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I am submitting herewith a dissertation written by Srividya Bhaskara entitled "Molecular mechanism of caffeine-induced expression of two cytochrome P450 genes, *Cyp6a2* and *Cyp6a8*, in *Drosophila melanogaster*". I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry, Cellular and Molecular Biology

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Vice Chancellor and
Dean of Graduate Studies

Molecular mechanism of caffeine-induced expression of two cytochrome P450 genes, *Cyp6a2* and *Cyp6a8*, in *Drosophila melanogaster*

A Dissertation

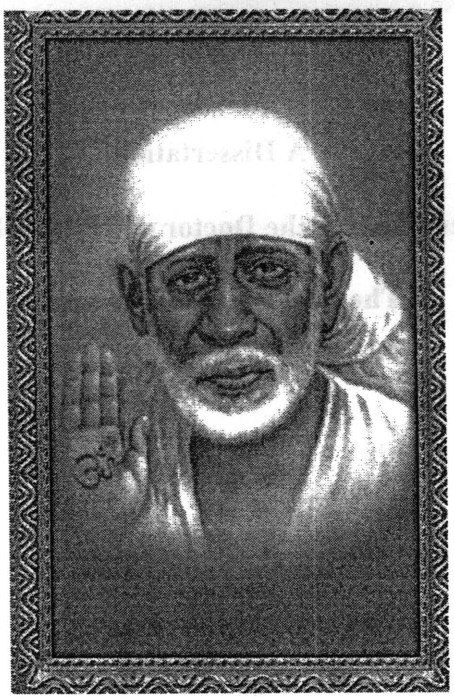
Presented for the Doctor of Philosophy Degree

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Srividya Bhaskara

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Dedication



This dissertation is dedicated to Sri Shirdi Saibaba,

my beloved parents and to all members of my family

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Abstract

Cytochrome P450 monooxygenases or CYPs comprise a large family of enzymes that are found in all classes of living organisms, from bacteria to man. These enzymes are involved in the metabolism of many endogenous and xenobiotic (foreign) compounds. In insects, CYPs confer resistance to various insecticides, and resistance-associated overexpression of multiple *CYP* genes in resistant insects is a common phenomenon. In *Drosophila*, multiple *Cyp* genes including *Cyp6a2* and *Cyp6a8* show higher level of expression in resistant strains than in the susceptible ones. To date, molecular basis of *CYP* gene overexpression has not been examined in detail. Barbiturate compounds such as phenobarbital and barbital induce both these genes. An unpublished observation from our laboratory showed that *Cyp6a2* as well as *Cyp6a8* are induced by over-the-counter caffeine tablet, Vivarin. In the present study, I used pure caffeine as a tool to better understand the mechanism of *Cyp6a2* and *Cyp6a8* gene regulation in *Drosophila*.

The specific objectives of this project has been to (1) map the upstream DNA of both genes for the sequences responsible for caffeine-induction; (2) examine whether adenosine receptors and/or cAMP-specific phosphodiesterase (cPDE) are involved in transduction of caffeine signal to induce the two *Cyp6* genes; and (3) investigate whether *Drosophila* AP-1 transcription factors (D-JUN and D-FOS) play a role in caffeine induction of *Cyp6a8* gene. For these objectives, several *Cyp-luc* reporter plasmids carrying firefly *luciferase* (*luc*) reporter gene under the control of different regions of *Cyp6a8* and *Cyp6a2* upstream DNAs were constructed to transfect *Drosophila* embryonic cells, Schneider line 2 (SL-2). Two transgenic reporter strains carrying firefly *luciferase*

(*luc*) reporter gene under the control of 0.8-kb or 0.2-kb upstream DNA of *Cyp6a8* gene were also used to study the mechanism of caffeine induction *in vivo*.

Results of Northern blot analysis showed that caffeine induces endogenous *Cyp6a2* and *Cyp6a8* genes at the steady-state mRNA levels both in reporter transgenic and wild-type flies. Transfection experiments with SL-2 cells showed that -983/-1 and -761/-11 upstream DNAs of *Cyp6a2* and *Cyp6a8*, respectively, have sequences for caffeine-induced expression. Further transfection experiments with reporter plasmids carrying *luc* reporter gene attached to truncated upstream DNAs of *Cyp6a2* and *Cyp6a8* genes showed that the regions between -265/-129 of *Cyp6a2* and -199/-109 of *Cyp6a8* have sequences that confer caffeine-induced expression. However, the level of both constitutive and induced expression was highest with -981/-1 DNA of *Cyp6a2* and -761/-11 DNA of *Cyp6a8* genes. Sequence analysis identified several putative binding sites for Activator Protein - 1 (AP-1) and cyclic AMP response element CRE binding protein (CREB) motifs in the upstream DNA of both genes. Moreover, when the four core bases of the single AP-1 site present in the -109/-11 DNA of *Cyp6a8* were mutated, constitutive expression decreased by 8-fold, suggesting the positive role of AP-1 in *Cyp6a8* gene expression.

To examine whether caffeine signaling is mediated *via* adenosine receptor (AdoR) and/or *via* cPDE inhibition, SL-2 cells transfected with *Cyp6a2* and *Cyp6a8* reporter constructs were treated with AdoR agonists or with antagonists or with cPDE inhibitors. The *Cyp6a8-luc* reporter transgenic lines were also treated with these chemicals. The results showed that caffeine signaling is mediated by PDE inhibition and *via* increase in the intracellular cAMP level. Indeed, treatment with dibutyryl cAMP induces *Cyp6a2* as

well as *Cyp6a8* promoters. Since induction of cAMP pathway is known to upregulate AP-1 transcription factors, effect of overexpression of *Drosophila* D-FOS and (or) D-JUN (components of AP-1) on *Cyp6a8* promoter activity in SL-2 cells was examined. Surprisingly, activity of *Cyp6a8-luc* reporter construct was inhibited when D-FOS or D-JUN proteins were overexpressed, suggesting that AP-1 proteins are inhibitory for *Cyp6* gene expression. In contrast, the *Cyp6a8* promoter activity was upregulated, when cells were transfected with anti-D-JUN plasmid or when cells transfected with D-JUN or D-FOS sense construct were treated with caffeine. When relation between caffeine and the two AP-1 proteins was examined, it was found that caffeine treatment significantly lowers the D-JUN protein level both in SL-2 cells as well as in adult flies. Reporter gene assays and Northern blot analysis showed that caffeine treatment has no effect on the transcriptional activity of the *D-jun* and *D-fos* genes. Taken together, it might be concluded that caffeine induction of *Cyp6a2* and *Cyp6a8* genes is mediated *via* degradation of D-JUN that acts as a repressor for the promoter of *Cyp6a8*. Induction of the cAMP pathway and subsequent phosphorylation of the AP-1 proteins may relieve the AP-1 mediated-repression by promoting the degradation of these proteins. Further investigation is required to resolve these possibilities.

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Chapter 1

General introduction

Cytochrome P450 enzymes and their biological functions

Cytochrome P450 monooxygenases (CYPs) represent a large family of heme proteins present in all forms of life (Guengerich, 1993). P450 derives its name from the discovery made 40 years ago of a liver microsomal pigment which when reduced and saturated with carbon monoxide (CO), shows an absorption peak of 450nm (Omura, 1999). The importance of CYPs is evidenced by the fact that is found in all cell types in the body except for RBC and skeletal muscle cells (Guengerich, 1993). As of January 2005, total number of CYP gene sequences reported to the nomenclature committee is 4504 (http://drnelson.utmem.edu). The distribution of these *CYP* gene sequences in different organisms are as follows: 1581 in animals, 1740 in plants, 508 in fungi, 203 in lower eukaryotes and 487 in bacteria. Because *CYPs* form a multigene family, most organisms have multiple *CYP* genes belonging to different families. A wide range of number of putatively functional *CYP* genes is found in different organisms. It is 57 in humans, 83 in *Drosophila melanogaster* and 2 in *Schizosaccharomyces pombe* (fission yeast). Highest number of putatively functional *CYP* gene sequences found in one species is 323 sequences in rice (http://drnelson.utmem.edu). No *CYP* sequences have been found in the malaria parasite, *Plasmodium falciparum*.

CYPs play a major role in oxidative transformation of endogenous and exogenous molecules (Omura, 1999). CYPs play major catalytic role in various metabolic pathways

for the biosynthesis of different biologically important compounds such as hormones, prostaglandin, pigments and etc (Guengerich, 2004; Coon, 2005; Gonzales 2005). CYPs also metabolize various xenobiotic (foreign) compounds such as drugs, synthetic organic chemicals, polycyclic aromatic hydrocarbons and many different environmental toxic chemicals (Porter and Coon, 1991; Coon 2005). Besides the monooxygenase reactions, CYPs also catalyze hydroxylation, epoxidation, peroxygenation, deamination, desulfuration, reduction, and dehalogenation (Porter and Coon, 1991). For this reason CYPs are also called mixed function oxidase or MFO that is capable of metabolizing structurally different chemicals (Guengerich, 1993).

CYPs contain an average of 500 amino acids and have a molecular weight of 40-55Kda (Fujii-Kuriyama et al., 1982). Xenobiotic-metabolizing forms of CYPs are predominantly found in the microsomes in eukaryotic cells to which these P450s are anchored via their N' terminal approximately 20 hydrophobic amino acid residues. The C-terminus of the protein has a signature amino acid sequence motif FXXGXXXCXG which forms the heme binding domain. The cysteine in this motif is highly conserved which binds with the heme (Omura, 1999). CYPs are also found in the mitochondria. The Mitochondrial and most bacterial P450s have three components, a FAD- and FMN containing flavoprotein called adrenodoxin reductase, an iron-sulphur protein, and P450 (Porter and Coon, 1991). The eukaryotic microsomal P450 contains only two components, a FAD and FMN containing NADPH-dependent P450 reductase, and P450 (Porter and Coon, 1991). For oxidation of the substrates, P450 splits the molecular oxygen into two atoms, and inserts one into the substrate (S), and combines the other with two hydrogen atoms to form a water molecule. Two electrons necessary for this

reaction are provided by NADPH as shown in the equation below. Since only one of the two oxygen atoms is incorporated into the substrate, P450s are called monooxygenases (Meunier et al., 2004). The detailed mechanism of oxidation reaction catalyzed by CYP is shown in Fig.1-1 in which S refers to substrate.

$$S + O_2 + 2e^- \text{ (from NADPH)} + 2\,H^+ \xrightarrow{\text{Cytochrome P450}} SO + H_2O$$

In insects, both mitochondrial and microsomal P450s have been shown to be present. The electrons provided to P450s in insects, are derived from NADPH cytochrome P450 reductase and/or cytochrome b5 for microsomal P450s and from ferredoxin coupled to adrenodoxin reductase in mitochondrial systems (Tijet et al., 2001).

Insect genomes probably carry about 100 P450 genes, each coding for a different P450 enzyme (Tijet et al., 2001). Recent genomic sequence data of 120Mb euchromatic genome of *Drosophila melanogaster* has identified the presence of 90 P450 genes, of which 83 code for functional genes whereas 7 are pseudogenes (listed by Tijet et al., 2001). More than half of the genes belong to only two families, *CYP4* and *CYP6*. The *CYP6* family is found only in insects, whereas the *CYP4* family genes are also found in vertebrates (Tijet et al., 2001).

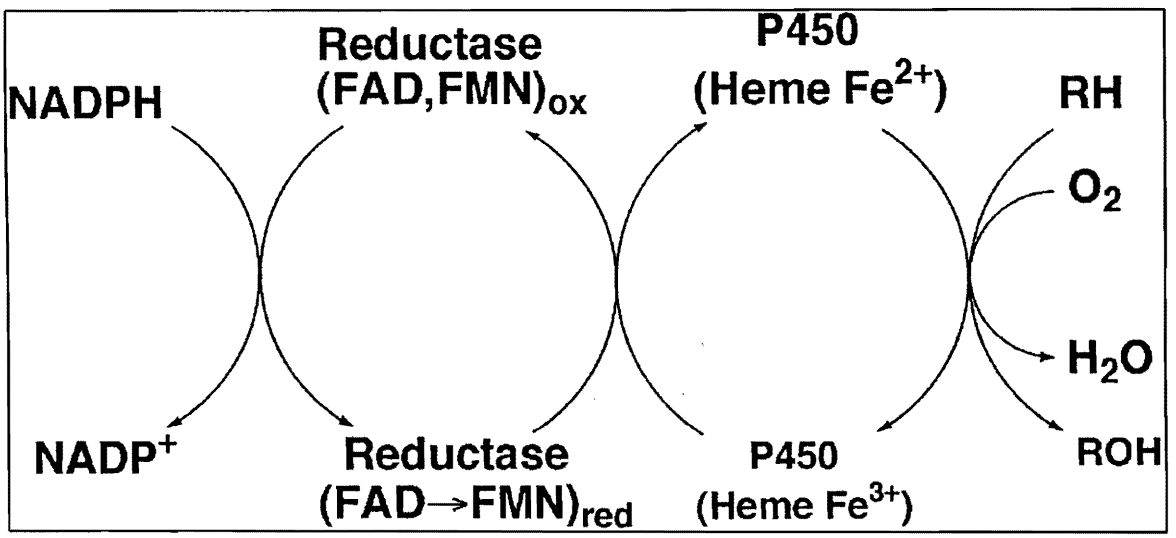


Figure1-1: Mechanism of substrate oxidation catalyzed by CYP.

Insect P450s and xenobiotic metabolism

In insects, P450s are also known to be involved in the metabolism of various endogenous and xenobiotic compounds. One of the major metabolic function of P450s in insect is to catalyze reactions in biosynthetic pathways involved in the synthesis of ecdysteroid and juvenile hormones, which are vital for insect growth, development, and reproduction (Feyereisen, 1999). In *Drosophila* five genes, *disembodied* (*dib*), *shadow* (*sad*), *shade* (*shd*), *spook* (*spk*) and *phantom* (*phm*) form a gene family called Halloween (Gilbert, 2004 for review). These five genes encode P450 enzymes and mutation of these genes is known to cause embryonic lethality. Recently it has been shown that *dib* (*Cyp302a1*), *sad* (*Cyp315a1*) and *shd* (*Cyp314a1*) are involved in ecdysone biosynthesis (Chavez et al., 2000; Gilbert, 2004).

Besides their role in hormone biosynthesis, P450s also play an important role for insects' survival. Their xenobiotic metabolizing capacity helps insect to feed on plants containing toxic compounds. For instance, tobacco hornworm, *Munduca sexta*, uses P450 enzymes to metabolize nicotine present in the tobacco plant that they feed on (Snyder and Glendinning, 1996). Larvae of the black swallowtail butterfly (*Papilio polyxenes*) ingest leaves of plants that produce a toxic chemical called as xanthotoxin. It was found that these larvae produce high level of CYP6B1 that catalyzes the oxidative destruction of xanthotoxins (Ma et al., 1994; Petersen et al., 2001).

P450s play another important role which helps insects protect themselves from the toxic effects of insecticides. P450 enzymes metabolize insecticides that might otherwise kill the insects (Feyereisen, 1999; Scott, 1999; Scott and Wen, 2001). Many

P450s capable of metabolizing insecticides and other xenobiotics are constitutively overexpressed in the resistant strains. Metabolic resistance to insecticides is found to be mostly due to the elevated levels of P450 because application of P450 inhibitor such as piperonyl butoxide, decreases the resistance factor significantly (Hodgson et al., 1993). Several studies on *Musca* (house fly) and *Drosophila Cyp* genes showed that insecticide resistance is associated with increased level of one or more *Cyps* (see Scott, 1999; Scott and Wen, 2001 for review). DDT-resistance associated overexpression has been observed for *Cyp6a2*, *Cyp6a8*, *Cyp12d1* and *Cyp6g1* genes of *Drosophila* (Waters et al., 1992; Maitra et al., 1996, 2000; Dombrowski et al., 1998; Daborn et al., 2002; Festucci-Buselli et al., 2005).

In *Drosophila*, *Cyp6a2* and *Cyp6a8* show higher level of constitutive expression in the DDT and diazinon resistant 91-R strain than in the susceptible 91-C and ry^{506} strains (Maitra et al., 1996). Also, xenobiotics like phenobarbital and barbital induces *Cyp6a2* and *Cyp6a8* genes in various strains of *Drosophila* (Maitra et al., 1996). CYP6A2 converts aflatoxin B1 into a recombinogenic product in *S.cerevisiae* (Saner et al., 1996). The CYP6A2 expressed in lepidopteran cells has been shown to catalyze metabolism of diazinon, an organophosphate insecticide (Dunkov et al., 1997). There are not many metabolic studies being done with CYP6A8 apart from a report where CYP6A8 was shown to metabolize lauric acid, a short-chain unsaturated fatty acid to produce 11-hydroxylauric acid in *S.cerevisiae* (Helvig et al., 2004). In the present investigation, *Cyp6a2* and *Cyp6a8* genes were used as the prototypes to understand the molecular mechanism of *Cyp6* family gene regulation as these genes are induced by a variety of xenobiotics and also have been shown to metabolize certain foreign compounds.

Cytochrome P450 gene regulation

The variety of CYPs necessitates the presence of considerable diversity in the mechanism of regulation of these enzymes as shown in Fig. 1-2. The most common mechanism of regulation is at the transcription level. Post-transcriptional mechanisms include mRNA and protein stabilization or degradation due to changes in the phosphorylation state of the enzyme. Interestingly, some CYPs are regulated at more than one level of expression. For example, CYP1A1 is regulated at the transcriptional level and by mRNA stabilization (Fig. 1-2). CYP2E1 is regulated at transcription, translation and post-translational levels (Fig. 1-2). Also, inducers of CYP2E1 like acetone, stabilize this protein by preventing it from getting phosphorylated, which would otherwise lead to degradation of the protein (Porter and Coon, 1991).

Induction studies have helped the scientists to better understand the mechanism of P450 gene regulation (Porter and Coon, 1991). The regulation of barbiturate induction of P450 is best understood in bacteria. The regulation of gene expression by barbiturates in bacteria provides a direction for examining similar regulation in eukaryotes as barbiturate induction is observed in a wide variety of species, ranging from bacteria to humans.

There is considerable information about the molecular mechanisms that regulate barbiturate-induced expression of $P450_{BM\text{-}1}$ (*CYP106*) and $P450_{BM\text{-}3}$ (*CYP102*) genes of *Bacillus megaterium*. BM3R1, the repressor molecule, binds to the operator sites of BM1 and BM3 genes which also have a *cis*-regulatory element called barbie box.

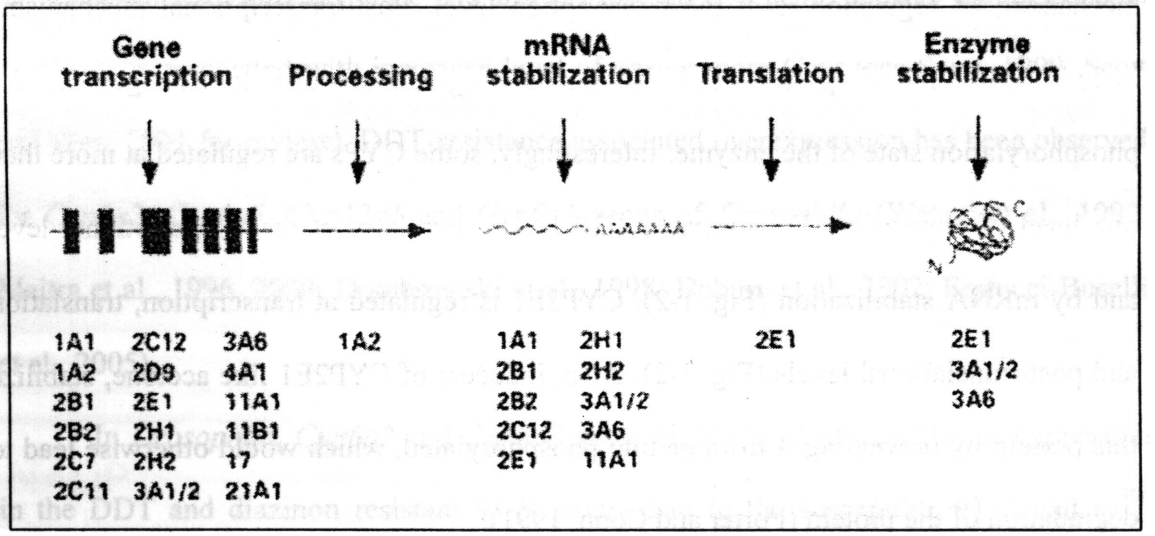


Figure 1-2: Multiplicity in the regulation of *CYP* gene expression. The names of the *CYP* genes that are regulated at different steps of gene expression are shown below each step. From Porter and Coon (1991).

The barbie box is found in the upstream region of these genes. When barbiturate compound is added to the medium, the repressor fails to bind to the operator site or the barbie box and therefore results in induction of *CYP106*. Barbiturates also induce synthesis of positive factors (BMP1, BMP2) that competes with the repressor to bind to the BM-1 operator site and to Barbie box and thereby increasing the expression of BM1 gene (Kemper, 1998).

The induction of human *CYP1A1* gene by polycyclic aromatic hydrocarbons is an ideal example showing how mammalian cells can cope with environmental toxicants (Whitlock, 1996). The regulation of *CYP1A1* has been studied extensively as its activity has been linked to lung cancer. Individuals with highly inducible forms of *CYP1A1* enzyme seem to be more susceptible to lung cancers. Dioxin (2,3,7,8-tetrachloro dibenzo-p-dioxin) also called as TCDD is the potent inducer of *CYP1A1* (Sanderson et al., 2001). The receptor for dioxin is aryl hydrocarbon receptor (Ah receptor). Ah receptor is normally bound to heat shock protein (hsp90) in the cytoplasm. Upon binding of dioxin to the Ah receptor, hsp-90 dissociates from the receptor and aryl hydrocarbon receptor nuclear translocater (ARNT) binds to the Ah receptor-dioxin. The complex of Ah receptor-dioxin-ARNT then enters the nucleus where it binds to the xenobiotic regulatory element (XRE) present in the upstream of *CYP1A1* gene and induces its transcription (Guengerich, 1993).

In contrast to the well-known Ah receptor-mediated regulation of the *CYP1A1* gene, the molecular mechanism of phenobarbital (PB) induction of *CYP* genes is still unclear. However, PB-responsive sequences have been identified in the upstream region of several mammalian *CYP* genes. The minimum sequence required for PB induction is

found to be a 51bp sequence called as Phenobarbital responsive enhancer module (PBREM)(Kemper, 1998). Recent evidence has shown that PB induction mechanism may also involve orphan receptors belonging to the nuclear receptor/steroid superfamily like CAR (constitutive androstane receptor), PXR (pregnane x receptor) and PPAR (peroxisome proliferator-activator receptor). These receptors bind to the dimerization partner, RXR (retinoid x receptor) and bind to DNA elements known as nuclear receptor binding sites (NR sites) and activate transcription (Honkakoski et al., 1998; Czekaj, 2000; Swales and Negishi, 2004). *CYP2B* and *CYP3A* genes appear to be targets for the CAR and PXR, respectively (Czekaj, 2000).

Caffeine and cytochrome P450

Caffeine is the most widely consumed beverage in the world. Caffeine is present in dietary sources like coffee, tea, chocolate bars, soft drinks and cocoa beverages (Lorist and Tops, 2003). Because of its widespread use, effect of caffeine on various physiological processes in humans, rats, mice and other mammals have been studied extensively (Fredholm et al., 1999). A significant proportion of the effects of coffee are related to the actions of caffeine, the best-known pharmacologically active constituent of coffee. Caffeine increases alertness and cognition in humans (Lorist and Tops, 2003). Caffeine has anti-carcinogenic effect as it inhibits cancer production by viral, chemical and physical agents. Stimulatory adenosine (A_{2A}) receptors induce enhanced nerve cell loss in ischemia, and blockade of A2A receptors cause neuroprotection. Thus caffeine is a good candidate that can be used to treat Parkinson's disease and offer neuroprotection due to its property to block A2A receptors (Chen, 2003).

In contrast to the above-mentioned beneficial effects of caffeine, it also has negative effects on physiology. Caffeine at very high concentrations induces chromosomal abnormalities and mutagenic effects as it inhibits DNA-repair process. Caffeine alters the G1, S and G2 checkpoints delay periods. When cells are treated with caffeine and DNA-damaging agent, the lethality of DNA-damaging agent is often stimulated (Porta et al., 2003). Caffeine may sensitize cells to DNA damage by either inhibition of repair by elimination of checkpoint controls or by binding to the site of damage and directly inhibiting repair function (Porta et al., 2003). Due to the wide role played by caffeine in various physiological processes, it is imperative to understand the mechanism of caffeine action.

Although the relation between caffeine and CYP gene regulation is not well studied, the role of CYPs in caffeine metabolism has been examined to a great extent. Caffeine is chemically a trimethyl xanthine. It is an alkaloid and is a xanthine derivative methylated at N-1, N-3 and N-7 positions. N-1, N-3, and N-7 demethylation reactions produce theobromine (TB), paraxanthine (PX), and theophylline (TP) respectively (Chung et al., 1998) (Fig.1-3). Caffeine is metabolized to paraxanthine in liver microsomes by CYP1A2 (Fredholm et al., 1999). Indeed the amount of PX produced from coffee is used to phenotype humans for their CYP1A2 activities. Flavin monooxygenase (FMO) present in liver microsomes together with CYPs, is also involved in the metabolism of caffeine. Studies using inhibitors of CYP1A2 that block the formation of TB, TP and trimethyl urate (TMU) are inhibited only marginally (Chung et al., 1998; Wittayalertpanya et al., 2003). These results showed that CYP2E1 is also involved in the formation of these metabolites (Chung et al., 1998).

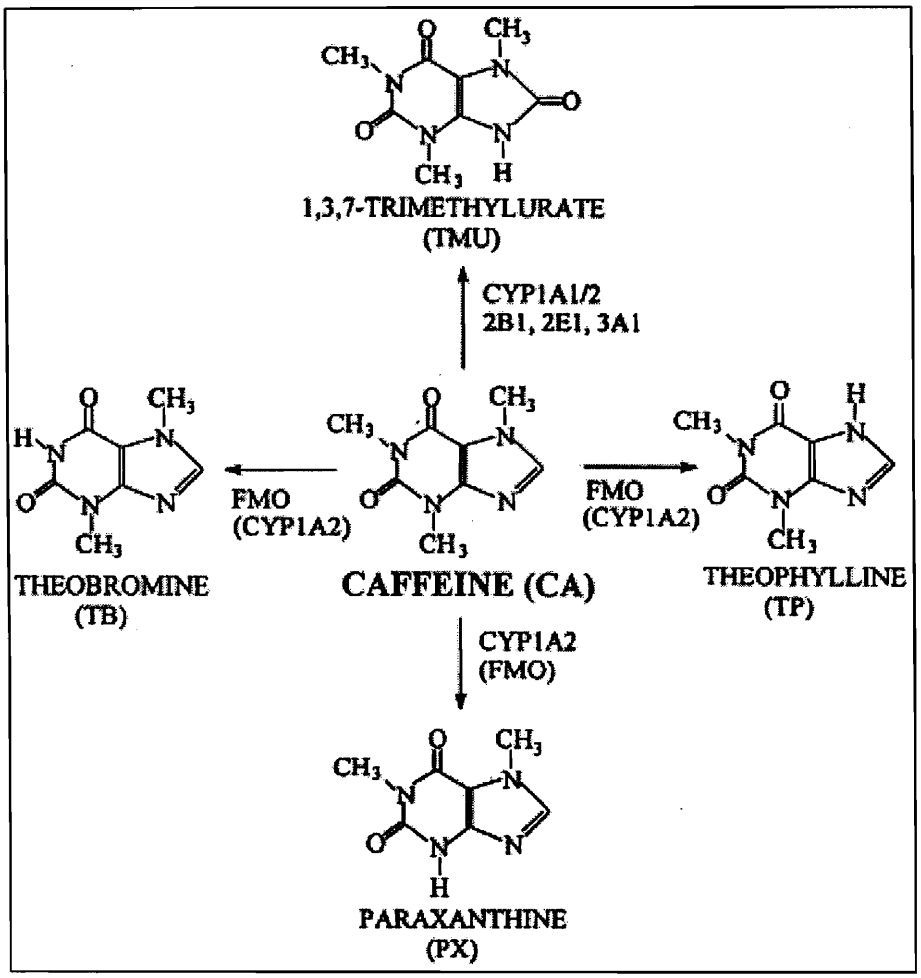


Figure 1-3: CYP-mediated metabolism of caffeine in rat liver microsomes. FMO refers to flavin-containing monooxygenase (Chung et al., 1998).

Since CYPs play a major role in caffeine metabolism, it would be vital to understand the mechanism by which *CYP* gene expression is upregulated by caffeine. There are few observations made on the effect of caffeine in insects. For instance, flies when fed with caffeine, show a dose-dependent decrease in rest (Shaw et al., 2000). *Cyp4e2* was found to be increased during waking period in *Drosophila* (Shaw et al., 2000). Caffeine that is naturally found in plants is shown to inhibit insect feeding and act as a pesticide (Nathanson, 1984). Although the above-mentioned studies are made, the effect of caffeine on gene expression is still not well explored except for few reports. Caffeine has been shown to induce CYP1A1/1A2 mRNA levels in rats (Goasduff et al., 1996). In PC-12 cells, caffeine upregulates the transcription of dopamine 2 receptor gene (D2R) (Stonehouse et al., 2003). A rapid, transient, and dose-dependent increase of c-fos, c-jun, and junB mRNAs was observed in rat striatum following caffeine treatment (Svenningsson et al., 1995). Even though, caffeine has been shown to alter the expression of certain genes including *CYP*s, the molecular mechanism by which caffeine induces *CYP* genes is still not understood.

Signal transduction mediated by caffeine

In mammals, the primary action of caffeine is to block adenosine receptors which are seven trans-membrane proteins (Fredholm, 1999). Adenosine is a neuromodulator that modulates the brain function by central inhibitory actions. So far, four adenosine receptors (purino receptors), A_1, A_{2a}, A_{2b}, and A_3 have been cloned and characterized in several species. The rat A_3 is not affected much by caffeine. The A_{2b} receptor requires very high concentrations of adenosine for its stimulation. Therefore, the likely candidates

for caffeine action are A_1 and A_{2a} receptors, which are activated at low concentrations of adenosine. A_1 and A_{2a} are both G-protein coupled receptors. A_1 is coupled to the pertussis toxin sensitive inhibitory G-proteins like Gi-1, Gi-2, Gi-3, Go1 receptors and A_{2a} receptor is coupled to the stimulatory G-protein like Gs (Fredholm et al., 1999; Fredholm et al., 2001). Activation of A_1 causes inhibition of adenylyl cyclase and decreases the cAMP level. Activation of A_{2a} leads to activation of adenylyl cyclase and increases the intracellular cAMP level. Interestingly, there are evidences for the presence of both types of receptors with opposing effects in the same cell (Fredholm et al., 2001).

Non-physiological doses of caffeine has been shown to block phosphodiesterases that hydrolyze cAMP (Fredholm et al., 1999). PDE inhibitors act by competing with cAMP to bind the catalytic site of the PDE enzyme, leading to an elevated level of cAMP (Corbin and Francis, 1999). Some PDEs are highly specific for hydrolysis of cAMP including PDE4, some are highly cGMP-specific, and some have mixed-specificity (Corbin and Francis, 1999). PDE4, the target for caffeine inactivation has a very high specificity for cAMP (Ye et al., 2001). In *Drosophila*, cAMP-specific PDE is encoded by the *dunce* (*dnc*) gene (Davis and Kiger, 1981). Mutation of this gene has a considerable effect on learning and memory of the fly (Byers et al., 1981).

Caffeine antagonism at adenosine receptor or its inhibition of PDE enzyme results in increased cAMP level in the cell (Fredholm et al., 1999). In mammals, cAMP has been shown to induce *CYP2A5* and *CYP11A1* transcription (Viitala et al., 2001; Guo et al., 2003). Increased cAMP levels enhance the expression of c-Jun and c-Fos in mammals (Xia and Goldstein, 1999). AP-1 (the heterodimer of D-jun and D-fos) much like its mammalian homolog has been shown to activate transcription of genes involved in

regulation of cell growth, differentiation, and development in *Drosophila* (Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997).

Objectives of the proposed research

Vivarin has been shown to induce *Cyp6a8* promoter activity in *Cyp6a8*-reporter transgenic flies (Dean and Ganguly, Unpublished data). These transgenic flies carry 0.2Kb or 0.8Kb upstream DNA of *Cyp6a8* attached to the luciferase reporter gene. Vivarin was also found to induce *Cyp6a2* and *Cyp6a8* gene expression in these transgenic flies (Dean and Ganguly, Unpublished data). The major component of Vivarin is caffeine. Therefore, the major focus of the proposed research is to elucidate the mechanism by which caffeine induces *Cyp6a2* and *Cyp6a8* gene expression.

The first objective is to map the upstream DNA of *Cyp6a2* and *Cyp6a8* genes for the sequences that are required for constitutive and caffeine-induced expression. In this context, putative binding sites for transcription activator protein AP-1 found in the upstream DNA of *Cyp6a8* (Maitra et al., 2002) are also relevant elements for investigation because in rat brain striatum caffeine increases AP-1 transcription factors (Svenningson et.al, 1995). The second objective is to determine whether caffeine induction is mediated *via* adenosine receptor and/or cAMP phosphodiesterase. The third objective is to examine the role (if any) of *Drosophila* AP-1 proteins, D-FOS and D-JUN in caffeine-induced expression of *Cyp6a8* gene.

Chapter 2

Investigation of the effect of caffeine on three CYP6 family genes in *Drosophila*

Introduction

Cytochrome P450 monooxygenases or CYPs comprise one of the largest family of enzymes that are found in all living organisms. CYPs catalyze reactions in various metabolic pathways that are involved in the biosynthesis of different biologically important compounds such as hormones, prostaglandin, pigments and etc (Guengerich, 2004; Coon, 2005). Besides their endogenous functions, CYPs metabolize a large number of foreign (xenobiotic) compounds that enter the living body everyday. Because multiple CYPs are found in most organisms and they are involved in diverse functions, CYPs have been studied in various organisms from different biological perspectives. One such perspective is the induction and regulation of *CYP* genes, especially in relation to xenobiotic metabolism in mammals including humans (Czekaj, 2000; Honkakoski and Negishi, 1997; Corcos and Lagadic-Gossmann, 2001; Wang and Negishi, 2003; Dickins, 2004; Nebert et al., 2004; Mandal, 2005). Majority of these studies used *CYP1A* and *CYP2B* family genes to understand the mechanism of induction by three xenobiotic compounds - phenobarbital, dioxin and benzo[a]pyrene. While phenobarbital is used as a drug, the other two chemicals are environmental carcinogens. These xenobiotic compounds helped identify the *cis*-regulatory elements, receptors and other factors that are normally involved in *CYP* gene regulation in mammals (Sueyoshi and Negishi, 2001; Nebert et al., 2004; Puga et al., 2005; Hankinson, 2005).

In insects, *CYP* genes have been studied mostly in relation to insecticide resistance phenotype. It has been found that resistant insects have higher levels of one or more CYP mRNA(s) compared to the susceptible insects (for review see Scott, 1999; Scott and Wen, 2001; Wilson, 2001). To understand the mechanism of *CYP* gene regulation in insects, phenobarbital (PB) has also been tried as a tool. Many insect *CYP* genes including *Cyp6a2* and *Cyp6a8* of *Drosophila* are induced by PB (Maitra et al., 1996; Dunkov et al., 1997; Liu and Scott, 1996; Dombrowski et al., 1998; Li et al., 2000; Stevens et al., 2000; Scott and Wen, 2001). It has been shown *via* germline transformation that the sequences present in -981/-129 DNA are required for constitutive and barbiturate-induced expression of *Cyp6a2* gene of *Drosophila* (Dombrowski et al., 1998). In the case of *Cyp6a8* of *Drosophila* sequences in the -199/-761 DNA are required for highest constitutive and barbiturate induction (Maitra et al., 2002).

Besides drugs and environmental toxicants, caffeine is probably another xenobiotic compound to which most people worldwide are exposed daily. Chemically caffeine is very similar to other purines and called trimethyl xanthine. Because of its effect on nervous system, caffeine has been studied extensively in relation to various physiological processes in humans and other mammals (Fredholm, 1999; Hartley et al., 2001; Nawrot et al., 2003; Papaioannou et al., 2005). In humans, caffeine is predominantly metabolized to paraxanthine by CYP1A2 (Butler et.al, 1989; Berthou at al., 1992). Although the role of CYPs in metabolism of caffeine is well-studied in humans and other mammals (Fredholm et al., 1999), very little is known about the effect of caffeine on the expression of *CYP* and other genes except that in one study it has been

shown that caffeine induces *CYP1A1* and *CYP1A2* genes in rat liver and kidney (Goasduff et al., 1996). *CYP1A2* expression is in turn induced by caffeine, supporting the fact that many P450s metabolize their own inducers (Goasduff et al., 1996). However, the link between caffeine and gene regulation is not fully understood. In another study, a rapid, transient, and dose-dependent increase of c-fos, c-jun, and junB mRNAs was observed in rat striatum following caffeine treatment and caffeine also upregulates the transcription of dopamine 2 receptor gene (D2R) in PC-12 cells (Svenningsson et al., 1995; Stonehouse et al., 2003).

Recently, effect of Vivarin, an over-the-counter caffeine tablet, on the *Cyp* gene expression has been examined in *Drosophila* (Dean et al., unpublished results). The results of this study showed that both *Cyp6a2* and *Cyp6a8* genes are induced by Vivarin. Although these studies suggest that caffeine may influence the expression of *CYP* and possibly other genes, the mechanism of caffeine induction has not been examined in a systematic way. In the present investigation induction of *Cyp6a2* and *Cyp6a8* genes of *Drosophila* by pure caffeine in adult flies and Schndeider-2 cell line (SL-2 cells) was examined and compared. The results showed that both genes are induced by caffeine similarly in adult flies and SL-2 cells suggesting that factors necessary for caffeine induction are present in the SL-2 cells. In addition, upstream DNA of both *Cyp6a2* and *Cyp6a8* genes were analyzed via reporter gene assay to identify sequence motifs that may play some role in caffeine-induced expression of these two genes.

Materials and methods

D. melanogaster strains and cell culture

In the present investigation ry^{506}, 91-C, 91-R, *0.2-luc4H-ry* and *0.8-luc110H-ry* strains of *D. melanogaster* were used. While 91-R strain is highly resistant, all other strains are susceptible to DDT. The *0.2-luc4H-ry* and *0.8-luc110H-ry* strains are reporter transgenic lines which carry 0.2luc-A8 and 0.8luc-A8 chimeric reporter constructs, respectively (Maitra et al., 2002). These reporter constructs have firefly *luciferase* (*luc*) gene as a reporter under the control of 0.2- or 0.8-kb upstream DNA of the *Cyp6a8* gene representing -11/-199 and -11/-761 positions, respectively (Maitra et al., 2002). Flies were reared at 25°C on standard cornmeal-agar-molasses medium (0.65% bactoagar, 5.5% cornmeal, 3% brewer's yeast, 5% (v/v) 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).

Drosophila melanogaster Schneider line SL-2 cells (Schneider, 1972) were obtained from Invitrogen (Carlsbad, CA). The cells were maintained at 25°C in Schneider's *Drosophila* medium (Gibco-BRL) supplemented with 10% heat-inactivated bovine calf serum and 0.01% penicillin-streptomycin (Sigma). The cells were transferred to fresh media every fourth day.

Construction of reporter plasmids

Recombinants plasmids carrying 0.9-kb (-983/-1) and 0.8-kb (-761/-11) upstream DNA of *Cyp6a2-91R* and *Cyp6a8-91R* alleles of 91-R strain (Dombrowski et al., 1998; Maitra et al; 2002) were used as the template for constructing additional plasmids used in

the present investigation. To map the *cis*-regulatory element(s) required for constitutive and caffeine-induced expression of *Cyp6a2* and *Cyp6a8* genes, these two template plasmids were used and different length of upstream DNA was amplified *via* PCR and cloned upstream of the firefly *luciferase* (*luc*) gene of pGL3 (basic) vector (Promega, Madison, WI). Sequences of the primers used for PCR amplification are shown in Table 2-1 and Table 2-2. To the 5'-end of all distal primers used for *Cyp6a2* DNA amplification a restriction site for *Mlu*I was added. The proximal primer A2-A, spanning between positions -1 and -17 of the *Cyp6a2-91R* allele, was used with each distal primer to amplify the upstream DNA. The A2-A primer had an added *Sal*I site at its 5'-end. The amplified DNAs were purified, digested with *Mlu*I and *Sal*I, and cloned in *Mlu*I-*Xho*I cut pGL3-basic vector (Promega Corporation, Madison, WI). This placed the upstream DNAs in front of the *luc* reporter gene in proper orientation. The amplified upstream DNA spanned -983/-1, -522/-1, -265/-1 and -129/-1 regions of the *Cyp6a2-91R* gene and the recombinant reporter plasmids were named 0.9luc-A2, 0.5luc-A2, 0.2luc-A2 and 0.1luc-A2, respectively. All PCR products were sequenced and found to match with the sequence of the upstream DNA reported earlier (Dombrowski et. al., 1998).

0.8luc-A8 and 0.2luc-A8 reporter plasmids with -766/-11 and -199/-11 DNA of *Cyp6a8-91R* allele cloned in front of the *luc* gene of pGL2(N)-basic vector (Promega, Madison, WI) have been described previously (Maitra et al., 2002). In the present investigation, 0.8luc-A8 plasmid was used as a template to amplify different regions of *Cyp6a8* upstream DNA. The distal primers, A8-5F, A8-1F and A8-0.6F, and the common proximal G10 primer used for amplification are described in Table 2-2.

Table 2-1

Primers used for the amplification of *Cyp6a2-91R* upstream DNA

Primer names	Region upstream DNA amplified	Sequence of the distal primer
A2-C	-983/-1	5' ctcacgcgtTTCATTCGTTTTATCGCCG 3' (-983/-965)
A2R-1M	-522/-1	5'ccacgcgtCAAGTGGGATCGTCCTGTAC 3' (-522/-503)
A2R-2M	-265/-1	5'ccacgcgtGCAGTGAAGCGTATGAGTATC 3' (-265/-245)
A2R-3M	-129/-1	5'ccacgcgtGCTAGCTAGCTCACATGCTGTC 3' (-129/-112)

Extra bases attached to the 5'-end of each distal primer are shown in lower case. The site for *Mlu*I is underlined. The same proximal primer, named A2-A was used with each distal primer to amplify a specific region of the *Cyp6a2* upstream DNA. The sequence of A2-A primer: 5'ctc*gtcgac*TTTGCGTAGCTGCTCCC 3' (-1/-17). The extra bases are in lower case and the site for *Sal*I is underlined.

Table 2-2

Primers used for amplification of *Cyp6a8-91R* upstream DNA

Primer name	Region upstream DNA amplified	Sequence of distal primer
A8-5F	-491/-11	5' ggcctcgagGTTGTGGTAGGTTAGTAGC 3' (-491/-473)
A8-1F	-109/-11	5' ggcctcgagCTAGCGACTGAGAATGCATC 3' (-109/-90)
A8-0.06F	-67/-11	5' ggcctcgagCAGCGTTTAAAAGCAGTTTGC 3' (-67/-47)
AP1/mutF	-109/-1(mAP-1)	5'gcctcgagCTAGCGACTGAGAATGCATCGTCATGCAAAGTA**TCAGT**ATTTC3'(-109/-67)

Extra bases attached to the 5'-end of each distal primer are shown in lower case. The site for *Xho*I is underlined. In the AP1/mutF distal primer, the core five bases of the putative AP1 site (Maitra et al., 2002) have been mutated. The mutated bases are shown in bold. The same proximal primer, named G10, was used with distal primer to amplify a specific region of the *Cyp6a8* upstream DNA. The sequence of the G-10 primer: 5'cgttcgaaATGATCTTCGAATACG 3' (-11/-26) (Maitra et al., 2002). The extra bases are shown in lower case and the *Hind*III site is underlined.

While an *Xho*I site was added to the 5'-end of all distal primers, a *Hind*III site was added to the 5'-end of the proximal G10 primer. The amplified products were cut with *Xho*I and *Hind*III, and cloned in *Xho*I/*Hind*III cut pGL2-basic vector (Promega, WI) in which the unique *Sal*I site was changed to *Not*I (Maitra et al., 2002). The resulting 0.5luc-A8, 0.1luc-A8, 0.06luc-A8 reporter plasmids respectively had -491/-11, -109/-11 and -67/-11 upstream DNA, respectively, cloned in front of the *luc* reporter gene. These plasmids, and 0.8luc-A8 and 0.2luc-A8 plasmids (Maitra et al, 2002) were used in the present study.

Mutagenesis of AP-1 site

The putative AP-1 site within -109/-11 region of *Cyp6a8* (Maitra et al., 2002), which is in the opposite orientation, was mutated using megaprimer PCR method (Sarkar and Sommer, 1990). The core sequence (AGTCA) of the putative AP-1 site in the *Cyp6a8* (-109/-11) was mutated to TCAGT. 0.1luc-A8 was used as the template for PCR amplification. The primers used for amplification were AP-1/MutF and G10 (Table 2-2). The DNA was amplified for 30 cycles under the following conditions: 1 cycle at 95°C for 2 minutes, 95°C for 45 seconds, 62°C for 45 seconds, and 72°C for 59 seconds. The amplified PCR product was visualized on a 1.5% agarose gel and the PCR product obtained was cloned into pGEM-T easy cloning vector. Base substitutions generated by PCR reaction were confirmed by sequencing with M13 primers. This plasmid was named as 0.1luc(APmut)/TA. The -109/-11 insert with the AP-1 mutation was then digested with *Xho*I and *Hind*III cloned into pGL2(N) basic vector to obtain 0.1luc(mAP1)-A8.

Treatment of flies, fly extract preparation, and luciferase assay

Flies to be treated with caffeine were etherized and groups of forty females were transferred into *vial*s containing normal media. The flies were left in the *vial*s overnight to recover from the ether shock. Next day the flies were transferred to *vial*s containing *Drosophila* media reconstituted with aqueous solution of caffeine (Sigma, MO) and kept in dark for 24h. After treatment, the flies were etherized and transferred in batches of ten into 1.5ml microfuge tubes. Upon recovery from the ether effect, the flies were immobilized by keeping the tubes on ice for few minutes and then they were homogenized in 100µl 1X Cell Culture Lysis Reagent (CCLR; Promega, WI). The homogenate was centrifuged twice at 14,000rpm for 5min at 4°C and the clear supernatant, free of any lipid layer, was collected. All extracts were stored frozen at –80°C until further use.

Luciferase assay of the fly extracts was performed using a commercially available kit (Promega). The luciferase assay reagent, which was stored at –20°C in 100µl aliquots, and the frozen fly extracts were thawed on ice and then they were equilibrated to room temperature. To assay LUC activity, 5µl fly extract was mixed with 100µl luciferase assay reagent, placed in a luminometer (Zylux) and readings were taken at 15s interval. Total protein in fly extract was quantified using the BCA protein assay kit (Pierce). To avoid interference from Triton X-100 present in the CCLR buffer, fly extracts and bovine serum albumin (used as standard) were adjusted to give a final CCLR concentration of 0.5X. For each sample, duplicate aliquots were assayed and the mean value was used for protein estimation. Luciferase activity was expressed as average Registered Light Units per µg of total protein (RLU/µg).

SL-2 cell transfection, extract preparation and dual luciferase assay

For SL-2 cell transfection, plasmid DNAs were reconstituted in 0.1M $CaCl_2$ and then mixed with equal volume of 2X HEPES buffer (Invitrogen, CA) at pH 7.1. Cells to be transfected were resuspended in fresh SL-2 medium at 1 X 10^6cells/ml density. To each well of a 48-well microtiter plate, 500µl of cells were dispensed and to each well 84µl of calcium phosphate-DNA mix was added in a drop-wise manner. After 18h incubation, cells were resuspended in 500µl of fresh media and allowed to recover for 24h at 25°C prior to treatment with caffeine. Following treatment, cells in the 48-well microtiter plate were pelleted and 250µl 1X Passive lysis buffer (Promega) was added to each well. The plates were agitated on a plate shaker for 15 min at room temperature to lyse the cells and then centrifuged at 1500 rpm for 5min at 4°C to pellet the debris. The lysate was collected from each well and assayed immediately for luciferase activity.

Since SL-2 cells were transfected with firefly as well as renilla luciferase plasmids (internal control), lysates were assayed by using a dual luciferase assay kit (Promega, WI). To 20µl lysate, 100µl luciferase assay reagent II was mixed, and placed in a luminometer within 10s to measure the firefly luciferase (F-luc) activity. Immediately after recording the F-luc activity, 100µl Stop and Glo reagent (Promega) was added, vortexed gently and the Renilla luciferase (R-luc) activity was measured within 10s. R-luc activity was used to normalize the data, which were expressed as the ratio of F-luc to R-luc activity.

Northern blot analysis

Total RNA was isolated from adult female flies by using Tri-reagent (Sigma, MO) and manufacturer's instructions. Before loading the samples, Ambion's

NorthernMax Formaldehyde load dye was added to the RNA samples and incubated at 65°C for 15 minutes to denature any RNA secondary structure. 20μg total RNA was dried and dissolved in 20μl formaldehyde loading dye (Ambion), and electrophoresed on a 1.2% formaldehyde-agarose gel. RNA was then blotted onto the nylon membrane and baked under vacuum for 3-4 h at 80°C. To check the integrity of the RNA samples, ribosomal bands were visualized on the blot by UV shadowing. Blots were prehybridized for 2 h at 37°C in Northern Max Prehybridization/Hybridization buffer. Hybridization with specific probes was also done in the same buffer at 37°C for 22-24h. Appropriate cDNA fragments were labeled by random priming using Strip-EZ DNA probe synthesis kit to examine the *CYP* gene expression. Specific activity of the random primer was 100X 10^6 cpm per 21ng of hybridizable DNA. RP49 used as the internal control for loading error, was labeled using nick translation kit (Gibco-BRL) using ^{32}P-αdATP. Following hybridization, the blots were washed in four changes of a buffer containing 2XSSC, 0.05% SDS at room temperature followed by four high stringency washes at 65°C with 0.2X SSC and 0.05% SDS. The blots were then exposed to X-ray films to visualize hybridization signals. Data was analyzed using instant imager and expressed as average *Cyp* over RP49 count.

Results

Three endogenous *Cyp6* family genes show variable level of caffeine-induced expression

Vivarin has been shown to induce *Cyp6a8* and *Cyp6a2* gene expression in 0.2luc4H-ry and 0.8luc110H-ry transgenic flies (Dean et al., unpublished data). Since the major component of Vivarin is caffeine, the present investigation was done to test whether pure caffeine also has the same effect on *Cyp6* gene expression. Moreover, similar to *Cyp6a2* and *Cyp6a8*, *Cyp6w1* gene is induced by other xenobiotic compounds, such as, barbital and phenobarbital (Maitra et al., 1996).

To examine induction of the above three *Cyp6* genes, total RNA isolated from different strains were used. For *Cyp6a2* and *Cyp6a8* induction *0.2-luc4H-ry* and *0.8-luc110H-ry* reporter transgenic lines, and the DDT-susceptible 91-C strains were used. Fig. 2-1 and Fig. 2-2 shows the expression of *Cyp6a8* gene in control and caffeine-treated flies. In all three strains caffeine caused significant induction of the endogenous *Cyp6a8* gene and the level of induction ranged between 3.4- and 9.3-fold. For *Cyp6a2* expression *0.8-luc110H-ry* and 91-C strains were used. Results showed that the induction of endogenous *Cyp6a2* gene in these strains by caffeine and the induction level varied between 2- to 10-fold (Fig. 2-3 and Fig. 2-4). To study the effect of caffeine on *Cyp6w1* gene expression, three non-transgenic strains were used: DDT-resistant 91-R, and DDT-susceptible ry^{506} and 91-C strains. In all three strains, significant but variable induction of *Cyp6w1* gene was observed (Fig. 2-5 and Fig. 2-6). In view of these results, induction of *0.8luc-A8* and *0.2luc-A8* transgenes by Vivarin in ry^{506} background observed previously (Dean et al., Unpublished data, 2003) must be due to caffeine, which is the major

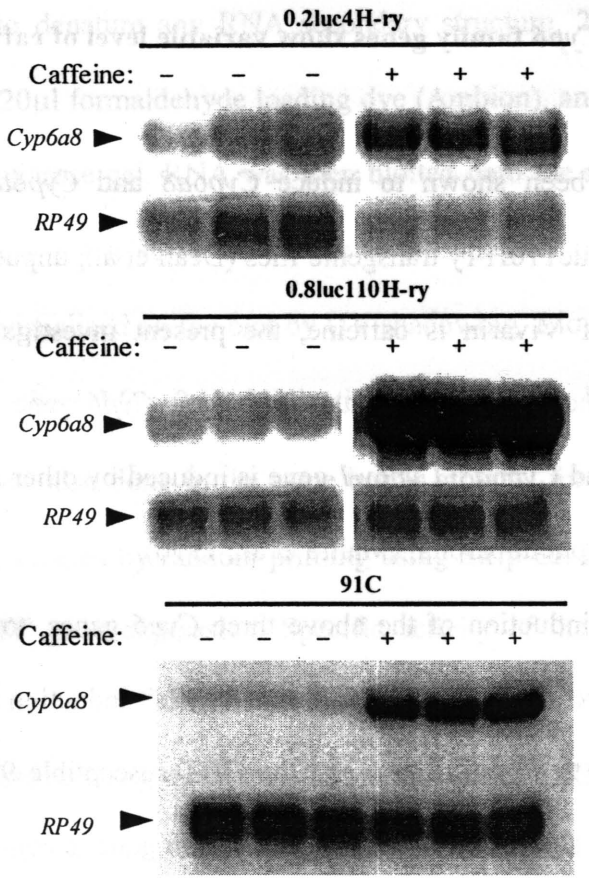


Figure 2-1: Expression of the endogenous *Cyp6a8* gene in untreated and caffeine-treated adult flies of *0.2-luc4H-ry*, *0.8-luc110H-ry* and 91-C strains. Total RNA isolated from untreated (-) and caffeine-treated (+) adult females of each strain was fractionated on 1.2% agarose-2.2 M Formaldehyde, blotted onto nylon membrane and hybridized with ^{32}P-labeled CYP6A8 cDNA under probe-excess condition as described in Materials and Methods. The blots were also hybridized with *RP-49* (ribosomal protein-49) gene probe to determine the RP-49 mRNA level, which was used to correct for RNA loading error. Quantitative analyses of the results are shown in Figure 2-2

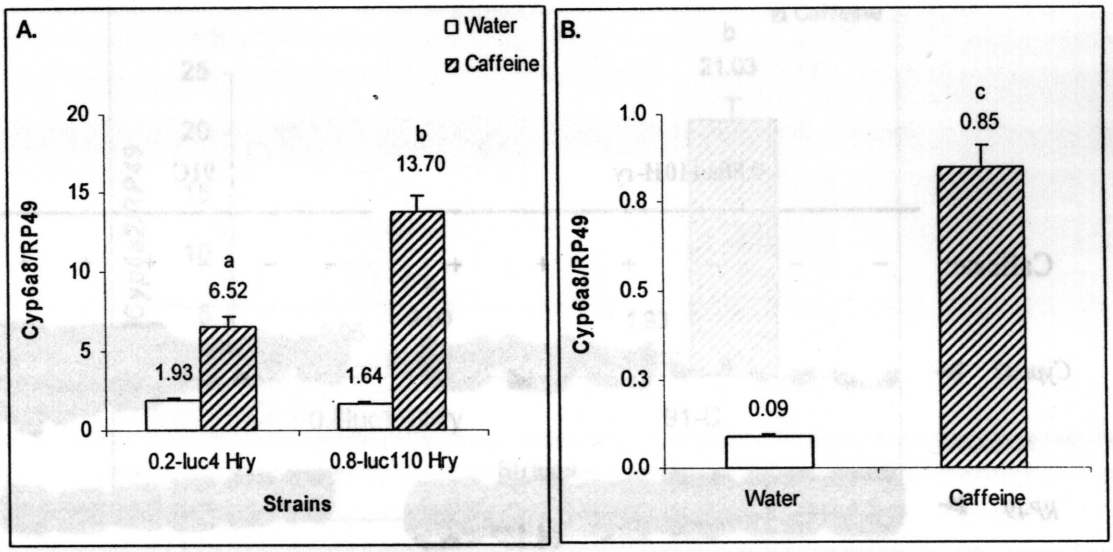


Figure 2-2: Induction of the endogenous *Cyp6a8* gene in (A) *0.2-luc4H-ry* and *0.8-luc110H-ry* strains and (B) 91-C strain. The blots shown in Figure 2-1 were scanned with a Packard Radioanalytical Imager. *Cyp6a8* mRNA levels were normalized by using RP49 mRNA level as internal control. Each bar is the mean ± S.D of *Cyp6a8/RP49* counts of three independent RNA samples examined via northern blot analysis (Fig. 2-1). The *p* values were calculated by Student's t-test. a < 0.0003; b < 0.0002; and c < 0.0002.

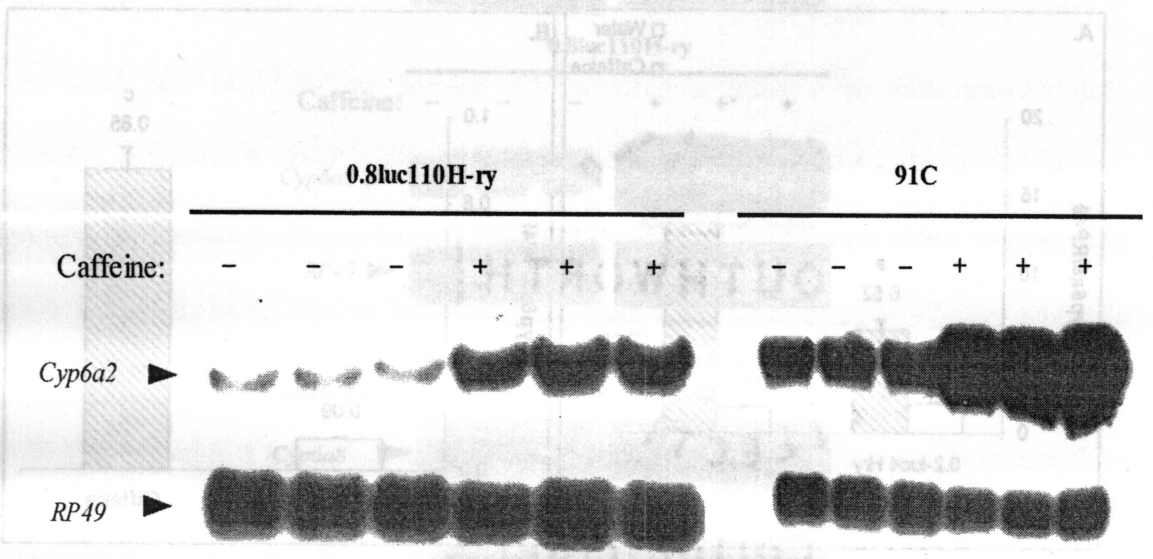


Figure 2-3: Expression of the endogenous *Cyp6a2* gene in untreated (-) and 16mM caffeine-treated (+) adult females of *0.8-luc110H-ry* and 91C strains. Total RNA samples were analyzed by northern blot analysis as described in Fig. 2-1.

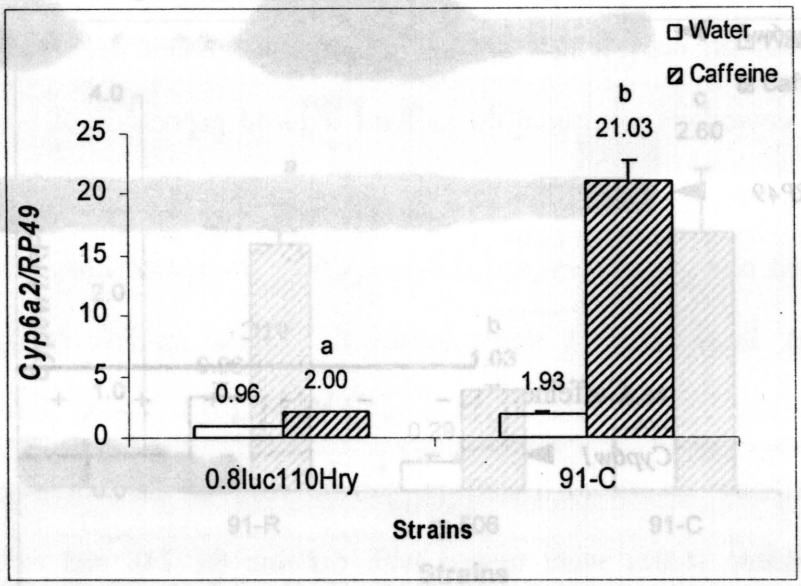


Figure 2-4: Quantification of the endogenous *CYP6A2* mRNA levels in *0.8-luc110H-ry* and 91C strains following caffeine treatment. Northern blots shown in Fig. 2-3 were analyzed with a radioanalytical imager as described in Fig. 2-2. Each bar is the mean ± S.D of *Cyp6a2/RP49* counts of three independent RNA samples examined via northern blot analysis (Fig. 2-3). Student's t-test was done to compare the means. Student's t-test was done to compare the means. *p:* $a < 0.0003$; and $b < 0.0002$.

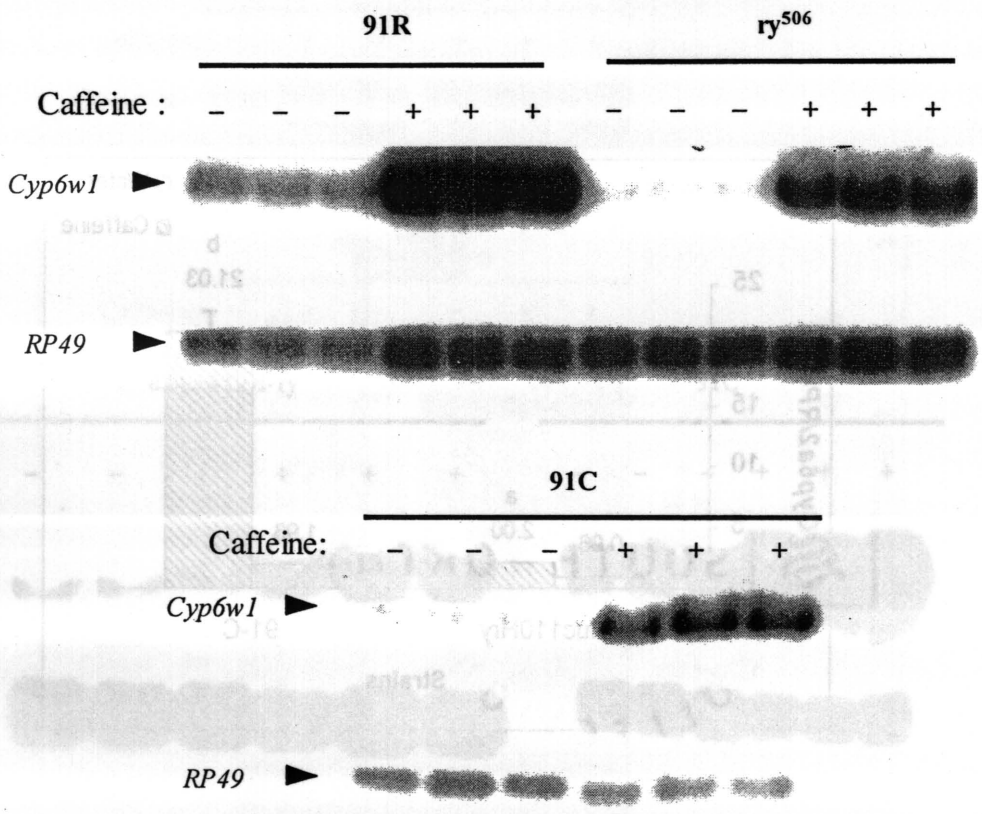


Figure 2-5: Northern blot analysis of *CYP6W1* mRNA levels in untreated (-) and caffeine-treated (+) adult females of 91-R, 91-C and ry^{506} strains.

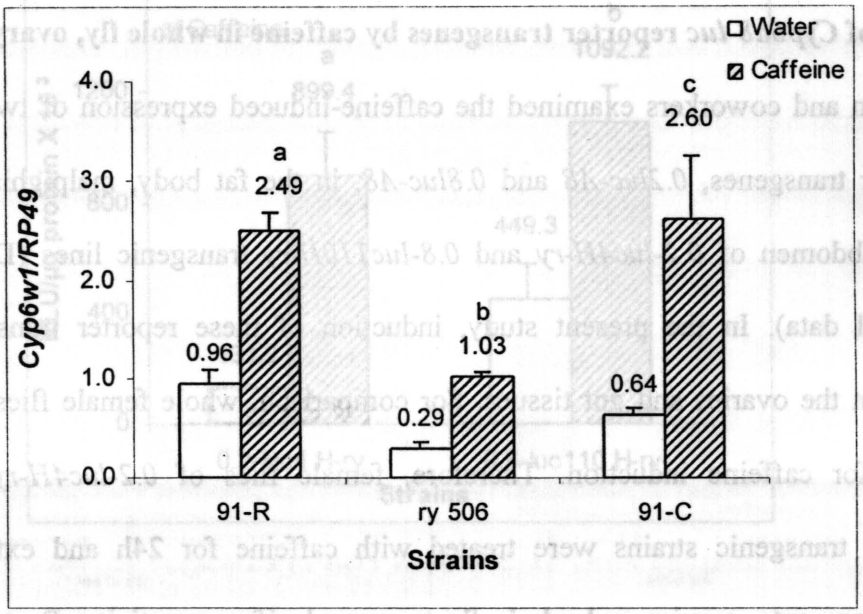


Figure 2-6: Quantification of the CYP6W1 mRNA in adult females of 91-R, 91-C and ry^{506} strains following caffeine treatment. Data were derived by scanning the blots shown in Fig. 2-5 with a Packard Radioanalytical Imaging system. RP-49 RNA level was used as internal control. Each bar shows the mean ± S.D of the data obtained with three isolates of RNA samples Student's t-test was done to compare the means. p: $a < 0.0005$; $b < 0.0002$; and $c < 0.007$.

component of Vivarin. Although all three genes were induced by caffeine, the level of induction of three *Cyp6* genes showed variation in the strains tested. In addition, the level of induction showed variation among the three genes. These variations may be due to the differences in the caffeine-responsive elements of these genes.

Induction of *Cyp6a8-luc* reporter transgenes by caffeine in whole fly, ovary and gut

Dean and coworkers examined the caffeine-induced expression of two *Cyp6a8-luc* reporter transgenes, *0.2luc-A8* and *0.8luc-A8*, in the fat body, malpighian tubules, head and abdomen of *0.2-luc4H-ry* and *0.8-luc110H-ry* transgenic lines (Dean et al., unpublished data). In the present study, induction of these reporter transgenes was examined in the ovaries and gut tissues. For comparison, whole female flies were also examined for caffeine induction. Therefore, female flies of *0.2-luc4H-ry* and *0.8-luc110H-ry* transgenic strains were treated with caffeine for 24h and extracts were prepared from guts, ovaries and whole fly to assay luciferase activity. Results showed that in all three samples both constitutive and caffeine-induced activities of *0.8luc-A8* transgene were higher than those of the *0.2luc-A8* transgene (Fig. 2-7 and Fig. 2-8). However, the fold difference between constitutive and caffeine induced activities of the two reporter transgenes showed tissue variation (Fig. 2-7 and Fig. 2-8). For example, in whole fly, *0.2luc-A8* showed almost 7-fold induction, whereas the *0.8luc-A8* reporter transgene showed about 2.3-fold induction (Fig. 2-7). Similarly, in the gut tissue, *0.2luc-A8* showed almost 20-fold induction, whereas about 10-fold induction was observed with the *0.8luc-A8* transgene (Fig. 2-8B). As a matter of fact, the constitutive expression of the *0.2luc-A8* reporter gene in the gut tissue is almost undetectable and hence, contributes to the huge fold-induction following caffeine treatment. In the ovary, while the *0.2luc-A8*

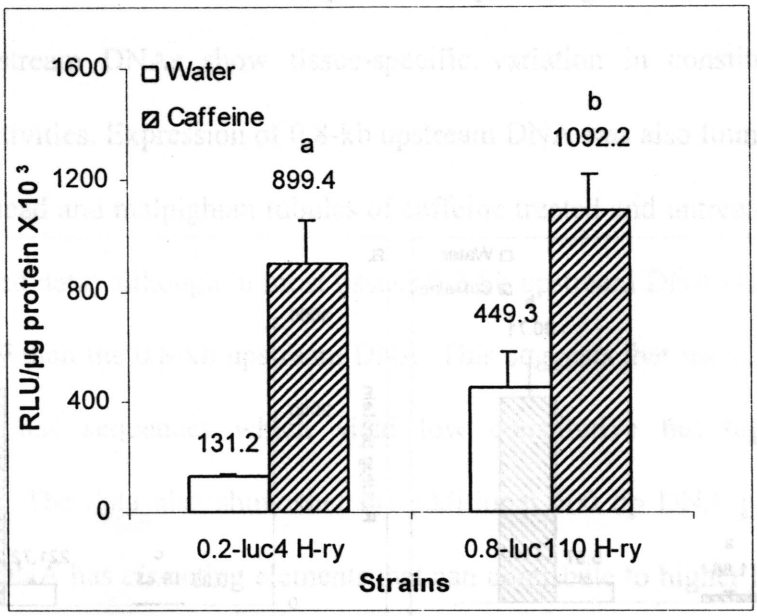


Figure 2-7: Caffeine induction of *O.2luc-A8* and *0.8luc-A8* reporter genes in *0.2-luc4H-ry* and *0.8-luc110H-ry* transgenic flies. Flies were treated with water (control) or with 16mM caffeine, and firefly luciferase activity per µg total protein was determined. Values represent mean ± S.D of triplicate samples. Student's t-test was done to compare the means. *p*: a < 0.003; and b < 0.005.

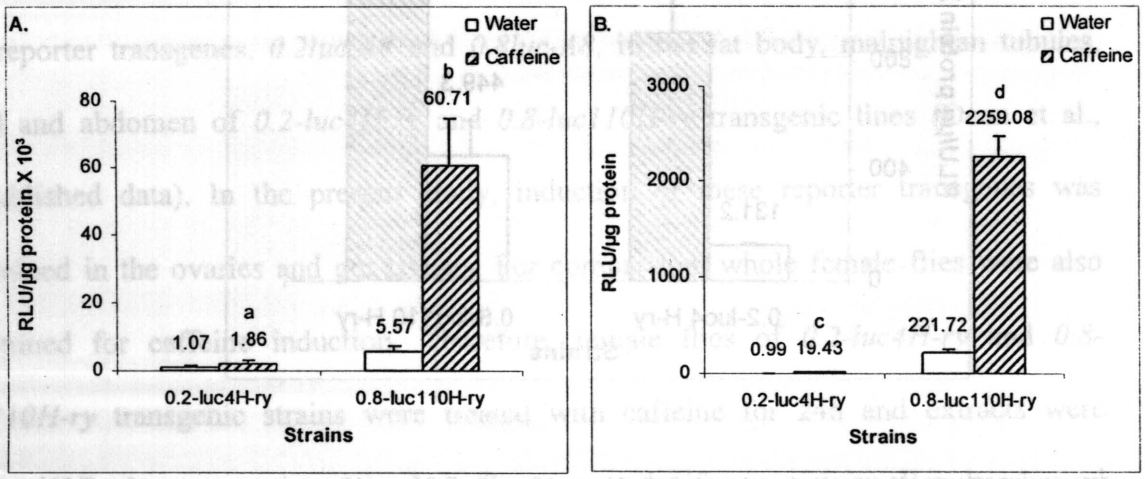


Figure 2-8: Constitutive and caffeine-induced expression of *0.2luc-A8* and *0.8luc-A8* transgenes in ovaries (Panel A) and guts (Panel B) of *0.2-luc4H-ry* and *0.8-luc110H-ry* flies. Firefly luciferase activity is expressed as the average luciferase activity per µg total protein. Values represent mean ± S.D of triplicate samples. Student's t-test was done to compare the means. p : a : 0.34; b < 0.005; c < 0.0003 and d < 0.0003.

reporter transgene did not show any induction with caffeine, about 12-fold induction was observed in case of the *0.8luc-A8* reporter transgene (Fig. 2-8 A). Thus, both the 0.2- and 0.8-kb upstream DNAs show tissue-specific variation in constitutive and caffeine-induced activities. Expression of 0.8-kb upstream DNA was also found to be higher in the fat body; head and malpighian tubules of caffeine treated and untreated flies (Dean et al., Unpublished data) although in most tissues 0.2-kb upstream DNA shows higher caffeine-inducibility than the 0.8-kb upstream DNA. This suggests that the 0.2-kb upstream DNA (-11/-199) has sequences which yield low constitutive but high caffeine-induced expression. The data also show that the additional 600 bp DNA present in the 0.8-kb upstream DNA has *cis*-acting elements that can contribute to higher levels of constitutive expression than the sequences present in the 0.2-kb DNA but these *cis*-elements do not respond to the caffeine to a large extent.

Caffeine induction of *Cyp6a8* and *Cyp6a2* promoter activities in *Drosophila* SL-2 cells

We wanted to further investigate if SL-2 cells have the necessary machinery for constitutive and caffeine-induced expression, as SL-2 cells will provide a powerful system to further dissect the mechanism of caffeine action. For this purpose, SL-2 cells were transfected with different amounts of 0.9luc-A2 DNA and extracts prepared from these cells were assayed for the firefly luciferase activity as described in material and methods section. Results showed that cells transfected with 1μg DNA displayed 100 times more luciferase counts when compared to the untransfected cells (Fig. 2-9).

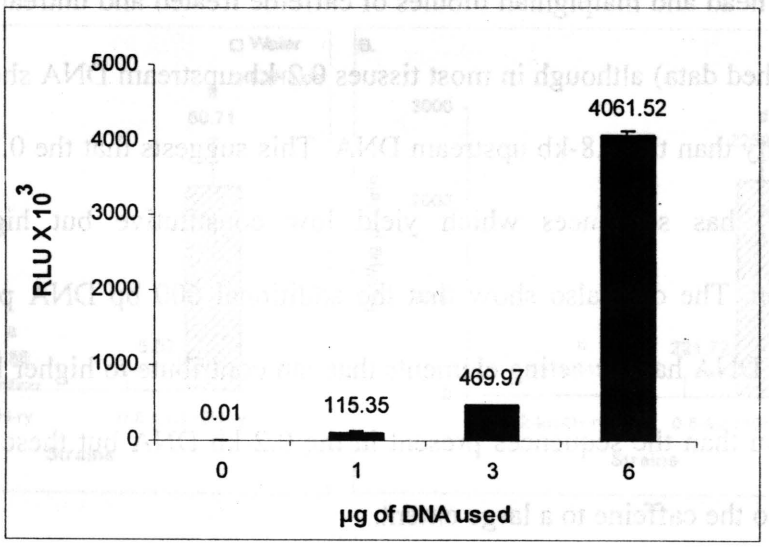


Figure 2-9: Relation between the amount of 0.9luc-A2 plasmid and activity of the *luciferase* reporter gene. SL-2 cells were transfected with different amounts of 0.9-lucA2 plasmid DNA. Extracts were prepared after 24h recovery period and assayed for luciferase activity. Values represent mean ± standard deviation of duplicate samples.

The absolute value of the luc activity further increased with the increasing amount of DNA, suggesting an increase in number of transfected cells with more DNA quantity. Although, maximum luciferase activity was observed with 6μg DNA, 1μg was used for transfection experiments as it displayed a detectable count that is several fold higher than the untransfected cells (Fig. 2-9). To control the variation in transfection efficiency between different samples, renilla luciferase plasmid (pRL-null) was used as an internal control. SL-2 cells were cotransfected with 0.9luc-A2 and pRL-null plasmids. Different ratios of the experimental (0.9luc-A2) to control plasmid (pRL-null) were used to optimize the proportion at which maximum firefly luciferase activity and renilla luciferase activity higher than the background could be achieved. Transfection with 1μg of 0.9luc-A2 and 500ng of pRL-null, gave highest activities for both firefly luciferase (F-luc) and renilla luciferase (R-Luc) enzymes (Fig. 2-10). Therefore, for subsequent transfections experiments, 1μg of the experimental plasmid and 500ng of the control (a ratio of 2:1) was used. Most importantly, these transfection experiments demonstrate that factors required for *Cyp6a2* expression are present in SL2 cells.

To further determine if SL-2 cells also have the necessary factors for caffeine-induced expression of *Cyp6a2*, SL-2 cells were transfected with 0.9luc-A2 reporter plasmid and treated with different concentrations of caffeine. Extracts prepared from the untreated and treated cells were assayed for firefly luciferase (F-luc) and renilla luciferase (R-luc) activities. Results showed a 4-fold increase in 0.9luc-A2 reporter activity when 1mM caffeine was added to the medium (Fig. 2-11). Thereafter, the reporter activity increased as the caffeine concentration was increased but it increased only about 30% when caffeine concentration was raised from 1mM to 8mM or 16mM. Nevertheless,

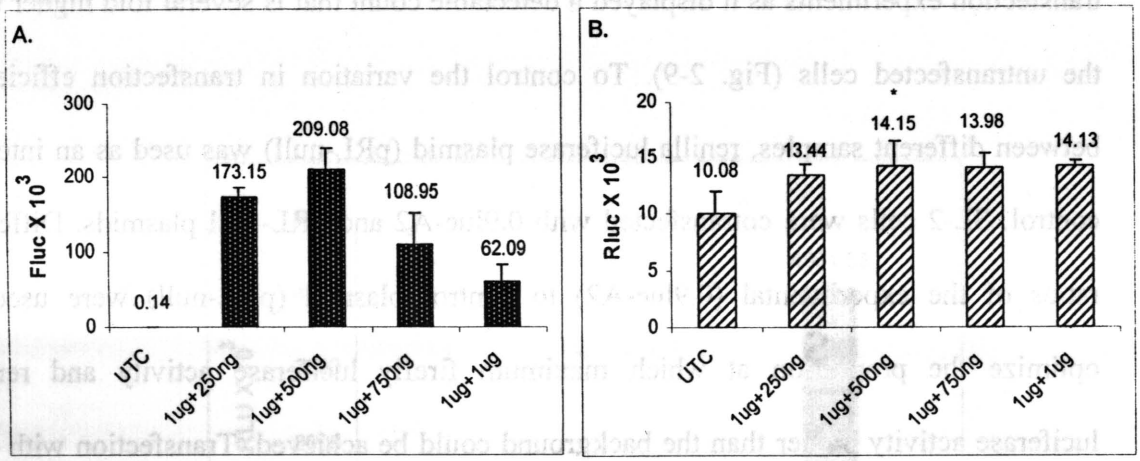


Figure 2-10: Determination of the optimum amounts of firefly (Fluc) and renilla luciferase (Rluc) plasmids to be used for dual luciferase assay. SL-2 cells were transfected with 1 µg of firefly luciferase plasmid (0.9luc-A2) and different amounts of renilla luciferase (pRL-null) plasmid ranging from 250ng to 1µg. Following 18h transfection, cells were transferred to fresh SL-2 media for 24h. Extracts were prepared. and assayed for firefly (A) and renilla luciferase (B) activities. Values represent the mean ± S.D. of triplicate samples. Student's t-test was done to compare the means. p : * < 0.07.

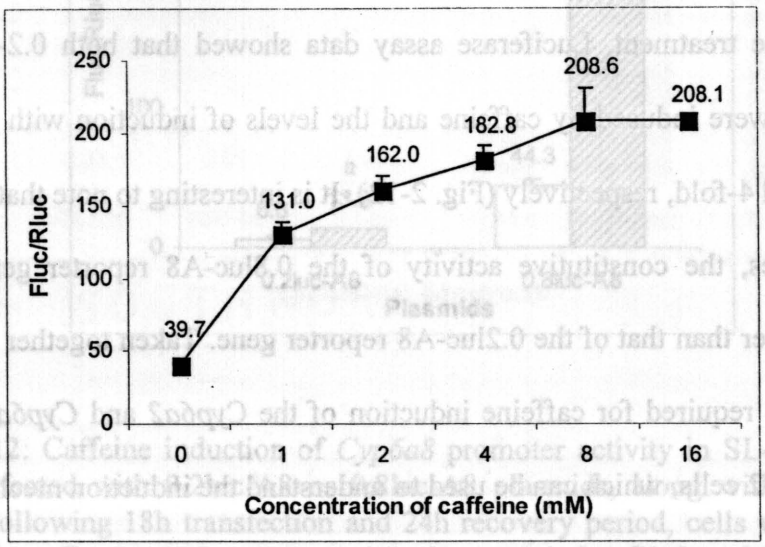


Figure 2-11: Dose response curve for caffeine induction of *Cyp6a2* promoter activity in SL-2 cells. SL-2 cells were transfected with 0.9luc-A2 reporter plasmid and treated with different concentrations of caffeine. Extracts were prepared and firefly and renilla luciferase activities were measured. Each data point represents the mean ± S.D of Fluc/Rluc ratios of triplicate samples. Student's t-test was done to compare the means. *p*: * < 0.0003.

since maximum induction was observed at 8mM concentration this concentration was used for all subsequent experiments.

We also wanted to determine if *Cyp6a8* promoter responds to caffeine treatment similar to that of *Cyp6a2* promoter. For this purpose, SL-2 cells were transfected with 0.2luc-A8 and 0.8luc-A8 reporter plasmids individually and extracts were prepared following caffeine treatment. Luciferase assay data showed that both 0.2- and 0.8-kb upstream DNAs were induced by caffeine and the levels of induction with these DNAs were about 2- and 4-fold, respectively (Fig. 2-12). It is interesting to note that as observed in transgenic flies, the constitutive activity of the 0.8luc-A8 reporter gene was also significantly higher than that of the 0.2luc-A8 reporter gene. Taken together these results show that factors required for caffeine induction of the *Cyp6a2* and *Cyp6a8* promoters are present in SL-2 cells, which can be used to understand the induction mechanism.

Mapping the *cis*-regulatory elements for constitutive and caffeine-induced expression of *Cyp6a8* gene

To map the *cis*-element(s) required for constitutive and caffeine-induced expression of the *Cyp6a8* gene, plasmids carrying *luciferase* reporter gene under the control of 0.06-, 0.1-, 0.2-, 0.5- and 0.8-kb upstream DNA of *Cyp6a8* were used for transient transfection assay with SL-2 cells. These upstream DNAs correspond to -67/-11, -109/-11, -199/-11, -491/-11 and -761/-11 regions of the *Cyp6a8* gene, respectively. Two independent transient transfection assay experiments were done with two isolates of each reporter plasmid DNA and two different batches of SL-2 cells. In each experiment, transfection with each plasmid was done in triplicate. The results of luciferase assays

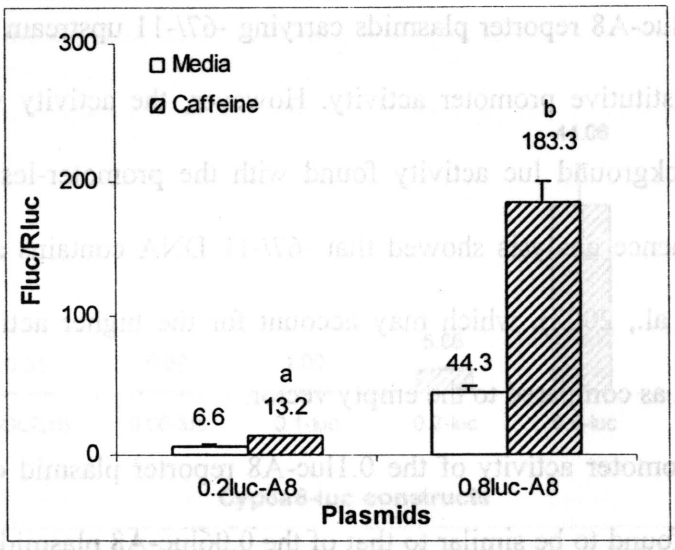


Figure 2-12: Caffeine induction of *Cyp6a8* promoter activity in SL-2 cells. SL-2 cells were transfected with 0.2luc-A8 or 0.8luc-A8 plasmids, along with pRL-null control plasmid. Following 18h transfection and 24h recovery period, cells were treated with 8 mM caffeine. Extracts were prepared and assayed for firefly and renilla luciferase activities. Each bar represents mean Fluc/Rluc ± S.D of triplicate samples. Student's t-test was done to compare the means. *p*: a <0.001; and b < 0.0002.

showed that the constitutive promoter activity (Fluc/Rluc activity) increased as the length of the *Cyp6a8* upstream DNA was increased in the reporter plasmid (Fig. 2-13). The activity of the 0.06luc-A8 reporter plasmids carrying -67/-11 upstream DNA of *Cyp6a8* showed lowest constitutive promoter activity. However, the activity was about 3-fold higher than the background luc activity found with the promoter-less pGL2-basic(N) empty vector. Sequence analysis showed that -67/-11 DNA contains a putative TATA element (Maitra et al., 2002), which may account for the higher activity obtained for 0.06luc-A8 plasmid as compared to the empty vector.

The constitutive promoter activity of the 0.1luc-A8 reporter plasmid carrying -109/-11 *Cyp6a8* DNA was found to be similar to that of the 0.06luc-A8 plasmid and about 3-fold higher than the activity seen with the empty vector. This suggests that the region between -67 and -109 does not have additional regulatory element(s) that can up regulate basal transcription of the *Cyp6a8* gene. When the length of the -109/-11 upstream DNA was increased by another 90 bases to -199, the promoter activity of the resulting reporter plasmid (0.2luc-A8) increased by about 5-fold (Fig. 2-13), suggesting the presence of additional *cis*-regulatory element(s) within this region that can positively influence the *Cyp6a8* transcription. A significant increase (about 8-fold) in the promoter activity was observed for 0.5luc-A8 plasmid (carrying -491/-11 upstream DNA). No significant increase in the constitutive activity was obtained when 0.8luc-A8 carrying -761/-11 upstream DNA was used. To compare caffeine responsiveness of different regions of the *Cyp6a8* upstream DNA, SL-2 cells transfected with the reporter plasmids were treated with caffeine and assayed for luciferase activity. The results showed that neither 0.06luc-

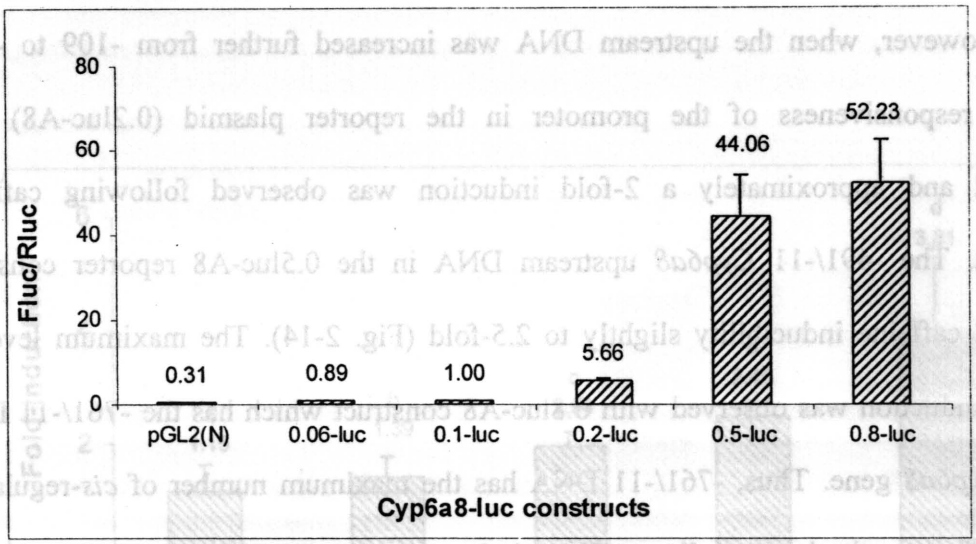


Figure 2-13: Constitutive promoter activity of different 5'-upstream DNA deletions constructs of *Cyp6a8* gene. SL-2 cells were transfected individually with five different firefly *luciferase* reporter plasmids carrying different length of *Cyp6a8* upstream DNA, and the pRL-null control plasmid. Extracts prepared from transfected cells were assayed for firefly (Fluc) and renilla luciferase (Rluc) activities. Values represent mean ± S.D. of triplicate samples.

A8 nor0.1luc-A8 reporter plasmid gave a significant level of caffeine induction (Fig. 2-14).

However, when the upstream DNA was increased further from -109 to -199, caffeine responsiveness of the promoter in the reporter plasmid (0.2luc-A8) also increased and approximately a 2-fold induction was observed following caffeine treatment. The -491/-11 *Cyp6a8* upstream DNA in the 0.5luc-A8 reporter construct increased caffeine inducibility slightly to 2.5-fold (Fig. 2-14). The maximum level of caffeine induction was observed with 0.8luc-A8 construct which has the -761/-11 DNA of the *Cyp6a8* gene. Thus, -761/-11 DNA has the maximum number of *cis*-regulatory elements for constitutive as well as caffeine-induced expression of the *Cyp6a8* gene.

Sequence analysis of the upstream DNA of the *Cyp6a8* gene has identified a putative AP-1 motif at -70/-80 in reverse orientation (Maitra et al., 2002). Since AP-1 family of transcription factors are known to positively modulate basal transcription of many promoters (Daniel et al., 1998), we wanted to test the contribution of this putative AP-1 motif, if any, on the basal *Cyp6a8* promoter activity. Therefore, a mutated -109/-11 upstream DNA was synthesized *via* PCR in which core five bases of the putative AP-1 element were substituted. The mutated DNA was cloned into pGL2-basic(N) plasmid to create 0.1luc-A8(mut) reporter plasmid. SL-2 cells were transfected with 0.1luc-A8(mut) and wild type 0.1luc-A8 plasmids, individually. Two independent experiments were done with two different DNA isolates and two batches of cells. The results show that the constitutive promoter activity of the 0.1luc-A8(mut) plasmid decreased about 8-fold relative to the activity of the wild type 0.1luc-A8 plasmid (Fig. 2-15). This suggests that

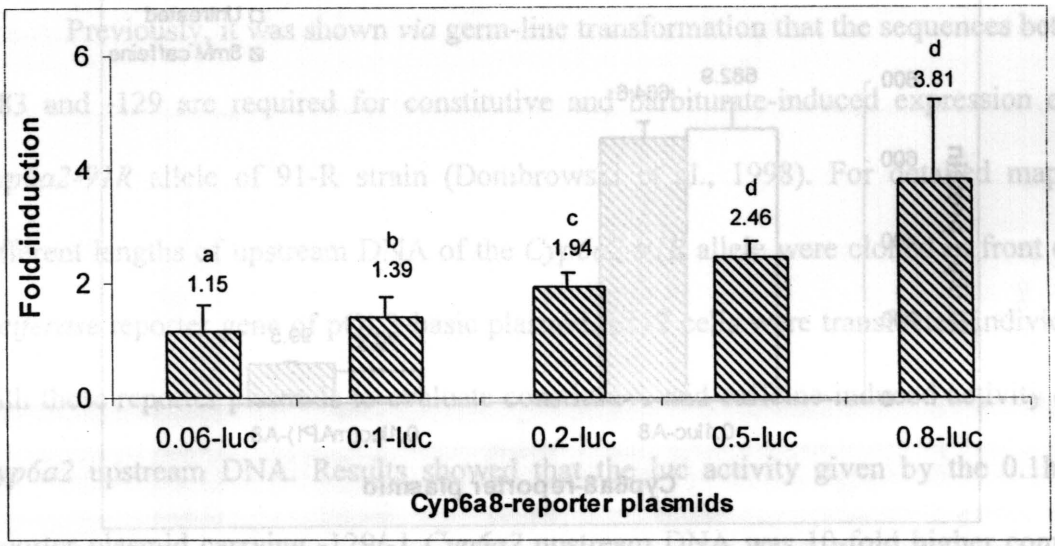


Figure 2-14: Caffeine induction of *Cyp6a8-luc* constructs in SL-2 cells. Cells were transfected individually with *Cyp6a8-luc* constructs along with the internal control plasmid pRL null. Transfected cells were treated in the absence (control,UT) or presence (treated, T) of 8mM caffeine. After 24h, cells were collected and the lysates were used for assaying firefly and renilla luciferase activities. Fold-induction represents the ratio of Fluc/Rluc activities obtained from caffeine-treated and untreated samples. Mean fold-difference ± standard deviation represents the average of fold-induction obtained in two independent experiments, each experiment being done in triplicate. Student's t-test was done to compare the means. *p*: a: 0.5, b: 0.14, c: 0.001, d: 0.0007 and e: 0.0001.

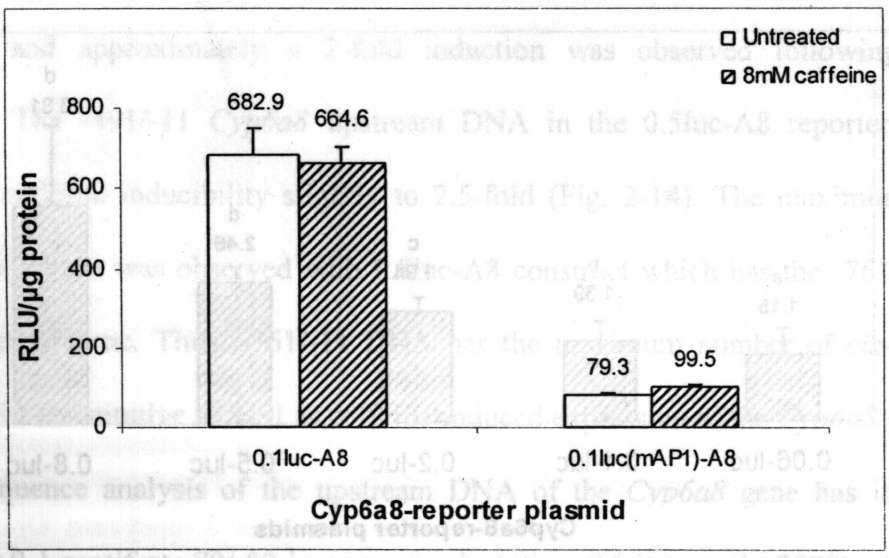


Figure 2-15: Effect of base substitution mutation of putative AP-1 site in 0.1-kb upstream DNA of *Cyp6a8* on its constitutive and caffeine-induced promoter activities. SL-2 cells were transfected individually with 0.1luc-A8 wild type or 0.1luc(mAP1)-A8 mutant reporter plasmid. Following 24 h post-transfection recovery period, cells were treated with 8mM caffeine. After 24h treatment extracts were prepared and luciferase activity/µg protein was determined. Untreated cells were used as control. Values represent mean ± S.D of triplicate samples.

the AP-1 motif found at position -70/-80 of the upstream DNA plays a major role in basal expression of the *Cyp6a8* gene.

Mapping the *cis*-regulatory element for constitutive and caffeine induced expression of *Cyp6a2* gene

Previously, it was shown *via* germ-line transformation that the sequences between -983 and -129 are required for constitutive and barbiturate-induced expression of the *Cyp6a2-91R* allele of 91-R strain (Dombrowski et al., 1998). For detailed mapping, different lengths of upstream DNA of the *Cyp6a2-91R* allele were cloned in front of the *luciferase* reporter gene of pGL3-basic plasmid. SL-2 cells were transfected individually with these reporter plasmids to evaluate constitutive and caffeine-induced activity of the *Cyp6a2* upstream DNA. Results showed that the luc activity given by the 0.1luc-A2 reporter plasmid carrying -129/-1 *Cyp6a2* upstream DNA was 10-fold higher compared to the activity given by the empty pGL3-basic vector (Fig. 2-16). This result was expected because the -129/-1 DNA contains the TATA box of the *Cyp6a2* gene (Waters et al., 1992). When reporter plasmid 0.2luc-A2 carrying -265/-1 upstream of *Cyp6a2* was used, the luc activity was found to be 40-fold greater than the activity observed with the empty vector. This suggests that the region between -129 and -265 has a strong positive effect on constitutive expression of the *Cyp6a2* gene. Increasing the upstream DNA further from -265 to -522 did not increase the promoter activity (Fig. 2-16). In fact, it remained more or less same as the activity found with 0.2luc-A2 plasmid. However, when the upstream DNA was increased from -522 to -981, promoter activity of the resulting reporter plasmid 0.9luc-A2 increased approximately by 4-fold. Compared to the empty vector luc activity given by 0.9luc-A2 plasmid increased about 98-fold.

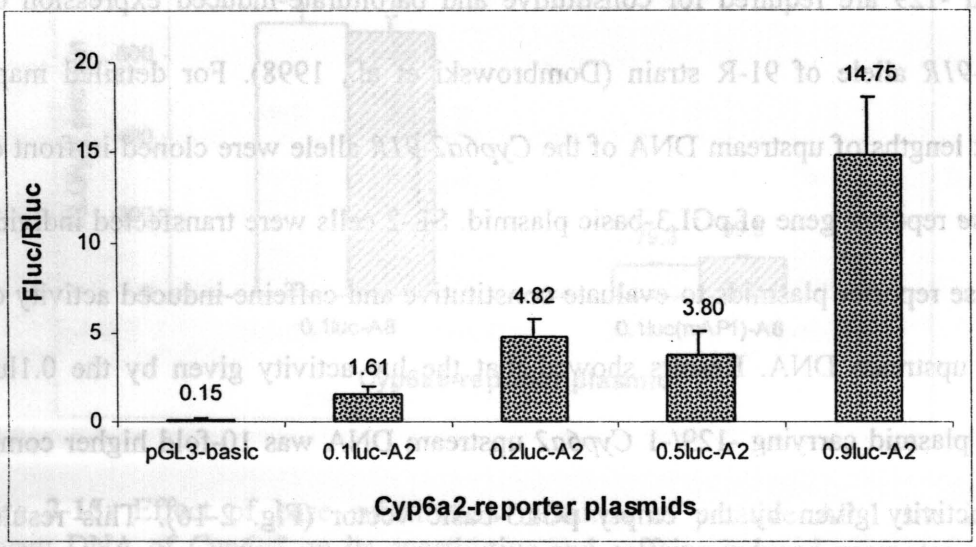


Figure 2-16: Constitutive promoter activity of different regions of *Cyp6a2* upstream DNA. SL-2 cells were transfected individually with four different firefly *luciferase* reporter plasmids carrying different length of *Cyp6a2* upstream DNA, and the pRL-null control plasmid. Extracts prepared from transfected cells were assayed for firefly (Fluc) and renilla luciferase (Rluc) activities. Values represent mean ± S.D. of triplicate samples.

To further evaluate the caffeine induction of these constructs, SL-2 cells transfected with each reporter plasmid were treated with caffeine and dual luciferase assay was performed. Two independent experiments with two different isolates of plasmids and two batches of SL-2 cells were done. In each experiment, triplicate determinations were done for each plasmid to calculate the mean fold-induction with caffeine. The averages of mean fold-inductions obtained in two independent experiments are shown in Fig. 2-17. The fold-induction with 0.1luc-A8 plasmid was 0.9, suggesting that -129/-1 DNA present in 0.1luc-A2 plasmid does not have caffeine responsive element and therefore, failed to show caffeine induction (Fig. 2-17). However, a robust induction (4.3-fold) with caffeine was observed with 0.2luc-A2 reporter plasmid, which has -265/-1 upstream DNA of the *Cyp6a2* gene. The 0.5luc-A2 reporter plasmid showed a 3-fold induction with caffeine and maximum induction (5-fold) was observed with the 0.9luc-A2 plasmid (5-fold), suggesting the presence of additional positive response elements for caffeine induction in the upstream between bases -522 and -983.

Discussion

Caffeine has been studied extensively in relation to different physiological processes in humans and many other mammals. However, except for a few studies, very (Svenningsson et al., 1995; Goasduff et al., 1996; Dassesse et al., 2001) limited investigation has been made on the effect of caffeine on the expression of specific genes including those coding for cytochrome P450 enzymes or CYPs. In one study, caffeine has

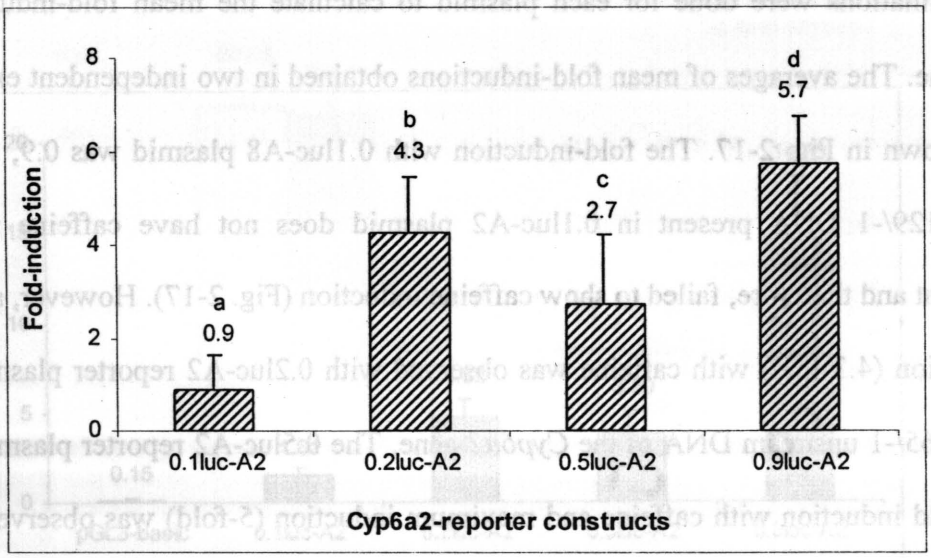


Figure 2-17: Caffeine induction of *Cyp6a2-luc* constructs in SL-2 cells. Cells were transfected individually with *Cyp6a2-luc* along with the internal control plasmid pRL-null. Transfected cells were treated in the absence (control, UT) or presence (treated, T) of 8 mM caffeine. After 24h, cells were collected and the lysates were used for assaying firefly and renilla luciferase activities. Fold-induction represents the ratio of Fluc/Rluc activities obtained from caffeine-treated and untreated samples. Mean fold-difference ± standard deviation represents the average of fold-induction obtained in two independent experiments. Student's t-test was done to compare the means. p: a: 0.1, b: 0.05, c: 0.04 and d:0.004.

been shown to increase the level of c-fos and c-jun mRNA in rat striatum (Svenningsson et al., 1995). In another study, effect of caffeine on the expression of a *CYP1A1/A2* genes has been examined. This study showed that caffeine treatment increases the CYP1A1/1A2 mRNA levels in rats (Goasduff et al., 1996). Recently, global effect of caffeine on gene expression has been examined *via* microarray analysis (Gore et al., 2000; Kucharski and Maleszka, 2002). Although these studies have identified various genes that are either up- or down-regulated by caffeine, the molecular mechanism involved is not known. The objective of the present investigation has been to use *Cyp* genes of *Drosophila* as prototype genes to understand the underlying molecular mechanism involved in caffeine-mediated gene expression.

Since cytochrome P450s are involved in the metabolism of various xenobiotics, it is possible that a xenobiotic compound may act as an inducer as well as a substrate for a P450 enzyme. In other words, a xenobiotic compound may induce a P450 enzyme for its own metabolism. This is found to be true in mammals and the best example is the induction of three *CYP* genes, *CYP1A1*, *CYP1A2* and *CYP1B1* (Nebert et al., 2004; Mandal, 2005). These enzymes are induced at the transcriptional level by various polyaromatic hydrocarbons, which are in turn metabolized by these enzymes. Interestingly, similar phenomenon is found with caffeine. It has been shown that caffeine treatment increases CYP1A2 mRNA level in rat liver and kidney (Goasduff et al., 1996) and CYP1A2 plays a major role in caffeine metabolism (Chung et al., 1998). Thus, it is possible that caffeine probably facilitates its own metabolism in flies by inducing the *Cyp6* genes. Although flies do not consume caffeine, it has purines that are caffeine-like molecules. Caffeine by itself is a purine and it is chemically called trimethylxanthine.

Therefore, enzymes that are involved in the metabolism of purines in *Drosophila* may also metabolize caffeine. Since *Cyp6a2* and *Cyp6a8* are induced by caffeine, it may be assumed that the enzymes encoded by these genes may be involved in purine and caffeine metabolism. However, the actual substrate metabolized by these enzymes in *Drosophila* is not known.

The pharmacological action of caffeine may be manifested by blocking the adenosine receptors and (or) inhibiting the cAMP phosphodiesterase enzyme that hydrolyzes cAMP (Fredholm et al., 1999; Fredholm et al., 2001). While adenosine receptors are transmembrane proteins, cAMP phosphodiesterase or PDE is an intracellular enzyme. In humans and in other mammals, very low dose of caffeine (at nanomolar concentration) inhibits adenosine receptors but higher dose (at millimolar concentration) leads to PDE inhibition (Fredholm et al., 1999). In the present investigation, it was found that 1 mM caffeine, the lowest dose tried, could induce the *Cyp6a2* gene promoter in SL-2 cells almost 3.5-fold (Fig. 2-11), which increased another 1.5-fold when the concentration was raised to 8mM or 16mM. Since caffeine dose used was much greater than nanomolar concentration there is a good possibility that caffeine induction of the *Cyp6a2* and *Cyp6a8* genes is mediated *via* PDE inhibition. Since both adenosine receptor antagonism and PDE inhibition by caffeine result in an increase in intracellular cAMP level (Fredholm et al., 1999), it may be assumed that caffeine treatment increases cAMP level in flies and in SL-2 cells. It has been shown that bovine *CYP11A1* transcription is positively modulated by cAMP signaling pathway (Ahlgren et al., 1999). Therefore, it is possible that caffeine-induced expression of the *Cyp6a2* and *Cyp6a8* genes is also mediated *via* cAMP signaling pathway.

In mammals, upstream DNA of many genes that are induced by the cAMP-mediated pathway has AP-1 binding sites for activator proteins (AP) and (or) cAMP response element (CRE) for the CREB protein (Karin, 1995; Karin et al., 1997; Montminy, 1997; Sng et al., 2004). The AP-1 family of transcription factors consists of several basic leucine zipper proteins (bZIP) which include proteins in Jun and Fos families. Genes encoding these AP-1 proteins constitute a family called immediate early genes or IEG because they are expressed immediately following extracellular stimuli which may increase cAMP (Karin et al., 1997; Sng et al., 2004). Therefore, genes that have AP-1 response element are expected to be induced by cAMP *via* AP-1 factors. On the other hand, genes that have CRE are induced by CREB. Interestingly, the Jun proteins can bind as a homodimer or heterodimer with Fos to three different consensus sequences: TGACATCA (an AP-1 site), TGACTCA (a 12-*O*-tetradecanoylphorbol-13-acetate {TPA}- response element or TRE) and TGACGTCA (a CRE) (Angel et al., 1987; Angel et al., 1988; Franza et al., 1988; Nakabeppu et al., 1988). Similarly, the CREB protein can bind to the AP-1 site in addition to the CRE (Hoeffler et al., 1988). In most cases, these three response elements are separate. However, in some mammalian genes, such as, dopamine β-hydroxylase, proenkephalin and prodynorphin, a composite CRE/AP1 site (TGCGTCA) is found which contains elements from both consensus sequences (Shaskus et al., 1992; Collins-Hicok et al., 1994; Messersmith et al., 1996; Swanson et al., 1998). Recently, a composite CRE/TRE element named SCAR has been described and it is found in the upstream DNA of human *CYP11A1* gene (Chen and Guo, 2000). This element appears to have higher affinity for CREB proteins that bind to CRE than the proteins that bind to TRE (Chen and Guo, 2000).

Analysis of the upstream DNA (-761/-11) of *Cyp6a8* gene using a web-based TFSEARCH program identified several sequence motifs (Fig. 2-18) in addition to those reported previously (Maitra et al., 2002). Besides seven putative AP-1 sites, three putative CREB sites are present in the upstream DNA. Reporter gene assays showed that the -67/-11 DNA of *Cyp6a8* DNA could confer only basal but not caffeine-induced promoter activity. Sequence analysis revealed the presence of a putative TATA box (-64/-75) in this DNA (Maitra et al., 2002), which may be responsible for the basal transcription of this reporter construct. The constitutive and caffeine-induced promoter activities of the 0.1luc-A8 construct with -109 /-11 upstream DNA were similar to those found with 0.06luc-A8 DNA despite the presence of a single putative AP-1 site which spans between positions -70 and -80 (Fig. 2-13 and Fig. 2-14). Although this putative AP-1 site does not confer caffeine-induced expression, results showed that mutation of this site drastically decreases the constitutive promoter activity (Fig. 2-15). These data suggest that the single AP-1 site in -109/-11 DNA is important for conferring the basal constitutive promoter activity of *Cyp6a8*. Interestingly, this AP-1 site is very close to the non-canonical TATA box and fully contained within a putative Barbie box identified by Maitra et al (2002). Barbie box is thought to be responsible for barbiturate-induced expression of *CYP* gene (Liang et al., 1995; Gaidamakova et al., 1996; Kemper, 1998). However, it has not been shown before that mutation of a putative Barbie box can drastically reduce transcription. Increasing the length of upstream DNA by another 90 bases, the resulting construct with -199/-11 region, gave a significant (1.9-fold) augmentation of caffeine-induced expression.

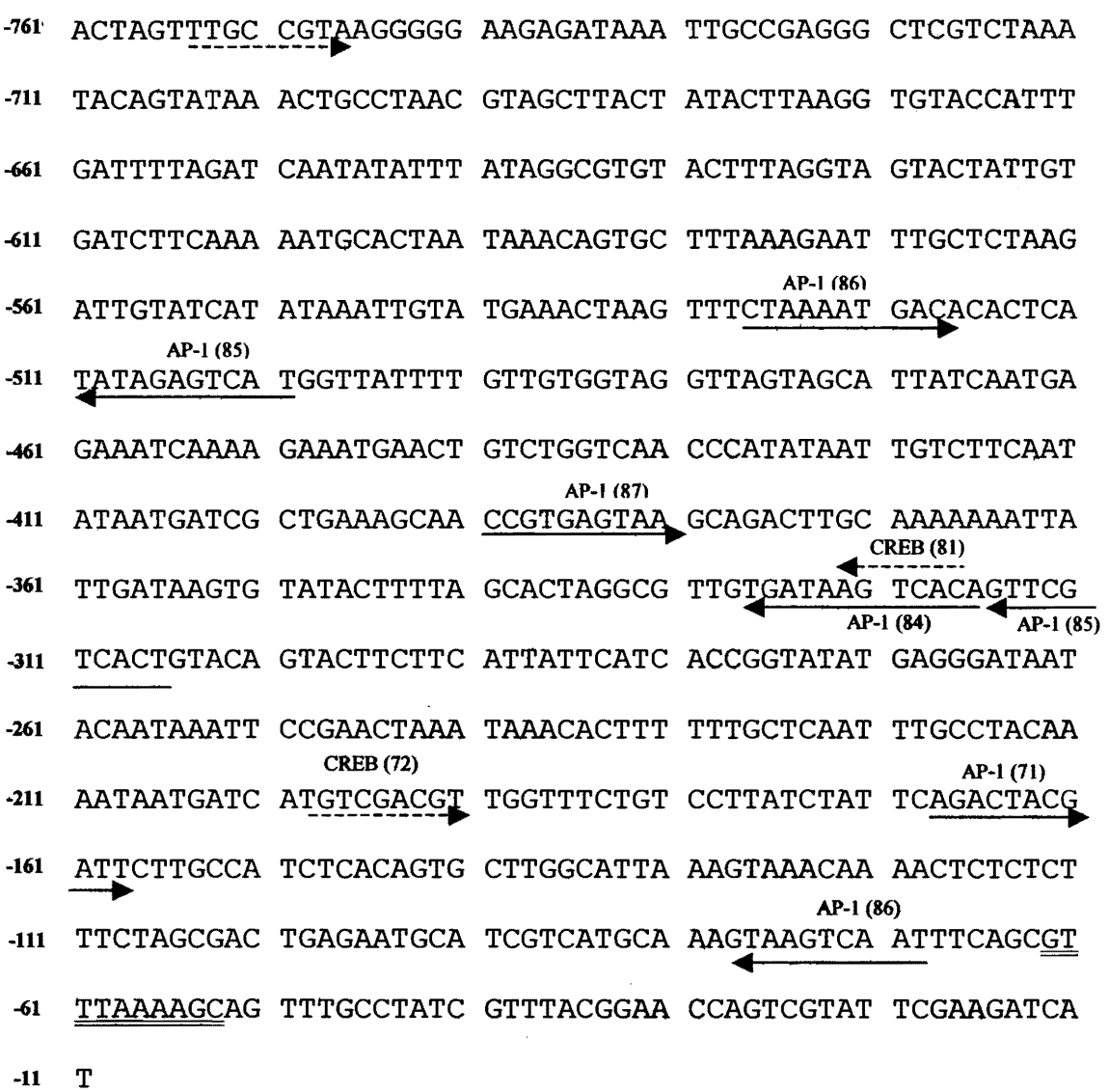


Figure 2-18: Nucleotide sequence analysis of the -761 to -11 region of the upstream DNA of *Cyp6a8-91R*. The putative AP-1 sites were reported previously by Maitra et al. (2002). Sequences that recognize AP-1 and CREB proteins were identified using 'TFSEARCH' web based software program. The AP-1 sites at positions -70/-80; -159/-169; -318/-328; -307/-318; -379/-389; -500/-510; -513/-523 are indicated by solid arrows. The dashed arrows indicate the putative CREB sites at positions -747/-754; -314/-321 and -198/-191. The putative TATA box (Maitra et al., 2002) is double-underlined. The percent identity is shown within parenthesis.

This region has a putative CREB (-198/-191) with 72% and a putative AP-1 site (-169/-159) with 71% identity to the respective consensus sequences. These sequence motifs may be functionally important because human GnRH receptor gene carrying CREB and AP-1 sites with 75% and 71% identity to their consensus sequences, respectively is induced by human chorionic gonadotropin-activated cAMP pathway (Cheng and Leung, 2002). Further increase of the upstream to -491 (0.5luc-A8 construct) increased both constitutive as well as caffeine-induced promoter activity compared to the 0.2luc-A8 reporter plasmid. Sequence analysis revealed that -491/-11 DNA has four putative AP-1 sites and two putative CREB sites. When the upstream DNA was increased from -491 to -761, two additional AP-1 sites and one CREB site were included in the resulting -761/-11 DNA. These extra motifs may be the reason for increase in the fold-induction with caffeine although not much difference in the constitutive promoter activity was observed. *Cyp6a2* upstream DNA also showed somewhat comparable expression. As observed with the -109/-11 *Cyp6a8* DNA, the -129/-1 *Cyp6a2* DNA did not show caffeine-inducibility although it gave significantly higher level of constitutive expression when compared to the pGL3-basic empty vector (Fig. 2-17). Lack of a putative AP-1 motif in this DNA may be the reason for lack of caffeine-inducibility. The modest and measurable constitutive promoter activity of the -129/-1 DNA is somewhat at odds with previous observation that this DNA failed to show any constitutive activity when used as a transgene in ry^{506} strain (Dombrowski et al., 1998). It is possible that in transgenic flies this DNA was under the influence of chromosomal position effect and got repressed. The -983/-1 DNA in transgenic flies showed very high activity as observed in the SL-2 cells in this investigation, but the activity varied in different transgenic lines due to chromosomal

position effect (Dombrowski et al., 1998). When the upstream DNA was increased to -265 bp, both basal and caffeine-induced promoter activities of the -265/-1 DNA increased significantly compared to the -129/-1 upstream DNA, which may be due the presence of a single AP-1 site between positions -150 and -160 of the upstream DNA (Fig. 2-16 and Fig. 2-17). Increasing the length of the upstream DNA further to bp -522 did not change the basal or induced expression although two additional AP-1 sites at -362/-370 and -472/-482 were added to the DNA (Fig. 2-19). This suggests that these two extra AP-1 sites do not have much role in *Cyp6a8* expression. However, a significant increase in both activities was observed when the 0.9luc-A8 construct was used. The extra DNA in this construct added one AP-1 (-853/-863) and two CREB sites (-639/-646 and -856/-863). Interestingly, the AP1 and one CREB site overlap with each other almost forming a composite CRE/TRE element that has been found in the upstream DNA of human *CYP11A1* gene (Chen and Guo, 2000). As mentioned before, one of the AP-1 factors, Jun can bind with both CRE and TRE as a homodimer or heterodimer with the Fos protein (Angel et al., 1987; Angel et al., 1988; Franza et al., 1988; Nakabeppu et al., 1988). Although the highest activity observed for the -981/-1 DNA might be a result of the cumulative effect of some of these putative *cis*-acting elements, the CREB and AP-1 sites present in the -983/-522 DNA may be functionally more important. In future, functional role of the putative AP-1 and CREB sites present in *Cyp6a2* and *Cyp6a8* upstream DNAs may be evaluated by performing gel-shift assays.

Figure 2-19: Nucleotide sequence analysis of the -983 to -1 region of the upstream DNA of *Cyp6a2-91R* for putative AP-1 and CREB transcription motifs. Sequences that recognize AP-1 and CREB proteins were identified using 'TFSEARCH', a web-based software program. The AP-1 sites at positions -150/-160; 362/-370; -472/-482; -853/-863 are indicated by solid arrows. The CREB sites at -856/-863; and -639/-646 are represented by arrows with dashed line. The putative TATA box (Waters et al., 1992) is double-underlined. The percent identity is shown within parenthesis.

-983 TTCATTCGTT TTATCGCCGT GGAACACCAA ACATTAACGT TGTAGTGGGG

-933 TGCTAATTAC TGAAATAAAT GGGTGATATT TTAAATGTGT ATGTCCGTGT

CREB (84)

-883 ATGTATTTAT CTACGGGAAA TTATGTCATT AACAATTTCA TGCGATAATG

AP-1 (82)

-833 TTCGCTCCGA TTCCCAAGGC TTAAAAACTC ATTTAATTAT ATTTGCTCTG

-783 TTTTCTAATA AATATTGAAT CGCATAGGAG AAAAACCTTT ATGCTCCTTA

-733 ACCAGCTTGT TTAATAACAA TCTTGTAATC GTTTGTTTGC ATGTTGATCC

CREB (84)

-683 ACTGCAATAG AACAGTAGAA CATCAAGAGA ATATCTCTTA TGTCAAATCT

-633 TGAATCTCTC ATAAAAAGCA AAAGAGCTTG GTGCTTGTCG CTGATAATGA

-583 AAGCTAGATG GAACGCTCGG CTATCAAATA CCCATTACTT AAACACCTTT

-533 ACCTTTAGTT ACAAGTGGGA TCGTCCTGTA CATTTGAAGC TACTTTGAAT

AP-1 (81)

-483 GACCGTGTCA TTGCGTTTTT ATAGTAATAC AATGATTTGA TACATTTTCC

-433 GCATATCGAT GTGTATTTAC CATTTAATGC TACCTCTACT TTTCGCACAT

AP-1 (83)

-383 GCCGAGCTTA TAAATGAGTG ATAAAACCTG ATGGGACACT TTTTACCCAA

-333 GCACCAACCG GTTTGTTTAA CAACAATCTC GTTATCATTT GTTTATATTT

-283 TGATTCGCGA GCATTTTTGC AGTGAAGCGT ATGAGTATCT CTTTGAAAGT

-233 GCTCTTAAAT CGGTTTGAAA AGTGCCTGCA TATTATCGCC GAGTCTGTAA

AP-1 (83)

-183 TCATGACAAC AACTTAAAAG TTGCGTAGTC ATGGTGATAG AAATATTTAG

-133 CTAGCTAGCT CACATGCTGT CATGCCTGTG CGTCGCAGGG GAATCTTATA

-83 AAAAGTGTGC GAACATTTTG TGGTGATCAG TAATTCGTCG TAGGTCGAGC

-33 ACGACGATTG CGAAACGGGA GCAGCTACGC AAA

Chapter 3

Examination of the signaling mechanism by which caffeine induces *Cyp6a2* and *Cyp6a8* gene expression

Introduction

Caffeine, the most commonly used psychostimulant, is found in coffee, tea, many soft drinks and several drug preparations. Because of its widespread use, effect of caffeine on various physiological processes in humans, rats, mice and other mammals have been studied extensively (Svenningsson et al., 1995a; Lorist and Tops, 2003; Porta et al., 2003b). Majority of these studies have been actually made on the effect of caffeine on sleep, hypertension and cardiac physiology. However, in modest number of studies, effect of caffeine on cell cycle checkpoints, DNA repair, apoptosis and carcinogen metabolism have also been examined (Porta et al., 2003a). Effect of caffeine on other animals has also been examined. When *Drosophila* are allowed to feed on caffeine containing food, a dose-dependent decrease in rest was observed (Shaw et al., 2000). Interestingly, during wake or active period expression of *Cyp4e2* gene increased about 2-fold compared to the rest or sleep period (Shaw et al., 2000) although the significance of this increased expression is not clear. Caffeine is also found to be effective in killing or repelling slugs and snails when applied to foliage (Hollingsworth et al., 2002). Caffeine that is naturally found in plants is shown to inhibit insect feeding and acts as a pesticide (Nathanson, 1984).

In mammals, caffeine exerts it action by antagonizing the A_1 and A_{2A} isoforms of adenosine receptor and (or) by inhibiting the cAMP phosphodiesterase enzyme (PDE) (Poulsen and Quinn, 1998; Fredholm et al., 1999; Fisone et al., 2004). Caffeine acts as an antagonist for A_1 and A_{2A} receptors, which are activated by low concentrations of adenosine (Poulsen and Quinn, 1998; Moreau and Huber, 1999; Fredholm et al., 2005). High concentrations of caffeine has been shown to act as a PDE inhibitor rather than A1 antagonist (Fredholm et al., 1999). Antagonism of adenosine receptor or PDE inhibition is known to elevate the intracellular cAMP level. The cAMP-mediated pathway has been shown to induce the expression of transcription factors, that constitute the activator protein-1 (AP-1) complex (Karin, 1995; Fredholm et al., 1999). In fact, caffeine has been shown to increase the expression of c-Fos, c-jun, and junB mRNAs or protein in the rat brain striatum (Svenningsson et al., 1995b; Bennet and Semba, 1998). It is possible that caffeine-induction of gene expression may be mediated via cAMP pathway, which has not been examined in a systematic way for a specific *CYP* gene.

Sequence analysis of *Cyp6a2* and *Cyp6a8* genes has identified multiple putative AP-1 sites in the upstream DNA of both genes (see Chapter 2 and Maitra et al., 2002). We wished to determine the signaling link between caffeine, cAMP and *Cyp* gene expression. Therefore, the present study was carried out to understand the signaling mechanism by which caffeine induces *Cyp6a2 and Cyp6a8* gene expression in *Drosophila*. Our results demonstrate that caffeine at doses used in the present study acts as a PDE inhibitor and it increases the intracellular cAMP level. The increase in cAMP level in turn increases the *Cyp6a8* and *Cyp6a2* promoter activities. Also, we found that

manipulation of the intracellular cAMP levels with various adenosine receptor agonists and antagonist did not alter the *Cyp6a2* and *Cyp6a8* promoter activities.

Materials and Methods

Fly stocks, cell culture and chemicals

Two transgenic lines of *Drosophila melanogaster*, *0.2-luc4H-ry* and *0.8-luc110H-ry*, and the mutant strain dnc^{1} were used in the present investigation. The transgenic lines have one copy of *0.2-luc* or *0.8-luc* reporter transgene located on chromosome 2 (Maitra et al., 2002). The reporter transgenes were created by cloning a 0.2- (-11/-199) or 0.8- (-11/-761) kb upstream DNA of *Cyp6a8* gene in front of the firefly *luciferase* (*luc*) gene. Details of the reporter constructs made and germ line transformation into ry^{506} host strain has been previously described (Maitra et al., 2002). By using appropriate balancer stocks, the original transgenic stocks were made homozygous for all three major chromosomes of the ry^{506} strain (Dean and Ganguly, Unpublished data). The dnc^{1} stock was received from Indrani Ganguly of Neuroscience laboratory, San Diego (CA). Schneider line SL-2 cells (Schneider, 1972) of *Drosophila melanogaster* were obtained from Invitrogen (Carlsbad, CA). The cells were maintained at 25°C in Scheneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated bovine calf serum and 0.01% penicillin-streptomycin, which were obtained from Sigma (St. Louis, MO). Every fourth day the cells were transferred to fresh media.

Caffeine, Adenosine, 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), N^{6} cyclopentyl xanthine (CPA), Theophylline, Rolipram and dibutyryl cAMP were purchased from Sigma (St. Louis, MO). Caffeine, theophylline and dibutyryl cAMP were

dissolved in sterile water, whereas CPA, DPCPX and Rolipram were dissolved in DMSO. Adenosine was dissolved in 250mM Tris pH 8.0.

Construction of 0.9luc-A2 reporter plasmid

To create *0.9luc-A2* chimeric reporter plasmid, a 0.9-kb upstream DNA of *Cyp6a2* from positions -983 to -1 (ATG at +1) was amplified by PCR. To the 5'-ends of the distal and proximal primers, sites for restriction enzymes *Mlu*I and *Sal*I sites were introduced, respectively. The PCR product was digested with *Mlu*I and *Sal*I, and cloned in front of the *luc* (*luciferase*) reporter gene between the *Mlu*I *and Xho*I sites of the pGL3-Basic vector (Promega, Madison, WI). All cloned PCR products were verified by sequencing at the Molecular Biology Resource Facility of the University of Tennessee.

Treatment of flies, fly extract preparation and luciferase assay

All flies were raised on standard corn meal-agar-molasses medium. Flies to be treated with different chemicals were etherized and groups of forty females were transferred into *vial*s containing normal media. The flies were left in the *vial*s for 12 h to recover from the ether shock before they were transferred live to *vial*s containing *Drosophila* media reconstituted with aqueous solution of caffeine, adenosine, or theophylline. To treat flies with DPCPX, CPA or Rolipram, solutions of these chemicals made in DMSO were dripped evenly on the surface of standard *Drosophila* medium in individual *vial*s, which were left at room temperature overnight for the solution to dry. Media dripped with solvent alone were used as control. Next morning, live flies were directly transferred to the *vial*s containing a chemical or solvent and kept in dark for 24 h. After treatment, flies were etherized and transferred in batches of ten into 1.5ml microfuge tubes. Upon recovery from the ether effect, the flies were immobilized by

keeping the tubes on ice for few minutes and then they were homogenized in 100µl 1X Cell Culture Lysis Reagent (CCLR) obtained from Promega (Madison, WI). The homogenate was centrifuged twice at 14,000 rpm for 5 min at 4^0C and the clear supernatant, free of any lipid layer, was collected. All extracts were stored frozen at –80°C until further use.

Luciferase assay of the fly extracts was performed using a kit from Promega (Madison, CA). The luciferase assay reagent, which was stored at –80°C in 100µl aliquots, and the frozen fly extracts were thawed on ice and then they were equilibrated to room temperature. To assay LUC activity, 5µl fly extract was mixed with 100µl luciferase assay reagent, placed in a luminometer (Zylux, Oak Ridge, TN) and readings were taken at 15s interval. Total protein in fly extract was quantified using the BCA Protein assay kit (Pierce). To avoid interference from Triton X-100 present in the CCLR buffer, fly extracts and bovine serum albumin (used as the standard) were adjusted to make final concentration of CCLR 0.5X. Duplicate aliquots of each extract were assayed and the mean value was to quantify protein concentration. Luciferase activity was expressed as Registered Light Units per µg of total protein (RLU/µg). All experiments were done in triplicate.

SL-2 cell transfection, extract preparation and dual luciferase assay

For cell transfection, plasmid DNAs were reconstituted in 0.1M$CaCl_2$ and then mixed with equal volume of 2X HEPES buffer, pH 7.5 (Invitrogen, Carlsbad, CA). Cells to be transfected were resuspended in fresh SL-2 medium at 1X 10^6 cells /ml density. To each well of a 48-well microtiter plate, 500µl of cells were dispensed and to each well 84 µl of calcium phosphate-DNA mix was added in a drop-wise manner. After 18h

incubation, cells were washed with SL-2 media, resuspended in 500 µl of fresh media and allowed to recover for 24 h at 25°C prior to treatment with any chemical. All treatments were done for 24h unless otherwise mentioned. Following treatment, cells in the 48-well microtiter plate were pelleted and 250µl 1X Passive lysis buffer (Promega, Madison, WI) was added to each well. The plates were agitated on a plate shaker for 15 min at room temperature to lyse the cells and then centrifuged at 1500 rpm for 5 min at 4^0C to pellet the debris. The lysate was collected from each well and assayed immediately for luciferase activity.

Experiments in which SL-2 cells were co-transfected with renilla luciferase plasmids (Promega, Madison, WI) as an internal control, cell lysates were assayed by using a dual luciferase assay kit obtained from Promega (Madison, WI). To 20µl lysate 100µl luciferase assay reagent II was added, mixed, and placed in a luminometer after 10 s to measure the firefly luciferase (F-luc) activity. Immediately after recording the F-luc activity 100µl Stop and Glo Reagent was added, vortexed gently and the *Renilla* luciferase (R-luc) activity was measured within 10 s. R-LUC activity was used to normalize the data, which were expressed as the ratio of F-LUC to R-LUC activity.

Phosphodiesterase assay

To measure total phosphodiesterase (PDE) activity in the extracts of transgenic flies or SL-2 cells, scintillation proximity Assay (SPA) was done by using a commercially available kit (Amersham Biosciences). Briefly, ten female flies or 0.5 X 10^6 SL-2 cells were homogenized in KHEM buffer (50mM KCl, 10mM EGTA, 1.92mM $MgCl_2$, 1mM dithiothreitol, 50mM HEPES, pH7.2) containing P8340 protease inhibitor

cocktail (Sigma, St. Louis) and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatants were collected and assayed for protein concentration using the Bradford protein assay kit (Bio-Rad, Hercules, CA). For PDE assay, fly or cell extract, equivalent to 20μg or 40μg protein, was mixed with 10X PDE assay buffer that contains 500 mM Tris-HCl pH7.5, 83 mM $MgCl_2$ and 17 mM EGTA. Caffeine and 0.05μci of [5', 8-^{3}H] adenosine 3',5'-cyclic phosphate (30Ci/mmol) was added and the final volume of the reaction mixture was adjusted to 100μl. After incubating at 30°C for 10 min, the reaction was terminated by adding 50μl (1mg) yttrium silicate beads, which were provided in the SPA kit (Amersham). A complex ion chelation mechanism enables the linear nucleotide (5'-AMP) to bind to the bead and allow radiation from the tritium to excite the scintillant within the bead. Following addition of the SPA beads, the reaction mixtures were incubated at room temperature for 20 min to facilitate the beads to settle down to the bottom of the tube. The radioactivity was measured with a liquid scintillation counter and PDE activity was expressed as counts per minute per μg protein (CPM/μg protein). The assay was done in triplicate.

Measurement of intracellular cAMP level

Intracellular cAMP level was measured by using the Biotrack cAMP enzyme immuno assay kit (Amersham Biosciences). Approximately, 10^5 cells suspended in SL-2 media were transferred to individual wells of a 96-well microtiter plate. The cells were treated with caffeine for 24 h then lysed in the lysis reagent (Biotrack EIA kit). The cell lysate (100μl) was transferred to the wells of a microtiter plate pre-coated with donkey anti-rabbit IgG (Biotrack EIA kit) and 100μl rabbit anti-cAMP antibody was then added to each well. After incubating at 4°C for 2h, 50μl of cAMP-peroxidase conjugate was

added to each well and incubated again at 4°C for 1h. The reaction mixture was discarded and wells were gently washed with a wash buffer (Biotrack EIA kit) and 150µl of 3, 3', 5, 5'-tetramethyl benzidine (TMB) was added to each well as substrate. Following 1h incubation at room temperature, the reaction was terminated by the addition of 100µl of 1M sulphuric acid. Within 30 min, absorbance at 450nm was measured using a plate reader (Labsystem Multiskan). Data was expressed as fmol cAMP/10^5 cells. Two independent experiments, each in duplicate, with different batches of cells were performed.

Results

Adenosine receptor antagonist and agonist have no effect on *Cyp6a2* and *Cyp6a8* promoter activities

In the previous chapter it was found that caffeine induces both *Cyp6a2* and *Cyp6a8* promoter in SL-2 cells as well as in adult flies. I therefore wanted to dissect the mechanism by which caffeine induces *Cyp6a2* and *Cyp6a8* gene expression. In mammals, caffeine is known to act as an antagonist to the inhibitory adenosine receptor A1, which normally decreases the intracellular cAMP level after it binds with its agonist, adenosine (Klinger et al., 2002). If caffeine induction of *Cyp6a2* and *Cyp6a8* promoters is mediated *via* antagonism of adenosine receptor, adenosine being an agonist should show an opposite effect and decrease the promoter activity. To test this hypothesis, SL-2 cells were co-transfected with 0.9luc-A2 and the internal control pRL-null plasmids. Transfected cells were then treated with various concentrations of adenosine or Tris

buffer as control. Extracts prepared from these cells were assayed for luciferase activity. The results showed that adenosine did not have any significant effect on the luc activity even at the highest concentration used 100μM (Fig.3-1A). The effect of adenosine on *Cyp6a8* promoter activity was also tested in SL-2 cells transfected with 0.2luc-A8 or with 0.8luc-A8 reporter plasmids. Again, no effect of 100μM adenosine on *Cyp6a8* promoter was observed (Fig. 3-1B). Thus adenosine does not influence the *Cyp6a2* and *Cyp6a8* promoter activities SL-2 cells.

Effect of adenosine on *Cyp6a8* promoter activity was also examined in flies by treating the 0.2luc4-*H-ry* and *0.8luc110-H-ry* transgenic lines with much higher concentration (8 mM) of adenosine than that was used to treat SL-2 cells. The results (Fig. 3-2) showed that there was no difference in the *Cyp6a8* promoter activity between adenosine-treated and control (Tris-treated) flies, further corroborating that adenosine does not alter the *Cyp6a8* promoter activity. Taken together these results suggest that adenosine does not affect the *Cyp6a2* and *Cyp6a8* promoter activities.

Like caffeine, dipropyl cyclopentyl xanthine (DPCPX) and cyclopentyl adenosine (CPA) also interact with adenosine receptor (for review see Poulsen and Quinn, 1998; Moreau and Huber, 1999; Klinger et al., 2002). While DPCPX is a selective antagonist for inhibitory adenosine (A1) receptor, CPA is a selective agonist for A1 receptor. Since adenosine has intermediary affinity for the A1 adenosine receptor, the effect of adenosine may not be as strong as expected for CPA, which has much higher affinity for the receptor (Poulsen and Quinn, 1998). Similarly, DPCPX has much higher affinity for the receptor than caffeine. Because these chemicals are more potent, it was thought that they might affect the *Cyp6a2* and *Cyp6a8* promoter activities. Therefore, SL-2 cells

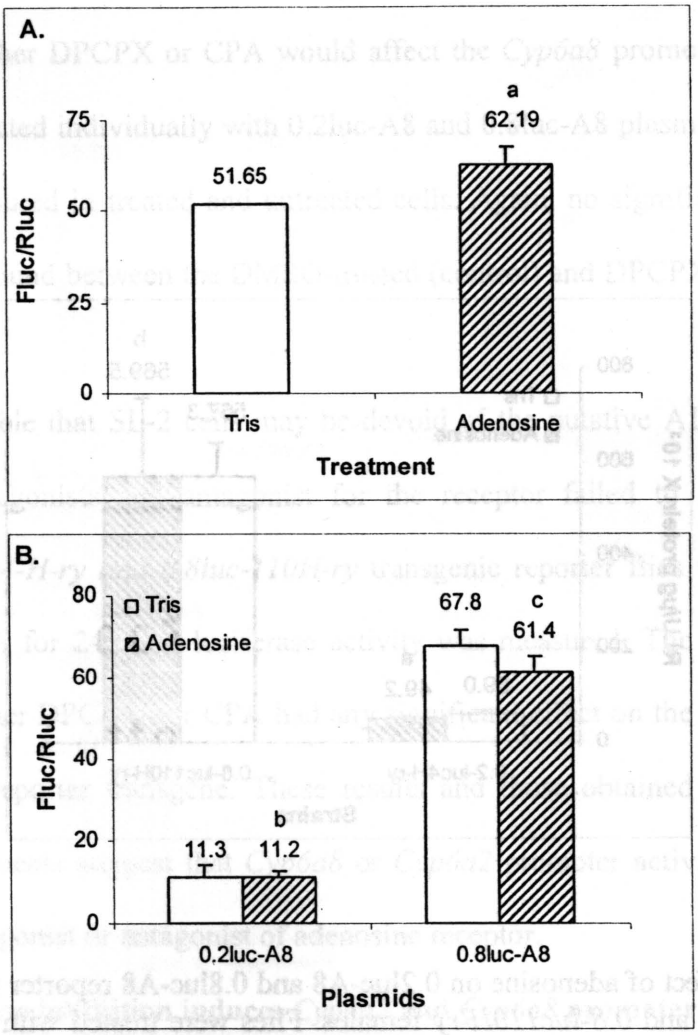


Figure 3-1: Effect of adenosine on *Cyp6a2* and *Cyp6a8* promoter activities in SL-2 cells. SL-2 cells were transfected with 0.9luc-A2 (Panel A) or with *Cyp6a8*-luc (0.2luc-A8 or 0.8luc-A8) (Panel B), individually, along with the internal control plasmid pRL null. Transfected cells were treated with either 8mM adenosine made in 250mM Tris, pH 8.0 or with 250mM Tris, pH8.0 as control. After 24h treatment, cells were collected and the lysates were assayed for firefly and renilla luciferase activities. Values represent mean ratio of firefly to renilla luciferase activities (Fluc/Rluc) ± S.D of triplicate samples. *p*: a < 0.04, b : 1.0 and c : 0.1.

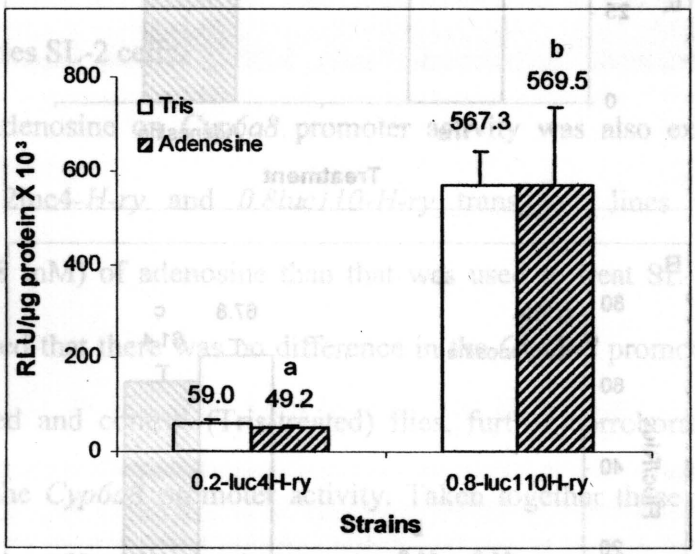


Figure 3-2: Effect of adenosine on 0.2luc-A8 and 0.8luc-A8 reporter transgene activities in *0.2-luc4H-ry* and *0.8-luc110H-ry* females. Flies were treated with 8mM adenosine or with 250mM tris (pH8.0) that was used to dissolve adenosine. Extracts prepared from adenosine-treated and Tris-treated flies were assayed for firefly luciferase activity. Firefly luciferase activity is expressed as average luciferase activity per µg total protein. Values represent mean ± S.D of triplicate samples. p: a : 0.18, and b : 0.98.

transfected with 0.9luc-A2 were treated with DPCPX or CPA and luciferase activity was measured. Neither DPCPX nor CPA had any effect on the 0.9luc-A2 activity (Fig. 3-3A). To examine whether DPCPX or CPA would affect the *Cyp6a8* promoter activity, SL-2 cells were transfected individually with 0.2luc-A8 and 0.8luc-A8 plasmids and luciferase activity was measured in treated and untreated cells. Again, no significant difference in luc activity was found between the DMSO-treated (control) and DPCPX- or CPA-treated cells (Fig. 3-3B).

It is possible that SL-2 cells may be devoid of the putative A1 receptor and for that reason the agonists and antagonist for the receptor failed to show any effect. Therefore, *0.2luc4-H-ry and 0.8luc-110H-ry* transgenic reporter flies were treated with DPCPX and CPA for 24h and luciferase activity was measured. The results (Fig. 3-4) showed that neither DPCPX nor CPA had any significant effect on the *Cyp6a8* promoter driving the *luc* reporter transgene. These results and those obtained in the adenosine treatment experiments suggest that *Cyp6a8* or *Cyp6a2* promoter activity is not affected by high affinity agonist or antagonist of adenosine receptor.

Phosphodiesterase inhibition induces *Cyp6a2* and *Cyp6a8* promoter activities

A high level of caffeine has been shown to inhibit cAMP phosphodiesterase or PDE that hydrolyzes cAMP (Svenningsson et al., 1995b). In mammals, caffeine inhibits PDE4, which is a cAMP-specific PDE (Ye et al., 2001). Thus, to examine whether other known PDE inhibitors can induce *Cyp6a2* and *Cyp6a8* promoter activities, SL-2 cells transfected with 0.9luc-A2 reporter plasmid were treated with PDE4- selective inhibitor rolipram or with 8mM theophylline which is a non-selective PDE inhibitor (Belibi et al., 2002). Extracts prepared from these untreated and treated cells were then assayed for luciferase

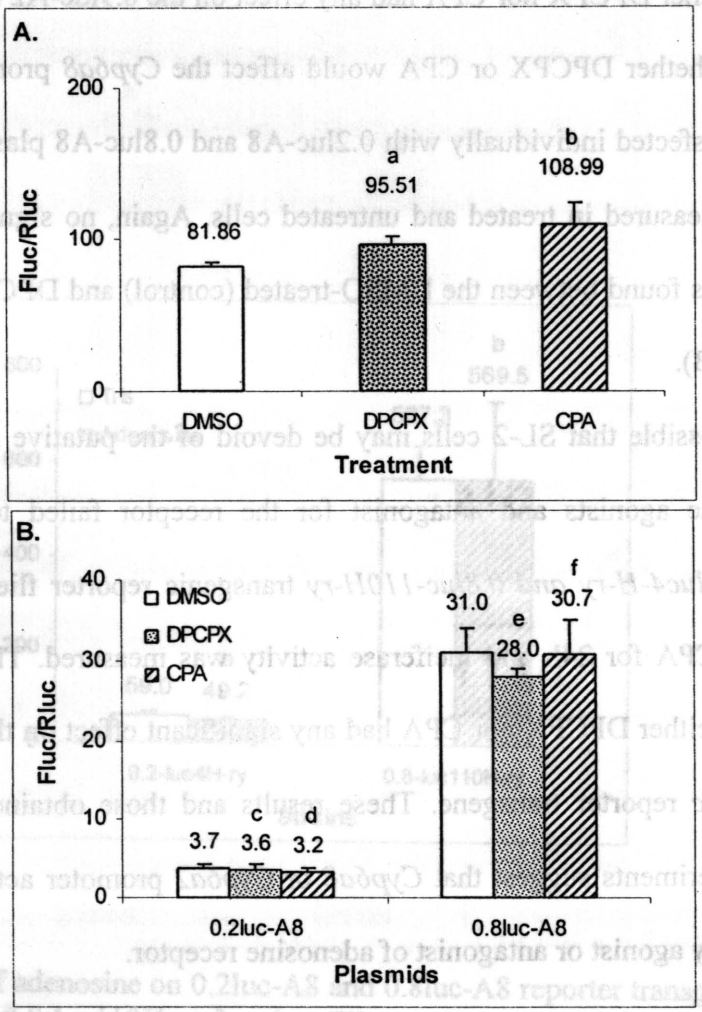


Figure 3-3: Effect of DPCPX and CPA on *Cyp6a2* and *Cyp6a8* promoter activities in SL-2 cells. SL-2 cells were transfected individually with 0.9luc-A2 (Panel A) or with *Cyp6a8*-luc plasmids (0.2luc-A8 or 0.8luc-A8) (Panel B) plasmids along with the internal control plasmid pRL null. Transfected cells were treated either with 5 μM DPCPX or 500μM CPA made in DMSO. Cells treated with DMSO was used as controls. After 24h treatment, cells were collected and the lysates were assayed for firefly and renilla luciferase activities. Values represent mean ratio of firefly to renilla luciferase activity (Fluc/Rluc) ± S.D of triplicate samples. *p*: a : 0.21, b : 0.03, c :0.84, d : 0.29, e : 0.18, and f : 0.93.

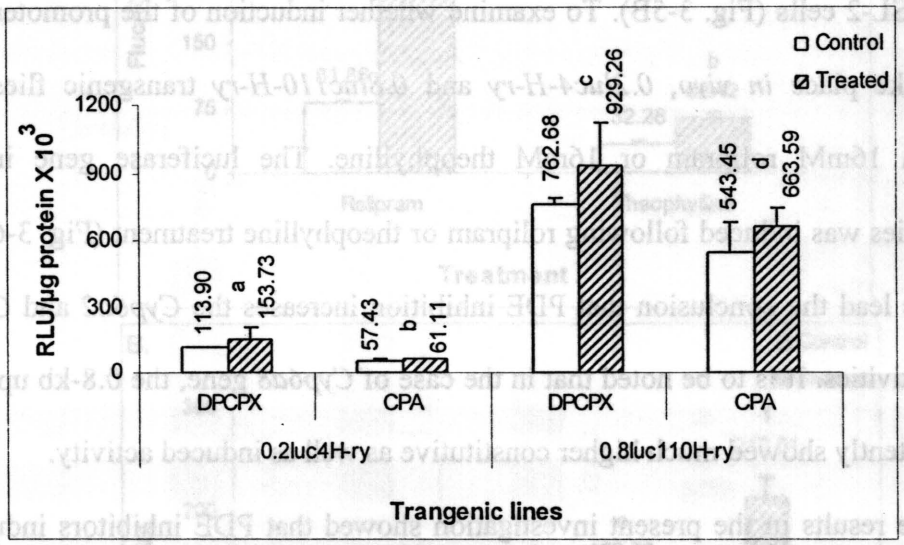


Figure 3-4: Effect of CPA and DPCPX on *Cyp6a8* promoter activity in transgenic reporter flies. *0.2-luc4H-ry* and *0.8-luc110H-ry* females were treated with 16mM CPA or with 1mM DPCPX or with DMSO (control) for 24 hours. The extracts prepared from these were assayed for firefly luciferase activity. Values represent mean ± S.D of three independent samples. p: a < 0.5, b < 0.5, c < 0.1 , d < 0.5.

activity. The results showed that both PDE inhibitors significantly induces the *Cyp6a2* promoter activity in SL-2 cells (Fig. 3-5A). Similarly, SL-2 cells transfected with *Cyp6a8* reporter plasmids, 0.2luc-A8 or with 0.8luc-A8, were also treated with rolipram or with theophylline. Results showed that both PDE inhibitors induced 0.2luc-A8 and 0.8luc-A8 plasmids in SL-2 cells (Fig. 3-5B). To examine whether induction of the promoter DNA may also take place *in vivo*, *0.2luc4-H-ry* and *0.8luc110-H-ry* transgenic flies were treated with 16mM rolipram or 16mM theophylline. The luciferase gene in both transgenic flies was induced following rolipram or theophylline treatment (Fig. 3-6). The observations lead the conclusion that PDE inhibition increases the *Cyp6a2* and *Cyp6a8* promoter activities. It is to be noted that in the case of *Cyp6a8* gene, the 0.8-kb upstream DNA consistently showed much higher constitutive as well as induced activity.

Since results in the present investigation showed that PDE inhibitors induce the *Cyp6a8* promoter activity, the effect of loss of function of PDE on *Cyp6a8* promoter activity *in vivo* was the tested. For this purpose, we used dnc^1, a hypomorphic allele of a *Drosophila* X-linked gene called *dunce*, which is involved in learning and memory, and codes for cAMP phosphodiesterase (Dudai et al., 1976; Davis et al., 1981). It has been shown previously that compared to the wild-type strains, dnc^1 mutant has lower level of cAMP-phosphodiesterase and higher amounts of intracellular cAMP (Davis et al., 1981). To examine if partial loss of endogenous PDE activity would increase the *Cyp6a8* promoter activity, dnc^1/dnc^1; +/+ females were crossed to the males of 0.8-luc110H-ry transgenic line which is homozygous for a 2nd chromosome-linked 0.8luc-A8 transgene. The genotype of these males is *+/Y; 0.8-luc/0.8luc*. The F1 males (0-3 d old) with genotype dnc^1/Y; *0.8luc/+* were collected. As a control, a reciprocal cross was done

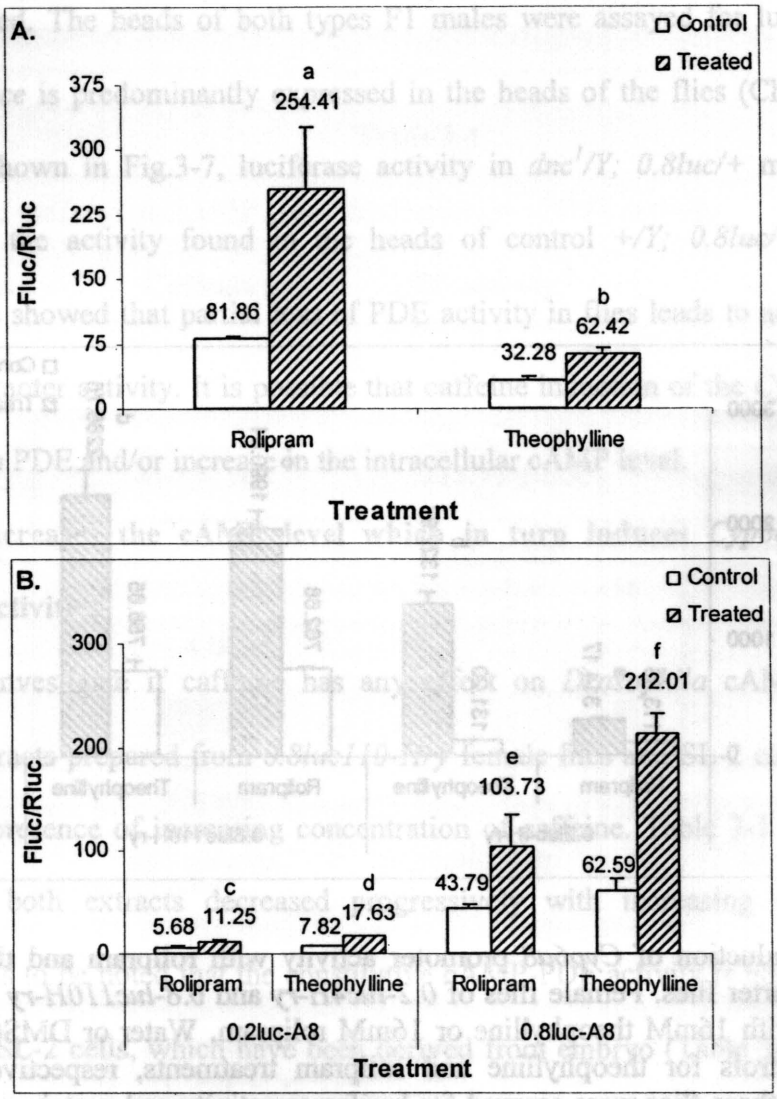


Figure 3-5: Effect of rolipram and theophylline on *Cyp6a2* and *Cyp6a8* promoter activities in SL-2 cells. SL-2 cells were transfected individually with 0.9luc-A2 (Panel A) or with 0.2luc-A8 or 0.8luc-A8 *Cyp6a8* reporter plasmids (Panel B), along with the internal control plasmid pRL null. Transfected cells were treated with 500μM rolipram or with 8mM theophylline. After 24h treatment, cells were collected, lysates were prepared and assayed for firefly and renilla luciferase activities. Cells treated with DMSO served as control for rolipram treatment and untreated cells in media served as the control for theophylline treatment. Values represent mean ratio of firefly to renilla luciferase activity (Fluc/Rluc) ± S.D of triplicate samples. *p*: a < 0.02, b < 0.005, c < 0.005, d <0.005, e <0.01, and f <0.01.

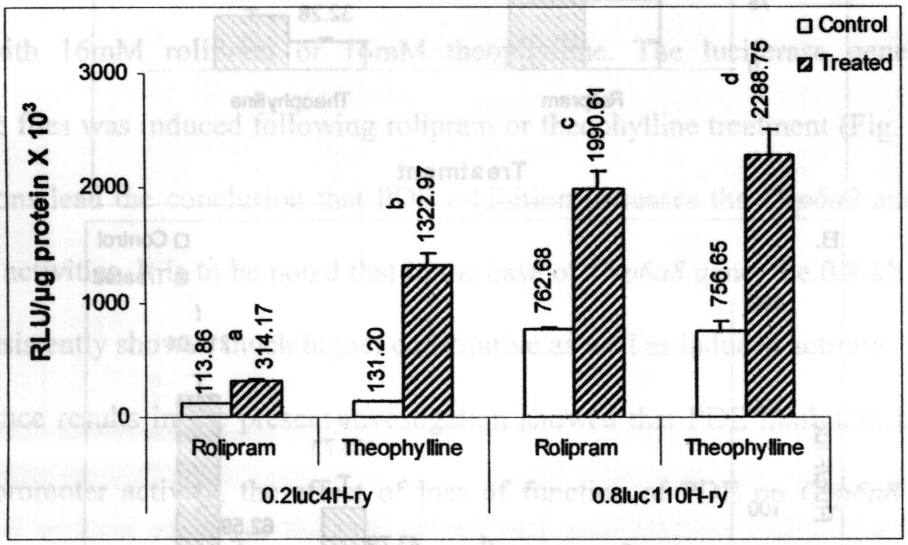


Figure 3-6: Induction of *Cyp6a8* promoter activity with rolipram and theophylline in transgenic reporter flies. Female flies of *0.2-luc4H-ry* and *0.8-luc110H-ry* reporter strain were treated with 16mM theophylline or 16mM rolipram. Water or DMSO treated flies served as controls for theophylline and rolipram treatments, respectively. Extracts prepared from these flies were assayed for luciferase activity and protein content. Values represent mean ± S.D of triplicate samples. a < 0.001, b < 0.001, c < 0.01, and d < 0.001.

between +/+; *0.8-luc/0.8luc* females and *dnc^{1}/Y; +/+* males, and F1 *+/Y; 0.8luc/+* males were collected. The heads of both types F1 males were assayed for luciferase activity because *dunce* is predominantly expressed in the heads of the flies (Cherry and Davis, 1995). As shown in Fig.3-7, luciferase activity in *dnc^{1}/Y; 0.8luc/+* males was 2-fold higher than the activity found in the heads of control *+/Y; 0.8luc/+* males. These observations showed that partial loss of PDE activity in flies leads to an increase in the *Cyp6a8* promoter activity. It is possible that caffeine induction of the *Cyp6* promoters is mediated *via* PDE and/or increase in the intracellular cAMP level.

Caffeine increases the cAMP level which in turn induces *Cyp6a2* and *Cyp6a8* promoter activities

To investigate if caffeine has any effect on *Drosophila* cAMP-specific PDE activity, extracts prepared from *0.8luc110-Hry* female flies and SL-2 cells were assayed *in vitro* in presence of increasing concentration of caffeine. Table 3-1 shows that PDE activity in both extracts decreased progressively with increasing concentration of caffeine. It is to be noted that the constitutive cAMP-PDE activity is higher in adult flies than in the SL-2 cells, which have been derived from embryo (Table 3-1). It is possible that PDE expression is developmentally regulated.

Since it was found that caffeine inhibits PDE activity, I thought caffeine treatment should increase the intracellular cAMP level. To investigate this, extracts of caffeine-treated and untreated SL-2 cells were assayed for intracellular cAMP level using Biotrack cAMP competitive enzyme immunoassay system. As shown in Fig. 3-8, the level of intracellular cAMP was about 2-fold higher in caffeine-treated cells than the level found in untreated ones. This is a direct demonstration that the cAMP level is increased upon

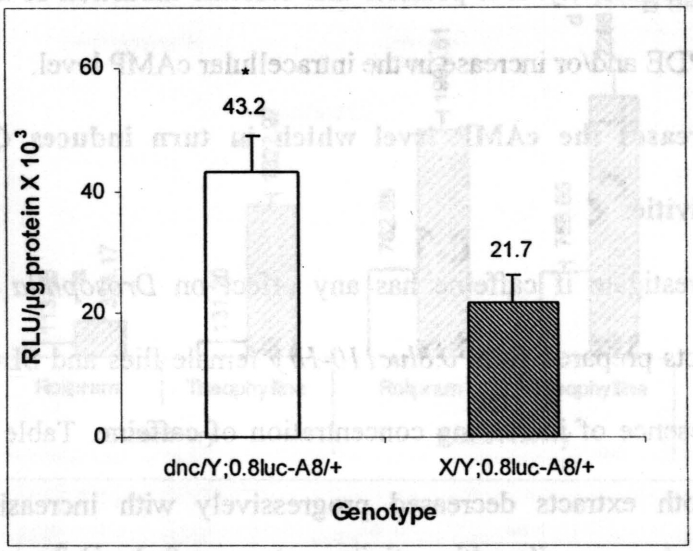


Figure 3-7: The effect of X-linked *dunce* mutation on *0.8luc-A8* reporter gene. Male flies of *0.8-luc110H-ry* transgenic line were crossed to *dnc*^{1} females. Heads of 0-3 d old F1 adult males (*dnc*^{1}/Y; *0.8luc-A8*/+) were collected, extracts were prepared and assayed for luciferase activity and protein content. F1 males of reciprocal cross with genotype *+/Y*; *0.8luc-A8*/+ were used as control. Values represent mean ± S.D of triplicate samples. *p* * :0.007.

Table 3-1

Effect of caffeine on cAMP phosphodiesterase (PDE) activity

Samples [a]	Caffeine (mM)	PDE activity [b] (cpm/μg protein)	p [c]
0.8-luc110 H-ry	0	462.18 ± 91.2	-
	1.6	278.63 ± 88.9	0.06
	3.2	211.75 ± 5.9	0.01
S2 cells	0	21.6 ± 3.5	-
	1.6	5.3 ± 0.6	0.02
	3.2	4.5 ± 0.1	0.02

[a] Extracts prepared from 0.8-luc110H-ry females or S2 cells were assayed for PDE activity.

[b] Extracts were treated with different concentrations of caffeine and the cAMP-PDE activity was measured by scintillation proximity assay. Values represent mean ± S.D of triplicate samples.

c p values obtained by two-sample t-test at 95% confidence level.

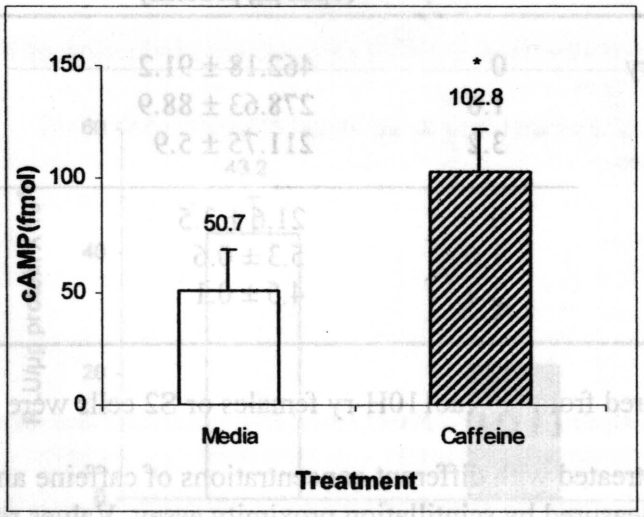


Figure 3-8: Intracellular cAMP level in caffeine-treated and untreated SL-2 cells. SL-2 cells (10^5 cells) were incubated for 24 h in 100 µl of 1mM caffeine containing medium. After incubation, extracts were prepared from treated and untreated cells, and cAMP levels were measured using ELISA. Values represent mean ± S.D of quadruplicate samples from two independent experiments. p* : 0.007.

caffeine treatment in SL-2 cells and it strongly suggests that caffeine induction of *Cyp6a2* and *Cyp6a8* promoter is mediated *via* cAMP pathway. To examine this possibility, SL-2 cells were individually transfected with 0.9luc-A2 and with 0.8luc-A8 reporter plasmids, and then treated with dibutyryl cAMP, a cell permeable and non-hydrolyzable analog of cAMP. Extracts prepared from these cells were assayed for luciferase activity. Results showed that dibutyryl cAMP increased both *Cyp6a2* and *Cyp6a8* promoter activities by about 3-fold (Fig.3-9).

To further examine the effect of dibutyryl cAMP on *Cyp6a8* promoter activity in flies, *0.2-luc4H-ry* and *0.8-luc110H-ry* reporter transgenic flies were treated with dibutyryl cAMP. Luciferase assay of the extracts prepared from untreated and cAMP-treated flies showed that dibutyryl cAMP induces *Cyp6a8*-luc activity by several fold in both 0.2-luc4H-ry and 0.8-luc110H-ry flies (Fig. 3-10). Taken together, these results support the hypothesis that caffeine inhibits cAMP-PDE and increases intracellular cAMP level, which in turn stimulates the *Cyp6a2* and *Cyp6a8* promoters.

Discussion

In humans and other mammals, caffeine act as an antagonist for adenosine receptor (Poulsen and Quinn, 1998; Fredholm et al., 1999; Fisone et al., 2004). At present, four different isoforms of adenosine receptor, A_1, A_{2A}, A_{2B} and A_3 from different mammalian species have been cloned and characterized (Klinger et al., 2002 for review). Caffeine acts as an antagonist for A_1 and A_{2A} receptors, which are activated by low concentrations of adenosine (Poulsen and Quinn, 1998; Moreau and Huber, 1999; Fredholm et al., 2001). A_1 and A_{2A} are both G-protein coupled receptors for adenosine. A_1 is coupled to

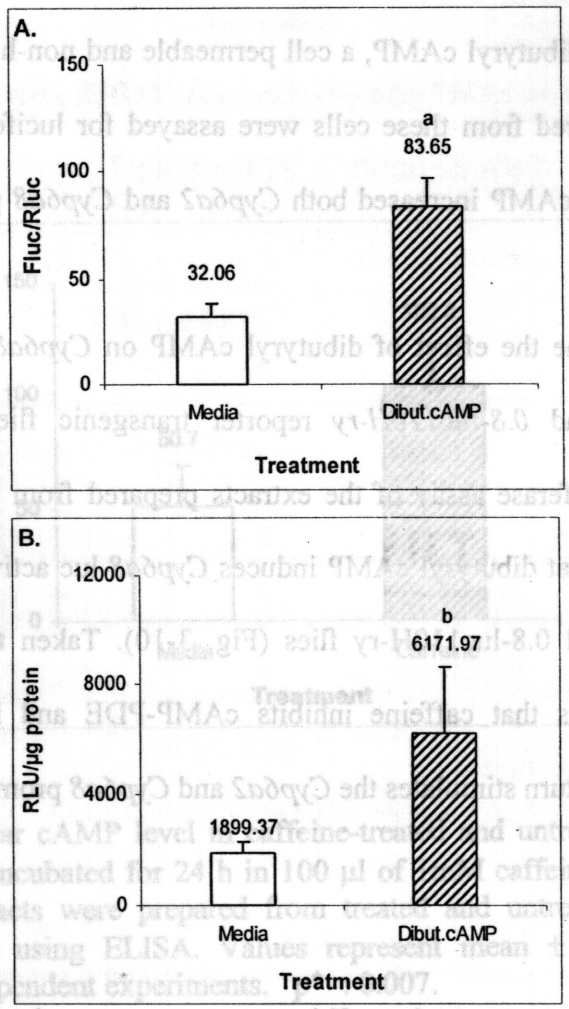


Figure 3-9: Effect of di-butyryl cAMP on *Cyp6a2* and *Cyp6a8* promoter activities in SL-2 cells. SL-2 cells were transfected with 0.9luc-A2 along with the internal control pRL null control plasmid (Panel A) or with *Cyp6a8*-luc (0.2luc-A8 or 0.8luc-A8) reporter plasmids (Panel B). Transfected cells were incubated for 24h in plain or 4mM di-butyryl cAMP containing media. After incubation, extracts were prepared and assayed for firefly (Fluc) luciferase activity and either normalized to Rluc activity (Panel A) or to the total protein quantity (Panel B). Each bar represents mean ± S.D of Fluc to Rluc activity ratio of three independent samples. *p*: a<0.005, and b<0.04.

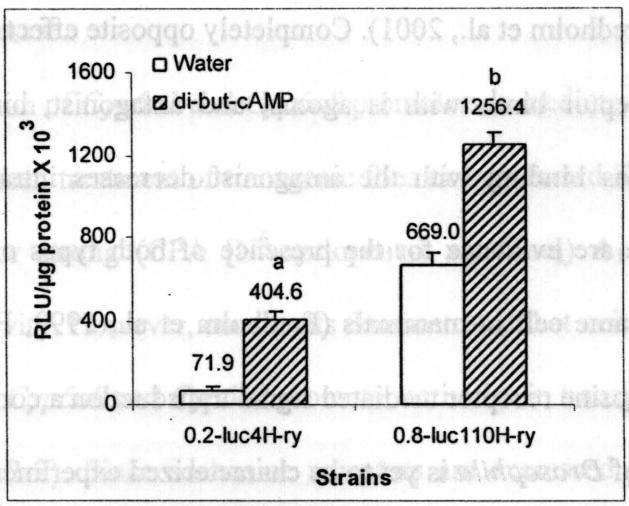


Figure 3-10: Effect of di-butyryl cAMP on *0.2luc-A8* and *0.8luc-A8* reporter transgene activities. *0.2-luc4H-ry* and *0.8-luc110H-ry* flies were treated with 4mM dibutyryl cAMP or water for 24h. Extracts were prepared from these flies, and firefly luciferase activity and protein content were measured. Each bar represents mean luciferase activity/µg protein ± S.D of triplicate samples. *p*: a <0.001, and b < .0003.

the pertussis toxin-sensitive inhibitory G-proteins, G_{i-1}, G_{i-2}, G_{i-3}, G_{o1} and G_{o2}, and activation of A_1 receptor causes inhibition of adenylyl cyclase as expected (Fredholm et al., 2001). On the other hand activation of A_{2A} receptor, which is coupled to stimulatory G protein (G_s), activates adenylyl cyclase (Fredholm et al., 2001). Because of this difference, binding of an agonists e.g., adenosine, to the A1 receptor lowers intracellular cAMP level, whereas binding of an antagonist such as caffeine increases the cAMP level (Fredholm et al., 1999; Fredholm et al., 2001). Completely opposite effect on cAMP level is found when A2A receptor binds with is agonist and antagonist; binding with the agonist increases, whereas binding with the antagonist decreases intracellular cAMP level. Interestingly, there are evidence for the presence of both types of receptor with opposing effects in the same cell in mammals (Fredholm et al., 1999; Fredholm et al., 2001), which makes adenosine receptor mediated signal transduction a complex process.

Adenosine receptor of *Drosophila* is yet to be characterized experimentally, although the presence of a putative adenosine receptor gene (ID: CG9753) has been reported in the *Drosophila* genome database (flybase.bio.indiana.edu), which shows 35% sequence similarity with mammalian A_1 and A_{2A} receptors. Since A_1 and A_{2A} receptors have opposite effect on the adenylyl cyclase enzyme two alternative hypotheses involving the putative adenosine receptors may be proposed to explain caffeine induction of *Cyp6a8* and *Cyp6a2* promoters. If caffeine acts as an antagonist for the stimulatory A_{2A} receptor, then treatment with caffeine would inhibit the A_{2A} receptor and in turn lower the cAMP level because inhibition of A_{2A} leads to a decrease in cAMP level (Fredholm et al., 2001). Since we found induction of the *Cyp6* gene promoters with caffeine treatment but not inhibition, the putative adenosine receptor identified in *Drosophila* may not be

functionally similar to the mammalian A_{2A} if it is assumed that caffeine-induction is mediated by antagonizing this putative receptor. Alternatively, the putative adenosine receptor may be functionally similar to the inhibitory A_1 receptor, which inhibits the adenylyl cyclase and lowers the cAMP level. Then, inhibition of this receptor by caffeine is expected to raise the intracellular cAMP level. It is possible that in *Drosophila* caffeine raises the intracellular cAMP level by inhibiting both the A_1-type receptor and cAMP phosphodiesterase.

If induction of *Cyp6a2* and *Cyp6a8* promoters is mediated *via* the putative inhibitory A_1 receptor, then treatment of transgenic flies or transfected SL-2 cells with intermediate (adenosine) or strong (CPA [N^6-cyclopentyl xanthine]) A_1 agonist should lower the promoter activity. However, our results demonstrated that neither adenosine nor CPA had any effect on *Cyp6a8* and *Cyp6a2* promoter activities in transgenic flies and in SL-2 cells (Fig.3-1 and 3-2). Since adenosine receptors are expressed predominantly in the brain region (Klinger et al., 2002), the effect of adenosine on *Cyp6a8*-luc activity in the heads of *0.8-luc110H-ry* flies was also examined. Again, no effect of adenosine on the *Cyp6a8* promoter activity was found in the heads of these flies (data not shown). These results suggest that either the putative *Drosophila* adenosine receptor is expressed at a very low level in *Drosophila* SL-2 cells or the signal mediated *via* the putative A_1 like adenosine receptors does not alter the *Cyp6a8* promoter activity. A third possibility could be based on the half-life of the reporter LUC enzyme. In all the experiments performed in the present investigation, SL-2 cells were allowed to recover for 24 hours after transfection before they were treated with any chemical. When these cells were assayed at the end of the recovery period, high level of constitutive luciferase activity was found (unpublished

observations). In other words, constitutive level of luciferase enzyme is already high in the transfected cells when 24 h adenosine or CPA treatment was started. If luciferase is a very stable protein with a very long half-life, its level may not change even if adenosine or CPA lowers the activity of the *Cyp6a8* promoters by stimulating the putative adenosine receptor. Conversely, stimulation of the promoter DNA by an antagonist such as caffeine is probably detectable because it would add more luciferase protein to the pool of enzyme already made constitutively. Although in mammalian cells, the half-life of firefly luciferase has been reported to be only 3 h (Thompson et al., 1991), the stability of this enzyme in SL-2 cells or in adult fly is not known. However, we found that in adult flies caffeine-induced luciferase enzyme is quite stable; the enzyme activity remains unchanged even after 24 hours after caffeine is withdrawn from the medium (data not shown). This may also hold true in the case of SL-2 cells.

In view of this, it is difficult to conclude whether lack of effect of adenosine or CPA is due to the absence of inhibitory A_1 type adenosine receptor in SL-2 cells or the effect on the receptor is obscured by the extended half-life of the luciferase enzyme. However, results obtained with the strong A_1 antagonist DPCPX are somewhat more useful to draw a probable conclusion. DPCPX (1,3-dipropyl-8-cyclopentyl xanthine) acts as a strong A_1 antagonist and shows higher affinity for the A_1 receptor than caffeine (Fredholm et al., 2001). Since DPCPX is an antagonist like caffeine, it should act the opposite way compared to that of adenosine or CPA. In other words, it should antagonize with the putative inhibitory A_1-like receptor, increase the intracellular cAMP level and up-regulate the *Cyp6* gene promoters. Theoretically, up-regulation should be detectable because it would add more luciferase enzymes to the pool of constitutively expressed enzyme.

However, no effect of DPCPX was observed on the luciferase activity. Taken together, all these results suggest that the SL-2 cells may not have any putative A_1 receptor. Therefore, induction with caffeine must be fully mediated by inhibiting the cAMP-phosphodiesterase enzyme.

Cyclic nucleotide phosphodiesterases or PDEs form a family of enzymes that are involved in the breakdown of second messengers, cAMP and cGMP. In humans, eleven families of PDEs, each with 1-4 genes, have been reported (Essayan, 2001). Some PDEs are highly specific for hydrolysis of cAMP including PDE4, some are highly cGMP-specific, and some have mixed-specificity (Corbin and Francis, 1999). PDE4, the target for caffeine inactivation has a very high specificity for cAMP (Ye et al., 2001). In *Drosophila*, *dunce* gene has been shown to code for cAMP-specific PDE, which is similar to mammalian PDE4 (Davis and Kiger, 1981). Recently, five additional PDE genes have been identified which are similar to mammalian genes belonging to families PDE1, PDE6, PDE8, PDE9 and PDE11 (Day et al., 2005). In the present study, effect of caffeine, rolipram and theophylline on *Cyp6a8* and *Cyp6a2* promoter activities was examined. Rolipram is a PDE4-selective inhibitor, whereas theophylline and caffeine are nonselective PDE inhibitor (Belibi et al., 2002). The results showed that all three PDE inhibitors induce *Cyp6a2* (Fig.3-5) and *Cyp6a8* (Fig.3-6) promoters.

We also investigated the effect of partial loss of function of PDE4 enzyme on the promoter activity by using dnc^1, a hypomorphic mutant for PDE4. As expected, partial loss of PDE4 activity stimulated the *Cyp6a8* promoter in the heads of dnc^1 flies. In agreement with these observations, we found that caffeine treatment decreases the PDE activity by several fold in flies as well as in SL-2 cells and increases the intracellular

cAMP levels in SL-2 cells. Moreover, treatment of flies and SL-2 cells with exogenous cAMP was found to increase both *Cyp6a8* and *Cyp6a2* promoter activities. Therefore, caffeine-induced expression of the two *Cyp6* gene is mediated by elevating cAMP level and triggering a signaling cascade that culminates in an increased *Cyp* promoter activity. Our next objective is to dissect the downstream events of this cAMP-mediated pathway. In mammals, increased cAMP levels enhance the expression of genes encoding AP-1 transcription factors c-Jun and c-Fos (Xia and Goldstein, 1999). Since there are putative AP-1 sites in the upstream DNA of both *Cyp6a8* and *Cyp6a2* genes (Chapter 2), it may be hypothesized that caffeine induction of *Cyp6a8* and *Cyp6a2* is probably mediated *via* the AP-1 complex made of D-fos and (or) D-jun. This possibility has been examined in the next chapter.

Chapter 4

Analysis of the role of D-fos and D-jun in constitutive and caffeine-induced expression of *Cyp6a8* gene

Introduction

The mechanisms involved in the transduction of environmental cues to stimulate gene transcription are multiple and complex. Cyclic adenosine monophosphate (cAMP)-dependent signaling pathway is one of the major pathways that is used to transduce extracellular signals to the nucleus and elicit alterations in gene transcription (Daniel et al., 1998). A large number of transcription factors have been identified that are modulated by this signaling pathway. A few examples of these transcription factors are the products of two cellular proto-oncogenes - c-Fos and c-Jun, cyclic AMP responsive element binding protein (CREB), the yeast GCN4 protein, and C/EBP enhancer binding protein (Ransone et al., 1990). Of these cAMP responsive transcription factors c-Jun and c-Fos belong to AP-1 or activator protein family. The members of this family are leucine zipper proteins which have a dimerization domain to form hetero or homodimers, and a basic region that allows them to bind with the DNA (Sng et al., 2004). The Jun and Fos proteins binds to the AP1 binding site on the DNA either as a homodimer or as a heterodimer using Fos as a binding partner. The Jun/Fos homodimer or Jun/Fos heterodimer also binds to TPA (12-o-tetradecanoylphorbol-13-acetate) and cAMP response elements known as TRE and CRE, respectively. Interestingly, CREB, a CRE binding protein, also recognizes AP-1 site (Ransone et al., 1990).

In mammals, the Fos and Jun families have many members, whereas in *Drosophila* AP-1 consists of only two proteins, one related to Fos and the other related to Jun family members. These proteins are called Fos- and Jun-related antigens or dFRA and dJRA respectively because they cross-react with the antibodies to mammalian Fos and Jun proteins (Perkins et al., 1990). AP-1 proteins show extensive functional diversities and involvement in various biological phenomena such as bone morphogenesis, cell death, differentiation, tissue remodeling and cancer (Kockel et al., 2001). In *Drosophila*, AP-1 factors, D-fos and D-jun are involved in embryonic dorsal closure, pupal thorax closure, endoderm patterning, wing patterning and eye development (Ciapponi and Bohmann, 2002). Although both proteins work in the same developmental processes, the *D-fos* and *D-jun* genes show difference in their expression patterns. While *D-fos* gene shows differential tissue and temporal expression, the *D-jun* gene is expressed uniformly throughout embryonic development (Xia and Goldstein, 1999).

Activation of cAMP pathway by various extracellular stimuli influences transcription of the *Fos* and *Jun* genes both in mammals and *Drosophila* in different ways. In mammalian cells, activation of cAMP pathway by a variety of external stimuli such as serum, growth factors, cytokines, phorbol esters, and viral infection induces transcription of both *c-fos* and *c-jun* genes transiently. In *Drosophila*, however, cAMP induces *D-fos* but negatively modulates the *D-jun* gene. The activity of the Fos and Jun proteins is also modulated *via* phosphorylation and dephosphorylation (Karin, 1995; Sng et al., 2004). Both in mammals and *Drosophila*, cAMP activates the mitogen-activated protein kinase (MAPK) pathway to phosphorylate Jun and Fos proteins. In *Drosophila*, MAP-kinase pathway phosphorylates D-Fos and D-Jun proteins *via* Jun-N-terminal

kinase (JNK) and Extracellular Regulated Kinase (ERK), respectively (Ciapponi et al., 2001). While phosphorylation increases the metabolic stability of Fos and Jun proteins (Karin, 1995), dephosphorylation makes these proteins highly unstable and shortens the half-life of c-fos to 10 min and c-jun to 60 min (Tsurumi et al., 1995).

Caffeine, a psychostimulant drug, has been shown to increase the levels of c-Fos protein in the rat brain (Bennet and Semba, 1998) and c-fos, c-jun, and junB mRNAs in rat striatum (Svenningsson et al., 1995). Results described in Chapter 3, showed that caffeine increases the intracellular cAMP levels *via* PDE inhibition, and cAMP treatment increases *Cyp6a8* promoter activity. In mammals, cAMP induces *Cyp2A5* and *Cyp11A1* transcription (Viitala et al., 2001; Guo et al., 2003) and activation of cAMP pathway increases transcription of *c-fos* and *c-jun* genes (Sng et al., 2004). Analysis of the upstream DNA sequence of *Cyp6a8* revealed the presence of putative binding sites for AP-1 proteins, Jun and Fos, and for cAMP response element binding protein (CREB)(see Chapter 2). Thus, I wished to investigate whether the *Cyp6a8* promoter activity is influenced by D-fos and D-jun and if caffeine has any effect on these two AP-1 proteins. Evidence showing that D-jun acts a negative regulator of *Cyp6a8* promoter and caffeine reduces D-jun protein levels is presented in this chapter. Two possible mechanisms for down regulation of D-jun expression following caffeine treatment and how they modulate the *Cyp6a8* promoter activity are also proposed.

Material and Methods

Plasmids and reagents for Western blot experiments

D-jun/HS plasmids carrying D-jun cDNA in sense (S) or antisense (AS) orientation under Hsp70 promoter in pCasper plasmid were obtained from Norbert Perrimon, Harvard University. In the present investigation these plasmids were named D-jun(S)/HS and D-jun(AS)/HS, respectively. The D-fos/pBSKS$^+$ and D-jun/pBSK$^-$ carrying full-length D-jun and D-fos cDNA with 6 X His-tag at the C' terminal end were from Dirk Bohmann, University of Rochester. FJF/pCasper-tub plasmid carrying tubulin (Tub) promoter was obtained from Laura Ciapponi, EMBL, Heidelberg. D-Jun (DB118) and D-Fos (DB152) cDNA expression plasmids, anti D-Jun and anti D-fos antibodies were generous gifts from Dirk Bohmann, University of Rochester. The BL21-codon plus cells and horse radish peroxidase (HRP) -conjugated anti-rabbit secondary antibody were obtained from Jeremy Myers and Zu-Wen Sun (Vanderbilt University), respectively.

Construction of D-jun and D-fos sense and antisense expression plasmids

All cDNA constructs used in the present investigation were cloned into pCasper vector carrying heat-shock (Hs) or tubulin (Tub) promoter. Besides D-jun(S)/HS and D-jun(AS)/HS plasmids mentioned above, following six expression plasmids driven by heat-shock or tubulin promoter were constructed and used for the present investigation: D-fos(S)/HS; D-fos(AS)/HS; D-jun(S)/Tub; D-jun(AS)/Tub; D-fos(S)/Tub and D-fos(AS)/Tub. The details of the construction of these plasmids are given in Fig. 4-1 and Fig. 4-2. To construct D-fos(S)/HS and D-fos(AS)/HS plasmids, the unique *Nde*I site located at the 5'-end of the D-fos cDNA in the D-fos/pBSKS$^+$ plasmid was converted to *Xba*I (Fig. 4-1). The reconstructed plasmid was named D-fos/pBSKS$^+$ Xba(Nde).

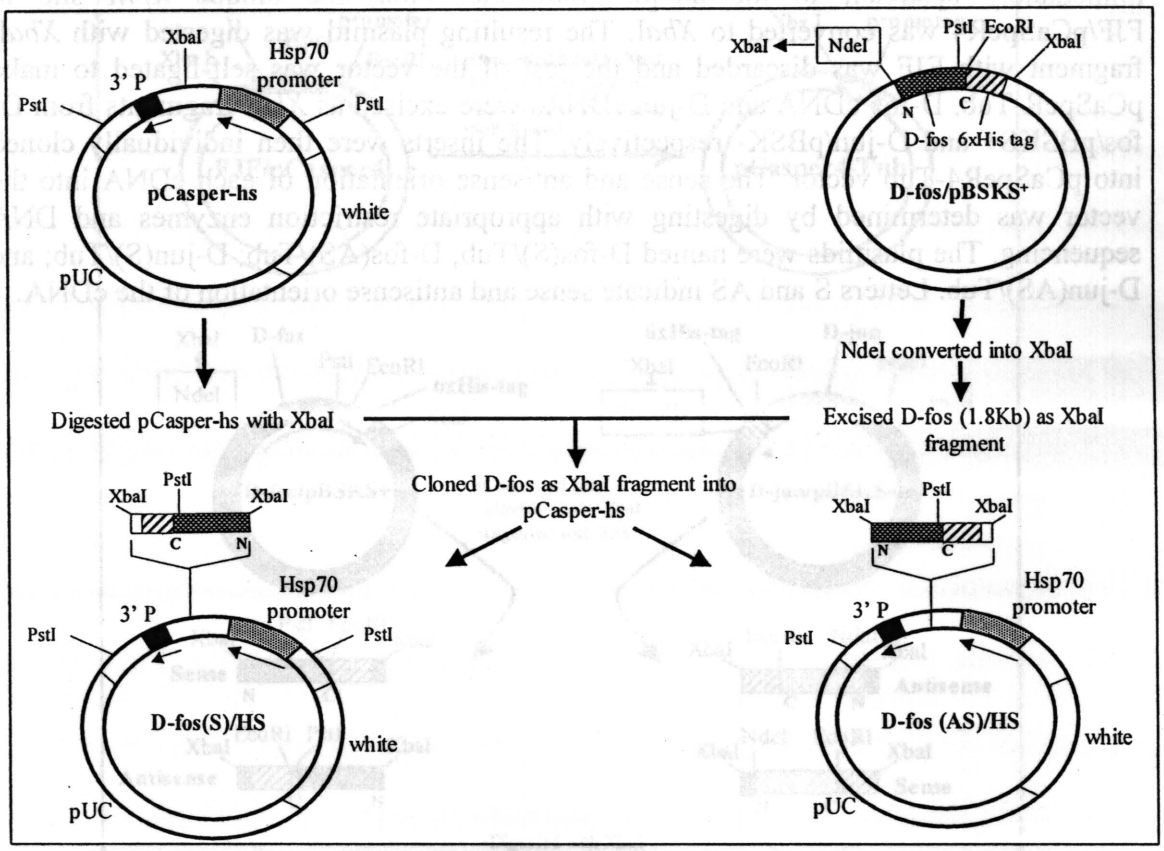
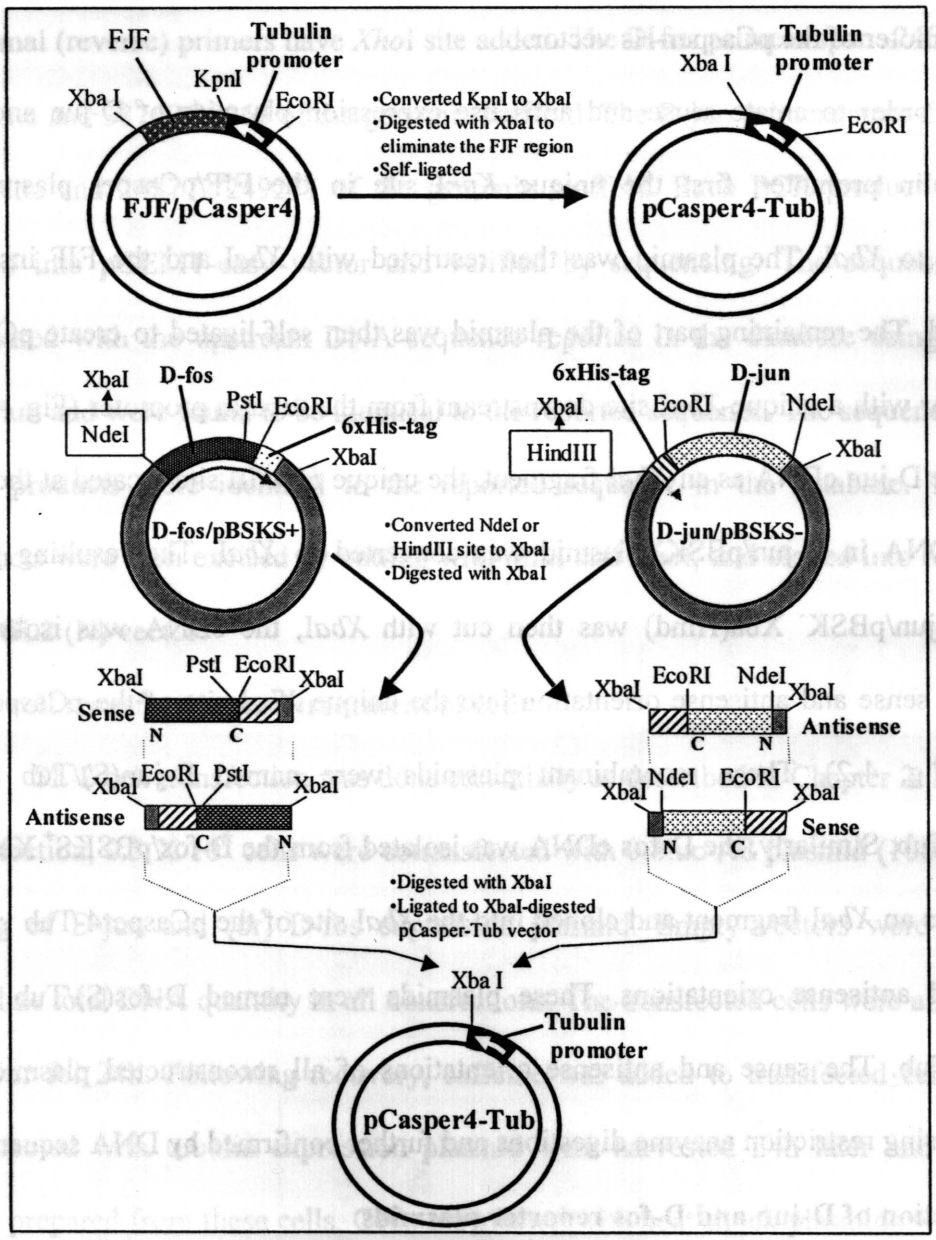


Figure 4-1: Construction of D-fos (S)/HS and D-fos (AS)/HS expression plasmids. In the pCaSpeR-HS vector the unique *XbaI* site is flanked by Hsp70 promoter with TATA element and Hsp70 3'-UTR with poly (A) signal. The D-fos cDNA has a 6xHis tag at the C' terminal end.

Figure 4-2: Construction of D-fos (S)/Tub and D-fos (As)/Tub plasmids. The FJF/pCaSpeR vector has tubulin promoter with TATA box and SV40 poly (A) signal immediately upstream to the unique *Xba*I site. First, the unique *Kpn*I site in FJF/pCaSpeR4 was converted to *Xba*I. The resulting plasmid was digested with *Xba*I, fragment with FJF was discarded and the rest of the vector was self-ligated to make pCaSpeR-Tub. D-fos cDNA and D-jun cDNAs were excised as *Xba*I fragments from D-fos/pBSKS+ and D-jun/pBSK- respectively. The inserts were then individually cloned into pCaSpeR4-Tub vector. The sense and antisense orientation of each cDNA into the vector was determined by digesting with appropriate restriction enzymes and DNA sequencing. The plasmids were named D-fos(S)/Tub; D-fos(AS)/Tub; D-jun(S)/Tub; and D-jun(AS)/Tub. Letters S and AS indicate sense and antisense orientation of the cDNA.


FJF
Tubulin promoter
KpnI
Xba I
EcoRI
FJF/pCasper4
•Converted KpnI to XbaI
•Digested with XbaI to eliminate the FJF region
•Self-ligated
Xba I
Tubulin promoter
EcoRI
pCasper4-Tub
XbaI
D-fos
NdeI
PstI
EcoRI
6xHis-tag
XbaI
D-fos/pBSKS+
6xHis-tag
D-jun
XbaI
EcoRI
NdeI
HindIII
XbaI
D-jun/pBSKS-
•Converted NdeI or HindIII site to XbaI
•Digested with XbaI
XbaI
PstI
EcoRI
XbaI
Sense
N
C
XbaI
EcoRI
NdeI
XbaI
Antisense
C
N
XbaI
EcoRI
PstI
XbaI
Antisense
C
N
XbaI
NdeI
EcoRI
XbaI
Sense
N
C
•Digested with XbaI
•Ligated to XbaI-digested pCasper-Tub vector
Xba I
Tubulin promoter
pCasper4-Tub

The 1.8-kb cDNA of this plasmid was then excised out as an *Xba*I fragment and cloned in sense or antisense orientation into the unique *Xba*I site located downstream from the heat shock promoter of the pCasper-Hs vector.

In order to create sense and antisense expression plasmids of D-jun and D-fos with tubulin promoter, first the unique *Kpn*I site in the FJF/pCasper plasmid was converted to *Xba*I. The plasmid was then restricted with *Xba*I and the FJF insert was eliminated. The remaining part of the plasmid was then self-ligated to create pCasper4-Tub vector with a unique *Xba*I site downstream from the tubulin promoter (Fig. 4-2). To isolate the D-jun cDNA as an *Xba*I fragment, the unique *Hind*III site located at the 5'-end of the cDNA in D-jun/pBSK$^-$ plasmid was converted to *Xba*I. The resulting plasmid called D-jun/pBSK$^-$ Xba(Hind) was then cut with *Xba*I, the cDNA was isolated and cloned in sense and antisense orientation into the unique *Xba*I site of the pCasper4-Tub vector (Fig. 4-2). These recombinant plasmids were named D-jun(S)/Tub and D-jun(AS)/Tub. Similarly, the D-fos cDNA was isolated from the D-fos/pBSKS$^+$ Xba (Nde) plasmid as an *Xba*I fragment and cloned into the *Xba*I site of the pCasper4-Tub vector in sense and antisense orientations. These plasmids were named D-fos(S)/Tub and D-fos(AS)/Tub. The sense and antisense orientations of all reconstructed plasmids were verified using restriction enzyme digestions and further confirmed by DNA sequencing.

Construction of D-jun and D-fos reporter plasmids

The D-fos and D-jun gene structures have been described previously (Wang and Goldstein, 1994; Rousseau and Goldstein, 2001). Based on these reports, PCR primers were designed to amplify D-fos and D-jun upstream DNA using the genomic DNA isolated from *0.8luc110H-ry* females. The sequence of the PCR primers and the

regions of the corresponding genes are shown in Table 4-1. The 5'-end of the distal (forward) primers used for each gene has *Mlu*I site, whereas the 5'-ends of the proximal (reverse) primers have *Xho*I site added. The D-fos primers span -240/+310 of the gene and 38769/39363 of the genomic scaffold. The D-jun primers span -510/+1 of the gene and 248772/249271 of the genomic scaffold. Each PCR product was first cloned into pGEMT-easy vector and verified by sequencing. The sequences were compared with the upstream DNA sequence reported in the database using BLAST program and were found to be identical to the reported sequence. The sequences of the PCR products were identical to the reported sequence in the database. The PCR products were then excised by cutting with *Mlu*I and *Xho*I, and cloned into *Mlu*I/*Xho*I cut pGL2 (N) vector.

SL-2 cell transfection and treatment of cells

SL-2 cell transfection was done essentially as described in Chapter 2. For each transfection, 0.5 X 10^6 cells were cotransfected with 0.8luc-A8 plasmid (100 ng) and 0.5μg of D-jun and (or) D-fos expression plasmid. Empty vectors were used to equalize total DNA quantity in all transfections. The transfected cells were allowed to recover for 24h. Following recovery, caffeine was added to transfected cells. Cells transfected with tubulin expression plasmid were harvested 24h later and extracts were prepared from these cells. Cells transfected with heat shock plasmids were heat-shocked in three 30 min pulses at 37°C as soon as they were transferred to caffeine or control media. The cells were allowed to recover for 30min at 25°C between heat shock pulses. Extracts were prepared following 12h final recovery period.

Table 4-1

Primers used for amplification of *D-fos* and *D-jun* upstream DNAs.

Primer name	Primer sequence (5'→ 3')	Tm
1. D-fosup(-240)	ggacgcgtCTTCTGCATCCCTGGCATCTAC	80.3
2. D-fosup(+310)	ggctcgagGTTGGGAATGTTCTGGCTTTGC	80.5
3. D-junup(-540)	ggacgcgtTCCTTTCCTATTTACCGACGCC	78.7
4. D-junup(+1)	ggctcgagGGGTGGGAACTTTG	73.5

Primers 1 and 2 were used to amplify -240/+310 *D-fos* DNA, and primers 3 and 4 were used to amplify -540/+1 *D-jun* DNA. Extra bases added to each primer are shown in lower case and the added restriction enzyme sites are underlined. Primers 1 and 3 have *Mlu*I, and primers 2 and 4 have *Xho*I sites.

Luciferase assay

Luciferase assays were done as described in the previous chapters. Luciferase activity was normalized to the total protein in the extract as described previously.

Northern blot hybridization

Total RNA was isolated from *0.8luc110H-ry*, 91R and 91C strains. Northern analysis was done with 20 μg total RNA as described in Chapter 2. 1.8kb *Xba*I cDNA fragment of D-fos and 1.2 Kb *Eco*RI cDNA fragment of Djun, excised from D-fos (S)/HS and D-jun(s)/HS, respectively, were used as probes to examine the expression of D-fos and D-jun. RP49 was used as the internal control to correct for loading errors. Hybridization signals were quantified by scanning the blots with a Packard Instant imager.

Bacterial expression of D-fos and D-jun proteins

D-jun and D-fos cDNAs were expressed in bacteria and the extracts prepared from these cells were used as positive controls for western analysis. The D-jun construct, DB118, with a His_6-tag at the c-terminal end and the D-fos construct, DB152, with a His_6-tag at the c-terminal end were used for the expression in bacterial cells. Each of these plasmids (50ng) was used to transform BL (21) codon plus cells using electroporation. Electroporation of cells was done using a gene pulser in 2mM cuvette for 5.2 millisec.

Following transformation, a single colony was inoculated in LB medium supplemented with chloramphenicol and ampicillin (final concentrations of 34 μg/ul and 100 μg/ul, respectively) and was grown over night at 37°C with constant shaking. The following day, the cultures were diluted 10-fold and a 2ml culture was grown at 30°C for

1hr in the absence of antibiotic, until the OD_{600} reached 0.8. An aliquot of uninduced culture was saved in a microcentrifuge tube. To the rest of the culture, 1.5mM IPTG was added and grown at 30°C for 3h. Extracts were prepared from uninduced and induced cells and the protein concentration of these extracts was determined using the Biorad Dc (detergent compatible) protein assay. To 25 µg protein, SDS-sample buffer and 2.5 µl of 6X loading dye was added and electrophoresed on a 12% SDS-PAGE. The gel was then stained with colloidal coomassie stain from Invitrogen for 3h. Gel picture (Fig.4-3) was taken the following day after extensive destaining of the gel for over 12h using water.

Western blot hybridization

Extracts prepared from SL-2 cells transfected with expression plasmids and from whole flies were examined for D-fos and D-jun expression levels. Extracts were prepared with Tris-NP40-NaCl (50mM TrispH 7.8, 150mM Nacl, 1% v/v NP-40) lysis buffer. P8340, a cocktail of protease from Sigma was added just before making the extracts (1µl/100µl cell lysis buffer) and stored at –80°C until use. Protein concentration was determined using D_C protein assay kit from Bio-Rad, Hercules, California. SL-2 cell extracts with equivalent amounts of total proteins were fractionated on SDS-polyacrylamide (12%) and transferred to the polyvinylidene diflouride (PVDF) membrane using semi-dry transfer apparatus. Following transfer, membranes were blocked in TBS+5% non-fat milk at 4°C for 12h with continuous shaking. The membranes were then incubated with D-jun primary antibody (1/5000 dilution) and D-fos primary antibody (1/500) for 1h with rocking at room temperature. The membranes were washed with TBST (TBS + 0.1% Tween-20) for two times, 15min each, with rocking

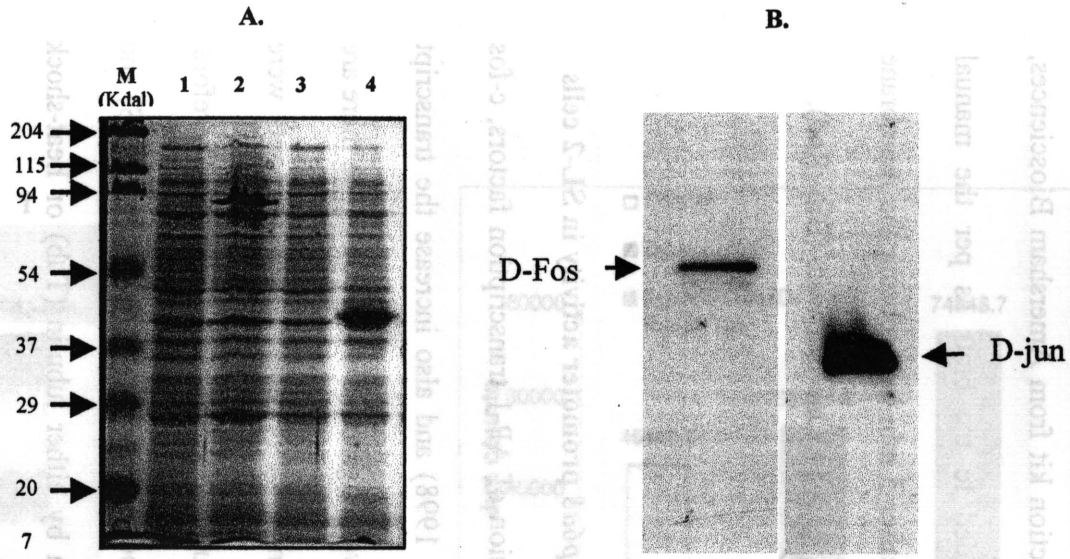


Figure 4-3: D-fos and D-jun expression in *E. coli*. Plasmids carrying full-length D-FOS (DB152) and D-JUN (DB118) cDNA were used to transform BL (21)-codon plus *E. coli*. The transformed bacteria were induced by IPTG to express D-FOS and D-JUN proteins as described in Materials and Methods. Panel A: Crude extracts prepared from induced and uninduced bacteria were electrophoresed on a 12% SDS-PAGE and stained with colloidal stain from Invitrogen. Full length D-fos (lane #2) (~90Kdal) and full length D-jun (lane # 4) (~40Kdal) are shown with dots. Lanes 1 and 3 had extracts from uninduced bacterial cells transformed with full-length D-FOS and D-JUN cDNA clones, respectively. M represents molecular weight marker (Kdal). Panel B: Western blot analysis. Crude bacterial extracts were fractionated on SDS-PAGE, blotted onto Nylon membrane and probed with α-Djun (1/5000) and α-Dfos (1/1000) antibodies. Protein from uninduced bacterial cells was also probed as negative controls and no non-specific bands were detected was detected in negative control lanes (not shown).

following primary antibody incubation. The secondary horseradish peroxidase-conjugated anti-rabbit antibody (1/7500 dilution) was then added to the membranes and incubated for 1h at room temperature. Following secondary antibody incubation, membranes were again washed with TBST for two times, 15 min each. Chemiluminesence was obtained using ECL detection kit from Amersham Biosciences, Piscataway, New Jersey. ECL reagent was added to the blots as per the manual instructions and chemiluminesence was detected after 5 min by exposing the membrane to an X-ray film. The blots were quantified using 'Quantity One' software (Bio- Rad).

Results

Effect of D-jun and D-fos overexpression on *Cyp6a8* promoter activity in SL-2 cells

Caffeine is shown to increase the expression of AP-1 transcription factors, c-fos and c-Jun, in the rat brain (Bennet and Semba, 1998) and also increase the transcript levels for c-fos, c-jun, and junB in rat striatum (Svenningsson et al., 1995). Since there are putative AP-1 binding sites in *Cyp6a8* upstream DNA (Chapter 2), experiments were undertaken to test whether the AP-1 proteins would induce *Cyp6a8* promoter. Therefore, SL-2 cells were cotransfected with 0.8luc-A8 reporter construct and D-jun sense (S) and (or) D-fos sense (S) expression plasmids driven by either tubulin (Tub) or heat-shock (HS) promoter. Extracts were prepared from these transfected cells and examined for D-jun and D-fos expression. Results (Fig 4-4) showed that cells transfected with D-jun(S) plasmid have higher level of D-jun protein compared to the cells transfected with 0.8-lucA8 plasmid alone. The level of D-jun protein was found to be much higher when SL-2

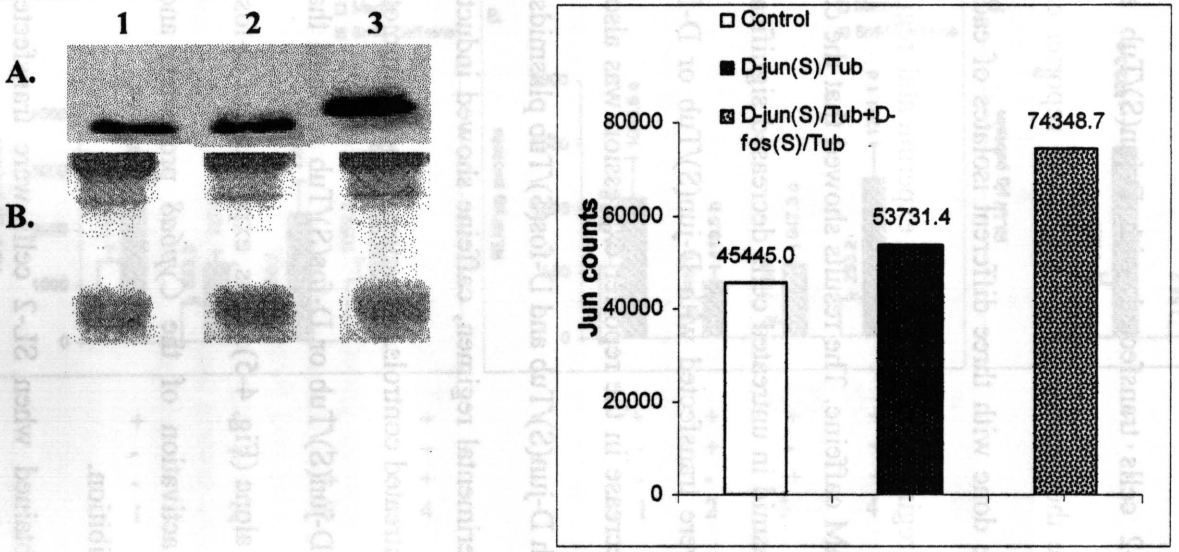


Figure 4-4: Western blot analysis of D-jun expression in SL-2 cells transfected with D-jun(S)/Tub alone or D-jun(S)/Tub and D-fos(S)/Tub plasmids together. Cell extracts were fractionated on SDS-PAGE and blotted onto PVDF membrane, which was first stained with Ponceau (panel B) and then probed with anti D-Jun antibody (panel A) as described in Fig. 4-3. Lanes: 1, pCaSpeR/Tub empty vector; 2, D-jun(S)/Tub; 3, D-jun(S)/Tub + D-fos(S)/Tub. In all transfections, equal amount of 0.8luc-A8 plasmid was used. Panel C: Quantitative analysis of the Western blot shown in panel A. Blots were analyzed by using a Bio-Rad "Quantity one" software program.

cells were transfected with both D-jun(S) and D-fos(S) plasmids (Fig. 4-4). Level of D-fos protein could not be determined because the anti-fos antibody failed to react with the cell extract although strong reaction was observed when D-fos was over-expressed in BL-21 codon plus bacterial cells shown in Fig. 4-3 (Materials and Methods).

Extracts prepared from SL-2 cells transfected with D-jun(S)/Tub and/or D-fos(S)/Tub expression plasmids were then examined for 0.8-lucA8 reporter expression. Three independent experiments were done with three different isolates of each plasmid following four (A-D) experimental regimen. Cells in each experimental regimen were either treated or not treated with 8 mM caffeine. The results showed that the constitutive expression of the 0.8-luc reporter plasmid in untreated cells decreased significantly in all three experiments when the cells were transfected with D-jun(S)/Tub or D-fos(S)/Tub plasmid (Fig. 4-5, A vs B or C). Decrease in the reporter expression was also observed when cells were transfected with both D-jun(S)/Tub and D-fos(S)/Tub plasmids (Fig. 4-5, regimen 4). Interestingly, in all experimental regimen, caffeine showed induction of the *Cyp6a8* promoter compared to the untreated controls. However, *Cyp6a8* promoter activity was lower in cells transfected with D-jun(S)/Tub or D-fos(S)/Tub plasmid than in cells transfected with the reporter plasmid alone (Fig. 4-5). Thus, expression of D-fos and/or D-jun causes inhibition rather than activation of the *Cyp6a8* promoter, and caffeine treatment somehow prevents this inhibition.

Comparable results were obtained when SL-2 cells were transfected with D-jun(S)/HS and D-fos(S)/HS plasmids carrying D-jun and D-fos sense cDNA under heat shock promoter (Fig. 4-6). When D-jun(S)/HS and D-fos(S)/HS cDNAs were expressed alone or together in SL-2 cells, 0.8-lucA8 reporter gene activity was downregulated.

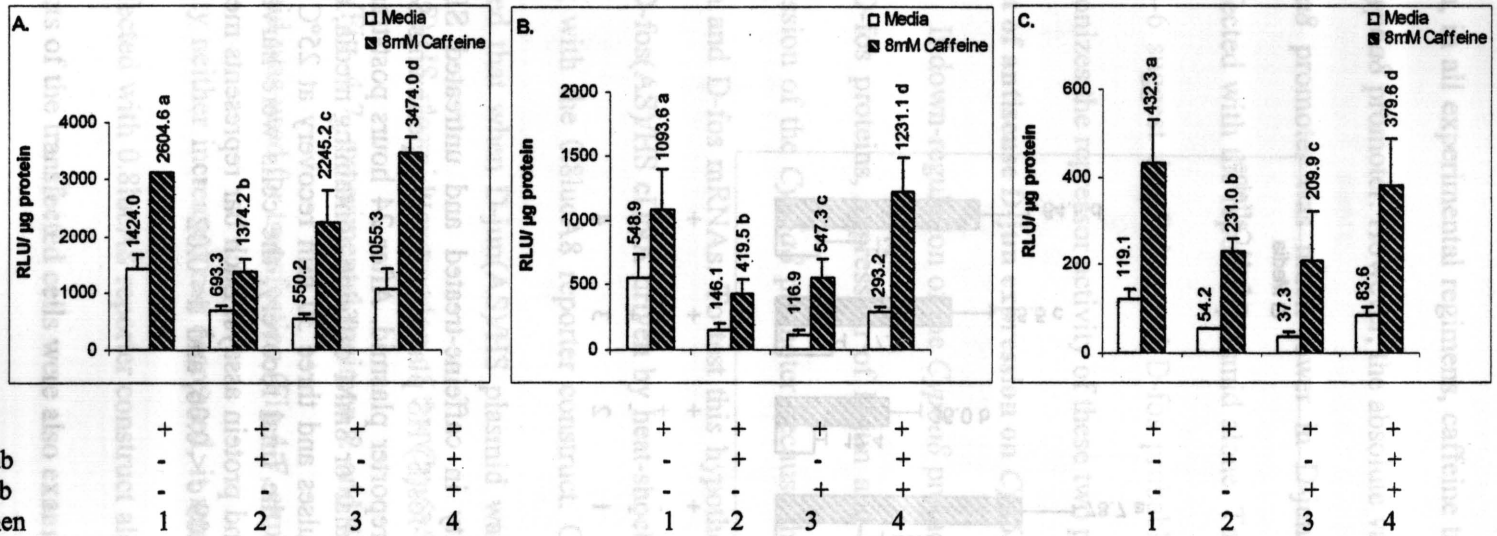


Figure 4-5: *Cyp6a8* promoter activity in presence or absence of caffeine in SL-2 cells transfected with D-jun(S)/Tub and/or D-fos(S)/Tub plasmids. Panels A-C show three independent experiments done with three different isolates of each plasmid and different batches of SL-2 cells. Four different experimental regimens (1-4) were used. However, in all plans, cells were transfected with the 0.8luc-A8 reporter plasmid. Following transfection with the plasmids indicated, the cells were allowed to recover for 24 hours and then they were treated with 8 mM caffeine for 24 hours. Each bar shows the mean ± S.D of three transfections. Control, open bars; treated, hatched bars. *p*: a <0.05 , b < 0.07, c <0.01 and d <0.01

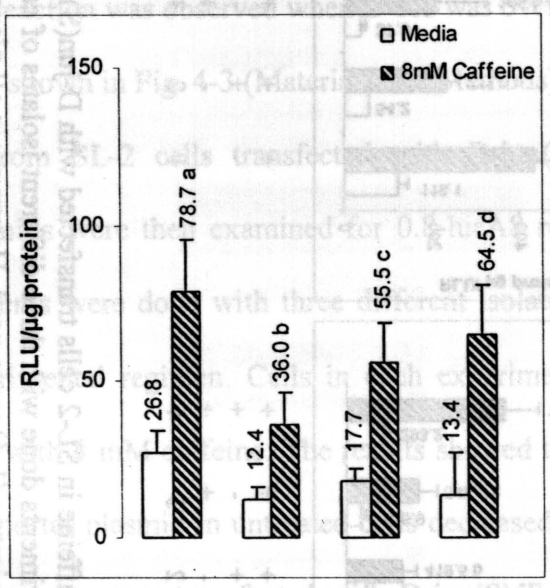


Figure 4-6: *Cyp6a8* promoter activity in caffeine-treated and untreated SL-2 cells transfected with D-jun(S)/HS and/or D-fos(S)/HS plasmids. In all experiments SL-2 cells were also transfected with 0.8luc-A8 reporter plasmid. After 24 hours post-transfection recovery, cells were transferred to regular or 8mM caffeine containing media, and heat-shocked at 37°C with three 30 min pulses and three 30 min recovery at 25°C between heat-shock pulses. Twelve hours after the final recovery, the cells were harvested and extracts were prepared for luciferase and protein assays. Each bar represents mean ± S.D of three transfections. *p*: a< 0.01 b< 0.09 c < 0.06, and d< 0.02

Again, in all experimental regimens, caffeine treatment induced the reporter gene activity *via Cyp6a8* promoter. However, the absolute values for caffeine-induced expression of the *Cyp6a8* promoter was much lower in D-jun(s)/HS transfected cells than in the cells transfected with the reporter plasmid alone. Taken together, the result shown in Figs. 4-5 and 4-6 suggest that D-jun and D-fos probably act as repressors and caffeine somehow antagonizes the repressor activity of these two proteins.

Effect of antisense D-jun expression on *Cyp6a8* promoter activity

If down-regulation of the *Cyp6a8* promoter was due to the overexpression of D-jun and D-fos proteins, expression of D-jun and D-fos antisense plasmids should stimulate expression of the *Cyp6a8* promoter because they would inhibit translation of the cellular D-jun and D-fos mRNAs. To test this hypothesis, antisense constructs of D-jun(AS)/HS and D-fos(AS)/HS cDNA driven by heat-shock promoter were introduced into SL-2 cells along with the 0.8lucA8 reporter construct. Consistent with this hypothesis, the results showed that when D-jun(AS)/HS plasmid was expressed alone or together with the D-fos(AS)/HS plasmid, expression of the *Cyp6a8* promoter increased in the untreated SL-2 cells (Fig. 4-7, experimental regimen 2 and 4). On the other hand, when D-fos(AS)/HS plasmid was used for transfection (Fig 4-7, experimental regimen 3), the *Cyp6a8* promoter activity neither increased nor decreased, it remained same as that observed in SL-2 cells transfected with 0.8lucA8 reporter construct alone (Fig 4-7, experimental regimen 1). The extracts of the transfected cells were also examined for D-jun protein level by western blot analysis. The results (Fig. 4-8) showed that transfection with D-jun(AS)/HS plasmid reduces the D-jun protein level. Taken together, these results suggest that there is an inverse correlation between D-jun level and *Cyp6a8* promoter activity.

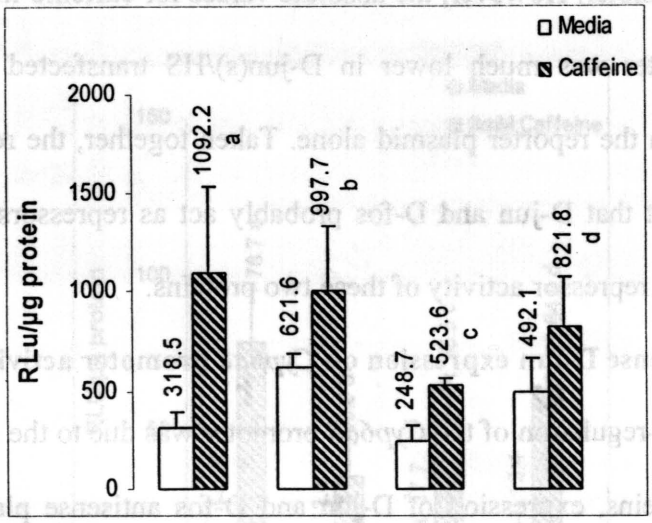


Figure 4-7: Effect of D-jun and D-fos antisense expression on constitutive and caffeine-induced *Cyp6a8* promoter activity in SL-2 cells. D-jun(AS)/HS and/or D-fos(AS)/HS expression plasmids were cotransfected with 0.8luc-A8 reporter plasmid into SL-2 cells. Cells were heat shocked and caffeine-treated as described in Fig. 4-6. Extracts were prepared from 8mM caffeine-treated and untreated cells, and luciferase activity and protein concentration were determined as described in the Methods. Each bar represents mean ± S.D of three transfections. p: $a < 0.08$, $b < 0.1$, $c < 0.005$, and $d < 0.1$.

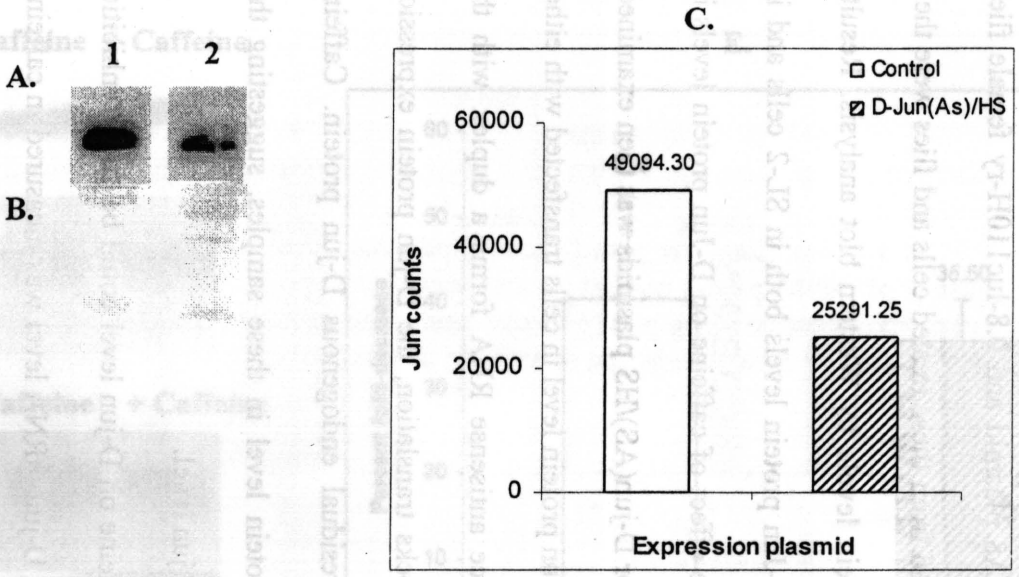


Figure 4-8: Effect of D-jun (AS)/HS antisense expression on D-Jun protein level in SL-2 cells. D-jun(AS)/HS was cotransfected with 0.8luc-A8 reporter plasmid into SL-2 cells. Extracts prepared from cells transfected with empty vector and with D-jun antisense plasmid were examined for D-JUN protein level by Western blot analysis as described. Panel A shows the Western blot. Lanes: 1, empty vector; 2, D-jun(AS)/HS. Panel B shows Ponceau stained blot. Panel C: quantitative analysis of the Western blot shown in panel A. Blots were analyzed using Bio-Rad "Quantity one" software program.

Effect of caffeine on *D-jun* expression

Since caffeine induces *Cyp6a8* promoter activity and down-regulates D-jun levels, it is tempting to postulate that the action of caffeine on promoter activity may be mediated *via* modulation of D-jun; caffeine may inhibit D-Jun protein level or *D-jun* gene transcription. To examine whether caffeine decreases the endogenous D-Jun protein levels, SL-2 cells transfected with 0.8luc-A8 plasmid and 0.8-luc110H-ry female flies were treated with caffeine. Extracts prepared from these treated cells and flies were then examined for the endogenous D-jun protein levels by Western blot analysis. Results showed that caffeine treatment reduces D-Jun protein levels both in SL-2 cells and in adult females (Fig. 4-9). Subsequently, the effect of caffeine on D-Jun protein levels in SL-2 cells transfected with D-jun(S)/Tub or D-jun(AS)/HS plasmids was then examined. Results showed that caffeine decreases D-jun protein level in cells transfected with either plasmids (Fig. 4-10 and Fig. 4-11). Since antisense RNA forms a duplex with the complementary mRNA sequence and blocks translation, the D-jun protein expression observed in this lane is probably the residual endogenous D-jun protein. Caffeine treatment further decreases the D-jun protein level in these samples, suggesting that caffeine down-regulates the endogenous D-jun level.

The down-regulatory effect of caffeine on D-jun level could be due to inhibition of *D-jun* transcription. To investigate this, D-jun mRNA level was measured in caffeine-treated *0.8luc110H-ry*, 91-R and 91-C strains, as *Cyp6a2* and *Cyp6a8* are induced by caffeine in all these strains (Chapter 2). Results showed that caffeine treatment does not affect steady state D-jun and D-fos mRNA levels significantly in all three strains examined (Fig. 4-12, panels A and B). Although a slight decrease in the D-jun transcript

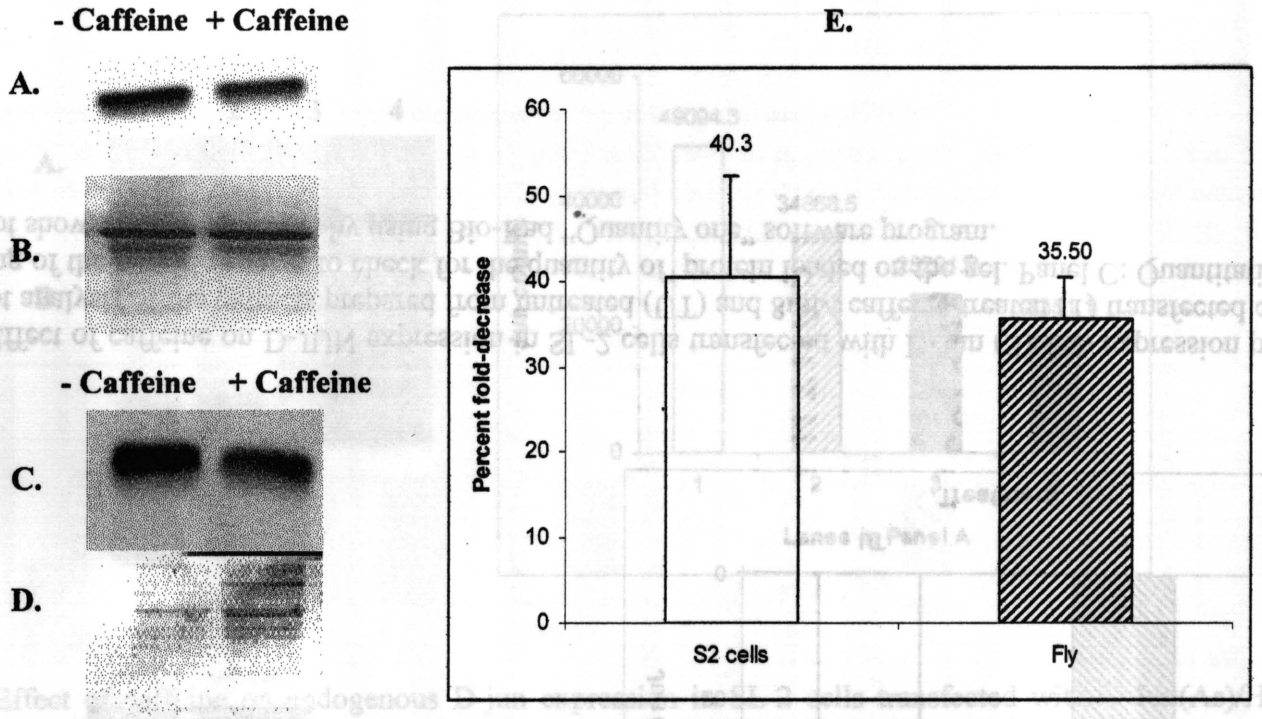


Figure 4-9: Effect of caffeine on the endogenous D-JUN protein level in SL-2 cells (Panel A and B) and in *0.8-luc110H-ry* female flies (Panel C and D). Panel-A: Western blot of the extracts of untreated (- caff.) and 24h 8mM caffeine-treated (+ caff.) SL-2 cells. Panel-C: Western blot of the extracts from *0.8-luc110H-ry* female flies treated with water (- caff.) or with 16mM caffeine (+ caff.) for 24h. Amounts of protein on blots shown in panels A and D were checked by Ponceau staining (panels C and D). Quantitative analysis of the Western blots were done by using Bio-Rad "Quantity one" software program and the results are shown in panel E. Each bar represents the mean percent decrease ± S.D relative to the untreated controls.

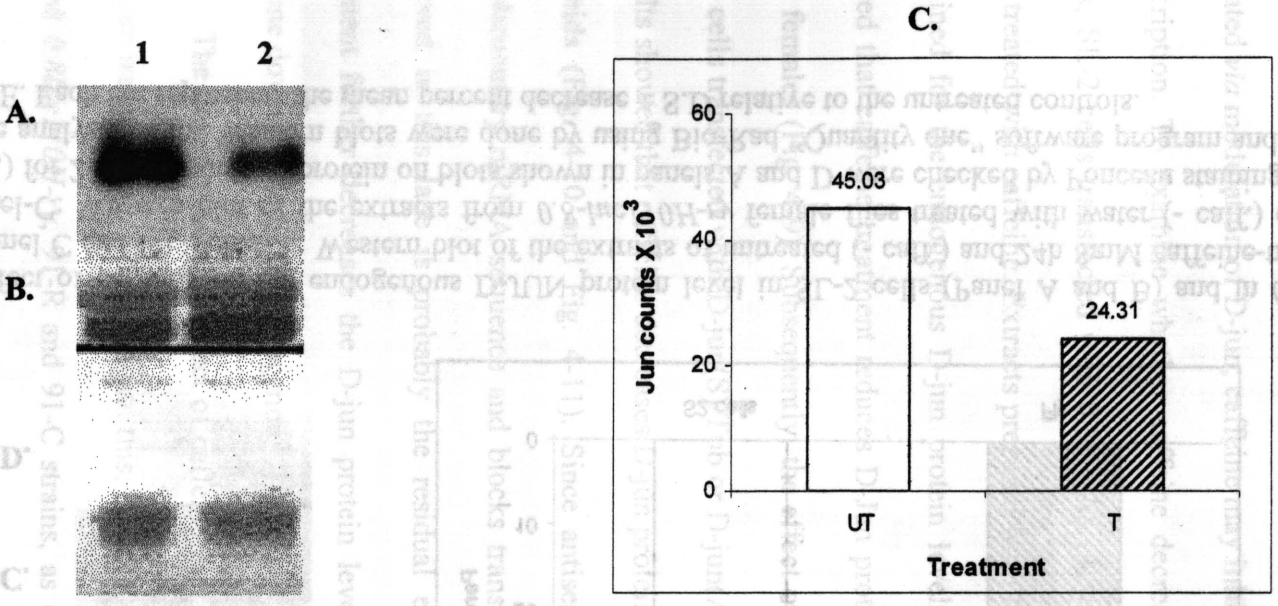


Figure 4-10: Effect of caffeine on D-JUN expression in SL-2 cells transfected with D-Jun (S)/Tub expression plasmid. Panel A: Western blot analysis of the extracts prepared from untreated (UT) and 8mM caffeine-treated (T) transfected cells. Panel B: Ponceau staining of the blot in panel A to check for the quantity of protein loaded on the gel. Panel C: Quantitative analysis of the Western blot shown in panel A done by using Bio-Rad "Quantity one" software program.

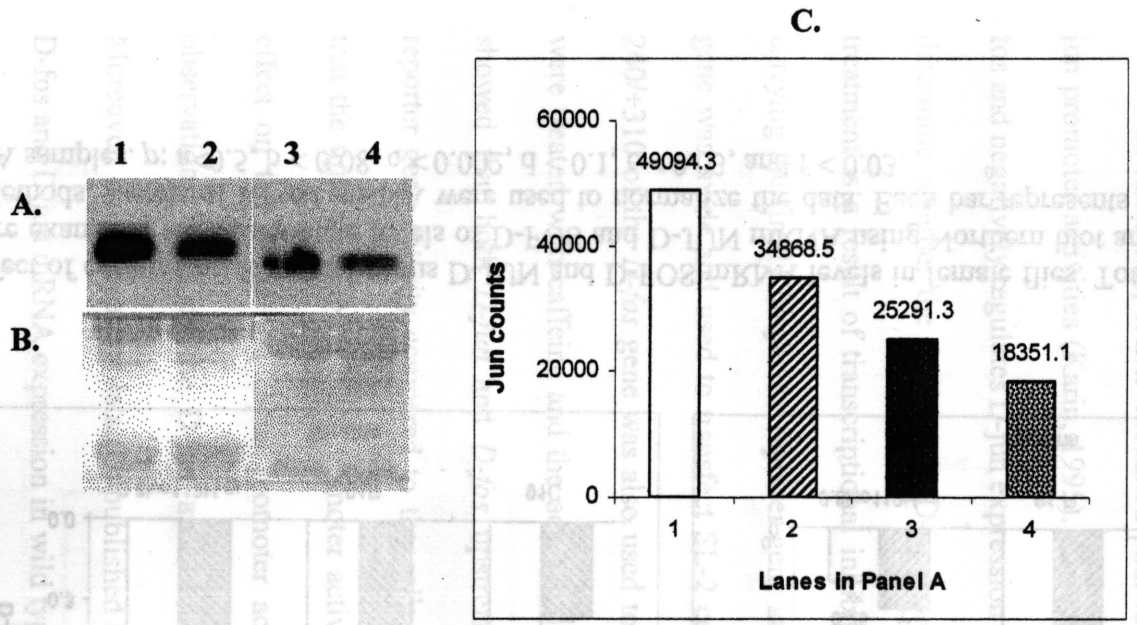


Figure 4-11: Effect of caffeine on endogenous D-jun expression in SL-2 cells transfected with D-Jun(As)/HS plasmid or empty vector. Panel A: Western blot analysis of the extracts prepared from SL-2 cells transfected with empty vector (lanes 1 and 2) or D-jun(AS)/HS plasmid (lanes 3 and 4). Transfected cells were incubated for 24 h in plain (lanes 1 and 3) or 8mM caffeine containing media (lanes 2 and 4). Panel B: Ponceau staining of the blot shown in panel A. Quantitative analysis of the blot shown in panel A was done using Bio-Rad "Quantity one" software program (panel C).

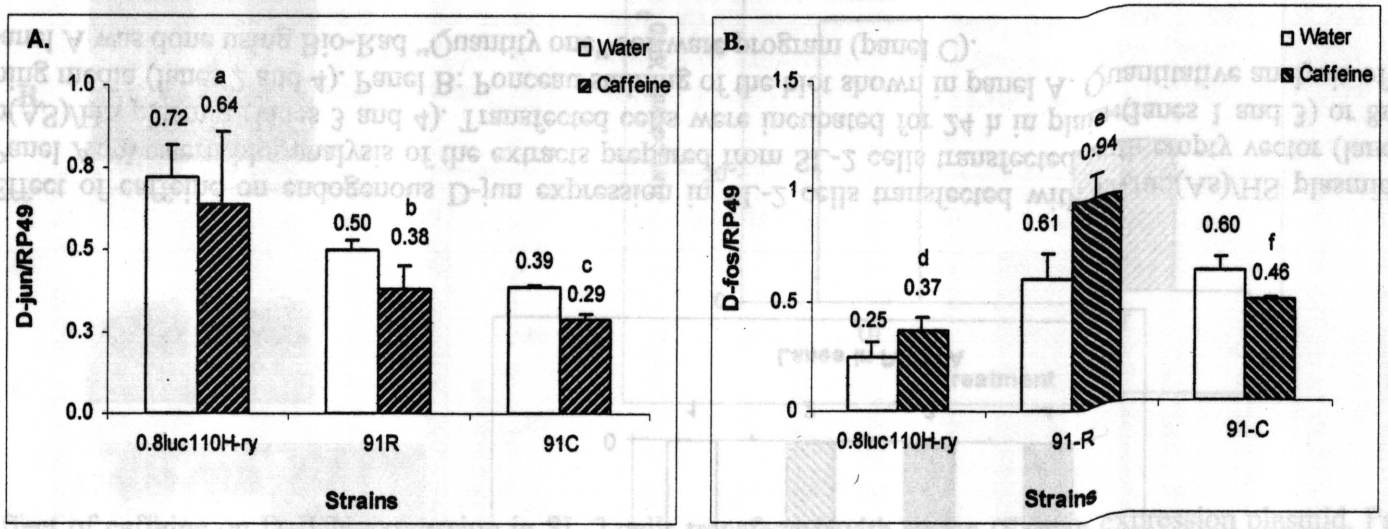


Figure 4-12: Effect of caffeine on the endogenous D-JUN and D-FOS mRNA levels in female flies. Total RNA isolated from females flies were examined for steady-state levels of D-FOS and D-JUN mRNA using Northern blot analysis as described in Materials and Methods. Levels of RP-49 mRNA were used to normalize the data. Each bar represents mean ± S.D. of three independent RNA samples. *p*: a<0.5, b < 0.08, c < 0.002, d < 0.1, e < 0.03, and f < 0.03.

level was observed in these strains, it was not statistically significant. Similarly, ~1.5-fold increase in D-fos RNA level in 91-R strain was not statistically significant (Fig. 4-12, panel B). Thus, once can conclude that the caffeine induction of *Cyp6a8* gene is not mediated *via* modulation of D-fos and D-jun mRNA levels.

In mammals, cAMP mediated pathway has been shown to increase c-fos and c-jun promoter activities (Karin, 1995). Also, the cAMP signaling positively regulates D-fos and negatively regulates D-jun expression in SL-2 cells (Xia and Goldstein, 1999). To determine whether the decrease in D-jun protein level in SL-2 cells following caffeine treatment is a result of transcriptional inhibition of the *D-jun* gene, a reporter plasmid carrying the firefly *luciferase* reporter gene attached to -510/+1 upstream DNA of *D-jun* gene was made and used to transfect SL-2 cells. A D-fos reporter plasmid made with -240/+310 of the *D-fos* gene was also used to transfect SL-2 cells. The transfected cells were treated with caffeine and the activity of the reporter gene was measured. Results showed that both *D-jun* and *D-fos* upstream DNA gave significantly high level of reporter gene activity compared to the cells transfected with empty vector, suggesting that the upstream DNAs have promoter activity (Fig. 4-13). However, caffeine had no effect on the *D-fos* and *D-jun* promoter activities in the SL-2 cells, supporting the observations made on the D-jun and D-fos mRNA levels in caffeine-treated flies. Moreover, the microarray data (Unpublished data) showed that caffeine has no effect on D-fos and D-jun mRNA expression in wild type Canton-S flies. Thus, the results strongly suggest that caffeine-mediated induction of *Cyp6a8* is probably not mediated *via* transcriptional regulation of the *D-fos* and *D-jun* genes in adult flies or in SL-2 cells. The

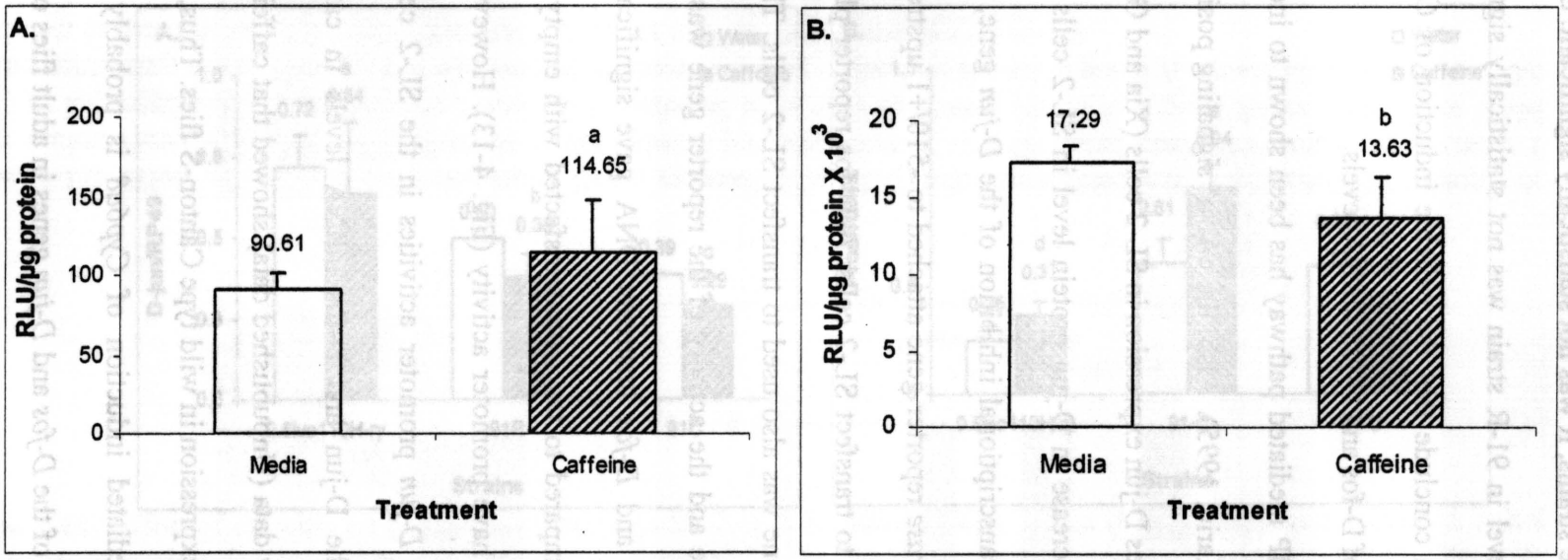


Figure 4-13: Effect of caffeine on the promoter activity of Dfos-up/pGL2 (N) and Djun-up/pGL2 (N) reporter plasmids in SL-2 cells. Transfected cells were incubated for 24 h in plain (control) or 8mM caffeine containing media. Extracts prepared from these cells were assayed for luciferase activity and protein content. Values represent mean ± S.D. of three transfections. [a]p:0.03, [b]p: 0.7

decrease in D-jun protein level observed in caffeine-treated cells and flies may be an effect of caffeine at the translational or post-translational level.

Discussion

Data presented in Chapter 3, showed that *Cyp6a8* gene of *Drosophila* is induced by caffeine *via* the cAMP-signaling pathway. It is known that cAMP stimulates various target genes including *c-jun* and *c-fos*, which are members of AP-1 family of transcription factors (Daniel et al., 1998; Sng et al., 2004). In Chapter 2, results showed that the upstream DNA of *Cyp6a8* gene has multiple AP-1 binding sites. Therefore, induction of *Cyp6a8* gene by caffeine may be mediated *via* upregulation of the *D-fos* and *D-jun* genes through cAMP pathway. In fact, caffeine has been shown to upregulate c-fos and c-jun mRNA levels in rat striatum (Svenningsson et al., 1995). If a similar mechanism is also operating in *Drosophila*, one would expect that overexpression of the D-fos and D-jun genes would induce the *Cyp6a8* promoter. Surprisingly, opposite results were observed; D-fos and D-jun proteins turned out to be negative modulators rather than positive ones. Thus, overexpression of these two AP-1 proteins in SL-2 cells caused downregulation of the *Cyp6a8* gene.

Although AP-1 proteins are known for their stimulatory function, there are reports which show that these factors may act as repressors for many genes, e.g., *c-fos*, *adipocyte P2*, human *insulin* gene, human aromatase gene, etc (Sassone-Corsi et al., 1988; Inagaki et al., 1992; Yubero et al., 1998; Ghosh et al., 2005). Transient expression of c-jun plasmid in JEG-3 cells has been shown to repress CGα and CGβ genes encoding

human chorionic gonadotropin hormone (Pestell et al., 1994; Johnson and Jameson, 2000). When the dimerization domain of c-jun protein was mutated, the repressor activity of the c-jun was abolished and the promoters of the CGαand CGβ genes were stimulated (Pestell et al., 1994). This suggests that c-jun represses the promoters of these two genes either by forming homodimers or by heterodimerizing with other factors. In case of CGβ gene, coexpression of c-jun and ETSL-2 protein belonging to ETS family of transcription factors blocks c-jun mediated transcription repression mediated *via* CRE site (Johnson and Jameson, 2000). Overexpression of c-jun has also been shown to inhibit the basal and cAMP induced *uncoupling protein 1(ucp-1)* gene expression in differentiated human brown adipocytes (Yubero et al., 1998). Repression in this case was blocked when CRE binding protein (CREB) was overexpressed in these cells, suggesting that CREB and c-jun compete for binding to the same cis-element on the *ucp-1* gene promoter. In the present investigation, results show that D-jun of *Drosophila* could also be inhibitory for transcription if it is overexpressed in the SL-2 cells. Conversely, if the level of D-jun was reduced, upon expressing the antisense D-jun plasmid, the *Cyp6a8* promoter was stimulated.

Transfection of SL-2 cells with D-fos(S)/TUB sense plasmid also caused downregulation of the *Cyp6a8* promoter (Fig.4-5). Inhibitory effect of c-fos on transcription has also been reported for many mammalian genes including muscle creatine kinase enhancer (Li et al., 1992), phosphoenolpyruvate carboxykinase gene (Gurney et al., 1992) and the gonadotropin-releasing hormone gene (Bruder et al., 1996). Recently, effect of c-jun and c-fos overexpression has been examined on the human *CYP2J2* gene promoter in HepG2 cells (Marden et al., 2003). The results of this study

showed that when HepG2 cells were transfected with c-jun expression plasmid, *CYP2J2* promoter was highly stimulated. However, this stimulation was abrogated when the cells were co-transfected with both c-Jun and c-Fos plasmids, suggesting that c-fos protein acts as a negative modulator of the *CYP2J2* gene. It was also shown that the *CYP2J2* promoter is highly active under normal condition but it is inhibited when the oxygen level becomes low (hypoxic). Interestingly, while the c-jun level remains moderate both at normal and hypoxic conditions, the c-fos level increases from undetectable level under normal condition to a very high level in hypoxic condition. Based on these findings and the cell transfection experiments it was proposed that the c-Jun/cFos heterodimer is inhibitory for *CYP2J2* promoter activity (Marden et al., 2003).

The above examples of c-jun and c-fos studies on various genes show that both these AP-1 proteins could be inhibitory or stimulatory for transcription and these effects vary from gene to gene. The AP-1 proteins may act as homodimers or heterodimers in mammals as well as in *Drosophila* (Gurney et al., 1992). In the present investigation constitutive activity of the *Cyp6a8* promoter was downregulated when D-jun or D-fos plasmid was expressed alone (Fig. 4-5), suggesting that these proteins are actually inhibitory rather than stimulatory for *Cyp6a8* transcription. When D-jun or D-fos plasmid is expressed alone in SL-2 cells, the level of these AP-1 proteins becomes higher than the steady-state condition. Overexpression may form homodimers of D-fos or D-jun, which in turn may downregulate the activity of the *Cyp6a8* promoter. If this was the case, co-expression of D-jun and D-fos plasmids would give higher promoter activity compared to that observed with overexpression of D-jun or D-fos alone. Fig. 4-5 shows that constitutive activity of the *Cyp6a8* promoter in untreated SL-2 cells indeed increased

when both expression plasmids were used for transfection. However, the promoter activity did not exceed the basal level found in cells transfected with empty vectors. This observation reiterates that D-jun/D-fos heterodimer keeps the promoter activity at a low steady-state basal level, which is expected to be downregulated when stoichiometry of these proteins shifts in favor of homodimer formation.

Conversely, condition that reduces the levels of D-jun and/or D-fos protein should increase the promoter activity above the basal level. This was observed when antisense constructs of D-jun and D-fos were used (Fig. 4-6), although the effect of D-Fos(AS)/HS was not conclusive. However, the promoter activity increased above the basal level when both antisense plasmids were used. Stimulation of the *Cyp6a8* promoter was also found when SL2 cells or *0.8-luc110 (H-ry)* transgenic flies were treated with caffeine (Fig. 4-5 and Chapter 2). Western blot analysis showed that the D-jun level in the treated cells and flies decreased by about 40-50% relative to the untreated samples (Fig. 4-9). This downregulatory effect is not mediated *via* RNA level because caffeine does not have any effect on the D-jun or D-fos gene transcription (Fig. 4-12 and Fig. 4-13). Since anti-D-fos antibody was not reactive to the cellular or fly extract, the level of this protein could not be measured. Nevertheless, reduction in D-jun level and concomitant increase in *Cyp6a8* promoter activity further supports the idea that D-jun is inhibitory rather than stimulatory for *Cyp6a8* promoter activity. Taken together, these data suggest that caffeine-induced expression of *Cyp6a8* gene is mediated *via* D-jun and D-fos proteins. It may be postulated that D-jun/D-fos homodimer or heterodimer downregulates *Cyp6a8* transcription, and caffeine may affect the half-life of these proteins. Caffeine may also

promote covalent modification of AP-1 proteins indirectly and thereby, relieve their inhibitory effect and stimulate *Cyp6a8* promoter activity.

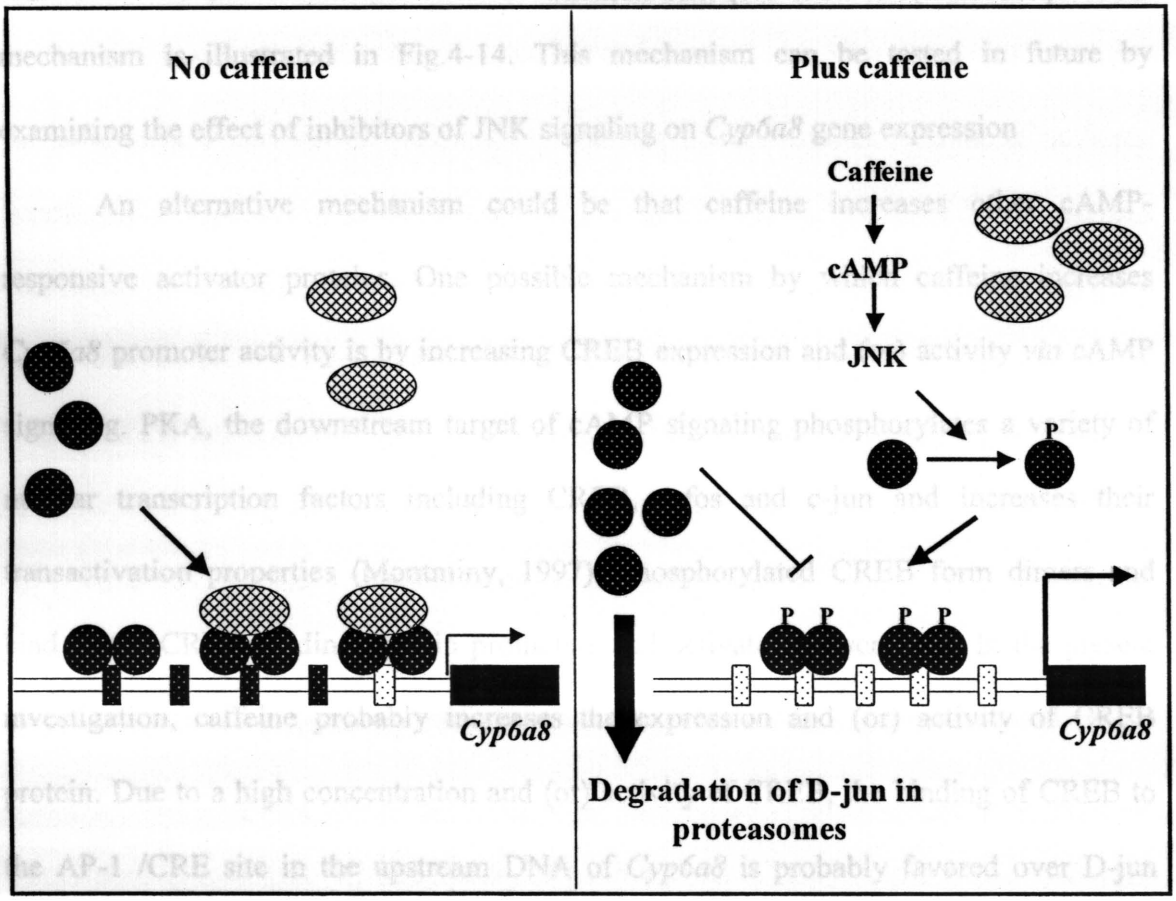
One possible mechanism by which caffeine action could be mediated is by modifying D-jun protein, by converting inactive D-jun protein into an active form. The activities of AP-1 components are mostly modulated *via* phosphorylation (Karin, 1995; Sng et al., 2004). In the case of c-Jun, phosphorylation at Ser-73 and Ser-63, located within its transactivation domain, potentiates its ability to activate transcription as a homodimer. Phosphorylation is thought to stabilize c-Jun by providing binding sites for molecules, which protect c-Jun against proteolysis. In *Drosophila*, a class of MAPK with substrate specificity for Jun was identified and is called as Jun N-terminal kinases (JNKs). D-jun in *Drosophila* is phosphorylated by a JNK named rolled (Peverali et al., 1996). Interestingly, it was found that the phosphorylation of c-jun by Jun-N-terminal kinase (JNK) relieves HDAC3-dependent suppression of the transcriptional activity of c-jun (Weiss et al., 2003). Thus, one can hypothesize that caffeine induction of *Cyp6a8* promoter activity is probably mediated through the JNK pathway.

JNK mediated signaling in response to cAMP, probably phosphorylates D-jun and releases D-jun from HDAC3 association. Phosphorylated D-jun probably then binds to the AP-1 site in the *Cyp6a8* upstream DNA and induces the promoter activity. Since there are only seven putative AP-1 and three putative CREB sites in the upstream DNA of *Cyp6a8* (Chapter 2), probably only a limited number of D-jun proteins are phosphorylated, which then bind to the promoter to cause gene activation. Also, it is possible that the half-life of the luciferase protein is high and that only few D-jun activator proteins are sufficient to elicit the response of elevated luc activity with caffeine

treatment. Therefore, it is possible that a fraction of D-jun protein molecules that are not bound to the promoter are still present in the cell. The D-jun proteins that are relieved from DNA binding and HDAC3 repression may be unphosphorylated and therefore, are highly unstable. Thus one can surmise that these proteins are probably targeted to degradation by proteasomes in the cell. A schematic diagram of the above-mentioned mechanism is illustrated in Fig.4-14. This mechanism can be tested in future by examining the effect of inhibitors of JNK signaling on *Cyp6a8* gene expression

An alternative mechanism could be that caffeine increases other cAMP-responsive activator proteins. One possible mechanism by which caffeine increases *Cyp6a8* promoter activity is by increasing CREB expression and (or) activity *via* cAMP signaling. PKA, the downstream target of cAMP signaling phosphorylates a variety of nuclear transcription factors including CREB, c-fos and c-jun and increases their transactivation properties (Montminy, 1997). Phosphorylated CREB form dimers and bind to the CREB binding sites in promoters and activates transcription. In the present investigation, caffeine probably increases the expression and (or) activity of CREB protein. Due to a high concentration and (or) activity of CREB, the binding of CREB to the AP-1 /CRE site in the upstream DNA of *Cyp6a8* is probably favored over D-jun binding (Fig. 4-15). The unbound D-jun proteins are probably targeted to degradation due to their unstable nature and thus low D-jun protein level. An observation in support of this hypothesis was made with the *uncoupling protein* (*ucp-1*) gene regulation. In response to adrenergic stimulation, cAMP stimulates PKA and regulates ucp-1 gene transcription, *via* CREB binding to the CRE motif. In this report it was also found that the

Figure 4-14: Hypothetical model for caffeine induction of *Cyp6a8* promoter activity mediated *via* D-jun stabilization. Caffeine probably activates JNK pathway *via* cAMP that in turn phosphorylates D-jun proteins. The phosphorylation of D-jun converts D-jun repressors into activators. The active D-jun proteins bind to the promoter and activate *Cyp6a8* transcription. As there are only limited AP-1/CRE sites in the upstream DNA of *Cyp6a8*, probably only a limited number of D-jun proteins are phosphorylated. The remaining D-jun proteins that are not phosphorylated are targeted to the proteasomal machinery, resulting in low D-jun levels. AP-1 denotes Activator Protein-1 motif and CRE represents cyclic AMP response element.



Histone deacetylase repressor complex

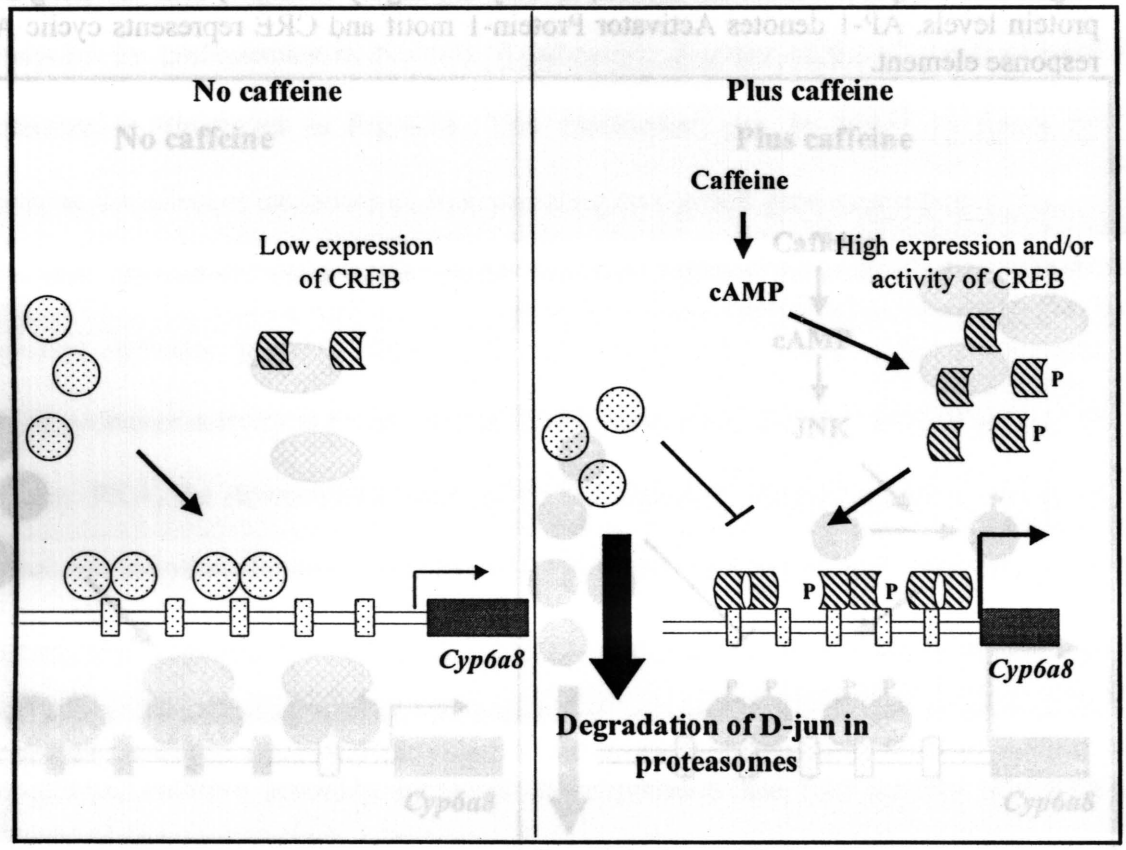
Unphosphorylated D-jun

P

Phosphorylated D-jun

AP-1 / CRE site

Figure 4-15: Possible role of CREB in caffeine-mediated induction of *Cyp6a8* promoter activity. Caffeine increases CREB levels and (or) activity *via* cAMP. CREB competes with D-jun to bind to the *Cyp6a8* promoter, and since CREB concentration exceeds the amount of D-jun in the cell, CREB binding is favored compared to the D-jun binding. The binding of CREB thus results in activation of the *Cyp6a8* promoter. Unphosphorylated D-jun molecules that are released from the promoter binding are targeted to the proteasomes in the cell, as they are highly unstable, thus lowering D-jun protein levels. AP-1 denotes Activator Protein-1 motif and CRE represents cyclic AMP response element.



Unphosphorylated D-jun

Unphosphorylated CREB

P Phosphorylated CREB

AP-1 / CRE site

expression of c-jun represses the transcriptional induction and overexpression of CREB blocks c-jun mediated repression (Yubero et al., 1998). Thus in future, one can examine the effect of CREB overexpression on *Cyp6a8* promoter activity to test the proposed mechanism.

Chapter 5

Conclusions

This dissertation aims to shed light on an unexplored area of caffeine induction of *Cyp* genes in *Drosophila melanogaster*. *Cyp6a2* and *Cyp6a8* genes were used as the prototype genes to understand the mechanism of *Cyp* gene regulation in *Drosophila* as they are induced by a variety of xenobiotics, including barbiturates (Maitra et al., 1996). A preliminary observation was made that Vivarin induces *Cyp6a2* and *Cyp6a8* gene expression in transgenic flies (Dean et al, unpublished data). Caffeine is the major component of Vivarin and so it was thought that Vivarin induction of *Cyp6a2* and *Cyp6a8* genes is the effect exerted by caffeine. With this line of thought, the present investigation was initiated to examine the mechanism by which caffeine induces *CYP* genes in *Drosophila*. Although caffeine has been widely studied in physiology due to its action as a neurostimulant and its effect on various biological processes, not many studies have been done on caffeine induction of *Cyp* genes, except for caffeine induction of *CYP1A1* gene expression in the rat striatum (Svenningsson et al., 1995). In this dissertation, experiments were designed and conducted to achieve three major objectives.

The first objective was to examine caffeine induction of *Cyp6a2* and *Cyp6a8* genes and to map the promoter region that is required for constitutive and caffeine-induced expression. It was found that caffeine induces *Cyp6a2* and *Cyp6a8* steady-state mRNA levels in flies (Chapter 2). It was also observed that SL-2 cells have the machinery for constitutive and caffeine-induced expression of *Cyp6a2* and *Cyp6a8* genes as the promoters of these genes respond to caffeine-treatment in SL-2 cells (Chapter 2).

These results establish SL-2 cell line as a suitable system to dissect the mechanism by which caffeine induces *Cyp6a2* and *Cyp6a8* expression. Mapping studies further helped me to delineate the minimum promoter regions required for constitutive and caffeine-induced expression of *Cyp6a2* and *Cyp6a8* genes (Chapter 2). Transient transfection studies in SL-2 cells revealed that the -129/-1 of *Cyp6a2* and -67/-11 of *Cyp6a8* are sufficient to confer the basal promoter activity to *Cyp6a2* and *Cyp6a8* genes in SL-2 cells (Chapter 2). Sequence analysis revealed the presence of a non-canonical TATA box within -129/-1 and -67/-11 regions of *Cyp6a2* and *Cyp6a8* promoters, respectively, that might be responsible for the observed basal constitutive promoter activity. Moreover, the region between -265/-1 of *Cyp6a2* and -199/-11 of *Cyp6a8* were found to be the minimum upstream DNAs required for caffeine-induced expression (Chapter 2). Sequence analysis of these upstream DNAs implicates the presence of putative activator protein -1 (AP-1) and putative cyclic AMP response element binding proteins (CREB) motifs (Chapter 2).

In mammals, AP-1 and CREB proteins have been shown to activate target gene expression in response to signals from cAMP (Daniel et al., 1998). Also, since caffeine has been shown to increase the intracellular cAMP level in other systems (Fredholm et al., 1999), one can hypothesize that caffeine induction of *Cyp6a2* and *Cyp6a8* is mediated *via* the putative AP-1 and CREB motifs present in the upstream DNA of these two genes via cAMP signaling. Interestingly, caffeine-induction increased with increasing length of upstream DNA from -265 to -983 of *Cyp6a2* and -199 to -761 of *Cyp6a8*, which have additional putative AP-1 and CREB motifs, that might be involved in caffeine-mediated

action (Chapter 2). This hypothesis was the basis for postulating the subsequent objectives.

The second objective was to examine the connection (if any) between caffeine induction and cAMP. In mammalian systems, caffeine has been shown to exert its effect by acting either as an adenosine receptor antagonist and or as a phosphodiesterase inhibitor, both of which result in an increased intracellular cAMP level. I therefore wanted to first examine if adenosine receptor - mediated signaling, affect the *Cyp6a2* and *Cyp6a8* promoter activities. My data showed that neither adenosine receptor antagonist (Dipropyl cyclopentyl adenosine-DPCPX) nor agonists (Adenosine and cyclopentyl adenosine- CPA) have any effect on the *Cyp6a2* and *Cyp6a8* promoter activities (Chapter 3). It was also found that caffeine inhibits PDE activity in SL-2 cells and in flies and also treatment with PDE inhibitors induce *Cyp6a2* and *Cyp6a8* promoter activities (Chapter 3). As a direct demonstration of the involvement of cAMP in caffeine action, it was found that caffeine increases the intracellular cAMP level and also treatment with dibutyryl cAMP; a cell-permeable cAMP analog induces *Cyp6a2* and *Cyp6a8* promoter activities. Hence, these results helped me to discover the key-signaling molecule, cAMP, which is involved in caffeine induction of *Cyp6a2* and *Cyp6a8* genes *via* PDE inhibition. I next wished to dissect the downstream signaling events, the transcription factors that respond to cAMP signaling and modulate *Cyp6a2* and *Cyp6a8* gene expression.

In the third objective, the role of D-fos and D-jun (if any) in caffeine-mediated induction of *Cyp6a8* gene was examined. As mentioned earlier, because the sequence analysis revealed the presence of putative AP-1 sites in the upstream DNA of *Cyp6a8*, I sought to investigate if D-fos and D-jun, the components of AP-1 complex, has any role

in caffeine-mediated expression of *Cyp6a8*. In mammals, c-fos and c-jun have been to shown to positively modulate its target gene expression in response to the signals from cAMP (Sng et al., 2004). Surprisingly, in this study, overexpression data showed that D-fos and D-jun negatively regulates the *Cyp6a8* promoter activity (Chapter 4). Moreover, caffeine induced expression decreased upon overexpression of either D-jun or D-fos in SL-2 cells. In contrast, inhibition of the endogenous D-jun expression by transfecting SL-2 cells with antisense construct of D-jun increased the *Cyp6a8* promoter activity for both constitutive and caffeine-induced expression. Interestingly, I also found that caffeine decreases the D-jun protein level in SL-2 cells and in flies (Chapter 4). These data together demonstrate a negative correlation between *Cyp6a8* promoter activity and D-jun protein expression. This observation is not an unprecedented one. There are reports in literature that show that D-jun acts as a repressor for the promoters of human chorionic gonadotropin (hCG) hormone and uncoupling protein-1 genes (Pestell et al., 1994; Yubero et al., 1998). In my study, it was found that caffeine *via* a hitherto unknown mechanism, overcomes the repressive effect of D-jun on the *Cyp6a8* promoter, and activates its transcription.

Two models are proposed that highlight on the probable mechanisms by which caffeine induces *Cyp6a8* promoter activity (Chapter 4). One model describes the possibility of caffeine increasing CREB protein expression *via* cAMP signaling, which in turn competes with D-jun to bind to the CREB site, and activates *Cyp6a8* gene expression. The other model highlights on the fact that caffeine stabilizes D-jun protein by activating the JNK pathway in response to cAMP signaling. JNK then phosphorylates

D-jun that results in the conversion of repressor form of D-jun into an activator. The activated D-jun then probably positively regulates *Cyp6a8* expression (Chapter 4).

The results presented in this dissertation have advanced our knowledge in caffeine-mediated induction of *Cyp6a2* and *Cyp6a8* genes. A major step of dissecting the signaling mechanism for caffeine induction was accomplished in the present investigation. Therefore, this dissertation opens new avenues for further delineating the link between caffeine induction and AP-1 and CREB proteins.

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Vita

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