BIOCHEMICAL PHARMACOLOGY 76 (2008) 1298–1306

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The CYP2B2 5′ flank contains a complex glucocorticoid response unit

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ARTICLE INFO

Article history:
Received 29 June 2008
Accepted 13 August 2008

Keywords:
Dexamethasone
CYP2B2
CYP2B10
Glucocorticoid response unit
Glucocorticoid receptor
Liver
Constitutive androstane receptor

ABSTRACT

Rat CYP2B1 and CYP2B2 and mouse CYP2B10 are dramatically induced by phenobarbital (PB) in liver. PB responsiveness requires the constitutive androstane receptor (CAR). However, dexamethasone treatment can also induce *CYP2B* genes in both rat and mouse liver. Three regions have been shown to be involved in conferring dexamethasone responsiveness on *CYP2B2* reporter constructs. They are the PB response unit, a functional glucocorticoid response element at −1.3 kb in the 5′ flank and a weak element in the basal promoter. We report here the identification, by deletion analysis of the *CYP2B2* 5′ flank, of new glucocorticoid response elements or accessory factor sites. Moreover, we show that CAR acts as an accessory factor in the dexamethasone response in vivo of CYP2B10 protein in mice, by increasing both the basal and induced levels. We propose a model to explain the dexamethasone responsiveness of the *CYP2B2* gene in which induction is mediated by a complex glucocorticoid response unit.

1. Introduction

Hepatic cytochrome P450 (CYP) enzymes are well known for their inducibility in response to xenobiotics [1,2]. In mouse liver the *Cyp2b10* gene and in rat liver the *CYP2B1* and *CYP2B2* genes are highly responsive to phenobarbital (PB) and PB-like inducers [1,2]. PB responsiveness is conferred by the PB response unit (PBRU), a 163-bp fragment located at −2317/−2155 in the rat *CYP2B2* 5′-flank [3,4]. Three DR-4 sites are located in the PBRU, NR1, NR2 and NR3, which are recognized by heterodimers of the constitutive androstane receptor (CAR) and the retinoid X receptor (RXR) [5–8]. CAR is normally retained in the cytoplasm of untreated hepatocytes and becomes concentrated in the nucleus in response to PB treatment [9]. There, it is thought to activate transcription of its target genes by binding to the NR sites [6,8,10,11].

Induction of *CYP2B* genes by dexamethasone (DEX) was described some time ago [12–14]. Although the specific mechanisms involved in DEX responsiveness are still unclear, the basal level of hepatic CYP2B10 protein is reduced in mice with a targeted glucocorticoid receptor (GR) gene disruption, and the CYP2B10 protein is not inducible by DEX in such

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Abbreviations: CAR, constitutive androstane receptor; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assays; FBS, fetal bovine serum; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GRU, glucocorticoid response unit; HNF-3, hepatocyte nuclear factor 3; oligo, oligonucleotide; PB, phenobarbital; PBRU, phenobarbital response unit; RXR, retinoid X receptor.

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doi:10.1016/j.bcp.2008.08.015

animals [15]. We have shown elsewhere that DEX induction of *CYP2B2* and *Cyp2b10* reporter genes is mediated by GR and that CAR acts as an accessory factor, by stimulating this response (Audet-Walsh and Anderson, submitted for publication). For *CYP2B2* reporters, the roles of the PBRU and of a previously reported glucocorticoid response element (GRE) situated at −1.3 kb [16], referred to here as GRE1.3, were studied. Both were found to be necessary for maximal DEX responsiveness. However, the results suggested that other sites, GREs or accessory sites, are also required for maximal responsiveness. We report here the identification of new elements in the *CYP2B2* 5′ flank that are necessary to obtain maximal DEX responsiveness, indicating that *CYP2B2* induction by DEX is mediated through a complex glucocorticoid response unit (GRU).

2. Materials and methods

2.1. Materials and animals

DEX was from Sandoz (Boucherville, Canada) and was diluted in dimethyl sulfoxide (DMSO) (1:100, v/v) for cell culture treatment. All enzymes were from Fermentas (Burlington, Canada). Waymouth's medium, minimum essential medium (α-MEM), fetal bovine serum (FBS), penicillin-streptomycin and gentamicin were from Invitrogen (Burlington, Canada). Oligonucleotide (oligo) primers, DMSO and other chemicals were from Sigma–Aldrich (Oakville, Canada). C57-Black/6 mice (20–25 g) were from Charles River (St-Constant, Canada). Mice in which the *Car* gene has been subjected to targeted inactivation [17] were provided by Dr. David Moore. Animals were treated in accordance with the requirements of the Comité de protection des animaux du CHUQ/CPAC.

2.2. Plasmids and plasmid constructs

pSG5-hGR, an expression vector for human GR, was from J.M. Pascussi. The pGL3-2B2X vector (herein referred as pGL3-2B2-Luc), based on the pGL3-Basic vector (Promega, Montréal, Canada) and containing 2.5 kb of the *CYP2B2* 5′-flank, including the natural promoter and the PBRU, subcloned upstream of the *luc* gene, has been described [18]. The different deletion constructs were prepared by amplification of pGL3-2B2-Luc with primers (see Table 1) flanking the region to be deleted, both having the same restriction site EcoRI [19]. The PCR products were digested with both EcoRI and DpnI and finally ligated with T4 DNA ligase to obtain the new constructs (Fig. 1). For the construction of the −1180/−1137 construct, the pGL3-120 plasmid [18] was amplified and the −1180/−1137 fragment was fused to the reverse primer; both primers (see Table 1) had the same restriction site (XhoI) and PCR products were treated as for the different deletion constructs. All plasmids were purified with purification kits (Qiagen, Mississauga, Canada), and the relevant regions were subjected to DNA sequencing by the DNA sequencing service of the Centre de recherche du CHUL (Québec, Canada).

2.3. Cells and transfection assays

HepG2 cells and H4IIEC3 cells were cultured and maintained (5% CO_2/37 °C) in medium B [20] supplemented with 10% (v/v) FBS plus antibiotics (gentamicin at 50 mg/ml or penicillin-streptomycin at 100 U/ml and 100 μg/ml, respectively). All transfections were performed in duplicate with GeneJuice® reagent (VWR International, Montréal, Canada) as described elsewhere (Audet-Walsh and Anderson, submitted for publication). Unless noted otherwise, each transfection was performed at least three times. DEX treatments were for 24 h for HepG2 cells and 48 h for H4IIEC3 cells; after 24 h the H4IIEC3 cells were washed once with HEPES buffer (6.4 mM KCl, 10 mM HEPES, 0.15 M NaCl, pH 7.6) and fresh medium was added. After the DEX treatments, the medium was removed, cells were washed once with HEPES buffer, 120 μl of passive lysis buffer (Promega) was added, and the cells were harvested by scraping and lysed by freeze–thaw treatment. Luciferase activity of each sample was assayed by luminometry with the Dual Luc kit (Promega). The firefly luciferase values were divided by the *Renilla* luciferase values to obtain the relative

Table 1 – Oligos used to make the different *CYP2B2* reporter constructs

Name	Sequence
Universal 2B2 forward primer	ATGAATTCTCGAGCCCGGGCTAGCACGC
−1759	ATGAATTCAGTTGAGGCAAGTTGACCACA
−1370	ATGAATTCCAATAATATCAGTTAGGGTACA
−1180	ATGAATTCAGGGAACCATTTGTCATTAGACA
−1148	ATGAATTCGAGACTATCTTTGTTAGGTTCACTATTTCT
−940	ATGAATTCCCTGATTTCTTACAGAACCCAA
−740	ATGAATTCAAGTTAGACCCGGGGCCCCAAC
−600	ATGAATTCCAGAGAGTGAAATGGGGACTC
−540	ATGAATTCAGGAACCAACAGACGGAGACAA
−500	ATGAATTCCTATTCTTGTCAACTCAAACAT
−440	ATGAATTCGCCCCAATAATTTAAGATTATA
−381	ATGAATTCCCAGTGCATCTAGACTCAGACAA
−240	ATGAATTCTAAGTAAACAGAGCTGACAAAA
−192	ATGAATTCACATAAAACAAGAGGCCTAAGT
−145	ATGAATTCCTGTTTCGTGGTTTCCTTGCC
Forward primer −1180/−1137	AACTCGAGCCCGGGCTAGCACGCGTAA
Reverse primer −1180/−1137	AACTCGAGAGGGAACCATTTGTCATTAGACACAGTGTTCAGAGAC-TATCTTTCTATGGTGTGGGTAAGGGAATGAG

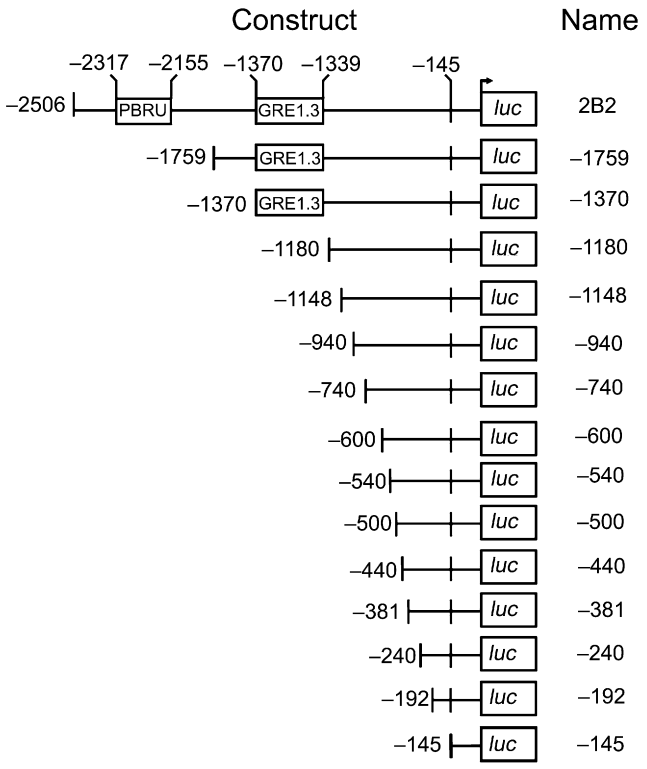

Fig. 1 – Schematic representation of the different *CYP2B2*-derived luciferase reporter constructs. The construct labelled 2B2 is the pGL3-2B2-Luc reporter, which contains 2.5 kb of the 5′ flanking region of *CYP2B2*. All the other constructs were derived from this vector, as described in Section 2.

luciferase activity. For all the experiments, DEX was added at 100 nM and DMSO was present at a dilution of 1:1000 (v/v) in the medium.

2.4. Microsomes and Western blotting

The mice were treated with DEX (50 mg/kg, i.p.) or vehicle (water with 0.15% methyl-4-hydroxybenzoate and 0.02% propyl-4-hydroxybenzoate, i.p.). Three injections were given, one every 24 h, and 24 h after the last injection the mice were sacrificed and liver microsomes were isolated [21]. Western blots were performed as described previously [22]. The anti-CYP2B1 antibody [23], which recognizes mouse CYP2B forms [24], was a gift from David Waxman. For detection of actin, the membranes were incubated with the H-196 antibody (sc-7210) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for 1 h at a 1:1000 dilution. Actin was detected as an 80-kDa covalent complex as described elsewhere (Audet-Walsh and Anderson, submitted for publication).

2.5. EMSA analysis

The nuclear extracts were prepared [25] from pooled livers of two vehicle-treated and two DEX-treated rats. The oligos in single-stranded form were end-labeled (approximately 10^5 c.p.m./μg) with [γ-^{32}P]ATP (PerkinElmer, Waltham, MA, USA) using T4 polynucleotide kinase and were subsequently annealed. The sequences of the oligos used are shown in Table 2. The labeled oligos were added to the reaction mixture [26] containing 9 μg of nuclear extract and incubated on ice for 30 min. Competitors (10 ng or 100 ng) were added as specified in the figures. Binding reaction mixtures (18 μl) were loaded on pre-run polyacrylamide gels (5%) and subjected to electrophoresis for 1.5 h at 10.5 V/cm. Products were revealed using a PhopshorImager screen and images were obtained using a Storm 860 PhosphorImager (Molecular Dynamics/GE Healthcare, Piscataway, NJ, USA).

Table 2 – Oligos used for EMSA analysis

Oligo	Sequence
−1180/−1148	AAAGGGAACCATTTGTCATTAGACACAG TGTTCAAAA
GRE1.3	ATTGGTACAAAGTGTTCAAAC
−192/−145	ACATAAAACAAGAGGCCTAAGTCCCA GTGCCCTTTTG TCCTGTGTAT
GRE consensus	AAGGTACAAAGTGTTCATG

Only the upper strand is shown.

2.6. Data analysis

Unless specified otherwise, data are shown as the average ± S.D. of at least three independent experiments. Differences were assessed by Student's *t*-test.

3. Results

3.1. Identification of a GRE in the CYP2B2 5′ flank, functional in HepG2 cells

In HepG2 cells, deletion of GRE1.3 only, or of GRE1.3 and the PBRU, reduces but does not abolish DEX responsiveness; the PBRU is required only for the subsequent stimulation of DEX induction by CAR (Audet-Walsh and Anderson, submitted for publication). Given that the reporter construct is still DEX-responsive when both GRE1.3 and the PBRU are deleted, we analyzed *CYP2B2* 5′ deletion constructs for their responsiveness to DEX in HepG2 cells. Successive deletions of the 5′ distal region between −2506 and −1180 (Fig. 1) led first to a reduction and then to a restoration of both the basal and induced levels (Fig. 2A), but did not affect appreciably the fold-induction (Fig. 2B). However, further deletion to −1148, as well as all subsequent deletions to −145, essentially completely abolished DEX responsiveness (Fig. 2A and B). Thus, two reporter constructs define a region of the *CYP2B2* 5′ flank making a major contribution to DEX responsiveness in HepG2 cells: the −1180 reporter construct retained DEX responsiveness but the −1148 reporter construct did not. These results indicate that a GRE or an accessory site lies in the 32-bp region between −1180 and −1148. There is a putative GRE in this region, similar to the GRE consensus sequence and where all the critical sites are conserved (Fig. 2C). When a fragment containing this putative GRE was fused to the *CYP2B2* basal promoter, it conferred DEX responsiveness, and indeed the response was greater than that conferred by the 2.5 kb *CYP2B2* 5′ flank (Fig. 2D).

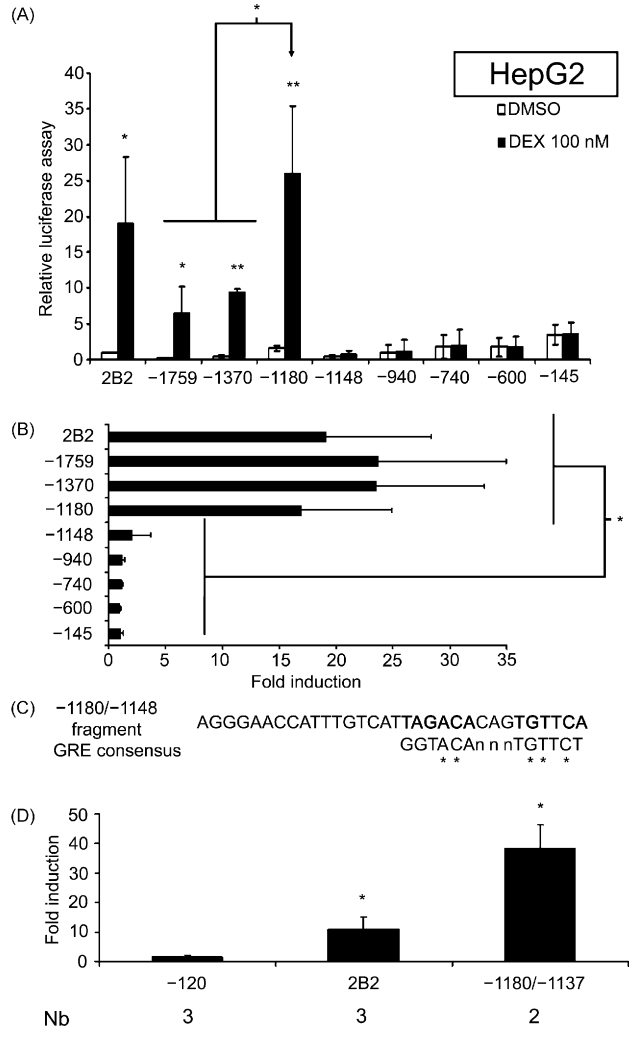

Fig. 2 – Identification of a putative GRE conferring DEX responsiveness in HepG2 cells. The GR expression vector was cotransfected with the different reporter constructs for each luciferase assay experiment in HepG2 cells. (A) Response to DEX for the different reporter constructs. The relative luciferase activity obtained for the pGL3-2B2-Luc construct cotransfected with the GR expression vector in the presence of DMSO was set at 1. The asterisks (*) and double asterisks (**) denote significant differences ($P < 0.05$ and 0.01, respectively) between the marked column and its basal level. The single asterisk (*) denotes a significant difference between the columns identified by the arrows ($P < 0.05$). (B) Fold induction after DEX treatment for the different reporter constructs. (C) Sequence of the −1180/−1148 fragment. The putative GRE is in bold. The GRE consensus sequence is at the bottom of the panel and the asterisks represent the critical nucleotides of this element [37]. (D) DEX responsiveness conferred by the −1180/−1137 fragment when it was fused to the basal *CYP2B2* promoter. The −120 construct is the pGL3-120 plasmid [18], the 2B2 construct is shown in Fig. 1, and the −1180/−1137 construct contains the −1180/−1137 fragment fused directly upstream of the −120 basal promoter as described in Section 2.2. The single asterisk (*) denotes a significant difference between the marked columns and the −120 reporter construct ($P < 0.05$). Nb refers to the number of independent transfection experiments performed.

3.2. *Identification of sequence elements in the CYP2B2 5′ flank required for maximal DEX responsiveness in H4IIEC3 cells*

As for the HepG2 cells, we analyzed the *CYP2B2* 5′ flank to look for GRE(s) or accessory sites responsible for conferring DEX responsiveness in H4IIEC3 cells. Substantial DEX responsiveness was retained in all deletion constructs up to −192 (Fig. 3A). It is noteworthy that the 32-bp region between −1180 and −1148 that conferred DEX responsiveness in HepG2 cells (Fig. 2) did not do so in H4IIEC3 cells, as demonstrated by the absence of an appreciable reduction of DEX responsiveness in the −1148 reporter construct (Fig. 3A). Deletion of the fragment between −192 and −145 led to a significant decrease in the fold induction (Fig. 3A). It thus appears that there is a response element or accessory site situated between these coordinates. The residual DEX responsiveness of the −145 construct (~4-fold) (Fig. 3A) corresponds to the level we observed with the basal promoter in H4IIEC3 cells (Audet-Walsh and Anderson, submitted for publication).

The DNA sequence of the −192/−145 fragment contains two putative GRE half sites, one on each strand, as well as a putative HNF-3 site (Fig. 3B).

3.3. *Proteins present in rat liver nuclear extracts bind to DNA sequence elements conferring DEX responsiveness in HepG2 and H4IIEC3 cells*

EMSA analyses with the −1180/−1148 fragment, the −192/−145 fragment and GRE1.3 were performed using liver nuclear extracts from vehicle-treated or DEX-treated rats (Fig. 4). For all three labeled fragments, retarded complexes were evident when liver nuclear extracts prepared from vehicle-treated animals were employed (Fig. 4A, compare lanes 1, 5 and 9 with lanes 2, 6 and 10). These retarded complexes, labeled a and b, were better defined and more prominent when liver nuclear extracts prepared from DEX-treated animals were employed (Fig. 4A, compare lanes 1, 5 and 9 with lanes 3, 7 and 11). Moreover, two new retarded complexes, labeled c and d, were evident with extracts prepared from DEX-treated animals for the −1180/−1148 and −192/−145 fragments (Fig. 4A, lanes 3 and 7), but not for GRE1.3 (Fig. 4A, lane 11). The nature and possible role of retarded complexes c and d is presently unclear. All retarded complexes were specific, as they were absent when the incubations were carried out in the presence of the corresponding unlabeled fragment (Fig. 4A and B). Performing the incubations in the presence of an unlabeled GRE consensus oligo eliminated essentially all of the retarded complexes a and b observed with fragments −1180/−1148 and GRE1.3 (Fig. 4B). This result suggests that GR does indeed bind to the putative GREs within the fragments, and this is in accordance with similar results obtained for the GRE1.3 fragment obtained by Jaiswal et al. [16]. The small amount of residual material present at the level of complex a in the presence of the GRE consensus oligo (Fig. 4B, lanes 6, 7, 19 and

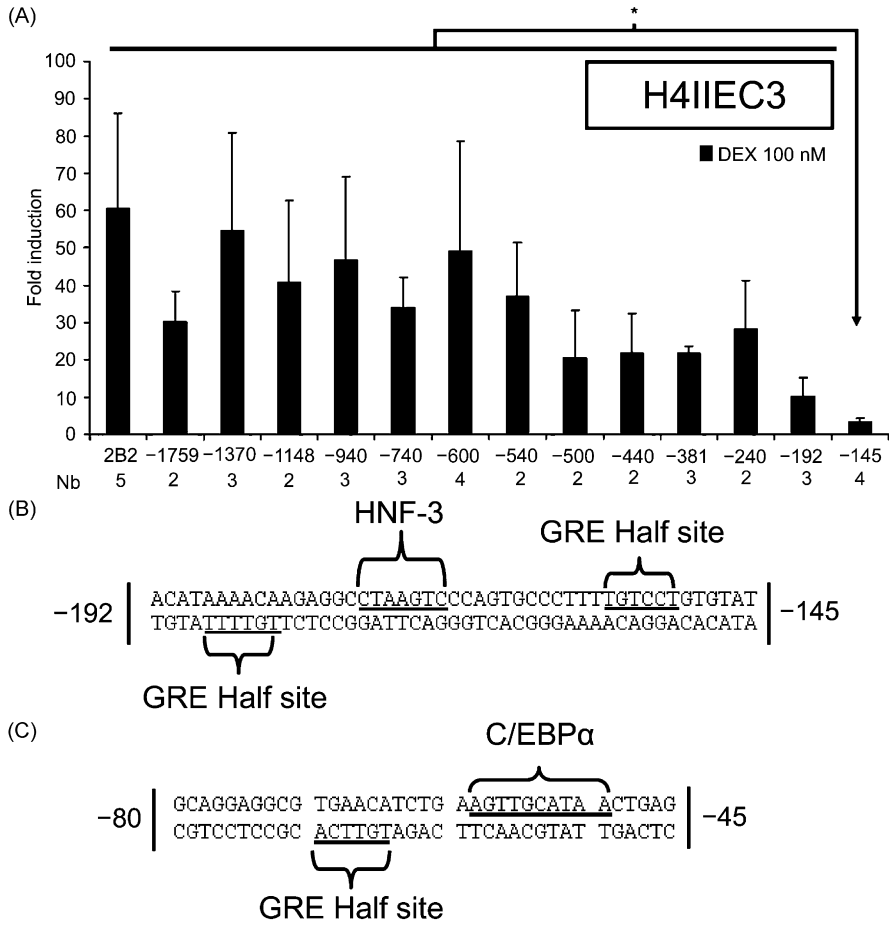

Fig. 3 – Response to DEX of the different reporter constructs in H4IIEC3 cells. (A) Fold induction after DEX treatment for the different reporter constructs. The single asterisk (*) denotes a significant difference between the two identified groups ($P < 0.05$). Nb refers to the number of independent transfection experiments performed. (B) Sequence of the −192/−145 fragment showing the different potential transcription factor binding sites. The putative HNF-3 site has been reported elsewhere [28]. (C) Sequence of part of the *CYP2B2* basal promoter showing the putative GRE-half site adjacent to a C/EBPα binding site [38].

20) presumably results from a complex that co-migrates with the GR complex and contains proteins other than GR that bind poorly to the GRE consensus. With the −192/−145 fragment, the most striking effect of the presence of unlabeled GRE consensus oligo was that the signal was poorly defined and tended to leave a smear (Fig. 4B, lanes 13 and 14). This may mean that in the absence of GR an unstable complex is formed. Similar results were obtained when the GRE1.3 oligo was included as an unlabeled competitor with the labeled −1180/−1148 fragment (data not shown).

3.4. *CAR is essential for maximal DEX responsiveness in vivo*

As mentioned above, CAR acts as an accessory factor by stimulating the DEX response mediated by GR in HepG2 and H4IIEC3 cells (Audet-Walsh and Anderson, submitted for publication). Therefore, we investigated the induction of CYP2B10 protein in mice lacking CAR in comparison to wild type mice. The results were similar to those obtained in the cultured cells. In the wild type CAR-positive mice, the level of CYP2B10 protein was higher than in CAR-negative mice (Fig. 5). Thus, CAR is not required for DEX induction of the *Cyp2b10* gene, as in its absence DEX induction is reduced but not abolished. Similar results have been reported for the induction of CYP2B10 mRNA by DEX in CAR-negative mice by Wei et al. [27].

4. Discussion

In other work (Audet-Walsh and Anderson, submitted for publication) we showed that *CYP2B2* and *Cyp2b10* reporter constructs are DEX-responsive in HepG2 and H4IIEC3 cells, that the response is mediated by GR, and that CAR acts as an accessory factor to stimulate the response. Here we describe new regions in the *CYP2B2* 5′ flank that contribute to DEX responsiveness, in addition to GRE1.3 reported by Jaiswal et al. [16] and the PBRU, and demonstrate that CAR also acts as an accessory factor in vivo.

The first new region, the 32-bp −1180/−1148 fragment containing a putative GRE (GRE2), was active in HepG2 cells but

not in H4IIEC3 cells. This may be due to the presence of accessory factor(s) present in HepG2 cells but absent from H4IIEC3 cells. It is noteworthy that when this fragment was fused to the *CYP2B2* basal promoter, not only did it confer higher DEX responsiveness than when it was in its natural sequence context at −1.15 kb, but it conferred higher DEX responsiveness than the pGL3-2B2-Luc reporter construct itself. This is so despite the fact that the pGL3-2B2-Luc reporter contains at least one additional element that can contribute to DEX responsiveness in HepG2 cells, namely GRE1.3 (Audet-Walsh and Anderson, submitted for publication). One possible explanation for this seemingly paradoxical observation is that the distance between GRE2 and the promoter may be important, and that by bringing it closer to the promoter, its capacity to confer DEX responsiveness was increased. Another possible explanation is that there are regulatory sequences in the *CYP2B2* 5′ flank that can act negatively on DEX induction.

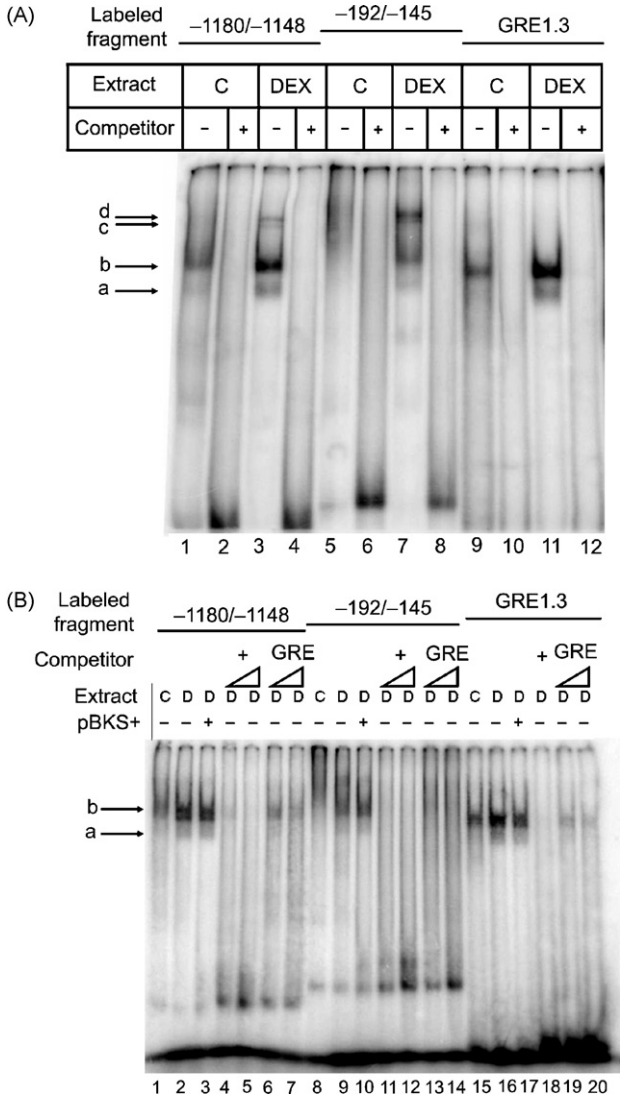

Fig. 4 – Binding of rat liver nuclear proteins to putative GREs or accessory sites and competition by the GRE consensus sequence. (A) Three fragments from the *CYP2B2* 5′ flank were used as labelled oligos: −1180/−1148, −192/−145 and GRE1.3 (see Table 2). Nuclear extracts were from livers of vehicle-treated rats (C) or from livers of DEX-treated rats (DEX). The four arrows labelled a–d indicate the four retarded complexes that were detected. The competitors were the three corresponding unlabelled oligos (100 ng). (B) Competition analysis with the three corresponding unlabeled oligos as well as with the GRE consensus oligo (identified as GRE). The smaller amount of unlabeled competitor was 10 ng and the larger was 100 ng; 100 ng of unlabeled GRE1.3 was added. Nuclear extracts were from livers of vehicle-treated rats (C) or from livers of DEX-treated rats (D). pBluescript SK+ (pBSK+) plasmid DNA was added as a non-specific competitor (100 ng) in lanes 3, 10 and 17. Complexes c and d seen in panel A were not well defined in this experiment due to the use of smaller wells.

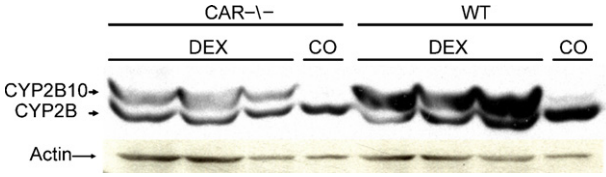

Fig. 5 – CAR is required for the maximal induced level of CYP2B10 protein in mouse liver. Western blot analysis of CYP2B10 protein in livers of vehicle-treated (CO) and DEX-treated wild type and CAR-negative mice. Each lane (10 μg of microsomal protein) corresponds to one animal. The results shown are representative of two independent experiments. CYP2B refers to constitutive immunoreactive CYP2B protein(s) present both in wild type and CAR-negative mice. Actin was used as loading control.

The possibility that there are negative regulatory sequences in the *CYP2B2* 5′ flank is supported by the observation that successive deletions of the *CYP2B2* 5′ flank between −2506 and −1180 led first to a reduction and then to a restoration of both basal and DEX-induced levels (Fig. 2A). Somewhat similar effects have been reported by others for the basal levels of transcription of *CYP2B1* and *CYP2B2* reporters in HepG2 cells [28] and of *Cyp2b10* reporters in primary mouse hepatocytes [29]. Our results suggest that an element acting positively to increase basal and DEX-induced expression is present between −2506 and −1759 and is deleted in the −1759 reporter construct, and that an element acting negatively is present between −1759 and −1180 and is deleted in the −1180 construct. The region between −2506 and −1759 contains the PBRU, but it is not likely to be the putative positive element, as deletion of the PBRU alone does not reduce reporter expression in HepG2 cells (Audet-Walsh and Anderson, submitted for publication).

Two other GREs or accessory sites are active in H4IIEC3 cells but not in HepG2 cells. Both are located within 192 bp of the *CYP2B2* transcription start site. One, within the basal promoter (Fig. 3C), was described elsewhere (Audet-Walsh and Anderson, submitted for publication). The other, which is described here, is located between −192 and −145. Once again the differences in the effects of the different GREs or accessory sites in the two cell lines may be a consequence of differences in their transcription factor or co-activator content. It is also conceivable that there is a species effect, HepG2 cells being of human origin and H4IIEC3 cells being of rat origin.

Whatever the explanation for the different results obtained with respect to DEX responsiveness conferred by the deletion constructs in the two cell lines, it is unlikely to be related to the level of CAR. HepG2 cells have vanishingly low levels of CAR mRNA [30] and furthermore they have long been used as a line essentially free of CAR to assess its capacity to activate transcription driven by putative CAR response elements [5,6]. Similarly for the H4IIEC3 cells, they do not appear to contain appreciable amounts of CAR either, because in these cells exogenous CAR activates CAR-response element-driven transcription by 10- to 24-fold over basal levels (Audet-Walsh and Anderson, submitted for publication). So, the conclusion is that neither HepG2 cells nor H4IIEC3 cells contain appreciable amounts of CAR and hence that we have to look elsewhere for the explanation for the differences in DEX responsiveness conferred by the deletion constructs.

Differences in RXR content are also unlikely to explain the different results obtained with respect to DEX responsiveness conferred by the deletion constructs in the two cell lines, Both contain RXR, easily detectable by Western blot analysis [31,32], consistent with the lack of a requirement for exogenous RXR in assays for CAR activation of CAR response element-driven reporter gene transcription [5,6] (Audet-Walsh and Anderson, submitted for publication).

With respect to GR, it has long been known that in H4IIEC3 cells DEX-induced GR-reponsive gene expression does not depend on the presence of exogenous GR [33], and it is well known that HepG2 cells require exogenous GR to be so responsive [34]. These observations are consistent with the presence of GR in H4IIEC3 cells and its absence from HepG2 cells.

Moreover, when saturating amounts of CAR and GR expression vectors are cotransfected into each cell line, DEX-induced luciferase levels are at least 10-fold higher in H4IIEC3 cells than in HepG2 cells (Audet-Walsh and Anderson, submitted for publication), suggesting that other transcription factors implicated in the response are present in H4IIEC3 cells. Finally, the −1180/−1148 fragment confers DEX responsiveness in the presence of GR in HepG2 cells but not in H4IIEC3 cells, whereas the −192/−145 region and the basal promoter confer DEX responsiveness in H4IIEC3 cells but not in HepG2 cells. These results are also consistent with the conclusion that additional transcription factors, present in HepG2 cells but not in H4IIEC3 cells, and vice versa, are needed for activation of transcription via these sites.

In the EMSA experiments with rat liver nuclear extracts as a source of proteins we observed two well defined retarded complexes with each of the labeled fragments, GRE1.3 and GRE2. The faster and slower moving complexes, referred to here as complexes a and b, probably represent GR monomers and dimers, respectively. Such GR monomer and dimer complexes have been observed by others [35,36]. The two complexes were effectively competed by a GRE consensus oligo, and furthermore both GRE1.3 [16] and GRE2 confer DEX responsiveness when placed adjacent to a basal promoter. Hence, we may conclude that they are indeed functional GREs, that they contribute to conferring DEX responsiveness on the *CYP2B2* reporter in cultured cells, and that they may similarly contribute to DEX responsiveness in rat liver.

The third labeled fragment used in the EMSA studies, −192/−145, does not contain a typical GRE, although it does contain two putative GRE half sites (Fig. 3B), as well as a putative hepatocyte nuclear factor-3 (HNF-3) site [28]. When this fragment was incubated with an extract from DEX-treated rats, four specific retarded complexes, migrating similarly to, but slightly slower than, complexes a, b, c and d, were generated. Their nature is unclear at this time. One possibility is that the two faster migrating complexes represent GR monomers and dimers, respectively. However, GRE half sites would not be expected to generate dimer complexes and, according to conventional wisdom, GRE half sites require the presence of an accessory factor bound to an adjacent site [37]. Perhaps HNF-3 could play that role for the GRE half sites present in the −192/−145 fragment. Perhaps too, GR and HNF-3 bind cooperatively. That could account for the apparent instability of the retarded complex observed in the presence of the GRE consensus oligo (Fig. 4B, lanes 13 and 14).

Use of liver nuclear extracts from DEX-treated rats led to much better defined retarded complexes in EMSA analyses, particularly for the −192/−145 fragment. This may reflect the formation with extracts from vehicle-treated rats of unstable complexes that tend to dissociate during the electrophoresis. This phenomenon could have several explanations, notably that DEX treatment leads to increased levels of other transcription factors [37] responsible for the formation of more stable retarded complexes or that DEX treatment leads to increased hepatic levels of GR itself. Thus, after DEX treatment, nuclear GR may participate in the formation of stable heterodimeric complexes. This interpretation is supported by the apparent destabilization of the retarded complexes formed with the −192/−145 fragment, in the presence of nuclear extracts from DEX-treated animals, when competed with the GRE consensus oligo. This gave rise to poorly defined complexes similar to those observed in the presence of nuclear extracts from vehicle-treated rats.

We reported elsewhere that CAR acts as an accessory factor in conferring DEX responsiveness in both HepG2 and H4IIEC3 cells (Audet-Walsh and Anderson, submitted for publication). Here we show that it appears to have the same effect for the induction of CYP2B10 protein in mouse liver. It was already known that CYP2B10 mRNA is inducible by DEX in the liver of CAR-negative mice [27]. In any event, CAR is clearly not essential for the expression of *CYP2B* genes, although it does act to amplify both basal and DEX-induced levels of expression.

In conclusion, we have obtained evidence for the existence in the *CYP2B2* 5′ flank of five regions that contribute to DEX responsiveness (Fig. 6). Although many details remain to be elucidated, it seems likely that they, as well as other elements yet to be defined, form part of a complex GRU (Audet-Walsh and Anderson, submitted for publication). The identification here of new sites contributing to DEX responsiveness, in addition to GRE1.3 and the PBRU (Audet-Walsh and Anderson, submitted for publication) supports this model. According to this model, DEX responsiveness depends on the GR pathway and CAR acts as an accessory factor through the PBRU. Other accessory factors may yet be identified. Questions that remain to be resolved include the determination of which of the GREs and accessory sites are functional in vivo to confer DEX

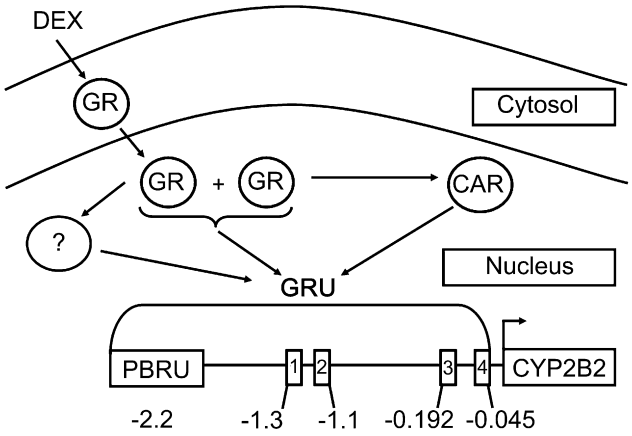

Fig. 6 – Model to explain DEX induction of the *CYP2B2* gene. The rectangles labeled 1–4 correspond to GRE1.3, the −1180/−1148 fragment, the −192/−145 fragment and the GRE half site present in the *CYP2B2* basal promoter, respectively. The *CYP2B2* gene and its 5′ flanking region are not drawn to scale. The question mark (?) represents possible accessory factors contributing to the DEX response.

responsiveness. The issue is particularly pertinent in view of the strikingly different responses observed in the two cell lines with respect to the GREs or accessory sites that confer DEX responsiveness. Another issue to be resolved is the relation of the sequence elements described here to the classical response to PB. Clearly the PBRU is involved in both, but in different ways. The role, if any, in PB induction of the other sequence elements involved in conferring DEX responsiveness remains to be elucidated. A yet more fundamental question relates to the physiological importance of the two pathways that can lead to induction of *CYP2B* genes in rodent liver.

Acknowledgments

The authors thank Sacha Auclair Vincent for reading the manuscript, Julie Dionne for the help with the figures, Denis Allard for providing the H4IIEC3 cells, Jean-Marc Pascussi for the GR expression vector, David Waxman for the anti-CYP2B antibody and David Moore for the CAR-negative mice. This work was supported by a grant from the Instituts de recherche en santé du Canada. This work has been submitted by EA-W to the Faculté des études supérieures, Université Laval, in partial fulfilment of the requirements for the degree of maître ès sciences. EA-W was the recipient of a studentship award from the Fondation J.-Arthur-Vincent.

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