To the Graduate Council:

I am submitting herewith a dissertation written by Susan M. Dombrowski entitled "Molecular Analysis of the Constitutive and Barbiturate-induced Expression of a *Drosophila melanogaster* Cytochrome P450 Gene, *Cyp6a2*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

Ranjan Ganguly

Ranjan Ganguly, Major Professor

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Associate Vice Chancellor and
Dean of The Graduate School

Molecular Analysis of the Constitutive and Barbiturate-Induced Expression of a *Drosophila melanogaster* Cytochrome P450 Gene, *Cyp6a2*

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

Susan M. Dombrowski
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DEDICATION

This dissertation is dedicated to the memory of my late parents,

Edward and Helen,

who made so many personal sacrifices so that I could pursue my educational goals.
May they rest in peace knowing that this long journey is finally over.

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ABSTRACT

Detoxification of endogenous compounds and environmental pollutants is mediated by cytochrome P450 monooxygenases (CYPs). In insects, CYPs may also play a role in the resistance to insecticides because the levels and enzymatic activities are generally higher in resistant strains than in susceptible ones. However, little is known about the regulation of resistance-associated insect *CYP* genes. Therefore, in the present investigation the *Cyp6a2* gene of *D. melanogaster* was used as a prototype to understand this phenomenon.

The 3'-untranslated region of the *Cyp6a2* gene of the underproducer 91C strain, but not the overproducer 91R strain, contains the long terminal repeat (LTR) of transposable element *17.6*. In the present study, the hypothesis that the LTR insertion influences the expression of *Cyp6a2* was tested. Quantitative RNA blot analysis of *Cyp6a2* expression in adults of different underproducer and overproducer strains showed that (i) the presence or absence of the LTR insertion does not correlate with the steady-state level of CYP6A2 mRNA; (ii) a constitutively low level of CYP6A2 mRNA is the wild-type phenotype and (iii) the level of CYP6A2 mRNA is higher in males than in females. Chromosome substitution experiments showed that the 2R-linked *Cyp6a2* gene is regulated by a locus (loci) on the third chromosome. Using P-element mediated germ line transformation, the *cis*-regulatory elements required for constitutive expression of *Cyp6a2* were localized between –985 and –129 relative to +1 of ATG. DNA sequence analysis of the –1331 to +1 region in an underproducer and overproducer strain showed that the upstream DNA sequences are identical except for six bases. This same region

also contains several interesting sequence motifs: an ecdysone response element, three binding sites for the ecdysone-inducible transcription factor, Broad Complex, and six "barbie boxes," *cis* elements that are found in the upstream DNA of barbiturate-inducible *CYPs*. Both barbital and phenobarbital induced the expression of *Cyp6a2*, but the pattern of induction with both compounds was different in underproducer and overproducer strains. Using germ line transformation, the sequences required for barbital-mediated induction were localized between –985 and –129. Collectively, these data shed new light on the molecular and genetic mechanisms that regulate insect *CYP* genes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

General Introduction

Introduction

Each day, all living organisms are confronted with innumerable environmental insults. Exposure to environmental pollutants, such as polycyclic hydrocarbons, pesticides, cigarette smoke and other noxious substances can threaten their very existence. Despite such environmental insults, living organisms survive and reproduce because they have a group of enzymes called cytochrome P450 monooxygenases (P450s or CYPs). P450s are microsomal, heme-thiolate proteins that are predominantly expressed in the liver of vertebrates and are integral components of a detoxification process that removes unwanted metabolites from an organism's intracellular environment. P450s are functionally diverse enzymes and play a major role in the oxidative metabolism of numerous endogenous compounds, including fatty acids, prostaglandins and steroids, as well as exogenous compounds that are foreign to an organism. Our present day understanding of the structure, function and diversity of P450s is based on more than 50 years worth of research contributions from scientific disciplines that are as diverse as the P450s themselves.

Cytochrome P450s: a historical review

In the early 1950's, the mechanism of steroid hormone biosynthesis from cholesterol and the biochemistry of lipid metabolism attracted the attention of many

researchers (see reviews by Lieberman and Dobriner, 1951; Samuels and Reich, 1952; Dorfman, 1957; Priess and Block, 1964). During this same time, Williams (1951) noted that various xenobiotic compounds could be converted from lipophilic forms to more water-soluble compounds that could be readily excreted from the body. Later, Axelrod (1955) and Brodie *et al.* (1958) demonstrated that the liver endoplasmic reticulum contained an enzyme system that was capable of catalyzing the oxidative metabolism of xenobiotic compounds. Soon thereafter, Hayaishi *et al.* (1955) and Mason *et al.* (1955) determined that dioxygenase and monooxygenase reactions were an integral process in liver microsomes. It was also observed that when certain chemical compounds were injected into laboratory animals they were metabolized into carcinogenic intermediates that promoted tumor formation (reviewed by Miller and Miller, 1959, 1976). While all of these studies demonstrated the existence of liver enzymes with diverse functional properties, their biochemical and molecular properties were not known.

During the course of an investigation on the oxidation-reduction kinetics of cytochrome b_5 in rat liver microsomes, Chance and Williams (1954) observed that when the NADPH-reduced suspension was gassed with carbon monoxide, a strong absorbance peak could be measured at 450 nm. This absorbance peak was enhanced further following the addition of the reducing agent sodium dithionite. This absorbance peak could not be attributed to cytochrome b_5 alone because its spectral profile did not change following the addition of carbon monoxide. This intriguing observation led Klingenberg (1958) to further define the carbon monoxide-binding properties of this unidentified microsomal protein with unique spectral properties. Later, Omura and Sato (1962, 1964a, 1964b)

determined that the carbon monoxide-binding pigment was a b-type cytochrome and they coined the term "P450" to define this hemoprotein.

The first enzymatic function that could be ascribed to P450s is credited to Estabrook *et al.* (1963) who demonstrated that the C-21 hydroxylation of 17α-hydroxyprogesterone in the microsomal fraction of the adrenal cortex was mediated by P450s. Later studies by Cooper *et al.* (1965) demonstrated that liver P450s also played a role in the demethylation and aromatic hydroxylation of drugs. It is now recognized that P450s exhibit broad and overlapping substrate specificities and are involved in more than 60 biochemical reactions including N- and S-oxidations, aliphatic oxidations, aromatic and aliphatic hydroxylations, epoxidations, dehalogenations and dealkylations (Nebert and Gonzalez, 1987; Lewis 1996). An estimated 800 compounds, and undoubtedly more, have been shown to serve as substrates for P450-mediated reactions (see reviews by Schenkman, 1993; Lewis, 1996). However, despite their functional diversity, the endogenous substrates for many P450s remain unknown.

The field of P450 research is vast and still in its infancy relative to other areas of scientific study. Between the early 1950's to late 1960's, P450 research focused on the identification and characterization of P450s. By the early 1970's, there were rapid advances in the isolation and purification of P450s from liver microsomes. Then, with the advent of recombinant DNA methodologies and molecular biological techniques in the late 1970's and 1980's, studies on P450s were soon directed toward the isolation and characterization of P450 genes from many different organisms. Today, the major emphasis is to identify the endogenous substrates of the many different P450s and to

understand how the genes that encode P450s are regulated constitutively and in response to various endogenous and exogenous inducing agents.

Structure, function and evolution of cytochrome P450 enzymes

CYPs are microsomal hemoproteins that contain between 400-500 amino acid residues and have molecular weights of 46- to 55-kD (Lewis, 1996). To date only three P450s have been analyzed by X-ray crystallographic studies. The results showed that P450s are composed of one α-helix and one β-sheet comprising approximately 70% and 22%, respectively, of the polypeptide (Poulos *et al.*, 1987; Ravichandran *et al.*, 1993; Hasemann *et al.*, 1995). Amino acid sequence comparison has shown that only three residues are absolutely conserved in all P450s analyzed. These are the cysteine in the heme binding region and a glutamic acid and an arginine located in the K helix of the polypeptide (Graham-Lorence and Peterson, 1996). The sequence analysis also showed that a sequence of 13 amino acids spanning the heme-binding region is highly conserved in the P450 superfamily. The consensus sequence of this conserved region is phe-(gly/ser)-x-gly-x-(his/arg)-x-cys-x-gly-x-(ile/leu/phe) (Graham-Lorence and Peterson, 1996).

Metabolic detoxification requires multiple enzymatic steps and is mediated by Phase I and Phase II enzymes. Phase I enzymes, which include P450s, use molecular oxygen and NADPH as the primary source of electrons to produce a hydroxylated substrate that can be further metabolized by Phase II enzymes. Phase II enzymes, which include UDP-glucuronosyltransferases, glutathione transferases, sulfotransferases and N-

acetyltransferases, catalyze conjugative and/or oxidative reactions that generate a water-soluble compound that can be excreted from the body (Guengerich, 1995). The general reaction catalyzed by Phase I enzymes, i.e. P450s, is summarized below.

$$RH + 0_2 \xrightarrow[CYP]{2e^-,\ 2H^+} ROH + H_20$$

In this reaction, RH is the substrate and ROH is the monooxygenated product. In this process, one atom of oxygen is incorporated into water, the other into the substrate. Two reducing equivalents that are required for this reaction are derived from NADPH and carried via an FAD-FMN-containing cytochrome P450 oxidoreductase (Lewis, 1996). It has been suggested that another microsomal carrier protein, cytochrome b_5, may also be used as a source of electrons (Backes, 1993). A schematic representation of the components of the P450 monooxygenase system in liver microsomes is shown in Figure 1.

The ultimate goal of Phase I and Phase II metabolism is to generate non-toxic, water-soluble compounds that can be excreted from the body. However, in some instances P450s can produce reactive biochemical intermediates that are toxic or carcinogenic and can alter normal cellular processes by binding with DNA. In mammals, it has been shown that polycyclic aromatic hydrocarbons are metabolized by CYP1A1 (also called aryl hydrocarbon hydroxylase or AHH) into cytotoxic and carcinogenic compounds (Conney, 1982). Interestingly, CYP1A1, which represents 1% of the total hepatic P450 complement in the mammalian liver is induced up to 50-fold by trace

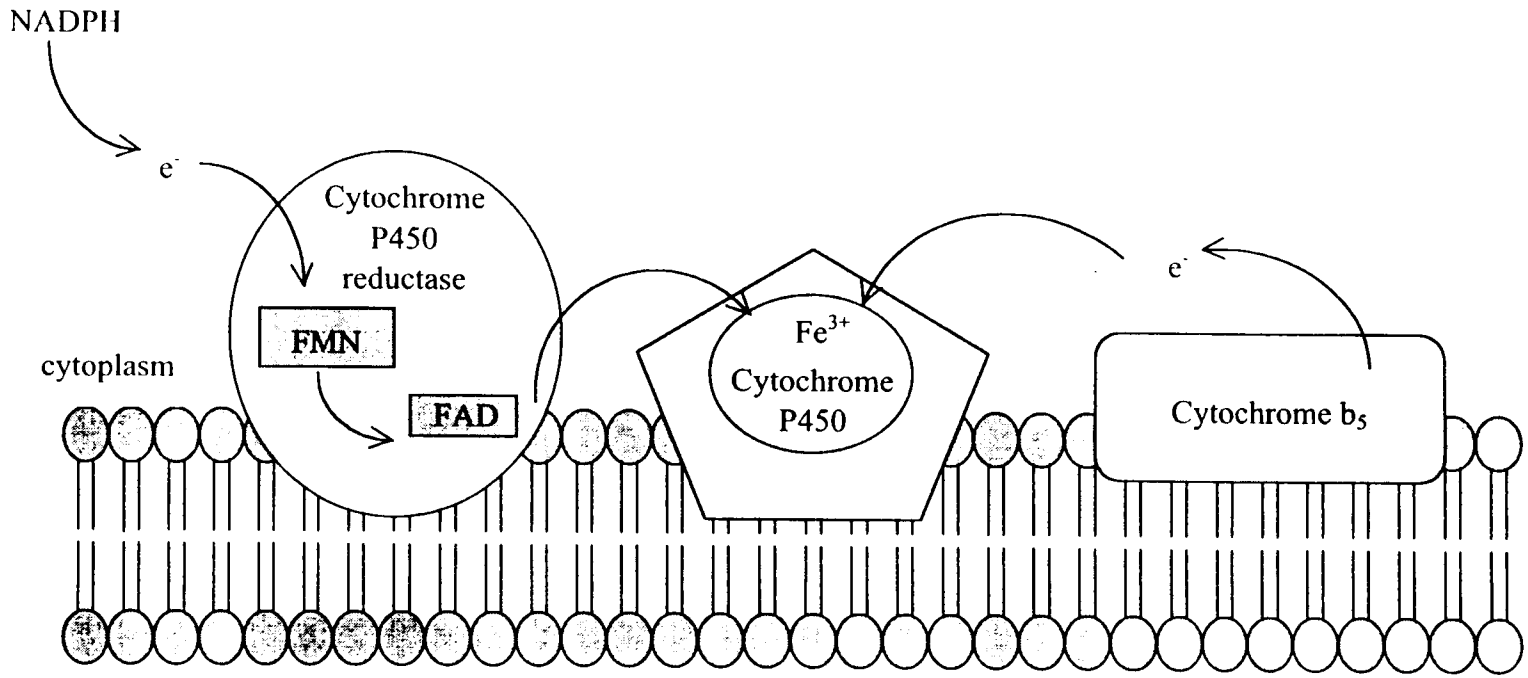

Figure 1: Schematic representation of the components of the cytochrome P450 monooxygenase system in liver microsomes. Before a cytochrome P450 can completely oxidize the substrate (Phase I metabolism), the iron atom in the cytochrome P450 enzyme must accept two reducing equivalents carried either by a microsomal flavin mononucleotide (FMN)-flavin adenine dinucleotide (FAD)-containing cytochrome P450 reductase and/or cytochrome b_5. Following the binding of the substrate to the P450, one reducing equivalent is used to reduce the ferric (Fe^{3+}) form of the enzyme to the ferrous (Fe^{2+}) form. This event is followed by the rapid binding of molecular oxygen to the reduced substrate-P450 complex. The addition of the second reducing equivalent and the subsequent loss of water yields a $Fe(O)^{3+}$ complex that can transfer its oxygen to the substrate. The oxidized substrate is released and the cycle repeats itself.

Adapted from Guengerich, F.P. 1993. *American Scientist* 81: 440-447.

amounts of genotoxic compounds that are also substrates of this enzymes, such as 3-methycholanthrene, TCDD and benzo[a]pyrene, a toxic compound that is found in cigarette smoke (Gonzalez, 1992; Ryan and Levin, 1993; Nebert *et al.*, 1996; Whitlock *et al.*, 1996). It is believed that differences in human responses to various kinds of procarcinogens, promutagens and toxic compounds may be related to heritable differences in various drug metabolizing enzymes including CYP1A1 (Pelkonen and Nebert, 1982; Nebert, 1991a,b,c).

CYPs represent an ancient and large superfamily, having evolved by divergent evolution from an ancestral P450 gene at least 1.4 billion years ago (Lewis, 1996 and references therein; Nelson *et al.*, 1996). Based on their amino acid sequence homologies CYPs have been grouped into families and subfamilies. CYPs that have < 40% amino acid sequence identity with one another are placed into different gene families (Nelson *et al.* 1993). Within a subfamily of a given gene family, CYPs generally share >40% identity with one another and, for a given species, there is >55% identity of the P450s within a given subfamily (Gonzalez *et al.*, 1993; Nelson *et al.*, 1993). In many genomes, each CYP subfamily appears to represent a cluster of tightly linked genes (see Nelson *et al.*, 1993 for review; Frolov *et al.*, 1994; Cohen *et al.*, 1995; Dunkov *et al.*, 1996; Maitra *et al.*, 1996). The total number of P450 genes and gene families that have been identified has grown tremendously since the first full-length P450 cDNA was reported in 1983 (Nebert and Gonzalez, 1987). In 1987, only ten P450 gene families were known (Nebert and Gonzalez, 1987). Between 1993 and 1996, the number of P450 genes and gene families more than doubled, increasing from 221 genes representing 36 gene families

(Nelson *et al.*, 1993) to 481 genes and 74 gene families (Nelson *et al.*, 1996). As of 1996, 14 families comprising 26 subfamilies have been reported in mammals of which 20 have been mapped in the human genome (Nelson *et al.*, 1996). There are currently more than 750 P450s that are partially or fully sequenced (Directory of P450-Containing Systems, October 1988).

Three *CYP* families have been identified in invertebrate systems: families 4, 6 and 9 (Snyder and Davidson, 1983; Gandhi *et al.*, 1992; Frolov *et al.*, 1994; Cohen *et al.*, 1995; Dunkov *et al.*, 1996; Maitra *et al.*, 1996). Two *CYP4* genes, *Cyp4d1* and *Cyp4e1*, have been fortuitously cloned during the course of unrelated investigations (Snyder and Davidson, 1983; Gandhi *et al.*, 1992). In insects, CYPs play important roles in the biosynthesis and metabolism of insect hormones and fatty acids, as well as provide protection against the toxic effects of plant allelochemicals (Frank and Fogelman, 1992; reviewed by Feyereisen, 1993; Prapaipong *et al.*, 1994; Hung *et al.*, 1996; Schuler, 1996). Of the three insect *CYP* gene families, the *CYP6* family is unique to insects. Interestingly, several members of the *CYP6* gene family are constitutively overexpressed in many insecticide resistant strains relative to susceptible ones, suggesting that these enzymes may be involved in metabolic resistance to insecticide and pesticide compounds (discussed below).

Mechanisms of insecticide resistance

The phenomenon of insecticide resistance is of extreme economic, medical and agricultural importance. Each year, billions of dollars of crops are lost due to the damage

caused by resistant insects. To overcome this problem, increasingly higher amounts of these toxic chemicals are sprayed on the agricultural fields. Despite such efforts to control insect populations, insects quickly become resistant to these chemicals and the battle goes on.

There are three general mechanisms that enable insects to become resistant to insecticides. These are decreased penetration of the insecticide, target site modification and increased metabolism of the insecticides (Oppenoorth, 1985; Russell *et al.*, 1990; Morton, 1993; Feyereisen, 1995). While substantial information is available regarding target site modification and metabolic resistance, little is known about decreased penetration as a mechanism of insecticide resistance. Decreased penetrance has been examined mostly in the house fly and a gene named *pen* on chromosome 3 has been implicated in decreased penetrance of insecticides (Plapp and Hoyer, 1968; Sawicki and Farnham, 1968). The rate of penetrance in *pen* mutants is generally 2-5 times lower than in wild-type strains, but it is also dependent on the type of insecticide compound (Sawicki and Lord, 1970).

In the case of target site modification, different components of the nervous system that interact with insecticides are altered. The target sites for most of these insecticides (e.g., organophosphates, DDT, cyclodienes, pyrethroids, etc.) are the GABA receptor, voltage-dependent sodium channels and the enzyme involved in the metabolism of the neurotransmitter acetylcholine, acetylcholinesterase (AchE or ACE)(Oppenoorth, 1985; Morton, 1993; Feyeriesen, 1995). It has been shown that organophosphates bind to the active site of AchE and prevent the enzyme from terminating the action of acetylcholine,

ultimately causing death to the insect. (Hutson and Roberts, 1985). Biochemical analysis has shown that in resistant houseflies and *Drosophila* AchE is insensitive to the organophosphate insecticides (see Hutson and Roberts, 1985 for review; Oppenoorth, 1985). Sequence comparison of the structural gene, *Ace*, in resistant and susceptible strains of *Drosophila* identified five point mutations in the *Ace* allele of the resistant strain (Mutero *et al.*, 1994). Presence of any one of these five mutations prevents the binding of AchE with organophosphates and the insect survives.

Resistance to cyclodiene compounds, such as dieldrin, is another example involving target site modification. The molecular basis for dieldrin resistance was found to be caused by a point mutation in the *Rdl* gene that codes for a $GABA_A$ receptor subunit (ffrench-Constant, 1994). Structurally, GABA receptors assemble as heteromultimers to form a chloride ion channel. DNA sequence analysis revealed a point mutation that changed alanine^{302} to serine in the second membrane-spanning region of the channel subunit (ffrench-Constant *et al.*, 1993). Analysis of resistant and susceptible strains from around the world revealed that the alanine to serine mutation was absent in 122 susceptible strains but present in 58 resistant strains (ffrench-Constant, 1994). Similar observations have been made in other dieldrin-resistant and –susceptible insects, including *Periplaneta americana* (cockroach), *Musca domestica* (house fly), *Tribolium castaneum* (flour beetle), *Hypothenemus hampei* (coffee berry borer) and *Bemisia tabaci* (whitefly). In two dieldrin-resistant insect species, *Aedes aegypti* (mosquito) and *Drosophila simulans* (fruit fly), the alanine^{302} residue is mutated to a glycine (ffrench-Constant, 1994).

Resistance to DDT and pyrethroids is also a result of target site modification. Flies that are susceptible are knocked down when they are exposed to these chemicals. In the 1950's, strains of house flythat did not show the knockdown phenotype were isolated and named *knock down resistant* or *kdr* (see Oppenoorth, 1985). Molecular and genetic analysis in *Drosophila* showed that the kdr phenotype maps to the *para* locus that encodes a Na^+-channel gene (Hall and Kasbekar, 1989). Other investigators showed that in the cockroach, tobacco budworm and house flythe *para* homolog is linked with the kdr phenotype in these species (see Feyereisen, 1995 for review).

The third mechanism of resistance, metabolic resistance, is mediated by the enzymes involved in the metabolism of insecticides. These enzymes, which include DDT-ase, glutathione S-transferase, oxidases, phosphatases and carboxylesterases, metabolize DDT and various types of organophosphate insecticides, such as malathion and paraoxon (Oppenoorth, 1985). In a malathion-resistant strain of mosquito, a point mutation in the gene encoding malathion carboxylesterase is thought to confer resistance to this insecticide compound (see Russell, 1990 for review; Whyard *et al.*, 1994, 1995).

In addition to the enzymes described above, CYPs have also been implicated in insecticide resistance. Clearly, an increase in the level and/or activity of these enzymes may render insects resistant to insecticides. While a gain-of-function mutation in the *cis*-acting element and /or the *trans*-acting factors controlling *CYP* gene expression, no such mutation has been reported for insect genes encoding enzymes involved in insecticide metabolism. However, gene amplification as a mechanism to increase enzyme level has been documented. It has been shown that in the malathion-resistant strain of the aphid,

Myzus percicae, a resistance-associated esterase gene (esterase E4) undergoes 64-fold amplification (Devonshire and Field, 1991). Amplification of esterase genes has also been observed in a number of insecticide-resistant strains of mosquitoes (see Feyereisen, 1995 for review).

CYPs and insecticide resistance

Based on the functional properties of CYPs it is reasonable to expect that these enzymes may also be involved in the metabolism of insecticides and pesticides. To test this hypothesis, resistant strains of house flywere treated with inhibitors of CYPs (e.g., piperonyl butoxide and other 1,3-dioxole compounds). The results showed that these compounds can render resistant strains susceptible to insecticide (Hodgson and Cassida, 1960; Sun and Johnson, 1960; Schonbrod *et al.*, 1973). Since these early observations, a large number of studies have been made in the house fly(*Musca domestica*), *Drosophila* and many other insects to find out if there is a correlation between the level of CYP enzymes and insecticide resistance. Numerous studies have shown that the amounts or activities of total and/or specific CYPs are greater in insecticide-resistant strains relative to their susceptible counterparts (Agosin, 1985; Oppenoorth, 1985; Sundseth *et al.*, 1990; Wheelock and Scott, 1990; Waters *et al.*, 1992a,b; Hodgson *et al.*, 1993; Feyereisen *et al.*, 1995). For example, the level of CYP6D1 was found to be higher in the pyrethroid-resistant LPR strain of house flythan in the susceptible strain (Wheelock and Scott, 1990; Scott and Lee, 1993). Similarly, the level of CYP6A1, another house flyCYP, is elevated in the diazinon resistant Rutgers strain of *Musca* but not in the susceptible aabys strain

(Cariño *et al.*, 1992). While these observations strongly suggest that CYPs may be involved in insecticide resistance, there is no direct evidence demonstrating the involvement of a specific CYP in conferring resistance to insecticide compounds. However, it has been shown *in vitro* and in a heterologous expression system that CYP6D1 can mediate the metabolism of pyrethroid and organophosphate compounds (Wheelock and Scott, 1992 a,b; Hatano and Scott, 1993). Another house flyCYP, CYP6A1, was expressed in *E.coli* and was found to metabolize the cyclodiene insecticides aldrin and hepatochlor (Anderson *et al.*, 1994). A *Drosophila* CYP, CYP6A2 (the subject of the studies described herein) was expressed in lepidopteran cells and was found to metabolize the cyclodiene insecticides aldrin and hepatochlor and the organophosphorus insecticide diazinon (Dunkov *et al.*, 1997). Although these observations strongly support the idea that these specific CYPs are involved in resistance, their *in vivo* roles in these two dipterans are not known.

Considerable amounts of work have also been done in *Drosophila* to examine whether there is a correlation between CYPs and insecticide resistance. Since CYPs show mixed function oxidase activity (MFO) and contain heme as a prosthetic group, Houpt *et al.* (1988) examined the MFO activity and amounts of heme-staining proteins (50- and 54-kDa) in malathion-resistant and susceptible strains of *Drosophila*. They found that both elevated levels of MFO activity and heme-staining protein content positively correlated with malathion resistance. Waters and his colleagues made a more in-depth analysis to examine the relationship between the activity of a specific CYP and insecticide resistance (see Waters *et al.*, 1992b for review). These investigators

examined the amounts of the 59.3- (P450A) and 55.8-kDa (P450B) heme-staining proteins and activity of P450-dependent dimethylnitrosamine demethylase (DMN-d) in the microsomes of various strains of *Drosophila*. It was observed that strains resistant to phenylurea had higher DMN-d activity and P450B content compared to the susceptible strains (Waters and Nix, 1988). Immunoblot analysis showed a positive correlation only between P450B content and insecticide resistance (Sundseth *et al.*, 1989). No such correlation was found with P450A (Sundseth *et al.*, 1989). While all of these studies show that the levels and activities of one or more CYPs are greater in resistant strains than in susceptible ones, the molecular and genetic basis of this upregulation is no clear.

CYP gene regulation in bacteria, mammals and housefly

Our current understanding about the molecular basis of *CYP* gene regulation is mainly based on what is known in bacteria and mammals. Fulco and his colleagues have shown that the $P450_{BM\text{-}1}$ and $P450_{BM\text{-}3}$ genes in *Bacillus megaterium* are controlled by genes encoding positive (BM1P1, BM1P2 and BM3P1) and negative (Bm3R1) *trans*-regulatory factors via a 15-17 base pair sequence known as a "barbie box" (Wen *et al.*, 1989; Fulco, 1991; He and Fulco, 1991; Shaw and Fulco, 1992, 1993; He *et al.*, 1995; Liang and Fulco, 1995; Liang *et al.*, 1995). The repressor molecule (Bm3R1) has been shown to bind to the barbie boxes and turn off the transcription of the $P450_{BM\text{-}1}$ and $P450_{BM\text{-}3}$ genes (Liang and Fulco, 1995). When pentobarbital is added to the medium, the genes encoding the above positive regulatory factors are induced (He *et al.*, 1995). This

event then inhibits the binding of the repressor protein to the barbie boxes and turns on $P450_{BM\text{-}1}$ and $P450_{BM\text{-}3}$ gene transcription (He *et al.*, 1995).

The best-studied *CYP* gene in mammals is the *CYP1A1* gene, which encodes aryl hydrocarbon hydroxylase (AHH)(Landers and Bunce, 1991; Nebert *et al.*, 1993, 1996; Swanson and Bradfield, 1993; Whitlock, 1993; Hankinson, 1995; Whitlock *et al.*, 1996). AHH is known to be involved in the bioactivation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and has been implicated in cancer (Nebert *et al.*, 1996; Whitlock *et al.*, 1996). *CYP1A1* is not usually expressed in the adult tissues, but it is induced several fold by polycyclic hydrocarbons and other agents. These ligands, which also serve as substrates for CYP1A1 bind to the cytosolic aromatic hydrocarbon receptor (AhR) which in turn interacts with a protein called Ah receptor nuclear translocator or Arnt (Hoffman *et al.*, 1991; Reyes *et al.*, 1992; see Whitlock, 1996 for review). The heteromeric complex of the AhR and Arnt then binds to several copies of *cis*-regulatory sequences, termed xenobiotic responsive element (XRE), in the 5' flanking DNA of the *CYP1A1* gene and induces the transcription of CYP1A1 (Denison *et al.*, 1988; Fisher *et al.*, 1990; Probst *et al.*, 1993; Denison and Whitlock, 1995).

The *CYP1A1* gene is also under the control of a negative regulatory mechanism which keeps the gene constitutively turned off (Hines *et al.*, 1988; Boucher *et al.*, 1993; 1995). In the human *CYP1A1* gene two negative regulatory elements (NREs) have been found, one of which contains a 21 base pair palindrome flanked by GC rich sequences (Boucher *et al.*, 1995). Specific binding of nuclear proteins to the palindrome has been

shown to cause suppression of transcription (Boucher *et al.*, 1995). Negative regulation via a *cis*-element has also been reported for the rat *Cyp1a1* gene (Imataka *et al.*, 1992).

The understanding that we have with regard to *CYP* gene regulation in the house flyis mainly based on studies of its *CYP6A1* and *CYP6D1* genes. *CYP6A1* was the first CYP6 cDNA to be cloned and sequenced from an insecticide-resistant strain (Rutgers) and two insecticide-susceptible strains (*aabys* and *sbo*) (Feyereisen *et al.*, 1989; Cohen *et al.*, 1994). The Rutgers strain, which has been reared under constant selection pressure with diazinon, also shows cross resistance to 2,2-bis(parachlorophenyl)-1,1,1-trichloroethane (DDT), organophosphorus and carbamate compounds (Forgash *et al.*, 1962). When the steady-state level of CYP6A1 mRNA was examined in different insecticide resistant- and -sensitive house fly strains, it was observed that the level of CYP6A1 mRNA level was highest in the resistant Rutgers strain. However, overexpression of *CYP6A1* could not always be correlated with an insecticide-resistant phenotype. In fact, the level of CYP6A1 mRNA in two resistant strains was found to be almost undetectable (Cariño *et al.*, 1992). This suggests that metabolic resistance to insecticide and pesticide compounds might involve multiple P450 enzymes. Southern and dot blot analysis of serially diluted genomic DNA from Rutgers and the insecticide-susceptible and underproducer *sbo* strain demonstrated that the constitutive overexpression of CYP6A1 in the Rutgers strain was not caused by amplification of the structural gene (Cariño *et al.*, 1994). Therefore, the most likely molecular mechanism leading to the constitutive overexpression would be due to an increased rate of transcription of the structural gene and/or increased stabilization of CYP6A1 mRNA. In

this context it must be mentioned that the *CYP6D1* gene of *M. domestica* is also expressed at higher levels in the pyrethroid-resistant LPR strain relative to its susceptible counterpart (Tomita *et al.*, 1995). Similarly, gene amplification has been ruled out to explain its constitutively high level of expression in the LPR strain (Tomita *et al.*, 1995).

To understand the genetic basis of *CYP6A1* and *CYP6D1* gene regulation in the housefly, several lines carrying chromosomes from resistant and susceptible strains were synthesized (Cariño *et al.*, 1994; Liu and Scott, 1996). In each line, the expression of the CYP6A1 and CYP6D1 genes was examined. The results of those studies suggested that a locus or loci on house fly chromosome 2 regulates the constitutive overexpression of the chromosome 5-linked *CYP6A1* gene (Cariño *et al.*, 1994; Cohen *et al.*, 1994). On the other hand, both chromosomes 2 and 1 influence the expression of chromosome 1-linked CYP6D1 gene (Liu and Scott, 1996).

The *Cyp6a2* gene of *D. melanogaster*: structure and expression

To further our understanding of *CYP* gene regulation in *Drosophila*, Waters *et al.* (1984b) identified two electrophoretically distinct subsets of P450 in *D. melanogaster*, termed P450A (~59-kDa) and P450B (~56-kDa). P450A was found in all strains examined while P450B was found only in those strains associated with resistance to insecticides. Monoclonal antibodies were prepared against a single form of P450 from each subsets (Sundseth *et al.*, 1989). The ubiquitous form from the P450A subset was named P450-A1 and the resistance-associated form from P450B was named P450-B1. The monoclonal antibodies were used to screen a cDNA expression library prepared from

the DDT-resistant 91R strain (Waters *et al.*, 1992a,b). Overlapping cDNA clones were isolated, restriction mapped and sequenced. On the basis of their deduced amino acid sequence, P450-A1 was named *Cyp4g1* and P450-B1 was named *Cyp6a2*. Using the 91R CYP6A2 cDNA as probe, *Cyp6a2* genomic clones were isolated from the genomic libraries of the 91R and the DDT-susceptible 91C strains (Waters *et al.*, 1992a,b).

Using *Cyp4g1* as a control, the expression of *Cyp6a2* was measured in 91C and 91R. cDNA probes specific for *Cyp4g1* and *Cyp6a2* were used on northern blots of total RNA isolated from adult flies (Waters *et al.*, 1992a,b). Densitometric analysis of the hybridization signals revealed that the steady-state level of CYP6A2 mRNA in the 91R strain was much greater than in the 91C strain (Waters *et al.*, 1992a,b). The amount of CYP4G1 mRNA was observed to be the same in the two strains. mRNA sizing, restriction mapping, Southern blot hybridization and DNA sequence analysis revealed two distinct structural differences of the *Cyp6a2* gene in the two strains (Figure 2). In 91R, but not in 91C, there is an additional 96 bp sequence that is located 560 bp downstream of the transcribed region of the *Cyp6a2* gene. This sequence is approximately 70% identical to a *D. melanogaster* genomic clone that contains the genes for U1b small nuclear RNA variants (Lo and Mount, 1990). However, it is not known if this 96 bp sequence in the *Cyp6a2* gene of the 91R strain is functionally significant. Waters *et al.* (1992a,b) also observed that the 3'-untranslated region (3'-UTR) of the *Cyp6a2* gene of the 91C strain, but not 91R, contained the 500 bp long terminal repeat (LTR) of transposable element *17.6* (Arkhipova *et al.*, 1995)(Figure 2). This LTR sequence contains three $A(T)_nA$ motifs, sequences that have been shown to promote rapid

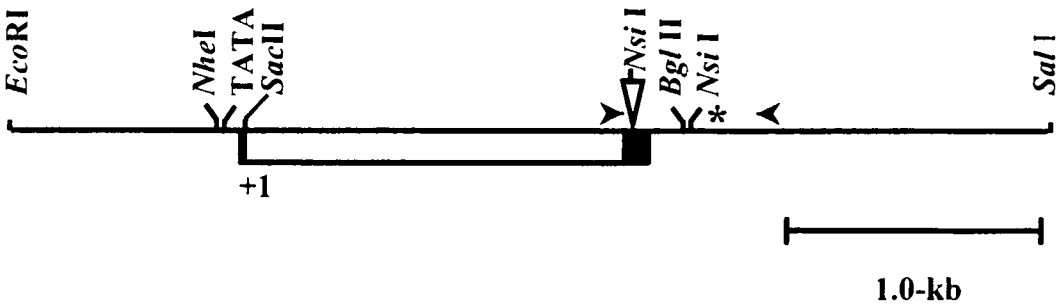

Figure 2: Organization of the *Cyp6a2* gene of *D. melanogaster*. The open rectangle is the coding region, where +1 represents the ATG start codon, and the solid boxes are the 5`- and 3`-untranslated regions. The location of the putative TATA box and pertinent restriction enzyme sites are shown. The inverted open triangle represents the long terminal repeat of transposable element *17.6* that is present in the 3`-untranslated region of the 91C strain. The "*" represents the 96-bp insertion that is present in 91R. Arrow heads denote the location of the forward and reverse primers used for PCR analysis of the genomic DNA in Chapter 2.

Adapted from Waters *et al.*, 1992a. *Proc. Natl. Acad. Sci. USA* 89: 4855-4859.

degradation of mRNA species (Shaw and Kamen, 1986; Sachs, 1993). There are also two putative polyadenylation signals located upstream of the $A(T)_nA$ sequences. Irrespective of whether the original poly(A) signal of the CYP6A2 transcript is used or if either poly(A) signal of the LTR is used during post-transcriptional processing, transcription of *Cyp6a2* in 91C could either produce a chimeric mRNA that may not be properly polyadenylated or a polyadenylated chimeric transcript that includes the $A(T)_nA$ sequences that are known to cause mRNA degradation.

Southern blot analysis of the genomic DNA from the two strains did not reveal any difference in the intensities of the hybridization signals. Thus, gene amplification as a mechanism to account for the overexpression of CYP6A2 mRNA and protein in the 91R strain was eliminated (Waters *et al.* 1992a,b). At the completion of these studies, it was hypothesized that the low level of CYP6A2 mRNA in the susceptible 91C strain may be caused by the presence of the LTR of transposable element *17.6* in the 3'-UTR (Waters *et al.*, 1992a,b). It was also postulated that the chimeric transcripts would be prone to rapid degradation because of the presence of the $A(T)_nA$ sequence (Waters *et al.*, 1992a,b). This hypothesis, however, has not yet been tested. Thus, it remains unknown whether other strains with or without the LTR insertion in the 3'-UTR of *Cyp6a2* also have a low or high level, respectively, of CYP6A2 mRNA.

Project goals and significance

The long-term objective of the proposed research is to elucidate the molecular and genetic mechanisms that regulate *CYP6* gene expression in invertebrate systems. Toward

this end, the studies described here focus on the *Cyp6a2* gene of *D. melanogaster* as a prototype for other *CYP6* genes that show a differential pattern of expression in insecticide-resistant and –susceptible insect strains. *D. melanogaster* has several advantages over *Musca* for the study of *CYP6* genes, including its well-defined genetics and the availability of a germ line transformation system (Wilson, 1988; ffrench-Constant *et al.*, 1992). The latter advantage permits *CYP6* gene expression to be studied *in vivo*, whereas examination of *Musca CYP* gene expression is limited to *in vitro* cell culture.

Since gene amplification of *CYP6* genes has not been observed in any insect species, the most probable molecular mechanism that regulates the constitutive overexpression of these genes in insecticide-resistant strains is at the level of transcription. Therefore, to better understand the regulation of *Cyp6a2* and other insect *CYP6* genes the following objectives have been proposed:

Objective 1A: Examine the constitutive expression of *Cyp6a2* in different strains of *D. melanogaster* and their hybrids.

Waters *et al.* (1992a,b) hypothesized that the presence of the LTR of transposable element *17.6* in the 3'-UTR of *Cyp6a2* makes the CYP6A2 mRNA unstable. This hypothesis was proposed after an examination of two *Drosophila* strains, the DDT-resistant 91R strain and its susceptible counterpart, 91C. Therefore, an examination of other strains with or without the LTR insertion is needed. This objective will determine if there is a positive correlation between the presence of the LTR insertion and a low level of CYP6A2 mRNA.

To examine the effect of genetic background on *Cyp6a2* and determine the genetic mechanisms that regulate the constitutive overexpression of *Cyp6a2, Cyp6a2* expression will be examined in the F1 hybrids of different strains that are overproducers or underproducers of *Cyp6a2*. In the housefly, chromosome substitution experiments have shown that its *CYP6A1* and *CYP6D1* genes are *trans*-regulated by a locus or loci on chromosome 2 (Cariño *et al.*, 1994; Cohen *et al.*, 1994; Feyereisen *et al.*, 1995; Tomita and Scott, 1995; Liu and Scott, 1996). Chromosome substitution analyses in different insecticide-resistant and susceptible strains of *D. melanogaster* have shown that the DMN-d activities of the P450B subset of proteins is regulated by a locus or loci on the third chromosome (Waters and Nix, 1988). Later, using monoclonal antibodies against one of these P450B proteins (P450-B1 or CYP6A2) Sundseth *et al.* (1989) demonstrated that the amounts of total P450B and the expression of CYP6A2 was influenced by loci on the second and third chromosomes. Experiments designed to look further into the effect of the third chromosome on the expression of the gene encoding CYP6A2 are presented in this dissertation.

Objective 1B: Examine *Cyp6a2* expression in male and female *D.melanogaster.*

Waters *et al.* (1983) have shown that the amount of total P450 is greater in males of the DDT-resistant 91R strain relative to females. However, an examination of DMN-d activities in the two sexes revealed that females have more DNM-d activity than males. To look into these phenomena further and examine the expression of a specific CYP,

CYP6A2 expression was examined in males and females of different strains of *D. melanogaster*.

Objective 2: Functional and sequence analysis of the upstream DNA of the *Cyp6a2* gene of an underproducer and overproducer strain of *D. melanogaster*.

P element-mediated germ line transformation will be used to identify the *cis*-regulatory sequences required for the expression of *Cyp6a2*. Since overexpression of *Cyp6a2* may be due to a mutation in the *cis*-regulatory elements of the gene, the promoter region of the *Cyp6a2* gene of the overproducer 91R strain will be sequenced and compared to the equivalent region of the *Cyp6a2* gene of the underproducer ry^{506} strain.

Objective 3: Examine the barbiturate-induced expression of *Cyp6a2* in different strains and map the *cis*-regulatory sequences required for barbiturate-mediated induction of *Cyp6a2*.

Barbiturate compounds are classical inducers of *CYP* gene expression (Conney, 1967). Dunkov *et al.* (1997) have shown that *Cyp6a2* is inducible with phenobarbital and that the *cis*-regulatory elements involved in this response are within 428 base pairs upstream of the ATG start codon. However, there is no information available regarding the effects of other barbiturate compounds on the expression of *Cyp6a2* in different strains. This objective will examine this phenomenon and used P element-mediated germ line transformation to map the *cis*-regulatory elements involved in barbital-mediated induction of *Cyp6a2*.

Chapter 2

Examination of the constitutive level of CYP6A2 mRNA in different strains of *D. melanogaster* and their hybrids

Introduction

In many insect species, the total cytochrome P450 content and/or the activity of specific P450 enzymes is generally higher in insecticide-resistant strains relative to their susceptible counterparts (Agosin, 1985; Oppenoorth, 1985; Sundseth *et al.*, 1989, 1990; Waters *et al.*, 1992a,b; Hodgson *et al.*, 1993; Feyereisen *et al.*, 1995). The genes that encode many of these resistance-associated enzymes belong to the *CYP6* gene family, which is unique to insects. Several *CYP6* genes are known to be overexpressed in insecticide-resistant strains of *M. domestica* and *D. melanogaster* (Cariño *et al.*, 1992; Waters *et al.*, 1992a,b; Tomita and Scott, 1995; Tomita *et al.*, 1995; Maitra *et al.*, 1996). As such, interest in the regulation of *CYP6* genes and their role in insecticide resistance have attracted the attention of many researchers within the P450 community.

Several molecular mechanisms can be proposed to explain the overexpression of *CYP* genes in insecticide-resistant strains: amplification of the structural gene, mutations and/or deletions in the *cis*-regulatory elements that regulate the expression of the *CYP* gene and mutations and/or deletions in the *trans*-acting protein(s) that interacts with the transcriptional control elements. Several studies have ruled out gene amplification to explain the constitutively high levels of mRNA encoded by the *CYP6A1*, *CYP6D1* and *Cyp6a2* genes of the house fly and *D. melanogaster* (Waters *et al.*, 1992a,b; Cariño *et al.*,

1994; Tomita *et al.*, 1995). Thus, other molecular mechanisms which are imposed at the transcriptional and/or post-transcriptional level must be in place to explain the overexpression of these resistance-associated *CYP6* genes.

While it is not completely understood why *CYP6* expression is lower in susceptible strains relative to resistant ones, efforts have been made to examine whether a specific chromosome(s) influences *CYP6* gene expression in *Musca*. It has been shown by RNA blot analysis that the expression of the *CYP6A1* and *CYP6D1* genes of this species are negatively regulated by a locus or loci located on chromosome 2 (Cariño *et al.*, 1994; Cohen *et al.*, 1994; Feyereisen *et al.*, 1995; Tomita and Scott, 1995; Liu and Scott, 1996). To date, similar studies have not been done on a specific *Cyp6* gene of *D. melanogaster*. However, it has been shown that chromosome 3 of this species has regulatory loci that influence the content and activity of P450 enzymes (Houpt *et al.*, 1988; Waters and Nix, 1988). In this context it should also be mentioned that chromosome 2 of *Musca* and chromosome 3 of *Drosophila* are thought to be homologous (Foster *et al.*, 1981). Thus, it is reasonable to assume that the above-mentioned *CYP6* genes of *Musca* and *Cyp6* genes of *Drosophila* may be regulated by similar molecular and genetic mechanisms. Studies designed to test this hypothesis are presented in this chapter.

Of the *D. melanogaster* P450s influenced by chromosome 3, Waters and Nix (1988) identified an electrophoretically defined band of proteins (~55-kDa) termed P450B. Later, using a monoclonal antibody against the P450B proteins, Waters *et al.* (1992a,b) isolated the gene that encodes one of these proteins and it was named *Cyp6a2*.

These investigators showed that the steady-state level of CYP6A2 mRNA is much higher in the DDT-resistant 91R strain than in the susceptible 91C strain. DNA sequence analysis showed that in 91C strain, but not 91R, the 3'-UTR of *Cyp6a2* contains a 0.5-kb DNA sequence which was identified as the LTR of transposable element *17.6* (Waters *et al.*, 1992a,b). Since the LTR DNA contains many $A(T)_nA$ sequences (Waters *et al.*, 1992a,b) and these sequences are known to cause mRNA degradation (Shaw and Kamen, 1986; Sachs, 1993). Waters *et al.* (1992a,b) proposed that these sequences may make the CYP6A2 mRNA unstable in 91C strain and prone to degradation. However, this hypothesis has not yet been tested. In fact, a comprehensive analysis of *Cyp6a2* expression in different strains, sexes and the hybrids of low and high producers of CYP6A2 mRNA has not been made. This chapter presents results of experiments that were undertaken to analyze these aspects of *Cyp6a2*.

Materials and Methods

D. melanogaster strains and culture conditions

The insecticide-resistant 91R and Hikone R-Bg strains and their susceptible counterparts, 91C and Oregon-R, respectively, were obtained from Dr. Larry C. Waters of Oak Ridge National Laboratories, Oak Ridge, TN. The insecticide-resistant MHIII-D23 and susceptible MHII-D5 strains were provided by Dr. R.A. Morton of McMaster University, Ontario, Canada. The MHIII-D23 strain is homozygous for the third chromosome from a malathion-resistant strain and the MHII-D5 strain is homozygous for the second chromosome from a malathion-resistant strain (R.A. Morton, personal

communication). Details of each strain and its origin have been described previously (Dapkus and Merrell, 1977; Singh and Morton, 1981; Waters *et al.*, 1984a,b; Morton and Holwerda, 1985; Halpern and Morton, 1987; Waters and Nix, 1988; Sundseth *et al.*, 1989; Waters *et al.*, 1992a,b). The insecticide-susceptible Canton-S and ry^{506} strains were obtained from Dr. John Lucchesi, Emory University, Atlanta, GA. The ry^{506} strain is described in Lindsley and Zimm (1992). Flies were reared at 23° C and 70% humidity on standard *Drosophila* medium (0.65% bactoagar, 5.5% cornmeal, 3% brewer's yeast, 5% (v/v) unsulfured molasses, 2% (v/v) light corn syrup, 0.25% (v/v) propionic acid, 0.3% ethanol and 0.1% Tegosept [p-hydroxy-benzoic acid, methyl ester].

Polymerase chain reaction

The original details of the polymerase chain reaction (PCR) have been described by Mullis *et al.* (1986). In the present study, the genomic DNA from different strains of *D. melanogaster* and the forward and reverse oligodexoynucleotide primers used to amplify the genomic DNA were from Dr. L.C. Waters. The two primers that were used for PCR amplification of *Cyp6a2* flank the point of insertion of the LTR insertional DNA (Figure 2). The sequence of the forward primer
(5' AGGAGCTCAGATCATCAGCCGGTT 3') corresponds to the sequence between 1663-1682 base pairs (bps) relative to +1 of ATG, and the sequence of the reverse primer (5' ATGCCCGTCTCGATGTCTGTCTG 3') is complementary to the sequence between 2583-2605 bps. See Genbank Accession Number M88009 for the sequence of *Cyp6a2* (Waters *et al.*, 1992a). The genomic DNA (500 ng) from each strain was amplified with

commercially available PCR reagents (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ). The PCR reaction mixture contained 1X PCR reaction buffer (10mM Tris-HCl, pH 8.3 and 50 mM KCl), 16mM $MgCl_2$, 200 μM each dNTP, 20 picomoles (0.5 μM) each primer and 2.5U AmpliTaq® DNA polymerase. The DNA was amplified for 32 cycles under the following conditions: 1 cycle at 95° C→2 minutes; 52° C→ 1 minute 30 seconds; 72° C-→3 minutes; 30 cycles at 94° C→1 minute 30 seconds; 52° C→1 minute 30 seconds; 72° C→3 minutes; 1 cycle at 72° C→5 minutes. The amplified DNA products were visualized on an ethidium bromide-stained 1% TBE (1X TBE is 89mM Tris, 89mM boric acid and 2mM EDTA) agarose gel.

Genetic crosses

To examine the effect of different genetic backgrounds on the constitutive level of CYP6A2 mRNA, crosses between overproducer (91R and MHIII-D23) and underproducer (ry^{506} and 91C) strains were carried out. Crosses between 91C and 91R, ry^{506} and 91R, and ry^{506} and MHIII-D23 strains were performed and the steady-state level of CYP6A2 mRNA was examined in the F1 hybrids.

The *Cyp6a2* gene of *D. melanogaster* is located on chromosome 2 (Maitra *et al.*, 1996). To examine the influence of the third chromosome on the expression of *Cyp6a2*, a *D. melanogaster* stock carrying the second chromosome of the overproducer 91R strain (2^R) and the third chromosome of the underproducer ry^{506} strain (3^{ry}), was synthesized. The base stock, $2^R/Cy$; $3^{ry}/3^{ry}$ was synthesized by Olé Raustol, an undergraduate student who worked under the supervision of Dr. Ranjan Ganguly. Flies with the $2^R/2^R$; $3^{ry}/3^{ry}$

genotype were collected from this base stock and outcrossed to the opposite sex of 91R strain ($2^R/2^R$; $3^R/3^R$) to generate flies with $2^R/2^R$; $3^R/3^{ry}$ chromosome composition. Poly(A)$^+$ RNA was isolated from $2^R/2^R$; $3^{ry}/3^{ry}$, $2^R/2^R$; $3^R/3^{ry}$, 91R ($2^R/2^R$; $3^R/3^R$) and ry^{506} ($2^{ry}/2^{ry}$ $3^{ry}/3^{ry}$) stocks, and the constitutive level of CYP6A2 mRNA in each stock was analyzed by northern blot hybridization.

Radiolabeling of cDNA probes

CYP6A2, Adh and RP49 cDNA fragments for RNA blot hybridization experiments were radiolabeled by random hexamer priming (Feinberg and Vogelstein, 1983) or nick translation using commercially available kits (Promega, Madison, WI and GIBCO BRL, Gaithersburg, MD, respectively) and according to the manufacturer's directions. The specific activity of the α^{32}P-dCTP (New England Nuclear, 3000 Ci/mmole) labeled probes ranged from 10^8-10^9 cpm/μg.

RNA isolation and blot hybridization

The steady-state level of CYP6A2 mRNA was examined by northern or slot blot hybridization following standard procedures (Maniatis, *et al.*, 1982; Ausubel *et al.*, 1994). Total RNA was isolated from the adult flies (unsexed or females only, fresh or frozen at –80° C) using the hot phenol method described by Jowett (1986). The poly(A)$^+$ fraction was isolated by oligo(dT)-cellulose chromatography as described by Maniatis *et al.* (1982).

For northern blot analysis poly(A)$^+$ RNA (1-5 μg) was denatured in 0.5X northern gel buffer [20mM MOPS (3-(N-Morpholino) propanesulfonic acid), 5mM sodium acetate, 0.5mM EDTA; pH 7.0], 6% formaldehyde and 50% formamide] at 68° C for 15 minutes. One-tenth volume of RNA loading dye (50% glycerol, 0.01M NaH_2PO_4, pH 7.0 and 0.4% bromophenol blue) was added and the denatured RNA was size-fractionated on a 2.2M formaldehyde-1.2% agarose gel. The RNA was transferred onto a nylon membrane (Hybond™-N, Amersham, Buckinghamshire, England) via capillary action using 20X SSC (3M NaCl and 300mM sodium citrate) as the transfer buffer. The blots were first UV-crosslinked (Stratalinker® UV Crosslinker Model 1800, Stratagene®, La Jolla, CA) and then baked for 2 hours in an 80° C oven. Hybridization of the CYP6A2 cDNA probe was carried out for 18-20 hours at 37° C in 50% formamide, 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrrolidine and 0.1% bovine serum albumin, Pentax Fraction V), 0.1M PIPES, pH 6.7, 0.1% SDS and 0.1 mg/ml sheared herring sperm DNA. After hybridization, blots were washed four times at room temperature with 2X SSC, 0.05% SDS and 0.05% sodium pyrophosphate then twice at 60° C with 0.2X SSC, 0.05% SDS and 0.05% sodium pyrophosphate. Hybridization signals were visualized by autoradiography and quantified with a scanning laser densitometer (LKB 2400 GelScan XL, LKB, Bromma, Sweden) or the Packard InstantImager Imaging System (Packard Instrument Company, Meriden, CT). To correct for differences in RNA loading, the same blot was hybridized with alcohol dehydrogenase (Adh)(Ganguly *et al.*, 1985; Krishnan *et al.*, 1991) or ribosomal protein 49 (RP49) cDNA probes (O'Connell and Rosbash, 1984).

For slot blot hybridization analysis, poly(A)$^+$ RNA (0.5 μg) was denatured in 3 volumes of denaturing solution (50% formamide, 6% formaldehyde, 20mM MOPS, 50mM sodium acetate and 1 mM EDTA) for 15 minutes at 68° C. Two volumes of ice-cold 20X SSC was added to each and the sample was spotted in triplicate under vacuum onto a nylon membrane immobilized on a Bio-Dot® SF Microfiltration Apparatus (Bio-Rad, Melville, NY). The sample chamber was rinsed twice with 1 ml of 10X SSC and the membrane was UV-crosslinked, baked and hybridized with a CYP6A2 cDNA probe as described for northern hybridization experiments. Hybridization signals were quantified with the Ambis Photoanalytical Imaging System (Ambis, Inc., San Diego, CA). To correct for loading error, the bound CYP6A2 cDNA probe was removed by incubating the membrane in stripping solution (50% formamide, 0.1% SDS and 0.1M PIPES, pH 6.7) at 68° C for 2 hours. The residual counts on the blots were quantified and then the blot was hybridized with the RP49 cDNA probe. These residual counts were then subtracted from the total counts obtained after hybridization with the RP49 cDNA probe.

Results and Discussion

Analysis of the 3'-UTR of *Cyp6a2* in different strains of *D. melanogaster*

Previous studies by Waters *et al.* (1992a,b) have shown that the steady-state level of CYP6A2 mRNA is greater in the DDT-resistant 91R strain relative to its susceptible counterpart, 91C. DNA sequence analysis of the gene in both strains revealed distinct structural differences within the 3'-UTR of *Cyp6a2* (Figure 2). In 91C, but not 91R, the 3'-UTR of *Cyp6a2* contains a 513 bp sequence that is the LTR of transposable element

17.6 (Waters *et al.*, 1992a,b; Arkhipova *et al.*, 1995). Sequence analysis showed that the LTR DNA contains multiple $A(T)_nA$ sequences (Waters *et al.*, 1992a,b) that are known to cause mRNA degradation (Shaw and Kamen, 1986; Sachs, 1993). This observation led Waters *et al.* (1992a,b) to hypothesize that the CYP6A2 mRNA in the 91C strain may be negatively influenced by the LTR insertion. In the studies described below, this hypothesis was tested by first determining if other strains of *D.melanogaster* contained this insertional DNA in the 3'-UTR of *Cyp6a2*. To do this, a PCR-based approach (Mullis *et al.*, 1986) that took advantage of the structural differences that exist within the 3'-UTR of *Cyp6a2* was employed. PCR of the genomic DNA with primers that flank the insertional DNA (see Figure 2) easily enables the two alleles to be distinguished from one another. Furthermore, this approach facilitates the screening of many different strains in a short period of time and eliminates the need to isolate, clone and characterize the *Cyp6a2* gene from each strain.

The 3'-UTR of *Cyp6a2* in 15 different strains of *D. melanogaster* was examined. As controls, the genomic DNA's of 91R and 91C were also amplified. The size of the resulting PCR product in 91R and 91C is approximately 947 and 1,368 bps, respectively. The results (Figure 3) showed that in 10/15 (67%) of the strains that were surveyed, the size of the amplified product was similar to that found in the 91R strain. Therefore, these strains do not carry any insertional DNA in the 3'-UTR of their *Cyp6a2* gene. Interestingly, in the Hikone AW and Hikone R-Swe strains the size of the amplified product was slightly smaller than that of the 91R control (Figures 3A and 3B), indicating a deletion of DNA sequences from within the 3'-UTR of *Cyp6a2*. On the other

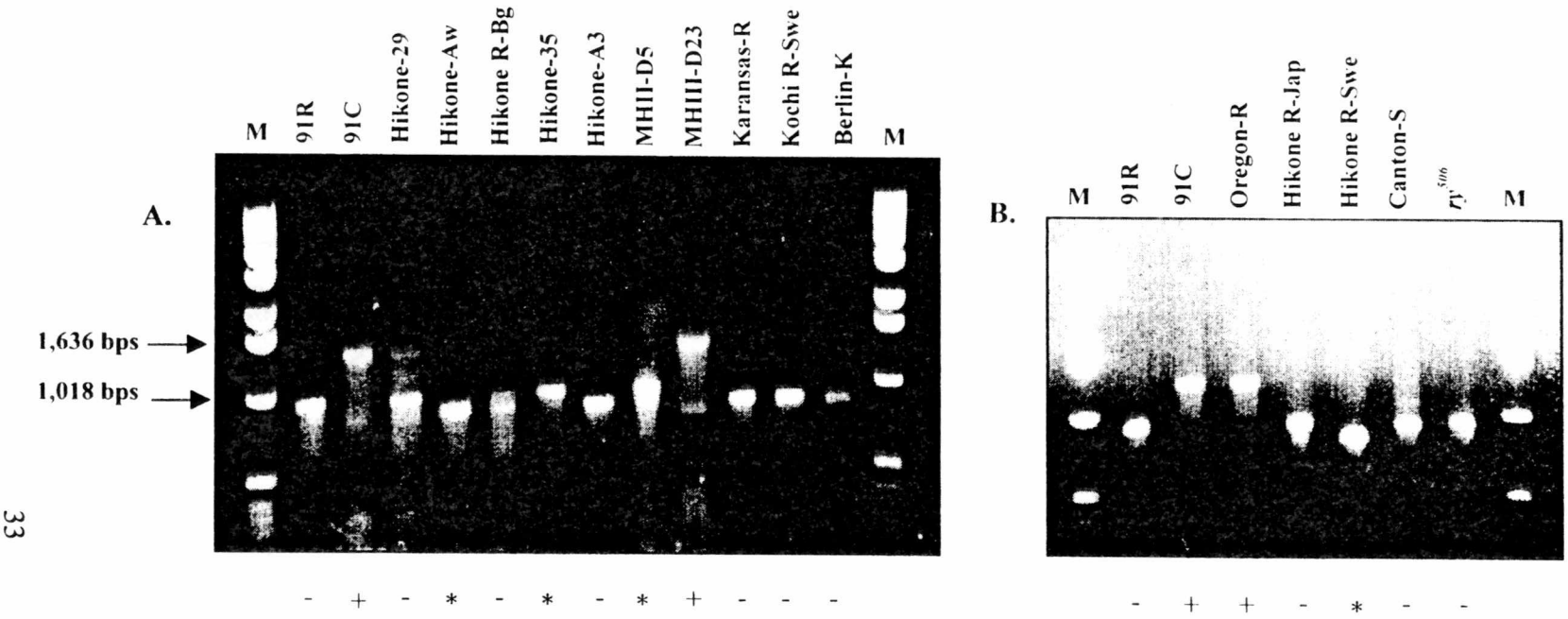

Figure 3: Polymerase chain reaction analysis of the 3'-untranslated region of *Cyp6a2* in different strains of *D. melanogaster*. (A, B). Ethidium-bromide stained 1% TBE-agarose gels. Genomic DNA from 15 different strains of *D. melanogaster* was amplified with primers that flank the point of insertion of the long terminal repeat of transposable element *17.6* (see Figure 2). The 1-kb molecular weight standard (M) was used as a reference and the pertinent sizes of the standard are indicated with arrows. In both panels, the size of the amplified DNA from each strain is compared to the 91R and 91C controls. A "+" or "-" below each lane denotes the presence or absence, respectively, of insertional DNA in the 3'-untranslated region. A "*" denotes an amplified product that is different from those of the 91R and 91C strains.

hand, the size of the amplified DNA in the Hikone 35 and MHII-D5 strains was slightly larger than that of the 91R control but less than 91C (Figure 3A). This suggests that in these two strains, the 3'-UTR of *Cyp6a2* contains extra DNA sequences. It is possible that the 0.5-kb LTR of transposable element *17.6* was present in the 3'-UTR of the *Cyp6a2* gene of these two strains, but a portion of the sequence was left behind when the insertional DNA was transposed to another part of the genome. Alternatively, the short extra sequence may be a part of a different mobile element. Sequence analysis of this region in the two strains would distinguish between these two possibilities.

In the MHIII-D23 and Oregon-R strains, the size of the amplified product appeared to be identical to 91C (Figure 3A and 3B), thus suggesting the presence of an insertional DNA in the 3'-UTR. DNA sequence analysis (Waters *et al.*, 1992a,b) showed that the *Cyp6a2* allele of 91C strain has two *Nsi*I sites. One is located in the LTR of transposable element *17.6* and the other is in the downstream DNA, approximately 180 bps downstream of the TAA stop codon. In between these two *Nsi*I sites there is a *Bgl*II site (Figure 2). Since the two primers used for PCR amplification flank all these sites, the amplified product is expected to have these sites. Therefore, restriction digestion of the amplified DNA with *Bgl*II and *Nsi*I can be used as a diagnostic test to determine if the type of insertional DNA in these two strains is identical to that which is present in the 91C strain. Restriction digestion of the amplified DNA of MHIII-D23 and Oregon-R produced DNA fragments that were identical to the fragments produced by the amplified product of 91C (data not shown). These data suggest that the 3'-UTR of *Cyp6a2* in

Oregon-R and MHIII-D23 contains an insertional DNA that appears to be similar to the LTR of transposable element *17.6*.

Examination of the steady-state level of CYP6A2 mRNA in *D. melanogaster* strains with or without insertional DNA in the 3'-UTR of *Cyp6a2*

The location of the LTR insertion in the 3'-UTR of *Cyp6a2* in the 91C strain moves the polyadenylation signal of the gene approximately 500 bases downstream (Waters *et al.*, 1992a,b). Furthermore, the LTR insertion contains two putative polyadenylation sites of its own. Irrespective of which poly(A) signal is used during the post-transcriptional processing of the CYP6A2 mRNA in this strain, it is possible that the LTR insertion may affect the polyadenylation status of the CYP6A2 transcript. One hypothesis proposed by Waters *et al.* (1992a,b) was that the low level of CYP6A2 mRNA and protein in the 91C strain may be a result of the synthesis of transcripts with a shortened or absent poly(A) tail, phenomena that are known to cause mRNA instability and degradation (see reviews by Bernstein and Ross, 1989; Beelman and Parker, 1995; Ross, 1996). If the presence of the LTR insertion in the 3'-UTR of the *Cyp6a2* gene of 91C shortens or prevents the addition of a poly(A) tail to the CYP6A2 transcript, then one would expect to see some CYP6A2 transcript in the poly(A)$^-$ fraction. To test this hypothesis, the level of CYP6A2 mRNA in the poly(A)$^+$ and poly(A)$^-$ RNA fractions of the 91R and 91C strains were examined by northern blot hybridization. No CYP6A2 mRNA was observed in the poly(A)$^-$ fraction (data not shown), suggesting that the low level of *Cyp6a2* gene expression in 91C was not due to sequestration of CYP6A2 mRNA in the poly(A)$^-$ fraction.

In an alternative hypothesis, Waters *et al.* (1992a,b) proposed that if the original poly(A) signal of the *Cyp6a2* allele or either poly(A) signal of the LTR sequence is used, then many of the $A(T)_nA$ sequences will be included in the chimeric RNA. They suggested that this may render the CYP6A2 mRNA unstable because $A(T)_nA$ sequences are known to cause mRNA degradation (Shaw and Kamen, 1986; Sachs, 1993). To test the hypothesis that this mobile element may influence the level of CYP6A2 mRNA, the steady-state level of CYP6A2 mRNA in seven different strains, with or without the insertional DNA, was examined by northern blot hybridization. To correct for error in RNA loading and normalize the data the same blot was simultaneously hybridized with cDNA probes for Adh and RP49.

Visual inspection of the autoradiogram (Figure 4) showed that the steady-state level of CYP6A2 mRNA varied between strains. The steady-state level of CYP6A2 mRNA in the four strains (ry^{506}, MHII-D5, Cantons S, Hikone R-Bg) that do not contain insertional DNA in the 3'-UTR, is less than the 91R control, which has a high level of CYP6A2 mRNA. The level of CYP6A2 mRNA in these strains ranged from barely detectable (e.g., ry^{506} and Hikone R-Bg) to moderate levels (MHII-D5 and Canton-S). This suggests that the absence of LTR insertional DNA does not always lead to a high steady-state level of CYP6A2 mRNA.

Analysis of the steady-state level of CYP6A2 mRNA in strains containing the LTR insertion also does not corroborate the idea that the LTR sequence may cause mRNA degradation. This is evident from the MHIII-D23 strain, which contains the insertional DNA. The steady-state level of CYP6A2 mRNA in this strain is the highest

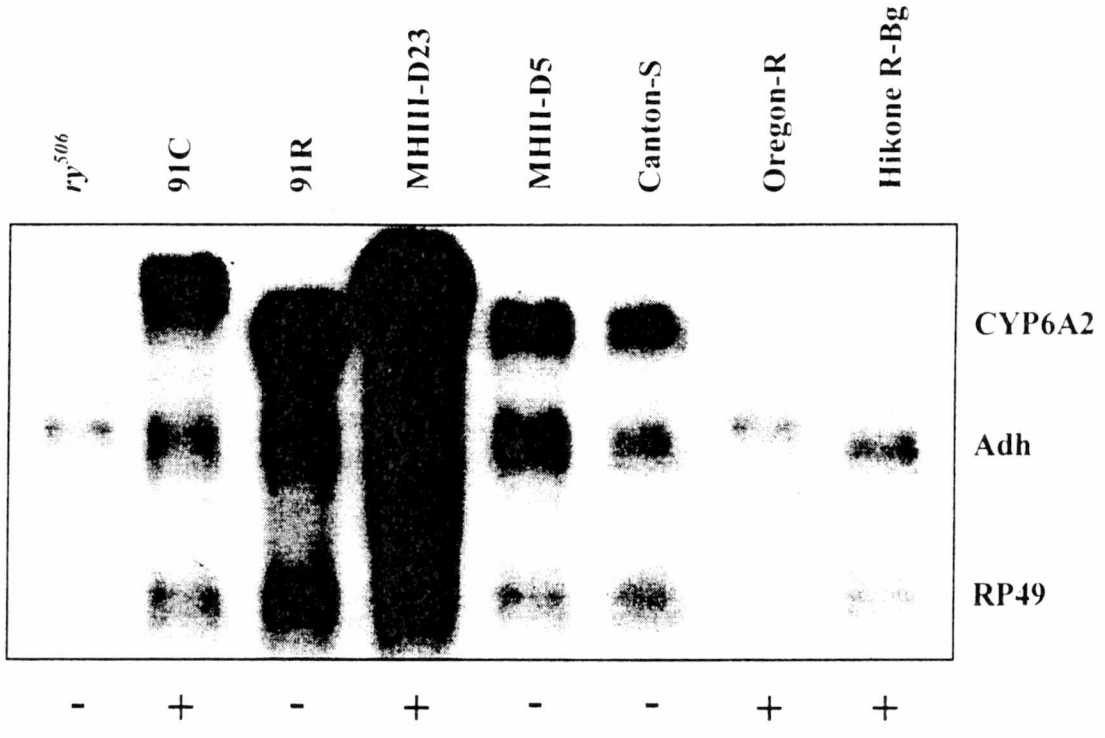

Figure 4: Northern blot analysis of the constitutive level of CYP6A2 mRNA in different strains of *D. melanogaster*. Poly(A)$^+$ RNA (5 µg) from a mixture (1:1) of adult males and females was size-fractionated on a 2.2M formaldehyde-1.2% agarose gel, blotted onto a nylon membrane and hybridized under stringent conditions (Ray and Ganguly, 1992) with a ^{32}P-labeled CYP6A2 cDNA probe. To check RNA loading, the blot was simultaneously hybridized with alcohol dehydrogenase (Adh) and ribosomal protein (RP49) cDNA probes. The presence (+) or absence (-) of the long terminal repeat of transposable element *17.6* in the 3'-untranslated region of *Cyp6a2* was determined by the polymerase chain reaction (see Figure 3) and the results are indicated below each lane.

of all the strains that were examined (Figure 4). It is to be noted that the ry^{506} and MHIII-D23 strains are comparable to 91R and 91C, respectively, with regard to the presence or absence of LTR insertion in the 3'-UTR of *Cyp6a2*. To better assess the relative difference in the level of CYP6A2 mRNA in these four strains, poly(A)$^+$ RNA from each strain was examined by slot blot hybridization. The data (Table 1 and Figure 5) show that there is 12.5-fold more CYP6A2 mRNA in the 91R strain than in ry^{506}, although both strains lack the LTR insertion. The MHIII-D23 and 91C strains, which carry the insertional DNA in the 3'-UTR of *Cyp6a2*, are also not comparable with respect to the level of CYP6A2 mRNA. The level of CYP6A2 mRNA in MHIII-D23 is 8.7-fold greater than in 91C. Collectively, these data do not support the hypothesis proposed by Waters *et al.* (1992a,b) and suggest that the LTR of transposable element *17.6* does not affect CYP6A2 mRNA stability. If it does make CYP6A2 mRNA unstable, it needs to be assumed that $A(T)_nA$-mediated mRNA degradation process works in 91C but not in the MHIII-D23 strain. Alternatively, the strain variation in the level of CYP6A2 mRNA may be a reflection of the mRNA turnover rate that is independent of the LTR insertion. Another possibility is the steady-state level of CYP6A2 mRNA in these strains may be influenced by other components involved in gene regulation, such as *cis*-regulatory elements and *trans*-regulatory factors.

Examination of *Cyp6a2* expression in male and female *D. melanogaster*

For practical purposes, most *CYP6* gene expression studies have been limited to females because the yield of total RNA or protein per fly is much greater than in males. Interestingly, in the mouse, rat and human, the levels of CYP enzymes involved in steroid

Table 1: Quantification of the constitutive level of CYP6A2 mRNA in different strains of *D. melanogaster*

Strain	Sample	Counts per minute*		CYP6A2/RP49	Mean ± S.D.
		CYP6A2	RP49		
ry^{506}	1	36	939	0.04	0.04 ± 0.01
	2	29	974	0.03	
	3	35	847	0.04	
91-C	1	111	564	0.20	0.21 ± 0.02
	2	98	513	0.19	
	3	89	384	0.23	
91-R	1	275	478	0.58	0.50 ± 0.06
	2	294	623	0.47	
	3	286	627	0.46	
MHIII-D23	1	880	486	1.81	1.82 ± 0.01
	2	887	485	1.83	
	3	474	261	1.82	

Poly(A)$^+$ RNA (0.5µg) from each strain was analyzed by slot blot hybridization with CYP6A2 and RP49 cDNA as probes. See Materials and Methods for further details.

* Counts per minute were determined by scanning the slot blots with a radioanalytical imager (Ambis).

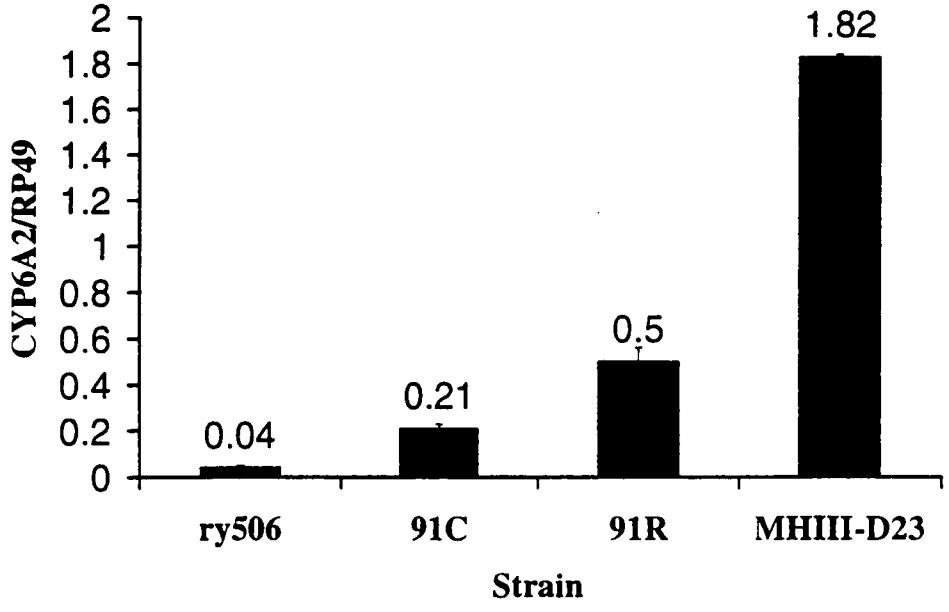

Figure 5: Quantification of the constitutive level of CYP6A2 mRNA in different strains of *D. melanogaster*. Poly(A)$^+$ RNA (0.5 μg) from four different strains of *D. melanogaster* was examined by slot blot hybridization with CYP6A2 and RP49 cDNA probes. See Materials and Methods for details. The hybridization signals were quantified with the Ambis Photoanalytical Imaging system and the graphical representation of the data presented in Table 1 is shown above.

hormone metabolism show sexual dimorphism and are either expressed in a gender-dominant or gender-specific manner (Lewis *et al.*, 1996 and references therein). For example, in the rat kidney, CYP2A4 catalyzes the 15α-hydroxylation of testosterone and is 6.2-fold more abundant in males than in females (Squires and Negishi, 1988). On the other hand, CYP2A5, which catalyzes the 7-hydroxylation of coumarin, is 6.6-fold more abundant in the kidneys of the female rat than in males (Squires and Negishi, 1988). A member of the *CYP3* family, CYP3A2, catalyzes the 6B-hydroxylation of testosterone and is male specific (Schenkman and Griem, 1993) while the 15B-hydroxylation of testosterone is catalyzed by the female-specific CYP2C12 enzyme (Schenkman and Griem, 1993). Thus, since there is precedence for sexual dimorphism of *CYP* gene expression in the rat, the possibility that it may exist in *D. melanogaster* and other invertebrates cannot be ignored. Both Saner *et al.* (1996) and Brun *et al.* (1996) examined the tissue-specific expression of *Cyp6a2* and reported that the highest level of expression was in the fat bodies. Thus, since females have more fat body tissue than males we hypothesized that the expression of *Cyp6a2* would be greater in females than in males. To investigate this further, northern blot hybridization was used to examine the steady-state level of CYP6A2 mRNA in male and female *D. melanogaster* strains ry^{506}, 91C and 91R. The results (Figure 6) showed that in all three strains CYP6A2 mRNA was more abundant in males than in females and that the relative difference between the two sexes was not the same in the three strains. In the ry^{506} strain, CYP6A2 mRNA was undetectable even with extended autoradiographic exposure time. However, radioanalytical imaging analysis of the blots (Figure 6B) indicated that ry^{506} males had

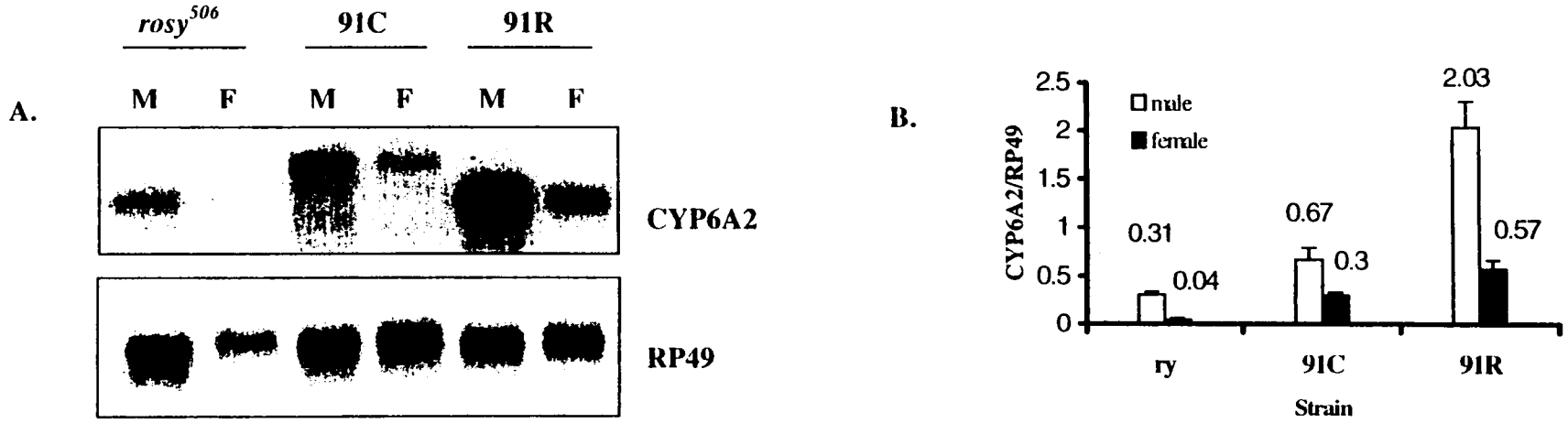

Figure 6: Constitutive level of CYP6A2 mRNA in males and females of different strains of *D. melanogaster*. (A) Representative northern blot showing the level of CYP6A2 mRNA in the two sexes. Poly(A)$^+$ RNA (1 μg) from adult males (M) and females (F) was electrophoresed on a 2.2M formaldehyde-1.2% agarose gel, transferred onto nylon and hybridized with CYP6A2 and RP49 cDNA probes as described in Materials and Methods. **(B)** Quantification of the level of CYP6A2 mRNA in males and females. Hybridization signals on representative northern blots were quantified with the Packard Radioanalytical Imaging system and the mean level of CYP6A2 mRNA in each strain was derived from three independent experiments. The RP49 signal in each lane was used to correct for the loading error and normalize the data.

7.8-fold mRNA than their female counterparts. In the 91C and 91R strains, males had 2.2 and 3.6 fold more CYP6A2 mRNA, respectively, relative to females. Waters *et al.* (1983) examined the amounts of total P450 in 91R males and females and found that males had more total P450 than females (0.42 and 0.28, respectively). However, females had more DMN-d activity than males (0.80 and 0.59, respectively). While the data of Waters *et al.* (1983) and the data presented in this section show that the levels of total P450, DMN-d activity and CYP6A2 mRNA are not the same in the two sexes, its biological significance remains unknown at this time.

Examination of the steady-state level of CYP6A2 mRNA in the F1 hybrids of low and high producer strains of CYP6A2

The results presented in Section 2 suggest that CYP6A2 mRNA stability is not influenced by the insertion of the LTR in the 3'-UTR of *Cyp6a2*. The observation that the level of CYP6A2 mRNA in 6 out of the 8 strains that were examined was less than 91R and MHIII-D23 suggests that a constitutively low level of CYP6A2 mRNA is the wild-type phenotype of the gene. Since the LTR insertion does not appear to be the molecular mechanism that regulates the level of CYP6A2 in overproducer and underproducer strains, a mechanism that imposes control at the level of the transcription of *Cyp6a2* is favored. Since transcriptional control requires the interaction of *trans*-regulatory proteins with the *cis*-acting elements of the gene, changes in the constitutive level of an mRNA may result from a mutation in the *cis*-elements of *Cyp6a2* and/or the regulatory gene. It is possible that there may be strain variation with respect to the *Cyp6a2* gene regulatory machinery. To test this hypothesis, northern hybridization was used to examine the level

of CYP6A2 mRNA in the F1 hybrids derived from crosses between overproducer strains (91R and MHIII-D23) and underproducer strains (91C and ry^{506}).

Since the 3'-UTR of the *Cyp6a2* gene of 91C (*Cyp6a2-91C*) contains the LTR of transposable element *17.6*, the size of the mRNA encoded by *Cyp6a2-91C* is approximately 0.5-kb larger than that transcribed in 91R (*Cyp6a2-91R*). This structural polymorphism makes it easy to distinguish the source of the *Cyp6a2* allele and examine their individual expression in the F1 hybrids derived from these two parental strains. Visual inspection of the autoradiogram (Figure 7) revealed that the expression of each *Cyp6a2* allele in the 91R x 91C hybrid (mixed sex) was approximately equal. Laser densitometric analysis of the hybridization signals (Table 2) from two independent experiments showed that the expression of the *Cyp6a2-91R* allele (R) in the hybrid was 1.8 – to 2.1-fold lower than that of the R allele in the 91R parental strain. On the other hand, the expression of *Cyp6a2-91C* allele (C) was nearly identical to that in the 91C parent.

The results of the above experiments prompted us to examine the steady-state levels of CYP6A2 mRNA in the hybrids of other overproducer and underproducer strains, including 91R and ry^{506} and MHIII-D23 and ry^{506}. In these experiments, RNA was isolated only from the F1 hybrid females. Figure 8 shows that in the 91R X ry^{506} hybrid, the intensity of the hybridization signals from the CYP6A2 mRNA is much lower than that observed in the case of the parental 91R strain. Radioanalytical analysis of the northern blot (Table 3) showed that the total amounts of CYP6A2 mRNA in the hybrid and parental 91R strain are 0.23 and 0.55, respectively. Because the size of the mRNA

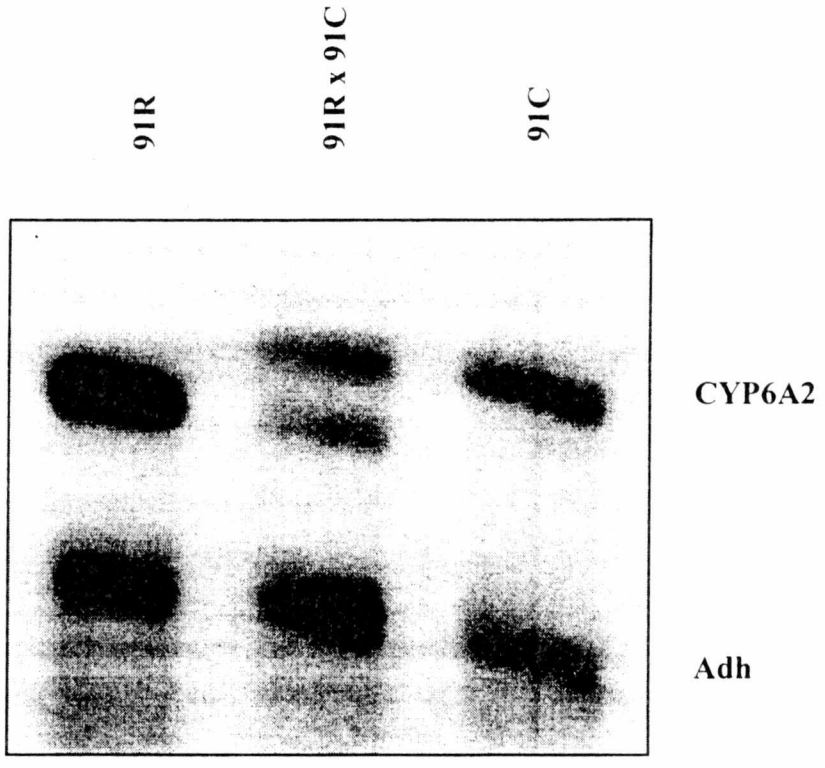

Figure 7: Northern blot analysis of CYP6A2 mRNA in the F1 hybrids of the 91R and 91C strains of *D. melanogaster*. Poly(A)$^+$ RNA (5 μg) from a 1:1 mixture of adult males and females was electrophoresed on a 2.2M formaldehyde-1.2% agarose gel, transferred onto a nylon membrane and hybridized with the CYP6A2 and Adh cDNA probes as described in Materials and Methods. Hybridization signals on the autoradiogram were scanned with an LKB scanning laser densitometer and the data from two independent experiments are presented in Table 2.

Table 2: Quantification of the steady-state level of CYP6A2 mRNA in the 91R x 91C hybrid

Strain	*Cyp6a2* Allele	Signal Density *		CYP6A2/Adh	*Cyp6a2* expression per gene dose
		CYP6A2	Adh		
91R	R	1.05	0.26	4.11	2.06
91R x 91C	C	1.32	1.53	0.86	0.86
	R	1.50	1.53	0.98	0.98
91C	C	1.64	0.97	1.69	0.85
91R	R	0.86	0.41	2.08	1.04
91R x 91C	C	0.15	0.41	0.35	0.35
	R	0.24	0.41	0.59	0.59
91C	C	0.33	0.47	0.70	0.35

* Signal density was determined by scanning the autoradiograms with a laser densitometer.

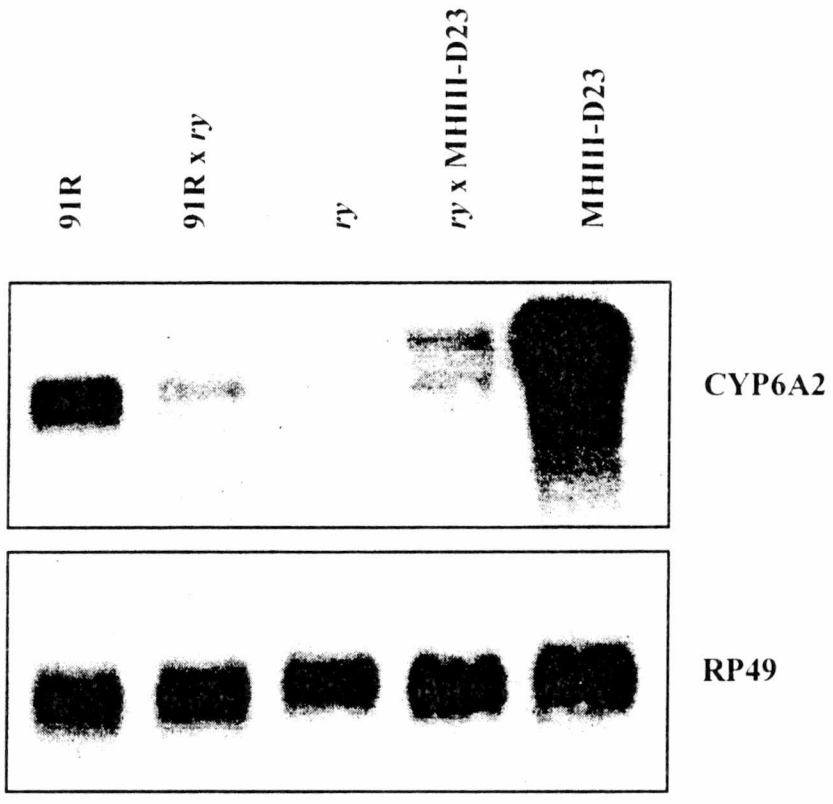

Figure 8: Northern blot analysis of the level of CYP6A2 mRNA in the F1 hybrids of different underproducer and overproducer strains of *D. melanogaster*. Poly(A)$^+$ RNA (3 μg) from adult females was electrophoresed on a 2.2M formaldehyde-1.2% agarose gel, transferred onto a nylon membrane and hybridized with CYP6A2 and RP49 cDNA probes as described in Materials and Methods. Hybridization signals from northern hybridization experiments were quantified with a radioanalytical imager (Packard) and the mean CYP6A2 mRNA level, derived from two independent experiments, is presented in Table 3.

Table 3: Quantification of the steady-state level of CYP6A2 mRNA in different overproducer and underproducer strains of *D. melanogaster* and their F1 hybrids

Strain	Cyp6a2 Allele	Experiment	Counts* CYP6A2	Counts* RP49	CYP6A2/RP49	Mean ± S.D.	CYP6A2 mRNA per gene dose
91R	R	1	2005	3265	0.61	0.55 ± 0.09	0.28
		2	2341	4836	0.48		
91R x *ry*	R + *ry* #	1	866	3524	0.25	0.23 ± 0.02	0.12
		2	1101	5193	0.21		
ry	ry	1	275	3214	0.09	0.06 ± 0.04	0.03
		2	160	4635	0.03		
ry x MHIII-D23	ry	1	844	4225	0.20	0.20 ± 0.0	0.20
		2	1078	5248	0.21		
	MHIII-D23	1	993	4225	0.24	0.19 ± 0.06	0.19
		2	775	5248	0.15		
MHIII-D23	MHIII-D23	1	8775	4590	1.91	2.37 ± 0.65	1.19
		2	15986	5636	2.84		

*Counts were determined by scanning the blots with a radioanalytical imager (Packard).

In this hybrid the size of the mRNA encoded by the *Cyp6a2-91R* and *Cyp6a2-ry* alleles is the same and cannot be distinguished from one another.

encoded by the *Cyp6a2* allele of the ry^{506} strain (*Cyp6a2-ry*) is identical to that of *Cyp6a2-91R*, it is not possible to ascertain whether the hybridizing band (Figure 8) in the hybrid contains RNA from both alleles or just one of the two. Nevertheless, the data show that per gene dose there is a two-fold inhibition of the *Cyp6a2* gene in the hybrid of overproducer 91R and underproducer ry^{506} strain (Table 2). If one assumes that both alleles are equally expressed in the hybrid, then one has to conclude that in the hybrid genome the *Cyp6a2-ry* allele is upregulated 4-fold because *Cyp6a2* activity per gene dose is 0.12 and 0.03 in the hybrid and parental ry^{506} strain, respectively (Table 2). On the other hand, it could also mean that the *Cyp6a2-91R* allele is downregulated 2.4-fold in the hybrid genome because *Cyp6a2* activity per gene dose is 0.12 and 0.28 in the hybrid and 91R strain, respectively. Whatever may be the case, the results demonstrate that the total activity of the *Cyp6a2* gene is downregulated in the hybrid genome and this pattern is very similar to that observed in the F1 hybrids of 91R and 91C strains.

When the *Cyp6a2* allele of another overproducer strain, MHIII-D23, was examined in the genome of the hybrid of ry^{506} and MHIII-D23 strains, a similar trend was observed. In these hybrids, RNA produced by the *Cyp6a2-MHIII* allele can be distinguished from the RNA produced by the *Cyp6a2-ry* allele because *Cyp6a2-MHIII* allele posseses the insertional DNA in the 3'-UTR of the gene. The results (Figure 8) showed that the level of expression of the *Cyp6a2-MHIII* allele in the hybrid was strongly inhibited. Interestingly, the *Cyp6a2-ry* allele is found to be upregulated in the hybrid genome. Quantitative analysis (Table 3) showed that per gene dose, *Cyp6a2* expression is dramatically different in the hybrid relative to the parental strains. The mean

expression of *Cyp6a2-ry*, which is undetectable in the parental strain, is upregulated 6.7-fold in the hybrid while *Cyp6a2-MHIII* is downregulated 6.3-fold. The cumulative level of expression of these two alleles (0.2 and 0.19) is also 6-fold lower than the total activity (2.37) in the parental MHIII-D23 strain.

Taken together, these results suggest that the genome of the ry^{506} and 91C strains may have a semi-dominant transcriptional repressor, and for this reason the *Cyp6a2-91R* allele is downregulated in the hybrids of 91R and 91C and 91R and ry^{506} strains. Alternatively, the high level of CYP6A2 expression in the 91R strain may be due to the activity of a transcriptional activator that is absent or mutated in the ry^{506} and 91C strains. This may explain why the *Cyp6a2-ry* allele shows expression in the F1 hybrid of the ry^{506} and 91R strains. The results observed with the hybrid of ry^{506} and MHIII-D23 strains, however, are a little bit more complex. In this hybrid, while the *Cyp6a2-MHIII* allele is drastically downregulated, the *Cyp6a2-ry* allele is dramatically upregulated. Similarly, the upregulation of the *Cyp6a2-ry* allele in this hybrid genome suggests that the MHIII-D23 genome may have a transcriptional activator(s) that partially neutralizes the effect of the semi-dominant transcriptional repressor present in the ry^{506} genome.

The effect of the third chromosome on the expression of *Cyp6a2*

The analysis of the level of CYP6A2 mRNA in the three F1 hybrids derived from crosses between an underproducer and overproducer strain strongly suggests that the genotypes of these strains differ from one another with respect to *Cyp6a2* gene regulation. Chromosome substitution experiments in overproducer and underproducer strains of *M. domestica* suggest that the *CYP6A1* gene in this species is negatively

regulated by a locus (loci) on chromosome 2 (Cariño *et al.*, 1994). Since chromosome 2 of *Musca* and chromosome 3R of *Drosophila* are thought to be homologous (Foster *et al.*, 1981), it is possible that the mechanisms that regulate the expression of *CYP6A1* and *Cyp6a2* are similar. Therefore, to gain more insight into the *trans*-regulation of the 2R-linked *Cyp6a2* gene, strains carrying two copies of the second chromosome of the overproducer 91R strain (2^R) and one or two copies of the third chromosome of ry^{506} were synthesized. The chromosome composition of these strains were $2^R/2^R$; $3^{ry}/3^{ry}$ and $2^R/2^R$; $3^R/3^{ry}$. As controls, 91R ($2^R/2^R$; $3^R/3^R$) and ry^{506} ($2^{ry}/2^{ry}$; $3^{ry}/3^{ry}$) were used. Poly(A)$^+$ RNA samples isolated from all these strains were examined by northern blot hybridization.

The results of these experiments (Figure 9) showed that relative to 91R, there was a 1.5-fold decrease in the mean CYP6A2 mRNA levels when one homologue of 3^R was replaced with 3^{ry} (i.e., $2^R/2^R$; $3^R/3^{ry}$) and a 2.4-fold reduction when both 3^R homologues were replaced with 3^{ry} (i.e., $2^R/2^R$; $3^{ry}/3^{ry}$). Statistical analysis of the data using the Student's T-test showed that these data are statistically significant ($p< 0.05$). These data suggest that *Cyp6a2-91R*, which is located on the second chromosome, is negatively regulated by a locus (loci) on the third chromosome of the ry^{506} strain, and in 91R this negative regulatory locus may be mutant. Another possibility is the third chromosome encodes a positive regulator and in the *ry506* strain it is mutant. This may explain why the level of expression in these chromosome substitution stocks is not as high as it is in the 91R strain. It should also be noted that the expression of *Cyp6a2-91R* in the $2^R/2^R$; $3^{ry}/3^{ry}$ strain is still 6-fold higher than the ry^{506} control. Three hypotheses are offered to

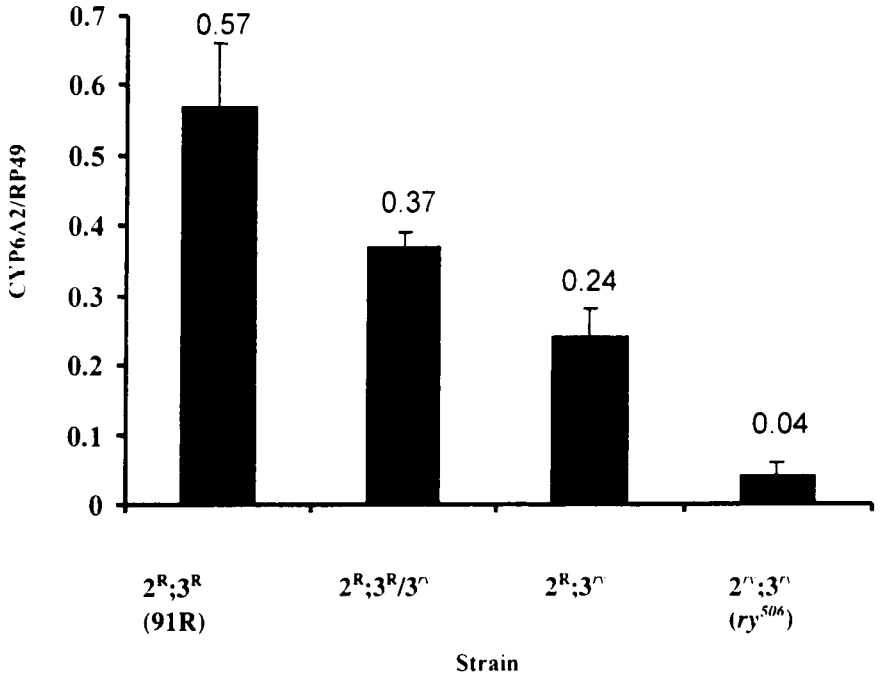

Figure 9: Effect of the third chromosome of the underproducer ry^{506} strain on the expression of the *Cyp6a2* allele of the overproducer 91R strain (*Cyp6a2-91R*). In this experiment, the second chromosome-linked gene of 91R (2^R) was placed under the influence of the third chromosome of the ry^{506} strain (3^{ry}). The level of CYP6A2 mRNA in the resulting stocks (2^R; 3^{ry}) and its derivative (2^R; $3^R/3^{ry}$) was compared to the 91R (2^R;3^R) and ry^{506} (2^{ry}; 3^{ry}) contols. Poly(A)$^+$ RNA (1 μg) was isolated from adult females and the constitutive level of CYP6A2 mRNA in each stock was examined by northern blot hybridization as described in Materials and Methods. Each column represents the mean CYP6A2 mRNA level derived from three independent experiments.

explain this observation. First, the second chromosome of the 91R strain may have loci that positively influence the expression of its *Cyp6a2* gene. Second, additional negative regulatory loci may be required to downregulate the expression of *Cyp6a2-91R* to the same level as *Cyp6a2-ry*. These loci may be located on the first (X), second or fourth chromosomes. Since the origins of the X and fourth chromosomes in the $2^R/2^R$; $3^{ry}/3^{ry}$ and $2^R/2^R$; $3^R/3^{ry}$ strains are not known in these two stocks, the effect of these chromosomes on *Cyp6a2* expression remains undetermined. To resolve this, *Cyp6a2-91R* expression can be examined in a strain where the inheritance of the first and fourth chromsomes can be traced. Third, it is possible that there is a mutation in the *cis*-regulatory elements of *Cyp6a2-91R* which prevents the putative transcriptional repressor encoded by 3^{ry} from further inhibiting its expression. Experiments designed to test this last hypothesis are presented in Chapter 3.

The results presented in this chapter provide new information on the molecular and genetic mechanisms that regulate the expression of the *Cyp6a2* gene of *D. melanogaster*. In this chapter, it is suggested that LTR of transposable element *17.6* and the associated $A(T)_nA$ sequences do not influence the steady-state level of CYP6A2 mRNA. Furthermore, in 6 of the 8 strains that were examined the steady-state level of CYP6A2 mRNA was less than in the 91R and MHIII-D23 strains, suggesting that a constitutive low level of CYP6A2 mRNA in the adult fly is the wild-type phenotype of the gene.

Several hypotheses are proposed to explain the constitutive overexpression of the *Cyp6a2-91R* and *Cyp6a2-MHIII* alleles. The overexpression could be due to a mutation

of the *cis*-regulatory element of the gene. Alternatively, it is possible that the putative transcriptional repressor that regulates the expression of *Cyp6a2* in these two strains has a mutation. The observation that *Cyp6a2-91R* and *Cyp6a2-MHIII* are downregulated in the hybrid genomes of two underproducer strains suggests that the genomes of the underproducer strains may encode a transcriptional repressor for the *Cyp6a2* gene. If in the 91R and MHIII-D23 genomes the transcriptional repressor becomes nonfunctional due to a loss-of-function mutation, then the *Cyp6a2* gene in these strains will be overexpressed. Alternatively, the high level of expression of *Cyp6a2-91R* and *Cyp6a2-MHIII* may be due to the actitivity of a positive regulator that is mutant or absent in the underproducer strains. If this is true, then the *Cyp6a2-91R* and *Cyp6a2-MHIII* alleles will not express in their respective hybrid genomes.

Chromosome substitution experiments indicate that *Cyp6a2-91R* may be under the influence of a negative regulatory locus or loci on the third chromosome. Another test of this hypothesis would be to take a transgenic approach and place the *Cyp6a2* allele from an overproducer strain in the genome of an underproducer strain and examine its ectopic expression in this different genetic background. The observation that the ry^{506} strain has a barely detectable level of CYP6A2 mRNA makes it an excellent host for germ line transformation studies involving the *Cyp6a2* allele from an overproducer strain. Since the *cis*-regulatory elements that regulate the expression of *Cyp6a2* have not been localized, the identification of the transcriptional control elements will be a signficant step in the elucidation of the molecular mechanism that regulates the expression of this gene in *D. melanogaster*. This information will also be benefical in understanding how

members of the *CYP6A* gene family are regulated in other invertebrate systems, such as *Musca domestica*.

Chapter 3

Functional and nucleotide sequence analysis of the upstream DNA of *Cyp6a2*

Introduction

The results described in the previous chapter suggest that the third chromosome of the underproducer ry^{506} strain has a locus or loci that downregulates the expression of the second chromosome-linked *Cyp6a2* gene of the overproducer 91R and MHIII-D23 strains. This suggests that the third chromosome of other underproducer strains, such as 91C, have a gene or genes that act as a transcriptional repressor of *Cyp6a2*. This may explain why *Cyp6a2* expression in ry^{506}, 91C and most other strains is low.

Several hypotheses can be proposed to explain the strain to strain variation in *Cyp6a2* expression. It is possible that in the 91R and MHIII-D23 strains, there is a mutation in the chromosome 3-linked gene that encodes the transcriptional repressor. If this is true, then this transcriptional repressor cannot downregulate the *Cyp6a2* gene of these two overproducer strains. Alternatively, it is possible that the gene encoding this transcriptional repressor does not carry the mutation, but the *cis*-regulatory elements of the *Cyp6a2-91R* and *Cyp6a2-MHIII* alleles is mutated and therefore fail to interact with the repressor molecule properly. A similar hypothesis can be put forward to explain the undetectable level of *Cyp6a2* expression in the ry^{506} strain. It is possible that in the ry^{506} strain, the positive factors needed for *Cyp6a2* expression are non-functional and for this reason, *Cyp6a2* expression is very low. This possibility may be remote because in most

strains that have been examined there is a constitutively low level of expression of *Cyp6a2* and this appears to be the wild-type phenotype of the gene. If a constitutively low level of expression is not the wild-type phenotype, then it has to be assumed that the majority of strains are mutants. This event seems unlikely because it is unreasonable to think that all strains would carry a mutation in the *cis*-regulatory elements or the regulatory genes that control the expression of *Cyp6a2*.

Although the molecular basis of *Cyp6a2* expression is not well understood, the data on the F1 hybrid flies (presented in Chapter 2) suggest that the genetic make-up of the high and low producer strains with respect to *Cyp6a2* expression must be different. Therefore, the objective of this chapter was to examine the expression of the cloned *Cyp6a2* gene of the high producer 91R strain (*Cyp6a2-91R*) in the genetic background of the underproducer ry^{506} strain. To do this, *Cyp6a2-91R* transgenes carrying different lengths of upstream DNA were constructed and placed into the genome of ry^{506} strain via P element-mediated germ line transformation. The results not only showed the effect of the ry^{506} genome on the expression of *Cyp6a2-91R*, but they also provide new information on the various *cis*-elements that may be involved in the regulation of *Cyp6a2*.

Materials and Methods

D. melanogaster stocks and culture conditions

The sources of ry^{506}, 91R and MHIII-D23 strains have been described in Chapter 2. The multiply-marked second (*Sco/CyO*; *Kar* ry^{506}) and third chromosome (*TM3 Sb ry*

e TM6B Tb e) balancer stocks were obtained from Dr. John Lucchesi, Emory University, Atlanta, GA and are described in Lindsley and Zimm (1992). Flies were reared on standard *Drosophila* medium as described in Chapter 2.

P-element vector, helper plasmid and *Cyp6a2-91R* constructs

The P-element transposon vector pDM30 (Mismer and Rubin, 1987) and the helper plasmid pπ25.7wc which encodes the transposase (Karess and Rubin, 1984) were obtained from Dr. Gerald Rubin, University of California, Berkley, CA. The pDM30 plasmid (Figure 10) contains the wild-type ry^+ gene, the structural gene for xanthine dehydrogenase, as the dominant selectable marker. The ry^+ gene gives wild-type (bright red) eye color in adult flies (Lindsley and Zimm, 1992). In addition, this plasmid contains unique *Sal*I and *Not*I sites that are located downstream of the ry^+ gene. These restriction sites and the ry^+ gene are flanked by inverted repeats of 31 bp (P elements). In the presence of the transposase enzyme (helper plasmid), the entire DNA between the two P elements is transposed onto the host chromosome. Here it should be noted that the P element in the pπ25.7wc plasmid is defective and therefore the transposase gene cannot be transposed onto the host chromosome (Karess, 1985).

The *Cyp6a2-91R* transgene carrying three different lengths of the upstream DNA (Figure 11) were isolated as *Sal*I fragments and individually cloned into the single *Sal*I site of pDM30. These recombinant P plasmids were then used to transform the DH5α strain of *Escherichia coli* (Gibco-BRL, Gaithersburg, MD). All plasmids used in this study were isolated by the alkaline lysis method (Maniatis *et al.*, 1982).

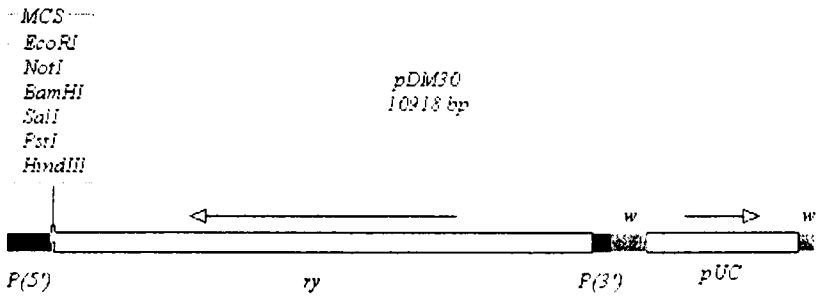

Figure 10: Schematic representation of the pDM30 transformation vector used for microinjection of ry^{506} embryos. The transposon vector has unique *Sal*I and *Not*I sites in the multiple cloning site (MCS) for cloning of genomic DNA fragments. In the present study, *Cyp6a2-91R* genomic fragments were cloned into the *Sal*I site. Arrows denote the direction of transcription of the ry^{+} dominant selectable marker and the ampicillin resistance gene (B-lactamase within the pUC vector sequence. The puC vector sequence also includes a bacterial origin of replication for propagation in *E.coli* bacterial cells. The ry^{+} gene is flanked by two P elements which allow the *Cyp6a2-91R* transgene and dominant selectable marker to be transposed onto the ry^{506} host chromosome.

Adapted from Mismer and Rubin, 1987. *Genetics* 116: 565-578.

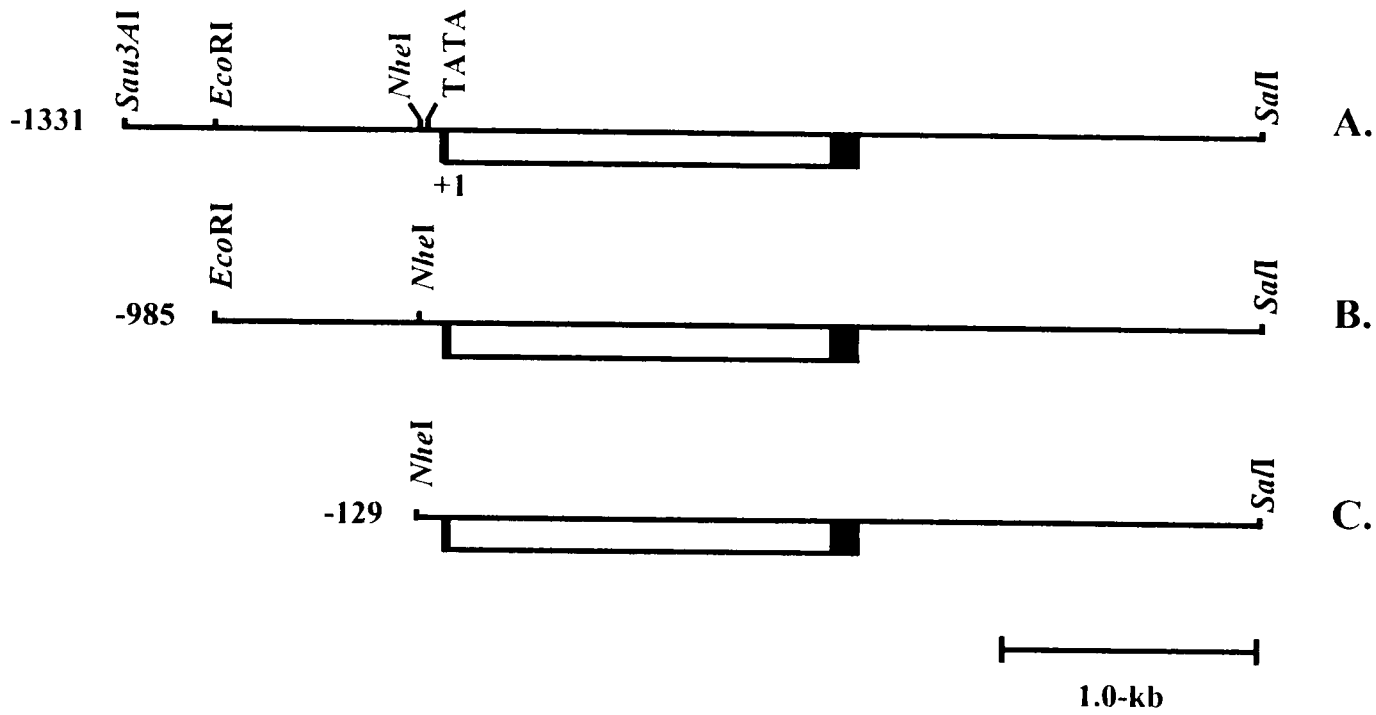

Figure 11: Schematic representation of the *Cyp6a2-91R* genomic DNA fragments used for P-element mediated germ line transformation of ry^{506}. The amount of upstream DNA present in each transgene construct is indicated at the left side of each construct. The coding region, which begins with +1 of ATG, is shown with an open rectangle The black boxes are the 5' and 3'-untranslated regions. The 5'-end restriction site in each fragment was converted to *Sal*I before cloning into the *Sal*I site of the pDM30 transformation vector (Figure 10)..

Germ line transformation and linkage analysis of the transgenes

For germ line transformation a 3:1 mixture of the recombinant P plasmids carrying the *Cyp6a2-91R* gene and pπ25.7wc helper plasmid was made in microinjection buffer (Karess, 1985). One-tenth volume of McCormick's green food dye was added in order to visualize the DNA mixture at the time of microinjection.

The DNA mixture was loaded into a glass capillary pipette (World Precision Instruments, Inc., Sarasota, FL) that was pulled to a fine point with a vertical pipette puller (Model 700C, David Kopf Instruments). Adult ry^{506} female flies (2-3 days old) were transferred to an egg-laying chamber for egg laying. Eggs were collected at 1 hour intervals to ensure that they were younger than preblastoderm stage. These embryos were mechanically dechorionated by gently rolling them over a piece of double-sided tape. Dechorionated embryos were then desiccated for several minutes at room temperature. The mixture of transposon and helper plasmid DNA was then injected into the posterior end of the embryo. The microinjected embryos were then overlaid with a thin layer of halocarbon oil (series 700; Halocarbon Products Corporation, Hackensack, NJ) and transferred to a petri dish containing standard *Drosophila* medium.

G_0 adults that emerged from the microinjected embryos were examined for ry^+ (wild-type) eye color. A small expression of the ry^+ gene present in the transposon is sufficient to produce wild-type eye color, but it does not mean that the ry^+ flies are germ line transformants. Therefore, the G_0 ry^+ flies were individually mated with the opposite sex of the host ry^{506} strain and the ry^+ G_1 progeny of these crosses were identified as

germ line transformants. A single, G1 germ line transformant derived from the G_0 cross was mated to the opposite sex of host ry^{506} strain to establish G_2 isolines.

Chromosomal linkage of the transgene was determined by crossing the ry^+ flies of each isoline with the opposite sex of second (*Sco/CyO*; *Kar ry*) and third (*TM3 Sb ry e/TM6B Tb e*) chromosome balancer stocks. The F1 ry^+ flies of these two crosses showing the second chromosome-linked curly (Cy) and third chromosome-linked stubble (Sb) phenotypes were mated with ry^{506}, and the phenotype of the F2 progeny was examined. Segregation between ry^+ and Cy phenotypes indicated that the transgene was linked to the second chromosome, whereas segregation between ry^+ and Sb phenotypes indicated linkage to the third chromosome. In this scheme, co-segregation with *Cy* as well as *Sb* means that the transgene is either on the X or on the fourth chromosome. To resolve this, transformed ry^+ males of such lines are crossed with ry^{506} females. If the ry^+ phenotype in the F1 flies is restricted to the females, it means that the transgene in this line is X-linked. Appearance of ry^+ phenotype in both male and female F1 progeny would mean that the transgene is linked to the fourth chromosome. Using balancer stocks for the X, second and third chromosomes, transgenes linked to the respective chromosomes were made homozygous.

In situ hybridization

The chromosomal location of the transgene in the transformant lines was determined by *in situ* hybridization of a biotinylated ry^+ or *Cyp6a2* gene probe to the polytene chromosomes as described by Ashburner (1989). Polytene chromosomes were

prepared from the salivary gland squashes of well-fed third instar larvae of each homozygous transformant line. The chromosomes were fixed in methanol-acetic acid (3:1), dehydrated and air-dried. Before hybridization chromosomes were treated with acetic anhydride to block non-specific binding sites. Genomic clones of *ry* and *Cyp6a2* genes were biotinylated using a commercially available kit (BioNick Labeling System, Gibco-BRL, Gaithersburg, MD). Hybridization signals were detected with a commercially available streptavidin-alkaline phosphatase kit (Gibco-BRL, Gaithersburg, MD) following the procedure described by Ashburner (1989). Polytene chromosomes were visualized and photographed with a phase contrast microscope.

Isolation of genomic DNA

To isolate genomic DNA, flies were homogenized in ice-cold nuclear isolation buffer (50mM Tris-HCl, pH 7.6, 25mM KCl, 5mM magnesium acetate and 0.35M sucrose). The homogenate was filtered through cheesecloth and centrifuged at 10,000 X g. The nuclear pellet was then resuspended in 1X DNA extraction buffer (DEB: 100 mM Tris-HCl, pH 8.0, 150mM NaCl and 10mM EDTA). The nuclei were then lysed by adding one-tenth volume of 5M sodium perchlorate and one-tenth volume of 10% SDS. The lysate was extracted twice with an equal volume of DEB-saturated phenol and twice with chloroform. To the final aqueous phase one-tenth volume of 2M sodium acetate, pH 7.5 and 2 volumes 95% ethanol were added and the genomic DNA was spooled onto a glass rod. The DNA was air dried briefly and dissolved in 1X TE.

Southern blot hybridization

Genomic DNA isolated from the transformant lines was digested with *Eco*RI and electrophoresed on an ethidium-bromide stained 0.8%-TBE agarose gel. The gel was then treated with 1.5M NaCl/0.5M NaOH for 1 hour at room temperature to denature the DNA. After neutralization in a solution containing 3M NaCl/0.5M Tris-HCl (pH 7.5) for 1.5 hours at room temperature, DNA was transferred via capillary action onto a nylon membrane with 20X SSC as the transfer buffer. The DNA on the blot was UV-crosslinked, baked and prehybridized in a buffer containing 6X SSC, 5X Denhardt's, 0.1% SDS and 50 μg/ml sonicated herring sperm DNA for 2 hours at 68° C.

To identify the number of transgene insertions in each transgenic line, the 5'-half of the ry^+ gene was radiolabeled by the random priming method (Feinberg and Vogelstein, 1983) and used as a probe for the Southern blot. Hybridization was carried out for 16-18 hours at 68° C in fresh prehybridization solution containing the probe. After hybridization the membrane was washed twice in 0.1X SSC and 0.1% SDS at 60°C then twice in 0.1X SSC at the same solution temperature. Hybridization signals were visualized by autoradiography.

RNA blot analysis

Total and poly(A)$^+$ RNA from a mixture of equal proportion of males and females of each transformant line was isolated and prepared for northern and slot blot hybridization as described in Chapter 2.

Construction and screening of genomic libraries

The 91R genomic library was obtained from Dr. Larry C. Waters and has been described previously (Waters *et al.*, 1992a,b). For construction of the ry^{506} and MHIII-D23 genomic libraries, genomic DNA from these strains was partially digested with *Sau*3AI, dephosphorylated and ligated with *Bam*HI digested DNA of EMBL3 lambda phage (Stratagene, La Jolla, CA). The ligated DNA was packaged into phage particles by using Gigapack II packaging extracts (Stratagene). The average size of the two libraries was approximately 5 X 10^5 plaque forming units.

Approximately 6 x 10^4 plaques from each genomic library were screened with a *Cyp6a2* DNA probe. To isolate *Cyp6a-91R* genomic clones that contain the upstream DNA, duplicate plaque lifts were prepared from the genomic libraries. One set of filters was hybridized with the 5' half of the *Cyp6a2* gene probe and the other set was probed with the 3' half of the *Cyp6a2* gene probe. Hybridization signals on the filters were visualized by autoradiography. Plaques that only hybridized with the 5' probe were isolated and purified through two more rounds of screening. DNA from each purified lambda clone was isolated by the plate lysate method as described by Helms *et al.* (1982).

DNA sequencing

Genomic DNA fragments containing the upstream DNA of *Cyp6a2-91R*, *Cyp6a2-ry* and *Cyp6a2-MHIII* were subcloned into the pBSKII+ (Stratagene) or pUC9 (Gibco-BRL, Gaithersburg, MD) plasmid vectors and transformed into DH5α bacterial cells. Plasmid DNAs were isolated using the standard alkaline-lysis method (Maniatis *et al.*,

1982). Double-stranded DNA templates were sequenced with overlapping synthetic oligodeoxynucleotide primers.

Manual sequencing, originally described by Sanger *et al.* (1977), was performed with ^{35}S-dATP and the Sequenase 2.0 DNA sequencing kit (United States Biochemical). The reactions were done according to manufacturer's protocol, and the samples were electrophoresed on a denaturing 8% polyacrylamide gel. To maximize the amount of DNA sequence that could be read from a single sequencing run, samples were electrophoresed in a salt gradient. The upper and lower buffer chambers contained 0.5X and 1X TBE, respectively. Approximately 60 minutes after the bromophenol blue dye had migrated out of the gel, sodium acetate, pH 5.0, was added to the lower buffer chamber to a final concentration of 1M. After gel electrophoresis, the sequencing gel was dried and exposed to autoradiographic film.

Automated sequencing was performed on an Applied Biosystems 373A automated sequencer at the Molecular Biology Resource Facility (MBRF) at the University of Tennessee, Knoxville.

Results and Discussion

Characterization of the *Cyp6a2-91R* transgenes in ry^{506} transformant lines

Three different constructs of the *Cyp6a2-91R* gene were made (Figure 11). All constructs included the entire *Cyp6a2-91R* transcription unit and approximately 2.3-kb of DNA downstream of the TAA stop codon. However, the upstream DNA of the gene

varied in these constructs and it extended up to -1331, -985 or -129 bp from the ATG at +1. For each construct, two or three independent transgenic lines were obtained.

To determine the number of transgene insertions in each transgenic line, Southern blot analysis of genomic DNA and *in situ* hybridization were used. For Southern blot analysis, genomic DNA isolated from all transgenic lines was digested with *EcoRI*, blotted and probed with the 5'-end of the ry^+ gene (Figure 12). *Eco*RI-digested genomic DNA of the host ry^{506} strain was used as a control. If a transformant carries one transgene insert, then two *Eco*RI fragments are expected to hybridize. One fragment originates from the transgene and the other from the endogenous *ry* gene. In other words, the total number of transgene inserts in each transformant line is equal to the total number of hybridizing bands minus one (the endogenous *ry* gene). Based on this formula, the results showed that lines 7B and 3A have one insert and line 11A1 has two inserts (Figure 13). The high molecular weight (~10-kb) hybridizing *Eco*RI DNA fragment found in all lines is a part of the endogenous *ry* gene because it is also present in the control *ry* strain.

No hybridization of any additional bands was seen in lines 2C, 7F, 9B and 1A. This may suggest that these lines do not have any transgene insert. However, genetic analysis and *in situ* hybridization (see below) showed that these lines do carry the *Cyp6a2-91R* transgene. The inability to visualize the transgene-specific fragment on the Southern blot (Figure 13) may be a function of incomplete transfer of the genomic DNA onto the membrane. Another possibility is that the transgene-specific *Eco*RI fragment in these lines comigrated with the *Eco*RI fragment of the endogenous ry^+ gene. To resolve

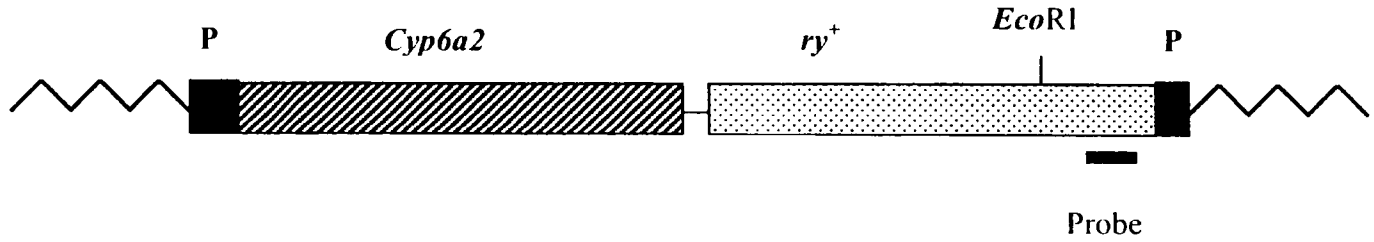

Figure 12: Schematic diagram showing the pDM30 P-element transposon with *Cyp6a2* and ry^+ genes integrated into the chromosomal DNA of a transformed fly. The wavy lines represent the chromosomal DNA of the ry^{506} host strain. The size of the *Eco*RI fragment that the probe (shown as a heavy dark line) hybridizes with depends on how far the first chromosomal *Eco*RI site is from the *Eco*RI site within the ry^+ gene.

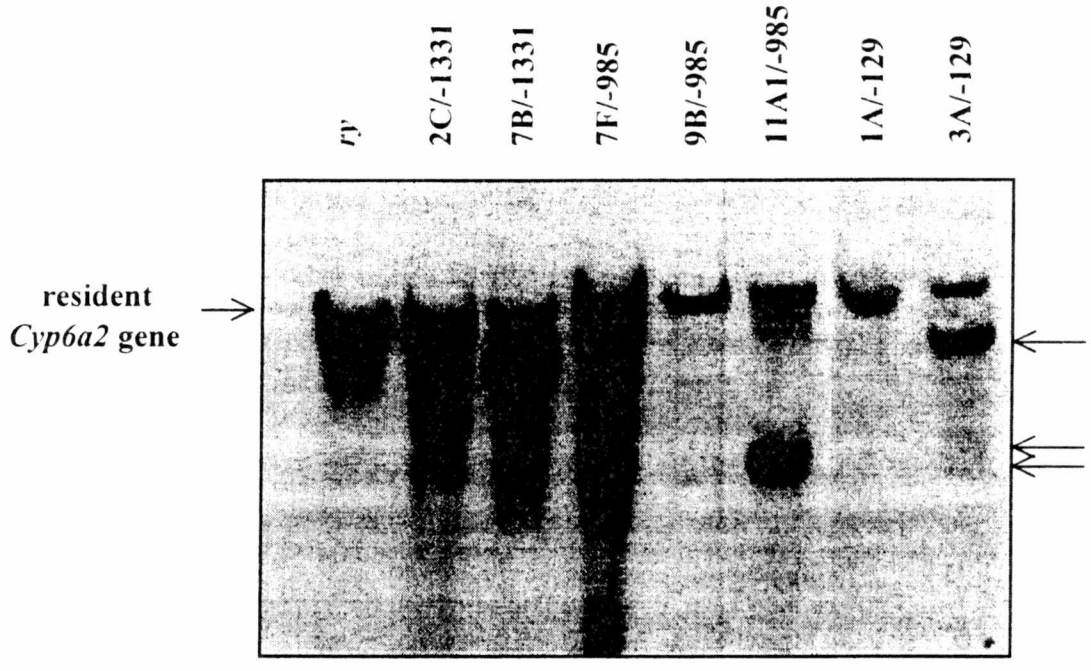

Figure 13: Southern blot hybridization of the genomic DNA of transformed flies. Genomic DNA isolated from the transgenic lines and the ry^{506} host strain was digested with *Eco*RI, electrophoresed on a 0.8% TBE-agarose gel and blotted onto a nylon membrane. The name of each line is shown above each lane. The blot was hybridized with a ^{32}P-labeled DNA fragment representing a portion of the 5'-end of the ry^{+} gene (see Figure 12 for details). The *Eco*RI fragment corresponding to the resident *Cyp6a2* gene is shown with an arrow on the left side of the panel. The arrows on the right side of the panel are the *Eco*RI fragments that correspond to the *Cyp6a2-91R* transgene.

this issue, the genomic DNA from each strain can be digested with a different restriction enzyme and reanalyzed by Southern blot hybridization.

The cytological position of each transgene insertion and the transgene copy number was determined by *in situ* hybridization of a biotinylated ry^+ or *Cyp6a2* gene probe to polytene chromosomes. Since the transposed DNA contains both the ry^+ dominant selectable marker and the *Cyp6a2-91R* transgene, *in situ* hybridization with a biotinylated ry^+ or *Cyp6a2* gene probe will identify the cytological position and transgene copy number of the transgene in each transformant line. Figures 14, 15 and 16 show the hybridization of the probe with the polytene chromosomes from each transgenic line. In all panels except Figure 15D, the endogenous ry^+ or *Cyp6a2* gene is also shown. The results, which are summarized in Table 4, show that each line carries at least one transgene insert, including the ones (2C/-1331, 7F/-985, 9B/-985 and 1A/-129) which could not be resolved by Southern blot hybridization. In line 11A1, two hybridization signals were found on the X chromosome, suggesting that this line has two transgene inserts. This observation agrees with the results of the Southern blot hybridization experiment.

Constitutive expression of the *Cyp6a2-91R* transgenes in the ry^{506} genetic background

The constitutive level of *Cyp6a2-91R* transgene expression in each transformant line was examined by northern and slot blot hybridization. Visual inspection of the autoradiogram (Figure 17A) showed that in all transformant lines the size of the mRNA encoded by the *Cyp6a2-91R* transgene was identical to the ry^{506} and 91R controls. The

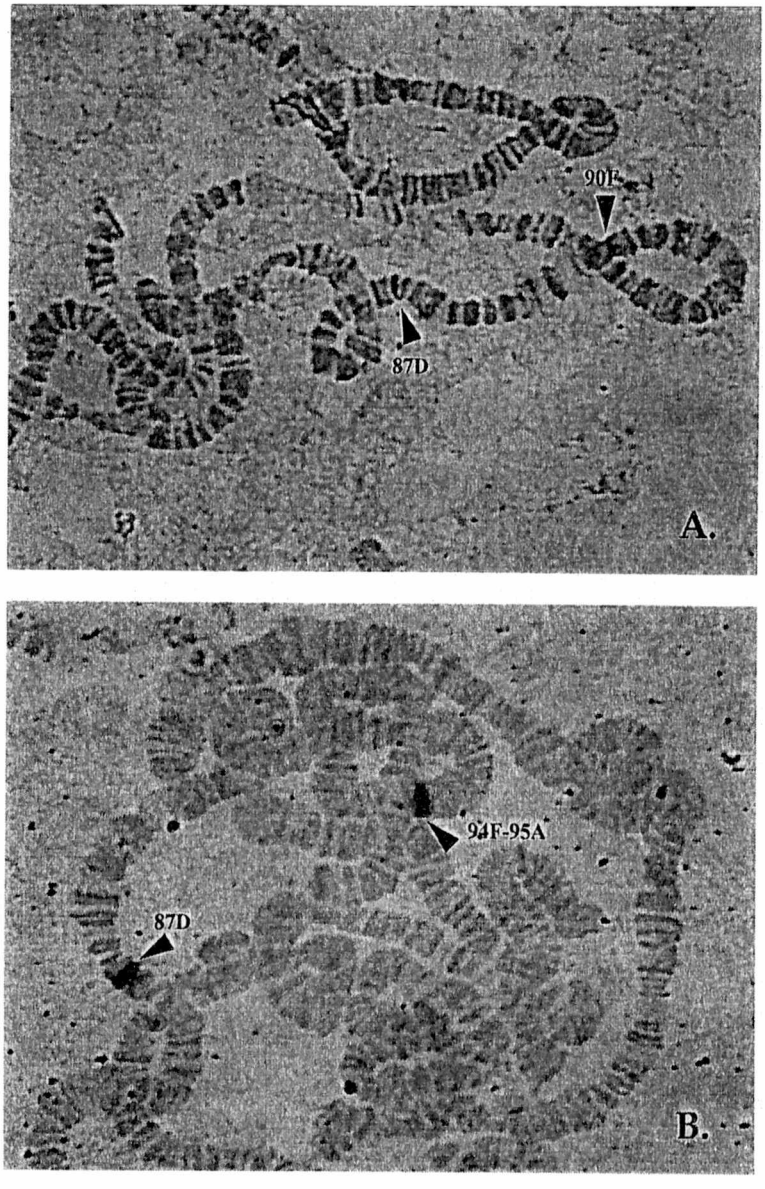

Figure 14: *In situ* hybridization of the biotinylated ry^+ probe to polytene chromosomes of transgenic lines with the *Cyp6a2-91R* transgene containing –1331 nucleotides of upstream DNA. **(A)** Transgenic line 2C/-1331; **(B)** 7B/-1331. In both panels, the endogenous ry^+ gene is shown with an arrowhead at polytene chromosome band 87D.

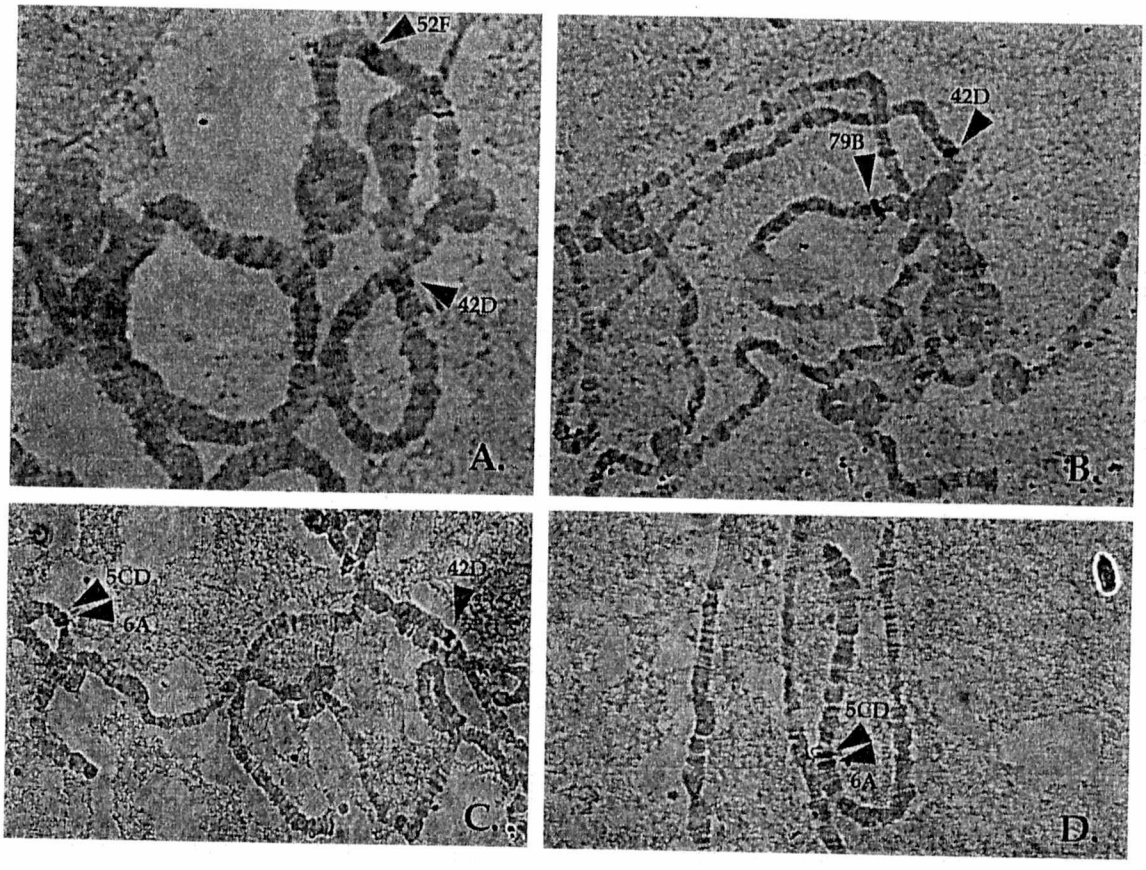

Figure 15: *In situ* hybridization of the biotinylated *Cyp6a2* probe to polytene chromosomes of transgenic lines with the *Cyp6a2-91R* transgene containing –985 bases of upstream DNA. **(A)** Transgenic line 7F/-985; **(B)** 9B/-985; **(C)** 11A1/-985 **(D)** 11A1/-985. In Panels A-C, the endogenous *Cyp6a2* gene at polytene chromosome band 42D is shown with an arrowhead.

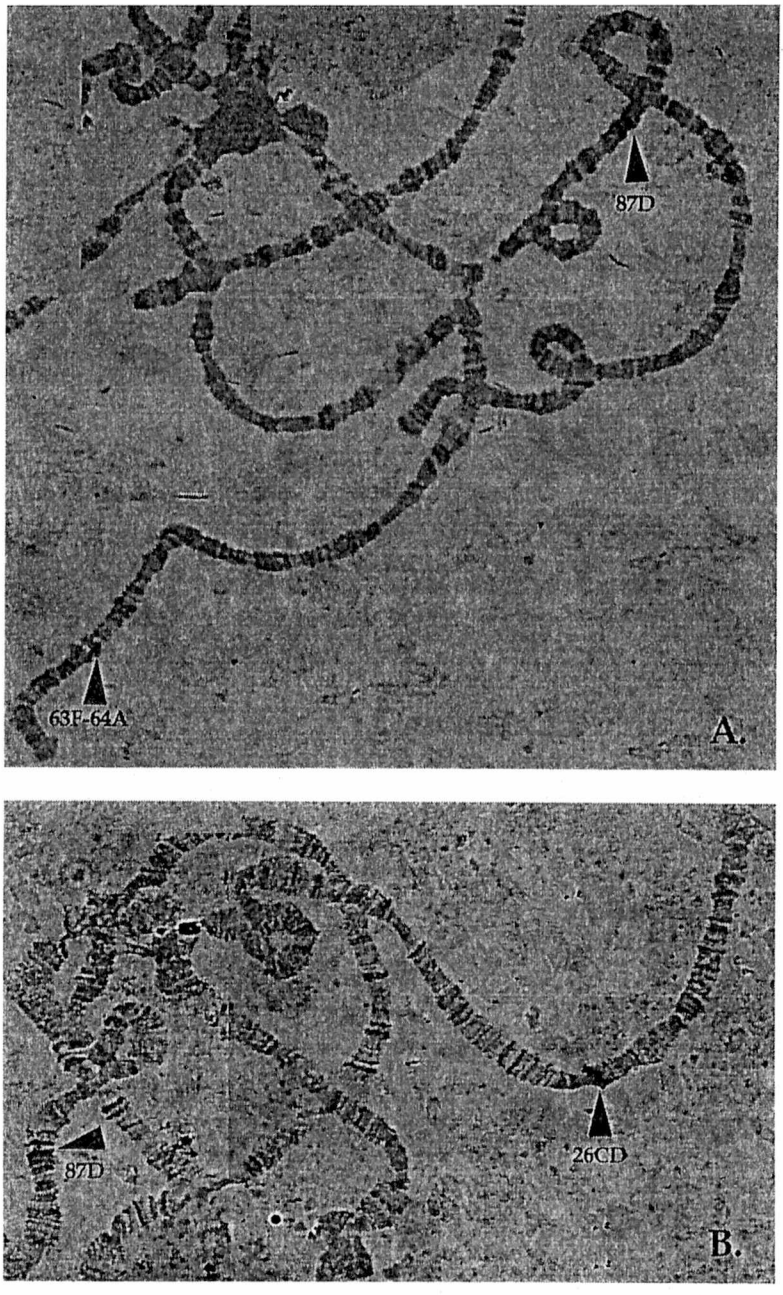

Figure 16: *In situ* hybridization of the biotinylated *Cyp6a2* probe to polytene chromosomes of transgenic lines with the *Cyp6a2-91R* transgene containing –129 bases of upstream DNA. **(A)** Transgenic line 1A/-129; **(B)** 3A/-129. In both panels, the endogenous ry^+ gene at polytene chromsome band 87D is shown.

Table 4: Characterization of *Cyp6a2-91R* transgenes in different transformant lines

Transgenic Lines[a]	Linkage Group	Cytological Position	Number of Transgenes
2C/-1331	3rd	90F	1
7B/-1331	3rd	94F-95A	1
7F/-985	2nd	52F	1
9B/-985	3rd	79B	1
11A/-985	X	5CD; 6A	2
1A/-129	3rd	63F-64A	1
3A/-129	2nd	26CD	1

[a] The negative number in the name of each line indicates the extent of upstream DNA present in the transgene.

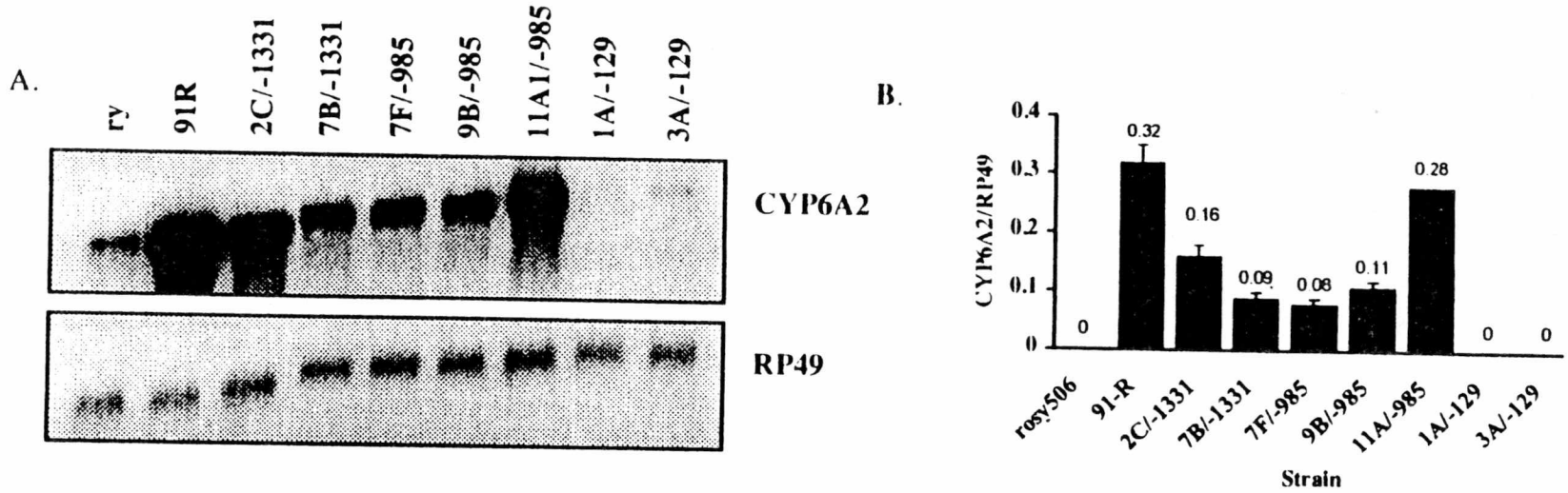

Figure 17: Expression of *Cyp6a2-91R* transgenes in ry^{506} transformants. (A) Sample northern blot. Poly(A)$^+$ RNA (5 µg) from a 1:1 proportion of male and female flies was electrophoresed on a 2.2M-1.2% formaldehyde agarose gel, transferred onto nylon and hybridized with CYP6A2 and RP49 cDNA probes as described previously. **(B)** Quantification of CYP6A2 mRNA levels in ry^{506} and transgenic lines. Poly(A)$^+$ RNA (1 µg) was analyzed by slot blot hybridization and hybridization signals were quantified with a radioanalytical imager (Ambis). Each column represents the average of three independent experiments. In each experiment, the non-specific background value was subtracted from the sample value. Since the constitutive level of CYP6A2 mRNA in the ry^{506} host and transgenic lines 1A/-129 and 3A/-129 is much lower than 91R and the other transgenic lines, the CYP6A2/RP49 value obtained for the ry^{506} host and transgenic lines 1A/-129 and 3A/-129 is "0" after the background subtraction.

expression of the *Cyp6a2-91R* transgenes carrying –1331 (2C/-1331 and 7B/-1331) and –985 (7F/-985, 9B/-985 and 11A1/-985) was observed to be greater than the expression of the endogenous *Cyp6a2* gene of the host ry^{506} strain. In transgenic lines 1A/-129 and 3A/-129, which carry a *Cyp6a2-91R* transgene with 129 bp upstream DNA, the expression of the transgene was very similar to that found in the ry^{506} strain.

For a better estimate of the differences in transgene expression relative to the ry^{506} host strain and 91R, the level of CYP6A2 mRNA in each line was examined by slot blot hybridization. In each experiment, the non-specific background value was subtracted from the sample value. The results (Figure 17B) show that transformant lines 2C, 7B, 7F, 9B and 11A carrying a *Cyp6a2-91R* transgene with 1331 or 985 bp upstream DNA have similar levels of CYP6A2 mRNA. Furthermore, these levels are higher than that of the endogenous *Cyp6a2* gene of the host ry^{506} strain. However, the levels of CYP6A2 mRNA in lines 2C, 7B, 7F and 9B were 50-75% lower than the level of CYP6A2 mRNA found in the 91R strain. In line 11A1/-985, the level of CYP6A2 mRNA was similar to that in 91R and this may be attributed either to the chromosomal environment that surrounds the transgene (i.e., position effect) or because this transformant line has two transgene inserts. The results also show that lines 1A/-129 and 3A/-129 carrying a *Cyp6a2-91R* transgene with 129 bp upstream DNA have almost undetectable levels of CYP6A2 mRNA like the host ry^{506} strain (Figure 17B). Since the constitutive level of CYP6A2 mRNA in the ry^{506} host and transgenic lines 1A/-129 and 3A/-129 is much lower than 91R and the other transgenic lines, the CYP6A2/RP49 value obtained for the ry^{506} host and transgenic lines 1A/-129 and 3A/-129 is "0" after the background

subtraction. Taken together, the results of these transgenic studies show that the *cis*-regulatory elements located between –985 and –129 base pairs allow the constitutive expression of *Cyp6a2-91R* in the ry^{506} genetic background.

The observation that the *Cyp6a2-91R* transgenes with 1331 or 985 bp upstream DNA show expression in the ry^{506} genome is interesting because the resident *Cyp6a2-ry* allele does not show much expression in this strain. Several hypotheses can be proposed to explain these observations. First, the experiments that have examined the expression of *Cyp6a2-91R* in the 91R x ry^{506} hybrid genome and in $2^R/2^R$; $3^{ry}/3^{ry}$ strains (see Chapter 2) suggest that ry^{506} genome contains a transcriptional repressor for *Cyp6a2* and that the locus (loci) encoding these repressors is located on the third chromosome. If the genome of the ry^{506} strain contains an active transcriptional repressor that downregulates *Cyp6a2* expression, then the addition of two extra copies of *Cyp6a2-91R* into the ry^{506} genome may cause the active transcriptional repressor to be limiting, thereby resulting in its inability to effectively downregulate the *Cyp6a2-91R* transgene. If this is the case, it needs to be assumed that the *cis*-elements for the repressor molecule are present between –1331 and –129 bp.

Second, it is possible that the *cis*-elements for the repressor are located upstream of the –1331 region and are not present in the *Cyp6a2-91R* transgenes used in this study. As a result, the repressor molecules present in the host ry^{506} strain cannot downregulate the transcription of the *Cyp6a2-91R* transgene. To test this hypothesis, *Cyp6a2-91R* constructs carrying longer than 1331 base pair upstream DNA can be placed into ry^{506} strain and the CYP6A2 mRNA level can be measured in the transformants.

A third possibility is that the DNA between –129 and -1331 base pairs does have the *cis*-elements for the repressor molecule, but in *Cyp6a2-91R* these elements have a gain-of-function mutation(s) which would prevent the active transcriptional repressor in the ry^{506} genome from fully downregulating the *Cyp6a2-91R* transgenes with 1331 or 985 bases of upstream DNA. This hypothesis is tested in the following section.

Nucleotide sequence analysis of the upstream DNA of *Cyp6a2* in different strains of *D. melanogaster*

One of the hypotheses discussed in the previous section proposes that the upstream DNA of *Cyp6a2-91R* may have a mutation. To test this, the upstream DNA of *Cyp6a2-91R* between +1 and –1331 region was sequenced and compared to the equivalent region from *Cyp6a2-ry*. The results showed that the upstream DNA of *Cyp6a2-ry* and *Cyp6a2-91R* are identical except for six bases located at positions –740, -689, -571, -570, -112 and –6 (Figure 18). Since the constitutive expression of *Cyp6a2-MHIII* is extremely high, the upstream DNA up to –985 base pair of this *Cyp6a2* allele was also sequenced and compared with the equivalent upstream DNA of the *Cyp6a2-91R* and *Cyp6a2-ry* alleles. The results (Figure 18) showed that the sequence of the upstream DNA of *Cyp6a2-MHIII* between +1 and –985 bases is identical to the sequences of the *Cyp6a2-91R* and *Cyp6a2-ry* alleles except at seven positions. In *Cyp6a2-MHIII*, the bases at –740, -689, -571 and –570 are identical to those found in *Cyp6a2-ry*. On the other hand, two nucleotides at –112 and –6 are identical to those present in *Cyp6a2-91R*. There is also a seventh base substitution at –328. The nucleotide at this position is not identical to the base found at this position in either *Cyp6a2-ry* or *Cyp6a2-91R*. While the

Figure 18: Nucleotide sequence analysis of the –1331 to +1 region of the upstream DNA of *Cyp6a2-91R*, *Cyp6a2-ry* and *Cyp6a2-MHIII*. Lower case letters represent the 5'-flanking DNA and non-coding sequences. Upper case letters are the coding sequences. The ATG start codon is indicated as +1. Arrows at –740, -689, -571, -570, -328, -112 and –6 denote the six bases that are different in the three *Cyp6a2* alleles. Barbie boxes at positions –623, -617, -588, -219, -171 and –86 are the boxed regions. The barbie box at –86 also overlaps with the putative TATA box (TATAAAAA) described by Waters *et al.* (1992a). The ecdysone repsonse element (EcRE) at –491 is underlined. The three Broad Complex (BR-C) motifs at –1183, -861 and –625 are also shown with underlined text. The nucleotide sequence of the upstream DNA of the *Cyp6a2-91R*, *Cyp6a2-ry* and *Cyp6a2-MHIII* alleles have been deposited into GenBank. The accession numbers are AF061081 (*Cyp6a2-ry*), AF061082 (*Cyp6a2-91R*) and AF061083 (*Cyp6a2-MHIII*).

```
                    .         .         .         .         .         .
91R      -1331  gatcttgtgttgtacattagcactgatttctgaaaataaaacgagataaggcaacaaac
rosy506         gatcttgtgttgtacattagcactgatttctgaaaataaaacgagataaggcaacaaac
MHIIID23


                    .         .         .         .         .         .
91R      -1272 taaatttagttgtcattttaaatgttggtgtgacatgtagatttaactacgcgacattgc
rosy506        taaatttagttgtcattttaaatgttggtgtgacatgtagatttaactacgcgacattgc
MHIIID23


                    .         .         . BR-C    .         .         .
91R      -1212 tttcgaatgaatacattttaatcacatatatgctaaatataatttaacacagcacagttg
rosy506        tttcgaatgaatacattttaatcacatatatgctaaatataatttaacacagcacagttg
MHIIID23


                    .         .         .         .         .         .
91R      -1152 atgcgttgtttccaaatgagcaataaaacccgatgcgattataatgtttaaaaccaaccg
rosy506        atgcgttgtttccaaatgagcaataaaacccgatgcgattataatgtttaaaaccaaccg
MHIIID23


                    .         .         .         .         .         .
91R      -1092 cgaccagtttaacgacaatcaagttatctactgtttatattttgatcgtcattcaaacag
rosy506        cgaccagtttaacgacaatcaagttatctactgtttatattttgatcgtcattcaaacag
MHIIID23


                    .         .         .         .         .         .
91R      -1032 tgaagcgtttaaatagctcttagaaagagttattggttctcccagcgaattcattcgttt
rosy506        tgaagcgtttaaatagctcttagaaagagttattggttctcccagcgaattcattcgttt
MHIIID23                                                   gaattcattcgttt


                    .         .         .         .         .         .
91R       -972 tatcgccgtggaacaccaaacattaacgttgtagtggggtgctaattactgaaataaatg
rosy506        tatcgccgtggaacaccaaacattaacgttgtagtggggtgctaattactgaaataaatg
MHIIID23       tatcgccgtggaacaccaaacattaacgttgtagtggggtgctaattactgaaataaatg


                    .         .         .         .         .    BR-C .
91R       -912 ggtgatattttaaatgtgtatgtccgtgtatgtatttatctacgggaaattatgtcatta
rosy506        ggtgatattttaaatgtgtatgtccgtgtatgtatttatctacgggaaattatgtcatta
MHIIID23       ggtgatattttaaatgtgtatgtccgtgtatgtatttatctacgggaaattatgtcatta


                    .         .         .         .         .         .
91R       -852 acaatttcatgcgataatgttcgctccgattcccaaggcttaaaaactcatttaattata
rosy506        acaatttcatgcgataatgttcgctccgattcccaaggcttaaaaactcatttaattata
MHIIID23       acaatttcatgcgataatgttcgctccgattcccaaggcttaaaaactcatttaattata
```

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                                                                  ↓
91R       -792 tttgctctgttttctaataaatattgaatcgcataggagaaaaacctttatgctccttaa
rosy506        tttgctctgttttctaataaatattgaatcgcataggagaaaaacctttatgatccttaa
MHIIID23       tttgctctgttttctaataaatattgaatcgcataggagaaaaacctttatgatccttaa

                                                        ↓
91R       -732 ccagcttgtttaataacaatcttgtaatcgtttgtttgcatgttgatccactgcaataga
rosy506        ccagcttgtttaataacaatcttgtaatcgtttgtttgcatgtagatccactgcaataga
MHIIID23       ccagcttgtttaataacaatcttgtaatcgtttgtttgcatgtagatccactgcaataga

                                                  BR-C
91R       -672 acagtagaacatcaagagaatatctcttatgtcaaatcttgaatctctcataaaaagcaa
rosy506        acagtagaacatcaagagaatatctcttatgtcaaatcttgaatctctcataaaaagcaa
MHIIID23       acagtagaacatcaagagaatatctcttatgtcaaatcttgaatctctcataaaaagcaa

                                                    ↓↓
91R       -612 aagagcttggtgcttgtcgctgataatgaaagctagatggaacgctcggctatcaaatac
rosy506        aagagcttggtgcttgtcgctgataatgaaagctagatggattgctcggctatcaaatac
MHIIID23       aagagcttggtgcttgtcgctgataatgaaagctagatggattgctcggctatcaaatac

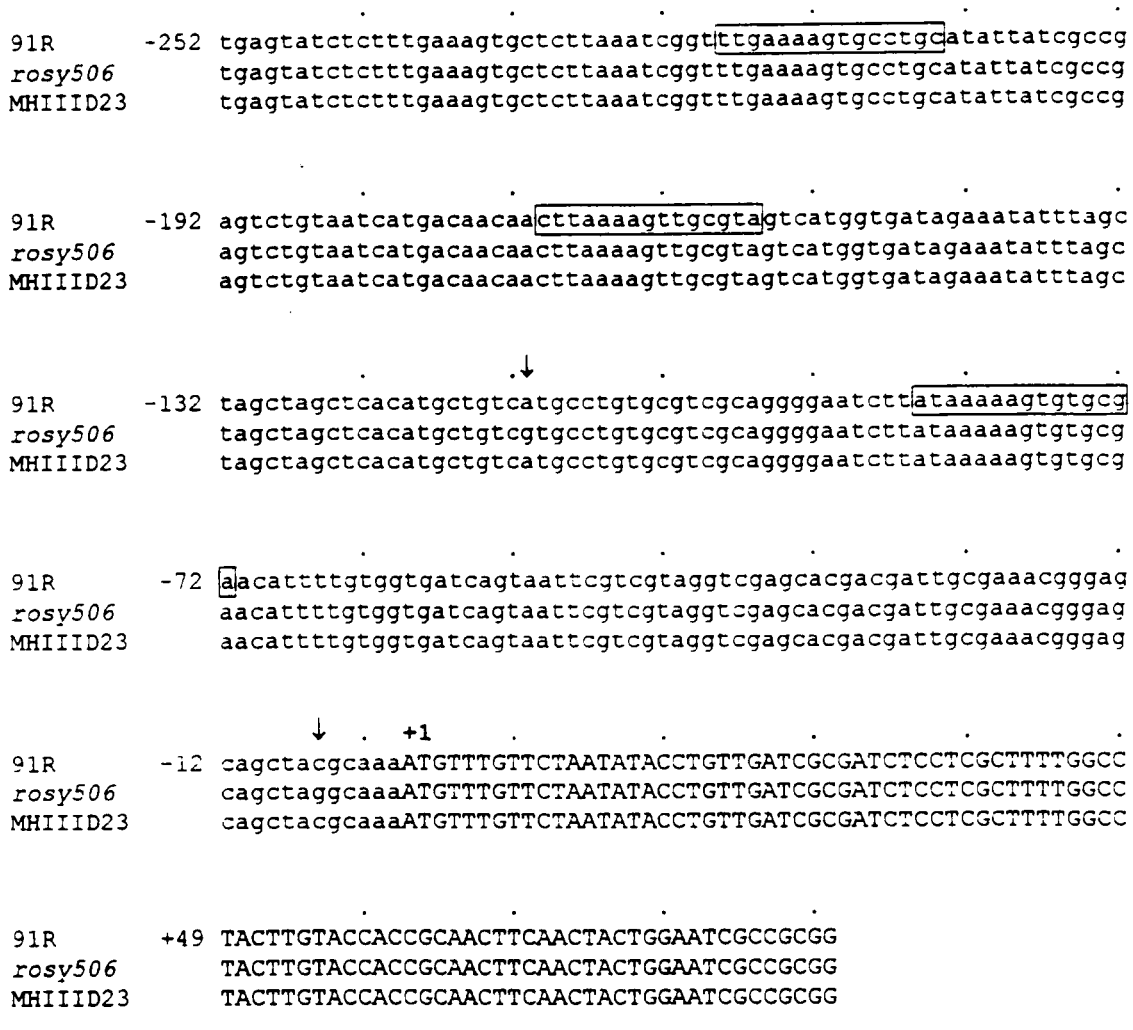
91R       -552 ccattacttaaacacctttacctttagttacaagtgggatcgtcctgtacatttgaagct
rosy506        ccattacttaaacacctttacctttagttacaagtgggatcgtcctgtacatttgaagct
MHIIID23       ccattacttaaacacctttacctttagttacaagtgggatcgtcctgtacatttgaagct

               EcRE
91R       -492 actttgaatgaccgtgtcattgcgtttttatagtaatacaatgatttgatacattttccg
rosy506        actttgaatgaccgtgtcattgcgtttttatagtaatacaatgatttgatacattttccg
MHIIID23       actttgaatgaccgtgtcattgcgtttttatagtaatacaatgatttgatacattttccg

91R       -432 catatcgatgtgtatttaccatttaatgctacctctacttttcgcacatgccgagcttat
rosy506        catatcgatgtgtatttaccatttaatgctacctctacttttcgcacatgccgagcttat
MHIIID23       catatcgatgtgtatttaccatttaatgctacctctacttttcgcacatgccgagcttat

                                                        ↓
91R       -372 aaatgagtgataaaacctgatgggacacttttacccaagcaccaaccggtttgtttaac
rosy506        aaatgagtgataaaacctgatgggacacttttacccaagcaccaaccggtttgtttaac
MHIIID23       aaatgagtgataaaacctgatgggacacttttacccaagcacccaccggtttgtttaac

91R       -312 aacaatctcgttatcatttgtttatattttgattcgcgagcatttttgcagtgaagcgta
rosy506        aacaatctcgttatcatttgtttatattttgattcgcgagcatttttgcagtgaagcgta
MHIIID23       aacaatctcgttatcatttgtttatattttgattcgcgagcatttttgcagtgaagcgta
```


91R -252 tgagtatctctttgaaagtgctcttaaatcggttttgaaaagtgcctgcatattatcgccg
rosy506 tgagtatctctttgaaagtgctcttaaatcggtttgaaaagtgcctgcatattatcgccg
MHIIID23 tgagtatctctttgaaagtgctcttaaatcggtttgaaaagtgcctgcatattatcgccg
91R -192 agtctgtaatcatgacaacaacttaaaagttgcgtagtcatggtgatagaaatatttagc
rosy506 agtctgtaatcatgacaacaacttaaaagttgcgtagtcatggtgatagaaatatttagc
MHIIID23 agtctgtaatcatgacaacaacttaaaagttgcgtagtcatggtgatagaaatatttagc
91R -132 tagctagctcacatgctgtcatgcctgtgcgtcgcaggggaatcttataaaaagtgtgcg
rosy506 tagctagctcacatgctgtcgtgcctgtgcgtcgcaggggaatcttataaaaagtgtgcg
MHIIID23 tagctagctcacatgctgtcatgcctgtgcgtcgcaggggaatcttataaaaagtgtgcg
91R -72 aacattttgtggtgatcagtaattcgtcgtaggtcgagcacgacgattgcgaaacgggag
rosy506 aacattttgtggtgatcagtaattcgtcgtaggtcgagcacgacgattgcgaaacgggag
MHIIID23 aacattttgtggtgatcagtaattcgtcgtaggtcgagcacgacgattgcgaaacgggag
+1
91R -12 cagctacgcaaaATGTTTGTTCTAATATACCTGTTGATCGCGATCTCCTCGCTTTTGGCC
rosy506 cagctaggcaaaATGTTTGTTCTAATATACCTGTTGATCGCGATCTCCTCGCTTTTGGCC
MHIIID23 cagctacgcaaaATGTTTGTTCTAATATACCTGTTGATCGCGATCTCCTCGCTTTTGGCC
91R +49 TACTTGTACCACCGCAACTTCAACTACTGGAATCGCCGCGG
rosy506 TACTTGTACCACCGCAACTTCAACTACTGGAATCGCCGCGG
MHIIID23 TACTTGTACCACCGCAACTTCAACTACTGGAATCGCCGCGG

functional significance of these differences in the base sequence remains unknown, the observation that the nucleotides at –112 and –6 are identical in two overproducer strains (91R and MHIII-D23) is interesting. These bases may be functionally important and play a role in the constitutive overexpression of *Cyp6a2* in these two strains.

In Chapter 2, the transgenic experiments showed that the genetic background of the ry^{506} strain could support the expression of *Cyp6a2-91R* transgenes with 1331 or 985 bases of upstream DNA. This implies that the upstream DNA between +1 and –1331 has sequence motifs that are recognized by transcriptional regulatory proteins present in ry^{506}. To gain insight into what transcription factors may regulate the expression of *Cyp6a2*, the nucleotide sequence between +1 and –1331 was analyzed with the MatInspector program (Quandt *et al.*, 1995) for binding sites for known *Drosophila* transcription factors reported in the TRANSFAC database (Wingender, 1994). Using a matrix similarity of ≥85%, the MatInspector program identified binding sites for many different transcription factors. Of these sequence motifs three are discussed. One is the binding site for an ecdysone-inducible transcription factor called Broad-complex (BR-C) (von Kalm *et al.*, 1994). Three BR-C motifs were identified on the non-transcribed strand (RNA strand) of *Cyp6a2* and they are located at positions –1183, -861 and –625. BR-C is a primary ecdysone response gene that encodes a class of TFIIIA-like zinc finger proteins that tightly regulate the spatial and temporal expression of many ecdysone-inducible genes during *Drosophila* metamorphosis (von Kalm *et al.*, 1994 and references therein). Interestingly, the work of Saner *et al.* (1996) suggests that *Cyp6a2* expression may be modulated by ecdysone. These investigators showed that the steady-state level of

CYP6A2 mRNA varies during the development of *D. melanogaster*. Qualitative northern blot analysis of total RNA isolated at different stages of development showed that the level of CYP6A2 mRNA was barely detectable in embryos, gradually increased during development, peaked at the third instar and pupal stages, then declined to low levels in the adult (Saner *et al.*, 1996). Interestingly, this pattern of expression is very similar to the ecdysteroid hormone titer during the development of *D. melanogaster* (Ashburner, 1989), thereby suggesting that ecdysone may regulate the expression of *Cyp6a2* during development. Later studies by Spiegelman *et al.* (1997) support this hypothesis. These investigators showed that at the non-permissive temperature (29°C) flies homozygous for the *ecd-1*^{ts} mutation fail to produce the precursor of 20-hydroxyecdysone and have two-fold lower level of CYP6A2 mRNA compared to the mutant flies when they were reared at the permissive temperature of 20° C. These observations suggest that the BR-C binding sites found in the upstream DNA may be functionally important.

In light of these observations, it was of interest to see if the upstream DNA from +1 to −1331 contained an ecdysone response element (EcRE) (Antoniewski *et al.*, 1993). A functional EcRE is an imperfect palindromic sequence that is very similar to the regulatory sequence that binds many vertebrate steroid hormone complexes (Riddihough and Pelham, 1987; Cherbas *et al.*, 1991; Luo *et al.*, 1991; Martinez *et al.*, 1991; Antoniewski *et al.*, 1993). In *Drosophila*, the EcRE binds a nuclear receptor complex that is a heterodimer of the ecydsone recepter (EcR) and Ultraspiracle (Usp), the *Drosophila* homologue of the vertebrate retinoid-X receptor (Antoniewski *et al.*, 1995; Antoniewski *et al.*, 1996; Crispi *et al.*, 1998). EcRE sequences have been reported in

many ecdysone inducible genes of *D. melanogaster*, including heat shock protein 27 (*hsp27*), fat body protein 1 (*Fbp1*), glue protein genes, and larval serum protein (*Lsp-2*) (Antoniewski *et al.*, 1993). Using the Genetic Computer Group (GCG) sequence analysis software package (Genetics Computer Group, 1994), a best-fit analysis of the EcRE sequence reported by Antoniewski *et al.* (1993) with the −1331 to +1 region of the upstream DNA of *Cyp6a2-91R* was performed. The results (Table 5) showed that a putative EcRE sequence is located at position –491 and is 77% identical to the reported consensus sequence. To see if the EcRE is found in the upstream DNA of other insect *CYP6* genes, the upstream DNA sequence of other *CYP6* genes of *D. melanogaster* (Maitra *et al.*, 1996), house fly(Cohen *et al.*, 1994; Cohen and Feyereisen, 1995) and *Papilio polyxenes* (black swallowtail butterfly)(Prapaipong *et al.*, 1994; Hung *et al.*, 1996) were examined. The results showed that like *Cyp6a2*, the upstream DNA of these nine *CYP6* family genes contain an EcRE motif showing 77-92% identity with the reported consensus sequence. Taken together, these observations suggest that *Cyp6a2* and possibly other *Cyp6* genes may be regulated either directly by a ecdysone receptor complex or indirectly via the action of an ecdysone-inducible transcription factor such as BR-C.

The MatInspector program also identified a 15-17 base pair sequence motif known as a "barbie box," a *cis*-regulatory element that is found in the upstream DNA of barbiturate-inducible cytochrome P450 genes of *Bacillus megaterium*, mouse and rat (Shaw and Fulco, 1993; Liang *et al.*, 1995 and references therein). Between the +1 and

Table 5: Sequence comparison of the putative ecdysone response element (EcRE) present in the upstream DNA of different insect *CYP6* genes

Organism (strain)	position from ATG (+1)	Putative EcRE[a]													% identity with consensus
Drosophila melanogaster															
Cyp6a2 (*ros*[UM] and 91-R)	-491	c	t	T	t	G	A	A	t	G	A	C	C	g	77
Cyp6a8 (91-R)	-102	c	t	G	a	G	A	A	t	G	C	A	T	c	77
Musca domestica															
CYP6A1 (Rutgers)	-204	A	a	T	g	C	c	A	t	G	A	C	T	T	77
CYP6A1 (aabys)	-204	A	G	T	g	C	c	A	t	G	A	C	T	T	85
CYP6A3 (sbo)	-80	c	a	G	T	C	A	T	t	G	C	g	C	C	77
CYP6A4 (sbo)	-197	t	G	t	T	C	A	t	t	a	A	t	T	C	77
CYP6A5 (sbo)	-141	G	G	G	t	G	A	G	a	G	A	A	C	a	85
CYP6C1 (sbo)	-54	G	G	G	g	C	A	T	T	G	A	C	a	a	77
Papilio polyxenes															
CYP6B1	-653	A	t	G	t	G	A	A	t	G	A	C	T	t	92
CYP6B3v2	-478	A	G	t	t	G	A	G	a	t	A	A	C	T	85
EcRE consensus[b]		A/G	G	G/T	T	G/C	A	N	T	G	A/C	A/C	C/T	C/T	

[a] Matches to the consensus sequence are shown in uppercase.

[b] Sequences were compared to the consensus sequence reported by Antoniewski *et al.*, 1995. *Mol. Gen Genet.* 249: 545-556.

–985 region of the upstream DNA of *Cyp6a2*, a distal and proximal group of three, closely clustered barbie boxes were identified. These putative barbie boxes are located at positions –623, -617, -588, -219, -171 and –86 (Figure 18). The similarities of these barbie boxes with the published consensus sequence (Liang *et al.*, 1995) are shown in Table 6. Interestingly, these barbie boxes are located in the vicinity of the six base substitutions that are found between in *Cyp6a2-91R* (Figure 18). Previous studies by Dunkov *et al.* (1997) and Brun *et al.* (1996) have shown that *Cyp6a2* is inducible with phenobarbital but the effect of other barbiturates on *Cyp6a2* expression has not been explored. Experiments designed to look into the phenomenon of barbiturate-mediated induction of *Cyp6a2* and the functional significance of the putative six barbie boxes found in the upstream DNA are presented in the next chapter.

Table 6: Sequence analysis of the putative barbie box sequences between –1331 and –129 of the upstream DNA of *Cyp6a2-91R*, *Cyp6a2-ry* and *Cyp6a2-MHIII*

Position from ATG (+1)	Putative Barbie Box Sequence															% identity with consensus
-623	A	T	A	A	A	A	A	G	C	A	A	A	A	G	A	67
-617	A	G	C	A	A	A	A	G	A	G	C	T	T	G	G	60
-588	A	A	T	G	A	A	A	G	C	T	A	G	A	T	G	67
-219	T	T	G	A	A	A	A	G	T	G	C	C	T	G	C	47
-171	C	T	T	A	A	A	A	G	T	T	G	C	G	T	A	60
-86	A	T	A	A	A	A	A	G	T	G	T	G	C	G	A	60
Barbie Box Consensus Sequence*																
	A	**T**	**C**	**A**	**A**	**A**	**A**	**G**	**C**	**T**	**G**	**G**	**A**	**G**	**G**	

* Sequences were compared with the consensus sequence published by Liang *et al.*, 1995 *J Biol Chem* 270(9): 4438-4450.

Chapter 4

Analysis of barbiturate-mediated induction of *Cyp6a2* in different strains and transgenic lines of *Drosophila melanogaster*

Introduction

Phenobarbital (PB) and other barbiturate compounds have long been recognized as classical inducers of *CYP* gene expression (Conney, 1967; see review by Waxman and Azaroff, 1992). There are numerous reports in the literature which indicate that the elevated level of CYP mRNA following exposure to barbiturate compounds is due to increased transcription from a barbiturate-inducible promoter rather than due to enhanced mRNA stability (see Fulco, 1991 for review; Waxman and Azaroff, 1992; Trottier *et al.*, 1995; Park *et al.*, 1996; Honkakoski and Negishi, 1997; Kim and Kemper, 1997).

There is considerable information available regarding the molecular mechanisms that regulate the barbiturate-induced expression of the $P450_{BM-1}$ (*CYP106*) and $P450_{BM-3}$ (*CYP102*) genes of *Bacillus megaterium* (Wen *et al.*, 1989; Fulco, 1991; Shaw and Fulco, 1993; Liang *et al.*, 1995; Liang and Fulco, 1995; He *et al.*, 1995; Gaidamakova *et al.*, 1996; Shaw *et al.*, 1998). However, less is known about this phenomenon in *Drosophila* and other invertebrates. In the housefly, both the *CYP6A1* and *CYP6D1* genes are induced by PB (Cariño *et al.*, 1992; Scott and Lee, 1993; Liu and Scott, 1997), and a locus on chromosome 2 is believed to regulate the PB-mediated induction of the *CYP6D1* gene located on chromosome 1 (Liu and Scott, 1997). However, the molecular basis of

barbiturate-induced expression of insect *CYP* genes is not well understood. It is not known whether barbiturate compounds act directly with the *cis*-regulatory elements of the *CYP* genes or via some other *trans*-regulatory proteins that control transcription of these genes.

Recently, Dunkov *et al.* (1997) analyzed the upstream DNA of *Cyp6a2* DNA for constitutive expression and PB inducibility by transfecting *Drosophila* embryonic cells with *luciferase* reporter gene fused with different lengths of *Cyp6a2* upstream DNA. Although this study has contributed some information with regard to the promoter activity of different regions of the upstream DNA of *Cyp6a2*, it is not known whether the promoter activity seen in the embryonic cell line is comparable to that in the adult fly.

In the present investigation, it was determined if there is any difference between the underproducer and overproducer strains of CYP6A2 mRNA with respect to their induction with two barbiturate compounds, phenobarbital and barbital. In addition, P element-mediated germ line transformation was used to functionally examine the upstream DNA of *Cyp6a2* for the DNA sequences involved in barbital-mediated induction of *Cyp6a2* in the adult fly.

Materials and Methods

Barbiturates and treatment of *D. melanogaster* strains and transgenic lines

Phenobarbital (5-ethyl-5-phenyl-2,4,6-(1H,3H,5H)-pyrimidinetrione monosodium salt) was purchased from Research Biochemicals International, Natick, MA and barbital (5, 5'-diethyl barbituric acid) was purchased from Sigma Chemical Company. The

chemical structure of these two barbiturate compounds is illustrated in Figure 19. Structurally, these two compounds are identical except that at carbon 5 barbital has an ethyl group and phenobarbital has a phenyl group. To examine the effect of PB and barbital on *Cyp6a2* expression, 4 gm of instant *Drosophila* medium (Carolina Biological Supply, Inc, Burlington, NC) was hydrated with 7 ml of freshly prepared 8 or 16 mM aqueous barbiturate solution. Adult females (2-3 days old) were allowed to feed on the barbiturate-containing medium for 24 hours. Instant medium prepared in water was used as the untreated control. To localize the *cis*-regulatory sequences required for barbital-mediated induction of *Cyp6a2*, a mixture of an equal proportion of adult males and females (2-3 days old) of the ry^{506} strain, and transgenic lines 7F/-985 and 1A/-129 were treated with 0.1M barbital solution for 48 hours.

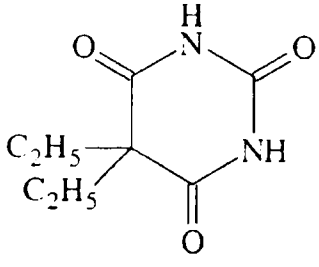
Nucleic acid isolation and RNA blot analysis

Total and poly(A)$^+$ RNA were isolated from adult flies as described in Chapter 2. The barbiturate-induced expression of *Cyp6a2* was analyzed by northern and slot blot hybridization with CYP6A2 and RP49 cDNA probes as described in Chapter 2.

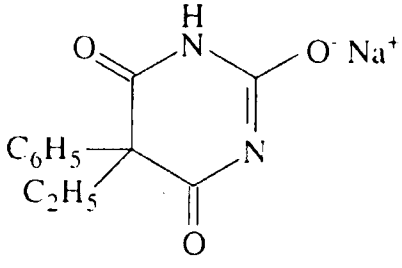
Results and Discussion

Examination of the barbital- and PB-induced expression of *Cyp6a2* in different underproducer and overproducer strains

To study the effect of different barbiturate compounds on the expression of different *Cyp6a2* alleles, adult females from two underproducer (ry^{506} and 91C) and two



Barbituric Acid (barbital)



Phenobarbital

Figure 19: Chemical structure of barbituric acid (barbital) and phenobarbital.

overproducer (91R and MHIII-D23) strains were treated with 8 and 16 mM barbital or PB for 24 hours. The levels of CYP6A2 mRNA in these four treated strains and the untreated controls were examined by northern blot hybridization. In the underproducer ry^{506} strain, it seems that the level of CYP6A2 RNA is higher in flies treated with barbital or PB (Figure 20). However, when RP49 RNA is used as an internal control to normalize the data, no difference in the level of CYP6A2 mRNA between the treated and untreated flies was found (data not shown). Therefore, it appears that in the ry^{506} strain the *Cyp6a2-ry* allele shows little induction with 8mM and 16mM barbital and PB. However, a greater molar concentration of either of these barbiturate compounds may be more effective on the induction of the *Cyp6a2-ry* allele.

Unlike in the ry^{506} strain, a much higher level of induction of *Cyp6a2* expression was seen in the 91C strain, which is also an underproducer of CYP6A2. Interstingly, of all the strains that were examined, the level of induction of *Cyp6a2* with these molar concentrations of barbital and PB was highest in the underproducer 91C strain (compare data in Figures 20, 21 and 22). Furthermore, both compounds increased the level of CYP6A2 mRNA in a dose-dependent manner (Figure 22). Taken together, these data suggest that the two underproducer strains, ry^{506} and 91C, differ with regard to the molecular components involved in inducibility of *Cyp6a2* to barbital and PB. Alternatively, the variation in the pattern of expression following treatment with these two barbiturate compounds may be a function of the mRNA turnover rate in each strain.

The expression of *Cyp6a2* in the overproducers (91R and MHIII-D23) of CYP6A2 was also found to be different following barbital and PB treatment. As in 91C,

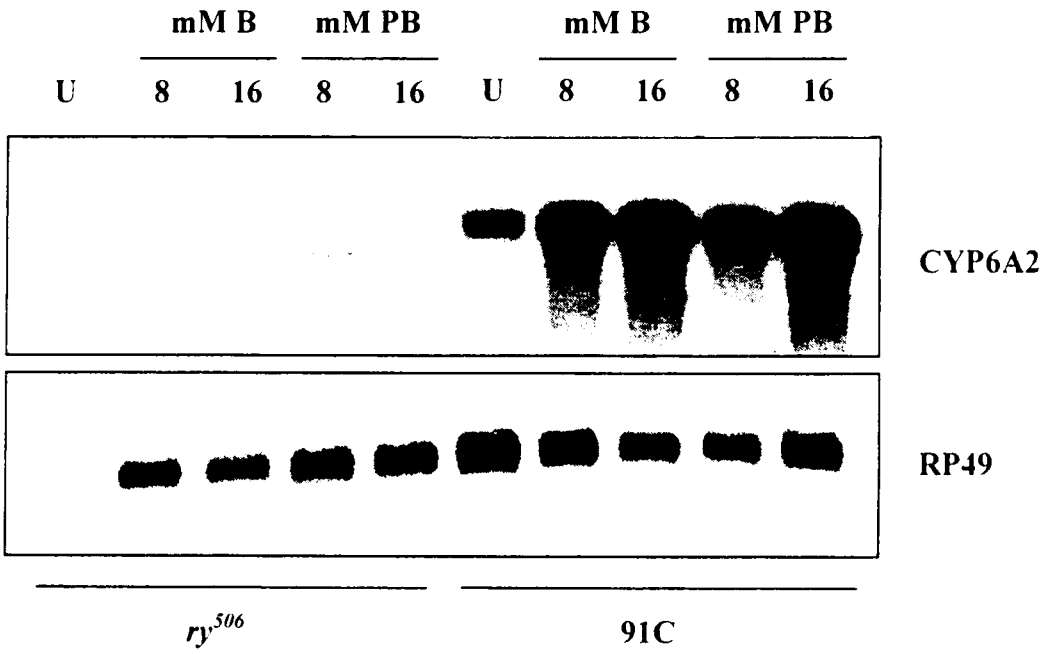


Figure 20: *Cyp6a2* expression in barbital- and phenobarbital-treated underproducer strains of *D. melanogaster*. Poly(A)$^+$ RNA (1 μg) from adult females treated with 8 or 16 mM barbital (B) or phenobarbital (PB) was electrophoresed on a 2.2M-1.2% formaldehyde agarose gel, transferred onto nylon and hybridized with CYP6A2 and RP49 cDNA probes as described in the Materials and Methods to Chapter 2. Poly(A)$^+$ RNA from flies allowed to feed on non-barbiturate medium was used as the untreated control (U).

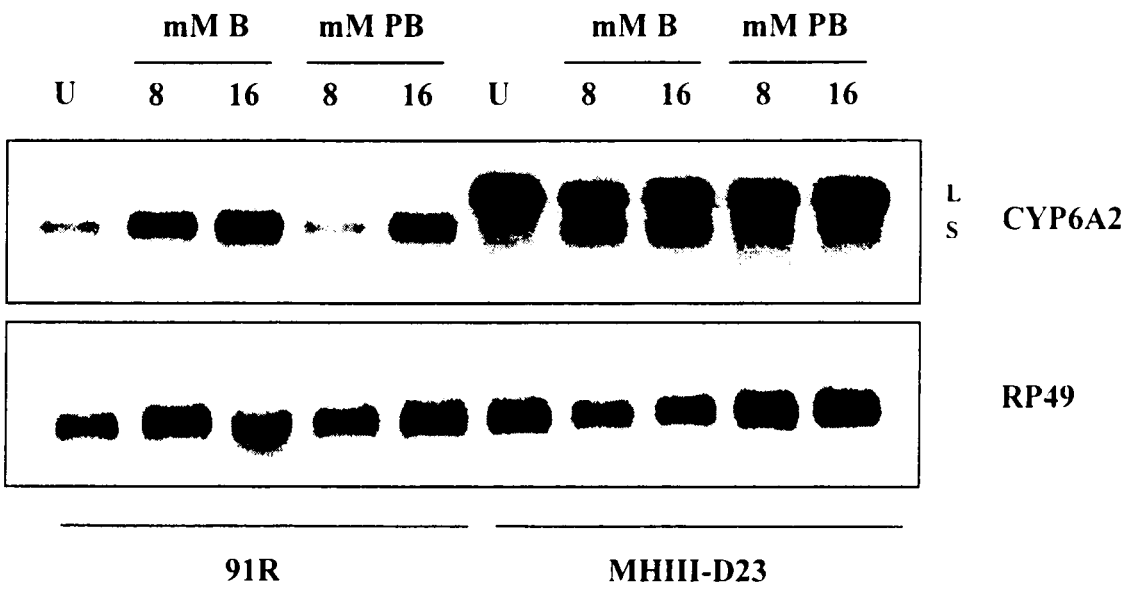


Figure 21: *Cyp6a2* expression in barbital- and phenobarbital-treated overproducer strains of *D. melanogaster*. Poly(A)$^+$ RNA (1 μg) from adult females treated with 8 or 16 mM barbital (B) or phenobarbital (PB) was electrophoresed on a 2.2M-1.2% formaldehyde agarose gel, transferred onto nylon and hybridized with CYP6A2 and RP49 cDNA probes as described in the Materials and Methods of Chapter 2. Poly(A)$^+$ RNA from flies allowed to feed on non-barbiturate medium was used as the untreated control (U). "L" and "S" indicate the large and small, respectively, CYP6A2 transcripts in the MHIII-D23 strain.

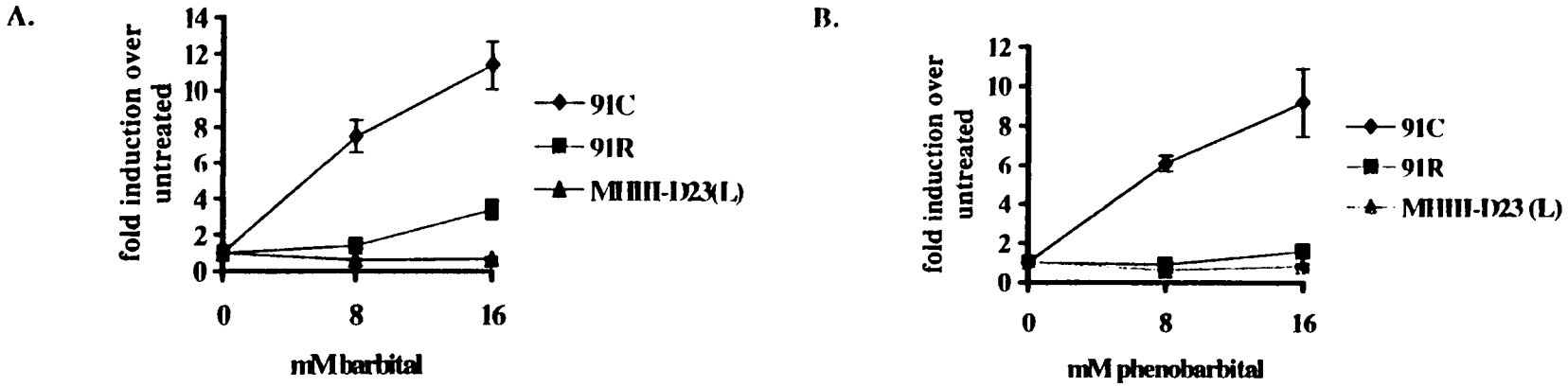


Figure 22: (A,B) Dose response curves of the effect of barbital and phenobarbital on *Cyp6a2* expression in different underproducer and overproducer strains of *D. melanogaster*. Hybridization signals from representative northern blots were quantified with the Packard InstantImager Radioanalytical Imaging System. Each point represents the average of three independent experiments. In Panels A and B , MHHH-D23(L) represents the larger transcript in barbital- and phenobarbital-treated flies (see Figure 21)

barbital-mediated induction of *Cyp6a2* was observed in the 91R strain (Figures 21 and 22), but at a much lower level. With 8 and 16mM barbital, the level of induction was 1.4 and 3.4 times that of the untreated control, respectively (Figure 22). These values were found to be statistically significant ($p \leq 0.02$) using the Student's t-test. Induction of the *Cyp6a2* allele of the 91R strain was also found to be much lower with PB. In fact, with 8mM PB the expression of the *Cyp6a2-91R* allele appeared to be inhibited. However, analysis of the data with the Student's t-test showed that this is not a statistically significant decrease ($p>0.10$). However, when the flies were treated with 16 mM PB, a slight, but significantly significant ($p<0.02$) increase (1.7-fold) in the level of CYP6A2 mRNA was observed relative to the untreated control (Figure 22).

In the other overproducer strain, MHIII-D23, barbital treatment produced an interesting observation. In this strain, two mRNA species hybridized with the CYP6A2 cDNA probe (Figure 21). One transcript (L) is identical in size to the CYP6A2 RNA found in the uninduced MHIII-D23 strain. The size of the shorter transcript (S) is similar to that found in strains (ry^{506} and 91R) that do not have LTR insertion in the 3'-UTR of *Cyp6a2*. In PB-treated flies the shorter transcript (S) is also present but it is barely visible. The S transcript, however, is not found in untreated MHIII-D23 flies (Figure 21.). Quantitative analysis showed that the levels of the L transcript in flies treated with 8mM and 16mM barbital or PB were significantly lower ($p \leq 0.05$) than the level found in untreated flies (Figure 22), suggesting that the barbiturates are inhibitory for the *Cyp6a2* allele producing L transcript. The *Cyp6a2* allele producing the S transcript, however, is

markedly induced by barbital because this transcript is not detectable in the untreated flies. Interestingly, this allele is barely induced by PB.

The expression of two transcripts (L and S) with barbiturate compounds suggests that this strain carries the LTR-minus S allele in the population. To explore this possibility further, the PCR data obtained in Chapter 2 (Figure 3) were reexamined. The results showed that in the lane containing the amplified genomic DNA of the MHIII-D23 strain (Figure 3A) there was another amplified DNA product that was similar in size to the PCR product obtained from 91R control, which does not have LTR insertion (Chapter 2, Figure 3B). The intensity of this amplified DNA product was much lower than the major amplified 1.4-kb PCR product. The unequal quantity of these two PCR products suggests that the MHIII-D23 strain does carry the S allele of *Cyp6a2* in the population but at a low frequency. Another explanation could be that only the L allele is present in the population but the S transcript represents a splice variant derived from post-transcriptional processing of the L transcript.

The results described above suggest that relative to the underproducer 91C strain, the *Cyp6a2* alleles in overproducer 91R and MHIII-D23 strains show little or no induction with 8 and 16mM PB. Similar observations have been made in PB treated underproducer and overproducer stains of the house fly(Cariño *et al.*, 1994). CYP6A1 mRNA is approximately 10-fold greater in the overproducer Rutgers strain relative to the underproducer *sbo* strain (Cariño *et al.*, 1994). Treatment of adult females of both strains with 4mM PB for 12 hours results in a 100-fold increase in CYP6A1 mRNA levels in the *sbo* strain but only a 22-fold increase in the Rutgers strain (Cariño *et al.*, 1992). In the

overproducer LPR strain, CYP6D1 mRNA is approximately 9-fold more abundant than in the underproducer *aabys* strain (Liu and Scott, 1996, 1997). Treatment of adult females of both strains with 12mM PB for 24 hours caused a 3-fold increase in the CYP6D1 mRNA levels in the *aabys* strain. The LPR strain was refractory to this dose of PB and showed no increase in CYP6D1 mRNA levels. A lower level of induction of a *Cyp6a2* allele in another overproducer strain was also observed when prolonged PB treatment was given (Brun *et al.*, 1996). The constitutive level of CYP6A2 mRNA is approximately 6-fold greater in the overproducer RDDT^R strain of *D. melanogaster* than in the wild-type, underproducer Canton S strain (Brun *et al.*, 1996). An examination of CYP6A2 mRNA levels in both strains after treatment with 10mM PB for 72 hours showed that CYP6A2 mRNA levels were increased 15-fold in Canton-S but only 2.5-fold in RDDT^R (Brun *et al.*, 1996). Collectively, the induction data from house flyand *Drosophila* indicate that at low doses of barbiturate compounds *CYP6* gene expression is induced more in underproducer than in overproducer strains. However, at higher doses of barbiturate compounds (i.e., >16mM) *CYP6* and specifically *CYP6A2* may show higher fold induction.

Analysis of the upstream DNA of *Cyp6a2* for barbital-mediated induction

In the previous chapter it was reported that the –1331 to +1 region of the upstream DNA of *Cyp6a2* contains six barbie-box sequence motifs, *cis*-regulatory elements that are believed to be involved in barbiturate-mediated induction of cytochrome P450 genes (Liang *et al.*, 1995; Gaidamakova, 1996). To examine whether these sequences are involved in barbital-mediated induction of *Cyp6a2*, a transgenic approach was used.

Therefore, transformant lines with –986 (7F/-986) and –129 (1A/-129) of upstream DNA that were created in the ry^{506} host strain (Chapter 3) were used for this purpose. Since the *Cyp6a2-ry* allele showed slight induction with 16mM barbital after 24 hours (Figure 20), the molar concentration of barbital and length of treatment were increased in the present investigation. A mixture of an equal proportion of male and female adults of lines 7F/-986 and 1A/-129 were allowed to feed on 100mM barbital-containing medium for 48 hours. As a control, the ry^{506} host strain was used.

Poly(A)$^+$ RNA isolated from the treated and untreated transgenic and host strain flies was analyzed by northern blot hybridization. In transgenic lines 7F/-985 and 1A/-129, the size of the CYP6A2 mRNA encoded by the endogenous *Cyp6a2-ry* gene and the *Cyp6a2-91R* transgene are identical and cannot be distinguished from one another (Figure 17A). Therefore, the observed level of CYP6A2 RNA in treated and untreated transgenic lines will be a cumulative effect of the expression of both the endogenous *Cyp6a2-ry* gene and the *Cyp6a2-91R* transgene. However, the levels of CYP6A2 mRNA in the treated and untreated ry^{506} host strain can be subtracted from the levels found in the treated and untreated transgenic flies to determine the level of CYP6A2 RNA produced by the *Cyp6a2-91R* transgene.

Laser densitometric analysis of the hybridization signals showed that in transgenic line 7F/-985, the level of CYP6A2 mRNA in treated and untreated flies was 4- and 6-fold higher than in the treated and untreated ry^{506} host strain, respectively (Figure 23). However, the levels of CYP6A2 mRNA in untreated or treated flies of line 1A/-129 and ry^{506} host strain were almost identical. These results suggest that the sequences between

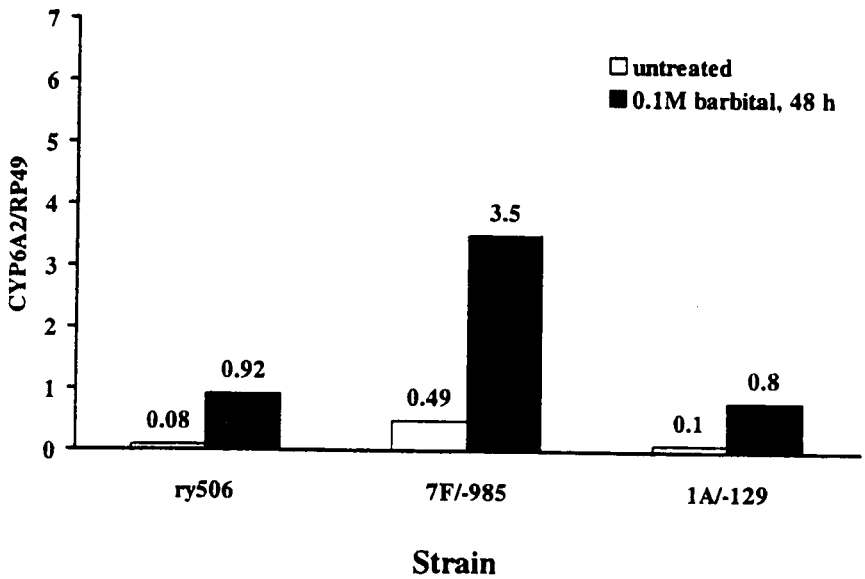


Figure 23: Quantification of CYP6A2 mRNA levels in untreated and barbital-treated *ry*^{506} host and transgenic flies. Flies (2-3 days old) were kept for 48 hours on instant *Drosophila* medium reconstituted in water (untreated) or 0.1M aqueous barbital solution. Poly(A)$^+$ RNA (5 μg) from the untreated and barbital-treated *ry*^{506} host and transgenic lines 7F/-985 and 1A/-129 was electrophoresd on a 2.2M formaldehyde-1.2% agarose gel, transferred onto nylon and hybridized with CYP6A2 and RP49 cDNA probes as described in the Materials and Methods of Chapter 2. Hybridization signals were quantified with a laser densitometer. See Figure 11 for schematic representation of the *Cyp6a2-91R* trangene constructs used in this experiment.

–985 and –129 are needed for constitutive as well as barbital-induced expression of the *Cyp6a2-91R* transgene. Chapter 3 has shown that five putative barbie boxes are present in the DNA between –989 and –129 bp. Therefore, these barbie boxes may be involved in the induction of the *Cyp6a2-91R* transgene. Since the induced expression of the *Cyp6a2-91R* transgene in transgenic line 1A/-129 was very similar to the induced expression in the ry^{506} host strain (0.8 compared to 0.92, respectively), this suggests that the barbie box sequence located at –86 may not be used for barbital-mediated induction of *Cyp6a2*.

Dunkov *et al.* (1997) reported that *Cyp6a2* promoter constructs with 1328 and 984 bases of upstream DNA showed slightly higher levels of reporter gene activity relative to a reporter gene construct with 428 base pairs of DNA upstream of +1 ATG. However, since no barbie boxes are present within –1328 to –984, it is possible that other sequences present between –1328 and –984 bases may also be involved in PB induction. In fact, there is some controversy regarding whether the same sequences that mediate the barbiturate-induced expression of the $P450_{BM-1}$ and $P450_{BM-3}$ genes of *B. megaterium* (Wen *et al.*, 1989; Fulco, 1991; Liang *et al.*, 1995; Liang and Fulco, 1995; He *et al.*, 1995) also play a role in the barbiturate-induced expression of eukaryotic *CYP* genes. For example, mutation of the single barbie box-like sequence located between –89 and –73 bases of the upstream DNA of the PB-inducible *CYP2B2* gene of the rat did not affect its responsiveness to PB (Park *et al.*, 1996). Rather, a distal PB-responsive element between –2318 and –2155 was found to be necessary for PB induction (Trottier *et al.*, 1995; Park *et al.*, 1996). In another study, a 132 bp PB-responsive element in the 5'-flanking DNA

of the mouse *Cyp2b10* gene was found to contain binding sites for nuclear factor 1 and nuclear receptor-like factors (Honkakoski and Negishi, 1997). Mutation of these binding sites was found to abolish PB-mediated induction (Honkakoski and Negishi, 1997). Therefore, it cannot be said definitively whether some of all of the barbie box sequences found in the upstream DNA of *Cyp6a2* play a role in barbital-induced expression. Site directed mutagenesis may resolve this issue.

Chapter 5

General Discussion

The CYP6 family of enzymes is exclusive to insects and includes at least 19 members comprising four different subfamilies (Directory of P450-containing Systems, October 1998). Several members of the CYP6A subfamily are constitutively overexpressed in insecticide resistant strains of *M. domestica* and *D. melanogaster* (Cariño *et al.*, 1992; Waters *et al.*, 1992a,b; Tomita and Scott, 1995; Tomita *et al.*, 1995; Liu and Scott, 1996; Maitra *et al.*, 1996; Dombrowski *et al.*, in press). However, the molecular and genetic mechanisms that regulate the constitutive overexpression of these resistance-related forms are not completely understood. To help bridge this gap in our knowledge of the regulation of insect *CYP6* genes, the results of the experiments presented in Chapters 2-4 have been published (Dombrowski *et al.*, in press). The work presented in this dissertation provides several leads on which future experiments can be designed to investigate *CYP6* gene regulation in *Drosophila* and *Musca.*

An examination of the constitutive level of CYP6A2 mRNA in the different strains of *D. melanogaster* used in the studies described herein suggest that the presence of the LTR of transposable element *17.6* in the 3'-UTR is not the reason for the low level of *Cyp6a2* expression. In fact, a constitutively low level of CYP6A2 mRNA appears to be the wild-type phenotype of *Cyp6a2* since the majority of the strains that were examined have a constitutively low level of *Cyp6a2* expression. This observation agrees with that made by Dunkov *et al.* (1997), Saner *et al.* (1996) and Brun *et al.* (1996), who

also found a low level of CYP6A2 mRNA in adults of several different wild-type strains. Similarly, the *CYP6A1* gene of the house flyis also constitutively expressed at reduced levels in adults (Cariño *et al.*, 1992). Here it should be stressed that all of these published studies have only examined the actual steady-state level of CYP6A2 mRNA. Thus, there is the possibility that the variation in the level of CYP6A2 mRNA between different strains may still be a function of mRNA stability. To test this hypothesis further, nuclear run-off assays will need to be performed to determine if the observed differences are due to changes in the mRNA turnover rate or an increase/decrease in the rate of transcription.

If low expression is considered as the wild-type phenotype of *Cyp6a2*, then overexpression in the 91R and MHIII-D23 strains needs to be viewed as a mutant phenotype of the gene. Two hypotheses that are not mutually exclusive have been proposed in this study to explain the constitutive overexpression of *Cyp6a2* in 91R and MHIII-D23. Both hypotheses are based on the idea that in the wild-type situation, *Cyp6a2* expression is downregulated by a transcriptional repressor. First, it was hypothesized that there may be a gain-of-function mutation in the *cis*-regulatory elements of the *Cyp6a2* gene and this may alter or prevent the binding of the transcriptional repressor. Alternatively, it is possible that the *cis*-acting elements are wild-type, but there may be a loss-of -function mutation in the gene encoding the repressor. In either case, the presence of this gain-of-function and/or loss-of-function mutation is expected to cause overexpression of *Cyp6a2*. Of these two mutations, one would favor the loss-of-function mutation of the repressor gene as a possible explanation because overexpression of another family 6 gene (*Cyp6a8*) is also found in 91R and MHIII-D23 strains (Maitra *et*

al., 1996; Maitra *et al.*, unpublished observations). In many respects, the pattern of expression of *Cyp6a8* is very similar that of *Cyp6a2* (Maitra *et al.*, unpublished observations), suggesting that these two genes may be under the control of a common transcriptional repressor. Therefore, a loss-of-function mutation in the gene encoding this repressor can affect both *Cyp6a* genes.

The transgenic experiments presented in this dissertation demonstrated that the *Cyp6a2-91R* allele shows expression when it is placed in the genome of ry^{506}. This observation is intriguing because the expression of the endogenous *Cyp6a2* gene in the ry^{506} host is barely detectable. This observation also suggests that the positive factors required for *Cyp6a2* transcription are present in the ry^{506} strain. Then, why does the *Cyp6a2-ry* allele show a barely detectable level of expression in the ry^{506} strain? Based on the transgenic data and the repressor hypothesis explained above, it can be proposed that *Cyp6a2* has a *cis*-acting silencer element which binds the repressor molecule and these sequences are not present in the *Cyp6a2-91R* transgene constructs that I have used in this investigation. The endogenous *Cyp6a2-ry* allele, however, has these silencer elements. Then according to this scenario, one would expect expression of the *Cyp6a2-91R* transgene in ry^{506} genome. To test this hypothesis, *Cyp6a2-91R* transgenes carrying greater lengths of upstream and downstream DNA's than the ones used in this investigation can be tested in the ry^{506} genome. If the longer constructs fail to show expression, it would indicate the presence of the putative silencer element. Alternatively, a *Cyp6a2-ry* construct identical to the *Cyp6a2-91R* constructs with 1331 bases of the upstream DNA can be placed in the ry^{506} genome and the expression of this transgene can

be measured. If this transgene shows expression in the ry^{506} genome, it would mean that the transgene is separated from the putative silencer elements.

Chromosome substitution experiments in the house flyhave shown that chromosome 2 negatively regulates the expression of both *CYP6A1* and *CYP6D1* genes. *CYP6D1* is also negatively regulated by chromosome 1. Since *Musca* and *Drosophila* are closely related insects and chromosome 3R of *Drosophila* and chromosome 2 of *Musca* are thought to be related, a similar approach was taken to identify which chromosome regulates the expression of the *Cyp6a2* gene. The results of the experiments with 2^{R}; 3^{ry} stocks presented in this dissertation (Chapter 2) showed that the third chromosome of ry^{506} inhibits the constitutive expression of *Cyp6a2-91R*. These data are also consistent with the findings of Waters and Nix (1988) and Sundseth *et al.* (1989). In their studies, it was shown that the third chromosome contains regulatory loci that repress the enzymatic activities of the structural gene. The level of expression in the 2^{R};3^{ry} stock, however, was not as low as it is in the underproducer ry^{506} strain. This may be due to the influence of other regulatory loci, both positive and negative, that may be present on the first, second and fourth chromosomes. In fact, the work of Sundseth *et al.* (1989) suggests that regulatory loci on the second chromosome also influence the expression of *Cyp6a2*. Biochemical and genetic studies by Houpt *et al.* (1988) also indicate the second and third chromosomes of *D. melanogaster* contain regulatory loci that influence the MFO activities in this organism. To further investigate the role of the first and fourth chromosomes on the expression of a specific CYP gene such as *Cyp6a2*, the origins of the first and fourth chromosomes will need to be controlled. Once established, CYP6A2

mRNA levels can be examined in a stock carrying the first, third and fourth chromosomes from ry^{506} and the second chromosome from the 91R strain. The level of CYP6A2 mRNA can also be examined in the reciprocal stock.

While the intracellular inducer of *Cyp6a2* remains to be identified, there is a growing body of evidence that suggests that *Cyp6a2* may be regulated by ecdysone or ecdysone-inducible genes. First, *Cyp6a2* is developmentally regulated, showing highest level of expression in larvae when the ecdysone titer is at its peak (Saner *et al.*, 1996). A similar observation was made with the *CYP6A1* gene of house fly(Cariño *et al.*, 1994). Second, relative to wild-type strains *Cyp6a2* expression and the level of ecdysone are lower in flies carrying $ecd\text{-}1^{ts}$ mutation when they are reared at the restrictive temperature (Spiegelman *et al.*, 1997). Third, analysis of the upstream DNA of *Cyp6a2* has revealed an ecdysone response element (EcRE) and binding sites for an ecdysone-inducible transcription factor, Broad Complex (BR-C). However, from these data it cannot be determined if *Cyp6a2* is directly regulated by ecdysone via binding of the ecdysone-receptor complex to the EcRE and/or indirectly through the action of a set of secondary response genes, such as *BR-C* (von Kalm *et al.*, 1994)

The 2R-linked and closely-clustered *Cyp6a8* and *Cyp6a9* genes of *D. melanogaster* are two other *Cyp6a* genes whose constitutive expression in different strains and in the 91R x 91C hybrid closely resembles that of *Cyp6a2* (Maitra *et al.*, 1996). *Cyp6a2*, *Cyp6a8* and *Cyp6a9* are constitutively expressed at reduced levels in the ry^{506} and 91C strains, but overexpressed in 91R. In the F1 hybrids of the 91R and 91C strains, per gene dose and relative to the parental 91R strain, the constitutive level of the

Cyp6a2 allele of 91R origin is reduced about two-fold. Similarly, per gene dose, expression of the *Cyp6a8* and *Cyp6a9* alleles of 91R origin is reduced in the F1 hybrid of 91R and 91C (Maitra *et al.*, 1996). Collectively, these observations suggest that the constitutive expression of these three *Cyp6a* genes may be regulated in a similar fashion. Additional similarities are also observed in their barbital-mediated induction. Both the *Cyp6a2* and the *Cyp6a8* alleles of ry^{506} and 91C, respectively, are almost transcriptionally silent but show induction with 0.1M barbital. On the other hand, like the *Cyp6a2* gene of the 91R strain, the *Cyp6a8 allele* of 91R shows higher induction with barbital than with phenobarbital (Maitra *et al.*, unpublished observations). Taken together, these observations suggest that *Cyp6a2* and *Cyp6a8* are under the control of a common regulatory locus (loci) that regulates the constitutive and barbiturate-induced expression of these two *Cyp* genes.

Based on their amino acid sequence, the CYP6A family of enzymes is most closely related to the mammalian CYP3A family (Feyereisen *et al.*, 1989; Waters *et al.*, 1992a,b; Lewis, 1996). Interestingly, the enzymes that are encoded by the genes belonging to these two evolutionarily divergent families have a number of functional features in common. *Cyp6a2* is inducible with ecdysone, while genes belonging to *CYP3A* family are inducible with many synthetic and endogenous steroids, including pregnenolone 16α-carbonitrile, dexamethasone, triacetyloleandomycin, cortisone and hydrocortisone (Lewis *et al.* 1996 and references therein). In the 5'-flanking DNA of *Cyp6a2* there is a putative ecdysone response element while the upstream DNA of the *CYP3* family genes has glucocorticoid response elements and estrogen response

elements. *Cyp6a2*, like *CYP3A1*, is expressed in a male-dominant manner (Schenkman and Griem, 1993; Lewis, 1996). CYP3A isoforms catalyze the 6B-hydroxylation of endogenous steroid hormones, such as testosterone and androstenedione (Funae and Imaoka, 1993; Maenpaa *et al.*, 1993; Zimniak and Waxman, 1993). Insect microsomes can also metabolize testosterone to 13 hydroxylated derivatives, including 4-androsten-3,17-dione, 6B-hydroxytestosterone and 16-hydroxytestosterone (Cuany *et al.*, 1990). Metabolism of eight of the thirteen metabolites were NADPH- and 0_2-dependent as well as CO-sensitive, indicating the involvement of CYP enzymes (Cuany *et al.*, 1990). While it is not known how many CYP enzymes exist in *D. melanogaster* microsomes, the observation that insect microsomes can carry out the oxidative metabolism of a mammalian steroid hormone indicates that one or more insect CYPs are functionally similar to mammalian CYP enzymes. It is possible that one of the unknown enzymes is CYP6A2. Lastly, both CYP6A2 and CYP3A can metabolize the chemical carcinogen aflatoxin B1 (Lewis *et al.*, 1996; Saner *et al.*, 1996).

Given the multiplicity of *CYP* genes in an organism and their differential pattern of induction with barbiturate compounds, the molecular mechanism of barbiturate-mediated induction of *CYP* gene is expected to be a complex phenomenon. While the exact mechanism of PB induction of mammalian *CYP* genes is unknown, a clearer picture is beginning to emerge. First, it appears that the enhanced level of CYP mRNA is due to increased transcription from a barbiturate-inducible promoter rather than due to increased mRNA stability (see Fulco, 1991 for review; Waxman and Azaroff, 1992; Trottier *et al.*, 1995; Park *et al.*, 1996; Honkakoski and Negishi, 1997; Kim and Kemper, 1997). There

is also speculation that PB induction is a receptor-mediated phenomenon (reviewed by Waxman and Azaroff, 1992; Shaw *et al.*, 1993). Studies in rat hepatoma cells suggest that PB-induced transcription of *CYP* genes may involve a steroid receptor (Shaw *et al.*, 1993; Denison and Whitlock, 1995). Transcriptional activation of the rat *CYP2C6* genes with PB was blocked with the glucocorticoid-progesterone antagonist RU486, as was the PB-induced transcription of reporter gene constructs carrying the 5'-flanking DNA of the rat *CYP2B1* or *CYP2B2* gene (Shaw *et al.*, 1993). However, to date no intracellular or intercellular receptor involved in PB induction has been identified. If PB mimics an endogenous steroid hormone, it may mediate its action by binding with the hormone receptor and the upstream responsive element of the *CYP* gene. Since the upstream DNA of many insect *CYP6* genes have an EcRE and the expression of some *CYP6* genes, including *Cyp6a2*, appears to be influenced by ecdysone, it is quite possible that barbiturate compounds like barbital and PB exert their actions via the ecdysone receptor or a similar intracellular protein. If this is the case, then the barbiturate-protein complex may bind with the upstream DNA of the *Cyp6a2* gene.

Additional studies are needed to identify the protein binding sites in the upstream DNA of *Cyp6a2*. Such information regarding the location of the protein binding sites and the DNA sequences can be determined from DNAaseI footprinting studies. Since the pattern of constitutive and barbiturate-mediated induction varies between different strains of *D. melanogaster* it is of interest to see if the *cis*-acting sequences and the *trans*-regulatory proteins involved in this response also differ between strains. To address this,

experiments that would involve electrophoretic mobility shift assays using nuclear extracts from different strains of *D. melanogaster* can be pursued.

In this dissertation, experiments designed to address two, complex biological phenomenona have been presented. First, what are the molecular and genetic mechanisms that regulate the expression of a family of *CYP* genes that are found only in insects and have been implicated in metabolic resistance to insecticides? Second, what are the molecular and genetic mechanisms that regulate their barbiturate-induced expression? These studies have examined both of these phenomena in a variety of *D. melanogaster* strains and the resulting data are as complex as the phenomena themselves. To incorporate the constitutive and barbiturate-induced data from all the strains into a working model is no easy feat and additional molecular and genetic studies are necessary. However, based on the data presented in this dissertation and comparison to published reports, it is apparent that several loci on different chromosomes regulate the constitutive expression of *Cyp6a2* and possibly the *Cyp6a8* gene of this organism. To advance the field further, experiments designed to further localize and ultimately characterize these transcriptional regulatory proteins are warranted. *D. melanogaster* is the ideal organism for such studies and has features not offered by *Musca*, including its well-established genetics and the availability of many deficiency stocks for genetic mapping studies (Wilson, 1988; ffrench-Constant *et al.*, 1992). Hence, once the map positions of the regulatory genes are known, they can easily be cloned. Toward this end, studies in our lab and others are now in progress.

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BIBLIOGRAPHY

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VITA

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