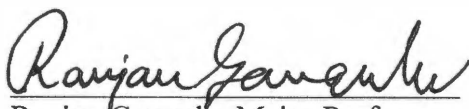


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Ranjan Ganguly, Major Professor

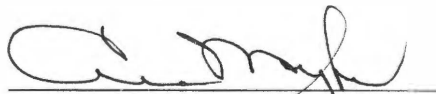
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Induction of the Cytochrome P450 gene, *Cyp6a8*, of *Drosophila melanogaster* by
caffeine

A Thesis
Presented for the
Master of Science
Degree
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Erika Danielle Dean
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This thesis is dedicated to my parents

For Mommy and Daddy

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I have always lived by the creed that life is what you make of it. Throughout my graduate study I have kept a little fortune I received several years ago after eating at my favorite local Chinese restaurant. It reads, "Stop searching forever; happiness is just next to you." Every morning, I wake up and read this little piece of inspiration that is taped to my bathroom mirror. I realize that the richest person in the world would wake up thankful to have all the wonderful friends and family in their life and less concerned with where the day was taking them. Therefore, I live each day with one simple thought. "It's not getting what you want, it's wanting what you've got."

Abstract

Cytochrome P450 monooxygenases (CYPs) are enzymes involved in the metabolism of a variety of endogenous and xenobiotic compounds including steroids and drugs. In insects, CYPs are involved in conferring resistance to insecticides. In mammals, CYP1A1/1A2 enzymes have been shown to metabolize the commonly consumed xenobiotic, caffeine. In addition, the CYP1A1/1A2 gene is induced by caffeine in both rat kidney and liver. Although common drugs such as barbitol and phenobarbitol induce *CYP* genes in insects, it is not known whether these genes are also induced by caffeine. In the present investigations, the effect of caffeine on the promoter of a *Drosophila Cyp* gene, *Cyp6a8*, was examined. For this purpose transgenic *Drosophila* carrying a *luciferase (luc)* reporter transgene, under the control of 0.8-kb (-11/-766) or 0.2-kb (-11/-199) upstream DNA of the *Cyp6a8* gene, were examined. Adult female flies were treated with Vivarin® containing caffeine or pure caffeine, and activity of the *luc* reporter gene was monitored at the enzyme level as well as at the RNA level. Different tissues of the treated and untreated female flies were also examined. Various other caffeine-related compounds were examined for their ability to induce the *Cyp6a8* promoter. The results showed that (a) both Vivarin and caffeine induce the *Cyp6a8-luc* transgene activity, (b) transgene activity was not induced by other purines (adenine, adenosine, hypoxanthine, and uric acid) tested, (c) the 0.8-kb construct gave higher constitutive expression as compared to the 0.2-kb construct, (d) the level of induced expression for both the 0.2-kb line and the 0.8-kb line was similar, (e) caffeine induced the transgenes at the RNA level, (f) the endogenous *Cyp6a8* gene

was induced by caffeine, as well as, the related *Cyp6a2* gene, (g) caffeine induction occurred in different tissues of *Drosophila* at varying levels, and (h) the highest level of constitutive reporter gene expression was found in the malpighian tubules with a 512 fold increase in transgene expression in the 0.8-kb line as compared to the 0.2-kb line. These results suggest that the 0.8- and 0.2-kb upstream DNA have sequences that can support caffeine-mediated induction of the *Cyp6a8* gene. Analysis of the *Cyp6a8* upstream DNA identified the presence of several putative binding sites for the transcription factor AP1 (Activator Protein 1). In rat striatum, caffeine treatment has been shown to increase mRNA levels of two members of AP1 complex, c-fos and junB, and their target genes. Therefore, we propose that a possible mechanism for caffeine-induced expression of *Cyp6a8* may be via caffeine-induced expression of *D-fos* and *Jra*, *Drosophila* homologues of c-fos and junB. Future analysis of *D-fos* and *Jra* expression in caffeine treated flies may reveal evidence to support this hypothesis.

Table of Contents

Section	Page
I. Introduction	1
General introduction	1
History of the cytochrome P450 enzymes	1
Taxonomy, nomenclature, and evolution	3
Structure and cellular distribution	4
Roles of P450s	6
Regulation of P450s	7
Regulation of <i>Cyp6a8</i> expression	9
Objectives	12
II. Materials and Methods	13
Fly strains and culture conditions	13
Synthesis of stocks homozygous for the transgene	15
Fly treatments	18
Extract Preparation for Luciferase Assay	18
Fly dissections	19
Luciferase Assays	19
Protein assay	20
RNA isolation	21
Radiolabeling of probe DNA	22
Northern blot hybridization	23
Statistics	24
III. Results	25
Caffeine-induced expression of <i>Cyp6a8-luc</i> transgene	25
Dosage dependency of transgene activity under caffeine-induced conditions	30
Luciferase activity of <i>0.8 luc 110 (H-ry)</i> females upon treatment with various purines	30
Northern blot analysis of caffeine (Vivarin®)-induced <i>luciferase</i> expression in transgenic flies	34
Northern blot analysis of caffeine (Vivarin®)-induced <i>Cyp6a8</i> expression in transgenic lines and <i>ry</i> ⁵⁰⁶ and 91-R strains	38
Northern blot analysis of caffeine (Vivarin®)-induced <i>Cyp6a2</i> expression in <i>0.8 luc 110 H</i> females	41

Constitutive and caffeine-induced expression of the luc transgene in different tissues	41
IV. Discussion	47
Caffeine, adenosine, and P450 gene expression in <i>Drosophila</i>	47
Activating protein 1 complex and gene expression	48
Mechanisms of adenosine receptor action	49
Investigating possible roles of CYP6A8 in the cell	52
References	54
Vita	62

List of Figures

Figures	Page
1 <i>Cyp6a8-luciferase</i> constructs used in the present investigation	14
2 Crosses to make <i>0.2 luc 30-4 H</i> and <i>0.8 luc 110 H</i> lines	16
3 Crosses to make <i>0.2 luc 30-4 H-ry</i> and <i>0.8 luc 110 H-ry</i> lines	17
4 Luciferase activity of three hemizygous <i>Cyp6a8-luc</i> transgenic lines upon vivarin® (caffeine) treatment	26
5 Luciferase activity of two homozygous <i>Cyp6a8-luc</i> transgenic lines upon vivarin® (caffeine) treatment	28
6 Comparison of luciferase activities of two homozygous <i>Cyp6a8-luc</i> transgenic lines upon vivarin® (caffeine) and caffeine (Sigma) treatments	29
7 Luciferase activity of different heterozygous <i>Cyp6a8-luc</i> transgenic lines upon 2.27 mM caffeine treatment	31
8 Luciferase activity in <i>0.8 luc 110 H</i> females upon treatment with increasing concentrations of vivarin®, caffeine (Sigma), and adenosine (Sigma)	32
9 Chemical structures of purines	33
10 Effect of different purines (2.27 mM each) on luciferase activity in <i>0.8 luc 110 (H-ry)</i> females	35
11 Northern blot analysis of LUC mRNA levels in <i>0.2 luc 30-4 H</i> and <i>0.8 luc 110 H</i> females treated with 2.27 mM vivarin® (caffeine)	36
12 Quantative analysis of normalized LUC mRNA levels in <i>0.2 luc 30-4 H</i> and <i>0.8 luc 110 H</i> females treated with 2.27 mM vivarin® (caffeine)	37
13 Northern blot analysis of CYP6A8 mRNA levels in <i>0.2 luc 30-4 H</i> , <i>0.8 luc 110 H</i> , <i>ry⁵⁰⁶</i> , and 91R females treated with 2.27mM vivarin® (caffeine)	39

14	Quantative analysis of normalized CYP6A8 mRNA levels in <i>0.2 luc 30-4 H</i> , <i>0.8 luc 110 H</i> , and 91-R females treated with 2.27mM vivarin® (caffeine)	40
15	Northern blot analysis of CYP6A2 mRNA levels in <i>0.8 luc 110 H</i> females treated with 2.27mM vivarin® (caffeine)	42
16	Quantative analysis of normalized CYP6A2 mRNA levels in <i>0.8 luc 110 homozygous</i> females treated with 2.27mM vivarin® (caffeine)	43
17	Constitutive transgene expression of <i>0.2 luc 30-4 (H-ry)</i> and <i>0.8 luc 110 (H-ry)</i> in different tissues	45
18	Caffeine-induced transgene expression in different tissues of <i>0.2 luc 30-4 (H-ry)</i> and <i>0.8 luc 110 (H-ry)</i> females	46

List of Tables

Tables		Page
1	Various animal P450s, their substrates, and their inducers	8
2	Putative AP1 binding sites within the -0.8 kb fragment of <i>Cyp6a8</i> upstream DNA	50

I. Introduction

General introduction

Cytochrome P450s are a superfamily of detoxifying enzymes that aid in the excretion of lipophilic compounds, which bombard our bodies everyday. Their functions are yet still so versatile that they also metabolize various endogenous compounds like steroids and fatty acids. The necessity of P450s to survival is affirmed by their presence in all living organisms, from bacteria to man. In addition to their beneficial metabolic functions, they are also involved in the toxic/mutagenic effects of several compounds, like polycyclic hydrocarbons and aromatic amines. Therefore, the oxidation reactions by which cytochrome P450 enzymes metabolize various compounds have been of an increasing interest since their discovery in the 1950's. Another interest is how P450s are regulated at the level of gene expression. Certain mammalian P450s have been linked to a genetic predisposition for cancer and other genetic disorders. By learning the mechanism by which these enzymes are controlled, we can harness this information for making better educated judgments on the prescription of certain pharmaceuticals and understand the underlying mechanisms behind P450s role in the pathogenesis of disease.

History of the cytochrome P450 enzymes

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

xenobiotic compounds. At that time, the identity and specific role of P450 enzymes in the catalysis of these reactions were unknown. It wasn't until 1958 when Klingenberg and Garfinkle, independently working with liver microsomes, first observed their presence. They found that when rat liver microsomes, treated with the reducing agent sodium dithionite, were gassed with carbon monoxide, a pigment produced a spectrophotometric peak at 450 nm (Klingenberg, 1958; Garfinkle, 1958; Guengerich, 1993; Estabrook, 1996). Later, Omura and Sato (1962, 1964 a,b) identified this pigment as a hemoprotein, and named it P450.

Enzymatic functions were first assigned to P450's in the 1960's. First, Estabrook *et al.* (1963) observed a P450-mediated hydroxylation of the steroid hormone 17 α -hydroxyprogesterone in microsomal fractions of the adrenal cortex. Later, Cooper *et al.* (1965) demonstrated the role of liver P450s in demethylation and aromatic hydroxylation of drugs such as codeine and acetanilide. P450s were assigned the title of the major detoxifying enzymes in liver microsomes based on the observation that increased metabolism of drugs is associated with elevated levels of CO-binding pigment. It is now estimated that P450s are involved in more than 60 types of biochemical reactions, including aromatic and aliphatic oxidation, hydroxylation, dealkylation, epoxidation, and desulphuration, with a broad range and overlapping substrate specificity of over 800 compounds (Agosin, 1985; Lewis, 1996) with new substrates being discovered constantly. P450s are largely responsible for the inactivation of xenobiotic (foreign) compounds, but sometimes also assist in the metabolic activation of some compounds (Guengreich, 1993).

Taxonomy, nomenclature, and evolution

Cytochrome P450 (*Cyp*) genes comprise one of the oldest and largest superfamily of genes known to man. Its importance for the sustenance of life is indicated by its presence in all living organisms, from the simplest to the most complex (Guengerich, 1993; Nelson et al., 1996, 1999; Feyereisen, 1999; Scott, 1999). As of September 25, 2002, this superfamily is reported to contain 281 different families and 2383 genes (<http://drnelson.utmem.edu/cytochromeP450.html>). Recent genome sequence analysis confirmed the presence of 80 *Cyp* genes in humans, 90 in *Drosophila melanogaster*, 80 in *Caenorhabditis elegans*, and 286 in *Arabidopsis thaliana* (Werck-Reichhart and Feyereisen, 2000; Tijet et al., 2001). Of the 90 *Cyp* genes in *Drosophila*, 83 are predicted to be functional genes while the rest are pseudogenes (Tijet et al., 2001).

Cytochrome P450 (CYP) proteins are classified into families and subfamilies based on similarities in their amino acid sequences. Members of a family share more than 40% identity at the amino acid sequence level, while members of a subfamily share more than 55% identity (Tijet et al., 2001). The universally accepted nomenclature, introduced by Nebert et al. (1987), appoints all gene members of the P450 superfamily with a *CYP* prefix (from Cytochrome P450) followed by a number for the family and a letter referring to the subfamily (e.g. *CYP1A*). Each individual gene is given a number to differentiate it from others in the subfamily (e.g. *CYP1A1*). In *Drosophila*, P450 genes are written in italics, whereas mRNA, cDNA, and protein are all denoted by capitals (e.g. *Cyp6a8* gene and CYP6A8 protein) (Tijet et al., 2001).

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

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

Structure and cellular distribution

CYPs are hemoproteins (46-55 kD) that consist of a single polypeptide chain of about 500 amino acids long and a single non-covalently bound heme prosthetic group. The amino acid chain provides sites for substrate and redox partner interaction, as well as

oxygen and heme binding. Generally, the C-terminal half of the protein, containing the heme-binding site, is more conserved among CYPs than is the N-terminal end. This site is at the heart of the protein where the oxidation reaction resulting in the splitting of an O₂ molecule and the subsequent oxidation of a substrate occurs. The heme-binding site contains the most characteristic consensus sequence (F-X-X-G-X-R-X-C-X-G) with the conserved cysteine that serves as fifth ligand to the iron atom (Graham-Lorence and Peterson, 1996; Werck-Reichhart and Feyereisen, 2000). Forming the mouth of the active site is a structure called the “meander,” which is a 14-15 residue stretch in the shape of a tunnel through which the substrate passes to the active site (Graham-Lorence and Peterson, 1996). The hydrophobicity of this region suits the hydrophobic nature of P450 substrates.

In prokaryotes, P450 proteins are water-soluble and float freely in the cell. In eukaryotes, on the other hand, they are attached to the cytoplasmic face of the endoplasmic reticulum by a 20 amino acid hydrophobic sequence in their N-terminal end. They are present abundantly in liver tissue where they play a major role in drug metabolism. However, there is no specific pattern for their tissue distribution. P450s can be found in all types of tissues and in all types of organisms. Examples of human tissue with notable levels of CYPs are liver, kidney, lung, pancreas, spleen, nasal epithelium, brain, adrenal gland, testis and ovary (Schenkman and Greim, 1993; Werck-Reichhart and Feyereisen, 2000).

Roles of P450s

An important metabolic role of P450s is the detoxification of insecticides, which enables certain pests to feed on agricultural crops. P450 enzymes are found to have both a higher activity and a higher expression in resistant insects than in insects susceptible to various insecticides (Scott, 1999). By studying the expression patterns of genes encoding P450s and the mechanisms by which they are regulated, it may be possible to devise methods to control insecticide resistance. Understanding this phenomenon of higher expression has been a major goal of several laboratories. Besides their roles in xenobiotic detoxification, P450s are also known to be involved in endogenous metabolism and survival of *Drosophila*. One P450 gene called *Cyp302a1(disembodied)* has been shown to have a role in maintenance of ecdysteroid levels and cuticle formation in embryos. This was discovered by the use of an embryonic lethal mutant allele (Chavez et al., 2000). Further analysis showed that CYP302A1 converts 2,22-dideoxyecdysone to 2-deoxyecdysone when expressed in *Drosophila* S2 cell culture (Warren et al., 2002). Additionally, another *Drosophila* P450 CYP315A1(*sad*) was shown to convert 2-deoxyecdysone to ecdysone and 2,22-dideoxyecdysone to 22-deoxyecdysone also in cell culture (Warren et al., 2002). The identification of other mutant alleles for P450s has been difficult due to the fact that they too are most likely lethal alleles.

In other insects, some P450s have been assigned a role in the survival of the insect. The black swallowtail butterfly, *Papilio polyxenes*, has a P450 called CYP6B1 that is necessary for it to feed on allelochemical containing plants (Table 1). The allelochemical called xanthotoxin is produced by the host plant P450, which is

metabolized in the gut of *Papilio* larvae as they feed on the plant. CYP6B1 gene has also been shown to be xanthotoxin-inducible by an unknown mechanism (Cohen et al., 1992).

Important roles for P450s in mammals have also been identified (Table 1). CYP1A1 encodes a P450 with aryl hydrocarbon hydroxylase activity. CYP1A2 performs arylamine oxidations. CYP2C19, CYP2D6, and CYP2E1 genes encode S-mephenytoin 4'-hydroxylase, debrisoquine hydroxylase, and N,N-dimethylnitrosamine N-demethylase activities, respectively (Nebert et al., 1996). CYP1A1 is perhaps the most well studied P450. It has been linked to metabolic activation of the procarcinogen benzo(a)pyrene commonly found in cigarette smoke and charbroiled foods. In fact, a genetic polymorphism within the CYP1A1 gene has been linked to genetic predisposition to lung cancer due to increased activity of CYP1A1 (Nakachi et al., 1993).

Regulation of P450s

Phenobarbital-induced expression has been documented for several P450s, including CYP106 and CYP102 in bacteria and CYP2B, CYP3A, CYP4D10, *Cyp6a2*, CYP6D1, and *Cyp28* in animals (Table 1) (Fulco, 1991; Aoki et al., 1997; Dupuy et al., 2001; Danielson et al., 1998; Dunkov et al., 1994; Liu and Scott, 1997; Danielson et al., 1997).

The mechanism for phenobarbital induction of CYP102 and CYP106 is fairly well characterized in *Bacillus megaterium* (Fulco, 1991). Sequences in the promoter of these genes have been identified to bind positive and negative factors for gene expression, influenced by the presence of phenobarbital. These 15-17 bp sequences are called barbie boxes (Liang et al., 1995). When phenobarbital or other barbiturate

Table 1. Various animal P450s, their substrates, and their inducers. (Adapted from Maitra, 2000).

Enzyme	Organism	Known Substrates	Known Inducers	Reference
CYP1A1 CYP1A2	Mammals	Planar polyaromatic hydrocarbons and caffeine	Caffeine and Beta-naphthoflavone	Nedelcheva and Gut, 1984 Kamataki, 1993 Goasduff et al., 1996 Aoki et al., 1997
CYP2A	Mammals	Aflatoxins, coumarins, pharmaceuticals, and procarcinogens	4-Vinylcyclohexene	Chang and Waxman, 1996 Raunio et al., 1999 Doerr-Stevens et al., 1999
CYP2B	Mammals	Barbituates, nicotine, DDT, aldrin, dieldrin, and parathion	Phenobarbital, Beta-naphthoflavone, alachlor, and 4-Vinylcyclohexene	Nims and Lubner, 1996 Dupuy et al., 2001 Aoki et al., 1997 Hanioka et al., 2002 Doerr-Stevens et al., 1999
CYP2D	Mammals	30% of drugs for human consumption		Gonzales, 1996
CYP2E	Mammals	Ethanol, benzene, diethyl ether, and acetone	IL-1alpha, ethanol and 4-Vinylcyclohexene	Ronis, et al., 1996 Peng and Coon, 2000 Upadhyay et al., 2000 Doerr-Stevens et al., 1999
CYP3A	Mammals	Erythromycin, cyclosporine, dexamethasone, and nifedipine	Glucocorticoid, ethanol, and phenobarbital	Maurel, 1996 El-Sankary et al., 2002 Kostrubsky et al., 1997 Dupuy et al., 2001
CYP4D10	<i>Drosophila mettleri</i>	unknown	Plant alkaloids and phenobarbital	Danielson et al., 1998
CYP6A1	Housefly	Cyclodiene, heptachlor, and aldrin	unknown	Andersen et al., 1994
CYP6A2	<i>Drosophila melanogaster</i>	Aflatoxin, DMBA, Trp-2, diazinon,	Phenobarbital and barbital	Saner et al., 1996 Dunkov et al., 1994
CYP6A8	<i>Drosophila melanogaster</i>	unknown	Phenobarbital and barbital	Maitra et al., 1996 Maitra et al., 2002
CYP6B1 CYP6B4	<i>Papilio polyxenes</i>	Plant furanocoumarins	Plant furanocoumarins (xanthotoxin)	Ma et al., 1994 Hung et al., 1997
CYP6B2	Bollworm	unknown	pyrethroids	Wang and Hobbs, 1995
CYP6D1	Housefly	Pyrethroids and phenanthrene	Phenobarbital	Tomita et al., 1995 Korytko et al., 2000 Liu and Scott, 1997
CYP9A	<i>Manduca sexta</i>	unknown	2-undecanone and xanthotoxin	Stevens et al., 2000
CYP28	<i>Drosophila</i>	unknown	Plant allelochemicals and phenobarbital	Danielson et al., 1997

compounds are added to the medium, positive *trans*-regulatory factors (BM1P1, BM1P2 and BM1P3) are induced. This event causes the repressor protein (Bm3R1) to dissociate from the barbie boxes, thereby inducing the transcription of CYP102 and CYP106 genes (He et al., 1995). Eukaryotes also contain sequences that act as putative phenobarbital response elements (Kemper, 1998). Though the mechanism for phenobarbital induction is not as well defined in animals, it is thought that phenobarbital enhances the binding of transcriptional coactivators to genes induced by phenobarbital (Kemper, 1998).

New discoveries in past years indicate two nuclear orphan receptors that appear to mediate phenobarbital responsiveness in mammals. The constitutive androstane receptor (CAR) has been implicated in mediating phenobarbital induction of CYP2B genes in the mouse when phenobarbital induction of those genes was abolished in mice carrying a null allele of CAR (Corcos and Lagadic-Gossmann, 2001). Additionally, another nuclear receptor the steroid and xenobiotic receptor/pregnenolone X receptor (SXR/PXR) was shown to mediate phenobarbital responsiveness when phenobarbital induction of CYP3A genes was deleted in PXR knockout mice (Xie et al., 2000; Corcos and Lagadic-Gossmann, 2001).

Regulation of *Cyp6a8* expression

Cyp6a2 in *Drosophila* has been shown to be induced by treatment with barbital and phenobarbital (Dombrowski et al., 1998; Dunkov et al. 1997). This gene has been the subject of study in our lab for several years now. *Cyp6a8* is a *Drosophila* P450 gene that is closely related to the *Drosophila* P450 gene *Cyp6a2* (Maitra et al., 1996). *Cyp6a8* was shown to be expressed constitutively at higher levels in the insecticide resistant strain

91-R as compared to insecticide susceptible strains *ry*⁵⁰⁶ and 91-C (Maitra et al. 1996, 2000). *Cyp6a8* was also shown to be induced by barbital in each strain (Maitra et al, 1996). However, *Cyp6a8* was phenobarbital responsive only in the *ry*⁵⁰⁶ and 91-C strains at the concentrations used. *Cyp6a8* of 91-R did not show induction with phenobarbital (Maitra, 2000). In fact, phenobarbital-induced *Cyp6a8* expression in 91-C was about the same as constitutive expression in 91-R.

Maitra et al. (2000) then addressed the mechanism of constitutive expression of *Cyp6a8* and its relation to factors on each chromosome of *Drosophila*. *Cyp6a8* is a located on the second chromosome of *Drosophila* at cytological location 51D6 (Maitra et al., 1996). Several consomic stocks were created using the 91-R and 91-C strains. Upon analysis of *Cyp6a8* expression of these consomic stocks, evidence was found that the third chromosome of the 91-C strain downregulates *Cyp6a8* expression of the 91-R allele (Maitra et al., 2000). This evidence suggested that the 91-R strain may have a mutant repressor locus (loci) that results in a higher constitutive expression level of *Cyp6a8*. Alternatively, it is also possible that the 91-R strain has a gain-of-function mutation linked to the third chromosome.

In the most recent publication on *Cyp6a8* gene expression, Maitra et al., 2002 explore the role of the upstream DNA of the 91-R allele of *Cyp6a8* on constitutive and barbital-induced expression. A putative noncanonical TATA box is present at position -64 (ATG at +1). Additionally, a putative CAAT box is found 30 bps upstream of this TATA box. Several putative transcriptional response elements have also been identified by sequence analysis (Maitra et al., 2002). These include twelve barbie boxes which are present in the upstream DNA between -1200 and -1 bps. Barbie boxes have the core

consensus sequence AAAG (He and Fulco, 1991). Two of these sites (-1123 and -62) have very high core matrix similarity (>90).

Using transgenic flies carrying varying lengths of *Cyp6a8-91R* upstream DNA attached to a *luciferase* (*luc*) reporter (Figure 1), Maitra et al. (2002) showed that the first 200 bps (-11/-199) of upstream DNA are sufficient to drive expression of the *luc* gene. However, an increased expression was observed with 800 bps (-11/-761) of upstream DNA. No significant increase was observed upon investigation of 3100 bps as compared to the 800 bps constructs (Maitra et al., 2002). Therefore, the region between -11 and -199 is sufficient to provide at least basal expression levels, while the region between -200 and -761 is necessary to provide higher expression levels.

Since other P450s have been shown to be regulated by phenobarbital, the *Cyp6a8-91R* luciferase transgenic lines were tested for their ability to respond to Phenobarbital (Maitra et al., 2002). All three transgenic constructs were induced by phenobarbital. The highest level of induction was in the 0.8-kb line. However, each of the lines showed comparable levels of induction (Maitra et al., 2002). Interestingly, the upstream DNA used for the constructs is from the 91-R allele of *Cyp6a8*. As you may recall though, *Cyp6a8* was not induced by phenobarbital in the 91R strain. A possible explanation for the lack of phenobarbital-induced *Cyp6a8* expression is that constitutive levels of *Cyp6a8* in 91-R are too high to accommodate any induction and mRNA levels are at a steady-state level. Alternatively, it has been proposed that phenobarbital treatment causes the removal of a transcriptional repressor from the upstream DNA of *Cyp6a8*, perhaps in a mechanism similar to that in *B. megaterium* (reviewed in Kemper, 1998). This repressor may be mutant in 91-R so that there is no repressor to remove in this strain. However,

since the *Cyp6a8-91R* constructs are in a ry^{506} genetic background, the repressor may be wild type and able to bind the 91-R upstream DNA. Therefore, induction would be observed in the transgenic lines. Regardless, xenobiotic regulation of *Cyp6a8* gene expression may be an important mechanism of control and mandates further investigation.

Objectives

Since many *CYP*'s expression, including *Cyp6a8*, have been shown to be induced by barbituates and other xenobiotics, I decided to see if I could use the transgenic lines created by S. Maitra (Maitra et al., 2002) to screen xenobiotics for their inductive or inhibitory ability upon P450s. Additionally, once a chemical is identified to have some effect on transgene expression, then the endogenous *Cyp6a8* gene and other P450 genes can be screened. *CYP1A1/1A2* genes in mammals have been shown to be induced by caffeine (Goasduff et al., 1996). Since caffeine had been shown to induce expression of other P450s though by an unknown mechanism, caffeine was the first xenobiotic tried. Caffeine is an important xenobiotic because of its vast consumption by humans. Therefore, it is of particular interest to understand the mechanism by which it regulates gene expression because caffeine consumption might have some beneficial or deleterious effects.

II. Materials and Methods

Fly strains and culture conditions

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

The DDT-resistant, 91-R strain and three different transgenic lines were used in the present investigation. The 91R strain evolved from *Drosophila* captured in the wild by D. J. Merrell in St. Paul, Minnesota in 1952. The original population was split into two populations. One population, named 91R, was raised on DDT containing medium for 14 years and the other population was maintained on normal medium (Dapkus and Merrell, 1977). The 91-R strain used in the present investigation was obtained in the early 1980's by Dr. Larry C. Water of Oak Ridge National Laboratory. Since then the 91-R strain has been maintained on normal medium. Periodic resistance assay showed that the strain still maintains DDT resistance phenotype (Ganguly, unpublished observations).

Three transgenic lines created by Maitra et al. (2002) in the ry^{506} host strain were also used in the present investigation. These transgenic lines have different lengths of *Cyp6a8* upstream DNA attached to the firefly luciferase (luc) reporter gene (Figure 1). The transgene in 0.2-luc and 3.1-luc transgenic lines are located at polytene bands 49E and 66D, respectively. The transgene in 0.8-luc 110 is located on chromosome 2, but its

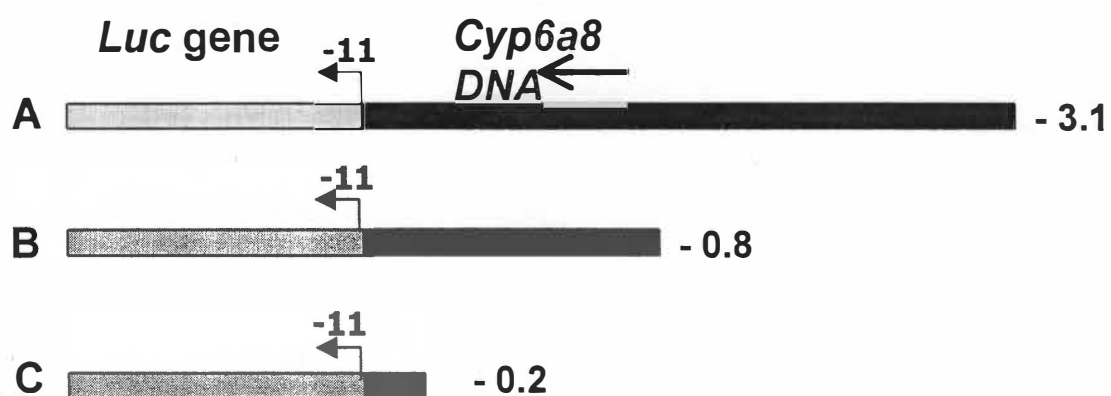


Figure 1– *Cyp6a8*-luciferase constructs used in the present investigation. Upstream DNA fragments are 3.1-, 0.8-, and 0.2-kb.

position has not been mapped (Maitra et al., 2002). However, Southern blot analysis showed that the 0.8-luc transgenic line has only one copy of the transgene (Maitra, 2000).

Synthesis of stocks homozygous for the transgene

Stocks homozygous for 0.2 luc and 0.8 luc transgenes were used in the present investigation. While the stock homozygous for 0.8 luc (0.8 luc 110 Homozygous) transgene was available (created by C. Price) in the lab, the other homozygous lines were synthesized in the present investigation. To synthesize stocks homozygous for 0.2 luc transgene, 0.2 luc 30-4 transgenic virgin females from the original lines with wild type (ry^+) eye color were crossed to males from the $+/Y; CyO/Sco; kar ry^{506}/ry^{506}$ balancer stock (Figure 2). Since it was known that the transgene was linked to the 2nd chromosome, virgin F1 females with curly wings (CyO) and wild type (ry^+) eyes (positive for presence of the transgene) were crossed to F1 males with the same phenotype. F2 progeny male and virgin female with normal (Cy^+) wings and wild type (ry^+) eye color were selected and crossed to make the homozygous stock. The resultant strains are called *0.2 luc 30-4 H* and *0.8 luc 110 H*.

Although the above stocks were homozygous for the 2nd chromosome-linked 0.8 luc or 0.2 luc transgenes, the genotype of this fly was not ry^{506} . To create homozygous transgenic lines in ry^{506} background a second set of crosses were done. In these crosses, virgin females from Line 5A (Maitra, 2000) with $r/r; R/R; ry^{506}/ry^{506}$ genotype (where r is from ry^{506} and R is from 91-R) were crossed to males from $X/Y; R/CyO; ry^{506}/ry^{506}$ strain available in the lab (Figure 3). The F1 males with rosy (ry^{506}) eyes and curly (CyO) wings were crossed to ry^+ virgin females from either the original 0.2 luc or 0.8 luc

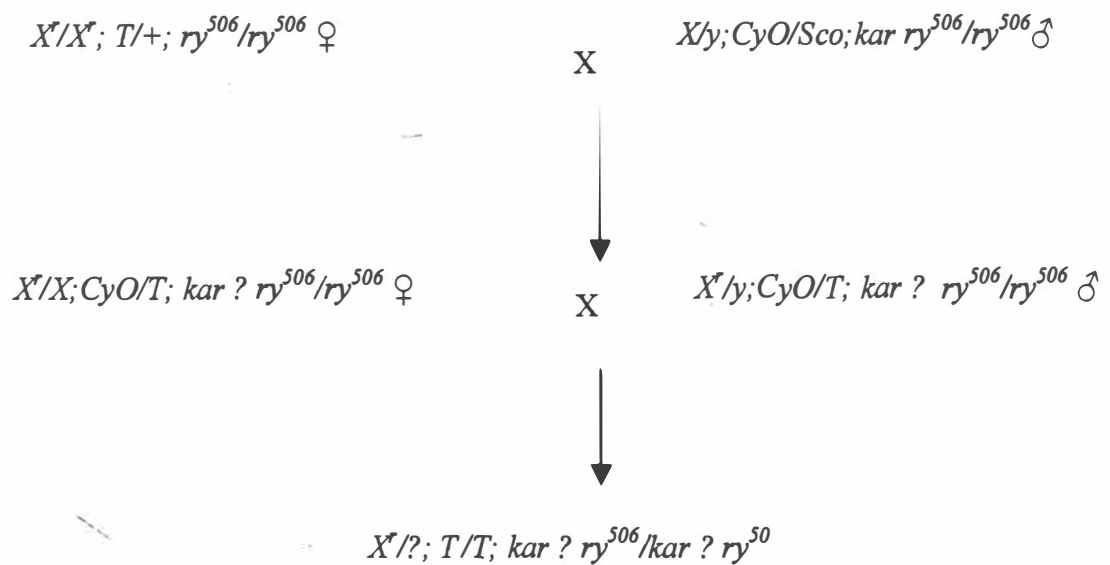


Figure 2. Crosses to make 0.2 *luc 30-4 H* and 0.8 *luc 110 H* lines. T- transgene located on the 2nd chromosome of *ry*⁵⁰⁶. The transgene carries both the *luc* reporter transgene and the *ry*⁺ allele. Xr indicates that this chromosome is from the *ry*⁵⁰⁶ strain. Alleles are separated by slashes and chromosome pairs are separated by semicolons. The initial cross was done with stocks all present in the lab. The secondary cross was done with flies created by the initial cross and screened for phenotype. 0.8 *luc 110 H* line was created by Charles Price in the Ganguly lab.

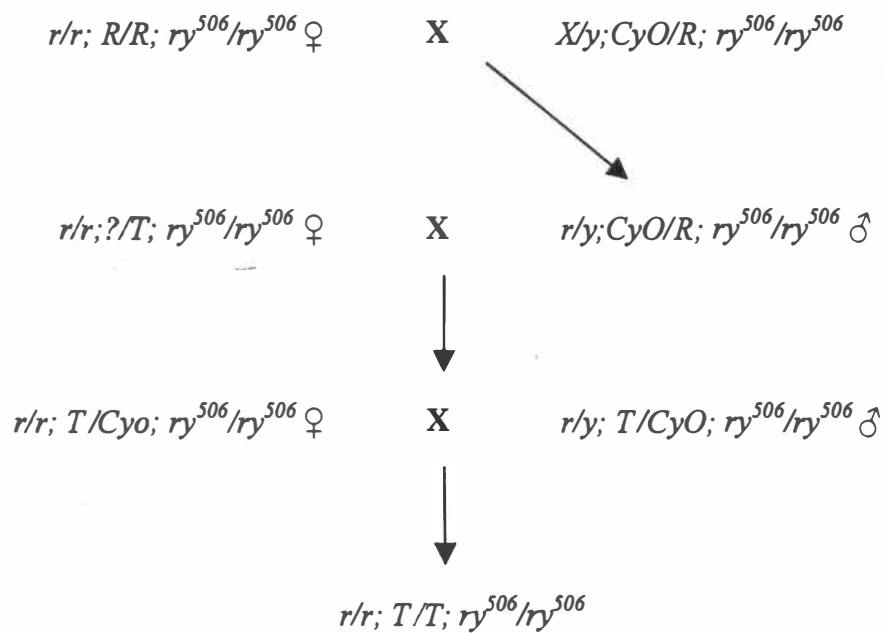


Figure 3. Crosses to make 0.2 *luc* 30-4 H-ry and 0.8 *luc* 110 H-ry lines. T- transgene located on the 2nd chromosome of ry^{506} . The transgene carries both the *luc* reporter transgene and the ry^+ allele. r indicates that this chromosome is from the ry^{506} strain. R indicates that this chromosome is from 91-R strain. Alleles are separated by slashes and chromosome pairs are separated by semicolons.

transgenic lines that was maintained by sibling mating in ry^{506} background. Virgin F2 females with ry^+ eyes and curly wings were crossed to F2 males with the same phenotype. F3 non-curly wing Cy^+ and ry^+ males and virgin females were selected and crossed to build up the homozygous stock. The resultant stocks are called *0.2 luc 30-4 (H-ry)* and *0.8 luc 110 (H-ry)*.

Fly treatments

Females, 48-96 hours of age, were used for all experiments. They were sorted and kept on normal medium for 24 hours to recover from ether treatment. Live flies were then transferred to potato media reconstituted with one of the following chemicals dissolved in water: Vivarin® containing caffeine, caffeine, adenosine, adenine, hypoxanthine, or uric acid. Except Vivarin®, all other chemicals were obtained from Sigma Chemicals (MO). Vivarin® was purchased from a local drug store. Each Vivarin® tablet consists of 200 mg caffeine and other inert ingredients (Carnauba wax, Colloidal Silicon dioxide, D+C Yellow #10 Al Lake, Dextrose, FD+C Yellow #6 Al Lake, Hydroxypropyl methylcellulose, Magnesium stearate, Microcrystalline cellulose, Polyethylene glycol, Polysorbate 80, Starch, and Titanium dioxide).

Extract Preparation for Luciferase Assay

Flies were etherized at the end of the treatment period and sorted into 1.5 ml eppendorf tubes in groups of 10 flies. After recovering from the effects of ether, the tubes were placed on ice for approximately 2 minutes to immobilize the flies, then 200 μ l

of 1X Cell Culture Lysis Reagent (CCLR) (Promega, WI) was added to each tube. Flies were homogenized on ice immediately upon addition of the buffer. The homogenates were kept on ice for 5 minutes and then centrifuged at 12,000 RPM for 8 minutes at 4°C. Supernatant (100 µl) was carefully removed to leave both insoluble and lipid fractions. Extracts were centrifuged again at 12,000 RPM for 8 minutes at 4°C. Supernatant was carefully removed, split into two 25 µl aliquots, and stored at -80°C. One aliquot was used for luciferase assay, and the other was used for protein assay.

Fly dissections

Female flies were etherized and dissected while asleep. Flies were dissected using two needles on a glass slide in a drop of Ringer's solution (Ashburner, 1989). The heads, malpighian tubules, and residual abdomen (after the removal of the ovaries, gut, and malpighian tubules) were used for assay. Respective body parts from 10 females were pooled in 200 µl of 1X CCLR on ice until homogenized and extracts were prepared and stored as described above.

Luciferase Assays

Luciferase activity was measured using a commercially available kit (Promega, WI). Luciferase Assay Reagent (LAR) containing beetle luciferin was prepared according to the manufacturer's protocol and aliquoted into single reaction volumes of 100 µl in 1.5-ml eppendorf tubes. The individual reagent tubes were stored at -80°C until use. For luciferase assays, LAR was left on ice in darkness until thawed and then

transferred to room temperature for 10 minutes before it was used. Fly or tissue extracts were thawed on ice for 15 minutes and then equilibrated at room temperature for 2 minutes. For luc assay, 5 μ l of extract was added to the prealiquoted 100 μ l of LAR, mixed gently and read in a luminometer (Zylux) for 1 minute at 15 second intervals with a 3 second delay. The second reading collected at approximately 33 seconds after reaction initiation was used for data analysis.

Protein assay

The total amount of protein in the extracts used for luc assays was quantified using the BCA protein assay kit (Pierce.) First, extracts were thawed on ice for 20 minutes. Then, 10 μ l (whole fly extract) or 50 μ l (tissue extract) of extract was used to quantify total protein content. Bovine Serum Albumin (BSA- Pierce) 2 mg/ml was used to perform the standard curve. All samples, including the BSA, were diluted to 0.5 X CCLR buffer conditions to prevent any excess of interference molecules in the reaction. Reactions were performed in duplicate according to manufacturer's protocol. The optical density for each reaction at 562 nm was read in a Shimadzu spectrophotometer model UV16U. The mean of the two readings was used to calculate the total protein content based on the equation of the standard curve that fell within the linear range of the spectrophotometer. Final data is expressed as Registered Light Units per μ g of total protein (RLUs/ μ g.)

RNA isolation

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

Poly (A)⁺ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as described by Maniatis et al. (1982). Briefly, an Oligo(dT) cellulose column was equilibrated with buffer A containing 0.5M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, and 0.1% SDS. Total RNA was heat-denatured for 10 min at 65°C , cooled to room temperature, and adjusted to 1 mg/ml or less concentration in buffer A. The RNA was then passed through the equilibrated oligo(dT) column, which was first washed with 5 volumes of buffer A and then with 5 volumes of buffer B (i.e. buffer A, without SDS). Poly(A)⁺ RNA was eluted with 10mM Tris, precipitated with ethanol in presence of 0.2M Na-acetate pH 7.5, and pelleted by centrifugation at 12,000 RPM. The RNA was dried and dissolved in water.

Radiolabeling of probe DNA

LUC and CYP6A8 cDNAs were isolated from respective cDNA clones via restriction digestion. *Cyp6a2* DNA was amplified by PCR with gene-specific primers from genomic DNA of adult flies. These DNAs were radiolabeled with ^{32}P by the random prime labeling method using the Prime-a-Gene® Labeling kit (Promega.) The reaction mixture consisted of a 1X random priming buffer with 5.2 A₂₆₀ u/ml random hexadeoxyribonucleotides, 0.4 mg/ml BSA, 0.06 mM each of dATP, dGTP, and dTTP, 50 μCi $\alpha^{32}\text{P}$ -dCTP, 0.66 μM dCTP, and 5 units of Klenow fragment of DNA polymerase I. DNA was heat denatured and labeling reactions were carried out for 2 hours at 25°C. The reactions were terminated by adding 50 μl of stop dye. Unincorporated nucleotides were removed from the reaction by fractionation through a Sephadex G50 column (Pharmacia).

Ribosomal protein 49 (RP49) DNA was amplified from a recombinant plasmid DNA (O'Connell and Rosbash, 1984) using gene-specific primers. RP49 was radiolabeled with ^{32}P by the nick translation method (Gibco-BRL). The reaction mixture contained 0.2 mM each of dATP, dGTP, and dTTP, 50 mM Tris-HCL (pH 7.8), 10mM MgCl₂, 20 mM β -mercaptoethanol, 25 μCi $\alpha^{32}\text{P}$ -dCTP (3000 Ci/mmol), and 2.5 units of Pol I/ DNase mix. The labeling reaction was carried out for 90 minutes at 15°C. Reactions were stopped by adding 50 μl of stop dye. Unincorporated nucleotides were removed from the reaction by fractionation through a Sephadex G50 column (Pharmacia).

Northern blot hybridization

Northern blot analysis was performed using 2 µg of Poly (A)⁺ RNA for all blots except LUC blots where 4 µg of Poly (A)⁺ RNA was used. The RNA sample was denatured at 68°C for 12 minutes in 0.5X northern gel buffer containing 20mM MOPS (3-(N-Morpholino) propane sulfonic acid), 5mM sodium acetate, 0.5mM EDTA, pH 7.0, 6% formaldehyde, and 50% formamide. After cooling on ice, one-tenth volume of RNA loading dye (50% glycerol, 0.01M NaH₂PO₄, pH 7.0, and 0.4% bromophenol blue) was added and samples were electrophoresed on a 2.2M formaldehyde-MOPS-1.2% agarose gel. Using 20X SSC as the transfer buffer, RNA was blotted onto a nylon membrane (Hybond-N, Amersham, England) by capillary action for 16-20 hours. To check for the efficiency of transfer and to determine the migration distance of RNA, ribosomal RNA bands were viewed under high wavelength UV light. Blots were cut 1 cm below the ribosomal RNA bands into upper and bottom sections. While the upper sections were hybridized with *Cyp* or *luc* gene probes, the bottom sections were hybridized with RP49 gene probe. All blots were UV-crosslinked (Stratalinker UV Crosslinker, Model 1800, Stratagene, CA) and baked at 80°C under vacuum for 2 hours.

Blots were first pre-hybridized at 42°C for 2 hours in 50% formamide, 0.1M PIPES pH 6.6, 0.8M NaCl, 5X Denhardt's solution (0.1% ficoll, 0.1% polyvinyl pyrrolidine, and 0.1% bovine serum albumin, Pentax fraction V), 0.1% SDS, and 0.1mg/ml denatured Herring Sperm DNA. The pre-hybridization solution was replaced with fresh pre-hybridization solution containing α³²P-labeled probe. Blots were hybridized at 42°C for 18-20 hours. After hybridization, blots were washed twice with

0.2X SSC-0.1% SDS at room temperature and twice at 65°C. Hybridization signals were quantified by using a Packard Instant Imager (Packard Instrument Company, Meriden, CT), and visualized by autoradiography. Hybridization signals from RP49 were used to correct RNA loading error.

Statistics

Statistical analysis (Student's t-test) was performed using the Excel 98 program of Microsoft Office 98. P-values are recorded in the figure legends.

III. Results

Caffeine-induced expression of *Cyp6a8-luc* transgene

Our pilot study to determine the effects of caffeine on *Cyp6a8* gene expression were performed in the transgenic lines created by S. Maitra (Maitra et al., 2002). Female flies that contained the 0.2-kb, 0.8-kb, or 3.1-kb *luc* constructs were used. Only females were used for all experiments because sexual dimorphism of both the endogenous *Cyp6a8* gene and of the luciferase lines has been observed (Maitra, 2000). At the time when this initial experiment was done, transgenic lines were not made homozygous for the transgene. Therefore, transgenic flies were crossed to the *ry*⁵⁰⁶ host strain and F1 progeny with *ry*⁺ (red) eyes were collected. This ensured that all flies had the same copy number of the transgene. Upon treatment of these hemizygous flies with a Vivarin® solution containing 2.27 mM caffeine, an approximate 2-fold induction was observed in each line (Figure 4). Since the basal expression of the 0.2-kb line is about 9-fold lower than the 0.8-kb line and the caffeine induced expression of all lines is maintained at 2-fold, it suggests that sequences necessary for caffeine induction are present within the 0.2- kb region of DNA located immediately upstream of position -11 (ATG at +1). However, sequences between 0.2- and 3.1-kb give much higher levels of luc expression in induced and uninduced flies. Since the basal and induced activities of the 0.8-kb and 3.1-kb constructs did not show much difference, the 3.1-kb construct was not studied subsequently.

To eliminate the need for fly crosses with each experiment, stable homozygous stocks were made for the 0.2-kb and 0.8-kb transgenic lines. Therefore, Vivarin®

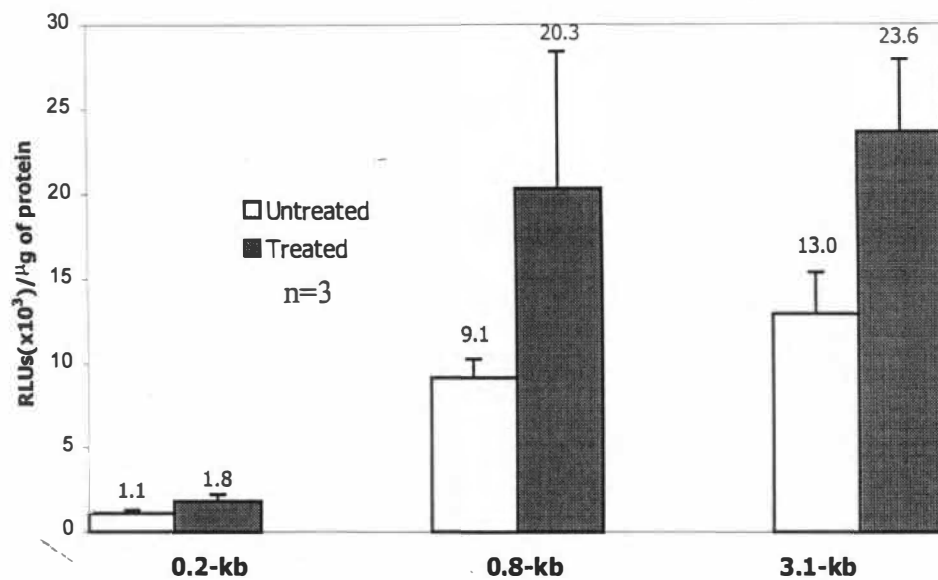


Figure 4 - Luciferase activity of three hemizygous *Cyp6a8-luc* transgenic lines upon vivarin® (caffeine) treatment. Flies were treated with 2.27 mM caffeine from vivarin®. Statistical analysis identified that only the 0.2-kb line is induced by caffeine significantly ($p < 0.009$). However, since this experiment was a pilot study further investigation was performed.

treatments were repeated in the newly created homozygous lines. Both *0.2 luc 30-4 H* and *0.8 luc 110 H* females showed a 4-fold increase in luciferase activity upon treatment with Vivarin® (Figure 5). This is double the amount of induction observed in the hemizygous lines. This increase can most likely be attributed to the gene dosage difference between the hemizygous and homozygous flies, since hemizygous flies contain only one copy of the transgene, while the homozygous flies have two copies of the transgene.

Since our confidence in the ability of Vivarin® to induce the transgene activity has been established, I raised the question whether caffeine in the Vivarin® solution was causing the actual induction or some other compound from the Vivarin® pills. Therefore, pure caffeine was used at the same molarity to test its' ability to induce the transgene. It was found that both caffeine and Vivarin® caused 2- to 3-fold induction of the *0.8 luc 110 H* line and 12- to 13-fold induction of the *0.2 luc 30-4 H* line (Figure 6). Transgene activity was slightly higher in both lines when treated with Vivarin® as compared to pure caffeine. However, the difference in luc activity between Vivarin® and caffeine treated flies is statistically insignificant and not likely due to some impurity in Vivarin® affecting transgene activity.

Additionally, I was concerned that perhaps the effect of caffeine on luciferase activity was unique to these two transgenic lines. Therefore, I used two additional *0.8 luc* lines with single third chromosomal transgene locations to eliminate this concern. All four original transgenic lines (*0.2 luc 30-4*, *0.8 luc 110*, *0.8 luc 14*, and *0.8 luc 121*) were crossed to *ry⁵⁰⁶* and F1 female flies with wild-type (*ry⁺*) eye color were selected. This insured the presence of a single copy of the transgene in each line, since flies made

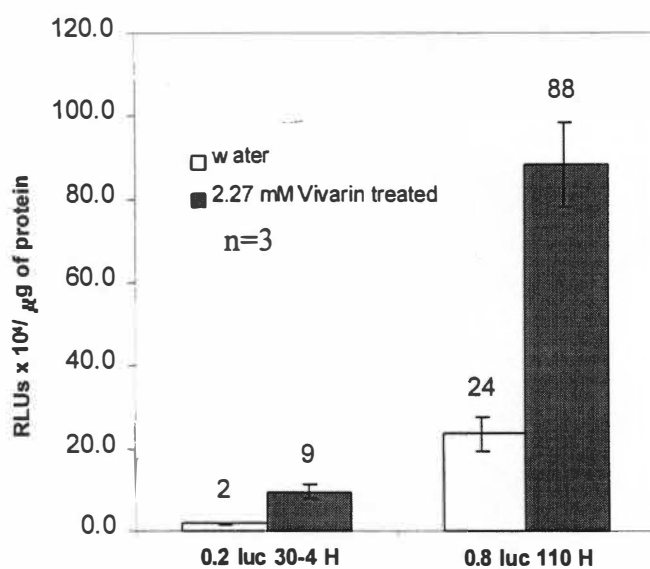


Figure 5- Luciferase activity of two homozygous *Cyp6a8-luc* transgenic lines upon vivarin® (caffeine) treatment. Flies were treated with 2.27 mM caffeine from vivarin®. Induction of both the 0.2 *luc 30-4 H* and the 0.8 *luc 110 H* lines are statistically significant ($p < 0.018$ and $p < 0.013$, respectively).

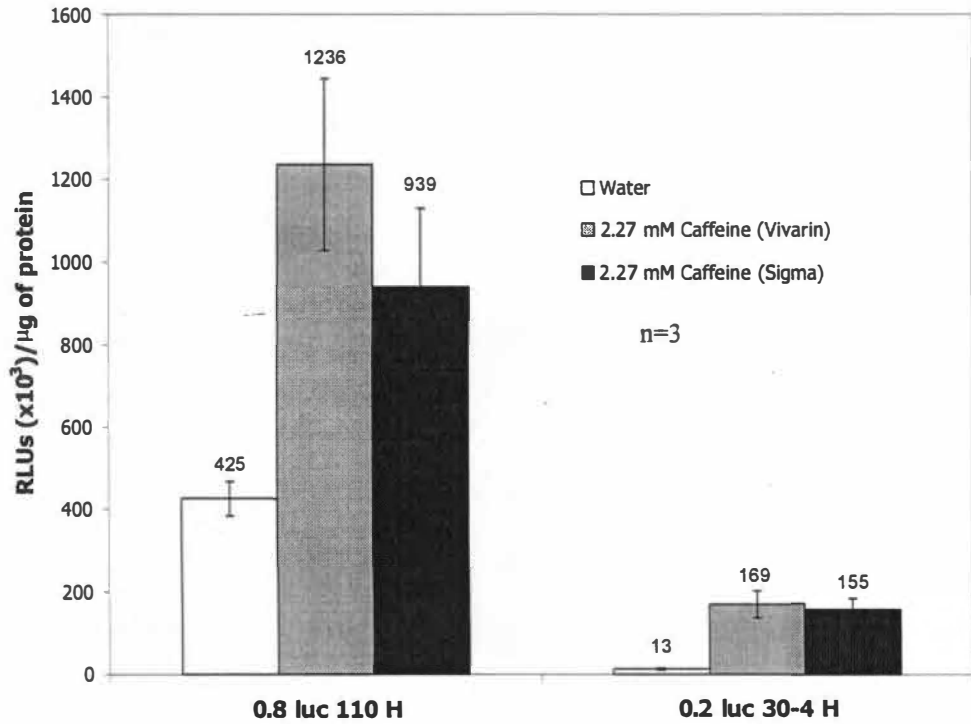


Figure 6- Comparison of luciferase activities of two homozygous *Cyp6a8-luc* transgenic lines upon vivarin® (caffeine) and caffeine (Sigma) treatments. Flies were treated with either water, 2.27 mM caffeine from vivarin®, or 2.27 mM caffeine (Sigma). Both caffeine (Sigma) and vivarin® induced the *0.2 luc 30-4 H* ($p < 0.009$ and $p < 0.014$, respectively) and the *0.8 luc 110 H* lines ($p < 0.05$ and $p < 0.017$, respectively) significantly. However, there was no significant variance between caffeine (Sigma) and vivarin® treatment for both the *0.2 luc 30-4 H* ($p < 0.47$) and the *0.8 luc 110 H* ($p < 0.17$) lines.

homozygous for the transgene did not exist for two of the lines. The F1 females were treated with 2.27 mM caffeine (Sigma) and assayed for luc activity. Each line was induced 4- to 7-fold upon caffeine treatment (Figure 7).

Dosage dependency of transgene activity under caffeine-induced conditions

I then decided to determine the effect of different dosages of vivarin and caffeine on one of the transgenic lines with both Vivarin® and caffeine. Since the basal level of luc expression is much lower in the *0.2 luc 30-4 H* line than in the *0.8 luc 110 H* line, the *0.2 luc 30-4 H* line was selected for this experiment. Both Vivarin® and caffeine induced *0.2 luc 30-4 H* luc activity in a concentration dependent manner. However, the activity level plateaus between 4 and 16 mM (Figure 8).

Additionally, females were treated with various concentrations (0-16 mM) of adenosine to determine its effect on transgene expression. This was done since caffeine is known to be an adenosine receptor antagonist (Svenningsson et al., 1997). Therefore, it was suspected that adenosine may have some negative effects on transgene expression. However, at all concentrations, adenosine has no effect on transgene expression.

Luciferase activity of *0.8 luc 110 (H-ry)* females upon treatment with various purines

Caffeine's chemical name is 1,3,7-trimethylxanthine. It is a purinic molecule similar to that of adenine, hypoxanthine, xanthine, and uric acid (Figure 9). Therefore, it was thought that perhaps caffeine mimics the endogenous function of one of these molecules. After all, caffeine is neither produced by the fly nor consumed by it naturally.

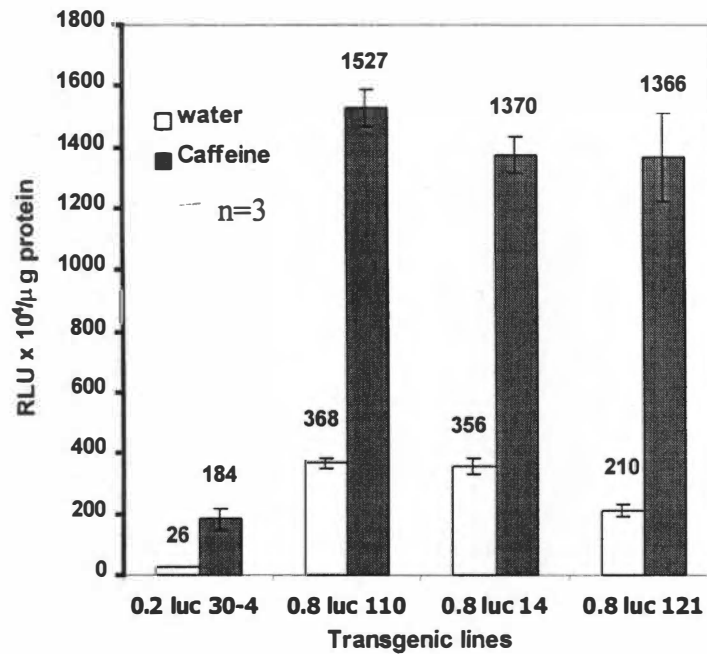


Figure 7. Luciferase activity of different heterozygous *Cyp6a8-luc* transgenic lines upon 2.27 mM caffeine treatment. 0.2 luc 30-4, 0.8 luc 110, 0.8 luc 14, and 0.8 luc 121 flies were all induced upon caffeine treatment ($p < 0.014$, $p < 0.0006$, $p < 0.0003$, and $p < 0.003$, respectively).

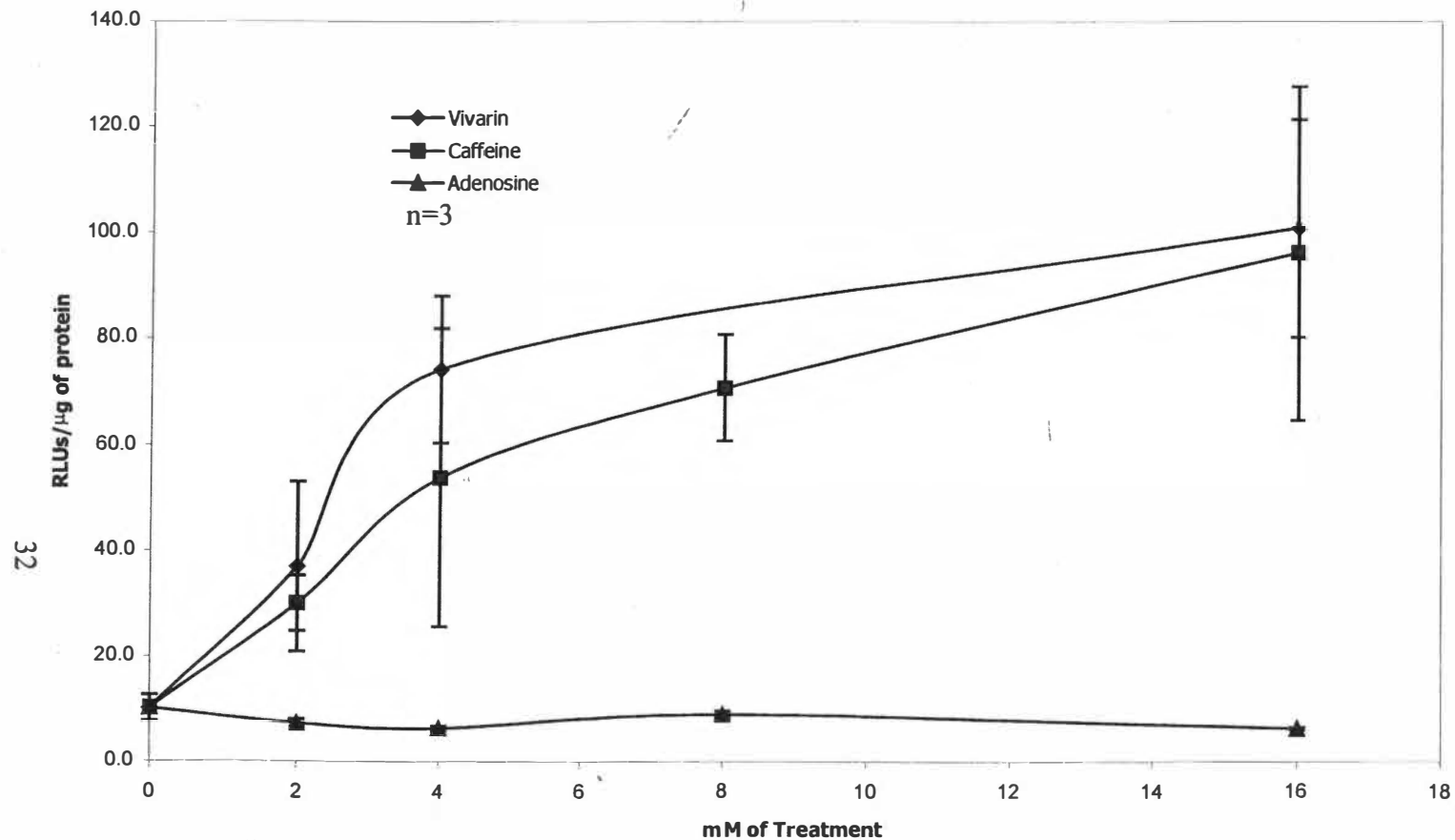


Figure 8- Luciferase activity in *0.2 luc 30-4 H* females upon treatment with increasing concentrations of vivarin®, caffeine (Sigma), and adenosine (Sigma). Induction with 16mM vivarin or caffeine shows a statistical significance ($p < 0.019$ and $p < 0.037$, respectively). However, decreased luciferase activity observed upon 16mM adenosine treatment is not significant ($p < 0.094$).

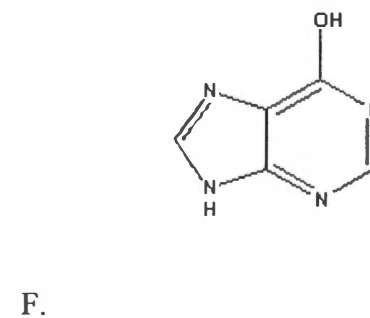
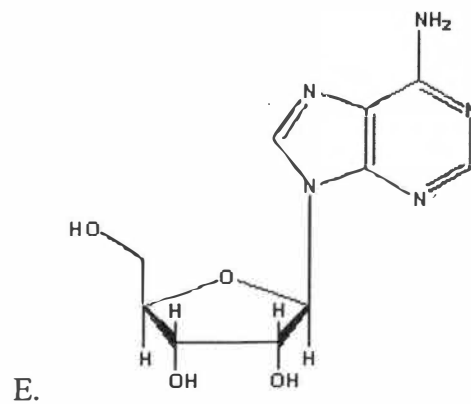
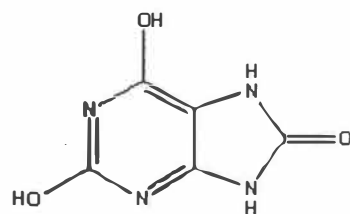
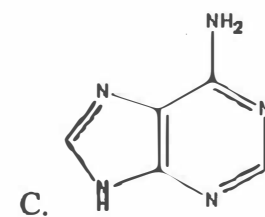
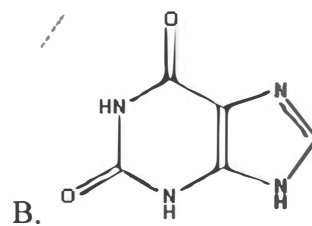
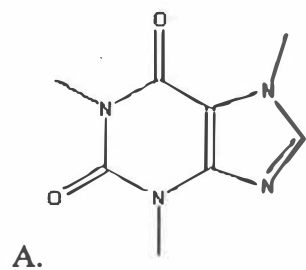


Figure 9- Chemical structures of purines. A- caffeine. B- xanthine. C- adenine. D-uric acid. E- adenosine. F- Hypoxanthine.

To test whether these chemicals might have an effect on *Cyp6a8* expression, females of *0.8 luc 110 (H-ry)* were treated with various purines. All chromosomes in *0.8 luc 110 (H-ry)* lines are from *ry*⁵⁰⁶. Except caffeine, no induction of the 0.8 luc reporter gene was observed with any of the purinic chemicals (Figure 10).

Northern blot analysis of caffeine (Vivarin®)-induced *luciferase* expression in transgenic flies

To determine whether caffeine-dependent increases in luc activity are accompanied with an increase in LUC mRNA levels, northern blot analysis was performed. Vivarin® treatment of *0.2 luc 30-4 H* and *0.8 luc 110 H* female flies increased the level of LUC transcripts in the fly by 2- to 3-fold in both transgenic lines (Figures 11-12). This is the same fold increase observed in luciferase activity upon treatment with 2.27 mM Vivarin® caffeine, the same concentration used for initial luciferase activity assays. Therefore, it is unlikely that caffeine is exerting any significant post-translation control on luciferase activity. However, these results do not prove conclusively that elements located within the *Cyp6a8* promoter are affecting caffeine induction.

Interestingly, two species of LUC mRNA can be observed in both lines under both control and Vivarin® caffeine treatment regimes. This could be attributed to several mechanisms, including cross-hybridization to another closely related gene, the presence of two transcriptional start sites in the promoter region, alternative splicing, or two different polyadenylation signals. Both cross-hybridization and alternative splicing are unlikely mechanisms because BLAST search (www.ncbi.nih.nlm.gov) did not identify

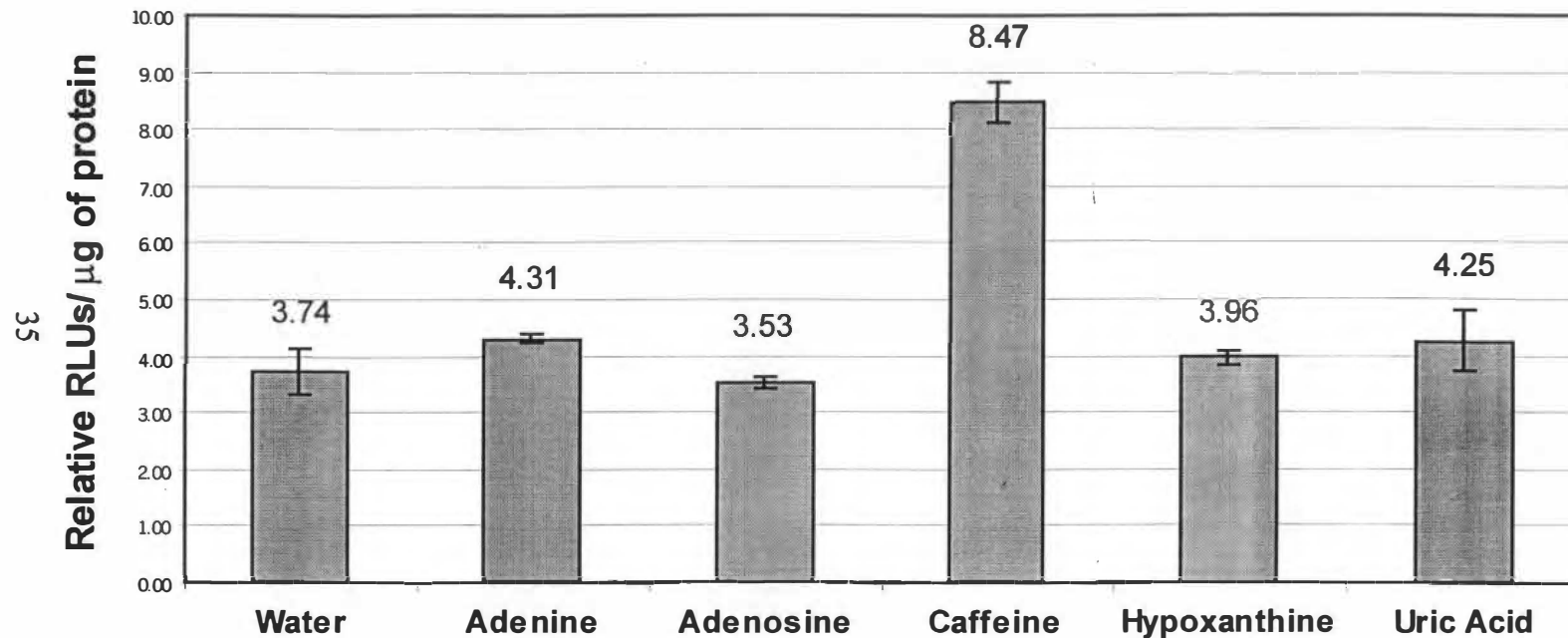
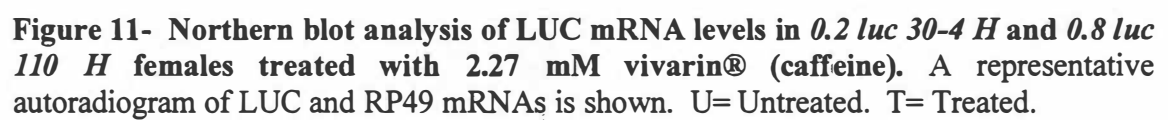


Figure 10– Effect of different purines (2.27 mM each) on luciferase activity in *0.8 luc 110 (H-ry)* females. (Adenine- $p < 0.1392$, Adenosine- $p < 0.3582$, Caffeine- $p < 0.0001$, Hypoxanthine- $p < 0.5339$, and Uric Acid- $p < 0.0687$).



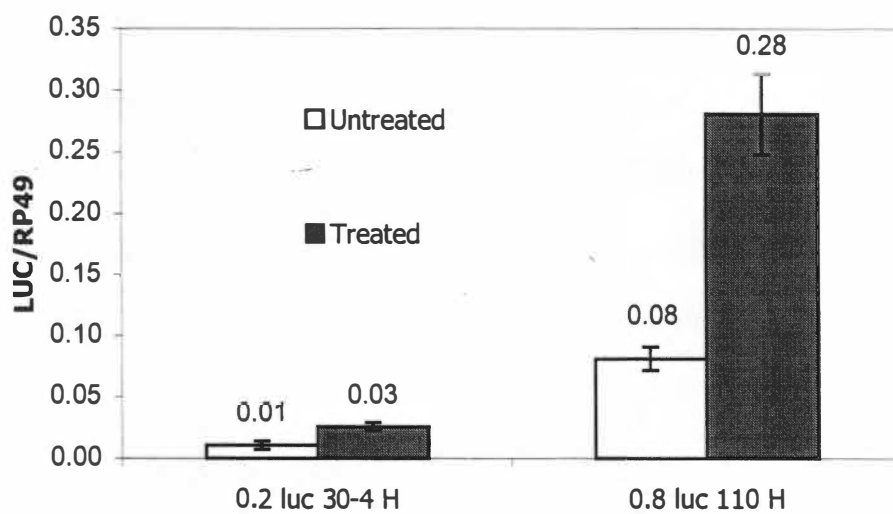


Figure 12- Quantative analysis of normalized LUC mRNA levels in *0.2 luc 30-4 H* and *0.8 luc 110 H* females treated with 2.27 mM vivarin® (caffeine). Induction of the *0.2 luc 30-4 H* and the *0.8 luc 110 H* lines are statistically significant ($p < 0.013$ and $p < 0.005$, respectively).

any *Drosophila* gene with significant sequence similarity with the *luciferase* cDNA and *luciferase* cDNA does not have any sequences to be spliced out. Therefore, the two transcripts are likely due to either alternative transcriptional start sites or alternative polyadenylation sites. Upon investigation of the latter possibility, two canonical polyadenylation site of AATAAA are observed in the 3' untranslated region (UTR) of the pGL2basic vector when visually scanned as well as several possible noncanonical polyadenylation sites. One of the non-canonical polyadenylation signals may produce a second RNA species with a different length of 3' UTR. It is necessary to mention that CYP6A8 mRNA has two isoforms-2.0-kb and 1.8-kb in the 91-R strain (Maitra et al., 1996; Maitra, 2000). However, primer extension and Northern blot analysis suggested that these two species are products of alternative polyadenylation sites in the 3' UTR.

Northern blot analysis of caffeine (Vivarin®)-induced *Cyp6a8* expression in transgenic lines and γ^{506} and 91-R strains

It is possible that caffeine induction of the *luc* transgene is due to chromosomal position effect. Alternatively, it may be due to sequences present in the upstream DNA of *Cyp6a8* attached to the *luc* gene. If caffeine-induced *luc* activity and expression are due to the *Cyp6a8* promoter, the endogenous *Cyp6a8* gene should also be induced by caffeine. Therefore, expression of the endogenous *Cyp6a8* gene of two transgenic lines and γ^{506} strains was examined. The results showed that upon treatment with 2.27 mM caffeine (Vivarin®), *Cyp6a8* expression increased 2- to 3-fold in female flies from 0.2 *luc* 30-4 H and 0.8 *luc* 110 H lines, 91-R and γ^{506} (Figure 13-14). Vivarin® treatment also induced the *Cyp6a8* allele in the 91-R strain (data not shown). This is consistent

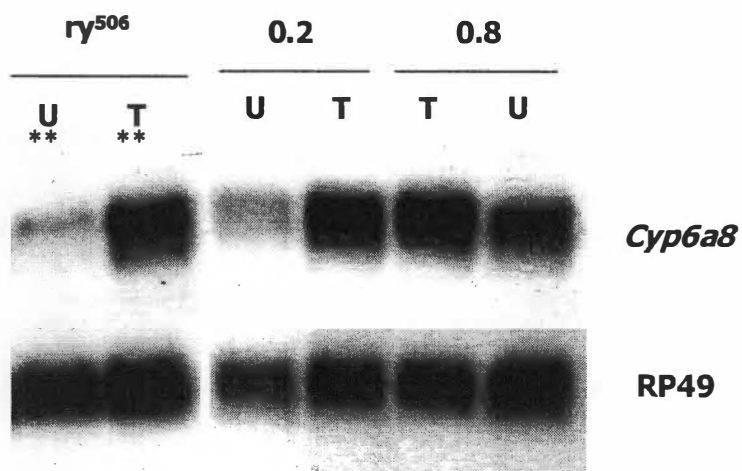


Figure 13- Northern blot analysis of CYP6A8 mRNA levels in *0.2 luc 30-4 H*, *0.8 luc 110 H*, and *ry⁵⁰⁶* females treated with 2.27mM vivarin® (caffeine). A representative autoradiogram of CYP6A8 and RP49 mRNAs is shown. U= Untreated. T= Treated. ** indicate that these blots were not hybridized in the same solution as the other blots displayed.

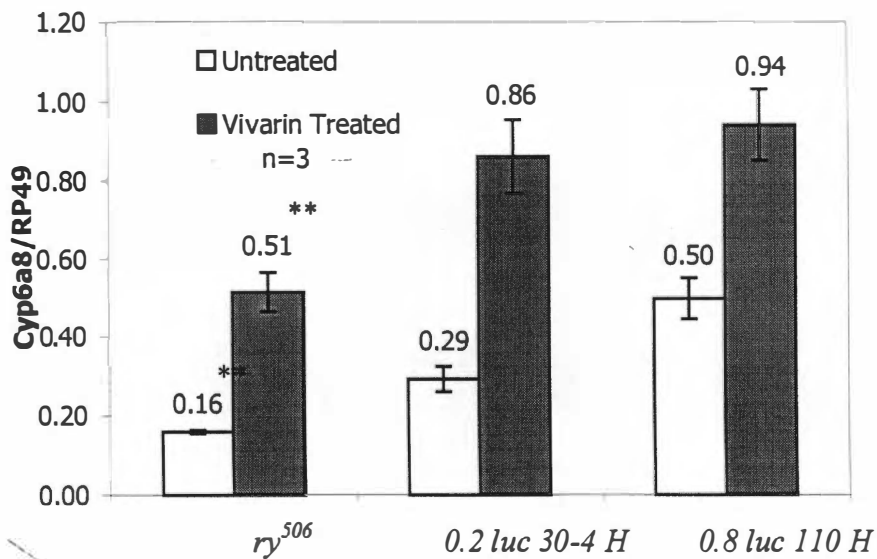


Figure 14- Quantative analysis of normalized CYP6A8 mRNA levels in 0.2 *luc* 30-4 *H*, 0.8 *luc* 110 *H*, and *ry*⁵⁰⁶ females treated with 2.27mM vivarin® (caffeine). ** indicate that these blots were not hybridized in the same solution as the other blots displayed. Induction of each transgenic line and *ry*⁵⁰⁶ was determined to be statistically significant. (0.2 *luc* 30-4 *H*- $p < 0.004$, 0.8 *luc* 110 *H*- $p < 0.007$, and *ry*⁵⁰⁶- $p < 0.0007$).

with both the luciferase activity assay data and the Northern analysis of LUC mRNA levels in the transgenic lines. From these experiments, I conclude that caffeine induction of the *luc* transgene is mediated through the upstream DNA of *Cyp6a8* present in the transgene. Additionally, two transcripts of *Cyp6a8* were observed in all lines and strains. This shows that the two alternative transcripts are not a unique polymorphism of the 91-R strain, since alternative transcripts were not observed in the *ry*⁵⁰⁶ strain by Maitra, 2000 due to faint hybridization signals. These two species of mRNA are thought to be the product of either alternative polyadenylation sites in the 3' UTR of the *Cyp6a8* (Maitra et al., 2002).

Northern blot analysis of caffeine (Vivarin®)-induced *Cyp6a2* expression in *0.8 luc 110 H* females

Since the *Cyp6a8* related gene *Cyp6a2* is regulated by similar mechanisms to *Cyp6a8* (Maitra, 2000, Maitra et al., 2002), it was asked if caffeine would have any effect on *Cyp6a2* expression. Vivarin® caffeine treatment (2.27 mM caffeine) of flies revealed a 3-fold increase in CYP6A2 transcripts in the *0.8 luc 110 H* line (Figure 15-16). This was predicted because *Cyp6a2* and *Cyp6a8* show some conservation of putative response elements, including those thought to be involved in mediating caffeine induction (Maitra et al., 2002).

Constitutive and caffeine-induced expression of the *luc* transgene in different tissues

Transgene expression in different tissues under caffeine induced and uninduced conditions were also investigated. Abdomen, head, and malpighian tubules of *0.2 luc 30-*

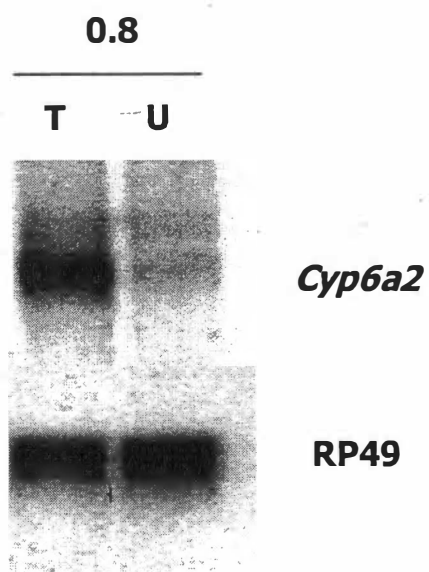


Figure 15- Northern blot analysis of CYP6A2 mRNA levels in *0.8 luc 110 H* females treated with 2.27mM vivarin® (caffeine). A representative autoradiogram of CYP6A2 and RP49 mRNAs is shown. U= untreated. T= treated.

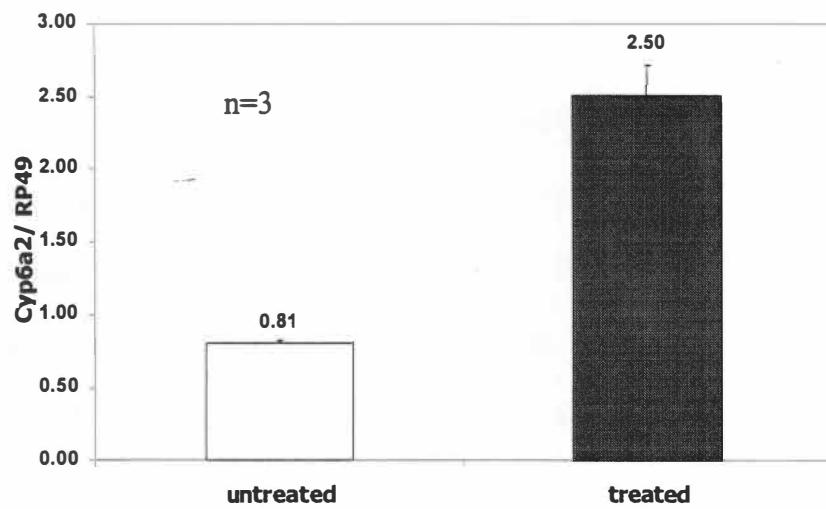


Figure 16- Quantative analysis of normalized CYP6A2 mRNA levels in *0.8 luc 110 H* females treated with 2.27mM vivarin® (caffeine). ($p < 0.004$).

4 (*H-ry*) and 0.8 *luc 110 (H-ry)* female flies were isolated and analyzed via luciferase activity assay (Figure 17). A 12-fold difference in expression between the 0.2 *luc 30-4 (H-ry)* and 0.8 *luc 110 (H-ry)* lines was observed in abdominal tissue compared to a 6.5-fold difference in whole females while head expression varied only 5.2-fold. However, malpighian tubule expression increased 510-fold between the 0.2 *luc 30-4 (H-ry)* and 0.8 *luc 110 (H-ry)* lines. These data suggest that there is an element between –199 and –761 bp that is giving malpighian tubule-specific expression.

For caffeine-induced expression, female flies were treated for 24 hours with 16 mM caffeine. Abdominal-specific caffeine-induced expression increased 10-fold and 20-fold for the 0.2 *luc 30-4 (H-ry)* and 0.8 *luc 110 (H-ry)* lines, respectively (Figure 18). The difference in fold expression may suggest that the sequences between –199 and –761 bp may be giving increased caffeine induction in abdominal tissues. In the head, caffeine treatment increased luciferase activity about 5.5-fold for both the 0.2 *luc 30-4 (H-ry)* and 0.8 *luc 110 (H-ry)* lines, while malpighian tubules expression increased only 3.5-fold and 1.5-fold, respectively.

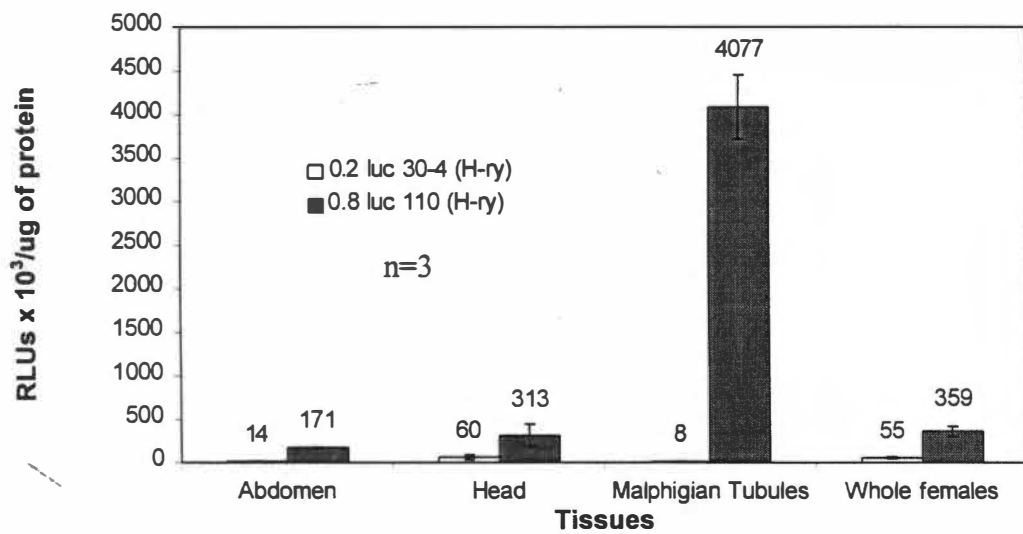


Figure 17- Constitutive transgene expression in different tissues of *0.2 luc 30-4 (H-ry)* and *0.8 luc 110 (H-ry)*. (Abdomen- $p < 0.0002$, Head- $p < 0.1128$, Malpighian Tubules- $p < 0.0027$, and Whole females- $p < 0.0093$.)

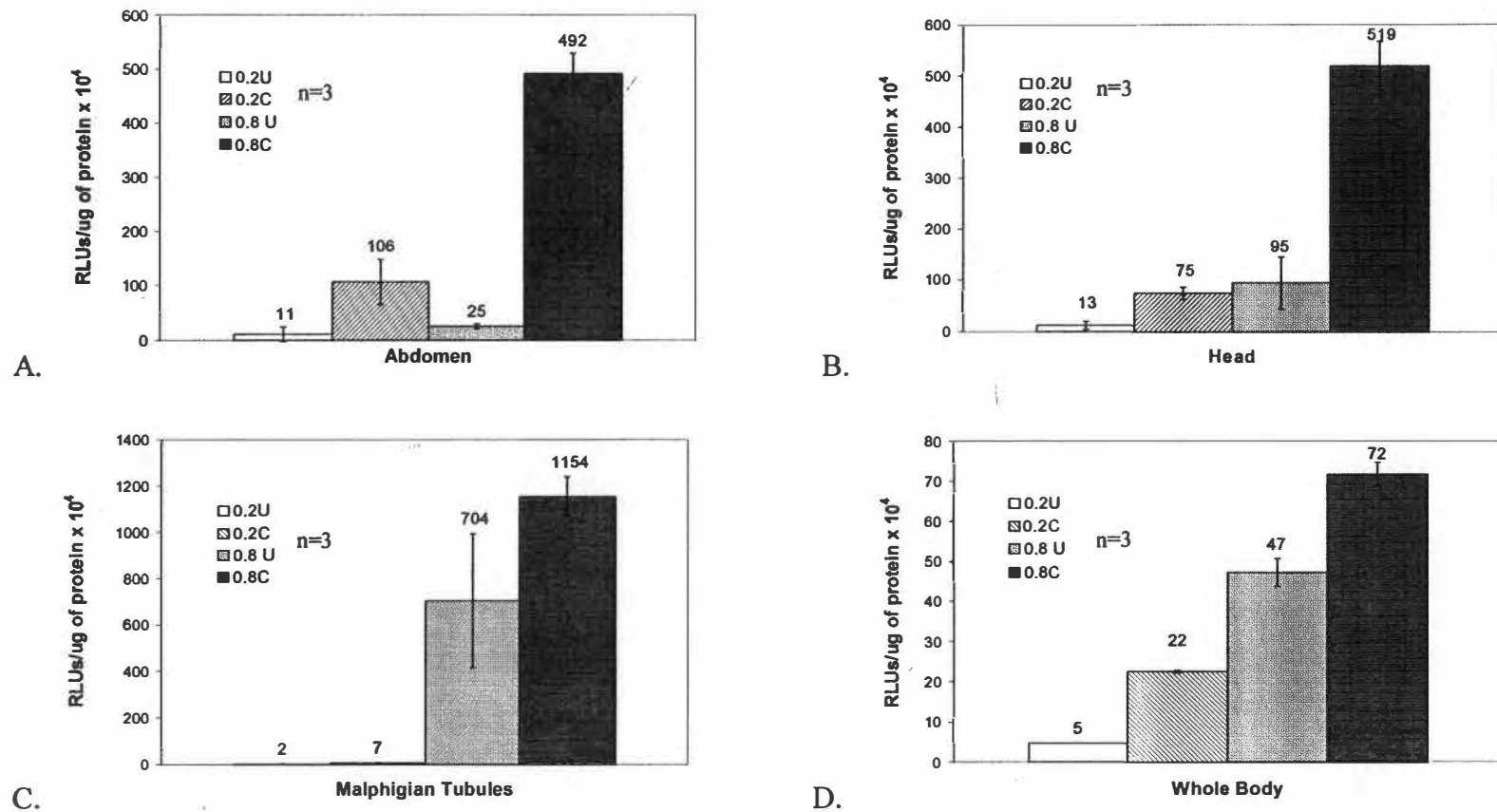


Figure 18- Caffeine-induced transgene expression in different tissues of *0.2 luc 30-4 (H-ry)* and *0.8 luc 110 (H-ry)* females. Females were treated with 16mM Caffeine (Sigma) for 24 hours prior to dissection and assay. A- Abdomen (0.2- $p < 0.0297$, 0.8- $p < 0.0017$), B- Head (0.2- $p < 0.0059$, 0.8- $p < 0.0013$), C- Malpighian Tubule (0.2- $p < 0.0067$, 0.8- $p < 0.1505$), D- Whole body (0.2- $p < 0.0912$, 0.8- $p < 0.0108$).

IV. Discussion

Caffeine, adenosine, and P450 gene expression in *Drosophila*

Caffeine and other methylxanthines have been reported to be adenosine receptor antagonists (Svenningsson et al., 1997). Therefore, it was predicted that adenosine might have an inhibitory effect on gene expression. Both *0.2 luc 30-4 H* and *0.8 luc 110 H* female flies were treated with varying concentrations (between 0-16mM) of adenosine solution. The results indicated that adenosine has no effect on luciferase activity (Figures 8 and 10). Since caffeine induces gene expression and is an adenosine receptor antagonist, then one would not anticipate that adenosine would induce gene expression. Rather, adenosine would inhibit gene expression or have no effect.

Though adenosine treatment did not induce *Cyp6a8* gene expression, caffeine increased activity of the *luc* gene under the *Cyp6a8* promoter. There are several mechanisms that could be proposed to explain this induction. First, caffeine could be causing some signaling event, which results in the increased transcription of the transgene. This would fall under the classic definition of induction. Second, caffeine could have some role in the post-transcriptional stability of mRNA produced from the transgene resulting in increased levels of *luc* mRNA in the cell and resultant increased activity. The possibility of mRNA stabilization by caffeine was discussed by Goasduff et al. (1996). Goasduff et al. (1996) showed that CYP1A1/1A2 mRNA levels increased upon treatment with caffeine. However, this increase in mRNA levels was attributed to transcriptional activation rather than mRNA stabilization because Gonzalez et al. (1993),

showed that CYP1A enzymes can be regulated by transcriptional activation and not mRNA stabilization. Finally, caffeine could be performing some form of post-translational regulation. There is no evidence from the literature that suggests that this is either a possibility or not. Therefore, it is not wise to completely rule out this effect.

Activating protein 1 complex and gene expression

The mechanism of caffeine induction for *Cyp6a8* is unknown. However, by literature review much has been learned about caffeine induction of other genes in other organisms that may shed some light on this phenomenon in *Drosophila*. Caffeine has been shown to upregulate expression of two immediate-early genes in rat striatum, c-fos and junB, as well as several downstream targets of c-fos and junB (Svenningsson et al., 1997). These two proteins are RNA polymerase II transcription factors and members of the bZip family of transcription factors. c-fos and junB heterodimerize to form the Activating Protein 1 (AP-1) complex. Upon heterodimerization, AP-1 complex binds DNA sequences (AP1) at the site of other genes and enhances transcription (reviewed in Karin et al., 1997). Analysis of the c-fos promoter shows that there are functional cAMP response elements (CREs), which respond via cAMP and Ca^{2+} dependent mechanisms in pancreatic β -cells (Schofl et al., 2002). Inhibition of these pathways via the voltage-sensitive Ca^{2+} channel blocker nifedipine or activation via forskolin, which enhances adenylate cyclase activity, was used to demonstrate this.

Drosophila have only one c-fos (*D-fos* or *kayak*) and one junB (*D-jun* or *jra*) homologue. There have been 16 *D-fos* alleles and 23 *D-jun* alleles reported (Flybase, 2002). AP-1 (i.e. the heterodimers of fos and jun) has been shown to be involved in

development in several organisms and mediate such events in *Drosophila* as, tissue closure processes (dorsal closure and head involution), patterning of eye, gut and wing, and apoptosis (Kockel et al., 2001). Since, AP-1 has been shown to be important for mediating other genes' expression in *Drosophila* and caffeine induction of genes in mammals has been shown to work via the AP-1 complex (Svenningsson et al., 1997), it causes me to ask whether AP-1 may be involved in mediating caffeine-induced expression of both *Cyp6a8* and *Cyp6a2*.

Upon sequence analysis of the upstream DNA of *Cyp6a8*, Maitra et al. (2002) identified several putative AP1 binding sites (Table 2). This finding offers a possible mechanism for caffeine-induced expression of *Cyp6a8*. If upon caffeine treatment of flies, *D-fos* and *D-jun* expression is induced as their homologues have been shown to do in mammals (Svenningsson et al., 1997), then AP-1 may be binding to the putative AP1 sites in the *Cyp6a8* upstream DNA and facilitating transcription of *Cyp6a8*.

Mechanisms of adenosine receptor action

Caffeine-mediated gene expression is thought to occur via the adenosine G protein-coupled receptor (GPCR) pathway. Adenosine receptors represent the smallest class of cloned GPCRs. There are four types of adenosine receptors: A₁, A_{2A}, A_{2B}, and A₃. A₁ receptors inhibit adenylate cyclase activity, while A₂ receptors stimulate adenylate cyclase activity (Poulsen and Quinn, 1998). Adenosine receptors are either coupled to the pertussis sensitive- G_i (inhibitory), as is the case for A₁ or G_s (stimulatory) proteins (Poulsen and Quinn, 1998).

Coordinates	Motif
-524	AP1
-510	
-441	
-389	
-327	
-317	
-291	
-80	

Table 2. Putative AP1 binding sites within the -0.8 kb fragment of *Cyp6a8* upstream DNA.

Drosophila have a single adenosine receptor homologue as determined by sequence prediction (Gene CG9753). Pairwise BLAST search (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>) identified that CG9753 is most similar to the human ($e=2e^{-41}$), rat ($e=7e^{-37}$), and mouse ($e=1e^{-37}$) A_{2A} receptors. Therefore, one would predict that this receptor would stimulate adenylate cyclase activity resulting in increased cAMP levels in the cell. If caffeine is mediating its effects via this receptor, then caffeine being traditionally an antagonist of the A₂ receptor would inhibit *Cyp6a8* expression by lowered cAMP titers in the cell. However, this hypothesis directly conflicts with the observed effect of caffeine in this investigation and the reports in the literature that increased cAMP levels result in *D-fos* and *D-jun* induction and induction of *Cyp6a8* by caffeine would then occur via the AP-1 complex binding to AP1 sites in the upstream DNA. Since *Drosophila* have only one adenosine receptor, further analysis of the *Drosophila* adenosine receptor may show that binding of adenosine in different cell types may either increase or decrease adenylate cyclase activity by its coupling to either a G_i or G_s subunit. This seems like a suitable hypothesis, since this single receptor may have to function as both a stimulant and an inhibitor of adenylate cyclase activity to mediate downstream effect. To test whether caffeine-induction is mediated by the effects of the adenosine receptor and cAMP, treatment of flies with other methylxanthines and forskolin may mimic the effects of caffeine on *Cyp6a8* expression, while competition assays with adenosine might diminish the effect of caffeine on *Cyp6a8* expression. These experiments would be more easily done in *Drosophila* S2 cell culture. Transfection of the 0.8-kb *Cyp6a8-luc* transgene indicates that it expresses in these cell lines (data not

shown) and these cells respond to caffeine treatment as measured by several *Cyp6a2-luc* constructs (Bhaskara and Ganguly, unpublished observations).

Another explanation might be that caffeine induction is mediated by events independent of adenosine receptors. There is no evidence that caffeine has an intracellular receptor in *Drosophila* or mammals. However, this may not be ruled out without further study, such as receptor binding experiments. Phenobarbital has been shown to mediate its inductive effects via nuclear receptors. It is plausible to suggest that caffeine is also able to bind to an orphan receptor to induce gene expression. If this is the case, then caffeine would induce gene expression by a novel mechanism. Caffeine is known to be an inhibitor of cAMP phosphodiesterase (Levin et al., 1981), which breaks down cAMP, and the endoplasmic reticulum targeted P450, CYP1A1/1A2 is responsible for caffeine metabolism. Therefore, since caffeine is likely to be internalized into the cell, it stands to reason that this could occur.

Investigating possible roles of CYP6A8 in the cell

Few *Drosophila* P450s have a known substrate (Table 1). CYP6A8 is a *Drosophila* P450 of unknown function. By understanding the circumstances and mechanisms by which this gene is expressed in the cell, we may gain insight as to the function of the enzyme in the cell. If *Cyp6a8* is regulated by the AP-1 complex, then perhaps this indicates a possible role for CYP6A8 in cell proliferation. Additional support for this hypothesis comes from a recent report that the absence of extracellular adenosine results in increased cell proliferation of *Drosophila* imaginal disc and embryonic cells in vitro (Zurovec et al., 2002). Therefore, if adenosine receptors prove to

be regulators of *Cyp6a8* expression, it is reasonable to pursue this line of investigation in discovering a role for CYP6A8.

Cyp6a8 gene expression has also been shown to be developmentally regulated (Jenkins, 2001). *Cyp6a8* expression peaks at the mid 3rd instar larval stage and again during adulthood. This may indicate a development role for CYP6A8. CYP6A8 may be involved in the metabolism of molecules necessary for the control of development.

Caffeine also deserves attention as a possible substrate for CYP6A8. Many P450s have been shown to metabolize their own inducers. This includes the self-induced metabolism of caffeine in mammals by CYP1A1/1A2 (Goasduff et al., 1996). Regulation of P450s by xenobiotics is most certainly an important way for an organism to combat the daily barrage of chemical assaults. By increasing our understanding of the regulation of P450 gene expression, we may be able to harness this information to design better pharmaceutical agents to prevent and treat disease, as well as increase agricultural productivity by manufacturing more effective means of controlling insect pests.

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Vita

Erika Danielle Dean was born in Nashville, Tennessee on July 20, 1978, the daughter of Larry Joe and Shirley Jo Dean. She attended school in the Nashville Metro Public School system until the age of 8, when she moved to Murfreesboro, TN and attended Murfreesboro City and Rutherford County schools. In May 1996, she graduated from Riverdale High School. She entered the Biomedical Engineering Program at Mississippi State University in August 1996. In August 1998, she transferred to the University of Tennessee, Knoxville to pursue an education in Molecular Biology and Genetics and received a Bachelor of Science degree in August 2000, with a major in Biochemistry and Cellular and Molecular Biology. She conducted undergraduate research in the laboratory of Ranjan Ganguly, Ph.D. on the molecular mechanisms governing Cytochrome P450 gene expression in *Drosophila melanogaster*. In August 2000, she entered the graduate program in Biochemistry and Cellular and Molecular Biology, continuing her research on Cytochrome P450 genes of *Drosophila melanogaster* in the lab of Dr. Ganguly. She received the Master of Science degree in Biochemistry and Cellular and Molecular Biology in December 2002.