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Comparison of caffeine-inducibility of 0.8-kb and 0.2-kb upstream DNA of the *Cyp6a8* gene in male *Drosophila melanogaster*

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Introduction

General Introduction

In the 1950's a very important discovery was made about different oxidation reactions by which cytochrome P450 enzymes metabolize various compounds. Cytochrome P450s, also known as CYPs, are a superfamily of detoxifying enzymes that are involved in the metabolism of various endogenous cellular metabolites and xenobiotic compounds (Agosin, 1985; Guengerich, 1993). Many of the CYPs in the body are found in the liver, since it is the main organ involved with the removal of drugs and toxins, however; CYPs can also be found in the small intestines as well. The significance of CYPs is seen in the fact that they exist on all living organisms, including bacteria. CYPs play a role in many processes such as the metabolism of fatty acids and bile salts; and it is vital in the formation of steroids, cholesterol, and arachidonic acid metabolites. It is speculated that certain P450s even play a role in the pathogenesis of some cancers by activating pro-carcinogens to carcinogens. Understanding the mechanism of cytochrome P450 is vital for those, like me who want to be pharmacists, and need to know potentially life threatening drug interactions. These enzymes are essential for the survival of organisms and learning the mechanism by which these enzymes are controlled can better give researchers and manufacturers better information to use when created certain prescriptions in the pharmaceutical industry.

Evolution, Taxonomy, and Nomenclature of Cytochrome P450

The enzyme was first named in 1961 as P450 because its pigment (P) had a spectral peak of 450 nm when it was reduced and bound to carbon monoxide. Cytochrome 450 was first thought to be only one enzyme. However, it is now known to be a family of enzymes. Extensive

research over the past several decades has shown that an organism generally has multiple P450s or CYPs. Humans have 55, Drosophila has 83, and rice has 453 CYP genes. It is suggested that the superfamily originated from a common ancestral gene around 3 billion years ago (Nebert and Russell 2002). Cytochrome P450 proteins are grouped into families and subfamilies based on similarities in their amino acid sequences. Those that share greater than 40% identity at the amino acid sequence level are assigned to a family specified by an Arabic numeral, while those that share greater than 55% identity are assigned a subfamily specified by a letter. Now, there are over 270 different CYP gene families (Nebert and Russel 2002).

Mechanism of Action and Function of CYPs

The major reaction CYPs catalyze is a monooxygenation. The overall reaction of substrate oxygenation is represented in the following equation where R represents the substrate:

$$NAD(P)H + R + O_2 \xrightarrow{P450} NAD(P)^+ + RO + H_2O$$

The chemistry involves the splitting of an oxygen molecule into two atoms. One of these atoms is inserted into the substrate, while the other is combined with two hydrogen atoms to form water.

CYPs are involved in diverse functions. Many foreign chemicals come into an organism's body every day. Many of these compounds can be toxic to the organism. Everyday humans are taking different compounds for various reasons. Many may be going to the local pharmacy to get an antibiotic for a sinus infection or some may be trying to fight other diseases. These compounds they are consuming are xenobiotics. Foreign chemicals that come into our systems are prone to get metabolized by cytochrome P450. One of its functions is to rid the body's system of any toxic chemicals. Xenobiotics are useful compounds and insects use this property of P450 to their advantage. They use P450 to metabolize insecticides and pesticides,

which are used to control the insects which would otherwise do much damage to the agriculture and propagate germs to various human and livestock diseases.

CYPs also play a major role in biosynthesis of many endogenous compounds. In plants CYPs are involved in biosynthesis of various pigments, sterols, and hormones. In humans and other mammals CYPs are also involved in biosynthesis of steroid hormones. For example, differential expression of P450's is also found in males and females in humans. Females have testosterone, but the P450 enzyme causes aromatase to convert it to estradiol. The estradiol helps develop female secondary sex characteristics. In males testosterone is converted by a non-P450 hormone, 5-α-reductase, to 5-α-dihydrotestoterone (DHT). DHT involves the development of male secondary sex characteristics. Thus it appears mutation of P450 genes may affect vital biosynthetic pathways and can cause abnormal physiology and sexual phenotypes.

Gene Induction

In mammals, P450s are induced by xenobiotic compounds and endogenous compounds (such as steroids). P450 gene induction is an important issue in humans because often patients are prescribed multiple drugs which may act on different P450s differently. This, of course, could lead to an adverse drug-drug interaction. Enzyme induction leads to the production of more enzyme, usually after three or more days of exposure to the inducer (Tredger and Stoll 2002). In humans and other mammals, expression of a P450 gene, CYP1A1, is induced by benzopyrene, which is found in cigarette smoke. There are other polycyclic hydrocarbons, which also induce CYP1A1. Interestingly, CYP1A1 enzyme is involved in the metabolism or detoxification of these inducer xenobiotics. This suggests that CYP1A1 protects mammals and humans from the ill effects of the inducer chemicals which are generally carcinogenic.

Insects are also known to utilize P450 enzymes to their advantage to protect themselves from the lethal effects of insecticides that are used heavily in agriculture. There are many examples which demonstrate that insects which are exposed to insecticides show overexpression of one or more P450 genes. For example, CYP6A1 gene of the housefly, Cyp6a2 and many other CYP genes of Drosophila are overexpressed in DDT resistant strains. Recently, CYP6G1 gene overexpression in Drosophila has been correlated with DDT resistance. There is also evidence in literature that exposure of Drosophila to DDT induces Cyp12d1 gene expression.

In insects, P450s are induced by xenobiotic compounds such as barbital and phenobarbital. There is also evidence in Drosophila that these compounds as well as DDT induce multiple genes. Most of these studies in Drosophila have been done on females. Female Drosophila also shows induction of P450 genes by the most common xenobiotic compound, caffeine. Very little is known about CYP gene induction in male flies. Male insects have never been examined for caffeine induction of P450 genes. To investigate this, I have used the luciferase gene as a reporter, but driven by the upstream DNA 0.2 KB and 0.8 KB of the Drosophila Cyp6a8 gene.

Many CYP's have shown to be induced by xenobiotics. Caffeine is considered a xenobiotic and an important one at that because of the large amounts that humans consume everyday. Therefore, knowing the mechanisms by which it regulates gene expression in fruit flies may be helpful for future studies. In this study, *0.2-luc 30-4 (H-ry) and 0.8-luc 110 (H-ry)* male flies were both treated with 16mM caffeine in order to study the effects of caffeine induction. Luciferase assays and protein assays were performed in order to obtain these results.

Materials and Methods

Fly Strains and Culture Conditions

0.2-luc 30-4 (H-ry) and *0.8-luc 110 (H-ry)* were the two lines of transgenic flies that were used in these experiments. Both of these lines were ready available for me in the Dr. Ganguly's laboratory. The flies were raised on standard cornmeal-agar-molasses medium which consisted of 0.65% bacto-agar, 5.5% cornmeal, 3% brewer's yeast, 5% unsulfured molasses, 2% light corn syrup, 0.25% propionic acid, 0.3% ethanol, and 0.1% tegosept at 23°C.

Both *0.2-luc 30-4 (H-ry)* and *0.8-luc 110 (H-ry)* have the firefly luc gene encoding the luciferase protein downstream from the promoter Cyp6a8 from the 91-R DDT resistant host strain. Therefore measuring the luciferase of these transgenic lines allowed the activity of Cyp6a8 to be easily examined.

Caffeine Treatment

Male flies of the following strains were used in both experiments: *0.2-luc 30-4 (H-ry)* and *0.8-luc 110 (H-ry)*. They were sorted and kept on normal fly food for 24 hours in order to recover from the ether treatment. The live flies were then transferred to a potato media reconstituted with 16mM caffeine. The flies were kept in the caffeine treatment for 24 hours. Following the treatment, extracts were made and luciferase assays and protein assays were performed.

Extract Preparation for Luciferase Assay

At the end of the treatment period, flies were etherized and sorted into 1.5 ml eppendorf tubes in groups of 10 flies. After the flies recovered from the effects of the ether, the tubes were placed on ice for approximately 2 minutes to paralyze the flies. Then 200 µl of 1X Cell Culture Lysis Reagent (CCLR) (Promega) was added to each tube. The flies were homogenized

immediately on ice upon the addition of the buffer for 20-30 seconds. The homogenates were kept on ice for 5 minutes and then the samples were centrifuged for 8 minutes at 12,000 RPM at 4°C. 100 µl of clear extract was removed from each sample and put into a fresh eppendorf tube leaving behind the entire insoluble fraction and as much lipid as possible. The extracts were then centrifuged again for 8 minutes at 12,000 RPM at 4°C. Supernatant was carefully removed and split into two 25µl aliquots. The samples were then stored at -80°C until one is ready to be used for luciferase assay and the other for protein assay.

Luciferase Assays

Luciferase activity was measured using a commercially available kit (Promega, WI). The Luciferase Assay Reagent contained beetle luciferin and was prepared according to the manufacturer's protocol into 100 µl aliquots. The appropriate number of tubes containing 100 ml of Luciferase Assay Reagent were removed from -80°C and stored on ice in darkness for 30 minutes. 15 minutes before the Luciferase Assay Reagent was completely thawed, the extracts for the luciferase extract were removed from -80°C and placed on ice to thaw. The Luciferase Assay Reagent was removed from the ice and placed at room temperature for 15 minutes. Next the luminometer was plugged in and set to PC-OPER. Then the Luminometer software was opened on the computer and Single Kinetics was selected as well as Danielle's Purine Treatment. The count was set to start with a 3 second delay, 15 seconds interval counting for 1 minute, and set it for two water blanks. Then the experiment was started by reading two water blanks. One extract at a time was removed to room temperature and allowed to equilibrate to room temperature for 2 minutes. 5 µl of extract was added directly to the prealiquoted 100 µl of Luciferase Assay Reagent and then flicked gently for 5 seconds before put into the Luminometer. The sample was then read for 1 minute at 15 second intervals with a 3 second

delay. This was repeated with all the samples. The data was saved to a disk after being transferred to an excel file. The reading used in the data analysis was the second reading collected.

Protein Assays

All extracts for protein assay were removed from -80°C and placed on ice to thaw. The appropriate number of baked test tubes for the assay were labeled. The assay for each sample was to be performed in duplicate. In addition to the test tubes for the samples, ten additional test tubes were labeled for the standards. In a baked test tube, BSA was diluted with 1X CCLR buffer (300 µl of BSA + 300 µl of 1X CCLR buffer). The BSA is now in 0.5 CCLR buffer and will be used later. After approximately 15 minutes when the extracts thawed, 25 µl of sterile water were added to each sample. The extract is now in 0.5X CCLR buffer. Next the 0.5X CCLR buffer was made fresh in a baked test tube by mixing 2 ml 1X CCLR buffer + 2 ml sterile water. This solution will be used later on. To prepare the samples for a standard curve, the BSA in 0.5X CCLR buffer was added to the appropriate pre-labeled, baked test tube. It was then brought up to 100 µl volume by adding the appropriate volume of 0.5X CCLR buffer. The following table was followed: *The liquid was always deposited to the bottom of the test tube.*

	BSA in 0.5X CCLR	0.5X CCLR buffer
Tubes 1 and 2	0µl	100µl
Tubes 3 and 4	25µl	75µl
Tubes 5 and 6	50µl	50µl
Tubes 7 and 8	75µl	25µl
Tubes 9 and 10	100µl	0µl

The extracts were further prepared by adding 20 µl of diluted extract to the appropriate pre-labeled, test tube. Then 80 µl of 0.5X CCLR buffer was added to bring the volume up to 100 µl. Once all the samples were prepared, the BCA protein assay reagent by Pierce was made. This was made by mixing 50 parts reagent A (clear liquid) with 1 part reagent B (blue liquid). 75 ml

was made by mixing 73.5 ml reagent A with 1.5 ml reagent B. The mixture turned green when mixed. Once mixed, 2 ml of the BCA protein assay reagent was added to each test tube (including both standards and samples). The pipeting mixed the samples well enough so that no further mixing was necessary. The reaction turned purple. The samples were then incubated for 30 minutes at 37°C. 20 minutes into the incubation the spectrophotometer was turned on in order to warm up. After incubation, the samples were removed and allowed to equilibrate to room temperature for 10 minutes. The absorbance was then read at 562nm in a disposable cuvette. Final data was expressed as Registered Light Units per μg of total protein (RLUs/μg).

Results and Discussion

The results of the experiments are shown in Figure 1. When two transgenic lines treated with water were compared, the level of luciferase activity was found to be 3.5- fold greater in the 0.8-luc line compared to the 0.2-luc line. Previous investigators have also found comparable differences between these transgenic lines in female flies. Since female flies were not used in

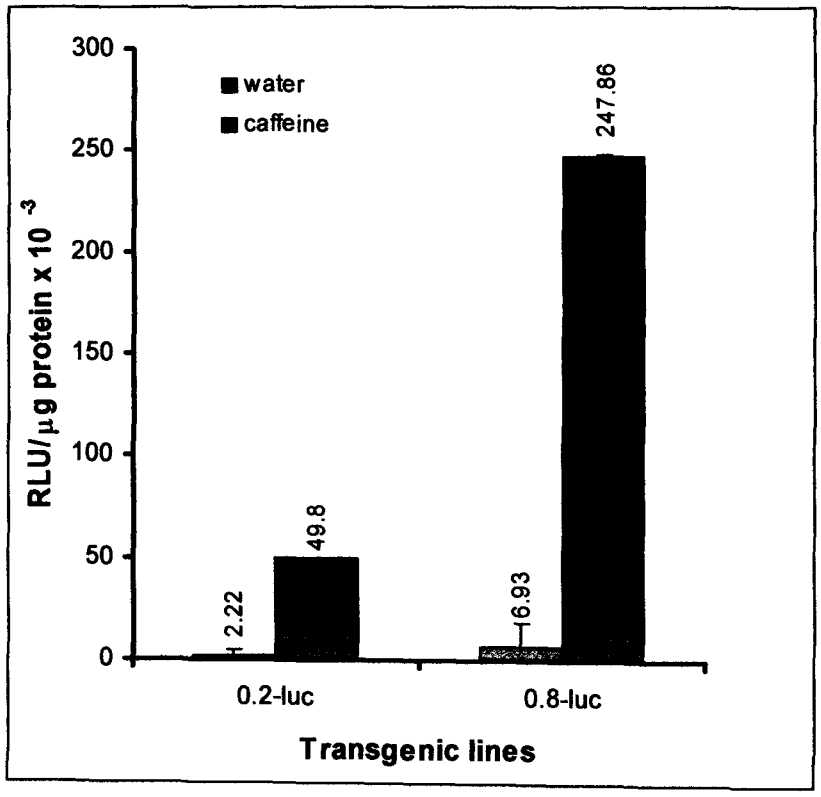

Figure 1 – The Effect of Caffeine on Transgenic Lines: 0.2-luc 30-4 (H-ry) and 0.8-luc 110 (H-ry)

the present investigation, the difference between the two sexes in each line could not be measured. However, Maitra (2002) found that male flies show higher level of CYP6a8 promoter activity.

When the male flies from two transgenic lines were treated with caffeine, a significant induction of luciferase reporter gene expression was observed. The level of induction in the 0.2-luc line was almost 25-fold, whereas in the 0.8-luc line it was about 32-fold. Higher levels of fold induction in the 0.8-luc line was also observed when female flies were examined by other investigators. These results suggest that inducibility of 0.2 KB and 0.8 KB upstream DNA of CYP6a8 is also observed in male flies, and thus the CYP6a8 gene does not show sexual dimorphism in expression. The sequences responsible for caffeine inducubility are present in both these upstream DNA fragments. However, since 0.8 KB DNA shows a higher level of expression than 0.2 KB, therefore 0.8 KB has more such sequences. The mechanism of caffeine induced expression of CYP genes has been examined by Bhaskara et al (2006). It has been shown that caffeine induction is mediated via cAMP pathway.

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