Regulation of Steroidogenesis in NCI-H295 Cells: A Cellular Model of the Human Fetal Adrenal

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NCI-H295 is a recently described human adrenocortical carcinoma cell line that makes a variety of steroid hormones. We sought to determine if steroidogenesis in these cells employs the same enzymes as those used in normal adrenal steroidogenesis, and if the genes encoding those enzymes exhibit characteristic responsiveness to activators of the protein kinase-A and -C pathways of intracellular second messengers. Northern blots show that NCI-H295 cells contain abundant mRNAs for three key steroidogenic enzymes, cytochrome P450scc, cytochrome P450c17, and cytochrome P450c21. These mRNAs accumulated in a time- and dose-dependent fashion in response to 8-bromo-cAMP (8Br-cAMP), forskolin, cholera toxin, and 3-isobutyl-1-methylxanthine, all activators of the protein kinase-A pathway. Nuclear run-on assays and actinomycin-D transcriptional inhibition experiments show that cAMP regulates the expression of all three genes primarily at the transcriptional level. Inhibition of protein synthesis with cycloheximide did not prevent the cAMP-induced accumulation of P450scc or P450c17 mRNAs, but did inhibit accumulation of P450c21 mRNA, suggesting that cAMP is acting through a mechanism dependent on protein synthesis to promote accumulation of P450c21 mRNA. Stimulation of the protein kinase-C pathway with phorbol ester decreased P450scc and P450c17 mRNAs, but stimulated the accumulation of P450c21 mRNA. RNase protection experiments, Northern blot hybridizations, and reverse transcription-polymerase chain reaction show that NCI-H295 cells express both the 11β-hydroxylase (P450c11β) encoded by the P450c11B1 gene and the aldosterone synthetase (P450c11AS) encoded by the P450c11B2 gene. 8Br-cAMP increased the abundance of both of these mRNAs with similar kinetics, with maximal accumulation of both after about 24 h. NCI-H295 cells also contain the mRNAs for aromatase and insulin-like growth factor-II. 8Br-cAMP increased the abundance of aromatase mRNA and decreased the abundance of IGF-II mRNA. These studies show that NCI-H295 cells express most of the enzymes needed for human adrenal steroidogenesis, and that the genes encoding these enzymes respond to stimulation of second messenger pathways in a manner similar to that of human adrenals. NCI-H295 cells appear to be a good model for studying the molecular regulation of human adrenal steroidogenesis. (Molecular Endocrinology 7: 423-433, 1993)

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INTRODUCTION

In adrenal steroidogenesis, cholesterol is converted to mineralocorticoids, glucocorticoids, and adrenal androgens by a series of well characterized steroidogenic enzymes (for review, see Ref. 1). The first and rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by cytochrome P450scc, which is thus the quantitatively regulating enzyme. P450scc is a single mitochondrial enzyme encoded by a single gene on human chromosome 15q23-q24 (2) that mediates three reactions, cholesterol 20α-hydroxylation, 22-hydroxylation, and C20,22 bond scission, all on a single active site, to yield pregnenolone. The next enzyme, cytochrome P450c17, determines the type of steroid produced. P450c17 is a single microsomal enzyme encoded by a single gene on human chromosome 10q24-q25 (2) that mediates both 17α-hydroxylase and 17,20-lyase activities. If pregnenolone is not metabolized by P450c17 (*e.g.* in the zona glomerulosa) 17-deoxy C-21 precursors of mineralocorticoids are produced. If pregnenolone is 17α-hydroxylated (*e.g.* in the zona fasciculata) 17-hydroxy C-21 precursors of glucocorticoids are made. If such 17α-hydroxylated steroids undergo the 17,20-lyase activity (*e.g.* in the adrenal zona reticularis or in testicular Leydig cells), 17-hydroxy C-19 androgens are produced. In the adrenal cortex, both 17-deoxy C-21 steroids in the zona glomerulosa and 17-hydroxy C-21 steroids in the zona fasciculata are 21-hydroxylated by the same cytochrome P450c21. P450c21 is a single enzyme encoded by the P450c21B gene on chromosome 6p21 that is

expressed solely in the adrenal cortex. Final metabolism of deoxycorticosterone to aldosterone requires 11- and 18-hydroxylations and 18-oxidation, all mediated by a single mitochondrial enzyme expressed solely in the zona glomerulosa and variously termed P450c11AS, aldosterone synthetase, P450c11aldo, and P450c11B2 (3–7). The 11-hydroxylation of 11-deoxycortisol to cortisol is catalyzed by a closely related enzyme expressed in the zonae fasciculata and reticularis and variously termed P450c11β and P450c11B1 (3–7). These two mitochondrial isozymes are encoded by the tandemly duplicated P450c11B1 and P450c11B2 genes on chromosome 8q22 that have 93% sequence identity (8).

The molecular biology of steroidogenesis has been studied in primary cultures of steroidogenic cells and in three transformed cell lines: mouse adrenocortical Y-1 cells, mouse Leydig MA-10 cells, and human JEG-3 choriocarcinoma cells. Studies with primary cultures of human adrenal cells are hampered by poor availability, rapidly diminishing responsiveness to ACTH and cAMP (9), and irreversible senescence (10). Transformed mouse Leydig MA-10 cells (11) make progesterone, but do not express P450c17 (12). JEG-3 choriocarcinoma cells (13) also make progesterone, closely resembling human cytotrophoblast cells (14), and are a good model for studying placental-specific steroidogenesis (15, 16), but this system is also confined to P450scc and its cofactors. A transformed rat granulosa cell line was described recently (17), but as expected for granulosa cells (26), these cells do not express steroidogenic enzymes distal to the synthesis of progesterone (18). Until recently, the only available adrenal steroidogenic cell line has been mouse Y-1 adrenocortical carcinoma cells (19). These have been widely used in transient transfection assays to study the transcription of genes for various steroidogenic enzymes (for reviews, see Refs. 20 and 22), but they do not express P450c21 or P450c17, and being mouse cells, they are sub-optimal for studying the expression of human genes.

Gazdar *et al.* (23) recently reported the establishment of NCI-H295 cells derived from a human adrenocortical carcinoma. These cells have been maintained in continuous culture for nearly 10 yr and make large amounts of the Δ^5-steroids (pregnenolone, 17-hydroxypregnenolone, and dehydroepiandrosterone), as is typical of human adrenal carcinomas, but also make small amounts of aldosterone and 11-deoxycortisol and trace amounts of cortisol and androstenedione (23), suggesting that these cells express all of the enzymes associated with adrenal steroidogenesis. However, it is well established that enzymes other than those normally functioning in adrenal steroidogenesis can have activities similar to those of the "authentic" adrenal enzymes (24, 25). Therefore, the studies of NCI-H295 cell steroidogenesis did not establish the identities of the responsible enzymes. To evaluate NCI-H295 cells as a model of the human adrenal, we examined their expression of the genes encoding several steroidogenic enzymes. NCI-H295 cells express the genes for P450scc, P450c17, and P450c21; furthermore, these genes respond to activators of the protein kinase-A (PKA) and protein kinase-C (PKC) pathways similarly to normal adrenals. These cells contain abundant mRNA for P450c11β, P450c11AS, and insulin-like growth factor-II (IGF-II), suggesting that they are less differentiated than the cells of the various zones of the adult human adrenal.

RESULTS

Expression of mRNAs for Steroidogenic Enzymes

Northern blots show that NCI-H295 cells contain the mRNAs for P450scc, P450c17, and P450c21, and that the abundances of these mRNAs increase when the cells are treated with 8-bromo-cAMP (8Br-cAMP) or other activators of the PKA pathway for 48 h (Fig. 1). The mRNAs for P450scc and P450c17 were more responsive to PKA agonists than the mRNA for P450c21, while the glyceraldehyde phosphate dehydrogenase (GAPDH) control was unresponsive. Stimulation with 3-isobutyl-1-methylxanthine (MIX), which inhibits phosphodiesterase, was less effective than the other PKA agonists, but elicited an additive effect when given in combination with cholera toxin, a stimulator of adenyl cyclase; however, the cAMP analog 8Br-cAMP always elicited the greatest response. The effect of 8Br-cAMP was maximal between 12–24 h of treatment, then remained essentially the same thereafter up to 48 h (Fig. 2). The mRNAs for P450scc, P450c17, and P450c21 responded to 8Br-cAMP in a dose-dependent fashion, showing maximal response in the range of 300–1000 μM (Fig. 3). Thus, both the kinetics and the dose-dependency of the responses of the steroidogenic

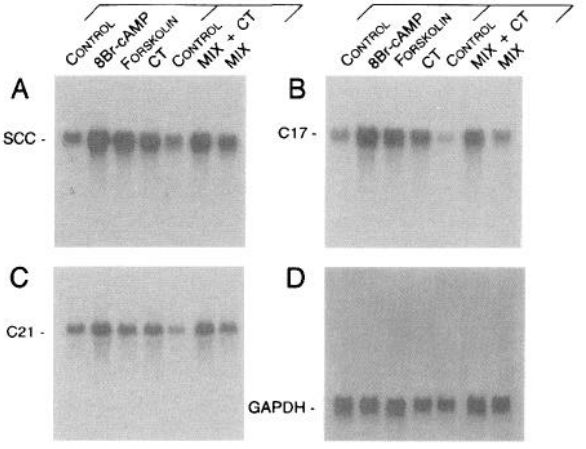

Fig. 1. Northern Blot

NCI-H295 cells were treated for 48 h with 100 μM 8Br-cAMP, 50 μM forskolin, 10 ng/ml cholera toxin (CT), 0.5 mM MIX, or 0.5 mM MIX and 10 ng/ml CT (MIX+CT). The control incubations were performed with medium alone or medium containing 0.1% ethanol (the solvent for forskolin). A single Northern blot containing 20 μg total RNA in each lane was hybridized sequentially with human cDNA probes for P450scc (A; SCC), P450c17 (B; C17), P450c21 (C; C21), and GAPDH (D).

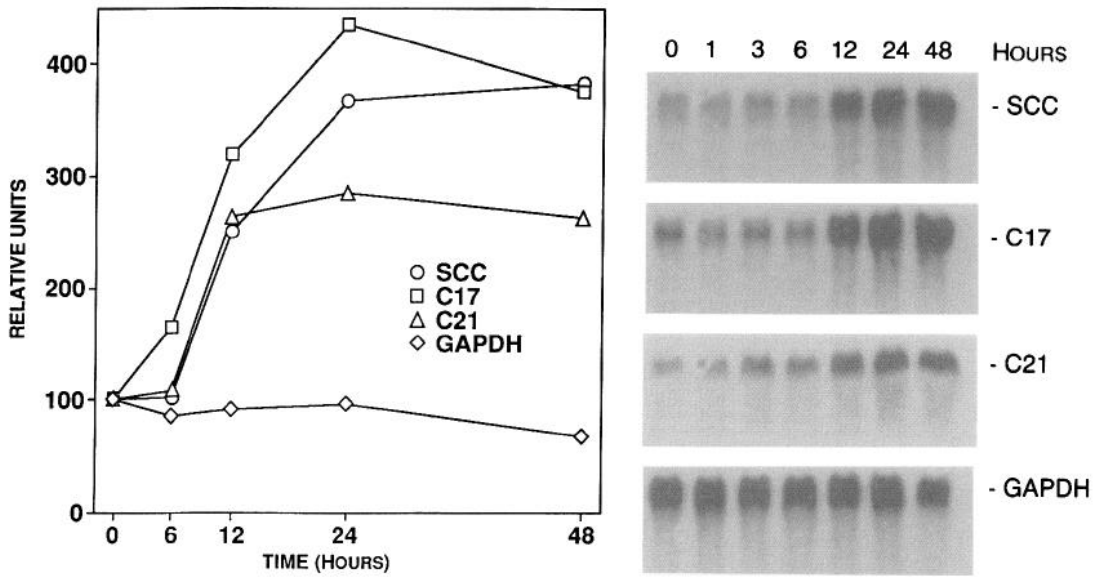

Fig. 2. Kinetics of Response to 8Br-cAMP

Cells were treated with 100 μM 8Br-cAMP for the times shown, and the relative abundance of the mRNAs for P450scc (SCC; ○), P450c17 (C17; □), P450c21 (C21; △), and GAPDH (◇) was determined by Northern blotting. The autoradiograms are shown at the *right*, and a graphic representation of the phosphorimager analysis is shown at the *left*.

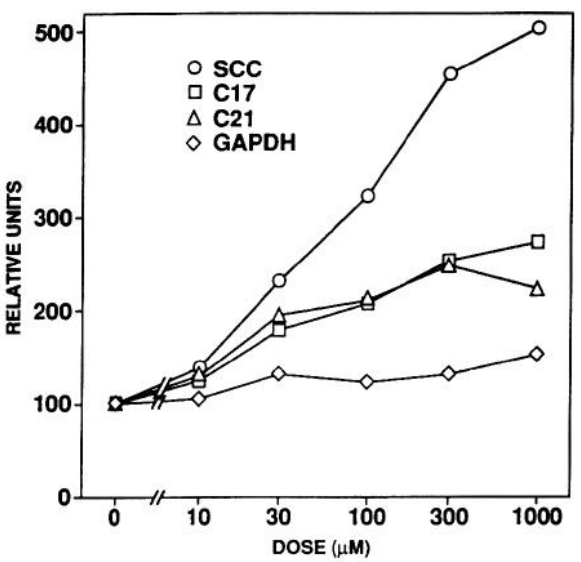

Fig. 3. Dose-Dependency of the Responses of the mRNAs for P450scc (SCC; ○), P450c17 (C17; □), P450c21 (C21; △), and GAPDH (◇) to Stimulation with 8Br-cAMP for 24 h

The graph shows the results of phosphorimager analysis of a single blot sequentially probed with each cDNA.

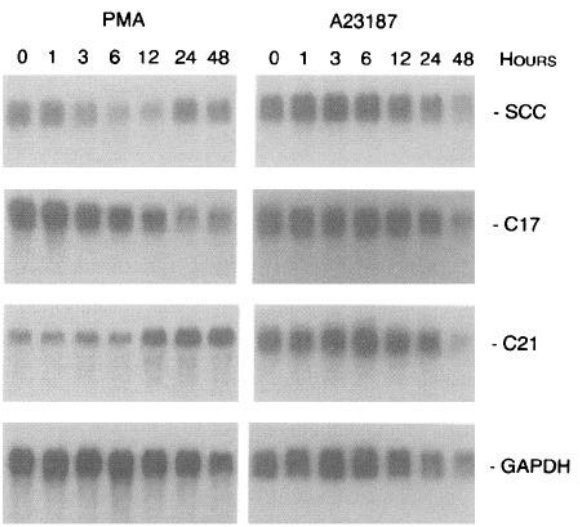

Fig. 4. Northern Blot Showing Kinetics of Response to Phorbol Ester and Calcium Ionophore

Cells were incubated with 100 ng/ml PMA (*left*) or 0.5 μM A23187 (*right*) for the times shown, and single Northern blots containing 20 μg RNA/lane were successively probed with cDNAs for P450scc (SCC), P450c17 (C17), P450c21 (C21), and GAPDH.

enzymes in NCI-H295 cells are similar to those previously found in primary cultures of both human fetal adrenal cells and luteinized human granulosa cells (9, 26, 27).

Effects of Phorbol Ester and Calcium Ionophore

To examine the influence of other intracellular second messenger pathways, we used the phorbol ester phorbol 12-myristate 13-acetate (PMA; also known as TPA) to activate PKC, and the calcium ionophore A23187 to mobilize intracellular calcium ion. These two pathways normally converge to mediate the response of the adrenal zona glomerulosa to angiotensin-II (for review, see Ref. 28). Incubating NCI-H295 cells with 100 ng/ml PMA diminished the abundance of P450scc mRNA within 3 h; this reached a nadir at 6 h (Fig. 4). However, by 12 h, a slight recovery was apparent, and by 24–48

Table 1. Influence of ACTH and Angiotensin-II on NCI-H295 Cells

Time (h)	P450scc	P450c17	P450c21
ACTH			
0	100	100	100
12	104	98	107
24	129	108	119
48	88	115	115
Angiotensin-II			
0	100	100	100
12	109	140	148
24	118	96	117
48	83	94	99

NCI-H295 cells were treated for the indicated periods of time with ACTH (0.1 μM) or angiotensin-II (1 μM). RNA was extracted, subjected to Northern blot analysis, and quantitatively analyzed on a Phosphorimager. Results are normalized to GAPDH mRNA and expressed in dimensionless units relative to the untreated control which was set equal to 100.

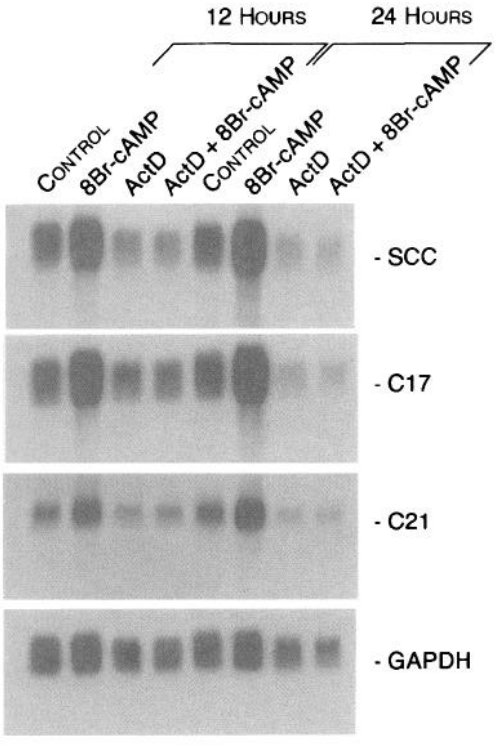

Fig. 5. Northern Blot

Cells were treated with 100 μM 8Br-cAMP and/or 5 μg/ml actinomycin-D (ActD; added 90 min before 8Br-cAMP) for 12 or 24 h. Ten micrograms of RNA were loaded in each lane, and the blot was probed sequentially with human cDNAs for P450scc (SCC), P450c17 (C17), P450c21 (C21), and GAPDH.

h, the abundance of P450scc mRNA had returned to basal levels. In sharp contrast, reprobing of the same Northern blot showed very different patterns of P450c17 and P450c21 mRNAs. There was a slow steady decrease in the abundance of P450c17 mRNA for 24 h, with minimal recovery by 48 h, while the mRNA for P450c21 accumulated slowly and steadily, reaching

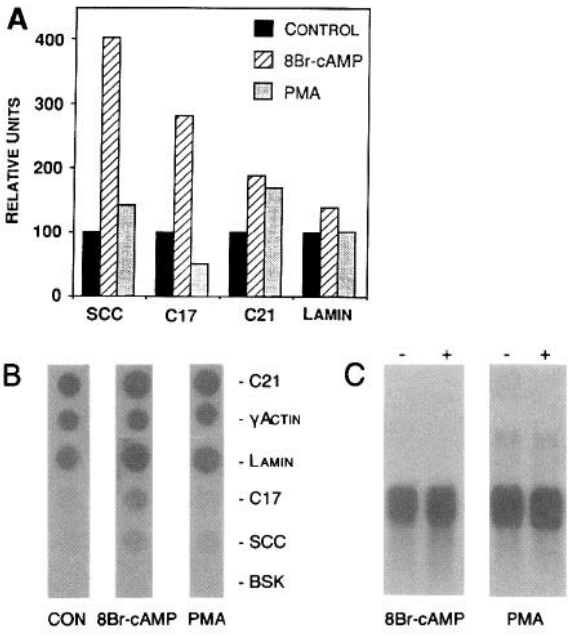

Fig. 6. Transcription of Genes for Steroidogenic Enzymes

Cells were incubated with 100 μM 8Br-cAMP or 100 ng/ml PMA for 24 h, and the transcription of the genes for P450scc (SCC), P450c17 (C17), P450c21 (C21), γ-actin, and nuclear lamin was assessed by RNA polymerase run-on assays. Control values (CON) are from cells treated with vehicle for 24 h. A, Results of phosphorimager analysis normalized to γ-actin and expressed relative to the untreated control value, which is set equal to 100. B, Autoradiograph of the filters used to generate A (BSK designates pBluescript DNA control). C, Northern blot analysis of γ-actin mRNA in NCI-H295 cells treated (+) or not treated (−) with 8Br-cAMP (100 μM) or PMA (100 ng/ml) for 24 h, showing that γ-actin is unchanged and appropriate for normalization.

a maximum after 48 h. The mRNA for GAPDH was slightly inhibited 48 h after PMA treatment.

Mobilization of intracellular calcium had little apparent effect on NCI-H295 cells. Incubation with 0.5 μM A23187 promoted a minimal accumulation of the mRNAs for P450scc, P450c17, and P450c21 after 6–12 h. However, by 48 h, the mRNAs for all three steroidogenic enzymes and the GAPDH control were substantially diminished, suggesting general cellular toxicity (Fig. 4).

Effects of ACTH and Angiotensin-II

To determine if NCI-H295 cells responded to extracellular stimulators of adrenal steroidogenesis, we incubated the cells with 10^{-7} M ACTH or 10^{-6} M angiotensin-II. As shown in Table 1, neither of these agents elicited changes in the mRNAs for P450scc, P450c17, or P450c21. Thus, NCI-H295 cells do not respond to ACTH or angiotensin-II, but the level of the defect is not known.

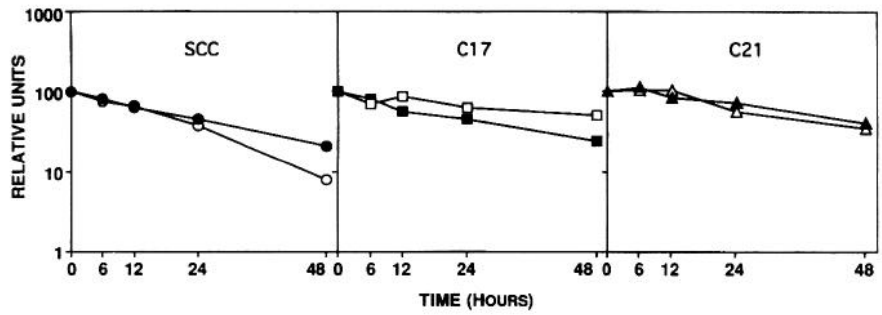

Fig. 7. Messenger RNA Stability

P450scc (SCC), P450c17 (C17), and P450c21 (C21) mRNAs were estimated by Northern blotting and phosphorimager analysis at various times after the addition of 5 μg/ml actinomycin-D to NCI-H295 cells that were treated for 24 h with 100 μM 8Br-cAMP (●, ■, and ▲) or vehicle (○, □, and △) for 24 h. The relative abundance of RNA before actinomycin-D treatment was set at 100%.

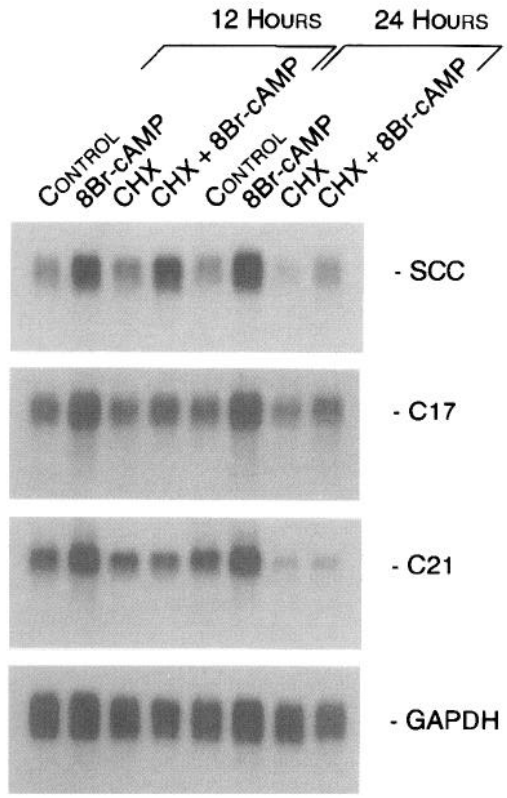

Fig. 8. Northern Blot

Cells were treated with 100 μM 8Br-cAMP and/or 10 μg/ml cycloheximide (CHX). Ten micrograms of RNA were loaded in each lane, and the blot was probed with human cDNAs for P450scc (SCC), P450c17 (C17), P450c21 (C21), and GAPDH.

Transcription of Genes for Steroidogenic Enzymes in NCI-H295 Cells

Transcription is the principal mechanism for the accumulation of the mRNAs for most human steroidogenic enzymes (for review, see Refs. 20–22). To determine the relative contributions of transcriptional and post-transcriptional processes to the increase in the abundance of the mRNAs for P450scc, P450c17, and P450c21, we measured rates of RNA synthesis and degradation. When nuclear transcription was interrupted with actinomycin-D (5 μg/ml) 90 min before treatment with 8Br-cAMP, the cAMP-mediated increases in P450scc, P450c17, and P450c21 mRNAs were abolished both 12 and 24 h after the addition of 8Br-cAMP (Fig. 5), suggesting that cAMP directly stimulates the transcription of these genes in NCI-H295 cells. Similarly, actinomycin-D reduced the abundance of GAPDH mRNA, consistent with its general effect to inhibit transcription.

To verify that 8Br-cAMP acts directly at the transcriptional level, we performed a nuclear run-on assay by hybridizing nuclear RNA labeled with [^{32}P]UTP to the cDNAs for P450scc, P450c17, P450c21, γ-actin, nuclear lamin, and control pBluescript vector. The filters were analyzed by phosphorimaging (Fig. 6A) and autoradiography (Fig. 6B). The phosphorimaging data are normalized to the transcriptional activity of γ-actin, as its mRNA was unaffected by 8Br-cAMP or PMA in NCI-H295 cells (Fig. 6C). These assays showed that transcription of the P450scc, P450c17, and P450c21 genes increased about 4-, 3-, and 2-fold, respectively, after incubation with 100 μM 8Br-cAMP for 24 h. Treatment with 100 ng/ml PMA for 24 h stimulated P450c21 gene transcription about 2-fold, but decreased transcription of the P450c17 gene consistent with the mRNA responses shown in Fig. 4, while the effect on P450scc was minimal.

To determine whether mRNA stability was altered by treatment with cAMP, RNA synthesis was blocked with actinomycin-D, and the abundances of the mRNAs for P450scc, P450c17, and P450c21 were examined in cells untreated or pretreated with 8Br-cAMP for 24 h (Fig. 7). The rates of disappearance of P450scc, P450c17, and P450c21 mRNAs were very similar in NCI-H295 cells treated with or without 8Br-cAMP, indicating that cAMP does not have a major influence on the stability of these mRNAs in NCI-H295 cells.

The role of protein synthesis in mediating basal and cAMP-induced transcription of genes for various steroidogenic enzymes differs among various cell types (12, 15, 20, 29). The addition of 10 μg/ml cycloheximide

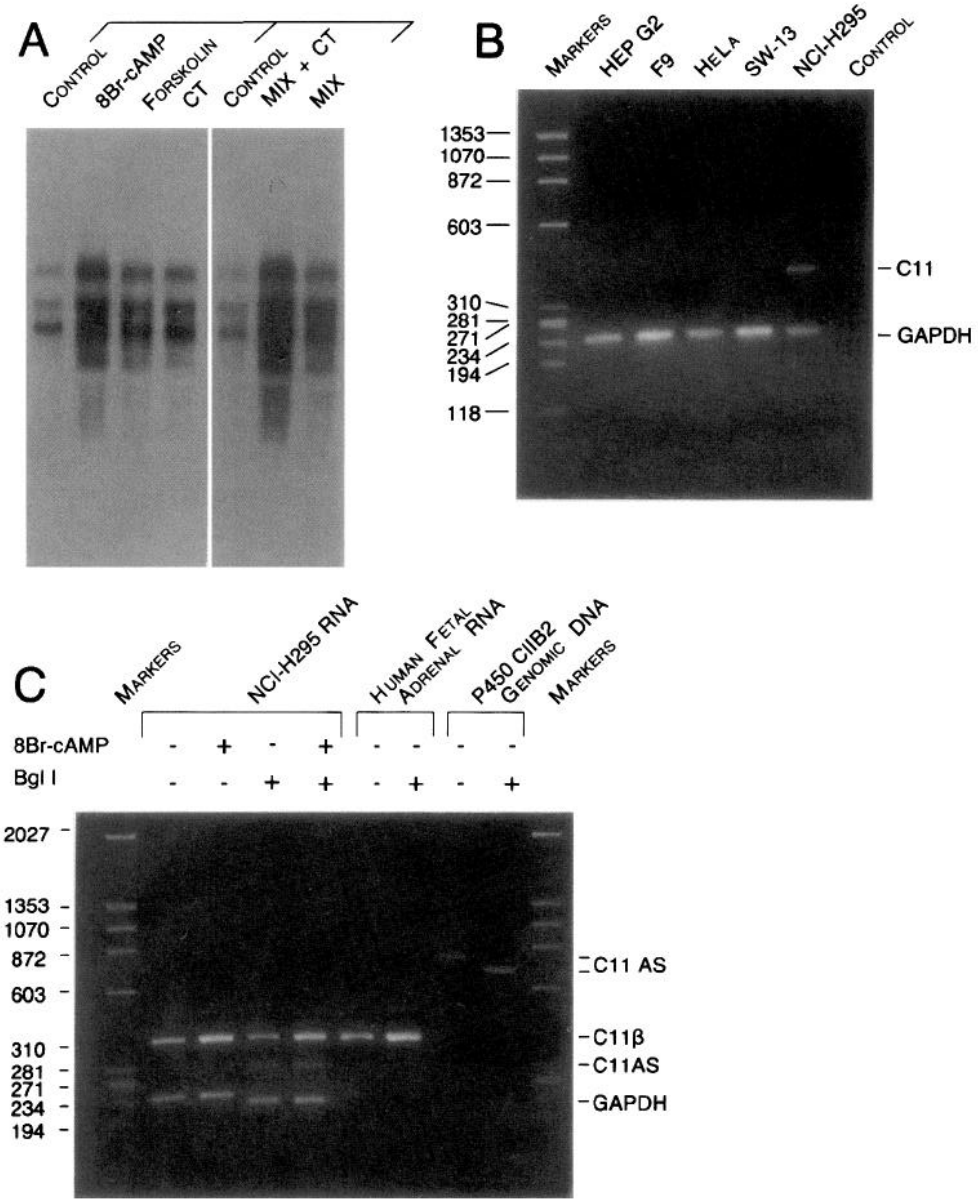

Fig. 9. Expression of P450c11β and P450c11AS mRNAs in NCI-H295 Cells

A, The Northern blot used in Fig. 1 was rehybridized to a human P450c11β exon 3 clone. B, One hundred nanograms of total RNA isolated from the indicated cell lines were reverse transcribed using random primers and then PCR amplified for 35 cycles using primers specific to exons 1 and 2 of both P450c11 genes. The PCR products were electrophoresed through 2% agarose gel and stained with ethidium bromide. GAPDH primers were used as internal controls for the RT-PCR reaction (control, no RNA added in the reaction). Note that a P450c11 band is amplified by RT-PCR only with RNA from NCI-H295 cells. C, PCR amplification of DNA made by RT of RNA from NCI-H295 cells or human fetal adrenals or DNA from a human genomic DNA clone of the P450c11B2 gene. The NCI-H295 cells were treated with (+) or without (−) 100 μM 8Br-cAMP for 24 h. PCR was performed with both the P450c11 and the GAPDH primers, and the resulting DNA was (+) or was not (−) digested with *BglI*. CT, Cholera toxin

for 12 h had little effect on P450scc or P450c17 mRNAs, whereas after 24 h of incubation, there was a 50% decrease in these two mRNAs, matched by a similar decrease in GAPDH mRNA, suggesting nonspecific cellular toxicity (Fig. 8). By contrast, P450c21 mRNA abundance decreased within 12 h and was less than 20% of the control value by 24 h. Cycloheximide in combination with 8Br-cAMP had no measurable effect on the cAMP-induced accumulation of P450scc mRNA, but it blunted the accumulation of P450c17 mRNA and completely prevented the cAMP-induced accumulation of P450c21 mRNA. Thus, transcription of the P450c21 gene appears to require ongoing protein synthesis in NCI-H295 cells, whereas transcription of the P450scc gene does not.

Expression of mRNAs for P450c11β and P450c11AS

The aldosterone synthase, P450c11AS, is expressed only in the zona glomerulosa, whereas the 11β-hydrox-

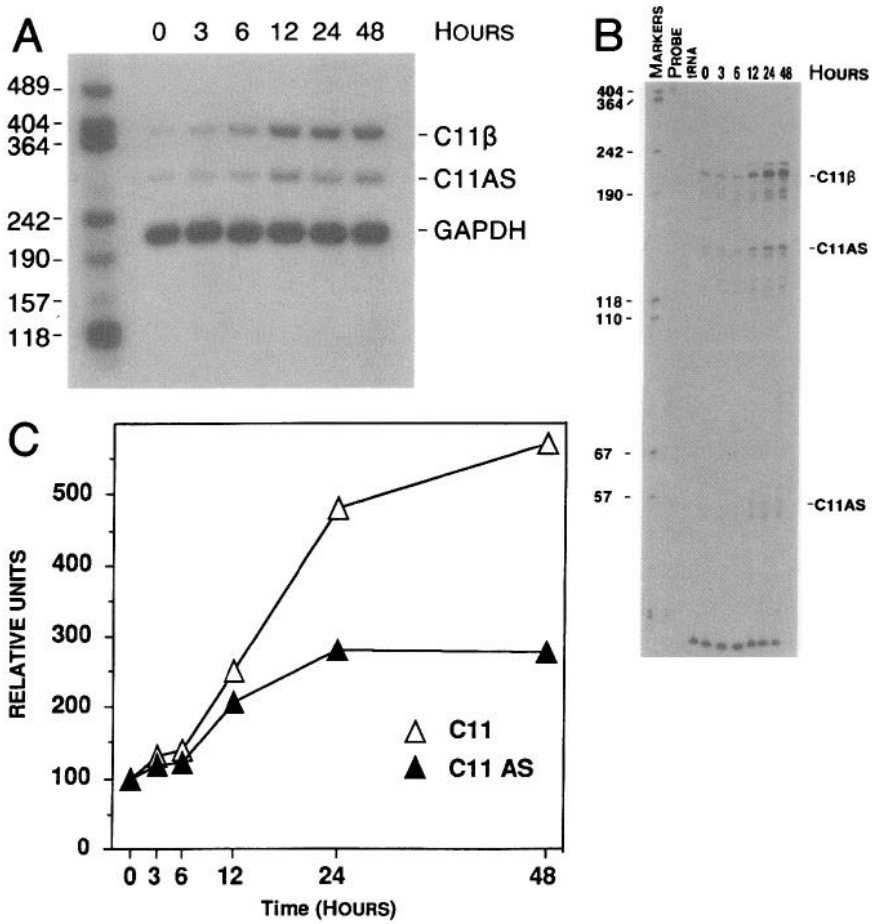

Fig. 10. Kinetics of Responses of P450c11β and P450c11AS to 8Br-cAMP

A, NCI-H295 cells were treated with 0.1 μM 8Br-cAMP for the times shown, and 50 ng total RNA were reverse transcribed and PCR amplified for 20 cycles, as described in Fig. 9B. The PCR amplification included 10^6 cpm end-labeled antisense P450c11 oligonucleotide and 10^6 cpm end-labeled sense GAPDH oligonucleotide as an internal control. The PCR products were then digested with *BglI* (to distinguish P450c11β from P450c11AS as in Fig. 9C) before electrophoresis through 2% agarose gel and autoradiography. B, RNase protection assay of total RNA extracted from NCI-H295 cells treated with 100 μM 8Br-cAMP for the times shown. Ten micrograms of each RNA sample were hybridized to a 386-base cRNA probe corresponding to exon 3 of P450c11β and digested with RNase-A. tRNA, Transfer RNA. C, Phosphorimager data of the experiment shown in B.

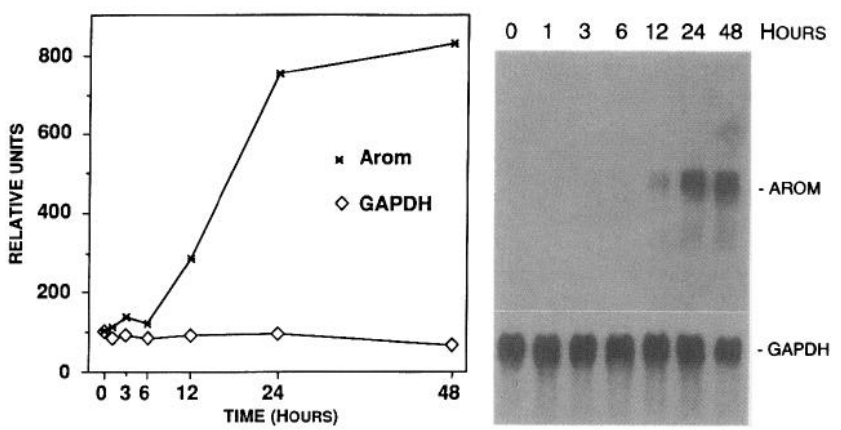

Fig. 11. Response of Aromatase mRNA to 8Br-cAMP

Cells were treated with 100 μM 8Br-cAMP for the times shown, and the relative amounts of mRNAs for aromatase (Arom) and GAPDH were determined by Northern blotting (*right*) and phosphorimaging (*left*).

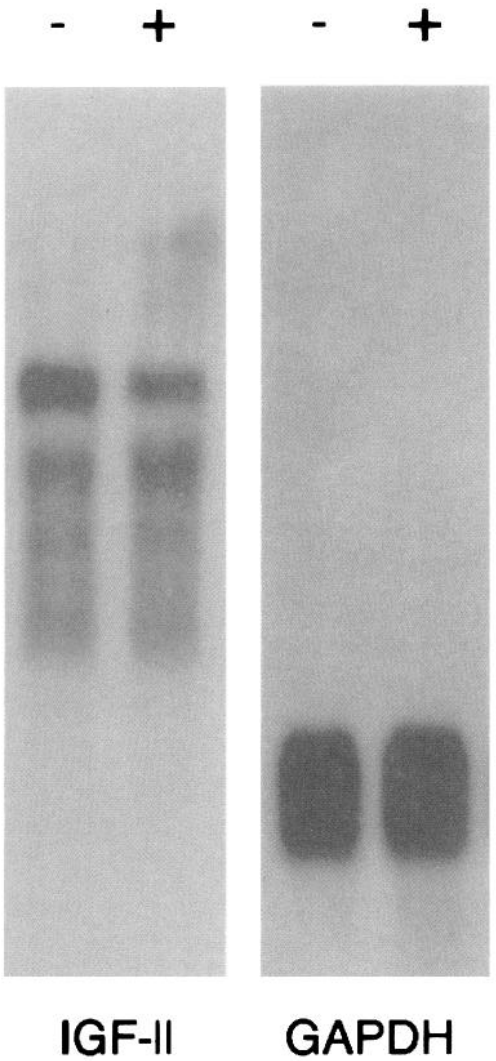

Fig. 12. Northern Blot

Cells were treated for 12 h without (−) or with (+) 100 μM 8Br-cAMP. Ten micrograms of RNA were loaded in each lane, and the blot was probed with human cDNAs for IGF-II (*left*) and GAPDH (*right*).

ylase, P450c11β, appears to be expressed only in the zonae fasciculata and reticularis (6, 7). To examine the expression of these two mRNAs (which bear 93% sequence identity) in NCI-H295 cells, we reprobed the Northern blot shown in Fig. 1 with a polymerase chain reaction (PCR)-amplified probe corresponding to exon 3 of the P450c11B1 gene. This probe detected the same pattern of bands seen in human fetal adrenals (3) and showed that all four RNA species accumulate in response to 8Br-cAMP or other activators of the PKA pathway (Fig. 9A). To confirm that these cells contain P450c11 mRNA, we used reverse transcription of mRNA, followed by PCR amplification (RT-PCR). Using PCR primers in exons 1 and 2 that do not distinguish between P450c11β and P450c11AS, Fig. 9B shows that NCI-H295 cells contain P450c11 mRNA, whereas non-adrenal human cell lines (HepG2, F9, and HeLa) and non-steroidogenic human adrenal SW-13 cells (30) do not contain P450c11 mRNA detectable by this very sensitive procedure. To distinguish P450c11β from P450c11AS mRNA, we cleaved the products of the RT-PCR reaction with *Bgl*I, which cleaves at the sequence encoding amino acid 29 of P450c11AS, but not in P450c11β (3, 8). Figure 9C shows that the NCI-H295 cells encode an RT PCR product that is cleaved to a 307-basepair (bp) band with *Bgl*I, as predicted for P450c11AS, and also encode an RT-PCR product of 392 bp that is not cleaved by *Bgl*I, as predicted for P450c11β. The completeness of the *Bgl*I digestion was demonstrated by an internal control using GAPDH, which contains a single *Bgl*I site that converts the 239-bp GAPDH RT-PCR fragment to 226 bp. Furthermore, PCR amplification of a clone of P450c11B2 genomic DNA containing exons 1 and 2 and the intervening intron yielded the expected 774-bp P450c11B2 fragment, which was cleaved to 689 bp by *Bgl*I. This ability to distinguish P450c11β from P450c11AS shows that NCI-H295 cells contain both mRNAs, whereas no known human adrenal cell type contains both of these mRNAs.

To examine the kinetics of the responses of P450c11β and P450c11AS to 8Br-cAMP, we performed RT-PCR reactions with a ^{32}P-labeled P450c11 antisense primer, which will label both P450c11β and P450c11AS, and with ^{32}P-labeled GAPDH sense primer in a single PCR reaction limited to 20 cycles. As shown in Fig. 10A, 8Br-cAMP increased the P450c11β signal within 3 h and was maximal by 24–48 h. Similarly, the P450c11AS signal was increased within 3–6 h and was also maximal by 24–48 h, although at a slightly lower level. Because this RT-PCR experiment is only semiquantitative, we also examined the abundance of P450c11β and P450c11AS mRNAs by solution hybridization/RNase protection, using a riboprobe generated from the P450c11β exon 3 cDNA probe used in Fig. 9A. RNA from NCI-H295 cells protected a 218-bp fragment corresponding to P450c11β and two internally cleaved fragments of 164 and 54 bp corresponding to P450c11AS (Fig. 10, B and C). Treatment with 8Br-cAMP increased the abundance of both P450c11β and P450c11AS. Both mRNAs increased between 6–12 h and reached maximal levels at 12–48 h, although P450c11AS rose only 3-fold, while P450c11β rose more than 5-fold.

Expression of Aromatase mRNA

Gazdar *et al.* (23) reported that NCI-H295 cells secrete small amounts of estrogen; therefore, we looked for aromatase mRNA in these cells. Northern blotting shows that NCI-H295 cells contain barely detectable amounts of aromatase mRNA, but that this RNA is readily induced by 8Br-cAMP (Fig. 11). Increases in aromatase mRNA were readily detectable after 12 h and were maximal at 24–48 h. By contrast, treatment with PMA elicited no detectable change in the very low basal levels of aromatase mRNA (not shown).

Expression of IGF-II mRNA

Human fetal adrenals express high levels of IGF-II mRNA, whereas no IGF-II mRNA is detected in adult adrenals (27, 31). Thus, in the adrenal, IGF-II mRNA may serve as a marker for fetal development. Northern blots show that IGF-II mRNA is very abundant in NCI-H295 cells, being detectable within 1 h of autoradiographic exposure (Fig. 12). Treatment of NCI-H295 cells with 8Br-cAMP for 12 h decreased IGF-II mRNA (Fig. 12); by contrast, ACTH increased IGF-II mRNA in primary cultures of human fetal adrenal cells (27, 31).

DISCUSSION

Studies of human adrenal steroidogenesis have been hampered by the lack of adequate cell culture systems; the establishment of the NCI-H295 cell system should change this dramatically. In their initial description of these cells, Gazdar *et al.* (23) provided steroidal evidence for the existence of all major pathways of adrenal steroidogenesis in these cells. Our data now show that these pathways are based on expression of the same enzymes found in the normal adrenal, and that the genes encoding these enzymes respond to intracellular second messengers in a physiologically meaningful fashion.

8Br-cAMP and other agonists of the PKA pathway stimulated P450scc, P450c17, and P450c21 mRNA accumulation similar to that in primary cultures of human fetal adrenal cells (9, 27, 31). Furthermore, the endogenous human P450scc, P450c17, and P450c21 genes in these cells were transcriptionally induced by cAMP, similar to the behavior of the corresponding human promoter/reporter constructions when transfected into mouse Y-1 adrenocortical carcinoma cells (for review, see Refs. 21 and 22). As in various systems, the cAMP-induced accumulation of these mRNAs is primarily a reflection of transcriptional, rather than post-transcriptional, induction. The cAMP induction of P450scc and P450c17 did not require ongoing protein synthesis, but the cAMP induction of P450c21 did. The role of protein synthesis in basal and cAMP-induced transcription has not been studied previously in human adrenal cells. Although the transcription of bovine P450scc, P450c17, and P450c21 genes appears to require ongoing protein synthesis in primary cultures of bovine adrenal cells (for review, see Ref. 20), transcription of the human P450scc, P450c17, and P450c21 promoters appears to be direct and not require protein synthesis when these promoters are put into mouse adrenal Y-1 cells (32–34). The difference in the cycloheximide sensitivity of P450c21 gene expression *vs.* the insensitivity of P450scc and P450c17 gene expression in NCI-H295 cells suggests that transcriptional activation of the human P450c21 gene is fundamentally different from activation of the P450scc and P450c17 genes, consistent with the view that some of the nuclear transcription factors that activate these genes are the same, and some are different (35).

Stimulating the PKC pathway in NCI-H295 cells with the phorbol ester PMA decreased P450scc mRNA accumulation after 3–12 h, but this recovered by 24 h (Fig. 4), a time at which no effect was seen on P450scc transcription (Fig. 6). Treatment with PMA for 6–12 h similarly suppresses transcription of human P450scc promoter/reporter constructions transiently transfected into mouse adrenal Y-1 cells (32). PMA also induced P450c21 gene transcription and mRNA accumulation, while decreasing P450c17 gene transcription and mRNA accumulation. Phorbol ester induces similar increases in P450c21 mRNA and decreases in P450c17 mRNA in primary cultures of human fetal adrenal cells (36), and PMA supresses transcription of the human P450c17 promoter/reporter constructions transiently transfected into Y-1 cells (33). By contrast, PMA increases P450c17 mRNA slightly in primary cultures of adult human adrenal cells (36), suggesting that NCI-H295 cells more closely resemble fetal than adult human adrenal cells. This is consistent with their relatively high production of Δ5-steroids and relatively low production of Δ4-steroids (23) and the expression of abundant IGF-II mRNA (27, 31), which are typical features of human fetal adrenal cells. Finally, the expression of large amounts of both P450c11β (11β-hydroxylase) and P450c11AS (aldosterone synthetase) mRNAs suggests that NCI-H295 cells represent an adrenocortical cell that has not fully differentiated into the phenotype of one of the three adrenocortical zones. This in combination with the various fetal characteristics of these cells suggest that they are zonally undifferentiated human fetal adrenal cells.

MATERIALS AND METHODS

Cell Culture

NCI-H295 cells were grown in RPMI-1640 supplemented with 2% fetal calf serum supplemented with selenium (5 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (10^{-8} M), and 17β-estradiol (10^{-8} M) at 37 C in 5% CO_2-95% air. To eliminate the influence of steroid hormones in the medium, cells were switched to RPMI-1640 supplemented with 10% fetal calf serum, selenium (5 ng/ml), insulin (5 μg/ml), and transferrin (5 μg/ml) 14 days before incubation with 8Br-cAMP at the indicated doses, 50 μM forskolin, 10 ng/ml cholera toxin, 0.5 mM MIX, 10 μg/ml cycloheximide, 100 ng/ml PMA, 0.5 μM calcium ionophore A23187, 0.1 μM ACTH, 1.0 μM angiotensin-II (all from Sigma, St. Louis, MO), or 5 μg/ml actinomycin-D (Boehringer Mannheim, Indianapolis, IN).

RNA Analysis

RNA isolated by acid guanidinium thiocyanate/phenol-chloroform extraction was electrophoresed through 1.2% agarose-formaldehyde gels and Northern-transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). Filters were successively hybridized to cDNAs for human P450scc (37), P450c17 (38), P450c21 (39), IGF-II (40), aromatase (41), and GAPDH (42), labeled with [^{32}P]deoxy-CTP by random primers. The P450c21 probe was the 0.7-kb *Kpn*I/*Eco*RI fragment of

cDNA that does not extend into the overlapping XB gene (43). A clone of exon 3 of the P450c11B1 gene encoding P450c11β (8) was isolated from a human fetal adrenal cDNA library by PCR amplification and subcloned in pBluescript SK (sense primer, TGAATGGGCCTGAATGGCGC; antisense primer, AAGTTGCTGGCTTCTATGGT) and used as probe for northern blots. Filters were hybridized to 1.0×10^6 cpm/ml of each probe and washed in 500 ml 0.1 × SSC and 0.1% sodium dodecyl sulfate for 10 min at room temperature and twice for 30 min at 65 C. Filters were scanned on a PhosphorImager (Molecular Dynamics, Eugene, OR) and quantitatively analyzed using Imagequant software before autoradiography with Kodak X-Omat-AR film (Eastman Kodak, Rochester, NY). Blots were then stripped twice for 30 min at 95 C in 0.1% sodium dodecyl sulfate and 5 mM Tris-HCl (pH 7.5) and reautoradiographed between hybridization of each probe to ensure that all radioactivity had been removed. P450c11β and P450c11AS expressions were analyzed by RNase protection and PCR amplification, followed by P450c11AS-specific restriction enzyme digestion. RNase protection assays were performed essentially as previously described (32), using a P450c11β exon 3 riboprobe synthesized from the cDNA clone. Hybridization of 20 μg total RNA to 5×10^5 cpm probe was performed at 50 C, followed by digestion with 15 μg DNase-free RNase-A for 1 h at 37 C. For PCR amplification, 50 ng total RNA were reverse transcribed using random hexamer primers, then P450c11 exons 1 and 2 were PCR amplified (sense primer, ATGGCACTCAGGGCAAAGGCA; antisense primer, CAAGAACACGCCACATTTGTGC), followed by P450c11B2-specific *Bgl*I digestion and 2% agarose gel electrophoresis. GAPDH-specific primers were used as internal controls. Each experiment was performed at least twice.

Nuclear Run-On Assay

Nuclei were isolated and labeled with [α-^{32}P]UTP (3000 Ci/mmol) for RNA polymerase run-on assays, as previously described (44). Nuclear RNA was isolated, and dot blots of nuclear RNA were hybridized and washed, as described above. Equivalent counts of labeled nuclear RNA were hybridized for 36 h at 42 C to 5 μg cloned cDNAs for human P450scc (37), P450c17 (38), P450c21 (39, 43), lamin-A (45), and γ-actin (46) or pBluescript vector DNA (Stratagene, La Jolla, CA). Filter hybridizations were quantitated by phosphorimaging (Fig. 6A) and autoradiography (Fig. 6B), as described for RNA blots.

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