Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1986–1990, April 1984
Biochemistry

Cloning and expression of cDNA encoding a bovine adrenal cytochrome P-450 specific for steroid 21-hydroxylation

(recombinant DNA/fusion protein/DNA sequence analysis/*in situ* immunoassay/21-hydroxylase)

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Communicated by Allan H. Conney, December 15, 1983

ABSTRACT **We isolated a cDNA clone encoding a bovine adrenal cytochrome P-450 specific for steroid 21-hydroxylation (P-450$_{C21}$). Serum from rabbits immunized with purified P-450$_{C21}$ precipitated a single protein from the products of an *in vitro* translation reaction using bovine adrenal mRNA. This protein migrated with P-450$_{C21}$ on NaDodSO$_4$/polyacrylamide gel electrophoresis. After sucrose gradient sedimentation, mRNA encoding P-450$_{C21}$ was found in the 19S fraction. This fraction was reverse transcribed into double-stranded cDNA and inserted into the *Pst* I site of pBR322 by the dC·dG tailing procedure. *Escherichia coli* cells transformed with recombinant plasmids were screened with an *in situ* immunoassay using anti-P-450$_{C21}$ serum and ^{125}I-labeled staphylococcal protein A. Two colonies consistently bound anti-P-450$_{C21}$ serum. They were identified as carrying the same plasmid by restriction mapping. This plasmid, pC21a, contains an insert of 520 base pairs. It hybridizes with mRNA encoding P-450$_{C21}$. The peptide encoded by the insert in pC21a is highly homologous to two peptides isolated from porcine P-450$_{C21}$ and shows limited homology to the P-450 induced by phenobarbital in rat liver. This clone may be useful in studying the molecular genetics of human congenital adrenal hyperplasia due to 21-hydroxylase deficiency.**

Cytochromes P-450 are heme-containing enzymes with molecular weights of about 50,000. They all act as terminal oxidases of NADPH-dependent electron transport pathways, but they vary as to substrate specificity and organ distribution. Several hepatic cytochromes P-450 can be induced to high levels by xenobiotics such as phenobarbital or methylcholanthrene, and it is these enzymes that have been best characterized structurally (1) and on the molecular genetic level (2, 3). A number of cytochromes P-450 in the liver, gonads, and adrenal cortex metabolize steroids. The conversion in the adrenal cortex of 17-hydroxyprogesterone to 11-deoxycortisol by 21-hydroxylation was, in fact, the first function assigned to a cytochrome P-450 (P-450) (4). Of the five steps required to synthesize cortisol from cholesterol, four require a P-450 (5): the C-22,27 side chain is cleaved by a P-450 to form pregnenolone; the 3β-hydroxyl is dehydrogenated, yielding progesterone, which is successively hydroxylated by three different cytochromes P-450 at the 17α, 21, and 11β positions to yield cortisol. These steps occur in two subcellular locations, the side-chain cleavage and 11β-hydroxylation steps in mitochondria, and the 17α- and 21-hydroxylations in microsomes.

In humans, genetic defects in each of the steps of cortisol biosynthesis have been described, although only in deficiency of cholesterol side-chain cleavage activity has a defective or deficient P-450 been documented (6). Of these inborn errors of metabolism, 21-hydroxylase deficiency is by far the most common, occurring in about 1/5000 individuals in most populations (7). It is inherited as a monogenic autosomal recessive trait closely linked to the HLA gene complex (8).

Because it is relatively common and has a well-defined mode of inheritance, 21-hydroxylase deficiency should be particularly amenable to study by using techniques of molecular genetics. As an initial step in such a study, we have isolated a cDNA clone corresponding to the P-450 responsible for steroid 21-hydroxylation in bovine adrenal cortex (P-450$_{C21}$). This clone encodes a polypeptide that carries antigenic determinants specific for this particular P-450.

MATERIALS AND METHODS

Production of Antiserum to P-450$_{C21}$. Bovine adrenal glands were obtained from a local slaughterhouse, frozen in liquid nitrogen, and stored at −70°C until used. P-450$_{C21}$ was purified by the method of Kominami *et al.* (9). Rabbits were immunized with four monthly injections, each containing 75 μg of purified P-450$_{C21}$ in complete (first injection) or incomplete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection.

Isolation of mRNA. Adrenal mRNA was isolated by serial ethanol precipitations from 6 M guanidine·HCl (Schwartz/Mann) (10). Poly(A)$^+$ mRNA was isolated on oligo(dT)-cellulose (11) (Collaborative Research, Waltham, MA).

In vitro translations were performed with the rabbit reticulocyte lysate system (12) (Bethesda Research Laboratories) containing [^{35}S]methionine (New England Nuclear). Reaction products were immunoprecipitated by using anti-P-450$_{C21}$ serum and formalin-fixed *Staphylococcus aureus* (13) (Enzyme Center, Boston, MA); the adsorbed antigen–antibody complexes were washed as described (14), eluted by boiling in sample buffer, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (15) and autofluorography.

Poly(A)$^+$ mRNA was fractionated on the basis of size by sucrose gradient sedimentation (16).

Construction of cDNA Clones. Size-fractionated mRNA was reverse-transcribed into cDNA in the presence of placental ribonuclease inhibitor (17) (Boehringer Mannheim), using an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (18) (Life Sciences, St. Petersburg, FL). The mRNA was removed by boiling and the second strand was synthesized with DNA polymerase I (19) (New England Biolabs). Hairpin loops at the 3′ ends of the first strand were removed with S1 nuclease (20) (New England Nuclear), and molecules longer than 500 base pairs (bp) were isolated by gel filtration through Sepharose 2B (Pharmacia). Terminal transferase (New England Nuclear) was used to add poly(dC) tails about 20 residues long to the recovered double-stranded (ds) cDNA (21).

Plasmid pBR322 was digested with *Pst* I restriction endo-

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Abbreviations: P-450, cytochrome P-450; P-450$_{C21}$, cytochrome P-450 specific for steroid 21-hydroxylation; P-450$_{pb}$, cytochrome P-450 induced by phenobarbital; ds, double-stranded; bp, base pair(s).

nuclease and tailed with poly(dG). Equimolar amounts of ds cDNA and linearized plasmid were annealed (22) and used to transform *E. coli* strain LE392, which had been rendered competent by a 3-hr incubation in 0.1 M $CaCl_2$ (23).

In an amount yielding about 500 colonies per 100-mm plate, transformed bacteria were plated onto nitrocellulose filters (HATF, Millipore) on L plates containing tetracycline at 10 μg/ml and were incubated overnight at 37°C.

Screening Transformants. An *in situ* immunoassay (24, 25) was used to screen for a clone expressing P-450$_{C21}$ antigenic determinants. Anti-P-450$_{C21}$ serum was adsorbed with boiled cells of *E. coli* strain LE392 (24). Staphylococcal protein A (Pharmacia) was labeled with ^{125}I (New England Nuclear) to a specific activity of 2×10^7 cpm/μg by the chloramine-T method (26). Tetracycline-resistant colonies were replicated onto additional nitrocellulose filters as described by Hanahan and Meselson (27), and the original and replica filters were incubated at 37°C for 24 hr.

The original filters were then stored at 4°C, and the replicas were tested by a modification of the procedure of Young and Davis (25), similar to the method of J. Ravetch and J. Unkeless (personal communication). All incubations were performed at room temperature with gentle shaking. The filters were exposed to chloroform vapor, transferred to individual Petri dishes, and treated with $NaDodSO_4$ and DNase I solutions as described (25). The filters were covered for 1 hr with "incubation buffer": 150 mM NaCl/50 mM Tris·HCl, pH 7.8/0.5% Nonidet P-40 (Sigma)/0.5% sodium deoxycholate/0.1% $NaDodSO_4$/2% calf serum. This was removed and replaced with adsorbed antiserum, in a 1:400 dilution in incubation buffer, plus 1% of a boiled 100× concentrated stationary-phase culture of *E. coli*. After 1 hr, the filters were washed twice for 5 min per wash in incubation buffer and incubated for 1 hr with 5×10^6 cpm per filter of ^{125}I-labeled protein A in incubation buffer containing 1% *E. coli* suspension. The filters were washed twice with each of the following three solutions: 0.5 M NaCl/50 mM Tris·HCl, pH 7.8/0.5% Nonidet-P40, 0.15 M NaCl/50 mM Tris·HCl/0.5% Nonidet-P40/0.1% $NaDodSO_4$, and 0.15 M NaCl/50 mM Tris·HCl/0.5% sodium deoxycholate. The filters were dried in air and autoradiographed overnight at −70°C by using an intensifying screen. Positive clones were selected, purified by streaking, and rescreened with the same technique.

Characterization of Plasmids. Plasmid DNA was prepared from consistently positive clones (28). Recognition sites for restriction endonucleases (all purchased from New England Biolabs and used according to the supplier's instructions) were determined in each cDNA insert; each intact plasmid was studied with several enzymes in order to determine the orientation of the insert. Appropriate restriction fragments were subcloned in the single-stranded phage M13 mp8 or mp9 (29) and their sequences were analyzed by the Sanger–Nicklen–Coulson "dideoxy" method (30), using reagents supplied by New England Biolabs.

RESULTS

Purification of P-450$_{C21}$. P-450$_{C21}$ was purified to homogeneity, as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1*A*), in a yield of 1 mg from 600 g of adrenal glands. The molecular weight as determined by electrophoresis was 52,000; other investigators using the same methodology have obtained M_r values of 47,000 (9) and 52,000 (31). This preparation displayed a difference spectrum characteristic of P-450$_{C21}$ (9) when the substrate 17-hydroxyprogesterone was added (Fig. 1*B*).

Characterization of Antiserum to P-450$_{C21}$. Sera were obtained from a single rabbit before and after immunization with P-450$_{C21}$. These were individually mixed with a sodium cholate extract of adrenal microsomes (9) and antigen–antibody complexes were removed with formalin-fixed *S. aureus*. The supernatants were tested for *in vitro* 21-hydroxylase activity (32). Whereas preimmune serum had no effect, increasing amounts of immune serum removed proportionately greater amounts of 21-hydroxylase activity, with 40 μl of immune serum removing half the activity present in 1 mg of microsomal protein.

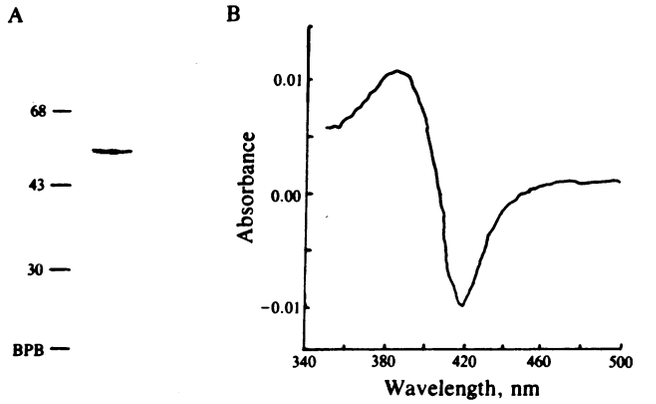

FIG. 1. Purification and characterization of P-450$_{C21}$. (*A*) Material purified by two rounds of chromatography on 8-amino-octylagarose was subjected to electrophoresis on a $NaDodSO_4$/8.5% polyacrylamide gel. M_r standards are bovine serum albumin (68,000), ovalbumin (43,000), and bovine carbonic anhydrase (30,000). The position of the bromphenol blue (BPB) dye is also indicated. (*B*) The spectrum of a 0.18 μM solution of P-450$_{C21}$ was determined in the presence and absence of 30 μM 17-hydroxyprogesterone and the difference is displayed.

Immune serum specifically precipitated a single protein from the products of an *in vitro* translation reaction; this protein migrated with the purified P-450$_{C21}$ used for immunization (Fig. 2*A*).

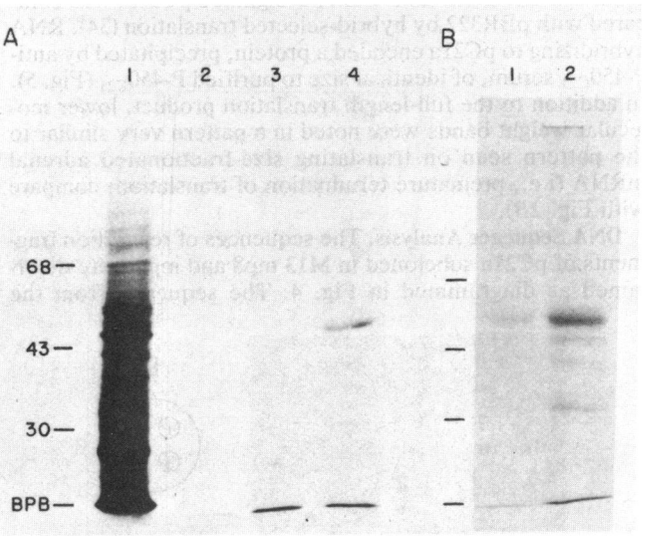

FIG. 2. *In vitro* translation of adrenal mRNA. (*A*) Translation of poly(A)$^+$ mRNA. Lane 1, translation products of mRNA, labeled with [^{35}S]methionine, separated on a $NaDodSO_4$/8.5% polyacrylamide gel, and autofluorographed. Standards are as described for Fig. 1. Lane 2, immunoprecipitation, using immune serum, of a control reaction containing no added mRNA. Lane 3, immunoprecipitation of translation products of adrenal mRNA, using preimmune rabbit serum. Lane 4, immunoprecipitation using anti-P-450$_{C21}$ serum. (*B*) Translation of size-fractionated mRNA. Portions of two adjacent fractions from a 5–25% sucrose gradient were translated and immunoprecipitated as in *A*. Lane 1 represents a heavier fraction than lane 2. Lane 2 is one of two fractions at about 19S with the peak amount of P-450$_{C21}$ mRNA. Note the presence of lower molecular weight translation products in both lanes.

RNA Purification. P-450$_{C21}$-specific mRNA was detected, by *in vitro* translation, predominately in 19S fractions after sucrose gradient sedimentation. Sedimentation values from 18S to 23S have been reported for other P-450 mRNAs (2, 18). While the size-fractionated mRNA was readily translatable, lower molecular weight proteins were precipitated from the *in vitro* translation reaction in addition to the full-length translation product (Fig. 2*B*). These low molecular weight bands were assumed to represent termination of translation at defined points. They were not seen when translating unfractionated mRNA and so were unlikely to be proteolytic degradation products. If mRNA were being degraded during fractionation, there should have been an increased proportion of the low molecular weight bands in translations of slower-sedimenting (smaller) mRNA; instead, in translations of different size fractions, the lower molecular weight bands varied in intensity in proportion to the amount of the full-length translation product. This phenomenon has been ascribed to aggregation of a particular mRNA with itself or with other mRNAs or to unknown contaminants that inhibit translation (33).

cDNA Cloning. One microgram of size-fractionated mRNA was reverse transcribed into ≈100 ng of ds cDNA. Twenty nanograms consisted of molecules longer than 500 bp. After poly(dC) tailing and annealing with 100 ng of linearized vector, this preparation transformed LE392 cells at an efficiency of ≈1000 transformants per ng of ds cDNA.

Approximately 8000 colonies were screened with the *in situ* immunoassay. Two colonies from a single plate consistently bound antiserum and ^{125}I-labeled protein A as detected by autoradiography (Fig. 3*A*). The amount of protein A bound by each colony was equivalent to that bound by less than 10 pg of purified P-450$_{C21}$ (Fig. 3*B*).

These two clones carried plasmids with inserts of identical size and pattern of recognition sites for several restriction enzymes (Fig. 4) and thus are presumably descendents of the same transformant. One clone, designated pC21a, was compared with pBR322 by hybrid-selected translation (34). RNA hybridizing to pC21a encoded a protein, precipitated by anti-P-450$_{C21}$ serum, of identical size to purified P-450$_{C21}$ (Fig. 5). In addition to the full-length translation product, lower molecular weight bands were noted in a pattern very similar to the pattern seen on translating size-fractionated adrenal mRNA (i.e., premature termination of translation; compare with Fig. 2*B*).

DNA Sequence Analysis. The sequences of restriction fragments of pC21a subcloned in M13 mp8 and mp9 were determined as diagrammed in Fig. 4. The sequence from the

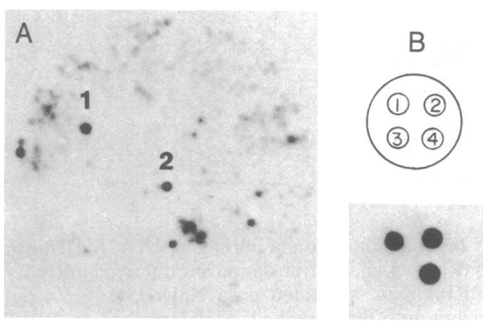

FIG. 3. *In situ* immunologic screening of bacterial colonies. (*A*) First round of screening. Autoradiogram of a replica of 1 of 16 filters after lysis with chloroform and incubation with immune serum and ^{125}I-labeled protein A. Numbers indicate positions of two colonies on this plate selected for further testing. (*B*) Testing of same two clones after purification. Positions 1 and 2 are the clones of *A*; position 3 is a clone carrying a different, uncharacterized plasmid. These colonies were 3 mm in diameter. Position 4, 10 pg of purified P-450$_{C21}$ spotted onto filter prior to chloroform treatment.

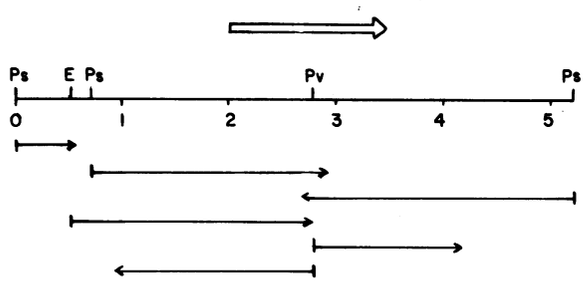

FIG. 4. Map of pC21a. Large arrow indicates direction of transcription in the β-lactamase gene of pBR322. Numbers indicate length in hundreds of bp. Recognition sites for restriction enzymes are indicated: Ps, *Pst* I; E, *Eco*RI; Pv, *Pvu* II. Fragments subcloned in M13 mp8 and mp9 are diagrammed below. Arrows indicate direction of transcription during sequencing reactions.

*Eco*RI site to the 5′ end of the insert was also determined by the Maxam–Gilbert method (35).

The nucleotide sequence of pC21a is displayed in Fig. 6. The cDNA insert of pC21a contains 520 bp, including homopolymer tails of 22 and 9 bp. There is a single open reading frame spanning the entire insert, which is in the same orientation and frame as the β-lactamase gene (22) into which the cDNA has been inserted.

There are no amino acid sequence data for bovine P-450$_{C21}$ available to compare with the amino acid sequence derived from the DNA sequence of pC21a. However, the sequences of the cysteine-containing peptides of the porcine enzyme have recently been determined (36), and in Fig. 7 the sequences of two peptides are compared with the corresponding derived amino acid sequence from pC21a. These two peptides show 90% homology to a single continuous sequence in the bovine enzyme consisting of 30 amino acids; each of the three differences between the bovine and porcine sequences can be accounted for by a single nucleotide substitution. The remaining five cysteine-containing peptides from porcine P-450$_{C21}$ exhibit no homology to the derived amino acid sequence of pC21a and may represent portions of the protein not encoded by pC21a.

Because the amino acid sequence data for porcine P-450$_{C21}$ are limited, we have also compared (Fig. 6) the nucleotide

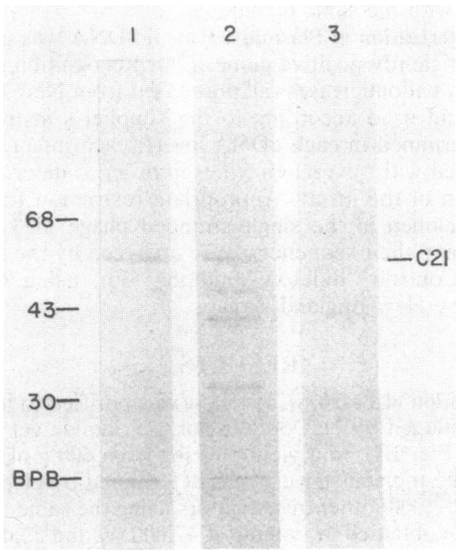

FIG. 5. Hybrid-selected translation by pC21a: Immunoprecipitations of products of *in vitro* translations, as in Fig. 2. The position of P-450$_{C21}$ is indicated. Lane 1, translation of adrenal mRNA. Lane 2, translation of mRNA selected by hybridization to pC21a (compare pattern to Fig. 2*B*). Lane 3, translation of mRNA selected by hybridization of pBR322.

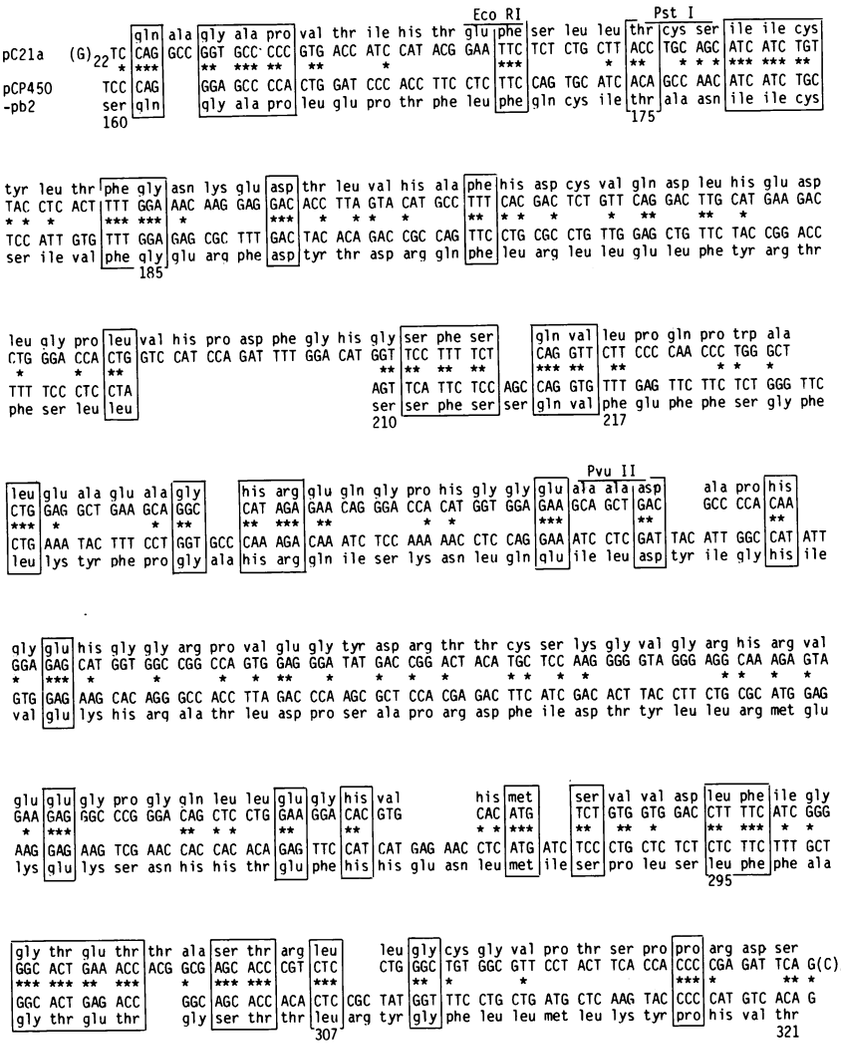

FIG. 6. Nucleotide sequence of pC21a and derived amino acid sequence. The positions of recognition sites of *Eco*RI, *Pst* I, and *Pvu* II are indicated. Also displayed are the nucleotide and derived amino acid sequences of a homologous region of pCP450-pb2 corresponding to amino acids 160–321 of P-450$_{pb}$, determined by Fujii-Kuriyama *et al.* (3). Asterisks indicate matching bases, and boxes, matching amino acids. Gaps have been introduced in both sequences to maximize alignment of homologous areas. The beginning and end of each homologous area are indicated by numbers below the pCP450-pb2 sequence, which refer to amino acids in P-450$_{pb}$.

and derived amino acid sequences of pC21a and a cDNA clone, pCP450-pb2 (3), encoding the P-450 induced by phenobarbital in rat liver [referred to here as P-450$_{pb}$, although there are in fact two closely related isozymes (1, 3)]. Significant homology is found between pC21a and the region of pCP450-pb2 encoding amino acids 160–321 of P-450$_{pb}$, which is approximately the middle third of the protein. This homology is concentrated in three limited areas, corresponding to amino acids 175–185, 210–217, and 295–307 in P-450$_{pb}$. There is approximately 65% nucleotide homology between pC21a and pCP450-pb2 within these areas. Overall, there is 35% nucleotide sequence homology, and 25% of the amino acids encoded by pC21a match those of pCP450-pb2.

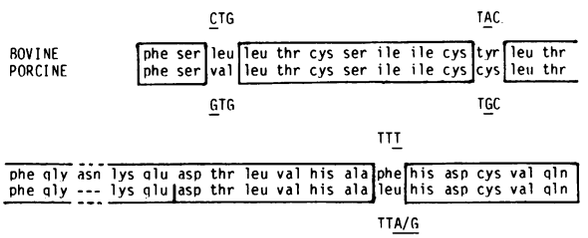

FIG. 7. Comparison of partial amino acid sequence of porcine P-450$_{C21}$ (36) with the corresponding sequence derived from the nucleotide sequence of pC21a (in Fig. 6, this region is immediately 3′ to the *Eco*RI site). Matching amino acids are boxed. Hypothetical nucleotide substitutions that could account for the observed differences in amino acid sequence are shown with underlines. The gap in the porcine sequence indicates an undetermined residue. The division between the two porcine peptides is indicated by the short vertical line.

DISCUSSION

We have isolated a cDNA clone, pC21a, that causes *E. coli* to express P-450$_{C21}$ antigenic determinants, presumably on a fusion protein encoded by a pBR322 β-lactamase gene containing a 500-bp cDNA insert. The insert in pC21a has been identified as a part of the P-450$_{C21}$ structural sequence because it hybridizes mRNA encoding a protein of the same size as P-450$_{C21}$ that is precipitated by anti-P-450$_{C21}$ serum. The DNA sequence of pC21a confirms this identification, because the derived amino acid sequence shows high homology to the sequences of two peptides isolated from porcine P-450$_{C21}$.

In principle, another adrenal P-450 might be sufficiently similar to P-450$_{C21}$ to crossreact antigenically and/or confuse the results of the hybrid-selected translation experiment and the sequence comparisons. Of the other known adrenal cytochromes P-450, P-450$_{scc}$, and P-450$_{11\beta}$ are mitochondrial in origin, do not crossreact antigenically with P-450$_{C21}$ (9), and are unlikely to be contaminating the purified microsomal protein preparation used for immunizations. P-450$_{17\alpha}$, a microsomal protein, is reported to be about 2000 daltons lighter than P-450$_{C21}$ and has a quite different amino acid composition (31). Thus, none of these cytochromes P-450 could be confused with P-450$_{C21}$.

The nucleotide and amino acid sequence homologies between pC21a and pCP450-pb2, 35% and 25%, respectively,

while significant, are limited; they are similar to the homologies between rat growth hormone and prolactin (39% and 24%) (37). Several regions of P-450 are in general more highly conserved, including, in rat $P\text{-}450_{pb}$, amino acids 145–158 (38), 346–358 (39), and 435–445 (36). None of these are within the area homologous to pC21a. Outside such regions, the homologies between different cytochromes P-450 seem to vary considerably. For example, cDNA clones encoding the two forms of rat $P\text{-}450_{pb}$ (1) differ in less than 3% of their sequence (3). A similar clone showed no hybridization to mRNA encoding two cytochromes P-450 induced, respectively, by methycholanthrene and isoafrole, which, however, were immunochemically related to each other (40). This clone ("R17," kindly provided by Milton Adesnik, New York University Medical School) also fails to hybridize in "northern" blots (41) to bovine adrenal mRNA, even under low-stringency conditions (50°C, 0.5 M NaCl) (unpublished observations). This is consistent with the 35% nucleotide homology noted between pC21a and pCP450-pb2. Nucleic acid sequence data are clearly indispensable in the study of relationships between cytochromes P-450, and they show in this case that although $P\text{-}450_{C21}$ and $P\text{-}450_{pb}$ are not part of a single "family" of genes, they are members of the same "superfamily" (42).

In contrast to the often limited homology between different cytochromes P-450, the structure of a P-450 with a particular substrate specificity does appear to be highly conserved in different species. Cloned cDNA for mouse methylcholanthrene-induced P-450 hybridizes to human genomic DNA and has been used to isolate genomic clones containing two separate genes (43). Limited comparison of bovine and porcine $P\text{-}450_{C21}$ reveals 90% homology of amino acid sequences. Because the bovine (31) and porcine (36) enzymes also have very similar amino acid compositions, the complete sequences will probably show a similar high level of homology. It seems likely that pC21a will hybridize to the homologous genes in other species, including man; it should therefore be a useful reagent in studying the molecular basis of 21-hydroxylase deficiency.

We thank Nancy Maruyama and Brenda Sirovich for excellent technical assistance. We had helpful discussions with many people, including Michael Sheffery, Walter Miller, Soo-Young Yang, Bob Knowles, Allan Oliff, and John Baxter. In particular, John Fiddes and Jeffrey Ravetch gave valuable advice on the application of *in situ* immunologic screening techniques. Members of the laboratory of Paul Marks and Richard Rifkind were generous with equipment and reagents. Milton Adesnik provided the R17 clone; he, Michael Sheffery, Ursula Mueller-Eberhardt, Abraham White, and Marge Boeck critically read the manuscript. P.C.W. was supported by the Fairchild Foundation and is a Fellow in Biomedical Research of the Norman and Rosita Winston Foundation. This work was supported by Grants CA-22507 and CA-08748 from the National Cancer Institute and HD00072 and RR47 from the National Institutes of Health.

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