UNDERSTANDING CYP6D1 TRANSCRIPTION: IMPLICATIONS FOR XENOBIOTIC INDUCTION AND INSECTICIDE RESISTANCE

by George Guan-Hua Lin

UNDERSTANDING *CYP6D1* TRANSCRIPTION: IMPLICATIONS FOR XENOBIOTIC INDUCTION AND INSECTCIDE RESISTANCE

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UNDERSTANDING *CYP6D1* TRANSCRIPTION: IMPLICATIONS FOR XENOBIOTIC INDUCTION AND INSECTICIDE RESISTANCE

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House fly cytochrome P450 CYP6D1 carries out the metabolism of xenobiotics. Expression of house fly *CYP6D1* is induced in response to the prototypical P450 inducer, phenobarbital (PB), in the insecticide susceptible strains, CS and *aabys*. In the permethrin resistant LPR strain, increased transcription of *CYP6D1* confers the metabolism-mediated resistance. *CYP6D1* is constitutively overexpressed without significant PB induction in LPR. A series of experiments were conducted to understand the transcriptional regulation of *CYP6D1*. The core promoter of *CYP6D1* is a dispersed type, as two transcription start sites were identified. Assays of the *CYP6D1v2* promoter from the CS strain in *Drosophila* S2 cells identified promoter regions critical for basal transcription and for PB induction. Using RNAi treatment of *Drosophila* S2 cells, HR96 (*hormone receptor-like in 96*) and BR-C (*broad-complex*) were identified to be transcription factors critical for PB induction of *CYP6D1v2*. HR96 and BR-C were an activator and repressor, respectively, of PB induction of *CYP6D1v2*. The same promoter region of *CYP6D1v1* from LPR was examined and shown to mediate PB induction to similar levels as C*YP6D1v2* from CS. This indicates variations in promoter sequences are not responsible for the lack of PB induction of *CYP6D1v1*. Therefore, constitutive overexpression without PB induction of *CYP6D1* in LPR is due to an unidentified *trans* acting factor. *HR96* was cloned and sequenced to examine if it is this *trans*

acting factor. Multiple *HR96* alleles were identified and alleles *v8-v10* were found to encode E28V and G110D amino acid substitutions in LPR. Permethrin selection of LPR showed *HR96* alleles *v8-v10* were not associated with permethrin resistance. Quantitative real-time RT-PCR showed no difference of *HR96* expression levels between LPR and CS. Thus *HR96* is not the *trans* acting factor responsible for constitutive overexpression of *CYP6D1* in LPR. The molecular basis of constitutive overexpression of *CYP6D1* in LPR is attributed to a *trans* acting factor responsible for PB induction in susceptible strains, but this *trans* acting factor remains unidentified.

BIOGRAPHICAL SKETCH

George Guan-Hua Lin was born on March 15, 1976 in Taipei, Taiwan. He was the first child in a family with two children. He showed strong interests in music and in book reading in his early childhood. He completed elementary, middle, and high school education in Taipei, Taiwan. In high school, he was selected for an advanced program in life sciences at Academia Sinica and he started showing strong enthusiasm for science and biology. Afterward, he entered National Taiwan University with a major in Plant Pathology and Entomology. At college, he received the National Taiwan University Presidential Award for his outstanding academic performance. In his sophomore year, he joined Dr. How-Jing Lee's lab and participated in a long-term ecological research project investigating the dynamic of aquatic insect populations in Hapen Nature Reserve in northern Taiwan for three years. He then joined Drs. How-Jing Lee and Ruey-Fen Liou to study for his Master's degree. There, he studied circadian clock gene, *period*, in German cockroaches.

In 2002, after two years mandatory military service, George continued his journey in scientific research and worked as a research assistant in the following three years in National Taiwan University and in Academia Sinica. He married Yu-Chin Yu on November 2002 and their first son, Dong-Ciao, was born on September 2004, in Taipei, Taiwan. In fall 2005, George came to Department of Entomology at Cornell University and joined Dr. Jeff Scott's lab to study for his Ph.D. His studies focused on the molecular basis dictating the constitutive overexpression of cytochrome P450 *CYP6D1* in the permethrin resistant LPR strain of house fly. He was a teaching assistant for a variety of courses and served as the Jugatae Seminar Coordinator. At the 2009 ESA meeting, he received the President's Prize for his ten-minute paper entitled "HR96 and BR-C modulate phenobarbital induced transcription

of cytochrome P450 *CYP6D1* in *Drosophila* S2 cells. Besides progress in scientific research, his second son, Jasper Yu-Sheng, was born in Ithaca, NY, on May 2009. During these five years of Ph.D. study, George was a Ph.D. student in the lab and was a husband and a father at home. The development of multiple lines in George's life and enormous support from his major advisor Dr. Jeff Scott and from Cornell provided George great and valuable lessons to build up his own management skill and philosophy. These five years of Ph.D. study and life in Ithaca have laid out the fundamental stepping stones which will lead George and his family to the land full of happiness in their following journey.

To my wife and children who have always believed in and supported me.

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LIST OF ABBREVIATIONS

AHR	aryl hydrocarbon receptor
aabys	susceptible house fly strain with visible recessive markers on chromosomes I-V
BR-C	broad-complex
CAR	constitutive active receptor
CS	Cornell Susceptible, house fly strain
DDT	dichlorodiphenyltrichloroethane
DFD	deformed
DTT	dithiothreitol
GST	glutathione *S*-transferase
HR96	hormone receptor-like in 96
LacZ	beta-*D*-galactosidase
LPR	Learn Pyrethroid Resistant house fly strain
OCR	a cyclodiene resistant (*Rdl*) house fly strain
P450	cytochrome P450
PB	phenobarbital
PCR	polymerase chain reaction
PXR	pregnane X receptor
R12	a laboratory strain has resistant alleles derived from chromosome 1 and 2 of LPR which are related to overexpression of *CYP6D1*
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
RT	reverse transcription
RXR	retinoid X receptor

S2 cells	*Drosophila* Schneider 2 cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TF	transcription factor
TSS	transcription start site
UGT	UDP-glucuronosyl transferase
USP	ultraspiracle

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

House fly *CYP6D1* is a cytochrome P450 involved in the metabolism of xenobiotics. Like other detoxification enzymes involved in xenobiotic metabolism, expression of *CYP6D1* is induced in response to the prototypical P450 inducer, phenobarbital (PB). In the permethrin resistant LPR strain, overexpression of *CYP6D1* confers the metabolism-mediated insecticide resistance. In LPR, *CYP6D1* is constitutively overexpressed, and lacks significant PB induction. My studies focus on the molecular basis responsible for transcriptional regulation of *CYP6D1* and the overexpression of *CYP6D1* in LPR. In this chapter, I will present the basic information needed to understand the dissertation by covering xenobiotic metabolism and detoxification enzymes, PB induction, insecticide resistance and mechanisms, and overexpression of *CYP6D1*.

1.2 Metabolism of xenobiotics

1.2.1 Phase I and phase II metabolism

Xenobiotics are chemicals which are not normally produced or expected to be present in an organism. Most xenobiotics are lipophilic. The metabolism of xenobiotics consists of series of biochemical reactions which process lipophilic foreign compounds into hydrophilic compounds which can be removed from the body through excretion. Xenobiotic metabolism can be divided into two major types, which are named with phase I and phase II metabolism (Willams 1949). In phase I metabolism, reactions such as oxidation, hydrolysis, or reduction, are employed in order to add a polar reactive group in the xenobiotic. Phase I metabolism can lead to

the formation of electrophilic (or oxygenated) intermediates (also called as reactive oxygen species, ROS) (Hodgson 1997, Yu 2008), which are able to react with or bind covalently to nucleic acids, causing chromosomal damage and resulting in genotoxicity. In addition, electrophilic intermediates may attack amino acids, damaging proteins, macromolecules and causing cell death (Nebert and Dalton 2006), although not all phase I enzymes cause the production of ROS. Phase II metabolism helps remove the risk of damage done by electrophilic intermediates. Enzymes participating in phase II metabolism conjugate a group, such as sugars, amino acids, and glutathione, with the substrate and thus produce a water-soluble product that can be readily excreted (Hodgson 1997, Yu 2008). The complete cycle of metabolism of xenobiotics often consists of phase I and II metabolism.

Phase I metabolism is carried out by numerous types of enzymes: cytochrome P450s (CYPs), flavin-containing monooxygenases (FMOs), alcohol dehydrogenases, aldehyde dehydrogenases, esterases, amidases, and epoxide hydrolases (Hodgson 1997). Among these enzymes, the cytochrome P450 enzymes appear to comprise the largest number of enzymes participating in phase I metabolism. In humans, P450 enzymes comprise approximately 70-80% of all enzymes involved in phase I metabolism (Evans and Relling 1999).

Phase II metabolism, or conjugation reactions can be classified into three types. Type I conjugation applies a reactive conjugating group to substrates which have OH, NH_2, COOH, and SH groups (Yu 2008). Reactive conjugating groups for type I conjugation can be glucose, sulfate, and phosphate. Type II conjugation deals with the substrate having COOH group by activating functional groups and combining with an amino acid moiety to produce conjugated products (Yu 2008). Therefore, type I and II conjugations require formation of high-energy intermediates before reactions proceed. In type III conjugation, reactions do not need any activation and can

directly combine a conjugating group (glutathione) with substrates having halogens, alkenes, NO_2, epoxides, ethers, and esters (Yu 2008). Major enzymes engaging in phase II metabolism are glutathione *S*-transferases (GSTs), uridine diphosphate glucuronosyltransferases (UGTs), sulphotransferases (SULTs), epoxidases, etc.

1.2.2 Detoxification enzymes

As mentioned above, there are many enzymes involved in phase I and II metabolism of xenobiotics. In terms of insecticide metabolism, three primary groups of detoxification enzymes are involved: cytochrome P450s, hydrolases, and glutathione *S*-transferases. These detoxification enzymes will be described below.

1.2.2.1 Cytochrome P450s

1.2.2.1.1 Biochemistry

Cytochrome P450 (CYP or P450) genes constitute a large gene superfamily and are found in most organisms. P450 proteins are typically 45-60 kDa and are heme-thiolate enzymes. Their name stems from their CO difference spectra having an absorbance maximum near 450 nm (Klingenberg 1958, Omura and Sato 1964). P450s catalyze the transfer of one atom of molecular oxygen (O_2) to the substrate and reduce the other atom to H_2O. The P450 reaction is commonly described as follows (RH stands for the substrate):

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

In eukaryotes, P450 proteins are membrane-bound in microsomes (smooth and rough endoplasmic reticulum) and mitochondria. They require redox partners to provide reducing equivalents (NADPH or NADH) for catalytic activity. Redox partners of

microsomal P450s are NADPH cytochrome P450 reductase and sometimes cytochrome b_5. The mitochondria P450s use adrenodoxin reductase as their redox partner. The basic catalytic cycle of P450 enzymes comprises four major steps (Werck-Reichhart and Feyereisen 2000, Feyereisen 2005). The first is the binding of substrate to the complex of heme-thiolate with displacement of water molecule. The second is the introduction of one electron to reduce the complex to a ferrous state (i.e. from Fe^{III} to Fe^{II} state). The third step is binding of molecular oxygen to give a superoxide complex. The fourth step is a second reduction step leading to the formation of activated oxygen species, which in turn results in the cleavage of the O-O bond and leads to the production of a water molecule and insertion of an oxygen atom to the substrate (which later turns out with OH group added at final). In addition to insertion of oxygen, P450s can be, reductases, desaturases, isomerases, etc (Werck-Reichhart and Feyereisen 2000, Feyereisen 2005).

1.2.2.1.2 Structure

P450s often encode proteins of approximately 500 amino acids. Peptide sequence comparison of diverse insect P450 genes reveals five conserved motif: WxxxR, GxE/DTT/S, ExLR, PxxFxPE/DRF, and PFxxGxRxCxG/A (Werck-Reichhart and Feyereisen 2000, Feyereisen 2005). Among these, the PFxxGxRxCxG/A motif serves as heme binding domain and is the most conserved region of P450 proteins (Werck-Reichhart and Feyereisen 2000, Feyereisen 2005). This conserved motif is characteristic of P450s. Microsomal and mitochondrial P450s are also characterized by their N-terminal sequence. P450 proteins found in microsomes have ~20 hydrophobic N-terminal amino acids which function as signal peptide to allow the protein to target to the endoplasmic reticulum (Feyereisen 2005). Mitochondrial P450s usually have longer N-terminal sequence containing several

charged residues (Feyereisen 2005).

The 3-dimentional crystal structure of diverse P450 proteins shows conserved secondary structure and folding patterns between soluble prokaryotic P450 proteins and membrane bound eukaryotic P450s proteins (Poulos and Johnson 2005). Twelve helices, A-L, and four β-sheets, 1-4, are generally conserved in both prokaryotic and eukaryotic P450 structures. There are additional and less conserved helices interspersed among the more highly conserved helices (Johnson and Stout 2005, Poulos and Johnson 2005). Comparison of two human P450 proteins, CYP2A6 and CYP3A4, which share less than 40% peptide sequence identity, showed that the most spatially conserved portions of P450 structures are helices E, I, J, K, and L as well as β-sheets 1 (Johnson and Stout 2005, Jordi 2005). These portions maintain a conserved binding site for the heme prosthetic group, which is probably necessary for catalytic activity.

1.2.2.1.3 Substrate selectivity

Substrates for P450s range from relatively small molecules, such as ethanol, to large antibiotics such as cyclosporine and erythromycin (Johnson and Stout 2005). It has been estimated that the total number of P450 substrates may exceed 200,000 (Porter and Coon 1991). Regions forming outer binding surfaces (Johnson and Stout 2005) and substrate recognition sites (SRS) of P450s (Gotoh 1992) determine substrate selectivity and specificity. Peptide sequences of the outer binding surfaces and SRSs are generally more divergent among P450s and determine the sizes, shapes, and chemical features of the active site that provide discrimination for different substrates. For example, CYP6B1 from *Papilio polyxenes* and CYP6B8 from *Helicoverpa zea* showed different substrate specificities. CYP6B1 appeared to have narrow substrate selectivity, but CYP6B8 appeared to be broad (Li et al. 2004).

Molecular modeling of both protein structures indicated that regions for outer binding surface and SRSs of CYP6B8 formed a more flexible overall folding and a more elastic catalytic pocket, which allowed more diverse substrates access to the catalytic site, compared to CYP6B1 (Li et al. 2004). Moreover, studies of human P450s also showed characteristics of substrate specificity for particular P450 subfamilies. For example, human CYP1A tends to prefer planar polyaromatic substrates, whereas human CYP2E prefers low molecular weight compounds, such as ethanol and acetone (Lewis 2000).

1.2.2.1.4 Nomenclature and classification

The nomenclature of P450 genes was first introduced at 1987 when there were only 65 P450 genes known (Nebert et al. 1987). In early 90s, the rapid increase in number of P450 genes led to a revision of nomenclature rules (Nelson et al. 1993, Nelson et al. 1996). Each P450 is assigned a CYP prefix, followed with an arabic numeral denoting the family, a capital letter denoting the subfamily, and an arabic numeral denoting the individual enzyme. Proteins are nonitalicized and genes are italicized. For example, CYP6A1 belongs to family 6, subfamily 6A. Members of P450s sharing >40% of peptide sequence identity are usually grouped in the same family. Members in the same subfamily are >55% identical in peptide sequences. This sequence identity-based classification is able to reflect the evolutionary relationships among P450 genes.

There are numerous P450 families identified so far. Based on the latest information (updated to 2006) in the Cytochrome P450 Homepage (http://drnelson.utmem.edu/CytochromeP450.html), there are 711 P450 families defined from bacteria to mammals, and 99 families were identified in animals (metazoan). The concept of higher order groupings (named as clans or clades) of

P450 families was first introduced by Nelson in 1998 (Nelson 1998) in order to provide phylogenetical and evolutionary perspectives of P450 families and genes. Based on several insect genomes, insect P450s can be classified into four major clades (i.e. CYP2, CYP3, CYP4 and mitochondrial clades) (Feyereisen 2006, Nelson 2006). Evolution and radiation of insect P450s will be addressed in Section 1.1.2.1.6.

1.2.2.1.5 Biological functions

Some P450s participate in the anabolism or catabolism of endogenous substrates such as hormones and lipids, and some are involved in the metabolism of xenobiotics such as drugs and pesticides. In this section, several examples of specialized biological functions of P450s in insect will be addressed.

20-Hydroxyecdysone is an important hormone which controls and coordinates the periodic molts and metamorphosis in insects. The biosynthesis of ecdysone is in the prothoracic gland. Studies in *Drosophila* showed CYP306A1, CYP302A1, and CYP315A1 catalyze the three sequential hydroxylations for the synthesis of ecdysone from dietary cholesterol in the prothoracic gland cells (Warren et al. 2002, Niwa et al. 2004) during which CYP306A1 catalyze the C25 hydroxylation (Niwa et al. 2004), and CYP302A1 and CYP315A1 catalyze the C22 and C2 hydroxylation (Warren et al. 2002), respectively. The conversion of ecdysone to 20-hydroxyecdysone (20HE) is carried out by CYP314A1 (Petryk et al. 2003).

The juvenile hormones (JHs) play roles in regulation of metamorphosis, caste determination, behavior, and diapause in insects (Goodman et al. 2005). P450s are involved in the anabolism and catabolism of JHs. For example, heterologous expression of CYP4C7 in *E. coli* showed CYP4C7 could metabolize JH precursors and JH III to a major metabolite (10E)-12-hydroxy-JH III (Sutherland et al. 1998), which was hypothesized as a first step in the inactivation of the very large amounts of

JH and JH precursors present in the CA after the peak of JH synthesis (Sutherland et al. 1998, Sutherland et al. 2000). Besides, insect P450s have also been indicated to be involved in the biosynthesis and clearance of insect hormones and allomones (Brattsten 1979, Hovemann et al. 1997, Plettner et al. 1998, Maibeche-Coisne et al. 2002).

Some insect P450s metabolize plant defense chemicals, which even can enable the specialized adaptation of insects to specific host plants. The specialized adaption of the back swallowtail *Papilio polyxenes* to furanocoumarin containing plants (the *Apiaceae* and the *Rutaceae*) was conferred by evolution of the specialized *CYP6B1*. CYP6B1 was demonstrated to metabolize linear furanocoumarins (Wen et al. 2003) and was detected in the midgut micorsomes of *P. polyxenes* (Cohen et al. 1992). Expression of CYP6B1 was selectively induced by furanocoumarins (Cohen et al. 1992, Hung et al. 1995). A DNA regulatory element in the *CYP6B1* promoter was identified as critical for furanocoumarin induction (McDonnell et al. 2004). In the fruit fly (*Drosophila pachea*) from the Sonoran desert, P450 was demonstrated to involved in specialized adaptation to specific columnar cactus hosts (Frank and Fogleman 1992).

1.2.2.1.6 Evolution of insect P450s

P450s are a large gene superfamily. P450s are found from bacteria, fungi and plants, to insects and mammals (Nelson 1999). The average number of P450s in each insect is around 100 P450s. The number of P450 genes found from species with sequenced genomes is listed in Table 1.1.

Insect P450s fall into four major clades. Among these four clades, the CYP3 and CYP4 clades appear to be abundant. In *Diptera*, CYP3 and CYP4 clades, each account for about 40% of total P450 genes (80% for CYP3 and CYP4 clades). In

Table 1.1: Numbers of validated P450s, esterases, and GSTs in the insect genomes.

species	Number of P450s	Number of esterases	Number of GSTs
Aedes aegypti	164	54	27
Anopheles gambiae	106	51	28
Apis mellifera	46	24	8
Bombyx mori	86	ND[a]	23
Drosophila melanogaster	85	35	37
Nasonia vitripennis	92	41	19
Tribolium castaneum	131	49	35

Data are taken from (Adams et al. 2000, Holt et al. 2002, Ranson et al. 2002b, Li et al. 2005, Claudianos et al. 2006, Tribolium castaneum Consortium 2008, Strode et al. 2008, Yu et al. 2008, Oakeshott et al. 2010).

[a] ND, not determined.

other insects, CYP3 and CYP4 clades still appear to be more numerous than the other two clades (i.e. CYP2 and mitochondrial clades), except the honeybee in which only CYP3 clade shows significant gene duplication and radiation (Claudianos et al. 2006, Feyereisen 2006). Moreover, members of CYP3 or CYP4 clades are often found in large clusters (Frolov and Alatortsev 1994, Cohen and Feyereisen 1995, Dunkov et al. 1996, Tijet et al. 2001, Ranson et al. 2002a) and are often conserved in intron-exon organization (Tijet et al. 2001). Expansion of the CYP3 clade appears to be insect specific (Feyereisen 2006). P450 genes belonging to CYP3 clade are often associated with xenobiotic metabolism and also insecticide resistance. In contrast,

members of CYP4 clade are often associated with metabolism of odorants and pheromones.

1.2.2.2 Hydrolases

Biochemically, hydrolases are enzymes catalyzing the hydrolysis of a chemical with introduction of one water molecule. Hydrolysis reactions include carboxylester hydrolysis, carboxyamide hydrolysis, and carboxythioester hydrolysis reactions (Hodgson 1997) which are represented in the following equations:

$RC(O)OR' + H_2O \rightarrow RCOOH + HOR'$	carboxylester hydrolysis
$RC(O)NR'R'' + H_2O \rightarrow RCOOH + HNR'R''$	carboxyamide hydrolysis
$RC(O)SR' + H_2O \rightarrow RCOOH + HSR'$	carboxythioester hydrolysis

Unfortunately, there is still no sequence based classification for hydrolases. It appears that a large number of esterases (carboxylesterase) are able to catalyze hydrolysis reactions (Hodgson 1997). Peptide sequence-based classification defined 8 esterase subfamilies: α-esterases, juvenile hormone esterases, β-esterases, gliotactins, acetylcholinesterases, neurotactins, neuroligins, and glutactin type (Ranson et al. 2002b). Three types of esterases (α-esterases, β-esterases, and acetylcholinesterases (AChEs)) have been associated with insecticide resistance (Oakeshott et al. 2005). The number of esterase genes identified from several insects with sequenced genome is shown in Table 1.1.

1.2.2.3 Glutathione *S*-transferases

Glutathione *S*-transferases (GSTs) play roles in protection against reactive oxygen species (ROS) and detoxification of xenobiotics. GSTs participate in phase II

metabolism and can inactivate the ROS by conjugating glutathione to the substrate. Besides the role in the phase II metabolism, they have been noticed to directly participate to the metabolism of xenobiotics. For example, DDT dehydrochlorination via a GST is a major route of DDT metabolism in insects (Clark and Shamaan 1984, Grant et al. 1991, Prapanthadara et al. 1993).

Most GSTs are cytosolic dimeric proteins with typical molecular weight of each subunit around 24-28kDa. Each subunit consists of two binding sites, the G site and H site. The G site binds the tripeptide glutathione and is highly conserved in amino acid residues residing in the N terminal of the protein. The H site in the C-terminal is responsible for the substrate binding and it is more variable in sequence and structure (Mannervik 1985). Their classification is based on amino acid sequence in which GSTs sharing >40% identity are assigned to the same class (Ranson et al. 2002b). They are named GST plus the class and an individual number that may reflect either the order of discovery or the genome organization. Two letters are added to indicate the species. For example, *Ag*GSTd12 is the twelfth member of delta class of *A. gambiae*.

Based on genome sequences of insects, plants, and mammals, a total of nine different classes of GSTs have been defined. At least six classes of insect GSTs have been identified (Table 1.1). *A. gambiae* and *D. melanogaster* (Ranson et al. 2002b), have 28 and 37 GSTs, respectively. The delta and epsilon classes are the two largest classes of insect GSTs and appear to be insect-specific classes. In *A. gambae* and *D. melanogaster*, there are 15 and 11 delta GSTs, and 8 and 14 epsilon GSTs, respectively (Ranson et al. 2002b). Delta and epsilon GSTs have been implicated to play an important role to detoxifying insecticides (Wang et al. 1991, Tang and Tu 1994, Wei et al. 2001, Ortelli et al. 2003).

1.3 Induction of P450s by phenobarbital

Phenobarbital (PB) is a prototypical P450 inducer, capable of increasing expression of numerous detoxification genes, including a subset of P450s (Gerhold et al. 2001, Hamadeh et al. 2002, King-Jones et al. 2006, Sun et al. 2006, Willoughby et al. 2006). PB induction of P450s was first described in 1967 (Conney 1967), and PB has been used to understand the molecular mechanisms of induction for more than 40 years. Studies have been carried out in diverse organisms, from bacteria to mammals. In this section the molecular models of PB induced transcription of P450s in bacteria, mammals, and insects will be reviewed.

1.3.1 PB induction of P450s in bacteria

PB induction in bacteria has been most thoroughly studied for two P450s in *Bacillus megaterium* $P450_{BM\text{-}1}$ and $P450_{BM\text{-}3}$. The regulatory sequence, named a Barbie box, required for PB induction was first determined in promoter areas (-318 to -302) and (-243 to -227) upstream of the translation start sites of $P450_{BM\text{-}1}$ and $P450_{BM\text{-}3}$, respectively (He and Fulco 1991). The Barbie box sequence interacts with a repressor, Bm3R1 (a helix-turn-helix DNA binding protein), to regulate the PB activated expression of $P450_{BM\text{-}1}$ and $P450_{BM\text{-}3}$ gene (Liang and Fulco 1995, Liang et al. 1995). In response to the stimulus of PB, dissociation of repressor Bm3R1 from Barbie box sequence was observed which in turn allowed for increase transcription (Liang and Fulco 1995, Liang et al. 1995). There were also two positive transcription factors identified, Bm1P1 and Bm1P2, which are involved in the regulation of PB activated-expression of $P450_{BM\text{-}1}$ by interfering with the binding of the repressor Bm3R1 (He et al. 1995).

1.3.2 PB induction of P450s in mammals

1.3.2.1 PB induction of *CYP2B* genes by PBREM and CAR

PBREM (PB responsive enhancer module) encompasses 51 bp at -2339 through -2289 in the mouse *CYP2B10* promoter (Honkakoski and Negishi 1997, Honkakoski et al. 1998b). This regulatory sequence element was also found in promoters of rat *CYP2B1*, *CYP2B2*, and human *CYP2B6* genes (Sueyoshi et al. 1999), which implies evolutionary conservation of PB induction of the PBREM in the *CYP2B* genes from mouse to human. The PBREM consisted of two nuclear receptor binding sites (NR1 and NR2) and an NF1 binding site (Honkakoski et al. 1998b). The NR1 site appeared to be the most conserved site among PBREMs identified in promoters of other *CYP2B* genes. Two different strategies were used to identify transcription factors interacting with the PBREM, especially the NR1 site. By using a transient transfection assay and DNA-affinity chromatography, it was shown that nuclear receptors CAR (constitutive active receptor) and RXR (retinoid X receptor) bound to the NR1 site of PBREM (Honkakoski et al. 1998a). CAR and RXR form a heterodimer in order to bind to the NR1 site, and the binding appeared to occur after the treatment with PB (Honkakoski et al. 1998a). In a CAR-null mutant mouse, PB did not induce *CYP2B10* (Wei et al. 2000), indicating CAR was required for PB induction of *CYP2B* genes.

Expression of CAR with a fluorescent protein-tag showed that it was retained in the cytoplasm (Kawamoto et al. 1999, Sueyoshi and Negishi 2001). The amount of CAR was increased and accumulated in the nucleus after treatment with PB (Kawamoto et al. 1999), indicating PB triggered the nuclear translocation of CAR. Treatment of rat and mouse primary hepatocytes with okadaic acid (OA, a inhibitor for protein phosphatase 2A) repressed *CYP2B* induction by PB (Sidhu and Omiecinski 1997, Honkakoski and Negishi 1998), and inhibited PB-dependent nuclear

translocation of CAR (Kawamoto et al. 1999), which implied the nuclear translocation of CAR was regulated by a phosphorylation and/or dephosphorylation mechanism. When mouse primary hepatocytes were pretreated with KN-62 and KN-93 (inhibitors of Ca^{2+}/calmodulin-dependent kinase), accumulated CAR in the nucleus failed to activate *CYP2B* induction by PB (Sueyoshi and Negishi 2001). Overexpression of CaMKII or CaMKIV resulted in enhancement of the CAR-dependent NR1 activity in HepG2 cells (Sueyoshi and Negishi 2001). These two observations indicated a regulatory mechanism involved in activation of CAR in the nucleus. In summary, there are multiple phosphorylation and/or dephosphorylaiton steps involved in order to regulate nuclear translocation of CAR and nuclear activation of CAR, which provide multiple check points to prevent CAR from activating genes in the absence of proper stimuli.

In addition to PBREM, Barbie boxes were also found in promoter regions in mammalian *CYP2B* genes. However, several studies indicated the Barbie box sequence did not mediate PB induced transcription in mammals (Park et al. 1996, Honkakoski and Negishi 1997, Stoltz et al. 1998).

1.3.2.2 PB induction of *CYP3A* genes by XREM and PXR

The XREM (xenobiotic responsive enhancer module) was first identified in the distal area (at -7836 through -7607) of the promoter of human *CYP3A4*. The XREM contained two NR binding sites which are separated by 29 bp (Goodwin et al. 1999). The XREM was shown to be able to respond to PB in human HepG2 cells (Goodwin et al. 1999). The nuclear receptor PXR (preganane X receptor) was first found to active inductions of *CYP3A* genes in response to preganane (Kliewer et al. 1998). Gel-shift assays showed that PXR and RXR formed a heterodimer to bind to the XREM in response to PB (Goodwin et al. 1999). Costransfection of PXR factor with

XREM sequence element showed increased expression of reporter gene in response to the treatment with PB (Goodwin et al. 1999). PXR is a constitutive inactive receptor and resides in the nucleus. Activation of PXR is ligand-dependent mechanism which means the direct binding of inducer to PXR activates this nuclear receptor (Sueyoshi et al. 1999). CAR-PBREM and PXR-XREM-mediated PB induction of *CYP2B* and *CYP3A* genes, respectively was illustrated in Figure 1.1.

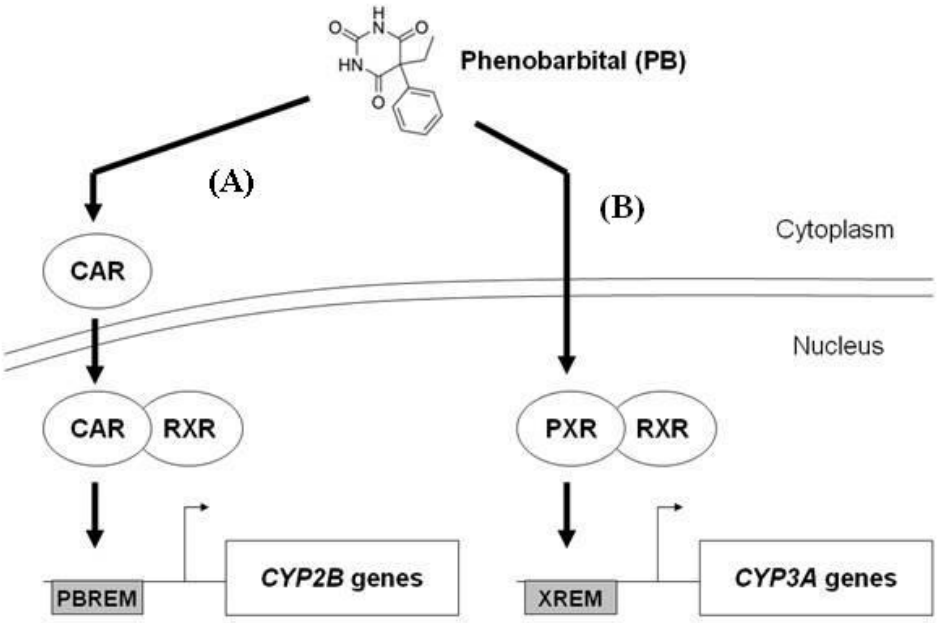


Figure 1.1: Signal transduction pathways of PB activation via CAR and PXR to *CYP2B* and *CYP3A* genes (Sueyoshi and Negishi 2001). (A) Association of PB and CAR remains unclear. The presence of PB activates CAR to enter the nucleus, associate with RXR, bind to the PBREM regulatory sequence, and activate transcription of target genes. (B) PB binds to and activates PXR. This allows PXR to associate with RXR, to bind to XREM regulatory sequence, and to activate transcription of target genes.

1.3.3 PB induction in insects

Studies to identify *cis* elements and associated transcription factors involved in PB induction have mainly focused on *Cyp6a2* and *Cyp6a8* of *D. melanogaster*. Promoter assays of the *Cyp6a2* promoter in *Drosophila* S2 cells identified the region within -428 bp of the translation start site as critical for mediating PB induction (Dunkov et al. 1997). Promoter assays of *Cyp6a8* promoter identified the region from -716 to -199 as critical for PB induction (Maitra et al. 2002, Morra et al. 2010). Using bioinformatics tools, putative binding sites for transcription factors BR-C (*broad-complex*), EcR, and AP1 have been found within promoter regions of *Cyp6a2* and *Cyp6a8* critical for PB induction (Dunkov et al. 1997, Maitra et al. 2002). However no functional assays have been carried out to examine the role of these putative transcription factors. The key transcription factor in insects regaling PB induced transcription remains unidentified.

Drosophila HR96 represents the single ortholog corresponding to mammalian nuclear receptors CAR and PXR (King-Jones and Thummel 2005, Laudet et al. 2005). Based on this evolutionary relationship, HR96 has been considered to play a role in regulating transcription in response to PB. A *D. melanogaster* HR96 null mutant has been generated and studied. Adults of the HR96 null mutant strain were more sensitive to DDT and PB (King-Jones et al. 2006), suggesting a role of HR96 in protection against these xenobiotics. Microarray results showed transcription of 29 P450s were induced in response to PB in wild type Canton-S strain. However, in the HR96 null mutant strain, these 29 P450s were still PB inducible to the same levels as in the wild type strain (King-Jones et al. 2006). It was suggested that the loss of HR96 (in the HR96 null strain) may be compensated by additional transcriptional regulators able to feed into this pathway (King-Jones et al. 2006); and a possible regulator of *Drosophila* ortholog of mammalian aryl hydrocarbon receptor was

suggested (King-Jones et al. 2006). The role of HR96 in PB induced transcription of P450s (and other PB-regulated genes) remains unclear.

1.4 Insecticide resistance

Insects destroy approximately 18% of the world annual crop production (Oerke and Dehne 2004) and 20% of stored food grains (Bergvinson and Garcia-Lara 2004). In addition, they transmit devastating human diseases such as malaria, dengue fever, yellow fever, encephalitis, filariasis, West Nile fever, and chikungunya (Hemingway and Ranson 2000, Nauen 2007). For example, malaria causes more than 1 million deaths per year and is due to infection with *Plasmodium falciparum* and *P. vivax* transmitted by *Anopheline* mosquitoes (Hemingway and Ranson 2000, Breman 2001). In addition, insects also transmit diseases to livestock and poultry (Dryden et al. 1993) causing deaths, as well as reduction in milk, egg, and meat production. Insecticides are widely used to control insects, but the evolution of resistance is challenging our long term use of insecticide.

Insecticide resistance leads to increased use, overuse, and misuse of insecticides which causes a greater impact to the environment and ecosystem, as well as to human health. Up to April 2007, the number of cases and species of insecticide resistance reported in the Arthropods Resistance to Pesticides Database (ARPD) was 7558 and 553, respectively (Mato-Sanchez et al. 2007). More than 62% of these cases were pests of agricultural, forest and ornamental plants. Another 35.5% occurred in medical, veterinary and urban pests (Mato-Sanchez et al. 2007). While the numbers in the ARPD database are certainly an underestimate of the scope of the resistance problem, the numbers document are increasing and challenging problem of insecticide resistance in crop protection, public health, and environmental protection.

Resistance is pre-adaptive and evolves following use of an insecticide against a

pest population, which selects resistant individuals. The World Health Organization defines resistance as "the inherited ability of a strain of some organism to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species" (WHO 1957). Failure of chemical control due to insecticide resistance decreases of crop production, increase loss of stored food, and increases the threat to human and animal health.

1.5 Major mechanisms of insecticide resistance

Toxicity is determined by five factors: penetration, distribution, metabolism, interaction with target site, and excretion. In theory, changes in any one of these factors could result in resistance. However, after nearly 80 years of study it is clear that insecticide resistance can be attributed to only two major mechanisms: metabolism and target site insensitivity. Reduced penetration has been frequently documented as a resistance mechanism, but generally confers low levels of resistance compared to metabolism and target site insensitivity (Yu 2008). Insecticide resistance mechanisms have been described in several reviews which can provide further information (Feyereisen 1995, Scott 1999, Hemingway et al. 2004, Li et al. 2007).

1.5.1 Metabolism-mediated resistance

Insects are armed with detoxification enzymes which are able to be induced in order to deal with xenobiotics or plant defense chemicals. Three major mechanisms can cause metabolism-mediated resistance: (1) Upregulated transcription of detoxification gene, (2) Gene duplication of detoxification gene, (3) Changes in the catalytic activity of a detoxification enzyme.

1.5.1.1 Upregulated transcription

Development of insecticide resistance due to upregulated transcription of a detoxification gene can be attributed to a mutation in the promoter region of the detoxification gene as well as to a mutation in a *trans* acting factor (or transcription factor) regulating the transcription of the detoxification gene. Several well studied examples were done in *Cyp6a2*, *Cyp6a8*, and *Cyp6g1* of *D. melanogaster* and *CYP6A1* and *CYP6D1* of *M. domestica*, and will be described in the following.

1.5.1.1.1 *D. melanogaster Cyp6a2*

The 91-R strain of *D. melanogaster* is a DDT resistant strain. In 91-R strain, *Cyp6a2* was 20-30 fold overexpressed compared to the insecticide susceptible 91-C strain (Waters et al. 1992). Southern blots indicated overexpression of *Cyp6a2* was not attributed to gene amplification (Waters et al. 1992). In $RDDT^R$ (also named as RaleighDDT) another DDT resistant strain of *D. melanogaster*, *Cyp6a2* was 6-fold overexpressed compared to an insecticide susceptible strain (Brun et al. 1996). Genetic linkage analysis indicated the overexpression of *Cyp6a2* mapped to chromosome 3 in the 91-R strain (Maitra et al. 2000), which *Cyp6a2* is on chromosome 2 (Dunkov et al. 1997). Thus overexpression of *Cyp6a2* was attributed to a mutation in a *trans* acting factor.

Transcription of *Cyp6a2* is about 15-fold PB inducible in insecticide susceptible strains (Brun et al. 1996, Dunkov et al. 1997, Dombrowski et al. 1998). However, PB induction of Cyp6a2 in the $RDDT^R$ strain was only 2.5-fold (Brun et al. 1996). PB induction of *Cyp6a2* has not been studied in the 91-R strain.

1.5.1.1.2 *D. melanogaster Cyp6a8*

D. melanogaster Cyp6a8 is located in the chromosome 2 and is within 4 kb of

Cyp6a9 (Maitra et al. 1996). In the DDT resistant 91-R strain, 8-fold overexpression of *Cyp6a8* was detected relative to the susceptible 91-C strain (Maitra et al. 1996). In response to PB treatment, *Cyp6a8* showed 40-fold PB induction in the 91-C strain, but only about 9-fold induction in the 91-R strain (Maitra et al. 1996). Genetic linkage analysis indicated that overexpression of *Cyp6a8* in the 91-R strain was attributed to a *trans* acting factor present in the chromosome 3 (Maitra et al. 2000). In the 91-R strain, overexpression of *Cyp6a2* and *Cyp6a8* has been speculated to be attributed to the same *trans* acting factor on chromosome 3 (Maitra et al. 2000).

1.5.1.1.3 *D. melanogaster Cyp6g1*

Overexpression of *Cyp6g1* was initially associated with DDT resistance in *D. melanogaster* (Daborn et al. 2001). This overexpression had been proposed due to an insertion of transposable element *Accord* in the promoter region of *Cyp6g1* (Daborn et al. 2001). However, subsequent studies found two strains, Hikone-RH and Canton-SH, with high expression of *Cyp6g1*, but without DDT resistance (Kuruganti et al. 2007), indicating overexpression of *Cyp6g1* was not associated with DDT resistance; although enhanced expression of *Cyp6g1* was still associated with the *Accord* insertion (Kuruganti et al. 2007).

1.5.1.1.4 *M. domestica CYP6A1*

CYP6A1 was mapped to chromosome 5 (Cohen et al. 1994). In the Rutgers strain, *CYP6A1* was 10-fold overexpressed in larvae and adults compared to insecticide susceptible *sbo* strain (Carino et al. 1994). Southern analysis showed overexpression of *CYP6A1* in the Rutgers strain was not attributed to gene duplication (Feyereisen et al. 1995). Co-expression of CYP6A1, P450 reductase, and cytochrome b_5 in *E. coli* showed the metabolism of diazinon to diazoxon and 2-

isopropyl-4-methyl-6-hydorxypyrimidine (Sabourault et al. 2001). It was hypothesized overexpression of CYP6A1 in Rutgers strain plays one of the causative factors of diazinon resistance (Sabourault et al. 2001).

In response to treatment with PB, adults of susceptible *sbo* strain showed about 100-fold induction of *CYP6A1*; whereas, adults of the Rutgers strain showed only 22-fold induction (Carino et al. 1992). Genetic linkage analysis demonstrated the overexpression of *CYP6A1* was attributed to a *trans* acting factor present in the chromosome 2 of the house fly (Carino et al. 1994, Feyereisen et al. 1995). The *ali-esterase* (*MdαE7*) was identified and mapped in the chromosome 2 and the mutant *Mdα*E7 bearing G137D substitution was proposed to be the *trans* acting factor to cause enhanced transcription of *CYP6A1* (Sabourault et al. 2001). In the proposed molecular model, metabolite products by wild type *Mdα*E7 acted as a "transcriptional repressor" to suppress transcription of *CYP6A1* and other target genes, such as *CYP6D1*. The G137D substitution of *Mdα*E7 disabled the generation of metabolite products which in turn released the negative control and allowed enhanced transcription (Sabourault et al. 2001). However, the association between the G137D substitution of *Mdα*E7 with insecticide resistance was lacking in other resistant strains LPR and NG98 of the house fly (Scott and Zhang 2003). In both resistant strains, the allele encoding G137 was identified accompanying with enhanced overexpression of *CYP6D1* genes (Scott and Zhang 2003). Therefore, the nature of *trans* acting factor determined to be present on chromosome 2 is still unclear.

In summary, common features were noticed among *Cyp6a2*, *Cyp6a8*, and *CYP6A1*: (1) they were overexpressed by a *trans* acting factor in resistant strains, (2) they were PB inducible in susceptible strains, and (3) they showed reduced PB induction in resistant strains.

1.5.1.1.5 Other detoxification enzymes

In the house fly LPR strain, increased transcription of *CYP6D1* confers permethrin resistance. Studies about *CYP6D1* will be addressed in Section 1.5.

In house flies resistant to organophosphates and carbamates, enhanced expression of GST-1 was reported (Plapp 1984). Genetic linkage studies showed the overexpression of GST-1 was *trans* regulated by a factor present in the chromosome 2 of the house fly (Plapp 1984). In *A. aegypti*, overexpression of GSTD1 was reported to confer DDT resistance (Grant et al. 1991, Grant and Hammock 1992), and the overexpression of GSTD1 was due to a loss-of-function mutation in an unidentified *trans* acting factor (Grant and Hammock 1992).

1.5.1.2 Gene duplication

An increase in the copy number of a gene encoding a detoxification enzyme can lead to the development of increased metabolism and the insecticide resistance. In *Myzus persicae*, enhanced degradation and sequestration of insecticides including organophosphates, carbamates, and pyrethroids is due to gene amplification of *E4* or *FE4* (Field and Devonshire 1998). Both *E4* and *FE4* share 97% nucleotide sequence identity and are adjacent to each other in a head-to-tail arrangement. In resistant strains (R1, R2, and R3), one of the two paralogs (*E4* and *FE4*) is amplified which in turn leads to overproduction of β-esterase and confers organophosphates resistance. The highly resistant R3 strain had the greatest amplification (up to 80 copies) (Field et al. 1999). Differences in resistance and esterase activity among resistant R1, R2, and R3 strains of aphids correlates with the number of copies of the esterase genes (Field et al. 1999). The mosquito *Culex pipiens pipiens* also shows increased gene copy number of *estα2* and *estα2*, which causes resistance of organophosphorus and carbamates insecticides. There is an approximate 20-fold amplification of the co-

amplicon of *estα2* and *estα2* in highly resistant strains (Gullemaud et al. 1997). *In situ* hybridization has revealed a tandem arrangement of the amplified genes in resistant mosquitoes (Nance et al. 1990). The same phenomenon of the same gene amplification has also been observed in *C. p. molestus* (Tomita et al. 1996). Gene amplification of *EST1* in the brown rice planthopper *Nilaparvata lugens* was reported to confer organophosphates resistance, based on Southern blots (Graham J. Small 2000) which showed 8- to 10-fold increase esterase activity in the resistant strain (Vontas et al. 2000). Gene amplification of *GSTs* associated with insecticide resistance has also been documented. In the organophosphate resistant Cornell-R strain of the house fly, increased copy number of *MdGSTD3* and *MdGSTD4* was associated with resistance to organophosphates (Zhou and Syvanen 1997).

1.5.1.3 Changes in the catalytic activity of a detoxification enzyme

Amino acid substitution of a detoxification enzyme, resulting from a non-synonymous mutation, might result in an enzyme with new substrate specificity and/or increased catalytic activity. Two reports have documented resistance of this type. In organophosphate resistant sheep blowfly, *L. cuprina*, a point mutation of G137D converted a carboxylesterase (*Lc*Eα7) to an organophosphorus hydrolase, conferring resistance to organophosphates (Newcomb et al. 1997). *In vitro* expression of natural and mutant enzymes confirmed that the mutant enzyme lost carboxylesterase activity and gained novel organophosphorus hydrolase activity (Newcomb et al. 1997). In the house fly, the same type of conversion of a carboxylesterase to organophosphorus hydrolase has also been noticed in the organophosphate resistant strain (Claudianos et al. 1999), in which the *Md*Eα7 acquired the same amino substitution (G137D) and displayed enhanced organophosphorus hydrolase activity (Claudianos et al. 1999). Point mutations resulting in DDT resistance have also been noticed in *Drosophila*

CYP6A2 (R335S, L336V, and V476L) in $RDDT^R$ strain (Amichot et al. 2004). The mutant CYP6A2 expressed in *E. coli* showed enhanced metabolism of DDT, with increased production of dicofol, dichlorodiphenyldichloroethane, and dichlorodiphenyl acetic acid, compared to wild type CYP6A2 (Amichot et al. 2004).

1.5.2 Target site insensitivity

Major target sites of conventional insecticides are acetylcholinesterase (AChE), γ-aminobutyric acid (GABA) receptors, voltage-gated sodium channels, and nicotinic acetylcholine receptors (nAChR). These target sites all modulate neural activities. Association of insecticides with these target sites devastates the normal physiological function of neurons which in turn disrupts the neural network, and leads to death. Insecticide resistance due to target site insensitivity develops through mutations that alter the target site protein, disrupting or weakening the association between the insecticide compound and the target site. For example, a mutation (L1014F) in the voltage-sensitive sodium channel confers resistance to DDT and pyrethroids (Ingles et al. 1996, Miyazaki et al. 1996, Williamson et al. 1996). Point mutations in AChE (Oakeshott et al. 2005), the GABA receptor (Buckingham and Satelle 2005), and nAChRs (Jeschke and Nauen 2005) have also been found to result in target site insensitivity and insecticide resistance.

1.5.3 Reduced penetration

The exoskeleton provides the first line of protection for insects against toxicants, and decreased cuticular penetration can limit insecticide toxicity. Insecticide resistance caused by reduced penetration although it generally provides only 2- to 4-fold resistance (Plapp 1986), which is much less than resistance due to target site insensitivity or increased metabolism. The possible mechanism of reduced

penetration has been studied by several investigators (Georghiou 1972, Plapp 1976) and it has been reported that there are higher total lipids, monoglycerides, diglycerides, fatty acids, sterols, and phospholipids in the cuticle of a resistant strain of house fly compared to a susceptible strain (Patil and Guthrie 1979), although the precise resistance mechanism is unknown.

1.6 Increased transcription of *CYP6D1* in the LPR strain of the house fly

1.6.1 The house fly

The house fly, *M. domestica*, is a vector of human and animal diseases. House flies carry pathogens which caused diseases such as typhoid fever, cholera, bacillary dysentery, infantile diarrhea, tuberculosis, plague, leprosy, yaws, samonellosis, anthrax, trachoma, and epidemic conjunctivitis (West 1951, Keiding 1986). In addition, house flies have been reported to transmit antibiotic resistant bacteria in hospitals (Graczyk et al. 2001, Maisnier-Patin and Andersson 2004). A recent report indicated that house flies can transmit the deadly strain of *E. coli* that caused 11 deaths in Japan (Iwasa et al. 1999). House flies also carry *streptococcus* and *staphylococcus* which are responsible for mastitis in dairy cattle as well as pathogens for numerous other animal diseases. The impact from house flies can result in decreased feed conversion and lower milk yields (Rutz et al. 1995). It has been estimated that costs of pesticides used for house fly control at poultry facilities were over $200 million annually in the USA (Geden et al. 1994).

1.6.2 The permethrin resistant LPR strain

The permethrin resistant LPR strain was collected in a dairy near horseheads, New York at 1982. After 22 generations of permethrin selection in the lab, the LPR strain became homozygous for resistance and showed a 6000-fold level of resistance

to permethrin (Scott et al. 1984). The LPR strain was highly resistant to pyrethroids having an unsubstituted phenoxybenzyl substituent (permethrin, deltamethrin, cypermethrin, etc). In addition, the LPR strain has multiple resistance to different insecticides due to the long history of insecticide use in New York State (Scott and Georghiou 1986b); although the levels of resistance vary considerably.

1.6.3 Mechanisms of resistance and genetic linkage study

Following treatment with the P450 inhibitor PBO (piperonyl butoxide), the permethrin resistance of the LPR strain was suppressed from 6000-fold to 32-fold (Scott and Georghiou 1986b), suggesting monooxygenase-mediated metabolism was the major mechanism of resistance. Expression of total cytochrome P450s, P450 reductase, and cytochrome b_5 were 4-, 3-, and 2-fold higher, respectively, in the LPR strain than in the susceptible strain (Scott and Georghiou 1986b). There were two additional mechanisms responsible for the resistance and they were *kdr* and reduced cuticle penetration (*pen*) (Scott and Georghiou 1986a, 1986b). Genetic linkage analysis of permethrin resistance in the LPR strain showed that monooxygenase-mediated mechanism was associated to autosome 1 and 2, and that *kdr* and *pen* were found in the autosome 3 (Liu and Scott 1995).

1.6.4 Overexpression of $P450_{lpr}$ in the LPR strain

Microsomes from the LPR strain analyzed by the SDS-PAGE showed a marked increase in a band with molecular mass around 54.4 kDa compared to insecticide susceptible strain (Lee and Scott 1989). The protein was HPLC purified and named $P450_{lpr}$ (Wheelock and Scott 1989). An antiserum specific to the $P450_{lpr}$ was used to demonstrate that $P450_{lpr}$ was expressed approximate 44-fold higher levels in the LPR strain compared to a susceptible strain (Wheelock and Scott 1990).

Immunoinhibition studies showed the $P450_{lpr}$ was able to mediate monooxygenase dependent metabolism of deltamethrin (Wheelock and Scott 1992a), and cypermethrin (Zhang and Scott 1996). The increased expression of $P450_{lpr}$, combined with its ability to detoxify pyrethroids, led to the conclusion that $P450_{lpr}$ was responsible for the monooxygenase-mediated resistance in the LPR strain (Wheelock and Scott 1992a).

In the insecticide susceptible strain, the abdominal expression of $P450_{lpr}$ was highest in fat bodies, proximal intestine, and reproductive organs (Lee and Scott 1992). PB induction of $P450_{lpr}$ in a susceptible strain was observed in most abdominal tissues (Lee and Scott 1992). In the LPR strain, higher expression level of $P450_{lpr}$ was found in various abdominal tissues (Scott and Lee 1993). $P450_{lpr}$ was also found to be expressed at higher level in head, thorax, abdomen, and thoracic ganglia of the LPR strain relative to the susceptible strain (Korytko and Scott 1998). However, in response to PB, substantial induction of $P450_{lpr}$ was not detected in the LPR strain (Scott and Lee 1993).

1.6.5 Cloning *CYP6D1*

Partial peptide sequences of purified $P450_{lpr}$ was determined and used to design degenerate primer pairs for PCR, which in turn came up with 1.3 kb partial nucleotide sequence coding part of $P450_{lpr}$ (Tomita and Scott 1995). RACE (Rapid Amplification cDNA Ends) based strategy was used to clone the 5' and 3' ends of cDNA encoding $P450_{lpr}$ (Tomita and Scott 1995). The resulting cDNA sequence consisted with 1816 nucleotides with an opening frame of 1548 nucleotides encoding 516 amino acid which was designated as CYP6D1 (Tomita and Scott 1995). Allelic specific PCR demonstrated *CYP6D1* was mapped to autosome 1 (Liu et al. 1995).

1.6.6 Expression profile of *CYP6D1*

The expression of *CYP6D1* was developmentally regulated, being found only in adults (Scott et al. 1996). High level expression (mRNA and protein) was observed in 1 to 6 days old adults (Wheelock et al. 1991, Tomita and Scott 1995). In the LPR strain, *CYP6D1* was overexpressed with 9-fold increase relative to susceptible strains (Scott et al. 1996, Liu and Scott 1997a, 1997b). In response to treatment with PB, adults of susceptible strain showed approximate 4- to 6-fold induction of *CYP6D1*, but no significant PB induction of *CYP6D1* was seen in the LPR strain (Scott et al. 1996, Liu and Scott 1997a).

1.6.7 Molecular basis of increased transcription of *CYP6D1* in the LPR strain

Southern analysis indicated *CYP6D1* was a single copy gene (Tomita and Scott 1995). The half life of *CYP6D1* transcripts of the susceptible strain and the LPR resistant strain did not show significant difference to each other (approximate 10 hr of half life) (Liu and Scott 1998). Both results suggested the overexpression of *CYP6D1* was not due to gene amplification or stabilized transcripts, but due to upregulated transcription. *In vitro* nuclear run-on transcription assay showed 10-fold greater transcription occurred in the LPR strain and confirmed the increased transcription in the LPR strain (Liu and Scott 1998).

Genetic linkage analysis demonstrated increased transcription of *CYP6D1* was attributed to factors present in chromosome 1 and 2 (Liu and Scott 1997b, 1997a). Further study showed that a *trans* acting factor on chromosome 2 was associated with the lack of PB induction of *CYP6D1* in the LPR strain (Liu and Scott 1997a).

The 5' flanking region of *CYP6D1* has been sequenced in various insecticide susceptible and resistant strains. One common transcription start site (TSS) was identified among CS, *aabys*, and LPR strains; and the TSS was located in 86 bp

upstream of the translation start site (Scott et al. 1999). Comparison of promoter sequences in house fly strains CS, *aabys*, OCR, Cornell-R, ISK, and LPR showed the presence of an 15 bp insertion in the promoter of LPR (Scott et al. 1999). This 15 bp insertion was located from -29 to -15 upstream of the TSS. It was noticed that the presence of 15 bp insertion in the promoter of LPR disrupts a binding site for transcriptional factor Gfi-1 (Scott et al. 1999). House fly *Gfi-1* was cloned, sequenced, and mapped to the chromosome 1 (Kasai and Scott 2001b). Gfi-1 contains 6 zinc finger domains for DNA interaction. Gfi-1 is a transcription repressor in mammals (Zweidler-McKay et al. 1996). Using gel-shift assays, it was shown that Gfi-1 was able to bind to the sequence lacking the 15 bp, but this binding was greatly reduced by the presence of 15 bp insertion (Gao and Scott 2006b). This suggests the 15 bp insertion found in LPR promoter disables the repression via Gfi-1.

1.6.8 Overexpression of other PB inducible P450s in LPR strain

CYP6D1 is overexpressed without significant PB induction in the LPR strain. Overexpression also occurs in other PB inducible genes in the LPR strain. *CYP6D3* was overexpressed in the LPR strain (with 12-fold increase) compared to the CS strain (Kasai and Scott 2001a), and the PB induction of *CYP6D3* was dramatically reduced in the LPR compared to the CS (Kasai and Scott 2001a). *CYP6A1* is PB inducible and also showed overexpression in the LPR strain (Carino et al. 1992), although PB inducibility of *CYP6A1* in the LPR strain has not yet been examined. These facts of overexpression among multiple PB inducible P450s indicated the systematic effect attributed to the malfunction and perturbation of an unidentified *trans* acting factor regulating PB induction in the LPR strain.

1.6.9 Is there a master *trans* acting factor of metabolism-mediated resistance?

In the house fly, metabolism-mediated resistance to DDT, organophosphorus insecticides, pyrethroids, and juvenoids has consistently been mapped to chromosome 2 of the house fly (Plapp 1984), and a postulated *trans* acting factor was located close to markers *aristapedia* and *carnation eye* (Wang and Plapp 1980). Other detoxification genes, such as *CYP12A1* (Guzov et al. 1998), *GST-1*, and *GST-3* (Fournier et al. 1992) appeared to under control by a *trans* acting factor mapped to this chromosomal area in the house fly. In the Rutgers strain, besides *CYP6A1*, overexpression was also noticed in *CYP12A1* and *GST-1* (Plapp 1984, Guzov et al. 1998), suggesting the perturbation of a common *trans* acting factor co-regulating their expression. In the LPR strain, overexpression of *CYP6D1* (Scott et al. 1996, Liu and Scott 1997a, 1997b), *CYP6D3* (Kasai and Scott 2001a), and *CYP6A1* (Carino et al. 1992) was detected; and a *trans* acting factor on chromosome 2 has been indicated to be involved in overexpression of *CYP6D1* (Liu and Scott 1997a). A master *trans* acting factor responsible for regulating expression of detoxification genes and metabolism-mediated resistance has been proposed (Plapp 1984). So far, the identity of the postulated master *trans* acting factor is still unknown. Lack of a genome sequence for the house fly presents a major impediment to the identification of this putative factor.

CHAPTER 2

RESEARCH GOALS

Cytochrome P450 *CYP6D1* encodes an enzyme involved in metabolism of xenobiotics in the house fly (*Musca domestica*). Expression of *CYP6D1* is phenobarbital (PB) inducible in insecticide susceptible strains, such as CS and *aabys*. In the permethrin resistant LPR strain of house fly, increased transcription of *CYP6D1* confers metabolism-mediated pyrethroid resistance. In LPR, *CYP6D1* is constitutively overexpressed, but expression is not further inducible with PB. Currently, not much is known about the transcriptional regulation of *CYP6D1*, especially PB induction. The primary goals of my Ph.D. research were to define the *CYP6D1* promoter and PB induction of *CYP6D1*. My secondary goals were to identify the molecular basis of overexpression (and lack of PB induction) of *CYP6D1v1* in LPR. The specific objectives are given below.

Specific Objective 1: Identify the transcription start sites (TSSs) and promoter region critical for basal transcription of *CYP6D1v2* from the insecticide susceptible CS strain. The TSSs of *CYP6D1* were determined using cap structure-selective 5' RACE (Rapid Amplification of cDNA Ends). The promoter region critical for basal transcription of *CYP6D1v2* was identified using the dual luciferase reporter assay system in *Drosophila* S2 cells. Results of these experiments are presented in Chapter 3.

Specific Objective 2: Identify the promoter region and transcription factors (TFs) critical for PB induction of *CYP6D1v2* from the CS strain. The promoter region critical for PB induction of *CYP6D1v2* was identified using the dual

luciferase reporter assay system with or without PB in *Drosophila* S2 cells. TFs expressed in S2 cells and critical for PB induction of *CYP6D1* were identified using RNAi treatment of S2 cells in conjunction with promoter assays. Results of these experiments are presented in Chapter 4.

Specific Objective 3: Investigate PB inducibility of the *CYP6D1v1* promoter from the LPR strain to determine whether or not lack of PB induction in LPR is due to differences in the promoter sequence. The experiment was conducted using the dual luciferase reporter assay system in S2 cells. These results are presented in Chapter 5.

Specific Objective 4: Examine if *HR96* is the transcription factor responsible for the constitutive overexpression of *CYP6D1* in LPR. The full length cDNA of *HR96* was cloned and sequenced from multiple strains to detect if a specific *HR96* allele is present and associated with resistance in LPR. Levels of *HR96* transcript were measured in CS and LPR using quantitative real-time RT-PCR to detect if *HR96* was associated with constitutive overexpression of *CYP6D1* in LPR. These results are presented in Chapter 5.

General knowledge about the transcription and PB induction of *CYP6D1* will be valuable information to understand transcriptional regulation of insect P450s involved in xenobiotic metabolism. In addition, my research will also examine if the PB induction pathway is involved in the increased transcription of *CYP6D1* in LPR, which would represent the first experimental evidence to indicate the role of PB induction pathway in the evolution of metabolism-mediated resistance conferred by overexpression of PB inducible P450s.

CHAPTER 3

IDENTIFICATION OF TWO TRANSCRIPTION START SITES AND A *CIS*-REGULATORY SEQEUNCE RESPONSIBLE FOR TRNASCRIPTION OF HOUSE FLY (*MUSCA DOMESTICA) CYP6D1*

3.1 Introduction

Cytochrome P450s constitute a large gene superfamily. The number of P450 genes in insect species ranges from 46 to 164 (Figure 1.1) (http://drnelson.utmem.edu/CytochromeP450.html). In insects, P450s are responsible for and involved in a great variety of biological functions and pathways, from the biosynthesis of endogenous hormones to the metabolism and detoxification of foreign compounds (xenobiotics)(Feyereisen 2005, Scott 2008). Insect P450s are grouped into four evolutionary clades (CYP2, CYP3, CYP4, and mitochondrial)(Nelson 2006), which are associated with general biological functions (Feyereisen 2006, Baldwin et al. 2009). For example, the CYP3 clade encompasses P450s associated with metabolism of xenobiotics. Despite the rapid pace at which insect P450 genes are being discovered, much less is known about their transcriptional regulation.

House fly (*Musca domestica*) *CYP6D1* belongs to the CYP3 clade. CYP6D1 is involved in metabolism of xenobiotics, such as benzo(a)pyrene (Wheelock and Scott 1992b), chlorpyrifos (Hatano and Scott 1993), cypermethrin (Zhang and Scott 1996), deltamethrin (Wheelock and Scott 1992a), methoxyresorufin (Wheelock and Scott 1992b), and phenanthrene (Korytko et al. 2000). *CYP6D1* is located on chromosome 1 (Liu and Scott 1995), it is expressed only in adults (Scott et al. 1996), but it is found in numerous tissues (Lee and Scott 1992, Korytko and Scott 1998). Sequences of the 5' flanking region of the *CYP6D1* gene have been determined in five strains of the

house fly and the *CYP6D1* promoter contains a putative vertebrate Gfi-1 binding site (Scott et al. 1999) capable of binding house fly Gfi-1 (Kasai and Scott 2001b, Gao and Scott 2006b). However, the region of the *CYP6D1* promoter responsible for basal transcription has not been identified.

The goal of this study was to better understand transcription of *CYP6D1* by identifying the transcription start site (TSS), and *cis*-regulatory DNA sequence(s). Using a cap structure selective 5' rapid amplification of cDNA ends (RACE), two *CYP6D1* TSSs were identified. The position of the proximal TSS is defined as +1, and the distal TSS is located at -26. The presence of two TSSs defined the core promoter of *CYP6D1* as a dispersed type. Promoter assays using progressive 5' serial deletions of the *CYP6D1* promoter expressed in *Drosophila* S2 cells identified promoter region from -280 to -246 was responsible for basal transcription of *CYP6D1*. Bioinformatic tools identified Adf-1, HSF, GAGA, and CTCF as putative *CYP6D1* TFs. The potential roles of these TFs in the regulation of *CYP6D1* are discussed.

3.2 Materials and methods

3.2.1 House flies

The CS and *aabys* strains (Hamm et al. 2005) of house fly were used. House fly larvae were reared on mixed media made of 500 g of calf manna (Agway, Ithaca, NY), 120 g of wood chips (Agway), 60 g of Baker's yeast (MP Biomedicals, Solon, OH), 1210 g of wheat bran (Agway) and 2000 ml of water. Adults were fed on powdered milk:granulated sugar (1:1) and water *ad libum*. Larvae and adults were reared at 28ºC, 60% relative humidity, with a 12: 12 hr light/dark photoperiod.

3.2.2 Preparation of mRNA

Eight to ten abdomens of three-day-old adult male house flies were used for

mRNA preparation using Illustra QuickPrep™ micro mRNA purification kit (GE healthcare, Little Chalfont, UK), 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, Waltham, MA).

3.2.3 Identification of the TSS

The TSS of *CYP6D1* was determined using FirstChoice® RLM-RACE (Ambion, Austin, TX) kit according to manufacturer's instructions. This technique generates a cap structure selective RACE product (generated from random decamer primers using 250 ng mRNA as template) coupled to a specific adapter. Next, PCR was performed in 50 µl of reaction volume containing 1 µl of Advantage 2 Polymerase (Clontech, Mountain View, CA), 2 µl of above 5'-RACE adaptor-coupled cDNA products, and 20 pmole of forward and reverse primers. Reactions were carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA) with the following temperature program: 95ºC for 2 min; 35 cycles of 95ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec; and 72ºC for 4 min. The first step PCR was performed using 5'-RACE outer primer (5'-GCTGA TGGCG ATGAA TGAAC ACTG-3') and *CYP6D1*-specific outer primer (5'-GAAGA ACAAA TAAAT GCCCA CCACC-3'). Nested PCR was performed using 1 µl of the first step PCR product with 5'-RACE inner primer (5'-CGCGG ATCCG AACAC TGCGT TTGCT GGCTT TGATG-3') and *CYP6D1*-specific inner primer (5'-ATACC TTTGC CATCG AGCCC A-3'). PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide (5 µg/ml). PCR products resolved on the agarose gel were individually excised and extracted using QIAEX II kit (Qiagen, Valencia, CA), according to manufacturer's instructions.

TA cloning of purified PCR products was performed using TOPO TA kit with pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) and TOP 10 competent cells

(Invitrogen), according to manufacturer's instructions. Plasmid DNA was purified using 2 ml of culture and a QIAprep Miniprep system (Qiagen), according to manufacturer's instructions.

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

Progressive serial 5' deletions of the *CYP6D1* promoter region from the CS strain were generated by PCR amplification. Restriction enzyme sites (*Sac* I and *Bgl* II) were added upstream and downstream, respectively, by the design of forward and reverse primers used in PCR. PCR products of promoter regions coupled with restriction sites were purified using QIAEX II kit (Qiagen) and sequentially digested by restriction enzymes *Sac* I and *Bgl* II (NEB, Ipswich, MA) at 37ºC overnight. Purified digested promoter regions were individually ligated into *Sac* I and *Bgl* II sites, located upstream of the firefly luciferase reporter gene, in the pGL3-Basic vector (Promega, Madison, WI) using T4 DNA ligase (Invitrogen). Ligation products were then individually transformed into TOP 10 competent cells (Invitrogen) by heat shock at 42ºC for 30 sec. Procedures of plasmid DNA purification, and sequencing were the same as described above. Promoter construct sequences were confirmed prior to use in the promoter assays. The concentration of each promoter construct was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific).

3.2.5 *Drosophila S2* cells

Drosophila S2 cells obtained from Dr. J. Lis (Molecular Biology and Genetics, Cornell University) were incubated in HyQ SFX-Insect cell culture medium (HyClone, Logan, UT) in a 75 cm^2 of tissue culture flask (BD Falcon, Bedford, MA) at 25±1ºC. Cells were subcultured every 2-3 days as they reached confluency. *Drosophila* S2

cells were used because they are well studied and no cell line is currently available from house flies.

3.2.6 Transfection and dual luciferase reporter promoter assay

To identify the *cis*-regulatory sequence(s) involved in the transcription of *CYP6D1*, we evaluated different lengths of the promoter using the dual luciferase reporter assay system (Promega) in *Drosophila* S2 cells. Individual promoter constructs (promoter region coupled to firefly luciferase reporter gene in pGL3-Basic vector) were co-transfected with pRL-TK vector (Promega) carrying *Renilla* luciferase reporter gene. For transfection, each well (bottom diameter: 22.09 mm) of a 12-well tissue culture plate (BD Falcon) was seeded with 1 x 10^6 S2 cells in 1 ml of HyQ SFX-Insect medium. The cell culture medium (including non-adhered cells) was removed after 30 min and 300µl of new cell culture medium was added along with 500 µl of transfection reagent (containing 10.377 fmole of the promoter construct, 3.746 fmole of pRL-TK vector and 7.75 µl of cellfectin® reagent (Invitrogen) in HyQ SFX-Insect medium). For all promoter constructs, equal molar amounts were applied for each transfection. After incubation for 3.5 hr, the transfection reagent was replaced with 1 ml of HyQ SFX-Insect medium, and the transfected cells were then incubated for 48 hr. Afterward, settled transfected cells were washed with 1X PBS buffer (1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 8 g of NaCl, and 0.2 g of KCl dissolved in 1 liter of ddH_2O) and lysed by incubating with 250 µl of 1X 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), which sequentially injected 50 µl of LAR II and 50 µl of Stop & Go Reagents (Promega) to the cell lysate, and then measured luminescences derived from firefly luciferase and *Renilla* luciferase. Luminescence derived from firefly luciferase was normalized by

luminescence derived from *Renilla* luciferase to correct for variation in transfection efficiencies. The normalized firefly luminescence represented the promoter activity driven by corresponding 5' deletion promoter region of *CYP6D1*. Three independent transfections of three replicates for each promoter construct (n = 9) were conducted. Relative luciferase activity represented in figures was derived by normalizing normalized firefly luminescence of each promoter construct to the mean derived from signals of pGL3-basic construct in the same replicate. Statistical analysis was conducted by pairwise comparison of relative luciferase activities from two adjacent serial promoter constructs by using Student's *t*-test.

3.2.7 Bioinformatic analyses

Prediction of putative TF binding site(s) within the 34 bp of the *cis*-regulatory sequence was conducted using TFsearch (version 1.3) (Heinemeyer et al. 1998), Match (version 1.0 public) (Kel et al. 2003), and Patch (version 1.0, developed from precursor PatSearch (Grillo et al. 2003)). These three tools correspond to two distinct types of searching strategies. TFsearch and Match use a weight matrix approach to look for any regional query sequence sharing high similarity/identity to the general consensus of a binding sequence weight matrix of a TF. Patch searches along the query sequence to identify a sequence exactly the same to any known TF binding sequence. Searches were done using the TRANSFAC database (Heinemeyer et al. 1998) limited to metazoa.

The expression of known or putative TFs in S2 cells was examined using public microarray datasets deposited in the Gene Expression Omnibus database (GEO) (Edgar et al. 2002) and FLIGHT (http://flight.licr.org) (Sims et al. 2006). *Drosophila* homologs of vertebrate TFs were identified using the HomoloGene database at NCBI. The BLAST search to define the best hit from *D. melanogaster* corresponding to

vertebrate putative TFs was performed using BLASTP (2.2.20+) program (BLAST at NCBI) searching against database of *D. melanogaster* RefSeq protein (gp/7227.9554/dm_refp).

3.3 Results and discussion

3.3.1 Identification of two *CYP6D1* TSSs

Cap structure selective RACE was used to determine the *CYP6D1* TSS. The PCR products of the CS and *aabys* strains showed two sharp bands with sizes of ~280 and ~250 bp (Figure 3.1A), indicating the presence of two *CYP6D1* TSSs. Clones obtained from these PCR products revealed two identical TSSs for *CYP6D1* in the CS and *aabys* strains. The proximal TSS was located 85 nt upstream of the translation start site and we defined the position of this TSS as +1 (Figure 3.1B). The distal TSS was located 111 nt upstream of the translation start site and we defined this position as -26 (Fig. 2). The presence of two TSSs within 27 nt indicates that *CYP6D1* has a dispersed type promoter (Juven-Gershon et al. 2008).

One *CYP6D1* TSS was previously reported in the CS and *aabys* strains using primer extension (Scott et al. 1999). This TSS is 1 nt upstream of the proximal TSS identified in our current study. The use of cap structure selective 5' RACE insures the identified TSS was derived from the full-length transcript, compared to the technique of primer extension. Our identification of two TSSs for *CYP6D1* probably reflects the greater sensitivity and resolution of the cap structure selective 5' RACE. The same results from CS and *aabys* flies suggest that two TSSs are not limited to a single strain.

3.3.2 The core promoter of *CYP6D1*

The core promoter region typically covers ~40 bp upstream and downstream of

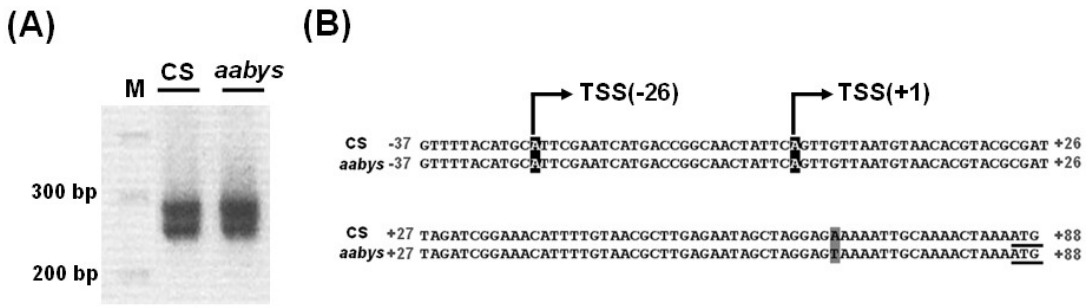


Figure 3.1: (A) Agarose gel (negative image) showing 5' RACE nested PCR products. Two major bands appear in both the CS and *aabys* strains indicating two *CYP6D1* TSSs. **M** indicates the lane containing the DNA standard ladder (1Kb plus DNA ladder, Invitrogen). (B) Positions of the two *CYP6D1* TSSs. Single nucleotide polymorphisms between the CS and *aabys* are shaded in grey, and locations of TSS are shaded in black. The proximal TSS identified in the CS and *aabys* strains was defined as +1. The position of the distal TSS is -26. The location of translation start site (ATG) is underlined.

the TSS. Dispersed type core promoter was reported to be associated with the lack of a TATA box in the core promoter (Carninci et al. 2006, Juven-Gershon et al. 2008). We examined the sequence (from positions -75 to +75) of *CYP6D1* covering both TSSs and did not detect the presence of a TATA box within the core promoter of *CYP6D1* using TFsearch (with positive control using core promoter sequences of *CecA1* and *Pdg* from *Drosophila* Core Promoter Database). Further studies will be needed to identify if different TSSs are used by different tissues, as is commonly found in mammals (Carninci et al. 2006).

P450s with dispersed type core promoters are not limited to *CYP6D1*. Two TSSs were also identified for human *CYP2F1*. The two TSSs of *CYP2F1* were

separated by 40 nt and the core promoter lacked a TATA box (Carr et al. 2003). Two distinct TSSs, separated by 75 nt, were found for rat *CYP11A1*. The distal TSS was used in the kidney and the proximal TSS was used in the testis (Shemer et al. 1992).

3.3.3 Identification of the *cis*-regulatory sequence responsible for basal transcription of *CYP6D1*

CYP6D1 promoter assays were conducted in *Drosophila* S2 cells using the dual luciferase reporter system (Promega) with serial 5' deletions (i.e. -451/+85, -344/+85, -246/+85, -211/+85, and -42/+85) of the promoter. A significant increase in the relative luciferase activity was detected in the promoter construct -344/+85 compared to the adjacent promoter construct -246/+85 (Figure 3.2), indicating the presence of *cis*-regulatory element(s) between positions -344 and -246 responsible for elevating transcription. To more specifically define the location of the *cis*-regulatory element(s), six promoter constructs (i.e. -344/+85, -330/+85, -312/+85, -298/+85, -280/+85, and -246/+85) were evaluated. A significant difference was detected between two adjacent promoter constructs (-280/+85 and -246/+85) (Figure 3.3) indicating the 34 bp between positions -280 and -246 contains a *cis*-regulatory sequence responsible for transcriptional activation of *CYP6D1*.

3.3.4 Putative TFs

Prediction of putative insect TF binding site(s) within *CYP6D1* promoter region (-280 to -246) was conducted using TFsearch (version 1.3), Match (version 1.0 public), and Patch (version 1.0). Putative binding sites of four *Drosophila* TFs, Bcd (*bicoid*), HSF (*heat shock factor*), Adf-1 (*adh transcription factor 1*), and the GAGA factor were identified (Table 3.1, 3.2 and 3.3) and their positions within this promoter region were shown in Figure 3.4.

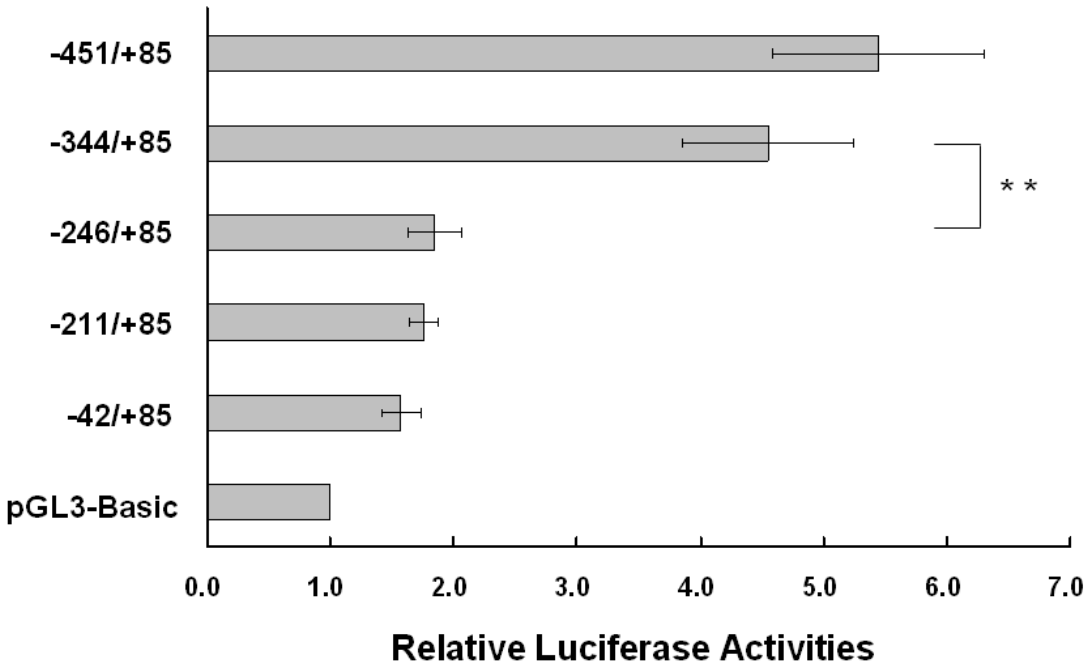


Figure 3.2: Promoter assay with progressive serial deletions of *CYP6D1* 5' flanking region. Promoter constructs are numbered relative to the TSS at +1. Signals derived from each promoter construct were normalized to the mean derived from signals of pGL3-Basic vector in the same replicate. Bars represent the mean ± S.D. of three independent transfections of three replicates (n = 9). A significant difference between two adjacent promoter constructs ($p < 0.01$, Student's *t*-test) is indicated by an asterisk.

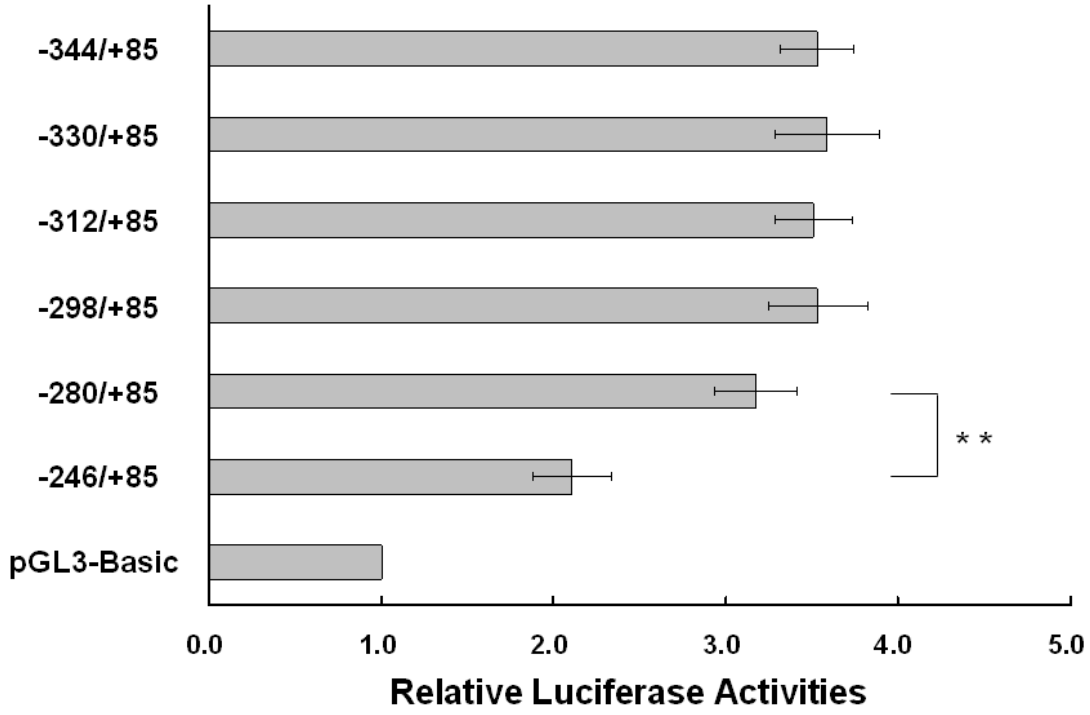


Figure 3.3: Promoter assay corresponding to promoter region between positions -344 and -246. Promoter constructs are numbered relative to the TSS at +1. Signals derived from each promoter construct were normalized to the mean derived from signals of pGL3-Basic vector in the same replicate. Bars represent the mean ± S.D. of three independent transfections of four replicates (n = 12). A significant difference between two adjacent promoter constructs ($p < 0.01$, Student's *t*-test) is indicated by an asterisk.

Table 3.1: Putative *Drosophila* TF binding sites within *CYP6D1* promoter region (-280 to -246) identified by TFsearch with cut off score 85.0.

Transcription factor	Consensus sequence[a]	Score	Positions
Bcd	SGGATTAN	87.3	-271/-264
HSF	AGAAN	94.3	-259/-255

[a] Consensus sequence used by TFsearch.

Table 3.2: Putative *Drosophila* TF binding sites within *CYP6D1* promoter region (-280 to -246) identified by Match with cutoff similarity 0.85 for the core and matrix.

Transcription factor	Consensus sequence[a]	Core similarity	Matrix similarity	Positions
Bcd	aTAATCgc	1.000	0.963	-271/-264

[a] Consensus sequence used by Match.

Table 3.3: Putative *Drosophila* TF binding sites within *CYP6D1* promoter region (-280 to -246) identified by Patch without mismatch to search pattern.

Transcription factor	Sequence (Search pattern)	Positions
Bcd	TATAATCGC	-272/-264
Adf-1	GCAGC	-265/-261
GAGA factor	CTCGC	-262/-258

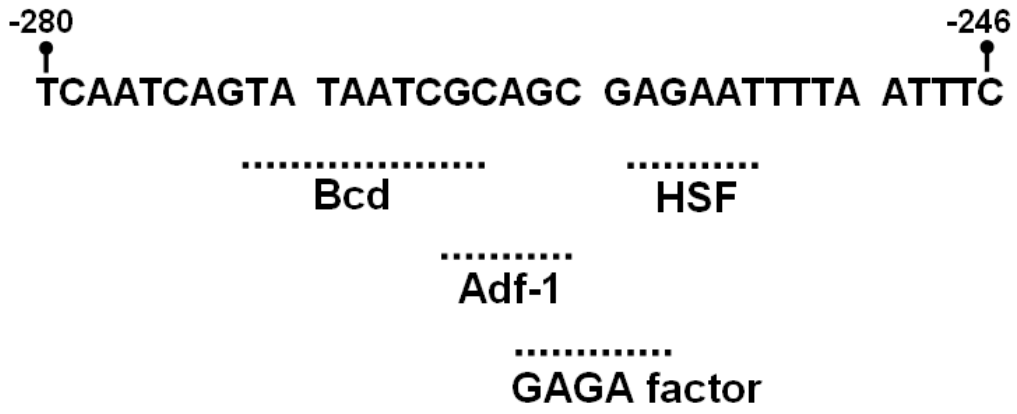


Figure 3.4: Positions of putative *Drosophila* TF binding site within *CYP6D1* promoter region (from -280 to -246) identified by TFsearch, Match, and Patch.

Table 3.4: Expression of putative *Drosophila* TFs in S2 cells based on microarray datasets from GEO and FLIGHT.

	Expression in S2 cells	
Transcription factor	By GEO[a]	by FLIGHT
Bcd	-	-
HSF	+	+
Adf-1	+	+
GAGA factor	ND[b]	+/-[c]

[a] Expression evidence is derived from dataset DGS1472 in GEO database at NCBI.

[b] ND, not determined, due to the absence of a probe to the gene of interest in the microarray.

[c] According to the two datasets found in the FLIGHT database, one called present (+) and the other called absent (-) for the expression in S2 cells.

Because promoter assays were conducted using *Drosophila* S2 cells, if the putative *Drosophila* TF identified is critical for *CYP6D1* transcription, it must be expressed in S2 cells. Expression of above four putative *Drosophila* TFs in S2 cells was examined using microarray data available from GEO and FLIGHT database and was shown on Table 3.4. Based on microarray data, Adf-1, HSF, and the GAGA factor are expressed in S2 cell, but Bcd is not. Therefore, roles of putative TFs Adf-1, HSF, and the GAGA factor in *CYP6D1* transcription are further discussed below.

In *D. melanogaster*, Adf-1 activates transcription of *adh* (*alcohol dehydrogenase*) (Heberlein et al. 1985). Expression of Adf-1 is ubiquitous in all life stages, based on *in situ* hybridization (England et al. 1992, DeZazzo et al. 2000). With the relatively close evolutionary distance between house fly and fruit fly, we speculate that house fly Adf-1 may have a similar expression pattern as the fruit fly, and could be involved in activating transcription of *CYP6D1*.

HSF (heat shock factor) is a TF responsible for activating transcription of heat shock proteins (Sorger 1991, Morimoto 1993). Given that we did not subject cells to conditions that would be expected to trigger activation of heat shock factors, it appears HSF is not independently involved in regulating the constitutive expression of *CYP6D1*. However, HSF and GAGA factors can serve as a cooperative regulatory mechanism as discussed below.

The GAGA factor was first characterized as a transcriptional activator required for *Ultrabithorax* (*Ubx*) and *engrailed* (*en*) genes (Biggin and Tjian 1988, Soeller et al. 1988). Subsequent studies found GAGA factor was involved in mediating the modification of chromatin structure, resulting in nucleosome disruption (Tsukiyama et al. 1994, Lehmann 2004). It is unlikely there is any involvement of a chromatin remodeling mechanism in our promoter assay. However, since chromatin remodeling is able to regulate genes expressed in a developmental stage specific

manner (Felsenfeld 1996), and since expression of *CYP6D1* is developmentally regulated (i.e. adult specific) (Scott et al. 1996), identification of the GAGA binding site suggests it might play a possible role in the developmental regulation of *CYP6D1* in the house fly. Another possibility is that HSF and the GAGA factor interact to control *CYP6D1* transcription. Co-occurrence of adjacent GAGA factor and HSF binding sites are required for DNase I hypersensitivity (Wall et al. 1995). It has also been reported that GAGA factor interacts with HSF to stabilize HSF binding to its cognate binding sequence (Mason and Lis 1997). Given the presence of adjacent GAGA factor and HSF binding sites in the *cis*-regulatory sequence of *CYP6D1*, we speculate both factors may be involved in controlling transcription of *CYP6D1*.

Putative binding sites of vertebrate TFs within *CYP6D1* promoter region (-280 to -246) were identified using TFsearch, Match, and Patch (Table 3.5, 3.6, 3.7, and Figure 3.5). *Drosophila* orthologs corresponding to these putative vertebrate TFs were searched using HomoloGene database at NCBI or using BLAST search (Table 3.8). Expression of respective putative *Drosophila* orthologs in S2 cells was examined using microarray datasets from GEO and FLIGHT (Table 3.8). The result showed only two of the vertebrate TFs GATA-3 (*Gallus gallus*) and CTCF (*Homo sapiens*) had *Drosophila* homologs (Grain and CTCF, respectively) identified in the HomoloGene database, but only CTCF showed evidence of expression in S2 cells (Table 3.8), which was further discussed in next paragraph. While the evolutionary distance between insects and vertebrates precludes unequivocal identification of the factors listed in Table 3.8 as regulators of *CYP6D1* transcription, the list is valuable as a rational starting point to examine *CYP6D1* TFs.

In vertebrates, studies showed that CTCF (CCCTC-binding factor) is required for the functioning of insulators (Bell et al. 1999). The role of insulators is to establish the euchromatin/heterochromatin boundaries (Gerasimova and Corces 2001), which

Table 3.5: Putative vertebrate TF binding sites within *CYP6D1* promoter region (-280 to -246) identified by TFsearch with cut off score 85.0.

Transcription factor (species)	Consensus sequence[a]	Score	Positions
CdxA (*G. gallus*)	WWTWMTR	86.4	-272/-266
CdxA (*G. Gallus*)	WWTWMTR	85.0	-275/-269

[a] Consensus sequence used by TFsearch.

Table 3.6: Putative vertebrate TF binding sites within *CYP6D1* promoter region (-280 to -246) identified by Match with cutoff similarity 0.85 for the core and matrix.

Transcription factor (species)	Consensus sequence[a]	Core/Matrix Similarity	Positions
Gfi-1 (*R. norvegicus*)	atcagtatAATCGcagcgagaatt	0.94/0.90	-277/-254
S8 (*M. musculus*)	agtaTAATCgcagcga	0.87/0.85	-274/-259
GATA-3 (*G. gallus*)	taTAATCgca	0.95/0.86	-272/-263
GATA-2 (*G. gallus*)	taTAATCgca	0.87/0.86	-272/-263
Nkx2-5 (*M. musculus*)	taTAATCg	1.00/0.87	-254/-247

[a] Consensus sequence used by Match.

Table 3.7: Putative vertebrate TF binding sites within *CYP6D1* promoter region (-280 to -246) identified by Patch without mismatch to search pattern.

Transcription factor (species)	Sequence (Search pattern)	Positions
GATA-1 (*M. musculus*)	TGATT	-278/-274
CTCF (*H. sapiens*)	GCTGC	-265/-261
C/EBP alpha (*G. gallus*)	AGAATTT	-259/-253
Gbx2 (*G. gallus*)	TTAAT / ATTAA	-253/-249

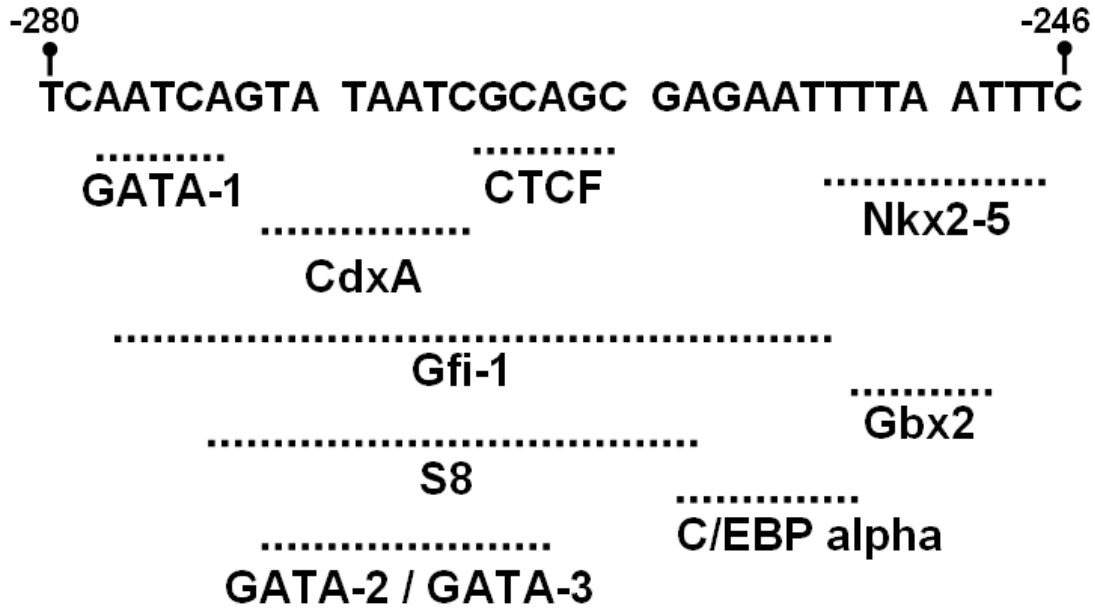


Figure 3.5: Positions of putative vertebrate TF binding site within *CYP6D1* promoter region (from -280 to -246) identified by TFsearch, Match, and Patch.

Table 3.8: *Drosophila* homologs or BLAST best hit corresponding to putative vertebrate TFs and their expression in S2 cells.

Vertebrate putative TFs (species)	*Drosophila* homolog[a] (HomoloGene entry ID)	BLAST best hit of *Drosophila*	BLAST E-value	BLAST score	Alignment coverage[b]	Expression in S2 cells by GEO[c]	Expression in S2 cells by FLIGHT
CdxA (*G. gallus*)	- (1366)	Caudal	8e-30	127	82/427	+/-	-
Gfi-1 (*R. norvegicus*)	- (3854)	Senseless-2	9e-83	304	165/746	-	ND[d]
S8 (*M. musculus*)	- (7524)	CG9876	2e-30	129	131/275	-	-
GATA-2 (*G. gallus*)	- (32030)	Grain (isoform A)	7e-64	241	306/486	-	-
GATA-3 (*G. gallus*)	Grain (1550)	Grain (isoform A)	8e-63	238	341/486	-	-
Nkx2-5 (*M. musculus*)	- (49239)	Forkhead 59A	8e-53	201	101/456	-	ND[d]
GATA-1 (*M. musculus*)	- (1549)	Grain (isoform B)	1e-54	210	119/699	-	-
CTCF (*H. sapiens*)	CTCF (4789)	CTCF	2e-79	293	363/818	+	+
C/EBP alpha (*G. gallus*)	- (3211)	Slow border cells	8e-09	57.6	75/449	+/-	-
Gbx2 (*G. gallus*)	- (1138)	Unplugged	1e-38	157	96/485	-	-

[a] *Drosophila* homologs indicated by HomoloGene at NCBI.

[b] Alignment coverage is equal to (number of amino acids aligned in the BLAST alignment) / (number of amino acids of the subject protein sequence in full length).

[c] Expression evidence is derived from dataset DGS1472 in GEO database at NCBI.

[d] ND, not determined, due to the absence of a probe to the gene of interest in the microarray.

plays an important role in the genomic organization of transcriptional regulation. *Drosophila* CTCF was identified and is required for the functioning of insulator Fab-8 (Moon et al. 2005), indicating similar biological function in insects and vertebrates. Consensus of the *Drosophila* CTCF binding sequence has been determined recently using a ChIP-array approach. The identification of 23 candidate CTCF binding fragments led to a strong AGG TGG CGC binding consensus towards its 3' end (Holohan et al. 2007). However, using the Blast 2 program (NCBI) and manual sequence comparison, there were no matches found with greater than 50% identity to the above binding consensus, which indicates the *Drosophila* CTCF consensus was not present within the 34 bp of the *cis*-regulatory sequence of *CYP6D1* and implies CTCF might not be involved in regulating transcription of *CYP6D1*.

One limitation to the study of transcription in house flies (and other non-model insects) is the lack of a cell line from this species. While *CYP6D1* has an identifiable Gfi-1 binding site in the promoter (Scott et al. 1999) which binds to house fly Gfi-1 (Gao and Scott 2006b), the lack of expression of Gfi-1 in S2 cells (Table 3.3) likely resulted in no effect of this region on our transcription assay. Thus, while analysis of house fly TFs using S2 cells is unquestionably valuable, the results will not always perfectly reflect the TFs used by the house fly.

In summary, the lack of a TATA box in the core promoter, together with presence of two TSSs, indicates that *CYP6D1* has a dispersed type promoter. Basal transcription of *CYP6D1* is controlled by a *cis*-regulatory sequence (from -280 to -246). Putative insect TFs binding to this sequence are Adf-1, HSF, and the GAGA factor, and these factors are all expressed in S2 cells. Adf-1 could be a potential activator for *CYP6D1* transcription. The adjacent co-occurrence of HSF and GAGA factor binding sites implies a regulatory mechanism of chromatin remodeling might be involved in the transcription of *CYP6D1* in the house fly. A human CTCF binding

site was identified within the *CYP6D1 cis*-regulatory sequence and the *Drosophila* CTCF ortholog is expressed in S2 cells. However, the recently identified binding consensus of *Drosophila* CTCF was not found in the *cis*-regulatory sequence. The identification of TSSs, the *cis*-regulatory DNA sequence, and putative TFs provides a fundamental understanding of transcriptional regulation of *CYP6D1*, and lays the foundation for further studies.

CHAPTER 4

HR96 AND BR-C MODULATE PHENOBARBITAL INDUCED TRANSCRIPTION OF CYTOCHROME P450 *CYP6D1* IN *DROSOPHILA* S2 CELLS

4.1 Introduction

Phenobarbital (PB) has been a prototypical inducer used for the study of xenobiotic responses in animals since it was discovered to cause induction of total cytochrome P450s more than 40 years ago (Conney 1967). In response to PB, animals show increases in expression of numerous genes, especially those involved in detoxification and metabolism, such as cytochrome P450s (P450s), glutathione *S*-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs) (Gerhold et al. 2001, Hamadeh et al. 2002, King-Jones et al. 2006, Sun et al. 2006, Willoughby et al. 2006). In mammals, CAR (constitutive androstane receptor) and PXR (pregnane X receptor) are key transcription factors (TFs) critical for regulating PB induced transcription of P450s (Sueyoshi and Negishi 2001, Timsit and Negishi 2007). In response to PB, CAR and PXR associate with their common TF partner RXR (retinoid X receptor) resulting in heterodimers of CAR-RXR and PXR-RXR, respectively, which in turn bind to target DNA sequences and activate transcription of regulated genes (Sueyoshi and Negishi 2001, Timsit and Negishi 2007). CAR and PXR are nuclear receptors, which are ancient ligand-activated transcription factors regulating pathways involved in metabolism, and are unique in Metazoa (Escriva et al. 2004, Baker 2005). Chicken and nematode orthologs of mammalian CAR and PXR have been shown to play a key role in regulating PB and other xenobiotic responses (Handschin et al. 2000, Handschin et al. 2001, Lindblom et al. 2001).

In insects, two general approaches have been used to identify TFs responsible for

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

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

The transcription of house fly (*Musca domestica*) cytochrome P450 *CYP6D1* is PB inducible (Scott et al. 1996, Liu and Scott 1997a). Given that *Drosophila* S2 cells are able to mediate PB induced transcription of *Cyp6a2* (Dunkov et al. 1997), we conducted promoter assays in *Drosophila* S2 cells to locate the PB responsive *cis*-

regulatory sequence of the *CYP6D1* promoter using progressive serial 5' deletions. The promoter sequence from position -330 to -280 (numbers are relative to the transcription start site, defined as +1) was found to be critical for PB induced transcription. Putative binding sites of *Drosophila* BR-C (*broad-complex*) and DFD (*deformed*) were found within the promoter sequence from -330 to -280. To identify TFs expressed in *Drosophila* S2 cells critical for PB induction of *CYP6D1*, RNAi treatment of S2 cells in conjunction with promoter assays was conducted and to examine if *Drosophila* HR96, BR-C, or DFD is critical for PB induction. Our results identified *Drosophila* HR96 and BR-C acting as positive and negative transcriptional regulators of PB induction of *CYP6D1*, respectively. Reaction of HR96 and BR-C were PB specific and PB dependent. This represents the first direct functional and *in vivo* evidence of the role of HR96 and BR-C in regulating PB induced transcription in insects.

4.2 Materials and methods

4.2.1 *Drosophila* S2 cells

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

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

Progressive serial 5' deletions of the *CYP6D1* promoter from the CS strain (Scott et al. 1999) were generated by PCR amplification. Restriction enzyme sites (*Sac* I and *Bgl* II) were added upstream and downstream by incorporation into the forward and reverse primers used in PCR, respectively. These PCR products were purified

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

4.2.3 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 x 10^6 S2 cells in 1 ml of HyQ

SFX-insect medium. Thirty minutes later, the cell culture medium (including non-adhered cells) was removed, 300µl of new cell culture medium and 500 µl of transfection reagent mix (containing 10.377 fmole of one *CYP6D1* promoter construct, 3.746 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 hr, the transfection reagent mix was replaced with 1 ml of HyQ SFX-insect medium containing ±0.5 mM PB (Sigma-Aldrich, St. Louis, MO), and the transfected cells were then incubated for 48 hr. This concentration (0.5 mM) of PB was chosen based on our preliminary test of concentration-response with serial concentrations of PB (0.5 mM of PB caused the greatest PB induction without causing toxicity to cells; data not shown), and because it had been used previously (Dunkov et al. 1997). Settled transfected cells were washed with 1X PBS buffer (1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 8 g of NaCl, and 0.2 g of KCl dissolved in 1 L of ddH_2O) and lysed by incubating with 250 µl of 1X 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), which sequentially injected 50 µl of LAR 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, done side-by-side) of three replicates for each promoter construct (n = 9) were conducted. Statistical analysis of pairwise comparisons of difference of [(PB induced promoter activity) – (basal promoter activity) relative to the next shorter *CYP6D1* promoter construct] was conducted using Student's *t*-test.

4.2.4 Design and preparation of dsRNA probes

RNAi probes were designed in exon regions of target genes and their specificity was confirmed using E-RNAi (Arziman et al. 2005). If multiple isoforms existed (according to Entrez Gene database at NCBI), the RNAi probe was selected for a region shared by all isoforms. A two-step PCR strategy was used to generate DNA template for dsRNA synthesis (Ramadan et al. 2007). In the first step, gene specific primers were used to amplify a fragment of the target gene (i.e. 252 bp of *hr96*, 254 bp of *br-c*, and 266 bp of *dfd*). In the second PCR step, gene specific primers tailed with T7 core promoter sequence (5'-TAA TAC GAC TCA CTA TAG GG-3') were used. Sequences of primers used in the first PCR step were: HR96-dsRNA-F: 5'-AAG CCA TTG CTG GAC AAG GA-3', HR96-dsRNA-R: 5'-GGG CTC GTC GTT GTA GTT GG-3', BR-dsRNA-F: 5'-CCT GCA GTC CCT ACT TCC GC-3', BR-dsRNA-R: 5'-AGC TTG TCG CTG ATG GAG AT-3', DFD-dsRNA-F: 5'-TCG GAG TAT GTG CAA TCC AA-3', and DFD-dsRNA-R: 5'-CAC TCA TAT GAC CCG TAG ATG C-3'. The dsRNA probe corresponding to *lacZ* (beta-*D*-galactosidase of *Escherichia coli*) was prepared using two primers: LacZ-dsRNA-F: 5'-GAA TTA ATA CGA CTC ACT ATA GGG AGA GAT ATC CTG CTG ATG AAG C -3', LacZ-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). To produce dsRNA probes, *in vitro* transcription was performed using AmpliScribe™ T7-Flash Transcription kit (Epicentre, Madison, WI). The reaction was carried out at 37ºC for 4 hr in a total reaction volume of 40 µl that included 1 µg of above purified DNA template, 3.6 µl of 100 mM ATP, 3.6 µl of 100 mM CTP, 3.6

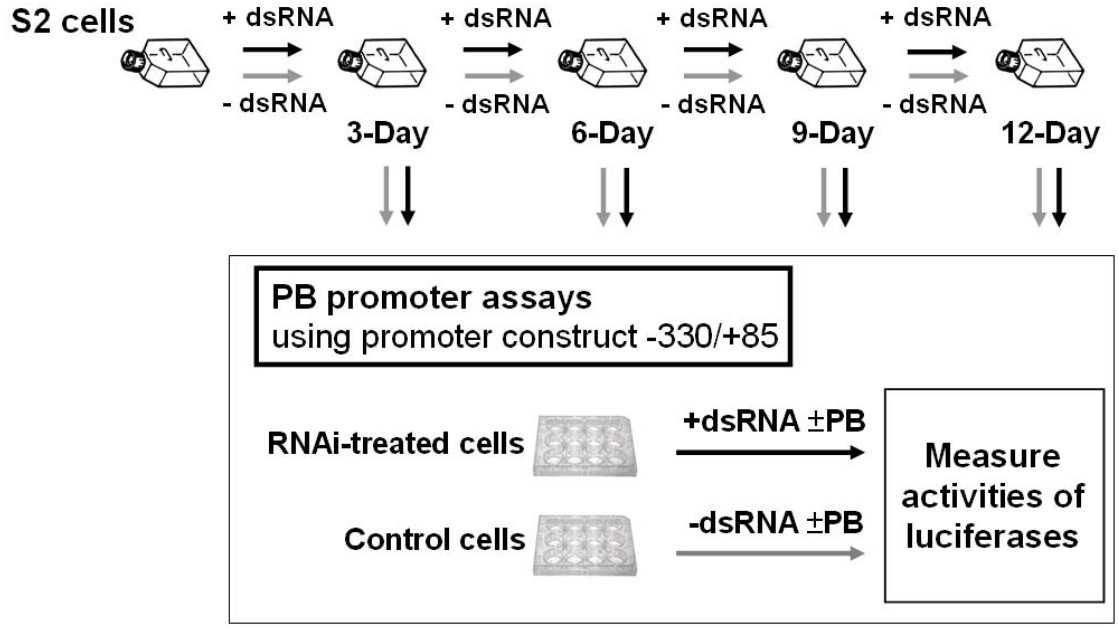
µl of 100 mM GTP, 3.6 µl of 100 mM UTP, 4 µl of 100 mM DTT, 4 µl of 10X buffer, and 4 µl of AmpliScribe^{TM} T7-Flash enzyme solution. Afterward, 2 µl of DNase I was added and incubated at 37ºC for 15 min. RNA was precipitated by adding 50 µl of ddH_2O, 10 µl of 3.0 M sodium acetate pH 5.2, and 250 µl of 95% ethanol and placed at -20ºC for 15 min. Following centrifugation at 13,000 rpm for 15 min at 4ºC, the RNA pellet was sequentially washed with 75% ethanol, air-dried, and resuspended in 400 µl of ddH_2O. The dsRNAs were annealed by incubating at 65ºC for 30 min followed by slow cooling to room temperature (~22ºC) (Clemens et al. 2000). The dsRNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific). Each DNA template used for producing dsRNA probes was sequenced and confirmed prior the use of *in vitro* transcription.

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

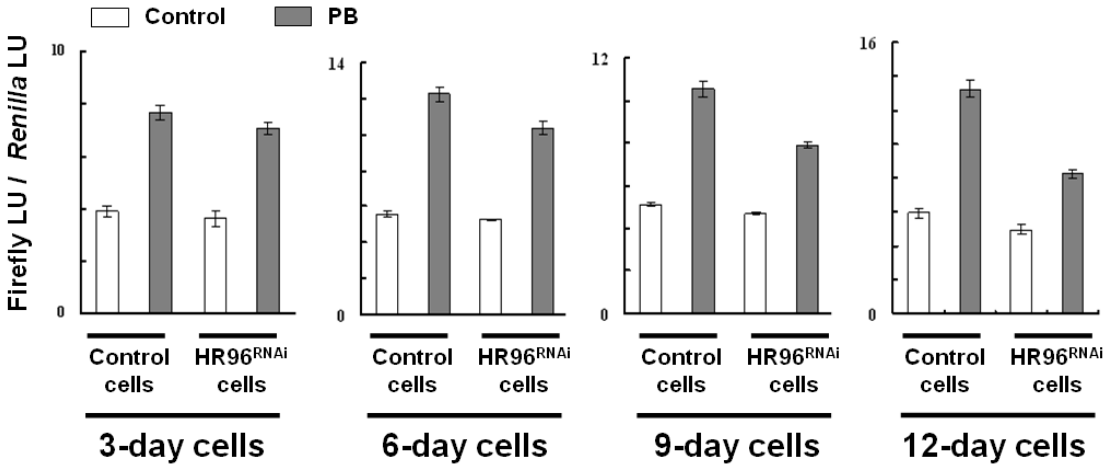
Drosophila S2 cells (4 x 10^6) were cultured in 3 ml of HyQ SFX-insect cell culture medium containing 23.2 µl of Cellfectin® (Invitrogen) and 30 µg of dsRNA probe in a 25 cm^2 of tissue culture flask (Corning, Corning, NY) and maintained in 22 ±1ºC. S2 cells were subcultured every 3 days by passing 4 x 10^6 cells to a new flask with addition of Cellfectin® and dsRNA to continue RNAi. Preliminary test was conducted using dsRNA probe of *hr96* to determine how many days of RNAi-treatment would be enough before the use of the following promoter assays (using *CYP6D1* promoter construct -330/+85) in order to see significant effect in PB induction compared to the use of control cells (without treatment of dsRNA probe). Promoter assays using 3-day, 6-day, 9-day and 12-day RNAi-treated cells (Figure 4.1A) showed, 1.5%, 10.5%, 19.4%, and 25.4% reduction of PB induction,

Figure 4.1: (A) RNAi treatment (using dsRNA probe of *hr96*) of S2 cells in conjunction with the PB promoter assay. *Drosophila* S2 cells were cultured in a 25T flask with Cellfectin with dsRNA probe (RNAi treated cells) or without dsRNA probe (control cells). Cells were subcultured every 3 days and Cellfectin ± dsRNA probe were added. RNAi-treated cells and control cells (without treatment of dsRNA probe) of 3-day, 6-day, 9-day, and 12-day were subjected to PB promoter assays using promoter construct -330/+85. Transfected RNAi-treated cells were continued with treatment of dsRNA probe. After 48 hr incubation with or without PB, firefly and *Renilla* luciferase activities were measured. (B) PB induced promoter activities (gray bars) and basal promoter activities (white bars) were derived from the use of 3-day, 6-day, 9-day, and 12-day of control cells (without treatment of dsRNA) and RNAi-treated cells (using dsRNA probe of *hr96*). Bars represent mean of promoter activity (firefly LU / *Renilla* LU) ± S.D. of three independent transfections (n=3). These results showed promoter assays using 3-day, 6-day, 9-day, 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.

(A)


S2 cells
+ dsRNA
- dsRNA
3-Day
6-Day
9-Day
12-Day
PB promoter assays
using promoter construct -330/+85
RNAi-treated cells
+dsRNA ±PB
Control cells
-dsRNA ±PB
Measure activities of luciferases

(B)


Control
PB
Firefly LU / Renilla LU
Control cells
HR96RNAi cells
3-day cells
6-day cells
9-day cells
12-day cells

respectively, compared to control cells (Figure 4.1B). These results indicated treatment of dsRNA probe for 12 days prior to the following promoter assays could result in enough depletion of target protein level to see significant and clear effect on PB induction. The RNAi-mediated promoter assays using 12-day RNAi-treated cells and control cells were conducted using *CYP6D1* promoter construct -330/+85 and following description above (Transfection and PB responsive promoter assay), except for the introduction of 10 μg (~10 μl) of dsRNA probe immediately before addition of 500 μl of transfection reagent mix into settled RNAi-treated cells for the 3.5 hr incubation. In the following 48 hr incubation of ±0.5 mM PB, 10 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. Mock controls using a dsRNA probe for *lacZ* of *E. coli* were also conducted and there was no significant effect in PB induction in response to the treatment of dsRNA of *lacZ*. Three independent transfections (±PB) with RNAi-treated cells or control cells were performed in each replicate of PB responsive promoter assay. Two or three replicates for each of the target genes were conducted. Statistical analysis of multiple pairwise comparisons was conducted using Student's *t*-test followed by Tukey's HSD (Honestly Significant Difference) test.

4.2.6 Purification of mRNA, synthesis of cDNA, and semi-quantitative RT-PCR

To indicate the effect of depletion of transcript level in response to treatment with dsRNA probe, 9-day and 12-day RNAi-treated cells and control cells were sampled to evaluate the transcript level of target gene. RNAi-treated cells or control cells (~ 2 x 10^6) were pelleted by centrifugation at 3,500 rpm for 3 min and were washed with 1X PBS buffer. The Illustra QuickPrep™ micro mRNA purification kit (GE Healthcare,

Little Chalfont, UK) was used according to the manufacture's instructions. The mRNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Scientific). The mRNA product was treated with DNase I using a DNA free^{TM} kit (Applied Biosystems, Foster City, CA). The reaction was carried out in a total reaction volume of 16.1 µl containing 1 µg of above mRNA, 1.6 µl of 10X buffer, and 1 µl of rDNase I, and was incubated at 37ºC for 20 min. The cDNA synthesis was conducted using SuperScript^{TM} III first-strand synthesis system for RT-PCR (Invitrogen). The RT reaction was in a total reaction volume of 20 µl including 8 µl of above DNase-treated mRNA, 1 µl of 50 µM oligo$(dT)_{20}$, 1 µl of 10 mM dNTP mix, 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT^{TM} (40 U/µl), and 1 µl of SuperScript^{TM} III RT (200 U/µl), and was carried out at 50ºC for 50 min, followed with incubation at 85ºC for 5 min to terminate the reaction.

Semi-quantitative RT-PCR was performed by monitoring PCR products following 15, 20, 25, 30, 35, and 40 PCR cycles. Each PCR reaction was in a total reaction volume of 20 µl containing 10 µl of 2X 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 of ddH_2O. PCR reactions were carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA) with the following temperature program: 95ºC for 3 min; 40 cycles of 95ºC for 30 sec, 54ºC for 30 sec, and 72ºC for 45 sec; and 72ºC for 5 min. Semi-quantitative RT-PCR of the housekeeping gene, *rpl3*, was conducted in parallel to determine the relative abundance of target gene cDNA in each sample. Forward and reverse primers for target genes *br-c*, *dfd*, and *rpl3* were designed in adjacent neighboring exons allowing detection of gDNA contamination. Primer pairs used were listed in the following: HR96-forward: 5'-AAG CCA TTG CTG GAC AAG GA-3', HR96-reverse: 5'-GGG CTC GTC GTT GTA GTT GG-3',

BRC-forward: 5'-ACG ACA CAC AGC ACT TCT GC-3', BRC-reverse: 5'-GGA ATT GGC CAG GTT CTG TA-3', DFD-forward: 5'-TGG ATC GGC AAA TGG ATA TT-3', DFD-reverse: 5'-GGA TCT TCT TCA TCC AGG GGT-3', RPL3-forward: 5'-CTC ATC GTA AGT TCT CGG CAC C-3', and RPL3-reverse: 5'-TAG AAG CGA CGA CGG CAC TC-3'. PCR products were analyzed in a 2% agarose gel containing ethidium bromide (5 μg/ml).

4.2.7 Quantitation using real-time RT-PCR

Relative quantitation of *hr96* and *br-c* transcript levels was measured by normalizing to *rpl3* transcript level using real-time RT-PCR with comparative C_T method. Purified mRNA (500 ng) derived from S2 cells was treated with DNase to remove gDNA (DNA free™ kit, Applied Biosystems, Foster City, CA), and cDNA was synthesized using the SuperScript™ III first-strand synthesis system (Invitrogen). Each real-time PCR reaction included 0.5 μl of cDNA product, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 7.5 μl of ddH_2O, and 10 μl of Power SYBR Green PCR Master Mix (2X) (Applied Biosystems). Primers used were HR96-forward: 5'-GCG GAC GTG GTG GAG TTC ATG-3', HR96-reverse: 5'-GCG GTC TGC TGT CTG CTG GG-3', BR-C-forward: 5'-GCA CAC CCT GCA AAC ACC CG-3', BR-C-reverse: 5'-TGC CTG CTG CTG CGT GAG TC-3', RPL3-forward: 5'-GAC GCC AGC AAG CCA GTC CA-3', and RPL3-reverse: 5'-GCC GAC AGC ACC GAC CAC AA-3'. Reactions were carried out using Applied Biosystems 7900HT Real-Time PCR system at the Cornell University Life Sciences Core Laboratories Center with following temperature program: 50ºC for 2 min; 95ºC for 10 min; 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Data was processed and analyzed using SDS software (version: 2.1). Three independent real-time PCR reactions of each target gene of each biological sample were acquired. PCR products were further

analyzed in a 2% agarose gel to confirm the size of product and were DNA sequenced.

4.3 Results

4.3.1 Identification of 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 (Figure 4.2), based on the significant increase of PB induction seen in promoter construct -344/+85 compared to -246/+85. To further define the PB responsive *cis*-regulatory 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 (Figure 4.3). These results indicate the PB responsive *cis*-regulatory sequence is located between positions -330 and -280 (Figure 4.3). The significant increases in PB induction across four promoter constructs suggests the binding sites for critical TFs span the junctions of the constructs and/or there are multiple TFs involved. Our results also showed promoter region from -280 to -246 was responsible for basal transcription in S2 cells (Figure 4.3). The promoter region from -900 to -344 was identified additionally to be able increase basal transcription of *CYP6D1* in S2 cells (Figure 4.2).

TFsearch of the TRANSFAC database (version 1.3) (Heinemeyer et al. 1998) was used to identify TF binding sites within the 51 nucleotides of PB responsive *cis*-regulatory sequence (from -330 and -280). Because promoter assays were conducted using *Drosophila* S2 cells, prediction of putative binding sites was focused on TFs of

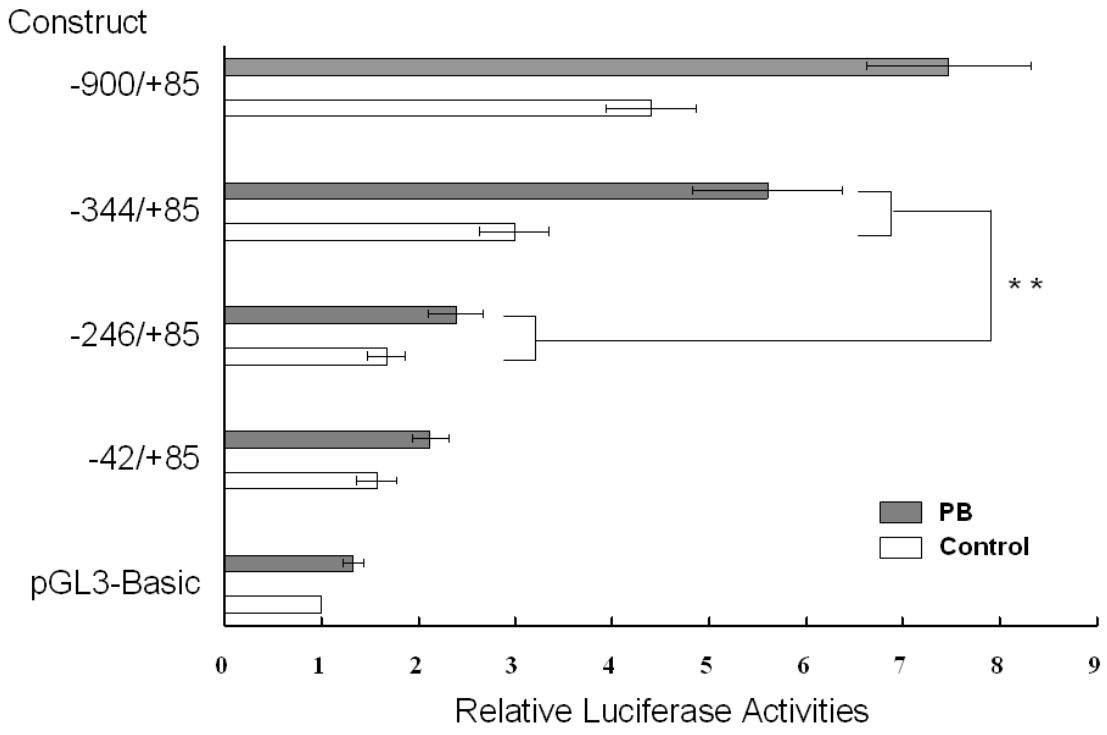


Figure 4.2: PB responsive promoter assay conducted with progressive 5' deletion of the *CYP6D1* promoter. Promoter constructs are numbered relative to the transcription start site (TSS) at +1. Relative luciferase activity was measured by normalizing the signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the average of the relative luciferase activity ± S.D. of three independent transfections of three replicates (n=9). Gray bars represent the signal in the presence of PB and white bars represent the control. Double asterisks indicate a greater PB induction relative to the next shorter promoter construct ($p < 0.01$, Student's *t*-test).

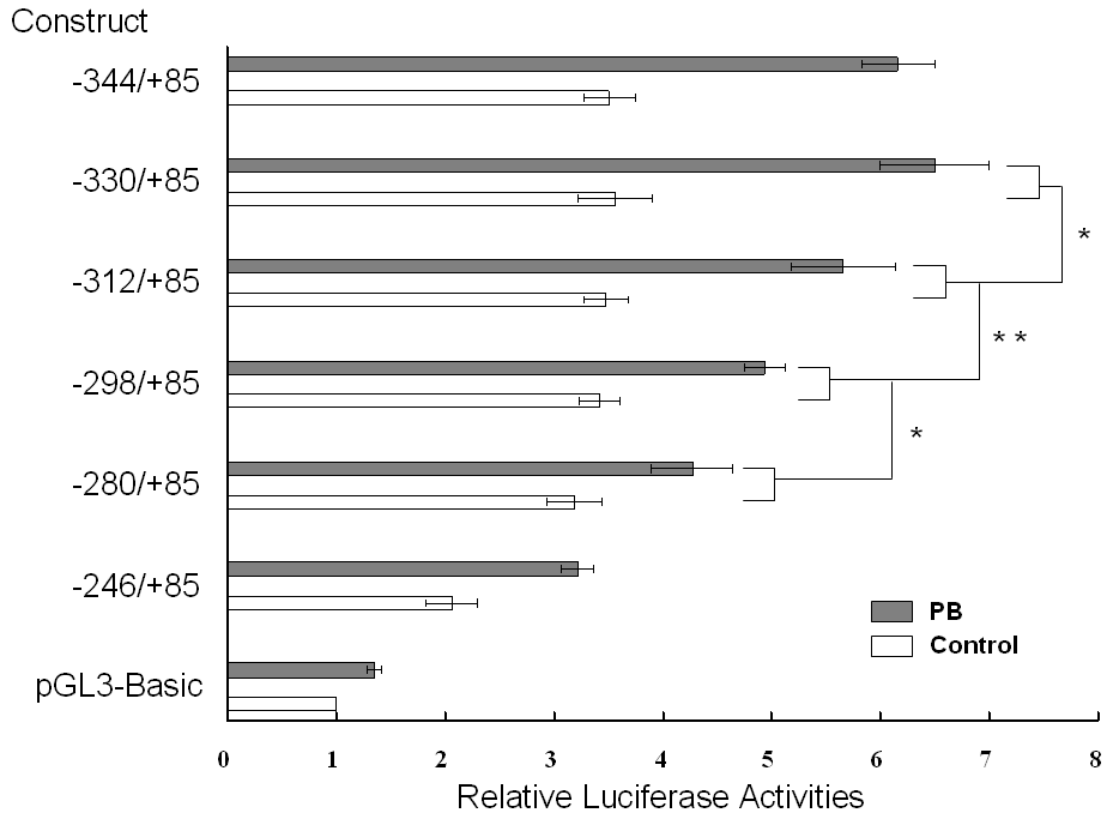


Figure 4.3: PB responsive promoter assay corresponding to the promoter region from -344 to -246 of *CYP6D1*. Promoter constructs are numbered relative to the TSS at +1. Relative luciferase activity was measured by normalizing the signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the average of the relative luciferase activity ± S.D. of three independent transfections of three replicates (n=9). Gray bars represent the signal in the presence of PB and white bars represent the control. Asterisks indicate a greater PB induction relative to the next shorter promoter construct (*****$p < 0.05$; ****** $p < 0.01$; Student's *t*-test).

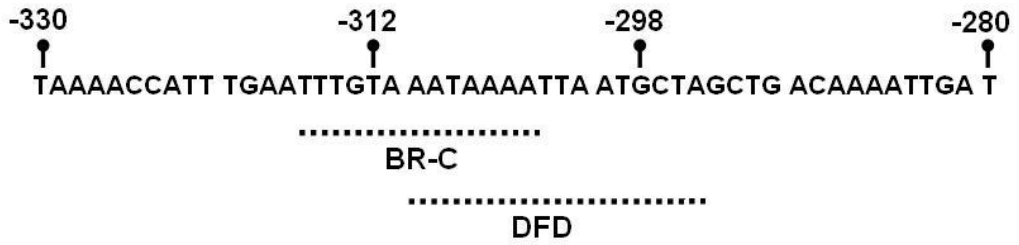


Figure 4.4: Sequence of the PB responsive promoter region from -330 to -280 of *CYP6D1* promoter and putative binding sites of *Drosophila* TFs. Numbers indicate position of 5' ends of serial deletion promoter constructs. The binding sites of *Drosophila* BR-C (*broad-complex*, isoform Z4) and DFD (*deformed*) were identified using TFsearch and are represented by dash lines beneath the sequence.

Drosophila and other insects. Based on the TFsearch with default cut-off score of 85.0, binding sites of *Drosophila* TFs [BR-C (*broad-complex*) and DFD (*deformed*)] were identified (Figure 4.4), with scores 92.5 and 90.0, respectively. BR-C is known to have isoforms, Z1-Z4 with different zinc-finger DNA binding domains produced by alternative splicing (Bayer et al. 1996). The putative binding site of BR-C identified by TFsearch belongs to isoform Z4.

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

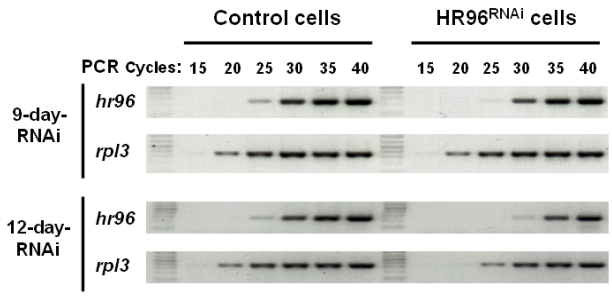
To identify TFs expressed in *Drosophila* S2 cells critical for regulating PB induction through the *CYP6D1* promoter, the roles of three *Drosophila* TFs, HR96, BR-C, and DFD were evaluated using RNAi in conjunction with promoter reporter

assays. Semi-quantitative RT-PCR result showed there was reduced abundance of *hr96* transcript at 9-day and 12-day HR96^{RNAi} cells compared to control cells (Figure 4.5A, 4.6A, and 4.7A), and reduced abundance of *br-c* transcript at 9-day and 12-day BR-C^{RNAi} cells compared to control cells (Figure 4.8A, 4.9A, and 4.10A). Relative quantitation of transcript levels of *hr96* or *br-c* in RNAi-treated cells compared to control cells were additionally measured using real-time RT-PCR (Figure 4.5B, 4.6B, 4.8B, and 4.9B). These results indicated treatment of dsRNA probe can result in reduction of transcript abundance of target gene in HR96^{RNAi} cells or in BR-C^{RNAi} cells compared to control cells. Our preliminary test (Figure 4.1) indicated 12-day treatment of dsRNA prior promoter assays could result in clear and significant effect in PB induction compare to control cells.

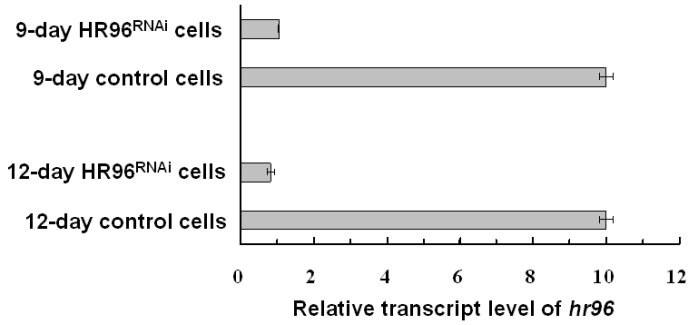
The 12-day RNAi-treated cells and control cells were subjected to PB responsive promoter assays using *CYP6D1* promoter construct -330/+85. HR96^{RNAi} cells showed a significant decrease in PB induced promoter activity (gray bars), but no significant change in basal promoter activity (white bars), compared to control cells (Figure 4.5C, 4.6C, and 4.7B). BR-C^{RNAi} cells showed significant increase in PB induced promoter activity compared to control cells; whereas, basal promoter activities did not show significant difference (Figure 4.9C and 4.10B). The mock control experiment of LacZ^{RNAi} cells (treatment with dsRNA probe corresponding to *LacZ* gene of *E. coli*) was conducted in parallel and no significant influence in basal or PB induced promoter activities were seen compared to control cells (Figure 4.9C and 4.10B). Our results indicate HR96 acts as a positive transcriptional regulator and BR-C acts as a negative transcriptional regulator for PB induction through the *CYP6D1* promoter in *Drosophila* S2 cells. The action of HR96 and BR-C were PB specific and PB dependent.

Semi-quantitative RT-PCR indicated the relative abundance of *dfd* transcript

(A)



(B)



(C)

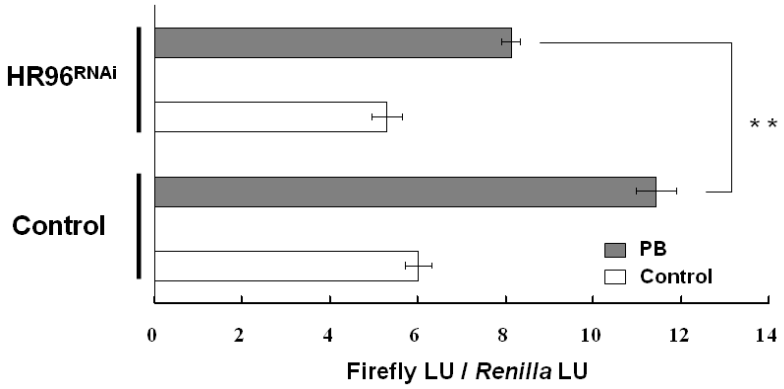
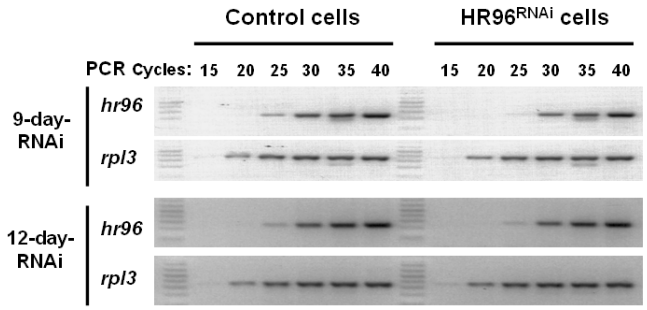
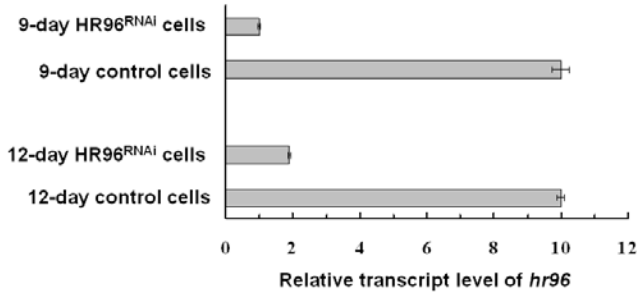


Figure 4.5: Experiment 1: (A) semi-quantitative RT-PCR and (B) real-time RT-PCR showed reduced transcript level of *hr96* in 9-day and 12-day HR96^{RNAi} cells compared to control cells. In the real-time RT-PCR result, values of relative transcript level of control cells were designated as 10. (C) HR96^{RNAi} cells showed reduced PB induced promoter activity (gray bar) compared to control cells (double asterisks, $p < 0.01$; Student's *t*-test with Tukey's HSD test). Bars represent mean of measurements ± SD.

(A)



(B)



(C)

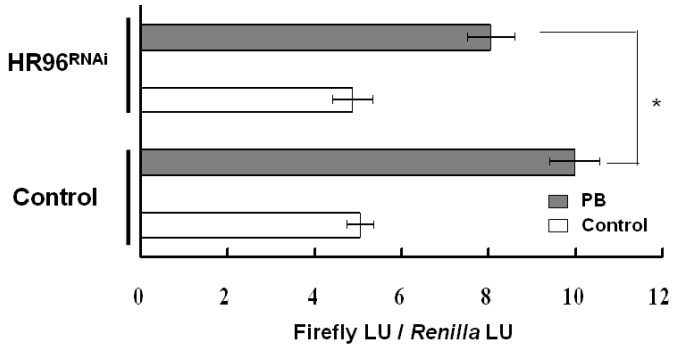
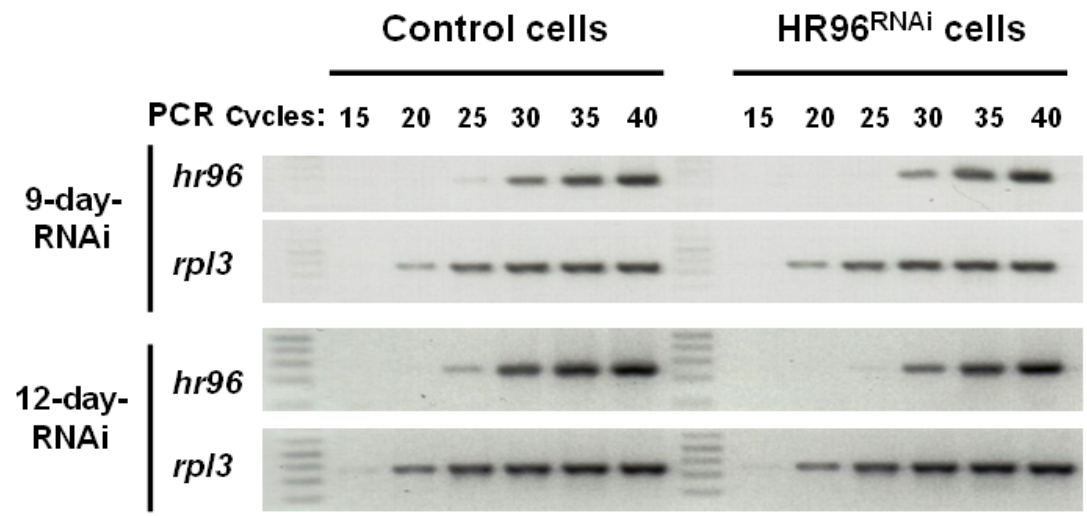


Figure 4.6: Experiment 2: (A) semi-quantitative RT-PCR and (B) real-time RT-PCR showed reduced transcript level of *hr96* in 9-day and 12-day HR96^{RNAi} cells compared to control cells. In the real-time RT-PCR result, values of relative transcript level of control cells were designated as 10. (C) HR96^{RNAi} cells showed reduced PB induced promoter activity (gray bar) compared to control cells (single asterisks, $p < 0.05$; Student's *t*-test with Tukey's HSD test). Bars represent mean of measurements ± SD.

(A)



(B)

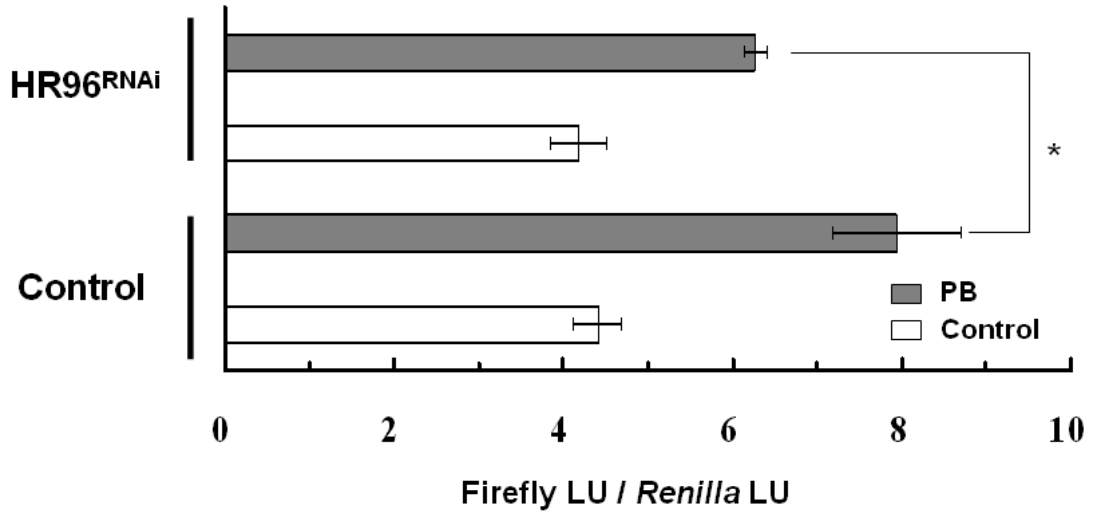
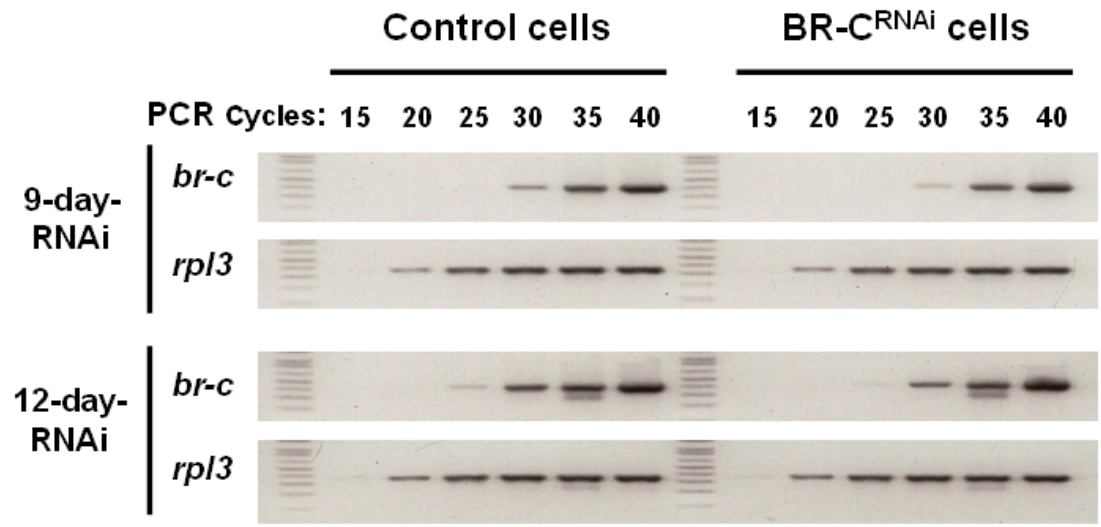


Figure 4.7: Experiment 3: (A) semi-quantitative RT-PCR showed reduced transcript level of *hr96* in 9-day and 12-day HR96^{RNAi} cells compared to control cells. (B) HR96^{RNAi} cells showed reduced PB induced promoter activity (gray bar) compared to control cells (single asterisks, $p < 0.05$; Student's *t*-test with Tukey's HSD test). Bars represent mean of measurements ± SD.

(A)



(B)

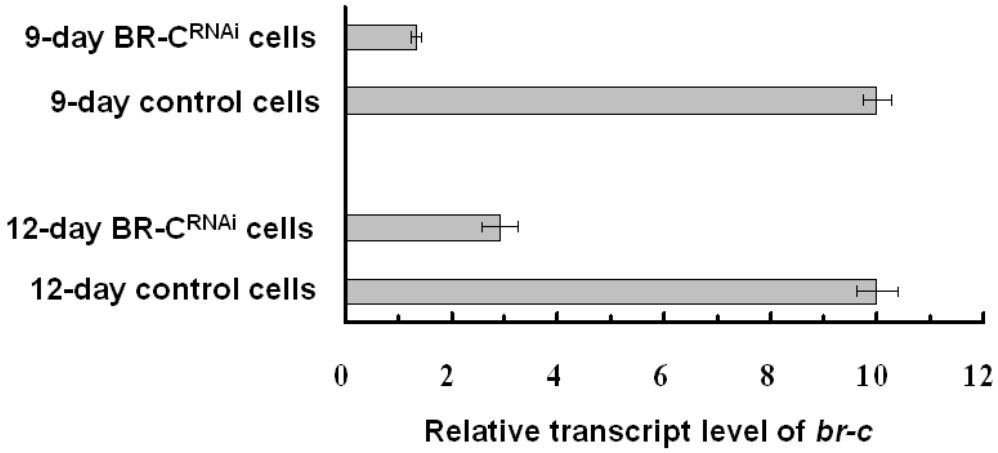
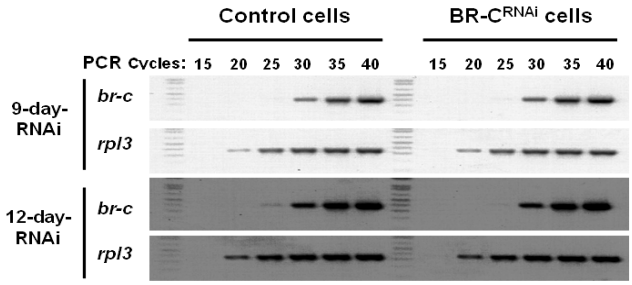
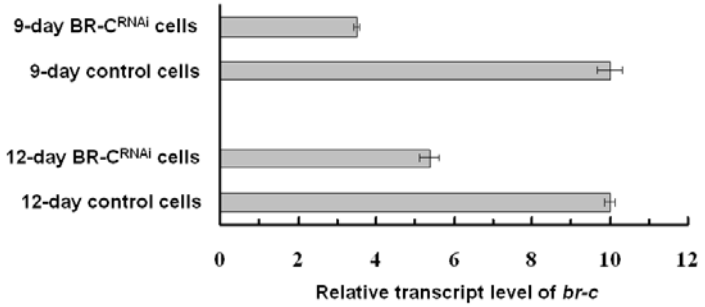


Figure 4.8: Experiment 4: (A) semi-quantitative RT-PCR and (B) real-time RT-PCR showed reduced transcript level of *br-c* in 9-day and 12-day BR-C^{RNAi} cells compared to control cells. In the real-time RT-PCR result, values of relative transcript level of control cells were designated as 10. Bars represent mean of measurements ± SD. These results indicate treatment with dsRNA probe of *br-c* can cause depletion of *br-c* transcript level.

(A)



(B)



(C)

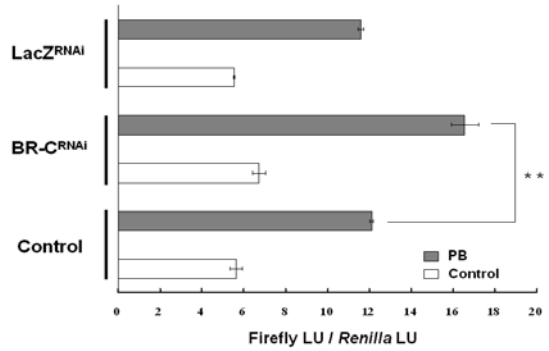
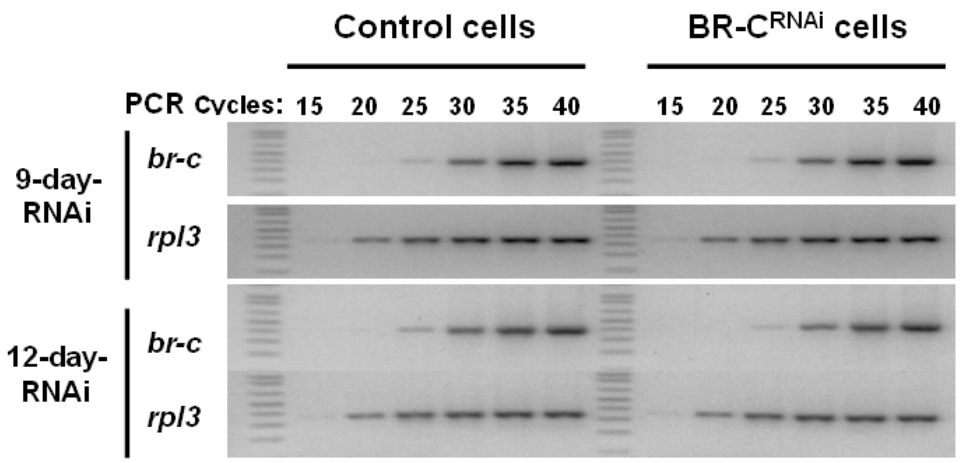


Figure 4.9: Experiment 5: (A) semi-quantitative RT-PCR and (B) real-time RT-PCR showed reduced *br-c* transcript level in 9-day and 12-day BR-C^{RNAi} cells compared to control cells. In the real-time RT-PCR result, values of relative transcript level of control cells were designated as 10. (C) BR-C^{RNAi} cells showed increased PB induced promoter activity (gray bar) compared to control cells (double asterisks, $p < 0.01$; Student's *t*-test with Tukey's HSD test). Bars represent mean of measurements ± SD.

(A)



(B)

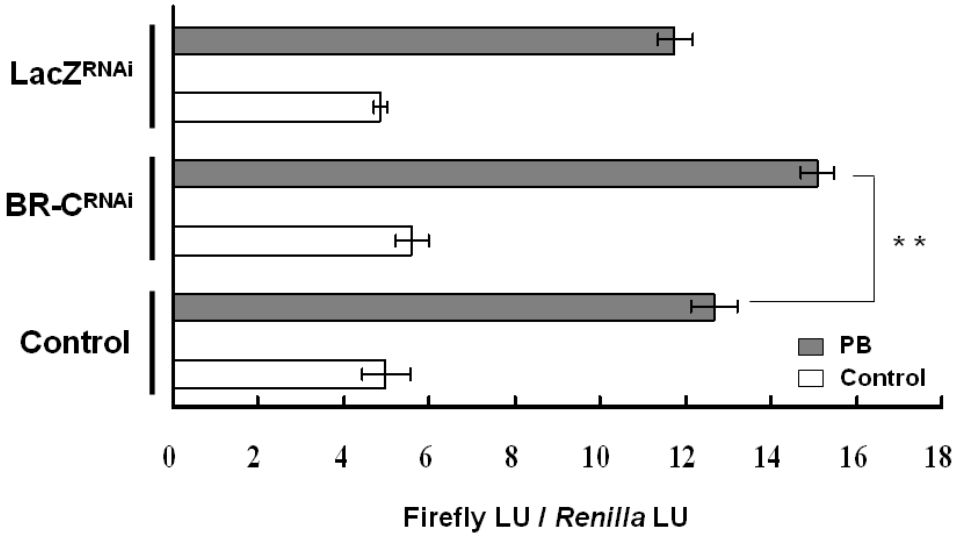


Figure 4.10: Experiment 6: (A) semi-quantitative RT-PCR showed reduced *br-c* transcript level in 9-day and 12-day BR-C^{RNAi} cells compared to control cells. (B) BR-C^{RNAi} cells showed increased PB induced promoter activity (gray bar) compared to control cells (double asterisks, $p < 0.01$; Student's *t*-test with Tukey's HSD test). Bars represent mean of measurements ± SD.

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

4.4 Discussion

4.4.1 *Drosophila* HR96 acts as an activator of PB induction

The depletion of HR96 in *Drosophila* S2 cells significantly reduced PB induction, indicating HR96 acts as a positive transcriptional regulator of PB induction. This is consistent with the expectation for the insect ortholog of vertebrate CAR and PXR, which function as transcriptional activators to regulate PB induced transcription of P450s (Handschin et al. 2000, Handschin et al. 2001, Sueyoshi and Negishi 2001, Timsit and Negishi 2007). Our results contrast with those using *Drosophila* HR96 null mutant. In those studies, PB induced transcription of 29 PB inducible P450s (and majority of PB-regulated genes) was not affected in the HR96 null strain, compared to the wild type (King-Jones et al. 2006). It was suggested that the loss of HR96 (in the HR96 null strain) may be compensated by additional transcriptional regulators able to feed into this pathway (King-Jones et al. 2006); and a possible regulator of *Drosophila* ortholog of mammalian aryl hydrocarbon receptor (AHR) was suggested (King-Jones et al. 2006). Interestingly, based on microarray dataset DGS1472 at GEO (Gene Expression Omnibus) at NCBI, the *Drosophila* ortholog of

mammalian AHR, *spineless*, is not expressed in S2 cells. In addition, a recent study indicated a high mortality in both *Drosophil*a male and female crosses of HR96 knockout by RNAi (Giraudo et al. 2010), which contrasts with HR96 null mutant strain generated by King-Jones et al (King-Jones et al. 2006). While our results demonstrated a role of HR96 in PB induction in *Drosophila* S2 cells, a complete list of genes for which HR96 has a role in PB induction will require further study.

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

For PB induction, mammalian CAR and PXR require association with RXR in order to bind to target DNA sequences (Baes et al. 1994, Kliewer et al. 1998, Sueyoshi and Negishi 2001, Timsit and Negishi 2007). The chicken ortholog CXR also requires RXR in order to bind to target DNA sequences (Handschin et al. 2000, Handschin et al. 2001). It is unknown what the TF partner of *Drosophila* HR96 is, although USP (*ultraspiracle*) represents the *Drosophila* ortholog of mammalian RXR and USP is expressed in S2 cells based on microarray dataset DGS1472 at GEO database. Two-hybrid system or DNA-chromatography could be used to examine if USP is the TF partner of HR96. The role of USP in PB induction in S2 cells could be evaluated using RNAi treatment in conjunction with promoter assays.

The cognate binding sequence of *Drosophila* HR96 remains unknown; although

it has been described that the DNA binding domain alone of *Drosophila* HR96 could shift oligonucleotides bearing a EcR binding site of the *Drosophila hsp27* promoter (Fisk and Thummel 1995). Within the *CYP6D1* promoter region (-330 to -280), the EcR binding site was not identified. While our studies identified a region of the *CYP6D1* promoter likely to bind *Drosophila* HR96, further studies will be needed to identify the DNA sequences to which HR96 binds.

4.4.2 *Drosophila* BR-C acts as 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 critical role in PB induction was unclear. Our results showed the depletion of BR-C in *Drosophila* S2 cells resulted in increase of PB induced promoter activity of *CYP6D1*, indicating BR-C acts as a negative transcriptional regulator of PB induction. BR-C has four types of isoforms, Z1-Z4, which have different zinc-finger DNA binding domains produced by alternative splicing (Bayer et al. 1996). These four isoforms appear to be present together in various types of tissues and cells but their relative abundance differs among tissue types (Emery et al. 1994, Bayer et al. 1996). TFsearch indicated the presence of BR-C Z4 binding site within *CYP6D1* promoter region (from -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 negative transcriptional factor. Using the RT-PCR protocol described by Tzolovsky et al. (Tzolovsky et al. 1999), we confirmed BR-C Z4 is expressed in *Drosophila* S2 cells (data not shown). In addition, binding sites of BR-C Z4 have also been identified in promoter regions critical for PB induction of *Cyp6a2* promoter (Dunkov et al. 1997) and *Cyp6a8* promoter (Maitra et al. 2002). These suggest BR-C Z4 may be involved in regulating PB induction of P450 genes in

multiple species.

Similar to HR96, 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. This suggests the PB-dependent reaction of BR-C in PB induction is not dependent on change of its abundance. Thus, the mechanism controlling the BR-C mediated PB induction remains unclear.

4.4.3 Conclusions

Identification and characterization of TFs involved in PB-induced gene expression may also help understand some cases of metabolism-mediated insecticide resistance. Many insecticide resistant strains having metabolism-mediated resistance, there is constitutive overexpression of multiple P450s and GSTs that are PB inducible in susceptible strains (Le Goff et al. 2003, Pedra et al. 2004, Vontas et al. 2005). It has been suggested that resistant strains may simply have detoxification genes are constitutively "induced" by an unknown *trans* acting factor (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 LPR strain is due to factors on chromosome 1 and 2 (Liu and Scott 1997a). *CYP6D1* expression is not induced by PB in LPR, and this trait has been mapped to chromosome 2 (Liu and Scott 1997a). Based on homology maps between *D. melanogaster* and *M. domestica* (Foster et al. 1981, Weller and Foster 1993), *HR96* and *BR-C* are expected to be present on chromosome 2 and 3, respectively, of *M. domestica*. Given our findings that *HR96* was a positive regulator of PB induced *CYP6D1* expression, and the

expectation that *HR96* is on house fly chromosome 2 makes *HR96* worth further study as a possible factor involved in the increased transcription of *CYP6D1* in LPR.

In summary, the *CYP6D1* promoter sequence from -330 to -280 was found to be critical for PB induction. *Drosophila* HR96 was shown to play a role in activating PB induction. This represents the first direct 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. 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.

CHAPTER 5

MOLECULAR BASIS OF THE CONSTITUTIVE OVEREXPRESSION WITHOUT PHENOBARBITAL INDUCTION OF CYTOCHROME P450 *CYP6D1* IN THE PERMETHRIN RESISTANT LPR STRAIN OF HOUSE FLY (*MUSCA DOMESTICA*)

5.1 Introduction

House fly (*Musca domestica*) cytochrome P450 CYP6D1 can metabolize numerous xenobiotics, including beno(a)pyrene (Wheelock and Scott 1992b), chlorpyrifos (Hatano and Scott 1993), methoxyresorufin (Wheelock and Scott 1992b), phenanthrene (Korytko et al. 2000), and phenoxybenzyl pyrethroids such as cypermethrin, deltamethrin, and permethrin (Wheelock and Scott 1992a, Zhang and Scott 1996). *CYP6D1* was sequenced (Tomita and Scott 1995) and mapped to chromosome 1 (Liu et al. 1995). Transcription of *CYP6D1* is phenobarbital (PB) inducible (~6-fold increase) in insecticide susceptible strains (Scott et al. 1996, Liu and Scott 1997a). *CYP6D1* is expressed in numerous tissues (Scott and Lee 1993, Korytko and Scott 1998) and expression of *CYP6D1* is developmentally regulated, being found only in adults (Scott et al. 1996).

Increased transcription of *CYP6D1* confers metabolism-mediated resistance to permethrin in the LPR strain of house fly. *CYP6D1* is overexpressed (~9-fold increase) in LPR relative to insecticide susceptible strains (Scott et al. 1996, Liu and Scott 1997a, 1997b). Overexpression of *CYP6D1* in LPR is not due to gene duplication in the LPR strain (Tomita et al. 1995) or increased transcript stability (Liu and Scott 1998). Increased transcription of *CYP6D1* was linked to factors on chromosome 1 and 2 (Liu and Scott 1997a, 1997b). The promoter sequences of *CYP6D1* alleles have been sequenced from five house fly strains. The comparison of

promoter sequences showed the LPR specific allele (*CYP6D1v1*) had a 15 nucleotide insertion located at -29 to -15 relative to the transcription start site (Scott et al. 1999). This insertion in the *CYP6D1* promoter of the LPR disrupts the binding site for a known transcriptional repressor Gfi-1. House fly *Gfi-1* is mapped to chromosome 1 (Gao and Scott 2006a). Thus, this appears to be the factor on chromosome 1 responsible for the elevated transcription in the LPR strain. However, the factor on chromosome 2 remains unidentified.

Unlike insecticide susceptible strains, *CYP6D1* in LPR is constitutively overexpressed without significant PB induction in response to treatment with PB (Scott et al. 1996, Liu and Scott 1997a), and this trait (lack of PB induction) was mapped to chromosome 2 (Liu and Scott 1997a). In LPR, there is constitutive overexpression *CYP6A1* (Carino et al. 1992) and *CYP6D3* (Kasai and Scott 2001a). PB induction of *CYP6D3* in LPR (~1.6 fold) was less than found in CS (~16 fold) (Kasai and Scott 2001a). *CYP6A1* is 100-fold inducible by PB in a susceptible strain of house fly (Carino et al. 1992). It is not known if *CYP6A1* is PB inducible in LPR. P450s (i.e. CYP6C1, C2, A3, A4, A5, and A6) which are not PB inducible in susceptible house flies do not show overexpression in LPR (Cohen and Feyereisen 1995). Thus, the constitutive overexpression (without significant PB induction) of *CYP6D1* in LPR has been proposed to be due to a mutation in the gene (the *trans* acting factor) responsible for regulating PB induction on chromosome 2 (Liu and Scott 1997a, Kasai and Scott 2001a). However, whether variation in *CYP6D1* promoter sequences is involved in the lack of PB induction is not clear.

The *CYP6D1v2* promoter sequence from -330 to -280 (numbers are relative to transcription start site, +1) of the CS strain has been identified to be critical for PB induction (Chapter 4). *Drosophila* HR96 (*hormone receptor-like in 96*) and BR-C (*broad-complex*) were identified as key transcription factors for PB induction of

CYP6D1v2 in *Drosophila* S2 cells (Chapter 4). Based on a chromosome homology map (Foster et al. 1981, Weller and Foster 1993), *HR96* is expected to be on chromosome 2 of house fly. We conducted PB responsive promoter assays using dual luciferase reporter assays in *Drosophila* S2 cells to examine the *CYP6D1v1* promoter of LPR. Our results showed the *CYP6D1v1* promoter of LPR was able to mediate PB induction, just as the *CYP6D1v2* promoter from CS (Chapter 4); although there are four SNPs in the PB responsive promoter region in LPR compared to CS (Scott et al. 1999). These results indicated variations in *CYP6D1* promoter sequences did not significantly affect of PB inducibility of *CYP6D1v1* promoter of LPR. Therefore, the lack of PB induction of *CYP6D1* in LPR appears due to an unidentified *trans* acting factor responsible for PB induction on chromosome 2. House fly *HR96* was cloned and sequenced in order to examine if it is this *trans* acting factor. Multiple *HR96* alleles (*v1*-*v10*) were identified. Three alleles (*v8*-*v10*) contained E82V and G110D amino acid substitutions and were only found in LPR. Genotyping LPR survivors of permethrin selection indicated *HR96* allele *v8*-*v10* were not associated with resistance. Transcript level of *HR96* in LPR was not different from CS. Thus *HR96* is not the *trans* acting factor responsible for the constitutive overexpression of *CYP6D1* in LPR. The identity of this *trans* acting factor remains unclear.

5.2 Materials and Methods

5.2.1 House flies

Four house fly strains were used. The CS and *aabys* are insecticide susceptible strains (Hamm et al. 2005). The LPR is a permethrin resistant strain and was originally collected in a dairy near Horseheads, New York at 1982 (Scott et al. 1984). The OCR is a cyclodiene resistant (*Rdl*) (Shono and Scott 2003) and pyrethroid

susceptible strain (Gao et al. 2007). House fly larvae were reared on mixed media made of 500 g of calf manna (Agway, Ithaca, NY), 120 g of wood chips (Agway), 60 g of Baker's yeast (MP Biomedicals, Solon, OH), 1210 g of wheat bran (Agway) and 2000 ml of water. Adults were fed on powdered milk:granulated sugar (1:1) and water *ad libum*. Larvae and adults were reared at 28ºC, 60% relative humidity, with a 12: 12 hr light/dark photoperiod.

5.2.2 *Drosophila* S2 cells

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

5.2.3 *CYP6D1* promoter constructs of the LPR strain and PB responsive promoter assays

Progressive 5' deletions of the *CYP6D1* promoter from the LPR strain (i.e. *CYP6D1v1*) (Scott et al. 1999) were generated by PCR amplification. Promoter regions regarding to -925/+85, -365/+85, -267/+85, and -57/+85 (numbers are relative to transcription start site, defined as +1) were constructed into restriction enzyme sites *Sac* I and *Bgl* II of pGL3-Basic vector (Promega, Madison, WI) according to previous description (Chapter 4). PB responsive promoter assays with above promoter constructs of LPR were performed using dual luciferase reporter assay system (Promega) in *Drosophila* S2 cells according to previous description (Chapter 4). 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 *CYP6D1v1* promoter. Three independent

transfections (PB or control) of each promoter construct were conducted in each replicate. Four replicates were acquired. 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 to indicate promoter regions critical for PB induction.

5.2.4 Isolation of gDNA or mRNA, gel extraction, TA cloning, plasmid DNA purification, and DNA sequencing

DNA isolated from individual adult house flies was conducted according to previous description (Hamm and Scott 2009). Purification of mRNA was using Illustra QuickPrep^{TM} micro mRNA purification kit (GE healthcare, Little Chalfont, UK). Gel extraction of PCR products, if needed, was done using a QIAEX II kit (Qiagen, Valencia, CA). Cloning of PCR products was performed using a TOPO TA kit with pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) and TOP 10 competent cells (Invitrogen). Plasmid DNA was isolated using QIAprep Miniprep system (Qiagen). Plasmid DNAs were sequenced at the Cornell University Life Sciences Core Laboratories Center.

5.2.5 Cloning of house fly *HR96* and PCR for genotyping

Degenerate primers were selected using CODEHOP (http://blocks.fhcrc.org/codehop.html) (Rose et al. 2003) with an alignment of HR96 peptide sequences of *Drosophila melanogaster* (NP_524493.1), *Drosophila yakuba* (XP_002099134.1), *Drosophila virilus* (XP_002054249.1), *Culex quinquefasciatus* (XP_001866050.1), and *Anopheles gambiae* (XP_313130.4). PCR reactions included 0.5 µl of genomic DNA (*aabys* adult), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 10 µl of ddH_2O, and 12.5 µl of 2X GoTaq® Green Master Mix

(Promega) was carried out in a iCycler thermal cycler (Bio-Rad, Hercules, CA) with following temperature program: 95ºC for 3 min; 35 cycles of 95ºC for 30 sec, 60ºC for 45 sec (decrease temperature by 0.3ºC every 1 cycle), and 72ºC for 1 min 20 sec; and 72ºC for 5 min. Three distinct degenerate primer pairs were successfully in acquiring partial nucleotide sequences of house fly *HR96* and were listed in the following: (i) forward: 5'-GTG GTG ATA AAG CCT TGG GTT AYA AYT TYA A-3' and reverse: 5'-GGC ATT AAA GGG GGA ATT CAT DAW YTT-3', (ii) forward: 5'-AAA ATT ACC GCC TTT AGA AAT ATG TGY CAR GA-3' and reverse: 5'-GGT AAT GGC ACA CAT AAT CAA AAT AAT RTT YTC RTC-3', and (iii) forward: 5'-CTT GTT GAA AGG TGG TTG TAC AGA RAT GAT GAT -3' and reverse: 5'-GGT AAT GGC ACA CAT AAT CAA AAT AAT RTT YTC RTC-3'. The FirstChoice® RLM-RACE kit (Ambion, Austin, TX) was used for 5' and 3' RACE of *HR96* according to manufacture's instruction using mRNA derived from 10 abdomens of 3-day-old male adult *aabys* flies. PCR for 5' RACE was performed using the 5' RACE outer primer: 5'-GCT GAT GGC GAT GAA TGA ACA CTG-3' and a gene-specific reverse primer: 5'-TCT CGC TCT TCA TGC CGA TGT CT-3' with the following thermal cycler program: 95ºC for 3 min; 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min; and 72ºC for 5 min. PCR for 3' RACE was conducted using the 3' RACE outer primer: 3'-GCG AGC ACA GAA TTA ATA CGA CT-3' plus a gene-specific forward outer primer: 5'-GCC AAG AGG ATC AGG TTG CCT T-3'. The nested PCR for 3' RACE was conducted using the 3' RACE inner primer: 5'-CGC GGA TCC GAA TTA ATA CGA CTC ACT ATA GG-3' plus a gene-specific forward inner primer: 5'-GCC AAG GGC AAT GTC TAT GAA GAA C-3'. The thermal cycler program for 3' RACE PCRs were identical to the 5' RACE PCR described above. PCR to amplify the complete coding sequence of *HR96* was performed using forward primer: 5'-CAA AGA TGT CAC CAA TTA ATA AAG TCT GTG C-3' and

reverse primer: 5'-ATG ATG TAG GAA TTA AGG ACA TTT GAG GTA AC-3' with the following thermal cycler program: 95ºC for 3 min; 40 cycles of 95ºC for 30 sec, 62ºC for 45 sec, and 72ºC for 2 min 30 sec; and 72ºC for 5 min. The cDNA product used for the above PCR was synthesized from mRNA of 10 abdomens of three-day-old male adults using the SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen). House fly strains of *aabys*, CS, OCR, and LPR were studied. PCR product of full length cDNA was analyzed on a 1.6% agarose gel and then subjected to gel extraction, TA cloning, plasmid DNA purification, and DNA sequencing according to description in above section.

Genotyping of *HR96* alleles regarding to allele *v4-v7* or *v8-v10* in LPR was performed using PCR to amplify a polymorphic region (from position -5 to 602, numbers are relative to translation start site ATG as +1) from gDNA of house fly individual. The PCR was performed in 50 µl of reaction volume including 1 µl of gDNA, 2 µl of 10 µM forward primer (5'-CAA AGA TGT CAA TTA ATA AAG TCT GTG C-3'), 2 µl of 10 µM reverse primer (5'-GCT TGT GAG GCA CGG TCC-3'), 20 µl of ddH_2O, and 25 µl of 2X GoTaq® Green Master Mix (Promega). Reactions were carried out in an iCycler thermal cycler (Bio-Rad) with following temperature program: 95ºC for 5 min; 30 cycles of 95ºC for 30 sec, 58ºC for 30 sec, and 72ºC for 50 sec; and 72ºC for 4 min. PCR products were purified using Wizard SV gel and PCR clean-up system (Promega) and sequenced at the Cornell University Life Sciences Core Laboratories Center using the forward primer described in above.

5.2.6 Permethrin selection of the LPR strain

Technical grade of permethrin, 60% *cis* and 39% *trans*, (ChemService, West Chester, PA) dissolved in acetone (AR grade) (Mallinckrodt Chemicals, Phillipsburg, NJ). Serial doses (3.35, 0.838, 0.209, and 0.052 µg/fly) were tested to determine the

proper dose which could result in 90-95% mortality in LPR strain. Doses of 3.35 and 0.052 μg/fly were also applied to insecticide susceptible *aabys* strain as a control and resulted in 100% mortality. For permethrin selection of the LPR strain, 300 male adults were treated with 6.25 μg/fly permethrin. Survivors (n = 11) were subjected to DNA isolation and PCR to amplify polymorphic region containing E82V and G110D substitutions as described in above section cloning of house fly *HR96* and PCR for genotyping.

5.2.7 Quantitative real-time RT-PCR of *HR96*

Relative quantitation of *HR96* transcript level was measured by normalizing to *Actin* (GenBank: ES652303.1) transcript level using real-time RT-PCR with comparative C_T method. Purified mRNA (500 ng) derived from 10 abdomens of 3-day-old male adults was treated with DNase to remove gDNA (DNA free™ kit, Applied Biosystems, Foster City, CA), and cDNA was synthesized using the SuperScript™ III first-strand synthesis system (Invitrogen). Each real-time PCR reaction included 0.5 μl of above cDNA product, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 7.5 μl of ddH_2O, and 10 μl of Power SYBR Green PCR Master Mix (2X) (Applied Biosystems). Primers used were HR96-forward: 5'-CGG ACC GTG CCT CAC AAG C-3', HR96-reverse: 5'-TCT TCA AAG CAT CGC CTG GAT AGT-3', ACTIN-forward: 5'-TCT GGC ATC ACG CTT TCT ACA ATG-3', and ACTIN-reverse: 5'-GGA GAG AAC AGC TTG GAA GGC A-3'. Reactions were carried out using Applied Biosystems 7900HT Real-Time PCR system at the Cornell University Life Sciences Core Laboratories Center with following temperature program: 50ºC for 2 min; 95ºC for 10 min; 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Data was processed and analyzed using SDS software (version: 2.1). Three independent real-time PCR reactions of three independent biological samples (i.e.

cDNA of 10 three-day-old male abdomens of each) of CS and LPR strains (n=9) were acquired. Statistic analysis was conducted using Student's *t*-test. PCR products were further analyzed in a 2% agarose gel to confirm the size of product and were DNA sequenced.

5.3 Results and Discussion

5.3.1 *CYP6D1v1* (LPR) promoter is able to mediate PB induction

The *CYP6D1v2* (CS) promoter sequence from -330 to -280 has been identified to be critical for mediating PB induction in *Drosophila* S2 cells (Chapter 4). The equivalent *CYP6D1v1* promoter sequence (from -351 to -301) from LPR contains four SNPs compared to *CYP6D1v2* promoter sequence from CS (Scott et al. 1999). Promoter constructs of progressive 5' deletions of the *CYP6D1v1* promoter of LPR (-925/+85, -365/+85, -267/+85, and -57/+85, numbers are relative to transcription start site, defined as +1) were used to determine if a region of *CYP6D1v1* controlled PB responsiveness. Four independent replicates indicated the promoter construct -365/+85 had increased PB induced luciferase activity compared to promoter construct -267/+85 (Figure 5.1), indicating the presence of critical cis-regulatory sequence within *CYP6D1v1* promoter region (-365 to -267) of LPR. The magnitude of the PB induced luciferase activity of LPR observed here (2.03 ± 0.27, n = 12) was equivalent to the PB induce luciferase activity found from the *CYP6D1v2* (CS) promoter (1.87 ± 0.07, n = 9) (Chapter 4). This indicates that despite polymorphisms between the *CYP6D1v1* and *CYP6D1v2* promoters, both are able to mediate induction in response to PB. Thus, the lack of PB induction of *CYP6D1* in the LPR strain appears to be attributed to an unknown *trans* acting factor, rather than sequence variations in the *CYP6D1v1* promoter rendering it unresponsive to PB.

Comparison of the current results with our previous study shows basal promoter

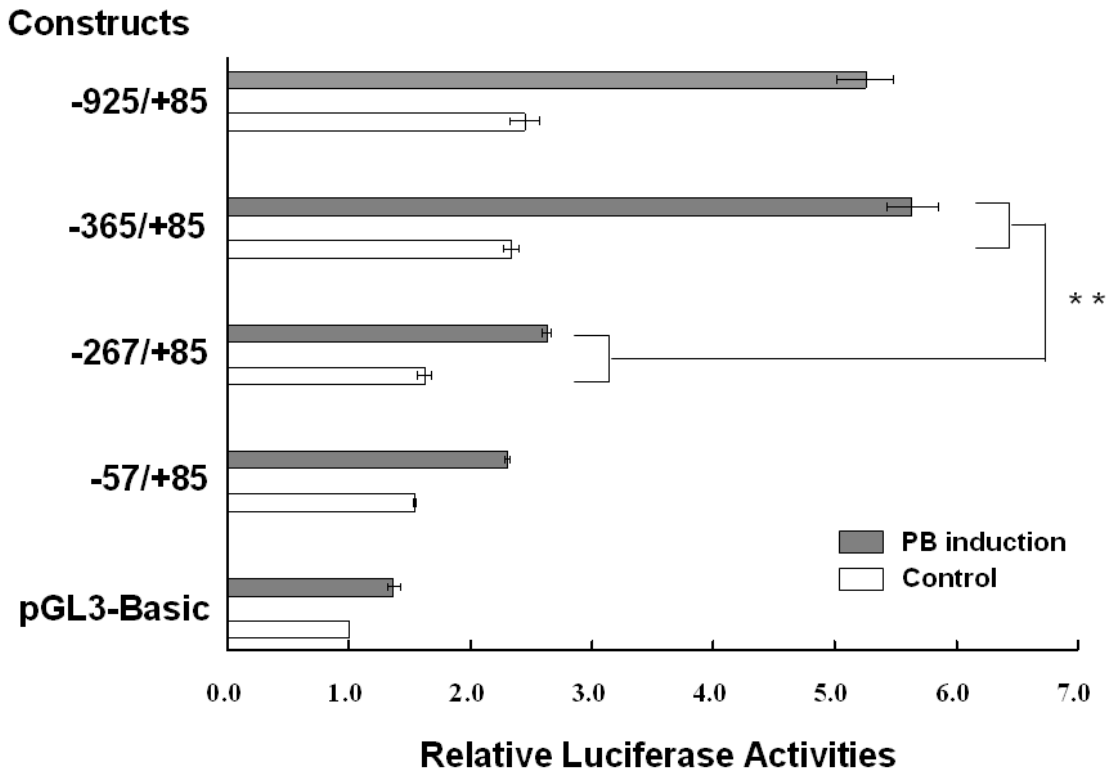


Figure 5.1: *CYP6D1* promoter sequence from -365 to -267 of the LPR is able to mediate PB induction. Promoter constructs are numbered relative to the transcription start site (TSS) at +1. Relative luciferase activity was estimated by normalizing each signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent mean of relative luciferase activity ± S.D. of three independent transfections. Gray bars represent the signal in the presence of PB and white bars represent the control. Double asterisks indicate a greater PB induction relative to the next shorter promoter construct ($p<0.01$, Student's *t*-test).

luciferase activities among parallel promoter constructs between CS and LPR did not significantly differ, except the -900/+85 construct from CS had significantly greater luciferase activities compared to the -925/+85 construct from LPR. This contrasts with the 9-fold greater transcription of *CYP6D1* found in LPR house flies, relative to CS. This suggests that *Drosophila* S2 cells lack the factor(s) found in LPR house flies that cause the enhanced transcription or that other region of DNA are responsible for the increased transcription of *CYP6D1* in LPR (e.g. other 5' flanking sequences, intron sequences, or 3' flanking sequences).

Expression of at least one of the PB inducible P450 in LPR is similar to *CYP6D1*. *CYP6D3* is 12-fold overexpressed in LPR (relative to CS) (Kasai and Scott 2001a). PB treatment increases expression of *CYP6D3* 16-fold in CS, but only 1.6-fold in LPR (Kasai and Scott 2001a). *CYP6A1* is also overexpressed in LPR (Carino et al. 1992), but PB inducibility has not been studied. P450s (i.e. CYP6C1, C2, A3, A4, A5, and A6) which are not PB inducible in susceptible house flies do not show overexpression in LPR (Cohen and Feyereisen 1995). These observations suggest the constitutive overexpression, as well as the lack of PB induction of *CYP6D1* in LPR, is due to a mutation of an unidentified *trans* acting factor responsible for PB induction. Identification of the *trans* acting factor responsible for PB induction and constitutive overexpression of *CYP6D1* may shed light on the transcriptional control of other P450s as well.

The *Drosophila* HR96 and BR-C were identified as a key transcription factors regulating PB induction of *CYP6D1v2* in *Drosophila* S2 cells (Chapter 4). Based on chromosome homology map (Foster et al. 1981, Weller and Foster 1993), *HR96* is expected to locate on chromosome 2 of house fly, while BR-C is expected to be on chromosome 3. Therefore, house fly *HR96* could be the *trans* acting factor responsible for constitutive overexpression of *CYP6D1* in LPR, and it was studied

further.

5.3.2 Cloning of house fly *HR96* complete coding sequence

The full length cDNA of house fly *HR96* gene contains the ORF of 2508 nucleotides encoding 836 amino acids, a 5' UTR of 6 nucleotides, and a 3' UTR of 440 nucleotides (GenBank accession number: HM150722). The DNA binding domain (aa: 6-76, zinc finger, C4 type) and ligand binding domain (aa: 638-810, Ligand-binding domain of nuclear receptor family 1) were 94% and 84 % identical to domains of HR96 of *D. melanogaster*, respectively (Figure 5.2).

5.3.3 *HR96* alleles

The complete coding sequence of *HR96* was cloned and sequenced from four house fly strains: *aabys*, CS, OCR, and LPR. Forty-five full length cDNA clones were sequenced and 10 *HR96* alleles (*v1*-*v10*) were identified (Table 5.1). Deduced amino acid sequences derived from full length cDNA of *HR96* alleles *v1*-*v7* were all identical. However, deduced amino acid sequences of alleles *v8*, *v9*, and *v10* all contained two non-synonymous SNPs with amino acid substitutions E82V and G110D (Table 5.1). Alleles *v8*, *v9*, and *v10* were only found in the LPR strain. The E82 residue is highly conserved among insect HR96 orthologs (even the local region is highly conserved) and is located right after the zinc finger DNA binding domain (aa: 6-76) (Figure 5.2A). The G110 residue is located in a region which is less conserved among insect HR96 orthologs. Given the conservation of the E82 residue, alleles with E82V (and G110D) mutation suggested a potential functional effect on the HR96 protein.

Since only LPR *had HR96* alleles *v8*-*v10* encoding E82V and G110D amino acid substitutions (Table 5.1), whether or not alleles *v8*-*v10* are associated to permethrin

Figure 5.2: Alignment of *D. melanogaster* (Dm) and *M. domestica* (Md) HR96 deduced protein sequences in DNA binding domain (DBD) and ligand binding domain (LBD). (A) Protein sequence alignment from DBD of Dm and Md. Peptide sequences representing the DBD were underlined. Locations of two amino acid substitutions (E82V and G110D) identified in alleles *v8-v10* from the LPR strain were denoted with asterisks above the alignment. (B) Protein sequence alignment of LBD of Dm and Md HR96. Peptide sequences representing LBD were underlined.

Table 5.1: *HR96* alleles, based on full length cDNA, from various house fly strains.

Strains	Resistance to permethrin	Alleles (number of clones)
aabys	No	*v1*(4), *v2*(1)
CS	No	*v1*(15), *v3*(1)
OCR	No	*v2*(4)
LPR	Yes	*v4*(6), *v5*(2), *v6*(1), *v7*(1), *v8*(5)[a], *v9*(4)[a], *v10*(1)[a]

[a] alleles encoding E82V and G110D amino acid substitutions.

Alleles (GenBank accession No.): *v1*(HM150723), *v2*(HM150724), *v3*(HM150725), *v4*(HM150726), *v5*(HM150727), *v6*(HM150728), *v7*(HM150729), *v8*(HM150730), *v9*(HM150731), *v10*(HM150732).

Table 5.2: Number of *HR96* alleles as *v4-v7* (encoding E82 and G110) or as *v8-v10* (encoding V82 and D110) identified in 10 LPR non treated males and in 11 LPR survivors after permethrin selection.

	HR96 alleles in LPR:	
	v4-v7	*v8-v10*
10 LPR non treated males[a]	14	6
11 LPR survivors after permethrin selection[b]	14	8

[a] These 10 LPR males were randomly sampled from the LPR strain.

[b] These 11 LPR survivors were obtained by permethrin selection of 300 LPR males.

resistance in LPR was further examined. Three hundred male adults of LPR strain were topically treated with permethrin (6.25μg/fly), which resulted in 96.3% mortality (11 survivors). To determine the *HR96* alleles regarding to E82V and G110D amino acid substitutions (i.e. *v8-v10* vs. *v4-v7*) in these 11 survivors, *HR96* gDNA region (from position -5 to +602, numbers are relative to translation start site ATG as +1) was amplified and sequenced. Genotypes of *HR96* alleles in 10 none treated and randomly sampled LPR males were determined to provide the background of allele frequencies of *v8-v10* and *v4-v7* alleles in the LPR strain. Our result showed among these 11 LPR survivors, the number of *HR96* allele *v8-v10* (encoding V82 and D110) was not greater than *HR96* allele *v4-v7* (encoding E82 and G110) (Table 5.2). Additionally, similar relative frequencies of *v8-v10* and *v4-v7* alleles were in permethrin selection survivors and in none treated flies (Table 5.2). These results indicated *H96* alleles *v8-v10* were not associated with permethrin resistance of LPR. The presence of multiple *HR96* alleles in LPR also indicated HR96 was not related to permethrin resistance and overexpression of *CYP6D1*.

5.3.4 Expression of *HR96* in the LPR and CS strain

Quantitative real-time RT-PCR was used to measure *HR96* transcript level in the LPR and CS strains. No significant difference in *HR96* expression was found between the LPR and CS strains (Figure 5.3). Therefore, difference of *HR96* expression was not associated with the constitutive overexpression of *CYP6D1* in the LPR strain.

5.3.5 Conclusion

In summary, the *CYP6D1v1* (LPR) promoter was shown to be able to mediate PB induction similar to the *CYP6D1v2* (CS) promoter. These results indicated variation

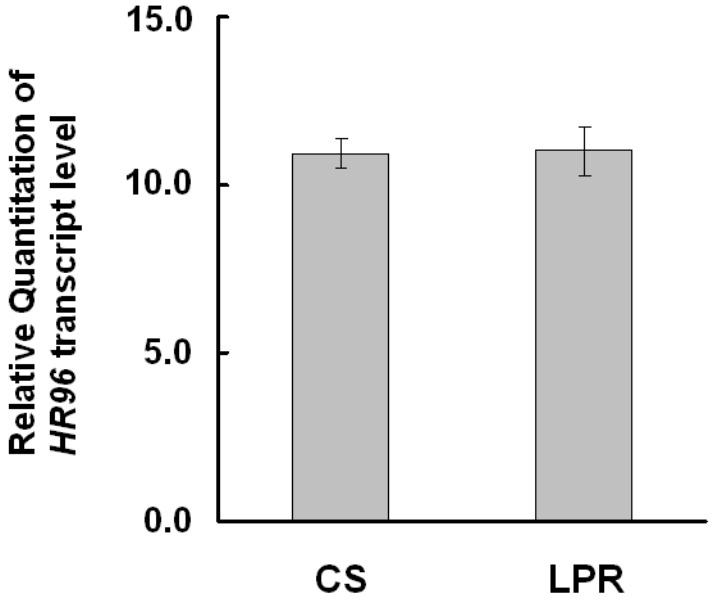


Figure 5.3: Relative quantitation of *HR96* transcript level of the CS and LPR strains. Abdomens of ten three-day-old male adults of CS and LPR were used to measure *HR96* transcript level relative to *Actin* transcript level using real-time RT-PCR with comparative C_T method. Bars represent means of relative quantitation of *HR96* (normalized by *Actin* transcript level) ± S.D. of three PCR reactions of three biological sample pools (n = 9). Difference of *HR96* transcript level between the CS and LPR was not significant (by Student's *t*-test).

in promoter sequence did not affect PB inducibility of *CYP6D1v1* of LPR. Thus, the constitutive overexpression of *CYP6D1* in LPR was attributed to a *trans* acting factor responsible for PB induction, which is consistent with facts that multiple PB inducible P450s were overexpressed in LPR (Carino et al. 1992, Kasai and Scott 2001a). Previous genetic linkage study indicated a *trans* acting factor on chromosome 2 was involved in the lack of PB induction of *CYP6D1* in LPR (Liu and Scott 1997a). House fly HR96 is a transcriptional activator of PB induction of *CYP6D1* (Chapter 4) and is expected to be on chromosome 2. House fly *HR96* were cloned and sequenced. Ten *HR96* alleles based on full length cDNA were identified among four house fly strains, and alleles (*v8*-*v10*) encoding E82V and G110D amino acid substitutions were only identified in LPR. Permethrin selection of LPR strain showed *HR96* alleles with amino acid substitutions were not associated with permethrin resistance. Presence of multiple HR96 *alleles* in LPR indicated HR96 is not associated to permethrin resistance. Additionally, *HR96* transcript level in LPR was measured and showed no difference compared to CS. Thus, there is no evidence indicating house fly *HR96* is associated with permethrin resistance and with the constitutive overexpression of *CYP6D1* in LPR. In conclusion, the molecular basis of constitutive overexpression of *CYP6D1* in LPR is attributed to a *trans* acting factor responsible for PB induction on chromosome 2, but the identity of this *trans* acting factor remains unclear.

CHAPTER 6

FUTURE DIRECTIONS

The ultimate goal of this study was to characterize the molecular basis for the constitutive overexpression of *CYP6D1* in the LPR strain of house fly. A series of experiments were performed and the major conclusions were presented in chapters 3-5. The molecular basis for constitutive overexpression (without PB induction) of *CYP6D1* in LPR was concluded to be due to a *trans* acting factor responsible for PB induction on chromosome 2. However, this *trans* acting factor remains unidentified. To identify other TFs responsible for PB induction of *CYP6D1* should be the first step. If the identified TF candidate is expected to be on chromosome 2, further study is to examine if there is any qualitative or quantitative change appearing in the candidate TF in LPR which could be associated to the overexpression of *CYP6D1* in LPR. Specific future directions are given in the following.

Future direction 1: Using DNA-chromatography technique, *trans* acting factors interacting with the *CYP6D1* promoter region for PB induction could be purified. The identity of each isolated *trans* acting factor can be determined using mass spectrometry following a SDS-PAGE separation. Each identified *trans* acting factor could be further evaluated to confirm if it is involved in mediating PB induction using RNAi treatment of S2 cells in conjunction with PB promoter assays. The respective *trans* acting factors critical for PB induction could be further examined whether or not it is expected to be on chromosome 2 of house fly. Qualified *trans* acting factors could then be studied to detect if there is any qualitative or quantitative change (by cloning its full length cDNA and by measuring its expression level, respectively) related to the constitutive overexpression of *CYP6D1* in LPR. The

limitation of this approach is that *trans* acting factors recruited in the higher hierarchies of serial signaling cascades would be less possibly purified and studied.

Future direction 2: RNAi treatment of S2 cells can be conducted in genome wide scale to identify *trans* acting factors critical for mediating PB induction of *CYP6D1*. This result could provide a list of *trans* acting factor candidates. These candidates could be further evaluated in animals (e.g. knock out in fruit fly by RNAi) to confirm their role in PB induction. *Trans* acting factor candidates will be further considered if it is expected to locate on chromosome 2 of house fly. Qualified *trans* acting factors could be evaluated to detect if there is any qualitative change of its peptide sequence (by cloning full length cDNA) or quantitative change of expression which could be associated to the constitutive overexpression of *CYP6D1* in LPR.

Future direction 3: The binding site of DFD was identified in the *CYP6D1* promoter region for PB induction. DFD is expected to locate on chromosome 2 of house fly. Because of the low expression level of DFD in S2 cells, whether DFD is able to play a critical role in PB induction remains unclear. Expressing DFD in S2 cells could be an approach to evaluate whether or not DFD is playing a role in mediating PB induction of *CYP6D1*. Knocking out or overexpression DFD (depends on whether DFD is expressed or not) in fruit fly (because of lack of similar techniques in house fly) could be a further step to confirm the role of DFD in PB induction. If the role of DFD in PB induction is positive, DFD can thus be studied to detect if there is any qualitative change of peptide sequence (by cloning its full length cDNA0 or quantitative change of its expression in LPR related to the overexpression without PB induction of *CYP6D1*.

APPENDIX A

TRANSCRIPTION START SITES OF *CYP6D1* IN LPR

A.1 Introduction

Two transcription start sites (TSSs) used for *CYP6D1* transcription have been identified in insecticide susceptible strains CS and *aabys* (Chapter 3). The same technique was used to identify the *CYP6D1v1* TSS in LPR.

A.2 Materials and methods

The identification of *CYP6D1v1* TSS in LPR was conducted using Cap structure selective 5' RACE kit (FirstChoice® RLM-RACE, Ambion, Austin, TX) as described in Chapter 3. Nested PCR products were analyzed on a 1.5% agarose gel. PCR products were subjected to gel extraction, TA cloning, and DNA sequencing as described in Chapter 3.

A.3 Results and discussion

Nested PCR products of 5' RACE of *CYP6D1v1* from LPR were analyzed on a 1.5% agarose gel and shown on Figure A.1A. The result showed a bright band about 280 bp and a very faint band about 250 bp. Both PCR products were further analyzed individually by TA cloning and DNA sequencing which led to identification of two respective TSSs located at 122 and 85 nt upstream of ATG (Figure A.1B). This result indicated two TSSs were used for *CYP6D1* transcription in LPR, although the distal TSS (122 nt upstream of ATG) was clearly the major TSS in LPR. The minor proximal TSS was defined as position +1. Relative to proximal TSS, the distal TSS was in position -37.

Two *CYP6D1* TSSs have been identified in insecticide susceptible strains CS and

aabys (see Chapter 3). Compared to results of CS and *aabys*, LPR also has two TSSs used for *CYP6D1* transcription. The proximal TSS (i.e. 85 nt upstream of ATG) of LPR was identical to the proximal TSS found in CS and *aabys* (Figure A.1B). However the distal TSS (122 nt upstream of ATG) of LPR was not identical to the distal TSS (i.e. 111 nt upstream of ATG) found in CS and *aabys* (Figure A.1B). In addition, the band intensity of PCR product on the agarose gel regarding to distal TSS of LPR was significantly greater than that of proximal TSS of LPR. This difference of band intensities of PCR products in LPR was different from the two TSS with similar band intensities found in CS and *aabys*. The molecular basis responsible for above distinct features found in LPR compared to CS and *aabys* remains unclear, although there is an insertion of 15 bp in the core promoter found in LPR which disrupt the binding site of house Gfi-1 (Scott et al. 1999). So far, there is no evidence indicating that distinct features about *CYP6D1* TSSs found in LPR are related and responsible for increased transcription of *CYP6D1* and respective insecticide resistance.

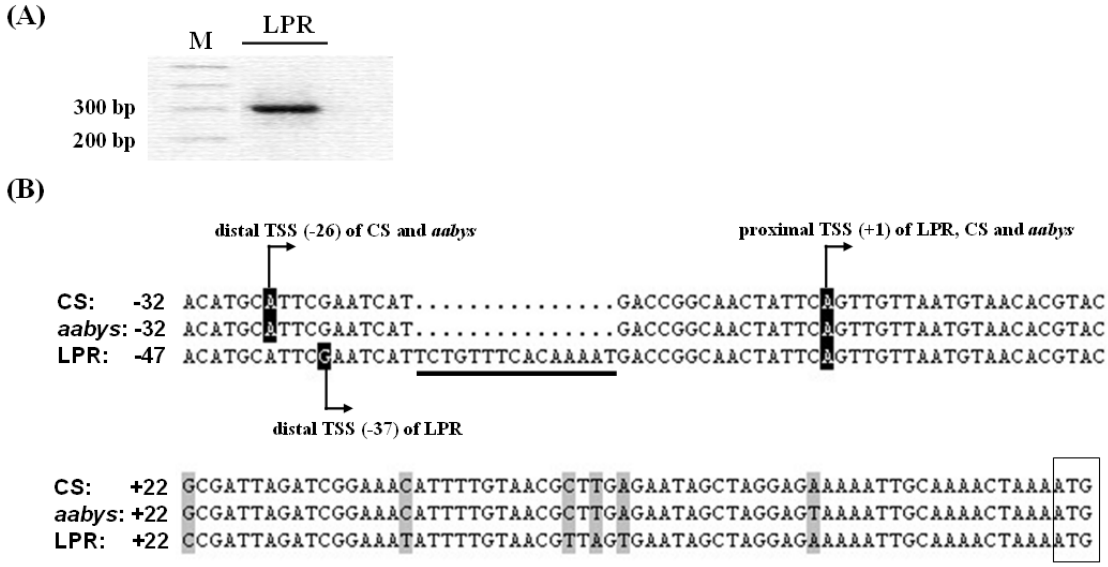


Figure A.1: (A) Nested PCR products of 5' RACE of *CYP6D1* from LPR were analyzed in an agarose gel (negative image). There was a very faint band beneath the bright band which was also cloned and sequenced. **M** denoted the lane of DNA ladder marker. (B) Two *CYP6D1* TSSs identified in LPR compared to CS and *aabys*. TSSs identified among LPR, CS, and *aabys* were denoted in black background colors. The proximal TSS was defined as +1, which was identical among LPR, CS, and *aabys*. The distal TSS of LPR was in position -37, but CS's and *aabys*'s were in position -26. SNPs among LPR, CS and *aabys* were denoted in gray background colors. The insertion of 15 bp identified only in LPR was underlined. Translation start site, ATG, was boxed and in position +86.

APPENDIX B

GENOTYPING OF HOUSE FLY DFD

B.1 Introduction

Based on the chromosome homology map (Foster et al. 1981, Weller and Foster 1993), house fly *Dfd* is expected to be on chromosome 2. Also on chromosome 2 is a *trans* acting factor is causing constructive overexpression of *CYP6D1* (Liu and Scott 1997a). Although whether or not DFD plays a role in transcription of *CYP6D1* remains unclear, a putative binding site of DFD was identified within the PB responsive promoter region (from -330 to -280) of *CYP6D1* (Chapter 4), suggesting a mutation of house fly *Dfd* in LPR could affect expression and PB induction of *CYP6D1*. Therefore, I examined if *Dfd* was linked to resistance in LPR.

A partial gDNA sequence (290 bp) of house fly *Dfd* was obtained using PCR with degenerate primers. This gDNA sequence region was used to genotype individual house flies from three strains: *aabys*, LPR, and R12. Ten *Dfd* alleles (*v1-v10*) were identified among house fly strains examined. The presence of multiple alleles, combined with a lack of time, resulted in several flies having genotypes that could not be determined. These results did not clearly indicate whether house fly *Dfd* is associated with resistance in LPR or not. Further work is needed to resolve whether or not *Dfd* is linked to resistance in LPR.

B.2 Materials and methods

B.2.1 Genotyping partial DNA sequence of house fly *Dfd*

PCR to obtain partial gDNA sequence of house fly *Dfd* was performed using degenerate primers (forward: 5'-CAA GAA TTG GGT ATG CGT TTG MGN TGY GAY GA-3' and reverse 5'-CAC CGG CCA CAT GAA TCT TYT TCA TCC A-3').

Degenerate primers were obtained using CODEHOP (http://blocks.fhcrc.org/codehop.html) (Rose et al. 2003) with an alignment of DFD peptide sequences of *Drosophila melanogaster* (NP_477201.1), *Drosophila yakuba* (XP_002096730.1), *Aedes aegypti* (XP_001660498.1), and *Anopheles gambiae* (XP_001688962.1). The PCR reaction included 0.5 µl of genomic DNA, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 10 µl of ddH_2O, and 12.5 µl of 2X GoTaq® Green Master Mix (Promega). PCR reactions were carried out in a iCycler thermal cycler (Bio-Rad, Hercules, CA) with following temperature program: 95ºC for 5 min; 35 cycles of 95ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 35 sec; and 72ºC for 3 min. Genotyping was performed using gDNAs of individual male flies (n=10) of each house strain (i.e. *aabys*, LPR, and R12). PCR products were purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI) and sequenced in both directions using the forward primer (5'-TGA TGA TGG CTC GGA AAA TGT-3') and the reverse primer (5'-CAC CGG CCA CAT GAA TCT TYT TCA TCC A-3'), respectively, at the Cornell University Life Sciences Core Laboratories Center.

B.2.2 R12 strain

The R12 strain is a strain having resistant alleles from chromosome 1 and 2 of the LPR strain, which are related to constitutive overexpression of *CYP6D1*. The rest of chromosomes (i.e. 3, 4, and 5) of R12 were derived from the *aabys* strain with susceptible genetic background. Genetic crosses for generating the R12 strain was conducted according to previous description (Liu and Scott 1997a). Female *aabys* flies were corssed with male LPR files. F_1 males were backcrossed to *aabys* females. Files with phenotype (++bys) were isolated. Permethrin selection was conducted to acquire homozygote R alleles on loci on chromosome 1 and 2 in R12 strain. Bioassays showed R12 strain was resistant to permethrin (LD_{50}= 235.53 ng;

RR=142.75, compared to *aabys*). Genotyping showed 100% (n=20) of homozygous R allele on *CYP6D1* locus in the R12 strain. Semi-quantitative RT-PCR showed *CYP6D1* is overexpressed in R12 strain compared to *aabys*.

B.3 Results and discussion

PCR using degenerate primers amplified a partial gDNA sequence of house fly *Dfd* with 290 bp. This sequence consists of partial regions of two exons and an intron (64 bp) (Figure B.1). Genotyping was performed to identify *Dfd* alleles in three house fly strains: *aabys*, LPR, and R12. Initially alleles and their sequences were determined from individuals having no SNPs (presumably homozygotes). Individuals having SNPs at positions corresponding variation between identified alleles were scored as heterozygotes of the known alleles. Individuals having SNPs at positions that did not correspond to variations between identified alleles were scored as "not determined" (ND). The alignment of identified alleles and ND sequences of *Dfd* is shown in Figure B.2. Table B.1 shows the genotype of *Dfd* alleles with the number of flies identified in each house fly strain. Table B.2 lists not determined sequences with the number of flies identified in each house fly strain. These results did not provide a clear evidence to indicate whether or not house fly *Dfd* is associated with resistance in LPR. Not determined sequences would require further cloning steps to acquire single sequence in order to determine respective alleles.

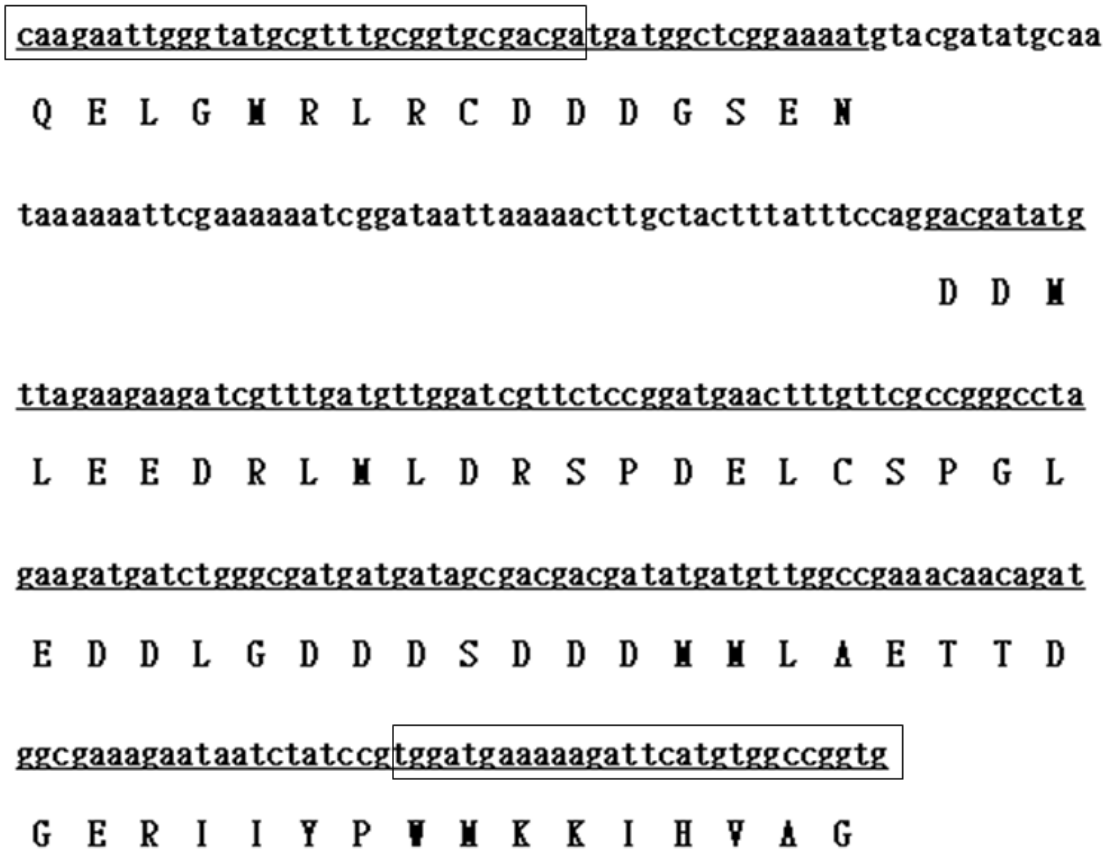


Figure B.1: Partial gDNA sequence (290 bp) of house fly *Dfd*. The coding regions (exons) are underlined and are denoted with amino acid letters beneath. Regions targeted by forward and reverse degenerate primers were boxed.

Figure B.2: Alignment of sequences of *Dfd* alleles (*v1*-*v10*) alleles and not determined sequences (ND1-ND14). These sequences do not include regions regarding to degenerate primers. The primary (100%), secondary (80%), and tertiary (60%) conservation are colored with black, gray, and white, respectively, in the background.


* 20 * 40 *
v1 : TGGCTCGGAAAATGTACGATATGCAATAAAAAATTCGAAAAAATCGGATA : 50
v2 : TGGCTCGGAAAATGTACGATATGCAATAAAAAATTCGAAAAAATCGGATA : 50
v3 : TGGCTCGGAAAATGTACGATATGCAATAAAAAATTCGAAAAAATCGGATA : 50
v4 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
v5 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
v6 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
v7 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
v8 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
v9 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATSGGATA : 50
v10 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATSGGATA : 50
ND1 : TGGCTCGGAAAATGTACGATATRCAATAAAAAATTCGAAAAAATCGGATA : 50
ND2 : TGGCTCGGAAAATGTACGATATGCAATAAAAAATTCGAAAAAATCGGATA : 50
ND3 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
ND4 : TGGCTCGGAAAATGTACGATATRCAATAAAAAATTCGAAAAAATCGGATA : 50
ND5 : TGGCTCGGAAAATGTACGATATRCAATAAAAAATTCGAAAAAATCGGATA : 50
ND6 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
ND7 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
ND8 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
ND9 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
ND10 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATCGGATA : 50
ND11 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATSGGATA : 50
ND12 : TGGCTCGGAAAATGTACGATATRCAATAAAAAATTCGAAAAAATSGGATA : 50
ND13 : TGGCTCGGAAAATGTACGATATRCAATAAAAAATTCGAAAAAATSGGATA : 50
ND14 : TGGCTCGGAAAATGTACGATATACAATAAAAAATTCGAAAAAATSGGATA : 50
60 * 80 * 100
v1 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
v2 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
v3 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
v4 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
v5 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
v6 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
v7 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
v8 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
v9 : ATTAAAAACTTGCTACTTTACTTCCAGGACGATATGTTAGAAGAAGACCG : 100
v10 : ATTAAAAACTTGCTACTTTACTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND1 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
ND2 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGATCG : 100
ND3 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND4 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND5 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND6 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND7 : ATTATAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND8 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND9 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND10 : ATTAAAAACTTGCTACTTTATTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND11 : ATTAAAAACTTGCTACTTTAYTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND12 : ATTAAAAACTTGCTACTTTAYTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND13 : ATTAAAAACTTGCTACTTTAYTTCCAGGACGATATGTTAGAAGAAGACCG : 100
ND14 : ATTAAAAACTTGCTACTTTAYTTCCAGGACGATATGTTAGAAGAAGACCG : 100

Figure B.2 (Continued)

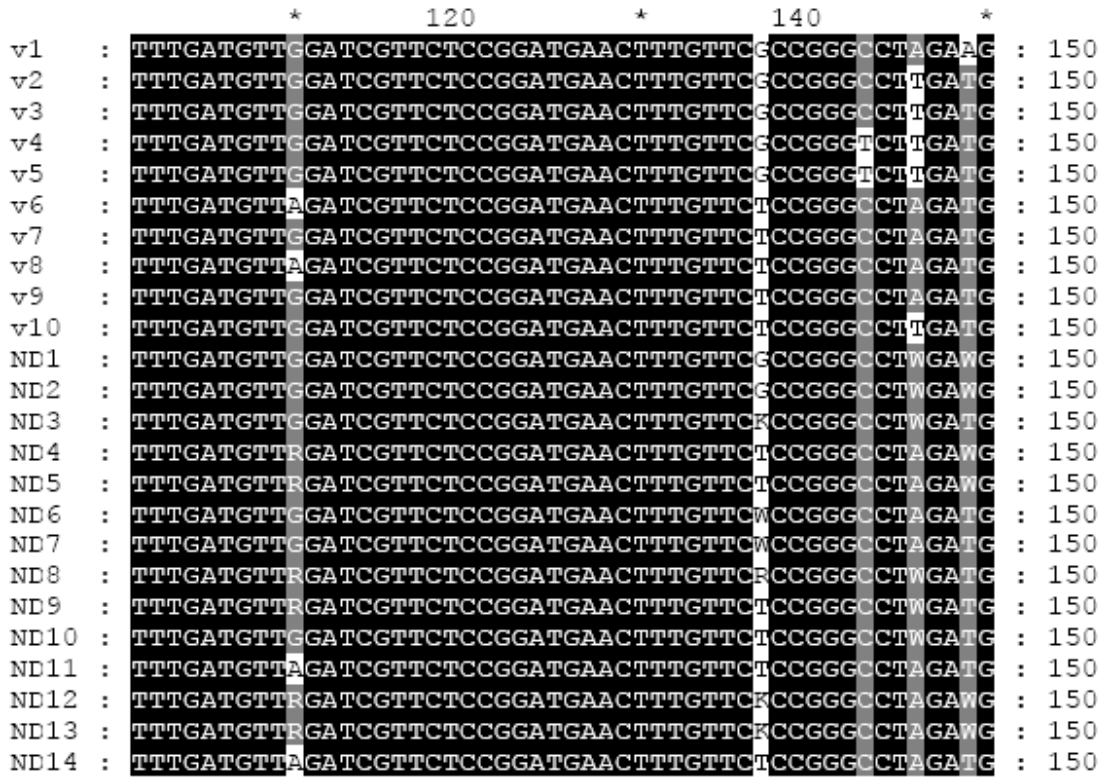


Figure B.2 (Continued)

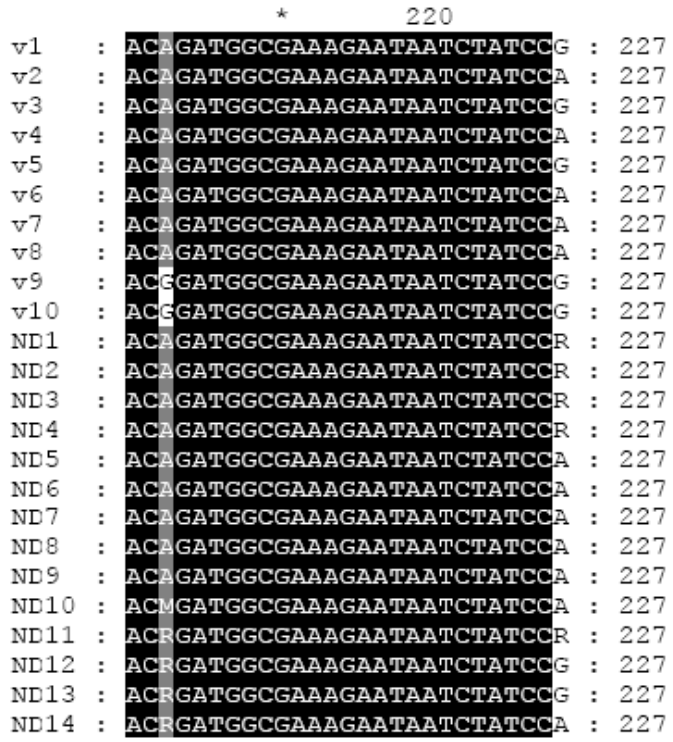


Table B.1: Genotype of *Dfd* alleles identified from three strains of house fly

Genotype and number of flies (in parenthesis) identified		
aabys	LPR	R12
v1/v1 (5)	*v4/v4* (1)	*v8/v8* (1)
v2/v3 (1)	*v5/v5* (2)	
v4/v4 (1)	*v6/v7* (1)	
	v9/v10 (1)	

Table B.2: *Dfd* not determined sequences identified from house fly strains

	Number of flies identified in each following strain		
	aabys	LPR	R12
ND1	2	-	-
ND2	1	-	-
ND3	-	1	-
ND4	-	-	1
ND5	-	-	1
ND6	-	1	-
ND7	-	1	-
ND8	-	1	-
ND9	-	-	1
ND10	-	-	1
ND11	-	1	1
ND12	-	-	2
ND13	-	-	1
ND14	-	-	1

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