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Effects of Metyrapone on Expression of CYPs 2C11, 3A2, and Other 3A Genes in Rat Hepatocytes Cultured on Matrigel

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ABSTRACT. Hepatocytes cultured on matrigel express many liver-specific functions, but the levels and activities of the predominant male-specific rat hepatic CYPs, 3A2 and 2C11, decline rapidly in culture. Metyrapone maintains the level of total cytochrome P450 of rat hepatocytes in primary culture, but the mechanism underlying this effect has not been completely elucidated. The present study sought to determine whether metyrapone acts solely to stabilise CYP proteins in rat hepatocytes cultured on matrigel, or whether it also influences mRNA levels of the encoding genes. Metyrapone maintained the level of total cytochrome P450 in cultured hepatocytes so that values were >200% of those found in untreated control cells 24 hr after isolation. At this time, CYP3A2-mediated testosterone 6β-hydroxylation was approximately 7-fold higher in hepatocytes cultured in the presence of metyrapone than in control cells, and CYP2C11-dependent testosterone 2α- and 16α-hydroxylation activities were between 2 and 3-fold greater. The results inferred from catalytic activities were supported by immunoquantitation of CYP3A and 2C11 proteins. The trend of increased CYP protein levels in metyrapone-treated cells continued throughout the 48-hr culture period. In control cells, CYP3A2 and 2C11 mRNA levels fell abruptly in culture to reach values at 24 hr that were <30% of those in freshly isolated cells; addition of metyrapone failed to arrest this fall. However, treatment of cells with metyrapone considerably elevated levels of one or more CYP3A subfamily mRNA species, as detected by a riboprobe based on the cDNA for *CYP3A1* ('CYP3A1-like mRNA') that were demonstrated, by another riboprobe, not to be CYP3A2 or RNCYP3AM. RT-PCR of mRNA prepared from cultured hepatocytes, followed by restriction mapping of the cloned cDNAs was used to characterise the *CYP3A* induced by metyrapone. This revealed that elevated levels of the CYP3A1-like mRNA were attributable to induction of RL33/cDEX mRNA; there were no *CYP3A1* cDNAs isolated from these cells. These data are interpreted as indicating that metyrapone stabilises the expression of cytochrome P450 in culture by both pre- and posttranslational mechanisms. The particular mechanism employed is gene-specific, whereby even the highly homologous genes CYP3A2, RL33/cDEX and, possibly, RNCYP3AM are subject to different types of regulation in the presence of metyrapone. BIOCHEM PHARMACOL 52;2:219–227, 1996.

KEY WORDS. cytochrome P450; CYP; CYP3A; CYP regulation; CYP2C11; hepatocyte; rat

Hepatic CYP§ are a superfamily of hemoproteins responsible for the oxidative metabolism of innumerable endogenous and xenobiotic chemicals [1]. Expression of CYP is subject to complex, multifactorial regulation [2–4] that renders the interpretation of *in vivo* studies inherently difficult. Theoretically, the use of isolated rat hepatocytes, placed in the controlled environment of monolayer cultures, would provide a more opportune system to examine the regulation of CYP-mediated hepatic biotransformation. In practice, however, maintaining cultured hepatocytes in a differentiated state has proven difficult, and dedifferentiation is associated with a rapid decline in expression of CYP mRNA and related proteins [5–8]. Others have noted that the rate of decline between individual CYP proteins is not homogenous and is highly dependent upon culture conditions [6, 7]. Moreover, some CYPs that are known to be inducible in the intact animal were no longer responsive to the same agents when hepatocytes were placed in culture [7, 8].

When hepatocytes were cultured on a more physiological substratum provided by matrigel, a reconstituted basement membrane-like material rich in laminin, type IV collagen, and proteoglycans, certain CYP isoforms were demonstrably inducible, notably CYP2B1/2 by phenobarbital, and CYP3A1 by corticosteroids, phenobarbital, isosafrole, and triacetyloleandomycin [7]. Moreover, hepatocytes grown on

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§ *Abbreviations:* CYP, cytochrome P450; IgG, immunoglobulin G; [^{35}S]UTP, uridine 5′-[α-^{35}S]triphosphate; ECL, enhanced chemiluminescence; tNA, total nucleic acids; HBSS, Hanks balanced salt solution; RT-PCR, reverse transcriptase-mediated polymerase chain reaction.

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matrigel remain viable and differentiated even in the absence of serum and hormonal supplements. Despite these advances, the diminished expression of constitutive CYPs has remained a major problem.

CYPs 2C11 and 3A2 are the predominant constitutive CYPs in adult male rat liver. At the mRNA and protein levels, expression of CYP3A2 declines rapidly and apparently irreversibly when hepatocytes from adult male rats are cultured on matrigel. CYPs 2C11, 2C6, and 2C7 exhibit similar patterns, but mRNA levels return to approximately 20–30% of preculture values after 5 days in culture [5, 9]. Loss of total cytochrome P450 in cultures of rat hepatocytes can be prevented by addition of certain pyridols, notably metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) [6, 10, 11]. The mechanism underlying this effect has not been completely elucidated, although Paine and colleagues have shown that metyrapone is capable of modulating the expression and degradation of CYP protein [12]. Thus, the ability of metyrapone to form a ligand with CYP may account for stabilization of the protein [13]. However, others have noted that the ability of metyrapone to bind purified CYPs does not correlate with its ability to maintain expression of these forms in cultured hepatocytes [6]. It has been suggested that metyrapone can induce expression of CYP3A mRNAs and proteins [14, 15], but which of the several CYP3A subfamily members known to be expressed in rat liver are upregulated by metyrapone has not been determined. Further, the relevance of this finding to the metyrapone effects in cultured hepatocytes is unclear. In the present study, we determined the effects of metyrapone on *CYP2C11*, *CYP3A2*, *CYP3A1*, and related *CYP3A* genes in hepatocytes maintained in well-differentiated culture, using the matrigel system.

MATERIALS AND METHODS

Chemicals

Collagenase (type IV), metyrapone (2-methyl-1,2,-di-3-pyridyl-1-propanone), polyoxyethylene-sorbitan monolaurate (Tween 20), peroxidase-labelled goat antirabbit IgG, cell culture medium components and other biochemicals were purchased from Sigma-Aldrich (Sydney, Australia). Glutamine and minimum essential medium vitamins were acquired from Gibco BRL (Grand Island, NY, U.S.A.). Proteinase K was supplied by Merck (Darmstadt, Germany) and RNase A and RNase T_1 were from Boehringer Mannheim Australia Pty., Ltd. Promega Biotech (Madison, WI, U.S.A.) provided restriction endonucleases, ligase, plasmid vectors, and all reagents for the *in vitro* transcription of cRNA probes. [^{35}S]UTP and ECL 'Western blot' detection reagents were purchased from Amersham International (Buckinghamshire, U.K.).

Isolation and Cell Culture of Hepatocytes

Hepatocytes were isolated from ether-anesthetized male Wistar rats (approximate weight 250–300 g) by *in situ* perfusion of the liver as described by Seglen [16] and modified by Bissell and Guzelian [17]. In brief, the liver was perfused with HBSS, pH 7.4, containing 0.5 mM EGTA, followed by 0.01% collagenase in HBSS, pH 7.4. The liver was excised and dispersed in modified Waymouth media [17] containing sodium bicarbonate (24 mM), penicillin (100 U/mL), HEPES (18 mM), and insulin (25 U/L) as the only hormonal supplement. Viability was determined by Trypan Blue exclusion, and only preparations in excess of 80% viable hepatocytes were used. Cells, 3.5×10^6 in a final volume of 3 mL, were inoculated into 60 mm culture dishes (A/S Nunc, Roskilde, Denmark) coated with 400 μL of matrigel that had been prepared, in house, from Engelbreth-Holm-Swarm tumor propagated in C57Bl/6J female mice [18]. Cultures of hepatocytes were incubated at 37°C in a humidified atmosphere of air containing 5% CO_2. After a 2-hr attachment period, media was renewed and metyrapone (0.5 mM) added. Metyrapone was dissolved directly into the culture media and passaged through a 0.2-μm filter unit. Media and supplements were replaced after 24 hr.

Hepatocytes were harvested from 0–48 hr, as indicated in legends to Figs. and Tables. Media was aspirated from culture trays and the cells were overlayed with 2 mL ice-cold 0.1 M PBS, pH 7.4, containing 5 mM EDTA. Hepatocytes (including matrigel) were scraped from the culture dishes with a rubber spatula and incubated on ice for 45 min to dissolve the matrigel. Cells were pelleted by centrifugation at $750 \times g$ for 5 min. Hepatocytes for tNA preparation were lysed in 4 mL of lysis buffer: 1% (w/v) SDS, 10 mM EDTA in 20 mM Tris-HCl, pH 7.5.

Solution Hybridization

tNA was prepared from cell lysates as previously described [19]. Nucleic acids were pelleted by centrifugation at $1000 \times g$, dissolved in 100 μL of 4 mM Tris-HCl, pH 7.5 containing 0.2% (w/v) SDS and 2 mM EDTA and stored at −70°C until solution hybridization assay. tNA concentration was determined spectrophotometrically and DNA was quantified by fluorometric assay using the DNA-binding compound Hoechst 33258 [20]. Total RNA concentration was calculated using values obtained from tNA and DNA determinations.

Solution hybridization assays for CYP2C11, CYP3A2, and CYP3A1-like mRNAs were performed using [^{35}S]UTP-labelled cRNA probes. CYP2C11 and CYP3A2 probes were prepared as described elsewhere [9]. cRNA probes were directed against regions of low sequence homology between closely related CYPs. The CYP3A1-like probe was a *Bsr*G1-*Pst*1 fragment corresponding to bases 840–1079 of the cDNA for *CYP3A1* [21]. This probe was unable to distinguish between four cDNA clones: P-450pcn1 (*CYP3A1*, [21]), pP450IGC2 (an allelic variant of *CYP3A1* isolated from phenobarbital-treated Wistar rat liver, [22]), RL33, a glucocorticoid-inducible *CYP3A* isolated from untreated male Sprague-Dawley rat liver [23] and cDEX (an

allelic variant of RL33 prepared from dexamethasone-treated Wistar and Sprague-Dawley rat livers [24]. However, this probe would not be expected to hybridize RNCYP3AM-type mRNA [25]. The fragment was ligated into the appropriate restriction sites of the polylinker region of the plasmid vector pGEM®-3Z. *In vitro* transcription of the cDNA inserts to produce ^{35}S-labelled cRNA probes was accomplished using a commercially available kit (Riboprobe, Promega Corp.).

Probe hybridization with aliquots of tNA was performed under the following conditions: 0.1% (w/v) SDS, 22 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.6 M NaCl, 20% (v/v) formamide, 0.75 mM dithiothreitol, and approximately 20,000 cpm of probe in a final volume of 40 μL. Optimum hybridization temperatures were experimentally determined and samples were incubated for 16 hr at 75°C for CYP2C11 and 65°C for CYP3A2. Solution hybridization using the CYP3A1-like probe was performed at 75°C with a final formamide concentration of 50% (v/v), to ensure no cross-reactivity with the highly homologous CYP3A2 mRNA. Unhybridized [^{35}S]UTP-labelled cRNA probe was removed by digestion of samples with RNase A and RNase T_1. The reaction was terminated after 45 min by the addition of ice-cold trichloroacetic acid (final concentration 0.6 M) and precipitated hybrids were collected on 24-mm Whatman GF/C filters (Whatman Far East, Singapore) that had been moistened with 4% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. Filters were washed as described [19] and added to 6 mL scintillation fluid (ACS II, Amersham Australia, Sydney, Australia). tNA prepared from untreated male rat liver was used to produce standard curves in CYP2C11 and CYP3A2 solution hybridizations and facilitate the calculation of inter-assay variability. Standard tNA for the CYP3A1-like solution hybridization probe was isolated from the liver of a dexamethasone-treated female rat (100 mg/kg body weight, prepared in corn-oil and injected intraperitoneally once daily for 3 days).

RT-PCR of mRNA Prepared From Tissue Culture Samples

Samples of total RNA were prepared from cultures of rat hepatocytes by standard methods [19]. mRNA was reverse transcribed using AMV reverse transcriptase (Promega Corp.) and oligo $(dT)_{15}$ (AMRAD Pharmacia Biotech., Australia) as primer, followed by heat-denaturation of the reverse transcriptase at 95°C for 5 min. The resultant cDNAs were amplified using Taq DNA polymerase (Boehringer Mannheim, Australia) and 'universal' *CYP3A* primers that recognize all reported members of the rat *CYP3A* subfamily. The primer sequences were as follows: upstream 5′-GCACATCATTTGGAGTGAA-3′, bases 638–656 of *CYP3A1*, downstream 5′-AATGCAGTTCCTGGGTCC-3′, bases 1396–1413 of *CYP3A1* [21]. Following 35 cycles of PCR; denature at 95°C for 1 min, anneal at 50°C for 1 min, extend at 75°C for 1 min; the resultant product, approximately 775 base pairs, was electrophoresed on a 1% agarose gel and purified from the gel using standard techniques [26]. The gel-purified DNA was directly cloned into a pGEM-T® vector (Promega Corp.), according to manufacturers instructions. Clones containing the RT-PCR product were restriction mapped using *Apa*I, *Afl*II and *Pst*I restriction endonucleases.

Preparation of Microsomes

Microsomes were prepared from cultured hepatocytes as follows: cell suspensions were homogenized in ice-cold 0.1 M potassium phosphate, pH 7.4, 0.25 M sucrose, and 1 mM EDTA in a Potter-Elvehjem homogenizer. The 10,000 × *g* (25 min) supernatant was centrifuged for 60 min at 105,000 × *g* and the pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4, containing 20% glycerol and 1 mM EDTA). Microsomal fractions were immersed in liquid nitrogen and stored at −70°C until use.

Purification of CYP Proteins and Preparation of Related Antisera

Rabbit anti-CYP3A1 IgG and purified CYP3A1, isolated from sodium phenobarbital-induced rat liver [27], were kindly provided by Dr. A. Åstrom, University of Stockholm, Sweden. This antisera is unable to distinguish between related CYP3A proteins. Rabbit anti-CYP2C11 was prepared and characterized as described elsewhere [28, 29].

Immunoblotting of Proteins on Nitrocellulose Membranes

Microsomal proteins prepared from cultured hepatocytes (1 μg protein/lane) were resolved on 7.5% polyacrylamide gels, according to the method of Laemmli [30] with buffer concentrations as described elsewhere [31]. After electrophoresis, proteins were transferred to nitrocellulose membranes using the method of Towbin *et al.* [32]. Nitrocellulose membranes were washed in Tris-buffered saline containing Tween 20 (TBS-T: 50 mM Tris-HCl, 200 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4) prior to incubation in blocking reagent (TBS-T, 5% (w/v) dry nonfat milk, pH 7.4). Membranes were placed in blocking reagent containing primary antibodies at the following dilutions: anti-CYP3A IgG, 1;10,000 dilution of 54 mg protein/mL and anti-CYP2C11 IgG, 1:2,000 of 37.5 mg protein/mL. Nitrocellulose membranes were washed in TBS-T and incubated with peroxidase-labelled goat antirabbit IgG diluted 1:3,000 in blocking reagent for 30 min. Blots were washed and protein visualized using ECL. ECL detection reagents were used as per supplier instructions and the signal generated was analysed using a densitometer (Molecular Dynamics, Inc.).

Testosterone Hydroxylase Assay

Testosterone hydroxylase assays, used to determine the catalytic activities of CYP2C11 (as the 2α- and 16α-

hydroxylases) and CYP3As (as the 6β-hydroxylases), were performed at 37°C for 10 min in 0.1 M potassium phosphate buffer (pH 7.4, with 1 mM EDTA) in the presence of 0.15 mg of microsomal protein; production of metabolites was linear with respect to time during this interval. The final concentration of [^{14}C]testosterone was 50 μM and the reaction was initiated by the addition of 1 mM NADPH. Assays were terminated by the addition of chloroform, and metabolites extracted as previously described [33]. TLC separation of products was performed according to the method of Waxman *et al.* [34] and identification of metabolites by autoradiography. Testosterone metabolite quantification was determined by scintillation counting.

Other Assays

Hepatic microsomal protein was estimated by the method of Lowry *et al.* [35] with bovine serum albumin as standard. Total cytochrome P450 was determined according to the method of Omura and Sato [36].

Statistics

All data are expressed as mean ± SD of at least 3 individual hepatocyte preparations. The significance of results between groups was determined using the Student's *t*-test.

RESULTS

Microsomal total cytochrome P450, CYP2C11, and CYP3A protein levels, catalytic activities, and mRNA levels in untreated primary cultures of hepatocytes

As previously reported [5–8], constitutive expression of total cytochrome P450 in primary cultures of rat hepatocytes declined within the first 24 hr of culture (Fig. 1). At this time, CYP2C11-mediated testosterone 2α- and 16α-hydroxylase activities [37] were reduced to 28 ± 4% and 29 ± 2% of preculture values (respectively, 1.16 ± 0.15 and 1.21 ± 0.25 nmol/min/mg microsomal protein {*n* = 5})

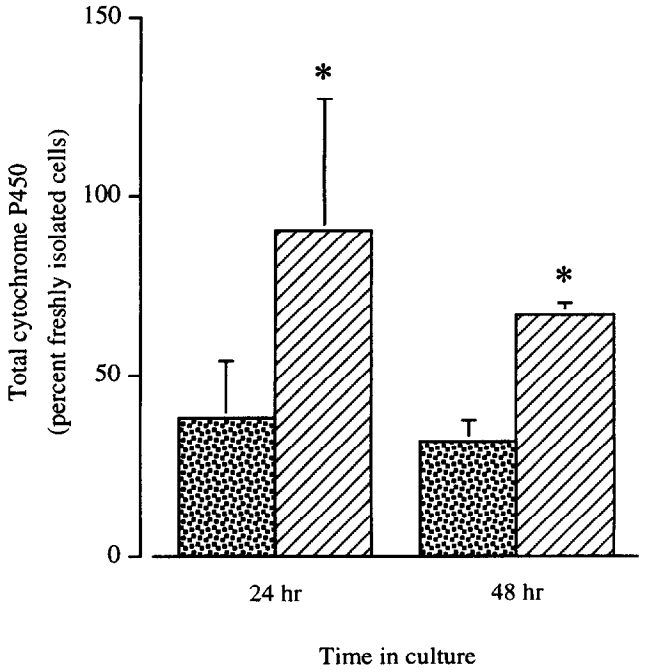

FIG. 1. Total microsomal cytochrome P450 in control (▨) and metyrapone-treated (▨) male rat hepatocytes in primary culture on matrigel. Hepatocytes were cultured for the times indicated prior to preparation of microsomes and determination of total cytochrome P450 content. Data are mean ± SD of at least 3 separate experiments. The value for total microsomal cytochrome P450 in freshly-isolated hepatocytes (n = 14) was 0.55 ± 0.14 nmol/mg microsomal protein. *significantly different from control ($P < 0.001$).

(Table 1). Similarly, testosterone 6β-hydroxylation, which is catalyzed predominantly by CYP3A2 in untreated male rat liver [4, 37], fell to 9 ± 3% of that in freshly isolated cells (1.36 ± 0.24 nmol/min/mg microsomal protein) (Table 1), and CYP2A1-mediated 7α-hydroxylation [37, 38] had declined to 24 ± 12% of that in freshly prepared cells. These CYP-mediated testosterone hydroxylation activities continued to decline over the entire 48-hr culture period (Table 1).

TABLE 1. Effect of metyrapone on CYP-mediated testosterone metabolism by microsomes prepared from hepatocytes cultured on matrigel

Testosterone hydroxylation pathway	Enzyme activity			
	24 hours in culture		48 hours in culture	
	Control	Metyrapone	Control	Metyrapone
2β (0.46 ± 0.30)	138 ± 52*	128 ± 39	112 ± 74	106 ± 12
2α (1.16 ± 0.15)	28 ± 4	64 ± 14†	21 ± 9	52 ± 18
6β (1.36 ± 0.24)	9 ± 3	70 ± 12‡	4 ± 2	48 ± 13‡
7α (1.04 ± 0.47)	24 ± 12	64 ± 29	14 ± 7	46 ± 7‡
16α (1.21 ± 0.25)	29 ± 2	70 ± 3§	26 ± 12	60 ± 16†

* Data expressed as mean ± SD from 3 individual cell culture experiments. All values are the percentage of those found in freshly isolated hepatocytes. Cells were harvested and microsomes prepared as described in Materials and Methods. Testosterone hydroxylation activity in microsomes prepared from freshly isolated hepatocytes, prior to culture, are given in parentheses and shown as mean ± SD nmol/min/mg microsomal protein. Metyrapone (0.5 mM) was added after a 2-hr attachment period. †Significantly different from control, $P < 0.05$. ‡Significantly different from control, $P < 0.01$.

In contrast, testosterone 2β-hydroxylation actually appeared to be elevated during the first 24 hr in culture, to 138 ± 52% of the value in freshly isolated cells. It should be noted that the results for this enzyme were (in contrast to all others studied) more variable, and the apparent difference was not significantly different from freshly prepared cells. As discussed later, this relatively minor pathway of testosterone hydroxylation is catalyzed by CYP3A subfamily proteins [39–41].

In parallel with the changes inferred by their catalytic activities, immunoquantifiable CYP3A and 2C11 proteins fell to 22 ± 3% and 11 ± 1%, respectively, of those in freshly isolated hepatocytes over the 48-hr culture period (Fig. 2). Likewise, CYP2C11, CYP3A2, and CYP3A1-like mRNA concentrations also declined to low levels over the 48-hr culture period. Thus, CYP2C11 mRNA was 4.7 ± 0.4% (Fig. 3A), CYP3A2 mRNA was 8.7 ± 0.9% (Fig. 3B), and CYP3A1-like mRNA (Fig. 3C) was 0.3 ± 1.3% of values for freshly isolated cells by 48 hr.

Effect of Metyrapone on CYP Expression in Cultured Hepatocytes

Treatment of freshly isolated hepatocytes with 0.5 mM metyrapone had a major effect on the maintenance of total cytochrome P450 levels in primary culture (Fig. 1). Similarly, catalytic activities attributable to at least 3 CYP enzymes (Table 1) were preserved in the presence of metyrapone. This appeared to be particularly noticeable for testosterone 6β-hydroxylase activity, which was still present at 70 ± 12% of preculture catalytic activity in metyrapone-treated cells at 24 hr, compared to 9 ± 3% in control cultures ($P < 0.002$, Table 1); similar observations were made after 48 hr in culture. The loss of testosterone 2α- and 16α-hydroxylation activities was also substantially prevented by the addition of metyrapone, as was that of CYP2A1-dependent testosterone 7α-hydroxylation [37, 38] (Table 1). Metyrapone had no definite effect on CYP3A-mediated testosterone 2β-hydroxylation at either 24 hr or

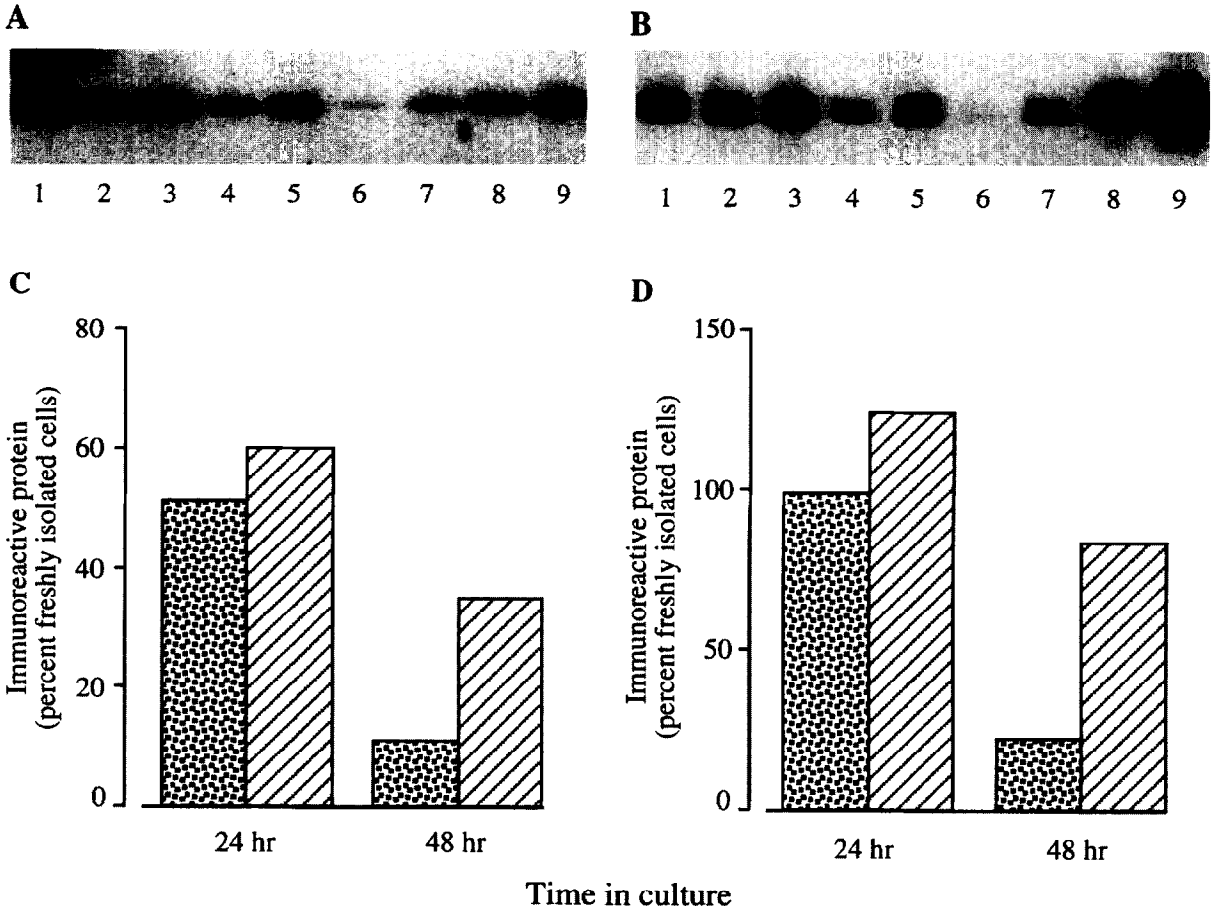

FIG. 2. Immunoblots of CYP2C11 (Panel A) and CYP3A (Panel B) proteins in microsomes prepared from cultured hepatocytes. Each lane was loaded with 1 μg of microsomal protein. Following electrophoresis and transfer to nitrocellulose membranes, bands were visualized using enhanced chemiluminescence and semiquantified using laser densitometry. Lane 1, freshly isolated hepatocytes; lanes 2 and 4, control cells at 24 and 48 hr, respectively; lanes 3 and 5, 0.5 mM metyrapone at 24 and 48 hr, respectively; lanes 6, 7, 8, and 9 contain 2, 4, 8, and 12 ng of standard protein, respectively. Graphic representations of densitometrically quantitated data for control (▒) and metyrapone-treated (▨) cells are shown in Panel C (CYP2C11) and Panel D (CYP3A). Data are expressed as a percentage of the amount of immunoquantifiable protein found in freshly prepared hepatocytes.

48 hr. The data for immunoquantification of CYP2C11 and total CYP3A proteins supported the observed maintenance of CYP enzyme expression by metyrapone (Fig. 2).

Determination of CYP-specific mRNA levels showed that metyrapone treatment failed to exert a pretranslational effect on either CYP2C11 (Fig. 3A) or CYP3A2 (Fig. 3B) expression in cultured rat hepatocytes. Indeed, by 48 hr in culture, mRNA concentrations for both CYPs had fallen to less than 10% of those observed in freshly isolated hepatocytes. In contrast, metyrapone caused a marked increase of CYP3A1-like mRNA throughout the culture period (Fig. 3C). The induction of this (or these) CYP-mRNA species occurred within the first 12 hr of culture and, despite a rapid decline, levels remained at least twice those found in control cells throughout the culture period.

RT-PCR Analysis of mRNA From Cultured Hepatocytes

The riboprobe used in this study to detect CYP3A1-like mRNA was unable to distinguish between closely related CYP3A1-like mRNAs. Therefore, a CYP3A cDNA library was constructed from mRNA isolated from cultured hepatocytes. Reverse transcription of this mRNA and subsequent PCR amplification using universal CYP3A primers yielded a single 775 base-pair fragment. Cloning of this fragment into pGEM-T® followed by restriction analysis of clones revealed that the observed increase in CYP3A1-like mRNA levels was attributable to RL33/cDEX-type mRNA. Thus, among the 20 clones produced from mRNA prepared from untreated hepatocytes that were restriction mapped, and 40 clones isolated from metyrapone-treated hepatocytes, all were either *CYP3A2* (25 clones), RNCYP3AM (23 clones) or RL33/cDEX-type (12 clones) cDNAs (Table 2). We were unable to isolate any clones that produced a *CYP3A1* restriction pattern.

DISCUSSION

The use of primary hepatocyte cultures for the study of hepatic drug metabolism and toxicity is limited by the early and major loss of cytochrome P450 [5, 6]. Many approaches have been explored to circumvent this problem. For example, use of a reconstituted basement membrane, such as matrigel, as the culture substratum can maintain the expression of some liver-specific genes and restore the inducibility of other CYPs [7]. Despite this, the expression of several major constitutive CYPs declines rapidly when rat hepatocytes are placed in culture [5–8], as indicated by the mRNA levels for CYPs 2C11 and 3A2 in the present study. The capability of metyrapone to maintain total cytochrome P450 levels in primary cultures of rat hepatocytes could provide another strategy to improve the utility of this *in vitro* system. Application of this system for studies of gene regulation, however, would require a clear understanding of the molecular mechanisms that underlie the apparent preservation of functional CYP proteins in hepatocytes maintained in the presence of metyrapone.

It has recently been determined that metyrapone treatment of male rats enhances the hepatic activity of CYP3A-dependent enzymes [15]. Prior to this, Padgham *et al.* [14] reported that rat hepatocytes cultured in the presence of metyrapone exhibited elevated levels of CYP3A1/2 mRNA, but the techniques used would not distinguish between CYP3A2, CYP3A1, and other CYP3A mRNAs that are closely related to CYP3A1. In the present study of hepatocytes cultured on matrigel, addition of metyrapone increased levels of total cytochrome P450 compared to untreated cells, as reported in other cell culture systems [6, 11–13]. In line with this, metyrapone also substantially ameliorated the decline in catalytic activity that is known to be mediated by the major male-specific CYP, CYP2C11. This interpretation of the enzyme changes was substantiated by determination of CYP2C11 protein levels. Metyrapone was also demonstrated to prevent the fall in catalytic activities attributable to CYP3A subfamily members [4, 37, 39–41], notably testosterone 6β- and 2β-hydroxylation. Metyrapone mitigated against the fall in microsomal content of total CYP3A protein, but it must be noted that the polyclonal antibody used in these studies is unable to distinguish between the several members of the rat CYP3A subfamily.

To clarify the specific effects of metyrapone on constitutive CYPs in cultured rat hepatocytes, studies of mRNA expression were undertaken. Two of the three cRNA probes used were gene-specific, for CYP2C11 and CYP3A2 mRNA. Metyrapone failed to maintain levels of CYP2C11 or CYP3A2 mRNA in cultured rat hepatocytes. Indeed, levels of the respective mRNAs were less than 10% of those in freshly isolated cells after 48 hr in culture. In contrast to this, the respective protein levels at the same time were maintained at approximately 80% and 40% of those in freshly prepared cells. In the case of CYP2C11, in particular, this is consistent with the proposal that a major mechanism for the effect of metyrapone in preventing the loss of CYP proteins in culture is posttranslational, most likely due to impairment of protein catabolism.

The third cRNA probe, designated 'CYP3A1-like,' was unable to distinguish between mRNAs produced by the following genes: *CYP3A1* [21], p450IGC2 which is an allelic variant of *CYP3A1* isolated from phenobarbital-treated Wistar rat liver [22], a recently reported glucocorticoid-inducible member of the *CYP3A* subfamily, RL33 [23], and it's allelic variant, named cDEX [24]. Use of the CYP3A1-like probe produced a striking novel finding. Thus, in contrast to CYP2C11 and CYP3A2 mRNAs, which fell irrespective of metyrapone, metyrapone-treated cells exhibited elevated levels of CYP3A1-like mRNA throughout the culture period. To examine the significance of this finding for individual *CYP3A1*-like genes, the mRNA isolated from cultured hepatocytes was further analyzed using *CYP3A* cDNA libraries constructed by RT-

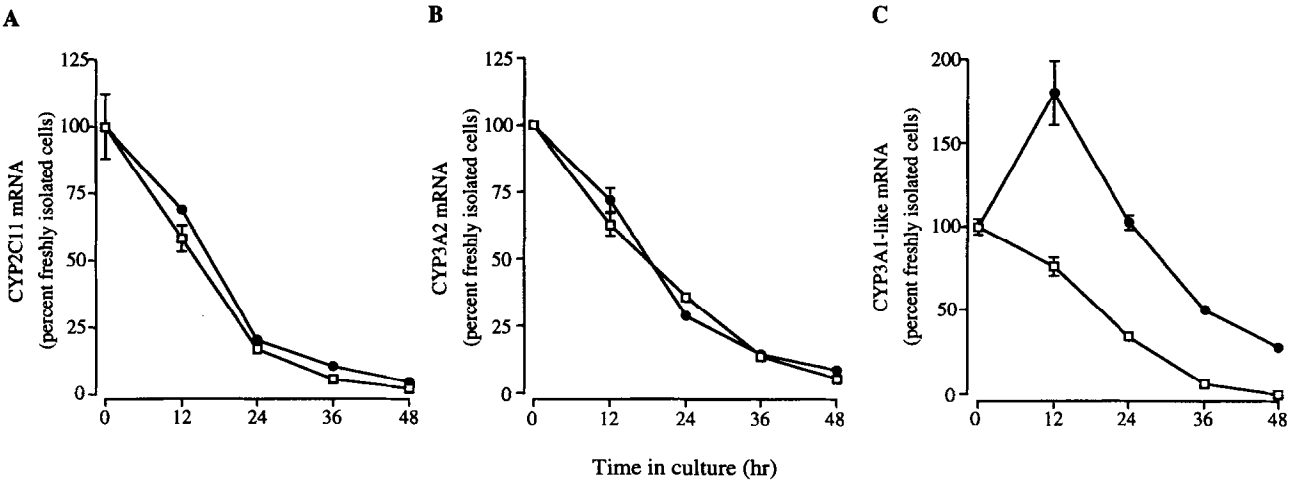

FIG. 3. Levels of CYP2C11 mRNA (Panel A), CYP3A2 mRNA (Panel B), and 'CYP3A1-like' mRNA (Panel C) in control (□) and metyrapone-treated (●) cultures of rat hepatocytes. Cells were maintained on matrigel in modified Waymouth medium and harvested for preparation of tNA at the times indicated. CYP mRNA was estimated by solution hybridization as described in Materials and Methods. CYP mRNA (expressed per microgram of DNA) is shown as a percentage of that obtained from freshly isolated hepatocytes (the values in freshly isolated cells were 2830 ± 350, 1760 ± 350, and 240 ± 10 cpm/μg DNA for CYP2C11, CYP3A2, and 'CYP3A1-like' mRNA, respectively, in the example shown). Each point represents the mean of triplicate determinations of tNA from 5 pooled culture dishes.

PCR. The primers employed for amplification of the cDNAs were universal for all reported rat *CYP3As* and the assumption was made that the primers were nonselective in their ability to amplify all *CYP3A* subfamily members. The results of restriction mapping of the cloned products indicated that the observed elevation in CYP3A1-like mRNA resulted from the induction or stabilization of RL33/cDEX mRNA. Thus, all constructs examined contained either *CYP3A2*, RNCYP3AM, or RL33/cDEX cDNAs. RNCYP3AM was isolated by Strotkamp *et al.* [25] from adult female Sprague-Dawley rat liver, but has also been shown to be present in male rats. In the present work, it was expressed in control and metyrapone-treated cells at unexpectedly high levels. It is important to note, however, that the CYP3A1-like riboprobe was constructed so as to be unable to hybridize with RNCYP3AM-type mRNA. The apparent elevated expression of this gene may be a result of the loss of *in vivo* regulatory factors when hepatocytes are placed in culture.

In uninduced male rat liver, CYP3A2 is responsible for 85% of testosterone 6β-hydroxytestosterone formation [4]. Administration of agents such as pregnenolone 16α-carbonitrile can change the pattern of steroid metabolism by inducing CYP3A1 which, then, becomes the predominant catalyst of testosterone 6β-hydroxylase activity [37]. In the male Wistar rats used for these studies, however, CYP3A1 was not expressed under a variety of experimental conditions (present results, and unpublished observations of M. Murray *et al.* 1994). As others have recently reported [23, 24], we found a relative abundance of the CYP3A1-like mRNA, RL33/cDEX in control hepatocytes. It is possible that increased levels of RL33/cDEX-type mRNA may contribute to the preservation of microsomal content of CYP3A protein in metyrapone-treated hepatocyte cultures. However, because the catalytic functions of RL33/cDEX are not yet characterized, the extent to which preservation of this protein contributes to the increase in CYP3A-mediated testosterone 6β-hydroxylation in metyrapone-treated cells compared with untreated cells is unclear.

It is evident from the above considerations that metyrapone can act at both a pretranslational level, as in the case of RL33/cDEX-type mRNA, and at a posttranslational level, as indicated by the protein and catalytic data for CYP2C11 (and probably CYP2A1). Whether or not metyrapone can modify the expression of CYP3A2 at a posttranslational level has not been demonstrated unambiguously in the present culture system because the antiserum used to immunoquantitate CYP3A protein is unable to distinguish between members of the CYP3A subfamily. Until a catalytic activity is assigned to the protein associated with the RL33/cDEX-like mRNA, it is difficult to formulate the precise mechanism for the stabilization of individual CYP3A proteins by metyrapone.

TABLE 2. Distribution of *CYP3A* RT-PCR clones prepared from mRNA isolated from cultured rat hepatocytes

	Number of clones	
***CYP3A* cDNA**	**Control cells**	**Metyrapone-treated cells**
*CYP3A2**	9	16
CYP3A1†	0	0
RL33/cDEX‡	5	7
p450IGC2§	0	0
RNCYP3AM‖	6	17
Total clones	20	40

* Gonzalez *et al.*, 1986 [42]. †Gonzalez *et al.*, 1985 [21]. ‡Komori *et al.*, 1994 [23] and Kirita *et al.*, 1993 [24]. §Ribeiro *et al.*, 1992 [22]. ‖Strotkamp *et al.*, 1995 [25].

In summary, the present results indicate complexities in the regulation of individual CYP proteins in cultured male rat hepatocytes, including those within the same gene subfamily. Depending on the gene, the level of expression may be enhanced at both pretranslational (as in the case of RL33/cDEX-type mRNA, and posttranslational levels (for CYP2C11). The data indicate the potential value of well-differentiated hepatocyte culture systems for the study of CYP gene regulation, but also demonstrate why it is important to appropriately integrate information about enzyme expression at multiple molecular levels when considering the mechanism for CYP enzyme regulation.

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