Pergamon

Insect Biochemistry and Molecular Biology •• (2001) ••–••

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Expression and regulation of *CYP6D3* in the house fly, *Musca domestica* (L.)

Shinji Kasai, Jeffrey G. Scott *

Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853-0901, USA

Received 1 November 2000; received in revised form 6 March 2001; accepted 12 March 2001

Abstract

Recently, a new cytochrome *P*450 gene, *CYP6D3*, was identified from house fly. *CYP6D3* was found upstream of a related gene (*CYP6D1*) on autosome 1. CYP6D3 cDNA sequences were obtained and compared from insecticide resistant (LPR) and susceptible (CS and Edinburgh) strains. Although each strain had a different *CYP6D3* allele, the deduced amino acid sequences revealed no consistent differences between the susceptible and resistant strains. There was approximately 12-fold more CYP6D3 mRNA detected in adult LPR flies compared to CS, and the elevated level of expression in LPR was not due to gene amplification. Northern blots indicate expression of CYP6D3 mRNA is developmentally regulated with no expression in eggs, yet it is readily detectable in larvae as well as male and female adults. Phenobarbital is a well studied inducer of *P*450s in insects and it induced expression of CYP6D3 mRNA in both the CS (16-fold) and LPR (1.6 fold) strains. The *CYP6D3* 5r flanking regions were sequenced from the resistant and susceptible strains. Possible regulatory sequences within this region are discussed. © 2001 Published by Elsevier Science Ltd.

Keywords: Cytochrome *P*450 monooxygenases; Insecticide resistance; *Musca domestica*; Insecta; Phenobarbital induction; Gene expression

1. Introduction

Although the cytochrome *P*450 monooxygenases are an important system for the metabolism of endogenous and exogenous compounds, the presence of multiple *P*450 isoforms makes it difficult to study these enzymes. We have recently reported on the identification of *CYP6D3* from the house fly, *Musca domestica* (Kasai and Scott, 2001). This *P*450 gene is located 4 kb upstream of the well studied *CYP6D1* on chromosome 1, and the deduced amino acid sequences of the two *P*450s have 78% identity. CYP6D1 is responsible for monooxygenase-mediated pyrethroid resistance in house flies from North America (Kasai and Scott, 2000). Previous studies showed that CYP6D1 mRNA was expressed at about a 10-fold increase in the permethrin resistant LPR strain compared to a susceptible strain (Liu and Scott, 1997; Tomita et al., 1995). Overexpression of *CYP6D1* is due to an increase in gene transcription and not to gene amplification nor to mRNA stabilization (Liu and Scott, 1998). Given the high degree of similarity between *CYP6D1* and *CYP6D3* there is a need to evaluate the expression of *CYP6D3*, determine the specificity of nucleotide probes for these related genes and to compare the alleles between resistant and susceptible strains. Furthermore, examination of the promoter regions of *CYP6D1* and *CYP6D3* might help to identify sequences that could be correlated with the expression of these two *P*450s.

Herein, we compare the alleles, probe specificity and promoter regions of *CYP6D1* and *CYP6D3* between an insecticide resistant (LPR) and two susceptible strains of house fly. The expression of *CYP6D3* is also examined at different life stages and following exposure to phenobarbital.

2. Materials and methods

2.1. House fly strains

Two strains of house flies were used. CS (Cornell susceptible) is an insecticide susceptible strain reared

* Corresponding author. Tel.: +1-607-255-7340; fax: +1-607-255-0939.

E-mail address: jgs5@cornell.edu (J.G. Scott).

0965-1748/01/$ - see front matter © 2001 Published by Elsevier Science Ltd.
PII: S0965-1748(01)00073-X

without exposure to insecticides (Scott et al., 1996). LPR is a pyrethroid resistant strain established from field collected flies that were subjected to permethrin selection (Scott and Georghiou, 1985). Synergism and in vitro metabolism studies showed that a major mechanism of pyrethroid resistance in this strain is cytochrome *P*450 monooxygenase detoxification of pyrethroids mediated by CYP6D1 (references in Scott, 1999).

2.2. Cloning of the full length CYP6D3 cDNA

Poly(A)+RNA was isolated using Quick Prep Micro mRNA purification kit (Amersham Pharmacia Biotech) from adult house flies. First strand cDNA was synthesized with isolated mRNA and an oligo(dT) primer. CYP6D3 cDNA sequences were amplified by RT–PCR using primers designed based on the *CYP6D3* sequence (Kasai and Scott, 2001) under the following conditions: the reaction mixtures were first kept at 94°C for 2 min, then 40 cycles of PCR (94°C for 45 s, 50°C for 45 s and 72°C for 1 min) were done, and the samples were finally kept at 72°C for 5 min. PCR products of the expected length were purified using a QIAquick Gel Extraction Kit (QIAGEN) and subcloned into TA-cloning vector (Invitrogen) as described by the manufacturer. Plasmid DNA from putative positive clones (i.e. those having the correct size insert) was isolated and sequenced by the Cornell Biotechnology Facility. A minimum of two independent clones were sequenced (in both directions and with internal primers). If there was any ambiguity in the sequence a minimum of two more clones was sequenced.

2.3. Evaluation of probe specificity

To evaluate the specificity of probes, Southern blots were carried out using purified CYP6D1 or CYP6D3 cDNA. CYP6D1 (1.4 kb) and CYP6D3 (1.6 kb) cDNAs were amplified by PCR, separated by agarose gel electrophoresis and purified as described above. Purified cDNA was separated by electrophoresis and transferred onto nylon membranes (GeneScreen Plus, NEN). CYP6D1 and CYP6D3 cDNAs were labeled with [α-^{32}P]dCTP using a RadPrime DNA labeling system (Life Technologies, Rockville, MD), and used as a hybridization probes. DNA blots were pre-hybridized at 68°C in QuickHyb solution (Stratagene, La Jolla, CA) for 3 h and then hybridized with probes in the presence of sheared salmon DNA for 16 h. The membranes were then washed three times with 2.0×SSC+0.1% SDS at room temperature followed by a 30 min wash with 0.2×SSC+0.1% SDS at 65°C.

2.4. Northern blotting

Using the Quick Prep mRNA Purification Kit (Amersham Pharmacia Biotech), mRNA was isolated directly from house fly tissues (0.1 g). Abdomens were used from adult house flies, otherwise whole eggs, larvae (fourth instar) or pupae were used. For the phenobarbital (PB) induction study, 2 day old female flies were exposed to 0.3% (w/v) of sodium phenobarbital (Sigma, St. Louis, MO) in 15% (w/v) sugar water (or sugar water only for the controls) as described previously (Liu and Scott, 1997). Flies were collected 24 h after exposure to PB, frozen in liquid nitrogen and stored at −80°C.

Three micrograms of mRNA from each sample were separated by formaldehyde denaturing gel electrophoresis and transferred onto nylon membrane (Sambrook et al., 1989). A 1.6 kb CYP6D3 cDNA was labeled with [α-^{32}P]dCTP (as described above) and used as the probe. RNA blots were pre-hybridized, hybridized and washed as described for the probe specificity test. Radioactive signal intensity from different samples was quantified using a phosphorimager (Molecular Dynamics) and compared to the signals from serial dilutions (1-, 2-, 4-, 8- and 16-fold) of mRNA from LPR. After quantification, the blots were stripped by washing two times (1 h each time) in a boiling solution of 0.1×SSC+0.1% SDS (Sambrook et al., 1989). To evaluate the amount of mRNA loaded into each lane the blots were re-hybridized at 68°C with a fragment of the ribosomal protein S3 cDNA (*RPS3*) from *M. domestica* (Kasai and Scott, 2000). Signal intensity was quantified as described above and used to correct for any differences in sample loading. For the developmental and sex specific expression studies, the *RPS3* probe was stripped and re-probed with *CYP6D1* cDNA as described above. All assays were replicated three times using RNA from different batches of houseflies.

2.5. Southern blotting

Genomic DNA was isolated from pupae as described previously (Tomita and Scott, 1995) and quantified by agarose gel electrophoresis using λDNA as a standard. DNA (10 μg) was digested with 200 units of *Eco*RI for 16 h at 37°C in 500 μl buffer solution. The digested DNA was ethanol precipitated, separated on a 1.0% agarose gel and transferred onto nylon membrane as described for probe specific study. The 800 bp *Eco*RI fragment of *CYP6D3* (Kasai and Scott, 2001) was labeled with [α-^{32}P]dCTP, hybridized with the membrane and washed as described above. The membrane was exposed to film (Kodak BioMax®) with an intensifying screen (Dupont Cronex®) for 6 h at −80°C and developed.

2.6. Cloning of the CYP6D3 5′ flanking sequence

The *CYP6D3* 5′ flanking sequences were cloned by PCR using primers D3G (tattagttccctctgaaacc) and AD3D (ggcaaagatgaagaaggcg) which were designed

based on the 5′ flanking sequence of *CYP6D3* previously reported (Kasai and Scott, 2001), and genomic DNA prepared from CS or LPR house flies as a template. The PCR reaction was heated to 95°C for 5 min, then put through 40 cycles of PCR amplification: 95°C for 45 s, 50°C for 45 s, 72°C for 1 min, followed by 72°C for 5 min. PCR products were purified, cloned into TA-cloning vector, and sequenced as described above. A minimum of two independent clones was sequenced (in both directions and with internal primers). If there was any ambiguity in the sequence a minimum of two more clones wwas sequenced.

2.7. Sequence analysis

Sequences were aligned and compared using the MegAlign (3.06 b) program from DNASTAR (Madison, WI). Searches for similar sequences were carried out using a Blast search (Altschul et al., 1997) of GenBank.

3. Results

3.1. Comparison of CYP6D3 deduced amino acid sequences

The CYP6D3 deduced amino acid sequences from the LPR (accession number AF285767), CS (accession number AF283257) and Edinburgh (accession number AF200191) strains revealed differences at positions 13, 190, 221, 339, 344 and 363, including two additional differences in the amino acids at the C-terminus in the CS strain (Table 1). None of the amino acid differences were exclusive to LPR. These results are in contrast to those of *CYP6D1* where the LPR allele is unique from the alleles of susceptible strains (Kasai and Scott, 2001; Tomita et al., 1995). Given that the CYP6D1 deduced amino acid sequence of LPR is uniquely different from susceptible strains, but that the CYP6D3 sequence is not, supports the suggestion (Tomita et al., 1995) that the *CYP6D1v1* allele in LPR codes for a *P*450 offers a selective advantage to this strain (i.e. is catalytically more active towards pyrethroid insecticides, and thus contributes to resistance). Although *Taq* polymerase errors can occur, they are very rare. For example, the *CYP6D1* sequence determined by PCR (Tomita and Scott, 1995) was 100% identical to the sequence subsequently obtained from a genomic clone (Scott et al., 1999). Thus, in the nearly 3000 bp which were sequenced there were no *Taq* errors. While we cannot rule out the possibility that one of the allele differences observed in this study could have been due to a *Taq* error, our past experience indicates this possibility is remote.

3.2. Probe specificity

Given that the CYP6D3 cDNA was 78% similar to CYP6D1, it became important to evaluate the specificity of the nucleotide probes used for Northern and Southern blotting. Fig. 1 shows that CYP6D3 does not cross-hybridize to CYP6D1 under high stringency conditions and *vice versa*. Given that each probe could detect 50 pg of its cDNA, but did not cross-hybridize with 5 ng of the other cDNA we conclude that the CYP6D1 and CYP6D3 probes are highly specific (by at least 100-fold). Therefore, these conditions were used for all subsequent analyses.

3.3. Expression of CYP6D3 and induction by PB

A comparison of the expression of CYP6D3 in CS and LPR house flies is shown in Fig. 2. There was approximately 12-fold more CYP6D3 mRNA detected in adult LPR flies compared to CS (Fig. 5). This is similar to CYP6D1 where there is approximately 10-fold more CYP6D1 mRNA in LPR compared to CS (Tomita et al., 1995).

CYP6D3 expression is developmentally regulated with no expression in eggs nor day 1 pupae, yet it is readily detectable in larvae and adults (both males and females) (Fig. 3). There was a faint level of expression in day 3 pupae which may be due to the presence of pharate adults in this sample. In contrast, CYP6D1 is expressed only in adults (Scott et al., 1996; Tomita et al., 1995), and CYP6A1 is expressed in all life stages (Carino et al., 1994).

PB is a well studied inducer of *P*450s in insects and at least two *P*450s (*CYP6A1* and *CYP6D1*) are inducible in adult house flies. PB induced expression of CYP6D3 mRNA in both the CS (16-fold) and LPR (1.6 fold)

Table 1
Differences in the CYP6D3 deduced amino acid sequences from three strains of house fly. Numbers indicate the position of each amino acid

Strain	Amino acid							
	13	190	221	339	344	363	519	520
LPR	Phe	Glu	Arg	Glu	Ala	Ile	—	—
CS	Ile	Glu	Gln	Glu	Ala	Ile	Arg	Arg
Edinburgh	Phe	Asp	Arg	Asp	Val	Leu	—	—

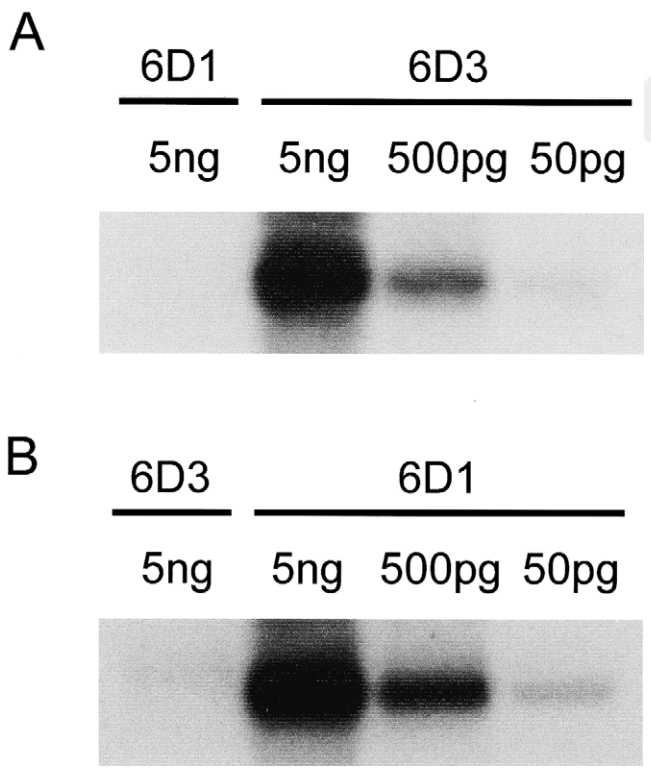

Fig. 1. Evaluation of probe specificity. *CYP6D1* and *CYP6D3* were amplified by PCR using TA-cloned plasmids as templates. Different amounts of each DNA were loaded on an agarose gel. Following electrophoresis, the separated DNA samples were transferred to a nylon membrane, denatured and hybridized with the *CYP6D3* (A) or *CYP6D1* (B) probe. Washing conditions are described in Materials and methods.

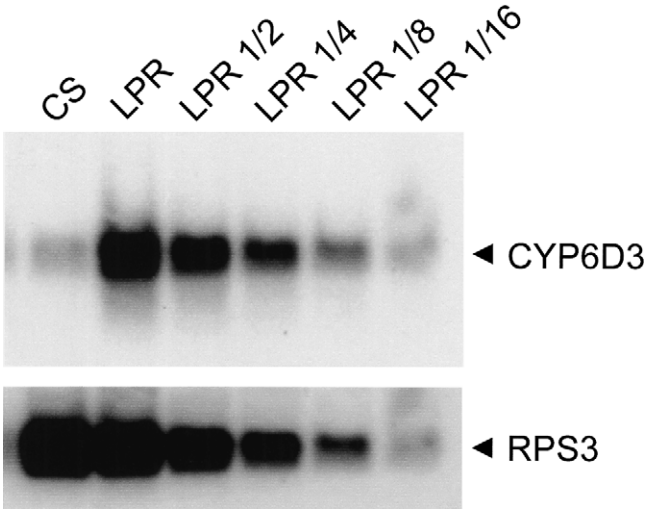

Fig. 2. Northern blot analysis of CYP6D3 mRNA from CS and LPR house flies. Three micrograms of mRNA from adult abdomens of house flies were separated by agarose gel electrophoresis, transferred to nylon membrane and hybridized with the CYP6D3 cDNA (top), and then stripped and rehybridized with ribosomal protein S3 (*RPS3*) cDNA (bottom). Serial dilutions (1-, 2-, 4-, 8- and 16-fold) of mRNA from LPR were loaded as a standard.

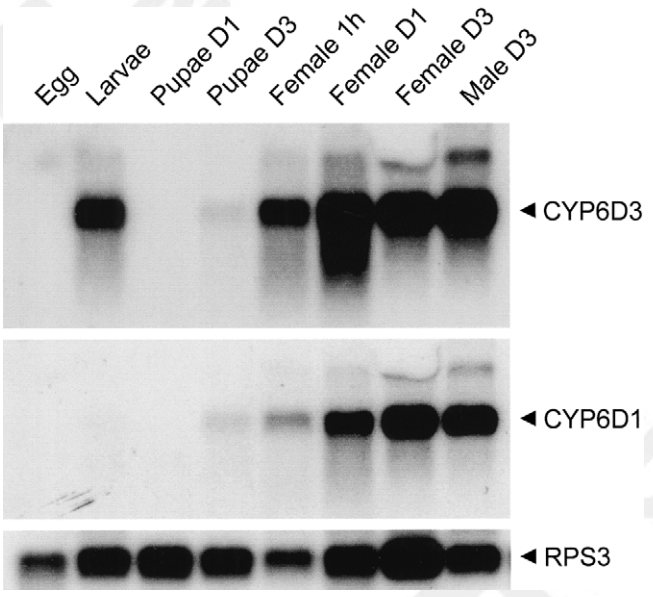

Fig. 3. Developmental changes in CYP6D1 and CYP6D3 mRNA expression in the LPR strain. Three micrograms of mRNA from LPR strain (eggs, larvae, pupae (day 1 and day 3), and adult females (1 h, day 1 and day 3)) were separated by agarose gel electrophoresis, blotted onto nylon membrane and hybridized with CYP6D3 cDNA probe. The probe was stripped and rehybridized with CYP6D1 (middle) and *RPS3* (bottom) cDNA.

strains (Fig. 4). This contrasts with the results for PB induction of CYP6D1 mRNA in CS which resulted in only a 3-fold increase (Scott et al., 1996). The different levels of induction of *CYP6D1* and *CYP6D3* suggest either multiple mechanisms are involved in PB induction or that some genes have more copies of PB responsive elements regulating their control.

3.4. Southern blotting

To determine whether the higher level of *CYP6D3* expression in LPR flies was due to gene amplification, Southern blots of LPR and CS genomic DNA were hybridized with a CYP6D3 cDNA probe. There was no difference in the intensity of the signals from LPR and CS DNA indicating that there was no gene amplification in LPR (Fig. 5). This suggests that the 12-fold higher level of expression of CYP6D3 mRNA in LPR is probably due to enhanced transcription, as has been found for *CYP6D1* (Liu and Scott, 1998), although increased mRNA stability cannot be ruled out for CYP6D3. The presence of a single band on the Southern blots supports our other results (above) that indicated the CYP6D3 probe was highly specific.

3.5. CYP6D3 5′ flanking sequences

CYP6D1 and *CYP6D3* have both similarities and differences in their patterns of expression. Therefore, the promoter regions of both genes were compared across

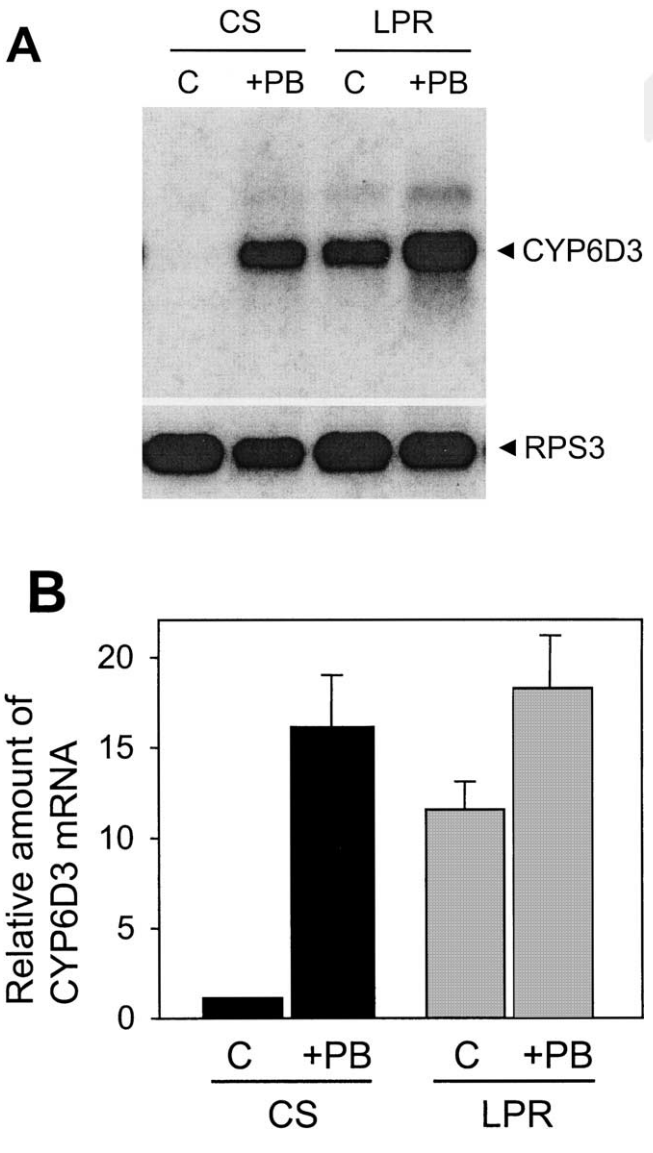

Fig. 4. Phenobarbital induction of the *CYP6D3* gene. (A) Three micrograms of mRNA isolated from two day old female flies, exposed to phenobarbital in sugar water as described in Materials and Methods, were separated by agarose gel electrophoresis, transferred to nylon membrane and hybridized with the *CYP6D3* cDNA probe (top) and then stripped and rehybridized with *RPS3* cDNA (bottom). Control flies (C) were given only sugar water without phenobarbital. (B) Relative CYP6D3 mRNA levels. The relative amount of CYP6D3 mRNA was calculated relative to CS (control)=1. Vertical bars indicate standard errors of the mean.

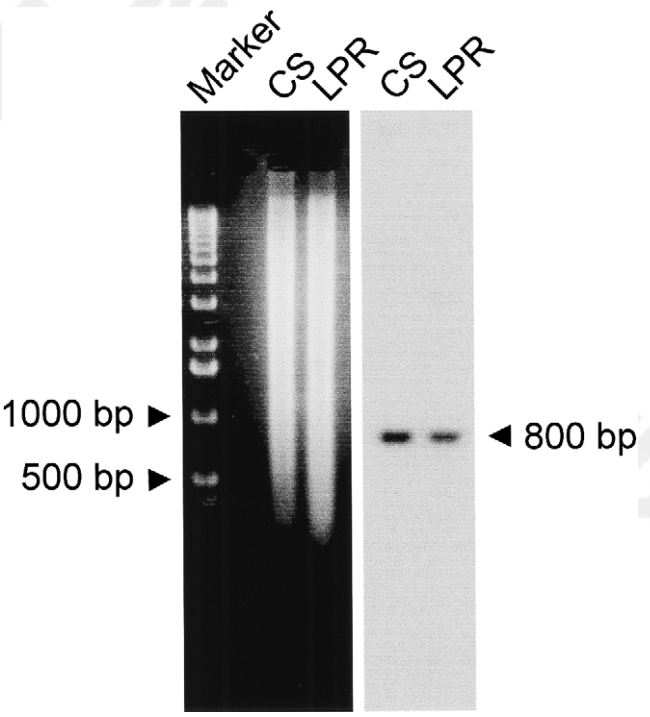

Fig. 5. Southern blot analysis of genomic DNA from LPR and CS house flies probed with CYP6D3 cDNA. Genomic DNA (10 μg) of pupal flies was digested with *Eco*RI, subjected to agarose gel electrophoresis, stained with ethidium bromide and photographed (left). Separated DNA was transferred to nylon membrane and hybridized with *Eco*RI digested *CYP6D3* probe (right).

and between strains to examine if any putative regulatory elements could be identified. An alignment of the *CYP6D3* promoter regions from three strains of house fly is shown in Fig. 6. One unique feature of these sequences is the presence of a repeated sequence (i.e. in LPR −485 to −441 is repeated at −436 to −384) in LPR and CS. The Edinburgh strain lacks the second repeat.

The transcription initiation site (TIS) of *CYP6D1* was previously identified at −86 nt from the translation start site (Scott et al., 1999) within a conserved arthropod promoter element (Cherbas and Cherbas, 1993). An identical sequence (TCAGT) was identified (at −92 to −88 from the translation start site of *CYP6D3* in LPR, Fig. 6) in all three strains suggesting this is the likely TIS. The putative *Gfi*-1 binding site previously identified in the 5′ flanking sequence of *CYP6D1* (Scott et al., 1999) was not found for *CYP6D3*.

If one assumes that the Edinburgh strain (not available for testing) is a typical susceptible strain (i.e. low CYP6D3 mRNA expression that is inducible with PB), then a comparison of the *CYP6D3* promoter regions might shed some light on the differential expression of this gene. Within this region there were several nucleotide differences that were unique to LPR and one of them was in Barbie box-like sequence (Fig. 6). There were also several palindromes found (data not shown), although none of these was specific for LPR. The susceptible CS and Edinburgh strains each had one more Barbie box-like sequence compared to LPR in this region (Fig. 6). Also present in the 5′ flanking region were GATA binding site consensus sequences which varied in position between strains (Abel et al., 1993).

```
LPR        aaactatagc tgct------ ---------- --------ac tgatctactt taaataattt cttttgttac -744
CS         aaactatagc tgct------ ---------- --------ac tgatctactt taaataattt cttttgttac -732
Edinburgh  gaattgtagc tgctctaaag ccaaatgttg ataggtagta tgatctacct taaataagtt cttttgttcg -684

LPR        gggagctcta ctaaatatgc atagtggtgt ggggtatcat atagtcggcc ccgcccgacc tttga-tttc -675
CS         gggagctcta ctaaatatgc atagtggtgt ggggtatcat atagtcggcc ccgcccgacc tttga-tttc -663
Edinburgh  ggagtt-ctg ctaaatgtgc atagtggtgt gggatatcat atagtcggcc ccgaccgatc tttgactttc -615

LPR        ccttactggt taacctactt tctttttttt gggaataaaa gcttctaaaa aaaaacacct ttatttaccc -605
CS         ccttactggt taacctactt tctttttttt- gggaataaaa gcttctaaaa aaaa-cacct ttatttaccc -595
Edinburgh  ccttactggt ttaactact- ---attttt ggcaataaaa gcttctaaag aaa--cacct ttatataccc -551

LPR        tcggtaggca tgtgtccgtg ggtataaata gaagggccgc tagacaaatc tctactcgaa ttcaaccgat -535
CS         tcggtaggca tgtgtccgtg ggtataaata gaagggccgc tagacaaatc tctactcgaa ttcaaccgat -525
Edinburgh  tcggtaggca tgtgtccgtg agtataaatg gaagggccgc tagaaaaatc tatactcgaa ttcaagcgat -481

LPR        tatttcggag ctctgaataa aatctctata tttatatttt gtgttcccct gtgttatcac agcagatatt -465
CS         tatttcggag ctctgaataa aatctctata tttatatttt gtgttcccct gtgttatcac agcagatatt -455
Edinburgh  catttcggtg ctctgaataa aatctctata tttttatttt atgttcccct gtgttatcac agcagatatt -411

LPR        caccttttct gttcggttac aatcccctgt gttcccctgt gttatcaaag cagatattca ccttttctat -395
CS         caccttttct gttcggttac aatcccctgt gttcccctgt gttatcacag cagatattca ccttttctat -385
Edinburgh  cccatttttt gttcgattac aa-------- ---------- ---------- ---------- ---------- -389

LPR        tcggttagaa ttaagtagat caataaaatt gttggaggga gcagatcaca aaattaattc aacctatcaa -325
CS         tcggttagaa ttaagtagat caataaaatt gttggaggga gcagatcaca aaattaattc aacctatcaa -315
Edinburgh  ---------- ttaagtagac caataaaatt gttggaggga gcagatcaca aaattaattt aacctatcaa -329

LPR        gataagacga ctatcatcgg ctaatgcgga gatttataaa t--taaattt taaaattatt atatattttc -257
CS         gataagacga ctatcatcgg ctaatgcgga ---------- ----------t taggattatt atctattttc -264
Edinburgh  gataagacga ctatcatcgg ctaatgcgga gatacggcta ccgaaaattt taaaattatt atctattttc -259

LPR        gcactgtcat gatgggtaag cataaacaaa tgacttgtgt tttggtttca actggacctt gtttacttcg -187
CS         gcactgtcat gatgggtaag cataagcaaa tgactcgtgt tttggtttta actggacatt gtttacttcg -194
Edinburgh  gcactgtcat gatgggtaag cataagcaaa tgacttgtgt tttggtttta actggacctt gtttacttcg -189

LPR        aagtagaaca agaagaacga atacagatta gaaacgtgag cg-------- ---------- ---ttttttg -133
CS         aagtagaaca agaagaacga atacagataa gaaacgtgag cgatttttg aatgtcatta aagttttttg -124
Edinburgh  aagtagaaca agaagaacga atacagatta gaaacgtgag cg-------- ---------- ---ttttttg -135

LPR        aatgtcataa aagtttagca tgtattttgt ttatgcccca agaattcagt tatcaatgag acccatagcc  -68
CS         aatgtcatta aagttt-gca tgtattt-gt ttatgcccca agaattcagt tatcaatgag acccacagcc  -56
Edinburgh  aatgtcatta aagtttagca tgtattttgt ttatgcgcct agaattcagt tatcaatgag acccacagcc  -70

LPR        gactagaaga ccccaagtga a-gtgaaaaa tagttgtcaa aataaaaccc aaattagaat ttgg-aaaaA   +1
CS         gactagaaga ccccaagtga a-gtgaaaaa tagttgtcaa atta------ ------gaat ttgg-aaaaA   +1
Edinburgh  gactagaaga ccccaagtga aagtgaaaaa tagttgtcaa aataaaaccc aaattagaat ttggaaaaaA   +1

LPR        TGTTATTATT TTTGTTAATT ACCCTGCTGA G                                          +32
CS         TGTTATTATT TTTGTTAATT ACCCTGCTGA G                                          +32
Edinburgh  TGCTATTATT TTTGTTAATT ACCCTGCTGA G                                          +32
```

Fig. 6. Alignment of the 5′-flanking sequences of *CYP6D3* alleles. The amino acid coding sequences are indicated by capital letters. The putative Barbie boxes were shown in bold. The nucleotides exclusive to LPR were boxed.

4. Discussion

CYP6D3 was found to be PB inducible in the susceptible and resistant strains, and was expressed at higher levels in the LPR strain compared to CS. This congruence between the subset of *P*450s that are PB inducible and those that are over expression in insecticide resistant strains (i.e. those having monooxygenase-mediated detoxification as a resistance mechanism) has been found in house flies and beyond. For example, CYP6D1, CYP6D3 and CYP6A1 mRNAs are all overexpressed in LPR relative to susceptible strains (Carino et al., 1992; Liu and Scott, 1996), and all three of these *P*450s are also PB inducible in susceptible strains (Carino et al., 1992; Scott et al., 1996). In addition, CYP6C1, C2, A3, A4, A5 and A6 mRNAs were not PB inducible nor expressed at higher levels in either the resistant LPR or Rutgers strains (Cohen and Feyereisen, 1995). CYP6A1 mRNA was PB inducible and over expressed in the Rutgers strain as well (Carino et al., 1992). These correlations have also been observed in *Drosophila* where DDT resistant strains expressed higher levels of CYP6A2 (Waters et al., 1992) and CYP6A8 mRNA (Maitra et al., 1996) and these *P*450s are inducible by PB (Brun et al., 1996; Maitra et al., 1996). This apparent congruence between PB inducibility and over expression in resistant strains, strongly suggests that these two events share a common mechanism; an idea that was originally proposed by Terriere (Terriere, 1983). This is supported by the fact that the gene for PB responsiveness (Liu et al., 1995), as well as overexpression of CYP6D1 and CYP6A1 in resistant strains (Carino et al., 1994; Liu and Scott, 1996) all map to autosome 2 in the house fly.

Another common feature of these studies is that in some insecticide resistant strains, the over expressed *P*450s are not affected by PB type inducers as highly as observed in the susceptible strains. For instance, *CYP6D1* and *CYP6D3* genes were induced by PB 3- and 16-fold, respectively, in the susceptible strain, yet less than 1.6-fold in LPR. In the case of *Drosophila*, CYP6A2 mRNA was induced 15-fold in the susceptible Canton-S strain, but only 2.5-fold in the resistant RDDT strain (Brun et al., 1996). *P*450 MA, purified and characterized from German cockroaches was over-produced in the cypermethrin resistant MA strain (Scharf et al., 1998). *P*450 MA mediated N-demethylase activity was measured using anti-*P*450 MA antiserum as an inhibitor and it was 2.5 times higher in the MA strain than the susceptible JWax strain. The activity was induced 8-fold in the JWax strain by pentamethylbenzene treatment, but only 3-fold in the MA strain (Scharf et al., 1998). Thus, some of the *P*450s over-expressed in resistant strains show less of an increase following induction compared to susceptible strains. Similarly, total *P*450 levels and monooxygenase activities showed greater percent increases following PB treatment in susceptible compared to resistant strains (i.e. strains resistant via increased monooxygenase activity). For example, two reports (Yu and Terriere, 1973; Vincent et al., 1985) noted the net increase in enzyme activity after PB treatment was greater in insecticide susceptible strains than in resistant strains. These workers (Vincent et al., 1985; Yu and Terriere, 1973) suggested that the difference in response to PB induction was due to the rapid metabolism of PB in the resistant strain. This proposed dependence of successful induction on stability of PB-type inducers has been demonstrated in rat liver microsomes (Tsyrlov et al., 1987). Alternatively, this difference in response to PB induction could be that the induction "switch" is always turned on (even in the absence of inducers) in the resistant strains. Further experiments will be needed to clarify this point.

CYP6D1 and *CYP6D3* are located within 20 kb of each other on house fly chromosome 1 and share 77% similarity based on nucleotide sequences. These genes likely diverged relatively recently (at least after the divergence of house fly and fruit fly). Both genes were expressed about 10 times higher in LPR than the susceptible CS strain, suggesting the possible involvement of a common mechanism(s) in the regulation of expression. However, it is noteworthy that there are some differences in the expression of these genes despite their nucleotide similarity.

CYP6D3 was more inducible by PB in the CS strain (Fig. 4) compared to *CYP6D1*, suggesting that these genes could be regulated by both common and unique mechanisms. Furthermore, *CYP6D3* was expressed in the larval stage while *CYP6D1* was not (Fig. 3) suggesting that these enzymes could have different homeostatic function(s).

One strategy for the identification of conserved regulatory elements is to sequence a given gene from closely related species. The premise of this approach is that the only sequences that will be conserved 5′ of the gene are those involved in transcription. The classic experiment of this type was done for the dopa decarboxylase gene (Scholnick et al., 1986). Previously the 5′ flanking region of *CYP6D1* was sequenced from LPR and five susceptible strains of house fly. The most notable feature about the sequence from the LPR strain was the presence of a 15 bp fragment close to the transcription initiation site (nt −15 to −29) which was absent in all of the susceptible strains. The sequences in this region from the pyrethroid susceptible strains (i.e. those lacking the 15 bp fragment) showed some homology with the consensus sequence of the *Gfi*-1 DNA binding site (Zweidler-McKay et al., 1996). Binding of *Gfi*-1 to this consensus sequence inhibits transcription in rats (Zweidler-McKay et al., 1996). Thus it is possible that the 15 bp fragment in the LPR strain acts to disrupt this *Gfi*-1-like repressor, leading to constitutively high levels of *CYP6D1* expression in this strain. This is consistent with the genetic analysis of *CYP6D1* where it was shown that the elevated level of *CYP6D1v1* transcription in LPR is due to factors on autosome 1 and 2. Although CYP6D1 and CYP6D1 mRNA levels are both elevated in LPR, there was no apparent *Gfi*-1 consensus sequence in the 5′ flanking sequence of *CYP6D3* in any of the strains examined. Thus, although *Gfi*-1 does not appear to regulate *CYP6D3* expression, more study will be needed to clarify the role of *Gfi*-1 in expression of *CYP6D1*.

Acknowledgements

We thank F. Harrison and J. Burnett for technical assistance. This work was supported by a grant from the National Institutes of Health (GM47835) and Hatch project 414.

References

Abel, T., Michelson, A.M., Maniatis, T., 1993. A *Drosophila* GATA family member that binds to *Adh* regulatory sequences is expressed in the developing fat body. Development 119, 623–633.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Brun, A., Cuany, A., LeMouel, T., Berge, J., Amichot, M., 1996. Inducibility of the *Drosophila melanogaster* cytochrome *P*450 gene, *CYP6A2*, by phenobarbital in insecticide susceptible or resistant strains. Insect Biochem. Molec. Biol. 26, 697–703.

Carino, F., Koener, J.F., Plapp, F.W.J., Feyereisen, R., 1992.

Expression of the cytochrome *P*450 gene *CYP6A1* in the housefly, *Musca domestica*. In: Mullin, C.A., Scott, J.G. (Eds.), Molecular Mechanisms of Insecticide Resistance: Diversity Among Insects. ACS Symposium Series, vol. 505. American Chemical Society, Washington, DC, pp. 31–40.

Carino, F.A., Koener, J.F., Plapp, F.W. Jr., Feyereisen, R., 1994. Constitutive overexpression of the cytochrome *P*450 gene *CYP6A1* in a house fly strain with metabolic resistance to insecticides. Insect Biochem. Molec. Biol. 24, 411–418.

Cherbas, L., Cherbas, P., 1993. The arthropod initiator: the capsite consensus plays an important role in transcription. Insect Biochem. Molec. Biol. 23, 81–90.

Cohen, M.B., Feyereisen, R., 1995. A cluster of cytochrome *P*450 genes of the CYP6 family in the house fly. DNA Cell Biol. 14, 73–82.

Kasai, S., Scott, J.G., 2000. Overexpression of cytochrome *P*450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. Pestic. Biochem. Physiol. 68, 34–41.

Kasai, S., Scott, J.G., 2001. Cytochrome *P*450 CYP6D3 from the house fly: genomic structure and deduced amino acid sequence. Insect Mol. Biol. (in press).

Liu, N., Scott, J.G., 1996. Genetic analysis of factors controlling elevated cytochrome *P*450, CYP6D1, cytochrome b_5, *P*450 reductase and monooxygenase activities in LPR house flies, *Musca domestica*. Biochem. Genet. 34, 133–148.

Liu, N., Scott, J.G., 1997. Phenobarbital induction of CYP6D1 is due to a *trans* acting factor on autosome 2 in house flies, *Musca domestica*. Insect Molec. Biol. 6, 77–81.

Liu, N., Scott, J.G., 1998. Increased transcription of CYP6D1 causes cytochrome *P*450-mediated insecticide resistance in house fly. Insect Biochem. Molec. Biol. 28, 531–535.

Liu, N., Tomita, T., Scott, J.G., 1995. Allele-specific PCR reveals that the cytochrome $P450_{lpr}$ gene is on chromosome 1 in the house fly, *Musca domestica*. Experientia 51, 164–167.

Maitra, S., Dombrowski, S., Waters, L., Ganguly, R., 1996. Three second chromosome-linked clustered *Cyp6* genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. Gene 180, 165–171.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Scharf, M.E., Neal, J.J., Marcus, C.B., Bennett, G.W., 1998. Cytochrome *P*450 purification and immunological detection in an insecticide resistant strain of German cockroach (*Blattella germanica* L.). Insect Biochem. Molec. Biol. 28, 1–9.

Scholnick, S.B., Bray, S.J., Morgan, B.A., McCormick, C.A., Hirsh, J., 1986. CNS and hypoderm regulatory elements of the *Drosophila melanogaster* dopa decarboxylase gene. Science 234, 998–1002.

Scott, J.G., 1999. Molecular basis of insecticide resistance: cytochromes *P*450. Insect Biochem. Molec. Biol. 29, 757–777.

Scott, J.G., Georghiou, G.P., 1985. Rapid development of high-level permethrin resistance in a field-collected strain of house fly (Diptera: Muscidae) under laboratory selection. J. Econ. Entomol. 78, 316–319.

Scott, J.G., Liu, N., Wen, Z., Smith, F.F., Kasai, S., Horak, C.E., 1999. House fly cytochrome *P*450 *CYP6D1*: 5 prime flanking sequences and comparison of alleles. Gene 226, 347–353.

Scott, J.G., Sridhar, P., Liu, N., 1996. Adult specific expression and induction of cytochrome $P450_{lpr}$ in house flies. Arch. Insect Biochem. Physiol. 31, 313–323.

Terriere, L.C., 1983. Enzyme induction, gene amplification, and insect resistance to insecticides. In: Georghiou, G.P., Saito, T. (Eds.), Pest Resistance to Pesticides. Plenum Press, New York, pp. 265–298.

Tomita, T., Liu, N., Smith, F.F., Sridhar, P., Scott, J.G., 1995. Molecular mechanisms involved in increased expression of a cytochrome *P*450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. Insect Molec. Biol. 4, 135–140.

Tomita, T., Scott, J.G., 1995. cDNA and deduced protein sequence of *CYP6D1*: the putative gene for a cytochrome *P*450 responsible for pyrethroid resistance in house fly. Insect Biochem. Molec. Biol. 25, 275–283.

Tsyrlov, I.B., Gerasimova, K.E., Gutkina, N.I., Lyakhovich, V.V., 1987. Directional modification of cytochrome P-450 substrate structure as a mode of obtaining new inducers of the microsomal monooxygenase system. Biokhimiya 52, 1307–1314.

Vincent, D.R., Moldenke, A.F., Farnsworth, D.E., Terriere, L.C., 1985. Cytochrome *P*-450 in insects. 6. age dependency and phenobarbital induction of cytochrome *P*-450, *P*-450 reductase, and monooxygenase activities in susceptible and resistant strains of *Musca domestica*. Pestic. Biochem. Physiol. 23, 171–181.

Waters, L.C., Zelhof, A.C., Shaw, B.J., Ch'ang, L.Y., 1992. Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated *P*450 gene in *Drosophila*. Proc. Natl. Acad. Sci. USA 89, 4855–4859.

Yu, S.J., Terriere, L.C., 1973. Phenobarbital induction of detoxifying enzymes in resistant and susceptible houseflies. Pestic. Biochem. Physiol. 3, 141–148.

Zweidler-McKay, P.A., Grimes, H.L., Flubacher, M.M., Tsichlis, P.N., 1996. *Gfi*-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. Molec. Cell. Biol. 16, 4024–4034.