Effect of First, Second and Third Chromosome on the Promoter Activity of Cyp6a8 Gene of Drosophila Melanogaster

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To the Graduate Council:

I am submitting herewith a thesis written by Anirban Mukherjee entitled "Effect of First, Second and Third Chromosome on the Promoter Activity of Cyp6a8 Gene of Drosophila Melanogaster." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Ranjan Ganguly, Major Professor

We have read this thesis and recommend its acceptance:

Mariano Labrador, Jae H. Park

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
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Effect of first, second and third chromosome on the promoter activity of *Cyp6a8* gene of *Drosophila melanogaster*

A Thesis Presented for the Master of Science Degree

The University of Tennessee

Anirban Mukherjee

December 2008
Abstract:

The mechanism of insect CYP gene regulation is largely unknown. In the present investigation, I used Drosophila as a model insect to understand the role of X, 2nd and 3rd chromosomes on the promoter activity of Cyp6a8 gene. Two reporter transgenic strains, 0.8luc110 H-ry and 0.8luc14, carrying 0.8luc-A8 reporter transgene (chimera of 0.8-kb upstream DNA of Cyp6a8 and the firefly luciferase gene) on the 2nd and 3rd chromosomes of ry506, respectively were used. The X, 2nd and 3rd chromosomes of these two transgenic lines were replaced with corresponding chromosomes from DDT-resistant 91-R strain (overproducer of Cyp6a8). The effect was determined by measuring luciferase activity. Results showed that the 3rd chromosome of the 91-R strain had a strong and the 2nd chromosome had a weak stimulatory effect on Cyp6a8 expression. The effect of the 1st chromosome was strongly inhibitory only if the 3rd chromosome from 91-R and the 2nd chromosome from the ry506 strains were present in the genome. Chromosomal effect on the Inducibility of 0.8-kb Cyp6a8 promoter DNA by phenobarbital and barbital was also examined. It was found that these compounds could induce the promoter significantly if the genome had X or second chromosome from the ry506 or 91-R strain. However, no induction was observed when the third chromosome of 91-R was present in the genome but the third chromosome of the ry506 strain supported barbiturate inducibility of the Cyp6a8 promoter.

To further investigate if Cyp6a8 is regulated by other genes, I studied the effect of third chromosome-linked DHR96 (mammalian CAR/RXR homolog) mutation on the promoter activity of the 0.8Kb upstream DNA of Cy6a8. I found that the DHR96 mutation gave 7-12 fold higher constitutive expression of Cyp6a8 compared to the wild type strain in all developmental stages. This investigation concludes that Cyp6a8 expression in Drosophila is influenced by all three major chromosomes and the third chromosome-linked DHR96 gene has a negative effect on Cyp6a8 expression.
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1. Introduction to Cytochrome P450

The survival of an organism depends majorly on the presence of an effective defense system that enables the organism to fight against the plethora of chemical and biological toxic substances present in the environment. Higher organisms and mammals defend themselves from the toxic effects by an elaborate immune system. Invertebrates such as insects do not possess a sophisticated immune system. However, every living organism contains a group of detoxification enzymes including glutathione S-transferase, esterase and cytochrome P450 monooxygenase which confer resistance to exogenous toxic attacks. The cytochrome P450 enzymes (or CYP) not only help in detoxification of toxic foreign elements but also take important role in diverse biological reactions including drug and steroid metabolism, plant hormone and flavonoids production, insect growth and development etc. which makes CYPs as one of the critical enzymes for the survival of the species.

Discovery of Cytochrome P450:

Cytochrome P450 monooxygenase comprises a large superfamily of hemotheolate proteins first discovered in pig liver microsome. Garfinkel et al. (1958) and Klienberg et al. (1958) independently, observed an unusual absorption band with an UV absorption maximum in the Soret region at 450 nm when the microsomes were reduced with carbon monoxide (CO). These CO binding pigments that showed absorption peak at 450 nm were thus referred to as P450. Omura and Sato (1962) classified this pigment as hemoprotein and named it as Cytochrome P450 (Fig. 1-1.A). Estabrook et al. (1963) showed that a CO-binding pigment from adrenal cortex and liver microsomes could hydroxylate steroids and
drugs. Estabrook’s lab by their classical photoactivation reaction proved that cytochrome P450 was the compound that functions to combine the molecular oxygen for the hydroxylation of steroid. It was found that treating rats with compounds such as phenobarbital increases the drug metabolizing ability of liver microsome (Conney et al., 1967). Later on several studies indicated a correlation between the increased amount of the CO-binding pigment and increased metabolism of drugs and steroids which made CYPs as one of the major enzymes studied in regards to exogenous detoxification process.

**Cytochrome P450 distribution and function:**

CYP enzymes are found in all lineages of life, including mammals, insects, worms, plants, fungi, bacteria etc. As of Sept. 7, 2007 a total of 7703 distinct CYP sequences have been reported (P450 Nomenclature Committee, 2007). CYPs represent 57 genes (and 58 pseudogenes) in human and 90 genes in *Drosophila melanogaster* (Ranson et al., 2002; Tijet et al., 2001). In bacteria CYPs are soluble proteins in the cytoplasm whereas in eukaryotes they remain anchored to the cytoplasmic face of the endoplasmic reticulum by their N-terminal signal anchor. Some CYPs are also found in the inner membrane of mitochondria, projecting towards the mitochondrial matrix. CYPs are present in all cell types except skeletal muscles and red blood cells.

So far sixty different reactions are tagged with this enzyme class including aromatic/aliphatic hydroxylation, dealkylation, oxydative deamination, desulphuration, dehalogenation etc. (Lewis et al., 1996) and involving a variety of activities such as monooxygenase, reductase, peroxydase, oxydase etc. (Guengerich et al., 1993). One of the major function of CYPs are the metabolism of harmful exogenous (not produced by the organism) compounds, more commonly referred to as the xenobiotics. The xenobiotics
include but not exclusive of drugs, pesticides, environmental pollutants, different phycotoxins (toxins produced by plants), aflatoxins (toxins produced by Fungi), environmental carcinogens etc. Living organisms deal with the xenobiotic compounds by a highly complex xenobiotic metabolism system which takes place in three phases; Phase I or modification phase, Phase II or conjugation phase and Phase III or excretion phase.

During the phase I reaction a variety of enzymes acts to attach a reactive polar group to the xenobiotics. During the phase II reaction the activated xenobiotic metabolites are further conjugated with a charged species such as glutathione (GSH) sulphate, glycine, glucuronic acid etc. The introduction of a large anionic group like GSH detoxifies the reactive electrophiles formed during the phase I process. Tagging of the conjugates also makes the xenobiotic compounds water soluble and thus easy to be excreted out through urine. The CYPs are thought to be involved in both Phase I and Phase II reaction of the xenobiotic metabolism. They are abundant in liver and also found in other tissues and organs that are likely to face xenobiotic onslaughts such as lungs, kidneys, pancreas etc. CYPs are also found in brain, adrenalin glands, ovary etc. which further indicate their role in biological processes other than xenobiotic metabolism.

**Mechanism of action of P450:**

The CYPs was first characterized as “mixed function oxydases” by Hayaishi et al. (1955) as it required an oxidant (molecular oxygen) and a reductant (NADPH+H+). CYPs form the terminal oxydases in a multicomponent electron transport chain that catalyzes the monooxygenase reaction. The basic reaction is very simple involving splitting of an oxygen molecule into two atoms. Unlike the dioxygenase system the monooxygenase system inserts one of these oxygen atoms to the substrate while the other oxygen atom is reduced
to form water. The reaction scheme is as follows where R-H is the substrate and ROH is the oxidized product.

\[ R-H + O_2 + H^+ + NADPH \rightarrow ROH + H_2O + NADP^+ \]

The eukaryotic microsomal monooxygenase reaction requires a NADPH dependent electron transport system (Fig. 1-1.B) involving two proteins; cytochrome P450 monooxygenase and NADPH-dependant cytochrome P450 reductase or simply cytochrome P450 reductase. The cytochrome P450 reductase is an FMN and FAD-containing flavoprotein. The heme iron in the inactive cytochrome P450 monooxygenase enzyme remains in its low spin ferric state (Fe$^{+3}$). The heme iron in this state is also bound to a water molecule. Substrate binding removes various water molecules including the one bound with the heme iron, which brings about a conformational change in the enzyme resulting in a shift from the low spin to a high spin state. This change in spin increases the interaction between cytochrome P450 reductase and cytochrome P450 monooxygenase and the first electron is transferred from NADPH to the heme iron of the monooxygenase enzyme. This changes the spin equilibrium of cytochrome P450 to ferrous (Fe$^{+2}$) state which in turn promote the binding of molecular oxygen to the heme group of the reduced P450-substrate. Next, another electron from NADPH is transferred to the ferrous ion of cytochrome P450 heme group. This addition of the second electron results in the formation of highly unstable iron-peroxy complex and the continuous flow of electrons ultimately make the oxygen-oxygen bond weaker to the extent that they split into two oxygen atoms. One of the oxygen atoms is then inserted in the monooxygenase-bound substrate while the other oxygen atom reacts with two protons and forms water.
Figure 1-1: Characterization of Cytochrome P450 enzyme

A. Finding of Omura and Sato: Cytochrome P450 is characterized by the distinct absorption at UV soret region at 450nm when the pigments are reduced with carbon monoxide (Omura and Sato, 1962).

B. Monoxygenase reaction of cytochrome P450: Cytochrome P450 electron transport system: Cytochrome P450 forms the terminal electron acceptor in this electron chain operative mainly in liver microsomes of higher organism. Electrons are transferred from the reduced NADPH to CYP through a flavine containing reductase system. These electrons are ultimately transferred to the molecular oxygen by CYP monoxygenase system thereby splitting the oxygen molecule into two component atoms. One of the oxygen atoms is transferred to the substrate while the other is reduced to form a molecule of water (Hayaishi et al., 1955).
Role of Cytochrome P450 in the metabolism of endogenous substances:

CYPs metabolize thousands of endogenous compounds and most CYPs can metabolize multiple substrates, and can catalyze multiple reactions. Mutations in CYP genes or deficiencies of the gene product are associated with various diseases.

Irmler et al. (2000) showed that CYP72A1 is expressed in epidermis of young leaves of *Catharanthus roseus* and were involved in converting loganin to secalogenin, a critical step in indole alkaloid biosynthetic pathway. *C. roseus* (or *Vinca rosea*) produces two very important indole alkaloids; vinblastine and vincristine which are anti-mitotic drug used to treat certain kinds of cancer, including Hodgkin's lymphoma, non-small cell lung cancer, breast cancer and testicular cancer. Kaltenbach et al. (1999) found that CYP75s are expresses in the epidermis and cotyledon of the flowering buds of *C. roseus* and were involved in biosynthesis of flavonoids such as flavone, flavonone, dehydroflavonols etc. The CYP75s family of CYP enzymes is thus thought to be involved in flowering of plants. CYP79 and CYP83B1 are involved in the conversion of indole glucosinolate to indole 3 acetic acid (IAA) or auxin (plant growth hormon) and arabidopsis plants mutant for these CYPs showed to have stunted growth. Other CYPs such as CYP701A3 and CYP88A are involved in Gibberelic acid biosynthesis. Gibberellic acid is a simple Gibberellin which promotes growth and elongation of cells, increase the rate of seed germination etc. CY72B1 and CYP72C1 are found to be involved in Brassinosteroid synthesis (Kim et al., 2002). Brassinosteroid are plant steroid hormones involved in pollen growth and pollen tube elongation, protection from draught and chill.

In animals CYPs are found to be involved in Biosynthesis of eicosanoids, cholesterol and bile acids, Steroid synthesis and metabolism, vitamin D3 synthesis and metabolism, the hydroxylation of retinoic acid etc.
CYP3A7 is involved in conversion of all-trans and 13-cis-retinoic acid to their 4-oxo and 4-hydroxo metabolites. This NADPH dependent reaction is one of the critical steps in conversion of all trans retinoic acid to vitamin A1 or all trans retinole. High or low amount of all trans retinole leads to severe birth defects (Chen et al., 2000). CYP27A, CYP27B1 and CYP24A are involved in formation of different dehydroxylated D3 metabolites including 1, 25 dehydroxy vitamin D3 which is a hormone involved in regulation of calcium and potassium metabolism (Guryev et al., 2003). Another important enzyme CYP11A1 is known to regulate the conversion of cholesterol to pregnenolone. Pregnenolone is a precursor to pregnane and progesteron. Chiang et al. (1998) showed that four CYP controls the 15 step enzymatic conversion of cholesterol to bile acid. Those are CYP7A, CYP7B CYP12 and CYP27 which convert cholesterol to cholic acid or to chenocholic acid (absence of one -OH group) which are the two main bile acids synthesized in human. Bile acids help to dispose of the cholesterol and helps in absorption of vitamins and fats in the intestine. Wang et al. (2000) found that CYP3A9 in female rat are involved in progesteron and other steroid hormon metabolism such as androstenol etc.

In insects CYPs are essential enzymes for biosynthesis and metabolism of steroid hormones and reproduction. CYP6L1 is found only in testes and the accessory glands in male German cockroaches and suggested to be involved in male specific reproduction (Wen et al., 2001). There are various Halloween genes found to be involved in regulation, production and metabolism of Ecdysone (E), the steroidal prohormone of the major insect molting (ecdysis) hormone 20-hydroxyecdysone (20E), which is secreted from the ring gland. In insects cholesterol is converted to 7-dhydroxycholesterol and enter ecdyson hormon byosynthesis pathway. In the prothoracic gland the 7-dhydroxycholesterol is converted to ketadiol. CYP306A1 or Phantom (Phm) is a 25-hydroxylase of Drosophila melanogaster and Bombix mori which converts Ketodiol to Ketotriol (Warren et al., 2004).
CYP302A1 or Disebodied (dib) and CYP315A1 or Shadow (sad) are C22 and C2 hydroxylases which converts Ketotriol to 2-deoxyecdysyson and 2-deoxyecdyson to ecdyson respectively (Gilbert, 2004). The dib mutants are embryonic lethal indicating the necessity of this enzyme for survival of the organism. After production the ecdysone leaves the prothorasic gland and transferred to various tissues where it is converted to the active form, 20E by CYP314A1 or Shade (shd) (Petryk et al., 2003). Other halloween genes such as CYP307A1 or Spook (Spo) does not express in larva and thought to be involved in stage specific componenet of E biosynthesis (Ono et al. 2006). The ecdysone biosynthetic pathway and the involvement of various CYPs are shown in Fig. 1-2.

Role of Cytochrome P450 in the metabolism of exogenous (xenobiotic) substances:

The Cytochrome P450 is the major enzyme that can metabolize exogenous compounds and are essential to survive through everyday xenobiotic insults. In human Liver and intestine are the two major sites for xenobiotic metabolism. Four families of CYPs are studied extensively for xenobiotic metabolism in liver namely, CYP1A, CYP2C, CYP3A and CYP4. These CYPS are involved in metabolism of hundreds of xenobiotics and drugs like Caffeine, estradiole (CYP1A1), Lansoprazole, omeprazole, anti-epileptic drugs as propranolol and hexobarbital (CYP2C19), Cocaine, quinine, cyclosporine, clarithromycin erythromycin (CYP3A, CYP5 and CYP7) etc. The CYP1 family is induced by aromatic or halogenated hydrocarbons such as benzo(a)pyrene and TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and involves a specialized receptor called AhR (or aryl hydrocarbon receptor) (Hankinson et al., 1995). The Ah receptor remains in the cytoplasm, bound with a heat shock protein 90 (hsp90) and other cytoplasmic proteins. Upon ligand binding the AhR dissociate from the Hsp90 and other co-chaperons but cannot enter the nucleus until it binds
with another protein called Arnt (or Ah receptor nuclear translocator). The AhR-Arnt heterodimer together binds with the upstream cis-element (the XRE-xenobiotic responsive elements) of CYP1A1 DNA to initiate active transcription. The AhR-Arnt heterodimer also activate various other genes that have the XRE cis-elements in their upstream DNA, including CYP1A2, CYP1B1, UDP glucuronosyltransferase etc. (Nebert and Gonzales, 1987). The CYP1 ligands are regarded as procarcinogens present in cigarette smoke and environmental pollutants which can be activated to carcinogens by CYP1. High levels of CYP1A2 have been linked to an increased risk of colon cancer. Another gene of the same family, CYP1B1 is often linked to primary congenital glaucoma. The normal substrate of this gene in mammals is not known, but it is thought that this P450 may be required to eliminate a signaling molecule. Defects in this gene lead to chronic high concentrations of the signaling molecule that lead to glaucoma. CYP2C19 metabolizes omeprazole, a common medication for ulcer. Polymorphisms in this gene cause a higher incidence of poor metabolizer phenotypes. Other CYPs are also considered as risk factors in several cancers since these enzymes can convert procarcinogens to carcinogens (Waxman et al., 1999).

Another Cytochrome P450 gene, CYP2E, is induced by phenobarbital in rat through a phenobarbital receptor called CAR (or constitutive androstane receptor). CAR belongs to nuclear receptor subfamily I which acts by forming heterodimer with another a receptor called RXR (or retinoid X receptor) and then the heterodimer binds with a phenobarbital response element in the upstream DNA to activate transcription of CYP2E.

Few CYP genes such as CYP4A1 are activated by certain drugs like clofibrate that bind to PPAR receptor (or peroxisome proliferator activated receptor). The PPAR was found to be activated by fibrates and other compounds previously known to increase the levels of peroxisomes in rodents and to increase expression of CYP genes in mice (Issemann et al., 1990; Rao et al., 1987; Lee et al., 1995). The drug when attached to this receptor, moves to
the nucleus, heterodimerize with the RXR and then binds with specific DNA sequence to activate transcription (reviewed by Waxman, 1999). The CYP4 family consists of 11 subfamilies (CYP4A–CYP4M), which encode constitutive and inducible isozymes expressed in both mammals and insects. CYP4 \( \omega \)-hydroxylate the terminal carbon of fatty acids and deficiency leads to metabolic disorders of lipid metabolism and inflammation.

CYPs also help insects to tolerate toxic chemicals that are found in their host plants. For instance, Hung et al. (1995) have found that Cytochrome P450 monooxygenases play important role in the detoxification of furanocoumarins, a toxin present in the host plant of *Papilio polyxenes* (black swallowtail butterfly) larvae. They have shown increased mRNA expression of *CYP6B1* and *CYP6B3* induced by both linear and angular furanocoumarins (xanthotoxin and bergapten respectively). The CYP6B1 was shown to metabolize the xanthotoxin when it is expressed in lepidopteron cells. In the Sonoran desert of southwestern United States and northern Mexico another interesting example of P450 mediated resistance to toxic plant allelochemicals was observed. Four endemic species of *Drosophila* (*D. mettleri, D. nigrospiracula, D. mojavensis* and *D. pachea*) live on necrotic tissues of five species of columnar cacti (sugaro, cardon, senita, agria and organ pipe). The larvae of those four species of *Drosophila* showed higher mRNA levels of different CYP alleles such as *Cyp28a1, Cyp28a2, Cyp28a3* and *Cyp4d10* indicating a possible role of those CYP in the toxin tolerance (Danielson et al., 1997).

Figure 1-2: CYP in biosynthesis of 20-hydroxyecdysone
The cytochrome P450 enzymes play essential role in the biosynthesis of 20E, the major hormone involved in molting and metamorphosis. 7-dhydroxycholesterol is converted to ketadiol and enters the Ecdysone biosynthesis.
Cytochrome P450 and Insecticide resistance:

The major xenobiotic insects are being challenged with are the insecticides. Molecular studies revealed that the increased level of CYP genes is directly associated with resistance. Most often the resistant insect showed a higher level of CYP expression than that of the susceptible ones.

It’s known that resistant insects can become susceptible if treated by CYP inhibitors like piperonyl butoxide (PBO), safrol and izosafrol etc. or by insecticides that acts on CYP enzyme system such as sesamex or pyrethroid compounds (Agosin et al., 1985; Hodgson et al., 1985; Kranthi et al., 2001; Kumar et al., 2002). In house flies the CYP6A1 and CYP6D1 alleles from the resistant strains produce higher levels of mRNA than the alleles from susceptible strains (Scott et al., 1999). Northern blot analysis of another Musca CYP6 gene, CYP6A2 showed about 3-fold higher mRNA expression in the resistant strains as compared to the susceptible strain. And that expression was about 20-fold higher when induced by phenobarbital (Carino et al., 1992). CYP6D1 showed about 10-times higher expression of mRNA (Scott et al., 1999) and about 8-fold higher expression in protein (Liu and Scott, 1996) in the resistant strain.

Historically two strains of Drosophila melanogaster were lab selected for DDT resistance viz. 91R (DDT resistant strain) and 91C (DDT susceptible strain) (Merrel and Underhill, 1956; Merrell; 1960). In Drosophila melanogaster, alleles of Cyp6a2 and Cyp6a8 from the resistant strains such as 91R show higher constitutive expression at mRNA level than the alleles from susceptible strains like 91C and rosy etc. (Waters et al., 1992; Maitra et al., 1996; Dombrowski et al., 1998). These studies indicated a correlation between insecticide resistance and Cytochrome P450 gene overexpression but it was not understood whether there is one major gene that is involved in the resistance or resistance is polygenic.
Daborn et al. (2001 and 2002) and Le Goffe et al. (2003) showed increased expression of Cyp6g1 in global population involved in resistance and suggested that this particular allele, Cyp6g1 has a broad range of cross-resistance and thus selected in field resistant flies due to the ability to incur resistance to a variety of insecticides. However, Le Goffe et al. (2003) also showed increased Cyp6a8 expression in the resistant strains and indicated that resistance might be conferred by complex synergism between more than just one gene. Daborn (2007) used GAL4/UAS system to overexpress Cyp6g1 and claimed that overexpression of this single P450 allele was sufficient to confer resistance to the susceptible strains. Daborn (2007) also suggested that an Accord transposable sequence present 291 bp upstream of this gene is responsible for the overexpression of the Cyp6g1 in the resistant strains.

Chung et al. (2007) found that Accord insertion in a transformant line can increase in Cyp6g1 mRNA level by 10 to 40 fold but that overexpressing strains showed susceptibility to even low dose (5ug) of Dicyclanil and Nitenpyram insecticides. Daborn et al. (2007) also used low DDT dose to measure resistance (10ug of DDT). Kuruganti et al. (2007) analyzed Cyp6g1 by using quantitative Northern and Western blot and found that the Cyp6g1 is produced in high amount in susceptible flies but not in DDT resistant flies such as 91R. Kuruganti et al. (2007) made recombinant lines where the Cyp6g1 allele of resistant strain 91R was substituted with the Cyp6g1 allele from the susceptible strain 91C. These recombinant lines had first and third chromosome from the resistant 91R and the second chromosome carried high producer Cyp6a2 allele of 91R strain and low producer Cyp6g1 allele of 91C. In the resulting Northern blot the Cyp6a2 showed higher expression in the recombinant lines similar to that of 91R and Cyp6g1 showed lower expression in the recombinant lines similar to that of 91C. But two of the three recombinant lines used showed higher level of DDT resistance than that of 91R and the third recombinant line showed
resistance equivalent to that of 91R. As compared to the susceptible 91C strain all those recombinant lines showed several hundred fold higher level of DDT resistance. Festucci-Buselli et al. (2005) also found that strain susceptible to DDT also had high levels of Cyp6g1 but not overexpressed in the highly resistant strain 91R. If Cyp6g1 a major gene involved in resistance it should also be overexpressed in laboratory selected resistant strains but that was not found to be the case. It seems likely that the field and the lab selected strains have preferences for one or more CYP gene expression in regards to resistance as those flies are subjected to different selection pressure and that the CYP gene expression is highly complex phenomenon involving more than one factor.

Cytochrome P450 gene regulation:

It was found that most of the wild type insecticide susceptible strains have lower mRNA level of many CYP genes than that of their resistant counterparts. In both Drosophila and Musca various Cyp6 genes seem to have low constitutive expression in the wild type flies than in the resistant flies (Dombrowski et al., 1998; Carino et al., 1992). For instance the Cyp6a8 and Cyp6a2 are upregulated in DDT resistant strain such as 91R and downregulated in DDT susceptible strain like 91C and ry 506. These genes are being studied in our lab as model genes to understand the genetic basis of the CYP gene overexpression in the insecticide resistant strains. So far four of the P450 genes, including Cyp6g1, Cyp6a2, Cyp6a8 and Cyp12d1 were mainly cited in the literature for their possible involvement with xenobiotic metabolism, especially with DDT resistance (Waters et al., 1992; Burn et al., 1996; Maitra et al.1996; Dombrowski et al., 1998; Brandt et al., 2002; Daborn et al., 2002; Le Goff et al., 2006, Festucci-Buselli et al. , 2005) but not much is understood about the mechanism by which those genes might be controlled. The general belief is that the DDT
resistance in *Drosophila* might be multifactorial and some factors are involved in DDT metabolism directly whereas others act as mere modifiers. Studies indicate that the number of DDT resistant genes might not be large but all three chromosomes might be involved in rendering resistance through CYP gene regulation by possible *trans*-regulatory control. For instance the mRNA levels of CYP6A1 and CYP6D1 genes located on chromosome 5 and 1 (respectively) of *Musca* were found to be *trans*-regulated by a locus or loci present on the second chromosome (Carino et. al., 1994; Liu and Scott, 1996). It was shown that the CYP6A1 and CYP6D1 alleles of the over producer strains of the housefly were downregulated by second chromosome from the underproducer flies. Interestingly chromosome 2 of *Musca* is thought to be homologous to the right arm of third chromosome of *Drosophila* (Foster et al., 1981). Waters et al. (1992) found 20-30 fold higher *Cyp6a2* mRNA level in the DDT resistant 91R flies than that of the susceptible 91C flies. Maitra et al. (1996) with the help of *Cyp6a2* cDNA probe had identified two new *Cyp6* gene sequences namely, *Cyp6a8* and *Cyp6a9*, by screening the cDNA library of 91R strain. The constitutive mRNA expression of these *Cyp6* genes was measured in various strains of *Drosophila*. It was found that both the genes had high mRNA expression in 91R (DDT resistant), moderate in ry^606^ (DDT susceptible), silent or low in 91C (DDT susceptible) and low in 91R-91C hybrids. However the mRNA level of *Cyp6a8* in the 91R-91C hybrids that carried the third chromosome from the wild type susceptible 91C flies was less than that was expected from the half dose of the *Cyp6a8* allele coming from the 91R strain. The downregulation of these two CYP genes in hybrid was explained by two hypotheses. The 91R is not a wild type fly and it is possible that during the lab selection it acquired new mutations or fixed a mutation that was already present in the population. It is possible that 1. The third chromosome from the susceptible wild type flies may contain factors that can produce active repressor molecules which in turn downregulate the expression of the two *Cyp6* genes on the second
chromosome. In 91R strain the active repressor is probably mutated and thus unchecked overexpression of the CYP genes occurs. Alternatively, 2. There might be a positive factor (s) which is normally turned off in wild type but in 91R due to mutation this factor (s) is constitutively turned on, stimulating the Cyp6a8 expression. Maitra et al. (2000) by chromosome substitution experiments confirmed the fact that factors on the third chromosome of Drosophila can regulate the expression of the Cyp6a8 allele present in the second chromosome. While the third chromosome from the underproducer strains of Drosophila downregulates the expression of Cyp6a2 and Cyp6a8, the third chromosome from the overproducer strains upregulates the expression of these genes. Maitra et al. (2000) found that the X chromosome can influence the expression of Cyp6a8 and Cyp6a2 genes. It was found that the X chromosome from 91R can downregulate the expression of the Cyp6a8 and Cyp6a2 genes. This indicates that there might be a common regulatory mechanism that can control the expression of both the CYP genes and this regulation involves factors not only on the third chromosome but also on the other chromosomes.

Beside the trans-regulation, Cyp6a8 may also be regulated by cis-acting elements present in its upstream promoter region and enhancer/silencer sequences. While the promoter regions regulate the basal transcription the enhancer/silencer sequences control temporal, tissue-specific and induced expression of a gene. However, mutation in trans-regulatory factors seems more plausible because the chance occurrence of a mutation in the cis-acting element that would give high constitutive overexpression in multiple CYP genes (Cyp6a2 and Cyp6a8) in resistant flies seems a rarity but again not impossible. Nevertheless, it seems that cis-regulatory factors have some role in the Cyp6a8 gene expression. Maitra et al. (2002) found several base substitutions in between the overproducer and the underproducer strains. The Cyp6a8 expression is perhaps a result of interaction between both cis-acting factors and trans-regulatory elements. In the soil
bacteria, *Bacillus megaterium*, it was found that both the *trans*-acting factors and *cis*-acting elements are involved in the repressor control of two CYP genes (Liang et al., 1995). These bacteria produce a repressor protein, Bm3R1 that turns off the expression of the CYP106 and CYP102 genes constitutively by binding with a highly conserved 5’ upstream promoter region called Barbie box. Mutation within these *cis*-regulatory Barbie box sequences results in reduction of the binding affinity of the Bm3R1 repressor protein to the Barbie box and causes constitutive overexpression of CYP106 and Cyp102 genes (Liang et al., 1995; Shaw et al., 1992).

**Research objectives:**

**Objective 1:**

The main objective of our study was to understand the mechanism of regulation of the *Cyp6a8* gene expression in *Drosophila*. Maitra et al. (2000) have found that the third chromosome from the underproducer strain of *Drosophila* downregulates the expression of *Cyp6a8* of the overproducer strain present in the second chromosome. The *Cyp6a8* sequence has been characterized and transgenic flies with different upstream DNA fragment of 91R-*Cyp6a8* sequence were constructed (Maitra et al. 2000). Maitra et al. (2002) showed that 200bp and 800bp upstream DNA fragment of *Cyp6a8* might have the regulatory sequences that control the *Cyp6a8* promoter expression and that the 800bp upstream fragment of *Cyp6a8* allele of overproducer strain 91R (91R-*Cyp6a8*) gives highest constitutive and PB induced expression. Bhaskara et al. (2006) showed that the same 800bp upstream DNA fragment of 91R-*Cyp6a8* gene gives high caffeine induced and constitutive expression both in adult flies and in SL-2 cell lines. Thus we chose transgenic reporter flies with 800bp upstream DNA fragments of *Cyp6a8* to study the effect of different
factor in Cyp6a8 promoter activity. Kuruganthi (unpublished data) showed that expression of another CYP gene, Cyp6g1 is regulated by the third and the first chromosome.

Maitra et al. (2000) also indicated that factors on X chromosome might regulate Cyp6a8 expression. Thus the major focus of the proposed research was to identify the role of first, second and third chromosomes on the promoter activity of Cyp6a8 and thereby gain understanding of CYP gene’s regulation. The reason to choose the Cyp6a8 is because this gene is overexpressed in resistance flies both constitutively and by induction of PB, Barbital and Caffeine (Maitra et al., 1996, 2000; Bhaskara et al., 2006).

Objective 2:

CYP gene expression seems to involve multiple factors and regulation by gene interaction is also a common phenomenon, another objective of our study has been to determine if Cyp6a8 expression is regulated by other genes. To date no studies have been done to understand if Cy6a8 expression is influenced by gene interaction. DHR96 (or Drosophila Hormone Receptor-like in 96 or Hr96) gene is present on the third chromosome and factors on the third chromosome seem to regulate the expression of the Cyp6a8 present in the second chromosome. King-Jones et al. (2006) showed that the DHR96 is involved in P450 gene transcription, especially in regards to phenobarbital induction. It was found that DHR96 mutation upregulates (Cyp6a2) or downregulates (Cyp12d) various CYP genes. DHR96 is also found to be selectively expressed in tissues that are highly active in xenobiotic metabolism such as gastric caeca, fat bodies and malpighian tubules etc.

Since DHR96 is the Drosophila homologue of mammalian CAR, and Cyp6a8 gene expression is influenced by phenobarbital, the effect of DHR96 mutation on Cyp6a8 expression was also studied.
2. Materials and Method:

Fly strains and culture condition:

Various *Drosophila melanogaster* strains were used in the present study. The 0.8luc110 H-ry and 0.8lu14 are transgenic reporter lines created by Maitra et al. (2002) in ry\textsuperscript{506} (rosy) host strain. These transgenic lines were made by inserting 800 bp (-11/-761) upstream DNA fragment of *Cyp6a8* on the upstream of a firefly (*Photinus pyralis*) luciferase reporter gene. Transgenic flies were created by P-element germ line transformation of the underproducer fly strain ry\textsuperscript{506} (rosy). Each of these strains has a single transgene with firefly luciferase (luc) reporter gene under the control of the 0.8kb upstream DNA of the *Cyp6a8* gene. The 0.8luc110 H-ry carries the transgene on the second chromosome while the 0.8lu14 has the transgene on the third chromosome.

The DHR96 and Canton-S-Utah stocks were provided by Prof. Carl S. Thummel at the University of Utah. The DHR96 stock is made by introducing a DHR96 mutated sequence in the Canton-S-Utah fly host. A 7.55 kb target DNA fragment was created containing DHR96 sequence with two deletions. The first deletion is 26bp long and removed the start codon while the second deletion is 331 bp long and removed the exon 4, downstream intron and the splice acceptor site for exon 5. A *pax6-EGFP* reporter sequence was inserted in that 7.55 kb fragment. This fragment containing the mutated DHR96 and a GFP sequence was inserted in the wild type DHR96 gene sequence of wild type Canton-S-Utah in reversed order (Fig. 5) thereby completely knocking down the gene (King-Jones et al., 2006).

The 91-R was selected by Merrell and Underhill (1956) and later Merrell and Dapkaus (1977) as described earlier (p.10) for DDT resistance. The 91R is the DDT resistant strain and also the overproducer of *Cyp6a8* mRNA.
The double balancer stock (described as DBL) was synthesized by Maitra et al. (2002) using three balancer stocks obtained from Dr. John Lucchesi (Emory University). The balancer stocks have multiple inversion in the same chromosome that makes them homozygous lethal. Each balancer has its own dominant and recessive markers. We used the double balancer stock with genotype w; SM6, Cy/ScO; MKRS, Sb/TM6, Tb. This stock has white eye homozygous recessive marker on the X chromosome, Cy/ScO (curly wing/Scutoid heterozygous and belongs to SM6 balancer) on the second chromosome and Sb/Tb (Stubble/Tubby and belongs to MKRS and TM6C balancers respectively) on the third chromosome.

All stocks were raised at 25°C on standard cornmeal-agar-molasses Drosophila medium. The base stocks with their respective genotypes are presented in table 2-1.

**Synthesis of Stocks Alpha (+c; 0.8; DHR96¹) and Beta (+c; 0.8; +c):**

To understand the effect of DHR96 mutation on the Cyp6a8 promoter activity we synthesized DHR96 mutant and DHR96 wild type flies with 0.8 luc reporter transgenic background.

DHR96 females, 0.8luc 110 H-ry females and Canton-S-Utah females were crossed with the double balancer males in three individual crosses to get intermediate stocks such as Pegasus (+c; Cy/Sco; DHR96¹), Pan (r/Y or w/Y; 0.8; Tb/Sb) and Echo (+c; Cy/Sco; +c) respectively. Pegasus female and Pan male were crossed to obtain Cy/Sb male (+c/Y; 0.8/Cy; DHR96¹/Sb) at F1. This male was backcrossed with Pegasus female to get Sco males (+c/Y; 0.8/Sco; DHR96) and Cy female (+c; 0.8/Cy; DHR96¹). This two F2 progenies are crossed to get F3 males (+c; 0.8; DHR96¹) and females (+c/Y; 0.8; DHR96¹) and were crossed to obtain Alpha flies (+c; 0.8; DHR96¹) at F4 generation. Similarly Beta (+c; 0.8; +c)
flies were made by crossing Echo and Pan. Pan male is obtained by maintaining the F2 cross and since we have always selected males in any cross with Pan it did not matter what the X chromosome of Pan carried. Echo females were crossed with the Pan males to get Cy-Sb males (+c/Y; 0.8/Cy; +c/Sb) in F1. These F1 Cy-Sb males were backcrossed with Echo female. From F2 generation Sco males (+c/Y; 0.8/Sco; +c) and Cy females (+c; 0.8/Cy; +c) were selected and crossed to obtain F3 males (+c/Y; 0.8; +c) and F3 females (+c; 0.8; +c). Those F3 progenies were crossed to maintain the Beta flies (+c; 0.8; +c).

**Substitution of the third chromosome of Alpha with the third chromosome of Beta:**

The third chromosome of Alpha (+c; 0.8; DHR96) carries the mutation in DHR96 gene. In order to confirm if the DHR96 mutation is the reason for differential expression between Alpha (DHR96 mutant) and Beta (DHR96 wild type) strains we have substituted the third chromosome of the Alpha strains to that of Beta. We mated Alpha females (+c; 0.8; DHR961) with DBL males (w/Y; SM6, Cy/ScO; MKRS, Sb/TM6, Tb) and selected the Sco-Sb males for the F1 progeny. These F1 males were crossed with Beta females (+c; 0.8; +c). In the F2 the Sb males (+c/Y; 0.8; +c/Sb) were selected and again crossed with the Beta females to produce a new strain, Alpha_Rev (+c; 0.8; +c) which has the Alpha third chromosome substituted to that of the Beta. This strain represented as a gain of function strain where the DHR96 mutation is replaced by the wild type allele.

To understand if the effect of DHR96 mutation on Cyp6a8 gene expression is dose dependant we have crossed the Alpha females and Beta males and crossed them to get Alpha_Beta F1 heterozygote (+c; 0.8; DHR96/+c) where the DHR96 mutation is present in half dose. These hybrid flies were still GFP positive as the single DHR96 mutant sequence with the EGFP reporter fragment still present in these flies.
Chromosome substitution stocks:

We have crossed the female flies of 91R, 0.8luc 110 H-ry and 0.8luc 14 with the double balancer males in three individual crosses to get intermediate stocks such as Sol (R; Cy/Sco; R), Eos (R; R; Sb/Tb), Iris (r; 0.8; Tb/Sb) and Hestia (r; Cy/Sco; 0.8) respectively. These stocks were used to synthesize the desired chromosome substitution stocks. For the chromosome substitution stocks we have used F1 hybrid males to minimize the time needed to obtain homozygous. Hence we had two types of fly strains; one with Cy-Sb phenotype and another with Sco-Tb. We made these two types of flies to understand if there is any effect of these markers on the Cyp6a8 expression. Twelve of such chromosome substitution stocks were prepared to measure the effect of the three major chromosomes in Drosophila on the promoter activity of Cyp6a8 gene (Table 2-2).

Sol (R; Cy/Sco; R) and Iris (r; 0.8; Tb/Sb) are used to obtain chromosome substitution stocks with reporter sequence on the second chromosome while Eos (R; R; Sb/Tb) and Hestia (r; Cy/Sco; 0.8) were used to synthesize substitution stocks with reporter gene on the third chromosome. Sol female and Iris males were crossed to obtain Strain#1a (R/Y; 0.8/Cy; R/Sb) and Strain#1b (R; 0.8luc/Sco; R/Tb). Transgenic line 0.8luc 110 H-ry females were crossed with the double balancer males to obtain Strain#2a (r; 0.8luc/Cy; r/Sb) and Strain#2b (r; 0.8luc/Sco; r/Tb). Iris females were crossed with Sol males to synthesize Strain#3a (r; 0.8luc/Cy; R/Sb) and Strain#3b (r; 0.8luc/Sco; R/Tb). Eos females were crossed with Hestia males to produce Strain#6a (R; R/Cy; 0.8luc/Sb) and Strain#3b (R; R/Sco; 0.8luc/Tb) flies. Transgenic 0.8 luc 14 females were crossed with double balancer males to get Strain#7a (r; r/Cy; 0.8luc/Sb) and Strain#7b (r; r/Sco; 0.8luc/Tb). Hestia females
were crossed with Eos males to produce Strain#8a (r; R/Cy; 0.8luc/Sb) and Strain#8b (r; R/Sco; 0.8luc/Tb).

Four types of heterozygous flies were also synthesized to further analyze the effect of the chromosomes. These flies were made by using the two previously mentioned transgenic lines and the over producer (91R) and the underproducer strain (ry^{506}). Males of the transgenic 0.8luc 110 H-ry flies were crossed with 91R females to synthesize Strain#9 (R/Y; R/0.8; R/r) and transgenic 0.8 luc 14 H-ry males were crossed with 91R females to synthesize Strain#10 (R/Y; R/r; R/0.8). In another set the ry^{506} males (r/Y; r; r) were crossed with the female flies of 0.8luc 110 H-ry and 0.8 luc 14 in two individual crosses to obtain Strain#13 (r/Y; r/0.8; r) and Strain#14 (r/Y; r; r/0.8) respectively.

<table>
<thead>
<tr>
<th>Fly Strains</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0.8luc 110 H-ry</td>
<td>r; 0.8luc; r</td>
</tr>
<tr>
<td>2. 0.8luc 14</td>
<td>r; r; 0.8luc</td>
</tr>
<tr>
<td>3. DHR96</td>
<td>+^{c}; _{c}; DHR96</td>
</tr>
<tr>
<td>4. Canton-S-Utah</td>
<td>+^{c}; _{c}; +^{c}</td>
</tr>
<tr>
<td>5. 91R</td>
<td>R; R; R</td>
</tr>
<tr>
<td>6. DBL (Double Balancer)</td>
<td>w; SM6,Cy/ScO; MKRS,Sb/TM6,Tb</td>
</tr>
</tbody>
</table>

Table 2-1: Names and genotypes of the base stocks.
Table 2-2: Names and genotypes of the chromosome substitution stocks.

<table>
<thead>
<tr>
<th>Fly Strains</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Strain#1a</td>
<td>R; 0.8luc/Cy; R/Sb</td>
</tr>
<tr>
<td>2. Strain #1b</td>
<td>R; 0.8luc/Sco; R/Tb</td>
</tr>
<tr>
<td>3. Strain #2a</td>
<td>r; 0.8luc/Cy; r/Sb</td>
</tr>
<tr>
<td>4. Strain #2b</td>
<td>r; 0.8luc/Sco; r/Tb</td>
</tr>
<tr>
<td>5. Strain #3a</td>
<td>r; 0.8luc/Cy; R/Sb</td>
</tr>
<tr>
<td>6. Strain #3b</td>
<td>r; 0.8luc/Sco; R/Tb</td>
</tr>
<tr>
<td>7. Strain #6a</td>
<td>R; R/Cy; 0.8luc/Sb</td>
</tr>
<tr>
<td>8. Strain #6b</td>
<td>R; R/Sco; 0.8luc/Tb</td>
</tr>
<tr>
<td>9. Strain #7a</td>
<td>r; r/Cy; 0.8luc/Sb</td>
</tr>
<tr>
<td>10. Strain #7b</td>
<td>r; r/Sco; 0.8luc/Tb</td>
</tr>
<tr>
<td>11. Strain #8a</td>
<td>r; R/Cy; 0.8luc/Sb</td>
</tr>
<tr>
<td>12. Strain #8b</td>
<td>r; R/Sco; 0.8luc/Tb</td>
</tr>
<tr>
<td>13. Strain #9</td>
<td>R/Y; R/0.8; R/r</td>
</tr>
<tr>
<td>14. Strain #10</td>
<td>R/Y; R/r; R/0.8</td>
</tr>
<tr>
<td>15. Strain #13</td>
<td>r/Y; r/0.8; r</td>
</tr>
<tr>
<td>16. Strain #14</td>
<td>r/Y; r; r/0.8</td>
</tr>
</tbody>
</table>

**Treatment of Flies:**

Caffeine, Barbital and Phenobarbital were used in various induction studies in our work and the doses, optimum for induction, were determined. We used instant fly food for the induction experiments. Caffeine, Barbital and Phenobarbital are water soluble so the respective treatment concentrations were made in aqueous solution. The 16ml disposable polypropylene fly culture tubes (Fisher) were used to culture the flies. About one third of the tubes were filled with the instant *Drosophila* medium (flakes) from Carolina Biological Supply Company, Burlington. The instant food was soaked with 2.5 to 3 ml of respective treatment solution and air dried for more than 6 hours. Fly food made with pure water was used as a control. Alpha and Beta adult female flies (5 days and 8 days old females) were fed on *Drosophila* instant food made with aqueous solution of 16mM caffeine (Sigma, St. Louis) for 24 hours and prepared for Luc assay. The chromosome substitution stocks (5 days old males) were treated with the appropriate chemical for 24 hours. Constitutive expression of
Alpha, Beta and the chromosome substitution stocks were done to measure the fold induction. All experiments were done in triplicate, at least in three different days with triplicate samples. Luciferase reporter gene activity and total protein in fly extracts were measured by Luc Assay Reagent kit (Promega) and BCA protein assay (Pierce), respectively as described earlier (p.27). For the developmental expression, 0 to 1 hour old embryo, 3 days old pupae, female third instar larvae, 4 days old adult female and 8 days old adult female Alpha and Beta flies were processed to measure their luciferase activity. About 40 to 50 female flies of Alpha and Beta were kept in an egg laying dish with untreated fly food for 1-2 hours and then the embryos were carefully collected using a fine needle without puncturing them and extracts were made for the expression assay. The pupae and the third instar larvae were collected from suitable, fresh fly cultures. Third instar larvae were identified by their size and denser pigmentation and were taken in a Petri dish with water and observed under microscope. The female larvae were chosen by the presence of smaller gonads under microscope. The 4 days old and 8 days old adults were taken from fly cultures maintained to have same age flies. The flies were grouped in groups of three with each group containing 5 flies and homogenized in 1X CCLR buffer (as described earlier in p.27) and prepared for Luc assay and expression analysis.

Luciferase Assay:

Flies were assayed by standard luciferase assay using Lusiferase Assay System (Promega). We typically took 5 female flies of similar age (4 days, 0-5 days and 8 days old age groups for the Alpha and the Beta strains) or 2 male flies of same age (5 days old flies for the chromosome substitution stocks) and homogenize them in 1X Cell Lysis Buffer (40ul buffer/fly) in 1.5ml eppendorf tubes using a battery operated homogenizer. The extracts were centrifuged in 4°C with 13,000 rpm for 8 minutes and 100ul of the supernatant were
carefully separated from the precipitated debris and kept in a fresh 1.5ml eppendorf tube. These supernatants were again centrifuged with same parameters of spinning to further purify the extract. After the second centrifugation 50ul of the supernatant is taken as the final purified fly extract in a fresh 1.5ml eppendorf tube and kept on ice. About 5ul of each extract was mixed with 25 ul of the Luciferase Assay Reagent (LAR) in a 1.5ml eppendorf tube in dark by tapping the tube with finger 2-3 times. Immediately after mixing the content of the tube with finger tapping, it was placed inside a FB12 single sample luminometer (Zylux Corporation). The promoter activity in the -800bp, region inserted upstream of the firefly luciferase reporter gene, initiate production of luciferase protein. The extract contains the luciferase protein. The luciferase protein catalyzes the oxidation of luciferin (a substrate for luciferase, present in the LAR) and light is generated in the reaction. The chemiluminiscence of this reaction was detected by the luminometer and interpreted as the relative activity of the reporter gene. Since *Drosophila* doesn’t have endogenous luciferase the background activity is low. The results are expressed in RLU (Relative Light Unit).

**Protein Assay:**

The protein content in the extract was measured by standard BCA (Bicinchoninic Acid) protein assay using BSA (Bovine Serum Albumin) standards using 595 nm wavelength of light in BioMate™ 3 series spectrophotometer (Thermospectronic). A 2mg/ml BSA stock (Pierce) was half diluted using 1X CCLR buffer. Five dilutions with known concentration of BSA protein were prepared from the 1mg/ml BSA working stock by mixing appropriate volume of 0.5X CCLR with the 1mg/ml BSA stock. These dilutions in duplicates were used as standards for the protein estimation.
Due to small sample volume the fly extracts were diluted by 3 fold by mixing 40ul of pure extract with 40ul of water and 40ul of 0.5X CCLR buffer. This 120ul diluted extract was divided into two 50ul aliquots in order to measure the protein content of each of the extracts in duplicate. The color developing agent was prepared by mixing BSA reagent ‘A’ and ‘B’ in 50:1 ratio respectively as instructed by the manufacturer’s protocol (Sigma). 1 ml of this developing solution is added in each of the 50ul sample and the absorbance of the sample was detected by the BioMate™ 3 series spectrophotometer. The RLU values were divided by the protein content and the final result is expressed in RLU/ ug of protein for each sample and compared for relative reporter gene activity.

**Isolation of Genomic DNA:**

The genomic DNAs of Alpha and Beta strains were isolated using DNAzol ® reagent (Invitogen). Eight female flies were homogenized in 500 ul of DNAzol and DNA is precipitated by absolute ethanol and finally solubilized in water. The DNAzol reagent uses a guanidine - detergent lysing solution. This solution hydrolyzes the RNA in the sample and enhances selective precipitation of the DNA. The genomic DNAs were detected in a 1.5% agarose gel using a 23.13 Kb Lambda DNA HindIII digest (New England Biolabs).

**PCR Amplification:**

The DHR96 mutant carries an EGFP (Enhance Green Fluorescence Protein) sequence under the control of *pax6* gene which expresses the GFP in the eye. So prior to PCR amplification we have observed the Alpha and Beta flies under Leica Fluo™ stereomicroscope 4000 FW (Leica) for GFP expression. About 20 flies of each strain, in triplicates were taken in different 1.5 ml eppendorf tubes and put to sleep by use of carbon
dioxide. The flies were observed for the GFP expression on their eyes and ocelli. Then we amplified the sequence in the deleted region of this gene to make sure if Alpha flies have the mutation and if Beta has the intact wild type DHR96 sequence. We have targeted the Del2 or the second deletion which is 331bp long so that the mutant strains would give a product 331bp lesser than that of the wild type (Fig. 2-1). The primer sequences are given in table 3. The PCR was done by EasyStart™ PCR Mix-in-a-Tube containing pre- aliquoted PCR mix from Molecular Bio Products. We have used 140ng of Alpha and 120ng of Beta DNA, 1ul (20uM) of each of the primers, 16units (0.3ul) of Taq DNA polymerase (Fischer Scientific) and millipore water to make 50ul of total volume. This 50ul reaction mixture was added to the EasyStart™ PCR tube containing 50ul of EasyStart™ solution. The total volume of the final PCR reaction was 100ul. Denaturation temperature was 94°C, annealing 57°C and extension temperature was 72°C (for 1 minute) and the reaction was set for 20 cycles. The products were analyzed on 1.5% agarose gel by using 50bp DNA ladder (New England Biolabs). Length of the product from Alpha was expected 183bp and from Beta 514bp. Alpha lied in between 150bp and 200bp marker bands while Beta lied in between the 500 - 550 bp bands.

Table 2-3: The sequence of forward primer (FWDHR96Del2) and reverse primer (RWDH96Del2) and their respective T_M.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>FWDH96Del2</td>
<td>5'-CGCCATTCGATTGAGTCACC-3'</td>
<td>62.45°C</td>
</tr>
<tr>
<td>RWDH96Del2</td>
<td>5'-CGTCCATGCGCCACTTCTC-3'</td>
<td>64.48°C</td>
</tr>
</tbody>
</table>
Figure 2-1. DHR96 mutation:
A 7.55 Kb DNA fragment was created containing the DHR96 mutated sequence with two deletions. The first deletion (Del1) is 26 bp long and removed the start codon while the second deletion (Del2) is 331 bp long and removed the exon 4, downstream intron and the splice acceptor site for exon 5. The pax6-EGFP reporter sequence is shown in the white box. The red arrows show the position of the primers we used to amplify the Del2 region. The forward primer was placed 123bp upstream of the Exon 4 and the reverse primer at 176 bp downstream of The Exon 4. The green box shows the region that was amplified to confirm the mutation.

(Modified after King-Jones et al., 2006)
3. Result:

Effect of the X, second and third chromosome on the promoter activity of the Cyp6a8 gene:

We measured the effect of three major chromosomes of Drosophila melanogaster on the Cyp6a8 promoter activity by using these chromosome substitution stocks showed previously in table 2-2 (p.27). We used the F1 heterozygous males to minimize the time to obtain pure homozygous flies. We saw a slight difference in luc expression among the Cy;Sb and Sco;Tb flies. To confirm this finding we did a student's t-Test to see if Cy;Sb and Sco;Tb genotypes have any significant effect on luc expression of the chromosome substitution stock.

The Chromosome substitution stocks with same genotype and only differing in Cy-Sb and Sco-Tb markers did not have any difference in their LUC expression (Fig. 3-1). We did a paired and an unpaired t-Test and found both the cases the \( P-value >0.05 \) so we accept the null hypothesis and we concluded that there is no significant difference between the luc expression of Cy-Sb and Sco-Tb. So any change in luc expression we observed would have been due to the 91R or rosy\textsuperscript{506} chromosomes and not the due to the markers.

We found that the third chromosome from 91R had stimulatory effect and the first chromosome from 91R had inhibitory effect on luc expression. Maitra et al. (2000) showed that the third chromosome of wild type flies have inhibitory effect on endogenous Cyp6a8 expression. Our result strengthens the finding as we saw similar inhibition of Cyp6a8 promoter activity by the underproducer ry\textsuperscript{506} third chromosome.

Changing the 91R first chromosome of the low expressing strains 1a and 1b (r; Cy/0.8luc; Sb/r and r; Sco/0.8luc; Tb/r genotypes respectively) to the third chromosome of
91R, the resulting stock 2a and 2b (r; Cy/0.8luc; Sb/R and r; Sco/0.8luc; Tb/R genotypes respectively) gave 6.5 fold increased luc reporter activity which indicates that the third chromosome from 91R have stimulatory effect on Cyp6a8 expression (Fig. 3-1). But when the first chromosome of overproducer strain 2 was substituted by 91R first chromosome the resulting strain 3 gave more than 4 fold downregulation in the promoter activity which indicates that the first chromosome of 91R plays stimulatory role in Cyp6a8 expression. If we compare strain 1 and 3 we can see that the stimulatory effect of 91R third chromosome is higher than the inhibitory effect of the 91R first chromosome as strain 3 shows still higher activity than that of strain 2 (Table 3-1 and Fig. 3-1). The opposite is also true if we consider the effect of ry<sup>506</sup> first and second chromosome. It seems the susceptible ry<sup>506</sup> third chromosome has inhibitory and the first chromosome has stimulatory effect on Cyp6a8 promoter activity.

This can be explained by model showed in Fig. 3-2. As we know 91R was synthesized by a long DDT selection in the lab. So it is not a wild type fly. During the selection process the 91R genome might have mutated. We hypothesize that the third chromosome from the susceptible wild type flies might produce an active repressor(s) which binds to the recognition site on the 0.8kb upstream DNA of Cyp6a8 and downregulates the expression of the reporter gene. In 91R the active repressor is probably absent or mutated. When we changed the third chromosome to 91R due to absence of the functional repressor Cyp6a8 promoter activity was increased. The alternative hypothesis is that wild type flies have various regulatory factors that are switched off in their adult stage. There might such a positive factor(s) which is normally turned off in wild type but in 91R due to mutation this factor(s) is constitutively turned on. This positive factor(s) might be stimulating the Cyp6a8 promoter and thereby giving the high LUC expression.
Table 3-1: LUC expression of the chromosome substitution stocks

<table>
<thead>
<tr>
<th>Fly strain used</th>
<th>Cross name</th>
<th>Mean RLU/ug protein</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>r; 0.8luc; r</td>
<td>1a</td>
<td>205228.4099</td>
<td>50294.96428</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>228502.1312</td>
<td>13675.65989</td>
</tr>
<tr>
<td>r; 0.8luc; R</td>
<td>2a</td>
<td>1,337,889.07</td>
<td>181,637.54</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>1,407,343.93</td>
<td>223,917.67</td>
</tr>
<tr>
<td>R; 0.8luc; R</td>
<td>3a</td>
<td>286,708.29</td>
<td>50,539.48</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>305,400.50</td>
<td>52,439.38</td>
</tr>
<tr>
<td>r; r; 0.8luc</td>
<td>6a</td>
<td>223,160.35</td>
<td>45,559.71</td>
</tr>
<tr>
<td></td>
<td>6b</td>
<td>284,637.34</td>
<td>55,455.38</td>
</tr>
<tr>
<td>r; R; 0.8luc</td>
<td>7a</td>
<td>358,248.95</td>
<td>57,808.89</td>
</tr>
<tr>
<td></td>
<td>7b</td>
<td>312,756.93</td>
<td>89,488.28</td>
</tr>
<tr>
<td>R; R; 0.8luc</td>
<td>8a</td>
<td>136,697.59</td>
<td>37,510.85</td>
</tr>
<tr>
<td></td>
<td>8b</td>
<td>205,139.45</td>
<td>89,412.07</td>
</tr>
</tbody>
</table>

Figure 3-1: Compilation of LUC assay for the chromosome substitution stocks to determine the effect of the first and the third chromosome:
The Cy-Sco balancer chromosomes do not have any significant effect. The genotype of strain 1 is shown in the inset. Changing the third chromosome to 91R increased the reporter gene expression (LUC activity) while changing the first chromosome to 91R decreased the expression.
Figure 3-2: Inhibitory effect of the third chromosome from wild type/susceptible strain:
The ry^{506} underproducer third chromosome might have a factor(s) which can bind to the Cyp6a8 upstream promoter region on the second chromosome and downregulate its activity resulting in low LUC expression. In 91R overproducer strain that factor(s) is mutated hence unchecked overexpression occurs.

Figure 3-3: Compilation of LUC assay for the chromosome substitution stocks to determine the effect of the first and the second chromosome:
The genotype of strain4 is shown in the inset. Changing the first chromosome to 91R decreased the LUC expression but second chromosome seems to have weak stimulatory or no effect on Cyp6a8 promoter activity.
We wanted to know the effect of the second chromosome on *cyp6a8* expression so we used the 0.8luc 14 transgenic lines with the luc reporter gene on the third chromosome to obtain another set of substitution stock with 0.8luc luciferase reporter gene on the third chromosome.

Changing the position of the 0.8luc fragment to another chromosome doesn’t seem to have much effect on luc expression as *r; 0.8luc; r* and *r; 0.8luc* do not show any significant change in luc expression (though the luc expression of the later is a little higher, data not shown here). All these strains have the stimulatory 91R third chromosome removed by the transgenic third chromosome that originally came from rosy flies (low producers of *Cyp6a8* mRNA) and as expected gave low luc expression. We synthesized six strains namely, 6a, 6b, 7a, 7b, 8a and 8b. If we compare strains 6 and 7 we can see that changing the second chromosome to 91R increases the LUC activity but not very significantly. So the second chromosome of 91R might have weak stimulatory or no effect on *Cyp6a8* promoter activity. However, when we changed the first chromosome of strain 7 to first chromosome of 91R the resulting strain 8 showed a 2.6 fold decrease in LUC expression which further confirms our previous finding that the first chromosome of 91R has inhibitory effect on *Cyp6a8* promoter activity (Fig. 3-3).

**Induction of *Cyp6a8* by Phenobarbital and Barbital:**

The *Cyp6a8* gene expression was shown to be induced by Phenobarbital and Barbital treatment (Maitra et al., 1996, 2002). To understand the regulatory control and to elucidate further our understanding on barbiturate mediated induction in *Drosophila*, we measured the phenobarbital and barbital induction in our chromosome substitution stocks. 4mM of each of the two chemicals were used for a treatment time of 24 hours. All the
chromosome substitution strains namely, 1, 2, 3, 6, 7 and 8 were induced by both PB and Barbital (Table 3-22). The flies which are constitutively highly expressed such as strain 3 gave the lowest induction by both the chemicals (Fig. 3-4). We discussed earlier that the second chromosome has no significant effect on \textit{Cyp6a8} promoter activity. Interestingly the second chromosome of 91R here seemed to confer high stimulatory effect for induction. The inhibitory 91R first chromosome also seemed to stimulate induction. From our result we could find that the third chromosome has the lowest effect on induction. This is perhaps because of the fact that the third chromosome already gives very high constitutive expression so the inducers have not much effect on further increase of \textit{Cyp6a8} expression. In fact the highest induction does not cross the highest basal level as produced by the strain 2 (\textit{r}; 0.8luc; \textit{R}). It seems that there remains an inverse relation in the regulation of the expression of this gene. The chromosomes that show lower constitutive expression give higher induced expression of \textit{Cyp6a8} gene whereas the chromosomes that gives higher constitutive LUC expression, gives very low or no induced expression. It also seems that all three of the chromosomes are controlled by presence of PB and Barbital. It is possible that the PB or Barbital is involved in the same pathway by which the for instance, the 91R third chromosome stimulates the \textit{Cyp6a8} expression. When \textit{Cy6a8} expression is already increased by the presence of stimulatory 91R third chromosome, PB or Barb has no further effect on the expression.

For Barbital induction the first chromosome has highest effect and third chromosome has very little stimulatory effect for both PB and Barbital induction. For PB induction the second chromosome alone does not gives higher expression it is only when with the first chromosome the effect is magnified (\textit{r}; \textit{R}; 0.8luc gives lower expression than that of \textit{R}; \textit{R}; 0.8luc).
### Table 3-2: Induction of Chromosome substitution stocks by PB and Barbital

<table>
<thead>
<tr>
<th>Fly strain used</th>
<th>Cross name</th>
<th>Treatment</th>
<th>Avg Mean RLU/ug protein</th>
<th>S.D.</th>
<th>Agy Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>r; 0.8luc; r</td>
<td>1a</td>
<td>Control</td>
<td>198,184.83</td>
<td>13,640.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbital</td>
<td>1,150,262.25</td>
<td>253,922.61</td>
<td>5.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>685,145.44</td>
<td>4,878.13</td>
<td>4.47</td>
</tr>
<tr>
<td>r; 0.8luc; R</td>
<td>2a</td>
<td>Control</td>
<td>1,216,096.47</td>
<td>38,335.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbital</td>
<td>1,531,021.13</td>
<td>285,394.19</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>1,369,715.46</td>
<td>33,621.45</td>
<td>1.12</td>
</tr>
<tr>
<td>R; 0.8luc; R</td>
<td>3a</td>
<td>Control</td>
<td>237,186.07</td>
<td>79,195.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbital</td>
<td>2,022,240.30</td>
<td>634,033.68</td>
<td>8.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>1,103,393.59</td>
<td>67,266.25</td>
<td>4.65</td>
</tr>
<tr>
<td>r; r; 0.8luc</td>
<td>6a</td>
<td>Control</td>
<td>180,794.47</td>
<td>2,369.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbital</td>
<td>855,379.88</td>
<td>181,373.13</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>1,305,702.79</td>
<td>212,441.02</td>
<td>7.22</td>
</tr>
<tr>
<td>r; R; 0.8luc</td>
<td>7a</td>
<td>Control</td>
<td>195,598.85</td>
<td>56,772.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbital</td>
<td>913,626.63</td>
<td>162,074.09</td>
<td>4.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>627,158.15</td>
<td>238,794.15</td>
<td>3.21</td>
</tr>
<tr>
<td>R; R; 0.8luc</td>
<td>8a</td>
<td>Control</td>
<td>150,515.84</td>
<td>7,535.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barbital</td>
<td>1,567,579.68</td>
<td>242,016.14</td>
<td>10.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB</td>
<td>935,546.86</td>
<td>242,752.55</td>
<td>6.22</td>
</tr>
</tbody>
</table>

#### Figure 3-4. Fold induction of chromosome substitution stocks by PB and Barbital:
The second chromosome of 91R showed high stimulatory effect and the third chromosome of the 91R showed lowest effect on induction. The first and the second chromosome of 91R seem to interact with each other to increase induction.
The effect of Barb is highest on induction of Cyp6a8 gene and it shows higher fold difference between different strains whereas the PB does not give so much differential expression between different genotypes. We know from previous observation that male flies give higher constitutive Cyp6a8 expression than the female flies and we wanted to know if similar pattern would follow in case of the induction since all the chromosome substitution flies we tested were males and we have found that for constitutive expression of the male flies were though higher than the females but there is no difference in their induced expression (data not shown). It can also be seen that the ry$^{506}$ low producer background gives higher fold induction than that of 91R high producer background the actual values of RLU/ug of protein for flies with 91R first chromosome is much higher. For example the R; 0.8luc; R genotype shows lower Barb induction than r; 0.8luc;r and no difference in PB induction but former gives two fold higher and more than two fold higher RLU values for PB and Barb induction respectively. The reason might be as discussed that the strains with 91R background already maintained in higher Cyp6a8 expression and thus further expression is checked. Similar result was shown by Maitra et al. (2002) where the low producer 91C gave higher fold induction than the overproducer 91R.

**Effect of DHR96 mutation on Cyp6a8 expression:**

CYP expression is often complex and requires multiple factors and epistasis is common in Drosophila where the expression of one gene is regulated by another gene(s). Recently a CYP gene, Cyp6g1 was shown to require cross talks between other genes to render its effect (Kuruganti et al., 2007). Cyp6g1 is cited in literature as one of the major genes to confer DDT resistant. We have analyzed the effect of DHR96 mutation on Cyp6a8 regulation using DHR96 wild type (Beta) and DHR96 mutant flies (Alpha) carrying the 0.8luc reporter transgene on the second chromosome as described earlier (p.24).
First of all we needed to know if the flies we were assaying have the DHR96 mutation. The DHR96 mutation does not have any known phenotype except that the mutation was created in such a manner that it carries a GFP sequence inserted in the DHR96 sequence under control of a pax6 promoter which expresses the green fluorescence specifically in the eye. We analyzed the flies by fluorescent microscopy and found that 100% of the Alpha flies were GFP positive whereas 100% of the Beta flies analyzed were GFP negative (Fig. 3-5). The expression is very prominent in the Ocelli of the Alpha flies. Drosophila sp. does not have endogenous GFP so Alpha flies have the DHR96 mutation. The DHR96 mutation was further confirmed by PCR amplification of part of the mutated DHR96 region. As described earlier (p.22 and Fig. 2-1) that the DHR96 mutation was created by inserting a 7.5KB fragment containing PGF sequence, DHR96 gene sequence with two deletions in a wild type DHR96 sequence in reversed order. We have targeted the bigger deletion, referred to as Del2 in this text. This deletion is 331 bp long and removed the exon 4, downstream intron and the splice acceptor site for exon 5. The Alpha flies as expected gave a product of 183bp (lane2 in Fig. 3-6) and Beta without the deletion gave a product of 514bp (lane3 in Fig. 3-6) which is exactly 331 bp longer than that of Alpha. Hence Alpha’s DHR96 gene is mutated.

To investigate if DHR96 mutation affects the expression of the Cyp6a8 gene we analyzed the DHR96 mutant and the wild type flies by LUC reporter gene assay. We found that in more than 150 flies analyzed within age group 0-5 days, all of them showed 7-12 fold upregulation of Cyp6a8 promoter activity in mutant flies (Fig. 3-7). If DHR96 can influence the expression of Cyp6a8 gene on 2nd chromosome this could be an interesting finding which might unveil DHR96 as one of the factors on the 3rd chromosome that is involved in the Cyp6a8 (or may be other CYP) gene regulations. DHR96 mutation was previously shown to regulate the transcription of different CYP genes including Cyp6a2 and Cyp12d which are believed to be involved in resistance.
Figure 3-5: GFP expression in DHR96 mutant vs. wild type flies
A: Alpha flies showing GFP in their Ocelli
B: Beta flies showing no GFP expression
C, D: Closer look at the GFP expression in the compound eyes of Alpha flies

Figure 3-6: PCR products ran on 1.5% agarose gel along with 50bp DNA ladder
Extreme left, out side the gel, the 50bp ladder fragments were shown. The band sizes were given beside each band and the product name is given under respective lanes.
Figure 3-7: LUC activity in 0-5 days old adult female (y-axis) shown for 11 individual experiments (x-axis)

All the flies tested showed at least 7 fold higher Luc expression in the DHR96 mutant flies. Red bars indicate RLU values of the DHR96 wild type flies and the blue bars indicate the RLU of DHR96 mutants.
To understand if this higher expression is age dependent, we did an expression profile of the DHR96 mutant (strain Alpha) vs. DHR96 wild type (strain Beta) flies. We measured the expression Alpha and Beta flies in various developmental stages namely, 0-2 hours old embryo, female 3’rd instar larvae, 3 days old pupae, 4 days old adult female and 8 days old adult females. The ages of the flies were strictly maintained throughout all the experiments. We found in all developmental stages Alpha flies give at least 7 fold higher expression than that of Beta flies. The highest fold difference in expression was seen in the 3’rd instars larvae (Fig. 3-8), which was more than 15 fold as compared to the Beta flies of same age. Next in lines were the 4 days old adults who showed 12 fold and then 8 days old adults who gave 10 fold differences in constitutive LUC expression. All those flies except the 0-2 hours embryo were taken as females. The sex of the embryos was not known. We wanted to know if there were differences in expression between the two sexes and found that both Alpha and Beta males ~3 fold higher in LUC expression than their female counterpart. We also found that like females the Alpha males showed ~7 fold higher LUC expression than that of the Beta males (Fig. 3-9).

Our results are consistent with the fact that DHR96 mutation affects the expression of \textit{Cyp6a8} gene. It also suggests that this gene might have a negative regulatory control over \textit{Cyp6a8} expression. In wild type the \textit{Cyp6a8} expression is checked to the basal level but when the DHR96 gene was mutated the negative control was removed leading to \textit{Cyp6a8} overexpression.

Finally, to confirm if the DHR96 mutation was the reason behind \textit{Cyp6a8} upregulation, perform an ectopic gain of function cross where the third chromosome of Alpha (carrying the DHR96 mutation) was replaced by that of Beta (carrying wild type DHR96). The new strain was named as Alpha_Reverse. This strain was studied for its luciferase activity.
Figure 3-8: Fold difference in LUC expression in different developmental stages of Alpha vs. Beta
The third instar showed the highest difference and the lowest difference was found in 0-2 hours old embryos but they are still eight fold higher in Alpha flies.

Figure 3-9: Effect of DHR96 mutation on Cyp6a8 promoter activity (male vs. female)
Male flies showed nearly three fold higher LUC activity, however the Alpha males are still 7 fold higher than the Beta males in LUC expression.
We amplified the DHR96, Del2 region of the Alpha Reverse as described earlier (p.30) and as we Alpha_reverse gave the same product size as that of Beta (Fig. 3-6) which helped us to conclude that the third chromosome of Alpha was successfully substituted by that of the wild type Beta flies. So now the Alpha_reverse flies had all the background of Alpha flies except that the third chromosome now had an intact DHR96 gene. We did a LUC assay and found that Alpha_reverse gave similar RLU values as that of Beta, so functionally as far as luc expression is concern Alpha_reverse behaves same as Beta (Fig. 3-10).

When we analyzed the Alpha-Beta F1 heterozygotes we found that lowering the dose of DHR96 by half did decrease the Luc activity which further confirmed that DHR96 does have some regulatory effect on $Cyp6a8$ expression. Interestingly in the half dose of DHR96 in Alpha-Beta hybrids the Luc activity was also halved (Fig. 3-11) which indicates that the effect of DHR96 is dose dependent.

We also wanted to study if there is any induction control by DHR96 but we did not find any induction effect on the DHR96 mutant Alpha flies by PB and Caffeine. This finding complies with King-Jones et al. (2006). They did not find any effect of PB induction of $Cyp6a8$ in the DHR96 mutant flies. Alpha flies showed very little or no effect by caffeine, no effect by PB, and little effect with PB + Caffeine combined dose. The wild type Beta flies showed 2 times higher induction by Caffeine, ~9 fold higher induction by PB and little more than 2 fold higher induction by PB + Caffeine combined treatment. So it seems that the DHR96 might be playing a stimulatory role in induction by PB or Caffeine. When DHR96 is mutated it cannot stimulate the induction but this need to be studied further to conclude anything. However, it might be said that DHR96 mutation has no major effect on $Cyp6a8$ regulation by induction.
Constitutive LUC expression in DHR96 mutant, Wild type and gain of function flies

**Figure 3-10: The LUC activity of Alpha, Beta and Alpha_reverse**
The Alpha flies still gave ~8 fold higher than Beta LUC activity where no difference was found in LUC activity of Beta and Alpha_reverse.

Constitutive LUC expression in DHR96 mutant, Wild type and mutant-wild type F1 hybrid: Dosage effect

**Figure 3-11: Effect of DHR96 mutation is dose dependent**
Alpha showed more than 8 fold higher Luc expression than Beta and Alpha-Beta hybrid showed more than 3 times higher Luc expression than Beta. Alpha-Beta F1 showed 2.7 times lower Luc expression than that of Alpha.
4. Discussion:

The major goal in our study was to understand the regulation of a prototype CYP gene, the \textit{Cyp6a8} with the broader aim to understand the mechanism of resistance by cytochrome P450 monooxygenase system. Four of the P450 genes, including \textit{Cyp6g1}, \textit{Cyp6a2}, \textit{Cyp6a8} and \textit{Cyp12d1} are studied in greater detail for their possible involvement with xenobiotic metabolism, especially with DDT resistance (Waters et al., 1992; Burn et al., 1996; Maitra et al., 1996; Dombrowski et al., 1998; Brandt et al., 2002; Daborn et al., 2002; Le Goff et al., 2006, Festucci-Buselli et al., 2005) but not much is understood about the mechanism by which those genes might be controlled and if those CYP genes interact with each other to confer resistance. It is thought that the DDT resistance in \textit{Drosophila} might be multifactorial and some of the factors are involved in DDT metabolism directly whereas the others act as mere modifiers. Studies also indicate that the number of DDT resistant genes might not be large but all three chromosomes might be involved in rendering resistance through CYP gene regulation by possible \textit{trans}-regulatory control. We found the third chromosome of 91R resistant strain has stimulatory effect on \textit{Cy6a8} expression where as the first chromosome has the inhibitory control. The third chromosome from the wild type flies rosy flies showed negative effect on \textit{Cyp6a8} expression. We did not find any effect of the second chromosome in \textit{Cyp6a8} expression. However, the second and the first chromosome seem to interact to enhance the effect on induction in both PB and Barbital treated flies. The flies that are constitutively highly expressed showed low induction but the highest induction does not cross the highest constitutive expression. This can be explained by the fact that the strains which have high \textit{Cyp6a8} level do not respond with additional induction which further indicates that PB or Barb might induce \textit{Cyp6a8} through a common pathway and the regulatory role played by the first and the third chromosome is also
operated by that same pathway. When the 91R third chromosome is already results in \textit{Cyp6a8} upregulation PB or Barb has no effect on its expression. The 91R first chromosome which is inhibitory to the constitutive expression of \textit{Cyp6a8} shows stimulatory role in the induced expression. It is possible that the first chromosome might produce factors/repressors that reduce the \textit{Cyp6a8} expression and PB or Barb might bind with those factors and thereby allosterically modify their repressible ability or PB or Barb might bind to the binding site for those repressors resulting in upregulation of \textit{Cyp6a8} gene. Nevertheless, it is evident that \textit{Cyp6a8} gene regulation is complex and involves all three chromosomes and there might be various other unknown factors. We also found that DHR96 gene mutation has profound effect on this regulation. The effect of DHR96 in wild type flies seems inhibitory as the DHR96 mutant flies shows at least 8-14 fold higher \textit{Cyp6a8} expression. This effect of DHR96 is consistent with different developmental stages and in both the sexes of the fly. DHR96 seems to have repressible control over the \textit{Cyp6a8} expression. We tested the role of DHR96 on the -800 bp upstream promoter region of \textit{Cyp6a8} King-Jones et al. (2006) however, did not find any effect of DHR96 mutation on endogenous \textit{Cyp6a8} gene. This disparity can be explained by a model (Fig. 4-1A and B). According to this model DHR96 acts as a repressor of \textit{Cyp6a8} but there might another repressor other than the DHR96. The endogenous \textit{Cyp6a8} gene has binding site for both DHR96 and the other repressor (Repressor X in Fig. 4-1A) and they both bind to upstream binding site of \textit{Cyp6a8} and thereby downregulate the gene expression. The truncated upstream region of \textit{Cyp6a8} does not have the binding site for the other repressor (Fig. 4-1B). So even if the other repressor(s) is present in cannot bind to the upstream region and thus cannot inhibit the promoter activity of the -800bp upstream region but it does have the binding site for the DHR96 which enable DHR96 to influence the expression of the reporter.
This indicates that CYP gene regulation requires multiple factors involving interaction between all three major chromosomes and probably other genes present in different chromosomes. If this complex mechanism of CYP gene regulation can be elucidated then that knowledge can be used to design tactics to control resistance to insecticide. Another vital issue is not only insects but all living organism including plants, animals and human rely of CYP enzyme system essential biological activities such as cholesterol breakdown and synthesis, steroid hormone biosynthesis, drug metabolism, synthesis of various eicosanoids, plant hormones, flavonoids etc. Increasing our knowledge in CYP gene regulation in Drosophila as a model organism might give us insight to number of such important life processes.

**Figure 4-1: Possible mechanism of Cyp6a8 regulation by DHR96**

A. The endogenous Cyp6a8 sequence showing binding site for DHR96 and another unknown repressor.

B. The truncated upstream region of Cyp6a8 lacking the binding site for the other unknown repressor.
Reference:


Appendix:

**Synthesis of stock with genotype +c; Cy/Sco; DHR96 (PEGASUS)**

<table>
<thead>
<tr>
<th>Generation</th>
<th>Male genotype</th>
<th>Female genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>♀ +c; +c; DHR96</td>
<td>♂ w/Y; Cy/Sco; Sb/Tb</td>
</tr>
<tr>
<td>(DHR'96)</td>
<td></td>
<td>(DBL)</td>
</tr>
<tr>
<td>(Cross A)</td>
<td></td>
<td>(Cross B)</td>
</tr>
<tr>
<td>F1</td>
<td>♂ +c/Y; Cy/+c; DHR96/Tb</td>
<td>♀ DHR96</td>
</tr>
<tr>
<td>(Cy-Tb males)</td>
<td></td>
<td>(Sco-Tb males)</td>
</tr>
<tr>
<td>F2</td>
<td>♀ +c; Cy/+c; DHR96</td>
<td>♂ F2 +c/Y; Sco/+c; DHR96</td>
</tr>
<tr>
<td>(Cy female)</td>
<td></td>
<td>(Sco male)</td>
</tr>
<tr>
<td>F3</td>
<td>♀ +c; Cy/Sco; DHR96</td>
<td>♂ F3 +c; Cy/Sco; DHR96</td>
</tr>
<tr>
<td>(Cy-Sco female)</td>
<td></td>
<td>(Cy-Sco male)</td>
</tr>
</tbody>
</table>

**A-1: Synthesis of stock with second chromosome carrying Cy and Sco markers and third chromosome carrying DHR96 mutated sequence**

The +c; Cy/Sco; DHR96 (PEGASUS) stock was synthesized by replacing the second chromosome of DHR96 mutant flies with that of the double balancer carrying both Cy and Sco phenotypic markers.
A-2: Synthesis of stock with second chromosome carrying 0.8luc reporter transgene and third chromosome carrying Tb and Sb markers

This stock was derived from 0.8luc 110 H-ry flies. We have substituted the third chromosome of this strain with DBL third chromosome carrying Sb and Tb markers. Only male flies of this strain were used.
Synthesis of stock with genotype +^c; Cy/Sco; +^c (ECHO)

A-3: Synthesis of stock with second chromosome carrying Cy and Sco markers and third chromosome carrying wild type DHR96 gene

The +^c; Cy/Sco; +^c (ECHO) stock was synthesized by replacing the second chromosome of Canton S-Utah flies with that of the double balancer carrying both Cy and Sco phenotype.
A-4: Synthesis of working stock Alpha (DHR96 mutant) with the second chromosome carrying 0.8luc reporter transgene and the third chromosome carrying mutant DHR96 gene

The +c; 0.8; DHR961 (Alpha) stock was synthesized by crossing Pegasus female and Pan male. In the resulting F1 progeny the first chromosome was not selected from Pan hence the genotype of the first chromosome of Pan was not important. Alpha flies are DHR96 mutants carrying the 0.8luc reporter on the second chromosome.
A-5: Synthesis of working stock Beta (DHR96 wild type) with the second chromosome carrying 0.8luc reporter transgene and the third chromosome carrying wild type DHR96 gene. The +c; 0.8; +c stock was synthesized by crossing Echo female and Pan male. Similarly as done in case of Alpha here also in the resulting F1 progeny the first chromosome was not selected from the Pan flies. Beta strains are DHR96 wild type fly carrying the 0.8luc reporter on the second chromosome.
Generation of working stock Alpha_Revers (\( +^c; 0.8; +^c \))

A-6: Synthesis of working stock Alpha_Revers (\( +^c; 0.8; +^c \)) with the second chromosome carrying 0.8luc reporter transgene and the third chromosome carrying wild type DHR96 gene

The \( +^c; 0.8; +^c \) stock was synthesized by replacing the DHR96 mutant third chromosome of Alpha with that of Beta. This strain is originally DHR96 mutant Alpha strain whose DHR96 mutation was reversed by chromosome substitution.
A-7: Synthesis of stock Sol (R; Cy/Sco; R) with the second chromosome carrying Cy and Sco markers and first and third chromosome from 91R flies
The Sol (R; Cy/Sco; R) stock was synthesized by replacing the second chromosome of 91R with that of the double balancer stock carrying Cy and Sco markers.

A-8: Synthesis of stock Eos(R; R; Sb/Tb) with the second chromosome from 91R and third chromosome carrying Tb and Sb markers
The Eos(R; R; Sb/Tb) stock was synthesized by replacing the third chromosome of 91R with the third chromosome of the balancer stock carrying both Tb and Sb markers.
A-9: Synthesis of stock Iris (r; 0.8; Tb/Sb) with 0.8luc reporter gene on the second chromosome and Tb and Sb markers on the third chromosome

The Iris (r; 0.8; Tb/Sb) stock was synthesized by replacing the third chromosome of the 0.8luc 110 H-ry reporter strain with that of the double balancer carrying Tb and Sb markers.

A-9: Synthesis of stock Hestia (r; Cy/Sco; 0.8) with the third chromosome carrying 0.8luc reporter gene and the second chromosome carrying Cy and Sco

The Hestia (r; Cy/Sco; 0.8) stock was synthesized by replacing the second chromosome of the 0.8luc 14 reporter strain with that of the double balancer carrying Cy and Sco markers.
Synthesis of working strain#1 (R/Y; 0.8/Cy; R/Tb and R/Y; 0.8/Sco; R/Sb)

P1  ♀ R; Cy/Sco; RX  ♂ r/Y; 0.8; Tb/Sb  
     (Sol)               (Iris male)  

F1  ♂ R/Y; 0.8/Cy; R/Tb (Strain#1a)  
     ♂ R/Y; 0.8/Sco; R/Sb (Strain#1b)  

A-10: Synthesis of chromosome substitution strain#1 (R/Y; 0.8/Cy; R/Tb and R/Y; 0.8/Sco; R/Sb)  
The strain1 flies were synthesized by crossing Sol females with Iris males. The strain 1 are male flies  
with first chromosome having half dose of 91R chromosome, second chromosome with half dose of  
0.8luc reporter gene and third chromosome with half dose of 91R chromosome.

Synthesis of working strain#2 (r/Y; 0.8/Cy; r/Tb and r/Y; 0.8/Sco; r/Sb)

♀ r; 0.8; r  
     (0.8luc 110 H-ry)  X  ♂ DBL  

F1  ♂ r/Y; 0.8/Cy; r/Tb (Strain# 2a)  
     ♂ r/Y; 0.8/Sco; r/Sb (Strain# 2b)  

A-11: Synthesis of chromosome substitution strain#2 (r/Y; 0.8/Cy; r/Tb and r/Y; 0.8/Sco; r/Sb)  
The strain2 flies were synthesized by crossing 0.8luc 110 H-ry females with double balancer males.  
The strain 2 are male flies with first chromosome having half dose of ry506 chromosome, second  
chromosome with half dose of 0.8luc reporter gene and third chromosome with half dose of ry506  
chromosome.
Synthesis of working strain#3 (r/Y; 0.8/Cy; R/Tb and r/Y; 0.8/Sco; R/Sb)

P1 ♀ r; 0.8; Sb/Tb (Iris) X ♂ R/Y; Cy/Sco; R (Sol male)

F1 ♀ r/Y; 0.8/Cy; R/Tb (Strain# 3a)
♀ r/Y; 0.8/Sco; R/Sb (Strain# 3b)

A-12: Synthesis of chromosome substitution strain#3 (r/Y; 0.8/Cy; R/Tb and r/Y; 0.8/Sco; R/Sb)
The strain3 flies were synthesized by crossing Iris females with Sol males. The strain 3 are male flies with first chromosome having half dose of ry506 chromosome, second chromosome with half dose of 0.8luc reporter gene and third chromosome with half dose of 91R chromosome.

Synthesis of working strain#6 (R/Y; R/Cy; 0.8/Tb and R/Y; R/Sco; 0.8/Sb)

P1 ♀ R; R; Sb/Tb (Eos) X ♂ r/Y; Cy/Sco; 0.8 (Hestia male)

F1 ♀ R/Y; R/Cy; 0.8/Tb (Strain#6a)
♀ R/Y; R/Sco; 0.8/Sb (Strain#6b)

A-13: Synthesis of chromosome substitution strain#6 (R/Y; R/Cy; 0.8/Tb and R/Y; R/Sco; 0.8/Sb)
The strain6 flies were synthesized by crossing Eos females with Hestia males. The strain6 are male flies with first and second chromosome having half dose of 91R chromosome and third chromosome with half dose of 0.8luc reporter gene.
A-14: Synthesis of chromosome substitution strain#7 (r/Y; r/Cy; 0.8/Tb and r/Y; r/Sco; 0.8/Sb)
The strain7 flies were synthesized by crossing 0.8luc 14 females with double balancer males. The strain7 flies are male flies with the first and the second chromosomes having half dose of \( r_y^{506} \) chromosome and the third chromosome with half dose of 0.8luc reporter gene.

A-15: Synthesis of chromosome substitution strain#8 (r/Y; R/Cy; 0.8/Tb and r/Y; R/Sco; 0.8/Sb)
The strain8 flies were synthesized by crossing Hestia females with Eos males. The strain8 flies are male flies with first chromosome having half dose of \( r_y^{506} \) chromosome, the second chromosome with half dose of 91R chromosome and third chromosome with half dose of 0.8luc reporter gene.
### Synthesis of working strain#9 (R/Y; R/0.8; R/r)

<table>
<thead>
<tr>
<th>P1</th>
<th>♂ r/Y; 0.8; r (0.8luc 110 H-ry)</th>
<th>X</th>
<th>♀ RRR (91R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>♂ R/Y; R/0.8; R/r (Strain# 9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A-16: Synthesis of hybrid strain#9 (R/Y; R/0.8; R/r)**
The strain9 flies were synthesized by crossing 91R females with 0.8luc110 H-ry males. The strain9 are male flies with the first chromosome having half dose of 91R chromosome, the second chromosome with half dose of 0.8luc reporter gene and half dose of 91R chromosome and the third chromosome having half dose of 91R and ry506 chromosome.

### Synthesis of working strain#10 (R/Y; R/r; R/0.8)

<table>
<thead>
<tr>
<th>P1</th>
<th>♂ r/Y; r; 0.8 (0.8luc 14)</th>
<th>X</th>
<th>♀ RRR (91R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>♂ R/Y; R/r; R/0.8 (Strain#10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A-17: Synthesis of hybrid strain#10 (R/Y; R/r; R/0.8)**
The strain10 flies were synthesized by crossing 91R females with 0.8luc 14males. The strain 10 are male flies with the first chromosome having half dose of 91R chromosome, the second chromosome having half dose of 91R and ry506 chromosome and the third chromosome with half dose of 0.8luc reporter gene and half dose of 91R chromosome.
A-18: Synthesis of hybrid strain#13 (r/Y; r/0.8; r)
The strain13 flies were synthesized by crossing 0.8luc 110 H-ry females with ry^{506} males. The strain 13 are male flies with the first chromosome having half dose of ry^{506} chromosome, the second chromosome having half dose ry^{506} and 0.8luc reporter gene and the third chromosome with ry^{506} chromosome.

A-19: Synthesis of hybrid strain#14 (r/Y; r/0.8; r)
The strain14 flies were synthesized by crossing 0.8luc 14 females with ry^{506} males. The strain14 are male flies with the first chromosome having half dose of ry^{506} chromosome, the second chromosome having ry^{506} chromosome and the third chromosome with half dose of 0.8luc reporter gene and ry^{506} chromosome.
Vita

I was born in Calcutta, India to a family of soldiers as a mental misfit. I have lived (and hope to continue that process for some more time) my life with one principle that having a principle restricts the mind to develop newer principles and thus, it is best for a soul not to have one. I am an ordinary man with little good, too much bad and way too much of happiness.

I like genetics, computer games, literature, classical music etc. and it is more likely that if one would tell me of any other things not in the list I shall like that too. I did my preliminary education in a convent amidst the Himalayas. Whenever I think of those hoary mountains with no sign of life for miles together, it gives me a feeling above happiness or sorrow. I feel that it is my destiny to be with those mountains. A year in school might boast for its implications in the earning industry but I found the teachings of the first ray of sun the loftier.

After getting my masters in India I wanted to visit amazing places and meet wonderful people and so I decided to come to the land where David Hasselhoff is still walking. During my stay I met amazing people; people with freedom, even if some cannot stay without coke (coca cola I mean). It helped me to express my true self to me without any prejudiced shame.

I did a second masters form the University of Tennessee, Knoxville. I learnt a lot from this place. The Smokey Mountain and the Tennessee River is almost heavenly but the people who live here are the most beautiful of all. And those people made Knoxville my second home. I learnt to be honest, to be simple and to find pleasure in small things.

My masters' research was in gene regulation of fruit fly which compelled me to peruse my career in flying. Thus I moved to airlines. It would be the beginning of another journey. I might find my third home in the sky or may be not. But I'll find out.