro Ala Gly Tyr Leu
1531 GCC CCT CGG GAC TTG GAG GCC AAG GAA GTG ACC CCC CGC ACC GCC CTG CTC ACT TGG ACT GAG CCC CCA GTC CGG CCC GCA GGC TAC CTG

541 Leu Ser Phe His Thr Pro Gly Gly Gln Asn Gln Glu Ile Leu Leu Pro Gly Gly Ile Thr Ser His Gln Leu Leu Gly Leu Phe Gly Ser
1621 CTC AGC TTC CAC ACC CCT GGT GGA CAG AAC CAG GAG ATC CTG CTC CCA GGA GGG ATC ACA TCT CAC CAG CTC CTT GGC CTC TTT GGG TCC

571 Thr Ser Tyr Asn Ala Arg Leu Gln Ala Met Trp Gly Gln Ser Leu Leu Pro Pro Val Ser Thr Ser Phe Thr Thr Gly Gly Leu Arg Ile
1711 ACC TCC TAC AAT GCA CGG CTC CAG GCC ATG TGG GGC CAG AGC CTC CTG CCG CCC GTG TCC ACC TCT TTC ACC ACG GGT GGG CTG CGG ATC

601 Pro Phe Pro Arg Asp Cys Gly Glu Glu Met Gln Asn Gly Ala Gly Ala Ser Arg Thr Ser Thr Ile Phe Leu Asn Gly Asn Arg Glu Arg
1801 CCC TTC CCC AGG GAC TGC GGG GAG GAG ATG CAG AAC GGA GCC GGT GCC TCC AGG ACC AGC ACC ATC TTC CTC AAC GGC AAC CGC GAG CGG

631 Pro Leu Asn Val Phe Cys Asp Met Glu Thr Asp Gly Gly Gly Trp Leu Val Phe Gln Arg Arg Met Asp Gly Gln Thr Asp Phe Trp Arg
1891 CCC CTG AAC GTG TTT TGC GAC ATG GAG ACT GAT GGG GGC GGC TGG CTG GTG TTC CAG CGC CGC ATG GAT GGA CAG ACA GAC TTC TGG AGG

661 Asp Trp Glu Asp Tyr Ala His Gly Phe Gly Asn Ile Ser Gly Glu Phe Trp Leu Gly Asn Glu Ala Leu His Ser Leu Thr Gln Ala Gly
1981 GAC TGG GAG GAC TAT GCC CAT GGT TTT GGG AAC ATC TCT GGA GAG TTC TGG CTG GGC AAT GAG GCC CTG CAC AGC CTG ACA CAG GCA GGT

691 Asp Tyr Ser Ile Arg Val Asp Leu Arg Ala Gly Asp Glu Ala Val Phe Ala Gln Tyr Asp Ser Phe His Val Asp Ser Ala Ala Glu Tyr
2071 GAC TAC TCC ATC CGC GTG GAC CTG CGG GCT GGG GAC GAG GCT GTG TTC GCC CAG TAC GAC TCC TTC CAC GTA GAC TCG GCT GCG GAG TAC

721 Tyr Arg Leu His Leu Glu Gly Tyr His Gly Thr Ala Gly Asp Ser Met Ser Tyr His Ser Gly Ser Val Phe Ser Ala Arg Asp Arg Asp
2161 TAC CGC CTC CAC TTG GAG GGC TAC CAC GGC ACC GCA GGG GAC TCC ATG AGC TAC CAC AGC GGC AGT GTC TTC TCT GCC CGT GAT CGG GAC

751 Pro Asn Ser Leu Leu Ile Ser Cys Ala Val Ser Tyr Arg Gly Ala Trp Trp Tyr Arg Asn Cys His Tyr Ala Asn Leu Asn Gly Leu Tyr
2251 CCC AAC AGC TTG CTC ATC TCC TGC GCT GTC TCC TAC CGA GGG GCC TGG TGG TAC AGG AAC TGC CAC TAC GCC AAC CTC AAC GGG CTC TAC

781 Gly Ser Thr Val Asp His Gln Gly Val Ser Trp Tyr His Trp Lys Gly Phe Glu Phe Ser Val Pro Phe Thr Glu Met Lys Leu Arg Pro
2341 GGG AGC ACA GTG GAC CAT CAG GGA GTG AGC TGG TAC CAC TGG AAG GGC TTC GAG TTC TCG GTG CCC TTC ACG GAA ATG AAG CTG AGA CCA

810 Arg Asn Phe Arg Ser Pro Ala Gly Gly Gly OP
2431 AGA AAC TTT CGC TCC CCA GCG GGG GGA GGC TGA GCTGCTGCCACCTCTCTCGACCCCACTATGACTGCCGAGCACTGAGGGGTGCCCCGAGAGAAGGCCAGGGT

2539 CCTTACCACCCAGCCGCTGGAGGAAGCCTTCTCTGCCAGGATCTCGACACTGTGTTACAGGGGGAGGGGGGTTCTGTACAGGAGCAATAAAGGAGAACTGAGGTACCCGA

FIG. 3. (Legend appears at the bottom of the opposite page.)

there are 6 nucleotide differences, all lying between bases 3074 and 3180 of the P450c21B gene, according to the numbering of Higashi *et al.* (5). However, DNA corresponding to bases 3022–3343 of the P450c21B gene, encompassing all 6 nucleotide differences with P450c21A in this region, were absent in the 2.7-kb cDNA (Fig. 2). The limits of this absent region have canonical intron/exon splice-junction sequences (19), consistent with P450c21B bases 3022–3343, constituting an intron in this newly described gene. Because all nucleotides distinguishing the P450c21A pseudogene from the P450c21B gene in this region are in the spliced intronic region, we cannot determine whether the 2.7-kb cDNA arises from the opposite strand of the P450c21A pseudogene or from the opposite strand of the P450c21B gene.

Structure of the 2.7-kb cDNA. The complete sequence of the 2.7-kb cDNA is shown in Fig. 3. It contains a single open reading frame of 2457 bases encoding 819 amino acids, followed by a TGA translational termination signal. It has a 193-base 3' untranslated region containing a typical AATAAA polyadenylation signal followed by a poly(A) tail 20 bases further downstream. The cDNA is clearly not full-length, as the first in-phase ATG codon is 500 bases from the 5' end (amino acid 148 in Fig. 3) and lacks a consensus translational initiation sequence (20). Furthermore, transfer blots of adrenal and other RNAs showed that a full-length cDNA should be at least 3.5 kb (see Fig. 4). Searching of several other cDNA libraries, including an Okayama–Berg human adrenal library that has yielded 5-kb cDNAs (21), did not yield a cDNA for this gene longer than 2.7 kb. The 5' end of the sequence is characterized by three repeated units lying between potential sites for proteolytic cleavage: Lys-Arg at amino acids 5 and 6, Lys-Arg at 100 and 101, Lys-Arg at 207 and 208, and His-Lys at 316 and 317. Of the 91 amino acids in the first repeat, 54 are identical in the second and 25 in the third. Sequencing of some genomic DNA fragments has revealed three additional homologous repeats lying 5' of the region encoding the 2.7-kb transcript (22). This structure suggests that the protein encoded by this gene may undergo proteolytic cleavage into a series of related peptides, plus a carboxyl-terminal peptide of unrelated sequence.

Expression of the Gene. We considered that this transcript from the opposite strand of the P450c21 locus might represent a fetal-specific transcript or might be an aberrant transcript associated with 21-hydroxylase deficiency. Therefore, we probed transfer blots of RNA prepared from a variety of human fetal tissues and from normal adult adrenals (Fig. 4). The 2.7-kb cDNA hybridized to mRNAs of 3.5 and 1.8 kb in normal adrenal RNA and in RNA from a human Leydig cell adenoma (35) but did not hybridize detectably to such mRNAs in heart, liver, brain, spleen, placenta, or fibroblasts (Fig. 4). Furthermore, transcription of the gene encoding the 2.7-kb cDNA is not specific to the fetus and is unrelated to CAH. However, the lack of significant amounts of this transcript in nonsteroidogenic tissues suggests it might be associated with steroidogenesis.

Role of this Gene. Overlapping genes encoded on opposite strands of DNA occur commonly in prokaryotes, bacteriophages, and viruses, but only a few examples have been reported in higher eukaryotes. These include a gene on the opposite strand of the gene for rat gonadotropin-releasing hormone (23), two *Drosophila* loci (24, 25), a mouse locus of unknown function (26), and a newly described member of the *c-erbA* gene family on the opposite strand from the gene encoding a rat (27) and human (28) thyroid hormone receptor. While the functional relationship, if any, between P450c21

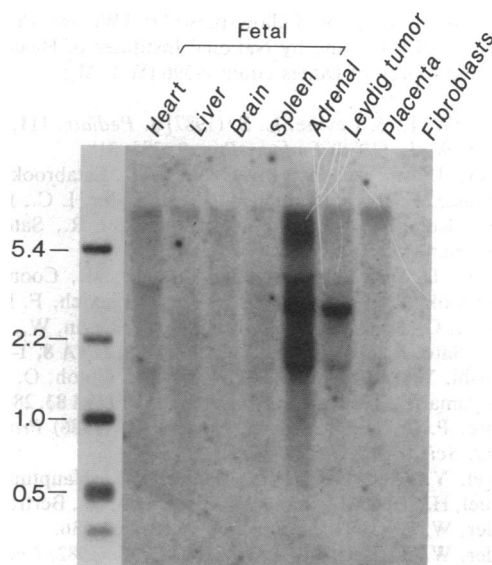


FIG. 4. Transfer blots of human RNA probed with the 1.8-kb *Bam*HI–*Eco*RI fragment of the 2.7-kb cDNA; this fragment lacks all P450c21 sequences. The tissue source of each 20- μ g RNA sample is shown. Molecular size (kb) markers are *Hind*III-cleaved bacteriophage PM2 DNA.

and the transcript from its opposite strand is unknown, it is obviously of interest that both mRNAs are found in the adrenal, suggesting a role in steroidogenesis. Potential roles in cholesterol transport or as a trans-acting regulatory protein cannot be evaluated at present, but the opposite-strand transcript we have described probably cannot encode another steroidogenic enzyme, as the genes for these are largely known (2), the available protein sequence lacks a steroid binding site (29), and only deficiency of 21-hydroxylase is linked to *HLA* (1, 2). P450c21 and the mRNA encoding the 2.7-kb cDNA are both found in the adrenal, but we do not know whether they are expressed in the same cells. If expressed in the same cell, the overlapping 3' sequences might regulate transcription or mRNA processing, translation, or half-life (30, 31).

Deletions extending from the P450c21A pseudogene to *C4B* are found in 14% of normal chromosomes (7), and a larger deletion encompassing the 5' half of the P450c21B gene occurs in 11% of patients with CAH (7, 12, 32). However, among 453 chromosomes bearing CAH alleles (reviewed in ref. 12), no deletion has been described extending into the 3' portion of the P450c21B gene. Therefore, we speculate that the 2.7-kb cDNA arises from a gene on the opposite strand of the 3' end of the P450c21B gene, and that it encodes a transcript whose absence causes early embryonic or fetal demise. Examination of the published sequences of the 3' ends of the murine (33) and bovine (34) P450c21 genes shows that these other mammals retain polyadenylation sites, open reading frames, translational stop signals, and canonical splice-junction sites in the same loci as shown in Fig. 2. The encoded amino acid sequence of the last exon of the opposite-strand transcript is very similar in all three species. Thus, it appears that this gene and its relationship to P450c21 may be a general mammalian feature.

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Fig. 3 (on opposite page). Sequence of the 2.7-kb cDNA. The longest open reading frame is shown. The potential proteolytic cleavage sites between amino acids 6 and 7, 100 and 101, 207 and 208, and 316 and 317 are boxed; the amino acids found between the first pair of potential cleavage sites that are conserved between the other pairs of potential cleavage sites are underlined. Note the AATAAA sequence 20 bases from the end of the cDNA. The poly(A) tail following the last base is designated A_n .

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