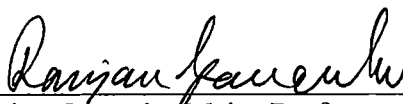
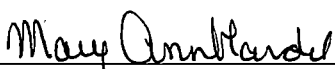


To the Graduate Council:

I am submitting herewith a thesis written by Rawan Awwad entitled "Expression of four Cytochrome P450 family six (*Cyp6*) genes in DDT-resistant and susceptible strains of *Drosophila melanogaster*." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

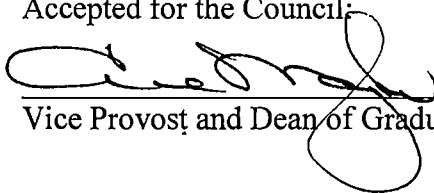

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and recommend its acceptance:


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Bruce McKee

Accepted for the Council:


Vice Provost and Dean of Graduate Studies

Expression of four Cytochrome P450 family six (*Cyp6*) genes in DDT-resistant and
susceptible strains of *Drosophila melanogaster*

A Thesis Presented
For the
Master of Science
Degree
The University of Tennessee, Knoxville

Rawan Awwad
December, 2001

This thesis is dedicated to my parents,
Raghida and Isam Awwad,
and to all those who contributed to my personal growth.

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ABSTRACT

Insecticide resistance has been a problem in both agriculture and public health. In most cases, resistance is mediated by the cytochrome P450 monooxygenases. One or more *Cyp* genes are generally found to be over-expressed in resistant strains compared to susceptible ones. In *Drosophila*, three *Cyp* genes (*Cyp6a2*, *Cyp6a8* and *Cyp6g1*) are known to show over- expression in the resistant 91-R relative to the susceptible 91-C strain. However, the total number of *Cyp* genes over-expressed in the 91-R strain is not known. This investigation was initiated to identify new *Cyp* genes that show a higher expression in 91-R relative to 91-C. The expression of four *Cyp6* family genes (*Cyp6w1*, *Cyp6u1*, *Cyp6a14*, and *Cyp6a13*) on chromosome 2 that lie close to a genetically mapped DDT resistance locus was examined in the resistant 91-R and susceptible 91-C strains. It was found that among all four, only *Cyp6w1* showed a significantly higher expression in 91-R relative to 91-C. Similar to *Cyp6a2* and *Cyp6a8* on chromosome 2, *Cyp6w1* was found to be inducible by barbital. It also shows a higher expression in males than in females. To understand how *Cyp6w1* is regulated, its expression was examined in several chromosome substitution stocks carrying different combinations of the three major chromosomes (X, 2nd, and 3rd) of 91-R and 91-C. It was found that, as for *Cyp6a2* and *Cyp6a8* genes, the 3rd chromosome negatively influences the expression of *Cyp6w1*, whereas the X chromosome has little or no effect. It was also found that the second chromosome of 91-C has an inhibitory influence on the expression of *Cyp6w1*-91C allele, whereas the second chromosome of 91-R has a positive influence on the expression of *Cyp6w1*-91R allele. The results of this investigation imply that *Cyp6w1*, *Cyp6a2*, and *Cyp6a8* genes may be regulated by the same negative *trans*-regulatory gene(s) on the third chromosome. A loss-

of-function mutation of this gene(s) may be responsible for their elevated expression in 91-R.

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I. INTRODUCTION

General introduction

The cytochrome P450s are a battery of detoxifying enzymes that rid our bodies from daily-introduced foreign compounds. Their functions are so versatile that they also metabolize various endogenous compounds like steroids and fatty acids. The importance of P450s in survival is indicated by their presence in all living organisms, from bacteria to man. In addition to their beneficial metabolic functions, they are also involved in the toxic/mutagenic effects of several compounds like polycyclic hydrocarbons and aromatic amines. Therefore, the oxidation reactions by which cytochrome P450 enzymes metabolize various compounds has been of an increasing interest since their discovery in the 1950's. A particularly important metabolic role of P450s is the detoxification of insecticides, which enables certain pests to feed on agricultural crops. P450 enzymes are found to have both a higher activity and a higher content in resistant insects. By studying the expression patterns of genes encoding P450s and the mechanisms by which they are regulated, it may be possible to devise methods to control insecticide resistance.

History of the cytochrome P450 enzymes

The cytochrome P450 enzymes were first discovered in the 1950s, when a class of oxidation reactions was found to take place in rat liver microsomes. These reactions proved to be significant in the metabolism of a wide array of chemicals, including drugs, chemical carcinogens, and steroids (Estabrook, 1996 and references therein). They were termed "mixed-function" oxidation reactions for their diverse role in the metabolism of

endogenous and xenobiotic compounds, in as diverse organisms as prokaryotes, plants, and insects. At that time, the identity and specific role of P450 enzymes in the catalysis of these reactions were unknown. It wasn't until 1958 when Klingenberg and Garfinkle, independently working with liver microsomes, first observed their presence. They found that when rat liver microsomes, treated with the reducing agent sodium dithionite, were gassed with carbon mono-oxide, a pigment produced a spectrophotometric peak at 450 nm (Klingenberg, 1958; Garfinkle, 1958; Estabrook, 1996 and references therein). Later, Omura and Sato (1962, 1964 a,b) identified this pigment as a hemoprotein, and named it P450.

The 1960's mark the period when the first enzymatic functions were assigned to P450's. First, Estabrook *et al.* (1963) observed a P450-mediated hydroxylation of the steroid hormone 17 α -hydroxyprogesterone in microsomal fractions of the adrenal cortex. Later, Cooper *et al.* (1965) demonstrated the role of liver P450s in demethylation and aromatic hydroxylation of drugs such as codeine and acetanilide. The observation that increased metabolism of drugs is associated with elevated levels of CO-binding pigment soon assigned P450s as the main detoxifying enzymes in liver microsomes. Now it is estimated that P450s are involved in more than 60 biochemical reactions with a broad range and overlapping substrate specificity of over 800 compounds (Lewis, 1996), and the list of substrates continues to grow.

Taxonomic distribution, nomenclature, and evolution

Cytochrome P450 (*Cyp*) genes comprise one of the oldest and largest superfamily of genes known to man. Its importance for the sustenance of life is indicated by its presence in all living organisms, from the simplest to the most complex (Guengerich, 1993; Nelson *et al.*, 1996, 1999; Feyereisen, 1999; Scott, 1999). As of January 2001, this superfamily is reported to contain 265 different families and 646 genes (<http://drnelson.utmem.edu/cytochromeP450.html>). Recent genome sequence analysis confirmed the presence of 80 *Cyp* genes in humans, 286 in *Arabidopsis thaliana*, and 90 in *Drosophila melanogaster* (Werck-Reichhart and Feyereisen, 2000). Of the 90 *Cyp* genes in *Drosophila*, 83 code for apparently functional genes and the rest are pseudogenes (Tijet *et al.*, 2001).

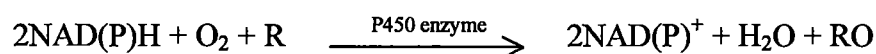
Cytochrome P450 (CYP) proteins are classified into families and subfamilies based on similarities in their amino acid sequences. Members of a family share more than 40% identity at the amino acid sequence level, while members of a subfamily share more than 50% identity (Feyereisen, 1999). The universally accepted nomenclature, introduced by Nerburt *et al.* (1987), appoints all gene members of the P450 superfamily with a *CYP* prefix (from Cytochrome P450) followed by a number for the family and a letter referring to the subfamily (e.g. *CYP1A*). Each individual gene is given a number to differentiate it from others in the subfamily (e.g. *CYP1A1*). In *Drosophila melanogaster*, *Cyp* genes are written with small letters in italics (e.g. *Cyp6w1*).

It is thought that members of this superfamily arose from a single P450 ancestral gene about 1.4-1.5 billion years ago (Nebert and Gonzales, 1987; Nelson and Strobel,

1987). The gene is thought to have arisen in a prokaryotic organism to utilize the increasing levels of atmospheric oxygen for the metabolism of carbon resources (Werck-Reichhart and Feyereisen, 2000). The gene was transferred from one generation to the next, and as the number of life forms began to rise, the number of P450 genes increased through divergent evolution to accommodate the increased complexity of organisms (Nebert and Gonzalas, 1987). For example, the branching of the CYP2 family of enzymes into CYP2A, B, C, D, and E subfamilies of related enzymes occurred at the time of mammalian radiation about 200 million years ago (Lewis, 1996). *CYP* gene evolution occurred by gene conversion events, (Adesnik and Atchison, 1985), gene duplication (Degtyarenko and Archakov, 1993), gene amplification, gene loss, and gene lateral transfer (Werck-Reichhart and Feyereisen, 2000), eventually leading to divergence and acquisition of new characteristics. Selection seemed to have favored gene duplication as the major method of CYP gene divergence, evidenced in the many *CYP* gene clusters in many organisms (Werck-Reichhart and Feyereisen, 2000). The sequence orientation and similarity between members of a gene cluster allow for tracing the evolutionary events that led to their divergence, although in most cases no functional link exists between members of a P450 gene cluster (Werck-Reichhart and Feyereisen, 2000). The polymorphic nature of CYP proteins in the human population is an indication that the CYP family is still evolving (Gonzalez and Gelboin, 1991).

Mechanism of action

CYPs have thousands of diverse substrates, but their mechanism of action is conserved for most of their metabolic functions. Embedded in microsomal membranes (Figure 1), they form the terminal oxidase of an electron transfer chain where they are involved in the splitting of an oxygen molecule (O_2). One oxygen atom gets incorporated into the substrate (R) and the other combines with two protons donated by 2 molecules of nicotinamide-adenine dinucleotide phosphate [NAD(P)H] to form a water molecule. The conserved oxidation reaction can be represented as follows (Guengerich, 1993):



In addition to a P450 enzyme, another key protein is involved in the oxidation of substrates in the eukaryotic microsomal system. This protein is cytochrome P450 reductase (CPR), a flavoprotein that contains a flavin adenine dinucleotide (FAD) domain with an NADPH binding site, and a flavin mononucleotide (FMN) domain (Figure 1). It is anchored in the cytoplasmic face of the endoplasmic reticulum in close proximity to the P450, and acts as the main electron donor in the oxidation reaction. Upon binding of NADPH, FMN is reduced and an electron is transferred to the FAD domain and eventually to the iron atom in the heme-binding site of P450 (Guengerich, 1993). In some cases, one electron is donated by CPR and the other is donated by Cytochrome b_5 (Figure 1).

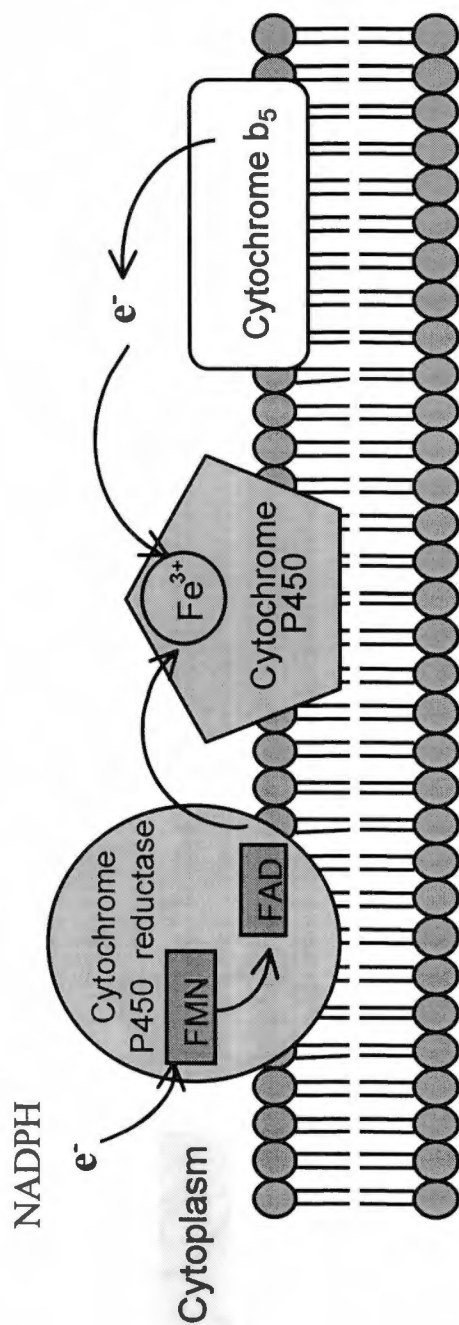


Figure 1 - Molecular components of the cytochrome P450 system in eukaryotic cells.

In order for a P450 enzyme to oxidize a substrate, its ferric (Fe^{3+}) group must be reduced to the ferrous (Fe^{2+}) form by accepting 2 electrons. Two molecules act as electron donors in this process: NADPH and cytochrome b_5 . The cytochrome P450 reductase -with the two subcomponents FMN and FAD- acts as an intermediary molecule in the transfer of electrons from NADPH. All components of the P450 system are embedded in the cytoplasmic face of the endoplasmic reticulum. (Adapted from Guengerich, 1993).

The catalytic action of the P450 enzymes in eukaryotes can be summarized in a few chemical steps (Figure 2). The substrate binds to the enzyme when the iron atom at the heme-binding site is in its ferric (Fe^{3+}) form. Binding of the substrate brings about a conformational change that facilitates the interaction of the enzyme with the nearby cytochrome P450 reductase. The first electron is transferred from NADPH (or sometimes from cytochrome b_5) to the iron atom resulting in its reduction to the ferrous (Fe^{2+}) form. An oxygen molecule then binds to the enzyme, and with the addition of a second electron and a proton donated by a second NAD(P)H^+ molecule, a water molecule is released leaving the enzyme with a $(\text{FeO})^{3+}$ complex that directly oxidizes the substrate (Guengerich, 1993; Lewis, 1996 and references therein).

Structure and cellular distribution

CYPs are hemoproteins (46-55 kD) that consist of a single polypeptide chain of about 500 amino acids long and a single non-covalently bound heme prosthetic group. The amino acid chain provides sites for substrate and redox partner interaction, as well as oxygen and heme binding. Generally, the C-terminal half of the protein, containing the heme-binding site, is more conserved among CYPs than is the N-terminal end. This site is at the heart of the protein where the oxidation reaction resulting in the splitting of an O_2 molecule and the subsequent oxidation of a substrate occurs.

Based on the 3-dimensional structures of six soluble bacterial P450 proteins, P450 in general seems to consist of 2 major domains: an α -helical domain accounting for approximately 70% of the protein, and a β -helical domain forming approximately 22% of

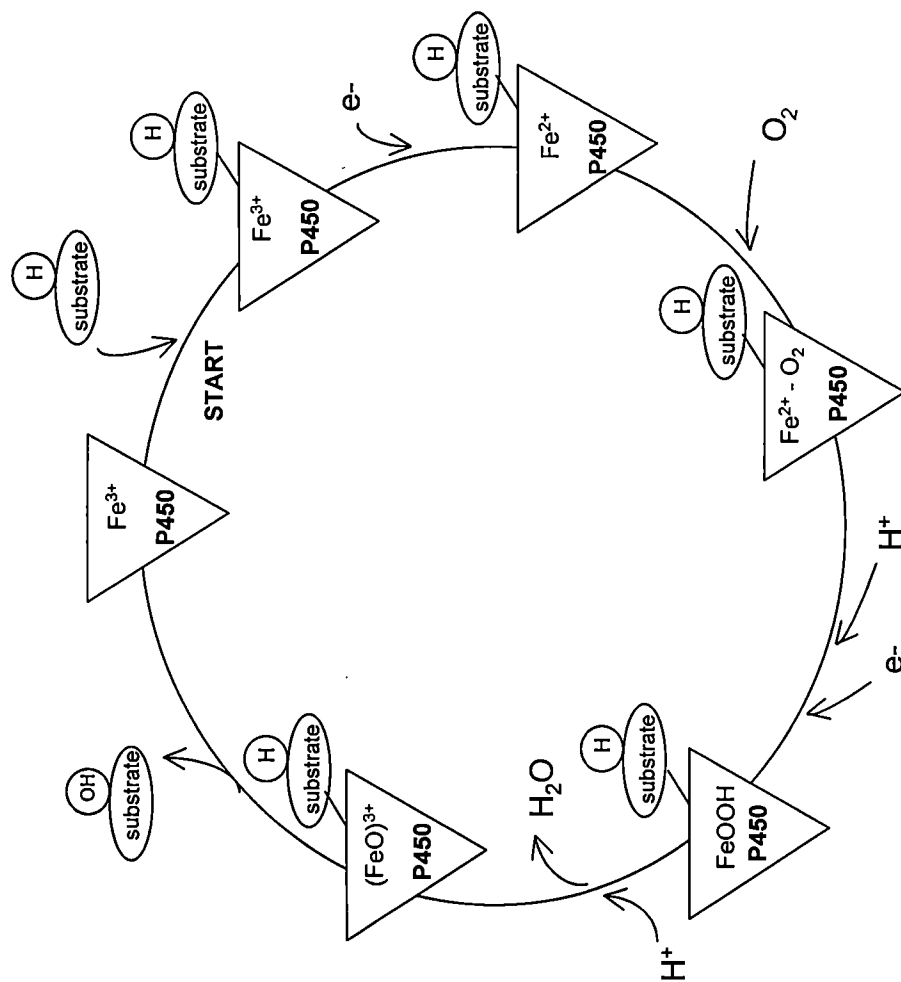


Figure 2- Cyclic mechanism for the oxygenation of a substrate by a cytochrome P450.

The initial step (start) in the reaction is the binding of the substrate to the P450. (Adapted from Guengerich, 1993).

the protein (Figure 3) (Graham-Lorence and Peterson, 1996). Helix I (Figure 3) borders one side of the heme pocket and contains what is considered the signature motif of P450s (A/G-G-X-E/D-T-T/S), which forms the proton transfer groove of the active site (Werck-Reichhart and Feyereisen, 2000). The helices are connected by random loops, of which the most important is the heme-binding loop, located between the K' and L helices. The heme-binding site contains the most characteristic consensus sequence (F-X-X-G-X-R-X-C-X-G) with the conserved cysteine that serves as fifth ligand to the iron atom (Graham-Lorence and Peterson, 1996; Werck-Reichhart and Feyereisen, 2000). Forming the mouth of the active site is a structure called the "meander," which is a 14-15 residue stretch in the shape of a tunnel through which the substrate passes to the active site (Graham-Lorence and Peterson, 1996). The hydrophobicity of this region suits the hydrophobic nature of P450 substrates.

In prokaryotes, P450 proteins are water-soluble and float freely in the cell. In eukaryotes, on the other hand, they are anchored in the cytoplasmic face of the endoplasmic reticulum by sequences in their N-terminal ends. They are present abundantly in liver tissue where they play a major role in drug metabolism. However, there is no specific pattern for their tissue distribution. Just as their functions are diverse, they can be found in all types of tissue and in all types of organisms, usually with a developmentally regulated expression. Examples of human tissue with notable levels of CYPs are kidney, lung, pancreas, spleen, nasal epithelium, brain, adrenal gland, testis and ovary (Schenkman and Greim, 1993; Werck-Reichhart and Feyereisen, 2000).

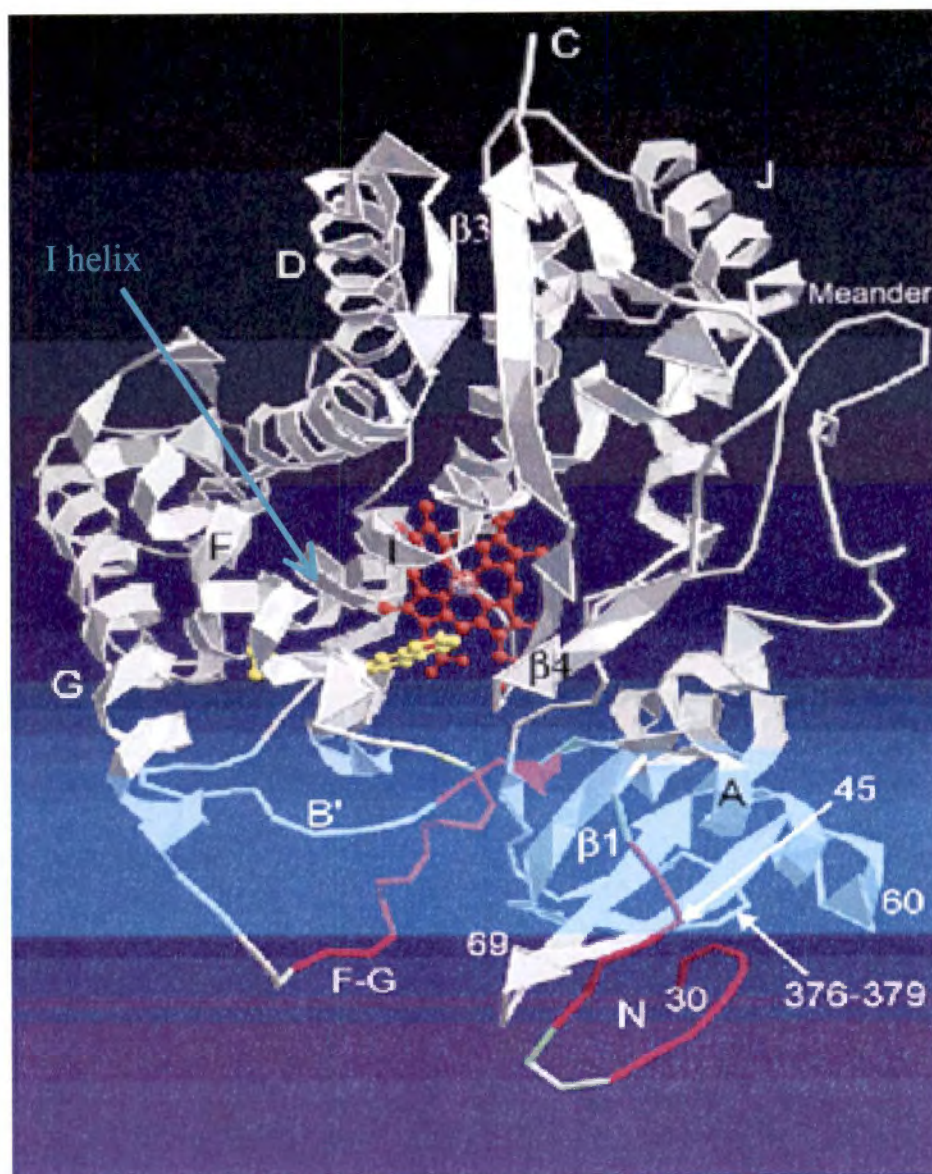


Figure 3- Ribbon representation of the folded structure of CYP25.

The protein is composed of two main domains: the α domain shown on the top left, and the β domain on the bottom right. The α helices (A-L, except K which is on the back side) and β sheets are labeled. The green arrow points at the I helix which contains the P450 signature sequence (A/G-G-X-E/D-T-T/S). The heme group is in orange, bound at the back with the heme binding loop, and the substrate is in yellow. Forming the mouth of the active site is the “meander,” a very conserved structure among CYPs (Werck-Reichhart and Feyereisen, 2000).

Metabolic functions

P450s are responsible for a variety of metabolic functions that are vital to the survival of living organisms. These include key reactions in steroidogenesis in animals (e.g. glucocorticoids, cortisols, estrogens, and androgens), xenobiotic (foreign compound)/drug metabolism in humans, insecticide resistance in insects, and herbicide resistance and flower coloring in plants (Graham-Lorence and Peterson, 1996). Some of the major endogenous compounds they metabolize are steroids, hormones, and fatty acids, while the list of xenobiotics includes drugs, plant allelochemicals, alcohol, anesthetics, pesticides, solvents, dyes, benzene, and nicotine (Porter and Coon, 1991; Gonzalas, 1996; Nims and Lubet, 1996; Ronis *et al.*, 1996). The chemical variability in structure and overall charge of the thousands of P450 substrates gives these enzymes their unique characteristic as the major metabolic enzymes vital for the survival of living things.

Metabolism of xenobiotic compounds occurs in a 2-phase reaction, using different enzymes in each phase. Phase I (functionalization) involves mostly P450 enzymes, where a functional group (eg. OH⁻) is added to the compound causing it to be more reactive (Figure 4A). In phase II (conjugation), enzymes like esterases, glutathione S-transferases, N-acetyltransferases use phase I reactive metabolites as substrates and conjugate them with other compounds to make them more hydrophilic and able to be excreted (Figure 4B) (Nebert *et al.*, 1996).

A unique example of an organism's dependence on P450s is the role CYP6B1 plays in the survival of larvae of the black swallowtail butterfly (*Papilio polyxenes*).

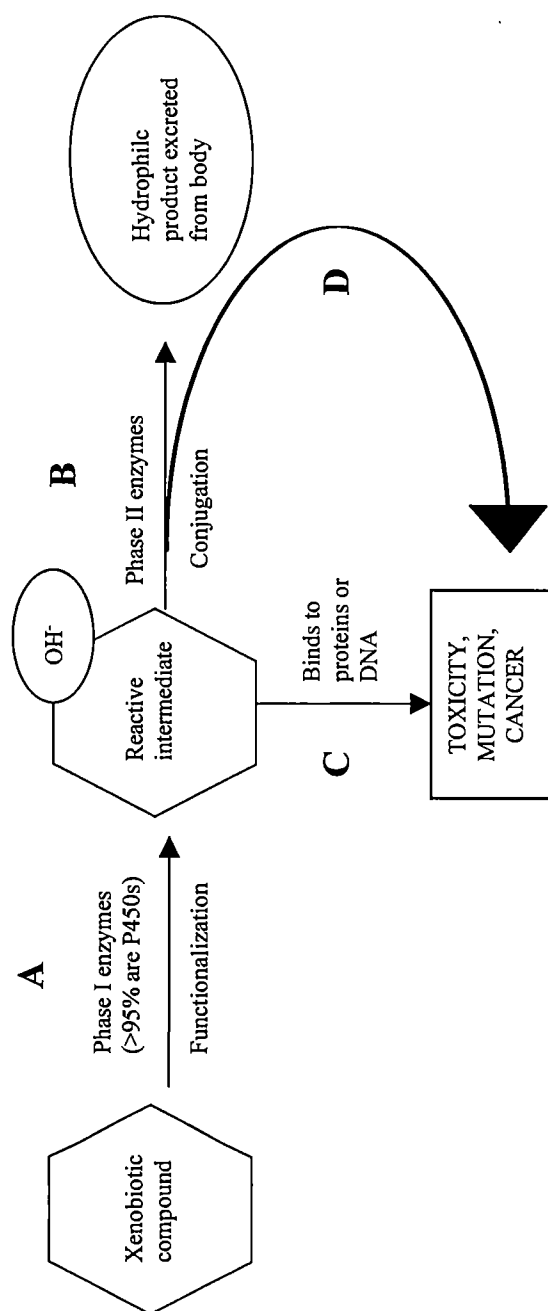


Figure 4- Metabolism of xenobiotic compounds is mediated by phase I and phase II enzymes.

A) Phase I enzymes, which are mostly CYPs, activate xenobiotic compounds by introducing a functional group (functionalization) like a hydroxyl (OH^-). B) Phase I metabolites act as substrates for Phase II enzymes, such as esterases, glutathione S-transferases, and N-acetyltransferases, where they are conjugated with other compounds to become more hydrophilic and able to be excreted (conjugation). C) Sometimes, Phase I reactive metabolites can bind to DNA and proteins resulting in mutations that may cause toxicity and cancer. D) Phase II hydrophilic metabolites can form conjugates that may also lead to toxic, mutagenic, and/or carcinogenic effects.

These larvae feed on plants of *Apiaceae* (carrot and parsley) and *Rutaceae* (citrus and other aromatic trees) species, which produce the poisonous chemical xanthotoxin (Ma *et al.*, 1994; Prapaipong *et al.*, 1994; Hung *et al.*, 1995). Upon the consumption of these plants, xanthotoxin induces the expression of CYP6B1 enzyme, thereby catalyzing its own oxidative destruction. Similarly, P450-mediated metabolism of nicotine allows the tobacco hornworm (*Manduca sexta*) to feed on tobacco plants (Snyder *et al.*, 1993), and metabolism of allelochemicals in cacti allows certain *Drosophila* species to feed on cactus trees in the Sonoran desert (Fogleman *et al.*, 1998).

Although P450 metabolism of xenobiotics usually leads to their detoxification, sometimes the reverse occurs. Several harmless chemicals are activated in Phase I to become potent mutagens. They can bind to DNA and proteins resulting in mutations that may lead to cancer or toxicity (Figure 4C). Examples of such chemicals are azo dyes, aflatoxin B1 and benzene. Heterocyclic amines present in burned food and cigarette smoke are also innocuous until they are acted upon by CYP1A1 (Guengreich, 1993). The extent to which they become carcinogenic depends largely on the activity of CYP1A1. A study showed that an RFLP in CYP1A1 present among Japanese ethnic groups is associated with a mutation in the heme-binding region of the protein, causing a 50% increase in its activity (Hayashi *et al.*, 1991). The increased activity is correlated with a higher incidence of lung cancer in those individuals who carry the mutation and smoke cigarettes (Nebert *et al.*, 1996 and references therein). Similarly, CYP1A1 and CYP3A sequentially activate benzo(a)pyrene into a product that is carcinogenic (Holder *et al.*, 1974; Shimada *et al.*, 1989). Bioactivation of such compounds by P450s is an important

pathway in chemical carcinogenesis, and forms the metabolic basis of the Ames test for testing mutagenic activities of chemicals (Ames, 1975). Conjugated hydrophilic compounds that are acted upon by Phase II enzymes can also form toxic, mutagenic, and/or carcinogenic metabolites (figure 4D). Examples are glutathione conjugates of halogenated carbons and hydroquinones, and glucuronide conjugates of benzo[a]pyrene (Monks *et al.*, 1990; Nebert *et al.*, 1996).

Cytochrome P450s and insecticide resistance

The main two types of insecticide resistance in insects are: 1) target site modification, where target molecules (usually proteins) of the toxin undergo a structural change thereby reducing its ability to bind and cause the pathological change in the insect; and 2) increased enzymatic detoxification by monooxygenases (P450s), glutathione S-transferases, and/or esterases (Scott, 1999; Hemingway, 2000; Wilson, 2001). Of these two types of resistance, increased detoxification is more common, with monooxygenase-mediated detoxification rated to be the most frequent type of metabolism-based insecticide resistance (Brattsten *et al.*, 1986; Wilson, 2001).

Several studies showed that increased amounts or activities of CYPs are present in insecticide-resistant strains compared to their susceptible counterparts (Agosin, 1985; Oppenoorth, 1985; Sundseth *et al.*, 1990; Waters *et al.*, 1992a,b; Hodgson *et al.*, 1993). Waters and Nix (1988) measured the P450-dependent nitrosodimethylamine demethylase (NDMA-d) activity and amounts of the heme-staining (P450) proteins in microsomes of several strains of *Drosophila*. They found that strains resistant to phenylurea had both a

higher NDMA-d activity and P450 content relative to the susceptible strains (Waters and Nix, 1988; Waters, 1992). Since CYPs show a mixed function oxidation (MFO) activity, Houpt *et al.* (1988) measured the MFO activity and amounts of heme-staining proteins in malathion resistant and susceptible strains of *Drosophila*. Similarly, they found that malathion resistance positively correlates with elevated levels of both MFO activity and heme-staining protein content in resistant strains (Houpt *et al.*, 1988). An earlier study examined the relationship between CYP proteins and insecticide resistance more directly by administering CYP protein inhibitors (e.g. piperonyl butoxide "PBO" or sesamex) to resistant strains of *Musca*. It was found that resistant strains were rendered susceptible by PBO (Wilkinson, 1983), suggesting that CYPs play a major role in insecticide resistance.

Since these early observations, several studies on *Musca* (house fly) and *Drosophila* CYP genes showed that insecticide resistance is associated with increased transcription of one or more CYP genes (Liu and Scott, 1996; 1998; Maitra *et al.*, 1996, 2000; Dombrowski *et al.*, 1998; Feyereisen, 1999; Scott, 1999). For example, *CYP6D1* gene in *Musca* shows a 10-fold higher expression in the insecticide resistant LPR strain than in the susceptible *aabys* strain (Liu and Scott, 1998; Scott, 1999). Similarly, the level of CYP6A1 in *Musca* is elevated in the diazinon resistant Rutgers strain compared to the susceptible *aabys* strain (Carino, 1992). In *Drosophila*, *Cyp6a2* and *Cyp6a8* show higher levels of expression in the resistant *91-R* strain than in the susceptible *91-C* and *ry*⁵⁰⁶ strains (Waters *et al.*, 1992 a, b; Dombrowski *et al.*, 1998; Maitra *et al.*, 1996, 2000). Protein levels of CYP6A2 are also higher in resistant strains (Waters *et al.*,

1992,b). While all these observations suggest that CYPs may be involved in insecticide resistance, there is no direct evidence that demonstrates the involvement of a specific CYP gene or genes in the resistance phenomenon.

Regulation of CYPs

While several studies draw a correlation between elevated levels of *CYP* gene expression and insecticide resistance, very little is known about the molecular basis of *CYP* gene regulation in insects. Most of our knowledge is based on studies in bacteria and mammals, which suggest the involvement of both *cis*- and *trans*-regulatory elements. In *Bacillus megaterium*, it has been shown that *P450_{BM-1}* and *P450_{BM-3}* genes are down regulated by a negative *trans*-regulatory element (Bm3R1), or a repressor, which binds to a 15-17 base pair sequence in the upstream DNA of these genes, known as a "barbie box." (Wen *et al.*, 1989; Fulco, 1991; He and Fulco, 1991; Shaw and Fulco, 1993; He *et al.*, 1995; Liang and Fulco, 1995; Liang *et al.*, 1995). When phenobarbital or other barbiturate compounds are added to the medium, positive *trans*-regulatory factors (BM1P1, BM1P2 and BM1P3) are induced. This event causes the repressor protein to dissociate from the barbie boxes, thereby inducing the transcription of *P450_{BM-1}* and *P450_{BM-3}* genes (He *et al.*, 1995).

In mammals, one of the best worked out pathways for *CYP* gene regulation is that for human *CYP1A1*, which encodes aryl hydrocarbon hydroxylase (AHH) (Nebert *et al.*, 1996; Swanson and Bradfield, 1993; see review by Whitlock, 1999). This gene is under the control of a negative regulatory mechanism that maintains its constitutive expression

at low levels (Hines et al., 1988; Boucher et al., 1993, 1995). In the presence of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), the expression of *CYP1A1* is induced to high levels (Denison and Whitlock, 1995). It was shown that the inducer binds to the aryl hydrocarbon receptor (AhR) in the cytoplasm, which is usually bound to the heat shock protein 90 (hsp90) and other cytoplasmic proteins. Binding of the inducer triggers the dissociation of AhR from hsp90, and the freed AhR relocates to the nucleus where it binds with the AhR nuclear translocating protein (Arnt). The AhR-Arnt heterodimer interacts with upstream *cis*-elements known as XREs (xenobiotic response elements) of the *CYP1A1* gene and induces its transcription (Denison and Whitlock, 1995; Whitlock, 1999).

Our understanding of Cyp gene regulation in insects is confined to what is known in the housefly (*Musca*) and *Drosophila*. Studies have shown that factors on chromosome II of *Musca* seem to negatively influence the expression of *CYP6A1* gene located on chromosome V (Carino et al., 1992). Similarly in *Drosophila*, the second chromosome-linked *Cyp6a2* and *Cyp6a8* genes are also negatively influenced by factors located on chromosome III (Maitra et al., 2000). It is possible that up-regulation of *CYP* genes in resistant strains of *Drosophila* is caused by a loss of function mutation of the negative factors (Maitra et al., 2000). Since chromosome II of *Musca* is thought to be homologous to the 3R of *Drosophila* (Foster et al., 1981), the evolutionary relationship between the two species predicts that the mechanism of *CYP6* (a CYP gene family found exclusively in insects) gene regulation may be conserved.

Objectives and Significance

In 1992, a DDT resistance locus (R2) was genetically mapped at 56 ± 1 m.u. (Dupkus, 1992). Based on the chromosomal and genetic map positions of various known genes, 56 ± 1 m.u. is close to region 42 of the polytene map. There are several *CYP* genes that lie close to this region which may be responsible for the resistance phenotype. One of these genes is *Cyp6a2*, which was previously found to have a higher expression in the insecticide resistant *91-R* strain than in the susceptible *91-C* and *ry*⁵⁰⁶ strains (Waters *et al.*, 1992 a,b; Maitra *et al.*, 1996, 2000; Dombrowski *et al.*, 1998). However, there is no evidence that suggests *Cyp6a2* gene to be solely involved in conferring resistance, although CYP6A2 could metabolize diazinon, aldrin and heptachlor in a heterologous expression system (Dunkov *et al.*, 1997). Some studies have led to the impression that resistance in *D. melanogaster* is a polygenic trait, not only involving several genes, but also involving all 3 major chromosomes (Dapkus and Merrell, 1977). Based on the observation that increased transcription of some *CYP* genes is associated with insecticide resistance (Liu and Scott, 1996; 1998; Feyereisen, 1999; Scott, 1999), measuring the differential expression between *91-R* and *91-C* of *CYP* genes that lie close to the resistance locus may allow us to identify one or more *CYP* genes whose higher expression in *91-R* may be attributed to the resistance phenotype. In addition, obtaining an expression profile of all *CYP* genes in *91-R* and *91-C* strains may identify other *CYP* genes showing higher expression in the resistant strain. Therefore, this project was initiated with the following 2 objectives:

- 1) To identify *Cyp* genes that are differentially expressed in 91-R and 91-C strains using microarray analysis technique. Those genes that are found to be over-expressed in 91-R, especially the ones located close to the DDT resistance locus, could be studied in greater detail.
- 2) To examine the expression of four family *Cyp6* genes that are located close to the DDT resistance locus (56 ± 1 m.u.). Family 6 genes are found exclusively in insects, and have been associated with insecticide resistance in *Musca*. Any genes found to be over-expressed in 91-R could be studied in greater detail.

II. MATERIALS AND METHODS

Fly strains and culture conditions

In the present investigation, DDT-resistant (91-R) and susceptible (91-C) strains were used. The original population was captured in the wild by D. J. Merrell in St. Paul, Minnesota in 1952, and was split into two populations. One population was raised on DDT containing medium for 30 generations to select and establish a resistant strain named 91-R (Merrell and Underhill, 1956). The other population was maintained on normal medium, and named 91-C. The 91-R strain used in the present investigation actually went through 14 years of DDT selection before it was obtained in the early 1980's by Dr. Larry C. Water of Oak Ridge National Laboratory. Since then the 91-R strain has been maintained on normal medium. Periodic resistance assay showed that the strain still maintains DDT resistance phenotype (Ganguly, unpublished observations).

The chromosome substitution stocks, RRC, CRC, RCR, and CCR, were constructed by Maitra (2000) using the chromosomes of 91-R and 91-C strains. R and C represent chromosomes from 91-R and 91-C strains, respectively. The order of chromosomes from left to right represents X (i.e., first), 2nd and the 3rd chromosomes. For example, in RRC, the X and 2nd chromosomes are from the resistant 91-R strain, and the 3rd chromosome is from 91-C.

Flies were raised on a standard cornmeal-agar-molasses medium (0.65% bacto-agar, 5.5% cornmeal, 3% brewer's yeast, 5% (v/v) unsulfured molasses, 2% (v/v) light corn syrup, 0.25% (v/v) propionic acid, 0.3% ethanol and 0.1% Tagosept [p-hydroxybenzoic acid, methyl ester]) at 23°C and 70% humidity.

Barbital treatment

91-C adult female flies (one week old) were allowed to feed on 4gm instant *Drosophila* medium (Carolina Biological Supply, Inc, Burlington, NC) wetted with a 7ml freshly prepared solution of 16mM barbital (5,5'-diethyl barbituric acid, from Sigma). Flies were left on medium for 24 hours before freezing at -80°C. As the untreated control, female flies from the same culture were allowed to feed on instant medium prepared with water. Total RNA from treated and untreated flies was isolated for northern blot analysis of *Cyp6w1*.

DDT treatment

A solution of acetone (Mallinckrodt) and DDT (Sigma) was prepared at two different concentrations of DDT: 50µg/ml and 150µg/ml. One tenth (0.1) of a milliliter of each solution was layered on top of standard cornmeal-agar-molasses media and dried in a hood overnight. Adult female flies of 91-C were allowed to feed on DDT-treated medium for 4.5 and 2 hours, respectively. To use as a control, female flies from the same culture were fed on standard cornmeal-agar-molasses medium treated with acetone only. Total RNA from treated and untreated flies was isolated and used for northern blot analysis of *Cyp6w1* and *Cyp6a2*.

RNA isolation

Total RNA was isolated from sexed adult flies by using Tri-reagent (Sigma) according to the manufacturer's instructions. Briefly, flies were homogenized in Tri-reagent, and the homogenate was centrifuged at 12K rpm to pellet the debris. The supernatant was transferred to a fresh tube, and protein was extracted with 1/5 volume of chloroform. The aqueous phase was transferred to a fresh tube, mixed with 2-volumes of isopropanol and RNA was precipitated overnight at -20°C . Next day, RNA was pelleted by centrifuging at 13,000 rpm. The pellet was washed with 75% ethanol, dried in a speed vacuum concentrator (Savant), and resuspended in water. RNA samples were quantified using a UV-visible spectrophotometer (model UV16U, from Shimadzu).

Poly (A)⁺ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as described by Maniatis *et al* (1982). Briefly, an Oligo(dT) cellulose column was equilibrated with buffer A containing 0.5M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, and 0.1% SDS. Total RNA was heat-denatured for 10 min at 65°C , cooled to room temperature and adjusted to buffer A. It was then passed through the equilibrated oligo(dT) column, which was first washed with 5 volumes of buffer A and then with 5 volumes of buffer B (made of the same constituents as buffer A, except for SDS). Poly(A)⁺ RNA was eluted with 10mM Tris and precipitated with ethanol in presence of 0.2M Na-acetate pH 7.5. Subsequently, RNA was pelleted, dried and dissolved in water.

Genomic DNA isolation

Unsexed flies were homogenized in ice-cold nuclear isolation buffer containing 50mM Tris-pH 7.6, 25mM KCl, 5mM magnesium acetate and 350mM sucrose. The

homogenate was centrifuged at 12,000 rpm and the resulting nuclear pellet was resuspended in 1X DNA extraction buffer (DEB) containing 100mM Tris pH 8.0, 150mM NaCl, and 10mM EDTA. Nuclei were lysed by adding one-tenth volume of both 5M sodium perchlorate and 10% SDS. The lysate was extracted twice with phenol saturated with 1X DEB, and twice with chloroform. The aqueous phase was transferred to a fresh tube and DNA was precipitated by adding one-tenth volume 2M sodium acetate and 2 volumes ice-cold 95% ethanol. After incubating overnight at -20°C the mixture was centrifuged at 12,000 rpm and the pellet was resuspended in 1X TE containing 10 mM Tris pH7.5 and 1mM EDTA. The contaminating RNA in the suspension was precipitated by adding one-half volume of 10 M lithium chloride. The suspension was centrifuged to pellet RNA, and genomic DNA in the supernatant was precipitated again by adding one-tenth volume 2M sodium acetate and 2 volumes 95% ethanol. The final genomic DNA pellet was dried in a speed vacuum concentrator (Savant) and resuspended in 1X TE.

Polymerase chain reactions

All 83 PCR primer pairs (18 – 20 mer) used in this investigation were designed in our laboratory. To lower the occurrence of non-specific amplification, each set of primers was designed to span a 400-700 bp fragment of the N-terminal region of each *Cyp* coding sequence where a low percentage of sequence similarity occurs between all 83 *Cyp* genes. Using computer software, primers were found to be negative for self-dimerization. BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) analysis showed

that the primers do not have significant similarities with other sequences in the *Drosophila* genome. The T_m of the primers ranged between 52-67°C.

All PCR reactions were carried out in EasyStart PCR-ready tubes (Molecular Bioproducts), containing 1X PCR buffer, 0.2mM dNTPs, and 2mM MgCl₂ in a 50µl reaction volume. To these tubes, 30-100 ng genomic DNA, 200ng of each primer, and 5 units of Taq DNA polymerase (Fisher) were added. The final volume was adjusted to 100µl.

Reactions were carried out using a gradient MasterCycler (eppendorf). The gradient PCR program allows for amplification of several genes at the same time, while maintaining a different and suitable annealing temperature (T_m) for each tube. The template DNA was first denatured by a 3-minute hot start incubation at 95°C. This was followed by 35 cycles of the following incubation steps: 1.5 minutes denaturation at 94°C, 1.5 incubation at the specific T_m per gene, and 3 minutes extension at 72°C. The PCR programs ended with a soak at 4°C.

Northern blot hybridization

Either 2 µg of poly (A)⁺ or 15 µg of total RNA was used for Northern blot analysis. RNA sample were denatured at 68°C for 12 minutes in 0.5X northern gel buffer containing 20mM MOPS (3-(N-Morpholino) propane sulfonic acid), 5mM sodium acetate, 0.5mM EDTA, pH 7.0], 6% formaldehyde, and 50% formamide. After cooling on ice, one-tenth volume of RNA loading dye (50% glycerol, 0.01M NaH₂PO₄, pH 7.0, and 0.4% bromophenol blue) was added and samples were size-fractionated on a 2.2M

formaldehyde-MOPS-1.2% agarose gel. Using 20X SSC as the transfer buffer, RNA was blotted onto a nylon membrane (Hybond-N, Amersham, England) by capillary action for 16-20 hours. To check for efficiency of transfer and to determine the migration distance of RNA, ribosomal RNA bands were viewed under high wavelength UV light. Blots were cut 1 cm below the ribosomal RNA bands into upper and bottom sections. While the upper sections were hybridized with *Cyp* gene probes, the bottom sections were hybridized with cDNA probes of RP49 (ribosomal protein 49) gene (O'Connell and Rosbash, 1984) to correct for RNA loading error. All blots were UV-crosslinked (Stratalinker UV Crosslinker, Model 1800, Stratagene, CA) and baked at 80°C under vacuum for 2 hours.

Blots were first pre-hybridized at 42°C for 2 hours in 50% formamide, 0.1M PIPES pH 6.6, 0.8M NaCl, 5X Denhardt's solution (0.1% ficoll, 0.1% polyvinyl pyrrolidine, and 0.1% bovine serum albumin, Pentax fraction V), 0.1% SDS, and 0.1mg/ml denatured Herring Sperm DNA. The pre-hybridization solution was replaced with a hybridization solution made of the same constituents with the addition of ³²P-labeled probe. Blots were hybridized at 42°C for 18-20 hours. After incubation was over, blots were washed twice with 2X SSC-0.1% SDS at room temperature, and twice with 0.2X SSC-0.1% SDS at 65°C. Hybridization signals were quantified using the Packard Instant Imager Imaging System (Packard Instrument Company, Meriden, CT), and visualized by autoradiography.

Microarray analysis

PCR products were purified using QIAquick PCR purification columns (Qiagen) according to the manufacturer's instructions. DNA was air dried and resuspended in water at a concentration of 15-230 ng/ μ l (the recommended concentration is 300 ng/ μ l in order for the spotted DNA to be about 150 ng/ μ l per spot). A spotting plate was prepared by adding 45-690 ng of each DNA sample into a microtiter plate and mixing it with an equal volume of dimethylsulfoxide (DMSO). An arm with 4X4 spotting pins (Virtex) was used to print samples on CMT-GAPS (Corning[®] Microarray Technology-Gamma Amino Propyl Saline) coated slides at 45-55% humidity. Spotted slides were crosslinked at 250mJ and air-dried. Unused slides were saved in a vacuum chamber until needed.

For hybridization, a spotted slide was first prehybridized at 42°C in 25% deionized formamide, 5X SSC, 0.1% SDS, and 1.0% bovine serum albumin (BSA). The slide was immediately washed with distilled water (Nanopure), and blown dry with compressed gas. A hybridization solution containing Cy3 and Cy5 labeled cDNA (see below), 25% deionized formamide, 5X SSC, and 0.1% SDA was heat denatured at 95°C and carefully dispensed onto the center of the slide. A cover slip was gently placed on top of the solution and the slide was hybridized in a humidifier over night at 42°C. Next day, the slide was washed once in 2X SSC- 0.1% SDS at 42°C, twice in 0.2X SSC at room temperature, and 4 times in 0.1X SSC at room temperature. Finally the slide was dried with compressed gas and fluorescence signals were detected by a confocal laser scanner (ScanArray, from Packard Biochip Technologies, model 4000XL). This scanner uses red and green Helium-Neon lasers to excite Cy5 (633nm) and Cy3 (543nm)

separately. For data analysis, Imagene (Biodiscovery) and Quantarray (Packard Biochip Technologies) software programs were used.

Radiolabeling of DNA fragments

Purified PCR products of Cyp genes were labeled with ^{32}P by using a commercially available random priming labeling kit (Promega). Appropriate PCR fragments were heat-denatured and incubated at 24°C in a reaction mixture containing 1X labeling buffer (including 26.0 A₂₆₀u/ml random hexadeoxyribonucleotides), 0.4 mg/ml BSA, 60 μM each of dATP, dGTP, and dTTP, 0.66 μM dCTP, 50 μCi $\alpha^{32}\text{P}$ -dCTP, and 5 units of Klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by fractionation through a G-50 Sephadex column (Pharmacia). The specific activity for probes used ranged between $10^8 \times 10^9$ cpm/ μg . PCR purified RP49 DNA fragments were ^{32}P -labeled by nick translation using a commercially available kit (Gibco-BRL).

Labeling total RNA with Cy3 and Cy5

Total RNA from adult female flies was labeled with Cy3 and Cy5 as described by Hedge *et al.* (2000). 80-100 μg total RNA was mixed with oligo(dT) (20 mer) fragments and heat denatured at 70°C . The mixture was adjusted to 2.5nM of either Cy3 or Cy5 (NEN), 1X first strand Buffer (BRL), 10mM DTT, 0.5mM each of dATP, dGTP, and dCTP, 0.4mM dTTP, 13.3 U/ μl Superscript II (BRL), and incubated at 42°C for 2 hours. The reaction was terminated by adding one-twentieth volume of 20mM EDTA. To

denature RNA, one-fourth volume of 0.1M NaOH was added, and the mixture was heated at 70°C. Finally, the mixture was neutralized to pH 7.5 by adding one-fourth volume of 0.1M HCl.

Unincorporated nucleotides were removed by passing cDNA probes through QIAquick PCR purification (Qiagen) at the manufacturer's instructions, with the exception that column washes with buffer PE were repeated twice. The purified Cy3 and Cy5-labeled probes were eluted in water, combined together, and mixed with poly(dA) and Cot-1 DNA repeat blockers at a final concentration of 0.25 µg/µl for each. The mixture was dried in a Savant speed vacuum and resuspended in hybridization buffer (25% deionized formamide, 5X SSC, 0.1% SDS).

III. RESULTS AND DISCUSSION

Microarray analysis of the expression of 83 *Cyp* genes in 91-R and 91-C strains – a preliminary study

The DDT resistant 91-R strain used in this study was derived from the susceptible 91-C strain by about 14 years of DDT selection (Dapkus and Merrell, 1977). Because they are closely related, gene expression patterns in these two strains are expected to be similar except for those genes that may confer resistance in 91-R, such as the *Cyp* genes. Various cloning techniques and genome sequence analysis have identified 83 functional *Cyp* genes in the *Drosophila* genome, many of which are predicted by computational programs. Only a few *Cyp* genes have been studied in the 91-R and 91-C strains, of which three (*Cyp6a2*, *Cyp6a8* and *Cyp6g1*) show significantly higher level of expression in the 91-R than in the 91-C strain. The first objective of the present investigation has been to study the relative expression of all *Cyp* genes between these two strains via microarray technique.

For this purpose, 83 genes (81 *Cyp* genes, Cytochrome P450 Reductase (CPR), and Adrenotoxin Reductase (ADXRDTASE)) were amplified via PCR using gene-specific primer pairs and genomic DNA of *ry*⁵⁰⁶ as a template. Tables 1-5 list the primers used here. To check for their size, all PCR products were electrophoresed on agarose gel (Figures 5-11), and were found to match the expected sizes. The PCR products were then printed onto glass slides and dried. Since no difference in hybridization efficiency is found between cDNA made from total and poly (A)⁺ RNA (Hedge et al., 2000), cDNA was synthesized from total RNA of 91-R and 91-C strains. Two hybridization

Table 1- List of gene specific primers used to amplify *Cyp* genes on the X-chromosome

Gene Name	Primer name	Primer Sequence	Tm (°C)	Fragment size (bp)
Cyp4g1	4G1-9	5' GGAAGTAGTTCAGGAGACGC 3'	60.4	682
	4G1-691	5' ACATGTCGACGACGGCTTGG 3'	70.7	
Cyp4d1	4D1-F	5' TTCTGCGGAACATGAGGACG 3'	58.38	
	4D1-R	5' TTCGGACCTTGGCCCAATGC 3'	61.78	
Cyp4d14	4D14-F	5' TTTGCGATTCTCCTGGCGACC 3'	61.25	
	4D14-R	5' CCGCAATGATATCCATGGCAC 3'	57.64	
Cyp4d2	4D2-F	5' ATGGTATATTGCCGGGTCCG 3'	57.96	
	4D2-R	5' GCTTCTGTTGGCTGCGTTAGC 3'	60.36	
Cyp4AE1-F	4AE1-F	5' TCCTGGTGACTCGCCTAGTG 3'	59.24	
	4AE1-R	5' GAGATGCGGCCTCATGAACC 3'	59.19	
Cyp4g15	4G15-5	5' AGGTGCTGAAGAAGGACGCC 3'	67.8	791
	4G15-796	5' AACAGCTGCGGGGATACACG 3'	69.3	
Cyp28c1	28C1-18	5' ACTGGGGATTGCCACCTTGC 3'	70.4	680
	28C1-698	5' CCAGGACGCAGTCAAACAGC 3'	68.2	
Cyp311a1	311A1-16	5' CTGCTACTGATCACCCTGAC 3'	59.9	852
	311A1-868	5' GATCACCGACTCCATTGGC 3'	66.7	
Cyp318a1	318A1-51	5' GAATCGCTCGGCCATTGTTG 3'	69.8	681
	318A1-732	5' GGCAGCCGTAAACTTAACCG 3'	65.3	
Cyp4S3	4S3-16	5' CTGGTGGCTTTCGTCTTTG 3'	66.8	687
	4S3-703	5' AGGATGCGGAAATGGAAGCC 3'	69.3	
Cyp306a1	306A1-19	5' GATATTGGCCACACCGTTG 3'	67.2	735
	306A1-754	5' TCCATGCGCGCATTCAATCG 3'	74.4	
Cyp18a1	18A1-21	5' GATCAAGTTTGTGCTGCGCC 3'	68.0	722
	18A1-743	5' ACTCCTCCCGACAATCTCAC 3'	63.1	
Cyp308a1	308A1-26	5' TATCCTATTGGCCGCCACGC 3'	70.8	669
	308A1-695	5' AAGAGCGGAGCGAGCCATTG 3'	69.9	
Cyp6v1	6V1-29	5' CGATAGTGACGATCCTGACG 3'	63.3	702
	6V1-731	5' ACGATATGCCGCGAAACAC 3'	69.0	
Cyp6t1	6T1-15	5' TTCACTCATTGCGGCTGCTC 3'	68.8	645
	6T1-660	5' CAGACTGTGCGCCTCTATGC 3'	66.0	

Table 2- List of gene specific primers used to amplify *Cyp* genes on chromosome 2L

Gene Name	Primer name	Primer Sequence	T _m (°C)	Fragment size (bp)
Cyp309a1	309A1-4	5' TTACCCTAGTGGGTTTGTGC 3'	61.3	677
	309A1-681	5' AGATGAAATCCGCCATCGC 3'	67.2	
Cyp28d1	28D1-251	5' AGTACAAGAACACCGACAGC 3'	59.4	690
	28D1-941	5' TTGGTGGCCATAGATTGGC 3'	65.7	
Cyp4ac1	4AC1-18	5' GGATCCCGATTTTGTGGC 3'	66.3	515
	4AC1-533	5' GCCTTTCGTCTGGAATGC 3'	63.4	
Cyp4ac2	4AC2-7	5' TGAAGTCCTGTTGCTGC 3'	66.6	528
	4AC2-535	5' GGGCTTTTCTTCTAGAGTGC 3'	59.6	
Cyp4ac3	4AC3-84	5' CTTTCATTATGCAAACGAGTGC 3'	63.3	613
	4AC3-697	5' GTTAAGTTCAAGCTCGAAGC 3'	58.3	
Cyp6a16	6A16-120	5' GGTCACTTCTTCAGGCTGC 3'	62.6	637
	6A16-757	5' ATATCCGGGTGAAGGATACGC 3'	65.7	
CPR	CPR-94	5' TCTGCACTCGTACACAGC 3'	58.6	531
	CPR-625	5' AGAAGCTCTACGTGCAGC 3'	58.0	
Cyp4d21	4D21-8	5' ATTGTTGGGTATCGCAGTGC 3'	64.7	578
	4D21-586	5' GCACGTTTTGCCATCAGC 3'	65.6	
Cyp4e3	4E3- 48	5' GTGTACTTTGAACGGAAGGC 3'	61.1	573
	4E3-621	5' CATCTTGCGGTGTTTATGCC 3'	65.2	
Cyp28a5	28A5-103	5' CCAAGCCAAAGCTATTGTGC 3'	64.4	608
	28A5-711	5' CGTCTTTAGAACCCAGACGC 3'	63.6	
Cyp303a1	303A1-163	5' AGGTGATCGATGTGTTTGC 3'	61.2	560
	303A1-723	5' TCCACGAAACCATTGTAGC 3'	61.1	
Cyp310a1	310A1-104	5' CATTACGTGGAGTTTCCTGC 3'	62.1	532
	310A1-636	5' TATCGAGGCCTGACTATGGC 3'	64.0	

Table 3- List of gene specific primers used to amplify *Cyp* genes on chromosome 2R

Gene Name	Primer name	Primer Sequence	T _m (°C)	Fragment size (bp)
Cyp6w1	6W1-61 6W1-613	5' CCTTATCGTTTTGGGAACGC 3' 5' TGGACTTCCGTACCACTGC 3'	65.2 63.8	552
Cyp6a2	6A2 -28 6A2 -703	5' CGATCTCCTCGCTTTTGGC 3' 5' ATGCGCATCTCAACCTGC 3'	67.1 66.3	675
Cyp6u1	6U1-97 6U1-794	5' GGATCCTGCACGAAAAGC 3' 5' CTGCGTTGGAATATAGCTGC 3'	60.7 60.6	697
Cyp9b1	9B1-49 9B1-577	5' TGTTCAAGTGGAGTACTGGC 3' 5' TTCATGTCCAGCTCAAAGGC 3'	64.5 65.1	528
Cyp9b2	9B2-123 9B2-708	5' GTGGGTAATATGGCTGCAGC 3' 5' TCCTCGGGAGAAAACCAGC 3'	64.2 66.5	585
Cyp4ad1	4AD1-165 4AD1-847	5' GGTGAGTACTGACCAAATAGC3' 5' GTTTGCGGAGCATTACAGC 3'	57.7 63.3	682
Cyp4e2	4E2-181 4E2-824	5' GAAAAGTCGTAGCGTTAGC 3' 5' AGGAGACCTAATCCCAGC 3'	56.7 58.1	643
Cyp4e1	4E1-53 4E1-749	5' TTTCGAGCTTGGACTACTGC 3' 5' GACCTCGTGAAAGTCCTGC 3'	62.1 62.5	696
Cyp6a14	6A14-287 6A14-886	5' CTACTTCCAGGATCGTGGC 3' 5' ATTTGCTCCAGGGTCAGGC 3'	62.0 65.5	599
Cyp6a13	6A13-25 6A13-602	5' AGTACCTCAACCCGTAGC 3' 5' TGCGCCAGAATTTGACGC 3'	61.4 62.1	577
Cyp49a1	49A1-65 49A1-747	5' CCCACACCAAAGACTTGGC 3' 5' CCACACACGTCGTATACGC 3'	65.5 62.7	682
Cyp12d1	12D1-51 12D1-719	5' CGATCTTCAAGATCAGC 3' 5' GCGGATTTCCCTTAATGCTGC 3'	53.7 65.5	668
ADR	ADR-19 ADR-715	5' ACATCTTCAGACGTGGC 3' 5' ATCTCTTAGCTCACGCAGGC 3'	64.3 63.5	696
Cyp6a22	6A22-183 6A22-845	5' AAGTTCAAGGGCAATGGAGC 3' 5' CCATTTCTCGATGGTGAGC 3'	65.2 66.7	662
Cyp6a17	6A17-57 6A17-728	5' CGACGTCATGGATACTGGC 3' 5' TGGTGTAAGTCTCTGC 3'	64.2 57.8	671
Cyp6a19	6A19-36 6A19-672	5' GTCTTCTATATCTGGCAGAGGC3' 5' GCCAAAGGCAATAGTCGC 3'	63.9 61.4	636
Cyp6a9	6A9-89 6A9-634	5' CCAGTCGATCATTTAGTGC 3' 5' ATGTGCAGTCTGCGTGC 3'	65.4 64.2	545
Cyp6a20	6A20-28 6A20-606	5' GTGTTATCACCTTCGTGCG 3' 5' CCCATTGGACAACTTCGC 3'	60.7 66.4	578
Cyp6a21	6A21-15 6A21-696	5' GTTCTACTAACGGCGCTGC 3' 5' TCATATGGAGGCGACGTGC 3'	62.5 67.2	681
Cyp6a8	6A8- 194 6A8- 865	5' ATTCAGGGAAAGCAAGGC 3' 5' TAATCCTCTGACCACATCGC 3'	64.1 62.6	671
Cyp317a 1	317A1-18 317A1-654	5' ATCATCGGATTGCTGGTGC 3' 5' GGCTATTCAAGGCCAGC 3'	66.1 69.0	636
Cyp12b2	12B2-64 12B2-672	5' GGAAGTCATCTGTTGGTGGC 3' 5' CCTTGTGCGAATATCAGC 3'	64.5 60.8	608
Cyp6d2	6D2-80 6D2-778	5' ATTGGGAGTCGATGAGGAGC 3' 5' GAATGCGGCTTTCGTAGC3'	65.4 63.4	698
Cyp9c1	9C1-104 9C1-639	5' CGAAAAGCCAATTCCTCTGC 3' 5' TCACTTGGATGCCAAAGGC 3'	65.7 65.9	535

Table 4- List of gene specific primers used to amplify Cyp genes on chromosome 3L

Gene Name	Primer name	Primer Sequence	Tm (°C)	Fragment size (bp)
CYP4D20	4D20-1	5' ATGTGGCTGACCTTGATCACG 3'	67.3	586
	4D20-587	5' TACTTGAGCATATCCACCGC 3'	62.6	
CYP302A1	302A1-16	5' TTAAAGATTAGCTGCACCTCG 3'	60.9	582
	302A1-598	5' ATTGCCACATTCACCATTTCC 3'	65.6	
CYP307A1	307A1-36	5' TTTACTGAGTGTCTTGCCAC 3'	61.7	519
	307A1-555	5' CCAGTGCTCCATTTCTCGC 3'	69.2	
CYP4D8	4D8-6	5' GCTGTTTCTACTGGTTGTGC 3'	59.9	522
	4D8-528	5' CGAACAGCTTTTCAGTTATGAG3'	60.4	
CYP316A1	316A1-15	5' CACATTCATTTGTTTCTGCCTG 3'	64.2	526
	316A1-541	5' ATCAAACACCTTTTGATCCGC 3'	64.8	
CYP314A1	314A1-37	5' TCGTACTTGGCTGCTACTGC 3'	63.1	508
	314A1-545	5' TGTACAGGTGCACTATTGGC 3'	61.2	
CYP312A1	312A1-42	5' CCTATACCTGCTATATGTCTTCG3'	58.4	515
	312A1-557	5' CAAATAGTGTCCAAAGTGAAGC 3'	60.2	
CYP12C1	12C1-8	5' GTCTCACAGTAAAGCACGGC 3'	62.4	512
	12C1-520	5' GAGATATATGAATTGCATTGGC 3'	59.8	
CYP305A1	305A1-5	5' GTGCCCTCATATTTCTGTGC 3'	62	491
	305A1-496	5' GGTGTCAGAAGTATGTGACGA 3'	60.2	

Table 5- List of gene specific primers used to amplify Cyp genes on chromosome 3R

Gene Name	Primer name	Primer Sequence	Tm (°C)	Fragment size (bp)
CYP313B1	313B1-36	5' TTTGGCTTGGTTATACTTCCTG 3'	61.7	439
	313B1-475	5' GAAGTTGCTGAGAATCTGGC 3'	61.5	
CYP12E1	12E1-45	5' GATCTACCAGCTATGCAGGG 3'	61.2	496
	12E1-541	5' GAAGTCAACCCATCATAGCC 3'	60.9	
CYP315A1	315A1-71	5' GAATCCTTAGCCTGAGCCTC 3'	61.8	501
	315A1-572	5' TCTTGCAGCTTTGATATTTCG 3'	62.9	
CYP313A2	313A2-45	5' GATCCGTTTTCTATGGAGTCG 3'	62.3	523
	313A2-568	5'CTGAAAGGACATCTTTTTCACC3'	61.4	
CYP313A3	313A3-8	5' CGTTTCAACTGCTTTTAGCCG 3'	65.5	549
	313A3-557	5' GAAGATGGGAAGAAACTCAGC 3'	62.9	
CYP313A5	313A5-84	5' GATGCTAAAGTTACCAGGACC 3'	59.1	502
	313A5-586	5'GTAAGGTCACCAGGAAATTGG 3'	62.1	
CYP9F2	9F2-127	5' GCTCTACTTTGGCAACATGG 3'	62.2	600
	9F2-727	5' CTTATCGTTATAGCACTTTCAGG 3'	59.7	
CYP9F3	9F3-152	5' ATGTTCTACGGAAGAGAGCC 3'	63.3	659
	9F3-811	5' AGGAACAAAAAGGAAACAGTCAC 3'	62.1	
CYP313A4	313A4-43	5' TGGATCTACTTTCTGTGGAGC 3'	60.8	580
	313A4-623	5' GTGGAATGAGATCCTTCTCG 3'	61.2	
CYP304A1	304A1-11	5' AAACCTCTCTGACGATCTGC 3'	61.0	553
	304A1-564	5' TTCTTTGACTGTGAAGTGCGC 3'	65.4	
CYP6D5	6D5-39	5' GCTATATGTCTATCTCAAGTGG 3'	54.6	442
	6D5-481	5' GTTGCTTCCTAATGCTGTCC 3'	61.2	
CYP313A1	313A1-80	5' TCCTGATGCTAAAGATTCCCG 3'	65.0	466
	313A1-546	5' TTAAACGATGGGTTAAAGTGCT 3'	61.2	
CYP12A5	12A5-19	5' GCACTTAATATACTTCAGTCGC 3'	57.2	557
	12A5-576	5' GGAATGATCCCTTGAACACC 3'	63.1	
CYP12A4	12A4-1	5' ATGCTAAAAGTTCGCAGTGCTC 3'	64.4	538
	12A4-539	5' ATAGTTGCCTGGACGATTGG 3'	63.7	
CYP6D4	6D4-42	5' GGCGTGGTTCTATCTGAAGC 3'	63.6	486
	6D4-528	5' CACTTCTTTATGTCCACCTCC 3'	61.3	
CYP6A18	6A18-80	5' CGTATTTTAAGAGGCGTGGC 3'	63.3	531
	6A18-611	5' GCCTTCTCATCTCGCAGACT 3'	63.6	
CYP4C3	4C3-12	5' CATACTGCTCTCAAAAGTGGG 3'	61.2	602
	4C3-614	5' AACAAAGAGGAACAGAAAATGC 3'	61.0	

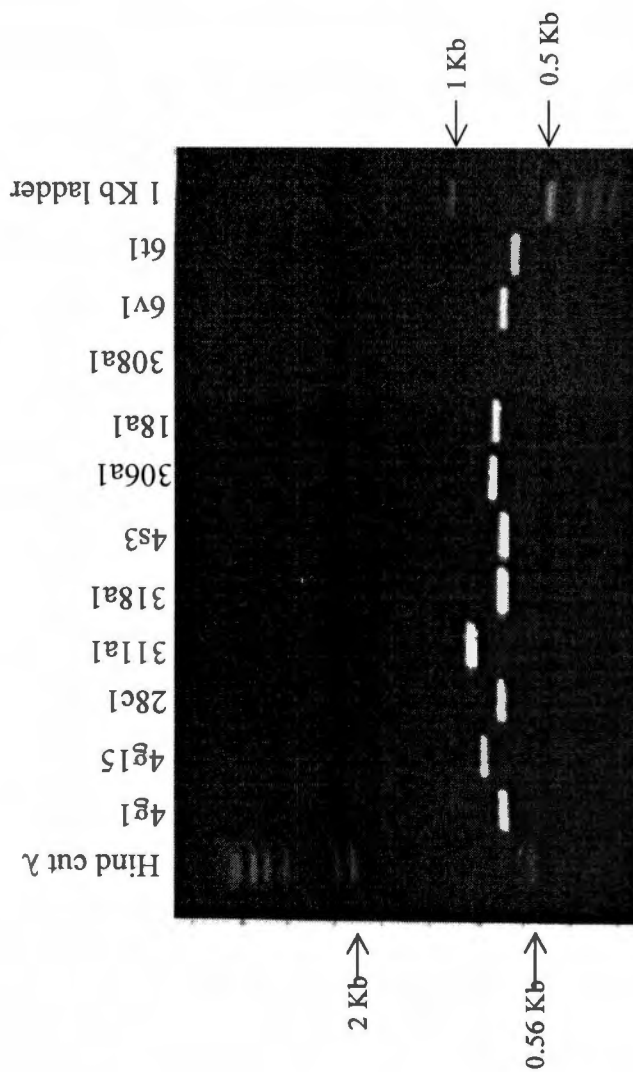


Figure 5- PCR products of *Cyp* genes on X chromosome of *D. melanogaster*

Gene specific primers listed in Table 1 were used to amplify *Cyp* genes on X chromosome for microarray analysis. The PCR products were fractionated on a 1% agarose gel to check for size. (See Figure 11 for products of *Cyp4dl*, *4dl4*, *4d2*, and *4ael*).

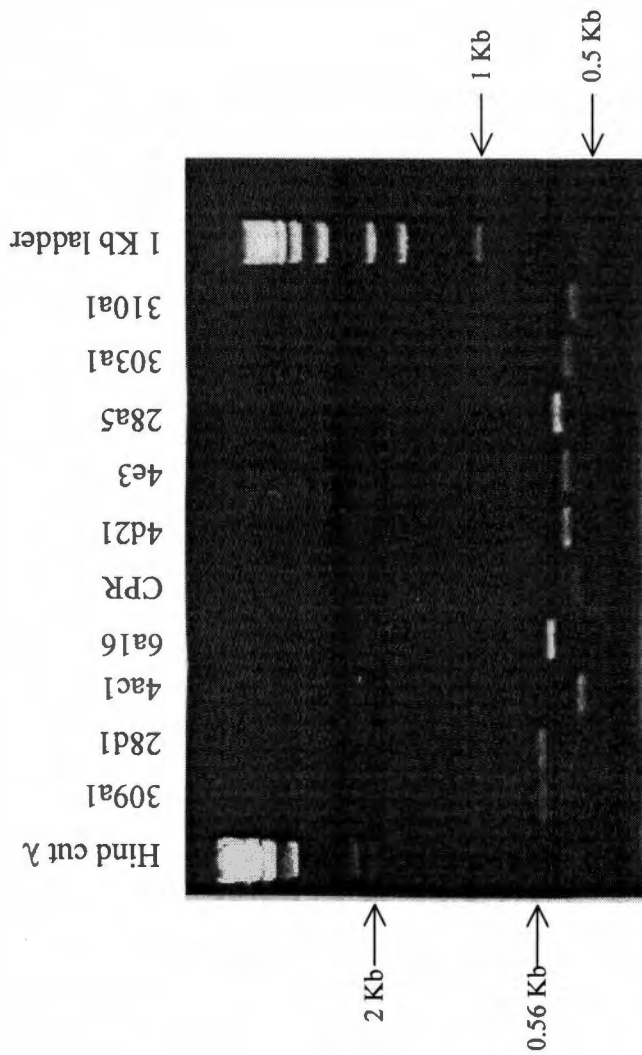


Figure 6- PCR products of *Cyp* genes on chromosome 2L of *D. melanogaster*
 Gene specific primers listed in Table 2 were used to amplify *Cyp* genes on chromosome 2L for microarray analysis. The PCR products were fractionated on a 1% agarose gel to check for size.

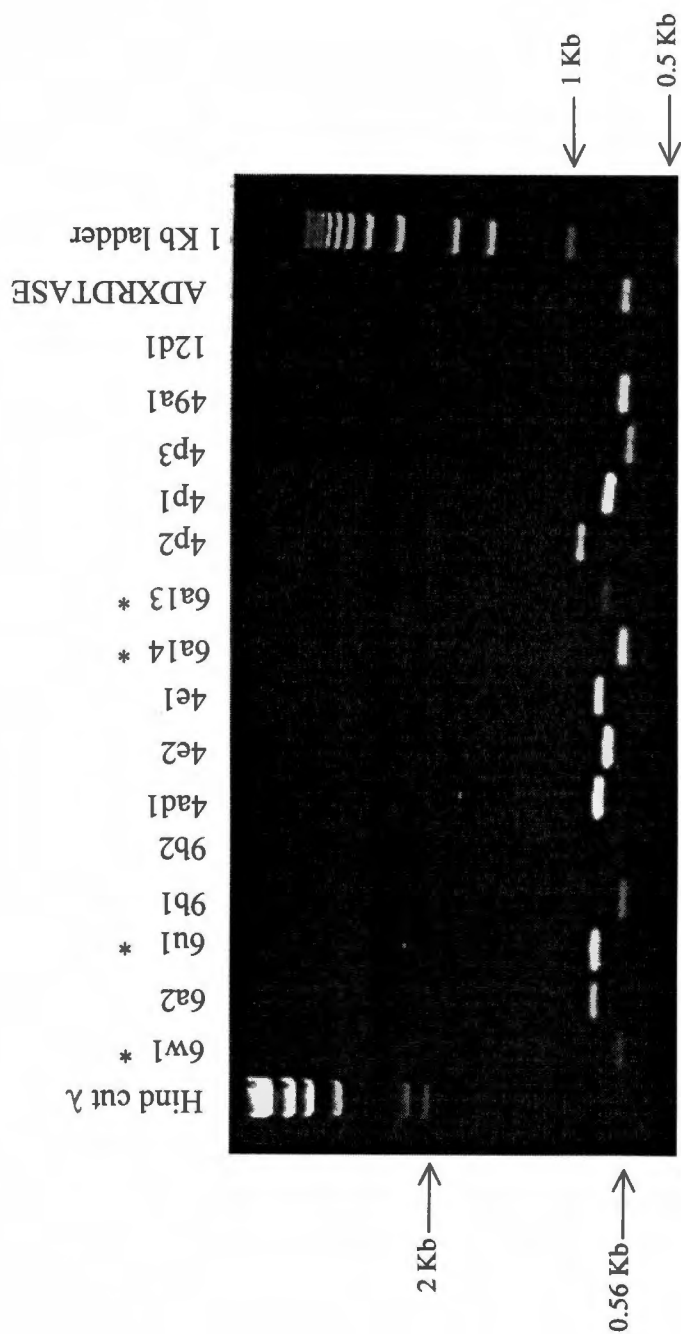


Figure 7- PCR products of *Cyp* genes on chromosome 2R of *D. melanogaster*, located close to the centromere

Thirteen gene specific primer pairs listed in Table 3 were used to amplify *Cyp* genes on chromosome 2R for microarray analysis. The PCR products were fractionated on a 1% agarose gel to check for size. Products marked with an asterisk were also used for Northern blot analysis.

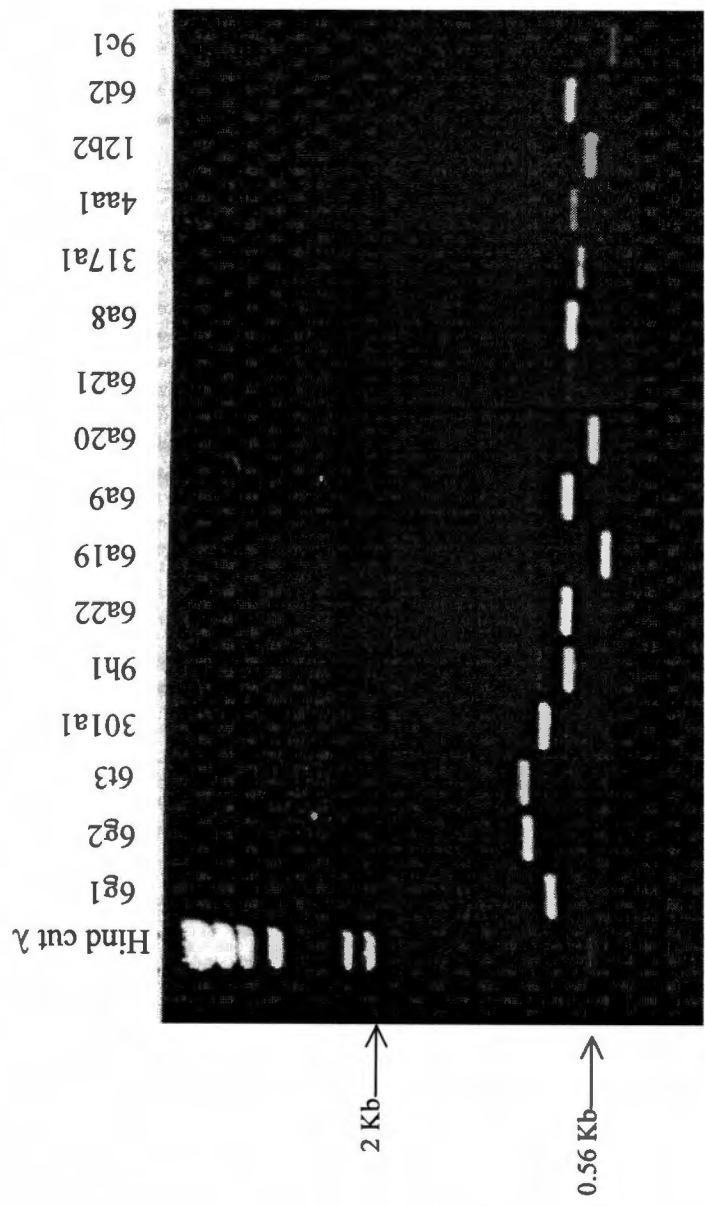


Figure 8- PCR products of *Cyp* genes on chromosome 2R of *D. melanogaster*, located distal to the centromere

Sixteen gene specific primer pairs listed in Table 3 were used to amplify *Cyp* genes on chromosome 2R for microarray analysis. The PCR products were fractionated on a 1% agarose gel to check for size.

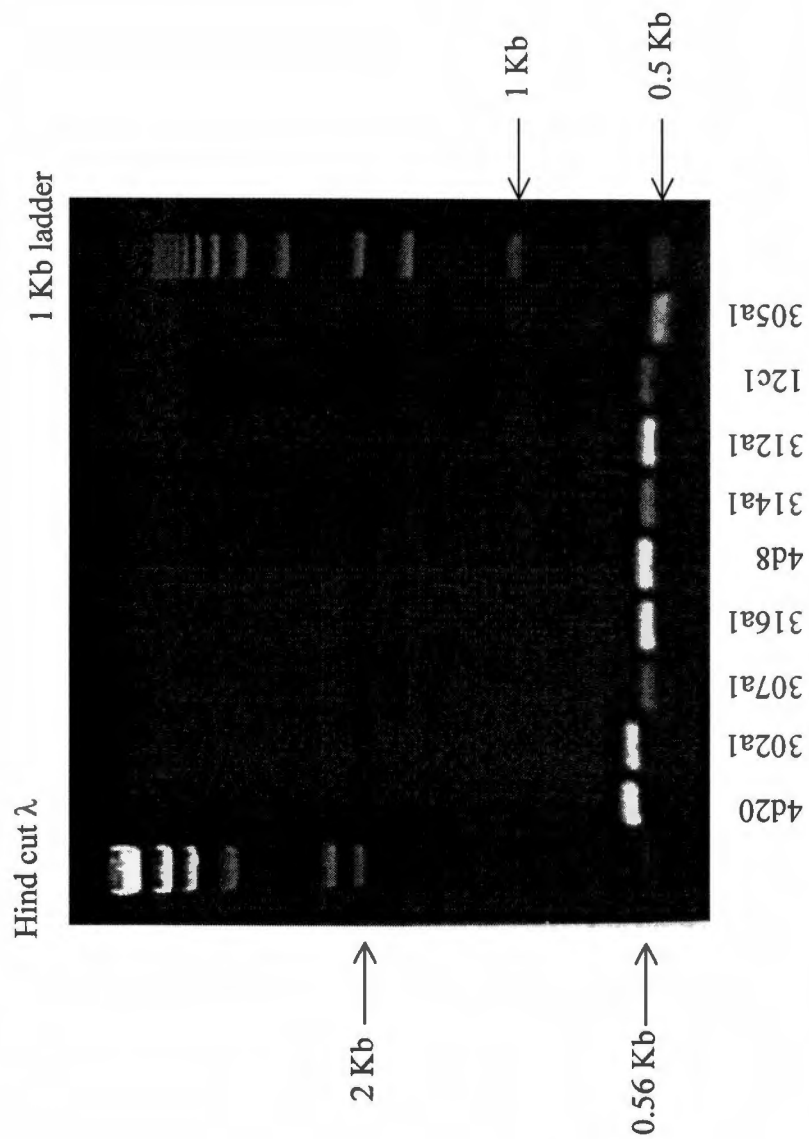


Figure 9- PCR products of *Cyp* genes on chromosome 3L of *D. melanogaster*

Gene specific primers listed in Table 4 were used to amplify *Cyp* genes on chromosome 3L for microarray analysis. The PCR products were fractionated on a 1% agarose gel to check for size.

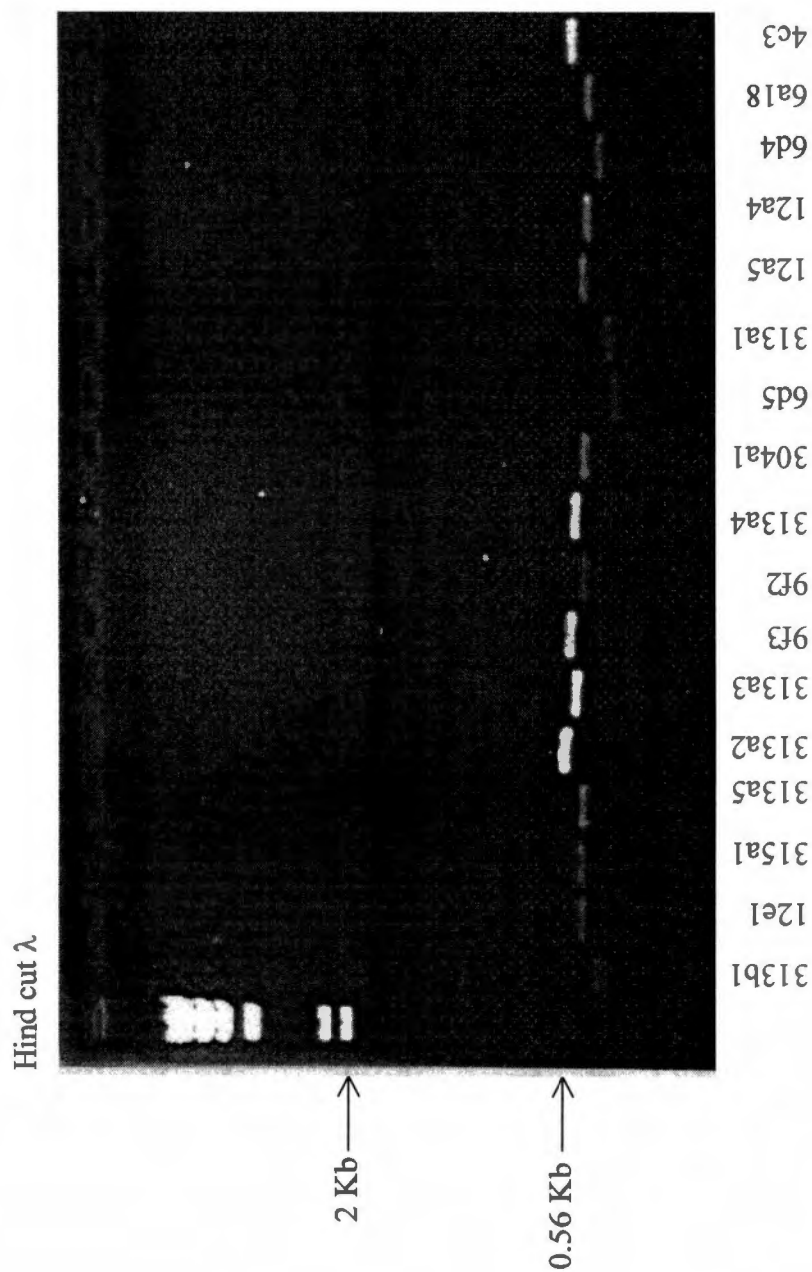


Figure 10- PCR products of *Cyp* genes on chromosome 3R of *D. melanogaster*

Gene specific primers listed in Table 5 were used to amplify *Cyp* genes on chromosome 3R for microarray analysis. The PCR products were fractionated on a 1% agarose gel to check for size.

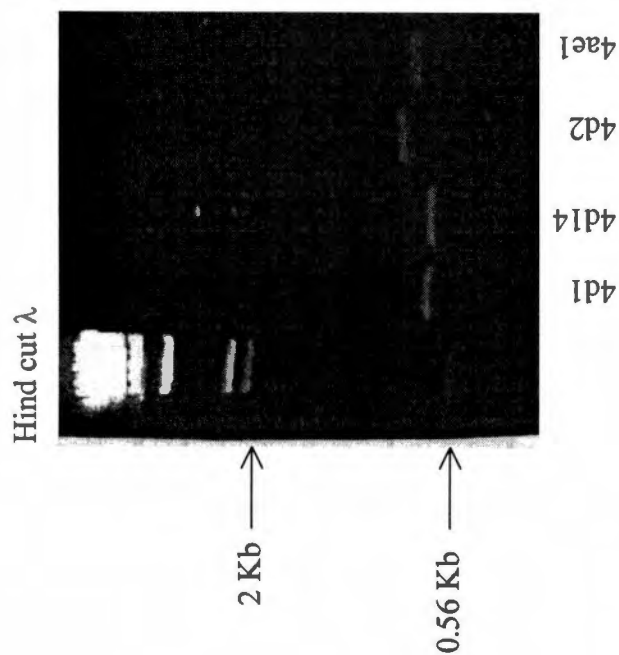


Figure 11- Four PCR products of *Cyp* genes on X chromosome of *D. melanogaster*.

Four pairs of gene specific primers listed in table 1 were used to amplify *Cyp4d1*, *4d14*, *4d2*, and *4ae1* genes on the X chromosome. The PCR products were fractionated on a 1% agarose gel to check for size.

experiments were done to determine the relative expression of each gene between 91-R and 91-C. In the first experiment, cDNA from 91-R was labeled with Cy3 whereas 91-C cDNA was labeled with Cy5. In the second experiment, the labeling pattern was switched. In each experiment, the labeled cDNAs from 91-R and 91-C were mixed and hybridized with the spotted arrays. Hybridization signals were detected by confocal fluorescent microscopy and an image of hybridization is shown in Figure 12. Genes that are expressed equally in two strains are expected to hybridize equivalent amounts of Cy3 (red) and Cy5 (green) cDNA molecules. These DNA spots give a yellow color (e.g. RP49). If a DNA spot hybridizes more with one cDNA (Cy3 or Cy5), the fluorescent color of that cDNA (red or green) will be seen. All three different color patterns were observed in the current investigation (Fig. 12), suggesting that some genes are equally expressed in both strains, whereas some genes are expressed more robustly in either the 91-R or the 91-C strain.

To determine the relative expression of each gene in 91-R and 91-C strains, ratios of fluorescence generated by 91-R cDNA to the fluorescence generated by 91-C were determined. Background fluorescence was subtracted locally at each spot and RP49 as used as an internal control. Table 6 shows the ratios of 91-R to 91-C fluorescence values obtained for each gene in two experiments. In both experiments, the ratio of RP49 expression in the two strains was 1.0, suggesting an equal expression. In the first experiment, most ratios were higher than 1, with *Cyp4g1* gene showing the highest ratio (3.30). This suggested that the expression of these genes is higher in 91-R relative to 91-C strain. Only two genes, *Cyp4ac1* and *Cpr*, showed significantly low ratios (0.5 and 0.69,

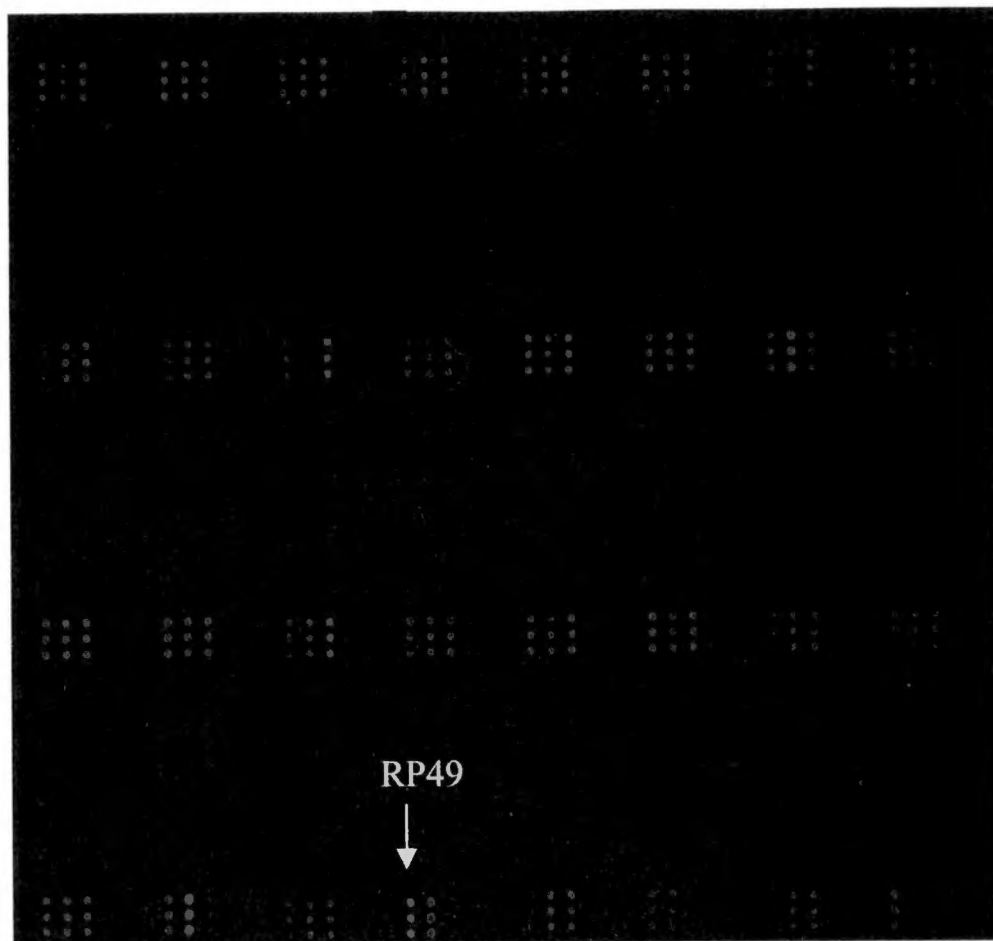


Figure 12- A scanned image of a microarray hybridization with Cy3 and Cy5 labeled cDNA

Eighty three *Drosophila Cyp* genes were spotted on a glass slide and hybridized with a mixture of Cy3 (red) and Cy5 (green)-labeled cDNA of 91-R and 91-C strains, respectively. Fluorescence intensities for Cy3 and Cy5 were measured separately at each spot and converted into computer images. Shown is a superimposed image of the results. RP49 is a control used for normalization of data, and shows an equal expression in both samples indicated by the yellow color.

Table 6- Mean ratios of arbitrary fluorescence units (AFU) for Cy3 and Cy5 in microarray analysis of *Cyp* genes of *Drosophila melanogaster*

In experiment 1, cDNA of 91-R was labeled with Cy3, and cDNA of 91-C was labeled with Cy5. In experiment 2, the dyes were switched. Ratios in both experiments represent the relative expression of each gene in 91-R compared to 91-C. The ratio values of six genes (*Cyp308*, *303*, *4p1*, *4p3*, *311a*, and *312*) were omitted due to their high standard deviation.

Gene Name	Experiment 1		Experiment 2	
	Mean AFU ratio of Cy3/Cy5	($\pm$)	Mean AFU ratio of Cy5/Cy3	($\pm$)
Cyp4g1	3.30	0.11	1.33	0.18
Cyp4s3	0.97	0.07	0.62	0.06
Cyp306a1	1.33	0.08	0.78	0.08
Cyp18a1	1.43	0.03	0.55	0.11
Cyp6v1	1.32	0.13	1.30	0.24
Cyp6t1	1.25	0.03	0.98	0.07
Cyp309a1	1.60	0.13	0.83	0.14
Cyp28d1	1.36	0.01	1.19	0.11
Cyp4d1	1.21	0.08	0.75	0.17
Cyp4ac1	0.50	0.21	0.56	0.33
Cyp4ac2	1.36	0.01	1.01	0.14
Cyp4ac3	1.78	0.19	0.75	0.06
Cyp6a16	1.38	0.13	1.21	0.10
CPR	0.69	0.03	0.58	0.04
Cyp4d21	1.30	0.02	1.35	0.08
Cyp4e3	2.10	0.18	0.62	0.02
Cyp28a5	1.38	0.03	1.48	0.10
Cyp4d14	1.33	0.15	0.84	0.08
Cyp310a1	1.38	0.12	1.47	0.12
Cyp6w1	1.92	0.39	1.30	0.22
Cyp6a2	1.53	0.09	1.25	0.03
Cyp6u1	1.49	0.09	1.05	0.05
Cyp9b1	1.44	0.09	1.30	0.07
Cyp9b2	2.20	0.05	1.17	0.04
Cyp4ad1	1.35	0.06	1.26	0.02
Cyp4e2	1.46	0.04	1.01	0.05
Cyp4e1	1.43	0.10	1.36	0.11
Cyp4d2	1.08	0.18	0.84	0.06
Cyp6a14	2.25	0.18	1.15	0.17
Cyp6a13	1.17	0.01	1.23	0.08
Cyp4p2	1.50	0.32	0.89	0.09
Cyp49a1	1.49	0.16	1.33	0.09
Cyp12d1	1.67	0.39	1.00	0.08
ADXRDASE	1.49	0.11	1.42	0.09
Cyp6g1	1.91	0.20	0.92	0.12
Cyp4ae1	1.10	0.19	0.93	0.10

Table 6- continued

Cyp6g2	1.41	0.09	0.88	0.11
Cyp6t3	1.74	0.36	1.06	0.09
Cyp301a1	1.52	0.10	1.30	0.09
Cyp9h1	1.47	0.24	1.21	0.05
Cyp6a22	1.57	0.04	0.85	0.07
Cyp6a19	1.85	0.08	0.92	0.16
Cyp6a9	1.54	0.06	0.86	0.07
Cyp6a20	2.06	0.14	0.76	0.06
Cyp4g15	1.25	0.11	0.86	0.09
Cyp6a21	1.45	0.09	0.72	0.08
Cyp6a8	1.27	0.03	0.70	0.04
Cyp317a1	1.29	0.11	0.69	0.06
Cyp4aa1	1.79	0.23	0.53	0.07
Cyp12b2	1.35	0.03	0.83	0.12
Cyp6d2	1.32	0.05	0.72	0.09
Cyp9c1	1.41	0.09	0.69	0.13
Cyp4d20	1.97	0.14	0.55	0.06
Cyp302a1	1.21	0.19	1.09	0.10
Cyp307a1	1.16	0.08	1.07	0.11
Cyp28c1	1.21	0.03	0.90	0.07
Cyp316a1	1.34	0.07	1.02	0.09
Cyp4d8	1.90	0.14	0.72	0.03
Cyp314a1	1.19	0.07	1.10	0.16
Cyp12c1	1.41	0.09	0.86	0.04
Cyp305a1	1.80	0.22	0.58	0.13
Cyp313b1	1.23	0.06	1.07	0.06
Cyp12e1	1.13	0.22	1.24	0.06
Cyp315a1	1.43	0.01	1.06	0.16
Cyp313a5	2.02	0.14	0.57	0.03
Cyp313a2	1.43	0.10	1.03	0.14
Cyp313a3	1.57	0.08	1.00	0.06
Cyp9f3p	1.45	0.04	1.58	0.09
Cyp9f2	1.97	0.22	1.12	0.17
Cyp313a4	1.61	0.04	1.02	0.02
Cyp304a1	1.57	0.08	0.94	0.01
Cyp6d5	1.90	0.09	2.07	0.08
Cyp313a1	2.33	0.25	0.87	0.17
Cyp12a5	1.19	0.01	0.96	0.05
Cyp12a4	1.49	0.17	1.04	0.10
Cyp318a1	1.36	0.04	0.63	0.09
Cyp6d4	1.60	0.13	1.45	0.11
Cyp6a18	2.55	0.13	0.57	0.08
Cyp4c3	1.31	0.22	1.10	0.18
RP49	1.00	0.09	1.00	0.05

respectively). Similar ratio values were observed for these two genes in both experiments, indicating that these genes are expressed at a higher level in 91-C than in 91-R strain. Unfortunately, however, ratios of many other genes did not agree between the two experiments. For example, the ratio of *Cyp4g1* (3.3), which was the highest in the first experiment, was reduced by 3-fold (1.3) in the second experiment. In addition, while there were only two genes (*Cyp4ac1* and *Cpr*) with ratios lower than 1 in the first experiment, the number increased to sixteen genes in the second experiment.

To examine the linear relationship between hybridization of 91-R and 91-C cDNA, and to better represent the ratios listed in Table 6, the logarithmic values of mean fluorescence intensities (n=3) were calculated for Cy3 and Cy5 at each spot. The data are represented in scatter plots (Figures 13-14). The diagonal line that goes through a 45° angle is called the “line of identity”. The slope of this line is always 1. Genes that are expressed equally in both samples fall in the line of identity and genes that are expressed significantly higher or lower in one sample compared to the other deviate accordingly from the line of identity. Distance of a spot from the line of identity represents the difference in expression of a given gene between the two samples. As observed, in both experiments most data points seem to fall on or very close to the slope line, indicating that their relative expression is similar in both strains. Notice that the data points for *Cyp4g1* and *Cyp4ac1* in the first experiment are clearly away from the line of identity (Figure 13) indicating differential expression of these two genes in two strains. However, data points for these genes do not stand out from the line of identity so clearly in experiment 2 (Figure 14).

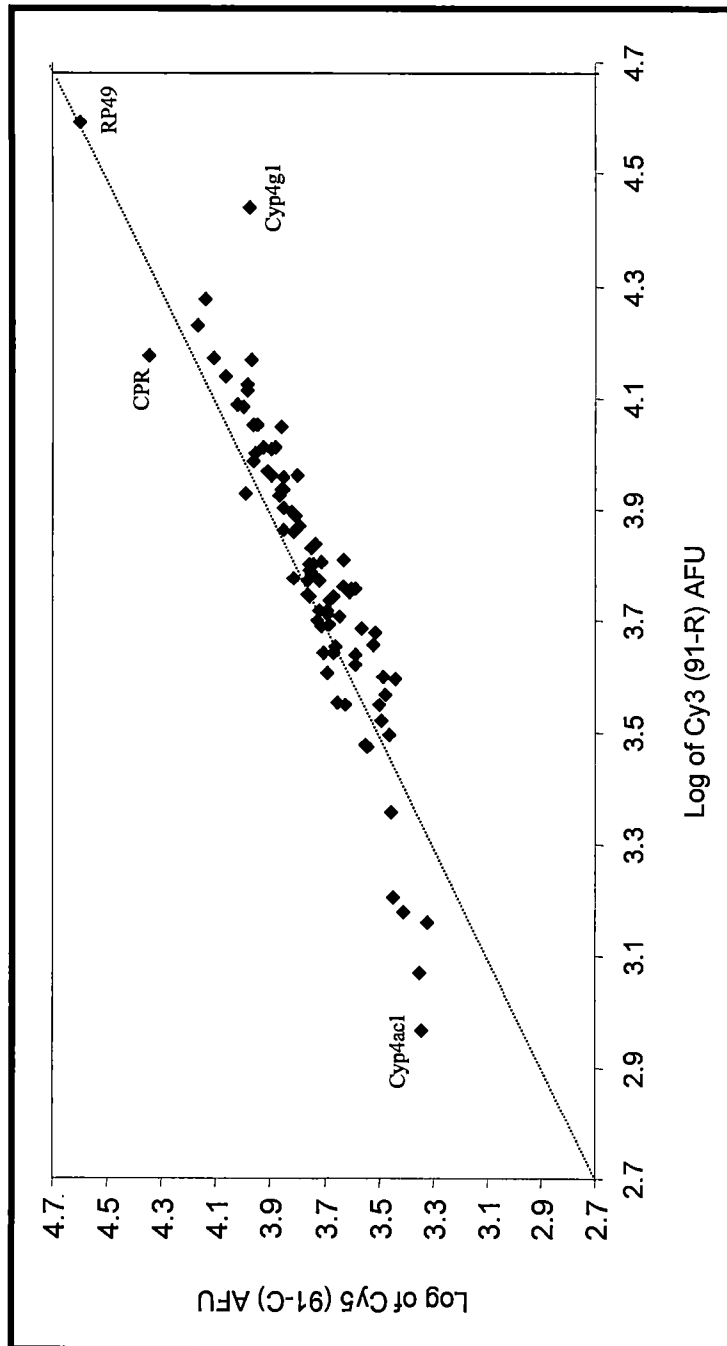


Figure 13- Scatter plot of logarithmic values for mean arbitrary fluorescence units (AFU) of Cy3 and Cy5 in experiment 1.

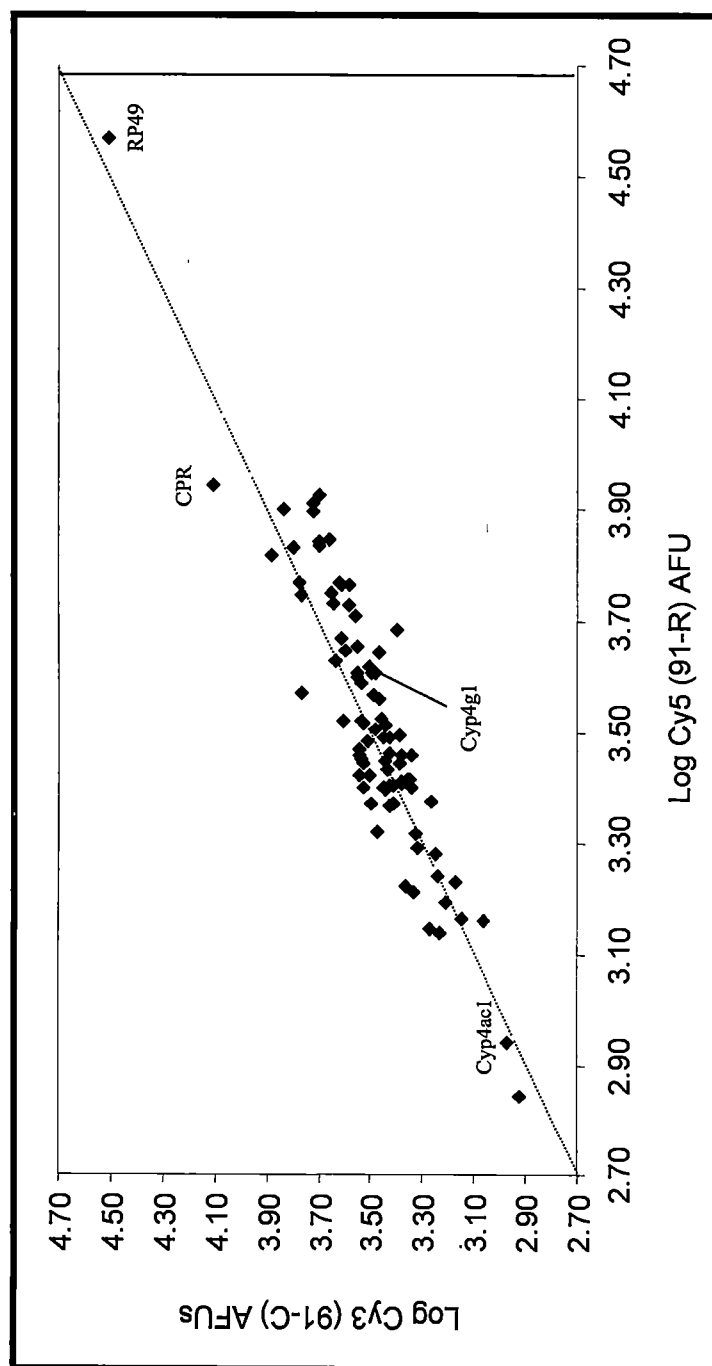


Figure 14- Scatter plot of logarithmic values for mean arbitrary fluorescence units (AFU) of Cy5 and Cy3 in experiment 2.

The observed discrepancy between the two experiments makes it difficult to draw any conclusion with regard to the differential *Cyp* gene expression in 91-R and 91-C strains. The results of microarray data presented here are also found to be at odds with previous Northern blot hybridization data. It has been shown consistently that *Cyp6a2*, *Cyp6a8* and *Cyp6g1* genes are expressed at much higher levels in 91-R than in 91-C strains (Waters et al., 1992 a; Maitra et al., 1996, 2001; Dombrowski et al., 1998). In the microarray experiments this does not seem to be the case (Table 6). In these experiments, *Cyp6a2* shows only 25-50% higher expression in 91-R. *Cyp6g1*, on the other hand, shows an inconsistent ratio between the two experiments. In experiment 1, it shows about 2-fold higher expression in 91-R, whereas an almost equal expression is observed in experiment 2. *Cyp6a8* shows more or less equal expression in both strains in both experiments. In view of these inconsistencies, *Cyp* genes showing a higher expression in one or the other strain could not be determined confidently, although such genes can be found in Table 6.

Since this is a preliminary experiment for *Cyp* gene expression profiles in 91-R and 91-C strains, several steps need to be improved in order to obtain more accurate data. First, the density of the spotted DNA should be higher. The average concentration of PCR products used for spotting the arrays was about 40ng/μl, whereas the recommended concentration is $\geq 150\text{ng}/\mu\text{l}$ (Peter Hoyt, personal communication). This may have affected the efficiencies of fluorescent measurement. Second, the number of controls used for normalization of data should be increased. Usually, a set of 5 to >20 controls with increasing concentrations are used, where the sum of their intensities should be

equal for both dyes (Ermolaeva *et al.*, 1998; Eisen and Brown, 1999; Hedge *et al.*, 2000; Peter Hoyt, personal communication). Third, these experiments must be repeated several times in order for the observed data to be statistically significant. Although this can be expensive and labor intensive for microarrays with thousands of spotted genes, it is manageable for the 83 *Cyp* genes studied here.

Constitutive expression of four family *Cyp6* genes located close to a DDT-resistance locus

The objective of the microarray experiment was to identify *Cyp* genes that show higher expression in 91-R than in 91-C strain, so that these genes could be studied in greater detail. Because the results of the microarray turned out to be inconclusive, a different strategy was used to identify *Cyp* genes showing differential expression in the two strains. Genetic mapping analysis on various strains of *Drosophila* has so far identified five loci (Figure 15) that confer resistance to several insecticides (Kikkawa, 1953; Ogita, 1958; Houpt *et al.*, 1988; Waters and Nix, 1988; Dapkus, 1992). Of these five, R1 is considered to be a major resistance locus because it confers resistance to multiple insecticides including DDT, malathion, and pyrethroids in several tested strains (Tsukamoto, 1958; Houpt *et al.*, 1988; Waters and Nix, 1988). Recently, Dapkus (1992) showed that DDT resistance in the 91-R strain is conferred by a second chromosome-linked locus (R2) that maps at 56 ± 1 mu. The biochemical nature of the products encoded by genes located at these five loci is still

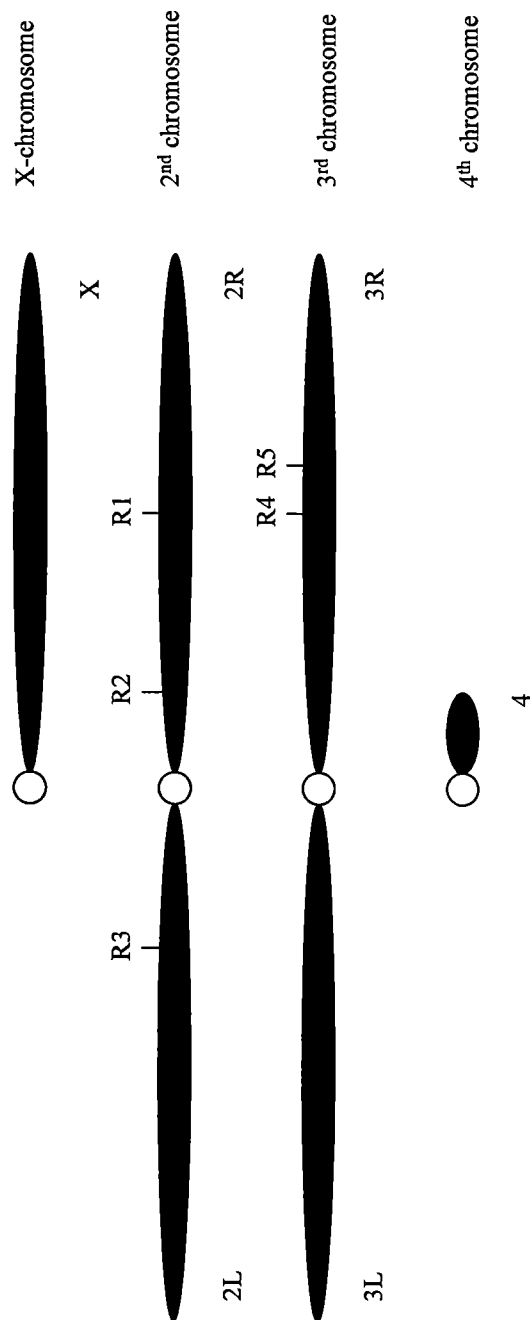


Figure 15- Linkage groups of *Drosophila melanogaster*, showing the approximate location of 5 loci associated with insecticide resistance.

Genetic mapping analysis in several insecticide resistant strains identified the presence of 5 loci associated with resistance, of which R1 is a major resistance locus (Kikkawa, 1953; Ogita, 1958; Hout et al., 1988; Waters and Nix, 1988; Dapkus, 1992). R2 is reported to confer resistance to DDT in the 91-R strain (Dapkus, 1992). All loci are associated with increased P450 content and/or activity (Ogita, 1985; Waters and Nix, 1988; Hout et al., 1988; Dapkus, 1992).

unknown. However, it has been shown that resistance conferred by some of them is associated with elevated levels of P450 content and/or activity (Agosin, 1985; Oppenoorth, 1985; Houpt et al., 1988; Waters and Nix, 1988; Sundseth et al., 1990; Waters, 1992 b). Therefore, in the present investigation I wished to determine whether the *Cyp* genes that are located close to the R2 locus show higher expression in the resistant 91-R than in the susceptible 91-C strain. Based on the cytological and genetic locations of known genes, the R2 locus appears to be close to polytene band 42D on the right arm of the second chromosome (Figure 16). *Drosophila* genome sequence analysis predicts the presence of several *Cyp* genes near and on polytene band 42D (Waters et al., 1992; Maitra, et al., 1996; Dombrowski et al., 1998; Tijet et al., 2001). If it is assumed that DDT resistance is due to increased amounts of a CYP, examination of the expression of *Cyp* genes located close to 42D region may be a worthwhile effort. Therefore, four family 6 genes (*Cyp6w1*, *Cyp6u1*, *Cyp6a14*, and *Cyp6a13*) that are close to 42D region were chosen for expression analysis. Family 6 genes are found in insects only.

To analyze the expression of these genes, gene-specific primers located within their less conservative N-terminal halves were used to amplify 500-700 bp DNA fragments using genomic DNA as a template. The PCR amplified products were labeled with ^{32}P and individually hybridized with poly (A)⁺ RNA from adult female flies of 91-R and 91-C strains. The results showed that only *CYP6w1* probe hybridized strongly with RNA from both strains (Figure 17). The other three probes showed very faint hybridization, suggesting that these genes are not very active transcriptionally in the adult

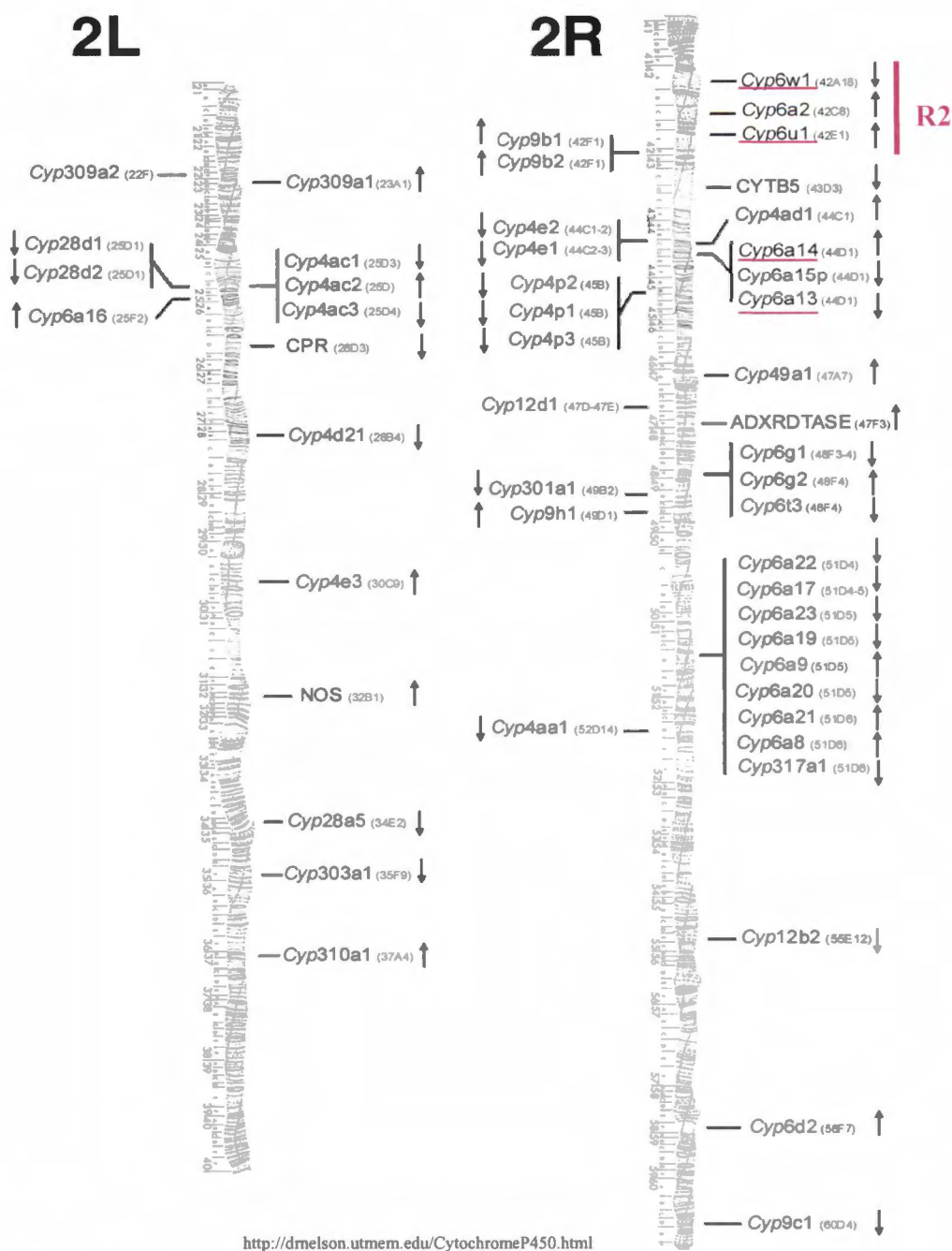


Figure 16- A cytological map of the second chromosome of *Drosophila melanogaster*.

A DDT-resistance locus (R2) was genetically mapped in 91-R to 56 ± 1 map units (Dapkus, 1992). The approximate location of this locus on the cytological map (42 region) is shown above. The four Cyp6 family genes underlined in pink were used for northern analysis to determine their relative expression in 91-R and 91-C strains.

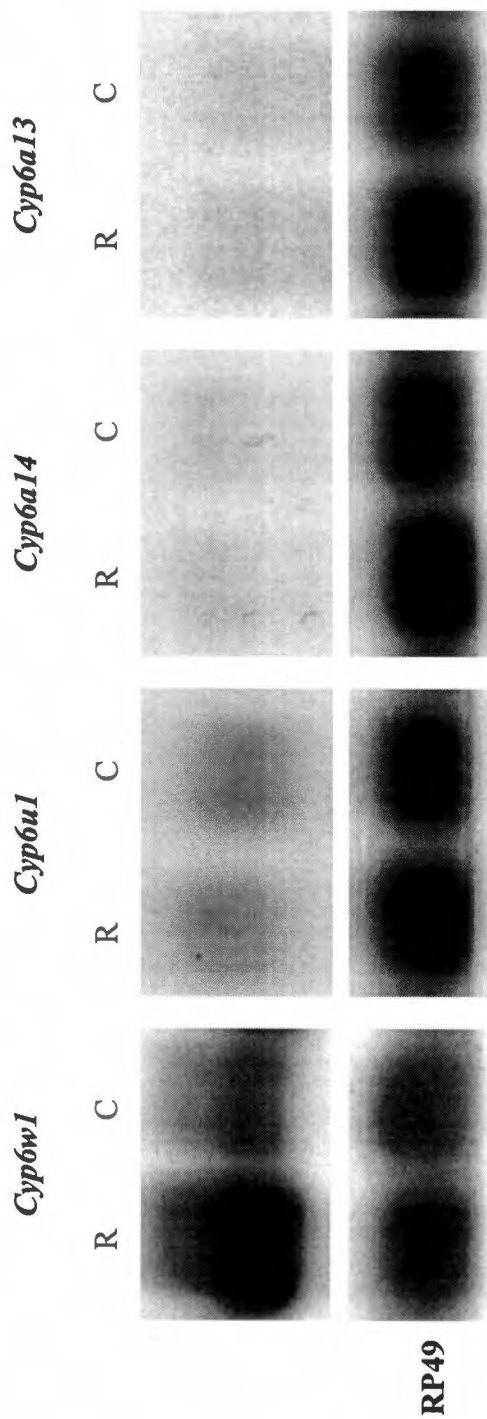


Figure 17- Constitutive expression of *Cyp6w1*, *Cyp6u1*, *Cyp6a14* and *Cyp6a13* genes in 91-R and 91-C strains

Poly (A)⁺ RNA from adult females of 91-R (R) and 91-C (C) strains were electrophoresed on 2.2M-1.2% formaldehyde agarose gel, transferred on nylon membranes, and hybridized with ³²P-labeled probes indicated on top. The bottom panels show hybridization of RP49, used as an internal control. Northern hybridization for each probe was done in triplicate (not shown).

signals of RP49 in each lane as an internal control, the results showed that CYP6W1 RNA is about 3-fold higher in 91-R than in 91-C strain (Figure 18). The observed difference was found to be statistically significant ($p \leq 0.0001$) as determined by the Student's t-test.

Cyp genes belonging to the same family show 40% amino acid sequence similarities (Nebert and Gonzales, 1987; Feyereisen, 1999). Therefore, it is possible that the primers designed for *Cyp6w1* might have amplified a different *Cyp* gene in the genomic DNA. If that was the case, then the observed hybridization could have been with a different RNA. To resolve this issue, the PCR product generated with the *Cyp6w1*-specific primers was sequenced and compared with *Drosophila* genomic sequence via NCBI Blast search. The results showed that the full-length sequence of the *Cyp6w1* PCR product matched with the expected region of the *Cyp6w1* gene reported in the genome sequence database, with 96% sequence identity. A pairwise comparison of the reported sequence and the sequence of the PCR fragment is shown in Figure 19. Several mismatches were observed between the two sequences, some of which were probably due to sequencing error. Other mismatches may be a reflection of nucleotide polymorphisms between 91-R strain and the strain used for *Drosophila* genome sequencing.

The BLAST search also showed that the *Cyp6w1* PCR product does not have much similarity with other *Drosophila* genome sequences in the database. Similarities

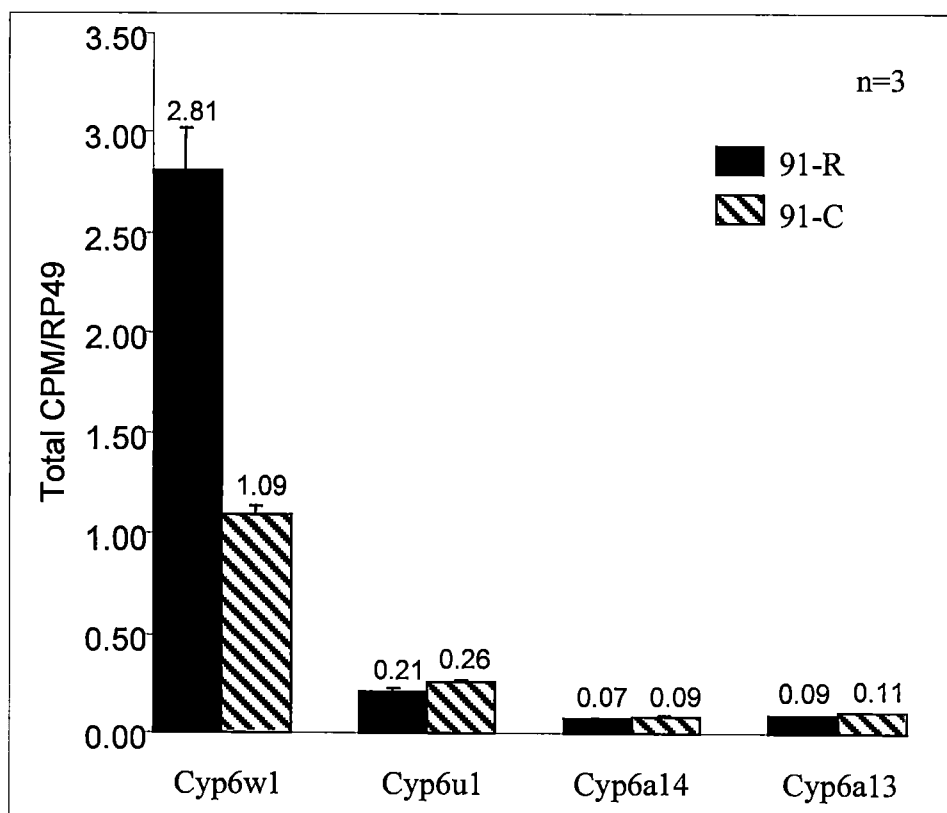


Figure 18- Quantitative expression of *Cyp6w1*, *Cyp6u1*, *Cyp6a14*, and *Cyp6a13* genes in 91-R and 91-C strains.

Poly (A)⁺ from 91-R and 91-C females were analyzed by northern blot as described in figure 17. Hybridization signals for each probe and the respective RP49 signals in each lane were measure by a radioanalytical imager. Bars represent the mean ratio of each probe/RP49 from three northern blots, were mean values are indicated on top. Among all four, *Cyp6w1* is the only gene showing a significantly higher expression in 91-R.

```

Query: 3   gngtaaaatacatcccgtccggttcccgtttaggctgcaccgggagttttgaccgc 62
          |||
Sbjct: 105 ggagtaaaatacatcc-gtccgtttcccgtttaggctgcaccgggagttttgaccgc 163

Query: 63   aaaagtgccgttcttcgagcagattcaaaaattccacgaagcgcttgcttcgagaacga 122
          |||
Sbjct: 164 aaaagtgccgttcttcgagcagattcaaaaattccacgaagcgcttgcttcgagaacga 223

Query: 123  gcctttcgttgaggtatacatgaccatcgccggcacttgctcattcgcgatttagaact 182
          |||
Sbjct: 224 gcctttcgttgaggtatacatgaccatcgccggcacttgctcattcgcgatttagaact 283

Query: 183  gatcaagacggtgatgatcaaaaagtttcaatacttcaacaaccgcgtccttcaaactga 242
          |||
Sbjct: 284 gatcaagacggtgatgatcaagaagtttcaatacttcaacaaccgcgtccttcaaactga 343

Query: 243  cccgcacaacgacgcgcttggtataacaacctcttcttgacgaagcccaggatggag 302
          |||
Sbjct: 344 cccgcacaacgacgcgcttggtataaaaacctcttcttgacgaagcccaggatggag 403

Query: 303  ggaattgcgcacaaaaatctctccggtttttacatctggcaagatcaagcaaatgtatcc 362
          |||
Sbjct: 404 ggaattgcgcacaaaaatctctccggtttttacatctggcaagatcaagcaaatgtatcc 463

Query: 363  tctaattggtgaaggaagccacagagagaatttggttgattcaaaaatgggtgctattt 422
          |||
Sbjct: 464 tctaattggtgaaggaagccacagagagaatttggttgattcaaaaatgggtgctattt 523

Query: 423  actgagttattattaagttgnnnnnnnnnnngtatattgtatctaagtttttaaattta 482
          |||
Sbjct: 524 actgagttattattaagttgttttttctttttgtatattgtatctaagtttttaaattta 583

Query: 483  atgctattttagattggaagaacttgacagacagcgcgagcgtctaggcagtggtac 542
          |||
Sbjct: 584 atgctattttagattggaagaacttgacagacagcgcgagcgtctaggcagtggtac 643

Query: 543  ngaa 546
          |||
Sbjct: 644 ggaa 647

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Figure 19- Pairwise comparison of *Cyp6w1* PCR probe with the *Cyp6w1* genomic sequence reported in NCBI.

Gene specific primers (table 1) were used to amplify *Cyp6w1* probe from *ry*⁵⁰⁶ genomic DNA. The probe was sequenced and aligned with the genomic sequence of *Cyp6w1* reported in the NCBI database. There is 96% identity between the two sequences. Basepair mismatches are indicated by a missing connector line between the query (PCR probe) and subject (genomic) sequences.

found with other sequences are confined to very short overlaps, ranging from 17 – 22 bp long. For example, the *Cyp6w1* PCR product shows a 100% sequence identity with a 19 bp fragment of the *fs(1)h* (female-sterile homeotic) gene, between base numbers 250037 and 250055 (Accession No. AE003442). Are such short sequence similarities capable of forming hybrids that can withstand the stringent hybridization and washing conditions used in the present investigation? If they are, then cross hybridization of the *Cyp6w1* PCR product with the 7.7-kb RNA of the *fs(1)h* gene would have been observed. However, no such hybridization was observed, suggesting that the *Cyp6w1* PCR product indeed hybridized with CYP6W1 mRNA on the Northern blot.

With these results, the number of *Cyp* genes on the second chromosome showing differential expression between 91-R and 91-C strains increased to four. These genes are *Cyp6a2*, *Cyp6a8*, *Cyp6g1* and *Cyp6w1*. As shown on the polytene map (Figure 16), both *Cyp6w1* and *Cyp6a2* are located in close proximity to each other, and to the DDT resistance locus (R2) at 56 ± 1 m.u. (42 region). *Cyp6g1*, on the other hand, is close to the R1 locus, while *Cyp6a8* is distal to R1. It should be noted here that not all over-expressed *Cyp* genes might be involved in the resistance phenotype (Scott, 1999). Some of these genes may be regulated by common regulatory genes. The common regulator may have undergone a mutation during DDT selection and become fixed in the population. This mutation may result in the up-regulation of all the genes that are controlled by the common regulator. A good example of how certain up-regulated *Cyp* genes may not be directly involved in resistance is found in a study by Bride *et al.* (1997) on the role of CYP6A2 in the metabolism of diazinon in five strains of *Drosophila*

melanogaster. In this study, levels of CYP6A2 protein content did not correlate with diazinon resistance levels. The Paquette strain had the highest amounts of CYP6A2, but it only had a 2-fold resistance to diazinon (Bride *et al.*, 1997). Another example is that of CYP6A1 in *Musca*. The mRNA levels of this gene are higher in the LRP than the Rutgers strain, yet Rutgers has higher levels of resistance to diazinon than LRP. Even though both these examples are for diazinon resistance, a similar situation may also be seen in the case of DDT resistance where a resistant strain showing high *Cyp* gene expression may not show the highest level of resistance.

Sexual dimorphism in *Cyp6w1* expression

Cyp genes have been shown to have higher mRNA content in *Drosophila* males than in females, observed for *Cyp6a2*, *Cyp6a8* (Maitra *et al.*, 1996), and *Cyp6g1* (unpublished data) genes. Usually, sexual dimorphism in *Cyp* gene expression is more apparent in resistant strains, especially since the constitutive expression levels in susceptible strains are usually too low to detect any real difference between the two sexes. To examine whether *Cyp6w1* also shows sexual dimorphism, total RNA from females and males of the resistant 91-R strain was isolated and hybridized with ³²P-labeled *Cyp6w1* probe. Figure 20 shows the autoradiogram of the northern blot. As observed, *Cyp6w1* expression is higher in males of 91-R. After data was quantified with a radioanalytical imager and *Cyp6w1* expression in each lane was corrected by the internal control (RP49), it was found that levels of *Cyp6w1* expression in males was

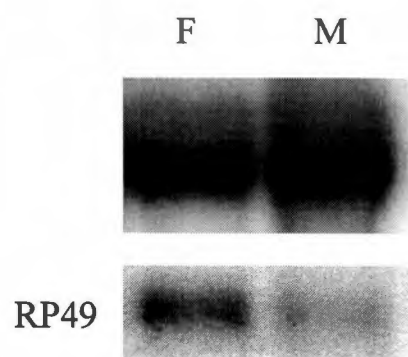


Figure 20- Expression of *Cyp6w1* in females and males of 91-R

Total RNA from female (F) and male (M) flies of 91-C were isolated and electrophoresed on 2.2M-1.2% formaldehyde agarose gel. RNA was transferred onto a nylon membrane and hybridized with ³²P-labeled *Cyp6w1* probe. As an internal control, ³²P-labeled RP49 probe was used to hybridize the bottom portion of the blot.

about 1.6 fold higher than in females (Figure 21). This difference is statistically significant ($p \geq 0.011$) as determined by the Student's t-test.

It is interesting to note that differences seen in male and female expression of *Cyp* genes have been found to negatively correlate with levels of resistance in *Drosophila*. Waters *et al.* (1983) observed that males had a higher total P450 content than females (0.42 and 0.28, respectively), yet females had more DMN-d activity than males (0.8 and 0.59, respectively). While sexual dimorphism is a common phenomenon among *Cyp* genes, the biological significance of these differences in expression is still unknown.

Induction of *Cyp6w1* with barbital

Barbiturate compounds are known to induce various *CYP* genes. They have been extensively used to analyze the molecular mechanisms of *CYP* gene regulation in *Bacillus megaterium*, *Musca*, and *Drosophila* (Liang and Fulco, 1995; He *et al.*, 1995; Dombrowski *et al.*, 1998; Kemper, 1998; Shaw *et al.*, 1998; Scott, 1999; Whitlock, 1999). In the present investigation, I wished to examine whether barbiturate compounds also induce *Cyp6w1* gene. Previously it has been observed that alleles of *Cyp6a2*, *Cyp6a8* and *Cyp6g1* genes showing high level of constitutive expression are refractory to barbiturate induction, whereas alleles that show low constitutive expression are highly inducible (Maitra *et al.*, 1996; Dombrowski *et al.*, 1998; Jenkins and Ganguly, unpublished results). In case of *Cyp6w1*, the alleles of 91-C were used because constitutive expression of *Cyp6w1-91C* is about 3-fold lower than *Cyp6w1-91R* allele. Therefore, adult females of the 91-C strain were allowed to feed on a medium made with

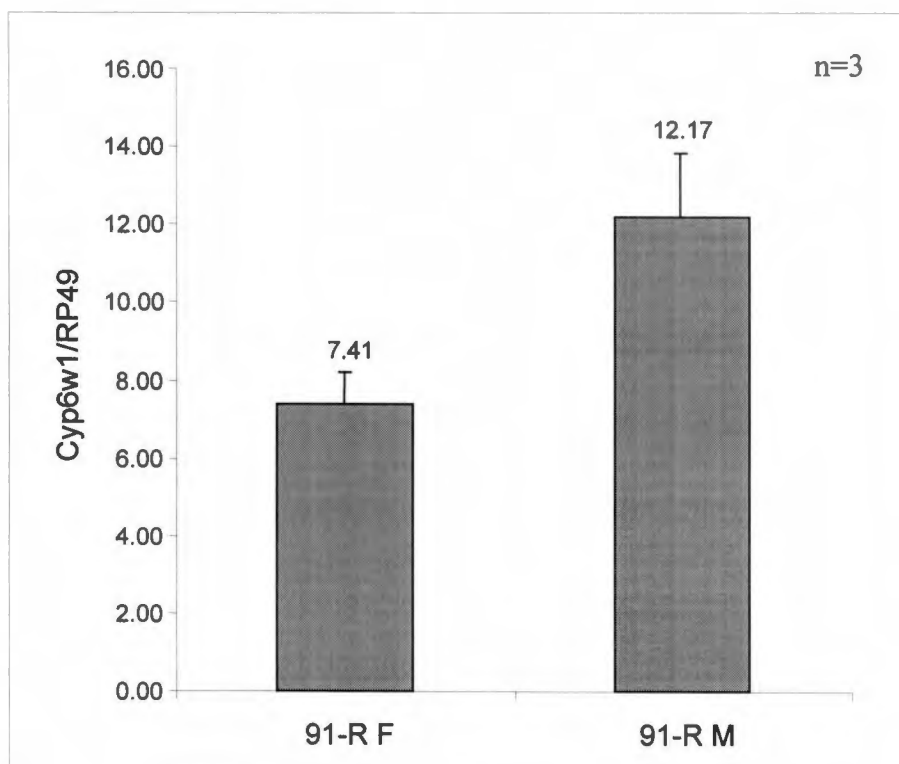


Figure 21- Quantitative analysis of *Cyp6w1* expression in females and males of 91-R

Total RNA from adult female and male flies of 91-R were isolated and analyzed with northern blot as described in Figure 20. Hybridization signals for *Cyp6w1* and RP49 probes were measured with a radioanalytical imager. Bars represent the mean ratios of CYP6W1/RP49 for triplicate northern blots. Males have a 1.6 fold higher expression than females.

16mM barbital solution for 24 hours. Total RNA was isolated from treated and untreated flies, and hybridized with a ^{32}P -labeled *Cyp6w1* probe. Visual scanning of the autoradiogram revealed that *Cyp6w1* was highly induced by barbital (Figure 22). Hybridization signals were quantified using a radioanalytical imager and normalized by using the RP49 signals as an internal control. The results showed that the barbital-treatment caused a 6-fold induction of *Cyp6w1* expression (Figure 23). The difference between means of treated and untreated samples was statistically significant ($p \leq 0.0003$). Since *Cyp6a2* and *Cyp6a8* of *Drosophila* are also highly induced by barbiturate compounds in 91-C (Maitra *et al.*, 1996; Dombrowski *et al.*, 1998), it is possible that these two genes and *Cyp6w1* are regulated by a common regulatory machinery.

Effect of DDT on expression of *Cyp6w1* and *Cyp6a2*

Cytochrome P450 enzymes are known to be involved in the metabolism of various xenobiotic compounds. It is interesting to note that some *CYP* genes are induced by the xenobiotic compounds they metabolize. This is true for *CYP1A1* gene in mammals, and *CYP6B1* and *CYP6B2* genes in *Papilio polyxenes* (Hung *et al.*, 1995, 1997). In the present investigation I examined DDT-inducibility of *Cyp6w1* and *Cyp6a2* genes, although the involvement of their protein products in DDT metabolism is not known. For this purpose, standard medium was coated with 0.1 ml of either 50 $\mu\text{g}/\text{ml}$ or 150 $\mu\text{g}/\text{ml}$ DDT solution made in acetone. The media were dried in a hood overnight, and female flies of 91-C strain were allowed to feed on them for 4.5 and 2 hours, respectively. As a control, some flies were allowed to feed on media coated with acetone

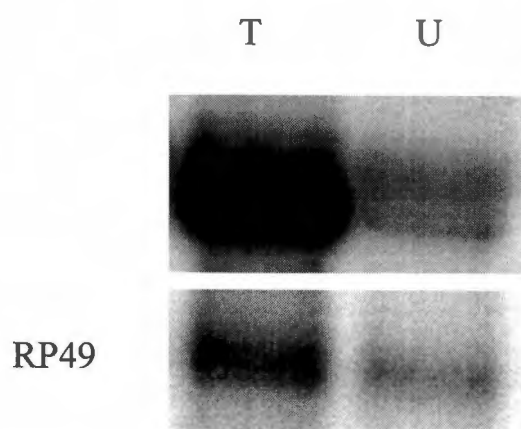


Figure 22- Induction of *Cyp6w1* with barbital

Females flies of 91-C were treated with 16mM barbital for 24 hours. Controls were kept on untreated medium for the same length of time. Total RNA from treated (T) and untreated (U) flies was isolated and electrophoresed on a 2.2M-1.2% formaldehyde agarose gel and transferred onto a nylon membrane. The blot was hybridized with ³²P-labeled *Cyp6w1* probe

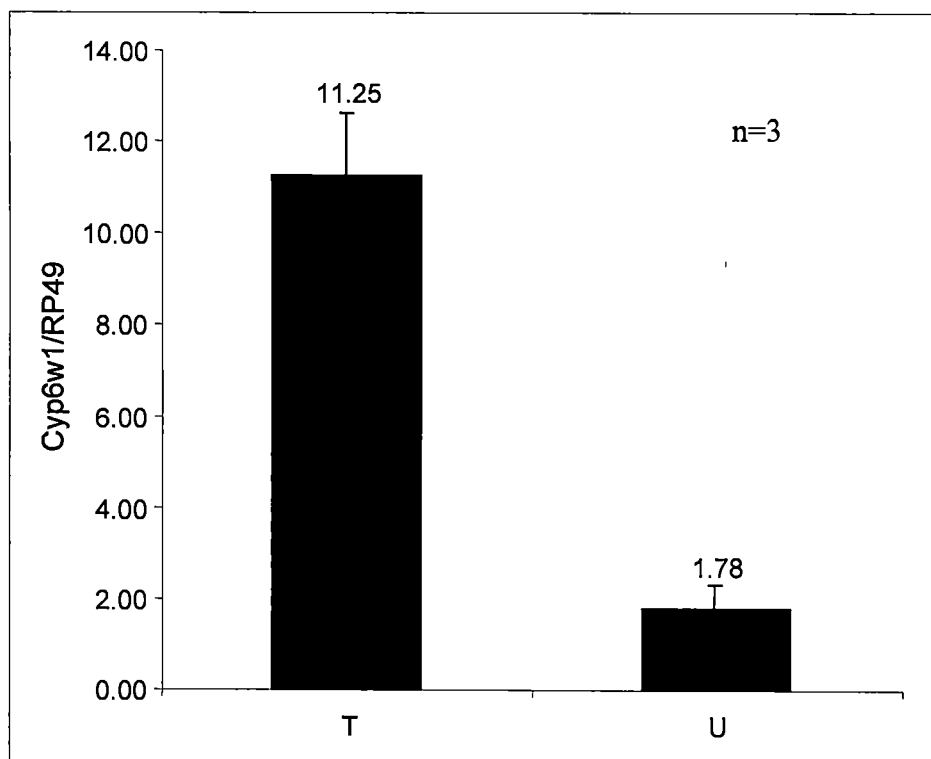


Figure 23- Barbital induction of *Cyp6w1* in 91-C adult females.

RNA from barbital treated (T) and untreated (U) female flies were analyzed by a northern blot as described in Figure 22. Hybridization signals of *Cyp6w1* and RP49 were measured with a radioanalytical imager. Bars represent the ratio of CYP6W1/RP49 from triplicate northern blots, where mean values are indicated on top of each bar. Barbital induces *Cyp6w1* by 6 fold in 91-C.

only. Northern blots of total RNA isolated from these flies were hybridized separately with ^{32}P -labeled *Cyp6w1* and *Cyp6a2* probes. Figure 24 is an autoradiogram of the blots, showing little difference in expression between control and treated flies. Using RP49 mRNA as internal controls, levels of CYP6W1 and CYP6A2 RNA were quantified. It was found that there is no significant difference between control and DDT-treated flies for CYP6W1 (Figure 25) and CYP6A2 (Figure 26) levels.

It is possible that *Cyp6w1* and *Cyp6a2* are inducible by DDT, but the length of treatment here was not enough to induce their expression. The flies were collected as soon as DDT negatively affected their balance and behavior. A chronic treatment with a lower concentration of DDT solution may be more useful. Alternatively, DDT may not induce these two genes, even if the treatment was for a longer time. It is possible that their higher expression in 91-R is due to one more mutations in a common regulator, resulting from DDT selection over time.

The effect of third chromosome on expression of *Cyp6w1*

The molecular mechanisms by which *Cyp* genes are regulated in *D. melanogaster* are not well understood. Studies in bacteria and the housefly showed the involvement of both cis- and trans-regulatory elements. For example, in *Bacillus megaterium* a repressor protein, BM3R1, binds to the upstream sequence of a *CYP* gene, *BM3* (*Cyp102*), and represses its expression (He *et al.*, 1995). In *Musca*, factors on chromosome II seem to negatively influence the expression of *CYP6A1* gene located on chromosome V (Carino *et al.*, 1992). Similarly, Maitra *et al.* (2000) found that expression of the second

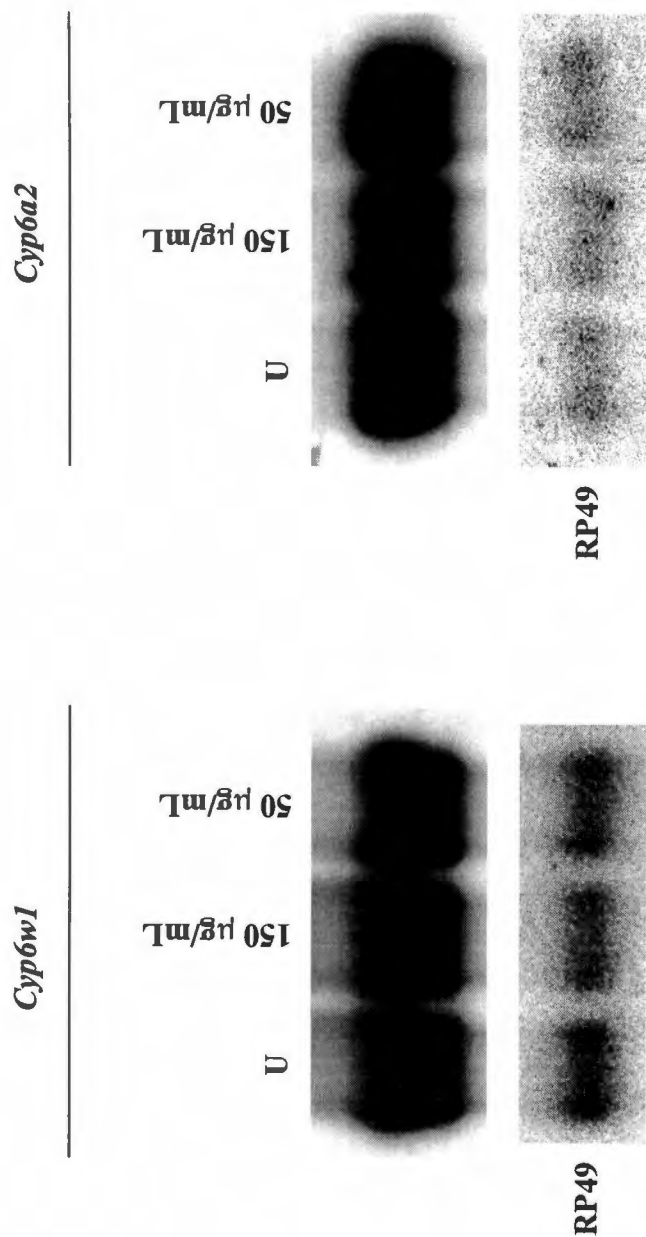


Figure 24- *Cyp6w1* and *Cyp6a2* gene expression in 91-C female flies treated with DDT

Adult female flies of 91-C were treated with either 50 µg/mL or 150 µg/mL of DDT solution made with acetone for 4.5 and 2 hours, respectively. Flies used as a control were kept on medium treated with acetone only for the same length of time. Total RNA was isolated from untreated (U) and DDT-treated flies and electrophoresed on 2.2M-1.2% formaldehyde agarose gel. RNA was transferred onto nylon membranes and hybridized with either ³²P-labeled *Cyp6w1* or *Cyp6a2* probes. To correct for loading errors, ³²P-labeled RP49 probe was hybridized to the bottom portion of each blot.

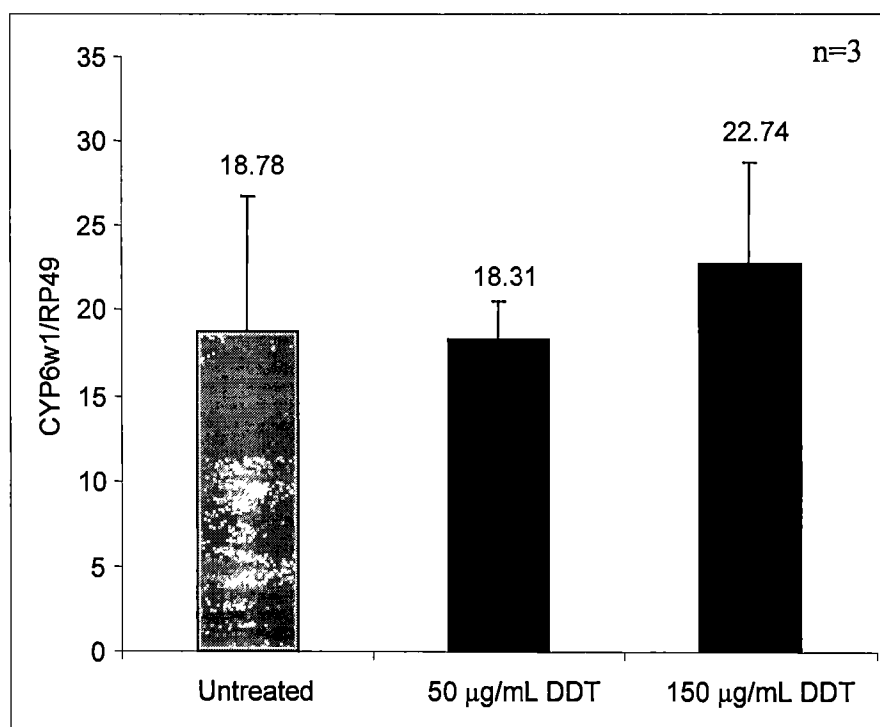


Figure 25- Quantitative analysis of *Cyp6w1* expression in DDT treated and untreated flies of 91-C.

Adult female flies of 91-C were treated with either 50 µg/mL or 150 µg/mL of DDT solution made with acetone. Total RNA from treated and untreated flies were analyzed by northern blot as described in Figure 24. Hybridization signals of *Cyp6w1* and RP49 were measured using a radioanalytical imager. Bars represent the mean ratio of CYP6W1/RP49 from three northern blots. DDT treatment has no significant effect on *Cyp6w1* expression.

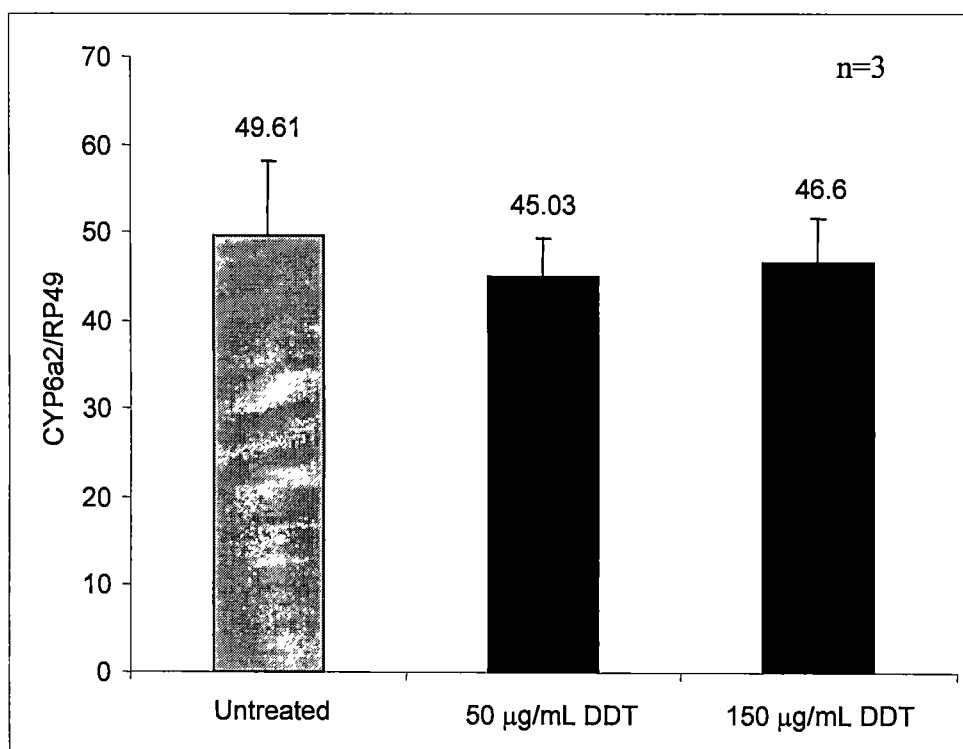


Figure 26- Quantitative analysis of *Cyp6a2* expression in DDT treated and untreated flies of 91-C.

Adult female flies of 91-C were treated with either 50 µg/mL or 150 µg/mL of DDT solution made with acetone. Total RNA from treated and untreated flies were analyzed by northern blot as described in Figure 24. Hybridization signals of *Cyp6a2* and RP49 were measured using a radioanalytical imager. Bars represent the mean ratio of CYP6W1/RP49 from three northern blots. DDT treatment has no significant effect on *Cyp6a2* expression.

chromosome-linked *Cyp6a2* and *Cyp6a8* genes in *Drosophila melanogaster* is influenced by a negative factor or factors on chromosome 3 which seem to down regulate their expression in susceptible strains compared to the resistant 91-R.

To investigate whether the 2nd chromosome-linked *Cyp6w1* is also influenced by the 3rd chromosome, its expression was examined in chromosome substitution stocks carrying various combinations of X, 2nd and 3rd chromosomes of 91-R and 91-C strains. The names of these stocks correspond to the order and source of each of the three chromosomes, where "R" is a chromosome from 91-R and "C" is a chromosome from 91-C. For example, the RRC strain has the X-chromosome from 91-R, the second chromosome from 91-R and the third from 91-C. The source of the fourth chromosome in all stocks is unknown. In this investigation, Northern blot analysis was used to compare constitutive expression of *Cyp6w1* in four chromosome substitution stocks (RRC, CRC, CCR, and RCR). Equal amounts of total RNA from adult females were hybridized with a ³²P labeled *Cyp6w1* probe. Results of the autoradiogram are shown in Figure 27. The hybridization signals were quantified by using a radioanalytical imager.

The results (Figure 28) showed that when the third chromosome of 91-R was substituted with the 3rd chromosome from 91-C (stock RRC), the expression of *Cyp6w1*-91-R allele decreased by about 1.6-fold (Figure 28, RRR vs RRC). Student's t-test showed that the difference between the two means was statistically significant ($p \geq 0.0018$). This suggests that *Cyp6w1*-91R allele is down-regulated by the 3rd chromosome of 91-C. When the X-chromosome of the RRC was substituted with the X chromosome from the 91-C strain, further decrease in *Cyp6w1*-91-R expression was

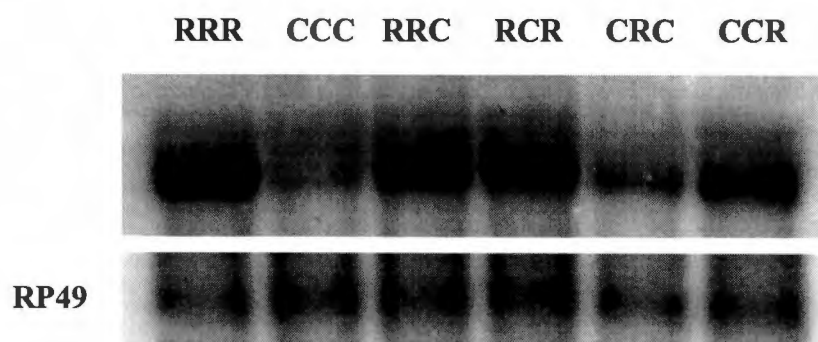


Figure 27- Constitutive expression of *Cyp6w1* in chromosome substitution stocks between 91-R (overproducer) and 91-C (underproducer) strains.

Total RNA from adult females of the above labeled stocks were isolated and electrophoresed on a 2.2M-1.2% formaldehyde agarose gel. RNA was transferred onto a nylon membrane and hybridized with ³²P-labeled *Cyp6w1*. To correct for loading errors, RP49 probe was hybridized to the bottom half of each blot.

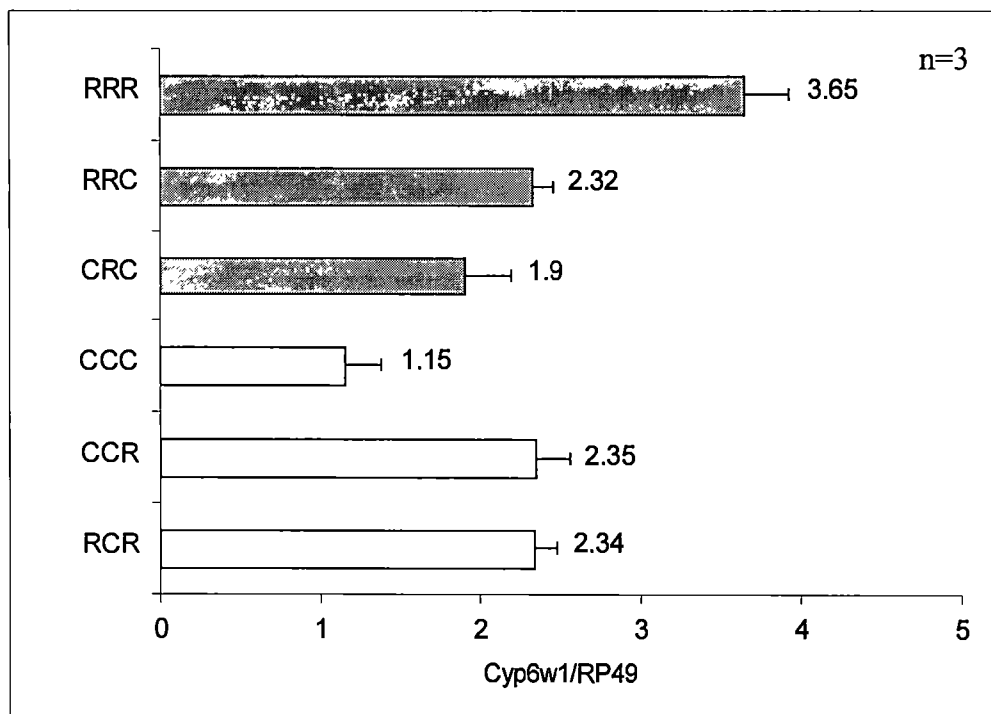


Figure 28- Quantitative measurements of *Cyp6w1* expression in chromosome substitution stocks between 91-R and 91-C strains.

Total RNA from female flies of the above labeled stocks were analyzed by northern blot as described in Figure 27. *Cyp6w1* and RP49 hybridization signals were quantified using a radioanalytical imager. Bars represent the mean ratios of CYP6W1/RP49 for three northern blots, where the mean values are indicated to the right of each bar. The third chromosome from 91-C has an inhibitory effect on *Cyp6w1* expression, whereas a positive effect is seen for chromosome 3 from 91-R.

observed (figure 28, RRC vs CRC). However, this decrease was not statistically significant ($p \leq 0.089$) as determined by the Student's t-test.

Whether the *Cyp6w1-91C* allele is also influenced by the 3rd chromosome was examined in CCR and RCR chromosome substitution stocks. The results showed that when the third chromosome of 91-C was substituted with that of 91-R, the expression of *Cyp6w1-91C* allele was increased by about 2-fold ($p \geq 0.0027$) (Figure 28, CCC vs. CCR). When the X-chromosome of CCR stock was substituted with the X from 91-R, no significant ($p \geq 0.97$) change in *Cyp6w1-91C* expression was observed.

Careful examination of the results in Figure 28 indicates that the second chromosome also has an influence on the expression of *Cyp6w1*. Comparison of the RRR and RCR stocks, which are similar except for their second chromosome, shows that the expression of *Cyp6w1* is about 1.6-fold lower in RCR than in RRR stock. These two stocks are similar except for their second chromosome. This indicates that the second chromosome of 91-C has an inhibitory influence on the expression of *Cyp6w1-91C* allele. In fact, the inhibitory influence observed for the second chromosome is almost equal to that for the third, indicated by the equal expression of *Cyp6w1* in both RRC and RCR (Figure 28). Similarly, the expression of *Cyp6w1* is about 1.6-fold higher in CCC relative CRC. This indicated that the third chromosome of 91-R has a positive effect on the expression of *Cyp6w1-91-R* allele. This positive influence is almost equal to that observed for the third chromosome, inferred by the similar expression of *Cyp6w1* in CCR and CRC stocks (Figure 28). This is especially true because the 1.2-fold difference in *Cyp6w1* expression between CCR and CRC is statistically insignificant.

Taken together, data presented in Figure 28 indicate that the second and third chromosomes of the 91-C strain have a negative influence on the expression of *Cyp6w1*, whereas the X-chromosome has low or no significant influence. On the other hand, the second and third chromosomes of 91-R show the opposite effect, and upregulate the expression of *Cyp6w1*. The X chromosome of 91-R does not have any significant effect. Because 91-C is the wild type strain, it can be concluded that in wild type flies, factors on the second and third chromosomes have an additive inhibitory effect on the expression of *Cyp6w1* gene located on the second chromosome. It can also be proposed that over-expression of *Cyp6w1* in 91-R may be due to mutation(s) in second and third chromosome-linked regulatory factor(s), rather than a mutation in the *cis*-acting elements of *Cyp6w1*. This is especially true because *Cyp6w1-91R* allele located on chromosome 2 of the RRC strain is down-regulated by the 3rd chromosome of 91-C. The pattern of regulation by the third chromosome observed here was previously observed for the 2nd chromosome-linked *Cyp6a2* and *Cyp6a8* genes (Maitra *et al.*, 1996; Dombrowski *et al.*, 1998), suggesting that a common repressor gene(s) located on the 3rd chromosome regulates these three *Cyp6* genes.

IV. SUMMARY AND CONCLUSIONS

The number and identity of genes responsible for insecticide resistance in *Drosophila melanogaster* is unknown. Previous studies gave the impression that resistance is a polygenic trait, not only involving several genes but all 3 major chromosomes (Crow, 1957; King & Somme, 1958; Dapkus & Merrell, 1977). Several expression studies, including the ones herein, have shown that so far there are four chromosome-2 linked *Cyp* genes of *Drosophila* that are overexpressed in the resistant 91-R relative to the susceptible 91-C strain. These genes are *Cyp6a2* (Waters *et al.*, 1992 a, b; Dombrowski *et al.*, 1998), *Cyp6a8* (Maitra *et al.*, 1996, 2001), *Cyp6g1* (Jenkins, 2001), and *Cyp6w1* studied in this investigation. Analysis of chromosome substitution stocks carrying a combination of the three major chromosomes (X, 2nd, and 3rd) of 91-R and 91-C strains showed that three out of the four *Cyp6* genes (*Cyp6a2*, *Cyp6a8* and *Cyp6w1*) are regulated similarly by the third chromosome. These genes seem to be under an inhibitory influence by the third chromosome of the wild type 91-C strain, suggesting the presence of negative *trans*-regulatory factors on the third chromosome that down-regulate their expression. Conversely, the third chromosome of the mutant 91-R strain shows a positive effect on their expression. This suggests that perhaps the putative *trans*-regulatory negative factors on the 3rd chromosome are mutated in the 91-R strain and they can no longer bind to *cis*-elements of the *Cyp6w1*, *Cyp6a2* and *Cyp6a8* genes on the second chromosome.

The results of chromosome substitution analysis in this investigation also show that the second chromosome influences the expression of *Cyp6w1*. Similar to the

inhibitory effect of the third chromosome of 91-C, the second chromosome of 91-C also shows an inhibitory effect on the expression of *Cyp6w1*-91C allele. In fact, quantitative analysis indicates the influence of the second and third chromosomes may be equal. Contrary to the negative effect of the second chromosome of 91-C, the second chromosome of 91-R has a positive effect on the expression of the *Cyp6w1*-91R allele. Similar influence of the second chromosome was also observed for *Cyp6a8*, but not for *Cyp6a2* expression (Maitra, 2000; unpublished data). Based on these observations, it is possible that there are *trans*-regulatory factors on the second chromosome that seem to influence the expression of these three *Cyp* genes. There are two possible models for the nature of regulatory action of such factors. In the first model, these factors may be inhibitory in the wild type (91-C) strain, causing a low constitutive expression. As a result of DDT selection, these factors may have undergone one or more loss-of-function mutations in the 91-R strain, causing an increase in *Cyp* gene expression. Alternatively, in the second model these second chromosome regulatory factors may be positive by nature, but are maintained at a relatively inactive state in the wild type strain. If that is the case, it is possible that induction of *Cyp6w1* by barbiturate compounds (observed in this investigation) occurs through activation of these factors in the wild type strain. This induction may mimic the effect of DDT selection, which may have caused the activation of these positive factors in the mutant strain over time, although no induction of *Cyp6w1*-91C allele by DDT was observed here.

To test the presence and effect of regulatory factors on the second chromosome, certain recombination studies can be used. For example, 91-R can be crossed to a

susceptible strain carrying several second-chromosome linked visible marker genes. Using these marker genes, *Cyp6w1-91R* allele can be separated from the factors on the 91-R 2nd chromosome via recombination. The recombinant progeny that show a low constitutive expression of *Cyp6w1-91R* can be analyzed to determine the second chromosomal region that may have a regulatory influence. One problem with this approach would be the genetic background of the susceptible strain. It would be hard to determine whether the observed low expression is due to the removal of regulatory genes on the second chromosome, or due to other factors in the genome of the susceptible strain affecting *Cyp6w1* expression. To avoid this problem, the recombinants must be made homozygous for the 3rd chromosome of 91-R strain.

Even though the position of the DDT resistance locus, R2, in 91-R strain appears to be close to polytene band 42D, other genetic analysis studies have shown that a major resistance locus (R1) located close to region 48-49 on the polytene map also confers resistance to DDT in resistant strains other than 91-R. *Cyp6g1* is the only *Cyp* gene in the 48-49 region which shows an overexpression in 91-R relative to 91-C. It is possible that DDT resistance in 91-R is conferred by several *Cyp* genes, each contributing a little to the observed phenotype. On that basis, the role of *Cyp6w1*, *Cyp6g1*, *Cyp6a2* and *Cyp6a8* genes in DDT resistance can be assessed by several ways. First, the protein content of these genes has to correlate with the resistance phenotype. Second, the 91-R alleles of these genes can be introduced into the genome of 91-C by a transgenic approach, using a marker other than eye color (e.g. the green fluorescence protein (GFP)). The transgenes can be introduced separately or in a combination (i.e. more than

one transgene at a time), and the level of resistance of transgenic lines to DDT and other insecticides can be tested. The results will indicate which one or more of the four genes are conferring resistance in the 91-R strain. An alternative to the “all for one” hypothesis, it is possible that not all over-expressed *Cyp* genes on the second chromosome are responsible for the resistance phenotype. Since some of these genes seem to be regulated by common regulatory factors on the second and third chromosomes, one or more mutations in these factors as a result of DDT selection may have caused an up-regulation of all *Cyp* genes regulated under their control. It is possible than only one *Cyp* gene is responsible for the phenotype. Future studies directly correlating overexpression of *Cyp* genes to DDT resistance will resolve this issue.

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V. REFERENCES

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