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# Investigations of the constitutive overexpression of *CYP6D1* in the permethrin resistant LPR strain of house fly (*Musca domestica*)

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ABSTRACT

House fly (*Musca domestica*) *CYP6D1* is a cytochrome P450 involved in metabolism of xenobiotics. *CYP6D1* is located on chromosome 1 and its expression is inducible in response to the prototypical P450 inducer phenobarbital (PB) in insecticide susceptible strains. Increased transcription of *CYP6D1* confers resistance to permethrin in the LPR strain, and this trait maps to chromosomes 1 and 2. However, the constitutive overexpression of *CYP6D1* in LPR is not further increased by PB and the non-responsiveness to PB maps to chromosome 2. It has been suggested that a single factor on chromosome 2 could be responsible for both the constitutive overexpression and lack of PB induction of *CYP6D1* in LPR. We examined the PB inducibility of *CYP6D1v1* promoter from LPR using dual luciferase reporter assays in *Drosophila* S2 cells and found the *CYP6D1v1* promoter was able to mediate PB induction, similar to the *CYP6D1v2* promoter from the insecticide susceptible CS strain. Therefore, variation in promoter sequences of *CYP6D1v1* and *v2* does not appear responsible for the lack of PB induction of *CYP6D1v1* in LPR; this suggests an unidentified *trans* acting factor is responsible. HR96 has been implicated in having a role in PB induction in *Drosophila melanogaster* and *M. domestica*. Therefore, house fly *HR96* cDNA was cloned and sequenced to examine if this *trans* acting factor is responsible for constitutive overexpression of *CYP6D1v1* in LPR. Multiple *HR96* alleles (*v1–v10*) were identified, but none were associated with resistance. Expression levels of *HR96* were not different between LPR and 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 elusive.



## 1. Introduction

House fly (*Musca domestica*) cytochrome P450 CYP6D1 can metabolize numerous xenobiotics, including beno(a)pyrene [1], chlorpyrifos [2], methoxyresorufin [1], phenanthrene [3], and phenoxybenzyl pyrethroids such as cypermethrin, deltamethrin, and permethrin [4,5]. *CYP6D1* was sequenced [6] and mapped to chromosome 1 [7]. Phenobarbital (PB) treatment of insecticide susceptible strains results in a 6- to 8-fold increase in CYP6D1 transcript and protein [8,9]. *CYP6D1* is expressed in numerous tissues [10,11] and expression of *CYP6D1* is developmentally regulated, being found only in adults [9].

Increased transcription of *CYP6D1* confers metabolism-mediated resistance to phenoxybenzyl pyrethroids (e.g. permethrin) in the Learn Pyrethroid Resistant (LPR) strain of house fly [12]. *CYP6D1* transcript and protein levels are overexpressed (~9-fold increase) in LPR relative to insecticide susceptible strains [8,9,13]. Overexpression of *CYP6D1* in LPR is not due to gene duplication [14] or increased transcript stability [12]. Increased transcription of *CYP6D1*, measured with nuclear run-on assays, was linked to factors on chromosomes 1 and 2 [8,13]. Multiple *CYP6D1* alleles have been identified in pyrethroid susceptible strains, but only one (*v1*) is found in resistant strains [15,16]. The promoters 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 [15]. This insertion in the *CYP6D1v1* promoter of the LPR disrupts the binding site for a known transcriptional repressor Gfi-1. House fly *Gfi-1* is on chromosome 1 [17]. Thus, this deletion in the *CYP6D1v1* promoter 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. The constitutive overexpression of *CYP6D1* in LPR is not further increased by PB and the non-responsiveness to PB maps to chromosome 2. Whether variation in *CYP6D1* promoter sequences is involved in the lack of PB induction in LPR is not known.

The *CYP6D1v2* promoter sequence from −330 to −280 (numbers are relative to transcription start site, +1) of the insecticide susceptible CS strain is critical for PB induction [18]. *Drosophila* HR96 (*hormone receptor-like in 96*) and BR-C (*broad-complex*) were

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identified as a PB dependent transcriptional activator and repressor, respectively, of *CYP6D1v2* in *Drosophila* S2 cells [18]. Based on a chromosome homology map [19,20], *HR96* and BR-C are expected to be on chromosomes 2 and 3, respectively, of house fly. Thus, properties of HR96 (activator on chromosome 2) were consistent with the hypothesis that this might be the factor responsible for the overexpression of *CYP6D1* in LPR.

The goals of this study were: (1) determine if the *CYP6D1v1* promoter retained its responsiveness to PB, (2) clone and sequence *HR96* from house fly, and (3) determine if *HR96* played a role in resistance. 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 like the *CYP6D1v2* promoter from CS [18]; although there are four single nucleotide polymorphisms (SNPs) in the PB responsive promoter region in LPR compared to CS [15]. These results indicated variations in promoter sequences did not significantly affect of PB inducibility of the *CYP6D1v1* promoter in S2 cells. Therefore, the lack of PB induction of *CYP6D1* in LPR appears due to an unidentified *trans* acting factor on chromosome 2. House fly *HR96* was cloned and sequenced in order to examine its role in resistance. Multiple *HR96* alleles (*v1–v10*) were identified, but neither a specific allele nor levels of expression were associated with resistance.

## 2. Materials and methods

### 2.1. House flies

Four house fly strains were used. The CS and *aabys* are insecticide susceptible strains [21]. The LPR is a permethrin resistant strain originally collected from a dairy near Horseheads, New York in 1982 [22]. The OCR is a cyclodiene resistant (*Rdl*) [23] and pyrethroid susceptible strain [24]. 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 libitum*. Larvae and adults were reared at 28 °C, 60% relative humidity, with a 12: 12 h light/dark photoperiod.

### 2.2. 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.

### 2.3. CYP6D1v1 promoter constructs and PB responsive promoter assays

Progressive 5′ deletions of the *CYP6D1v1* promoter from the LPR strain [15] were generated by PCR amplification. Promoter regions −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) as previously described [18]. PB responsive promoter assays were performed using a dual luciferase reporter assay system (Promega) in *Drosophila* S2 cells as previously described [18]. Three independent transfections (PB or control) of each promoter construct were conducted in each replicate. The entire experiment was replicated four times. 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.

### 2.4. Isolation of gDNA or mRNA, gel extraction, TA cloning, plasmid DNA purification, and DNA sequencing

DNA was isolated from individual adult house flies as previously described [25]. Purification of mRNA was done using an Illustra QuickPrep™ micro mRNA purification kit (GE healthcare, Little Chalfont, UK). Gel extraction of PCR products 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 a QIAprep Miniprep system (Qiagen). Plasmid DNAs were sequenced at the Cornell University Life Sciences Core Laboratories Center.

### 2.5. Cloning of house fly HR96

Degenerate primers were selected using CODEHOP (http://blocks.fhcrc.org/codehop.html) [26] with an alignment of the 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* adults), 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) and was carried out in a iCycler thermal cycler (Bio-Rad, Hercules, CA) with following temperature program: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 60 °C for 45 s (decrease temperature by 0.3 °C every 1 cycle), and 72 °C for 1 min 20 s; and 72 °C for 5 min. Three distinct degenerate primer pairs produced partial sequences of *HR96*: (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′. A FirstChoice® RLM-RACE kit (Ambion, Austin, TX) was used for 5′ and 3′ RACE of *HR96* according to the manufacture's instructions 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: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and 72 °C 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. The complete coding sequence of *HR96* was obtained from four strains (*aabys*, CS, OCR, and LPR) by PCR using forward primer 5′-CAA AGA TGT CAC CAA TTA ATA AAG TCT GTG C-3′, reverse primer 5′-ATG ATG TAG GAA TTA AGG ACA TTT GAG GTA AC-3′ and the following thermal cycler program: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 62 °C for 45 s, and 72 °C for 2 min 30 s; and 72 °C 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). The PCR products were analyzed on a 1.6% agarose gel and then subjected to gel extraction, TA cloning, plasmid DNA purification, and DNA sequencing as described above.