S. (1982) *Nature (London) 299*, 793-797.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature (London) 302*, 528-532.
Ratnam, M., & Lindstrom, J. (1984) *Biochem. Biophys. Res. Commun. 122*, 1225-1233.
Ratnam, M., Le Nguyen, D., Rivier, J., Sargent, P., & Lindstrom, J. (1986a) *Biochemistry* (in press).
Ratnam, M., Sargent, P., Sarin, V., Fox, J., Le Nguyen, D., Rivier, J., Criado, M., & Lindstrom, J. (1986b) *Biochemistry* (in press).
Smart, L., Meyers, H.-W., Hilgenfeld, R., Saenger, W., & Maelicke, A. (1984) *FEBS Lett. 178*, 64-68.
Souroujon, M., Mochly-Rosen, D., Gordon, A., & Fuchs, S. (1983) *Muscle Nerve 6*, 303-311.
Souroujon, M., Neumann, D., Pizzighella, S., Safran, A., & Fuchs, S. (1986) *Biochem. Biophys. Res. Commun. 135*, 82-89.
Tzartos, S., & Lindstrom, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755-759.
Tzartos, S., Rand, D., Einarson, B., & Lindstrom, J. (1981) *J. Biol. Chem. 256*, 8635-8645.
Tzartos, S., Langeberg, L., Hochschwender, S., Swanson, L., & Lindstrom, J. (1985) *Neuroimmunology 10*, 235-253.
White, M. (1985) *Trends Biochem. Sci. (Pers. Ed.) 10*, 364-368.
Wilson, P., Gershoni, J., Hawrot, E., & Lentz, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2553-2557.
Wilson, P., Lentz, T., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790-8794.
Wolosin, J., Lyddiatt, A., Dolly, O., & Barnard, E. (1980) *Eur. J. Biochem. 109*, 495-505.
Young, E. F., Ralston, E., Blake, J., Ramachandran, J., Hall, Z. W., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626-630.

Bovine Steroid 21-Hydroxylase: Regulation of Biosynthesis†

Maliyakal E. John,‡ Takashi Okamura,‡,§ Albert Dee,‡ Beverly Adler,‡ Manorama C. John,‡ Perrin C. White,‖ Evan R. Simpson,‡ and Michael R. Waterman*,‡

Departments of Biochemistry and Obstetrics and Gynecology and The Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Health Science Center at Dallas, Dallas, Texas 75235, and Laboratory of Human Immunogenetics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Received October 10, 1985; Revised Manuscript Received February 4, 1986

ABSTRACT: A recombinant cDNA clone, PBC21-1, specific for bovine steroid 21-hydroxylase cytochrome P-450 (P-450$_{C21}$) was identified in a bovine adrenocortical cDNA library, and this identity was confirmed by nucleotide sequencing which revealed significant amino acid homology (77%) with human P-450$_{C21}$ cDNA. The pBC21-1 insert is 1.7 kilobases in length and includes a 1128 base pair region that encodes the C-terminal 376 amino acids of bovine P-450$_{C21}$ as well as 535 base pairs of 3′-untranslated sequence. A novel feature of this insert is a 20 base pair intervening sequence near the 5′ end, apparently the result of an aberrant splicing event. Northern blot analysis reveals that bovine P-450$_{C21}$ is encoded by two transcripts, 2.3 and 2.0 kilobases in length which are detected in adrenal cortical RNA. Bovine liver, heart, kidney, and corpus luteum do not contain detectable P-450$_{C21}$ transcripts. Regulation of P-450$_{C21}$ gene expression by adrenocorticotropin was investigated with pBC21-1 and bovine adrenocortical cells in primary, monolayer culture. Treatment with ACTH or analogues of cAMP increases the steady-state levels of P-450$_{C21}$ RNA in such cell cultures. In vitro transcription run-on assays suggest that this increase is, at least in part, due to the enhanced transcriptional activity of the P-450$_{C21}$ gene.

Biosynthesis of a number of physiologically active compounds such as glucocorticoids, mineralocorticoids, sex hormones, 1,25-dihydroxycholecalciferol, and bile acids requires mixed-function oxidase activity, catalyzed by various forms of cytochrome P-450 (Waterman et al., 1986). The particular forms of cytochrome P-450 localized in steroidogenic tissues (i.e., adrenal cortex, ovary and testis) are under both acute and chronic regulation by peptide hormones [either adrenocorticotropin (ACTH),[1] follicle-stimulating hormone (FSH), or luteinizing hormone (LH)], which thereby control the synthesis of steroid hormones, which in turn regulate a wide variety of metabolic and biosynthetic activities. In the adrenal cortex two mitochondrial forms of cytochrome P-450, cholesterol side-chain cleavage cytochrome P-450 (P-450$_{scc}$) and 11β-hydroxylase cytochrome P-450 (P-450$_{11\beta}$), and two microsomal forms of cytochrome P-450, 17α-hydroxylase cytochrome P-450 (P-450$_{17\alpha}$) and 21-hydroxylase cytochrome P-450 (P-450$_{C21}$), are components of the steroidogenic pathway

† This work was supported by U.S. Public Health Service Grants AM 28350, HD 13234, and HD 11149 and Grant I-624 from The Robert A. Welch Foundation.
‡ University of Texas Health Science Center at Dallas.
§ Permanent address: First Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan.
‖ Memorial Sloan-Kettering Cancer Center.

[1] Abbreviations: P-450$_{C21}$, cytochrome P-450 specific for steroid 21-hydroxylation; P-450$_{scc}$, cytochrome P-450 specific for side-chain cleavage reaction of cholesterol; P-450$_{17\alpha}$, cytochrome P-450 specific for steroid 17α-hydroxylation; P-450$_{11\beta}$, cytochrome P-450 specific for steroid 11β-hydroxylation; SDS, sodium dodecyl sulfate; SSC, 1 × SSC = 0.15 M NaCl/15 mM sodium citrate; DTT, dithiothreitol; ACTH, adrenocorticotropin; RNase, ribonuclease; bp, base pair; kb, kilobase.

0006-2960/86/0425-2846$01.50/0

leading from cholesterol to cortisol. P-450$_{C21}$ catalyzes the 21-hydroxylation of progesterone and 17α-hydroxyprogesterone, ultimately leading to steroid products such as cortisol, corticosterone, and aldosterone. A deficiency in the activity of any one of these adrenocortical steroid hydroxylases results in disorders referred to collectively as congenital adrenal hyperplasia (New et al., 1982). In humans, the most common congenital adrenal hyperplasia is the impairment of 21-hydroxylation mediated by P-450$_{C21}$. Thus, the gene structure as well as its regulation of expression by ACTH is relevant to the understanding of the basis of congenital adrenal hyperplasia, as well as being of general interest with respect to regulation of eukaryotic gene expression by peptide hormones.

In the present study we have isolated and characterized by nucleotide sequencing a recombinant cDNA clone specific for P-450$_{C21}$, pBC21-1, and thus derived the partial amino acid sequence of bovine P-450$_{C21}$. Using bovine adrenocortical primary monolayer cell cultures and this recombinant DNA clone, we have examined the effect of ACTH on P-450$_{C21}$ gene expression. These studies suggest that ACTH, via cAMP, modulates transcriptional activity of the P-450$_{C21}$ gene, resulting in the accumulation of P-450$_{C21}$ mRNA.

Materials and Methods

A bovine adrenocortical cDNA library (John et al., 1984; Okamura et al., 1985) was screened with a 520-bp insert of the bovine P-450$_{C21}$ hydroxylase specific clone, pC21a (White et al., 1984a). One of the positive clones, (pBC21-1) containing the longest insert (1.7 kb) was used in subsequent studies. A restriction map of the insert of pBC21-1 was obtained by the method of Smith and Birnstiel (1976). DNA sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977) using [^{35}S]deoxyadenosine 5′-(α-thiotriphosphate) (New England Nuclear; 500 Ci/mmol). Preparation and manipulation of adrenocortical monolayer cell cultures have been described previously (Funkenstein et al., 1983). Autoradiographic signals were quantified by a scanning densitometer with an attached computing integrator (Transidyne 2955).

RNA Isolation and Blotting. RNA from cell cultures and fresh tissue was isolated in the presence of guanidinium thiocyanate (Fluka) (Chirgwin et al., 1979) or by the lithium chloride precipitation method (Cathala et al., 1983). Polysomes were isolated from cell cultures by lysing the cells in 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 10 mM KCl, 0.25 M sucrose, 100 μg/mL heparin, 0.1% Triton X-100, and 0.1% diethyl pyrocarbonate (Sigma). Nuclei and mitochondria were removed by centrifugation and total polysomes isolated by centrifugation at 45 000 rpm for 3 h at 4 °C. Polysomal RNA was isolated by the method of Palmiter (1974). RNA transfer to nitrocellulose paper (Schleicher & Schuell) was accomplished after electrophoresis on agarose/formaldehyde gels or electrophoresis on agarose gels following incubation with glyoxal (John et al., 1985). DNA was labeled with phosphorus-32 by using a nick translation kit from Bethesda Research Labs (BRL) and [^{32}P]dCTP (New England Nuclear). Plasmid isolation, restriction endonuclease (BRL, Biolabs, and P-L Biochemicals) digestions, and agarose and polyacrylamide gel electrophoresis were all carried out as described by Maniatis et al. (1982). Filter prehybridizations were carried out in 4 × SSC, 10 × Denhardt's solution, 1 mM EDTA, 0.5% SDS, 100 μg/mL sonicated denatured salmon sperm DNA, and 10 μg/mL poly(adenylic acid) at 65 °C for about 12 h. Hybridizations were in the same solution except that ^{32}P-labeled probe (0.5–1 × 10^6 cpm/mL) was added, and incubations were carried out for between 12 and 18 h. Low stringency washings of the filters were carried out in 3 × SSC, 1 mM EDTA, and 0.1% SDS at room temperature for 20 min. The washing was repeated in 2 × SSC (2 times) at 50 °C and then in 1 × SSC (2 times) at 50 °C as above. High stringency washings were in 0.5 × SSC followed by 0.1 × SSC at 50 °C, 2 times each. All washing solutions contained 1 mM EDTA and 0.1% SDS. Hybridized ^{32}P-labeled probe was removed from filters by pouring boiling 0.1 × SSC and 0.1% SDS over the filters and leaving them in this solution for about 4 h. If necessary this step was repeated before reusing the filter.

In Vitro Transcription Run-On Assays. Nuclei were isolated from cell cultures by homogenizing the cells in 0.25 M sucrose, 3 mM $CaCl_2$, 10 mM Tris (pH 8.0), 2 mM magnesium acetate, 0.1 mM EDTA, 0.1% Triton X-100 (Sigma), and 1 mM DTT. The homogenate was mixed with 2 volumes of 2.3 M sucrose, 5 mM magnesium acetate, 10 mM Tris (pH 8.0), 0.1 mM EDTA, and 1 mM DTT. The mixture was then centrifuged (128000g for 30 min, 4 °C) through a 2 M sucrose cushion in the above buffer. The nuclei were stored in 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT, and 50 mM Tris (pH 8.0) at −70 °C. Transcription run-on assays were carried out with 100–200 μCi of [α-^{32}P]GTP in a solution containing 0.5 mM ATP, CTP, and UTP, 0.025 mM *S*-adenosylmethionine (Sigma), 120 mM KCl, 5 mM magnesium acetate, 2.5 mM DTT, 25 mM Tris (pH 8.0), 0.05 mM EDTA, 12.5% glycerol, and (5–15) × 10^6 nuclei for 30 min at 25 °C. RNA was then isolated by phenol–SDS extraction or by cesium chloride centrifugation. Plasmid DNA was bound to nitrocellulose by the sodium iodide method (Bresser & Gillespie, 1983). Hybridizations [(3–5) × 10^6 cpm] were carried out in 50% formamide, 5 × SSC, 0.1% SDS, 1 mM EDTA, 2 mg/mL each of BSA, poly(vinylpyrrolidine), and Ficoll (Sigma), 10 μg/mL poly(adenylic acid), and 50 μg/mL sonicated denatured salmon sperm DNA at 37 °C for 90 h. Filters were washed with 2 × SSC, 0.1% SDS, and 1 mM EDTA at room temperature and then incubated with 10 μg/mL RNase for 10 min at 37 °C. They were further washed in 0.3 M NaCl at room temperature, dried, and autoradiographed by using intensifying screens (Du Pont).

Results

Identification and Characterization of cDNA Clones. A recombinant cDNA clone, pC21a, specific for bovine P-450$_{C21}$ was identified by in situ immunologic screening of bacterial colonies (White et al., 1984a). The cDNA insert from this clone (520 bp) was used to identify corresponding clones from bovine adrenal cDNA libraries constructued by using Okayama and Berg vectors (John et al., 1984; Okamura et al., 1985). The clone containing the longest insert (1.7 kb), pBC21-1, was used in the studies described herein. A restriction map and the sequencing strategy of the insert are shown in Figure 1. The complete nucleotide sequence (1683 bp) of the insert of pBC21-1 is shown in Figure 2A. Computer analysis of this sequence showed that the second reading frame is the longest one having a stop codon at position 1151 and as such can encode 383 amino acids. Comparisons were made between amino acids derived from this reading frame with those obtained from (a) a portion of the bovine P-450$_{C21}$ gene (Chung et al., 1985), (b) several porcine P-450$_{C21}$ peptides (Yuan et al., 1983; Bienkowski et al., 1984), and (c) the human P-450$_{C21}$ cDNA sequence (P.C. White, unpublished results). A high degree of homology (76%) was observed between the 346 carboxy terminal amino acids of bovine and human P-450$_{C21}$. Nevertheless, the 36 N-terminal residues derived from pBC21-1 contained only three matches with the corresponding human amino acid sequence. However, at the nucleotide level

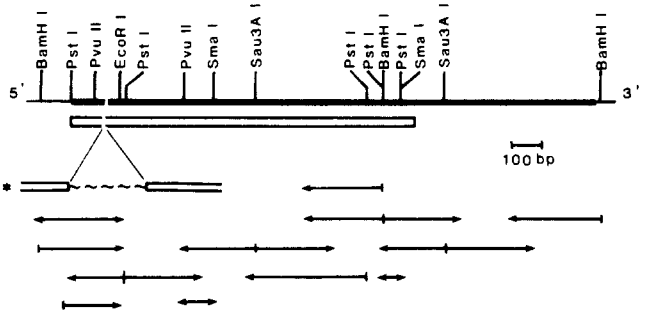

FIGURE 1: Restriction endonuclease map and sequencing strategy of the P-450$_{C21}$ cDNA insert from pBC21-1. The open box indicates the coding region and the filled box the complete insert. The thin lines at either end of the complete insert represent vector DNA. The 5′ and 3′ orientations are indicated. The coding region of the insert is interrupted (asterisk) by a 20 bp intervening sequence (see Figure 2B). Arrows indicate extent and direction of sequencing.

there were 79 matches and only 10 mismatches in this segment. Moreover, there was a 20 bp region in the bovine sequence that was absent in the human sequence (Figure 2). When amino acids derived from all three reading frames for the first 109 bp of pBC21-1 were compared with this region of the human sequence, it was found that the third reading frame rather than the second reading frame matches well (86%) with the human sequence provided the above-mentioned 20 bp fragment is not considered. Moreover, the known sequence of porcine P-450$_{C21}$ cysteinyl peptide T-29 (Yuan et al., 1983) matches with the amino acids derived from the third reading frame with one exception (Figure 2B). However, the third reading frame contains a stop codon beginning at nucleotide position 114. These apparent discrepancies were resolved when we compared the 5′-cDNA sequence with the corresponding bovine genomic sequence (Walter L. Miller, personal communication). From these limited sequence comparisons, it was found that residues GAG at position 87–89 are the 3′ end of an exon and residues CGC at position 110–112 are the 5′ end of the following exon. In the gene, an intron of about 200 bp lies between these two exons (Walter L. Miller, personal communication). The 20 bp segment in our cDNA is identical with the sequence of the 3′ end of this intron. As can be seen from Figure 2B the consensus splice sequences AG and CAG (Breathnach et al., 1978; Sharp, 1981) are at the 3′ ends of the exon and intron sequences, respectively. Hence, we assume that, in a majority of cases, the splicing should have occurred at this site with the removal of the 20 bp sequence. Screening of other cDNA clones to evaluate the frequency of this unusual splicing event in P-450$_{C21}$ RNA is under way.

Thus, when the sequence of bovine P-450$_{C21}$ is read beginning at the 5′ end of pBC21-1 in the third reading frame and is shifted to the second reading frame at position 110 following removal of the 20 bp intronic sequence, 86% homology with the coding region of the human P-450$_{C21}$ cDNA is obtained, as is 77% homology with the human amino acid sequence. By comparison with the human sequence which is 494 amino acids long, it is estimated that about 115 amino acids at the N-terminus of the protein are missing from our bovine sequence. Limited amino acid sequence data (Amor et al., 1985) available for mouse P-450$_{C21}$ show about 63% homology with the bovine protein. Similarly, a partial porcine sequence (Bienkowski et al., 1984) shows 87% homology with the reported bovine sequence.

A hexanucleotide, AATAAA, is found 16 bp upstream from the poly(A) tail and is presumed to be the poly(A) addition signal. Comparison of amino acid sequences of a number of forms of cytochrome P-450 has led to the identification of a segment in the carboxy-terminal region, designated HR2, which shows considerable conservation throughout the evolutionary time scale (Grotoh et al., 1983; Morohashi et al., 1984). The HR2 sequence contains a cysteine residue, the thiol group of which is thought to be the fifth ligand of the heme iron (Kawajiri et al., 1984). Amino acid residues of the HR2 peptides from selected steroid hydroxylases are compared in Figure 3. Bovine, mouse, porcine, and human P-450$_{C21}$ hydroxylases show striking homology in this sequence (≅95%).

Multiple Transcripts and Tissue Specificity of P-450$_{C21}$. The clone pBC21-1 hybridizes to two transcripts in RNA from bovine adrenal cortex (Chung et al., 1985), which differ by about 300 bases in length. The longer, minor transcript is approximately 2300 bases while the shorter, major one is about 2000 bases long (Figure 4). It is not known from which transcript the cDNA described in Figure 2 arises. Steroid hydroxylases exhibit tissue-specific expression. Thus, P-450$_{11\beta}$ is expressed only in the adrenal cortex, while the P-450$_{scc}$ is common to adrenal cortex and corpus luteum (John et al., 1984, 1985). Extraadrenal 21-hydroxylation of progesterone has been reported in a number of species (Acevedo et al., 1963; Winkel et al., 1980; Dieter et al., 1982a). However, the identities of these enzymes and their relationship to the adrenal P-450$_{C21}$ remain to be established. To examine the tissue specificity of P-450$_{C21}$, RNA isolated from bovine heart, corpus luteum, and adrenal cortex was blotted to nitrocellulose paper and probed for the presence of P-450$_{C21}$ transcripts. As seen in Figure 5, only adrenal cortex contained RNA for P-450$_{C21}$. When the same blot was probed for P-450$_{scc}$ using a specific clone, pBSCC-2 (John et al., 1984), both corpus luteum and adrenal cortex RNA showed hybridization. Thus, it is clear that heart and corpus luteum do not contain detectable levels of transcripts that are homologous to adrenal P-450$_{C21}$ RNA. We also examined kidney and liver for the expression of homologous P-450$_{C21}$ transcripts. A Northern filter containing RNA from kidney, liver, and adrenal cortex was hybridized to the insert of pBC21-1, and binding only to adrenocortical RNA was observed (Figure 6). A few plasmids have been isolated from our cDNA library which hybridize with RNA from a number of tissues. By restriction mapping and differential hybridization studies, we have established that they do not correspond to steroid hydroxylases (unpublished results). To check the integrity of the kidney and liver RNA used in Figure 6, the insert of one such plasmid (pBARP-B11) was used as a control. After removal of the [^{32}P]P-450$_{C21}$ probe by washing, the blot was rehybridized to the ^{32}P-labeled insert of pBARP-B11. This plasmid shows hybridization to an RNA band about 18S in size in all three tissues which demonstrates both presence and integrity of kidney and liver RNAs (Figure 6).

pBC21-1 cross-hybridizes with rat, rabbit, and human adrenal RNA (Figure 7). In all these three species the prominent P-450$_{C21}$ RNA size is about 2.0 kb, the same as that of bovine. Both rat and rabbit RNAs show weak hybridization to a second band at about 2.3 kb in length while human RNA shows a single band as noted previously (Carroll et al., 1985).

Previous work in this laboratory has demonstrated by in vitro translation and immunoisolation that ACTH treatment increases the level of translatable RNA for P-450$_{C21}$ in bovine adrenocortical cell cultures (Funkenstein et al., 1983). When RNA isolated from ACTH-treated and control cells was probed for P-450$_{C21}$ transcripts, an increase was observed upon ACTH treatment (Figure 8A). The analogue of cAMP, dibutyryl-cAMP, was also able to increase the P-450$_{C21}$ transcript concentration in cell cultures (Figure 8A). Slot blot

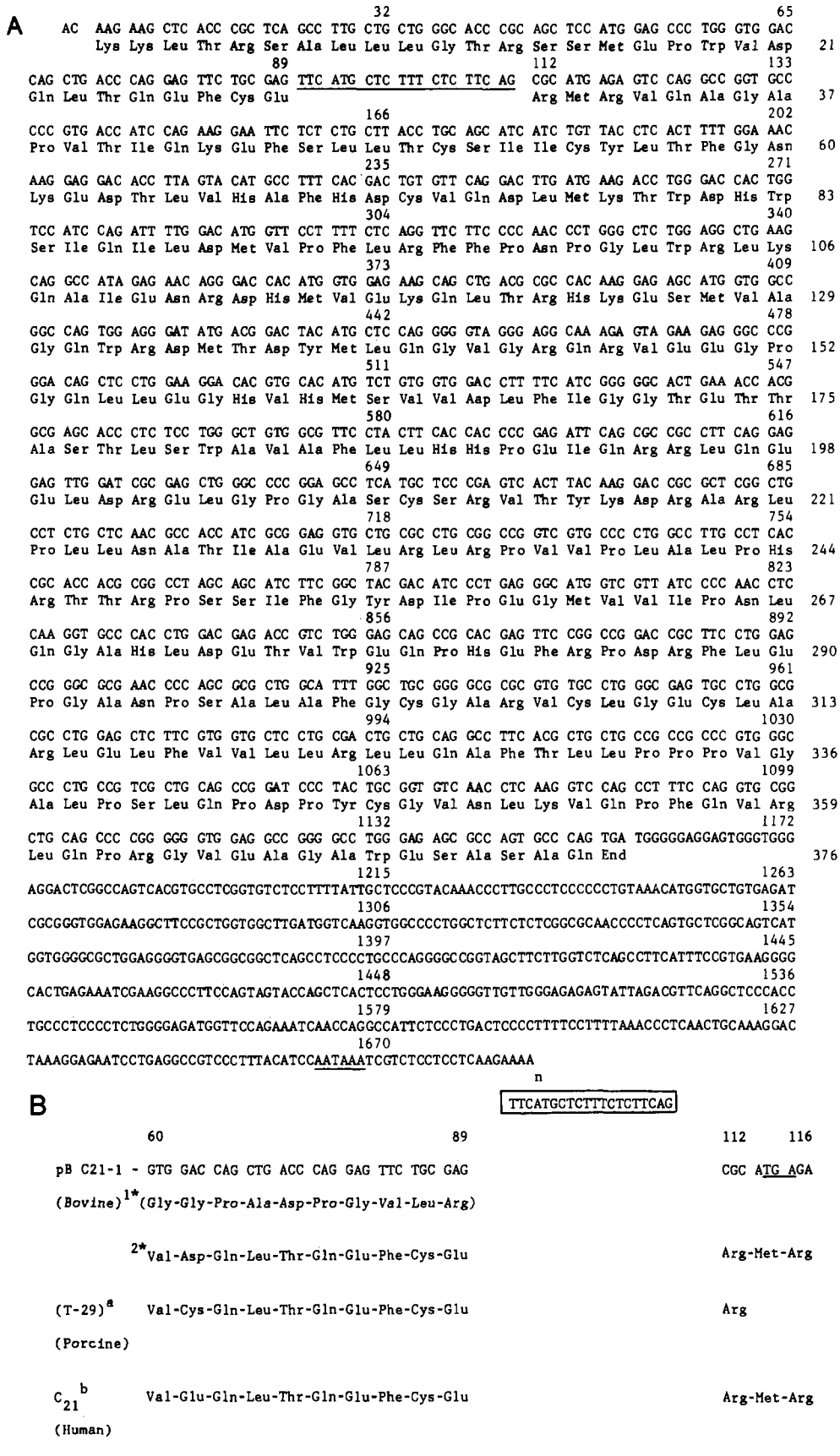

FIGURE 2: (A) Partial nucleotide sequence with derived amino acid sequence for bovine P-450$_{C21}$. Nucleotides are numbered at the top of each line and amino acids at the right margin. Since the sequence lacks about 115 amino acids at the N-terminus, the numbering is arbitrary. The hexanucleotide, AATAAA, and the 20-base intron are underlined. (B) Presence of an intervening sequence in pBC21-1. A 20-bp intervening sequence (in box) with 5′ and 3′ flanking exonic sequences. (1*) Amino acids derived from the second reading frame (in parentheses) starting with a G at position 59 (not shown). This sequence is not homologous with porcine or human P-450$_{C21}$. Note the stop codon at position 114 (underlined). (2*) Amino acids derived from the third reading frame starting from position 60. Numbers are nucleotide positions from (A). Note the homology with the analogous porcine and human sequences. In human P-450$_{C21}$ the 20 bp region is not present. (a) Cysteine containing peptide, T-29. Data from Yuan et al. (1983). (b) Data from P. C. White et al. (unpublished results).

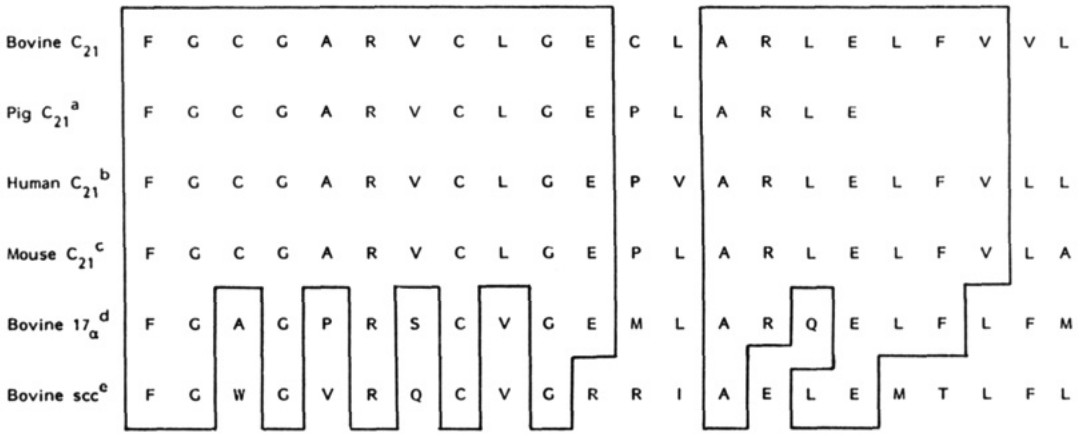

FIGURE 3: Comparison of the conserved heme binding peptide (HR-2) of steroid hydroxylases. Boxed region shows 100% matches. (a) Sequence from peptides T.37 and *Tsa*23 (Yuan et al., 1983). Comparison with our sequence shows that these two peptides are contiguous. (b) P. C. White (unpublished results). Cysteine at position 428. (c) Amor et al. (1985). (d) Zuber et al. (1986). Cysteine at position 442. (e) Morohashi et al. (1984). Cysteine at position 461.

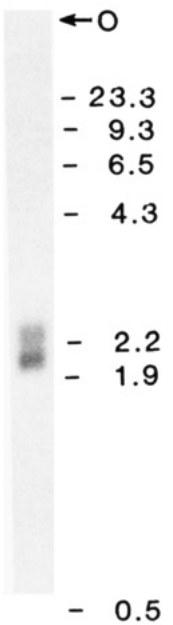

FIGURE 4: Multiple transcripts for bovine P-450$_{C21}$. Bovine adrenocortical poly(A+) RNA (5 μg) was transferred to nitrocellulose paper after electrophoresis on a 1.25% agarose/formaldehyde gel. The blots were hybridized (1×10^6 cpm/mL) with nick-translated insert of plasmid, pBC21-1 (2×10^8 cpm/μg). It was then washed under high stringency conditions. Autoradiography was for 3 h with intensifying screens. A *Hin*dIII digest of λDNA was used as size markers.

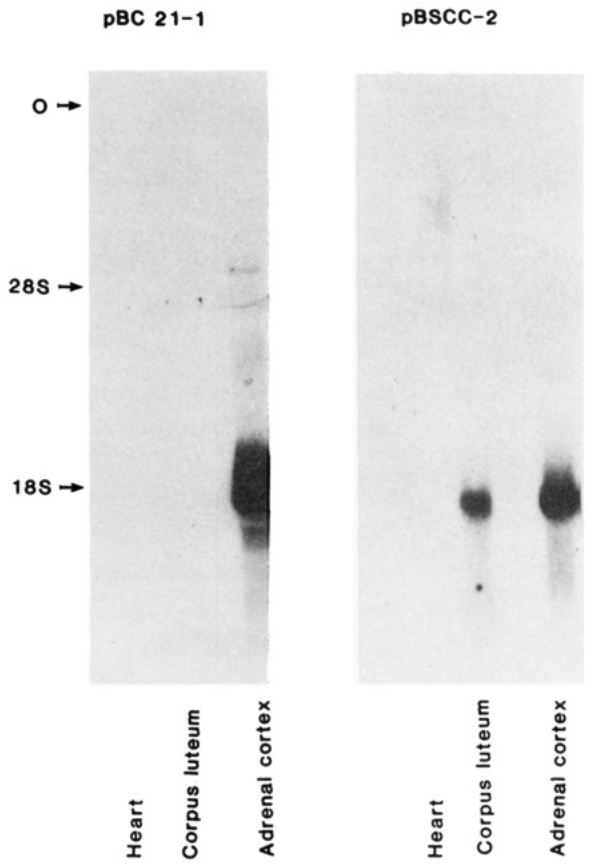

FIGURE 5: Tissue-specific expression of P-450$_{C21}$. A total of 20 μg each of RNA from bovine corpus luteum, heart, and adrenal cortex was size-fractionated on agarose/formaldehyde gels and transferred to nitrocellulose. The filter was hybridized to ^{32}P-labeled insert of pBC21-1 (left panel) as described for Figure 4. The broad hybridization signal indicates hybridization to both the 2 and 2.3 kb bands. The 18S and 28S RNA positions were detected by acridine orange staining of the gel. The origin (O) is marked. The filter was then washed to remove the ^{32}P-labeled probe and rehybridized (right panel) to the ^{32}P-labeled insert of the P-450$_{scc}$ specific clone, pBSCC-2 (John et al., 1984) using the same conditions. Hybridization is seen to both corpus luteum and adrenocortical RNA.

analysis of the temporal pattern of induction by ACTH and dibutyryl-cAMP shows that the increase in P-450$_{C21}$ transcript level is detectable between 6 and 8 h after the initiation of treatment. Quantification of the autoradiographic signals by densitometric scannings indicate a 3–5-fold increase in the steady-state levels of P-450$_{C21}$ RNA (data not shown). The increase in transcript level in response to ACTH treatment can also be observed in isolated polysomes (Figure 8B), indicating that the additional transcripts are functional.

Regulation of the P-450$_{C21}$ Gene at the Transcriptional Level. Modulation of P-450$_{C21}$ gene expression by ACTH was investigated in transcription run-on assays using nuclei isolated from untreated and treated cells. In this type of assay the RNA chains initiated in vivo are allowed to elongate in vitro, and quantification of radiolabeled P-450$_{C21}$ transcripts is achieved by hybridization to pBC21-1. The results of assays carried out with nuclei from ACTH-treated and control cells are shown in Figure 9. Densitometric scannings of these results show only a modest increase of 2–3-fold. On the other hand, the increase in steady-state levels of RNA from a number of cell cultures by Northern and dot blot analysis shows a P-450$_{C21}$ RNA increase in the range of 7–10-fold at 24 h (see Figure 8; quantitation not shown). Pulse-labeling the cells using [^{3}H]uridine followed by determination of the

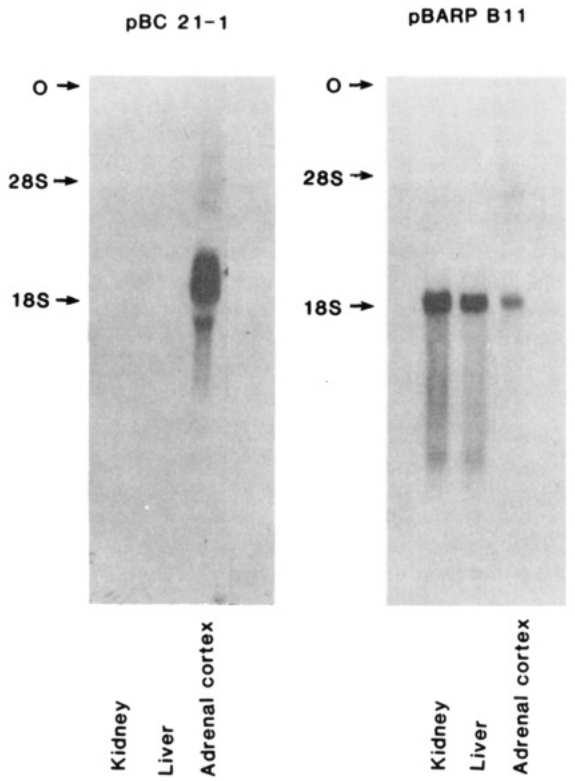

FIGURE 6: Adrenal P-450$_{C21}$ homologous transcripts are not expressed in nonadrenal bovine tissues. A total of 15 μg each of poly(A+) RNA from kidney and liver and 25 μg of total RNA (corresponding to about 0.5 μg of poly(A+) RNA from adrenal cortex) were size-fractionated on agarose/formaldehyde gels and transferred to nitrocellulose. The filter was hybridized to the nick-translated insert of pBC21-1 and washed under low stringent conditions (left panel). The filter was then strip washed and rehybridized to the nick-translated insert of a control plasmid, pBARP-B11 (right panel). All three tissue RNA species show hybridization.

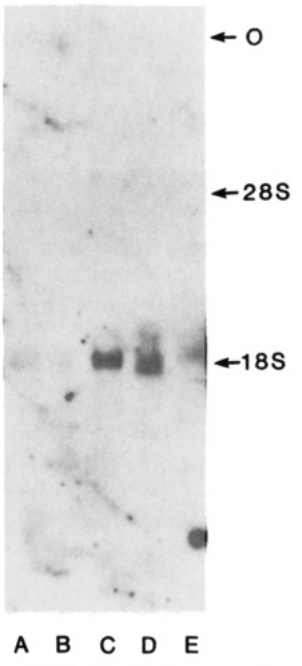

FIGURE 7: P-450$_{C21}$ RNA sizes in human, rat, and rabbit adrenal. Northern blotting and hybridization conditions were similar to those described for Figure 4. Following hybridization the blot was washed 2 times in 2 × SSC, 0.1% SDS, and 1 mM EDTA, 50 °C, and 1 time in 1 × SSC followed by one wash in 0.5 × SSC for 15 min at 50 °C. Autoradiography was for 48 h at −70 °C with intensifying screens. Lanes A and B, 25 μg of total and 25 μg of poly(A−) RNA of human fetal adrenal, respectively; lane C, 2 μg of poly(A+) RNA from human fetal adrenal; lane D, 25 μg of rabbit adrenal RNA; lane E, 25 μg of rat adrenal RNA.

relative stability of P-450$_{C21}$ RNA in the presence and absence of ACTH shows no significant changes in stability induced by ACTH (V. Boggaram, unpublished results). The discrepancy in the fold increase in P-450$_{C21}$ mRNA between steady-state levels and transcription measurements could be due to low rates of P-450$_{C21}$ transcription. Difficulty in measuring low rates of transcription have also been encountered by others in different systems (Mayo & Palmiter, 1981; Israel & Whitlock, 1984). Alternatively ACTH may induce a short burst of transcription that is subsequently attenuated. A similar phenomenon is observed for prolactin gene transcription induced by epidermal growth factor (Murdoch et al., 1982). The transcriptional activation of P-450$_{C21}$ gene was also probed by using the transcriptional inhibitor actinomycin D. Cell cultures were treated with actinomycin D (1 μg/mL of medium) plus ACTH, or ACTH alone while a third set contained no modulators. After 24 h of incubation RNA was isolated and probed for P-450$_{C21}$ transcripts. Actinomycin D inhibited the ACTH-induced increase of P-450$_{C21}$ transcripts (not shown), further implicating the role of ACTH in regulation of the P-450$_{C21}$ gene at the transcriptional level. A similar effect was also obtained for another adrenocortical steroid hydroxylase, P-450$_{11\beta}$ (John et al., 1985).

Discussion

The adrenal cortex is the sole site of glucocorticoid and mineralocorticoid synthesis from cholesterol and as such is the principal site of 21-hydroxylase activity. The molecular mechanism by which the peptide hormone ACTH maintains optimal 21-hydroxylase activity remains to be elucidated. Impairment of 21-hydroxylase activity results in overproduction of cortisol precursors and sex steroids leading to congenital adrenal hyperplasia. In both man (White et al., 1985; Carroll et al., 1985) and mouse (White et al., 1984c), two potential P-450$_{C21}$ genes have been identified, and both are located in the major histocompatibility complex (the HLA and H-2 regions, respectively). Gene deletion has been suggested as one of the causes of congenital adrenal hyperplasia (White et al., 1984b). Studies utilizing hypophysectomized rats (Mitani, 1979) as well as the bovine adrenocortical cell culture system (Waterman et al., 1986) imply that ACTH influences steroid hydroxylase gene expression, and a cyclic AMP-mediated pathway has been proposed for this regulation (Kramer et al., 1984). Treatment of cell cultures with ACTH or dibutyryl-cAMP increases the concentrations of P-450$_{C21}$ transcripts (Figure 8). From both transcription run-on assays and actinomycin D experiments we conclude that this is at least in part due to increased initiation of RNA synthesis in the nuclei of such cells (Figure 9). Thus, P-450$_{C21}$ gene expression is regulated by ACTH via cyclic AMP at the transcriptional level. Further studies are under way to elucidate the mechanism of this transcriptional regulation. Recent studies on the regulation of gene expression of components of steroid hydroxylases such as P-450$_{11\beta}$ (John et al., 1985), P-450$_{17\alpha}$ (Zuber et al., 1985), and adrenodoxin (Okamura et al., 1985) have provided evidence to suggest roles for labile inducer protein factors. P-450$_{C21}$ gene expression may also follow such a pattern. In that event, in addition to mutations in the structural gene, a mutation in a trans-acting regulatory element of the P-450$_{C21}$ gene could also lead to congenital adrenal hyperplasia.

Comparison of the partial bovine P-450$_{C21}$ amino acid sequence with the human sequence confirms the identity of the clone and suggests strong conservation within this protein. The

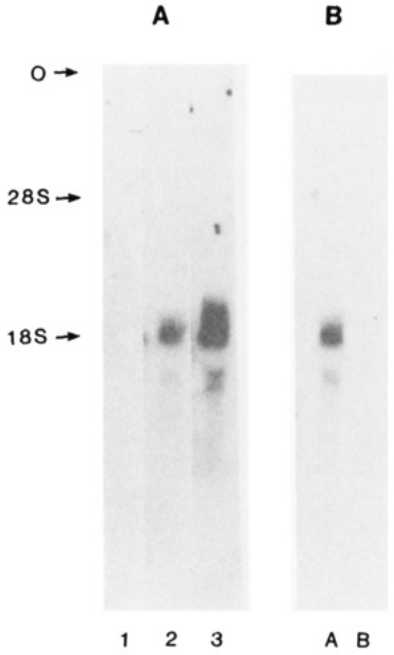

FIGURE 8: Increase in P-450$_{C21}$ transcript concentration in cultured bovine adrenocortical cells following treatment with ACTH or dibutyryl-cAMP. (A) Bovine adrenocortical monolayer cell cultures were treated with dibutyryl-cAMP (1 mM) or ACTH (10^{-6} M) for 12 and 24 h, respectively. Control cell cultures contained no modulators. Total RNA (20 μg each) was isolated and Northern blotted. The blot was hybridized to the nick-translated insert of pBC21-1. Lane 1, control; lane 2, dibutyryl-cAMP, 12 h; land 3, ACTH, 24 h. (B) Total polysomes were isolated from cell cultures treated with or without ACTH (10^{-6} M) for 36 h. A total of 25 μg of RNA from polysomes from treated (A) and control (B) cells was Northern blotted and probed with pBC21-1.

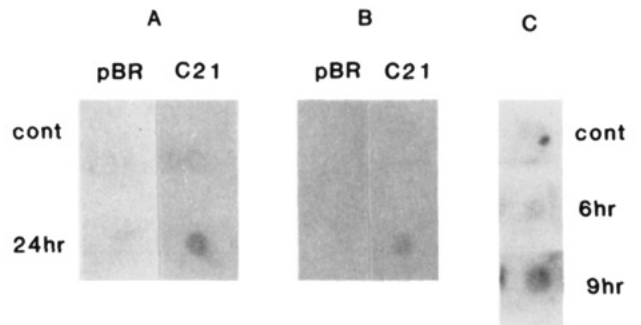

FIGURE 9: In vitro transcription run-on assays. Nuclei were isolated from cells treated with ACTH for 6 and 9 h (C) and 24 h (A and B) or without ACTH (cont), and in vitro transcription run-on assays were carried out. ^{32}P-labeled RNA (3×10^6 cpm) was hybridized to pBC21 (10 μg) bound on filters. pBR 322 was used to estimate nonspecific hybridization. The fold increase is 5.7 at 24 h in panel A, 2.7 at 24 h in panel B, 2.4 at 6 h in panel C, and 9.7 at 9 h in panel C. In data not shown, a 3-fold increase was observed at 10 h of ACTH treatment. When α-amanitin (1 μg/mL) is included in the assay, the incorporation of ^{32}P label into total RNA decreased by 35–50%. Additional run-on assays were carried out by using nuclei of cells treated with ACTH for 1, 3, 4, and 12 h. In none of these assays was the fold increase in transcription different from those shown. Comparisons of RNA initiations in nuclei of cells isolated from adrenal gland vs. those in the primary cell culture treated with ACTH shows a 3-fold increase in gland nuclei (not shown). This result is consistent with the observation that are about 2–3-fold more P-450$_{C21}$ RNA in the gland compared to cell culture and also indicates that competition of unlabeled mRNA is not the basis of low signal intensity. In addition, signal intensity is linear with input RNA. Control experiments using genes that show high steady-state levels of RNA also show high levels of initiations in both control and ACTH-treated cells. These genes were identified by cDNA library screenings (unpublished results).

presence of a portion of an intervening sequence in pBC21-1 is a novel feature (Figure 2). During splicing the ligation has occurred 20 bp upstream from the normal position. The majority of the P-450$_{C21}$ mRNA transcripts must be devoid of this intervening sequence, and we assume that this is a rare occurrence.

Among the components of the adrenocortical steroid hydroxylase system, P-450$_{scc}$ (John et al., 1984), P-450$_{11\beta}$ (John et al., 1985), and P-450$_{17\alpha}$ (M. X. Zuber, personal communication) show tissue-specific gene expression. Adrenodoxin, the iron–sulfur protein, is expressed in both adrenal cortex and corpus luteum, and also adrenodoxin or a related gene is expressed in kidney and liver (Okamura et al., 1985). Extraadrenal 21-hydroxylase activity has been demonstrated in human fetal testicular tissue, abnormal ovarian tissue, cortical tissue of kidney, and rabbit liver (Winkel et al., 1980; Dieter et al., 1982a; Senciall et al., 1983). Furthermore, 21-hydroxylation of progesterone to deoxycorticosterone can occur in adrenalectomized persons (Winkel et al., 1979). Thus, the possibility exists that P-450$_{C21}$ or a homologous gene is expressed in these tissues. Hybridization studies shown in Figures 5 and 6 clearly indicate that bovine kidney, liver, or corpus luteum do not express transcripts homologous to adrenal P-450$_{C21}$ in any appreciable amounts. We estimate that we could have detected 100-fold lower concentrations of P-450$_{C21}$ transcripts compared to the adrenal tissue (not shown). Weak hybridization signals as in the human total RNA lane A, Figure 7, could be detected. In studies using rabbits, monoclonal antibodies prepared against rabbit liver 21-hydroxylase show no cross-reactivity with rabbit adrenocortical 21-hydroxylase (Johnson et al., 1985). Moreover, comparison of amino acid sequence of rabbit hepatic C-21 hydroxylase, P-450-1 (Tukey et al., 1985), with bovine and human C-21 sequences shows only 34% and 35% homology, respectively. However, a recent study has demonstrated adrenocortical type P-450$_{C21}$ gene expression in mouse liver (Amor et al., 1985). The level of expression is estimated to be 50-fold less than that of mouse adrenal gland. Using 3.8-fold higher concentrations of bovine liver poly(A+) RNA (15 μg) and less stringent filter washing conditions than used in the mouse study, we could not detect cross-hybridization to pBC21-1. Species or individual differences within species could account for this discrepancy. In rabbits, for example, a 300-fold variation in the enzymatic activity of liver microsomal 21-hydroxylase was observed between individuals (Dieter et al., 1982b).

Steroid hydroxylase genes have been generally perceived to be under both tissue-specific and peptide hormonal control. Thus, expression of adrenal P-450$_{C21}$ in mouse liver has interesting implications. As demonstrated herein P-450$_{C21}$ gene expression in the adrenal cortex is regulated by ACTH (Figures 8 and 9). Since hepatic cells lack receptors for this hormone, ACTH will be ineffective in regulating P-450$_{C21}$ gene expression in this tissue. However, cyclic AMP mediated regulation could occur. Whether or not such regulation is of physiological significance remains to be determined. Further studies are required in different animal species including man to elucidate the expression of the steroid 21-hydroxylase gene.

ACKNOWLEDGMENTS

We appreciate the technical assistance of Leticia Cortez and Bill Rainey in the preparation of cell cultures. We thank Walter L. Miller, Department of Pediatrics, University of California, San Francisco, for providing unpublished information on intronic and exonic sequences near the 5′ end of bovine P-450$_{C21}$ gene and D. Russell and T. Yamamoto of this institution for providing a bovine adrenocortical cDNA library. We also thank Dr. E. F. Johnson for providing the sequence of rabbit liver P-450 form 1 prior to its publication.

Registry No. P-450$_{C21}$, 9035-51-2; ACTH, 9002-60-2; cAMP, 60-92-4; dibutyryl-cAMP, 362-74-3; steroid 21-hydroxylase, 9029-68-9.

REFERENCES

Acevedo, H. F., Axelrod, L. R., Ishikawa, E., & Takaki, F. (1963) *J. Clin. Endocrinol. Metab. 23*, 885–890.

Amor, M., Tosi, M., Duponchel, C., Steinmetz, M., & Meo, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4453–4457.

Bienkowski, M. J., Haniu, M., Nakajin, S., Shinoda, M., Yanagibashi, K., Hall, P. F., & Shively, J. E. (1984) *Biochem. Biophys. Res. Commun. 125*, 734–740.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F., & Chambon, P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4853–4857.

Bresser, J., & Gillespie, D. (1983) *Anal. Biochem. 129*, 357–364.

Carroll, M. C., Campbell, D. C., & Porter, R. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 521–525.

Cathala, G., Savounlet, J. F., Mendez, B., West, B. L., Karim, M., Mortial, J. A., & Baxter, J. D. (1983) *DNA 2*, 329–335.

Chrigwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chung, B.-C., Matteson, K. J., & Miller, W. L. (1985) *DNA 4*, 211–219.

Dieter, H. H., Muller-Eberhard, U., & Johnson, E. F. (1982a) *Biochem. Biophys. Res. Commun. 105*, 515–520.

Dieter, H. H., Muller-Eberhard, U., & Johnson, E. F. (1982b) *Science (Washington, D.C.) 217*, 741–743.

Funkenstein, B., McCarthy, J. L., Dus, K. M., Simpson, E. R., & Waterman, M. R. (1983) *J. Biol. Chem. 258*, 9398–9405.

Gotoh, O., Tagashira, Y., Iizuka, T., & Fujii-Kuriyama, Y. (1983) *J. Biochem. (Tokyo) 93*, 807–817.

Israel, D. I., & Whitlock, J. P. (1984) *J. Biol. Chem. 259*, 5400–5402.

John, M. E., John, M. C., Ashley, P., MacDonald, R. J., Simpson, E. R., & Waterman, M. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5628–5632.

John, M. E., John, M. C., Simpson, E. R., & Waterman, M. R. (1985) *J. Biol. Chem. 260*, 5760–5767.

Johnson, E. F., Finlayson, M. J. P., Tukey, R. H., & Rancy, J. L. (1985) in *Microsomes and Drug Oxidations* (Boobis, A. R., Caldwell, J., DeMatteis, F., & Elcombe, C. R., Eds.) pp 3–12, Taylor and Francis, London.

Kawajiri, K., Gotoh, O., Sogawa, K., Tagashira, Y., Muramatsu, M., & Fujii-Kuriyama, Y. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1649–1653.

Kramer, R. E., Rainey, W. E., Funkenstein, B., Dee, A., Simpson, E. R., & Waterman, M. R. (1984) *J. Biol. Chem. 259*, 707–713.

Maniatis, G., Fritsch, E. F., & Sambrook J. (1982) in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mayo, R. E., & Palmiter, R. D. (1981) *J. Biol. Chem. 256*, 2621–2624.

Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T., Inayama, S., & Omura, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4647–4651.

Murdoch, G. H., Potter, E., Nicolaisen, A. K., Evans R. M., & Rosenfeld, M. G. (1982) *Nature (London) 300*, 192–194.

New, M. I., Dupont, B., Grumbach, K., & Levine, L. S. (1982) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., & Brown, M. S., Eds.) pp 973–1000, McGraw-Hill, New York.

Okamura, T., John, M. E., Zuber, M. X., Simpson, E. R., & Waterman, M. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5705–5709.

Palmiter, R. D. (1974) *Biochemistry 13*, 3606–3615.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Senciall, I. R., Bullock, G., & Rahal, S. (1983) *Can. J. Biochem. Cell Biol. 61*, 722–730.

Sharp, P. A. (1981) *Cell (Cambridge, Mass.) 23*, 643–646.

Smith, H. O., & Birnstiel, M. L. (1976) *Nucleic Acids Res. 3*, 2387–2398.

Tukey, R. H., Okino, S., Barnes, H., Griffin, K. J., & Johnson, E. F. (1985) *J. Biol. Chem. 260*, 13347–13354.

Waterman, M. R., John, M. E., & Simpson, E. R. (1986) in *Cytochrome P-450; Structure, Mechanism and Biochemistry* (Ortiz de Mantellano, P. R., Ed.) Plenum, New York (in press).

White, P. C., New, M. I., & Dupont, B. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1986–1990.

White, P. C., New, M. I., & Dupont, B. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7505–7509.

White, P. C., Chaplin, D. D., Weis, J. H., Dupont, B., New, M. I., & Seidman, J. G. (1984c) *Nature (London) 312*, 465–467.

White, P. C., Grossberger, D., Onufer, B. J., Chaplin, D. D., New, M. I., Dupont, B., & Strominger, J. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1089–1093.

Winkel, C. A., Milewich, L., Parker, C. R., Gant, N. F., Simpson, E. R., & MacDonald, P. C. (1979) *Proc. Soc. Gynecol. Invest. 26*, 140 (Abstr.).

Winkel, C. A., Simpson, E. R., Milewich, L., & MacDonald, P. C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7069–7073.

Yuan, P. M., Nakajin, S., Haniu, N., Shinoda, M., Hall, P. F., & Shively, J. E. (1983) *Biochemistry 22*, 143–149.

Zuber, M. X., John, M. E., okamura, T., Simpson, E. R., & Waterman, M. R. (1986) *J. Biol. Chem. 261*, 2475–2482.