Dexamethasone Induction of Murine CYP2B Genes Requires the Glucocorticoid Receptor

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ABSTRACT:

Hepatic cytochrome P450 (P450) enzymes metabolize exogenous and endogenous compounds, and many are inducible by xenobiotics. Their synthesis is tightly regulated, particularly through nuclear receptors. Expression of murine CYP2B genes is strongly activated by treatment with phenobarbital or phenobarbital-like inducers, and a detectable response requires the presence of the constitutive androstane receptor (CAR). However, other compounds can also induce murine CYP2B proteins. For example, dexamethasone is known to induce rat CYP2B1 and CYP2B2 and mouse CYP2B proteins. Using human HepG2 and rat H4IIEC3 hepatoma cell lines, we found that dexamethasone induction of CYP2B2 and Cyp2b10 luciferase reporters required the glucocorticoid receptor. Given the well-known observation that CYP2B genes are not phenobarbital-responsive in cultured cell lines, the dexamethasone responsiveness of CYP2B reporter constructs in cell lines demonstrates in itself that the mechanism of dexamethasone induction is distinct from that of phenobarbital. We also analyzed the relative importance of the phenobarbital response unit (PB RU) and of a known glucocorticoid response element in this response. Both sites contributed to the response, but other sites were required for maximal induction. CAR was also found to act as an accessory factor to stimulate the response to dexamethasone by the glucocorticoid receptor. Furthermore, in H4IIEC3 cells, CAR activated the PB RU in the natural sequence context of the CYP2B2 and Cyp2b10 5′ flanks. In summary, there are at least two independent mechanisms of CYP2B induction: one involving phenobarbital and phenobarbital-like inducers and another involving glucocorticoids that induce via the glucocorticoid receptor with CAR acting as an accessory factor.
response elements (GREs), and activate transcription of target genes (Schoneveld et al., 2004). Glucocorticoid regulation of many genes involves glucocorticoid response units (GRUs), which consist of GREs associated with one or more binding sites for accessory transcription factors. The paradigmatic GRU of the gene for rat phosphoenolpyruvate carboxykinase (PEPCK) (Imai et al., 1990) consists of four accessory factor binding sites, (gAF1, gAF2, and gAF3 and a cAMP response element) and two GREs (Scott et al., 1996). By themselves, neither the accessory factor binding sites nor the GREs confer DEX responsiveness on the PEPCK promoter in H4IIE cells. Mutation or deletion of any one of the accessory factor sites leads to an approximately 2-fold reduction in DEX responsiveness and mutation of any two gAF sites essentially abolishes DEX responsiveness (Imai et al., 1990; Scott et al., 1996).

DEX is a moderately effective inducer of rat CYP2B1 and CYP2B2 (Lake et al., 1998) and is about as effective as PB in inducing mouse CYP2B10 mRNA and protein (Corcos, 1992; Jarukamjorn et al., 1999). Jaiswal et al. (1990) described a functional GRE located approximately 1.3 kb upstream of the CYP2B2 gene (referred to here as GRE1.3) (Figs. 1B and 2), and Stoltz et al. (1998) identified a putative GRE within the CYP2B2 promoter in H4IIE cells. Furthermore, the basal level of hepatic CYP2B10 protein is reduced in mice with a targeted GR deletion, and the CYP2B10 protein is not inducible by DEX of such animals (Schuetz et al., 2000). These clues led us to investigate whether GR could activate transcription of CYP2B2 reporter genes in response to DEX and whether CAR or PXR had a role to play in this response.

We report here that in two hepatoma cell lines, human HepG2 and rat H4IIEC3, CYP2B2 reporter constructs are highly responsive to DEX. We used these lines to demonstrate GR dependence of DEX induction and to investigate the role therein of the PBRE and GRE1.3. Moreover, we show that CAR acts as an accessory factor in this induction.
The rats were treated with DEX (40 mg/kg i.p.), vehicle (water with 0.15% methyl-4-hydroxybenzoate and 0.02% propyl-4-hydroxybenzoate i.p.), or PB (80 mg/kg i.p.). Three injections were given, one every 24 h, and 24 h after the last injection the rats were sacrificed and liver microsomes were isolated (Desrochers et al., 1996). Western blots were performed as described previously (Roberge et al., 2004). The anti-CYP2B1 antibody was a gift from David Waxman, and its capacity to recognize rat CYP2B proteins has been described elsewhere (Waxman, 1984; Desrochers et al., 1996). For detection of actin, the membranes were incubated with the H-196 antibody (sc-7210; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at a 1:1000 dilution. Actin was detected as an 80-kDa covalent complex with 17β-estradiol dehydrogenase (Leenders et al., 1994). This complex is present in liver (Renwick et al., 1981) and can be isolated with microsomes (Henderson and Warren, 1984).

Data Analysis. Data are shown as the average ± S.D. of results of at least three independent experiments. Differences between test and control conditions were assessed by Student’s one-tailed t test, assuming unequal variances.

Results

Response of CYP2B Reporter Genes to DEX Requires GR.

Figure 3 illustrates the induction in rat liver by DEX and PB of CYP2B1 and CYP2B2 proteins, which are not well resolved under our electrophoresis conditions. The faster migrating form recognized by the anti-CYP2B1 antibody is the constitutive CYP2B3 protein (Desrochers et al., 1996). Although not as high as after PB treatment, the DEX-induced levels of CYP2B1/CYP2B2 proteins are clearly higher than the basal level, which is barely perceptible. Note that PB and DEX treatments diminished the level of the constitutive CYP2B3 form.

We exploited cultured cell lines as models to study the mechanism of DEX induction. In HepG2 cells without added GR, there was no detectable response to DEX of the pGL3-2B2-Luc reporter (Fig. 4A, series 1). However, after cotransfection of a GR expression vector, there was an approximately 12-fold induction by DEX (Fig. 4A, series 2). Induction by DEX was not detected after cotransfection of expression vectors for CAR or for PXR (Fig. 4A, series 3 and 5). Thus, in HepG2 cells the addition of exogenous GR is necessary and sufficient to confer DEX responsiveness on the pGL3-2B2-Luc reporter. The
same pattern was seen in HepG2 cells for the pGL3-2b10-Luc reporter, which contains 2.5 kb of the Cyp2b10 5′ flank. In the absence of added GR, there was no response to DEX (Fig. 4B, series 1). Cotransfection with a GR expression vector again conferred DEX responsiveness (Fig. 4B, series 2), and again no induction by DEX was detected after cotransfection of expression vectors for CAR or for PXR (Fig. 4B, series 3 and 5). Hence, in HepG2 cells the absence of DEX responsiveness of the CYP2B reporter seems to be caused only by the lack of GR, which, if present, can confer it. This conclusion is in agreement with the requirement in HepG2 cells for exogenous GR to detect DEX responsiveness of human CYP2C9 reporters (Gerbal-Chaloin et al., 2002).

In H4IIEC3 cells, on the other hand, there was an approximately 60-fold response to DEX of the pGL3-2B2-Luc reporter in the absence of the GR expression vector (Fig. 5A, series 1). Addition of exogenous GR by cotransfection increased the fold induction by a factor of 2 (Fig. 5A, series 2). Here again, the results argue against a presence of the GR expression vector (Fig. 5A, series 1). Addition of GR by cotransfection enhanced the response to DEX, up to approximately 40-fold (Fig. 5B, series 2). Hence, H4IIEC3 cells contain sufficient GR to permit DEX induction of CYP2B2 reporters but insufficient to permit a maximal response. A similar conclusion was reached by Imai et al. (1990) with respect to DEX induction of a PEPCK reporter. The response to DEX was dependent on GR in H4IIEC3 cells, as shown by its inhibition by the GR antagonist RU486 (Fig. 6). This indicates that the DEX induction seen in H4IIEC3 cells without addition of exogenous GR is mediated by endogenous GR.

**Stimulation of DEX Induction by CAR.** In HepG2 cells, exogenous CAR had no appreciable effect on the basal levels of transcription for the pGL3-2B2-Luc or pGL3-2b10-Luc reporter constructs (Fig. 4, A and B, compare series 1 and 3). Surprisingly, however, exogenous CAR stimulated the response to DEX in presence of GR, by increasing the induced levels approximately 3-fold for pGL3-2B2-Luc and approximately 1.7-fold for pGL3-2b10-Luc (Fig. 4, A and B, compare series 2 and 4). In H4IIEC3 cells, in the absence of exogenous GR, CAR increased DEX-induced levels of transcription by 4.3- or 19-fold for the pGL3-2B2-Luc and pGL3-2b10-Luc reporters, respectively (Fig. 5, A and B, compare series 1 and 3). In the presence of exogenous GR, CAR also increased DEX-induced levels of transcription for both reporters (Fig. 5, A and B, compare series 2 and 4). Taken together, these results show that CAR alone does not mediate induction by DEX, but that it acts to stimulate DEX induction mediated by GR in both cell lines.

Because CAR-retinoid X receptor heterodimers bind to the NR1, NR2, and NR3 sites of the PBRU (Honkakoski et al., 1998b; Tzameli et al., 2000; Beaudet et al., 2005; Zhang et al., 2006), the impact of exogenous CAR on induction by DEX was evaluated with the PBRU reporter construct. When added by cotransfection to H4IIEC3 cells, CAR reduced significantly both the basal and induced levels of transcription for the PBRU reporter construct (Fig. 7, compare series 4 and 5). Hence, in H4IIEC3 cells, the stimulation of DEX-induced transcription by CAR clearly depends on the PBRU, because when it was deleted not only was the stimulation no longer observed but also DEX responsiveness was actually reduced. In HepG2 cells, the -fold induction for the PBRU reporter construct was approximately the same as that for the pGL3-2B2-Luc reporter, approximately 13- to 15-fold (Fig. 8, compare series 1 and 3). For the PBRU reporter, the addition of CAR did not increase the induced level (Fig. 8, compare series 3 and 4). Thus, in HepG2 cells, when the PBRU was deleted, CAR was no longer able to increase the DEX-induced levels.

**In H4IIEC3 Cells CAR Activates Reporter Gene Transcription Driven by the CYP2B PBRUs in Their Natural Sequence Contexts.** In HepG2 cells, exogenous CAR activates reporter gene transcription driven by the murine CYP2B PBRU or PBREM when the enhancers are placed directly adjacent to an enhancerless basal promoter such as that of "k (Honkakoski et al., 1998b), CYP2C1 (Kim et al., 2001), or CYP2R2 (Beaudet et al., 2005). We confirmed these results here, where CAR activated the PBREM-120 construct by 24-fold in H4IIEC3 cells (Fig. 7, compare series 2 and 3, without DEX) and by 38-fold in HepG2 cells (Fig. 9, compare series 1 and 3, without DEX). These results are consistent with the known absence from HepG2 cells of appreciable levels of functional CAR (Honkakoski et al., 1998b) and of CAR mRNA (Rencurel et al., 2005), and they indicate that H4IIEC3 cells also lack functional CAR. With the PBREM-120 construct in H4IIEC3 cells, in the presence of exogenous CAR, a modest 2.5-fold further increase in transcription was observed in the presence of DEX (Fig. 7, series 3); but with this construct in HepG2 cells, in the presence of CAR or of CAR plus GR, no further increase in transcription was observed in the presence of DEX (Fig. 9, series 3 and 4).

In agreement with earlier results (Beaudet et al., 2005), no activation by CAR was detectable when the CYP2B2 PBRU in its natural sequence context in the pGL3-2B2-Luc construct was tested in HepG2 cells (Fig. 4A, compare series 1 and 3, without DEX). Likewise, no activation by CAR was detected when the Cyp2b10 PBRU in its natural sequence context in the pGL3-2B10-Luc construct was tested in HepG2 cells (Fig. 4B, compare series 1 and 3, without DEX). For both reporters, similar results were obtained in HepG2 cells in the presence of exogenous GR (Fig. 4, A and B, series 2 and 4, without DEX).

However, in H4IIEC3 cells CAR did activate transcription driven by the CYP2B2 PBRUs when they were in their natural sequence contexts. This activation was approximately 14-fold for the CYP2B2 reporter construct and approximately 13-fold for the Cyp2b10 reporter construct (Fig. 5, A and B, compare series 1 and 3, without DEX). The activation by CAR was essentially unchanged when exogenous GR was also present (Fig. 5, A and B, compare series 3 and 4, without DEX). This activation by CAR depends on the PBRU, because, as for the stimulation of DEX induction, when the PBRU was deleted, the activation by CAR was abolished, and indeed the basal level was reduced by approximately 2-fold in the presence of exogenous CAR (Fig. 7, compare series 4 and 5, without DEX).
Sites Other Than the PBRU and GRE1.3 Are Needed for Maximal Response to DEX.

To begin the characterization of the sequence elements involved in the DEX response, we analyzed DEX responsiveness of CYP2B2 reporter constructs with different deletions. In H4IIEC3 cells, the effect of deleting the PBRU in the absence of exogenous CAR was to reduce the fold-induction by DEX by a factor of 2 (Fig. 7, compare series 1 and 4), indicating that the PBRU plays a role in DEX induction in these cells. Furthermore, the basal CYP2B2 promoter construct retained the capacity for a modest response, approximately 4-fold, in H4IIEC3 cells (Fig. 7, series 7), but...
not in HepG2 cells (Fig. 9, series 5). In both cell lines, with the PBREM-120 construct, the DEX response was increased compared with that of the basal CYP2B2 promoter alone (Fig. 7, compare series 2 and 7 for H4IIEC3 cells; Fig. 9, compare series 2 and 5 for HepG2 cells). Thus, sequences within the PBREM can contribute to the DEX response in both cell lines. However, in HepG2 cells the deletion of the PBRU in the natural sequence context did not diminish the response to DEX (Fig. 8, compare series 1 and 3), although, as we have seen, it eliminated the stimulation of the DEX-induced levels by CAR (Fig. 8, compare series 3 and 4). Therefore, sequences within the PBRU can contribute to DEX induction mediated by GR in H4IIEC3 cells and are necessary for stimulation of this induction by CAR in both cell lines.

We also investigated the possible role of the GRE reported by Jaiswal et al. (1990), GRE1.3, in the response to DEX. In both cell lines its deletion reduced but did not eliminate DEX responsiveness (Fig. 7, compare series 1 and 6 for H4IIEC3 cells; Fig. 8, compare Series 1 and 5, for HepG2 cells). In H4IIEC3 cells, deletion of both the PBRU and GRE1.3 had the same effect as deletion of the PBRU only (Fig. 7, compare series 4 and 8), indicating that their effects are not additive. In HepG2 cells, deletion of GRE1.3 and the PBRU had the same effect as deletion of GRE1.3 only (Fig. 8, compare series 5 and 7). In both cell lines, deletion of both the PBRU and GRE1.3 reduced DEX responsiveness by approximately 2-fold (Fig. 7, compare series 1 and 8 for H4IIEC3 cells; Fig. 8, compare series 1 and 7 for HepG2 cells). Taken together, these results show that in H4IIEC3 cells the role of GRE1.3 is secondary to that of the PBRU in conferring DEX responsiveness whereas in HepG2 cells, GRE1.3 but not the PBRU contributes to DEX responsiveness. Moreover, when GRE1.3
was deleted, the stimulation by CAR of the DEX-induced levels was conserved (Fig. 8, compare series 5 and 6).

**Discussion**

We have demonstrated that GR is responsible for the induction of *CYP2B2* and *Cyp2b10* luciferase reporters by DEX treatment in HepG2 cells, wherein induction by DEX was only seen with the addition of exogenous GR. In H4IIEC3 cells on the other hand, DEX responsiveness was observed without addition of exogenous GR. The abrogation of DEX responsiveness in H4IIEC3 cells by the addition of RU486 indicates that GR is also required for DEX responsiveness in H4IIEC3 cells. Furthermore, given the well known observation that *CYP2B* genes are not PB-responsive in cultured cell lines, the DEX responsiveness of *CYP2B* reporter constructs in cell lines demonstrates in itself that the mechanism of DEX induction is distinct from that of PB.

The data obtained argue against a role for PXR in the response to DEX under our experimental conditions. However, at the concentration of DEX used (100 nM), human or mouse PXR are not expected to be activated (Klierwer et al., 1998; Lehmann et al., 1998), and it is possible that at higher concentrations PXR would be activated by DEX and then could activate transcription of the *CYP2B* reporters.

Our results are in accord with those obtained with GR-null mice by Schuetz et al. (2000), indicating that GR is necessary for DEX induction of *Cyp2b10* in mice and, by extension, of *CYP2B1* and *CYP2B2* in rats. However, the absence of GR after targeted inactivation of its gene may have affected the level of other transcription factors or coactivators (Huss and Kasper, 2000; Schoneveld et al., 2004). For example, the levels of CAR are increased by the addition of GR and DEX to human hepatocytes (Pascussi et al., 2000), so absence of GR could lead to lower levels of CAR in mice lacking GR.

The advantage of the cell culture system is that it is possible to add, one by one, specific transcription factors and thus to obtain direct evidence that GR is responsible for DEX responsiveness.

The putative GRE of the PBRU (GRE-A; Fig. 1A) and GRE1.3 (Figs. 1B and 2) are candidates for conferring DEX responsiveness on rat *CYP2B2*. Our results demonstrate that GRE1.3, although it contributed to DEX responsiveness for the *CYP2B2* reporter in both cell lines, is clearly not the only sequence element required, because its deletion reduced but did not abolish DEX responsiveness. GRE-A apparently has no role to play in the DEX response in HepG2 cells when it is in its natural context, because deletion of the PBRU did not influence the response to DEX. However, the deletion of the PBRU did diminish DEX responsiveness in H4IIEC3 cells, indicating that it contains an element that can contribute to DEX responsiveness. Moreover, the PBREM itself, when fused to the basal promoter, conferred DEX responsiveness in both cell types. Because GRE-A is ablated in the PBREM (Fig. 1A), these results suggest that there is another GRE or accessory site within the PBREM. GRE-B, another putative GRE in the PBRU (Fig. 1A) (Stoltz et al., 1998), is a candidate for this site.

Given that after deletion of both the PBRU and GRE1.3 DEX responsiveness was only reduced by approximately 2-fold, other sites are clearly needed to confer maximal induction. The DEX response therefore seems to be modulated through several GREs or accessory sites that are spread throughout the *CYP2B2* 5’ flank. One or more of these additional sites are outside the basal promoter, because the construct in which GRE1.3 and the PBRU were deleted retained higher DEX responsiveness than the −120 construct in both cell types. Another site that can contribute to DEX responsiveness is located in the basal promoter, because the −120 construct responded to DEX in H4IIEC3 cells. A putative GRE half-site (5’−TGTTCA−3’, −70/−65, lower strand) is present in the *CYP2B2* basal promoter. For GRE half-sites to be functional, they require the presence of an accessory factor bound to an adjacent site (Schoneveld et al., 2004). CCAAT/enhancer-binding protein α may act as such an accessory factor because there is a binding site for it adjacent to the putative GRE half-site in the basal *CYP2B2* promoter (Luc et al., 1996; Park and Kemper, 1996). We have identified two additional regions in the *CYP2B2* 5’ flank that can contribute to DEX responsiveness. One, active in HepG2 cells, is between −1180 and −1148 and contains a putative GRE; the other, active in H4IIEC3 cells, is between −192 and −145 and contains a putative GRE half-site on each strand (Aulet-Walsh et al., 2008). Taken together, these observations suggest that the *CYP2B2* 5’ flank contains a GRU (Imai et al., 1990). Moreover, the high level of DEX responsiveness observed in our experiments is in agreement with this conclusion, as the fold induction is often higher for GRUs than for simple GREs (Schoneveld et al., 2004). It remains to be determined which GREs or accessory sites function in rat liver to confer DEX responsiveness. This issue is particularly pertinent given, for example, the different effects on DEX responsiveness observed here in the two cell lines after deletion of the PBRU or GRE1.3.

The role of CAR in activating *CYP2B2* transcription is interesting for several reasons. To our knowledge, ours are the first results for which the *CYP2B2* PBRU placed in its natural sequence context at −2317/−2155 in *CYP2B2* 5’ flank is activated by CAR, as observed in H4IIEC3 cells. As might have been expected, this was true for the *Cyp2b10* reporter construct as well. In HepG2 cells, only when the PBRU or the PBREM was placed at −120 in *CYP2B2* 5’ flank were they activated by exogenous CAR (Beaudet et al., 2005). Because CAR is active in the absence of an agonist ligand (Baes et al., 1994) and goes directly to the nucleus in HepG2 cells (Kobayashi et al., 2003), the question arises as to why it is not able to activate the PBRU in its natural sequence context in these cells. It seems likely that H4IIEC3 cells but not HepG2 cells contain transcription factors or coactivators that are required for DEX activation of murine PBUR-driven transcription when the enhancer is in its natural sequence context. Although the factors(s) responsible for this effect are as yet unknown, one transcription factor, GR, can be ruled out: HepG2 cells to which GR was added by cotransfection were as refractory to CAR activation of the PBRU in its natural sequence context as were HepG2 cells not cotransfected.

CAR is usually thought of as being a transcriptional activator in its own right, but in HepG2 cells, as we have seen, it is unable to activate PBUR-driven transcription of the *CYP2B2* or *Cyp2b10* PBURs in their natural sequence contexts. However, CAR is capable, in the presence of GR, of activating their DEX-induced transcription. Hence, under these conditions in HepG2 cells, CAR clearly acts as an accessory factor for DEX-induced transcription. The abrogation, in both HepG2 and H4IIEC3 cells, of the stimulatory effects of CAR on DEX induction by deletion of the PBRU suggests that CAR does not act by binding to GR, but rather by binding to sequence elements within the PBRU, presumably NR1, NR2, and NR3, to increase DEX responsiveness. Thus, with respect to DEX induction of murine *CYP2B* genes, CAR behaves as a classic accessory factor in the sense in which that term is applied to the *PECK* GRU and other GRUs (Imai et al., 1990; Schoneveld et al., 2004). The interactions between accessory factors and GRs seem to contribute to the *PECK* glucocorticoid response by facilitating GR binding or interaction with coactivators or both (Stafford et al., 2001a,b), and CAR may act similarly to stimulate DEX induction of *CYP2B* genes.

GR is not only essential for the DEX response of the *CYP2B* reporters. It is also necessary for maximal PB induction of *Cyp2b10* in vivo, as GR-null mice have approximately two thirds of the...
wild-type PB-induced levels of CYP2B10 protein (Schuetz et al., 2000). As demonstrated by our results, CAR and GR act independently to activate transcription of CYP2B reporters, but CAR can act as an accessory factor in the DEX response and GR may play a similar role in PB-responsiveness. This finding could explain the decrease seen in the PB-induced level of the CYP2B10 protein in GR-null mice. Moreover, the synergistic effect seen by cotransfection of expression vectors for CAR and GR, which, for the pGL3-2b10-Luc reporter in H4IIE3 cells gave induced levels more than 400 times the basal level, is in agreement with previously reported synergistic CYP2B induction by cotreatment with DEX and PB in primary hepatocytes (Waxman et al., 1990).

Contrary to the effect of DEX in inducing CYP2B1 and CYP2B2 proteins, CYP2B3 levels decreased in response to treatment with DEX. This is not a surprising result, as the mouse ortholog of CYP2B3, CYP2B9, has also been reported to be suppressed in liver by DEX treatment (Jarukamjorn et al., 1999). Furthermore, PB treatment also decreased CYP2B3 in rat liver, which is in agreement with previously reported results (Desrochers et al., 1996). The mechanism by which CYP2B3 is decreased remains unknown.

In conclusion, murine CYP2B genes are regulated not only by exogenous compounds but also by a pathway that is normally regulated by endogenous compounds. CYP2B proteins are well known to possess androgen hydroxylase activity (Waxman, 1984), and glucocorticoid hormones may influence androgen levels by regulating CYP2B transcription. In addition, H4IIE3 cells, should be useful for studying CAR activation of CYP2B transcription, because they seem to contain factors necessary for this activation that are lacking in HepG2 cells. In summary then, there are at least two independent mechanisms of CYP2B induction, one involving PB and PB-like inducers and another involving glucocorticoids that induce via GR with CAR acting as an accessory factor.

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