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Vivari Induction of Expression of Cuplea 8 in Drosophila Melanogaster Larvai

Katherine Elizabeth Fleming
University of Tennessee - Knoxville

Appendix E - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL

Name: Katherine E. Fleming

College: Arts + Sciences Department: Biochemistry, Cellular + Molecular Biology

Faculty Mentor: Ranjan Ganguly, PhD

PROJECT TITLE: Vivarin® Induction of Expression of Cyplea8 in Drosophila Melanogaster larvae

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Ranjan Ganguly, Faculty Mentor

Date: May 6, 2003

General Assessment - please provide a short paragraph that highlights the most significant features of the project.

Comments (Optional):

Vivarin® Induction of Expression of *Cyp6a8* in *Drosophila melanogaster* larvae

Senior Honors Thesis
University Honors Program
University of Tennessee, Knoxville

Katherine E. Fleming
Mentor: Dr. Ranjan Ganguly
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Abstract

Cytochrome P450 monooxygenases or CYPs are an important superfamily of enzymes that are involved in the metabolism of many endogenous and xenobiotic (foreign) compounds. In *Drosophila melanogaster*, CYPs have been implicated in insecticide-resistance mechanisms. Studying the regulation of *CYP* genes in *Drosophila melanogaster* is important because it can illuminate the mechanisms by which resistant insects detoxify insecticides. In *Drosophila*, the *Cyp6a8* gene shows higher level of expression in insecticide-resistant strains compared to the susceptible ones. In a previous investigation, caffeine was shown to induce *Cyp6a8* expression in adult flies. The present investigation was initiated to examine whether caffeine tablet, Vivarin®, also induces *Cyp6a8* expression in larvae. For this purpose, two transgenic lines, *0.2luc30-4(H-ry)* and *0.8luc110(H-ry)*, carrying *luciferase* (*luc*) reporter gene were used. In these lines, the *luc* gene was under the control of a 0.2-kb (-11/-199) and 0.8-kb (-11/-766) upstream DNA of *Cyp6a8*, respectively. Third instar larvae (90 hours) from each line were allowed to feed on medium containing 16-mM caffeine (Vivarin®) for 24 hours. Inducibility of *Cyp6a8* promoter in each line was determined assaying extracts of the larvae for luciferase activity. The results showed that caffeine-treated larvae of *0.2luc30-4(H-ry)* and *0.8luc110(H-ry)* lines had 45 and 5.5 times higher luciferase activity respectively compared to the untreated larvae. These observations suggest that –11/-766 DNA of *Cyp6a8* has sequences that give caffeine-induced transcription of the *Cyp6a8* gene. Higher inducibility in *0.2luc30-4(H-ry)* line compared to the *0.8luc110(H-ry)* line suggests that sequences for maximum caffeine-Induced expression are present In -11/-199 DNA.

I. Introduction

Cytochrome p450 monooxygenases are a superfamily of enzymes found in all living organisms from bacteria to man. They are involved in the metabolism of endogenous and exogenous compounds, in particular the detoxification of xenobiotics. These enzymes are hemoproteins that have many isoforms and can have a very broad substrate specificity within a particular isoform (Scott 2001). In eukaryotes, these enzymes are usually associated with the endoplasmic reticulum, and the reaction they perform involves a cofactor, usually NADPH, which is used as a reducing equivalent. The overall reaction is as follows, where RH is the substrate:

$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$ (Scott 2001).

These enzymes are named according to their degree of amino acid similarity. All enzymes in the superfamily are given the CYP prefix. Families, denoted by a number, indicate greater than 40% identity in the amino acid sequences. Subfamilies, denoted by a letter, correspond to greater than 55% identity. Each enzyme within the subfamily is then given a number. For example, CYP6A2 and CYP6A8 are in the same family and subfamily and share a greater than 55% identity in their amino acid sequences (Maitra 1996). In *Drosophila melanogaster*, proteins, mRNA, and cDNA are written in capital letters, while the genes are written in italics, e.g. *Cyp6a8* (Dean 2002).

In mammals, mutations in CYP genes increase the risk of toxicity from certain medications and possibly the risk of cancer as well. Alternate alleles of CYP1A1 have been linked to an increased risk of lung cancer. CYP1A1 is a structural gene that encodes aryl hydrocarbon hydrolylase activity (Nebert 1996). CYP1A2 is also a structural gene encoding an arylamine hydroxylase, an enzyme involved in the

metabolism of aromatic amine procarcinogens. Furthermore, CYP2C19 (*s*-mephenytion 4'-hydroxylase), CYP2D6 (debrisoquine hydroxylase), and CYP2E1 (dimethylnitrosamine *n*-demethylase) are also involved in the metabolism of drugs and procarcinogens (Nebert 1996).

In insects, cytochrome p450s show an incredible diversity. Due to their role in detoxification of xenobiotic compounds, they have been implicated as a possible mechanism for insecticide resistance. *Drosophila melanogaster* has been used as a model organism for studying the mechanisms of *Cyp* gene regulation in insects, especially in relation to mechanisms of insecticide resistance. Maitra et al. (1996) showed that insecticide-resistant strains of *Drosophila melanogaster* have higher levels of *Cyp* expression than susceptible strains. In particular, *Cyp6a2* and *Cyp6a8* are overproduced in the 91-R DDT-resistant strain versus the 91-C DDT-susceptible strain. In addition, *Cyp6a8* was shown to be induced by barbital treatment in overproducer and underproducer strains and hybrids of these two strains (Maitra 1996). *Cyp6a8*, located on the second chromosome, appears to be regulated by loci on the third chromosome that probably produce repressors which downregulate the activity of *Cyp6a8*. Barbital probably intervenes with the repressors' ability to downregulate *Cyp6a8* (Maitra 2000).

Maitra el al. (2002) created transgenic lines that have upstream regulatory DNA from the 91-R strain *Cyp6a8* allele of the 91R strain attached to the luciferase reporter gene. These transgenic lines also showed barbital-induction of *Cyp6a8*. Furthermore, the upstream region between base pairs –199/–761 appear to be required for maximum constitutive expression and barbital-induced expression (Maitra 2002). The data indicate

that the overproduction of CYP6A8 in resistant strains is due to a mutation in the *trans*-repressor sequence rather than in the *cis*-regulatory sequences of *Cyp6a8* (Maitra 2002).

In a further study of the *Cyp6a8* gene, Dean (2002) showed that caffeine induced expression of *Cyp6a8* in adult transgenic flies created by S. Maitra (Maitra 2002). Caffeine has been previously shown to induce CYP1A1 and CYP1A2 genes in rats, genes that are involved in the metabolism of caffeine (Goasduff 1996). The present investigation examined the induction of the *Cyp6a8* gene by caffeine in larvae from the transgenic lines created by S. Maitra and E. Dean (Maitra 2002, Dean 2002). Studying the regulation of *Cyp* genes in *Drosophila melanogaster* can illuminate the mechanisms by which resistant organisms detoxify insecticides. Moreover, *Drosophila melanogaster* can act as a model organism for understanding CYP regulation in humans, where cytochrome p450s have been shown to be involved in certain cancers and genetic disorders (Dean 2002).

II. Materials and Methods

Fly Strains and Culture Conditions

Two lines of transgenic flies were used in this study: *0.2 luc 30-4 (H-ry)* and *0.8 luc 110 (H-ry)*, which were available in the laboratory. S. Maitra (2002) made the transgenic 0.2 *luc* and 0.8 *luc* lines that served as the basis for the lines used in the experiment. These transgenic lines have the firefly *luc* gene encoding the luciferase protein placed downstream of the *Cyp6a8* promoter from the 91-R DDT-resistant host strain. The 0.2-luc line contains about 200 base pairs of the promoter while the 0.8-luc line has about 800 base pairs of the *Cyp6a8* promoter (see Figure 1). The *0.2 luc 30-4 (H-ry)* and *0.8 luc 110 (H-ry)* were created by E. Dean (2002) in order to create strains of flies that were homozygous for the transgene and had ry^{506} host strain genetic background. Measuring the luciferase allowed the activity of the *Cyp6a8* promoter to be easily assayed.

The two strains of flies were cultured at 23°C±1°C . The medium used in fly cultures consisted of 0.65% bacto-agar, 5.5% cornmeal, 3% brewer's yeast, 5% unsulfered molasses, 2% light corn syrup, 0.25% propionic acid.

Larval Treatments

Adult flies were cleared for one hour and then left for egg-laying for three hours. The flies were placed in a round plastic cylinder closed at one end by a Petri dish with the cornmeal-agar-molasses medium and at the other end by a cheesecloth and cotton stopper. The egg-laying canisters were placed in a dark cabinet with a large beaker filled with hot water to provide extra humidity. One canister was used for each of the

transgenic lines. The medium in the petri dishes was scored, and then mashed banana and dry yeast were added to induce the adult flies to lay eggs. The flies laid eggs at an optimal rate in the afternoon. After three hours the adult flies were removed, and the larvae was allowed to develop for 90 hours in covered petri dishes at room temperature.

After 90 hours, the petri dishes containing the larvae were flooded with water until the larvae floated to the top. This method allowed for easy removal of the larvae and separation into two dishes, one with Vivarin®-food and one with control food. This method was performed for each transgenic line. The treated food was prepared directly before use with dry potato flakes that were reconstituted with either a Vivarin® solution (in the case of the experimental treatment) or water (in the case of the control treatment). The Vivarin® solution consisted of one pill of Vivarin®, crushed with a mortar and pestle, and mixed with 64-ml distilled water. Vivarin® contains 200 mg of caffeine as well as other inert ingredients (carnuba wax, colloidial silicon dioxide, D+C Yellow #10 Al Lake, dextrose, FD+C Yellow #6 Al Lake, hydroxypropyl methylcellulose, magnesium sulfate, microcrystalline cellulose, polyethylene glycol, Polysorbate 80, starch, and titanium dioxide). The Vivarin® solution was 16 mM, and the Vivarin® pills came from the local pharmacy as an over-the-counter medication. The larvae were exposed to the treated medium for 24 hours (see Figure 2).

Preparation of the Extracts

After 24 hours, the larvae were again removed by drowning and sorted into 1.5-ml Eppendorf tubes for extract preparation. Nine to ten larvae were placed into each tube

with 20-µl 1X Cell Culture Lysis Reagent (CCLR, Promega). Extracts were made in triplicate. The larvae were immediately homogenized for 20 seconds and placed on ice for 5 minutes. The homogenates were centrifuged at 13K, 4°C, for 8 minutes. Following centrifugation, 100 µl of the clear extract were removed, carefully leaving behind the soluble and lipid portions, and placed into clean 1.5-ml Eppendorf tubes. The extracts were again centrifuged for 8 minutes at 13K, 4°C. Again leaving behind the lipid fraction, 50 µl were removed and placed into clean 1.5-ml Eppendorf tubes, and 25 µl of these portions were placed in additional clean 1.5-ml Eppendorf tubes. The two portions were stored at -80°C until they could be respectively assayed for luciferase activity and protein content.

Luciferase Assay

A commercially available kit (Promega, WI) was used to measure luciferase activity. The Luciferase Activity Reagent (LAR) contained beetle luciferin and was prepared ahead according to the manufacturer's instructions. Single reaction aliquots of 100 µL were stored at –80°C in 1.5-ml Eppendorf tubes until use. For the luciferase assay, one aliquot of LAR was used for each tube of extract. These LAR aliquots were stored on ice in darkness for 45 minutes and then at room temperature for 15 minutes prior to use. The extracts were allowed to thaw on ice for 15 minutes and then at room temperature for 2 minutes prior to use. Once the extracts and aliquots were thawed, 5 µl of the extract were added to the LAR aliquot and read in a luminometer (Zylux) for 1 minute at 15 second intervals after a 3 second delay. The second reading was used for

this investigation, which was collected about 33 seconds after the reaction initiation. Readings were given in Registered Light Units (RLUs).

Protein Assay

The BCA protein assay kit (Pierce) was used to assay the amount of protein in the extracts used for the luciferase assay. Bovine Serum Albumin (BSA) at 2mg/ml was diluted with 1X CCLR to a BSA concentration of 1mg/ml and 0.5X CCLR. Adding BSA to 0.5X CCLR in varying percentages generated the samples used to create the standard curve. Extracts were thawed on ice for 15 minutes and then diluted to 0.5XCCLR. The dilution of the standard samples and the extracts reduces the number of interference molecules in the reaction. All reactions were performed in duplicate and according to the manufacturer's protocol. The absorbance of the standard samples and extracts were read at 562 nm in a disposable cuvette on a Shimadzu spectrophotometer model UV16U. The two readings for each sample were averaged, and this value was used to calculate the total protein content using the standard curve equation. The final values were expressed in RLUs per μg of total protein.

Statistics

A Student's t-test was performed to analyze the statistical significance of the data (http://www.physics.csbsju.edu/stats/).

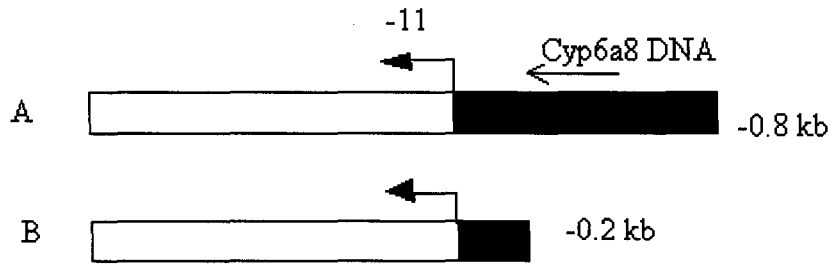

Figure 1. Diagram of transgenes. 0.8 luc transgene (A) has the upstream regulatory DNA bases -11 to -761 from *Cyp6a8*. 0.2 luc transgene (B) has upstream regulatory DNA from bases -11 to -199 from *Cyp6a8*.

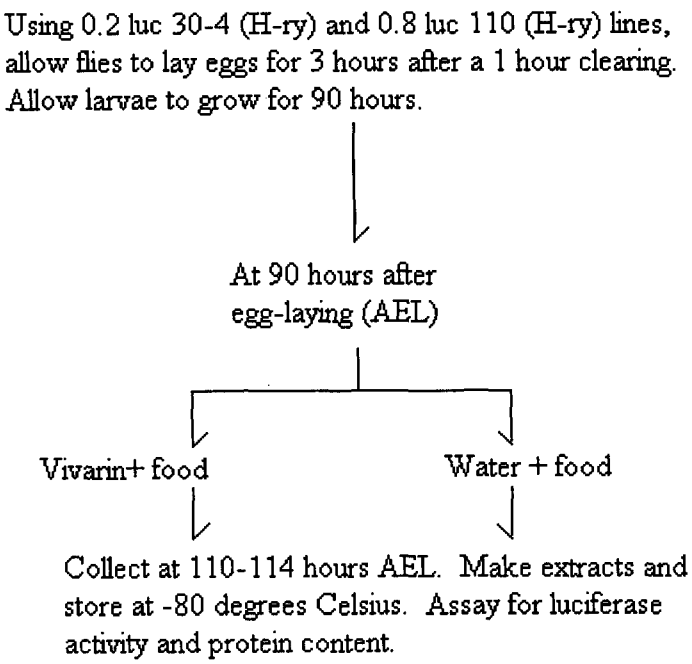

Figure 2. Flow chart of method used to treat larvae with Vivarin®.

III. Results

This investigation of caffeine induction of *Cyp6a8* in larvae was performed using transgenic lines created by S. Maitra and E. Dean (Maitra 2002, Dean 2002). The 0.2 *luc* and 0.8 *luc* lines used were homozygous for the transgene. Dean (2002) used these two lines and an additional transgenic line with a 3.1-kb upstream DNA of *Cyp6a8* in front of the luciferase gene. The difference in the baseline expression and the caffeine-induced expression in adults showed that the region of upstream DNA between bases –199 and –761 (between the 0.2 and 0.8 constructs) is the most important region for maximum induction by caffeine. The expression values of the 3.1 and the 0.8 lines showed no statistical difference. Therefore, the 3.1 *luc* transgenic line was no longer studied (Dean 2002). As this investigation was designed to mirror the investigation by Dean (2002), the 3.1 *luc* line was not used.

In this investigation, Vivarin® was shown to induce expression of *Cyp6a8* in both the 0.2 and 0.8 transgenic lines in larvae. The difference in baseline expression between the 0.8 line and the 0.2 line was 8-fold, with the 0.8 line showing higher baseline expression(see Table 1 and Figure 3). The 0.8 line showed slightly higher Vivarin®-induced expression than the 0.2 line. The difference in the 0.2 untreated- and treated-expression was 45-fold, with a Student's t-test p-value of less than or equal to 0.0098, a statistically significant difference. The difference in the 0.8 untreated- and treated-expression was 5.5-fold, with a Student's t-test p-value of less than or equal to 0.010, also a statistically significant difference. Clearly, Vivarin® induces expression of *Cyp6a8* in these two transgenic lines (see Table 1 and Figure 3).

Table 1. Table showing results of Vivarin® induction in transgenic lines.

Line	Sample	RLUs	Protein mg/ml	RLUs/mg	Average	Treated/Untreated
0.2 Untreated	1	85087200	0.013	6.35E+09		
	2	91674400	0.017	5.35E+09		
	3	57163400	0.014	4.19E+09	5.30E+09	
0.2 Vivarin	4	3459072600	0.017	2.00E+11		
	5	3724779200	0.021	1.78E+11		
	6	3697855000	0.011	3.39E+11	2.39E+11	45.10
0.8 Untreated	7	687182000	0.030	2.32E+10		
	8	557420800	0.007	8.44E+10		
	9	580165800	0.027	2.14E+10	4.30E+10	
0.8 Vivarin	10	3546196800	0.015	2.39E+11		
	11	3722782600	0.021	1.78E+11		
	12	3722942000	0.012	3.12E+11	2.43E+11	5.65

Dean (2002) examined the possibility that an inert ingredient in Vivarin® rather caffeine caused the induction of *Cyp6a8* in adults flies. An identical experiment was performed using caffeine instead of Vivarin®, and no statistical difference was found between the induced-expression of the two chemicals. Therefore, it was concluded that the caffeine, and not an inert ingredient, in Vivarin® caused the induction of *Cyp6a8* seen in adult flies (Dean 2002). Due to these results, Vivarin® was the only treatment used in this investigation of larval induction of *Cyp6a8*.

Furthermore, only the 0.2 *luc* 30-4 (H-ry) and the 0.8 *luc* 110 (H-ry) transgenic lines were used in this experiment. Dean (2002) addressed the concern that induction by caffeine was an artifact of these two transgenic strains by testing additional transgenic strains. These additional transgenic lines showed a similar level of induction as the original transgenic lines (Dean 2002). Therefore, in this study only the 0.2 *luc* 30-4 (H-ry) and the 0.8 *luc* 110 (H-ry) lines were used, as similar mechanisms of induction were probably at work in adult flies and larvae.

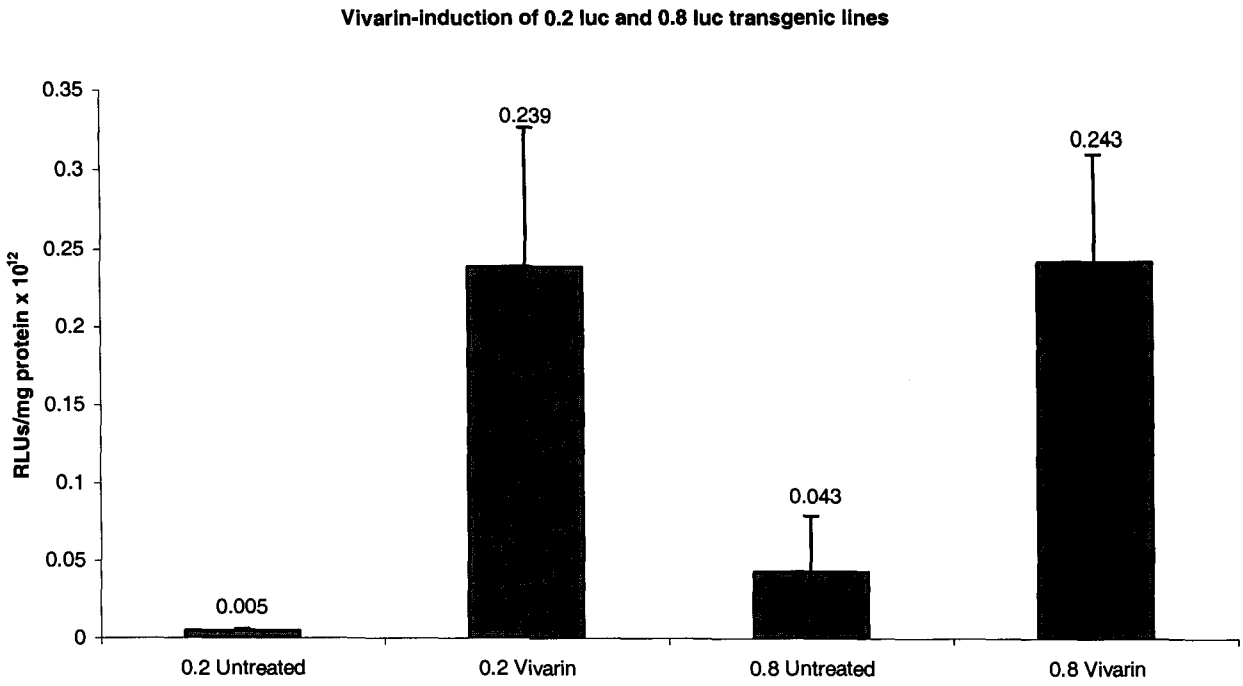

Figure 3. Graph showing Vivarin® induction of *Cyp6a8* in 0.2 and 0.8 transgenic lines. P value for 0.2 untreated versus treated was less than or equal to 0.0098. P value for 0.8 untreated versus treated was less than or equal to 0.010.

IV. Discussion

Caffeine induces expression of *Cyp6a8* in larvae of the 0.2 and 0.8 *luc* transgenic lines used in this investigation. However, the two lines displayed differences in baseline and inducible expression. The 0.8 transgenic line had an 8-fold higher baseline expression than the 0.2 line, but the 0.2 transgenic line exhibited a much higher fold change in expression due to caffeine induction. The 0.2 line appears to be much more inducible than the 0.8 line. Nevertheless, the 0.8 line showed slightly higher levels of caffeine-induced expression.

According to Maitra et al. (2000), wild-type flies exhibit a low level of *Cyp6a8* expression due to repressor molecules rather than a weak promoter. The repressor genes appear to be located on the third chromosome while *Cyp6a8* is located on the second chromosome. Overproduction *Cyp6a8* in insecticide-resistant strains is thought to be due to a mutation in the *trans*-regulatory repressor rather than a mutation in the upstream *cis*-regulatory sequences of *Cyp6a8* (Maitra 2000). Maitra et al. (2002) showed that the DNA sequences between –199 and –761 are necessary for maximum constitutive and barbital-induced expression. These sequences contain six barbie-boxes and other transcription factor binding sites. Furthermore, the region between –11 and –761 was shown to respond to *trans*-regulatory factors from the ry^{506} genome, an underproducer strain (Maitra 2002). The presence of these regulatory sequences in the region of –11/–761 indicates that this region is the most important promoter region. These findings support the hypothesis that the DNA sequences of –199/–761 are necessary for maximum constitutive and induced expression and thus explain why the 0.8 transgenic line shows a higher level of baseline and induced expression.

However, the differences in fold change in expression due to caffeine induction between the 0.2 and 0.8 transgenic lines are not explained by the presence of regulatory regions in the –199/–761 DNA sequence. Dean (2002) also saw a higher fold change in the 0.2 transgenic line than in the 0.8 transgenic line in adults, but the 0.8 transgenic lines in adults showed significantly higher levels of caffeine-induced expression. It is unlikely that larvae and adults have different mechanisms by which caffeine induces *Cyp6a8* expression. Therefore, the differences in the level of fold change in induced expression between larvae and adults should be further explored. The mechanism by which caffeine induces may interact with a regulatory sequence present in the –11/–199 region. In such a case, the higher induced expression seen in the 0.8 transgenic lines would be due to the difference in constitutive expression between the two transgenic lines.

Finally, the mechanism of caffeine induction is unknown and should be explored further. Caffeine induces greater activity of the *Cyp6a8*-luciferase transgene. This result could be due to several factors including an increase in transcription, a chromosomal position effect, a post-transcriptional stabilization of the mRNA, or a post-translational change in luciferase activity or stability. Increased activity due to a post-translational change in luciferase or to a chromosomal position effect can be ruled out. Dean (2002) compared the level of luciferase activity in the caffeine-induced expression in transgenic adults to the level of mRNA in caffeine-induced expression in the ry^{506} and 91-R strains and found consistent levels of induction among host and transgenic strains. Thus, caffeine induction involves either an increase in transcription levels or a stabilization of mRNA. Goasduff et al. (1996) demonstrated in rats that caffeine increases the level of the homologous mammalian genes CYP1A1/1A2 mRNA and ruled out the possibility

that mRNA stabilization led to these increased levels. According to Gonzales et al. (1993) as reviewed by Goasduff (1996), CYP1A1 enzymes cannot be regulated through stabilization of mRNA levels but rather only through activation of transcription. As similar mechanisms are likely at work in rats and *Drosophila melanogaster*, caffeine induction of *Cyp6a8* is probably due to transcriptional activation, not stabilization of mRNA.

Dean (2002) reviewed possible mechanisms for caffeine induction of *Cyp6a8* as the actual mechanism of caffeine induction is unknown. Svenningsson et al. (1997), as reviewed by Dean (2002), showed that caffeine upregulates c-fos and junB. These two early gene products heterodimerize to form Activating Protein 1 (AP-1) complex, which is a DNA binding transcriptional activator. Dean (2002) suggests that caffeine induction of *Cyp6a8* may occur through *Drosophila* homologues of c-fos and junB. Maitra et al. (2002) identified several putative binding sites for AP-1, several of which are in the –11/–761 region. These putative AP-1 binding sites may be the sequences through which caffeine induces expression of *Cyp6a8*.

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