

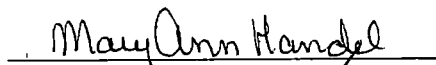
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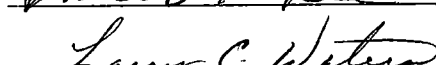
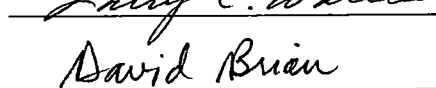
I am submitting herewith a dissertation written by Sushmita Maitra entitled "Molecular and Genetic Analysis of Cytochrome P450 gene regulation in *Drosophila melanogaster*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.



Ranjan Ganguly, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the council:



Associate Vice Chancellor and
Dean of The Graduate School

Molecular and Genetic Analysis of Cytochrome P450 Gene Regulation in
Drosophila melanogaster

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Sushmita Maitra
May 2000

This dissertation is dedicated to my parents

For Baba and Ma

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ABSTRACT

Metabolic detoxification of insecticides is a major factor that causes resistance in insects. Cytochrome P450s (CYPs) play an important role in the process as these detoxifying enzymes are expressed at high levels in resistant insects. However, not much is known about the mechanisms responsible for the high level of *CYP* gene expression in resistant insects. Such knowledge is important in the understanding and development of 'biorational' strategies to control insecticide resistance. This study was initiated to gain an understanding about the mechanisms of *CYP* gene regulation using *Drosophila* as a model organism. Two new clustered *CYP* genes (*Cyp6a8* and *Cyp6a9*) were isolated from *Drosophila*, of these *Cyp6a8* was found to be highly expressed in insecticide resistant strains. Hence, this gene was used as a prototype to study insect *CYP* gene regulation. Analysis of *cis*-acting sequences of *Cyp6a8*, showed the presence of basal promoter elements within 0.2-kb of the upstream DNA. This region also had sequences that could confer the barbiturate inducibility and sexual dimorphism shown by *Cyp6a8*, to a heterologous *luciferase* reporter gene. However, analysis of the upstream region did not reveal sequences that could be responsible for the overexpression of *Cyp6a8* in resistant strains. Chromosome substitution studies, on the other hand, showed that the 3rd chromosome from wild type, susceptible, *CYP*-underproducer strains carry negative *trans*-regulatory loci (locus) that downregulate the 2nd chromosomal *Cyp6a8* allele from resistant, *CYP*-overproducer strains. A similar effect was also observed for *Cyp6a2*, another 2nd chromosomal gene that shows overexpression in resistant strains, implying that the regulatory effects are global, affecting more than one *CYP* gene. The 2nd

chromosome was found to influence the phenobarbital response of *Cyp6a8* and *Cyp6a2* in the same way, implying that common regulatory controls exist that influence both constitutive and inducible expression of at least two *CYP* genes. The *CYP*-regulatory mechanisms indicated in this study were found to be similar to the ones shown for the housefly, implying a conservation of such mechanisms between insect species. This study also generated several molecular reagents that can be used to further dissect the mechanisms of *CYP* gene regulation in insects.

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Chapter 1

General Introduction

"The P450s are remarkable because there are so many of them and they do so many things."

Ronald W. Estabrook, 1996

In: The remarkable P450s: a historical overview of these versatile hemeprotein catalysts
FASEB J. 10, 202-204.

Organisms have an amazing capacity to survive the daily onslaught of biological and chemical insults. This resilience is due to an innate defense mechanism, which involves the well-known immune system and the not so well known battery of detoxifying enzyme systems. These enzymes include esterases, glutathione S-transferases and cytochrome P450s or CYPs. Presence of detoxifying enzymes was inferred even before the discovery of these enzymes, as it was known that drugs and other foreign toxic compounds could be metabolized in the endoplasmic reticulum of the liver. In most cases, this led to the excretion of the xenobiotics as innocuous compounds. However, in case of some compounds like aromatic amines and polycyclic aromatic hydrocarbons such metabolism resulted in biotransformation of the compounds to toxic reactive intermediates. It was not until much later that it was discovered that the enzymes involved in both these processes were cytochrome P450 enzymes or CYPs. These enzymes are versatile as they are involved in several other processes like metabolism of steroids, fatty acids and bile acids; pathogenesis of several diseases including cancer; insect growth, development and reproduction and in production of biocides and even flower color in plants (Estabrook, 1996). These enzymes are thus vital for the survival of organisms as not only are they involved in normal cellular physiology, they also act as molecular guardians in the interaction of the organism with the environment.

Discovery of Cytochrome P450 proteins.

Klingenberg (1958) and Garfinkle (1958) independently, working with liver microsomes observed an unusual absorption band with a peak at 450 nm when microsomes were reduced with carbon monoxide (CO). This CO-binding pigment had an UV absorption maximum in the Soret region at 450 nm. Omura and Sato (1962, 1964a, b) identified it to be a hemoprotein and named this pigment – *Cytochrome P450*. Pioneering work from the Estabrook laboratory showed that a CO-binding pigment from adrenal cortex and liver microsomes could hydroxylate steroids and drugs (Estabrook et al., 1963; Cooper et al., 1965). It was also found that the drug metabolizing activity of liver microsomes could be enhanced by treating rats with phenobarbital or polycyclic hydrocarbons like benzopyrene (Remmer and Merker, 1965; Conney et al., 1967). Correlation of increases in the CO-binding pigment with increased metabolism of drugs and steroids soon established the CYPs as the major detoxifying enzymes in liver microsomes.

In bacteria, these enzymes are soluble and are found in the cytoplasm. In eukaryotes these enzymes are mostly found in the endoplasmic reticulum (or microsomes). They are anchored to the cytoplasmic face of the endoplasmic reticulum by sequences in their N terminal end. Some CYPs are also found in the mitochondria and are present in the inner membrane projecting into the mitochondrial matrix. CYPs are present in all cell types except skeletal muscles and red blood cells (Omura, 1992; Guengerich, 1993a). They are most abundant in the liver and are found in lower amounts in other tissues like lungs, kidneys, nasal epithelium, pancreas, spleen, gastrointestinal tract, that is, organs that are likely to encounter xenobiotic compounds. However, CYPs are also found in the brain, pancreas, adrenal glands, testis, and ovary, implying their role in endogenous metabolism (Schenkman and Greim, 1993).

Taxonomic distribution and nomenclature

The importance of CYPs is indicated by the fact that they are found in all living organisms from bacteria to humans (Nelson et al., 1996; Nelson, 1999). In addition to the widespread taxonomic distribution, CYPs comprise one of the largest families of enzymes in the biotic world. CYPs are classified into families and subfamilies based on amino acid sequence similarities. Generally, all P450 proteins showing greater than 40% identity at the amino acid level are said to belong to the same gene family and proteins showing greater than 55% identity are classified as members of the same subfamily. The genes encoding P450 proteins have the root symbol *CYP* representing 'cytochrome P450'. An Arabic numeral designates the family, followed by a letter indicating the subfamily. A number indicates the individual gene in the subfamily.

Currently the superfamily of *CYP* genes is made up of 70 families and 127 subfamilies (Nelson, 1999). The last update on the total number of *CYP* genes indicated that there were 481 genes and 22 pseudogenes (Nelson et al., 1996). The most recent reports indicate that there are 49 *CYP* genes described in humans, 77 in bacteria, 80 in *C. elegans*, 33 in the slime mold, 186 in *Arabidopsis*. CYP proteins have also been reported from the parasite *Trypanosoma cruzi*. It is predicted that *Arabidopsis* will have about 350 CYP proteins when the entire genome gets sequenced, which is the maximum number described for any species (Nelson, 1999). The most recent reports have indicated that there are 90 P450 genes in *Drosophila*, belonging to 25 families (February, 2000; <http://ag.arizona.edu/p450/INSECTLIST.html>).

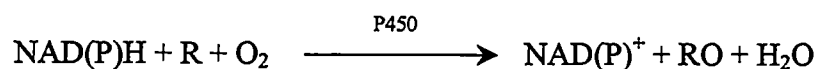
Evolution of CYPs

It is speculated that all members of this superfamily arose from a single ancestral gene about 1.4 – 1.5 billion years ago (Nebert and Gonzales, 1987; Nelson and Strobel, 1987). The gene had been retained by the evolving organisms and radiated along with the

organisms. Evolution of the P450 proteins was not only phylogenetic but also horizontal, within species. This occurred by gene conversion events (Adesnik and Atchison, 1985) and also by gene duplication, subsequent divergence and acquisition of new characteristics (Degtyarenko and Archakov, 1993). It is speculated that the P450 enzyme probably arose in a prokaryotic organism to utilize the increasing amounts of atmospheric oxygen to metabolize carbon resources. Increases in complexity of life forms correlate with the increase in diversity of the P450 phylogenetic tree. There was a rapid increase in life forms at the beginning of the Cambrian era, about 570 million years ago (mya) along with increased levels of oxygen in the atmosphere. This event is thought to coincide with the divergence of xenobiotic metabolizing CYPs from steroid metabolizing ones. The diversification of the CYP2 family is thought to coincide with the colonization of land by animals and the subsequent phase of coevolution between plants and animals about 400 mya (Nebert and Gonzales, 1987). Divergence of CYP1 family into CYP1A1 and CYP1A2 appears to be around the time of mammalian radiation about 200 mya. The branching of CYP2A, B, C, D and E subfamilies into related enzymes in different mammalian species also appeared to occur around this time (Lewis, 1996a). Thus, there appears to be a good correlation between increase in diversity of CYPs and evolutionary radiation of organisms. Presence of *CYP* polymorphism in human population seems to indicate that the CYP family is still evolving (Gonzales and Gelboin, 1991). Over the course of 1400 million years, selection has favored the divergence of CYPs by duplication and subsequent evolution. The result of this process is the presence of a multigene family that can metabolize almost all kinds of chemical compounds that an organism may encounter.

Mechanism of CYP action.

CYPs form the terminal oxidase of an electron transfer chain and despite the diversity of the chemical compounds that CYPs metabolize, the chemistry of metabolism is the same, involving conserved components (Fig. 1-1). The basic chemistry involves splitting of an oxygen molecule into two atoms, of which one is inserted into the substrate and the other is combined with two hydrogen atoms to form water. The overall reaction for substrate oxygenation can be represented as follows where R represents the substrate



In the eukaryotic microsomal system, two proteins are involved in the monooxygenase reaction. One is a CYP protein and the other is a FMN and FAD-containing flavoprotein, called NADPH-dependent cytochrome P450 reductase or cytochrome P450 reductase.

When CYP enzymes are not bound to substrates, the heme iron is in a low-spin ferric state (Fe^{3+}) and the sixth ligand to the heme iron is water. Binding of substrate displaces several water molecules, including the one bound to the heme iron. This brings about a conformational change in the enzyme and a conversion of the spin equilibrium to the high spin form. These changes facilitate CYP interaction with the cytochrome P450 reductase and transfer of the first electron from NADPH to the heme iron. This converts the CYP protein to the ferrous state (Fe^{2+}) and facilitates binding of molecular oxygen to the heme moiety of the reduced P450-substrate complex. In the next step, a second electron from NADPH (or from cytochrome b_5 in some cases) is added to the oxyferrous moiety resulting in a iron peroxy complex. The iron porphyrin system in P450 is thought to transfer electrons to molecular oxygen, which makes the oxygen-oxygen bond progressively weaker, ultimately resulting in breakage of the bond. One atom of oxygen gets inserted in the bound substrate while the other atom reacts with two protons and

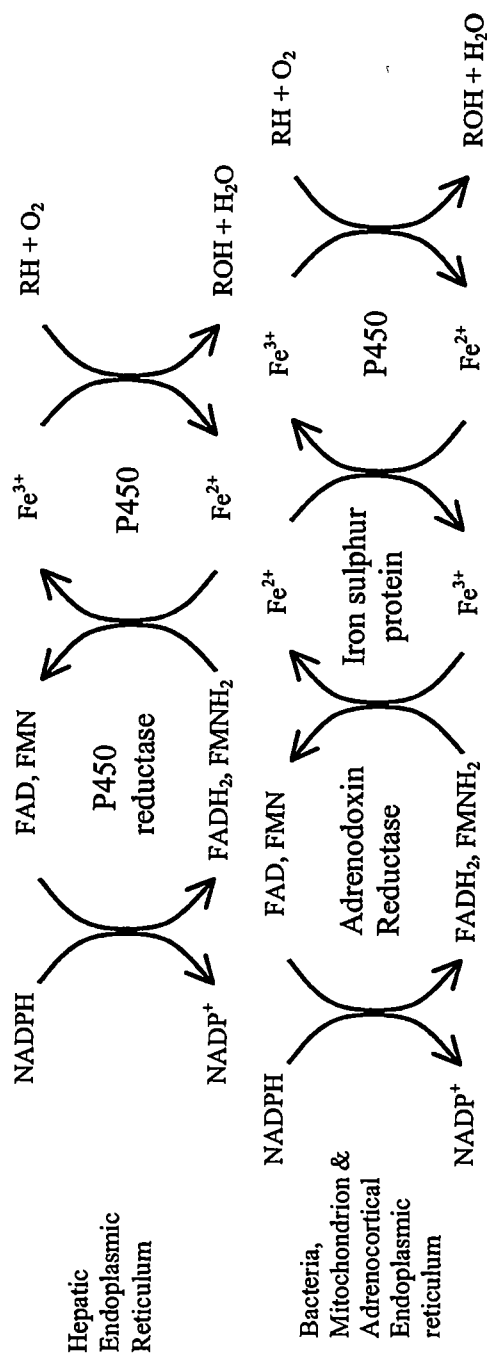


Fig 1-1. Components and electron transport pathways of the P450 system

Cytochrome P450 enzymes form the terminal oxidase in an electron transport chain. Electrons are transferred from NADPH or NADH through a flavine containing reductase to the P450 enzyme as shown for the monooxygenase system in the hepatic endoplasmic reticulum. In the bacterial and mitochondrial systems there is an additional component, an iron-sulphur containing ferredoxin, in between the reductase and the P450 enzyme. Such a monooxygenase system is also found in the adrenocortical endoplasmic reticulum. The P450 enzyme finally transfers the electrons to oxygen, forming water. In the process, the oxygen molecule is split and while one atom is reduced to water, the other atom gets inserted into the P450 substrate. (Adapted from Lewis 1996b).

form one molecule of water (Guengerich, 1996; Lewis, 1996a and references therein) (Fig. 1-2).

Structure of CYPs

CYPs are hemoproteins containing a non-covalently bound heme prosthetic group (iron protoporphyrin IX) and a single polypeptide of about 500 amino acids (apoprotein with molecular weights ranging from 46 to 57 kD as estimated by SDS-PAGE). The apoprotein provides sites for substrate interaction, oxygen binding and heme binding. The heme prosthetic group is the heart of the enzyme, which splits oxygen molecules into atoms with their subsequent insertion into substrates to be oxidized. Thus, the apoproteins provide the broad range of substrate specificity while the basic chemistry of metabolism remains similar in all CYPs.

To date, crystal structures of 6 soluble bacterial CYP proteins have been determined (Graham and Petersen, 1999). Comparison of these structures and correlation with the presence of conserved or invariant residues in P450 proteins have led to the current concept about CYP structure. The shape of the protein is like an asymmetrical triangular prism with helical segments (named A to L) and several β -sheet structures (β 1- β 5). These elements are connected together by random coils and loops. The α -helical segments are concentrated on the right side and comprise about 70% of the molecule whereas the predominantly β -sheet region (about 22%) is on the left side of the molecule (Makino and Shimada, 1993; Graham-Lorence and Petersen, 1996). All P450s appear to have a similar structural fold although the proteins may have less than 20% sequence identity (Graham and Petersen, 1999).

There are several structural motifs that tend to be conserved among the P450 proteins and these are involved in heme binding, formation of an oxygen-binding pocket, and membrane binding region in case of microsomal P450s. The most important of the

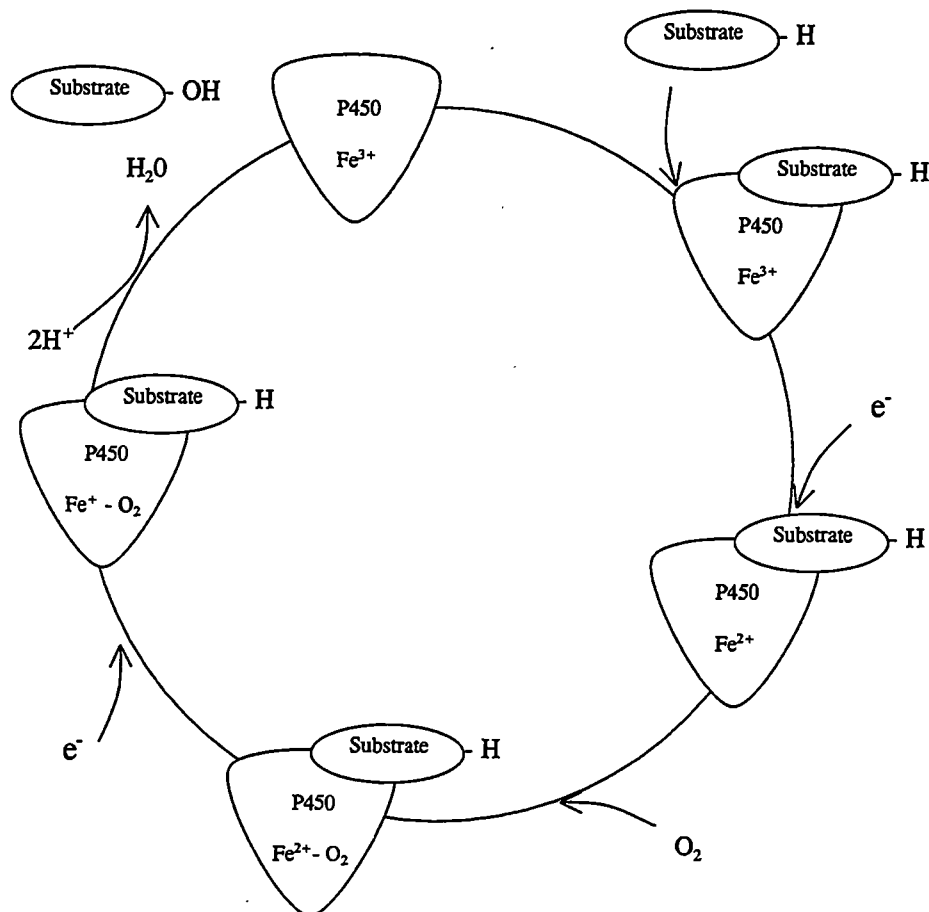


Fig. 1-2: Mechanism for oxygenation of substrates by CYPs

P450 enzymes or monooxygenases (Phase I enzymes) mediate metabolism of complex chemical compounds by inserting a single oxygen atom into the substrate. There are several steps involved in the process which is diagrammed above. The heme moiety of the P450 enzyme is involved in splitting the oxygen molecule, one of which is inserted into the substrate, while the other is reduced to water. The reducing equivalents (2 electrons and 2 protons) are obtained from NADPH or NADH through a flavoprotein known as the reductase in the system. The insertion of the oxygen atom makes the substrates more reactive to the Phase II enzymes. The action of the latter enzymes results in the excretion or bioactivation of the P450 substrates. (Adapted from Lewis 1996a, b and Guengrich, 1993a).

conserved motifs is the 10-residue P450 signature motif – F * * G * R * C * G, found towards the C terminal end. The invariant cysteine residue in this motif has the thiolate (S⁻) group, which forms the fifth ligand of the heme iron. The I helix, which is also highly conserved, forms a part of the heme binding pocket and also has a conserved threonine residue which is the key amino acid responsible for forming the oxygen binding pocket. Another motif is a stretch of 15-20 hydrophobic residues at the N terminal end of the protein that forms a membrane anchor for microsomal CYPs (Makino and Shimada, 1993; Lewis, 1996b). Generally, the C terminal half of the proteins tend to be more conserved than the N terminal half.

The variable regions of the proteins are involved in substrate recognition and redox partner binding. Homology is low in the substrate recognition sequences or SRS (Gotoh, 1992) and variability is also introduced depending on the nature of the redox partners. Class I P450s accept electrons from small iron-sulfur proteins whereas Class II P450s have to accommodate the larger FAD/FMN containing NADPH dependent P450 reductase (Graham-Lorence and Petersen, 1996). However, most microsomal P450s have a conserved tryptophane residue that may have an important function in the transfer of electrons from the reductase to the heme (Lewis, 1996a).

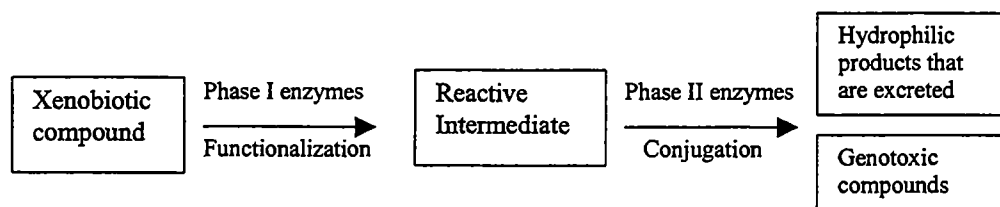
Substrate specificity

Although the highly conserved heme binding region and heme moiety determine the reactions catalyzed by CYPs, substrate specificity is determined by the substrate recognition sequences or SRS (Gotoh, 1992). Only few amino acid residues in the SRS actually determine the substrate specificity. The influence of amino acid residues on activity of the enzyme can be illustrated by the example of CYP2A4 and CYP2A5 in the mouse. The catalytic activity of CYP2A5 (coumarin 7-hydroxylase) gets converted to a testosterone 15 α -hydroxylase (CYP2A4) activity if there is a substitution at a single

position (Phe-209-Leu) (Negishi et al., 1996). Similarly, CYP2B1 has androgen 16 β -hydroxylase activity. Mutation of two residues converts the specificity of CYP2B1 to progesterone 15 α -hydroxylase activity (Negishi et al., 1996). It appears that residues in the substrate-heme pocket that determine substrate specificity may not affect the tertiary structure of the CYPs thus conserving the chemistry of CYP action while allowing for recognition of a large number of substrates.

Diverse metabolic functions of CYPs

Metabolism of exogenous compounds: CYPs metabolize almost all kinds of xenobiotic chemicals that enter an organism through air, water or food it ingests. The list includes anesthetics, alcohol, antioxidants, solvents, dyes, pesticides, procarcinogens and plant products like biocides. It is estimated that over 200,000 chemicals are metabolized by the P450 enzymes (Porter and Coon, 1991). Metabolism of chemical compounds generally comprises of two phases of reaction – Phase I (functionalization) and Phase II (conjugation).



Most of the Phase I enzymes are CYPs which introduce or reveal a functional group like an –OH (hydroxyl) radical in the xenobiotic compounds (Nebert et al., 1996). In most cases this makes the parent metabolites less lipophilic and more reactive. The Phase I metabolites act as substrates for Phase II enzymes like esterases, glutathione S-transferases, *N*-acetyltransferases (Nebert et al., 1996). The latter enzymes conjugate the phase I metabolites to other compounds and make them hydrophilic so that they can be

excreted. A few examples of involvement of CYPs in the metabolism of various compounds are shown in Table 1-1.

Bioactivation and drug metabolism: Metabolism of xenobiotics by CYPs may not always lead to detoxification. In many cases Phase I reactions result in metabolic activation or biotransformation of relatively innocuous compounds into potent mutagens. The reactive Phase I intermediate metabolites can bind to proteins and DNA resulting in mutations. A variety of chemicals like azo dyes, aflatoxin B₁, benzene have to be biotransformed by P450 enzymes before they can become genotoxic. Heterocyclic amines present in charred food and cigarette smoke are not harmful until they are acted upon by CYP1A1 (Guengerich, 1993a). Similarly, benzo(a)pyrene is a procarcinogen which is activated by sequential epoxidation by CYP1A1 and CYP3A to a product that is carcinogenic (Holder et al., 1974; Shimada et al., 1989). Thus, it appears that bioactivation of compounds by P450 proteins is an important process in chemical carcinogenesis. This forms the metabolic basis for the Ames test for testing mutagenic activity of chemicals (Ames, 1975).

Metabolism of endogenous compounds: CYPs can also modify endogenous substrates like steroids, cholesterol, fatty acids, bile acids, vitamins (A and D) and their metabolites. In contrast to the xenobiotic metabolizing CYPs, these forms have narrower substrate specificity. Biosynthesis of cholesterol and its conversion to different metabolites like bile acids, steroid hormones and vitamin D precursors involves hydroxylation reactions catalyzed by specific CYPs (Zimniak and Waxman, 1993). Conversion of cholesterol to steroid hormones is initiated in steroidogenic tissues of the adrenals and gonads by CYP11A and involves subsequent steps catalyzed by CYPs belonging to families 17, 21, 11B and 19. Formation of bile acids involves the actions of CYP7 and CYP27. In case of some endogenous substrates like testosterone, hydroxylation involves the action of CYPs belonging to family 1, 2 and 3 which are primarily involved in metabolism of exogenous

Table 1-1
Some common substrates of CYP enzymes belonging to different families

Enzyme	Organism	Compounds metabolized	Reference
CYP1A1 CYP1A2	Mammals	Planar polyaromatic hydrocarbons	Nedelcheva and Gut, 1984 Kamataki, 1993
CYP2A	Mammals	Aflatoxins and coumarins	Chang and Waxman, 1996
CYP2B	Mammals	Barbiturates, nicotine, DDT, aldrin, dieldrin, parathion	Nims and Lubet, 1996
CYP2D	Mammals	30% of drugs of human consumption	Gonzales, 1996
CYP2E	Mammals	Ethanol, benzene, diethyl ether, acetone	Ronis et al., 1996
CYP3A	Mammals	Erythromycin, cyclosporin, dexamethasone, nifedipine	Maurel, 1996
CYP6A1*	Housefly	Cyclodiene, heptachlor, aldrin	Andersen et al., 1994
CYP6A2*	<i>Drosophila</i>	Aflatoxin, DMBA, Trp-2, diazinon	Saner et al., 1996 Dunkov et al., 1997
CYP6D1*	Housefly	pyrethroids	Tomita et al., 1995
CYP6B1*	<i>Papilio</i>	Plant furanocoumarins	Ma et al., 1994
CYP6B4*	<i>Papilio</i>	Plant furanocoumarins	Hung et al., 1997

Some common substrates are listed in the table. It is to be noted that these chemicals are not the only ones metabolized by the CYP enzymes indicated. The enzymes marked with an asterix are from insects that are resistant to insecticides or plant allelochemicals

compounds. Other reactions involving endogenous substrates include ω oxidation of fatty acids catalyzed by CYP4 enzymes and oxidation of ethanol and other low molecular weight compounds by CYP2E proteins (reviewed in Lewis, 1996a).

Plant-animal interactions: A unique outcome of the catalysis of xenobiotics by CYPs is that such metabolism may allow animals to occupy exclusive ecological niches. A case in point is the metabolism of plant furanocoumarins by CYP6B1. This enzyme is expressed in high amounts in the black swallowtail butterfly (*Papilio polyxenes*), which allows the insect to metabolize xanthotoxins (a linear furanocoumarin) found in plants of the *Apiaceae* (carrot and parsley) and *Rutaceae* (citrus species) family (Ma et al., 1994; Prapaipong et al., 1994; Hung et al., 1995). P450 mediated metabolism of nicotine allows *Manduca sexta* (tobacco hornworm) to feed on tobacco plants (Snyder et al., 1993). It has also been shown that several *Drosophila* species in the Sonoran desert can live on columnar cacti as they are capable of metabolizing the cactus allelochemicals that are toxic to other insects. This capacity has been shown to be due to the detoxification of the allelochemicals present in the cacti, by P450 enzymes (Fogleman et al., 1998). A correlation has also been shown between a high aldrin epoxidase activity which is a P450 dependent reaction and polyphagy in insects (insects feeding on a large number of plant species) (Krieger et al., 1971). It is speculated that the extent and diversity of members belonging to the CYP2 family was a result of the plant-animal warfare which began with the colonization of land by animals in the Devonian era. Animals had to develop enzymes to detoxify plant products which were a part of their diet and in order to protect from being eaten, plants had to develop an arsenal of biocides. This is thought to have led to the rapid radiation of the CYP2 family of detoxifying enzymes (Nebert and Gonzales, 1987).

Regulation of *CYP* activity

The expression of CYP proteins is regulated by a variety of mechanisms, almost as diverse as the CYPs themselves. Expression of CYPs is influenced by developmental stage, tissue, sex, stress and disease conditions, nutritional status and is regulated by a variety of exogenous and endogenous compounds like drugs, chemicals, hormones and cytokines. Most studies have focused on the interaction between *cis*-regulatory elements and *trans*-acting factors (Gonzales et al., 1993) and the effect is generally at the level of transcription. Induction studies have helped understand the mechanisms of P450 gene regulation (Okey, 1990; Denison and Whitlock, 1995; Whitlock, 1999) several of which involve interaction of xenobiotic ligands like benzo(a)pyrene, phenobarbital, clofibrate with their respective receptors (Waxman, 1999).

Induction studies: One of the best understood pathways of *CYP* gene regulation is that for *CYP1A1* as studied in the mammals like the mouse (reviewed in Whitlock, 1999). *CYP1A1* is induced to high levels by polycyclic aromatic hydrocarbons like benzo(a)pyrene and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), whereas the basal constitutive level of expression is low (Denison and Whitlock, 1995). The inducer acts by binding to the aryl hydrocarbon receptor (AhR) which is present in the cytoplasm, bound to heat shock protein 90 (hsp90) and other cytoplasmic proteins. Upon binding the inducer, AhR dissociates from hsp90 and enters the nucleus where it interacts with Arnt (AhR nuclear translocating protein). The AhR-Arnt heterodimer interacts with upstream *cis*-elements (XRE – xenobiotic responsive elements) of the *CYP1A1* gene and activates transcription. The AhR-ARNT complex also activates other genes that have XREs in the upstream DNA. This includes genes like *CYP1A2*, *CYP1B1*, UDP glucuronosyltransferase and glutathione transferase (Nebert and Gonzales, 1987). Such a coordinate regulation would seem to have a selective advantage as both Phase I and Phase II enzymes are induced upon exposure to xenobiotics.

Phenobarbital induction studies have been more elucidative in *Bacillus megaterium*, where *cis*-acting sequences (barbie boxes) and both positive and negative *trans*-acting protein factors have been identified that interact to mediate induction (reviewed in Kemper, 1998).

Constitutive expression: Many of the inducible P450 proteins are also expressed constitutively. Since several CYPs metabolize endogenous compounds, there must be endogenous ligands like cytokines and growth factors to which the genes respond (Morgan et al., 1998). These *CYP* genes have been shown to be regulated by sex-specific, developmental stage specific or tissue specific factors (reviewed in Nebert and Gonzales, 1987). Thus, high expression of *CYP2E1* in the liver has been correlated with the presence of a *cis*-acting HNF-1 α binding element (hepatocyte nuclear factor binding element) (Ueno and Gonzales, 1990). Generally, regulation occurs at the level of transcription but other mechanisms have also been reported like the silencing of *CYP1A1* expression by DNA methylation (Takahashi et al., 1998) and regulation of *CYP1A2* expression by proteins binding to the 3'UTR of the mRNA (Raffalli-Mathieu et al., 1997). Regulation of enzyme activity can also occur at the post translational level by phosphorylation (reviewed in Jansson, 1993).

Polymorphism in CYPs and drug metabolism in humans

It is a well known fact that individuals in a population show different response to drugs. One of the reasons may be attributed to polymorphism in their CYP profile. In the human population, individuals have been shown to have different capacities to metabolize drugs like debrisoquine and other common antidepressant, cardiovascular drugs (debrisoquine panel) due to polymorphisms in *CYP2D6*. 'Poor metabolizers' are homozygous for the loss of function alleles of *CYP2D6*, while 'ultrarapid metabolizers' carry an amplification of *CYP2D6* genes (Meyer, 1994). Similarly, polymorphism in

CYP2C9 and CYP2C10 result in variation in the metabolism of the anticonvulsant drug mephenytoin (Kupfer et al., 1982) and this is transmitted as an autosomal recessive trait (Kupfer and Preisig, 1984). While variation in the nature of the gene itself can bring about such a polymorphism, variation in the levels of P450 protein(s), caused by regulatory mutations or induction by chemicals can also cause a similar effect. In insects, such an effect is manifested in the ability of some individuals in the population to resist the toxic effects of insecticides. (Agosin, 1985).

CYP gene regulation and insecticide metabolism.

One of the mechanisms of insecticide resistance is increased detoxification, which has often been accompanied by elevated levels of P450 enzymes (Agosin, 1985). Functional involvement of CYPs in insecticide resistance has been indicated by the use of P450 inhibitors like piperonyl butoxide (PBO) or sesamex (reviewed in Wilkinson, 1983), where it has been demonstrated that resistant insects can be rendered susceptible by treating them with PBO. Overexpression of several P450 genes has been found in resistant insects. These are *CYP6A1* and *CYP6D1* of *Musca* (housefly) and *Cyp6a2* of *Drosophila* (Feyereisen et al., 1989; Sundseth et al., 1990; Waters et al., 1992; Wheelock and Scott, 1992 a, b). The CYPs encoded by these genes have been shown to metabolize various xenobiotic compounds including insecticides (see Table 1-1). Of these three CYPs, only CYP6D1 has been shown to be specifically involved in resistance to pyrethroid insecticides (Korytko and Scott, 1998). Genetic mapping experiments have identified four loci (R1-R4) in *Drosophila* that are associated with insecticide resistance (Fig. 1-3, Kikkawa, 1953; Ogita, 1958, Houpt et al. 1988, Waters and Nix, 1988; Dapkus, 1992). These loci and another locus (R5) have also been shown to influence the level of P450 enzymes. However, it is not known whether variation in the level of the enzymes is due to mutation(s) in a structural gene or a regulatory gene. In *Musca*, there is evidence

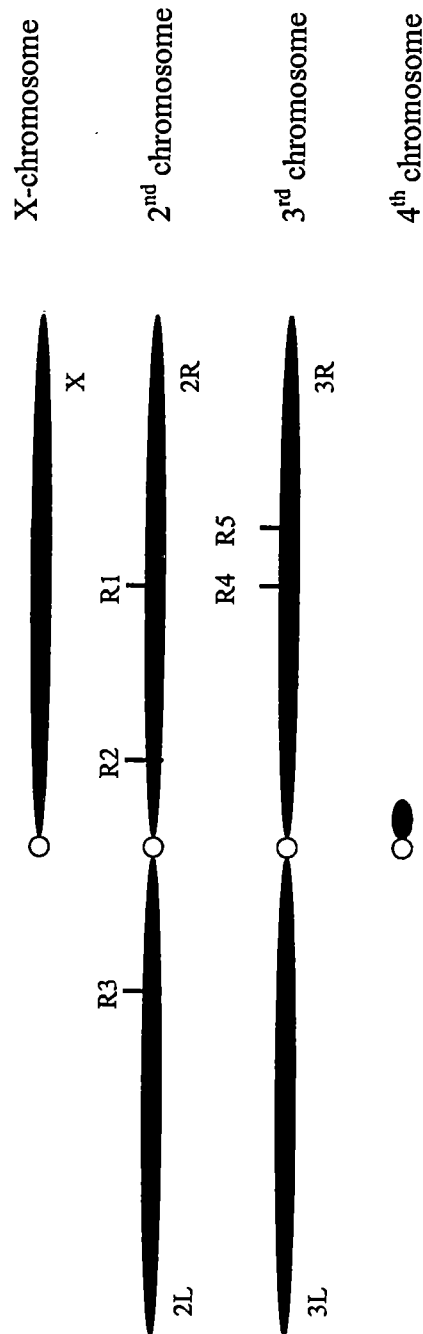


Fig 1-3: Linkage groups of *Drosophila melanogaster*, showing the position of the major loci implicated in insecticide resistance and expression of heme proteins.

Several studies in *Drosophila* have shown that there are some major loci that are associated with insecticide resistance (R1-R4) of which R1 is the major resistance locus (Kikkawa, 1953). All these five loci (R1-R5) have been reported to influence P450 protein levels and/or activity (Ogita, 1958; Waters and Nix, 1988; Houtpt et al., 1988; Dapkus, 1992).

of regulatory gene(s) that act(s) in *trans* and upregulates two *CYP* genes – *CYP6A1* and *CYP6D1* (Carino et al., 1992; Cohen et al., 1994; Feyereisen et al., 1995; Tomita and Scott, 1995; Liu and Scott, 1996). It has been proposed that several detoxifying enzymes (P450 proteins, glutathione S transferase, DDT dehydrochlorinase) are under control of a master regulatory gene on chromosome 2 in the housefly (Plapp, 1984).

Most of the work done on *CYP* gene expression and regulation has been done in mammalian systems and in bacteria. It is only in recent years that several *CYP* genes have been cloned from insects. Some of the CYP6 family genes that have been isolated are shown in Table 1-2. Interest in the mechanism of *CYP* gene regulation in insects is due to the association of CYP activity with development of resistance, involvement of CYPs with metabolic processes in insects like moulting, pheromone synthesis, ecdysteroid and juvenile hormone biosynthesis and fatty acid metabolism (Feyereisen, 1999). However, not much is known about *CYP* gene regulation in insects. Studies have mapped *cis*-regulatory elements involved in expression of the *Papilio* gene *CYP6B1* and the *Drosophila* gene *Cyp6a2* (Prapaipong et al., 1994; Dunkov et al., 1997; Dombrowski, 1998). *Trans*-regulation has been shown to be also involved in *CYP* gene expression by chromosome substitution studies in the housefly (Carino et al., 1994; Liu and Scott, 1996, 1997a).

Use of *Drosophila* as a model system to study *CYP* gene regulation.

Study of regulation of *CYP* gene expression has a direct utility in elucidation of the molecular mechanisms of insecticide resistance. Even though *Drosophila* is not a 'real world' pest, it provides an excellent model system for genetic and molecular resistance studies (Wilson, 1988; French-Constant et al., 1992). Studies have shown that *Drosophila* is similar to *Musca* in terms of overexpression of *CYP* genes in resistant strains. Homologous chromosome and genetic maps between *Drosophila* and other

Table 1-2
Cytochrome P450 genes belonging to different subfamilies of the CYP6 family.

Subfamily	Number of members	Genes	Insect species
6A	18	<i>CYP6A1</i> to <i>Cyp6a17</i>	<i>Musca domestica</i> (housefly) <i>Drosophila melanogaster</i> (fruit fly) <i>Ceratitis capitata</i> (Mediterranean fruit fly)
6B	10	<i>CYP6B1</i> to <i>CYP6B10</i>	<i>Papilio polyxenes</i> (black swallowtail butterfly) <i>Papilio glaucus</i> (butterfly) <i>Helicoverpa armigera</i> (Australian cotton bollworm) <i>Helicoverpa zea</i> <i>Heliothis virescens</i>
6C	2	<i>CYP6C1</i> <i>CYP6C2</i>	<i>Musca domestica</i>
6D	3	<i>CYP6D1</i> to <i>CYP6D3</i>	<i>Musca domestica</i> (housefly) <i>Drosophila melanogaster</i> (fruit fly)
6E	1	<i>CYP6E1</i>	<i>Culex quinquefasciatus</i> (mosquito)
6F	1	<i>CYP6F1</i>	<i>Culex quinquefasciatus</i> (mosquito)
6G	1	<i>Cyp6g1</i>	<i>Drosophila melanogaster</i> (fruit fly)
6H	1	<i>CYP6H1</i>	<i>Locusta migratoria migratorioides</i> (locust)
6J	1	<i>CYP6J1</i>	<i>Blattella germanica</i> (German cockroach)
6K	1	<i>CYP6K1</i>	<i>Blattella germanica</i> (German cockroach)
6L	1	<i>CYP6L1</i>	<i>Blattella germanica</i> (German cockroach)
6M	1	<i>CYP6M1</i>	<i>Anopheles gambiae</i> (mosquito)
6N	1	<i>CYP6N1</i>	<i>Anopheles gambiae</i> (mosquito)
6P	1	<i>CYP6P1</i>	<i>Anopheles gambiae</i> (mosquito)
6Q	1	<i>CYP6Q1</i>	<i>Anopheles gambiae</i> (mosquito)
6R	1	<i>CYP6R1</i>	<i>Anopheles gambiae</i> (mosquito)
6S	1	<i>CYP6S1</i>	<i>Anopheles gambiae</i> (mosquito)

All the cytochrome P450 genes that have been classified as belonging to the CYP6 family have been found to be from insects. The most recent update (December 1999) shows that there are seventeen subfamilies in the CYP6 family with a total of 46 members (<http://drnelson.utmem.edu/P450db.html>).

dipterans (housefly, sheep blowfly) are present (Foster et al., 1981). In addition, *Drosophila* has many advantages as a model system. The genetics of the system is well known, there are a lot of molecular biology and genetic tools available, the transformation system is well established and the *Drosophila* genome sequencing project is almost complete (Spradling and Rubin, 1982; Ashburner, 1989; Lindsey and Zimm, 1992; Berkeley genome project).

Resistance studies using *Drosophila*, both laboratory-based and in the field, have resulted in generation of several strains that show resistance to insecticides (Merrell and Underhill, 1956; Singh and Morton 1981). In one such study, *Drosophila melanogaster* flies were collected from the wild around St. Paul, Minnesota, USA and the population was split into two sub populations. Of the two, one was exposed to DDT and selected for resistance (91-R) while the other was maintained as a control population (91-C). This selection regime resulted in two strains of *Drosophila*, 91-R being highly resistant to DDT and malathion while the control strain (91-C) was susceptible to the two chemicals (Merrell and Underhill, 1956; Dapkus and Merrell, 1977; Sundseth et al., 1989). Like other resistant insects, 91-R was found to have high levels of P450 proteins (Sundseth et al., 1989). *Cyp6a2* was cloned by screening an expression library from 91-R and was found to be overexpressed in the 91-R strain compared to 91-C (Waters et al., 1992). *Cyp6a2* shows polymorphism in the level of expression in different strains of *Drosophila* and was used as a prototype to study regulation of *CYP* gene activity (Dombrowski, 1998).

Objectives of the proposed research

The major focus of the proposed research has been to elucidate the mechanisms of *CYP6* gene regulation in *Drosophila*. The focus on the *CYP6* family is due to the fact that members of this family are not only overexpressed in resistant strains of insects but also

associated with resistance or metabolism of specific insecticides. Thus, CYP6A1 in the housefly has been associated with metabolism of aldrin and heptachlor (Andersen et al., 1994). In *Drosophila*, CYP6A2 has been shown to metabolize aflatoxin B1, DMBA and Trp-2 (Saner et al., 1996). It has also been shown that CYP6A2 can metabolize diazinon, a common insecticide, when expressed in a *Drosophila* cell line (Dunkov et al., 1997). Overexpression of CYP6D1 in the housefly has been associated with pyrethroid resistance (Korytko and Scott, 1998) and CYP6B1 in *Papilio* has been shown to metabolize xanthotoxins (Ma et al., 1995).

Overexpression of *CYP* genes in insects is associated with development of resistance. There is evidence that several *CYP* genes are coordinately overexpressed in resistant insects (Feyereisen, 1999). Though it has been speculated that in the housefly, there is a master regulator gene that controls the expression of several detoxifying enzymes (Plapp, 1984), not much is known about the molecular mechanisms behind *CYP* gene overexpression in resistant insects. *Cyp6a2* shows a high level of expression in 91-R, a strain of *Drosophila* that was selected for resistance to DDT (Waters et al., 1992). This strain also shows cross-resistance to malathion (Sundseth et al., 1989). Could this imply that there are more *CYP* genes that are overexpressed in 91-R, one of which conferred resistance to malathion? In that case the regulatory mechanism(s) responsible for the upregulation of *CYP* genes in 91-R could be similar to that speculated for the housefly, involving a regulator gene(s) that controls the expression of several *CYP* genes.

A proper understanding of regulatory mechanisms involved in overexpression of *CYP* genes in resistant insects is necessary to elucidate the molecular mechanisms behind insecticide resistance. Only then can strategies be devised to control resistance, like the development of 'biorational' insecticides.

The current research was undertaken with the objective to gain an understanding about *CYP* gene regulation in *Drosophila*. For this purpose, we wanted to isolate more

CYP genes belonging to family 6 and study their structure and organization and also expression in several insecticide resistant and susceptible strains of *Drosophila*. The final goal of this study was to use the genes isolated as prototype genes to gain an understanding about the mechanisms that were responsible for the high level of expression of *CYP* genes in resistant strains of insects.

Chapter 2

Isolation, organization and expression of three CYP6 family genes in *Drosophila melanogaster*.

"How many more P450s are there? – Will we approach infinity?"

Ronald W. Estabrook, 1996

In: *Cytochrome P450 – Metabolic and Toxicological Aspects*,
Ed. C. Ioannides, CRC Press Inc, 3-28.

Abstract

Insecticide resistance has been correlated with a high level of cytochrome P450 enzymes especially those belonging to family 6. In *Drosophila*, *Cyp6a2* was found to be highly expressed in the DDT resistant 91-R strain compared to the susceptible 91-C strain (Waters et al., 1992, Proc. Natl. Acad. Sci. USA, 89, 4855-4859). For this purpose, an adult cDNA library was screened under low stringency, with CYP6A2 cDNA as a probe. The screen yielded several cDNA clones of which two were found to encode for CYP6A8 and CYP6A9 as determined by conceptual translation of DNA sequence data. Both these polypeptides contained the heme binding motif which is a signature motif for CYP proteins. Database searches showed that these proteins were most similar to CYP6A2. Isolation of the corresponding genomic clones showed that *Cyp6a8*, *Cyp6a9* and another putative *CYP* gene (*Cyp6a9*-related) are closely clustered within 4 kb and they map to the polytene chromosome band 51C on the second chromosome. *Cyp6a8* is highly expressed in the resistant 91-R strain and produces 2.0- and 1.8-kb transcripts. In the DDT sensitive 91-C strain, expression of this gene is almost undetectable. *Cyp6a8* is also transcriptionally very active in MHIII-D23, another strain of *Drosophila*, where the 3rd chromosome is from a malathion resistant strain. *Cyp6a9* also produces two

transcripts of 2.1-kb and 1.8-kb. While the 2.1-kb transcript is equally expressed in both insecticide resistant and susceptible strains, the 1.8-kb transcript appears to be expressed more in 91-R and MHIII-D23 compared to the susceptible strains. Southern blot analysis showed that gene amplification is not the basis of overexpression of *Cyp6a8* in 91-R. Therefore, it is hypothesized that there may be a regulatory mutation in the *cis*-acting elements of *Cyp6a8* or strain specific *trans*-acting factors that bring about the high level of expression of *Cyp6a8* in 91-R.

Introduction

Insects may become resistant to insecticides through a variety of mechanisms (Oppenoorth, 1985; Feyereisen, 1995). It may be as simple as a behavioral phenomenon, which makes insects avoid insecticides or it may be a result of reduced cuticular penetration of the chemicals. Many chemicals bind to ion channels, disrupt cellular function and kill insects. Mutations may change these target channel proteins such that they may still be functional but cannot bind to the chemicals, thereby bringing about resistance. Of all the mechanisms, however, metabolic detoxification seems to be the most effective and widespread path to resistance. Since CYPs are known to metabolize various kinds of endogenous and xenobiotic compounds, they have the potential to metabolize insecticides and confer resistance. The best supportive evidence for involvement of CYPs in metabolic resistance comes from the observations that resistant insects could be rendered susceptible when they are treated with inhibitors of CYPs such as piperonyl butoxide (PBO) and sesamex (reviewed in Wilkinson, 1983). In addition, a positive correlation between insecticide resistance and high levels of CYP enzymes has been documented in insects for a long time (Hodgson, 1985; Agosin, 1985; Cohen et al., 1992).

Typically, in most insects, resistant strains have higher amounts of one or more CYP protein than their susceptible counterparts (Agosin, 1985; Oppenoorth, 1985). Higher levels of CYP6A1 and CYP6D1 are found in the insecticide resistant Rutgers and Learn-Pyr strains of housefly, respectively, compared to their susceptible counterparts (Carino et al., 1992, 1994; Scott et al., 1996). In *Drosophila*, the level of CYP6A2 is higher in the DDT resistant 91-R strain than in the susceptible 91-C strain (Waters et al., 1992). Overexpression of several *CYP* genes have also been reported in black swallow tail butterfly (*Papilio polyxenes*) and *Drosophila* that feed on plants producing toxic allelochemicals (Prapaipong et al., 1994; Hung et al., 1995; Danielson et al., 1997, 1998). In all these cases, increased amounts of CYPs in the resistant insects are accompanied with increased steady-state levels of the respective CYP mRNAs, suggesting that metabolic resistance may be mediated at the level of transcription of *CYP* genes.

Knowledge about mechanisms leading to the high expression of detoxifying enzymes has obvious implications in designing strategies to control resistance in insects. However, given the background that CYPs are a superfamily of enzymes, the question was which CYP or CYP family would be appropriate to study. Fortunately, at the time when this investigation began it became clear that CYPs belonging to family 6 is found only in insects and that two of them, *CYP6A1* and *Cyp6a2* were expressed at high levels in resistant insects (Feyereisen et al., 1989; Waters et al., 1992). Therefore, it was rationalized that family 6 enzymes could be involved in resistance to various insecticides. Another hypothesis was also in place at the time when the present investigation was initiated. This hypothesis proposed that the second chromosome of *Drosophila* has a major resistance locus that confers resistance to various insecticides (Kikkawa, 1953; Ogita, 1958; Tsukamoto, 1958; Houpt et al., 1988; Waters and Nix, 1988). Increased CYP activities were also associated with this locus (Hallstrom, 1985; Hallstrom and Blanc, 1985; Houpt et al., 1988). At that time, Waters et al. (1992) had cloned the

Cyp6a2 gene from *Drosophila* and shown that the expression of this gene is higher in the DDT resistant 91-R strain than in the susceptible 91-C strain. All these observations led to the hypothesis that CYPs belonging to family 6 may be involved in insecticide resistance, and also that genes encoding these enzymes could be regulated by a common mechanism at the transcriptional level. To gain an understanding behind mechanisms of regulation of *CYP* genes that may be involved in resistance it became necessary to isolate more *CYP* genes belonging to family 6 of *Drosophila* and examine whether these genes are under the influence of a common regulatory mechanism.

With this objective in mind, Ganguly et al. (unpublished observations) hybridized a *Cyp6a2* gene probe to *Eco*R1 digested genomic DNA from the 91-R strain under conditions of reduced stringency. Besides the *Cyp6a2* gene fragments, many other genomic DNA fragments were found to hybridize to the probe, implying the presence of genes similar to *Cyp6a2* in the *Drosophila* genome. Therefore, a cDNA library made from 91-R adults (Waters et al., 1992) was also screened under similar hybridization conditions using *Cyp6a2* cDNA as a probe. Seventeen clones that hybridized faintly to the probe and thought to contain *Cyp6a2*-related cDNAs were picked for further characterization (Ganguly et al., unpublished observation). The present investigation was initiated to characterize these 17 *Cyp6a2*-related cDNA clones with the hope to discover new CYP6 family genes and to study their pattern of expression in various insecticide resistant and susceptible strains of *Drosophila*. At the time when this investigation began, *Cyp6a2* (Waters et al., 1992) was the only known family 6 gene of *Drosophila* that was studied in relation to insecticide resistance. It was not known whether *Drosophila* has other family 6 genes and whether they are also expressed at high levels in resistant strains. It was also not known how these family 6 genes are organized in the genome and whether they are regulated by a common mechanism. Therefore, the 17 isolated, putative *Cyp6a2*-related cDNA clones were the ideal materials to investigate these issues.

Materials and Methods

Drosophila stocks and culture conditions

Flies were raised on standard cornmeal-agar-molasses medium at 23°C and 70% humidity. The insecticide resistant 91-R and susceptible 91-C strains used in the present investigation were derived from a common large collection of wild flies captured in St. Paul, Minnesota, in 1952 (Merrell and Underhill, 1956). The original population was split into two parts of which one part (91-R) was subjected to DDT selection and the other part (91-C) was maintained on normal medium (Merrel and Underhill, 1956). DDT selection of the 91-R strain continued for another 20 years (Dapkus and Merrell, 1977). The 91-R strain that has been used in the present investigation was obtained from Larry C. Waters (Oak Ridge National Laboratory). Although this strain has not been under DDT selection since 1985 (L. C. Waters, personal communication), it still shows resistance to malathion and DDT (Sundseth et al., 1989; and Ganguly et al., unpublished observations). MHIII-D5 and MHIII-D23 strains were kindly provided by R. A. Morton (McMaster University, Canada). These strains are homozygous, respectively, for the 2nd and 3rd chromosomes from a malathion resistant strain called MH-110 (Halpern and Morton, 1987). Insecticide susceptible Oregon R, Canton S and *ry*⁵⁰⁶ strains were obtained from Larry Waters (Oak Ridge National Laboratory, Oak Ridge, TN) and John Lucchesi (Emory University, Atlanta, GA).

Screening of genomic DNA library

Genomic library of adult flies of the 91-R strain made in λ GEM-11 phages (Waters et al., 1992) was kindly provided by Dr. Larry Waters (Oak Ridge National Laboratory). Phages were allowed to infect LE392 bacterial cells (Gibco BRL) and plated on LB-agar plates. Plaque lifts were done according to the method of Benton and Davis

(1977) and hybridized with appropriate ^{32}P -labeled DNA probes at 68°C in 6X SSC, 5X Denhardt's solution, 0.1 mg/ml sonicated herring sperm DNA and 0.1% SDS for 18 hours. Blots were then washed under high stringent conditions at 60°C in 0.1 X SSC and 0.1% SDS. Clones that hybridized with the probe were picked and the phages were dispersed in 1 ml SM (50mM Tris-HCl, pH 7.5, 100mM NaCl, 8mM MgSO_4 and 0.01% gelatin). Three more rounds of screening were done to make the clones plaque pure.

Plasmid and phage DNA isolation

Plasmid DNA was isolated using the alkali lysis method (Maniatis et al., 1982). Bacteria carrying recombinant plasmid were grown overnight in appropriate amounts of LB (Luria broth) medium (1% Tryptone, 0.5% yeast extract, 1% NaCl, 0.2% MgSO_4 and 1% Casamino acid) containing 50 $\mu\text{g/ml}$ ampicillin. Cells were pelleted by centrifugation at $1800 \times g$, resuspended in solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 7.5) and incubated at room temperature for 5 minutes. Two volumes of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, mixed gently and incubated in ice for 5 minutes. One and half volume of solution III (made by titrating 14.25 ml glacial acetic acid with 10 N KOH to pH 4.8 and adjusting the volume to 50 ml with water) was added and mixed by inverting three time and incubated in ice for another 5 minutes. Cellular debris was removed by centrifuging at $15000 \times g$. The supernatant was filtered through four layers of cheesecloth and nucleic acids were precipitated by adding equal volume of 95% ethanol at room temperature. Nucleic acids were pelleted by centrifugation, resuspended in 1 X TE (10 mM Tris, 1 mM EDTA, pH 7.5) and mixed with half volume of 7.5 M ammonium acetate. After incubating in ice for 15 min, high molecular weight RNAs were pelleted by centrifugation at $15000 \times g$. Two volumes of chilled 95% ethanol was added to the supernatant, mixed well and incubated at -20°C overnight to precipitate DNA. DNA was pelleted by centrifugation, dried and

resuspended in an appropriate volume of 1X TE. Phage DNA was isolated by using a plate lysate method (Helms et al. 1982). Overnight culture (0.1 ml) of *E. coli* were infected with 5×10^5 recombinant λ phages and plated on LD agar (5% tryptone, 2.5% yeast extract, 2.5% NaCl, 1.5% bactoagar). Phages were allowed to grow at 37°C until confluent lysis was reached. The lysed bacteria were then overlaid with 6 ml of ice-cold lambda diluent (10mM Tris pH 8.0, 2mM MgCl₂) and incubated overnight at 4°C. The diluent containing the phages was collected from each plate and passed individually through 2 ml DE52 cellulose columns equilibrated with 10mM Tris pH 8.0. The columns were washed with 3-5 ml chase buffer (10mM Tris pH 8.0, 10mM magnesium acetate, 60mM sodium acetate). The phages were then eluted from each column with 0.9 ml elution buffer containing high magnesium (10mM Tris pH 8.0, 50mM magnesium acetate). The protein coat of the phages were removed by incubating the eluted phages for 5 minutes at room temperature in a mixture of 0.8mg/ml proteinase K and 0.4% SDS. Potassium acetate was added to the lysate to a final concentration of 1 M and the mixture was incubated at 88°C for 15 minutes. After cooling in ice for 15 minutes, debris was removed by centrifugation at 15,000 x g. Two and half volume of chilled 95% ethanol was added to the supernatant and DNA was precipitated overnight at -20°C. Subsequently, the DNA was pelleted, dried and resuspended in 1X TE.

RNA and DNA Isolation from *Drosophila*

Total RNA was isolated by the hot phenol method as described by Jowett (1986). Unsexed adult flies were used in the present investigation. The isolated RNA was fractionated through oligo(dT)-cellulose chromatography to obtain the poly A⁺ RNA (Maniatis et al., 1982).

Genomic DNA was isolated from unsexed flies as described by Ganguly et al., 1985. Flies were homogenized in ice cold nuclear isolation buffer containing 50mM Tris-

HCl, pH 7.6, 25mM KCl, 5mM magnesium acetate and 350mM sucrose. The homogenate was centrifuged at 10,000 x g and the resulting nuclear pellet was suspended in 1X DEB (DNA extraction buffer) containing 100mM Tris-HCl, pH 8.0, 150 mM NaCl, 10mM EDTA. Nuclei were then lysed by adjusting the suspension to 0.5 M sodium perchlorate and 1% SDS. The lysate extracted twice with DEB-saturated phenol and twice with chloroform. The aqueous phase was collected and mixed with one-tenth volume of 2M sodium acetate (pH 7.5). Two volumes of chilled 95% ethanol was gently layered on the aqueous phase and DNA was spooled onto a glass capillary tube. After air drying DNA was dissolved in 1X TE.

Southern blot hybridization

DNA samples were digested with desired restriction enzymes and electrophoresed on a 0.8% agarose gel made in 1X TBE buffer (90mM Tris, pH 8.0, 90 mM boric acid and 2mM EDTA). After electrophoresis the gel was incubated for an hour in denaturing solution containing 0.5N NaOH and 1.5M NaCl and 45 min (twice) in neutralizing solution containing 3M NaCl, 0.5M Tris-HCl, pH 7.5. DNA was blotted onto a nylon membrane (Amersham) using 6X SSC (1X SSC is 150mM NaCl, 15mM sodium citrate) as the transfer buffer. The DNA fragments were then UV crosslinked to the nylon membrane using an UV crosslinker (Stratalinker[®] UV Crosslinker Model 1800, Stratagene) and baked under vacuum for 3-4 hours at 80°C.

The blots were prehybridized for 2 hrs at 68°C in a solution containing 6X SSC, 0.1% SDS, 0.1 mg/ml sonicated herring sperm DNA and 5X Denhardt's solution (1X Denhardt's solution is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine serum albumin – fraction V). Before hybridization the prehybridization solution was replaced with fresh solution containing ³²P-labeled DNA probes and hybridization was done for 16-18 hours at 68°C (high stringency) or 51°C (low stringency). After hybridization, the

blots were washed in two changes of 0.1X SSC, 0.1% SDS at 60°C (high stringency) or 0.5X SSC, 0.1% SDS at 54°C (low stringency) followed by two washes in 0.1X SSC at the respective temperatures. Blots were dried and exposed to X-ray films (Kodak XOMAT) to visualize hybridization signals.

Northern blot hybridization

2.5 to 3.5 µg poly A⁺ RNA, isolated from different strains of *D. melanogaster*, was size fractionated through a 1.2% agarose - 2.2M formaldehyde - MOPS denaturing gel (MOPS - 3-N-Morpholino propanesulfonic acid). Before loading, the RNA samples were denatured in 0.5X northern buffer (20mM MOPS, 5mM sodium acetate, 0.5mM EDTA, pH 7.0) by heating at 68°C for 10 minutes. RNA transfer was set up in 20X SSC and was blotted onto nylon membranes (Hybond-N, Amersham, England) by capillary action for 16-18 hours. The blots were crosslinked (Stratalinker[®] UV Crosslinker Model 1800, Stratagene) and baked under vacuum for 3-4 hours at 80°C. To check the integrity of the RNA samples, the ribosomal bands were visualized on the blot by UV shadowing using long wavelength UV light before baking.

Blots were prehybridized for 2 hours at 37°C in a buffer containing 50% formamide, 5X Denhardt's solution, 0.1M PIPES (Piperazine-N-N'-bis 2-ethanesulphonic acid) pH 6.7, 0.8M NaCl, 0.1% SDS and 0.1mg/ml sonicated, denatured, herring sperm DNA. Hybridization with specific probes was carried out for 18-20 hours at 37°C in fresh buffer with the same composition as the prehybridization buffer. After hybridization the blots were washed in four changes of a buffer containing 2X SSC, 0.05% SDS and 0.05% sodium pyrophosphate at room temperature followed by two high stringency washes at 60°C with 0.2X SSC, 0.05% SDS and 0.05% sodium pyrophosphate. The blots were exposed to X-ray films to visualize hybridization signals.

Radiolabeling of DNA fragments

Appropriate cDNA fragments were labeled by random priming to use as a probe for *CYP* gene activity. The 0.3-kb and 1.0-kb *Eco*R1 cDNA fragments were used to study the expression of the gene encoding the AF4 cDNA and the 0.8-kb *Eco*R1 cDNA fragment was used to study the expression of the gene encoding the AF5 cDNA (diagrammed on page 48). Gel purified cDNA fragments were denatured and the labeling reactions were carried out at 25°C using a random priming buffer with random hexamers (Promega), 2.5 mg/ml BSA, 0.5 mM each of dATP, dTTP and dGTP, 50µCi ³²P-αdCTP and 5 units of Klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by passing the reaction mix over a G50 Sephadex column (Pharmacia). Specific activity of such random primed labeled probes ranged from 10⁸ – 10⁹ cpm/µg of DNA. Alcohol dehydrogenase (*Adh*) (Ganguly, et al., 1985; Krishnan et al., 1991) or ribosomal protein 49 (RP49) (O'Connell and Rosbash, 1984) was used as an internal control to correct for loading errors. These cDNA fragments were labeled by nick translation using ³²P-αdCTP and commercially available kits (Gibco-BRL).

***In situ* hybridization to polytene chromosomes.**

In situ hybridization of DNA probes to the polytene chromosomes was done as described (Ashburner, 1989). Briefly, polytene chromosome spreads were made by squashing salivary glands of late third instar larvae of the 91-R strain in 45% acetic acid. The chromosome preparations were kept overnight at 4°C in an acetic acid vapor chamber. Next day, the coverslips were removed, and the chromosomes were fixed in methanol: acetic acid (3:1) and dehydrated by passing through 70% and 95% ethanol. The chromosomes were then alkali denatured, acetylated and hybridized overnight with biotinylated DNA probes at 53°C. Biotinylation of the DNA fragments was done by using a nick translation kit (Gibco-BRL). After hybridization the chromosomes were

processed as described (Ashburner, 1989) and the biotinylated probes were visualized by using a streptavidin-alkaline phosphatase detection kit (Gibco-BRL).

DNA sequencing

DNA fragments to be sequenced were sub-cloned into pBSKII⁺ plasmid (Stratagene, CA). Recombinant plasmids were isolated by the alkali lysis method as described earlier. The insert DNA was sequenced using the dideoxy chain termination method (Sanger et al., 1977) with a commercially available DNA sequencing kit (Sequenase II, United States Biochemicals) and the manufacturer's protocol. Both universal and gene-specific oligonucleotide primers were used for sequencing. DNA sequences were analyzed by the GCG software package (Genetics Computer Group, University of Wisconsin). cDNA sequences were conceptually translated to get the protein products which were then compared with each other and CYP6A2. Programs used to analyze the DNA sequences included the MAP, TRANSLATE, BESTFIT and PILEUP programs of the GCG software package. The protein sequences were submitted to the Cytochrome P450 nomenclature committee for classification of the genes.

Results and Discussion

Isolation of new *Cyp6a2*-related cDNA clones

Initial screening of the 91-R adult cDNA library made in λ gt11 phage yielded seventeen clones that hybridized faintly with a CYP6A2 cDNA probe under low stringent conditions (Ganguly et al., unpublished observations). Seven of these clones (cAF4, cAF5, cAF6-1, cBS2, cBF6-1, cBF6-2, cCF3) were randomly chosen and analyzed by Southern blot hybridization to examine the size of the cDNA insert, and also to see whether the intensity of hybridization, as seen during library screening, is also maintained

on Southern blots. In the λ gt11 vector, cDNA fragments are cloned into an unique *Eco*RI site using synthetic *Eco*RI linker. Therefore, to determine the size of the cDNA inserts and whether they have any internal *Eco*RI site, DNA isolated from each cDNA clone was digested with *Eco*RI and Southern blotted. The resulting blot was hybridized to ^{32} P-labeled CYP6A2 cDNA under low stringent conditions. Fig. 2-1 shows that the clones cAF6-1 and cBF6-1 failed to hybridize with the probe. Thus, either these clones had generated false-positive signals during library screening or they had shorter than 0.1-kb cDNA inserts that ran off the gel during electrophoresis. These clones were not examined further.

Clones cBS2 and cCF3 appears to be two independent isolates of the same cDNA clone because both had a 0.2-kb cDNA insert fragment which hybridized to the probe with a similar intensity (Fig. 2-1, lanes 4 and 7). In addition, hybridization intensities of these two cDNA clones were almost similar to the signal generated by homologous hybridization of the CYP6A2 cDNA probe (data not shown), suggesting that cBS2 and cCF3 were probably cDNA clones of *Cyp6a2* gene. Because of this possibility and small inserts, these two clones were eliminated from further analysis.

Ethidium bromide staining of agarose gels had shown that *Eco*RI digestion of the remaining three clones, cAF4, cAF5 and cBF6-2, produced more than one DNA fragment (data not shown). This suggested that these cDNA inserts had internal *Eco*RI site(s). *Eco*RI digestion of clone cAF4 produced 1.0-, 0.3- and 0.2-kb fragments. However, on the Southern blot shown in Fig 2-1 only the 1.0-kb fragment hybridized with the CYP6A2 cDNA probe. Clone cAF5 produced 0.8- and 0.7-kb fragments of which the 0.8-kb fragment hybridized with the probe (Fig. 2-1). These results suggest that the 1.0-kb and 0.8-kb fragments of cAF4 and cAF5 clones have sequences that are more homologous to the CYP6A2 cDNA probe than the other fragments that did not hybridize with the probe. In case of clone cBF6-2, *Eco*RI digestion produced 0.6- and 0.3-kb

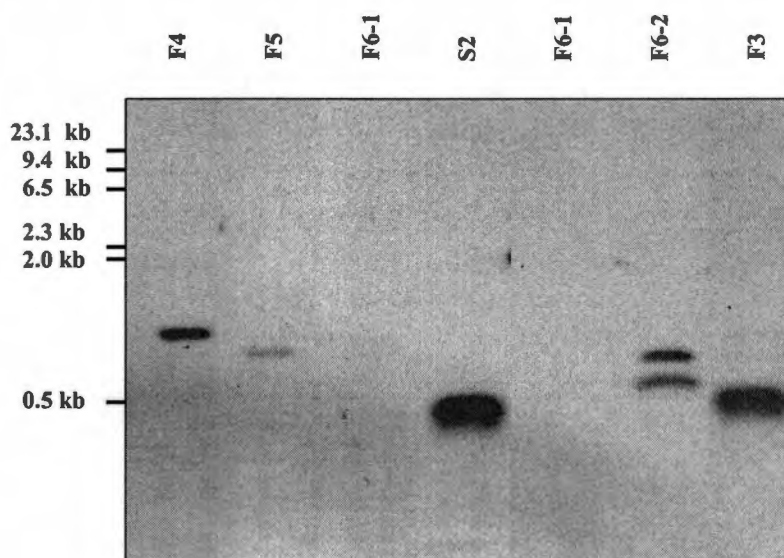


Figure 2-1: Southern blot analysis of *Cyp6a2*-related cDNA clones.

cDNA clones (AF4, AF5, AF6-1, BS2, BF6-1, BF6-2, CF3) were isolated by screening a cDNA library made from adult flies (Waters et al., 1992), using *Cyp6a2* cDNA as a probe under reduced stringent conditions. DNA was isolated from these λ clones and digested with *EcoRI*. The digests were fractionated through a 0.8% TBE-agarose gel and blotted onto nylon membrane. The blot was probed with a cDNA fragment of *Cyp6a2* under the same conditions used for library screening. Molecular weight markers (*HindIII* cut λ DNA) are shown on the left.

fragments. Sequences homologous to CYP6A2 cDNA probes seem to be present in these two *EcoRI* fragments because both fragments hybridized with the CYP6A2 cDNA probe (Fig. 2-1).

To further verify the internal *EcoRI* sites, the cDNA inserts in cAF4, cAF5 and cBF6-2 lambda clones were amplified by PCR. The unique *EcoRI* cloning site in λ gt11 vector is flanked by sequences that are complementary to universal primers. Therefore, these primers were used for PCR amplification. The resulting PCR fragments obtained from the three cDNA clones were digested with *EcoRI* and electrophoresed on agarose gel (Fig. 2-2). As expected, *EcoRI* digestion produced three fragments (1.0-, 0.3- and 0.2-kb) in case of cAF4, and two fragments in case of cAF5 (0.8- and 0.7-kb) and cBF6-2 (0.6- and 0.3-kb) clones. These results confirmed that these cDNA clones have internal *EcoRI* site(s). Since the CYP6A2 cDNA does not have internal *EcoRI* site(s) (Waters et al., 1992), cAF4, cAF5 and cBF6-2 are not cDNA clones of *Cyp6a2* gene. Thus, the *EcoRI* fragments of each cDNA insert were sub-cloned into pBSKII⁺ plasmid for further analysis.

Genomic analysis of the *Cyp6a2*-related cAF4, cAF5 and cBF6-2 cDNA clones

To characterize the cDNA clones further, the pattern of hybridization of cAF4, cAF5 and cBF6-2 cDNA clones to genomic DNA was studied. For this purpose, equal amounts of genomic DNA from adult 91-R flies were digested with *EcoRI*, fractionated on agarose gel and blotted onto nylon membranes. These blots were individually probed with ³²P-labeled 1.0-kb *EcoRI* fragment of cAF4, 0.8-kb *EcoRI* fragment of cAF5, 0.3-kb + 0.6-kb *EcoRI* cDNA fragments of cBF6-2 and a full-length (1.6-kb) CYP6A2 cDNA fragment. Hybridization and washing were done under both low and high stringent conditions. Under both conditions the CYP6A2 cDNA probe hybridized strongly with a 5.8-kb *EcoRI* genomic DNA fragment (Fig 2-3) suggesting, that the cDNA is fully

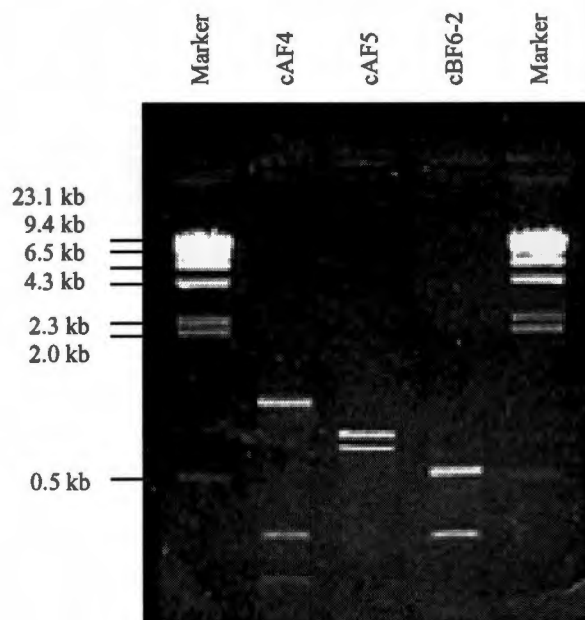


Figure 2-2: Restriction digestion pattern of PCR products containing *Cyp6a2*-related cDNA.

PCR products obtained by amplifying cDNA inserts in λ gt11 (cAF4, cAF5, cBF6-2) were digested with *Eco*RI and fractionated on a 0.8% TBE-agarose gel. All three products, that is, cAF4, cAF5 and cBF2-6 have internal *Eco*RI sites, indicating that these clones are not reisolates of *Cyp6a2*, which does not have any *Eco*RI sites in the cDNA (Waters et al., 1992). Molecular weight standards (*Hind*III cut λ DNA) are shown on the left.

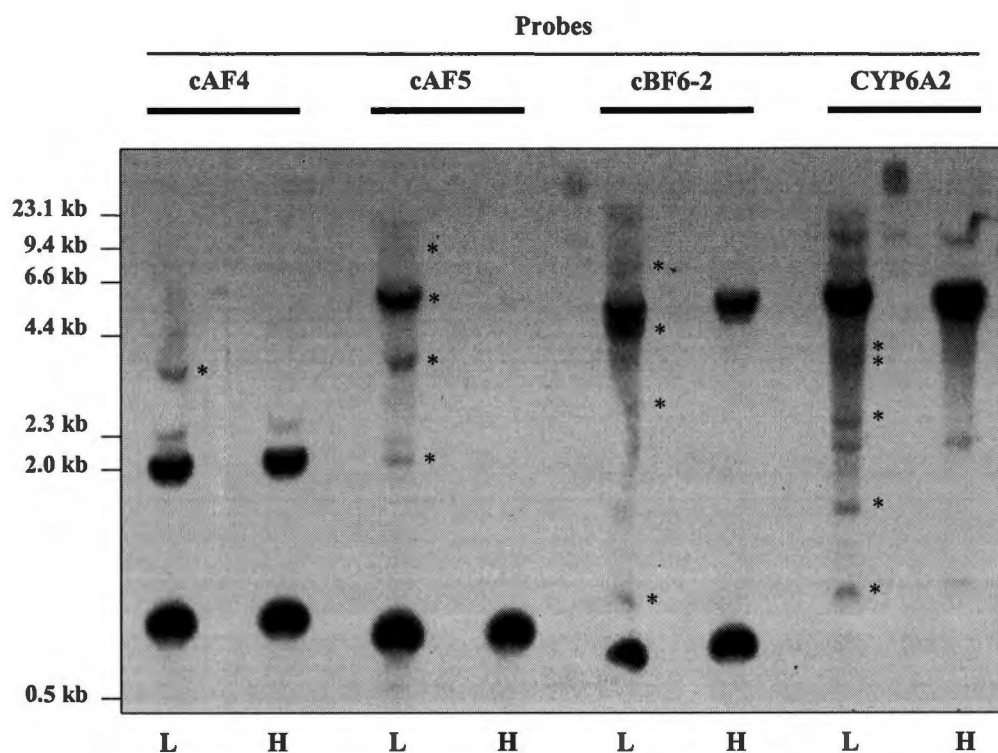


Figure 2-3: Southern blot analysis of 91-R genomic DNA showing pattern of hybridization of *Cyp6a2*-related DNA clones cAF4, cAF5 and cBF6-2.

Genomic DNA from 91-R adult flies was digested with *EcoRI*, fractionated through a 0.8% TBE-agarose gel and blotted onto nylon membrane. Four such blots, in two sets, were individually probed with cDNA from AF4, AF5, BF6-2 and *Cyp6a2*, the last being used as a positive control. One set (all L lanes) was probed under low stringent conditions and the other set was probed using high stringent conditions (all H lanes). Fragments marked with an asterisk are thought to be cross hybridizing bands that disappear under high stringent conditions of probing. Molecular weight standards (*HindIII* cut λ marker) are shown on the left.

contained within this genomic DNA fragment. This agrees with the restriction map of *Cyp6a2* gene published by Waters et al. (1992). The results also showed that the pattern of hybridization of all four cDNA probes to the genomic DNA differed from one another (Fig. 2-3). Therefore, the new cDNA clones are different from one another and they are not reisolates of CYP6A2 cDNA clones. Interestingly, however, under low stringent conditions all four cDNA probes hybridized faintly with several DNA fragments (Fig. 2-3, bands with asterisks), suggesting the presence of other related sequences in the 91-R genome. Such cross hybridization under low stringent condition is expected because *CYP* genes are thought to evolve by gene duplication and subsequent divergence. Therefore, some sequence conservation between such duplicated gene sequences could be expected.

Sequence analysis of the cAF4, cAF5 and cBF6-2 cDNAs.

The cAF4, cAF5 and cBF6-2 cDNA clones were sequenced by the dideoxy chain termination method. The DNA sequence of each cDNA clone was conceptually translated in six reading frames using the MAP and TRANSLATE program of the GCG software. In case of cAF4 and cAF5, such translation showed the presence of a single open reading frame that yielded polypeptides of 504 and 494 amino acids, respectively. Since the size of the cBF6-2 cDNA insert sequenced was small, it yielded a short (162) sequence of amino acids. These three CYPs and CYP6A2 were compared by using the PILEUP program of GCG software package.

As shown in Fig. 2-4, the AF4, AF5 and BF6-2 polypeptides have the F**G***C*G decapeptide sequence towards their C terminal ends. This sequence is similar to the highly conserved heme binding motif found in other cytochrome P450 proteins (Omura and Sato, 1964a). BLAST analysis showed that AF4 and AF5 polypeptides are most closely related to CYP6A2. While the AF5 polypeptide is 77% similar and 60% identical to CYP6A2, the AF4 polypeptide is 75% similar and 55%

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Cyp6a2  ...MFV IYL LIAISSL IAY LYHENFNYWN RRGL EHDAPH PLYENMVGFR
Cyp6a8  MALAYI FQV AVALLAI TY YIHPKLT YFK RRGIEFVAPH LIRSNMEELQ
Cyp6a9  MGVYSV LAI VVVLGVY LLL KWRPALH IHWQ NLDI CCEPH ILMESLTGVQ
Bf6     .....

Cyp6a2  KNRVMHDFFY DYNNKY KSG FPFVGFYFLH KPAAFIVDTQ LAENILIKDF
Cyp6a8  KTKNIHEIFQ DEYNKFRFSK APFVGSFFFQ SBAAFVIDLE LAKQLLIKDF
Cyp6a9  TSRSFSAIWM DYNNKFRGTG .PFAGFYWFQ RPSILVLDIS LAKQLLIKDF
Bf6     .....

Cyp6a2  SNFADR SQFH NGRDDDELTOH LENLDGKKWK DMRQRLTPTF TSGKMKFMFE
Cyp6a8  SNFSNKSI FY NEDDDPISAH LENLDGAQWR LLRNKLSSTF TSGKMKLMYF
Cyp6a9  NKITDR GFYH NEDDDPLSGQ LELLDCQKWK SMRSKLSSTF TSGKMKMYFE
Bf6     .....

Cyp6a2  TVIKVSEEFV KITEQVPAA QNGAVL I IKE LMARFTTQVI GTCRFGIECN
Cyp6a8  TVVSVANEFM TMHEKVP . KNSVL IIRD LVARFTTQVI GTCASAIQCN
Cyp6a9  TVVKVGHEHI EHFQ . .AM EKSPIVSVRD ILARFTTQVI GTCARFGIES
Bf6     .....

Cyp6a2  TTRTPVSDER TMQKVFTDM RHGKLLTMFV FSFPKLASRL RMRMPEDVH
Cyp6a8  SRDEKABRL YFKRSLVDK RHGTLNNGFM RSYPKLARKL GAVRTAPHIQ
Cyp6a9  SKDPEABER VMRRRAIFEQ RHGPIGIAFI NSFQNLARRI HMKITLEEAE
Bf6     .....

Cyp6a2  QFFMRLNNDT INLRERENFK RNDFMNLLIE LRQKGSSTFL DNGEVIEGMD
Cyp6a8  EFYSRIITET VAVREKEHIK RNDFMOMLIE LFNQ.KEMIL ENGDDVVRGLT
Cyp6a9  HFFLRIVRET VAFREKKNIR RNDFMQDLID LFN..SPLIK SESGESVNLT
Bf6     .....

Cyp6a2  IGE LAACVFV FYVAGFETSS STMSYCLYEL AQNDIQDRL RNEIQTVLEE
Cyp6a8  MEEVLAQAFV FFIAGFETSS STMGFALYEL AKNPDI....EQ
Cyp6a9  IEBMAAQAFV FFGAGFETSS TTMGFALYEL AQHQDIQDRV RKECQEVIGK
Bf6     .....

Cyp6a2  QEGQLTVESI KAMTYLNQVI SETLRITVLV PHLERKALND YVVPGEKLV
Cyp6a8  HDQNFTVECT KDLKYLNQVL DETLRITIV PNLDRMAKR YVVPCHPNFV
Cyp6a9  YNGEITYESM KDMVYLDQVI SETLRITVL PDLNRECLD YEVPGHPKV
Bf6     ...EFTYDSM QELRYMELVI AETLRKYPIL PDLTRISRHL YAAKGRHIFY

Cyp6a2  IEKGTQVILP ACAYHRDEDL YPNPETEDPE RFSPEKVAAR ESVEWLPFGD
Cyp6a8  IEAGQSVILP SSAIHHDPSI YPEPFERPE RFSPEESAGR PSVAWLPFGD
Cyp6a9  IKKGMPVILP CGAMHRDEKL YANPNTENPI .FFARTSEGS DSVWLPFGD
Bf6     IEPQMLLIP VYGIHDPAL YPEPHKFIPE RFLADQLAQR PTAWLPFGD

Cyp6a2  GPRNCIGMRF GOMQARIGLA QIISRFVSV GDTTEIPLKY SPMSIVLGTV
Cyp6a8  GPRNCIGLRF GOMQARIGPA LLIRNEKFSV GSKTPNPLVY DEKSFVLGVK
Cyp6a9  GPRICIGMRF GOMQARSCLA LLINREKFSV CEQTTIPVY SKKTFLLISSE
Bf6     GPRNCIGMRF GKMOTTIGLV SLLRNEHFSV GPRTDPKIEF LKSNILLCPA

Cyp6a2  GGIYLRVERI *.....
Cyp6a8  DGIYLLKQETV *.....
Cyp6a9  TGIHLKVERV *.....
Bf6     HGIYLLKQQQL SQMSS*

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Figure 2-4: Comparison of amino acid sequences of the polypeptides encoded by *Cyp6a2* and the *Cyp6a2*-related cDNA clones cAF4, cAF5 and cBF6-2.

The PILEUP program of the GCG software was used to create the alignment of the conceptual translates of the new *Cyp6a2*-related cDNA clones. Dark boxes indicate identical amino acid sequences. The heme binding region is indicated by the overlying line and the invariant cysteine residue is within this region. The ETLR motif and the conserved threonine residue in the I helix is also marked. GenBank accession numbers for the new CYP genes are L46859 for *Cyp6a8*, L46860 for *Cyp6a9* and L46858 for BF6-2.

identical to the same. The AF4 and AF5 polypeptides also show strong similarities (61-70%) with seven housefly CYPs of family 6 (Cohen and Feyereisen, 1995; Cohen et al., 1994). Moderate similarity (56-59%) is also found with CYP6B1 of black swallowtail butterfly, *Papilio* (Cohen et al., 1992). Based on these similarities and recommendation of the Nomenclature Committee the AF4 and AF5 polypeptides were classified under family 6 and subfamily A, and designated as CYP6A9 and CYP6A8, respectively. The corresponding genes encoding these polypeptides were named *Cyp6a9* and *Cyp6a8*. The amino acid sequence encoded by cBF6-2 cDNA was too short for it to be classified into a particular subfamily. However, the presence of the heme-binding motif with invariant amino acid sequence F**G***C*G at the C terminal end indicates that this cDNA is also a part of a *CYP* gene.

Comparison and analysis of the amino acid sequences (Fig. 2-4) showed the presence of conserved regions which included several motifs. These include the *heme binding motif* (residues 433 to 442) mentioned above, the *I helix* with the conserved threonine residue (residue 314) which is important for oxygen binding and the *ETLR motif* (residues 356 to 359). The amino acid residue numbers correspond to that of CYP6A8. The last motif appears to be conserved in all CYP6 family members and many members of the CYP3 family (von Wachenfeldt and Johnson, 1995). It is to be noted that of all the CYP families, proteins coded for CYP6 family genes are closest to proteins coded for by the CYP3 family genes at least with respect to amino acid similarity. Hydrophobicity profiles of CYP6A8 and CYP6A9 showed the presence of a hydrophobic stretch of amino acids at the N terminal end (data not shown). A hydrophobic N' terminal end is a common feature among microsomal P450s, which serves as a membrane anchor. CYP6A8 was subjected to MaxHom, a multiple sequence alignment program that finds similar sequences in the database (predictprotein@dodo.cpmc.columbia.edu). The result

showed that CYP6A8 is most closely related to CYP6A2 than to other members of the CYP family 6 enzymes (data not shown).

Genomic organization of the *Cyp6a8* and *Cyp6a9* genes.

To analyze the *Cyp6a8* and *Cyp6a9* gene organization, a genomic library of 91-R adult flies (Waters et al., 1992) was screened with cAF4 and cAF5 cDNAs. As mentioned above, cAF4 and cAF5 are cDNA clones of *Cyp6a9* and *Cyp6a8* genes, respectively. Two genomic clones, λ A1-1 and λ A3-1, hybridized with both cDNA probes while a third genomic clone (λ A2-1) hybridized only with the cAF4 cDNA probe. Since both cAF4 and cAF5 cDNAs hybridized to the same genomic clones (λ A1-1 and λ A3-1), it implied that *Cyp6a8* and *Cyp6a9* genes are located close to each other in the genome. The results also suggested that λ A2-1 clone overlaps with the regions of λ A1-1 and λ A3-1 clones that have cAF4, that is, *Cyp6a9* sequences.

The λ A3-1 genomic clone that hybridized with both the cDNA probes was chosen for further analysis. Restriction mapping showed that this clone has a 14-kb genomic DNA insert (Fig. 2-5). To localize the transcribed regions, multiple Southern blots of λ A3-1 DNA restricted with various enzymes were made. These blots were individually hybridized with the ^{32} P-labeled 0.7- and 0.8-kb *Eco*RI fragments of cAF5 (*Cyp6a8*) and three fragments (1.0-, 0.3- and 0.2-kb) of the cAF4 (*Cyp6a9*) cDNAs. The results showed that both *Eco*RI cDNA fragments of *Cyp6a8* (0.7kb and 0.8kb) were present completely in the λ A3-1 clone (Fig. 2-5, fragment 4, 5). However for *Cyp6a9*, while 0.2- and 0.3-kb fragments were completely present in the λ A3-1 clone, only a part of the 1.0-kb fragment was present in this genomic clone (Fig. 2-5). The arrangements of these cDNA fragments (Fig. 2-5, fragments 1-3) in the genomic clone were determined by

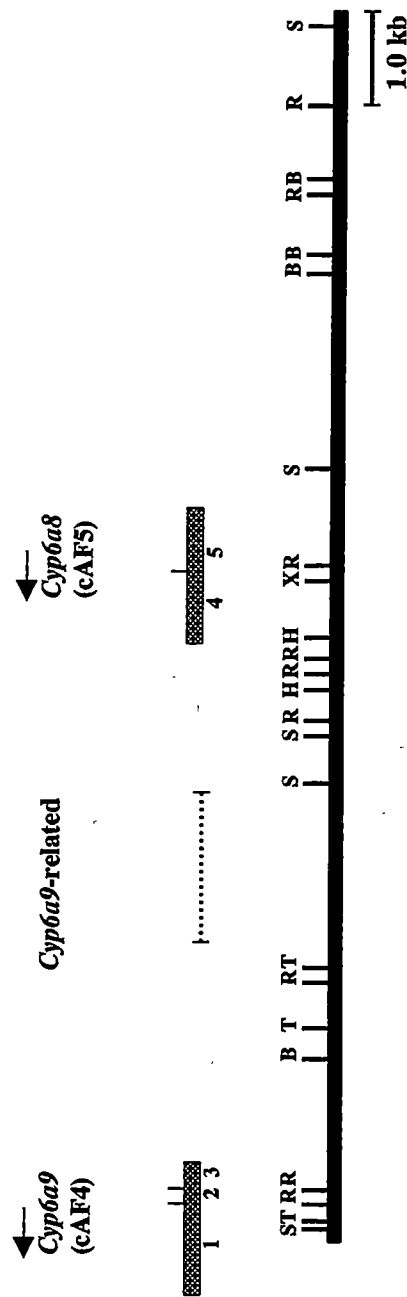


Figure 2-5: Restriction map of the λA3-1 genomic clone.

The location of *Cyp6a8* and *Cyp6a9* are shown above the restriction map. The direction of transcription shown by the arrows was determined by aligning the conceptual translation products with CYP6A2. The 1.0 kb cDNA fragment of *Cyp6a9* cross hybridizes very strongly to the *EcoRI* – *SalI* fragment, represented by the dashed line, present in between the two genes, implying the presence of another gene very similar to *Cyp6a9*. B, *Bam*HI; R, *Eco*RI; H, *Hind*III; S, *Sal*I, T, *Sst*I; X, *Xba*I.

restriction mapping of both genomic and cDNA clones, and by Southern blot hybridization.

A surprising result was obtained in course of mapping the transcription units. When the 1.0-kb *Eco*R1 fragment of CYP6A9 cDNA (Fig. 2-5, fragment 1) was used as a probe, it showed very strong hybridization with a 2.0-kb *Eco*RI-*Sal*II fragment located between *Cyp6a8* and *Cyp6a9* gene (Fig. 2-5, dotted line). Interestingly, however, the 0.3- and 0.2-kb *Eco*RI fragments of the same cDNA (Fig. 2-5, fragments 2 and 3) did not hybridize with the 2.0-kb *Eco*RI-*Sal*II fragment or any contiguous fragments in the λ A3-1 clone. Therefore, the λ A3-1 clone does not seem to have a duplicate copy of the *Cyp6a9* gene but it has *Cyp6a9*-related sequences within the 2.0-kb *Eco*RI-*Sal*II fragment (Fig. 2-5). Since multigene families, including *CYP* gene families, are thought to have expanded via gene duplication or gene conversion events, it is not surprising to find similar sequences (*Cyp6a8*, *Cyp6a9* and *Cyp6a9*-related) so closely clustered in the genome. Clustered *CYP* genes belonging to other families have also been identified in *Drosophila*. These are *CYP4* and *CYP28* gene clusters located on the X and the 2L chromosomes, respectively (Frolov and Alatortsev, 1994; Dunkov et al., 1996; Feyereisen, 1999). Closely clustered *CYP6* family genes have also been identified in *Musca* (Cohen and Feyereisen, 1995), *Papilio* (Hung et al., 1996) and *Helicoverpa* or cotton bollworm, an insect pest of considerable economic importance (Ranasinghe and Hobbs, 1998).

In situ hybridization of biotinylated phage DNA (λ A3-1) to polytene chromosomes from 91-R showed that the λ clone hybridized to a single polytene band, 51C, on the right arm of the second chromosome (Fig. 2-6). A large number of *CYP* genes have been identified in *Drosophila* and their chromosomal positions are known (Feyereisen, 1999), however, the three genes located on λ A3-1 form a part of a cluster that had not been reported prior to this investigation.

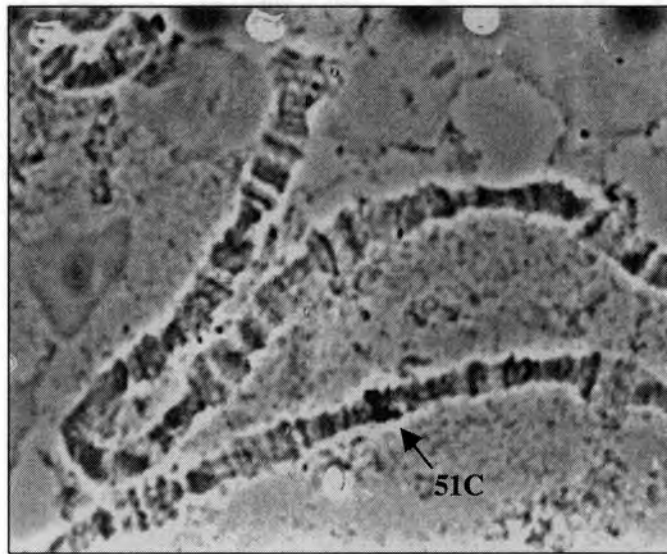


Figure 2-6: *In situ* hybridization of the λ A3-1 genomic clone to polytene chromosomes of 91-R.

λ A3-1 phage DNA was biotinylated and hybridized to polytene chromosome squashes made from salivary glands of third instar larvae from 91-R. The clone hybridized to a single polytene band at 51C on the right arm of the second chromosome. The position of hybridization is shown by an arrow.

Expression of *Cyp6a2*-related genes encoding CYP6A8, CYP6A9 and BF6-2 polypeptides in 91-R and 91-C strains of *Drosophila melanogaster*

One of the objectives of this investigation was to study whether other members of the CYP6 gene family have similar patterns of expression as *Cyp6a2*. It has been well documented that insecticide resistant strains of the housefly have higher levels of expression of genes belonging to the CYP6 family compared their susceptible counterparts (Feyereisen, 1999; Scott, 1999). In *Drosophila* too, the DDT resistant 91-R strain shows a higher level of *Cyp6a2* expression than the susceptible 91-C strain (Waters et al., 1992). Therefore, expression of the newly isolated *Cyp6a2*-related genes encoding CYP6A8, CYP6A9 and BF6-2 polypeptides was measured in the 91-R and 91-C strains by Northern blot analysis (Fig. 2-7). After correcting for the RNA loading error using RP49 mRNA as an internal control, it was found that the levels of BF6-2 mRNA in two strains were similar (Fig. 2-7, panel A). In case of the *Cyp6a8* gene, a striking difference between 91-R and 91-C strains was observed when the 0.8-kb cDNA fragment was used as a probe. While among all three newly isolated genes *Cyp6a8* showed the highest level expression in the 91-R strain, its expression in the 91-C strain was barely detectable (Fig. 2-7, panel B). The same pattern of hybridization was observed when the 0.7-kb DNA fragment of cAF5 was used as a probe (data not shown). Although it is not visible in the autoradiograph shown in Fig. 2-7 (panel B), a shorter exposure revealed that the 0.8-kb DNA probe hybridized with two transcripts of 2.0- and 1.8-kb. Many genes, such as *Sxl* (Bell et al., 1991) and *DGγ1* (Ray and Ganguly, 1992), have been identified in *Drosophila* which are known to transcribe more than one mRNA species. Multiple mRNAs may be generated via alternative transcription initiation, alternative splicing or alternative polyadenylation. One of these mechanisms may be used by the *Cyp6a8* gene to produce the 2.0- and 1.8-kb mRNAs. Alternatively, it may be surmised that either of the two RNAs is a transcript of another CYP gene that has significant sequence

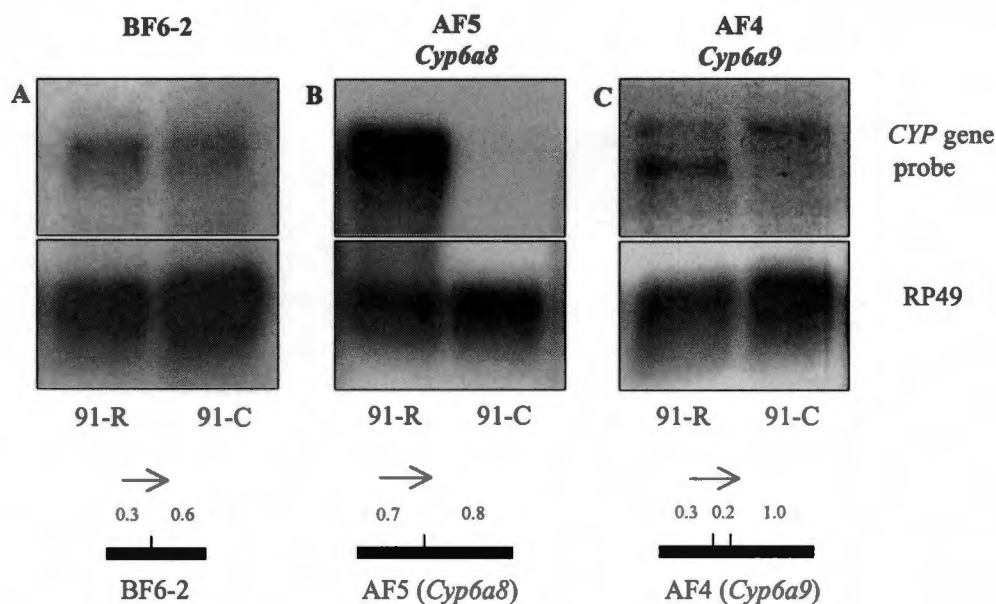


Figure 2-7: Expression of the *Cyp6a2*-related cDNA clones in the 91-R and 91-C strains of *Drosophila melanogaster*.

2.5 µg poly A⁺ RNA was fractionated through a 1.2% denaturing agarose gel containing formaldehyde and MOPS. The blots were probed with the appropriate ³²P-labeled cDNA probes. In panel A - BF6-2, both 0.6- and 0.3-kb fragments were used together to probe the blots. Similarly, in panel B, the AF5 - 0.8-kb fragment and in panel C, the AF4 - 1.0-kb fragment was used to hybridize to the RNA on the blot. To control for variations in RNA loading the same blots were also probed with RP49. The figures at the bottom of each panel show the *Eco*R1 cDNA fragments present in the insert in each λ clone. The arrows on top of the inserts indicate direction of transcription.

similarities with the 0.8- and 0.7-kb DNA fragments of the cAF5 cDNA. The latter possibility can be ruled out firstly because high stringent hybridization and washing condition were used. Secondly, the C terminal regions of CYP proteins that contains the heme binding region are generally found to be more conserved than the N terminal regions (Lewis, 1996a). Since the 0.7-kb cDNA fragment which represents the 5' region of *Cyp6a8* and codes for the N terminal half of CYP6A8, also hybridizes strongly to both transcripts, the presence of the two transcripts may not be due to cross hybridization. Sequence analysis suggested that alternative transcription initiation or polyadenylation could be the mechanism that produces the two CYP6A8 mRNAs. These are discussed in Chapter 3 in the context of gene organization.

In case of the *Cyp6a9* gene, hybridization of two different sized RNAs (2.1- and 1.8-kb) was seen when the 1.0-kb cDNA fragment representing the 3' terminal portion of the gene was used as a probe (Fig. 2-7, panel C). When the hybridization signals were normalized using RP49 RNA as an internal control, the level of expression of the 2.1-kb transcript was found to be similar in 91-R and 91-C. However, the 1.8-kb transcript was found to express to a higher extent in 91-R compared to 91-C.

Expression of *Cyp6a8* and *Cyp6a9* genes in seven different strains of *Drosophila*

Since the *Cyp6a8* and *Cyp6a9* genes show differential expression in resistant 91-R and susceptible 91-C strains, we wanted to study the expression of these two genes in other strains of *Drosophila* which have differential responses to insecticides. Therefore, poly A⁺ RNA from seven different strains were hybridized with the 0.8-kb cDNA fragment (fragment 4, Fig. 2-5) of the *Cyp6a8* gene. Of the seven strains examined, 91-R and MHIII-D23 express *Cyp6a8* at a very high levels whereas *ry*⁵⁰⁶ shows a lower level of expression (Fig. 2-8, panel A). No detectable expression was observed in the other four strains. It is to be noted that both 91-R and MHIII-D23 strains are associated with

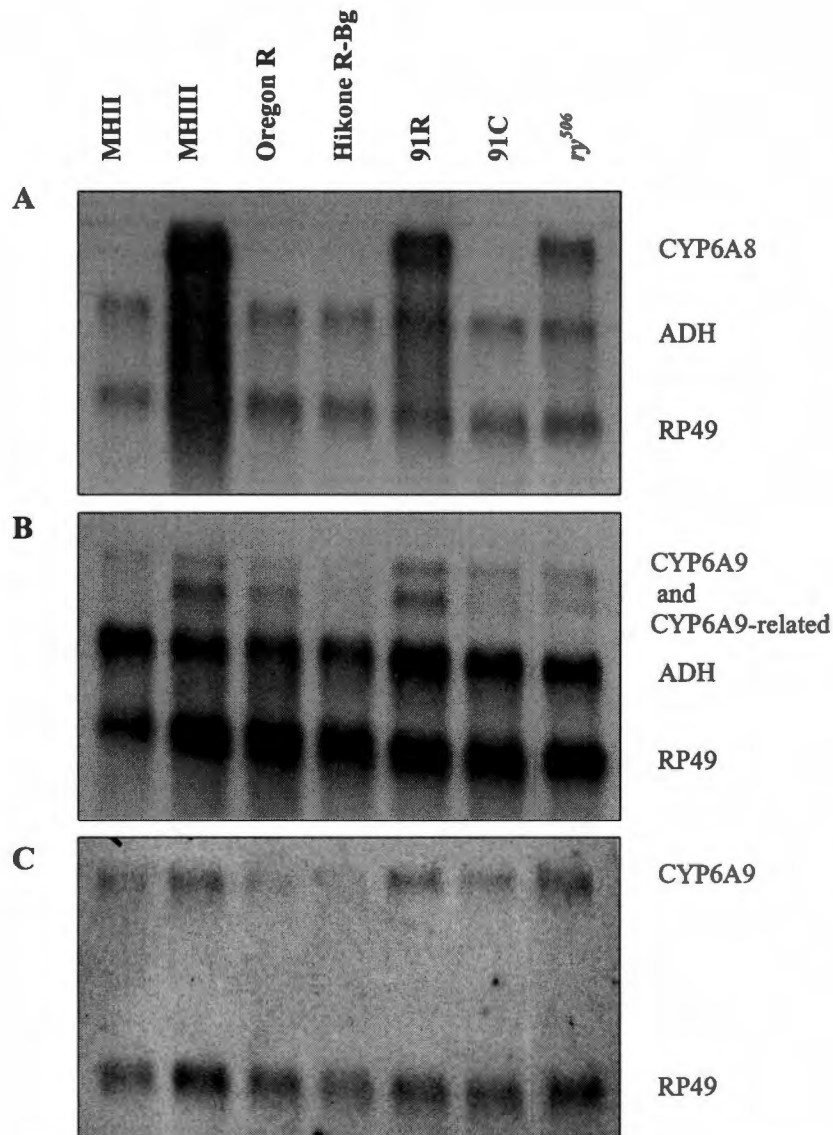


Figure 2-8: Expression of *Cyp6a8*, *Cyp6a9* and *Cyp6a9*-related genes in several strains of *Drosophila melanogaster*.

3.5 μ g poly A⁺ RNA was fractionated through a 1.2% agarose denaturing gel containing MOPS and 2.2M formaldehyde, blotted onto nylon membrane and hybridized successively under stringent conditions to ³²P labeled cDNA probes. The cDNA probes used were *Cyp6a8* - 0.8 kb (panel A), *Cyp6a9* - 1.0 kb (panel B) and *Cyp6a9* specific - 0.3 kb (panel C). The blots were stripped after hybridization with each probe and exposed to autoradiographic film to ensure that all the previous probe had been removed. To normalize for RNA loading, both ADH and RP49 were used.

resistance to insecticides (Merrell and Underhill, 1956; Halpern and Morton, 1987). Thus, high level of *Cyp6a8* expression in these strains follows the general dogma that resistance is associated with overexpression of *CYP* genes. However, Hikone R-Bg does not follow this general trend. This strain is highly resistant to several insecticides (Tsukamoto, 1958), yet it shows almost no expression of the *Cyp6a8* gene (Fig. 2-8A). The level of another *CYP* mRNA, *CYP6A2*, is also undetectable in this strain (Dombrowski, 1998). Interestingly, however, the amount of *CYP6A2* protein is found to be very high in Hikone R-Bg strain (Sundseth et al., 1989). Therefore, it is thought that in Hikone R-Bg strain, *CYP* gene expression may be regulated at the translational level. At present, it is not known whether *Cyp6a8* is also regulated at the translational level. However, this could be determined in future by using *CYP6A8*-specific antisera.

Since only two of the seven strains examined show overexpression (Fig. 2-8A), a low level of expression appears to be the wild type phenotype for the *Cyp6a8* gene. A similar pattern of *Cyp6a2* expression has also been reported, that is, high in 91-R and MHIII-D23 and lower in the other strains that were studied here (Dombrowski, 1998). It is to be noted that low level of expression of *CYP* genes is generally accompanied by susceptibility to insecticides. Oregon R, 91-C and *ry*⁵⁰⁶ strains, which are known to be susceptible to DDT and malathion (Sundseth et al., 1989; R. Ganguly, unpublished observations), appear to be low producers of *CYP6A8* (Fig. 2-8A) as well as *CYP6A2* (Dombrowski et al., 1998). MHIII-D5, however, does not follow the general norm. It is a strain with an isogenic second chromosome from a malathion resistant strain and other chromosomes from a susceptible strain (L. C. Waters, Personal communication). However, *Cyp6a8* expression is almost nonexistent in this strain (Fig. 2-8A) although major resistance loci are known to be on the second chromosome, as is also the locus for *Cyp6a8*. It is possible that the high levels of *Cyp6a8* expression in resistant strains are due to regulatory loci not located on the second chromosome. Consequently, the MHIII-

D5 strain shows a low level of *Cyp6a8* expression. Whether other chromosomes indeed influence the expression of *Cyp6a8*, which is on the second chromosome, was examined and the results are presented in Chapter 4.

Figures 2-8B and 2-8C show the expression of *Cyp6a9* in the *Drosophila* strains mentioned above. When the 1.0-kb cDNA fragment of *Cyp6a9* (fragment 1, Fig. 2-5), corresponding to the 3' terminal portion of the gene, was used as a probe, hybridization of 2.1 and 1.8-kb transcripts was observed (Fig. 2-8B). Interestingly, the level of the 1.8-kb transcript was higher in the 91-R and MHIII-D23 strains than in the other five strains examined (Fig. 2-8B). When the 0.3-kb cDNA fragment (fragment 1, Fig. 2-5) corresponding to the 5' terminal portion of the *Cyp6a9* gene was used as a probe, hybridization of a single 2.1-kb RNA was observed (Fig. 2-8C). The level of the 2.1-kb CYP6A9 RNA was found to be similar in all strains except Oregon R and Hikone R-BG, both of which showed a lower level of expression. The 1.8- and 2.1-kb CYP6A9 RNAs probably differ at the 5' end because the 5'-end or N' terminal probe (0.3-kb fragment of *Cyp6a9*) hybridizes with the 2.1-kb RNA but not with the 1.8-kb RNA (Fig 2-8C). It is possible that the 2.1-kb RNA may have an alternative 5' exon that is spliced out to generate the 1.8-kb RNA. Alternatively, the 1.8-kb RNA may not be a product of the *Cyp6a9* gene at all. It may be a product of the *Cyp6a9*-related gene instead. In that case the recognition of two transcripts by the 1.0-kb probe (Fig. 2-8B) is not unlikely because the 1.0-kb cDNA fragment of the *Cyp6a9* gene (fragment 1, Fig. 2-5) shows strong cross-hybridization with the 2.0 *EcoRI-SalI* fragment in the λ A3-1 genomic clone (Fig 2-5). This region is thought to contain another gene related to *Cyp6a9*. Sequence comparison of the CYP6A9 cDNA, and the *Cyp6a9* and *Cyp6a9*-related genomic DNAs along with primer extension analyses will be needed to determine the origin of the 2.1- and 1.8-kb RNAs.

Two possibilities can be invoked to explain the high level of expression of *Cyp6a8* in 91-R and the almost null expression in 91-C. It could either be a result of gene amplification or involve a structural modification of the *Cyp6a8* gene. In insects like the peach potato aphids and the mosquito (*Culex* species), higher amounts of esterase enzyme are found to be a result of gene amplification (Fields et al., 1988; Hemingway et al., 1998). To examine this possibility, Southern blots of equivalent amounts of genomic DNA from the 91-R and 91-C strains were individually probed with the 0.7- and 0.8-kb cDNA fragments of the *Cyp6a8* gene (fragments 4 and 5, Fig. 2-5). Fig. 2-9 shows no restriction fragment length polymorphism between two strains with respect to the restriction enzymes used. In addition, both probes hybridized with a 2.8-kb *SaII* fragment (Fig. 2-9 A, B), as was expected from the restriction map of *Cyp6a8* (Fig. 2-5). The hybridization intensity was also similar in the 91-R and 91-C strains. Taken together, these results imply that the gene structure, at least in the coding region, is similar in 91-R and 91-C and that gene amplification is not the mechanism of *Cyp6a8* overexpression in 91-R. As a control to check for presence of equal amount of genomic DNA from the two strains, one of the blots was reprobed with a cDNA fragment of the *Drosophila* alcohol dehydrogenase (ADH) gene after stripping off the first probe. The hybridization intensity of the ADH specific band (Fig. 2-9C) was the same in both 91-R and 91-C, implying that equal amount of DNA was present from the two strains on the Southern blots.

Mechanisms of overexpression of *Cyp6a8*

In most cases expression of any particular gene, at the level of transcription, is regulated by both *cis*-acting elements and *trans*-acting factors. The *cis*-acting elements include 5' promoter regions and enhancer/silencer sequences. While the promoter regions regulate the basal level of transcription, the enhancer or silencer sequences are involved in temporal, tissue-specific and induced expression of the gene. Transacting factors also

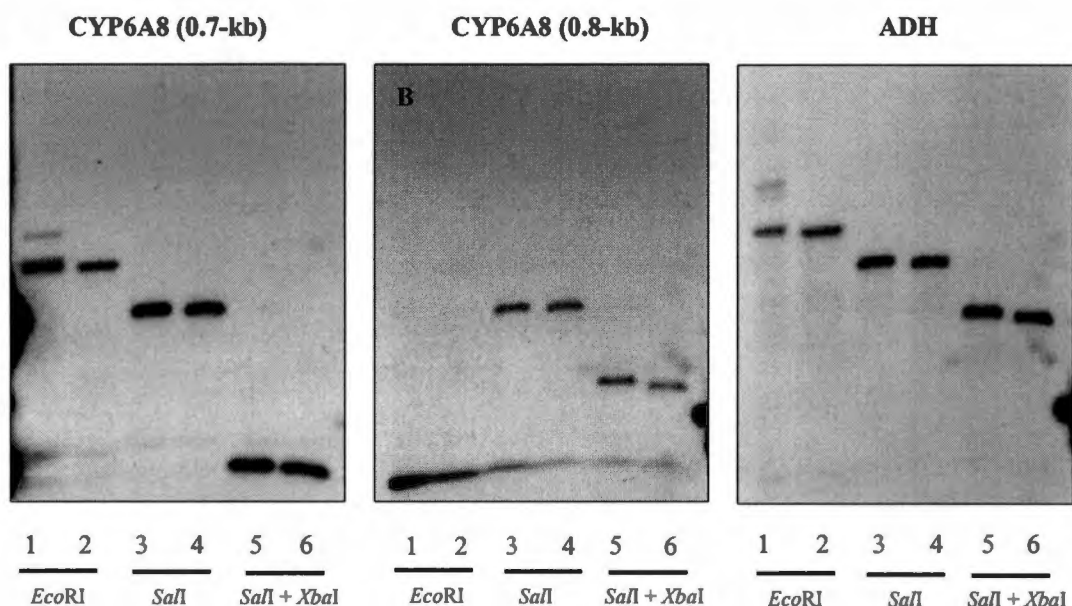


Figure 2-9: Genomic southern blot analysis of 91-R and 91-C to study presence of restriction polymorphism in the coding region of *Cyp6a8*.

10 µg genomic DNA was digested with *EcoRI*, *SalI* or double digested with *XbaI* and *SalI*. *EcoRI* and *XbaI* cut in the middle of the coding region and *SalI* sites flank the gene as shown in figure 2-5. The blots were separately probed with the 5' half (0.7-kb *EcoRI* cDNA fragment; panel A) and the 3' half (0.8-kb *EcoRI* cDNA fragment; panel B) of the *Cyp6a8* coding region. The blot in panel B was stripped and reprobed with a cDNA fragment containing the ADH coding region (Panel C). All odd numbered lanes contain DNA from 91-R and all even numbered lanes contain DNA from 91-C. Lanes 1, 2 – *EcoRI* digested; lanes 3, 4 – *SalI* digested; lanes 5, 6 – double digested with *XbaI* and *SalI*.

play a major role in basal and modulated transcription. These factors include both general and regulatory transcription factors. One of the major objectives of this dissertation has been to study the mechanisms behind the overexpression of *CYP* genes in resistant insects. Since *Cyp6a8* is overexpressed in the resistant 91-R strain and is not expressed in the susceptible 91-C strain, this gene was used as a model to study *CYP* gene regulation. A high level of expression could be caused by gene amplification but this was not found to be the case with *Cyp6a8*. Therefore, it appears that high expression of *Cyp6a8* in 91-R is probably due to a regulatory mutation. Merrell and Underhill (1956) subjected part of a wild type *Drosophila* population to 30 generations of DDT treatment. The resistant strain that evolved during this selection process was named 91-R. The rest of the population was maintained on normal food and named 91-C. It is possible that during DDT selection (Merrell and Underhill, 1956), a *de novo* mutation occurred, giving high levels of *CYP* gene expression. Alternatively, a similar mutation that was already segregating in the population could have become fixed during DDT selection. In either case, this resulted in a high level of expression of at least two *CYP* genes, *Cyp6a2* and *Cyp6a8* (Waters et al., 1992; Maitra et al., 1996). It is possible that the *cis*-regulatory elements of each *CYP* gene were mutated during selection resulting in high levels of constitutive expression of these genes. Alternatively, the genes encoding *trans*-acting factors that regulate multiple *CYP* genes could be mutated. The latter possibility seems more probable because the chance of occurrence of upregulatory mutation in the *cis*-acting regions of multiple *CYP* genes is expected to be rare. To understand the molecular basis of the upregulation of *Cyp6a8* in 91-R, the next goal was to analyze the *cis*-regulatory elements of *Cyp6a8*.

Chapter 3

Analysis of *Cyp6a8* upstream DNA

"...secondly, they were a paradox, like God, being one in its functional unity but infinite in its diversity of purpose..."

Dennis V. Parke, June 1995

commenting on why the P450 superfamily of enzymes were not regarded with more interest by scientists and the general public

In: *Cytochromes P450 – Structure, function and mechanism*,

D. F. V. Lewis (1996) Taylor and Francis, Bristol, PA

Abstract

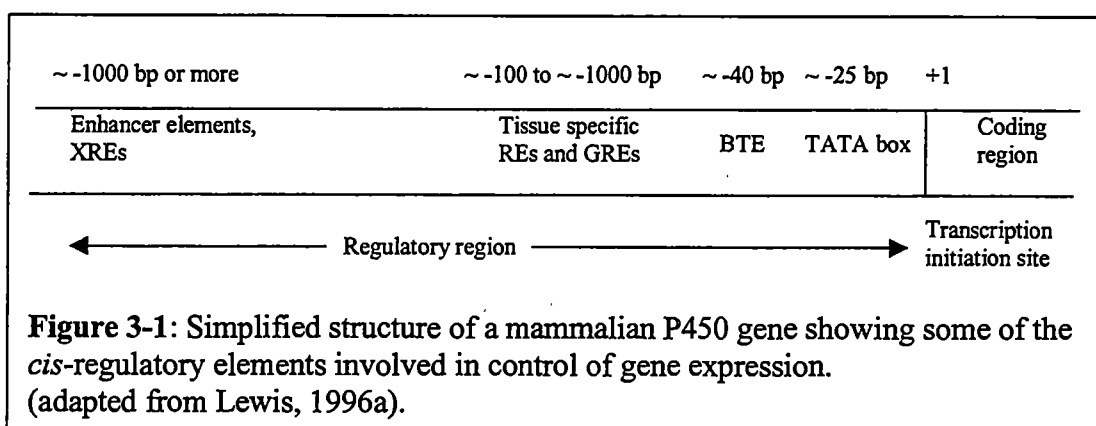
Cyp6a8, a *Drosophila* CYP gene, follows the general dogma that cytochrome P450 genes are overexpressed in resistant strains of insects. It is expressed at high levels in the DDT resistant 91-R strain and MHIII-D23, a strain carrying the 3rd chromosome from malathion resistant flies. The expression is low in the susceptible *ry*⁵⁰⁶ strain and 91-C strains. This investigation aimed at defining the gene structure of *Cyp6a8*, including the upstream DNA sequences that could be responsible for high expression in insecticide resistant strains of *Drosophila*. *Cyp6a8* was found to have an open reading frame of 1460 nucleotides interrupted by two introns. Sequences preceding the first ATG codon matched consensus Kozak sequences, signifying that this could be the start site for translation. The two transcripts of 2.0-kb and 1.8-kb, coded for by *Cyp6a8* appeared to be due to two alternative sites for polyadenylation. There appears to be a TATA box at -64 position from the first ATG codon in the cDNA. This, along with an initiator-like sequence probably defines the basal promoter region of the gene. Functional studies with the *luciferase* reporter gene showed promoter elements for basal transcription are located within -0.2-kb upstream DNA of *Cyp6a8* (ATG at +1). Upstream DNA between -200 to

-700 nucleotides was found to contain sequences that were responsible for a five fold higher level of constitutive expression. These elements could be binding sites for transcription factors like AP1, ATF and Oct1 that were identified by sequence analysis. Comparison of *Cyp6a8* upstream DNA sequences from overproducer (91-R, MHIII-D23) and underproducer (91-C) strains for *Cyp6a8* identified differences at 24 places. Most of these sequence differences were not in the region of the basal promoter. An insertion of three bases (TAC) was found in the underproducer 91-C strain which could be significant as the insertion is very close to binding sites for transcription factors AP1, ATF and Oct1. The functional significance of these sequence differences is not known.

Introduction

CYPs comprise a superfamily of enzymes that oxidatively metabolize diverse endogenous and exogenous substrates. The variety of enzymes is matched by the diversity of regulatory controls that are involved in *CYP* gene expression. Genes encoding these enzymes may be differentially influenced by species, chemicals, developmental stage, sex or tissues, implying the existence of different modes of regulation. The same gene may show variable expression in different species, sexes and tissues or closely related genes belonging to the same subfamily may show differences in constitutive or inducible expression within the same species. This variation may be due to different transacting factors like ligand activated receptors, second messengers, tissue specific factors or hormones which have been shown to regulate the expression of several P450 genes (Nebert and Gonzales, 1987; Fujii-Kuriyama, 1993; Gonzales et al., 1993). *Cis*-regulatory elements of the P450 genes may vary too, thereby providing another mechanism of gene regulation.

Regulatory elements in P450 genes include general promoter elements that control the basal level of expression. Superimposed on this level of control are other elements that modulate expression according to the requirements of the organism. Most mammalian P450 genes contain a TATA box about 20 to 40 bp upstream from the transcription start site and a BTE (basal transcription element) about 20 bp upstream of the TATA box (Fujii-Kuriyama, 1993; Lewis, 1996a), that control basal level of expression (Fig 3-1).



However, some response elements are present that are specific to certain classes of CYPs (Table 3-1). The presence of these response elements (both the number and position) determines the way these genes behave in response to endogenous and exogenous ligands.

Induction studies with ligands like TCDD and phenobarbital have helped to identify regulatory controls that exist for *CYP* gene expression. One of the best-studied examples is the regulation of the mammalian *CYP1A1* gene. This *CYP* gene is induced by benzo (a) pyrene and TCDD (Poland and Knutson, 1982; Negishi and Nebert, 1981, Gonzales et al., 1984). The inducer binds to a cytosolic receptor (Ah receptor) and makes it competent to interact with xenobiotic response elements (XREs) found in the upstream DNA of the gene. This brings about conformational changes in the DNA that removes a

Table 3-1
List of response elements found in the upstream DNA of *CYP* genes

Response elements (RE)	CYPs or CYP families where REs present	Factors involved in binding to or regulating response involving the REs.	References
CRE (cAMP response element)	steroidogenic CYPs (CYP11A, 11B, 17, 19 and 21)	cAMP	Kemper, 1993
GRE (Glucocorticoid response element)	CYP1A1	glucocorticoids	Hines, 1994
Barbie boxes (Barbiturate response elements)	All barbiturate inducible genes CYP102, CYP106, CYP2B1, CYP2B2	Barbiturate compounds	Fulco, 1991 Padmanaban and Nirodi, 1994
XRE (Xenobiotic response element)	CYP family 1, 2 and 3 CYP1A1	Xenobiotic compounds TCDD & Ah receptor	 Whitlock, 1999
PPRE (Peroxisome proliferator response element)	CYP family 4	Drugs that cause proliferation of peroxisomes and induction of peroxisomal enzymes, like Clofibrate	Kimura et al., 1989

Several *cis*-elements have been found in the upstream DNA of *CYP* genes that are involved in regulating *CYP* gene expression. These are characteristic of the gene families where found and may be also used to functionally classify the P450 genes.

repressor protein bound to the BTE, thereby allowing transcription to occur (reviewed in Whitlock, 1999). There are about three to five XREs in the 5' flanking region of *CYP1A1* in mouse, rats and humans, of which one or two of the distal motifs are important for binding the Ah receptor (reviewed in Gonzales et al., 1993). Another pathway that has been worked out involves the BM1 (*CYP106*) and BM3 (*CYP102*) genes from *Bacillus megaterium*. In absence of inducers, such as barbiturate compounds, transcription is inhibited by a repressor protein BM3R1. The repressor binds to sequences in the operator regions in front of both the genes as well as specific sequences known as barbie boxes found in the upstream DNA (Liang and Fulco, 1995). When barbiturate compounds are added, not only is the repressor removed from the upstream DNA sequences, but positive regulatory factors (BMP1 and BMP2) are also synthesized which enhance transcription (reviewed in Kemper, 1998). Thus, as in the case of any gene, *CYP* gene expression is influenced by *cis*-acting regulatory elements.

It has been shown that at least two *CYP* genes (*Cyp6a2* and *Cyp6a8*) are expressed at a very high level in the DDT resistant 91-R strain (Waters et al., 1992; Maitra et al., 1996). The upregulation of *Cyp6a2* probably had a selective advantage because CYP6A2 has been shown to metabolize aldrin, heptachlor and diazinon in a heterologous system (Andersen et al., 1994). Although the substrate(s) for CYP6A8 are not yet known, this gene may have played a role in development of resistance as it is expressed at high levels in the 91-R strain. To study the mechanisms behind upregulation of *CYP* genes in insecticide resistant strains, we first wanted to study the *cis*-regulatory sequences that could be involved in the high level of *Cyp6a8* expression in 91-R.

The main objective of this work was to identify sequences that could control *Cyp6a8* expression. Sequence analysis of genomic DNA was done to study the gene structure of *Cyp6a8* and identify transcription factor binding motifs present in the upstream DNA of the gene. Functional analysis of the upstream DNA sequences of the

Cyp6a8 gene was done by using a *luciferase* reporter gene system and P-element mediated germ line transformation. Since this gene is overexpressed in 91-R and MHIII-D23 and is silent in 91-C, the *Cyp6a8* upstream DNA from these three strains were compared to see if there were any sequence differences in the promoter regions between the strains.

Materials and Methods

Drosophila stocks

Four strains of *Drosophila melanogaster* – 91-R, 91-C, MHIII-D23 and *ry*⁵⁰⁶ were used for the present study, all of which have been described in the previous chapter. The *ry*⁵⁰⁶ stock was used as a host strain for transformation studies. Balancer stocks that were used for linkage analysis of the transgene in transformant lines included a second chromosome balancer stock - *Sco/CyO; kar ry*⁵⁰⁶ and a third chromosome balancer stock - *TM3 Sb ry*⁵⁰⁶ *e*/TM6B *Tb e* (Lindsley and Zimm, 1992). Both the stocks were obtained from Dr. John Lucchesi, Emory University, Atlanta, GA. All stocks and transformant lines were maintained on standard *Drosophila* medium under conditions described in Chapter 2.

Screening of 91-C and MHIII-D23 genomic libraries.

91-C genomic DNA library in λ GEM-11, made from adult flies, was kindly provided by Dr. Larry Waters (Waters et al., 1992). The MHIII-D23 genomic library, also from adult fly DNA, was constructed in the phage EMBL3 (Stratagene) as described (Dombrowski, 1998).

Approximately 5×10^4 plaques from each genomic library were screened with a 2.0-kb *SaII-BamHI* fragment containing the upstream DNA of *Cyp6a8* from 91-R

(*Cyp6a8-91R*) (Fig 3-2, fragment 6). The plaques that hybridized to the probe were made plaque pure by two more rounds of screening. Phage DNA was isolated according to the methods described in Chapter 2.

Cloning of genomic DNA fragments

Restriction fragments (Fig. 3-2, fragments 1, 2, 3, 7 and 8) from the λ A3-1 genomic clone of the 91-R strain were cloned into the pBSKII⁺ plasmid (Stratagene). The resulting recombinant plasmids were then used to transform DH5 α strain of *E. coli* (Gibco-BRL). The upstream DNA of the *Cyp6a8* alleles of 91-C (*Cyp6a8-91C*) and MHIII-D23 (*Cyp6a8-MHIII*) strains were first identified via Southern blot hybridization. These DNAs were then isolated and cloned into the pBSKII⁺ plasmid. All plasmids used in this study were isolated using the alkali lysis method (Maniatis, 1982). Plasmids used for automated sequencing were purified by a subsequent step of PEG (polyethylene glycol) precipitation.

The genomic DNA fragments carrying the entire *Cyp6a8* transcription unit and the upstream DNA were sequenced manually or by using an automated sequencer (Applied Biosystem) using the dideoxy chain termination method (Sanger et al., 1977).

Sequence motifs present in the upstream DNA of *Cyp6a8* were identified by using MatInspector (Quandt et al., 1995), TF Search and WWW Signal Scan software programs available on the internet. The databases searched by the programs for transcription factor binding sites included the Transfac database, TFMATRIX database (Wingender et al., 1998) and the TFD database. Sequence comparison of the upstream DNA from *Cyp6a8-91R*, *Cyp6a8-91C* and *Cyp6a8-MHIIID23* was done using the PILEUP program of GCG (Genetics Computer Group, University of Wisconsin, Madison).



Figure 3-2: Restriction map of the λA3-1 genomic clone from 91-R.

The location of *Cyp6a8* and *Cyp6a9* are shown below the map. The dashed line shows the regions of the genomic clone that have been sequenced. The lines on top of the map represent the fragments that were subcloned into pBSKII⁺. The *Hind*III site indicated by a dotted line was generated artificially by incorporating sequences coding for a *Hind*III site in primers used to amplify this fragment. The 2.8-kb *Sal*I fragment (#3) and the 2.0-kb *Sal*I-*Bam*HI fragments (#6) were used to sequence the *Cyp6a8* genomic DNA. The 3.6-kb *Eco*RI fragment (#7) was used in all reconstructions for obtaining *Cyp6a8* upstream DNA to make the *Cyp6a8* upstream DNA-*luciferase* chimeric constructs. The 8.3-kb *Bam*HI fragment (#8) was used for germline transformation. There is a single *Spe*I site in the *Cyp6a8* upstream DNA up to the first *Eco*RI site. Other *Spe*I sites have not been mapped on the λ clone. B, *Bam*HI; R, *Eco*RI; H, *Hind*III; S, *Sal*I; Sp, *Spe*I.

Construction of recombinant plasmids with *Cyp6a8-91R* upstream DNA and the *luciferase* reporter gene

Cyp6a8-91R upstream DNA fragments (0.2 kb, 0.8 kb, and 3.1 kb) were individually cloned in front of the firefly *luciferase* (*luc*) gene in the pGL2 basic plasmid (Promega) in which a *NotI* site was created downstream to the *luc* gene (Fig 3-3, Step IV). The resulting chimeric plasmids were named p0.2/*luc*, p0.8/*luc* and p3.1/*luc*. The details of the cloning strategy are shown Fig. 3-3.

The *Cyp6a8-luc* cassettes carrying the *luc* gene with *Cyp6a8* upstream DNA were removed from the above chimeric plasmids as *XhoI/NotI* fragments (Fig. 3-3, step V). These fragments were then individually cloned between *NotI* and *SalI* sites of pDM30, a *Drosophila* P-element transformation vector (Mismer and Rubin, 1987). The resulting plasmids, pDM30/* series (Fig. 3-3, step V), were used for germ line transformation.

P-element mediated transformation and chromosomal localization of transgenes.

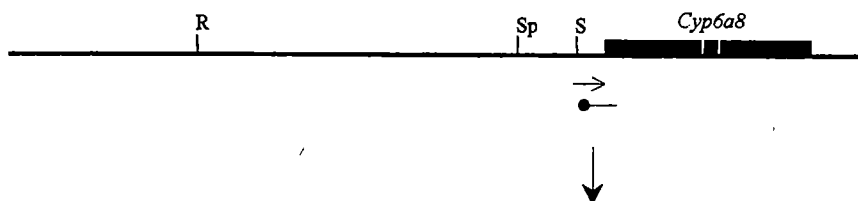
The pDM30/* series plasmids (pDM30/0.2-91R*luc*, pDM30/0.8-91R*luc* and pDM30/3.1-91R*luc*) were used for germ line transformation. Mixtures of each of these plasmids and p π 25.7wc helper plasmid (Karess and Rubin, 1984) were individually injected into preblastoderm embryos of the *ry*⁵⁰⁶ strain as described (Spradling and Rubin, 1982; Karess and Rubin, 1984; Karess, 1985). The helper plasmid produces transposase that is necessary for the transposition of the transposon from the pDM30 plasmid to any chromosome. Because the helper plasmid has defective P-element, it cannot induce its own transposition (Karess and Rubin, 1984) and is eventually degraded in the cell.

The transposon in the pDM30 transformation plasmids used in the present investigation has the wild type allele (*ry*⁺) of the *ry*⁵⁰⁶ mutation besides the *Cyp6a8-luc* cassette. Therefore, the embryos that survived and became adults (G₀) had wild type

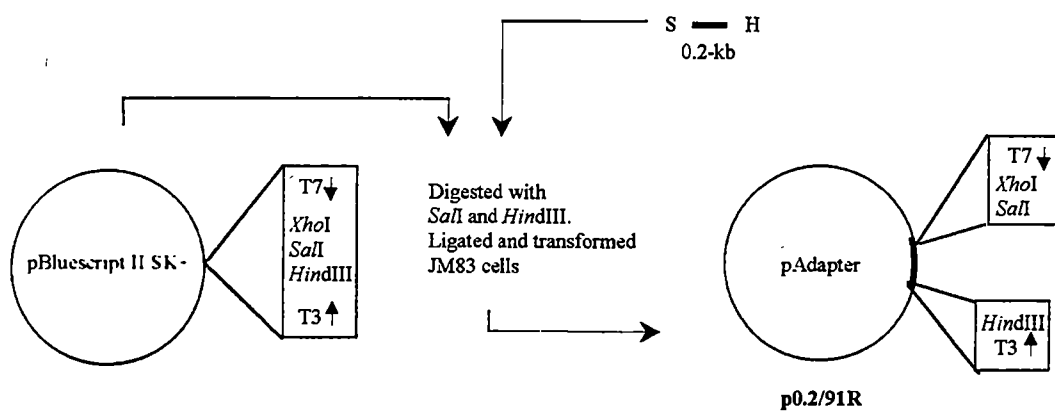
Figure 3-3: Construction of recombinant plasmids with *Cyp6a8*-91R upstream DNA and the *luciferase* reporter gene.

- I) Schematic diagram of *Cyp6a8* gene structure with the relevant upstream restriction enzymes sites indicated. R, *EcoRI*; Sp, *SpeI*; S, *SalI*; H, *HindIII*.
- II) Construction of pAdapter: The immediate *Cyp6a8* 5' flanking DNA was PCR amplified from a 91-R genomic DNA clone, using primers which had sequences for a 5' *SalI* and 3' *HindIII* site. The resulting fragment was cloned into pBSKII⁺. This plasmid is also referred to as p0.2/91R.
- III) Construction of p*/91R series plasmids: Longer upstream fragments were obtained as *XhoI*-*SalI* fragments from appropriate 91-R genomic DNA containing plasmids, and cloned into the *XhoI*-*SalI* sites of the pAdapter. This resulted in p0.8/91R and p3.1/91R. The *XhoI* (X*) site at the end of the 2.9-kb *EcoRI*-*SalI* and 0.6-kb *SpeI*-*SalI* fragment is from the MCS (multiple cloning site) of the plasmids from which these fragments have been obtained.
- IV) Construction of p*/luc series plasmids: pGL2basic (Promega) was first modified by generating a *NotI* site (underlined) downstream of the *luciferase* coding region. *Cyp6a8* upstream DNA was excised as *XhoI*-*HindIII* fragments from the p*/91R series of plasmids and as a *SalI*-*HindIII* fragment in case of 0.2/91R and cloned into the MCS (*XhoI*, *HindIII*) of pGL2basic in front of the *luciferase* reporter. This resulted in p0.2/luc, p0.8/luc and p3.1/luc plasmids.
- V) Construction of pDM30/* series plasmids: The *Cyp6a8* upstream DNA-*luciferase* cassette was excised as *XhoI*-*NotI* fragments from the p*/luc series of plasmids and cloned into *SalI*-*NotI* cut pDM30 to generate the transformation plasmids, pDM30/0.2(91R)luc, pDM30/0.8(91R)luc and pDM30/3.1(91R)luc.

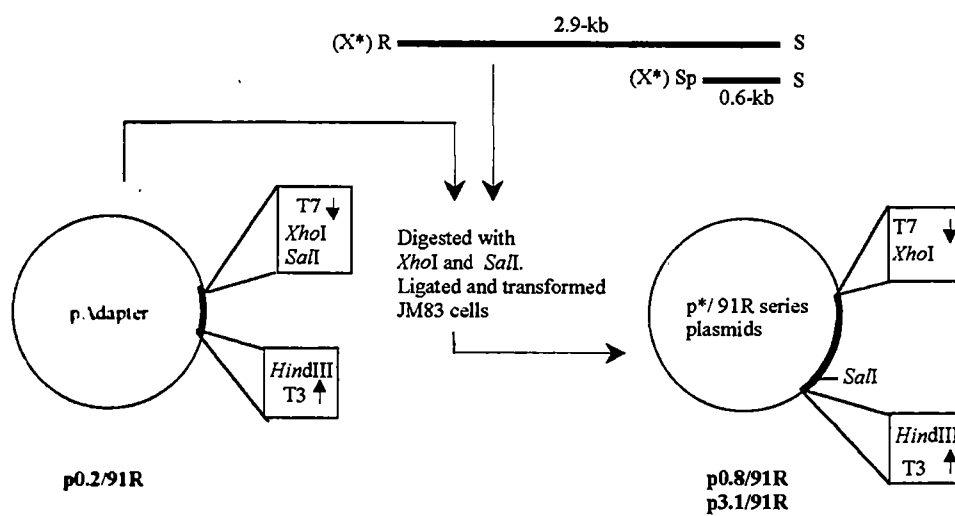
I



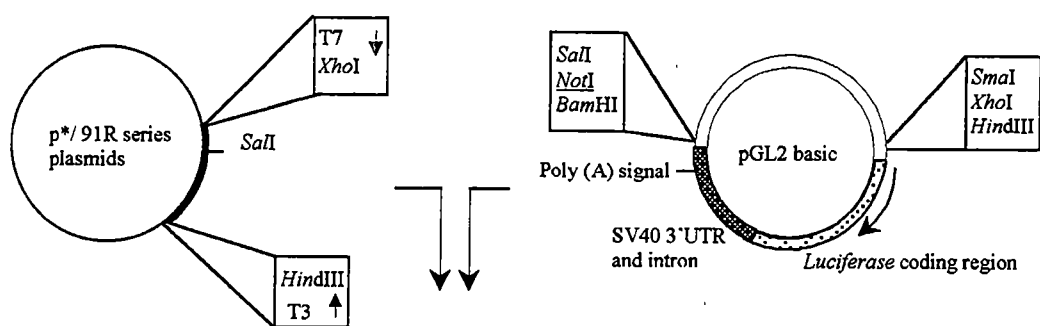
II



III

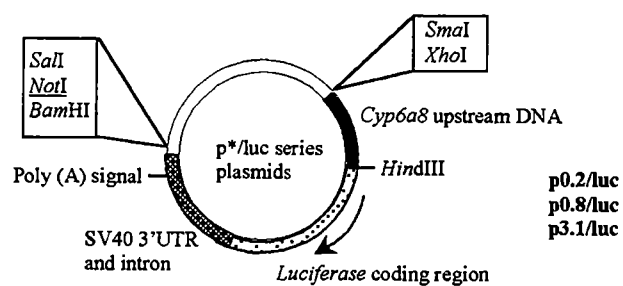


IV

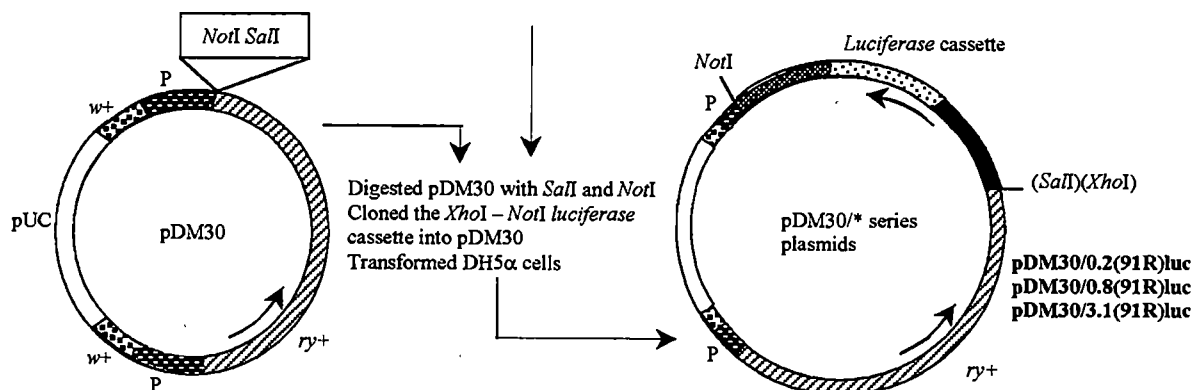
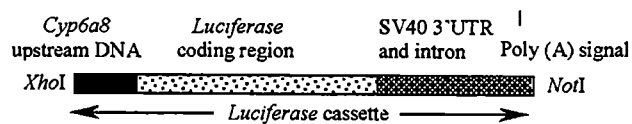


Removed insert from p*/91R series of plasmids as *XhoI* - *HindIII* fragments and cloned into pGL2 basic vector

V



Excised luciferase cassette as *XhoI* - *NotI* fragments



(bright red) eye color only if the transposon was retained in the cell. To determine whether a stable integration had occurred, G_0 adults with wild type eye color were individually backcrossed to ry^{506} flies of the opposite sex. If, the progeny (G_1 flies) showed the wild type eye color phenotype, it implied that a germ line transposition had occurred. A single G_1 fly with the wild type eye color was crossed to ry^{506} flies to establish a transformant line.

A transformation plasmid pDM30/8.3*Bam* was created by cloning the 8.3-kb *Bam*HI fragment from λ A3-1 (Fig. 3-2, fragment 8) into pDM30. P-element mediated germline transformation was done as mentioned above. Chromosomal linkage of the transposon carrying the 8.3-kb *Bam*HI fragment and the ry^+ gene was determined genetically by using appropriate 2nd and 3rd chromosome balancer stocks and dominant visible markers (Karess and Rubin, 1984).

Chromosomal location of the above transgene as well as transgenes carrying the *Cyp6a8-luc* cassettes and the ry^+ gene was determined by *in situ* hybridization technique as described (Ashburner, 1989). The cloned 8.3-kb *Bam*HI fragment or *rosy* DNA was biotinylated using a nick translation kit (Gibco-BRL) and hybridized with the polytene chromosomes of the transformant lines. The hybridization was detected by using a streptavidin-alkaline phosphatase system (Gibco-BRL).

Luciferase assay.

Since the transformant lines carrying the *Cyp6a8-91R-luc* transgene was not made homozygous, males of each line were crossed to ry^{506} virgin females and the F1 females with ry^+ eye color were collected. These F1 hybrid flies had one copy of the *Cyp6a8-91R-luc* transgene. All crosses were set up in large pint bottles. The parents were transferred to fresh bottles after 3-4 days, 4-5 times so that F1 hybrid flies could be collected over several days.

Only 24-28 h old F1 females with wild type (*ry*⁺) eye color were used for the luciferase assay. The flies were etherized and placed in eppendorf tubes in batches of 12. After the flies recovered from the effects of ether they were homogenized on ice, in 240 μ l of 1X CCLR buffer (Promega). (CCLR – Cell culture lysis reagent; 1X concentration has 25mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). The homogenates were kept on ice for 15-30 minutes and then centrifuged for 7 minutes at 14,000 rpm at 4°C. The supernatant was removed and re-centrifuged for 5 minutes under the same conditions. The clear extract was carefully removed so that most of the lipid layer on top was left behind. Extracts obtained this way were frozen at -80°C until analyzed.

The amount of luciferase enzyme in cellular extracts from different transformant lines was measured by analyzing light activity, using luciferin as a substrate. The Promega luciferase assay system and protocol (Promega Technical Bulletin 101) was used for this analysis. Extracts were taken out from -80°C, thawed on ice for an hour, and then kept at room temperature for about 15 minutes before the assay. Luciferase assay buffer (100 μ l) was equilibrated to room temperature and mixed with 20 μ l of the cellular extract in a glass vial. The glass vial was placed in a luminometer (Azur) and readings were taken at about 15 seconds after mixing. Two aliquots of each sample were assayed for the measurement of light emission and a mean of the two readings was taken as the light activity of the sample. The luciferase activity was expressed as light units/ μ g of total protein. Three such readings were obtained with independent homogenates of a particular transgenic line, which were averaged to get a mean reading for each transgenic line. All statistical calculations and graphical representations were done using the Microsoft Excel program.

Protein assay

The amount of total protein in the cellular extracts was quantified by using the BCA protein assay kit (Pierce). BSA (Bovine serum albumin 2mg/ml) supplied in the kit was used to make the standard curve. All samples, including the BSA standard, were adjusted to $\frac{1}{2}$ X CCLR buffer conditions. Ten μ l of the cellular extract was used for the assay according to the manufacturer's protocol. Optical density was measured at 562 nm using a Shimadzu Spectrophotometer. Two aliquots of each sample were assayed and the mean OD₅₆₂ reading was used to calculate the amounts of protein using the BSA standard curve.

Results and Discussion

Analysis of the *Cyp6a8* gene structure.

Genomic DNA fragments 3 and 6 (Fig. 3-2), that contain the transcription unit and upstream DNA of the *Cyp6a8-91R* allele, were sub-cloned into the pBSKII⁺ plasmid vector and used for sequencing with universal and gene-specific primers. The sequence data were compared with the sequence of the 1576 bp cDNA that was obtained previously. Genomic DNA sequencing yielded about 1.2-kb upstream and 0.3-kb downstream sequences in addition to the sequences in the cDNA. The composite sequence data are shown in Fig. 3-4. Because the transcription start point (TSP) could not be determined with accuracy, A in the first ATG in the cDNA was designated as +1 (Fig. 3-4). This ATG codon is preceded by the sequence CAAC (Fig. 3-4) which matches the consensus (C/A)AA(A/C) sequences present immediately before translational start sites in *Drosophila* (Cavener, 1987; Kozak, 1987). This ATG codon is presumed to code for the first methionine in CYP6A8.

Figure 3-4: Nucleotide and deduced amino acid sequence of *Cyp6a8*.

Nucleotides are numbered on the left and amino acids on the right. All cDNA sequences are in lower case letters while genomic DNA sequences and introns are in uppercase letters. The introns start at +993 and +1102 are also delineated with vertical arrows on top of the sequence. The putative TATA box, CAAT box, arthropod initiator (Inr) sequences and cap sites are boxed. Putative barbie boxes in the upstream DNA are double underlined and numbered 1-12. Promoter elements believed to be a part of the basal promoter are outlined in bold lines. Upstream *SalI* site at -199 and *SpeI* site at -761 are underlined for reference. Sequencing the genomic DNA revealed two differences with the cDNA sequences obtained and these are marked by bold letters within the coding region. The start codon (**atg**) and the stop codon (**taa**) are in bold and underlined. Putative polyadenylation signals are bold and double underlined. Sequences showing similarity to 'terminator' sequences found proximal to the 2nd poly A signal are bold and underlined with a wavy line. Some of the conserved regions of the CYP6A8 protein, like the heme binding region, are boxed.

-1237 AACCGGAATTTAGGTAGGAAAATTTGTTTGGGGGTATATAAATTTTGGGGGGGAATTTT
 -1177 TATGGGAAACTTATGGATGTCCTTCTAATACTCATTTTTTAAACAAATAAAGCTGGTTGCT
 -1117 GGATAATATGCAAAATATAAACTTTTCGTTCTTTGTTTATGTAACCTTTTCATTGATAAC
 -1057 ATTTTAACGCCCAATTAACGTTTAAATGGCATAGAAAATAAAAAACAAATGAACAAAAGA
 -997 AAAGCTCAATCGCAAAGCTGAAATAAAGCCAAAATAAATAAAAAATAGAAAAGCTACCTG
 -937 TTTTAATTACGCGTTGTTGTTGCCGATATCCGCACTTGAAATCTAATCAAATGTCAAACA
 -877 AATATAACTGAAAAGGTAAACAATGTGCTTGCCAGATCCGTCAAATATCAGGAGAAGTAA
 -817 TGCCCATATCATGTTACGCTGTCAATAGAACACGAATCCCACTGATGAAGTAGCCACTA *SpeI*
 -757 GTTGTCCGTAAGGGGGAAGAGATAAATTGCCGAGGGCTCGTCTAAATACAGTATAAACTG
 -697 CCTAACGTAGCTTACTATACTTAAGGTGTACCATTTGATTTTAGATCAATATATTTATAG
 -637 GCGTGTACTTTAGGTAGTACTATTGTGATCTTCAAAAATGCACTAATAAACAGTGCTTTA
 -577 AAGAATTTGCTCTAAGATTGTATCATATAAATTGTATGAACTAAGTTTCTAAAATGACA
 -517 CACTCATATAGAGTCATGGTTATTTTGTGTTGGTAGGTTAGTAGCATTATCAATGAGAAA
 -457 TCAAAAGAAATGAACTGTCTGGTCAACCCATATAATTGTCTTCAATATAATGATCGCTGA
 -397 AAGCAACCGTGAGTAAGCAGACTTGCAAAAAATTATTGATAAGTGTATACTTTTAGCAC
 -337 TAGGCGTTGTGATAAGTCACAGTTCGTCACTGTACAGTACTTCTTCATTATTCATCACCG
 -277 GTATATGAGGGATAATACAATAAATTCCGAATAAATAAACACTTTTTTGCTCAATTTGC
 -217 CTACAAAATAATGATCATGTGACGTTGGTTTCTGTCCTTATCTATTACAGACTACGATTC
 -157 TTGCCATCTCACAGTGCCTTGGCATTAAAGTAAACAAAACCTCTCTCTTTCTAGCGACTGAG
 -97 AATGCATCGTCATGCAAAGTAAGTCAATTTCAAGCGTTTAAAAAGCAGTTTGCCTATCGTTT
 -37 ACGGAATCAGTTCGATTTCGAAGATCATccgcctcaacatggcggttgacttacatcctttt
 23 ccaagtggcggtgcgcctactggcgatcttaacctactatattcaccgcaagctaacata
 Q V A V A L L A I L T Y Y I H R K L T Y

Cap site Inr
 CAAT box
 Inr
 Cap site
 Sali
 TATA box
 +1
 M A L T Y I L F

8 28

83 tttcaagcgtcgagggattcccttcgttgcaccgcatctcatcaggggaacatggagga
 F K R R G I P F V A P H L I R G N M E E 48
 143 attacaaaagacgaaaaatatccacgagatattccaagatcactataacaaattcagggga
 L Q K T K N I H E I F Q D H Y N K F R E 68
 203 aagcaaggcaccttttgttggatttttctttttccaaagtcccgtgcatttgtgataga
 S K A P F V G F F F F Q S P A A F V I D 88
 263 tcttgaattggccaagcaaatattattaaggacttctcgaatttctcgaacaagggtat
 L E L A K Q I L I K D F S N F S N K G I 108
 323 attctacaatgaaaaagatgatcccatttctgcccattctcttcaatctggacgggtgccca
 F Y N E K D D P I S A H L F N L D G A Q 128
 383 gtggcgtttgttaaggaacaaattatcatcgacctttacatccggtaaaatgaagttaat
 W R L L R N K L S S T F T S G K M K L M 148
 443 gtatccgaccgtagtgcagtgaggccaatgaatttatgaccgtgatgcacgagaaagtgcc
 Y P T V V S V A N E F M T V M H E K V P 168
 503 gaaaaattcggttctggagatacgtgatctggtggccagggtttacggtggatgtgatcgg
 K N S V L E I R D L V A R F T V D V I G 188
 563 aacctgcgccttttggcatccaatgcaacagtctgcgcgatgaaaaggccgagtttctgta
 T C A F G I Q C N S L R D E K A E F L Y 208
 623 ctttggaaagaggtcttttggttgacaagagacatgggactctattgaatggcttttatgcg
 F G K R S L V D K R H G T L L N G F M R 228
 683 cagctatcccaaattggccaggaaattgggtatggttcgaactgcaccgcatattcagga
 S Y P K L A R K L G M V R T A P H I Q E 248
 743 attctatagtagaattgtcacagaaacagtggcgggtgcgagaaaaagagcacatcaaacg
 F Y S R I V T E T V A V R E K E H I K R 268
 803 caacgatttcatggacatgctgattgaacttaaaaaatcaaaaggaaatgactctagagaa
 N D F M D M L I E L K N Q K E M T L E N 288
 863 tggcgatgtggtcagaggattaaccatggaggaggttttggcacaagcctttgtgttctt
 G D V V R G L T M E E V L A Q A F V F F 308
 923 cattgctggcttttgagacatcctcctccaccatgggatttgcctatacgaactggcaaa
 I A G F E T S S S T M G F A L Y E L A K 328

Comparison of genomic and cDNA sequences identified differences at two places within the coding region of *Cyp6a8* (Fig. 3-4). The first one involves the fourth amino acid. According to the cDNA sequence, the fourth codon codes for alanine whereas according to the genomic DNA sequence the amino acid coded for is a threonine. The second site is associated with the 193rd codon, which is GGC in the genomic DNA but GCG in the cDNA, coding for glycine and alanine, respectively. These differences may be due to sequencing error or polymorphism of *Cyp6a8* in the 91-R population. It is possible that the cDNA and the genomic clones used for sequence comparison are from two different alleles that may be present in the population.

Comparison of the genomic and cDNA sequences also revealed the presence of two introns. The first intron is 36 bp long and starts at position +993. The second intron is 59 bp long and starts at position +1102 (Fig. 3-4). The position and the phase (Gotoh, 1993) of the second intron coincides exactly with introns of similar lengths reported for *CYP6A1* (Cohen et al., 1994) and *Cyp6a2* (Dunkov et al., 1997). The 5' splice donor site (tg/GTATGT) and the 3' splice acceptor site (TATCCTTGCAg/a) also match well with the consensus splice sites reported for eukaryotes (Shapiro and Senapathy, 1987; Fig. 3-5). The first intron, however, does not have sequences similar to either consensus or non-conforming splice sites nor does it have a pyrimidine rich region that is generally found towards the 3' end of introns (Shapiro and Senapathy, 1987). It is possible that *Cyp6a8* is polymorphic in the 91-R population. One allele has an intron, while the other does not. The cDNA that was studied in this investigation may not have originated from the genomic clone that was sequenced. This possibility can be verified by RT-PCR.

The presence of first intron in *Cyp6a8* also raises an interesting point with respect to the evolution of CYP6 family genes in insects. Generally, organization of *CYP* genes belonging to the same family tends to be conserved as has been observed in case of mammalian *CYP* genes (Lewis, 1996a; Gotoh, 1993). *Cyp6a8* departs from this general

	5' ss	3' ss
Consensus	ag/GTAAGT-----TTTTTCTTTTTCAG/g	
Intron1	ac/ATTCAA-----AGGTGGAGGAGGTC/a	
Intron2	tg/GTATGT-----TAATATCCTTGACAG/a	

Figure 3-5: Splice junctions of the two introns present in *Cyp6a8*

Lower case letters represent exon sequences and the upper case letters represent intron sequences. While the second intron (at +1102) carries nucleotides similar to the consensus sequence for both the 5'ss (splice site) and the 3'ss, this is not the case with the first intron (at + 993) (Fig. 3-4).

trend because its closest relatives, the *Drosophila Cyp6a2* and the housefly *CYP6A1*, lack an intron at equivalent positions. Interestingly, conceptual translation of the intron-I sequences gives a 12 amino acid long open reading frame (ORF) that is in-frame with the CYP6A8 polypeptide. The presence of weak splice sites and in-frame ORF in intron-I may signify one of two opposing evolutionary trends. It could either signify that the *Cyp6a8* gene is in the process of gaining the in-frame ORF as an exon or it is in the process of losing this ORF by placing it in an intron.

Transcription start point and basic promoter sequences

DNA sequences immediately upstream to a gene contain basic promoter elements that control basal level of transcription. Primer extension experiments were done first to determine the transcription start point (TSP). Although an extended product was observed, the intensity of the signal was too faint to draw a definite conclusion (data not shown). Therefore, a different approach was taken to determine the approximate location of TSP. For this, two genomic DNA fragments were used as probes for Northern blots of 91-R poly A⁺ RNA. The first probe was a 0.19-kb genomic DNA fragment located between -11 and the *SaII* site at -199 position (ATG at + 1) of the upstream DNA (Fig. 3-4; Fig 3-2, fragment 4). The second probe was the 0.6-kb *SaII-SpeI* fragment (Fig 3-2, fragment 5) located immediately upstream of the 0.19-kb fragment. While a very faint hybridization was observed with the 0.19-kb probe, no hybridization signal was seen with the 0.6-kb probe. This suggests that the 5'-end of the mRNA definitely does not extend into the 0.6-kb DNA and the TSP is located within the 0.19-kb DNA, which is immediately upstream to the 5'-end of the cDNA. Faint hybridization suggests that the 0.19-kb DNA has a very short sequence that is complimentary to the CYP6A8 mRNA.

That the TSP is located within the 0.19-kb upstream DNA became a strong possibility when the genomic DNA upstream to the cDNA sequence was analyzed by

using internet software programs (Methods) to identify motifs important in transcription. This analysis identified a putative non-canonical TATA box (5'-CGTTTAAAA-3', Fig. 3-4) at the -64 position, with a score of 86.5 (TF Search program, 100 being a perfect match). Several pertinent sequences were then discovered with reference to this putative TATA box, all of which are marked in the upstream DNA sequences in Fig. 3-4. About 30 bases upstream to the putative TATA box a sequence (5'-ATGCATCGT-3') was found, which may be a CAAT box. Although this does not have much similarity with the eukaryotic CAAT box sequences, it is similar to the *Drosophila* consensus sequence [5' AA/TGCAA/TNA/TN 3'] as defined by O'Connell and Rosbash (1984). An ATF (Activator Transcription Factor) binding site and an AP1 (Activator Protein 1) site was found upstream to the putative TATA box at -92 and -80 respectively, on the non-template strand. About 34 nt downstream to the putative TATA box was a consensus CAP site (CANYYY) at the -30 position. At -31, a CCAGT sequence was found, that is very similar to TCAGT, a consensus sequence for an arthropod transcription initiator element (Cherbas and Cherbas, 1993). Generally, RNA polymerase II transcription units have a TATA box and an initiator element (Weis and Reinberg, 1992; Smale and Baltimore, 1989). While the TATA box is more important, the initiator element is also important for transcription initiation especially for genes that lack a TATA box. These initiator elements are situated -10 to +10 from the TSP for arthropod promoters (Cherbas and Cherbas, 1993). In case of *Cyp6a8*, the CCAGT sequence found at -31 is only 1 bp upstream to a putative cap site at -30. Although CCAGT is not the consensus initiator sequence, nor is it a cognate sequence, this sequence has nevertheless been found in some arthropod promoters (Cherbas and Cherbas, 1983). It has been postulated that in case of a weak TATA box, initiator sequences may increase the affinity of TFIID for the promoter and hence increase transcription. The presence of an initiator element thus becomes significant, as the putative TATA box does not show strong similarities with the

canonical TATA box. Such initiator elements seem to be conserved in CYP6 family genes because the consensus initiator sequence (TCAGT) is found close to the TSP in *CYP6A1*, *Cyp6a2* and *CYP6D1* genes (Cohen et al., 1994; Dombrowski, 1998; Scott, 1999).

Analysis of sequences 3' to the coding region

cDNA probes for *Cyp6a8* recognize two transcripts of 2.0-kb and 1.8-kb (Fig. 2-7) implying that there may be alternative TSP or alternative polyadenylation signals. If it is assumed that both RNAs have the same polyadenylation site, TSP for the 2.0-kb RNA should be 0.2-kb upstream of the TSP of the 1.8-kb RNA. This would place the TSP of the larger RNA approximately 200 nt upstream of the putative TSP at -30. Visual scanning of the upstream sequence showed that was a consensus cap site (CATCTC) at -153 followed immediately by a cognate initiator site (ACAGT) at -147, both of which together could specify a TSP. However, extremely poor hybridization of the 0.19-kb DNA fragment spanning between -11 and -199 bp (Fig 3-2, fragment 4) to poly A⁺ RNA argues against this possibility. If the capsite at -153 was functional a much stronger hybridization should have been observed with the 0.19-kb probe on Northern blots.

Analysis of the cDNA sequences revealed a poly A tail of 18 adenine residues at the 3' end. A non-canonical poly A signal (AATAAC) is found 29 nt upstream from the site of addition of the adenine residues. Analysis of the genomic DNA identified a canonical poly A signal (AATAAA) that is located 163 nt downstream of the non-canonical poly A signal (Fig. 3-4). This implied that the cDNA clone isolated was one that was derived from the shorter mRNA transcript. The difference in the position of the two poly A signals could account for the difference in the size of the two *Cyp6a8* transcripts.

Several reports have indicated that in addition to the poly A signal, terminator

sequences may play an important role in the termination of transcription (Wahle and Keller, 1992). Reports have indicated that sequences like TTTTATA, present upstream to the poly A signal may act as a partial termination signal resembling rho-mediated termination in bacteria (Henikoff and Cohen, 1984). In the light of these reports, it is interesting that a TTTTAT sequence is found upstream to the distal poly A signal and that there is a complementary sequence TAAAA which has the potential to form a hairpin structure, albeit a very weak one (Fig. 3-4). Since the distal canonical poly A signal is presumably stronger than the proximal one and also has terminator-like sequences upstream to it, this site is probably the preferred site for polyadenylation. This may account for the fact that the 2.0-kb transcript of *Cyp6a8* is more abundant than the 1.8-kb transcript (data not shown).

The ORF for the isolated *Cyp6a8* cDNA is 1460 nt. With the addition of a 5'UTR of about 30 nt, a 3'UTR of 83 nt (when the non-canonical poly A signal is used) and a poly A tail of about 200 residues, the size of the CYP6A8 mRNA is expected to be 1773 nt. This value corresponds well with the size of the shorter CYP6A8 mRNA, which is estimated to be 1.8-kb. An additional 163 nt in the 3'-UTR would yield the higher molecular weight transcript that is estimated to be 2.0-kb by Northern blot hybridization.

Identification of sequence motifs relevant to *CYP* genes

Analysis of the 1235 bp upstream DNA of *Cyp6a8* using the programs mentioned earlier identified a sequence at -62 position that contains an AAAG sequence (Fig. 3-4). This is the same as the core sequence of a motif known as the barbie box. This 15 bp sequence motif was originally described by He and Fulco (1991) and is found in the upstream DNA of barbiturate inducible *CYP102* and *CYP106* of *Bacillus megaterium*. Later, similar sequences were found in many other barbiturate inducible genes (Kemper, 1998). Interestingly, the barbie-box-like sequence found at -62 position of the *Cyp6a8*

gene overlaps with the putative TATA box (Fig. 3-4). Such an arrangement has also been seen for the *Cyp6a2* gene (Dombrowski, 1998). Since multiple barbie boxes are found in the *Cyp6a2* gene, the upstream DNA of *Cyp6a8* was also visually scanned for the presence of the core barbie box motif (AAAG). Eleven additional sequences were identified in the upstream that have the core sequence motif and may act as potential barbie box elements (Fig. 3-4). However, later experiments indicated that only some of these putative barbie box sequences are functionally important.

Although the substrate for *Cyp6a8* is not known, the fact that it is upregulated in two insecticide resistant strains of *Drosophila* (Chapter 2) may indicate that this enzyme could have xenobiotic metabolizing functions. Several genes including murine *CYP1A1* and glutathione-S transferase genes, that are involved in the metabolism of chemicals, have sequence motifs called XRE (xenobiotic response element) and ARE (antioxidant response element) associated with the upstream sequences (Whitlock, 1999; Rushmore et al., 1991). With this in mind the upstream DNA of *Cyp6a8* was searched for consensus XRE and ARE using the bestfit program of GCG (Genetics Computer Group, University of Wisconsin, Madison). This search identified a sequence (CATGCAA) between -87 and -81 bp which is similar to the complement of the XRE sequences (TNGCGTG) found in the xenobiotic responsive *CYP1A1* gene of mouse (Denison et al., 1988; Denison and Whitlock, 1995). Another close-by sequence (GCATCGTCAT), located between -94 and -85 bp, was found to be perfectly complementary to the ARE consensus sequence present in the upstream DNA of genes involved in xenobiotic metabolism (Rushmore et al., 1991). These sequences have also been found in the upstream DNA of some CYP6B subfamily genes (Hung et al., 1996). The fact that these sequences are found so close and upstream to the basal promoter elements suggests that these sequences may have some functional significance.

Binding sites for several transcription factors with known functions in development are also found in the upstream DNA of *Cyp6a8*. Some of these sequences are also found in the upstream DNA of *Cyp6a2* (Dombrowski, 1998). In the *Cyp6a8* DNA these include five BRCZ motifs (Broad-Complex binding sites) with matrix similarities more than 0.94 as identified by the MatInspector analysis (Fig. 3-6, at positions -1145, -1025, -970, -961, and -133). Broad-Complex (BR-C) is a family of zinc-finger proteins that act as transcriptional regulators in *Drosophila*. BR-C genes are primary ecdysone response genes that play an important role in metamorphosis by transmitting the ecdysone signal to its target genes. Binding sites for another ecdysone induced transcription factor called E74A is found in the upstream DNA of *Cyp6a8* at position -1243 (Fig. 3-6). Under reduced stringency of comparison (core similarity - 1; matrix similarity - 0.87) a second E74A binding site is found at -260 on the template (sense) strand (Fig. 3-6). BR-C and E74 are thought to regulate the expression of salivary gland glue protein genes and some other genes that act late in *Drosophila* metamorphosis (Fletcher and Thummel, 1995). To date, only one ecdysone-inducible *Drosophila* CYP gene, called *Eig17-1*, has been isolated via subtraction hybridization (Hurban and Thummel, 1993). Sequence analysis suggests that this gene is much closer to *CYP2D* rather than *CYP6* or *CYP4* family genes (Hurban and Thummel, 1993). Presence of sequence motifs similar to the BR-C and E74 binding sites may make *Cyp6a8* ecdysone responsive. A simple way to examine this possibility would be to examine whether CYP6A8 mRNA levels change concomitantly with the ecdysone level during larval development.

Using the BESTFIT program in GCG (Genetics Computer Group), a third ecdysone related sequence called ecdysone response element (EcRE) was found at -102 position of the *Cyp6a8* gene (Fig. 3-6). This sequence shows a 77% identity to the consensus EcRE sequence (Dombrowski, 1998). The same study (Dombrowski, 1998)

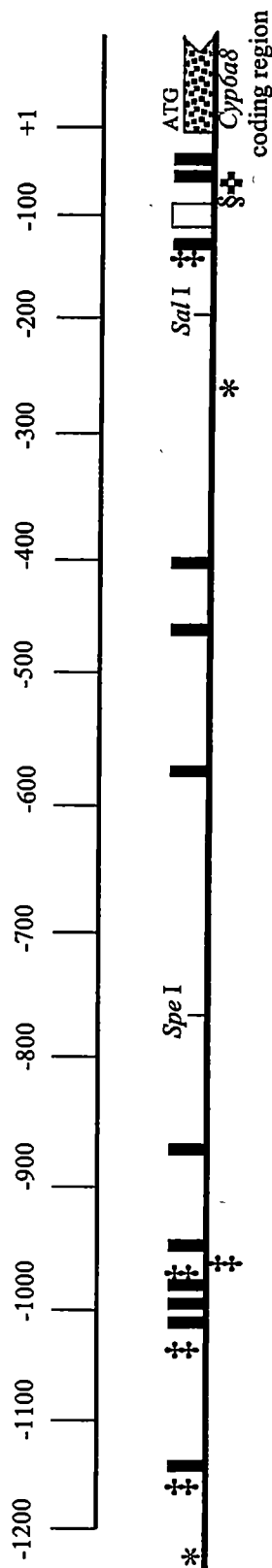


Figure 3-6: Position of transcription motifs in *Cyp6a8* upstream DNA.

Cyp6a8 upstream DNA was analyzed using World Wide Web based software programs. Some of the transcription motifs that are associated with the upstream DNA of *CYP* genes were also found to be present in the *Cyp6a8* upstream DNA. The *SalI* and *SpeI* sites have been kept as points of reference. The following symbols represent the position of various motifs - ■, barbie box, ‡ BRCZ (Broad-complex binding site), □ EcRE (ecdysone response element), and * E74A (E74A binding site; E74A is an ecdysone inducible transcription factor), § ARE (antioxidant response element), † XRE (xenobiotic response element). Position of the symbols on the top of the line indicate that they are on the non transcribed DNA strand whereas position of the symbols below the line indicate that the binding sequence is on the template strand.

also showed the presence of an EcRE sequence at -491 position of *Drosophila Cyp6a2* and other family 6 genes, e.g. *CYP6A1*, *CYP6A3-5*, *CYP6C1*, *CYP6B1* and *CYP6B3v2*. This suggests that family 6 genes may be regulated by ecdysone titer during development. In this context it is interesting to note that in *Drosophila*, the expression of *Cyp6a2* is lower in a temperature sensitive ecdysone deficient mutant (*ecd^l*) than in the wild type strain (Spiegelman et al., 1997). Therefore, presence of the EcRE in the upstream DNA of *Cyp6a8* may be functionally meaningful.

Functional analysis of *Cyp6a8* upstream DNA sequences

Sequence analysis of the *Cyp6a8* upstream DNA revealed several motifs that may act in facilitating and regulating expression of *Cyp6a8*. To delineate the actual regions that may be involved, an 8.3-kb *Bam*HI fragment from λ A3-1 (Fig. 3-2, fragment 8) was used for germline transformation into *ry⁵⁰⁶* embryos. Germline transformants were selected and made homozygous. *In situ* hybridization of a biotinylated probe carrying the 8.3-kb *Bam*HI fragment showed that in the transformant line Tp7.5A1, the transgene maps at band 100C of the polytene chromosome (data not shown). Poly A⁺ RNA isolated from this homozygous transformant line was analyzed by Northern blotting and subsequent hybridization with a *Cyp6a8* cDNA fragment as probe. Fig. 3-7 shows that both the transcripts of *Cyp6a8* are produced at high levels in the transformant line. Densitometric analysis showed that the levels of the *Cyp6a8* transcripts are about two times greater in Tp7.5A1 than the *ry⁵⁰⁶* host strain. Thus, the *Cyp6a8-91R* transgene is transcriptionally active in the *ry⁵⁰⁶* genome, and the *cis*-acting sequences required for the expression of *Cyp6a8* are present in the 8.3-kb *Bam*HI fragment.

Since the previous experiment showed that *Cyp6a8-91R* could express in the *ry⁵⁰⁶* background, this strain was used as a host for transformation to further delineate the *cis*-regulatory elements of the *Cyp6a8* gene. For this purpose a reporter gene system was

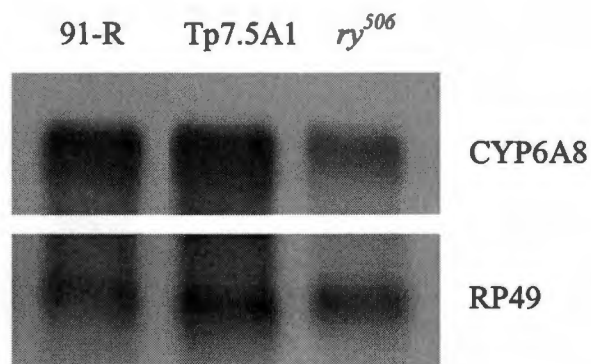


Figure 3-7: Expression of *Cyp6a8-91R* transgene in the *ry*⁵⁰⁶ host strain.

A Northern blot was prepared by electrophoresing poly A⁺ RNA isolated from unsexed flies, homozygous for the transgene from the transformant line Tp7.5A1. Poly A⁺ RNA from 91-R and *ry*⁵⁰⁶, unsexed adults were run as controls. The blot was hybridized with ³²P-labeled 0.8-kb CYP6A8 cDNA fragment. RP49 was used as an internal control to correct for RNA loading.

used. Different lengths (0.2- 0.8- and 3.1- kb) of upstream DNA from *Cyp6a8* were individually cloned in front of the *luciferase* reporter gene from the firefly. The *Cyp6a8-luc* chimeric gene was then cloned into a transforming vector (see Methods) which has the wild type allele (ry^+) of the ry^{506} gene. These recombinant plasmids were microinjected into ry^{506} embryos and a total of seventeen transformant lines were obtained. Chromosomal locations of the *Cyp6a8-luc* transgene in each line were determined by *in situ* hybridization. The position and number of transgene insertions in each transgenic line is indicated in Table 3-2. These transformant lines were backcrossed to the ry^{506} host strain and the F1 females with wild type eye color were used for luciferase assay. This ensured that all flies carried the transgene in a single copy. Since sexual dimorphism had been reported for gene expression in case of several mammalian *CYP* genes, such dimorphism could be also present for *Drosophila* *CYP* genes. This has been discussed in a greater detail in the next chapter. Therefore, only female flies were assayed for luciferase activity.

The levels of luciferase activity observed in the different transgenic lines are shown in Fig. 3-8. An average activity was calculated for each construct by taking the mean of the activity shown by all the lines in the set, excepting the obvious outlying lines (Table 3-3). Lines 3.1luc-36 and 0.8luc-109 express at much lower levels than the other lines in the group and were excluded from the analysis (Fig. 3-8).

The results (Fig. 3-8; Table 3-3) show that luciferase activity was lowest (3.8×10^3 RLU/ μ g protein) in transformants carrying 0.2-kb upstream DNA (-11 to -199 nt) of *Cyp6a8*. The fact that this construct expresses the luciferase gene suggests that this DNA fragment has sequences that have promoter activity. However, a 5-fold increase in luciferase activity (18.8×10^3 RLU/ μ g protein) was observed when 0.8-kb (-11 to -761 nt) upstream DNA was used to drive the expression of the *luciferase* reporter gene (Table 3-

Table 3-2
Characterization of chimeric *Cyp6a8-luciferase* transgenes in *Drosophila*.

Transgenic Lines	Linkage Group	Cytological position	Number of transgenes
0.2-4 luc	2R	49E	1
0.2-6 luc	X and 3R	10F and 98E	2
0.2-7 luc	3L	62C and 70B	2
0.8-c luc	2L	21C	1
0.8-14 luc	3R	95C	1
0.8-109 luc	3R	99E	1
0.8-121 luc	3R	86A	1
0.8-123 luc	3R	91F	1
3.1-2 luc	3L	66D	1
3.1-4 luc	2R	49A	1
3.1-36 luc	3L	79F	1

The position of the luciferase transgenes was mapped by *in situ* hybridization in the transgenic lines by using *ry*⁺ biotinylated probes.

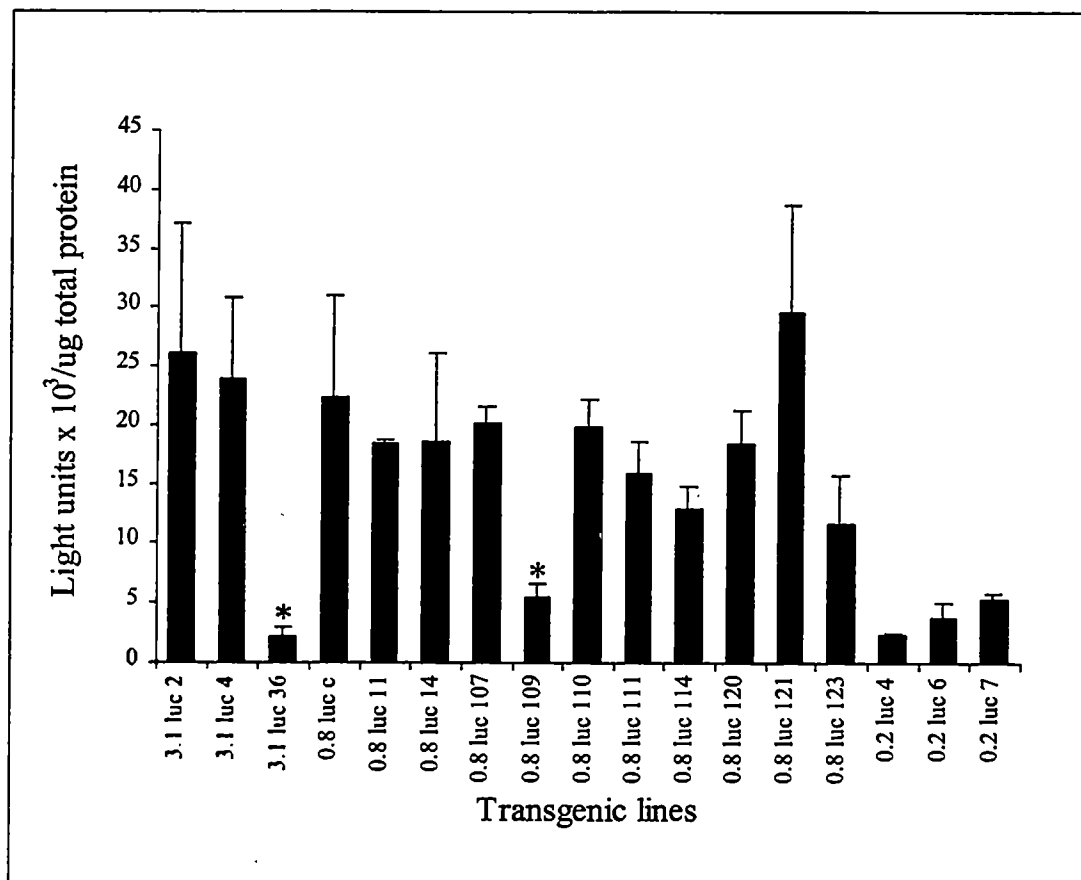


Figure 3-8: Luciferase activity in transgenic lines of *Drosophila* with chimeric *Cyp6a8-luc* constructs.

Seventeen transgenic lines were generated that carried chimeric luciferase constructs with 0.2-kb, 0.8-kb and 3.1-kb upstream DNA from *Cyp6a8*. They are designated as 0.2luc*, 0.8luc* and 3.1luc*. Males from each transgenic lines were crossed to *ry*⁵⁰⁶ females and the F1 wild type (red eyed) adult females were collected. The total body homogenate obtained from such females was used to assay luciferase activity. Light activity was expressed per microgram of total protein used for the assay. Each bar in the histogram represents a mean of three samples (light units/μg total protein). The low level of activity in transgenic lines (0.8luc109, 3.1luc36) marked with an asterisk is probably due to the position of insertion of the transgene.

Table 3-3
Mean luciferase activity in transgenic lines of *Drosophila*
carrying *Cyp6a8-luc* chimeric constructs

Constructs present in transgenic lines	Number of transgenic lines	Mean luciferase activity*	Standard deviation [#]
<i>3.1Cyp6a8-luc</i>	2	24.97 x 10 ³	±1.6
<i>0.8Cyp6a8-luc</i>	10	18.77 x 10 ³	±5
<i>0.2Cyp6a8-luc</i>	3	3.82 x 10 ³	±1.5

*The average luciferase activity shown by transgenic lines carrying a particular construct, was calculated by averaging the mean activity shown by all the transgenic lines with the same construct. The numbers represent the relative light units/μg of total protein. Transgenic lines that show obvious deviation from the general trend of activity were not included in the calculations.

[#] This represents the variation between the activity shown by different transgenic lines that have the same construct.

3). This suggests that DNA sequences between -761 and -194 nt have some additional *cis*-regulatory elements that confer higher promoter activity. Therefore, this region was scanned for potential binding sites for transcription factors using sequence analysis programs (Methods). Five potential AP1 binding sites were found to be present in this region, between the *SpeI* and *SalI* sites (Fig. 3-9). Two of these are present on the non-template strand at -524 and -388 nt, three are present on template strand at positions -328, -319 and -290 (Fig. 3-9). In addition to these, a single ATF binding site was found at -315 and an Oct 1 site at -295. These sequence motifs may be transcriptionally important and responsible for increased promoter activity of the 0.8-kb fragment. The presence of four TFIID binding sites was indicated at -706, -644, -552 and -216 (Fig. 3-9). Since there was no hybridization when the 0.6-kb *SpeI-SalI* fragment was used as a probe on a Northern blot implying that the TSP was further downstream, the significance of these binding sites is not clear.

Only 40% (1.4-fold) increase in luciferase activity (24.9×10^3 RLU/ μ g protein) was observed when the upstream DNA length was increased from 0.8-kb to 3.1-kb (Table 3-3). This suggests that the extra upstream DNA present in the 3.1-kb fragment may not have many *cis*-elements that are transcriptionally meaningful. Indeed, analysis of the upstream DNA sequence obtained (upto 1.1-kb) did not identify any transcriptionally important sequence motif except a potential Sp1 binding site at position -1054 (data not shown).

Comparison of upstream DNA of *Cyp6a8* alleles from three different strains of *Drosophila melanogaster*

In Chapter 2 it was shown that *Cyp6a8* exhibits variable levels of expression in different strains of *Drosophila melanogaster*. The level of expression of *Cyp6a8* is highest in MHIII-D23 and moderate in 91-R strains, whereas is barely detectable in 91-C

-817 TGCCCATATCATGTTACGCTGTCAATAGAACACGAATCCCAACTGATGAAGTAGCCACTA^{SpeI}
 -757 GTTTGCCGTAAGGGGAAGAGATAAATTGCCGAGGGCTCGTCTAAATACAGTTATAAACTG^{TFIID}
 -697 CCTAACGTAGCTTACTATACTTAAGGTGTACCATTGATTTTAGATCAATATATTTATAG^{TFIID}
 -637 GCGTGTA~~CTTTAGGTAGTACTATTGTGATCTTCAAAAATGCACTAATAAACAGTGCCTTA~~
 -577 AAGAATTTGCTCTAAGATTGTATCATTATAAATTGTATGAACTAAGTTTCTAAATGACA^{API}
 -517 CACTCATATAGAGTCATGGTTATTTTGTGTGGTAGGTTAGTAGCATTATCAATGAGAAA
 -457 TCAAAAGAAATGAAGTGTCTGGTCAACCCATATAATTGTCTTCAATATAATGATCGCTGA
 -397 AAGCAACCGTGAGTAAGCAGACTTGCAAAAAAATTATTGATAAGTGTATACTTTTAGCAC^{API}
 -337 TAGGCGTTGTGATAAGTCACAGTT~~CGTCACTGTACAGTACTTCTTCATTATTTCATCACC~~G^{API ATF}
 -277 GTATATGAGGGATAATACAATAAATTCCGAACATAAATAAACACTTTTTTGTCTCAATTGCT^{API}
 -217 CTACAAAATAATGATCATGTCGACGTTGGTTTCTGTCCTTATCTATTCAGACTACGATTC^{TFIID Sall}
 -157 TTGCCATCTCACAGTGCTTGGCATTAAAGTAAACAAAACCTCTCTCTTTCTAGCGACTGAG
 -97 AATGCATCGTCATGCAAAGTAAGTCAATTTCAGCGTTTAAAGGCAGTTTGCCTATCGTTT^{CAAT box ATF Oct1 TATA box}
 -37 ACGGAATCAGTTCGTATTTCGAAGATCATccgcctcaacatg^{Inr CAPsite API +1}

Figure 3-9: Transcription motifs in *Cyp6a8* upstream DNA that may enhance transcription from the basal promoter.

Sequences that recognize transcription factors that may have a role in enhancing transcription were identified using web based software programs. Such motifs are highlighted in the above sequence. The relevant motifs are written above the sequence, if the motif is on the non-transcribed strand. If they are on the template strand, they are written below the sequence. The *Sall* and *SpeI* sites are marked for reference. Sequences that are thought to be involved in basal promoter activity are boxed in. API sites are underlined by a wavy line. ATF sites are marked by strikethrough lines in the sequence. Oct1 sites are marked by bold letters. The TFIID binding sites mentioned in the text are in bold and double underlined.

strain (Fig 2-8). Since variation in the levels of expression may be due to differences in the *cis*-regulatory elements, the upstream DNA sequence of *Cyp6a8* alleles of 91-R, 91-C and MHIII-D23 strains were compared. To do so, the 2.0-kb *SalI*-*Bam*HI DNA (Fig. 3-2, fragment 6) representing the upstream region of *Cyp6a8-91R* allele was used to screen the 91-C and MHIII-D23 genomic libraries. DNA from the genomic clones that hybridized with the probe was isolated, restriction mapped and examined by Southern blot hybridization. This helped identification of the upstream DNA of *Cyp6a8-91C* and *Cyp6a8-MHIII* alleles, which were then isolated and sub-cloned into the pBSKII⁺ plasmid.

These DNA fragments were sequenced and compared with the upstream DNA sequence of the *Cyp6a8-91R* allele. This comparison identified many base substitutions between strains. These substitutions can be classified into three types. In type I (91-R/MHIII), the bases are identical in 91-R and MHIII-D23 strains but different in the 91-C strain. In type II (91-R/91-C), bases are identical in 91-R and 91-C, but different in MHIII-D23 strain. Type III (91-C/MHIII) involves the bases that are the same in 91-C and MHIII-D23 but different in 91-R strain.

There were 24 places in the upstream DNA where Type I substitutions are found (Fig. 3-10). This may be functionally significant because the bases at these sites are identical in 91-R and MHIII-D23 strains, which are overproducers of the CYP6A8 RNA. Seventeen of these 24 sites coincide with sequences that are potential binding sites for various transcription factors including Oct 1. Substitutions in the *Cyp6a8-91C* allele (with respect to Oct1 binding) occurred at the -304 and -1117 position (Fig 3-10). The nucleotide numbers correspond to those of *Cyp6a8-91C*. Interestingly, there is a 3 nucleotide (TAC) insertion five bases upstream to the proximal Oct1 binding site (at -304) in *Cyp6a8-91C*, while this insertion is absent in both *Cyp6a8-91R* and *Cyp6a8-*

Figure 3-10: Nucleotide sequence analysis of -1.1 kb upstream DNA of *Cyp6a8-91R*, *Cyp6a8-91C* and *Cyp6a8-MHIII*.

Uppercase letters represent the 5' flanking genomic DNA sequences while lower case letters represent sequences in the cDNA. The ATG in the cDNA is designated as +1. Shaded boxes mark regions of nucleotide substitution between the three alleles of *Cyp6a8*. The medium shade  represents Type I substitution (the nucleotide in alleles from the overproducer 91R and MHIII strains are the same while the same residue is different in the underproducer 91C strain). The darkest shade  represents Type II substitution (where the nucleotide is different in MHIII compared to 91R and 91C). The lightest shade  represents Type III substitutions (where the nucleotide is the same in 91C and MHIII but different in 91R). Only the places where such substitutions coincide with putative transcription motifs are boxed. Binding sites for AP1, ATF, flanking the 3 nucleotide insertion in *Cyp6a8-91C* are also boxed in. There may be other regions in the upstream DNA where such motifs are present but they have not been marked in the figure. Arrows beside the motifs indicate the DNA strand on which the motif is present. Thus, → represents motifs on the non transcribed strand, and ← represents motifs on the template strand.

91R	AAGCTGGTTG	CTGGATAATA	TGCAATAATA	AAAACCTTTT	GTTCTTTT	-1081
MHIII	AAGCTGGTTG	CTGGATAATA	TGCAATAATA	AAAACCTTTT	GTTCTTTT	-1092
91C	AAGCTGGTTG	CTGGATAATA	TGCAATAATA	AAAACCTTTT	GTTCTTTT	-1097

← Oct1

91R	TATGTAACTT	TTCATTGATA	ACATTTTAAC	GGCCCAATTA	ACGTTTAAAT	-1031
MHIII	TATGTAACTT	TTCATTGATA	ACATTTTAAC	GGCCCAATTA	ACGTTTAAAT	-1042
91C	TATGTAACTT	TTCATTGATA	ACATTTTAAC	GGCCCAATTA	ACGTTTAAAT	-1047

		BRCZ →		Barbie box →	
91R	GGCATAGAAA	TAAAAAAA	CAAATGAACA	AAAGAAAAGC	TCAATCAGAA
MHIII	GGCATAGAAA	TAAAAAAA	CAAATGAACA	AAAGAAAAGC	TCAATCAGAA
91C	GGCATAGAAA	TAAAAAAA	CAAATGAACA	AAAGAAAAGC	TCAATCAGAA

			→ Barbie box	
91R	AGCTTAAATA	AAGCCAAAAT	AAATAAAAAA	TAGAAAAGCT
MHIII	AGCTTAAATA	AAGCCAAAAT	AAATAAAAAA	TAGAAAAGCT
91C	AGCTTAAATA	AAGCCAAAAT	AAATAAAAAA	TAGAAAAGCT

BRCZ ←

91R	ATTACGCGTT	GTTGTTGCG	ATATCCGCAC	TTGAAATCTA	ATCAAATGTC	-883
MHIII	ATTACGCGTT	GTTGTTGCG	ATATCCGCAC	TTGAAATCTA	ATCAAATGTC	-894
91C	ATTACGCGTT	GTTGTTGCG	ATATCCGCAC	TTGAAATCTA	ATCAAATGTC	-897

91R	AAACAAATAT	AACTGAAAAG	GTAACAATG	TCGTTGCCAG	ATCCGTCAAA	-833
MHIII	AAACAAATAT	AACTGAAAAG	GTAACAATG	TCGTTGCCAG	ATCCGTCAAA	-844
91C	AAACAAATAT	AACTGAAAAG	GTAACAATG	TCGTTGCCAG	ATCCGTCAAA	-847

91R	TATCAGGAGA	AGTAATGCCC	ATATCATGTT	ACGCTGTCAA	TAGAACACGA	-783
MHIII	TATCAGGAGA	AGTAATGCCC	ATATCATGTT	ACGCTGTCAA	TAGAACACGA	-794
91C	TATCAGGAGA	AGTAATGCCC	ATATCATGTT	ACGCTGTCAA	TAGAACACGA	-797

SpeI

91R	ATCCCAACTG	ATGAAGTAGC	CACTAGTTTG	CCGTAAGGGG	GAAGAGATAA	-733
MHIII	ATCCCAACTG	ATGAAGTAGC	CACTAGTTTG	ACTCAAGGGG	GAAGAGATAA	-744
91C	ATCCCAACTG	ATGAAGTAGC	CACTAGTTTG	ACTCAAGGGG	GAAGAGATAA	-747

91R	ATTACCGAGG	GTCGTCTAA	ATACAGTATA	AACTGCCTAA	CGTAGCTTAC	-683
MHIII	ATTACCGAGG	GTCGTCTAA	ATACAGTATA	AACTGCCTAA	CGTAGCTTAC	-694
91C	ATTACCGAGG	GTCGTCTAA	ATACAGTATA	AACTGCCTAA	CGTAGCTTAC	-697

91R	TATACTTAAG	GTGTACCATT	TGATTTTAGA	TCAATATATT	TATAGGCGTG	-633
MHIII	TATACTTAAG	GTGTACCATT	TGATTTTAGA	TCAATATATT	TATAGGCGTG	-644
91C	TATACTTAAG	GTGTACCATT	TGATTTTAGA	TCAATATATT	TATAGGCGTG	-647

91R	TACTTTAGGT	AGTACTATTG	TGATCTTCAA	AAATGCACTA	ATAAAGGTG	-583
MHIII	TACTTTAGGT	AGTACTATTG	TGATCTTCAA	AAATGCACTA	ATAAAGGTG	-594
91C	TACTTTAGGT	AGTACTATTG	TGATCTTCAA	AAATGCACTA	ATAAAGGTG	-597

→ Barbie box

91R	CTTTAAGAA	TTGCTCTAA	GATTGTATCA	TATAAATTGT	ATGAAACTAA	-533
MHIII	CTTTAAGAA	TTGCTCTAA	GATTGTATCA	TATAAATTGT	ATGAAACTAA	-544
91C	CTTTAAGAA	TTGCTCTAA	GATTGTATCA	TATAAATTGT	ATGAAACTAA	-547

91R	GTTTCTAAAA	TGACACACTC	ATATAGAGTC	ATGGTTATTT	TGTTGGGTA	-483
MHIII	GTTTCTAAAA	TGACACACTC	ATATAGAGTC	ATGGTTATTT	TGTTGGGTA	-494
91C	GTTTCTAAAA	TGACACACTC	ATATAGAGTC	ATGGTTATTT	TGTTGGGTA	-497

→ Barbie box

91R	GGTTAGTAGC	ATTATCAATG	AGAAATCAAA	AGAAATGAAC	TGTCTGGTCA	-433
MHIII	GGTTAGTAGC	ATTATCAATG	AGAAATCAAA	AGAAATGAAC	TGTCTGGTCA	-444
91C	GGTTAGTAGC	ATTATCAATG	AGAAATCAAA	AGAAATGAAC	TGTCTGGTCA	-447

91R	ACCCATATAA	TTGTCTTCAA	TATAATGATC	GCTGAAAGCA	ACCGTGAGTA	-383
MHIII	ACCCATATAA	TTGTCTTCAA	TATAATGATC	GCTGAAAGCA	ACCGTGAGTA	-394
91C	ACCCATATAA	TTGTCTTCAA	TATAATGATC	GCTGAAAGCA	ACCGTGAGTA	-397

91R	AGCAGACTTG	CAAAAAAAT	ATTGATAAGT	GTATACTTTT	AGCACTAGGC	-333
MHIII	AGCAGACTTG	CAAAAAAAT	ATTGATAAGT	GTATACTTTT	AGCACTAGGC	-344
91C	AGCAGACTTG	CAAAAAAAGT	ATTGATAAGT	GTATACTTTT	AGCACTAGGC	-347

→ AP1

→ Oct1

91R	GTTGTGATAA	GTCACAGTTC	GTCACGTGAC	AGTACTT	CTTCATTATT	-286
MHIII	GTTGTGATAA	GTCACAGTTC	GTCACGTGAC	AGTACTT	CTTCATTATT	-297
91C	GTTGTGATAA	GTCACAGTTC	GTCACGTGAC	AGTACTT	CTTCATTATT	-297

← AP1

← Oct1

91R	CATCACCGGT	ATATGAGGGA	TAATACAATA	AATTCCGAAC	TAAATAAACA	-236
MHIII	CATCACCGGT	ATATGAGGGA	TAATACAATA	AATTCCGAAC	TAAATAAACA	-247
91C	CATCACCGGT	ATATGAGGGA	TAATACAATA	AATTCCGAAC	TAAATAAACA	-247

Sali

91R	CTTTTGTGCT	CAATTTGCCT	ACAAAATAAT	GATCATGTCG	ACGTTGGTTT	-186
MHIII	CTTTTGTGCT	CAATTTGCCT	ACAAAATAAT	GATCATGTCG	ACGTTGGTTT	-197
91C	CTTTTGTGCT	CAATTTGCCT	ACAAAATAAT	GATCATGTCG	ACGTTGGTTT	-197

91R	CTGTCCTTAT	CTATTCAGAC	TACGATTCTT	GCCATCTCAC	AGTGCTTGGC	-136
MHIII	CTGTCCTTAT	CTATTCAGAC	TACGATTCTT	GCCATCTCAC	AGTGCTTGGC	-147
91C	CTGTCCTTAT	CTATTCAGAC	TACGATTCTT	GCCATCTCAC	AGTGCTTGGC	-147

91R	ATTAAAGTAA	ACAAAAC	TC	TCTCTTTCTA	GCGACTGAGA	-97
MHIII	ATTAAAGTAA	ACAAAACCTA	AGATTAACTC	TCCCTTTCTA	GCGACTGAGA	-97
91C	ATTAAAGTAA	ACAAAACCTA	AGATTAACTC	TCCCTTTCTA	GCGACTGAGA	-97

91R	ATGCATCGTC	ATGCAAAGTA	AGTCAATTTT	AGCGTTTAAA	AGCAGTTTGC	-47
MHIII	ATGCATCGTC	ATGCAAAGTA	AGTCAATTTT	AGCGTTTAAA	AGCAGTTTGC	-47
91C	ATGCATCGTC	ATGCAAAGTA	AGTCAATTTT	AGCGTTTAAA	AGCAGTTTGC	-47

91R	CTATCGTTTA	CGGAACCACT	CGTATTTCGAA	GATCATccgc	ctcaacatg	+3
MHIII	CTATCGTTTA	CGGAACCACT	CGTATTTCGAA	GATCATccgc	ctcaacatg	+3
91C	CTATCGTTTA	CGGAACCACT	CGTATTTCGAA	GATCATccgc	ctcaacatg	+3

MHIII (Fig. 3-10). It may also be mentioned that there are two AP1 sites and one ATF binding site identified immediately (5-8 bases) upstream to this 3 nucleotide insertion.

Only six, type II substitutions (91-R/91-C) are found in the upstream DNA and all of them are located upstream of -973 nt (Fig. 3-10). Bases at these six sites are identical in 91-R and 91-C but different in *MHIII*-D23. Presence of different bases in *MHIII*-D23 is of interest because *Cyp6a8* expression is extremely high in *MHIII*-D23 strain. Interestingly, four of these six bases are associated with a putative barbie box sequence at -988. It has been reported that in *Bacillus megaterium* binding of a repressor protein to the barbie box results in a low constitutive expression of both the *CYP102* and *CYP106* genes (He and Fulco, 1991; Liang et al., 1995). It is possible that a repressor-like protein may bind to the putative barbie box at -988 and downregulate the *Cyp6a8* gene. If this is the case, four base changes found in *MHIII*-D23 may prevent repressor interaction with the putative barbie box resulting in high level of constitutive expression of *Cyp6a8*.

There are six bases that show Type III substitutions. These bases are identical in *MHIII*-D23 and 91-C but different in the 91-R strain (Fig. 3-10). In addition, 11 bases that are present in the *Cyp6a8-91C* and the *Cyp6a8-MHIII* alleles (-129 to -119) are missing in the *Cyp6a8-91R* allele. Although this is substantial difference in the upstream DNA between 91-R and the other two strains, it does not seem to have a significant contribution in determining the level of *Cyp6a8* expression because *MHIII*-D23, which is the highest producer of CYP6A8 mRNA, does not have this deletion.

Although several differences have been found in the upstream DNA of three *Cyp6a8* alleles, it is difficult to predict whether these differences have any functional significance. It is expected that the coding regions of a gene will be under a greater selective pressure to conserve regions of the gene that are functionally important while the major regions of the upstream DNA may be less constrained. The series of substitutions observed in the upstream DNA of *Cyp6a8* is probably a reflection of such a

trend. In this light, it seems significant that most substitutions occur beyond the *Sa*I site, well away from the basal promoter region.

Since the *cis*-acting elements did not appear to be dramatically different in the three alleles of *Cyp6a8* from the overproducer and underproducer strains, the next goal was to study the effect of *trans*-acting factors that may have an effect of modulating the constitutive levels of *Cyp6a8* expression.

Chapter 4

Effect of chromosome substitutions between *CYP* underproducer and overproducer strains of *Drosophila melanogaster* on expression of *Cyp6a8* and *Cyp6a2*

"Cytochrome P450 has much to do with our chemical individuality. However, cytochrome P450 is equally important to our individuality as a species."

David R. Nelson, 1999

In: Cytochrome P450 and the Individuality of the species,
Archives of Biochemistry and Biophysics, 369,1-10.

Abstract

Most wild type insecticide susceptible insect strains show a low level of *CYP* gene expression, while resistant strains show overexpression of one or more *CYP* genes. In *Drosophila*, *Cyp6a8* is upregulated in the DDT resistant 91-R strain and also in MHIII-D23 which carries the 3rd chromosome from a malathion resistant strain. This gene was used as a prototype to study the genetic basis behind *CYP* gene overexpression in the insecticide resistant strains of *Drosophila*. Hybrids were generated between overproducer (91-R and MHIII-D23) and underproducer strains (91-C and *ry*⁵⁰⁶). Northern blot analysis showed that the expression of *Cyp6a8* alleles from the overproducer strains was downregulated in the F1 hybrids, implying the presence of negative regulators in the underproducer, susceptible strains. To analyze the linkage group of these loci several chromosome substitution lines were synthesized between the overproducer (91-R, MHIII-D23) and the underproducer (91-C, *ry*⁵⁰⁶) strains. It was found that the 3rd chromosome of both underproducer strains carried negative regulators that could reduce the expression of the second chromosomal *Cyp6a8-91R* allele as well

as the *Cyp6a8-MHIII* allele. The X chromosome was also found to influence the expression of *Cyp6a8* on the 2nd chromosome. Similar chromosomal effects were also observed on *Cyp6a2*, another *Drosophila CYP* gene that is overexpressed in resistant strains. This implied that there might be a common regulatory mechanism that can regulate the expression of more than one CYP6 family gene. In the resistant overproducer strains, the 3rd chromosomal negative regulators could be mutated leading to high levels of expression of both *Cyp6a2* and *Cyp6a8*. A similar mechanism of regulation has also been shown for housefly *CYP* genes, implying that mechanisms regulating *CYP* gene expression may be conserved between insect species.

Introduction

All cytochrome P450 enzymes catalyze a basic chemical reaction of inserting a single oxygen atom into complex organic compounds. This basic chemistry of monooxygenation, however, has been utilized very effectively by nature to catalyze reactions in innumerable physiological processes. Of these processes, perhaps the one that cytochrome P450s are most famous for, includes metabolism of xenobiotics. Since any insecticide is a 'xenobiotic' that the insect encounters in its environment, it is not surprising that these enzymes have been recruited to help insects metabolize insecticides leading to the development of resistance. Consequently, a positive correlation has been found between resistance and overexpression of one or several *CYP* genes in resistant insects (Agosin, 1985; Hodgson, 1985, General introduction). The fact that resistance is reduced in these insects by treatment with general inhibitors of CYP enzymes, like piperonyl butoxide, implicate CYPs in insecticide resistance.

Since the introduction of DDT in 1945, there has been a rapid increase in resistant species of arthropods (Forgash, 1984). This started a cycle where more potent

insecticides were developed followed by evolution of resistant insect species. In this scenario, CYPs offer a unique entry point for controlling insect populations. This is firstly due to the obvious role that CYPs play in conferring resistance and secondly due to the fact that many aspects of the insects' life cycle, like growth, development, reproduction and determination of trophic niches involve CYP enzymes (Feyereisen, 1999). Consequently, the study of *CYP* gene regulation could offer unique ways by which resistant insect populations could be controlled.

In *Drosophila melanogaster* also, several *CYP* genes have been shown to be upregulated in strains that are resistant to insecticides or plant toxins (Waters et al., 1992; Maitra et al., 1996; Danielson, 1997; Dombrowski et al., 1998). One such gene, *Cyp6a8* is expressed at high levels in the insecticide resistant 91-R and MHIII-D23 strains of this species. In most susceptible strains this gene is expressed at a lower level as in the *ry*⁵⁰⁶ strain, and it shows almost no expression in 91-C (Chapter 2). Analysis of the *cis*-regulatory elements of *Cyp6a8* revealed the presence of upstream DNA sequences close to the putative transcription start site, which constitute the basal promoter. In addition, several motifs were also found in the upstream DNA, which indicated that *Cyp6a8* is not just a housekeeping gene and that it could be classified as a regulated gene.

While a lot of work has been done on transacting factors involved in the regulation of mammalian and bacterial *CYP* genes (reviewed in Gonzales et al., 1993; Whitlock, 1999; Kemper, 1998), very little is known about the molecular and genetic basis of *CYP* gene regulation in insects. Genetic studies in the housefly have shown that *CYP6A1* and *CYP6D1* genes, located on chromosome 5 and 1, respectively, are *trans*-regulated by loci present on chromosome 2 (Carino, et al., 1994; Liu and Scott, 1996). *CYP6D1* expression is also influenced by chromosome 1 (Liu and Scott, 1997a, 1998). Since chromosome 2 of the housefly and chromosome 3 of *Drosophila* are thought to be evolutionarily related (Foster, et al., 1981), it is possible that the third chromosome of

Drosophila has similar regulatory loci that influence the activity of second chromosomal CYP genes like *Cyp6a8*. Indeed, Waters and Nix (1988) have shown that in *Drosophila*, the level of P450B1, a subset of 58-kD heme binding polypeptides, is regulated by loci present on the third chromosome.

The objective of the work reported in this chapter was to determine the chromosomal linkage of factors that could regulate *Cyp6a8* expression. Several chromosome substitution stocks were constructed with various combinations of chromosomes from overproducer (91-R and MHIII-D23) and underproducer (91-C and *ry*⁵⁰⁶) strains and the expression of *Cyp6a8* was measured in these strains through Northern blot analysis. Since *Cyp6a2* has a pattern of expression similar to that of *Cyp6a8*, that is, higher in resistant strains than in susceptible ones, the expression of *Cyp6a2* was also measured in the chromosome substitution stocks.

Materials and Methods

Fly stocks and culture condition

Several strains of *Drosophila melanogaster* were used for the present investigation. 91-R and MHIII-D23 were representative of strains which show a high level of CYP gene expression. 91-C and *ry*⁵⁰⁶ were used as representative underproducer strains of CYP6A2 and CYP6A8 mRNAs. These strains have been described in chapter 2. Three balancer stocks were used to synthesize the chromosome substitution stocks. These were: (1) *Sp*/SM5 *al*² *Cy*¹ *sp*²; (2) *Sco*/CyO, *Cy*; *kar ry*⁵⁰⁶; (3) TM3 *Sb ry e*/TM6 *Tb e*. While the first one was obtained from the Bowling Green Mid America Stock Center, the other two were obtained from Dr. John Lucchesi (Emory University). The details about the stocks are described in Lindsley and Zimm (1992). All stocks were raised on standard cornmeal-agar-molasses medium at 23°C.

Generation of hybrid stocks

Hybrid stocks were generated between the underproducer (91-C, ry^{506}) and overproducer (91-R, MHIII-D23) strains. The following combinations were made – 91-C x 91-R, ry^{506} x 91-R, and ry^{506} x MHIII-D23. F1 hybrid female flies were collected and stored at -80°C for RNA isolation.

Synthesis of chromosome substitution stocks between overproducer and underproducer strains

Stocks carrying different combinations of three major chromosomes (X, 2nd and 3rd) from the underproducer (ry^{506} and 91-C) and overproducer (91-R and MHIII-D23) strains of CYP6A2 and CYP6A8 mRNAs were synthesized (Table 4-1 and 4-2). Stocks listed in groups A-C (see below) were synthesized by combining chromosomes from the ry^{506} and 91-R strains, where the 2nd and the 3rd chromosomes were followed in the cross (Table 4-1), while all other stocks D-F (listed in Table 4-2) were synthesized by using pair-wise combinations of the four different strains. In these crosses all three major chromosomes were followed. A brief description for each of these stocks is given below.

- A. Non-iso 1 ($2^R/2^R; 3^Y/3^Y$) - Olé Raustol, an undergraduate student in the laboratory, synthesized this stock. This stock was homozygous for the second chromosome from 91-R. It was also homozygous for the third chromosome from ry^{506} but the chromosomes could have undergone crossing over with the 91-R 3rd chromosome during synthesis. Neither the 2nd nor the 3rd chromosomes are isogenic (See appendix, A-1 for crossing scheme).
- B. Iso-2, iso-3, iso-6 ($2^R/2^R; 3^Y/3^Y$) - These stocks were derived from the non-iso 1 stock and all three had the same chromosomal constitution as non-iso 1 (Table 4-1). However, unlike the non-iso 1 stock, these stocks were isogenic for three different 2nd chromosomes from 91-R (present in the non-iso 1 population). The third chromosome

Table 4-1
Chromosome composition of stocks with the 2nd chromosomes
from 91-R and 3rd chromosomes from either 91-R or ry^{506} strains

Stock names	Chromosome sources		Chromosome composition
	2nd	3rd	
Non-iso 1*	91-R	ry^{506}	2R;3ry
Iso-2 [#]	91-R	ry^{506}	2R;3ry
Iso-3 [#]	91-R	ry^{506}	2R;3ry
Iso-6 [#]	91-R	ry^{506}	2R;3ry
Iso-22**	91-R	91-R	2R;3R
Iso-25**	91-R	91-R	2R;3R

* The second chromosome is not isogenic and the third chromosome may have undergone crossing over during synthesis of the stock.

These stocks are isogenic for three different 2nd chromosomes from the 91R strain. The 3rd chromosome is from ry^{506} and has not undergone crossing over with chromosomes of any other strain and is not isogenic.

** Both stocks were derived from the Iso-2 stock by replacing the 3rd chromosome of ry^{506} origin with that of the 91R strain. They are isogenic for two different 3rd chromosomes from the 91R strain. Iso-2 and these stocks are isogenic for the same second chromosome.

Table 4-2
Chromosome substitution stocks involving the X, 2nd and 3rd
chromosomes from the *ry*⁵⁰⁶, 91-C, 91-R and MHIII-D23 strains

Stock No.	Chromosomes sources			Chromosome compositions*
	X	2nd	3rd	
1	91-R	91-R	<i>ry</i>	RRr
2	91-R	<i>ry</i>	91-R	RrR
3	<i>ry</i>	91-R	<i>ry</i>	rRr
4	91-R	91-R	91-C	RRC
5	91-R	91-C	91-R	RCR
6	91-C	91-R	91-C	CRC
7	91-C	91-C	91-R	CCR
8	MHIII	MHIII	91-C	MMC
9	MHIII	91-C	MHIII	MCM
10	91-C	MHIII	91-C	CMC
11	91-C	91-C	MHIII	CCM

*All stocks were non isogenic and synthesized by using at least 10-15 parental flies in each generation.

from ry^{506} was non-isogenic and had not undergone crossing over with chromosomes of any other stock (appendix A-2)

- C. Iso-22 and iso-25 ($2^R/2^R; 3^R/3^R$) – These stocks were derived from iso-2 by maintaining the same isogenic second chromosome from 91-R but re-substituting the third chromosome from ry^{506} with two different third chromosomes from 91-R. In this case, the replaced third chromosomes were isogenic (appendix A-3).
- D. Three stocks (RRr, RrR and rRr) were synthesized by combining chromosomes from the 91-R and ry^{506} strains. The chromosome constitutions of the stocks are shown in Table 4-2. In these crosses, the chromosomes were not isogenic and care was taken to see that a large number of flies were sampled in each generation to set up the crosses so that the effect of individual genetic variations could be minimized (appendices A-5, A-6, A-9).
- E. RRC; RCR; CRC and CCR stocks were synthesized by combining chromosomes from the 91-R and 91-C strains (Table 4-2; appendices, A-5, A-7, A-8, A-10).
- F. Chromosome substitution stocks between 91-C and MHIII-D23 was synthesized as the previous cases with 91-R/ ry^{506} or 91-R/91-C. The names of the stocks were MMC, MCM, CMC and CCM (Table 4-2; appendices, A-5, A-6, A-8, A-10).

RNA isolation and Northern blot hybridization

Steady state CYP6A8 mRNA level was studied by Northern blot hybridization techniques described in the previous chapters. Briefly, poly A⁺ RNA was isolated from unsexed or female adult flies. A set of RNA samples to be compared was loaded on the same 2.2M formaldehyde-1.2% agarose gel, electrophoresed and blotted onto nylon membrane. Three sets of Northern blots were prepared for all samples that were analyzed. Since there was a good separation between the *Cyp6a8* (or *Cyp6a2*) transcripts and the one coded for by RP49, the top 2/3 and bottom 1/3 of the nylon membrane were

separated. The top parts of three sets of blots were placed in the same bag and hybridized with ^{32}P -labeled *Cyp6a8* cDNA probe (0.8-kb *EcoRI* fragment; Fig. 2-6B). Similarly, the lower portions of three sets of blots were hybridized together with labeled ribosomal protein 49 (RP49) gene probe. DNA fragments to be used as probes were labeled with ^{32}P either by random priming or nick translation and all hybridizations were done under probe excess conditions (next section). Hybridization signals were quantified by scanning the blots with a Packard InstantImager Imaging system. RP49 mRNA level in each lane was used as an internal control to correct for RNA loading errors between lanes.

Calculation of probe excess

An inherent assumption in the comparison of hybridization signals on Northern blots is that the probe used is not limiting. The variation in signal intensity would then solely be a function of the amount of target RNA present on the blot. To test for conditions of probe excess, different amounts of poly A⁺ RNA from MHIII-D23 (1 μg , 2 μg and 4 μg) was electrophoresed, blotted and hybridized with a 1.7 fold excess of a *Cyp6a8* probe labeled by random priming. If the amount of probe *was* in excess then there would be a linear increase in the signal intensity. Fig. 4-1 shows results of the hybridization where the increase in the hybridization signal is linear. Since a 1.7 fold excess of probe brought about a linear increase in signal intensity with MHIII-D23 poly A⁺ RNA where expression of *Cyp6a8* was the highest (Fig. 2-7), it would be expected that a similar fold probe added with A⁺ RNA from any other strain would surely be in excess. Therefore, in case of hybridization with *CYP* probes an approximately 1.5 fold excess probe was added for all hybridizations. Similarly, conditions for probe excess with RP49 was tested by hybridizing a blot which had increasing amounts of MHIII-D23 poly A⁺ RNA (1 μg , 2 μg and 4 μg), with a nick translated probe for RP49. It was found that a linear increase in hybridization was obtained with a 2/3rd fold probe excess (Fig 4-1).

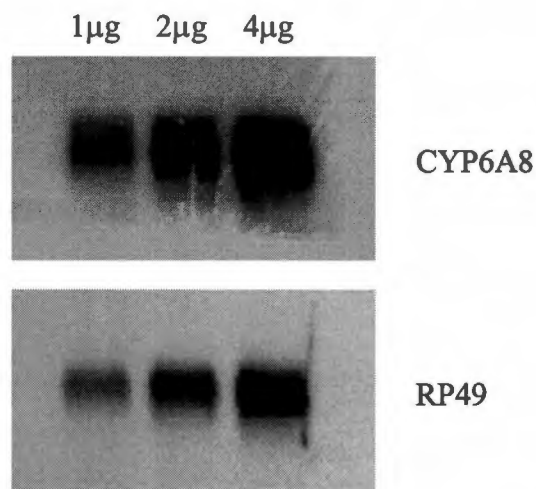


Figure 4-1 : Northern blot analysis to establish conditions of probe excess

Increasing amounts of poly A⁺ RNA from MHIII-D23 adult females (1μg, 2μg and 4μg) was fractionated on a 2.2M formaldehyde-1.2% agarose gel and transferred onto nylon membrane. The membrane was hybridized with *Cyp6a8* and RP49 cDNA probes under assumed conditions of probe excess as described under Materials and Methods. Hybridization signals show a linear increase with increase in the amount of RNA, thereby establishing the conditions of probe excess.

Since a linear increase in hybridization was seen using this probe amount, this experiment established the conditions of probe excess for RP49.

Luciferase assay

Luciferase assays were done as described in the previous chapter. However, the amount of sample and the assay buffer used were reduced by half. The light readings were quantitated using the FB12 luminometer from Zylux Corporation (Maryville).

Results and Discussion

Sexual dimorphism in *Cyp6a8* expression.

Waters et al. (1983) had shown that the cytochrome P450 content was 1.5 times more in males than in females in the Hikone-R strain of *D. melanogaster*. In light of this observation it seemed logical to study whether sexual dimorphism also existed for *Cyp6a8* expression. Therefore, *Cyp6a8* expression was measured in adult males and females by Northern blot analysis. Fig. 4-2A shows the steady state mRNA levels of *Cyp6a8* in males and females from the 91-R, 91-C and *ry*⁵⁰⁶ strains. In all three strains, the expression of *Cyp6a8* is higher in the males compared to females. Consequently, all further analysis was restricted to one sex and adult females were used in subsequent Northern blot experiments.

Since *Cyp6a8* was found to show a gender bias in expression, we wanted to see whether this sexual dimorphism is also exhibited by the *luciferase* transgenic lines that carried the *Cyp6a8-luc* constructs. Therefore, *Drosophila* transgenic lines which have the *luciferase* reporter gene with 0.2-kb, 0.8-kb or 3.1-kb upstream DNA of *Cyp6a8* were examined for luciferase activity in two sexes. The results showed that luciferase activity was significantly higher in male transgenic flies than in the females irrespective of the

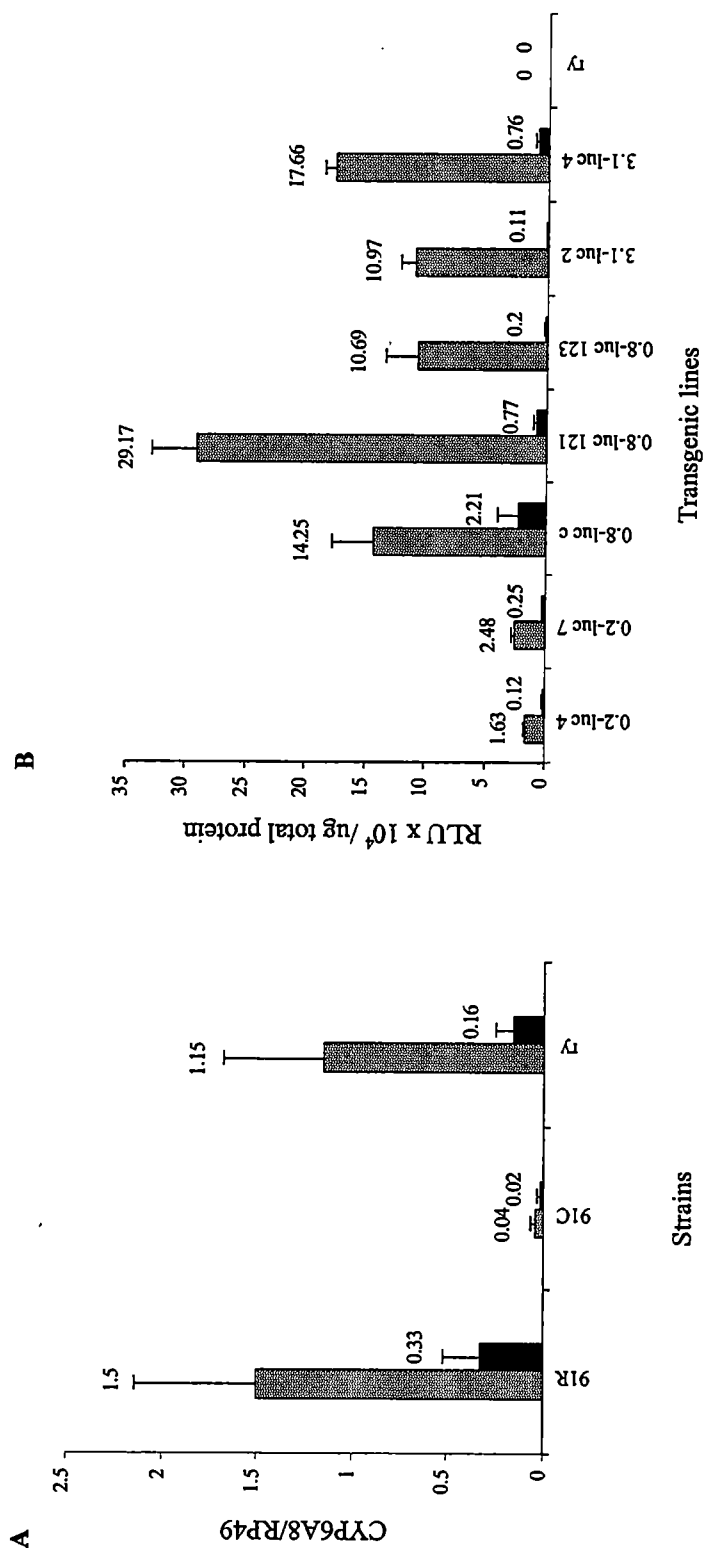


Figure 4-2 : Expression of *Cyp6a8* in adult males and females of *Drosophila melanogaster*.

A) Constitutive levels of CYP6A8 mRNA was compared in males and females of 91-R, 91-C and *ry*⁵⁰⁶ strains by Northern blot analysis. 2 µg of poly A⁺ RNA was fractionated on a 2.2M formaldehyde-1.2% agarose gel, transferred onto nylon membrane and hybridized with CYP6A8 and RP49 cDNA probes. The data are presented as CYP6A8/RP49 ratios and each value is a mean of three readings. Stippled columns represent expression in males while solid columns represent expression in females.

B) Expression of luciferase from chimeric luciferase constructs in transgenic adult males and females. The amount of luciferase activity in total body homogenates from adult flies was measured using luciferin as a substrate. The light emitted in the reaction was quantitated using a FB12 Zylux luminometer and expressed as RLUs (relative light units)/ µg of total protein. Each column in the histogram represents a mean of three individual readings.

length of the *Cyp6a8* upstream DNA present in the transgene (Fig. 4-2B). The fact that transgenic lines carrying the *0.2Cyp6a8-luc* constructs show a 10-13 fold higher level of luciferase activity in males compared to females indicates that the proximal 0.2-kb region of *Cyp6a8* was sufficient for the higher level of *Cyp6a8* expression in males. A *cis*-acting transcription element, known as SDI (Sex Difference Information), has been identified in the male-specific P450 16 α gene and the sequence was shown to be important for the sex-specific expression of the gene (Yoshioka et al., 1990). This sequence was located in between -102 and -84 in the 5' upstream DNA of the P450 16 α gene. However, analysis of the upstream DNA sequences of *Cyp6a8* did not reveal any sequences similar to the SDI sequences. In this context it needs to be mentioned that *Cyp6a2* also shows higher level of expression in males than in females (Dombrowski, 1998). It was also apparent from the data that the variations in constitutive levels of luciferase activity observed in females of transgenic lines carrying *luciferase* constructs with different lengths of *Cyp6a8* upstream DNA (Fig. 3-8, Table 3-2) was also maintained in the males (Fig. 4-2B).

Expression of *Cyp6a8* in F1 hybrids of overproducer and underproducer strains

1) Expression of *Cyp6a8* in 91-R x 91-C hybrids.

The wild type phenotype of *Cyp6a8* appears to be a low level of expression as most strains show such a phenotype. Thus, overexpression could be considered to be a mutant phenotype. If this is the case, it seemed logical to determine whether overexpression is dominant or recessive to the low level of expression. Therefore, CYP6A8 mRNA levels in the female F1 hybrids of underproducer (*ry*⁵⁰⁶ or 91-C) and overproducer (91-R or MHIII-D23) strains were measured by Northern blot analysis. The results in Fig. 4-3 showed that the levels of CYP6A8 mRNA in the 91-R and 91-C parental strains were 1.77 and 0.09 respectively. This means that the expression per

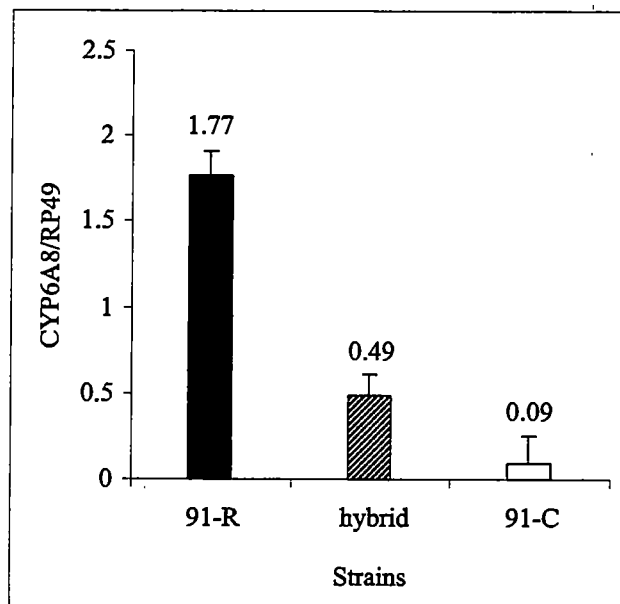


Figure 4-3: Expression of *Cyp6a8* in F1 hybrids of 91R and 91C

Constitutive levels of CYP6A8 mRNA in 91-R x 91-C F1 hybrid flies were analyzed by northern blot analysis using equivalent amounts poly A⁺ RNA. Three such blots were hybridized with *Cyp6a8* and RP49 gene probes as described in Materials and Methods. The level of hybridization was quantified by scanning the blot with an InstantImager imaging system. RP49 was used as internal control to correct for RNA loading error.

allele was 0.89 for the *Cyp6a8-91R* allele and 0.045 for the *Cyp6a8-91C* allele. On the other hand, total *Cyp6a8* activity in the F1 hybrid was 0.49 (Fig 4-3). Since the *Cyp6a8-91R* and *Cyp6a8-91C* mRNAs have the same molecular weights they could not be distinguished from one another in the RNA from the hybrid flies. It was assumed that each allele of *Cyp6a8* expressed equally in the hybrid flies. Thus, the expression of the *Cyp6a8-91R* allele in the hybrid would be half of 0.49 or 0.25. This would mean that there is a 3.6-fold (72%) downregulation of the *Cyp6a8-91R* allele in the hybrid flies when compared with its expression in the parental 91-R strain where per allele expression was 0.89. Such evidence suggests that the chromosomes of the underproducer 91-C strain have a negative regulatory influence on the expression of the *Cyp6a8-91R* allele. Conversely, it can also be argued, by the same criteria of equal contribution by both alleles to total CYP6A8 mRNA level in the F1 hybrids, that the *Cyp6a8-91C* allele becomes upregulated by the influence of 91-R chromosomes. If chromosomes from 91-R had a positive regulatory influence, *Cyp6a8* expression in the F1 hybrids would be expected to be at least half of that in 91-R, if not more, by additional contribution of the upregulated *Cyp6a8-91C* allele. However, the *Cyp6a8* expression in the F1 hybrid was lower than half the level of expression found in 91-R (Fig. 4-3) suggesting a downregulation of *Cyp6a8-91R* expression in the F1 hybrid flies. This lends credence to the hypothesis that 91-C chromosomes have negative factors, which can downregulate the *Cyp6a8-91R* expression in the F1 hybrids of 91-R and 91-C.

II) Expression of *Cyp6a8* in *MHIII-D23* x *ry*⁵⁰⁶ and *91-R* x *ry*⁵⁰⁶ F1 hybrids

Since chromosomes from the underproducer 91-C strain were found to downregulate the *Cyp6a8-91R* allele, it was important to determine whether this phenomenon was restricted to 91-C alone or also shown by chromosomes from other underproducer strains. For this purpose, *ry*⁵⁰⁶ (underproducer) strain was crossed to overproducer strains 91-R and MHIII-D23, and the F1 hybrid females of each cross were

examined for the steady-state levels of CYP6A8 mRNA. Table 4-3 shows the expression of *Cyp6a8* per gene dose in the three parental strains and their F1 hybrids. Because the size of the CYP6A8 mRNA is identical in all strains, it was again assumed that the two different *Cyp6a8* alleles in the F1 hybrids contributed equally to the total CYP6A8 mRNA content. Using this assumption, the expression levels of *Cyp6a8-MHIII* and *Cyp6a8-91R* alleles in the MHIII-D23 x *ry*⁵⁰⁶ and 91-R x *ry*⁵⁰⁶ hybrids were calculated to be 0.88 and 0.69, respectively (Table 4-3). However, expression values of the same alleles, *Cyp6a8-MHIII* and *Cyp6a8-91R*, were 3.23 and 0.97 in the parental MHIII-D23 and 91-R strains. Thus, *Cyp6a8-MHIII* and *Cyp6a8-91R* alleles were downregulated in the F1 hybrids and the levels of downregulation were 73% and 30%, respectively (Table 4-3). These data suggest that like the 91-C genome, the *ry*⁵⁰⁶ genome also downregulates the *Cyp6a8* alleles from the overproducer strains. This indicates that the genomes of underproducer strains may have regulatory loci that downregulate or repress the *Cyp6a8* gene. If this is the case, the underproducer strains must be carrying the wild type alleles of these loci because most strains of *Drosophila* show a low or barely detectable level of *Cyp6a8* expression. This would mean that the overproducer strains carry mutant alleles for these loci. Because the *Cyp6a8* alleles are downregulated in the hybrids, it appears that the negative regulatory loci of the underproducer strains are dominant to their alleles present in the overproducer strains. The dominance, however, is not complete because the *Cyp6a8-91R* and *Cyp6a8-MHIII* alleles only show a partial downregulation in the hybrid flies. Interestingly, similar observations were also made with *CYP6A1* and *CYP6D1* expression in the F1 hybrids of underproducer and overproducer strains of *Musca* (Carino et al., 1994; Liu and Scott, 1997a). Therefore, the genetic mechanism of CYP6 gene regulation appears to be conserved between *Drosophila* and *Musca*.

Table 4-3
Expression of *Cyp6a8* in F1 hybrids of the underproducer *ry*⁵⁰⁶ strain
with the overproducer MHIII-D23 and 91-R strains

Strains	Cyp alleles*	n	Cyp/RP49 Mean + S.D. [#]	Expression per allele	percent downregulation ^{\$}
MHIII-D23	MHIII	3	6.56 + 0.11	3.28	
MHIII x <i>ry</i> ⁵⁰⁶	MHIII + <i>ry</i>	3	1.75 + 0.18	0.88	73%
<i>ry</i>	<i>ry</i>	3	1.19 + 0.14	0.59	
91R x <i>ry</i> ⁵⁰⁶	R + <i>ry</i>	3	1.38 + 0.16	0.69	30%
91R	R	3	1.93 + 0.05	0.97	

Steady state mRNA levels of *Cyp6a8* was determined by Northern blot analysis of poly A⁺ RNA with RP49 as the internal control. A mean of three readings (CYP6A8/RP49) was used to compare the expression of *Cyp6a8* in different samples. In the F1 hybrids, equal expression of both alleles was assumed to calculate the expression per allele.

R- *Cyp6a8*-91R; *ry* - *Cyp6a8*-*ry*; MHIII - *Cyp6a8*-MHIII. Percent downregulation was calculated as [1 - (expression in hybrids/expression in the parental strain)]x100.

Effect of the third chromosome on expression of *Cyp6a8-91R*

The results in the previous section indicated that chromosomes from the underproducer strains could negatively regulate *Cyp6a8* alleles from the overproducer strains. Since loci on the third chromosome have been shown to affect total P450 content in *Drosophila* (Hallstrom, 1985; Houpt et al., 1988; Waters and Nix, 1988), it was rationalized that this chromosome could also influence *Cyp6a8* expression. Therefore, several stocks carrying the second chromosome from 91-R and hence the *Cyp6a8-91R* allele, and the third chromosome from *ry*⁵⁰⁶ or 91-R were synthesized (Table 4-1). Poly A⁺ RNA was isolated from adult females of all these stocks and analyzed by Northern blot hybridization.

1) Effect of the third chromosome of *ry*⁵⁰⁶ on *Cyp6a8-91R* expression

Fig. 4-4 shows *Cyp6a8* expression in 91-R, *ry*⁵⁰⁶ and the non-iso 1 (*2^R/2^R; 3^{ry}/3^{ry}*) chromosome substitution stocks. The expression of *Cyp6a8* was highest in the 91-R strain, followed by the *ry*⁵⁰⁶ strain and lowest in the non-iso 1 stock. Compared to the 91-R parental stock, the expression of *Cyp6a8* dropped by 75% in the non-iso 1 stock even though the stock is homozygous for *Cyp6a8-91R*. Thus, the presence of homozygous third chromosomes from *ry*⁵⁰⁶ reduces the expression of *Cyp6a8-91R* on the second chromosome. These results imply that repressive factors are present on the third chromosome of *ry*⁵⁰⁶ that downregulate the expression of *Cyp6a8*. A point to be noted is that expression of *Cyp6a8* is higher in the *ry*⁵⁰⁶ strain compared to the non-iso 1 stock, despite the fact that both these stocks carry the *ry*⁵⁰⁶ third chromosomes. This may be due to differences in the nature of interaction of the repressive factors produced by the *ry*⁵⁰⁶ third chromosome with the *Cyp6a8-ry* and *Cyp6a8-91R* alleles present in the *ry*⁵⁰⁶ strain and non-iso 1 stock respectively.

In a population of flies, a gene may be polymorphic, and different alleles of the gene may respond differentially to various regulatory factors. This may be true also for

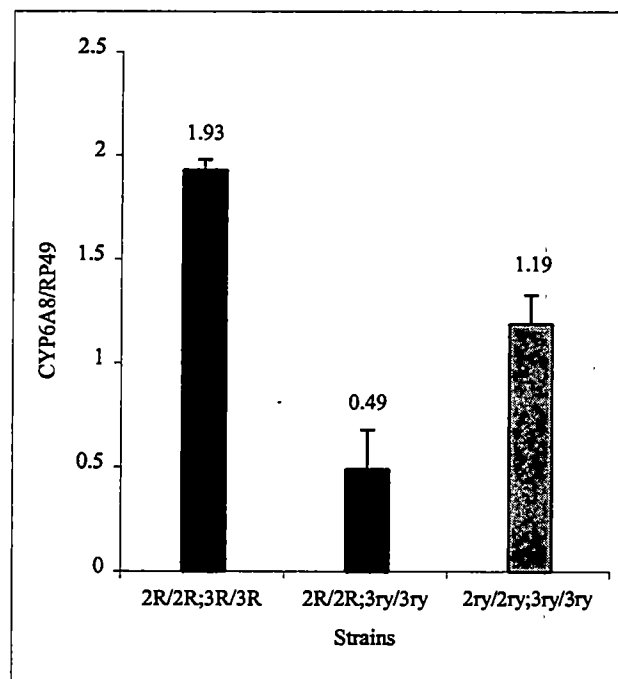


Figure 4-4: Effect of the third chromosome of the underproducer ry^{506} strain on expression of *Cyp6a8* from the overproducer 91-R strain.

Chromosome substitution stocks were made between 91-R (2R/2R;3R/3R) and ry^{506} (2ry/2ry;3ry/3ry) strains to obtain a stock which was homozygous for second chromosomes from 91-R and third chromosomes from ry^{506} referred to as non-iso 1 (2R/2R; 3ry/3ry). Expression of *Cyp6a8* was analyzed by Northern blots of poly A⁺ RNA from adult females, with RP49 as the internal control. Each bar in the histogram represents the mean of three readings.

Cyp6a8 in the 91-R strain. To test this hypothesis, three stocks (iso-2, iso-3 and iso-6) were synthesized. All the three stocks had the same chromosome constitution as the non-iso 1 stock ($2^R/2^R; 3^{ry}/3^{ry}$). However, these stocks were isogenic for three different second chromosomes from the 91-R strain. Consequently, these strains were isogenic for three different *Cyp6a8-91R* alleles. These strains were also homozygous, but not isogenic, for the third chromosome of the ry^{506} strain. The results of Northern blot analysis (Fig. 4-5) showed that all three *Cyp6a8-91R* alleles were downregulated and the levels of downregulation were 86%, 64% and 79% in the iso-2, iso-3 and iso-6 stocks respectively. Thus, within the variability present in a biological system, all the *Cyp6a8-91R* variants appear to respond in a similar manner to the negative regulators present on the third chromosome of ry^{506} .

II) Effect of resubstituting the ry^{506} third chromosome in the iso-2 stock by third chromosomes from 91-R

To confirm that the effect of downregulation of the *Cyp6a8-91R* allele in the above lines was indeed due to the presence of the third chromosome from ry^{506} , re-substitution experiments were done. Iso-2 was chosen as the representative line for the re-substitution cross as the extent of downregulation was maximum in this line. Two isogenic stocks, iso-22 and iso-25, were derived from iso-2 by replacing the third chromosome of the ry^{506} origin in iso-2 with a third chromosome from 91-R. Thus, iso-22 and iso-25 carried the same second chromosome from the iso-2 stock, but were isogenic for two different third chromosomes of the 91-R strain (Table 4-1). The results of Northern blot analysis with poly A⁺ RNA from the iso-22 and iso-25 lines are shown in Fig. 4-6. There was a 3 to 5 fold increase in *Cyp6a8* expression in these two lines compared to the expression in the control (iso-2) line (Fig. 4-6). Thus, downregulation of the *Cyp6a8-91R* allele in the iso-2 stock was due to the presence of the ry^{506} third chromosomes. It follows from these experiments that the wild type phenotype of low

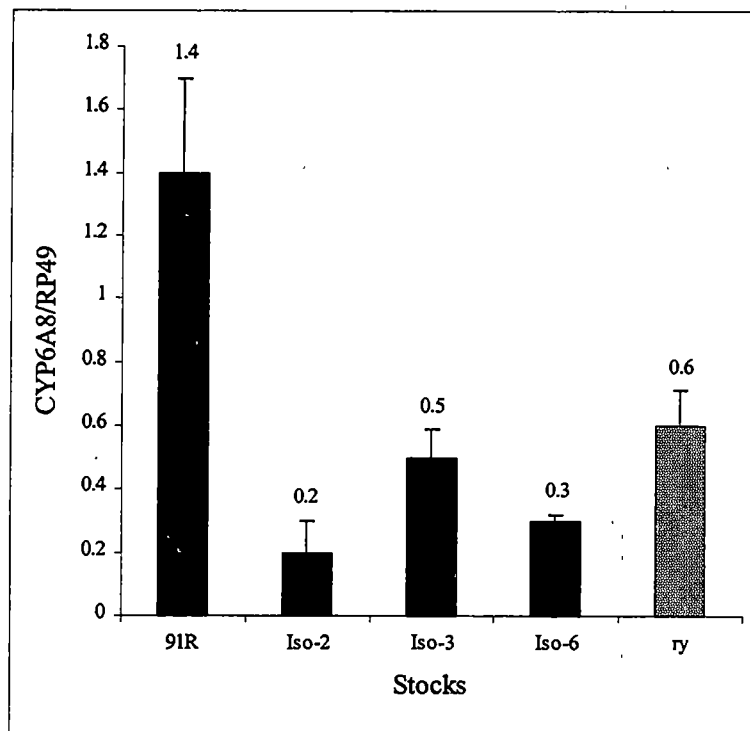


Figure 4-5 : Expression of *Cyp6a8-91R* alleles in three different isogenic stocks.

CYP6A8 mRNA levels were quantified by RNA blot analysis in triplicate as described in Fig 4-1. The iso stocks used were isogenic for three different 2nd chromosome from the 91-R strain, and homozygous for the 3rd chromosome of *ry*⁵⁰⁶ strain. For details see Table 4-1.

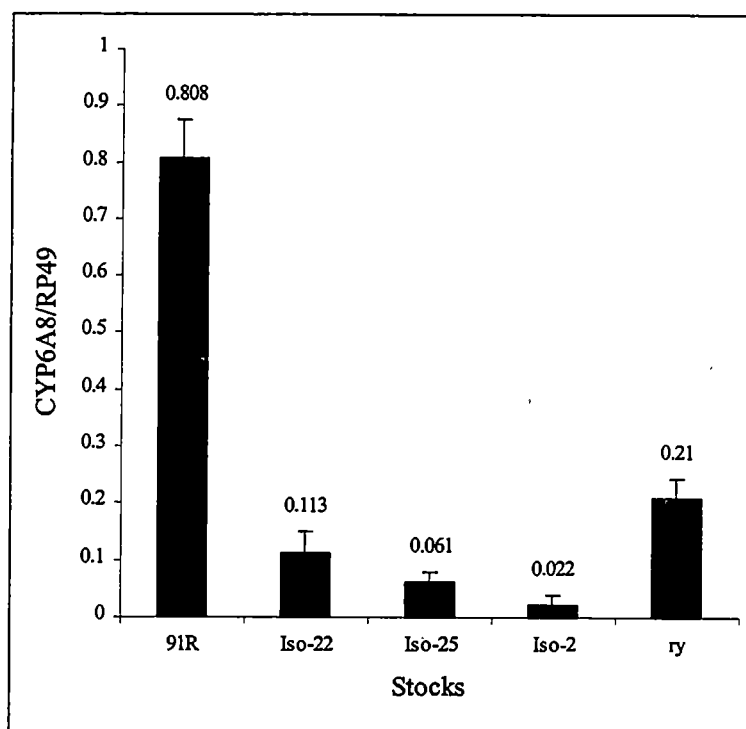


Figure 4-6: Expression of *Cyp6a8-91R* in iso-2 and two chromosome re-substitution stocks.

CYP6A8 mRNA levels were quantified by RNA blot analysis in triplicate as described in Fig 4-1. All three stocks are isogenic for the 2nd chromosome from 91-R and hence carry the same allele for *Cyp6a8* (*Cyp6a8-91R*). Iso-25 and iso-22 stocks were derived from the iso-2 stock by substituting its 3rd chromosome of *ry*⁵⁰⁶ origin with two different 3rd chromosomes of 91-R strain. For details see Table 4-1.

expression may be caused by the presence of negative regulatory factors in the genome. A corollary to this hypothesis is that in 91-R these negative regulatory factor(s) are mutant and this is probably one of the reasons for upregulation of *Cyp6a8* in 91-R. Therefore, when the third chromosomes of *ry*⁵⁰⁶ origin were replaced with the third chromosome from the 91-R strain in the iso-22 and iso-25 lines, the repression on *Cyp6a8* was removed. Consequently, *Cyp6a8-91R* expression increased 3 to 5 fold compared to the expression seen in the iso-2 line.

However, the phenotype of overexpression in 91-R appears to be more complex than just the presence of mutant negative factors on the third chromosomes. Though the chromosomal constitution of the iso-22 and iso-25 lines are the same as the parental 91-R strain with respect to the 2nd and 3rd chromosomes, the expression level in these stocks is much less than the parental 91-R strain (Fig. 4-6). The high level of *Cyp6a8* expression in 91-R strain has probably been achieved by coevolution and selection of several regulatory factors, which may be scattered over the genome. Consequently, the replacement of just two chromosomes may not provide the optimum combination of all the factors necessary for the high level of expression seen in 91-R. Despite all these caveats the result that emerges from these experiments is that in *Drosophila*, a wild type third chromosome carries a repressor gene(s) that negatively regulates the expression of *Cyp6a8* located on the second chromosome. Mutation of this repressor locus may be a reason for the high level of *Cyp6a8* expression in overproducer strains like 91-R.

Effect of chromosome substitutions between *ry*⁵⁰⁶ and 91-R strain on *Cyp6a2* and *Cyp6a8* expression

The results in the previous section showed that the third chromosomes from underproducer strains like *ry*⁵⁰⁶ carry negative regulators that bring about a low level of *Cyp6a8* expression. This negative effect could be gene specific for *Cyp6a8*. However,

results from several laboratories point to a more global effect of the regulators thought to be present on the third chromosome (Hallstrom, 1985; Waters and Nix, 1988; Houpt et al., 1988). To study whether other *CYP* genes are also under a similar control, analysis of *Cyp6a2* expression was examined in chromosome substitution stocks made from the 91-R and *ry*⁵⁰⁶ strains. Expression of *Cyp6a2* has been shown to be higher in the resistant 91-R than in the susceptible *ry*⁵⁰⁶ strain (Dombrowski et al., 1998). While 91-R is highly resistant to malathion and DDT (Sundseth et al., 1989; Delpuech et al., 1993), *ry*⁵⁰⁶ is susceptible to both these chemicals (Ganguly et al., unpublished observations).

In the chromosome substitution studies described in the previous section, the source of the first (X) and the fourth chromosome was not known. The 4th chromosome may not be important because this chromosome does not have any major effect on P450 activity and insecticide resistance (Waters and Nix, 1988, Dapkus and Merrell, 1977; Houpt et al., 1988,). The X chromosome, however, cannot be ignored because it is a major chromosome and several reports have suggested that this chromosome might have modifiers that influence DDT resistance (Dapkus and Merrell, 1977). Therefore, it was important to study whether the X chromosome also plays a role on regulating *Cyp6a2* and *Cyp6a8* expression in *Drosophila*.

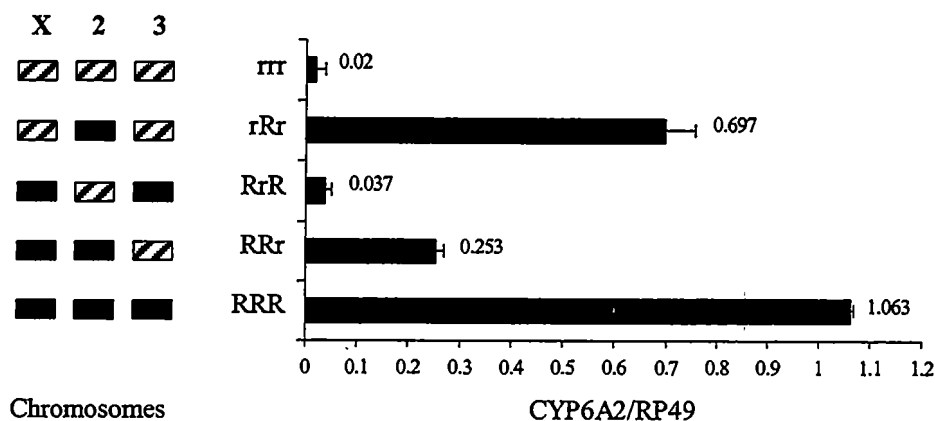
Three chromosome substitution stocks (RRr, RrR and rRr) were synthesized by combining chromosomes from the 91-R (R) and *ry*⁵⁰⁶ (r) strains. In the previous experiments (Fig. 4-5 and 4-6), a small variation in *Cyp6a8-91R* expression was seen in the isogenic stocks (iso-2, iso-3, iso-6, iso-22 and iso-25). This implied that the chromosomes in the population of the parental stocks were not genetically homogeneous and during the synthesis of the iso-lines, different chromosome samples might have been selected. To minimize a founder effect, at least 10-15 males and an equal number of females were used to set up crosses in every generation to synthesize the RRr, RrR and rRr stocks. Adult females were collected from these three stocks and *ry*⁵⁰⁶ and 91-R

parental stocks for RNA isolation. CYP6A2 and CYP6A8 mRNA levels in the various stocks were examined by Northern blot analysis.

Results (Fig. 4-7A and B) showed that the levels of both mRNAs were highest in the RRR (91-R) strain while they were much lower in the *rrr* (ry^{506}) strains. When the 3rd chromosome of the RRR strain was substituted with the 3rd chromosome from the ry^{506} strain (*rrr*), an approximately 4-fold reduction of CYP6A2 mRNA was observed (Fig. 4-7A, RRr). This suggested that loci on the 3rd chromosome of ry^{506} repress the expression of *Cyp6a2-91R* on the 2nd chromosome. When the X chromosome of the RRr stock was replaced with the X from the ry^{506} strain (in the rRr stock), expression of *Cyp6a2-91R* increased almost 2.5-fold in the rRr stock (Fig. 4-7A, rRr vs RRr). Interestingly, a similar variation in the pattern of expression of the other *CYP* gene, *Cyp6a8-91R*, was also found when the RRR, RRr and rRr stocks were compared (Fig. 4-7B). This suggests that both the *Cyp6a2-91R* and *Cyp6a8-91R* alleles are similarly influenced by the X and 3rd chromosomes of the ry^{506} strain. Based on the results observed with the RRR, RRr and rRr stocks it may be concluded that the 3rd chromosome of the ry^{506} strain represses the expression of both *Cyp6a2* and *Cyp6a8* while the X chromosome from the same strain enhances the expression of these genes. The net effect of these two opposing forces in ry^{506} may be downregulation of the *CYP* genes.

In contrast to the observations made with the chromosomes of ry^{506} , the X and 3rd chromosomes from 91-R show an opposite effect on *Cyp6a2* and *Cyp6a8* expression. While the X chromosome of 91-R appears to be inhibitory, the 3rd chromosome appears to stimulate transcription. This conclusion can be reached if comparisons are made between expression of *Cyp6a8-ry* in the RrR stock and the parental ry^{506} strain (*rrr*) where the expression is found to be higher in the former stock (Fig. 4-7B). This implies that presence of chromosomes (X and 3rd) from 91-R increases *Cyp6a8-ry* expression. Such a trend is also seen in case of *Cyp6a2-ry* (Fig. 4-7A; RrR vs *rrr*), although the

A.



B.

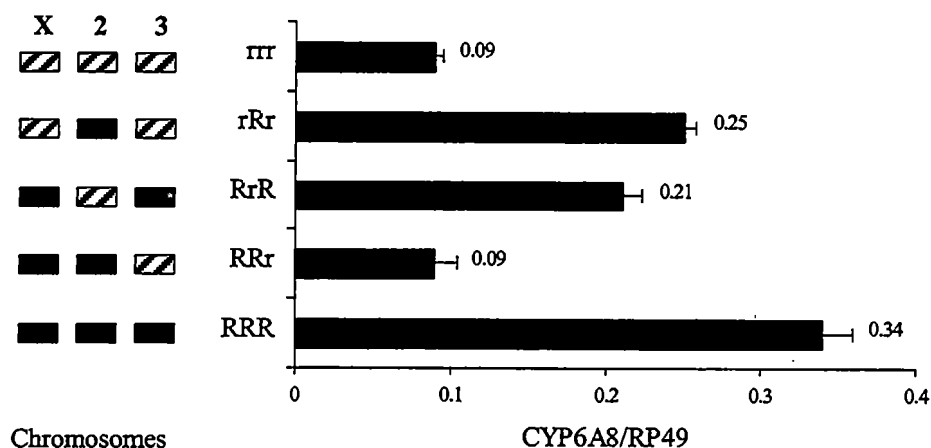


Figure 4-7: Expression of *Cyp6a2* (A) and *Cyp6a8* (B) in various chromosome substitution stocks between the underproducer *ry*⁵⁰⁶ and overproducer 91-R strains. Steady state mRNA levels of the two second chromosomal *CYP* genes were assayed by Northern blot analysis of poly A⁺ RNA as described in Fig 4-1. The bars in the histogram represent a mean of three CYP/RP49 ratios. The small solid bars represent chromosome from 91-R and the small cross hatched bars represent chromosomes from the *ry*⁵⁰⁶ strain.

increase is slight. The inhibitory effect of the X chromosome of 91-R can be seen if the expression of *Cyp6a2-91R* and *Cyp6a8-91R* is compared between the RRr and rRr stocks where expression of both the genes is found to be lower in the RRr stock (Fig. 4-7A and B). Since the X chromosome of 91-R appears to inhibit expression, the upregulation of the *CYP* genes in the RrR stock can be attributed to the 3rd chromosome from 91-R. Taken together, the net effect of these two chromosomes of 91-R (X and 3rd) appears to be one of positive regulation.

These effects may be due to the presence of negative regulatory loci on the 3rd chromosome from *ry*⁵⁰⁶, which code for negative factors which downregulate *CYP* genes. In that case the 91-R 3rd chromosome could be presumed to have a mutant locus for the negative factor. Upregulation of *CYP* genes in 91-R is thus due to a lack of negative factors. Alternatively, the results can also be explained by postulating that upregulation is the active phenomenon caused by synthesis of positive factors in 91-R and that low expression in the *ry*⁵⁰⁶ strain is due to a lack of such positive factors. Although both these mechanisms are possible, the results obtained with the hybrids of overproducer and underproducer strains suggest that the former possibility is more likely.

Even though the regulatory effects of the 91-R (stimulatory) and *ry*⁵⁰⁶ (inhibitory) chromosomes are apparent on the non-homologous alleles, the level of upregulation or downregulation observed was not complete. Consequently, expression of both the *CYP* genes in the rRr stock was not as low as it was in the parental rrr (*ry*⁵⁰⁶) strain. Similarly, their expression in the RrR stock was not as high as seen in the 91-R strain.

One possible explanation for the lack of complete downregulation (or upregulation) could be the nature of the *cis*-acting sequences of the *CYP* alleles. It is possible that the *cis*-elements for the alleles of the two *CYP* genes are different in the *ry*⁵⁰⁶ and the 91-R strain. For this reason perhaps the expression of the *Cyp6a2-ry* allele remained very low in the RrR stock even though the X and 3rd chromosomes are from the

91-R strain (Fig. 4-7A). Only a 2-fold upregulation was seen in the RrR stock when compared to the rrr stock. In the case of the *Cyp6a8-ry* allele too, complete upregulation was not observed. This gene showed a 2.7-fold upregulation in the RrR stock compared to the control rrr stock (Fig. 4-7B). This suggests that the *cis*-acting sequences of the *Cyp6a2-ry* and *Cyp6a8-ry* alleles may limit the extent of upregulation. Therefore, even though the putative negative regulators are mutant or lacking in the RrR stock, the extent of expression of the *CYP* genes in this stock is not to the levels as seen in 91-R.

A second possibility that may explain the incomplete upregulation is that besides the *Cyp6a2-ry* and *Cyp6a8-ry* alleles, a negative regulatory locus (loci) may be present on the second chromosome of *ry*⁵⁰⁶ which prevents upregulation to the level seen in 91-R.

In summary, it can be proposed that in wild type flies, e.g., *ry*⁵⁰⁶ the third chromosome has negative regulatory loci that downregulate the *Cyp6a2* and *Cyp6a8* genes located on the 2nd chromosome. The X chromosome of the *ry*⁵⁰⁶ strain appears to have factors that have a positive influence on the *Cyp6a2* and *Cyp6a8* genes. In addition, the second chromosome may also have negative regulatory loci (*cis*-elements or *trans*acting factor) that influence these *CYP* genes. Though the *cis*-regulatory sequences of these genes are also expected to play a role, these experiments do not shed any light in this regard. A transgenic approach may help determine how the alleles of these genes from the underproducer and overproducer strains compare with respect to the *cis*-acting sequences.

Effect of chromosome substitutions between 91-C and 91-R strains on *Cyp6a2* and *Cyp6a8* expression

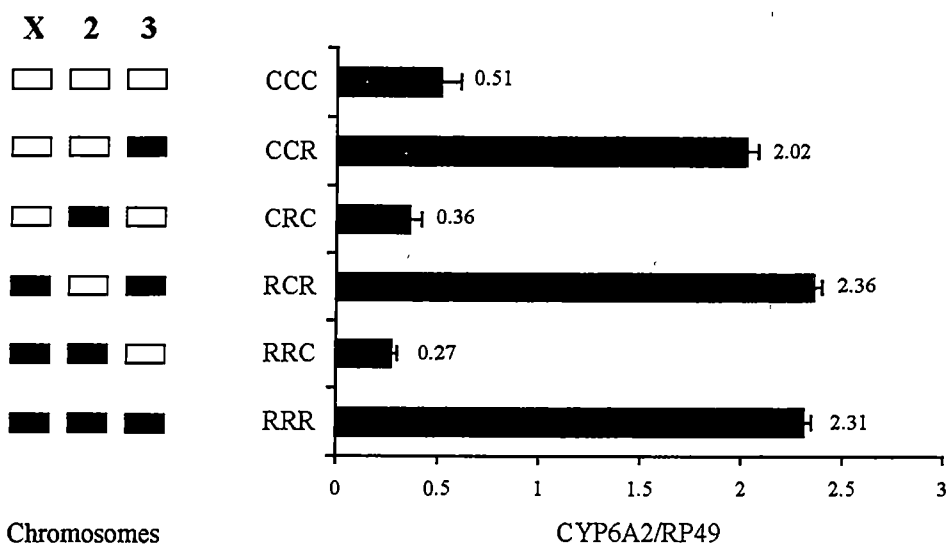
Like the *ry*⁵⁰⁶ strain, 91-C is a DDT-susceptible strain and an underproducer of CYP6A2 and CYP6A8 mRNAs (Waters et al., 1992; Maitra et al., 1996; Dombrowski et al., 1998). We wanted to study whether the pattern of *CYP* gene regulation shown by the

chromosomal loci from the underproducer ry^{506} strain is also conserved in the 91-C strain. For this purpose, four chromosome substitution stocks were synthesized using 91-C and 91-R as the parental strains. This combination had more biological significance than the 91-R/ ry^{506} combination as 91-R evolved from 91-C through DDT selection (Merrill and Underhill, 1956) and so 91-R would be genetically more similar to 91-C than to ry^{506} .

Poly A⁺ RNA was isolated from the females of 91-R, 91-C and the four chromosome substitution stocks (RRC, RCR, CRC and CCR) and was analyzed by Northern blot hybridization. In these stocks, R and C represent chromosomes from the 91-R and 91-C strains (Fig. 4-8). The results showed that the level of both CYP6A2 and CYP6A8 mRNA dropped about 10-fold when the 3rd chromosome of the 91-R (RRR) strain was replaced with the 3rd chromosome from 91-C (Fig. 4-8A and B, RRC). This suggests that the 3rd chromosome of the 91-C strain has loci that repress the expression of the *Cyp6a2-91R* and *Cyp6a8-91R* alleles located on the 2nd chromosome of RRC stock. The level of repression could be considered to be complete in case of the *Cyp6a2* gene because the activity of this gene in the RRC stock was lower than that seen in the 91-C (CCC) strain (Fig. 4-8A). However, *Cyp6a8-91R* was not completely repressed (to the levels seen in the 91-C strain) in presence of the 3rd chromosomes from 91-C (Fig. 4-8B, RRC vs CCC). When the X chromosome of the RRC stock was replaced with the X from 91-C (in the CRC stock) activity of both *Cyp6a2-91R* and *Cyp6a8-91R* increased slightly (Fig. 4-8A and B, CRC vs RRC). This suggests that the X chromosome of 91-C has a small positive effect on the expression *Cyp6a2* and *Cyp6a8* genes from 91-R. Conversely, this could also mean that the X chromosome from 91-R has a slight inhibitory effect on both the *CYP* genes from the same strain.

The results in Fig. 4-8 also show that the presence of third chromosomes from the 91-R strain brings about a high level of constitutive expression of the *Cyp6a2* and

A.



B.

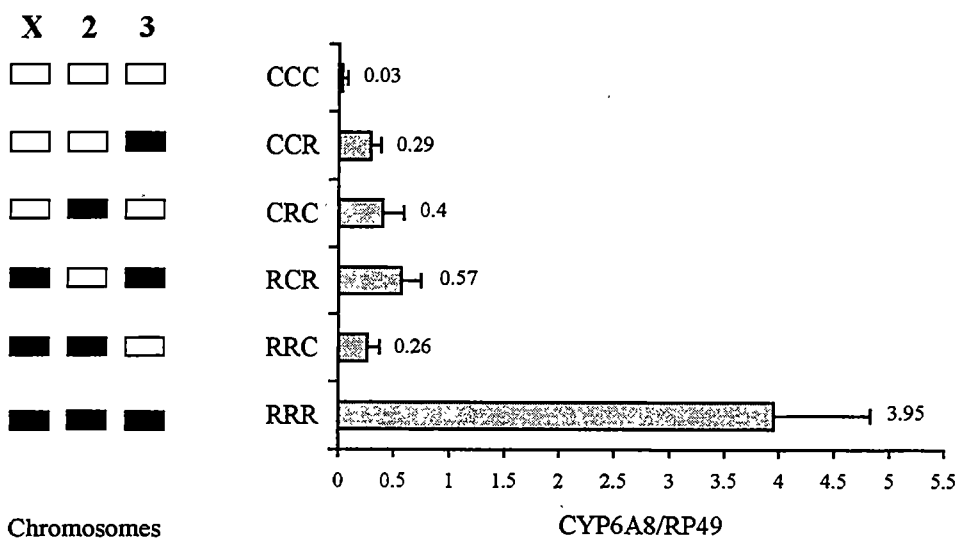


Figure 4-8: Expression of *Cyp6a2* (A) and *Cyp6a8* (B) in chromosome substitution stocks between the underproducer 91-C strain and the overproducer 91-R strain. Steady state mRNA levels were assayed by Northern blot analysis. Bars in the histogram represent a mean of three readings. The small solid bars represent chromosomes from 91-R and the small open bars represent chromosomes from 91-C.

Cyp6a8 genes. Thus, when the 3rd chromosome of the 91-C strain (CCC) was replaced with the 3rd chromosome of the 91-R strain (Fig. 4-8, CCR), expression of the underproducer *Cyp6a2-91C* and *Cyp6a8-91C* alleles increased by 4 and 10-fold respectively (Fig. 4-8A and B). If the negative repressor theory is true, this increase could be explained by a lack of repressors in the flies.

To study the effects of the X chromosome from 91-C, expression of the *CYP* genes was compared between the stocks CCR and RCR. The activity of the *Cyp6a2-91C* allele decreased a little when the X chromosome of RCR stock was replaced with the X chromosome of the 91-C strain (Fig. 4-8A, CCR vs RCR). There was a 2-fold decrease in *Cyp6a8-91C* activity when the same stocks were compared (Fig. 4-8B, CCR vs RCR). However, this inhibitory effect of the 91-C chromosome is not observed when the CRC and RRC stocks are compared. In case of both *Cyp6a2* and *Cyp6a8*, the effect of the 91-C X chromosome seems to be opposite, that is, one of upregulation (Fig. 4A and B, CRC vs RRC). Thus, the X chromosome seems to influence the expression of the *CYP* genes on the 2nd chromosome but the effect is slight.

Based on the above observations it can be concluded that there is a locus (or loci) on the 3rd chromosome in the wild type flies like 91-C, that acts as a repressor and downregulates the expression of *Cyp6a2* and *Cyp6a8* genes located on the second chromosome. In the 91-R strain, this locus is probably mutant and the product of this locus (repressor) is not functional. For that reason the constitutive expression of the alleles of *Cyp6a2* and *Cyp6a8* genes from both 91-C and 91-R strains increased whenever the 3rd chromosome of the 91-R strain was present in the genome. This increase was more dramatic in the case of *Cyp6a2* gene (Fig. 4-8A). In the presence of the 3rd chromosome of 91-R (in CCR), activity of the underproducer *Cyp6a2-91C* allele was upregulated almost to the level of *Cyp6a2* expression found in the 91-R strain (Fig. 4-8A). However, the *Cyp6a8-91C* allele did not show similar level of upregulation in the

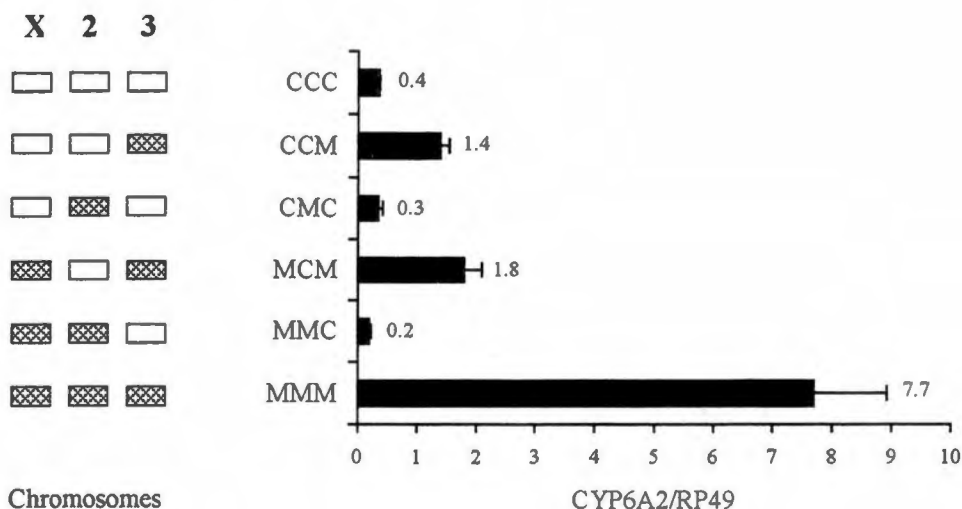
CCR stock. It is clear from the results that high level of expression of *Cyp6a2* and *Cyp6a8* in the 91-R strain is not due to *cis*-regulatory mutations in the individual *CYP* genes because these alleles can respond to the negative regulatory effects of the 3rd chromosome from 91-C. It seems more likely that the upregulation in resistant flies is due to a mutation in a *trans*-acting repressor. Consequently, the genes on the second chromosome are upregulated whenever the third chromosomes from 91-R are present in the genome. Another interesting point is that the stimulatory effect observed in case of the underproducer *ry*⁵⁰⁶ X chromosome (Fig 4-7A and B; rRr vs RRr), is also seen with the X chromosome of 91-C. However, this effect seems to act only on the *CYP* alleles from 91-R. The X chromosome of 91-C seems to have a slight effect of downregulating *CYP* alleles from the same strain, that is, from 91-C.

Effect of chromosome substitutions between 91-C and MHIII-D23 on expression of *Cyp6a2* and *Cyp6a8*

MHIII-D23 strain of *D. melanogaster* shows an unusually high level of CYP6A2 and CYP6A8 mRNAs (Dombrowski et al., 1998, Fig. 2-7). This led to an obvious question whether the superactive *CYP* alleles of this strain could be influenced by the chromosomes from an underproducer strain. To answer this question, expression of *Cyp6a2* and *Cyp6a8* was studied in stocks with combination of chromosomes from MHIII-D23 and the underproducer 91-C strain. Poly A⁺ RNA isolated from females flies of these stocks (MMC, MCM, CMC and CCM) and MHIII-D23 (MMM) and 91-C (CCC) were analyzed by Northern blot hybridization for *Cyp6a2* and *Cyp6a8* expression.

The results (Fig. 4-9A and B, MMM vs MMC) show that the level of CYP6A2 and CYP6A8 mRNAs dropped about 38 and 31-fold when the 3rd chromosome of MHIII-D23 strain was substituted with the third chromosome from the 91-C strain. Therefore, the *Cyp6a2*-MHIII and *Cyp6a8*-MHIII alleles, which show the highest level of activity

A.



B.

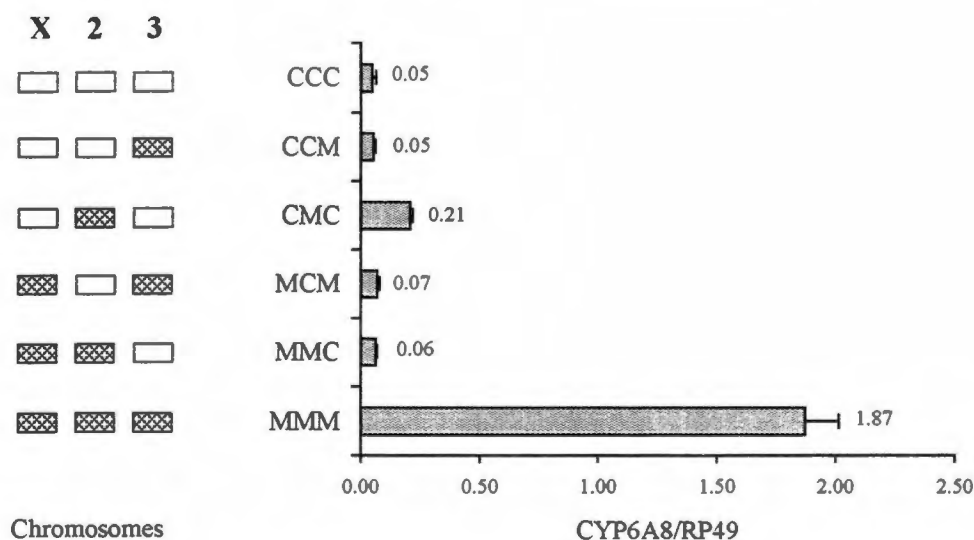


Figure 4-9: Expression of *Cyp6a2* (A) and *Cyp6a8* (B) in chromosome substitution stocks between the underproducer 91-C strain and the overproducer MHIII-D23 strain.

Northern blot analysis was used to study the influence of 91-C and MHIII-D23 chromosomes on *CYP* alleles from the reciprocal strain. The CYP/RP49 ratios are a mean of three readings. Open bars represent chromosomes from 91-C whereas cross hatched bars represent chromosomes from the MHIII-D23 strain.

among all strains, are also repressed by loci on the third chromosome of 91-C.

To examine the effects of the X chromosome, expression of the two *CYP* genes were compared between the CMC and MMC stocks. While this did not affect the expression of *Cyp6a2-MHIII*, there was a 3.5 fold increase in *Cyp6a8-MHIII* expression in the CMC stock (Fig. 4-9A and B). This trend is similar to what was found with the 91-C/91-R combinations in the results shown in Fig. 4-8, as the presence of the X chromosome from 91-C was found to increase the expression of *CYP* alleles from 91-R. The inhibitory effect of the X chromosome from 91-C was also reflected in the slightly lower expression of *Cyp6a2-91C* in the CCM stock compared to the MCM stock (Fig. 4-8 AB; Fig 4-9A).

In this investigation, four strains of *Drosophila* were examined, which showed differing levels of *CYP* gene expression with respect to *Cyp6a2* and *Cyp6a8* located on the second chromosome. Chromosome substitution studies showed that the expression of these genes could be modified when put in a genetic background with chromosomes from other strains. The third chromosomes from insecticide susceptible, underproducer strains like *ry*⁵⁰⁶ and 91-C could downregulate alleles of both *Cyp6a2* and *Cyp6a8* from insecticide resistant, overproducer strains like 91-R and MHIII-D23. Thus the obvious conclusion was that the high level of expression of *CYP* genes observed in the overproducer strains was due to the mutation of the presumed negative regulators present on the 3rd chromosome. However, although the dominant mechanism seems to be the one exerted by the third chromosome regulators, other loci appear to be present on the X chromosome, which could impose additional levels of control. The X chromosomal effect could be caused either by the presence of positive factor(s) on the X chromosome of the underproducer strains or negative factor(s) on the X from overproducer strains. There also appeared to be a polymorphism in the X chromosomal loci in the underproducer strains. Thus, the extent of upregulation by the X chromosome of the *ry*⁵⁰⁶ strain on *CYP*

gene transcription was much more than a similar effect by the X chromosome of 91-C. In case of alleles that had negligible expression in the parental strains, *Cyp6a2-ry* in the *ry*⁵⁰⁶ strain and *Cyp6a8-91C* in the 91-C strain, also showed the influence of the *trans*-acting factors. However, there was a limit to the expression level, which was probably defined by allele-specific *cis*-regulatory elements.

Plapp (1984) had suggested that metabolic resistance in the housefly is controlled by a few major loci and individual detoxifying enzymes are scattered over the genome, which are controlled by a master regulator gene on chromosome 2. Later studies showed that two housefly *CYP* genes, *CYP6A1* and *CYP6D1*, are *trans*-regulated by loci on chromosome 2 (Carino et al., 1994; Liu and Scott, 1996). The nature of the regulation suggested that the *trans*-regulatory factor is a repressor (Feyereisen et al., 1995). These results and speculations match well with the results of this investigation. Chromosome 2 of the housefly and the 3rd chromosome of *Drosophila* are thought to be homologous (Foster et al., 1981). Hence, the negative regulatory effects of the third chromosome indicate the presence of a conserved mechanism of *CYP* gene regulation that exists between *Drosophila* and the housefly.

Metabolic resistance has been correlated with high levels of monooxygenase activity. The mechanisms outlined in this chapter define some of the regulatory controls that exist for constitutive levels of *CYP* gene activity which can be modulated to achieve high levels of CYP enzymes that confer resistance. However, other regulatory controls also exist on expression of detoxifying enzymes, which involve induction of these enzymes upon exposure to xenobiotics like insecticides, pollutants, plant allelochemicals and drugs like barbiturate compounds. To get a holistic picture of the mechanisms that operate in regulating the expression of CYP6 family genes, barbiturate mediated induction of these genes was analyzed next.

Chapter 5

Analysis of barbiturate-mediated expression of *Cyp6a8* and the effect of chromosomes from overproducer and underproducer strains in modulating the barbiturate response

"He speculated that individual variations in the liver enzymes might influence the response of a patient to a particular drug"

From Beam's biography of Archibald Garrod, 1993
In: *Archibald Garrod and the Individuality of Man*,
Oxford Univ. Press, Oxford.

Abstract

Many xenobiotic cytochrome P450 enzymes are inducible. This helps the organism to metabolize toxic chemicals in the environment, as basal levels of such xenobiotic metabolizing enzymes are generally low. Insect P450 genes are induced by compounds like plant allelochemicals and certain drugs. Thus, induction is an important mechanism of regulatory control in metabolic resistance. To study whether similar regulatory controls exist for *Cyp6a8* expression in *Drosophila*, induction studies were carried out using barbiturate compounds. As barbiturates are known to induce *CYP* genes and sequence analysis had shown the presence of barbiturate response elements (barbie box) in the upstream DNA of *Cyp6a8*, adult flies were separately treated with two barbiturate compounds, barbital and phenobarbital. Northern blot analysis showed that both compounds induced *Cyp6a8* expression. However, the extent of induction was found to be strain-specific. There was a negative correlation between constitutive levels of expression and the extent of induction. The fold-induction was lowest in MHIII-D23, which has a very high level of constitutive expression, and highest in 91-C where the basal level of *Cyp6a8* is negligible. Phenobarbital treatment of transgenic lines carrying

chimeric *luciferase* reporter constructs indicated that sequences required for barbiturate induction are present within -0.2-kb of the upstream DNA of *Cyp6a8*. Two motifs that are associated with the barbiturate response, the barbie box and NF1 binding site present in the PBRUs of mammalian *CYP* genes were found in this region by sequence analysis. Steroid binding half sites that are thought to be important elements of the PBRU are also found within -0.2 kb. The functional significance of these sites has yet to be determined. Analysis of barbiturate inducibility in several chromosome substitution lines showed that the 2nd chromosome has factors that are involved in the regulation of *Cyp6a8* phenobarbital induction. This response appears to be also modulated by factors on the X chromosome. Study of barbiturate response of another *CYP* gene, *Cyp6a2*, showed similar trends in the regulation as observed for *Cyp6a8*, implying that the regulatory effects of chromosomes on barbiturate-mediated *CYP* gene expression may be global, affecting several barbiturate responsive genes.

Introduction

Regulatory controls on gene expression determine characteristic patterns of expression for any gene. In case of *CYP* genes such controls are manifested in the low basal level of constitutive expression in insecticide susceptible wild type strains of *Drosophila*. Insecticide resistant strains which show overexpression of *CYP* genes are thus constitutive mutants. This phenotype of *CYP* overexpression is thought to be one of the factors for metabolic resistance.

Induction is important as a mechanism of regulatory control in metabolic resistance, as many detoxifying enzymes have been shown to be inducible by xenobiotics and plant allelochemicals that an organism is exposed to (reviewed in Okey, 1990 and Feyereisen, 1999). The inducible nature of these enzymes can be regarded as an adaptive

response to the chemical environment of an organism since the basal levels of these enzymes are low. Induction may occur in response to exogenous chemicals and in many cases the inducers are metabolized (Conney, 1967, Okey, 1990). In several instances it has been found that the mechanism of induction is coordinate, inducing several detoxifying enzyme at the same time (Okey, 1990; Plapp, 1984). The implication of such coordinate induction is that a single genetic mechanism may be involved. The mechanisms behind induction of detoxifying enzymes have been the subject of intense research as there are implications in several processes like drug interactions, bioactivation of compounds, 'chemoprophylaxis' to name a few (Gonzales, 1989; Okey, 1990).

Most of the work done on the mechanisms regulating *CYP* gene induction has been in the bacterial and mammalian systems. In the bacteria, *Bacillus megaterium*, a series of studies have worked out the mechanisms for barbiturate induction of two CYP genes – BM1 (*CYP106*) and BM3 (*CYP102*) (He and Fulco, 1991; Liang and Fulco, 1995, He et al., 1995, Liang et al., 1995). The critical *trans*-acting factor is a repressor molecule, BM3R1, which binds to the operator sites for BM1 and BM3 and also barbie box sequences found in the 5' region of both genes. This results in a low level of constitutive expression. In presence of barbiturates, the repressor does not bind to the operator site or the barbie box of the BM3 gene and hence results in induction. Barbiturates also induce synthesis of positive factors (BMP1, BMP2). These compete with the repressor to bind to the BM1 operator sites and also inhibit binding of the repressor to the BM1 barbie box, thereby increasing expression of the BM1 gene (reviewed in Kemper, 1998).

In the case of mammals the mechanism of *CYP* gene induction is best understood for *CYP1A1*. This gene is induced by polycyclic aromatic hydrocarbon (PAH) compounds that include TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), 3MC (3-Methylcholanthrene) and benzo(a)pyrene while the basal level of expression of this gene

is almost undetectable (Poland and Knutson, 1982; Nebert, 1989). Studies have shown that ligands like TCDD bind to the cytosolic Ah receptor (Aromatic hydrocarbon receptor) which dissociates from hsp 90 and other Ah-R interacting proteins and enters the nucleus. The liganded receptor then heterodimerizes with Arnt (Ah receptor nuclear translocator) and forms an active transcription factor. This binds to XREs (xenobiotic response elements) in the upstream DNA of *CYP1A1* and effects transcription of the gene (Whitlock, 1999). Other xenobiotic chemicals like phenobarbital, rifampicin, clofibric acid induce CYP enzymes of the CYP2, CYP3 and CYP4 family, respectively. Recent evidence has shown that these induction mechanisms involve "orphan" receptors belonging to the nuclear receptor/steroid receptor superfamily like CAR (constitutive androstane receptor), PXR (pregnane X receptor) and PPAR (peroxisome proliferator-activator receptor) for the respective xenobiotics and families. The receptors share a common dimerization partner, RXR (retinoid X receptor) and bind to DNA elements known as NR sites which contain two imperfect repeats of the AGGTCA sequence spaced by a few nucleotides (reviewed in Waxman, 1999).

Several studies have documented that insect *CYP* genes are induced by drugs and plant chemicals but not much is known about the mechanism of induction of insect *CYP* genes. Most induction studies have concentrated on *CYP* genes that are involved in host-plant interaction. It has been shown that larvae of the *Papilio* species express *CYP6B1* and *CYP6B4* to high levels. Exposure of the insects to toxic chemicals like xanthotoxins, found in plants like carrots, induce these P450 genes considerably (Cohen et al., 1992; Hung et al., 1995, 1997). Similarly, in *Drosophila mettleri*, a cactophilic species of *Drosophila* found in the Sonoran desert, the alkaloids found in columnar cactus were found to induce three genes of the CYP28 family and also *CYP4D10* (Danielson et al., 1997, 1998). In the tobacco hornworm, *Manduca sexta*, nicotine induction of P450 genes allows the insect to feed on tobacco leaves despite the toxic nature of nicotine (Snyder et

al., 1993). The above observations imply that induction of specific *CYP* genes allows for the utilization of particular plants as hosts. Insecticide metabolism has been associated with some members of the CYP6 family. Thus, CYP6A1 has been shown to metabolize aldrin and heptachlor (Andersen et al., 1994), CYP6D1 is associated with metabolism of pyrethroid insecticides (Wheelock and Scott, 1992a, b) and CYP6A2 metabolizes certain promutagens like DMBA, Trp-2 and insecticides like aldrin, heptachlor and diazinon (Saner et al., 1996, Dunkov et al., 1997). However, it is not known whether these CYP6 family genes are induced by the xenobiotic compounds they metabolize. In separate studies the above three CYP6 family genes were found to be induced by phenobarbital (PB), a generic inducer of *CYP* genes (Carino et al., 1992; Brun et al., 1996; Dunkov et al., 1997; Liu and Scott, 1997b; Dombrowski, 1998).

A study on the mechanisms of induction may give an insight on the regulatory controls that exist on a gene. To analyze whether *Cyp6a8* is subjected to additional levels of control, two barbiturate compounds were used in the present investigation to examine the effects on gene expression. Barbiturate compounds were chosen for induction studies as these compounds are known to induce *CYP* genes (Okey, 1990; Waxman and Azaroff, 1992). Analysis of the *Cyp6a8* upstream DNA in Chapter 3 revealed the presence of several barbie boxes. Although such elements are found in the upstream DNA of several barbiturate inducible genes, the functional significance of these elements have been clearly delineated only in bacteria (Wen et al., 1989; He and Fulco, 1991; Liang and Fulco, 1995; Liang et al., 1995). There are conflicting reports as to the significance of these sequences in the mammalian system (Kemper, 1998). The functional significance of these elements, present in the *Cyp6a8* upstream DNA, was analyzed using the transgenic lines carrying chimeric *luciferase* constructs with different lengths of the *Cyp6a8* upstream DNA (Chapter 3). Although the phenomenon of barbiturate induction was known for a long time, not much is known about the mechanism of induction. To

study the genetic factors that may be involved in induction, barbiturate induced expression of *Cyp6a8* was studied in strains exhibiting variations in the constitutive levels of expression. Induction studies were also done in chromosome substitution stocks carrying different combinations of the X, 2nd and 3rd chromosomes from *Drosophila* strains that show either a high expression or a low expression of *CYP* genes.

Materials and Methods

Drosophila stocks

Four strains of *Drosophila melanogaster* were used for the present investigation. These included two insecticide susceptible underproducer strains, 91-C and *ry*⁵⁰⁶, and two overproducer strains associated with insecticide resistance, 91-R and MHIII-D23. All the strains have been described in Chapter 2. Six transgenic lines were used to map the *cis*-elements for barbiturate induction. These carried *luciferase* constructs with varying amounts of *Cyp6a8* upstream DNA. They were obtained by P-element mediated transformation of *ry*⁵⁰⁶ embryos with the chimeric constructs as described in chapter 3. Three stocks with chromosome combinations between 91-R and *ry*⁵⁰⁶ were used for barbiturate induction studies. Two of the stocks, non-iso 1 and iso-6 have been described in Chapter 4. Iso-34 (*2^v/2^v;3^R/3^R*) was synthesized such that the second chromosome was isogenic for a chromosome from the *ry*⁵⁰⁶ strain, while the third chromosome was isogenic from 91-R. The chromosome substitution lines between 91-R and 91-C (RRC, RCR, CRC and CCR), described in the previous chapter were also used for barbiturate treatment. All fly stocks were maintained on the standard *Drosophila* medium (cornmeal-agar-molasses) at 23°C.

Barbiturate treatment of adult flies

Barbital (5, 5'-diethyl barbituric acid) was purchased from Sigma Chemical Company and Phenobarbital (5-ethyl-5-phenyl-2,4,6,(1H,3H,5H)-pyrimidinetrione monosodium salt) was purchased from Research Biochemicals International, Natick, MA. In all the studies for barbiturate induction adult females (2-3 days old) were used for treatment. After collection, the flies were kept in an empty bottle for 2-3 hours to starve them and allow them to recover from the effects of ether. Barbiturates were administered by reconstituting *Drosophila* instant medium (Carolina Biological Supplies) in an aqueous solution of the barbiturate compound.

I) Treatment of 91-R, 91-C, ry^{506} and MHIID-223 flies

Flies were treated to various concentrations of barbital and phenobarbital (PB). For the initial experiment to study the inducibility of *Cyp6a8*, flies from the 91-R, 91-C and ry^{506} strains were treated to 100 mM barbital and two concentrations of PB, 4mM and 8mM. To compare the effect of barbiturate induction in the four strains of *Drosophila*, two concentrations (8mM and 16 mM) of both barbital and phenobarbital were used. In both experiments, flies were treated for 24 hours, frozen at -80°C and subsequently used for RNA isolation.

II) Treatment of luciferase transgenic lines

Adult flies were collected as described for the assay for constitutive expression in chapter 3. Briefly, F1 hybrids of the *luciferase* transgenic lines with ry^{506} were collected. Only wild type (red eyed) females (0-4 hours old) were collected and kept in an empty vial for 4 hours. This was done to allow recovery from ether and to starve the flies before treatment. The flies were then transferred to instant food reconstituted in an aqueous solution of 16mM phenobarbital and treated for 24 hours. These flies were homogenized immediately after treatment and the extracts were frozen at -80°C . Control flies were

kept on instant *Drosophila* medium reconstituted in water for the same amount of time as the treated flies.

III) Treatment of chromosome substitution lines

The 91-R/*ry*⁵⁰⁶ chromosome substitution lines, non-iso 1, iso-6 (Table 4-1; appendix A-1, A-2) and iso-34 (appendix, A-11) were treated to barbital and phenobarbital at 16mM concentrations for 24 hours. The chromosome substitution lines, RRC, RCR, CRC and CCR were treated with 4mM barbital and 4mM phenobarbital for 24 hours to study the effect of chromosomal substitution on barbiturate induction. After treatment, flies were stored at -80°C and used for RNA isolation subsequently.

RNA isolation and Northern blot hybridization.

Poly A⁺ RNA was isolated as described in chapter 2 and fractionated through 2.2M formaldehyde-1.2% agarose gel, blotted onto nylon membrane and hybridized with a CYP6A8 cDNA probe to study the expression of *Cyp6a8*. The RNA amount varied from 2 – 3.5 µg between experiment, however, the same amount of RNA was fractionated to compare the expression of *Cyp6a8* in samples within an experiment. RP49 was used as the internal control to correct for loading errors.

Luciferase assay

After treatment with barbiturates, flies were separated in batches of 12 flies, allowed to recover from the effects of ether and homogenized in 1X CCLR (Cell Cluture Lysis Reagent, Promega). Homogenates were processed as described in Chapter 3 and stored at -80°C till all samples were collected. Luciferase assay was done as described in Chapter 3. However, the amount of the sample used for the assay was different. Five µl of the undiluted homogenate was mixed with 50 µl of the Promega luciferase assay

buffer and light emission was measured using an FB12 luminometer (Zylux Corporation). Luciferase activity in between samples was compared as RLU/ μ g of total protein.

Results and Discussion

Induction of *Cyp6a8* by barbiturates

Several members of the CYP6 subfamily of genes can be induced with various xenobiotic chemicals that include barbiturate compounds (Scott, 1999). To study whether *Cyp6a8* could be induced by barbiturate compounds, the effect of two barbiturate compounds, barbital and phenobarbital, were studied. Adult flies were treated to 0.2% (8 mM) phenobarbital or 100mM barbital and *Cyp6a8* mRNA levels were assayed in the flies. Similar concentration of phenobarbital had been used to analyze barbiturate induction of *Cyp6a2* in other studies (Fuchs et al., 1994; Dunkov et al., 1997). A much higher concentration of barbital was used in this investigation as barbital is less lipophilic than phenobarbital and a report had shown that there was a positive correlation between the lipophilicity and potency of barbiturate inducers on monooxygenase activity in bacteria (Kim and Fulco, 1983).

Figure 5-1 and 5-2 show the results of a Northern blot analysis with poly A⁺ RNA samples isolated from barbital and phenobarbital treated and untreated adult flies from 91-R, 91-C and *ry*⁵⁰⁶ strains. *Cyp6a8* is induced by barbital in all three strains examined (Fig 5-1). Densitometric scan of the autoradiogram showed that barbital treatment increases RNA levels by about 9 fold in 91-R and by about 40 fold in *ry*⁵⁰⁶. This difference in the fold induction could be due to the difference in their constitutive expression. The results in Fig. 5-1 also show that the induced level of CYP6A8 mRNA in *ry*⁵⁰⁶ is slightly greater than the induced level in 91-R. Barbital treatment also induces the *Cyp6a8-91C* allele, the constitutive levels of which are almost non-existent in the 91-C

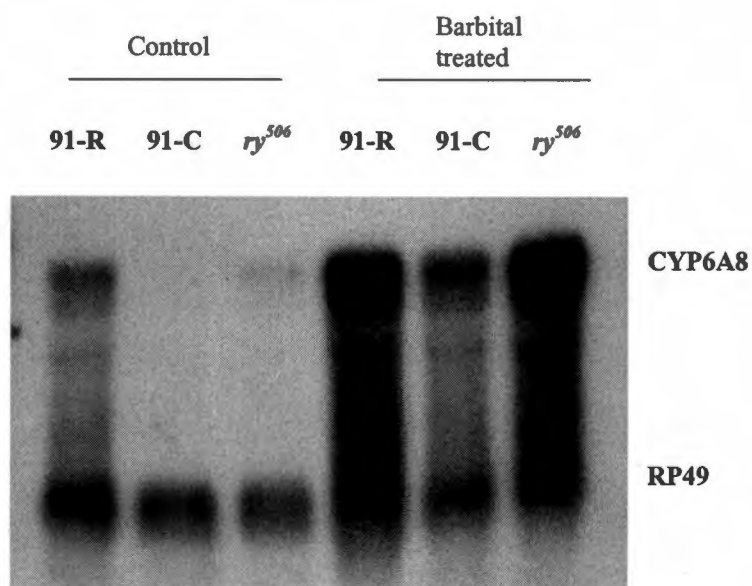


Figure 5-1: Induction of *Cyp6a8* with barbital

Poly A⁺ RNA (3.5μg) from adult flies treated with 0.1M barbital for 24 hours was electrophoresed on a 2.2M-1.2% formaldehyde agarose gel, transferred onto nylon membrane and hybridized with cDNA probes for *Cyp6a8* and RP49 as described in Materials and Methods of Chapter 2. As controls, poly A⁺ RNA was isolated from flies kept on medium without barbital for the same length of time.

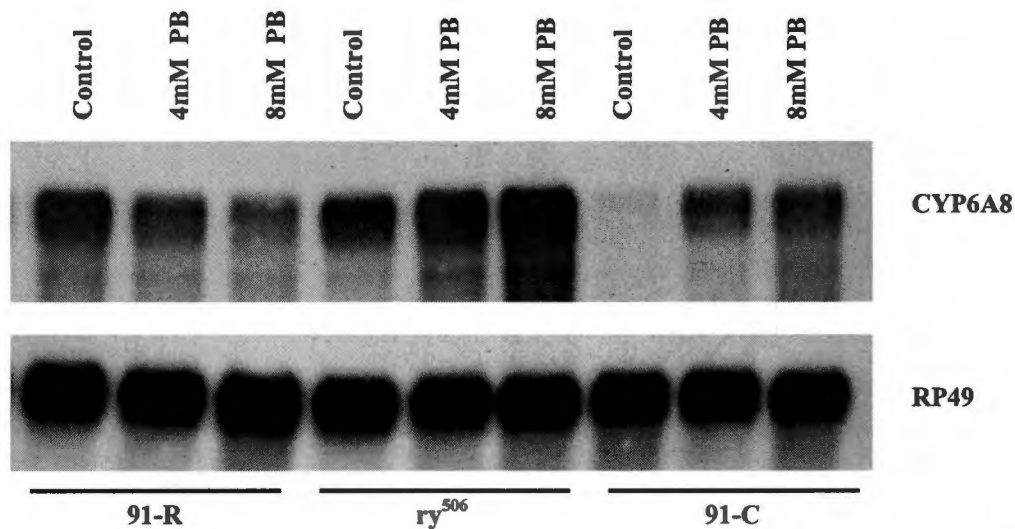


Figure 5-2: Induction of *Cyp6a8* with phenobarbital

Adult females were treated to two concentrations (4mM and 8mM) of phenobarbital (PB) for 24 hours. Poly A⁺ RNA (3μg) from such flies was electrophoresed on a 2.2M-1.2% formaldehyde agarose gel, transferred onto nylon membrane and hybridized with cDNA probes for *Cyp6a8* and RP49 as described in Materials and Methods in Chapter 2. As controls, poly A⁺ RNA was isolated from females kept on medium without phenobarbital for the same length of time.

strain. The results in Fig 5-2 show that, like barbitol, phenobarbital induces *Cyp6a8* in the 91-C and *ry*⁵⁰⁶ strains. The levels of the *Cyp6a8* transcripts are the highest in the induced *ry*⁵⁰⁶ strain. In case of 91-C, the levels of the transcripts are increased almost to the levels found in the uninduced 91-R strain as was seen in case of the barbitol treatment. However, unlike the effect of barbitol, phenobarbital does not seem to induce *Cyp6a8* in the 91-R strain, at least the concentrations used. With 8 mM phenobarbital, the effect seems to be one of repression (Fig. 5-2).

Phenobarbital is known to induce several drug and steroid metabolizing enzymes in the liver, including P450s and it was also found to induce several CYP6 family genes. Thus, it was not an unexpected result to find that *Cyp6a8* was also barbiturate responsive. An unexpected observation was that *Cyp6a8-91C* is induced to a considerable extent, that is, to levels comparable or even a little higher than seen in the uninduced 91-R strain (Fig. 5-1 and 5-2). Results in the previous chapter had indicated that the low expression of *CYP* genes in 91-C could be due to the presence of a repressor molecule. Thus, the induction of *Cyp6a8-91C* with barbiturates could mean that these compounds could directly or indirectly antagonize the repressor. In that respect, the mechanism of barbiturate induction in *Drosophila* may be similar to that found in bacteria, involving the inactivation of the 3rd chromosomal negative regulatory factor(s) that are presumably bound to the promoter regions (Results and Discussion, Chapter 4). There is evidence that barbiturate response in bacteria also involves synthesis of positive factors (He et al., 1995). It may be that the induction of *Cyp6a8* in the strains examined (Fig. 5-1 and 5-2) is the result of both the processes, which involves inactivation of the repressor and stimulation by a positive factor(s).

It appears from the results in Fig. 5-2, that *Cyp6a8* has a different response to barbitol and phenobarbital in the 91-R strain. The difference could be due to the relative amounts of the barbiturate compounds used, as the barbitol concentration was about 12 to

25 fold greater than the phenobarbital concentrations. However, if the effect of phenobarbital on *Cyp6a8* induction is considered in the three different strains analyzed, that is 91-R, 91-C and *ry*⁵⁰⁶, the lack of induction is only seen in case of 91-R which is an overproducer strain compared to the other two strains (91-C and *ry*⁵⁰⁶) which are underproducer strains (Fig. 5-2). Similar results have been observed in studies with *Cyp6a2* (Fuchs et al., 1994, Brun et al., 1996) and the housefly *CYP* genes, *CYP6A1* and *CYP6D1* (Carino et al., 1992; Scott and Lee, 1993; Scott et al., 1995). Phenobarbital treatment of insect strains which show a high level of constitutive *CYP* gene expression is not very effective in inducing *CYP* gene expression or does so to a moderate amount. The extent of induction is far greater in insect strains where the constitutive level of expression is lower. It may be that in the overproducer strains, transcription of the *CYP* genes is occurring near maximum levels, so that there is not much induction upon exposure to barbiturates.

Functional analysis of *Cyp6a8* upstream DNA in mediating barbiturate inducibility.

Analysis of the upstream DNA of *Cyp6a8* identified several putative barbie box sequences (Fig. 3-4). Barbie boxes have been found in the upstream DNA of several barbiturate inducible genes (Liang and Fulco, 1995). Given the fact that *Cyp6a8* is inducible with two barbiturate compounds, the barbie box -like sequences found in the upstream DNA of *Cyp6a8* take on a definite functional significance. The invariant AAAG barbie box signature was found in 12 places in the upstream DNA of which only one (at -62) was identified in the MatInspector search. To assess the functional significance of these sequences and define *cis*-elements that may be responsible for barbiturate induction, several *luciferase* transgenic lines that were generated in previous experiments (Chapter 3) were studied for barbiturate inducibility. Two representative transgenic lines were selected for each construct (0.2-luc, 0.8-luc and 3.1-luc) for

induction. In these constructs, 0.2-kb, 0.8-kb or 3.1-kb of upstream DNA of *Cyp6a8* is attached to the 5' end of the *luciferase* reporter gene.

Results of the luciferase assay (Fig. 5-3) show that phenobarbital induces expression of the reporter gene in all transgenic lines. The magnitude of the induction over constitutive levels seems to be similar in all lines, although the absolute activity is variable. There is an approximately 8-fold induction of activity in the 0.2-luc constructs. This implies that *cis*-elements responsible for phenobarbital induction are present within – 0.2-kb of the upstream DNA. Sequence analysis shows that there are three barbie box sequences (at – 62, – 87 and – 137) within this region. Thus, either one or all three of them together may be responsible for the phenobarbital inducibility of the reporter construct. Compared to the induced levels in the 0.2-luc transgenic lines, the induced levels (absolute amounts) in the 0.8-luc transgenic are about 4-fold higher (Fig. 5-3). Sequence analysis showed that the 0.8-kb upstream DNA has 6 putative barbie boxes, including the ones found in the 0.2 kb DNA. These sequences in the 0.8-kb DNA may have an additive effect and bring about the higher expression in the induced 0.8-luc lines. However, it is also likely that the higher expression is due to the more efficient transcriptive capacity of the construct itself. If the constitutive levels of expression are studied (solid bars, Fig. 5-3), a similar 4-fold increase in can be seen in between the 0.2-luc and 0.8-luc transgenic lines. It may be recalled that computer analysis of the upstream DNA identified several transcription factor binding sites between the upstream *SpeI* and *SalI* sites which could bring about more efficient transcription (Fig. 3-9). The 3.1-luc transgenic lines appear to have a lower level of induced expression (absolute levels) compared to the induced expression in the 0.8-luc transgenic lines (Fig. 5-3). Thus, the cluster of barbie boxes found between – 0.8-kb and – 3.1-kb upstream DNA (Fig. 3-6) does not seem to have a role in enhancing the response to phenobarbital. The lower level of expression could also

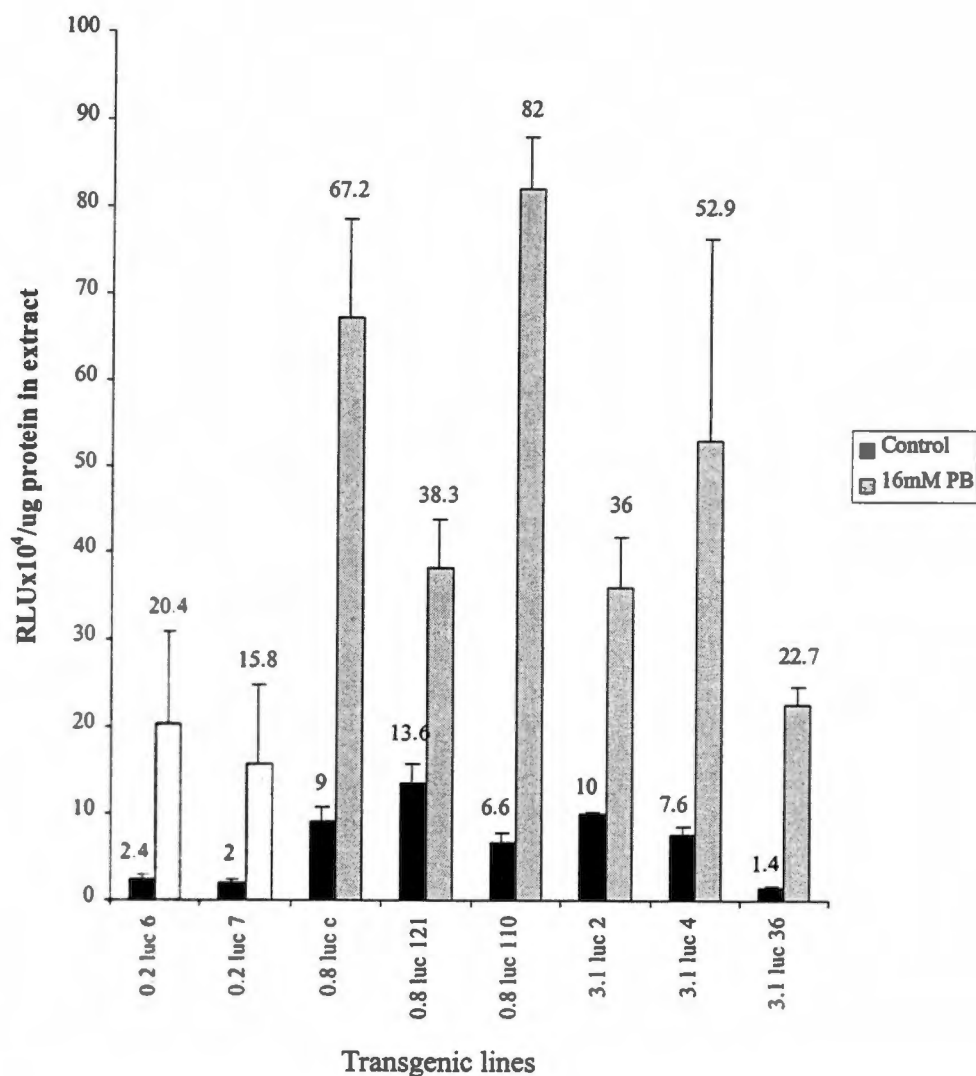


Figure 5-3: Phenobarbital induction of luciferase activity in transgenic lines.

Transgenic hybrid females were treated to 16 mM phenobarbital for 24 hours. Luciferase activity was measured in total body homogenates using luciferin as a substrate. The light emitted in the reaction was quantitated using a Zylux FB12 luminometer and expressed as RLUs (relative light units)/ μ g of total protein. Each column in the histogram represents a mean of three individual readings.

mean that the *Cyp6a8* upstream region between -764 (*SpeI* site) and -3.1 kb (*EcoRI* site) has potential sequences that could bind factors that attenuate the PB responsiveness. This putative negative factor, however, would be specific for barbiturate responsiveness because the constitutive levels of expression of the 3.1-luc lines appear to be similar to that shown by the 0.8-luc lines (Fig. 5-3, solid bars and Fig. 3-8). All these results taken together imply that the sequences that can confer phenobarbital responsiveness on the *luciferase* reporter gene are located within -0.2-kb region of the *Cyp6a8* upstream DNA.

NNNNAAAGNNNDN was formulated to be the consensus sequence for the barbie box (Fulco et al., 1994; Gaidamakova, 1996). A search of the EMBL nucleotide sequence database found a 2 to 5 fold higher occurrence of this consensus in cytochrome P450 genes compared to all the nucleotide sequences in the database (Gaidamakova, 1996). This implied that presence of these sequences is a characteristic feature of cytochrome P450 genes. Barbie box sequences have been found in the 5' regulatory regions of several barbiturate inducible genes including mammalian and insect *CYP* genes (Fournier, et al., 1994; Cohen et al., 1994; Liang and Fulco, 1995; Dunkov et al., 1997, Scott, 1999). Studies with barbie box sequences from bacteria and rat showed that these sequences can bind to proteins from both bacteria and rat liver extracts and that the protein binding is different with barbiturate treated and untreated extracts (He and Fulco, 1991). This implied that the barbie box sequences may have a role in mediating the barbiturate response.

However, in the mammalian system there seems to some doubt about the relevance of these sequences with respect to barbiturate induction. Although barbie box sequences appears to be conserved in the proximal promoters of the mammalian *CYP2B1/2* and *CYP2C1* genes, transgenic analysis in the mouse showed that the proximal promoter sequences did not confer phenobarbital responsiveness (Ramsden et al., 1993). A distal sequence, -5.9 to -1.1 of the chicken *CYP2H1* gene could confer

phenobarbital responsiveness to a heterologous SV40 promoter (Hahn et al., 1991). Similarly, a fragment between -2.5 to -2.0 of the *CYP2B2* gene could confer PB inducibility to the CAT (chloramphenicol acetyl transferase) reporter gene (Trottier et al., 1995). Such experiments identified a region of 163 bp known as PBRU (phenobarbital response unit) which is thought to be important in PB mediated induction of mammalian *CYP* genes (Kemper, 1998). The PBRU contains an NF-1 site, which is the major protein binding site for a nuclear factor and also putative nuclear receptor binding sites (NR sites) that flank the NF-1 site (Honkakoski et al., 1998; Kemper, 1998). Each NR site includes two imperfect direct repeats of the AGGTCA sequence separated by 4 bp (Forman and Evans, 1995), which is a steroid binding half site and has been proposed to be critical in mediating the phenobarbital response (Honkakoski et al., 1996). In the light of these observations it seems significant that an NF-1 site was identified by the MatInspector analysis at -143 of *Cyp6a8*. Visual scanning identified four regions that contain imperfect AGGTCA sites. (TGTCCT at -184, AAGTCA at -77, TTGCCT at -50, AGATCA at -17, the ones at -184 and -50 being on the template strand, Fig. 3-4). It is to be noted that these sequences in the - 0.2-kb region of the *Cyp6a8* upstream DNA, are present as single sites and hence the significance as to acting as a bonafide NR sites is questionable. However, the presence of sequences like barbie boxes, the NF1 site or the receptor half sites, within 0.2-kb of the upstream region of *Cyp6a8* seem significant as this fragment confers barbiturate inducibility to a heterologous reporter gene. Similar sequences have been shown to be functionally important in bacterial or mammalian *CYP* genes for phenobarbital induction (Liang et al., 1995; Honkakoski, et al., 1998; Kemper, 1998). However, the real significance of the NF1 site, steroid binding half sites or the barbie box sequences seen in the *Cyp6a8* upstream DNA can only be definitely determined if site directed mutagenesis of the critical residues are carried out and the effect on barbiturate responsiveness is studied.

Strain specific differences in *Cyp6a8* expression in response to barbiturates

Cyp6a8 shows variation in constitutive levels of expression in different strains of *Drosophila*. This variation could be due to differences in the *cis*-elements of the strain-specific *Cyp6a8* alleles or a variation in the strain-specific *trans*-acting regulatory factors. The evidence obtained up to now favors the latter hypothesis. Such evidence includes the effect of the third chromosomes from underproducer strains like 91-C and *ry*⁵⁰⁶ on *Cyp6a8* alleles from overproducer strains like 91-R and MHIII-D23 (Chapter 3, Results and Discussion). Evidence that the almost null expression of *Cyp6a8-91C* may be due to a negative *trans*-acting factor is also provided by the barbiturate inducibility of this allele (Fig. 5-1), where induction with barbiturate compounds increased the level of *Cyp6a8-91C* expression to levels of expression of the *Cyp6a8-91R*. Since barbiturates could reduce the repression present on constitutive levels of expression of *Cyp6a8* in 91-C, it was important to determine whether barbiturate inducibility of *Cyp6a8* was strain specific. This would not only help to understand the mechanism of barbiturate induction in eukaryotes but would also show whether the machinery for barbiturate induction interacts with controls that exist for constitutive levels of CYP expression in *Drosophila*.

Poly A⁺ RNA samples isolated from adult flies from four strains of *Drosophila melanogaster*, treated with two different concentrations (8mM and 16 mM) of barbital or phenobarbital, was examined by Northern blot analyses. Expression of *Cyp6a8* in the control and treated samples were represented as ratios of CYP6A8 to RP49 hybridization. The magnitude of the excess over control samples was calculated to provide an estimate about the extent of induction and these values were compared between the strains. Fig. 5-4 A shows the induction of *Cyp6a8* over control values after barbital treatment of flies from MHIII-D23, 91-R, 91-C and *ry*⁵⁰⁶. Treatment with 16mM barbital results in maximum induction of *Cyp6a8* in the 91-C strain, which is about 45-fold. This is

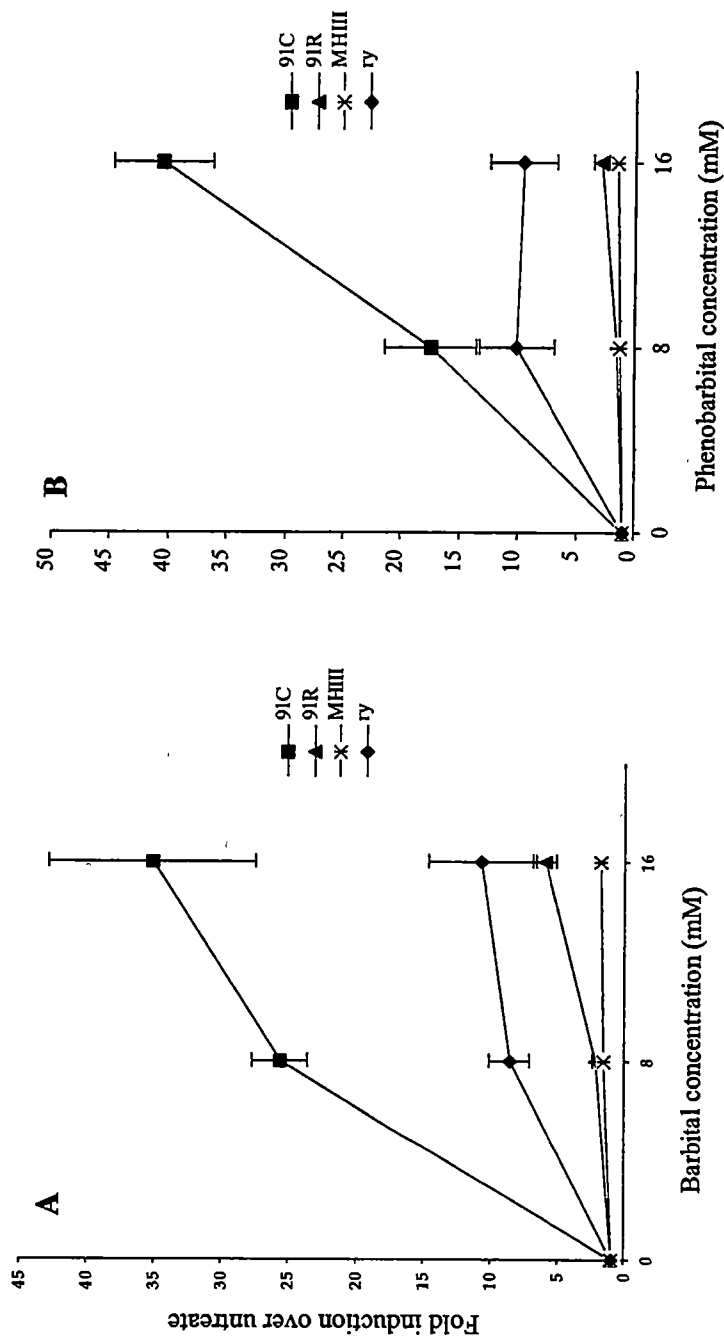


Figure 5-4: Dose response graphs for the effect of barbital (A) and phenobarbital (B) on *Cyp6a8* expression.

Effect of barbital and phenobarbital on induction of *Cyp6a8* was studied in two underproducer (91-C and *ry*⁵⁰⁶) and two overproducer (91-R and MHIII-D23) strains of *D. melanogaster*. *Cyp6a8* hybridization signals on Northern blots were quantified and corrected for RNA loading using RP49 as the internal control. Fold induction was calculated for each treatment over values obtained for the untreated samples. Each data point represents a mean reading obtained from three Northern blots.

followed by a 10-fold induction in γ^{506} . The least levels of induction are shown by the overproducer 91-R and MHIII-D23 strains. In case of 91-R there is a 6-fold induction with 16mM barbital, however, very little induction is shown with the 8mM treatment. MHIII-D23 shows very modest levels of induction, a little less than 2-fold, at both the concentrations tested. A similar picture is reflected in case of phenobarbital induction (Fig. 5-4 B).

The results from Fig. 5-4 (A and B) show that there is a perfect *negative* correlation between constitutive levels of *Cyp6a8* expression in the four strains of *D. melanogaster* and extent of *Cyp6a8* induction at both concentrations of barbital and phenobarbital used. This seems to imply that regulatory mechanisms involved in barbiturate induction may be linked to regulatory mechanisms that control constitutive levels of expression. In overproducer strains like MHIII-D23 where the basal level of *Cyp6a8* expression is very high, the gene expresses at near maximum levels. Hence when barbiturates are added, the extent of induction is not very high. Similarly in 91-C, where expression of *Cyp6a8* is almost non-existent, inducer chemicals can relieve the repression and cause induction of expression. There is also an increase in the extent of induction with an increase in the amount of the barbiturate compound used. This seems to imply that the inducer chemicals could act either directly by titering away the negative factors that maintain a low basal level of expression or act by synthesis of positive factors that can alleviate repression in a quantitative manner. Barbiturate induction in bacteria seems to involve both these mechanisms, though there is some controversy regarding the binding of one of the positive factors (reviewed in Kemper, 1998; Shaw et al., 1998). It has been hypothesized that high expression of *CYP* genes in strains like MHIII-D23 and 91-R was due to a lack of repression by negative factors (Chapter 3, Results and Discussion). If barbiturates act by removal of the negative factors, the increase in *Cyp6a8* expression would not be expected to be very high in the overproducer strains as there

maybe a paucity of negative factors in such strains. This may be one explanation for the low level of induction in overproducer strains. Superimposed on such controls could be further increase in expression by barbiturate mediated synthesis of positive factors. If positive control were the only operational mechanism, it would be expected that the extent of increase would be the same in all four strains, unless there was a strain specific polymorphism for the barbiturate regulated positive factors.

Similar results have been obtained in phenobarbital induction studies with *CYP6A1* and *CYP6D1* (Carino et al., 1992; Scott and Lee, 1993). In both cases the *CYP* genes are phenobarbital inducible. However, the extent of induction is not much in the overproducer Rutgers and LPR strains of the housefly. It is much higher in the underproducer strains. In case of *CYP6A1*, the level of induction was found to be 22-fold in the insecticide resistant, Rutgers strain and about 100-fold in the insecticide sensitive, *sbo* strain (Carino et al., 1992). Tissue distribution studies of *CYP6D1* in phenobarbital treated house flies showed that tissues from LPR strains flies showed a low response to phenobarbital, while tissues from phenobarbital treated susceptible flies showed increase up to the levels found in flies of the LPR strain (Scott and Lee, 1993). Measurement of CYP dependent enzyme activity, like *p*-nitroanisole (PNA) demethylation in resistant strain also showed a similar lack of responsiveness to phenobarbital induction (Hallstrom et al., 1984).

Effects of chromosomes from *ry*⁵⁰⁶, 91-R and 91-C strains on barbiturate mediated induction of *Cyp6a8*

To understand regulatory controls that may exist on *Cyp6a8* expression and with the larger goal of elucidating the mechanisms of phenobarbital induction in insects, barbiturate induction of *Cyp6a8* was studied in some of the chromosome substitution stocks synthesized in the previous chapter.

1) Barbiturate induction of *Cyp6a8* in stocks with chromosomes from 91-R and ry^{506}

Initial experiments that were done to study whether barbiturate compounds induced *Cyp6a8* also indicated that strain-specific differences existed in the extent of induction (Fig 5-1 and 5-2). Since the amount of the induced transcripts was more in the ry^{506} strain than 91-C strain, the first set of an overproducer/underproducer chromosome combination stocks to be analyzed was between 91-R and ry^{506} . Previous experiments had showed that the 3rd chromosome from under-producer strains exerted the major effect on constitutive expression of *Cyp6a8*. To see whether such an effect was shown for barbiturate mediated expression, the 3rd chromosomes from 91-R and ry^{506} were analyzed for any effect on barbiturate-mediated induction of *Cyp6a8*.

Fig. 5-5 shows the results of Northern blot analyses of poly A⁺ RNA from control and barbiturate treated flies from three 91-R/ ry^{506} combination stocks – non-iso 1, iso-6 and iso-34. The chromosome constitution of these stocks are $2^R/2^R$; $3^{ry}/3^{ry}$ for the first two stocks and $2^{ry}/2^{ry}$; $3^R/3^R$ for the last one. It should be mentioned that the two latter stocks carry isogenic chromosomes. Flies from 91-R and ry^{506} were used as control samples. *Cyp6a8* is shown to be induced by both barbital and phenobarbital in all the stocks examined. The extent of induction is more with barbital than phenobarbital in 91-R, non-iso 1 and iso-6, that is, all stocks with the 2nd chromosome from the 91-R strain. The two barbiturate compounds induce *Cyp6a8* to similar levels in iso-34 and ry^{506} , both of which carry the 2nd chromosome from the ry^{506} strain. It is to be noted that *Cyp6a8* is located on the 2nd chromosome.

If barbiturate induction of *Cyp6a8* is compared between the 91-R and ry^{506} strains, it appears that the two strains show a similar response to barbital. Hence, induction with barbital cannot be used as a probe to analyze strain-specific chromosomal effects. However, the response of *Cyp6a8* to phenobarbital is different in the two strains.

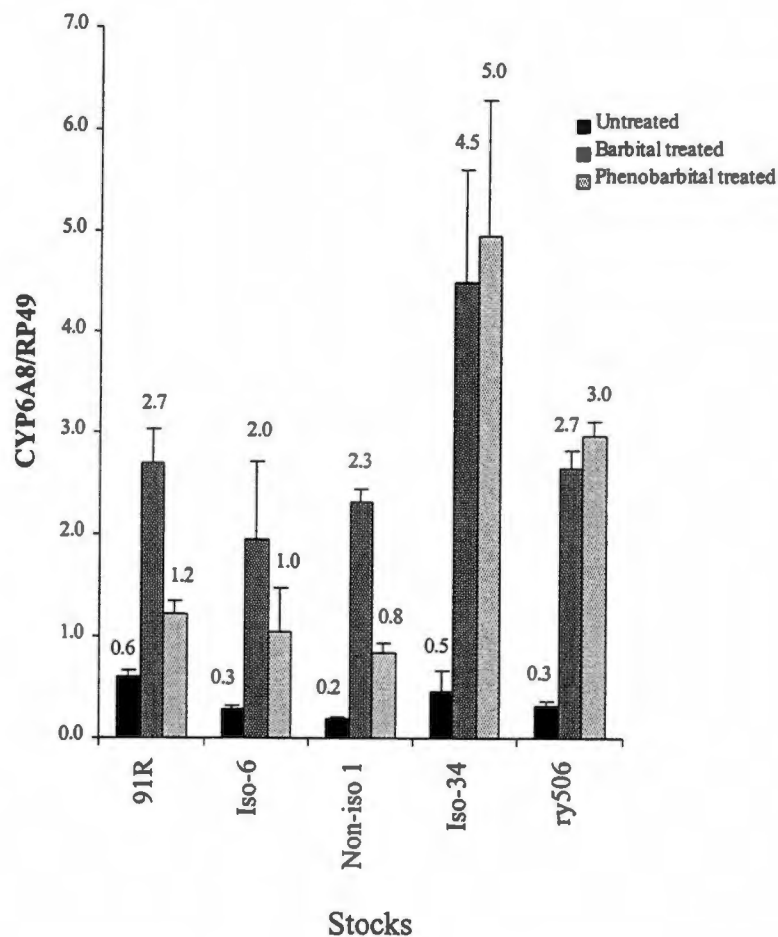


Figure 5-5: Barbiturate induction of *Cyp6a8* in 91-R/*ry*⁵⁰⁶ chromosome combination stocks.

Adult females were treated to 16mM barbital or phenobarbital for 24 hours. Poly A⁺ RNA isolated from such flies was used for Northern blot analysis. Expression of *Cyp6a8* was studied using a CYP6A8 cDNA probe and RNA loading errors were corrected using RP49. Each bar represents the mean CYP6A8/RP49 ratios for readings obtained from three Northern blots. Untreated control flies were kept on a medium reconstituted with water for the same length of time.

This drug induces *Cyp6a8* to about 10-fold in the ry^{506} strain while induction is only 2-fold in 91-R (Fig. 5-5). Strain-specific differences could be a reflection of a difference in the nature of regulatory *trans*-acting factors or a difference in the nature of *cis*-elements specific to each allele. To analyze whether the difference in the phenobarbital induction pattern was due to differences in strain-specific *trans*-acting factors, the barbiturate induction pattern of *Cyp6a8* was compared between non-iso 1 and iso-6 stocks with the parental 91-R strain. It was seen that the overall pattern of *Cyp6a8* induction with phenobarbital in both stocks resembled the pattern seen in case of the parental 91-R strain. The extent of induction of the *Cyp6a8-91R* alleles after phenobarbital treatment was 4.4 in case of non-iso 1 and 3.6 in case of iso-6 which is more similar to the induction pattern observed in 91-R than observed in the ry^{506} strain. Since non iso 1 and iso 6 carry the 3rd chromosome from ry^{506} , this implied that the third chromosome from ry^{506} could not modulate the phenobarbital inducibility of the *Cyp6a8-91R* allele and increase the induction to the levels seen in the ry^{506} strain. Similarly, when *Cyp6a8* induction was compared between iso-34 (carrying *Cyp6a8-ry*) and ry^{506} , it was found that *Cyp6a8-ry* followed the same pattern of induction as seen in the parental ry^{506} strain. The induction after treatment with phenobarbital was about 10-fold over control, which was similar to that seen in ry^{506} . Hence, it appears that the third chromosome does not have much effect in modulating the phenobarbital induction response of *Cyp6a8*. Rather, the patterns seen in the combination strains seem to resemble the pattern of the parental strain from which the 2nd chromosome has been derived. The lower response of *Cyp6a8-91R* to phenobarbital at the concentration used (16 mM), thus could be a function of either an allele specific feature of the gene or could be due to *trans*-acting factors on the second chromosome of 91-R.

To study whether barbiturate induction patterns of *Cyp6a8* are influenced in a similar manner by chromosomes from another under-producer strain, the 91-R/91-C chromosome combination stocks (Chapter 4, Materials and Methods) were analyzed next.

II) Barbiturate induction of *Cyp6a8* in stocks with chromosomes from 91-R and 91-C

The results in the previous section had shown that there was a strain-specific difference in phenobarbital inducibility of *Cyp6a8* between 91-R and *ry*⁵⁰⁶. To magnify these differences between 91-R and 91-C, a lower concentration of the barbiturate compounds was chosen. It was expected, by extrapolation of the results shown in Fig. 5-4, at 4mM there would be no induction of *Cyp6a8* with the 91-R related factors but the 91-C related factors would still be responsive.

Fig. 5-6 shows the results of Northern blot analyses of 91-R/91-C chromosome substitution stocks (RRC, RCR, CRC, CCR) treated with 4 mM barbital or phenobarbital. If the barbital induction of *Cyp6a8* is compared between 91-R and 91-C, the gene is induced in both strains, but the extent of induction is greater in 91-C (7-fold) than 91-R (1.6-fold) (Fig. 5-6A). However, the phenobarbital inducibility is about 8-fold in 91-C in contrast to 91-R where *Cyp6a8* is not induced (Fig. 5-6B). Thus, the low or non-inducibility of *Cyp6a8* to phenobarbital (at 4mM) appears to be a characteristic feature of the 91-R strain as *Cyp6a8* is induced by the drug in two other strains (*ry*⁵⁰⁶ and 91-C).

To study the effects of the 91-C chromosomes on phenobarbital inducibility of *Cyp6a8-91R*, induction of *Cyp6a8* in the RRC and CRC stocks was compared with the induction of the same allele in the parental 91-R strain. In both the combination stocks, *Cyp6a8-91R* was not induced by phenobarbital, implying that the 3rd chromosome from 91-C does not have *trans*-acting regulators for modulating phenobarbital inducibility. On the other hand, phenobarbital induction of *Cyp6a8-91C* in the RCR and CCR stocks is 5 fold and 2.7 fold, respectively. In both stocks, the 3rd chromosome is from 91-R. If the

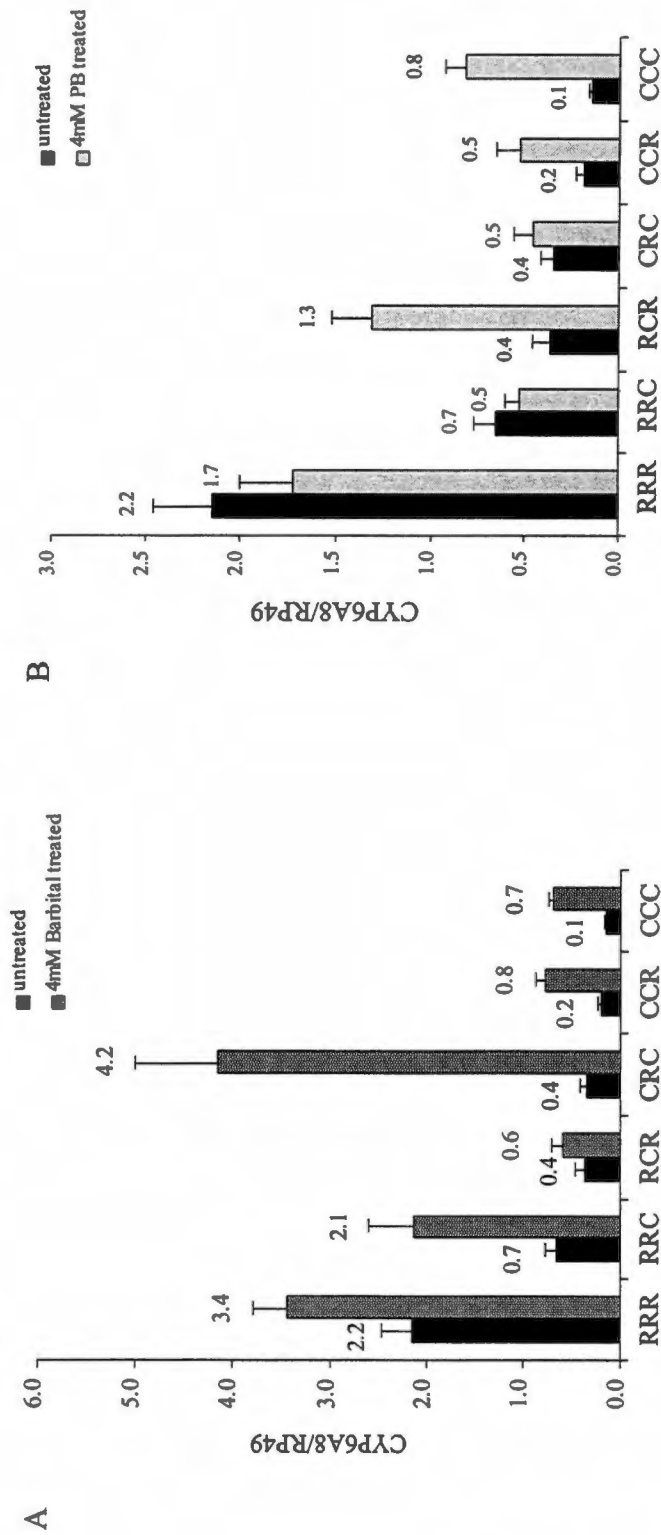


Figure 5-6: Induction of *Cyp6a8* with barbitol and phenobarbital in 91-R/91-C chromosome substitution stocks

Adult females were treated to 4mM barbitol or 4mM phenobarbital (PB) for 24 hours. Poly A⁺ RNA extracted from these flies was subjected to Northern blot analysis as described in Materials and Methods in Chapter 2. Hybridization signals were quantitated using a Packard InstantImager Radioanalytical Imaging system and RP49 was used as internal control to correct for RNA loading. Each bar in the histogram represents a mean of CYP6A8/RP49 ratios from three Northern blots.

lack of response of *Cyp6a8-91R* was a result of *trans*-acting factors on the 3rd chromosome, the effect would probably have been seen on *Cyp6a8-91C* in the RCR and CCR stocks. But *Cyp6a8-91C* is induced by phenobarbital in these stocks indicating that the lack of response of *Cyp6a8-91R* is not due to factors on the 3rd chromosome of 91-R.

Comparison of phenobarbital induction of *Cyp6a8-91R* in between the RRC and CRC stocks showed that phenobarbital induces the gene to a similar extent. This implies that the X chromosome of 91-C does not affect phenobarbital inducibility of *Cyp6a8*. Similar comparison of *Cyp6a8-91C* induction in the RCR and CCR stocks shows that the 91-R X chromosome does not bring about a reduction of PB responsiveness. However, in presence of the 2nd chromosome of 91-C, the X chromosome of 91-R seems to have a positive effect as the phenobarbital response of *Cyp6a8-91C* is about 3.5 times more in RCR compared to CCR. Rather than a simple effect of some locus (loci) on the X chromosome of 91-R enhancing the phenobarbital response, these data seem to indicate that the mechanism of phenobarbital responsiveness may be also regulated by interactions between loci on the X chromosome and the 2nd chromosome. If the higher level of induction of *Cyp6a8-91C* in RCR (compared to the level in CCR) was indeed due to a positive factor on the X chromosome of 91-R, the effect should have been reflected in a similar increase in *Cyp6a8-91R* induction in the stock RRC compared to that in CRC. However, that does not appear to be the case. The results taken together indicate that both the X and the 3rd chromosome are not responsible for the lack of *Cyp6a8* response to phenobarbital. Thus this phenotype is primarily caused by factors on the 2nd chromosome. However, it cannot be distinguished whether these factors are the *Cyp6a8* allele-specific *cis*-elements or *trans*-acting factors on the chromosome. Since similar results were obtained with the 91-R/*ry*⁵⁰⁶ chromosome combination stocks, it seems that the lack of response is caused by factors specific to the 91-R chromosome.

An interesting result was obtained with respect to barbital induction. Four mM barbital induces *Cyp6a8* in 91-R and 91-C, though the level of induction is about 1.6-fold in 91-R and 7-fold in 91-C. When the extent of barbital induction of *Cyp6a8-91R* is compared between the RRC and CRC stocks it is seen that the induction is about 3.3-fold in the former and about 12-fold over the basal level in the latter stock (Fig. 5-6A). The chromosomal difference between RRC and CRC is the origin of the X chromosome, which is from 91-C in CRC. Since the induction is about 4 times greater in CRC than RRC, it appears that barbital inducibility may be enhanced by loci on the X chromosome from 91-C. A similar effect of the 91-C X chromosome is also reflected in the barbital induction of *Cyp6a8-91C* in the RCR and CCR stocks where there is a 1.6-fold induction in the former stock and a 4.1-fold induction in the latter stock (Fig. 5-6A). These data suggest that the X chromosome has factors that may influence the barbital response. However, it cannot be distinguished whether the effect seen is due to positive factors on X chromosome from 91-C or presence of negative factors on the same chromosome from 91-R.

There also seems to be a component of the second chromosome in the enhanced barbital induction response. This becomes apparent when the stock CRC is compared with the parental 91-C strain (CCC), where the induction is much higher in the former stock. However, in this case not only are the 2nd chromosomes different but the *Cyp6a8* alleles are different too, so allele specific differences may complicate the picture. The main conclusions that can be drawn from these experiments are as follows

- 1) The major contribution in regulation of phenobarbital responsiveness appears to be from the 2nd chromosome. Phenobarbital inducibility does not seem to be affected by the X chromosome or the 3rd chromosome. But there seem to be some interactions between the 91-R X chromosome and 91-C 2nd chromosome, which enhance the PB response to a small extent. Since the expression of only one gene (*Cyp6a8*) has been

analyzed, it is not clear whether the lack of phenobarbital response is due to the *Cyp6a8-91R* allele or trans-acting factors on the 2nd chromosome.

2) Barbital inducibility of *Cyp6a8* appears to be influenced by loci or a locus on the X chromosome but second chromosomal and allele specific factors may also contribute to the extent of induction.

To try and resolve these issues, barbiturate inducibility of another CYP gene, *Cyp6a2*, was analyzed in the 91-R/91-C combination stocks.

Effects of chromosomes from 91-R and 91-C on barbiturate induction of reciprocal alleles of *Cyp6a2*

The 91-R/91-C combination stocks, RRC, RCR, CRC, CCR, were used for examining the effects of the 91-R and 91-C chromosomes on barbiturate inducibility of *Cyp6a2*. Fig. 5-7 shows the effect of 4mM barbital and phenobarbital on *Cyp6a2* expression in the chromosome combination stocks. 91-R and 91-C were also treated to the same concentration of the drugs and used as controls. As in the case of *Cyp6a8*, phenobarbital does not induce *Cyp6a2* in 91-R, and the RRC and CRC combination stocks but induction is seen in the 91-C strain as well as the RCR and CCR combination stocks. Barbital, on the other hand, induces *Cyp6a2* in all the stocks used for the experiment.

Viewed in a holistic manner, the trends in barbiturate induction of *Cyp6a2* appear to be similar to those seen for *Cyp6a8* (Fig. 5-6 and 5-7). Like PB induction of *Cyp6a8*, 4 mM phenobarbital does not induce *Cyp6a2* in 91-R but induces the gene about 7-fold in 91-C (Fig.5-7B). Analysis of PB induction of *Cyp6a2-91R* in RRC and CRC stocks, when compared with the parental 91-R strain shows that the presence of the 3rd chromosome from 91-C does not reverse the lack of PB responsiveness of the *Cyp6a2-91R* allele. Similarly, the 3rd chromosome of 91-R does not make the *Cyp6a2-91C* allele

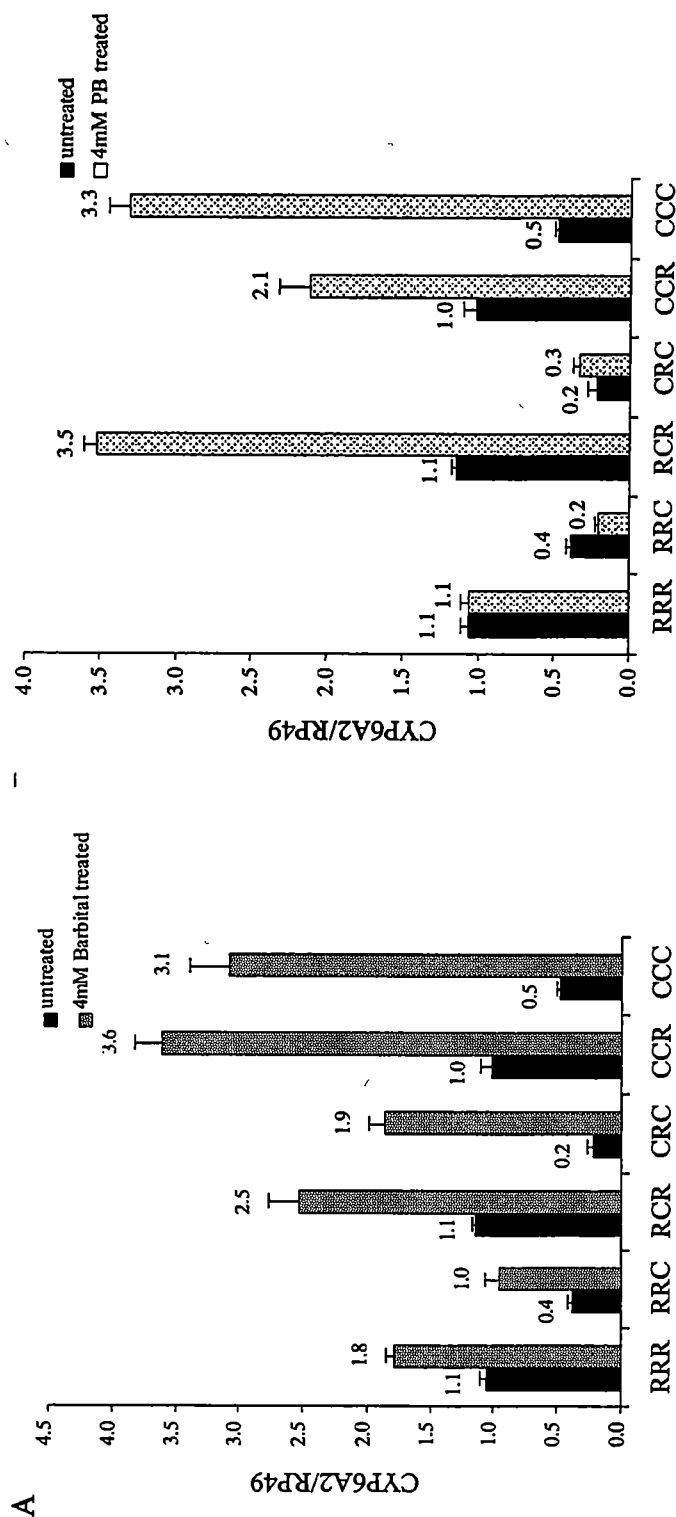


Figure 5-7: Induction of *Cyp6a2* with barbitol and phenobarbital in 91-R/91-C chromosome substitution stocks
 Adult females were treated to 4mM barbitol or 4mM phenobarbital (PB), poly A⁺ RNA was isolated and analyzed by Northern blotting as described in Fig. 5-6. Each bar in the histogram represents a mean of CYP6A2/RP49 ratios from three Northern blots.

refractory to PB in the RCR and CCR stocks. These results taken together prove that the 3rd chromosome does not have any effect in modulating PB induction, at least at the concentration tested. As in case of *Cyp6a8*, the X chromosome from 91-C does not influence PB response of *Cyp6a2-91R* in the CRC stock (compared to the expression of *Cyp6a2-91R* in the RRC stock) nor does the X chromosome from 91-R bring about a *lack* of PB response in the RCR stock (compared to the trend of *Cyp6a2-91C* expression in the CCR stock). However, in presence of the 91-R X chromosome, the PB inducibility of *Cyp6a2-91C* appears to be enhanced. Whether this is an interaction of the 91-R X chromosome *trans*-acting factors with the *Cyp6a2-91C* allele or with 2nd chromosomal factors from 91-C cannot be distinguished. The fact that a similar trend is observed with *Cyp6a8-91C* seems to suggest that the latter mechanism may be more likely. Since the X chromosome and the 3rd chromosome do not seem to confer the refractive pattern of PB response to the *Cyp6a2-91C* allele, by default, factors on the 2nd chromosome appear to be important in regulating the response to PB. These factors could be either *trans*- or *cis*-acting. However, since two genes (*Cyp6a2* and *Cyp6a8*) show similar responses to the chromosomal effects on PB responsiveness, it is more probable that a single *trans*-acting factor on the 91-R 2nd chromosome is responsible for the lack of phenobarbital responsiveness of the 91-R *CYP* alleles, rather than analogous *cis*-acting elements for the two independent genes.

With respect to barbital induction, the response of *Cyp6a2* is higher in 91-C compared to 91-R (6.4-fold to 1.7-fold). Barbital induces *Cyp6a2* in all the combination stocks and hence is not very effective as a probe to study the 91-R or 91-C specific chromosomal effects. The induction of *Cyp6a2-91R* in the CRC stock is about 9-fold. Compared to the barbital induction seen in the parental 91-R and 91-C stocks, the trend (extent of induction) seems to be more like that seen in the 91-C strain. Comparison of the *Cyp6a2-91R* expression in between the RRC and CRC stocks shows that barbital

induction is about 2-fold more in the latter. This may imply that the X chromosome of 91-C has factors that can enhance the barbital response. A comparison of the barbital induced expression of *Cyp6a2-91C*, in the RCR and CCR stocks, seems to support this hypothesis as the expression is higher in the latter stock which has the X chromosome from 91-C.

It appears from the results obtained with phenobarbital induced expression of *Cyp6a2* as well as *Cyp6a8*, that chromosome 2 has factor(s) that can regulate the response to phenobarbital. Liu and Scott (1997b) have mapped a *trans*-acting factor that regulates phenobarbital induction of *CYP6D1* in the house fly to chromosome 2. However, chromosome 2 of *Musca* is homologous to chromosome 3 of *Drosophila*. In two independent sets of crosses (91-R/*ry*⁵⁰⁶ and 91-R/91-C), the 3rd chromosome did not appear to affect phenobarbital response of the second chromosomal *Cyp6a8* gene. Studies with *Cyp6a2* in the 91-R/91-C combination stocks also showed this effect. Therefore, the factor that regulates PB responsiveness of *CYP6D1* appears to have a different chromosomal location from that in *Drosophila*. Terriere (1983) had proposed that the same regulatory genes were involved in both induction and biochemical resistance. That may well be the case in *Drosophila* as a major locus that is involved in insecticide resistance was localized to map position 64-66 on the second chromosome, as were other loci conferring DDT (56 ± 1) and malathion resistance (48.5) (Kikkawa, 1953; Tsukamoto, 1958; Houpt et al., 1988; Waters and Nix, 1988; Dapkus, 1992).

If all the regulatory influences on *CYP* gene expression are considered, it is seen that the effect of the 3rd chromosome regulators dominate and determine the level at which the *CYP* genes will be expressed. Under these conditions, when barbiturates are added, gene expression is enhanced over basal levels. All the regulatory trends for barbiturate-mediated expression do not supercede the controls for constitutive levels of expression but seem to be superimposed on them. A case that may be taken for

illustration is barbital induction of *Cyp6a8-91R* or *Cyp6a2-91R* in the RRC substitution stock (Fig. 5-6A and 5-7A). If the constitutive levels of expression are seen, the expressions of both *CYP* genes are about 3-fold lower compared to the parental 91-R strain. This can be explained by the fact that the RRC stock carries the 3rd chromosome from 91-C that has the putative negative regulators. Barbital induces both *Cyp6a8* and *Cyp6a2* in this stock but the absolute level of induction is lower than in the parental 91-R strain. This implies that the regulatory mechanisms for barbiturate induction may interact with and modulate the regulatory mechanisms for constitutive expression. If barbiturate induction were dependent on a mechanism, genetically independent to that regulating constitutive levels, then the absolute levels of barbiturate induction would probably not follow the trend of constitutive expression.

In conclusion, analyses of barbiturate inducibility of *Cyp6a8* in several chromosome substitution lines showed that 2nd chromosome has factors that can regulate the phenobarbital induction response, and that this response may be modulated by factors on the X chromosome. The X chromosome was also found to influence barbital induction of *Cyp6a8*. Study of the barbiturate response of another *CYP* gene, *Cyp6a2*, showed very similar trends in regulation as observed for *Cyp6a8*, implying that the regulatory effects of the chromosomes on barbiturate-mediated *CYP* gene expression may be a global effect, affecting several barbiturate responsive genes.

Chapter 6

General Discussion

“ In the end it should be apparent, however, that despite intensive study and despite dramatic advances in conceptual and technical approaches, sufficient intrigue remains concerning the evolution, mechanisms, and consequences of P-450 induction to preoccupy at least one more generation of diverse biological scientists. ”

Allan B Okey, 1990

In: Enzyme induction in the cytochrome P450 system.

Pharmacology and Therapeutics. 45, 241-298.

Xenobiotic metabolism by CYP enzymes has a special relevance in the life of an insect as it has an adaptive significance in interactions with the environment. Firstly, CYPs have been shown to be involved in metabolism of toxic plant chemicals which allows insect species to exploit several trophic niches (Krieger et al., 1971; reviewed in Feyereisen, 1999). Secondly, CYP-mediated metabolic detoxification has been shown to be an important factor in helping insects overcome the effects of a hostile chemical environment and become resistant to insecticides (Feyereisen, 1999). The observation that led to the above conclusions was that overexpression of a particular or several CYP enzymes was associated with resistance or utilization of plant resources which contained toxic chemicals (Schuler, 1996; Fogelman et al., 1998; Scott, 1999). In many cases such effects were reversed by application of general CYP inhibitors like piperonyl butoxide, implying that the mechanism of resistance or plant chemical metabolism was CYP-dependent.

CYP-mediated insecticide resistance

Many mechanisms may be involved in development of insecticide resistance, like gene amplification, target site insensitivity, and metabolic detoxification involving

enzymes like cytochrome P450 monooxygenases, esterases and glutathione-S-transferases. In a large number of cases, elevated levels of CYP enzymes are the cause of resistance (Agosin, 1985; Oppenoorth, 1985).

Resistance may be defined as the ability of a population to survive a dose of toxic chemical that would kill most individuals of the species. This phenotype is thought to be *preadaptive*, implying that resistance develops due to selection of factors already present in the population (Oppenoorth, 1985). Insects are exposed to a large variety of plant allelochemicals, and have evolved detoxification systems to break down toxic chemicals encountered in food plants. Thus, a genetic system already exists for detoxification of insecticides (Fraenkel, 1959). Survival of organisms, upon exposure to toxic chemicals depends on the rapid metabolism of the chemicals. Of the systems present in an organism, the recruitment of the monooxygenase enzyme system seems to be a logical choice for detoxification. The choice is not only due to the fact that these enzymes are involved in metabolism of complex organic compounds but also to the fact that several of the enzymes are inducible. Increased detoxification could be brought about either by a *modification* of CYP enzymes already present in the susceptible wild type strains or an increase in the *amounts* of the enzyme present in wild type insects. Under a constant insecticide selection pressure, genetic mechanisms facilitating increased detoxification could be selected for, leading to resistant populations.

Development of resistance in insects has obvious disadvantages in the control of arthropod pests. In most cases, development of resistance has been countered by increasing the amount of insecticide used, with detrimental effects on both human health and the environment. Resistance in insect vectors has also adversely affected the control of vector-borne diseases (WHO, 1976).

To design strategies to control resistance, a thorough understanding of the molecular mechanisms governing insecticide resistance is required as also the factors that

are responsible for the evolution of resistance. Since monooxygenase-mediated resistance is an important mechanism for development of resistance, strategies to regulate monooxygenase levels may be an important device to control resistance. In fact, several P450 inhibitors have been used in the design of insecticides (Feyereisen et al., 1990). Any study that contributes to the understanding of the molecular basis of CYP gene regulation in insects, thus, fulfils the long-term goal of devising strategies to control resistance.

Regulation of *CYP* genes in *Drosophila*

The main focus of this dissertation was a study of the regulation of *CYP* genes in *Drosophila*. As with other studies involving *CYP* gene regulation, the larger goal was in contributing to the understanding of the molecular mechanisms of insecticide resistance.

Regulation of constitutive expression

Most insect strains belonging to different genera like *Musca* (housefly), *Blatella* (cockroach), *Heliothis* and *Helicoverpa* (butterfly), *Culex* (mosquito) show a low level of constitutive expression of *CYP* genes (see Scott, 1999 for review). The first *CYP* gene to be isolated from *Drosophila* was *Cyp6a2* (Waters et al., 1992) and like the general trend in most insects, this gene was found to be expressed at low levels in the insecticide susceptible, wild type 91-C strain. In contrast, the expression of *Cyp6a2* was higher in the insecticide resistant 91-R strain. Since the constitutive level of *CYP* gene expression in most insect strains is low, this is thought to be the wild type phenotype, while the overexpression encountered in the resistant strains is regarded to be the mutant phenotype.

Studies done during this dissertation identified three new *CYP* genes, *Cyp6a8*, *Cyp6a9* and *Cyp6a9*-related from *Drosophila*. Two of them, *Cyp6a8* and *Cyp6a9*-related, were found to express at higher levels in 91-R compared to 91-C. Analysis of *Cyp6a8*

expression in several insecticide susceptible and resistant strains of *Drosophila* showed that, like *Cyp6a2* (Dombrowski et al., 1998), *Cyp6a8* expresses at low levels in insecticide susceptible strains while the expression is higher in 91-R. Since all three *CYP* genes, *Cyp6a2*, *Cyp6a8* and *Cyp6a9*-related are upregulated in the resistant 91-R strain, it may be postulated that these genes are under the control of a common regulatory mechanism. This feature is also found in MHIII-D23, another resistant strain of *D. melanogaster*, where both *Cyp6a2* (Dombrowski, et al., 1998) and *Cyp6a8* (Chapter 2, this dissertation) show very high expression. In the housefly, the insecticide resistant LPR strain shows high level of expression of *CYP6A1* and *CYP6D1*, implying that the upregulation of more than one *CYP* gene in resistant strains is not restricted to *Drosophila* (Carino et al., 1992; Liu and Scott, 1996). Thus, difference in the level of *CYP* gene expression between resistant and susceptible strains of insects seems to involve regulatory mutations, rather than *cis*-acting mutations in several individual *CYP* genes in the resistant species of insects.

The possibility that high expression in resistant strains may be due to a regulatory mutation is also suggested by analysis of the expression of *Cyp6a8* in the F1 hybrids between resistant (overproducer) and susceptible (underproducer) strains. In all three combinations examined (91-R/91-C, 91-R/*ry*⁵⁰⁶, and MHIII-D23/91-C), the expression of the allele from the overproducer strain was downregulated implying that the underproducer wild type strains carried a repressor. Similar observations were made in case of the *Cyp6a2* in the same hybrid combinations (Dombrowski, 1998) and also in case of *CYP6A1* from the house fly in F1 hybrids of the overproducer (Rutgers) and underproducer (*sbo*) strains (Carino et al., 1994). The results imply that the low level of constitutive expression of *CYP* genes (including *Cyp6a8*) in wild type flies may be due to the presence of a repressor. The high expression observed in the 91-R and MHIII-D23 strains may be due to the lack or mutation of such a negative factor(s).

Genetic studies in housefly suggest that expression of both *CYP6A1* on chromosome 5 and *CYP6D1* on chromosome 1 are regulated by *trans*-acting factors produced by loci present on chromosome 2 (Carino et al., 1994; Liu and Scott, 1996). In addition to chromosome 2, loci on chromosome 1 also influence *CYP6D1* (Liu and Scott, 1997a). The presence of a 'master regulator' gene on the 2nd chromosome of the housefly had been proposed by Plapp in 1984, based on resistance studies in the housefly. The regulatory effect of the housefly 2nd chromosome on *CYP* gene expression thus correlates well with the resistance studies. Since chromosome 2 of the house fly is thought to be homologous to chromosome 3 of *Drosophila* (Foster et al., 1981), one of the first experiments done to study the effect of *trans*-acting factors was to analyze the expression of *Cyp6a8-91R* in presence of the 3rd chromosomes from the underproducer *ry*⁵⁰⁶ strain. The results showed that the expression of *Cyp6a8-91R* is downregulated in presence of the 3rd chromosome from *ry*⁵⁰⁶. Two stocks that were derived independently (stock non-iso 1 and rRr, Chapter 3) showed the same effect. This repressive effect on *Cyp6a8-91R* was also seen with the 3rd chromosome of 91-C, which is another underproducer strain (stocks RRC and CRC, Chapter 4). Thus the 3rd chromosome from two underproducer strains showed a similar repressive effect on *Cyp6a8-91R*. Analysis of *Cyp6a8* expression in 91-C/MHIII-D23 combination stocks showed that this effect was not restricted to the *Cyp6a8* allele from 91-R but was also seen on *Cyp6a8-MHIII*. These data indicate that not only is there a negative factor present in two underproducer strains, but the effect of such a factor is similar on two alleles of *Cyp6a8* from the overproducer 91-R and MHIII-D23 strains. Effect of this negative factor is also seen on another *CYP* gene – *Cyp6a2*. In combination strains between 91-C/91-R and 91-C/MHIII-D23, the presence of the 3rd chromosome of 91-C was found to repress the expression of *Cyp6a2-91R* and *Cyp6a2-MHIII*. All these results taken together imply that there is a locus (loci) on the 3rd chromosome from underproducer strains which can reduce the expression of two *CYP*

genes (*Cyp6a2* and *Cyp6a8*) belonging to the same subfamily. Thus in the underproducer strains, the low level of constitutive expression is probably a function of this negative factor. The fact that two genes are affected in the same manner implies that *CYP* genes belonging to the same subfamily could be coordinately regulated. The expression of alleles from the underproducer strains (*Cyp6a2-91C* and *Cyp6a8-91C*) is higher in the presence of the 3rd chromosome from either 91-R or MHIII-D23, compared to the parental underproducer 91-C strain. These results can be explained if we assume that the high level of expression in the resistant strains is due to a mutation in the 3rd chromosomal negative factors. Since the negative factors are probably attenuated or absent, the 3rd chromosome from 91-R and MHIII-D23 bring about a higher level of expression of the *Cyp6a2-91C* and the *Cyp6a8-91C* alleles.

There is indirect evidence in support of the hypothesis of a 3rd chromosome regulator. Waters and Nix (1988) showed in *Drosophila*, that the level of a heme containing polypeptide band on SDS-polyacrylamide gels found in resistant strains (P450-B), and the activity of dimethylnitrosamine demethylase (DMN-d), a P450 dependent activity is higher in insecticide resistant strains of *Drosophila* like Hikone R. The same study suggested that while the structural genes are present on the 2nd chromosome, the 3rd chromosome from the insecticide resistant Hikone R strain was required for high levels of the heme containing polypeptide band. Recombination analysis showed that two loci on the 3rd chromosome, one near *hairy* (3-26) and the other near *curled* (3-50) regulate DMN-d activity (Waters and Nix, 1988). In a similar study, Houpt et al. (1988) mapped loci responsible for malathion resistance in *Drosophila*. Their results showed that stocks carrying the 3rd chromosome from a malathion resistant strain have more cytochrome P450 content and heme staining protein bands than control stocks. Mapping experiments showed that the locus responsible was near *striped* (3-62). One study by Hallstrom (1985) identified a regulatory locus on the 2nd chromosome, at ~ 65

map units, which was also the major locus for insecticide resistance mapped by other investigators (Kikkawa, 1953; Ogita, 1958; Tsukamoto, 1958). Taken together these data indicate that there may be several loci that can regulate P450 content and P450 activity, however, they are mostly present on the 3rd chromosome. In the above studies, the presence of 3rd chromosome regulators was inferred based on modulations of CYP-mediated enzymatic activity and variation in the amounts of heme containing protein bands on SDS polyacrylamide gels. The difference with the study done for this dissertation lies in the fact that this was the first study showing that in *Drosophila*, two specific 2nd chromosomal *CYP* genes (*Cyp6a2* and *Cyp6a8*) were *trans*-regulated by loci on the 3rd chromosome.

The regulation of constitutive levels of *CYP* gene expression does not appear to be as simplistic as just the presence of a negative factor on the 3rd chromosome of wild type strain, although the major effect seems to be exerted by the 3rd chromosome regulators. The X chromosome seems to have an effect as *CYP* gene expression is elevated in stocks that carry the X chromosome from *ry*⁵⁰⁶ (Stocks RRr and rRr, Chapter 4). However, this effect does not seem to extend to the X chromosome from 91-C (Stocks RRC and CRC, Chapter 4). Except in case of *Cyp6a2* in the 91-C/91-R combination stocks, the upregulation of the *CYP* alleles (*Cyp6a2-91C* and *Cyp6a8-91C*) in presence of the 3rd chromosome from the overproducer 91-R or MHIII-D23 strain does not occur up to the endogenous levels in 91-R or MHIII-D23. This seems to imply that an interaction between loci on different chromosomes from the same strain is probably required to achieve the high levels seen in 91-R or MHIII-D23. A result along similar lines was found by Dapkus and Merrell (1977), when they tried to map DDT resistance in 91-R. They concluded that resistance was multifactorial and that all three major chromosomes were involved. 91-R and MHIII-D23 evolved due to selection pressure by DDT and malathion, respectively. The final product of the resistant over-producing strains (91-R or

MHIII-D23) could be expected to be the result of co-selection of several factors. However, these factors may not have equal contributions to the phenotype of resistance or *CYP* gene overexpression. The results in this study indicate that one such major factor is on the 3rd chromosome. The chromosome substitution stocks probably did not have the correct combination(s) for the factors required for over-expression, hence 91-R levels (in case of *Cyp6a8*) and MHIII-D23 levels (in case of both *Cyp6a2* and *Cyp6a8*) of *CYP* expression were not achieved in the substitution stocks (Chapter 4 Fig. 4-8 and 4-9).

Based on the results from this study a very simplistic model can be proposed for the constitutive expression of two *Drosophila CYP* genes belonging to the CYP6a subfamily (Fig. 6-1). The wild type strains like 91-C and *ry*⁵⁰⁶ have a negative regulator(s) on the 3rd chromosome. This repressor interacts with the *cis*-elements of the *CYP* structural genes like *Cyp6a2* and *Cyp6a8* which are on the 2nd chromosome and thus brings about a low constitutive level of expression. In insecticide resistant strains, which generally show higher levels of *CYP* gene expression, one of the ways in which the high level of expression could be achieved is by mutation of the repressor locus (loci) on the 3rd chromosome. The mutation could be very severe, resulting in a total absence of the negative factor thereby bringing about the extremely high levels of expression of both *Cyp6a2* (Dombrowski et al., 1998) and *Cyp6a8* (Chapter 2) in MHIII-D23. The mutation in 91-R may result in an attenuated form of the negative factor, which results in a high level of *CYP* gene expression in 91-R but not as high as in MHIII-D23. This mechanism may set the basic framework of *CYP* gene regulation. Variations of this theme could be provided by interaction of strain-specific factors on the X chromosome, *cis*-acting elements of the individual genes or homologous interaction between chromosomes of the same strain.

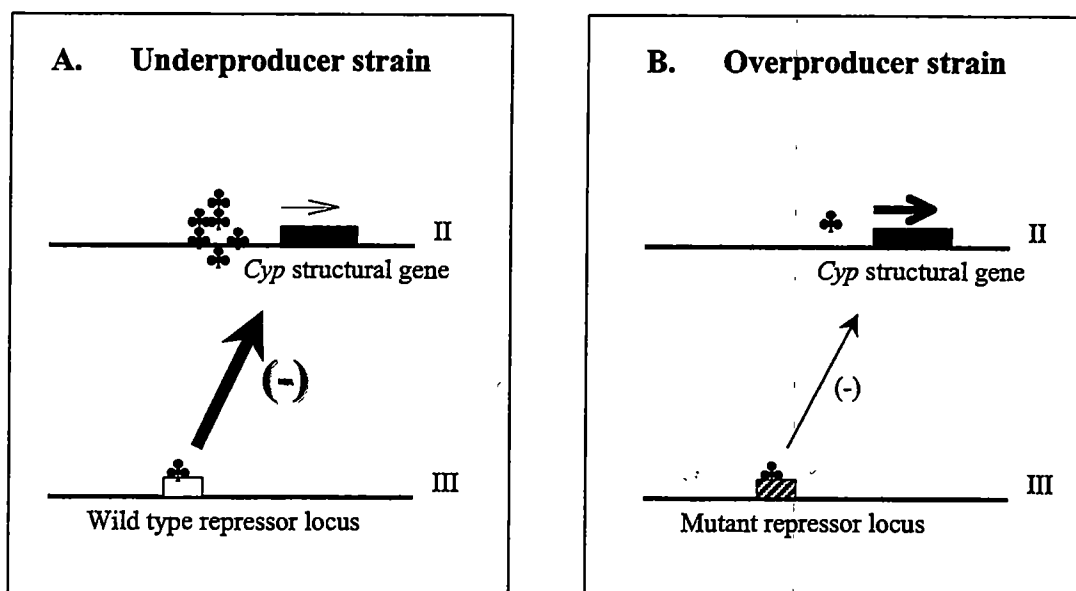


Figure 6-1: Model showing a possible mode of regulation for constitutive levels of expression of CYP6 genes in *Drosophila*.

The model is based on results obtained by analysis of *Cyp6a2* and *Cyp6a8* expression in various chromosome combination stocks of *Drosophila*. The 3rd chromosome of under-producer stocks like 91-C and *ry*⁵⁰⁶ are thought to carry negative factor(s) which interact with *cis*-elements of *CYP* genes on the 2nd chromosome and bring about a low level of expression. In insecticide resistant strains such as 91-R and MHIII-D23, the overexpression of several *CYP* genes may be brought about by a mutation in the 3rd chromosome repressor locus resulting in either a lower amount of the negative factor or a form that binds weakly to the upstream DNA of *CYP* genes, thereby bringing about a high level of constitutive expression.

II and III represent the 2nd and 3rd chromosomes from *Drosophila* while the symbol ♣ represents the putative negative factor synthesized by loci on the 3rd chromosome.

Regulation of inducible expression

Barbiturate compounds have been known to induce drug metabolizing enzymes in mammals (Remmer, 1962). Insect CYPs which have been directly or indirectly implicated in insecticide resistance, like *CYP6A1*, *CYP6D1*, *Cyp6a2* have been shown to be inducible by phenobarbital (Carino et al., 1992; Scott and Lee, 1993; Brun et al., 1996, Dunkov et al., 1997). Since there was evidence that *Cyp6a2* and *Cyp6a8* were coordinately regulated with respect to constitutive levels of expression, the possibility existed that there could be a coordinate regulatory mechanism for induced expression too. Thus, it was not very surprising to find that *Cyp6a8* was also induced by barbiturates compounds like barbital and phenobarbital. The extent of induction was found to be different in the four strains of *Drosophila* studied. The fold induction in the strains showed a negative correlation with the constitutive level of *Cyp6a8* expression in the strains. While the constitutive level of *Cyp6a8* expression increased in the following order, 91-C < γ^{506} < 91-R < MHIII-D23, the extent of induction with both barbital and phenobarbital decreased in the reverse order, that is, 91-C > γ^{506} > 91-R > MHIII-D23. In a strain where the constitutive level of expression is very high, as in MHIII-D23, transcription probably occurs at a very high level beyond which there is little increase possible, hence induction levels are low. Conversely, in 91-C, where the constitutive expression of *Cyp6a8* is barely detectable, barbiturate induced expression is considerable. A similar situation is also seen with *Cyp6a2* (Dombrowski, 1998) and *CYP6A1* and *CYP6D1* of the housefly (Carino et al., 1992; Scott and Lee, 1993) where the induction is lower in strains with high levels of constitutive expression.

Not much work has been done on the mechanisms of barbiturate-mediated induction in insects. Studies with the transgenic *luciferase* lines showed that the proximal most 0.2 kb upstream DNA of *Cyp6a8* contained the elements required for phenobarbital-induced expression. Sequence analysis of this region showed that there were three barbie

box sequences at -62, -87 and -137. These barbie boxes were shown to be critical elements for phenobarbital induction in the bacteria (He and Fulco, 1991; Liang and Fulco, 1995). However, in mammalian *CYP* genes like *CYP2B2* and *CYP2B1*, even though barbie box sequences are conserved in the upstream DNA of these genes and have also been shown to bind several barbiturate regulated proteins, they *do not* confer phenobarbital inducibility on heterologous genes (Kemper, 1998). A more distal element, known as the PBRU (phenobarbital responsive unit) seems to be required for phenobarbital induction in these genes. Critical sequences in the PBRU include a NF1 site and NR sites. Interestingly, sequence analysis identified a NF1 site and four imperfect AGGTCA steroid receptor binding half sites within 0.2 kb of the *Cyp6a8* upstream DNA. It is not known which of these elements are critical for phenobarbital induction in insects. Only a more critical examination of these sequences will determine whether the mechanism of barbiturate induction in insects resembles the bacterial or mammalian mechanisms or is a combination of both.

One of the striking observations in the study of barbiturate-mediated induction was that barbiturate compounds induce *Cyp6a8-91C*. This implies that in presence of barbiturates the postulated 3rd chromosomal negative factors are probably removed to allow for transcription of *Cyp6a8-91C*. In case of bacteria, barbiturate compounds had been shown to interact with the BM3R1 repressor protein and reduce binding to the promoter/operator DNA sequences of genes regulated by the repressor (Shaw and Fulco, 1993). In case of the *Drosophila CYP* genes, while the repressor has to be removed or dislodged in presence of barbiturates to allow for transcription, a direct interaction model (of the repressor with barbiturates) will not explain the differential effect of 4mM phenobarbital on *CYP* gene induction in the 91-R and 91-C (Chapter 5, Fig 5-6 and 5-7). At a concentration of 4 mM, phenobarbital fails to induce both *Cyp6a2* and *Cyp6a8* in 91-R, while at the same concentration both genes are induced about 6-7 fold in 91-C.

This effect was mapped to the 2nd chromosome of 91-R (Chapter 5). This second chromosomal factor could be analogous to the positive factors BMP1 and BMP2 synthesized in bacteria in presence of barbiturate compounds or to a receptor like molecule that could respond to phenobarbital. Since a differential response to 4 mM phenobarbital is seen in 91-R and 91-C, this 2nd chromosomal factor has to be polymorphic in 91-R and 91-C. A possible model of phenobarbital induction of *Cyp6a8* is shown in Fig. 6-2. In the absence of phenobarbital, the positive factor is present in an inactive state so that transcription occurs at basal levels. In presence of barbiturates, the positive factor is activated and interacts either directly or indirectly with the repressor(s), which results in removal of the repressor(s) from the *cis*-elements of the *CYP* genes, thereby inducing gene transcription. The differential effect of 4 mM phenobarbital in 91-R and 91-C can be explained if we assume that the 91-C factor gets activated at low concentrations of phenobarbital, while the 91-R factor requires a higher concentration. Thus at 4mM, the 91-R factor is not activated and does not bring about induction in presence of phenobarbital. At higher concentrations, the factor gets activated and so induction of *Cyp6a8* is seen in 91-R at 8mM and 16mM concentrations (Chapter 5, Fig. 5-4).

Relevance of studies in *Drosophila* with respect to insecticide resistance

CYP-mediated metabolic detoxification is a common way by which insects could become resistant to insecticides. Increased detoxification could be caused by a mutant form of a CYP enzyme that is very effective in clearing a particular insecticide or may be caused by higher amounts of the enzymes involved in removal of insecticides. In one instance, in the evolution of resistance in *Drosophila* that resulted in the 91-R strain, the latter mechanism seems to have been selected. The DDT resistant 91-R strain evolved from a wild type population of flies through DDT selection in the laboratory and resulted

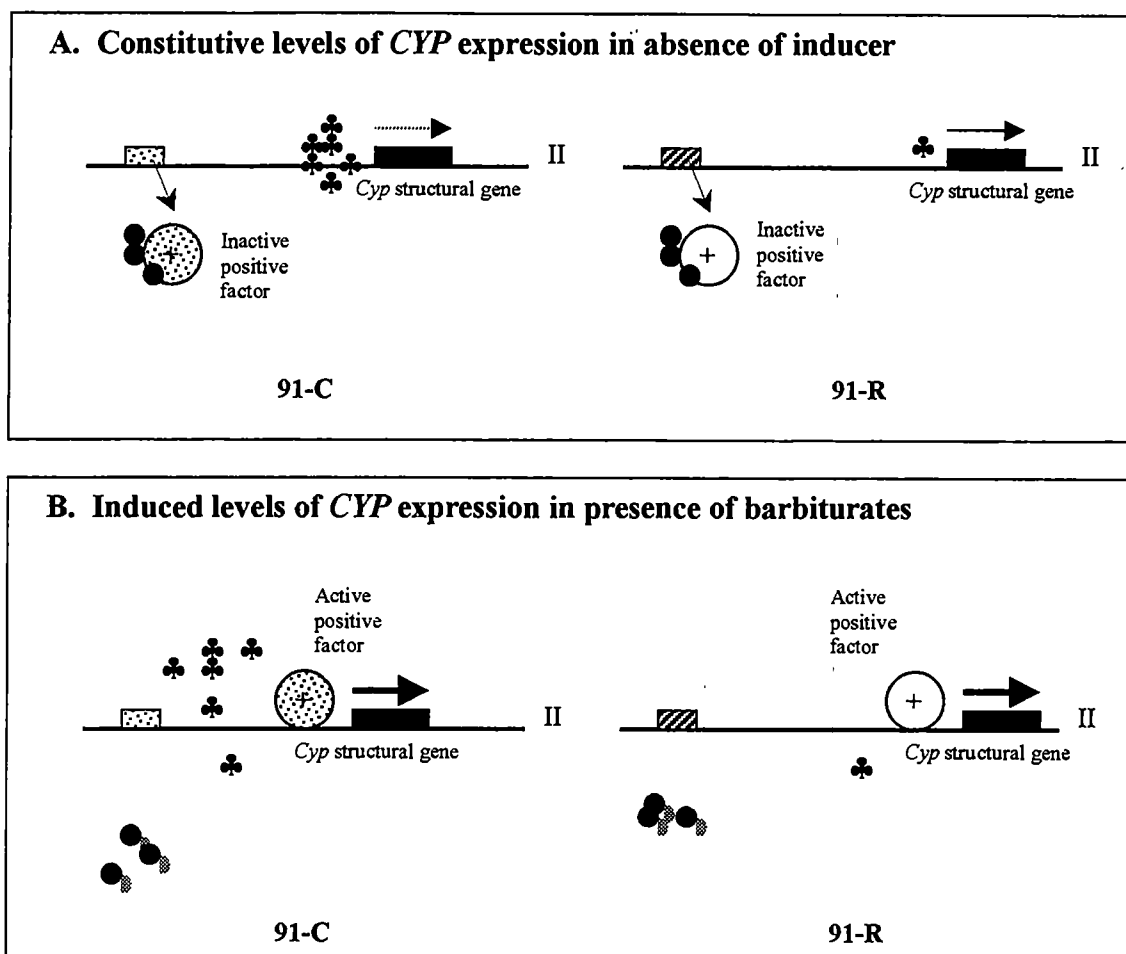


Figure 6-2: Model showing possible mechanisms involved in barbiturate-mediated induction of cytochrome P450 genes in *Drosophila*.

Barbiturates induce expression of *Cyp6a8* in 91-C. The model proposes that barbiturate-mediated induction probably occurs by the removal of the negative factors (♣) that maintain the low basal level of expression. This removal is hypothesized to be effected by a positive factor (⊕). This factor is presumably maintained in an inactive state in absence of barbiturates. The symbol (●) refers to factors that may be involved in keeping the positive factor in an inactive state. In presence of barbiturates (▨), the positive factor may get activated, remove the repressors and bring about higher levels of induced expression.. The 91-R strain-specific effect of a lack of *Cyp6a8* and *Cyp6a2* response to low concentrations of phenobarbital was mapped to the 2nd chromosome (Chapter 5). The locus (loci) involved could encode such putative positive factor as described above. II refers to the 2nd chromosome of *Drosophila*. Solid boxes refer to *CYP* structural genes on the 2nd chromosome, while stippled boxes (91-C specific) and hatched boxes (91-R specific) refer to a locus that may code for the positive factor.

in a strain that was about 70 times more resistant to DDT than the control 91-C flies (Merrell and Underhill, 1956, 1960, 1966). From then, 91-R was subjected to DDT selection for 23 years (Dapkus and Merrell, 1977) but was not subjected to further selection. However, 91-R still remains very resistant to DDT even though there has been no selection pressure for several years. As in other resistant insects, there is a high level of expression of more than one *CYP* gene, that is, *Cyp6a2* and *Cyp6a8* (Waters et al., 1992; Maitra et al., 1996). The fact that 91-R is resistant and shows a high level of *CYP* gene expression even in the absence of any insecticide selection pressure, indicates that the genetic mechanisms responsible for both the phenotypes of resistance and *CYP* overexpression have been fixed in the population. The evolution of metabolic resistance in 91-R could have occurred by a modification of the inducible cytochrome P450 system that already existed in the flies. Under DDT selection, what could have been selected and eventually fixed in the population could be a mutant form of the 3rd chromosomal repressor. If this repressor was a common regulatory factor, involved in regulation of several genes, a mutation in the factor would result in an increase in constitutive levels of expression of those genes. Thus, there may be other *CYP* genes that have a high level of expression in 91-R. Since neither *Cyp6a2* nor *Cyp6a8* seem to be involved in DDT resistance (unpublished observation, R. Ganguly), there may be an as yet unidentified *CYP* gene that is upregulated and responsible for the DDT resistant phenotype. This theme of upregulation of several *CYP* genes in resistant insects seems to be a recurrent one as a similar scenario is seen in the MHIII-D23 strain of *Drosophila* and the LPR strain of *Musca*. Thus, the *mechanism* of overexpression and resistance seem to be conserved among species. In the light of these observations, it may be possible that similar mechanisms may be responsible for resistance in insect pests of economic importance or insect vectors of disease.

The present studies were an attempt to elucidate the molecular mechanisms

involved in *CYP* gene regulation in insects using *Drosophila* as a model organism. The work has generated tools that can be used to further elucidate the mechanisms behind the high level of *CYP* gene expression in resistant strains of *Drosophila*, with the ultimate goal of devising strategies to control CYP-mediated resistance in insects. The results obtained agree with what has been found in the housefly, implying that regulatory mechanisms for *CYP* gene expression are conserved between insect species. Such results reiterate the utility of *Drosophila* as a model organism (Wilson, 1988; ffrench-Constant et al., 1992). Once the mechanisms of regulatory controls and interacting genes are worked out in *Drosophila*, homologous genes and regulatory mechanisms that are involved in insecticide resistance can be studied in insects of economic importance. These can then be used to devise strategies to control resistance in insects pests and disease vectors.

LIST OF REFERENCES

REFERENCES

- Adesnik, M and Atchison, M (1985). Genes for Cytochrome P-450 and their regulation. *Crit. Rev. Biochem. CRC.* 19(3): 247-305.
- Agosin, M. (1985). Role of microsomal oxidations in insecticide degradation. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Vol. 12: Insect Control. Eds., G. A. Kerkut and L. I. Gilbert, Pergamon Press, LTD, Oxford. 647-712.
- Ames, B. N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mut. Res.*, 31: 347-364.
- Andersen, J. F., Utermohlen, J. G. and Feyereisen, R. (1994). Expression of house fly CYP6A1 and NADPH-Cytochrome P450 reductase in *Escherichia coli* and reconstitution of an insecticide-metabolizing P450 system. *Biochemistry*, 33: 2171-2177.
- Ashburner, M. (1989). *Drosophila: A Laboratory Handbook*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Bell, L. R., Horabin, J. I., Schedl, P. and Cline, T. W. (1991). Positive autoregulation of sex-lethal by alternative splicing maintains the female determined state in *Drosophila*. *Cell*, 65(2): 229-239.
- Benton, W. D. and Davis, R. W. (1977). Screening lambda gt recombinant clones by hybridization to single plaques in situ. *Science*, 196: 180-182.
- Brun, A., Cuany, A., Le Mouel, T., Berge J. and Amichot, M. (1996). Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, *Cyp6a2*, by phenobarbital in insecticide susceptible or resistant strains. *Insect. Biochem. Molec. Biol.* 26(7): 697-703.
- Carino, F. Koener, J. F., Plapp, F. W., and Feyereisen, R. (1992). Expression of the cytochrome P450 gene *CYP6A1* in the housefly, *Musca domestica*. In: *Molecular Mechanisms of Insecticide Resistance: Diversity Among Insects*, Eds., J. Scott and C. Mullin, American Chemical Society Books, Washington DC, 31-40.
- Carino, F.A., Koener J.F, Plapp, Jr. F.W. and Feyereisen, R. (1994). Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides. *Insect Biochem. Molec. Biol.* 24(4): 411-418.
- Cavener, D. R. (1987). Comparison of the consensus sequence flanking translation start sites in *Drosophila* and vertebrates. *Nucl. Acids Res.*, 15: 1353-1361.

- Chang, T. K. H and Waxman D. J. (1996). The CYP2A subfamily. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 99-134.
- Cherbas, L. and Cherbas, P. (1993). The arthropod initiator: The capsite consensus plays an important role in transcription. *Insect Biochem. Mol. Biol.*, 23: 81-90.
- Cohen, M.B. and Feyereisen. R. (1995). A cluster of cytochrome P450 genes of the CYP6 family in the house fly. *DNA and Cell Biology* 14(1): 73-82.
- Cohen, M. B., Schuler, M. A. and. Berenbaum, M. R (1992). A host-inducible cytochrome P450 from a host-specific caterpillar: Molecular cloning and evolution. *Proc. Natl. Acad. Sci. USA*, 89: 10920-10924.
- Cohen, M.B., Koener, J.F. and R. Feyereisen. (1994). Structure and chromosomal localization of CYP6A1, a cytochrome P450-encoding gene from the house fly. *Gene* 146: 267-272.
- Conney, A. H. (1967) Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* 19: 317-366.
- Cooper, D. Y., Levin, S., Narasimhulu, S., Rosenthal, O. and Estabrook, R. W. (1965). Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science*, 147: 400-402.
- Danielson, P.B., MacIntyre R.J. and Fogelman, J.C. (1997). Molecular cloning of a family of xenobiotic-inducible drosophilid cytochrome P450s: evidence for involvement in host-plant allelochemical resistance. *Proc. Natl. Acad. Sci. USA* 94: 10797-10802.
- Danielson, P.B., Foster, J. L. M., McMahon, M. M., Smith, M. K. and Fogelman, J.C. (1998). Induction by alkaloids and phenobarbital of family 4 cytochrome P450s in *Drosophila*: evidence for involvement in host plant utilization. *Mol. Gen. Genet.* 259: 54-59.
- Dapkus, D. (1992). Genetic localization of DDT resistance in *Drosophila melanogaster* (Diptera: Drosophilidae). *J. Econ. Entomol.*, 85: 340-347.
- Dapkus, D. and Merrell, D. J. (1977). Chromosomal analysis of DDT-resistance in a long-term selected population of *Drosophila melanogaster*. *Genetics*, 87: 685-697.
- Degtyarenko, K. N. and Archakov, A. I. (1993). Molecular evolution of P450 superfamily and P450 containing monooxygenase systems. *FEBS Letters*, 332: 1-8.
- Delpuech, J.M., Aquadro C.F. and Roush. R.T. (1993). Noninvolvement of the long terminal repeat of transposable element 17.6 in insecticide resistance in *Drosophila*. *Proc Natl. Acad. Sci. U.S.A.* 90: 5643-5647.

- Denison, M.S. and Whitlock, Jr. J.P. (1995). Xenobiotic-inducible transcription of cytochrome P450 genes. *J. Biol. Chem.* 270(31): 18175-18178.
- Denison, M.S., Fisher J.M. and J.P. Whitlock, Jr. (1988). The DNA recognition site for the dioxin-Ah receptor complex. *J. Biol. Chem.* 263: 17221-17224.
- Dombrowski, S. M. (1998). Molecular analysis of the constitutive and barbiturate-induced expression of a *Drosophila melanogaster* cytochrome P450 gene, *Cyp6a2*. Ph.D. dissertation, University of Tennessee, Knoxville.
- Dombrowski, S. M., Krishnan, R., Witte, M., Maitra, S., Diesing, C., Waters L. C. and R. Ganguly. (1998). Constitutive and barbituric acid-induced expression of the *Cyp6a2* allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain. *Gene*, 221(1): 69-77.
- Dunkov, B. C., Rodriguez-Arnaiz, R., Pittendrigh, B., French-Constant, R. H. and Feyereisen, R. (1996). Cytochrome P450 gene clusters in *Drosophila melanogaster*. *Mol. Gen. Genet.* 251: 290-297.
- Dunkov, B. C., Guzov, V. M., Mocelin, G., Shotkoski, F., Brun, A., Amichot, M., French-Constant, R. H. and Feyereisen, R. (1997). The *Drosophila* cytochrome P450 gene *Cyp6a2*: Structure, localization, heterologous expression and induction by phenobarbital. *DNA and Cell Biol.* 16(11): 1345-1356.
- Estabrook, R. W. (1996a). The remarkable P450s: a historical overview of these versatile hemeprotein catalysts. *FASEB. J.*, 10: 202-204.
- Estabrook, R. W. (1996b). Cytochrome P450: From a single protein to a family of proteins – with some personal reflections. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 3-28.
- Estabrook, R. W., Cooper, D. Y. and Rosenthal, O. (1963). The light-reversible carbon monoxide inhibition of the steroid C-21 hydroxylation system in the adrenal cortex. *Biochem. Zeit.*, 338: 741-755.
- Feyereisen, R. (1995). Molecular biology of insecticide resistance. *Toxicol. Lett.*, 82/83, 83-90.
- Feyereisen, R. (1999). Insect P450 enzymes. *Ann. Rev. Entomol.* 44: 507-533.
- Feyereisen, R., Koener, J. F., Farnsworth, D. E. and Nebert, D. W. (1989). Isolation and sequence of cDNA encoding a cytochrome P450 from an insecticide resistant strain of the house fly, *Musca domestica*. *Proc. Natl. Acad. Sci. USA.* 86: 1465-1469.

Feyereisen, R., Koener, J. F., Carino, F. A. and Daggett, A. S. (1990). Biochemistry and molecular biology of insect cytochrome P450. In: *Molecular Insect Science*. Eds., H. H. Hagedorn, J. G. Hildebrand, M. G. Kidwell and J. H. Law, Plenum Press, NY, 263-272.

Feyereisen, R., Andersen, J. F., Carino, F. A., Cohen, M. and Koener, J. F. (1995). Cytochrome P450 in the housefly: Structure, catalytic activity and regulation of expression of CYP6A1 in an insecticide resistant strain. *Pestic. Sci.* 43: 233-239.

Ffrench-Constant, R.H., Roush R.T. and F.A. Carino. (1992). *Drosophila* as a tool for investigating the molecular genetics of insecticide resistance. In: *Approaches to Fundamental and Applied Entomology*. Eds, J. Oakhurst and M. J. Whitte. Springer-Verlag, NY, 1-37.

Field, L. M., Devonshire, A. L. and Forde, B. G. (1988). Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem. J.*, 251: 309-312.

Fletcher, J. C. and Thummel, C. S. (1995). The ecdysone-inducible *Broad-Complex* and *E74* early genes interact to regulate target gene transcription and *Drosophila* metamorphosis. *Genetics*, 141: 1025-1035.

Fogleman, J. C., Danielson, P. B. and MacIntyre, R. J. (1998). The molecular basis of adaptation in *Drosophila*. In: *Evolutionary Biology*, Vol 30. Eds., M. K. Hecht, R. J. MacIntyre, and M. T. Clegg, Plenum Press, NY, 15-77.

Forgash, A. J. (1984). History, evolution and consequences of insecticide resistance. *Pestic. Biochem. Physiol.*, 22: 178-186.

Forman, B. M. and Evans, R. M. (1995). Nuclear hormone receptors activate direct, inverted, and everted repeats. *Ann. N. Y. Acad. Sci.*, 761: 29-37.

Foster, G.G., Whitten, M.J., Konovalov, C., Arnold J.T.A. and Maffi., G. (1981). Autosomal genetic maps of the Australian sheep blowfly, *Lucilia cuprina dorsalis* R.-D. (Diptera: Calliphoridae) and possible correlation with the linkage maps of *Musca domestica* L. and *Drosophila melanogaster* (Mg). *Genet. Res.* 37: 55-69.

Fournier, T., Mejdoubi, N., Lapoumeroulie, C., Hamelin, J., Elion, J., Durand, G and Porquet, D. (1994). Transcriptional regulation of rat α_1 -acid glycoprotein gene by phenobarbital. *J. Biol. Chem.*, 269(44): 27175-27178.

Fraenkel, G. S. (1959). The raison d'être of secondary plant substances. *Science*, 129: 1466-1470.

- Frolov, M. V. and Alatortsev, V. E. (1994). Cluster of cytochrome P450 genes on the X chromosome of *Drosophila melanogaster*. *DNA Cell Biol.* 13(6): 663-668.
- Fuchs, S. Y., Spiegelman, V. S. and Belitsky, G. A. (1994). Inducibility of various cytochrome P450 isozymes by phenobarbital and some other xenobiotics in *Drosophila melanogaster*. *Biochem. Pharmacol.*, 47(10): 1867-1873.
- Fujii-Kuriyama, Y. (1993). Drug-inducible P-450s. In: *Cytochrome P450*. Eds., T. Omura, Y. Ishimura, and Y. Fujii-Kuriyama, VCH Publishers, NY, 224-230.
- Fulco, A. J. (1991). P450_{BM3} and other inducible bacterial P₄₅₀ cytochromes: Biochemistry and regulation. *Ann. Rev. Pharmacol. Toxicol.*, 31: 177-203.
- Fulco, A. J., He, J. -S. and Liang, Q. (1994). The role of conserved 5'-flanking (Barbie box) DNA sequences and barbiturate-responsive DNA-binding proteins in the mechanism of induction by barbiturates of P450 cytochromes in *Bacillus megaterium* and other prokaryotic and eukaryotic organisms. In: *Cytochrome P450 - Biochemistry, Biophysics and Molecular Biology*. Ed., M. Lechner, John Libbey Eurotext, Paris, 37-42.
- Gaidamakova, E. K., Alpatov, I. V., Ischenko, I. V., Kovalenko, S. P. and Lyakhovich, V. V. (1996). Phenobarbital dependent protein binding to barbie box-like sequences in the coding region of cytochrome P450_{BM-3} gene from *Bacillus megaterium*. *Gene*, 183: 97-101.
- Ganguly, R., Ganguly, N. and Manning, J. E. (1985). Isolation and characterization of the glucose-6-phosphate dehydrogenase gene of *Drosophila melanogaster*. *Gene*, 35(1-2): 91-101.
- Garfinkle, D. (1958). Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. *Arch. Biochem. Biophys.*, 77: 493-509.
- Genetics Computer Group. (1994). Program manual for the Wisconsin Package, Version 8. Genetics Computer Group, Madison, WI.
- Gonzales, F. J. (1989). The molecular biology of Cytochrome P450s. *Pharmacol. Rev.*, 40(4): 243-288.
- Gonzales, F. J. (1996). The CYP2D subfamily. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 183-210.
- Gonzales, F. J. and Gelboin, H. V. (1991). Human cytochromes P450: evolution, catalytic activities and inter-individual variations in expression. *Prog. in Clin. Biochem. Res.*, 372: 11-20.

Gonzales, F. J., Tukey, R. H. and Nebert, D. W. (1984). Structural gene products of the Ah locus. Transcriptional regulation of cytochrome P₁-450 and P₃-450 mRNA levels by 3-methylcholanthrene. *Mol. Pharmacol.*, 26: 117-121.

Gonzales, F. J., Liu, S-Y., Yano, M. (1993). Regulation of cytochrome P450 genes: molecular mechanisms. *Pharmacogenetics*, 3: 51-57.

Gotoh, O. (1992). Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem.*, 267: 83-90.

Gotoh, O. (1993). P-450 genes and their expression. In: *Cytochrome P-450*. In *Cytochrome P450*. Eds., T. Omura, Y. Ishimura, and Y. Fujii-Kuriyama, VCH Publishers, NY, 207-223.

Graham, S. E. and Peterson, J. A. (1999). How similar are P450s and what can their differences teach us? *Arch. Biochem. Biophys.*, 369: 24-29.

Graham-Lorence, S. and Peterson, J. A. (1996). P450s: Structural similarities and functional differences. *FASEB J.*, 10: 206-214.

Guengerich, F. P. (1993a). Cytochrome P450 enzymes. *Amer. Sci.* 81: 440-447.

Guengerich, F. P. (1993b). Metabolic reactions: Types of reactions of Cytochrome P450 enzymes. In: *Handbook of experimental Pharmacology*, Vol. 105: Cytochrome P450. J. B. Schenkman and H. Greim, Springer-Verlag, Berlin, Heidelberg, 89-103.

Guengerich, F. P. (1996). The chemistry of cytochrome P450 reactions. In: *Cytochromes P450 Metabolic and Toxicological aspects*. Ed C. Ionnides. CRC Press, Inc., 55-74.

Hahn, C. N., Hansen, A. J. and May, B. K. (1991). Transcriptional regulation of the chicken CYP2H1 gene. Localization of a phenobarbital-responsive enhancer domain. *J. Biol. Chem.*, 266: 17031-17039.

Hallstrom, I. (1985). Genetic regulation of the cytochrome P450 system in *Drosophila melanogaster*. II. Localization of some genes regulating cytochrome P450 activity. *Chem-Biol. Interact.*, 56: 173-184.

Hallstrom, I. and Blanc, A. (1985). Genetic regulation of the cytochrome P450 system in *Drosophila melanogaster*. I. Chromosomal determination of some cytochrome P450 dependent reactions. *Chem-Biol Interact.*, 56: 157-171.

Hallstrom, I., Blanck, A. and Atuma, A. (1984). Genetic variation in cytochrome P-450 and xenobiotic metabolism in *Drosophila melanogaster*. *Biochem. Pharmacol.*, 33(1): 13-20.

Halpern, M. E. and Morton, R. A. (1987). Reproduction and developmental defects in a malathion-resistant, laboratory-selected population of *Drosophila melanogaster*. *Pestic. Biochem. Physiol.*, 28: 44-56.

Hatano, R. and J.G. Scott. (1993). Anti-P450_{lpr} antiserum inhibits the activation of chlorpyrifos to chlorpyrifos oxon in house fly microsomes. *Pest. Biochem. Physiol.* 45: 228-233.

He, J. -S. and Fulco, A. J. (1991). A barbiturate-regulated protein binding to a common sequence in the cytochrome P450 genes of rodents and bacteria. *J. Biol. Chem.* 266(12): 7864-7869.

He, J., Liang, Q. and Fulco, A. J. (1995) The molecular cloning and characterization of BM1P1 and BM1P2 proteins, putative positive transcription factors involved in barbiturate-mediated induction of the genes encoding cytochrome P450_{BM-1} of *Bacillus megaterium*. *J. Biol. Chem.* 270: 18615-18625.

Helms, C., Graham, M. Y., Dutchik, J. E. and Olson, M. (1982). A new method for purifying lambda DNA from phage lysates. *DNA*, 4: 39-49.

Hemingway, J., Hawkes, N., Prapanthadara, L., Indrananda Jayawardenal, K. G. and Ranson, H. (1998). The role of gene splicing, gene amplification and regulation in mosquito insecticide resistance. *Phil. Trans. R. Soc. Lond. B* 353: 1695-1699.

Henikoff, S. and Cohen, E. H. (1984). Sequences responsible for transcription termination on a gene segment in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, 4: 1515-1520.

Hines, R. N., Piechocki, M. and Boncher, P. D. (1994). Molecular mechanisms controlling *CYP1A* gene expression. *Frontiers in Biotransformation*, 2: 85-110.

Hodgson, E. (1984). The significance of cytochrome P-450 in insects. *Insect Biochem.*, 13: 237-246.

Holder, G., Yagi, H., Dansette, P., Jerina, D. M., Levin, W., Lu, A. Y. H. and Conney, A. H. (1974). Effects of inducers and epoxide hydase on the metabolism of benzo(a)pyrene by liver microsomes and a reconstituted system: analysis by high pressure liquid chromatography. *Proc. Natl. Acad. Sci. USA*, 71: 4356 - 4360.

- Honkakoski, P. and Negishi, M. (1997). Characterization of a phenobarbital-responsive enhancer module in mouse P450 *Cyp2b10* gene. *J. Biol Chem.* 272: 14943-14949.
- Honkakoski, P., Moore, R., Gynther, J. and Negeishi, M. (1996). Characterization of phenobarbital-inducible mouse *Cyp2b10* gene transcription in primary hepatocytes. *J. Biol. Chem.*, 271: 9746-9753.
- Honkakoski, P., Zelko, I., Sueyoshi, T. and Negeishi, M. (1998). The nuclear orphan receptor CAR-Retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the *CYP2B* gene. *Mol. Cell. Biol.*, 18(10): 5652-5658
- Houpt, D. R., Pursey, J.C. and Morton, R.A. (1988). Genes controlling malathion resistance in a laboratory selected population of *Drosophila melanogaster*. *Genome*, 30: 844-853.
- Hung, C.-F., Berenbaum, M. R. and Schuler, M. A. (1997). Isolation and characterization of CYP6B4, a furanocoumarin-inducible cytochrome P450 from a polyphagous caterpillar (Lepidoptera: Papilionidae). *Insect Biochem. Mol. Biol.*, 27: 377-385.
- Hung, C.-F., Prapaipong, H., Berenbaum, M. R. and Schuler, M. A. (1995). Differential induction of cytochrome P450 transcripts in *Papilio polyxenes* by linear and angular furanocoumarins. *Insect Biochem. Mol. Biol.* 25: 89-99.
- Hung, C.-F., Holzmacher, R., Connolly, M. R., Berenbaum, M. R. and Schuler, M. A. (1996). Conserved promoter elements in the CYP6B family suggest common ancestry for cytochrome P450 monooxygenases mediating furanocoumarin detoxification. *Proc. Natl. Acad. Sci. USA*, 93: 12200-12205.
- Hurban, P. and C. S. Thummel. (1993). Isolation and characterization of fifteen ecdysone-inducible *Drosophila* genes reveal unexpected complexities in ecdysone regulation. *Mol. Cell. Biol.* 13(11): 7101-7111.
- Jansson, I. (1993). Posttranslational modification of cytochrome P450. In: *Handbook of experimental Pharmacology*, Volume 105: Cytochrome P450. Eds., J. B. Schenkman and H. Greim. Springer Verlag, Berlin, Heidelberg, 561-580.
- Jowett, P. (1986). Preparation of nucleic acids. In: *Drosophila: A practical approach*. Ed., D. B. Roberts. IRL Press, Oxford, 275-286.
- Karess, R.E. (1985). P element mediated germ line transformation of *Drosophila*. In: *DNA Cloning: A Practical Approach* (Vol. II). Ed., D.M. Glover, IRL Press, 121-141.
- Karess, R. E. and Rubin, G. E. (1984). Analysis of P transposable element functions in *Drosophila*. *Cell*, 38: 135-146.

Kamataki, T. (1993). Metabolism of xenobiotics. In: *Cytochrome P450*. Eds., T. Omura, Y. Ishimura, and Y. Fujii-Kuriyama, VCH Publishers, NY, 141-158.

Kawajiri, K. and Hayashi, S. I. (1996). The CYP1 family. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 77-97.

Kemper, B. (1993). Mammalian cytochrome P-450 genes. *Frontiers in Biotransformation*, 8: 1-58.

Kemper, B. (1998). Regulation of cytochrome P450 gene transcription by phenobarbital. *Prog. Nucleic Acid Res. Mol. Biol.* 61: 25-64.

Kikkawa, H. (1953) *Japan J. Gen.*, 28: 171

Kim, B. H. and Fulco, A. J. (1983). Induction by barbiturates of a cytochrome P-450-dependent fatty acid monooxygenase in *Bacillus megaterium*: relationship between barbiturate structure and inducer activity. *Biochem. Biophys. Res. Comm.*, 116(3): 843-850.

Kimura, S., Hanioka, N., Matsunaga, E. and Gonzales, F. J. (1989). The rat clofibrate-inducible CYP4A gene subfamily. I. Complete intron and exon sequence of the CYP4A1 and CYP4A2 genes, unique exon organization, and identification of a conserved 19-bp upstream element. *DNA*, 8: 503-516.

Klingenberg, M. (1958). Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* 75: 376-386.

Koener, J.F., Carino F.A. and Feyereisen. R. (1993). The cDNA and deduced protein sequence of house fly NADPH-cytochrome P450 reductase. *Insect Biochem. Mol. Biol.* 23: 439-447.

Korytko, P. J. and Scott, J. G. (1998). CYP6D1 protects thoracic ganglia of houseflies from the neurotoxic insecticide cypermethrin. *Arch. Insect Biochem. Physiol.* 37: 57-63.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucl. Acids Res.*, 15: 8125-8148.

Krieger, R. I., Feeny, P. O. and Wilkinson, C. F. (1971). Detoxification enzymes in the guts of caterpillars: an evolutionary answer to plant defenses. *Science*, 172: 579-581.

Krishnan, R., Swanson, K. D. and Ganguly, R. (1991). Dosage compensation of a retina-specific gene in *Drosophila miranda*. *Chromosoma*, 100(2): 125-133.

Kupfer, A. and Preisig, R. (1984). Pharmacogenetics of mephenytoin: a new drug hydroxylation polymorphism in man. *Eur. J. Clin. Pharmacol.*, 26: 753-759.

Kupfer, A., Dick, B. and Preisig, R. (1982). A new drug hydroxylation polymorphism in man: the incidence of mephenytoin hydroxylation deficient phenotypes in a European population study. *Naunyn Schmiedebergs Arch. Pharmacol.*, 321-333.

Lewis, D.F.V. (1996a). Cytochromes P450: Structure, Function and Mechanism. Taylor and Francis, Bristol, PA.

Lewis, D. F. V. (1996b). Molecular modeling of mammalian cytochrome P450. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 355-398.

Liang, Q. and Fulco, A. J. (1995). Transcriptional regulation of the genes encoding cytochromes P450 BM-1 and P450BM-3 in *Bacillus megaterium* by the binding of Bm3R1 repressor to barbie box elements and operator sites. *J. Biol. Chem.* 270: 18606-18614.

Liang, Q., He, J.-S. and Fulco, A. J. (1995). The role of barbie box sequences as *cis*-acting elements involved in the barbiturate-mediated induction of cytochromes P450BM-1 and P450 BM-3 in *Bacillus megaterium*. *J. Biol. Chem.* 270: 4438-4450.

Lindsley, D. L. and Zimm G. G. (1992). The genome of *Drosophila melanogaster*. Academic Press, Inc. San Diego, CA.

Liu, N. and Scott, J.G. (1996). Genetic analysis of factors controlling high level of expression of cytochrome P450, CYP6D1, cytochrome b5, P450 reductase and monooxygenase activities in LPR house flies, *Musca domestica*. *Biochem Genet.* 34: 133-148.

Liu, N. and Scott, J.G. (1997a). Inheritance of CYP6D1-mediated pyrethroid resistance in house fly (Diptera: Muscidae). *J. Econ. Entomol.*, 90: 1478-1481.

Liu, N. and Scott, J.G. (1997b). Phenobarbital induction of CYP6D1 is due to a trans acting factor on autosome 2 in house flies, *Musca domestica*. *Insect Molec. Biol.*, 6(1): 77-81.

Liu, N. and Scott, J. G. (1998). Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly. *Insect Biochem. Molec. Biol.* 28: 531-535.

Ma, R., Cohen, M. B., Berenbaum, M. R. and Schuler, M. A. (1994) Black swallowtail (*Papilio polixenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins. *Arch. Biochem. Biophys.* 310: 332-340.

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

Makino, R. and Shimada, H. (1993). Structures of cytochrome P-450. In: *Cytochrome P450*. Eds., T. Omura, Y. Ishimura, and Y. Fujii-Kuriyama, VCH Publishers, NY, 17-30.

Maniatis, T., Fritsch, D. F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maurel, P. (1996). The CYP3 family. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides CRC Press Inc., 241-270.

Merrell, D. J. and Underhill, J. C. (1956). Selection for DDT resistance in inbred, laboratory, and wild type stocks of *Drosophila melanogaster*. *J. Econ. Entomol.*, 49(3), 300-306.

Meyer, U. A. (1994). Pharmacogenetics: The slow, the rapid, and the ultrarapid. *Proc. Natl. Acad. Sci. USA*, 91: 1983-1984.

Misner, D. and Rubin, G. M. (1987). Analysis of the promoter of the *ninaE* opsin gene in *Drosophila melanogaster*. *Genetics*, 116: 565-578.

Morgan, E. T., Sewer, M. B., Iber, H., Gonzales, F. J., Lee, Y-H., Tukey, R. H., Okino, S., Vu, T., Chen, Y-H., Sidhu, J. S., Omiecinski, O. (1998). Physiological and pathophysiological regulation of cytochrome P450. *Drug Metab. Dispo.*, 26(12), 1232-1240.

Nebert, D. W. (1989). Regulation of the mouse dioxin-inducible P₁450 gene. In: *Cytochrome P-450: Biochemistry and Biophysics*. Ed., I. Schuster, Taylor and Francis, London, 484-490.

Nebert, D. W. and Gonzales, F. J. (1987). P450 genes: Structure, evolution and regulation. *Ann. Rev. Biochem.* 56: 945-993.

Nebert, D. W., McKinnon, R. A. and Puga, A. (1996). Human drug-metabolizing enzyme polymorphisms: Effect on risk of toxicity and cancer. *DNA. Cell. Biol.* 15: 273-280.

- Nedelcheva, V. and Gut, I. (1994). P450 in the rat and man: methods of investigation, substrate specificities and relevance to cancer. *Xenobiotica*, 24: 1151-1175.
- Negeishi, M. and Nebert, D. W. (1981). Structural gene products of the Ah complex. Increases in large mRNA from mouse liver associated with cytochrome P₁-450 induction by 3-methylcholanthrene. *J. Biol. Chem.*, 256: 3085-3091.
- Negeishi, M., Uno, T., Darden, T. A., Sueyoshi, T. and Pedersen, L. G. (1996). Structural flexibility and functional versatility of mammalian P450 enzymes. *FASEB J.* 10: 683-689.
- Nelson, D. R. (1999). Cytochrome P450 and the individuality of the species. *Arch. Biochem. Biophys.*, 369: 1-10.
- Nelson, D. R. and Strobel, H. W. (1987). Evolution of Cytochrome P-450 proteins. *Mol. Biol. Evol.* 4: 572-593.
- Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C. and Nebert, D. W. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6: 1-42.
- Nims, R. W. and Lubet, R. A. (1996). The CYP2B family. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 135- 160.
- O'Connell, P. and Rosbash, M. (1984). Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Research* 12(13): 5495-5513.
- Ogita, Z. (1958). The genetical relation between resistance to insecticides in general and that to phenylthiourea (PTU) and Phenylurea (PU) in *Drosophila melanogaster*. *Botyu-Kagaku*, 23, 188-205.
- Okey, A. (1990). Enzyme induction in the cytochrome P450 system. *Pharmac. Ther.* 45, 241-298.
- Omura, T. and Sato, R. (1962). A new cytochrome in liver microsomes. *J. Biol. Chem.* 237: 1375-1376.
- Omura, T. and Sato, R. (1964a). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 239: 2370-2378.
- Omura, T. and Sato, R. (1964b). The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J. Biol. Chem.* 239: 2378-2385.

Omura, T. (1992). History of Cytochrome P450 In: *Cytochrome P450*. Eds., T. Omura, Y. Ishimura, and Y. Fujii-Kuriyama, VCH Publishers, NY, 1-15.

Oppenoorth, F.J. (1985). Biochemistry and genetics of insecticide resistance. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 12: Insect Control. Eds., G.A. Gilbert and L.I. Gilbert, Pergamon Press, Ltd. Oxford. 731-773.

Padmanaban, G. and Nirodi, C. S. (1994). Regulation of phenobarbitone-inducible cytochrome P-450 gene expression. In: *Frontiers in Biotransformation*, Vol. 9: Regulation and control of complex biological processes by biotransformation. Eds., K. Ruckpaul and H. Rein, Berlin, Akademie Verlag, 60-84.

Plapp, F. W. Jr. (1984). The genetic basis of insecticide resistance in the house fly: evidence that single locus plays a major role in metabolic resistance to insecticides. *Pestic. Biochem. Physiol.*, 22:194-201.

Poland, A. and Knutson, J. C. (1982). 2,3,7,8-Tetrochlorodibenzo-p-dioxin and related aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann. Rev. Pharmacol. Toxicol.*, 22: 517-554.

Porter, T. D. and Coon, M. J. (1991). Cytochrome P-450: multiplicity of isoforms, substrates and catalytic and regulatory mechanisms. *J. Biol. Chem.*, 266: 13469-13472.

Praipapong, H., Berenbaum, M. R. and Schuler, M. A. (1994) Transcriptional regulation of the *Papilio polyxenes* CYP6B1 gene. *Nucleic Acids Res.* 22: 3210-3217.

Quandt, K., Frech, H., Karas, E., Wingender, E. and Warner, T. (1995). MatInd and MatInspector – new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucl. Acids Res.* 23: 4878-4884.

Raffalli-Mathieu, F., Geneste, O and Lang, M. A. (1997). Characterization of two nuclear proteins that interact with cytochrome P-450 1A2 mRNA: Regulation of RNA binding and possible role in the expression of the *Cyp1a2* gene. *Eur. J. Biochem.* 245: 17-24.

Ramsden, R., Sommer, K. M. and Omiecinski, C. J. (1993). Phenobarbital induction and tissue-specific expression of the rat CYP2B2 gene in transgenic mice. *J. Biol. Chem.*, 268: 21722-21726.

Ranasinghe, C. and Hobbs, A. A. (1998). Isolation and characterization of two cytochrome P450 cDNA clones for CYP6B6 and CYP6B7 from *Helicoverpa armigera* (Hubner): possible involvement of CYP6B7 in pyrethroid resistance. *Insect Biochem. Molec. Biol.*, 28: 571-580.

Ray, K. and Ganguly, R. (1992). The *Drosophila* G protein γ -subunit gene (D-G γ 1) produces three developmentally regulated transcripts and is predominantly expressed in the central nervous system. *J. Biol. Chem.*, 267: 6086-6092.

Remmer, H. (1962). Drugs as activators of drug enzymes. In: Metabolic Factors Controlling Duration of Drug Action. In: *Proceedings of First International Pharmacological Meeting*, Vol. 6. Eds., B. B. Brodie and E. G. Erdoes, Macmillan, NY.

Remmer, H. and Merker, H. J. (1965). The effect of drugs on the formation of smooth endoplasmic reticulum and drug-metabolizing enzymes. *Ann. N. Y. Acad. Sci.*, 123: 79

Ronis, M. J. J., Lindros, K. O. and Ingelman-Sundberg, M. (1996). The CYP2E subfamily. In: *Cytochromes P450 - Metabolic and toxicological aspects*. Ed., C. Ioannides, CRC Press Inc., 211-239.

Rushmore, T. H., Morton, M. R. and Pickett, C. B. (1991). The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* 266: 11632-11639.

Saner, C., Weibel, B., Wurgler, F. E., and Sengstag, C. (1996). Metabolism of promutagens catalysed by *Drosophila melanogaster* CYP6A2 enzyme in *Saccharomyces cerevisiae*. *Environ. Mol. Mutagen.*, 27: 46-58.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain - terminating inhibitors. *Proc. Natl Acad Sci. USA.*, 74(12), 5463-5467.

Schenkman, J. B. and Greim, H. (1993). Handbook of experimental Pharmacology, Vol. 105: Cytochrome P450. Springer-Verlag, Berlin, Heidelberg.

Schuler, M. A. (1996). The role of cytochrome P450 monooxygenases in plant-insect interactions. *Plant Physiol.*, 112: 1411-1419.

Scott, J. G. (1999). Cytochromes P450 and insecticide resistance. *Insect Biochem and Molec. Biol.* 29: 757-777.

Scott, J. G. and Lee, S. S. T. (1993). Tissue distribution of microsomal cytochrome P-450 monooxygenases and their inducibility by phenobarbital in the insecticide resistant LPR strain of house fly, *Musca domestica* L. *Insect Biochem. Molec. Biol.*, 23: 729-738.

Scott, J. G., Sridhar, P. and Liu, N. (1996). Adult specific expression and induction of cytochrome P450_{1pr} in house flies. *Arch. Insect Biochem. Physiol.* 31: 313-323.

Shapiro, M. B. and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucl. Acids Res.*, 15: 7155-7174.

Shaw, G-C and Fulco, A.J. (1993). Inhibition by barbiturates of the binding of Bm3R1 repressor to its operator site on the barbiturate-inducible cytochrome P450BM-3 gene of *Bacillus megaterium*. *J. Biol. Chem.* 268(4): 2997-3004.

Shaw, G. -C., Sung, C. -C., Liu, C. -H. and Lin, C. -H. (1998). Evidence against the Bm1P1 protein as a positive transcription factor for barbiturate-mediated induction of cytochrome P450_{BM-1} in *Bacillus megaterium*. *J. Biol. Chem.*, 273(14), 7996-8002.

Shimada, T., Martin, M. V., Pruess-Schwartz, D., Marnett, L. J. and Guengerich, F. P. (1989). Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo (a) pyrene, 7, 8 - dihydroxy- 7, 8 - dihydrobenzo (a) pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res.* 49: 6304 - 6312.

Singh, R. S. and Morton, R. A. (1981). Selection for malathion-resistance in *Drosophila melanogaster*. *Can. J. Genet. Cytol.* 23: 355-369.

Smale, S. T. and Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell*, 57: 103-113.

Snyder, M. J., Hsu, E. L. and Feyereisen, R. (1993). Induction of cytochrome P450 activities by nicotine in the tobacco hornworm, *Manduca sexta*. *J. Chem Ecol.*, 19: 2903-2916.

Spiegelman, V.S., Fuchs, S.Y. and Belitsky, G.A.. (1997). The expression of insecticide resistance-related cytochrome P450 forms is regulated by molting hormone in *Drosophila melanogaster*. *Biochem. Biophys. Res. Comm.* 232: 304-307.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germline chromosomes. *Science*. 218: 341-347.

Sundseth, S.S., Kennel, S.J. and Waters, L.C. (1989). Monoclonal antibodies to resistance-related forms of cytochrome P450 in *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* 33(2): 176-188.

Sundseth, S.S., Nix, C.E. and Waters, L.C. (1990). Isolation of insecticide resistance-related forms of cytochrome P450 from *Drosophila melanogaster*. *Biochem J.* 265: 213-217.

Takahashi, Y., Suzuki, C. and Kamataki, T. (1998). Silencing of CYP1A1 expression in rabbits by DNA methylation. *Biochem. Biophys. Res. Comm.* 247: 383-386.

Terriere, L. C. (1983). Enzyme induction, gene amplification, and insect resistance to insecticides. In: *Pest resistance to pesticides*. Eds., G. P. Georgiou and T. Saito, Plenum Press, NY, 265-298.

Tomita, T. and Scott, J.G. (1995). cDNA and deduced protein sequence of CYP6D1: the putative gene for a cytochrome P450 responsible for pyrethroid resistance in house fly. *Insect Biochem. Mol. Biol.* 25(2): 275-283.

Tomita, T., Liu, N., Smith, F.F., Sridhar, P. and Scott J.G. (1995). Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. *Insect Mol. Biol.* 4: 135-140.

Trottier, E., Belzil, A., Stolz, C. and Anderson, A. (1995). Localization of a phenobarbital-responsive element (PBRE) in the 5'-flanking region of the rat *CYP2B2* gene. *Gene*. 158: 263-268.

Tsukamoto, M. (1958). *Drosophila Information Service.*, 32: 87.

Ueno, T. and Gonzales, F. J. (1990). Transcriptional control of the rat hepatic CYP2E1 gene. *Mol. Cell. Biol.*, 10: 4495-4505.

Von Wachenfeldt, C. and Johnson, E. F. (1995). Structure of eukaryotic cytochrome P450 enzymes. In: *Cytochrome P450: Structure, Mechanism and Biochemistry*. Ed., P. R. Ortiz de Montellano, Plenum Press, NY, 183-223.

Wahle, E. and Keller, W. (1992). The biochemistry of 3'-end cleavage and polyadenylation of messenger RNA precursors. *Ann. Rev. Biochem.*, 61: 419-440.

Waters, L.C. and Nix, C. E. (1988). Regulation of insecticide resistance-related cytochrome P-450 expression in *Drosophila melanogaster*. *Pestic. Biochem. Physiol.* 30(3): 214-227.

Waters, L. C., Nix, C. E. and Epler, J. L. (1983). Studies on the relationship between Dimethyl-nitrosamine-demethylase activity and Dimethyl-nitrosamine-dependent mutagenesis in *Drosophila melanogaster*. *Chem. Biol. Interactions*, 46: 55-66.

Waters, L.C., Zelhof, A. C., Shaw, B.J. and Ch'ang, L-Y. (1992). Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 89: 4855-4859.

Waxman, D. J. (1999). P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR and PPAR. *Arch. Biochem. Biophys.* 369(1), 11-23.

Waxman, D. J. and Azaroff, L. (1992). Phenobarbital induction of cytochrome P-450 gene expression. *Biochem*, 281: 577-592.

Weis, L. and Reinberg, D. (1992). Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. *FASEB J.*, 6: 3300-3309.

Wen, L. P., Ruettinger, R. T. and Fulco, A. J. (1989). Requirement for a 1-kilobase 5'-flanking sequence for barbiturate-inducible expression of the cytochrome P-450_{BM-3} gene in *Bacillus megaterium*. *J. Biol. Chem.*, 264: 10996-11003.

Wheelock, G. D. and Scott, J. G., (1992a). Anti-P450_{Ipr} antiserum inhibits specific monooxygenase activities in LPR housefly microsomes. *J. Exp. Zool.* 264: 153-158.

Wheelock, G. D. and Scott, J. G. (1992b). The role of cytochrome P450_{Ipr} in deltamethrin metabolism by pyrethroid-resistant and susceptible strains of house flies. *Pest. Biochem.*, 43: 67-77.

Whitlock, J. P. (1999). Induction of cytochrome P450A1. *Ann. Rev. Pharmacol. Toxicol.*, 39: 103-125.

Wilkinson, C. F. (1983). Role of mixed function oxidases in insecticide resistance. In: *Pest resistance to pesticides*. Eds., G. P. Georgiou and T. Saito. Plenum Press Inc., 175-205.

Wilson, T.G. (1988). *Drosophila melanogaster* (Diptera: Drosophilidae): A model insect for insecticide resistance studies. *J. Econ. Entomol.* 81(1): 22-27.

Wingender, E. (1994). Recognition of regulatory regions in genomic sequences. *J. Biotechnol.*, 35: 273-280.

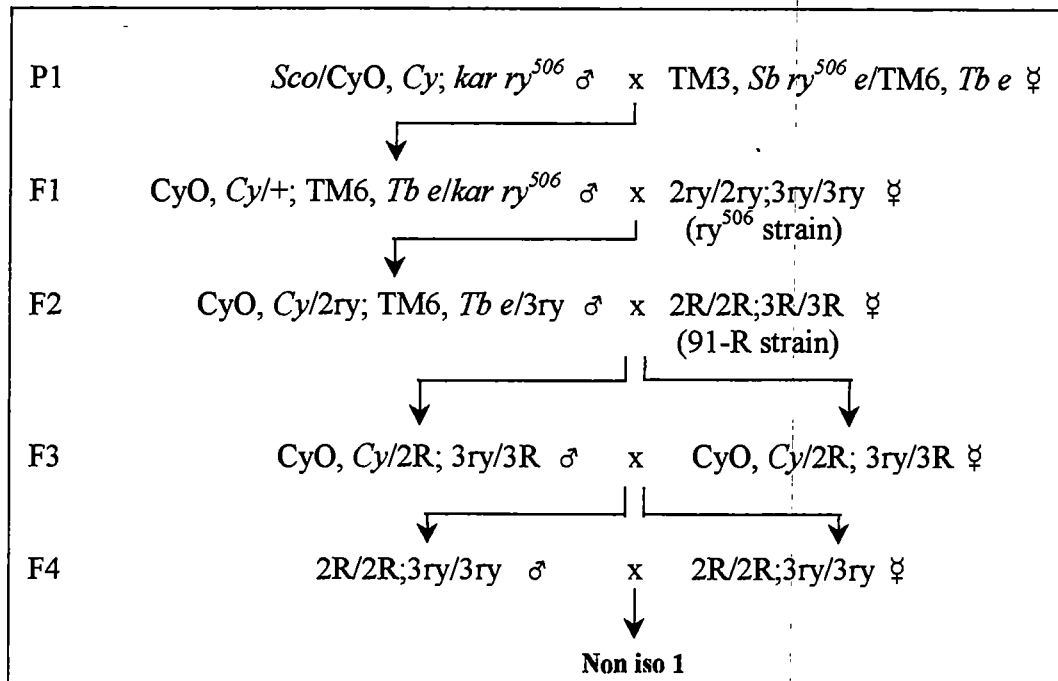
World Health Organization (WHO) Expert Report on Insecticides (1976). 22nd report. In: *WHO Tech. Rpt. Ser.* Vol. 585, 77.

Yoshioka, H., Lang, M., Wong, G. and Negeishi, M. (1990). A specific *cis*-acting element regulates in vitro transcription of sex-dependent mouse steroid 16 alpha-hydroxylase (C-P450(16 alpha)) gene. *J. Biol Chem.*, 265: 14612-14617.

Zimniak, P and Waxman, D. J. (1993). Liver cytochrome P450 metabolism of endogenous steroid hormones, bile acids and fatty acids. In: *Handbook of experimental Pharmacology*, Vol. 105: Cytochrome P450. Eds., J. B. Schenkman and H. Greim., Springer-Verlag, Berlin, Heidelberg, 123-144.

APPENDIX

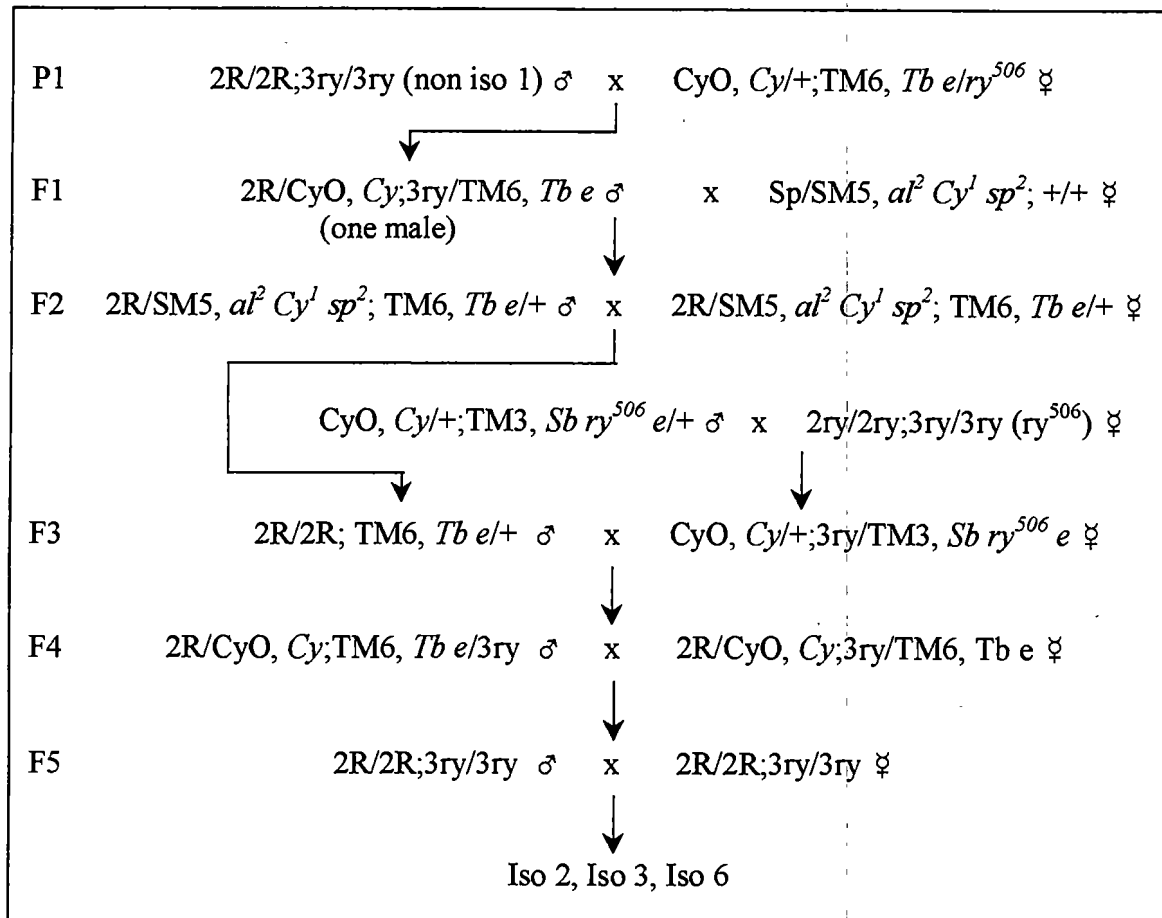
Generation of non iso 1 stock



A-1: Generation of non iso 1 stock with the 2nd chromosome from 91-R and the 3rd chromosome from the ry⁵⁰⁶ strain.

R represents chromosomes from the 91-R strain and ry represents chromosomes from the ry⁵⁰⁶ strain. In each generation crosses were set up with several males and females. In F3, non curly, rosy eyed males and females were selected and crossed to get the non iso 1 stock. Consequently, the 3rd chromosome from ry⁵⁰⁶ in the non iso stock may have undergone crossing over in the F3 females.

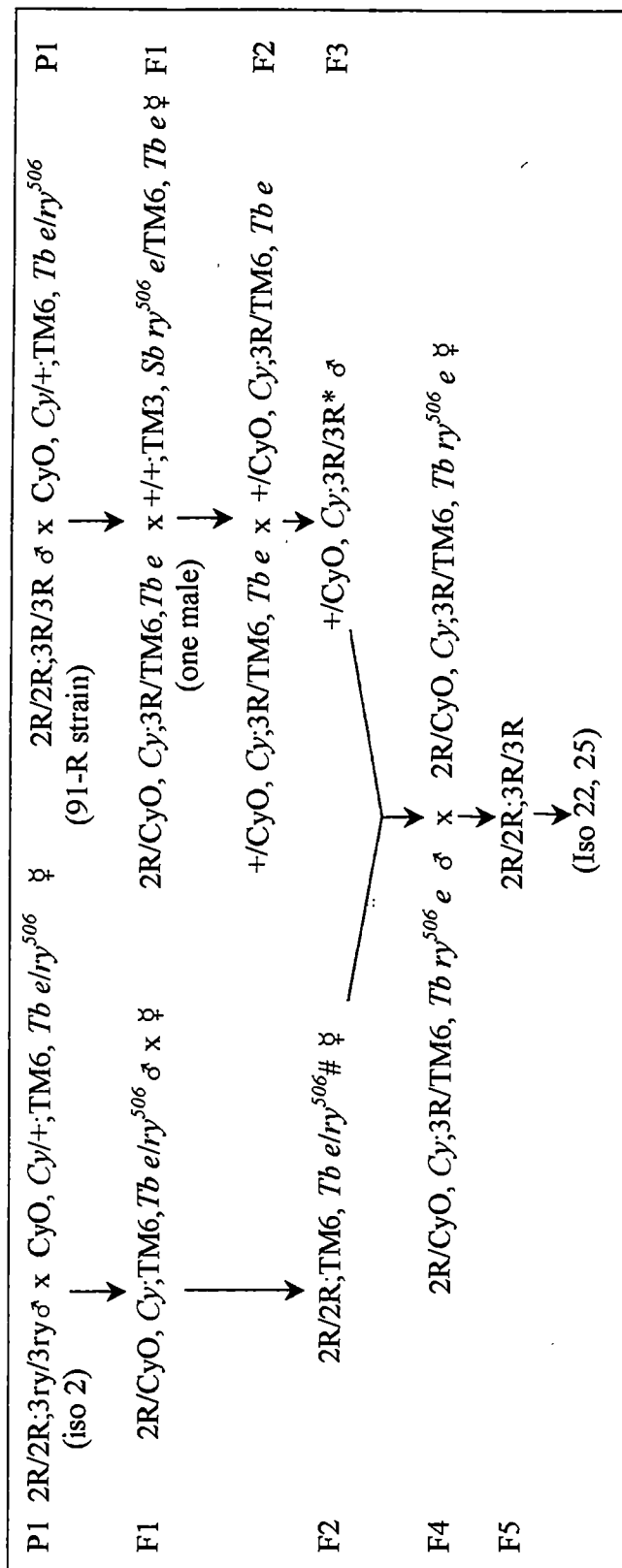
Generation of Iso lines 2, 3 and 6



A-2: Generation of iso lines with 2nd chromosome from the 91-R strain and 3rd chromosome from the ry⁵⁰⁶ strain.

The non iso 1 stock was used to synthesize the iso lines, 2, 3 and 6. In the F1 generation a single male was taken to synthesize a particular iso line. The chromosome constitution of these iso lines is the same as the non iso 1 stock. However, the 2nd chromosome in the latter stocks is isogenic. R represents chromosomes from the 91-R strain while ry represents chromosomes from the ry⁵⁰⁶ strain. A number prefix in front of R or ry represents the chromosome, that is, 2 and 3 represent the 2nd and the 3rd chromosome respectively.

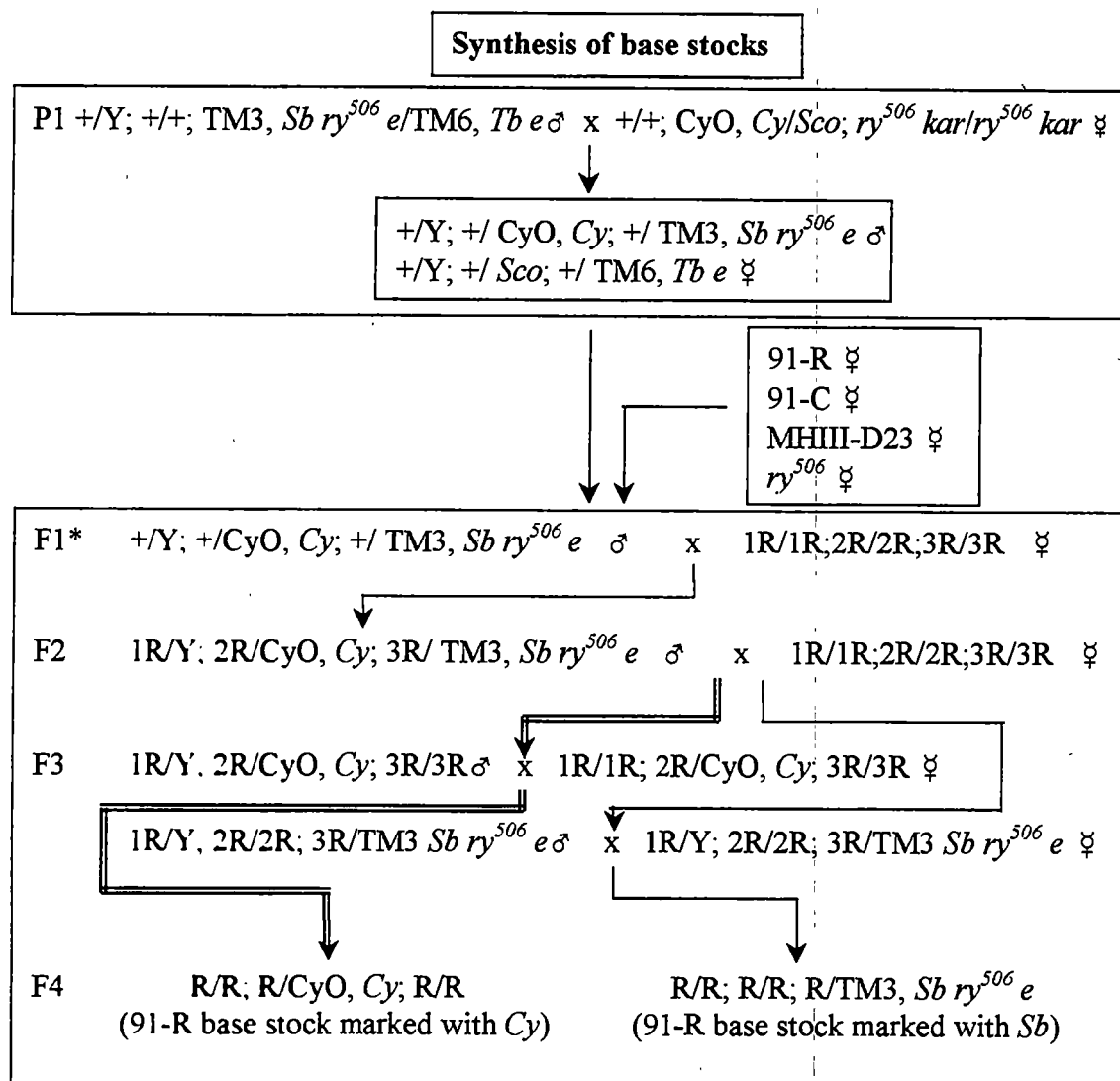
Generation of resubstituted stocks, iso 22 and 25



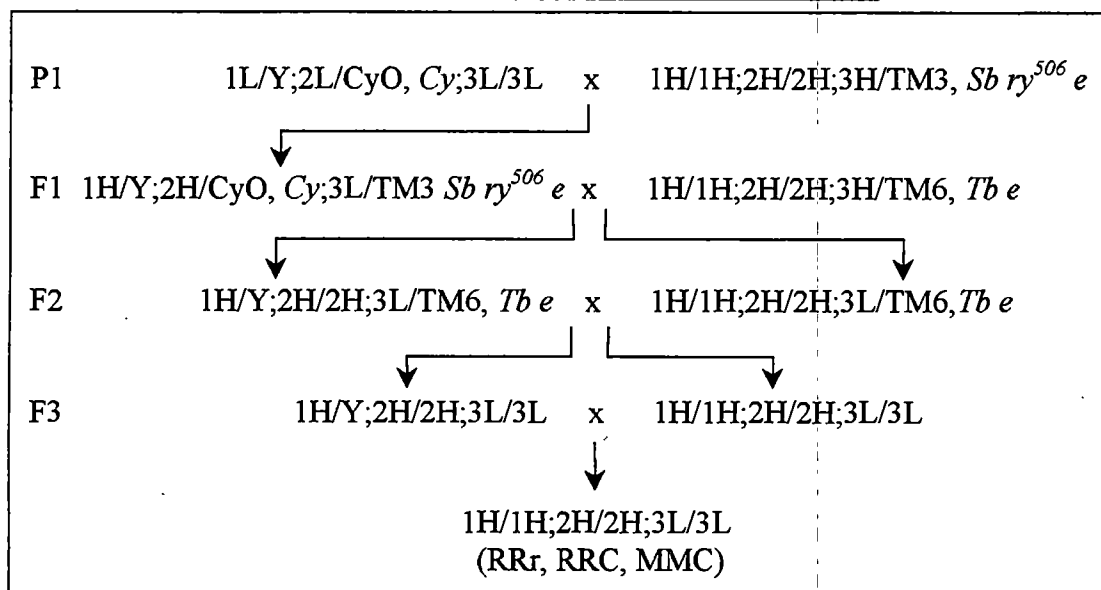
A-3: Synthesis of resubstituted stocks where the 3rd chromosome of the ry⁵⁰⁶ strain in the iso 2 stock is replaced by the 3rd chromosome from 91-R

This stock (2R/2R;TM6, Tb e/ry⁵⁰⁶) was derived from the iso 2 stock (appendix A-2) and hence carries an isogenic 2nd chromosome from 91-R.

*The stock (+/CyO;3R/3R) was derived using single males in the F1 generation of the cross and hence is isogenic for the 3rd chromosome from 91-R. Several lines were made of which two lines (# 2, 5) were used for resubstituting the 3rd chromosome. The above stocks, #2R/2R;TM6, Tb e/ry⁵⁰⁶ and *+/CyO;3R/3R were combined to yield two resubstituted stocks, iso 22 and iso 25, which carried isogenic 2nd and 3rd chromosomes from 91-R. The 2nd chromosome is the same as the one in the iso 2 stock (appendix A-2).



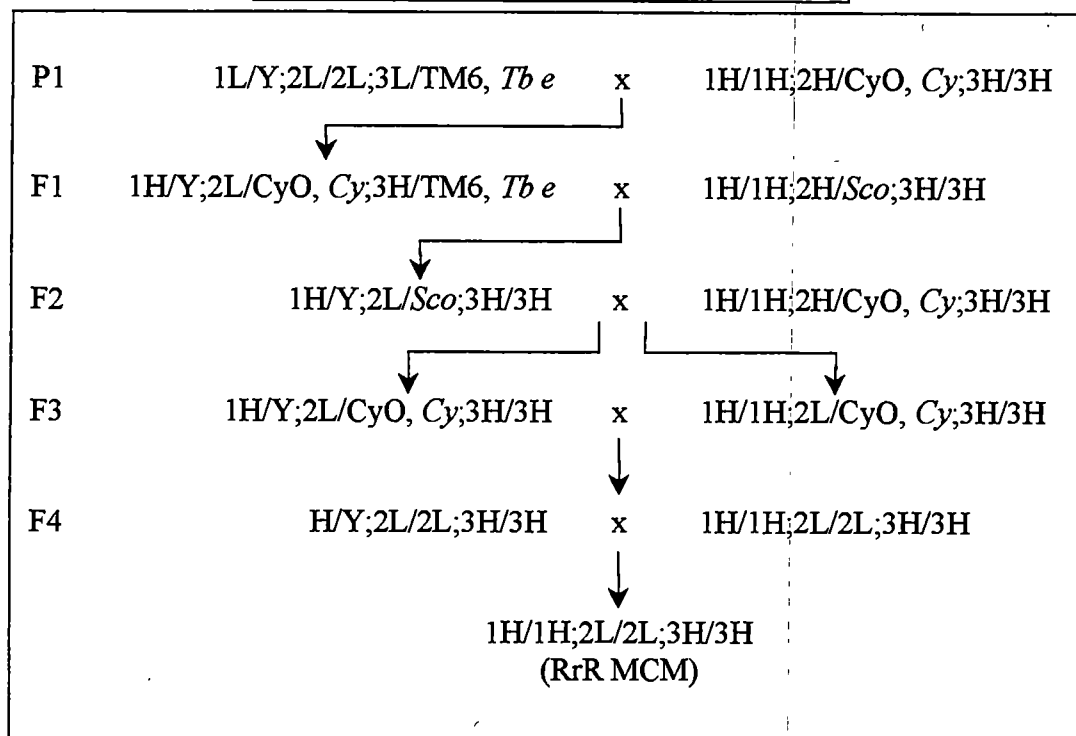
Synthesis of RRr, RRC and MMC stocks



A-5: Synthesis of stocks with X and 2nd chromosome from CYP overproducer strains and the 3rd chromosome from CYP underproducer strains

Three stocks (RRr, RRC, MMC) were synthesized using base stocks of the 91-R, 91-C, MHIII-D23 and ry⁵⁰⁶ strains, where one of the chromosome was replaced by a balancer chromosome carrying a dominant marker. H represents chromosomes from CYP overproducer strains (91-R and MHIII-D23) and L represents chromosomes from CYP underproducer strains (91-C and ry⁵⁰⁶). The prefixes 1, 2 or 3 in front of the letters H or L represent the X, 2nd or 3rd chromosome from the overproducer or underproducer strain. CyO, TM3, TM6 are balancer chromosomes with dominant markers that can be followed in the cross (Lindsley and Zimm, 1992).

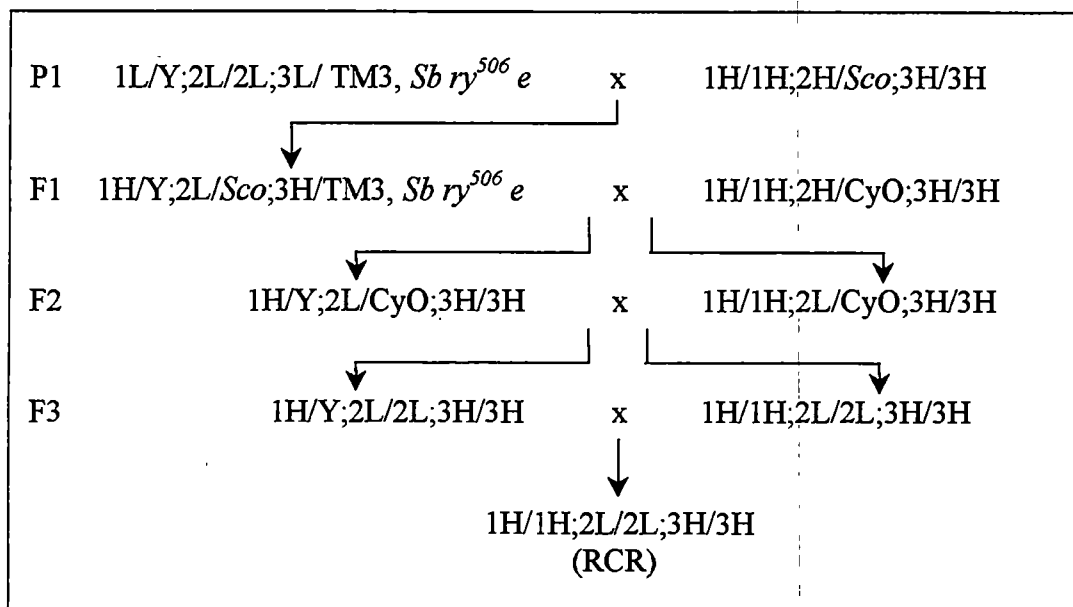
Synthesis of RrR and MCM stocks



A-6: Synthesis of stocks with X and 3rd chromosome from CYP overproducer strains and the 2nd chromosome from CYP underproducer strains

Three stocks (RrR, RCR, MCM) were synthesized using base stocks of the 91R, 91C, MHIII-D23 and *ry*⁵⁰⁶ strains, where one of the chromosome was replaced by a balancer chromosome carrying a dominant marker. H represents chromosomes from CYP overproducer strains (91R and MHIII-D23) and L represents chromosomes from CYP underproducer strains (91C and *ry*⁵⁰⁶). The prefixes 1, 2 or 3 in front of the letters H or L represent the X, 2nd or 3rd chromosome from the overproducer or underproducer strain. CyO, TM3, TM6 are balancer chromosomes with dominant markers that can be followed in the cross (Lindsley and Zimm, 1992).

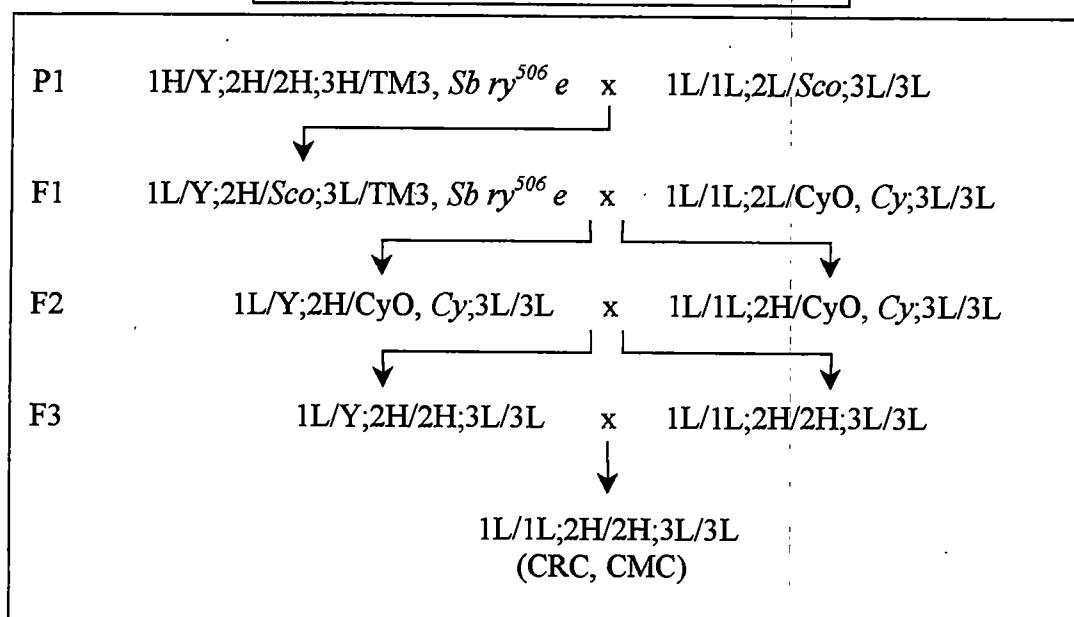
Synthesis of the RCR stock



A-7: Synthesis of the RCR stock with X and 3rd chromosome from CYP overproducer strain (91-R) and the 2nd chromosome from CYP underproducer strain (91-C)

RCR was synthesized using base stocks of the 91-R and 91-C strains. H represents chromosomes from CYP overproducer 91-R strain and L represents chromosomes from CYP underproducer 91-C strain. The prefixes 1, 2 or 3 in front of the letters H or L represent the X, 2nd or 3rd chromosome from the 91-R or 91-C strain. CyO and TM3 are balancer chromosomes with dominant markers that can be followed in the cross (Lindsley and Zimm, 1992). *Sco* is a dominant marker which was used in males of the F1 generation to study the segregation of the 2nd chromosome of the 91-C strain.

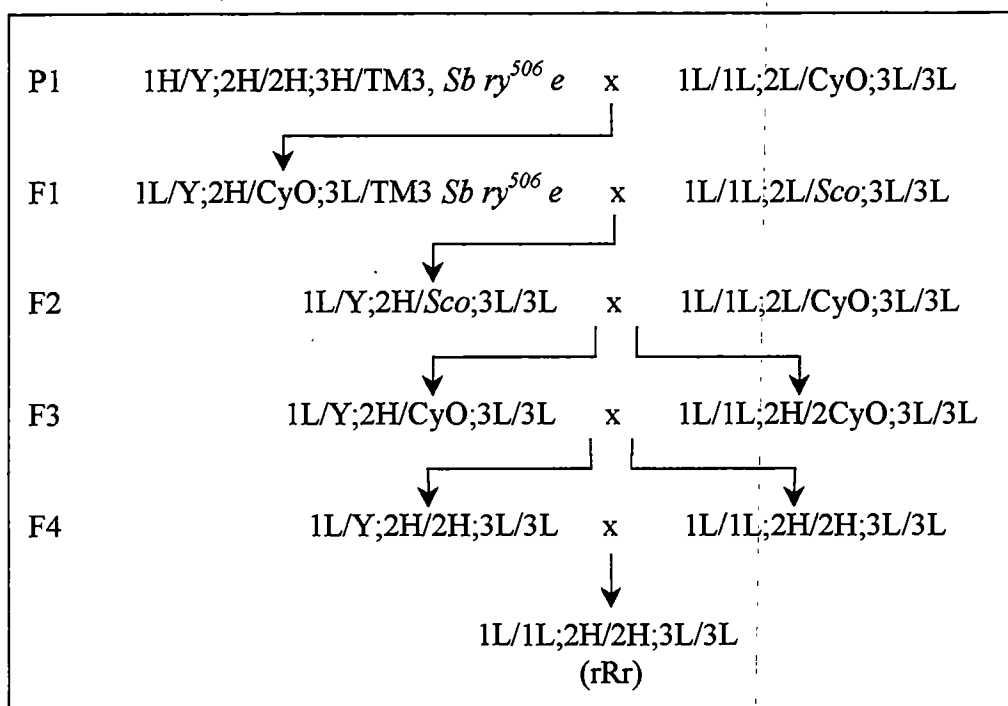
Synthesis of CRC and CMC stocks



A-8: Synthesis of stocks with X and 3rd chromosome from CYP underproducer strains and the 2nd chromosome from CYP overproducer strains

Two stocks (CRC, CMC) were synthesized using base stocks of the 91-R, 91-C, MHIII-D23 strains, where one of the chromosomes was replaced by a balancer chromosome carrying a dominant marker. In case of the stocks marked with *Sco*, the chromosome carrying *Sco* is not a balancer. Hence crosses involving *Sco* stocks were designed that the outcrosses with the *Sco* chromosome occurred in males in the F1 generation. H represents chromosomes from CYP overproducer strains (91-R and MHIII-D23) and L represents chromosomes from the CYP underproducer strains 91-C. The prefixes 1, 2 or 3 in front of the letters H or L represent the X, 2nd or 3rd chromosome from the overproducer or underproducer strain. *CyO* and *TM3* are balancer chromosomes with dominant markers (Lindsley and Zimm, 1992).

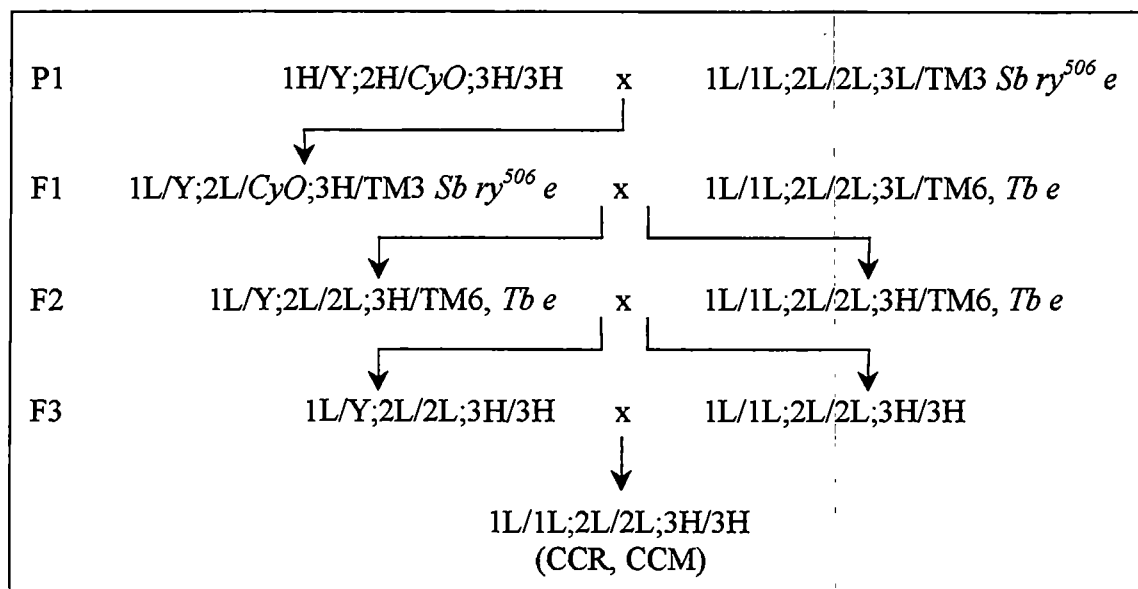
Synthesis of the rRr stock



A-9: Synthesis of stocks with X and 3rd chromosome from a CYP underproducer strain (*ry⁵⁰⁶*) and the 2nd chromosome from a CYP overproducer strain (91-R)

The rRr stock was synthesized using base stocks of the 91-R and the *ry⁵⁰⁶* strains. The cross involving the *ry⁵⁰⁶* stock marked with *Sco* was designed that the outcross with the *Sco* carrying chromosome occurred in males in the F2 generation. H represents chromosomes from the CYP overproducer 91-R strain while L represents chromosomes from CYP underproducer 91-C strain. The prefixes 1, 2 or 3 in front of the letters H or L represent the X, 2nd or 3rd chromosome from the overproducer or underproducer strain. CyO and TM3 are balancer chromosomes with dominant markers that can be followed in the cross (Lindsley and Zimm, 1992).

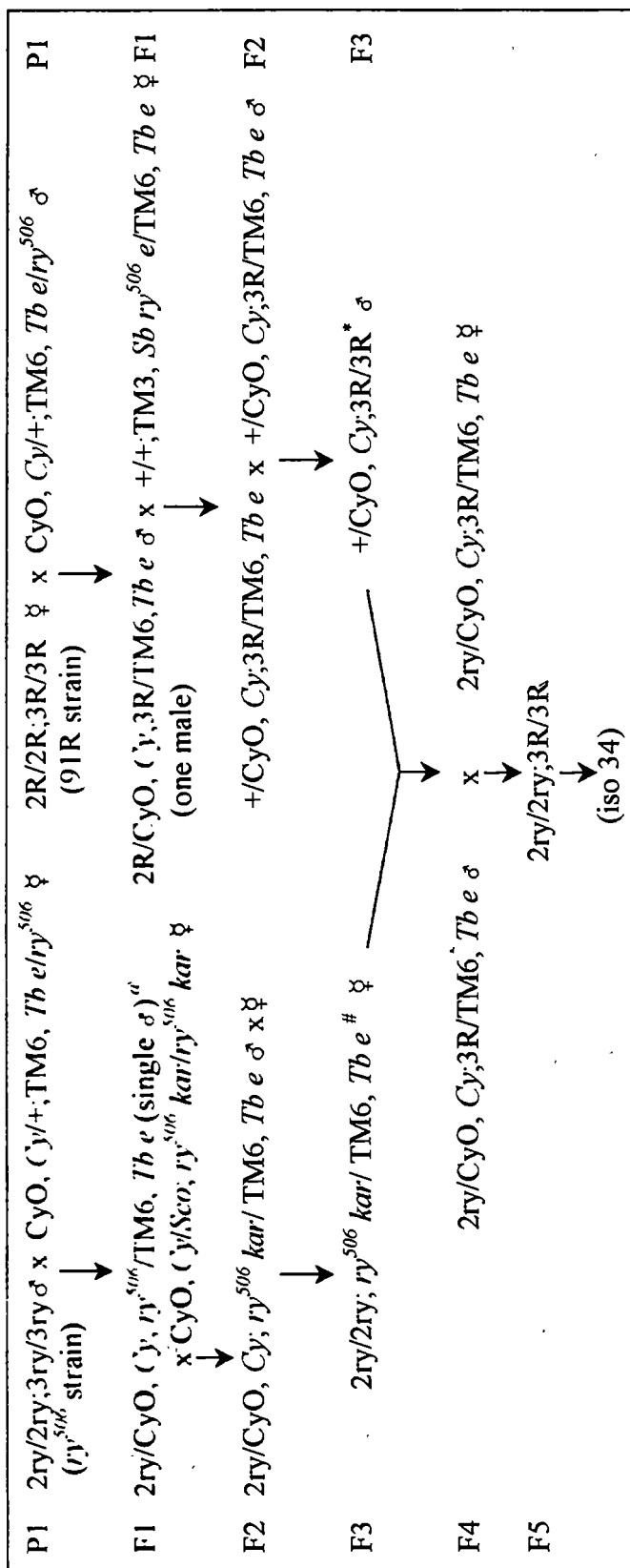
Synthesis of CCR and CCM stocks



A-10: Synthesis of stocks with X and 2nd chromosome from CYP underproducer strains and the 3rd chromosome from CYP overproducer strains

Two stocks (CCR, CCM) were synthesized using base stocks of the 91-R, 91-C and MHIII-D23 strains. One chromosome in each stock was replaced by a balancer chromosome carrying a dominant marker. H represents chromosomes from CYP overproducer strains (91-R and MHIII-D23) and L represents chromosomes from the CYP underproducer strain, 91-C. The prefixes 1, 2 or 3 in front of the letters H or L represent the X, 2nd or 3rd chromosome from the overproducer or underproducer strain. CyO, TM3, TM6 are balancer chromosomes with dominant markers (Lindsley and Zimm, 1992).

Generation of Iso 34



A-11: Synthesis of a stock where the 2nd chromosome from the ry⁵⁰⁶ strain and the 3rd chromosome from the 91-R strain.

[#]This stock (2ry/2ry;TM6, ry⁵⁰⁶ kar /Tb e) was derived from a single male in the F1 generation[@] and hence it carries an isogenic 2nd chromosome from the ry⁵⁰⁶ strain.

^{*}The stock (+/CyO;3R/3R) was derived using single males in the F1 generation of the cross and hence is isogenic for the 3rd chromosome from 91-R. Several lines were made of which one line (# 4) was used for substituting the 3rd chromosome of the 2ry/2ry;ry⁵⁰⁶ kar/TM6, Tb e stock[#].

The above stocks, [#]2ry/2ry;TM6, Tb e/ry⁵⁰⁶ and ^{*}+/CyO;3R/3R were combined to yield the iso 34 stock, which carried an isogenic 2nd chromosome from the ry⁵⁰⁶ strain and an isogenic 3rd chromosomes from the 91-R strain.

VITA

Sushmita Maitra was born on the 5th of April, 1962 to Mira and Arup Maitra, in Pune, India. She attended various schools, all over India. Her final secondary school was Loreto Convent, Asansol, India. She did her higher secondary education at La Martiniere, Calcutta. She received her Bachelor of Science degree in Zoology from the University of Calcutta in 1983, where she ranked second. She was a gold medallist for the Master of Science degree in Zoology, which she received in 1986, also from the University of Calcutta. She started her graduate studies in Dr. A. S. Mukherjee's laboratory, at the University Colleges of Science, Technology and Agriculture, Calcutta, in 1987. She worked on the phenomenon of dosage compensation in *Drosophila* and was awarded a Ph.D. Degree in Zoology and Comparative Anatomy in 1996. In August 1993, she was admitted to the University of Tennessee for another Ph.D. degree. She joined Dr. Ranjan Ganguly's laboratory where she worked on the molecular mechanisms of insecticide resistance in *Drosophila*.