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Hormone receptor-like in 96 and Broad-Complex 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, the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) have been identified as key transcription factors regulating PB induced transcription of xenobiotic responsive genes. In insects, much less is known about the transcription factors involved in regulating PB induced transcription, although CAR and PXR have a single orthologue hormone receptor-like in 96 (HR96) in *Drosophila melanogaster*. Using dual luciferase reporter assays in *Drosophila* Schneider (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* Broad-Complex (BR-C) and deformed (Dfd) were identified within this promoter region using TFsearch. RNA interference (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 PB dependent. This represents new functional evidence that *Drosophila* HR96 and BR-C can act as an activator and repressor, respectively, in regulating PB induced transcription in insects.

Keywords: xenobiotic induction, cytochrome P450, BR-C, Dfd, HR96, transcription.

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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 uridine diphosphate-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, constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are key transcription factors (TFs) critical for regulating PB induced transcription of P450s (Sueyoshi & Negishi, 2001; Timsit & Negishi, 2007). In response to PB, CAR and PXR associate with their common TF partner retinoid X receptor (RXR) 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 & Negishi, 2001; Timsit & Negishi, 2007). CAR and PXR are nuclear receptors, which are ligand-activated transcription factors regulating pathways involved in metabolism, and are unique in Metazoa (Escriva *et al*., 2004; Baker, 2005). Chicken and nematode orthologues of mammalian CAR and PXR have been shown to play a key role in regulating PB and other xenobiotic responses (Handschin *et al*., 2000, 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 uses 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, ecdysone receptor (EcR) and activator protein 1] 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 hormone receptor-like in 96 (HR96). *Drosophila* HR96 represents the single orthologue corresponding to mammalian nuclear receptors CAR and PXR (King-Jones & Thummel, 2005; Laudet & Bonneton, 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 dichlorodiphenyltrichloroethane and PB (King-Jones *et al*., 2006), suggesting a role of HR96 in protection against these xenobiotics. Microarray results showed that transcription of 29 P450s was 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 (Giraudo *et al*., 2010; Morra *et al*., 2010).

The transcription of house fly (*Musca domestica*) cytochrome P450 *CYP6D1* is PB inducible (Scott *et al*., 1996; Liu & Scott, 1997). Given that *Drosophila* Schneider (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* Broad-Complex (BR-C) and deformed (Dfd) were found within the promoter sequence from –330 to –280. To identify TFs expressed in *Drosophila* S2 cells critical for PB induction of *CYP6D1*, RNA interference (RNAi) treatment of S2 cells in conjunction with promoter assays was conducted 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. Reactions 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 (ie without PB) transcription in S2 cells (Fig. 1).

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 that 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 (ie without PB) transcription in S2 cells (Fig. 2).

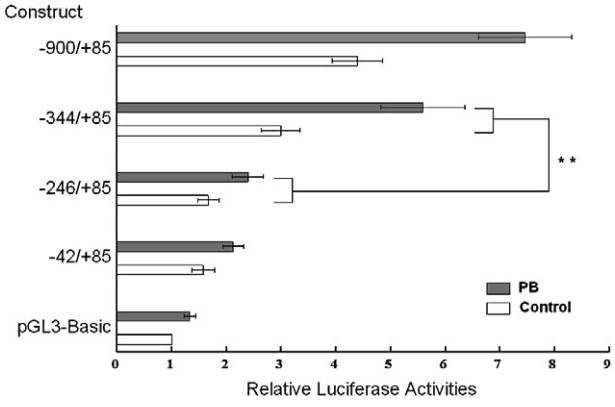

Figure 1. Phenobarbital (PB) responsive promoter assay conducted with progressive 5′ deletion of the *CYP6D1* promoter. Promoter constructs are numbered relative to the transcription start site (TSS) at +1. Relative luciferase activity was measured by normalizing the signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the average of the relative luciferase activity ± SD of three independent transfections of three replicates ($n = 9$). Grey 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).

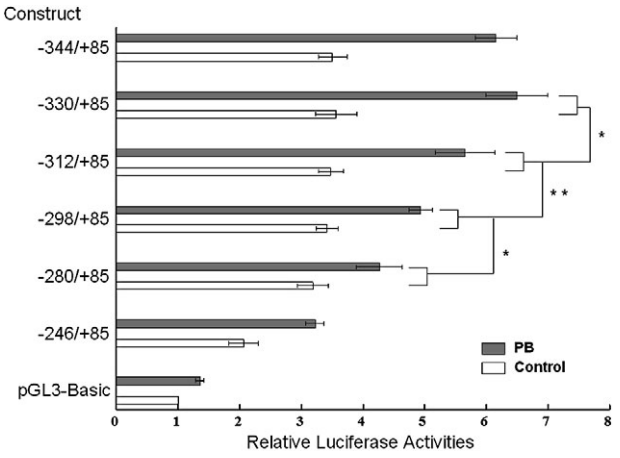

Figure 2. Phenobarbital (PB) responsive promoter assay corresponding to the promoter region from −344 to −246 of *CYP6D1*. Promoter constructs are numbered relative to the transcription start site (TSS) at +1. Relative luciferase activity was measured by normalizing the signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the average of the relative luciferase activity ± SD of three independent transfections of three replicates ($n = 9$). Grey 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).

TFsearch of the TRANSFAC database (v. 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 and Dfd) were identified (Fig. 3), with scores of 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 (v. 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 orthologue of mammalian CAR and PXR. Preliminary experiments suggested maximal silencing of transcripts after 12 days of culturing S2 cells with double-stranded RNA (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 (ie *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 (grey 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 whereas 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 the *LacZ* gene of *Escherichia 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 that 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 that 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.

Semiquantitative RT-PCR indicated the relative abundance of *Dfd* transcript level in *Drosophila* S2 cells was approximately >1000 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

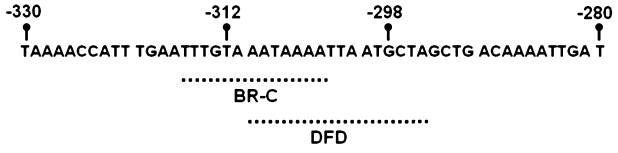

Figure 3. Sequence of the phenobarbital (PB) responsive *cis*-regulatory sequence from −330 to −280 of *CYP6D1* promoter and putative binding sites of *Drosophila* transcription factors (TFs). Numbers indicate position of 5′ ends of serial deletion promoter constructs. The binding sites of *Drosophila* Broad-Complex (BR-C, isoform Z4) and deformed (Dfd) were identified using TFsearch and are represented by dashed lines beneath the sequence.

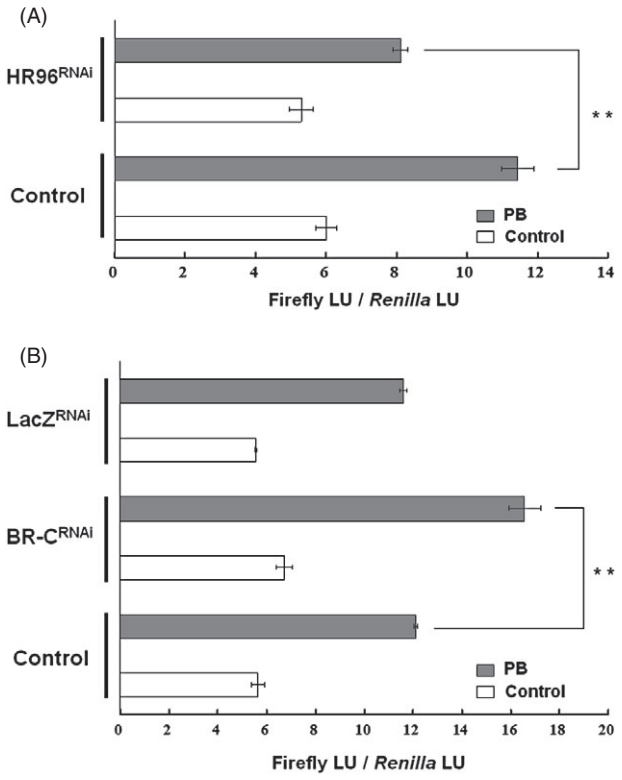

Figure 4. Effect of RNA interference (RNAi) on phenobarbital (PB) induction of *CYP6D1* in *Drosophila* Schneider (S2) cells. The luciferase activities of RNAi-treated cells [hormone receptor-like in 96^{RNAi} (HR96^{RNAi}) and Broad-Complex^{RNAi} (BR-C^{RNAi})] and control cells in the presence and absence of PB were measured. Grey bars represent PB induced promoter activities and white bars represent basal promoter activities of the *CYP6D1* promoter construct −330/+85. Bars represent mean of promoter activity [firefly luminescence units (LU)/*Renilla* LU] ± SD 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 honestly significant difference (HSD) test]. No significant difference appeared in basal activities between HR96^{RNAi} cells and control cells. (B) BR-C^{RNAi} cells showed significant increase 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 BR-C^{RNAi} cells and control cells. The control LacZ^{RNAi} cells (with treatment of double-stranded RNA probe corresponding to the sequence of the *LacZ* gene of *Escherichia coli*) revealed no significant change in PB induced and basal promoter activities compared to control cells. This indicates that the RNAi effects seen for HR96 and BR-C were specific, and not simply the result of the RNAi treatment. Three independent replicates for HR96 and BR-C were carried out.

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 because of 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 that 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 that HR96 acts as a positive transcriptional regulator of PB induction. This is consistent with the expectation for the insect orthologue of vertebrate CAR and PXR, which function as transcriptional activators of PB induced P450s (Handschin *et al*., 2000, 2001; Sueyoshi & Negishi, 2001; Timsit & 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 has been 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). A *Drosophila* orthologue of mammalian aryl hydrocarbon receptor (AHR) was suggested to have this role (King-Jones *et al*., 2006). Based on the microarray dataset DGS1472 at Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI), the *Drosophila* orthologue 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 cross (Giraudo *et al*., 2010). The reason for these differences is not known. Although our results demonstrate 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 & Negishi, 2001; Timsit & 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 & Negishi, 2001; Timsit & Negishi, 2007). The chicken orthologue CXR also requires RXR in order to bind to target DNA sequences (Handschin *et al*., 2000, 2001). It is unknown what the TF partner of *Drosophila* HR96 is, although *ultraspiracle* (USP) represents the *Drosophila*

orthologue 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 & Thummel, 1995). However, no putative EcR binding site was identified with TFsearch in the *CYP6D1* promoter (–330 to +85). Although our studies have 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 that 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 amongst tissue types (Emery *et al*., 1994; Bayer *et al*., 1996). 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 that 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 that 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 with metabolism-mediated resistance, there is constitutive over-expression 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 (Plapp, 1984; Liu & Scott, 1997; Maitra *et al*., 2002; King-Jones *et al*., 2006; 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 Learn pyrethroid resistant (LPR) strain is a result of factors on chromosome 1 and 2 (Liu & Scott, 1997). *CYP6D1* expression is not induced by PB in LPR, and this trait has been mapped to chromosome 2 (Liu & Scott, 1997). Based on homology maps between *D. melanogaster* and *M. domestica* (Foster *et al*., 1981; Weller & Foster, 1993), *HR96* and *BR-C* are expected to be present on chromosome 2 and 3, respectively, of *M. domestica*. Our finding that *HR96* is a positive regulator of PB induced *CYP6D1* expression, and the expectation that *HR96* is on house fly chromosome 2, make *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. As a result of 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, USA) in 75 cm^2 of tissue culture flask (BD Falcon, Bedford, MA, US–A). 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 a QIAEX II kit (Qiagen, Valencia, CA, USA) and sequentially digested by the restriction enzymes *Sac*I and *Bgl*II (NEB, Ipswich, MA, USA) at 37 °C overnight. The resulting products were individually ligated into *Sac*I and *Bgl*II sites of the pGL3-Basic vector (Promega, Madison, WI, USA) in order to drive the expression of the firefly luciferase reporter gene using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA). Ligation products were individually transformed into TOP 10 competent cells (Invitrogen) by heat shock at 42 °C for 30 s. Transformed competent cells were grown in 250 μl Super Optimal Broth medium (Invitrogen) at 37 °C for 1 h. Next, 40 μl of 40 mg/ml X-gal and 50 μl 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 LB liquid medium containing 150 μg 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 to 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, USA).

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 HyQ SFX-insect medium. Thirty minutes later, the cell culture medium (including non-adhered cells) was removed, 300 μl new cell culture medium and 500 μl transfection reagent mix [containing 10.4 fmole of one *CYP6D1* promoter construct, 3.75 fmole of pRL-TK vector and 7.75 μl of Cellfectin reagent (Invitrogen) in the HyQ SFX-insect medium] were sequentially added. After incubation for 3.5 h, the transfection reagent mix was replaced with 1 ml HyQ SFX-insect medium containing ± 0.5 mM PB (Sigma-Aldrich, St. Louis, MO, USA), and the transfected cells were then incubated for 48 h. 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× phosphate-buffered saline (PBS) [1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 8 g NaCl and 0.2 g KCl dissolved in 1 L double-distilled H_2O (ddH_2O)] and lysed by incubating with 250 μl of 1× Passive Lysis Buffer (Promega) for 20 min. Cell lysate (10 μl) was placed in a 1.6 ml microtube and loaded into the $20/20^n$ luminometer (Turner BioSystems, Sunnyvale, CA, USA), which sequentially injected 50 μl luciferase assay reagent II and 50 μl 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, carried out 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 (ie 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 *E. 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-dsRNR-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, USA). To produce dsRNA probes, *in vitro* transcription was performed using AmpliScribe T7-Flash Transcription kit (Epicentre, Madison, WI, USA). The reaction was carried out at 37 °C for 4 h in a total reaction volume of 40 μl that included 1 μg of the above purified DNA template, 3.6 μl of 100 mM ATP, 3.6 μl of 100 mM CTP, 3.6 μl of 100 mM GTP, 3.6 μl of 100 mM uridine triphosphate, 4 μl of 100 mM dithiothreitol (DTT), 4 μl of 10× buffer and 4 μl of AmpliScribe T7-Flash enzyme solution. Afterward, 2 μl DNase I was added and incubated at 37 °C for 15 min. RNA was precipitated by adding 50 μl ddH_2O, 10 μl of 3.0 M sodium acetate pH 5.2 and 250 μl of 95% ethanol and placed at –20 °C for 15 min. Following centrifugation at 10 000 *g* for 15 min at 4 °C, the RNA pellet was sequentially washed with 75% ethanol, air-dried and resuspended in 400 μl ddH_2O. 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 absor-

bance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific). Each DNA template used for producing dsRNA probes was sequenced and confirmed prior to *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 HyQ SFX-insect cell culture medium containing 23.2 μl of Cellfectin (Invitrogen) and 30 μg dsRNA probe in a 25 cm^2 of a tissue culture flask (Corning, Corning, NY, USA) and maintained in 22 ± 1 °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. A preliminary test was conducted using the 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 a significant effect in PB induction compared to the use of control cells. Promoter assays using 3-, 6-, 9- and 12-day RNAi-treated cells (Fig. S1A) showed 1.5, 10.5, 19.4 and 25.4% reduction of PB induction, respectively, compared to control cells (Fig. S1B). These results indicated that treatment of dsRNA probe for 12 days prior to the following promoter assays could result in enough depletion of target protein level to see a significant and clear effect on PB induction. The RNAi-mediated promoter assays using 12-day RNAi-treated cells and control cells were conducted using the *CYP6D1* promoter construct −330/+85 and following the description above (Transfection and PB responsive promoter assay), except for the introduction of 10 μg (~10 μl) of dsRNA probe immediately before the addition of 500 μl transfection reagent mix into settled RNAi-treated cells for the 3.5 h incubation. In the following 48 h incubation of ±0.5 mM PB, 10 μg 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 (±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 Honestly Significant Difference test.

Purification of mRNA, synthesis of cDNA and quantification of transcripts

RNAi-treated cells and control cells were sampled to determine the transcript levels of the target genes. Cells (~2×10^6) were pelleted by centrifugation at 1200 *g* for 3 min and were washed with 1× PBS buffer. The Illustra QuickPrep micro mRNA purification kit (GE Healthcare, Little Chalfont, UK) was used according to the manufacturer'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, USA). The reaction was carried out in a total reaction volume of 16.1 μl containing 1 μg mRNA, 1.6 μl of 10× buffer and 1 μl 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 μl including 8 μl DNase-treated mRNA, 1 μl of 50 μM oligo$(dT)_{20}$, 1 μl of 10 mM dNTP mix, 2 μl of 10× reverse transcription (RT) buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl RNaseOUT (40 U/μl) and 1 μl SuperScript III RT (200 U/μl), 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 free kit, Applied Biosystems), and cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen). Each qPCR reaction included 0.5 μl cDNA product, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 7.5 μl ddH_2O and 10 μl Power SYBR Green PCR Master Mix (2×) (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 the following temperature program: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were processed and analysed using SDS software (v. 2.1). Three independent qPCR reactions of each target gene of each biological sample were acquired. PCR products were analysed in a 2% agarose gel to confirm that a single band was obtained, and were DNA sequenced to confirm that the expected product was obtained.

Semiquantitative 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 μl containing 10 μl of 2× GoTaq Green Master Mix (Promega), 0.5 μl of above cDNA, 0.5 μl of 10 μM forward primer, 0.5 μl of 10 μM reverse primer and 8.5 μl ddH_2O. PCR reactions were carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) with the following temperature program: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s; and 72°C for 5 min. Semiquantitative 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 neighbouring exons allowing detection of gDNA contamination. Primer pairs used were as follows: 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 analysed in a 2% agarose gel containing ethidium bromide (5 μg/ml).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2583.2010.01047.x

Figure S1. (A) RNA interference (RNAi) treatment [using double-stranded RNA (dsRNA) probe of *hormone receptor-like* in 96 (*hr96*)] of S2 cells in conjunction with the phenobarbital (PB) promoter assay. *Drosophila* S2 cells were cultured in a 25 cm^2 flask with Cellfectin ± dsRNA probe. Cells were subcultured every 3 days and Cellfectin ± dsRNA probe were added. RNAi-treated cells and control cells of 3, 6, 9 and 12 days were subjected to PB promoter assays using the promoter construct −330/+85. Transfected RNAi-treated cells were continued with treatment of dsRNA probe. After 48 h incubation with or without PB, firefly and *Renilla* luciferase activities were measured. (B) PB induced promoter activities (grey bars) and basal promoter activities (white bars) derived from the use of control cells and RNAi-treated cells of 3-, 6-, 9- and 12-day RNAi treatments (using dsRNA probe of *hr96*). Bars represent mean of promoter activity [firefly luminescence units (LU)/*Renilla* LU] ± SD of three independent transfections ($n = 3$). These results show that promoter assays using 3-, 6-, 9- and 12-day RNAi-treated cells resulted in 1.5, 10.5, 19.4 and 25.4% reduction of PB induction, respectively, compared to control cells.

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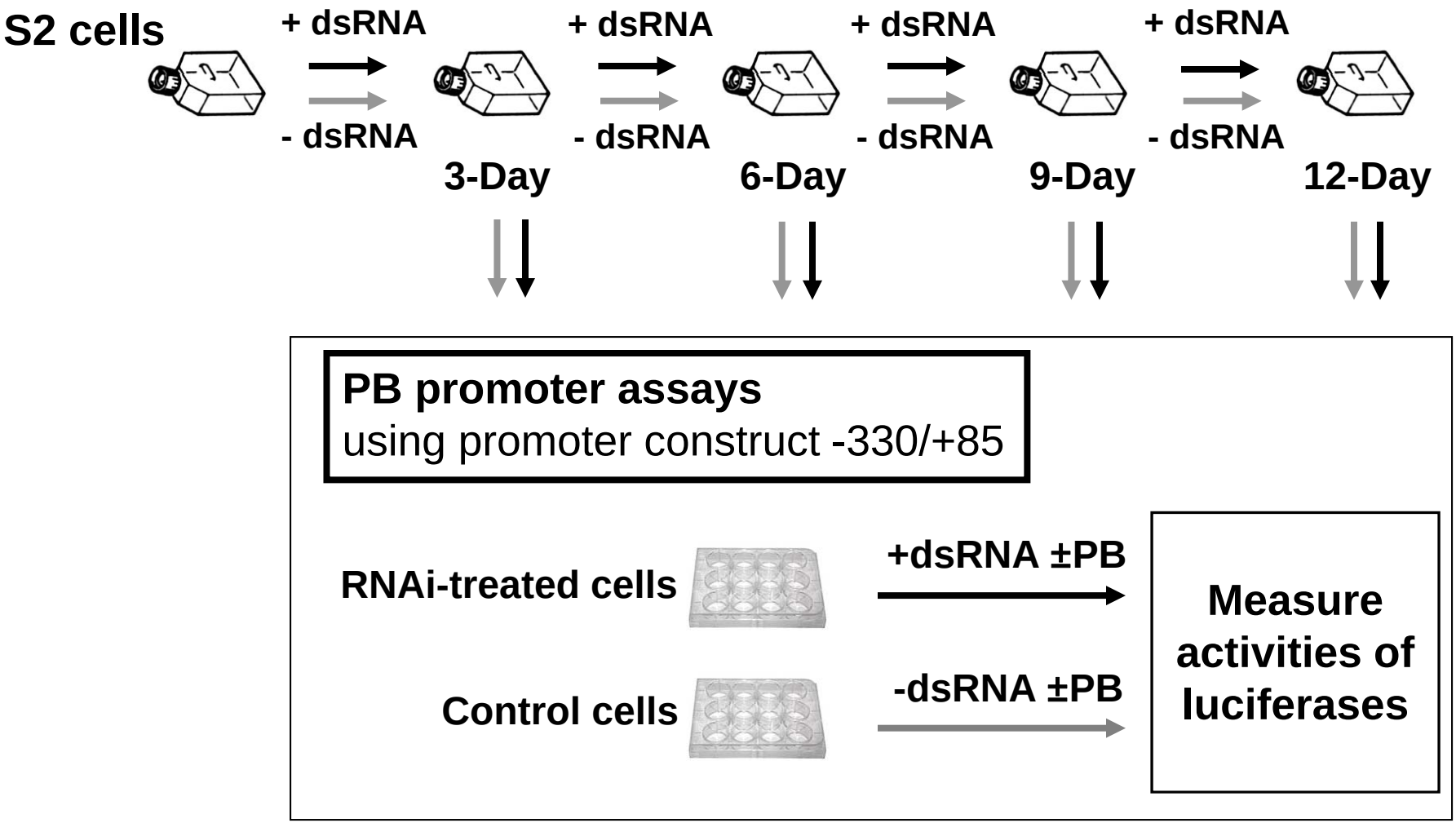
Fig. S1A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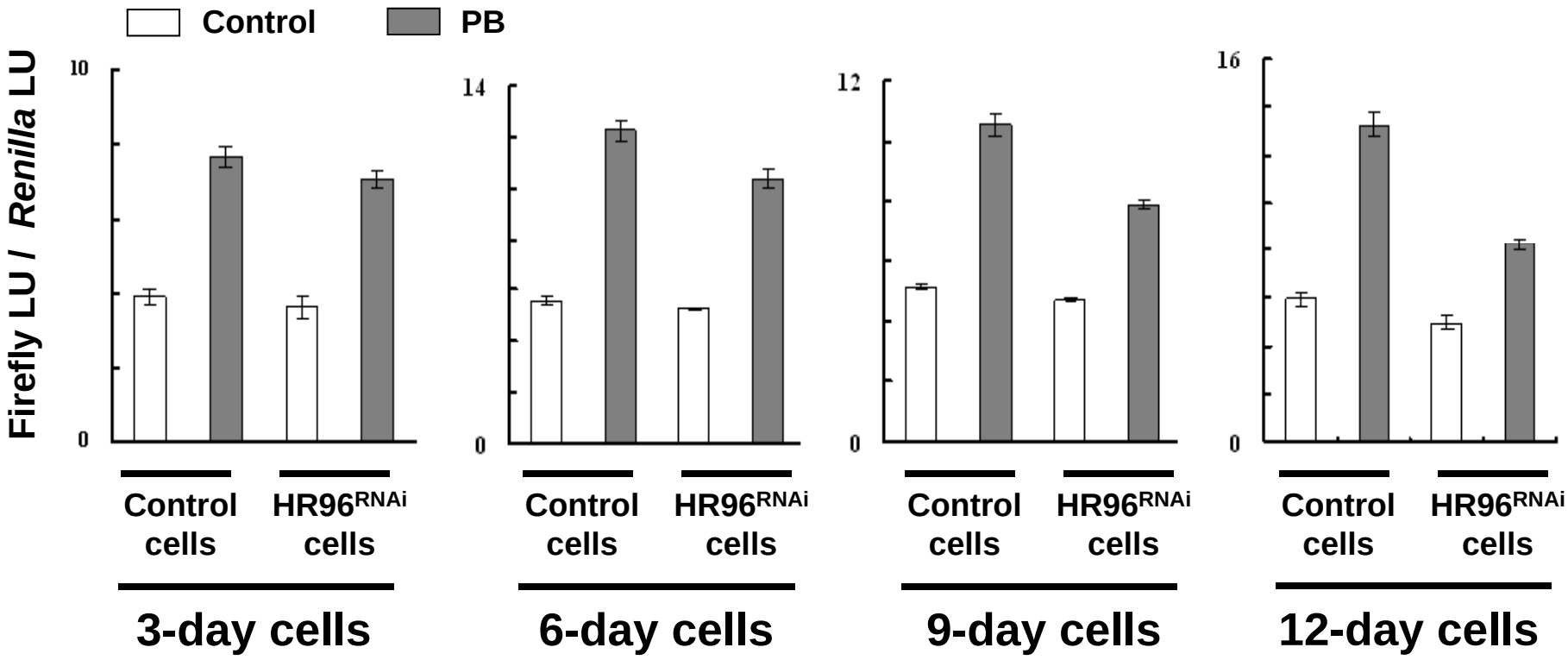
Control cells
-dsRNA ±PB
Measure activities of luciferases

Fig. S1B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