Biochemical Pharmacology, Vol. 56, pp. 473–481, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00163-4

Differential Inducibility of Specific mRNA Corresponding to Five CYP3A Isoforms in Female Rat Liver by RU486 and Food Deprivation

COMPARISON WITH PROTEIN ABUNDANCE AND ENZYMIC ACTIVITIES

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ABSTRACT. The induction of cytochrome P450 3A (CYP3A) protein and mRNA by RU486 [17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-1-propyl-estra-4,9-dien-3-one] treatment and food deprivation in female rat liver was studied using Western blotting and competitive reverse transcription–polymerase chain reaction (RT-PCR). CYP3A apoprotein levels increased in response to food deprivation and to RU486 treatment, and the combination of RU486 treatment plus food deprivation had an apparent additive effect. Food deprivation and RU486 treatment also caused increases in CYP3A1, CYP3A18, and CYP3A23 mRNA, and the combined effects of these treatments on each of these mRNA forms were synergistic. CYP3A2 mRNA was not detected in any of the treatment groups, and there was a lack of concordance between CYP3A9 mRNA levels and the specific messages corresponding to the other CYP3A isoforms. CYP3A9 mRNA levels were highest in food-deprived animals, whereas RU486 inhibited CYP3A9 mRNA expression and suppressed the induction effect of food deprivation. Food deprivation and RU486 treatment each separately caused increased microsomal diazepam C_3-hydroxylase activity, and the combined effects of these treatments on this monooxygenase were additive. In contrast, the [*N-methyl*-^{14}C]erythromycin demethylase activity of the fasted, RU486-treated group of rats did not differ from that of the untreated group, and kinetic analyses revealed that both groups of animals exhibited similar K_m and V_{max} values. These results suggest that CYP3A9 may be primarily responsible for erythromycin *N*-demethylation and that the isoforms induced by the combination of fasting and RU486 administration are CYP3A1, CYP3A23, and, to a lesser extent, CYP3A18. BIOCHEM PHARMACOL **56**;4: 473–481, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. CYP3A; RT–PCR; RU486; mifepristone; food deprivation; mRNA; diazepam C_3-hydroxylase; erythromycin *N*-demethylase

The superfamily of CYP† heme thiolate monooxygenases is important in the oxidative metabolism of many endogenous and xenobiotic substrates [1, 2]. One particular subfamily, CYP3A, metabolizes many endogenous and xenobiotic steroids [3] and is involved in the clearance of many clinically important, structurally unrelated drugs [4]. Members of the CYP3A subfamily are inducible; their abundance in tissue increases as the result of exposure to a number of steroid hormones [5–11] and other chemicals [12–14]. The signalling and regulatory pathways mediating control of CYP3A expression remain unidentified.

We previously reported a strong induction of CYP3A enzyme protein in female rat liver caused by food deprivation and by the administration of RU486 [15], a synthetic steroid that possesses both antiprogestin and antiglucocorticoid properties [16]. Although developed as an abortifacient, the potential for other applications of RU486 has become apparent and includes treatment for Cushing's syndrome [17] and various types of cancer [18–20]. Due to the critical roles played by CYP3A in the liver concerning the clearance of RU486 [21], and the expanding potential uses of RU486, we considered it important to conduct further studies into RU486 induction mechanisms of hepatic CYP3A monooxygenases and to further characterize synergism with the effects of food deprivation. We chose to study these induction effects in adult female rats for reasons of sensitivity of detection of isoforms that are at low abundance in the naive animal [15].

At the present time, five CYP3A genes have been detected in rat liver, *CYP3A1* [22], *CYP3A2* [23], *CYP3A9* [24], *CYP3A18* [25], and *CYP3A23* [26, 27]. The most recently discovered, *CYP3A9*, was first isolated from rat brain [28]. In the present study, competitive RT-PCR was used to quantitate the specific mRNA gene products corresponding to each of these CYP3A isoforms in female rat liver to examine their differential regulation by RU486 treatment and food deprivation. Total CYP3A apoprotein levels were also measured, and catalytic studies were carried

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† *Abbreviations:* CYP, cytochrome P450; RT-PCR, reverse transcription–polymerase chain reaction; and TMB, tetramethylbenzidine.

Received 31 December 1997; accepted 4 May 1998.

out using substrates considered to be CYP3A-specific to further explore their relationships with gene expression.

MATERIALS AND METHODS

Materials

RU486 [17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-1-propyl-estra-4,9-dien-3-one] was a gift from Roussel-Uclaf. D-Glucose-6-phosphate (disodium salt), NAD, and glucose-6-phosphate dehydrogenase (yeast enzyme, grade 1) were from Boehringer Mannheim Erythromycin was a gift from Fauldings Pty. Ltd., and diazepam and camazepam were gifts from Roche Products Pty. Ltd. [*N-methyl*-^{14}C]-Erythromycin was purchased from DuPont/NEN Australia Ltd., and dimedone was obtained from Merck. Ultima Gold scintillation fluid was from Canberra Packard Pty. Ltd. HPLC grade methanol was from Mallinckrodt Pty. Ltd. Acrylamide was purchased from BDH Chemicals Ltd. Immobilon-P polyvinylidene fluoride (PVDF) membrane (0.45 μm pore size) was a product of the Millipore Corp. The primary antibody was rabbit IgG raised against heterologously expressed *N*-terminally truncated human CYP3A4 protein [29], and was prepared in our laboratory. Anti-rabbit IgG (from donkey) linked to horseradish peroxidase (secondary antibody) was purchased from Amersham Australia Pty. Ltd. TMB membrane peroxidase substrate was a product from Kirkegaard & Perry Laboratories Inc. obtained from Bio-Mediq DPC Pty. Ltd. TRI REAGENT™ was from Molecular Research Center, Inc., and diethyl pyrocarbonate (DEPC) was from the Sigma Chemical Co. All primers were synthesized at The Centre for Molecular and Cellular Biology. Ready-To-Go You-Prime First-Strand beads and 2′-deoxynucleoside 5′-triphosphate (dNTP) mix were purchased from Pharmacia Biotech. AmpliTaq Gold DNA polymerase was from Perkin Elmer. The neutral DNA fragment used in the synthesis of the competitor fragment was obtained from a PCR MIMIC Construction kit (Integrated Sciences). PCR was performed on a PT-100 Programmable Thermal Controller (MJ Research, Inc.) obtained from Bresatec. All reagents were of molecular biology grade (for RT-PCR) or reagent grade, and water purified using a MilliQ Plus 185 system having a specific resistance of 18.2 MΩ · cm was used throughout.

Choice of Vehicle for Drug Administration

Studies of CYP induction with lipophilic xenobiotics have traditionally employed a plant seed oil or a water-miscible low-polarity solvent for administration of such compounds to experimental animals. For our study, the use of plant seed oils was contraindicated because any nutritional effect would confound our experimental design involving food deprivation. The use of water-miscible solvents often employed as vehicles for the administration of lipophilic compounds was also contraindicated because many have been demonstrated consistently to be CYP inducers in their own right. This is the case for ethanol [30, 31], methanol and propylene glycol [32, 33], and DMSO [15, 34]. The use of these chemicals would confound our experimental design due to their potential as monooxygenase inducers, and for these reasons we administered microcrystalline RU486 by intraperitoneal injection as a suspension in sterile pyrogen-free 0.9% saline to obviate these problems.

Animal Treatments

Female Wistar rats (random outbred), 5 weeks of age, were obtained from the University Central Animal Breeding House. Animals were maintained for 1 week at 21 ± 2° on a 12-hr light/dark cycle prior to experimentation. During this time, rats were allowed unlimited access to normal drinking water and standard cubed rodent food (NORCO). Rats were divided into four groups with seven animals in each group (groups A–D). Forty-eight hours prior to being killed, two groups were fed normally (A and C) while the remaining groups were denied access to food (B and D). During this period all animals were allowed *ad lib*. access to water as required. Groups C and D were injected intraperitoneally with RU486 suspended in 0.9% saline (25 mg/kg, twice daily), while groups A and B received 0.9% saline alone. At the end of the experimental period, animals were decapitated, and the livers were removed. Several small portions (100 mg) from each liver were frozen immediately in liquid nitrogen and stored at −80° until required. Liver microsomes were prepared from the remaining tissue [35], microsomal protein and CYP contents were determined [36, 37], and microsomal suspensions were frozen, using liquid nitrogen, and stored at −80°.

CYP3A Immunodetection

Microsomal fractions containing 10 pmol total CYP were subjected to SDS–PAGE (10% acrylamide) according to the method of Laemmli [38]. Each gel included three calibration lanes containing 0.1 pmol of purified human CYP3A4 protein as positive control standards. Following electrophoresis, Western blotting was performed [39] and CYP3A apoprotein detected using the primary and secondary antibodies described above. Colour development was achieved using TMB substrate, and stained protein was digitally quantified using a Bio-Rad imaging densitometer (model GS-670) operating in the reflectance mode. Then microsomal CYP3A band densities on individual membranes were quantified with reference to the standard positive control lanes to normalize for inter-blot variation in staining development.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from 100 mg of rat liver using TRI REAGENT™. RNA yield was determined spectrophotometrically at 260 nm. Denaturing gel electrophoresis (1% agarose containing 2.2 M formaldehyde) was carried out to

confirm that RNA was of high quality. cDNA synthesis was performed using Ready-To-Go You-Prime First-Strand beads to ensure equal efficiency of reverse transcription between samples. RNA samples were heated at 65° for 10 min and then were cooled on ice for 2 min. Five micrograms of total RNA and 30 pmol of antisense gene-specific primer were added to the reverse transcription mixture and made up to a final volume of 35 μL with RNase-free water. Samples were incubated at 37° for 1 hr and then heated at 90° for 5 min to inactivate the enzyme.

Validation of Quantitative PCR Methodology

The competitive PCR protocol used in the present study involved single-tube coamplification of a *CYP3A* gene segment from cDNA and a synthetic internal standard that shares the same primer binding sequences as the target gene. Hence, only one primer pair is included in the PCR. In this way, the internal standard imitates the primer binding and amplification characteristics of the target, even though the sequences between their respective primer binding sites are heterologous. The use of synthetic internal standards prepared in this way eliminates the problems associated with using endogenous (housekeeping) sequences as the internal standard, as genes containing these sequences have been shown not to be expressed in tissues in a constitutive, uniformly invariant manner [40–44].

Primers and Design of Competitor Fragment for PCR

The sequences of the gene-specific primer pairs used in amplifying fragments of the five known CYP3A isoforms and in the synthesis and amplification of the competitor fragments are published elsewhere [24]. The competitor fragments were constructed using the gene-specific primers, a set of composite primers, and a neutral DNA fragment. Syntheses of the competitors were performed according to manufacturer's instructions (Integrated Sciences). Each composite primer contained the target gene-specific primer sequence attached to a 20 bp sequence designed to anneal to the opposite strands of the neutral DNA fragment. In this way, the target primer sequence was incorporated into the competitor fragment.

Conditions for Competitive PCR

The cDNA sample was coamplified in the presence of serial dilutions of competitor fragment using the gene-specific primers. PCR was performed in a total reaction volume of 50 μL containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each sense and antisense primer, 1 μL of cDNA, 2 μL of competitor (10^{-3}–100 amol/μL), and 2.5 U of AmpliTaq Gold DNA polymerase. Standardization of PCR conditions between samples was established by using a PCR master mix. Reaction mixtures were heated to 95° for 9 min followed by 30 cycles at 94° for 45 sec, 57° for 45 sec, and 72° for 1 min with a final extension step for 7 min at 72°. Control reactions containing no template, cDNA only, or competitor only, were routinely subjected to this amplification procedure to assess contamination.

Quantification of Amplified Products

A 5-μL sample from each reaction tube was electrophoresed on a 1.6% TBE agarose gel containing 0.5 μg/mL of ethidium bromide. Gels were photographed using a Sony type IV UPP-110HA superior density printing paper. The photograph was scanned as a negative image using a model GS-670 densitometer operating in reflectance mode. Bands were quantified, and O.D. measurements of both target and competitor for each reaction were obtained.

Catalytic Assays

Microsomal suspensions were analysed for diazepam C_3-hydroxylase by a previously described method [45], using camazepam as internal standard. [*N-methyl*-^{14}C]Erythromycin demethylase assays were conducted using 1.0 μM microsomal CYP and were based on a previously published method [46] using dimedone as the formaldehyde trapping agent.

Analyses of Kinetic Parameters

The kinetic parameters describing substrate saturation data were determined for the erythromycin demethylase assays using the Scientist™ (MicroMath Scientific Software) statistics program. The most appropriate analysis, giving minimal error estimates, was fitted to the two-component model $v = (A \cdot S) + (V_{max} \cdot S)/(K_m + S)$, indicating a contribution from a non-saturable component in catalysis (v = velocity, A = slope value of linear term, and S = substrate concentration). V_{max} values were expressed as turnover numbers normalized on CYP (pmol product/min/nmol microsomal CYP) to allow comparison of activities between different microsomal preparations of varying CYP specific content. Derived values for these kinetic parameters are given in the legend to Fig. 3.

RESULTS

Panels A–D of Fig. 1 show the western blots obtained for liver microsomes from animal groups A–D. Figure 1E shows the hepatic microsomal CYP3A apoprotein levels determined for each of these groups. Saline control (group A) rat samples contained low but detectable CYP3A protein. The abundance of CYP3A protein was increased significantly by food deprivation (B > A) and by RU486 administration (C > A). Food deprivation and exposure to RU486 evoked a much greater induction response than was obtained by fasting or RU486 alone (D > C, D > B). The combined effects of fasting and RU486 treatment (group D animals) on CYP3A apoprotein appeared to be additive.

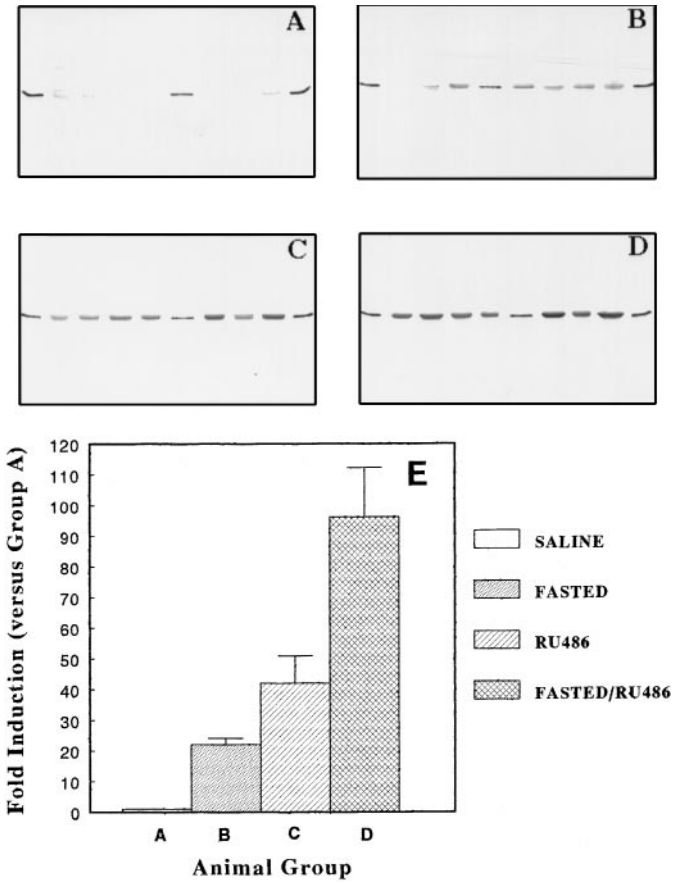

FIG. 1. Western blotting and immunoquantitation of CYP3A apoprotein in rat liver samples. Panels A–D show the results for the animal treatment groups A–D, respectively. Group A rats, saline treated; Group B rats, 48-hr fasted; Group C rats, RU486 treated; and Group D rats, RU486 + fasted. In panels A, C, and D, lanes 1, 6, and 10 contained 0.1 pmol of purified heterologously expressed human CYP3A4 protein; the other gel lanes contained 10 pmol of microsomal CYP protein from livers of rats in group A, C, and D, respectively. In panel B, lanes 1, 5, and 10 contained 0.1 pmol of purified heterologously expressed human CYP3A4 protein; the other gel lanes contained 10 pmol of microsomal CYP protein from the livers of group B rats. The primary antibody IgG preparation was diluted to a concentration of 0.5 μg of protein/mL, and the secondary antibody (horseradish peroxidase linked, donkey anti-rabbit IgG) was used according to the manufacturer's instructions. The histogram shown in panel E displays the CYP3A protein abundances for these treatment groups relative to group A (saline-treated control). Values are means ± SEM, N = 7. The non-parametric Mann-Whitney rank sum test was used to determine levels of significance of difference between groups. The results of these tests were: A vs B: $P < 0.001$; A vs C: $P < 0.001$; B vs C: $P < 0.05$; B vs D: $P < 0.001$; C vs D: $P < 0.05$.

Results for the immunodetection data were closely reflected in the diazepam C_3-hydroxylase measurements (Fig. 2). This suggests identity between the CYP3A protein induced by RU486 and fasting and this monooxygenase. Activity was low in group A (saline-treated) animals and was increased by fasting (B > A) and by RU486 administration (C > A). The combined effect of RU486 treatment and food deprivation (group D) evoked a more marked increase in the monooxygenase reminiscent of the response obtained for CYP3A apoprotein (compare group D results in Figs. 1E and 2).

Microsomes from group A (saline control) and group D (food deprived, RU486 treated) were analysed for [*N-methyl*-^{14}C]erythromycin demethylase in order to further study the induction effect noted for diazepam C_3-hydroxylase and CYP3A apoprotein. Substrate saturation curves were obtained (Fig. 3), which demonstrated similar catalytic profiles and similar K_m and V_{max} values for both groups. These results indicate that the combined treatment schedule did not induce this monooxygenase.

Hepatic CYP3A isoform-specific mRNA levels were examined using competitive RT-PCR. An example of typical results using data obtained for CYP3A18 mRNA are displayed in Fig. 4 and 5. The gel photographs obtained for each animal treatment group are shown in Fig. 4, panels A–D; and the corresponding graphical analyses of O.D. values are depicted in Fig. 5, panels A–D. The mRNA abundances of the five different isoforms for each of the treatment groups are summarized in Table 1, and the values are expressed as fold induction or suppression relative to group A (saline-treated) rats. CYP3A2 mRNA was not detectable in any of the groups. Food deprivation (group B) and RU486 treatment (group C) each separately induced specific message for CYP3A23 > CYP3A1 > CYP3A18 (in order of fold induction), and the combination of food deprivation and RU486 treatment (group D) greatly stim-

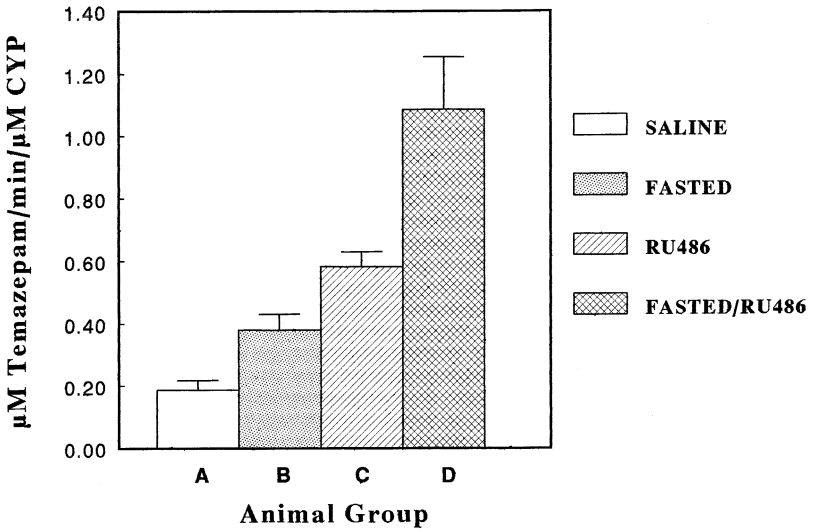

FIG. 2. Microsomal diazepam C_3-hydroxylase activities for the four animal treatment groups. Group A rats, saline treated; Group B rats, 48-hr fasted; Group C rats, RU486 treated; and Group D rats, RU486 + fasted. Values are means ± SEM, N = 7. Data were analysed using the non-parametric Mann-Whitney rank sum test. Results of this analysis were: A vs B: $P < 0.05$ A vs C: $P < 0.005$; B vs C: $P < 0.05$; B vs D: $P < 0.005$; and C vs D: $P < 0.05$.

ulated the expression of these specific messages. The combined effects of these treatments on CYP3A1, CYP3A18, and CYP3A23 mRNA were much greater than the added effect of either treatment alone, suggesting a synergistic interaction of the two treatments.

In contrast to these findings, CYP3A9 mRNA levels lack concordance with the other CYP3A isoforms. Fasting alone (group B) was the strongest inducer of hepatic CYP3A9 mRNA, whereas RU486 treatment (group C) suppressed *CYP3A9* gene expression and also reduced the induction response in fasted rats (group D). This inhibition of *CYP3A9* gene expression by RU486 and its strong induction by food deprivation clearly demonstrate CYP3A9 to be an isoform whose expression is controlled quite differently from CYP3A1, CYP3A18, and CYP3A23.

DISCUSSION

In the present work, female rat liver CYP3A enzyme protein and diazepam C_3-hydroxylase activities were increased significantly in response to food deprivation and RU486 treatment. Inspection of Figs. 1 and 2 shows that the effects of these treatments were equivalent, strongly suggesting that this monooxygenase is a good marker activity for CYP3A protein. This result is in keeping with previously published observations [15, 45, 47–50]. Compet-

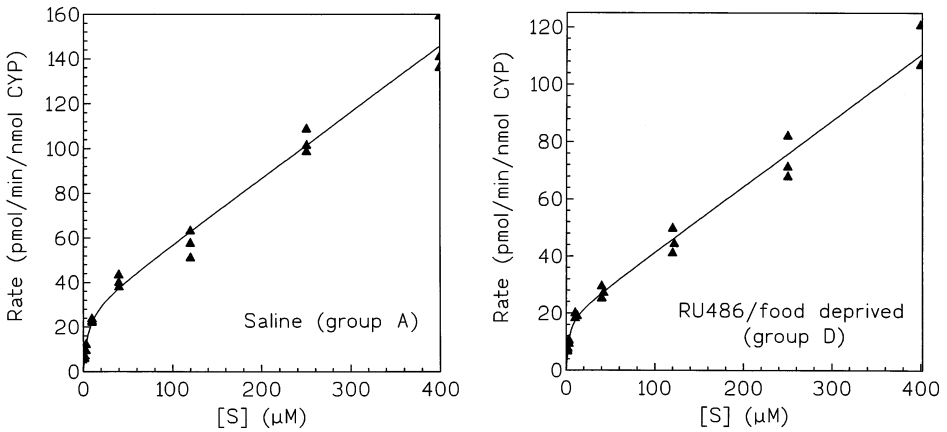

FIG. 3. [*N-methyl*-^{14}C]Erythromycin *N*-demethylase substrate saturation curves for treatment groups A (saline-treated control) and D (food deprivation combined with RU486 treatment). Seven different substrate concentrations were used, each conducted in triplicate. Kinetic parameters were determined by fitting the data to the two-component model $v = (A \cdot S) + (V_{max} \cdot S)/(K_m + S)$ using the computer program Scientist™ (Micro Math Scientific Software). The results of these analyses are: Group A: K_m (erythromycin) = 4.84 ± 2.15 µM, V_{max} = 28.62 ± 3.84 pmol formaldehyde/min/nmol CYP; Group D: K_m (erythromycin) = 2.60 ± 1.26 µM, V_{max} = 18.89 ± 2.40 pmol formaldehyde/min/nmol CYP. The value of the slope parameter (A) calculated from each of the substrate saturation data sets was: Group A rats, 0.293 ± 0.014; Group D rats, 0.228 ± 0.009.

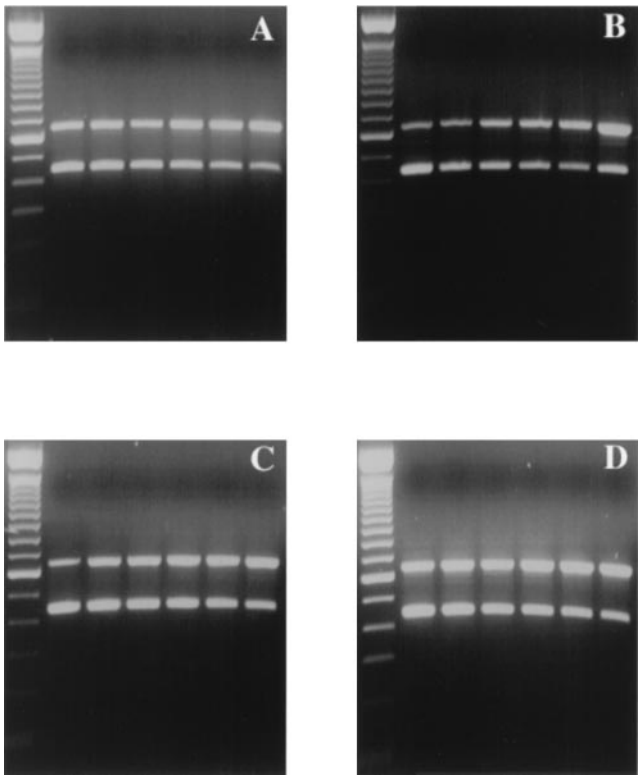

FIG. 4. Analysis of female rat liver CYP3A18 mRNA levels by competitive RT-PCR. The animal treatment group is indicated in the top right corner of each gel photograph. Group A rats, saline treated; Group B rats, 48-hr fasted; Group C rats, RU486 treated; and Group D rats, RU486 + fasted. Two-fold serial dilutions of the competitor were co-amplified in the presence of constant amounts of cDNA. The upper band corresponds to the amplified target (649 bp), whereas the lower band corresponds to the amplified competitor (420 bp). The leftmost lane in each gel contains 100-bp DNA markers (Life Technologies).

itive RT-PCR studies showed that RU486 and food deprivation each increased CYP3A1, CYP3A18, and CYP3A23 mRNA and that a synergistic effect of the two treatments was observed in fasted, RU486-treated rats. Such synergism contrasts with the apparent additive effect of these treatments on CYP3A apoprotein. Information on the kinetics of protein and mRNA turnover in response to these treatments would be required to resolve this observation; differential rates of transcription and translation may explain the lack of congruence between effects on protein and message abundances.

Recent studies have confirmed that RU486 administration increases hepatic immunoreactive CYP3A apoprotein, with a concomitant increase in microsomal testosterone 6β-hydroxylase [51], and CYP3A18 protein has been shown to have high testosterone 6β-hydroxylase activity [52]. In the present study, RU486 has been shown to cause an increase in CYP3A18 mRNA, and we are continuing studies to confirm increased microsomal testosterone 6β-hydroxylase levels in RU486-treated animals.

The expression levels of CYP3A9 in the treatment groups used in the present study contrasted with those of CYP3A1, CYP3A18, and CYP3A23. Food deprivation was the most potent inducer of CYP3A9. RU486 administration actually lowered constitutive levels of this isoform and almost abolished the induction caused by fasting. In the present study, [*N*-methyl-^{14}C]erythromycin demethylase activities for the saline and food-deprived, RU486-treated groups were comparable, which may indicate CYP3A9 to be the isoform primarily responsible for erythromycin demethylation at the *N*-methyl sites. The expression pattern for CYP3A9 observed in the present study is supported further by previous results obtained in our laboratory [15], which document fasting as a strong inducer of erythromycin demethylase and suppression of this activity by RU486. Control of CYP3A9 expression in the present study is clearly distinct from that of other CYP3A enzymes in agreement with previous studies showing distinctive patterns of age-, hormone- and gender-dependent regulation [24, 53].

The administration of RU486 to food-deprived animals produced striking increases in hepatic CYP3A apoprotein and diazepam C_3-hydroxylase levels. These combined treatments also caused marked elevations in CYP3A1, CYP3A18, and CYP3A23 mRNA levels, and the magnitude of induction was greater than the sum of the separate effects of food deprivation and RU486 administration. This apparent synergism may result from dual actions of RU486 and adrenal hormones released during food deprivation. This is suggested on the basis of previous work where synergism was reported for the induction of CYP3A by the glucocorticoid dexamethasone plus the antiglucocorticoid pregnenolone 16α-carbonitrile [5, 54–57]. In addition, hormones such as glucagon and adrenaline released during

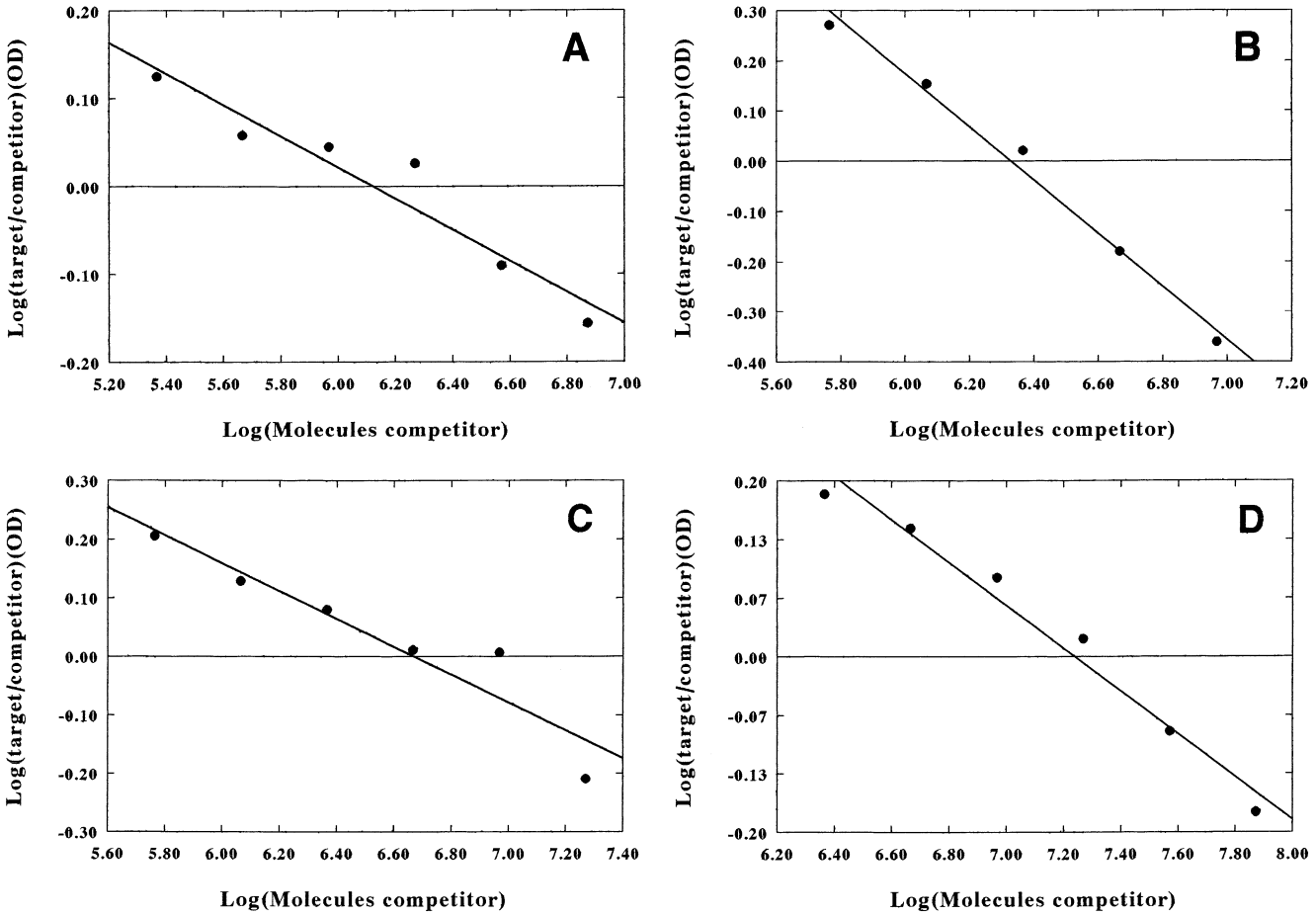

FIG. 5. Logarithmic plots of the results of competitive RT–PCR shown in Fig. 4, A–D. The logarithms of the ratios of target band O.D. to competitor band O.D. in each lane were plotted as a function of the initial amount of competitor added to the PCR. The initial abundance of target cDNA present in each sample was extrapolated from the point on each curve where the O.D. values (and, therefore, the amount) of the amplified competitor and target are equal. The animal treatment groups are shown in the top right corner of each panel; Group A rats, saline treated; Group B rats, 48-hr fasted; Group C rats, RU486 treated; and Group D rats, RU486 + fasted.

food deprivation, which cause increased hepatocellular concentrations of cyclic AMP, may enhance induction of CYP3A by RU486 in fasted animals. Such synergism was reported for CYP1A1 induction in primary cultures of adult mouse hepatocytes incubated with cyclic AMP and 3-methylcholanthrene [58]. Studies to characterize the interactions between food restriction and RU486-mediated induction are continuing in our laboratory.

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TABLE 1. Liver CYP3A mRNA abundances expressed as fold induction relative to amounts in fed, saline-treated rats

	Fold induction				
Treatment	**CYP3A1**	**CYP3A2**	**CYP3A9**	**CYP3A18**	**CYP3A23**
Saline treated	1.0	ND	1.0	1.0	1.0
48-hr Fasted	1.7	ND	6.0	1.6	4.6
RU486 treated	4.6	ND	0.5	2.2	7.7
RU486 + fasted	46.3	ND	1.8	13.6	44.3

Results shown are for liver samples from single rats within each group whose CYP3A apoprotein abundance was closest to the mean for the group. Saline treated, group A rats; 48-hr fasted, group B rats; RU486 treated, group C rats; and RU486 + fasted, group D rats. ND = not detectable.

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