

# Dexamethasone Induces Pregnane X Receptor and Retinoid X Receptor- $\alpha$ Expression in Human Hepatocytes: Synergistic Increase of CYP3A4 Induction by Pregnane X Receptor Activators

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## ABSTRACT

In this report we show that submicromolar concentrations of dexamethasone enhance pregnane X receptor (PXR) activator-mediated CYP3A4 gene expression in cultured human hepatocytes. Because this result is only observed after 24 h of co-treatment and is inhibited by pretreatment with cycloheximide, we further investigated which factor(s), induced by dexamethasone, might be responsible for this effect. We report that dexamethasone increases both retinoid X receptor- $\alpha$  (RXR $\alpha$ ) and PXR mRNA expression in cultured human hepatocytes, whereas PXR activators such as rifampicin and clotrimazole do not. Accumulation of RXR $\alpha$  and PXR mRNA reaches a maximum at a concentration of 100 nM dexamethasone after treatment for 6 to 12 h and is greatly diminished by RU486. A similar pattern of expression is observed with tyrosine aminotransfer-

ase mRNA. Moreover, the effect of dexamethasone on PXR mRNA accumulation seems to be through direct action on the glucocorticoid receptor (GR) because the addition of cycloheximide has no effect, and dexamethasone does not affect the degradation of PXR mRNA. Furthermore, dexamethasone induces the accumulation of a RXR $\alpha$ -immunoreactive protein and increases the nuclear level of RXR $\alpha$ :PXR heterodimer as shown by gel shift assays with a CYP3A4 ER6 PXRE probe. This accumulation of latent PXR and RXR $\alpha$  in the nucleus of hepatocytes explains the synergistic effect observed with dexamethasone and PXR activators together on CYP3A4 induction. These results reveal the existence of functional cross talk between the GR and PXR, and may explain some controversial aspects of the role of the GR in CYP3A4 induction.

Efficient detoxification of harmful xenobiotics is essential to the survival of living organisms. Members of the cytochrome P450 (CYP) superfamily of monooxygenases play a crucial role in this regard, by converting pollutants, plant toxins, carcinogens, and drugs to products that can be excreted either in urine or bile (Guengerich, 1991). The human CYP3A forms (CYP3A4 and CYP3A7) are of particular significance in this respect because they are involved in the metabolism of approximately two-thirds of clinically used drugs (Lewis et al., 1998). A number of these drugs, including antibiotics (rifampicin), antimycotics (clotrimazole), and glucocorticoids (dexamethasone) activate the transcription of CYP3A genes both in vivo in the liver and in vitro in cultured

human hepatocytes (Pichard et al., 1992; Backman et al., 1996). This process of induction, coupled to the broad substrate specificity of the CYP3A enzymes, constitutes the molecular basis for a number of important drug-drug interactions in patients taking multiple medication.

The 9-*cis*-retinoic acid receptors (RXRs) belong to the steroid/thyroid hormone receptor superfamily. Three isoforms of RXR, i.e., RXR $\alpha$ , RXR $\beta$ , and RXR $\gamma$ , have been identified (Levin et al., 1992), RXR $\alpha$  being the dominant isoform in the liver (Berrodin et al., 1992). This receptor acts as an auxiliary protein, heterodimerizing with other nuclear receptors, including the retinoic acid receptors, vitamin D receptor, thyroid hormone receptors, and peroxisome-proliferator-activated receptor. These heterodimers then transactivate target genes in a ligand-dependent manner (Yu et al., 1991; Kliewer et al., 1992a,b; Levin et al., 1992). Thus, the level of RXR $\alpha$  may be a crucial parameter in determining the effects of hormones. Recently, an orphan nuclear receptor termed

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**ABBREVIATIONS:** CYP, cytochrome P450; RXR, retinoid X receptor; PXR, pregnane X receptor; GR, glucocorticoid receptor; PCN, pregnenolone 16 $\alpha$ -carbonitrile; RU486, mifepristone; GADPH, glyceraldehyde phosphate dehydrogenase; TAT, tyrosine aminotransferase; DTT, dithiothreitol; MDR, multidrug resistance (or resistant) glycoprotein; CHX, cycloheximide; Pgp, P-glycoprotein.

pregnane X receptor (PXR), steroid and xenobiotic receptor, or pregnane-activated receptor has been identified as a new RXR $\alpha$  partner (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). Transient cotransfection experiments have shown that this receptor is activated by high (pharmacological) concentrations of a range of drugs known to induce CYP3A gene expression. It binds as a heterodimer with RXR $\alpha$  to a xenobiotic response element containing two everted repeats TGA(A/G)CT separated by six nucleotides (ER6 motif) in the CYP3A4 (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998) and CYP3A7 gene promoters (Pascussi et al., 1999).

In this report we show that CYP3A4 mRNA induction in response to PXR activators is markedly enhanced by submicromolar concentrations of dexamethasone in cultured human hepatocytes. Our results suggest that this is the consequence of the induction of RXR $\alpha$  and PXR gene expression by dexamethasone and thus reveal the existence of functional cross talk between the glucocorticoid receptor (GR) and the PXR receptor-signaling pathway.

## Experimental Procedures

**Drugs and Materials.** Ham F-12 and Williams E culture media, medium additives, collagenase (type IV), dimethyl sulfoxide, rifampicin, clotrimazole, cycloheximide, actinomycin D, pregnenolone 16 $\alpha$ -carbonitrile (PCN), and dexamethasone were purchased from Sigma (St. Louis, MO). Mifepristone (RU486) was obtained from Roussel-Uclaf (Paris, France). Collagen-coated culture dishes were obtained from Corning (Iwaki Glass, Iwaki, Japan).  $\alpha$ -[<sup>32</sup>P]dUTP,  $\alpha$ -[<sup>32</sup>P]dCTP, and  $\gamma$ -[<sup>32</sup>P]dATP were from Amersham International (Amersham, England).

**Preparation of Riboprobe Plasmids.** PGEMT-hPXR pvuII: After PCR amplification, cDNA encoding amino acids 1-434 of hPXR from psg5-hPXR (S. Kliewer, Glaxowellcome, Research Triangle Park, NC) with oligonucleotides 5'-GGGTGTGGGGAATTCACCATGGAGGTGAGACCCAAAGAAAGC-3' (sense) and 5'-GGGTGTGGGGGATCCTCAGCTACCTGTGATGCCG-3' (antisense) were inserted into pGEMT easy vector (Promega, Charbonnières, France). To prepare the PXR RNase protection probe, the plasmid was digested with *PvuII* and the antisense probe was synthesized with SP6 polymerase. The native probe was 293 nucleotides long and the protected probe was 216 nucleotides long (from 1092 to 1308). pB-SKII-hGR *DdeI*: the 0.747-kb fragment of *EcoRI* digested psg5-hGR plasmid (1627 to 2374) was cloned in reverse orientation in the *EcoRI* site of pBluescript II KS + plasmid (Stratagene, La Jolla, CA). To prepare the hGR RNase protection probe, the plasmid was digested with *DdeI* and the antisense probe was synthesized with T3 polymerase. The native probe was 246 nucleotides long and the protected probe was 215 nucleotides long (from 2159 to 2374).

**Cell Cultures.** Liver samples were obtained from adult patients who had undergone liver lobectomy resection for medical reasons totally unrelated to our research program (Table 1). The use of these specimens for scientific purposes has been approved by the French National Ethics Committee. Human hepatocytes were isolated and plated in ISOM medium as previously described (Pichard et al., 1990). Forty-eight hours after plating, dexamethasone was withdrawn from the culture medium for 16 h. Cells were then cultured in the presence or absence of the indicated inducers for 6 to 48 h. Total RNA and protein were isolated, with Trizol reagent (Gibco BRL, Cergy-Pontoise, France), from 10<sup>7</sup> cultured hepatocytes according to the manufacturer's instructions. For quality control, 30  $\mu$ g of total RNA was analyzed by Northern blot with a rat glyceraldehyde phosphate dehydrogenase (GADPH) cDNA probe (J. M. Blanchard, Institut de Génétique Moléculaire de Montpellier, Montpellier, France).

**Ribonuclease Protection Assays and Northern Blot.** Total RNA (30  $\mu$ g) was analyzed by the RNase protection assay with specific riboprobe as previously described (Greuet et al., 1996) with minor modification. Total RNA was hybridized with radiolabeled antisense RNA probe (100,000 to 150,000 cpm) overnight at 42°C after incubation for 10 min at 95°C. For Northern blot experiments, 30  $\mu$ g of total RNA was analyzed with  $\alpha$ -[<sup>32</sup>P]dCTP-labeled rat GADPH, mouse tyrosine aminotransferase (TAT; T. Grange, Institut J. Monod, Paris, France), cDNA probes, and T4 polynucleotide kinase- $\gamma$  [<sup>32</sup>P]ATP-labeled 40 mer antisense synthetic RXR $\alpha$  DNA probe (Geneka Biotechnology Inc., Euromedex, France) and autoradiography was carried out by exposing the dried gel to Kodak X-AR film. The signals were quantified by analyzing the radioactivity with a PhosphorImager apparatus and ImageQuant software.

**Extraction of Nuclear Proteins.** Nuclear extracts were prepared according to the method of Schreiber et al. (1989), with minor modifications. Hepatocytes (10<sup>7</sup>) were washed, harvested in ice-cold PBS, and pelleted by centrifugation at 1500g for 5 min. The pellet was resuspended in 500  $\mu$ l of cold buffer A [10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol (DTT); 0.5 mM phenylmethylsulfonyl fluoride] and cells were allowed to swell on ice for 15 min, after which 32  $\mu$ l of a 10% Nonidet NP-40 was added and the tube was vortexed for 30 s. After centrifugation, the nuclear pellet was resuspended in 75  $\mu$ l of ice-cold buffer C (20 mM HEPES, pH 7.9; 0.2 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride) and the tube was vigorously rocked at 4°C for 30 min. Finally, the nuclear extract was centrifuged for 5 min and the supernatant was frozen at -80°C.

**Immunoblot Analysis.** One hundred micrograms of total proteins or 50  $\mu$ g of nuclear extracts from 10<sup>7</sup> hepatocytes were separated by SDS-polyacrylamide gel electrophoresis (10%), then electroblotted onto Immobilon P (Millipore, Bedford, MA). Membranes were incubated with specific antibodies against multidrug resistance glycoprotein (MDR; Ab-1; Oncogene Research Products, Cambridge, MA), RXR $\alpha$  ( $\Delta$ N 197; Santa Cruz Biotechnology, Santa Cruz, CA), or CYP3A6 (Greuet et al., 1997) and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia, Cardiff, England).

**Electrophoretic Mobility Shift Assays.** Electrophoretic mobility shift assays were performed with mPXR-1 (S. Kliewer, Glaxowellcome) and mRXR $\alpha$  (Prof. P Chambon, Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Stasbourg, France), in vitro translated proteins (Transcription and Translation System; Promega), or 25 to 40  $\mu$ g of hepatocyte nuclear extracts. Proteins were incubated for 15 min at room temperature with 200,000 cpm of T4 polynucleotide kinase-labeled probes in 10 mM Tris (pH 8), 6% glycerol, 1 mM DTT, 500 ng/ $\mu$ l poly(dI-dC) (Pharmacia, Orsay, France) and then submitted to electrophoresis with a 4% polyacrylamide gel in 0.5X TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA). The following oligonucleotides were used either as radiolabeled probes or as competitors (sense strand is shown): CYP3A4 ER6: 5'-TAGAATATGAACCTCAAAGGAGGTCAGTGAGT-3'; ER6-mut: 5'-TAGAATATTAACCTCAATGGAGGCAGTGAGT-3'. Anti-RXR $\alpha$  ( $\Delta$ N197, s.c. 774 X; Santa Cruz Biotechnology) were used in super shift essays.

TABLE 1

Clinical characteristics of livers and lobectomy donors

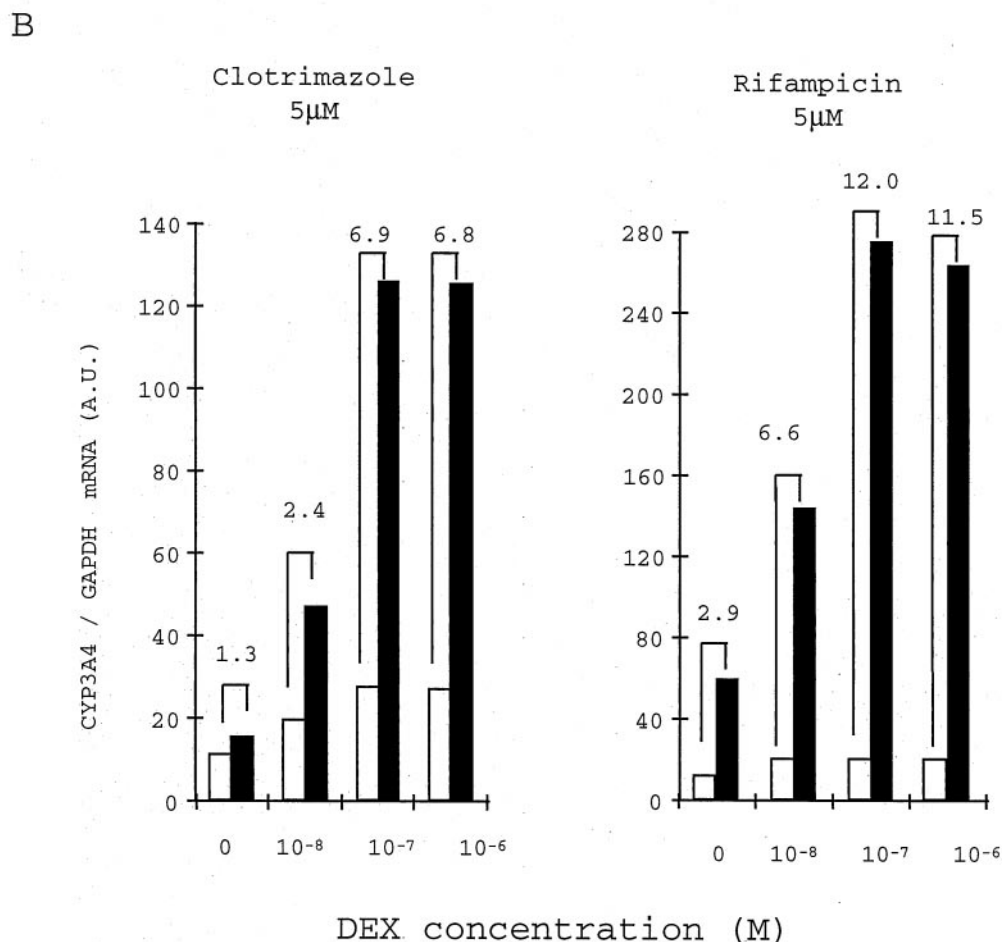
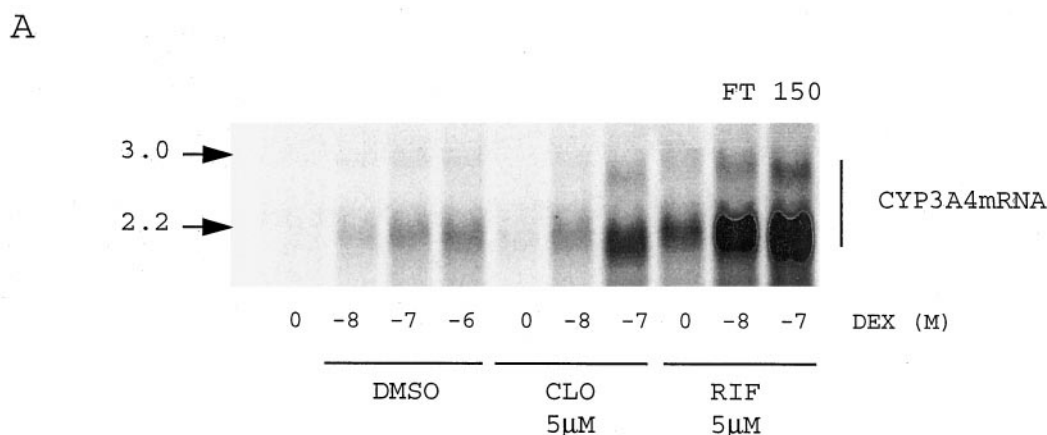
| Donor Identification | Sex | Age | Diagnosis                   |
|----------------------|-----|-----|-----------------------------|
| FT152                | M   | 67  | Metastasis of colon cancer  |
| FT155                | F   | 55  | Metastasis of colon cancer  |
| FT156                | M   | 77  | Metastasis of colon cancer  |
| FT157                | M   | 53  | Metastasis of colon cancer  |
| FT158                | F   | 58  | Metastasis of rectum cancer |
| FT159                | F   | 50  | Metastasis of colon cancer  |
| FT160                | M   | 41  | Adenoma on normal liver     |
| FT164                | F   | 47  | Metastasis of colon cancer  |
| FT166                | F   | 67  | Metastasis of colon cancer  |

Autoradiography was carried out by exposing the dried gel to Kodak X-AR film.

## Results

**Effect of Dexamethasone on PXR Activator-Mediated Induction of CYP3A4 mRNA in Cultured Human Hepatocytes.** Human hepatocytes cultured in a dexamethasone-free medium for 16 h were exposed for 48 h to 5  $\mu$ M

rifampicin or clotrimazole, two potent PXR activators (Lehmann et al., 1998), either alone or in association with increasing concentrations of dexamethasone (10 nM–1  $\mu$ M), after which the levels of CYP3A4 mRNA were measured. As shown in Fig. 1, rifampicin or clotrimazole alone only slightly induced CYP3A4 mRNA (2.9- to 6-fold and 1.3- to 1.5-fold induction, respectively, depending on the culture). Concomitant addition of dexamethasone, however, enhanced both rifampicin- and clotrimazole-dependent CYP3A4 mRNA in-



**Fig. 1.** PXR activator-dependent induction of CYP3A4 mRNA is markedly enhanced by submicromolar concentration of dexamethasone in human hepatocytes. Forty-eight hours after plating of hepatocytes, dexamethasone was withdrawn from the medium for 16 h. Cells were then cultured in the absence or presence of 5  $\mu$ M rifampicin or clotrimazole and in the presence of increasing concentration of dexamethasone. Forty-eight hours later, total RNA was extracted and 30  $\mu$ g was subjected to Northern blot analysis. A, culture FT150. Northern blot analysis of CYP3A4 mRNA; note the presence of two bands at 3.3 and 2.2 kb revealing two transcripts with 3'-untranslated region of different length. B, culture FT155. Radioactivity associated to the bands representing CYP3A4 and GAPDH (data not shown) mRNAs was measured by PhosphorImager apparatus and ImageQuant software. The ratio of CYP3A4 to GAPDH mRNA in arbitrary units (A.U.) is represented.  $\square$  refer to the absence, and  $\blacksquare$  to the presence of 5  $\mu$ M clotrimazole (left) or 5  $\mu$ M rifampicin (right). Numbers bridging pairs of bars indicate fold induction by the inducer.

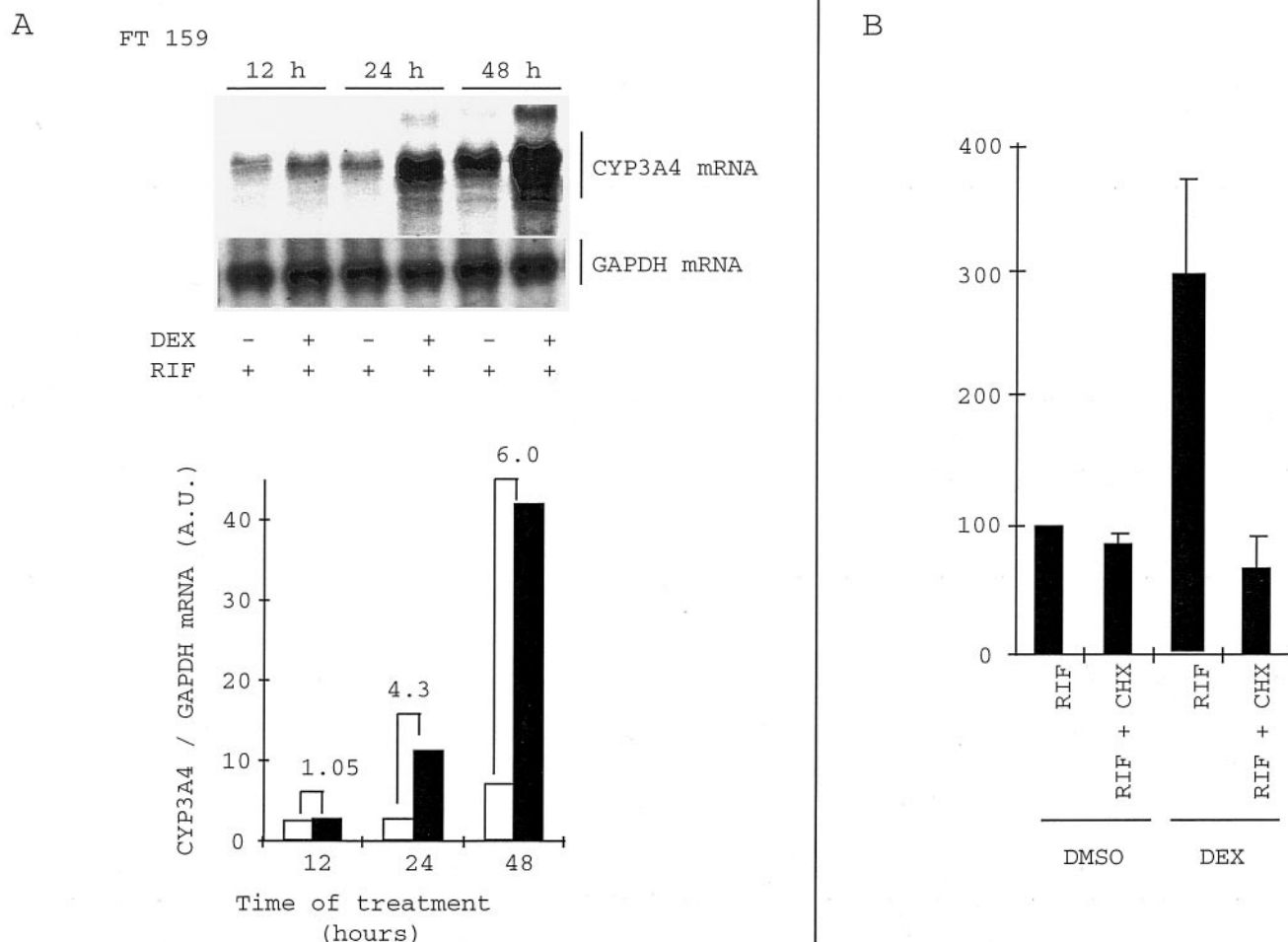


duction in a concentration-dependent manner, the maximum being reached at 100 nM, a concentration that fully activates the GR. Similar results were obtained with five different cultures prepared from five different patients (FT150, FT152, FT157, FT156, FT159). Depending on the culture, addition of 100 nM dexamethasone produced a 3- to 6-fold or a 12- to 21-fold increase of CYP3A4 mRNA induction, in response to clotrimazole and rifampicin, respectively, with respect to baseline levels.

**Enhancement of CYP3A4 mRNA Induction by Dexamethasone Is Dependent on De Novo Protein Synthesis.** We next investigated the time course of the enhancement of PXR activator-mediated induction of CYP3A4 mRNA by dexamethasone. Cultured human hepatocytes were treated with 5  $\mu$ M rifampicin, with or without 100 nM dexamethasone for 12, 24, or 48 h. A significant enhancement of rifampicin-mediated induction of CYP3A4 mRNA was observed only when cells were cotreated with dexamethasone for at least 24 h (Fig. 2A). To evaluate the role of de novo protein synthesis in this effect, the same experiments were repeated

in hepatocytes pretreated with cycloheximide (CHX), an inhibitor of protein synthesis. The enhancement of rifampicin-dependent induction of CYP3A4 mRNA by dexamethasone (after 24 h of treatment) was drastically inhibited by CHX (Fig. 2B). The level of CYP3A4 mRNA observed with the association rifampicin-dexamethasone was decreased by 60 to 70% in response to CHX, dropping back to the level obtained in response to rifampicin alone. Similar results were observed in four different cultures prepared from four different patients ( $p$  test = 0.0163,  $n$  = 4; FT152, FT154, FT157, FT159). In contrast, CYP3A4 mRNA induction mediated by rifampicin alone was not affected by CHX. Together, these results indicate that de novo synthesis of one or several protein factor(s) is required for mediating the enhancement of PXR activator-dependent CYP3A4 mRNA induction by submicromolar concentration of dexamethasone.

**Enhancement of CYP3A4 mRNA Induction by Dexamethasone Is Not Related to a Decreased Efflux of Inducers from Cells.** P-glycoprotein (Pgp), and notably MDR-1 Pgp efflux pump, has been shown to decrease the

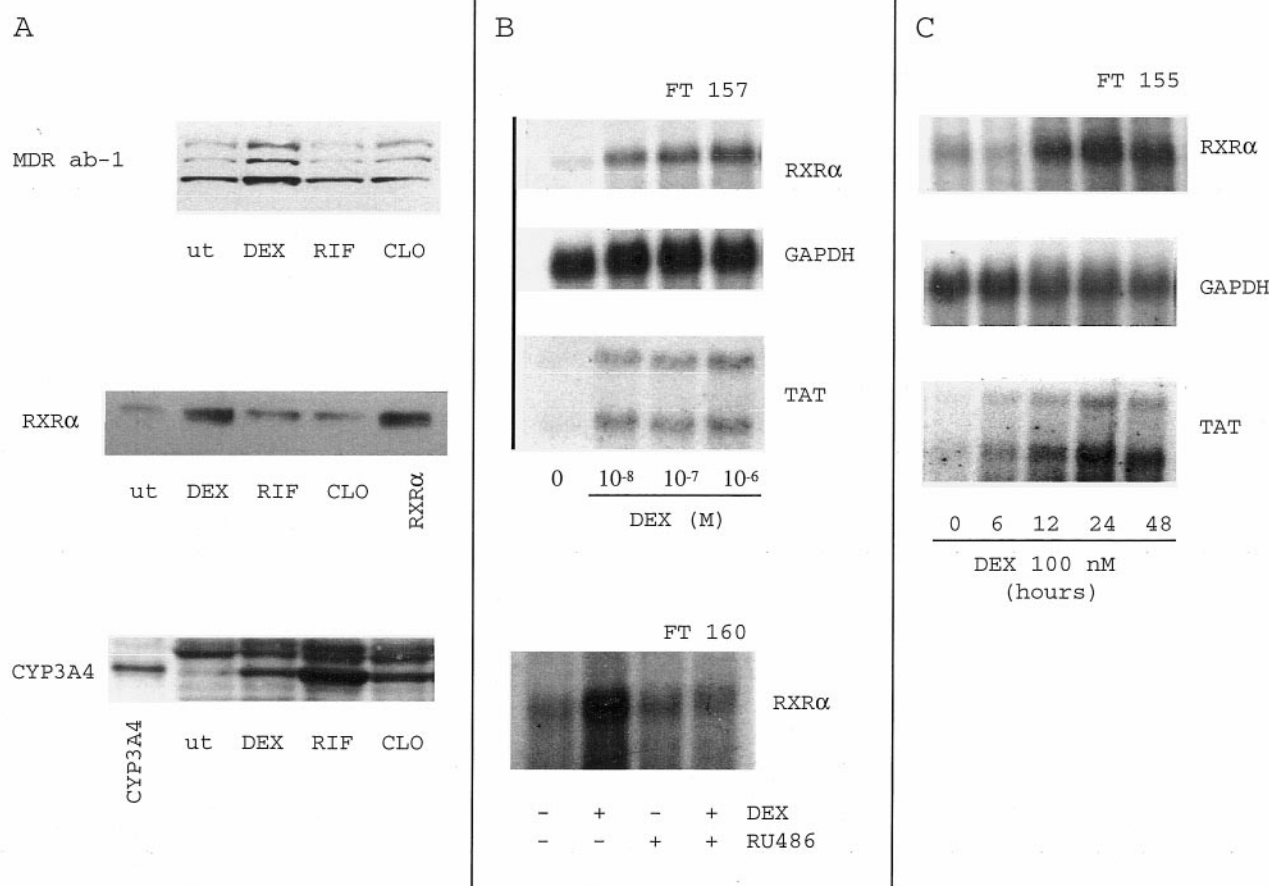


**Fig. 2.** Enhancement of rifampicin induction of CYP3A4 mRNA by dexamethasone: time dependence and effect of CHX. A, 48 h after plating of hepatocytes (FT159), dexamethasone was withdrawn from the medium for 16 h. Cells were then cultured with 5  $\mu$ M rifampicin in the absence ( $\square$ ) or presence of 100 nM dexamethasone ( $\blacksquare$ ) for 12, 24, or 48 h. Total RNA was extracted and 30  $\mu$ g was subjected to Northern blot analysis. Radioactivity associated to the bands representing CYP3A4 and GAPDH mRNAs was measured by PhosphorImager apparatus and ImageQuant software. B, 48 h after the plating of hepatocytes (FT152, FT154, FT157, FT159), dexamethasone was withdrawn from the medium for 16 h. Cells were then pretreated or not with 25  $\mu$ M CHX for 2 h, and then cultured in the presence of 5  $\mu$ M rifampicin in the absence or presence of 100 nM dexamethasone. Twenty-four hours later, total RNA was extracted and 30  $\mu$ g was subjected to Northern blot analysis. Radioactivity associated to the bands corresponding to CYP3A4 and GAPDH mRNA was measured by PhosphorImager apparatus and ImageQuant software. Data presented are the ratio CYP3A4/GAPDH and are normalized at 100 for rifampicin (RIF) treatment alone ( $p$  test = 0.0163,  $n$  = 4).

cellular concentration of CYP3A4 inducers and particularly rifampicin (Schuetz et al., 1996). We therefore wondered whether the enhancement of CYP3A4 induction by dexamethasone could result from a decreased efflux of the inducers from the cells. For this purpose we investigated the expression of MDR-1 protein in cultured human hepatocytes in response to dexamethasone. Although the antibody used (MDR ab-1) does not discriminate among the members of the MDR family, there is substantial evidence that MDR-1 is the primary Pgp subtype expressed in hepatocytes (Fojo et al., 1987; Thiebaut et al., 1987). As already reported for hepatocyte cell lines (Zhao et al., 1993), dexamethasone induced the Pgp protein in our hepatocyte cultures (Fig. 3A). However, we failed to detect any change in MDR protein expression in response to rifampicin and clotrimazole, in contrast to previous observations in a human colon adenocarcinoma cell line, LS180 (Schuetz et al., 1996). Because our results imply that efflux of xenobiotics from human hepatocytes should be increased and not reduced by dexamethasone, modulation of

the cellular concentration of inducers cannot account for the observed enhancement of CYP3A4 induction.

**Effect of Dexamethasone on RXR $\alpha$  Protein and mRNA Expression in Cultured Human Hepatocytes.** Because glucocorticoids have been reported to increase RXR $\alpha$  expression and enhance thyroid hormone action in cultured rat hepatocytes (Yamaguchi et al., 1999), we wondered whether the expression of this factor also could be increased by dexamethasone in cultured human hepatocytes. We anticipated that, if this was the case, the response to compounds acting through receptors heterodimerizing with RXR $\alpha$ , such as PXR, should be greater. The results reported in Fig. 3A show that 100 nM dexamethasone increased the expression of RXR $\alpha$  immunoreactive protein, whereas PXR activators rifampicin or clotrimazole only induced the CYP3A4 protein. Induction of RXR $\alpha$  mRNA by dexamethasone is shown in Fig. 3, B and C. In parallel, we analyzed the expression of TAT, a prototypical target gene of the GR. The levels of both RXR $\alpha$  and TAT mRNAs were increased in response to treatment



**Fig. 3.** Dexamethasone increases RXR $\alpha$  protein and mRNA expression in cultured hepatocytes. A, 48 hours after plating of hepatocytes (FT157), dexamethasone was withdrawn from the medium for 16 h. Cells were then cultured in the presence of 100 nM dexamethasone, 5  $\mu$ M rifampicin or clotrimazole for 48 h. Cells were harvested and total protein was extracted as described in *Experimental Procedures*. One hundred micrograms of total protein was subjected to Western blot analysis with specific antibody against MDR Ab-1, RXR $\alpha$ , and rabbit CYP3A6. Complementary DNA-expressed form of CYP3A4 (Genetex, Woburn, MD) was used as migration standard, and RXR $\alpha$  protein was present as a 56-kDa band identical with that synthesized in vitro. Similar results were obtained with four other cultures tested (FT154, FT157, FT159, FT160). B, 48 hours after plating of hepatocytes, dexamethasone was withdrawn from the medium for 16 h. Cells were then cultured 48 h in the presence of dexamethasone at the indicated concentrations, ranging from 10 nM to 1  $\mu$ M in absence (top) or presence of 1  $\mu$ M RU486 (bottom). Total RNA was extracted and 30  $\mu$ g was subjected to Northern blot analysis with RXR $\alpha$ , GAPDH, or TAT  $^{32}$ P-labeled cDNA probes. These Northern blots were obtained from cultures FT157 and FT160. C, 48 hours after plating of hepatocytes, dexamethasone was withdrawn from the medium for 16 h. Cells were then cultured in the presence of dexamethasone at the indicated concentrations for different periods as mentioned. These Northern blots were obtained from culture FT155 and similar results were obtained with two other cultures tested (FT157, FT160). Total RNA was extracted and 30  $\mu$ g was subjected to Northern blot analysis with RXR $\alpha$ , GAPDH, or TAT  $^{32}$ P-labeled cDNA probes.

with dexamethasone for 48 h, in a similar concentration-dependent manner (Fig. 3B, top). Dexamethasone did not significantly affect the relative concentration of GAPDH mRNA, used as a control. Cotreatment with RU486, a glucocorticoid antagonist, inhibited the dexamethasone-mediated RXR $\alpha$  mRNA accumulation (Fig. 3B, bottom). In addition, the time course induction of RXR $\alpha$  mRNA paralleled that of TAT mRNA, and these experiments revealed that a significant accumulation of these messengers was obtained after 12 h of treatment (Fig. 3C). These results are close to those previously described by others with primary cultures of rat hepatocytes or rat hepatoma cell lines where the direct action of dexamethasone on the transcription of the RXR $\alpha$  gene was noted (Wan et al., 1994; Yamaguchi et al., 1999). These authors reported that dexamethasone induces RXR $\alpha$  mRNA accumulation, the increase of which was significant at  $10^{-8}$  M dexamethasone and was not affected by pretreatment with CHX. Finally, because dexamethasone did not affect the degradation of RXR $\alpha$  mRNA, they proposed that this effect was through enhanced transcription (Yamaguchi et al., 1999).

**Effect of Dexamethasone on PXR mRNA Expression in Cultured Human Hepatocytes.** Next, we analyzed the effect of dexamethasone and other glucocorticoid agonists on the expression of PXR in our cultures. For this purpose, an RNase protection assay was performed, with an hPXR-specific probe encoding part of the ligand-binding domain. This riboprobe allows the detection of both PXR-1 and PXR-2 mRNAs because they only diverge in their 5' region (Bertilsson et al., 1998). As shown in Fig. 4A, PXR mRNA was induced specifically upon exposure to the GR agonists tested, i.e., dexamethasone, prednisolone, and to a lesser extent, hydrocortisone. In contrast, rifampicin, clotrimazole, RU486, PCN, and phenobarbital did not affect its expression at micromolar concentrations (Fig. 4, A and B), not even in the presence of 100 nM dexamethasone (data not shown).

Regulation of gene expression by glucocorticoids is known to occur through two distinct mechanisms involving the GR. The primary response is immediate and does not depend on de novo protein synthesis, as previously reported in particular for RXR $\alpha$  (Yamaguchi et al., 1999). The secondary response requires the synthesis of a factor in response to the ligand, which is necessary for subsequent gene activation by the GR (Nebes and Morris, 1987; Fan et al., 1992). The nature of the response of the PXR gene to dexamethasone in cultured hepatocytes was further investigated by analyzing the following: 1) the concentration- and time-dependence of PXR mRNA induction; 2) the effect of actinomycin D, an inhibitor of transcription; 3) the effect of CHX, an inhibitor of protein synthesis; and 4) the effect of a glucocorticoid agonist, RU486. As shown in Fig. 4, B and C, dexamethasone was a very potent inducer of PXR mRNA, as observed with RXR and TAT mRNAs. The induction of PXR mRNA in response to 100 nM dexamethasone ranged from a 2.3- to 6-fold increase, depending on the culture. In addition, the increase of the PXR mRNA level could be detected as early as 6 to 8 h after beginning the treatment, the maximum level being reached between 8 and 12 h (depending on the culture), similar to the result with TAT and RXR $\alpha$  mRNAs. The experiments with actinomycin D (Fig. 5A) showed that dexamethasone did not influence the subsequent decay of the PXR mRNA after it has been induced by dexamethasone (i.e., 24 h with 1  $\mu$ M dexa-

methasone). This suggests that dexamethasone does not affect the degradation of PXR mRNA, and that the increase in PXR mRNA in response to dexamethasone is not through the stabilization of its mRNA. Moreover, pretreatment with CHX did not affect the increase of PXR mRNA accumulation in response to dexamethasone (Fig. 5B). Finally, a concomitant treatment with RU486 significantly decreased both PXR and TAT mRNA accumulation in a similar way (70% of inhibition; Fig. 5C). These observations are consistent with a direct transcriptional regulation of PXR expression by the activated GR.

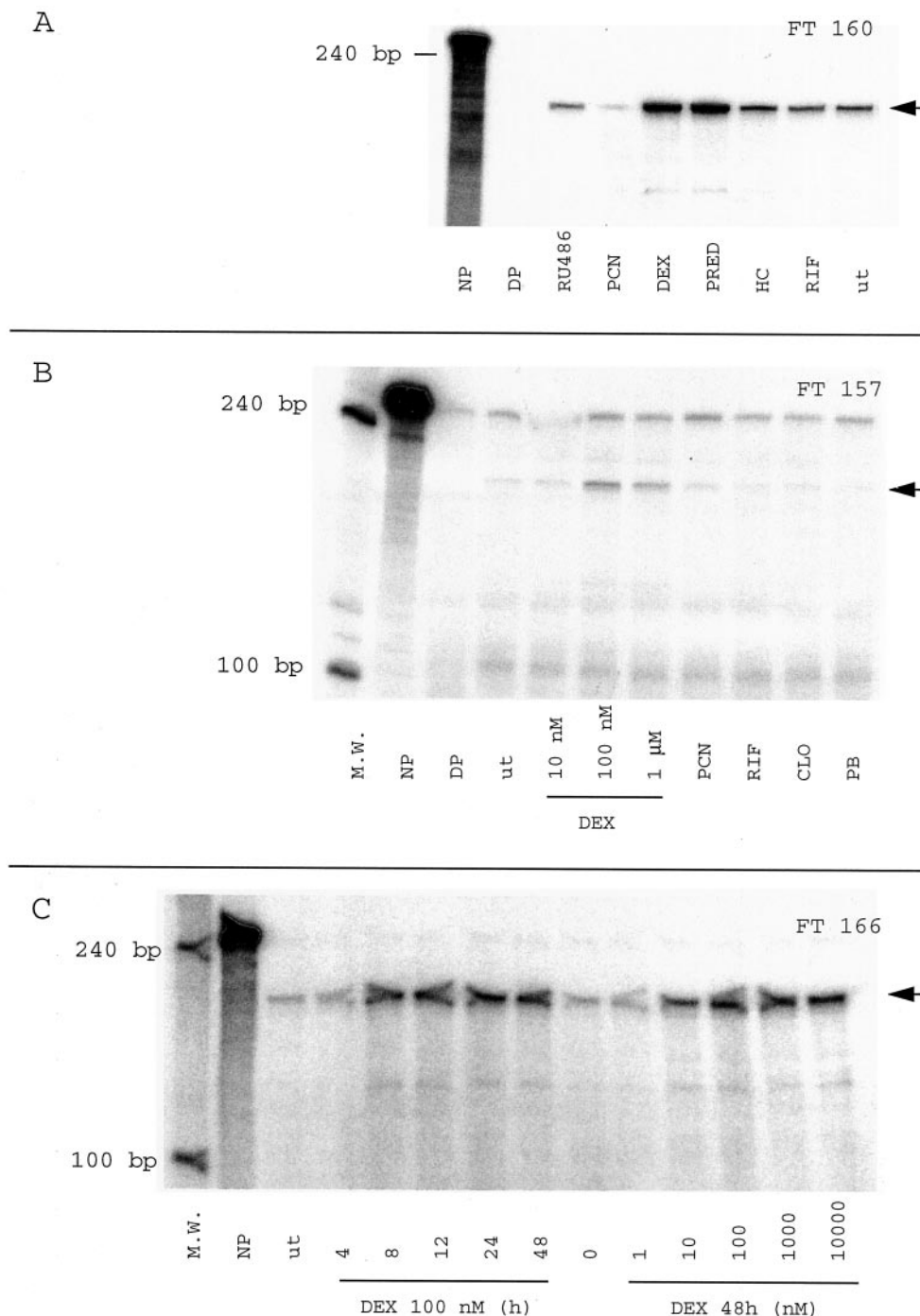
**Effect of Dexamethasone on ER6-Binding Activity in Human Hepatocyte Nuclear Extracts.** The functional activity of RXR $\alpha$  and PXR proteins in our cultures was investigated by electrophoretic mobility shift assay, with nuclear extracts prepared from hepatocytes exposed to 100 nM dexamethasone and radiolabeled CYP3A4 ER6. In vitro synthesized mRXR $\alpha$  and mPXR were used as positive controls in these experiments. As shown in Fig. 6A, a complex was present in nuclear extracts from untreated and dexamethasone-treated hepatocytes; however, its amount was significantly greater in the nuclear extracts from dexamethasone-treated hepatocytes (2- to 4- fold induction depending on cultures, mean = 3.6,  $P < .05$ ,  $n = 5$ ; FT155, FT157, FT160, FT164, FT166). This complex comigrated with the one formed with the in vitro synthesized RXR $\alpha$ :PXR heterodimer. The nature of this complex was characterized further by competition experiments and supershift analysis. An excess of wild-type CYP3A4 ER6 displaced most of the complex formed in hepatocyte extracts, whereas an excess of mutated ER6 oligonucleotide did not (Fig. 6A). In addition, this complex was retarded with anti-RXR $\alpha$  antibodies because the RXR $\alpha$ :RXR $\alpha$  homodimer bound to a DR-1 probe (Fig. 6B), but not with anti-GR antibodies (data not shown). Next, we analyzed the kinetics of the formation of PXR:RXR $\alpha$  heterodimer by electrophoretic mobility shift assays with an ER6 oligonucleotide as a probe. In parallel, we evaluated the kinetics of the formation of RXR $\alpha$  homodimer in the same nuclear extracts, by the same method with a DR-1 oligonucleotide as a probe. The results are presented in Fig. 6C. RXR homodimers reached a maximal level in the nucleus after 12 to 24 h of dexamethasone treatment. Western blot analysis of the same extracts with an RXR $\alpha$ -specific antibody nicely confirmed this observation. Thus, there is no significant lag between the production of RXR $\alpha$  mRNA (reaching a maximum after 12 to 24 h of dexamethasone treatment; Fig. 3C) and the formation of the homodimer in the nucleus. In comparison, although the accumulation of PXR mRNA reached a maximum after 12 h (Fig. 4C), the formation of the PXR:RXR $\alpha$  heterodimer was detectable in the nucleus only after 12 h and increased continuously between 24 and 48 h. Interestingly, this kinetics fits nicely with the kinetics of CYP3A4 mRNA induction presented in Fig. 2A. We therefore conclude that formation of the PXR:RXR $\alpha$  heterodimer in the nucleus is the rate-limiting step in CYP3A4 up-regulation by dexamethasone.

**Expression of PXR, RXR $\alpha$ , and GR in Human Liver Tissue Compared with Cultured Hepatocytes.** To evaluate the relevance of our observations to the in vivo situation, we compared the levels of PXR, RXR $\alpha$ , and GR mRNAs in our cultures with those measured in the corresponding tissue and freshly isolated hepatocytes. For this purpose,



hepatocytes were plated and cultured in our standard conditions (i.e., ISOM medium, in the presence of 100 nM dexamethasone) for 2 days. The culture was then continued, either in the absence or presence of dexamethasone for 2 more days (between days 3 and 4 after plating). Total RNA was extracted from the liver tissue (before collagenase perfusion), freshly isolated hepatocytes (before plating), and cultured hepatocytes (at days 1, 2, 3, and 4 after plating), and analyzed by RNase protection or Northern blot. The results are reported in Fig. 7 for patient FT166. PXR, RXR $\alpha$ , and GR mRNAs were expressed significantly in the tissue and their levels exhibited no major change when measured in freshly

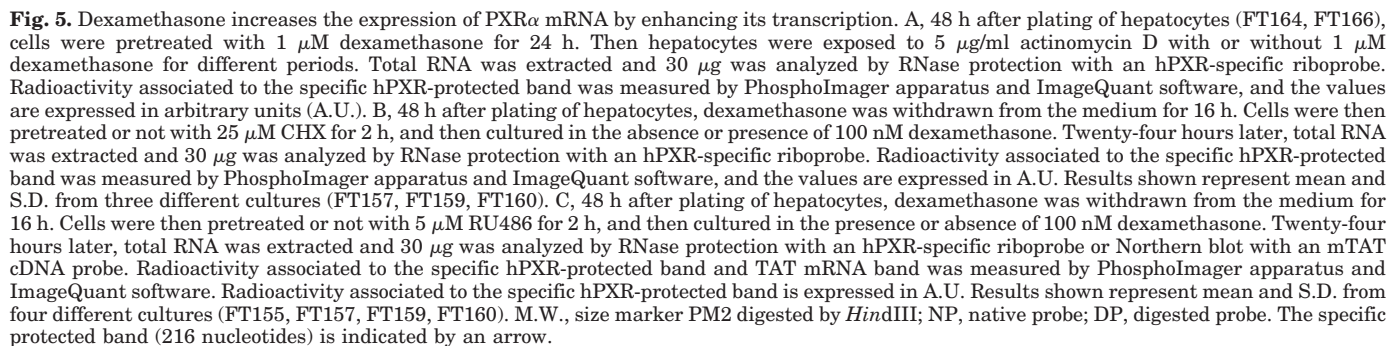
isolated hepatocytes. In the standard culture conditions (i.e., in the presence of 100 nM dexamethasone), PXR mRNA exhibited a transient decrease at day 1 after plating, but returned to a level close to that observed in the tissue at day 2, and this level was maintained up to day 4. RXR $\alpha$  exhibited a similar behavior. The transient decrease observed at day 1 is thought to result from the stress due to cell isolation and plating, as this is routinely observed with other phenotype markers such as  $\alpha$ 1-antitrypsin, for example (J. B. Ferrini, personal communication). As expected, when cells were cultured in the absence of dexamethasone (between days 2 and 4), the levels of both PXR and RXR $\alpha$  mRNA exhibited a



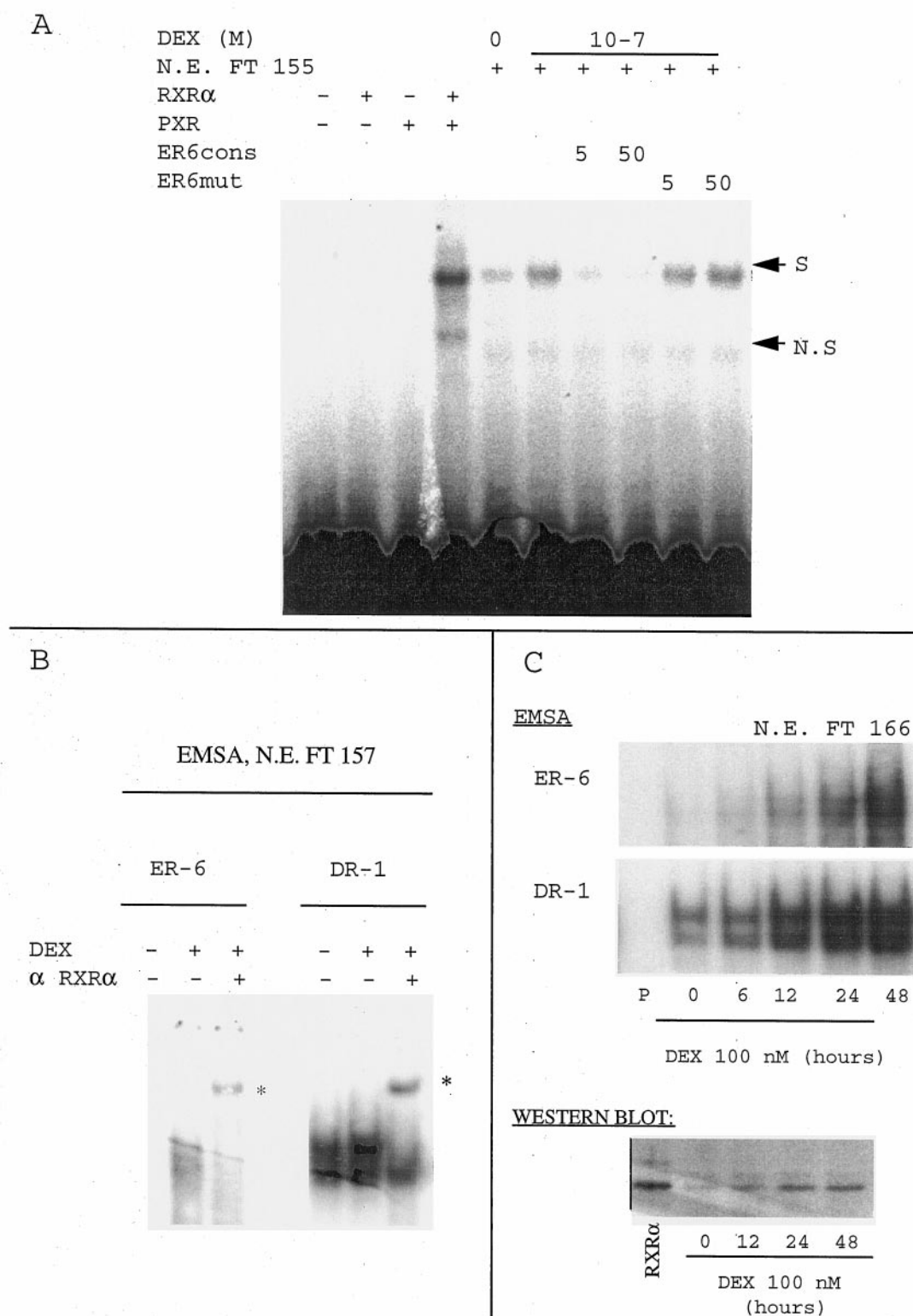
**Fig. 4.** Dexamethasone increases PXR mRNA expression in cultured hepatocytes. Forty-eight hours after plating of hepatocytes, dexamethasone was withdrawn from the medium for 16 h. **A**, cells from FT160 were then cultured in the presence of 1  $\mu$ M dexamethasone (DEX), prednisolone (PRED), hydrocortisone (HC), or 5  $\mu$ M RIF, RU486, and PCN for 48 h. Total RNA was extracted and 30  $\mu$ g was analyzed by RNase protection with an hPXR-specific riboprobe. **B**, cells from patient FT157 were then cultured 48 h in the presence of 5  $\mu$ M rifampicin (RIF), 5  $\mu$ M clotrimazole (CLO), 5  $\mu$ M PCN, 50  $\mu$ M phenobarbital (PB), or dexamethasone at the indicated concentrations ranging from 10 nM to 1  $\mu$ M. Total RNA was extracted and 30  $\mu$ g was analyzed by RNase protection with an hPXR-specific riboprobe. **C**, cells from patient FT166 were cultured in the presence of dexamethasone at the indicated concentrations ranging from 1 nM to 10  $\mu$ M for different periods. Total RNA was extracted and 30  $\mu$ g was analyzed by RNase protection with an hPXR-specific riboprobe. M.W., size marker PM2 digested by *Hind*III; NP, native probe; DP, digested probe. The specific protected band (216 nucleotides) is indicated by an arrow.

sponse to dexamethasone parallels that of TAT mRNA in terms of time course and concentration dependence. Because the dexamethasone-mediated induction of RXR $\alpha$  gene transcription has been thoroughly investigated by others (Yamaguchi et al., 1999), we focused our attention on the molecular mechanism leading to PXR gene control by glucocorticoids, and notably dexamethasone. Although run-on experiments have not been performed in this study, our results suggest that this control occurs at the transcriptional level because 1) dexamethasone did not affect the degradation of PXR $\alpha$  mRNA; 2) PXR mRNA induction is partially blocked by the glucocorticoid antagonist RU-486; and 3) CHX does not affect the expression of this mRNA, whereas it significantly blocks the enhancement of CYP3A4 induction. The finding that the GR controls positively the expression of

In this work we have investigated the mechanism by which dexamethasone, at submicromolar concentrations, enhances the induction of CYP3A4 in response to PXR activators in human hepatocytes in primary culture. Our results show that the expression of both RXR $\alpha$  and PXR mRNA in re-







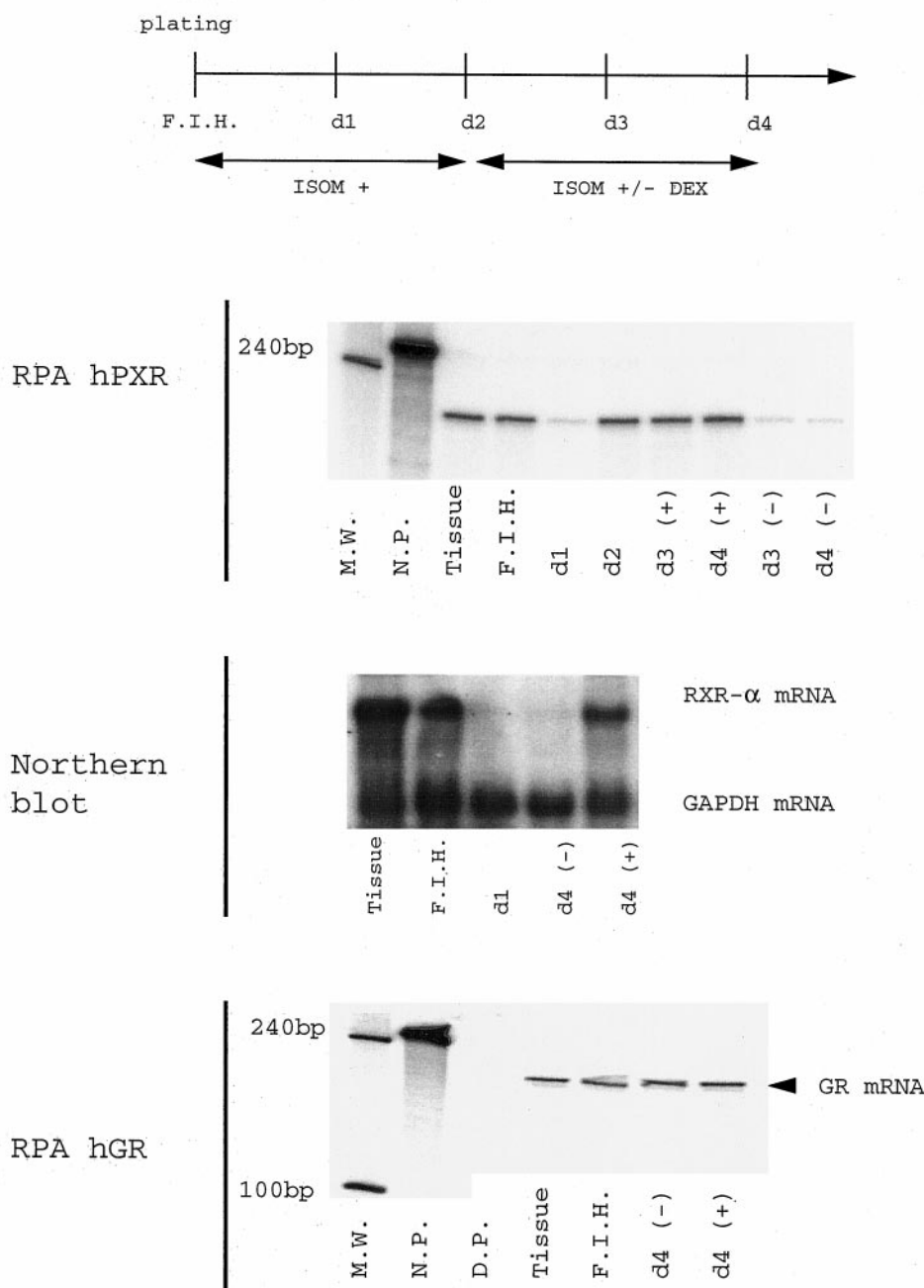
**Fig. 6.** Dexamethasone increases ER6-binding activity in nuclear extracts from cultured hepatocytes. Forty-eight hours after plating of hepatocytes, dexamethasone was withdrawn from the medium for 16 h. Cells were then cultured in the presence or absence of 100 nM dexamethasone for different periods and then harvested for the preparation of nuclear extracts as described in *Experimental Procedures*. A, in vitro synthesized mPXR.1, mRXR $\alpha$ , or 25  $\mu$ g of nuclear extracts from hepatocytes treated or not with 100 nM dexamethasone for 48 h (NE, FT157) were used for electrophoretic mobility shift assays with a <sup>32</sup>P-labeled CYP3A4 ER6 oligonucleotide in the presence of either a 5- or 50-fold excess of unlabeled oligonucleotide containing the CYP3A4 ER6 (ER6 cons) or a mutated CYP3A4 ER6 (ER6 mut) as indicated. Specific (S) and nonspecific (NS) bands are indicated. B, hepatocytes from FT157 were treated for 48 h with or without 100 nM dexamethasone and 40  $\mu$ g of nuclear extracts was used for electrophoretic mobility shift assays with a <sup>32</sup>P-labeled CYP3A4 ER6 or DR-1 oligonucleotides in the presence of 1  $\mu$ g of anti-RXR $\alpha$  antibody as indicated. \*, supershifted associated bands. C (top), hepatocytes from FT166 were treated for different periods with 100 nM dexamethasone and 30  $\mu$ g of nuclear extracts was used for electrophoretic mobility shift assays with a <sup>32</sup>P-labeled CYP3A4 ER6 or DR-1 oligonucleotides as indicated. Bottom, 50  $\mu$ g of the same nuclear extract was subjected to Western blot analysis with an anti-RXR $\alpha$  antibody.

both RXR $\alpha$  and PXR thus accounts for the synergistic effect of dexamethasone and PXR activators on CYP3A induction observed herein. In addition, this provides an explanation of previous observations reported by others, such as the potentiation of CYP3A induction in response to metyrapone or PCN by submicromolar concentrations of dexamethasone in cultured rat hepatocytes (Wright, 1996; Pereira et al., 1998). We recognize that some of the experimental conditions used, in particular the depletion of glucocorticoid from the medium (herein dexamethasone as a substitute), may appear far from physiological. However, the advantage of this *in vitro* model is to enable such modulation of gene expression in a way that would not be possible *in vivo*. Interestingly, these results

show that the constitutive levels of PXR, RXR $\alpha$ , and GR mRNAs in our standard culture conditions (i.e., in the presence of 100 nM dexamethasone) are close (if not identical) to those observed in the liver *in vivo*. Our data provide further arguments in favor of the critical role of glucocorticoids such as dexamethasone for the maintenance of differentiated phenotype in cultured hepatocytes. This explains *a posteriori* why these cultures represent a relevant *in vitro* model for investigating the transduction pathway controlling CYP3A4 gene induction.

The main physiological role of RXR $\alpha$  is to heterodimerize with several nuclear receptor partners, thus allowing the transduction of specific signals in response to specific li-

## Culture and sampling protocol



**Fig. 7.** PXR, RXR $\alpha$ , and GR mRNA receptors levels in human liver compared with cultured hepatocytes. Hepatocytes from patient FT166 were plated and cultured in the standard conditions (ISOM medium, 100 nM dexamethasone) for 2 days. Cells were cultured either in the absence (-) or presence (+) of 100 nM dexamethasone until day 4. Total RNA was extracted from the liver tissue, freshly isolated hepatocytes (F.I.H.), or cultured hepatocytes at the time indicated. Thirty micrograms of RNA was analyzed by RNase protection with hPXR- or hGR-specific riboprobes or Northern blot with RXR $\alpha$  or GAPDH  $^{32}$ P-labeled cDNA probes.

gands. Therefore, it is to be expected that under the conditions in which the amount of RXR becomes rate limiting, increased expression of this factor would lead to the enhancement of the transcriptional activity of these nuclear receptors. Indeed, it has been shown that dexamethasone enhances the differentiating effects of c-retinoic acid in rat hepatoma cell lines (Wan et al., 1994) and the T3-responsiveness of several T3-regulated genes in primary cultured rat hepatocytes (Yamaguchi et al., 1999), by increasing the expression of RXR $\alpha$  in these cellular systems. However, peroxisome-proliferator-activated receptor has been shown to inhibit selectively the transcriptional activity of thyroid receptors by competition for RXR $\alpha$  (Juge et al., 1995). In addition, although most cells contain endogenous RXRs and significant activity is obtained when a receptor such as the vitamin D receptor is expressed, this activity is much greater when exogenous RXR $\alpha$  is provided by transfection (Weigel, 1996).

In contrast to the action of dexamethasone, RU486, rifampicin, clotrimazole, or phenobarbital, all known inducers of CYP3A4 and hPXR activators, failed to induce PXR expression in our cultures, even in the presence of dexamethasone (data not shown). Zhang et al. (1999) recently reported that accumulation of rPXR mRNA in rat liver was increased by dexamethasone and PCN but not by troleandomycin, a potent CYP3A inducer. Interestingly, they observed that two non-CYP3A inducers, isoniazide and perfluorodecanoic acid, were the strongest inducers of PXR mRNA among 10 compounds tested. These observations suggest therefore that PXR activators are not necessarily involved in the positive control of PXR gene expression, either in cultured human hepatocytes or in the rat in vivo.

Our finding (Fig. 1) that dexamethasone induces CYP3A4 mRNA expression at submicromolar concentration in cultured human hepatocytes is puzzling. Indeed, it has been reported by Lehmann et al. (1998) that this compound is a weak activator (if at all) of hPXR, with an ED<sub>50</sub> in the range of 10  $\mu$ M. One possibility is that hGR is able to activate directly the transcription of CYP3A4. This is unlikely because the time course of this induction requires at least 24 to 48 h (data not shown), in contrast to the rapid induction (within 12 h) of TAT, RXR $\alpha$ , or PXR mRNAs. Two other possibilities can be proposed. Either PXR is able to activate CYP3A4 expression even in the absence of a specific activator (basal expression), or a weak PXR activator is present in the culture medium or synthesized in the cells. At the moment we cannot discriminate between these two alternatives. It is likely that concomitant treatment with compounds that increase the expression of PXR and RXR $\alpha$  and compounds that activate PXR will produce a synergistic increase of CYP3A4 expression. This classification provides a molecular basis for the structural diversity of CYP3A inducers and may explain the apparent discrepancies between the capacity of xenobiotics to activate PXR in transfection studies and their effectiveness as CYP3A4 inducers in human hepatocytes.

Because GR controls the expression of both PXR and RXR $\alpha$ , it suggests a possible explanation for previous observations in animals and humans that CYP3A gene expression is negatively regulated during inflammation and/or infections. These pathological situations are known to result in the increased release in the blood of cytokines, including interleukin-6 and tumor necrosis factor- $\alpha$ . We and others

have observed that expression of CYP3A4 mRNA (and protein) is strongly repressed in cultured human hepatocytes exposed to physiological concentrations of interleukin-6 and tumor necrosis factor- $\alpha$  (Muntane-Relat et al., 1995). These cytokines are known to activate a cascade of genes and factors that leads to the transcriptional activation of c-Jun and c-Fos. These genes have been shown to interact with and inhibit the transcriptional activity of GR (Schule et al., 1990; McKay and Cidlowski, 1998). It is therefore likely that in hepatocytes exposed to cytokines, inactivation of a portion of GR molecules by these proto-oncogenes could result (at least partly) in a decreased expression of both RXR and PXR that eventually leads to a decrease in the expression of CYP3A4 gene.

In conclusion, our results reveal the existence of a functional cross talk between the GR- and PXR-signaling pathways, with potential implications in the regulation of steroid hormone and sterol homeostasis.

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