Divergent Regulation of the Class II P-Glycoprotein Gene in Primary Cultures of Hepatocytes versus H35 Hepatoma by Glucocorticoids

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Abstract
We investigated whether the glucocorticoid-mediated mechanisms controlling P-glycoprotein (pgp2 or mdr1b) are similar in normal hepatocytes compared with the H35 hepatoma cell line. In primary rat hepatocytes, dexamethasone (DEX) caused a dose- and time-dependent decrease in the amount of the pgp2 mRNA, which correlated with functional pgp2 expression (intracellular accumulation of [3H]vincristine). The suppression of pgp2 mRNA was specific for glucocorticoids because a representative estrogen and progesterin were without effect, and DEX suppression of pgp2 mRNA could be reversed by cotreatment with an anti-glucocorticoid. DEX suppression of pgp2 mRNA appears to be posttranscriptional because following actinomycin D inhibition of new RNA synthesis, the pgp2 transcript disappeared at a faster rate in DEX treated versus untreated hepatocytes. Moreover, transcriptional activity of chloramphenicol acetyltransferase plasmids containing the pgp2 promoter in primary rat hepatocytes was unaffected by DEX treatment. Thus, suppression of pgp2 mRNA by glucocorticoids in primary hepatocytes is due to a decrease in pgp2 mRNA stability. In contrast, in the H35 hepatoma cell line, DEX dose dependently increased pgp protein and pgp2 mRNA, effects which parallel transcriptional activation of the pgp2 promoter. Activation of the pgp2 promoter was specific for glucocorticoids since a representative estrogen had no significant effect on transcription of the pgp2 promoter and RU486 blocked DEX activation of pgp2 transcription. Transcriptional activation of the pgp2 promoter was not due to a global up-regulation of basal transcription factors because DEX treatment did not activate either a herpes simplex virus thymidine kinase promoter or the SV40 early gene promoter. Further studies with a panel of pgp2 5’ sequence deletion plasmids revealed that the minimal promoter (~66 bp) was not activated by DEX. In contrast, inclusion of sequences up to ~177 bp restored DEX-dependent transcriptional activation. These are the first studies to demonstrate that glucocorticoids regulate pgp2 by different mechanisms in normal rat hepatocytes compared to the H35 hepatoma cell line.

Introduction
The multidrug resistance gene encodes a plasma membrane transporter, pgp, which is a phosphoglycoprotein. The pgp genes are part of a small gene family that is composed of three members in rodents and two in humans (reviewed in Ref. 1) for which cDNAs have been isolated and characterized. Full-length cDNAs for mouse mdr1 (2), mouse mdr-3 (3), and human MDR1 (4), but not mouse mdr2 (5) or human MDR2 (MDR3 (6), can confer the multidrug-resistant phenotype when transfected and overexpressed in drug-sensitive cells. Since pgps function as ATP-dependent drug efflux transporters (7), many studies have causally linked overexpression of pgp with the development of the multidrug resistant phenotype (8), and in many human tumors which are refractory to treatment, overexpression of pgp is associated with drug resistance (9). Despite its clear importance in the development of drug resistance, its physiological role has not been elucidated. The pgps are expressed in a polarized fashion on the apical membrane of epithelial cells lining the luminal space (10), suggesting that these may function in normal tissues as transporters of naturally occurring toxic substances and endogenous cellular products, such as steroids.

Early studies suggested that the transcriptional activity of the mouse mdr1 promoter was differentially active, depending upon the lineage of the recipient cell (11). These studies clearly formed the basis for the formal possibility that differential activation of the pgp gene between the parental drug-sensitive cells and the multidrug-resistant cells might be dependent on the types and amounts of transcription factor proteins and their activation of different pgp cis-regulatory elements. Thus, current studies have focused on cloning the 5’-flanking sequences of the pgp genes and elucidating the cis-regulatory elements and transcriptional factors controlling their basal expression (12–15). However, the expectation of finding differential transcriptional acti-

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1 The abbreviations used are: pgp, P-glycoprotein protein product; MDR, multidrug resistance gene; pgp2, rat class II pgp gene (also known as mdr1b); CAT, chloramphenicol acetyltransferase; DEX, dexamethasone, 16a-fluoro-16a-methyl-11β,17α,21-trihydroxyprogna-1,4-diene-3,20-dione; MMTV, mouse mammary tumor virus; GRE, glucocorticoid response element; GCR, glucocorticoid receptor; CYP, cytochrome P450; LTR, long terminal repeat.
viation of ppg cis-regulatory elements between parental drug-sensitive cells and the multidrug-resistant cells has not been widely realized (16). Nevertheless, recent studies have reported that hepatic ppg is suppressed by DEX in primary cultures of rat hepatocytes (17) but increased in mouse and human hepatoma cells (18). While these reported differences in glucocorticoid regulation of hepatic ppg could reflect species differences in ppg regulation (19), it was also possible that the differences in DEX regulation of ppg in normal hepatocytes versus hepatomas is due to differences in transcription factors acting upon different cis-regulatory elements in the ppg gene.

Our initial investigations in this report demonstrate that glucocorticoids suppress ppg2 expression in normal rat hepatocytes while increasing ppg2 expression in the continuously replicating rat hepatoma cell line, Reuber H35. These findings represented the first opportunity to explore for the ppg gene family the mechanisms involved in the divergent glucocorticoid regulation of ppg in normal hepatocytes compared with hepatoma cells. Although there is evidence for posttranscriptional regulation of hepatic genes by dexamethasone (20), glucocorticoids such as dexamethasone have been shown to increase the transcription of many liver proteins, such as tyrosine aminotransferase, by binding to consensus GRE palindrome or GRE half-sites (reviewed in Ref. 21). Glucocorticoids can also suppress the transcription of some liver genes, such as type I collagen (22), by binding to nGREs or to GREs (23). Indeed, a putative GRE half-site exists in the 5′-flanking sequences of the rat ppg2 gene, which we have described recently (24), which could participate in glucocorticoid regulation of ppg. In this report, we have used a ppg2 gene-specific probe and transient transfection of a series of ppg2CAT and ppg2Luc constructs into normal rat hepatocytes and the H35 rat hepatoma cell line to determine the mechanistic basis for the divergent regulation of ppg2 by glucocorticoids in normal rat hepatocytes compared to a hepatoma cell line.

**Results**

**Effect of DEX on ppg2 mRNA Levels in Primary Rat Hepatocytes.** Fardel et al. (17) reported that DEX decreased ppg expression in primary rat hepatocytes cultured on serum-coated plastic. However, we and others routinely culture primary rat hepatocytes on the basement membrane matrix, Matrigel, which can have dramatic effects on hepatic gene expression. Therefore, we first determined whether DEX would alter ppg expression in rat hepatocytes cultured on Matrigel. Because we (25) and others (26) have demonstrated that ppg2 mRNA and immunoreactive protein increase during hepatocyte culture (and that ppg2 is the predominant family member expressed), we assessed whether DEX treatment prevented this increase in ppg2 mRNA. Hepatocytes were cultured for a total of 72 h in either the absence of DEX, or DEX was added for the intervals shown before being harvesting all cells at 72 h of culture for RNA. The RNA was analyzed by Northern blot probed successively with probes that are gene-specific for ppg2, the glucocorticoid inducible CYP3A1, and cyclophilin. The amount of ppg2 mRNA was 8-fold higher in the untreated 72-h control cells (Fig. 1, labeled C) compared with 72-h cells, which received DEX for 24 h prior to harvest. Longer incubation with DEX (total, 48 h) resulted in further suppression in the amount of ppg2 mRNA at 72 h to 6% of the 72-h control cells. Interestingly, the size of the ppg2 transcript decreased with increasing time of DEX treatment (Fig. 1). The suppression of ppg2 mRNA by DEX was specific because mRNA for cytochrome P4503A (CYP3A) was dramatically increased by DEX. Moreover, ethidium bromide staining of the Northern blot before and after transfer to membrane revealed uniform loading and transfer of the RNA (data not shown). To further control for possible RNA loading variation, we probed for cyclophilin mRNA because it has been reported to be unperturbed by a variety of conditions in hepatocytes (27). In these experiments, the level of cyclophilin mRNA actually appeared to slightly increase with longer DEX treatments. Further studies revealed that for hepatocytes cultured on type I collagen, DEX similarly suppressed ppg2 mRNA levels.4 Thus, regardless of the substrate on which primary rat hepatocytes are cultured [serum-coated plastic (17), type I collagen,4 or Matrigel (Fig. 1)], DEX treatment prevents the increase in ppg2 mRNA.

**Effect of DEX on Functional ppg Expression in Primary Rat Hepatocytes.** To determine whether changes in expression of ppg2 mRNA were paralleled by changes in ppg2 functional protein, we assessed the effect of DEX treatment on the uptake of [3H]vincristine (25), a ppg substrate. Because pgp3 does not transport drugs (5, 6) and pgp1 mRNA is not expressed in cultured rat hepatocytes (26, 28), in 72-h hepatocytes [3H]vincristine uptake will directly assess ppg2 uptake.

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4 J. Schuetz, unpublished observation.
function. [3H]Vincristine accumulation was measured in 72-h hepatocytes cultured on Matrigel in the absence or presence of a 24- or 48-h pretreatment with DEX. As the length of DEX pretreatment increased from 24 to 48 h, [3H]Vincristine accumulation increased to 183 and 380% of untreated cultures, respectively (Fig. 2). The addition of DEX (10^{-5} M) to cultures 15 min before the addition of [3H]Vincristine had no effect upon [3H]Vincristine uptake compared to untreated control cultures, suggesting DEX may not be a substrate for pgp2. To determine if DEX produced a dose-dependent increase in the accumulation of [3H]Vincristine, hepatocytes were treated with various concentrations of DEX for 48 h, washed, and resuspended into drug-free medium, followed by the addition of [3H]Vincristine (Fig. 2, inset). Pretreatment with DEX caused a concentration-dependent increase in hepatic [3H]Vincristine accumulation. DEX treatment of hepatocytes cultured on type I collagen, followed by an evaluation of [3H]Vinc-

Fig. 2. Effect of dose and duration of DEX pretreatment on vincristine accumulation in primary rat hepatocytes. Hepatocytes were cultured for 72 h on Matrigel. Some cells received either no treatment throughout culture (○) or were treated with 10^{-5} M DEX from 24 to 72 h (△) or 48 to 72 h (▲) of culture. At 72 h, all hepatocytes were washed and incubated in fresh medium. The time course of [3H]vincristine accumulation was evaluated throughout a 2-h incubation in [3H]vincristine (200 nM) as described in "Materials and Methods" and Ref. 25. Bars, SD of four independent determinations. Inset, hepatocytes were cultured for 48 h in the absence (cont, control) or presence of varying concentrations of DEX and then washed with drug-free medium; [3H]Vincristine accumulation was assessed as described above. The values are expressed as a percentage of the maximum drug accumulation.

Fig. 3. Effect of DEX on the stability of pgp2 mRNA in hepatocytes. Hepatocytes were cultured for 24 h in control medium. At 24 h, pgp2 mRNA decay was determined by the addition of actinomycin D (10 μg/ml) in the absence (○) or presence of 10^{-5} M DEX (▲). Total RNA was harvested from the untreated "zero h" time control cultures and from the treated cultures after Actinomycin; 8 μg were analyzed by slot blot. The blot was sequentially hybridized with a specific rat pgp2 oligonucleotide (53), then stripped and rehybridized with radiolabeled β-actin. The autoradiographs were densitometrically quantified, and the rat pgp2 signal was normalized by the signal to β-actin. The rate of pgp2 mRNA turnover (normalized to actin) is depicted and expressed as a percentage of the signal at "zero h.

Effect of DEX on pgp2 mRNA Stability. Because DEX treatment prevented the increase in pgp2 mRNA (Fig. 1) typically observed in culture (25, 28), and because DEX appeared to decrease the size of the pgp2 mRNA transcript (Fig. 1), we determined whether DEX affected the stability of pgp2 mRNA (Fig. 3). Hepatocytes were cultured for 24 h, then RNA synthesis was inhibited with actinomycin-D, and one-half of the cultured cells were treated with DEX (10^{-5} M). DEX treatment resulted in a rapid increase in the rate of decay of pgp2 mRNA (an estimated overall pgp2 mRNA half-life of 15.5 h with DEX versus 35.5 h in untreated cultures). In contrast, DEX had no effect on the stability of β-actin.

Specificity of DEX Suppression of pgp2 mRNA. To determine if the suppression of pgp2 mRNA was specific for glucocorticoids, we compared the effect of the glucocorticoid DEX with several additional glucocorticoids (triamcinolone acetonide and corticosterone), a progestin (R5020, promegestone), an anti-glucocorticoid (RU486), and vincristine. Primary rat hepatocytes were cultured for 24 h in the absence of drug, followed by treatment for 48 h with these agents. Subsequently, RNA was isolated, and slot blot analysis was performed (Fig. 4). As positive controls, we measured the effect of these agents on two hepatic genes known to be regulated by glucocorticoids, tyrosine aminotransfer-
The concentration of DEX that inhibited pgp2 mRNA by 50% was estimated as 10^{-6} M. Paradoxically, RU486 more readily reversed DEX suppression of pgp2 at the higher concentrations of DEX. A possible explanation might be the low affinity of RU486 for the glucocorticoid receptor compared to other receptors expressed in the liver (e.g., the progesterone receptor). Nevertheless, the finding that only steroids of the glucocorticoid class suppress pgp2 mRNA, and this effect is reversed by the anti-glucocorticoid RU486, demonstrates that the inhibition of pgp2 mRNA expression is specific for glucocorticoids.

**DEX Has No Effect on Run-On Transcription of pgp2 or Transcriptional Activity of a pgp2 Promoter Plasmid.** Although these studies indicate that the stability of the pgp2 mRNA is decreased by DEX treatment, it is possible that DEX treatment disrupts the rate of pgp2 transcription. We performed run-on transcriptional analysis in nuclei isolated from rat hepatocytes cultured on Matrigel with or without 10^{-5} M DEX from 24 to 48 h of culture. These studies demonstrated that the ongoing transcription of pgp2 was unaffected by DEX treatment (negative result not shown). To further explore the possibility that suppression of pgp2 mRNA might be due to a decrease in pgp2 transcription, we developed a pgp2CAT plasmid containing the pgp2 promoter (see "Materials and Methods"; Ref. 24). These pgp2 promoter sequences have been demonstrated to contain all the necessary elements for basal transcription of pgp (Ref. 24; Fig. 10). The plasmid pgp2CAT containing the pgp2 promoter (−369 to +151) fused to the reporter gene CAT was transiently transfected into primary rat hepatocytes (Fig. 5).
6). Although it would be optimal to perform the transfections in rat hepatocytes cultured on Matrigel, it is technically impossible to transfect, by lipofection, hepatocytes cultured on this matrix (the Matrigel heparan sulfate proteoglycans adsorb the DNA-lipofectin complex; Refs. 31–33). Therefore, hepatocytes were cultured on serum-coated plastic since DEX suppresses expression of pgp2 protein and mRNA equally well in rat hepatocytes cultured on this matrix (17). As a positive control for glucocorticoid receptor-mediated gene activation, we have transfected in parallel cultures MMTVCAT, a plasmid containing the glucocorticoid-inducible promoter from the mouse mammary tumor virus LTR (34). MMTVCAT was readily induced by DEX in the primary hepatocytes with the estimated dose for producing 50% of maximal induction, $5 \times 10^{-8}$ M. Untreated rat hepatocytes transfected with pgp2CAT had readily detectable CAT activity (Fig. 6). Indeed, we found in rat hepatocytes, similar to reports in other cultured cells for mdr genes, that the basal promoter activity of pgp2CAT approaches that of plasmids with the SV40 promoter (see “Materials and Methods”) and thus, any suppression by DEX should have been readily detected. However, DEX treatment, even at concentrations as high as 10 μM had no significant effect on transcriptional activation of pgp2CAT. These results indicate that, regardless of dose, DEX does not alter transcription of the pgp2 promoter, despite the fact the MMTV LTR is readily activated by DEX in the same hepatocytes. Further, these findings recapitulate the findings in the run-on transcription assay demonstrating that DEX has no effect on nuclear transcription of pgp2 in primary cultures of rat hepatocytes.

**Effect of DEX on pgp2 mRNA and Protein in the H35 Hepatoma.** We next analyzed pgp2 gene expression in the Reuber H35 hepatoma cell line to determine whether glucocorticoids regulate pgp2 differently in these cells compared to primary rat hepatocytes. Total RNA was isolated from the H35 cell line after treatment with either no drug or varying concentrations of DEX for 24 h. Northern blot analysis showed that DEX significantly increased pgp2 mRNA in H35 cells (Fig. 7). The increase in the amount of pgp2 mRNA was quantified densitometrically and found to increase over 10-fold as the concentration of DEX was raised from $10^{-9}$ to $10^{-6}$ M, with the estimated concentration required to produce 50% of maximal induction being $7 \times 10^{-7}$ M. The blot was stripped and rehybridized with a liver-specific and glucocorticoid-regulated gene, tyrosine aminotransferase. The amount of tyrosine aminotransferase hybridizable mRNA was also dose-dependently increased by DEX, albeit at lower concentrations of steroid. These studies demonstrate that in the H35 hepatoma, unlike in the primary cultures of hepatocytes, pgp2 mRNA is increased by DEX. Additional studies showed that DEX treatment did not affect the stability of pgp2 mRNA in these cells (negative result not shown).

We next determined whether immunodetectable pgp increases in parallel with pgp2 mRNA. Crude membranes were isolated from cultures of H35 that were either untreated or treated with varying concentrations of DEX. Western blot analysis was then performed on these membranes (Fig. 8). Consistent with results on pgp2 mRNA, we found that low doses of DEX were relatively ineffective in inducing pgp, but higher doses produced a dose-related increase in the amount of immunoreactive pgp protein. Thus, both immunoreactive pgp protein and pgp2 mRNA increase after DEX treatment of the H35 hepatoma.

**Ligand and Promoter Specificity of DEX Activation of the pgp2 Promoter.** To test if the increase in hybridizable pgp2 mRNA by DEX was attributable to an increase in transcriptional activity of the pgp2 promoter, we assessed CAT activity in the H35 cells after transfection with the pgp promoter plasmid, pgp2CAT (described above; Fig. 6). pgp2CAT activity was readily detectable in untreated H35 cells and similar to that found in primary rat hepatocytes (see “Materials and Methods”). Concentrations of DEX below $10^{-8}$ M slightly increased pgp2CAT activity, while higher DEX concentrations dose-dependently activated the pgp2 promoter, with the half-maximal dose being $10^{-6}$ M (data not shown), which is similar to the amount of DEX required to activate the endogenous gene. To determine the hormone specificity of pgp2 promoter activation, we tested representative agonists of several other steroid receptor classes for their ability to activate the pgp2 promoter (Fig. 9A). Plasmids containing the mouse mammary tumor virus glucocorticoid response element (MMTVCAT) and the vitellogenin estrogen response enhancer linked to the thymidine promoter show considerable cross-reactivity with glucocorticoids, although this effect is highly dependent on cell type (Fig. 9B).
kinase promoter (VIT-TKCAT) were included as controls. Only the glucocorticoids (corticosterone, trimcinolone acetonide, and DEX) increased the activity of the pgp2 promoter, while the representative progestin, RU5020 and representative estrogen, β-estradiol, produced no significant changes from control. The H35 cells were estrogen responsive because the vitellogenin estrogen responsive enhancer was induced by β-estradiol. Likewise, for the MMTV LTR, only the glucocorticoids induced transcription from this promoter. As has been noted previously, β-estradiol actually decreased transcription from the MMTV LTR. The lack of progesterone activation of the MMTV LTR in H35 (but activation in other cell types; Ref. 35) suggested the H35 cells might be deficient in progesterone receptor. In H35 cells cotransfected with a progesterone receptor expression vector, 10^{-7} M RU5020 induced the MMTV LTR 10-fold, and Pgpp2CAT was activated 3.1-fold (SD, 0.9; n = 3). Thus, when the appropriate receptor is constitutively present, a ligand-dependent hormonal activation of Pgpp2CAT transcription can be elicited. Additional studies revealed that the anti-glucocorticoid RU486 could block the DEX-induced transcriptional activation of the pgp2 promoter and MMTV LTR. Thus, transcriptional activation of pgp2 in H35 cells is specific to the glucocorticoid class of hormone receptor ligands. Moreover, these results demonstrate that this segment of the pgp2 promoter can accurately recapitulate glucocorticoid-dependent pgp2 gene regulation of the endogenous gene and that the inability of DEX to activate the pgp2 promoter in primary rat hepatocytes (Fig. 6) is not because this promoter is DEX unresponsive.

Finally, we evaluated the DEX responsiveness of two control promoters, the herpes simplex virus thymidine kinase promoter and the SV40 gene promoter (Fig. 9B). For either promoter, a 24-h DEX treatment produced, on average, no greater than a 32% increase in promoter activity. Cumulatively, these data demonstrate that activation of the pgp2 promoter is specific for glucocorticoids, requires the
constitutive presence of the receptor, and is not due to DEX producing a general up-regulation in transcription factors.

**Localization of the DEX Responsive Region in the pgp2 Promoter.** To localize the sequences that allow DEX to activate the pgp2 promoter, we prepared a series of plasmids (Fig. 10A) containing variable amounts of the pgp2 promoter and 5′-flanking DNA, including from 66 to 369 bp upstream and from 151 to 608 bp downstream from the start of transcription (+1). These pgp2 fragments were cloned upstream of the luciferase reporter (instead of CAT) to minimize the problem of the nonsignal, sequence-dependent export of the CAT into the medium of some cells (36), a phenomenon which can account for up to 85% of total CAT activity being released into the medium of cultured hepatocytes and hepatomas. A 24-h exposure to 10^-5 m DEX resulted in greater than a 3.5-fold increase in luciferase activity for constructs containing sequences from 369 to 177 bp 5′ to the transcriptional start site. In addition, deletion of the first intron (+151 to +569) had no effect on the DEX induction (compare constructs −369 to +150 with −351 to +608). Further deletion of the glucocorticoid receptor half site (Fig. 10A) at position 277 upstream of the transcriptional initiation site had no effect on DEX induction, despite the fact that GRE half sites can weakly bind the glucocorticoid receptor (21). It was not until all but 66 bp 5′ to the start of transcription were removed that glucocorticoid responsiveness ceased (Fig. 10B). These studies localize, in H35 cells, the region conferring DEX responsiveness in the pgp2 promoter and show that it is not simply DEX causing an increase in the transcriptional activity of the basal (66 bases upstream from the transcription site) pgp2 promoter.

**Discussion**

These studies demonstrate that pgp2 expression is suppressed by glucocorticoids in the normal rat hepatocyte, whereas pgp2 mRNA is induced by glucocorticoids in the H35 hepatoma cell line. The pgp2 promoter provided a useful tool to dissect the mechanisms responsible for the differential regulation of pgp2 by DEX in primary hepatocytes versus the hepatoma cell. These results demonstrate that despite the pgp2 promoter being very active in both primary hepatocytes and H35 hepatoma cells, transcription from the transfected pgp2 promoter construct was selectively activated by DEX in the rat hepatoma cell and required 5′-sequences between bp −66 to −177 of the promoter. Indeed, this report identifies, for the first time, pgp2 promoter sequences that are selectively regulated in a hepatoma cell compared with a normal hepatocyte. Furthermore, this report demonstrates that in primary rat hepatocytes, the mode of pgp2 control by glucocorticoids is fundamentally different from regulation in H35 hepatoma cells. In primary hepatocytes, glucocorticoids had no effect on the rate of pgp2 run-on transcription, nor did they affect pgp2 promoter activity but appear to regulate pgp2 posttranscriptionally by decreasing the stability of the pgp2 mRNA transcript.

Our data also strongly indicate that steroid hormone-dependent transcriptional activation of the pgp2 promoter in the H35 hepatoma cell line is specific for glucocorticoids. Supporting evidence includes the findings that: (a) DEX dose-dependently activates the pgp2 promoter, whereas the anti-glucocorticoid RU486 blocks this activation; and (b) DEX-dependent activation of the pgp2 pro-
moter occurs in the presence of glucocorticoids, not estrogens. Moreover, DEX activation is specific for the pgp2 promoter and is not a general activation of all plasmid promoter vectors transfected into the H35 hepatoma cells. Furthermore, the transcriptional activation of the pgp2 promoter is not indirect and due to a general up-regulation of basal transcription factors because other promoters (both simple and complex) were not transcriptionally activated by DEX.

Typically, physiological doses of glucocorticoids bind to and activate the glucocorticoid receptor, and the ligand-activated receptor then binds to a specific 15-bp DNA consensus GRE in the 5'-flanking sequences of specific genes, rapidly stimulating their transcription. Unlike many glucocorticoid-regulated genes in liver, pgp2 appears "atypical" in requiring higher steroid doses and longer duration of steroid treatment to be affected. Moreover, while glucocorticoids as a class can increase pgp2 promoter activity, the 5'-flanking sequence of pgp2 (−369 to +150 bp) lacks the prototypical 15-bp GRE (24). Although a GRE "half-site" (TGTCTC at 277 bases upstream from the transcriptional start site; Ref. 22) is present in the pgp2 5'-flanking sequence and these GRE half-site sequences can weakly bind the GCR (21), deletion of this site has no effect

Fig. 10. The pgp2 promoter and reporter constructs (A) and effects of deletions of the pgp2 promoter on DEX responsiveness (B). H35 cells were transfected with the indicated pgp2Luc vectors. The pgp2 promoter deletion constructs are not drawn to scale (A). Following transfection, cells were incubated in the absence (−) or presence (+) of 10−5 M DEX for 24 h and harvested; then reporter activities were measured. The values are the average of three independent experiments, each performed in duplicate; bars, SD.
on DEX induction of pgp2 transcription. Furthermore, the region that confers DEX responsiveness (located between -177 and -66 bp upstream from the transcription initiation site) does not contain any sequence related to other non-consensus glucocorticoid receptor binding sites. In total, the current findings are consistent with the pgp2 promoter being transcriptionally activated by glucocorticoids in an “atypical” manner, most likely requiring ancillary DNA regulatory proteins. Indeed, glucocorticoid induction by ancillary proteins, in contrast to direct sequence binding by the GR, has been proposed as the mechanism for DEX induction of other genes, including a2 microglobulin (37) and a1-acid glycoprotein (38) genes. Further studies are under way to evaluate this possibility for pgp2.

In the absence of exogenous progesterone receptor, the H35 cells were refractory to R5020 activation of pgp2. The inability of the rat pgp2 promoter to respond to progesterone would have been surprising because its mouse orthologue, mdr1b, is progesterone responsive (39), and comparison of the 5'-flanking region of the two genes reveals an overall 84% sequence identity. The restoration of pgp2 progesterone responsiveness to H35 cells upon transfer of an exogenous progesterone receptor illustrates the importance of a cell’s receptor complement in eliciting hormonally regulated transcription of pgp2. Moreover, the absence or differential expression of hormone receptors may play a critical role in determining whether and how much pgp2 is expressed or ligand activatable in different tissues.

The results in the primary rat hepatocytes have established that suppression of pgp2 mRNA is specific for the glucocorticoid class of hormones since: (a) suppression of pgp2 occurs only for hormones with glucocorticoid properties, such as DEX, but not of other steroid classes (estrogens or progesterone); and (b) the potently anti-glucocorticoid RU486 reversed DEX suppression of pgp2. These results have also established that glucocorticoid suppression of pgp2 mRNA correlates with functional pgp expression since the dose-dependent decrease in the pgp2 mRNA by DEX is directly related to an increase in vincristine accumulation. Furthermore, these studies have shown that DEX can suppress pgp2 expression in primary cultures of hepatocytes, regardless of the matrix substrata.

Glucocorticoids have been demonstrated previously to destabilize the liver specific gene, α1-glycoprotein, mRNA through enhanced mRNA de-adenylation, a critical step in mRNA turnover (40). In fact, the decrease in size of the pgp2 mRNA transcript (Fig. 1) is consistent with a decrease in poly(A) tail length; moreover, preliminary results suggest that the poly(A) tail is shorter for pgp2 mRNA from DEX treated-cultures compared with control rat hepatocyte cultures.4 This mechanism does not exclude the possibility that the sequences within the pgp2 mRNA contribute to its instability. Although the consensus AAUAA Shaw-Kamen motif (41) is not present in the 3′-untranslated region of pgp2, the 3′-untranslated region of pgp2 does contain a DNA element (an AU-rich region) homologous to that involved in the glucocorticoid-induced instability of the IFN-β gene (42). Glucocorticoids could also cause mRNA instability by activating an RNase which degrades mRNAs (42). Other potential sites of mRNA destabilization, such as a CRE half-site (sequence motif TGGTCY; Ref. 22) exist in the 3′-untranslated region of pgp2 and might influence pgp2 mRNA stability, perhaps through altering the rate of export of pgp2 mRNA from the nucleus to the cytoplasm. Indeed, this mechanism has been reported to contribute to glucocorticoid-induced instability of a number of liver-derived genes (43). Since the current studies show that the stability of the pgp2 mRNA is decreased by DEX in primary rat hepatocytes, it is possible that these 3′ GR-binding sequences are important in the regulation of pgp2 mRNA stability. Indeed, such GR-binding sites have been shown to be important for GCR-regulated turnover of some genes (44).

What are the molecular mechanisms responsible for divergent DEX regulation of pgp2 in normal hepatocytes versus a hepatoma cell line? (a) While the most obvious difference, the state of hepatocyte differentiation is one possibility, this seems unlikely because we have found that, irrespective of whether the primary hepatocyte was cultured on type I collagen or serum-coated plastic versus on Matrigel (matrices which maintain less or more differentiated hepatic gene expression, respectively; Ref. 25), DEX suppressed pgp2 expression; and (b) mutations in the endogenous pgp2 promoter do not explain the differential regulation by DEX because the transfected promoter plasmids, which have the identical DNA sequence, recapitulate the response of the recipient cell. These studies, therefore, indicate that divergent regulation of pgp2 by DEX in the normal rat hepatocyte versus the rat hepatoma cell is qualitatively dominated by cellular factors indigenous to these cells rather than to structural differences in the promoter sequences in these genes. Further studies are needed to determine if differential activation of the pgp2 promoter by DEX in these cells is the consequence of differences in transcriptional proteins that interact with the promoter regulatory segments. It is unlikely that possible variations in the amounts of glucocorticoid receptor between primary rat hepatocytes and H35 hepatoma alters the glucocorticoid regulation of hepatic pgp2 because glucocorticoid induction of MMTV-CAT was actually greater in primary rat hepatocytes (in which DEX suppresses pgp2) than the H35 cells (in which DEX induces pgp2).

The possibility that glucocorticoids regulate pgp gene expression in general and that glucocorticoids might differentially regulate pgp in the normal hepatocyte and hepatoma cells has important clinical implications. Glucocorticoids are given concurrently in many chemotherapeutic regimens for their lympholytic effects, antiemetic properties, as antiinflammatory agents, as mild euphorians, as appetite stimulants, for their antihypercalcemic effects (reviewed in Ref. 45), and for their hematoprotective effect against antimetabolic cytotoxic agents (46). They are also used for their antitumor activity in chronic lymphocytic leukemias, in Hodgkin’s and non-Hodgkin’s lymphomas, in multiple myelomas, and in breast cancer (reviewed in Ref. 45). Therefore, whether glucocorticoids regulate pgp is an important consideration, both in the development of drug resistance in tumors and in cytotoxicity to the normal hepatocyte. If glucocorticoids differentially suppress pgp in normal hepatocytes, this would effectively increase the intracellular concentration of chemotherapeutic agent and could increase hepatotoxicity. In contrast, if glucocorticoids enhance pgp expression in hepatoma cells, this would facilitate the pgp-mediated chemotherapeutic resistance of the tumor. Moreover, while a limited survey of cell types (LMTK- and NIH3T3 cells) concluded that glucocorticoid induction of pgp is liver specific (18), in fact, DEX has been reported to decrease mdr1b (mouse orthologue to rat pgp2) in J7.V1–1 cells (a macrophage-like cell; Ref. 14). The high frequency with which glucocorticoids are give in combina-
tion with chemotherapy suggests that a more comprehensive survey of the tissue specificity of glucocorticoid regulation of pgp is warranted.

Materials and Methods

Plasmids

The cDNA clone tyrosine aminotransferase and pBLCAT2 vector were kindly provided by Dr. Gunther Schutz (German Cancer Research Center, Heidelberg, Germany: Refs. 47 and 48). The CYP3A1 cDNA has been described previously (49). pMMTVCAT (34) was kindly provided by Dr. R. Micsicek (German Cancer Research Center). pRSV-βgal was kindly provided by Dr. W. J. Rutter (Hormone Research Institute, University of California at San Francisco, San Francisco, CA). VIT-TKCAT (50) and the progesterone receptor expression plasmid (B isofrom) were kindly provided by Dr. P. Chambon (Institut de Chimie Biologique, Cedex, France). pGL2-prom was purchased from Promega (Madison, WI). The TK(f)Luc luciferase expression vector was constructed by excising the herpes simplex thymidine kinase promotor from pBLCAT2 (48) with BglII and BamHI. The thymidine kinase promotor was then ligated into the BglII site of the pGL2 basic vector (Promega). Orientation was determined by using restriction enzyme analysis, and these results were confirmed by DNA sequence analysis.

Oligonucleotides

Oligonucleotides were synthesized by the Center for Biotechnology, St. Jude Children's Research Hospital. The sequence and hybridization conditions for the cyclophilin oligonucleotide have been described previously (51). The rat pgp2 oligonucleotide, 5'-GAAATACCTAGCACCT-CAAATACTCCCCAC-G-3', is based on the rat genomic DNA sequences (52), and the specificity of this oligonucleotide has been published (53).

Animals

Male Sprague-Dawley rats from Harlan Laboratories (Indianapolis, IN) weighing 200–210 g were the donor animals used for preparing hepatocyte cultures. They were housed in wire-bottomed cages with access to food and water.

Cultured Cells

Preparation of Male Rat Hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats by collagenase perfusion as described (54) and cultured in standard serum-free medium, which is a modification of Waymouth MB-752 medium, containing insulin (0.157 mM) as the only hormone. The freshly isolated hepatocytes (3.5 × 10⁷) in a total volume of 3.0 ml of culture medium were placed into 60-mm culture dishes coated with Matrigel (Life Technologies, Gaithersburg, MD: Ref. 54) for all experiments except transfection (see below) and were incubated at 37°C in a humidified atmosphere of air containing 5% CO₂. Culture medium was renewed every 24 h.

Reuber H35 Hepatoma Cells. H35 cells obtained from American Type Culture Collection were maintained as per ATCC instructions.

Cellular Uptake of [³²P]Vincristine

[Vincristine (Amersham, Arlington Heights, IL) uptake was measured in primary rat hepatocytes cultured for 72 h in Waymouth MB-752 medium on Matrigel (25).

Northern Blot Analysis

Total RNA was isolated from cells pooled from five to seven culture dishes and analyzed by Northern blot or slot blot analysis (25, 54). The ethidium bromide pattern of the 18S and 28S rRNA was routinely assessed, both before and after transfer to a positively charged membrane to confirm the evenness and integrity of the RNA that was loaded onto the gel, and then transferred to the membrane.

PCR, Subcloning, and DNA Sequencing

We used PCR to amplify a genomic fragment containing the promoter of rat pgp2. Rat genomic DNA was used as a template for two primers. The first primer was synthesized after we compared musmdr1 (GenBank accession numbers M60348, M34426, M9000) with musmdr3 (accession number S1971) and pgp2 hamster (accession number L03287) and identified a conserved oligonucleotide sequence from the 5'-flanking region of these genes with the sequence 5'-AAA GCA ATG TCG TCA GAA GG-3'. This oligonucleotide was paired with an oligonucleotide from the 5' portion of the pgp2 (mdrb) cDNA, which contained the sequence 3'-TTT GGG CCT CCA AAT TGC ACT CCA C-5'. and was used to amplify Sprague-Dawley rat genomic DNA. PCR reactions (final volume, 100 μl) contained 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, and 0.2 mM of each deoxynucleotide triphosphate, 100 pmol each of sense and antisense primer, and 1 μl of genomic DNA. DNA was heated for 5 min at 95°C and quick chilled; then 2.5 units Taq polymerase were added. The reaction was overlayed with 100 μl mineral oil, heated to 94°C for 2 min, and immediately cycled in an Omniphotom thermocycler for 30 cycles consisting of 30 s annealing at 42°C, 1 s extension at 72°C, and 60 s denaturation at 94°C. The final step was 5 min of extension at 72°C. The reaction product was separated on a 0.5% NuSieve agarose/0.5% agarose gel (w/v). The single 519-bp pgp2 PCR product was subcloned and sequenced as described previously (55). Multiple independent clones were sequenced and analyzed and found to be identical. By best fit alignment, the PCR product represented nucleotides from −369 to +150 bp of the rat pgp2 gene (Ref. 56; accession number L16546) and was 99.2% identical to the 5' sequences of pgp2. This variation probably represents allelic differences in the pgp2 gene. This PCR product was subcloned in either orientation into the promoterless expression vector pBLCAT3 (48) to generate pgp2CAT.

Transfections

H35. One day prior to transfection, the cells were subcultured by trypsinization and plated at 3–4 × 10⁵ cells/60-mm culture dish. When cultures attained 25–35% confluence, the cells were transfected by standard calcium phosphate coprecipitation using 10 μg of purified test plasmid DNA (Qiagen Corp., Chatsworth, CA). Following an 18-h transfection, the cells were treated for 36 h with steroids, and cells were harvested for CAT assay. The H35 cells were transfected in medium containing 10% dialyzed fetal bovine serum. This medium was used in all transfections.
because many promoters, including pgp2, have very low basal (in most cases, undetectable) activity using charcoal-stripped serum.4

Primary Rat Hepatocytes. Cells (1.3 × 10⁶/60-mm dish) were cultured on serum-coated plastic for 24 h and then transfected with DNA using Lipofectin reagent (Life Technologies; Ref. 33). Following a 6-h transfection, cells were changed to fresh medium. After a 12-h interval, the medium was renewed and supplemented with or without steroid and harvested for CAT or luciferase activity 36 h later, as described previously (33).

CAT Assays
Cells were scraped from 60-mm dishes after washing once with PBS [80 mM Na₂HPO₄, 20 mM KH₂PO₄, and 100 mM NaCl (pH 7.4)] and a brief incubation in a harvest buffer [150 mM NaCl, 40 mM Tris (pH 7.4), and 5 mM EDTA] as described (33). Cellular CAT activity was assayed exactly as described in detail previously for primary rat hepatocytes (33, 57), except that H35 cell extracts (60 μg) were heat inactivated for 15 min at 65°C prior to CAT assay and the assay was run overnight. After subtraction of background activity obtained from mock-transfected control dishes and normalization for β-galactosidase (see below), the data were calculated as relative CAT activity as described in the figure legends for the individual experiments. Duplicate assays performed on a given cell extract varied by less than 12% for either cell type. The average specific activity of the CAT assay for untreated primary hepatocytes transfected with pgp2CAT was 57 ± 9 dpnmic/g h (n = 5), whereas for H35, it was 22 ± 13 dpnmic/g h (n = 6). MMTV/CAT was induced 128-fold in the H35 hepatoma and 240-fold in the primary rat hepatocyte culture by 10⁻³ M DEX. This value was comparable to our previous finding for DEX induction of MMTV/CAT in primary cultures of rat hepatocytes (33).

Luciferase Assay
Cells were washed twice in PBS and then incubated for 15 min in Reporter Lysis buffer (Promega) before being scraped from the culture dishes. The assay was routinely performed upon 20 μg of lysate with reagents used from the kit supplied by the manufacturer (Promega). The assay was performed on a Optitcomp 1 luminometer (MGM Instruments, Hamden, CT) using a counting window of 10 s, according to the manufacturer’s instructions.

β-Galactosidase Assay
The β-galactosidase assay was performed upon cells which had been cotransfected with 1 μg of the RSV-βgal expression plasmid. The β-galactosidase activity was used to normalize for transfection efficiency (58).

Western Blot Analysis
Crude membranes were prepared from hepatocytes by a modification of the method described by Lee et al. (26). All procedures were carried out at 4°C. Briefly, cells were removed from the plate by scraping in PBS. The cells were pelleted at 10,000 × g for a refrigerated microtube, and the pellet was resuspended in membrane storage buffer (MSB; 100 mM potassium phosphate (pH 7.4), 1.0 mM EDTA, 20% glycerol, 1 mM DTT, 20 μM butylated hydroxytoluene, and 2 mM phenylmethylsulfonyl fluoride) and lysed for 35 s at

30% power with a Ultrasonic homogenizer (Cole Parmer Corp, Chicago, IL). The crude membranes were isolated by centrifugation at 10,000 × g for 5 min in a refrigerated microtube. The pellet was resuspended in a small volume of MSB. Protein was determined by the method of Lowry (59). The crude membrane proteins (35 μg) were resuspended in standard Laemmli sample preparation buffer (60), loaded immediately onto a 6% Laemmli polyacrylamide gel, and resolved overnight. Proteins were immunoblotted to nitrocellulose filters as described previously (26, 49), and the filters were incubated sequentially with primary polyclonal rabbit anti-mdr(ab-1) IgG (Oncogene Science, Uniondale, NY), peroxidase conjugated anti-rabbit IgG, and developed with the Amersham ECL detection system following the manufacturer’s instructions. The relative amount of pgp was determined by densitometric analysis.

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References


