



2005

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COMBINED EFFECT OF THE X AND THIRD CHROMOSOMES ON THE
PROMOTER ACTIVITY OF THE *CYP6A8* GENE OF *DROSOPHILA*
MELANOGASTER

by

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College Scholars Senior Thesis

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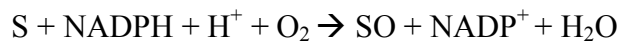
December 7, 2005

Abstract

Cytochrome P450 monooxygenases or CYPs are a superfamily of enzymes that are responsible for many metabolic processes in the body and the degradation of many exogenous toxic compounds. Insects use CYPs to protect themselves from various insecticides. In many insects, insecticide resistant strains show overexpression of one or more *Cyp* genes. Past studies have indicated that the 91R, a DDT resistant strain of *Drosophila melanogaster*, demonstrates higher expression of many P450 genes, including *Cyp6a8* which has been used in the present investigation. Converse to the 91R strain the *ry*⁵⁰⁶, a DDT susceptible strain, shows low expression of *Cyp6a8* and other *Cyp* genes. The present investigation aims to determine what effect, if any, the X and 3rd chromosomes have on the promoter activity of the *Cyp6a8* gene, which is located on the 2nd chromosome. To track this activity, a luciferase (*luc*) reporter gene was positioned downstream from the 0.8-kb upstream DNA of *Cyp6a8* and the chimeric reporter gene was transformed into the *ry*⁵⁰⁶ strain. In the transgenic strain, the reporter transgene was located on the 2nd chromosome. Via genetic crosses, the X and 3rd chromosomes of the transformed *ry*⁵⁰⁶ strain (r; 0.8luc; r) were substituted with the X and 3rd chromosomes from the 91-R strain to make the R; 0.8luc; R strain. Activity of the luciferase gene in these two strains was determined by reporter *luc* gene assay. The results showed that the R; 0.8; R strain had much higher luciferase activity than the r; 0.8; r strain. Thus, activity of the *Cyp6a8* gene promoter is under the influence of the X and 3rd chromosomes.

I. Introduction

Cytochrome P450 monooxygenases or CYPs form a family of enzymes, which are present in all living organisms, from bacteria to mammals. These enzymes are responsible for the metabolism of endogenous and exogenous compounds, as well as the synthesis and degradation of insect hormones and pheromones (Guengerich, 2004). P450 enzymes catalyze oxidative reactions in the body, with NADPH often serving as the reducing equivalent. In the following P450 reaction, the substrate is denoted by S:



Before beginning a discussion about any member of the P450 superfamily, it is important to first outline the nomenclature of this class of enzymes as well as of their corresponding genes. All enzymes of the P450 superfamily are assigned the prefix CYP (Nelson et al., 1996). Within the CYP superfamily, there are families, which are denoted by numerals, and among these are subfamilies, denoted by letters. Finally, the individual genes are assigned numerals as well and are expressed in italics. Proteins of these genes are expressed in capital letters. An example of the full name of a gene of the CYP superfamily is thus *Cyp6a8*, while the protein is CYP6A8. While families share greater than 40% identity at the amino acid sequence level, subfamilies indicate greater than 55% similarity (Nelson et al., 1996).

CYPs are implicated in a number of functional roles, including growth, development, and protection against foreign chemical agents known as xenobiotics. Many xenobiotics are toxic and carcinogenic. CYPs detoxify these chemicals and protect organisms from their lethal effects. As part of this latter role, CYPs confer a level of resistance to the organism, which, in insects, translate to a resistance to nearly all

insecticides, one of which is DDT, the first modern pesticide designed to control insects and pests (see Scott, 1999 for review). Resistant insects have developed an ability to survive under treatment with foreign compounds that, following the same dose, would kill the majority of the susceptible population of that species. There exists a variety of means by which insects may develop this resistance; however, P450 mediated detoxification stands out as one of the foremost methods (see Scott, 1999 for review), and thus, deserves some investigation.

In the study of P450 expression and its consequences, the *Drosophila melanogaster*, a species of the fruit fly, is considered a model organism. Through extensive experimentation with *Drosophila*, scientists aim to apply what they learn to help understand the internal workings of other organisms. This is possible because certain biological processes, such as metabolic or developmental pathways, are believed to be conserved through evolution. *Drosophila* is utilized for genetic experiments based on the ease and speed of growing a culture of this organism, and the availability of innumerable mutations that can be used to map genes on chromosomes. *Drosophila* is often used to study CYP activity, as the species is thought to use CYPs to detoxify insecticides including DDT because resistant strains show overexpression of multiple *Cyp* genes. Overexpression of multiple *CYP* genes has been observed in the resistant strains of other insects also (Maitra et al., 1996; Dombrowski et al, 1998; Scott, 1999). The implication of CYPs involvement in conferring resistance is supported by an earlier study showing that treatment of these strains with a P450 monooxygenase inhibitor, such as piperonyl butoxide, may strip the organism of some or even all of its DDT-resistance (Scott, 1999).

When compared with a susceptible strain of the same species, such as 91-C or *ry*⁵⁰⁶, the resistant 91-R strain in *Drosophila melanogaster* shows high expression of several P450 genes, one of which is *Cyp6a8*, which is marked by an elevation of the RNA of *Cyp6a8* alleles (Maitra et al., 1996). Although there remains speculation on this matter, chromosome substitution experiments showed that expression of *Cyp6a8* and other P450 genes located on the right arm of the second chromosome, seem to be trans-regulated by loci on the 3rd chromosome (Maitra et al., 2000). These factors are believed to be repressors implicated in the downregulation of *Cyp6a8* activity (Maitra et al., 2000). It is also proposed that these repressors on the 3rd chromosome are somehow mutated in the resistant strain, thus resulting in unhampered expression and overproduction of CYPs. Effect of X chromosome, however, has not been examined in this regard. Therefore, the objective of the present investigation has been to examine the effect of the X and the 3rd chromosomes together on *Cyp6a8* promoter activity. For this purpose, I created a chromosome substitution stock (R; 0.8luc; R) in which the X and 3rd chromosomes were from the DDT-resistant 91-R strain, while the second chromosome carrying the *Cyp6a8-luc* chimeric reporter gene was from the DDT susceptible *ry*⁵⁰⁶ strain. Activity of the reporter gene in this strain was compared with the reporter activity in another transgenic strain (r; 0.8luc; r) in which the X and the 3rd chromosomes were from the *ry*⁵⁰⁶ strain. By comparing these two strains, I could detect whether the X and 3rd chromosomes have any impact on the *Cyp6a8* promoter activity. An increase in activity of the gene promoter in the chromosome substitution stock R; 0.8luc; R would indicate that X and 3rd chromosomes of 91R strain have a positive, stimulatory effect on the promoter, while a decrease would signify that these chromosomes inhibit the promoter activity.

II. Materials and Methods

Fly Strains and Culture Conditions

All fly stocks were cultured at 24°C in a medium consisting of 0.65% bacto-agar, 5.5% cornmeal, 3% brewer's yeast, 5% unsulfured molasses, 2% light corn syrup, and 0.25% propionic acid. For this investigation, the transgenic line 0.8 *luc* 110 (Maitra et al., 2002) was used. In this stock, the second chromosome, carrying a *Cyp6a8-luc* chimeric reporter, was from *ry*⁵⁰⁶ strain. The chimeric reporter gene had firefly *luciferase* (*luc*) gene positioned downstream from the *Cyp6a8* gene promoter, a roughly 800 base pair sequence of DNA, between bases -11 and -761 (Figure 1). Since the X and 3rd chromosomes of the 0.8 *luc*110 stock were from the *ry*⁵⁰⁶ strain, this stock is referred as *r*; 0.8*luc*; *ry*. In addition, the following stocks were also used: (1) *R*; *Sco*/*R*; *Sb*/*R*; (2) *R*; *Sco*/*R*; *Tb*/*R*; (3) *R*; *Cy*/*R*; *R* and (4) *R*; *Cy*/*R*; *Tb*/*R*. The letter R denotes chromosomes from 91-R strain. *Sco* (scutoid); *Cy* (curly), *Sb* (stubble) and *Tb* (tubby) are dominant markers. While *Sco* and *Cy* are on the second chromosome, *Tb* and *Sb* are located on the 3rd chromosome. The *Cy* and *Tb* chromosomes are true balancer chromosomes meaning that they have multiple inversions which prevent crossing over with their homologues. The *Sb* chromosome also has inversions, but they are not extensive enough to suppress crossing over completely. The *Sco* chromosome is not a balancer chromosome. However, it can be used in males and its dominant phenotype helps in tracking the homologous chromosome.

Synthesis of Chromosome Substitution Stock of Flies

The major part of this investigation has been to synthesize a stock that is homozygous for the X and third chromosomes from a DDT-resistant strain, 91-R, and the

second chromosome carrying a luciferase reporter gene under the control of the Cyp6a8 gene promoter. The crosses and the results of the crosses are described in the Results section (Figure 2).

Preparation of the Extracts

For extract preparation, flies were etherized and groups of ten female flies were sorted into 1.5-ml Eppendorf tubes. After the flies recovered, the tubes were set on ice to knockdown the flies. To each tube 200 μ l 1X Cell Culture Lysis Reagent (Promega) was added and homogenized on ice for 20-30 seconds. The homogenate was then left to stand on ice for an additional 5 minutes. The homogenates were then centrifuged for 8 minutes at 13 K, 4 $^{\circ}$ C, and 100 μ l of the resulting clear extract from each tube were removed to new 1.5-ml Eppendorf tubes. Care was taken to ensure that as much lipid was left behind as possible. These extracts were centrifuged, again for 8 minutes at 13 K, 4 $^{\circ}$ C. From these tubes, 50 μ l were removed, leaving behind as much lipid as possible, and transferred to fresh 1.5-ml Eppendorf tubes. Of this final extract, 5 μ l was removed from each tube and reserved for the luciferase assays. All extracts were stored at -80 $^{\circ}$ C until use.

Luciferase Assay

Luciferase Activity Reagent (LAR) is stored at -80 $^{\circ}$ C in 100 μ l aliquots. When needed, the LAR tubes were brought out from the -80 $^{\circ}$ C freezer and kept at room temperature for 15 minutes in dim light. Once all samples were completely thawed, 5 μ l of fly extract were added to the 100 μ l aliquots of LAR, taking care to perform each transfer quickly with minimal exposure to light, as it accelerates luciferase activity decay.

Readings were taken after a 3 second delay at 15 second intervals for 1 minute total duration and were expressed in Registered Light Units (RLUs).

III. Results and Discussion

The first task for this investigation has been to synthesize R; 0.8*luc*; R stock. This involved three generation genetic crosses which are shown in Figure 2. In order to synthesize this stock, a transgenic line had to act as a parent strain. The strain used was a *ry*⁵⁰⁶ strain that also carried a luciferase reporter gene on its second chromosome. This 0.8 *luc* 110 transgenic line was named r; 0.8*luc*; *ry*. Males from this stock were crossed to female virgins from a resistant R; *Sco*/R; *Sb*/R stock. From the F1 generation, males were selected, again showing the *Sco*-*Sb* phenotype. This strain, named R/Y; 0.8/*Sco*; *Sb*/*ry*, now carried the luciferase reporter gene. This stock was crossed to females with the *Cy*-*Tb* phenotype, a strain whose parent strains were male with the genotype R/Y; *Sco*/R; *Tb*/R and female with the genotype R/R; *Cy*/R; R/R. Males and females from the F2 generation, with the genotype R; 0.8*luc*/*Cy*; R/*Sb*, were then crossed to get the final stock, the F3 generation. The genotype of this chromosome substitution stock was R; 0.8*luc*; R. Luciferase activity of this synthesized stock was compared with its susceptible counterpart, the r; 0.8*luc*; *ry* strain, again a 0.8 *luc* 110 transgenic line, though homozygous for the X and third chromosomes of the *ry*⁵⁰⁶ strain.

This investigation of the influence, if any, of the X and 3rd chromosomes on the reporter gene located on the 2nd chromosome was performed using the chromosome substitution stock R; 0.8*luc*; R and the transgenic susceptible stock, r; 0.8*luc*; *ry*. Both lines were homozygous for the luciferase (*luc*) reporter transgene. This *luc* reporter gene was placed downstream from the 0.8-kb upstream DNA of the *Cyp6a8* gene, the DNA sequence whose activity was being investigated. Luciferase activity assay showed that the synthesized stock R; 0.8*luc*; R had much higher activity than the transgenic line r;

0.8luc; ry. In fact, there was an average 4.44-fold increase in activity from the susceptible r; 0.8luc; ry strain to the resistant R; 0.8luc; R strain (Figure 3). This increase in activity thus illustrates that the presence of X and 3rd chromosomes homozygous for the 91R strain not only affects activity of the *Cyp6a8* gene promoter on the second chromosome, but that this effect is stimulatory in nature. Whether this activation occurs due to the presence of stimulatory factors or the impedance of inhibitory factors remains to be seen, though many studies indicate that it is the latter mechanism.

The regulatory effect of the third chromosome on the *Cyp6a8* gene located on the chromosome was examined in a past study by Maitra et. al (2000). In this study, the third chromosome of the *ry*⁵⁰⁶ strain, a known underproducer of *Cyp6a8* mRNA, was found to downregulate the *Cyp6a8* gene. Besides this finding, the study did not determine exactly how many and which bases of the upstream DNA of the resident gene were actually required for the response to the inhibition by the third chromosome.

For the present investigation, the luciferase reporter transgene included only 0.8 kb upstream DNA of the *Cyp6a8* gene promoter. In the resistant chromosome substitution stock R; 0.8luc; R, *luc* assays confirmed that the reporter transgene was upregulated by the X and third chromosomes, which were natural to the 91R strain. In the r; 0.8luc; ry transgenic line, the *luc* reporter gene was shown to be downregulated by the X and third chromosomes, homozygous for the *ry*⁵⁰⁶ strain. Thus, these experiments confirm that the roughly 800 bases upstream DNA must contain the cis-regulatory elements required for *Cyp6a8* to respond to the trans-regulatory signals from the X and third chromosomes. Results from this investigation thus allow for a better definition of the area of upstream DNA that is necessary for transregulation of the *Cyp6a8* gene to

occur. However, further investigations must be conducted to allow a finer truncation of the area of upstream DNA involved in this regulation.

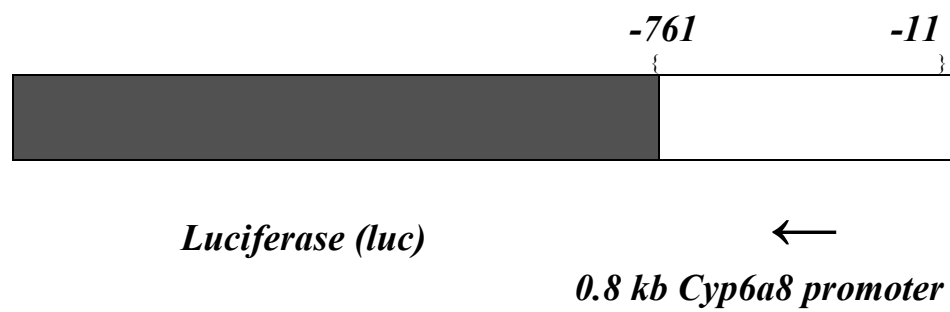


Figure 1. Diagram of the 0.8 luciferase reporter transgene. The 0.8kb DNA promoter sequence, upstream from the *luc* transgene, contains the basal promoter and transcription start signal for the *Cyp6a8* gene.

P1: ♂ *r*; **0.8luc**; *ry* x ♀ **R**; *Sco/R*; *Sb/R* ♂ *R/Y*; *Sco/R*; *Tb/R* x ♀ **R**; *Cy/R*; **R**



F1: ♂ **R/Y**; **0.8/Sco**; *Sb/ry* x ♀ **R**; *Cy/R*; *Tb/R*



F2: ♂ x ♀ **R**; **0.8/Cy**; *R/Sb*



F3: ♂ & ♀ **R**; **0.8luc**; **R**

Figure 2: Flowchart of the series of genetic crosses resulting in the final F3 generation, the chromosome substitution stock *R*; **0.8luc**; **R**. All chromosomes are homozygous unless otherwise indicated.

Comparison of Luc Activity Between R;0.8luc;R and r;0.8luc;ry

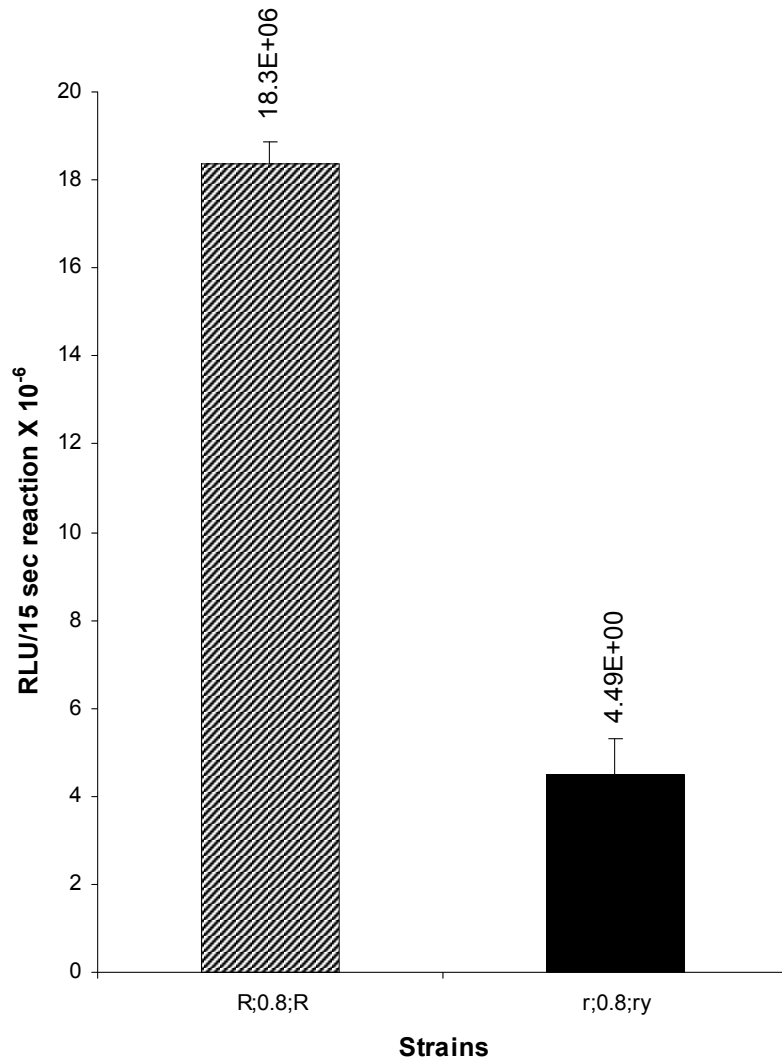


Figure 3. Bar graph depicting difference in luciferase activity between R; 0.8luc; R and r; 0.8luc; ry strains. Fly extracts were mixed with luciferase assay reagent, and readings were taken at different time intervals.

IV. References

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