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HR96 and BR-C modulate phenobarbital induced transcription of cytochrome P450 *CYP6D1* in *Drosophila* S2 cells

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Abstract

Phenobarbital (PB) is a prototypical inducer for studies of xenobiotic responses in animals. In mammals, nuclear receptors CAR and PXR have been identified as key transcription factors regulating PB induced transcription of xenobiotic responsive genes. In insects, much less is known about transcription factors involved in regulating PB induced transcription, although CAR and PXR have a single ortholog, HR96 (*hormone receptor-like in 96*), in *Drosophila melanogaster*. Using dual luciferase reporter assays in *Drosophila* S2 cells, constructs containing variable lengths of the promoter of the PB inducible cytochrome P450 *CYP6D1* were evaluated in the presence and absence of PB. The promoter region between –330 and –280 (relative to the position of transcription start site, +1) was found to be critical for PB induction. Putative binding sites for *Drosophila* BR-C (*broad-complex*) and DFD (*deformed*) were identified within this promoter region using TFsearch. RNAi treatment of S2 cells in conjunction with *CYP6D1* promoter assays showed that suppression of *Drosophila* HR96 and BR-C transcription in S2 cells resulted in a significant decrease and increase, respectively, of PB induction. Effects of HR96 and BR-C in mediating PB induction were PB specific and dependent. This represents new functional evidence that *Drosophila* HR96 and BR-C can act as an activator and repressor, respectively, regulating PB induced transcription in insects.

Keywords

xenobiotic induction; cytochrome P450; BR-C; Dfd; HR96; transcription

Introduction

Phenobarbital (PB) has been a prototypical inducer used for the study of xenobiotic responses in animals since it was discovered to cause induction of total cytochrome P450s more than 40 years ago (Conney 1967). In response to PB, animals show increases in expression of numerous genes, especially those involved in detoxification and metabolism, such as cytochrome P450s (P450s), glutathione *S*-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs) (Gerhold et al. 2001; Hamadeh et al. 2002; King-Jones et al. 2006; Sun et al. 2006; Willoughby et al. 2006). In mammals, CAR (constitutive androstane receptor) and PXR (pregnane X receptor) are key transcription factors (TFs) critical for regulating PB induced transcription of P450s (Sueyoshi and Negishi 2001; Timsit and Negishi 2007). In response to PB, CAR and PXR associate with their common TF partner RXR (retinoid X receptor) resulting in heterodimers of CAR-RXR

and PXR-RXR, respectively, which in turn bind to target DNA sequences and activate transcription of regulated genes (Sueyoshi and Negishi 2001; Timsit and Negishi 2007). CAR and PXR are nuclear receptors, which are ligand-activated transcription factors regulating pathways involved in metabolism, and are unique in Metazoa (Baker 2005; Escriva et al. 2004). Chicken and nematode orthologs of mammalian CAR and PXR have been shown to play a key role in regulating PB and other xenobiotic responses (Handschin et al. 2000; Handschin et al. 2001; Lindblom et al. 2001).

In insects, two general approaches have been used to identify TFs responsible for regulating PB induced transcription. The first approach used promoter assays to identify regions critical for PB induced transcription. Studies of the promoter sequences of PB inducible cytochrome P450 *Cyp6a2* and *Cyp6a8* of *Drosophila melanogaster* located PB responsive regions to within 428 bp upstream from the translation start site of *Cyp6a2* (Dunkov et al. 1997) and between positions -716 and -199 (numbers are relative to translation start site) of *Cyp6a8* promoter (Maitra et al. 2002). Within these regions, putative binding sites for three TFs (BR-C, EcR, and AP1) were found (Dunkov et al. 1997; Maitra et al. 2002), although the role of these TFs in PB induction remains unclear.

The second approach used to identify TFs involved in PB induced transcription is based on *D. melanogaster* nuclear receptor HR96 (*hormone receptor-like in 96*). *Drosophila* HR96 represents the single ortholog corresponding to mammalian nuclear receptors CAR and PXR (King-Jones and Thummel 2005; Laudet et al. 2005). Based on this evolutionary relationship, HR96 has been considered to be important for regulating transcription in response to PB. A *D. melanogaster* HR96 null mutant has been generated and studied. Adults of the HR96 null mutant strain were more sensitive to DDT and PB (King-Jones et al. 2006), suggesting a role of HR96 in protection against these xenobiotics. Microarray results showed transcription of 29 P450s were induced in response to PB in wild type Canton-S strain. However, in the HR96 null mutant strain, these 29 P450s were still as PB inducible as in the wild type strain (King-Jones et al. 2006). Thus, the role of HR96 in PB induced transcription of P450s (and other PB-regulated genes) remains unclear (Giraud et al. 2010; Morra et al. 2010).

The transcription of house fly (*Musca domestica*) cytochrome P450 *CYP6D1* is PB inducible (Liu and Scott 1997; Scott et al. 1996). Given that *Drosophila* S2 cells are able to mediate PB induced transcription of *Cyp6a2* (Dunkov et al. 1997), we conducted promoter assays in *Drosophila* S2 cells to locate the PB responsive *cis*-regulatory sequence of the *CYP6D1* promoter using progressive serial 5' deletions. The promoter sequence from position -330 to -280 (numbers are relative to the transcription start site, defined as +1) was found to be critical for PB induced transcription. Putative binding sites of *Drosophila* BR-C (*broad-complex*) and DFD (*deformed*) were found within the promoter sequence from -330 to -280. To identify TFs expressed in *Drosophila* S2 cells critical for PB induction of *CYP6D1*, RNAi treatment of S2 cells in conjunction with promoter assays was conducted and to examine if *Drosophila* HR96, BR-C, or DFD is critical for PB induction. Our results identified *Drosophila* HR96 and BR-C acting as positive and negative transcriptional regulators of PB induction of *CYP6D1*, respectively. Reaction of HR96 and BR-C were PB specific and PB dependent. This represents new functional evidence of the role of HR96 and BR-C in regulating PB induced transcription in insects.

Results

Identification of a PB responsive *cis*-regulatory sequence in the *CYP6D1* promoter

Evaluation of the *CYP6D1* 5' serial deletion promoter constructs, -900/+85, -344/+85, -246/+85, and -42/+85 (numbers are relative to the position of transcription start site, +1)

located the PB responsive *cis*-regulatory region to be between -344 and -246 (Fig. 1), based on the significant increase of PB induction seen in promoter construct -344/+85 compared to -246/+85. These results also showed the promoter regions from -900 to -344 and from -344 to -246 were responsible for basal (i.e. without PB) transcription in S2 cells (Fig. 2).

To further define the PB responsive *cis*-regulatory region (and the proximal basal promoter region), additional promoter assays were conducted using promoter constructs -344/+85, -330/+85, -312/+85, -298/+85, -280/+85, and -246/+85. Significant increases in PB induction were seen in promoter constructs -330/+85, -312/+85, and -298/+85 in comparison to the next shorter promoter construct (Fig. 2). These results indicate the PB responsive *cis*-regulatory sequence is located between positions -330 and -280 (Fig. 2). The pattern of significant increases in PB induction across four promoter constructs suggests the binding sites for TFs involved in PB induction span the junctions of the constructs and/or there are multiple TFs involved. Our results also further defined the promoter region from -280 to -246 as responsible for basal (i.e. without PB) transcription in S2 cells (Fig. 2).

TFsearch of the TRANSFAC database (version 1.3) (Heinemeyer et al. 1998) was used to identify putative TF binding sites within the 51 nucleotides of the PB responsive *cis*-regulatory sequence (from -330 and -280). We focused on the TFs of *Drosophila* (and other insects) because promoter assays were conducted using *Drosophila* S2 cells. Based on the TFsearch cut-off score of 90.0, putative sites of *Drosophila* TFs [BR-C (*broad-complex*) and DFD (*deformed*)] were identified (Fig. 3), with scores 92.5 and 90.0, respectively. BR-C is known to have isoforms, Z1–Z4 with different zinc-finger DNA binding domains produced by alternative splicing (Bayer et al. 1996). The putative binding site of BR-C identified by TFsearch belongs to isoform Z4. The binding site of HR96 remains unclear and is not in the TRANSFAC database (version 1.3).

RNAi treatment of *Drosophila* S2 cells and PB responsive promoter assays

To identify TFs expressed in *Drosophila* S2 cells critical for regulating PB induction through the *CYP6D1* promoter, the roles of three *Drosophila* TFs, HR96, BR-C, and DFD were evaluated using RNAi in conjunction with promoter reporter assays. BR-C and DFD were selected because their putative binding sites were found in the PB responsive promoter region (above). HR96 was chosen because it represents the ortholog of mammalian CAR and PXR. Preliminary experiments suggested maximal silencing of transcripts after 12 days of culturing S2 cells with dsRNA (Fig. S1) (Lin 2010). Therefore, we used this duration for all subsequent experiments. To demonstrate the silencing effect of transcription in response to treatment of dsRNA, 12-day RNAi-treated cells and control cells were sampled to measure target gene (i.e. *hr96* and *br-c*) transcript abundance (by normalizing to *rpl3*) using quantitative real time PCR (qPCR). There was an average 6.5-fold reduced abundance of *hr96* transcript in 12-day HR96^{RNAi} cells compared to control cells, and an average 2.7-fold reduced abundance of *br-c* transcript in 12-day BR-C^{RNAi} cells compared to control cells.

The 12-day RNAi-treated cells and control cells were subjected to PB responsive promoter assays using *CYP6D1* promoter construct -330/+85. HR96^{RNAi} cells showed a significant ($P < 0.01$) decrease in PB induced promoter activity (gray bars, Fig. 4A), but HR96^{RNAi} cells in the absence of PB (white bars Fig. 4A), were not different from control cells. BR-C^{RNAi} cells showed a significant increase ($P < 0.01$) in PB induced promoter activity compared to control cells while the BR-C^{RNAi} cells in the absence of PB were not different from control cells (Fig. 4B). Promoter assays using LacZ^{RNAi} cells (treatment with dsRNA probe corresponding to *LacZ* gene of *E. coli*) were conducted in parallel (to test the specificity of the RNAi treatment) and no significant effect of this RNAi treatment was seen (with or without PB) compared to control cells (Fig. 4B). This indicates the effects seen for HR96

and BR-C were specific, and not simply the result of the RNAi treatment. The same results were found in three independent replicates for each target gene. Our results indicate HR96 acts as a positive transcriptional regulator and BR-C acts as a negative transcriptional regulator for PB induction through the *CYP6D1* promoter in *Drosophila* S2 cells. The action of HR96 and BR-C were PB specific and PB dependent.

Semi-quantitative RT-PCR indicated the relative abundance of *dfd* transcript level in *Drosophila* S2 cells was approximately >1,000 fold less than *hr96* or *br-c* transcript level (based on approximate difference of >10 PCR cycles). In fact, we could not reproducibly amplify a significant PCR product for *dfd*, even after 40 cycles (data not shown). *DFD*^{RNAi} cells showed no significant change ($P=0.3$) in PB responsiveness (data not shown), although suppression of *dfd* transcript by RNAi treatment could not be confirmed due to its low abundance. Thus, the effect of RNAi treatment on *dfd* expression levels could not be unequivocally determined. The low abundance of *dfd* transcripts (compared to *hr96* and *br-c*) in S2 cells suggests DFD may not be involved in regulating PB induction in S2 cells.

Discussion

Drosophila HR96 is an activator of PB induction

The depletion of HR96 in *Drosophila* S2 cells significantly reduced PB induction, indicating HR96 acts as a positive transcriptional regulator of PB induction. This is consistent with the expectation for the insect ortholog of vertebrate CAR and PXR, which function as transcriptional activators of PB induced P450s (Handschin et al. 2000; Handschin et al. 2001; Sueyoshi and Negishi 2001; Timsit and Negishi 2007). Our results contrast with those in *Drosophila*, where 29 PB inducible P450s (and the majority of PB-regulated genes) were still PB inducible in an HR96 null strain, compared to a wild type strain (King-Jones et al. 2006). It was suggested that the loss of HR96 (in the HR96 null strain) may be compensated by additional transcriptional regulators able to feed into this pathway (King-Jones et al. 2006); and a possible *Drosophila* ortholog of mammalian aryl hydrocarbon receptor was suggested (King-Jones et al. 2006). Based on the microarray dataset DGS1472 at GEO (Gene Expression Omnibus at NCBI), the *Drosophila* ortholog of mammalian AHR, *spineless*, is not expressed in S2 cells. In contrast to the isolation of a HR96 null strain, a recent study found high mortality in *Drosophila* when HR96 silencing was driven by a tubulin-GAL4 promoter crosses (Giraud et al. 2010). The reason for these differences is not known. While our results demonstrated a role of HR96 in PB induction in insects, a complete list of genes for which HR96 has a role in PB induction will require further study.

Reaction of *Drosophila* HR96 was PB specific and dependent. Studies in mammals have revealed the presence of regulatory cascades controlling the activation of mammalian CAR and PXR in response to PB (Sueyoshi and Negishi 2001; Timsit and Negishi 2007). Whether or not similar regulatory cascades or mechanisms control PB specific and dependent reaction of *Drosophila* HR96 remain unclear. Based on microarray dataset DGS2071 (King-Jones et al. 2006) at GEO, the *Drosophila* *hr96* transcript levels in wild type adults do not change significantly in response to PB, which indicates the PB specific and dependent reaction of *Drosophila* HR96 is not attributed to change of its abundance.

For PB induction, mammalian CAR and PXR require association with RXR in order to bind to target DNA sequences (Baes et al. 1994; Kliewer et al. 1998; Sueyoshi and Negishi 2001; Timsit and Negishi 2007). The chicken ortholog CXR also requires RXR in order to bind to target DNA sequences (Handschin et al. 2000; Handschin et al. 2001). It is unknown what the TF partner of *Drosophila* HR96 is, although USP (*ultraspiracle*) represents the *Drosophila* ortholog of mammalian RXR and USP is expressed in S2 cells (dataset DGS1472 at GEO).

The cognate binding sequence of *Drosophila* HR96 remains unknown; although it has been described that the DNA binding domain of *Drosophila* HR96 could shift oligonucleotides bearing an EcR binding site of the *Drosophila hsp27* promoter (Fisk and Thummel 1995). However, no putative EcR binding site was identified with TFsearch in the *CYP6D1* promoter (−330 to +85). While our studies identified a region of the *CYP6D1* promoter likely to bind *Drosophila* HR96, further studies will be needed to identify the DNA sequences to which HR96 binds.

Drosophila BR-C is a repressor of PB induction

BR-C has been suggested to be involved in PB induction of *Drosophila Cyp6a2* (Dunkov et al. 1997) and *Cyp6a8* (Maitra et al. 2002), but whether or not BR-C plays a role in PB induction remains unclear. Our results showed the depletion of BR-C in *Drosophila* S2 cells resulted in an increase of PB induced *CYP6D1* promoter activity, indicating BR-C is a negative transcriptional regulator of PB induction. BR-C has four types of isoforms, Z1–Z4, which have different zinc-finger DNA binding domains produced by alternative splicing (Bayer et al. 1996). These four isoforms appear to be present together in various types of tissues and cells, but their relative abundance differs among tissue types (Bayer et al. 1996; Emery et al. 1994). TFsearch indicated the presence of a putative BR-C Z4 binding site within the PB responsive promoter region of *CYP6D1* (−330 to −280). This is consistent with results from *Aedes aegypti* (Zhu et al. 2007) and *D. melanogaster* (Crossgrove et al. 1996), where BR-C Z4 has been reported to function as a transcriptional repressor. Using an RT-PCR protocol (Tzolovsky et al. 1999), the expression of BR-C Z4 in *Drosophila* S2 cells was confirmed (data not shown). In addition, binding sites of BR-C Z4 have also been identified in PB responsive promoter regions of *Cyp6a2* (Dunkov et al. 1997) and *Cyp6a8* (Maitra et al. 2002). These results suggest BR-C Z4 may be involved in regulating PB induction of P450 genes in multiple species.

The abundance of *br-c* transcript in wild type adults of *D. melanogaster* does not change significantly in response to PB, based on the microarray dataset DGS2071 (King-Jones et al. 2006) at GEO, similar to HR96. This suggests the PB-dependent control of transcription by BR-C is not dependent on change of its abundance.

Induction and Resistance

Identification and characterization of TFs involved in PB-induced gene expression may also help understand some cases of metabolism-mediated insecticide resistance. In many insecticide resistant strains having metabolism-mediated resistance, there is constitutive overexpression of multiple P450s and GSTs that are PB inducible in susceptible strains (Le Goff et al. 2003; Pedra et al. 2004; Vontas et al. 2005). It has been suggested that resistant strains may simply have detoxification genes that are constitutively “induced” by an unknown *trans* acting factor (King-Jones et al. 2006; Liu and Scott 1997; Maitra et al. 2002; Plapp 1984; Sun et al. 2006). Theoretically, a mutation in any component of the transcriptional machinery or regulatory cascades of PB induction could underlie this phenomenon. Therefore, identifying these components could further our understanding of the molecular basis of metabolism-mediated insecticide resistance.

Increased transcription of *CYP6D1* in the permethrin resistant LPR strain is due to factors on chromosome 1 and 2 (Liu and Scott 1997). *CYP6D1* expression is not induced by PB in LPR, and this trait has been mapped to chromosome 2 (Liu and Scott 1997). Based on homology maps between *D. melanogaster* and *M. domestica* (Foster et al. 1981; Weller and Foster 1993), HR96 and BR-C are expected to be present on chromosome 2 and 3, respectively, of *M. domestica*. Given our findings that HR96 was a positive regulator of PB induced *CYP6D1* expression, and the expectation that HR96 is on house fly chromosome 2

makes *HR96* worth further study as a possible factor involved in the increased transcription of *CYP6D1* in LPR.

In summary, the *CYP6D1* promoter sequence from −330 to −280 was found to be critical for PB induction. *Drosophila* HR96 was demonstrated to play a role in activating PB induction. This represents new functional and *in vivo* evidence for the role of HR96 in regulating PB induced transcription in insects. *Drosophila* BR-C was found to act as a repressor of PB induction, which represents a unique aspect of the transcriptional regulation of PB induction in insects. Due to the lack of a house fly cell line, the experiments reported herein relied on the use of S2 cells. Thus, care must be taken in drawing conclusions from these studies and extrapolating them to house flies. Future studies are needed to identify the target DNA sequences of HR96, TF partner(s) associated with HR96, and the regulatory mechanisms for PB-dependent reactions of HR96 and BR-C.

Experimental procedures

Drosophila S2 cells

Drosophila S2 cells were maintained and grown in serum free cell culture medium of HyQ SFX-Insect (HyClone, Logan, UT) in 75 cm² of tissue culture flask (BD Falcon, Bedford, MA). Cells were subcultured every 2–3 days as they reached confluency.

Constructs of progressive 5' deletions of the *CYP6D1* promoter

Progressive serial 5' deletions of the *CYP6D1* promoter from the CS strain (Scott et al. 1999) were generated by PCR amplification. Restriction enzyme sites (*Sac* I and *Bgl* II) were added upstream and downstream by incorporation into the forward and reverse primers used in PCR, respectively. These PCR products were purified using QIAEX II kit (Qiagen, Valencia, CA) and sequentially digested by restriction enzymes *Sac* I and *Bgl* II (NEB, Ipswich, MA) at 37°C overnight. Resulting products were individually ligated into *Sac* I and *Bgl* II sites of the pGL3-Basic vector (Promega, Madison, WI) in order to drive the expression of the firefly luciferase reporter gene using T4 DNA ligase (Invitrogen, Carlsbad, CA). Ligation products were individually transformed into TOP 10 competent cells (Invitrogen) by heat shock at 42°C for 30 sec. Transformed competent cells were grown in 250 µl of SOC medium (Invitrogen) at 37°C for 1 hr. Next, 40 µl of 40 mg/ml X-gal and 50 µl of transformed competent cells were sequentially spread on a Luria Broth (LB) plate containing ampicillin (50 µg/ml) and incubated at 37°C overnight. Single colonies were selected and individually grown overnight in 3 ml of LB liquid medium containing 150 µg of ampicillin. Plasmid DNA was purified using the QIAprep Miniprep system (Qiagen). Plasmid DNA of each promoter construct was sequenced at the Cornell University Life Sciences Core Laboratories Center prior the use for transfection. The concentration of each promoter construct was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA).

Transfection and PB responsive promoter assay

To identify the *cis*-regulatory sequence responsible for PB induced transcription, *CYP6D1* promoter constructs were evaluated using the dual luciferase reporter assay system (Promega) in *Drosophila* S2 cells. Individual promoter constructs were co-transfected with pRL-TK vector (Promega), carrying the *Renilla* luciferase reporter gene, into *Drosophila* S2 cells to serve as an internal control for transfection efficiency. For transfection, each well (bottom diameter: 22.09 mm) of a 12-well tissue culture plate (BD Falcon) was seeded with 1.2×10^6 S2 cells in 1 ml of HyQ SFX-insect medium. Thirty minutes later, the cell culture medium (including non-adhered cells) was removed, 300 µl of new cell culture medium and 500 µl of transfection reagent mix (containing 10.4 fmole of one *CYP6D1* promoter

construct, 3.75 fmole of pRL-TK vector, and 7.75 μ l of Cellfectin® reagent (Invitrogen) in the HyQ SFX-insect medium) were sequentially added. After incubation for 3.5 hr, the transfection reagent mix was replaced with 1 ml of HyQ SFX-insect medium containing ± 0.5 mM PB (Sigma-Aldrich, St. Louis, MO), and the transfected cells were then incubated for 48 hr. This concentration (0.5 mM) of PB was chosen based on our preliminary test of concentration-response with serial concentrations of PB (0.5 mM of PB caused the greatest PB induction without causing toxicity to cells; data not shown), and because it had been used previously (Dunkov et al. 1997). Settled transfected cells were washed with 1 $\times$ PBS buffer (1.15 g of Na₂HPO₄, 0.2 g of KH₂PO₄, 8 g of NaCl, and 0.2 g of KCl dissolved in 1 L of ddH₂O) and lysed by incubating with 250 μ l of 1 $\times$ Passive Lysis Buffer (Promega) for 20 min. Cell lysate (10 μ l) was placed in a 1.6 ml microtube and loaded into the 20/20ⁿ luminometer (Turner BioSystems, Sunnyvale, CA), which sequentially injected 50 μ l of LAR II and 50 μ l of Stop & Go Reagents (Promega) to the cell lysate, and measured luminescences derived from firefly luciferase and *Renilla* luciferase, respectively. Luminescence of firefly luciferase was normalized by luminescence of *Renilla* luciferase. The normalized firefly luminescence represented the promoter activity driven by corresponding 5' deletion of *CYP6D1* promoter. Three independent transfections (PB and control, done side-by-side) of three replicates for each promoter construct (n = 9) were conducted. Statistical analysis of pairwise comparisons of difference of [(PB induced promoter activity) – (basal promoter activity) relative to the next shorter *CYP6D1* promoter construct] was conducted using Student's *t*-test.

Design and preparation of dsRNA probes

RNAi probes were designed in exon regions of target genes and their specificity was confirmed using E-RNAi (Arziman et al. 2005). If multiple isoforms existed (according to Entrez Gene database at NCBI), the RNAi probe was selected for a region shared by all isoforms. A two-step PCR strategy was used to generate DNA template for dsRNA synthesis (Ramadan et al. 2007). In the first step, gene specific primers were used to amplify a fragment of the target gene (i.e. 252 bp of *hr96*, 254 bp of *br-c*, and 266 bp of *dfd*). In the second PCR step, gene specific primers tailed with T7 core promoter sequence (5'-TAA TAC GAC TCA CTA TAG GG-3') were used. Sequences of primers used in the first PCR step were: HR96-dsRNA-F: 5'-AAG CCA TTG CTG GAC AAG GA-3', HR96-dsRNA-R: 5'-GGG CTC GTC GTT GTA GTT GG-3', BR-dsRNA-F: 5'-CCT GCA GTC CCT ACT TCC GC-3', BR-dsRNA-R: 5'-AGC TTG TCG CTG ATG GAG AT-3', DFD-dsRNA-F: 5'-TCG GAG TAT GTG CAA TCC AA-3', and DFD-dsRNA-R: 5'-CAC TCA TAT GAC CCG TAG ATG C-3'. The dsRNA probe corresponding to *lacZ* (beta-*D*-galactosidase of *Escherichia coli*) was prepared using two primers: LacZ-dsRNA-F: 5'-GAA TTA ATA CGA CTC ACT ATA GGG AGA GAT ATC CTG CTG ATG AAG C -3', LacZ-dsRNA-R: 5'-GAA TTA ATA CGA CTC ACT ATA GGG AGA GCA GGA GCT CGT TAT CGC -3' (the T7 promoter is underlined), and the plasmid DNA bearing *lacZ* gene. Primers and the plasmid DNA were from Drs. J. Lis and N. Fuda (Molecular Biology and Genetics, Cornell University). PCR products coupled with T7 core promoter sequences on both ends were purified using Microcon YM-30 centrifugal filters (Millipore, Billerica, MA). To produce dsRNA probes, *in vitro* transcription was performed using AmpliScribe™ T7-Flash Transcription kit (Epicentre, Madison, WI). The reaction was carried out at 37°C for 4 hr in a total reaction volume of 40 μ l that included 1 μ g of above purified DNA template, 3.6 μ l of 100 mM ATP, 3.6 μ l of 100 mM CTP, 3.6 μ l of 100 mM GTP, 3.6 μ l of 100 mM UTP, 4 μ l of 100 mM DTT, 4 μ l of 10 $\times$ buffer, and 4 μ l of AmpliScribe™ T7-Flash enzyme solution. Afterward, 2 μ l of DNase I was added and incubated at 37°C for 15 min. RNA was precipitated by adding 50 μ l of ddH₂O, 10 μ l of 3.0 M sodium acetate pH 5.2, and 250 μ l of 95% ethanol and placed at -20°C for 15 min. Following centrifugation at 13,000 rpm for 15 min at 4°C, the RNA pellet was sequentially washed with 75% ethanol, air-dried, and

resuspended in 400 μ l of ddH₂O. The dsRNAs were annealed by incubating at 65°C for 30 min followed by slow cooling to room temperature (~22°C) (Clemens et al. 2000). The dsRNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific). Each DNA template used for producing dsRNA probes was sequenced and confirmed prior the use of *in vitro* transcription.

RNAi treatment of *Drosophila* S2 cells and the PB responsive promoter assay

Drosophila S2 cells (4×10^6) were cultured in 3 ml of HyQ SFX-insect cell culture medium containing 23.2 μ l of Cellfectin® (Invitrogen) and 30 μ g of dsRNA probe in a 25 cm² of tissue culture flask (Corning, Corning, NY) and maintained in $22 \pm 1^\circ\text{C}$. S2 cells were subcultured every 3 days by passing 4×10^6 cells to a new flask with addition of Cellfectin® and dsRNA to continue RNAi. Preliminary test was conducted using dsRNA probe of *hr96* to determine how many days of RNAi-treatment would be enough before the use of the following promoter assays (using *CYP6D1* promoter construct -330/+85) in order to see significant effect in PB induction compared to the use of control cells. Promoter assays using 3-day, 6-day, 9-day and 12-day RNAi-treated cells (supplemental information, Fig. S1A) showed, 1.5%, 10.5%, 19.4%, and 25.4% reduction of PB induction, respectively, compared to control cells (supplemental information, Fig. S1b). These results indicated treatment of dsRNA probe for 12 days prior to the following promoter assays could result in enough depletion of target protein level to see significant and clear effect on PB induction. The RNAi-mediated promoter assays using 12-day RNAi-treated cells and control cells were conducted using *CYP6D1* promoter construct -330/+85 and following description above (Transfection and PB responsive promoter assay), except for the introduction of 10 μ g (~10 μ l) of dsRNA probe immediately before addition of 500 μ l of transfection reagent mix into settled RNAi-treated cells for the 3.5 hr incubation. In the following 48 hr incubation of ± 0.5 mM PB, 10 μ g of dsRNA probe was applied to RNAi-treated cells to continue the RNAi suppression. Luminescence was measured as described above (Transfection and PB responsive promoter assay). Controls lacking dsRNA were conducted in parallel. Controls using a dsRNA probe for *lacZ* of *E. coli* were also conducted. Three independent transfections ($\pm$ PB) with RNAi-treated cells or control cells were performed in each replicate of PB responsive promoter assay. Three replicates for each of the target genes were conducted. Statistical analysis of multiple pairwise comparisons was conducted using Student's *t*-test followed by Tukey's HSD (Honestly Significant Difference) test.

Purification of mRNA, synthesis of cDNA, and quantitation of transcripts

RNAi-treated cells and control cells were sampled to determine the transcript levels of the target genes. Cells ($\sim 2 \times 10^6$) were pelleted by centrifugation at 3,500 rpm for 3 min and were washed with $1 \times$ PBS buffer. The Illustra QuickPrep™ micro mRNA purification kit (GE Healthcare, Little Chalfont, UK) was used according to the manufacture's instructions. The mRNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific). The mRNA product was treated with DNase I using a DNA free™ kit (Applied Biosystems, Foster City, CA). The reaction was carried out in a total reaction volume of 16.1 μ l containing 1 μ g of mRNA, 1.6 μ l of $10 \times$ buffer, and 1 μ l of rDNase I, and was incubated at 37°C for 20 min. The cDNA synthesis was conducted using SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen). The RT reaction was in a total reaction volume of 20 μ l including 8 μ l of DNase-treated mRNA, 1 μ l of 50 μ M oligo(dT)₂₀, 1 μ l of 10 mM dNTP mix, 2 μ l of $10 \times$ RT buffer, 4 μ l of 25 mM MgCl₂, 2 μ l of 0.1 M DTT, 1 μ l of RNaseOUT™ (40 U/ μ l), and 1 μ l of SuperScript™ III RT (200 U/ μ l), and was carried out at 50°C for 50 min, followed with incubation at 85°C for 5 min to terminate the reaction.

The *hr96* and *br-c* transcript levels were measured by normalizing to *rpl3* transcript level using qPCR by the comparative C_T method. Purified mRNA (500 ng) derived from S2 cells was treated with DNase to remove gDNA (DNA freeTM kit, Applied Biosystems, Foster City, CA), and cDNA was synthesized using the SuperScriptTM III first-strand synthesis system (Invitrogen). Each qPCR reaction included 0.5 μ l of cDNA product, 1 μ l of 10 μ M forward primer, 1 μ l of 10 μ M reverse primer, 7.5 μ l of ddH₂O, and 10 μ l of Power SYBR Green PCR Master Mix (2X) (Applied Biosystems). Primers used were HR96-forward: 5'-GCG GAC GTG GTG GAG TTC ATG-3', HR96-reverse: 5'-GCG GTC TGC TGT CTG CTG GG-3', BR-C-forward: 5'-GCA CAC CCT GCA AAC ACC CG-3', BR-C-reverse: 5'-TGC CTG CTG CTG CGT GAG TC-3', RPL3-forward: 5'-GAC GCC AGC AAG CCA GTC CA-3', and RPL3-reverse: 5'-GCC GAC AGC ACC GAC CAC AA-3'. Reactions were carried out using Applied Biosystems 7900HT Real-Time PCR system at the Cornell University Life Sciences Core Laboratories Center with following temperature program: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data was processed and analyzed using SDS software (version: 2.1). Three independent qPCR reactions of each target gene of each biological sample were acquired. PCR products were analyzed in a 2% agarose gel to confirm a single band was obtained, and were DNA sequenced to confirm the expected product was obtained.

Semi-quantitative RT-PCR was performed by monitoring PCR products following 15, 20, 25, 30, 35, and 40 PCR cycles. Each PCR reaction was in a total reaction volume of 20 μ l containing 10 μ l of 2X GoTaq[®] Green Master Mix (Promega), 0.5 μ l of above cDNA, 0.5 μ l of 10 μ M forward primer, 0.5 μ l of 10 μ M reverse primer, and 8.5 μ l of ddH₂O. PCR reactions were carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA) with the following temperature program: 95°C for 3 min; 40 cycles of 95°C for 30 sec, 54°C for 30 sec, and 72°C for 45 sec; and 72°C for 5 min. Semi-quantitative RT-PCR of the housekeeping gene, *rpl3*, was conducted in parallel to determine the relative abundance of target gene cDNA in each sample. Forward and reverse primers for target genes *dfd* and *rpl3* were designed in adjacent neighboring exons allowing detection of gDNA contamination. Primer pairs used were listed in the following: DFD-forward: 5'-TGG ATC GGC AAA TGG ATA TT-3', DFD-reverse: 5'-GGA TCT TCT TCA TCC AGG GGT-3', RPL3-forward: 5'-CTC ATC GTA AGT TCT CGG CAC C-3', and RPL3-reverse: 5'-TAG AAG CGA CGA CGG CAC TC-3'. PCR products were analyzed in a 2% agarose gel containing ethidium bromide (5 μ g/ml).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PB responsive promoter assay conducted with progressive 5' deletion of the *CYP6D1* promoter. Promoter constructs are numbered relative to the transcription start site (TSS) at +1. Relative luciferase activity was measured by normalizing the signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the average of the relative luciferase activity $\pm$ S.D. of three independent transfections of three replicates (n=9). Gray bars represent the signal in the presence of PB and white bars represent the control. Double asterisks indicate a greater PB induction relative to the next shorter promoter construct ($p < 0.01$, Student's *t*-test).

Figure 2.

PB responsive promoter assay corresponding to the promoter region from -344 to -246 of *CYP6D1*. Promoter constructs are numbered relative to the TSS at $+1$. Relative luciferase activity was measured by normalizing the signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the average of the relative luciferase activity $\pm$ S.D. of three independent transfections of three replicates ($n=9$). Gray bars represent the signal in the presence of PB and white bars represent the control. Asterisks indicate a greater PB induction relative to the next shorter promoter construct (* $p < 0.05$; ** $p < 0.01$; Student's t -test).

-330 -312 -298 -280
 ↑ ↑ ↑ ↑
 TAAAACCAT TGAATTTGTA AATAAAATTA ATCTAGCTG ACAAAATGA T

 BR-C

 DFD

Figure 3.

Sequence of the PB responsive *cis*-regulatory sequence from -330 to -280 of *CYP6D1* promoter and putative binding sites of *Drosophila* TFs. Numbers indicate position of 5' ends of serial deletion promoter constructs. The binding sites of *Drosophila* BR-C (*broad-complex*, isoform Z4) and DFD (*deformed*) were identified using TFsearch and are represented by dash lines beneath the sequence.

Figure 4.

Effect of RNAi on PB induction of *CYP6D1* in *Drosophila* S2 cells. The luciferase activities of RNAi-treated cells (HR96^{RNAi} and BR-C^{RNAi}) and control cells in the presence and absence of PB were measured. Gray bars represent PB induced promoter activities and white bars represent basal promoter activities of *CYP6D1* promoter construct -330/+85. Bars represent mean of promoter activity (firefly LU / *Renilla* LU) $\pm$ S.D. of three independent transfections (n=3). (A) HR96^{RNAi} cells showed significant decrease in PB induced promoter activity compared to control cells (double asterisks, $p < 0.01$; Student's *t*-test with Tukey's HSD test). No significant difference appeared in basal activities between HR96^{RNAi} cells and control cells.