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Expression and activity of a house-fly cytochrome P450, CYP6D1, in *Drosophila melanogaster*

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Abstract

The cytochrome P450 system of animals comprises many individual cytochromes P450 in addition to a single cytochrome P450 reductase and cytochrome b_5. Although individual genes of the cytochrome P450 superfamily are highly diverged, the P450 reductase and cytochrome b_5 remain more conserved across taxa. Here, we describe the transformation of *Drosophila melanogaster* with a house-fly-specific cytochrome P450, CYP6D1. Functional activity of ectopically expressed cytochromes P450 requires successful interaction between the transgenic P450 and the requisite coenzymes of the host organism. Transformed *Drosophila*, but not controls, contained CYP6D1 protein as identified by protein immunoblotting, elevated total P450 and elevated CYP6D1 enzymatic activity. These data demonstrate that house-fly CYP6D1 can interact with low to moderate efficiency with *Drosophila* P450 reductase and cytochrome b_5.

Keywords: cytochrome P450 monooxygenases, house fly, *Drosophila*, expression, activity, P450 reductase, cytochrome b_5.

Introduction

Cytochromes P450 (P450s) comprise a gene superfamily (Nelson *et al.*, 1996) with each cytochrome P450 designated CYP followed by a family, subfamily and isoform number (Nelson *et al.*, 1996). P450s metabolize xenobiotics and endogenous compounds (Sipes & Gandolfi, 1991; Mansuy, 1998). Most species have multiple P450 isoforms (eighty-six in *Drosophila melanogaster* (Adams *et al.,* 2000)) and each P450 may have several substrates (Rendic & Di Carlo, 1997). Since the P450s in any one species may have overlapping substrate specificity, it remains difficult to identify the functions of individual P450s. Transgenic technology allows for examination of metabolic contributions of individual P450s *in vitro* and possibly *in vivo* in a system where the enzyme may be absent.

P450 isoforms are the catalytic agents of the P450 monooxygenase system. For functional activity, all P450 isoforms require the cofactor NADPH-dependent P450 oxidoreductase (P450 reductase) and sometimes cytochrome b_5. P450 reductase transfers one or more electrons to the P450 (Backes, 1993; Lewis & Pratt, 1998). In a substrate-specific manner, some P450s also require cytochrome b_5 to either donate the second electron or to allosterically interact with the P450 (Bonfils *et al.*, 1989; Schenkman, 1989; Zhang & Scott, 1996). For example, CYP6D1, a house-fly-specific P450 (Wheelock *et al.*, 1991), requires cytochrome b_5 for aryl hydrocarbon hydroxylase activity (AHH activity, i.e. metabolism of benzo[*a*]pyrene), but not for methoxyresorufin-*O*-demethylase (MROD activity) (Wheelock & Scott, 1992).

Although P450 reductase and cytochrome b_5 are well conserved (Hovemann *et al.*, 1997), the conservation does not guarantee that P450s of one species will interact with the P450 reductase or cytochrome b_5 of any other species. Ectopic expression (i.e. expression where it is not normally present) and activity of P450s in transgenic organisms provides evidence that P450s from one species can interact with the P450 reductase of closely as well as distantly related taxa. For example, human CYP3A7 is active in transgenic mice (Li *et al.*, 1996), while mammalian CYP1A1 expressed in plants is metabolically active with or without fusion to P450 reductase (Shiota *et al.*, 1994; Inui *et al.*, 1999). In yeast, house-fly CYP6D1 and *Drosophila* CYP6A2 are active when coexpressed with P450 reductase of yeast (Smith & Scott, 1997) and humans (Saner *et al.*, 1996), respectively. Furthermore, the microsomal activity of baculovirus-expressed *Drosophila* CYP6A2 is enhanced when incubated with purified P450 reductase of house flies (Dunkov *et al.*, 1997). These studies indicate that P450s can interact with the P450 reductase of other species. Less is known of the interactions of P450s and the cytochrome b_5 of other species. In one study, house-fly CYP6D1

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did not interact efficiently or at all in the presence of endogenous yeast cytochrome b_5 (Smith & Scott, 1997); while in another study baculovirus-expressed *Drosophila* CYP6A2 activity was enhanced by the addition of purified house-fly cytochrome b_5 (Dunkov *et al.*, 1997).

Drosophila remains the most practical transgenic insect system (Ashburner *et al.*, 1998), and P-element-mediated transformation of *Drosophila* is an extensively used method (Rubin & Spradling, 1982). This transformation system provides potential for identifying the specific functions of P450 isoforms (particularly for the growing number of cloned insect P450s), because ectopic P450s can be studied *in vivo* in a eukaryote that may lack that isoform. Evidence from transgenic studies indicates that the P450 reductase of *Drosophila* can interact with two P450s of distantly related species; rat CYP2B1 was directly shown to be active in *Drosophila* microsomes (Jowett *et al*., 1991), while indirect evidence demonstrates that canine CYP1A1 was active in *Drosophila* (Komori *et al.*, 1993). It remains unclear if the cytochrome b_5 of *Drosophila* could also interact with ectopically expressed P450s.

Here, we describe the genetic transformation of *Drosophila* with the cDNA of a well-studied house-fly-specific P450, CYP6D1 (Scott *et al*., 1998; Scott, 1999a,b). We used two enzyme activity assays to determine if CYP6D1 was active in *Drosophila* (i.e. interacts with the P450 reductase and cytochrome b_5 of *Drosophila*). The utility of this type of expression system is discussed in light of the many insect P450s that are presently known.

Results

Embryo microinjections of *Drosophila* with the pUAST-CYP6D1 plasmid resulted in at least three individual strains transformed with *UAS–CYP6D1* in which *CYP6D1* could be expressed after activation of the upstream activator sequence (UAS) elements by GAL4, a transcriptional activator (Brand & Perrimon, 1993). DNA blots (Fig. 1) revealed that the *CYP6D1* insert was in the transformed strains, but not control strains. The insertion position of the *CYP6D1* gene was different between transgenic strains A, B and C because the band size was different for each strain. Also, the single band identified in the DNA of the *CYP6D1*-transformed strains indicated that there was probably only one *CYP6D1* insert each for strains A–C. Linkage experiments verified that strains A, B, and C were genetically distinct because the insertion positions were on chromosomes 2, 3 and X (Table 1).

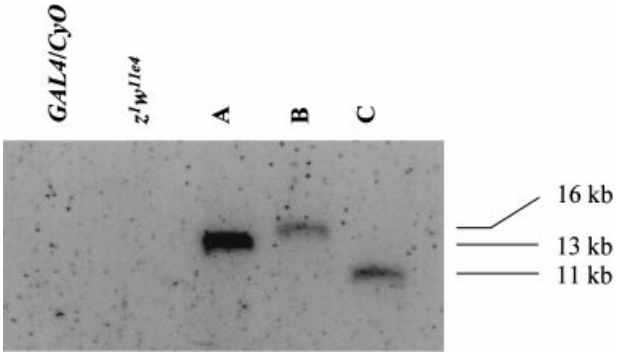

Figure 1. DNA blots of strains *GAL4/CyO*, z^1w^{11e4} and *CYP6D1*-transformed strains A, B and C. Genomic DNA (3 µg) was restriction digested with *Eco*RI prior to Southern blotting (see text) using a 800 bp probe specific to *CYP6D1*. Intensity of bands was not reproducibly different between strains. Approximate sizes of DNA fragments (kb) shown to right of the figure.

CYP6D1 can be expressed in the transformed *Drosophila* via the *GAL4–UAS* expression system only if GAL4 is expressed in flies transformed with the *UAS–CYP6D1* element. Crossing strains A, B and C with *GAL4/CyO* resulted in F_1 animals with either a complete or incomplete expression system that were either capable of expressing *CYP6D1* or not, respectively (Table 1). Protein immunoblotting (Western blotting) with a CYP6D1-specific antibody (Wheelock & Scott, 1990) confirmed that no CYP6D1 was present in *Drosophila* with an incomplete expression system regardless of heat-shock treatment (Fig. 2). The blots also showed that low levels of CYP6D1 were present in

Table 1. Chromosomal linkage of *CYP6D1* insertion in three *Drosophila* strains and genotypes of the F_1

Strain	Chromosomal linkage[a]	Genotype of F_1[b]	
		Complete expression system[c]	Incomplete expression system[d]
A	2	*GAL4/UAS-CYP6D1*	*CyO/UAS-CYP6D1*
B	3	*GAL4/+; UAS-CYP6D1/+*	*CyO/+; UAS-CYP6D1/+*
C	X	*UAS-CYP6D1/+; GAL4/+*	*UAS-CYP6D1/+; CyO/+*
z^1w^{11e4}	–	–	*GAL4/+OR; CyO/+*

[a]Linkage of *UAS–CYP6D1* insert in transformed strains.
[b]F_1 are progeny of females of the transgenic lines (strains A–C) or z^1w^{11e4} and males of *GAL4/CyO*.
[c]All *Drosophila* with a complete expression system should have enhanced *CYP6D1* expression after a heat shock.
[d]All *Drosophila* with an incomplete expression system (i.e. lack either *GAL4* or *UAS–CYP6D1* element) should not be capable of *CYP6D1* expression regardless of heat-shock treatment.

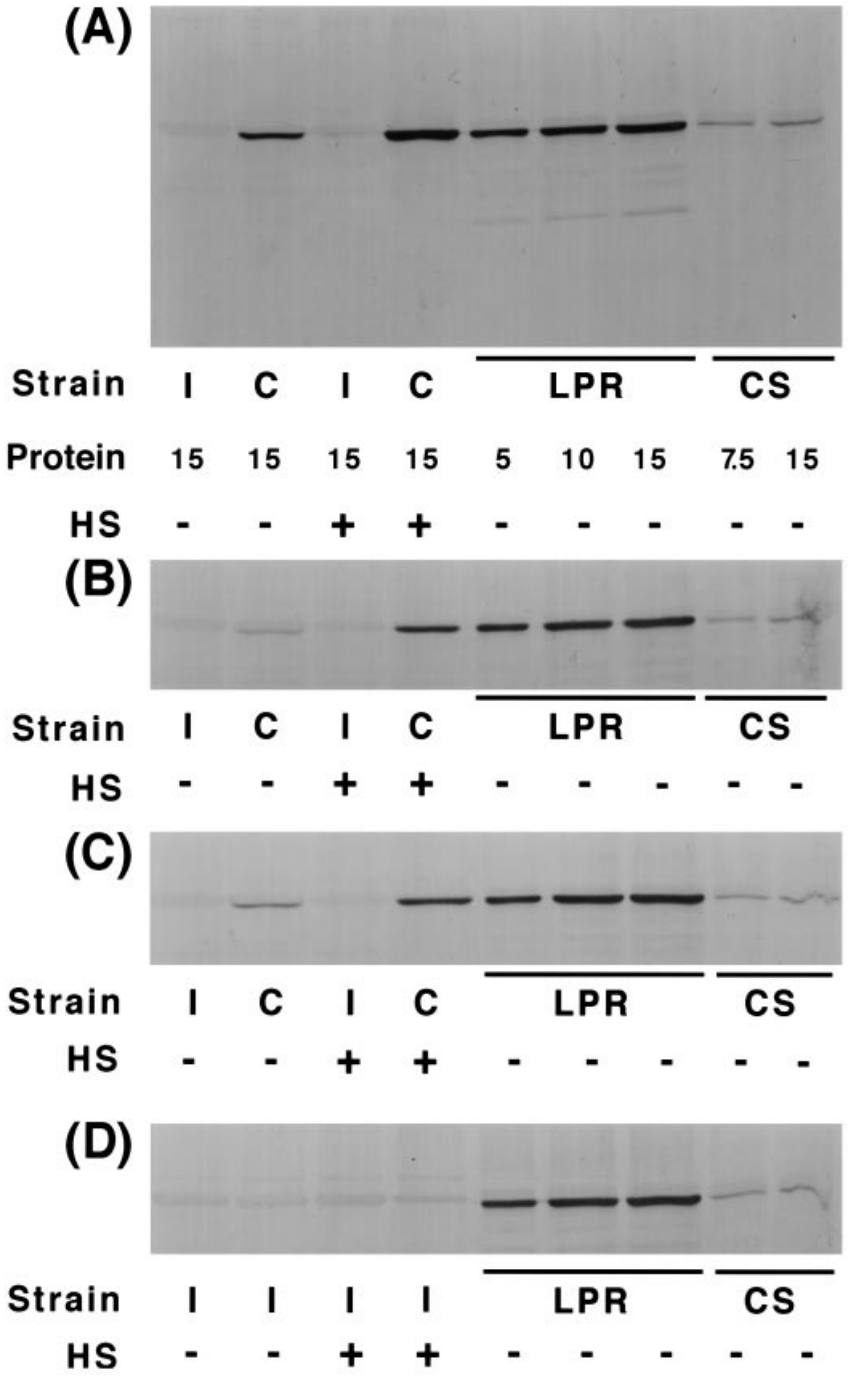

Figure 2. Protein immunoblotting with a CYP6D1-specific antibody. The antibody bound to only protein bands from microsomes of house flies or transformed *Drosophila* with a complete expression system. Microsomes from LPR and CS house flies are shown in each panel as a positive control. Microsomes of *Drosophila* are designated complete (C) or incomplete (I) regarding the expression system. *Drosophila* with an incomplete expression system were incapable of expressing *CYP6D1* because they lacked either the *GAL4* component of the expression system or *UAS–CYP6D1*, the target gene. Protein content in each lane is expressed as micrograms of microsomal protein/lane. Heat shock (HS) is indicated by + or – (control). Panels A, B, C and D show immunoblots of microsomes of progeny of strains A, B, C, or $z^{1}w^{11e4}$, respectively, crossed with *GAL4/CyO* (see Table 1 for genotype of each group of *Drosophila*). All proteins that cross-reacted with the antibody were approximately 59.5 kDa, the estimated size of CYP6D1 (Korytko & Scott, 1998). Each blot was replicated at least twice with similar results.

Drosophila with a complete *GAL4/UAS–CYP6D1* expression system, and that *CYP6D1* expression was dramatically enhanced in the *Drosophila* with a complete expression system that were heat shocked. The low level of CYP6D1 in *Drosophila* with a complete expression system that were not heat shocked is probably due to the leaky nature of the hsp70 promoter, while the enhanced expression following heat shock is clearly a result of the induced *GAL4/UAS–CYP6D1* expression system. More immunodetectable CYP6D1 was found in heat-shocked *Drosophila* with a complete expression system than in CS house flies, and this was about one- to two-thirds the amount of CYP6D1 found in LPR house flies.

AHH activity is a known metabolic marker activity of CYP6D1 (Wheelock & Scott, 1992). Microsomes from *Drosophila* with an incomplete *CYP6D1* expression system had no or barely detectable AHH activity (< 20 pmol/min/mg protein; data only shown for *GAL4/CyO* genotype),

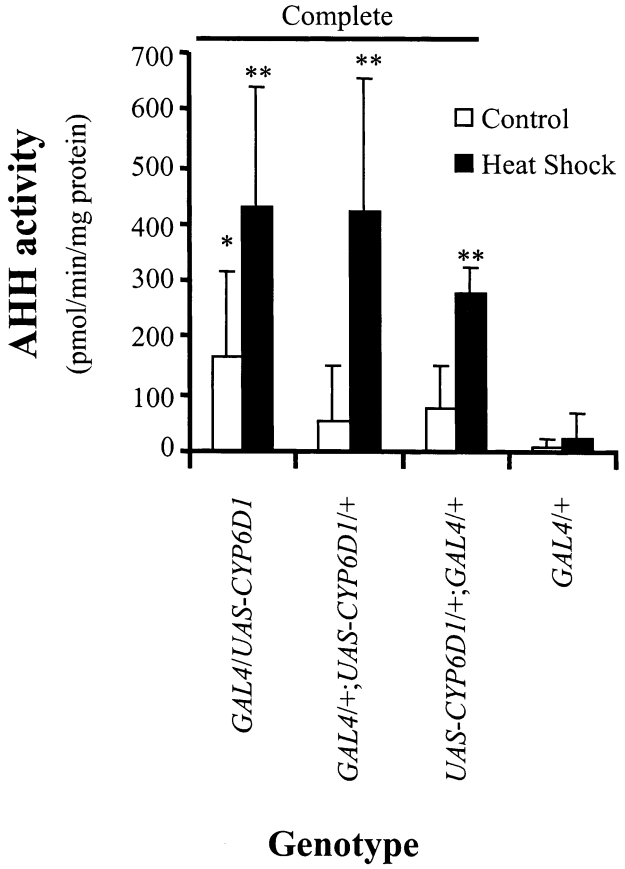

Figure 3. AHH activity of microsomes from *Drosophila* with or without heat shock. AHH activity of microsomes from *Drosophila* that could express CYP6D1 was significantly greater than AHH activity of microsomes that lacked CYP6D1 (*GAL4*/+), at $P < 0.05$ (*) and $P < 0.01$ (**) (*t*-test).

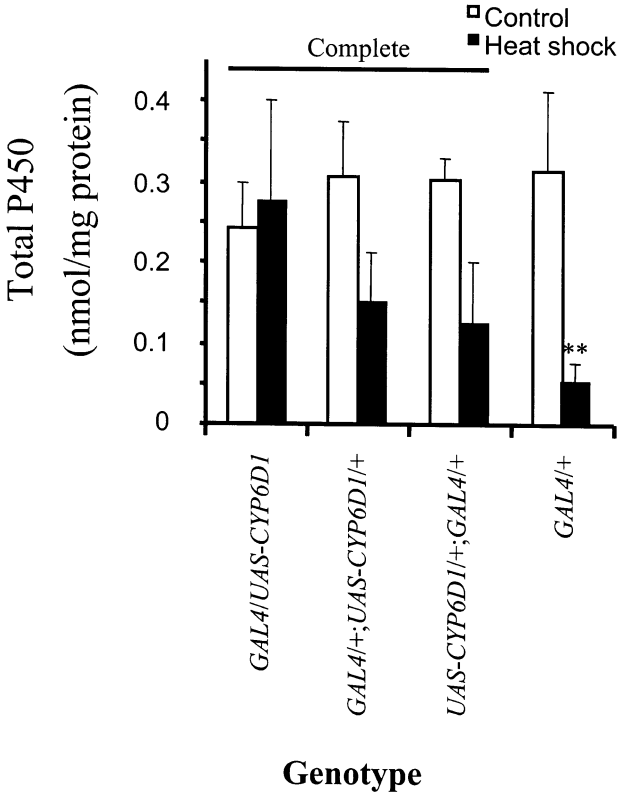

Figure 4. Total P450 levels in microsomes of *Drosophila* with and without heat-shock treatment. Total P450 levels declined after heat shock in microsomes of *Drosophila* that could not express CYP6D1 (*GAL4*/+) relative to sibling controls that were not heat shocked (*t*-test, $P < 0.01$ (**)).

but AHH activity was readily detected in all strains with a complete *CYP6D1* expression system (Fig. 3). Also, there were 2.6–7.6-fold higher levels of AHH activity in *Drosophila* with a complete expression system that were heat shocked relative to non-heat-shocked siblings. Since AHH is a known activity of CYP6D1 (Wheelock & Scott, 1992) and substantial AHH activity was detected only in microsomes that had high levels of CYP6D1 (as determined by the protein immunoblots above), the AHH activity appears due to *CYP6D1* expression. The AHH activity in *Drosophila* with a complete *CYP6D1* expression system demonstrates that CYP6D1 is active in *Drosophila* microsomes; it interacts at some level of efficiency with P450 reductase and cytochrome b_5. However, the maximum AHH activity in microsomes of *Drosophila* was only about 50% of the AHH activity of microsomes from CS house flies (CS strain: 1034 ± 230; mean ± SD pmol/min/mg protein).

Total P450 is the sum of all P450 isozymes in the microsomes of an organism, including ectopically expressed P450s. After heat shock, a control strain that could express *GAL4*, but not *CYP6D1* (*GAL4*/+) had lower total microsomal P450 levels relative to siblings that were not heat shocked (Fig. 4). However, the strains with a complete expression system did not suffer the reduction of total microsomal P450 relative to sibling controls that were not heat shocked (Fig. 4). These data indicate that the heat shock in flies that express *GAL4* reduces total P450 levels, but some of the total P450 appeared to be replaced by CYP6D1 in *Drosophila* that have a complete *CYP6D1* expression system (*GAL4* and *CYP6D1* expression). In *Drosophila* that were not heat shocked, there was no difference in total P450 between flies with a complete or incomplete expression system (data not shown).

MROD is a second CYP6D1-mediated activity in LPR house-fly microsomes (Wheelock & Scott, 1992). Unfortunately, the background MROD activity of the normal *Drosophila* P450s and the general decrease of total P450 after heat shock gave a declining trend of MROD activity in all *Drosophila* after heat shock. The moving baseline of this activity made the CYP6D1-specific component of MROD activity impossible to measure. The decline in total P450 was not a problem for measuring AHH activity because

there was no P450-mediated background AHH activity in control flies.

Discussion

We demonstrated that the house-fly-specific P450, CYP6D1, was active when expressed in *Drosophila*, yet the activity was lower than found in wild-type house flies (CS strain). This remains true even when protein immunoblotting (Western) demonstrated that the microsomes of *Drosophila* with induced *CYP6D1* expression contained similar levels or more CYP6D1 protein than the microsomes of CS house flies. We did not quantify the amount of CYP6D1 in the microsomes of *Drosophila* using rocket immunoelectrophoresis (Hagedorn *et al*., 1978; Korytko & Scott, 1998) because the amount of CYP6D1 in these samples was near the limit of detection using this analysis method. The limited activity of CYP6D1 in the transgenic system may be due in part to suboptimal interactions of CYP6D1 with the *Drosophila* P450 cofactors or to a limited supply of cofactors in the microsomes of *Drosophila* relative to the LPR strain of house fly (i.e. house-fly strains having elevated CYP6D1 also have elevated P450 reductase and b_5). Alternatively, a difference in endoplasmic reticulum membrane structure in *Drosophila* relative to house fly may not fully support P450s of house flies (Stewart & Strother, 1999). Furthermore, the direct comparison of microsomes of house flies and *Drosophila* as equal is not valid because house-fly microsomes are prepared from only abdomens where P450s (Scott *et al*., 1998) including CYP6D1 (Lee & Scott, 1992; Korytko & Scott, 1998) are concentrated. In contrast, the microsomes of *Drosophila* were prepared from whole animals. The preparation of microsomes from the abdomens avoids dilution of the microsomes with proteins from body regions that are high in protein but relatively low in P450 such as the muscular thorax. For these reasons, the microsomes that we isolated from *Drosophila* that express CYP6D1 may have a lower level of total P450, CYP6D1 protein and CYP6D1-mediated activity than if we prepared microsomes exclusively from *Drosophila* abdomens. Unfortunately, production of microsomes from only *Drosophila* abdomens proved intractable.

The moderate ectopic activity of CYP6D1 demonstrated in these experiments encourages our pursuit of further enhancing the CYP6D1 activity in *Drosophila*. At present, the best method to increase the activity of ectopic CYP6D1 is to express more *CYP6D1* in *Drosophila*. This may be accomplished by altering the heat-shock regime to further increase CYP6D1 production, using different strains that express *GAL4* to higher levels, and by increasing the copy number of *CYP6D1* and *GAL4* genes in the *Drosophila* from one copy each to two or more each.

The ability of P450 reductase and cytochrome b_5 of *Drosophila* to support the activity of P450s from other species has telling implications for the evolution of the P450 system. Although P450 gene sequences are widely divergent (Nelson *et al*., 1996), the P450 reductase (Hovemann *et al*., 1997) and cytochrome b_5 are much more conserved in sequence and can still function as cofactors with P450s of different species. The evolution of the structures of P450 reductase and b_5 may be constrained because domains of these cofactors must interact with several P450s. Small changes in P450 reductase or cytochrome b_5 may have dramatic effects on the activity of many or all P450s in an organism potentially resulting in diminished fitness. In contrast, P450s may be less constrained to evolve because there is a pool of P450 monooxygenases that have overlapping substrate specificity. Furthermore, many P450 monooxygenases may function as general detoxifying enzymes with no specific substrate. Mutations of one P450 merely alter that P450, not a subset or all of the P450s, and there may be no deleterious effect. For example, humans that lack a functional *CYP2D6* gene (Dalen *et al*., 1998) or mice that lack either a *CYP1A1*, *1A2*, *1B1* or *2E1* gene (Buters *et al*., 1999), but have an otherwise normal complement of P450 genes, are developmentally, physiologically and pathologically normal.

Drosophila remains a practical transgenic insect system (Ashburner *et al*., 1998) for the study of P450s. Since the P450 reductase and b_5 of this species support the general activities of ectopically expressed P450s to at least a moderate degree, this system could be useful to discover the functions of a variety of P450s, particularly those of insects (Dombrowski *et al*., 1998). Using a *Drosophila* expression system to examine the *in vivo* functions of insect P450s could be useful in identifying P450s that are involved in insect hormone metabolism, homeostasis or xenobiotic metabolism.

Experimental procedures

Strains and reagents

J. Lis and M. Wolfner (Cornell University) supplied the z^1w^{11e4} and *GAL4/CyO* strains. The pUAST plasmid (Brand & Perrimon, 1993) was a gift from M. Wolfner. Unless noted, all reagents were from Sigma-Aldrich Co. (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). All *Drosophila* were reared at 25 °C on media provided by the Cornell *Drosophila* media services, while house-flies, *Musca domestica*, were reared as described (Scott *et al*. 2000). CS is a wild-type strain of house-fly (Scott *et al*., 1996). LPR is a mutant strain of house-fly that overexpresses CYP6D1 (Liu & Scott, 1996).

GAL4–UAS expression system

Brand & Perrimon (1993) developed a two-component GAL4–UAS expression system that allows for selective activation of any cloned gene or cDNA in transformed *Drosophila* (Brand & Perrimon, 1993). Excellent and detailed descriptions of this system are

available (Brand & Perrimon, 1993; Kaiser, 1993; Brand & Dormand, 1995; O'Kane, 1998). The first component is a *Drosophila* strain transformed with a vector that contains a transcription element coupled to a *GAL4* gene. Many strains have been developed, each with different transcription elements (Brand & Dormand, 1995; Kaiser, 1993) that allow for tissue-specific, cell-specific and/or temporal expression of *GAL4*. In the *GAL4*/*CyO* strain, *hsp70* heat-shock elements enhance *GAL4* expression such that there is a high level of GAL4 protein after a heat shock.

The second component of the expression system contains the *UAS* DNA element. The *Drosophila* transformation vector pUAST (Brand & Perrimon, 1993) is commonly used for GAL4-mediated expression of exogenous genes. Within the flanking transposable P-elements, this plasmid contains a w^+ gene, five optimized *UAS* elements, an *hsp70* transcriptional start site, and a multiple cloning site. Even potentially lethal genes (Brand & Perrimon, 1993; Kaiser, 1993) could be expressed in *Drosophila* using this *GAL4–UAS* expression system. When *GAL4* is expressed in cells that have a *UAS* element, the GAL4 binds the element and strongly enhances the expression of the target gene controlled by the *UAS* element (Brand & Perrimon, 1993; Kaiser, 1993; O'Kane, 1998).

Subcloning of CYP6D1 *and* Drosophila *transformation*

A cDNA of *CYP6D1* was created from messenger RNA (mRNA) of the LPR house-fly strain (Scott & Georghiou, 1985, 1986a) by reverse transcription followed by polymerase chain reaction (PCR) amplification. Both primers contained a 5′ GG clamp, a restriction site (*Not*I or *Xho*I – underlined) and base pairs specific to the 5′ and 3′ ends of the *CYP6D1* cDNA (sense primer: 5′-GGGCGGCCGC ATG TTG TTA TTA CTG CTG ATT; antisense primer: 5′-GGCTCGAG TCA CCA CCT CTT CGA CAG GCC CAC). The PCR product was digested with *Not*I and *Xho*I and inserted into the multiple cloning site of the pUAST vector. Competent Top10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA) were transformed with the new plasmid construct and incubated on LB-amp agar plates.

Plasmid DNA from the resultant colonies were amplified by culturing the bacterial colonies, purified, then screened by PCR in addition to restriction mapping. The PCR screen used primers specific to pUAST on either side of the multiple cloning site (sense primer: 5′-AGA ATC TGA ATA GGG AAT TGG G; antisense primer: 5′-CCT CAT TAA AGG CAT TCC ACC). The second screen used *Not*I and *Xho*I restriction enzymes to digest the plasmid. Both screens were evaluated by assessing the size of the products using 1% agarose gel electrophoresis. Of the 12 plasmids that contained a cDNA insert, one plasmid contained the correct *CYP6D1* sequence (100% identity, Cornell DNA Sequencing Facility). This plasmid, pUAST–CYP6D1, was amplified by culturing the bacterial colony and extensively purified; purification steps included three extractions each with phenol : chloroform, chloroform and ethyl ether followed by ethanol precipitation. Plasmid DNA was resuspended (0.1 mM sodium phosphate pH 7.8, 5 mM potassium chloride) to a concentration of 0.7 μg/μl.

J. Werner of Cornell University microinjected 343 embryos of the *w*;P(2Δ3) *Drosophila* strain with purified pUAST–CYP6D1 plasmid using an established transformation protocol (Rubin & Spradling, 1982). Seventy insects pupated, sixty-five eclosed and sixty were fertile. Two G_0 flies were transformants; together, they produced fifty transformed G_1 flies that were identified by the w^+ eye marker. Stable lines of the transformants were developed by crossing each of these fifty flies with the z^1w^{11e4} strain that lacks transposase. Of these lines, twenty-four appeared to have stable P-elements after four generations of crossing back to the z^1w^{11e4} strain. The z^1w^{11e4} strain was used as a control strain for the remaining experiments. Since the transformed strains were crossed back to z^1w^{11e4} for several generations, this strain is genetically most similar to the experimental strains, but lacks the *UAS–CYP6D1* genetic element.

DNA hybridization

Genomic DNA was isolated (Jowett, 1998) from mixed sex *Drosophila*. DNA concentration was determined spectrophotometrically (Sambrook *et al.*, 1989a). Genomic DNA (3 μg) was completely digested (Sambrook *et al.*, 1989b) with *Eco*RI restriction endonuclease enzymes (Life Technologies, Grand Island, NY), separated by agarose gel electrophoresis (Smith, 1996) using a 1% agarose gel, and transferred (Smith & Murphy, 1996) to a GeneScrene Plus membrane (NEN Life science Products, Inc., Boston, MA). The membrane was probed with an 800-bp DNA probe that was prepared by PCR amplification of a portion of the *CYP6D1* cDNA with the pUAST-6D1 plasmid as template (sense primer 5′-TAT GGC ATG ACG TTG AGT CG, antisense primer 5′-CCT CAT TAA AGG CAT TCC ACC). The probe was purified by gel electrophoresis (QIAGEN gel extraction kit, QIAGEN, Valencia, CA) and labelled with a random primer labelling kit (Life Technologies) with α-^{32}P-dCTP (Amersham Life Science, Arlington, IL). The DNA hybridization procedure was completed using a Stratagene QuikHyb solution according to manufacturers instructions (La Jolla, CA) followed by exposure of the membrane to Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY). The size of the detected DNA bands was estimated with DNA size standards (Life Technologies).

Linkage of CYP6D1 *insert*

Chromosomal linkage of the *CYP6D1* inserts were determined by crossing each line with *ywf*; *CyO*/*Gla* and *w*;*TM6,Tb*/*XBB70* stocks (Karess, 1985). *CyO* and *Gla* represent balancers for chromosome 2; *TM6,Tb* and *XBB70* represent balancers for chromosome 3. These balancer stocks were also used to make those strains with inserts on the second and third chromosome homozygous for *CYP6D1* (Karess, 1985). Homozygous lines of the X-linked transgenes were developed by single pair sibling crosses for three generations.

Genetic crosses to establish a complete CYP6D1 expression system

Expression of *CYP6D1* in the transformed strains requires combining a *GAL4* component with the *UAS–CYP6D1* genetic component. This was accomplished by crossing the *GAL4*/*CyO* strain with virgin female homozygous *UAS–CYP6D1* transformants. The *GAL4*/*CyO* strain has a lethal insertion of the *GAL4* gene on chromosome 2, which is balanced with a *CyO* chromosome. Expression of *GAL4* protein is enhanced in this strain with *hsp70* promoter elements upstream of the *GAL4* gene. The progeny of this cross (Table 1) will have either a complete expression system (*GAL4* and *UAS–CYP6D1*) that should express *CYP6D1* or an incomplete expression system (lacks either *GAL4* or *UAS–CYP6D1*) that should not express *CYP6D1*.

Those flies with an incomplete expression system were easily separated from the flies with a complete expression system

because the former were marked with *Curly* wings or were progeny of z^1w^{11e4}. Here, flies with an incomplete expression system are the best controls because they are similar to the experimental animals except that one chromosome is different (i.e. *GAL4* insert or *CyO* balancer). Although the heat-shock enhancer elements are 'leaky' and should enhance *GAL4* expression in all strains with the *GAL4* element, we increased *CYP6D1* expression by heat shocking adult flies for 90 min at 37 °C two times daily for 3 consecutive days. Preliminary evidence showed that this heat-stress regime provided strong expression of *CYP6D1*. On day 4, microsomes were prepared from the adult flies.

Protein preparation

To prepare microsomal protein fractions from *Drosophila*, 200–300 mixed-sex adult flies were homogenized in 5 ml of homogenization buffer (10% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM phenylmethyl sulphonyl fluoride, 1 mM phenylthiocarbamide, 0.1 M sodium phosphate, pH 7.5) (Lee & Scott, 1989a) in a glass/Teflon homogenizer by ten strokes of a motorized homogenizer (Caframo, Wiarton, Ontario). The homogenate was centrifuged for 20 min at 10 000 ***g*** (13 000 rpm) in a JA-20 rotor with a Beckman (Fullerton, CA) J-21C centrifuge. The supernatant was centrifuged for 60 min at 100 000 ***g*** (37 000 rpm) in a Beckman 70-ti rotor with a Beckman L8-M ultracentrifuge. The resulting pellet of microsomes was resuspended in 0.5 ml resuspension buffer (20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM phenylmethyl sulphonyl fluoride, 0.1 M sodium phosphate, pH 7.5) (Lee & Scott, 1989a). Microsomes of LPR (Scott & Georghiou, 1986b) and CS (Scott *et al*., 1996) house-fly strains were prepared as above, but from abdomens of females that were previously frozen. Protein was assayed (Bradford, 1976) with bovine serum albumin (BSA) as the standard. Total P450 and cytochrome b_5 was quantified spectrophotometrically (Omura & Sato, 1964).

Protein immunoblotting

The microsomal protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, CA) with 4% and 10% acrylamide for the stacking and separation gels, respectively (Laemmli, 1970) and transferred to nitrocellulose membrane (Kao *et al*., 1986). Immunoblotting with the CYP6D1-specific antibody 7–735 was completed as previously described (Wheelock & Scott, 1990).

AHH and MROD activities of CYP6D1 in Drosophila

To determine if the CYP6D1 expressed in *Drosophila* was active and could interact with *Drosophila* P450 reductase and cytochrome b_5, the CYP6D1-specific (Wheelock & Scott, 1992) AHH activity was examined in the *Drosophila* microsomes. AHH activity was assayed with an Aminco SPF 500 spectrofluorometer (excitation 387, slit 2 nm; emission 407, slit 0.2 nm). Each assay consisted of 0.2 mg microsomal protein in a total of 200 µl resuspension buffer (above), 1.8 ml of reaction buffer (0.1 M potassium phosphate pH 7.8, 0.1 mM EDTA and 0.5 mM $MgCl_2$) (Lee & Scott, 1989b), 0.8 µl of 2 mM benzo[*a*]pyrene in DMSO (substrate), and 10 µl of 10 mM NADPH. For each assay, NADPH was added after verifying that the background activity was zero. Then, each reaction was assayed for 6–8 min at 28 °C. To quantify the activity, a linear standard curve of benzo[*a*]pyrene was developed by adding 0–0.8 µl (in 0.2-µl increments) of 2 mM benzo[*a*]pyrene to a reaction mixture that did not contain NADPH.

MROD is also a marker of CYP6D1 activity in LPR microsomes (Wheelock & Scott, 1992), but this activity requires only P450 reductase and not cytochrome b_5 (Zhang & Scott, 1994). The MROD activity was measured as described for the AHH assays except for the following: (i) excitation and emission wavelengths were set at 530 (slit 4) and 580 (slit 0.5), respectively; (ii) 4.0 µl of 1 mM methoxyresorufin was used as the substrate; and (iii) the linear standard curve was developed by adding 0–5 µl of 0.005 mM resorufin to reaction buffer.

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