Pesticide Biochemistry and Physiology 100 (2011) 130–134

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

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

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ARTICLE INFO

Article history:
Received 5 January 2011
Accepted 24 February 2011
Available online 2 March 2011

Keywords:
Pyrethroid resistance
CYP6D1 transcription
Phenobarbital induction
HR96
Musca domestica
S2 cells

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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doi:10.1016/j.pestbp.2011.02.012

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.

2.6. Permethrin selection of the LPR strain

Technical grade permethrin, 60% *cis* and 39% *trans*, (ChemService, West Chester, PA) was 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 would result in 90–95% mortality in LPR. 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 the polymorphic region containing E82V and G110D substitutions as described in Section 2.5.

2.7. HR96 genotyping

The polymorphic region of *HR96* (from position −5 to 602, numbers are relative to translation start site ATG as +1) was amplified and sequenced using genomic DNA of individual flies to determine if *HR96* was at the permethrin resistance locus on chromosome 2. 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: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 50 s; and 72 °C 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.

2.8. Quantitative real-time RT-PCR of HR96

Relative quantitation of *HR96* transcript level was measured by normalizing to *Actin* (GenBank: ES652303.1) using real-time RT-PCR with the comparative C_T method. Purified mRNA (500 ng), derived from 10 abdomens of three-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: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data 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 prepared independently) of CS and LPR strains ($n = 9$) were acquired. Statistical 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.

3. Results and discussion

3.1. CYP6D1v1 (LPR) promoter is able to mediate PB induction in S2 cells

The *CYP6D1v2* (CS) promoter sequence from −330 to −280 has been identified to be critical for mediating PB induction in *Drosophila* S2 cells [18]. The equivalent *CYP6D1v1* promoter sequence (from −351 to −301) from LPR contains four SNPs compared to the *CYP6D1v2* promoter sequence from CS [15]. 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 (Fig. 1), indicating the presence of critical *cis*-regulatory sequence for PB induction within *CYP6D1v1* promoter region (−365 to −267) of LPR. The fold change in PB induction of *CYP6D1v1* (2.03 ± 0.27, $n = 12$) did not show significant difference ($p < 0.3774$, Student's *t*-test) compared to the fold change in PB induction of *CYP6D1v2* (1.87 ± 0.07, $n = 9$) [18]. This indicates that despite polymorphisms between the *CYP6D1v1* (from LPR) and *CYP6D1v2* (from CS) 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 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 another region of DNA is responsible for the increased transcription of *CYP6D1* in LPR (e.g. other 5′ flanking sequences, intron sequences, or 3′ flanking sequences).

Previously, *Drosophila* HR96 and BR-C were identified as a key transcription factors regulating PB induction of *CYP6D1v2* in *Drosophila* S2 cells [18]. Based on a chromosome homology map [19,20], *HR96* is expected to be 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.

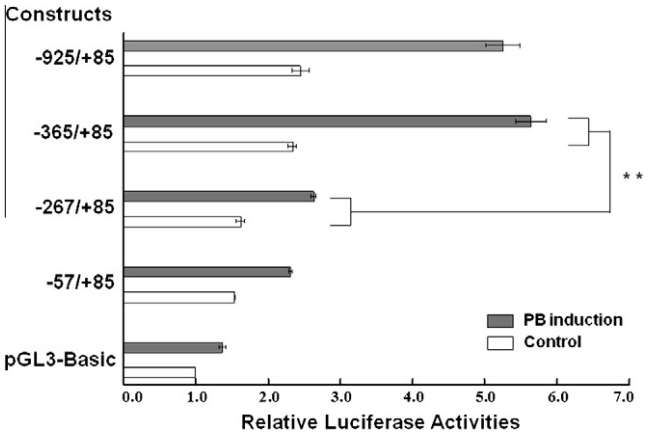

Fig. 1. *CYP6D1v1* promoter sequence from −365 to −267 (from LPR) is able to mediate PB induction. Promoter constructs are numbered relative to the transcription start site (TSS) at +1. Relative luciferase activity was estimated by normalizing each signal of each promoter construct to the mean of signals of pGL3-Basic vector in the same replicate. Bars represent the mean 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).

Fig. 2. Alignment of *D. melanogaster* (Dm) and *M. domestica* (Md) HR96 deduced protein sequences. (A) Protein sequence alignment of the DNA binding domain (DBD). Sequences representing the DBD are underlined. Locations of two amino acid substitutions (E82V and G110D) identified in alleles *v8–v10* from the LPR strain are denoted with asterisks above the alignment. (B) Protein sequence alignment of the ligand binding domain (LBD). Sequences representing the LBD are underlined.

Table 1
HR96 alleles, based on full length cDNA, from four 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]

Alleles (Accession No.): *v1*(HM150723), *v2*(HM150724), *v3*(HM150725), *v4*(HM150726), *v5*(HM150727), *v6*(HM150728), *v7*(HM150729), *v8*(HM150730), *v9*(HM150731), *v10*(HM150732).
[a] Alleles encoding E82V and G110D amino acid substitutions.

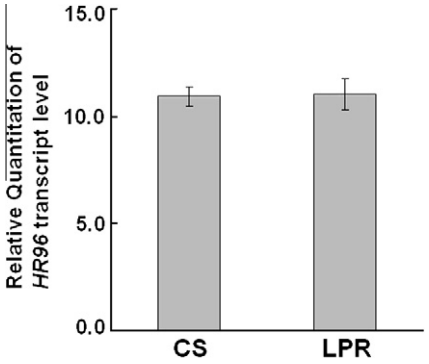

Fig. 3. Relative quantitation of *HR96* transcript level of the CS and LPR strains. Abdomens of ten three-day-old male adults of CS and LPR were used to measure *HR96* transcript level relative to *Actin* transcript level using real-time RT-PCR by the comparative C_T method. Bars represent means of relative quantitation of *HR96* (normalized by *Actin* transcript level) ± S.D. of three PCR reactions of three biological sample pools ($n = 9$). The *HR96* transcript levels were not significantly different between CS and LPR (by Student's *t*-test).

3.2. Cloning of house fly HR96 complete coding sequence

The full length cDNA of house fly *HR96* contains an ORF of 2508 nucleotides encoding 836 amino acids, a 5′ UTR of six nucleotides, and a 3′ UTR of 440 nucleotides (GenBank Accession No. HM150722). The DNA binding domain (aa: 6–76, zinc finger, C4 type, Fig. 2A) and ligand binding domain (aa: 638–810, ligand-binding domain of nuclear receptor family 1, Fig. 2B) were 94% and 84% identical to domains of HR96 of *D. melanogaster*, respectively.

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 1). The deduced amino acid sequences of alleles *v1–v7* were all identical (alleles were identified by synonymous SNPs and/or intron polymorphisms). However, alleles *v8*, *v9*, and *v10* all contained two non-synonymous SNPs (E82V and G110D; Table 1). 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) (Fig. 2A). The G110 residue is located in a region which is less conserved among insect HR96 orthologs. Alleles *v8*, *v9*, and *v10* were found only in the LPR strain, although LPR also contained the *v4–7* alleles which were found in pyrethroid susceptible strains (Table 1).

The presence of multiple *HR96* alleles in LPR (including those found in susceptible strains) suggested that either this locus was not linked to resistance or that the LPR strain had been inadvertently contaminated. To test these competing hypotheses, 300 adult male LPR flies were topically treated with permethrin (6.25 μg/fly), which resulted in 96.3% mortality (11 survivors). The HR96 alleles present in these 11 survivors were compared to the alleles found in 10 non-treated and randomly sampled LPR flies. There was no difference in the frequency of the alleles between the permethrin treated survivors and the non-treated flies. The permethrin survivors had 14 wild-type alleles (*v4–7*) and eight alleles containing the E82V and G110D mutations (*v8–10*), compared to 14 *v4–7* and 6 *v8–10* alleles in the non-treated flies. These

results indicate there was no selective sweep at the *HR96* locus associated with permethrin resistance in LPR.

3.4. Expression of HR96 in LPR and CS

Quantitative real-time RT-PCR was used to measure *HR96* transcript level in the resistant LPR and susceptible CS strains. No significant difference in *HR96* expression was found between these strains (Fig. 3). Therefore, differences in *HR96* expression are not associated with constitutive overexpression of *CYP6D1* (nor resistance) in the LPR strain.

4. Conclusion

The *CYP6D1v1* (LPR) promoter is able to mediate PB induction similar to the *CYP6D1v2* (CS) promoter. Thus, the constitutive overexpression of *CYP6D1* in LPR is attributed to a *trans* acting factor on chromosome 2, rather than differences in the promoter. House fly *HR96* was cloned, sequenced and 10 alleles were identified. However, analysis of the alleles present, and of *HR96* levels in LPR compared to CS, did not support the hypothesis that HR96 was the transcription factor responsible for the constitutive overexpression of *CYP6D1* in LPR. The identification of this factor remains elusive. It has recently been proposed that the house fly genome should be sequenced [27]. Such an effort would offer tremendous new insights into the biology of the house fly, including offering potential genes that could be investigated for their role in regulating *CYP6D1* (and other P450s).

Acknowledgments

We thank Dr. John Lis (Molecular Biology and Genetics, Cornell University) for providing the *Drosophila* S2 cells and valuable advice. We thank Dr. Brian Lazzaro (Entomology, Cornell University) for providing valuable advice and Dr. Toshinori Kozaki for providing some of the constructs used. This work was supported by a Sarkaria Fellowship (to G.G.H.L.), a Griswold Fellowship (to G.G.H.L.), and the National Institutes of Health (GM47835).

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