The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*

Kirst King-Jones,[1,2,3] Michael A. Horner,[1,2] Geanette Lam,[1] and Carl S. Thummel*

[1] Howard Hughes Medical Institute, Department of Human Genetics, University of Utah School of Medicine, 15 N 2030 E 5100, Salt Lake City, Utah 84112
[2] These authors contributed equally to this work.
[3] Present address: University of Alberta, CW-405 Biological Sciences Building, Edmonton, Alberta TGG 2E9, Canada.
*Correspondence: carl.thummel@genetics.utah.edu

Summary

Exposure to xenobiotics such as plant toxins, pollutants, or prescription drugs triggers a defense response, inducing genes that encode key detoxification enzymes. Although xenobiotic responses have been studied in vertebrates, little effort has been made to exploit a simple genetic system for characterizing the molecular basis of this coordinated transcriptional response. We show here that ~1000 transcripts are significantly affected by phenobarbital treatment in *Drosophila*. We also demonstrate that the *Drosophila* ortholog of the human SXR and CAR xenobiotic receptors, DHR96, plays a role in this response. A *DHR96* null mutant displays increased sensitivity to the sedative effects of phenobarbital and the pesticide DDT as well as defects in the expression of many phenobarbital-regulated genes. Metabolic and stress-response genes are also controlled by *DHR96*, implicating its role in coordinating multiple response pathways. This work establishes a new model system for defining the genetic control of xenobiotic stress responses.

Introduction

Higher organisms are constantly challenged by a wide range of toxins in their environment. These compounds, referred to as xenobiotics, enter the body by physical contact, inhalation, or ingestion and can originate from many sources including pharmaceuticals, pesticides, plant toxins, and pollutants. In order to deal with the deleterious effects of xenobiotics, higher organisms induce enzymes that metabolize these compounds into less harmful substances, aiding in their inactivation and excretion. This detoxification machinery includes four classes of enzymes that are conserved from insects to humans: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs). The most abundant class of xenobiotic metabolizing enzymes is the P450s, represented by 57 genes in humans and 90 genes in the fruit fly *Drosophila melanogaster* (Maurel, 1996; Ranson et al., 2002). They are referred to as phase I enzymes because they catalyze the first step in the detoxification process, decreasing the biological activity of a broad range of substrates. The other classes of detoxifying enzymes, including GSTs, carboxylesterases, and UGTs, are classified as phase II enzymes. Carboxylesterases catalyze the hydrolysis of ester-containing xenobiotics leading to their detoxification, while GSTs and UGTs add bulky side groups onto toxic compounds to increase their hydrophilicity, facilitating their excretion from the organism.

Detailed studies in vertebrates have defined a central role for two nuclear receptors in sensing xenobiotic compounds and regulating detoxification gene expression: the human Steroid and Xenobiotic Receptor (SXR, PXR in mice, NR1I2), and Constitutive Androstane Receptor (CAR, NR1I3) (Chawla et al., 2001; Francis et al., 2003; Willson and Kliewer, 2002). SXR/PXR and CAR are most abundantly expressed in the liver and small intestine, organs that provide a first line of defense against xenobiotics. SXR/PXR directly bind a wide range of lipophilic xenochemicals, while CAR interacts with a more restricted set of compounds and can be activated indirectly (Goodwin and Moore, 2004; Kliewer et al., 2002). These receptors appear to function in a partially redundant manner, with a range of overlapping target genes, including genes that encode phase I and phase II detoxification enzymes, multidrug-resistance enzymes, sulfotransferases, and proteins involved in the transport and conjugation of small lipophilic compounds (Maglich et al., 2002; Rosenfeld et al., 2003; Ueda et al., 2002).

In contrast to these studies in humans and mice, relatively little effort has been made to characterize the regulation of insect xenobiotic responses. Rather, studies in insects have been largely restricted to insecticide-resistant strains that are the result of extreme selective pressures, analyzing populations that have adapted to the presence of specific compounds in their environment (Ffrench-Constant et al., 2004; Wilson, 2001). For example, overexpression of a single P450 gene, *Cyp6g1*, is sufficient to confer DDT resistance in *Drosophila* (Daborn et al., 2002), and resistance to organophosphates, malathion, and carbamates have been linked to overproduction of carboxylesterases (Hemingway et al., 2004; Zhu et al., 2004). There are also examples of acquired resistance to naturally occurring toxins. For example, some *Drosophila* species that live in the Sonoran Desert use hazardous cactus species as a food source due to their expression of appropriate detoxification enzymes (Danielson et al., 1997, 1998; Fogleman, 2000). This ability of insects to adapt to specific xenobiotic compounds remains the greatest impediment to the development of effective insecticides, with serious consequences for human health and welfare. Better forms of insect population control are critical for increasing agricultural crop yields as well as combating lethal insect-borne human diseases such as malaria.

Table 1. Occurrence of gene families in microarray results based on InterPro domains

	ABC transp. (51)		UDP-Gluc. (38)		GST (45)		Carboxylesterase (39)		Cytochrome P450 (92)		DUF227 (41)		Peptidase S1 (196)		JHBP (27)		ML domain (8)		Turandot (8)	
array results (n)	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value
1 *CS*±PB ↑ (503)	4	2.2E-02	**7**	**1.8E-09**	**16**	**1.5E-42**	**7**	**3.4E-09**	**29**	**4.3E-66**	**13**	**1.0E-30**	10	3.5E-02	2	>0.05	0	>0.05	**4**	**1.1E-16**
2 *CS*±PB ↓ (484)	3	>0.05	2	>0.05	1	>0.05	2	>0.05	3	>0.05	4	3.7E-03	**25**	**1.6E-19**	2	>0.05	1	>0.05	0	>0.05
3 *CS*+PB:*DHR96*[1] +PB ↑ (326)	**5**	**1.0E-05**	2	>0.05	1	>0.05	0	>0.05	5	6.5E-03	0	>0.05	6	>0.05	**4**	**1.9E-07**	1	2.0E-02	0	>0.05
4 *CS*+PB:*DHR96*[1] +PB ↓ (199)	1	>0.05	0	>0.05	1	>0.05	2	1.3E-02	0	>0.05	0	>0.05	5	4.1E-02	1	>0.05	**2**	**3.8E-11**	**4**	**1.2E-41**
5 *CS:DHR96*[1] ↑ (183)	0	>0.05	0	>0.05	**3**	**1.0E-04**	0	>0.05	**4**	**9.7E-04**	2	1.1E-02	4	>0.05	**2**	**6.6E-04**	**1**	**9.1E-04**	0	>0.05
6 *CS:DHR96*[1] ↓ (386)	3	>0.05	1	>0.05	1	>0.05	**4**	**3.0E-04**	2	>0.05	2	>0.05	**12**	**5.5E-05**	2	5.0E-02	**3**	**1.6E-12**	0	>0.05
7 *w:hsDHR96* ↑ (174)	0	>0.05	1	>0.05	2	1.4E-02	0	>0.05	1	>0.05	0	>0.05	3	>0.05	0	>0.05	0	>0.05	0	>0.05
8 *w:hsDHR96* ↓ (500)	**6**	**5.3E-05**	3	4.5E-02	2	>0.05	**7**	**3.0E-09**	**9**	**2.1E-05**	**7**	**9.7E-09**	12	2.5E-03	**4**	**8.7E-05**	**3**	**9.3E-10**	0	>0.05

Pairwise comparisons between microarray gene lists (in rows) and gene lists that represent different protein families as determined by InterPro domains (in columns). The number of overlapping genes is shown in each cell (n) along with the significance of this overlap as represented by a p value determined by a χ^2 test (bold indicates p value $< 10^{-3}$). The p values show the significance of the difference between the observed number of genes in the overlap and the number of genes that would be expected on average when two equally sized lists of randomly picked *Drosophila* genes are compared. Arrows indicate up- or downregulated gene sets. The number of genes in each data set is shown in parentheses. *CS*, *Canton S* control stock; PB, Phenobarbital; *w*, w^{1118} control stock; transp., transporter; UDP Gluc., UDP-glucuronosyl transferase; GST, glutathione S-transferase; DUF227, Domain of Unknown Function 227; JHBP, Juvenile Hormone Binding Protein; ML, MD-2-related Lipid-recognition.

SXR/PXR and CAR are represented by a single ortholog in *Drosophila*, DHR96 (NR1J1), and by three family members in *C. elegans*, DAF-12, NHR-8, and NHR-48 (Escriva et al., 2004). Genetic studies in *C. elegans* have shown that *nhr-8* mutants display reduced resistance to colchicine and chloroquine, suggesting a role in xenobiotic responses (Lindblom et al., 2001). Overall, however, little is known about the roles for nuclear receptors in invertebrate xenobiotic responses. We show here that treatment with phenobarbital results in a dramatic reprogramming of *Drosophila* gene expression, affecting genes that encode both phase I and phase II enzymes as well as novel xenobiotic response genes that are likely to facilitate drug detoxification and excretion. DHR96 is selectively expressed in the primary digestive, metabolic, and excretory organs of the animal, providing the capacity to respond to toxic compounds. Consistent with this expression pattern, a *DHR96* null mutant displays increased sensitivity to the sedative effects of phenobarbital and defects in the expression of many phenobarbital-regulated genes, defining a role for this gene in responses to a xenobiotic compound. These studies establish *Drosophila* as a genetic model system for characterizing the regulation of xenobiotic responses and provide new possible directions for the rational design of more effective pesticides.

Results

Phenobarbital treatment results in global changes in detoxification gene expression

The GABA agonist phenobarbital (PB) is one of the most widely studied xenobiotic drugs and provides a highly effective inducer of detoxification genes in organisms ranging from bacteria to humans (Waxman, 1999; Zelko and Negishi, 2000). PB appears to have a similar effect in *Drosophila*, leading to significant transcriptional induction of several cytochrome P450 genes (Brun et al., 1996; Danielson et al., 1997; Dombrowski et al., 1998; Dunkov et al., 1997; Maitra et al., 1996; Waxman, 1999). We thus selected this drug as a means of determining whether a xenobiotic compound is sufficient to direct a global reprogramming in insect detoxification gene expression. Staged wild-type *Canton S* (*CanS*) flies were treated for 10 hr with either sucrose alone or sucrose supplemented with 0.3% PB, a concentration known to induce *Drosophila* P450 gene transcription (Brun et al., 1996; Dunkov et al., 1997). RNA was extracted from these animals, labeled, and hybridized to Affymetrix *Drosophila* 2.0 microarrays. Raw data was analyzed using gcRMA (Wu et al., 2004), and significant gene expression changes were determined by SAM 2.0 (Tusher et al., 2001).

A total of 503 genes are upregulated and 484 genes are downregulated upon PB treatment, most of which encode enzymes (Table 1, lines 1 and 2). The overall effects on transcript levels are remarkable, with over 250 genes displaying from 3-fold to 700-fold changes in expression level. Members of the four classic detoxification gene families are significantly overrepresented in the upregulated gene set. These include 29 P450 genes (Table 1, line 1), with eight such genes among the 30 top PB-inducible genes, including *Cyp6a8* (Figure 1A), *Cyp6a21*, *Cyp12d1-p*, and *Cyp6a2*. Lower levels of PB-induced expression are seen for *Cyp6g1* and *Cyp12a4*. In addition, 35 other oxidoreductase genes are induced by PB, with a total of 64 upregulated genes in this class (Table 2, line 1). Sixteen GST genes (e.g., *GstD2*, Figure 1A), seven UGT genes, and seven carboxylesterase genes are also upregulated by PB.

In addition to the classic detoxification genes, several other gene sets are over-represented among the PB-regulated genes as revealed by protein families with common InterPro domains (Table 1) or searches for enriched gene ontology terms (Table 2). These include 13 genes characterized by a putative choline kinase domain known as DUF227, which is implicated in insecticide resistance in *Drosophila* (Aminetzach et al., 2005) (Table 1, rows 1 and 2). Members of this family are induced as well as repressed; for example, *CG6908* is induced ~26-fold while *CG10514* is downregulated ~31-fold by PB (Figure 1B). The *Turandot* stress-response genes and two acyl-CoA synthetase genes, *CG4500* (Figure 1A) and *CG6300/CG11659*, are also highly induced by PB. Acyl-CoA synthetases have been implicated in xenobiotic metabolism in mammals (Knights and Drogemuller, 2000). The only large group based on InterPro domains that is significantly downregulated includes 25 members of the serine peptidase S1 gene family (Table 1, line 2), although many other peptidase genes are also downregulated (Table 2, line 2).

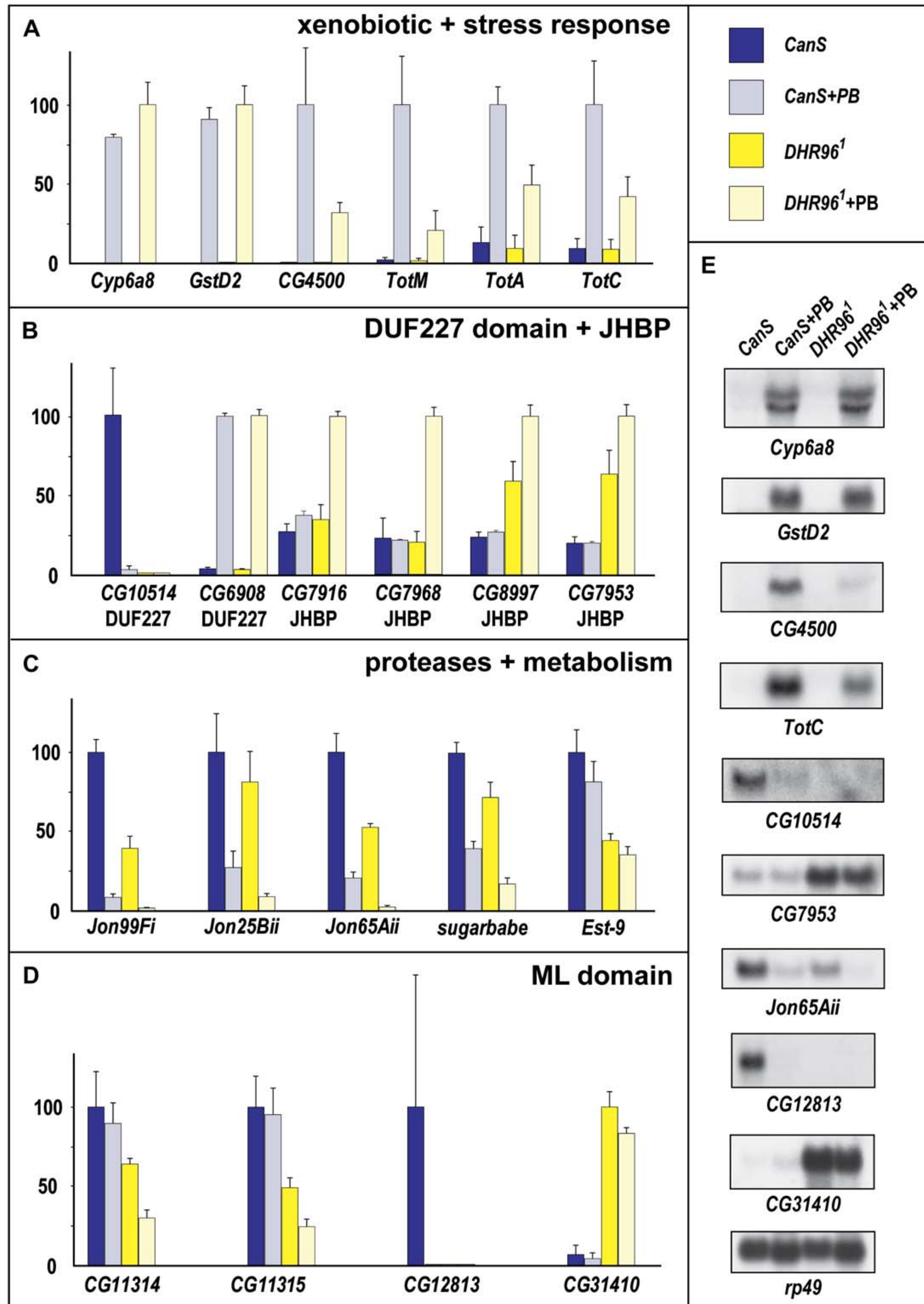

Figure 1. Expression profiles and microarray validation of genes that are significantly affected by PB treatment and/or the *DHR96*^{1} mutation

A–D) Data from three microarray replicates were averaged for each data point, with error bars representing the standard deviation. The highest expression level was set to 100% for each group. *CanS* controls, untreated (dark blue) or PB-treated (light blue), and *DHR96*^{1} mutants, untreated (dark yellow) or PB-treated (light yellow), are depicted. (**A**) Xenobiotic and stress-response genes. *Cyp6a8*, *GstD2*, and *CG4500* are the three most highly PB-induced genes in wild-type animals. The *TotM*, *TotA*, and *TotC Turandot* genes are stress-response genes that depend on *DHR96* for their maximal response to PB. (**B**) Genes that encode either DUF227 domain proteins or members of the Juvenile Hormone Binding Protein (JHBP) family. (**C**) Three genes encoding *Jonah* family proteases, the transcription factor gene *sugarbabe*, and the *Est-9* carboxylesterase gene. (**D**) Four of the eight ML domain (MD-2-related lipid recognition) encoding genes found in the fly genome are regulated by PB and *DHR96*.

E) Northern blot analysis of selected genes shown in panels (**A**)–(**D**). Blots were hybridized with *rp49* as a control for loading and transfer. Of 27 PB-regulated genes tested in this manner, all but two were validated on Northern blots.

Table 2. Occurrence of gene ontology terms in microarray results

	lipid metab. (249)		carbohydrate metab. (179)		amino acid metab. (98)		purine metab. (60)		cholesterol metab. (30)		Nucleus (1328)		proteolysis (726)		oxidoreductase (293)		ion transport (96)	
array results (n)	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value	n	p value
1 *CS*±PB ↑ (503)	**24**	**7.7E-12**	**36**	**1.0E-47**	**12**	**4.1E-09**	**11**	**5.5E-14**	**6**	**4.1E-09**	**5**	**-7.0E-08**	25	>0.05	**64**	**3.9E-93**	3	>0.05
2 *CS*±PB ↓ (484)	**26**	**3.3E-15**	10	1.1E-02	2	>0.05	0	>0.05	2	>0.05	22	-2.8E-02	**57**	**6.2E-20**	15	5.7E-03	7	3.5E-03
3 *CS*+PB:*DHR96¹* +PB ↑ (326)	7	>0.05	5	>0.05	4	>0.05	**5**	**9.0E-05**	0	>0.05	**47**	**1.8E-07**	17	>0.05	**14**	**5.9E-05**	2	>0.05
4 *CS*+PB:*DHR96¹* +PB ↓ (199)	7	6.6E-03	3	>0.05	1	>0.05	0	>0.05	2	2.7E-03	19	>0.05	16	2.2E-03	4	>0.05	3	4.8E-02
5 *CS:DHR96¹* ↑ (183)	3	>0.05	3	>0.05	0	>0.05	1	>0.05	0	>0.05	22	8.7E-03	14	7.7E-03	6	>0.05	0	>0.05
6 *CS:DHR96¹* ↓ (386)	7	>0.05	4	>0.05	1	>0.05	1	>0.05	0	>0.05	**48**	**3.3E-05**	23	3.1E-02	7	>0.05	3	>0.05
7 *w:hsDHR96* ↑ (174)	3	>0.05	1	>0.05	3	2.7E-02	1	>0.05	0	>0.05	15	>0.05	11	>0.05	5	>0.05	0	>0.05
8 *w:hsDHR96* ↓ (500)	**19**	**9.6E-07**	**16**	**1.6E-07**	7	5.8E-03	4	5.4E-02	3	1.3E-02	28	>0.05	**35**	**2.3E-04**	**20**	**8.2E-06**	**8**	**5.4E-04**

Pairwise comparisons between microarray gene lists (in rows) and gene lists that represent different protein families as determined by gene ontology terms (in columns). The number of overlapping genes is shown in each cell (n) along with the significance of this overlap as represented by a p value determined by a χ^2 test (bold indicates p value $<10^{-3}$). The p values show the significance of the difference between the observed number of genes in the overlap and the number of genes that would be expected on average when two equally sized lists of randomly picked *Drosophila* genes are compared. Arrows indicate up- or downregulated gene sets. The number of genes in each data set is shown in parentheses. *CS*, *Canton S* control stock; PB, Phenobarbital; *w*, w^{1118} control stock; metab., metabolism.

Searches for enriched gene ontology terms revealed overrepresentation of pathways associated with carbohydrate, amino acid, purine, and cholesterol metabolism among the upregulated genes and lipid metabolic pathways among both the up- and downregulated genes, indicating that toxin exposure alters the metabolic state of the animal (Table 2, lines 1 and 2). For example, 26 genes involved in fat breakdown, including genes encoding triacylglycerol lipases, hydrolases, and dehydrogenases, are coordinately downregulated (Table 2, line 2). Interestingly, the only group that is statistically underrepresented in the PB-regulated gene set are those genes encoding DNA and RNA binding proteins (represented by the term "nucleus" in Table 2, lines 1 and 2), suggesting that the genomic response to PB relies upon existent transcription factors rather than de novo synthesis of these regulatory proteins. Taken together, this study demonstrates that insects can mount a coordinated transcriptional response to a xenobiotic compound and identifies many new genes that are likely to play a role in detoxification pathways.

DHR96 is selectively expressed in tissues that monitor and metabolize xenobiotics

As a first step toward determining whether DHR96 might act in *Drosophila* xenobiotic responses, we determined its spatial pattern of expression. Organs were dissected from wandering third instar larvae and stained with affinity-purified antibodies. DHR96 protein is restricted to the nucleus of all expressing cells, consistent with its proposed function as a transcription factor (Figure 2). It is most abundantly expressed in four tissues: the fat body (Figure 2A), salivary glands (Figure 2C), gastric caeca of the midgut (Figure 2D), and Malpighian tubules (Figure 2E). Upon prolonged staining, DHR96 can also be detected in the proventriculus and anterior region of the midgut. No specific expression was detected in the imaginal discs, muscle, epidermis, brain, or trachea (data not shown). The gastric caeca represent the primary site for secretion of digestive enzymes and absorption of dietary components into the circulatory system. Nutrients are metabolized and stored in the fat body, the insect equivalent of the mammalian liver. Waste products, along with metabolized toxic compounds, are then transferred back into the circulatory system and absorbed by the Malpighian tubules, the principal osmoregulatory and excretory organ of the insect. The spatial pattern of DHR96 expression thus tracks the course of toxic compounds as they are absorbed through the gut epithelium (gastric caeca), metabolized (fat body), and eliminated from the body (Malpighian tubules). The function of DHR96 in the larval salivary glands is less clear. This organ opens onto the esophagus and thus could be exposed to toxic compounds that enter through the diet or it could provide enzymes for digestion.

Generation of *DHR96* mutations

"Ends-in" gene targeting was used to introduce specific mutations into the *DHR96* locus in an effort to determine if this gene

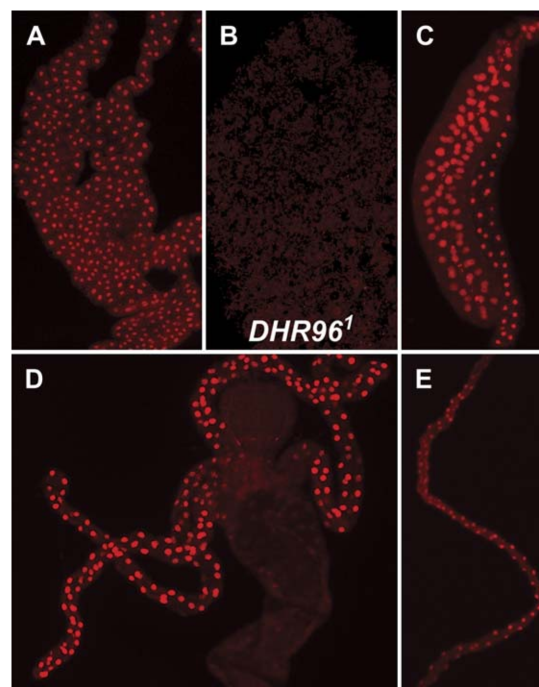

Figure 2. DHR96 protein is expressed in a restricted subset of tissues

Tissues were dissected from wild-type late third instar larvae and stained with affinity-purified antibodies directed against DHR96. The protein is restricted to the nucleus and detected in the fat body (**A**), salivary glands (**C**), gastric caeca of the midgut (**D**), and the Malpighian tubules (**E**). No protein is detected in the *DHR96¹* mutant, as depicted for the fat body (**B**).

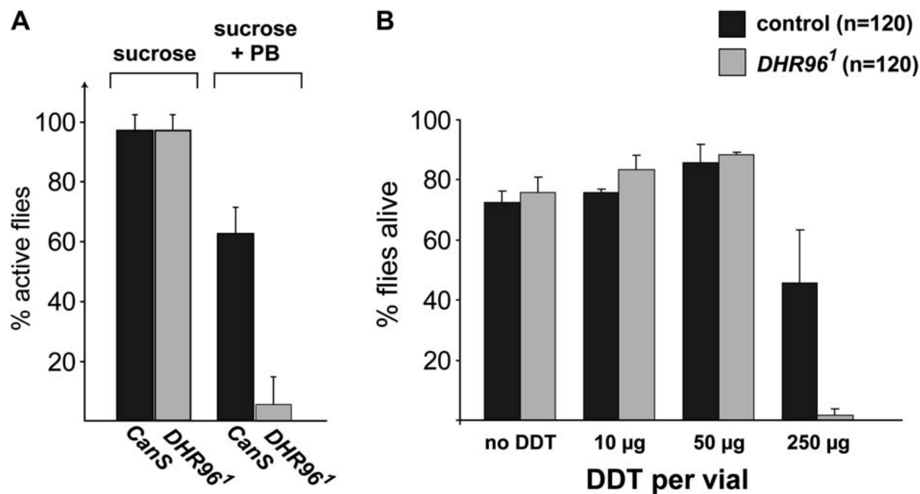

Figure 3. *DHR96* mutants are sensitive to phenobarbital and DDT

A) Staged young adult flies were fed either sucrose alone or sucrose and 1% phenobarbital (PB) for 18 hr. A negative geotaxis assay was used to quantitate activity, showing the percentage of the total population that remained active after treatment. Each bar represents the average from three repeats on each of three vials (a total of nine samples), with error bars depicting the standard deviation.

B) Young adult flies were maintained on medium containing either 10, 50, or 250 μg DDT per vial. The percentage of surviving flies was determined after three weeks. Each bar depicts the average from four vials with 30 flies each, with the error bars showing standard deviation.

might function in xenobiotic response pathways (Rong and Golic, 2000). Two deletions were introduced into the donor *DHR96* sequences, one of which removes the translational start codon and the second of which removes exon four, the downstream intron, and the splice acceptor site for exon 5, disrupting the ligand binding domain-coding region (Figure S2A in the Supplemental Data available with this article online.).

A screen for specific targeted mutations resulted in the isolation of *DHR96^1*, which carries a GFP reporter gene between two nonfunctional copies of *DHR96* (Figure S2A). Southern blot analysis of DNA isolated from homozygous *DHR96^1* mutants revealed a restriction pattern that is consistent with a duplication event (Figures S2A and S2B). In addition, DNA sequencing of PCR-amplified fragments revealed that both mutations are present in the inserted sequences in *DHR96^1*, with the second deletion also present in the 3′ duplicated copy of the gene (Figure S2A). Similar unpredicted sequence changes have been reported for *Drosophila* ends-in gene targeting (Rong et al., 2002). Western blot analysis using affinity-purified antibodies directed against DHR96 demonstrated that the protein is undetectable in mutant animals (Figure S2C). Similar results were seen in antibody stains of tissues dissected from *DHR96^1* mutant larvae (Figure 2B). Taken together, the molecular defects in the mutant and the antibody studies demonstrate that *DHR96^1* is a strong loss-of-function allele and most likely a null mutation.

To minimize the effect of genetic background on xenobiotic sensitivity (Miyo et al., 2001, 2003; Pyke et al., 2004), we outcrossed the *DHR96^1* mutation to wild-type *CanS* flies through nine generations of free recombination. *CanS* is widely used as a control for pesticide resistance studies (Bogwitz et al., 2005; Brandt et al., 2002; Daborn et al., 2001; Pedra et al., 2004). All subsequent studies were conducted using the outcrossed *DHR96^1* mutant in combination with a *CanS* control.

DHR96 mutants are sensitive to phenobarbital and DDT

The *DHR96^1* mutant is viable and fertile when raised under standard conditions, similar to *PXR*, *CAR*, and *nhr-8* mutants (Lindblom et al., 2001; Wei et al., 2000; Xie et al., 2000). *DHR96^1* mutants, however, display increased sensitivity to PB. Staged *CanS* and *DHR96^1* mutant flies were fed either sucrose alone or sucrose and 0.1%–10% PB for 6 to 24 hr. No significant effect was seen in mutant or wild-type animals treated with 0.1% PB, but both mutant and wild-type displayed reduced activity and uncoordination at higher PB concentrations. *DHR96^1* mutants, however, were markedly less active than their wild-type counterparts, as quantified by a negative geotaxis assay (Figure 3A). *DHR96* is thus required for proper resistance to the sedative effects of PB.

We also tested sensitivity to the pesticide DDT as a model insect xenobiotic. Although acute exposure to DDT did not reveal a significant difference in the sensitivity of control and *DHR96* mutant animals, chronic exposure to lower concentrations of pesticide resulted in a reproducibly lower survival rate for mutant animals relative to the controls. This is depicted for a range of DDT concentrations in Figure 3B, where only a few *DHR96^1* mutant flies survive chronic exposure to a higher concentration of DDT relative to the survival of about half of the *CanS* controls.

Xenobiotic gene transcription is affected in *DHR96* mutants

Studies of PXR and CAR mutant mice focus primarily on the transcription of specific P450 target genes, with whole-animal experiments largely involving hepatic damage and sensitivity to paralytic sedatives (Wei et al., 2000; Xie et al., 2000; Zhang et al., 2004). We thus chose to restrict our initial studies to characterizing possible roles for *DHR96* in the regulation of genes affected by PB, attempting to link the PB sensitivity of *DHR96^1* mutants with changes in the expression of specific detoxification genes. For this purpose, microarray analysis was used to compare the transcriptional responses of PB-treated *CanS* wild-type flies with PB-treated *DHR96^1* mutants. A total of 525 genes displayed significantly different levels of expression between these data sets (Tables 1 and 2, lines 3 and 4). Of these differentially expressed genes, 102 genes are present in the list of 987 genes regulated by PB in wild-type animals (p value = 2.9×10^{-49}, Figure 4A). Among these 102 genes are 21 genes that are PB-induced but which require *DHR96* for their maximal response to the drug. These include four members of the *Turandot* (*Tot*) gene family (Table 1, line 4). *TotM* and *TotX* are ~5-fold lower in *DHR96* mutants relative to controls, whereas *TotC* and *TotA* reach ~50% of wild-type induction levels (Figure 1A). This list also includes the *CG4500* acyl-CoA synthetase gene (Figure 1A), which is the third most highly PB-induced gene and

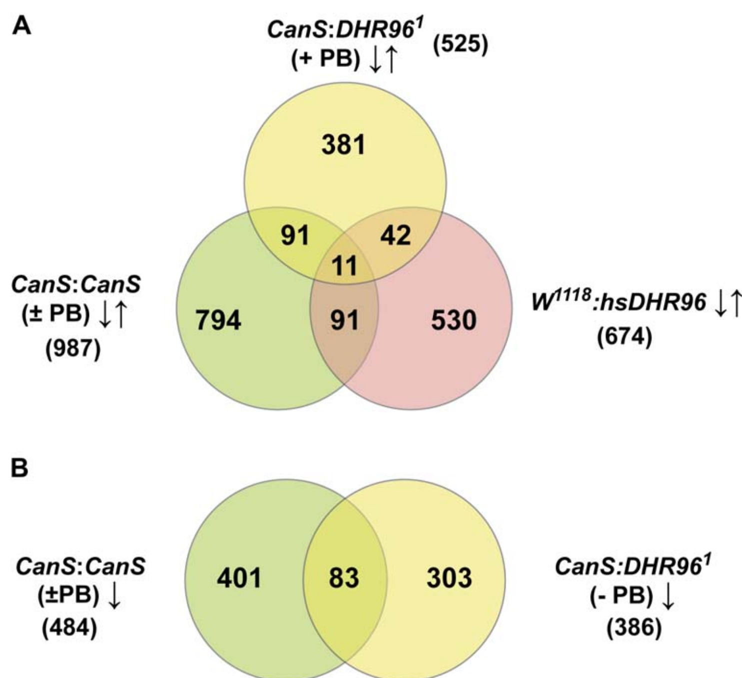

Figure 4. Many PB-regulated genes depend on *DHR96* for their proper expression

A) Comparison of genes that change their expression in wild-type flies treated with PB (*CanS:CanS* ±PB) with genes that are differentially expressed between PB-treated wild-type controls and $DHR96^1$ mutants (*CanS*:$DHR96^1$ +PB), or genes that are differentially expressed between w^{1118} controls and heat-induced *hsDHR96* transformants (w^{1118}:*hsDHR96*). Arrows indicate up- and downregulation.

B) Comparison of genes that are downregulated by PB in wild-type flies (*CanS:CanS* ±PB) and genes that are downregulated in untreated $DHR96^1$ mutants (*CanS*:$DHR96^1$ –PB).

which is likely to contribute to xenobiotic responses (Knights and Drogemuller, 2000).

A group of 20 genes, including the *Jonah* protease genes and *sugarbabe* (Figure 1C), are repressed by PB but are further downregulated in *DHR96* mutants, suggesting that this class of drug-responsive genes requires *DHR96* for its normal expression. The remaining genes either fail to be properly repressed (26 genes) or are superinduced (35 genes) in PB-treated mutants. Interestingly, the latter group harbors numerous potential xenobiotic genes, including the *Cyp309a2*, *Cyp4s3*, *Cyp6a14* P450 genes, two additional oxidoreductase genes (*CG9509* and *CG3597*), *Ugt86Dd*, *GstD7*, and the *CG6300/CG11659* acyl-CoA synthetase genes. Taken together, these findings support the proposal that *DHR96* contributes to xenobiotic transcriptional responses.

We also identified genes that are normally unresponsive to PB treatment but can be induced by PB in $DHR96^1$ mutants. These include four genes that encode so-called Juvenile Hormone Binding Proteins (JHBPs): *CG7916*, *CG7968*, *CG8997*, and *CG7953* (Table 1, lines 3 and 4) (Figure 1B). This finding indicates that *DHR96* is required for the repression of some genes under normal conditions and may also inhibit their PB-responsiveness.

Untreated *DHR96* mutants reveal links to xenobiotic responses

We next asked whether differential gene expression patterns between untreated *CanS* controls and $DHR96^1$ mutants revealed regulatory pathways that require *DHR96* under normal conditions. Interestingly, this comparison identified a number of genes encoding likely xenobiotic enzymes (Table 1, lines 5 and 6). These include moderate upregulation of three P450 genes, *Cyp18a1*, *Cyp9h1*, and *Cyp4s3*, and reduced expression of *Cyp4ac1* and *Cyp4e3*, the latter being strongly induced by PB in wild-type animals. The array data also indicated reduced expression of four carboxylesterase genes, *Nrt*, *Ace*, *Est-8*, and *Est-9*, in $DHR96^1$ mutants (*Est-9* depicted in Figure 1C), which are not strongly affected by PB but may play a role in the detoxification of other compounds (Menozzi et al., 2004).

Half of the eight genes in the fly genome that encode ML domain proteins are also significantly affected in both untreated and PB-treated *DHR96* mutants (Table 1, lines 3–6). The ML domain (MD-2-related lipid-recognition, also known as Def2/Der2) is a widespread protein motif, found in plants, fungi, and animals, that has been implicated in binding to specific lipids (Inohara and Nunez, 2002). The most strikingly affected ML domain genes in *DHR96* mutants are *CG12813* and *CG31410*. *CG12813* mRNA is virtually absent in $DHR96^1$ flies, resulting in expression that is >100-fold below wild-type (Figure 1D). In contrast, *CG31410* expression levels are ~20-fold higher in mutant animals than in controls, representing the most strongly derepressed gene in $DHR96^1$ flies (Figure 1D). A second gene pair encoding ML domain proteins, *CG11314* and *CG11315*, display almost identical patterns in mutant animals, as both are submaximally expressed and display a moderate downregulation when PB is added to the diet (Figure 1D).

We also identified a highly significant overlap of 83 genes that are downregulated in both untreated $DHR96^1$ mutants and PB-treated wild-type flies (Figure 4B). Interestingly, these genes display similar fold changes in expression levels, suggesting that a loss of *DHR96* function in untreated flies is functionally equivalent to PB-dependent downregulation (Table S1). For example, the DUF227 and ML domain-encoding genes *CG10514* and *CG12813* are significantly downregulated by PB in wild-type animals but are expressed at an equally low level in untreated $DHR96^1$ mutants (Figures 1B and 1D). In addition, we find that this set harbors 11 peptidase-encoding genes, including three *Jonah* protease genes (*Jon99Fi*, *Jon25Bii*, and *Jon65Aii*), that are significantly reduced in $DHR96^1$ mutants (Figure 1C and Table 1, line 1). This set of 83 genes also contains a significant cluster of 13 DNA/RNA binding proteins, demonstrating that more than half of the 22 PB-repressed genes associated with the

ontology term "nucleus" (Table 2, line 2) are identical to the 48 "nuclear" genes that are submaximally expressed in *DHR96* mutants (Table 2, line 6). This observation suggests that the detoxification pathway and the *DHR96* regulatory network converge on a common set of regulatory factors.

Ectopic *DHR96* expression represses genes associated with xenobiotic and metabolic pathways

As a final step in our analysis of *DHR96* function, we determined the effects of ectopic *DHR96* expression on the patterns of genome-wide transcription and compared these effects with those seen in *DHR96*[1] loss-of-function mutants as well as our list of PB-regulated genes. Staged transgenic animals that carry a heat-inducible promoter fused to the *DHR96* gene (*hsDHR96*) and control wild-type animals of the same genetic background (*w*[1118]) were subjected to a brief heat treatment and allowed to recover for 4 hr. RNA was extracted from these animals and analyzed on Affymetrix microarrays, essentially as described above. Comparison of expression levels in these data sets revealed 500 genes that are downregulated upon ectopic *DHR96* expression and 174 genes that are upregulated (Tables 1 and 2, lines 7 and 8). Among the 500 repressed genes are six genes encoding ABC transporters, including the *Mdr49* multidrug resistance family member, three UGTs, two GSTs, seven carboxylesterases, nine P450s, seven DUF227 domain proteins, 12 S1 peptidases, four JHBPs, and three ML domain proteins (Table 1, line 8). The observation that these same gene families are regulated by PB (Table 1, lines 1 and 2) supports the proposal that ectopic *DHR96* expression affects at least part of the xenobiotic response pathway. Additional evidence for this arises from a comparison of the genes that change their expression upon ectopic *DHR96* expression with the genes regulated by PB in wild-type animals. This study reveals a significant overlap of 102 genes (p value = 1.3×10^{-31}, Figure 4A). These genes include four P450 genes, all of which are induced by PB treatment and downregulated upon ectopic *DHR96* expression, along with two carboxylesterase genes, one GST gene, two genes harboring a DUF227 domain, one ML domain gene, and four genes involved in lipid metabolism. Taken together with the transcriptional profiles of the *DHR96* loss-of-function mutant, this study indicates that this receptor participates in the control of PB-regulated genes. In addition, analysis of the *hsDHR96* gain-of-function dataset using gene ontology terms revealed that the downregulated genes display a significant enrichment for terms that include lipid and carbohydrate metabolism, proteolysis, oxidoreductases, and ion transporters (Table 2, line 8), suggesting that *DHR96* contributes to metabolic gene regulation.

Overlaps between xenobiotic and stress-response pathways

The coordinate regulation of the *Turandot* stress-response genes under most conditions tested prompted us to examine the possibility that PB-treatment, and/or *DHR96*, contribute to stress-response pathways. To do this, we compared our PB-regulated microarray data sets with the stress-response microarray study of Girardot et al. (2004), identifying 69 genes that are upregulated and 78 genes that are downregulated under both conditions (Table 3, lines 1 and 2). Of the 25 top PB-induced genes, only two are identified as general stress-response genes, *GstD2* and *Cyp309a1*, while the next 25 PB-induced genes harbor 11 such stress-inducible genes, including *DptB*, *GstD5*,

Table 3. *DHR96* and Phenobarbital microarray data compared to stress responses

		stress ↑ (222)		stress ↓ (211)	
array results (n)		n	p value	n	p value
1	*CS*±PB ↑ (503)	**69**	**1.5E-140**	6	>0.05
2	*CS*±PB ↓ (484)	2	>0.05	**78**	**1.9E-187**
3	*CS*+PB:*DHR96*[1] + PB ↑ (326)	11	5.8E-04	**16**	**2.6E-08**
4	*CS*+PB:*DHR96*[1] + PB ↓ (199)	3	>0.05	9	6.4E-05
5	*CS:DHR96*[1] ↑ (183)	8	3.7E-04	**17**	**6.1E-19**
6	*CS:DHR96*[1] ↓ (386)	5	>0.05	**18**	**2.3E-09**
7	*w:hsDHR96* ↑ (174)	**18**	**6.2E-32**	4	>0.05
8	*w:hsDHR96* ↓ (500)	5	>0.05	**21**	**3.5E-08**

The stress microarray data from Girardot et al. (2004) was condensed to sets of 222 upregulated genes and 211 downregulated genes (see Experimental Procedures) and compared to our microarray data sets. The number of overlapping genes is shown in each cell (n) along with the significance of this overlap as represented by a p value determined by a χ^2 test (bold indicates p value < 10^{-6}). The p values show the significance of the difference between the observed number of genes in the overlap and the number of genes that would be expected on average when two equally sized lists of randomly picked *Drosophila* genes are compared. Arrows indicate up- or downregulated gene sets. The number of genes in each data set is shown in parentheses. *CS*, *Canton S* control stock; PB, Phenobarbital; *w*, *w*[1118] control stock.

and *GstE6*. If one includes the four *Turandot* genes, roughly one third of the top 50 PB-induced genes represent a general stress response.

When the *DHR96*[1] mutant data is compared to the stress array data, we find that *DHR96* mutants, whether treated or not treated with PB, display significant misregulation of genes that are normally downregulated in response to stress, while a moderate but significant number of stress upregulated genes have elevated expression levels in *DHR96* mutants (Table 3, lines 3–6). Comparison of *DHR96* gain-of-function gene sets with the stress microarray data further indicates that these animals execute a partial stress response (Table 3, lines 7 and 8).

Discussion

The studies described here represent a first step toward using *Drosophila* to define the coordinate transcriptional regulation of defensive responses to toxic compounds. We show that insect xenobiotic responses are not limited to the adaptive effects of insecticide resistance but also include a highly inducible transcriptional response of key detoxification enzymes, analogous to that found in mammalian systems. We also provide evidence that the *Drosophila* ortholog of the mammalian SXR/PXR and CAR nuclear receptors, DHR96, plays a role in insect xenobiotic responses, providing resistance to the sedative PB and contributing to the proper regulation of detoxification genes. Below, we expand upon our studies of PB-regulated transcription and *DHR96* functions, comparing and contrasting our work with studies in vertebrates and in other insects.

Drosophila execute a massive transcriptional response to a xenobiotic compound

Although two studies have used genome-wide microarrays to define the transcriptional profile of insecticide-resistant strains of *Drosophila* (Pedra et al., 2004) and the mosquito *Anopheles gambiae* (Vontas et al., 2005), none have characterized the inducible transcriptional response to a toxic compound in wild-type insects. The data presented here, examining the effects of PB on

transcription in wild-type flies, provide insights into how insects mount a defense response to toxic compounds. Approximately 1000 transcripts are affected by PB treatment, with many showing high-fold changes in expression. The vast majority of these genes encode enzymes, and many of these correspond to known detoxification pathways, including multiple P450s, GSTs, carboxylesterases, and UGTs (Table 1, lines 1 and 2). Although relatively few genes have been linked to specific xenobiotic functions in insects, the second most highly PB-induced gene (677-fold), *Cyp6a8*, can metabolize organophosphates, cyclodiene insecticides (Dunkov et al., 1997), and promutagens (Saner et al., 1996). Similarly, the PB-inducible *Cyp6g1* and *Cyp12a4* genes are sufficient to confer resistance to the pesticide Lufenuron (Bogwitz et al., 2005; Daborn et al., 2002). The overall types of enzymes regulated by PB in *Drosophila* resemble those seen in insecticide-resistant strains of *Anopheles* and *Drosophila* (Pedra et al., 2004; Vontas et al., 2005), as well as the enzymes affected by xenobiotics in mammalian studies (Kume et al., 2005; Maglich et al., 2002; Ueda et al., 2002), providing further evidence that the core detoxification machinery has been conserved through evolution, from insects to humans.

In addition to the main classes of detoxification genes, several gene families emerge from searches for overrepresented InterPro or gene ontology terms. These include 13 PB-inducible genes that encode proteins with a DUF227 domain (Table 1, line 1). This apparently insect-specific domain is proposed to act as a choline/ethanolamine kinase (InterPro IPR004119). A recent study reported that a specific mutation in a DUF227 domain-encoding gene (*CG10618*) confers resistance to organophosphates in *Drosophila* (Aminetzach et al., 2005). Two acyl-CoA synthetase genes are also among the ten most abundantly induced PB-responsive genes (*CG4500* and *CG6300/CG11659*). Acyl-CoA synthetases have been implicated in xenobiotic metabolism in mammals (Knights and Drogemuller, 2000), and an acyl-CoA synthetase is upregulated in an insecticide-resistant strain of *Anopheles* (Vontas et al., 2005).

Remarkably, many PB-regulated genes not associated with overrepresented gene families may also contribute to general detoxification responses. For example, *Jheh1*, which is induced 16-fold by PB, encodes an epoxide hydrolase. These enzymes can detoxify epoxides by increasing their solubility and aiding in their excretion (Lu and Miwa, 1980). Similarly, the glycine N-methyltransferase encoded by *CG6188*, which is induced 77-fold by PB, can bind polyaromatic hydrocarbons and contribute to mammalian *Cyp1A1* induction (Bhat and Bresnick, 1997). Thus, the genome-wide transcriptional response to PB has revealed not only new members of the known classes of detoxification enzymes but also a number of other pathways that are likely to contribute to xenobiotic responses, providing a new basis for understanding how insects defend themselves against environmental toxins. Our recent studies have also shown that many of these genes are regulated in an identical manner by a different drug, the dopamine antagonist chlorpromazine, suggesting that this transcriptional pattern reflects a general defense response to xenobiotics (M.H., unpublished data).

DHR96 is selectively expressed in tissues that function in xenobiotic detoxification

DHR96 protein is ideally positioned to monitor the entry and exit of dietary nutrients and foreign compounds, coordinating xenobiotic stress and metabolic responses within the animal. DHR96 displays a highly restricted pattern of expression, limited primarily to organs that are involved in nutrient and xenobiotic absorption (gastric caeca), metabolism (fat body), and waste elimination (Malpighian tubules) (Figure 2).This pattern is similar to that of PXR and CAR, which are most highly expressed in the liver and intestine. This expression pattern also reflects that of the two PB-inducible P450 transcripts that have been spatially localized. *Cyp6a2* is expressed in the midgut, fat bodies, and Malpighian tubules of adult flies while *Cyp12a4* is expressed primarily in the midgut and Malpighian tubules of third instar larvae (Bogwitz et al., 2005; Brun et al., 1996). Unlike its mammalian orthologs, however, which reside in the cytoplasm and translocate to the nucleus upon xenobiotic challenge (Kobayashi et al., 2003; Squires et al., 2004), DHR96 protein appears to be restricted to the nucleus.

DHR96 is required for proper responses to a xenobiotic compound

DHR96 mutants display a significant increase in their sensitivity to the sedative effects of PB (Figure 3A). This observation is similar to the prolonged sleep phenotype of PB-treated CAR mutant mice (Swales and Negishi, 2004) and the effects of PB treatment on resistance to the paralytic effects of the muscle relaxant zoxazolamine in CAR mutants (Wei et al., 2000). Of 144 genes that change their expression upon intraperitoneal injection of PB in mice, about half are dependent upon CAR for their proper response to the drug (Ueda et al., 2002). This is similar to the effect we see in *Drosophila*, where 102 PB-regulated genes are affected by either the *DHR96*^{1} loss-of-function mutation or ectopic *DHR96* gain-of-function (Figure 4A). In addition, many of these genes encode members of the four classic detoxification enzyme families, demonstrating that the insect xenobiotic response depends upon *DHR96* for its proper implementation. Similar effects on xenobiotic gene regulation were seen in PXR-VP16 gain-of-function studies and with *PXR* mutant mice (Maglich et al., 2002; Rosenfeld et al., 2003).

Interestingly, some genes are only PB-inducible in a *DHR96* mutant background. The most strongly affected genes in this group are members of the Juvenile Hormone Binding Protein family (JHBPs, Figure 1B). JHBPs are hemolymph carrier proteins that are capable of binding lipophilic compounds such as juvenile hormone, facilitating their transport within the animal and protecting them against nonspecific esterases (Touhara and Prestwich, 1993). Whereas JHBP genes are not responsive to PB in wild-type animals, they are induced by PB in *DHR96* mutants, indicating that *DHR96* normally acts to block their expression. This could reflect a protective function for *DHR96* in that JHBPs may normally protect lipophilic compounds from degradation, thus interfering with drug clearance. Several other genes are also induced by PB only in a *DHR96* mutant background, including *Glutathione Synthetase* and the putative transcription factor *CG14965*. It remains unclear, however, how these genes might contribute to the detoxification response. Remarkably, a similar effect has been reported for CAR function in mice, where a subset of genes is only responsive to PB in a CAR mutant background (Ueda et al., 2002). These genes include two P450 genes and genes encoding a calcium binding protein and glucosamine phosphate N-acetyl transferase. This so-called CAR-dependent blocking of gene expression may reflect an evolutionarily conserved aspect of the xenobiotic response pathway.

Untreated *DHR96* mutants display significant changes in detoxification gene expression

Further evidence of a role for *DHR96* in xenobiotic response pathways arises from the finding that many potential detoxifying enzymes change their expression in untreated *DHR96* mutants (Table 1, lines 5 and 6). Among this set, we found 83 genes that are downregulated in both untreated *DHR96* mutants and PB-treated wild-type flies, including genes that encode transcription factors and numerous proteases and peptidases (Table S1). Curiously, the relative fold changes of these genes are almost identical, in spite of their responding to two very different conditions, suggesting that untreated *DHR96* mutants display some aspects of a toxin response (data not shown). There are two possible models to explain this observation. First, *DHR96* may regulate a specific branch of the xenobiotic network and the loss of *DHR96* may result in the accumulation of toxic metabolites (endobiotics) that activate *DHR96*-independent xenobiotic pathways. Although at first glance, this ''endobiotic model'' is attractive, we note that relatively few PB-inducible genes are upregulated in untreated *DHR96* mutants (18 genes, Table S1). Thus, the mutants do not appear to display a xenobiotic response, but rather misregulate a set of genes that are normally repressed by PB treatment. As a result, we favor an alternative model which proposes that *DHR96* is required for normal expression of a subset of genes involved in the xenobiotic response pathway (exemplified by the 83 genes described here). PB then acts to inhibit this function of *DHR96*, resulting in the reduced expression of these *DHR96* target genes. In addition, as discussed below, other xenobiotic receptors are likely to provide input to this pathway.

PB and DHR96 regulate stress-response and metabolic pathways

Given that treatment with a toxic compound is likely to impose stress on the animal, it is not surprising that we observe a strong correlation between genes up- and downregulated by PB and genes regulated in the same manner by stress (Table 3). Our studies with loss-of-function and gain-of-function *DHR96* mutations also indicate a role for this receptor in regulating stress responses (Tables 1 and 2). Stress-response genes are upregulated in insecticide-resistant strains of *Anopheles* and some insecticide-resistant strains of insects are more resistant to oxidative stress, providing a functional link between these two pathways (Abdollahi et al., 2004; Vontas et al., 2005; Vontas et al., 2001). Our results suggest that this link is conferred at the level of specific stress-response gene regulation. In addition, PXR has been recently implicated in mediating oxidative stress responses, suggesting that at least some aspects of this regulation have been conserved through evolution (Gong et al., 2006).

PB treatment is also associated with a significant upregulation of genes involved in energy and sugar metabolism. The first two steps in gluconeogenesis are catalyzed by pyruvate carboxylase (*CG1516*) and phosphoenolpyruvate carboxykinase (*PEPCK*), enzymes encoded by genes that are upregulated by PB. Other glucose-generating processes show a similar response to PB, including six α-amylase genes that are induced more than 3-fold by the drug. Amylases are secreted by the salivary glands and midgut epithelia to break down dietary starch and glycogen into dextrins. Two genes encoding glucosidases, which can further degrade dextrins into monosaccharides, are also upregulated upon PB treatment. This effect is the opposite of that seen in mammals, where gluconeogenesis is downregulated by PB (Kodama et al., 2004). It makes sense, however, that this pathway would be upregulated as part of the detoxification response. As noted by Reichert and Menzel (2005), toxin metabolism is energetically costly. P450s consume NADPH or NADH for their oxidation of xenobiotics, while UGTs consume glucose and GSTs consume glutathione. Thus, part of the metabolic response to xenobiotics appears to provide the appropriate energetic requirements for detoxification.

Future directions

Although our loss-of-function and gain-of-function genetic data indicate a role for *DHR96* in mediating xenobiotic responses, it is interesting to note that the majority of PB-regulated genes are unaffected by *DHR96* mutations. This is similar to studies in mice, where at least half of the detoxification gene network is unaffected by *PXR* or *CAR* null mutations (Maglich et al., 2002; Ueda et al., 2002). Given the massive coordinate regulation of the PB response, it seems likely that one or more additional transcriptional regulators feed into this pathway. These could include PAS-bHLH family members, analogous to the role of the mammalian aryl hydrocarbon receptor in regulating xenobiotic responses (Rowlands and Gustafsson, 1997). Functional studies of PB-regulated promoters should provide insights into how their activity is controlled by the drug and identify additional players in their regulation. Similarly, the identification and characterization of direct targets for DHR96 transcriptional control will allow us to define how this receptor exerts its regulatory functions. Finally, DHR96 provides a potential target for the rational design of novel pesticides. By developing compounds that alter DHR96 activity it may be possible to increase the effectiveness of pesticide treatment for insect population control. Taken together, the studies described here provide a basis for using *Drosophila* as a genetic model for dissecting the regulation of xenobiotic responses, with implications for better understanding how these pathways are controlled in all higher organisms.

Experimental procedures

Antibody studies

Histidine-tagged DHR96 protein was purified from pET24c-DHR96 bacteria, resolved by preparative SDS-PAGE, excised from the gel, injected into three rabbits (Covance), and antisera were screened by Western blotting. Antiserum was affinity-purified as described (Carroll and Laughon, 1987), using protein from pMAL-DHR96 bacteria. Wandering third instar larval tissues were dissected and fixed as described (Boyd et al., 1991). DHR96 protein was detected using affinity-purified DHR96 antibodies diluted 1:100 and incubated overnight at 4ºC. Donkey anti-rabbit CY3 secondary antibodies (Jackson) were used at a 1:200 dilution. The stains were visualized on a Biorad confocal laser scanning microscope. For Western blots, protein from adult flies was extracted in SDS sample buffer. The equivalent of approximately one adult fly was loaded in each lane of an 8% polyacrylamide gel, separated by electrophoresis, and transferred to a PVDF membrane. *hsDHR96* transformants were treated at 37.5ºC for 30 min followed by a 3 hr recovery at room temperature. DHR96 protein was detected by incubating the membrane with a 1:500 dilution of affinity-purified anti-DHR96 antibodies, followed by a 1:1000 dilution of goat anti-rabbit HRP secondary antibody (Pierce). A supersignal chemiluminescence kit was used to detect the antibody (Pierce).

Negative geotaxis assays and DDT sensitivity

Five-day-old adult flies were raised on standard cornmeal/agar food and starved overnight under humid conditions at 25ºC before treatment with

phenobarbital (PB). Treatment was conducted in plastic vials that contained a strip of Whatman filter paper soaked with 500 μl of either 5% sucrose or 5% sucrose and 1% PB. Eight adult flies were placed in each vial. Activity was scored 18 hr later at room temperature. To test for activity, the flies were banged to the bottom of the vial and, after 30 s, the number of flies that climbed 6.5 cm from the bottom was determined. Each experiment consisted of three trials on each vial, with three vials tested for each condition, for a total of nine data points. DDT resistance was determined by transferring newly eclosed flies to medium that was supplemented with either 10, 50, or 250 μg DDT (Sigma) per vial. For each vial, 3 g of instant medium (Carolina 4-24) was mixed with 10 ml of deionized water that contained the appropriate DDT amounts. Flies were transferred to fresh medium once a week, and scored after three weeks.

Microarray analysis

Total RNA was isolated from staged animals using phenol/chloroform extraction or Trizol (Gibco) and purified on RNAeasy columns (Qiagen). One- to two-day-old adult flies were raised on standard cornmeal/agar food and starved overnight under humid conditions at 25ºC before treatment with either 5% sucrose or 5% sucrose and 0.3% PB for 10 hr. This is a nonlethal concentration of PB that is sufficient to direct significant upregulation of *Cyp6a2* and *Cyp6a8* transcription. For *DHR96* overexpression, third instar larvae staged at 4 hr after the second-to-third instar larval molt were treated at 37ºC for 1 hr and allowed to recover at 25ºC for 4 hr. All samples were prepared in triplicate to allow subsequent statistical analysis. Probe labeling, hybridization to Affymetrix GeneChip® *Drosophila* 2.0 Genome Arrays, and scanning were performed by the University of Maryland Biotechnology Institute Microarray Core Facility. Background/signal correction, normalization, and calculation of probe set expression values was determined by gcRMA (Wu et al., 2004). Calculation of fold changes and t-statistics was performed by SAM 2.0 (Tusher et al., 2001). Data sets were ranked by significance and the top 3% upregulated and downregulated genes (563 genes each) that also displayed fold changes ≥ 1.3 were used for further analysis. Microarray datasets were compared using Microsoft Access. Gene ontology terms are based on Affymetrix annotation files (June 2005). To compare our data to the stress microarray study of Girardot et al. (2004), we created a condensed stress data set that included all stress-responsive genes independent of treatment (the authors used five different stress conditions). For this, we calculated the geometric mean of the fold changes and filtered for gene expression changes that were on average >1.5, obtaining 222 upregulated and 211 downregulated genes.

Microarray data from this study can be accessed at NCBI GEO, with the following accession numbers: GSE5096 for the PB-treated studies and GSE5097 for the *hsDHR96* gain-of-function studies. Excel spreadsheets for the array data described in this paper can be accessed at the Thummel lab website: http://thummel.genetics.utah.edu/.

Supplemental data

Supplemental data include Supplemental Experimental Procedures, Supplemental References, two figures, and two tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/1/37/DC1/.

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Accession numbers

Microarray data from this study can be accessed at NCBI GEO, with the following accession numbers: GSE5096 for the PB-treated studies and GSE5097 for the *hsDHR96* gain-of-function studies.