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Selective Induction of Cytochrome P450 3A1 by Dexamethasone in Cultured Rat Hepatocytes

Analysis With a Novel Reverse Transcriptase–Polymerase Chain Reaction Assay

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ABSTRACT. The study of drug metabolism in cultured rat hepatocytes is hampered by the rapid loss of the expression of cytochrome P450 enzymes. Nevertheless, the activity of cytochrome P450 3A (CYP3A), one of the most important isoenzymes for drug metabolism, can be elevated by chemical inducers. In the present study, we investigated in cultured rat hepatocytes the induction of all four currently identified CYP3A isoforms by dexamethasone, and compared the results obtained *in vitro* with the induction profile of dexamethasone *in vivo*. To this end, CYP3A mRNA levels were quantified with a novel, radioactive reverse transcriptase–polymerase chain reaction (RT–PCR) assay, and CYP3A enzymatic activity was measured by a testosterone hydroxylation assay. In the RT–PCR assay, CYP3A isoforms were co-amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of radioactively labeled nucleotides. This resulted in an extremely sensitive and accurate determination of CYP3A expression levels, relative to those of GAPDH. Using this RT–PCR assay, it was found that the expression of all CYP3A isoforms in rat hepatocytes, cultured on a collagen matrix, was decreased by 80–90% within one day of cultivation. After addition of dexamethasone, at one day after isolation, CYP3A1 mRNA levels were elevated to levels comparable to those in freshly isolated hepatocytes within two days. In contrast, CYP3A2, CYP3A9, and CYP3A18 mRNA levels were not affected by dexamethasone treatment, and were hardly detectable after three days of cultivation. CYP3A enzymatic activity was also induced in cultured hepatocytes (approximately 6-fold) after addition of dexamethasone. *In vivo*, CYP3A1 mRNA levels increased 45-fold after dexamethasone administration. However, in contrast to the situation in cultured hepatocytes, CYP3A2 and CYP3A18 were also induced, albeit to a lesser extent (4- and 7-fold elevated mRNA levels, respectively). We conclude that the selective induction of CYP3A1 in dexamethasone-treated rat hepatocytes allows the study of biotransformation reactions by CYP3A1, without interference by any of the other CYP3A isoenzymes. BIOCHEM PHARMACOL **60**;10:1509–1518, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. primary rat hepatocyte; cytochrome P450; drug metabolism; gene expression; RT–PCR; testosterone hydroxylation

Primary cultures of rat hepatocytes constitute an attractive model for the study of drug metabolism. The identification of biotransformation reactions *in vitro* is favored over the *in vivo* analysis of metabolic pathways both because of the reduced complexity of the system, and the reduction of the number of animals used in the experiments. Unfortunately, the use of rat hepatocyte cultures is hampered by the rapid loss of expression of CYP§ isoenzymes [1], the main catalysts of phase I biotransformation and bioactivation reactions [2]. Several approaches have been followed in attempts to maintain differentiated hepatocyte functions in culture, including hormonal supplementation [3], application of special culture matrices [1, 4], and co-culture with non-parenchymal cells [5, 6]. However, culture conditions that maintain, for extended time periods, expression levels of biotransformation enzymes that are comparable to the *in vivo* situation have not yet been reported.

Of all CYP families, the CYP3A subfamily is of uttermost importance for biotransformation in humans. Approximately 50 percent of all new drugs are metabolized by the human CYP3A4 and CYP3A5 isoenzymes. In the rat, five *CYP3A* genes have been identified: *CYP3A1* [7], *CYP3A2* [8], *CYP3A9* [9, 10], *CYP3A18* [11, 12], and *CYP3A23* [13–16]. As it appeared recently that *CYP3A23*, and not the originally discovered *CYP3A1* gene [7], encodes the active CYP3A1 protein [17], we refer to CYP3A23 as

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§ *Abbreviations:* CYP, cytochrome P450; RT–PCR, reverse transcriptase–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DEPC, diethylpyrocarbonate; DMEM, Dulbecco's modified Eagle's medium; HNF, hepatocyte nuclear factor; DexRE, dexamethasone response element; DR-3, direct repeat-3; RXR, retinoid X receptor; PXR, pregnenolone X receptor; and COUP-TF, chicken ovalbumin upstream promoter-transcription factor.

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CYP3A1 in our study. The rat CYP3A isoenzymes show a high homology to the members of the human CYP3A subfamily, both in amino acid sequence and in function. Various studies, performed with cultured hepatocytes as well as *in vivo*, demonstrated that in the rat CYP3A activity can be induced by glucocorticoids (e.g. dexamethasone) and antiglucocorticoids (e.g. pregnenolone 16α-carbonitrile) [1, 7, 10, 18–22]. However, in none of these studies was the induction of all four presently identified CYP3A isoenzymes in cultured hepatocytes investigated separately.

In the present study, we examined the effects of dexamethasone on the expression of CYP3A isoenzymes in cultured rat hepatocytes and correlated the effects found *in vitro* with the induction of CYP3A isoenzymes *in vivo*. To be able to detect changes in the expression of both high and low abundant CYP3A mRNAs in small amounts of cells and tissue, we developed a novel, quantitative RT–PCR assay. By adding radioactively labeled nucleotides to the PCR reactions, the sensitivity and accuracy of the assay were increased compared to conventional RT–PCR assays. The mRNA levels of the CYP3A isoenzymes were quantified by relating them to the simultaneously amplified mRNA of the housekeeping gene *GAPDH*. In addition to measuring effects at the mRNA level, CYP3A enzymatic activity was determined by assay of testosterone 6β-hydroxylation.

MATERIALS AND METHODS

Materials

Collagenase type IV, BSA fraction V, insulin, dexamethasone, testosterone, corticosterone, glucose 6-phosphate, NADP, DEPC, and yeast tRNA were obtained from Sigma. Amylym (Lintner's Starch) was from J.T. Baker. [α-^{32}P]dCTP was purchased from Amersham. 6β-OH-testosterone was from Steraloids. Guanidinium isothiocyanate was purchased from Promega. Collagen-S type I and glucose-6-phosphate dehydrogenase were from Boehringer Mannheim. DMEM, fetal bovine serum, and penicillin/streptomycin were obtained from Bio Whittaker. Tris-saturated biophenol, pH 8 was purchased from Biosolve. Ribogreen™ was from Molecular Probes. Superscript™ II reverse transcriptase and RNaseH were obtained from Life Technologies. Oligonucleotides and Goldstar DNA polymerase were from Eurogentec. All other chemicals were of analytical grade.

Animals

Male Wistar WU rats were used for all experiments. The animals were fed *ad lib*. on regular chow and had free access to drinking water.

Isolation and Culture of Rat Hepatocytes

Hepatocytes were isolated between 9 a.m. and 10 a.m. from male Wistar rats (strain code: Crl: (WI) WU BR), weighing between 200 and 300 g, by collagenase perfusion according to the method of Seglen [23]. After isolation, cells were washed 4 times with DMEM, containing 0.2% (w/v) BSA, 140 mU mL^{-1} of insulin, 2 mM l-glutamine, 100 U mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin. The cells (viability >80%, as judged by trypan blue exclusion) were seeded in 12-well plates, coated with collagen-S type I (4 μg/cm^2), at a density of 8×10^4 cells/cm^2. To allow adherence, the cells were initially cultured for 3.5 hr in DMEM containing 10% (v/v) fetal bovine serum, 140 mU mL^{-1} of insulin, 2 mM L-glutamine, 100 U mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin in a humidified 5% CO_2 atmosphere at 37°. Thereafter, non-adhering cells were washed away and the incubation medium was changed to serum-free DMEM, containing 0.2% (w/v) BSA, 140 mU mL^{-1} of insulin, 2 mM L-glutamine, 200 U mL^{-1} of penicillin, and 200 μg mL^{-1} of streptomycin. The hepatocytes formed a confluent monolayer within 1 day after seeding. The medium was refreshed every day. For induction of CYP3A expression, dexamethasone (as a 10-mM solution in DMSO; final concentration: 10 μM) was added to the medium at 24 hr after seeding of the cells.

Induction of CYP3A In Vivo

Male Wistar rats (strain code: HsdCpb: WU), weighing between 220 and 240 g, were, for three consecutive days at 10 a.m., intraperitoneally injected with dexamethasone at a dose of 100 mg/kg body weight. The injected dexamethasone was dissolved in amylym suspension (1% (w/v) starch, boiled in 50 mM potassium phosphate buffer, pH 7.45). Control rats received only amylym suspension. Animals were killed by decapitation 24 hrs after the last injection. A part of the liver was frozen in liquid nitrogen for RNA isolation. Microsomes were isolated from the remaining part of the liver.

Isolation of RNA

RNA was isolated according to Chomczysnki *et al.* [24] with a few modifications. For RNA isolations from total liver, pieces of approximately 0.1 g of frozen liver tissue were homogenized in 2 mL of GTC solution (4 M guanidinium isothiocyanate in 25 mM sodium citrate buffer, pH 7.0, containing 0.5% *N*-lauroylsarcosine and 0.1 M β-mercaptoethanol) by beating for 2 × 60 sec in a mini-bead beater (Biospecs Products) in the presence of 0.4 g of glassbeads (Ø 1.0 mm). The liver homogenates were divided into 3 aliquots of 0.5 mL, and RNA was extracted from the samples in triplicate. For RNA isolation from cultured hepatocytes, cells were dispersed in GTC solution (0.5 mL/well) and agitated briefly. The dissolved samples were stored at −80° for up to one week. RNA was extracted by the sequential addition of 0.1 vol. of 2 M sodium acetate pH 4.0, 1 vol. of Tris-saturated phenol (pH 8), and 0.2 vol. of chloroform:isoamylalcohol 49:1 (v/v). After each addition, the mixtures were agitated for 30 sec and placed on ice

TABLE 1. Primers used in PCR reactions

Messenger	Forward primer	Reverse primer	Fragment size	Ref
CYP3A1	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC	329	[10]
CYP3A2	TACTACAAGGGCTTAGGGAG	CTTGCCTGTCTCCGCCTCTT	348	[10]
CYP3A9	GGACGATTCTTGCTTACAGG	ATGCTGGTGGGCTTGCCTTC	373	[10]
CYP3A18	TCCTGTCTCCAACCTTCACC	CACTCGGTTCTTCTGGTTTG	413	
GAPDH	TTCAACGGCACAGTCAAG	CACACCCATCACAAACAT	240	

for 15 min. Samples were centrifuged in a microcentrifuge at 10,000 *g* for 20 min to allow phase separation. The aqueous phase was subjected to a second phenol/chloroform extraction. RNA was precipitated by adding 2.5 vol. of ice-cold ethanol. The mixture was placed at −20° for at least 1 hr and centrifuged at 10,000 *g* for 45 min. Pellets were washed twice with 70% ethanol in DEPC-treated water and dissolved in 20 μL of DEPC-treated TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The RNA concentration in the samples was determined with the RNA-specific fluorescent dye Ribogreen™. Yeast tRNA was used to construct a calibration curve.

Reverse Transcriptase–Polymerase Chain Reaction: cDNA Synthesis

cDNA synthesis was performed on 1 μg of total RNA dissolved in 11 μL of DEPC-treated water. After the addition of 1 μL of 0.1 mM oligo$(dT)_{15}$ primer, samples were heated to 70° for 10 min and subsequently placed on ice. Then, 2 μL of 10 × PCR buffer (500 mM Tris–HCl pH 8.3, 750 mM KCl), 2 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, and 2 μL of 0.1 M dithiothreitol were added, and the samples were incubated at 42° for 5 min. Then, 1 μL of superscript II™ reverse transcriptase (40 U/μL) was added, and the reverse transcription reaction was allowed to proceed for 50 min at 42°. The reverse transcriptase was inactivated at 70° for 15 min. The mixture was not subjected to a subsequent RNaseH treatment, as it was found that treatment with 0.5 U of RNaseH did not result in significant changes in the amounts of PCR product formed in the subsequent PCR reactions.

Reverse Transcriptase–Polymerase Chain Reaction: PCR Reaction

A PCR reaction was performed using 0.5% of the amount of reverse-transcribed RNA and the primer sets depicted in Table 1. Primers specific for a CYP3A isoform and those specific for GAPDH were added to the same reaction tube. For detection of CYP3A2 and CYP3A9 mRNA, primers described earlier by Mahnke *et al.* were used [10]. CYP3A1 was amplified using primers earlier reported to be specific for CYP3A23 [10], since it appeared recently that CYP3A1 is probably identical to CYP3A23 [17]. A novel set of primers was designed for the detection of CYP3A18 mRNA, as the primers described by Mahnke *et al.* [10] might interfere with GAPDH amplification. All PCR primers were chosen to span at least one intron, so that the amplification products of mRNA and possibly present genomic DNAA contaminations would appear as distinct bands after polyacrylamide gel electrophoresis. Primers for 3A1 and 3A2 were checked for the absence of cross-reactivity using isolated cDNA clones, which were kindly provided by K. Nagata, Tohoku University, Sendai, Japan. The PCR reactions were performed in 50-μL reaction mixtures, containing 25 mM TAPS (*N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid, pH 9.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 μM of each dNTP, 1 μCi of [α-^{32}P]dCTP, 2 ng/μL of each primer, and 0.5 U GoldStar DNA polymerase. After 5 min of denaturation at 94°, the reaction mixtures were subjected to 18 PCR cycles, consisting of 1 min at 94°, 1 min at 52°, and 1 min at 72°, followed by a final extension step of 10 min at 72°. Aliquots of 20 μL of the amplified mixtures were mixed with 2.5 μL of loading buffer (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol). The samples were subjected to electrophoresis in 10% polyacrylamide gel (1.5-mm thickness) under non-denaturing conditions in 1 × TBE buffer (0.1 M Tris, 0.1 M boric acid, 2.5 mM EDTA, pH 8.0) at 100 V until the xylene cyanol disappeared from the gel. The gels were fixed with 7% acetic acid, washed five times with water, and exposed overnight to a phosphorimager screen. The radioactivity present in the CYP3A and GAPDH bands was quantified using ImageQuant™ software (Molecular Dynamics). Amplification efficiencies were calculated from plots of the ^{2}logs of the intensities of PCR product bands versus the number of PCR cycles using the formula: $E = (2^{slope} - 1) \times 100\%$.

Isolation of Microsomes

Liver tissue was homogenized in 2 volumes of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.155 M NaCl. The homogenate was centrifuged for 20 min at 12,000 *g*, and subsequently the microsomes in the supernatant were pelleted by centrifugation for 60 min at 100,000 *g*. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 25% (v/v) glycerol. Microsomes were stored at −80° until analysis of testosterone hydroxylation activity. Protein concentrations in the microsomal preparations were determined by the method of Lowry with a BSA standard [25].

Assay of Testosterone Hydroxylation

Testosterone hydroxylation was measured in cultured rat hepatocytes and in isolated microsomes. Cultured rat hepatocytes were incubated with 100 μM testosterone (added as solution in DMSO; final DMSO concentration in medium: 0.1% v/v). After 1 hr of incubation at 37°, medium was collected and stored at −80° until analysis. The testosterone hydroxylation activity in microsomal preparations was determined as described by Wortelboer *et al.* [26], with some minor adjustments. Approximately 2 mg of microsomal protein was preincubated for 10 min at 37° in 2 mL of 50 mM HEPES buffer (pH 7.4), containing 5 mM $MgCl_2$, 38 mM KCl, 1 mM NADP, 8 mM glucose 6-phosphate, and 4 U glucose-6-phosphate dehydrogenase. After addition of testosterone to a final concentration of 1 mM (as a methanolic solution; final methanol concentration: 1% v/v), an aliquot of 1 mL was taken for background determination. The remaining reaction mixture was incubated for 20 min at 37°. The amounts of testosterone and its metabolites in the microsomal reaction mixtures and the hepatocyte medium were determined as follows. Aliquots of 0.5–1.0 mL were supplemented with 20 nmol of corticosterone as an internal standard, and proteins were precipitated by the addition of 1 vol. of 3% $HC1O_4$ and centrifugation for 30 min at 10,000 *g* at 4°. Testosterone, its metabolites, and the internal standards were extracted from the supernatant by the addition of 1.5 vol. of dichloromethane. The dichloromethane layer was evaporated by nitrogen stream at 65°. The residue was dissolved in 300 μL of HPLC mobile phase (60% water, 39% methanol, 1% acetic acid), and 100 μL of the solution was injected onto a Chrompack C18 reversed-phase column (3 × 100 mm, particle size 5 μm). Testosterone, its metabolites, and the internal standard were separated by isocratic elution with the mobile phase described above. The flow rate was 0.6 mL min^{-1} and the compounds were detected at 254 nm. In each experiment, a calibration curve ranging from 0.1 to 20 nmol of 6β-OH-testosterone was constructed. The retention time of 6β-OH-testosterone under these conditions was approximately 4 min.

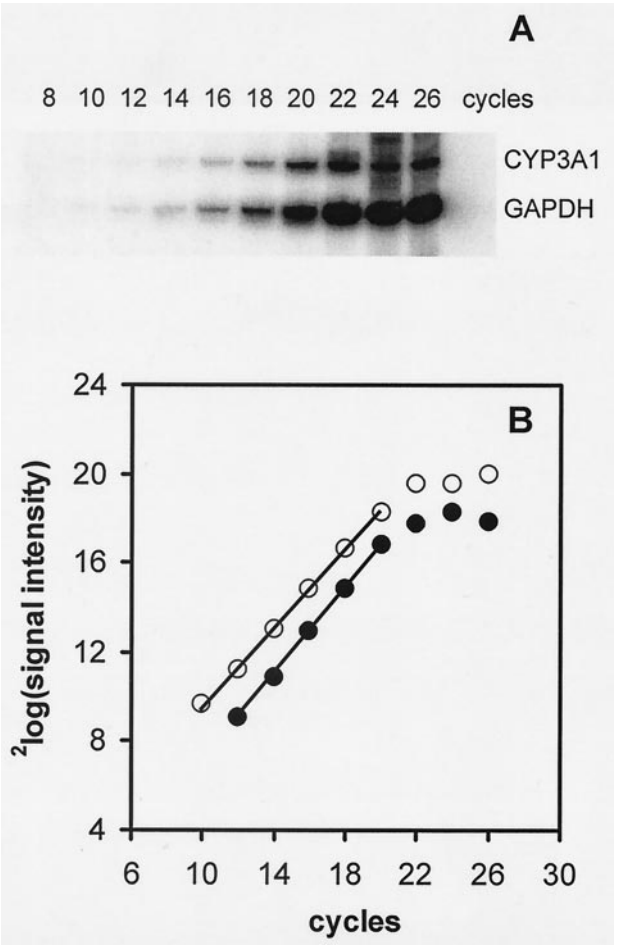

FIG. 1. Determination of PCR product formation at increasing PCR cycles. A multiplex PCR was performed on reverse-transcribed RNA from freshly isolated rat hepatocytes with CYP3A1- and GAPDH-specific primers in a total reaction volume of 200 μL. After every 2 cycles, samples of 4 μL were taken, subjected to polyacrylamide gel electrophoresis, and exposed to a phosphorimaging screen (Panel A). The intensities of the bands of the CYP3A1 (●) and GAPDH (○) amplification products were quantified with ImageQuant software, and the ^{2}log values of the intensities are plotted against the number of the PCR cycles (Panel B). Regression lines were calculated taking into account 10–20 (GAPDH) or 12–20 (CYP3A1) cycles.

RESULTS

Development of a sensitive and quantitative RT–PCR assay

To detect changes in cytochrome P450 levels in small amounts of cells or tissue, a sensitive RT–PCR assay was developed. The expression of the different CYP3A isoenzymes was quantified by relating their expression to that of the housekeeping protein GAPDH. For this purpose, cDNA was synthesized from total cellular RNA by reverse transcriptase in the presence of oligo(dT) primers. Thereafter, a multiplex PCR reaction was performed on the reverse-transcribed RNA with two sets of primers, one being specific for a CYP3A isoform and the other specific for GAPDH. The primers used in the PCR reactions are shown in Table 1. The mRNA levels of the GAPDH internal control were found to be comparable under all tested conditions. Radiolabeled [α-^{32}P]dCTP was added to the PCR reaction mixtures to enable, especially at a low number of PCR cycles, a highly sensitive detection of polyacrylamide gel-separated PCR products with phosphorimaging techniques.

For a quantitative RT–PCR assay, it is essential to stop the reaction in the exponential phase of the PCR amplification. For all CYP3A/GAPDH combinations, we measured the PCR product formation as a function of the number of PCR cycles. As an example, Fig. 1 shows for the multiplex PCR reaction of CYP3A1 and GAPDH that the PCR amplification was exponential between 12 and 20 PCR cycles and leveled off at higher numbers of PCR

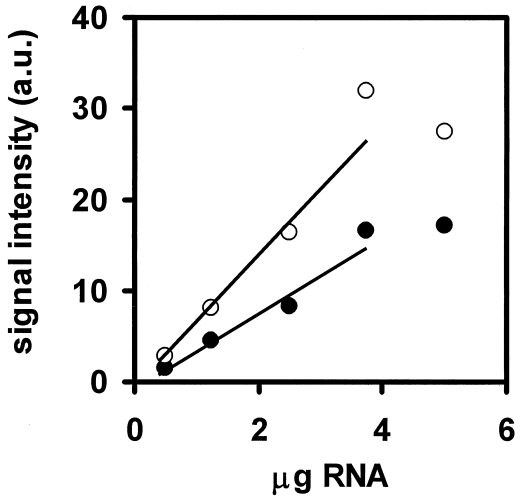

FIG. 2. PCR product formation from CYP3A1 and GAPDH mRNAs at different amounts of RNA added to the RT reaction. Reverse transcription reactions were performed on 0.5–5 μg of total RNA from freshly isolated hepatocytes. Aliquots of 0.5% of the RT reaction mixture were amplified in a multiplex PCR with CYP3A1- and GAPDH-specific primers. The PCR mixtures were subjected to polyacrylamide gel electrophoresis and the products were quantified with a phosphorimager. The intensities of the signals derived from the CYP3A1 (●) and GAPDH (○) PCR products are expressed in arbitrary units (a.u.). In the range of 0.5–3.75 μg RNA, squared linear regression coefficients for CYP3A1 and GAPDH are 0.969 and 0.975, respectively.

cycles. Amplification of CYP3A2, CYP3A9, and CYP3A18 was also exponential for up to 20 PCR cycles (not shown). When a multiplex PCR is to be used quantitatively by relating PCR products, it is necessary that the replication efficiencies be similar for all templates. From the slopes of the semilogarithmic plots of the amounts of PCR product against the number of amplification cycles (Fig. 1B), it appears that CYP3A1 cDNA and GAPDH cDNA were amplified with similar efficiencies. Also, when the low abundant CYP3A9 cDNA was amplified together with the high abundant GAPDH cDNA, the replication efficiencies were highly similar, although the absolute amounts of formed PCR products largely differed. The amplification efficiencies of the four different CYP3A cDNAs and the GAPDH cDNAs were all in the range of 85 ± 4%. The similar replication efficiencies result in CYP3A/GAPDH ratios that remain constant for up to 20 PCR cycles.

For a quantitative determination of mRNA levels by RT–PCR, it is essential that the amount of PCR product be proportional to the amount of RNA added to the assay. We therefore determined the relation between the amounts of radiolabeled PCR product and the amounts of RNA added to the RT reaction. In Fig. 2, the signals derived from the radiolabeled CYP3A1 and GAPDH PCR products are plotted against the amounts of RNA added. The amounts of PCR products increased linearly with the amounts of RNA added in the RT reaction provided that no more than 3.75 μg of RNA was used. In the range of 0.5–3.75 μg RNA, the ratio of the CYP3A and GAPDH amplification products also remained constant. Furthermore, PCR product formation was found to be linear with respect to the amount of reverse-transcribed RNA added to the PCR reaction provided that no more than 0.75% of the reaction mixture, obtained after reverse transcription of 1 μg of total RNA, was used (not shown). Based on the described experiments, the following conditions were used in all further RT–PCR experiments. The RT reaction was performed with 1 μg of total RNA. For the subsequent PCR reaction, 0.5% of the reverse-transcribed RNA was utilized and the amplification was allowed to proceed for 18 cycles.

Induction of CYP3A in Cultured Rat Hepatocytes

To determine cytochrome P450 levels in cultured hepatocytes and to assess the inducibility of the various CYP3A isoforms by dexamethasone, parenchymal cells were isolated from rat liver and cultured in collagen-S-coated wells in serum-free medium containing insulin. At different time points, total RNA was isolated from the cells and the mRNA levels of the different CYP3A isoenzymes were evaluated with the RT–PCR assay described above. As shown in Fig. 3, all CYP3A mRNAs could be readily detected in freshly isolated hepatocytes. However, already after 3.5 hr of cultivation, mRNA levels were approximately 15–25% lower than the levels before plating (not shown). At 24 hrs after seeding of the hepatocytes, all CYP3A mRNA levels were decreased to 10–20% of the initial values. In order to induce CYP3A expression in the cultured rat hepatocytes, dexamethasone was added 24 hr after seeding of the hepatocytes to a final concentration of 10 μM. Figure 3 shows that 24 and 48 hrs after addition of dexamethasone, CYP3A1 mRNA levels were raised to levels comparable to those found in freshly isolated hepatocytes. CYP3A2, CYP3A9, and CYP3A18 mRNAs, however, were not induced by dexamethasone, and were hardly detectable at 48 and 72 hrs after isolation. The induction of CYP3A1 mRNA by dexamethasone was already observed after 2 hr, and was maximal after two days.

To examine whether the increase in CYP3A1 mRNA levels observed in cultured hepatocytes after addition of dexamethasone was accompanied by an induction of enzymatic activity, testosterone 6β-hydroxylation activity, a specific marker for CYP3A enzymatic activity, was measured. The levels of 6β-OH-testosterone found in the medium after incubation with 100 μM testosterone were increased after exposure of the hepatocytes to dexamethasone. When 10 μM dexamethasone was added to the medium 24 hrs after seeding of the cells, the testosterone 6β-hydroxylation activity in the hepatocytes was increased 6 times by exposure to dexamethasone for 48 hrs. Without dexamethasone treatment, testosterone 6β-hydroxylation activity was hardly detectable at 72 and 96 hr after seeding of the hepatocytes (Fig. 4). In the presence of dexamethasone, CYP3A enzymatic activity remained constant for up to 11 days (not shown). It should, therefore, be concluded

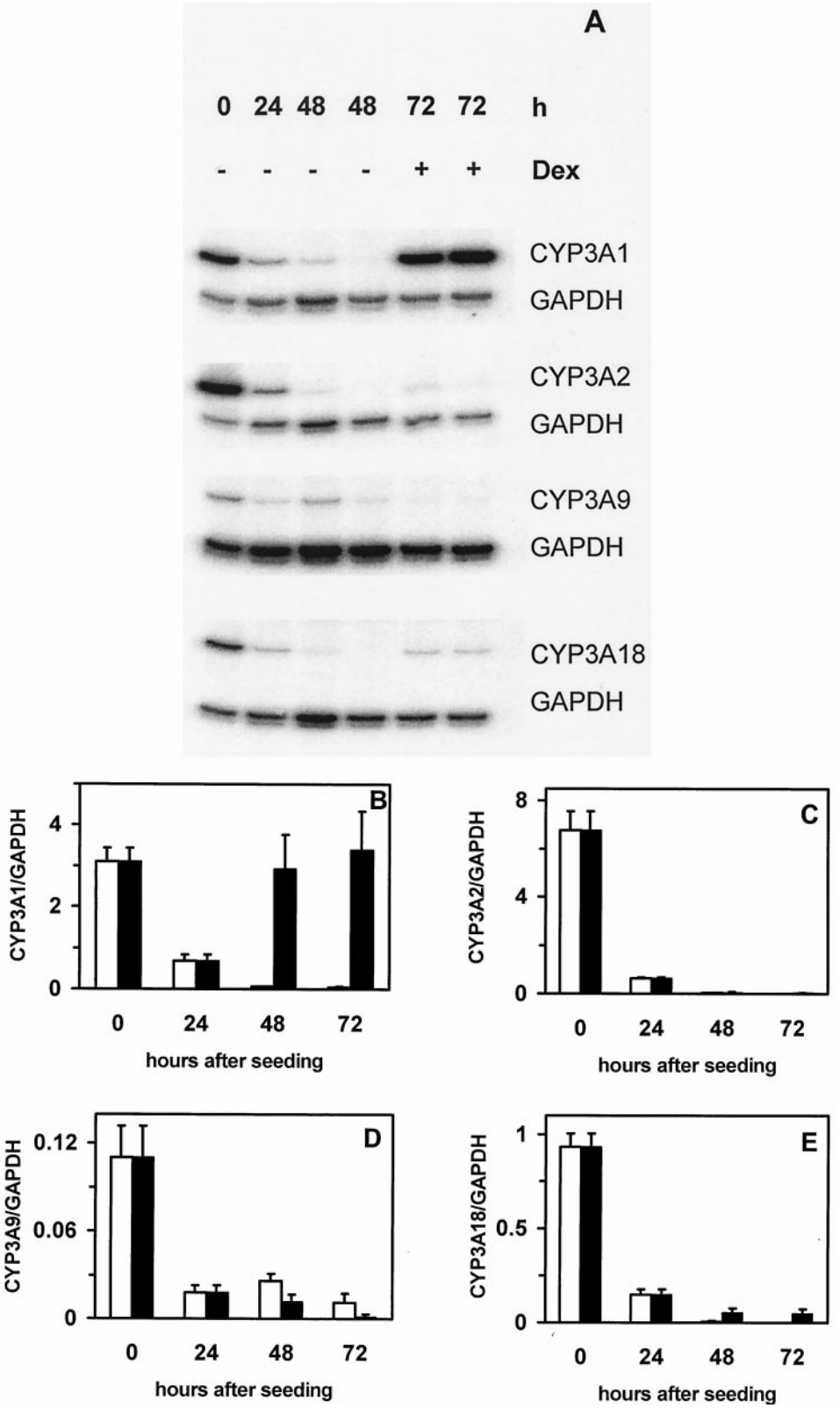

FIG. 3. Expression of CYP3A isoenzyme mRNAs in cultured rat hepatocytes: effects of stimulation with dexamethasone. Rat hepatocytes were isolated and seeded on collagen-S-coated plates. At 24 hr after seeding, hepatocytes were either incubated with 10 μM dexamethasone (closed bars) or left untreated (open bars). RNA was isolated from freshly isolated hepatocytes (0 hr), from hepatocytes cultured for 24 hr, and from hepatocytes cultured for a further 24 and 48 hr (48 and 72 hr after seeding) in the absence or presence of dexamethasone. Quantitative RT–PCR reactions on the four different CYP3A mRNAs were performed. Samples were subjected to gel electrophoresis and scanned with the phosphorimager (Panel A). The expressions of CYP3A1 (Panel B), CYP3A2 (Panel C), CYP3A9 (Panel D), and CYP3A18 (Panel E) mRNAs are plotted as relative to the expression of GAPDH mRNA. Means ± SEM of 6 different RNA isolations from 2 different hepatocyte cultures are shown.

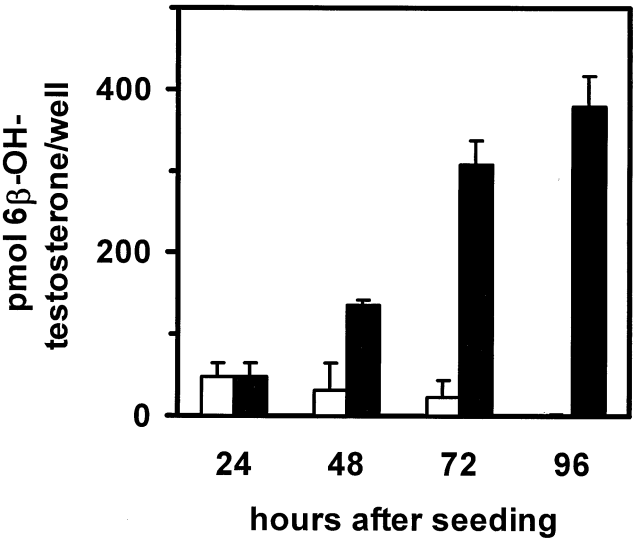

FIG. 4. Formation of 6β-OH-testosterone in cultured hepatocytes: effects of stimulation with dexamethasone. Rat hepatocytes were isolated and seeded on collagen-S-coated plates. After 24 hr of culture, hepatocytes were either incubated with 10 μM dexamethasone (closed bars) or left untreated (open bars). At the indicated time points, cells were incubated with 100 μM testosterone. The formation of 6β-OH-testosterone was analyzed by HPLC with corticosterone as an internal standard. Results are means ± SEM of 9 measurements from 3 different hepatocyte cultures.

that dexamethasone is an inducer of both CYP3A1 mRNA and protein, and that CYP3A1 is the only inducible CYP3A isoenzyme in cultured rat hepatocytes.

In Vivo *Induction of CYP3A by Dexamethasone*

To investigate whether the pattern of induction of CYP3A isoenzymes by dexamethasone that was observed in cultured hepatocytes reflects the induction pattern *in vivo*, male Wistar rats were daily injected with dexamethasone (100 mg/kg intraperitoneally) for 3 consecutive days. At 24 hrs after the last injection, total liver RNA and liver microsomes were isolated. Quantification of mRNA levels by RT–PCR indicated that CYP3A1 is also the major inducible CYP3A isoenzyme *in vivo* (Fig. 5). The level of CYP3A1 mRNA was increased 45-fold after dexamethasone administration. However, unlike in cultured hepatocytes, CYP3A2 and CYP3A18 were also up-regulated by dexamethasone, albeit to a smaller extent (approximately 4- and 7-fold, respectively). The expression of CYP3A9 was not significantly affected by dexamethasone treatment. The increase of the CYP3A mRNAs was paralleled by an induction of CYP3A enzyme activity, which was assayed by measuring testosterone 6β-hydroxylation in microsomes. The formation of 6β-OH-testosterone in microsomes from dexamethasone-treated rats was increased compared to control rats: from 220 ± 30 pmol/min mg protein to 703 ± 121 pmol/min mg protein (means ± SD of 3 rats).

DISCUSSION

In the present study, the mRNA levels of all four currently identified CYP3A isoenzymes were determined in isolated hepatocytes and in rat liver using a novel, quantitative RT–PCR assay. RT–PCR assays have several advantages over conventional assays for RNA quantification such as Northern blotting and nuclease protection assays. Both the sensitivity and specificity of the RT–PCR assay are higher [27, 28]. We further improved the sensitivity and accuracy of the RT–PCR assay by including radioactively labeled nucleotides in the PCR reaction. The detection limit of radiolabeled PCR products by phosphorimaging is orders of magnitude lower than that of conventional ethidium bro-

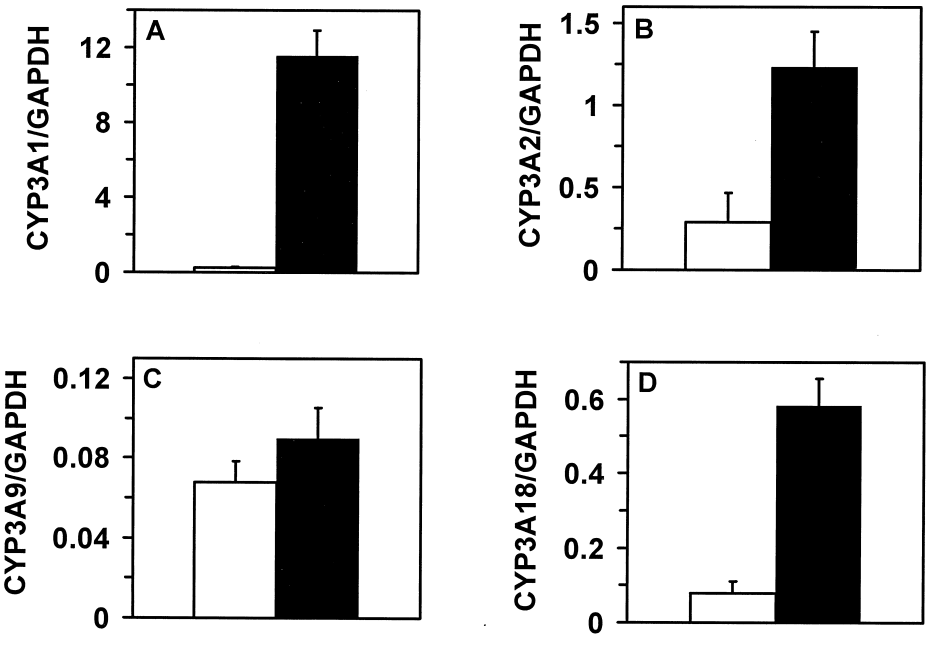

FIG. 5. Induction of CYP3A mRNAs by dexamethasone *in vivo*. Male Wistar rats were treated with 100 mg/kg dexamethasone in amylym suspension for three consecutive days (closed bars). Control rats received only amylym suspension (open bars). Twenty-four hours after the last injection, rats were decapitated. Liver tissue was frozen in liquid nitrogen and total RNA was isolated. Quantitative RT–PCR reactions were performed on CYP3A1 (Panel A), CYP3A2 (Panel B), CYP3A9 (Panel C), and CYP3A18 (Panel D) mRNA with GAPDH mRNA as an internal standard. Results are means ± SEM of 3 rats (3 separate RNA isolations and RT–PCR reactions per rat).

mide staining, while the linear detection range is larger. Even low abundant mRNAs could thus be quantified after 18 PCR cycles, whereas in other reports 26–33 PCR cycles were applied [10, 18, 29]. A low number of PCR cycles is advantageous, because the PCR reaction is then in the phase of exponential amplification and because exponentially amplified experimental errors are minimized [27, 28]. In the present assay, mRNA of the housekeeping gene *GAPDH* was taken as an internal control. It was co-amplified with the mRNA of the gene under investigation, in this case CYP3A, in a single multiplex PCR reaction. As the expression of GAPDH is very constant under the applied conditions, the ratio of CYP3A mRNA over GAPDH mRNA can be used to determine, in different samples, differences in expression levels of CYP3A. The stable expression of GAPDH is in contrast to the expression of β-actin, which has been reported to be highly variable during cultivation of hepatocytes [1]. Application of the GAPDH standard is fast and ensures that tube-to-tube variations are minimized and that gel-loading differences are directly corrected for [27]. It was ascertained that several prerequisites for accurate quantification of gene expression with a co-amplified mRNA were met: equal amplification efficiencies for the different transcripts, detection in the exponential phase of the PCR reaction, and linearity of PCR product formation with respect to the added amount of RNA and cDNA. Also, the CYP3A over GAPDH ratio remained constant with increasing amounts of added RNA. The use of GAPDH as an endogenous standard resulted in good reproducibility of the RT–PCR assay. Although absolute amounts of formed PCR product varied significantly between different RNA isolations from identical cells or tissues, the variability in the CYP3A to GAPDH ratios did not exceed 20%.

With the quantitative RT–PCR assay, we clearly demonstrated that in cultured hepatocytes only CYP3A1 mRNA levels were increased by dexamethasone. CYP3A2, CYP3A9, and CYP3A18 mRNA levels were not affected by dexamethasone treatment. As only very little CYP3A1 mRNA was present before dexamethasone induction, the increase in the levels of CYP3A1 was due to induction at the transcriptional level and not due to decreased degradation of the mRNA. The induction of CYP3A1 mRNA by dexamethasone was accompanied by an increase in CYP3A enzyme activity, as reflected by an enhanced testosterone 6β-hydroxylation in hepatocytes. As none of the other CYP3A mRNAs was up-regulated by dexamethasone, CYP3A1 was most likely the only isoenzyme responsible for this enzymatic activity. This is the first demonstration of the selective induction of CYP3A1 by dexamethasone in cultured hepatocytes, as the oligonucleotide and cDNA probes used in earlier studies after dexamethasone-mediated induction of CYP3A were not sufficiently specific to discriminate between the different CYP3A isoforms [19, 20].

The profile of CYP3A induction by dexamethasone in cultured rat hepatocytes significantly differed from the effect of dexamethasone on CYP3A expression *in vivo*. Although CYP3A1 is also the major dexamethasone-inducible CYP3A isoenzyme *in vivo* (45-fold increase in mRNA levels) CYP3A2 and CYP3A18 mRNA levels were, in contrast to the situation in cultured hepatocytes, also elevated by dexamethasone, albeit to a lesser extent than CYP3A1 mRNA. This finding is in agreement with other studies on the inducibility of CYP3A isoenzymes in rats. In an early study, it was found that dexamethasone only induced CYP3A1 mRNA and CYP3A1 protein, but not CYP3A2 [8]. In later studies, however, it became clear that CYP3A2 expression is, in males, also induced upon treatment with dexamethasone, albeit to a lesser extent [21, 22, 30]. Similarly, in a more extensive study by Mahnke *et al.*, CYP3A2 was found to be induced 2-fold, whereas CYP3A1 (referred to as CYP3A23) was induced 13-fold [10]. In the same study, CYP3A9 and CYP3A18 mRNA levels were also shown to be increased considerably upon dexamethasone treatment (7- and 15-fold, respectively) [10]. In the study by Mahnke *et al.*, Sprague-Dawley rats were used. Our studies were done in Wistar rats, but the expression pattern of the different CYP3A isoenzymes after dexamethasone treatment was comparable to the pattern found in Sprague–Dawley rats with the exception of CYP3A9, which was induced in Sprague–Dawley rats but not in Wistar rats.

Induction of CYP3A mRNA and protein by dexamethasone has also been observed in cultures of human hepatocytes [31]. Human hepatocytes are presently used for the screening of drugs for their potential to induce CYP3A expression, as induction of CYP3A expression may lead to drug–drug interactions [32]. From our experiments in cultured rat hepatocytes and in rats, it appears that careful comparison of the *in vitro* and *in vivo* induction profiles of all isoenzymes, e.g. by RT–PCR analysis, is important when using human hepatocytes as a model system.

The differential induction of CYP3A1 and CYP3A2 expression by dexamethasone reflects a remarkable difference in regulation of *CYP3A1* and *CYP3A2* transcription by glucocorticoids, despite their highly comparable promoter structure. The *CYP3A1* and *CYP3A2* promoters both contain HNF4-binding sites and two dexamethasone response elements, referred to as DexRE-1 and DexRE-2 [33–36]. The DR-3 sequence in DexRE-2, 5′-*AGTTCAN*$_3$*AGTTCA*-3′, can be bound by a heterodimer of PXR and RXRα [37, 38]. Since glucocorticoids serve as ligands for the PXR [37], transcriptional induction of the *CYP3A* genes by dexamethasone is apparently mediated by binding of the PXR/RXRα to DexRE-2 [39]. However, in a recent paper by Huss *et al.*, it was shown that the presence of a protein complex B at the DexRE-1 is an important accessory factor for glucocorticoid induction because it stimulates the binding of the PXR/RXRα to the DexRE-2 [38]. Protein complex B has to compete with COUP-TF for binding to the DexRE-1. Due to small sequence differences, the DexRE-1 of *CYP3A2* is mainly occupied by COUP-TF, whereas the DexRE-1 of *CYP3A1* is preferentially bound by the protein complex B. Therefore, the transcriptional

induction of *CYP3A2* is lower than that of *CYP3A1* [38]. The observation that *CYP3A2* is not induced in cultured hepatocytes forms the first evidence that the DR-3 sequence in the DexRE-2 of *CYP3A2* is not bound by PXR/RXRα in cultured hepatocytes. This can be explained by assuming that the concentration of protein complex B in hepatocytes is lower than *in vivo*. Accordingly, protein complex B is not able to compete with COUP-TF for binding to the DexRE-1 of *CYP3A2 in vitro*, whereas some binding to the DexRE-1 of *CYP3A1* occurs, thereby enabling binding of PXR/RXRα to the DexRE-2 and induction of CYP3A1, although to a lesser extent than *in vivo*.

In summary, we developed a novel RT–PCR assay that allows the accurate quantification of high and low abundant mRNA levels in small amounts of cells or tissue. When primers are chosen appropriately, the assay can be universally applied for the fast and sensitive quantification of gene expression relative to the expression of a housekeeping gene. Using the RT–PCR assay, we demonstrated that CYP3A1 is the only CYP3A isoenzyme that is induced by dexamethasone in rat hepatocytes cultured on collagen-coated plates. Dexamethasone-stimulated hepatocytes can, therefore, be used to study drug metabolism by CYP3A1 without interference by any of the other CYP3A isoforms.

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